Mechanism of DNA Homologous Recombination through Studies of DNA Sliding Clamps, Clamp Loaders, and DNA Polymerases

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This dissertation titled
Mechanism of DNA Homologous Recombination through Studies of DNA Sliding
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ABSTRACT

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Mechanism of DNA Homologous Recombination through Studies of DNA Sliding Clamps, Clamp Loaders, and DNA Polymerases

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DNA double-strand breaks (DSBs) are introduced into the genome by several factors including ionizing radiation, mutagenic chemicals, reactive oxygen species, and stalled DNA replication. Without appropriate repair, DSBs may lead to cell lethality or cancer. Homologous recombination (HR) is a widely conserved essential mechanism for high-fidelity repair of DSBs. Available evidence suggests that DNA sliding clamps and DNA polymerases are playing important roles in HR, but the biochemical details of their roles are unclear. This study focused on the functions of two DNA sliding clamps, proliferating cell nuclear antigen (PCNA) and the DNA damage checkpoint clamp (9-1-1 complex), and three DNA polymerases (Pol), Pol δ, Pol η, and Pol ζ, in HR processes. PCNA is required for DNA homologous recombination, but its exact role is unclear. The loading of PCNA onto a synthetic D-loop intermediate of HR and the functional interactions of PCNA with Rad51 recombinase and Pol δ, η, and ζ were investigated. PCNA was loaded onto the synthetic D-loop as efficiently as it was loaded onto a primed DNA substrate. Efficient PCNA loading requires Replication Protein A (RPA), which is associated with the displaced single-stranded DNA loop and provides a binding site for the clamp-loader Replication Factor C (RFC). Loaded PCNA greatly stimulates DNA synthesis by Pol δ within the D-loop, but does not affect primer recognition by Pol δ. This suggests that the
essential role of PCNA in HR is not recruitment of Pol δ to the D-loop, but is stimulation of Pol δ to displace a DNA strand during D-loop extension. Both Pol η and Pol ζ extended the D-loop more efficiently than Pol δ in the absence of PCNA, but little or no stimulation was observed in the presence of PCNA. Rad51 strongly inhibited both the loading of PCNA onto the D-loop and the extension of the D-loop by Pol δ and η. However, pre-loaded PCNA on the D-loop counteracts the Rad51-mediated inhibition of the D-loop extension. This suggests that the inhibition of post-invasion DNA synthesis by Rad51 occurs mostly at the step of PCNA loading. Even though the 9-1-1 complex has been suggested to be potentially involved the post invasion synthesis of HR, neither its loading on the DNA substrate nor the stimulation of polymerase activity were detected in this study. This study contributes to our understanding of the mechanisms of HR-mediated DNA repair and maintenance of genome integrity.
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LIST OF ABBREVIATIONS

DSBs: double-strand breaks
HR: homologous recombination
PCNA: proliferating cell nuclear antigen
Pol: polymerase
RPA: Replication Protein A
RFC: Replication Factor C
DDR: DNA damage response
NHEJ: non-homologous end joining
SSA: single strand annealing
BIR: break induced replication
DSBR: double-strand break repair
SDSA: synthesis-dependent strand annealing
D-loop: displacement-loop
dHJ: double Holliday junction
IDCL: inter-domain connecting loop
PIP: PCNA-interaction peptide
BER: base excision repair
NER: nucleotide excision repair
MMR: mismatch repair
SUMO: small ubiquitin-like modifier
TLS: translesion synthesis
MCM: minichromosome maintenance
CHAPTER 1: INTRODUCTION

1.1. DNA Double-strand Breaks

Cells are subjected to a variety of DNA damage that pose a constant threat to the structural and genetic integrity of the genome [1]. The maintenance of genomic integrity and faithful DNA replication during the cell cycle are essential to prevent mutations and chromosomal rearrangements, because even a single nucleotide change in an essential gene or physical discontinuity of the chromosome can have severe effects on the health and viability of an organism [1, 2]. DNA damage is generated as a result of exposure to different endogenous and exogenous genotoxic agents. These agents cause many types of DNA damage, ranging from base damage (missing, altered, incorrect), deletion or insertion of a nucleotide, cross-linked strands, to single or double-strand breaks [3].

DNA double-strand breaks (DSBs) are considered to be one of the most harmful types of DNA lesion for two reasons [4]. First, even one unrepaired DSB in a cell causes cell lethality. Second, through inappropriate repair, DSBs may lead to gross chromosomal rearrangements and cause deleterious mutations that promote tumourigenesis [5]. In a DSB, both polynucleotide backbones of the dsDNA are broken, so that one dsDNA molecule is physically broken into two separate pieces, which often causes loss of genetic information [4]. In addition, compared with an intact DNA strand, the exposed broken DNA ends of a DSB are more vulnerable to further physical and chemical attack, which can result in loss or damage to the bases or formation of abnormal DNA structures. On average, a single human cell is estimated to repair approximately 50 DSBs per day [6].
DSBs are introduced into the genome by many different endogenous and exogenous mutagenic agents [7]. DSBs may occur as a consequence of exposure to harmful exogenous agents, such as ionizing radiation and mutagenic chemicals. One example of a chemical that induces DSBs is camptothecin, which inhibits type II topoisomerases and leads to the formation of DSBs [8]. Reactive oxygen species, such as hydrogen peroxide and superoxide, generated during normal aerobic respiration, are examples of endogenous mutagenic agents that may also introduce DSBs to the genome [9]. Ionizing radiation is a frequent exogenous cause of DSB [10]. Many other reagents can introduce DSBs more indirectly by blocking DNA replication. A stalled replication fork often initiates a complex repair pathway that converts the fork structure into a DSB [11].

Even though DSBs are generally considered to be detrimental to cell health, tightly controlled DSBs are required to initiate DNA rearrangement during some cellular processes. For example, DSBs are introduced and repaired during V(D)J recombination and class-switching at immunoglobulin gene loci, thereby contributing to the diversity of antibody repertoire in the immune system [12]. DSBs are also actively introduced in chromosomes to initiate the pairing of homologous chromosomes during meiosis [13]. Meiotic DSB formation and repair is crucial for equal distribution of chromosomes into gametes. In these processes, the generation and repair of DSB are strictly controlled to avoid the potentially detrimental effects of these lesions.
DNA damage response (DDR) refers to comprehensive mechanisms exploited by the cells to monitor and maintain the integrity of genome [14]. When cells are under stress caused by different kinds of DNA damage, they can arrest the progression of the cell cycle by different mechanisms depending on the nature of the damage. While the cell cycle is being arrested, DNA repair machinery is activated to repair the damage. If the damage is not repaired, cells may activate the apoptosis pathway to cause cell-death [1].

Since DSBs induce cell-cycle arrest, failure to repair DSBs can lead to prolonged cell cycle arrest and ultimately cell death by apoptosis [15]. Without appropriate repair, even a single DSB is enough to cause cell lethality. Furthermore, inappropriate repair of DSBs may result in genomic rearrangements including the DNA translocations and chromosome fusions that are common in many different types of cancer [16-18]. Under some conditions, cells may escape cell cycle arrest and continue to divide with unrepaired DSBs, which may eventually cause the failure of mitosis or development of cancer [4]. Due to all the harmful properties of DSBs discussed above, proper repair of DSBs is vital for the preservation of genomic and cell integrity.

1.2. DNA Double-strand Break Repair

Cells have several different pathways to repair DSBs. In one major pathway, the DNA ends are processed and re-ligated together (non-homologous end joining (NHEJ)) [4]. Alternatively, DSB can be repaired by DNA recombination with a homologous DNA template (homologous recombination (HR)) [4]. Single strand annealing (SSA) is another
DSB repair pathway that promotes recombination between tandemly repeated DNA sequences, and involves reannealing of processed ssDNA [19]. HR and NHEJ are the two main pathways for DSB repair and have been studied extensively [20]. DSBs occur in all organisms from bacteria to human. Even though the genomic complexity of these organisms varies extensively, the fundamental principle of DSBs repair mechanisms are conserved [21, 22].

1.2.1. Pathway choice for DSB repair

NHEJ, HR, and SSA are three known pathways for DSB repair, and the pathway choice among the three is determined by the species, cell type, cell-cycle stage, and the nature of DNA damage [23]. Since a sister chromatid is available as a homologous template only in the S and G2 phases of the cell cycle, a DSB in G1 phase is mainly repaired by NHEJ [24-27]. In a G2 cell, a homologous partner is available, and HR is the favored pathway for DSB repair [28]. Even though a homologous chromosome is always available in a diploid cell, studies in S. cerevisiae have demonstrated that the sister chromatid is the preferred template over a homolog, when both are available [29]. In diploid budding yeast, HR between homologous chromosomes has also been demonstrated to occur in the G1 phase, in very low frequency [30]. In higher eukaryotes, HR between homologous chromosomes is normally suppressed in non-germ-line cells, since non-reciprocal HR between paternal and maternal chromosomes can lead to loss of heterozygosity. However, such inter-homolog pairing is facilitated during meiosis. These observations
suggest that HR is mostly restricted to the S- and G2 phases of the cell cycle. The regulatory mechanisms of partner-choice are not completely elucidated.

Processing of the broken ends is another important factor determining the pathway in NHEJ, HR, and SSA. Both SSA and HR depend on ssDNA formation by DSB resection. HR needs long (200 nt in average) resected broken ends to generate a 3′ ssDNA tail onto which the key Rad51 recombinase can bind [31]. SSA probably requires a similar length ssDNA tail for Rad52-mediated ssDNA annealing to occur. Interestingly, a nuclease needed for HR (CtIP for vertebrate, Sae2 for *S. cerevisiae*) is only active in S/G2 phase, restricting HR and SSA to these cell cycle phases [32]. Knock-down of CtIP dependent end resection activity alleviates DSBs repair defects [33], suggesting that inhibition of extensive end resection prevents HR and repair of these DSBs is by NHEJ.

NHEJ, which is predominant in a G1 phase, requires relatively short and local processing of the broken ends for ligation. This processing requires distinct proteins including the Ku7/80 complex [34]. This NHEJ machinery occupies the DSB ends in G1 cell, so that the HR-specific end resection machinery can work only when NHEJ fails to repair the damage [24, 25, 32, 35, 36].

The HR/NHEJ ratio of DSB repair (even in G2 phase) varies greatly in different species. For example, while HR is dominant in *S. cerevisiae*, NHEJ occurs at a much higher frequency in mice. Remarkably, human cells show even higher preference for the NHEJ
pathway, which makes gene targeting much less efficient in human cells. It is important to understand why organisms prefer one pathway over the other and how one pathway is chosen over the other when both are available. It is unknown how the balance between HR and NHEJ pathways is determined.

1.2.2. Homologous recombination (HR)

HR is a widely conserved essential mechanism for high-fidelity repair of DSBs [19, 37-39]. HR is a highly coordinated multi-step biochemical process. In *S. cerevisiae*, HR repairs the majority of DSBs. Much of the fundamental understanding about the mechanism of HR has been achieved in studies using yeast. According to knowledge accumulated from studies in various organisms, the biochemical process of HR can be summarized into the following five steps: 1) detection of DSBs; 2) DSB end processing; 3) strand invasion; 4) post-invasion DNA synthesis; 5) resolution of the repair product (Figure 1.1). Each step is overviewed below.

Step 1: DSBs are first detected by the MRX complex (composed of Mre11-Rad50-Xrs2 proteins in *S. cerevisiae*), which compete for binding to unprocessed DSB ends with the Ku70-80 complex [35, 40-44].

Step 2: DSB ends are then processed by specialized exonucleases to generate 3’ overhangs [45-47], which are then coated with the single-strand DNA (ssDNA) binding protein, RPA.

Step 3: The Rad52 recombination mediator interacts with RPA and recruits Rad51 recombinase onto the ssDNA to form a helical nucleoprotein filament [48-51].
The Rad51-ssDNA filament mediates strand invasion into a homologous double-stranded DNA to produce a DNA structure that is referred to as the D-loop [52].

Step 4: The 3'-end of the invading strand is used as the primer by DNA polymerases for DNA synthesis (post-invasion DNA synthesis), extending the D-loop.

Step 5: After post-invasion DNA synthesis, repair may be completed by break induced replication (BIR), double-strand break repair (DSBR), or synthesis-dependent strand annealing (SDSA) pathway [37].

The detailed biochemical process of HR will be discussed in the following sections.
1.2.2.1. Detecting DNA double-strand breaks and initiating the signaling (Step 1)

Accurate and fast repair of DSBs relies on efficient detection of damaged DNA, which is the very first step in the DNA damage response (DDR) [42]. In the initial steps of the DSB repair pathway, phosphorylation and activation of a series of structural and enzymatic proteins are important for efficient damage repair. There are two major DDR signaling pathways in yeast, the Tel1-dependent and Mec1-dependent pathways [15].

Figure 1.1. DNA double-strand break repair by homologous recombination.
Both Tel1 and Mec1 are damage-induced kinases, but they are activated by different forms of damage. While the Tel1 pathway is triggered by DSB ends and MRX, the Mec1 pathway is triggered by ssDNA-RPA-Dpb11, which is generated by end-resection. In *S. cerevisiae*, Tel1 is a key player of the initial DSB response. The Xrs2 subunit of the MRX complex can directly interact with Tel1 and recruit Tel1 to the DSB damage site [53], which leads to the activation of the Tel1-dependent DNA damage response signaling pathway [54-56]. Tel1 phosphorylates Xrs2, Mre11 and Sae2, to function in DNA repair and checkpoint activation [54, 55, 57, 58]. However, a TEL1 knockout mutant is proficient in DNA damage checkpoint activation and does not exhibit increased sensitivity to DSBs inducing agents. This is because of another checkpoint activation pathway that involves Mec1. TEL1 deletion in the context of Mec1 mutants enhances the sensitivity to mutagens, suggesting some degree of functional redundancy between Mec1 and Tel1 [59-62]. Tel1 kinase activity is stimulated by binding of the MRX complex to the DNA–protein complexes at DNA ends [63]. Mammalian ATM, which is the homolog of *S. cerevisiae* Tel1, also recognizes unprocessed DSBs. Unlike Tel1, ATM undergoes damage induced autophosphorylation that leads to a conformational change from inactive dimer to partially active monomers [64]. Partially activated ATM monomers are recruited to DSB sites through their interaction with the NBS1 subunit of the MRN (the homolog of *S. cerevisiae* MRX complex, composed of the MRE11-RAD50-NBS1) complex [65-67]. ATM is then fully activated by the binding to DNA.
1.2.2.2. Broken end processing (Step 2)

The prerequisite of DSB repair by homologous recombination is the processing (typically about 200 nt long) of the broken ends by a nuclease to generate a ssDNA region with 3’ end, essential for the formation of the Rad51 recombinase filament. DSB broken ends are processed in two steps [46, 47].

In *S. cerevisiae*, the first step of the processing is carried out by Sae2 (CtIP for human) and MRX (MRN for human) [68-71]. Removal of approximately 50–100 nucleotides from the 5’ ends of the break gives rise to short 3’ ssDNA tails [46, 47]. This initial processing prevents DSB repair by NHEJ and facilitates extensive end processing to prepare a properly structured DNA substrate for HR [35].

The second step further resects the DNA ends via one of two pathways. One pathway depends on the exonuclease Exo1. Exo1 nuclease activity is stimulated by the MRX complex and Sae2 [44, 72], partially by suppressing NHEJ Ku complex accumulation at DSBs and promoting Exo1 recruitment to DNA ends [44, 72, 73]. The other pathway is mediated by the helicase-topoisomerase complex Sgs1-Top3-Rmi1 in collaboration with the 5’ flap endonuclease Dna2 [46, 47, 74]. The MRX complex and Top3-Rmi1 facilitate the recruitment of Dna2 to DNA ends, increasing the DNA-binding affinity of Sgs1, which stimulates the helicase activity of Sgs1 and promotes DNA unwinding [44, 45, 72, 75]. Sgs1 also has a function in defining the directionality of DNA resection. RPA inhibits Dna2-dependent degradation of 3’ ends while stimulating degradation of 5’ ends.
This specificity ensures only 3’ ssDNA overhangs will be generated for HR. Recent in vitro studies have demonstrated that vertebrate homologues of S. cerevisiae EXO1, BLM (Sgs1), and DNA2 (Dna2) process the broken ends of DSB by two similar pathways [76, 77].

1.2.2.3. Strand invasion (Step 3)

During the end resection, the 3’-single stranded DNA tail is protected by Replication Protein-A (RPA). RPA-ssDNA binding also removes the secondary structure within the ssDNA [78-80]. In order for HR to occur, Rad51 displaces RPA on the ssDNA and forms the Rad51-ssDNA filament. This process is facilitated by yeast Rad52 [81], which mediates the replacement of RPA by RAD51 to form a continuous Rad51-ssDNA nucleoprotein. Because of its role in Rad51-recruitment, Rad52 is defined as a recombination mediator. The Rad51-ssDNA filament searches for a homologous dsDNA sequence and promotes the exchange of homologous DNA strands between the ssDNA and the dsDNA (strand invasion). As a result, one strand of the dsDNA template is displaced, while the other strand is paired with the invading strand. This specific recombination structure is referred to as a displacement-loop (D-loop) (Figure 1.1, c).

1.2.2.4. Post-invasion DNA synthesis (Step 4)

After strand invasion catalyzed by Rad51, the 3’ end of the invading DNA strand is used as a primer for DNA synthesis that extends the invading DNA strand on the homologous template (Figure 1.1, e). This process is called post-invasion DNA synthesis. Since it
copies information that might be missing at the break site, fidelity of the synthesis directly affects the accuracy of the HR-mediated DSB repair. Therefore, post-invasion DNA synthesis is crucial to high fidelity repair of DSBs.

Post-invasion DNA synthesis resembles DNA replication, but is significantly different in several aspects. First, it occurs independent of the activation of S-Cdk or replication origin, which is essential for S-phase DNA replication. Second, it is not associated with several protein factors that are considered essential in S-phase DNA replication. For example, the Pol α-primase complex is dispensable in HR, indicating that the invading 3’ end is the only primer used for DNA synthesis and that lagging strand synthesis is not required for HR. More importantly, post-invasion DNA synthesis does not need Mcm2-7 replicative helicase. The Mcm helicase is activated at the replication origin at the beginning of S-phase, and unwinds the dsDNA template ahead of the replication fork. Post-invasion DNA synthesis, however, can occur without Mcm helicase [82]. Currently no helicase has been identified as an essential component in post-invasion synthesis. Therefore, it is likely that post-invasion DNA synthesis can extend the D-loop without a helicase. DNA synthesis itself should accompany the strand displacement. However, eukaryotic DNA polymerases normally have only limited strand-displacement activity. Mammalian Pol ε is incapable of displacement synthesis [83], and hence is an unlikely candidate for first end DNA synthesis during HR. Third, the DNA structure of the HR intermediate (D-loop) is distinct from the replication intermediate. In S-phase DNA replication, the 3’ end of the leading strand is normally followed by a downstream
ssDNA region that is created by Mcm helicase and complexed with RPA. Such a ssDNA region does not exist in D-loop. This raises a question whether or not polymerases can access such a 3’ end. Fourth, the D-loop intermediate is associated with specific recombination proteins including the Rad51 recombinase, which might affect post-invasion DNA synthesis. Several DNA polymerases are identified to be involved in this process, but the mechanisms of polymerase selection, recruitment, and regulation are largely unknown.

1.2.2.5. Resolution of the repair product (Step 5)

After post-invasion DNA synthesis, the majority of HR intermediates are resolved by either DSBR or SDSA pathways to complete the repair process. In the DSBR pathway (Figure 1.2), a double Holliday junction (dHJ) is produced that can be resolved to generate either crossover or non-crossover products[84]. Resolution by crossover requires a symmetric cleavage of both Holliday junctions in opposite orientations by Holliday junction resolvase. Resolution to non-crossover products can also be achieved by cleaving of both junctions in the same orientation and by collapsing the dHJ, followed by resolution involving a type I topoisomerase activity [84]. In mammalian cells resolution of the Holliday junction is most likely achieved through the action of the Slx1–Slx4 nuclease complex [85-87]. Mammalian cells also have another Holliday junction resolving protein called Gen1 (the orthologue of Yen1 in yeast) [88]. The symmetry of Holliday junctions enables them to be resolved in either of two orientations, which determines whether the DNA sequences flanking the Holliday junction are exchanged or
not. Nevertheless, in each case the DSB and the genetic information lost at a break in one chromatid is faithfully restored using its sister chromatid.

An alternative pathway used to resolve the post invasion synthesis products is called synthesis dependent strand annealing (SDSA), which does not involve the formation of Holiday junctions and results in non-crossover products only (Figure 1.2) [89]. In this pathway the invading DNA strand can be displaced from the homologous dsDNA after the post-invasion synthesis, and re-anneal to the other broken chromosome end [84]. Since the extension occurs on a sister chromatid in most cases, missing DNA sequence on the broken chromatid can be restored accurately.

Break-induced replication (BIR) is another DSB repair pathway that repairs DSB by strand invasion into a homologous duplex DNA followed by replication to the chromosome end (Figure 1.1) [90].
1.2.3. NHEJ

Ideally, DSBs need to be repaired in a faithful way, i.e., through homologous recombination. However, when the sister chromatids are not available to serve as homologous template, cells will take other pathways like NHEJ to survive (Figure 1.3) [23]. NHEJ simply rejoins the two broken ends together without extensive processing of the DNA sequence at the break. This pathway requires several proteins and a specialized DNA ligase (DNA ligase IV). NHEJ repair is initiated by the Ku complex (Ku70/Ku80 heterodimer) that recognizes broken ends. The Ku complex binds to both ends of the

Figure 1.2. Resolution of HR intermediates leads to the formation of either crossover or non-crossover products.
break and then recruits the catalytic subunit of the DNA dependent protein kinase (DNA-PKcs) [1]. Depending on the nature of the broken ends, the ends can be either trimmed by nucleases or filled in by DNA polymerases to create compatible ends (~2 nucleotides of microhomology at both ends) for efficient rejoining [91]. The final step of NHEJ is the ligation of the two ends. The ligation complex consists of DNA ligase IV, X-Ray Cross-Complementation group 4 (XRCC4) and Xrcc4 like factor (XLF) [92, 93].

As mentioned above, DSBs also occur during the V(D)J recombination at the immunoglobulin locus, where V (variable), D (diversity) and J (joining) DNA segments are randomly selected from multiple arrays of the repeated segments, and joined together to produce a coding sequence of an immunoglobulin heavy chain [94]. NHEJ is essential for this process. Furthermore, an assembled variable region is combined with a DNA segment encoding one of several constant domains, which determine the type of the antibody (IgM, IgD, IgG, IgE or IgA) [95]. This process, called class switch recombination, also requires NHEJ.
Figure 1.3. DNA double-strand break repair by non-homologous end joining.

DSB repair by NHEJ very often leads to the loss of genetic information. If a DSB were to be created with complementary overhangs, and if they were re-ligated directly with NHEJ [96], then the NHEJ mechanism could repair the DSB without any loss of sequence information. However, this occurs rarely; in majority of cases, the ligation occurs after loss of a length of DNA from the break sites. Therefore, NHEJ is by definition error-prone and considered to be mutagenic. While NHEJ plays a critical role in maintaining the structural integrity of DNA, it does not guarantee the preservation of genetic integrity.
1.2.4. DNA Damage-induced signaling and chromatin modification

DSB repair is a complicated cellular process that requires the collaboration of many different processes, including intracellular signaling. This emphasizes the significance of Tel1/Mec1-dependent (ATM/ATR for human) damage checkpoint that leads to changes in chromatin conformation. In *S. cerevisiae*, the phosphorylation of histone H2A on S129 (\(\gamma\)-H2A) by Tel1 is a critical event in the activation of the DDR pathway after detection of DSBs [97-101]. This modification is most prominent at the DSB ends, but it also spreads over a lengthy chromatin region flanking the break [98, 101, 102]. The same modification is conserved in mammalian cells where ATM-dependent phosphorylation occurs on S139 of the H2AX (\(\gamma\)-H2AX, a variant of yeast H2A) [103].

The significance of H2A phosphorylation in DDR and DSB repair has been demonstrated by many experiments done with H2A phosphorylation defective mutants. For example, yeast H2A-S129 mutants are very sensitive to DSB-inducing agents and are impaired for DSB repair [98, 104, 105]. H2A-S129 mutants are defective in G1 checkpoint activation in response to DSB due to impaired recruitment of the Rad9 checkpoint protein to sites of damage, which binds \(\gamma\)-H2A via its tandem BRCT domain [106, 107]. Mammalian cells that are deficient in H2AX are radiosensitive and display elevated genomic instability and defects in sister chromatid recombination [108-111]. In addition, H2AX \(^{-/-}\) mouse embryonic fibroblasts are defective in G2-M checkpoint activation following low doses of ionizing radiation [112]. These and other studies revealed that \(\gamma\)-H2A(X) promotes efficient DSB repair through several different
mechanisms, including the recruitment of cohesin, histone modifiers, and chromatin remodeling complexes in both yeast and vertebrates [113, 114].

Phosphorylated H2AX provides the assembly sites for checkpoint and repair proteins and promotes their retention on the damage sites [115]. This is critical for the activation of a robust checkpoint response as suggested by the fact that co-localization of checkpoint and repair proteins on chromatin is sufficient to activate checkpoint response in the absence of DNA damage [116, 117].

1.2.5. Physiological significance of HR

Accumulation of DNA damage can lead to genomic instability within the cell and is closely linked to cancer generation. Due to this property, many anticancer treatments are targeting DNA damage response and repair pathways [15]. DSBs are the most cytotoxic form of DNA damage among diverse types of DNA lesions and pose a serious threat to genome stability and cell viability. Cells exposed to DSBs initiate a series of events to promote repair of the lesion in order to survive and restore chromosomal integrity [118]. Failed or inaccurate repair of DSBs can have a number of consequences, such as gross chromosomal rearrangements and eventually cell death [119]. The essential mechanism for faithful repair of DSB, homologous recombination, is a complicated biochemical process that requires a large number of proteins. It is well-known that many mutations in DSB repair genes have been implicated in cancer pre-disposition diseases such as ataxia telangiectasia, Nijmegen breakage syndrome, and Bloom syndrome [120]. Although it is
difficult to estimate the exact percentage of HR defective cancers in overall cancer incidence, recent studies suggest that it may be as high as 50% in certain cancers [121]. The proteins required for DSB repair are highly conserved in eukaryotes from yeast to humans, which highlight the importance of the study of repair in lower eukaryotes.

Besides their detrimental impact on genome stability, DSBs are also required for normal cellular processes such as chromosome segregation during meiosis and V(D)J recombination in immunoglobulin genes [2, 37]. In meiosis, diploid germ-line cells produce haploid gametes by two rounds of cell division, i.e. homologous chromosomes segregate at meiosis I and sister chromatids separate at meiosis II. In order to assure proper chromosomal segregation at meiosis I, homologous chromosomes normally undergo at least one crossing-over event. This homolog-pairing leads to a high frequency of reciprocal recombination between homologous chromosomes (not between sister chromatids) [13]. Different from a cross-over event, gene conversion introduces a small segment of DNA from one background into the other. Both gene conversion and cross-over are initiated by meiosis-specific DSBs [13].

The activity of HR has to be tightly regulated, as either too much or too little HR can be toxic to a cell. For example, a cell that undergoes too much HR can result in gross chromosomal rearrangements including duplications, deletions, and translocations [122]. On the other hand, too little HR may causes cell death, chromosome deletion or fusion by
NHEJ, immunological defects, meiotic nondisjunctions, cancer predisposition, and premature aging [4, 23, 123].

1.3 Proliferating Cell Nuclear Antigen (PCNA)

1.3.1. PCNA is an evolutionally conserved DNA sliding clamp

DNA sliding clamps are ring-shaped protein complexes that encircle and slide along the DNA [124, 125]. DNA sliding clamp was originally identified as an elongation factor (β subunit) for DNA polymerase III of *E. coli* [126]. During *in vitro* DNA replication, the DNA polymerase III core enzyme tends to fall off the template frequently in the absence of the sliding clamp, making replication of DNA very inefficient. The incompletion of DNA replication causes cell lethality. In the presence of the β subunit, the DNA polymerase III core enzyme is tethered to the DNA through its interaction with the clamp and synthesizes thousands of bases in a few seconds without detaching from the DNA template [127, 128]. The function of β subunit requires another protein (γ subunit), which loads the sliding clamp onto the primer-template complex using the energy of ATP hydrolysis [129]. This function is referred to as the clamp loader activity. Later, the two β subunits were found to form a ring-shaped structure [124], providing a perfect mechanistic explanation to how it can increase the processivity of DNA synthesis.

Sliding clamps and their clamp loaders are structurally and functionally conserved from bacteriophage to humans [130] (Figure 1.4). The PCNAs of yeast and human are well-studied examples of eukaryotic sliding clamps [130-133]. Although the bacterial β clamp...
is a homo-dimer and PCNA of eukaryotes is homo-trimer, the sliding clamp from
different species form a conserved pseudo-six-fold symmetry ring structure [130, 134].
PCNA is functionally conserved in all three domains of life [135]. In some cases, the
function of PCNA as a processivity factor is not species-specific: plant PCNA can
stimulate human DNA polymerase δ [136, 137], and both yeast and Drosophila PCNA
can stimulate mammalian DNA polymerase [138, 139].

Figure 1.4. PCNA stimulates processive DNA synthesis by Pol δ/ε during
chromosomal DNA replication.
Bottom. Current model of PCNA function at replication fork.
The clamp loader of PCNA is the Replication Factor C complex (RFC complex) that is composed of Rfc1, Rfc2, Rfc3, Rfc4, and Rfc5. The RFC complex binds to PCNA, opens it at one inter-subunit surface, and loads it onto the DNA substrate in an ATP-dependent manner [140-143]. Once the PCNA is loaded, it encircles and slides freely along the dsDNA, serving as an anchoring site for DNA polymerases to prevent them from falling off the template during DNA synthesis (Figure 1.4) [144-146].

1.3.2. Structure of PCNA

Since the demonstration of the first crystal structure of S. cerevisiae PCNA [147], structural data obtained from yeast, archaea, plant, and human PCNAs have shown similar structures to the E. coli β clamp (DNA pol III β subunit). PCNA, forms a pseudo-six-fold symmetry ring consisting of three identical subunits [147] (Figure 1.4). The internal diameter of the ring is 3.4 nm allowing for the free sliding once loaded on the DNA substrate [148]. The two sides of the PCNA ring are different, with one side having the protruding C-terminal end (C-face; defined as the front side) [147, 149-151]. During DNA replication, PCNA is loaded onto the DNA primer-template junction with the front side facing the 3′ end of the elongating DNA strand, which ensures that DNA polymerases are positioned to synthesize the growing end [135]. PCNA monomers interact with each other in a head-to-tail fashion[124], which is similar to that of the E. coli β clamp. The N-terminal region of PCNA contributes to its interaction with human α-helicase and cyclin-D [152]. The C-terminal region of PCNA is involved in the interaction with proteins such as Pol ε, RFC, and cyclin-dependent kinase 2 [152].
Another very important structural component within each PCNA monomer is the inter-domain connecting loop (IDCL), which connects the N- and C-terminal domains of each monomer. IDCL serves as the docking site for different PCNA interacting proteins such as Pol δ, p21, DNA ligase 1, and flap endonuclease (Fen1) [153].

Many proteins bind to PCNA via the conserved PCNA-interaction peptide (PIP) motif [154], which is usually located at the extreme N- or C-terminus of the monomers. The PIP-box consensus motif in both eukaryotes and archaea has been defined as $q x x a x x h$ $h$ [154]. The two $h$’s represent a small hydrophobic amino acid (Ile/Leu/Met) and an aromatic hydrophobic amino acid (typically Phe/Trp) ($x$ can be any amino acids). The crystal structure of human PCNA with the cell cycle checkpoint protein shows that p21 interacts with PCNA through the glutamine of the PIP-box motif directly and via solvent molecules [149]. The hydrophobic section of the PIP-box motif is inserted into the hydrophobic cleft on the PCNA surface, and the extended peptide interacts with the IDCL [149].

1.3.3 Multiple function of PCNA

After its initial discovery as a DNA polymerase processivity factor, the PCNA sliding clamp has been shown to be involved in almost every process dealing with DNA metabolism, including replication, modification, and repair [152, 155-157]. PCNA serves as a docking station for many other proteins involved in DNA processing, including DNA repair enzymes, DNA modulating enzymes, and translesion DNA polymerases [135].
Because of its versatility in interacting with different proteins, PCNA is considered to be a master controller allowing proteins to access the DNA, thereby coordinating functions of enzymes depending on the physiological condition of the cells.

1.3.3.1. Function of PCNA in DNA replication

The most well know function of PCNA is to stimulate the processivity of replicative DNA polymerases, such as DNA polymerase δ. During DNA replication, the DNA template primed with a short fragment of RNA primer is recognized by the RFC clamp loader complex at the 3′ junction with the primer [158], causing the dissociation of the primase complex and the loading of PCNA onto the template using the energy generated from ATP hydrolysis [135, 159]. After loading polymerases on DNA, PCNA slides along DNA with synthesis by Pol ε/δ occurring concomitantly on the leading and lagging strand. Since the leading strand is synthesized in a continuous manner while the lagging strand is synthesized in a discontinuous manner, more PCNA loading events are required on the lagging strand [135].

According to the current understanding of DNA replication in eukaryotes, Pol α/primase synthesizes the first primer on the leading strand. Then, together with the DNA polymerase switch initiated by RFC, Pol ε with PCNA performs continuous leading-strand synthesis, whereas pol α/primase is involved in RNA priming and discontinuous DNA synthesis at the lagging strand. However, completion of Okazaki fragment synthesis requires the action of a processive Pol δ. Thus, both initiation of leading-strand
synthesis and discontinuous lagging-strand synthesis require a DNA polymerase switch. PCNA has been shown to play a central role in coordinating this process. Binding of PCNA to the 3’ end of the primer prevents re-loading of DNA pol α and acts as a recruitment signal for Pol δ/ε [152].

The identification of PCNA as a processivity factor for replicative polymerases placed it at the heart of the replisome [135]. However, additional roles of this protein emerged later, changing our view of PCNA from the simple processivity factor to a platform that coordinates the complex network of interactions occurring at the replication fork. Due to its physical localization in the DNA replication machinery and its ability to interact with numerous proteins, the PCNA clamp functions as a master control in DNA replication to recruit, stabilize, and exchange of various proteins, coordinating the DNA replication process [152].

1.3.3.2. Function of PCNA in DNA repair

PCNA is essential for many DNA repair pathways, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR).

BER is the primary DNA repair pathway that is responsible for the repair of base modifications including oxidation, alkylation, deamination, depurination, and depyrimidination [160]. In BER, damaged bases are recognized by DNA glycosylases that remove the base from the polynucleotide backbone. The resulting abasic sugar (AP)
is then cleaved by AP endonucleases (APE). The exposed DNA gap is then filled by DNA synthesis mediated by either Pol β (short patch BER) or Pol δ or ε (long patch BER). One obvious function of PCNA is to recruit Pol δ/ε. However, the function of PCNA in BER is not limited to DNA synthesis after removal of the damaged DNA segment, but also in other steps of BER. This was demonstrated by analyzing the repair of abasic sites by *Xenopus laevis* egg extract (long-patch BER) [161, 162]. Moreover, PCNA has been shown to interact with many other enzymes that are involved in yeast and mammalian BER pathways, including AP endonuclease 1 [163], AP endonuclease 2 [164, 165], uracil-DNA glycosylase 2 [166, 167], 3-methyladenine-DNA glycosylase [168], the human homologue of *E. coli* endonuclease III [169], and a MutY homologue [170]. It has been proposed that PCNA serves as a platform to recruit all required enzymes for BER. In addition, XRCC1, a scaffolding protein in both BER and single-strand break repair [171], has been shown to be recruited to replication foci by PCNA and to interact with DNA polymerase β, which is another DNA polymerase that participates in BER synthesis (short-patch BER) [171, 172].

In NER, the damaged DNA strand (thymine dimer, for example) is recognized by a protein complex containing XP (xeroderma pigmentosum)-C, and is incised on both sides of the lesion by XPG and ERCC1-XPF nuclease, resulting in the removal of a fragment approximately 25-30 nucleotides in length [173]. The generated gap is then repaired by DNA synthesis and ligation [173]. The involvement of PCNA in NER has been demonstrated by studies of human cell lines *in vitro* [174, 175]. As in the BER, a major
function of PCNA in NER is in gap-filling DNA synthesis after removal of damaged segment through interaction with Pol δ/ε, RFC, and DNA ligase 1. An additional function of PCNA that is unique to NER, is the recruitment of XPG endonuclease by direct interaction [176]. The XPA protein is a component of the core preincision complex of NER, interacting with DNA and other NER protein factors. Immunofluorescence studies on wild-type and XPA mutant cells showed that after UV treatment, PCNA localization to the damaged sites in the nucleus is dependent on the functional XPA protein [177-179].

MMR is an evolutionarily conserved DNA repair pathway that corrects base mismatches occurred during DNA replication that escaped proofreading by replicative DNA polymerases [160]. In eukaryotic MMR, the mismatch is detected by MSH (prokaryotic MutS homolog) proteins that recruit MLH (prokaryotic MutL homolog). MLH proteins are matchmakers that coordinate multiple steps in MMR. In prokaryotes, an endonuclease, MutH, nicks the nascent DNA strand at hemimethylated GATC sites. As in BER and NER, PCNA functions as a Pol δ processivity factor for the re-synthesis of the excised DNA fragment. In addition, PCNA also contributes to early repair events including the recognition and DNA incision process [180]. PCNA can form a complex with MSH2-MSH6 to stimulate their preferential binding to the mismatched DNA bases [181]. Moreover, PCNA can interact with MMR core proteins, including MSH3 and MSH6 to enhance their specific binding to mismatched sites [182-185]. PCNA, RFC and Exonuclease 1(EXO1) are necessary to activate the endonuclease activity of the MutLα complex (MLH1-PMS2), which is essential for MMR [186]. PCNA also interacts with
other members of the MMR pathway, such as MutL homologue 1 (MLH1) and exonuclease 1 (EXO1) [185].

1.3.3.3. Function of PCNA in damage tolerance by translesion synthesis.

Translesion synthesis bypasses DNA lesions using specialized DNA polymerases (TLS polymerases) [187]. The TLS polymerases are recruited to lesions by interacting with PCNA that has been modified by monoubiquitilation [188-190]. Since these polymerases do not necessarily repair the lesion but simply synthesize over it, TLS is not considered as a repair pathway, but referred to as a damage tolerance pathway (Figure 1.5) [187]. In this pathway, the lesion site is first encountered by replicative polymerases that cannot synthesize over the lesion, causing the replication fork to stall [187]. The stalled replication fork recruits a special ubiquitin conjugation system (Rad6/Rad18 ubiquitin ligase) that adds a single ubiquitin molecule onto PCNA [191]. Based on the current model, monoubiquitinated PCNA facilitates the switching from a replicative polymerase to a TLS polymerase [192]. The major role of PCNA in TLS is not to increase the general processivity of the TLS polymerase, but to stimulate its efficiency of nucleotide incorporation at sites opposite to the DNA damage [187, 193].
PCNA, at least in yeast, is also modified by SUMO (small ubiquitin-like modifier) [194]. Both ubiquitination and sumoylation take place on the same Lys164 residue in response to a stalled DNA replication fork [194]. Therefore, sumoylation counteracts the ubiquitination-dependent TLS pathway of damage tolerance. In addition, the SUMO modification can also occur on PCNA at the Lys127 residue [195]. Sumoylation of PCNA on Lys127 inhibits interaction with certain PCNA-binding proteins, e.g. Eco1, which is responsible for establishing sister-chromatid cohesion in S phase [195]. PCNA also goes through polyubiquitination, which is thought to promote error-free damage avoidance through template switching [194].

1.3.3.4. Function of PCNA in HR

PCNA has been shown to be required for HR in several aspects. First, both genetic and biochemical studies demonstrated the participation of PCNA in the post invasion DNA synthesis of HR [82, 196-199]. Wild type haploid *S. cerevisiae* cells are capable of switching mating type between “a” and “α” (the two mating types of haploid cells), which is essentially a gene conversion event. The mating type switching is initiated by a
specific DSB at the mating type locus by HO endonuclease. This DSB is repaired by the gene conversion (HR without crossover) using the DNA segment that has the opposite mating type sequence. This system has been used experimentally to induce a unique double-strand break in the yeast genome for repair by homologous recombination [200].

PCNA was shown to be required for mating-type switching [82]. This requirement is most likely due to the interaction between PCNA and DNA polymerases to increase their processivity, because PCNA was found to be required to synthesize even as little as 30 nucleotides following strand invasion [198]. Consistently, in vitro biochemical studies using purified yeast or human proteins indicated that PCNA is required for DNA synthesis following Rad51-mediated strand invasion [196, 197, 199]. In these studies, PCNA greatly stimulates processive DNA synthesis by Pol δ on a DNA recombination substrate. The role of PCNA in HR is probably more than an accessory factor of DNA polymerases. After sumoylation on Lys-164, PCNA can recruit the Srs2 helicase to block the formation of Rad51-ssDNA filament to prevent inappropriate homologous recombination [194, 201].

Several questions remain unanswered regarding the molecular functions of PCNA in HR. For example, due to the apparent structural difference of DNA replication and recombination substrates, it is not clear how PCNA is loaded onto the recombination intermediates. Some essential protein factors present in S-phase DNA replication are not available during recombination, but the impact of their absence on PCNA function in HR has not been assessed.
1.4. The 9-1-1 Clamp: Alternative Sliding Clamp

1.4.1. The role of the 9-1-1 complex in damage checkpoint

The damage checkpoint is composed of complex signaling pathways that are initiated by DNA damage or replication stress [202, 203], resulting in several cellular responses to deal with DNA damage. These responses include blockage of the cell cycle, expression of proteins that are responsible for repair, and tethering sister chromatids to facilitate error-free repair [202, 203]. The 9-1-1 complex is one of the important players in checkpoint activation. The name “9-1-1 complex” came from the names of its three subunits, Rad9, Hus1, and Rad1 of humans. However, in *S. cerevisiae*, the components of the same complex are Rad17, Mec3 and Ddc1[204]. The structure of the complex is conserved from yeast to humans (Figure 1.6) [205-207], where the heterotrimeric complex of Rad9, Hus1, and Rad1 forms a stable ring shaped complex [206, 208-214]. Importantly, the entire structure of the 9-1-1 complex is similar to PCNA.

![Figure 1.6. The 9-1-1 clamp is structurally similar to PCNA.](image-url)
Not only does the structure of 9-1-1 complex itself resemble the PCNA clamp, the clamp loader for the 9-1-1 complex is also related to the PCNA loader, RFC complex [204, 212, 214-217]. The RFC complex is composed of five subunits (Rfc1, 2, 3, 4, and 5), four of which are shared with the loader of the 9-1-1 complex. The largest subunit of RFC complex (Rfc1) is substituted by Rad24 in the 9-1-1 loader. Thus, this loader is termed Rad24-RFC. *In vitro* analyses showed that the 9-1-1 complex is loaded on DNA by Rad24-RFC using the energy from ATP hydrolysis, similar to that of PCNA loading [204, 215, 216]. Consistent with this biochemical observation, knockout of the loader function in human and yeast cells and in *Xenopus* egg extracts blocks damage induced chromatin binding by the 9–1–1 complex [218-221]. The loaded 9-1-1 complex is involved in a series of signaling events to facilitate DNA damage repair, such as G2/M cell cycle arrest, and replication fork stabilization [222].

### 1.4.2. The role of the 9-1-1 complex in TLS

Accumulating evidence suggests that the 9-1-1 complex also participates in TLS. For example, studies in yeast have indicated a role of the 9–1–1 complex in the regulation of translesion DNA synthesis (TLS) by DNA polymerase ζ [223, 224]. Since Pol ζ is an error-prone polymerase, its activity is genetically monitored by a mutator phenotype after UV-irradiation. In the cells that lacks 9-1-1 function, UV-induced mutagenesis is suppressed, indicating that the 9-1-1 complex is required for Pol ζ translesion synthesis [224]. It has been shown that the 9-1-1 clamp physically interacts with Pol ζ and is partially required for spontaneous Pol ζ dependent mutagenesis in *S. cerevisiae* [225]. A
similar observation was made in *Schizosaccharomyces pombe* with DNA polymerase κ (kappa) [223].

1.4.3. The role of the 9–1–1 complex in double-strand break repair by homologous recombination

Studies of *Rad9−/−* embryonic stem cells have shown that the 9-1-1 complex plays a critical role in survival of cells treated with ionizing radiation to induce DNA double-strand breaks [226]. In *S. cerevisiae*, deletion of genes encoding the Rad24 clamp loader and the Rad17 clamp subunit decreases cell survival rate following the introduction of enzyme produced double-strand breaks [227]. These increases in DSB sensitivity is not due to the lack of cell-cycle arrest by the loss of the 9-1-1 complex, since it is observed in artificially arrested cells. Therefore, these results indicate that the 9-1-1 complex plays a direct role in the DSB repair.

The biochemical roles of the 9-1-1 complex in HR are still largely unknown. Because the Rad1 subunit of the 9-1-1 complex has been shown to have nuclease activity [228, 229], some groups proposed that the 9-1-1 complex may directly participate in the DSB processing as a nuclease to produce the ssDNA that is a prerequisite for HR. An alternative hypothesis is that the 9–1–1 complex serves as a sliding clamp that anchors one or more proteins which are involved in the DSB repair. For example, DNA processing enzymes like the MRX complex might interact with the 9-1-1 complex, promoting its processing activity. Interaction with DNA polymerases is another important
candidate interaction with the 9-1-1 clamp. Currently, none of the hypotheses are supported by conclusive experimental evidence.

1.5. DNA Polymerases

1.5.1. Classification of eukaryotic DNA polymerases

DNA polymerases are critical not only for DNA replication, but DNA repair, damage-tolerance, and recombination [230]. Since there are many cellular processes that DNA polymerases are involved in, it is not surprising that multiple DNA polymerases with unique enzymatic activities have been identified in different organisms. To date, there are at least 5, 9, and 15 different DNA polymerases that have been discovered in *E. coli*, budding yeast, and humans, respectively [231]. All of these polymerases share the same structure at their catalytic centers, but are significantly different in other parts of the protein (Figure 1.7) [231, 232].
Based on their roles in bulk DNA replication in S-phase, DNA polymerases are divided into replicative and non-replicative polymerases [230]. The replicative DNA polymerases are responsible for the major part of DNA synthesis at the replication fork. Polymerases in this group have robust DNA synthesis activity with very high fidelity (synthesize ~500 nt/sec/enzyme at 10^{-4} to 10^{-5} error rate) [233-235]. Pol α (associated with primase), Pol δ (lagging strand synthesis), and Pol ε (leading strand synthesis) have been identified as the replicative polymerases in eukaryotic cells [230]. Pol δ and ε possess 3′–5′ exonuclease activity that allows proof-reading of the newly synthesized fragment during strand
elongation, thereby increasing the fidelity of replication [236, 237]. None of the other DNA polymerases are responsible for extensive DNA replication and are termed non-replicative polymerases [230]. They carry out a variety of cellular functions in DNA metabolism that are described below. Non-replicative polymerases are not used in bulk DNA synthesis and have higher error rate than replicative polymerases. They do not require the exact DNA structure of the primer-template complex and often incorporate a nucleotide at mismatched or damaged template bases [238]. Although such non-replicative polymerases are not major components of the replication fork, increasing evidence suggests that some of them are at least occasionally associated with the replication fork. This may allow quick switching from a replicative polymerase to a specialized polymerase when replication fork encounters damaged DNA site [238]. However, the biochemical details of the polymerase switching is not clear.

DNA polymerases can are also classified based on the sequence homology. Mammalian polymerases have four families: the A family (Pol γ, ν, θ), the B family (Pol α, δ, ε, ζ), the X family (Pol β, λ, μ, σ), and the Y family (Pol η, κ, λ, and Rev1) [239, 240]. These DNA polymerases vary considerably in processivity and fidelity. Their structural and biochemical details will be discussed in the following sections.

The A family DNA polymerases includes Pol γ (gamma), Pol θ (theta), and Pol ν (nu) [239, 240]. Polymerases belonging to the A family are found from bacteria to higher eukaryotes. Pol γ is the only DNA polymerase that is found in mitochondria [241]. Pol θ
is proposed to function in the repair of interstrand cross-links and base excision repair [242, 243].

The B family DNA polymerases includes Pol α (alpha), Pol δ (delta), Pol ε (epsilon) and Pol ζ (zeta) [239, 240]. The first three are replicative DNA polymerases responsible for the replication of chromosomal DNA, while Pol ζ has been shown to be involved in translesion synthesis.

The X family of DNA polymerases, including Pol β (beta), Pol λ (lambda), Pol μ (mu), and Pol σ (sigma), share structural similarities and limited sequence homology [244]. X family polymerases are found in archea, bacteria, and eukaryotes [239, 240]. X family polymerases have been shown to be involved in base excision repair and DSB repair, and (VDJ) recombination for generating antibody diversification [245]. Pol β was the first X family polymerase identified, and it has been extensively studied for correlation with human cancers [246].

The Y family DNA polymerases, all found to have translesion synthesis (TLS) activity, include Pol η (eta), κ (kappa), ι (iota), and Rev1 [239, 240]. The Y family polymerases are highly conserved and share structural homology with members within the family and other TLS polymerases [247, 248]. Due to their ability to synthesize over various DNA lesions, the Y family polymerases directly contribute to DNA damage tolerance by cells. The catalytic domains of the Y family polymerases are structurally more relaxed and
therefore are able to accommodate bulky DNA lesions, allowing incorporation of nucleotides at the opposite to the DNA lesions.

1.5.2. Functions of DNA polymerases in DNA damage tolerance

Apart from their involvement in DNA replication and repair, some DNA polymerases have been shown to be able to synthesize over unrepaired DNA lesions to temporarily tolerate damages, which is described as DNA damage tolerance pathway [248, 249]. Even though the cells have a very efficient and sophisticated DNA repair system, it is impossible for the cells to repair all lesions immediately after they occur. Some of these unrepaired sites of DNA damage may persist into the S phase of the cell cycle. These sites may block the migration of replicative polymerases on the template, resulting in replication fork stalling and collapse. A collapsed replication fork is extremely harmful to genome stability and cell viability. Therefore, cells have to prevent replication fork stalling by either repairing or tolerating damage [11, 250]. The damage tolerance pathways allow cells to temporarily tolerate DNA damage until the lesions are removed by DNA repair pathways.

Damage tolerance or lesion bypass is carried out by specialized translesion synthesis (TLS) DNA polymerases (Figure 1.5) [193, 248], including the Y family polymerases η, ι, κ, and Rev1, as well as DNA polymerases from other families, such as Pol ζ and β [251, 252]. Among these TLS polymerases, Pol η is probably the most well studied enzyme and its activity is tightly linked to the development of cancer [253]. In addition to Pol η,
the expression patterns of many other TLS polymerases have been found to be altered in various tumor tissues and their activities have been linked to mutagenesis and anticancer drug resistance [233, 236, 254, 255].

The polymerase-switch model is a commonly accepted mechanism of TLS [256, 257]. Depending on the properties of the DNA lesion, the TLS pathway may require sequential association of multiple DNA polymerases to extend from the lesion site. A specialized TLS polymerase can insert nucleotides opposite to the lesion sites, whereas another TLS polymerase suggested to be mainly Pol ζ and Pol κ [258-260], will extend a few nucleotides from the insertion site followed by switching back to the replicative polymerase. During this switching process, PCNA probably interacts with both the replicative and the TLS polymerases sequentially or simultaneously, controlling their access to the DNA template. PCNA is normally associated with replicative polymerases in DNA replication. Upon mono-ubiquitination by the E2-conjugating enzyme Rad6 and E3 ubiquitin ligase Rad18, PNCA recruits the TLS polymerases to the DNA lesion site [191, 261]. After lesion bypass, the TLS has to be disengaged from the DNA template, followed by a switch to a replicative polymerase to continue high-fidelity DNA synthesis. Similar to the first switch, it has been suggested that deubiquitination of PCNA may be involved in this process [262]. Alternatively, the DNA damage checkpoint clamp, 9-1-1 clamp, has been found to be loaded onto damaged DNA strands and interact with the TLS enzymes, pol ζ and pol κ, suggesting a role of the 9-1-1 clamp in regulating the activity of these polymerases during TLS [225, 263].
1.5.3. Function of DNA polymerase in DNA repair and recombination

Accumulation of DNA lesions causes instability to the genome, which can lead to cancer generation and progression [255, 264, 265]. Therefore, it is critical to repair DNA damage in a timely and accurate manner. Depending on the nature of the lesion, cells will activate different repair pathways that very often require the activity of DNA polymerases. For example, base damage is repaired by the base excision repair (BER) pathway. The main polymerases involved during BER are Pol β, Pol γ, and Pol λ [266, 267]. Distorted DNA helices and base adducts are removed by the nucleotide excision repair (NER) pathway, which requires the participation of Pol δ and Pol ε [268, 269]. Pol δ has been shown to participate in the mismatch repair (MMR) pathway that corrects the DNA mismatch [270]. Much remains to be known about the mechanisms of polymerase selection.

Double-strand break (DSB) is the type of DNA lesion that is considered to be most toxic to the genome and can be generated by exogenous and endogenous mutagenic agents [249, 271]. DSBs can be repaired by nonhomologous end-joining (NHEJ) or homologous recombination (HR); both require the participation of DNA polymerases. DNA polymerases, including Pol ε, Pol β, Pol ζ, Pol η, Pol θ, Pol λ, Pol μ, have been suggested to function in DSB repair by HR or NHEJ [272-281]. For example, several DNA polymerases, including Pol δ, η, and ζ, are possibly involved in HR post-invasion DNA synthesis. Genetic studies in yeast indicate that Pol δ is involved in mitotic gene conversion [282], meiotic recombination [283], repair of γ-ray-induced DNA damage
[284], and HO endonuclease-induced gene conversion [82]. Studies of HR in vertebrate systems have shown that translesion polymerases Pol η and Pol ζ also contribute to HR. Pol η-deficient chicken DT40 cells have defects in gene conversion at the immunoglobulin locus [285], and purified human Pol η can catalyze DNA synthesis within a synthetic D-loop [286]. Loss of Pol ζ function (REV3−/−) resulted in increased sensitivity to DNA damaging reagents in mouse [287] and chicken cells [288, 289]. Although Pol ζ is not essential for HR in yeast, the deletion of yeast REV3 gene greatly decreases the mutation rate near DSB [272]. This is presumably because of the low fidelity of Pol ζ.

1.6. Specific Aims of the Research

The study described in this dissertation is aimed at understanding the function of PCNA and the 9-1-1 clamp during post invasion DNA synthesis of HR. Specifically, is the PCNA/9-1-1 clamp loaded on the D-loop and how does it affect the activity of the DNA polymerases that are possibly involved in recombination DNA synthesis? Based on the current understanding I have reviewed above, I set two major hypotheses for my PhD study.

The hypotheses are:

1. PCNA and the 9-1-1 clamp are loaded onto the D-loop, the homologous recombination intermediate.
2. Loaded PCNA or the 9-1-1 clamp stimulates Pol δ, η, or ζ mediated post-invasion DNA synthesis of HR.

![Figure 1.8. Hypotheses.](image)

PCNA and 9-1-1 clamp are possibly involved in homologous recombination to regulate the activity of DNA polymerase δ, η, and ζ activity during post-invasion DNA synthesis.

As unrepaired DSBs are potential causes for human diseases like cancer, understanding the molecular mechanisms regulating the response to DSBs will not only further our knowledge of these intricate signaling networks, but it may also identify directions for developing therapeutics with the potential to significantly impact human health. Even though numerous studies have been carried out to understand the biochemical details of HR, several critical questions remain unanswered. One question to be answered is the mechanism and regulation of the post-invasion DNA synthesis, which my study focused on.
2.1. Introduction

PCNA has been shown to be required in several aspects of HR. Both genetic and biochemical studies have demonstrated the participation of PCNA in post invasion DNA synthesis of HR [82, 197-199, 290].

Early evidence supporting the involvement of PCNA in DNA recombination came from genetic studies using the *S. cerevisiae* MAT-switching system [82, 198]. Wild-type haploid yeast cells are capable of switching mating type between *a* and *α* (the two mating types of haploid yeast cell) by what is essentially a gene conversion event. Switching is initiated by Homothallic (HO) endonuclease cleavage that introduces a DSB that is later repaired by homologous recombination (Figure 2.1) [200]. In one experiment, after HO induced DSBs at MATα and MATa loci, yeast cells carrying a cold temperature sensitive allele of PCNA were incapable of accumulating switched product even 20 h after HO induction at the restrictive temperature of 14°C [82], whereas switching at the permissive temperature of 30°C was nearly 100% efficient.
Figure 2.1. Yeast *S. cerevisiae* mating type switch mechanism.

HO endonuclease creates a site-specific DSB in MAT, which is repaired by gene conversion, using one of two unexpressed donor loci, HMLα and HMRα, to provide the template for new DNA synthesis. Normally, MATa recombines with HMLα and MATα interacts with HMRα, so that the gene conversion replaces approximately 700 bp of sequences[198].

The requirement of PNCA for mating-type switch may not be limited to its interaction with DNA polymerases increasing their processivity, because PCNA was found to be required to synthesize even as little as 30 nucleotides following strand invasion [198]. Therefore, loading of PCNA onto the DNA within the D-loop is likely to serve as a signaling platform necessary for recruiting or signaling the proper DNA polymerase and/or repair proteins to the site of damage.

Consistently, biochemical studies using purified yeast and human proteins indicated that PCNA is required for DNA synthesis following Rad51-mediated strand invasion [197, 199, 290]. One of the biochemical studies using purified yeast proteins showed that
PCNA is specifically required to initiate recombination-associated DNA synthesis at the 3' end of the invading strand in a D-loop [290]. PCNA seems to recruit Pol δ to the D-loop formed by Rad51 mediated strand invasion, allowing efficient recognition of the 3' end of invading strand [197, 290]. A similar observation was made in another study using purified human proteins [199].

![Diagram](image)

**Figure 2.2. Structures of replication and recombination DNA substrate.**

Even though PCNA is required for both DNA recombination and chromosomal DNA replication, the structure of DNA recombination and replication substrates is significantly different (Figure 2.2), leading to the possibility that PCNA loading and function in HR and DNA replication is mechanistically different. The most noticeable difference between the two substrates is the presence of a displaced ssDNA loop in the recombination substrate, but not in the replication substrate. During DNA recombination, Rad51 recombinase nucleates on ssDNA and forms a nucleoprotein filament, promoting the invasion of the 3' single-stranded end into a homologous DNA duplex. The invading strand base pairs with its complementary strand and displaces the non-complementary
strand, forming a three-stranded structure that is termed as “displacement loop” (D-loop) (Figure 2.2). The D-loop of the recombination substrate may have functional implications, e.g. providing a site for the assembly of DNA synthesis machinery including PCNA. Such a displaced ssDNA loop is not present in the replication substrate. DNA synthesis on the lagging strand during chromosomal DNA replication requires a primer that is synthesized by Pol α-primase complex [291]. The polymerase switch from Pol α to Pol δ, necessary for processive DNA synthesis, is believed to be regulated by PCNA [291]. Unlike replication, HR does not need Pol α-primase complex. The 3’ end of the invading strand is utilized as a primer. Therefore, the PCNA mediated polymerase switch that occurs on the lagging strand synthesis is not required in HR.

The protein factors required for chromosomal replication and recombination DNA synthesis are also different. During S-phase replication, the six minichromosome maintenance (MCM) proteins (Mcm2-7 complex) function as a replicative helicase that uses ATP binding and hydrolysis to fuel the unwinding of the template DNA strands at the replication fork [292, 293]. However, a genetic study with an mcm7 temperature sensitive mutant showed that MAT switching occurred normally when the Mcm7 protein was degraded below the level of detection, suggesting the replicative Mcm2-7 helicase is not essential during gene conversion [82]. One related observation is that during DSB repair, the repair efficiency is related to the size of the DNA segment to be copied [294]. As the length of the template for gap repair increases from a few base pairs to 9 kb, the efficiency of repair decreases by four-fold. This observation implies that recombination
DNA synthesis is not intrinsically as efficient or processive as chromosomal replication. One explanation for this result is that repair synthesis lacks the Mcm2-7 helicase, so that DNA polymerase tends to dissociate from the template, as it has to traverse longer distances. Mammalian Pol ε is incapable of displacement synthesis [83], and hence is an unlikely candidate for DNA synthesis during HR. DNA synthesis of the second end is also of the leading strand type in that it can be continuous but does not require displacement synthesis. Based on the differences in DNA structure and the requirement for Mcm2-7 replicative helicase, the DNA synthesis during homologous recombination is mechanistically different from S-phase replication [82].

PCNA loading has been extensively analyzed on replication substrates. In vitro, RFC can load PCNA onto DNA with 5’ junctions (i.e., junctions between the ssDNA template and 5’ end of the primer) and nicked dsDNA in the absence of RPA [295-298]. However, in the presence of RPA, PCNA is uniquely loaded to 3’ junctions i.e., the junction between the ssDNA template and the 3’ end of the primer. RPA directly interacts with RFC to facilitate its specific binding to the 3’-junction, thereby directing RFC to load PCNA onto that specific DNA structure [141, 145, 299, 300]. Important to this study, PCNA loading on the DNA recombination substrate has not been directly observed.

The structures and functions of clamp and clamp loader proteins are highly conserved through evolution reflecting their critical role in DNA metabolism [159]. Besides PCNA and RFC, other clamp and clamp loader complexes have been identified [159] in which
the clamp loaders share four small subunits (Rfc2-5) but have a different large subunit that replaces Rfc1. An example of one such alternative clamp and clamp loader complex is the DNA damage checkpoint clamp (9-1-1 clamp) and its clamp loader (Rad24-RFC complex in *S. cerevisiae* and Rad17-RFC complex in human) [204, 216]. The mammalian 9-1-1 clamp and its loader have been suggested to contribute to some HR reactions, e.g., repair of X-ray induced DSBs and gene targeting [301, 302]. Mammalian *RAD9*−/−, and *RAD17*−/− cells are hypersensitive to a wide variety of DNA-damaging agents, including γ rays, UV exposure, alkylating agents, and chemical cross-linkers [303]. The yeast 9-1-1 complex has been proposed to control the access of translesion DNA polymerases to the 3’ termini of a growing strand [223, 225]. In the absence of a functional 9-1-1 complex, *S. cerevisiae* delays repair of meiotic double-strand breaks (DSBs) which results in an altered ratio of crossover-to-noncrossover products [304]. *In vitro*, the yeast Rad24-RFC complex loads the 9-1-1 complex onto the 5’ end of a primed DNA [159, 295, 305, 306], and similarly, the human 9-1-1 complex can be loaded onto DNA by its specific clamp loader [216, 307]. However, all reported biochemical studies used simple primer-template complex as a DNA substrates.

Despite its importance, PCNA and 9-1-1 clamp loading onto the DNA recombination substrate has never been characterized. Such a study shall provide important information for understanding the regulation of the post invasion DNA synthesis step of HR. To understand the role of PCNA and the 9-1-1 complex in HR, I expressed and purified yeast PCNA, the RFC complex, the 9-1-1 clamp, and the Rad24-RFC complex. Then I
established an in vitro assay system to analyze the loading of 9-1-1 and PCNA on various DNA substrates. Using this system, the loading efficiencies of PCNA on HR intermediates and simple primer-template complex were compared and the effects of RPA, Rad51, and the displaced ssDNA loop on PCNA loading were investigated.

2.2. Material and Methods

2.2.1. Construction of plasmids for PCNA, 9-1-1 clamp, RFC complex, and Rad24-RFC complex expression

Two plasmids (pET11(a)-RFC2,3,4 and pLANT-RFC1,5) for expression of the RFC heteropentamer were generously provided by Dr. John Kuriyan (University of California, Berkeley) [308]. The plasmid for PCNA expression (pET28pp-PCNA) was obtained from the same source. PCNApka is a variant of PCNA that can be labeled by \(^{32}\)P, allowing the detection of trace amount of PCNA loading. To modify pET28pp-PCNA to pET28pp-PCNApka, three repeats of synthetic dsDNA that encodes a cAMP dependent kinase (PKA) recognition site (annealed PKA-F and PKA-R, see Table 1 for sequence) was inserted into NdeI site that is upstream of the original start codon of POL30 of pET28pp-PCNA. For expression of PCNA without the kinase recognition site, pET28-PCNA, which has the POL30 ORF cloned between the Ndel and Xhol sites of pET28a (Novagen), was also constructed.

The 9-1-1 complex of yeast is composed of Rad17, Mec3, and Ddc1 subunits. RAD17 (Ndel and Xhol, fused with a His\(_6\) tag at its C terminus), MEC3 (Ndel and Xhol), and
DDC1 (BamHI and XhoI) ORFs were first cloned into the pET21(a) vector (Novagen). pET21-RAD17 was then digested with NcoI and partially filled with dTTP, dATP, and dCTP using Klenow fragment, followed by digestion with XhoI. The released RAD17 containing fragment was cloned into pESC-URA vector (BamHI-partially filled with dGTP, dATP, dTTP and cut by XhoI). pET21-MEC3 and pET21-DDC1 were digested with BamHI and XhoI and cloned into the same restriction digestion sites of pESC-TRP and pESC-LEU vector, respectively. For 9-1-1pka complex expression, the RAD17 ORF was first cloned into the pET28pp(3pka) vector between the NdeI and NotI sites to generate pET28pp-RAD17pka. The resulting plasmid was digested with BlnI and BglII to release Rad17pka that was then cloned into the pLANT vector to give pLANT-RAD17pka. The Rad24-RFC complex expression system in E.coli is composed of pLANT-RAD24, RFC1 and pET11-RFC2,3,4. The RAD24 ORF was first cloned into pET28pp vector between the NheI and NotI sites to generate pET28pp-RAD24, which was later sub-cloned into pLANT-RFC1, RFC5 between NheI and NotI to replace the Rfc1 subunit and make pLANT-RAD24, RFC5. All clones were sequenced.
2.2.2. Expression and purification of PCNA, PCNA\textsubscript{pka}, the RFC complex, the 9-1-1 clamp, the 9-1-1\textsubscript{pka} clamp, and the Rad24-RFC complex

Rad51 and RPA were prepared as described previously [48, 309]. RFC, the complex of Rfc1-5, was expressed and purified as follows. Freshly transformed \textit{Escherichia coli} BL21(DE3) cells harboring pET11(a)-RFC2,3,4 and pLANT-RFC1,5 were grown overnight at 37°C in 30 ml of LB medium containing kanamycin and ampicillin. Then 25 ml of the culture was inoculated into 2.5 liter of TB broth (10 g/l tryptone, 20 g/l yeast extract, 0.4% glycerol, and 20 mM K-phosphate buffer pH 7.2) and cultivated at 37°C in a BIOFLO 110 Bioreactor system (New Brunswick). When the OD$_{600}$ reached about 0.5, the culture was chilled to 20°C, which normally took about 15 min, and then IPTG was added to a final concentration of 0.5 mM. After RFC production was induced at 20°C for 8 hours, the cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C with a Beckman TS-5.1-500 rotor and stored at -80°C. For purification, the frozen cells were thawed on ice and suspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 5% glycerol, 1000 mM NaCl, 10 mM Imidazole) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). All subsequent steps were carried out on ice or at 4°C. Cells were lysed by sonication (1 min X 3 times, with 1 min interval on ice) using a Branson Sonifier model 250 and cleared by centrifugation at 20,000 rpm for 30 min with JA-25.50 rotor (Beckman). The supernatant was loaded onto an 8 ml Ni-Chelating Sepharose column pre-equilibrated with cell lysis buffer. The column was then washed sequentially with 150 ml of lysis buffer, 50 ml of wash buffer-1 (50 mM Tris-HCl (pH 7.5), 5% glycerol, 1000 mM NaCl, 50 mM Imidazole), and 50 ml of wash buffer-2 (50 mM Tris-HCl (pH
RFC was eluted with elution buffer (50 mM Tris-HCl (pH 7.5), 5% glycerol, 100 mM NaCl, 200 mM Imidazole). The Ni-Sepharose eluate was directly loaded on 5 ml of SP-Sepharose FF (GE-Healthcare) connected to an FPLC system, which was pre-equilibrated with TGEB buffer (30 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM EDTA, and 5 mM β-mercaptoethanol), containing 100 mM NaCl. The column was then subjected to a 40 ml linear gradient from 100 to 500 mM NaCl in TGEB. RFC, which was eluted at about 250-330 mM NaCl, was divided into small aliquots and stored at -80°C.

*E.coli* strain (BL21, DE3) harboring Rad24, Rfc2, Rfc3, Rfc4, Rfc5 expressing plasmids (pLANT-RAD24, RFC5 and pET11(a)-RFC2, 3, 4) was cultivated and induced as described for the RFC complex, except that the protein expression induction was done at 28°C with 0.5 mM of IPTG for 6 hours. After induction, the cells were harvested, purified, and stored as described for the RFC complex.

PCNA with the PKA recognition site (PCNApka) was expressed in BL21 (DE3) containing pET28pp-PCNApka plasmid. Protein expression and Ni-Sepharose fractionation were performed as described in RFC preparation, except that the lysis buffer and wash buffer-1 for PCNA contained 500 mM NaCl. The eluate from the Ni-Sepharose column was directly loaded on 5 ml Q-Sepharose FF (GE-Healthcare) that was pre-equilibrated with TGEB buffer containing 100 mM NaCl. Then the column was subjected to 40 ml linear gradient from 100 to 500 mM NaCl in TGEB. PCNApka was eluted at
about 300-400 mM NaCl from Q-Sepharose. Peak fractions were pooled, divided into small aliquots and stored at -80°C. PCNA without the PKA recognition site was expressed in BL21 (DE3) harboring pET28-PCNA and purified using the same procedure.

The wild-type 9-1-1 clamp was expressed in yeast. Rad17, Mec3, Ddc1 expressing plasmids (pESCUra-RAD17, pESCTrp-MEC3, pESCLeu-DDC1) were introduced into yeast strain BJ5465. Transformants were selected and purified on SCD – UTL plate, and grown in 5 ml SCD – UTL liquid medium. This 5 ml pre-culture was mixed with one liter of SCGal-UTL medium (contained 3% (w/v) galactose) to induce protein expression. After about 48 hours of incubation, cells were harvested at 3500 rpm for 15 min with Beckman TS-5.1-500 rotor and stored at -80°C. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 10% glycerol, 300 mM NaCl) supplemented with 1 mM PMSF and a protease inhibitor cocktail by glass bead beating for 10X30 sec with a 30 sec interval on ice, and cleared two times by centrifugation at 20,000 rpm for 15 min with Beckman JA25.50 rotor. The supernatant was equilibrated to 5 mM imidazole and incubated with 2.5 ml of Ni-Sepharose affinity resin at 4°C for 2 hours. The resin was then sequentially washed with buffer containing 5 mM and 30 mM imidazole (25 ml each), and eluted with 3 ml of buffer containing 200 mM imidazole. The Ni-Sepharose eluate was diluted with buffer without NaCl and loaded onto a 5 ml Heparin column pre-equilibrated with TGEB buffer (30 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, and 5 mM β-mercaptoethanol), containing 100 mM NaCl. After loading, the column was washed with the loading buffer until the OD280 reached the baseline. The bound protein was then
eluted with a 40 ml linear NaCl gradient (100 mM to 500 mM). The 9-1-1 complex was eluted between 350 mM and 480 mM NaCl. The 9-1-1pka complex was expressed and purified as described for Rad24-RFC complex.

Concentrations of Rad51 and RPA were determined using extinction coefficients of 1.29 x 10^4 and 8.8 x 10^4 M^-1 at 280 nm, respectively. Concentrations of the other proteins were determined using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific).

2.2.3. DNA substrates for clamp loading assays

Structures of the synthetic DNA substrates used in this study are summarized in Figure 2.3. Synthetic oligonucleotides (sequences in Table 1) were purchased from Invitrogen and purified by polyacrylamide gel electrophoresis, followed by ethanol precipitation. To generate the synthetic D-loop substrate “DL” and control substrate “C2”, equal molar concentrations (1 µM) of oligonucleotides (TSO317, 318, 319 for DL and TSO318, 319 for C2, Table 2) were mixed in buffer containing 30 mM Hepes-Na (pH 7.5) and 5 mM MgCl₂. The mixtures were incubated at 95°C for 5 min and then 80°C for 30 seconds in a heat block. Then the heat block was removed from the incubator and left at room temperature for 2 hours to allow annealing. The DL and C2 substrates were then stored at 4°C until use. Derivatives of DL (DL-L, DL-R, DL-CAP, DL2-L, DL2-R, Figure 2.3) were generated in the same way, using oligonucleotides listed in Table 2.
Figure 2.3. Structure of synthetic DNA substrates used in this study.
The structure of different synthetic DNA substrates used for clamp loading assays.
Numbers are the length of DNA substrate in oligonucleotides.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KpnI-F</td>
<td>TTGGGTACCGGG</td>
</tr>
<tr>
<td>KpnI-R</td>
<td>CCCGGTACCCAA</td>
</tr>
<tr>
<td>PKA-F</td>
<td>TAGTCTTAGACGAGCTTCTGTGCA</td>
</tr>
<tr>
<td>PKA-R</td>
<td>TATGCACAGAGCTCGTCTAAGAC</td>
</tr>
<tr>
<td>TSO-236</td>
<td>TGGTTTAGTACTCACCAGTCAAGAAAAAGCATCTTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCGCTGGTACCC</td>
</tr>
<tr>
<td>TSO-237</td>
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Table 2. Synthetic oligonucleotides used to produce substrates for clamp loading.

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<th>Substrate</th>
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</tr>
<tr>
<td>C3</td>
<td>TSO354, TSO365</td>
</tr>
</tbody>
</table>

2.2.4. Clamp loading analyzed by gel filtration

Purified PCNApka was labeled using γ-32P-ATP and cAMP dependent kinase, thereby allowing the detection of a trace amount of PCNA loading. 5 pmol of PCNApka was labeled using 750 unit of cAMP dependent kinase (New England Biolabs) in a 20 µl reaction mixture containing 20 mM Hepes-Na (pH 7.5), 1 mM DTT, 12 mM MgCl₂, 0.1 M NaCl, and 5 µCi of γ-32P-ATP (3,000 Ci/mmol, Perkin Elmer) at 37°C for 20 min. Then, free γ-32P-ATP was removed by passage through a G-25 spin column. In the complete loading reaction, primed Bluescript ssDNA (1 pmol) was first incubated with RPA (29.5 pmol) at 30°C for 5 min in loading buffer containing 25 mM Hepes-Na (pH 7.5), 50 mM NaCl, 1 mM DTT, 5 mM MgCl₂, 0.05 mg/ml BSA, and 1 mM ATP. 32P-PCNA (1 pmol) and RFC (4 pmol) were then added to the reaction to carry out PCNA loading and incubated at 30°C for 5 min. The reaction mixture (20 µl) was loaded onto a Sephacryl S-400 gel filtration column (~1 ml bed volume, 30 cm in length, and 3 mm inner diameter). Gravity flow was used to run the sample through the column (~1.5 min/drop), and one-drop fractions were collected. Column fractions containing labeled PCNApka were identified using a liquid scintillation analyzer (Packard, Model 1900TR).
Fractions were then analyzed by a 12% SDS-PAGE dried on DE81 chromatography paper (Whatman) under vacuum and the labeled products were visualized by a Personal Molecular Imager FX (Bio-Rad).

2.2.5. Clamp loading analyzed by affinity pull down

Pull-down experiments with immobilized DNA were carried out as follows. Biotinylated TSO318 (90 mer) was annealed with TSO319 (29 mer) to make Biotin-C2 substrate (see Figure 2.3). Streptavidin beads (Bangs Laboratories Inc; 2 µl) were incubated with the C2 substrate (0.2 pmol) in buffer containing 25 mM Hepes-Na (pH 7.5), 50 mM NaCl, and 5 mM MgCl₂, for 30 min on ice to allow the DNA substrate to bind to the beads. The reaction mixture was centrifuged at 6,000 RPM for 5 min and the supernatant was carefully removed. RPA (0.4 pmol) and ATP (1 mM) were added to the reaction and incubated at 30°C for 5 min in clamp loading buffer (25 mM Hepes-Na (pH 7.5), 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.05 mg/ml BSA, and 0.025% NP-40). RFC (0.4 pmol) and PCNA (1 pmol) were then added to the reaction to start PCNA loading and incubated at 30°C for 30 min. After incubation, the reaction mixture was centrifuged at 6,000 RPM for 5 min. The supernatant was carefully removed and saved for analysis. The beads were washed with 50 µl of wash buffer three times. Then 20 µl of TE buffer was added followed by 4 µl of 6XSDS sample loading buffer to release protein from the DNA and beads. The pellet fraction and 5% of the supernatant were loaded on a 12% SDS-PAGE, dried on DE81 chromatography paper (Whatman) under vacuum, and the labeled products were visualized by a Personal Molecular Imager FX (Bio-Rad).
Pull-down experiments with immobilized PCNA were carried out as follows. The oligonucleotide TSO319 was labeled with $^{32}$P using T4 polynucleotide kinase and $\gamma$-$^{32}$P-ATP, and then annealed with TSO318 (for C2) or with both TSO317 and 318 (for DL, Figure 2.3). In a 20 µl reaction, the DNA substrate (1 pmol of DL or C2) and RPA (4 pmol) were incubated for 4 min at 30°C in binding buffer (30 mM Hepes-Na (pH 7.5), 5 mM MgCl$_2$, 1 mM DTT, 0.2 mg/ml BSA) in the presence or absence of 1 mM ATP as indicated. The NaCl concentration was adjusted to 100 mM prior to the PCNA loading reaction. RFC (0.4 pmol) and PCNA (1 pmol) were then added to carry out PCNA loading at 30°C for 4 min. Then, 10 µl (50% slurry) of His-select (Sigma-Aldrich) beads (prewashed by 1X binding buffer) was added to the mixture, which was then incubated on ice for 10 min with gentle mixing by pipetting every 5 min. Then the reaction mixture was centrifuged at 1,000 RPM for 3 min, the supernatant was carefully removed and saved for analysis. The resin was washed with 400 µl of wash buffer (30 mM Hepes-Na (pH 7.5), 100 mM NaCl, 5 mM MgCl$_2$, and 20 mM imidazole) three times; 20µl of 1X SDS loading buffer was added to release the bound protein from the resin. The amount of DNA that was associated with the immobilized PCNA was quantified using a liquid scintillation analyzer (Packard, Model 1900TR). Immobilized PCNA was then then resolved on a 12% SDS-PAGE.

2.2.6. Clamp loading analyzed by non-denaturing PAGE

The DNA substrate (1 pmol of C2 or DL) was incubated with RPA (4 pmol) in the presence or absence of ATP (1 mM) at 30°C for 5 min in clamp loading buffer (25 mM
Hepes-Na (pH 7.5), 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA, and 0.1% NP-40). RFC (0.4 pmol) and PCNA (1 pmol) were then added to the reaction to start PCNA loading. After incubation at 30°C for 15 min, the reaction products were separated by 8% non-denaturing PAGE. The gel was dried on DE81 chromatography paper (Whatman) under vacuum and the labeled products were visualized using a Personal Molecular Imager FX (Bio-Rad).

2.2.7. Clamp loading analyzed by SDS-agarose gel mobility shift assay
Phosphorylated PCNApka (³²P-PCNA, as described in section 2.2.4) was kept on ice and directly used for loading experiments. PCNA loading was analyzed essentially as described previously [310]. In standard reactions, plasmid DNA (200 ng) or synthetic DNA substrate (2.5 pmol) was incubated with RPA (50 pmol or 10 pmol, respectively) in 20 mM Hepes-Na (pH 7.5), 1 mM DTT, 10 mM MgCl₂, 0.2 mg/ml BSA, and 1 mM ATP for 5 min on ice. Then 2 pmol of RFC and 0.5 pmol of ³²P-PCNA were added to the reaction in a final volume of 25 µl. The mixtures were incubated at 37°C for 3 min, mixed with glutaraldehyde to a final concentration of 0.1% (w/v), and further incubated for 10 min at 37°C. The reaction was stopped by chilling on ice and mixed with 7.5 µl of TBE dye (50% glycerol, 0.05% bromophenol blue in 0.5x TBE). Products were separated by electrophoresis through 0.8% (for plasmid substrates) or 2% (for synthetic substrates) agarose gels in 0.5x TBE buffer containing 0.01% SDS. The gels were dried on DE81 chromatography paper (Whatman) under vacuum and the labeled products were
visualized by a Personal Molecular Imager FX (Bio-Rad). The amounts of free and loaded $^{32}$P-PCNA were quantified by using ImageLab software (Bio-Rad).

### 2.3. Results

#### 2.3.1. PCNA and RFC expression and purification

The RFC complex is composed of five subunits-Rfc1, Rfc2, Rfc3, Rfc4, and Rfc5. To purify the functional RFC complex in a large amount, I tried to overexpress all of the five subunits together, one of which (Rfc1) was tagged with a His$_6$ affinity tag for efficient purification. Originally, I designed the RFC complex to be expressed in *S. cerevisiae*, as full length proteins. However, this attempt was not successful probably because, as already reported [308, 311], the N-terminus of the largest subunit, Rfc1, is susceptible to proteolytic attack in the process of induction and purification. To overcome this problem, I obtained plasmids expressing a truncated Rfc1 (amino acids 295-785) and full length Rfc2, Rfc3, Rfc4, and Rfc5 in *E. coli*, from Dr. John Kuriyan (University of California). The truncated Rfc1 is expressed as a fusion protein with a His$_6$ tag on its N-terminus. The expression of the RFC complex in *E. coli* utilized two plasmids. One plasmid, pLANT-RFC1,5, contained RFC1 and RFC5 genes on the pLANT expression vector. The other plasmid, pET11(a)-RFC2,3,4, contained RFC2, RFC3, and RFC4 genes on pET11(a) expression vector. The expression of each of these genes is under the control of an inducible T7 promoter. The pLANT vector contains a p11A replication origin, which is compatible with the CoIE1 replication origin of pET11 vector. The pLANT vector also
contains a RIL region, which encodes rare tRNAs that facilitate the expression of eukaryotic proteins in *E. coli* system.

The two plasmids were introduced into *E. coli* strain BL 21 (DE3) and co-transformants (Ap\(^R\) and Km\(^R\)) were isolated and used for RFC complex overexpression (see Materials and Methods for more details). When the culture reached OD\(_{600}\) of about 0.6, expression of the RFC complex was induced by addition of 0.5 mM (final concentration) of IPTG for 8 hours at 20°C. Cells were harvested, lysed, and separated into pellet and supernatant fractions by centrifugation. SDS-PAGE of these fractions (Fig. 2.4) indicated that proteins with sizes corresponding to some Rfc subunits were clearly made. Even though a large amount of the four smaller subunits (Rfc2, Rfc3, Rfc4, and Rfc5) was an insoluble (Figure 2.4, pellet lane), a significant amount of the Rfc subunits was detected in the supernatant of the cell lysate (Figure 2.4, supernatant lane). Rfc1 expression was not clearly detected by comparing the uninduced and induced sample. This might be because of the relatively higher molecular weight of Rfc1, making the expression of this subunit more difficult than smaller subunits.

The supernatant fraction was then subjected to Ni-Sepharose affinity chromatography. Protein bands with molecular weight corresponding to Rfc2-5 subunits were clearly present in the eluate from the Ni-affinity column (Fig. 2.4). Since only Rfc1 was His-tagged, this confirmed that His\(_6\)-Rfc1 was made and complexed with the other RFC subunits. To further confirm the expression of Rfc1, a Western blot analysis using
HisProbe-HRP (Thermo Scientific) was carried out. HisProbe-HRP is a nickel activated derivative of horseradish peroxidase (HRP) that enables direct detection of His$_6$-tagged proteins in Western blots. The Ni-eluate from above gave a positive signal at the molecular weight corresponding to Rfc1 (Figure 2.5). The other samples did not give any signal at the size of Rfc1. This was likely due to the very low expression level of Rfc1. Only after an enrichment procedure by affinity purification, did Rfc1 become detectable under the experimental conditions used.

**Figure 2.4. RFC complex expression and purification by Ni Sepharose affinity chromatography.**
Rfc1, Rfc2, Rfc3, Rfc4, Rfc5 was co-expressed in *E. coli*. Harvested cells were lysed by sonication and the lysate was cleared by centrifugation at 20,000 RPM for 30 min, 4°C. The supernatant was loaded on a Ni-Sepharose affinity column. The column was washed with the binding buffer contained 10 mM imidazole and the bound proteins were eluted with 200 mM imidazole. Different fractions of the purification procedure were analyzed by 12% SDS-PAGE. The predicted molecular weights of the five subunits of RFC complex are: 58kD (truncated Rfc1), 39 kD(Rfc2), 38 kD(Rfc3), 36 kD(Rfc4), 40 kD(Rfc5), respectively. As a control, cell lysate from the *E.coli* cells transformed with the same plasmids but not induced with IPTG was loaded (uninduced).
Ni-affinity chromatography purification is an efficient method to purify a large amount of His$_6$-tagged recombinant protein. However, some native proteins from the expression host are also histidine rich, so they often bind to the affinity resin during the Ni-affinity purification procedure. The presence of these contaminating proteins can be reduced by extensive washing with buffer containing a relatively higher concentration (20-50 mM) of imidazole, which may also release some of the His$_6$ tagged protein from the resin. To determine the optimal imidazole concentration for RFC complex purification, an imidazole step gradient elution test was conducted (Figure 2.6). After protein binding, buffers containing 50, 75, 100, 125, 150, 200, and 250 mM imidazole were used to elute bound protein from the Ni affinity resin. With 50 mM imidazole, approximately 50% of the smaller subunits (Rfc2, Rfc3, Rfc4, and Rfc5) were eluted from the resin, together with majority of contamination proteins. When imidazole concentration reached 150 mM, almost all the proteins bound to the resin were eluted. There was no visible protein band in 200 mM and 250 mM imidazole eluted fractions on coomassie blue stained SDS-PAGE gel (Figure 2.6).
Figure 2.5. Western Blot analysis of RFC complex expression.
Fractions of RFC complex Ni-Sepharose affinity purification were analyzed by Western blotting to confirm the expression of His\textsubscript{6}-Rfc1. The same samples in Figure 2.4 were probed by HisProbe, which directly recognizes His\textsubscript{6} peptide. Rfc1 signal and molecular mass standards are indicated. There were prominent signals in the Marker lane (75 kD and 100 kD markers). These crossreaction occurred probably because these molecular weight standard were purified as His\textsubscript{6} tagged recombinant proteins.
Figure 2.6. RFC complex purification by Ni Sepharose affinity chromatography with step gradient of imidazole.

RFC complex was expressed as described in Material and Method section of this chapter. The cell lysate (supernatant) was loaded on Ni-Sepharose affinity resin in the presence of 10 mM imidazole, followed by extensive wash with binding buffer. The bound proteins were eluted with buffer (3 times of bed volume) containing different concentration of imidazole as indicated. Fractions were analyzed with 12% SDS-PAGE.

However, this SDS-PAGE analysis did not clearly indicate the imidazole concentrations at which Rfc1 was eluted. Therefore, I carried out a Western blot analysis with HisProbe to determine the elution profile of the Rfc1 subunit (Figure 2.7). The result indicated that the Rfc1 signal started to show up in the 75 mM imidazole fraction. A protein with very similar mobility to Rfc1 on SDS-PAGE (Fig. 2.6) in the 50 mM imidazole fraction did not cross react with HisProbe, suggesting it was a contaminating protein. This information was important to establish a large scale RFC complex purification protocol, because the wash with 50 mM imidazole can eliminate most of the contaminating proteins but retain the majority of complete RFC complex.
Figure 2.7. **Western blot analysis of Ni-sepharose affinity chromatography.** Fractions containing different imidazole concentrations were probed by HisProbe to detect His$_6$-Rfc1.

The purification was then repeated on a larger scale (Figure 2.8). After cell lysis by sonication and centrifugation, the supernatant was loaded on to an 8 ml Ni affinity column equilibrated with binding buffer containing 10 mM imidazole. After loading, the column was washed extensively with the same binding buffer and then washed with wash buffer containing 50 mM imidazole. The RFC complex was eluted from the Ni affinity resin with buffer containing 200 mM imidazole. Even though a significant amount of the Rfc2-5 subunits were lost during the wash step, a relatively pure five subunit RFC complex was eluted from the resin.

To further purify the RFC complex, the eluate from Ni-Sepharose was separated by SP-Sepharose ion exchange chromatography (Figure 2.9). The majority of contaminating proteins and some Rfc subunits did not bind and were found in the flow through fraction.
Then, bound proteins were eluted using the linear gradient of NaCl. The RFC complex was purified to near homogeneity in the fractions containing 250-330 mM NaCl (Figure 2.9, fractions 9-14).

![Figure 2.8. Large scale RFC complex purification by Ni-Sepharose affinity chromatography.](image)

*E. coli* strain expression RFC complex was cultivated, induced and harvested as described in the Material and Method section of this chapter. The supernatant was loaded on Ni-Sepharose column in the presence of 10 mM imidazole, followed by extensive washing with the same binding buffer until the OD280 absorbance back to the baseline. The resin was then washed with buffer containing 50 mM imidazole and eluted with buffer containing 200 mM imidazole. Fractions were analyzed by 12% SDS-PAGE. The eluate was saved for further purification by ion exchange chromatography.
Figure 2.9. RFC complex purification by SP-Sepharose ion exchange chromatography.

Eluate from Ni-Sepharose affinity chromatography containing 100 mM of NaCl was directly loaded on a 5 ml SP-Sepharose cation exchange column, followed by extensive wash with the binding buffer until the UV$_{280}$ reached the baseline. The bound proteins were eluted with a linear gradient from 100 to 500 mM of NaCl. The eluate was collected in 2 ml fractions and analyzed by 12% SDS-PAGE. Fractions 12, 13, and 14 were pooled, divided into small aliquots, and stored at -80°C for future biochemical assays.

The PCNApkα expression plasmid (pET28pp-PCNApkα) was constructed to express a variant of wild-type PCNA, which can be labeled by γ-$^{32}$P-ATP using cAMP-dependent protein kinase (NEB) in an in vitro phosphorylation reaction. The radiolabeled PCNA has a great advantage in clamp loading assays, because it provides for very sensitive detection of even a trace amount of loading. To introduce the kinase recognition site, I
obtained a plasmid pET28pp-PCNA from Dr. John Kuriyan of University of California, which had an NdeI site at the upstream of the start codon of PCNA open reading frame. Three repeats of a double-strand DNA fragment encoding a protein kinase recognition site (RRXS/TY, where X can be any common amino acid and Y tends to be a hydrophobic residue) were inserted into the NdeI site. Upstream of the kinase recognition site, a His$_6$ tag was fused in frame with the PCNApka gene to allow affinity purification by Ni-Sepharose. The generated plasmid (pET28pp-PCNApka) was introduced into E. coli BL21 (DE3). The cultivation and induction procedure were essentially the same as that of the RFC complex. PCNApka was expressed at very high level in soluble form after 0.5 mM IPTG induction at 20°C for 8 hours. The harvested cells were lysed by sonication, cleared by centrifugation, and PCNApka partially purified by Ni-Sepharose (Figure 2.10). After Ni-affinity purification, PCNApka was about 95% homogenous. The eluate from Ni-Sepharose column was subjected to further purification by Q-Sepharose ion exchange chromatography. PCNApka was eluted at about 300-400 mM NaCl from Q-Sepharose. Peak fractions were pooled, divided into small aliquots, and stored at -80°C.
Figure 2.10. PCNApka purification by Ni affinity chromatography.

A. BL21(DE3) harboring pET28pp-PCNApka was cultivated at 37°C until the OD$_{600}$ reached ~0.6. Then PCNApka was induced with IPTG at 20°C for 8 hours. Harvested cells were lysed by sonication and cleared by centrifugation. The supernatant was loaded on a Ni-Sepharose affinity purification column. The column was washed with the binding buffer contained 10 mM imidazole and the bound proteins were eluted with 200 mM imidazole. Fractions of the purification procedure were analyzed by 12% SDS-PAGE. The predicted molecular weight of PCNApka protein is 29 kDa. Ni-FT stands for Ni-affinity purification flow through. B. Purified PCNA and PCNApka were loaded on lanes 2 and 3.

2.3.2. Rad24-RFC and 9-1-1 complex purification

Rad24-RFC is the clamp loader of the yeast 9-1-1 sliding clamp complex (Rad17-Mec3-Ddc1 complex). Because Rad24-RFC and the RFC complex share four subunits (Rfc2-5), Rad24-RFC expression system was constructed based on the RFC complex expression system. Essentially, theRFC1 gene in the pLANT-RFC1,5 plasmid was replaced by the full length RAD24 gene with a His$_6$ tag on its N-terminus. During a small scale induction and purification test, the Rad24 complex was found to be less stable than the RFC
complex, likely due to the expression of Rad24 as a full length protein. Therefore, Rad24 complex expression was induced at 28°C for 6 hours, a relatively shorter time compared with induction of the RFC complex. Harvested cells were lysed by sonication and the Rad24 complex was purified by Ni affinity purification (Figure 2.11) as described for the RFC complex. Both the expression level and the amount of soluble protein were significantly lower than that of RFC complex.

Figure 2.11. Rad24-RFC purification by Ni Sepharose affinity chromatography. E.coli strain (BL21, DE3) that was harboring Rad24, Rfc2, Rfc3, Rfc4, Rfc5 expressing plasmids (pLANT-Rad24, RFC5 and pET11(a)-RFC2, 3, 4) was cultivated, induced for protein production and RFC complex was purified by affinity chromatography as described in the Material and Methods section. Fractions of the purification procedure were analyzed by 12% SDS-PAGE. The predicted molecular weights of His6-tagged Rad24 subunit is 78 kDa.

To further purify the Rad24-RFC complex, the Ni eluate was loaded onto SP-Sepharose ion-exchange column (Figure 2.12). The Rad24-RFC complex eluted in multiple fractions. Because Rad24-RFC was expressed at a relatively low level and was diluted
during ion exchange chromatography, the fractions were pooled and concentrated by Amicon Ultra filtration (Figure 2.13).
To express the 9-1-1 complex in *S. cerevisiae* the *RAD17*, *MEC3*, and *DDC1* genes were cloned into pESC-URA (between NdeI and XhoI sites), pESC-TRP (between BamHI and XhoI sites), and pESC-LEU (between BamHI and XhoI sites), respectively. The Rad17 subunit was tagged with His$_6$. The other subunits were untagged. These three plasmids were introduced into yeast and expression of the 9-1-1 complex was induced by galactose addition. About 6 grams of wet cells were harvested from one liter of induced culture. The cell were lysed by glass-bead beating, the supernatant was cleared by centrifugation and subjected to Ni-affinity chromatography. After Ni affinity purification, multiple protein bands were identified in the Ni-eluate fraction (Figure 2.14). Three of these bands correspond to the predicted molecular weights of Rad17, Mec3, and Ddc1 proteins. Western blot using the HisProbe confirmed the expression of His$_6$ tagged Rad17 (Figure 2.15). As shown in the coomassie blue stained SDS-PAGE, the Rad17 band was thicker.
than bands of the other two subunits. This is probably because Rad17 is the only subunit having the affinity purification tag and every Rad17 protein molecule may not be complexed with Mec3 and Ddc1. This observation also suggested the necessity of further purification by other chromatography procedure to isolate the complete functional three subunits complex.

The 9-1-1 complex in the Ni-Sepharose eluate was further purified by Heparin affinity chromatography (Figure 2.16). During elution with a NaCl gradient, excess Rad17 monomer was eluted at a lower NaCl concentration (fractions #28-34) and therefore separated from the complex. Band intensity on a coomassie stained SDS-PAGE gel indicated that the ratio of Rad17, Mec3, and Ddc1 subunits in the fractions #36-41 was approximately 1:1:1, suggesting formation of the three protein complex. The peak fractions from Heparin purification were pooled and stored for later biochemical analysis.

In order to detect the loading of the 9-1-1 complex onto a DNA substrate, a modified form of the 9-1-1 complex (9-1-1pka) was also produced, in which a cAMP-dependent protein kinase recognition site was inserted between the His6 tag and the start codon of the RAD17 open reading frame (RAD17pka). Then, RAD17pka was cloned between the BglI and BglIII site of pLANT vector. MEC3 and DDC1 genes were cloned on pET21 expression vector. Both plasmids were introduced into E.coli to overexpress the 9-1-1pka complex. The 9-1-1pka complex was purified using essentially the same procedure as that of Rad24-RFC purification (Figure 2.17). However, the recovery of the protein was
very low (Figure 2.17, Ni-Eluate), mainly because of the low solubility of the Rad17pka subunit. The 9-1-1pka was further purified by Heparin affinity chromatography (Figure 2.18). Somehow the protein complex was concentrated during the procedure. The peak fractions of Heparin purification gave an approximately 1:1:1 ratio of the three subunits of the complex, suggesting the formation of functional complex.

![Image of gel electrophoresis](image.png)

**Figure 2.14. The 9-1-1 complex expressed in yeast and purified by Ni-Sepharose affinity chromatography.**

Rad17, Mec3, Ddc1 expressing plasmids (pESCU-Rad17, pESCT-Mec3, pESCL-Ddc1) were introduced into yeast strain BJ5465. The cultivation and induction of the strain and the purification of induced protein are described in detail in the *Material and Methods* section.
Figure 2.15. Western Blot analysis of His6-Rad17 subunit in Ni Sepharose fractions.
Fractions of Ni-Sepharose affinity chromatography were analyzed by Western blot using
HisProbe that directly recognizes His6 tagged proteins. Strong signal in Ni-eluate was
observed (Rad17). Signals in the marker lane were likely due to the cross-reaction of
HisProbe with His6 tagged recombinant marker proteins.
Figure 2.16. Heparin affinity chromatography of the 9-1-1 complex. The eluate of Ni-Sepharose chromatography was directly loaded on a 5 ml heparin affinity chromatography column, followed by extensive wash with the binding buffer until the absorbance at 280nm reached the baseline. The bound proteins were eluted with a linear gradient from 100 to 500 mM NaCl. The eluate was collected in 1 ml fractions and analyzed by 12% SDS-PAGE. Fractions 38, 39, and 40 were pooled, divided into small aliquots, and stored at -80°C.
Figure 2.17. The 9-1-1pka complex was expressed in *E. coli* and purified by Ni Sepharose affinity chromatography.

Rad17pka, Mec3, Ddc1 were overexpressed in *E. coli* strain (BL21, DE3) to produce the 9-1-1pka complex. The cells were cultivated at 37°C until the OD<sub>600</sub> reached about 0.6, then the proteins were induced by 0.5 mM IPTG at 28°C for 6 hours. Harvested cells were lysed by sonication and cleared by centrifugation at 20,000 RPM for 30 min at 4°C. The supernatant was loaded on a Ni-Sepharose affinity purification column. The column was washed with binding buffer contained 10 mM imidazole and the bound proteins were eluted with buffer containing 200 mM imidazole. Fractions of the purification procedure were analyzed by 12% SDS-PAGE.
Figure 2.18. Heparin affinity chromatography purification of the 9-1-1pka complex. Eluate from Ni-Sepharose chromatography was loaded on a 5 ml heparin affinity chromatography column, followed by extensive wash with the binding buffer until the UV$_{280}$ reached the baseline. The bound proteins were eluted with a linear gradient from 100 to 500 mM NaCl. The eluate was collected in 2 ml fractions and analyzed by 12% SDS-PAGE. Fraction #10 was collected and concentrated by Amicon ultra-filtration, divided into small aliquots, and stored at -80°C.
Figure 2.19. PCNApka labeling by T4 protein kinase with $\gamma^{\text{32P}}$-ATP.

Purified PCNApka (5 pmol) was incubated with 750 unit of cAMP dependent kinase in a 20 µl reaction mixture containing 20 mM Hepes-Na (pH 7.5), 1 mM DTT, 12 mM MgCl$_2$, 0.1 M NaCl, 5 µCi of $\gamma^{\text{32P}}$ ATP (3,000 Ci/mm, Perkin Elmer) at 37°C. Aliquots were retrieved at time points indicated. The samples were analyzed by a 12% SDS-PAGE and the gel was dried on DE81 chromatography paper (Whatman) under vacuum and the labeled products were visualized by exposing to an X-ray film. As a control, PCNA without kinase recognition site was treated under the same procedure.

2.3.3. Clamp loading analyzed by gel filtration.

PCNApka was labeled with $^{32}$P to make a sensitive, quantitative loading assay. Because PCNApka was fused with a protein kinase recognition site at its N terminus, it can be labeled by $^{32}$P using cAMP-dependent protein kinase (PKA) *in vitro*. To do this, purified PCNApka was incubated with $\gamma^{\text{32P}}$-ATP and PKA as indicated in the manufacturer’s instructions. To determine the optimal incubation time for PCNA labeling, a reaction time course was done. PCNApka or PCNA (as a control) was incubated with $\gamma^{\text{32P}}$-ATP and PKA, and samples were taken after 15, 30, 45, 60 min and separated by SDS-PAGE.
The dried SDS-PAGE gels were exposed to an x-ray film to visualize radioactive signal (Figure 2.19). Due to the lack of a kinase recognition site, wild-type PCNA was not phosphorylated by the kinase. In the reaction containing PCNApka, a radioactive signal corresponding to the molecular weight of PCNApka was detected. The signal strength was similar in the 15 min to 60 min samples, suggesting that 15 min of incubation was sufficient for the labeling reaction.

Gel filtration chromatography separates molecules by sizes. Large molecules are excluded from the pores of the gel filtration resin, thus eluting in early fractions. Smaller molecules are included in the pores and migrate more slowly during chromatography. Since the resin has pores with a wide variety of sizes, the smaller the molecule is, the later it is eluted from the column. The molecular masses of PCNA and Bluescript ssDNA are approximately 29 kDa and 990 kDa, respectively. Bluescript ssDNA is too large to be included in the Sephacryl S-400 gel filtration resin (separation range for globular proteins is $2 \times 10^4$–$8 \times 10^6$ Dalton). Therefore, Bluescript ssDNA should elute in the excluded volume. Because PCNA is small enough to be included in the resin, it should be eluted in later fractions. If PCNA is loaded on the DNA, labeled signal should be observed in the excluded volume. As a test, I first loaded a simple mixture of primed Bluescript ssDNA (with multiple primers among which TSO237 was labeled with $^{32}$P) and free $\gamma$-$^{32}$P-ATP on the column (Figure 2.20). Two separate radioactive peaks were observed, indicating that the ssDNA template and ATP were separated. The peak at fraction 17/18
corresponded to the DNA substrate. The other peak at fraction 34/35 corresponded to the free $\gamma^{\cdot}$-32P-ATP.

**Figure 2.20. Sephacryl S-400 gel filtration analyses of $32^P$-ssDNA and free $\gamma^{\cdot}$-32P-ATP.**

Bluescript ssDNA (circular) was annealed with oligonucleotides, TSO237, TSO236, pJN58R, and the T3 promoter primer. The TSO237 primer was labeled with $32^P$. The annealed ssDNA (0.4 pmol) and 0.5 µl (5 µCi) of free $\gamma^{\cdot}$-32P-ATP were loaded onto a S400 column (~1 mL bed volume, 30 cm in length, and 3 mm inner diameter). Each drop of eluate (about 50 µl) was separated as a fraction. Radioactivity of each fraction was measured by a liquid scintillation analyzer (Packard, Model 1900TR). The radioactivity (counts per minute) was plotted against fraction number.

Next, as a control, free PCNApka was first labeled with $32^P$ as described above, and then loaded onto the same S-400 column. A radioactive signal was observed around fraction 29 (Figure 2.21, B). Corresponding fractions were collected and analyzed by SDS-PAGE and autoradiography (Figure 2.21, A). The band intensities of $32^P$-PCNA on the gel and the cpm measured by the scintillation analyzer were parallel (comparing Figure 21, A and B), indicating that the radioactivity peak observed was generated by $32^P$-PCNA.
Figure 2.21. Sephacryl S-400 gel filtration analysis of $^{32}$P-PCNA.
Purified PCNApk was labeled with $^{32}$P with PKA in vitro. Then, free $\gamma$-$^{32}$P-ATP was removed by passing through a G-25 spin column (GE Healthcare). $^{32}$P-PCNA was diluted to 50 nM final concentration and was loaded onto Sephacryl S-400 gel filtration column, and drop fractions were collected. A. Fraction 22-31 was analyzed by a 12% SDS-PAGE and the gel was dried on DE81 chromatography paper (Whatman) under vacuum and the labeled products were visualized by a Personal Molecular Imager FX (Bio-Rad). B. The radioactivity of each fraction was measured by a liquid scintillation analyzer (Packard, Model 1900TR).

Then, I carried out the PCNA loading reaction and tried to detect the PCNA-DNA complex by S-400 gel filtration assay (Figure 2.22). $^{32}$P-PCNA and the Bluescript ssDNA that was hybridized with multiple synthetic oligonucleotides were incubated with RFC clamp loader and ATP for 5 min at 30°C. After the reaction, the entire mixture was loaded on the S-400 gel filtration column for separation. Collected fractions were
analyzed for radioactivity (Figure 2.22, B) and resolved through 12% SDS-PAGE gels to visualize $^{32}\text{P}$-PCNA (Figure 2.22, A). The free $^{32}\text{P}$-PCNA peak was clearly identified at around fraction 30 (Figure 2.22, B). However, a peak corresponding to the $^{32}\text{P}$-PCNA-DNA complex (fraction fraction #10-20) was not clear (Figure 2.22, B). Consistently, autoradiography of the SDS-PAGE gel showed that only little $^{32}\text{P}$-PCNA was eluted in the early fractions (Figure 2.22, A, top panel).

Figure 2.22. $^{32}\text{P}$-PCNA loading onto plasmid based substrate analyzed by Sepahcryl S-400 gel filtration.

Purified PCNApk was labeled with $^{32}\text{P}$ to generate $^{32}\text{P}$-PCNA. PCNA loading and gel filtration were performed as described in Material and Methods. A. Fractions 10-19 and 26-35 were analyzed by a 12% SDS-PAGE and the gel was dried on DE81 chromatography paper (Whatman) under vacuum and the labeled products were visualized by a Personal Molecular Imager FX (Bio-Rad). B. Radioactivity of fractions collected.
One possible reason of the apparent inefficient PCNA loading was protein degradation during incubation period. To test this possibility, the same PCNA loading reaction and the gel filtration were carried out and the fractions were analyzed by SDS-PAGE. The gels were first stained with coomassie blue to visualize all proteins (Figure 2.23, bottom panel). The RFC complex and RPA were identified in fractions around 15, 16, and 17, whereas no $^{32}$P-PCNA was observed in these fractions. $^{32}$P-PCNA was detected in fractions 23-32 using SDS-PAGE, matching the radioactivity signal from autoradiography (Figure 2.23, top panel). According to the result shown in Figure 2.23, proteins, including the RFC complex, $^{32}$P-PCNA, and RPA, were not degraded during the incubation for clamp loading, suggesting that one or more of these proteins was not biologically active, or that the PCNA on the DNA was not stable enough to be detected under these experimental conditions.
Figure 2.23. $^{32}$P-PCNA loading onto plasmid based substrate analyzed by Sephacryl S-400 gel filtration, and comparison with corresponding SDS-PAGE fractions.

$^{32}$P-PCNA was incubated with primed Bluscript ssDNA, RPA, and RFC under the same conditions shown in Figure 2.22, and the reaction mixture was loaded on a Sephacryl S-400 gel filtration column. Fraction 6-41 was separated through 12% SDS-PAGE and stained by Coomassie Brilliant blue. The gel was then dried on DE81 chromatography paper (Whatman) under vacuum (bottom panels) and the labeled products were visualized by a Personal Molecular Imager FX (Bio-Rad) (top panels).

PCNA loading is a multistep biochemical process dependent on the ATPase activity of RFC complex. Omitting ATP or RFC complex from the reaction should abolish the loading. To investigate whether the weak radioactive signal in the early fractions was due to PCNA loading on the DNA, three parallel experiments (complete reaction, -ATP reaction, and –RFC reaction) were carried out (Figure 2.24). In the complete reaction, consistent to Figures 2.22 and 2.23, weak but detectable radioactive signals appeared around fraction 16. In –ATP or –RFC reaction, no such signals were detected, indicating that the signals in the complete reaction was due to the loading of the $^{32}$P-PCNA to the DNA substrate. This also indicates that at least a portion of my protein preparations have the expected biochemical activities. However, less than 5% of the total $^{32}$P-PCNA was
loaded on the DNA substrate, indicating either very low efficiency of PCNA loading or very low stability of PCNA-DNA complex.

**Figure 2.24. Effect of ATP and RFC on the $^{32}$P-PCNA loading.**
The same PCNA loading assays as shown Figure 2.23, including all components (complete), or in the absence of ATP (-ATP) or RFC (-RFC) were repeated. Fractions 14-33 were analyzed by 12% SDS-PAGE. The gel was dried on DE81 chromatography paper (Whatman) under vacuum and the labeled products were visualized by a Personal Molecular Imager FX (Bio-Rad).

All the DNA substrates tested above mimicked DNA replication intermediates (primer-template complex). Because the structure of DNA recombination intermediates is significantly different from that of DNA replication (Figure 2.3), it is possible that the PCNA loading mechanism on a DNA recombination substrate is quite different from loading on a replication substrate. To test this possibility, a D-loop intermediate of DNA recombination was generated from synthetic oligonucleotides as described in the
Materials and Methods. Then, the PCNA loading reaction was carried out using the D-loop substrate, and products were analyzed by gel filtration (Figure 2.25).

Because the length of the D-loop substrate is one hundred nucleotides, it is likely to be included in the Sephacryl S-400 gel filtration resin, causing an overlap of loaded and free $^{32}$P-PCNA signals. To minimize the overlap, I used Sephacryl S-200 resin that has a separation range able to separate free and loaded $^{32}$P-PCNA.

First, the radiolabeled D-loop substrate, DL2-L (see Materials and Methods section for illustration) and free $\gamma^{32}$P-ATP was analyzed separately with the Sephacryl S-200 (Figure 2.25, A). The radioactivity eluted between fractions 15-25, and the peak from free $\gamma^{32}$P-ATP was observed around fraction 32. Using the same S-200 column, 5 µg of un-labeled PCNA-pka was then analyzed by SDS-PAGE (Figure 2.25, B). Comparison of Figure 2.25 A and Figure 2.25 B showed that fractions containing PCNA-pka (#21-35) and DL2-L (#15-25) had some overlap. This overlap made the differentiation of free $^{32}$P-PCNA and $^{32}$P-PCNA-DNA complex very difficult. Therefore, I decided not to pursue this method to analyze PCNA loading on the DNA recombination substrate.
2.3.4. Clamp loading analyzed by affinity pull-down

Another approach I took to analyze the PCNA loading onto DNA was an affinity pull-down assay using synthetic oligonucleotides that had biotin labeling at their ends (Figure 2.26, A). The biotinylated DNA can be pulled down with streptavidin-beads. If $^{32}$P-PCNA is loaded on the synthetic DNA substrates, the radioactivity signal from $^{32}$P-PCNA can be pulled down by the streptavidin beads.
Using the simple substrate C2 (annealed from TSO318 and TSO319), PCNA loading was analyzed by the biotin-streptavidin pull down assay (Figure 2.26, B). In the absence of DNA substrate and the RFC complex (lane 1), no radioactive signal was associated with streptavidin beads. In the complete reaction (lane 3) (the reaction contained DNA, PCNA, RFC, RPA, and ATP), $^{32}$P-PCNA signal was detected in the pellet fraction, suggesting the loading of $^{32}$P-PCNA onto the DNA substrate. However, a comparable amount of signal was also observed in the reaction that did not contain ATP (lane 4). Because PCNA loading by the RFC complex requires ATP, ATP-independent association should not be functional PCNA loading. I suggest that the result may be due to the interaction of PCNA with the RFC complex that is associated with the DNA. Although some PCNA loading might be occurring in the reaction in lane 3, this assay could not distinguish PCNA loading from the indirect PCNA association to the DNA substrate. Therefore, PCNA loading and the affinity pull-down assay needed to be optimized to differentiate non-specific binding and PCNA loading. Therefore, I tried to improve the wash step to eliminate non-functional association of PCNA to DNA.
Figure 2.26. Analysis of $^{32}$P-PCNA loading onto biotin labeled C2 substrate and affinity pull-down by streptavidin beads.

(A) Illustration of reaction. Biotinylated TSO318 (60 mer) was annealed with TSO319 (29 mer) to generate C2 substrate. The detailed $^{32}$P-PCNA loading and result analysis procedure were presented in the *Material and Methods* section.

The wash step was optimized with buffers containing different amounts of NaCl (Figure 2.27) or EDTA (Figure 2.28), in the hope of abolishing the non-specific binding. In the experiment shown in Figure 2.27, the pellet fractions from three parallel experiments were washed with buffers containing 250 mM, 500 mM, and 1000 mM NaCl. Even with the 1000 mM NaCl wash, a significant amount of $^{32}$P-PCNA signal was observed in the pellet fraction. Because the oligonucleotide $^{32}$P-TSO319 was also labeled, the localization
of DNA substrate was also determined (indicated by “P-319” in the figure). In the reaction washed with lower NaCl concentrations (250 mM and 500 mM), TSO319 was still associated with the beads. In a reaction washed with 1000 mM NaCl, only a very low signal from the DNA was detected in the pellet fraction, suggesting the disruption of dsDNA or biotin-streptavidin interaction by high NaCl. In the same reaction, the $^{32}$P-PCNA signal in the pellet fraction was not reduced, indicating that $^{32}$P-PCNA was associated with the streptavidin beads in a dsDNA-independent manner. Because this non-specific binding required RFC, it is very likely that RFC associated with PCNA directly binds to the beads.
PCNA loading on $^{32}$P-labeled and biotinylated C2 substrate was performed as described in Figure 2.26. After the loading reaction, beads were washed with 50 µl of wash buffer containing various concentration of NaCl (250 mM, 500 mM, or 1000 mM) for three times. Proteins were discharged from the beads by adding 20 µl of TE buffer and 4 µl of 6XSDS sample loading buffer. Pellet and 5% of the supernatant were analyzed by a 12% SDS-PAGE and the gel was dried on DE81 chromatography paper (Whatman) under vacuum and the radiolabeled products were visualized by a Personal Molecular Imager FX (Bio-Rad).

Since the highest NaCl concentration did not disrupt the non-specific PCNA-bead interaction, I tested a wash buffer containing EDTA (Figure 2.28). PCNA was found in the pellet fractions in all reactions that contained RFC, even without ATP or DNA (lane 2), indicating that PCNA was associated with streptavidin beads via RFC. Comparing the last two lanes in Figure 2.28, the EDTA (5 mM) wash did not reduce the non-specific binding of RFC to the streptavidin beads. Because of this stable non-specific binding of
RFC, it was impractical to analyze PCNA loading with this Biotin-Streptavidin affinity pull down assay.

**Figure 2.28.** $^{32}$P-PCNA loading onto $^{32}$P-C2-biotin substrate and affinity pull-down by streptavidin beads, followed by washing with EDTA.

DNA substrate generation and PCNA loading was performed as described in Figure 2.26. After the loading reaction, beads were washed with 50 µl of wash buffer containing 5 mM EDTA for three times. Proteins were discharged from the beads by adding 20 µl of TE buffer and 4 µl of 6XSDS sample loading buffer. Pellet and 5% of the supernatant were analyzed by a 12% SDS-PAGE and the gel was dried on DE81 chromatography paper (Whatman) under vacuum and the radiolabeled products were visualized by a Personal Molecular Imager FX (Bio-Rad).

In addition to the biotinylated DNA pull-down, I also tried the pull-down assay with Ni-affinity beads. If His6-PCNApka is loaded onto the $^{32}$P-DNA substrate, then the labeled DNA should be pulled down by Ni affinity resin, which can be detected by radioactivity in the bead fraction. As shown in Figure 2.29, PCNA loading was tested with C2 (lane 1-6) and DL (lane 7 and 8). Comparing lane 5 (-ATP reaction) with lane 6 (complete
reaction), no significant increase of radioactivity signal was detected in the complete reaction. Similar result was also observer for the DL substrate (comparing Lane 7, -ATP reaction, with lane 8, complete reaction).

2.3.5. Gel-mobility shift assay of the PCNA loading

PCNA loading onto the DNA substrate should result in the formation of a protein-DNA complex, which would decrease the mobility of PCNA during electrophoresis. $^{32}$P-PCNA loading reactions were performed as described using non-labeled C2 or DL substrate. After the reaction, the reaction mixtures were resolved on an 8% non-denaturing PAGE (Figure 2.30). Radiolabeled C2, DL, and PCNA were loaded as mobility references (last three lanes). Even though some low mobility (migration slower than the PCNA trimer) species were identified on the gel (indicated by * in the Figure), there was no significant difference between the –ATP reaction with the complete reaction on either C2 (Figure 2.30, compare lane 4 and 5) or DL (compar lane 9 and 10). The reduced mobility was consistently observed in the reactions contained DNA and RPA, suggesting the mobility shift change was due to the RPA binding to the DNA substrates.
Figure 2.29. Ni-Sepharose affinity pull down assay of PCNA loading onto $^{32}$P-labeled C2 or DL.

PCNA loading and affinity pull down were performed as described in the Material and Methods section. Proteins associated with the resin were discharged by adding 20μl of 1X SDS loading. Radioactivity of each sample was measured by a liquid scintillation analyzer (Packard, Model 1900TR) and then entire sample was resolved on a 12% SDS-PAGE.
Figure 2.30. $^{32}$P-PCNA loading onto oligonucleotide based substrate (C2 or DL) analyzed by gel mobility shift assay.

$^{32}$P-PCNA loading onto C2 and DL substrate was performed as described in the Material and Methods section. The reaction mixture was analyzed by an 8% non-denaturing PAGE and the gel was dried on DE81 chromatography paper (Whatman) under vacuum and the labeled products were visualized by a Personal Molecular Imager FX (Bio-Rad).

2.3.6. Modified gel-mobility shift after glutaraldehyde treatment successfully detected PCNA loading

I then adopted the method published by Podust et al. [310] for quantitative analysis of PCNA-loading onto DNA substrates with various structures. Essentially, we constructed
\(^{32}\)P-labeled PCNApka (\(^{32}\)P-PCNA) and tested its loading onto un-labeled DNA molecules in vitro. In a standard reaction, the pre-annealed DNA substrate was first incubated with RPA followed by RFC and \(^{32}\)P-PCNA. Three minutes after the addition of PCNA, the reaction mixture was treated with glutaraldehyde to fix the PCNA ring structure in its closed conformation on the DNA substrate and subjected to agarose gel electrophoresis (Figure 2.31). To disrupt non-specific protein-protein or protein-DNA interactions, the electrophoresis contained SDS. Free \(^{32}\)P-PCNA should migrate faster than the \(^{32}\)P-PCNA loaded on the DNA substrate. I first tested the loading of \(^{32}\)P-PCNA on a primed Bluescript ssDNA substrate (Figure 2.31). As expected, a low-mobility PCNA-DNA complex was produced in the presence of RFC and ATP. The complete reaction (lane 6) showed nearly 100% shift, indicating the reaction was very efficient. The gel-shift signal depends on ATP (lane 5), RFC (lane 4), and RPA (not shown). The reaction was much less efficient with unprimed ssDNA or dsDNA (Figure 2.31). All these results are consistent with the previous reports [312, 313], indicating that functional PCNA loading occurred in the reaction and that the loaded complex was successfully detected by this assay method.

2.3.7. PCNA is loaded on the D-loop and primed ssDNA with similar efficiency

Since the SDS-agarose gel electrophoresis of the glutaraldehyde treated sample successfully detected PCNA loading on the plasmid based simple substrate, I next analyzed the loading of the \(^{32}\)P-PCNA on a synthetic substrate that mimicked the D-loop (DL) and on a primed ssDNA substrate (C2) (Figure 2.32, B). When I used the C2
substrate (Figure 2.32, lanes 1-4), $^{32}$P-PCNA mobility was shifted in the gel in the presence of RPA, RFC and ATP (lane 4), indicating that it was loaded on C2. Although C2 is a linear DNA molecule, loaded PCNA did not slide off from the substrate because both ends of the DNA were complexed with RPA, as previously reported [300, 314]. Likewise, when the DL substrate was used (Figure 2.32, lanes 5-8), a mobility shift was observed in the complete reaction (lane 8), but not in the absence of RFC, ATP, or RPA (lane 5 to 7) indicating that PCNA was loaded on the D-loop substrate. PCNA loading efficiency in the complete reactions on the C2 and DL substrates was comparable (Figure 2.32, D).

**Figure 2.31.** $^{32}$P-PCNA loading onto Bluescript ssDNA based substrate analyzed by agarose gel electrophoresis and autoradiography. (A) Illustration of PCNA-loading reaction. DNA substrate (ssDNA, primed ssDNA, or dsDNA) was incubated with RPA and then with RFC and $^{32}$P-PCNA for clamp-loading. Then, glutaraldehyde was added to cross-link the PCNA ring structure. (B) Loading reactions of PCNA onto single-stranded DNA (ss), primed ssDNA (primed), or dsDNA (ds) were performed with or without RFC or ATP as indicated. Products were separated by agarose gel electrophoresis followed by ethidium bromide staining (lanes 10-18) and phosphor-imaging (lanes 1-9).
Figure 2.32. $^{32}$P-PCNA loading onto synthetic DNA substrate analyzed by agarose gel electrophoresis and autoradiography.

(A) Illustration of the PCNA loading assay on synthetic DNA substrate. DNA substrate (C2 or DL) was incubated with RPA and then with RFC and $^{32}$P-PCNA for clamp-loading. Then, glutaraldehyde was added to cross-link the PCNA ring structure. (B) Structures of synthetic DNA substrates (DL and C2) used for PCNA loading analysis. Numbers are lengths of DNA segments in nucleotide. (C) The loading reaction of $^{32}$P-PCNA on C2 and DL were performed in the presence or absence of ATP, RFC, and RPA as indicated. (D) Percentages of loaded PCNA were calculated from (C) with repeated experiments (n=3). Error bars represent the standard deviations.

In the experiment shown in Figure 2.32, the PCNA loading efficiency on C2 and DL were quite similar. This result was unexpected, because DL does not have 3' junction (the
junction between the 3’ end of the primer and the template) that is believed to be the most efficient DNA structure for the PCNA loading (see below). To investigate the difference in loading efficiency onto C2 and DL more in detail, I did an RFC titration to determine the optimal RFC/PCNA ratio for efficient PCNA loading \textit{in vitro}. In the RFC titration experiment, increasing amounts of RFC were incubated with a fixed amount of $^{32}$P-PCNA and DNA substrate, either C2 or DL, in PCNA loading reactions (Figure 2.33). The result indicated that the $^{32}$P-PCNA loading on both C2 and DL increased in the presence of increasing amounts of RFC, until saturated at around the RFC/PCNA ratio of four to six (Figure 2.33, B). More importantly, the two curves in Figure 2.33, B (PCNA loading on C2 and DL) were very similar, suggesting a comparable efficiency of PCNA loading on the two substrates. This conclusion is also supported by the observation that PCNA loading onto C2 and DL had comparable kinetics (Figure 2.34).

**Figure 2.33. $^{32}$P-PCNA loading on C2 and DL required similar amount of RFC.**
(A) PCNA loading reaction was performed as described. Increasing amount of RFC (0, 0.005, 0.01, 0.02, 0.04, 0.08, or 0.12 μM) and 0.02 μM of $^{32}$P-PCNA were added to 0.1 μM of DNA substrate (C2, lane 1-7, or DL, lane 8-14). (B) Percentages of loaded PCNA were calculated from (A) with repeated experiments (n=3). Error bars are standard deviations.
Next, I investigated where in the DL substrate that PCNA was loaded. There are three distinct dsDNA regions in the DL substrate. PCNA can be either loaded onto the two ends or onto the internal region of DL (Figure 2.32, B). This is important because only the PCNA loaded on the internal region of the DL can stimulate DNA polymerase activity. To identify the region of the D-loop where PCNA was loaded, I treated the product of the complete loading reaction with \textit{KpnI} to cleave the dsDNA region in the D-loop (Figure 2.35, A). Five minutes of \textit{KpnI} digestion did not release the loaded PCNA from the substrate, likely because the clamp loading reaction buffer was not optimal for \textit{KpnI} digestion. After thirty minutes, most of the \textsuperscript{32}P-PCNA was released from the complex (Figure 2.35), indicating that \textit{KpnI} digestion opens the D-loop and allows the loaded PCNA to slide off. These data show that the PCNA is efficiently loaded onto the dsDNA region inside the D-loop.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure2_34.png}
\caption{Time course of \textsuperscript{32}P-PCNA loading on C2 and DL.} \label{fig:34}
PCNA loading reaction was performed as described in Figure 2.29. RFC (0.08 \textmu M) and \textsuperscript{32}P-PCNA (0.02 \textmu M) were added to 0.1 \textmu M of DNA substrate (C2 or DL). The reactions were stopped at the time points indicated. Percentages of loaded PCNA were calculated from repeated experiments (\textit{n}=3). Error bars are standard deviations.
Figure 2.35. *KpnI* digestion confirmed the loading position of ³²P-PCNA.
The complete PCNA loading reaction with DL substrate was followed by incubation with (lane 2 and 4) or without (lane 1 and 3) *KpnI* for indicated times.

Previous studies using primed ssDNA substrates indicated that efficient PCNA loading required a ssDNA region beyond the 3’ junction of the primer and template [295, 296]. This ssDNA region is believed to be complexed with RPA, which provides a binding site for RFC and for subsequent PCNA loading. However, The DL substrate I used for PCNA loading had only a one-nucleotide ssDNA region at the 3’-end of the invading DNA strand, which is too small for RPA binding [80, 309], yet required RPA for PCNA-loading. Therefore, I did a series of experiments to identify the location of RPA on the D-loop substrate in PCNA loading using different DNA structures (Figure 2.36).

Interestingly, the loading of PCNA on the D-loop substrates containing 3’ junctions (DL-L and DL2-L) was not better than the D-loop substrates containing 5’ junctions (DL-R and DL2-R). All of these data indicate that PCNA loading on D-loop was not dependent on a 3’-junction, in contrast to the previous studies using primed ssDNA substrates [140-143]. Therefore RPA most likely binds to the displaced ssDNA loop to stimulate the PCNA-loading reaction (Figure 2.36, C).
2.3.8. RPA on the ssDNA loop stimulates PCNA loading

To confirm that RPA associated with the displaced ssDNA loop provided the PCNA loading machinery assembly site, I analyzed PCNA-loading on a modified DL substrate where the ssDNA loop was annealed to another oligonucleotide to make it double-stranded (Figure 2.37, DL-CAP). The loading of $^{32}$P-PCNA on the DL-CAP was greatly reduced, supporting the notion that RPA binding to the displaced ssDNA region of the D-loop is necessary for PCNA loading.

Figure 2.36. Comparison of 32P-PCNA loading efficiency on different DL substrates. PCNA loading reactions were carried out by using DNA substrates with different structures (DL, DL-L, DL-R, DL2-L, DL2-R), which are illustrated in (B). Percentages of loaded PCNA were calculated from (A) with repeated experiments (n=3). Error bars
represent the standard deviations. (C) RPA associated with the displaced ssDNA loop provides the assembly site for PCNA loading machinery.

Figure 2.37. \(^{32}\text{P}\)-PCNA loading onto the DL requires RPA binding to the displaced ssDNA loop.

PCNA-loading was performed on the DL (lane 3 and 4) and its derivative that had an additional oligonucleotide covering the ssDNA loop (DL-CAP; lane 1 and 2).

Since studies have shown that RPA-RFC protein-protein interaction is involved in the PCNA-loading on simple primed substrates [141, 299, 300], I investigated whether this is also the case in the PCNA-loading on D-loop. To do this, I replaced RPA with \textit{E. coli} SSB protein in the loading protocol (Figure 2.38). SSB is the single strand DNA binding protein of \textit{E. coli} and only poorly stimulated the loading of PCNA on the DL (Figure 2.38, lane 5) and C2 (lane 2), indicating the RFC-RPA species-specific interaction plays crucial role in the PCNA loading on D-loop.

Additionally, RPA-titration indicated that the amount of RPA that was required for efficient PCNA loading roughly corresponded to that required for full occupancy of the displaced ssDNA loop (Figure 2.39, lane 5 and lane 6). RPA gel mobility shift assays with C2 and DL suggested one or two RPA molecules can bind to the DNA substrate (Figure 2.40). Based on these results, I conclude that RPA on the ssDNA loop provides
the binding site for the RFC, leading to PCNA loading onto the dsDNA region within the D-loop.

Figure 2.38. RPA on the ssDNA loop stimulates PCNA loading on D-loop.
(A) PCNA-loading on the C2 (lane 1-3) and the DL (lane 4-6) were performed under the standard conditions in the presence or absence of RFC (0.08 μM), RPA (0.8 μM), and E. coli SSB protein (0.8 μM) as indicated. (B) Percentages of loaded PCNA were calculated from (A) with repeated experiments (n=3 to 4). Error bars represent the standard deviations.

Figure 2.39. PCNA loading requires RPA.
(A) 32P-PCNA loading on DL substrate was performed in the absence (lane 2) or presence of 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 μM of RPA (lane 3 to 9). Lane 1 was a control reaction without RPA or RFC. (B) Quantification of loaded PCNA percentage in (A). Percentage of loaded PCNA was average of at least three repeated experiments. Error bars represent the standard deviations.
Figure 2.40. RPA binding to C2 and DL substrate analyzed by gel mobility shift assay.

(A) Gel shift of C2 by RPA. RPA (0, 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 μM from lane 1-7) was added to 32P labeled C2 substrate (0.1 μM). RPA binding was carried out at 37°C for 3 min followed by glutaraldehyde (0.1% final concentration) cross-linking at 37°C for 10 min. The products were analyzed by 2% agarose gel electrophoresis. (B) The same experiment as described in (A) using DL.

2.3.9. Rad51 inhibits PCNA loading on D-loop

Although Rad51-dependent DNA strand invasion is crucial for HR, an increasing number of studies have indicated that Rad51 inhibits HR after DNA strand invasion [315-317]. Therefore, it is of special interest to investigate the effect of Rad51 on PCNA-loading on D-loop (Figure 2.41). When the RPA-DL complex was incubated with an increasing amount of Rad51 and then subjected to the PCNA-loading reaction, the loading of 32P-PCNA was inhibited in a concentration-dependent manner (Figure 2.41, A and D, red open circle). At the concentration of Rad51 that was sufficient to saturate all dsDNA regions of the DL substrate (Rad51/DNA = 32), almost no loading was observed. In contrast, when Rad51 was added to the DL after the PCNA-loading reaction, a super-shift of the signal was observed (Figure 2.41, B), indicating the formation of a Rad51-PCNA-DNA complex. No significant effect on the percentage of the loaded PCNA was observed
These results indicate that Rad51 inhibits the loading of PCNA, but does not affect the stability of the loaded PCNA.

The experiments in Figure 2.41 showed that Rad51 inhibited PCNA-loading onto the DL, but which region of the DL Rad51 associated with was unclear. Since the reaction
contained RPA that occupies the ssDNA loop, Rad51 should preferentially bind to the 
dsDNA region of DL (see illustration of Figure 2.41). This was confirmed by measuring 
the ATPase activity of Rad51 under the same conditions (Figure 2.42). This was done 
because a previous study showed that the ATPase activity of a Rad51- ssDNA complex is 
3 to 5-fold higher than that of Rad51-dsDNA complex (4). Therefore, I used ATPase 
activity to probe the Rad51-DNA binding status on the D-loop. The DL was incubated 
with increasing amounts of Rad51 under the same conditions used to generate Figure 
2.41, A, and the ATP hydrolysis rate was measured by the spectrophotometric method as 
described [80]. As controls, the same reactions were done in the absence of DNA (no-
DNA) or in the presence of excess dsDNA (dsDNA) and ssDNA (ssDNA). The DL and 
dsDNA stimulated ATP hydrolysis rates were very similar and both were significantly 
lower than that of ssDNA, indicating that the majority of Rad51 molecules in the Rad51-
DL complex were associated with dsDNA.

All the results, therefore, indicate that the Rad51-mediated inhibition of PCNA-loading 
was not due to the removal of RPA from the D-loop or the melting of the D-loop 
structure into ssDNA, but due to Rad51 complexed with dsDNA in the D-loop. Most 
likely, Rad51 bound to the invading DNA strand acts as physical obstruction preventing 
the loading of PCNA.
Figure 2.42. Comparison of ATPase activity of Rad51 on dsDNA, ssDNA and DL. DL (20 pmol) was incubated with increasing amounts of Rad51 under the same conditions in Figure 2.41, A, containing 80 pmol of RPA, 10 units/ml pyruvate kinase, 10 units/ml lactate dehydrogenase, 0.3 μM phosphoenolpyruvate, 256 μM NADH. Reaction (200 μl) was done in a cuvette set in a Cary50 spectrophotometer, and ATP hydrolysis rate was measured. As controls, the same reactions were done in the absence of DNA (no-DNA) or in the presence of excess dsDNA (dsDNA) and ssDNA (ssDNA).

It was unexpected that Rad51-ssDNA preincubation (Figure 2.41, C) was not more inhibitory than incubation of Rad51 with pre-annealed DL (Figure 2.41, D, bottom two curves). I speculate that this is because Rad51 on the invading DNA strand quickly redistributes along the dsDNA region after strand invasion. Consistently, a time course of the reaction revealed that PCNA-loading onto the Rad51-DL complex that was produced from the Rad51-ssDNA complex was slower than was that produced by incubation of Rad51 and preannealed DL (Figure 2.43, compare bottom two curves).
Figure 2.43. Time course of PCNA loading on Rad51-DL complex.

(A) $^{32}$P-PCNA and RFC were added to Rad51-DL complexes that were produced under different procedures as follows. Blue square and black triangle: The 29-mer oligonucleotide (TSO319, 0.1 μM) was incubated with Rad51 (at indicated molecular ratio) for 5 min, then with 0.1 μM of preannealed TSO317 and TSO318 for 15 min, and then with 0.4 μM of RPA for another 3 min at 37°C. Green open circle: Pre-annealed DL was incubated with RPA for 3 min then with Rad51 for 5 min. Red circle (no Rad51): Pre-annealed DL (0.1 μM) was incubated with 0.4 μM of RPA at 37°C for 3 min. (B) PCNA loading reaction was stopped at indicated times after addition of PCNA, and (C) the percentages of loaded PCNA were quantified and plotted against the incubation time. Error bars are the standard deviations (n=3).
2.3.10. The 9-1-1 complex was not loaded on the plasmid based DNA substrates

Since PCNA loading was detected on the simple and HR substrates, I applied the same method to analyze the loading of the yeast 9-1-1 complex (complex of Rad17, Mec3, and Ddc1) by the Rad24-RFC alternative clamp loader. The loading of the 9-1-1 complex on the plasmid based substrates was analyzed using the same procedure as described for PCNA loading (Figure 2.44). However, no strong gel-mobility shift signal was obtained for any substrate tested (ssDNA, primed, or dsDNA). I found faint signals when the image was over-exposed (Figure 2.44, B), but these signals were not dependent of Rad24-RFC or ATP, indicating that they were the result of non-functional association.
Figure 2.44. \(^{32}\)P-9-1-1 loading onto Bluescript ssDNA based substrate analyzed by agarose gel electrophoresis and autoradiography. (A) Illustration of the \(^{32}\)P-9-1-1 complex loading reaction. DNA substrate (ssDNA, primed ssDNA, or dsDNA) was incubated with RPA and then with Rad24 complex and \(^{32}\)P-9-1-1 complex for the clamp-loading. Then, glutaraldehyde was added to cross-link the 9-1-1 ring structure. (B) Loading reactions of PCNA onto single-stranded DNA (ss), primed ssDNA (primed), or dsDNA (ds) were performed with or without Rad24-RFC complex or ATP as indicated. Products were separated by agarose gel electrophoresis followed by ethidium bromide staining (lanes 11-20) and phosphor-imaging (lanes 1-10).

2.4. Discussion

PCNA loading on the replication substrate has been studied extensively and a consensus model has been proposed (Figure 2.45, A). Even though PCNA has been known to be required for DNA synthesis during HR, PCNA loading on the DNA recombination substrate has never been characterized. In this chapter, I characterized the mechanism of PCNA loading onto the recombination substrate, the D-loop, and compared it with replication substrate, using synthetic DNA substrates and purified recombinant proteins.
PCNA was loaded on the primed substrate more efficiently than the ssDNA or dsDNA substrates (Figure 2.31), which is consistent with the previous reported results [312, 313]. PCNA is well known for its function as the processivity factor of replicative DNA polymerases during DNA replication. Because the primed substrate used in the experiment described in Figure 2.31 mimicked the replication intermediate, it is reasonable to see the highest PCNA loading efficiency on this substrate.

![Figure 2.45](image)

**Figure 2.45. PCNA loading is loaded on the replication and recombination substrates by different mechanisms.**

Different from the structure of the DNA replication intermediate, the recombination intermediate has a displaced ssDNA loop that migrates forward as post-invasion synthesis occurs (Figure 2.45). PCNA loading on the recombination substrate turned out to be as efficient as it was on the replication substrate (Figure 2.32, C and D), and the kinetics of PCNA loading on the two substrates was also very similar (Figure 2.34). In addition, PCNA-loading onto the D-loop required the binding of RPA to the displaced ssDNA loop, thereby providing a binding site for RFC which mediates the PCNA-loading (Figure 2.37, 2.38, and 2.39). This observation was surprising since previously reported results showed that RPA bound to the ssDNA at the 3’ junction of the primer-
template, allowing for efficient PCNA loading (Figure 2.45, A) [295, 296]. According to the results observed in this study, the displaced ssDNA loop is bound by RPA, providing an alternative assembly site for PCNA loading machinery (Figure 2.45, B).

Additionally, analysis of PCNA loading efficiency on different DL derivatives also supported that a ssDNA region beyond the 3’ junction of the primer and template is not absolutely required for efficient PCNA loading on recombination substrate (Figure 2.36). The loading of PCNA on the D-loop substrates containing 3’ junctions (DL-L and DL2-L) was not better than the D-loop substrates containing 5’ junctions (DL-R and DL2-R). All of these data indicate that PCNA loading on the D-loop was not dependent on a 3’-junction, instead, the displaced ssDNA loop provides the binding site for RFC and PCNA loading (Figure 2.45, B), in contrast to the previous studies using primed ssDNA substrates [140-143]. Whether there is competition between the classical PCNA loading site (3’ junction of the primer-template) and the newly observed PCNA loading site (displaced ssDNA loop) is unclear. My observation that PCNA loading used RPA associated with the displaced ssDNA loop is novel and needs to be further elucidated. One hypothesis that can be made from the observation is that the ssDNA region 3’ of the invading strand might not be available under certain conditions, requiring another mechanism of PCNA loading.

Rad51 is a key factor of DNA recombination. It assembles on ssDNA and mediates DNA strand invasion, a crucial step of recombination. However, its prolonged presence on the
invading strand after invasion has a negative effect on DNA recombination. It has been shown that Rad51 needs to be removed from the invading strand to expose the 3’-OH for DNA polymerase access [315-317]. According to the results in this chapter, Rad51 on the DL also inhibits PCNA loading (Figure 2.41, 2.42, and 2.43). Therefore, Rad51 inhibits two steps that are crucial for progression of the recombination process: PCNA loading and primer polymerase interaction. Is the inhibition of PCNA loading by Rad51 more biologically significant? This question will be addressed in next chapter.

Figure 2.46. Rad51 inhibits PCNA loading.

In order to produce the DL substrate efficiently in vitro, I preannealed the synthetic oligonucleotides before the addition of Rad51 and PCNA loading. In vivo, however, Rad51 binds to the ssDNA first and then invades into the homologous dsDNA template. Two Rad51-DL complexes, one produced with preannealed DL and Rad51 and the other produced by Rad51-mediated strand invasion, were used to analyze the inhibitory effect of Rad51 on PCNA loading. My experiment revealed that the PCNA loading efficiency on the two DL substrates was similar (Figure 2.41 and 2.43). This raises a new question
about the stoichiometry of Rad51 inhibition of PCNA loading: Even when Rad51 and ssDNA were preincubated before reconstituting the Rad51–DL complex, maximum inhibition of PCNA loading required a level of Rad51 that could saturate the entire dsDNA region of the DL (Rad51/DL ratio = 32; Figure 2.41, C and D, Figure 2.43). This might suggest that Rad51 molecules not present on the invading DNA strand may also contribute to inhibition. Alternatively, Rad51 on the invading DNA strand may quickly redistribute along the dsDNA region after DNA strand invasion. Consistent with the later idea, Rad51-ssDNA preincubation inhibited the PCNA loading more strongly during the first 2 min of the loading reaction (Figure 2.40 C; compare lower two curves).

I could not establish a system where the 9-1-1 complex was loaded on the DNA substrate. Only, some non-specific binding to RPA or ssDNA was observed under my experimental conditions (Figure 2.44, B). Even though the 9-1-1 clamp and Rad24-RFC complex appeared to be of good quality on SDS-PAGE, the proteins might have been inactivated during the process of preparation. Alternatively, the experimental procedure needs to be modified for the 9-1-1 loading analysis. However, due to time constrain, this was not pursued.

In this chapter, RFC dependent loading of PCNA onto multiple DNA recombination substrates was reconstituted \textit{in vitro} using purified proteins and model DNA substrates. The significance of RPA and Rad51 recombinase interaction with PCNA loading machinery was also analyzed. The reconstitution of PCNA clamp loading was
challenging because *in vitro* biochemical study required the purification of these proteins to homogeneity without losing their biological activities. This is especially true for protein complexes composed of multiple subunits. Several protein factors, including the RFC complex, the Rad24-RFC complex, and the 9-1-1 complex, described in this chapter contain multiple heterologous subunits. Without high quality preparation of these protein complexes, the biochemical analyses described here would be impossible.

Originally, the native RFC complex was designed to be expressed in *S. cerevisiae*. However, full length Rfc1 subunit expression in yeast was not successful. This was most likely because the Rfc1 subunit is susceptible to proteolysis. It has been reported that the N-terminal region of Rfc1 (1-283 AA) can be deleted in both yeast and human RFC without decreasing RFC clamp loader function with PCNA [312, 318]. A genetic study indicated that the truncated RFC1 gene had an indistinguishable phenotype to its wild type allele [318], indicating that it encoded all essential functions. The deletion of the C terminal fragment (786-861 AA) also did not affect the PCNA loading activity of the RFC complex. Accordingly, I changed the construct and successfully expressed the RFC complex with an Rfc1 protein that had truncations on both N and C termini. All my *in vitro* experiments were done using an RFC complex containing the truncated Rfc1. However, I cannot exclude the possibility that a nonessential activity of the RFC complex was affected by the truncation so that my results may not be completely consistent with the *in vivo* event. Also, the RFC complex was expressed in *E. coli* instead of yeast, allowing for short cultivation and higher expression level (Figure 2.4). However, the *E.*
coli system may not provide unknown post-translational modifications that might occur in yeast affecting the biochemical activities of the RFC complex. Currently, post-translational modification of the RFC complex in yeast has not been reported.

The Rad24-RFC complex was expressed by the modified dual vector expression system used for RFC complex production. Because the Rad24-RFC complex and the RFC complex share four common subunits, the only modification needed was replacing the RFC1 gene with the full length RAD24 gene. However, the expression level of Rad24-RFC complex was greatly reduced in comparison with the expression of the RFC complex (Figure 2.11). This was likely due to Rad24 being very large and expressed as full length protein, making it more difficult to transcribe or translate. Another protein complex that was expressed from the pLANT dual vector system was the 9-1-1pka complex. The wild type 9-1-1 complex was expressed in yeast (Figure 2.14). However, the insertion of the PKA recognition site upstream of the open reading frame in the yeast expression system lowered the expression level significantly (data not shown). Even with the pLANT expression system, the 9-1-1pka yield was still low (Figure 2.17). The major reason for this was the low solubility of the Rad17pka subunit of the complex. It is not easy, and generally impossible, to predict or control protein expression and solubility under inducing conditions.

Because PCNA loading and unloading is a dynamic process, loaded PCNA normally accounts for about only 10% of the total input in most in vitro systems. That makes it
difficult to analyze PCNA loading on different DNA substrates quantitatively. Radio-labeling of PCNA with $^{32}$P enabled the detection of even trace amount of PCNA loading on the DNA substrate (Figure 2.19). Most often, radio-labeling of a protein substrate is achieved by incorporating $^{35}$S- methionine during the process of translation using a cell free system. The \textit{in vitro} assays described in this chapter require a large amount of highly purified protein, making the $^{35}$S labeling system inappropriate. $^{32}$P-labeling of protein by cAMP dependent protein kinase (PKA) mediated \textit{in vitro} phosphorylation reaction is an efficient mechanism of protein radio-labeling. PKA is a serine/threonine kinase that recognizes the motif RRXS/TY, where X can be any amino acid and Y tends to be a hydrophobic residue. PCNApka was efficiently labeled by PKA to high specificity (Figure 2.16). In theory, any protein containing this motif can be labeled in the same way. This \textit{in vitro} radio labeling system will be useful for the detection of low amounts of protein-protein or protein-nucleic acid interaction. Since the PKA recognition motif contains only five amino acids, it is not likely to change the biochemical properties of the target protein. However, there is still a possibility that the addition of the motif and/or its phosphorylation will change the activity of the target proteins, including PCNA.

Gel filtration chromatography separates different molecules based on their sizes. Free PCNA and PCNA-plasmid DNA complexes have a significant size difference that can be separated by the proper gel filtration resin. In the complete reaction described in Figure 2.24, PCNA was found in the fractions corresponding in size to the PCNA-DNA complex. However, due to the extremely low amount of the complex compared with the total
PCNA in the reaction, I was not able to conclude that signal resulted from PCNA loading. I speculated that the low mount of complex was due to the instability of the PCNA-DNA complex.

The affinity pull-down method is very commonly used to study protein-protein or protein-DNA interaction. Therefore, PCNA loading was analyzed by Ni-Sepharose or streptavidin-biotin affinity pull-down. These attempts were unsuccessful, because the RFC complex bound non-specifically to the resins (Figure 2.27). The RFC complex associated with resin can bind to PCNA, which makes the differentiation of loaded PCNA from non-specifically bound PCNA impossible. Even 1000 mM NaCl (Figure 2.27) or 5 mM EDTA (Figure 2.28) in the wash buffer did not eliminate RFC non-specific binding to the resin. PCNA loading was also examined by gel mobility shift assay using native PAGE (Figure 2.30). After the PCNA loading reaction, the products were resolved on 8% native PAGE. No specific PCNA loading signal was observed with this method. In the later agarose gel electrophoresis, 0.01% of SDS was included in the electrophoresis buffer, which should eliminate the non-specific binding among PCNA, affinity resin, and DNA substrate. Another difference between the native PAGE gel shift assay and the agarose gel shift assay was that in the agarose gel shift assay, the PCNA ring structure was not crosslinked after the loading reaction. The fixation, as discussed above, stabilizes the loading products.
Based on the information accumulated from gel filtration and affinity pull-down assays, the keys for successful observation of PCNA loading onto the DNA substrate is the efficiency of PCNA loading and maintenance of the PCNA-DNA complex during the process of product resolution. Glutaraldehyde cross-links proteins allowing the protein-protein interaction to be observed, but it does not cross-link protein to DNA. After the loading, glutaraldehyde treatment fixes the PCNA ring structure and therefore maintains its association with the DNA substrate, allowing for relatively harsh treatment of the reaction product for analysis. All other protein-DNA interactions should be eliminated by the presence of SDS. As shown in Figure 2.31, the PCNA loading product was resolved by agarose gel electrophoresis after glutaraldehyde treatment. Crosslinking and gel shift assay enabled the analysis of PCNA loading on the recombination substrate generated from synthetic oligonucleotides, using 2% agarose gel.
CHAPTER 3 : LOADED PCNA REGULATES THE ACTIVITY OF DNA POLYMERASE δ, η, AND ζ

3.1. Introduction

Depending on the nature of DNA damage, lesions are repaired by different DNA repair pathways. Ideally, DNA damage should be repaired faithfully to avoid mutation and the disturbance of genome integrity that can lead to the development of diseases. In general, accurate repair of DNA damage requires a complementary DNA strand that can be used as a template for DNA polymerization. For example, if only one strand is broken, such as occurs with base damage and nucleotide damage, the other strand of the double helix can serve as the template for accurate repair. However, this is impractical for DSB repair due to the simultaneous break of both DNA strands [4]. During DSB repair, the accuracy of the repair is determined by 1) the availability of homologous template 2) the degree of homology between broken DNA and the template DNA, and 3) the fidelity of the DNA polymerase utilized [319]. If a DSB is repaired by HR between sister chromatids, the first two points above are not an issue. Therefore, the last point pertaining to post invasion DNA synthesis is the crucial step determining the fidelity of DSB repair [319, 320]. The DNA polymerases involved in HR have not been clearly defined. Several DNA polymerases, including Pol δ, η, and ζ, have been implicated in this process.

Pol δ is well known as a major player in the DNA replication machinery. It is responsible for the synthesis of Okazaki fragments on the lagging strand [321, 322]. In addition to its
role during DNA replication, many studies have suggested the involvement of Pol δ in HR-mediated DSB repair. For example, genetic studies in yeast indicate that Pol δ is involved in mitotic gene conversion [282], meiotic recombination [283], repair of γ-ray-induced DNA damage [284], and HO endonuclease-induced gene conversion [82]. A genetic study suggests that DSB repair in yeast requires Pol δ [198]. Pol32, a subunit of Pol δ, is apparently required for break-induced replication and telomerase-independent telomere maintenance [323].

![Figure 3.1. Pol η and Pol ζ are involved in translesion DNA synthesis.](image)

Pol η was the first identified translesion synthesis (TLS) polymerase (Figure 3.1), and it is also the most extensively studied polymerase associated with TLS. Defects in Pol η activity are linked to the development of cancer [253]. Like other TLS polymerases, Pol η can polymerize across UV-induced damage, such as thymidine dimers (cyclobutane pyrimidine dimers [CPDs]) and (6-4) photoproducts in an almost error-free manner [324, 325]. Pol η is also able to bypass a variety of other DNA lesions, including 8-oxoguanine, O6-methylguanine, benzo[a]pyrene-N2-deoxyguanine DNA adducts, as well as DNA
damage created by treatment with anticancer drugs, like cisplatin, gemcitabine, and oxaliplatin [326-330]. The catalytic polymerase domain of Pol η is located at its N-terminus and is highly conserved among the Y family DNA polymerases [331]. The Pol η catalytic domain has a more structurally open configuration than that of replicative polymerases. This feature allows the enzyme to accommodate bulky DNA lesions, like thymidine dimers [187, 332]. The C-terminus of Pol η contains a nuclear localization signal and PCNA binding motif, which allows the enzyme to be recruited to DNA lesion sites [253, 333]. Pol η lacks a 3’ to 5’ exonuclease proofreading activity and it is generally considered to be a low processivity polymerase. During translesion synthesis, Pol η bypasses the lesion site and then only synthesizes the complement of few adjacent nucleotides before the polymerase switch removes the enzyme, allowing the replicative polymerase to continue strand elongation [334, 335].

In humans, Pol η was first described as the enzyme lacking in a variant form of xeroderma pigmentosum (XPV), a rare autosomal-recessive disorder that is characterized by extreme sensitivity to UV light [336-338]. The symptoms of XPV patients resemble those suffering from other forms of xeroderma pigmentosum (XPA to XPG). The XPA to XPG genes are all defective in the nucleotide excision repair (NER) pathway in XPA to XPG, XPV gene is not defective for the NER pathway [339], indicating that Pol η is involved in the repair of UV-damage independent of NER pathway.
Pol ζ is believed to be a heterodimeric complex comprised of a catalytic subunit, Rev3, and an accessory subunit, Rev7 that has been shown to considerably increase the polymerase activity Pol ζ [248]. The catalytic domain of Pol ζ is located at the C terminus of Rev3, while the N terminus of Rev3 possesses a protein interaction domain. Different from other B family DNA polymerases, Pol ζ lacks the 3′ to 5′ exonuclease activity for proofreading. On the contrary, it has been suggested that inaccurate synthesis by Pol ζ contributes significantly to mutagenesis in the majority of eukaryotic organisms [340, 341]. In fact, inaccurate translesion synthesis by Pol ζ is responsible for a majority of spontaneous mutagenesis and virtually all induced mutagenesis in mammals [342].

Like Pol η, Pol ζ plays a critical role in DNA damage tolerance and the bypass of UV irradiation-induced lesions (Figure 3.1) [343, 344]. However, unlike Pol η, Pol ζ only inefficiently incorporates nucleotides opposite to DNA lesions. Therefore, it is suggested that Pol ζ might actually perform primer extension following the insertion of a nucleotide across the lesion by another TLS polymerase [345-347]. Pol ζ is also involved in recombination-independent repair of intra-strand crosslinks, which requires Rev1 and monoubiquitinated PCNA [238, 343, 348]. Additionally, it has been demonstrated that the mismatch repair system regulates Pol ζ-dependent mutagenesis in S. cerevisiae [349]. Homozygous knockout of mouse REV3 resulted in embryonic lethality [350-352]. To overcome this, conditional knockout mice were created, that showed that deficiency of Pol ζ resulted in elevated incidence of cancer [353]. Pol ζ protein expression levels are
reportedly low in most cell types, and studies done with *S. cerevisiae* showed increased mutagenesis and resistance upon UV irradiation when Pol ζ was upregulated [354].

The results from studies in various species suggest the contribution of Pol η and Pol ζ in DSBs repair by HR. Studies of HR in vertebrate systems have shown that translesion polymerases Pol η and Pol ζ contribute to HR. Pol η-deficient chicken DT40 cells have defects in gene conversion at the immunoglobulin locus [285], and purified human Pol η can catalyze DNA synthesis within a synthetic D-loop [286]. Loss of Pol ζ function (*REV3*−/−) resulted in increased sensitivity to DNA damaging reagents in mouse [287] and chicken cells [288, 289]. Although Pol ζ is not essential for HR in yeast, the deletion of the yeast *REV3* gene greatly decreases the mutation rate near DSB sites [272], likely because Pol ζ is a low fidelity polymerase and its utilization introduces mutations. These observations implicate that Pol ζ is involved in HR.

Since DNA polymerization is an essential step in HR repair of DSBs, identification of the DNA polymerases that carry out post invasion DNA synthesis is important to understand the molecular mechanisms of DSB repair (Figure 3.2). In this chapter, DNA synthesis on the homologous recombination intermediates, the D-loop, by Pol δ, η, and ζ was analyzed and compared.
3.2. Material and Methods

3.2.1. Construction of plasmids for DNA polymerase δ, η, and ζ expression

A plasmid for expression of Pol η with a C-terminus His6 tag (pET-RAD30) was constructed by cloning the RAD30 ORF between the NdeI and XhoI sites of pET21a (Novagen). A plasmid for coexpression of Rev3 and Rev7 (pESCURA-REV3/7) was constructed by cloning REV3 between SalI-KpnI and REV7 between BclI-PacI in pESC-URA (Stratagene). REV7 was fused with a FLAG affinity tag at its N-terminus in this construct. Plasmids for expression of Pol δ (Pol3, Pol31, Pol32 complex) were constructed as follows. POL3 (in EcoRI/SacI) and POL31 (in XmaI/XhoI) were cloned into pESC-TRP (Stratagene) to produce pESCTRP-POL3/31. POL32 gene was first cloned into pET28pp to introduce an N-terminal His6-tag that is cleavable by PreScission protease (GE Healthcare). Then the tagged POL32 construct was amplified by PCR and re-cloned into pESC-URA (BamHI and XhoI) to produce pESCURA-POL32. All the DNA constructs were sequenced.

3.2.2. Expression and purification of Pol η, Pol δ, and Pol ζ

Pol η was expressed in E. coli BLR (DE3) cells harboring pET-RAD30 and pCodonPlus (Agilent Technologies) plasmids. The procedure used for protein expression and Ni-
Sepharose fractionation were the same as for PCNA purification (see Materials and Method of Chapter 2). Then, SP-Sepharose fractionation was carried out as described for RFC preparation (see Materials and Method of Chapter 2). Pol η was eluted from SP-Sepharose at about 200-350 mM NaCl. Peak fractions were pooled and diluted with 2 volumes of TGEB buffer without NaCl to decrease the NaCl concentration to about 100 mM. The sample was then loaded on a 5 ml Q-Sepharose FF pre-equilibrated with TGEB buffer containing 100 mM NaCl. The flow-through from Q-Sepharose was collected and directly loaded onto 5 ml of SP-Sepharose pre-equilibrated with TGEB containing 100 mM NaCl. The column was subjected to a 40 ml linear gradient from 100 to 500 mM NaCl in TGEB. Pol η was eluted at about 220-340 mM NaCl. The peak fractions were pooled, concentrated by Amicon ultrafiltration (Millipore), divided into small aliquots, and then stored at -80°C.

Pol δ was overexpressed in yeast BJ5465 (MATa ura3–52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1) that was freshly co-transformed with pESCURA-POL32 and pESCSTR-POL3/31. A single colony was picked from a selective SCD plate and inoculated into 5 ml SCD medium. After 18-24 hours of cultivation at 30°C with vigorous shaking, 3 ml of the culture was transferred into 30 ml SCD selective medium. The next day 25 ml of the culture was inoculated into 500 ml of selective SCGL medium (6.7 g/l yeast nitrogen base, 3% (v/v) glycerol, 2% (v/v) lactic acid, and required amino acids). When the OD_{600} reached 0.8-1 (after about 24 hours), 500 ml of YPGL (10 g/l yeast extract, 20 g/l peptone, 3% (v/v) glycerol, and 2% (v/v) of lactic acid) was added
and the cultivation was continued for an additional 3 hours. Then solid galactose (final 2% (w/v)) was added to induce Pol δ expression. Cells were harvested after 4 hours and stored at -80°C. For Pol δ purification, thawed cells were resuspended in lysis buffer (50 mM Tris-HCl pH7.5, 5% glycerol, 1 mM EDTA, and 5 mM β-mercaptoethanol) containing 500 mM NaCl and 1 mM PMSF. Cells were lysed by vortexing with glass beads (10 times repeat of 30 sec mixing followed by 30 sec on ice). The cell lysate was cleared by centrifugation at 20,000 rpm for 30 min at 4°C using a JA-25.50 rotor. Pol δ was purified by Ni-Sepharose and SP-Sepharose FF as described for RFC purification (see Chapter 2 Material and Methods for details), except that the lysis buffer and wash buffer-1 for Ni-Sepharose contained 500 mM NaCl. Pol δ was eluted from SP-Sepharose at 280-350 mM NaCl. The peak fractions were pooled, divided into small aliquots and stored at -80°C.

Pol ζ (Rev3-Rev7 complex) was overexpressed in BJ5465 harboring pESCURA-REV3/7 by the same protocol as used for Pol δ expression. Thawed cells were suspended in 20 ml of TGE_{500} buffer (30 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, and 500 mM NaCl) containing 1 mM PMSF and lysed with glass beads as described for Pol δ purification. TritonX-100 was then added to a final concentration of 1%, followed by incubation on ice for 15 min. The sample was then cleared by centrifugation at 20,000 rpm for 30 min using JA-25.50 rotor. The supernatant was mixed with 0.1 ml of EZview Red ANTI-FLAG M2 Affinity Gel (Sigma) that was pre-equilibrated in TGE_{500} and incubated at 4°C for 2.5 hours with gentle shaking. The resin was recovered in an Econo-
column (BioRad) and washed sequentially with 25 ml of TGE500, 10 ml of TGE500 containing 1 mM ATP and 5 mM MgAc, and then with another 25 ml of TGE500 buffer. Pol ζ was eluted from the resin with 3 ml of TGE500 buffer containing 150 μg/ml of FLAG peptide after incubation at 4°C for 30 min with gentle mixing. The eluate was dialyzed against TGEB buffer containing 50 mM NaCl, and stored at -80°C as small aliquots. Concentrations of the purified proteins were determined by using Coomassie (Bradford) Protein Assay Kit (Thermo Scientific).

3.2.3. DNA substrates for polymerase assay

DL and C2 were generated in the same way as described in Chapter 2 for the clamp loading assay, except for that TSO319 was labeled with γ-32P-ATP (Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs). To analyze longer DNA synthesis, a plasmid-sized dsDNA with 457 unpaired nucleotides “bubble” was produced as follows (Figure 3.3). First, BluescriptSK- and SK+ circular ssDNA were separately annealed with oligonucleotides that were complementary to the KpnI sites (KpnI-F to SK-, KpnI-R to SK+). The annealing products were then linearized by KpnI digestion and purified from agarose gel. To generate short-Y substrate, Bluescript SK- and SK+ circular ssDNA were separately annealed with oligonucleotide (TSO418, see Chapter 2 Material and Methods for sequence) that was complementary to the DraIII site. The annealed products were then linearized by DraIII digestion. Linearized SK+ and SK- ssDNA were mixed and annealed as described above. KpnI was then added to the annealing product. Then short-Y substrate was purified from 1.5% agarose gel.
3.2.4. DNA polymerase assay

Complete reactions were carried out in buffer containing 20 mM Na-Hepes (7.5), 1 mM DTT, 10 mM MgCl₂, 0.1 mg/ml BSA, and 0.1 mM (each) dNTP. 0.2 pmol of synthetic DNA substrate containing a ^{32}P-labeled oligonucleotide primer (29-mer; TSO319) was incubated with 0.8 pmol of RPA for 5 min on ice. Then 0.2 pmol of PCNA and 0.8 pmol of RFC were added and incubated for 3 min at 37°C to carry out clamp loading. Then 0.2 pmol of DNA polymerase (Pol δ, η, or ζ) was added to start DNA synthesis. For the Pol ζ reaction, the NaCl concentration was adjusted to 100 mM before addition of the polymerase. The final volume of the reaction was 10 μl. In the reactions using Bluescript-based substrates, the DNA substrate (0.05 pmol of Bubble, 0.1 pmol of Y-shape, or 0.1 pmol of linear Bluescript SK+ ssDNA) was first incubated with 0.1 pmol of ^{32}P-labeled oligonucleotide primer (40-mer; TSO400) to allow primer annealing (65°C for 3 min
followed by 37°C 5 min), 1.5 pmol of RPA was then added and incubated for 3 min at 37°C. PCNA (0.1 pmol) and RFC (0.4 pmol) were then added and incubated for 3 min. Pol η, ζ, or δ (0.1 pmol, or 0.5 U of Klenow exo- for control reactions) was added to start DNA synthesis. The reactions were stopped at indicated times by adding 40 µl of formamide dye (95% formamide, 0.05% bromophenol blue, 10 mM EDTA). Products were separated by electrophoresis through 6% (plasmid substrates) or 12% (synthetic substrates) polyacrylamide gel in TBE buffer containing 7 M urea. Radioactive DNA products were analyzed as described above for clamp loading (see Chapter 2 Material and Methods).

3.3. Results

3.3.1. DNA polymerase expression and purification

Pol δ of S. cerevisiae is a complex of Pol3, Pol31, and Pol32 subunits (predicted molecular weight are 125 kDa, 55 kDa, 43 kDa, respectively), in which the largest subunit (Pol3) is the catalytic subunit. Both Pol3 and Pol32 have been shown to interact with PCNA. To purify the heterotrimeric complex from yeast, the Pol32 subunit was tagged with a His6 tag that can be released by PreScission protease cleavage. The other two subunits were expressed as untagged native forms. The expression of these genes was under the control of galactose inducible promoters. Because Pol δ is one of the major replicative polymerases, its overexpression is likely toxic to the host strain. Therefore, the induction time was tightly controlled. To achieve a relatively high level of protein expression, the Pol δ expression strain was first cultured in non-inducing SCGL medium
that contained glycerol and lactic acid as carbon sources. When the OD$_{600}$ of the culture reached about 1, rich medium (YPD) was added to boost cell growth for 3 hours. Galactose was then added to a final of 2% (w/v) followed by 4 hours of induction of Pol $\delta$ expression. About 5 grams of wet cells were produced from one liter of culture. The harvested cells were lysed by beating with glass beads, and cleared by centrifugation. The Pol $\delta$ complex was purified by Ni-Sepharose affinity chromatography (Figure 3.4, See Materials and Methods for more details). In the Ni-Eluate, three bands corresponding to the predicted molecular weight of Pol3, Pol31, and Pol32 were identified, showing that Pol 3 and Pol 31 co-purified with Pol 32 and formed a complex in the cell.

Figure 3.4. Pol $\delta$ (Pol 3, Pol 31, and Pol 32 complex) purification by Ni-Sepharose affinity chromatography.
Pol $\delta$ was overexpressed in yeast strain that was freshly co-transformed with pESCURA-POL32 and pESCTR-POL3/31. Harvested cells were lysed by vortexing with glass beads and the cell lysate was cleared by centrifugation. The supernatant was loaded onto Ni-Sepharose affinity resin with 10 mM imidazole in the binding buffer. The bound proteins were eluted with buffer contained 200 mM of imidazole. Different fractions from the purification procedure were analyzed by 12% SDS-PAGE.
Due to the presence of contaminating proteins in the Ni-Eluate, the Pol δ was further purified by SP-Sepharose ion exchange chromatography (Figure 3.5). After loading and extensive washing with low NaCl buffer, Pol δ was eluted by NaCl gradient. The three subunits of Pol δ eluted between 280-350 mM NaCl, suggesting a stable interaction among the subunits. Although the Pol δ subunits in the gel looked free from contamination, the band intensities of the subunits were not stoichiometric, suggesting that free subunits or partial complexes were still included in the peak fractions. Therefore, I saved only the main fraction (fraction 13), which was stored for further biochemical analysis. Fractions 11, 12, 14-16 were pooled as a side fractions and used for further purification.

**Figure 3.5. Pol δ purification by SP-Sepharose ion exchange chromatography.**
The eluate from Ni-Sepharose affinity chromatography was directly loaded on a 5 ml SP-Sepharose column, and bound proteins were eluted with a linear gradient of NaCl concentration from 100 to 500 mM. The eluate was collected as 3 ml fractions and fractions around Pol δ peak were analyzed by 12% SDS-PAGE. The approximate NaCl concentrations in the fraction 9 and 17 were 250 and 400 mM, respectively. Fraction 13 was divided into small aliquots and store at -80°C. In lane 17, there was a band with very close mobility to Pol31. Later analysis revealed that this band was slightly smaller than Pol31 (data not shown) and probably a contaminating protein.
PreScission protease is a fusion protein consisting of human rhinovirus 3C protease and GST moiety. It specifically cleaves between the Gln and Gly residues of the recognition sequence of LeuGluValLeuPheGln/GlyPro. Proteolytic treatment should release the His\textsubscript{6} tag from Pol32 and leave only three amino acid residues upstream of the native start codon of Pol32 minimizing the unwanted impact from the affinity purification tag on the biochemical activity of the recombinant protein.

After PreScission protease treatment, the side fractions of SP-Sepharose chromatography were loaded onto a 5 ml Heparin column (Figure 3.6). The column was then washed with a linear NaCl concentration gradient; Pol31 and Pol32 were eluted at lower NaCl (around 400 mM NaCl). Then, the three subunits of Pol \( \delta \) co-eluted at around 500 mM to 600 mM NaCl. The ratio of Pol3, Pol31 and Pol32 in Fraction 18, 19, and 20 was approximately 1:1:1 (Figure 3.6). Therefore, these fractions were pooled and concentrated by Amicon ultra filtration. Different fractions collected during the Pol \( \delta \) purification process were analyzed by Western blot using HisProbe (Figure 3.7). His\textsubscript{6} tagged Pol32 was detected in the eluates from the Ni-affinity column any SP-Sepharose column, but not in the eluate from the Heparin column, indicating that the PreScission protease treatment successfully removed the His\textsubscript{6} tag from Pol32.
Side fraction of Ni-Sepharose chromatography was treated with PreScission protease (GE Healthcare) at 4°C for 12 hours. The sample was then loaded on a 5 ml Heparin affinity chromatography column, followed by extensive wash with the binding buffer until the UV$_{280}$ reached the baseline. The bound proteins were eluted with a linear gradient of NaCl concentration from 100 to 650 mM. The eluate was collected as 1 ml fractions and analyzed by 12% SDS-PAGE. Fractions 18, 19 and 20 were pooled, concentrated by Amicon ultra-filtration, divided into small aliquots, and stored at -80°C.
Figure 3.7. Western blot analysis of Pol δ purification fractions.
Fractions from Pol δ Ni-Sepharose affinity purification, SP-Sepharose ion exchange purification, and Heparin affinity purification were analyzed by Western blot with HisProbe that directly interacts with His-tagged proteins.

Pol η of *S. cerevisiae* is a single polypeptide polymerase encoded by the *RAD30* gene. To overexpress Pol η in *E. coli*, the *RAD30* gene was cloned into the pET21 expression vector. *RAD30* was fused with a His6 tag at its C terminus. The plasmid was transformed into *E. coli* BL21 (DE3) strain for protein expression. After induction, harvested cells were lysed by sonication and the soluble fraction was separated by centrifugation. Ni-Sepharose affinity was then carried out to purify Pol η (Figure 3.8, top panel). Under these conditions, Pol η was largely soluble and bound to Ni-Sepharose efficiently. One step of Ni affinity purification gave a Pol η preparation with higher than 90% of homogeneity. The Ni eluate was further purified by SP-Sepharose ion exchange chromatography (Figure 3.8). Pol η was eluted from the SP-Sepharose between 230-350 mM NaCl.
The SP-Sepharose column efficiently concentrated Pol η from the Ni eluate, however, some contaminationing proteins were still present. To further purify the Pol η protein, pooled Pol η fractions from SP-Sepharose were diluted to decrease the NaCl concentration and loaded onto a 5 ml Q-Sepharose column (Figure 3.9). A significant amount of Pol η bound to the resin. Fractions 6, 7, 8, and 9, eluting at 240-320 mM NaCl, were pooled and saved. The double band observed on the gel shown in Figure 3.5 was likely an electrophoresis artifact, because the 75 kDa marker also showed a double band. Some Pol η was also detected in the column flow through (~ 120 ml), as predicted from the pI of Pol η (8.25).

Because the flow through from Q-Sepharose contained Pol η protein, it was loaded on a second SP-Sepharose ion exchange chromatography, under the same conditions as the first loading (Figure 3.10). Following elution, the Pol η homogeneity was higher than 95%. Fractions 5-10 shown in Figure 3.10 were pooled and concentrated by Amicon ultra filtration.
Figure 3.8. Pol η (Rad30) purification by Ni-Sepharose affinity chromatography and SP-Sepharose ion-exchange chromatography.

The Pol η expressing plasmid (pET-RAD30) was transformed into *E. coli* strain (BLR, DE3). The cells were cultivated at 37°C and induced with 0.5 mM IPTG for protein expression at 20°C for 8 hours. Harvested cells were lysed by sonication and the lysate was cleared by centrifugation. The supernatant was loaded on Ni-Sepharose affinity purification column. After washing with the binding buffer contained 10 mM of imidazole, the bound proteins were eluted with buffer contain 200 mM imidazole. The eluate, containing 100 mM NaCl, was directly loaded SP-Sepharose ion exchange column, followed by extensive washing with binding buffer until the OD280 reached the baseline. The bound proteins were eluted with a linear gradient from 100 to 500 mM NaCl. The eluate was collected as 2 ml fractions and analyzed by 12% SDS-PAGE. Fractions 7-18 were pooled for further purification.
Figure 3.9. Pol η purification by Q-Sepharose ion-exchange chromatography.
Pooled fractions from SP-Sepharose ion exchange purification were diluted three fold with buffer without NaCl to decrease NaCl concentration. The diluted sample (~100 ml) was loaded on a 5 ml Q-Sepharose ion exchange column. The flow-through was collected for further purification by SP-Sepharose. The Q-Sepharose resin was washed with the binding buffer until the OD280 reached the baseline. The bound proteins were eluted with a linear gradient from 100 to 500 mM NaCl. The eluate was collected as 4 ml fractions and analyzed by 10% SDS-PAGE. Fractions 6, 7, 8, and 9 were pooled as a side fraction and stored at -80°C.

Figure 3.10. Pol η purification by SP-Sepharose ion-exchange chromatography.
The flow-through of Q-Sepharose chromatography was directly loaded on SP-Sepharose and was washed extensively with the binding buffer until the OD280 reached the baseline. The bound proteins were eluted with a linear gradient from 100 to 500 mM NaCl, as described in Figure 3.8. The eluate was collected as 2 ml fractions and analyzed by 10% SDS-PAGE. Fractions 5-10 were pooled, concentrated, divided into small aliquots, and stored at -80°C.
The Pol ζ used in this study is a protein complex composed of Rev3 and Rev7 protein from *S. cerevisiae*. Rev3 is the catalytic subunit and Rev7 is an accessory protein. Rev7 was tagged with a FLAG affinity tag to enable the affinity purification using a FLAG resin. The pESCU-REV3/7 plasmid was transformed into yeast strain BJ5465 and transformants were selected by growth on synthetic complete (SC)-dextrose medium without uracil (SCD-U medium). Two transformants were picked and inoculated into 5 ml of SC-galactose (SC medium containing galactose) in which expression of both subunits should be induced. The harvested cells were lysed, centrifuged, and fractions tested for FLAG-Rev7 expression by Western blot with anti-FLAG antibody (Figure 3.11). FLAG-Rev7 was only detected in the insoluble fraction from both transformants, suggesting a very low solubility of the Rev7 protein. Another signal of approximately 100 kDa molecular weight was observed in all fractions, likely due to a non-specific cross reaction of an endogenous yeast protein with anti-FLAG antibody.
Figure 3.11. Western blot analysis of FLAG-Rev7 expression.
The Pol ζ (Rev3-Rev7 complex) expressing plasmid pESCURA-REV3/7 was introduced into yeast strain BJ5465. Two transformants were inoculated into SC-galactose medium and cultivated for 36 hours at 30°C. Cells were harvested and lysed with glass beads. The soluble and insoluble fractions were separated by centrifugation and analyzed by Western blotting with anti-FLAG antibody.

Then, the expression of Pol ζ was expanded to a one liter culture. The expression of FLAG-Rev7 was confirmed by Western blot analysis using Anti-FLAG antibody (Figure 3.12, B). The harvested cells were lysed by vortexing with glass beads, cleared by centrifugation, and the supernatant was then subjected to FLAG affinity purification. The FLAG resin has very high specificity to FLAG tagged protein. This one step of affinity purification produced a Pol ζ preparation that was nearly homogenous, although the total amount of soluble protein was not very high (Figure 3.12, A).
Figure 3.12. Pol ζ (Rev3-Rev7 complex) purification by anti-FLAG affinity purification.

(A) Cells (~5 g) expressing Pol ζ were suspended in cell lysis buffer and lysed by vortexing with glass beads and cleared by centrifugation. The supernatant was mixed with 0.1 ml of EZview Red ANTI-FLAG M2 Affinity Gel (Sigma) that was pre-equilibrated with lysis buffer and incubated at 4°C for 2.5 hours with gentle shaking. Resin was recovered in an Econo-column (BioRad) and washed. Pol ζ was eluted from the resin with lysis buffer containing 150 μg/ml of FLAG peptide by incubating at 4°C for 30 min with gentle mixing. The eluate was dialyzed, divided into small aliquots, and stored at -80°C. (B) Western analysis with Anti-FLAG antibody.

3.3.2. PCNA stimulates Pol δ during the post-invasion DNA synthesis

Genetic and biochemical studies in yeast indicated that both PCNA and Pol δ play major roles in HR [82, 282-284]. Studies with vertebrate systems showed that Pol η and ζ are also involved in HR [285, 288]. To further characterize the roles of these polymerases in
HR, I investigated the ability of Pol δ (Pol3-Pol31-Pol32 complex), Pol η (Rad30), and Pol ζ (Rev3-Rev7 complex) to extend the invading primer strand within a synthetic D-loop in the presence and the absence of loaded PCNA (Figure 3.14 and 3.15).

To distinguish primer recognition and strand displacement in D-loop extension, I used the DL substrate that had a one-nucleotide ssDNA region beyond the 3’-end of the primer (See Figure 3.13, B). The polymerase can extend the primer by 1-nt prior to encountering the dsDNA region of the DL substrate. Pol δ showed the poorest ability to extend the D-loop (DL) in the absence of the loaded PCNA (Figure 3.14, compare lane 2, 4 and 6; and Figure 3.15), consistent to the previous observations [197, 286]. Interestingly, however, Pol δ extended almost all the primers for 1 to 2-nt (Figure 3.14, lane 4), indicating that Pol δ efficiently recognized the primer within the D-loop in the absence of PCNA, but processive synthesis was obstructed by dsDNA. When Pol δ was added to DL that had loaded with PCNA (Figure 3.14, lane 5 and Figure 3.15, lane 11-13), the majority of the primers were fully extended to the end of the template, indicating that PCNA greatly stimulated Pol δ’s ability to displace the complementary DNA strand during DNA synthesis. Both the inability of Pol δ to extend the D-loop in the absence of PCNA and the stimulation of processive DNA synthesis by PCNA were reproduced with the plasmid-sized substrate with a 457-nt loop (Figure 3.13 "Bubble" and “Y-shape, and Figure 3.16, lane 7, 8 and 11), indicating that these activities are unrelated to the loop-size.
Figure 3.13. Structure of oligonucleotide and plasmid based substrates used in DNA polymerase assay.

(A) Illustration of DNA polymerase assay on DL containing ³²P-labeled invading DNA strand (red arrow). Non-radiolabeled PCNA was loaded on DL, and then Pol δ, η, or ζ was added to start DNA synthesis. (B) Structure of synthetic oligonucleotides based DNA substrates (DL and C2) used for polymerase assay. (C) Plasmid-sized substrates that were used in polymerase assay. ³²P-labeled primer (40-mer, red arrow) was annealed to 3.0 kb dsDNA containing 457-nt non-complementary region (Bubble), which was produced from Bluescript SK+ and SK- ssDNA molecules (see details in Materials and Methods). The same primer was annealed to a Y-shaped derivative (Y-shape), and linearized Bluescript SK+ ssDNA (Linear).
Figure 3.14. Comparison of D-loop extension by Pol δ, Pol η, and Pol ζ in the presence or absence of PCNA.
The DL substrate was incubated with PCNA and RFC for 3 min to allow PCNA loading, and then with Pol η, δ, or ζ for 15 min. DNA products were analyzed by denaturing polyacrylamide gel electrophoresis.

Figure 3.15. Time course of C2 and DL extension by Pol δ, Pol η, and Pol ζ in the presence or absence of PCNA.
(A) Time courses of the DNA synthesis in DL substrate by indicated polymerases were analyzed in the presence or absence PCNA/RFC. (B) The same reactions as (A) were repeated except that simple primed substrate (C2) was used instead of DL.
Figure 3.16. Plasmid based D-loop extension by Pol δ, Pol η, and Pol ζ.
Plasmid-sized DNA substrates shown in Figure 3.13, C were used for the PCNA loading and DNA synthesis reactions by Pol δ, η, ζ, or klenow fragment (K). Lane 1, 10, and 13 contained size markers that were produced by labeling 100bp DNA ladder (New England Biolabs) with γ-32P-ATP by T4 polynucleotide Kinase followed by denaturing in formamide.

I detected an unexpected premature termination product in the Pol δ extension reactions on the simple primed substrate C2 (indicated by * in Figure 3.15, B). It was not detected on the DL that had the same sequence as C2 (Figure, 3.15, A), or on other primed DNA substrates (Figure 3.17, substrate C3). I thought that this sequence-specific termination was due to the secondary structure of the C2 substrate. In the PCNA loading experiment described in Chapter 2, I observed that loading of PCNA on the D-loop substrates containing 3’ junctions (DL-L and DL2-L) was not better than the D-loop substrates containing 5’ junctions (DL-R and DL2-R), indicating that efficient PCNA loading on D-
loop was not dependent on a 3’-junction. That was also confirmed by analyzing the PCNA-dependent extension of the D-loops by Pol δ (Figure 3.17), where DL, DL2-L, DL2-R gave comparable amounts of full length products, indicating that PCNA was loaded onto these substrates with similar efficiency.

Figure 3.17. PCNA-dependent and independent DNA syntheses by Pol δ on DNA substrates with different structures.
(A) PCNA was loaded onto different D-loops (DL, DL2-L, and DL2-R) and simple primed ssDNA substrates (C2, and C3) as indicated, and then Pol δ was added to start DNA synthesis. DL and C2 shared the same sequence, and so are DL2-L and C3. However, DL and DL2-L have no sequence homology. (B) Quantification of the full length products shown lanes 2, 3, 7, 9, 10, 11, 12, and 16 in (A).
3.3.3. Pol δ, η, and ζ respond differently to PCNA on the D-loop

When yeast Pol η was added, the primer within the D-loop was extended efficiently without the loaded PCNA (Figure 3.14, lane 2; Figure 3.15, A, lane 2-4), consistent with the previous observation of human Pol η [286]. Extension was not significantly enhanced by loaded PCNA (Figure 3.14, lane 3, Figure 3.15, A, lane 5-7). However, on the plasmid-sized D-loop, extension by Pol η was moderately but clearly stimulated by the loaded PCNA (Figure 3.16, lane 3 and 4). This may suggest that Pol η does not efficiently interact with PCNA, but that once they interact, a Pol η-PCNA complex can extend a primer more than 100nt. In control reactions (Fig. 3.15, B), both Pol η and δ extended the primer on simple primed substrate both in the presence and the absence of the loaded PCNA. Pol ζ extended the simple primed substrate less efficiently than Pol η and δ (Figure 3.15, B). Interestingly, Pol ζ extended the DL substrate slightly more efficiently than Pol δ in the absence of PCNA (Figure 3.14 lane 6, and Figure 3.15, A, lane 14-16), indicating that Pol ζ extension was not strongly inhibited by D-loop structure. However, no stimulation was observed in the presence of PCNA (Figure 3.14, 3.15, and 3.16 “Pol ζ”).

I also observed a small fraction of unused primer in the presence of PCNA and RFC (Figure 3.14, 3.15, and 3.16). This was observed consistently in the reactions containing RFC and was independent of PCNA or polymerase type. Since the reaction contained four times more RFC than DNA substrate to allow efficient PCNA loading in short
reaction time, the excess RFC on the DNA might inhibit primer recognition by polymerases. I do not believe that this is biologically significant.

### 3.3.4. Loaded PCNA counteracts the Rad51-mediated inhibition of DNA synthesis

A previous study has shown that Rad51 on the invading DNA strand inhibits DNA synthesis [355]. I have shown here that Rad51 inhibits the loading of PCNA on the D-loop. Therefore, I expected that Rad51 should also inhibit PCNA-dependent D-loop extension by polymerases. However, it was unclear whether Rad51 would inhibit the D-loop extension if PCNA had been already loaded (Figure 3.18, A). To address this question, I examined the effect of Rad51 on DNA synthesis by Pol δ and η in the D-loop, in the presence or the absence of loaded PCNA (Figure 3.18). As expected, in the absence of PCNA, Rad51 strongly inhibited both Pol δ and η extension of the DL substrate (Figure 3.18 B and C, lanes 2-8, blue curves in D and E). Importantly, if PCNA was pre-loaded, Rad51 inhibition of DNA synthesis by Pol η (Figure 3.17 B, lanes 9-15 and D) and Pol δ (Figure 3.18 C, lanes 9-15 and E) was less pronounced. These results indicate that loaded PCNA lessens the inhibition by Rad51.
Figure 3.18. Pre-loaded PCNA counteracts Rad51 inhibitory effect on Pol δ and Pol η.

(A) Rad51 was added to DL preloaded with PCNA. DNA synthesis was then started by the addition of Pol δ or η. (B) and (C) Rad51 (0, 0.2, 0.4, 0.6, 0.8, 1.2 and 1.6 µM) was added to 0.02 µM of DL (lane 2-8), or DL pre-loaded with PCNA (lane 9-15). Then DNA synthesis was started by adding 0.02 µM of Pol η (B) or Pol δ (C). (D) and (E) Quantification of the relative percentage of primer usage in (B) and (C), respectively. The values were normalized by setting the value of no-Rad51 reaction to be 100%. Error bars represent standard deviations (n=3).
3.3.5. The 9-1-1 complex did not stimulate the DL synthesis by Pol δ or Pol ζ

Even though the 9-1-1 clamp loading onto the plasmid based substrates was not detected (Figure 2.44), its effect on the activities of Pol δ and Pol ζ was tested. Loading of the 9-1-1 complex onto the DL substrate was performed as described for PCNA loading, followed by addition of DNA polymerase to start DNA synthesis within the D-loop. The activities of Pol δ or Pol ζ were compared in the presence or the absence of the 9-1-1 complex and Rad24-RFC clamp loader complex. DNA synthesis by Pol δ and Pol ζ within the D-loop were unaffected by the presence or the absence of these proteins.
Figure 3.19. The 9-1-1 complex did not stimulate the DL synthesis by Pol δ, and Pol ζ.
DL substrate was incubated with 9-1-1 complex and Rad24-RFC complex for 3 min as indicated, and then with Pol ζ or δ for 15 min. DNA products were analyzed by denaturing polyacrylamide gel electrophoresis.

3.4. Discussion
Homologous recombination is one of the major pathways utilized for the repair of DSBs. The D-loop is one of the early intermediates of HR. HR post strand invasion DNA synthesis is essential for the restoration of lost genetic information. Even though numerous studies have been done to identify the DNA polymerases involved in HR DNA synthesis, which polymerases are used is rather uncertain. Genetic and biochemical evidence support the involvement of the DNA polymerase δ, η, and ζ, which are characterized and compared by the biochemical studies described in this chapter.
The three polymerases studied in this chapter were overexpressed either in *S. cerevisiae* (Pol δ and Pol ζ) or *E. coli* (Pol η). Because these three DNA polymerases are involved in important cellular process, e.g. extensive DNA synthesis during replication by Pol δ, their over-expression is very likely to be toxic to the host cell. This toxicity was indeed observed for Pol δ and Pol ζ overexpression. When the POL3 gene, encoding for the catalytic subunit of Pol δ, was cloned and transformed into an *E. coli* strain for plasmid amplification, no colonies were present after overnight incubation at 37°C, likely due to leaky expression of the polymerase. This problem was overcome by incubating the transformation plate at a lower temperature, 28°C. Even though the cells grow significantly slower at 28°C, the production of the presumably toxic polymerase was presumably reduced. Pol δ induction in yeast culture was also found to be toxic to the host cell. After the addition of galactose to induce Pol δ expression, the yeast cells began to die. Therefore, the cells were cultivated in medium using glycerol and lactic acid as carbon source, and the induction was conducted for short time beginning at relative high cell density.

The studies in this chapter were designed to reconstitute post invasion DNA synthesis during HR *in vitro*. Synthetic DNA substrates and purified recombination factors, including Rad51, PCNA, RFC, Pol δ, η, and ζ were used in these studies. This system allowed me to compare the activity of the three DNA polymerases and their interaction with PCNA during DNA synthesis that occurs at the recombination intermediate, the D-
loop. It has been suggested that Pol δ plays a major role in DNA repair synthesis, and it is considered to be the main polymerase associated with DNA repair primer extension [82, 356]. *In vitro*, as well as *in vivo*, data indicate that a translesion polymerase Pol η is also capable of mediating D-loop primer extension [286]. The interaction of loaded PCNA with DNA polymerases δ, η, and ζ were analyzed here (Figure 3.14, 3.15, and 3.16). In the presence of PCNA, Pol δ activity was greatly stimulated; Pol η activity was stimulated slightly on the plasmid based substrate, consistent with the observation that PCNA interacts more efficiently with Pol δ than with Pol η [357]. When interacting with PCNA, Pol δ extended the primer more than 1 kb, Pol η produced (as expected) short extension products, reflecting the differences in the biochemical properties of these polymerases, including their processivity and strand-displacement activities [358-360]. Pol η can extend a D-loop without loaded PCNA (Figure 3.20, B). Without PCNA, Pol δ was very poor at extending the primer in the D-loop. The D-loop structure itself did not interfere with the polymerase-primer interaction. Therefore, the major role of PCNA in the D-loop extension is to stimulate Pol δ displacement of the interfering DNA strand. In contrast, Pol η extended the D-loop much more efficiently than Pol δ in the absence of PCNA, but the majority of the products were shorter than 100-nt. In the presence of PCNA, the reactions were extended a much longer distance. Taken together, these results suggest that Pol δ and η may have different roles in HR that are dependent on the availability of PCNA. Pol ζ demonstrated only limited ability to extend the primer in the D-loop and extension was not enhanced by PCNA. However, my Pol ζ preparation did not include the Rev1 subunit previously suggested to have a critical role in Pol ζ function.
in HR [361]. Recently, three groups reported that Pol31-Pol32, which was considered as subunits of Pol \( \delta \), is essential subunits of functional Pol \( \zeta \) [362-364]. The potential roles of these proteins in D-loop extension by Pol \( \zeta \) remain to be elucidated.

![Figure 3.20. Pol \( \delta \) and Pol \( \eta \) can synthesize over the D-loop.](image)

My \textit{in vitro} system showed that Pol \( \delta \) and Pol \( \eta \) were more processive in D-loop extension than Pol \( \zeta \) (Figure 3.20). However, my results did not suggest that any of these polymerases had a higher affinity for the D-loop. In this sense, no polymerase was “selected” for association with D-loop \textit{in vitro}, but some polymerases were more capable of primer extension within the DL substate. It is unclear how, or if, DNA polymerases are selected for access at the D-loop \textit{in vivo}.

The identification of Pol \( \delta \) and Pol \( \eta \) as DNA polymerases involved in D-loop extension is a critical step in developing a complete mechanistic understanding of homologous recombination. This will help to unravel the mechanisms that govern the differences between various recombination modes (synthesis-dependent strand annealing, gap repair, double Holliday junction formation, and break-induced replication) that are distinguished by their DNA synthesis reactions [365]. In experiments here, a combination of Pol \( \delta \),
PCNA and RFC was found to be absolutely required for the extension of the primer from the D-loop substrate (Figure 3.20, A), in good agreement with previous biochemical and genetic studies [290, 323].

The effect of Rad51 on post invasion DNA synthesis was also investigated (Figure 3.18). A previous study indicated that Rad51 inhibited primer extension by Klenow enzyme [355]. We found that Rad51 recombinase strongly inhibited PCNA-loading on the D-loop (Figure 2.41) and inhibited DNA synthesis as well (Figure 3.18). Therefore, Rad51 inhibits two essential biochemical processes essential to post-invasion DNA synthesis. It is not clear which of these two activities of Rad51 is more important in regulating HR. However, it seems more likely that PCNA-loading is a key regulatory target of Rad51, because PCNA-loading precedes polymerase-primer interaction [322]. Two pieces of data in this paper are consistent with this idea. First, in Rad51-titration experiments (Figure 2.41, D, Figure 3.18, D and E), the Rad51 concentration needed to inhibit PCNA-loading was lower than that required to inhibit primer extension, indicating that Rad51 is a more effective inhibitor of PCNA-loading than of DNA synthesis per se. Second, when PCNA was pre-loaded on the D-loop, prior to addition of Rad51, the polymerases extend the D-loop more efficiently compared to the reactions without pre-loaded PCNA (Figure 3.18). Although I do not know whether PCNA is or can be loaded before removing Rad51 from the invading strand in vivo, this in vitro result suggests that inhibition of PCNA-loading is more crucial step for inhibition of post-invasion DNA synthesis.
Rad51-mediated inhibition of HR can be abolished by Rad54, which is known to remove Rad51 from dsDNA [355, 366].

In the experiment described in Figure 2.36, PCNA was shown to be loaded onto D-loops with 3’ junctions and 5’ junctions with comparable efficiency. This result was also supported by Pol δ DNA synthesis on different DL substrates in the presence and absence of loaded PCNA (Figure 3.17). Loaded PCNA greatly stimulated Pol δ ability to extend to the end of the template. The more PCNA is loaded, the more full-length product was synthesized. As shown in Figure 3.17, DL2-L and DL2-R gave comparable amounts of full-length product in the presence of loaded PCNA. Because the loaded PCNA clamp can slide freely on the DNA template, the PCNA loaded from the 5’ junction must be able to travel to the primer end and interact with DNA polymerase.

Unexpectedly, Pol δ mediated DNA synthesis on the C2 substrate prematurely terminated in the absence of PCNA. As one of the major replicative DNA polymerases, Pol δ has the greatest polymerase activity among the three polymerases tested. This termination was not observed in reaction using C3 or DL substrates, indicating substrate specificity not related to the activity of Pol δ. The observed premature termination was likely caused by the secondary structure unique to C2 substrate.

My preparation of the 9-1-1 complex did not stimulate the D-loop synthesis by Pol δ or Pol ζ (Figure 3.19). Because no clamp-loading was detected with this complex (Figure
2.44), the lack of polymerase activity stimulation was most likely due to the lack of loaded functional 9-1-1 complex on the DNA substrate. However, it also possible that 9-1-1 was loaded on the DL substrate, but was not able to stimulate the activity of Pol δ and Pol ζ.

In summary, the studies in this chapter provided novel information about the post invasion DNA synthesis step of HR. After strand invasion, Rad54 dissociates Rad51 from the duplex DNA to allow the loading of PCNA and polymerase access to the 3’ end of the invading strand [355]. Loading of PCNA by RFC targets DNA Pol δ to the D loop, leading to efficient and processive DNA synthesis from the invading 3’ end. Pol η might be the preferred DNA polymerase for D-loop synthesis in some cases, because it can catalyze D-loop extension for short distances without loaded PCNA. These findings consolidate the genetic evidence in yeast that suggested a role for Pol δ and Pol η in homologous recombination with biochemical data. The in vitro system described in this chapter offers the opportunity to unravel the molecular mechanisms and regulation of HR downstream of D-loop formation, particularly the post invasion DNA synthesis step. It will, therefore, be enlightening to study further the differences between these processes, and thus elucidate their specific regulatory elements, including the roles of post-translational modifications and protein–protein interactions.
Failures of DSB repair in humans are related to many severe genetic diseases. Post-invasion DNA synthesis is essential for high fidelity repair. Attempts to identify the DNA polymerase involved in this process have been many, but without clear conclusive results. Loss of human Pol $\eta$ causes a xeroderma pigmentosum variant (XP-V) and a predisposition to skin cancer [367]. Pol $\zeta$ is error-prone when repairing UV-damage, and is involved in the UV-induced mutagenesis [287, 368]. Both polymerases have been suggested to be involved in post-invasion DNA synthesis. However, the molecular mechanism of polymerase selection and action is still unclear. Very little is known about the mechanisms that recruits a specific DNA polymerase to recombination intermediates. Recombinases which direct homology search and strand invasion, may have key roles, but the results from research regarding this question are contradictory. One in vitro study shows that Rad51 interacts with Pol $\eta$ and stimulates DNA synthesis [286], suggesting that Rad51 recruits Pol $\eta$ for post-invasion DNA synthesis. However, another in vitro study shows that Rad51 on the D-loop inhibits access of the Klenow fragment, and needs to be removed by Rad54 for DNA synthesis to occur [355]. In vivo observation of post-invasion DNA synthesis during meiosis showed that DNA synthesis starts when Rad51 is removed from the DSB site [286], supporting the observation that removal of Rad51 is required for post-invasion DNA synthesis. This apparent inconsistency needs to be clarified. Furthermore, the meiosis-specific recombinase Dmcl may have a role in recruiting polymerases, thereby defining the specific pathway used in meiotic inter-
homolog recombination. DNA synthesis in a Dmc1 mediated D-loop has never been studied.

Another intriguing aspect regarding post invasion DNA synthesis in HR is the function of the sliding clamps, especially the 9-1-1 clamp (Radl7-Mec3-Ddcl complex). It was reported recently that PCNA is loaded onto D-loop structure and stimulates DNA synthesis by Pol δ [197, 290]. However, the loading of 9-1-1 clamp onto HR intermediate has never been reported. The choice of DNA polymerases affects the accuracy and the extent of post-invasion DNA synthesis and may affect "pathway-choice" of HR together with sliding clamp. My study was designed to explore the possibility that DNA sliding clamps are involved in the D-loop extension step of HR. Although a genetic study indicated that PCNA is involved in HR [82], it remains unclear at which biochemical step PCNA is functioning. The 9-1-1 clamp is also involved in both mitotic and meiotic HR processes [222, 369], but its biochemical contribution to HR remains elusive.
Figure 4.1. Model of PCNA functions in HR.
DNA strand invasion by Rad51 requires RPA, which occupy the ssDNA loop (b). After mediating the strand invasion, Rad51 needs to be removed by Rad54 (c). RPA remaining on the ssDNA recruits RFC, which mediates the loading of PCNA onto the invading DNA strand (d). Loaded PCNA stimulates the activity of Pol δ and η to carry out the post invasion DNA synthesis (f). In the absence of PCNA, Pol η may extend the DNA in lower efficiency (e).

In this study, I described new findings about the functional interactions of RFC, PCNA, DNA polymerases, and Rad51 recombinase on the DNA recombination intermediate.

Based on the results, I propose an updated DSB repair model, focusing on the events after Rad51-mediated strand invasion (Figure 4.1). First, I showed that PCNA loading on the D-loop structure was very efficient. In addition, PCNA-loading onto the D-loop required
the binding of RPA to the displaced ssDNA loop, thereby providing a binding site for RFC which mediates PCNA-loading. Loaded PCNA greatly stimulated Pol δ primer extension within the D-loop. Without PCNA, Pol δ was very poor at extending the D-loop. The D-loop structure itself did not interfere with the polymerase-primer interaction. Therefore, the major role of PCNA in the D-loop extension is to stimulate Pol δ to displace DNA strand. In contrast, Pol η extended the D-loop much more efficiently than Pol δ in the absence of PCNA, but the majority of the products were shorter than 100-nt. PCNA stimulated Pol η to extend the primer for much longer distances. Taken together, these results suggest that Pol δ and η may have different roles in HR, dependent on the availability of PCNA (Figure 4.1, d and e). In this study, Pol ζ demonstrated only a limited ability to extend the D-loop that was not stimulated by PCNA. However, my Pol ζ preparation did not include the Rev1 subunit previously suggested to have a critical role in Pol ζ function in HR [361]. Recently, three groups reported that Pol31-Pol32, which were considered as subunits of Pol δ, were essential subunits of functional Pol ζ [362-364]. The potential roles of these proteins in D-loop extension by Pol ζ remain to be elucidated.

It is known that only one side of the PCNA ring can interact with polymerases [135, 145]. Therefore, if the PCNA-loading is oriented randomly, only half of the loaded PCNA can stimulate polymerase. As shown in Figure 3.15, the majority of the DL substrate produced the full-length product in the presence of PCNA. Importantly, the reaction contained the same amounts (0.2 pmol) of DNA substrate and PCNA, indicating that
majority of PCNA in the reaction was loaded on the D-loop in the right orientation (oriented to the 3’ end of the primer). Previous studies using simple primed ssDNA substrates showed that 3’-oriented loading of PCNA depends on RPA, which directionally binds to ssDNA template 3’ of the invading DNA strand [135, 296]. However, in the D-loop analyzed in this study, RPA binds to the displaced ssDNA, that has the opposite polarity as the template strand, which does not fit into the classic PCNA loading model. The precise molecular architecture of the RPA-RFC-PCNA complex on the D-loop needs to be demonstrated to reconcile this apparent inconsistency.

A previous study indicated that Rad51 inhibited primer extension by Klenow enzyme [355]. I found that Rad51 recombinase strongly inhibited PCNA-loading on the D-loop (Figure 2.38) and inhibited DNA synthesis as well (Figure 3.18). Therefore, Rad51 can inhibit two essential biochemical processes of the post-invasion DNA synthesis. It is not clear which is more important in regulating HR. However, it seems more likely that PCNA-loading is a key regulatory target of Rad51, because PCNA-loading precedes polymerase (Pol δ)-primer interaction [322]. Two pieces of data in this paper are consistent with this idea. First, in Rad51-titration experiments (Figure 3.15 A, Figure 3.18 D and E), the Rad51 concentration needed to inhibit PCNA-loading was lower than that was required to inhibit primer extension, indicating that Rad51 is a more effective inhibitor of PCNA-loading than of DNA synthesis per se. Second, when PCNA was pre-loaded on the D-loop, the Rad51 inhibitory effect was alleviated and the polymerases extended the D-loop more efficiently compared with the reaction without pre-loaded
PCNA (Figure 3.18). Although I do not know whether PCNA can be loaded before removing Rad51 \textit{in vivo}, my \textit{in vitro} result suggests that PCNA-loading is more crucial for the inhibition of post-invasion DNA synthesis. Rad51-mediated inhibitions can be relieved by Rad54, which is known to remove Rad51 from dsDNA [355, 366] (Figure 4.1 C).

The loading of the 9-1-1 complex on the DNA was not detected using the same conditions used for PCNA loading. Instead, some non-specific binding to the RPA or ssDNA was observed (Figure 2.41, B). Even though the 9-1-1 clamp and Rad24 complex were detected by SDS-PAGE, any of the constituent proteins may have lost activity during the process of preparation. Alternatively, the experimental procedure needs to be modified for 9-1-1 loading analysis. The 9-1-1 clamp also did not stimulate the D-loop synthesis by Pol $\delta$ or Pol $\zeta$ (Figure 3.19). Because no 9-1-1 clamp loading was detected (Figure 2.42), the lack of polymerase activity stimulation was most likely due to the lack of loaded functional 9-1-1 on the DNA substrate. However, it also possible that 9-1-1 was loaded on the DL substrate, but was not able to stimulate the activity of Pol $\delta$ and Pol $\zeta$.

HR is essential pathway for faithful repair of DSBs. Post invasion DNA synthesis is the step responsible for the recovery of genetic information lost because of the DSB. This study contributes to our understanding of the mechanisms of HR-mediated DNA repair and maintenance of genome integrity.
REFERENCES


