Regulation of Nucleosome Dynamics: Mechanisms for Chromatin Accessibility and Metabolism

Dissertation

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By

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The DNA in eukaryotic cells is organized into a tightly-regulated structural polymer called chromatin that ultimately controls crucial functions of the genome, including gene expression, DNA synthesis, and repair. The basic unit of chromatin is the nucleosome in which 147bp of DNA wraps 1.7-times around eight "core" histone proteins (two copies each of H2A, H2B, H3, H4). Repeats of this structural unit have been shown to fold into higher order structures, which play a central role in controlling DNA accessibility for transcription regulation. However, at the individual nucleosome level, DNA-histone interactions that wrap DNA into the nucleosome also control DNA accessibility. A significant number of factors have been shown to regulate nucleosome accessibility, including variants and post-translational chemical modifications of to the core histone proteins, chromatin remodeling complexes that reposition and disassemble nucleosomes, and histone chaperones that deposit or remove histones. Ultimately, these chromatin regulatory factors must physically alter nucleosomes to change DNA accessibility to transcription, replication, and DNA repair machinery.

This work encompasses a detailed study of the integral relationship between histone post-translational modifications (PTMs) and DNA accessibility. There are more than 100 reported PTMs throughout the nucleosome, many of which serve as binding sites for chromatin regulatory proteins. However, a subset of these PTMs are buried beneath the DNA-histone interface and are seemingly inaccessible to regulatory proteins. Given that nucleosomes in vivo typically possess multiple PTMs, until recently it has been difficult to determine the precise function of PTMs residing in the DNA-histone interface. Using fully-synthetic and semi-synthetic protein ligation strategies in conjunction with the Ottesen Lab, we have
engineered and incorporated histones bearing precise PTMs into nucleosomes for biophysical characterization. Additionally we have employed analogs and amino acid substitution mimics of these PTMs commonly used in biophysical and genetic screening assays.

We find that PTMs located in the DNA-histone interface between the entry-exit region (where DNA enters and exits the nucleosome) and the Loss of Ribosomal Silencing (LRS) region 45 base pairs into the nucleosome function by controlling spontaneous, partial DNA unwrapping to increase DNA accessibility. Conversely, we find that PTMs located within the nucleosome dyad, which is the furthest point that DNA wraps into the nucleosomes, do not alter nucleosome unwrapping. Rather, these PTMs function to regulate DNA accessibility by increasing nucleosome mobility and decreasing nucleosome stability to facilitate disassembly. Additionally, we find that PTMs and DNA base pair sequence within the nucleosome entry-exit independently and additively regulate DNA unwrapping to enhance or suppress DNA accessibility. Moreover, we find that PTM mimics typically do not capture the biophysical function of the precise PTM. Finally, we find that PTMs in the DNA-histone interface function synergistically with the SWI/SNF chromatin remodelling complex and the MSH2/MSH6 DNA mismatch recognition complex to reposition and disassemble nucleosomes due to the reduction in DNA-histone binding and increased unwrapping imposed by the PTM. Taken together, our results suggest that PTMs in the DNA-histone interface can function independently or synergistically with other external factors to regulate transcription, replication, and DNA repair.
To the Poirier Lab. May there always be more questions than answers.
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INTRODUCTION

"Roads were made for journeys; not destinations."
— Confucius

0.1 Overview and Historical Perspective

When Anton van Leeuwenhoek (Netherlands, 1632-1723) and Robert Hooke (England, 1635-1703) recorded the first observations of biology at the microscopic level in the mid 1600’s, they made the fundamental discovery that all living organisms, from the complex to the simple, are comprised of sub-millimeter sized compartments that today we call "cells". In fact, it was the chamber-shaped organization of cork cells that reminded Hooke of the cell-like sleeping quarters of Celibate Monks to which he gave his discovery its name. Since then, cellular biology and microbiology have continuously striven to dissect the inner-workings of how cells function to grow, replicate, and survive. Fast forwarding 200 years, in the late 1800’s Walter Flemming (Germany 1843-1905) observed within the nucleus of eukaryotic cells thread-like substances that were darkly stained, which he termed chromatin (or Chromosomes; meaning "colored bodies") (Figure 1A). Flemming observed that chromatin was segregated between each daughter cell during cell division, which he published in his seminal work, Zellsubstanz, Kern und Zelltheilung ("Cell Substance, Nucleus and Cell Division", [1]). It was also during this time that the Augustinian Friar, Gregor Mendel (Austria-Hungary, 1822-1888), performed his ground-breaking work on the inheritance of characteristics in pea plants, which he published in 1866. Today, Mendel’s laws of inheritance are the foundation of modern genetics. However, unaware of each other’s work, neither Mendel nor Flemming
would realize the connection between chromatin and inheritance.

In a cascade of experiments throughout the early- to mid- twentieth century, inheritance was postulated to be transferred from parents to offspring by some physical particles termed "genes" (Hugo de Vries), possibly by chromosomes (Walter Sutton and Theodore Boveri). It was the ground-breaking work of Thomas Hunt Morgan (United States, 1866-1945) and his student Alfred Sturtevant that linked inheritance of genes to chromosomes (1910) and led to the development of the first genetic map (1913). However, during this time the chemical nature of genetic information was in serious debate. Some claimed that it was the weakly acidic nuclein molecules localized to the nucleus (nucleic acid; now known to be deoxyribonucleic acid), which were first observed and isolated by Johann Freidrich Miescher in 1869 (Sweden, 1844-1895) and determined to be the key component of chromatin by Eduardo Zacharias in 1881. Others disputed that it was protein in nature, since the 20 primary amino acid building blocks are more complex than the 4 nucleotide bases of DNA, and hence, more likely to store genetic information. It wasn’t until the work of Oswald Avery, Colin MacLeod, and Maclyn McCarty in 1944, which demonstrated deoxyribonucleic acid (DNA) was the basis of genetic transformation in bacteria, and the conclusive experiments of Alfred Hershey and Martha Chase in 1952, which demonstrated DNA is the molecule of genetic inheritance in viruses, that DNA was established as the molecule in which genetic information is stored and transferred.

It was here the 1950’s, when the DNA era of genetics took shape, that modern physics made significant inroads into determining the structure and inherent function of proteins and DNA (Figure 2). In 1951 Linus Pauling (1901-1994) and colleagues used X-ray diffraction of short amino acid peptides to determine the α-helix and β-sheet nature of protein secondary structure. Similarly, in 1950 Maurice Wilkins (1916-2004) and Rosalind Franklin (1920-1958) measured the X-ray diffraction pattern of crystallized DNA, which enabled James Watson (1928- ) and Francis Crick (1916-2004) to deduce the 3-dimensional structure of DNA. Again in 1960, Maurice Wilkins provided the first insights into the structure of chromatin using x-ray diffraction of purified chromatin fibers. He observed that chromatin possesses a repeated structural subunit that is not present in purified DNA or
DNA-associated proteins alone. In 1975, the first electron microscope image of chromatin was obtained by Ada Olins and Donald Olins to reveal that chromatin appears as a series of tiny particles attached to one another by thin filaments (Figure 1B). This “beads-on-a-string” appearance led to the suggestion that the beads are made of a protein core (histone proteins) and the thin filament connecting each bead is DNA. Today, we refer to each bead, along with its associated stretch of DNA as the "Nucleosome” and the short connecting DNA segments as "Linker DNA”.

While seemingly simple in nature, the tiny nucleosome is the essential structural and regulatory subunit of chromatin. While nucleosomes not only aid in structuring the approximately one meter length of DNA each human cell possesses inside the ∼10 micron diameter environment of the nucleus, nucleosomes impose an inherent level of regulation on the flow of the genetic information stored by the DNA itself. Nucleosomes control crucial functions of the genome, including gene expression, DNA synthesis, and DNA repair. Today, in the "genomics era” of genetic research, where genome wide DNA sequencing and nucleosome positioning technology allows high resolution mapping of where nucleosomes reside within gene and inter-genic regions of the DNA, our goal is to understand how each individual nucleosome functions to regulate the genetic process associated with the DNA surrounding it. This is not only because the DNA surrounding each nucleosome is different, but because the protein core of one nucleosome to the next varies as well. These variations as discussed shortly seemingly allow for the function of each nucleosome to be dynamically regulated. Our stretch of the road on this journey comprises a key question: how do both intrinsic nucleosome properties and extrinsic chromatin metabolic machinery, which alter and maintain chromatin, function together to regulate gene expression, DNA synthesis, and DNA repair? Determining these relationships is an essential piece of the genetics story that provides critical insight into genetic disorders, cancer, and many other diseases. In this work we will analyze how both DNA sequence and modifications to the nucleosome protein core function both independently and synergistically with proteins that remodel and disassemble chromatin to alter the accessibility of genomic DNA to gene transcription and DNA repair machinery.
Figure 1: **History of Chromatin** (A) Early mitotic cells stained for observing DNA. The dark thread-like structures in the nucleus are chromatin fibers [2]. (Image Courtesy Michael Poirier). (B) Electron micrograph of chicken erythrocyte chromatin showing a "beads-on-a-string" structure for extended chromatin fibers. Each bead is a nucleosome and the short stretches between them are linker DNA [3]. Figure reproduced under: Science Magazine License Number: 3042070983684. Date: Dec 04, 2012. Olins A.L. and Olins D.E. *Spheroid chromatin units (v bodies)*. Science. 25 January 1974 183(4122): 330-332. DOI: 10.1126/science.183.4122.330
0.2 Biology of the Cell

At the core of all genetic cellular operations is the double-helix structured deoxyribonucleic acid (DNA) (Figure 2A). DNA is an anti-parallel, double stranded deoxyribose sugar-phosphate polymer of only 2nm in diameter containing specific sequences of four nucleotide bases, guanine (G), adenine (A), thymine (T), and cytosine (C). Between the two anti-parallel strands, each A base of one strand pairs with a corresponding T base on the other strand via two hydrogen bonds, and similarly, G pairs with C via three hydrogen bonds (Chargraff’s rules). Particular stretches of the DNA nucleotide sequence encode for genes (See Overview Above) that control the operations of every cell, including cell growth, division, differentiation, and repair. In the ”Central Dogma” of molecular biology (Figure 2) information contained within the genes sequentially flows between information-carrying polymers. First, the DNA sequence encoding for a specific gene is copied into a single-stranded version called messenger RNA (mRNA). mRNA is a ribose sugar-phosphate polymer containing the same nucleotide sequence information as DNA using the four nucleotide bases guanine (G), adenine (A), uracil (U, replaces T), and cytosine (C). This allows for the information originally contained within the DNA to be further processed while minimizing the risk of detrimentally altering the DNA itself. Finally the mRNA is used as a template for protein synthesis in a process called translation. Every three nucleotide bases of the mRNA molecule constitutes a codon which codes for one of twenty amino acids, the building blocks of protein (Figure 3). A large RNA-protein complex called the ribosome systematically reads each codon along the mRNA molecule and incorporates each corresponding amino acid in a step-wise fashion to a growing amino acid peptide chain. This peptide chain then folds into higher order structures and may form complexes with other folded peptides to form the final functioning protein for which one or more genes encoded. After translation these proteins can be further modified and processed, including chemical alterations (post-translational modifications) to the amino acid residues. In the context of the histone proteins that comprise chromatin, it is just such modifications, their physical effects on nucleosomes, and their biological roles that are the focus of this work.
0.3 Nucleosomes Structure and Function

Our entire genome is organized into chromatin by wrapping stretches of DNA approximately ~1.7 times around a histone octamer protein core to form nucleosomes at periodic intervals [4, 5]. This DNA-protein complex contains 2 copies of each core histone protein: H2A, H2B, H3 and H4; and ~146 base pairs of DNA [6, 7] for which the x-ray crystal structure is well known (Figure 4A, [8, 9]). Nucleosomes are connected to each other by about 10 to 50 base pairs of linker DNA depending on the species, and they tend to be spaced in integrals of 5+10n base pairs between each other [10, 11]. Nucleosomes are positioned throughout the genome [12, 13] in long strings that compact into dynamic, higher-order structures [14, 15], which are proposed to be organized into solenoids of nucleosomes, creating a fiber of 30nm in diameter (Figure 5) [16–22]. However, the precise higher-order arrangement of nucleosomes is still unresolved [23].
Figure 3: **Amino Acid structures.** Names, 3-letter abbreviations and single letter code of all 20 primary amino acids. Image courtesy of New England Biolabs website: http://www.neb.com
The nucleosome structure contains distinct regions that are integrally related to its function (Figure 4). The following definitions and nomenclature will be extensively used through the course of this work, and are central to understanding the inherent function of nucleosomes. In regards to the histone proteins, each histone contains a well conserved ∼70 amino acid "histone-fold" motif of three α-helices connected by short loops near the C-terminal end [9]. Histones H2A and H2B associate to form the H2A/H2B dimer (Figure 4C). Similarly, histones H3 and H4 associate to form dimers that further associate to form H3/H4 tetramer via extensive interactions between the C-terminal α-helix of each H3 protein (Figure 4B). One H3/H4 tetramer and two H2A/H2B dimers associate via H2B-H4 and H2A-H3 interactions to form the complete histone octamer. The DNA associates with the histone octamer lateral surface via numerous ionic and hydrogen bond interaction, a majority of which reside within the histone-fold motifs. These interactions function to wrap and stabilize the DNA around the histone octamer, and experiments estimate that DNA-histone interactions stabilize the nucleosome structure by 40-50 k_BT, or 24-30 kcal/mol ([24, 25], k_B is the Boltzmann constant and T is temperature). Both the exposed face of the histone octamer and the histone tails that extend beyond the DNA-histone interface function to further stabilize the nucleosome and/or mediate inter-nucleosomal interactions for higher-order chromatin folding [21, 26, 27].

The nucleosome DNA-histone interface can be divided into three distinct regions: (A) the Entry-Exit region, (B) Dyad region, and (C) Loss of Ribosomal Silencing region (Figure 4). The entry-exit region is where the DNA first wraps in and out of the nucleosome and is the most accessible region of nucleosomal DNA [28, 29]. Nucleosomes themselves are inherently dynamic, by which DNA wrapped into the nucleosome spontaneously, partially unwraps to temporarily expose the DNA that was buried within the nucleosome [29–32]. The equilibrium between the partially unwrapped state and the fully wrapped state is termed site-exposure, and DNA in the entry-exit region is accessible to protein binding about 2 to 5 percent of the time [29, 30]. This is the key intrinsic mechanisms by which nucleosomal DNA is made accessible to extrinsic chromatin regulatory proteins. Conversely, the Dyad is the furthest point by which the DNA wraps into the nucleosome, and hence, is the least
accessible with site exposure probabilities of $\sim 10^{-5}$ [30]. This region contains a large number of interactions between the histone surface and the DNA [33], and is where the H3/H4 tetramer is first loaded onto the DNA, making it essential for nucleosome assembly and stability. Finally, the Loss of Ribosomal Silencing (LRS) region is highly similar in structure to the Dyad region [34]. It is located between the entry-exit and dyad regions about 40-50 base pairs into the nucleosome. Amino acid substitutions within each of these regions have shown to alter transcription regulation and DNA repair in yeast genetic studies [34]. It has been proposed that alterations in these regions function by perturbing DNA-histone interactions to alter the stability and dynamics of nucleosomes to chromatin regulatory factors [35, 36]. Our goal is to understand the physical nature by which alterations to the DNA-histone surface physically function both intrinsically and synergistically with extrinsic chromatin regulatory factors to alter nucleosome structure and DNA accessibility.

Figure 4: **Nucleosome Structure.** (A) Nucleosome crystal structure [37] of 146 bases of DNA wrapped around the histone octamer. DNA in gray, H3 in blue, H4 in green, H2A in yellow, and H2B in red. Entry-exit region (base pairs 1-10), LRS region (base pairs 40-50) and dyad (base 73) are indicated. (B) DNA base pairs of the entry-exit region (orange) and surrounding the dyad region (cream) associated with the H3/H4 tetramer. (C) DNA base pairs associated with the H2A/H2B dimers (orange). PDB ID: 1KX5.
Figure 5: **Model of Chromatin Compaction.** Nucleosomes assembled onto genomic DNA associate with one another to form solenoid-like chromatin fibers of 30nm in diameter. Chromatin is further structured and compacted inside the cell nucleus. (Drawing courtesy Kristi North)
0.3.1 Chromatin modification and accessibility

The organization of DNA within chromatin has long been thought to limit access to DNA sites. Modulation of chromatin structure in order to dynamically regulate access to DNA, and thus regulate cellular processes such as RNA transcription, is thought to occur by two primary mechanisms: reorganization of nucleosomes by chromatin remodeling factors [38] that function to reposition and/or disassemble nucleosomes from DNA, and chemical modifications to histone proteins (post-translational modifications) [35, 39]. Histone post-translational modifications (PTMs) are critical for many cellular processes such as regulation of transcription, DNA replication, and DNA repair. However, the molecular functions these modifications play in such processes are not well-understood.

Until recently, studies have focused on the large number of PTMs on the unstructured and exposed N-terminal tail domains of the core histone proteins (Figure 4) [40–42]. These modifications are known to alter chromatin structure [43] by decompacting folded chromatin fibers (Figure 5) [44] and to serve as sites for the binding of non-histone proteins [45] that function to regulate gene activation for RNA transcription. These PTMs are typically studied in vivo with amino acid substitutions that mimic the modified residues [46–48] and in vitro by the removal of histone tails [49, 50]. While these studies have been vital in determining the importance of tail modifications, they don’t reproduce the precise molecular structure of a specific modification. Recently, techniques such as Expressed Protein Ligation (EPL), which combines the control of solid phase peptide synthesis with the advantages of cellular protein expression, have proven to be powerful tools for the introduction of precise PTMs into proteins that still maintain the native amino acid sequence [51–53]. In the histone field, this has been used to introduce chemical modifications into the N-terminal tails of several core histones [54–57]. Significantly, studies using EPL were used to demonstrate that the acetylation of H4 Lysine 16 functions by decompacting chromatin [44], enhancing chromatin remodelling activity of the RSC complex [57], and inhibiting the activity of the ACF remodelling complex [58].
0.3.2 Histone Post-Translational Modifications of the DNA-histone interface

Several years ago, mass spectrometry revealed that there are over 30 modified amino acid residues in histone fold regions of the nucleosome core [59–61]. Unlike the unstructured tail regions that extend beyond the nucleosome, the histone fold domains have a well-defined structure that is likely to be functionally altered by post-translation modifications [47, 62]. This work is focused on PTMs that reside between the lateral histone surface and the DNA that wraps around it (Figure 6). These PTMs are buried within the DNA-histone interface, rendering them seemingly inaccessible. Therefore, in order for them to serve as binding sites for regulatory proteins, nucleosomes must be physically altered to expose these PTMs. An alternate hypothesis is that these PTMs function to directly alter the physical structure and/or dynamics of individual nucleosomes (Figure 7). PTMs in the DNA-histone interface are poised to disrupt DNA-histone interactions involved in nucleosome binding and DNA wrapping to regulate DNA accessibility [35]. Additionally, many extrinsic factors, including numerous chromatin remodelers and other chromatin regulatory proteins can exploit nucleosome unwrapping [30, 63, 64] to reposition and disassemble nucleosomes to expose nucleosomal DNA.

While PTMs to all four histones are found within the DNA-histone interface, this work focuses on Lysine acetylation/methylation and Threonine/Serine/Tyrosine phosphorylation of the H3 and H4 histone proteins (Figure 8). Such PTMs of H3 and H4 are found in all three DNA-histone interface regions (entry-exit, LRS, and dyad), and the genetics of which are better understood than for H2A and H2B PTMs. Therefore, the study of H3/H4 PTMs provides a strong rationale for understanding the integral relationship between the physical effects of histone acetylation and phosphorylation within the DNA-histone interface on nucleosome structure/dynamics and their biological role in regulating gene expression, DNA synthesis, and DNA reparation. The four modifications in the nucleosome dyad region are acetylation of H3 Lysine 115 [H3(K115ac)], phosphorylation of H3 Threonine 118 [H3(T118ph)], acetylation of H3 Lysine 122 [H3(K122ac)], and phosphorylation of H4 Serine 47 [H4(S47ph)]
These modifications occur near by each other (Figure 6) and are involved in transcription regulation and DNA repair \cite{46, 59}. These residues are found both individually and simultaneously modified \cite{60}, which suggests that multiple modifications may function in a coordinated manner. In the LRS region, H4 Lysines 77 and 79 are found simultaneously acetylated $[\text{H4(K77ac), H4(K79ac)}]$ \cite{60}, and are implicated in regulation of ribosomal RNA expression \cite{34, 65}. Additionally, H3 Serine 86 can be phosphorylated $[\text{H3(S86ph)}]$ in the LRS region as well \cite{66}. The three modifications in the entry-exit region are phosphorylation of H3 Tyrosine 41 $[\text{H3(Y41ph)}]$, phosphorylation of H3 Threonine 45 $[\text{H3(T45ph)}]$, and acetylation of H3 Lysine 56 $[\text{H3(K56ac)}]$ \cite{67–69}. Just outside of the H3 $\alpha$-N helix where the tail comes up between the entry-exit and dyad DNA gyres, H3 Lysine 36 can be trimethylated $[\text{H3(K36me3)}]$ as well \cite{70}. Each of these PTMs are directly involved in transcription \cite{67, 70} and DNA synthesis dependnet DNA repair \cite{68, 71}. Save for H4(S47ph) and H3(S86ph), this study examines the physical effects of each of these modifications on nucleosome accessibility, mobility, and stability. Accordingly, we study the synergistic effects of these PTMs on nucleosome repositioning and disassembly by chromatin remodelling and DNA replication complexes.

### 0.4 Experimental Methodology

There are three critical aspects of this work that allow us to make biologically relevant contributions to the understanding of histone post-translational modifications within the DNA-histone interface. First, we construct nucleosomes and chromatin molecules with well-defined histone PTMs and/or amino acid substitution mimics. Most histone modification studies are carried out only using mutations that mimic modifications (Figure 8), which leaves open the possibility that the observed effects are not representative of actual modifications. Here, in collaboration with Ottesen and Colleagues, we have constructed synthetic histone proteins with Native Chemical Ligation and Expressed Protein Ligation \cite{51} that bear the precise modification to determine how each modification functions biologically. Second, we reconstitute single nucleosomes \cite{73} and nucleosome arrays \cite{74} using
Figure 6: **Histone Post-Translational Modifications within the DNA-histone interface.** (A) Face view of nucleosome structure [37] indicating location of H3 and H4 PTMs that reside in the DNA-histone interface: H3(K36me3), forest; H3(Y41ph), mustard; H3(T45E), royal; H3(K36ac), orange; H4(K77ac), yellow; H4(K79ac), cream; H3(S86ph), salmon, H4(S47ph), magenta; H3(T118ph), red; H3(K115ac), blue; H3(K122ac), teal. (B) Top view of nucleosome lateral surface of nucleosome in (A). (C) Nucleosome structure of H2A and H2B PTMs located within the DNA-histone interface. PDB ID: 1KX5
Figure 7: Mechanisms by which Nucleosomes can be altered to expose DNA. (A) Nucleosomes undergo spontaneous thermal fluctuations in which the DNA partially unwraps from the histone surface to expose a buried DNA site (blue hashed) and then rewraps [72]. (B) Nucleosomes, typically mediated by chromatin remodelers, can be repositioned along the DNA to expose a buried site. (C) Nucleosome, typically mediated by histone chaperones and other chromatin regulatory factors, can be disassembled to remove all or part of the nucleosome.
Figure 8: Types of Histone Post-Translational Modifications. While there are many different types of histone modifications, the three types studied in this work are shown here. (A) Acetylated Lysine and corresponding Glutamine mimic. (B) Trimethylated Lysine and corresponding Methionine mimic; can also be mono- and di-methylated. (C) Phosphorylated Serine, Threonine, and Tyrosine and corresponding Glutamic Acid mimic.
high affinity nucleosome positioning sequences. These sequences allow for the assembly of homogenously positioned nucleosomes [75], so we are able to investigate specific effects without being obscured by heterogeneity of the sample. Third, our experimental approach is to combine biochemical and biophysical studies that probe either equilibrium and/or dynamics of nucleosome and chromatin structure: restriction enzyme digestion, fluorescence resonance energy transfer, stopped flow fluorometry, nucleosome mapping and footprinting, histone affinity measurements, and AFM, chromatin remodeling assays, and nucleosome disassembly assays. Due to the specific synthesis strategies and nucleosome properties that each PTM imparts, variations of many of these experimental techniques were required to study each PTM. Therefore, for clarity the specific experimental procedures for the characterization of each PTM are summarized at the end of each corresponding chapter. For the interested reader, detailed master protocols are referenced and given in the appendices. Here in the introduction, we present the theory of the key biophysical assays employed in this work.

0.4.1 Histone Affinity Measures

In vivo nucleosome assembly and disassembly is primarily mediated by histone chaperones [76–78]. However, nucleosomes can also be assembled from purified DNA and histone octamer components [50, 79] in a process called ”Reconstitution”. Under high monovalent salt conditions, the monovalent ions screen DNA-histone interactions to prevent nucleosome formation. As the salt concentration is lowered, H3/H4 tetramer first deposits onto the DNA via numerous interactions between the DNA and histone lateral surface at the dyad. The H2A/H2B dimers then associate with the tetramer via H2B-H4 interactions to form the full nucleosome. During this process in which the salt concentration is gradually lowered, DNA and histone components form a dynamic equilibrium with formed nucleosomes. Once the salt concentration is low enough, conversion between nucleosomes and free components stops.

In ”Competitive Reconstitutions” of histone affinity measures developed by the Widom Lab [75, 80], a DNA sample of interest and a separate ”competitor” DNA are allowed to
compete for a limiting amount of histone octamer such that when under equilibrium conditions the fraction of free histone octamer is negligible. Separately, a reference DNA molecule is also competed against the pool of competitor DNA for limiting amounts of histone octamer. From this, the free energy of nucleosome formation, $\Delta G_{\text{nuc}}^{\text{sample}}$, of the sample DNA can be measured relative to the free energy of nucleosome formation for the reference DNA molecule, $\Delta G_{\text{nuc}}^{\text{reference}}$. This change in free energy, $\Delta \Delta G_{\text{nuc}}^{\text{sample}} = \Delta G_{\text{nuc}}^{\text{sample}} - \Delta G_{\text{nuc}}^{\text{reference}}$, is a direct measure of the relative affinity of the DNA-histone complex compared to the reference system. In biological terms, this measurement is an indicator of the relative stability of nucleosomes to chromatin regulatory complexes that reposition and disassemble nucleosomes. Note that due to unknown variables in the reconstitution process, the free energy of formation of the individual DNA sample or reference DNA cannot be determined. However, by measuring the relative free energy, the unknowns are accounted for.

The calculation is performed as follows, assuming that the amount of free histone octamer during and after reconstitution is negligible. First the equilibrium, $K_{\text{eq-sample}}^{\text{nuc}}$, between free sample DNA and nucleosomes containing the sample DNA is determined by electrophoresis methods (See Appendix E). From this we assume the corresponding equilibrium between free competitor DNA and competitor DNA within nucleosomes, $K_{\text{eq-comp}}^{\text{nuc}}$, is given by:

$$K_{\text{eq-comp}}^{\text{sample}} = \frac{1}{\left(\frac{[C]}{[H]-[D]}\left(1+K_{\text{eq-sample}}^{\text{nuc}}\right)\right)}$$

where $[C]$ is the concentration of the competitor DNA, $[D]$ is the concentration of the sample DNA, and $[H]$ is the concentration of the histone octamer used in the experiment.

The same is done for the competitive reconstitution containing reference and competitor DNA to determine $K_{\text{eq-reference}}^{\text{nuc}}$ and $K_{\text{eq-comp}}^{\text{reference}}$. The total change in free energy relative to the reference sample is then:
\[ \Delta \Delta C_{sample}^{nuc} = -K_B T \left[ \ln \left( \frac{K_{eq-sample}^{nuc}}{K_{eq-reference}^{nuc}} \right) - \ln \left( \frac{K_{eq-comp}^{sample}}{K_{eq-comp}^{reference}} \right) \right] \]  

(2)

where \( K_{\text{extrmB}} \) is the Boltzmann constant, and \( T \) is the temperature at which the experiments were conducted. For full details, limitations, and applications of this method see [75, 80]. In the context of histone PTMs we do not compare different DNA samples as above. Rather, we compare the competitive reconstitution of limiting amounts of histone octamer bearing a precise PTM as our "sample" in the presence of high affinity and competitor DNA to the competitive reconstitution of unmodified histone as our "reference" under the same conditions. By comparing the equilibrium reconstitution of sample nucleosomes relative to reference nucleosomes using equation 1.2 we can measure the change in free energy of nucleosome formation induced by the precise PTM for the high affinity DNA molecule.

0.4.2 Restriction Enzyme Measures of Nucleosome Accessibility

Restriction Enzymes are proteins that recognize and bind specific DNA sequences of 4 or more base pair in length. These enzymes can then cleave the phosphate backbone on both DNA strands to produce two separate DNA molecules containing either blunt ends or ends containing a single-stranded DNA overhang. The ability of restriction enzymes to cleave DNA makes them an ideal probe of DNA accessibility throughout the nucleosome [72, 81]. DNA containing a unique restriction enzyme (RE) site wrapped into the nucleosome can be cleaved with the RE to determine the apparent rate of digestion, \( k_{\text{RE nuc-app}} \) (Figure 9). In practice this is done by quantifying the kinetic evolution of digested DNA fragments resolved by electrophoresis techniques (see Appendix E). The same RE digestion is separately performed on naked DNA to determine the apparent rate of digestion, \( k_{\text{RE DNA-app}} \) in the absence of the nucleosome. For naked DNA the observed forward rate of digestion is solely dependent upon the forward and reverse rates of RE binding to the RE site, \( k_{23} \) and \( k_{32} \), and the rate of cleavage, \( k_{34} \) (Figure 9C). However, RE sites within DNA wrapped
into the nucleosome are only exposed for digestion a fraction of the time. This is regulated by the nucleosome site exposure equilibrium of the RE, $K_{eq}^{RE} = k_{12}^{RE} / k_{21}^{RE}$, which is the equilibrium between the partially unwrapped nucleosome state to expose the RE site and the fully wrapped nucleosome state in which the RE site is inaccessible. Additionally, it has been shown that the site exposure equilibrium for RE digestion is via DNA unwrapping mechanisms and not arising from nucleosome repositioning or disassembly [32].

![Figure 9: Mechanics of Restriction Enzyme Kinetics Measures.](image)

Following the methods of Baldwin and Shore [82] and Polach and Widom [81], we determine the apparent RE digestion rate of its DNA site, D, within the nucleosome:

$$k_{nuc-app}^{RE} = -\frac{1}{[D]} \frac{d[D]}{dt}$$  \hspace{1cm} (3)
Under the steady state assumption that the rate limiting step is exposure of the RE site for formation of the restriction enzyme - DNA substrate complex, then the apparent rate, \( k_{nuc-app}^{RE} \), is given by:

\[
\begin{align*}
  k_{nuc-app}^{RE} &= \frac{k_{34}[RE]}{K_M} \frac{k_{12}^{RE}}{k_{12}^{RE} + k_{21}^{RE} + \frac{k_{34}[RE]}{K_M}} \\
                    &= k_{nuc-app}^{RE}
\end{align*}
\]

(4)

where \([RE]\) is the restriction enzyme concentration, and

\[
K_M = \frac{k_{32} + k_{34}}{k_{23}}
\]

(5)

In the case that the site exposure equilibrium, \( K_{eq}^{RE} = \frac{k_{12}^{RE}}{k_{21}^{RE}} \), is small (\( \ll 1 \)), as is the case for nucleosomes [81], and if the cleavage of the exposed RE site occurs in the pre-equilibrium regime in which the concentration of the exposed RE site is \( \ll K_M \), then \([RE] \approx [RE]_o\), the total concentration of added restriction enzyme to the experiment, and the apparent digestion rate becomes:

\[
\begin{align*}
  k_{nuc-app}^{RE} &= \frac{k_{34}[RE]_o}{K_M} K_{eq}^{RE} \\
                    &= k_{nuc-app}^{RE}
\end{align*}
\]

(6)

If in a separate experiment the naked DNA is digested under the identical conditions save for possibly \([RE]_o\), then the apparent rate of DNA digestion is

\[
\begin{align*}
  k_{DNA-app}^{RE} &= \frac{k_{34}[RE]_o}{K_M} \\
                    &= k_{DNA-app}^{RE}
\end{align*}
\]

(7)
Comparing the two experiments yields:

\[ K_{RE}^{eq} = \left( \frac{k_{RE}^{nuc-app}}{k_{RE}^{DNA-app}} \right) \left( \frac{[RE]_{o-DNA}}{[RE]_{o-nuc}} \right) \]  

(8)

To determine the effect of post-translational modifications on site accessibility at different DNA positions within the nucleosome, we determine the equilibrium constant of site exposure for modified nucleosomes relative to that of unmodified nucleosomes:

\[ \frac{K_{RE}^{eq-modified}}{K_{RE}^{eq-unmodified}} = \frac{k_{modified-nuc-app}^{RE}}{k_{unmodified-nuc-app}^{RE}} \]  

(9)

The relative equilibrium constant does not depend on the rate of naked DNA digestion and the concentrations of restriction enzyme. Thus, we only need to determine the rate of nucleosomal DNA digestion with and without the post-translational modifications.

### 0.4.3 Fluorescence Resonance Energy Transfer Measures of Protein Binding within Nucleosomes

A complementary method for measuring nucleosome site accessibility is by fluorescence based detection of protein binding within a nucleosome [30, 31]. In this technique the DNA recognition sequence of a transcription factor protein is inserted into nucleosomal DNA such that when wrapped into the nucleosome, the site is blocked from transcription factor binding, but exposed for binding when the DNA partially unwraps (Figure 10A). From this, the site exposure equilibrium for nucleosome unwrapping to expose the site, \( K_{eq^{wrap}} = k_{12}/k_{21} \), can be determined [81].

In the case of LexA binding to naked DNA, the affinity or dissociation constant, \( K_{D-DNA} \), of transcription factor binding to its site within naked DNA is given by:
\[ K_{D-DNA} = \frac{k_{23}}{k_{32}} \]  

(10)

However, for the nucleosome the apparent affinity or dissociation constant of the transcription factor binding to its site within the nucleosome, \( K_{D-nuc}^{app} \), is given by:

\[ K_{D-nuc}^{app} = K_{D-DNA} \left(1 + \frac{1}{K_{eq}^{wrap}}\right) \approx \frac{K_{D-DNA}}{K_{eq}^{wrap}} \]  

(11)

in the limit that \( K_{eq}^{wrap} \ll 1 \) as is the case for nucleosomes.

By independently measuring the affinity of the transcription factor to naked DNA and to its site when wrapped into the nucleosome, the site exposure equilibrium of transcription factor accessibility within the nucleosome is determined:

\[ K_{eq}^{wrap} = \frac{K_{D-DNA}}{K_{eq}^{app}} \]  

(12)

For histone post-translational modifications, we can directly compare the observed binding affinities of the transcription factor to modified and unmodified nucleosomes to obtain the relative site exposure equilibrium:

\[ \frac{K_{eq}^{wrap}_{\text{modified}}}{K_{eq}^{wrap}_{\text{unmodified}}} = \frac{K_{D-DNA}^{app}_{\text{modified}}}{K_{D-DNA}^{app}_{\text{unmodified}}} \]  

(13)

The relative equilibrium constant does not depend on the affinity of the transcription factor
to naked DNA. Thus, we only need to determine the apparent binding of the transcription factor to its site within nucleosomes with and without post-translational modifications.

To monitor transcription factor binding within the nucleosome, we monitor the relative distance between the DNA near the transcription factor binding site and the surface of the histone octamer using Fluorescence Resonance Energy Transfer (FRET, Figure 10). A donor fluorescent molecule (D) is placed on the DNA while an acceptor fluorescent molecule (A) is placed on the histone octamer. FRET is a dipole-dipole interaction between the two fluorescent molecules in which energy from the donor fluorophore is non-radiatively transferred to the acceptor. The two requirements for FRET are (1) the energy needed to excite the acceptor is the same as that emitted by the donor, and (2) there is energetic coupling between the two molecules [83]. The first criterion is met if there is sufficient overlap between the emission spectra of the donor and excitation spectra of the acceptor. The second criterion is dependent upon the distance and orientation between the two dipoles. The rate of energy transfer is [83]:

$$k_{FRET} = k_D \left(\frac{R_o}{R}\right)^6, \quad R_o^6 = (8.785 e^{-25})Q_D \kappa^2 \eta^{-4} J \text{ (cm}^6\text{)}$$  \hspace{1cm} (14)$$

where $k_D$ is the rate of donor fluorophore emission in the absence of the acceptor, $R$ is the scalar distance between the donor/acceptor pair, and $R_o$ is the Förster radius where $Q_D$ is the quantum yield of the donor, $\kappa$ is the average orientation factor between the two dipoles, $\eta$ is the index of refraction of the media, and $J$ is the amount of overlap between the donor emission and acceptor excitation spectra. If the donor and acceptor are free to rotate, the molecular rotational correlation time is on the order of $10^{-10}$s, allowing the two dipoles to average over all orientations during the donor excitation lifetime of $\tau_D \sim 10^{-8}$s. This results in $\kappa^2 = 2/3$. If the donor and acceptor molecules are not free to rotate, then $\kappa^2$ can range from 0-4 [83]. As the rate of energy transfer is sensitive to the spatial proximity of the two fluorescent molecules, it is a highly versatile tool for monitoring inter- and intra-molecular
interactions in biomolecules.

In practice, the FRET efficiency of energy transfer, $E$, is typically measured, which is given by:

$$E = \frac{k_{FRET}}{k_D + k_{FRET}} = \frac{1}{1 + \left(\frac{R_o}{R_E}\right)^6}$$  \hspace{1cm} (15)

Measurement of $E$ can be performed by multiple methods in which various aspects the donor and acceptor fluorescence emission spectra are analyzed (see [83] for more details). In this work we use the (Ratio)$_A$ method [83] to accurately measure the FRET efficiency, which is given by:

$$E = 2[\epsilon_A(\lambda'')F_A(\lambda', \lambda_D)/F_A(\lambda'', \lambda_A) - \epsilon_A(\lambda')] / [\epsilon_D(\lambda')d^+]$$  \hspace{1cm} (16)

where $\lambda'$ is the wavelength of light at which the donor (D) fluorophore is excited, and $\lambda''$ is the subsequent wavelength of light at which the acceptor (D) fluorophore is directly excited at. The prefactor of 2 reflects the presence of two acceptor molecules on the histone octamer (due to two copies of each histone) per donor molecule on the DNA. $F_A(\lambda', \lambda_A)$ is the fluorescence emission of the acceptor at $\lambda_A$ when the donor is excited at $\lambda'$. $F_A(\lambda'', \lambda_A)$ is the fluorescence emission of the acceptor at $\lambda_A$ when directly excited at $\lambda''$. $\epsilon_D(\lambda')$, $\epsilon_A(\lambda')$, $\epsilon_A(\lambda'')$ are the molar extinction coefficients of the donor (D) and acceptor (A) molecules at $\lambda'$ and $\lambda''$. $d^+$ is the fractional labeling of the donor molecule.

0.5 Outline and Summary of Results

The first underlying principle that this work reveals is that PTMs in the DNA-histone interface function by regulating spontaneous, partial DNA unwrapping to facilitate DNA accessibility and/or decreasing nucleosome stability for regulating nucleosome mobility and
Figure 10: **Kinetic model of Transcription Factor Binding.** (A) Transcription factor (blue sphere) binding to its DNA recognition sequence (blue hashes) within naked DNA. (B) Transcription factor binding to its DNA recognition sequence when wrapped into the nucleosome. The nucleosome is labeled with a FRET fluorophore pair with the donor fluorophore (green) attached to the DNA and the acceptor fluorophore (red) attached to the histone surface at H2A(K119C). Relative changes in the average distance between the two fluorophores causes a measurable difference in FRET efficiency.
assembly/disassembly. We find that there are two distinct regions of the DNA-histone interface. As shown Chapters 1-2, PTMs in the nucleosome dyad region control nucleosome mobility and stability without directly impacting DNA unwrapping. These results suggest that alterations in the dyad region create nucleosomes that are poised for disassembly or remodeling without increasing DNA unwrapping. Additionally, in Chapter 3 we show that H3(T118ph) potentially alters nucleosome assembly pathways to form alternate DNA-histone structures that significantly change DNA accessibility and chromatin structure. Conversely, in Chapter 4 we demonstrate that PTMs between the DNA entry-exit region and the Loss of Ribosomal Silencing region enhance partial DNA unwrapping. This creates nucleosomes that on average are more accessible to regulatory proteins that rely on thermal DNA unwrapping fluctuations to access DNA sites buried within the nucleosome.

The second principle this work reveals is that PTMs of the DNA-histone interface can function synergistically with other intrinsic and extrinsic chromatin factors to regulate nucleosomes accessibility and disassembly. Within the DNA entry-exit region, we observe that DNA sequence and PTMs function independently and additively to regulate DNA unwrapping (Chapter 5). In Chapter 6 we determine that the homologous DNA repair protein, RAD51, disassembles nucleosomes by iteratively trapping DNA unwrapping fluctuations to unwrap nucleosomes. In Chapter 7 we observe that the chromatin remodelling complex, SWI/SNF, exhibits enhanced repositioning and disassembly of nucleosomes bearing PTMs within the nucleosome dyad. Similarly, PTMs both in the DNA entry-exit and dyad regions enhanced nucleosomes disassembly (Chapter 7) by the DNA-mismatch recognition complex, HsMSH2-HsMSH6. Further experiments will be required to determine if these PTMs also function synergistically with Rad51 to disassemble nucleosomes. Taken together our results suggest that Rad51 and other extrinsic regulatory proteins that function by utilizing inherent nucleosome fluctuations will be synergistically regulated by PTMs in the DNA-histone interface.

As a whole, these studies embody a significant advancement in our understanding of core histone post-translational modifications of histones H3 and H4 that lie within the DNA-histone interface. PTMs of H2A and H2B that reside in the DNA-histone interface are
located near the entry-exit and LRS regions that define the boundaries of the unwrapping zone. Our results suggest that these H2A/H2B PTMs will function similarly to increase nucleosome accessibility. Moreover, the PTMs studied in this work are integrally involved or implicated in numerous genomic processes as discussed in each chapter. Our results indicate that each of these PTMs may carry out their observed role in the regulation of gene transcription, DNA repair, and DNA synthesis by directly altering the inherent structure and dynamics of nucleosomes. However, since most in vivo studies that characterize the role of these PTMs use amino acid substitution mimics, further studies are required to determine the precise role of each of these PTMs in vivo.
Chapter 1

ACETYLYATION OF HISTONE H3 AT THE NUCLEOSOME DYAD ALTERS DNA-HISTONE BINDING

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Acetylation of histone H3 at the nucleosome dyad alters DNA-histone binding
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1.1 Introduction

The nucleosome dyad is a distinct region that encompasses several key interactions that include essential protein-protein contacts at the H3/H4 tetramer interface and histone-DNA contacts at the center of the positioned DNA sequence [9]. The DNA-histone interface at the nucleosome dyad contains only four amino acid residues that are known to have post-translational modifications (PTMs): phsophorylated H4 Serine 47 [H4(S47ph)], acetylated H3 Lysine 115 [H3(K115ac)], phosphorylated H3 Threonine 118 [H3(T118ph)], and acetylated H3 Lysine 122 [H3(K122ac)] [60, 61, 66]. Of these four residues, Lysines 115 and 122 [H3(K115) and H3(K122)] were found to be acetylated, either simultaneously or individually. Lysine acetylation both reduces the positive charge of the histone octamer surface
and increases the steric bulk of the residue. The H3(K115) and H3(K122) residues are positioned between two histone-DNA binding motifs at the nucleosome dyad (Figure 1.1). The side chain amines of these Lysine residues are poised for electrostatic interactions with the DNA backbone. Because of the inherent nucleosome symmetry, two copies of each amino acid exist in close spatial proximity. Simultaneous modifications at these Lysine residues would therefore create a patch of four histone modifications that could alter a large surface area of DNA-histone interactions in the assembled nucleosome [9].

Figure 1.1: **H3 Lysines 115 and 122 are buried beneath the DNA at the nucleosome dyad.** (A) Face view of the nucleosome [9]. H3 residues 1-109 are shown in yellow; residues 110-135 are shown in red. H3(K115) is shown in blue spheres and H3(K122) is shown in teal spheres. (B) Top view of the nucleosome, and (C), close-up view of the nucleosome dyad at 45° angle relative to (B), illustrates that H3(K115) and H3(K122) are positioned to interact with the DNA phosphate backbone. PDB ID: 1KX5 (Reproduced with permission)

Yeast genetic screenings show that mutations H3(K115Q) and H3(K122Q), which mimic constitutively acetylated lysine, reduces transcriptional silencing at ribosomal DNA (rDNA) and telomeric loci [46]. H3(K115R), which mimics constitutively unacetylated Lysine, exhibits wild type silencing in both rDNA and telomeric regions. However, H3(K122R) ex-
hibits wild type silencing in ribosomal DNA but reduced silencing in telomeres [46]. Another study showed that H3(K115A) and H3(K122A), which essentially eliminates the lysine residue, separately displays reduced expression of the PHO5 gene in budding yeast, while H3(K122Q) exhibited wild-type expression [84]. DNA repair also appears dependent on H3(K115) and/or H3(K122) acetylation. H3(K115Q) increases yeast sensitivity to hydroxyurea, while H3(K115R) does not [46]. Moreover, H3(K115A), H3(K122A) and H3(K122Q) were all shown to be highly sensitive to Zeocin, a DNA double strand break (DSB) mimetic [84]. These results suggest that the modification state of the Lysine residue is important for transcription regulation and DNA repair. However, whether the Lysine →Glutamine mutations capture the entire features of Lysine acetylation is a significant unknown.

Residues H3(K115) and H3(K122) are also of interest as they are located near two SWI/SNF-independent (SIN) histone mutations, H3(R116H) and H3(T118I) [85]. SIN alleles of histones are isolated point mutations that partially alleviate the requirement of the SWI/SNF ATP-dependent chromatin remodeling complex for transcriptional activation of a subset of yeast genes [86]. Thermal repositioning assays show that H4(R116H) and H3(T118I) increase the rate of nucleosome repositioning following thermal heating [87, 88] and show reduced DNA-histone interactions [87, 89].

Given that acetylation of H3 Lysine 115 [H3(K115ac)] and/or H3 Lysine 122 [H3(K122ac)] is poised to alter DNA-histone contacts and that mutations to these and nearby residues alter gene expression, DNA repair, and nucleosome structure, these histone PTMs may function to alter nucleosome dynamics. They may enhance nucleosome mobility or "sliding" of nucleosomes between different positions along the DNA, often with the assistance of chromatin remodeling factors [35]. Therefore, DNA regulatory sites might be significantly more exposed as a result of such nucleosome movement [90]. Alternatively, H3(K115ac) and H3(K122ac) may alter transient nucleosome unwrapping fluctuations [28, 30, 81] that expose buried DNA regulatory sites within the nucleosome. Reduction of DNA-histone binding interactions by these PTMs may modify the dynamics of the nucleosome structure and alter the proportion of DNA that is transiently unwrapped and accessible to regulatory factors.
In order to examine the precise role of Lysine acetylation at the nucleosome dyad, full-length histone H3 containing acetylated Lysine 115 [H3(K115ac)], acetylated Lysine 122 [H3(K122ac)] or both acetylated Lysines 115 and 122 [H3(K115ac,K122ac)] were synthesized by Ottesen and colleagues using expressed protein ligation (EPL) [56, 91, 92]. For more information on EPL, synthesis of H3(K115ac) and/or H3(K122ac), and recent advancements in ligation strategies see the following resources [51, 93]. Purified histone octamers bearing H3(K115ac) and/or H3(K122ac) were then reconstituted with a well-defined nucleosome positioning sequence (NPS). An NPS is an ~147 base pair sequence of DNA that positions a nucleosome to one or a few locations along the DNA. These studies indicate that nucleosome positions on a well-defined NPS is not altered by H3(K115ac) and/or H3(K122ac). Additionally both transcription factor binding and restriction enzyme kinetic studies (See Chapter: Introduction) suggest that dynamic DNA site accessibility to regulatory factors is not altered by the Lysine modifications. However, competitive reconstitution analysis (See Chapter: Introduction) suggests that these modifications reduce the free energy of binding between the histone octamer and DNA substrates. Additionally, the H3(K122ac) and H3(K115ac,K122ac) nucleosomes demonstrate increased mobility during thermally induced repositioning. Interestingly, Lysine →Glutamine substitution of these residues does not alter DNA-histone binding or nucleosome mobility, suggesting that glutamine does not capture all the essential features of H3(K115) and H3(K122) acetylation. These results are consistent with the conclusion that modifications of the nucleosome dyad alter DNA-histone binding and control nucleosome mobility.
1.2 Results

1.2.1 H3(K115ac) and/or H3(K122ac) can be refolded into histone octamer

In order to synthesize full-length protein bearing H3(K115ac) and/or H3(K122ac), a truncated version of H3 containing residues 1-109 and a C-terminal thioester [H3(1-109)] is expressed and purified from E. coli and ligated with an excess of synthesized C-terminal H3(110-135) peptide [H3Pep] bearing the precise acetyl modification H3Pep(K115ac): CAIHAK(Ac)RVTIMPKDIQLARRIRGERA; H3Pep(K122ac): CAIHAKRVTIMPK(Ac)DIQLARRIRGERA; H3Pep(K115ac,K122ac): CAIHAK(Ac)RVTIMPK(Ac)DIQLARRIRGERA. X. laevis histone H3 naturally contains a Cysteine at residue 110. Ligation at this site creates a fully native H3 protein sequence bearing the precise acetylated Lysine modification. Due to details of the purification process, H3(1-109) is copurified with the full-length H3 protein (Figures 1.2-1.3) at purities of 50:50 to 70:30 full-length H3 to H3(1-109). However, H3(1-109) lacks the C-terminal helix that forms the essential protein-protein interface in the H3/H4 tetramer. When histone octamer is refolded from equimolar amounts of H2A, H2B, H3 + H3(1-109), and H4, only the full-length H3 is incorporated into histone octamer and the efficiency of refolding appears to be solely dependent on the full-length component of the H3 mixture.

Histone octamer was refolded using the wild-type unmodified H3, three Glutamine acetyl-lysine mimics H3(K115Q), H3(K122Q), H3(K115Q,K122Q), and three semi-synthetic acetylated H3 variants H3(K115ac), H3(K122ac), H3(K115ac,K122ac). Both SDS-PAGE (Figure 1.3) and MALDI-TOF mass spec analysis (Figure 1.2) demonstrate that the H3(1-109) fragment is completely eliminated from the histone octamer in each of the semi-synthetic preparations, and that the acetylation modifications survive purification. Interestingly, the fact that histone octamer is refolded and purified with either or both modifications suggests that H3(K115ac) and H3(K122ac) do not inhibit the formation of histone octamer.
Figure 1.2: Preparation of semi-synthetic histone octamers by expressed protein ligation. (A) EPL strategy for constructing histone H3 with acetylated K115 and/or K122. (B) SDS-PAGE analysis a representative H3(K115ac) preparation. Lane 1, molecular weight standard (MWS). Lane 2, cleavage of the H3(1-109)-Intein fusion protein to generate H3(1-109) thioester with 90% efficiency. Lane 3, H3Pep(K115ac) was added to the concentrated cleavage mixture, and overnight ligation yielded 60% full-length H3(K115ac). (C) MALDI-TOF mass spectrometry analysis of purified unmodified histone octamer: H3 observed m/z 15273, expected 15271. (D) MALDI-TOF MS of purified histone octamer containing H3(K115ac,K122ac): observed m/z 15362, expected 15355. H3(1-109) would occur at m/z 12285 if present. (Reproduced with permission)
1.2.2 H3(K115ac) and H3(K122ac) do not prohibit nucleosome formation

Nucleosomes were reconstituted with histone octamer containing H3(K115ac) and/or H3(K122ac) and DNA containing the high affinity nucleosome positioning sequence Mp2, a variant of the 601 sequence (Figure 1.4A) [94]. The reconstitutions were characterized by electrophoresis mobility shift assay (EMSA) on a 5% native polyacrylamide gel and by sucrose gradient centrifugation (Figure 1.4B-D). EMSA is sensitive to changes in the overall structure of the nucleosome, such as changes in the position of the nucleosome on the DNA sequence [87, 95, 96]. We observed the electrophoretic mobility of the nucleosomes with H3(K115ac) and/or H3(K122ac) to be similar to unmodified nucleosomes. Coordinate, the rate of nucleosome sedimentation through a sucrose gradient depends on the mass and shape of the molecule. Nucleosomes containing H3(K115ac) and/or H3(K122ac) sediment at the same rate as unmodified nucleosomes. These results suggest that the H3(K115ac) and/or H3(K122ac) modifications do not dramatically alter the nucleosome structure.
Figure 1.4: Gel shift and sucrose gradient characterization of nucleosomes reconstituted with H3 acetylated K115 and/or K122. (A) The two DNA constructs used throughout the experiments, which contain the altered 601 nucleosome positioning sequence Mp2. Mp2-192 is used in the competitive reconstitutions and the restriction enzyme experiments. Mp2-247 is used in the thermal shifting and exonuclease III experiments. (B) PAGE analysis of seven different nucleosomes reconstituted with Mp2-247 prior to purification; each lane is labeled with the histone octamer used. The two bands represent two nucleosome positions. (C) PAGE gel shift characterization of nucleosomes reconstituted with Mp2-247 DNA and purified by sucrose gradient fractionation to remove free DNA. (D) Elution from the sucrose gradient was visualized by the Cy5 fluorescent label on the DNA. DNA in the absence of histone octamer is included as a control. (Reproduced with permission)
1.2.3 H3(K115ac) and H3(K122ac) reduces the free energy of DNA-histone binding

Nucleosome competitive reconstitutions (See Chapter: Introduction) performed by Alex Mooney examined the effect of H3(K115ac) and/or H3(K122ac) on the binding affinity of the histone octamer to DNA. Competitive reconstitutions were carried out as previously described [80] with the high affinity nucleosome positioning sequence Mp2 [94] in the presence of an excess of low affinity competitor DNA, which in this case was a 168 base pair fragment from the Amp\(^r\) gene in pUC19. Under these conditions the high-affinity DNA competes against the low-affinity DNA for a limiting amount of histone octamer, and thus, a dynamic equilibrium between free DNA and nucleosomes is established. The equilibrium constant, or "binding equilibrium", between nucleosomes and free DNA can be determined by EMSA on a 5% native PAGE [80]. Comparison of the binding equilibrium of specific histone octamer modifications to the binding equilibrium of the unmodified octamer determines the change in binding free energy or $\Delta \Delta G_{\text{nuc}}^{\text{K115ac}}$ of nucleosome formation. Competitive reconstitutions with octamer containing H3(K115ac), H3(K122ac), or H3(K115ac,K122ac) were performed at least three times, each in triplicate. In addition, for each semi-synthetic construct the reconstitution was repeated with histone octamer refolded from a separate EPL purification in order to confirm that the results were sample independent.

An example of a competitive reconstitution analysis is shown in Figure 1.5A. The background was first corrected for by subtracting from each pixel within a box drawn around a particular band the local median pixel value of the perimeter of that box. The $K_{eq}^{\text{nuc}}$ of nucleosome formation for each sample was then calculated from the ratio of gel-shifted nucleosomes to the free high-affinity DNA band. Variations in $\Delta \Delta G_{\text{nuc}}^{\text{ac}}$ due to differences in each round of reconstitutions were eliminated by determining the relative binding equilibrium of the acetylated sample versus the binding equilibrium of the unmodified reference used in each specific reconstitution only.

Alex found that H3(K115ac) induces a $\Delta \Delta G_{K115ac}^{\text{nuc}} = 0.4 \pm 0.2$ kcal/mol, H3(K122ac) induces a $\Delta \Delta G_{K122ac}^{\text{nuc}} = 0.2 \pm 0.2$ kcal/mol and the combination H3(K115ac,K122ac) in-
Figure 1.5: Competitive reconstitution experiments determine how acetylation or glutamine substitution at the dyad alters DNA-histone binding affinity. Native PAGE of a single reconstitution experiment using Mp2-192 DNA for unmodified (A) and H3(K115Ac,K122Ac) (B) nucleosomes. For each experiment, an unmodified reconstitution was included as a reference. (C) The ratio of the nucleosome band divided by the DNA band was used to determine the $K_{\text{eq}}^\text{nuc}$ of nucleosome formation for each modified sample and unmodified reference. Each experiment was repeated at least three times, each time in triplicate, such that each bar in plot C represents the relative equilibrium, $K_{\text{eq-\text{modified}}}^\text{nuc}/K_{\text{eq-\text{unmodified}}}^\text{nuc}$ over at least 9 data points. The error bars are the standard deviation. (Reproduced with permission)

Reduced a $\Delta\Delta G_{\text{K115ac,K122ac}}^\text{nuc} = 0.6 \pm 0.2$ kcal/mol (Figure 1.5B). These results demonstrate that the acetylation of H3(K115) and the simultaneous acetylation H3(K115ac,K122ac) reduce the free energy of histone octamer binding by 0.4 and 0.6 kcal/mol, respectively. The acetylation of K122 may also reduce the histone octamer affinity but appears unaffected within the measurement uncertainty.

1.2.4 Mutations that mimic H3(K115ac) and H3(K122ac) do not reduce DNA-histone binding free energy

Lysine to Glutamine substitution mutations are often used to mimic Lysine acetylation in vivo [59]. In fact, Lysine $\rightarrow$ Glutamine substitutions in yeast studies were used to demonstrate the importance of H3(K115) and H3(K122) in transcription regulation and DNA repair [46, 84]. In order to clarify the differential influences of charge and steric bulk for locations buried at the histone-DNA interface, Alex Mooney examined histone octamer containing H3(K115Q) and/or H3(K122Q) by competitive reconstitution analysis. He found
that the binding equilibrium, $K_{eq\text{-mutated}}$, of nucleosome formation for H3(K115Q) and/or H3(K122Q) was not altered relative to the binding equilibrium, $K_{eq\text{-unmodified}}$, for wild-type unmodified histone octamer (Figure 1.5B). These results demonstrate that the Lysine → Glutamine substitution does not fully replicate the role of Lysine acetylation. Moreover, it appears that Lysine acetylation plays a role beyond simple electrostatic effects in the stability of the nucleosome, and that the details of the side chain structure contribute as well.

**1.2.5 H3(K115ac) and H3(K122ac) do not alter DNA unwrapping within the nucleosome**

To investigate the influence of H3(K115ac) and/or H3(K122ac) on nucleosome unwrapping, restriction enzyme digestion kinetics assays (See Chapter: Introduction) were employed using unique restriction enzyme (RE) sites that are located throughout the Mp2 NPS (Figure 1.6A). This method quantifies site exposure for a particular RE site as the equilibrium constant, $K_{eq}^{RE}$, between the unwrapped and wrapped states of the nucleosome, which is proportional to the forward rate of RE digestion. The restriction enzyme kinetics studies were performed as previously described [81, 94] to determine relative site exposure equilibrium, $K_{eq\text{-modified}}^{RE}/K_{eq\text{-unmodified}}^{RE}$, for modified nucleosomes containing H3(K115ac) and/or H3(K122ac) compared to unmodified nucleosomes for a particular RE (Figure 1.6B-E).

We measured the forward rates of restriction enzyme digestion for sites throughout the nucleosome with and without H3(K115ac) and/or H3(K122ac) (Figure 1.6B-D). From this we determined the relative equilibrium, $K_{eq\text{-modified}}^{RE}/K_{eq\text{-unmodified}}^{RE}$, for H3(K115ac) and/or H3(K122ac) versus unmodified H3 at each RE site (Table 1.1).

The HhaI site, which is located directly above residues H3(K115) and H3(K122) at the midpoint of the wrapped DNA sequence, might be anticipated to exhibit directly altered site exposure by the acetylation of these residues. However, the $K_{eq\text{-rel}}^{HhaI}$’s for H3(K115ac), H3(K122ac) and H3(K115ac,K122ac) show no measurable change in HhaI digestion rate compared to unmodified nucleosomes (Table 1.1 and Figure 1.6). This result implies that there is no significant increase in the site exposure near the acetylated Lysine residues.
Figure 1.6: Restriction enzyme kinetics determine the site accessibility of DNA throughout the nucleosome. (A) Crystal structure of the nucleosome [37] showing the location of the restriction enzyme sites (red), H3(K115) (blue), and H3(K122) (teal); PDB ID: 1KX5. Representative PAGE analysis of TaqαI digestion with unmodified (B) or H3(K115Ac,K122Ac) (C) nucleosomes reconstituted with Mp2-192. Lanes are labeled with the time in minutes at which a time point was quenched. (D) The bands from each gel were quantified, and the course of the reaction was plotted as the fraction of DNA remaining uncut. Shown are HindIII digestion of unmodified (squares) and H3(K115ac,K122ac) (circles) nucleosomes, and TaqαI digestions of unmodified (inverted triangle) and H3(K115ac,K122ac) (triangle) nucleosomes. (E) Digestions were carried out at six different restriction sites, for unmodified and acetylated nucleosomes, in at least duplicate. The plot illustrates the average relative $K_{RE}^{eq}$ for H3(K115ac) and/or H3(K122ac) nucleosomes relative to unmodified at each restriction site; error bars represent standard deviation. Each bar is placed at the relative position of the restriction site along the DNA sequence.
### Table 1.1: Relative rates of RE accessibility for H3 acetylated K115 and/or K122 compared to unmodified nucleosomes for each indicated restriction enzyme site. See 1.6 for RE location within DNA sequence.

<table>
<thead>
<tr>
<th>RE</th>
<th>$K_{eq-rel-K115ac}^{RE}$</th>
<th>$K_{eq-rel-K122ac}^{RE}$</th>
<th>$K_{eq-rel-K115ac/K122ac}^{RE}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PstI</td>
<td>-</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>HindIII</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>HaeIII</td>
<td>1.3 ± 0.3</td>
<td>0.9 ± 0.5</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>TaqI</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>HhaI</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.4</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>PmlI</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

Interestingly, the HindIII site exhibits an increase rate of digestion for H3(K122ac), and H3(K115ac,K122ac) compared to unmodified nucleosomes (Table 1.1 and Figure 1.6). These results are consistent with an increased site exposure for H3(K122ac) and H3(K115ac,K122ac). However, the HindIII site is located about 4nm from the acetylated Lysine residues, making it unlikely that these modifications directly alter the DNA contacts that control DNA unwrapping in the vicinity of the HindIII site. Additionally, the PstI and HaeIII sites, which are located in the entry/exit region 5 base pairs before and 15 base pairs after the HindIII site, respectively, do not show any change in site exposure. This indicates that the observed increase in site exposure of the HindIII site may be due to small differences in transient nucleosome repositioning undetectable with the PstI site that would place the HindIII site outside of the nucleosome, or due to the details of how the HindIII restriction enzyme binds to its site. From these results we conclude that site exposure is not altered in nucleosomes containing H3(K115ac) and/or H3(K122ac).

### 1.2.6 (K115ac) and H3(K122ac) do not facilitate transcription factor binding within the nucleosome entry-exit

We also investigated the influence of H3(K115ac,K122ac) on transcription factor protein binding (See Chapter: Introduction) to a DNA target site buried within the nucleosome as
previously reported [30, 31, 97, 98]. We prepared the 601L-end DNA molecule, which contained a 147 base pair 601 nucleosome positioning sequence with a LexA binding site located between base pairs 8-27 and Cy3 labeled to the 5′ end of the first base pair (Figure 1.7A, 601L-end). 601L-end was then reconstituted into histone octamer containing Cy5-labeled H2A(K119C) (Figure 1.7B) and either unmodified H3 or H3(K115ac,K122ac). Upon reconstitution, the Cy3 and Cy5 molecules are juxtaposed at the nucleosome entry-exit region to produce a measurable FRET efficiency. Following reconstitution, nucleosomes were purified by sucrose gradient. Both nucleosomes containing unmodified H3 and H3(K115ac,K122ac) have the same initial fret efficiency (E$_{0-\text{unmod}}$ = 0.61 ± 0.01; E$_{0-K115ac,K122ac}$ = 0.61 ± 0.01). This suggests that H3(K115ac,K122ac) does not alter inherent nucleosomes unwrapping.

We performed LexA binding studies [30] by detecting the reduction in FRET efficiency that is due to LexA binding to its target sequence located within the nucleosome between base pairs 8 and 27 of the 147 base pair 601 nucleosome positioning sequence. We initially titrated LexA from 0µM to 1µM in the presence of 1.0mM Na+ and found that the FRET efficiency reduces to approximately 0.2 at high concentrations of LexA (Figure 1.7C-E). Such a nonzero FRET efficiency at high LexA concentrations is concordant with previous site accessibility measurements [30]. These results are consistent with the conclusion that unmodified nucleosomes and nucleosomes containing H3(K115ac,K122ac) are not disassembled by LexA binding.

The FRET efficiencies in the presence of LexA were fitted to a non-cooperative binding curve, and the LexA concentration of half-saturation, (S$_{0.5-nuc}$), in which 50% of the nucleosomes are bound by LexA was determined for nucleosomes containing unmodified H3 (S$_{0.5-unmod}$ = 49 ± 4 nM), and H3(K115ac,K122ac) (S$_{0.5-unmod}$ = 56 ± 4 nM) (Figure 1.7E). The concentration of half-saturation by LexA binding to its site within naked DNA was determined by gel shift analysis [30] to be S$_{0.5-DNA}$ = 0.14 ± 0.02nM (See Chapter 4). We determined the site exposure equilibrium constant K$_{eq}^{\text{wrap}}$ from the half-saturation value of LexA binding to its target sequence within the nucleosome and to naked DNA, since S$_{0.5-nuc} = S_{0.5-DNA}/K_{eq}^{\text{wrap}}$, in the limit that K$_{eq}^{\text{wrap}}$ is much less than 1. From this equa-
Figure 1.7: **H3 Lysine K115/K122 acetylation does not facilitate transcription factor binding** (A) DNA constructs used for FRET measurements: 601 NPS (gray), 50 base pairs of additional linker DNA (black), 20 base pair LexA binding site between bases 8 and 27 (red), and Cy3 attached to DNA end (601L-end) or 4th base (601L-50-E) (green). (B) Nucleosome crystal structure [37] with 601L-end DNA construct: H2A(K119C) (pink) location for Cy5 attachment, H3(K115) (blue), H3(K122) (teal). (C) Fluorescence emission spectra of 601L-end nucleosomes containing unmodified H3 (left) of H3(K115ac,K122ac) (right) excited at 510nm (donor, Cy3 excitation) in the presence of 0nM lexA (black line), 30nM LexA (gray line), or 1000nM LexA (light gray line). (D) Fluorescence emission spectra of 601L-end nucleosomes containing unmodified H3 (left) of H3(K115ac,K122ac) (right) excited at 610nm (acceptor, Cy5 excitation) in the presence of 0nM lexA (black line), 30nM LexA (gray line), or 1000nM LexA (light gray line). (E) FRET efficiency calculated from C,D and two additional independent titrations as a function of [LexA]. Error bars are the standard deviation. 601L-end data is fit to a non-cooperative binding curve while 601L-50-E data is fit to a cooperative binding curve.
tion, we determined the equilibrium constant for site exposure for nucleosomes containing unmodified H3 ($K_{eq-unmod}^{\text{wrap}} = 0.00028 \pm 0.0005$) and H3(K115ac,K122ac) ($K_{eq-K115ac,K122ac}^{\text{wrap}} = 0.00025 \pm 0.0004$) at low ionic strength (1.0mM Na+).

The change in the site exposure equilibrium of modified nucleosomes relative to unmodified nucleosomes is equal to the change in the probability that LexA can bind to its site within the nucleosome. There is no measurable difference in the probability of LexA binding for H3(K115ac,K122ac) nucleosomes versus unmodified ($K_{eq-unmod}^{\text{wrap}}/K_{eq-K115ac,K122ac}^{\text{wrap}} = 0.88 \pm 0.20$). This result is consistent with Restriction Enzyme measures, which indicate that H3(K115,K122ac) does not facilitate nucleosome unwrapping.

To control for the possibility that transient nucleosome repositioning could facilitate LexA binding, or vice versa, we prepared a DNA template with 50 base pairs of additional linker DNA on each side of the 601 NPS to allow for nucleosome repositioning. Again, the LexA binding sequence was inserted between base pairs 8-27 within the 601 NPS and a Cy3 fluorescent molecule was attached to an amine-modified thymine at the 4th base of 601 (Figure 1.7A, 601L-50-E). Like with the 601L-end construct, after reconstitution with histone octamer containing Cy5-labeled H2A(K119C) and either unmodified H3 or H3(K115ac,K122ac), we observe that the initial FRET efficiency is the same for both nucleosomes containing H3 and H3(K115ac,K122ac) ($E_{o-unmod} = 0.68 \pm 0.02$; $E_{o-K115ac,K122ac} = 0.68 \pm 0.02$). This is consistent with the conclusion that at low ionic strength (1.0mM Na+), there is no significant difference in nucleosome positioning between nucleosomes containing unmodified H3 and H3(K115ac,H122ac) on the 601 NPS. Additionally the higher initial FRET observed for the 601L-50-E nucleosomes versus the 601L-end nucleosomes is consistent with the fact that the 4th base at which Cy3 is attached in 601L-50-E is oriented toward and 18.1Å from H2A(K119C) whereas the end-labeled Cy3 of 601L-end is oriented away and 18.8Å from H2A(K119C).

We again titrated LexA from 0µM to 1µM in the presence of 1.0mM Na+ and found that the FRET efficiency reduces to approximately 0.25 at high concentrations of LexA (Figure 1.7E). The FRET efficiencies in the presence of LexA were fit to a cooperative binding curve with cooperativity parameter, p, and the concentration of half-saturation by LexA ($S_{0.5-nuc}$)
was determined for nucleosomes containing unmodified H3 ($S_{0.5-unmod} = 49 \pm 8$ nM, $p_{unmod} = 1.5 \pm 0.3$), and H3(K115ac,K122ac) ($S_{0.5-unmod} = 50 \pm 6$ nM, $p_{K115ac,K122ac} = 1.7 \pm 0.4$) (Figure 1.7E). From this we determined the equilibrium constant for site exposure as before for nucleosomes containing unmodified H3 ($K_{wrap-eq-unmod} = 0.00028 \pm 0.0006$) and H3(K115ac,K122ac) ($K_{wrap-eq-K115ac,K122ac} = 0.00028 \pm 0.0005$) at low ionic strength (1.0mM Na+). As for nucleosomes without additional linker DNA, nucleosomes containing 50 base pairs additional linker DNA do not show any change in the probability for LexA binding nucleosomes containing H3(K115ac,K122ac) versus unmodified ($K_{wrap-eq-unmod}/K_{wrap-eq-K115ac,K122ac} = 1.0 \pm 0.3$).

Taken together, these experiments demonstrate that H3(K115ac,K122ac) neither increases nucleosome unwrapping nor facilitates protein binding within the nucleosome. Additionally binding of LexA within the nucleosome does not appear to measurably induce nucleosome repositioning. Interestingly, it appears that while additional linker DNA extending from a mononucleosome does not alter the site exposure equilibrium, $K_{wrap-eq}$ ($S_{0.5-601L-end}/S_{0.5-601L-50-E} = 1.0 \pm 0.2$), it does increase the cooperativity by which LexA binds ($S_{0.5-601L-50-E}/S_{0.5-601L-nd} = 1.3 \pm 0.2$). However, these results require further investigation to determine the effects of linker DNA on nucleosome accessibility.

1.2.7 H3(K122ac) increases the rate of nucleosome repositioning

The competitive reconstitutions demonstrated that acetylation of H3(K115) and/or H3(K122) reduce the histone-DNA binding free energy, which may lead to increased mobility during thermally driven nucleosome repositioning assays. To investigate this possibility, thermal repositioning studies were performed as previously described [88, 96, 99] to measure the relative increase in nucleosome mobility by H3(K115ac) and/or H3(K122ac). The thermal shifting experiments were carried out with purified nucleosomes reconstituted with the Cy3 and Cy5 labeled Mp2-247 DNA construct and histone octamer containing either unmodified H3, H3(K113ac), H3(K122ac), or H3(K115ac,K122ac) (Figure 1.8A,B). The Mp2 positioning sequence required higher temperatures to produce thermal shifting than previous studies using the S. variegatus NPS [87] or the mouse mammary tumor 3'
The long terminal repeat [96, 99]. The rate of repositioning was quantified by EMSA on a 5% polyacrylamide gel, since changes in nucleosome positions alter electrophoretic mobility (Figure 1.8A,B). Upon thermal heating of nucleosomes at 53°C, the slowest mobility nucleosome species (top shifted band) decreases in intensity, while a new band appears just below it. The slowest nucleosome species (bottom shifted band) and the free DNA band (bottom band) remain relatively constant throughout the thermal shifting experiment (Figure 1.8C-D).

In order to characterize the nucleosome positions before and after thermal shifting, exonuclease III (ExoIII) mapping (Figure 1.9) [100] was performed on unshifted and shifted nucleosome samples. The forward and reverse strands of the Mp2-247 DNA construct were independently 5′-labeled with Cy3 (forward strand) and Cy5 (reverse strand) to allow simultaneous visualization of ExoIII digestion from the 3′-end of both the forward and reverse strands in the same sequencing gel. The location of nucleosome-dependent ExoIII pause sites were determined by comparing the ExoIII digestions of nucleosomes to sequencing ladders that were prepared with a sequencing reaction that contained the same DNA molecule and either the Cy3 or Cy5 labeled DNA oligonucleotide.

Prior to thermal shifting, ExoIII stalled at 197 base pairs from the Cy5 5′-label (50 base pairs from the Cy3 end; Figure 1.9A) and 187, 197 and 201 base pairs from the Cy3 5′-label (60, 50, and 46 base pairs from the Cy5 end; Figure 1.9A). These positions imply that 147 base pairs are protected from ExoIII digestion, and determine that the dominant nucleosome position of the Mp2 positioning sequence is centrally positioned on the DNA. The 187 and the 201 base pair stall sites that occur on the Cy3 labeled top strand do not have corresponding stall sites on the Cy5 labeled bottom strand. This data can be interpreted to suggest that the 201 base pair stall position results from steric clash between the nucleosome and ExoIII, preventing it from reaching the dominant 197 base pair stall site. The 187 base pair stall site is a likely result of partial nucleosome invasion by ExoIII.

Based on the relative percentage of nucleosome species comprising the top and bottom shifted bands seen by EMSA (Figure 1.4C), this dominant position can be assigned to the top band.
Figure 1.8: **Thermal shift experiments determine rates of heat-induced nucleosome repositioning.** PAGE gel shift analysis of thermal shifting at 53°C for unmodified (A) and H3(K115ac,K122ac) (B) nucleosomes reconstituted with Mp2-247. Lanes are labeled with the time (min) at which a point was acquired. The relative position of the nucleosome for each band is illustrated to the right. (C) and (D) The fraction of DNA in each band in A and B was quantified based on the Cy5 fluorescent label. Circles show the fraction in the dominant central nucleosome position. Squares show the fraction in the nucleosome position that appears with time, 20 base pairs towards the Cy5-end. Diamonds show the fraction of nucleosomes located at the end of the DNA. Triangles show the fraction of free DNA that remains. (E) Since free DNA and end-positioned nucleosomes remain constant, only the nucleosome fractions in the middle position (open circle, unmodified nucleosomes; open square, H3(K115Ac); open diamond, H3(K122Ac); open triangle, H3(K115Ac/K122Ac)) and new shifted position (filled circle, unmodified nucleosomes; filled square, H3(K115Ac); filled diamond, H3(K122Ac); filled triangle, H3(K115Ac/K122Ac)) were plotted. (Figure reproduced with permission)
Figure 1.9: Exonuclease III mapping to determine positions of unmodified nucleosomes. Representative denaturing PAGE of exonuclease III mapping of unmodified nucleosomes reconstituted with Mp2-247 before (A) and after (B) heating to 53°C for 60 minutes, visualized using the Cy5 fluorescent label. Lanes are labeled with the time at which the exonuclease digestion was quenched. (C) and (D) Visualization of the gels from A and B using the Cy3 fluorescent label, which illustrates digestion from the other end of the DNA; a positioned nucleosome should protect 147 base pairs of DNA between the two digests. The new position that appears after heating is shifted 20 base pairs towards the Cy5-labeled end of the DNA relative to the Mp2 positioning site.
Figure 1.10: Exonuclease III mapping to determine positions of H3(K115ac,K122ac) nucleosomes. Representative denaturing PAGE of exonuclease III mapping of H3(K115ac,K122ac) nucleosomes reconstituted with Mp2-247 before (A) and after (B) heating to 53°C for 60 minutes, visualized using the Cy5 fluorescent label. Lanes are labeled with the time at which the exonuclease digestion was quenched. (C) and (D) Visualization of the gels from A and B using the Cy3 fluorescent label, which illustrates digestion from the other end of the DNA. The new position that appears after heating is shifted 20 base pairs towards the Cy5-labeled end of the DNA relative to the Mp2 positioning site as observed for unmodified nucleosomes (Figure 1.9)
Also, we observe a faint full-length 247 base pair band that survives initial ExoIII digestion for both the Cy5 and Cy3 labeled strands, as well as a Cy3 band that suggests ExoIII stalls 155 base pairs from the Cy3 end (Figure 1.9A,C). These additional ExoIII stall positions appear to indicate that a small fraction of the nucleosomes are positioned at the ends of the Mp2 DNA 50 base pairs away from the center. We can assign this position to the lower shifted band in the EMSA gels (Figure 1.4C). In addition, both gel shifted bands sediment on a sucrose gradient at the same rate (Figure 1.4D), which suggests that they have similar mass. This combined with 147 base pairs of protection strongly indicates that these bands contain canonical nucleosomes. We observe identical ExoIII mapping of H3(K115ac,K122ac) nucleosomes before thermal shifting (Figure 1.10A,C).

Following thermal shifting, we found new ExoIII stall positions at 177 and 182 base pairs from the Cy5-labeled end (70 and 65 base pairs from the Cy3-labeled end) and at 217 base pairs from the Cy3-labeled end (30 base pairs from the Cy5-labeled) for both unmodified (Figure 1.9B,D) and H3(K115ac,K122ac) nucleosome (Figure 1.10B,D). These new pause sites suggest that the nucleosome is shifted 20 base pairs from the center of the Mp2 sequence toward the Cy5-labeled end and are consistent with protection of 147 base pairs of DNA, indicative of a canonical nucleosome at the new position. Additionally this new position may be assigned to the middle nucleosome species band observed by EMSA that appears in time during thermal shifting (Figure 1.8A-B). The mobility of each of the different nucleosome positions is consistent with previous reported thermal shifting experiments [99].

We determined the rate of shifting to this new position by quantifying the bands containing each of the nucleosome positions and free DNA for unmodified nucleosomes and for nucleosomes containing H3(K115ac) and/or H3(K122ac) (Figure 1.8C-D). The fraction of nucleosomes positioned within the Mp2 sequence decreased with time while the fraction of nucleosomes shifted by 20 base pairs toward the Cy5-end increased concurrently. While there were variations in the fraction of nucleosomes initially positioned at the end of the Mp2 DNA molecule in different nucleosome preparations, both the end-positioned fraction and the fraction of free DNA template remained constant after the first minute (Figure
We conclude that the primary alteration in nucleosome position following thermal shifting results in depositioning from the central location to 20 base pairs towards the Cy5-labeled end.

We fit the fractions of centrally positioned nucleosomes and nucleosomes shifted by 20 base pairs to a single exponential decay with an initial and final offset (Figure 1.8E). We found unmodified nucleosomes shift with a rate of $0.026 \pm 0.002$ min$^{-1}$. Nucleosomes containing H3(K115ac) shifted with a rate of $0.025 \pm 0.002$ min$^{-1}$, nucleosomes containing H3(K122ac) shifted with a rate of $0.046 \pm 0.003$ min$^{-1}$, and nucleosomes containing H3(K115ac,K122ac) shifted with a rate of $0.041 \pm 0.004$ min$^{-1}$. Thus, nucleosomes with H3(K122ac) and with or without H3(K115ac) shift 1.6 $\pm$ 0.2 and 1.8 $\pm$ 0.2 times faster than unmodified nucleosomes, respectively. In contrast, nucleosomes only containing H3(K115ac) shifted at nearly the same rate as unmodified nucleosomes. In addition, we observe that the fraction of repositioned nucleosomes is increased such that 20-25% more nucleosomes shift to the new position when they contain H3(K122ac) compared to unmodified. Together these results are consistent with the conclusion that acetylation of H3(K122) increases nucleosome mobility.

1.2.8 Modifications that mimic H3(K115ac) and H3(K122ac) do not alter the rate of nucleosome repositioning

To investigate if the lysine acetylation mimic, Lysine $\rightarrow$ Glutamine, used in yeast genetic studies captures the effects of lysine K115 and/or K122 acetylation on nucleosome mobility, we carried out thermal repositioning studies with purified nucleosomes reconstituted with the Cy3 and Cy5 labeled Mp2-247 DNA construct and histone octamer containing either unmodified H3, H3(K113Q), H3(K122Q), or H3(K115Q,K122Q) (Figure 1.11). Again, upon thermal heating of nucleosomes at 53°C, the slowest mobility nucleosome species (top shifted band) decreases in intensity, while a new band appears just below. Coordinately, the slowest nucleosome species (bottom shifted band) and the free DNA band (bottom band) remain relatively constant throughout the thermal shifting experiment (Figure 1.11A-B).

We determined the rate of shifting to this new position by quantifying the bands con-
aining each of the nucleosome positions and free DNA for unmodified nucleosomes and for nucleosomes containing H3(K115Q) and/or H3(K122Q) (Figure 1.11C). The fraction of nucleosomes positioned within the Mp2 sequence decreased with time while the fraction of nucleosomes shifted by 20 base pairs toward the Cy5-end increased concurrently. We fit the fractions of centrally positioned nucleosomes and nucleosomes shifted by 20 base pairs to a single exponential decay with an initial and final offset (Figure 1.11C). We found unmodified nucleosomes shift with a rate of $0.026 \pm 0.002 \text{ min}^{-1}$. Nucleosomes containing H3(K115Q) shifted with a rate of $0.023 \pm 0.002 \text{ min}^{-1}$, nucleosomes containing H3(K122Q) shifted with a rate of $0.029 \pm 0.003 \text{ min}^{-1}$, and nucleosomes containing H3(K115Q,K122Q) shifted with a rate of $0.026 \pm 0.002 \text{ min}^{-1}$. Thus, nucleosomes with H3(K122Q) and H3(K115Q) have no measurable effect on nucleosome mobility compared to unmodified nucleosomes (Figure 1.11D). In contrast, nucleosomes containing H3(K122ac) with or without H3(K115ac) shifted at $\sim 1.7$-fold faster than unmodified nucleosomes. Together these results are consistent with the conclusion that in the case of H3(K115ac) and H3(K122ac), the Lysine → Glutamine acetyl-lysine mimic does not capture the physical effects of lysine acetylation on nucleosome mobility.
Figure 1.11: H3 K112/K122 acetyl-lysine mimics do not influence nucleosome repositioning. PAGE gel shift analysis of thermal shifting at 53°C for unmodified (A) and H3(K115Q,K122Q) (B) nucleosomes reconstituted with Mp2-247. Lanes are labeled with the time (min) at which a point was acquired. The relative position of the nucleosome for each band is illustrated to the right. (C) The fraction of the dominant central nucleosome position (open circle, unmodified nucleosomes; open square, H3(K115Ac); open diamond, H3(K122Ac); open triangle, H3(K115Ac/K122Ac)) and the fraction of the new shifted position by 20 base pairs (filled circle, unmodified nucleosomes; filled square, H3(K115Ac); filled diamond, H3(K122Ac); filled triangle, H3(K115Ac/K122Ac)) as determined by EMSA. (D), Relative rate of nucleosome repositioning of modified nucleosomes versus unmodified nucleosomes obtained from the exponential fit for the data in Figure (C) and Figure 1.8E.
1.3 Discussion

Histone post-translational modifications (PTMs) play key roles in essentially all biological processes that involve DNA-protein interactions. In this study, we have focused on two of the PTMs that are located in the DNA-histone interface of the nucleosome dyad. While genetic data demonstrates that these PTMs are important for transcription regulation and DNA repair, there is no information on how these modifications might alter nucleosome function(s).

Chemical tools to introduce distinct modifications into histone anc chromatin associated proteins [101–103] and the use of EPL to introduce histone modifications into unstructured histone tails [44, 57, 92] have proven to be an essential part of the study of chromatin structure. Here, we report the first use of EPL to introduce modified residues into a histone-fold domain of the nucleosome core. Because our approach generates the native protein sequence of histone H3, it has enabled us to precisely characterize the effects of buried modifications at the histone-DNA interface.

We find that histone octamers acetylated at H3(K115) and H3(K122) reduce the binding free energy, $\Delta \Delta G_{\text{nuc}}^{\text{K115ac,K122ac}}$ to a DNA positioning sequence by 0.6 kcal/mol. Remarkably, histone octamers containing H3(K115ac) alone appear to account for the majority of the reduced binding free energy (0.4 kcal/mol). This $\Delta \Delta G_{\text{nuc}}^{\text{K115ac,K122ac}}$ of 0.6 kcal/mol is similar to the difference in free energy between average genomic DNA and the natural, higher-affinity $S$. variegatus 5S nucleosome positioning sequence [80]. In fact, the typical variation in binding free energy for genomic DNA is approximately 0.5 - 1 kcal/mol [12]. This implies that the $\Delta \Delta G_{\text{nuc}}$ induced by these modifications are on the same scale as the typical variation in histone binding affinity caused by the DNA sequence in vivo. Taken as a whole, these observations suggest that the reduced affinity of nucleosomes containing H3(K122ac) and/or H3(K115ac) may directly influence the positioning of nucleosomes within the genome [12, 13].

Not only could the reduction in DNA-histone binding influence where nucleosomes are positioned in vivo, it may also influence DNA unwrapping from the histone octamer and/or
nucleosome repositioning [59]. Our LexA transcription factor binding and restriction enzyme kinetics studies demonstrate that the acetylation of H3(K115) and/or H3(K122) in general do not alter the probability of DNA site exposure DNA binding proteins. We do note a modest increase in site accessibility of the HindIII restriction enzyme site near the DNA entry-exit region for nucleosomes containing H3(K115ac) and/or H3(K122ac). It is formally possible that the dyad modifications directly influence DNA unwrapping near the DNA entry-exit region. We view this as unlikely because H3(K115) and H3(K122) are over 4nm from the DNA entry-exit region of the nucleosome, and restriction sites 5 base pairs and 15 base pairs before and after the HindIII site do not show increased accessibility. Dramatic changes in the nucleosome structure would be required for the acetylation of these residues to directly reduce DNA wrapping and accessibility at the entry-exit region. Instead, we consider the possibility that small changes in the nucleosome position could transiently expose sites near the DNA entry-exit region.

We found that the rate of thermal repositioning was primarily increased by nucleosomes containing H3(K122ac). While two-fold effects can be significant in gene expression as is the case with dosage compensation and haplo-insufficiency diseases, it seems more likely that these modifications do not singularly reposition nucleosomes in vivo. More likely is the probability that H3(K122ac) and potentially H3(K115ac) facilitate nucleosome repositioning in the presence of associated chromatin remodeling factors [90, 104].

Nucleosome assembly occurs in a stepwise fashion where the deposition of H3/H4 tetramer is followed by the deposition of H2A/H2B heterodimer [78]. Nucleosome disassembly occurs in the reverse order [78]. DNA-histone interactions in the dyad region of the nucleosome are likely to play an important role during the assembly/disassembly process. In general, histone modifications could be introduced prior to nucleosome formation to alter the rate of nucleosome assembly, or within chromatin to alter the rate of nucleosome disassembly. Given that H3(K115ac) primarily alters DNA-histone binding as measured by competitive reconstitution, dynamic modification of H3(K155) and potentially H3(K122) may serve as potential regulator of the assembly/disassembly process. The histone chaperone Asf1 is known to assemble and disassemble nucleosomes by loading and unloading.
H3/H4 dimers [76, 105]. In this regard it is worth noting that the H3(K115) and H3(K122) residues are located near the Asf1-histone interface [84].

Lysine acetylation is often mimicked in vivo by the Lysine → Glutamine substitution. Our comparison of acetylated-lysine with the Lysine → Glutamine substitution in competitive reconstitution and thermal repositioning studies found significant difference between the modification and the mimic. Our results appear to suggest that the Lysine → Glutamine substitution mimics the change in charge, but is a very poor mimic of the steric effects of acetylation. Previous studies have compared the effect of Glutamine and acetylated Lysine located in the unstructured tail domains of histone proteins in higher order chromatin structure. In this context, Glutamine proved an effective mimic of the acetylated Lysine [106]. However, in the structured core domains of histones, Lysine residues are restricted by contacts with the DNA backbone and within the histone octamer, and steric effects could play a more significant role in influencing the structure and stability of the nucleosome.
1.4 Experimental Procedures

1.4.1 DNA constructs

The Mp2-192 and Mp2-247 DNA molecules were prepared by PCR from the plasmid pMp2 [94] (see Appendix D for DNA and oligo sequences). The Mp2-192 molecule contains the Mp2 Nucleosome Positioning Sequence (NPS) with 35 and 10 base pairs of linker DNA upstream and downstream of the NPS, respectively, and a Cy5 fluorophore attached to each 5′ end. The Mp2-247 molecule contains the Mp2 NPS with 50 bases of linker DNA upstream and downstream of the NPS, a Cy5 fluorophore attached to the upstream 5′ end and a Cy3 fluorophore attached to the downstream 5′ end. Each oligonucleotide (Sigma-Aldrich) was labeled with a Cy3 or Cy5 NHS ester (GE Healthcare) at an amino group attached to the 5′ end and then purified by RP-HPLC with a 218P®C18 (Grace/Vydac) column. The unlabeled Mp2-192 and Mp2-247 DNA molecules were prepared by PCR with the same unlabeled oligonucleotides. The 601L-end and 601L-50-E DNA molecules containg the 601 NPS with the LexA transcription factor binding site inserted at bases 8-27 and labeled with Cy3 at the 5′ end of base 1 or to an amine-modified thymine at base 4 was prepared by PCR from the plasmid pNucB3-LexAL. The Cy3-labeled oligonucleotide (Sigma-Aldrich) was labeled as described above. Following PCR amplification, each DNA molecule was purified by HPLC with a Gen-Pak Fax column (Waters). Additionally, 168 base pair competitor DNA was prepared by PCR from the Ampr gene in pUC19, and then purified by HPLC with a Gen-Pak Fax column.

1.4.2 Expression and Purification of Wild Type and Mutant Histones

All histones were expressed and purified following [79, 107] as detailed in Appendix A. Mutations H3(K115Q), H3(K122Q), and H3(K115Q,K122Q) were introduced by site directed mutagenesis (Stratagene).
1.4.3 Histone Labeling

Histone H2A(K119C) for FRET measures of LexA transcription factor accessibility assays was first labeled in denaturing conditions with a 20-fold excess of Cy5-maleimide, purified over a Sephadex G20 (GE healthcare) gel filtration column at 0.5 ml/min equilibrated with TUF1000, extensively dialyzed into water, quantified by UV-Vis absorption to measure the protein concentration, lyophylized, and stored at -80°C until ready to be unfolded. See Appendix B for full details. By this labeling method, protein it typically 60-80% as determined by UV-vis and verified by Maldi-TOF mass spec.

1.4.4 Histone octamer preparation and LexA purification

Histone octamer refolding and purification were performed following [79, 107] as described in Appendix A. After unfolding, the absorption at 276nm was measured for each unfolded histone to determine the concentration. UV-Vis absorption spectra of acetylated histones H3(K115ac), H3(K122ac), and H3(K115ac,K122ac) contain a background peak centered at 230nm that overlaps with the protein absorption peak centered at 276nm. This peak is of unknown origin, but presumably due to a remnant chemical contaminant from the synthesis process. To correct for this, the overlap between the anomalous 230nm peak and 276nm protein peak was estimated by hand as described in Appendix A. Similarly, for Cy5-labeled H2A(K119C), the faction of Cy5 absorbance at 276nm is subtracted from the protein absorbance as detailed in Appendix B.

The four core histones were combined at equal molar ratio with total histone concentration adjusted to 5 mg/ml in 200µl. In the case of histone octamer containing Cy5-labeled H2A(K119C), equal molar ratio of H2A(K119C) and H2B was added in 2-fold excess to equal molar ratio of H3 and H4 to ensure complete incorporation of H2A(K119C) into full histone octamer. Additionally, due to the lack of the H3 C-terminal tail which interacts to from H3/H4 tetramer, H3(1-109) forms dimer with H4 but is not incorporated into full histone octamer. Therefore, all truncated H3(1-109) is purified away as H3(1-109)/H4 from histone octamer during gel filtration. The purity of each octamer was confirmed by
SDS-PAGE (Figure 1.3) and mass spectrometry (Figure 1.2).

LexA protein was expressed and purified from the pJWL288 plasmid (gift from Dr. Jonathan Widom) as previously described [108] and detailed in Appendix F.

1.4.5 Nucleosome Reconstitutions

Nucleosomes for restriction enzyme analysis, thermal shifting and exonuclease III mapping were reconstituted by salt double dialysis [80] (Appendix C) with 0.5 µg of Cy3 and Cy5 labeled Mp2-192 or Mp2-247 DNA, 4.5 µg of competitor DNA and 4 µg of purified histone octamer that contained unmodified H3, H3(K115ac), H3(K122ac), or H3(K115ac,K122ac).

Nucleosomes for FRET measures of LexA transcription factor accessibility were reconstituted with 5 µg of Cy3 labeled 601L-end or 601L-50-E and 4.5 µg of purified HO that contained Cy5-labeled H2A(K119C) and either unmodified H3 or H3(K115ac,K122ac). Reconstitution was performed in a 200mL reservoir by salt gradient pumping (Appendix C) from 2M to 1mM NaCl over 36 hours. After dialysis samples were extracted from the dialysis chamber and purified by sucrose gradient centrifugation (Appendix C).

1.4.6 Competitive Reconstitutions

Competitive reconstitutions (See Chapter: Introduction) were modified from published protocols to measure the change in DNA-histone binding induced by H3(K115Ac) and/or H3(K122Ac) [75, 80]. Reconstitutions were prepared in 2 M NaCl, 0.5x TE, 1mM BZA with 1 ng/µl labeled Mp2-192 DNA, 9 ng/µl unlabeled Mp2-192 DNA, 40 ng/µl 168 base pair buffer DNA from the Amp<sup>r</sup> gene, and 8 ng/µl of histone octamer (either unmodified, modified or mutated) in a total volume of 40 µl. To minimize variation in DNA and HO concentrations, a DNA and buffer master mix was first prepared that was then combined with each HO stock at a concentration of 96 ng/µl in 2 M NaCl, 0.5x TE and 1mM BZA. Each DNA-HO sample was then split into thirds and dialyzed separately.

Each sample was reconstituted in the same 1.5L reservoir by salt gradient pumping (Appendix C) from 2M NaCl to 200mM NaCl over 24 hours. The samples were then dialyzed overnight against 0.5x TE and 1mM BZA to reduce the final NaCl concentration.
to <1 mM NaCl. The reconstitution products were examined by EMSA on a 5% native 29:1 acrylamide gel with 0.3x TBE in the gel and running buffer; gels were prerun for 1 hour at 20V/cm (300V) on a GibcoBRL V16 vertical rig, Ficol was added to 2.5% to each sample, samples were applied to the gel while running at 300V, and samples were allowed to resolve for 1 hour at 300V (Appendix E). Gels were scanned with a Typhoon 8600 variable mode imager (GE Healthcare) and analyzed with ImageQuant (Molecular Dynamics) using local median background correction.

1.4.7 Restriction Enzyme Kinetics Method for Site Accessibility

The restriction enzyme kinetics method (See Chapter: Introduction) was used to determine the nucleosomal DNA accessibility at six separate restriction enzyme sites: PstI, HindIII, HaeIII, TaqI, HhaI and PmlI. The reactions were initiated by mixing 50 µl of 2x restriction enzyme mixture 400-20000 units/ml restriction enzyme (New England Biolabs), 0.2 mg/ml BSA, 2x concentration of recommended NEB buffer, 10% glycerol and 50 µl of 2nM nucleosomes. TaqI digestion was carried out at 65°C, 47°C, and 37°C, and all three temperatures resulted in the same relative equilibrium constant for site exposure; all other reactions were carried out at 37°C. Time points at 1, 2, 4, 8, 16, 24 and 32 min were acquired by quenching 10 µl of the reaction with EDTA at a final concentration of 20mM. Proteinase-K (1 mg/ml final concentration) and SDS (0.02% final concentration) were added to each time point to remove the histone octamer from the DNA. Each time point was examined by EMSA on a 5% 29:1 acrylamide gel with 0.3x TBE in the gel and running buffer; gels were prerun for 1 hour at 20V/cm (300V) on a GibcoBRL V16 vertical rig, Ficol was added to 2.5% to each sample, samples were applied to the gel while not running, and samples were allowed to resolve for 1 hour at 300V (Appendix E). The DNA was visualized with a Typhoon 8600 variable mode imager (GE Healthcare) and quantified by ImageQuant with local median background subtraction.

The digestions were fit to a single exponential to determine the initial digestion rate. An initial drop in undigested DNA between the zero and 1 minute time point is due to a small fraction of nucleosomes that dissociate during the rapid mixing. We therefore neglect
this initial time point as has been done in previous studies [81, 94].

1.4.8 Fluorescence Resonance Energy Transfer Measures of LexA Binding within Nucleosomes

The equilibrium constants for site accessibility were determined from the reduction in FRET efficiency as LexA binds to its target site buried within the nucleosome. LexA was titrated from 0µM to 3µM with 5nM Cy3/Cy5-labeled nucleosomes in 0.5TE buffer. Nucleosomes were excited at 510nm (donor excitation) and emission spectra were collected from 530 to 750 nm; nucleosomes were then excited at 610nm (acceptor excitation) and emission spectra were collected from 630 to 750 nm using a Fluoromax-4 (Horiba) steady state spectrophotometer. The FRET efficiency was determined by the (Ratio)ₐ method [83], performed in triplicate, for each LexA concentration (See Chapter: Introduction). The average FRET efficiency versus the LexA concentration was fitted to a non-cooperative or cooperative binding isotherm to determine the LexA concentration at which the FRET efficiency has been reduced by half (S₀.₅⁻nung), which is the LexA concentration when its target site is bound 50% of the time by LexA. Additionally, we used EMSA to measure the LexA concentration at which its target site within naked DNA is 50% bound by LexA S₀.₅⁻DNA (See Chapter 4).

1.4.9 Nucleosome Thermal Repositioning

Nucleosomes were thermally repositioned within the Mp2-247 DNA substrate exactly as previously described [88]. Sucrose gradient purified nucleosomes reconstituted with Mp2-247 were diluted to a concentration of 200nM in 50mM Tris (pH 8.0). The nucleosomes were heated in a thermal cycler (Eppendorf) to 47, 50, 53, 59 or 65°C. Nucleosome shifting occurred above 50°C and dissociated above 59°C before significant thermal shifting occurred. At 53°C approximately 20% of the nucleosomes shifted position while very little dissociated. Thermal shifting experiments were performed at 53°C for 1, 2.5, 5, 10, 15, 20, 30, 45 and 60 min and the reaction was stopped by transferring 10µl of the heated nucleosomes to an ice water bath. Samples were analyzed by EMSA on a 5% 59:1 acrylamide PAGE gel with 0.2x
TBE in the gel and running buffer; gels were prerun for 3 hour, 4°C, at 20V/cm (300V) on a GibcoBRL V16 vertical rig. Ficol was added to 2.5% to each sample, samples were applied to the gel while running, and samples were allowed to resolve for 3 hour, 4°C, at 300V (Appendix E). Buffer was continuously circulated between the top and bottom reservoirs at ∼ 5 ml/min. Following electrophoresis, the gels were imaged with a Typhoon 8600 variable mode imager (GE Healthcare) and quantified by ImageQuant with local median background subtraction.

1.4.10 Exonuclease III Mapping

The nucleosome positions within the Mp2-247 DNA molecule were determined with ExoIII mapping. Reactions were carried out in an initial volume of 100 µl with 20nM nucleosomes and with 30 or 100 units/ml of ExoIII (NEB) in Buffer 1 (NEB). At each time point, a 10 µl aliquot of the reaction was quenched with EDTA to a final concentration of 20mM EDTA. A final concentration of 1mg/ml of proteinase K and 0.02% of SDS was added to each time point to remove the histone octamer from the DNA. Samples were sequenced by an 8% PAGE-urea denaturing gel in 7M Urea and 1x TBE. The sequence markers were prepared with a SequiTherm Excel II DNA sequencing kit (Epicentre) using Cy3 or Cy5 labeled primers, an Mp2-247 DNA template and either ddATP or ddTTP (Appendix E). Results were imaged by a Typhoon 8600 variable mode imager (GE Healthcare), which detects Cy3 and Cy5 separately in the same gel. The Cy3 and Cy5 ladders could be loaded in the same lanes to increase accuracy of the mapping gel readout. In parallel ExoIII digestions with naked DNA were carried out to confirm that none of the positions observed with nucleosomal DNA were due to exonuclease pause sites.
Chapter 2
PHOSPHORYLATION OF HISTONE H3 THREONINE 118 ALTERS NUCLEOSOME STABILITY AND MOBILITY

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Bartholomew B., Ottesen J.J., Fishel R., Poirier M.G.
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2.1 Introduction

In Chapter 1 we examined the role of Lysine acetylation within the DNA-histone interface of the nucleosome dyad. The nucleosome dyad is a key structural region that includes essential protein-protein contacts to form the H3/H4 tetramer and DNA-histone contacts at the center of the positioned DNA sequence. We found that acetylation H3 Lysine 115 [H3(K115)ac] with or without acetylated H3 Lysine 122 [H3(K122ac)] alter DNA-histone binding by increasing the free energy of nucleosome formation by at least 0.4 kcal/mol compared to nucleosomes not bearing the modification. Additionally, we found that H3(K122ac) with or without H3(K115ac) increased nucleosome mobility during thermal repositioning by
approximately 2-fold. These results suggested that the acetylation state of H3(K115) and H3(K122) could play a role in vivo in regulating nucleosome assembly/disassembly process and chromatin remodeler mediated repositioning. These conclusions are consistent with the observation the residues H3(K115) and H3(K122) are important in yeast for transcription regulation and DNA repair [46, 84].

In this chapter we turn our attention to histone phosphorylation within the DNA-histone interface of the nucleosome dyad. Phosphorylation increases the negative electrostatic charge of the amino acid residue and increases steric bulk. Of the four histone residues that are known to bear post-translational modifications (PTMs) in this region, Histone H4 Serine 47 and H3 Threonine 118 can be phosphorylated [H4(S47ph) and H3(T118ph)]. A number of histone phosphorylation sites lie within the DNA-histone interface (See Chapter: Introduction) [60, 66, 68, 69]. However, among the putative histone phosphorylation sites, H3(T118ph) and H4(S47ph) stand out. Their side chain hydroxyl is located within 3Å of the DNA phosphate backbone at the nucleosome dyad (Figure 2.1A-B). Modification of the hydroxyl moiety with a phosphate could bring this negatively charged group in close proximity to the negatively charged DNA phosphate backbone [87]. Additionally, like with acetylation of H3(K115) and H3(K122), simultaneous phosphorylation of each copy of H3(T118) and H4(S47) could alter a large surface area of DNA-histone interaction in the assembled nucleosome. It is interesting to note that mass spectroscopy techniques indicate that H3(T118ph) is observed only occasionally and typically occurs in conjunction with H3(K115ac) and/or H3(K122ac) acetylation. H4(S47) phosphorylation, however, is observed at a higher frequency and is typically accompanied by mono- or di-methylation at a site between H4(H35) and H4(R51) [60, 66].

Mutations H3(T118E) and H3(T118A), which are used to mimic constitutively phosphorylated and de-phosphorylated states, respectively, are lethal in haploid yeast while mutation H4(S47E) exhibits a slow-growth phenotype [46]. Heteroallelic expression of H3(T118E) and H3(T118A) mutations with 'wild-type' H3 eliminates telomeric DNA silencing. Conversely, H4(S47E) and H4(S47A) lead to repression of telomeric and ribosomal DNA expression. Additionally these mutations showed defects in DNA double strand break
repair pathways near replication forks induced by hydroxyurea [46]. These results suggest that the H3(T118) and H4(S47) residues, and their modification state, are essential for appropriate chromatin metabolism [59].

Residue H3(T118) and residue H4(V35), which is located near H4(S47), also influence the requirement for SWI/SNF chromatin remodelling [85]. H3(T118I), H3(R46C), and H3(R45H) are SWI/SNF independent (SIN) alleles that return expression from the HO mating-type recombination locus to near wild-type levels in a mutant yeast strains defective in SWI/SNF remodeling [85, 86]. A number of studies subsequently showed that nucleosomes containing one of these mutations exhibit increased mobility [87, 88], accessibility [89], and/or chromatin remodelling [88]. Furthermore, H4(R45H) decreases chromatin higher order structure [109]. This suggests that alterations at H3(T118) may also impact higher order structure. Taken as a whole, these results suggest that modification of residues H3(T118) and H4(S47) may influence nucleosomes mobility, chromatin compaction, and chromatin remodeling activity.

Here we have examined the influence of H3(T118) phosphorylation on nucleosome dynamics in vitro. Histone H3 bearing H3(T118ph) was prepared by Ottesen and colleagues using expressed protein ligation (EPL, [51, 55, 56]) as discussed in Chapter 1. We found that H3(T118ph) dramatically decreases DNA-histone binding, increases thermally induced nucleosome repositioning and increases DNA accessibility near the nucleosome dyad. Our results suggest that H3(T118ph) may function to destabilize nucleosomes in vivo by regulating their mobility, disassembly and remodeling.
Figure 2.1: **Location of H3(T118ph) within the nucleosome structure.** (A) and (B) The side and top view of the nucleosome structure [37] with histone H3 in blue and H3(T118) in red; PDB ID: 1KX5. (C) The DNA constructs contain the 147 base pair nucleosome positioning sequence (NPS) Mp2 [94] with no additional DNA (Mp2-147), with 20 base pairs of DNA flanking each side (Mp2-187) or with 50 base pairs of DNA flanking each side (Mp2-247). Each of these DNA constructs were 5'-labeled with Cy3 and Cy5 on the left and right ends, respectively. (Reproduced with permission)
2.2 RESULTS

2.2.1 Construction of defined nucleosomes containing H3(T118ph)

We examined the consequences of H3(T118ph) (Figure 2.1A-B) on nucleosome stability and mobility by semi-synthetic construction of this modified histone using expressed protein ligation (EPL) [51, 93]. In order to synthesize full-length H3 protein bearing phosphorylated Threonine 118, a truncated version of H3 containing residues 1-109 and an C-terminal thioester [H3(1-109)] was expressed and purified from *E. coli*. This was ligated with an excess of synthesized C-terminal H3(110-135) peptide [H3Pep] bearing the precise phosphate modification H3Pep(T118ph): CAIHAKRVT(ph)IMPKDIQLARRIRGERA. We used the native Cysteine at residue H3(110) as a ligation site to generate the native protein sequence and purified the final product by ion exchage chromatography. H3(1-109) co-purifies with full-length H3(T118ph) with typical purities of 70:30 to 80:20 full length H3 to H3(1-109).

We refolded equal molar ratios of recombinant *X. laevis* H2A, H2B and H4 with H3(T118ph) + H3(1-109) into histone octamers (Figure 2.2). As discussed in Chapter 1, H3(1-109) refolds into H3/H4 dimer, but lacks the C-terminal helix that forms the essential protein-protein interface in the H3/H4 tetramer. Therefore, only the full-length H3 is incorporated into histone octamer and the efficiency of refolding appears to solely depend on the full-length component of the H3 mixture.

Full histone octamers containing H3(T118ph) were purified by gel filtration and used for nucleosome reconstitutions (Figure 2.3) with DNA molecules containing the Mp2 nucleosome positioning sequence (NPS, [94]) (Figure 2.1C), which is a derivative of the 601 NPS [73]. For DNA molecules reconstituted with H3(T118ph) histone octamer we observe a secondary nucleosome band with significantly slower electrophoretic mobility (Figure 2.3A). These species migrate twice as far on a sucrose gradient compared to mononucleosomes containing unmodified H3 or H3(T118ph). Therefore, this secondary species is purified away during sucrose gradient purification and not characterized in these subsequent experiments. However, this secondary species has been characterized as an alternate DNA-histone complex and is the subject of Chapter 3.
Figure 2.2: Preparation of histone octamer containing H3(T118ph). (A) Scheme for the synthesis of H3(T118ph). A fusion of truncated H3(1-109) with Intein-CBD was expressed as a recombinant protein and then refolded by dialysis into high salt buffer. Thiolysis was initiated with the addition of 100 mM MESNA, which generated H3(1-109) with a thioester terminus. This was then ligated to a peptide containing the phosphothreonine in 6M urea, 1M NaCl, 50mM, HEPES (pH 7.5), 1mM EDTA, 20mM tris(2-carboxyethyl)phosphine and the product purified by ion-exchange chromatography. (B) SDS-PAGE analysis of the thiolysis and ligation. (Lane 1) molecular weight standard, (Lane 2) the generation of H3(1-109) following thiolysis, and (Lane 3) the generation of H3(T118ph) by ligation of H3(110-135) to H3(1-109). (C) MALDI-TOF mass spectrometry analysis of purified histone octamer containing H3(T118ph). (D) SDS-PAGE analysis of unmodified, H3(T118ph) and H3(T118E). (Reproduced with permission)
Figure 2.3: **Preparation of H3(T118ph) nucleosomes.** (A) Cy5 fluorescence image of a native PAGE analysis of fractions from a sucrose gradient purification of a nucleosome reconstitution with histone octamer containing H3(T118ph). Lane U is the sample before purification; Lanes 4-22 are the fraction numbers from the sucrose gradient. (B) Cy5 fluorescence image of an electrophoretic mobility shift analysis (EMSA) of purified nucleosomes containing Mp2-147 with: (Lane 1) unmodified, (Lane 2) H3(T118E), and (Lane 3) H3(T118ph) histone octamer. (C) Cy5 fluorescence image of EMSA of: (Lane 1) free Mp2-187 DNA, (Lane 2) unmodified, (Lane 3) H3(T118E) modified, and (Lane 4) H3(T118ph) histone octamer. (D) Cy5 fluorescence image of EMSA of: (Lane 1) free Mp2-287 DNA, (Lane 2) unmodified, (Lane 3) H3(T118E) modified, and (Lane 4) H3(T118ph) histone octamer. (Reproduced with permission)
2.2.2 H3(T118ph) reduces DNA-histone binding free energy

The influence of H3(T118ph) on the free energy of DNA-histone binding to form nucleosomes, which is a measure of nucleosome stability, was examined by competitive reconstitutions (See Chapter: Introduction) [93]. H3(T118ph) reduced the nucleosome formation free energy by $2.1 \pm 0.2$, $1.6 \pm 0.2$ and $1.7 \pm 0.3$ kcal/mol at $12^\circ$C, $25^\circ$C, and $33^\circ$C, respectively (Figure 2.4). These results indicate that the probability for nucleosome formation is reduced by $\sim 16$-fold at physiological temperatures. In contrast, the H3(T118E) amino acid substitution, which adds a negative charge and is often used to mimic phosphothreonine [59], has no influence on the nucleosome formation free energy (Figure 2.4). Together these findings suggest that the precise moiety introduced by the phosphorylation of H3(T118) is more important than simply the presence of negative charge for the reduction in the DNA-histone binding free energy.

Figure 2.4: H3(T118ph) reduces the free energy of nucleosome formation. (A) Electrophoretic mobility shift analysis (EMSA) of competitive reconstitutions with unmodified, H3(T118ph) and H3(T118E) histone octamers with the Mp2-147 NPS in triplicate. The ratio of the nucleosome band to the DNA band is used to determine the equilibrium constant, $K_{eq}^{nuc}$, of each octamer. (B) The difference in nucleosome formation free energy, $\Delta \Delta G_{sample}^{nuc}$, between H3(T118ph) or H3(T118E) containing histone octamer and unmodified octamer at $12^\circ$C, $25^\circ$C and $33^\circ$C. The change in the nucleosome formation free energy was determined from $\Delta \Delta G_{sample}^{nuc} = -k_B T \ln\left(\frac{K_{eq}^{nuc, sample}}{K_{eq}^{nuc, reference}}\right)$, where $k_B$ is the Boltzmann constant and $T$ is 285K, 298K or 306K. (Reproduced with permission)
2.2.3 H3(T118ph) dramatically enhances nucleosome mobility

The reduction in nucleosome formation free energy could result in increased nucleosome mobility as has been reported for histone SWI/SNF independent (SIN) mutants including H3(T118I) [87, 88], and nucleosomes acetylated at the dyad axis as discussed in Chapter 1. We examined the effect of H3(T118ph) on nucleosome mobility by heating nucleosomes to 53°C and then measuring the kinetic evolution of nucleosome positions by electrophoretic mobility shift analysis (EMSA) [88] within the Mp2-187 (Figure 2.5) and the Mp2-247 (Figure 2.6) DNAs. Upon heating at 53°C, we observe both by EMSA and by ExoIII mapping Figures 2.8-2.9) that centrally positioned unmodified and H3(T118ph) nucleosomes within the Mp2-187 DNA reposition by 20-21 base pairs (Figure 2.5). Coordinately, we observe a similar repositioning of unmodified and H3(T118ph) nucleosomes within the Mp2-247 DNA from a central position to a new position 20 base pairs away (Figure 2.6). Additionally there is negligible change in the fraction of end-positioned nucleosomes or free DNA for both Mp2-187 and Mp2-247 DNA molecules. This is identical to the repositioning observed for unmodified, H3(K115ac) and/or H3(K122ac) nucleosomes within the Mp2-247 NPS as detailed in Chapter 1.

We quantify the fraction of centrally positioned nucleosomes as a function of time and fit it to a single exponential to determine the characteristic rate of nucleosome repositioning (Figure 2.6, Table 2.1). We find that H3(T118ph) containing nucleosomes reposition 12 ± 2 times faster than unmodified nucleosomes on the Mp2-187 sequence and 23 ± 4 times faster on the Mp2-247 sequence. In contrast, nucleosomes containing H3(T118E) repositioned slower than unmodified nucleosomes.

We also investigated the influence of H3(T118ph) on nucleosome mobility at the physiological temperature of 37°C. We find that H3(T118ph) allows for 20 base pair repositioning with a decay of \( \tau_{187-T118ph,37°C} = 37 \pm 10 \) min, while unmodified nucleosome repositioning was undetectable (Figure 2.7, Table 2.1). These results indicate that the histone octamer within a repositioned nucleosome remains intact and that the reduction in nucleosome formation free energy increases nucleosome mobility by 10-20 fold. Additionally, the increase
in nucleosome mobility appears to facilitate thermally induced repositioning at physiological temperatures.

<table>
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<th>Histone</th>
<th>$K_{MP2-187,37^\circ C} \text{ (min}^{-1}\text{)}$</th>
<th>$K_{MP2-187,53^\circ C} \text{ (min}^{-1}\text{)}$</th>
<th>$K_{MP2-247,53^\circ C} \text{ (min}^{-1}\text{)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmod</td>
<td>$1.6e-4 \pm 1.6e-4$</td>
<td>$0.043 \pm 0.008$</td>
<td>$0.047 \pm 0.005$</td>
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<tr>
<td>H3(T118E)</td>
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<td>$0.029 \pm 0.005$</td>
<td>$0.026 \pm 0.014$</td>
</tr>
<tr>
<td>H3(T118ph)</td>
<td>$0.029 \pm 0.007$</td>
<td>$0.493 \pm 0.019$</td>
<td>$1.094 \pm 0.152$</td>
</tr>
</tbody>
</table>

Table 2.1: **Thermally induced rates of nucleosome repositioning.** Rates are determined from the exponential fit to the kinetic evolution of positioned nucleosome fraction during thermal heating at $37^\circ C$ and $53^\circ C$. Rates are in change of positioned fraction per minute.
Figure 2.5: H3(T118ph) increases the rate of nucleosome mobility on Mp2-187. (A) and (B) Cy5 fluorescence images of thermally (53°C) induced shifts in unmodified and H3(T118ph) nucleosome positions within Mp2-187 NPS, respectively. The top band is centrally positioned nucleosomes, the second band is nucleosomes positioned at the ends of the DNA 20-21 base pairs away from the central position toward the Cy5 end, and the bottom band is naked DNA (diagramed on the right). (C) and (D) Quantification of the fraction of center positioned nucleosomes (squares), end positioned nucleosomes (circles) and naked DNA (diamonds). The error bars were determined from the standard deviation from at least three separate experiments. The kinetic evolution of each nucleosome position was fit to a single exponential decay. The inset shows the first 6 minutes of plot (D). (E) Diagram of the predicted change in nucleosome positions at 53°C as determined by nucleosome mapping (Figures 2.8-2.9). (Reproduced with permission)
Figure 2.6: H3(T118ph) increases the rate of nucleosome mobility on Mp2-247. (A) and (B) Cy5 fluorescence images of thermally (53°C) induced repositioning visualized by EMSA of unmodified and H3(T118ph) Mp2-247 NPS nucleosomes, respectively. The top band is centrally positioned nucleosomes, the second band is nucleosomes shifted toward the Cy5 end from the center by 20 base pairs, the third band are nucleosomes positioned at the Cy3 or Cy5 end of the DNA and the bottom band is naked DNA (diagramed on the right). (C) and (D) Quantification of the fraction of center positioned nucleosomes (squares), 20 base pairs shifted nucleosomes (circles), end positioned nucleosomes (triangles) and naked DNA (diamonds). The error bars were determined from the standard deviation of each time point from at least three separate experiments. The kinetic evolution of each nucleosome position was fit to a single exponential decay. The inset shows the first 6 min of plot (D). (E) Summary of the characteristic decay times for repositioning of the central nucleosome position within the Mp2-187 NPS (see Figure 2.5) and Mp2-247 NPS. (F) Diagram of the change in nucleosome positions at 53°C as determine by nucleosome mapping (Figures 2.10-2.11). (Reproduced with permission)
Figure 2.7: H3(T118ph) provokes nucleosome repositioning at physiological temperatures. (A) and (B) Cy5 fluorescence images of thermally (37°C) induced shifts in unmodified and H3(T118ph) nucleosome positions within the Mp2-187 NPS, respectively. The top band is centrally positioned nucleosomes, the second band is nucleosomes positioned at the ends of the DNA and the bottom band is naked DNA (diagramed on right). (C) and (D) Quantification of the fraction of center positioned nucleosomes (squares), end positioned nucleosomes (circles) and naked DNA (diamonds). The error bars were determined from the standard deviation from at least three separate experiments. The kinetic evolution of each nucleosome position was fit to a single exponential decay. The decay of the unmodified nucleosomes is so slow that it is immeasurable within the error of the experiment, while the time decay of the H3(T118ph) nucleosomes was 37 ± 10 minutes. (Reproduced with permission)
Figure 2.8: Exonuclease III mapping of the position of unmodified nucleosomes within the Mp2-187 NPS. Denaturing PAGE analysis of exonuclease III mapping of unmodified nucleosomes reconstituted with the Mp2-187 NPS before (A) and after (B) heating to 53°C for 90 min and visualized using the Cy3 fluorescent label. Lanes are labeled with the time at which the exonuclease digestion was quenched. (C) and (D), visualization of the gels from (A) and (B) using the Cy5 fluorescent label, which illustrates digestion from the other end of the DNA; a positioned nucleosome will protect 147 base pairs of DNA between the two digests. The new position that appears after heating is shifted 20 base pairs toward the Cy5-labeled end of the DNA relative to the Mp2 NPS. (Reproduced with permission)
Figure 2.9: Exonuclease III mapping of the position of H3(T118ph) nucleosomes within the Mp2-187 NPS. Denaturing PAGE analysis of exonuclease III mapping of unmodified nucleosomes reconstituted with the Mp2-187 NPS before (A) and after (B) heating to 53°C for 90 min and visualized using the Cy3 fluorescent label. Lanes are labeled with the time at which the exonuclease digestion was quenched. (C) and (D), visualization of the gels from (A) and (B) using the Cy5 fluorescent label, which illustrates digestion from the other end of the DNA; a positioned nucleosome will protect 147 base pairs of DNA between the two digests. The new position that appears after heating is shifted 20 base pairs toward the Cy5-labeled end of the DNA relative to the Mp2 NPS. (Reproduced with permission)
Figure 2.10: Exonuclease III mapping of the position of unmodified nucleosomes within the Mp2-247 NPS. Denaturing PAGE analysis of exonuclease III mapping of unmodified nucleosomes reconstituted with the Mp2-247 NPS before (A) and after (B) heating to 53°C for 90 min and visualized using the Cy3 fluorescent label. Lanes are labeled with the time at which the exonuclease digestion was quenched. (C) and (D), visualization of the gels from (A) and (B) using the Cy5 fluorescent label, which illustrates digestion from the other end of the DNA; a positioned nucleosome will protect 147 base pairs of DNA between the two digests. The new position that appears after heating is shifted 20 base pairs toward the Cy5-labeled end of the DNA relative to the Mp2 NPS. (Reproduced with permission)
Figure 2.11: Exonuclease III mapping of the position of H3(T118ph) nucleosomes within the Mp2-247 NPS. Denaturing PAGE analysis of exonuclease III mapping of unmodified nucleosomes reconstituted with the Mp2-247 NPS before (A) and after (B) heating to 53°C for 90 min and visualized using the Cy3 fluorescent label. Lanes are labeled with the time at which the exonuclease digestion was quenched. (C) and (D), visualization of the gels from (A) and (B) using the Cy5 fluorescent label, which illustrates digestion from the other end of the DNA; a positioned nucleosome will protect 147 base pairs of DNA between the two digests. The new position that appears after heating is shifted 20 base pairs toward the Cy5-labeled end of the DNA relative to the Mp2 NPS. (Reproduced with permission)
2.2.4 H3(T118ph) increases DNA accessibility near the nucleosome dyad

To determine whether H3(T118ph) influences nucleosomal DNA accessibility, we quantified the DNA site exposure equilibrium of nucleosomes containing H3(T118ph) relative to unmodified nucleosomes with Restriction Enzyme (RE) kinetics assays (See Chapter: Introduction) [81]. The DNA site exposure equilibrium, $K_{eq}^{RE}$, is the equilibrium between nucleosome states that are accessible for RE binding and nucleosome states that are inaccessible to RE binding. Four of the five restriction sites analyzed displayed no significant increase in site exposure equilibrium in nucleosomes containing H3(T118ph). However, the HhaI site near the dyad symmetry axis of the nucleosome and residue H3(T118) displayed a 6-fold increase in site exposure (Figure 2.12). This suggests that H3(T118ph) increases DNA site accessibility only near the dyad symmetry axis.

We also investigated the influence of H3(T118ph) on DNA accessibility with DNase I footprinting. DNase I is an endonuclease that cleaves both strands of the DNA backbone and whose activity is highly DNA sequence dependent leading to DNase hypersensitive sites. This provides for the quantification of the level of protection imposed by DNA-histone interactions from DNase I activity. After DNase I digestion of free Mp2-187 DNA and Mp2-187 nucleosomes containing either unmodified H3 or H3(T118ph), DNase I hypersensitive sites were resolved by denaturing PAGE (Figures 2.13-2.14). The Mp2-187 DNA molecule contains a Cy3 attached to the 5′ end of the forward DNA strand and a Cy5 molecule attached to the 5′ end of the reverse strand. This allows for simultaneous visualization of DNase digestion fragments from both DNA ends. After resolving the DNase I digestion pattern by denaturing PAGE, we determined the line trace of the 2 minute lane for both the Cy3 (Figure 2.13) and Cy5 (Figure 2.14) images, representing detection of both the top (Cy3) and bottom (Cy5) strands of Mp2-187. We find that nucleosomes with and without H3(T118ph) protect the central 147 base pairs of Mp2-187 from DNase I cleavage and display the periodic 10 base pair cleavage pattern that is typical of nucleosome footprinting by DNase I.

The DNase I cleavage is enhanced near the nucleosome dyad at the 95th bp of both
the top (Cy3) and bottom (Cy5) DNA strands of Mp2-187 for H3(T118ph) nucleosomes relative to unmodified nucleosomes (Figure 2.14D). This result independently confirms the RE measurement that DNA accessibility is increased near the dyad symmetry axis region by H3(T118ph). The region near the dyad symmetry axis typically contains the least accessible nucleosomal DNA with a site exposure equilibrium constant of $\sim 10^{-5}$ [28, 29].

While the 6-7 fold increase in the site accessibility by H3(T118ph) as measured by RE and DNase I digestion is significant, the overall site accessibility equilibrium constant remains much less than naked DNA. The enhanced accessibility could be due to alterations in DNA unwrapping where rare unwrapping fluctuations that extend close to the nucleosome dyad more often continue to unwrap past the dyad when H3(T118) is phosphorylated. Alternatively, H3(T118ph) could increase the probability that a DNA bulge forms near the dyad [110] or that the DNA near the dyad slips off the side of the histone octamer. Each of these models would only increase accessibility near the dyad region without increasing site accessibility closer to the DNA entry-exit region.

H3(T118ph) also enhanced DNase I cleavage in the DNA entry-exit region at the 20th base pair of Mp2-187 relative to unmodified nucleosomes (Figure 2.14D). However, the DNase I cleavage site near the opposite entry-exit region at the 160th base pair of Mp2-187 is not altered by H3(T118ph). This is consistent with our results from ExoIII nucleosome mapping (Figures 2.8-2.9), which indicate that a fraction of nucleosomes are positioned off the Mp2 sequence toward the Cy5-labeled end of Mp2-187.
Figure 2.12: H3(T118ph) increases DNA accessibility to Restriction Enzymes near the dyad symmetry axis. (A) and (B) PAGE analysis of unmodified and H3(T118ph) nucleosomes, respectively, digested with HhaI. The lanes are labeled with the digestion quench time in minutes. (C) and (D) Plots of the fraction of HaeIII and HhaI undigested DNA, respectively. Digestions of unmodified (squares) and H3(T118ph) (circles) nucleosomes were fit with a single exponential decay. (E) Plot of the site accessibility of H3(T118ph) nucleosomes, $K_{eq}^{RE}_{PT118}$ relative to the site accessibility of unmodified nucleosomes, $K_{eq-unmod}^{RE}$ for five separate restriction enzyme sites. The error bars were determined from the standard deviation of at least 3 separate experiments. (F) The nucleosomes crystal structure [37] with the 5 restriction enzyme sites shown in blue and the two H3(T118) residues shown in red. (Reproduced with permission)
Figure 2.13: DNase I accessibility of nucleosomes with unmodified H3 and H3(T118ph). (A) Cy3 fluorescence image of a denaturing PAGE analysis of a DNase digestion with naked Mp2-187 DNA. The first 2 lanes are A and T ladders of the Mp2-187 DNA molecule. Lanes 3 through 8 are labeled with the time in min. (B) Cy3 fluorescence image of a denaturing PAGE analysis of a DNase digestion with unmodified nucleosomes containing the Mp2-187 DNA molecule. The labels 95 and 160 indicate cleavage at the 160th and 95th base pair positions within the Mp2-197 DNA molecule, which are located in the nucleosome DNA entry-exit and dyad regions, respectively. (C) Cy3 image of a denaturing PAGE analysis of a DNase digestions with H3(T118ph) containing nucleosomes. (Reproduced with permission)
Figure 2.14: **H3(T118ph) increases DNase I accessibility near the dyad symmetry axis.** (A) Cy5 fluorescence image of a denaturing PAGE analysis of a DNase digestion with naked Mp2-187 DNA. The first 2 lanes are A and T ladders of the Mp2-187 DNA molecule. Lanes 3 through 8 are labeled with the time in min. (B) Cy5 fluorescence image of a denaturing PAGE analysis of a DNase digestion with unmodified nucleosomes containing the Mp2-187 DNA molecule. The labels 20 and 95 indicate cleavage at the 20th and 95th base pair positions within the Mp2-187 DNA molecule, which are located in the nucleosome DNA entry-exit and dyad regions, respectively. (C) Cy5 image of a denaturing PAGE analysis of a DNase digestions with H3(T118ph) containing nucleosomes. (D) Line trace of the 2 minute DNase digestion lane from images A-C for both Cy3 and Cy5 images. The location of a peak in each trace corresponding to a DNase cut position is calibrated using the A and T ladders. Each trace is background corrected and normalized by the intensity of the uncut DNA in the 0 minute lane. The decrease in digestion of unmod and H3(T118ph) nucleosomes with respect to naked DNA from 167-140 base pairs is due to the ∼147 base pair footprint of the nucleosome. Both unmodified and H3(T118ph) containing nucleosomes exhibit a similar 10n phasing in digestion intensity except at the dyad (95bp) where H3(T118ph) containing nucleosomes are ∼7 times more sensitive than unmodified nucleosome to DNase cleavage. (Reproduced with permission)
2.3 Discussion

From these results we find that H3(T118ph) dramatically reduces the DNA-histone binding free energy and increases nucleosome mobility by $\sim 25$ times. Furthermore, we observe a significant increase in DNA accessibility near the modification site in the nucleosome dyad. These results indicate that H3(T118ph), which is located in the DNA-histone interface near the dyad symmetry axis, directly impacts nucleosome dynamics. The side chain hydroxyl of H3(T118) is within 3Å of the DNA phosphate backbone [87]. The phosphorylation of H3(T118) would place significant negative charge in close proximity to the DNA phosphate backbone. One possibility is that electrostatic repulsion between the phosphothreonine and the DNA phosphate backbone is responsible for the observed alterations in nucleosome properties. However, the H3(T118E) substitution also introduces a negative charge, but does not reduce the DNA-histone binding free energy or nucleosome mobility. Moreover, the H3(T118I) SIN mutant induces similar but less pronounced alterations to nucleosome mobility [87, 88] and DNA accessibility [89] compared to the H3(T118ph). The substitution of a Threonine with an Isoleucine replaces a hydrophilic residue with a hydrophobic residue and slightly increases steric bulk. The crystal structure of nucleosomes containing H3(T118I) and the much bulkier substitution H3(T118H) illustrate a distortion of the nucleosome DNA and a reduction in the number of hydrogen bonds in the nucleosome dyad region [87].

Taken as a whole these observations suggest that specific properties of H3(T118ph) are responsible for the dramatic alterations in nucleosome stability and dynamics and that it is the combination of sterics and the precise negative charge of the phosphate moiety that significantly disrupts the DNA-histone interactions. Furthermore, the fact that H3(T118E) does not reproduce the effects of H3(T118ph) but that both H3(T118E) and H3(T118A) are lethal in budding yeast is consistent with the notion that switching between the phosphorylated and dephosphorylated states of H3(T118) is required for yeast viability. Finally, since H3(T118ph) is observed at low levels [60] and dramatically perturbs nucleosome dynamics, it might be anticipated that this histone PTM would occur only transiently and be tightly
Regulated.

Residue H4(S47) is analogous to H3(T118) in its location within the DNA-histone interface of the nucleosome dyad and in its close proximity to the DNA backbone [9]. Yeast genetic studies indicate that mutations to H4(S47) exhibit similar phenotypes of altered transcription regulation and DNA repair [46]. H4(S47) is near H4(R45) which is the location of the SIN alleles H4(R45C) and H4(R45H) [85]. Analogous to the SIN mutant H3(T118I), these mutations increase nucleosome mobility [87, 88], and nucleosome crystal structures containing these mutations show a loss of key DNA-histone contacts [87]. Our results indicated that phosphorylation of H4(S47) may function in a similar manner as H3(T118ph) to influence nucleosome dynamics, facilitate nucleosome disassembly and regulate or target chromatin-remodeling mechanisms.
2.4 Experimental Procedures

2.4.1 DNA constructs

The Mp2-187 and Mp2-247 DNA molecules were prepared by PCR from the plasmid pMp2 [94] (See Appendix D for DNA and oligo sequences). The Mp2-187 and Mp2-247 molecules contain the Mp2 NPS with 20 and 50 bases, respectively, of linker DNA upstream and downstream of the NPS. Each molecule also contains a Cy5 fluorophore attached to the 5’ end of the forward DNA strand and a Cy3 fluorophore attached to the 5’ end of the reverse strand. Each oligonucleotide (Sigma-Aldrich) was labeled with a Cy3 or Cy5 NHS ester (GE Healthcare) at an amino group attached to the 5’ end and then purified by RP-HPLC with a 218P®C18 (Grace/Vydac) column. Following PCR amplification, each DNA molecule was purified by HPLC with a Gen-Pak Fax column (Waters). Additionally, 201 base pair competitor DNA was prepared by PCR from the AmpR gene in pUC19, and then purified by HPLC with a Gan-Pak Fax column.

2.4.2 Expression and Purification of Wild Type and Mutant Histones

Mutation H3(T118E) was introduced by site directed mutagenesis (Stratagene). All histones were expressed and purified following [79, 107] as detailed in Appendix A.

2.4.3 Histone octamer refolding and purification

Histone octamer refolding and purification were performed following [79, 107] as described in Appendix A. The absorption unfolded histones at 276nm was measured for each unfolded histone to determine the concentration. UV-Vis absorption spectra of histone H3(T118ph) contain a large background peak centered at 230nm that overlaps with the protein absorption peak centered at 276nm. This peak is of unknown origin, but presumably due to a remnant chemical contaminant from the synthesis process. To correct for this, the overlap between the anomalous 230nm peak and 276nm protein peak was estimated by hand as described in Appendix A.
The four core histones were combined at equal molar ratio with total histone concentration adjusted to 5 mg/ml in 200µl. The octamer was refolded by double dialysis in refolding buffer. The recovered refolded octamer was centrifuged to remove large aggregates and then purified by gel filtration over a Superdex 200 (GE healthcare) column to remove any H3/H4 tetramer and H2A/H2B dimer proteins. Additionally, due to the lack of the H3 C-terminal tail which interacts to form H3/H4 tetramer, H3(1-109) forms dimer with H4 but is not incorporated into full histone octamer. Therefore, all truncated H3(1-109) is purified away as H3(1-109)/H4 from histone octamer during gel filtration. The purity of each octamer was confirmed by SDS-PAGE and mass spectrometry (Figure 2.2).

2.4.4 Nucleosome Reconstitutions

Nucleosomes were prepared with 4µg of purified histone octamer and 5µg DNA in 50µg of 2M NaCl, 0.5x TE, 1mM benzamidine (BZA) by the salt double dialysis method (See Appendix C). Nucleosomes were purified by sucrose gradient centrifugation (See Appendix 3) with 0.5mM MgCl₂ added to the sucrose gradient. The addition of 0.5mM MgCl₂ was required to maintain stability during the purification of H3(T118ph) nucleosomes.

2.4.5 Competitive Reconstitutions

Competitive reconstitutions were modified from published protocols to measure the change in DNA-histone binding induced by H3(T118ph) [93]. Reconstitutions were prepared in 2M NaCl, 0.5 xTE, 1mM BZA with 0.2µg of Cy3/Cy5-labeled Mp2-147 DNA, 2µg of 201 base pair competitor DNA, and either 0.5µg unmodified, 0.5µg H3(T118E), or 1.2µg of H3(T118ph) histone octamer in a total volume of 50µl. To minimize variation in DNA and histone octamer concentrations, a DNA and buffer master mix was first prepared that was then combined with each histone octamer stock at a concentration of 250ng/µl for unmodified H3 and H3(T118E) and 600ng/µl for H3(T118ph) in 2M NaCl, 0.5x TE and 1 mM BZA. Each DNA-histone octamer sample was then split into thirds and placed in an engineered dialysis chamber.

Each sample was reconstituted in the same 0.2L reservoir by salt gradient pumping (See
Appendix C) from 2M NaCl to <1mM NaCl over 36 hours, keeping 0.5x TE and 1mM BZA fixed throughout. Competitive reconstitutions were performed at 12°C, 25°C, and 33°C. The reconstitution products were examined by EMSA on a 5% native 29:1 acrylamide gel with 0.3x TBE in the gel and running buffer; gels were prerun for 1 hour at 20V/cm (300V) on a GibcoBRL V16 vertical rig, Ficol was added to 2.5% to each sample, samples were applied to the gel while running at 300V, and samples were allowed to resolve for 1 hour at 300V (See Appendix E). Gels were scanned with a Typhoon 8600 variable mode imager (GE Healthcare) and analyzed with ImageQuant (Molecular Dynamics) using local median background correction.

2.4.6 Nucleosome Thermal Repositioning

Thermal shifting experiments were modified from previously described methods [88, 93, 96]. Sucrose gradient purified nucleosomes at 50nM in 20mM Tris (pH 8.0) and 0.5mM MgCl₂ were incubated at 53°C of 37°C for 0.5, 1, 2, 5, 15, 30, 60, and 90 minutes and the reaction stopped by transferring 10µl of the heated nucleosomes to an ice water bath. Electrophoretic mobility shift assays (EMSAs) were used to determine the fraction of each nucleosome position within either the mp2-187 and mp2-247 DNAs over 90 min. EMSA was performed on a 5% 59:1 acrylamide PAGE gel with 0.2x TBE in the gel and running buffer; gels were prerun for 3 hour, 4°C, at 20V/cm (300V) on a GibcoBRL V16 vertical rig, sucrose and TBE were added to 5% and 0.3x, respectively, to each sample, samples were applied to the gel while running, and samples were allowed to resolve for 3 hour, 4°C, at 300V (See Appendix E). Buffer was continuously circulated between the top and bottom reservoirs at ~5 ml/min. Following electrophoresis, the gels were imaged with a Typhoon 8600 variable mode imager (GE Healthcare) and quantified by ImageQuant with local median background subtraction.

2.4.7 Exonuclease III Mapping

The nucleosome positions within the Cy3/Cy5-labeled Mp2-187 and Mp2-247 DNA molecule were determined with ExoIII mapping. Reactions were carried out in an initial volume of
50µl with 10nM nucleosomes and 50U/ml of ExoIII (NEB) in 20mM Tris pH8, 0.5mM MgCl\textsubscript{2} at 16°C to prevent H3(T118ph) nucleosome disassembly (data not shown). At each time point, a 7µl aliquot of the reaction was quenched with EDTA to a final concentration of 20mM. A final concentration of 1mg/ml of proteinase K and 0.02% of SDS was added to each time point to remove the histone octamer from the DNA and samples were separated by 8% denaturing PAGE in 7M Urea and 1x TBE (See Appendix E). The sequence markers were prepared with a SequiTherm Excel II DNA sequencing kit (Epicentre) using Cy3 or Cy5 labeled primers, an Mp2-187 or Mp2-247 DNA template and either ddATP or ddTTP (See Appendix E). Results were imaged by a Typhoon 8600 variable mode imager (GE Healthcare), which detects Cy3 and Cy5 separately in the same gel. The Cy3 and Cy5 ladders could be loaded in the same lanes to increase accuracy of the mapping gel readout.

2.4.8 Restriction Enzyme Kinetics Method for Site Accessibility

The restriction enzyme kinetics method was used to determine the nucleosomal DNA accessibility (See Chapter: Introduction), at five separate restriction enzyme sites: HindIII, HaeIII, Taq\textalpha{}I, HhaI and PmlI. The reactions were initiated by mixing 50µl of 2x restriction enzyme mixture 400-20000U/ml restriction enzyme (New England Biolabs), 0.2mg/ml BSA, 2x concentration of recommended NEB buffer, 10% glycerol and 50µl of 2 nM of mp2-147 nucleosomes with either unmodified or H3(T118ph) containing histone octamer. Taq\textalpha{}I digestion was carried out at 65°C; all other reactions were carried out at 37°C. Time points at 1, 2, 4, 8, 16, 24 and 32 min were acquired by quenching 7µl of the reaction with EDTA at a final concentration of 20 mM. Proteinase-K (1 mg/ml final concentration) and SDS (0.02% final concentration) were added to each time point to remove the histone octamer from the DNA. Each time point was examined by EMSA on a 5% 29:1 acrylamide gel with 0.3x TBE in the gel and running buffer; gels were prerun for 1 hour at 20V/cm (300V) on a GibcoBRL V16 vertical rig, Ficol was added to 2.5% to each sample, samples were applied to the gel while running, and samples were allowed to resolve for 1.25 hours at 300V (See Appendix E). The DNA was visualized with a Typhoon 8600 variable mode imager (GE Healthcare) and quantified by ImageQuant with local median background subtraction.
The digestions were fit to a single exponential to determine the initial digestion rate. An initial drop in undigested DNA between the 0 and 1 min time point is due to (i) a small fraction of DNA that copurifies with the mononucleosomes and (ii) a fraction of nucleosomes that dissociates during the rapid mixing. We therefore neglect this initial time point as has been done in previous studies [81, 94].

### 2.4.9 DNase I footprinting

DNase I sensitive sites within Cy3/Cy5-labeled Mp2-187 nucleosomes containing unmodified or H3(T118ph) histone octamer relative to naked DNA were determined by mild DNase I digestion. Reactions were carried out in an initial volume of 50µl with 10nM nucleosomes or naked DNA, 22 U/ml DNase I (Invitrogen), 20mM Tris pH 8, and 0.5mM MgCl$_2$ at 16°C. Standard DNase I reaction buffer consists of 0.1M sodium acetate and 5mM MgCl$_2$ (Invitrogen). We removed the monovalent salt and adjusted MgCl$_2$ to 0.5mM to prevent H3(T118ph) nucleosome disassembly during DNase I digestion (data not shown) due to the decreased stability of H3(T118ph). Each time point at 1, 2, 5, 10, and 15 minutes was acquired by quenching 7µl of the reaction with a final concentration of 5 mM EDTA, 1 mg/ml of proteinase K and 0.02% SDS. Digestions were analyzed 8% acrylamide, 7M Urea denaturing PAGE as described for ExoIII mapping above (See Appendix E).
Chapter 3

PHOSPHORYLATION OF H3 THREONINE 118 ALTERS CHROMATIN STRUCTURE

3.1 Introduction

In Chapters 1-2 we focused on the impact of histone post translational modifications (PTMs) that reside within the nucleosome dyad [H3(K115ac), H3(T118ph), and H3(K122ac)] on nucleosome stability, mobility and DNA unwrapping. These core histone modifications are observed to increase nucleosome mobility during thermally induced repositioning, locally increase nucleosomal DNA accessibility, and/or decrease nucleosome stability for nucleosome assembly/disassembly. Additionally, nucleosome arrays containing the SIN mutant, H4(R45C), which is located near the H4(S47) phosphorylation site, completely lose the ability to undergo magnesium-dependent intramolecular chromatin folding, suggesting that histone modifications may affect chromatin structures beyond the nucleosome [111]. These results demonstrate that histone PTMs in the DNA-histone interface can directly alter nucleosome physical properties and support the model that certain histone PTMs can function by directly impacting nucleosome structure and/or dynamics versus serving as binding sites for chromatin metabolic proteins [35]. In this chapter we characterize for the first time a histone PTM that distinctly alters the nucleosome assembly pathway to regulate nucleosome structure and chromatin folding.

As discussed in Chapter 2, Histone H3 at Threonine 118 is located in the DNA-histone interface near the nucleosome dyad symmetry axis (Figure 3.1B) where significant DNA-
histone interactions occur, and it can be phosphorylated as was determined by mass spectrometry \[H3(T118ph), \text{citebib279}\]. Genetic studies of this residue found that either a Glutamic Acid or Alanine substitution is lethal in haploid yeast, while low level expression of H3(T118E) and H3(T118A) resulted in defects in transcriptional silencing and DNA repair \[46\]. In addition, H3(T118I) is a SWI/SNF Independent (SIN) mutation, where this substitution relieves the requirement of the SWI/SNF chromatin remodeling complex for mating type switching in budding yeast \[85\]. In Chapter 2 we found that H3(T118ph) enabled nucleosome mobility and reduced DNA-histone binding by \(\sim 16\) fold at physiological temperatures. This suggested that phosphorylation within the DNA-histone interface significantly impacts nucleosome stability. In addition, we noted that a secondary DNA-histone complex was detected by electromobility gel shift assay (EMSA) following nucleosome reconstitutions, which we purified away by sucrose gradient centrifugation for our previous studies.

Interestingly, there have been a number of nucleosome crystal structures solved with histone PTMs \[112\] and histone mutants \[87, 113\]. However, none of these histone alterations have significantly altered nucleosome structure. Here, we report characterization of a distinct DNA-histone complex induced by H3(T118ph). EMSA, sucrose gradient sedimentation, thermal disassembly, micrococcal nuclease digestion, and atomic force microscopy measurements all indicate that H3(T118ph) induces the formation of an alternate DNA-histone (ADH) complex that involves DNA wrapping around two histone octamers. These complexes form during nucleosome reconstitution by salt dialysis, during nucleosome assembly with Nap1 and repeatedly on 3 kilobase DNA molecules. These results suggest that phosphorylation within the nucleosome dyad can significantly impact nucleosome assembly and structure, which could play a role in the regulation of RNA transcription and DNA repair.
3.2 Results

3.2.1 H3(T118ph) induces the formation of DNA-histone complexes with a significant change in gel mobility and sucrose gradient sedimentation.

During our initial studies of the impact of H3(T118ph) on nucleosome stability and dynamics, we found that nucleosomes reconstituted using salt dialysis produced both canonical nucleosomes and an alternate DNA-histone (ADH) complex with a significantly altered electrophoretic mobility (Figure 3.1). We were able to purify canonical nucleosomes from the ADH complexes by sucrose gradient centrifugation. The ADH complexes sediment about 2 times further on a sucrose gradient than canonical nucleosomes (Figure 3.2). This allowed us to separately characterize the impact of H3(T118ph) on the stability, mobility and remodeling of canonical nucleosomes (Chapter 2) and the nature of the ADH complexes.

Alterations in electrophoretic mobility within polyacrylamide gels are sensitive to DNA length, DNA sequence, and position of the histone octamer along the DNA molecule [96, 99, 114]. Therefore, we investigated the influence of DNA sequence on the electrophoretic mobility of the ADH complexes (Figure 3.1C). We find that the Low Mobility ADH (LM-ADH) complex formed with six different nucleosome positioning sequences (NPS). This indicates that the DNA sequence is not responsible for the LM-ADH complex.

We also investigated the influence of DNA length on the formation of ADH complexes by reconstituting nucleosomes with 147, 187, 247 base pair DNA molecules containing the Mp2 or L. variegatus 5S NPS in the center (Figure 3.1A). The Mp2 NPS is a variant of the 601 NPS [73, 94]. We find that the LM-ADH complex is formed irrespective of DNA length (Figure 3.1D-F). In addition, we observed an additional band with samples prepared with 247 base pair DNA molecules. This band has a faster electrophoretic mobility than center positioned nucleosomes and has a similar mobility to that of a depositioned nucleosome. However, this DNA-histone complex sediments through a sucrose gradient twice as far as canonical nucleosomes (Figure 3.2) suggesting that this High Mobility ADH (HIM-ADH) complex is a non-nucleosomal species.
Figure 3.1: Phosphorylated H3 T118 forms altered DNA-histone complexes. (A) DNA constructs for quantifying effects of H3(T118ph) on DNA-histone interactions. DNA’s with a single nucleosomes positioning sequence (NPS, pink) contain either no linker DNA (NPS-147), 20 base pairs of linker DNA (black) on both sides (NPS-187), 10 and 35 base pairs on opposite sides (NPS-192), or 50 base pairs on both sides (NPS-247); and contain a Cy3 (green) fluorophore attached to the 5'-end of the forward strand and Cy5 (red) to the 5'-end of the reverse strand. 601-17mer [94] contains a tandem repeat of seventeen nucleosome positioning sequences based on the 601 NPS [73] each separated by 30 base pairs of linker DNA. (B) Nucleosome crystal structure [37] with histone H3 in blue and H3(T118ph) in red; PDB ID: 1KX5. (C) EMSA of NPS-192 DNA’s reconstituted with either unmodified or H3(T118ph) histone octamer (HO). "Nuc" is a canonical nucleosome species; "LM-ADH" is a Low Mobility Altered DNA-Histone complex; the DNA pictogram is free DNA. (D) EMSA of NPS-147 DNA’s containing either the Mp2 or L. variegatus 5S NPS reconstituted with unmodified, H3(T118E) or H3(T118ph) HO. The gray sphere superimposed on the DNA pictogram indicates a nucleosome and its position on the DNA. (E) EMSA of NPS-187 DNA’s reconstituted with unmodified, H3(T118E) or H3(T118ph) HO. (F) EMSA of NPS-247 DNA’s reconstituted with unmodified HO or HO containing H3(T118ph). "HM-ADH" is a High Mobility Altered DNA-Histone complex.
Figure 3.2: ADH Complexes Sediment Faster than Mononucleosomes. EMSA of 5%-40% sucrose gradient fractions of (A) Mp2-187 and (B) Mp2-247 reconstituted with H3(T118ph) histone octamer (HO). Lanes are marked with the sucrose fraction number; U is the unpurified sample. Both LM-ADH and HM-ADH species sediment faster than mononucleosomes. (C) Integrated fluorescence intensity of each fraction for sucrose gradient purification of Mp2-247 DNA alone (gray), Mp2-247 reconstituted with unmodified HO (red), and Mp2-247 reconstituted with H3(T118ph) HO (blue). Fractions numbers in (C) correspond to the fractions in (B) for H3(T118ph) sample. EMSA of sucrose gradient purified nucleosomes reconstitute on (D) Mp2-187 and (E) Mp2-247 DNA’s. H3(T118ph) mononucleosomes and AHD complexes are almost completely purified from each other.
H3(T118ph) could function to alter nucleosome formation by irreversibly changing the histone octamer structure during refolding. We investigated this possibility by treating refolded H3(T118ph) containing histone octamer with arctic phosphatase to remove the phosphate group from T118 within histone octamer. The removal of the phosphate was confirmed by mass spectrometry (Figure 3.3A). We find that nucleosomes reconstituted with the dephosphorylated histone octamer only formed canonical nucleosomes (Figure 3.3B-C). This indicates that the phosphate is necessary and sufficient for the formation of both LM-ADH and HM-ADH complexes.

Figure 3.3: ADH complexes are H3(T118ph) Dependent. (A) Mass spec (Z = +2) of unmodified histone octamer (HO) before CIP phosphatase treatment (Top), H3(T118ph) HO before CIP phosphatase treatment (Middle), and H3(T118ph) HO after CIP phosphatase treatment (Bottom) (see methods for details). (B) and (C) EMSA of Mp2-187 and Mp2-247 DNA’s, respectively, reconstituted with unmodified HO without CIP phosphatase treatment (Lane 1), H3(T118ph) HO without CIP (Lane 2), unmodified HO after CIP (Lane 3), and H3(T118ph) HO after CIP (Lane 4).
3.2.2 DNA length impacts the altered DNA-histone complex’s stability and DNA footprint

H3(T118ph) impacts the stability and mobility of canonical nucleosomes (Chapter 2). Therefore, we investigated the stability of the ADH complexes by incubating them at 53°C for increasing time periods and then determining the fraction of ADH complexes by EMSA (Figure 3.4). We find that the LM-ADH complexes convert to canonical nucleosomes during a 90 minute incubation at 53°C irrespective of DNA length. In contrast the HM-ADH complexes were stable at 53°C. They did not convert to canonical nucleosomes or fall apart into free histones and naked DNA (Figure 3.4E-F).

These thermal disassembly results suggest that the LM-ADH complexes contain mis-wrapped nucleosomes that convert to correctly wrapped nucleosomes upon heating to 53°C. Therefore, we investigated the DNA footprint of the ADH complexes with micrococcal nuclease (MNase) digestion (Figure 3.5) and Exonuclease III (ExoIII) nucleosome mapping (Figures 3.6-3.7). We find that the ADH complex containing 187 base pair DNA had a footprint that is similar to the 147 base pair footprint of canonical nucleosomes both before and after 90 minutes at 53°C. In contrast, the ADH complexes with 247 base pair DNA molecules had both a 147 and 247 base pair footprint. We confirmed that the altered DNA-histone complexes did not fall apart during MNase and ExoIII digestions (data not shown). These results, combined with the observation that the ADH complexes containing 187 base pairs have only the slow mobility species while ADH complexes containing 247 base pair DNA form both a slow and fast mobility species, suggest that the LM-ADH complexes contain DNA that is wrapping similarly to nucleosomes. In contrast, the HM-ADH complexes contain a DNA organization that appears to be distinct from nucleosomal DNA organization with a larger DNA footprint.
Figure 3.4: LM-ADH Complexes are Decoupled into Mononucleosomes. (A) EMSA of purified LM-ADH complexes containing H3(T118ph) histone octamer (HO) and Mp2-147 after heating at 53°C for the indicated amount of time. LM-AHD complexes convert to species consistent with positioned mononucleosomes as determined by EMSA. (B) Quantification of fraction of LM-ADH (squares), nucleosome (circles), and free DNA (diamond) species for the gel in (A) versus time. Error bars are the standard deviation of three independent experiments. (C) EMSA of purified LM-ADH complexes containing H3(T118ph) HO and Mp2-187 after heating at 53°C for the indicated amount of time. LM-AHD complexes convert to positioned and depositioned mononucleosomes as determined by MNase (Figure 3.5) and ExoIII nucleosome mapping (Figure 3.6). (D) Quantification of fraction of LM-ADH (squares), nucleosome (circles), and free DNA (diamond) species for the gel in (A) versus time. Error bars are the standard deviation of three independent experiments. (E) EMSA of purified LM-ADH and HM-ADH complexes containing H3(T118ph) HO and Mp2-247 after heating at 53°C for the indicated amount of time. LM-AHD complexes convert in part to positioned and depositioned mononucleosomes as determined by MNase (Figure 3.5) and ExoIII nucleosome mapping (Figure 3.7). (F) Quantification of fraction of LM-ADH (squares), HM-ADH (triangles), nucleosome (circles), and free DNA (diamond) species for the gel in (E) versus time. Error bars are the standard deviation of three independent experiments.
Figure 3.5: MNase Footprinting. Representative EMSA visualized by CYBR-gold stain of (A) purified unmodified nucleosomes reconstituted with Mp2-187; (B) purified H3(T118ph) nucleosomes reconstituted with Mp2-187; (C) purified H3(T118ph) ADH complexes reconstituted with Mp2-187; (D) purified unmodified nucleosomes reconstituted with Mp2-247; (E) purified H3(T118ph) nucleosomes reconstituted with Mp2-247; (F) purified H3(T118ph) ADH complexes reconstituted with Mp2-247, each before thermal heating ("0min"). Lane 1 is DNA ladder of indicated length; Lanes 2-10 are the MNase concentration in U/ml.
Figure 3.6: Exonuclease III Mapping of Mp2-187 Species. Representative denaturing PAGE of exonuclease III mapping of purified H3(T118ph) mononucleosomes before thermal heating (“0min”) (A) imaged using the Cy3 fluorescent label and (B) imaged using the Cy5 fluorescent label; H3(T118ph) nucleosomes after thermal heating at 53°C for 90 minutes (“90min”) (C) imaged using the Cy3 fluorescent label and (D) imaged using the Cy5 fluorescent label; H3(T118ph) AHD complexes before thermal heating (E) imaged using the Cy3 fluorescent label and (F) imaged using the Cy5 fluorescent label; H3(T118ph) ADH complexes after thermal heating at 53°C for 90 minutes (G) imaged using the Cy3 fluorescent label and (H) imaged using the Cy5 fluorescent label. Lanes 1 and 2 are A and T sequencing ladders of the DNA base sequence; Lanes 3-8 are labeled with the time in minutes at which the exonuclease digestion was quenched. Visualization of the gels using the Cy3 and Cy5 fluorescent labels illustrates digestion from both ends of the DNA; a positioned nucleosome should protect 147 base pairs of DNA between the two digests.
Figure 3.7: Exonuclease III Mapping of Mp2-247 Species. Representative denaturing PAGE of exonuclease III mapping of purified H3(T118ph) mononucleosomes before thermal heating ("0min") (A) imaged using the Cy3 fluorescent label and (B) imaged using the Cy5 fluorescent label; H3(T118ph) nucleosomes after thermal heating at 53°C for 90 minutes ("90min") (C) imaged using the Cy3 fluorescent label and (D) imaged using the Cy5 fluorescent label; H3(T118ph) AHD complexes before thermal heating (E) imaged using the Cy3 fluorescent label and (F) imaged using the Cy5 fluorescent label; H3(T118ph) ADH complexes after thermal heating at 53°C for 90 minutes (G) imaged using the Cy3 fluorescent label and (H) imaged using the Cy5 fluorescent label. Lanes 1 and 2 are A and T sequencing ladders of the DNA base sequence; Lanes 3-8 are labeled with the time in minutes at which the exonuclease digestion was quenched. Visualization of the gels using the Cy3 and Cy5 fluorescent labels illustrates digestion from both ends of the DNA; a positioned nucleosome should protect 147 base pairs of DNA between the two digests.
3.2.3 The ratio of histones to DNA of altered DNA-histone complexes depends on DNA length

The observation that the LM-ADH complexes convert to mononucleosomes, while the HM-ADH complexes did not convert to nucleosomes, could be due to the complexes containing different ratios of histone proteins. Therefore, we determined the ratio of H2A/H2B heterodimers and H3/H4 tetramers relative to DNA molecules. We introduced an additional Cysteines at the N-termini of H2A and H4 by site directed mutagenesis and then labeled H2A(C0) with Cy5 and H4(C0) with Alexa488 (Figures 3.8A). Histone octamers were refolded with the same preparation of fluorophore labeled H2A and H4, and either with unmodified H3 or H3(T118ph), and H2B (3.9A). Following gel filtration purification, nucleosomes were reconstituted with each histone octamer and Cy3-only labeled DNA.

The canonical nucleosomes and ADH complexes were analyzed by EMSA and the Alexa488, Cy3 and Cy5 fluorophore emissions were detected with a Typhoon fluorescence scanner. We determined the emission from H2A/H2B heterodimers and H3/H4 heterodimers relative to DNA and relative to each other (Figure 3.8B-D). We confirmed that each fluorophore is spectrally separable by the Typhoon imager (Figure 3.9B) and that none of these complexes undergo significant fluorescence resonance energy transfer (FRET, Figure 3.9C-E) that would bias the fluorescence ratio between each fluorophore pair. We find that the canonical nucleosomes and the LM-ADH complexes contain the same ratio of H2A/H2B heterodimers, H3/H4 tetramer, and DNA. In contrast, the HM-ADH complexes contain twice the amount of H2A/H2B heterodimers and H3/H4 tetramers relative to DNA compared to unmodified mononucleosome. This data confirms the conclusion that the Low Mobility Alternate DNA-Histone complexes contain mis-wrapped nucleosomes that can be converted to canonical nucleosomes, while the High Mobility Alternate DNA-Histone complexes are distinct from canonical nucleosomes with twice as many histones to DNA as in canonical nucleosomes.
Figure 3.8: **AHD Complexes Contain Equimolar Ratios of Histones.** (A) Crystal structure [37] of fluorescence labeled nucleosome containing 5' end-labeled DNA with Cy3 (Green), Alexa488-labeled H4(C0) (Blue), and Cy5-labeled H2A(C0) (magenta); H3(T118ph) in Red; PDB ID: 1KX5. EMSA of (B) Cy3-labeled Mp2-187 and (C) Cy3-labeled Mp2-247 DNA’s reconstituted on fluorescent-labeled unmodified or H3(T118ph) histone octamer. Each EMSA gel was soaked in 1M NaCl and imaged by the Cy3-DNA label (left, excitation at 532nm, emission at 610 ± 15nm), Alexa488-H4(C0) label (middle, excitation at 488nm, emission at 520 ± 20nm), and Cy5-H2A(C0) label (right, excitation at 633nm, emission at 670 ± 15nm). (D) Fluorescence ratio of two fluorophore-labeled components (Blue, Cy5-H2A(C0) vs. Cy3-DNA; Orange, Alexa488-H4(C0) vs. Cy3-DNA; Pink, Cy5-H2A(C0) vs. Alexa488-H4(C0)) for H3(T118ph) nucleosomes ("Nuc"), LM-ADH, and HM-ADH species relative to the same two fluorophore-labeled components of the unmodified nucleosome species for each indicated DNA. Error Bars are the standard deviation of three independent reconstitutions. (EMSA for Mp2-147 not shown)
Figure 3.9: N-terminal fluorophore-labeled nucleosomes are spectrally separable (A) SDS-PAGE gel of purified histone octamer (left) with unlabeled H2A(C0), unlabeled H4(C0), H2B, and either unmodified H3 ("WT") or H3(T118ph), or with purified histone octamer (right) with Cy5-H2A(C0), Alexa488-H4(C0), H2B, and either unmodified H3 ("WT") or H3(T118ph). The hypershifted band above H4(C0) is Alexa488-H4(C0); Cy5-H2A(C0) cannot be resolved from unlabeled. (B) EMSA of 3 DNA oligos separately labeled with Cy3 (Lane 1), Alexa488 (Lane 2), and Cy5 (Lane 3) and imaged by a Typhoon 8600 variable mode imager by the Cy3-DNA label (left, excitation at 532nm, emission at 610 ± 15nm), Alexa488-H4(C0) label (middle, excitation at 488nm, emission at 520 ± 20nm), and Cy5-H2A(C0) label (right, excitation at 633nm, emission at 670 ± 15nm). Fluorescence emission spectra of nucleosomes containing fluorescent labeled histone octamer and Cy3-labeled Mp2-147 (C) excited at 488nm, (D) excited at 532nm, and (E) excited at 633nm in the absence and presence of 1M NaCl, which causes nucleosome dissociation. Black arrows indicate emission due to fluorescence resonance energy transfer (FRET) that is abolished upon addition of 1M NaCl.
3.2.4 AFM images of the altered DNA-histone complexes reveal they have twice the volume as canonical nucleosomes

The observation that both the LM-ADH and HM-ADH complexes sediment twice as far within a sucrose gradient as canonical nucleosomes suggested the particle’s mass could be larger than canonical nucleosomes. To investigate the size of the ADH complexes relative to canonical nucleosomes we used atomic force microscopy (AFM) in conjunction with Marek Simon. We imaged sucrose gradient purified canonical nucleosomes containing either unmodified or H3(T118ph) histone octamers, and ADH complexes. We studied complexes that contained Mp2-247 (Figure 3.10A-C) and Mp2-147 (Figure 3.11A-C). We analyzed the AFM images to quantify the average area and height of ∼200 particles of each sample type.

The height distribution of unmodified canonical nucleosomes, H3(T118ph) containing canonical nucleosomes and ADH complexes with either Mp2-247 or Mp2-147 had a maximum of about 3 nm. This is the typical mononucleosome height measured by AFM [115]. In contrast, we found that the area distribution maximum of the ADH complexes with Mp2-247 was twice as large as both unmodified and H3(T118ph) containing canonical nucleosomes with Mp2-247 (Figure 3.10D-E). In addition, the area distribution of the ADH complexes with the Mp2-147 DNA molecule had two peaks (Figure 3.11D-E). One peak maximum was about double the area distribution maximum of canonical nucleosomes. The second peak maximum was equal the canonical nucleosome distribution maximum. This second peak is likely due to the destabilization of the ADH complexes when they are diluted for AFM imaging and that they were not completely purified by sucrose gradient centrifugation. The observation that ADH complexes are the same height and twice the area of canonical nucleosomes implies that the volume of ADH complexes are double that of canonical nucleosomes. We also found that the shape of the ADH complexes were elliptical, while the unmodified and H3(T118ph) containing nucleosomes were circular.

These results, combined with our observation that LM-ADH complexes can be converted to canonical nucleosomes by heating them to 53°C and that they contain equal numbers of DNA molecules and histone octamers, suggest that the LM-ADH complexes contain
two DNA molecules and two histone octamers, where the two DNA molecules partially wrap around each of the histone octamers. In contrast, these AFM measurements of the ADH complexes containing Mp2-247, combined with the observations that the HM-ADH complexes cannot be converted to canonical nucleosomes, that they have an increased DNA footprint, and that they contain twice as many histone octamers as DNA molecules, suggest that the Mp2-247 HM-ADH complexes contain one DNA molecule wrapped around two histone octamers.

Figure 3.10: Mp2-247 ADH Complexes are Twice the Size as Mononucleosomes. AFM images of purified (A) Mp2-247 nucleosomes with unmodified histone octamer (Mp2-247 unmod Nuc), (B) Mp2-247 nucleosomes with H3(T118ph) (Mp2-247 T118ph Nuc), and (C) Mp2-247 ADH complexes with H3(T118ph) (Mp2-247 T118ph ADH). Inset is 60nm in scale. Histograms of (D) surface area and (E) height of Mp2-247 containing unmodified nucleosomes (black square, n=173), H3(T118ph) nucleosomes (blue circle, n=140), and H3(T118ph) ADH complexes (red triangle, n=175). AFM images of 601-17mer arrays reconstituted with (G) unmodified and (H) H3(T118ph) histone octamer.
3.2.5 Altered DNA-histone complexes form on 3000 base pair DNA molecules

Our observation that increasing the DNA length to 247 base pair allowed for two histone octamers to form on one DNA molecule raised the question of whether a further increase in DNA length would result in increasingly larger complexes. Therefore, we reconstituted
nucleosomes with a 3 kilobase DNA molecule that contained a tandem repeat of seventeen 601-like nucleosome positioning sequences (601-17mer) with unmodified and H3(T118ph) histone octamer. We used limiting amounts of histone octamer with a ratio of 1 histone octamer to 2 NPS to prevent aggregation. We then analyzed the nucleosome arrays by AFM. We find that nucleosomes form with unmodified histone octamers and have similar dimensions to that of mononucleosomes (Figure 3.10G). In contrast, the 3 kilobase DNA molecule reconstituted with H3(T118ph) histone octamer form numerous DNA-histone complexes that are significantly larger than single nucleosomes (Figure 3.10H) and comparable in size to AHD complexes formed on Mp2-147 and Mp2-247.

To verify that the difference seen between unmodified H3 and H3(T118ph) histone octamer reconstituted on the 3 kilobase DNA molecule are not due to the subsaturating conditions in which the arrays were reconstituted, we reconstituted nucleosomes with a 424 base pair DNA molecule that contained two 601-like nucleosome positioning sequences separated by 30 base pairs of linker DNA (601-2mer, Figure 3.12A) and either unmodified H3 or H3(T118ph) histone octamer. 601-2mer molecules were reconstituted under the saturating conditions of 1µg 601-2mer, 3µg low-affinity λ-phage DNA, and 3µg histone octamer per nucleosome positioning sequence such that DNA molecules reconstituted with unmodified histone octamer contained one nucleosome at each NPS as determined by EMSA and SacI restriction enzyme digests (Figure 3.12B-C). When digested in the linker DNA by SacI, we find that 601-2mer molecules reconstituted with histone octamer containing unmodified H3 are converted to mononucleosomes with negligible free DNA, indicating that a majority of the NPS are occupied by a nucleosome. Interestingly, under the same condition we observe that 601-2mer molecules reconstituted with H3(T118ph) octamer are marginally digested at the SacI site. These results are consistent with the conclusion that ADH structures formed on longer DNA’s have altered DNA-histone wrapping and an increased footprint as seen for ADH species formed on Mp2-247 DNA molecules.

We sucrose gradient purify the 601-2mer nucleosomes and image the unmodified H3 and H3(T118ph) containing species by AFM (Figure 3.11G-H). We find that 94% of the positioning sequences within the 601-2mer arrays containing unmodified H3 are occupied.
by a nucleosome (n = 119 nucleosomes out of n₀ = 127 NPS). Additionally, for saturated 601-2mer molecules containing unmodified H3 histone octamer, nearly every individual nucleosome can be resolved from the adjacent one (Figure 3.11G). Conversely, for each 601-2mer array reconstituted with H3(T118ph) histone octamer under the same conditions as unmodified, the molecules larger size and absence of distinguishable mononucleosomes is consistent with ADH structures observed both for Mp2-247 and 601-17mer molecules containing H3(T118ph). Taken together, these results suggest that the ADH complexes that form on Mp2-247 DNA molecules can also form on significantly longer DNA molecules and are negligibly influenced by the conditions (saturating versus subsaturating) under which the longer DNA molecules are reconstituted.

Figure 3.12: AHD Complexes form on Long DNA Molecules (A) DNA molecule (601-2mer) containing two 601-like positioning sequences (“mp1”) separated by 30 base pairs of linker DNA with and additional 50 base pairs of DNA on each end, Cy3 labeled on the 5′-end of the forward strand Cy5 labeled on the 5′-end of the reverse strand. Blue line indicates location of SacI site. (B) EMSA of 1µg of 601-2mer DNA molecule reconstituted with 3µg of low-affinity DNA and increasing amounts (1.33-3µg) of unmodified histone octamer. “*” indicates the conditions (2.33µg histone octamer) in which both NPS of 601-2mer appear to be occupied; DNA pictogram to left indicates nucleosome occupancy (gray sphere). (C) EMSA of SacI digests of 601-2mer DNA (Lanes 1 and 2), DNA reconstituted with unmodified histone octamer (Lanes 3 and 4) and DNA reconstituted with H3(T118ph) histone octamer (Lanes 5 and 6). Pictogram of mononucleosome DNA fragments indicate SacI digestion products.
3.2.6 Nucleosome assembled by the Nap1 histone chaperone results in the formation of the ADH complexes

Nucleosomes \textit{in vivo} are formed by the deposition of histones onto DNA by histone chaperones [77, 78]. Therefore, we determined if the ADH complexes could be formed with the histone chaperone Nap1, which can deposit both H3/H4 tetramers and H2A/H2B heterodimers to form nucleosomes \textit{in vitro}. We find that unmodified histone deposition by Nap1 onto Mp2-187 (Figure 3.13) and Mp2-247 (Figure 3.13) forms nucleosomes that have the same electrophoretic mobility as nucleosomes formed by salt dialysis. Histone deposition by Nap1 with H3(T118ph) containing histone octamer onto Mp2-187 forms nucleosomes and ADH complexes (Figure 3.13) and deposition onto Mp2-247 forms nucleosomes and LM-ADH complexes (Figure 3.14), each with the same electrophoretic mobility as their respective species formed by salt dialysis. This result suggests that the ADH complexes could be formed \textit{in vivo} by histone chaperones.

We also determined if the ADH complex formation depended on Nap1 concentration. We find that H3(T118ph) histone octamer requires significantly less Nap1 than unmodified histone octamer to assemble both canonical nucleosomes and ADH complexes. In addition, the same Nap1 concentration was required to competitively prevent the formation of both the H3(T118ph) containing canonical nucleosomes and ADH complexes (Figure 3.13-3.14). This suggests that ADH complexes have a similar stability to that of canonical nucleosomes containing H3(T118ph). We were unable to determine if H3(T118ph) histone deposition by Nap1 forms HM-ADH complexes with Mp2-247. This was due to the different Nap1 concentrations required to competitively prevent the formation of unmodified canonical nucleosomes versus ADH complexes, and that the formation of well-positioned nucleosomes is strongly Nap1 dependent.
Figure 3.13: ADH complexes are assembled by yNap1 on Mp2-187. EMSA of (A) unmodified histone octamer (HO) and (B) H3(T118ph) HO reconstituted on Mp2-187 by salt dialysis (lane 1, "R"), unmodified HO mixed with DNA in the absence of yNap1 (lane 2,"-"), and unmodified HO assembled on DNA in the presence of increasing amounts of yNap1 (lanes 3-7, "+"). (C) Quantification of the fraction of yNap1 assembled nucleosomes ("nuc", squares) and remaining free DNA ("DNA", diamonds) as a function of [Nap1] for unmodified (black) and H3(T118ph) HO (gray) from the data in (A) and (B). (D) Fraction of positioned versus depositioned nucleosomes assembled by yNap1 as a function of [Nap1] for unmodified nucleosomes (black square) and H3(T118ph) nucleosomes (gray squares).
Figure 3.14: **ADH complexes are assembled by yNap1 on Mp2-247.** EMSA of (A) unmodified histone octamer (HO) and (B) H3(T118ph) containing HO reconstituted on Mp2-247 by salt dialysis (lane 1, "R"), unmodified HO mixed with DNA in the absence of yNap1 (lane 2, ",-"), and unmodified HO assembled on DNA in the presence of increasing amounts of yNap1 (lanes 3-7, "+"). (C) Quantification of the fraction of yNap1 assembled nucleosomes ("nuc", squares) and remaining free DNA ("DNA", diamonds) as a function of [Nap1] for unmodified HO (black) and H3(T118ph) containing HO (gray) from the data in (A) and (B).
3.3 Discussion

We find that H3(T118ph) can significantly influence nucleosome structure and DNA wrapping around the histone octamer. We observe two types of altered DNA-histone complexes: a low electrophoretic mobility complex, LM-ADH, and a high electrophoretic mobility complex, HM-ADH. Both complexes have twice the volume compared to canonical nucleosomes, a height similar to canonical nucleosomes, and an elliptical shape. These results combine to suggest that the ADH complexes have 2 histone octamers that are positioned side by side (Figure 3.15). There are differences between the LM-ADH and HM-ADH complexes, however. The LM-ADH complexes have the same ratio of histone octamer to DNA, they have the same DNA footprint as canonical nucleosomes, and they can be converted to canonical nucleosome when heated to 55°C. In contrast, the HM-ADH complexes have twice the amount of histone octamer to DNA, they have a larger DNA footprint than canonical nucleosomes, and they cannot be converted to canonical nucleosomes by heating. Therefore, we conclude that the LM-ADH complexes contain two histone octamers positioned side by side with two DNA molecules wrapped around the two histone octamers. Furthermore, we conclude that the HM-ADH complexes contain 1 DNA molecule wrapped around 2 histone octamers.

It appears that the two phosphates on H3(T118) near the nucleosome dyad cause the DNA to avoid contact with the histone octamer near the dyad. The DNA phosphate backbone is within 3Å of H3(T118), so the addition of a phosphate at this residue could introduce a strong electrostatic repulsion between the phosphate on the Threonine side chain and the DNA phosphate backbone. Our results suggest that the DNA molecule avoids the phosphothreonine by partially wrapping around two separate histone octamers. For short DNA molecules it appears that two DNA molecules are required to make most of the DNA-histone contacts while DNA molecules of about 250 base pairs and longer can do this with one DNA molecule (Figure 3.15). Additionally our observation that HM-ADH, and possibly LM-ADH complexes are assembled both during salt gradient dialysis and via yeast Nap1 mediated nucleosome assembly supports the conclusion that these species form by
altered DNA wrapping around the histone octamer. In contrast, lysine acetylation near the nucleosome dyad does not impact nucleosome structure. This suggests that the addition of a negative charge with a phosphorylation has a much more dramatic impact on nucleosome structure than the removal of a positive charge by lysine acetylation.

Our studies indicate that phosphorylation in the DNA-histone interface can significantly impact nucleosome structure and DNA wrapping. There are 4 additional histone residues within the DNA histone interface that have been identified as phosphorylation sites [H4(S47), H3(S86), H3(T45), and H3(Y41)] [60, 66, 67, 69]. To date there is little understanding of these buried phosphorylations. Future studies will be required to determine if these additional histone PTMs impact nucleosome structure and how these modifications function in vivo.
3.4 Methods

3.4.1 DNA constructs

The Mp2-147, Mp2-187, Mp2-192, and Mp2-247 DNA molecules were prepared as previously described in Chapter 1 from the pMp2 plasmid [94]. The 5S-147, 5S-187, 5S-192, and 5S-247 were prepared by PCR from the plasmid p5S in which the Mp2 positioning sequence in pMp2 was replaced with the *L. variegatus* 5S RNA positioning sequence [116] (See Appendix D for DNA and oligo sequences). The 5SX-192, Pho5-N1, Pho5-N2, and Gal4-192 molecules were prepared from plasmids containing the *X. borealis* 5S RNA positioning sequence [117], the Pho5 promoter containing the first (N1) and second (N2) nucleosome positions of the upstream activator sequence (UASg) [118], and the Gal1-Gal10 UASg containing the nucleosome position over the Gal4 binding site [119], respectively (See Appendix D for DNA and oligo sequences). The 601-17mer array was prepared as previously described [120], and 601-2mer molecules were prepared by PCR from the plasmid p601.2mp1 in which the MP2 positioning sequence in pMp2 was replaced with a tandem repeat of Mp1 and Mp2 NPS separated by 30 base pairs of linker DNA (See Appendix D for DNA and oligo sequences).

3.4.2 Expression and Purification of Wild Type and Mutant Histones

Mutations H4(C0) and H2A(C0) were introduced by site directed mutagenesis (Stratagene). All histones were expressed and purified following [79, 107] as detailed in Appendix A. Histones H4(C0) and H2A(C0) expressed as a mixture of three different species that were resolved by cation exchange chromatography by standard histone purification procedures (Appendix A). One of the three species exhibited a mass consistent with H4(C0) (m/z observed = 11341, expected = 11339) or H2A(C0) (m/z observed = 14054, expected = 14056). The other mass species observed were consistent with S-methylated Cysteine and Methionine-Cysteine substitution. We attribute this to impaired Methionine editing from inserting the Cysteine by site directed mutagenesis into the histone sequence between the N-terminal Methionine that gets edited off after expression and H2A or H4 Serine 1.
3.4.3 Nucleosome Preparation

Nucleosomes were prepared by salt dialysis with purified histone octamer and DNA and purified by sucrose gradient centrifugation as detailed in Appendix C. The addition of 0.5mM MgCl$_2$ was required to maintain stability during the purification of H3(T118ph) nucleosomes. H3(T118ph) was prepared by expressed protein ligation as previously described [93, 121].

3.4.4 Phosphatase Treatment

Dependence of ADH complex formation on H3(T118) phosphorylation was determined by treating unmodified or H3(T118ph) histone octamer with Arctic phosphatase (NEB). Ocatamers at 1mg/ml were incubated with 1U/µl Arctic phosphatase in 50mM Tris-HCl, 1mM MgCl$_2$, 0.1mM ZnCl$_2$, 1M NaCl, pH 8.0 at 37°C for 40 minutes. Reactions were quenched by addition of EDTA to 2.5mM final concentration. Treated samples were then assayed by MALDI-TOF mass spectroscopy and used for reconstitution.

3.4.5 Fluorescence Measures of Histone:DNA content

The ratio of H2A/H2B hetordimer to DNA or H3/H4 tetramer to DNA of unmodified nucleosomes compared to H3(T118ph) nucleosomes was quantified using Cy5-labeled H2A(C0), Alexa488-labeled H4(C0), and Cy3-labeled DNA. Cy5-H2A(C0) and Alexa488-H4(C0) were labeled before refolding into the histone octamer (See Appendix B). Labeling efficiency was 50-60% as determined by UV-Vis absorbance and mass spec (data not shown). Fluorescent octamer was reconstituted onto Cy3-end-labeled Mp2-147, Mp2-187, and Mp2-247. Reconstituted samples were verified for absence of FRET between the three fluorescent tags on a Fluoromax 3 spectrofluorometer (Horiba). The three fluorescent dyes were validated to be spectrally separable by a Typhoon 8600 variable mode imager by end-labeling 3 different DNA molecules with Alexa488, Cy3, or Cy5 and resolving by native page gel. Alexa488 fluorescence is resolved by 488nm laser excitation and imaging with a 520 ± 20nm bandpass emission filter; Cy3 by 532nm excitation laser and 610 ± 15nm emission filter; Cy5
by 633nm excitation laser and 670 ±15nm emission filter. Reconstituted nucleosomes were resolved by 5% Native Page in 0.3x TBE at 20V/cm (See Appendix E), soaked for at least 20 minutes in 1M NaCl, imaged by for each florescent molecule as described above on a Typhoon 8600 variable mode imager, and quantified by ImageQuant.

3.4.6 Thermal Decoupling

Purified Mp2-187 and Mp2-247 ADH complexes containing H3(T118ph) were thermally decoupled by heating samples at 50nM in 20mM Tris (pH 8.0) at 53‰ for 0-90 minutes. Decoupling reactions were quenched by transferring 10µl of the heated nucleosomes to 2µl of 15% sucrose in 0.6x TBE (3% sucrose, 0.2x TBE final) on ice. Samples were analyzed by 5% PAGE in 0.2x TBE. The gel was pre-run for 3 hours before running the samples for 3 hours at 20 volts/cm at 4°C with continuous buffer recirculation (See Appendix E). Gels were imaged with a Typhoon 8600 variable mode imager (GE Healthcare) and quantified by ImageQuant.

3.4.7 Nap1 Assembly

Unmodified and H3(T118ph) nucleosomes were assembled from DNA and purified histone octamer by yNap1. His6 tagged yNap1 (generous gift from Toshio Tsukiyama) was expressed and purified as previously reported [122] (See Appendix F). DNA at 12.5 ng/µl and histone octamer at 12.5 ng/µl were incubated with 0-7.2µM yNap1 dimer at 30°C for 60 minutes in 130mM NaCl, 0.5mM MgCl2, 7.5mM Tris pH 7.5, 0.25mM EDTA, 0.25mM DTT, 0.1mg/ml BSA, 2.5% glycerol, 0.01% NP40, 0.01% Tween20. Reactions were then resolved by 5% Native PAGE in 0.2x TBE at 20V/cm at 4°C for 3 hours with continuous buffer recirculation (See Appendix E).

3.4.8 Exonuclease III Mapping

The nucleosome positions within the Cy3/Cy5-labeled Mp2-187 and Mp2-247 DNA molecule were determined with ExoIII mapping. Reactions were carried out in an initial volume of 50µl with 10nM nucleosomes and 50U/ml of ExoIII (NEB) in 20mM Tris pH8, 0.5mM
MgCl₂ at 16°C to prevent H3(T118ph) nucleosome disassembly (data not shown). At each time point, a 7µl aliquot of the reaction was quenched with EDTA to a final concentration of 20mM. A final concentration of 1 mg/ml of proteinase K and 0.02% of SDS was added to each time point to remove the histone octamer from the DNA and samples were separated by 8% denaturing PAGE in 7 M Urea and 1x TBE (See Appendix E). The sequence markers were prepared with a SequiTherm Excel II DNA sequencing kit (Epicentre) using Cy3 or Cy5 labeled primers, an Mp2-187 or Mp2-247 DNA template and either ddATP or ddTTP (See Appendix E). Results were imaged by a Typhoon 8600 variable mode imager (GE Healthcare), which detects Cy3 and Cy5 separately in the same gel. The Cy3 and Cy5 ladders could be loaded in the same lanes to increase accuracy of the mapping gel readout.

3.4.9 MNase Footprinting

Histone protection of the Mp2-187 and Mp2-247 DNA molecules was determined by MNase digestion. Reactions were carried out in an initial volume of 10µl with 10nM nucleosomes and 0-40U/ml of MNase (NEB) in 20mM Tris pH8, 0.5mM CaCl₂ at 16°C to prevent H3(T118ph) nucleosome disassembly. After 20 minutes, reactions were quenched with 15mM EDTA final concentration and resolved by 5% 29:1 native acrylamide gel with 0.3x TBE at 300V for 1 hour (See Appendix E). Gels were stained with Cybr Gold (Invitrogen) and imaged by a Typhoon 8600 variable mode imager (GE healthcare) using 488nm excitation laser and 520 ± 20nm emission filter.

3.4.10 AFM imaging

For imaging of Mp2-147 and Mp2-247, purified nucleosomes were diluted to 0.5nM in 0.5x TE, 0.5mM MgCl₂, and 0.01% Glutaraldehyde and incubated on ice for 30 minutes; for 601-2mer and 601-17mer, purified arrays were diluted to 0.5nM in 0.5x TE. Samples were then deposited on poly-D-lysine-treated mica surface as previously described [120]. Samples were then imaged with a Dimension Icon with ScanAsyst SPM (Bruker) using Peak Force Mode and ScanAsyst Air tips (Bruker) with a scan rate of 1 Hz and 0.1 pN peak force. Images were processed and analyzed with Gwyddion 2.19 open source software.
Chapter 4
POST-TRANSITIONAL MODIFICATIONS OF THE DNA-HISTONE INTERFACE THAT CONTROL DNA UNWRAPPING

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Shimko J.C., North J.A., Bruns A.N., Poirier M.G., Ottesen J.J.
Preparation of fully synthetic histone H3 reveals that acetyl-lysine 56 facilitates protein binding within nucleosomes

4.1 Introduction

In Chapters 1-3 we explored the physical consequences of histone post-translational modifications (PTMs) of the nucleosome dyad on DNA-histone binding affinity, nucleosome mobility, DNA accessibility, and chromatin structure. We found that while the histone PTMs H3(K115ac), H3(K122ac), and H3(T118ph) negligibly altered DNA accessibility of individual nucleosomes, these modifications can tune the DNA-histone binding energy of nucleosome formation, $\Delta \Delta G^{\text{nuc}}_{\text{sample}}$ by $\sim 0.4 \text{ kcal/mol}$ to $\sim 2.0 \text{ kcal/mol}$. These PTMs also facilitate nucleosome mobility by 2-28 fold faster than unmodified nucleosomes. Additionally H3(T118ph) induces the formation of alternate DNA-histone complexes that consist of DNA assembled around a core of two histone octamers. Given that point mutation
of H3(K115), H3(T118), H3(K122), and H4(S47) lead to defects in transcription, regulation and DNA repair refs, these results suggest that nucleosome dyad PTMs may regulate nucleosome assembly, disassembly, and repositioning by histone chaperones, chromatin remodelers, and other regulatory complexes.

In this chapter we turn our focus to the nucleosome entry-exit region where the DNA wraps into and out of the nucleosome [9]. The entry-exit region encompasses a large number of key DNA-histone contacts in which the first 10-12 base pairs of the DNA wrapped within the nucleosome interact with the H3/H4 tetramer; the subsequent ∼30 base pairs of DNA interact with the H2A/H2B dimer at which point the DNA returns to interacting with the H3/H4 tetramer as it proceeds toward the dyad. There are at least 15 PTMs that are distributed along the DNA-histone interface beginning at the DNA entry-exit region and extending 45-50 base pairs into the nucleosome at the Loss of Ribosomal Silencing Region (See Chapter: Introduction). There are currently no known acetylation/phosphorylation/methylation PTMs in the DNA-histone interface between base pair 50 where H3 Serine 86 phosphorylation occurs and base pair 65 near the dyad where H3 Threonine 118 and H4 Serine 47 phosphorylation occurs. As with the dyad PTMs, these PTMs within the first 50 bases of the DNA-histone interface are poised to alter DNA-histone binding. While these PTMs may function to regulate nucleosomes stability and mobility, they may also control DNA unwrapping.

Histone acetylation of the unstructured DNA tails has been shown to regulate nucleosome site exposure [49], and in addition, acetylation of H3 Lysine 56 [H3(K56ac)], which is located at the DNA-histone interface 10 base pairs into the nucleosome entry-exit, was recently shown to shift the first 10-20 bases of the nucleosome entry-exit toward the unwrapped state [103]. These data suggest that histone PTMs in the nucleosome entry/exit region could function to control DNA site exposure for the access of DNA-binding proteins that must interact with nucleosomal DNA to perform their prescribed function [30, 97]

Histone PTMs: trimethylated H3 Lysine 36 [H3(K36me3)], phosphorylated H3 Tyrosine 41 [H3(Y41ph)], phosphorylated H3 Threonine 45 [H3(T45ph)], acetylated H3 Lysine 56 [H3(K56ac)], and acetylated H4 Lysines 77 and 79 [H4(K77ac,K79ac)] are of particular
interest due to their recently discovered roles in transcription regulation and DNA repair. Starting at the very beginning of the nucleosome entry-exit, H3(K36me3) is located just outside of the H3 α-N helix between the entry-exit DNA gyre and the dyad DNA gyre as the H3 tail comes up between these two regions and out of the nucleosome. H3(K36me3) is a mark of active transcription elongation [70], and targets the ISW1b remodelling complex to chromatin for transcription activation [123]. The Proline-Tryptopan-Tryptophan-Proline (PWWP) domains of ISW1b, LEDGF/p75, Dmnt3a, Brpf1, MSH6, NSD1, NSD2 and N-PAC have been shown to preferentially bind H3(K36me3) [123–126], but have been shown to also bind DNA as well [127–129]. These studies suggest that while H3(K36me3) may function similarly to H3 tri-methylated Lysine 4 on the histone tail as a binding module [70], it may also facilitate DNA unwrapping for PWWP domain binding to H3(K36me3) and the nucleosomal DNA for transcription regulation and DNA repair [123–126, 130].

H3(Y41ph) and H3(T45ph) are slightly further into the DNA entry-exit, located under base pairs 4-5. In mammalian cells H3(Y41ph) plays a role in gene regulation and chromatin remodelling [67]. This mark controls transcription of genes connected to normal hematopoiesis and leukemia [54], and is important for embryonic stem cell self-renewal [131]. Similarly, in humans H3(T45ph) is important mark for cell apoptosis [69] and in yeast is a replication-associated PTM required for genomic integrity and DNA repair [68].

H3(K56ac), located 10 base pairs within the DNA entry-exit, is the most well understood, and is required for a number of DNA processes [132, 133]. H3(K56) appears to be acetylated prior to deposition onto newly replicated DNA [134] and is important for DNA repair [135, 136], maintenance of genomic stability [71], and transcriptional regulation [62, 134, 137]. It has been suggested that H3(K56ac) alters chromatin structure and dynamics, allowing accessibility to DNA metabolic proteins [134] and acting as a signal to DNA damage checkpoints [135]. The preparation of H3(K56ac) using the evolved pyrrolysine incorporation machinery was recently reported [103]. This study suggested that H3(K56ac) modestly increased SWI/SNF and RSC-catalyzed nucleosome repositioning and increased DNA unwrapping at the entry-exit region. They also found that H3(K56ac) did not influence chromatin compaction. However, a separate study of the acetylation mimic
H3(K56Q) found that it inhibited interactions between chromatin fibers [138]. Interestingly, structural studies of the acetylation mimic H3(K56Q) observe minimal changes in the fully wrapped nucleosome structure [139]. However, such crystallography experiments are a static picture and DNA dynamics cannot be resolved. In yeast, the acetylation mimic, H3(K56Q), leads to increased sensitivity to MNAse digestions and derepresses rDNA and telomeric DNA silencing, while mutation H3(K56R), which mimics de-acetylation, does not lead to defects in MNAse protection and rDNA/telomeric silencing [46, 140]. Taken together these results are consistent with the notion that H3(K56ac) may function in part as a DNA entry-exit gate for access to nucleosomal DNA [62, 103]. It is also possible that H3(K56ac) functions to alter nucleosome stability and mobility. Recently, it was reported that H3(K56ac) reduces H3/H4 tetramer binding by the histone chaperone Nap1 [141]. In addition H3(K56Q) increases nucleosome mobility during thermal repositioning by nearly 2-fold [142]. However, the precise influence of H3(K56ac) on DNA site exposure and protein binding within the nucleosome remains a significant unknown.

Finally, H4(K77ac) and H4(K79ac), which are located 45 base pairs into the nucleosome, occur simultaneously [60], and reside in a structurally interesting region of the nucleosome called the Loss of Ribosomal DNA Silencing (LRS) region [34, 65]. The histone fold loops, H3-L1 and H4-L2, of the LRS are structurally similar to the H3-L2 and H4-L1 loops of the nucleosome dyad where H4(S47ph), H3(K115ac), and H3(T118ph), and H3(K122ac) are located [9]. The acetyllysine mimics H4(K77Q) and H4(K79Q) alter telomeric silencing and exhibit LRS phenotypes [65]. Additionally, mutations near H4(K79) also induce LRS phenotypes as well [65]. However, these LRS mutants do not result in SWI/SNF Independent (SIN) phenotypes nor influence chromatin higher-order structure [34], indicating that these DNA histone interface PTMs in a structurally similar region outside of the dyad influence transcription differently than dyad PTMs [34].

We have tested the hypothesis that H3(K36me3), H3(Y41ph), H3(T45ph), H3(K56ac), and H4(K77ac,K79ac) function in part to regulate nucleosome site exposure using a combination of Restriction Enzyme (RE) kinetics and transcription factor accessibility assays. Histone H3(K,c36me3,C110A) was produced by engineering H3(K36C,C110A) and incorpar-
ing Lysine 36 trimethyl with a methyl-Lysine analog [101] that resulted is the replacement of the γ-carbon with sulfur (hence the designation with "Kc"). H3(Y41ph) and H3(T45ph) were mimicked using the phospho-mimetic amino acid substitution, Glutamic Acid, to produce H3(Y41E,C110A) and H3(T45E,C110A). H3(K56ac,C110A) was produced by Ottesen and colleagues by fully-synthetic native chemical ligation and the simultaneously acetylated H4 Lysines 77 and 79 [H4(K77ac,K79ac)] were produced semi-synthetically by expressed protein ligation [51, 93]. We find that all of these histone PTMs except H3(K36me3) increase nucleosome unwrapping and facilitate transcription factor binding to a transcription factor site located between base pairs 8 and 27 within the nucleosome by a factor of nearly 2-fold each. Additionally, we find that modifications H3(K56ac) and H4(K77ac,K79ac) increase DNA site exposure throughout the entire nucleosome, which is explained by the fact that DNA unwrapping of the nucleosome is cooperative [143, 144]. These results suggest that the nucleosome has a distinct "unwrapping" region that begins at the DNA-histone interface of the nucleosome entry-exit region with H3(Y41) and proceeds at least 45 base pairs into the nucleosome to the LRS region with H4(K77)/H4(K79). This is in distinct contrast to the nucleosome dyad in which PTMs appear to primarily control nucleosome stability, mobility, and chromatin structure (Chapters 1-3). Our results suggest that histone PTMs that are located in this unwrapping region may function to regulate transcription, replication, and DNA repair by controlling nucleosome site exposure and accessibility to metabolic proteins.
4.2 Results

4.2.1 The introduction of nonnative cystienes in histone H3 alters nucleosome structure

Due to the location of H3 entry-exit PTMs, H3(K56ac), H3(T45ph), H3(Y41ph) within the H3 protein sequence, these modifications are not amenable to synthesis using expressed protein ligation as are the dyad PTMs. Therefore, a synthesis strategy had to be devised in which 3 separate peptides, an N-terminal, a middle bearing the precise modification, and a C-terminal, are synthesized and ligated together using Native Chemical Ligation (NCL). This method requires a Cystiene at each ligation point. However, *X. laevis* histone H3 bears only one native Cystiene at the 110th amino acid. To achieve this, the modified *X. laevis* H3(C110A) sequence, which is commonly used in biophysical studies [50], was selected as the basis for the ligation strategy. The C110A substitution occurs in yeast and has not previously been reported to affect nucleosome structure, positioning, and DNA unwrapping [95, 97]. Cystiene residues were then introduced into H3(C110A) at Arginine 40 and Serine 96 based on homology alignments that found H3(R40C) in *Cairina moschata* [145] and H3(S96C) in the H3.1 variants of *Homo sapiens*, *Mus musculus* [146], and *Caenorhabditis elegans* [147]. These Cystiene residues could be crafted into a two-step NCL strategy in which the longest synthetic segment would be a central 56-residue peptide containing the PTM of choice (Figure 4.1A, see [148] for full details). As proof of principle we began with synthesis of H3(R40C,S96C,C110A) and (R40C,K56Q,S96C,C110A).

Nucleosomes were reconstituted with the 601L-end DNA molecule which contained a Cy3 5′-end-labeled 147 base pair 601 nucleosome positioning sequence with a LexA binding site located between base pairs 8 and 27 (Figure 4.2A, 601L-end), and histone octamer containing Cy5-labeled H2A(K119C) (Figure 4.2B) and either wild-type unmodified H3, H3(R40C,S96C,C110A), H3(R40C,K56Q,S96C,C110A), or synthetic H3(R40C,K56ac,S96C,C110A). Following reconstitution, nucleosomes were purified by sucrose gradient (Figure 4.2D).

To determine if the Cystienes introduced at R40 and S96 alter nucleosome structure,
we measured the initial FRET efficiency of each nucleosome construct. Unfortunately, we found that the FRET efficiency decreased from 0.62 ± 0.02 to 0.43 ± 0.01 when wild-type unmodified H3 containing nucleosomes were compared to the control with H3(R40C,S96C,C110A) nucleosomes (Figure 4.3). This observation suggests that the H3(R40C,S96C) containing nucleosomes display altered structure and/or dynamics. Nucleosomes containing H3(R40C,K56Q,S96C,C110A) or H3(R40C,K56ac,S96C,C110A) resulted in a further decrease in FRET efficiency to 0.38 ± 0.02 and 0.35 ± 0.04, respectively (Figure 4.3). However, this additional reduction in FRET efficiency is significantly less than that induced with the Cystiene substitutions alone. These results reveal the potential pitfalls associated with the introduction of non-native histone sequence substitutions in nucleosome structure and/or dynamics.

Figure 4.1: Assembly of modified histone H3 by NCL used in the analysis of reconstituted nucleosomes. (A) Schematic representation of total synthesis of H3(K56ac) using sequential NCL in solution phase. Synthesis of (R40C,K56ac,S96C,C110A) proceeded through the second ligation step; synthesis of H3(K56ac) used new ligation sites and added the final desulfurization step to generate the native sequence. (B) Proteins generated and characterized: H3(R40C,K56ac,S96C,C110A), H3(K56ac,C110A), H3(K115ac,K122ac), H4(K77ac,K79ac).
Figure 4.2: DNA substrates and reconstituted nucleosomes containing Cy3 and Cy5 for Fluorescence Resonance Energy Transfer (FRET) measurements. (A) The 147 base pair DNA molecules, 601L-end and 601-end, contain the 601 positioning sequence with and without a LexA binding site at base pairs 8-27, respectively, and a Cy3 fluorophore attached to the 5′-end. (B) Structure of the nucleosome [37] reconstituted for FRET analysis (49); PDB ID: 1KX5. Histone H3 is depicted in blue ribbons with Lysine 56 highlighted in orange. Histone H2A(K119C) (magenta) has been modified with Cy5. DNA construct specifically labeled at the 1st base with Cy3 (green) and containing a LexA binding site (red). (C) and (D) are Cy3 fluorescence images of PAGE analysis of nucleosome reconstitutions prior and after purification by sucrose gradient centrifugation, respectively. (Reproduced with permission)

4.2.2 Acetylation of H3(K56) reduces DNA wrapping at the entry/exit region of the nucleosome

Because the introduction of non-native Cystiene residues significantly influenced nucleosome structure and/or dynamics, we improved our method for preparing fully synthetic native histones containing defined PTMs. In this second-generation approach, we combined sequential NCL with a desulfurization step [149] (Figure 4.1A). This scheme allows the more common Alanine residue to be used as a ligation site. We selected the native Alanine residues H3(A47) and H3(A91) as ligation sites which allowed for the synthesis of H3(K56ac,C110A).
Figure 4.3: Fluorescence Resonance Energy Transfer (FRET) efficiency and LexA binding are impacted by introduced Cystenes in H3. (A) Fluorescence emission spectrum from nucleosomes containing wild-type unmodified H3 (purple), H3(R40C,S96C,C110A) (blue), H3(R40C,K56Q,S96C,C110A) (red) or H3(R40C,K56ac,S96C,C110A) (green) when excited at 510nm (donor excitation). (B) Fluorescence emission spectrum from nucleosomes in (A) when exited at 610nm (acceptor only excitation). (C) The fluorescence energy transfer (FRET) efficiency for nucleosomes containing wild-type unmodified H3, H3(R40C, S96C,C110A), H3(R40C,K56Q,S96C,C110A) or H3(R40C,K56ac,S96C,C110A) determined from the \( \text{ratio}_A \) method (See Chapter: Introduction) (63). The error bars were determined from the standard deviation of at least three separate measurements. (Reproduced with permission)
The H3(A47) residue was incorporated as Thiazole in the middle peptide, and H3(A91) was incorporated as Cystiene in the C-terminal peptide to allow sequential ligation (Figure 4.1). After ligation, free-radical desulfurization [149] was carried out directly on the crude ligation mixture to convert the ligation site Cystienes into Alanines as found in the native H3 sequence.

Sequential NCL was repeated using a synthetic middle segment which bore the unmodified Lysine 56 residue to generate synthetic H3(C110A) [H3(C110A)\text{syn}] (Figure 4.2D). Nucleosomes containing this synthetic unmodified protein were directly compared to nucleosomes reconstituted with recombinant H3(C110A) [H3(C110A)\text{rec}] to demonstrate that the synthetic process did not introduce any undesired modifications.

We examined the biophysical properties of H3(K56ac) and H3(K56Q) using the FRET system described above (Figure 4.2A). We determined the FRET efficiency at low ionic strength [0.5x TE buffer with 1mM NaCl from disodium ethylenediamine tetraacetic acid (EDTA)] for nucleosomes containing wild-type unmodified H3, H3(K56Q), H3(C110A)\text{rec}, H3(C110A)\text{syn}, H3(K56Q,C110A), and H3(K56ac,C110A), and at physiological ionic strength (0.5x TE buffer with 75mM or 130mM NaCl) for nucleosomes containing H3(C110A)\text{rec}, H3(C110A)\text{syn}, H3(K56Q,C110A), and H3(K56ac,C110A) (Figure 4.4, Table 4.1). We find that the FRET efficiency is reduced by about 15-20% for nucleosomes containing H3(K56ac,C110A), H3(K56Q), and H3(K56Q,C110A) compared to unmodified H3. In contrast, the FRET efficiency is increased by only 6% with H3(C110A)\text{rec} and is unaltered for H3(C110A)\text{syn} with respect to unmodified H3 (Figure 4.4C). These results indicate that H3(K56ac) increases the average distance between the DNA and the histone surface at the entry-exit region under low and physiological ionic strengths. In addition, H3(K56Q) appears to quantitatively mimic the effects of H3(K56ac) on the steady-state structure, and this difference does not depend on ionic strength.

Nucleosomes containing H3(K56ac) or H3(K56Q) showed a slight shift in electrophoretic mobility (Figure 4.2C,D). Altered mobility could be explained by the increase in DNA unwrapping which is consistent with our FRET measurements. Alternatively, it could be attributed to a shift in nucleosome position. We therefore determined the positions
of nucleosomes containing H3(C110A)$_{rec}$ and H3(K56Q,C110A) by hydroxyl radical cleavage [95] using Fe(III) (s)-1-(p-bromoacetamidobenzyl) ethylenediamine tetraacetic acid (FeBABE, Figure 4.5A). This label did not alter the gel mobility of nucleosomes containing H3(C110A)$_{rec}$ or H3(K56Q,C110A) (Figure 4.5B). We found that the cleavage pattern was indistinguishable between these nucleosomes (Figure 4.5C,D). This indicates that the observed altered mobility and reduced FRET of nucleosomes containing H3(K56Q) and, by extension, H3(K56ac) are not due to nucleosome repositioning but rather due to increased DNA unwrapping.

**4.2.3 Histone PTMs H3(Y41ph), H3(T54ph), H4(K77ac), and H4(K79ac) may regulate DNA unwrapping**

Given that H3(K56ac) appears to reduce the FRET efficiency of the Cy3/Cy5-labeled nucleosomes system (Figure 4.2A-B) via increased DNA unwrapping instead of nucleosome repositioning, we sought to determine if other histone PTMs or their amino acid substitution mimics in the DNA-histone interface may possess a similar function. We reconstituted 601L-end DNA molecules with H2A(K119C) Cy5-labeled histone octamer containing unmodified H3(C110A), H3 trimethyl-lysine 36 analog H3(K$_{36me3}$,C110A), H3 Tyrosine 41 phosphomimetic H3(Y41E,C110A), H3 Threonine 45 phospho-mimetic H3(T45E,C110A), or acetylated H4 Lysines 77 and 79 [H4(K77ac,K79ac)]. Nucleosomes were then purified by sucrose gradient and checked for purity by EMSA (Figure 4.6A).

As with H3(K56ac) we observe by EMSA a hypershift in nucleosome mobility of nucleosomes bearing H3(Y41E) and H3(T45E), but not with nucleosomes bearing H3(K$_{36me3}$) (Figure 4.6C). These results are consistent with the conclusion that within the first 10bp of the nucleosome entry-exit region histone PTMs H3(Y41ph), H3(T45E), and H3(K56ac), function in part to shift the inherent nucleosomes site exposure equilibrium toward the unwrapped state. Conversely, H3(K36me3) does not appear to alter nucleosome unwrapping. For H4(K77ac,K79ac) 45 base pairs into the nucleosome and H3(K115ac,K122ac) at the dyad we do not observe any hypershift in nucleosome mobility.

As an independent verification we measured the steady-state FRET efficiency of each
Figure 4.4: **Fluorescence Resonance Energy Transfer (FRET) efficiency is reduced by H3(K56Q) and H3(K56ac).** (A) Fluorescence emission spectra from nucleosomes containing unmodified H3(C110A) (blue), H3(K56Q,C110A) (red) and H3(K56ac,C110A) (blue) when excited at 510nm (donor excitation) with 130mM NaCl. (B) Fluorescence emission spectrum from nucleosomes in (A) when exited at 610nm (acceptor excitation). (C) The FRET efficiency as determined by the (ratio)$_A$ method (See Chapter: Introduction) [83] of nucleosomes containing unmodified H3 (purple) and H3(K56Q) (magenta) at 1mM NaCl, and unmodified H3(C110A)$_{rec}$ (blue), unmodified H3(C110A)$_{syn}$ (orange), H3(K56Q,C110A) (red) and H3(K56ac,C110A) (green) at 1mM, 75mM and 130mM NaCl. The error bars were determined from the standard deviation of three separate measurements. (Reproduced with permission)
Figure 4.5: Nucleosome positioning is not influenced by K56Q. (A) The crystal structure of the nucleosome [37] with H3(K56) shown in orange, H4(S47), which is replaced with a Cystiene and labeled with FeBABE, shown in blue, and the bases that are cleaved by FeBABE shown in red. (B) EMSA of nucleosomes labeled with FeBABE at H4(S47C). Lane 1 contains the DNA substrate. Lanes 2 and 4 contain nucleosomes with H3(C110A)rec with the top and bottom DNA strand 5’-labeled with Cy3, respectively. Lanes 3 and 5 contain nucleosomes with H3(K56Q,C110A) with the top and bottom DNA strand 5’-labeled with Cy3, respectively. (C) and (D) Denaturing polyacrylimide gel electrophoresis where the top and bottom DNA strands, respectively, are visualized by Cy3 fluorescence of the nucleosomal DNA cleaved by FeBABE for 0, 5 and 10 minutes. Within each gel, lanes 1-3 and 10-12 contain sequencing tracks terminated with ddGTP, ddATP, and ddTTP respectively, lanes 4-6 contain nucleosomes with H3(C110A)rec and lanes 7-9 contain nucleosomes with H3(K56Q,C110A). (Reproduced with permission)
nucleosome at low ionic strength (1 mM NaCl, Figure 4.6C). We observe a ∼26% decrease in initial FRET efficiency compared to unmodified nucleosomes for H3(Y41E), H3(T45E), and H3(K56ac), whereas for H3(K,36me3) we observed no change in FRET. These results further implicate that H3(Y41ph) and H3(T45ph) function similarly to H3(K56ac) to increase nucleosomes site exposure toward the unwrapped state. For H4(K77ac,K79ac) we only observe a small 5% decrease in the initial FRET efficiency and we observe no change in FRET for H3(K115ac,K122ac) compared to unmodified, indicating little or no change in inherent DNA unwrapping. This supports the conclusion that histone PTMs in the DNA-histone interface of the nucleosomes entry-exit region function in part to increase inherent nucleosome unwrapping.

4.2.4 Acetylation of H3 lysine 56, and H4 Lysines 77 and 79 facilitates protein binding within the nucleosome at low ionic strength

We determined the influence of H3(K56ac), H3(K56Q), H4(K77ac,K79ac), and H3(K115ac,K122ac) on DNA accessibility using Fluorescence Resonance Energy Transfer Measures of Protein Binding within Nucleosomes (See Chapter: Introduction). We reconstituted nucleosomes with modified and unmodified histone octamer containing Cy5-labeled H2A(K119C) and the 601L-end DNA molecule (4.2A), which contains a LexA binding site between base pairs 8 and 27 of the 601 nucleosome positioning sequence and a 5'end labeled Cy3 fluorophore on the 1st base pair. We performed LexA binding studies by detecting the reduction in FRET efficiency that is due to LexA binding to its target sequence located within the nucleosome (Figure 4.7A) at low ionic strength, since previous FRET studies of DNA unwrapping have been carried out under these conditions [30, 31, 103]. We initially titrated LexA from 0 µM to 3 µM in the presence of 1.0 mM NaCl and found that the FRET efficiency reduces to approximately 0.2 at high concentrations of LexA (Figures 4.7B-E and 4.8). Such a nonzero FRET efficiency at high LexA concentrations is concordant with previous site accessibility measurements [30]. These results are consistent with the conclusion that unmodified nucleosomes and nucleosomes containing H3(K56ac), H3(K56Q), H4(K77ac,K79ac), and H3(K115ac,K122ac) are not
Figure 4.6: Other PTMs in the nucleosome entry-exit may increase nucleosome unwrapping (A) Face view (left) and Top view (right) of nucleosome structure (Luger) indicating location of LexA binding site (red) and Cy3-label (green) within the 601L-end DNA molecule; H2A(K119C) location of Cy5-label (magenta). Location of Histone PTMs indicated: H3(K36me3) (forest), H3(Y41ph) (mustard), H3(T45E) (royal), H3(K56ac) (orange), H4(K77ac) (yellow), H4(K79ac) (cream), H3(K115ac) (blue), H3(K122ac) (teal); PDB ID: 1KX5. (B) EMSA of sucrose gradient purified nucleosomes reconstituted with the Cy3-labeled 601L-end DNA molecule and histone octamer bearing Cy5-labeled H2A(K119C) and the indicated modification. Note: H4(K77ac,K79ac) histone octamer contains unmodified H3(C110). (C) Initial FRET efficiency determined by the (Ratio)A method (See Chapter: Introduction) [83] for Cy3/Cy5-labeled nucleosomes in (B) bearing the indicated modification. Values and error bars are the average and standard deviation of three independent measurements. (D) FRET efficiency of modified nucleosomes (Eo-mod) relative to nucleosomes lacking the particular modification (Eo-unmod) for the nucleosomes in (C).
disassembled by LexA binding.

The FRET efficiencies in the presence of LexA were fitted to a non-cooperative binding curve, and the concentration of half-saturation in which LexA is bound to 50% of its site within nucleosome, \(S_{0.5-nuc}\), was determined for nucleosomes containing unmodified H3, H3(K56Q), H3(C110A)\(_{rec}\), H3(C110A)\(_{syn}\), H3(K56Q,C110A), H3(K56ac,C110A), H4(K77ac,K79ac), and H3(K115ac,K122ac) (Figures 4.7D-F, 4.8C, Table 4.1). The concentration of half-saturation in which LexA is bound to 50% of its site within naked DNA, \(S_{0.5-DNA}\), was determined by gel shift analysis (Figure 4.10A) [30]. We used two separate preparations of LexA with \(S_{0.5-DNA}\) values of 0.14 ± 0.02 nM and 0.32 ± 0.04 nM.

We determined the site exposure equilibrium constant \(K_{eq}^{\text{wrap}}\) from the half-saturation value of LexA binding to its target sequence within the nucleosome and to naked DNA, since \(S_{0.5-nuc} = S_{0.5-DNA}/K_{eq}^{\text{wrap}}\), in the limit that \(K_{eq}^{\text{wrap}}\) is much less than 1 (See Chapter: Introduction for details). From this we determined the equilibrium constant of site exposure for nucleosomes containing unmodified H3, H3(K56Q), H3(C110A)\(_{rec}\), H3(C110A)\(_{syn}\), H3(K56Q,C110A), H3(K56ac,C110A), H4(K77ac,K79ac), and H3(K115ac,K122ac) (Figure 4.9A, Table 4.1) at low ionic strength (1.0mM NaCl).

The change in the site exposure equilibrium of modified nucleosomes relative to unmodified nucleosomes is equal to the change in the probability that LexA can bind to its site, which extends 27 base pairs into the nucleosome (Figure 4.9B). H3(K56ac,C110A) increases this value by 1.8 ± 0.4 times. The H3(K56Q) substitution, which has been used in numerous genetic studies as a mimic of H3(K56ac), increases the probability of LexA binding by 1.8 ± 0.4 and 1.9 ± 0.5 with and without H3(C110A), respectively. This demonstrates that the H3(C110A) mutation does not alter the influence of H3(K56Q) on nucleosomal DNA unwrapping, consistent with FRET efficiency measurements in the absence of LexA. Interestingly, H3(C110A) does appear to modestly increase the absolute value of DNA site accessibility. However, by comparing H3(K56ac,C110A) and H3(K56Q,C110A) to H3(C110A)\(_{rec}\) and H3(C110A)\(_{syn}\), we control for this effect. Similarly, we observed that H4(K77ac,K79ac) increases the probability of LexA binding by 1.7 ± 0.4 times, while H3(K115ac,K122ac) does not affect LexA binding as we reported in Chapter 1. The fact that we see a 1.7-fold increase
in site exposure equilibrium for LexA binding to nucleosomes containing H4(K77ac,K79ac) but do not see altered nucleosome mobility and decreased initial FRET efficiency can be explained by the observation that unwrapping fluctuations extending 40-50 base pairs into the nucleosome occur with a probability of $10^{-4}$ [28, 98], and therefore, a factor of 2 change in unwrapping probability in unlikely to be detectable by gel mobility and FRET efficiency in the absence of LexA. These results indicate that at low ionic strength 1mM NaCl, Lysine acetylation in the DNA-histone interface beginning at the nucleosome entry-exit and extending at least 45 base pairs into the nucleosome LRS region facilitates protein binding within the nucleosomes by shifting the nucleosomes site exposure equilibrium toward the unwrapped state.
Figure 4.7: H3(K56Q) and H3(K56ac) facilitate LexA binding within nucleosomes at 1 mM NaCl. (A) A three state model for LexA binding to its target site within a nucleosome. (B) and (C) are fluorescence emission spectra at 0nM (black), 10nM (blue) and 1000nM (red) of LexA with nucleosomes containing unmodified H3(C110A)$_{rec}$ or H3(K56ac,C110A), respectively. The samples were excited at 510nm (donor excitation). (D) Energy transfer efficiency, as determined by the (ratio)$_A$ method (See Chapter: Introduction) [83], versus LexA concentration for nucleosomes containing unmodified H3 (purple) and H3(K56Q) (magenta). (E) Energy transfer efficiency, as determined by the (ratio)$_A$ method, versus LexA concentration for nucleosomes containing recombinant H3(C110A)$_{rec}$ (blue), synthetic H3(C110A)$_{syn}$ (orange), H3(K56Q,C110A) (red) and H3(K56ac,C110A) (green). Plots in (D) and (E) are the average of three LexA titrations and the error bars were determine from the standard deviation of the three measurements. The data were fit to a non-cooperative binding curve, which determines $S_{0.5-nuc}$, the LexA concentration at which 50% of the nucleosomes are bound by LexA. (Reproduced with permission)
Figure 4.8: H4(K77ac,K79ac) facilitates LexA binding within nucleosomes at 1mM NaCl. (A) and (B) are the fluorescent spectra of H4(K77ac,K79ac) containing nucleosomes that are excited by 510 and 610 nm, respectively, with 0nM (black), 30nM (blue), and 1000nM (red) LexA. (D) Energy transfer efficiency, as determined by the (ratio)A method (See Introduction: Chapter) [83], versus LexA concentration for nucleosomes containing unmodified H3 (purple), H4(K77ac,K79ac) (yellow), and H3(K115ac,K122ac) (magenta). The experiments were done in triplicate and the error bars are the standard deviation.

Figure 4.9: Lysine acetylation of the DNA-histone interface within the entry-exit and LRS regions increase site exposure equilibrium. (A) Equilibrium constants of LexA site exposure for nucleosomes in Figures 4.7D,E, and 4.8C. The equilibrium constants were determined from the ratio: \( K_{eq}^{\text{wrap}} = \frac{S_{0.5-DNA}}{S_{0.5-nuc}} \). (B) Relative site exposure equilibrium of modified nucleosomes relative to unmodified nucleosomes. Modified nucleosomes containing residue H3(C110A) were compared to unmodified H3(C110A)rec nucleosomes; modified nucleosomes containing residue H3(C110) were compared to unmodified H3 nucleosomes.
### Summary of nucleosome site exposure equilibrium for LexA binding

<table>
<thead>
<tr>
<th>H3 histone</th>
<th>$E_0$</th>
<th>$S_{0.5-Nuc}$</th>
<th>$K_{\text{wrap}}^{-1}$</th>
<th>$\frac{K_{\text{eq-mod}}}{K_{\text{eq-unmod}}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>$0.62 \pm 0.02$</td>
<td>$43 \pm 3$</td>
<td>$0.0033 \pm 0.0005$</td>
<td>1</td>
</tr>
<tr>
<td>H3(K56Q)</td>
<td>$0.47 \pm 0.02$</td>
<td>$22 \pm 2$</td>
<td>$0.0063 \pm 0.0010$</td>
<td>$1.9 \pm 0.4$</td>
</tr>
<tr>
<td>H3(C110A)$_{rec}$</td>
<td>$0.68 \pm 0.01$</td>
<td>$58 \pm 6$</td>
<td>$0.0056 \pm 0.0009$</td>
<td>1</td>
</tr>
<tr>
<td>H3(C110A)$_{syn}$</td>
<td>$0.61 \pm 0.01$</td>
<td>$62 \pm 7$</td>
<td>$0.0052 \pm 0.0008$</td>
<td>$0.9 \pm 0.2$</td>
</tr>
<tr>
<td>H3(K56Q,C110A)</td>
<td>$0.45 \pm 0.01$</td>
<td>$32 \pm 3$</td>
<td>$0.0102 \pm 0.0014$</td>
<td>$1.8 \pm 0.4$</td>
</tr>
<tr>
<td>H3(K56ac,C110A)</td>
<td>$0.43 \pm 0.01$</td>
<td>$32 \pm 3$</td>
<td>$0.0102 \pm 0.0015$</td>
<td>$1.8 \pm 0.4$</td>
</tr>
<tr>
<td>H3</td>
<td>$0.61 \pm 0.01$</td>
<td>$49 \pm 4$</td>
<td>$0.0029 \pm 0.0005$</td>
<td>1</td>
</tr>
<tr>
<td>H4(K77ac,K79ac)</td>
<td>$0.59 \pm 0.01$</td>
<td>$29 \pm 3$</td>
<td>$0.0047 \pm 0.0008$</td>
<td>$1.66 \pm 0.41$</td>
</tr>
<tr>
<td>H3(K115ac,K122ac)</td>
<td>$0.61 \pm 0.01$</td>
<td>$56 \pm 3$</td>
<td>$0.0025 \pm 0.0004$</td>
<td>$0.88 \pm 0.20$</td>
</tr>
</tbody>
</table>

### 75mM NaCl

<table>
<thead>
<tr>
<th>H3 histone</th>
<th>$E_0$</th>
<th>$S_{0.5-Nuc}$</th>
<th>$\frac{K_{\text{wrap}}^{-1}}{K_{\text{eq-unmod}}}$</th>
<th>$E_0$</th>
<th>$S_{0.5-Nuc}$</th>
<th>$\frac{K_{\text{eq-mod}}}{K_{\text{eq-unmod}}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3(C110A)$_{rec}$</td>
<td>$0.73 \pm 0.01$</td>
<td>$3240 \pm 181$</td>
<td>1</td>
<td>$0.74 \pm 0.01$</td>
<td>$13122 \pm 536$</td>
<td>1</td>
</tr>
<tr>
<td>H3(C110A)$_{syn}$</td>
<td>$0.66 \pm 0.01$</td>
<td>-</td>
<td>-</td>
<td>$0.67 \pm 0.01$</td>
<td>$11849 \pm 876$</td>
<td>$1.1 \pm 0.2$</td>
</tr>
<tr>
<td>H3(K56Q,C110A)</td>
<td>$0.53 \pm 0.01$</td>
<td>$1276 \pm 150$</td>
<td>$2.5 \pm 0.6$</td>
<td>$0.52 \pm 0.01$</td>
<td>$5176 \pm 524$</td>
<td>$2.5 \pm 0.5$</td>
</tr>
<tr>
<td>H3(K56ac,C110A)</td>
<td>$0.52 \pm 0.01$</td>
<td>$991 \pm 115$</td>
<td>$3.3 \pm 0.7$</td>
<td>$0.50 \pm 0.01$</td>
<td>$3975 \pm 488$</td>
<td>$3.3 \pm 0.7$</td>
</tr>
</tbody>
</table>

Table 4.1: **Summary of nucleosome site exposure equilibrium for LexA binding.** $(E_0)$ is the FRET efficiency without LexA; $(S_{0.5-Nuc})$ is the concentration of half saturation by LexA to the nucleosome in nM concentration.
4.2.5 LexA specifically binds to its target site within a nucleosome

The differences in LexA binding observed for nucleosomes containing H3(K56ac) and H4(K77ac,K79ac) compared to unmodified nucleosomes may be due to several factors. Modified nucleosomes could facilitate LexA binding due to increased DNA unwrapping, due to LexA induced nucleosome repositioning to move the LexA site outside of the nucleosome, or due to alterations in the nonspecific binding of LexA at high LexA concentrations as observed by EMSA with naked DNA Figure 4.10A,B.

In order to verify that the reduction in FRET upon addition of LexA to nucleosomes containing the 601L-end construct was due to specific LexA binding to its target site within the nucleosome, we performed EMSA assays of LexA bound to the nucleosome containing H3(C110A)red, H3(K56Q,C110A), or H3(K56ac,C110A). Nucleosomes were incubated with 0-3 µM LexA in 0.5x TE for 2 minutes at 20°C before resolving on a 5% native PAGE. As previously reported [30], we did not observe a detectable gel shift until a LexA concentration much greater than the $S_{0.5-nuc}$ for binding of 601L-end observed by FRET (Data not shown). Therefore we employed glutaraldehyde fixation to trap specifically bound LexA. To demonstrate that glutaraldehyde does not affect the specific binding of LexA to naked DNA we performed EMSA on 601L-end naked DNA. DNA at 0.1nM was incubated with 0-1 µM LexA in 0.5x TE or 5mM HEPES, pH 8.0 for 2 minutes at 20°C. Glutaraldehyde was added to 0.05% final concentration to the reactions containing HEPES, incubated for 2 minutes at 20°C before quenching with a final concentration of 50mM Tris pH 8.0, and resolved by EMSA (Figure 4.10A,B). The fraction of free DNA as a function of LexA concentration was quantified for three independent experiments (Figure 4.10C) and fit to a cooperative binding curve to determine the concentration of half saturation ($S_{0.5-DNA}$) and cooperativity parameter (p) without glutaraldehyde fixation ($S_{0.5-DNA-glut} = 0.32 \pm 0.04$, $p = 2.1 \pm 0.2$, $R^2 = 0.9995$) and with glutaraldehyde fixation ($S_{0.5-DNA+glut} = 0.36 \pm 0.04$, $p = 1.4 \pm 0.2$, $R^2 = 0.9984$). $S_{0.5-DNA}$ is unaffected by glutaraldehyde fixation and the only apparent effects glutaraldehyde has on LexA binding is that it decreases the cooperativity parameter by $\sim 25\%$ and alters the mobility of non-specific DNA-LexA complex at higher
For binding to 601L-end nucleosomes, nucleosomes at 5 nM in 5 mM HEPES pH 8.0 were incubated with LexA, fixed with 0.05% glutaraldehyde and resolved by 5% native PAGE as described above (Figure 4.10D). A well-defined hyper-shifted band, absent without glutaraldehyde fixation, was observed from 30-300 nM LexA. As this is the range of LexA concentrations in which fluorescence measurements show a decrease in FRET, we attribute this band to a specific LexA-nucleosome complex. The fraction of free nucleosomes as a function of LexA concentration was quantified for three independent experiments (Figure 4.10E) and fit to a cooperative binding curve for nucleosomes containing H3(C110A)rec (S0.5−C110A = 88 ± 13 nM, R² = 0.9995), H3(K56Q,C110A) (S0.5−K56Q,C110A = 64 ± 4 nM, R² = 0.9984), or H3(K56ac,C110A) (S0.5−K56ac,C110A = 56 ± 3 nM, R² = 0.9984).

We determined the site exposure equilibrium constant, Kwrapeq = S0.5−DNA/S0.5−nuc, from LexA half-saturation binding concentration to its target site both within the nucleosome and to naked DNA as measured by EMSA. We observed equilibrium constants of Kwrap−C110A,rec = 0.0036 ± 0.0007, Kwrap−K56Q,C110A = 0.0050 ± 0.0007, and Kwrap−K56ac,C110A = 0.0058 ± 0.0007 for nucleosomes containing H3(C110A)rec, H3(K56Q,C110A), and H3(K56ac,C110A), respectively, which are similar to those measured by FRET (Table 4.1). From this we measure the change in the site exposure equilibrium for LexA binding to its site within the nucleosome containing H3(K56Q) and H3(K56ac) relative to unmodified nucleosomes to be 1.4 ± 0.3 and 1.6 ± 0.3 times, respectively, which, within the uncertainty of the measurements, agree with the FRET measurements (Figure 4.10F). Taken together, these results demonstrate that LexA specifically binds to its target site within the nucleosome, thus decreasing the FRET efficiency.

4.2.6 Non-specific LexA binding does not contribute to fluorescence measures of LexA accessiblitily within nucleosomes

EMSA of naked DNA and nucleosomes bound with LexA at concentrations above 100nM LexA show hypershifted bands that are consistent with non-specific binding as previously
Figure 4.10: EMSA of LexA binding to naked DNA and nucleosomes containing the 601L-end DNA construct. (A) EMSA of LexA binding to naked 601L-end DNA without glutaraldehyde fixation. (B) EMSA of LexA binding to naked 601L-end DNA with 0.05% glutaraldehyde fixation in 5mM HEPES pH 8.0. (C) Average fraction of unbound DNA as a function of [LexA] with (gray) and without (black) glutaraldehyde fixation for the experiments in (A) and (B) and two additional experiments each. The error bars are the standard deviation of the three measurements. (D) EMSA of LexA binding to 601L-end nucleosomes containing H3(C110A) and H3(K56ac,C110A) with 0.05% glutaraldehyde fixation in 5mM HEPES pH 8.0. (E) Average fraction of unbound nucleosomes as a function of [LexA] for nucleosomes containing H3(C110A) (blue), H3(K56Q,C110A) (red) and H3(K56ac,C110A) (green) for the experiment in (D) and two additional experiments. The error bars are the standard deviation of the three measurements. The data was fit to a cooperative binding curve to determine $S_{0.5} - nuc$. (F) Equilibrium constants of H3(K56Q,C110A) relative to H3(C110A)$_{rec}$ (1.4 ± 0.3), and H3(K56ac,C110A) relative to H3(C110A)$_{rec}$ (1.6 ± 0.3)
reported [30]. Therefore a component of the measured FRET change induced by LexA could be due to non-specific LexA binding. To control for this effect on FRET efficiency, we determined the FRET efficiency of nucleosomes reconstituted with H3(C110A), H3(K56Q,C110A) or H3(K56ac,C110A) with DNA that did not contain the LexA target sequence (Figure 4.2A, 601-end). We find no decrease in the FRET efficiency in the presence of up to 1µM LexA (Figure 4.11A-E). This concentration of LexA fully reduces the FRET efficiency of nucleosomes that contain the LexA target sequence (Figure 4.7).

Additionally, to demonstrate the elimination of LexA specificity to the 601-end DNA lacking the LexA recognition sequence, we performed EMSA of naked 601-end DNA incubated with LexA from 0-1µM as described above. We do not observe any distinct hypershifted band indicative of specific LexA-DNA binding as observed for the 601L-end DNA (Figure 4.11F). This demonstrates that there is no specific LexA-DNA interaction with DNA lacking the LexA site, and by extension with nucleosomes containing the 601-end DNA. These results, combined with the FRET measures with nucleosomes containing 601-end DNA confirm that the reduction in the FRET efficiency of 601L-end containing nucleosomes is due to LexA specifically binding to its target sequence within the nucleosome.

4.2.7 Histone PTMs H3(K56ac) and H4(K77ac,K79ac) facilitate protein binding by increasing DNA unwrapping

The increased protein accessibility induced by H3(K56ac) and H4(K77ac,K79ac) could result from changes in DNA unwrapping, nucleosome repositioning, or both. To resolve these possibilities, we determined the position of nucleosomes containing H3(C110A)$_{rec}$ and H3(K56Q,C110A) in the presence of 1µM LexA by hydroxyl radical mapping. We found that nucleosomes in the presence of 1µM LexA retained a cleavage pattern identical to that of nucleosomes without LexA, as measured by denaturing PAGE (Compare Figures 4.5 and 4.12). LexA at this concentration of 1µM is bound to its target sequence within nucleosomes, as measured by FRET efficiency (Figure 4.7) and EMSA (Figure 4.10).

We also carried out FRET efficiency studies with nucleosomes that were labeled at the 80th base pair with Cy3 (Figure 4.13A, 601L-dyad). Based on the nucleosome crystal
Figure 4.11: **LexA binding to nucleosomes without a LexA binding site.** (A) and (B) Fluorescence emission traces for H3(C110A) nucleosomes containing the 601-end DNA construct in the presence of LexA at 0nM (black), 10nM (red), 1000nM (blue), excited at 510nm and 610nm, respectively. (C) and (D) Fluorescence emission traces for H3(K56ac,C110A) nucleosomes containing the 601-end DNA construct in the presence of LexA at 0nM (black), 10nM (red), 1000nM (blue), excited at 510nm and 610nm, respectively. (E) Energy transfer efficiency, as determined by the (ratio)_A method (See Chapter: Introduction) [83], versus LexA concentration for nucleosomes containing H3(C110A) (blue), H3(K56Q,C110A) (red), and H3(K56ac,C110A) (green). The error bars are the standard deviation of the separate measurements. (F) EMSA of naked 601-end DNA incubated with the indicated amount of LexA in 0.5x TE and resolved by 5% native PAGE. (Reproduced with permission)
structure [9] the distance between the Cy3 molecule and the nearest Cy5 molecule is about 2.3 nm, which converts to a FRET efficiency of 0.99. We anticipate that this efficiency would be slightly reduced due to the 6-carbon linker used to attach Cy3 to the thymine base. If the LexA site were exposed only by repositioning of ~30 base pairs, the distance between Cy3 and the nearest Cy5 would increase to 6.2 nm, which converts to a FRET efficiency of 0.45. We find that the FRET efficiency remains constant at 0.8 for nucleosomes containing unmodified H3, H3(K56Q), H3(C110A)$_{rec}$, H3(K56Q,C110A), and H3(K56ac,C110A) under conditions that are consistent with a full occupancy of the LexA binding site (Figure 4.13).

Under certain circumstances it is possible that a small nucleosome repositioning that would partially expose the LexA site cannot be detected using the 601L-dyad construct. Based on the nucleosome crystal structure [Js45], a nucleosome repositioning event that would move the LexA site partially outside the nucleosome would proceed by first moving the Cy3 fluorophore of 601L-dyad away from the Cy5 fluorophore of the proximal H2A(K119C) amino acid residue, but in the process will initially move the Cy3 toward the other Cy5 of the distal H2A(K119C). An ~10 base pair repositioning could result in such a movement of the Cy3 molecule where its distance from one Cy5 fluorophore after repositioning is the same as it was to the other before repositioning, leading to an undetectable change in FRET.

To control for this possibility, we reconstituted nucleosomes containing the 601R-end DNA construct Figure 4.14A in which the Cy3 molecule remains attached to the 5'-end of the forward DNA strand, but the LexA site is moved to the opposite entry-exit side of the nucleosome between bases 121 and 140. LexA binding to its site in the entry-exit opposite to the entry-exit side containing the Cy3 fluorophore can only produce a change in FRET in the event of nucleosome sliding and repositioning (Figure 4.14B). For nucleosomes containing H3(C110A)$_{rec}$, H3(K56ac,C110A), and H4(K77ac,K79ac) we observed no change in FRET upon LexA binding up to 1µM in [LexA] (Figure 4.14C), which, based on EMSA (Figure 4.10) and FRET measures (Figure 4.7), under these conditions the LexA site is fully occupied.

The combination of FRET studies and hydroxyl radical mapping suggests that
H3(K56Q), H3(K56ac), and H4(K77ac,K79ac) do not increase DNA site accessibility via a nucleosome repositioning model. Instead, acetylation of H4 Lysines 77 and 79, acetylation of H3 Lysine 56 and its mimic appear to increase LexA accessibility by increasing the probability that the nucleosome is partially unwrapped.

Figure 4.12: Hydroxyl Radical mapping shows LexA binding within the nucleosome does not reposition the nucleosome. (A) and (B) Denaturing polyacrylimide gel electrophoresis of the nucleosomal DNA cleaved by FeBABE for 0, 5 and 10 minutes in the presence of 1000nM LexA. Within each gel, lanes 1-3 and 10-12 contain sequencing tracks terminated with ddGTP, ddATP and ddTTP respectively, lanes 4-6 contain nucleosomes with H3(C110A)$_{rec}$ and lanes 7-9 contain nucleosomes with H3(K56Q,C110A). (A) and (B) are images of denaturing gels with the top and bottom DNA strands, respectively, are visualized by Cy3 fluorescence. The cleavage patterns remain unchanged compared to FeBABE cleavage without LexA (Figure 4.5).

4.2.8 Acetylation of H3(K56) facilitates accessibility to DNA within nucleosomes at physiological ionic strength

Our initial studies were carried out at low ionic strength, but the physiologically relevant concentration of monovalent ions is 130150 mM. Therefore, we carried out LexA binding studies with 601L-end nucleosomes at both 75mM and 130mM NaCl, as described above.
Figure 4.13: LexA binding does not reposition nucleosomes as monitored by FRET at the Dyad  
(A) The 147 base pair DNA molecule, 601L-dyad, contains the 601 positioning sequence with LexA binding site at base pairs 8-27 and a Cy3 fluorophore attached to 80th base pair of the DNA molecule.  
(B) The FRET efficiency as determined by the (ratio)\textsubscript{A} method (See Chapter: Introduction) \cite{83} of nucleosomes containing unmodified H3, H3(K56Q), H3(C110A), H3(K56Q,C110A) and H3(K56ac,C110A). Each nucleosome contained the 601L-dyad DNA molecule. The error bars were determined from the standard deviation of three separate measurements.  
(C) The energy transfer efficiency determined by the \textsubscript{(ratio)}\textsubscript{A} method versus LexA concentration for H3(C110A), H3(K56Q,C110A) and H3(K56ac,C110A) nucleosomes containing the 601-dyad DNA construct. Each plot is the average of at least three LexA titrations and the error bars are the standard deviation.  
(Reproduced with permission)
Figure 4.14: LexA binding does not reposition nucleosomes as monitored by FRET at the entry-exit. (A) 601R-end DNA construct to determine if LexA binding to its target sequence occurs via DNA unwrapping or DNA repositioning. The LexA site is inserted between the 121st and 140th base pairs of the 601 nucleosome positioning sequence while the Cy3-label remains attached to the 5'-end of the leading DNA strand. (B) Nucleosome structure [37] indicating location of LexA binding site (red) and Cy3 fluorophore (green) in the 601R-end DNA molecule; H2A(K119C) in magenta, H3(K56) (orange), H4(K77) (yellow), H4(K79) (cream), H3(K115) (blue), and H3(K122ac) (teal); PDB ID: 1KX5. The target sequence and the Cy3 fluorophore are near the opposite DNA entry-exit regions. A change in FRET due to LexA binding to its target sequence can only occur by nucleosome repositioning and not DNA unwrapping. (C) LexA titrations of nucleosomes containing 601R-end with H3(C110A)$_{rec}$, H4(K77ac,K79ac), and H3(K115ac,K122ac) histone octamers. We find that none of the nucleosomes have a reduction in FRET efficiency as LexA is increased to concentrations that dramatically reduce the FRET efficiency of nucleosomes with the LexA target sequence and Cy3 on the left end of the 601 sequence (Figure 4.7).
for 1.0mM NaCl. We determined the $S_0.5\text{-nuc}$ values for H3(C110A)$_{rec}$, H3(C110A)$_{syn}$, H3(K56Q,C110A), and H3(K56ac,C110A) at 75mM and 130mM NaCl (Figure 4.15A,B, Table 4.1). We were unable to determine the $S_0.5\text{-DNA}$ value for naked DNA by EMSA because of the increase in NaCl. Therefore, we determined the $K_{\text{eq}}^{\text{wrap}}$ values for H3(K56Q,C110A) and H3(K56ac,C110A) nucleosomes relative to unmodified nucleosomes. At 75mM, $K_{\text{eq}}^{\text{wrap}}\text{-H3(K56ac)}/K_{\text{eq}}^{\text{wrap}}\text{-unmod} = 3.3 \pm 0.4$ and $K_{\text{eq}}^{\text{wrap}}\text{-H3(K56ac)}/K_{\text{eq}}^{\text{wrap}}\text{-unmod} = 2.5 \pm 0.3$, while at 130mM, $K_{\text{eq}}^{\text{wrap}}\text{-H3(K56ac)}/K_{\text{eq}}^{\text{wrap}}\text{-unmod} = 3.3 \pm 0.4$ and $K_{\text{eq}}^{\text{wrap}}\text{-H3(K56Q)}/K_{\text{eq}}^{\text{wrap}}\text{-unmod} = 2.5 \pm 0.3$ (Figure 4.15C, Table 4.1).

These results imply that at the physiological ionic strength of 130mM NaCl, H3(K56ac) increases DNA unwrapping fluctuations that expose the LexA target site 3-fold, in turn resulting in a 3-fold increase in LexA binding to its target site. Furthermore, we find that H3(K56Q) increases DNA site exposure similarly to H3(K56ac) at physiological ionic strength, suggesting that H3(K56Q) is a good acetylation mimic of H3(K56ac) for in vivo studies. Further experiments are required to determine the effects of physiological ionic strength on the DNA site exposure and LexA accessibility of nucleosomes containing H4(K77ac,K79ac).

4.2.9 Histone PTMs H3(K56ac) and H4(K77ac,K79ac) increase nucleosome unwrapping throughout the nucleosome

As an independent measure of the effects of H3(K56ac) and H4(K77ac,K79ac) on DNA accessibility to regulatory proteins, we performed Restriction Enzyme kinetics measures of nucleosomes site exposure (See Chapter: Introduction). In these studies, we reconstituted modified and unmodified histone octamers onto an NPS that places restriction enzyme target sites at specific positions along the histone-DNA interface (Figure 4.16, [94]). Because H3(K56ac) contains the amino acid substitution C110A, we compared H3(K56ac) measurements to unmodified nucleosomes containing H3(C110A)$_{rec}$, whereas measurements with H4(K77ac,K79ac) and H3(K115ac,K122ac) were compared to unmodified nucleosomes containing H3(C110). H3(K56ac) and H4(K77ac,K79ac) increase digestion rate by three-fold near the DNA entry-exit region and by 1.5-2 fold throughout the rest of the nucleosomes.
Figure 4.15: H3(K56ac) and H3(K56Q) enhances LexA binding to its DNA target sequence within nucleosomes at physiological ionic strength. (A) and (B) Energy transfer efficiency in the presence of 75mM and 130mM NaCl, respectively, as determined by the (ratio)_A method (See Chapter: Introduction) \[83\], versus LexA concentration for nucleosomes containing unmodified and recombinant H3(C110A)$_{rec}$ (blue), unmodified and synthetic H3(C110A)$_{syn}$ (orange), H3(K56Q,C110A) (red), and H3(K56ac,C110A) (green). Plots in (A) and (B) are the average of three LexA titrations and the error bars are the standard deviation. The data were fit to a non-cooperative binding curve to determine $S_{0.5-\text{nuc}}$, the LexA concentration at which 50% of the nucleosomes are bound by LexA. (C) Relative equilibrium constants of modified nucleosomes with respect to unmodified nucleosomes at 1mM NaCl, 75mM NaCl, and 130mM NaCl. (Reproduced with permission)
(Figure 4.17, Table 4.2). Conversely, H3(K115ac,K122ac) does not impact digestion rate save for a 1.6-fold increase at the HindIII site (See Chapter 1 for further details). These results are consistent with our findings using the FRET system for quantification of LexA accessibility, in which H3(K56ac) and H4(K77ac,K79ac) enhances nucleosome site exposure by increasing the probability of DNA unwrapping by 2-3 fold.

\[ \text{Table 4.2: Relative Restriction enzyme site exposure equilibrium of modified versus unmodified nucleosomes. RE is the restriction enzyme used for each RE site within nucleosome as shown in Figure 4.16A,B.} \]

<table>
<thead>
<tr>
<th>RE</th>
<th>( \frac{K_{eq-H3(K56ac,C110A)}}{K_{eq-H3(C110A)}} )</th>
<th>( \frac{K_{eq-H4(K77ac,K79ac)}}{K_{eq-H3}} )</th>
<th>( \frac{K_{eq-H3(K115ac,K122ac)}}{K_{eq-H3}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PstI</td>
<td>2.9 ± 0.1</td>
<td>2.4 ± 0.3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>HindIII</td>
<td>1.9 ± 0.4</td>
<td>3.0 ± 0.7</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>HaeII</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.4</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>TaqI</td>
<td>2.0 ± 0.1</td>
<td>1.8 ± 0.4</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>HhaI</td>
<td>1.5 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>PmlI</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.4</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

4.2.10 Histone PTM analogs and mimics of H3(K36me3), H3(Y41ph), and H3(T45ph) implicate their role in DNA unwrapping

Our observation that H3(K56ac) within the entry-exit region increases DNA unwrapping to facilitate protein binding indicates that other PTMs in this region could have a similar function. To investigate if the histone PTMs: H3(K36me3), H3(Y41ph), and H3(T45ph) (Figure 4.6) facilitate protein binding within the nucleosome by increasing DNA unwrapping, we performed the same LexA binding studies as with H3(K56ac) near physiological ionic strength (75mM NaCl, Figure 4.18A) using the trimethyl-lysine analog, H3(K5,36me3) and phosphomimetics, H3(Y41E), and H3(T45E). We measure the FRET efficiency of nucleosomes containing the particular analog or mimic as a function of LexA concentration.
Figure 4.16: Nucleosome construct for restriction enzyme digestion analysis. (A) The DNA construct, Mp2-192, was used for the restriction enzyme digestion analysis of DNA unwrapping site accessibility within the nucleosome. It contains restriction enzyme cleavage sites located throughout the NPS. The Mp2-192 DNA molecule is 5′-labeled with Cy5 to allow for visualization by fluorescence. (B) The nucleosome crystal structure [37] shows the position of the restriction enzyme cleavage sites in red and the modifications are indicated by the same colors used in Figure 4.14; PDB ID: 1KX5. (C) Electrophoretic mobility shift assay of sucrose gradient purified nucleosomes imaged with Cy5 fluorescence from the Mp2-192 DNA molecule (lane 1), unmodified wild-type nucleosomes (lane 2), H3(C110A),rec containing nucleosomes (lane 3), H3(K56ac,C110A) containing nucleosomes (lane 4), H4(K77ac,K79ac) containing nucleosomes (lane 5), and H3(K115ac,K122ac) containing nucleosomes (lane 6). (Reproduced with permission)
Figure 4.17: Restriction enzyme digestion analysis shows that H3(K56ac) and H4(K77ac,K79ac) enhance DNA accessibility throughout the nucleosome. (A) 5% native PAGE analysis of PstI restriction enzyme digests of Mp2-192 nucleosomes (Figure 4.16) containing H3(C110A)$_{rec}$ and H3(K56ac, C110A). The time in minutes is the duration for which the digestion was allowed to proceed before quenching. (B) Quantification of the digests in (A) for nucleosomes containing H3(C110A)$_{rec}$ (blue circle) and H3(K56ac,C110A) (green circle). The Fraction Uncut is measured as the intensity of the uncut band relative to the sum of the uncut and cut band intensities. The solid line is a least-squares fit of a single exponential to the data. (C) Five percent native PAGE analysis of PstI restriction enzyme digests of Mp2-192 nucleosomes containing H3, H3(K115ac,K122ac), and H4(K77ac,K79ac). (D) Quantification of the digests in (C) for nucleosomes containing H3 (purple circle), H3(K115ac,K122ac) (magenta circle), and H4(K77ac,K79ac) (yellow circle). (E) Summary of the DNA unwrapping equilibrium constant, $K_{eq}^{RE}$, of nucleosomes with H3(K56ac,C110A) relative to nucleosomes with H3(C110A)$_{rec}$ (green), H4(K77ac,K79ac) relative to unmodified H3 (yellow) and H3(K115ac,K122ac) relative to unmodified H3 (magenta).
and fit each series to a non-cooperative binding curve to determine the $S_{0.5-nuc}$ at which 50% of the nucleosomes are bound by lexA (Figure 4.18A). From this we determine the relative change in nucleosome site exposure equilibrium of LexA binding for each modification relative to unmodified H3(C110A)$_{rec}$ using the relationship $K_{\text{wrap eq-modified}}/K_{\text{wrap eq-unmodified}} = S_{0.5-nuc-unmodified}/S_{0.5-nuc-unmodified}$. We observe that similar to H3(K56ac), both H3(Y41E) and H3(T45E) increase nucleosomes unwrapping by $2.5 \pm 0.7$ and $2.2 \pm 0.5$, respectively. Conversely, H3(K$_{c36}$me3) does not alter nucleosome unwrapping. These results suggest that the histone PTMs H3(Y41ph) and H3(T45ph) may also function to increase nucleosome unwrapping.

Figure 4.18: Phosphomimetics of PTMs in the entry-exit increase DNA unwrapping. (A) FRET efficiency of nucleosomes bearing H3(C110A)$_{rec}$ (blue), H3(K$_{c36}$me3) (forest), H3(Y41E) (mustard), and H3(T45E) (royal) measured by the (Ratio)$_A$ method (See Chapter: Introduction) [83] as a function of LexA concentration. Error bars are the standard deviation of three separate titrations. Solid lines are the fit to a non-cooperative binding curve to determine LexA concentration of half saturation, $S_{0.5-nuc}$, at which nucleosomes are 50% bound by LexA. (B) Site exposure equilibrium, $K_{\text{wrap eq-modified}}/K_{\text{wrap eq-unmodified}}$ of modified nucleosomes relative to unmodified nucleosomes, $K_{\text{wrap eq-modified}}/K_{\text{wrap eq-unmodified}}$ for LexA binding.
4.3 Discussion

Post-Translational Modifications (PTMs) of histones occur throughout the protein sequence with multiple disparate types of modifications often detected on a single histone [150, 151]. Current methods of preparing modified histones are limited to the site-specific introduction of a single type of modification within a histone protein [101, 103, 152], or a variety of modifications within a localized region of the histone protein [44, 55, 57, 91–93, 153]. This can be accomplished using a single native Cystiene as the ligation site [e.g. H3(C110)] as was employed in the construction of H3(K115ac,K122ac) [93]. Similarly, desulfurization can be coupled with ligation at a single introduced non-native Cystiene which is converted back to a native Alanine as was employed in the construction of H4(K77ac,K79ac) [120]. However, these strategies are limited to a single ligation site in the context of introducing N-terminal or C-terminal histone PTMs.

In order to study the biophysical properties of PTMs that are located throughout the histone sequence, Ottesen and Colleagues have established a method for incorporating one or several PTMs into a histone protein by sequential NCL, targeting the common Alanine residue as a ligation junction. The procedure thus generates a native histone sequence containing only the PTM(s) of interest with no non-native residues other than the well-studied H3(C110A). In other studies, the introduction of select non-native Cystiene residues through ligation in the unstructured nucleosome tail has had a negligible effect on nucleosome dynamics [44, 55, 57]. This work demonstrates that the semi-conservative introduction of Cystiene residues into the nucleosome core can perturb DNA wrapping and underpins the importance of considering the impact of these substitutions when interpreting biochemical or biophysical measurements that introduce non-native Cystiene sites throughout the nucleosome. Moreover, these synthesis strategies in which spurious effects from non-native Cystienes are mitigated by conversion to native Alanines have enabled detailed quantification of the effects of Lysine acetylation in the DNA-histone interface on nucleosome site exposure equilibrium and accessibility to regulatory proteins.

Although this initial study is restricted to the fully-synthetic synthesis and characteri-
zation of H3(C110A) and H3(K56ac,C110A), this method is limited only by the synthesis of individual peptide segments and would allow for the introduction of PTMs throughout histone H3, particularly H3(Y41ph) and H3(T45ph), whose phosphomimetics we studied in this work. Additionally, as H3 is the largest of the core histone proteins, our success suggests that the total synthesis strategy may be applied to all of the histone proteins, including rare variants. These methods should therefore be useful in determining the function and biophysical properties associated with the voluminous numbers of Histone PTMs.

4.3.1 Histone PTMs define nucleosome unwrapping region

In this study we have characterized the effects of a subset of PTMs throughout the DNA-histone interface on nucleosome unwrapping by either synthesizing histones with the precise modification or employing analogs and amino acid substitution mimics of the precise modifications. These include H3 trimethylated Lysine 36 analog H3(Kc36me3), H3 Tyrosine 41 and Threonine 45 phosphomimetics H3(Y41E) and H3(T45E), and fully-synthetic H3 acetylated Lysine 56 [H3(K56ac,C110A)] within the entry-exit region; semi-synthetic H4 acetylated Lysines 77 and 79 [H4(K77ac,K79ac)] within the Loss of Ribosomal Silencing (LRS) region; and semi-synthetic H3 acetylated Lysines 115 and 122 [H3(K115ac,K122ac)] within the nucleosome dyad region.

Our measurements of nucleosomes containing K56ac are in agreement with the results of Neumann et al. [103], which show that K56ac increases the population of nucleosomes that are partially unwrapped at the DNA entry-exit region by up to seven times at low ionic strength. We extend these studies to demonstrate that DNA unwrapping in the DNA entry-exit region facilitates protein binding 27 base pairs into the nucleosome by a factor of 1.8 under low ionic conditions (0.5x TE buffer). In addition, we determined the influence of K56ac on DNA unwrapping and protein binding within the nucleosome at physiological ionic strength (130mm NaCl). We find that this enhances the influence of K56ac on DNA unwrapping such that protein binding is increased 3.3 times to 27 base pairs into the nucleosome. These studies are consistent with enhanced accessibility to transcription factors and DNA repair components in chromatin regions containing K56ac.
Interestingly, Neumann et al. found that the FRET distribution was not altered 27 base pairs into the nucleosomes with K56ac. This appears to be in contrast to our result that K56ac facilitates protein binding to a site that extends 27 base pairs into the nucleosome. Additionally, Restriction Enzyme measures of site exposure equilibrium indicate that DNA accessibility is increased throughout the nucleosomes. We can understand this apparent discrepancy by considering the previously reported cooperativity of adjacent DNA target sites within a nucleosome [143, 144]. Protein binding to the outer DNA target site within the nucleosome facilitates binding to the inner target site. In our case, K56ac appears to act as the outer adjacent site that facilitates protein binding to its target site within the nucleosome.

Our quantitative measurements of LexA protein accessibility by K56ac and K56Q are in agreement with multiple studies. During DNA replication, nucleosomes are assembled with H3(K56ac) [134]. Polymerase misincorporation errors and DNA lesions result in mismatched nucleotides, replication fork collapse, and DNA double-strand breaks that must be repaired to ensure genomic stability [154]. Deletion of rtt109, which acetylates H3(K56), or mutation of H3(K56) to Arginine [H3(K56R)], which mimics unacetylated H3, causes large defects in postreplication DNA repair [136] and leads to genomic instability [71]. Genetic studies that found phenotypes in both gene expression and DNA repair, use H3(K56Q) substitution to mimic lysine acetylation [134, 135]. Our study suggests that similar phenotypes will result in cells that are constitutively acetylated at H3(K56). Recently, the crystal structure of nucleosomes containing H3(K56Q) was reported by Watanabe et al. [138]. They found that K56Q did not impact the structure of the fully wrapped state of the nucleosome, consistent with a role for K56 acetylation in nucleosome dynamics. They also reported that H3(K56Q) did not influence the compaction of nucleosome arrays, regardless of nucleosome density. However, H3(K56Q) dramatically reduced interactions between multiple arrays of nucleosomes; thus, H3(K56ac) may function to reduce chromatin-chromatin interactions to help keep nucleosome-free regions accessible for DNA replication and repair. Their studies relied on the assumption that H3(K56Q) accurately mimics H3(K56ac). Our studies, which
demonstrate that H3(K56Q) mimics H3(K56ac), indicate that this assumption is correct.

Phosphorylation of H3 Tyrosine 41 and H3 Threonine 45 are poised to function similarly to H3(K56ac). Phosphomimetics H3(Y41E) and H3(T45E) exhibit a 2.5- and 2.3- fold increase in DNA accessibility to LexA, respectively at 75mM NaCl. This is consistent with the 3.3-fold increase observed for H3(K56ac) and suggests that the PTMs H3(Y41ph) and H3(T45ph) may function as a DNA entry-exit gate to regulate nucleosome unwrapping like H3(K56ac). However, given that the phosphomimetic H3(T115E) does not capture the 40-fold reduction in nucleosome stability observed with the precise PTM, H3(T118ph) (See Chapter 2), further studies using the precise modifications, H3(Y41ph) and H3(T45ph), are necessary to determine the amount by which they increase nucleosome unwrapping. Since H3(K56ac) reduces the probability of DNA-tetramer interaction by only 7-12 fold [103, 141], phosphorylation in the nucleosome entry-exit could have an even larger impact on nucleosome accessibility. Interestingly, our studies using the methyl-lysine analog of H3 trimethylated Lysine 36, which is located just outside of the H3 α-N helix between the entry-exit DNA gyre and the dyad DNA gyre as the H3 tail comes up through these two regions, indicates that this PTM does not function to increase nucleosome site exposure. Finally, we observe that in the LRS region 40-50 base pairs into the nucleosome, H4(K77ac,K79ac) both increases DNA accessibility to LexA binding and site exposure throughout the nucleosomes by nearly 2-fold. These results suggest that phosphorylated H3(S86) located just beyond these two PTMs may also function to increase nucleosome unwrapping.

Interestingly, histone PTMs H3(K115ac), H3(K122ac), and H3(T118ph) in the nucleosome dyad do not increase nucleosome accessibility to LexA binding or DNA site exposure (See Chapters 1-2). Rather these dyad modifications are observed to decrease nucleosome stability and facilitate nucleosome mobility. Taken together with the observations above, we observe that the DNA-histone interface has two distinct regions of PTM function. We find that modifications from the DNA entry-exit region to at least 45 base pairs into the nucleosome enhance partial DNA unwrapping. This is consistent with previous studies where alterations in this region by histone PTMs [103], point mutations, and mutations in the H3 α-N helix [142] enhance DNA unwrapping. Secondly, as shown chapters 1-2, modifications
in the nucleosome dyad region control nucleosome mobility and stability without directly impacting DNA unwrapping. These results are consistent with the observations of Simon et al. in which PTMs of the nucleosome dyad facilitate mechanically induced nucleosome disassembly whereas PTMs in other regions of the DNA-histone interface do not. Taken as a whole, these results suggest that alterations in the dyad region create nucleosomes that are poised for disassembly or remodelling without increasing DNA accessibility. External factors that facilitate DNA unwrapping would then be required in order to initiate nucleosome disassembly.

The observation that nucleosomes have a distinct region of the DNA histone interface that regulates unwrapping and another region that primarily regulates nucleosome mobility/stability is consistent with the nucleosome crystal structure [9]. In fact, it has previously been proposed that the numerous histone PTMs within the DNA-histone interface [60] may function to alter nucleosome stability, mobility [35, 59], and unwrapping [103, 142]. By quantifying the influence of histone PTMs on DNA unwrapping and stability/mobility, we demonstrate that histone PTMs spanning from the entry-exit region to the LRS region 40-50 base pairs into the nucleosomes can significantly impact DNA unwrapping while PTMs in the dyad separately tune nucleosome stability, mobility, and assembly/disassembly. However, these distinct processes are functionally related because DNA unwrapping is likely to occur before histone proteins dissociate from the DNA in a stepwise mechanism for nucleosome disassembly [155]. This is supported by the observation that histone mutations of the α-N helix region of H3 [H3(V35)-H3(S57)] near the nucleosome entry exit not only increase nucleosome unwrapping, but also increase nucleosome mobility and decrease nucleosome stability [142]. Similarly, the acetylation mimic H3(K56Q) increases nucleosomes thermal mobility by 1.8-fold [51]. Therefore, PTMs near the entry-exit region such as H3(Y41ph), H3(T45ph), H3(K56ac) or H4(K77ac,K79ac) that enhance DNA unwrapping could also indirectly facilitate nucleosome disassembly. Further studies will be required to determine if these histone PTMs function synergistically with histone PTMs in the dyad region that facilitate histone release to enhance nucleosome disassembly. Fully synthetic native chemical ligation, which was successfully demonstrated in this work to synthesize and quantify the
function of H3(K56ac), will enable such a study of multiple, simultaneous PTMs throughout the entire nucleosome.
4.4 Materials and Methods

4.4.1 DNA constructs

The DNA molecules 601-end, 601L-end, 601L-dyad, and 601R-end were prepared by PCR with Cy3-labeled oligonucleotides from a plasmid containing the 601 positioning sequence with or without a LexA binding site at bases 827 or bases 121-140 (See Appendix D for DNA and oligo sequences). The Mp2-192 DNA molecule for restriction enzyme assays was prepared by PCR from the plasmid pMp2 [94]. The Mp2-192 molecule contains the Mp2 NPS with 35 and 10 bases of linker DNA upstream and downstream of the NPS, respectively, and a Cy5 fluorophore attached to each 5’-end. Each oligonucleotide (Sigma-Aldrich) was labeled with a Cy3 or Cy5 NHS ester (GE Healthcare) at an amino group attached to either the 5’-end or to a modified internal thymine. Oligos were then purified by RP-HPLC with a 218P®C18 (Grace/Vydac) column. Following PCR amplification, each DNA molecule was purified by HPLC with a Gen-Pak Fax column (Waters).

4.4.2 Expression and Purification of Wild Type, Mutant, and Modified Histones

Recombinant histones were expressed and purified following [79, 107] as detailed in Appendix A. Mutations H3(R40C), H3(S96C), H3(C110A), H3(K56Q), and H4(S47C), H3(C110A), H3(K36E), H3(Y41E), H3(T45E) were introduced by site-directed mutagenesis (Stratagene). H2A(K119C) was labeled before or after histone octamer refolding with Cy5 maleamide (GE Healthcare) as detailed in Appendix B. We achieved a labeling efficiency of 75-90%, as determined by mass spectrometry and UV absorption.

Mutation H3(K115ac,K122ac) was synthesized as detailed in [93] and Chapter 1. Mutations H3(K56ac,C110A) and H3(R40C,K56ac,S96C,C110A), and fully synthetic H3(C110A)_{syn} were synthesized as detailed in [148]. Mutation H4(K77ac,K79ac) was synthesized as detailed in [120]. Due to details of the purification process, truncated H4(1-75) which is ligated onto H4Pep = C(Kac)R(Kac)TVTAMDVVYALKRQGRTLYGFGG and desulfurized to convert the introduced Cystiene at residue 76 into a native alanine,
is co-purified with the full length ligated H4 at purities of 70:30 to 80:20 full length to truncated H4(1-75). However, H4(1-75) lacks the C-terminal domain that interacts with H2B, preventing incorporation into full histone octamer. Trimethyl-lysine analog was incorporated as (2-bromoethyl) trimethylammonium bromide (Sigma-Aldrich) into H3(K36C,C110A) exactly following [101]

4.4.3 Histone Octamer Preparation and LexA protein purification

Histone octamer refolding and purification were performed following [79, 107] as described in Appendix A. Briefly, each histone was unfolded for 1 to 3 hours in unfolding buffer at a histone concentration of 2 to 20 mg/ml, and then spun to remove aggregates. The absorption at 276 nm was measured for each unfolded histone to determine the concentration. UV-Vis absorption spectra of acetylated histones H3(K56ac,C110A), H4(K77ac,K79ac), and H3(K115ac,K122ac) contain a background peak centered at 230nm that overlaps with the protein absorption peak centered at 276nm. This peak is of unknown origin, but presumably due to a remnant chemical contaminant from the synthesis process. To correct for this, the overlap between the anomalous 230nm peak and 276nm protein peak was estimated by hand as described in Appendix A. Similarly, for Cy5-labeled H2A(K119C), the faction of Cy5 absorbance at 276nm is subtracted from the protein absorbance as detailed in Appendix A.

Histones H2A(K119C), H2B, H4 and either H3(K56Q,C110A), H3(K56ac,C110A), H3(C110A)$_{rec}$, or H3(C110A)$_{syn}$, were combined at equal molar ratios. In the case of histone octamer containing Cy5-labeled H2A(K119C), equal molar ratio of H2A(K119C) and H2B was added in 2-fold excess to equal molar ratio of H4 or H4(K77ac,K79ac) and either unmodified H3 or H3(K56Q) to ensure complete incorporation of H2A(K119C) into full histone octamer. The octamer was refolded by double dialysis in refolding buffer. The refolded octamer was centrifuged to remove large aggregates and then purified by gel filtration over a Superdex 200 (GE healthcare) column to remove any H3/H4 tetramer and H2A/H2B dimer proteins (See Appendix A). The purity of each octamer was confirmed by SDS-PAGE and mass spectrometry.
LexA protein was expressed and purified from the pJWL288 plasmid (gift from Dr. Jonathan Widom) as following [108] as detailed in Appendix F.

4.4.4 Nucleosome reconstitution

Nucleosomes were reconstituted by salt double dialysis (See Appendix C) with 7µg of DNA and 5µg of histone octamer in a total volume 50µl of 0.5x TE buffer (10mM Tris, 1mM EDTA, pH 8.0), 2M NaCl, and 1mM benzamidine (BZA). Nucleosomes were then sucrose gradient purified (Appendix C) and their purity was verified by 5% 29:1 native PAGE.

4.4.5 Mapping nucleosome positions with hydroxyl radical cleavage

Nucleosome positions were mapped using FeBABE protein cutting reagent (Thermo Scientific) conjugated to H4(S47C). Histones H2A, H2B, and H4(S47C), and either H3(C110A)$_{rec}$ or H3(K56Q,C110A) were combined at equal molar ratios and refolded as detailed in Appendix A. Histone octamer was conjugated with FeBABE using protocols modified from manufacturer’s instruction (See Appendix B). Nucleosomes containing 601L-end with Cy3 on the 5'-end of the forward strand or nucleosomes containing 601L with Cy3 on the 5'-end of the reverse strand were reconstituted by salt double dialysis (Appendix C) with 1mM BZA omitted from all reconstitution buffers.

To perform hydroxyl radical mapping, we resuspended nucleosomes to 25nM on ice in degassed 20mM Tris (pH 7.5), 0.1mM EDTA, and 10% glycerol. Then 40mM L-ascorbic acid in degassed 20mM Tris (pH 7.5), 10mM EDTA, and 80mM hydrogen peroxide in degassed 20mM Tris (pH 7.5) and 10mM EDTA were added in quick succession to the nucleosomes with thorough mixing to final concentrations of 4mM and 8mM, respectively. The reaction was allowed to proceed for 10 minutes and 20 minutes before 7µl of the reaction mixture was transferred to 3µl of 1.3M Tris (pH 7.5). Samples were resolved by 12% denaturing PAGE in 7M urea and 1x TBE buffer. The sequence markers were prepared with a SequiTherm Excel II DNA sequencing kit (Epicenter) using Cy3-labeled forward or reverse primer, a 601L DNA template, and either ddGTP, ddATP, or ddTTP (See Appendix E). Results were imaged by a Typhoon 8600 variable mode imager (GE Healthcare).
4.4.6 Fluorescence Resonance Energy Transfer Measures of LexA Binding within Nucleosomes

The FRET efficiency of nucleosome in the absence of LexA was determined by the \((\text{Ratio})_A\) method (See Chapter: Introduction) \[83\]. The equilibrium constants for site accessibility were determined from the reduction in FRET efficiency as LexA binds to its target site buried within the nucleosome. LexA was titrated from 0\(\mu\)M to 3\(\mu\)M with 5 nM Cy3/Cy5-labeled nucleosomes in 0.5x TE buffer or from 0\(\mu\)M to 100\(\mu\)M with 5 nM Cy3/Cy5-labeled nucleosomes in 0.5x TE buffer with 75mM or 130mM NaCl. Nucleosomes were excited at 510nm (donor excitation) and emission spectra were collected from 530 to 750 nm; nucleosomes were then excited at 610nm (acceptor excitation) and emission spectra were collected from 630 to 750 nm using a Fluoromax-4 (Horiba) steady state spectrophotometer. The FRET efficiency was determined by the \((\text{Ratio})_A\) method, performed in triplicate, for each LexA concentration (See Chapter: Introduction). The average FRET efficiency versus the LexA concentration was fitted to a non-cooperative or cooperative binding isotherm to determine the LexA concentration at which the FRET efficiency has been reduced by half \(S_{0.5-nuc}\), which is the LexA concentration when its target site is bound 50% of the time by LexA. Additionally, we used EMSA to measure the LexA concentration at which its target site within naked DNA is 50% bound by LexA \(S_{0.5-DNA}\).

4.4.7 Restriction Enzyme Kinetics Method for Site Accessibility

The restriction enzyme kinetics method (See Chapter: Introduction) was used to determine the nucleosomal DNA site exposure equilibrium at six separate restriction enzyme sites: PstI, HindIII, HaeIII, TaqAl, HhaI and PmlII. The reactions were initiated by mixing 50\(\mu\)l of 2x restriction enzyme mixture 400-20000 units/ml restriction enzyme (New England Biolabs), 0.2mg/ml BSA, 2x concentration of recommended NEB buffer, 10% glycerol and 50\(\mu\)l of 2nM nucleosomes. TaqAl digestion was carried out at 65\(^\circ\)C; all other reactions were carried out at 37\(^\circ\)C. For PstI, time points at 0, 1/6, 1/3, 2/3, 1, 2, 3, 4, and 6 min were acquired by quenching 10\(\mu\)l of the reaction with EDTA at a final concentration of 20mM;
for all other restriction enzymes time points were acquired at 0, 1, 2, 4, 8, 16, 32, 48 min. Proteinase-K (1 mg/ml final concentration) and SDS (0.02% final concentration) were added to each time point to remove the histone octamer from the DNA. Each time point was examined by EMSA on a 5% 29:1 acrylamide gel with 0.3x TBE in the gel and running buffer; gels were prerun for 1 hour at 20V/cm (300V) on a GibcoBRL V16 vertical rig, Ficol was added to 2.5% to each sample, samples were applied to the gel while not running, and samples were allowed to resolve for 1 hour at 300V (See Appendix E). The DNA was visualized with a Typhoon 8600 variable mode imager (GE Healthcare) and quantified by ImageQuant with local median background subtraction.

The digestions were fit to a single exponential to determine the initial digestion rate. An initial drop in undigested DNA between the zero and 1 minute time point is due to a small fraction of nucleosomes that dissociate during the rapid mixing. We therefore neglect this initial time point as has been done in previous studies [81, 94].
Chapter 5

REGULATION OF DNA UNWRAPPING CONTROLS NUCLEOSOME ACCESSIBILITY

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Regulation of the nucleosome unwrapping rate controls DNA accessibility
First published online: September 10, 2012 doi:10.1093/nar/gks747

5.1 Introduction

Up to this point we have focused on the regulation of nucleosome stability, mobility, and accessibility by histone post-translational modifications (PTMs). However, not only do histone PTMs regulate DNA site exposure equilibrium, but the DNA sequence bound by the core histones does as well [28, 156, 157]. However, the mechanisms that regulate nucleosome site exposure remain undetermined. These intrinsic factors of histone PTMs and DNA sequence could influence the rate of nucleosome unwrapping to expose the site, the rate of nucleosome rewrapping to bury the site, or both. Histone H3 lysine 56 acetylation [H3(K56ac)], which helps regulate eukaryotic transcription, replication, and repair [62, 71,
is located in the DNA-histone interface near the nucleosome entry-exit region. Structural studies of the acetylation mimic H3(K56Q) observe minimal changes in the fully wrapped nucleosome structure [113], while replacing histone H3 with its variant, CENP-A, does significantly alter the DNA in the crystal structure [139]. As shown in Chapter 4, H3(K56ac) shifts the site exposure equilibrium toward partially unwrapped nucleosome states [103], which enhances DNA accessibility for TF binding within the nucleosome. These results suggest that H3(K56ac) reduces DNA-histone interactions that enhance site exposure without altering the nucleosome structure in the fully wrapped state.

Determining the mechanisms by which both PTMs and DNA sequence influence site exposure is central to understanding how transcription and DNA repair complexes gain access to nucleosomal DNA. Here we examine the influence of H3(K56ac) and DNA sequence on the nucleosome unwrapping and rewrapping rates in order to determine the changes in nucleosome dynamics associated with changes in site exposure equilibrium. We find that H3(K56ac) and DNA sequence within the nucleosome entry-exit region separately influence the nucleosome unwrapping rate without altering the rewrapping rate. H3(K56ac) and DNA sequence additively influence the nucleosome unwrapping rate by at least an order of magnitude and result in an equivalent change in TF occupancy within the nucleosome. These results are consistent with the conclusion that modulation of nucleosome unwrapping by PTMs and DNA sequence is a general mechanism for regulating DNA accessibility for transcription and DNA repair.
5.2 Results

5.2.1 H3(K56ac) enhances site accessibility by increasing the nucleosome unwrapping rate

In Chapter 4 we showed that H3(K56ac) increases the site exposure equilibrium for LexA binding by 3-fold. To investigate the kinetic mechanism of H3(K56ac) on nucleosome site exposure, we used stopped flow fluorescence resonance energy transfer (FRET). FRET was used to detect the binding of the model TF, LexA, to its target sequence within the nucleosome as previously described in Chapter 4. The nucleosomes contained the 147-bp high-affinity 601 NPS (Jb40), with the LexA target sequence inserted between base pairs 8-27 (601L-end, Figure 5.1A). The DNA was labeled with Cy3 at the 5′-end nearest the LexA target sequence, while Cy5 was linked to the histone octamer at H2A(K119C) (Figure 5.1B). The distance between Cy3 and Cy5 within the nucleosome is significantly less than the Cy3-Cy5 Forster radius (Figure 5.1C) and results in efficient FRET. We chose as our unmodified wild-type histone H3 sequence *X. laevis* H3(C110A) as used in previous studies refs. This allows for direct comparison to the fully synthetic H3(K56ac,C110A), which was introduced into H3 by Ottesen and colleagues using sequential native chemical ligation [148] (Figure 5.1B). Additionally, this allows site specific labeling of Cy5 to H2A(K119C) when incorporated into fully formed histone octamer.

Sucrose gradient-purified nucleosomes (Figure 5.2A) were reconstituted with and without H3(K56ac). In the presence of LexA, the nucleosome can be trapped in a partially unwrapped state (Figure 5.1C), which results in a reduction in the FRET efficiency. To measure the nucleosome unwrapping rate, k_{12} (Figure 5.1C), the LexA TF was rapidly mixed with Cy3/Cy5-labeled nucleosomes, and LexA binding to the nucleosome was detected by the measured reduction in Cy5 FRET-stimulated emission (Figure 5.1D-E). LexA concentrations (300-900 nM) were used to confirm that the rate of FRET reduction was independent of LexA concentration (Figure 5.2D). Under these conditions the rate-limiting step of LexA binding is the nucleosome unwrapping rate, k_{12}, and the observed rate of reduction in Cy5 FRET-stimulated fluorescence is equal to k_{12} [31]. We determined at low
ionic strength (0.5x TE, 1mM NaCl) the unwrapping rates for nucleosomes containing unmodified H3 ($k_{12-unmod} = 8 \pm 1 \text{ s}^{-1}$), H3(K56Q)($k_{12-K56Q} = 15 \pm 2 \text{ s}^{-1}$) and H3(K56ac) ($k_{12-K56ac} = 15 \pm 1 \text{ s}^{-1}$; Figure 5.1F). The unwrapping rate for unmodified nucleosomes is similar to previous measurements [31], while H3(K56ac) and H3(K56Q) increase the unwrapping rate by 1.9 ± 0.3- and 2.0 ± 0.3-fold, respectively (Figure 5.1G). The changes in unwrapping rates are identical to the changes in site exposure equilibrium, $K_{eq}^{wrap}$, we previously measured (See Chapter 4 for details), which implies that H3(K56ac) and H3(K56Q) do not alter the nucleosome rewrapping rate ($k_{calc}^{21} = k_{12}/K_{eq}^{wrap}$, Figure 5.1G, Table 5.1).

We next investigated the influence of H3(K56ac) on the nucleosome unwrapping rate at physiological ionic strength (0.5x TE, 130mM NaCl). We rapidly mixed Cy3/Cy5-labeled nucleosomes in the presence of 130mM NaCl with LexA ranging between 20 and 50µM. At these TF concentrations the decay rate of Cy5 fluorescence is independent of LexA concentration (Figure 5.2E) and therefore equal to the nucleosome unwrapping rate. We determined the unwrapping rates for nucleosomes containing unmodified H3 (16 ± 5 s$^{-1}$), H3(K56Q) (43 ± 11 s$^{-1}$), and H3(K56ac) (50 ± 11 s$^{-1}$) (Figures 5.1H and 5.2B-C). H3(K56ac) and H3(K56Q) increased the nucleosome unwrapping rate by 3.0 ± 1.1- and 2.6 ± 1.0-fold, respectively (Figure 5.1I). This change in unwrapping rates is nearly identical to the increase in the site exposure equilibrium induced by H3(K56ac) and H3(K56Q) (See Chapter 4 for details), providing additional support for the conclusion that the nucleosome rewrapping rate is unchanged by H3(K56ac) and H3(K56Q) (Figure 5.1I, Table 5.1). Taken together, these results suggest that H3(K56ac) enhances TF binding within the nucleosome by increasing the nucleosome unwrapping rate without altering the rewrapping rate.
Figure 5.1: **H3(K56ac) increases the rate of nucleosome unwrapping.** (A) DNA constructs for FRET measurements of nucleosome unwrapping kinetics. Both higher affinity 601 NPS or lower affinity *X. borealis* 5S ribosomal DNA NPS contain a LexA protein binding site from bases 827 and a Cy3 molecule on the 5′-end. (B) Structure of FRET-labeled nucleosome [37] containing 601L-end or 55L-end DNAs; the LexA-binding site in red, Cy3 in green, Cy5 on H2A(K119C) in magenta and H3(K56Ac) in orange; PDB ID: 1KX5. (C) A three-state model for LexA binding to its target site within a nucleosome. (D) and (E) Stopped Flow Cy5 FRET-stimulated emission versus time of 601L-end nucleosomes containing unmodified H3 or H3(K56Ac) nucleosomes, respectively, at 1mM NaCl mixed with 0900nM LexA. (F) Normalized stopped flow Cy5 FRET-stimulated emission versus time at 900nM LexA for nucleosomes containing unmodified H3 (blue circles), H3(K56Q) (red circles) and H3(K56ac) (orange circles) at 1mM NaCl. (G) Relative unwrapping and calculated rewrapping rates of nucleosomes containing H3(K56Q) and H3(K56ac) versus unmodified at 1mM NaCl. (H) Normalized stopped flow Cy5 FRET-stimulated emission versus time at 30µM LexA for nucleosomes containing unmodified H3 (blue circles), H3(K56Q) (red circles) and H3(K56ac) (orange circles) at 130mM NaCl (Also See Figure 5.2B-C). (I) Relative unwrapping and calculated rewrapping rates of nucleosomes containing H3(K56Q) and H3(K56ac) versus unmodified H3 at 130mM NaCl. (Reproduced with permission)
Figure 5.2: -

Nucleosome unwrapping rate is [LexA]-independent at high-LexA concentration. (A) Cy3 fluorescence image of native PAGE analysis of purified FRET-labeled nucleosomes containing the 601L-end DNA molecule and unmodified H3, H3(K56Q), or H3(K56ac). (B) and (C) Stopped Flow Cy5 FRET-stimulated emission versus time of 601L-end nucleosomes containing unmodified H3 or H3(K56Ac) nucleosomes, respectively, at 130mM NaCl mixed with 0-50µM LexA. (D) and (E) Apparent unwrapping rate, $k_{12-app}$, for nucleosomes in 1mM NaCl and 130mM NaCl, respectively as a function of [LexA]. At 1mM NaCl in the presence of 300-900nM LexA and at 130mM NaCl in the presence of 20-50µM LexA, the apparent unwrapping rate is independent of [LexA], indicating that in this regime the apparent unwrapping rate is the actual nucleosome unwrapping rate, $k_{12}$. (Reproduced with permission)
<table>
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<th>DNA</th>
<th>Histone</th>
<th>Na⁺ (mM)</th>
<th>E₀</th>
<th>S₀.₅ (nM)</th>
<th>( \frac{K^{\text{wrap}}<em>{\text{eq}}}{K^{\text{wrap}}</em>{\text{eq}-601\text{L}-\text{unmod}}} )</th>
<th>( k_{12} ) (s⁻¹)</th>
<th>( \frac{k_{12}}{k_{12-601\text{L}-\text{unmod}}} )</th>
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<td>6700±300</td>
<td>0.36±0.03</td>
<td>5.4±0.7</td>
<td>0.33±0.07</td>
<td>1.1±0.3</td>
<td>-</td>
</tr>
<tr>
<td>5SL(1-7)</td>
<td>unmod</td>
<td>75</td>
<td>0.48±0.02</td>
<td>4600±300</td>
<td>0.53±0.05</td>
<td>7±2</td>
<td>0.40±0.1</td>
<td>1.3±0.4</td>
<td>-</td>
</tr>
<tr>
<td>5SL(28-27)</td>
<td>unmod</td>
<td>75</td>
<td>0.54±0.02</td>
<td>2600±200</td>
<td>0.96±0.08</td>
<td>17±3</td>
<td>1.0±0.2</td>
<td>0.9±0.2</td>
<td>-</td>
</tr>
<tr>
<td>5SL(1-47)</td>
<td>unmod</td>
<td>75</td>
<td>0.48±0.02</td>
<td>5300±400</td>
<td>0.46±0.05</td>
<td>7±1</td>
<td>0.40±0.09</td>
<td>1.1±0.3</td>
<td>-</td>
</tr>
<tr>
<td>601L</td>
<td>H3(K56Q)</td>
<td>75</td>
<td>0.52±0.01</td>
<td>870±100</td>
<td>2.8±0.4</td>
<td>50±10</td>
<td>3.1±0.8</td>
<td>0.9±0.3</td>
<td>-</td>
</tr>
<tr>
<td>5SL(1-7)</td>
<td>H3(K56Q)</td>
<td>75</td>
<td>0.45±0.01</td>
<td>1400±100</td>
<td>1.2±0.2</td>
<td>20±3</td>
<td>1.3±0.3</td>
<td>1.3±0.3</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.1: Summary of the TF binding equilibrium and nucleosome unwrapping measurements. Column 1 is the DNA construct. Column 2 is the type of histone octamer. Column 3 is the concentration of sodium. Column 4 is the average FRET efficiency without LexA. Column 5 is the half saturation concentration of LexA. Column 6 is the site exposure equilibrium relative unmodified nucleosomes containing 601L-end. Column 7 is the nucleosome unwrapping rate. Column 8 is the nucleosome unwrapping rate relative to unmodified nucleosomes containing 601L-end. Column 9 is the calculated rewrapping rate relative to unmodified nucleosomes containing 601L-end.
5.2.2 The influence of H3(K56ac) on the nucleosome unwrapping equilibrium is independent of DNA sequence

To test the possibility that the nucleosomal DNA sequence impacts the influence of H3(K56ac) on the site exposure equilibrium, we prepared Cy3/Cy5 nucleosomes with the *Xenopus borealis* 5S rDNA positioning sequence [162]. This is a naturally occurring sequence containing part of the somatic 5S ribosomal DNA gene and has been used as a model NPS in numerous biophysical studies [75, 89, 163]. As with the 601 sequence, we inserted the LexA target sequence between base pairs 8-27 of the 5S NPS (5SL-end, Figure 5.1A). We then reconstituted nucleosomes with the 5SL-end DNA labeled with Cy3 at the 5′-end nearest the LexA target sequence and histone octamer labeled with Cy5 at H2A(K119C) (Figure 5.1B). H3(K56ac) and H3(K56Q) induced a slight shift in 5SL nucleosome electrophoretic mobility relative to unmodified nucleosomes (Figure 5.3A), as was previously observed for the 601 NPS (See Chapter 4 for details). To determine if the change in electrophoretic mobility was due to a position change, we mapped the nucleosome positions on 5SL-end by hydroxyl radical mapping [95] using the FeBABE label as previously detailed (See Chapter 4 for details). We find that the H3(K56Q) does not impact the cleavage pattern compared to unmodified H3 (Figure 5.4). Furthermore, the cleavage pattern with 5SL-end nucleosomes is indistinguishable from nucleosomes containing 601L-end (See Chapter 4 for details). This indicates that the nucleosomes were well positioned within the 5S NPS and that nucleosome position was not altered by modifying H3(K56).

To determine the influence of H3(K56ac) on the 5S NPS site exposure equilibrium, we carried out LexA titrations with unmodified, H3(K56ac) and H3(K56Q) nucleosomes containing the 5SL-end sequence (Figure 5.3B-D). We determined the FRET efficiency of unmodified and modified nucleosomes at each LexA concentration in triplicate and then fit the FRET efficiency as a function of [LexA] to a non-cooperative binding isotherm. This analysis determines the LexA concentration at which half of the nucleosomes are bound by LexA, $S_{0.5-nuc}$. From this we use, $\frac{K_{eq-modified}^{\text{wrap}}}{K_{eq-unmodified}^{\text{wrap}}} = \frac{S_{0.5-unmodified}}{S_{0.5-modified}}$. 

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to infer the relative nucleosome site exposure equilibrium [30, 148]. The resulting increase in 5SL-end site accessibility matches that observed for the 601L-end NPS

\[ \frac{K_{\text{wrap}}^{\text{eq}-5\text{SL}-K56ac}}{K_{\text{wrap}}^{\text{eq}-5\text{SL}-\text{unmod}}} = 1.8 \pm 0.3 \text{ and } \frac{K_{\text{wrap}}^{\text{eq}-5\text{SL}-K56ac}}{K_{\text{wrap}}^{\text{eq}-5\text{SL}-\text{unmod}}} = 2.0 \pm 0.4; \text{ Figure 5.3E and Table 5.1}. \]

To verify that the increase in the site exposure equilibrium was due to unwrapping and not repositioning, we monitored LexA-induced nucleosome sliding by placing the Cy3 fluorophore on the 80th bp of the DNA (5SL-dyad; Figure 5.5A) [148]. This results in juxtaposition of the Cy3-Cy5 FRET pair near the nucleosome dyad (Figure 5.5B). Repositioning induced by LexA binding would result in a FRET change, while unwrapping will not. We do not observe any change in FRET, even at saturating concentrations of LexA (Figure 5.5C).

As an independent verification, we conducted hydroxyl radical mapping [95] using the FeBABE label [148] with 5SL-end nucleosomes pre-incubated with saturating concentrations of LexA (1µM). There was no observable difference between the cleavage of 5SL-end and 601L-end nucleosomes in the presence and absence of LexA (Figure 5.4). The combination of the site exposure equilibrium measurements for TF binding within 5SL and 601L containing nucleosomes as well as the nucleosome position mapping strongly suggests that H3(K56ac) increases the nucleosome site exposure equilibrium independent of the underlying nucleosomal DNA sequence.

### 5.2.3 DNA sequence within the nucleosome entry-exit region influences accessibility by modulating the DNA unwrapping rate

We investigated the influence of DNA sequence changes within the 601 NPS on nucleosome unwrapping and rewrapping rates. First, we determined the LexA concentrations at which half of the nucleosomes are bound by LexA, \( S_{0.5-nuc} \), for nucleosomes containing 601L-end and 5SL-end. We find at low ionic strength (0.5x TE with 1mM NaCl, 5.1) that \( S_{0.5-nuc} \) of unmodified nucleosomes containing 5SL-end is reduced relative to unmodified nucleosomes with 601L-end, indicating a lower site exposure, \( K_{\text{eq}}^{\text{wrap}} \), in the entry-exit region

\[ \frac{K_{\text{eq}}^{\text{wrap}-5\text{SL}-\text{unmod}}}{K_{\text{eq}}^{\text{wrap}-601\text{L}-\text{unmod}}} = 0.6 \pm 0.1. \] 

Changing the DNA sequence from 601L-end
Figure 5.3: DNA sequence does not influence H3(K56ac) enhancement of TF binding. (A) Cy3 fluorescence image of native PAGE analysis of purified FRET-labeled nucleosomes containing the 5SL-end NPS and unmodified H3 (lane 2), H3(K56Q) (lane 3), or H3(K56ac) (lane 4). (B) and (C) Fluorescence emission spectra of 5SL-end FRET-labeled nucleosomes containing unmodified H3 or H3(K56ac), respectively, excited at 510nm (donor excitation) in the presence of 0.5x TE, 1mM NaCl and LexA at 0nM (black), 30nM (red), or 1000nM (blue). (D) Steady state FRET efficiency, as determined by the (ratio)_A method (See Chapter: Introduction) [83], versus LexA concentration for nucleosomes containing unmodified H3 (blue), H3(C110) (purple), H3(K56Q) (red), and H3(K56a) (orange) at 1mM NaCl. Plots are the average of three LexA titrations and the error bars were determined from the standard deviation of the three measurements. The data were fit to a non-cooperative binding curve, which determines S_{0.5—nuc}, the LexA concentration at which 50% of the nucleosomes are bound by LexA. (E) Relative change in S_{0.5—nuc} for nucleosomes with the 601L-end (14) or 5SL-end NPS containing H3(K56Q) or H3(K56ac) versus unmodified H3 at 1mM NaCl. This is equal to the relative change in site exposure equilibria. (Reproduced with permission)
Figure 5.4: Hydroxyl Radical mapping of nucleosome position. (A) The crystal structure of the nucleosome [37]. H3(K56) is shown in orange. H4(S47), which is replaced with a cysteine and labeled with FeBABE, is shown in blue. The bases that are cleaved by FeBABE are shown in red; PDB ID: 1KX5. (B) EMSA of nucleosomes labeled with FeBABE at H4(S47C) and containing 601L-end (left) or 5SL-end (right) DNA. Lane 1 contains the DNA substrate, Lane 2 contains nucleosomes with unmodified H3 and Lane 3 contains nucleosome with H3(K56Q). (C) and (D) Cy3 image of denaturing PAGE of 601L-end and 5SL-end nucleosomal DNA, respectively, cleaved by FeBABE for 0, 5 and 10 minutes. Within each gel, lanes 1-3 and 10-12 contain sequencing tracks terminated with ddGTP,ddATP and ddTTP respectively, lanes 4-6 contain nucleosomes with unmodified H3 and lanes 7-9 contain nucleosomes with H3(K56Q). (E) and (F) Cy3 image of denaturing polyacrylimide gel electrophoresis of 601L-end and 5SL-end nucleosomal DNA, respectively, cleaved by FeBABE for 0, 5 and 10 minutes after pre-incubation with 1µM LexA. Lanes are the same as in (C) and (D). The cleavage pattern is identical to that of nucleosome in the absence of LexA, indicating that LexA binding does not reposition nucleosomes. (Reproduced with permission)
Figure 5.5: **Nucleosomal DNA is not repositioned upon LexA binding.** (A) DNA constructs for FRET measures of LexA-induced nucleosome repositioning. The *X. borealis* 5S NPS contains a LexA protein binding site from bases 8-27 and a Cy3 molecule on the 80th base. (B) Structure of FRET-labeled nucleosome [37] containing 5SL-dyad DNA; the LexA binding site in red, Cy3 in green, and Cy5 on H2A(K119C) in magenta; PDB ID: 1KX5. (C) Steady state FRET efficiency, as determined by the (ratio)A method (See Chapter: Introduction) [83], versus LexA concentration for nucleosomes containing unmodified H3 (blue) or H3(K56Q) (red). Plots are the average of three LexA titrations and the error bars were determined from the standard deviation of the three measurements. The FRET is constant over the range of [LexA] in which the LexA site is occupied within the nucleosome, indicating that the DNA is not repositioned by LexA binding. (Reproduced with Permission)
to 5SL-end had a similar influence on the $K_{eq}^{\text{wrap}}$ for LexA binding within nucleosomes containing either H3(K56Q) [$K_{eq}^{\text{wrap}}(5\text{SL}−5\text{K56Q})/K_{eq}^{\text{wrap}}(601\text{L}−5\text{K56Q}) = 0.8 \pm 0.2$] or H3(K56ac) [$K_{eq}^{\text{wrap}}(5\text{SL}−5\text{K56ac})/K_{eq}^{\text{wrap}}(601\text{L}−5\text{K56ac}) = 0.7 \pm 0.1$].

We also investigated the influence of DNA sequence on $K_{eq}^{\text{wrap}}$ closer to physiological ionic strength. While we were unable to fully saturate LexA binding to nucleosomes containing 5SL in 130mM NaCl (Figure 5.6A,D), and hence get adequate changes in fluorescence for accurate stopped flow kinetics measures (Figure 5.6B-D), qualitatively the differences in LexA binding at 130mM NaCl between the 601L and 5SL NPS are consistent with accessibility differences at low salt: $K_{eq}^{\text{wrap}}(5\text{SL}−\text{unmod})/K_{eq}^{\text{wrap}}(601\text{L}−\text{unmod}) = 0.2 \pm 0.1$ (130mM NaCl) and $K_{eq}^{\text{wrap}}(5\text{SL}−\text{unmod})/K_{eq}^{\text{wrap}}(601\text{L}−\text{unmod}) = 0.2 \pm 0.1$ (130mM NaCl).

However, LexA binding to nucleosomes did saturate at 75mM NaCl (Figure 5.8B). We used these conditions (0.5x TE with 75mM NaCl) to further examine the DNA sequence dependence of $K_{eq}^{\text{wrap}}$. Under these conditions, changing the NPS from 601L to 5SL significantly increased $S_{0.5−\text{nuc}}$ which implied that the site exposure equilibrium constant, $K_{eq}^{\text{wrap}}$, was reduced by $\sim 3$-fold [$K_{eq}^{\text{wrap}}(5\text{SL}−\text{unmod})/K_{eq}^{\text{wrap}}(601\text{L}−\text{unmod}) = 0.36 \pm 0.03$].

The observation that the *X. borealis* 5S NPS reduced the site exposure equilibrium relative to the 601 NPS was unexpected because the sea urchin 5S NPS has a significantly higher free energy for nucleosome formation (lower histone octamer affinity) relative to the 601 positioning sequence [75]. To determine which region(s) of the DNA sequence is responsible for this 3-fold reduction in $K_{eq}^{\text{wrap}}$, we created three DNA chimeras where segments of 601L-end DNA was replaced with segments from 5SL-end DNA (Figure 5.7A). These chimeric DNAs were labeled with Cy3 and reconstituted into nucleosomes with Cy5-labeled histone octamer (Figure 5.8A). We carried out LexA-binding analysis with nucleosomes containing the chimeric DNA sequences to determine the relative changes in $S_{0.5−\text{nuc}}$ to detect alterations in the site exposure equilibrium, $K_{eq}^{\text{wrap}}$ (Figures 5.7B-C, 5.8B). We found that most of the increase in $S_{0.5−\text{nuc}}$ and therefore reduction in $K_{eq}^{\text{wrap}}$ is induced by changing base pairs 1-7, located between the nucleosome entry-exit and the LexA target sequence [$K_{eq}^{\text{wrap}}(5\text{SL}(1−7)−\text{unmod})/K_{eq}^{\text{wrap}}(601\text{L}−\text{unmod}) = 0.53 \pm 0.04$]. Changing base pairs 28-47, located on the opposite side of the LexA target sequence, does not reduce the $K_{eq}^{\text{wrap}}$. 

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\[ \frac{K_{\text{wrap}}^{5SL(28-47)-\text{unmod}}}{K_{\text{wrap}}^{601L-\text{unmod}}} = 0.98 \pm 0.08 \]. Furthermore, the combined influence of changing base pairs 1-7 and 28-47 on \( K_{\text{wrap}}^{eq} \) is similar to the influence of changing only base pairs 1-7 \[ \frac{K_{\text{wrap}}^{eq-5SL(1-47)-\text{unmod}}}{K_{\text{wrap}}^{eq-601L-\text{unmod}}} = 0.46 \pm 0.05 \]. This suggests that the DNA sequence located between the nucleosome entry-exit and the TF-binding site can significantly influence the site exposure equilibrium.

We performed stopped flow experiments with nucleosomes containing each of the 601L-5SL chimeric DNAs to examine the mechanism by which DNA sequence modulates site accessibility (Figures 5.7D, 5.8C). The change in Cy5 fluorescence was measured at LexA concentrations between 10 and 30 \( \mu \)M where the rate of change of Cy5 fluorescence was independent of LexA concentration (Figure 5.8D) and equal to the nucleosome unwrapping rate. We determined the nucleosome unwrapping rate, \( k_{12} \), for each DNA chimera (Figure 5.7D and Table 5.1). Replacing base pairs 1-7 of 601L with 5SL significantly reduced the unwrapping rate \[ \frac{k_{12-5S(1-7)-\text{unmod}}}{k_{12-601L-\text{unmod}}} = 0.4 \pm 0.1 \], while replacing base pairs 28-47 of 601L with 5SL did not influence the unwrapping rate \[ \frac{k_{12-5S(28-47)-\text{unmod}}}{k_{12-601L-\text{unmod}}} = 1.0 \pm 0.2 \]. Additionally, the combined change of base pairs 1-7 and 28-47 reduced the unwrapping rate \[ \frac{k_{12-5S(1-47)-\text{unmod}}}{k_{12-601L-\text{unmod}}} = 0.4 \pm 0.1 \], which appears indistinguishable to changing only base pairs 1-7.

Importantly, the DNA sequence-induced changes in the unwrapping rates are nearly identical to the induced changes in site exposure equilibrium (Figure 5.7C,E), which implies that the changes in DNA sequence do not influence the calculated nucleosome rewrapping rates, \( k_{21}^{calc} \) (Figure 5.7F). Taken together, these results demonstrate that alterations in DNA sequence influence TF binding within nucleosomes by changing the DNA unwrapping rate without influencing the rewrapping rate. Note that while the \( K_{\text{wrap}}^{eq} \) measured by LexA binding increased for nucleosomes containing 5SL-end relative to 601L-end, the average FRET efficiency without LexA was lower for nucleosomes containing 5SL-end (0.47 \( \pm 0.02 \)) compared to 601L-end (0.67 \( \pm 0.02 \)). This contradictory result could be due to increased nucleosome unwrapping of the first few base pairs of 5SL. However, the average FRET efficiency is influenced by numerous parameters [83]. In particular, the structure of a nucleosome containing the 601 positioning sequence was recently reported to contain
145 base pairs as compared to 147 base pairs for an alpha satellite DNA sequence \cite{164}. This could significantly alter the Cy3-Cy5 distance. In contrast, determining changes in the site exposure equilibrium from the $S_{0.5-nuc}$ measurements of LexA binding to nucleosomes does not rely on the absolute average FRET efficiency. Furthermore, the agreement between the DNA sequence-induced changes in nucleosome unwrapping and the changes to the equilibrium of LexA binding to a nucleosome confirms the reliability of the LexA-binding measurements. Therefore, we used the $S_{0.5-nuc}$ measurements from LexA titrations to determine the relative change in site exposure of the LexA target sequence.

5.2.4 DNA sequence and modification of H3(K56) additively regulate TF binding within nucleosomes

Our observation that DNA sequence does not alter the influence of either H3(K56ac) or H3(K56Q) on nucleosome site exposure equilibrium suggests that the influence of DNA sequence is independent of the modification state of H3(K56). To test this hypothesis, we prepared Cy3-Cy5-labeled nucleosomes that contained the DNA chimera 5S(1-7) and the acetyl-lysine mimic H3(K56Q). We found that the influence of replacing the first 7 base pairs of 601L-end with 5SL-end did not alter the impact of H3(K56Q) on the LexA concentration to bind half of the nucleosomes, $S_{0.5-nuc}$, and therefore the site exposure equilibrium, $K_{wrap}^{eq}$

$$
\frac{K_{wrap}^{eq-5S(1-7)\text{-K56Q}}}{K_{wrap}^{eq-601L\text{-K56Q}}} = 0.36 \pm 0.06 \text{ and } \frac{K_{wrap}^{eq-5S(1-7)\text{-unmod}}}{K_{wrap}^{eq-601L\text{-unmod}}} = 0.36 \pm 0.03; \text{ Figure 5.7B,C}.\]

We then determined that the influence of replacing the first 7 base pairs of 601L with 5SL on the unwrapping rate was unaffected by H3(K56Q) $[k_{12-5S(1-7)\text{-K56Q}}/k_{12-601L\text{-K56Q}} = 0.4 \pm 0.1 \text{ and } k_{12-5S(1-7)\text{-unmod}}/k_{12-601L\text{-unmod}} = 0.33 \pm 0.07; \text{ Figure 5.7D,E}].$ These results are consistent with the conclusion that H3(K56) modification state and the DNA sequence influence nucleosome unwrapping rate and ultimately TF binding independently. This implies that the DNA sequence and the modification of H3(K56) may additively combine to significantly influence TF binding. In fact, the unwrapping rate of nucleosomes modified at H3(K56) within the 601 NPS is $10 \pm 2$ times larger than unmodified nucleosomes within the 5S NPS. This difference enhances TF binding by $8 \pm 2$ times.
Figure 5.6: **5SL-end nucleosome are not fully bound at physiological ionic strength** (A) FRET efficiency, as determined by the (Ratio)_A method (See Chapter: Introduction) [83], of 5LS-end nucleosomes containing unmodified (blue) or H3(K56Q) (red) histone octamer as a function of LexA concentration. Plots are the average and error bars are the standard deviation of three separate titrations. FRET efficiencies were fit to a non-cooperative binding isotherm to determine the concentration of half saturation, S_{0.5-nuc} at which 50% of the nucleosomes are bound by LexA. (B) Normalized stopped flow fluorescence decay of unmodified (blue) or H3(K56Q) (red) containing 5SL-end nucleosomes rapidly mixed with LexA. Each trace exhibits a fast (LexA independent due to specific binding) and slow (LexA dependent due to non-specific binding) kinetic. The black line is the fit of a double exponential to the data. (C) Measured rates of nucleosome unwrapping from the exponential fit of the fast kinetic in (B). Error bars are the standard deviation of at least 7 independent experiments. (D) Relative equilibrium accessibility (white), unwrapping rate (light gray) and rewinding rate (gray) of 5SL-end nucleosomes containing H3(K56Q) versus unmodified H3 from the data in (A) and (B).
Figure 5.7: DNA sequence between the nucleosome entry-exit and the TF binding site impacts nucleosome unwrapping. (A) DNA constructs with the 601 sequence (blue) iteratively replaced by the 5S sequence (lavender), where the parenthetical numbers represents the bases in 601 replaced by 5S; "end" nomenclature indicating Cy3 5′-end label 1st base pair omitted for brevity; LexA site in red; 5SL(1-147) is the same as 5SL for clarity. (B) Steady State FRET efficiency, as determined by the (ratio)_A method (See Chapter: Introduction) [83], versus lexA concentration for nucleosomes containing 601L and unmodified H3 (blue), 601L and H3(K56Q) (red), 5SL(1-7) and unmodified H3 (green), and 5SL(1-7) and H3(K56Q) (olive) at 75mM NaCl. Plots are the average of three LexA titrations and the error bars were determined from the standard deviation of the three measurements. The data were fit to a non-cooperative binding curve. (C) Relative S_{0.5-nuc} for the indicated nucleosome versus nucleosomes containing 601L and unmodified H3 at 75mM NaCl. (D) Normalized stopped flow Cy5 emission versus time at 15µM LexA for nucleosomes containing 601L and unmodified H3 (blue), 601L and H3(K56Q) (red), 5SL(1-7) and unmodified H3 (green), and 5SL(1-7) and H3(K56Q) (olive) at 75mM NaCl. (E) and (F) Relative unwrapping and calculated rewrapping rates, respectively, for the indicated nucleosomes versus nucleosome containing 601L and unmodified H3 at 75mM NaCl (See also Figure 5.8)(Reproduced with permission.)
Figure 5.8: DNA sequence between the nucleosome entry-exit and the TF binding site impacts nucleosome accessibility. (A) Cy3 fluorescence image of native PAGE analysis of purified FRET-labeled containing unmodified H3 and the 601L, 5SL(28-47), 5SL(1-7), 5SL(1-47), or 5SL(1-147) NPS. (B) Steady state FRET efficiency, as determined by the (ratio)$_A$ method (See Chapter: Introduction) [83], versus LexA concentration for nucleosomes containing unmodified H3 and 601L (blue), 5SL(28-47) (purple), 5SL(1-7) (green), 5SL(1-47) (orange), and 5SL(1-147) (violet) at 75mM NaCl. Plots are the average of three LexA titrations and the error bars were determined from the standard deviation of the three measurements. The data were fit to a non-cooperative binding curve, which determines $S_{0.5 \text{-nuc}}$, the LexA concentration at which 50% of the nucleosomes are bound by LexA. (C) Normalized stopped flow Cy5 emission versus time at 15µM LexA for nucleosomes containing unmodified H3 and 601L (blue), 5SL(28-47) (purple), 5SL(1-7) (green), 5SL(1-47) (orange), and 5SL(1-147) (violet) at 75mM NaCl. (D) The LexA binding rate for nucleosomes in 75mM NaCl as a function of [LexA]. In the presence of 7.5-30µM LexA, the apparent unwrapping rate is independent of [LexA], indicating that in this regime the LexA binding rate is the equal to the nucleosome unwrapping rate, $k_{12}$. (Reproduced with permission)
5.2.5 The 601 positioning sequence has a higher binding affinity but lower nucleosome unwrapping equilibrium than a 5S positioning sequence

The observation that substitution of the first 7 base pairs of the 601 NPS with the X. borealis 5S NPS reduces the site exposure equilibrium was unexpected. To further investigate this, we determined the difference in free energies for nucleosome formation between the 601-5S chimeras with competitive reconstitutions (See Chapter: Introduction), [75, 80]. This method determines the free energy difference, $\Delta \Delta G_{\text{nuc}}^\text{sample}$, for nucleosome formation between distinct DNA sequences. Nucleosomes are reconstituted with a fluorophore-labeled NPS in the presence of low-affinity competitor DNA and histone octamer. The naked DNA and histone octamer establish a dynamic equilibrium with assembled nucleosomes during the reconstitution by gradual salt dialysis. The equilibrium constant, or "binding equilibrium", is then determined by EMSA (Figure 5.9A) for the high-affinity sample DNA ($K_{\text{eq-sample}}^{\text{nuc}}$) and the reference DNA ($K_{\text{eq-reference}}^{\text{nuc}}$) from which the the free energy difference of nucleosome formation is determined. We then compared this to the difference in free energy for the nucleosome to remain fully wrapped (Figure 5.9), which we determined from site exposure equilibrium measurements:

$$\Delta \Delta G_{\text{wrap-sample}} = k_B T \ln \left[ \frac{K_{\text{eq-sample}}^{\text{wrap}}}{K_{\text{eq-reference}}^{\text{wrap}}} \right].$$  (5.1)

We find that substitution of the first 7 base pairs of the X. borealis 5SL sequence into the 601L sequence reduced the nucleosome formation free energy ($\Delta G_{5\text{SL}(1-7)}^{\text{nuc}} - \Delta G_{601L}^{\text{nuc}} = -0.5 \pm 0.3 \text{ kcal/mol}$). This observation is in agreement with the reduced site exposure equilibrium induced by this chimeric NPS ($\Delta G_{\text{wrap}}^{\text{nuc}}(1-7) - \Delta G_{\text{wrap}}^{\text{nuc}}(601L) = -0.35 \pm 0.05 \text{ kcal/mol}$).

The insertion of 5SL base pairs 28-47 into the 601L sequence increased the nucleosome formation free energy ($\Delta G_{5\text{SL}(28-47)}^{\text{nuc}} - \Delta G_{601L}^{\text{nuc}} = 0.3 \pm 0.2 \text{ kcal/mol}$), but did not impact the site exposure free energy ($\delta G_{\text{wrap}}^{\text{nuc}}(5\text{SL}(28-47)) - \Delta G_{\text{wrap}}^{\text{nuc}}(601L) = -0.03 \pm 0.05 \text{ kcal/mol}$). The insertion of both 5SL base pairs 1-7 and 28-47 into the 60L sequence displayed a free energy change equal to the sum of the free energy changes of the separate substitutions ($\Delta G_{\text{wrap}}^{\text{nuc}}(5\text{SL}(1-47)) - \Delta G_{\text{wrap}}^{\text{nuc}}(601L) = -0.2 \pm 0.2 \text{ kcal/mol}$). In contrast, the $\Delta \Delta G_{\text{wrap-sample}}$ induced by changing both
base pairs 1-7 and 28-47 ($\Delta G_{\text{5SL}(1-47)}^{\text{wrap}} - \Delta G_{\text{601L}}^{\text{wrap}} = -0.43 \pm 0.06$ kcal/mol) and between 601L and 5SL(1-147) ($\Delta G_{\text{5SL}(1-147)}^{\text{wrap}} - \Delta G_{\text{601L}}^{\text{wrap}} = -0.57 \pm 0.03$ kcal/mol) is similar to the free energy difference induced by base pairs 1-7 alone. These comparisons are consistent with the conclusion that the influence of base pairs 1-7, 28-47 and 48-147 on the nucleosome stability free energy is additive but that only base pairs 1-7 influence the exposure of the LexA transcription factor binding site at bases 8-27 within the nucleosome. Taken as a whole these results suggest that changes in the nucleosomal DNA sequence can separately tune TF-binding site exposure as well as overall nucleosome stability.

### 5.2.6 TF-binding sites reside in the entry-exit regions of \textit{S. cerevisiae} nucleosome positions

Our observation that H3(K56ac) and DNA sequence have a combined influence on transcription factor (TF) binding within nucleosomes suggests that the regulation of DNA unwrapping could be a major regulator of TF occupancy. To investigate if TF binding sites are poised for this regulatory mechanism \textit{in vivo}, Alex Mooney determined the fraction of TF-binding sites that reside within \textit{S. cerevisiae} nucleosome entry-exit regions. He examined the consensus maps of \textit{S. cerevisiae} nucleosome occupancy [165] and the map of TF-binding sites [166] to determine the distance from the center of a TF-binding site to the dyad center of the nearest nucleosome (Figure 5.10A). The nucleosome dyad and entry-exit regions were defined to be 0-36 and 37-74 base pairs from the nucleosome dyad center, respectively. We found that 31% and 33% of TF-binding sites are within the dyad and entry-exit regions, respectively (Figure 5.10B). Approximately 36% of TF-binding sites are located in linker DNA between nucleosomes. Variation in the criteria for TF and nucleosome occupancy modestly altered these results (Figure 5.11). We compared the observed distance distribution of TF-binding sites from the nearest nucleosome dyad to the distance distribution of every position in the genome to the nearest nucleosome dyad (Figure 5.10A, black line). The expected fractions of TF-binding sites within the dyad, entry-exit and linker DNA regions for randomly positioned nucleosome are 36%, 37% and 22%, respectively (Figure 5.10B). These observations suggest that while TF-binding sites appear somewhat biased to-
Figure 5.9: Relation between DNA-histone binding and DNA unwrapping free energies.  
(A) Cy3 fluorescence image of native PAGE analysis of competitive reconstitutions performed in triplicate for unmodified histone octamer with the DNA chimeras used for site exposure measures.  
(B) Change in free energy of nucleosome formation ($\Delta \Delta G_{\text{nuc sample,601L}}^\text{nuc}$, light gray) relative to 601L as determined by competitive reconstitution from the gel in (A), and the change in nucleosome free energy for wrapping ($\Delta \Delta G_{\text{wrap sample,601L}}^\text{wrap}$, dark gray) relative to 601L as determined from site accessibility measures (Figure 5.7B-C). The error bars for $\Delta \Delta G_{\text{nuc sample}}^\text{nuc}$ are the standard deviation of the three independent measurements for each nucleosome type.  
(C) Table of the change in free energy values in kcal/mol for nucleosome formation of each DNA species relative to L. variegatus 5S NPS ($\Delta \Delta G_{\text{nuc sample,5S-Lv}}^\text{nuc}$), for nucleosome formation of each DNA species relative to 601L DNA ($\Delta \Delta G_{\text{nuc sample,601L}}^\text{nuc}$), and for nucleosome wrapping for each DNA species relative to 601L DNA ($\Delta \Delta G_{\text{wrap sample,601L}}^\text{wrap}$). (Reproduced with permission)
ward being in linker DNA between nucleosomes [167–169], there is rather little suppression of TF-binding sites within the nucleosome; thus a significant fraction of TF-binding sites are located within nucleosome entry-exit regions and hence poised to be regulated through nucleosome unwrapping. Furthermore, these observations are consistent with the report that within *S. cerevisiae* the positions of TF-binding sites at gene promoters are correlated with the nucleosome entry-exit region for nucleosomes containing the histone variant H2A.Z [169].

Figure 5.10: **TF binding sites predominantly reside within nucleosomes.** (A) Distribution of TF binding site distances from the nearest nucleosome dyad (black circles), with distances of 0-36 bases considered within the dyad region, distances of 37-74 bases considered within the entry-exit region, and distance greater than 74 bases considered to be in naked DNA (see methods and Figure 5.11 for details). The black line represents the expected TF binding site distribution if they were distributed randomly throughout the genome. (B) Measured and expected percentages of TFs in each region calculated from the distribution in (A). (Reproduced with permission)
Figure 5.11: **Transcription factor binding site distances from nearest nucleosome dyad.** (A) and (B) Distribution of nearest nucleosome dyad distance from a TF binding site for only nucleosomes with occupancy >10% or for all nucleosomes in the consensus map of nucleosome positions reported by Jiang and Pugh ref 3, respectively. The black line represents the distribution of TF binding site distances to the nearest nucleosome dyad if TF binding sites are randomly distributed. Red circles represent all mapped TF binding sites with P<0.005, while blue circles represent only mapped TF binding sites with P<0.001, which are evolutionarily conserved ref 4. These data sets have been normalized such that they have the same integral as the random distribution. (Reproduced with permission)
5.3 Discussion

Site exposure is an inherent property of the nucleosome that appears to provide transcription [30, 31, 120, 143, 144, 148] and DNA repair [24, 154, 170] complexes direct access to DNA. Here, we demonstrate that H3(K56ac) and DNA sequence influence TF occupancy by altering the nucleosome unwrapping rate, but not the rewrapping rate. We find that DNA sequence and H3(K56ac) additively influence the nucleosome unwrapping rate, which allows these two factors to function together to enhance or counteract their influence on TF occupancy (Figure 5.12). We observe 3-fold changes in TF occupancy induced by either H3(K56ac) or DNA sequence alone and a 10-fold effect in combination. These results, in combination with the observation that 30% of S. cerevesia TF-binding sites are located within nucleosome entry-exit regions, suggests that the modulation of the nucleosome unwrapping rate could be a mechanism for regulating TF occupancy in vivo.

The observation that the H3(K56ac) and DNA sequence influence the nucleosome unwrapping rates but not the rewrapping rates suggests that they influence the free energy of the fully wrapped state while not affecting the free energies of the unwrapped and the transition states. This could be caused by a reduction in DNA-histone binding. The influence of H3(K56ac) removes a negative charge, which could disrupt water-mediated hydrogen bonding near K56 as is observed for H3(K56Q) [113]. The first 7 base pairs of the 601 DNA sequence contain a GG dinucleotide that is in phase with the GC/GG dinucleotides, which are important for the strong positioning. However, this predicts that the first 7 base pairs of the 601 sequence should be more tightly wrapped than the 5S sequence, which is not what we observe. Instead, the DNA sequence may alter the precise DNA structure near the entry-exit region as is observed for nucleosomes containing the 601 and alpha satellite DNA molecules [164]. This could alter direct and water-mediated hydrogen bonding in the entry-exit region and influence the free energy of the fully wrapped nucleosome state. In contrast, it appears the DNA bending involved in nucleosome rewrapping is not significantly different between the 601 and 5S sequences since the rewrapping rate is unaffected.

Our conclusion that the nucleosome rewrapping rate is not influenced by DNA sequence
Figure 5.12: Kinetic model of H3(K56Ac) and DNA sequence modulation of nucleosome unwrapping rate. (A) and (B) Nucleosomes containing TF binding sites at two different loci within the genome (nucleosome 1 with entry-exit in blue, nucleosome 2 with entry-exit in magenta). The DNA sequence between the entry-exit and TF binding site influences the inherent nucleosome unwrapping rate to regulate TF binding within each nucleosome. Acetylation/de-acetylation of H3 lysine 56 at nucleosome 1 enhances-suppresses the DNA unwrapping rate to adjust TF occupancy. This influences both the TF occupancy within nucleosome 1 but also TF occupancy relative to nucleosome 2. (Reproduced with permission)
and H3(K56ac) relies on our observation that the relative changes in the rate of unwrapping and the LexA-binding equilibrium are identical. This observation implies that the combined influence of k21, k23 and k32 (Figure 5.1C) does not alter the LexA-binding equilibrium to nucleosomes. If the rewrapping rate, k21, were to change, k23 and k32 would need to change to exactly compensate. Instead, the simplest explanation is that these rates are not influenced by H3(K56ac) and DNA sequence. However, further studies that directly measure k21, k23 and k32 are required to confirm this interpretation.

Our studies provide additional insight into the influence of DNA sequence on site exposure equilibrium. A previous study showed introduction of a poly-A track placed at the first 16 base pairs within a 601-like sequence increased nucleosome site accessibility $\sim$1.5-fold [156]. Here we find that changing only the first 7 bases of the 601 DNA sequence to the X. borealis 5S sequence decreases the rate of DNA unwrapping by 2.5-fold. In contrast, the central 80 base pairs of the 601 NPS are largely responsible for the enhanced binding free energy relative to the 5S sequence [97]. These results, in combination with $\Delta\Delta G$ measurements, indicate that while 601 was selected for optimal nucleosome stability [73], it is not optimized to suppress partial DNA unwrapping at the entry-exit region.

In vivo, nucleosomes are embedded within chromatin where higher order compaction could impact the influence of nucleosome unwrapping/rewrapping kinetics on TF occupancy. Recent studies of nucleosome unwrapping/rewrapping fluctuations indicate that higher order chromatin compaction does not significantly impact TF occupancy within the nucleosome but does impact occupancy in linker DNA [97, 171]. While this suggests that the influence of H3(K56ac) and DNA sequence on TF occupancy within the nucleosome will occur in the context of chromatin, additional studies are required to investigate the influence of chromatin higher order structure.

Other histone PTMs in the nucleosome entry-exit region could function like H3(K56ac) to enhance site accessibility by altering the nucleosome unwrapping rate. H4(K77ac) and H4(K79ac), which are located at the DNA-histone interface 40-45 base pairs within the nucleosome, enhanced TF binding by 2-fold to their site between base pairs 8-27 within the nucleosome [120]. Furthermore, histone H3 PTMs at Proline 38 [171], Tyrosine 41
and Threonine 45 reside at the nucleosome entry-exit. As shown in Chapter 4, phosphomimetics H3(Y41E) and H3(T45E) also enhance TF binding by \( \sim 2 \)-fold. Further studies are required to determine if additional histone PTMs in the nucleosome entry-exit regulate the DNA unwrapping rate to control protein binding within nucleosomes.
5.4 Experimental Procedures

5.4.1 DNA constructs

The 601L-end [also called 601L], 5SL-end [also called 5SL(1-147) or 5SL], 5SL-dyad, 5S(1-7), 5S(28-47) and 5S(1-47) molecules for fluorescence studies were prepared by PCR from plasmid containing the 601 nucleosome positioning sequence (NPS) or the *Xenopus borealis* 5S rDNA NPS with a LexA-binding site (TACTGTATGAGCATACAGTA) cloned into bases 8-27. The 5S and 5S-Lv molecules were prepared by PCR from plasmid containing the X. borealis and L. variegatus 5S rDNA NPS, respectively. Oligonucleotides for PCR (Sigma-Aldrich) (see Appendix D for DNA and oligo sequences) were conjugated to a 5-prime or internal amine with Cy3-NHS (GE Healthcare) and purified by RP-HPLC on a 218TP® C18 (Grace/Vydac) column. Following PCR amplification, each DNA molecule was purified by HPLC with a Gen-Pak Fax column (Waters). Additionally, 201 base pair competitor DNA was prepared by PCR from the Amp" gene in pUC19 and then purified by HPLC with a Gen-Pak Fax column.

5.4.2 Expression and Purification of Wild Type and Mutant Histones

*Xenopus laevis* recombinant histones were expressed and purified following [79, 107] as detailed in Appendix A. Mutations H3(C110A), H3(K56Q,C110A), and H4(S47C) were introduced by site-directed mutagenesis (Stratagene). Histone H3(K56Ac,C110A) was prepared as previously described [148].

5.4.3 Preparation of histone octamers and LexA protein

Histone octamer refolding and purification were performed following [79, 107] as described in Appendix A. Briefly, each histone was unfolded for 1 to 3 hours in unfolding buffer at a histone concentration of 2 to 20 mg/ml, and then spun to remove aggregates. The absorption at 276nm was measured for each unfolded histone to determine the concentration. UV-Vis absorption spectra of acetylated histone H3(K56ac,C110A) contains a background peak centered at 230nm that overlaps with the protein absorption peak centered at 276nm. This
peak is of unknown origin, but presumably due to a remnant chemical contaminant from the synthesis process. To correct for this, the overlap between the anamalous 230nm peak and 276nm protein peak was estimated by hand as described in Appendix A.

Each of the four histones were combined at equalmolar ratios, refolded and purified as previously described in Appendix A. H2A(K119C) containing histone octamer was labeled with Cy5-maleamide (GE Healthcare) as detailed in Appendix B.

LexA protein was expressed and purified from pJWL288 plasmid as detailed in Appendix F.

5.4.4 Nucleosome reconstitutions

Nucleosomes were reconstituted from DNA and purified histone octamer by salt double dialysis and purified by sucrose gradient (see Appendix C). Nucleosomes containing Cy3 labeled DNA for fluorescence studies were reconstituted with histone octamer containing Cy5-labeled H2A(K119C). Nucleosomes reconstituted with 5S-Lv, 5S and 5SL resulted in two nucleosome positions as previously reported [95]. The central positioned 5SL nucleosomes used in FRET measures were purified from the depositioned nucleosomes by sucrose gradient purification.

5.4.5 Fluorescence Resonance Energy Transfer Measures of LexA Binding within Nucleosomes

The FRET efficiency of nucleosome in the absence of LexA was determined by the (Ratio)A method (See Chapter: Introduction) [83]. The equilibrium constants for site accessibility were determined from the reduction in FRET efficiency as LexA binds to its target site buried within the nucleosome. LexA was titrated from 0µM to 3µM with 5 nM Cy3/Cy5-labeled nucleosomes in 0.5x TE buffer or from 0µM to 100µM with 5 nM Cy3/Cy5-labeled nucleosomes in 0.5x TE buffer with 75mM or 130mM NaCl. Nucleosomes were excited at 510nm (donor excitation) and emission spectra were collected from 530 to 750 nm; nucleosomes were then excited at 610nm (acceptor excitation) and emission spectra were collected from 630 to 750 nm using a Fluoromax-4 (Horiba) steady state spectrophotometer.
The FRET efficiency was determined by the (Ratio)_A method, performed in triplicate, for each LexA concentration (See Chapter: Introduction). The average FRET efficiency versus the LexA concentration was fitted to a non-cooperative or cooperative binding isotherm to determine the LexA concentration at which the FRET efficiency has been reduced by half (S₀.₅−nuc), which is the LexA concentration when its target site is bound 50% of time. Additionally, we used EMSA to measure the LexA concentration at which its target site within naked DNA is 50% bound by LexA S₀.₅−DNA.

We previously determined that non-specific DNA binding of LexA does not reduce the FRET efficiency and that binding of LexA to its target sequence within the nucleosome does not induce dissociation of H2A-H2B heterodimers (See Chapter 4).

5.4.6 Stopped flow nucleosome kinetics measurements

Stopped flow experiments were performed on a KinTek 2004-SF instrument at room temperature as previously described [31]. Samples were excited by a XeHG arclamp with a 525 ±22nm excitation filter (Omega); simultaneous Cy3 and Cy5 emission was followed using a 570±5nm bandpass filter (Newport) and 680±15nm bandpass filter (Chroma), respectively. After rapid mixing, samples contained 7nM Cy3/Cy5-labeled nucleosomes in 0.5x TE with 1, 75 or 130mM NaCl and [LexA] varying from 0 to 50µM. Data were smoothed by 10 point forward averaging and fit to a single exponential decay, except for unmodified nucleosomes in 0.5x TE, 1mM NaCl, which was fit to a double exponential due to an additional slower process, which can be attributed to non-specific LexA binding [31].

5.4.7 Nucleosome competitive reconstitutions

Competitive reconstitutions (See Chapter: Introduction) were modified from published protocols to measure the change in DNA-histone binding induced by altering the DNA sequence [93]. Briefly, 0.6µg of unmodified histone octamer were combined with 0.5µg high-affinity sample DNA and 2µg low-affinity 201 base pair competitor DNA in 2M NaCl, 0.5x TE, 1mM benzamidine (BZA). To minimize variation in DNA and histone octamer concentrations, a DNA and buffer master mix was first prepared that was then combined with each
histone octamer stock at a concentration of 250ng/µl in 2 M NaCl, 0.5x TE and 1 mM BZA. Each DNA-histone octamer sample was then split into thirds and placed in an engineered 50µl dialysis chamber.

Each sample was reconstituted in the same 0.2L reservoir by salt gradient pumping (See Appendix C) from 2M NaCl to <1mM NaCl at 4°C over 36 hours, keeping 0.5x TE and 1mM BZA fixed throughout. The reconstitution products were examined by EMSA on a 5% native 29:1 acrylamide gel with 0.3x TBE in the gel and running buffer; gels were prerun for 1 hour at 20V/cm (300V) on a Gibco BRL V16 vertical rig, Ficol was added to 2.5% to each sample, samples were applied to the gel while running at 300V, and samples were allowed to resolve for 1 hour at 300V (See Appendix E). Gels were scanned with a Typhoon 8600 variable mode imager (GE Healthcare) and analyzed with ImageQuant (Molecular Dynamics) using local median background correction.

5.4.8 Analysis of nucleosome position and TF-bindingsite databases

The fraction of TF-binding sites within the nucleosome dyad, entry-exit and linker regions were determined with the reported consensus map of nucleosome positions [165]: (http://refnucl.atlas.bx.psu.edu/)

and TF-binding sites [166]: (http://fraenkel.mit.edu/improved_map/)

in *S. cerevisiae*. Nucleosomes with occupancy > 10 and TF-binding sites with binding P-values < 0.005 and no limitation on conservation were used. For each TF-binding site we determined the number of bases between the nearest nucleosome dyad and the center of the TF-binding site rounded down to the nearest base. The random distribution simply treats every base pair in the yeast genome as the center of a potential TF-binding site while retaining the same consensus map of nucleosome positions and is therefore the probability density for the distance to the nearest nucleosome from any position. The number of sites exactly on top of a nucleosome dyad was doubled to account for the two possible ways to be any distance greater than zero base pairs from the dyad. Distributions of TF-binding sites relative to nucleosome positions were also determined with different criteria on nucleosome
occupancy and TF-binding sites.
Chapter 6
HsRAD51 DNA REPAIR PROTEIN DISASSEMBLES NUCLEOSOMES BY TRAPPING DNA UNWRAPPING FLUCTUATIONS

6.1 Introduction

In the preceding chapters we examined the influence of intrinsic nucleosome factors, including nucleosome thermal fluctuations, histone post-translational modifications, and DNA sequence, on nucleosome stability, mobility, and DNA accessibility. In these final chapters we turn our attention to extrinsic factors in the form of chromatin remodelers and chromatin associated proteins that facilitate nucleosome assembly/disassembly, repositioning, and DNA accessibility. We examine how intrinsic factors synergistically function with such extrinsic factors to regulate their function and activity *in vivo*. In this chapter we focus on the mechanisms by which the Rad51/RecA family of DNA repair proteins trap nucleosome thermal unwrapping fluctuations to disassemble nucleosomes.

Double strand breaks (DSB) of genomic DNA occur in chromatin following replication fork collapse and chemical or physical damage [172]. Recombinational DNA repair of DSBs engenders a complex cascade of responses that include cellular signaling integrated with the physical processes of DSB repair [173, 174] which can result from high fidelity homologous recombination (HR) or from lower fidelity non-homologous end joining (NHEJ) [175–177]. HR uses homologous chromatids or chromosomal sequences to bridge the DSB
with an undamaged template. Numerous biochemical reactions must manage a disrupted dsDNA/ssDNA chromatin composite of the broken donor DNA in order to search and pair with the assembled chromatin template of the bridging homologous acceptor DNA [176–178]. However, in order for DSB repair to occur, nucleosomes must be redistributed or disassembled [36]. Genome instability resulting from defects in recombinational repair of DNA double strand breaks (DSBs) have been linked to hereditary breast cancer (BRCA1/2) as well as hematopoietic and other solid tumors (Ataxia telangiectasia mutated, ATM; Nijmegen breakage syndrome, NBS1; Fanconia anemia, FANC; Bloom’s syndrome, BLM; among others) [179–183].

RAD51 plays a central role in eukaryotic and archael HR and is homologous to the prototypical bacterial RecA [176, 178, 184]. RecA/RAD51 form and maintain a stable nucleoprotein filament (NPF) that catalyzes the initial homologous pairing (or ”end capture”) and strand exchange (recombinase) of HR between the damaged donor and template acceptor chromosomes or chromatids [178, 184, 185]. RAD51 may nucleate an NPF on both ssDNA and dsDNA with near equal affinity [186, 187]. However, unusual cationic salts appear to bias a stable ATP-bound RAD51 NPF to ssDNA that then promotes recombinase initiation functions [188–190]. Substitution of a conserved Aspartic Acid (D) residue in the ATP cap domain with a Lysine (K) residue that is generally conserved in the RecA family [RAD51(D316K)] relieves the unusual salt requirements, enhances discrimination between ssDNA and dsDNA, and induces the formation of a stable and active NPF for HR [191]. The conservation of D316 appears to suggest that the RAD51 family may be poised to regulate the ssDNA and dsDNA NPF via this residue.

RAD51 recombinase activity has been observed with chromatin substrates reconstituted from uncharacterized HeLa and Drosophila cellular histones as well as recombinant Xenopus laevis histones [192–196]. Three consistent observations have emerged: (1) RAD51 may catalyze the homology search and pairing reaction even if the homology site is within a nucleosome, (2) RAD54 appears to convert a paranemic RAD51 pairing structure that can be disrupted by RAD51 protein removal to a plectonemic strand exchange product that cannot be disrupted, and (3) RAD51 recombinase may stimulate a RAD54 chromatin
remodeling activity. Even though RAD51 appears capable of catalyzing recombination initiation within a nucleosome, the process presents a topological problem at locations where the wrapped DNA makes multiple contacts with the histone octamer. These observations suggest that recombination is likely to require synchronized and/or regulated nucleosome processing. However, the mechanics of nucleosome processing are largely unknown.

The over-expression of *Saccharomyces cerevisiae* RAD51 (ScRad51) induces spontaneous RAD foci on yeast chromatin and appears toxic in the absence of SWI/SNF translocases that include ScRad54, ScRdh54 and Uls1 [197]. These results suggest that cellular dsDNA binding by RAD51 may be significant and tightly regulated. However, the precise function of the RAD51-dsDNA NPF is largely unknown. ScRAD51 polymerization activity was recently suggested to "push" or reposition nucleosomes along dsDNA, and that nucleosome destabilization from this activity may facilitate disassembly [198]. This observation was surprising since the multiple contacts between the core histones within the nucleosome DNA would seem to engender a substantial energetic barrier against repositioning [72]. Here we demonstrate that *Homo sapien* Rad51 (HsRAD51) dsDNA NPF disassembles nucleosomes by unwrapping the DNA from the core histones. HsRAD51 that has been constitutively or biochemically activated for recombinase functions displays significantly reduced nucleosome disassembly activity. These results suggest HsRAD51 may switch between ATP hydrolysis-independent recombinase and ATP hydrolysis-dependent nucleosome disassembly functions during DSB repair.
6.2 Results

6.2.1 HsRAD51 disassembles nucleosomes

To examine the interplay between HsRAD51 and chromatin we constructed DNA substrates containing either the 60l [73] or Xenopus borealis 5S rDNA (5S) [117] nucleosome positioning sequence (NPS) as well as a Cy3 fluorophore located near the entry-exit (E) or dyad regions (D; Figure 6.1A; see Methods) [8]. We examined the possibility that HsRAD51 might require the linker DNA between nucleosomes to form an NPF by constructing DNA substrates that either contained only the 147 base pair NPS sequence or the NPS plus a 100 base pair 3’-extension (linker, Figure 6.1A). These DNA substrates were assembled into nucleosomes with histone octamer containing H2A(K119C) labeled with Cy5 (Figure 6.1D) as in previous chapters. Upon nucleosome formation, the Cy5 acceptor and Cy3 donor are juxtaposed for efficient fluorescence resonance energy transfer (FRET; Figure 6.1B,C) [8]. Fluorescence emission spectra of the nucleosome substrates excited at 510nm resulted in quenched Cy3 emission at 570nm and Cy5 FRET-stimulated emission at 670 nm (Figure 6.2B,C, black).

HsRAD51 catalyzes homologous pairing and strand exchange in the presence of divalent calcium (Ca$^{+2}$) but not divalent magnesium (Mg$^{+2}$) cations [188]. This is because Ca$^{+2}$ appears to maintain an active ATP-bound HsRA51 filament while Mg$^{+2}$ promotes ATP hydrolysis resulting in either protein turnover or a mixed adenosine nucleotide NPF [191]. First, to determine if HsRAD51 can disassemble nucleosomes under physiological ionic strength (130mM KCl) and in the presence of magnesium (Mg$^{+2}$), we performed electrophoretic mobility shift assays (Figure 6.2). In these studies we incubated 601D-linker nucleosomes with increasing HsRAD51 and quantified the fraction of DNA bound in nucleosomes (Figure 6.2A, upper band) compared to free DNA (Figure 6.2A, lower band). We observed an HsRAD51 concentration-dependent decrease in the nucleosome band by EMSA (Figure 6.2E, pink) with a concomitant increase in free DNA. This demonstrates that HsRAD51 in the presence of Mg$^{+2}$ disassembles 601D-linker nucleosomes into free DNA. Unfortunately we were unable to perform HsRAD51 nucleosome disassembly assays by EMSA in the presence of Ca$^{+2}$ due to the stability of the NPF filament which could not
be fully removed from the DNA substrate. Therefore we developed a FRET based assay to
determine the fraction of nucleosomes disassembled by HsRAD51 under differing divalent
ion conditions.

We incubated 601D-linker nucleosomes with increasing HsRAD51 in the presence of
M$^{+2}$ as before and measured the steady-state fluorescence emission of nucleosomes excited
at 510nm (Cy3 donor excitation) for increasing concentrations of HsRad51 (Figure 6.2B),
and the emission spectra from the nucleosome substrates when directly excited at 610nm
(Cy5 acceptor excitation, Figure 6.2D) [83]. We then determined the FRET efficiency as
a function of HsRad51 concentration from these spectra using the (ratio)$_A$ method (See
Chapter: Introduction and Figure 6.2E; black, plotted as a fraction of the starting FRET
in the absence of HsRAD51). When the FRET efficiency of nucleosomes incubated with
HsRAD51 are compared to the remaining nucleosome fraction independently determined
by EMSA, the dependence on HsRAD51 concentration coincide (Figure 6.2E). Together,
these results indicate that the reduction in FRET observed by the 601D-linker nucleosome
construct may be used as a direct measure of nucleosome disassembly.

To determine the role of ATP hydrolysis and NPF stability on nucleosome disassembly
we incubated 601D-linker nucleosome in presence of HsRAD51 and Ca$^{+2}$ (Figure 6.2C,D) or
the ATP-cap substitution mutation, HsRAD51(D316K), and Mg$^{+2}$ [data not shown], which
naturally maintains an ATP-bound filament [191]. We observed a significantly smaller de-
crease in FRET efficiency of the 601D-linker nucleosomes in the presence of HsRad51 with
Ca$^{+2}$ or HsRAD51(D316K) with Mg$^{+2}$ (Figure 6.2E; plotted as the fraction of starting
FRET in the absence of HsRAD51). These observations are consistent with the conclusion
that HsRAD51 disassembles nucleosomes and that there appears to be a significant differ-
ence in the extent and/or kinetics of nucleosome disassembly between HsRAD51-Mg$^{+2}$ and
HsRAD51-Ca$^{+2}$ or HsRAD51(D316K)-Mg$^{+2}$.

### 6.2.2 HsRAD51 unwraps DNA from nucleosomes

There are two possible mechanisms for the observed disassembly of these mononucleosome
templates: (1) sliding of the histone octamer along the DNA until it becomes destabilized
Figure 6.1: DNA templates for HsRAD51 nucleosome disassembly (A) Diagram of Cy3-labeled DNA’s containing either the 147 base pair 601 or X. borealis 5S rDNA nucleosome positioning sequence (NPS) with or without a 100 base pair linker that may be used for quantifying HsRAD51 interactions with a nucleosome. (B) Nucleosome structure [8] showing the location of the Cy3-label (green) within the 601E (left) and 601D (right) DNA molecules. Location of Cy5 label on H2A(K119C) in red; PDB ID: 1KX5. (C) Schematic of the FRET system using the Cy3-labeled 601E-linker (Top) or 601D-linker (Bottom) DNAs reconstituted into nucleosomes with Cy5-labeled histone octamer. (D) EMSA using the Cy3 label of purified nucleosomes containing the DNA molecules in (A) and histone octamer containing Cy5-labeled H2A(K119C).
Figure 6.2: **HsRAD51 disassembles nucleosomes** (A) EMSA of 601D-linker nucleosomes incubated in the indicated amount of HsRad51 at 37°C for 30 minutes in the presence of 130mM KCl, 2mM Mg⁺², and 250µM ATP. Nucleosomes (upper band) are disassembled into free DNA (lower band) in a HsRAD51-dependent manner. (B) Representative fluorescence emission spectra of 601D-linker nucleosomes excited at 510nm in the presence 130mM KCl and 2mM Mg⁺² with HsRAD51 at 0nM (black), 100nM (blue), 200nM (green), and 500nM (orange). (C) Representative fluorescence emission spectra of 601D-linker nucleosomes excited at 510nM in the presence 130mM KCl and 2mM Ca⁺² with HsRAD51 at 0nM (black), 100nM (blue), 200nM (green), and 500nM (orange). (D) Fluorescence emission spectra of 601D-linker nucleosomes excited at 610nm in the presence 130mM KCl and 2mM Mg⁺² (left) or 2mM Ca⁺² (right) and incubated with HsRAD51 at 0nM (black), 100nM (blue), 200nM (green), and 500nM (orange). (E) Nucleosome fraction determined by gel shift analysis for HsRad51 + 2mM Mg⁺² (pink square); FRET efficiency as a fraction of the initial FRET in the absence of HsRAD51 (“Nucleosome fraction”) as a function of HsRAD51 concentration for HsRad51 + 2mM Mg⁺² (black square), HsRad51 + 2mM Ca⁺² (red circle) and HsRad51(D316K) + 2mM Mg⁺² (blue triangle). Each point represents the average and standard of deviation from at least three independent experiments.
or dissociates from an open end, or (2) unwrapping the DNA from the histone octamer. These mechanisms produce different kinetic predictions for the DNA substrates containing FRET pairs at the entry-exit (E) or dyad (D) regions of the nucleosome (Figure 6.1A,B). For example, the kinetics of Cy5 FRET-stimulated emission reduction during nucleosome sliding should be virtually identical for the E and D nucleosome substrates since histone octamer movement relative to these two sites should occur at the same rate. In contrast, during nucleosome unwrapping, FRET reduction in the E substrate should occur significantly faster than FRET reduction in the D substrate due to the increased time required to unwrap from the nucleosome entry-exit to the dyad.

We examined the Cy5 fluorescence kinetics by rapidly mixing HsRAD51 with nucleosomes in a fluorometer (Figure 6.3) and following the change in Cy5 stimulated emission via FRET. Representative fluorescence decay analysis of 601E-linker and 601D-linker nucleosome substrates in the presence of HsRAD51 with Mg$^{+2}$, HsRAD51 with Ca$^{+2}$ and HsRAD51(D316K) with Mg$^{+2}$ are shown in Figure 6.3. The reduction in FRET fluorescence at each concentration of HsRAD51 was fit to a single exponential decay. Visual inspection shows a clear difference in the kinetics and extent of the FRET decay curves between the 601E-linker and 601D-linker nucleosome substrates (compare Figure 6.3A with 6.3B, Figure 6.3C with 6.3D, and Figure 6.3E with 6.3F). These differences translated into different rates of FRET decay (Figure 6.4A,B) and are consistent with a model where the nucleosome is unwrapped before it is disassembled. An approximate 25-fold difference between $k_{\text{unwrap}}$ and $k_{\text{off}}$ was consistently observed regardless of the conditions (Figure 6.4C).

These results are consistent with previous studies that suggest the entry-exit region of the nucleosome is capable of significant "breathing", or DNA unwrapping, independent of nucleosome disassembly [30, 81] until the DNA is unwrapped through the nucleosome dyad promoting disassembly [199]. In the presence of HsRAD51 nucleosome breathing seems to allow occupation of the entry-exit region by the NPF that ultimately results in the FRET decay first observed by the 601E-linker substrate. Unwrapping-mediated NPF occupation appears to then proceed through the dyad resulting in the subsequent FRET decay observed by the 601D-linker substrate, and ultimately nucleosome disassembly. This mechanism is
further supported by the 25-fold difference between $k_{\text{unwrap}}$ and $k_{\text{off}}$. The nucleosome contains $\sim 75$ base pairs of DNA wrapped from entry-exit to the dyad. With a footprint of 3 base pairs per HsRAD51 molecule, simple stoichiometry arguments indicate it would require a NPF filament of $\sim 25$ HsRAD51 monomers to span from the entry-exit to the dyad to maximally destabilize the nucleosome, and hence, result in a $k_{\text{off}}$ that is 25-fold slower than $k_{\text{unwrap}}$. However, further experiments are required to determine if this is the case.

We determined that the rate of nucleosome disassembly was 4-fold faster with HsRAD51 in the presence of Mg$^{+2}$ compared to HsRAD51 in the presence of Ca$^{+2}$ or the HsRAD51(D316K) substitution (Figure 6.4D). These results suggest that HsRAD51 may switch between an active ATP-bound filament capable of recombinase functions and ATP hydrolysis-dependent nucleosome disassembly functions.

### 6.2.3 HsRAD51 nucleosome disassembly does not require linker DNA

We examined nucleosome disassembly with DNA substrates that did not contain linker DNA. Representative steady-state HsRAD51-dependent FRET reduction of the 601D and 5SD nucleosome substrates are shown in Figure 6.5A-B. The normalized decrease in FRET was plotted and compared to the 601D-linker nucleosome substrate (Figure 6.5C). We found that the concentration of HsRAD51 required to disassemble half of the nucleosomes was virtually identical for nucleosomes that do not contain a linker and nucleosomes that do contain a linker ($S_{0.5-601D} = 370 \pm 40\text{nM}$; $S_{0.5-5SD} = 400\pm 50\text{nM}$; $S_{0.5-601D \text{-linker}} = 340 \pm 50\text{nM}$; Figure 6.5D). Since the cellular concentration of HsRAD51 appears to be near 6\mu M [35], there is likely to be sufficient protein to disassemble nucleosomes in vivo. Moreover, these results suggest that HsRAD51 may initiate the disassembly of wrapped DNA within a nucleosome independent of associated linker DNA regions. We also note that there appears to be little or no difference in extent of nucleosome disassembly between the 601 and 5S rDNA NPS (Figure 6.5C). This observation suggests that relative localization stability is unlikely to significantly influence HsRAD51 catalyzed nucleosome disassembly.
Figure 6.3: **RAD51 Unwraps the DNA from the Histone Core.** (A) Representative FRET fluorescence decay of 601E-linker nucleosomes incubated with HsRAD51 at 0nM (black), 50nM (red), 100nM (blue), 200nM (green), 500nM (orange), 900nM (purple) in the presence of 130mM KCl and 2mM Mg$^{+2}$. (B) Representative FRET fluorescence decay of 601D-linker nucleosomes incubated with HsRAD51 at 0nM (black), 50nM (red), 100nM (blue), 200nM (green), 500nM (orange), 900nM (purple) in the presence of 130mM KCl and 2mM Mg$^{+2}$. (C) Representative FRET fluorescence decay of 601E-linker nucleosomes incubated with HsRAD51 at 0nM (black), 50nM (red), 100nM (blue), 200nM (green), 500nM (orange), 900nM (purple) in the presence of 130mM KCl and 2mM Ca$^{+2}$. (D) Representative FRET fluorescence decay of 601D-linker nucleosomes incubated with HsRAD51 at 0nM (black), 50nM (red), 100nM (blue), 200nM (green), 500nM (orange), 900nM (purple) in the presence of 130mM KCl and 2mM Ca$^{+2}$. (E) Representative FRET fluorescence decay of 601E-linker nucleosomes incubated with HsRAD51(D316K) at 0nM (black), 100nM (blue), 200nM (green), 500nM (orange), 900nM (purple) in the presence of 130mM KCl and 2mM Mg$^{+2}$. (F) Representative FRET fluorescence decay of 601D-linker nucleosomes incubated with HsRAD51(D316K) at 0nM (black), 100nM (blue), 200nM (green), 500nM (orange), 900nM (purple) in the presence of 130mM KCl and 2mM Mg$^{+2}$. 

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Figure 6.4: **ATPase activity regulates HsRAD51 function**

(A) Graph of FRET fluorescence decay rates for 601E-linker nucleosomes versus HsRAD51 concentration: HsRAD51 with Mg$^{+2}$ (black), HsRAD51 with Ca$^{+2}$ (blue), and HsRAD51(D316K) with Mg$^{+2}$ (red). Each point represents the average and standard of deviation from at least two independent experiments.

(B) Graph of FRET fluorescence decay rates for 601D-linker nucleosomes versus HsRAD51 concentration: HsRAD51 with Mg$^{+2}$ (black), HsRAD51 with Ca$^{+2}$ (blue), and HsRAD51(D316K) with Mg$^{+2}$ (red). Each point represents the average and standard of deviation from at least two independent experiments.

(C) The rate of entry-exit unwrapping, $k_{\text{unwrap}}/k_{\text{off}}$, divided by the rate of nucleosome disassembly, $k_{\text{off}}$. $k_{\text{unwrap}}$ was determined from the FRET fluorescence decay of 601E-linker nucleosomes and $k_{\text{off}}$ was determined from the FRET fluorescence decay of 601D-linker nucleosomes.

(D) Relative rate of disassembly determined by dividing the rate of HsRAD51 nucleosome disassembly in the presence of Mg$^{+2}$ ($k_{\text{off}}-\text{HsRAD51-Mg}$) by the rate of HsRAD51 nucleosome disassembly in the presence of Ca$^{+2}$ ($k_{\text{off}}-\text{HsRAD51-Ca}$) (Top blue) or the rate of HsRAD51(D316K) nucleosome disassembly ($k_{\text{off}}-\text{HsRAD51(D316K)-Mg}$) in the presence of Mg$^{+2}$ (Bottom red).
Figure 6.5: Nucleosome disassembly is independent of linker DNA and nucleosome positioning stability. (A) Representative fluorescence emission spectra of 601D nucleosomes incubated with HsRAD51 at 0nM (black), 100nM (blue), 500nM (orange) in the presence of 130mM KCl and 2mM Mg^{2+}. (B) Representative fluorescence emission spectra of 5SD nucleosomes incubated with HsRAD51 at 0nM (black), 100nM (blue), 200nM (green), 500nM (orange) in the presence of 130mM KCl and 2mM Mg^{2+}. (C) Normalized FRET efficiency of 601D (black circle) and 5SD (blue circle) nucleosomes compared to 601D-linker nucleosomes (red square) as a function of HsRAD51 concentration. (D) Concentration of HsRAD51 (nM) at which 50% of nucleosomes are disassembled ($S_{0.5-nuc}$). Values represent the average and standard of deviation from at least three independent experiments.
6.2.4 RecA and ScRAD51 are unable to disassemble nucleosomes in HsRAD51 conditions

A recent report suggested that NPFs formed with either yeast Rad51 (ScRAD51) or RecA may slide and destabilize nucleosomes [198]. These studies required high concentrations of recombinase and low salt conditions to stabilize the NPF and allow nucleosome repositioning [198]. We examined ScRad51 and RecA catalyzed nucleosome repositioning using our fluorophore labeled nucleosome substrates (Figure 6.6). A modest decay in Cy5 fluorescence was observed from the entry-exit reporter (601E-linker) under the physiologically relevant conditions that were optimized for HsRAD51 catalyzed disassembly, and where ScRad51 and RecA may retain some active recombinase functions (Figure 6.6A,C,E). However, we did not observe a change in Cy5 fluorescence from the dyad reporter (601D-linker; Figure 6.6B,D,F). The lack of any kinetic FRET fluorescence decay of the 601D-linker nucleosome reporter suggests that ScRad51 and RecA were less optimized to directly disassemble nucleosomes under conditions where HsRAD51 was capable of nucleosome disassembly. We cannot rule out nucleosome destabilization or repositioning by RecA or ScRad51 since we restricted our studies to HsRAD51 solution conditions, there was no linker DNA 5’ of the nucleosome on which to slide, and the previous studies used uncharacterized histones isolated from cells to reconstitute nucleosomes.
Figure 6.6: ScRad51 and RecA partially invade but do not disassemble nucleosomes. (A) Representative FRET fluorescence decay of 601E-linker nucleosomes incubated with ScRad51 at 0nM (black), 200nM (green), 500nM (orange), 1000nM (purple) in the presence of 130mM KCl and 2mM Mg$^{2+}$. (B) Representative FRET fluorescence decay of 601D-linker nucleosomes incubated with ScRad51 at 0nM (black), 200nM (green), 500nM (orange), 1000nM (purple) in the presence of 130mM KCl and 2mM Mg$^{2+}$. (C) Representative FRET fluorescence decay of 601E-linker nucleosomes incubated with RecA at 0nM (black), 1µM (purple), 2µM (blue), 5µM (magenta), 10µM (cyan) in the presence of 25mM KCl and 2mM Mg$^{2+}$. (D) Representative FRET fluorescence decay of 601D-linker nucleosomes incubated with RecA at 0nM (black), 1µM (purple), 2µM (blue), 5µM (magenta), 10µM (cyan) in the presence of 25mM KCl and 2mM Mg$^{2+}$. (E) Representative FRET fluorescence decay of 601E-linker nucleosomes incubated with RecA at 0nM (black), 1µM (purple), 2µM (blue), 5µM (magenta), 10µM (cyan) in the presence of 130mM KCl and 2mM Mg$^{2+}$. (F) Representative FRET fluorescence decay of 601D-linker nucleosomes incubated with RecA at 0nM (black), 1µM (purple), 2µM (blue), 5µM (magenta), 10µM (cyan) in the presence of 130mM KCl and 2mM Mg$^{2+}$. 
6.3 Discussion

Our studies demonstrate that HsRAD51 may disassemble nucleosomes under physiologically relevant conditions by a dynamic "occupation mechanism" of DNA regions that are undergoing transient thermal unwrapping fluctuations from the histone octamer (Figure 6.7). This activity appears to be separate from its recombinase functions since it requires ATP hydrolysis.

![Figure 6.7: Model for HsRAD51 NPF catalyzed nucleosome disassembly. Nucleosome unwrapping by HsRAD51 occurs by gradual occupation of the entry-exit and dyad regions until the histone octamer is destabilized.](image)

We observed a similar activity for the human mismatch repair initiation heterodimer HsMSH2-HsMSH6 [154]. Interestingly, unmodified nucleosome disassembly by HsRAD51 appears 3-4 fold more efficient at equivalent protein concentrations than HsMSH2-HsMSH6. Moreover, nucleosome disassembly by HsMSH2-HsMSH6 is dramatically stimulated by the stability of the NPS DNA sequence and histone modifications that destabilize the nucleosome [154]. This does not appear to be the case for HsRAD51 where nucleosomes reconstructed with 601 and 5S appear to be disassembled equivalently in spite of their significant stability differences [75]. In addition, HsMSH2-HsMSH6 is at least 100-fold less efficient at nucleosome disassembly than HsRAD51 at physiological protein concentrations ($k_{off}(HsMSH2-HsMSH6)_{200nM} = 0.009 \text{ min}^{-1}$; $k_{off}(HsRAD51)_{1M} = 0.15^{-1}$). These results may be an indicator of when and where these DNA repair components encounter nucleosomes. HsMSH2-HsMSH6 is largely associated with the replication fork [200, 201] where partially assembled and/or acetylated nucleosomes occur that appear more easily disas-
sembled [154]. In contrast, HsRAD51 may catalyze HR genome-wide where a variety of chromatin structures, nucleosome types and modifications may occur.

Both ssDNA and dsDNA RAD51 NPFs occur \textit{in vivo} and appear to be regulated by accessory factors [202]. For example, BRCA2 appears to guide the assembly of a RAD51 ssDNA filament while excluding a dsDNA filament at the dsDNA/ssDNA interface of the resected ssDNA tail during homologous pairing and strand exchange [203, 204]. In contrast, spontaneous ScRad51 foci form on yeast chromosomes in the absence of the SWI/SNF translocases ScRdh54, ScRad54 and ScUls1 that ultimately lead to chromosomal losses [197]. These spontaneous foci were not due to dsDNA breaks or accumulated ssDNA since they were not accompanied by RPA foci [197]. Moreover, elevated expression of ScRAD51 is lethal in the absence of ScRdh54 [25]. Taken as a whole, these results suggest that both ssDNA and dsDNA RAD51 NPFs occur \textit{in vivo} and that accessory proteins may dictate timing and location.

Rad51 mediated nucleosome disassembly might be required at several stages of HR. The first is shortly after the introduction of a DSB where clearing the DNA ends near the DSB of nucleosomes would facilitate end processing prior to homologous pairing and strand exchange. Since RAD51 generally appears at the DSB after end resection and the arrival of other repair and chromatin remodelling proteins including INO80 [ref], this possibility seems relatively remote [205, 206]. Alternatively, nucleosome disassembly may occur during replication fork collapse where a RAD51 ssDNA NPF that appears to protect against nuclease digestion [207] might extend into the adjacent dsDNA to clear nucleosomes prior to repair and/or replication restart. In addition, nucleosome disassembly might occur on the acceptor DNA during homologous pairing and strand exchange. In this case, the initiation of homologous pairing or "end capture" produces a ssDNA D-loop in the non-pairing strand of acceptor homologous chromosome or chromatid that may be bound by RPA, while RAD51 remains bound to the heteroduplex between the donor strand and pairing strand of the acceptor homologous chromosome or chromatid as a dsDNA NPF [208, 209]. Extension of the heteroduplex dsDNA NPF during end capture and subsequent strand exchange might result in nucleosome disassembly by a similar "occupation mechanism" of transiently un-
wrapped nucleosome DNA in the absence of other chromatin remodeling factors. However, we cannot neglect the possibility that before or during strand exchange, the formation and extension of the ssDNA and dsDNA HsRASD51 NPFs could be tightly regulated by BRCA2 and the SWI/SNF translocases [195–197, 203, 204], either by preventing HsRAD51 occupation of nucleosomal DNA and subsequent disassembly or by facilitating and enhancing Rad51 chromatin remodelling activity.

The central role of ATP hydrolysis in nucleosome disassembly is intriguing since homologous pairing and strand exchange by RecA/RAD51 appear to require only ATP binding and largely occurs independent of ATP hydrolysis [210, 211]. Several observations indicate a mechanistic ability for HsRAD51 to switch between nucleosome disassembly and recombinase activity. RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) play an essential role in HR by promoting RAD51 NPF formation and mediating checkpoint signaling [212]. Recently, HsRAD51C has been shown in vivo to order with HsRAD51 in NPF formation at DBS sites [213], and paralog-deficient hamster, chicken, and human cell lines lack the ability to form a HsRAD51 NPF at IR irradiation induced DSBs [214, 215]. While RAD51 homologues (HsRad51, ScRAD51, HsDCM1, MvRADA) contain a conserved Aspartate that forms a salt bridge with the ATP γ-phosphate at the nucleotide-binding interface between RAD51 subunits known as the ATP cap, E. coli RecA and most of the HsRAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2) contain a conserved Lysine instead [191]. Replacement of the conserved Aspartate with Lysine [HsRAD51(D316K)] (as found in the RAD51 paralogs) significantly decreases the rate of ATP hydrolysis and RAD51 turnover, and eliminates the requirement of Ca²⁺ and non-physiological monovalent salts for stable ATP-bound NPF formation and strand exchange (recombinase) activity. Given that in vitro studies indicate HsRAD51 NPF disassembles beginning from the filament end [216], taken together these observations suggest that HsRAD51 paralogs containing an ATP cap Lysine may function to stabilize the HsRAD51 filament. Indeed, such a RAD51 NPF stabilization has recently been demonstrated for the yeast paralogs Rad55 and Rad57 [217]. Additionally, differential binding of the MRE repeats of HsBRAC2 to HsRAD51 function to decrease HsRAD51 ATPase activity, stabilize binding of the NPF to ssDNA, and enhance
recombinase activity [203, 218]. These results suggest that HsRAD51 activity in the absence of such mediators could facilitate nucleosome disassembly. However, additional studies will be necessary to understand the regulatory switch that governs any choice between HsRAD51 recombinase and nucleosome disassembly activities.
6.4 Experimental Procedures

6.4.1 HsRAD51 and ScRad51 expression and purification

HsRAD51 and ScRAD51 were expressed and purified as previously described [219, 220]. E.coli RecA was purchased from Epicenter Biotechnologies (RE44200). All RecA/RAD51 proteins were active for strand exchange under their respective published optimal conditions.

6.4.2 DNA Constructs

The 601E, 601D, 5SD, 5SE, Mp2-147, and components for the 601D-linker and 601E-linker molecules for fluorescence studies were prepared by PCR from plasmid containing the 601 or *Xenopus borealis* 5S rDNA nucleosome positioning sequence (NPS) and a TspRI restriction site 10bp downstream of the NPS. Oligonucleotides (Sigma, See Appendix D for oligo and DNA sequences) were labeled with a Cy3 NHS ester (GE healthcare) at an amino group attached at the 5′-end or to a modified internal thymine and then purified by RP-HPLC on a 218TP—C18 column (Grace/Vydac). Following PCR amplification, each DNA molecule was purified by HPLC with a Gen-Pak Fax column (Waters). 601D-linker and 601E-linker were synthesized by separately digesting with TspRI in NEB buffer 4 (New England Biolabs) PCR product containing the 601 NPS and TspRI site and PCR product containing the TspRI site and a 90 base pair linker DNA. Digested NPS and linker fragments were purified by Gen-Pak Fax column, ligated with T4 ligase in supplied buffer plus 2mM ATP (New England Biolabs) and purified by Gen-Pak Fax column to remove unligated fragments.

6.4.3 Histone Expression and purification

*X. laevis* recombinant histones were expressed and purified following [79, 107] as detailed in Appendix A. Mutation H3(C110A) was introduced by site-directed mutagenesis (Stratagene).
6.4.4 Histone Octamer formation and labeling

Histones: H2A(K119C), H2B, H3(C110A), H4 or H2A, H2B, H3, H4 were unfolded, combined at equal molar ratios, and refolded following [79, 107] as detailed in Appendix A. H2A(K119C) was labeled after octamer refolding with Cy5-maleamide (GE Healthcare) and purified over a Superdex 200 gel filtration column (See Appendix B). The purity of each octamer was confirmed by SDS-PAGE and mass spectrometry. The labeling efficiency was 70-80% as determined by UV-VIS and mass spectroscopy.

6.4.5 Nucleosome Reconstitution

Nucleosomes for fluorescence measurements were reconstituted salt gradient pumping (See Appendix C) from 2µg Cy3-labeled DNA, 6µg of lambda DNA, and 2µg of Cy5-labeled histone octamer and purified by sucrose gradient (See appendix C). Non-labeled nucleosomes were reconstituted from 12µg Mp2-147 DNA a 10µg unlabeled histone octamer by salt gradient double dialysis (See Appendix C) and purified by sucrose gradient. Purity of each nucleosome was checked by 5% native PAGE with 0.3x TBE in the gel and running buffer, prerun for 1 hr at room temperature and 20V/cm, loaded while running with 1x ficol, and resolved for 1 hr at room temperature and 20V/cm (See Appendix E). Gels were imaged using a typhoon 8600 variable mode imager. Nucleosome position mapping studies using ExoIII and hydroxy radical cleavage of nucleosomes reconstituted with these and similar substrates have been published previously [93] (See chapters 1,5), and indicate that a significant fraction of the fluorescent-labeled substrates are localized to the 601 and 5S NPS.

6.4.6 HsRad51 Nucleosome Disassembly by Gel Shift

Fluorescent-labeled 601D-linker nucleosomes (2nM) and unlabeled Mp2 nucleosomes (10nM) in 20mM Tris pH 8.0, 2mM MgCl2, 250µM ATP, 3% glycerol, 1mM dithiothreitol (DTT), 0.2mg/ml acetylated bovine serum albumin (BSA) and 130 mM NaCl, or 130 mM KCl, or 65mM (NH4)2SO4 were incubated at 37°C for 30 minutes with 0 - 500nM
HsRAD51 in 10\mu l total reaction volume. Reactions were quenched with 10mg/ml oligo-dA ssDNA and 20mM ethylenediamine-tetraacetic acid pH 8.0 (EDTA) and incubated at 42°C for 90 min to destabilize the HsRAD51 filament. Reactions were resolved by 5% native PAGE gel in 0.3x TBE plus 10mM EDTA, prerun at 7V/cm for 1hr and run at 7V/cm for 2 hrs with continuous buffer circulation (See Appendix E). Gels were imaged by the Cy3 fluorophore using a Typhoon Trio variable modeimager (GE Healthcare). Images were quantified using Image Quant software (Invitrogen) using local median background subtraction.

6.4.7 Nucleosome disassembly kinetics by FRET

Fluorescent-labeled nucleosomes (2 nM) and unlabeled mp2-147 nucleosomes (10 nM) were preincubated at 37°C for 5 min in 130mM KCl or 20mM KCl, 2mM MgCl2 or CaCl2, 250\mu M ATP or ATPγS, 20mM Tris pH 8.0, 3% glycerol, 1mM DTT, 0.05mg/ml acetylated BSA, and 1x COT/NBA as a triplet state quencher (1mM 1-3-5-7-Cyclooctatetraene + 1mM nitrobenzoic acid). Unlabeled nucleosomes were included to enhance the stability of the fluorescent-labeled nucleosomes as previously described [120]. The total concentration of nucleosomes was 12nM or 2\mu M DNA nucleotides. HsRAD51, HsRad51(D316K), ScRad51, or RecA was then rapidly mixed with the nucleosomes and placed into a submicro quartz cuvette (Starna, 16.12F-Q-1.5/Z8.5) in a Fluoromax-4 (Horiba) photon counting steady state fluorometer with circulating waterbath at 37°C. The Cy3 label was excited at 510nm, and the changes in fluorescence resonance energy transfer (FRET) between Cy3 and Cy5 was monitored by measuring Cy5 fluorescence emission at 670nm. The Cy5 fluorescence emission as a function of time, F(t), from 0 to 300 seconds was fit to a single exponential decay, F(t) = Ae^{(-t/\tau)} + Fo, where A is the amplitude of fluorescence decay, \tau is the rate of fluorescence decay, and Fo is the final Cy5 fluorescence. The equilibrium FRET efficiency between Cy3 and Cy5 was determine by the (ratio)_A method as previously described [83].
Chapter 7

POST-TRANSLATIONAL MODIFICATIONS
OF THE DNA-HISTONE INTERFACE
REGULATE CHROMATIN REMODELLING
AND NUCLEOSOME DISASSEMBLY
COMPLEXES

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7.1 introduction

In Chapter 6 we observed that the *Homo sapien* DNA homologous repair protein, HsRad51, functions to disassemble nucleosomes by trapping intrinsic DNA unwrapping fluctuations unwrap the nucleosomes beginning at the entry-exit region and proceeding through the dyad. Our observations that intrinsic histone PTMs throughout the DNA-histone interface function in part to regulate DNA unwrapping, nucleosome mobility and/or stability indicate that they may function synergistically with HsRAD51 and other extrinsic chromatin regulatory complexes to facilitate chromatin remodelling and nucleosome disassembly. We investigated the mechanisms by which Post-Translation Modifications (PTMs) of the DNA-Histone interface influence nucleosome remodelling and disassembly in the context of DNA repair by the *Homo sapien* HsMSH-HsMSH6 DNA mismatch recognition complex and in the context of transcription regulation using the SWI/SNF chromatin remodelling complex. Both of these complexes are observed *in vivo* to reposition and/or disassemble nucleosomes [63, 154].

Histone point mutations have been isolated that result in SWI/SNF Independent (SIN) phenotypes that alleviate the requirement of SWI/SNF activity for mating type switching at the yeast HO locus in strains defective in SWI/SNF activity [85, 86]. Many SIN mutations, including H3(T118I), have been shown to increase nucleosome mobility [87, 88] and facilitate SWI/SNF chromatin remodelling activity. Both histone residues H3(T118) and H4(R45) are locations of SIN alleles and H3(T118) and H4(S47) are know to be phosphorylated *in vivo* [60, 66]. These results suggest that histone PTMs of the DNA-Histone interface that regulate nucleosome unwrapping, stability, and mobility may function to regulate SWI/SNF activity.

Coordinately, for HsMSH2-HsMSH6, DNA mismatch recognition and repair often occurs behind replication forks during DNA synthesis [221]. In yeast, newly assembled nucleosomes are found with the histone PTM, H3(K56ac) [134]. HsMSH2-HsMSH6 has been observed *in vitro* to disassemble nucleosomes in the vicinity of a DNA mismatch [154]. HsMSH2-HsMSH6 functions in part to recognize and bind DNA mismatches [222]. Upon hydrolysis
of the bound APT cofactor to ADP, HsMSH2-HsMSH6 forms a sliding clamp that moves along the DNA [223]. It has been proposed that the concerted action of multiple sliding clamps along the DNA function to disassemble nucleosomes [154].

We observe that both H3(K56ac) within the nucleosome entry-exit and H3(T118ph) within the nucleosome dyad facilitate HsMSH2-HsMSH6 dependent nucleosome disassembly by ~2 and 25 fold, respectively, compared to unmodified nucleosomes. Coordinately, we observe that H3(T118ph) induces SWI/SNF dependent nucleosome disassembly whereas unmodified nucleosomes are primarily repositioned by SWI/SNF. Our results are consistent with the conclusion that PTMs in the DNA-histone interface regulate the activity and function of chromatin regulatory complexes in vivo that reposition and disassemble nucleosomes.


7.2 Results

7.2.1 H3(K56ac) facilitates nucleosome removal by the DNA-mismatch recognition complex HsMSH2-HsMSH6

The modulation of nucleosome unwrapping is also a potential mechanism for regulating the interaction between nucleosome disassembly complexes and nucleosomal DNA. We investigated this mechanism in the context of DNA repair by determining the influence of H3(K56ac) on nucleosomal disassembly by the DNA-mismatch recognition complex, HsMSH2-HsMSH6 [24, 154]. Recent studies have demonstrated that HsMSH2-HsMSH6 disassembles nucleosomes by passively unwrapping and/or repositioning nucleosomes [154]. Additionally, model simulations indicate that HsMSH2-HsMSH6 functions to disassemble nucleosomes by applying a 1-dimensional pressure to iteratively unwrap nucleosome DNA through the dyad. The H3 Lysine 56 acetylation mimic H3(K56Q) has been demonstrated to increase the rate of HsMSH2-HsMSH6 dependent nucleosome disassembly by 2-fold [154]. Given that H3(K56Q) appears to capture the biophysical effects of H3(K56ac) on nucleosome unwrapping, these results suggest that H3(K56ac) facilitates invasion into the nucleosome by chromatin remodelling and nucleosome disassembly complexes.

To investigate this hypothesis, we reconstituted unmodified and H3(K56ac)-containing nucleosomes with DNA containing a mismatch 55 base pair from the X. borealis 5S rDNA positioning sequence [154] (Figure 7.1A). Incubation with HsMSH2-HsMSH6 showed that these nucleosomes were disassembled by HsMSH2-HsMSH6 1.7 ± 0.3 times faster ($\tau_{H3(K56Ac)} = 112 ± 5$ min) than unmodified nucleosomes ($\tau_{H3(C110)} = 189 ± 33$ min; Figure 7.1B-D]. This difference is statistically significant with a P-value of 0.005. The preparation of H3(K56ac) required the mutation H3(C110A), while unmodified H3 contained the native Cystiene. To control for this difference, we confirmed that the conversion of the Cystiene at the 110th amino acid of H3 to an Alanine did not alter the nucleosome site accessibility with the X. borealis 5S NPS (Chapter 4). As a control we showed that nucleosomes without a mismatch were not disassembled significantly by HsMSH2-HsMSH6 (Figure 7.2). Furthermore, H3(K56Q) with the native Cystiene at the 110th amino acid,
enhances HsMSH2-HsMSH6-induced nucleosome disassembly by 2-fold [154], similarly to H3(K56ac). The combination of these measures indicates that the increase in DNA unwrapping by H3(K56ac) enhances HsMSH2-HsMSH6-induced nucleosome disassembly adjacent to a DNA mismatch. Additional studies are required to determine if this 2-fold increase is biologically significant. However, 2-fold changes often have significant biological consequences as in dosage compensation and haploinsufficiency diseases.

7.2.2 Nucleosome Disassembly by HsMSH2-HsMSH6 is significantly enhanced by Dyad Modifications

Previous studies of HsMSH2-HsMSH6 dependent nucleosome disassembly demonstrated that H3 acetylated Lysines 115 and 122 [H3(K115ac,K122ac)] significantly increased the rate of nucleosome disassembly from the *X. borealis* 5S rDNA nucleosome positioning sequence by 5-fold compared to unmodified nucleosomes [154]. These studies were consistent with the experiments of Simon, et al. [120] which observed that H3(K115ac,K122ac) increases the frequency of nucleosome disassembly by 5-fold compared to unmodified nucleosomes during mechanically induced chromatin stretching studies using single molecule magnetic tweezers [120]. Additionally, in Chapters 1 we demonstrate by competitive reconstitution measure that H3(K115ac,K122ac) reduce DNA-Histone binding affinity by ∼0.6 kcal/mol. These results indicate that histone PTMs in the nucleosome dyad are poised to facilitate nucleosomes disassembly complexes.

Histone H3 phosphorylated Threonine 118 [H3(T118ph)] is located between these two acetylation PTMs in the dyad region. In Chapter 2 we observe that H3(T118ph) decreases DNA-histone binding affinity by ∼2kcal/mol and increases nucleosome mobility by 10-20 fold which is consistent with the binding affinity measures. Combined with our observations for H3(K115ac,K122ac), these results suggested that phosphorylation might also influence HsMSH2-HsMSH6 induced nucleosome disassembly.

We initially examined the influence of H3(T118ph) on HsMSH2-HsMSH6 nucleosome disassembly using the 5S rDNA NPS for comparison to experiments using H3(K56ac) and H3(K115ac,K122ac) and found the disassembly kinetics to be too rapid for quantification by
Figure 7.1: **H3(K56ac) facilitates nucleosome dissociation by the mismatch recognition complex HsMSH2-HsMSH6.** (A) DNA constructs containing the *X. borealis* 5S NPS with (5S-GT) or without (5S-GC) a DNA mismatch and a 5′-biotin. (B) and (C) Electrophoretic mobility shift analysis of H3(C110) nucleosomes adjacent to a GT mismatch, and H3(K56ac) nucleosomes adjacent to a GT mismatch disassembled by HsMSH2-HsMSH6, respectively. Lane 1: sucrose gradient-purified nucleosomes, Lane 2: nucleosomes bound by streptavidin, Lane 3: nucleosomes bound by streptavidin and HsMSH2-HsMSH6, Lanes 4-9: kinetic analysis of streptavidin-bound nucleosome disassembly by HsSH2-HsMSH6 in the presence of 1mM ATP. (D) The fraction of unmodified 5S-GC NPS nucleosomes (black square), H3(C110) 5S-GT NPS nucleosomes (lavender diamond) and H3(K56ac) 5S-GT NPS nucleosomes (orange triangle) versus time in the presence of HsMSH2-HsMSH6 (250nM) and ATP (1mM). The error bars were determined from the standard deviation of at least three separate experiments. The fraction of nucleosomes versus time were fit excluding the zero time point to a single exponential decay. (Reproduced with permission)
Figure 7.2: **Disassembly of nucleosomes from 5S NPS is HsMSH2-H2MSH6 dependent.** (A) Electrophoretic mobility shift analysis of H3(K56ac,C110) nucleosomes on homoduplex DNA (5S-GC) without a GT mismatch in the presence of HsMSH2-HsMSH6. Lane 1: sucrose gradient-purified nucleosomes, Lane 2: nucleosomes bound by streptavidin, Lane 3: nucleosomes bound by streptavidin and HsMSH2-HsMSH6, Lanes 4-9: kinetic analysis of streptavidin-bound nucleosome disassembly by HsSH2-HsMSH6 in the presence of 1 mM ATP. (Reproduced with permission)

Gel shift analysis (data not shown). Using the significantly more stable 601 NPS derivative, Mp2, we found that H3(T118ph) nucleosomes were disassembled by HMSH2-HsMSH6 25 ± 7 times faster ($t_{1/2} = 55 ± 4$ min) than unmodified nucleosomes ($t_{1/2} = 1400 ± 400$ min) (Figure 7.3). This increase in rate is nearly identical to the increase in mobility induced by H3(T118ph) nucleosomes using the same Mp2 NPS substrate (See Chapter 2).

### 7.2.3 H3(T118ph) Enables Nucleosome Disassembly During the SWI/SNF Remodeling Reaction

The report that H3(T118A) and H3(T118E) impact transcriptional regulation [46] and our observation that H3(T118ph) enhances nucleosome sliding and reduces DNA-histone binding suggests that this modification could influence chromatin remodeling. Moreover, the H3(T118) residue was found to contribute to the SWI/SNF independent (SIN) phenotype [85]. We examined the influence of H3(T118ph) on chromatin remodeling by the purified
Figure 7.3: H3(T118ph) dramatically enhances nucleosome dissociation by the mismatch recognition complex HsMSH2-HsMSH6. (A) DNA constructs containing the MP2 NPS which is a derivative of the 601 NPS with (Mp2-GT) or without (Mp2-GC) a DNA mismatch and a 5′-biotin. (B) and (C) Electrophoretic mobility shift analysis of unmodified H3 nucleosomes adjacent to a GT mismatch, and H3(T118ph) nucleosomes adjacent to a GT mismatch disassembled by HsMSH2-HsMSH6, respectively. Lane 1: sucrose gradient-purified nucleosomes, Lane 2: nucleosomes bound by streptavidin, Lane 3: nucleosomes bound by streptavidin and HsMSH2-HsMSH6, Lanes 4-9: kinetic analysis of streptavidin-bound nucleosome disassembly by HsMSh2-HsMSh6 in the presence of 1mM ATP. (D) The fraction of unmodified Mp2-GC NPS nucleosomes (black square), unmodified Mp2-GT NPS nucleosomes (red triangles) and H3(T118ph) Mp2-GT NPS nucleosomes (blue circles) versus time in the presence of HsMSh2-HsMSh6 (250nM) and ATP (1mM). The error bars were determined from the standard deviation of at least three separate experiments. The fraction of nucleosomes versus time were fit excluding the zero time point to a single exponential decay. (Reproduced with permission)
Figure 7.4: **Disassembly of nucleosomes from Mp2 NPS is HsMSH2-H2MSH6 dependent.** (A) Electrophoretic mobility shift analysis of H3(T118ph) nucleosomes on homoduplex DNA (Mp2-GC) without a GT mismatch in the presence of HsMSH2-HsMSH6. Lane 1: sucrose gradient-purified nucleosomes, Lane 2: nucleosomes bound by streptavidin, Lane 3: nucleosomes bound by streptavidin and HsMSH2-HsMSH6, Lanes 4-9: kinetic analysis of streptavidin-bound nucleosome disassembly by HsSH2-HsMSH6 in the presence of 1 mM ATP. (Reproduced with permission)

SWI/SNF complex [224]. We reconstituted unmodified and H3(T118ph) nucleosomes on the Mp2-187 DNA molecule and purify by sucrose gradient as detailed in Chapter 2 (Figure 7.5A). In the presence of ATP, SWI/SNF repositioned unmodified nucleosomes without inducing nucleosome disassembly as previously observed [225, 226] (Figure 7.5B-C). In contrast, SWI/SNF remodeling of H3(T118ph) containing nucleosomes resulted in a dramatic increase in free DNA, suggesting that SWI/SNF directly disassembles H3(T118ph) containing nucleosomes. We fit the total unmodified nucleosome fractions and the total H3(T118ph) nucleosome fractions to:

\[
\text{nucleosome fraction} = 1 - \frac{[\text{SWI/SNF}]}{K_{12} + [\text{SWI/SNF}]}
\]  

(7.1)

where \(K_{12}\) is the SWI/SNF concentration at which half of the nucleosomes are disassembled. We find that there is not a decrease in the fraction of total unmodified nucleosomes
(K$_{1/2-unmod}$ < 2x10$^{-5}$ nM), while for total H3(T118ph) nucleosomes, K$_{1/2-H3(T118ph)}$ = 26 ± 4 nM. This is consistent with our HsMSH2-HsMSH6 studies that show modifications at the nucleosome dyad enhance the dissociation or disassembly of partially wrapped nucleosomes [154].

To determine if these differences are due to changes induced by H3(T118ph) in SWI/SNF affinity to the nucleosome as has been reported for other histone modifications [44, 95], we bound unmodified and H3(T118ph) nucleosomes in the absence of ATP (Figure 7.6). We observe that there is no quantifiable difference in the binding of SWI/SNF to nucleosomes with and without H3(T118ph). These results are consistent with the conclusion that H3(T118ph) increases the repositioning and/or disassembly activity SWI/SNF.

We also analyzed the influence of H3(T118ph) on repositioning of intact nucleosomes (Figure 7.6). We fit the fraction of positioned nucleosomes compared to total nucleosomes as a function of SWI/SNF concentration to determine the concentration K$_{1/2-positioned}$ at which 50% of the initially positioned nucleosomes are depositioned (Figure 7.5D). We find H3(T118ph) decreases the observed amount in which nucleosomes reposition by 6-fold compared to unmodified nucleosomes (K$_{1/2-unmod-positioned}$ = 80 ± 3 nM and K$_{1/2-H3(T118ph)-positioned}$ = 500 ± 100 nM). Interestingly, for H3(T118ph) nucleosomes in the presence of SWI/SNF, the free DNA band increased and the total nucleosome content decreased. Taken together these results indicate that H3(T118ph) reduces the repositioning of nucleosomes to other DNA sites by SWI/SNF by 6-fold (Figure 7.5D). However, H3(T118ph) nucleosomes are already distributed to two separate positions in the absence of SWI/SNF, yet retain a 147 base pair DNA footprint (See Chapter 2). We cannot rule out the possibility that the 2 dominant nucleosome positions are in dynamic equilibrium and that SWI/SNF repositions the nucleosomes rapidly between these two positions.

There appears to be two nonexclusive mechanisms by which H3(T118ph) enables nucleosome disassembly by SWI/SNF in our experiments. The first model is that SWI/SNF allows the histone octamer to directly disassociate from the DNA. This model relies on our observation that H3(T118ph) significantly reduces the DNA-histone binding free energy,
which combined with the 50 base pairs of DNA that are unwrapped from the histone octamer by SWI/SNF could directly release the histone octamer. Alternatively, H3(T118ph) could allow SWI/SNF to slide the histone octamer off the end of the DNA. This model is consistent with our observation that H3(T118ph) dramatically enhances the rate of nucleosome thermal repositioning. Since DNA ends are rare in vivo, this alternate model would be consistent with the conclusion that H3(T118ph) increases SWI/SNF repositioning in vivo.
Figure 7.5: H3(T118ph) switches SWI/SNF remodeling from nucleosome sliding to nucleosome disassembly. (A) DNA construct containing the Mp2 NPS and 20 base pairs of linker DNA on each end. (B) EMSA of ATP dependent chromatin remodeling with unmodified and H3(T118ph) nucleosomes in the presence of increasing concentrations of SWI/SNF. Lanes are labeled with the nanomolar (nM) concentration of SWI/SNF. Each reaction was incubated for 30 min with or without 1mM ATP. The bracket indicates the range of nucleosome electrophoretic mobility and the arrow indicates the location of central positioned nucleosomes. (C) The change in the fraction of total unmodified (red triangles) or H3(T118ph) (blue circles) nucleosomes versus SWI/SNF concentration. The error bars were determined from the standard deviation of 3 separate experiments. The values were determined from the ratio of the total nucleosome band (bracket) to the total nucleosome and free DNA bands and normalized to the fraction of total nucleosomes at zero concentration of SWI/SNF. (D) The change in the fraction of centrally positioned nucleosomes relative to the initial fraction of centrally positioned nucleosome for nucleosomes containing unmodified H3 (red triangles) and H3(T118ph) (blue circles) as a function of SWI/SNF concentration. The values were determined from the ratio of the top band (arrow) to the entire nucleosome band (bracket) and normalized by the fraction of positioned nucleosomes at zero concentration of SWI/SNF. (Reproduced with permission)
Figure 7.6: H3(T118ph) does not influence SWI/SNF binding to nucleosomes. (A) and (B) are native PAGE analysis of SWI/SNF binding to unmodified and H3(T118ph) nucleosomes, respectively. The lanes are labeled with the concentration of SWI/SNF (nM). (C) Quantification of the fraction of unmodified (red triangles) and H3(T118ph) (blue circles) nucleosomes bound by SWI/SNF. The error bars were determined from the standard deviation of 3 separate experiments. (Reproduced with permission)
7.3 Discussion

We have quantified the effects of histone PTMs in the DNA-histone interface on nucleosome remodelling and disassembly in the context of DNA repair and transcription regulation. We observe that enhanced DNA unwrapping by H3(K56ac) within the DNA entry-exit region facilitates nucleosome disassembly by the DNA mismatch recognition complex, HsMSH2-HsMSH6. Additionally we observe that H3(T118ph) in the nucleosome dyad dramatically enhances HsMSH2-HsMSH6 dependent nucleosome disassembly and switches SWI/SNF chromatin remodelling function for repositioning to disassembly of nucleosomes.

SWI/SNF appears to remodel nucleosomes by binding the histone octamer face creating a ~50 base pair DNA bulge near the entry exit region of the nucleosome that propagates to the opposite entry-exit region, ultimately resulting in nucleosome sliding without disassembly [64, 227]. The presence of histone chaperones or adjacent nucleosomes appears to facilitate histone octamer release [228–231]. Our observation that H3(T118ph) enables nucleosome disassembly by SWI/SNF suggests that H3(T118ph) may intrinsically regulate the outcome of a SWI/SNF remodeling reaction. These results are consistent with the hypothesis that the H3(T118ph) PTM may target the SWI/SNF chromatin disassembly reaction to distinct chromatin sites.

Previously, we proposed two mechanisms by which HsMSH2-HsMSH6 disassembles nucleosomes [154]. The first model is that HsMSH2-HsMSH6 traps unwrapping fluctuations and eventually traps enough unwrapped DNA that the nucleosome spontaneously disassembles. In this model, the histone octamer does not shift relative to the DNA molecule. The second model is that HsMSH2-HsMSH6 traps nucleosome repositioning fluctuations such that the histone octamer slides off the end of the DNA. In this model, the histone octamer moves with respect to the DNA. Our findings that H3(T118ph) enhances restriction enzyme and DNase I accessibility near the dyad is consistent with increased DNA unwrapping out to the nucleosome dyad region. An increase in DNA unwrapping that extends to the nucleosome dyad could enhance nucleosome disassembly by reducing the fraction of DNA required to disassemble a nucleosome, which appears to be just beyond the nucleosome dyad [199].
This is also consistent with the observation that H3(K115ac,K122ac) within the nucleosome dyad increases by 5-fold both the rate of HsMSH2-HsMSH6 dependent nucleosome disassembly and the probability for nucleosome disassembly during mechanically induced DNA unwrapping [120, 154]. We also observed that H3(T118ph) and H3(K115ac,K122ac) enhances thermally induced nucleosome repositioning. This enhanced sliding could facilitate nucleosome disassembly within the framework of the nucleosome sliding model. Since these models are nonexclusive, both mechanisms could be contributing to the H3(T118ph) enhancement of nucleosome disassembly by HsMSH2-HsMSH6. Interestingly, model simulations of HsMSH2-HsMSH6 nucleosome disassembly can account for the differences in activity for unmodified and modified nucleosomes based on the energetics of DNA unwrapping alone [24].

The mechanism of enhanced nucleosome unwrapping rate by which H3(K56ac) enhances HsMSH2-HsMSH6 disassembly could be the case for other histone PTM’s in the entry-exit region. H4(K77ac,K79ac), which is located at the DNA-histone interface 40-45 base pair within the nucleosome, enhanced TF binding 2-fold to its site between base pairs 8 - 27 within the nucleosome. Furthermore, phosphomimetics of histone H3 PTMs Y(41ph) and T(45ph) also increased nucleosome unwrapping as well. Further studies are required to determine if additional histone PTMs in the nucleosome entry-exit regulate the DNA unwrapping rate to control nucleosome disassembly by HsMSH2-HsMSH6.

Taken as a whole, these results suggest that histone PTMs in the DNA interfaces are positioned to synergistically function with chromatin remodelers, histone chaperones, and other chromatin regulatory complexes to unwrap, reposition, and/or disassemble nucleosomes. Further studies are required to determine the precise function relationship between these PTMs and chromatin regulatory complexes in vivo.
7.4 methods

7.4.1 DNA Constructs

Unlabeled Mp2-147 and Cy3/Cy5 labeled Mp2-187 DNA molecules were synthesized by PCR from the pMp2 plasmid containing the Mp2 NPS, a derivative of the 601 NPS, and unlabeled of 5'-labeled DNA oligos (Sigma, See Appendix D for DNA and Oligo sequences). Molecules Mp2-GT, Mp2-GC, 5S-GT, or 5S-GC were prepared as previously described [154] from P$^{32}$ labeled oligos and pBluescript plasmid containing the Mp2 or *X. borealis* NPS. These molecules contain 20 base pairs of extra DNA to the left of the NPS and 100 base pairs to the right with a 5' biotin label and a G/T mismatch or G/C homoduplex at the 20th base pair before the biotin label.

7.4.2 Expression and Purification of Wild Type and Mutant Histones

*Xenopus laevis* recombinant histones were expressed and purified following [79, 107] as detailed in Appendix A. Histones H3(K56Ac,C110A) and H3(T118ph) were prepared as previously described [121, 148].

7.4.3 Preparation of histone octamers

Histone octamer refolding and purification were performed following [79, 107] as described in Appendix A. Briefly, each histone was unfolded for 1 to 3 hours in unfolding buffer at a histone concentration of 2 to 20 mg/ml, and then spun to remove aggregates. The absorption at 276 nm was measured for each unfolded histone to determine the concentration. UV-Vis absorption spectra of acetylated histone H3(K56ac,C110A) and phosphorylated histone H3(T118ph) contains a background peak centered at 230nm that overlaps with the protein absorption peak centered at 276nm. This peak is of unknown origin, but presumably due to a remnant chemical contaminant from the synthesis process. To correct for this, the overlap between the anomalous 230nm peak and 276nm protein peak was estimated by hand as described in Appendix A. Each of the four histones were combined at equalmolar ratios, refolded and purified as previously described in Appendix A.
7.4.4 Nucleosomes Reconstitution

All nucleosomes were reconstituted by the salt gradient double dialysis method (See Appendix C) and purified by sucrose gradient purification. Inclusion of 0.5mM MgCl₂ in all reconstitution and purification buffers was required to maintain the stability of nucleosomes containing H3(T118ph).

7.4.5 HsMSH2-HsMSH6 nucleosome remodeling assay

Nucleosome disassembly reactions were carried out at 37°C as previously described [154] with 0.25nM of unmodified or H3(T118ph) nucleosomes reconstituted with the Mp2-GT or Mp2-GC NPS and 5nM of unlabeled unmodified nucleosomes reconstituted with the mp2-147 DNA molecule. Reactions with H3(K56ac,C110A) were carried out at 37°C with 0.25nM of unmodified or H3(T118ph) nucleosomes reconstituted with the 5S-GT or 5S-CG NPS. Kinetic analysis was performed by staggering the time ATP was added. The fraction of disassembled nucleosome were analyzed by gel shifts on polyacrylamide gels as previously described [154]. A small fraction of free DNA appears because we do not completely purify naked DNA away from the nucleosome and a fraction of nucleosomes fall apart during the rapid mixing of the nucleosomes. We control for this as we do with the restriction enzyme digestion experiments where we do not include the zero time point in the exponential decay fit. Furthermore, the GC duplex control experiments show no change in the fraction of H3(T118ph) of H3(K56ac,C110A) containing nucleosomes from the second to the last time point. This demonstrates that we observe HsMSH2-HsMSH6 dependent nucleosome disassembly as we previously reported [154].

7.4.6 SWI/SNF Nucleosome Binding and Remodeling assays

SWI/SNF binding reactions were performed in 7µl with 28nM of unmodified or H3(T118ph) nucleosomes with mp2-187 and 3.5nM - 56nM of SWI/SNF at 30°C for 30 min. Half of the binding reactions were examined by 4% (79:1 acrylamide:bis-acrylamide) native PAGE in 0.5x TBE at 200V for 4 hr in 4°C (See Appendix E). The remaining half was cooled down
to 18°C and ATP was added to 800µM final concentration. The remodeling reactions were incubated at 18°C for 30 min and stopped with 1µl of a 1:1 mix of 10mg/ml salmon sperm DNA and 10mM γ-thio-ATP as previously described [225]. For gel shift analysis of the remodeling reactions, 4µl samples were loaded on a 4% (35:1 acrylamide: bis-acrylamide) native PAGE in 0.5x TBE at 200V for 4 hrs in 4°C (See Appendix E).
There are more than 100 reported PTMs throughout the nucleosome. Many of these PTMs reside on the histone tails that extend beyond the core of the nucleosome, or on the nucleosome face. These PTMs primarily serve as binding sites for chromatin regulatory proteins or perturb intermolecular interactions between nucleosomes that alter folding and compaction of higher order chromatin structure to regulate DNA accessibility.

This work encompasses a detailed study of the integral relationship between histone post-translational modifications (PTMs) that reside in the nucleosome DNA-histone interface and DNA nucleosomes accessibility, mobility, and stability. There are 19 reported methylation, acetylation, or phosphorylation PTMs that reside throughout the DNA-histone interface \([H3(K36me3), H3(Y41ph), H3(T45ph), H3(K56ac), H3(S86ph), H3(K115ac), H3(T118ph), H3(K122ac); H4(S47ph), H4(K77ac), H4(K79ac); H2A(K36ac), H2A(K74me), H2A(K75me), H2A(K77me); H2B(K43me1), H2B(K85ac), H2B(K120ac), H2B(K125ac)], [60, 61, 66, 67, 69]\). However, while histone PTMs in the DNA-histone interface have been proposed to function by regulating nucleosomes unwrapping, mobility, and stability \([35, 59]\), the overall function of these PTMs have remained mostly unknown.

Using a combination of semi- and fully-synthetic protein ligation strategies developed by Ottesen and colleagues, we have engineered and incorporated histones bearing one or more of the precise PTMs: \(H3(K56ac), H4(K77ac), H4(K79ac), H3(K115ac), H3(T118ph),\) or \(H3(K122ac)\) into nucleosomes for biophysical characterization. Additionally, for proteins
that have not yet been engineered with the precise PTMs, we have employed analog and amino acid substitution mimics of H3(K36me3), H3(Y41ph), and H3(T45ph) to study their putative role in nucleosome regulation. Together, save for the H3(T118ph) homologue, H4(S47ph), and H3(S86ph), these studies quantify the biophysical roles of each known acetylation/methylation/phosphorylation PTM of the H3/H4 tetramer that reside within the DNA-histone interface. Not only do our results demonstrate the power of native chemical ligation and expressed protein ligation for preparing histones with well define PTMs, they also underpin the need to carefully interpret biophysical genetic studies that employ histone amino acid substitutions (Glutamine mimic of acetylation, Glutamic Acid mimic of phosphorylation, Methionine mimic of methylation) to characterize the role of precise PTMs. We observe that while certain mimics such as H3(K56Q) capture the biophysical effects of H3(K56ac), neither H3(K115Q), H3(T118E), nor H3(K122Q) capture any of the effects observed with the precise modification.

The first underlying principle that this work reveals is that PTMs in the DNA-histone interface can function by (1) regulating spontaneous, partial DNA unwrapping to facilitate DNA accessibility and/or (2) alter DNA accessibility by decreasing nucleosome stability and increasing mobility for nucleosome repositioning and disassembly. We find that there are two distinct regions of the DNA-histone interface (Figure 8.1). As shown in Chapters 1-2, modifications in the nucleosome dyad region control nucleosome mobility and stability without directly impacting DNA unwrapping. These results are consistent with the observations of Simon et al. [120] in which PTMs of the nucleosome dyad facilitate mechanically induced nucleosome disassembly whereas PTMs in other regions of the DNA-histone interface do not. Taken as a whole, these results suggest that alterations in the dyad region create nucleosomes that are poised for disassembly or remodelling without increasing DNA accessibility. Conversely in Chapter 4 we demonstrate that modification between the DNA entry-exit region and the Loss of Ribosomal Silencing region 45 base pairs into the nucleosome enhance partial DNA unwrapping. This is consistent with previous studies where alterations in this region by histone PTMs [103], point mutations [142], and mutations in the H3 α-N helix [142] enhance DNA unwrapping. This creates nucleosome that on aver-
age are more accessible to regulatory proteins that rely on thermal nucleosome unwrapping fluctuations to access DNA sites buried within the nucleosome.

However while the function of these two regions are distinct, these processes of nucleosome unwrapping and nucleosomes mobility/disassembly are inherently related. DNA unwrapping is likely to occur before histone proteins disassociate from the DNA in a stepwise mechanism for nucleosome disassembly [155]. This is supported by the observation that histone mutations of the α-N helix region of H3 [H3(V35)-H3(S57)] near the nucleosome entry exit not only increase nucleosome unwrapping, but also increase nucleosome mobility and decrease nucleosome stability [142]. Similarly, the acetylation mimic, H3(K56Q), increases nucleosomes thermal mobility by 1.8-fold [142]. Therefore, PTMs near the entry-exit region such as H3(Y41ph), H3(T45ph), H3(K56ac) or H4(K77ac,K79ac) that enhance DNA unwrapping could also indirectly facilitate nucleosome disassembly. Further studies will be required to determine if these histone PTMs function synergistically with histone PTMs in the dyad region that facilitate histone release to enhance nucleosome disassembly.

Moreover, in Chapter 3 we see that alterations in the path by which DNA wraps around the histone octamer induced by H3(T118ph) lead to the assembly of altered DNA-histone complexes both by salt dialysis and Nap1 histone chaperone mediated assembly. H3(T118ph) leads to the wrapping of a single stretch of DNA around two adjacent histone octamers, presumably due to the electrostatic repulsion induced by the presence of the phosphothreonine in close proximity to the DNA-phosphate backbone. These altered structures have an altered DNA footprint of protection and significantly alter folding of nucleosome arrays. These results also suggest that alterations in DNA wrapping introduced by histone PTMs can also regulate higher order chromatin structure.
Figure 8.1: **Structural Functions of PTMs in the DNA-Histone Interface.** Structure of the nucleosome [ref] indicating distinct regions in which histone PTMs regulate nucleosome unwrapping (orange region) and nucleosome disassembly/mobility (purple region). Location of H3 and H4 PTMs in the DNA-Histone interface are indicated; H3(K36me3) (forest), H3(Y41ph) (mustard), H3(T45E) (royal), H3(K36ac) (orange), H4(K77ac) (yellow), H4(K79ac) (cream), H3(S86ph) (salmon), H4(S47ph) (magenta), H3(T118ph) (red), H3(K115ac) (blue), H3(K122ac) (teal).
The second principle that this work reveals is that PTMs of the DNA-histone interface can function synergistically with other intrinsic and extrinsic chromatin factors to regulate nucleosomes accessibility and disassembly. Within the DNA entry-exit region, we observe that DNA sequence and PTMs function independently and additively to regulate DNA unwrapping to enhance or suppress DNA accessibility (Chapter 5). Similarly, at the nucleosome Dyad where the H3/H4 tetramer make the first contacts with DNA during nucleosome assembly, we find that both the type of histone PTM (Chapter 1, acetylation; Chapter 2, phosphorylation) and the DNA sequence [75, 96, 99] contribute to the stability and mobility of the nucleosomes. Take together, these results demonstrate that both intrinsic factors of DNA sequence and histone PTMs in the DNA-histone interface synergistically regulate nucleosomes dynamics and stability. Further studies will be required to determine if and to what extent histone variants also intrinsically contribute to these effects.

Extrinsic factors can also work synergistically with intrinsic factors of thermal DNA unwrapping fluctuations and PTMs. We observe that the DNA homologous repair protein HsRAD51 (Chapter 6) disassembles nucleosomes by iterative trapping DNA unwrapping fluctuations beginning at the DNA entry-exit region and proceeding through the Dyad. Our results suggest that histone PTMs throughout the DNA-histone interface are poised to facilitate HsRAD51 disassembly of nucleosomes. However further experiments are required to determine such a functional relationship for HsRAD51. We do observe that the chromatin remodelling complex, SWI/SNF (Chapter 7), exhibits enhanced repositioning and disassembly of nucleosomes bearing PTMs within the nucleosome dyad. Other reports also demonstrate that H3(K56ac) in the entry-exit region also facilitate SWI/SNF activity [103]. Given that SWI/SNF appears to reposition nucleosomes via a mechanism by which DNA is partially unwrapped from the histone surface to translocate the histone octamer along the DNA [64], these results are consistent with the conclusion that PTMs in the DNA-histone interface function synergistically to enhance SWI/SNF activity by increasing nucleosome unwrapping and altering nucleosome mobility/stability. Similarly, PTMs both in the DNA entry-exit and dyad regions enhanced MSH2-MSH6 dependent nucleosomes disassembly (Chapter 7). MSH2-MSH6 appears to function by trapping transiently unwrapped
nucleosome states to iteratively unwrap nucleosomes through the Dyad, causing them to disassemble [24]. Again, these results are also consistent with the conclusion that PTMs in the DNA-histone interface function synergistically to enhance MSH2/MSH6 activity by increasing nucleosome unwrapping and altering nucleosome mobility/stability. Further experiments will be required to determine if these PTMs also function synergistically with Rad51 to disassemble nucleosomes. We have demonstrated that Rad51 (Chapter 5) functions by trapping nucleosomes unwrapping fluctuations to unwrap the nucleosomes through the DNA to promote disassembly. Taken together our results suggest that Rad51 and other extrinsic regulatory proteins that function by utilizing inherent nucleosome fluctuations will be synergistically regulated by PTMs in the DNA-histone interface.

As a whole, these studies embody a significant advancement in our understanding of core histone post-translational modifications of H3 and H4 that lie within the DNA-histone interface. PTMs of H2A and H2B that reside in the DNA-histone interface are located near the entry-exit and LRS regions that define the boundaries of the unwrapping zone. Our results suggest that these H2A/H2B PTMs will function similarly to increase nucleosome accessibility. Moreover, the PTMs studied in this work are integrally involved or implicated in numerous genomic processes as discussed in each chapter. Our results indicate that each of these PTMs may carry out their observed role in the regulation of gene transcription, DNA repair, and DNA synthesis by directly altering the inherent structure and dynamics of nucleosomes. However, since most in vivo studies that characterize the role of these PTMs use amino acid substitution mimic, further studies are required to determine the precise role of each of these PTMs in vivo.
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mines nucleosome positioning of the transcriptionally repressed pho5 gene of saccha-

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Appendix A

HISTONE EXPRESSION AND OCTAMER REFOLDING

A.1 Introduction

This appendix details all methods for expressing and purifying histones, and refolding and purifying histone octamers, dimers, and tetramers from purified histones. Following this protocol will result in high-purity octamers, tetramers, and dimers for reconstitution with desired DNA constructs. Where necessary, refolding with labeled and methyl-lysine analog histones will be referenced to methods in Appendix B on conjugating histones with various functional moieties that must be performed before refolding.

The following procedures are adapted from:


A.2 Histone expression vectors

See Table A.1 for a summary of expressed histone molecular weights and extinction coefficients.

DNA sequences encoding H2A, H2B, and H4 recombinant *Xenopus laevis* histones are cloned into the pET3a expression vector, containing the Amp\(^r\) gene. The DNA sequence encoding the H3 recombinant *Xenopus laevis* histone is cloned into the pET3d expression vector, containing the Amp\(^r\) gene. All DNA sequences encoding the human hH2A, hH2A.Z, hH2B, hH4, and hH3.3 histones are cloned into unknown pET vectors containing the Amp\(^r\) gene.

*Xenopus* histones: H2A, H2B, H3, and H4; and human histone hH2A.Z were gifts from Karolin Luger (Colorado State).

*Xenopus* histones: H2A(K119C), H4(S47C), H3(C110A) were generous gifts from Jonathan Widom (Northwestern)

Human histones: hH2A, hH2B, hH3.3, and hH4 were generous gifts from Jeff Parvin (Ohio State University)

Note: Human H4 and *Xenopus* H4 proteins are completely homologous, and thus can be equivalently expressed from either the human or xenopus encoding plasmids. Additionally Human H3.3 and *Xenopus* H3.3 are completely homologous and Human H3.2 and *Xenopus* H3.2 are completely homologous as well. Xenopus like H3, which we colloquially denote as H3, is H3.2(G102A); see alignment below.

hH3.1

\[
\begin{align*}
\text{hH3.1:} & \quad \text{MARTKQTARK STGGKAPRKQ LATKAARKSA P} & \text{TGGVKKPH RYRPGTVALR}
\end{align*}
\]
EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQS SAV M ALQEA CEAY LVGLFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA

**hH3.2** Same as Xenopus laevis H3.2

MARTKQTARK STGGKAPRKQ LATKAARKSA PATGGVKKPH RYRPGTVALR EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQS SAV M ALQEA SEAY LVGLFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA

**hH3.3** Same as Xenopus laevis H3.3

MARTKQTARK STGGKAPRKQ LATKAARKSA PATGGVKKPH RYRPGTVALR EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQS SAV M ALQEA SEAY LVGLFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA

**Xenopus-like H3** recombinant H3.2 with (G102A) mutation, colloquially called H3

MARTKQTARK STGGKAPRKQ LATKAARKSA PATGGVKKPH RYRPGTVALR EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQS SAV M ALQEA SEAY LV A LFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA

All histone mutations have been incorporated by site directed mutagenesis (Stratagene), and therefore are in the same plasmid as its corresponding unmodified histone.

DNA sequencing of the vectors can be performed using the T7P and T7T as the forward and reverse primers, respectively.

**H3-pET3,** *Xenopus laevis-like* (GenBank: AJ556872.1) tctagaata attttgttta actttaagaa ggagatatac cagatgcctg acccagcga cccggttaa atccaccgga gggagaggttc ccgcaagca
getggccacc aaggcagcca ggaagtccgc tcctgctacc ggcggagtca agaaacctca ccgttaccgg ccccggcacag tcgctctccg cgagatccgc cgctaccaga aatccaccga gctgctcatc cgcaaactgc cttttccagcg cctggtccgg gagatcgctc aggacttcaa gaccgacctg cgcttccaga gctcggccgt tatggctctg caggaggcca ggcaggtta tctgtgcteg etetttgagg acaccaacet gtgegeccat caegccaaga ggcagtcaccat catgccaag gacatecage tggcagcag aacacaactg cacagggactt agatecggct gctaataagag cccgaagagc acgtgagttggct gctgccaccg ctcggccgt

MARTKQTARK STGGKAPRKQ LATKAARKSA PATGGVKKPH RYRPGTVALR EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQSSAV MALQEASEAY LVALFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA

Note: N-terminal methionine is edited off during expression; amino acid numbering begins with A designated as residue 1.

**H2A-pET3, Xenopus laevis-like** (GenBank: AJ556870.1) tctagaata attttgttta acttcggcttgct aagaggcaga caagcggaga aacccgcgc taagcgaacatct agaggtctagct ctcggctttc gctacagttc cctgttggcc gtcgctccag gctgttaagg aacaactgtcat ggtgcggct ctcatcttgg cgcttcgctcttctgg gagattcctg ccccatatg cccggggatg gcgccccgta ccccaacaactgt catgctctgga gactgtccga actgtccttc ccacatctac tctcgttctg cccgagcag aacccggag tctcagttgc aacacgagc acggacgct cccgcctgg acgtgcttgct gcgctgcactgctcgagc ggtgtgcctc gcctggtgcagactgtgca actgcggatgc cgggttcgcttgctcgagc acgtcgtcagct ccagcagcag aacccggct tcgtggtcgt cggcggcgc agtgcctggct gctgccaccg ctcggccgt

MSGRGKQGGK TRAKAKTRSS RAGLQFPVGR VHRLLRKGNY AERVGAGAPV YLAAVLEYLT AEILELAGNA ARDNKKTRII PRHLQLAVRN DEELNKLLGR VTI-AQGGVLP NIQSVLLPKK TESSKSASKK

Note: N-terminal methionine is edited off during expression; amino acid numbering begins with S designated as residue 1.
H2B-pET3, *Xenopus laevis*-like (GenBank: AJ556871.1) tctagaaata attttgttta actttaagaa
ggagatatat atggccaagt cctgctcacc cccgataacc ggcatactcgt ccaagggcag
ggaggatatac atgtctgg tcgtgtaaa ggtgtaaag gttctgggttc actgctgtgtg
tcaacgtgt atggtagctga agcaggtgccg cccctgccag cccgataacc ggcatactcgt ccaagggcag
ggaggatatac atgtctgg tcgtgtaaa ggtgtaaag gttctgggttc actgctgtgtg
tcaacgtgt atggtagctga agcaggtgccg cccctgccag cccgataacc ggcatactcgt ccaagggcag
Note: N-terminal methionine is edited off during expression; amino acid numbering begins
with A designated as residue 1.

H4-pET3, *Xenopus laevis*-like (GenBank: AJ556873.1) tctagaaata attttgttta actttaagaa
ggagatatat atatgtctgg tcgtgtaaa ggtgtaaag gttctgggttc actgctgtgtg
tcaacgtgt atggtagctga agcaggtgccg cccctgccag cccgataacc ggcatactcgt ccaagggcag
Note: N-terminal methionine is edited off during expression; amino acid numbering begins
with S designated as residue 1.
**hH2A.1B/E-pET3, human**

```plaintext
TTTGTAAAC
aggggcaaa gcggcggcct agaggagaac ccggccccag gcggcggcct

TTTGTAAAC
aggggcaaa gcggcggcct agaggagaac ccggccccag gcggcggcct
```

**MSGRKQGGK ARAKASKRSS RAGLQFPVGR VHRLRKGNY AERVGAGPVL AAVYSAILE YLTAERLELA GNASKDLKVKG ITPRHLQLQIA IRGDERDELSL IKATIAGGGV PIIIHKLSTL KKGQKTV**

Note: N-terminal methionine is edited off during expression; amino acid numbering begins with S designated as residue 1.

**hH2A.Z-pET3, human**

```plaintext
ATGGCTGGC
GGTACTGAGT

ATGGCTGGC
GGTACTGAGT
```

**MAGGKAGKDS GKAKTKAVSR SQRAGLQFPVG RIHRBLKSR TTSHEGRVGAT AAVYSAILE YLTAERLELA GNASKDLKVKG ITPRHLQLQIA IRGDERDELSL IKATIAGGGV PIIIHKLSTL KKGQKTV**

272
Note: N-terminal methionine is edited off during expression; amino acid numbering begins with A designated as residue 1.

hH2B.1O-pET3, human
ttagaataat tttgttaac ttaagaagg agatatatac atgcctgaac
cagctaagtctc agctctgtgct ccaagaagg gttcaagaa ggccttgacc aaggegcaga agaaggatgg
cagaagcgc aaggecagtc gtaaggagag ctaacctgctg tataagtatac aggtgctaaa acaggttcac
cccgatactg gcatctcact caaggccatg ggcatcatga attctctgtg taacgacatc ttgcaacgca
tgcagggca ggccttgcgt ctggccceact caaccaagcgt ctcgaccatt acctccaggag acatcagac
cgctgtccgct ctcgactctt ccggagagct ggccaagcac gcagtgtccg aaggtaccaa ggctgtcacc
aagttataaca gctcagagta aagatccgaa ttcgagctcc gtcgacaagc ttgcggccgc actcgagcac caccacc
acc accactgaga tccggctg

Note: N-terminal methionine is edited off during expression; amino acid numbering begins with P designated as residue 1.

hH3.3-pET3, human
ttagaataat ttaacttttaa gaaggagata tataatggc ccaagaaggagg cagactgttc
tgaagttccac gccttgagaa gcccceccga aacagctgge cagaaagcc gcccagaaaa ggcctccctc
tactccgggg gtaggaagage ctccagctga cagccggggg cgcgtggtgc ttgcaagatg tgctcttat
cagaagtcga cccgagcct gtcgactcctt cttgaggttg gtaggaagaatgc ggcggaggatt
tctaaacccga cctgaggttt cagacggcag gtcgaggtgc gctgagggag ctgactctggtt
gggctgttc gaagatacct cactgttggt gcctcaagctc aagagactc cccagctgcc ccaagaccctc
cagttggctc gcgcggtacgc gggagagaga gcttacagat ccaattcgc gcctgctgca aagttggcgc
ggcgttgctc gcgcggtacgc gggagagaga gcttacagat ccaattcgc gcctgctgca aagttggcgc

ggcgttgctc gcgcggtacgc gggagagaga gcttacagat ccaattcgc gcctgctgca aagttggcgc

cgcctgctgca aagttggcgc

273
MARTKQTARK STGGKAPRKQ LATKAARKSA PSTGGVKKPH RYRPGTVALR EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQSAAI GALQEASEAY LVGLFEDTNL CAHHAKRTI MPKDIQLARR IRGERA

Note: N-terminal methionine is edited off during expression; amino acid numbering begins with A designated as residue 1.

hH4.1A-pET3, human atgtccggca gaggaaaggg cggaagaagc tttaggcaaag ggggcgc-taa gcgccacgcc aaggtcttga gagacaacat tcagggcatc accaagcctg ccattcggcg tctagcgcgg cgtggcggcg ttaagcggat ctctggcctc atttacgagg agacccgagg tgtgtcgaag gtgttcctgg agaatgtgat tcgggacgca gtcacctaca ccgacgaca cagagcgcag acgcagacag ccatggtatgt ggtgtacgcg ctcaacgcc cgggaagcgtc ctcgggaaaggt ctggaagctg ag

MSGRGKKGGK LGKGAKHR HRKVLRDIQGI TKPAIRRLAR RGGVKRISGL IY-EETRGVLK VFLENVIRDA VTYTEHAKRK TVTAMDVVYA LKRQGRFLYG FGG

Note: N-terminal methionine is edited off during expression; amino acid numbering begins with S designated as residue 1.
A.3 Histone molecular weights and extinction coefficients

Molecular Weights for Full-Length Recombinant *Xenopus laevis* and human histones are bases off of the amino acid sequence obtained for each histone in the above section. Extinction coefficients are from [79, 107].

<table>
<thead>
<tr>
<th>Histone</th>
<th>Avg. MW</th>
<th>MonoIso MW</th>
<th>$e$ (cm$^{-1}$ M$^{-1}$)</th>
<th>$e$ (cm$^{-1}$ mg/ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>15270.8</td>
<td>15261.5</td>
<td>4040</td>
<td>0.2646</td>
</tr>
<tr>
<td>H3(C110A)</td>
<td>15238.7</td>
<td>15229.4</td>
<td>4040</td>
<td>0.2646</td>
</tr>
<tr>
<td>H2A</td>
<td>13950.1</td>
<td>13941.8</td>
<td>4050</td>
<td>0.2903</td>
</tr>
<tr>
<td>H2B</td>
<td>13493.6</td>
<td>13485.4</td>
<td>6070</td>
<td>0.4498</td>
</tr>
<tr>
<td>H4</td>
<td>11236.1</td>
<td>11229.3</td>
<td>5400</td>
<td>0.4806</td>
</tr>
<tr>
<td>hH3.2</td>
<td>15270.8</td>
<td>15261.5</td>
<td>4040</td>
<td>0.2646</td>
</tr>
<tr>
<td>hH3.3</td>
<td>15196.7</td>
<td>15187.4</td>
<td>4040</td>
<td>0.2658</td>
</tr>
<tr>
<td>hH3.3(C110A)</td>
<td>15164.6</td>
<td>15155.5</td>
<td>4040</td>
<td>0.2664</td>
</tr>
<tr>
<td>hH2A</td>
<td>13974.2</td>
<td>13965.9</td>
<td>4050</td>
<td>0.2898</td>
</tr>
<tr>
<td>hH2B</td>
<td>13774.9</td>
<td>13766.5</td>
<td>6070</td>
<td>0.4407</td>
</tr>
<tr>
<td>hH4</td>
<td>11236.1</td>
<td>11229.3</td>
<td>5400</td>
<td>0.4806</td>
</tr>
<tr>
<td>hH2A.Z</td>
<td>13421.5</td>
<td>13413.5</td>
<td>2700</td>
<td>0.2012</td>
</tr>
</tbody>
</table>

Table A.1: Histone molecular weight and extinction coefficients. Average molecular weight is the expected weight by mass spectrometry due to the average natural abundance of isotopes. MonoIso molecular weight is the calculated molecular weight if one were to add up the molecular weight of each residue using the most abundant isotope molecular weight.
A.4 Transformation of recombinant histone expression vectors

A.4.1 Materials

- LB + agarose culture plates with 50µg/ml ampicillin (See Appendix H)
- LB + agarose culture plates with 50µg/ml ampicillin + 34µg/ml chloramphenicol (See Appendix H)
- Culture Tubes containing 5ml 2xYT growth media, autoclaved (See Appendix H)
- 50mg/ml ampicillin stock (See Appendix H)
- 34mg/ml chloramphenicol stock (See Appendix: Buffers and Materials)
- DH5a competent bacteria (or equivalent for plasmid storage)
- BL21(DE3)pLysS expression host (Noavgen cat no. 69451 or Sigma-Aldrich cat no. B3310)
- 80% glycerol in dH2O, autoclaved

A.4.2 Expression Plasmid purification and DH5a glycerol stocks

1. Transform desired recombinant histone plasmid into DH5a-type competent cells by standard procedures:

* Thaw aliquot of competent cells on ice for 5 minutes; gently flick to redistribute.
* add 1ng of plasmid; gently flick to mix; incubate on ice for 5 minutes.
* heat shock cells at 42°C for 90 seconds - home made calcium competent cells; 60 seconds - home made ultracompetent cells; 45 seconds - XL1 blue or XL10 gold on heat block with water in each well; return cells to ice.
• Incubate cells on ice for 5 minutes. Open bottle of S.O.C. media (Invitrogen-Life Technologies) under bunsen burner flame; add 450µl of S.O.C. to cells by pipetting media down side of tube to mix will cells; invert to mix.

• Incubate cells at 37°C on New Brunswick shaker table at 200RPM for 1 hour.

• Plate 200µl onto LB + 50µg/ml ampicillin agarose culture plates and incubate at 37°C for 16 hours in incubator.

2. Inoculate single colonies from transformation into 5ml LB + 50µg/ml ampicillin media; shake at 200RPM, 37°C until the optical density measures > 0.8 at 600nm, 1cm pathlength (OD$_{600nm,1cm}$).

3. Mix 2 parts culture to 1 part 80% glycerol to make a 20% glycerol stock; immediately flash freeze in liquid nitrogen and store at -80°C.

4. Harvest cells and extract plasmid by standard Mini-Prep (Qiagen) procedures

A.4.3 Expression host [BL21(DE3)pLysS] Transformation

1. Transform desired recombinant histone plasmid into BL21(DE3)pLysS expression host (Noavgen cat no. 69451 or Sigma-Aldrich cat no. B3310) following manufacturer protocol.

2. Plate cells onto LB + 50µg/ml ampicillin + 34µg/ml chloramphenicol agarose culture plates and incubate at 37°C for 16 hours. Wrap plates with parafilm and store at 4°C until ready for Induction Trial.
A.5 Induction trial and optimization

In this section it will be determined which transformed BL21(DE3)pLysS clones express the highest level of histone, and if desired, which parameters optimize yield.

A.5.1 Materials

- Culture Tubes containing 5ml 2xYT growth media, autoclaved (See Appendix H)
- 50mg/ml ampicillin stock (See Appendix H)
- 34mg/ml chloramphenicol stock (See Appendix H)
- 100mM IPTG (Isopropyl B-D-1-thiogalactopyranoside, USB 17886) Dissolve in water and syringe filtered; store at -20°C
- 80% glycerol in dH2O, autoclaved
- 16% SDS-PAGE gel (See Appendix E)
- 1x Tris-Glycine-SDS buffer (See Appendix E)
- 6x SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- 0.5x TE (See Appendix H)

A.5.2 First induction and BL21(DE3)pLysS glycerol stock

1. Inoculate 3-5 single transformed BL21(DE3)pLysS colonies into separate tubes of 5ml 2xYT + 50µg/ml ampicillin + 34µg/ml chloramphenicol and shake at 280RPM, 37°C until the optical density of a given clone measures 0.2 at 600nm, 1cm pathlength (OD_{600nm,1cm}).

2. Mix 2 parts culture for a given clone to 1 part 80% glycerol to make a 25% glycerol
stock; immediately flash freeze in liquid nitrogen and store at -80°C.

3. Allow remaining culture for a given clone to continue to shake at 280RPM, 37°C until OD$_{600nm,1cm}$ = 0.5.

4. Transfer 50µl of culture to a microcentrifuge tube on ice ("0 hour timepoint").

5. Induce remaining culture with 10µl of 100mM IPTG to 0.2mM final concentration; allow induced culture to continue to shake at 280RPM, 37°C.

6. At 1, 2, 3 and 4 hours post induction, transfer 50µl of culture to a microcentrifuge tube on ice for each clone ("1,2,3,4 hour timepoints").

7. When each clone has completed its 4 hours of induction, discard remaining culture.

8. Centrifuge each time point tube (0,1,2,3,4 hours) at 3000g for 2 minutes. Remove supernatant and resuspend pellet in 10µl of 0.5x TE; add 10µl of 6x SDS loading dye; boil at 95°C for 5 minutes; centrifuge at 5000g for 10 seconds; return tubes to ice or store in refrigerator.

A.5.3 SDS-PAGE analysis of expression

1. Pour a 16% SDS-page gel. Assemble gel into BioRad Mini-Protean Gel Rig and fill both inner and outer chamber with 1x Tris-Glycine-SDS; purge wells. Load 10µl of each sample onto gel and run at 20mA per gel while loading dye is in the stacking gel; increase current to 30mA per gel while loading dye is in the resolving gel. Stop current when loading dye just runs out of bottom of the gel.

2. Disassemble gel rig and remove gel from glass plates; place gel in tip box with gel stain. Microwave 15 seconds then swirl box to wash stain over gel; repeat microwave and swirl
two more times; allow to cool for 10-15 minutes on shaker table. Move gel to 6x9 pyrex dish with destain; destain ~20 minutes on shaker table to expose protein bands.

3. Based on the SDS-Page results, determine which clones expressed and at what level with respect to background proteome (Figure A.1).

![Histone expression induction trial](image)

Figure A.1: **Histone expression induction trial.** 16% SDS-PAGE gel of trial induction. Clone 2 has the highest expressed H3 to background proteome ratio, so it was chosen as the best clone for bulk expression, with 4 hours being the required time. The HO lane contains 1µg of histone octamer.

### A.5.4 Optimizing induction parameters

The optical density and the concentration of IPTG at which the expression culture is induced can influence expression levels and can be optimized if desired. Typically induction at $\text{OD}_{600nm,1cm} = 0.5$ with 0.2mM IPTG final concentration works well and produces 20-30mg histone per liter of starting culture for H3, H2A and H2B, and 2-4mg H4 per liter of starting culture. While this normally is sufficient for H3, H2A, and H2B, it is generally of interest to optimize conditions for H4. We find that inducing H4 at $\text{OD}_{600nm,1cm} = 0.5$ with 0.5mM IPTG and shaking at 300RPM, 37°C produces ~8 mg H4 per liter of starting
culture. We also find that expressing H4 in single volumes greater than 500ml growth media reduces yield.

Perform all of the following under the bunsen burner flame using sterile techniques:

1. Prewarm as many tubes of 5ml 2xYT to 37°C as desired for the number of combinations to be tested. Typically one may consider 12 conditions to test induction: \( \text{OD}_{600nm,1cm} = 0.4, 0.5, 0.6, 0.8 \) in combination with IPTG final concentration at 0.2mm, 0.5mm, 0.8mm

2. To begin, inoculate 5ml 2xYT + 50µg/ml ampicillin + 34µg/ml chloramphenicol with BL21(DE3)pLysS glycerol stock with desired histone expression vector chosen from Induction Trial above. Shake at 300RPM, 37°C until the OD600nm,1cm = 0.2

3. To each tube add antibiotics to a final concentration of 50µg/ml ampicillin + 34µg/ml chloramphenicol and then add 200µl of culture at OD600nm,1cm = 0.2 to each tube. Reserve 50µl of culture in a microcentrifuge tube on ice ("0 hour timepoint").

4. Shake at 300RPM, 37°C until the optical density of a given tube reaches the desired level for induction; induce with the desired amount of IPTG. Continue to shake at 300RPM, 37°C.

5. At 2, 3, and 4 hours post induction for each sample, transfer 50µl of culture to a microcentrifuge tube on ice. When each sample has completed its 4 hours of expression, discard remaining culture.

6. Follow the procedure from Induction Trial above to prepare gel samples and perform SDS-PAGE analysis.

7. Based on the SDS-PAGE results, choose the induction optical density, IPTG concentra-
tion, and expression time that optimizes yield.

A.5.5 Notes

1. To take effective optical density measurements, transfer 50-100µl of culture to a microcentrifuge tube. Blank spectrophotometer with 2xYT + 50µg/ml ampicillin + 34µg/ml chloramphenicol. If using a 1cm cuvette, dilute sample by 10-fold in microcentrifuge tube, vortex briefly, immediately transfer to cuvette and spec, taking dilution factor into account. If using a Nanodrop™ with 1mm pathlength, briefly vortex undiluted sample, immediately apply to instrument and spec, taking pathlength into account.

2. For quick and efficient staining of SDS-PAGE gel, place gel in a microwaveable container and add enough stain to cover by 3-5mm. Microwave gel on medium power for 15 seconds with the container lid cocked. Remove the container and swirl to move stain over the gel. Perform the heating and swirling procedure two more times. Place container with gel and stain on a benchtop or shaker table for 10 minutes until cool.

3. For quick and efficient destaining, transfer gel from staining container to a fresh container and cover gel with destain to about 1/4 inch depth. Place a paper towel or Kimwipe in the container to help with dye absorption. Place container with gel, destain, and paper towel on the benchtop or shaker table for 30-60 minutes until protein bands are exposed.
A.6 Histone Bulk Expression and Harvest

A.6.1 Materials

- Culture Tubes containing 5ml 2xYT growth media, autoclaved (See Appendix H)
- 250ml Erlenmeyer flask containing 60ml 2xYT growth media, autoclaved (See Appendix H)
- 2L Erlenmeyer flasks containing 500ml 2xYT growth media OR 4L Erlenmeyer flasks containing 1L 2xYT growth media, autoclaved (See Appendix H)
- 50mg/ml ampicillin stock (See Appendix: Buffers and Materials)
- 34mg/ml chloramphenicol stock (See Appendix: Buffers and Materials)
- 1M IPTG stock (Isopropyl B-D-1-thiogalactopyranoside, USB 17886) Dissolve in water and syringe filtered; store at -20°C
- Wash Buffer at room temperature (See Appendix H)
- 16% SDS-PAGE gel (See Appendix E)
- 1x Tris-Glycine-SDS buffer (See Appendix E)
- 6x SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- 0.5x TE (See Appendix H)

A.6.2 Expression

During any part of the expression, the cell OD_{600,1cm} should not go above the OD at which the cells will be induced (typically 0.5). At this point cells start to become sick and eject their plasmid, resulting in low yield of expression. Therefore there are two options in growing up the cells overnight and still ensuring that the cells do not overgrow:
Serial Dilution Method, ”The fail safe” (Poirier Lab)

Perform the following under bunsen burner flame using sterile techniques:

1. Begin by inoculating 5ml 2xYT + 50µg/ml ampicillin + 34µg/ml chloramphenicol culture media from the desired BL21(DE3)pLysS glycerol stock.

2. Allow the cells to shake at 280-300RPM, 37°C for 20-30 minutes in order to equilibrate and start producing their antibiotic resistance.

3. Perform a serial dilution of the cells by transferring 100-500µl of the cells from the first 5ml of culture tube to a second tube of 2xYT + 50µg/ml ampicillin + 34µg/ml chloramphenicol culture media; mix well by swirling. Repeat by transferring 100-500µl of cells form the second 5ml culture media to a third tube of 2xYT + 50µg/ml ampicillin + 34µg/ml chloramphenicol culture media; mix well by swirling. Repeat as desired. Typically having 3-4 total tubes in the serial dilution is sufficient such that in the next morning, one of the tubes will visibly have cells but not be overgrown.

4. Shake overnight (12-16hrs) at 280-300RPM, 37°C. Also shake overnight the 250ml flasks of 2xYT without antibiotics to prewarm.

5. In the morning determine which tube has the optimal growth of cells (cells are visible but OD$_{600nm,1cm}$ <0.5). Add 50µg/ml ampicillin + 34µg/ml chloramphenicol final concentration to the prewarmed 60ml 2xYT in 250mL Erlenmeyer flasks. Inoculate with 1-2ml of the cells. Continue to shake at 280-300RPM, 37°C. Also, place the 2L and/or 4L flasks with 2xYT without antibiotics onto the shaker table to prewarm.

5. When OD$_{600nm,1cm}$ is 0.2-0.5 in the 250ml flask add antibiotics to a final concentration of 50µg/ml ampicillin + 34µg/ml chloramphenicol to each of the prewarmed 2L and/or 4L
flasks and divide the cell culture among them. For optimal growth it is advised at this point not to dilute cells (especially those containing H4 plasmid) by more than 50-fold into the new flask.

6. Continue to shake at 280-300RPM, 37°C until the optical density of a given culture reaches the desired level determined from the Induction Optimization performed above (default is $\text{OD}_{600nm,1cm} = 0.5$). Transfer 50µl of the culture to a microcentrifuge tube on ice ("pre-induction timepoint"; the sample used to determine the OD can be used). Induce with the desired amount of IPTG determined from the Induction Optimization performed above (default is 0.2mM IPTG final concentration).

7. Continue to shake at 280-300RPM, 37°C for the amount of time determined from the Induction Optimization performed above (typically 4 hours). Proceed immediately to Harvest

**Erlenmeyer Flask Method (Most Lab’s Standard Method)**

Perform the following under bunsen burner flame using sterile techniques:

1. Begin by inoculating 60ml 2xYT + 50µg/ml ampicillin + 34µg/ml chloramphenicol culture media in a 250mL Erlenmeyer flask from the desired BL21(DE3)pLysS glycerol stock.

2. Shake overnight (~10hrs) at 280-300RPM, 37°C. Also shake overnight the 2L and/or 4L flasks of 2xYT without antibiotics to prewarm.

3. In the morning, check the $\text{OD}_{600nm,1cm}$ of the cells in the 250ml flask. If $\text{OD}_{600nm,1cm}$ is higher than the level at which the cells will be induced (typically 0.5) then abort the expression and start over. Otherwise, when $\text{OD}_{600nm,1cm}$ is 0.2-0.5 in the 250ml flask add antibiotics to a final concentration of 50µg/ml ampicillin + 34µg/ml chloramphenicol to each of the prewarmed 2L and/or 4L flasks and divide the cell culture amongst them. For
optimal growth it is advised at this point not to dilute cells (especially those containing H4 plasmid) by more than 50-fold into the new flask.

4. Continue to shake at 280-300RPM, 37°C until the optical density of a given culture reaches the desired level determined from the Induction Optimization performed above (default is OD$_{600nm,1cm} = 0.5$). Transfer 50µl of the culture to a microcentrifuge tube on ice (“pre-induction timepoint”; the sample used to determine the OD can be used). Induce with the desired amount of IPTG determined from the Induction Optimization performed above (default is 0.2mM IPTG final concentration).

5. Continue to shake at 280-300RPM, 37°C for the amount of time determined from the Induction Optimization performed above (typically 4 hours). Proceed immediately to harvest.

**A.6.3 Harvest**

1. Place Beckman JLA 10.5 rotor into Avanti centrifuge and set centrifuge to 20°C.

2. When each flask has completed the desired time for expression, transfer 50µl of the culture to a microcentrifuge tube on ice (“post-induction timepoint”).

3. Fill Beckman 500ml plastic bottles up to shoulder with cells. Reserve any cells that cannot fit into bottles. Cap and place bottles in JLA 10.5 rotor. Centrifuge cells at 4000g, 10 minutes at room temperature. It may be prudent to keep each flask worth of cells (500ml from 2L flask or 1L from 4L flask) separated until expression is verified. However, it is rare that expressions levels in different flasks vary significantly when started from the same initial starting culture.

4. If supernatant is not translucent brown, repeat spin. If supernatant is translucent, decant off supernatant. If there is remaining un-centrifuged cell culture from step 3, pour
culture in bottles on top of current cell pellet. Cap and place bottles in JLA 10.5 rotor with current pellet positioned near the spindle. Centrifuge cells at 4000g, 10 minutes at room temperature.

5. After all cells have been pelleted and supernatant has been decanted off, turn bottles upside down on a paper towel for 5 minutes to drain remaining growth media.

6. Resuspend cells to 12-15ml total volume in Wash Buffer at room temperature per each liter of starting culture. Transfer to 50ml tubes. Flash freeze in liquid nitrogen and store at -80°C.

7. For SDS-PAGE analysis, centrifuge each pre- and post-induction timepoint at 3000g for 2 minutes. Remove supernatant and resuspend pellet in 10µl of 0.5x TE; add 10µl of 6x SDS loading dye; boil at 95°C for 5 minutes; centrifuge at 5000g for 10 seconds; return tubes to ice or store in refrigerator.

8. Follow the procedure in section on SDS-PAGE analysis to verify histone expression.

A.6.4 Notes

1. BL21(DE3)pLysS containing H3, H2A, or H2B grow well in 500mL 2xYT + 50µg/ml ampicillin + 34µg/ml chloramphenicol culture media in a 2L Erlenmeyer flask or 1L media in a 4L flask.

2. BL21(DE3)pLysS containing H4, hH2A.Z and any of their mutant strains grow optimally in a 500ml media in a 2L flask. Significant reduction in yield can be seen using 1L media in a 4L flask.

3. When resuspending cells in Wash Buffer, it may be prudent to keep each flask worth of cell pellet separate until the pre- and post-induction samples are analyzed by SDS-PAGE to verify expression. However, it is rare that expressions levels in different flasks
vary significantly when started from the same initial starting culture. Also, for subsequent purification steps, it is rather easy to work in terms of 1L worth of cell pellet per 50ml falcon tube.

4. For thorough and efficient suspension of cell pellet:

- Start with the first centrifuge bottle of cell pellet: add \( \sim 4 \text{ml} \) of Wash Buffer at room temperature.

- Use a 10ml serological pipette on a motorized pipette aid to continually wash over pellet until all of the pellet is dissolved. If pellet is split between several different centrifuge bottles, transfer resuspended cells to the next centrifuge bottle and continue resuspension process.

- Move resuspended pellet to a 50ml tube. Use \( \sim 3 \text{ml} \) of fresh Wash Buffer to clean out the centrifuge bottles of residual cells.

- Bring total volume of cells in 50ml tube up to 12-15ml with Wash Buffer.
A.7 Inclusion body isolation

Typically, preparing the inclusion bodies is the most difficult and imprecise section in the purification process. The most important step is shearing of the genomic DNA by sonication. If the DNA is not sheared enough, then clogging of the gel filtration column and loss of resolution (very broad DNA and protein peaks) in the subsequent section may result. Likewise, if the DNA is over-sheared then there will be poor separation of DNA from histones (tight but overlapping DNA and protein peaks). Subsequent dialysis of histones contaminated with large amounts of DNA will result in histone binding to the DNA and aggregating out. This is particularly problematic for H4, which has the lowest yields and highest isoelectric point.

A.7.1 Materials

- Wash Buffer, refrigerated (See Appendix H)
- TW Buffer, refrigerated (See Appendix H)
- Bronson sonicator microtip

A.7.2 Cell Lysis

1. Set water bath to 37°C.

2. Remove frozen harvest cell suspension from -80°C freezer and place in a 37°C water bath. Continually rotate and invert to promote even thawing. Cells will begin to lyse and viscosity will increase.

3. Re-freeze cells at -80°C for ~30 minutes (do not flash freeze in liquid nitrogen). Re-thaw cells as before.

4. Repeat this freeze/thaw cycle 1-2 more times until lysate is very viscous and looks like
"snot".

5. If desired, lysates from 500ml worth of starting culture that were kept separate during Section IV until histone expression could be verified can be combined at this point into 1L worth of starting culture pools. For subsequent steps, it is difficult to work with samples from more than 1L worth of starting culture per 50ml tube. If more than 1L was expressed, it is recommended to keep samples divided in terms of 1L worth of starting culture.

**A.7.3 Shearing and sonication**

Perform all of the following on ice:

1. Adjust the volume of the lysate with refrigerated Wash Buffer to 25-30ml.

2. Reduce the viscosity by shearing with a 30ml BD syringe (do not use a needle): Pull up lysate and expel back into the tube. Repeat 4-5 times until the lysate flows smoothly from the syringe (not chunky).

3. Sonicate:

The following is optimized for a QSONICA Sonicator with long micro-tip tip installed (Magliery and Jeronic Labs). Obtain training from respective lab members before using.

- Turn the sonicator controller power on with the switch located at the back of the unit.
- From home menu select: "Are you using a Microtip": YES
- Press "HERE" under "To Select of Modify a Program or Sequence:
- Press "Select/Modify a Program"
- Press "0" to select "Program 0"
• Press black field by "Amplitude" and press "CLR" - "6" - "ENT" to enter an amplitude of 6.

• Press black field by "Process Time" and press "CLR" - "1" - "0" - "0" - "ENT" to enter a Process Time of 1 minute.

• Press black field by "Pulse-ON Time" and press "CLR" - "1" - "5" - "ENT" to enter a Pulse-ON time of 15 seconds.

• Press black field by "Pulse-OFF Time" and press "CLR" - "1" - "0" - "ENT" to enter a Pulse-OFF time of 10 seconds.

• Press "SAVE". This method will sonicate for 15 seconds with ~4W power, pause for 10 seconds; it will repeat this sonicate-pause cycle three more times for a total of 4 cycles with 1 minute total of Pulse-ON time and 40 seconds total of pause time.

• Press "RUN" to go to program start/stop screen for this program "0"

• Install and prewash sonicator probe with ethanol then dH2O.

• Place the 50ml falcon tube with cell lysate in a 250ml beaker filled with ice. Put the beaker on the adjustable stage and raise the beaker with tube until the tip is ~1/2 inch from bottom of the tube.

• Press "START" to begin program. Allow program to complete all 4 Pulse-ON/OFF cycles.

• Use a glass Pasteur pipette to mix the sonicated lysate to break up any long strands.

• Check the viscosity by capping tube and tipping back and forth on its side to monitor flow. Before sonication, when the lysate it tipped back and forth the lysate will appear visco-elastic: the lysate will flow back and forth looking like there is a "clump" in the middle. When the DNA becomes fully sonicated, the lysate will freely flow back and forth: the motion of one section of the lysate will not look like it is affecting the motion of another section. The DNA is optimally sonicated when the lysate just reaches this
stage. Also with tube on its side, gently shake tube to coat. If lysate films span to the top of tube and persist for several seconds then DNA is not optimally sonicated either.

- If the DNA is not optimally sheared, press "START" to start Program "0" again. After 2 Pulse-ON/OFF cycles, press "PAUSE" to pause the program.

- Check the viscosity as detailed above.

- Press "Resume" to continue sonicating 2 more Pulse-ON/OFF cycles if necessary. It normally takes 6-8 Pulse-ON/OFF cycles of sonication to optimally shear the DNA.

- When complete, fully "EXIT" out of program menus to main menu "Are you using a Microtip?". Turn the sonicator controller power off. Clean tip with ethanol and water and remove from sonicator.

The following is optimized for a Branson Sonifier 450 with long micro-tip tip installed (Dongping Zhong Lab). Obtain training from lab members before using.

- Set sonicator to: Output Control = 2.5 or 3, Duty cycle = 50%

- Install and prewash sonicator probe with ethanol then dH2O.

- Place the 50ml falcon tube with cell lysate in a 250ml beaker filled with ice. Put the beaker on the adjustable stage and raise the beaker with tube until the tip is \( \sim \frac{1}{2} \) inch from bottom of the tube.

- Turn the sonicator on; cycle for 30 sec; turn off.

- Use a glass Pasteur pipette to mix the sonicated lysate to break up any long strands.

- Check the viscosity by capping tube and tipping back and forth on its side to monitor flow. Before sonication, when the lysate it tipped back and forth the lysate will appear visco-elastic: the lysate will flow back and forth looking like there is a "clump" in the middle. When the DNA becomes fully sonicated, the lysate will freely flow back and forth: the motion of one section of the lysate will not look like it is affecting the motion.
of another section. The DNA is optimally sonicated when the lysate just reaches this stage. Also with tube on its side, gently shake tube to coat. If lysate films span to top of tube and persist for several seconds then DNA is not optimally sonicated either.

- If the DNA is not optimally sheared, Turn the sonicator on; cycle for 30 sec; turn off.
- Check the viscosity as detailed above.
- Repeat 30 second shearing cycles until DNA is optimally sonicated. It normally takes 4-6 rounds of sonication to optimally shear the DNA.
- When complete, wash the microtip with ethanol and water and remove from sonicator.

**A.7.4 Centrifugation**

1. Place JA-17 rotor into Beckman Avanti centrifuge and equilibrate to 4°C.

2. After sonication, centrifuge for 20 minutes at 4°C, 23000g in 50ml polycarbonate or polypropylene round bottom tubes. For effective separation, the volume of lysate in each tube should be 20-34ml.

3. After centrifugation, the samples should have a cloudy supernatant and a pellet with 2 layers. The top brown layer contains cell debris, the bottom white/cream layer contains the inclusion bodies and genomic DNA.

4. Decant the supernatant and discard. If possible, carefully scrape off the brown layer from the inclusion bodies and discard brown layer. Typically the brown layer can easily be removed from H3, H2B, and H2A inclusion bodies. Typically it cannot be removed from H4 and H2A.Z.

5. Carefully transfer the inclusion bodies to a 15ml conical bottom tube.
A.7.5 Washing inclusion bodies when the brown layer has been removed

1. Place JA-17 rotor into Beckman Avanti centrifuge and equilibrate to 4°C.

2. (TW Buffer 1) Wash the inclusion body pellet by resuspending it in 10ml refrigerated TW Buffer per liter of starting culture. Use a microspatula to macerate the pellet and disperse the inclusion bodies throughout the TW Buffer. The pellet will not resuspend to homogeneity.

3. Centrifuge for 10 minutes at 4°C, 10000g; decant and discard supernatant.

4. (TW Buffer 2) Again wash the inclusion body pellet by resuspending it in 10ml refrigerated TW Buffer per liter of starting culture as before; centrifuge for 10 minutes at 4°C, 10000g; decant and discard supernatant.

5. (Wash Buffer 1) Wash the inclusion body pellet by resuspending it in 10ml refrigerated Wash Buffer per liter of starting culture as before; centrifuge for 10 minutes at 4°C, 10000g; decant and discard supernatant.

6. (Wash Buffer 2) Again wash the inclusion body pellet by resuspending it in 10ml refrigerated Wash Buffer per liter of starting culture as before; centrifuge for 10 minutes at 4°C, 10000g; decant and discard supernatant.

A.7.6 Washing inclusion bodies when the brown layer was NOT removed

1. Place JA-17 rotor into Beckman Avanti centrifuge and equilibrate to 4°C.

2. Perform 4 rounds of TW Wash as described in steps 2 and 3 above.

   - TW Buffer 1 (as above)
   - TW Buffer 2 (as above)
• TW Buffer 3 (as above)

• TW Buffer 4 (as above)

By this point the cell debris should be washed away and the pellet after spinning down should be creamy white without any residual brown layer on top from the cell debris. If not, continue to perform TW Buffer washes until cell debris are gone. Typically 6 washes are the maximum number required.

3. Perform 2 rounds of Wash Buffer as described in step 5 above.

• Wash Buffer 1 (as above)

• Wash Buffer 2 (as above)

A.7.7 Inclusion body storage

1. When TW and Wash Buffer washing of inclusion body pellet is complete, decant and drain the pellet as much as possible.

2. Flash freeze inclusion bodies in liquid nitrogen and store at -80°C. While cell pellets containing expressed histones are stable for only 4-6 months, isolated inclusion body preps are stable for ~2 years.
A.8 Histone unfolding and gel filtration

In this section, genomic DNA will be removed from histone and other cellular proteins by size exclusion chromatography on a gel filtration column.

A.8.1 Materials

- DMSO (Dimethylsulfoxide, Sigma D8418)
- Unfolding Buffer, 100ml refrigerated (See Appendix H)
- SAU1000, 1L-2L filtered and degassed at room temperature (See Appendix H)
- dH2O, 2L-4L degassed at room temperature
- 0.2M NaOH, filtered and degassed at room temperature
- 20% Ethanol, 1L degassed at room temperature
- 2mM BME (2-mercaptoethanol, Sigma M3148) in dH2O, 5 - 2L beakers refrigerated
- 6000-8000MWCO dialysis tubing, boiled (See Notes section below)
- Whatman filter paper (Whatman 3030-866)
- 16% SDS-PAGE gel (See Appendix E)
- 1X Tris-Glycine-SDS buffer (See Appendix E)
- 6X SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- GE Healthcare HiPrep 26/60 Sephacryl S-200 High Resolution column for 1L starting culture worth of inclusion bodies (Cat no. 17-1195-01), OR
GE XK 26/70 column hand packed with Sephacryl S-200 High Resolution media for 1L-2L starting culture worth of inclusion bodies. For larger preps, larger columns can be used.

A.8.2 Unfold histones

1. Remove 15ml tube of isolate inclusion bodies from from -80°C freezer; thaw on ice.

2. Add 160µl of DMSO per 1L of starting culture. Use microspatula to macerate the pellet. Allow to incubate in DMSO for 30-60 minutes on ice.

3. Add 6ml of refrigerated Unfolding Buffer per 1L of starting culture. Use a microspatula to macerate and mix the pellet.

4. Transfer the entire volume to a 50ml tube with a small stirbar and adjust volume to 15-20ml with refrigerated Unfolding Buffer. If there is a considerable amount of inclusion bodies clinging to the side of the 15ml tube, it can be washed out with the Unfolding Buffer used to bring the volume up to 20ml.

5. Mix on a stirplate at room temp for at least 1 hour or overnight at 4°C. Monitor the consistency of the unfolding inclusion bodies. If the suspension becomes so viscous due to DNA that the stir bar does not actively mix sample, add 20%-50% more in volume of Unfolding Buffer until the viscosity is appropriately reduced. If possible keep the total volume less than or equal to 5-8% of the total volume of the gel filtration column to which the histones will be applied. If the sample volume is significantly above this, it is wise to split the sample into appropriate fractions and perform multiple gel filtration runs to ensure proper resolution between DNA and histones.

A.8.3 Centrifuge histones

1. Place JA17 rotor in Beckman Avanti centrifuge and equilibrate to 4°C.
2. Transfer all volume to a 50ml round bottom polycarbonate or polypropylene tube. Centrifuge at 4°C, 32000g for 20 minutes.

3. Observe the clarity of the supernatant:
   - If supernatant is a light transparent yellow to slightly translucent, carefully decant the supernatant to a fresh 15ml or 50ml tube.
   - If the supernatant is visibly cloudy with strong gradients in density due to suspended debris, centrifuge again at 4°C, 32000g for 30-60 minutes longer.
   - If centrifugation at 32000g is unsuccessful, which is rare, sample can be spun on a Beckman TI-45 swinging bucket type ultracentrifuge rotor at 4°C, 41000RPM for 20-30 minutes.

A.8.4 Gel filtration column equilibration, loading, and elution

While the histones are unfolding and centrifuging, equilibrate the Gel Filtration column. Do all of the following at room temperature to prevent running buffer precipitation.

1. Sephacryl 200 column is always stored in 20% Ethanol. Prime P1 pump with degassed dH2O. With pump running at ~0.5ml/min, connect P1 pump outlet to column inlet. Immediately open column outlet to prevent pressure buildup.

2. Wash column with 2 column volumes (CV) of degassed dH2O at 2ml/min.

3. Equilibrate column with 1CV of SAU1000 buffer at 2ml/min.

4. To apply sample, stop flow to column and close column outlet. Disconnect column inlet from P1 pump and place inlet in 50ml tube with sample. Open column outlet and connect to P1 pump outlet. Turn on pump at 1ml/min and reverse pump direction so that buffer is being pulled from the bottom of the column to ”suck” sample onto column. Remove pump
inlet line from bottle of SAU1000 and allow to drip into waste to prevent contamination of SAU1000 buffer stock.

5. When sample is nearly loaded, stop pump. Replace pump inlet line to bottle of SAU1000. Disconnect column outlet from pump and close outlet. Reconnect column inlet to pump outlet. Open pump outlet and connect pump outlet to fraction collector.

6. Elute sample at 1.3ml/min with SAU1000, collecting 5ml fractions across 1CV. Typically DNA and remaining debris will elute around \( \sim \)1/3 CV and the sample elutes around \( \sim \)1/2 CV.

7. When done, wash column with 2CV of degassed dH2O. If necessary clean column with 1CV of 0.2M NaOH followed by 2CV of degassed dH2O. Store column by washing with 2CV of 20% EtOH.

A.8.5 Fraction quantitation

1. Measure the optical density of each fraction in the range from 220nm - 350nm (Figure A.2A). If separation between the DNA and protein is good, the OD signal should have a peak at 260nm for fractions eluted at \( \sim \)1/3 CV corresponding to DNA and then the OD signal should transition to a peak at 276nm for fractions eluted at \( \sim \)1/2 CV corresponding to protein. However, depending on resolution of the column and the amount of DNA loaded onto the column, a small amount of DNA (< 10%) may cross-contaminate the protein fractions and mask the protein signal at 280nm. If this is the case, one can at least get an idea of where the bulk of the DNA eluted. Additionally a small amount of contaminating DNA will not adversely affect subsequent steps. Only if a majority of the DNA is still present will histones tend to aggregate onto the DNA during dialysis.

2. For better resolution of which fractions contain protein, apply 30-50\( \mu \)l of each fraction onto Whatman filter paper either by directly blotting onto a 2cm x 2cm grid or by using
a vacuum manifold blotter. Microwave for 15 seconds to dry. Briefly stain with gel stain ~30 seconds (old stain can be used); destain for ~30 minutes in fresh destain. Spots with the darkest intensity typically correspond to eluted histones. Faint spots at fractions before eluted histones typically are high molecular weigh proteins from the proteome. Faint to moderate spots at fractions after eluted histones typically are low molecular weight proteins from the proteome.

3. For the best resolution of which fractions contain protein, combine 2μl of 6x SDS loading buffer to 10μl of each sample. Boil for 5 minutes at 95°C. Apply to 16% SDS-PAGE gel as described in above Section on SDS-PAGE analysis (Figure A.2).

Figure A.2: Histone gel filtration. A UV-Vis spectra of gel filtration fractions for H2A. Note how fractions 28-30 contain primarily DNA while fractions 38-44 contain primarily protein. The transition from DNA to protein occurs from fractions 32-35 (not shown). B 16% SDS-PAGE of gel filtration fractions for H2A. The HO lane contains 1μg of histone octamer. Fractions 31-35 overlap with the tail of the eluted DNA peak, and were thus pooled separately from fractions 36-46 to minimize DNA contamination.

A.8.6 Pool fractions and dialyze

1. Separate gel filtration fractions containing histone protein into two separate pools:
(A) Protein + DNA and (B) Protein only based upon the above quantitation.

2. Dialyze each pool in 6000-8000 MWCO dialysis tubing (See Note 1 below) against at least 5 - 2L changes of water + 2mM BME at 4°C for at least 6 hours per change, with the last one going overnight. During the dialysis process, DNA will typically aggregate out and the sample may become cloudy. This will not adversely affect the yield unless the DNA is a significant fraction of the sample (> 10-20%). This may or may not be the case for Pool A, which should have significantly less protein than Pool (B) anyway.

3. When dialysis is complete, transfer each pool to a 50ml polycarbonate or polypropylene round bottom centrifuge tube and centrifuge at 4°C, 30000g for 20 minutes to pellet out aggregates.

4. Carefully remove supernatant from DNA pellet. At this point supernatant from all pools can be combined if desired or kept separate.

5. Transfer supernatant to 50ml falcon tubes; flash freeze with liquid nitrogen; lyophilize. Store at -20°C after lyophilization.

A.8.7 Column cleaning and storage

1. After sample has eluted, wash column with 1CV of SAU1000. This is the best way to remove protein.

2. Wash column with 1-2CV of degassed dH2O to remove SAU1000

3. Wash column with 1CV of filtered and degassed 0.2M NaOH. This will remove DNA and other debris.

4. Wash column with 1-2CV of degassed dH2O
5. To store column, wash column with 1CV of degassed 20% EtOH to prevent bacterial growth.

A.8.8 Notes

1. To remove trace metals and other substances from manufacturing process, dialysis tubing should be boiled at a medium boil for 15 minutes in 1mM EDTA pH 8.0; decant off buffer. Repeat boiling and decanting procedure 2 more times. Cover dialysis tubing for a 4th time with 1mM EDTA and bring to medium boil; remove from heat; cover. Dialysis tubing can be stored up to 6 months covered in a refrigerator. Wash dialysis tubing thoroughly with dH2O before use.

2. If large pieces of DNA are unsuccessfully spun out of solution before loading unfolded histones onto the gel filtration column, DNA and other debris may become lodged in the first few millimeters of the column and may compress the media by 1-2cm. If this happens, during the 0.2M NaOH cleaning step reverse the flow through the column and slowly pump 1CV of 0.2M NaOH (∼0.5 ml/min) through the column. Typically this will resolve the issue. Reverse the flow back to normal direction and pump at least 2CV of degassed dH2O through the column to recondition.

3. If air bubbles get lodged within the top 10-15cm of a 26/60 or larger gel filtration column, during the dH2O wash step reverse the flow through the column and slowly pump 1-2CV of degassed dH2O (∼0.5 ml/min) through the column. Reverse the flow back to normal and pump at least 2CV of degassed dH2O through the column to recondition.

4. If a column gets pumped dry, the Sephacryl 200 High Resolution (S200HR) media can be removed from the column, washed well with degassed 20% ethanol and repacked in degassed 20% ethanol (See note 6).

5. If there is suspect of bacteria growth in the column, the S200HR media can be removed,
washed well with water and autoclaved with a standard 20 minute wet cycle. Afterward, wash the media well with degassed 20% ethanol and repack the column (See note 6).

6. To repack a column:

- Disassemble column following manufacturer instructions and thoroughly wash all components; allow to dry.
- Wash the S200HR media thoroughly by resuspending the media in 3 volumes of degassed 20% ethanol using a glass or plastic stir rod and allowing the media to settle completely (∼1 hour). Decant the ethanol supernatant and repeat wash cycle 2 more times.
- After media has settled the 3rd time, adjust ethanol volume above media to 20% of media volume.
- Reassemble the column and either attach a packing adaptor sold by the manufacturer or attach an additional piece of tubing to the top of the column that is the same diameter as the column and at least 1/3 of the length. To attach the extension tube, wrap from 3 inches below the joint to 3 inches above the joint between the column and extension tube with Teflon tape to prevent contaminants from entering through the joint. Next do the same with electrical tape to seal the joint. Finally do the same with masking or duct tape to prevent the joint from expanding during packing.
- Resuspend the media to uniform consistency with a glass or plastic stir rod. With the outlet of the column closed, immediately pour ALL of the media at a steady rate down the inside of the column wall to avoid air from becoming entrained in the media. Allow the media to begin to settle. *** Do NOT top off with more media as the media settles. This will cause uneven packing of the media and result in poor resolution***
- After the media has settled for ∼15-20 minutes, connect the column inlet to the top of the extension tube. Open the column outlet and begin pumping degassed 20% ethanol through the column at a low flow rate (∼0.5 ml/min). *** Do not pump on
the column via the outlet. This will cause uneven packing and loss of resolution. The column must be packed by flowing buffer in the forward direction.

- Slowly increase the flow rate to 4 times the expected operating flow rate (The GE 26mm I.D. columns with S200HR media run at 1.3 ml/min for purification, therefore it is packed at 5 ml/min).

- Allow the media to pack at the above flow rate until no further settling is observed. At this point the media is almost optimally packed.

- Slowly increase the flow rate to 5.5 times the expected operating flow rate (The GE 26mm I.D. columns with S200HR media run at 1.3 ml/min for purification, therefore it is packed at 7 ml/min). During this step the media closer to the inlet will become slightly denser than the medial toward the outlet, increasing purification resolution. Carefully monitor the settling of the media to ensure that it accidentally does not become compressed. If this occurs a spike in the pressure on the column and a rapid increase in the settling of the bed is observed. If this happens immediately return the flow rate to 4 times the expected operating rate then gradually bring the flow down to 0.5 ml/min. During this time the medial will relax and the bed height will come back up; go to the next step. If the media does not accidentally compress, wait until no further settling in the media is observed; bring the flow rate down gradually to 0.5 ml/min.

- Once the media is optimally packed, close the column outlet and immediately turn off the flow to the column. Remove either the packing adaptor or the extension tube from the column. Top off the top of the column with degassed 20% ethanol and insert the column inlet to the top of the column being careful not to trap any air between the inlet and the media. If the inlet has a flow adaptor that allows the sample to be applied directly to the media, push the flow adaptor down to the surface of the media and then gently push the flow adaptor down 1-2mm more to slightly compress the top 1-2mm of media. This will enhance even application of the sample to the media and
concomitantly increase purification resolution.

- After the column is reassembled, pump 2 column volumes of degassed 20% ethanol through the column at the expected operating flow rate to equilibrate column.
A.9  Ion exchange chromatography

A.9.1  Materials

- SAU0, 1L filtered and degassed at room temperature (See Appendix H)
- SAU1000, 1L filtered and degassed at room temperature (See Appendix H)
- dH2O, 1L degassed at room temperature
- 0.2M NaOH, filtered and degassed at room temperature
- 20% Ethanol, 1L degassed at room temperature
- 2mM BME (2-mercaptoethanol, Sigma M3148) in dH2O, 5 - 2L beakers refrigerated
- 6000-8000MWCO dialysis tubing, boiled (See Section: Histone Unfolding and Gel Filtration, Note 1)
- 16% SDS-PAGE gel (See Appendix E)
- 1X Tris-Glycine-SDS buffer (See Appendix E)
- 6X SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- TSK-GEL®SP-5PW 21.5mm ID x 15cm, 10µm particle size Ion Exchange HPLC Column with TSK guard-gel kit (Tosoh Bioscience, Cat no. 07575 & 16093) for 10-60mg protein purification scale, OR
- TSK-GEL®SP-5PW 7.5mm ID x 7.5cm, 10µm particle size Ion Exchange HPLC Column with TSK guard-gel kit (Tosoh Bioscience, Cat no. 07161 & 07211) for 1-5mg protein purification scale
A.9.2 Column equilibration

Do all of the following at room temperature using Agilent 1200 series HPLC

1. Fill seal wash bottle with 10% isopropanol and open seal wash outlet so that it drips ~5 times per minute.

2. With purge valve open: equilibrate Line A = SAU0, Line B = SAU1000, Line C = dH2O, Line D = 20% ethanol at 5 ml/min for 5-10 minutes. Turn flow rate to and leave purge valve open.

3. Using "zero volume" tubing (green color coded metal tubing; Agilent) or 0.007” I.D. yellow PEEK tubing (Micro SOLV 47305-07) with 1/16” ferrule connectors, connect the HPLC purge valve outlet to the Rheodyne injector port 2. Connect a 12 inch length of tubing with 1/16” ferrule connectors to the Rheodyne injector port 3.

4. Close purge valve and let tubing fill with ethanol until it comes out of line connected to the Rheodyne injector port 2 at 0.5 ml/min.

5. Remove plugs from column and connect column inlet to line from the Rheodyne injector port 3. Allow to flow at 0.5 ml/min, 100% D (ethanol) until buffer comes out of column outlet.

6. Connect column outlet to UV-VIS flow cell, and connect UV-VIS flow cell outlet to a short piece of tubing for collection.

7. Bring flow rate up to 2 ml/min in 0.5 ml/min increments. Wash column at 2 ml/min with 2CV of degassed dH2O.
8. Slowly transition column at 1-2% per minute into 100% SAU0 buffer at 2 ml/min.

9. Slowly transition column at 1-2% per minute into 80% SAU0 buffer, 20% SAU1000 buffer at 2 ml/min and allow column to equilibrate with 1-2 column volumes.

A.9.3 Sample Preparation

1. Place JA-17 rotor into Beckman Avanti centrifuge and equilibrate to 4°C.

2. Remove lyophilized protein from -20°C. Dissolve lyophilized protein pellet in just enough SAU200 (80% SAU0, 20% SAU1000) so that no aggregates are visible. Thoroughly wash down the sides of the tube to remove protein stuck to the tube wall. The goal is to resuspend histones to between 10 and 15 mg/ml.

3. Centrifuge sample at 4°C, 5000g for 2 minutes to collect liquid from tube walls.

4. Measure the optical density of the sample using SAU200 as a blank. If the OD$_{276nm,1cm} > 7.5$, add enough SAU200 to bring the OD $\sim 7.5$ or less. This will ensure that the histones are fully denatured. At this point it is impossible to accurately determine the concentration of histones in solution due to contaminating proteome background and residual DNA with unknown concentration and extinction coefficients. However, in our experience if one were to "measure the concentration" of histones using the histone extinction coefficient, the actual histone concentration is about 50% of the "measured" concentration.

5. Allow protein to denature on ice for 1 hour. Centrifuge sample at 4°C, 5000g for 10 minutes to precipitate any residual aggregates.

A.9.4 Sample application and elution

1. With the Rheodyne in the "load" position, wash the injection loop with 10ml of SAU200 (80% SAU0, 20% SAU1000).
2. When the column is equilibrated into SAU200, keep the Rheodyne in the "load" position and load no more than 3ml of sample onto the 21.5mm ID x 15cm column or 500µl of sample onto the 7.5mm ID x 7.5cm column. These are the limits at above which the column can become overloaded.

3. Inject sample onto column by turning Rheodyne injector to "inject" position. Elute sample at 2 ml/min using the appropriate program in Table A.2. Histones typically elute within the last 15 minutes of the program.

4. Collect fractions by hand based on real-time UV-VIS spectra at 280nm or collect 2ml fractions across the entire gradient.

<table>
<thead>
<tr>
<th>H3</th>
<th>-</th>
<th>Time</th>
<th>%B</th>
<th>H2A</th>
<th>-</th>
<th>Time</th>
<th>%B</th>
<th>H2B</th>
<th>-</th>
<th>Time</th>
<th>%B</th>
<th>H4</th>
<th>-</th>
<th>Time</th>
<th>%B</th>
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<td>11</td>
<td>36</td>
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<td>36</td>
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<td>43.6</td>
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<td>36</td>
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</tbody>
</table>

Table A.2: Histone Ion Exchange Purification Gradients. Gradients in terms of %B buffer (SAU1000); Remainder of 100% is %A buffer (SAU0). The gradients are given as what state the system should be in at the indicated time in minutes from the point of injection.

A.9.5 Pool fractions and dialyze

1. Combine 10µl of each fraction collected with 2µl of 6x SDS loading buffer; boil for 5 minutes at 95°C; centrifuge at 10000g for 2 minutes. Apply to 16% SDS-PAGE gel as described in Section: Histone Unfolding and Gel Filtration on SDS-PAGE analysis.
2. Based on purity of fractions as determined by SDS-PAGE, pool fractions from multiple runs and dialyze in 6000-8000 Da MWCO dialysis tubing (See Section: Histone Unfolding and Gel Filtration, Note 1) against at least 5 - 2L changes of water + 2mM BME at 4°C for at least 6 hours per change, with the last one going overnight.

3. When dialysis is complete, transfer sample to a 50ml conical bottom tubes; flash freeze with liquid nitrogen; lyophylize.

4. After lyophylization, take a small scraping of purified histone pellet for quantitation by mass spec and/or SDS-PAGE gel.

5. Store purified histone at -20°C after lyophylization.

A.9.6 Column cleaning and storage

1. After purification is complete, slowly transfer column at 1-2% per minute into SAU0 and then into degassed water.

After every 30 injection perform the following, otherwise go to step 11.

2. Bring the flow down to 0.5 ml/min in 0.5 ml/min increments and remove column from HPLC; separate guard column from main column and plug the inlet and outlet of both.

3. With the flow rate at 0.5 ml/min place main column onto the HPLC in the reverse direction; bring flow rate up to 2 ml/min in 0.5 ml/min increments.

4. Set Rheodyne to load position and fill entire 5ml loop with of filtered and degassed 0.2M NaOH. Turn Rheodyne injector to inject position and inject NaOH onto the column; continue to flow water at 2 ml/min for 1CV.
5. Repeat NaOH injection and 1CV dH2O wash two more time for a total of three injections.

6. Bring flow rate down to 0.5 ml/min in 0.5 ml/min increments; remove main column and plug with caps; connect guard column to HPLC in reverse direction and bring flow rate up to 2 ml/min in 0.5 ml/min increments.

7. Inject 1ml of filtered and degassed 0.2M NaOH onto the column and continue to flow water at 2 ml/min for 1CV.

8. Repeat NaOH injection and 1CV water wash two more time for a total of three injections.

9. When complete with cleaning, reduce flow rate to 0.5 ml/min in 0.5 ml/min increments; remove guard column, reassemble the guard and main column; connect reassembled unit onto the HPLC in the forward direction; bring flow rate up to 2 ml/min in 0.5 ml/min increments.

10. Pump 2CV degassed water through the reassembled column in the forward direction at 2 ml/min to re-equilibrate.

11. Slowly exchange column into 20% ethanol by 1-2% per minute. Wash the column with 1-2CV of 20% ethanol for column storage. When done, bring flow rate down to 0.5 ml/min in 0.5 ml/min increments; remove column and cap ends.
A.10 Histone aliquoting and long term storage

For convenient histone refolding, purified histone can be aliquotted and stored lyophilized at -80°C for many years. Possible aliquot amounts are given in Table A.3. A 1x aliquot for refolding produces 125-175µg of purified histone octamer and a 10x aliquot for refolding produces 1.5-2mg of purified histone octamer. Aliquots much smaller than 1x see significant loss in yield during refolding and purification steps.

<table>
<thead>
<tr>
<th>Histone</th>
<th>1x (µg)</th>
<th>10x (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>H2A</td>
<td>91</td>
<td>0.91</td>
</tr>
<tr>
<td>H2B</td>
<td>92</td>
<td>0.92</td>
</tr>
<tr>
<td>H4</td>
<td>74</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Table A.3: Histone Aliquots: for 100µg and 1mg octamer purification scale

1. Dissolve lyophilized protein pellet in just enough water so that no aggregates are visible. Thoroughly wash down the sides of the tube to remove protein stuck to the tube wall.

2. Centrifuge sample at 4°C, 5000g for 2 minutes to collect liquid from tube walls.

3. Measure the optical density OD$_{276nm,1cm}$ of the sample using water as a blank. If the measured concentration of the histone is > 10 mg/ml, add enough water to reduce concentration to ~10 mg/ml. This will ensure that the histone is fully resuspended.

4. Aliquot histones in desired amounts into 1.5ml centrifuge tubes.

5. SpeedVac or lyophilize sample; store at -80°C
A.11 Histone octamer refolding

A.11.1 Materials

- Refolding Buffer, 3 - 2L beakers refrigerated (See Appendix H)
- Unfolding Buffer, 100ml refrigerated (See Appendix H)
- 50mm x 50cm, 6000-8000 MWCO dialysis tubing (See Section: Histone Unfolding and Gel Filtration, Note 1)
- 23mm x 10cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Section: Histone Unfolding and Gel Filtration, Note 1) OR
- 10mm x 20cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Section: Histone Unfolding and Gel Filtration, Note 1)
- Dialysis Chambers (See Table C.1 below)
- Refolding Buffer w/o BME, 1L filtered and degassed, refrigerated
- Water, 1L degassed, refrigerated
- 0.2M NaOH, 1L filtered and degassed, refrigerated
- 20% Ethanol, 1L degassed, refrigerated
- 16% SDS-PAGE gel (See Appendix E)
- 1X Tris-Glycine-SDS buffer (See Appendix E)
- 6X SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- Superdex 200 10/300 GL gel filtration column (GE Healthcare, cat no. 17-5175-01) OR
• Superdex 200 16/60 pg gel filtration column (GE Healthcare, cat no. 17-1069-01)
<table>
<thead>
<tr>
<th>Total Histone Scale</th>
<th>Sample Volume</th>
<th>Concentration Range</th>
<th>Dialysis Chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>250ug-500ug (3/4x-1.5x)</td>
<td>50ul</td>
<td>5 - 10 mg/ml</td>
<td>BrandTec 0.5ml PCR tube (781310)</td>
</tr>
<tr>
<td>500ug-2000ug (1.5x-5x)</td>
<td>200ul</td>
<td>2.5 - 10 mg/ml</td>
<td>Eppendorf 1.5mL PCR Tube (022364120)</td>
</tr>
<tr>
<td>&gt; 2000ug (&gt;5x)</td>
<td>&gt;500ul</td>
<td>1 - 10 mg/ml</td>
<td>10mm 6000-8000MWCO Dialysis tubing</td>
</tr>
</tbody>
</table>

Table A.4: Dialysis Chambers
1. The minimal total histone mass that can be put on a GE Superdex 200 10/300 GL gel filtration column without complete loss of sample to the column is 250µg. The largest total histone mass that can be put on a GE Superdex 200 10/300 GL gel filtration column without overloading is 4000µg (10x) scale.

2. The type of PCR tube used for dialysis of 50µl and 200µl samples is very important. The tubes listed here will not rip the dialysis membrane when the dialysis chamber is assembled. If doing a > 2000µg scale use clips to seal ends of the diaysis tubing. Also see Section: Histone Unfolding and Gel Filtration, Note 1 on how to prepare dialysis tubing.

A.11.2 Refolding

Unfolding Histones

Perform all of the following on ice:

1. Resuspend histones: H2A, H2B, H3, and H4 individually in Unfolding Buffer at a concentration of ∼10 mg/ml (histone concentrations above 10mg/ml produce histone aggregates).

2. Allow histones to unfold for 1 hour but no more than 4 hours on ice.

3. Centrifuge at 20000g for 2 min, 4°C to precipitate any aggregates.

Measuring Histone Concentration

Measure OD_{276nm,1cm} for each histone and calculate the concentration in mg/ml.

UV-Vis absorption spectra of semi- and fully- synthetic acetylated and phosphorylated histones contain a background peak centered at 230nm that overlaps with the protein absorption peak centered at 276nm. This peak is of unknown origin, but presumably due to a remnant chemical contaminant from the synthesis process. To correct for this, the overlap
between the anomalous 230nm peak and 276nm protein peak is estimated by hand.

For fluorophore-labeled histones to be refolded into histone octamer, the absorption spectra of most fluorescent dyes contains a primary absorption peak at a characteristic wavelength and a secondary absorption plateau from 250 to 290nm with a molar extinction of a defined percentage of the molar extinction coefficient at the primary absorption peak. For example for Cy5, which has a primary absorption peak at 650nm, $\epsilon_{650nm,1cm} = 250000cm^{-1}M^{-1}$ and $\epsilon_{276nm,1cm} = 0.05*\epsilon_{650nm,1cm}$ (see GE Healthcare Cy5 maleimide product manual). Histones have an even smaller molar extinction coefficient (See Section: Histone Molecular Weights and Extinction Coefficients). Therefore, the UV absorption at 276nm of fluorophore-labeled histones after gel filtration and dialysis is typically comprised of 5 parts due to fluorophore absorption and 1 part due to histone absorption. Because of this, it is absolutely necessary to subtract out the fluorophore absorption component and verify if the calculated amount of protein is reasonable using the correction. For example for Cy5-labeled H2A(K119C):

$$[H2A(K119C)] = \left( A_{276} - 0.05 * A_{650} \right) / \left( p(cm) * \epsilon_{276,1cm} = 4050cm^{-1}M^{-1} \right)$$  \hspace{1cm} (A.1)

where $p$ is the spectrophotometer pathlength and $A$ is the measured absorption at the given wavelength.

Typically after fluorophore labeling and purification of individual histones, 50% of the protein is lost to gel filtration and dialysis. Therefore, one should not have a final calculated protein yield that is much more than 50% of the protein mass that was initially added to the labeling reaction. As an additional check, given that the protein is typically 60-80% labeled and that there is 50% free dye and 50% protein after gel filtration and dialysis, then a close approximate of the protein concentration can be obtained from the primary fluorophore absorption peak.

If these two calculations are in close agreement, then one can be confident in the measured protein concentration. The labeling efficiency is qualitatively verified by MALDI-TOF
mass spec, which gives a conservative estimate of the labeling efficiency due to the lower efficiency at which labeled histone is ionized and detected versus unlabeled. Additionally, the amount of free dye remaining after labeling, gel filtration, and dialysis can be confirmed by running \( \sim 50 \) fmoles of sample on a 16% SDS-PAGE gel and imaging Cy5 fluorescence using a Typhoon 8600 variable mode imager.

**Combining Histones**

Perform the following on ice:

- **In general, for histone octamers containing unlabeled and unmodified histones**, combine histones in equimolar ratio (1\( \mu \)g H3 : 0.92\( \mu \)g H2B : 0.91\( \mu \)g H2A : 0.74\( \mu \)g H4).

- **For histone octamers containing modified acetylated or phosphorylated histones**, which are always in limiting quantities, combine the modified histone in equimolar ratio with the complementary histone (i.e. equimolar H3:H4 or equimolar H2A:H2B), and then with 1.2-fold molar excess of remaining histones. For example, for H3(K56ac) combine histones in a molar ratio of 1*H3(K56ac) : 1*H4 : 1.2*H2A : 1.2*H2B. This will ensure complete incorporation of H3(K56Ac) into histone octamer for minimal losses.

- **For fluorophore labeled histones**, efficient incorporation of histone into octamer complex appears to be inhibited by the fluorophore. To ensure complete incorporation, combine the fluorophore-labeled histone in equimolar ratio with the complementary histone and then add this in 2-fold molar excess of remaining histones. For example, for H2A(K119C-Cy5) combine histones in a molar ratio of 1*(H3) : 1*H4 : 2*H2A(K119-Cy5) : 2*H2B.

- **For H2A/H2B heterodimer or H3/H4 tetramer only** simply combine H2A and H2B in equimolar ratio, or H3 and H4 in equimolar ratio. For modified or fluorophore-labeled histones that are typically in limiting quantities, 1.2-fold molar excess of the
complementary histone can be added to ensure complete incorporation of the limiting histone. For example, combine a molar ratio of $1^*H2A(K119C-Cy5) : 1.2^*H2B$ to ensure complete incorporation of H2A(K119C-Cy5).

- **For histone octamers, tetramers, and dimers to be labeled after refolding**
  
  Ensure that the only histone carrying a cystiene is the one to which a functional group will be conjugated (i.e. fluorophore, hydroxyl radical Cyst-EDTA, spin label, etc). Even cystienes buried within the histone core, such as H3(C110), are accessible in the fully refolded histone octamer and can be labeled. Therefore, they must be removed by site directed mutagenesis [i.e. use H3(C110A)]. If the histones are labeled prior to refolding, then other cystienes such as H3(C110) do not have to be removed. However, the linkage between the label and histone must be a non-reducible covalent bond because of the presence of reducing agents in the refolding and purification process.

**Refolding Dialysis**

1. Adjust the final volume of combined histones with Unfolding Buffer to a volume suitable for one of the dialysis chambers listed in Table C.1.

2. Place histone sample into selected dialysis chamber:

   - **If using PCR tubes as the dialysis chamber**, cut the camber as depicted in Figure A.3A. Next take a piece of 32mm x 5cm dialysis tubing and cut along the crease so that the dialysis tubing opens into a single sheet of dialysis membrane. Cut a 3in x 3in square of parafilm an place it on the benchtop and place the cap of the PCR tube on the parafilm. Place the sample in the cap of the PCR tube, hold membrane over the cap, and press the ring from the cut PCR tube down over the dialysis membrane as shown in Figure A.3B. Practice this step with buffer as the "sample" and then disassemble the chamber to ensure that the membrane is not being ripped during assembly.
• If doing > 2000µg scale use 10mm 6000-8000 MWCO dialysis tubing. Place the sample in the tubing and clip the ends with dialysis tubing clips.

3. Pour 50-80ml of Unfolding Buffer into a 50mm x 50cm dialysis tubing. Place dialysis chamber into the dialysis tubing and clip the ends as shown in Figure A.3C.

4. Dialyze at 4°C against at least three changes of 2L Refolding Buffer. The third dialysis should be performed overnight.

5. Reclaim refolded histone octamer from dialysis chamber: for PCR caps, place cap on a piece of Parafilm, puncture membrane near rim with pipette tip suck out sample with pipetter; for 10mm dialysis tubing, unclip and extract sample. Store samples on ice.

A.11.3 Purification

Perform all of the following on a GE Healthcare Akta HPLC at 4°C (Jeronic Lab, primary or Zhong Lab, secondary). Obtain training from respective lab members before using.

1. Wash Superdex 200 10/300 GL column at 0.5 ml/min for 10/300 GL column or at 1 ml/min for 16/60 pg column with 1-2CV of degassed water at 4°C. 1CV = 24ml for 10/300 GL column and 1CV = 117ml for 16/60 pg column.

2. Equilibrate column at 0.5 ml/min for 10/300 GL column or at 1 ml/min for 16/60 pg column with 1-2CV of Refolding Buffer without BME at 4°C.

3. Spin octamer sample at 20000g for 2 minutes at 4°C to remove aggregates.

4. Inject sample onto column and elute at 0.5 ml/min collecting 0.25ml fractions for 10/300 GL column or at 1 ml/min collecting 1ml fractions for 16/60 pg column over 1CV. Remaining aggregates elute at fraction ~15, octamer at fraction ~45, tetramer at fraction ~52,
Figure A.3: **Schematic Drawing for Histone Refolding.** (A) Construction of a micro-dialysis button from a PCR tube; (B) Assembly of the micro-dialysis button with sample; and (C) General double-dialysis set-up. Note: the buffer inside the large 50mm dialysis tubing is the same as the buffer inside the smaller dialysis chambers. If using the 10mm 6000-8000MWCO dialysis tubing for sample, place clipped 10mm tubing inside the 50mm tubing filled with buffer just as would be done for the 50µl or 150µl button samples. Clip an air bubble between the middle and top clip of the 50mm dialysis tubing so that entire assembly floats.
dimer at fraction \( \sim 60 \).

5. Check stoichiometry and purity of fractions on 16% SDS-page gel.

6. Pool desired fractions and concentrate using an Amicon 30 (millipore) at 3000g \(^\circ\)C for \( \sim 60 \) minutes to reduce volume to \( \sim 100\mu l \). It is recommended to prewash Amicon with Refolding Buffer without BME at 7500g, 15 minutes, \( 4^\circ C \) to remove glycerol from membrane.

**A.12 Storage**

1. Measure octamer absorbance at \( \text{OD}_{276\text{nm},1\text{cm}} \). \( \text{OD}_{276\text{nm},1\text{cm}} = 0.45 \) results in 1 mg/ml octamer solution.

2. Store octamer on ice in the refrigerator. Change ice weekly when ice begins to melt and becomes slushy. Octamer is stable on ice for 4-6 months before H3 begins to degrade, OR

3. Combine concentrated octamer with equal volume of 80% glycerol. Mix thoroughly and store at -20\(^\circ\)C in Nalgene LabTop Cooler freezer boxes to prevent freeze/thaw during freezer autodefrost. Octamer is stable for 1-2 years before H3 begins to degrade. Note that concentration of histone octamer is difficult to measure after placing in glycerol stock. Accurately measure histone octamer before combining with glycerol and assure the concentration is reduced by half. Additionally, we have observed that octamers placed into glycerol stock are conducive for general reconstitutions to produce nucleosomes for biophysical studies, however, histone octamers placed into glycerol stock are not conducive for competitive reconstitutions, leading to large errors in \( \delta\delta G \) measures, presumably due to the inaccuracy in pipetting from the glycerol stock and potential changes in stock concentration over time.
Appendix B

HISTONE LABELLING

B.1 (Introduction)

Histone labeling is generally accomplished by conjugating a functionalized reagent (typically fluorescent dye, spin label, hydroxyl radical label, or modified amino acid analog) to a cysteine that is site specifically engineered into to histone sequence by site directed mutagenesis. As the only native cysteine in *Xenopus laevis* and *Homo sapien* is H3.2(C110) (colloquially called H3 in Xenopus) and H3.3(C110A), histones are convenient for site specific engineering of labeled amino acid residues for biophysical and biochemical studies. Only H3(C110) needs to be altered to H3(C110A) by site directed mutagenesis in order to site-specifically label other engineered locations. Also note that Homo Sapien H3.1 contains H3.1(C96,C110); however save for C96, H3.1 is identical in sequence to H3.2 and therefore H3.2(C110A) can easily be employed.

Labels are typically functionalized with one of several moieties: maleimide, acyl-halide (e.g. bromoacetamide), alkyl-halide (e.g. methylchloride) or disulfide, which are reactive with the free thiol of the engineered cysteine site in the histone protein. The specificity and/or reactivity of these groups is extremely pH dependent and care must be taken to ensure proper buffer conditions to prevent unwanted side or dead-end products. Additionally, even though the anion form of cysteine at higher pH (pK\(_{a_{\text{cysteine}}}} = 8.3\) is more reactive and a better nucleophile than the protonated form, the \(\varepsilon\)-amine of lysine can also react with these labeling groups at pH levels above 8.5 (pK\(_{a_{\text{lysine}}} = 10.5\)). Therefore the following
considerations should be observed when selecting appropriate labeling chemistry:

- In all protein samples and labeling buffers, common free thiol-containing reagents should be removed and avoided (e.g. beta-mercapto ethanol, ammonium thiosulfate, glutathione) as well as amine-containing compounds such as Tris and benzamidine.

- Maleimide groups react by S-alkylation with the free thiol to generate a stable thioester. They are reactive in pH range from 6.5-7.5 under these conditions ~1000-fold more reactive toward thiol versus amine [232]. Therefore, maleimides are an ideal species for specifically labeling cysteines. However, this group is highly susceptible to hydrolysis, particularly outside of pH 6.5-7.5 and can therefore encounter challenges in achieving high labeling efficiency. Conjugation buffer containing 100mM - 1M HEPES is recommended for effective amine-free pH 6.5-7.5 buffering of this group to prevent pH drop upon proton release during thioester formation.

- Alkyl-halide and acyl-halide groups also react by S-alkylation with the free thiol to generate a stable thioester. These species are optimally reactive at pH ranges from 8.0-9.0 and are less susceptible to hydrolysis, making them very efficient at labeling (typically 95-100%). However, their high pH range makes them prone to unwanted side-reactions with primary amines. To avoid this, conjugation buffer containing 50-100mM HEPES pH 8.2 or 100mM-1M HEPES pH 7.8 is recommended for effective amine-free buffering at the lower end of these groups’ reactivity pH ranges and to prevent pH drop upon proton release during thioester formation.

- Disulfide reagents react by thiol-disulfide interchange to produce a new disulfide between the cystiene free thiol and the disulfide label. Typically the disulfide label contains one RS-SR’ structure in which the -SR’ group is a good leaving group that promotes interchange of the cystiene thiolate form (-S− instead of -SH) to the -SR group. Therefore, pH range of 8.0-9.0 is recommended for efficient exchange (pKa$_{\text{cysteine}}$ = 8.3). While this disulfide interchange chemistry is 100% site specific and allows use of Tris buffers (i.e Tris pH 8.0) for conjugation reaction, reduction of the disulfide bond
after interchange by trace reducing reagents in subsequent experimental buffer and equipment poses considerable difficulties in preventing label loss and prohibits the use of reducing reagents in experimental buffers. Additionally, after disulfide interchange, the sample should be dialyzed into low pH buffer containing 5mM PIPES pH 6.1 to stabilize the disulfide bond until sample is ready to be used. Therefore, this chemistry has limited application to highly constrained condition.

- Regardless of the chemistry selected, all cysteines need to be reduced with TCEP before labeling to eliminate any thiol-capping reagents (e.g. BME) and oxidized sulfur staes. Samples need to then be dialyzed into low pH buffer containing 5mM PIPES pH 6.1 to stabilize the free thiol form and remove residual TCEP reducing agent (see procedures below).

### B.2 Labeling Refolded Histone Octamer, Tetramer, or Dimer with Maleimide-functionalized groups

This procedure is optimized to label 150-600µg of refolded histone complex with 0.25mg or 1mg of maleimide functionalized fluorescent dye for a 20-50 fold molar excess of dye during the labeling reaction. Final labeling efficiencies typically are between 80% and 95% as determined by UV-vis after labeled histone complex has been fully purified by gel filtration.

#### B.2.1 Materials

- 0.2M TCEP [Tris(2-carboxyethyl)phosphine hydrochloride, Sigma C4706]; store unopened dry powder in refrigerator for up to 1 year. Dissolve 1.72g powder in 20ml water and pH to 7.1 with concentrated NaOH. Bring total volume up to 25ml; immediately aliquot into 0.5ml fractions and flash freeze in liquid nitrogen. Store aliquots at -80°C for up to 2 years. Thaw and use each aliquot once. Do not refreeze or use thawed TCEP after more than 6 hours.

- 3 - 300ml beakers of 5mM PIPES pH 6.1, 2M NaCL. Dissolve 1.62g PIPES [Piperazine-N,N’-bis(2-ethanesulfonic acid), Sigma P1851] and 116.82g NaCL in 900ml water. Stir
to dissolve and slowly bring pH to 6.1 with concentrated NaOH. Solubility of PIPES is pH dependent and will not go into solution until above pH 5. Slowly add NaOH dropwise; there is a long relaxation time for pH to come back down after adding each drop of NaOH. Bring volume up to 1L; filter and degas; split between 3 beakers refrigerate at 4°C. Can be made on previous day.

- 2M HEPES pH7.1 [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, Sigma-Aldrich H4034]. Dissolve 11.9g HEPES in 20ml water. pH to 7.1 with NaOH and bring total volume up to 25ml with water. Aliquot into 0.5ml aliquots; flash freeze in liquid nitrogen; store at -80°C for up to 2 years. Thaw and use each aliquot once. Do not refreeze.

- 23mm x 10cm 6000-8000MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1) OR

- 10mm x 20cm 6000-8000MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1)

- Dialysis Chambers (See Table C.1 below)
- argon tank with regulator
- test tube bubbler with rubber stopper and inlet/outlet lines
- 1mm x 6in glass capillary
- 1.0mm inner diameter tubing (GE healthcare 19-0040-01)
- small gauge hypodermic needle
- parafilm
- maleimide functionalized dye [Cy (GE healthcare), Alexa (Invitrogen/Life technologies), ATTO (Sigma-Aldrich/AttoTech)] or other maleimide functionalized moiety
• 1ml syringe

• DMF, anhydrous [N,N-dimethyl formamide, sigma-aldrich]

• VortexGenie vortexer
<table>
<thead>
<tr>
<th>Total Histone Scale</th>
<th>Sample Volume</th>
<th>Concentration Range</th>
<th>Dialysis Chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>250µg-500µg (3/4x-1.5x)</td>
<td>50µl</td>
<td>5 - 10 mg/ml</td>
<td>BrandTec 0.5ml PCR tube (781310)</td>
</tr>
<tr>
<td>500µg-2000µg (1.5x-5x)</td>
<td>200µl</td>
<td>2.5 - 10 mg/ml</td>
<td>Eppendorf 1.5mL PCR Tube (022364120)</td>
</tr>
<tr>
<td>&gt; 2000µg (&gt; 5x)</td>
<td>&gt; 500µl</td>
<td>1 - 10 mg/ml</td>
<td>10mm 6000-8000 MWCO Dialysis tubing</td>
</tr>
</tbody>
</table>

Table B.1: Dialysis Chambers
B.2.2 Procedure

1. Following standard protocols for Histone Refolding (See Appendix A: Histone Octamer Refolding), refold a 3-6x scale (300µg - 600µg of each histone) of histone octamer, (H3/H4)$_2$ tetramer or H2A/H2B dimer, where the only histone containing a cystiene is the histone to be labeled. All other cystienes must be removed by site directed mutagenesis [i.e. H3(C110) must be expressed as H3(C110A) for labeling other histones]. Histone complexes should **NOT** be purified by gel filtration after refolding, and refolded histone stocks should be stored at concentrations above 1.5mg/ml.

2. Before labeling, aliquot suitable amount of refolded histone octamer/tetramer/dimer from stock tube. 300-600µg (3-6nmol) of histone octamer, 150-300µg (3-6nmol) of tetramer, or 150-300µg (6-12nmol) of dimer contains 6-12nmol of free thiol that will be conveniently labeled by 25-50 fold molar excess of 300nmol (typically 0.25mg) of maleimide functionalized reagent.

3. Remove an aliquot of TCEP from -80°C and allow to thaw on ice for 10-20 minutes. Reduce histone octamer/tetramer/dimer by adding TCEP pH 7.1 to 10mM final concentration and incubating on ice for 30 minutes.

4. Place histone sample into selected dialysis chamber:

   - **If using PCR tubes as the dialysis chamber**, cut the camber as depicted in Figure B.1A. Next take a piece of 32mm x 5cm dialysis tubing and cut along the crease so that the dialysis tubing opens into a single sheet of dialysis membrane. Cut a 3in x3in square of parafilm and place it on the benchtop; place the cap of the PCR tube on the parafilm. Place the sample in the cap of the PCR tube, hold membrane over the cap, and press the ring from the cut PRC tube down over the dialysis membrane as shown in Figure B.1B. Practice this step with buffer as the "sample" and then disassemble the chamber to ensure that the membrane is not being ripped during assembly.
If doing > 2000µg scale use 10mm 6000-8000MWCO dialysis tubing. Place the sample in the tubing and clip the ends with dialysis tubing clips.

5. Dialyze chamber containing histone octamer/dimer/tetramer by single dialysis against 3 changes of 300ml of 5mM sodium PIPES pH 6.1, 2M NaCl buffer for 2-3 hours per change. This promotes the free thiol state of the Cystiene residue and removes any reducing agents. Reclaim samples from dialysis chamber and place on in test tube on ice.

6. Measure the absorption of the octamer/tetramer/dimer at OD$_{276nm,1cm}$. For histone octamer OD$_{276nm,1cm} = 0.45$ gives a concentration of 1mg/ml; for tetramer and dimer use the approximation of OD$_{276nm,1cm} = 0.40$ gives a concentration of 1 mg/ml.

7. Dilute histone octamer to 1.5-3 mg/ml (14-28µM octamer complex = 28-56µM free thiol); dilute tetramer to 0.75-1.5 mg/ml (14-28µM complex = 28-56µM free thiol); dimer to 0.75-1.5 mg/ml (28-56µM dimer = 28-56µM free thiol) with 5mM PIPES pH 6.1, 2M NaCl buffer.

8. Set up argon purge system (Figure B.2) with 1/4in I.D. tubing from regulator reducing to 1mm I.D. tubing. Connect to bubbler inlet to 1mm I.D. tubing from argon tank and connect 1mm I.D. tubing to bubbler outlet. Insert 1mm glass capillary tube into the end of 1mm I.D. tubing. Turn on argon flow from tank so that argon bubbles through bubbler fast enough that there is no delay between successive bubbles. Use back of hand or arm to feel that argon is flowing out of each capillary at a gentle flow.

9. Remove test tube containing sample to be labeled, uncap, and wrap opening of tube with parafilm to seal tube. With small gauge needle, poke two holes in the parafilm (inlet and outlet). Place test tube rack in deli fridge by argon bubbler. Take glass capillary connected argon bubbler outlet and gently insert capillary through one of the two holes in parafilm covering tube opening. Push capillary down until it is 0.5-1cm above sample. Do
not allow capillary to go down into sample as sample will foam out of tube.

10. Purge sample for 15-20 minutes under argon at 4°C to remove O₂. When done, quickly remove capillary and cap tube by pressing lid through parafilm; keep on ice. While sample is purging, remove aliquot of maleimide functionalized reagent from refrigerator/freezer and place at room temperature to equilibrate. Additionally, remove aliquot of 2M HEPES pH7.1 from -80°C freezer and thaw at room temperature.

11. When sample is done purging, immediately start bubbling 500µl 2M HEPES pH 7.1 in argon for 5 minutes to remove O₂. Perform same procedure as above by wrapping opening of tube with parafilm, poking two holes with needle, and inserting capillary through one of the holes. However in this case push capillary all the way into the sample so that argon actively bubbles through. When done, cap tube by pressing lid through parafilm and place on rack at room temperature.

12. While HEPES is bubbling; place test tube containing histone octamer in test tube rack at room temperature to equilibrate. Also remove 0.5ml aliquot of DMF from stock bottle using syringe and place in test tube at room temperature. Dissolve aliquot of maleimide functionalized reagent (typically 300nmol or 0.25mg) in 14µl of anhydrous DMF per 0.25mg reagent to 22mM (18 mg/ml) final concentration. For resuspending more than 0.25mg of label, add DMF in 14µl aliquots with thorough mixing between each addition to prevent micelle formation of hydrophobic labels.

13. Turn on vortexer to lowest setting so that it agitates and mixes sample, but does not violently swirl and churn sample. Add 5µl 2M HEPES pH 7.1 per 100µl of histone sample to 100mM final concentration to rapidly bring pH back up to 7.1. Immediately touch tube to vortexer to mix in HEPES. Immediately begin adding maleimide functionalized reagent in DMF by 2-4µl additions; mix sample by touching to vortexer for 30 seconds between each addition. Continually add dye until all is used or has been added to a 25-50 molar
excess of the free thiol containing histone.

14. When all reagent has been added, cover test tube in foil and incubate on rotisserie 1hr at room temperature. Then transfer rotisserie with sample to 4°C refrigerator and allow to incubate overnight.

15. Quench reaction with 10mM DTT final concentration at store at 4°C on ice until ready to purify.

B.2.3 Purification

1. Purify labeled histone complex from free dye by standard gel filtration on Superdex 200 column as detailed in Appendix A. Concentrate by Amicon 30, measure UV-Vis absorption, and store as described in Appendix A. Note that for fluorophore-labeled histones, the absorption spectra of most fluorescent dyes contains a primary absorption peak at a characteristic wavelength and a secondary absorption plateau from 250 to 290nm with a molar extinction of a defined percentage of the molar extinction coefficient at the primary absorption peak. For example for Cy5, which has a primary absorption peak at 650nm, \(\varepsilon_{650nm,1cm} = 250000cm^{-1}M^{-1}\) and \(\varepsilon_{276nm,1cm} = 0.05*\varepsilon_{650nm,1cm}\) (see GE Healthcare Cy5 maleimide product manual). Histones and histone complexes have an even smaller molar extinction coefficient. Therefore, the UV absorption at 276nm of fluorophore-labeled histones after gel filtration must be corrected to account the for absorption component from the fluorescent dye. For example for Cy5-labeled H2A(K119C):

\[
[H2A(K119C)] = (A_{276} - 0.05 * A_{650})/(p(cm) * \varepsilon_{276,1cm} = 4050cm^{-1}M^{-1})
\]  

(B.1)

where \(p\) is the spectrophotometer pathlength and \(A\) is the measured absorption at the given wavelength.

Typically after fluorophore labeling and purification of refolded histone complexes, 80-95% of the protein is labeled and there is no free dye remaining.
B.2.4 Notes

1. The maleimide group is extremely sensitive to moisture and hydrolysis. Always store maleimide functionalized labels at 4°C in original and unopened container. When ready to use, remove from 4°C and allow to equilibrate to room temperature to avoid moisture condensation upon opening. Always use anhydrous DMF to resuspend dye and use immediately. If not all of the dye can be used at once, purge tube containing remaining resuspended dye with argon or nitrogen; cap and seal with parafilm. Flash freeze in liquid nitrogen and store at -20°C for up to 2 weeks or -80°C for up to 6 months. When ready to use, remove from freezer and allow to fully equilibrate to room temperature before opening.

2. If several distinct fluorescent or other labels are required within the histone octamer complex, there are two options for achieving this:

- If one label resides in the (H3/H4)$_2$ tetramer and one label in the dimer, individually refold tetramer and dimer with histones containing the desired site specific cysteines. Label each histone complex separately with their respective labels and purify separately by gel filtration as detailed above. Concentrate to ~3 mg/ml and keep stored in Refolding Buffer without BME (See Appendix H) on ice at 4°C. Measure the concentration of each purified complex by performing a 1:4 dilution of purified complex into 7M guanidine and using a 1:4 dilution of Refolding Buffer without BME into 7M guanidine as the blank. Use the exact extinction coefficients of $\epsilon_{\text{tetramer,276nm}} = 18880$ M$^{-1}$cm$^{-1}$ with FW = 53014Da for tetramer and $\epsilon_{\text{dimer,276nm}} = 10120$ M$^{-1}$cm$^{-1}$ with FW = 27435Da for dimer. Correct for absorption due to fluorophore labels as described above. To form histone octamer, combine 1.2 moles of purified dimer per 1 mole of tetramer. Purified complexes should be used due to inhibition of interaction by high concentration of free label, particularly for fluorescent dyes. Depending on how accurately the concentration of the dimer and tetramer was able to be measured (certain dyes such as ATTO have strong absorption at 275m, and can lead to errors in concentration greater than 25%), one may need to add an even higher molar excess
of dimer to ensure complete incorporation of tetramer into histone octamer. Allow combined dimer and tetramer to incubate on ice overnight and purify by gel filtration on a superdex 200 column as done for purifying dimer, tetramer, and octamer.

- If multiple labels reside in the dimer and/or tetramer, individual histones must be first labeled prior to refolding as described below.
B.2.5 Labeling of individual histones before refolding

This procedure is optimized to label 1mg to 5mg of histone with 1mg to 5mg of maleimide functionalized fluorescent dye for a 20-fold molar excess for dye during the labeling reaction. Final labeling efficiencies typically are between 65% and 85% as determined by UV-vis after labeled histone has been fully refolded into histone octamer complex. This procedure is useful if histone complex contains multiple different labels that are not amenable to separate labeling of tetramer or dimer, or if one of the histones contains a superfluous cystiene that cannot be removed (i.e. ligation point of semi- and fully-synthetic modified histones).

B.2.6 Materials

- 0.2M TCEP [Tris(2-carboxyethyl)phosphine hydrochloride, Sigma C4706]; store unopened dry powder in refrigerator for up to 1 year. Dissolve 1.72g powder in 20ml water and pH to 7.1 with concentrated NaOH. Bring total volume up to 25ml; immediately aliquot into 0.5ml fractions and flash freeze in liquid nitrogen. Store aliquots at -80°C for up to 2 years. Thaw and use each aliquot once. Do not refreeze or use thawed TCEP after more than 6 hours.

- 3 - 200ml beakers of 5mM PIPES pH 6.1, 2M guanidine. Dissolve 1.07g PIPES [Piperazine-N,N'-bis(2-ethanesulfonic acid), Sigma P1851] and 114.6g guanidine (MP biomedicals; ultrapure 820539) in 500ml water. Stir to dissolve and slowly bring pH to 6.1 with concentrated NaOH. Solubility of PIPES is pH dependent and will not go into solution until above pH 5. Slowly add NaOH dropwise; there is a long relaxation time for pH to come back down after adding each drop of NaOH. Bring volume up to 600ml; filter and degas; split between 3 beakers refrigerate at 4°C. Can be made on previous day.

- 7M guanidine (MP biomedicals; ultrapure 820539), filtered, degassed, stored at 4°C.
- 2M HEPES pH 7.1 [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, Sigma-Aldrich H4034]. Dissolve 11.9 g HEPES in 20ml water. pH to 7.1 with NaOH and bring total volume up to 25ml with water. Aliquot into 0.5ml aliquots; flash freeze in liquid nitrogen; store at -80°C for up to 2 years. Thaw and use each aliquot once. Do not refreeze.

- 23mm x 10cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1) OR

- 10mm x 20cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1)

- Dialysis Chambers (See Table C.1)

- argon tank with regulator

- test tube bubbler with rubber stopper and inlet/outlet lines

- 1mm x 6in glass capillary

- 1.0mm inner diameter tubing (GE healthcare 19-0040-01)

- small gauge hypodermic needle

- parafilm

- maleimide functionalized dye [Cy (GE healthcare), Alexa (Invitrogen/Life technologies), ATTO (Sigma-Aldrich/AttoTech)] or other maleimide functionalized moiety

- 1ml syringe

- DMF, anhydrous [N,N-dimethyl formamide, sigma-aldrich]

- glass 13 x 100mm or 20 x 150mm test tube for labeling reaction

- stir bar to fit in bottom of glass tube

- 20µl capillary pipette tips (VWR 53509-015)
- TU1000 buffer (See Appendix H)
- Hand packed GE C10/20 (GE 19-5002-01) or C10/40 (GE 19-5003-01) column with or custom packed GE XK26/20 column containing 20cm sephadex G25 medium (GE 17-0030-01).
- 16% SDS-PAGE gel (See Appendix E)
- 1x Tris-Glycine SDS (See Appendix E)
- 6x SDS loading buffer without dye (See Appendix E) OR
- 6x SDS loading dye (See Appendix E)
- Gel stain (See Appendix E)
- Gel destain (See Appendix E)
- 23mm x 10cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1) OR
- 10mm x 20cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1)
- 3-500ml beakers of 5mM BME (beta-mercaptoethanol) in water at 4°C.

B.2.7 Labeling

1. Resuspend 1mg - 5mg (70-360nmol) of histones in 7M guanidine at 4-5 mg/ml final concentration.

2. Remove an aliquot of TCEP from -80°C and allow to thaw on ice for 10-20 minutes. Reduce histones by adding TCEP pH 7.1 to 10mM final concentration and incubating on ice for 30 minutes.

3. Place histone sample into selected dialysis chamber:
– **If using PCR tubes as the dialysis chamber**, cut the camber as depicted in Figure B.1A. Next take a piece of 32mm x 5cm dialysis tubing and cut along the crease so that the dialysis tubing opens into a single sheet of dialysis membrane. Cut a 3in x 3in square of parafilm and place it on the benchtop; place the cap of the PCR tube on the parafilm. Place the sample in the cap of the PCR tube, hold membrane over the cap, and press the ring from the cut PCR tube down over the dialysis membrane as shown in Figure B.1B. Practice this step with buffer as the "sample" and then disassemble the chamber to ensure that the membrane is not being ripped during assembly.

– **If doing > 2000µg scale** use 10mm 6000-8000 MWCO dialysis tubing. Place the sample in the tubing and clip the ends with dialysis tubing clips.

4. Dialyze chamber containing histones by single dialysis against 3 changes of 200ml of 5mM sodium PIPES pH 6.1, 2M guanidine buffer for 2-3 hours per change. This promotes the free thiol state of the Cystiene residue and removes any reducing agents. Reclaim samples from dialysis chamber and place in test tube on ice.

5. Measure the absorption of the histones at OD$_{276nm,1cm}$ and dilute to 150µM (∼2 mg/ml) with 5mM PIPES pH 6.1, 2M guanidine buffer.

6. Set up argon purge system (Figure B.2) at room temperature with 1/4in I.D. tubing from regulator reducing to 1mm I.D. tubing. Connect 1mm I.D. tubing from argon tank to bubbler inlet and connect 1mm I.D. tubing to outlet. Insert 1mm glass capillary tube into end of 1mm I.D. tubing. Turn on argon flow from tank so that argon bubbles through bubbler fast enough that there is no delay between successive bubbles. Use back of hand or arm to feel that argon is flowing out of each capillary at a gentle flow. Place glass 13mm x 100mm tube (for 1mg labeling scale) or 20mm x 150mm tube (for 5mg labeling scale) in test tube holder on stir plate. Place stir bar in bottom of test tube and turn on stir plate to medium-low for gentle, continuous
mixing.

7. Remove test tube containing sample to be labeled, uncap, and transfer sample to glass test tube on stir plate. Wrap opening of glass tube with parafilm to seal tube. With small gauge needle, poke two holes in the parafilm (inlet and outlet). Take glass capillary connected to argon bubbler outlet and gently insert capillary through one of the two holes in parafilm covering tube opening. Push capillary down until it is 1cm above sample. Do not allow capillary to go down into sample as sample will foam out of tube.

8. Purge sample for 20-30 minutes under argon to remove O\textsubscript{2}. While sample is purging, remove aliquot of maleimide functionalized reagent from refrigerator/freezer and place at room temperature to equilibrate. Additionally, remove aliquot of 2M HEPES pH 7.1 from -80°C freezer and thaw at room temperature.

9. When sample has 5 minutes left of purging, start bubbling 500µl 2M HEPES pH 7.1 with unused argon line for 5 minutes to remove O\textsubscript{2}. Perform same procedure as above by wrapping opening of tube with parafilm, poking two holes with needle, and inserting capillary through one of the holes. However in this case push capillary all the way into the sample so that argon actively bubbles through.

10. While sample and HEPES are finishing purging/bubbling; remove 0.5ml aliquot of DMF from stock bottle using syringe and place in test tube at room temperature. Dissolve aliquot of maleimide functionalized reagent (typically 1.2µmol or 1mg dye for 1mg histone and 6µmol or 5mg dye for 5mg histone) in 56µl of anhydrous DMF per 1mg reagent to 22mM (18 mg/ml) final concentration. Add DMF in 14µl aliquots with thorough mixing between each addition to prevent micelle formation of hydrophobic labels.
11. When ready to label, remove argon line from HEPES, cap, and keep at room temperature. Keep argon line in glass test tube containing sample and continue flowing argon. Using a needle, poke a third hole in the parafilm that is large enough to easily insert capillary pipette tip. Using a capillary pipette tip, add 5µl of 2M HEPES pH 7.1 per 100µl of histone sample to 100mM final concentration to rapidly bring pH back up to 7.1. Using a capillary pipette tip, immediately begin adding maleimide functionalized reagent in DMF by 5µl additions; allow sample to mix for 30 seconds between each addition. Continue adding dye until all is used or has been added to a 25-50 molar excess of the free thiol containing histone.

12. When all reagent has been added, leave argon line in test tube with sample and continue to flow argon. Cover test tube in foil and continue to mix on stir plate for 1hr at room temperature.

13. Quench reaction with 10mM DTT final concentration and store at 4°C until following day for gel filtration purification.

### B.2.8 Purification

If the molecule used to label histones is small and polar (< 800Da) or very small non-polar (< 500Da) then excess label can be mostly removed by extensive single dialysis against 3 - 500ml changes of 5mM BME in water at 4°C a least 12 hours per change. Proceed to next section. However, if molecules are large or very hydrophobic (particularly flouroescent dyes) perform the following:

1. For gel filtration removal of free label, follow general protocols for gel filtration as described in Appendix A.8.4: Gel Filtration. Briefly, at room temperature wash Sephadex G25 column (C10/20 or C10/40 for 1mg histone; XK26/20 for 5mg histone) at 1 ml/min with 2CV filtered and degassed water to wash out 20% ethanol storage
buffer. Equilibrate column with 2CV of TU1000 buffer at 1 ml/min. Load sample onto column by inserting line to column inlet into sample and line from column outlet to peristaltic pump; "suck" sample onto column by pumping on column outlet with peristaltic pump at 0.5 ml/min. When sample is loaded, reconnect line to column inlet to pump and flow sample across column at 0.5 ml/min. For C10/20 or C10/40 column collect 0.25ml fraction across 1CV; for XK26/20 collect 0.5ml fraction across 1CV. Histones will just run ahead of free label with about 40-50% contamination of histone peak with tail of free dye peak.

2. Quantify fractions by SDS-Page gel as described in Appendix E. Combine 15µl of each fraction (typically fractions 8-23) with 3µl 6x SDS loading buffer without dye if labels are fluorescent molecules, or 6x SDS loading dye if labels are not fluorescent. Run gel until free label and histone bands are resolved in the case of fluorescent labels, or just until the loading dye runs off the gel in case of non-fluorescent labels. Determine fractions containing fluorophore labeled histone by eye or with Typhoon 8600 imager; determine fractions containing non-fluorescent labeled histones by staining and destaining gel.

3. Pool and dialyze histone fractions by single dialysis extensively against 500ml of 5mM BME at 4°C with at least two additional changes at 12 hours per change.

B.2.9 Histone quantification and storage

1. Reclaim labeled histones from dialysis and lyophylize until dry.

2. Resuspend histones with a small amount of water to ~10mg/ml. Measure UV-vis absorption. Note that for fluorophore-labeled histones, the absorption spectra of most fluorescent dyes contains a primary absorption peak at a characteristic wavelength and a secondary absorption plateau from 250 to 290nm with a molar extinction of a de-
fined percentage of the molar extinction coefficient at the primary absorption peak. For example for Cy5, which has a primary absorption peak at 650nm, $\epsilon_{650\text{nm},1\text{cm}} = 250000\text{cm}^{-1}\text{M}^{-1}$ and $\epsilon_{276\text{nm},1\text{cm}} = 0.05*\epsilon_{650\text{nm},1\text{cm}}$ (see GE Healthcare Cy5 maleimide product manual). Histones and histone complexes have an even smaller molar extinction coefficient. Therefore, the UV absorption at 276nm of fluorophore-labeled histones after gel filtration must be corrected to account for absorption component from fluorescent dye. For example for Cy5-labeled H2A(K119C):

$$[\text{H}2\text{A}(\text{K}119\text{C})] = (A_{276\text{nm}} - 0.05 * A_{650\text{nm}})/(p(\text{cm}) * \epsilon_{276\text{nm},1\text{cm}} = 4050\text{cm}^{-1}\text{M}^{-1})$$

(B.2)

where p is the spectrophotometer pathlength and A is the measured absorption at the given wavelength.

Typically after fluorophore labeling and purification of individual histones, 50% of the protein is lost to gel filtration and dialysis. Therefore, one should not have a final calculated protein yield that is much more than 50% of the protein mass that was initially added to the labeling reaction. As an additional check, given that the protein is typically 60-80% labeled and that there is 50% free dye and 50% protein after gel filtration and dialysis, then a close approximate of the protein concentration can be obtained from the primary fluorophore absorption peak. If these two calculations are in close agreement, then one can be confident in the measured protein concentration. The labeling efficiency is qualitatively verified by MALDI-TOF mass spec, which gives a conservative estimate of the labeling efficiency due to the lower efficiency at which labeled histone is ionized and detected verses labeled. Additionally, the amount of free dye remaining after labeling, gel filtration, and dialysis can be confirmed by running ~50 fmoles of sample on a 16% SDS-PAGE gel and imaging Cy5 fluorescence using a Typhoon 8600 variable mode imager.

3. Aliquot histones into reasonable amounts (See Appendix A) and speed vac to
dryness. Store at -80°C.
B.3 Labeling Histone Octamer after refolding with acyl-
halide and alkyl-halide functional groups

This protocol is optimized for labeling histone octamer with the FeBABE (Fe(III) 
(s)-1-(p-bromoacetamidobenzyl) ethylenediamine tetraacetic acid) hydroxyl radical 
cleavage group for nucleosome mapping reactions. However, this protocol can be used 
in general for any halide-based S-alkylating reagent.

B.3.1 Materials

– 0.2M TCEP [Tris(2-carboxyethyl)phosphine hydrochloride, Sigma C4706]; store 
unopened dry powder in refrigerator for up to 1 year. Dissolve 1.72g powder into 
20ml water and pH to 7.1 with concentrated NaOH. Bring total volume up to 
25ml; immediately aliquot into 0.5ml fractions and flash freeze in liquid nitrogen. 
Store aliquots at -80°C for up to 2 years. Thaw and use each aliquot once. Do 
not refreeze or use thawed TCEP after more than 6 hours.

– 3 - 300ml beakers of 5mM PIPES pH 6.1, 2M NaCL, 4mM EDTA. Dissolve 1.62g 
PIPES [Piperazine-N,N’-bis(2-ethanesulfonic acid), Sigma P1851] and 116.82g 
NaCL in 900ml water. Add 8ml 0.5M EDTA pH8.0 (Ethylenediaminetetraacetic 
acid). Stir to dissolve and slowly bring pH to 6.1 with concentrated NaOH. 
Solubility of PIPES is pH dependent and will not go into solution until above pH 
5. Slowly add NaOH dropwise; there is a long relaxation time for pH to come 
back down after adding each drop of NaOH. Bring volume up to 1L; filter and 
degas; split between three beakers; refrigerate at 4°C. Can be made on previous 
day.

– 2M HEPES pH 8.2 [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, Sigma-
Aldrich H4034]. Dissolve 11.9 g HEPES in 20ml water. pH to 8.2 with NaOH 
and bring total volume up to 25ml with water. Aliquot into 0.5ml aliquots; flash 
freeze in liquid nitrogen; store at -80°C for up to 2 years. Thaw and use each
aliquot once. Do not refreeze.

- 23mm x 10cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1) OR

- 10mm x 20cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1)

- 50% glycerol, syringe filtered

- 3-300ml beakers of 50mM Tris, pH 8.2, 2M NaCl, 0.1mM EDTA, 50% glycerol. Make 1L of buffer; split between 3 beakers; refrigerate at 4°C. Do not filter and degas as 50% glycerol does not go through vacuum filter.

- Dialysis Chambers (See Table C.1)

- argon tank with regulator

- test tube bubbler with rubber stopper and inlet/outlet lines

- 1mm x 6in glass capillary

- 1.0mm inner diameter tubing (GE healthcare 19-0040-01)

- small gauge hypodermic needle

- parafilm

- FeBABE reagent (1 tube = 50µg = 85nmol; Thermo 20332) or other acyl-halide and alkyl-halide labeling reagent.

- 1ml syringe

- VortexGenie vortexer

**B.3.2 Procedures**

1. Following standard protocols for Histone Refolding (See Appendix A: Histone Octamer Refolding), refold a 3-6x scale (300µg - 600µg of each histone) of histone
octamer, where the only histone containing a cystiene is the histone to be labeled. All other cystienes must be removed by site directed mutagenesis [i.e. H3(C110) must be expressed as H3(C110A) for labeling other histones]. Histone complexes should be purified by gel filtration and concentrated into Refolding Buffer without BME; refolded histone stocks should be stored at concentrations above 1.5 mg/ml on ice at 4°C.

2. Before labeling, aliquot suitable amount of refolded histone octamer to a new tube on ice: 50-100 µg of histone octamer, which contains 0.9-1.8 nmol of free thiol, is conveniently labeled by an 85 nmol aliquot of FeBABE reagent for a 50-100 fold excess labeling reaction.

3. Remove an aliquot of TCEP from -80°C and allow to thaw on ice for 10-20 minutes. Reduce histone octamer by adding TCEP pH 7.1 to 10 mM final concentration and incubating on ice for 30 minutes.

4. Place histone sample into selected dialysis chamber:

- **If using PCR tubes as the dialysis chamber**, cut the camber as depicted in Figure B.1A. Next take a piece of 32 mm x 5 cm dialysis tubing and cut along the crease so that the dialysis tubing opens into a single sheet of dialysis membrane. Cut a 3 in x 3 in square of parafilm and place it on the benchtop; place the cap of the PCR tube on the parafilm. Place the sample in the cap of the PCR tube, hold membrane over the cap, and press the ring from the cut PCR tube down over the dialysis membrane as shown in Figure B.1B. Practice this step with buffer as the "sample" and then disassemble the chamber to ensure that the membrane is not being ripped during assembly.

- **If doing > 2000 µg scale** use 10 mm 6000-8000 MWCO dialysis tubing. Place the sample in the tubing and clip the ends with dialysis tubing clips.
5. Dialyze chamber containing histone octamer by single dialysis against 3 changes of 300ml of 5mM sodium PIPES pH 6.1, 2M NaCl, 4mM EDTA buffer for 2-3 hours per change. This promotes the free thiol state of the Cystiene residue and removes any reducing agents. Reclaim samples from dialysis chamber and place on in test tube on ice.

6. Before sample is almost done dialyzing, remove aliquot of FeBABE or other acyl- or akyl-halide functionalized reagent from refrigerator/freezer and place at room temperature to equilibrate. Additionally, remove aliquot of 2M HEPES pH 8.2 from -80°C freezer and thaw at room temperature.

7. Measure the absorption of the octamer at OD_{276nm,1cm}. For histone octamer OD_{276nm,1cm} = 0.45 gives a concentration of 1mg/ml.

8. Dilute histone octamer to 1.1-1.7 mg/ml with 5mM PIPES pH 6.1, 2M NaCl, 4mM EDTA buffer. Then add 50% glycerol 5% final concentration (histone octamer will be between 1.0-1.5mg/ml) and place at room temperature to equilibrate. Additionally, to 0.5ml of 5mM PIPES pH 6.1, 2M NaCl, 4mM EDT buffer, add 50µl of 50% glycerol and 25µl of 2M HEPES pH 8.2 to 5% and 100mM final concentration respectively to make "Conjugation Buffer". Keep both on ice.

9. Set up argon purge system (Figure B.2) in deli fridge with 1/4in I.D. tubing from regulator reducing to 1mm I.D. tubing. Connect 1mm I.D. tubing from argon tank to bubbler and connect 1mm I.D. tubing to bubbler outlet. Insert 1mm glass capillary tube into end if 1mm ID tubing. Turn on argon flow from tank so that argon bubbles through bubbler fast enough that there is no delay between successive bubbles. Use back of hand or arm to feel that argon is flowing out of each capillary at a gentle flow.

10. Remove test tube containing sample to be labeled, uncap, and wrap opening
of tube with parafilm to seal tube. With small gauge needle, poke two holes in the parafilm (inlet and outlet). Place test tube rack in deli fridge by argon bubbler. Take glass capillary connected to the argon bubbler outlet and gently insert capillary through one of the two holes in parafilm covering tube opening. Push capillary down until it is 0.5-1cm above sample. Do not allow capillary to go down into sample as sample will foam out of tube.

11. Purge sample for 15-20 minutes under argon at 4°C to remove O₂. When done quickly remove capillary and cap tube by pressing lid through parafilm; keep on ice. While sample is purging, remove aliquot of FeBABE or other similar reagent from refrigerator/freezer and place at room temperature to equilibrate.

12. When sample is done purging, immediately start bubbling the aliquots of 2M HEPES pH 8.2 and "conjugation buffer" [100mM HEPES pH 8.2, 5mM PIPES, 2M NaCl, 4mM EDTA, 5% glycerol] in argon for 5 minutes to remove O₂. Perform same procedure as above by wrapping opening tube with parafilm, poking two holes with needle, and inserting capillary through one of the holes. However in this case push capillary all the way into the sample so that argon actively bubbles through. When done, cap tube by pressing lid through parafilm and place on rack at room temperature.

13. While HEPES and conjugation buffer are bubbling; place test tube containing histone octamer in test tube rack at room temperature to equilibrate. When HEPES and conjugation buffer are done bubbling, cap and place at room temperature to equilibrate.

14. Turn on vortexer to lowest setting so that it agitates and mixes sample, but does not violently swirl and churn sample. Resuspend aliquot of FeBABE or similar labeling reagent in 14µl of degassed conjugation buffer to 8-10mM final concentration.
Immediately add 5µl 2M HEPES pH 8.2 per 100µl of histone sample to 100mM final concentration to rapidly bring pH back up to 8.2. Immediately touch tube to vortexer to mix in HEPES. Immediately begin adding labeling reagent by 2µl additions; mix sample by touching to vortexer for 30 seconds between each addition. Continually add dye until all is used or has been added to a 25-50 molar excess of the free thiol containing histone.

15. When all reagent has been added, cover test tube in foil and incubate on rotisserie 1hr at room temperature. Then transfer rotisserie with sample to 4°C refrigerator and allow to incubate overnight.

16. Quench reaction with 10mM BME final concentration and dialyze extensively against 3-300ml changes of 10mM Tris pH 8.0, 2M NaCl, 0.1mM EDTA, 50% glycerol ”storage buffer” at 4°C for at least 12 hours per change to remove free FeBabe. Store conjugated octamer at -20°C or at 4°C on ice until ready to purify.

B.3.3 Notes

1. Addition of glycerol to ”conjugation buffer” and ”storage buffer” in this protocol is specific to the FeBABE and other hydroxyl-radical producing labels. Glycerol serves as a free-radical scavenger preventing protein degradation. It can be omitted for other acyl-halide and alkyl-halide reagents.

2. In general, acyl-halide and alkyl-halide reagents are not susceptible to hydrolysis and therefore can easily be resuspended in most thiol-free buffers for addition to labeling reaction. Here, the FeBABE is resuspended in conjugation buffer containing 5% glycerol to prevent degradation by trace hydroxyl-radical. HEPES buffer is suitable for resuspending most of these reagents.
B.4 Labeling individual histones with acyl-halide and alkyl-halide functional groups

This protocol is optimized for labeling cysteines with methy-lysine analogs as described in Matthew D. Simon, et.al. Site specific installation of methy-lysine analogs into recombinant histones Cell 2007. March 9; 128(5):1003-1012. However, this method is valid for labeling with most acyl-halide and alkyl-halide functional groups.

B.4.1 Materials

- monomethy-lysine analog me1 = (2-chloroethyl)-methylammonium chloride OR
- dimethyl-lysine analog me2 = (2-chloroethyl)-dimethylammonium chloride OR
- trimethyl-lysine analog me3 = (2-bromoethyl) trimethylammonium bromide [Sigma-Aldrich 117196]
- Alkylation Buffer: 7.46g HEPES (310mM Sigma 4034), 17.98g HEPES sodium salt (690mM, Sigma H7006), 149mg D/L-Methionine (10mM, Sigma M9500); add 55ml water and briefly mix on stir plate. Slowly add 38.21g Guanidine hydrochloride (4M, Ultrapure MP biomedicals 820539) while stirring until completely dissolved. Adjust pH to 7.8 with HEPES or HEPES sodium salt. Bring volume to 100 mL with water. Filter and degas; make 1.5ml aliquots and flash freeze in liquid nitrogen. Store at -80°C and remove aliquots for single use just before labeling.
- 1M DTT (dithiothreitol)
- 37°C heat block
- Heated stir plate
- Digital thermocouple
- 1.5ml tube heat block insert
- flea stirbar
B.4.2 Procedure for monomethyl- and dimethyl lysine analog

1. To label with mono or dimethyl-lysine (me1 or me2) analog, resuspend 5-10mg of histone with cystiene at residue to be labeled in 900µl or 930µl alkylation buffer, respectively. Place in fresh 1.5ml centrifuge tube.

2. Add 20µl 1M DTT to 20mM final concentration and reduce for 1hr on 37°C heat plate.

3a. For me1, add 100µl of 1M of me1 label dissolved immediately before use in alkylation buffer. Allow reaction to proceed for 4hrs at room temperature. Add 10µl of 1M DTT (10mM final) and allow reaction to proceed for at least 10hr at room temperature.

3b. For me2, add 50µl of 1M of me2 label dissolved immediately before use in alkylation buffer. Allow reaction to proceed for 2hrs at room temperature. Add DTT (10µl of 1M) and incubate at room temperature for 30min. Treat reaction with an additional 50µl of 1M of me2 dissolved immediately before use in alkylation buffer. Allow to proceed for an additional 2hrs at room temperature.

4. In either case quench the reaction with 50µl of 14.2M BME

5. Extensively dialyze against 3mM BME. Lyophilize protein, resuspend in water, aliquot into 1x-5x aliquots, speed vac to dryness and store at -80°C.

B.4.3 Procedure for trimethyl-lysine analog

1. To label with trimethyl-lysine (me3) analog resuspend 5-10mg of histone with cystiene at residue to be labeled in 980µl alkylation buffer. Place in fresh 1.5ml centrifuge tube.
2. Add 20µl 1M DTT to 20mM final concentration and reduce for 1hr on 37°C heat plate.

3. Meanwhile, set 1.5ml tube heat block insert on top of heated stir plate. Place thermocouple in one of the test tube holes so that it is in good contact with the metal block. Set the heated stir plate to 60-65°C and allow to equilibrate until thermocouple reads 50°C.

4. Remove sample from 37°C heat block. Place flea bar in bottom of reaction mixture. Add 100mg of solid me3 analog reagent [(2-bromoethyl) trimethylammonium bromide]; place in block on heated stir plate and set stirrer to medium speed. Cover block with foil to shield from light.

5. Allow reaction to proceed for 2.5hrs at 50°C. Add 10µl of 1M DTT (10mM final) and allow reaction to proceed for another 2.5hrs at 50°C.

6. Quench the reaction with 50µl of 14.3M BME

7. Extensively dialyze against 3mM BME. Lyophilize protein, resuspend in water, aliquot into 1x-5x aliquots, speed vac to dryness and store at -80°C.

B.4.4 Notes

1. As these are methyl-lysine analogs with a sulfur replacing the γcarbon of the methylated lysine, the short hand nomenclature is KCme1, KCme2, and KCme3 for mono, di, and trymethylated analogs. Fore example trimethylated H3 Lysine 36 analog would be denoted as H3(KC-36me3).

2. Methyl groups appear to come off of the amine after labeled histones are resus-
pended and/or octamer is refolded into aqueous environment over the course of 1-2 months. Therefore it is advised to always make fresh histone octamer from lyophilized, labeled histones stored at -80°C. It is unknown how long these histones are stable at -80°C.
B.5 Histone octamer labeling by disulfide interchange

This protocol is applicable to labeling any histone complex with a disulfide interchange labeling group. Due to the reducing agents used for histone complex refolding and trace reducing agents present in chromatography equipment, this type of labeling is not amenable to labeling histones prior to refolding into histone complex.

B.5.1 Materials

- 0.2M TCEP [Tris(2-carboxyethyl)phosphine hydrochloride, Sigma C4706]; store unopened dry powder in refrigerator for up to 1 year. Dissolve 1.72g powder into 20ml water and pH to 7.1 with concentrated NaOH. Bring total volume up to 25ml; immediately aliquot into 0.5ml fractions and flash freeze in liquid nitrogen. Store aliquots at -80°C for up to 2 years. Thaw and use each aliquot once. Do not refreeze or use thawed TCEP after more than 6 hours.

- 3 - 300ml beakers of 5mM PIPES pH 6.1, 2M NaCL. Dissolve 1.62g PIPES [Piperazine-N,N'-bis(2-ethanesulfonic acid), Sigma P1851] and 116.82g NaCL in 900ml water. Stir to dissolve and slowly bring pH to 6.1 with concentrated NaOH. Solubility of PIPES is pH dependent and will not go into solution until above pH 5. Slowly add NaOH dropwise; there is a long relaxation time for pH to come back down after adding each drop of NaOH. Bring volume up to 1L; filter and degas; split between 3 beakers refrigerate at 4°C. Can be made on previous day.

- 2M TRIS pH8.5

- 23mm x 10cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1) OR

- 10mm x 20cm 6000-8000 MWCO dialysis tubing cut along the crease to create a single sheet of membrane (See Appendix A: Histone Unfolding and Gel Filtration, Note 1)
• Dialysis Chambers (See Table C.1 below)

• argon tank with regulator

• test tube bubbler with rubber stopper and inlet/outlet lines

• 1mm x 6in glass capillary

• 1.0mm inner diameter tubing (GE healthcare 19-0040-01)

• small gauge hypodermic needle

• parafilm

• disulfide exchange label i.e. (1-Oxyl-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl) Methanethiosulfonate (MTSL) spin label, FW = 264.3, Toronto Research Chemical O875000; N-[S-(2-Pyridylthio)cysteaminyl]ethylenediamine-N,N,N’,N’-tetraacetic Acid [Cyst(EDTA)NPS], FW = 460, Toronto Research Chemical P996250

• 1ml syringe

• DMF, anhydrous [N,N-dimethyl formamide, sigma-aldrich]

• VortexGenie vortexer

• 3 - 300ml beakers of 5mM PIPES pH 6.1, 2M NaCl, 0.5mM EDTA

B.5.2 Procedure

1. Following standard protocols for Histone Refolding (See Appendix A: Histone Octamer Refolding), refold a 3-6x scale (300µg - 600µg of each histone) of histone octamer, where the only histone containing a cystiene is the histone to be labeled. All other cystienes must be removed by site directed mutagenesis [i.e. H3(C110) must be expressed as H3(C110A) for labeling other histones]. Histone complexes should be purified by gel filtration after refolding, and refolded histone stocks should be stored at concentrations above 1.5 mg/ml.

2. Before labeling, aliquot suitable amount of refolded histone octamer from stock tube.
300-600µg (3-6nmol) of histone octamer contains 6-12nmol of free thiol that will be conveniently labeled by 100-200 fold molar excess of 1200nmol (typically 0.5mg) of labeling reagent.

3. Remove an aliquot of TCEP from -80°C and allow to thaw on ice for 10-20 minutes. Reduce histone octamer by adding TCEP pH 7.1 to 10mM final concentration and incubating on ice for 30 minutes.

4. Place histone sample into selected dialysis chamber:

- **If using PCR tubes as the dialysis chamber**, cut the camber as depicted in Figure B.1A. Next take a piece of 32mm x 5cm dialysis tubing and cut along the crease so that the dialysis tubing opens into a single sheet of dialysis membrane. Cut a 3in x 3in square of parafilm and place it on the benchtop; place the cap of the PCR tube on the parafilm. Place the sample in the cap of the PCR tube, hold membrane over the cap, and press the ring from the cut PCR tube down over the dialysis membrane as shown in Figure B.1B. Practice this step with buffer as the "sample" and then disassemble the chamber to ensure that the membrane is not being ripped during assembly.

- **If doing > 2000µg scale** use 10mm 6000-8000 MWCO dialysis tubing. Place the sample in the tubing and clip the ends with dialysis tubing clips

5. Dialyze chamber containing histone octamer by single dialysis against 3 changes of 300ml of 5mM sodium PIPES pH 6.1, 2M NaCl buffer for 2-3 hours per change. This promotes the free thiol state of the Cystiene residue and removes any reducing agents. Reclaim samples from dialysis chamber and place on in test tube on ice.

6. Measure the absorption of the octamer at OD$_{276nm,1cm}$. For histone octamer OD$_{276nm,1cm}$ = 0.45 gives a concentration of 1 mg/ml.

7. Dilute histone octamer to 1.5-3 mg/ml (14-28µM octamer complex = 28-56µM free thiol)
with 5mM PIPES pH 6.1, 2M NaCl buffer.

8. Set up argon purge system (Figure B.2) in deli fridge with 1/4in I.D. tubing from regulator reduced to 1mm I.D. tubing. Connect 1mm I.D. tubing from argon tank to bubbler inlet and connect 1mm I.D. tubing to bubbler outlet. Insert 1mm glass capillary tube into end of 1mm I.D. tubing. Turn on argon flow from tank so that argon bubbles through bubbler fast enough that there is no delay between successive bubbles. Use back of hand or arm to feel that argon is flowing out of each capillary at a gentle flow.

9. Remove test tube containing sample to be labeled, uncap, and wrap opening of tube with parafilm to seal tube. With small gauge needle, poke two holes in the parafilm (inlet and outlet). Place test tube rack in deli fridge by argon bubbler. Take glass capillary connected to argon bubbler outlet and gently insert capillary through one of the two holes in parafilm covering tube opening. Push capillary down until it is 0.5-1cm above sample. Do not allow capillary to go down into sample as sample will foam out of tube.

10. Purge sample for 15-20 minutes under argon at 4°C to remove O₂. When done quickly remove capillary and cap tube by pressing lid through parafilm; keep on ice. While sample is purging, remove aliquot of disulfide interchange labeling reagent from refrigerator/freezer and place at room temperature to equilibrate. Additionally, aliquot 500µl of 2M Tris pH 8.5 in a 1.5ml centrifuge tube.

11. When sample is done purging, immediately start bubbling 500µl 2M Tris pH 8.5 in argon for 5 minutes to remove O₂. Perform same procedure as above by wrapping opening of tube with parafilm, poking two holes with needle, and inserting capillary through one of the holes. However in this case push capillary all the way into the sample so that argon actively bubbles through. When done, cap tube by pressing lid through parafilm and place on rack at room temperature.
12. While Tris is bubbling; place test tube containing histone octamer in test tube rack at room temperature to equilibrate. Also remove 0.5ml aliquot of DMF from stock bottle using syringe and place in test tube at room temperature. Dissolve aliquot of labeling reagent (typically 1200nmol or 0.5mg) in 28µl of anhydrous DMF per to 22mM (~9 mg/ml) final concentration. For resuspending more than 0.5mg of label, add DMF in 14µl aliquots with thorough mixing between each addition to prevent micelle formation of hydrophobic labels.

13. Turn on vortexer to lowest setting so that it agitates and mixes sample, but does not violently swirl and churn sample. Add 5µl 2M Tris pH 8.5 per 100µl of histone sample to 100mM Tris final concentration to rapidly bring pH back up to 8.5. Immediately touch tube to vortexer to mix in HEPES. Immediately begin adding labeling reagent in DMF by 5µl additions; mix sample by touching to vortexer for 30 seconds between each addition. Continually add dye until all is used or has been added to a 100-200 molar excess of the free thiol containing histone.

14. When all reagent has been added, cover test tube in foil and incubate on rotisserie 1hr at room temperature. Then transfer rotisserie with sample to 4°C refrigerator and allow to incubate overnight.

15. Dialyze labeled histone octamer extensively against 3 changes of 300ml 5mM PIPES pH 6.1, 2M NaCl, 0.5mM EDTA for at least 6 hours per change. Store histone octamer at 4°C on ice.

B.5.3 Notes

1. Due to trace amounts of reducing agents (sulfites) in dialysis tubing, thoroughly rinse dialysis tubing stored in 1mM EDTA with water before use.

2. Additionally, it appears that there is always a fixed amount (~0.5µg) of octamer that loses label during dialysis, presumably due to trace reducing agents. It is advised to do
larger scale reconstitutions of 5-10μg of octamer to avoid appreciable losses. Additionally remove benzamidine from reconstitution buffer.

Figure B.1: Schematic Drawing for Histone Refolding. (A) Construction of a micro-dialysis button from a PCR tube; (B) Assembly of the micro-dialysis button with sample; and (C) General double-dialysis set-up. Note: the buffer inside the large 50mm dialysis tubing is the same as the buffer inside the smaller dialysis chambers. If using the 10mm 6000-8000MWCO dialysis tubing for sample, place clipped 10mm tubing inside the 50mm tubing filled with buffer just as would be done for the 50µl or 150µl button samples. Clip an air bubble between the middle and top clip of the 50mm dialysis tubing so that entire assembly floats.
Figure B.2: Argon purge system setup
Appendix C

Nucleosome Reconstitution and Purification

C.1 Nucleosome Reconstitution

C.1.1 Introduction and Key Parameters

Nucleosome reconstitution is the procedure by which purified DNA and histone complex (octamer, tetramer, or dimer + tetramer) are added under high NaCl (2M) conditions to keep DNA-histone interactions screened. The NaCl concentration is then slowly lowered to low levels (~1mM) allowing for DNA-histone interactions to form complete nucleosome, hexasome, or tetrasome complexes.

Nucleosomes, Tetrasomes, and Hexasomes This Protocol is a general guideline of appropriate DNA and histone amounts for quality nucleosome reconstitution and purification. Formation of tetrasomes using DNA and purified tetramer only or formation of tetrasome/hexasome/nucleosome mixtures using purified tetramer and heterodimer combinations is less precise and optimal conditions need to be found empirically for each DNA-histone complex. However, for short mononucleosome positioning sequences (< 247 base pairs) these complexes can be reproducibly formed using the same general procedures as for using full histone octamer.

Buffered Reconstitutions For many short mononucleosomal DNAs, buffering DNA is
not required as indicated in Table C.2. However, for most reconstitutions of nucleosome arrays and DNA with long linker DNA (i.e. linker DNA length is equal to or longer than nucleosome positioning sequence) a low affinity "buffering" DNA is required as a sink for excess histones and to prevent non-specific DNA-histone interactions. In these cases reconstitutions of DNAs shorter than 8-mer arrays (< 1050 base pairs) typically employ lambda DNA as the best choice. It is commercially available and during sucrose gradient purification lambda is completely separated form the desired nucleosome construct. For larger arrays (> 8mer), then short low affinity DNA fragments (147-600 base pairs) such as core particle DNA (cpDNA) from chicken erythrocytes or a PCR product from the Amp\(^r\) gene (168 or 201 base pair competitor DNA, See Appendix D) should be used to achieve full separation during purification. Alternatively, if the array DNA is restriction enzyme digested out of the plasmid in which it is cloned, the remaining plasmid piece may be conducive for digestion into 150-600 base pair fragments by DdeI restriction enzyme. The array DNA and digested fragments should then be phenol extracted to remove restriction enzymes. During reconstitution the fragments serve as buffering DNA for the array. Finally, if buffer DNA does not need to be removed, than any buffering DNA described above can be used.

**Optimization** For large arrays (trimucleosome and greater) and for long DNA’s with large amounts of linker DNA with respect to the positioning sequence(s), then it is advised to perform an initial test reconstitution titration by varying histone octamer ratios at [0.33, 0.5, 0.67, 0.75, 1.0] compared to total DNA. Then for bulk reconstitution, select which histone octamer to total DNA ratio gives the best saturated mononucleosomes and arrays without additional histones loading onto the linker DNA.

**Dialysis Methods** In general for most reconstitutions, use of double dialysis (sample chamber is placed in a secondary bag containing the same buffer conditions as the sample and is then dialyzed against the desired buffer) is sufficient to produce well-formed and well-positioned nucleosomes. In this setup, the rate of dialysis is highly non-linear within the sample chamber and goes to completion in 12-18 hours depending on how often dialysis
buckets are changed. For mononucleosomes and arrays in which the histone octamer does not contain fluorescent labels or large amounts of linker DNA, then use of double dialysis is sufficient and in general preferred for its ease of setup. In the case of fluorophore labeled histones or unusual DNA geometries with large amounts of linker DNA, then dialysis via peristaltic pumping is necessary. In this case, sample chambers are placed in a beaker of high NaCl buffer and then the concentration in the beaker is slowly changed over the course of 24 hours by pumping in low NaCl buffer and pumping out high NaCl buffer via peristaltic pump. This method appears to suppress hexasome formation in which some heterodimer is evidently lost either to the dialysis membrane or non-specific stickage to DNA.

C.1.2 Materials

- 0.5mM Benzamidine stock (BZA), filtered and degassed. Store 4° up to 6 months
- 100x TE (1M Tris, 100mM EDTA, pH 8.0), filtered and degassed
- 10x TE, 20mM BZA solution
- 5M NaCl (Sigma 71376 Ultrapure preferred), filtered and degassed;
- 200ml 2M NaCl, 0.5x TE, 1mM BZA made from above components before use, refrigerated or chilled on ice [for double dialysis or pumping reconstitution].
- 2-4 L Nalgene buckets with 0.5x TE, 1mM BZA made from above components before use, refrigerated [for double dialysis only].
- 2L 0.5x TE, 1mM BZA made from above components before use, refrigerated [for pumping dialysis only].
- two-channel peristaltic pump [for pumping dialysis only]
- 50mm x 50cm 6000-8000 molecular weight cut off dialysis tubing (See Appendix A: Histone Unfolding and Gel Filtration, Note 1).
- 23mm x 20cm 6000-8000 molecular weight cut off dialysis tubing (See Appendix A: Histone Unfolding and Gel Filtration, Note 1).
• 10mm x 20cm 6000-8000 molecular weight cut off dialysis tubing (See Appendix A: Histone Unfolding and Gel Filtration, Note 1).

• 50mm and 10mm dialysis tubing clips

• suitable dialysis chamber from Table C.1 below

• Purified high affinity DNA

• Low affinity buffering DNA typically lambda (life technologies), core particle DNA, or buffer DNA PCR product from Amp\textsuperscript{r} gene, see Appendix D

• Purified histone octamer, tetramer, or heterodimer
<table>
<thead>
<tr>
<th>Total Histone Scale</th>
<th>Sample Volume</th>
<th>Concentration Range</th>
<th>Dialysis Chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>250µg-500µg (3/4x-1.5x)</td>
<td>50µl</td>
<td>5 - 10 mg/ml</td>
<td>BrandTec 0.5ml PCR tube (781310)</td>
</tr>
<tr>
<td>500µg-2000µg (1.5x-5x)</td>
<td>200µl</td>
<td>2.5 - 10 mg/ml</td>
<td>Eppendorf 1.5mL PCR Tube (022364120)</td>
</tr>
<tr>
<td>&gt; 2000µg (&gt;5x)</td>
<td>&gt;500µl</td>
<td>1 - 10 mg/ml</td>
<td>10mm 6000-8000 MWCO Dialysis tubing</td>
</tr>
</tbody>
</table>

Table C.1: Dialysis Chambers
C.1.3 Procedure

1. Thoroughly rinse with water all dialysis tubing required for reconstitution (50mm for double dialysis, 23mm and/or 10mm for dialysis chambers) to remove storage buffer (1mM EDTA). Additionally, if using PCR tubes as dialysis chambers cut off 2cm squares and for each square cut open along one of the creases so that tubing opens into a single sheet of dialysis membrane. Place all tubing in beaker of 2M NaCl, 0.5x TE, 1mM BZA on ice or refrigerator.

2. Next determine the amount of DNA (high affinity and buffering) and Histone octamer required. Use the following Table C.2 to assist in selection of correct ratios.
   3. Next, on ice in a PCR or falcon tube combine the following:
      - 5M NaCl to 2M final concentration
      - 10x TE, 20mM BZA solution to 0.5x TE, 1mM BZA final concentration
      - DNA (selected from Table C.2)
      - Purified Histone Octamer (selected from Table C.2)
      - Water to bring volume up to scale suitable for a chamber listed in Table C.1.

4. Place samples into selected dialysis chambers: For using PCR tubes as dialysis chambers, prepare tubes as depicted in Figure C.1A-B.
   - Using animal nail clippers, cut off the cap/tube hinge. Then place capped tube into cutters and cut off tube about 2-3mm from bottom of cap; discard bottom of tube. Separate cap from top of tube which now serves as a rim to seal the dialysis membrane over the cap.
   - Place a sheet of Parafilm on the lab bench and place the rim and cap on the Parafilm. Label cap then invert so the well of the cap is facing upward.
Table C.2: **Nucleosome and Array Reconstitution Parameters.** 

<table>
<thead>
<tr>
<th>HA DNA-length</th>
<th>HA-DNAb</th>
<th>B-DNAb</th>
<th>HO : {HA-DNA : B-DNA}\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>147-167 (S or A)</td>
<td>mononuc</td>
<td>none</td>
<td>0.90 : {1 : 0}</td>
</tr>
<tr>
<td>167-197 (S or A)</td>
<td>mononuc</td>
<td>none</td>
<td>0.82 : {1 : 0}</td>
</tr>
<tr>
<td>197-217 (S or A)</td>
<td>mononuc</td>
<td>none</td>
<td>0.71 : {1 : 0}</td>
</tr>
<tr>
<td>217-247 S</td>
<td>mononuc</td>
<td>lambda</td>
<td>0.63 : {1 : 0}</td>
</tr>
<tr>
<td>217-247 A</td>
<td>mononuc</td>
<td>lambda</td>
<td>0.33 : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>&gt;247 (S or A)</td>
<td>mononuc</td>
<td>lambda</td>
<td>x.xx : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>300-350</td>
<td>dinuc</td>
<td>lambda</td>
<td>0.88 : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>350-400</td>
<td>dinuc</td>
<td>lambda</td>
<td>0.75 : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>400-450</td>
<td>dinuc</td>
<td>lambda</td>
<td>0.67 : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>&gt;450</td>
<td>dinuc</td>
<td>lambda</td>
<td>x.xx : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>&gt;450</td>
<td>trinuc</td>
<td>lambda</td>
<td>x.xx : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>&gt;600</td>
<td>tetranuc</td>
<td>lambda</td>
<td>x.xx : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>&gt;900</td>
<td>hexanuc</td>
<td>lambda</td>
<td>x.xx : {1 : 2}\textsuperscript{d}</td>
</tr>
<tr>
<td>&gt;1200</td>
<td>octanuc</td>
<td>cpDNA\textsuperscript{e}</td>
<td>x.xx : {1 : 1}\textsuperscript{d}</td>
</tr>
<tr>
<td>&gt;1350</td>
<td>N-mer (N&gt;8)</td>
<td>cpDNA\textsuperscript{e}</td>
<td>x.xx : {1 : 1}\textsuperscript{d}</td>
</tr>
</tbody>
</table>

**Notes:**

- HA-DNA is high affinity DNA (i.e. 601 or 5S); S denotes symmetric DNA about positioning sequence, A denotes asymmetric.  
- B-DNA is buffering DNA.  
- HO is histone octamer. Histone octamer mass is given as a ratio with respect to total DNA (HA-DNA plus B-DNA). For example a HO : HA-DNA : B-DNA ratio of 0.9 : 1 : 2 contains 0.9\(\mu\)g of histone octamer per 1ug of total DNA mass. Of the total DNA mass there is 1ug of HA-DNA per 2\(\mu\)g of B-DNA. 
- Perform initial test reconstitution titration of histone octamer against DNA by varying histone octamer ratios at [0.33, 0.5, 0.67, 0.75, 1.0] compared to total DNA. 
- Can also use DdeI digested pUC19 plasmid that gives 147-600pb fragments.

- Remove sample from ice and pipette into the center of the cap.

- Take a piece of 23mm dialysis tubing that has been cut open to form a single sheet and hold over top of cap containing sample.

- Take cut rim from PCR tube and in one clean, gentle motion, press rim down over membrane and cap to seal membrane over cap; trim off excess membrane and place in 2M NaCl with 0.5x TE, 1mM BZA on ice.

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For using 10mm dialysis tubing, fill with sample and clip as usual. Place in 2M NaCl with 0.5x TE, 1mM BZA on ice.

5a. To perform double dialysis:

- Double clip end of 50mm dialysis bag and add 50ml of 2M NaCl, 0.5x TE, 1mM BZA to bag.

- Place samples in bag and slightly compress bag so that buffer raises a few centimeters above its normal level before compression. Clip bag right at the buffer level so no air bubbles are trapped in the dialysis bag where the sample are. This prevents bubbles from getting into the dialysis caps and blocking dialysis.

- Next place another clip at the top of the bag so that an air bubble is formed between the clip placed at the buffer level and the top of the bag. This will allow the bag to float in the dialysis buffer (Figure C.1C).

- Place dialysis bag into 4L bucket of 0.5x TE, 1mM BZA on stir plate and dialyze at least 8 hours. Change into second 4L bucket and dialyze at least 12 hours, preferable overnight. If samples contain fluorescent molecules, cover bucket with aluminum foil.

5b. To perform dialysis by pumping:

- Place dialysis chambers in beaker with 200ml 2M NaCl, 0.5x TE, 1mM BZA so that they float. For PCR tube chambers, place so that membrane side is facing down and in full contact with buffer. Make sure air bubbles are not trapped between buffer and membrane.

- Place beaker with samples on stir plate with small stir bar and stir slowly so that chambers are not inverted by stirring action.
- Using a two-channel peristaltic pump, place and clip the inlet of one line into the 2L beaker of 0.5x TE, 1mM BZA and place and clip the outlet at the bottom of the beaker containing sample chambers in 200ml high salt buffer. Then, place and clip the inlet of the second line at the opposite side of the bottom of the beaker containing sample chambers in 200ml high salt buffer, and place and clip the outlet into a 2L waste beaker.

- Pump at 0.8 ml/min for 36 hours until almost all of the low salt buffer in the 2L beaker has been pumped through the beaker containing sample chambers.

6. To reclaim reconstituted samples, remove caps and 10mm dialysis chambers from dialysis container.

- For 10mm dialysis tubing, unclip and remove sample with a pipette and transfer to a tube on ice.

- For PCR caps, place a piece of Parafilm on the benchtop and place cap on parafilm with membrane facing up. Using a pipette tip suck off any remaining buffer on outside of membrane and discard. Next take pipette with new pipette tip and depress piston. Puncture membrane along edge of rim and carefully suck out all of sample from the cap. Transfer to a tube on ice.

10. Store reconstitutions on ice until ready to purify.
Figure C.1: Schematic Drawing for Histone Refolding. (A) Construction of a micro-dialysis button from a PCR tube; (B) Assembly of the micro-dialysis button with sample; and (C) General double-dialysis set-up. Note: the buffer inside the large 50mm dialysis tubing is the same as the buffer inside the smaller dialysis chambers. If using the 10mm 6000-8000MWCO dialysis tubing for sample, place clipped 10mm tubing inside the 50mm tubing filled with buffer just as would be done for the 50µl or 150µl button samples. Clip an air bubble between the middle and top clip of the 50mm dialysis tubing so that entire assembly floats.
C.2 Sucrose Gradient Purification

Sucrose gradient purification is the means by which reconstituted nucleosomes and arrays are purified from free DNA and potentially unwanted byproducts. In general this method can separate nucleosomes and arrays from free high affinity positioning DNA and buffering DNA, providing the buffering DNA or buffering DNA-containing nucleosomes are not of similar mass to the nucleosome or array wanting to be purified. Additionally, this procedure can resolve but not fully separate multiple nucleosome positions and different levels of array saturation. Therefore, optimization of nucleosome positioning and array saturation by reconstitution techniques is necessary before sucrose gradient purification to achieve high-quality, well-define samples.

C.2.1 Materials

- 15ml Gradient Maker (GE Healthcare SG15)
- peristaltic pump
- stir plate
- flea stir bar
- 5% (w/v) sucrose with 0.5x TE, filtered and degasses; store in refrigerator up to 6 months.
- 30% (w/v) sucrose with 0.5x TE, filtered and degasses; store in refrigerator up to 6 months.
- 40% (w/v) sucrose with 0.5x TE, filtered and degasses; store in refrigerator up to 6 months.
- Beckman SW41 swinging bucket rotor
- Beckman Pollyallomer 14mm x 89mm tubes (cat no. 331372)
- Fraction collector
• Amicon 30 (Millipore)
• 1mm x 6in glass capillary
• 1.5ml PCR tubes
• 0.5x TE, refrigerated (use ultrapure filtered through Amicon 5 if using nucleosomes for AFM)

C.2.2 Procedure

1. Set up the gradient maker apparatus as shown in Figure C.2 below.

   - Place gradient maker on stir plate with outlet valve (green) open and valve between chambers (red) closed.

   - Place flea stir bar in chamber near outlet and turn stir plate to maximum speed.

   - Connect tube from outlet of gradient maker to peristaltic pump and then place end of tube at bottom of Beckman 14mm x 89mm centrifuge tube.

   - Set peristaltic pump to flow at 1 ml/min.

2. Place higher density sucrose (see Table C.3) in chamber closest to outlet and place lower density sucrose in chamber farthest from outlet.

   3. In quick succession turn on pump, open center valve (red) between chambers, and gently push with finger on top of chamber farthest from outlet to expel any air between chambers.

4. As sucrose drips into centrifuge tube, slowly raise tubing so that it is always above the level of the sucrose rather than below. This will ensure good layering of the gradient without violent mixing compared to if sucrose were to drip down all the way from the top of the
<table>
<thead>
<tr>
<th>Length</th>
<th>Type</th>
<th>Hi-Density</th>
<th>Vol</th>
<th>Lo-Density</th>
<th>Vol</th>
<th>Run Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>147-197 S or A</td>
<td>mononuc</td>
<td>30%</td>
<td>5.3</td>
<td>5%</td>
<td>5.9</td>
<td>22hrs</td>
</tr>
<tr>
<td>197-247 S</td>
<td>mononuc</td>
<td>30%</td>
<td>5.3</td>
<td>5%</td>
<td>5.9</td>
<td>24hrs</td>
</tr>
<tr>
<td>197-247 A</td>
<td>mononuc</td>
<td>35%</td>
<td>5.3</td>
<td>5%</td>
<td>5.9</td>
<td>24hrs</td>
</tr>
<tr>
<td>&gt;247 S or A</td>
<td>mononuc</td>
<td>40%</td>
<td>5.2</td>
<td>5.25%</td>
<td>6.0</td>
<td>22hrs</td>
</tr>
<tr>
<td>200-450</td>
<td>dinuc</td>
<td>40%</td>
<td>5.2</td>
<td>5%</td>
<td>6.0</td>
<td>18hrs</td>
</tr>
<tr>
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<td>dinuc</td>
<td>40%</td>
<td>5.2</td>
<td>5%</td>
<td>6.0</td>
<td>???</td>
</tr>
<tr>
<td>&gt;450</td>
<td>trimuc</td>
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<td>5.2</td>
<td>5%</td>
<td>6.0</td>
<td>18</td>
</tr>
<tr>
<td>&gt;600</td>
<td>tetranuc</td>
<td>40%</td>
<td>5.2</td>
<td>%</td>
<td>6.0</td>
<td>17</td>
</tr>
<tr>
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<td>5%</td>
<td>6.0</td>
<td>16</td>
<td></td>
</tr>
<tr>
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<tr>
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<td>5%</td>
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<td>5.2</td>
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<td>&gt;2200</td>
<td>15-16-mer</td>
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<td>5.2</td>
<td>5%</td>
<td>6.0</td>
<td>9</td>
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<tr>
<td>&gt;2400</td>
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<td>5%</td>
<td>6.0</td>
<td>8</td>
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<tr>
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<td>5.2</td>
<td>5%</td>
<td>6.0</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Table C.3: Sucrose Gradient Concentration and Centrifuge Times. S denotes symmetric DNA about positioning sequence, A denotes asymmetric.
centrifuge tube. As sucrose drips in, one should see gradients in density depress below the meniscus of the sucrose gradient and the rise to the top. Each sequential drip of sucrose should occur after the previous drip has risen to the top of the gradient.

5. When gradient is completely poured, mass tube to between 13.7g and 14.0g. To add mass, gently layer 5% sucrose on top with pipette; to remove, gently suck off from top of gradient. The meniscus of the gradient should be between 10mm and 5mm from the top. Greater than 10mm leads to tube collapse; less than 5mm leads to sucrose wicking out of tube during run. Each pair of tubes for centrifugation must be massed to within 0.05g.

6. Incubate sucrose gradients in refrigerator for at least 30 minutes and no more than 4 hours. Do not agitate gradients during incubation process. Also at this time, equilibrate ultracentrifuge, SW41 rotor, and rotor buckets to 4°C.

7. To apply sample to gradient, remove sample from PRC tube with pipette and hold tip about 2-3mm above sucrose gradient. Apply sample drop by drop to prevent mixing with the gradient. A maximum of 500µl can be applied to the column and still maintain resolution. Less than 250µl is preferred.

8. Place gradients into Beckman swinging buckets in pairs [i.e. bucket 1 and 4, bucket 2 and 5, bucket 3 and 6] and tighten down caps. Hang buckets on SW41 rotor.

9. Place SW41 rotor with buckets into ultracentrifuge. Centrifuge at 41000RPM, 4°C for the time listed in Table 1.

10. After centrifugation is complete, remove rotor from centrifuge and place buckets containing gradients in refrigerator until ready to fractionate (no greater than 2 hours).

11. Set up fraction collector in deli-refrigerator as depicted in Figure C.3.
- Connect 1mm glass capillary to end of 1mm I.D. tubing and clamp tubing with a 90 degree bracket where capillary is inserted so that capillary protrudes down between test tube clamp fingers.

- Connect other end of tubing to pump inlet.

- Connect pump outlet to fraction collector.

- Label and place 30 - 1.5ml PCR tubes in fraction collector

- Set pump to 1 ml/min; set fraction collector to collect 0.4 minute fractions

12. With pump off, remove sucrose gradient from bucket. Gently slide gradient up between test tube clamp so that capillary slides down along the side of the 14mm x 89mm all the way to the bottom of the gradient. Try to avoid mixing of the gradient with the capillary as much as possible. Clamp the sucrose gradient in place with the test tube clamp.

13. Turn on the pump at 1ml per minute. When sucrose begins to elute from pump outlet into fraction collector, turn on fraction collector and collect 30 - 0.4ml fractions.

14 When done, transfer fractions to ice and clean pump lines with water before applying next gradient.

15. Determine peak fractions containing purified nucleosomes. Peak fractions will typically occur around fractions 4-12 (near bottom of gradient):

- If samples are fluorescent labeled mononucleosome that are not asymmetric and greater than 200 base pairs or fluorescent labeled arrays, then apply 30-50µl of each fraction to 384 well Costar plate and measure fluorescence from each sample using a fluorescence plate
reader.

- If a plate reader is not available or if labeled asymmetric mononucleosomes are greater than 200 base pairs, run mononucleosomes on a 5% native gel; dinucleosomes on a 4% native gel; for all other arrays digest away histone octamer with 1 mg/ml proteinase K + 0.1% SDS for 15 minutes at 37°C; heat kill 5 minutes at 65°C. Run on a 0.8% agarose gel and image by Typhoon 8600 imager. As nearly all DNA on gel will be from nucleosomal DNA due to optimization of reconstitution conditions for array saturation, the saturated arrays will correspond to the peak in the DNA bands on the gel.

- If samples are unlabeled, run mononucleosomes on 5% native gel and dinucleosomes on 4% native gel. Ethidium stain and image by UV trans-illuminator. For all other arrays, digest away histone octamer with 1 mg/ml proteinase K + 0.1% SDS for 15 minutes at 37°C; heat kill 5 minutes at 65°C. Run on a 0.8% agarose gel with ethidium and image by UV trans-illuminator.

16. Pool peak fractions and concentrate in an Amicon 30 at 3000g, 30 minutes, 4°C. Wash 3 times with 3ml of refrigerated 0.5x TE at 3000g, 30 minutes, 4°C. The last centrifugation run may take 46-60 minutes to concentrate sample to <100µl.

17. Reclaim sample and store on ice at 4°C.
Figure C.2: Sucrose Gradient Maker Setup

Figure C.3: Sucrose Gradient Fraction Collector Setup
Appendix D
DNA and Oligo Sequences

All oligos are given in 5′ to 3′ orientation. For alignment purposes the reverse primers given here are the reverse complement of the actual primer that was synthesized.

D.1 DNA constructs from pMp2 Plasmid

Mp2 nucleosome positioning sequence underlined

tgtaaaacga cggccagtga attccggtg aacaggttgc gggatgatca ctgcagaagc ttggtgccgg ggc-
cgctcaa ttggtcgtag caagctctgg atccgcttga tcgaacgtac gcgctgtccc ccgcgtttta aacgc-
caagg ggattactcc ctagtctcca ggcacgtgtc agatatatac atcctgtcgg accgagctcg ataag-
gacga gcccgacata gatctgcatg caagtt

D.1.1 Mp2-147

Forward Primer: ctgcagaagcttggtgccgg

Reverse Primer: ccaggcacgtgtcggaccgatctgtgctecgg accagctcg ataag-gacga gcccgacata gatctgcatg caagtt

D.1.2 Mp2-192

Forward Primer: agtgaattccggtgaacaggtcg Reverse Primer: tacatctgtgaccgccgag
D.1.3 Mp2-187
Forward Primer: acgaggtgcggggatgatca
Reverse primer: ggacccgagctcgataagga

D.1.4 Mp2-247
Forward Primer: tgtaaaacgacggccagtgaattccggttg
Reverse primer: agcccgacatagatctgcatgcaagctt

D.2 DNA constructs from *L. variegatus* 5S rDNA p5S plasmid

*L. variegatus* 5S rDNA nucleosome positioning sequence underlined

tgttaaaacgagccagtgaattccggttg acgaggtgcggggatgatca ttccaggat ttataagccg atgacgctcat aacatccctg accetttaaa tagctaact ttcatcaage aagagctac gaccatacctatgtgaatat accgggttc gtcgcataacgcaagtcaag cagcatagggctcggttc aggacagctc ataaggacgag ccgcacata gatctgcatg caagctt

D.2.1 5S-147
Forward Primer: ttccaggattataagccgatgacgtcat
Reverse Primer: caagcagcatagggctcggtt

D.2.2 5S-192
Forward Primer: agtgaattccggttcggaggtg
Reverse Primer: ggcctcgttcggacagcage
D.2.3 5S-187

Forward Primer: acgaggtgcggggatgatca

Reverse primer: ggaccgagctcgataagga

D.2.4 5S-247

Forward Primer: tgtaaaacgacggccagtgaattccggttg

Reverse primer: agcccgacatagatctgcatgcaagctt

D.3 Dinucleosome DNA construct from plasmid pMp2-Mp1

Mp2 sequence is 1st underline; Mp1 sequence is 2nd underline

tgtaaaacgacggccagtga attccggttg acgaggtgc gggatgatca ctgcagaagc ttggtgccgg ggc-
cgctcaa ttggtcgtag caagctctgg atccgcttga tcgaacgtac gcgctgtccc ccgcgtttta aacgc-
caagg ggattactcc ctagtctcca ggcacgtgtc agatatatac atcctgtctgg accgagctcc tccgat-
ca tccgcccttg gagaatcttg gtgccgaagc cgctcaattg gtcgtagcaa getetaacc cgettaaacg
cacgtaagg gcgtgccctg ccgttttaacc gcacagagga ttactccccca gtetecaggc acgcgtcaga
tatatatac ctgtgcagtg attgacgccg acatagatct gcatgcaagc tt

Forward Primer: tgtaaaacgacggccagtgaattccggttg

Reverse primer: agcccgacatagatctgcatgcaagctt

380
D.4 186-192 base pair DNA constructs from *X. laevis* 5s rDNA and *S. cerevisae*a pho5 and gal4 genes

D.4.1 5Sx-192

*X. laevis* 5S rDNA nucleosome positioning sequence underlined

tgtaaaacga cggccagtga attcgggttg acgaggtgcg gggatgatca agtactaacc aggcccgacc ct-
gcttggtct tcggagatca gacgatagc ggcaacctca ggggtggatg gcggtaggac agcacaaggc
tgacttttcc ttccctggtg ccgtcagcggcttcttg gggggggcct ccctccctccc aatgaccagggc
tgacttttcc ttccctggtg ccgtcagcggcttcttg gggggggcct ccctccctccc aatgaccagggc
gcggatcctgt ctaggacga ccggacgagct
gcgttggttc gcggagcgg

Forward Primer: gtgaattccggttgacgaggtg

Reverse Primer: catgccagccggacgag

D.4.2 pho5-N1

*S. cerevisae*a pho5 N1 nucleosome positioning sequence underlined

aacattggta atctcgaatt tgcctgactc atttgtttgtg ttcttttctc ttcgagagct tgaagccctc
taaacgctg cttacccgaa cttcagccgg ggtatagtc ctgggccagcc tgggcttgag cccgacg

Forward Primer: aacattggta atctcgaatt

Reverse Primer: tgggtatagtc ctgggccagcc tgggcttgag cccgacg

D.4.3 pho5-N2

*S. cerevisae*a pho5 N2 nucleosome positioning sequence underlined

aacattggta atctcgaatt tgcctgactc atttgtttgtg ttcttttctc ttcgagagct tgaagccctc
taaacgctg cttacccgaa cttcagccgg ggtatagtc ctgggccagcc tgggcttgag cccgacg

Forward Primer: aacattggta atctcgaatt

Reverse Primer: tgggtatagtc ctgggccagcc tgggcttgag cccgacg
caagtaaggt gaccaatttg ataatttgcc atgtgctgatc tcttccgaaa cagggaccag aatcataaat tttaaattgt ctagtcccac gtgtgagttgc caagggattga tcggattcatt tagattcatt tagagattgc ctattcaatt aactctttta atcaatttta ctcttttggc tegttcggga ccgacg

Forward Primer: caagtaaggtgaccaattt

Reverse Primer: ggctcggttcggaccgacg

D.4.4 gal4

*S. cerevisae*a gal4 nucleosome positioning sqeuence underlined

tgctcattgc tatatttgaag tacggattag aagccgccga ggcggccgaca gcctccgac ggaagactct cctcgtcggcc tctcgtcttt caceggctgct gcggctaaac gcagatgtgc cctcgccgcca cactgctccg aacaataag attctacaat actagctggc teggtcggga cccgacg

Forward Primer: tgctcattgctatttgaag

Reverse Primer: ggctcggttcggaccgacg

D.5 Buffer DNA

D.5.1 201bp buffer DNA

AT rich region in *Amp*\(^{r}\) gene of puc19

cattgtacg gggctgacgc attcgtggaa cggaaactca ggttaagggc tttgggtcat gagattatca aaaaaggatct tcacatcgact ctttaaat taaaaaatgaa gtttaaatc aatctaaagt atatatgagt aaacttggtc tcagttac

cattgtaaa tcagtgaggc acctatctca gcgatctgtc t

Forward Primer: cttttctacg gggctgacgc

382
Reverse Primer: cctatctcagctgtctgtct

D.5.2 168bp buffer DNA

region in Amp\(^{\prime}\) gene of puc19 acgttgtttgc cattgctaca ggcategtgg tgcacgctc gtcgttttgt atg-
gtctct ctgctcgg ttccttcaacga tcagcggcag ttacatgtgct ccccatgttg tgcataaaag cggtagtcg ctctgt-
gtctct cctgctct tccagaatg tttggccg

Forward Primer: acgttgtttgcccattgctacaggc

Reverse Primer: gtcctccgatctgttcagaagtaagttggccg
Appendix E

Electrophoresis Techniques

This section details all of the electrophoresis techniques employed in this work. It is split into three main categories: Acrylamide, Agarose, and SDS-PAGE methods. Applications for each method are detailed with the respective protocol.

E.1 Acrylamide Gel Techniques

E.1.1 Pouring an Acrylamide Gel

All acrylamide gels listed below, except for the composite gel, are poured in the following fashion and are conducive for fluorescent quantitation:

Take a large (18.5cm x 19.7cm) and small (16.0cm x 19.7cm) glass plate (Labrebco E11074010) and clean with 1M KOH. If glass plates are exceptionally dirty, soak in 1M KOH overnight.

Rinse thoroughly with water and set vertically against gel rig (Whatman V16 or V16-2, Labrepco E21070010 or E31070010) to drain. Using a large kimwipe gently dry. Do not rub excessively as static electricity build-up will cause lint to adhere to glass. Inspect the small glass plate to see if one side is treated with ”gel slick” (Cambrex 50640); there should be an arrow on the plate pointing toward the side that is treated. If plate produces friction when gently rubbed with a Kimwipe, then apply ~500μl of gel slick solution to the side to be treated and thoroughly spread solution over surface with Kimwipe until completely dry.
Return dried glass plate to standing vertically against the rig.

Place a fresh large Kimwipe on benchtop and place large plate on Kimwipe

Take a set of 0.8mm spacers (Labrepco E41077011), two vertical with foam blocks and one horizontal with interlocking tabs, and interlock vertical spacers with horizontal spacers to form a U. Place the small plate on top with the gel slick side facing inward toward the large plate.

Carefully pick up sandwiched assembly and set along bottom (horizontal spacer) edge. Hold sandwich together with one hand and with the other adjust glass plates and spacers so that the edges of each glass plate and spacers are all evenly aligned with one another along the three sides containing the spacers.

Carefully holding aligned plates in place, take large binder clips and clamp sandwich together with three clips along each side containing spacers to secure.

With nitrogen air line, blow out space between glass plates to remove any lint or dust.

Set gel vertically on benchtop and make appropriate gel mix for the column (see gel types and recipes below). Also get out an appropriate 0.8mm comb (10 well, 12 well, 20 well), two additional binder clips, motorized pipetter with 25ml serological pipette, and TEMED (Tetramethylethylenediamine, Sigma T9281)

In quick succession: - Add TEMED to acrylamide gel mixture,

- Cap and gently invert several times to mix without aerating mixture

- Pipette up mixture with motorized pipetter; with one hand hold assembled plates
vertically and slightly tilted toward one of the vertical spacer sides

- With the motorized pipetter, place pipette tip containing gel mixture in the corner of the gap between the glass plates a the vertical spacer that is tilted downward

- In a steady stream pipette the gel mixture into the sandwich so that it flows down along the vertical spacer preventing entrainment of air bubbles (if an air bubble gets in the gel, stop pipetting briefly until bubble rises to top and pops)

- As the plates fill with gel mixture toward the top, tilt sandwich back upright so the top of the sandwich is parallel with the benchtop in order to completely fill the plates

- Place comb parallel to gap between plates and touch just the bottom of combs to gel mix; move comb around a little to dispel and air that might be trapped between the bottom of the combs and the gel mix. When no bubbles are observed, push combs down into gel mix about 2/3-3/4 of the way

- Take binder clips and clip glass plates together where they are in contact with the combs to dispel any acrylamide from between glass plate and comb; wick out any excess acrylamide above level of glass plates with a Kimwipe

Allow gel to thoroughly polymerize for ~10 minutes

Disassemble all binder clips from glass plates and gently pull out horizontal spacer

Place glass plates with polymerized gel into single (V16) or double (V16-2) gel rig and clip gel into rig with two binder clips on each side

Pour appropriate running buffer into top and bottom reservoirs of gel rig and purge out
any air bubbles in the gap between the glass plates left behind from the horizontal spacer with a needle and syringe.

At this point gently pull out combs, this prevents air suction from collapsing wells. Pure out any unpolymerized acrylamide and air bubbles from wells to prevent secondary polymerization of acrylamide in bottom of wells leading to loss of resolution.

The Gel is now ready to prerun and resolve samples. Poured gels assembled in buffer filled rigs can be kept up to 1 day before using except in the case of Urea-danaturing gels.

### E.1.2 Native Acrylamide Gels

Native acrylamide gels are conducive for resolving dsDNA lengths up to 500 base pairs, and mononucleosomes and dinucleosomes reconstituted on DNA’s up to 500 base pairs. In certain instances (e.g. LexA binding) the are conducive for resolving DNA-protein and nucleosome-protein complexes. Acrylamide with a linear:bis ratio of 29:1 is used at concentrations of 4% for resolving dinucleosomes and 5% for resolving mononucleosomes. These gels are generally run at room temperature at 15-20V/cm in a Tris-borate buffer which can be supplemented with 0.5-1mM magnesium for nucleosome stability or up to 10mM EDTA (20mM NaCl) to prevent DNA-protein/nucleosome-protein interactions and chelate magnesium present in the sample. Addition of NaCl above 20mM to electrophoresis buffer drastically increases gel current and distorts resolution of sample. Additionally, samples loaded onto the gel containing more than 300mM NaCl are significantly distorted. If glycerol above 1% is present in the sample, then a Native Glycerol-Acrylamide gel should be used.

**Materials:**

- 40% 29:1 linear:bis acrylamide (National Diagnostics EC852)
- 5x TBE or suitable electrophoresis buffer
- 10% APS (Ammonium Persulfate) solution less than 1 week old
TEMED (Tetramethylethylenediamine, Sigma T9281) less than 6 months old

6x ficol w/o Oragne G

Procedure:

For 30ml (one gel) of 4% or 5% native gel mixture add:

- 3ml (4% final) or 3.75ml (5% final) of 40% 29:1 acrylamide,
- 1.8ml 5x TBE (0.3x final; 1x = 90mM Tris, 90mM boric acid, 2mM EDTA, pH 8.3)
  or suitable electrophoresis buffer
- 300µl 10% APS (0.1% final)
- 24.9ml (for 4%) or 24.15ml (for 5%) of water

For 2 gels make 45ml or 1.5 times the above recipe.

Add 45-75µl of TEMED just before pouring gel.

Pour the gel and assemble into the gel rig according to procedures detailed above and fill gel reservoirs with same electrophoresis buffer as placed in gel (i.e. 0.3x TBE)

Prerun the gel at 300V (20V/cm) for 60 minutes until current running through gel drops by half of the starting current to ensure polymerization agents have been run out of gel.

Add 6x ficol to 1x final concentration to samples for loading onto gel.

Before loading samples, purge wells with needle and syringe to remove any air bubbles and unpolymerized acrylamide that may have formed in the wells during the prerun.

With gel running at 300V, apply sample to gel; 20 well combs require at least 5µl of sample
per well, 10-12 well combs require at least 10µl of sample per well.

Run gel for 60-90 minutes at 300V to resolve bands.

### E.1.3 Native Glycerol-Acrylamide Gels

Many enzymatic and chromatin remodelling/disassembly/exchange assays require glycerol for optimum protein performance. Glycerol concentration above 1% can cause significant distortion and loss of resolution in DNA and nucleosome bands. To eliminate these issues, 5% glycerol can be added to the gel mix before polymerization. Additionally the boric acid in TBE electrophoresis buffer must be replaced by taurine (TTE buffer) as boric acid esterifies with glycerol to form a species that distorts DNA and nucleosome bands.

**Materials:**

- 40% 29:1 linear:bis acrylamide (National Diagnostics EC852)
- 20x TTE buffer (National Diagnostics EC-871)
- 50% glycerol, (Sigma 191612; replaced by 9012)
- 10% APS (Ammonium Persulfate) solution less than 1 week old
- TEMED (Tetramethylethylenediamine, Sigma T9281) less than 6 months old

**Procedure:**

For 30ml (one gel) of 4% or 5% native gel mixture add:

- 3ml (4% final) or 3.75ml (5% final) of 40% 29:1 acrylamide,
- 0.45ml 20x TTE (0.3x final; 1x = 90mM Tris, 30mM taurine, 0.5mM EDTA, pH 8.3)
- 3ml 50% glycerol (5% final)
- 300µl 10% APS (0.1% final)
- 23.25ml (for 4%) or 22.5ml (for 5%) of water
For 2 gels make 45ml or 1.5 times the above recipe.

Add 45-75µl of TEMED just before pouring gel.

Pour the gel and assemble into the gel rig according to procedures detailed above and fill gel reservoirs with 0.3x TTE.

Prerun the gel at 300V (20V/cm) for 60 minutes until current running through gel drops by half of the starting current to ensure polymerization agents have been run out of gel.

Typically no loading buffer needs to be added to the sample as it already contains ~5% glycerol.

Before loading samples, purge wells with needle and syringe to remove any air bubbles and unpolymerized acrylamide that may have formed in the wells during the prerun.

Change voltage to 200V (15V/cm), apply sample to gel; 20 well combs require at least 5µl of sample per well, 10-12 well combs require at least 10µl of sample per well.

Run gel for 90-120 minutes at 200V to resolve bands.

**E.1.4 High resolution acrylamide or Glycerol-Acrylamide gels**

The relative mobility of different nucleosomes positions on a particular DNA substrate is known to be affected by the linear:bis acrylamide ratio used for polymerizing a gel. A ratio of 59:1 linear:bis acrylamide results in mononucleosomes positioned toward the end of a DNA substrate to migrate faster than those positioned toward the center [95]. However, due to the lower amount of bis-acrylamide, gels must be run at 4°C to prevent matrix collapse and gel distortion. Therefore, while optimal, this method can prove rather challenging and time-consuming.
Materials:

- 40% 29:1 linear:bis acrylamide (National Diagnostics EC852)
- 40% linear acrylamide (Sigma A4058)
- 5x TBE or suitable electrophoresis buffer
- 10% APS (Ammonium Persulfate) solution less than 1 week old
- TEMED (Tetramethylethylenediamine, Sigma T9281) less than 6 months old
- 1.2x TBE, 15% (w/v) sucrose loading buffer
- two-channel peristaltic pump

Procedure:

For 30ml (one gel) of 4% or 5% native gel mixture add:

- 1.5ml (4% final) or 1.9ml (5% final) of 40% 29:1 acrylamide,
- 1.5ml (4% final) or 1.9ml (5% final) of 40% linear acrylamide,
- 1.2ml 5x TBE (0.2x final; 1x = 90mM Tris, 90mM boric acid, 2mM EDTA, pH 8.3) or suitable electrophoresis buffer
- 300µl 10% APS (0.1% final)
- 25.5ml (for 4%) or 24.75ml (for 5%) of water

For 2 gels make 45ml or 1.5 times the above recipe.

Add 45-75µl of TEMED just before pouring gel.

Pour the gel and assemble into the gel rig according to procedures detailed above and fill gel reservoirs with same electrophoresis buffer as placed in gel (i.e. 0.2x TBE). Gently pull
out comb and purge wells immediately with needle and syringe.

Place gel in 4°C deli fridge along with 2-channel peristaltic pump loaded with tubing in both channels. Place the inlet of channel 1 tube in the top reservoir of the gel rig and the outlet in the bottom reservoir. Conversely, place the inlet of channel 2 tube in the bottom reservoir and the outlet in the top reservoir. Hold tubing in place with binder clips or rubber bands. Circulate buffer at ~5 ml/min.

Prerun the gel at 4°C in deli fridge at 300V (20V/cm) for 3 hours minutes with continuous buffer circulation until current running through gel drops by half of the starting current to ensure polymerization agents have been run out of gel.

Add 1.2x TBE, 15% sucrose (6x) to 0.2x TBE, 2.5% sucrose (1x) final concentration to samples for loading onto gel.

Before loading samples, purge wells with needle and syringe to remove any air bubbles and unpolymerized acrylamide that may have formed in the wells during the prerun.

With gel running at 300V, apply sample to gel; 20 well combs require at least 5µl of sample per well, 10-12 well combs require at least 10µl of sample per well.

Run gel for 3 hours at 300V, 4°C with continuous buffer circulation to resolve bands.

E.1.5 Composite Gel

For nucleosome samples with DNA larger than 500 base pairs or for nucleosome arrays larger than dinucleosomes, then acrylamide concentrations lower than 4% must be used. To keep the acrylamide matrix from collapsing at low concentrations, agarose is used as a support to form an agarose-acrylamide composite gel. Additionally, the pouring and running method is altered from the standard acrylamide to prevent the composite gel from sliding out from
between the glass plates. Additionally, due to interactions of EDTA with chromatin to alter compaction, Tris-borate only (TB) buffer should be used; if samples contain more than 1% glycerol then Tris-Taurine only (TT) buffer should be used.

Materials:

- 40% 29:1 linear:bis acrylamide (National Diagnostics EC852)
- Agarose powder (Lonza SeaKem 50001)
- 5x TB (or 20x TT)
- 10% APS (Ammonium Persulfate) solution less than 1 week old
- neoprene gel casting boot (Labrepco 21070010)
- TEMED (Tetramethylethlenediamine, Sigma T9281) less than 6 months old
- 6x Ficol
- heated stirplate
- two-channel peristaltic pump

Procedure:

Turn on the heated stirplate to 90°C and place a fresh Kimwipe over the stirplate.

Assemble the gel plates as described above with the following exceptions:

1. Do not use the horizontal spacer between the glass plates;

2. When laying down the vertical spacers on the large glass plate, angle the bottom edge of the spacers toward the center of the glass plate by 0.5cm so that the spacers are at a slight upright V-shape;
3. Place the class glass plate on top to form the sandwich and clip only the sides of the glass plate sandwich along the vertical spacers with 2 binder clips.

Next insert the bottom edge (without binder clips) of the glass plate sandwich into the bottom of the neoprene casting boot and ensure that the bottom of the boot is flush with the edges glass plates. Also, ensure that the drain plug is closed.

Carefully unclip the binder clips along the sides of the vertical spacers but DO NOT insert these sides if the sandwich into the boot at this point.

Place the glass plate - boot assembly onto the heated stirplate so that the large glass plate is in contact with the heatplate to warm.

At this point prepare the acrylamide-agarose gel mix for one gel at a time:

Add 0.6g agarose to 60ml of 0.2x TB or 0.2x TT buffer in a glass beaker and microwave for 45 seconds; swirl to mix and microwave for 45 seconds more until agarose is fully melted and clarified.

Place beaker with melted agarose mix on benchtop and allow to cool until no longer steaming but skin is not stating to form on top of agarose yet.

While agarose is cooling add 1.5ml 40% 29:1 agarose and 300µl 10% APS to a 50ml falcon tube.

When the agarose is almost cooled off, place TEMED stock, motorized pipetter with 25ml serological pipette, and 10 or 12 well comb on benchtop.

In quick succession:
- Take glass plate - boot assembly off of heat plate and insert remaining edges firmly into casting boot and set bottom edge of assembly on the benchtop with the top edge propped up on a pipette tip box.

- Add 28.5ml of the agarose mixture to the acrylamide and immediately add 50µl of TEMED. Cap tube and gently invert several times to mix.

- Pipette up mixture with motorized pipette; with one hand hold assembled plates vertically and slightly tilted toward one of the vertical spacer sides

- With the motorized pipetter, place pipette tip containing gel mixture in the corner of the gap between the glass plates a the vertical spacer that is tilted downward

- In a steady stream pipette the gel mixture into the sandwich so that in flows down along the vertical spacer preventing entrainment of air bubbles (if an air bubble gets in the gel, stop pipetting briefly until bubble rises to top and pops)

- As the plates fill with gel mixture toward the top, tilt sandwich back upright so the top of the sandwich is parallel with the benchtop in order to completely fill the plates

- Place comb parallel to gap between plates and touch just the bottom of combs to gel mix; move comb around a little to dispel any air that might be trapped between the bottom of the combs and the gel mix. When no bubbles are observed, push combs down into gel mix only 5-7mm so that comb does not adhere to gel when it solidifies.

- Take binder clips and clip glass plates together where they are in contact with the combs to dispel any acrylamide from between glass plate and comb; wick out any excess acrylamide above level of glass plates with a Kimwipe
Lean assembly back down so that top edge is resting against the top of the piette tip box to prevent mixture from leaking out of casting boot as it polymerizes. Allow gel to thoroughly polymerize for \( \sim 10 \) minutes. Transfer assembly to 4°C deli fridge for 5-10 minutes until thoroughly cold.

Disassemble all binder clips from glass plates and gently pull off casting boot. Agarose tends to adhere to boot and fast removal can tear the gel.

Assemble gel into the gel rig according to procedures detailed above and fill gel reservoirs with same electrophoresis buffer as placed in gel (i.e. 0.2x TB or TT). Very carefully remove comb; using a scalpel cut off any agarose films and balls extending from wells that might block application of sample to the wells. Gently purge wells with a needle and syringe.

Place gel in 4°C deli fridge along with 2-channel peristaltic pump loaded with tubing in both channels. Place the inlet of channel 1 tube in the top reservoir of the gel rig and the outlet in the bottom reservoir. Conversely, place the inlet of channel 2 tube in the bottom reservoir and the outlet in the top reservoir. Hold tubing in place with binder clips or rubber bands. Circulate buffer at \( \sim 5 \text{ ml/min} \).

Prerun the gel at 4°C in deli fridge at 300V (20V/cm) for 3 hours minutes with continuous buffer circulation until current running through gel drops by half of the starting current to ensure polymerization agents have been run out of gel.

Add 6x ficol w/o orange G to 1x final concentration to samples for loading onto gel.

Before loading samples, purge wells with needle and syringe to remove any air bubbles and unpolymerized acrylamide that may have formed in the wells during the prerun.

With gel running at 300V, apply sample to gel; 20 well combs require at least 5\( \mu l \) of sample.
per well, 10-12 well combs require at least 10µl of sample per well.

Run gel for 3 hours at 300V, 4°C with continuous buffer circulation to resolve bands.

E.1.6 Urea Denaturing Gels

Denaturing gels inhibit dsDNA duplex and ssDNA secondary structure from forming so that ssDNA (or dsDNA) runs as a function of length on the gel. A combination of 7M urea, 50% formamide, and heat are used to keep DNA denatured both during gel loading and resolving. 20% Acrylamide denaturing gels are useful for assaying DNA oligo purity from the manufacturer or after purification/labeling procedures. To resolve DNA fragments from mapping reactions (e.g. ExoIII, DNase, and hydroxyl radical) 12% acrylamide denaturing gels are optimal for DNA lengths less up to 187 base pairs, while 8% acrylamide denaturing gels are optimal for lengths greater than 247 base pairs. Always make gel mixes containing urea just before use and use poured gels within several hours after polymerization to prevent urea crystals.

Materials:

- 40% 29:1 linear:bis acrylamide (National Diagnostics EC852)
- Urea (Sigma U1250)
- 5x TBE or suitable electrophoresis buffer
- mixed bed resin (Sigma M8032)
- Heated stirplate
- 10% APS (Ammonium Persulfate) solution less than 1 week old
- TEMED (Tetramethylethylenediamine, Sigma T9281) less than 6 months old
- Whatman filter paper (Whatman 3030-866)
- formamide
• 95°C heatblock

Procedure:

For 30ml (one gel) of 20%, 12% or 8% denaturing gel mixture add:

• 12.62 g urea

• 15ml (20% final) or 9ml (12% final) or 6ml (8% final) of 40% 29:1 acrylamide,

• 6ml 5x TBE (1x final; 1x = 90mM Tris, 90mM boric acid, 2mM EDTA, pH 8.3)

• 0ml (for 4%) or 6ml (for 12%) or 9ml (8% final) of water

• pinch mixed be resin

Combine above into a beaker with stir bar and mix on stir plate at 90°C until completely dissolved, about 10 minutes. Filter and degas through steri-cup and transfer to 50ml falcon tube to cool completely.

When ready to pour gel add 300µl of 10% APS (0.1% final) and only 30µl of TEMED to denaturing gel mix.

Pour the gel and assemble into the gel rig according to procedures detailed above and fill gel reservoirs with same electrophoresis buffer as placed in gel (i.e. 0.3x TBE).

Cut filter paper so that its width is the same as the exposed surface of the front glass plate and several inches longer. Wet the filter paper with 1x TBE in the bottom gel rig reservoir. Lay the paper against the front glass plate so all of the plate 2cm from the bottom of the wells and below are covered and the bottom of the paper is in the bottom reservoir to wick the buffer.

Prerun the gel at 48W for 30 minutes until current running through gel drops by half of
the starting current to ensure polymerization agents have been run out of gel.

While gel is pre-running, add formamide to 50% (v/v) final concentration to each sample; close cap tightly and heat at 95°C on heat plate for 15 minutes until ready to load samples.

Before loading samples, purge wells with needle and syringe to remove any air bubbles and urea gradients that may have formed in the wells during the prerun.

With gel running at 48W, remove sample one by one from heat block apply sample immediately to gel; 20 well combs require at least 5µl of sample per well, 10-12 well combs require at least 10µl of sample per well. Also off to the side in an unused well load 5µl of 1x ficol w/ orngnge G to follow progression of gel.

Run gel for 30-45 minutes at 48W until orange dye just runs off bottom to resolve bands.

E.1.7 Staining Gels

Addition of ethidium bromide or Cybr dyes (Invitrogen, Life Technologies) to the gel before polymerization in order to stain and image DNA is not feasible due to migration of the dye out of the gel during the 60-90 minute running time. Therefore, acrylamide gels need to be stained after running in 50µL/L ethidium bromide (Sigma E1510) or 1x Cybr Gold stain (Invtirogen, Life Technologies S-11494) in 0.3x TBE for 30 minutes before imaging.
E.2 Agarose Gel Techniques

Agarose gels are faster and more convenient than acrylamide gels but compromise resolution. They are very useful to check PCR products, plasmid size, restriction enzyme digestion, ligation products, and sucrose gradient fractions when approximately 50ng or more of DNA can be loaded in a lane.

DNA lengths less than 1000 base pairs and mononucleosomes are resolved by 2.0% (w/v) agarose gels while DNA lengths longer than 1000 base pairs are resolved by 0.8% agarose gels. In the case of DNA gels, 50µg/L ethidium bromide (Sigma E1510) or 1x Cybr Gold stain (Invitrogen, Life Technologies S-11494) can be added to the gel buffer before solidifying for efficient imaging. For gels resolving nucleosomes, dyes must be omitted from the gel during running due to interference with the nucleosomes and then stained afterwards as for acrylamide gels.

E.2.1 Nucleosome agarose gels

Materials:

- 100ml 0.3x TBE (1X = 90mM Tris, 90mM Boric acid, 2mM EDTA, pH 8.3)
- X grams agarose per 100ml 0.3x TBE for X% w/v gel (AquaPor LE, National diagnostics EC-202)
- gel casting plate and rig (Owl, Thermo scientific)
- gel combs
- 6x ficol w/o orange G
- 100 base pair or 1000 base pairs ladder (NEB N3231, N3232) w/ orange G

Procedures:

Combine agarose and buffer in 500ml beaker. Microwave for 60 seconds. Swirl beaker and
microwave for 45 seconds more. Allow to cool until no longer steaming. Pour into casting plate and insert combs. Allow to fully solidify.

Remove combs from gel and remove gel from casting rig. Place gel in rig and cover with 0.3x TBE buffer to ∼3mm above level of gel. Purge wells.

Add 6x ficol w/o orange G to 1x final concentration and apply to gel. Also apply appropriate ladder to gel. Run at 200V until orange G marker is 3/4 down the gel.

Stain after running in 50µl/L ethidium bromide (Sigma E1510) or 1x Cybr Gold stain (Invitrogen, Life Technologies S-11494) in 0.3x TBE for 30 minutes before imaging.

E.2.2 DNA agarose gels

Materials:

- 100ml 0.5x TAE (1X = 40mM Tris-acetate, 2mM EDTA, pH 8.3) with 50µg/L ethidium bromide (Sigma E1510)

- X grams agarose per 100ml 0.5x TAE for X% w/v gel (AquaPor LE, National diagnostics EC-202)

- gel casting plate and rig (Owl, Thermo scientific)

- gel combs

- 6x ficol w orange G

- 100 base pairs or 1000 base pairs ladder (NEB N3231, N3232) w/ orange G

Procedures:

Combine agarose and buffer in 500ml beaker. Microwave for 60 seconds. Swirl beaker and microwave for 45 seconds more. Allow to cool until no longer steaming. Pour into casting
plate and insert combs. Allow to fully solidify.

Remove combs from gel and remove gel from casting rig. Place gel in rig and cover with 0.3x TBE buffer to ~3mm above level of gel. Purge wells.

Add 6x ficol w orange G to 1x final concentration and apply to gel. Also apply appropriate ladder to gel. Run at 200V until orange G marker is 3/4 down the gel.

If ethidium was not added to the gel before solidifying, stain after running in 50µl/L ethidium bromide (Sigma E1510) or 1x Cybr Gold stain (Invitrogen, Life Technologies S-11494) in 0.3x TBE for 30 minutes before imaging.
E.3 SDS-PAGE protein gles

SDS-PAGE consists of two components, the stacking gel and the resolving gel. Regardless of sample type, the stacking gels remains unchanged to concentrate samples as they move into the gel. The resolving gel varies from 10%-16% depending on the sample type. Small molecules (e.g. histones) should be resolved by 15-16% acrylamide while larger proteins should be resolved by 10-12%.

Materials:

- 40% 29:1 acrylamide (National Diagnostics EC-852)
- 1M Tris pH 6.8
- 1.5M Tris pH 8.8
- 10% APS (Ammonium Persulfate) solution less than one week old
- 10% SDS solution (Sodium dodecyl sulfate)
- TEMED (N,N,N',N'-Tetramethyethylene-diamine, Sigma T9281)
- Biorad Mini Protean gel system (Biorad 165-8000)
- 100% ethanol
- 5X Tris-Glycine-SDS buffer
- 6X SDS loading buffer w/ or w/o bromophenol blue
- 95°C heat block
- Gel stain
- Gel destain

Procedure:
For the stacking gel combine the following except APS and TEMED:

- 3.4ml water
- 850µl 40% 29:1 acrylamide
- 630µl 1M Tris pH 6.8
- 50µl 10% w/v SDS (Sodium dodecylsulfate, Sigma L3771)
- 50µl 10% APS
- 10µl TEMED

For the resolving gel 12% or 16% combine the following except APS and TEMED:

- 4.30ml (for 12%) or 3.55ml (for 16%) water
- 3.00ml (for 12%) or 4.00ml (for 16%) of 40% 29:1
- 2.5ml 1.5M Tris-HCL pH 8.8
- 100µl 10% w/v SDS
- 100µl 10% APS
- 20µl TEMED

Combine all ingredients except APS and TEMED for resolving gel and stacking gel in separate falcon tubes. If using a Biorad Mini Protean gel kit, one batch fills two gels. If using a standard 16x19cm gel rig make a double batch.

For mini protean gels, sandwich together thin front plate and thick back plate with built in spacers and lock into casting rig. For standard 16x19cm plates assembly as described in the section of acrylamide electrophoresis.

Add APS to the Resolving Gel mixture and invert to mix. Next add TEMED, invert to
mix, and immediately pipette into casting rig until 3/4 of the way full. Immediately overlay 100% ethanol on top of the poured resolving gel to remove bubbles and form a level interface.

After polymerization is complete, pour off ethanol and use a Kimwipe to absorb any remaining ethanol.

Add APS to the Resolving Gel mixture and invert to mix. Next add TEMED, invert to mix, and immediately pipette into casting rig until full. Immediately insert gel comb. Use a kimwipe to wick up excess acrylamide solution.

When polymerization is complete place gel into gel rig and fill rig with 1X Tris-Glycine-SDS running buffer.

Remove comb from gel and purge wells with needle and syringe.

Combine sample with 6X SDS loading buffer w/ bromophenol blue to desired final concentration (typically 3X for cell pellets, 1X for protein in solution); boil at 95°C for 5 minutes; centrifuge at 5000g for 1 minute to spin down evaporated water if necessary. If resolving fluorescent labeled protein from free dye, use 6x SDS loading buffer w/o bromophenol blue.

Apply sample to gel then run at 20mA per gel while bromophenol blue loading dye is in the stacking gel. In this case use free fluorescent dye as a visual indicator for when protein passes into resolving gel.

Once loading dye transitions into resolving gel, run at 30mA per gel until loading dye just runs off gel.

Remove gel from plates and place in microwaveable container. Cover gel with 2-3mm of gel
stain. Microwave gel for 15 seconds, swirl container to wash over with stain; repeat 2 more times.

Place gel in stain on the benchtop or on a shaker table for 10 minutes until cool.

Transfer gel to a fresh container and cover with inch of destain.

Place gel in destain on the benchtop or on a shaker table for 30-60 minutes until bands evolve.
E.4 Electrophoresis buffers

E.4.1 10% APS solution
Combine 300mg ammonium persulfate (APS, amresco K833) per 3ml of water. Vortex to dissolve. Write expiration date on tube for 1 week from day it was dissolved. Store at 4°C.

E.4.2 5x TBE
Combine 54g Tris Base (Sigma T1503), 27.5g Boric Acid (Sigma B7901), and 20ml 0.5M EDTA pH 8.0. Bring volume up to 1L total with water. Mix thoroughly for 30-60 minutes. Filter and degas. Solution is stable for up to 6 months. EDTA is omitted for TB buffer.

E.4.3 50x TAE
Combine 242g Tris Base (Sigma T1503), 57.1ml glacial acetic acid, 100ml 0.5M EDTA pH 8.0. Bring volume up to 1L total volume with water. Mix thoroughly for 30-60 minutes. Filter and degas.

E.4.4 20x TT
For 20x TTE (1.78M Tris, 0.57M Taurine, 10mM EDTA) use National Diagnostics 20x TTE buffer. For TT buffer combine 53g Tris Base (Sigma T1503) and 17.8g taurine (Sigma T0625). Bring volume up to 250ml total with water. Thoroughly mix to dissolve. Filter and Degas

E.4.5 XM Tris pH Y solution
Combine 121.14g times X molar Tris Base (Sigma T1503) per 1L water. Stir to dissolve completely and pH to desired pH with concentrated hydrochloric acid. Filter and degas.

E.4.6 10% SDS solution
Combine 100g (Sodium dodecylsulfate, Sigma L3771) per 1L water. Stir to completely dissolve. Do NOT filter and degas.
E.4.7 5X Tris-Glycine-SDS Buffer

- 15.1g Tris Base (Sigma T1503, 125mM final)
- 94g Glycine (Sigma G8898, 1.25M final)
- 50ml 10% w/v SDS solution (0.5% final)

Stir all together. Do NOT filter or degas

E.4.8 Gel Destain

Combine 4L Methanol (Sigma 179957, 50% final) and 800ml glacial acetic acid (10% final) and bring volume up to 8L total with water.

E.4.9 Gel Stain

1g/L brilliant blue in destain (Sigma B0149) stir for several hours then filter

E.4.10 6X SDS Loading Buffer

- 625µl 4% w/v Bromophenol Blue (Sigma B0126, 0.25% final)
- 600µl 10% w/v SDS
- 1.5ml 1M Tris-HCl pH 6.8
- 20µl BME (2-mercaptoethanol, Sigma M3148, 30mM final)
- 3.75ml 80% glycerol in dH2O, autoclaved (30% final)

Combine in PCR tube and store at 4°C. Omit bromophenol blue if desired.

E.4.11 6x Ficol w/ or w/o Orange G

Combine 15g Ficol 400 (Sigma F9378) and 250mg Orange G (Sigma O3756). Bring volume to 100ml total with water and thoroughly dissolve. Autoclave. Omit orange G if desired.
Appendix F

TRANSCRIPTION FACTOR, NAP1, AND PFU PURIFICATION

This section includes detailed protocol for LexA transcription factor, Nap1 histone chaperone, and PFU DNA polymerase. While several of these proteins were not employed in this work, their expression and purification were optimized for other experiments, and encompass three major types of purification: native protein, His$_6$ tagged, and glutathione S-transferase tagged. All buffer recipes specific to these procedures are provided at the end of this section due to their application in multiple protocols.

F.1 Expression Vectors and Protein Properties

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (Da)</th>
<th>complex</th>
<th>$\epsilon_{270nm,1cm}$</th>
<th>plasmid</th>
<th>resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LexA</td>
<td>22356</td>
<td>dimer</td>
<td>6970</td>
<td>pET</td>
<td>Amp</td>
</tr>
<tr>
<td>VP16(413-490)</td>
<td>8904</td>
<td>monomer</td>
<td>2900</td>
<td>pGEX-5x</td>
<td>Amp</td>
</tr>
<tr>
<td>yNap1-His6</td>
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<td>dimer</td>
<td>36390</td>
<td>pET28</td>
<td>Kan</td>
</tr>
<tr>
<td>PFU</td>
<td>90113</td>
<td>monomer</td>
<td>126100</td>
<td>???</td>
<td>Amp</td>
</tr>
</tbody>
</table>

Table F.1: Protein Expression Vectors and Properties

E. coli LexA repressor native protein:

TTTGTTTAAC TTTAAGAAGG AGATATACAT ATGAAAGCGT TAACGGCCAG

409
GCAACAAGAG GTTTTTGATC TCATCCGTGA TCACATCAGC CAGACAGGTA TGCCGCCGAC GCGTGCGGAA ATCGCGAACC ACTTCTGGCG CAACACGATA TTGAAGGTCA TTATCAGGTC GACCGCATTG GAAGAGGAAG AAGGGTTGCC GCTGTAGAGT CTGGTGGCTG CCGGTGAACC ATCGCGAACC ACTTCTGGCG CAACACGATA TTGAAGGTCA TTATCAGGTC GACCGCATTG GAAGAGGAAG AAGGGTTGCC GCTGTAGAGT CTGGTGGCTG CCGGTGAACC ATCGCGAACC ACTTCTGGCG

Protein Sequence:

MKALTARQQE VFDLIRDHIS QTGMPPPRAE IAQRLGFRSP NAAEEHLKAL ARKGvieiVS GASRGlRLQ EEEEGPVLVGV RVAAGEPLLAA QQHIEGHYQV DP-
SFLKPNAD FLLRVSGMAM KDIGMDGDGL LAVHKTQDVR NGQVYVVARID. DE-
VTVKLKK QGNKVELELPE NSEPKPIVVD LRQQSFTIEG LAVGVIIRNGD WL

Note: N-terminal methionine not edited off of protein

Herpesvirus VP16(413-490) activation domain with Factor Xa cleavable IEGR— site for

GST tag removal CTGATCGAAG GTCGTGGGAT CTCTCGAGCC GCCCCCCCAGA CCGATGTCAG CCTGGGGGAC GAGCTCCACT TAGACGGCGA GGACGTGGCG ATGGCGCATG CCGACGCGCT AGACGATTTTC GATCTGGACTC TGTTGGGGGA CGGGGGATCC CCGGGTCCGG GATTTACCCC CCACGACTCC GCCCCCTACG GCGCTCTGGA TATGGCCGAC TTCGAGTTTG AGCAGATGTT TACCGATGCC CTTGGAATTG ACGAGTACGG TGGGTAGCGG ATCCCCGGGA ATTCATCGTG
Protein Sequence (N-terminal IEGRGISRA from cloning vector):

IEGRGISRAA PPTDVSGLGDE LHLDGEDVAM AHADALDDFDL DMLGDGDSPG
PGFTPHDSAPY GALMDADFEF EQMTDIALGI DEYGG

Yeast Nap1 with His$_6$ tag, thrombin cleavable LVPR—GS site for His$_6$ tag removal:

TTAACTTTAA GAAGGAGATA TACCA$^\text{T}$GGC AGCAGCCATC ATCATCATCA
TCACAGCAGC GGCCTGGTGCC CGCGCGCGAG CCATATGGCT AGCATGACTG
GTGGACAGCA AATGGGGTCGG ATCATGACAG ACCCTATCAG AACGAAACCC
AAGTCGTCGA TGCAAATCGA CAACGCCCTC ATGCCTACGA ATACCCCGAG
ATCGGTCTCTG AATCCCGAGCT ATTTGAAAA CGGCAACCC AGTTAGGGCC
CAGGCACAGG AGCAGGATGA CAAGATCGGC ACCATCAACG AGGAGGACAT
CTTGGCTTACG CAACCCCTGT TGGTACAGCT ATCCAGGAC AGACTTGGCT
CGCTTGGTGGG CCAGGACAGC GGGTATGGTG GGGTCTTCAG CAAGAACGTT
AAGGAAAAAGC TGCTGATGTCG GAAGACTTTA CAAAGCGAGC TATTCGAAGT
TGAAAAAGAA TTTCAGGTGG AGATGTTTGA GCTAGAGAA ACAGTTTCTGC
AGAGAGTAGA GCCCATTTTG GAGCAGCGGT CCAGGATCAT TTCAGGACAA
GAGGAACCCCA ACGCGGAACA GATCGCAAGG GGCAAGAGA TTGTGAGGTC
ACTCAATGAG ACGGAGTTGT TGGTGACGCA AGAAGAGAG GCCAAATATG
ATTCCCGAGGA GGAACAGGTG AAGGAATTC CCTAATGCTG GCTAAAGCGC
TTAGACAGCT TGCCCATCGT TGGCGACACC ATCAGTACC GCAGTGCGGA
AGTTCTGGAG TACCTGCAAG ATATTGTCTT GGAATACCTG ACGGATGGTA
GACCCGTTTT CAAGCTGTTT TTCAGATTGAG AGTCTTCCGC CAACCCCTTC
TTCACCAACG ACATTCTGGA CAAGACCTAC TTCTACGAGA AGGAGCTCGG
TTACTCGGAG GACTTTATCT ACGACCACCAG AGAAGGGCTGC GAGATGAGTT
GGAAGAGACA CGCCCAACAC GTACCTGGTG ACGTGAAGAT GCGCAAGCAG
AGAAACAAGA CCACCAAGCA GGTGAGGACC ATCGAGAGA TCACTCCCAT
AGAATCCTTT TTCAACTTCT TCGACCCTCC CAAGATCCAA AACGAAGACC
AAGACGAGGA GTTGGAGGAG GACCTCGAGG AACGTCTAGC TTATAGACTAC
TCCATCGGGG AACAACCTAA GGACAAGCTA ATTCCTAGAG CCGTGGACTG
GTTTACCCTGC GCAGCCTTGG AATTGAGATT CGAGGAGGAC GAAGAAGAAG
CGGACGAGGA CGAAGACGAA GAAGATGATG ATGATCAGGG CTTGGAGGAT
GACGACGGGG AATCTGGCCGA AGAGCAAGAC GATTTTGCTG GCAGGCGGGA
ACAGGCTCCT GAATGCAAGC AGTCATAATC CCAACGC ACT TCAGAAGAGT
GGCATATAACC GTATTCATAT ATTTATATAC ATATGGCTAG CATGACTGGT
GGACAGCAAA TGGGTGCAGG ATCAGAATTC GAGCTCCGTC GACAAGCTTG
CGGCCGCACCT CGAGCCACAC CACCACCCAG ACTGAGAT

Protein Sequence (N-terminal MGSSHIIIIIISSGLVPRGSHMASMTGGQQM from cloning vector):

MGSSHIIIIIISSGLVPRGSH MASMTGGQQM GRIMTDPIRT KPKSSMQIDN
APTPHNTPSAV LNPSYLKNQ PVRAQAQEQLDQ DKITNEEILANQPLLQ SQI-
DRLGSVL QGDGYSVGL PKNVKEKLLS LKLQSELFEV EKEFQEMFE LENK-
FLQKYK PIWEQRSRII SGEQPQPKEQ IAKGEIVES LNETELLVDE EEEAQNSEE
EQVKGIPSFWL TALENPIC DTITDRDAEV LEYLQDIGLE YLTDGRPGFK
LLFRFESSAN PFFTNDLCK TYYFYQKELGY SGDFTYDHAEG CEISWKNNAH
NTVVDLEMRK QRNKTTKQVR TIEKITPIES FFNFPDPKIK QNEDQDEELE
EDLEERLALD YSIEQKLDKL IPRAVDFWTG AALEFEFEED EEEADEDEDE ED-
DDEHGED DDGESAEEQD DFAGRPEQAP ECKQS

Note: N-terminal methionine edited off of protein

*Pyrococcus furiosus* DNA polymerase in unknown vector

gagcataatg attttagatg tggattacat aactgaagaa gggaaaaacg ttattaggt attccaaaaa gaggaacggga
aatattaagat agagcatgat agaactttta gaccatacat ttacgtcctt ctcagggatg attcaagat tgaagaagtt
Protein Sequence: MILDVYITE EGKPVIRLFK KENGKFKIEH DRTFRPYIYA LLRDDSKEIE VKKITGERHG KIVRIVDVEK VEKKGFLGKPI TVWKLYLEHP QD-VP TIREKV REHPAVVDIF EYDIPFAKRY LIDKGLIPME GEEELKILAF DIETLY-HEGE EFGGPPIIMI SYADENEAKV ITWKNIDLPY VEYVSSEREM IKRFLRIIRE KDPIIVTYN GDSFDFPYLA KRAEKLGIKL TIGRDSEP K MQRIGDMTAV EVKGRIHFDL YHVITRTINL PTVTLEAVYE AIFGKPKEK V YADEIKAWE SGENLRAVAK YSMEADAKATY ELGKEFLPME IQLSRLVGQP LWDSRSSTG NLVEWFLLIRK AYERNEVAPN KPSEEYYQRR LRESYTGGGF KEPEKGLWEN IVYLDFRALY PSIIITHNVS PDTLNLEGCK NYDIAPQVGH KFCKDIPGFI PSLLGH- LLEE RQKIKTKME TQDPIEKILL DyrQKAIKLL ANSFYGYGGY AKARWYCKEC AESVTAWGRK YIELVWKELE EKFGFKVLYI DTDGLYATIP GGESEEIJKK ALE- FVKYINS KLPGLLELEY EGFYKRGFFV TKKRAYVIDE EGKVITRGLLE IVRRDWS- SEIA KETQARVLET ILKHGVEDAA VRIVKEVIQK LANYEIPPEK LAYEITRNR LHEYKAIQPH VAVAKLAAK GVKIKPGMVI GYIVLRGDGP ISNRAILAEY YDP- KKHKYDA EYYIENQVLP AVLRILEFGF YRKEIDLYQK TRQVLTSWL NIKKS

F.2 General Protein Expression

The expression procedure applies to all BL21(DE3)pLysS E. coli expression strains expressing T7 RNA polymerase under lac operator control and carrying the pLysS lysozyme plasmid, which suppresses basal expression from the T7 promoter and aids in cell lysis. All expression vectors (e.g. pET, pGEX, pET-His6 type) must contain the T7 promoter or the Ptac T7/lac fusion promoter which is under control of the lac operator.

F.2.1 Materials

- Culture Tubes containing 5ml 2xYT growth media, autoclaved (See Appendix H)
- 250ml Erlenmeyer flask containing 60ml 2xYT growth media, autoclaved (See Appendix H)
- 2L Erlenmeyer flasks containing 500ml 2xYT growth media OR 4L Erlenmeyer flasks
containing 1L 2xYT growth media, autoclaved (See Appendix H)

- 50 mg/ml ampicillin or kanamycin stock (See Appendix H; See Table F.1)
- 34 mg/ml chloramphenicol stock (See Appendix H)
- 1M IPTG (Isopropyl β-D-1-thiogalactopyranoside, USB 17886) Dissolve in water and syringe filtered; store at -20°C
- Buffer A + 10% sucrose + 1mM PMSF (see Purification Buffers Section below) chilled to 4°C
- Lysozyme (Sigma L6876)
- 16% SDS-PAGE gel (See Appendix E)
- 1x Tris-Glycine-SDS buffer (See Appendix E)
- 6x SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- 0.5x TE (See Appendix E)
- 1M MgCl₂

### F.2.2 Transformation and Trial Induction

To transform plasmid into BL21(DE3)pLysS and perform trial inductions of expression. Follow the methods outlined for histone expression in Appendix A.4-A.5 with the single alteration that the antibiotics used in the growth media must be specific to the expression plasmid used. Growth of cell culture at 37°C, 300RPM to an OD₆₀₀nm,₁cm of 0.5 before induction with 1mM IPTG and then expression for 3-4 hours before harvesting is sufficient for both trial induction and bulk harvest. Typically, optimization of growth conditions does not have to be performed for transcription factor and histone chaperone expression.
They are expressed as native proteins (not in inclusion bodies) at yields of 15-30mg per 1L cell culture. After induction trial is complete and a viable clone has been stored in -80°C glycerol stock, then cells are ready for bulk expression.

F.2.3 Grow and Induce Cells

This methods uses the overnight serial dilution method (See Appendix A.6) optimized for growth of native expressed proteins (i.e. proteins not expressed and packaged into inclusions bodies)

1. Warm to room temp 3 - 5ml culture tubes of 2xYT media.

2. Add 5µl of expression plasmid specific antibiotic (See Table F.1) to 50 µg/ml final concentration and 5µl of 34 mg/ml chloramphenicol to 34 µg/ml final concentration to each tube.

3. From glycerol stock tubes stored -80°C inoculate a small clump of frozen stock on a sterile 200µl pipette tip under Bunsen burner flame, eject tip into first media tube, and return stock to -80°C before thaw.

4. Shake tube inoculated with cells at 37°C, 300RPM for 20-30 minutes to equilibrate cells and start growth phase. After incubation, perform a 25-fold serial dilution (200µl into 5ml) of the inoculated media into the second tube; swirl to mix; then perform a 25-fold dilution (12500-fold final) from the second tube into the third; swirl to mix.

5. Grow cell overnight at 300RPM, 37°C. The next morning choose which of the serial dilutions is cloudy but not above an OD₆₀₀nm,₁cm = 0.6.

6. Inoculate 60ml 2xYT media in a 250ml flask by adding 5ml of starting culture, 60µl expression vector specific antibiotic (See Table F.1) to 50 µg/ml final concentration and
60µl of 34 mg/ml chloramphenicol to 34 µg/ml final concentration.

7. Grow until slightly cloudy at 300RPM, 37°C, ~2hrs, but not above an OD$_{600nm,1cm}$ = 0.6.

8. Inoculate 500ml 2xYT media in 2L flasks by adding culture from steps 6-7 above, 500µl of 50 mg/ml expression vector specific antibiotic (See Table F.1) to 50µg/ml final concentration and 500µl of 34 mg/ml chloramphenicol to 34 µg/ml final concentration.

9. Grow at 300RPM, 37°C, until OD$_{600nm,1cm}$ = 0.5. Reserve 100µl for pre-induction gel sample.

10. When OD$_{600nm,1cm}$ = 0.5, induce with 1M IPTG to 1mM final concentration.

11. Grow at 300RPM, 37°C for 2-3hr depending on results of induction trial. Typically 2hr results in higher protein activity and less truncated products for smaller proteins (e.g. LexA, VP16). Larger proteins typically require 3 hours for good expression (e.g. Nap1, PFU)

12. After expression is complete, reserve 100µl of cells for post induction timepoint.

13. Gel Sample Preparation: Take the 100µl of reserved cells in 2xYT media, centrifuge 6800g for 1 minute, remove supernatant, resuspend pellet in 10µl 0.5x TE, and add 10µl 6x SDS Loading Buffer; refrigerate for later.

**F.2.4 Harvest and Store cells**

1. After expression is complete, chill cultures rapidly by immersing flask into an ice-water slurry and shaking on shaker table or by covering in ice. Meanwhile ensure Avanti centrifuge and JLA 10.5 rotor are chilled to 4°C.
2. Fill and balance Beckman centrifuge 500-ml bottles up to just below the shoulder. Spin in Avanti centrifuge in JLA 10.5 at 4°C, 4000g for 10 minutes.

3. Pour off supernatant if clear. Add on top of pellet any remaining cells; repeat balance, load bottles into centrifuge so that pellets are nearest to the center of the rotor, and spin 4°C, 4000g for 10 minutes; pour off supernatant.

4. Invert bottles on paper towel and allow to drain for 3-5 minutes so that all residual media is removed.

5. Resuspend each pellet from 500ml culture in 20ml Buffer A + 10% sucrose + 1mM PMSF (approximately 0.2g cell paste per ml buffer), keeping 500ml bottle on ice during resuspension. Transfer resuspended cells to 50ml falcon tube on ice.

6. Dissolve lysozyme into Buffer A + 10% sucrose + 1mM PMSF to 10 mg/ml final concentration. Centrifuge 20000g for 2 minutes to remove aggregates.

7. Add 20µl of 10 mg/ml lysozyme per ml of resuspended cells to 0.2mg/ml final concentration. Incubate on ice for 1 hour.

8. Flash freeze cells in liquid nitrogen and store at -80°C. Cell pellets are stable for up to 2 months before purification. After 2 month appreciable loss in lysozyme and expressed protein activity can be observed, presumably due to background protease activity.

**F.2.5 Cell Lysis**

When lysozyme is produced endogenously inside the cell (from pLysS plasmid) and introduced exogenously after harvest, cell lysis is extremely efficient and only takes one thawing of the cell suspension after harvest. For cell lysis, perform the following:
1. Turn water bath to 37°C to equilibrate.

2. Remove cells from -80°C and thaw at room temperature with occasional mixing by inverting tubes until just thawed.

3. Add 1M MgCl₂ to 10mM final concentration to cells and thoroughly mix by inverting tubes. At this point cell suspensions should be fairly fluid and mobile.

4. Place capped tubes of cell suspensions in 37°C water bath; continuously keep tubes immersed by hand while tilting back and forth to gently mix. After 1-2 minutes, tubes of cell suspension should feel equilibrated in temperature to the water bath. At this point, keep cells immersed for 1-2 minutes longer so that cells fully reach 37°C for 1 minute.

5. Place tubes on ice and incubate for 30 minutes. During this time cells will fully lyse and become extremely viscous. If this does not occur, the lysozyme added to the cell suspension during harvest has gone bad. If this happens, refreeze the cells by placing at -80°C for 30 minutes and then re-thawing in the 37°C water bath for 3-5 minutes until just thawed; transfer to ice. This should cause cells to fully lyse.
F.3 LexA Purification

This protocol is based off of Little J.W., et.al. Cleavage of LexA Repressor. Methods in Enzymology. 1994 244: 266-284. This protocol yields 4-5mg of protein per gram of cell paste. 1L of growth media will produce 8-10g of cell paste for a total of 40-50mg protein. Note that LexA is expressed as a monomer that dimerizes at concentrations above several picomolar. Therefore, when quantifying LexA concentration based on the monomer extinction coefficient, the active LexA dimer complex is half of the measured monomer concentration.

F.3.1 Cell Debris Pelleting

Materials:

- Buffer A + 10% sucrose
- 30ml BD syringe
- 50ml round bottom polycarbonate tube (minimum volume = 20ml; maximum volume = 34ml)

1. Grow, express, harvest, and lyse cells as described in Section: General Protein Expression above.

2. After lysis, dilute lysate 2-3 times in Buffer A + 10% sucrose to decrease viscosity so that the lysate is pipetteable. If necessary, use a 30ml syringe without a hypodermic needle to pipette lysate up and down to mix.

3. Centrifuge resulting solution at 30000g, 4°C for 30 minutes on Beckman JA-17 rotor in round bottom 50ml tubes. The DNA and cell debris form a pellet.

4. Pool together the non-viscous supernatant and the fairly viscous material just overlying the pellet by decanting into a 250ml beaker on ice (Fraction I).
F.3.2 DNA and Protein Precipitation

Materials:

- 10% Polymin P solution (See Purification Buffers Section below)
- Stir Plate in 4°C Deli Fridge
- 50ml round bottom polycarbonate tube (minimum volume = 20ml; maximum volume = 34ml)
- ammonium sulfate (Sigma A4418)
- Buffer A + 10% sucrose (See Purification Buffer Section below)
- 3 - 1L beakers of Buffer B + 500mM NaCl (See Purification Buffer Section Below)

1. Slowly add 3.62ml of 10% Polymin P dropwise per 100ml Fraction I to 0.35% final concentration with stirring on a stir plate over the course of 10-15 minutes at 4°C. During this time genomic DNA will aggregate and begin to precipitate out.

2. Continue to stir 10 minutes more a 4°C.

3. Centrifuge at 15000g, 4°C for 10 minutes on Beckman JA-17 rotor in round bottom 50ml tubes. Decant and pool supernatant into 250ml beaker on ice.

4. Slowly add 0.4g ammonium sulfate per ml of supernatant with stirring at 4°C on a stir plate over the course of 10-15 minutes. Rapid addition of ammonium sulfate will cause local aggregation and premature precipitation of protein.

5. Continue to stir 20 minutes more at 4°C.

6. Centrifuge precipitate at 20000g, 4°C for 20 minutes on Beckman JA-17 rotor in round bottom 50ml tubes. Decant off and discard supernatant.
7. Redissolve pellet in 0.5-1x the original volume before precipitation with buffer A + 10% sucrose.

8. Re-precipitate with ammonium sulfate (0.4g ammonium sulfate/ml sample) as in steps 4-5 above.

9. Centrifuge precipitate at 20000g, 4°C for 20 minutes on Beckman JA-17 rotor in round bottom 50ml tubes. Decant off and discard supernatant.

10. Dissolve pellets in a small volume of Buffer B + 500 mM NaCl (5-10ml; approximately 1ml per gram of cell paste collected from the harvest)

11. Dialyze against 3 changes of 1L Buffer B + 500mM NaCl for at least 8 hours per change with the last one going overnight.

12. Centrifuge dialyzed protein in 15mL conical bottom tube at 1000g for 10 minutes on Beckman JA-17 rotor to precipitate any aggregates formed during dialysis. Collect supernatant (Faction II).

F.3.3 Phosphocellulose Column

See Whatman Note IL3: Use of Whatman Cellulose Phosphate Ion-Exchangers for care and handling of cellulose phosphate. Typically, Sigma Cellulose Phosphate (C3145) is used at 8g of dry powder per 1-2L of cell culture. Dry, unused powder has a 6-12 month shelf life when stored in the refrigerator. Cellulose phosphate should only be used once.

Materials

- XK26/70 chromatography column (GE 28-9889-50) with additional Adapter (GE 28-9898-77)
• 1mM I.D. tubing (GE 19-0040-01)
• Amersham P1 pump
• Cellulose Phosphate (Sigma C3145)
• 0.5L of 0.5M NaOH (See Purification Buffers Section Below)
• 0.5L of 0.5M HCl (See Purification Buffers Section Below)
• 1L of 0.5M potassium phosphate buffer pH 7.0 (See Purification Buffers Section Below)
• 0.5L of Buffer B (See Purification Buffers Section Below)
• 0.5L of Buffer B + 200mM NaCl (See Purification Buffers Section Below)
• 0.25L of Buffer B + 800mM NaCl
• Akta FPLC
• GE 50ml superloop (18-1113-82)
• Whatman Filter Paper (Whatman 3030-866)

Pre-Cycling

The precycling method has proved to be the most efficient and failsafe of typical methods. Filtering fluid from cellulose using filter paper and a vacuum buchner funnel causes the cellulose to pack too densely, running risk of clogging of the filter.

When stirring cellulose phosphate to resuspend, use a glass pasteur pipette or plastic serological pipette. Use of metal spatula can result in fines.

Unused, equilibrated media can be stored in 0.5M phosphate buffer, pH 7.0 at 4°C for up to one week.

1. For use in the Amersham C26/70 column, weigh out 8g of cellulose phosphate powder
(sigma C3145). Note that when hydrated, cellulose phosphate occupies approximately 4ml packed resin per dry gram. Use 25-30 ml bed volume of hydrated cellulose phosphate per 1-2 liter of LexA starting culture.

2. Put weighed cellulose phosphate in a 500ml beaker and gently stir in 200ml (20 volumes of cellulose phosphate) of 0.5M NaOH. Let stand for 5 minutes.

3. Using a pipette, suck off the supernatant as far down to the settled cellulose phosphate as possible. It helps to tip the beaker onto its bottom edge so more of the supernatant pools above the cellulose. If some of the cellulose is sucked up, it is OK. You should have 40-50ml cellulose + residual supernatant in the beaker. pH of supernatant should be ∼13.

4. Immediately add 200ml (to bring back up to 20 volumes) of 0.5M phosphate buffer pH 7.0. Gently stir and let stand for 5 minutes.

5. Repeat pipetting procedure to remove supernatant. pH of supernatant should be ∼7.5.

6. Immediately add 200ml (to bring back up to 20 volumes) of 0.5M HCl. Gently stir and let stand for five minutes.

7. Repeat pipetting procedure to remove supernatant. pH of supernatant should be ∼1.0.

8. Immediately add 200ml (to bring back up to 20 volumes) of 0.5M phosphate buffer pH 7.0. Gently stir and let stand for five minutes.

9. Repeat pipetting procedure to remove supernatant. pH of supernatant should be ∼6.5.

10. Immediately add another 200ml (to bring back up to 20 volumes) of 0.5M phosphate buffer pH 7.0. Gently stir and let stand for five minutes.
11. Repeat pipetting procedure to remove supernatant. pH of supernatant should be \( \sim 7.0 \).

**Column Packing**

1. Set up the GE XK26/70 column on a ring stand. Place XK26 flow adapter into bottom of GE XK26/70 column and push adapter plunger all the way down and tighten to seal. Connect the outlet of the bottom flow adapter to 1mm I.D. tubing and place tubing into a waste beaker. Keep the top flow adapter removed from the column at this point.

2. Dilute settled cellulose phosphate 3-fold with \( \sim 100ml \) Buffer B. Gently stir and let stand for 5 minutes.

3. Note the settled volume occupied by the cellulose phosphate after settling and adjust the volume of liquid above the cellulose phosphate so that it is 20\% of the settled cellulose phosphate volume. This should be 15-20 ml.

4. Pour a couple of ml of Buffer B into the column and allow most of it to flow out; plug the outlet.

5. Stir the cellulose phosphate and pipette into the column with the outlet open. Allow the buffer to flow from the column into waste, but don’t let the buffer level drop below the cellulose phosphate level. Continue to add slurry until all is added; plug the column outlet to stop the flow.

6. Fill column to top with Buffer B. Let set for 5 minutes to allow the column to settle.

7. Insert the top XK26 flow adapter at an angle to prevent air from being trapped. Lower the adapter until the support screen is about 0.5cm from the bed; tighten down the flow adapter to seal. As the plunger is pushed down, fluid will spill out from the top of the column. Keep paper towels under the column to absorb extra buffer.
Column Equilibration

1. Prime the P1 pump with Buffer B + 200mM NaCl. Attach the outlet of the P1 pump to the inlet of the upper flow adapter with 1mm I.D. tubing.

2. Equilibrate column by running 3-4CV of Buffer B + 200mM NaCl through the column at a flow rate of 0.75 ml/min (in general at least 45 ml/hr/cm² of the internal cross-sectional area of the column) until the column bed height is constant and the pH of the eluate is stabilized. Note: This normally takes 3 column volumes; it typically works best to equilibrate overnight at 0.1-0.2 ml/min and finish in the morning.

3. When equilibrated, stop the pump, loosen the top XK26 flow adapter and gently lower flow adapter until it contacts the resin; tighten down the flow adapter to seal.

4. Connect column to Akta FPLC system at 4°C (GE healthcare, use system in Jaroneic Lab after appropriate training) equilibrated with Buffer B + 200mM NaCl in line A1 and Buffer B + 800mM NaCl in line B1. Also, fill GE superloop with Buffer B + 200mM NaCl.

5. Equilibrate column on FPLC with 0.5-1CV of Buffer B + 200mM NaCl at 0.75 ml/min until conductivity and UV-Vis are stable. During this time the bed volume may decrease due pressure of the FPLC. If this happens simply lower the flow adapter to the new bed height.

Sample Loading and Elution

1. Dilute Fraction II 2.5-fold with Buffer B at 4°C to give a final NaCl concentration of 200mM NaCl.

2. Load diluted Fraction II into superloop.
3. Apply sample to column at 0.75 ml/min, collecting flowthrough in case protein does not bind to column.

4. Wash sample with 1.5CV (∼45ml) of Buffer B + 200mM NaCl at 0.75 ml/min to remove any unbound protein. UV-vis should return to baseline. Collect flowthrough during wash.

5. Elute protein with a linear gradient in Buffer B from 200mM NaCl to 800mM NaCl at 0.75 ml/min over 2.5 column volumes (∼75ml) followed by a 1CV (∼30ml) wash at 800mM NaCl. Collect 1.3ml fractions during the gradient and wash. LexA elutes at about 500mM NaCl.

6. For quantitation of which fractions contain protein, apply 30-50µl of each fraction onto Whatman filter paper either by directly blotting onto a 2cm x 2cm grid or by using a vacuum manifold blottter. Microwave for 15 seconds to dry. Briefly stain with gel stain ∼30 seconds (old stain can be used); destain for ∼30 minutes in fresh destain. Spots with the darkest intensity typically correspond to eluted LexA.

7. Pool fractions containing LexA into 50ml falcon tube and store on ice (Fraction III).

**F.3.4 Hydroxyapatite Column**

The following protocol is designed based on the BioRad CHT Ceramic Hydroxyapatite Instruction Manual. Hydroxyapatite [Ca$_5$(PO$_4$)$_3$(OH)] is a dual or "mixed mode" ion exchanger due to sample interactions with both calcium cations and phosphate anions leading to unique binding properties that, depending on the characteristics of the protein being purified, may or may not aid in separation compared to strict anion or cation exchangers. As LexA is very positively charged at neutral pH due to high lysine content, hydroxyapatite proves to be a cost-effective and easy resin to use for FPLC applications.

However, short shelf life of resuspended resin (6-12 months) can result in unpredictable
binding capacity of older resin, requiring column repacking with fresh resin. Additionally, hydroxyapatite is sensitive to both EDTA and light, therefore buffers with low EDTA (~0.1mM) concentrations should be used, and both unused resin and packed column should be stored away from direct light.

Note that recent advances in hydroxyapatite chemistry and handling have made many practices employed in older protocols obsolete. First, use of hydrated salts (potaasium phosphate monobasic and potassium phosphate dibasic trihydrate) should be used as the anhydrous forms contain trace elements that degrade hydroxyapatite. Secondly, inclusion of 0.5mM CaCl₂ (20ppm Ca) in binding and elution buffers prevents calcium leaching from hydroxyapatite and promotes column longevity. Thirdly, while columns used to be stored in 1M NaOH or 200mM phosphate dibasic, it is now recommended to store columns in 0.1M NaOH + 10mM phosphate. The following protocol reflects these advances.

Materials

- CHT ceramic hydroxyapatite Type II, 20µm particle size (Biorad 158-2200)
- 200mM Potassium Phosphate dibasic (See Purification Buffers Section below)
- 1M NaOH, filtered and degassed
- water, filtered and degassed
- 500mM Potassium Phosphate Buffer, pH 7.0 (See Purification Buffers Section below)
- Buffer C + 50mM Potassium Phosphate, pH 7.0 (See Purification Buffers Section below)
- Buffer C + 400mM Potassium Phosphate, pH 7.0 (See Purification Buffers Section below)
- GE C10/10 column (GE 19-5001-01)
- GE C10 flow adapter (GE 19-5006-01)
• 1mm I.D. tubing (GE 19-0040-01)

• Amersham P1 pump

• GE Akta FPLC system

• GE 50ml superloop (18-1113-82)

• 0.1M NaOH + 10mM potassium phosphate buffer

**Pre-Cycling and Column Packing**

1. For use in the Amersham C10/10 column, weigh out 4g CHT Ceramic Hydroxyapatite. Note: 1g hydrated CHT hydroxyapatite makes ~2.3ml bed volume. Use 8-10ml bed volume of hydrated hydroxyapatite per liter of LexA starting culture

2. Put weighed hydroxyapatite in a 50ml beaker and gently stir in 30ml of 200mM potassium phosphate dibasic to make a 10% slurry. Use a plastic serological pipette or glass pasteur pipette to stir to avoid production of fines. Allow to settle for 10 minutes.

3. Set up the Amersham C10/10 column vertically with the top end piece and flow adapter removed, the bottom end piece with bed support attached to the column, and 1mm I.D. tubing running from the outlet into waste.

4. Fill the column with 5ml of 200mM potassium phosphate dibasic, and let 4ml flow into waste. Plug column outlet to stop the flow so that the column is 10% full. 5. Note the volume of the settled hydroxyapatite and using a pipette, suck off the excess sodium phosphate dibasic until its volume above the settled resin is 20% of the settled resin volume. Swirl the slurry and carefully pipette mixture into the column until full. Allow hydroxyapatite to settle for 5 minutes.

6. Open the column outlet and allow fluid to elute until supernatant level is just above the
bed; plug the column outlet.

7. Resuspend remaining hydroxyapatite in beaker and add more of the slurry into the column. Repeat settling, eluting, pipetting procedure until all hydroxyapatite slurry is used or resin height is within 1cm of the top of the column.

8. Plug the column outlet and fill column to the top with 200mM potassium phosphate dibasic.

9. Insert the C10 flow adapter at an angle to prevent air from being trapped. Lower the adapter until the support screen is about 1mm from the bed. As the plunger of the flow adapter is pushed down, fluid will elute from the open end of the column inlet tubing connected to the plunger.

Note: The initial pressure felt when inserting the C10 adapter is due to fitting the o-ring into the column. Apply force liberally as at this point you will not crush the bed. As soon as the o-ring slips into the column, stop applying force. Now slowly move the plunger down so that the fluid elutes at about 1 ml/min out of the 1mm I.D. tubing inserted into the flow adapter. Pushing the column down too fast may crush the bed.

10. Plug the column inlet and screw the locking ring onto the flow adapter and tighten the locking nut.

11. Prime the P-1 peristaltic pump with 200mM potassium phosphate dibasic, unplug the column inlet and attach the column inlet to the pump. Open the column outlet and pump 50ml (5 column volumes) through the column at 2 ml/min (200% operating flow rate) until the bed height is constant.

12. Stop the flow to the column, plug the column outlet, and detach the column inlet from
the pump.

13. Lower the C10 adapter until the support screen contacts the bed, but do not apply excessive force resulting in crushing of the bed. As the plunger is pushed down, fluid will elute from the open end of the tubing connected to the plunger. Note: Additional pumping may be required for final adjustment of the flow adapter.

14. Plug the inlet of the column and proceed to column equilibration.

**Column Equilibration or waking a stored column**

Whether column has just been packed or is a previously packed and stored column, perform the following:

1. Wash column using P1 pump with 1CV (~10ml) of water, filtered and degassed at 1 ml/min.

2. Sanitize column using P1 pump with 5CV (~50ml) of 1M NaOH at 1 ml/min.

3. Wash column using P1 pump with 1CV (~10ml) of water, filtered and degassed at 1 ml/min.

4. Pre-equilibrate column using P1 pump with 3CV (~30ml) of 500mM Potassium Phosphate buffer, pH 7.0 at 1 ml/min. At this point stop flow and plug inlet and outlet of column and proceed to sample loading and elution.
Sample Loading and Elution

1. Set up column on the Akta FPLC system at 4°C with GE 50ml superloop attached to injector port (Use system in Jeroneic lab). Equilibrate line A1 with Buffer C + 50mM phosphate and line B1 with Buffer C + 400mM phosphate.

2. Equilibrate column with 3CV (~30ml) of Buffer C + 50mM phosphate at 1 ml/min until both UV-vis and conductivity are stable.

3. Dilute Fraction III by 2-2.5 fold with Buffer C + 50mM phosphate to bring NaCl concentration down to ~200mM from the 400-500mM NaCl from cellulose phosphate procedure.

4. Load sample onto superloop.

5. Inject sample onto column at 1.5ml/min collecting flowthrough in case protein does not bind.

6. Wash column with 1.5CV (~15ml) of Buffer C + 50mM phosphate 1ml/min until UV returns to baseline. Collect flowthrough.

7. Elute sample over a 4CV (~40ml) linear gradient from Buffer C + 50mM phosphate to 400mM phosphate at 1ml/min. Wash column with 1CV of Buffer C + 400mM phosphate. Collect 1ml fractions during entire gradient and wash. Store fractions on ice.

8. Before running another injection perform: wash with water 1CV, sanitize with 1M NaOH 5CV, wash with water 1CV, pre-equilibrate with 500mM phosphate buffer pH 7.0 3CV, equilibrate with Buffer C + 50mM phosphate 3CV.

9. To store column wash with water 1CV, sanitize with 1M NaOH 5CV, wash with water
1CV, store with 3CV 0.1M NaOH + 10mM phosphate. Store column at 4°C away from light.

F.3.5 Determining LexA fraction and Dialysis

Materials

- 12% SDS-PAGE gel (See Appendix E)
- 1x Tris-Glycine-SDS buffer (See Appendix E)
- 6x SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- 3 - 1L beakers of Protein Storage Buffer (See Purification Buffers Below)
- Amicon 10K centrifuge concentrator (Millipore)

LexA fraction analysis and dialysis

1. Based on UV-VIS chromatogram from PFLC purification, combine 10µl of each fraction potentially containing LexA with 2µl of 6x SDS loading dye. Boil 95°C for 5 minutes and spin down on microcentrifuge.

2. Resolve fractions on 12% SDS-page gel; stain and destain when done (See Appendix E).

3. Determine which fractions contain pure LexA with minimal contaminants.

4. Pool desired fractions and dialyze against 3 changes of 1L each of Protein Storage Buffer (10% glycerol, 10mM HEPES pH 7.5, 1mM EDTA, 1mM DTT, 200mM NaCl) at 4°C for at least 8 hours per change with the last change going overnight.
Storage of LexA

1. After dialysis, reclaim samples and store in 15ml falcon tube on ice.

2. Quantify LexA concentration by UV-Vis. The LexA monomer has a molar extinction
coefficient of \( \epsilon_{(275\text{nm}, 1\text{cm})} = 6970 \text{ M}^{-1} \text{cm}^{-1} \) and a molecular weight of 22371Da. However, LexA occurs as a dimer above picomolar concentrations. Therefore, concentrations
of LexA aliquots should be expressed as the full dimer complex. LexA monomer concentra-
tions at 70\( \mu \)M (35\( \mu \)M dimer complex) are typical after purification and dialysis. However,
nucleosome binding experiments at physiological salt concentrations require LexA monomer
concentrations of 300\( \mu \)M (150\( \mu \)M dimer complex). LexA can easily be concentrated by an
Amicon 10 to monomer concentrations between 300-500\( \mu \)M without aggregation.

3. Aliquot LexA in 200\( \mu \)l aliquots and flash freeze in liquid nitrogen. Store at -80\(^\circ\)C.

4. LexA is a relatively stable protein and can be stored at 4\(^\circ\)C on ice for several weeks
or for several years at -80\(^\circ\)C. Wild type LexA slowly autodigests under these conditions,
therefore, SDS-page analysis should be periodically performed to verify LexA purity. Ad-
ditionally, wild type LexA does not contain native cysteines, however, inclusion of 1mM
DTT throughout the entire purification process is for good practice, to prevent aggregation
of other proteins, and to make the procedure compatible with LexA cystiene mutants.
F.4 Purification of Glutathione Tagged Proteins (GEX vectors)

The following protocol is optimized for purification and cleavage of Glutathione S-transferase tagged proteins (See Table F.1), which is derived from cloning the gene of interest into the pGEX glutathione-S-transferase fusion protein system (GE Healthcare).

F.4.1 Cell Debris Pelleting

Materials:

- 30ml BD syringe
- sonicator micro-tip
- Buffer A + 10% sucrose + 1mM PMSF chilled to 4°C (See Purification Buffers Section below)
- Beckman SW41 rotor and Beckman S-class ultracentrifuge chilled to 4°C
- Beckman 14mm x 89mm ultra-clear tubes (Beckman 344059)

1. Grow, express, harvest, and lyse cells as described in Section: General Protein Expression above.

2. After cells are lysed, reduce viscosity by shearing with 30ml syringe. Suck entire lysate up and expel back into tube on ice 5-6 times.

3. Sonicate cells as detailed in Appendix A, Section: Inclusion Body Isolation. Briefly, sonicate cells on ice with micro tip on a Qsonica sonicator at power 6 (4-5 watts) for 6-8 rounds of 15 seconds on, 10 seconds off until cells are no longer viscous.

4. Load sonicated lysate into pairs of 14mm x 89mm centrifuge tubes massed to within
0.05g of each other with Buffer A + 10% sucrose + 1mM PMSF and filled to within 10mm-5mm from the top of the tube to prevent tube collapse or overflow.

5. Centrifuge at 41000 RPM, 4°C for 45 minutes in SW41 rotor on the Beckman Optima ultracentrifuge. When done collect supernatant and store on ice (Fraction I).

F.4.2 Glutathione Agarose Purification

There are several methods for purification of proteins by glutathione agarose. They can be purely batch methods where all protein binding and cleavage is performed in a test tube, purely by column where all binding and cleavage is performed on resin packed in a column, or a combination of both. While column methods yield the highest purity, they are notorious for inefficient binding and cleavage whereas batch processes are the most efficient but harder to remove contaminants resulting in lower purity. This protocol is designed to strike a balance of both efficiency and purity due to the high cost of both glutathione resin and cleavage agents.

The method is as follows:

1. Bind protein (Fraction I) by batch process to glutathione agarose in order to get efficient binding.

2. Fully remove background proteome and non-specifically bound protein by extensive washing on a column.

3. Purge resin with bound protein from column and batch cleave with appropriate cleavage agent to efficiently cleave protein.

4. Batch bind cleavage agent with benzamidine agarose to efficiently bind cleavage agent.
5. Collect supernatant carrying protein cleaved protein. 6. Perform further purification if necessary.

Materials:

- glutathione agarose (Qiagen 1057865)
- Buffer A, refrigerated at 4°C (do not include any protease inhibitors as this will inhibit efficient cleavage)
- GE C10/10 column (GE 19-5001-01) with flow adapter (GE 19-5006-01)
- 1mM I.D. tubing (GE 19-0040-01)
- P1 pump

1. Remove glutathione agarose from refrigerator and gently swirl to resuspend. Transfer 1.5ml of slurry per 1L of starting culture to a 50ml falcon tube.

2. Add 30ml of Buffer A at 4°C to the resin and gently invert to mix.

3. Centrifuge at 5000g for 5 minutes at 4°C on Beckman JA17 rotor in Avanti centrifuge. Turn tube 180 degrees and centrifuge again for 5000g, 5 minutes to fully pellet resin. Decant supernatant and discard.

4. Add another 30ml of Buffer A. Repeat centrifugation and decanting as in step 3.

5. Add clarified protein sample (Fraction I) to resin and gently invert to mix. Add a small stir bar to the tube and stir at medium low on stir plate at 4°C for 30 minutes to actively mix resin with protein sample for efficient binding.

6. Meanwhile, set up C10/10 column in 4°C deli fridge with bottom piece with bed support screen attached and with C10 adapter removed. Attach 1mm tubing to column outlet and
place tubing line into waste beaker; plug outlet. Prime P1 pump with Buffer A in deli fridge at 4°C.

6. When protein is done binding to resin, centrifuge at 5000g for 5 minutes at 4°C on Beckman JA17 rotor in Avanti centrifuge. Turn tube 180 degrees and centrifuge again for 5000g, 5 minutes to fully pellet resin. Decant supernatant and discard.

7. Add 8ml of Buffer A to protein-bound resin and gently swirl to mix.

8. Using a 10ml pipette, gently pipette resin into C10/10 column until column is full or all resin is used. Unplug outlet and allow buffer to flow through column to gently pack the resin. Add any additional resin as column settles and packs. Do not let the buffer level go below the resin height in the column; add additional Buffer A if necessary to prevent this. When all resin has been added, plug the column outlet.

9. Fill the column to the top with Buffer A. Carefully insert the C10 flow adapter at an angle to prevent trapping of air and then apply liberal force to get end of flow adapter with O-ring into column. Once O-ring goes into column, stop applying excessive force; gently push flow adapter down until flow adapter is 0.5cm from the top of the resin. As the flow adapter is pushed down, buffer will elute from open end of flow adapter. Tighten nut to lock down flow adapter.

10. Attach column inlet to P1 pump and unplug column outlet. Wash column with 5CV (50ml) of Buffer A at 1.5-2ml/min to completely remove all unbound and non-specifically bound protein. This is gentler on the resin-bound protein and much more efficient than multiple rounds of batch washing and centrifugation.

11. Stop flow to column and plug outlet. Carefully unscrew the bottom piece from the column exposing the bottom of the column and the resin. Place a 15ml falcon tube under
the column and the resin. Unlock the flow adapter and push the flow adapter all the way
down to expel the resin into the 15ml tube. Turn on P1 pump and flow \( \sim 0.5 \text{ml} \) of buffer A
to flush out any residual resin. There should be about 2ml total of resin plus buffer in the
tube. Store on ice.

**F.4.3 Cleavage**

Materials:

- 5mM CaCl\(_2\) (Sigma C3306)
- Rotisserie or nutator
- Thrombin (MP biomedicals 154163) or Factor Xa (NEB 8010S) protease
- Benzamidine Agarose (Sigma A8332)
- 12% SDS-PAGE gel (See Appendix E)
- 1x Tris-Glycine-SDS buffer (See Appendix E)
- 6x SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- 3 - 1L beakers of Protein Storage Buffer (See Purification Buffers Below)
- Amicon 10K centrifuge concentrator (Millipore)

1. Add an equal volume (\( \sim 2\text{ml} \)) of 5mM CaCl\(_2\) to dilute resin and buffer by 2-fold to
give 25mM Tris pH 8.0, 100mM NaCl, 0.25mM EDTA, 2.5mM CaCl\(_2\). This is the optimum
cleavage conditions for Thrombin and Factor Xa.

2. 1\( \mu \text{g} \) of Factor Xa or 10U of Thrombin will cleave 100-200\( \mu \text{g} \) of protein at room temper-
ature (23°C) overnight with slow agitation on the rotisserie.
• For Factor Xa remove a new 50µg tube (NEB P8010S) from -20°C and add entire stock to resin-bound protein.

• For Thrombin, combine 500µl of Buffer A with 500µl of 80% glycerol at 4°C and thoroughly mix. Resuspend entire 1KU vial (MP biomedicals) with 1ml Buffer A/glycerol mixture, aliquot into 100µl samples. Flash freeze aliquots and store at -80°C. Take one 100µl aliquot and add entire stock to resin-bound protein.

3. After adding Thrombin or Factor Xa to resin-bound protein, cap and cover 15ml falcon tube with aluminum foil. Place on rotisserie and incubate at room temperature overnight for 12 hours. Cleavage should go to completion during this time depending on the activity of the protein and the accessibility of the cleavage site for the particular fusion protein being cleaved.

4. Remove Benzamaidine agarose from refrigerator and gently swirl to resuspend. Remove 2ml and transfer to 15ml falcon tube. Add 5ml of Buffer A and 5ml water to resin.

5. Centrifuge resin in Beckman JA17 rotor on Avanti Centrifuge at 5000g, 4°C for 5 minutes. Turn tube 180 degrees and centrifuge again 5000g, 5 minutes to fully pellet resin. Decant supernatant.

6. Add another 5ml of Buffer A and 5ml water to the resin. Gently invert to resuspend. Centrifuge and decant supernatant as in step 5 above.

7. Add 0.25ml of Buffer A and 0.25ml water to resin and gently swirl to resuspend. Carefully pipette Benzamidine Agarose slurry into glutathione agarose/protease/cleaved protein sample from overnight cleavage. Recap tube and return to rotisserie. Allow to incubate at room temperature for 20-30 minutes to fully bind protease to benzamidine agarose.

8. Centrifuge mixture in Beckman JA17 rotor on Avanti Centrifuge at 5000g, 4°C for 5
minutes. Turn tube 180 degrees and centrifuge again 5000g 5 minutes to fully pellet resins. Carefully remove supernatant containing cleaved protein and store on ice.

9. Assay protein purity by 12% SDS-PAGE. Additional treatment with more benzamidine agarose may be required to remove remnant Thrombin or Factor Xa protease. If protein is sufficiently pure, the protein can be dialyzed into protein storage buffer by 3 - 1L changes at 4°C for at least 8 hours per change with the last change going overnight. Protein can be concentrated before storage by Amicon 10 centrifugation concentration device.

However, additional purification by ion exchange chromatography is typically required to remove remnant glutathione S-transferase that releases from glutathione-agarose during cleavage and to remove truncated protein products of the protein being purified.
F.5 Yeast NAP1-His6 Purification

This protocol is adapted from:


This protocol is optimized for purification of His6 tagged S. cerevisae nucleosome assembly protein 1 (yNap1). However, the Nickel chromatography protocol developed here for batch biniding and column elution, which is optimized for maximum yield and purity, should be applicable to most His6-tagged transcription factors and histone chaperones. The monoQ anion exchange polishing step is specific for yNap1.

F.5.1 Cell Debris Pelleting

Materials:

- 30ml BD syringe
- sonicator micro-tip
- Buffer A + 10% sucrose + 1mM PMSF chilled to 4°C (See Purification Buffers Section below)
- Beckman SW41 rotor and Beckman S-class ultracentrifuge chilled to 4°C
- Beckman 14mm x 89mm ultra-clear tubes (Beckman 344059)
1. Grow, express, harvest, and lyse cells as described in Section: General Protein Expression above.

2. After cells are lysed, reduce viscosity by shearing with 30ml syringe. Suck entire lysate up and expel back into tube on ice 5-6 times.

3. Sonicate cells as detailed in Appendix A, Section: Inclusion Body Isolation. Briefly, sonicate cells on ice with micro tip on a Qsonica sonicator at power 6 (4-5 watts) for 6-8 rounds of 15 seconds on, 10 seconds off until cells are no longer viscous.

4. Load sonicated lysate into pairs of 14mm x 89mm centrifuge tubes massed to within 0.05g of each other and filled to within 10mm-5mm from the top of the tube to prevent tube collapse or overflow.

5. Centrifuge at 41000RPM, 4°C for 45 minutes in SW41 rotor on the Beckman Optima ultracentrifuge. When done collect supernatant and store on ice (Fraction I).

F.5.2 Nickel-NTA Agarose Purification

There are several methods for purification of proteins by nickel agarose. They can be purely batch methods where all protein binding and elution is performed in a test tube, purely by column where all binding and elution is performed on resin packed in a column, or a combination of both. While column methods yield the highest purity, they are notorious for inefficient binding whereas batch process are the most efficient but harder to remove contaminants resulting in lower purity. This protocol is designed to strike a balance of both efficiency and purity due to the high cost of both glutathione resin and cleavage agents.

The method is as follows:

1. Bind protein by batch process to nickel agarose in order to get efficient binding.
2. Fully remove background proteome and non-specifically bound protein by extensive washing on a column with low concentration imidazole.

3. Elute sample with a step elution of high concentration imidazole.

4. Perform additional purification to remove truncated protein contaminants.

Materials:

- Nickel-NTA agarose (Qiagen 1018244)
- TTD Buffer + 1mM Imidazole, refrigerated at 4°C (20mM Tris pH 7.5, 0.2% Tween 20, 1mM DTT, See Purification Buffers below)
- TTD Buffer + 100mM Imidazole, refrigerated at 4°C (See Purification Buffers below)
- 3-1L beakers of TED200 buffer (20mM Tris pH 7.5, 1mM DTT, 1mM EDTA, 200mM NaCl, see Purification Buffers below)
- GE C10/10 column (GE 19-5001-01) with flow adapter (GE 19-5006-01)
- 1mM I.D. tubing (GE 19-0040-01)
- P1 pump
- Fraction Collector

1. Remove Nickel NTA agarose from refrigerator and gently swirl to resuspend. Transfer 1.5ml of slurry per 1L of starting culture to a 50ml falcon tube.

2. Add 30ml TTD Buffer + 1mM imidazole at 4°C to the resin and gently invert to mix. Do not use buffers with EDTA as they will remove nickel from the resin. While Fraction I in Buffer A has 0.5mM EDTA, addition of 10mM MgCl₂ for cell lysis effectively bound up all free EDTA.
3. Centrifuge at 5000g for 5 minutes at 4°C on Beckman JA17 rotor in Avanti centrifuge. Turn tube 180 degrees and centrifuge again for 5000g, 5 minutes to fully pellet resin. Decant supernatant and discard.

4. Add another 30ml of TTD Buffer + 1mM Imidazole. Repeat centrifugation and decanting as in step 3.

5. Add clarified protein sample (Fraction I) to resin and gently invert to mix. Add a small stir bar to the tube and stir at medium low on stir plate at 4°C for 30 minutes to actively mix resin with protein sample for efficient binding.

6. Meanwhile, set up C10/10 column in 4°C deli fridge with bottom piece with bed support screen attached and with C10 adapter removed. Attach 1mm I.D. tubing to column outlet and place tubing line into waste beaker; plug outlet. Prime P1 pump with TTD Buffer + 1mM Imidazole in deli fridge at 4°C.

6. When protein is done binding to resin, centrifuge at 5000g for 5 minutes at 4°C on Beckman JA17 rotor in Avanti centrifuge. Turn tube 180 degrees and centrifuge again for 5000g, 5 minutes to fully pellet resin. Decant supernatant and discard.

7. Add 8ml of TTD Buffer + 1mM Imidazole to protein-bound resin and gently swirl to mix.

8. Using a 1ml pipette, gently pipette resin into C10/10 column until all resin is added. Unplug outlet and allow buffer to flow through column to gently pack the resin. Do not let the buffer level go below the resin height in the column; add additional TTD Buffer + 1mM Imidazole if necessary to prevent this. When resin has mostly packed and settled plug the column outlet.
9. Fill the column to the top with TTD Buffer + 1mM Imidazole. Carefully insert the C10 flow adapter at an angle to prevent trapping of air and then apply liberal force to get end of flow adapter with O-ring into column. Once O-ring goes into column stop applying excessive force; gently push flow adapter down until flow adapter is just at the top of the resin. As the flow adapter is pushed down, buffer will elute from the open end of flow adapter. Tighten nut to lock down flow adapter.

10. Attach column inlet to P1 pump and unplug column outlet. Wash column with 50ml of TTD Buffer + 1mM Imidazole at 1.5-2 ml/min to completely remove all unbound and non-specifically bound protein. This is gentler on the resin-bound protein and much more efficient than multiple rounds of batch washing and centrifugation.

11. When washing is complete, elute protein with a single step elution of 10ml TTD Buffer + 100mM Imidazole at 0.5 ml/min. Collect 0.5ml fractions using fraction collector.

12. Determine protein fractions using UV-vis absorption. Pool fractions and dialyze against 3 - 1L changes of TED200 buffer at 4°C on stir plate for at least 8 hours per change with the last change going overnight.

13. When done dialyzing, reclaim sample and store on ice (Fraction II)

**F.5.3 MonoQ Ion Exchange Purification**

**Materials:**

- TED0 (25mM Tris, 1mM EDTA, 1mM DTT, pH 7.5, See Purification Buffers section)
- TED1000 (25mM Tris, 1mM EDTA, 1mM DTT, 1000mM NaCl, pH 7.5, See Purification Buffers section)
- water, filtered and degassed
- 20% Ethanol, filtered and degassed
- MonoQ anion exchange column (GE 17-5166-01)
- GE 50ml superloop (18-1113-82)
- HPLC with Rheodyne Injector
- Deli Fridge
- 12% SDS-PAGE gel (See Appendix E)
- 1x Tris-Glycine-SDS buffer (See Appendix E)
- 6x SDS loading dye (See Appendix E)
- Gel Stain (See Appendix E)
- Gel Destain (See Appendix E)
- 3-1L beakers Protein Storage Buffer (See Purification Buffers section below)

1. Configure HPLC so that:
   - Place TED0, TED1000, and water, and 20% ethanol bottles are in lines A, B, C, and D, respectively. Fill lines with their respective buffer.
   - Connect purge valve outlet directly to inlet of heat block of HPLC and set block to 8°C.
   - Fill superloop with TED200 buffer (80% TED0, 20% TED1000)
   - Place Rheodyne injector, MonoQ column, and superloop in Deli fridge at 4°C. Connect outlet of 8°C heat block to inlet of rheodyne injector (Port 2). Do not connect Rheodyne Remote Control to HPLC. Set injector to Load position. Connect superloop inlet (top) to Rheodyne injector Port 1 and superloop outlet (bottom) to Port 4.
   - Flow 20% ethanol through system at 0.2 ml/min until buffer comes out of Rheodyne injector outlet (Port 3). Connect MonoQ column inlet to Rheodyne outlet, and connect MonoQ outlet to UV-vis module.
Connect a collection line to the UV-vis outlet to collect sample by hand or run line back to fraction collector in deli fridge at 4°C.

- With the Rheodyne still in Load position, equilibrate MonoQ with water at 0.75 ml/min, and then with TED200 (80% TED0, 20% TED1000) at 0.75 ml/min.

- When column is in TED200, turn Rheodyne to inject position and allow superloop plunger to move down until it is all the way to the bottom of the superloop.

2. After system and column are equilibrated; spin down sample at 5000g, 4°C for 5 minutes in Avanti JA-17 rotor to remove aggregates.

3. Carefully remove supernatant and load into a 50ml BD syringe with needle for Rheodyne injector; expel air from needle and syringe.

4. Stop HPLC pump and turn Rheodyne back to load position. Insert syringe into injector port. Push sample into superloop. White plunger in superloop will move up as sample is loaded.

5. Balance HPLC UV-vis; set flow rate to 0.75 ml/min with 80% TE0, 20% TE1000; and turn Rheodyne to inject position. As sample loads onto the column the white superloop plunger should move back down. Monitor UV-Vis as sample loads onto the column. Positive charged proteins that do not bind to the will elute from the column at an OD of around 100-200mAU. Watch for sudden spikes in UV-Vis and pressure as this indicates overloading of the column. If this occurs, turn Rheodyne back to load position immediately and continue to Step 7.

6. After all of sample has been loaded, turn Rheodyne back to inject position.

7. Wash column with 1-2CV of 80% TE0, 20% TE1000 until UV-Vis returns to baseline.
8. Elute Nap1 protein on a linear gradient of 20% TE1000 to 60% TE1000 over 30 minutes followed by a constant elution of 60% TE1000 for 10 minutes before returning to initial conditions.

9. Collect Nap1 peak by hand. yNap1 elutes around 40% TE1000. The gradient goes to 60% to clean column of other more tightly bound proteins.

10. Run fractions of yNap1 on a 12% SDS-page gel to check purity. Pool fractions.

11. Dialyze protein with 3 - 1L changes of Protein Storage buffer (10% Glycerol, 250mM NaCl, 10mM Tris pH 7.5, 1mM EDTA, 1mM DTT ) at 4°C for at least 8 hours per change with the last one going overnight.

12. Reclaim protein and quantify concentration by UV-vis. yNap1-His6 monomer has a molar extinction coefficient of \( \epsilon_{276\text{nm},1\text{cm}} = 36390 \text{ M}^{-1}\text{cm}^{-1} \) and a molecular weight of 51263Da. At concentrations above 1nM, yNap1 is a homodimer. Therefore the concentration of the functional Nap1 homodimer complex is half of the monomer concentration.

13. Aliquot yNap1 in 200\text{µl} aliquots with molar concentration of Nap1 homodimer indicated on tube. Flash freeze in liquid nitrogen; store at -80°C. Note: At -80°C Nap1 is stable for ~6 months. Aliquots thawed on ice are stable for 1 week.
F.6 Pyrococcus fruiosus Polymerase Purification

This protocol is based off of Erickson HP, and Lu C. Expression in Escherichia coli of the thermostable DNA polymerase from Pyrococcus furiosus. Protein Expression and Purification. 1997 11(2):197-84.

Pyrococcus furiosus (pfu) polymerase is easily expressed and purified by a single step over cellulose phosphate resin. This protein is the unmodified gene from Pyrococcus furiosus and contains 3′ exonuclease proofreading activity. For PCR with this protein care must be taken to use 3′ thiophosphate linkage in the PCR oligo in prevent 3′ exonuclease digestion of the oligo, particularly for oligos shorter than 30 base pair and/or containing modifications and sequences different from the parent template.

F.6.1 Cell Debris Pelleting and Protein Precipitation

Materials:

- 30ml BD syringe
- sonicator micro-tip
- Buffer A + 10% sucrose + 1mM PMSF chilled to 4°C (See Purification Buffers Section below)
- DNaseI (Sigma D4527 or D5025)
- Beckman SW41 rotor and Beckman S-class ultracentrifuge chilled to 4°C
- Beckman 14mm x 89mm ultra-clear tubes (Beckman 344059)
- water bath at 72°C
- 50ml round bottom polycarbonate tubes
- 0.22µm syringe filter
1. Grow, express, harvest, and lyse cells as described in Section: General Protein Expression above with the following exception: After cells are thawed for lysing, add 1 mg/ml DNase I in Buffer A + 10% sucrose + 1mM PMSF to 0.4 µg/ml final concentration in conjunction with adding MgCl₂ to 10mM final concentration. Additionally, after heat shock at 37°C for 1-2 minutes, incubate at room temperature on rotisserie for 30 minutes instead of on ice.

2. After cells are lysed, reduce viscosity by shearing with 30ml syringe. Suck entire lysate up and expel back into tube on ice 5-6 times.

3. Sonicate cells as detailed in Appendix A, Section: Inclusion Body Isolation. Briefly, sonicate cells on ice with micro tip on a Qsonica sonicator at power 6 (4-5 watts) for 6-8 rounds of 15 seconds on, 10 seconds off until cells are no longer viscous.

4. Load sonicated lysate into pairs of 14mm x 89mm centrifuge tubes massed to within 0.05g of each other and filled to within 10mm-5mm from the top of the tube to prevent tube collapse or overflow.

5. Centrifuge at 41000RPM, 4°C for 30 minutes in SW41 rotor on the Beckman Optima ultracentrifuge. When done collect supernatant and aliquot 10ml volumes into 15ml falcon tubes on ice.

6. Incubate sample in 15ml falcon tubes in a 72°C water bath for 10 minutes to denature proteins.

7. Centrifuge in round bottom 50ml polycarbonate centrifuge tube at 25000PRM, 4°C for 15 minutes to remove denatured proteins. Collect supernatant.

8. Filter-sterilize supernatant using a 0.22µm syringe filter to remove residual aggregates.
(Fraction I) and store on ice.

**F.6.2 Cellulose Phosphate Chromatography**

See Whatman Note IL3: Use of Whatman Cellulose Phosphate Ion-Exchangers for care and handling of cellulose phosphate. Typically Sigma Cellulose Phosphate (C3145) is used at 8g of dry powder per 1-2L of cell culture. Dry, unused powder has a 6-12 month shelf life when stored in the refrigerator. Cellulose phosphate should only be used once.

**Materials**

- XK26/70 chromatography column (GE 28-9889-50) with addition Adapter (GE 28-9898-77)
- 1mM I.D. tubing (GE 19-0040-01)
- Amersham P1 pump
- Cellulose Phosphate (Sigma C3145)
- 0.5L of 0.5M NaOH (See Purification Buffers Section Below)
- 0.5L of 0.5M HCl (See Purification Buffers Section Below)
- 1L of 0.5M potassium phosphate buffer pH 7.0 (See Purification Buffers Section Below)
- 0.5L of TED0 (See Purification Buffers Section Below)
- 0.5L of TED1000 (See Purification Buffers Section Below)
- Akta FPLC
- GE 50ml superloop (18-1113-82)
- 12% SDS-PAGE gel (See Appendix E)
- 1x Tris-Glycine-SDS buffer (See Appendix E)
- 6x SDS loading dye (See Appendix E)
• Gel Stain (See Appendix E)

• Gel Destain (See Appendix E)

• 3 - 1L beakers pfu storage buffer (50mM Tris pH 8.2, 0.1mM EDTA, 1mM DTT, 0.1% NP40, 0.1% TWEEN 20) refrigerated

**Pre-Cycling**

The precycling method has proved to be the most efficient and failsafe of typical methods. Filtering fluid from cellulose using filter paper and a vacuum buchner funnel causes the cellulose to pack too densely running the risk of clogging the filter.

When stirring cellulose phosphate to resuspend, use a glass pasteur pipette or plastic serological pipette. Use of metal spatualaa can result in fines.

Unused, equilibrated media can be stored in 0.5M phosphate buffer, pH 7.0 at 4°C for up to one week.

1. For use in the Amersham C26/70 column, weigh out 8g of cellulose phosphate powder (sigma C3145). Note that when hydrated, cellulose phosphate occupies approximately 4ml packed resin per dry gram. Use 25-30 ml bed volume of hydrated cellulose phosphate per 1-2 liter of starting culture.

2. Put weighed cellulose phosphate in a 500ml beaker and gently stir in 200ml (20 volumes of cellulose phosphate) of 0.5M NaOH. Let stand for 5 minutes.

3. Using a pipette, suck off the supernatant as far down to the settled cellulose phosphate as possible. It helps to tip the beaker onto its bottom edge so more of the supernatant pools above the cellulose. If some of the cellulose is sucked up, it is OK. You should have 40-50ml cellulose + residual supernatant in the beaker. pH of supernatant should be ~13.5.
4. Immediately add 200ml (to bring back up to 20 volumes) of 0.5M phosphate buffer pH 7.0. Gently stir and let stand for 5 minutes.

5. Repeat pipetting procedure to remove supernatant. pH of supernatant should be ~7.5.

6. Immediately add 200ml (to bring back up to 20 volumes) of 0.5M HCl. Gently stir and let stand for five minutes.

7. Repeat pipetting procedure to remove supernatant. pH of supernatant should be ~1.

8. Immediately add 200ml (to bring back up to 20 volumes) of 0.5M phosphate buffer pH 7.0. Gently stir and let stand for five minutes.

9. Repeat pipetting procedure to remove supernatant. pH of supernatant should be ~6.5.

10. Immediately add another 200ml (to bring back up to 20 volumes) of 0.5M phosphate buffer pH 7.0. Gently stir and let stand for five minutes.

11. Repeat pipetting procedure to remove supernatant. pH of supernatant should be ~7.0.

**Column Packing**

1. Set up the GE XK26/70 column on a ring stand. Place XK26 flow adapter into bottom of GE XK26/70 column and push adapter plunger all the way down and tighten to seal. Connect the outlet of the bottom flow adapter to 1mm I.D. tubing and place tubing into a waste beaker. Keep the top flow adapter removed from the column at this point.

2. Dilute settled cellulose phosphate 3-fold with ~100ml TED200 (80% TED0 + 20% TED1000). Gently stir and let stand for 5 minutes.
3. Note the settled volume occupied by the cellulose phosphate after settling and adjust the volume of liquid above the cellulose phosphate so that it is 20% of the settled cellulose phosphate volume. This should be 15-20 ml.

4. Pour a couple of ml of TED200 into the column and allow most of it to flow out; plug the outlet.

5. Stir the cellulose phosphate and pipette into the column with the outlet open. Allow the buffer to flow from the column into waste, but don’t let the buffer level drop below the cellulose phosphate level. Continue to add slurry until all is added; plug the column outlet to stop the flow.

6. Fill column to top with TED200. Let set for 5 minutes to allow the column to settle.

7. Insert the top XK26 flow adapter at an angle to prevent air from being trapped. Lower the adapter until the support screen is about 0.5cm from the bed; tighten down the flow adapter to seal. As the plunger is pushed down, fluid will spill out from the top of the column. Keep paper towels under the column to absorb extra buffer.

**Column Equilibration**

1. Prime the P1 pump with TED200. Attach the outlet of the P1 pump to the inlet of the upper flow adapter with 1mm I.D. tubing.

2. Equilibrate column by running 3-4CV of TED200 through the column at a flow rate of 0.75 ml/min (in general at least 45 ml/hr/cm² of the internal cross-sectional area of the column) until the column bed height is constant and the pH of the eluate is stabilized. Note: This normally takes 3 column volumes; it typically works best to equilibrate overnight at
0.1-0.2 ml/min and finish in the morning.

3. When equilibrated, stop the pump, loosen the top XK26 flow adapter and gently lower flow adapter until it contacts the resin; tighten down the flow adapter to seal.

4. Connect column to Akta FPLC system (GE healthcare, use system in Jaroniec Lab after appropriate training) equilibrated with TED0 in line A1 and TED1000 in line B1. Also, fill GE superloop with TED200 (80% TED0, 20% TED200).

5. Equilibrate column on FPLC with 1.5CV of TED200 at 0.75 ml/min until conductivity and UV-Vis are stable. During this time the bed volume may decrease due back pressure from the FPLC. If this happens simply lower the flow adapter to the new bed height.

**Sample Loading and Elution**

1. Load diluted Fraction I into superloop.

2. Apply sample to column at 0.75 ml/min, collecting flowthrough in case protein does not bind to column.

3. Wash sample with 1.5CV (∼45ml) of TED200 at 0.75 ml/min to remove any unbound protein. UV-vis should return to baseline. Collect flowthrough during wash.

5. Elute protein with a linear gradient in from TED200 to TED1000 at 0.75 ml/min over 2.5 column volumes (∼75ml) followed by a 1CV (∼30ml) wash at TED1000 NaCl. Collect 1.3ml fractions during the gradient and wash. pfu elutes at about 500mM NaCl.

6. Run fraction on at 12% SDS-page gel to determine which fractions contain pfu (∼90kDA band)
7. Pool fractions containing pfu into 50ml falcon tube.

8. Dialyze against 3-1L Changes of pfu Storage buffer at 4°C for at least 8 hours per change with the last one going overnight.

**F.6.3 pfu Activity Assay and Storage**

Materials:

- pfu storage buffer
- 10x pfu reaction buffer (See Purification Buffers section below)
- 10mM each deoxynucleotide triphosphate mixture (dNTP’s, NEB N0447)
- 10 ng/µl pMP2 plasmid (See Appendix D)
- 10 µM mp2-147 forward primer (See Appendix D)
- 10 µM mp2-147 reverse primer (See Appendix D)
- Thermocycler
- 2% agarose gel with ethidium (See Appendix E)
- Amicon 50 centrifugal concentrator (Millipore)
- 100% glycerol (Sigma G5516)

1. Reclaim purified pfu from dialysis. Carefully perform a 2-fold serial dilution across at least an order of magnitude 2,4,8,16,32,64,128-fold dilutions into pfu storage buffer.

2. In 0.2ml PCR tubes, combine in a total volume of 100µl: 1x pfu reaction buffer, 0.2mM each dNTP, 1µM forward primers, 1µM reverse primer, 0.8 ng/µl plasmid. To each tube add 1µl of pfu stock and one of the dilutions. Thoroughly mix and perform PCR for 24 cycles of: 1 minute 94°C, 0.5 minutes 55°C, 0.5 minutes 72°C.
3. Run samples on a 2% agarose gel with ethidium to resolve PCR produces.

4. Determine which pfu dilution optimally amplifies the 147 base pair product by using ~75% of the primers and does not produce side products.

5. If pfu stock is not concentrated enough to optimally produce the 147 base pair product under these conditions, concentrate stock by 5-10 fold with amicon 50 and repeat activity assay. If one of the dilutions optimally produces the 147 base pair product, this dilution is the "Unit Definition" in which 1µl of pfu at this concentration in a 100µl standard reaction as performed above is enough to convert ~75% of primers into PCR product with minimal side products in 24 cycles.

6. Dilute pfu stock by 1/2 of the dilution factor determined for optimal PCR with pfu storage buffer and mix thoroughly to create a 2U/µl stock. Then add an equal volume of 100% glucero and mix thoroughly to create a 1U/µl stock.

7. Aliquot in 200µl aliquots labeled at 1 U/µl concentration. Flash freeze in liquid nitrogen and store at -80°C for up to 2 years.
F.7 Protein Purification Buffers

F.7.1 Buffer A

- 25ml 2M Tris pH 8.0 per L (50 mM Tris-HCl final, Sigma T1503)

- 1ml 0.5m EDTA pH 8.0 per L (0.5 mM Ethylenediaminetetraacetic acid final, Sigma E1644)

- 1ml 1M DTT per L (1 mM dithiothreitol final, Sigma D9779)

- 40ml 5M NaCl per L (200 mM NaCl final, Sigma S3014)

Add above constituents to beaker. Bring up to final amount with water. Stir until thoroughly mixed. Filter and degas, store at 4°C.

- To add 10% sucrose combine 100g sucrose (Sigma S0389) per L of Buffer A and mix thoroughly. Filter and degas.

- To add 1mM PMSF (polymethylsulfonyl fluoride, Sigma P7626) add 100mM PMSF in DMF (dimethylformamide, sigma 227056) stored at -20°C while rapidly mixing. Some of the PMSF may aggregate out, this is typical. When thawed, PMSF is active for up to 2 hours. Always add PMSF immediately thawed from -20°C to buffer just before use. As this is for cell suspension and lysis, buffer does not need to be refiltered.

- To add 100mM Imidazole add 6.8g of imidazole Sigma I5513 per L Buffer A and mix thoroughly. Filter and degas. Refrigerate at 4°C.

- To add 1mM Imidazole add 10ml of Buffer A + 100mM imidazole per L buffer A and mix thoroughly.
F.7.2 Buffer B

- 40ml 0.5M Potassium Phosphate buffer pH 7.0 per L (20mM final)
- 200µl 0.5M EDTA pH 8.0 per L (0.1 mM Ethylenediaminetetraacetic acid final, Sigma E1644)
- 100mL 100% glycerol per L (10% final, Sigma G5516)
- 1ml 1M DTT per L (1 mM dithiothreitol final, Sigma D9779)
- For 500mM NaCl add 100mL 5M NaCl per L
- For 200mM NaCl add 40mL 5M NaCl per L
- For 800mM NaCl add 160mL 5M NaCl per L

Fill beaker with above constituents. Bring up to final volume with water. Mix thoroughly, filter and degas, and store at 4°C.

F.7.3 Buffer C

This buffer set is designed for optimal use with hydroxyapatite chromatography. It does not include EDTA which can lead to resin degradation, and it includes 0.5mM CaCl₂ for resin stability during purification.

- 100ml 100% glycerol per L (10% final, Sigma G5516)
- 1ml 1M DTT per L (1 mM dithiothreitol final, Sigma D9779)
- 0.5M 1M CaCl₂ per L (0.5mM calcium chloride dihydrate, Sigma C3306)
- For 50mM phosphate add 100ml 0.5M potassium phosphate buffer pH 7.0 per L
- For 400mM phosphate add 800ml 0.5M potassium phosphate buffer pH 7.0 per L

Fill beaker with above constituents. Bring up to final volume with water. Mix thoroughly, filter and degas, and store at 4°C.
F.7.4 TED buffers

For TED0:

- 12.5ml 2M Tris pH 7.5 per L (25mM Final, Sigma T1503)

- 2ml 0.5m EDTA pH 8.0 per L (1 mM Ethylenediaminetetraacetic acid final, Sigma E1644)

- 1ml 1M DTT per L (1 mM dithiothreitol final, Sigma D9779)

For TED 1000 add 58.44g NaCL per L (1000mM final).

Combine designated ingredients in a beaker and bring up to final volume with water. Mix thoroughly, filter and degas, and store at 4°C.

F.7.5 Polyethyleneimine (polymin P)

For 10ml 10% ployminP add 2.12g polyethyleneimine (Sigma-Aldrich P3143) to 8ml dH2O with stirring. pH to 7.9 with concentrated HCl. Bring up to final volume with water. Store at 4°C for up to 24 hours.

F.7.6 2M Potassium Phosphate Dibasic

Add 456.46g potassium phosphate dibasic tetrahydrate (K2HPO4*3H2O, FW = 228.23, Sigma P9666) per liter of solution. Add designated amount of K2HPO4, bring up to final volume with water and mix thoroughly. Filter and degas. Store at room temperature.

F.7.7 0.5M Phosphate Buffer pH 7.0

Add 21.8g Potassium Phosphate Monobasic (KH2PO4, Sigma P9791) per L and add 77.6g Potassium Phosphate Dibasic Trihydrate (K2HPO4*3H2O, Sigma P9666) per L solution. Add designated constituents to beaker and bring up to final volume with water. Thoroughly mix, filter and degas.
F.7.8  1M Sodium Hydroxide

Add 40g NaOH (Sigma 221465, FW = 40.00) per liter buffer. Add designated amount of NaOH to beaker. Bring up to volume with water. Mix thoroughly, filter and degas.

F.7.9  0.5M Sodium Hydroxide

Add 20g NaOH (Sigma 221465, FW = 40.00) per liter buffer. Add designated amount of NaOH to beaker. Bring up to volume with water. Mix thoroughly, filter and degas.

F.7.10  0.5M Hydrochloric acid

Add 41.7ml HCL (Sigma 320331, 12M) per liter buffer. Add required amount of water first to beaker then add acid. Mix thoroughly, filter and degas.

F.7.11  Protein Storage Buffer

- 100ml 100% glycerol per L (10% glycerol, Sigma G5516)
- 10ml 1M HEPES pH 7.5 per L (10mM final, Sigma H3375)
- 2ml 0.5m EDTA pH 8.0 per L (1 mM Ethylenediaminetetraacetic acid final, Sigma E1644)
- 1ml 1M DTT per L (1 mM dithiothreitol final, Sigma D9779)
- 40ml 5M NaCl per L

Fill beaker with above constituents. Bring up to final volume with water. Mix thoroughly and store at 4°C.

F.7.12  pfu Storage Buffer

- 25ml 2M Tris pH 8.2 per L (50mM final, Sigma T1503)
- 0.2ml 0.5m EDTA pH 8.0 per L (0.1 mM Ethylenediaminetetraacetic acid final, Sigma E1644)
• 1ml 1M DTT per L (1 mM dithiothreitol final, Sigma D9779)

• 1ml 10% NP40 per L (0.1% final, Sigma NP40)

• 1ml 10% TWEEN 20 per L (0.1% final, P9416)

Fill beaker with above constituents. Bring up to final volume with water. Mix thoroughly and store at 4°C.

F.7.13 10x pfu reaction buffer

• 100ml 2M Tris pH 8.8 per L (200mM Final, Sigma T1503)

• 20ml 1M MgSO₄·7H₂O per L (20mM final magnesium sulfate heptahydrate, Sigma M1880)

• 100ml 1M KCl per L (100mM Final, Sigma P9541)

• 100ml 1M (NH₄)₂SO₄ per L (100mm ammonium sulfate final, Sigma A4418)

• 10ml 100% Triton X-100 (1% final, Fisher BP-151)

• 1000mg BSA per L (1mg/ml final, Sigma A7638)

Combine all ingredients and bring up to final volume with water. Filter through 0.22μm syringe filter.
Appendix G

Nucleosome Mapping and Footprinting Techniques

G.1 Introduction

This section details all nucleosome position mapping and nucleosomal DNA footprint assays employed in this work. Depending on the DNA length and nucleosome positioning sequence properties, each method has its benefits and drawbacks as described below.

Mapping of nucleosome position is performed using Exonuclease III (ExoIII) to determine digestion stall sites relative to the DNA 3’-end caused by DNA wrapped into the nucleosome at each entry/exit region. Based on the location of stall sites at each entry/exit region, both the nucleosome footprint and position of the nucleosome can be inferred. However, due to transient unwrapping of the nucleosome that allows progression of ExoIII further into the nucleosome, typically multiple stall sites at 10 base pair intervals are observed at each entry/exit region. Therefore, only nucleosome positioning sequences that wrap well at the entry/exit and only have a few positions are amenable to ExoIII mapping. Additionally, in order to observe stall sites, DNAs with at least 20 base pair extra linker DNA on each side of the positioning sequence is required, excluding the use of this method for 147 base pair DNAs.

Alternatively, precise positioning of the nucleosome dyad to within near base pair resolution regardless of DNA length can be achieved using site-specific hydroxyl radical cleavage based on the Fenton reaction (also known as Haber-Weiss cycle). In this cycle,
ascorbic acid is used to oxidize iron from Fe$^{+2}$ to Fe$^{+3}$; Fe$^{+3}$ then reduces hydrogen peroxide to form a hydroxyl anion (HO$^-$) and a hydroxyl radical (HO*) which can cleave amide and phosphodiester bonds of protein and DNA backbones [95]. Localization of the iron species for site specific cleavage is done using an EDTA-based chelating group functionalized with a thiol-specific labeling group (Fe(III) (s)-1-(p-bromoacetamidobenzyl) ethylenediamine-N,N,N',N'-tetraacetic acid [FeBABE]; Thermo 20332, or N-[S-(2-Pyridylthio)cystaminyl]ethylenediamine-N,N,N',N'-tetraacetic Acid [Cyst(EDTA)NPS]; Totonto Research Chemical P996250). For DNA cleavage near the nucleosome dyad, H4(S47C), located at the DNA-histone interface 3 bp from the dyad, is typically used [88, 95, 96, 99]. Production of hydroxyl radicals from the label via the Fenton reaction cleaves the DNA phosphate backbone only in the immediate vicinity of the label. Cleavage products can then be resolved to determine the location of the nucleosome dyad.

The FeBABE bromoacetamide attachment chemistry provides the advantage of irreversible and high labeling efficiency. Additionally, the affinity of iron to the FeBABE EDTA group is higher than to free EDTA in solution. This allows for inclusion of EDTA in mapping buffers to chelate up trace metal ions that may interact with the DNA-phosphate backbone, which essentially eliminates background cleavage. However, the larger size of the FeBABE attachment group provides a larger cleavage radius that cleaves the DNA phosphate backbone near the nucleosome dyad at 3 locations: 77th base (+3 from dyad), 70th base (-4 from dyad), and 62nd base (-12 from dyad). The additional -12 cleavage site makes it difficult to resolve nucleosome positions less than 20 base pairs apart from each other. Therefore, this method is amenable only to well-positioned nucleosomes with multiple positions greater than 20 base pairs apart.

For nucleosomes with multiple positions less 20 base pairs apart, then the Cyst(EDTA)NPS label must be used. This smaller label only cleaves at the 72nd base (-2 from dyad) and 79th/80th bases (+5/+6 from dyad) [95]. However, the disulfide linkage connecting the label to H4(S47C) is prone to reduction and label loss due to trace reducing reagents. Additionally, the affinity of iron to the Cyst(NPS)EDTA label is significantly lower than free EDTA in solution. Therefore, all trace metals must be eliminated

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from all buffers by passing them over EDTA chelating resin (Chelex, BioRad), and free EDTA cannot be used to reduce interaction of iron with the DNA-phosphate backbone. Therefore, this protocol is significantly more difficult and optimum concentrations of iron added to the reaction must be determined empirically.

Nucleosome footprinting to determine the amount of DNA protected by the histone octamer is performed by micrococcal nuclease (MNase), deoxyribonuclease I (DNaseI), or free hydroxyl radical cleavage (not described here). Micrococcal nuclease, which is around 17kDa, is a relatively non-specific double- or single-stranded endo/exo nuclease with only a slight preference for AT/AU rich regions. Due to its larger size and low sequence specificity for digestion, this enzyme is ideal for isolating and quantifying the DNA footprint protected by a nucleosome from digestion. This has the added benefit of being able to resolve digested fragments using native PAGE electrophoresis and imaging using simple ethidium of Cybr (Invitrogen) stain.

Conversely, DNase I, which is larger at around 30kDa, has significant DNA sequence bias in which purine-pyrimidine (A-C, A-T, G-C, G-T) dinucleotides are preferentially cleaved compared to all other dinucleotides [233]. Additionally, increased helical twist in the DNA backbone enhances DNAse I cleavage [234]. Therefore in the nucleosome structure, when the DNA minor groove is facing away from histone surface, this region exhibits enhanced or "hypersensitive" DNase I cleavage. While this does not make DNase I as amenable to nucleosome footprinting for isolation of 146-147 base pair fragments protected by the nucleosome, comparison of nucleosome hypersensitive sites to naked DNA provides detailed information about the helical phasing and relative protection of DNA throughout the entire nucleosome.

G.2 DNA Sequencing Ladders

Materials:

- Sequitherm Excel II DNA Sequencing Kit (Epicentre Biotechnologies, SEM79100) replaced by Sequenase Version 2.0 DNA Sequencing Kit (USB 70770), untested but
according to USB is the identical Kit

- Nucleosomal DNA template (not plasmid but PRC product used for nucleosome reconstitution)

- Nucleosomal DNA template forward primer 5′-cy3-labeled (labeled forward primer used for PCR of Nucleosomeal DNA template above)

- Nucleosomal DNA template reverse primer 5′-cy5-labeled (labeled forward primer used for PCR of Nucleosomeal DNA template above)

- thermocycler

- 3x stop buffer (10mM EDTA in Formamide)

- 50% formamide + 1x ficol with orage G (See Appendix E)

Typically for these kits, A and T ladders produced using ddATP or ddTTP in the deoxynucleotide PCR mix is efficient at making large amounts of quality ladder for a standard reaction. However G and C ladders tend to have very low yields and require an altered recipe. Therefore depending on the ladder type use one of the two following recipes. Also note that the forward and reverse sequencing ladder reaction cannot be done in the same tube. Rather, individual reactions containing the template plus forward primer and template plus reverse primer must be done as separate sequencing reactions.

For A or T sequencing ladders combine the following in a 0.2ml PCR tube

- 7.5 pmol cy3-labeled-forward or cy5-labled-reverse primer

- 100 fmoles template

- 4.6µl 3.5x Sequitherm Buffer

- 1.5µl 5U /µl Sequitherm polymerase

- water to bring volume up to 16µl

- 8µl 3x Sequitherm A-termination or T-termination mix
Cycle on thermocycler at: 95°C for 30 seconds, 50°C for 60 seconds, 70°C for 45 seconds for 30 cycles. Quench reaction when done with 12µl 3x stop buffer. Dilute sequencing ladder 4-fold with 108µl of 50% formamide + 1x ficol with Orange G. At this point, if the forward and reverse sequencing ladders for a given nucleotide (A or T) are labeled with different fluorescent dyes (e.g. cy3 and cy5) then the forward and reverse sequencing ladders can be combined in order to run in the same lane on a Urea Denaturing gel for sequencing, thus increasing resolution. Store sequencing ladders at -20°C.

For applying ladders to a sequencing gel, remove sample from -20°C and heat at 95°C on heat block for 20 minutes. Remove sample from heat block immediately before loading and load 20µl of forward/reverse combined ladders or 10µl of individual ladder per lane on Urea Denaturing gel (See Appendix E). // For G or C sequencing ladders combine the following in a 0.2ml PCR tube

- 45 pmol cy3-labeled-forward or cy5-labeled-reverse primer
- 100 fmols template
- 4.6µl 3.5x Sequitherm Buffer
- 1.5µl 5 U/µl Sequitherm polymerase
- water to bring volume up to 16µl
- 8µl 3x Sequitherm A-termination or T-termination mix

Cycle on thermocycler at: 95°C for 30 seconds, 50°C for 60 seconds, 70°C for 45 seconds for 30 cycles. Quench reaction when done with 12µl 3x stop buffer. Dilute sequencing ladder 3-fold with 72µl of 50% formamide + 1x ficol with Orange G. At this point, if the forward and reverse sequencing ladders for a given nucleotide (A or T) are labeled with different fluorescent dyes (e.g. cy3 and cy5) then the forward and reverse sequencing ladders can be combined in order to run in the same lane on a Urea Denaturing gel for sequencing, thus increasing resolution. Store sequencing ladders at -20°C.

For applying ladders to a sequencing gel, remove sample from -20°C and heat at 95°C on heat block for 20 minutes. Remove sample from heat block immediately before loading
and load 20\mu l of forward/reverse combined ladders or 10\mu l of individual ladder per lane on Urea Denaturing gel (See Appendix E).
G.3 Exonuclease III Mapping

Mapping reactions should be done with a final Exonuclease III concentration of 30 U/ml, 50 U/ml, or 100 U/ml. The concentration required for optimum digestion depends upon the activity of the ExoIII stock, the length of the DNA being mapped, and how tightly the DNA is wrapped around the nucleosome at the entry/exit. This concentration must be found empirically for each nucleosome construct and ExoIII stock. Therefore, for initial mapping reactions, it is recommended to perform test reactions at each concentration above for 0, 5, 15, 30 minutes before performing the full timecourse. The optimal concentration is the one in which complete digestion of Naked DNA occurs in 5-10 minutes while digestion of nucleosomal DNA stalls at the entry/exit region up through 30 minutes with minimal digestion further into the nucleosome.

Materials:

- Histone octamer
- Nucleosomal DNA template labeled on one 5'-end with cy3 or cy5 for mapping in one direction using the forward or reverse strand, or DNA labeled with cy3 on one 5'-end and cy5 on the other for simultaneous mapping using the forward and reverse strand.
- Exonuclease III (NEB M0206)
- Supplied 10x NEB buffer 1 or 2x TM buffer (40mM Tris pH 8.0, 1mM MgCl$_2$).
- Heat block
- 160mM EDTA pH 8.0
- 10 mg/ml proteinase K (NEB P8102)
- 0.2% SDS (Sodium Dodecyl Sulfate, Sigma L4390)
- 8-12% acrylamide-Urea denaturing gel
- Sequencing Ladder with ficol w/ ornge G loading dye (See Section: DNA Sequencing Ladder above)
Formamide (Fisher BP228-100)

1. Reconstitute nucleosomes with desired DNA substrate as detailed in Appendix C. Purify nucleosomes by sucrose gradient and concentrate into 0.5x TE to concentrations > 100nM.

2. On ice, combine nucleosomes or naked DNA template to 20mM final concentration and 10x NEB buffer 1 to 1x final concentration (or 2x TM buffer to 1x final) in 35µl total volume.

3. In a separate tube on ice combine ExoIII to 60 U/ml, 100 U/ml, or 200 U/ml final concentration and 10x NEB buffer 1 to 1x final concentration (or 2x TM buffer to 1x final) in 35µl total volume.

4. Prepare Quench tubes by placing 1µl of 160mM EDTA in each tube.

5. Prepare 0 minute timepoint by adding 3.5µl nucleosome mixture to quench tube followed by adding 3.5µl of ExoIII mixture to same quench tube.

6. Incubate nucleosome and ExoIII mixtures at 37°C or 16°C for 2 minutes to equilibrate temperature.

7. Initialize reactions by combining together 30µl of nucleosome mixture and 30µl of ExoIII mixture. Note that mapping reactions of multiple samples can be performed in parallel by staggering the start of each sample every 6 minutes.

8. At 1, 2, 5, 10, 15, 20, 30 minutes, transfer 7µl of reaction mixture to a quench tube.

9. When all timepoints are collected, remove protein from samples by adding 1µl of 10mg/ml
proteinase K and 1 µl 0.2% SDS to each sample and incubating at 37°C for 15 minutes.

10. Add 10µl formamide to 50% final concentration.

11. Resolve DNA by 7M Urea, 8-12% acrylamide denaturing gel in 1x TBE as described in Appendix E. Run sequencing ladders alongside mapping reaction samples to determine length of digestion fragments. For 187-247 base pair DNAs, run 12% gel until orange G dye runs out of bottom of gel plus 4-8 minutes more, respectively. For longer DNA’s use an 8% gel.
G.4 FeBABE Hydroxyl Radical Mapping

Materials:

- H4(S47C)H3(C110A) histone octamer labeled with FeBABE label (See Appendix B.3: Histone Labeling)
- Nucleosome DNA labeled on one 5′-end with cy3 or cy5 for mapping in one direction using the forward or reverse strand, or DNA labeled with cy3 on one 5′-end and cy5 on the other for simultaneous mapping using the forward and reverse strand.
- 2x Cutting Buffer (40mM Tris pH 7.5, 0.2mM EDTA, 20% glycerol), degassed
- 1x EDTA Buffer (20mM Tris pH 7.5, 10mM EDTA), degassed
- Quench Buffer (1.3M Tris pH 7.5)
- L-ascorbic acid (Sigma A92902)
- 50% Hydrogen Peroxide (Sigma 516813)
- Quench Buffer (1.3M Tris pH 7.5)
- 8-12% acrylamide-Urea denaturing gel
- Sequencing Ladder with ficol w/ orange G loading dye (See Section: DNA Sequencing Ladder above)
- Formamide (Fisher BP228-100)

1. Label refolded histone octamer with FeBABE reagent as detailed in Appendix B.3: Labeling Histone Octamer after refolding with acyl-halide and akyl-halide functional groups. Purify histone octamer after labeling.

2. Reconstitute nucleosomes with desired DNA substrate as detailed in Appendix C. Purify nucleosomes by sucrose gradient and concentrate into 0.5x TE to concentrations > 100nM.
3. Degas Cutting and EDTA buffers by placing 1ml aliquots in the bottom of a millipore steri-cup chamber in a foam tube holder. Attach steri-top vacuum manifold to chamber and vacuum for 10 minutes to degas.

4. On ice, combine nucleosomes to 25nM final concentration and 2x Cutting Buffer to 1x final concentration in 20µl total volume (e.g. 5µl 100nM Nucleosomes, 5µl dH20, 10µl 2x Cutting Buffer).

5. In separate tubes on ice, make a 40mM stock of ascorbic acid in 1x EDTA buffer (7mg L-ascorbic acid, 1mL 1x EDTA buffer) and an 80mM Hydrogen peroxide stock (5µl 50% H2O2, 1mL 1x EDTA buffer)

6. Prepare timepoint quench tubes by adding 3µl of Quench Buffer to each tube on ice. Transfer 5µl of nucleosome sample to the first quench tube before mapping for a 0 minute timepoint to validate quenching.

7. To remaining nucleosomes add in quick succession ascorbate stock to 4mM final concentration (2.5µl to 15µl of nucleosomes) then hydrogen peroxide stock to 8mM final concentration (2.5µl to 15µl nucleosomes), with thorough mixing between each addition.

8. At each time point transfer 7µl of reaction mixture to a quench tube.

9. Prepare samples for electrophoresis by adding an equal volume (10µl) of formamide to 50% final concentration.

10. Resolve DNA by 7M Urea, 8-12% acrylamide denaturing gel in 1x TBE as described in Appendix E. Run sequencing ladders alongside mapping reaction samples to determine length of digestion fragments. For 147, 187, 247 base pair DNAs run 12% gel until orange G dye runs out of bottom of gel plus 0, 4, 8 minutes more, respectively. For longer DNA’s
use an 8% gel.
G.5 Cyst(EDTA)NPS Hydroxyl Radical Mapping

Materials:

- H4(S47C)H3(C110A) histone octamer labeled with N-[S-(2-Pyridylthio)cystaminyl] ethylenediamine-N,N,N',N'-tetraacetic Acid (Cyst(EDTA)NPS FW = 460, Totonto Research Chemical P996250) (See Appendix B: Histone Labeling)

- Nucleosome DNA labeled on one 5′-end with cy3 or cy5 for mapping in one direction using the forward or reverse strand, or DNA labeled with cy3 on one 5′-end and cy5 on the other for simultaneous mapping using the forward and reverse strand.

- Amicon 30 (millipore)

- Chelex Resin (BioRad 142-1253)

- 5M NaCl, ultrapure (Sigma 71376)

- 2M Tris pH 7.5

- 0.5M EDTA pH 8.0

- 600ng/µl salmon sperm DNA (Sigma D1626) sheared to 5000bp

- 23mm x 10cm 6000-8000 MWCO dialysis tubing, boiled as detailed in Appendix A.8.8. Soak membranes in water overnight at 4C prior to using in the protocol.

- 50mm x20cm 6000-8000 MWCO dialysis tubing

- 0.1N Nitric acid (Sigma 258121)

- Steri-Cup (millipore)

- Ammonium Iron(II) sulfate hexahydrate (Sigma 203505)

- L-ascorbic acid (Sigma A92902)

- 50% Hydrogen Peroxide (Sigma 516813)
• Quench Buffer (30mM EDTA, 30mM DTT, 15% glycerol, 600mM Tris)

• 8-12% acrylamide-Urea denaturing gel

• Sequencing Ladder with ficol w/ ornge G loading dye (See Section: DNA Sequencing Ladder above)

• Formamide (Fisher BP228-100)

**G.5.1 Nucleosome Reconstitution**

1. Label refolded histone with FeBABE reagent as detailed in Appendix B.3: Labeling Histone Octamer after refolding with acyl-halide and akyl-halide functional groups. Purify histone octamer after labeling. After labeling, be sure to omit reducing agents from all buffers to prevent loss of label.

2. Prepare Stock Buffers and Glassware for reconstitution:

   • Acid wash 2 - 2L glass beakers and 2 - 250ml glass beaker with 0.1M Nitric acid. Fill beakers completely with 0.1M acid and let stand covered overnite. Thoroughly wash glassware the next morning with water.

   • Additionally, remove required amount of boiled 23mm and 50mm dialysis tubing for reconstitution from storage container and soak overnight in 1L water at 4°C to remove as many remaining trace contaminants as possible. Rinse thoroughly the next morning with water.

   • Place 1g Chelex resin in the bottom of 3 separate 50ml falcon tubes and 0.5g Chelex resin in the bottom of a 15ml falcon tube. Add 25ml 5M NaCl to one 50ml tube and 25ml of 2M Tris pH 7.5 to each of the other two tubes. Add 10ml of 0.5M EDTA to the 15ml falcon tube. Place tubes on rotisserie for 1 hour to thoroughly mix and bind trace multivalent metals.
- Centrifuge tubes at 5000g for 10 minutes on Beckman Avanti centrifuge in JA17 rotor. Rotate tubes by 180 degrees and centrifuge again at 5000g for 10 minutes to completely precipitate resin. Carefully transfer buffers to new 50ml falcon tubes.

3. Prepare Reconstitution Buffers:

- **Low Salt Buffer**: Fill 2 acid washed 2L beakers with 1.7L water and add Chelex equilibrated 2M Tris pH 7.5 to 20mM final concentration and Chelex equilibrated 0.5M EDTA to 1mM EDTA final. Refrigerate in 4°C deli refrigerator.

- **High Salt Buffer**: In a 50mL Falcon tube (do Not Use Glass) combine 20mL Chelex equilibrated 5M NaCl (2M final), 0.5ml Chelex equilibrated 2M Tris pH 7.5 (20mM final), 100µl Chelex equilibrated 0.5M EDTA (1mM final) and 833µl salmon sperm DNA (10 ng/µl final). Chill on ice.

- **Tris Buffer**: Fill 1 acid washed 250mL beaker with 145mL water and add Chelex equilibrated 2M Tris pH 7.5 to 20mM Tris pH 7.5 + 5g chelex resin and chill to 4°C.

4. In a 1.5ml tube on ice prepare sample by combining:

- 8µg Cyst(EDTA)-labeled octamer

- 7µg unlabeled nucleosome DNA template and 2µg labeled nucleosome DNA template or 12µg salmon sperm DNA and 2µg labeled nucleosome DNA template.

- Chelex equilibrated 5M NaCl to 2M final concentration

- Chelex equilibrated 500mM Tris pH 7.5 to 20mM final concentration

- Chelex equilibrated 50mM EDTA to 1mM final concentration

- water to 50µl final volume

5. Place sample in cap of 0.8ml centrifuge tube (DNase, RNAse, pyrogen free) and seal with 6000-8000 MWCO dialysis tubing as detailed in Appendix C to create the sample chamber.
6. Place 50mL of High Salt Buffer in 50mm 6000-8000 MWCO dialysis tubing and place sample chamber in High Salt Buffer. Seal 50mm dialysis tubing with clips as detailed in Appendix C and place in 2L beaker of Low Salt Buffer at 4°C. Dialyze 8hrs against 1st volume of Low Salt Buffer and then overnite against the second.

7. Remove sample chamber from 50mm dialysis tubing and place in 250mL beaker of Tris Buffer with chelex resin for 6 hours at 4°C.

8. Reclaim samples from sample chamber.

G.5.2 Mapping

1. In the remaining acid-washed 250ml beaker add 198 ml water and Chelex equilibrated 2M Tris pH 7.5 to 20mM final concentration.

2. Equilibrate 2 Steri-Cups by running 200ml water through each filter and discarding.

3. In one Steri-cup filter 200ml water through filter and degas for 10 minutes. In the other Steri-cup filter 200ml of the 20mM Tris pH 7.5 buffer from Step 1 and degas for 10 minutes. Store at 4°C for up to 1 week. Just prior to use, degas for 5 minutes by attaching Steri-cup filter top to Steri-cup and placing under vacuum.

4. In a 1.5ml tube on ice dissolve 3.4mg Ammonium Fe(II) sulfate in 1ml degassed water from Step 3 to create a 10mM Stock. Dilute 100µl of 10mM stock into 900µl of deagssed water to create a 1mM stock. Dilute 20µl of 1mM stock into 980µl of degassed 20mM Tris pH 7.5 to create a 20µM working stock.

5. Dilute nucleosome stock for mapping by 2.5-fold in degassed 20mM Tris pH7.5 from Step 3 to ~400nM final nucleosome concentration in 20µl total volume (e.g. 8µl nucleosomes, 12µl 20mM Tris pH 7.5)
6. Add 2µl of 20µM Fe(II) solution to nucleosomes and incubate on ice 10 minutes.

7. Meanwhile in separate 1.5ml tubes on ice make a 48mM ascorbate solution in 20mM Tris pH 7.5 by combining 8.4mg L ascorbic acid and 1mL degassed 20mM Tris pH 7.5 from Step 3; and make a 0.5% hydrogen peroxide solution in 20mM Tris pH 7.5 by combining 10µl 50% hydrogen peroxide and degassed 1mL 20mM Tris pH 7.5 from Step 3.

8. Additionally to each quench tube add 3µl Quench Buffer.

9. After 10 minute Fe(II) incubation, transfer 6µl of nucleosome sample to the first quench tube before initializing reaction.

10. To the remaining nucleosome sample add in quick succession 2µl 48mM ascorbate solution to 4.8mM final concentration and 2µl 0.5% peroxide solution to 0.05% final concentration with thorough mixing by flicking tube after each addition.

11. At 30 and 60 minutes transfer 7µl of reaction mixture to a quench tube.

12. Prepare samples for electrophoresis by adding an equal volume (10µl) of formamide to 50% final concentration.

13. Resolve DNA by 7M Urea, 8-12% acrylamide denaturing gel in 1x TBE as described in Appendix E. Run sequencing ladders alongside mapping reaction samples to determine length of digestion fragments. For 147, 187, 247 base pair DNAs run 12% gel until orange G dye runs out of bottom of gel plus 0, 4, 8 minutes more, respectively. For longer DNA’s use an 8% gel.
G.6 DNaseI footprinting

Mapping reactions should be done with a final DNase I concentration between 10 U/ml and 50 U/ml. The concentration required for optimum digestion depends upon the activity of the DNase I stock, the length of the DNA being mapped, and buffer conditions. This concentration must be found empirically for each nucleosome construct and DNase I stock. Therefore, for initial mapping reactions, it is recommended to preform test reactions for several concentrations in the above range for 0, 5, 15, 30 minutes before performing the full timecourse. The optimal concentration is the one in which after 10 minutes of digestion, Naked DNA accumulates ~1 cut per molecule. Under these conditions exposed nucleosomal DNA will be cut approximately one time per molecule while protected DNA will have less cuts.

Materials:

- Purified Histone octamer
- Nucleosome DNA labeled on one 5'-end with cy3 or cy5 for mapping in one direction using the forward or reverse strand, or DNA labeled with cy3 on one 5'-end and cy5 on the other for simultaneous mapping using the forward and reverse strand.
- DNaseI (Invitrogen, Life Technologies 18068-015)
- Supplied 10x DNase I reaction buffer (200mM Tris pH 8.4, 20mM MgCl2, 500mM KCl) or 2x TM buffer (40mM Tris pH 8.0, 1mM MgCl2) for low stability nucleosomes.
- Heat block
- 160mM EDTA pH 8.0
- 10 mg/ml proteinase K (NEB P8102)
- 0.2% SDS (Sodium Dodecyl Sulfate, Sigma L4390)
- 8-12% acrylamide-Urea denaturing gel
• Sequencing Ladder with ficol w/orange G loading dye (See Section: DNA Sequencing Ladder above)

• Formamide (Fisher BP228-100)

1. Reconstitute nucleosomes with desired DNA substrate as detailed in Appendix C. Purify nucleosomes by sucrose gradient and concentrate into 0.5x TE to concentrations > 100nM.

2. On ice, combine nucleosomes or naked DNA template to 20mM final concentration and 10x DNase I buffer to 1x final concentration (or 2x TM buffer to 1x final) in 30µl total volume.

3. In a separate tube on ice combine DNase I to 22 U/ml final concentration (or optimized concentration from test digestions) and 10x DNase I buffer to 1x final concentration (or 2x TM buffer to 1x final) in 30µl total volume.

4. Prepare Quench tubes by placing 1µl of 160mM EDTA in each tube.

5. Prepare 0 minute timepoint by adding 3.5µl nucleosome mixture to quench tube followed by adding 3.5µl of DNase I mixture to same quench tube.

6. Incubate nucleosome and DNase I mixtures at 37°C or 16°C for 2 minutes to equilibrate temperature.

7. Initialize reactions by combining together 30µl of nucleosome mixture and 30µl of DNase I mixture. Note that mapping reactions of multiple samples can be performed in parallel by staggering the start of each sample every 6 minutes.

8. At 1,2,5,10,15,20,30 minutes, transfer 7µl of reaction mixture to a quench tube.
9. When all timepoints are collected, remove protein from samples by adding 1µl of 10mg/ml proteinase K and 1 µl 0.2% SDS to each sample and incubating at 37°C for 15 minutes.

10. Add 10µl formamide to 50% final concentration.

11. Resolve DNA by 7M Urea, 8-12% acrylamide denaturing gel in 1x TBE as described in Appendix E. Run sequencing ladders alongside mapping reaction samples to determine length of digestion fragments. For 187, 247 base pair DNAs run 12% gel until orange G dye runs out of bottom of gel plus 4, 8 minutes more, respectively. For longer DNA’s use an 8% gel.
G.7 MNase footprinting

Micrococcal Nuclease footprinting is different in practice from DNAse I as we are only concerned with the DNA footprint protected by the nucleosome instead of hypersensitive site protection throughout the nucleosome. Therefore, nucleosomes are titrated with increasing amounts of MNase to reveal the protected DNA, and hence, empirical determination of optimal MNase concentrations are not required like for DNAse I and ExoIII.

Materials:

- Purified Histone octamer
- Unlabeled nucleosome DNA template
- labeled on one 5′-end with cy3 or cy5 or DNA labeled with cy3 on one 5′-end and cy5 on the other for simultaneous mapping using the forward and reverse strand.
- MNase (NEB M0247)
- Supplied 10x MNase reaction buffer (500mM Tris pH 7.9, 50mM CaCl2) or 2x TC buffer (40mM Tris pH 8.0, 1mM CaCl2) for low stability nucleosomes.
- Heat block
- 160mM EDTA pH 8.0
- 10 mg/ml proteinase K (NEB P8102)
- 0.2% SDS (Sodium Dodecyl Sulfate, Sigma L4390)
- 5% native acrylamide
- 100 base pair ladder (NEB N2321) at 20 ng/µl, 50 base pair ladder (NEB N3236) at 20 ng/µl or custom made ladder using 147, 187, 247 base pair PCR products at 4ng/µl in 1x ficol with orange G
- 6x ficol with orange G (See Appendix E)
• 2x Cybr Gold stain in 0.3x TBE (Invitrogen, Life Technologies S-11494)

1. Reconstitute nucleosomes with a 9:1 ratio of unlabeled and labeled DNA substrate as detailed in Appendix C so that there is enough labeled DNA to follow nucleosome reconstitution and purification by the fluorescent label. Purify nucleosomes by sucrose gradient and concentrate into 0.5x TE to concentrations > 100nM.

2. On ice, combine nucleosomes or naked DNA template to 20mM final concentration and 10x MNase I buffer to 1x final concentration (or 2x TC buffer to 1x final) in 50µl total volume.

3. In separate tubes on ice perform a serial dilution of 2x MNase stocks to 80, 40, 10, 4, 2, 1, 0.4, 0.2, 0 U/ml final concentration and 10x DNAse I buffer to 1x final concentration (or 2x TM buffer to 1x final) in 20-30µl total volume.

4. Additionally label sample tubes for 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 40 U/ml reactions and place on 37°C or 16°C heatblock.

5. Incubate nucleosomes and 2x MNase stocks at 37°C or 16°C for 2 minutes to equilibrate temperature.

6. Initialize reactions by combining together 5µl of nucleosome mixture (∼10ng DNA) and 5µl of 2x MNase mixture in the appropriate reaction tube. Note that multiple reactions can be performed in parallel by staggering the start of each reaction every 30 seconds.

7. Incubate reactions for 20 minutes at 37°C or 16°C and then quench with 1µl of 160mM EDTA to 15mM final concentration.

8. When all timepoints are collected, add 2µl of 6x ficol with orange G to 1x final concen-
9. Resolve DNA by 5% native acrylamide gel in 0.3x TBE as described in Appendix E. Run ladders alongside mapping reaction samples to determine length of digestion fragments.

10. Carefully remove small glass plate from acrylamide gel to expose gel surface. Place ~250ml of freshly made 2x cyber gold stain in 0.3x TBE in a clean, shallow plastic container and gently place bottom glass plate with gel into the stain bath to completely immerse. Stain for 30 minutes.

11. Destain gel for 15 minutes in a clean, shallow plastic container with ~250ml of freshly made 0.3x TBE.

12. Image stained gel using Typhoon variable mode imager using 488nm excitation laser and 570 ± 20 nm emission filter.
Appendix H
BUFFERS AND MATERIALS

H.1 Growth Media and Culture Plates

H.1.1 Antibiotics

50 mg/ml Ampicillin sodium salt (Sigma A0166) dissolved in water and filter sterilized through 0.22µm syring filter. Store at -20°C.

50 mg/ml Kanamycin sulfate (Gibco 11815-024) dissolved in water and filter sterilized through 0.22µm syring filter. Store at -20°C.

34 mg/ml Chloramphenicol (Sigma C0378) dissolved in 95% molecular biology grade ethanol. Store at -20°C.

H.1.2 LB Media

25 g/L 1Mohm water of powdered LB broth (Sigma L3152)

Mix on stir plate until dissolved. Pour media at a rate of 5ml per 30ml culture tube, 60ml per 250ml Erlenmeyer flask, 500ml per 2L flask, and 1L per 4L Erlenmeyer flask. Loosely cap or cover opening with aluminum foil. Autocalve on 20 minute wet cycle. Store culture tubes in refrigerator, store flasks at room temperature.
H.1.3 LB Agarose Plates

25g/L 1Mohm water of powdered LB broth (Sigma L3152)

15g/L 1Mohm water of Agar (Fisher BP1423)

50 mg/ml ampicillin

34 mg/ml chloramphenicol

1. Heat large waterbath to 80°C.

2. Meanwhile, mix LB on a stir plate until dissolved. Place agarose in the flask that is to be autoclaved. Pour media into the flask and swirl to suspend agarose. Loosely cover with aluminum foil. Autoclave on 20 minute wet cycle.

3. After autoclaving, allow flask to cool slightly then place in 80°C heat bath until flask reaches the temperature of the bath.

4. Add antibiotics to 50 µg/ml ampicillin or 50 µg/ml Kanamycin and/or 34 µg/ml chloramphenicol and swirl flask to mix.

5. Pour media into sterile culture plates under open flame or in a sterile room until plates are half full (~20ml). Remove bubbles by sucking up bubble with sterile serological pipette.

6. Cock culture plate lid over media until solidifies. Cover plates with lids and store in refrigerator.
H.1.4  2xYT Media

16 g/L 1Mohm water of Tryptone (Sigma T9410)

10 g/L 1Mohm water of Yeast Extract (Sigma Y1625)

5 g/L 1Mohm water of NaCl (Sigma S3014)

Mix on stir plate until dissolved. Pour media at a rate of 5ml per 30ml culture tube, 60ml per 250ml Erlenmeyer flask, 500ml per 2L Erlenmeyer flask, 1L per 4L Erlenmeyer flask. Loosely cap or cover opening with aluminum foil. Autoclave on 20 minute wet cycle. Store culture tubes in refrigerator, store flasks at room temperature.

H.2  Histone and Nucleosome Purification Buffers

H.2.1  Wash Buffer, 500mL

12.5ml 2M Tris-HCl pH 7.5, filtered and degassed (Sigma T1503, 50mM final)

10ml 5M NaCl, filtered and degassed (Sigma S3014, 100mM final)

1ml 0.5M EDTA pH 8.0, filtered and degassed (Ethylenediaminetetraacetic acid disodium salt, Sigma E1644, 1mM final)

1ml 0.5M BZA, filtered and degassed (Benzamidine, Sigma B6506, 1mM final)

water to 500mL total volume

Mix on stir plate at room temperature. Refrigerate overnight at 4°C.
**H.2.2 TW Buffer, 500ml**

12.5ml 2M Tris-HCl pH 7.5, filtered and degassed (Sigma T1503, 50mM final)

10ml 5M NaCl, filtered and degassed (Sigma S3014, 100mM final)

1ml 0.5M EDTA pH 8.0, filtered and degassed (Ethylendiaminetetraacetic acid disodium salt, Sigma E1644, 1mM final)

1ml 0.5M BZA, filtered and degassed (Benzamidine, Sigma B6506, 1mM final)

5ml Triton X-100 (Fisher BP-151, 1% v/v final)

water to 500mL total volume

Mix on stir plate at room temperature. Refrigerate overnight at 4°C.

**H.2.3 Unfolding Buffer, 100mL**

66.85g Guanidine HCL (Sigma G3272 or MP Biomedical Ultrapure 820539, 7M final)

1ml 2M Tris-HCl pH 7.5, filtered and degassed (Sigma T1503, 10mM final)

1ml 1M DTT (Sigma 646563, 10mM final)

water to 100ml total volume

Mix on stir plate at room temperature. Filter and degas. Refrigerate overnight at 4°C.
**H.2.4 Refolding Buffer, 1.8L for dialysis**

210g NaCl (Sigma S3014, 2M final)

9ml 2M Tris-HCl pH 7.5, filtered and degassed (Sigma T1503, 10mM final)

3.6ml 0.5M EDTA pH 8.0, filtered and degassed (Ethylenediaminetetraacetic acid disodium salt, Sigma E1644, 1mM final)

643µl 14.3M BME (2-mercaptoethanol, Sigma M3148, 2mM final)

water to 1.8L total volume in 2L Beaker

Mix on stir plate at room temperature then refrigerate overnight at 4°C.

**H.2.5 Refolding Buffer w/o BME, 1L for gel filtration**

117g NaCl (Sigma S3014, 2M final)

5ml 2M Tris-HCl pH 7.5, filtered and degassed (Sigma T1503, 10mM final)

2ml 0.5M EDTA pH 8.0, filtered and degassed (Ethylenediaminetetraacetic acid disodium salt, Sigma E1644, 1mM final)

water to 1L total volume

Mix on stir plate at room temperature then filter and degas. Refrigerate overnight at 4°C.

**H.2.6 SAU0, 1L**

420.4g Urea (Sigma U1250, 7M final)
6.67ml 3M Sodium Acetate pH 5.2, filtered and degassed (Sigma 236500, 20mM Final)

2ml 0.5M EDTA pH 8.0, filtered and degassed (Ethylenediaminetetraacetic acid disodium salt, Sigma E1644, 1mM final)

water to 1L

Small scupula of mixed bed resin (Sigma M8032)

357µL 14.3M BME (2-mercaptoethanol, Sigma M3148, 5mM final)

Combine all ingredients except BME. Allow to dissolve ~1 hr with stirring at 100°C. Monitor the color of the mixed bed resin. If all beads turn yellow, then add more. When done mixing, add BME. Filter and degas. The mixed bed resin deionizes the urea; SAU0 lasts for about 24 hrs until the urea reionizes.

H.2.7 SAU1000, 1L

420.4g Urea (Sigma U1250, 7M final)

58.44g NaCl (Sigma S3014, 1M final)

6.67ml 3M Sodium Acetate pH 5.2, filtered and degassed (Sigma 236500, 20mM Final)

2ml 0.5M EDTA pH 8.0, filtered and degassed (Ethylenediaminetetraacetic acid disodium salt, Sigma E1644, 1mM final)

Small scupula of mixed bed resin (Sigma M8032)

357µL 14.3M BME (2-mercaptoethanol, Sigma M3148, 5mM final) water to 1L
Combine all ingredients except BME. Allow to dissolve ~1 hr with stirring a 100°C. Monitor the color of the mixed bed resin. If all beads turn yellow, then add more. When done mixing, add BME. Filter and degas. The mixed bed resin deionizes the urea; SAU1000 lasts for about 24 hrs until the urea reionizes.

**H.2.8 TU1000, 1L**

420.4g Urea (Sigma U1250, 7M final)

58.44g NaCl (Sigma S3014, 1M final)

10ml 2M Tris pH 9.0, filtered and degassed (Sigma T1503, 20mM final)

2ml 0.5M EDTA pH 8.0, filtered and degassed (Ethylenediaminetetraacetic acid disodium salt, Sigma E1644, 1mM final)

Small scupula of mixed bed resin (Sigma M8032)

357µL 14.3M BME (2-mercaptoethanol, Sigma M3148, 5mM final) water to 1L

Combine all ingredients except BME. Allow to dissolve ~1 hr with stirring a 100°C. Monitor the color of the mixed bed resin. If all beads turn yellow, then add more. When done mixing, add BME. Filter and degas. The mixed bed resin deionizes the urea; SAU1000 lasts for about 24 hrs until the urea reionizes.

**H.2.9 100x TE, 1L**

500ml 2M Tris pH 8.0, filtered and degassed (Sigma T1503, 1M final)

200ml 0.5M EDTA pH 8.0, filtered and degassed (Ethylenediaminetetraacetic acid disodium salt, Sigma E1644, 100mM final)
water to 1L total volume

Combine all ingredients. Filter and degas. Store at room temperature.