I, David R Friedmann, hereby submit this original work as part of the requirements for the degree of:

Doctor of Philosophy

in Molecular Genetics, Biochemistry, & Microbiology

It is entitled:

Thermodynamic and structural insights into CSL mediated transcription complexes

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Thermodynamic and structural insights into CSL mediated transcription complexes

A dissertation submitted to the Division of Graduate Studies and Research Of the University of Cincinnati

In partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY (Ph.D.)

In the Department of Molecular Genetics, Biochemistry, & Microbiology of the College of Medicine

2009

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B.A., Miami University, 2004

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Abstract

The Notch pathway is an indispensable signaling mechanism, in which contacts between neighboring cells result in changes in gene regulation that dictate cellular development, proliferation, and differentiation. The necessity for proper signaling is self-evident, as mutation of Notch pathway components has been shown to result in cancer, development defects, and congenital diseases. Notch signals are transduced into transcriptional outputs by the pathway’s sole transcription factor, CSL (CBF-1, Su(H), Lag-1), which regulates expression from Notch responsive genes. In the absence of a Notch signal, CSL functions as a repressor by forming complexes with transcriptional corepressor proteins. This dual functionality of CSL highlights its importance in signaling and the need to understand at the molecular level its interactions with coactivators and corepressors. The work described herein aims to characterize the thermodynamic, structural, and functional details of CSL mediated transcription complexes. In Chapter II, using ITC and X-ray crystallography, we provide a quantitative description of the Notch RAM domain and its interaction with CSL. Based on these findings, we propose an allosteric model, in which RAM binding facilitates ternary complex formation. In Chapter III, ITC and X-ray crystallography are also used to investigate the molecular details of CSL recognizing consensus and nonconsensus DNA binding sites. These experiments give a detailed thermodynamic and structural explanation for CSL binding to two known in vivo binding sites, highlighting the differences between the two sites and the overall moderate affinity of CSL for DNA. In Chapter IV, we investigated the binding of the corepressor MINT to CSL, which is the first quantitative study of a CSL-corepressor complex. This study provided molecular
insights into the proposed competition between coactivators and corepressors for binding sites on CSL. Finally, in Chapter V, knowledge gleaned from our structural studies of CSL mediated transcription complexes was used to develop CSL mutants that were tested in cellular transcriptional reporter assays. While the aim of these studies was to disrupt transcriptional activation, interestingly, some of these CSL mutants were also defective in transcriptional repression as well. These data seem to indicate that both coactivators and corepressors bind similar interfaces on CSL. In sum, the dissertation work presented here provides substantially new insights into the structure and function of Notch pathway transcription complexes - details that could aid in the development of therapeutics designed to target Notch signaling related diseases.
Acknowledgments

I would like to take this opportunity to specifically acknowledge a number of people who were instrumental during my time in graduate school. Their scientific and moral support made this process one of the greatest experiences of my life, and for that I am extremely grateful.

I would first like to thank my advisor and mentor, Rhett Kovall. Rhett had only been at UC for a few weeks when I began my rotation in his lab, and some people told me that I was taking a risk in joining the lab of a new faculty member; however, I always thought Rhett was taking more of a risk taking me into his lab. I was young and scientifically unskilled but I guess Rhett was able to look past these shortcomings. Rhett never turned me away when I had a question, was always willing to look at my failed experiments and suggest a solution, would take the time to read my writings and really help me become a better writer, and push me to be the best scientist I could be. His enthusiasm in the lab is contagious and this energy is what kept me going over the past five years. I will always remember our coffee hour discussions about all things science, as well as life in general, and hope that some day I can pass this enthusiasm on to a graduate student of my own. I would also like to thank the other members of my committee, Drs. Andy Herr, Gary Dean, Carol Caperelli, and Carolyn Price. Your guidance, suggestions, and criticisms have been influential in bringing about the work presented here.

I would next like to thank the past and present members of the Kovall Lab, in no particular order: Dr. Jeff Wilson, Dr. Zhenyu Yuan, Xaimoi Chai, Andy Russell, Brad
VanderWielen, Kelly Reneau, and Ashley Reyer. Thank you so much for putting up with me for all this time. All of you made coming to work so much fun, and we learned some stuff too! I would like to extend a special thank you to Dr. Wilson for taking the time to teach me so much about crystallography and computers. I would also like to thank Dr. Yuan for all of his help in the lab. Without Zhenyu, half of this work may never have been completed.

To all of my fellow graduate students, thank you. I really believe that as a scientist you learn as much from your experiments as you do from those around you. Your different perspectives on the scientific questions at hand always reminded me to keep my mind open and consider other views. I would like to thank two students in particular, Mike Flagler and Olivia Schneider. Mike and Olivia have been a constant source of support since our undergraduate days together at Miami University.

I would especially like to thank my family. Although my Mom and Dad may have been somewhat confused about what I have been doing these past five years, they have never wavered in their support of my goal. Having my parents and my brother right here in Cincinnati throughout my studies provided me with mental stability when my experiments were not working, and whether they know it or not, they helped me earn this degree.

And finally I would like to say thank you to my new wife, Katie. Your support of my scientific endeavors is unbelievable, and has given me the freedom to explore great opportunities. Where this adventure will take us, I do not know. But I am so glad to have you by my side for the journey.
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<th>Description</th>
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<tbody>
<tr>
<td>DSL</td>
<td>Delta, Serrate, Lag-2</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF-1, Su(H), Lag-1</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>LNR</td>
<td>Lin12/Notch repeats</td>
</tr>
<tr>
<td>HD</td>
<td>heterodimerization domain</td>
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<tr>
<td>NRR</td>
<td>negative regulator region</td>
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<tr>
<td>T-ALL</td>
<td>T-cell acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>NotchIC</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>RAM</td>
<td>RBP-jκ associated molecule</td>
</tr>
<tr>
<td>ANK</td>
<td>ankyrin repeats</td>
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<tr>
<td>PEST</td>
<td>proline, glutamine, serine, threonine</td>
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<tr>
<td>MINT</td>
<td>Msx2 interacting nuclear target</td>
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<tr>
<td>SHARP</td>
<td>SMRT/HDAC associated repressor protein</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator of retinoid and thyroid hormone receptors</td>
</tr>
<tr>
<td>CIR</td>
<td>CBF-1 interacting repressor</td>
</tr>
<tr>
<td>SKIP</td>
<td>Ski-interacting protein</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
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<tr>
<td>SPOC</td>
<td>Spen paralog and ortholog C-terminal domain</td>
</tr>
<tr>
<td>CtBP</td>
<td>C-terminal binding protein</td>
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<td>CtIP</td>
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<td>NTD</td>
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<td>BTD</td>
<td>beta-trefoil domain</td>
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<tr>
<td>CTD</td>
<td>C-terminal domain</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>RMSD</td>
<td>root mean square deviation</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<table>
<thead>
<tr>
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<tr>
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<td>nano</td>
</tr>
<tr>
<td>°C</td>
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</tr>
<tr>
<td>ΔG</td>
<td>change in free energy</td>
</tr>
<tr>
<td>ΔH</td>
<td>change in enthalpy</td>
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<tr>
<td>ΔS</td>
<td>change in entropy</td>
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Chapter I

Notch signaling: overview of components, function, implications in disease, and structures
The Notch signaling pathway is a highly conserved cell-to-cell signaling pathway amongst metazoa. First identified in genetic studies of *Drosophila melanogaster*, the pathway derives its name from the notched wing phenotype observed in flies heterozygous for a mutation in the gene encoding the Notch receptor (1). The signaling mechanism has been shown to be vital in embryonic development, as well as cellular differentiation and proliferation (2,3). Proper regulation of Notch signaling is essential, as errant Notch signals have been associated with developmental defects, neurological disorders, and various cancers (4-6). Here we will provide a brief, but comprehensive overview of the pathways major components, the signaling mechanism, processes regulated by Notch signaling, involvement in disease states, and structures of pathway components.

**Pathway components**

The Notch signaling pathway is composed of three major components: the ligand, DSL; the receptor, Notch; and the transcription factor, CSL. The ligand DSL (Delta, Serrate, Lag-2) draws its name from the mammalian, *D. melanogaster*, and *C. elegans* orthologs, respectively. While organisms encode different numbers of ligands – mammals five ligands (Jagged 1 and 2, Delta 1,3, and 4), flies two (Delta and Serrate), and worms two as well (Lag-2 and Apx-1) – much of the structure and function of these proteins has been evolutionarily maintained (7,8). All are Type I transmembrane proteins with a single pass transmembrane domain, and all contain EGF-like (Epidermal Growth Factor) repeats near the N-terminus (Figure 1). The EGF-repeats are essential for making protein-protein interactions with the Notch receptor and the activation of signaling. The number of EGF-repeats is the distinguishing feature between the DSL paralogs and orthologs. The synthesis of the ligand and its presentation on the cell surface alone is not
**Figure 1: Domain schematics of Notch pathway components.** (A) The ligand DSL is composed mainly of EGF-like repeats (purple circles) and a transmembrane domain (TM). (B) The receptor Notch is composed of an extracellular domain (Notch ECD), a short transmembrane domain (TM), and the intracellular domain (NotchIC). The ECD can be subdivided into the EGF-like repeats which make contacts with the ligand, and the Lin12/Notch repeats (LNR, colored green ovals) and the heterodimerization domain (HD, green rectangles), which together form the negative regulatory region (NRR). NotchIC is composed of the RAM domain (red rectangle), the ankyrin repeats (yellow squares), and the PEST domain (green oval). (C) The transcription factor CSL is composed of a N-terminal domain (NTD, colored cyan) and C-terminal domain (CTD, colored orange), which share structural homology to the Rel family of transcription factors, and the Beta-trefoil domain (BTD, colored green).
enough to render the ligand active for signaling. The intracellular domain of the ligand must be recognized by the E3 ubiquitin ligase Mind-bomb or Neuralized, which leads to its mono-ubiquitination (9,10). Without this modification the ligand is incapable of signaling and will be degraded prior to Notch activation. However, upon modification, the ligand is presented on the membrane with its EGF-repeats exposed to the extracellular space, poised to interact with and activate a Notch receptor.

Similar to the ligand, the receptor Notch is a Type I transmembrane protein, with different numbers of receptors encoded by various organisms – mammals four (Notch 1-4), fly one (Notch), and worms two (Lin-12 and Glp-1) (11,12). The receptor is synthesized as a single polypeptide, but is cleaved during transport to the cell surface by a furin-like convertase, resulting in an extracellular domain and an intracellular domain held together noncovalently, and forming the heterodimerization domain (Figure 1) (13,14). The major feature of the extracellular domain is the EGF-like repeats that are crucial for interacting with the ligand and activating signaling (15). During maturation of Notch, the EGF-like repeats are glycosylated by O-fucosyltransferase 1, which not only glycosylates Notch, but also aids in the proper folding of the receptor (16-18). Deletion of this enzyme has been shown to cause the accumulation of misfolded Notch in the endoplasmic reticulum (ER). The EGF-like repeats can also be glycosylated by Fringe, which also has the ability to glycosylate the EGF-repeats on the ligand (19). This modification of Notch by Fringe has been observed to dictate which ligand the receptor preferentially interacts with (20). C-terminal to the EGF-like repeats, but still on the extracellular side of the membrane, is the LNR (Lin/Notch repeats) domain and the heterodimerization domain (HD). Together these two domains are referred to as the NRR (Negative Regulatory Region) and have been shown to be vital for keeping the pathway turned
off until a proper signal is received, as disruption of this domain results in constitutively active signaling (21,22). Furthermore, this region has also been shown to be the site of many activating mutations in human T-ALLs (T-cell acute lymphoblastic leukemia) (23). C-terminal to the NRR is a short transmembrane domain followed by the Notch intracellular domain (NotchIC). The intracellular domain is composed of the RAM (RBP-jκ associated molecule) domain, the ankyrin repeats domain, and the PEST (rich in proline, glutamine, serine, and threonine) domain. The entire intracellular domain is important for activating transcription, with each domain providing different functions. RAM was the first domain shown to interact with the Notch pathway transcription factor, CSL, and has been demonstrated to be essential for appropriate signaling to occur (11,24,25). The ankyrin repeats have been shown to coordinate protein-protein interactions necessary for signaling, and the PEST domain facilitates degradation of the Notch receptor (25-29). The intracellular domain is also the target of several enzymes whose activity has been shown to modulate signaling. The E3 ligases Itch and NEDD4 were shown to ubiquitinate the RAM-ankyrin repeat region, subsequently targeting Notch for degradation (30-32). Another E3 ligase, Deltex, which binds Notch in the ankyrin repeats, promotes Notch signaling (33).

The third and final major component of the pathway is the transcription factor CSL, which derives its name from the mammal, fly, and worm orthologs, respectively (CBF-1/RBP-jκ, Su(H), Lag-1) (34-36). While organisms encode multiple ligands and receptors, all organisms only encode one CSL molecule, meaning that all signals are converted into transcriptional outputs by CSL. CSL orthologs are highly conserved through evolution with approximately 84% identity at the primary sequence between the fly and human proteins, and 99% identity between the mouse and human proteins. CSL is absolutely essential, as CSL knockout mice are shown to
be embryonic lethal (37,38). CSL is a sequence specific DNA binding protein that is somewhat unique as a transcription factor, as it has been characterized as both a transcriptional activator and repressor, and observed to interact with several transcriptional regulatory proteins (39). Structural elucidation of CSL complexes has revealed the domain structure of CSL as well as how it interacts with some of the Notch pathway regulatory proteins, the specific details of which will be covered in the following sections and chapters.

**Signaling mechanism**

Canonical Notch signaling is activated when the EGF-like repeats of the ligand on the signaling cell interact with the EGF-like repeats of the Notch receptor on the receiving cell (Figure 2). This interaction allows for the second cleavage of the Notch receptor on the extracellular side of the membrane by the metalloprotease TACE (TNF-alpha converting enzyme) (40). This also releases the extracellular domain of Notch, which is endocytosed in complex with the ligand by the signaling cell. This cleavage renders the receptor susceptible to a third and final cleavage by gamma-secretase, which releases NotchIC from the membrane (41,42). Once released, NotchIC is localized to the nucleus by its two nuclear localization signals, where it interacts with CSL to activate transcription.

Many of the details regarding the activation, repression, and regulation of transcription by CSL are explored in greater length in the following chapters. Therefore, a general model of CSL regulated transcription will be presented here to establish the major components and mechanisms. In the absence of a Notch signal, the generally accepted model in the field suggests that CSL is bound at the promoters of Notch responsive genes, keeping these genes transcriptionally repressed (43). CSL has not been observed to operate alone as a repressor, but
Figure 2: Overview of the Notch signaling pathway. (continued next page)
Figure 2: Overview of the Notch signaling pathway. The Notch pathway involves a number of essential receptor cleavage events, protein-protein interactions, and protein-DNA interactions. The first cleavage (1) occurs in the endoplasmic reticulum (ER) by the Furin-like convertase as the receptor is being transported to the plasma membrane. The two portions of the receptor remain non-covalently associated through the heterodimerization domain and the receptor is presented on the receiving cell membrane. When the receptor EGF-like repeats makes contact with the ligand EGF-like repeats the receptor is susceptible to cleavage by the TACE enzyme (2). This activity releases the extracellular domain, which in complex with the ligand can be endocytosed by the signaling cell. With the extracellular domain removed, the receptor can be cleaved for a third and final time by the gamma-secretase complex (3). Once cleaved, NotchIC is released from the membrane and translocates to the nucleus (4). In the absence of a Notch signal, CSL is bound to the DNA in complex with corepressors keeping transcription of Notch responsive genes repressed (5). The presence of a Notch signal is believed to cause the displacement of corepressors, recruitment of coactivators (i.e. Mastermind), allowing for the formation of an active transcription complex, which subsequently activates transcription (6).
rather in complex with proteins identified as having transcriptional repressive functions. Some of the proteins identified as members of the CSL repression complex are: SMRT (Silencing mediator of retinoid and thyroid hormone), Cir (CBF-1 interacting corepressor), SKIP (Ski interacting protein), and MINT (Msx2 interacting nuclear target protein) (44-47). The localization of NotchIC to the nucleus is thought to displace the corepressors from CSL, by out-competing them for binding to CSL. This complex recruits the coactivator Mastermind to CSL, as well as other general transcriptional coactivators p300, GCN5/PCAF, which contain histone acetyltransferase domains, in order to activate transcription of Notch genes (48-51). The localization of Mastermind to the transcription complex also recruits kinases to the ternary complex that phosphorylate Notch, which facilitate its turnover (52). This marking recruits the ubiquitin ligase Fbw7/Sel10, which ultimately tags Notch for degradation by the proteosome. The current model then suggests that the removal of Notch allows for the corepressors to return to CSL and establish a transcriptional repression complex once again. This description of the CSL “transcriptional switch” mechanism is rather limited in detail, but as mentioned previously, work described here will explore different aspects of CSL’s role in transcription and provide details that will expand our knowledge of this process.

**Notch regulated cellular processes**

Notch signaling has been extensively studied for its pleiotropic functions in differentiating cells and self-renewing tissues (53). The developing nervous system is heavily reliant on Notch signals for proper differentiation and distribution of the various cell fates. A Notch signal will inhibit the differentiation of a neuronal progenitor cell into a neuron (54). Conversely, a Notch signal that acts upon a glial progenitor cell will promote the differentiation
of that progenitor to the astrocyte cell fate (55). Notch signals have also been shown to inhibit the differentiation of the oligodendrocyte precursor to an oligodendrocyte (56). Hematopoetic cellular maintenance and differentiation is similarly dependent upon Notch signaling. Gain of function studies have shown that the hematopoetic stem cells (HSC) found in the bone marrow are inhibited from differentiation by Notch, which seems to increase self-renewal of the HSC population (57). Once these HSC’s are initially differentiated and begin the process of cellular maturation toward their terminal cell fate, Notch is a crucial determinant in the appropriate division of both T-cells and B-cells. Thymic expression of Notch1 has been shown to induce the T-cell fate from the lymphoid progenitor cells at the expense of the B-cell fate (58). Further specification of T-cell types is both inhibited and promoted by various Notch signals as well. The splenic development of B-cell types is contingent upon the presence of Notch2, which will promote marginal zone B-cells and inhibit follicular B-cells (59). While neurogenesis and hematopoesis have provided much of our knowledge about the biological function of Notch signaling, it is important to note that Notch has crucial functions in a number of other tissues, including intestinal cellular differentiation and skin cell maturation (53).

**Notch and human disease**

Aberrant Notch signaling has been identified in cancers, congenital defects, cardiovascular disease, and herpesvirus infections (60). The most well understood example of this is the chromosomal translocation, which results in T-cell acute lymphoblastic leukemia (T-ALL) (61). This genetic rearrangement results in the C-terminus of the Notch receptor being fused to the TCR Beta-enhancer, which when synthesized, produces a constitutively active Notch receptor. While not all T-ALLs are a result of chromosomal translocations, it has been observed
that roughly 50% of all human T-ALL’s contain Notch activating mutations (23). This high percentage suggests that Notch inhibitors may be good lead components for novel T-ALL therapeutics. To this end, studies have shown that inhibitors of Notch can suppress the growth of T-ALL cells in culture, and Notch inhibitors are currently being pursued for use in clinical treatment of patients with T-ALL that are recalcitrant to conventional treatments (62).

Genetic mutations of other Notch pathway components have been shown to be the causative factor of other inherited diseases. A mutation in the Notch ligand Jagged1 has been linked to the autosomal dominant disorder Alagille syndrome, which is characterized by multiple developmental defects (63). Likewise, a point mutation in Jagged1 has been connected to the congenital heart disease tetralogy of Fallot (4). CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), a neurological disorder that displays vascular defects as well, has been associated with over 70 different mutations in the EGF-like repeats of the Notch3 receptor (64). And most recently, clinical studies have identified mutations in the Notch1 receptor, which seem linked to developmental defects in the aortic valve that can lead to progressive aortic valve disease (5).

Potentially, due to Notch’s ubiquitous nature and direct involvement in cellular proliferation, viruses have developed means to co-opt Notch pathway components for their benefit. Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA-2) is capable of relieving the transcriptional repressive function of CSL, allowing for the activation of genes necessary to promote B-cell immortalization (65). It has been proposed that EBNA-2 acts in a similar manner to Notch, displacing corepressors from CSL. The Kaposi’s sarcoma associated herpesvirus (KSHV) encoded RTA (replication and transcription activator) protein has been shown to interact with CSL and control the switch from latent to lytic viral replication. This process is a crucial
event in the life cycle of the virus and progression of the disease state (66,67). The ability and need for viral proteins to interact with CSL for viral subsistence suggests that disrupting or preventing these interactions may be an effective target for antiviral therapeutics.

**Structural biology of Notch signaling**

Genetic and biochemical experiments over the past twenty years have elucidated Notch pathway components, their functional mechanisms, and their involvement in disease states. However, with regards to our structural understanding of the proteins and protein complexes of the pathway, we have only recently begun to illuminate the molecular details of Notch pathway components. The first protein component of the Notch pathway to be structurally characterized was the ankyrin (ANK) repeats domain of Notch from *D. melanogaster* (Figure 3) (68). The structure revealed that the domain contains seven iterative ankyrin repeat motifs, correcting the earlier prediction of only six repeats that was made based on primary sequence analysis (69). Moreover, this work highlighted how previously isolated mutations of the ankyrin repeats disrupt Notch function by destabilizing secondary structural elements necessary to form an active transcription complex. Subsequently, the structure of the human ankyrin repeats domain was solved and showed a high degree of structural conservation between the two organisms (70).

The structural determination of CSL through the use of x-ray crystallography has been vital to our understanding of how Notch signals are converted into transcriptional outputs (71). This structure provided the first high-resolution description of the three domains of CSL bound to DNA, clarifying previous incorrect assumptions regarding CSL domain architecture and function (27). CSL is composed of an N-terminal domain (NTD) and C-terminal domain (CTD), which share structural homology to the Rel family of transcription factors (NF-κB). The NTD
Figure 3: Structures of the Notch ankyrin repeats, CSL-DNA complex, and the CSL transcriptional activation ternary complex. (A) Structure of *D. melanogaster* ankyrin repeats (1OT8). The seven repeats identified from the structure are individually colored and numbered below the structure. Note the partially unstructured ankyrin repeat 1 at the N-terminus (blue). (B) Structure of worm CSL bound to DNA (1TTU). N-terminal domain colored cyan, beta-trefoil domain (BTD) colored green, and C-terminal domain (CTD) colored orange. DNA represented as grey, orange, and blue sticks. (C) Structure of worm ternary activation complex (2FO1). CSL colored as in panel B; Mastermind (MM) colored as a black helix. NotchIC ankyrin repeats (ANK) represented by yellows cartoon helices and the RAM domain by yellow, blue, and red spheres. Ankyrin repeats in this structure contained seven complete ankyrin repeats as well as an N-terminal cap repeat.
was observed to make specific and nonspecific contacts with the DNA. Inserted between these two domains is the beta-trefoil domain (BTD), which also makes specific and non-specific DNA contacts. The BTD is a capped beta-barrel structure with three-fold symmetry that is similar to the structure found in some cytokines (72). It was also proposed that a surface exposed hydrophobic pocket on the BTD was the interface where the RAM domain of NotchIC bound. Subsequently, this structure led to the simultaneous determination of the worm and human transcriptionally active ternary complex structures (73,74). Both structures contained CSL bound to DNA in complex with the ankyrin repeats of Notch and the coactivator Mastermind; however, the worm structure contained the RAM domain of NotchIC, which was not included in the human structure. These structures revealed several important details about transcriptional activation by the CSL-NotchIC-Mastermind complex. First, the DNA contacts observed in the absence of NotchIC and Mastermind were conserved in the ternary complex structure. Second, the CTD, whose function was somewhat unclear from the previous CSL-DNA structure, was shown to form a large interface with the ankyrin repeats of NotchIC and a portion of Mastermind. Mastermind, which adopts a bent alpha helical structure, also makes contacts with the NTD of CSL through its C-terminus. Finally, the hydrophobic tetrapeptide motif of the RAM domain was observed to be buried in the surface-exposed hydrophobic pocket of the BTD, validating earlier proposals. In sum, the resolved structures of nuclear Notch signaling components have provided us with an overall greater understanding of how transcription is regulated and how mutations may perturb this process. In spite of these advances, we are still lacking molecular descriptions for a multitude of Notch pathway components and complexes; and while the determination of these structures may not answer all of our questions, they will certainly offer valuable insight into this complicated process.
Chapter II

RAM Induced Allostery Facilitates Assembly of a Notch Pathway Active Transcription Complex

Abstract

The Notch pathway is a conserved cell-to-cell signaling mechanism, in which extracellular signals are transduced into transcriptional outputs through the nuclear effector CSL. CSL is converted from a repressor to an activator through the formation of the CSL-NotchIC-Mastermind ternary complex. The RAM domain of NotchIC avidly interacts with CSL; however, its role in assembly of the CSL-NotchIC-Mastermind ternary complex is not understood. Here we provide a comprehensive thermodynamic, structural, and biochemical analysis of the RAM-CSL interaction for components from both mouse and worm. Our binding data shows that RAM and CSL form a high affinity complex in the presence or absence of DNA. Our structural studies reveal a striking distal conformational change in CSL upon RAM binding, which creates a docking site for Mastermind to bind to the complex. Finally, we show that the addition of a RAM peptide in trans facilitates formation of the CSL-NotchIC-Mastermind ternary complex in vitro.
Introduction

The Notch pathway mediates signaling between neighboring cells and plays important roles in cellular differentiation, proliferation, apoptosis, and stem cell renewal (2,75). Notch signaling is required during embryonic development and patterning as well as for cell fate decisions during adult homeostasis. Proper regulation of Notch is essential, as errant signaling results in congenital defects, cardiovascular disorders, and cancer (4,5,76). As first identified and characterized in *D. melanogaster* and *C. elegans* (77), the canonical Notch signaling pathway is activated when the cell surface ligand DSL (Delta, Serrate, Lag-2) interacts with the receptor Notch on an adjacent cell (78). This receptor-ligand interaction initiates two proteolytic cleavages of the receptor Notch, whereupon the intracellular domain of Notch (NotchIC) is released from the membrane and translocates to the nucleus. Once inside the nucleus, NotchIC interacts with the DNA binding transcription factor CSL (CBF-1, Su(H), Lag-1), and together they form a ternary complex with the transcriptional coactivator Mastermind in order to activate transcription from Notch target genes. A detailed description of the conversion of CSL from a repressor to an activator and its interactions with transcriptional coregulators is shown in Figure 1.

Previously, using Notch pathway components from *C. elegans*, we determined crystal structures for DNA-bound CSL and the CSL-NotchIC-Mastermind ternary complex bound to DNA (Figure 2A and 2B) (71,73). Concurrent with our structural studies of the worm complex, the human ternary complex was also determined (74). These structural studies revealed the overall architecture of CSL and the transcriptionally active ternary complex, which have been remarkably conserved through evolution (82). As shown in Figure 2, the three domains of CSL,
Figure 1. Overview of transcriptional regulation mediated by CSL. Top, prior to pathway activation, CSL engages transcriptional corepressors (CoR), which recruit multiprotein repressor complexes that function to silence Notch target gene transcription through the action of histone deacetylases (HDAC) (79). Right, upon pathway activation, nuclear NotchIC binds to CSL through its RAM (RBP-jk associated molecule) (24) and ankyrin repeats domain (ANK) (27), which is thought to displace corepressors from CSL (44,46). Bottom, the subsequent binding of Mastermind to CSL-NotchIC renders the ternary complex poised to activate transcription (49,80). The DNA bound CSL-NotchIC-Mastermind complex recruits general transcription factors such as PCAF/GCN5 and CBP/p300 (50,81), which contain histone acetylase (HAT) domains, to upregulate transcription of Notch target genes. Left, transcription is terminated by the degradation of NotchIC, which is mediated by an E3 ubiquitin ligase that recognizes the phosphorylated C-terminal PEST domain, leading to disassembly of the activation complex (52).
Figure 2. Overview of previously determined CSL structures. (continued next page) Ribbon diagrams for the coregulator-free worm CSL-DNA complex (A), and the worm CSL-NotchIC-Mastermind ternary complex (B)(71,73). CSL domains NTD, BTD, and CTD are colored cyan, green, and orange, respectively. A β-strand that bridges all three domains is colored magenta. For the ternary complex in (B), the NotchIC RAM domain (RAM) and ankyrin repeats (ANK) are colored red and yellow, respectively; Mastermind (MM) is colored grey. The DNA is represented as a stick model. (C) Domain schematics for core CSL, NotchIC, and Mastermind are colored according to ribbon diagrams. (D) Structural alignment of CSL from the worm and human ternary complex structures (73,74). Cα backbone representation of worm CSL (2FO1) in tricolor (cyan, green, and orange) overlayed onto human
CSL (2F8X) colored gray. Alignment was performed over the NTD, highlighting the interdomain differences in BTD and CTD between the two CSL structures. Worm CSL in the context of the ternary complex structure undergoes large interdomain movements that are not observed in the human CSL structure. Despite these interdomain differences, a loop structure within NTD (henceforth referred to as the NTD-loop) is in a similar open conformation in both CSL structures. In the coregulator-free worm CSL-DNA structure the NTD-loop is in a closed conformation. The structure-function explanation for this conformational change is evident, as opening of the NTD-loop removes steric hindrances, which would otherwise block the C-terminal helix of Mastermind binding to CSL, preventing ternary complex formation. Also shown are the site of RAM (red) binding to the BTD and the location of the NTD-loop.
which constitute “core” CSL - N-terminal domain (NTD), beta-trefoil domain (BTD), and the C-terminal domain (CTD) - simultaneously mediate interactions with both NotchIC and Mastermind. The RAM and ANK domains of NotchIC interact with the BTD and CTD of CSL, respectively (Figure 2B and 2C). Mastermind adopts an elongated bent helical conformation in the complex, with the N-terminal helical region of Mastermind forming a tripartite interaction with ANK of NotchIC and the CTD of CSL, and the C-terminal helical region of Mastermind interacts with the NTD of CSL (Figure 2B).

While these ternary complex structures were a milestone in the field, several unanswered questions remain regarding what role individual domains of NotchIC play in binding to CSL and subsequent assembly of the active ternary complex. In particular, the function of the NotchIC RAM domain is a point of contention. On one hand, RAM is absolutely conserved in all Notch receptors; and in contrast to ANK, RAM avidly interacts with CSL both in vitro and in vivo (11,24,25). On the other hand, RAM is dispensable for signaling in cells under non-physiological conditions when ANK is overexpressed (83-85). Similarly, in vitro, RAM is not necessary for reconstituting the CSL-NotchIC-Mastermind ternary complex when ANK is present in large excess (27), and indeed, the structure of the human CSL-NotchIC-Mastermind ternary complex did not contain RAM (74). Furthermore, there are significant conformational differences within CSL between the two ternary complex structures (Figure 2D), raising questions as to the significance and contribution of RAM to these observed conformational differences (82).

In the present study, we endeavored to characterize the CSL-RAM interaction and address the question of whether the isolated RAM domain of NotchIC would trigger any conformational changes in CSL in the absence of ANK and Mastermind, suggesting that
allostery is a component of the mechanism that converts CSL from a repressor to an activator. Therefore, we pursued a structural, thermodynamic, and biochemical characterization of the NotchIC RAM domain interaction with CSL, using Notch components from both mouse and worm. Our structural studies afford unprecedented higher resolution models of CSL, which include two crystal forms of worm CSL-RAM complexes bound to DNA, and a crystal structure of mouse CSL bound to DNA. Using electrophoretic mobility shift assays (EMSA), we analyzed the contribution of RAM to assembly of the CSL-NotchIC-Mastermind ternary complex. Using isothermal titration calorimetry (ITC), we determined the affinity and energetics of the CSL-RAM binding reaction. Our results reveal that NotchIC RAM binding to the BTD of CSL triggers a distal conformational change in the NTD of CSL; a peptide corresponding to RAM facilitates formation of the CSL-NotchIC-Mastermind ternary complex; and NotchIC RAM binds to core CSL with nanomolar affinity. Taken together, our studies provide molecular snapshots of intermediary Notch pathway transcription complexes and the energetics that underlie their formation, which clarify the role of NotchIC RAM in signaling.
Materials and Methods

Cloning, Expression, and Protein Purification. The cloning, expression, and purification for the C. elegans orthologs CSL (Lag-1), NotchIC (Lin-12), and Mastermind (Sel-8/Lag-3) were described previously (71,73). The M. musculus CSL ortholog, residues 53-474 (core) or residues 203-393 (BTD) was cloned into the pGEX-6P-1 vector; murine Notch1 protein, encoding residues 1744-2113 (RAMANK) and 1827-2113 (ANK), were also cloned into the pGEX-6P-1. Transformed bacteria were grown at 37°C in LB media, cooled to 20°C, induced with 0.1mM IPTG, and grown overnight at 20°C. Bacteria were harvested by centrifugation, resuspended in PBS, and frozen. Cell pellets were lysed by sonication, cleared by centrifugation and filtration, and subsequently loaded onto a glutathione sepharose column (GE Healthcare). The column was washed with PBS, and the GST-fusion proteins were eluted using reduced glutathione. The eluants were dialyzed, and the GST tag cleaved with Precision Protease (GE Healthcare) per manufacturer’s protocol. A subsequent GST affinity column removed the GST moiety. All protein constructs were further purified to homogeneity using ion exchange and size exclusion chromatography. Peptides encoding the RAM domain were generated by cloning mouse Notch1 residues 1744-1771, Lin-12 residues 930-957, and Glp-1 residues 788-817 into a modified pET 28b(+) vector (generous gift of Dr. Christopher Lima). This vector encodes a fragment of SMT3, producing a His-SMT3-RAM fusion protein. The fusion protein was overexpressed as described above. The lysate was run over a nickel affinity column. The column was washed, and fusion protein eluted with imidazole. The fusion protein was then cleaved to remove His-SMT3 from the RAM moiety using the Ulp1 protease (gift of Dr. Christopher Lima), which leaves only an N-terminal serine residue attached to RAM following
cleavage. The RAM peptide was separated from the fusion by size exclusion chromatography in 30% Acetonitrile, 0.1% TFA, dried down in a vacuum centrifuge, and stored at -80°C.

**Crystallization and Data Collection.** A 13mer DNA duplex with TT/AA single-stranded overhangs was co-crystallized with all complexes (71). Mouse CSL-DNA complexes were set up in a 1:1.1 molar ratio and the Hampton Research Index Screen was used to identify initial crystallization conditions, using an Art Robbins Phoenix Crystallization Robot. Crystals were optimized at 4°C in 100 mM Bis-Tris pH 5.5, 27% PEG 3350, with and without 100 mM NaCl. Crystals were cryoprotected by soaking in mother liquor solution containing increasing amounts of xylitol and flash frozen in LN2 for transport. Diffraction data were collected at the Advanced Photon Source, beamline 22-ID. The crystals belong to the orthorhombic space group P2\(_1\)2\(_1\)2\(_1\), with unit cell dimensions a = 66.77Å, b = 95.39Å, c = 113.70Å (Supplemental Table 1).

Worm CSL-RAM-DNA complex crystals were obtained using similar methods. Two orthorhombic crystal forms were obtained at 4°C, the C22\(_2\)_1 form (a = 62.94Å, b = 95.97Å, c = 126.31Å) was grown using a chemically synthesized RAM peptide corresponding to Lin-12 residues 938-950, and the P2\(_1\)2\(_1\)2\(_1\) (a = 60.153Å, b = 98.866Å, c = 126.313Å) form using the recombinant RAM peptide (930-957). The C22\(_2\)_1 crystals were grown in 100mM Bis-Tris pH 6.0, 200mM MgCl\(_2\), and 26% PEG 3350, cryoprotected in 20% sorbitol, flash frozen, and data collected at the NSLS beamline X6A. The P2\(_1\)2\(_1\)2\(_1\) crystals were grown in 100mM Bis-Tris pH 5.5, 100mM ammonium sulfate, and 21% PEG 3350, cryoprotected in 15% ethylene glycol, flash frozen, and data collected at APS beamline 22-ID. All data was integrated and scaled using HKL2000 (86).
Table S1. Diffraction data and structure refinement statistics

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<th>mCSL (RBP-κκκ) + DNA</th>
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<th>wCSL (Lag-1) + wRAM (Lin12) + DNA</th>
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<td>a = 62.944, b = 95.972, c = 223.630</td>
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<td>98.2 (87.3)</td>
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<td>Disallowed regions (%)</td>
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Structure Determination, Model Building, and Refinement. The known structures of *C. elegans* CSL-DNA (1TTU) and human CSL (2F8X) were used with Phaser (87) to obtain molecular replacement solutions for the worm CSL-DNA-RAM and mouse CSL-DA structures. Model bias was reduced through the use of prime-and-switch from RESOLVE (88) and Coot (89) was used for manual rebuilding of models. Refinement of the structures was carried out using Refmac with TLS parameters (90) and composite-omit maps were generated using CNS to further validate models and areas of weak density (91). The quality of the structure was assessed by PROCHECK (92). The mouse CSL-DNA structure is completely resolved, containing all three domains of CSL and the 15mer DNA duplex, with the exception of two short unresolved regions: amino acids 256-261 of the BTD, and amino acids 198-199 in the NTD. The structure has been refined to an $R_{work} = 21.9\%$ and $R_{free} = 25.6\%$ (Table S1).

For both worm CSL-RAM structures, the RAM peptide is well resolved. For the P2_12_12 data, while the peptide included residues 930-957, only residues 937-951 were resolved. The P2_12_12 structure was refined to a final $R_{work} = 22.1\%$ and $R_{free} = 26.4\%$ (Table S1). For the C222_1 structure, which used the RAM peptide that encoded residues 938-950, all of the residues were resolved. The C222_1 structure was refined to an $R_{work} = 22.3\%$ and $R_{free} = 27.6\%$ (Table S1). Pymol was used for creation of all figures involving structures (http://pymol.sourceforge.net).

*Isothermal Titration Calorimetry.* ITC experiments were performed using a Microcal VP-ITC microcalorimeter. All ITC experiments were performed at 25°C in a buffer containing 50mM sodium phosphate pH 6.5, 150mM NaCl, and 1mM TCEP. All RAM peptides, and CSL and NotchIC proteins, were either resuspended or dialyzed into a buffer-matched solution. For experiments that contained CSL bound to DNA, two DNA oligos were annealed resulting in a 19mer blunt-ended DNA duplex containing one CSL binding site and purified by size exclusion...
chromatography in a matched buffer for ITC. The experiments were performed with either core CSL or the BTD of CSL in the microcalorimeter cell (10 µM), with or without the 19mer DNA (11 µM), and titrated with Notch peptides or RAM-ANK constructs (100 µM). An approximate 60 nanomolar K_d for the interaction of CSL with the 19mer DNA was determined under our experimental conditions, ensuring that all of our RAM binding to CSL-DNA experiments were performed under stoichiometric CSL-DNA conditions. Protein concentrations were calculated using spectrophotometry at UV_{280} as well as BCA assay (Pierce). Data were analyzed using the ORIGIN software, and fitted to a one-site binding model. Data reported are the sum of at least three individual experiments. The c-value (c= K_a[M]N) for all experiments was between 5 and 300.

*EMSA.* Electrophoretic mobility shift assays were performed using the 19mer DNA duplex stated above. Protein components were in a binding buffer: 10mM Tris pH 8, 150mM NaCl, 100ug/ml BSA, 1mM DTT, 5mM MgCl_2, 0.1% NP-40, and 5% glycerol. Various protein components were mixed with DNA on ice and then allowed to form complexes at room temperature for 15 minutes, followed by incubation on ice prior to electrophoresis. Complexes were then separated on a 10% non-denaturing PAGE in 0.5x TBE or 1x TG at 4°C, and visualized using SYBR-GOLD stain (Invitrogen). Statistical analysis was performed using GraphPad.
Results

Structures of Worm CSL-RAM-DNA and Mouse CSL-DNA Complexes. In order to obtain high-resolution structural data for complexes of core CSL with the RAM domain of NotchIC and characterize any structural changes associated with RAM binding to CSL, we used recombinantly purified CSL and RAM constructs from mouse and worm, and crystallized these complexes bound to a short piece of duplex DNA, containing a single CSL binding site (see Experimental Procedures). We obtained two orthorhombic crystal forms of worm CSL-RAM complexes bound to DNA that diffracted to 2.2 Å and 2.4 Å resolution, respectively (Figure 3A). Despite the significantly different crystal lattices, the two worm CSL-RAM complexes are nearly structurally equivalent, overlaying with a high degree of similarity (RMSD ~0.8 Å for all Cα atoms) (Figure S1). While we were able to crystallize a mouse CSL-RAM complex bound to DNA, these crystals nominally diffracted to only ~7 Å, which precluded determination of the structure; however, we were able to grow an orthorhombic crystal of the mouse CSL-DNA complex that diffracted to 2.2 Å (Figure 3B). The data collection, structure determination, and refinement statistics for both worm and mouse CSL structures are summarized in Experimental Procedures and Table S1.

Comparison of our two newly determined complexes of CSL-RAM from worm against previously determined CSL structures reveals similarities to both the original structure of CSL bound to DNA (coregulator-free), as well as the structure of CSL in the context of the worm ternary complex structure. As shown in Figure 3C, the RAM-BTD interactions for the worm CSL-RAM complexes determined here display a high degree of
Figure 3. Structures of worm CSL-RAM-DNA and mouse CSL-DNA complexes. Ribbon diagrams corresponding to worm CSL-RAM-DNA (A) and mouse CSL-DNA (B) structures reported here. The domain coloring for CSL is the same as in Figure 1. The RAM domain in (A) is represented as a stick model colored by atom. (C) Cα overlays of BTD-RAM interactions determined here with BTD-RAM interaction from worm ternary complex structure, highlighting high degree of correspondence. The BTD is colored green and RAM colored yellow with hydrophobic tetrapeptide residues (VWMP) displayed as sticks. (D) Molecular surface representation of BTD-RAM interaction with BTD surface colored according to electrostatics: red-negative; blue-positive; and grey-nonpolar. RAM is in a stick representation colored by atom. RAM binds in an extended conformation across a large hydrophobic surface and an electronegative patch on BTD. RAM also forms a β-stranded structure with a large loop on BTD that is also implicated in corepressor binding. All of the nonpolar and polar RAM-BTD interactions as well as the β-structure formed with the BTD loop are maintained in the worm CSL-RAM complexes determined here.
Figure S1. Structural alignment of worm CSL-RAM complexes. Figure shows C\(\alpha\) overlay of P2\(_1\)2\(_1\)2\(_1\) CSL-RAM structure (tricolor) with C222\(_1\) CSL-RAM structure (grey). 430 C\(\alpha\) atoms align with an RMSD of 0.8 Å. For comparison, a structurally equivalent C\(\alpha\) atom is denoted as a sphere in the NTD-loops.
similarity with the RAM-BTD interactions from the worm ternary complex structure, essentially maintaining equivalent interactions. In contrast, the overall domain arrangements (NTD, BTD, and CTD) in the worm CSL-RAM complexes correspond more closely with the domain arrangements from the coregulator-free structure of CSL, exhibiting very little correlation with the sizeable interdomain movements observed for CSL in the worm ternary complex structure (Figure 4C and 4D). Interestingly, closer inspection of the structural comparisons reveals an additional observation: the conformation of a loop structure within the NTD of the CSL-RAM complexes, which we define as the “NTD-loop”, assumes an open conformation strikingly similar to the conformation of the NTD-loop when Mastermind is bound to CSL in both worm and human CSL-NotchIC-Mastermind ternary complexes (Figure 4D). The significance of this observation is described in greater detail in subsequent sections.

Our murine CSL construct reported here is 99% identical to human CSL with only three conservative amino acid changes, and our structure of mouse CSL bound to DNA represents the only structure of mammalian CSL determined in a coregulator-free form (Figure 3B). Overall, there is a high degree of correspondence when the Cα atoms are overlayed between mouse and human CSL structures with an RMSD of ~1 Å (Figure 4B). The primary areas of structural difference between the mouse and human structures are localized to the BTD and the conformation of the NTD-loop. For the BTD structural difference, a large loop within BTD that engages RAM is disordered in the mouse CSL structure, but in the human ternary complex structure, even though RAM is not present, assumes an ordered structure similar to what is observed in all of the worm CSL-RAM complexes. For the primary structural difference in NTD, the NTD-loop, which is in an open conformation in the human ternary complex structure to
Figure 4. Comparison of CSL structures. Figure shows Cα overlays for mouse and worm CSL structures determined here against previously determined worm and human CSL structures, highlighting differences in domain dispositions and conformation of the NTD-loop. CSL domains are colored as in Figure 1. For comparison, a structurally equivalent Cα atom is denoted as a sphere in the NTD-loops. For overlays performed in panels (B) and (C), the alignment was done over the entire CSL molecule; for overlays in (A) and (D), the alignment was done only over the NTD of CSL, due to the substantial interdomain movements of BTD and CTD about the NTD. (A) Overlay of the coregulator-free form of worm CSL (apo) in tricolor with worm CSL from the ternary complex (NIC+MM) colored gray, with RMSD greater than 2.4 Å for all Cα atoms, note open and closed conformations of the NTD-loop. (B) Overlay of coregulator-free mouse CSL (apo) in tricolor with human CSL from the ternary complex (NIC+MM) colored gray, with RMSD of ~0.8 Å for corresponding Cα atoms, note open and closed conformations of the NTD-loop similar to worm structures in (A). (C) Overlay of coregulator-free worm CSL (apo) in tricolor with worm CSL-RAM colored gray, with RMSD of less than 0.8 Å for corresponding Cα atoms, note open and closed conformations of the NTD-loop. (D) Overlay of worm CSL-RAM in tricolor with worm CSL from ternary complex (NIC+MM) colored gray, with RMSD greater than 2.4 Å for all Cα atoms, note similar open conformations of the NTD-loop.
accommodate Mastermind binding, is in a closed conformation similar to what is observed for
the corresponding NTD-loop in the coregulator-free worm CSL-DNA structure (Figure 4B).

**RAM Induced Conformational Changes.** As previously shown in the CSL-NotchIC-
Mastermind ternary complex structures (Figure 2B), approximately one half of the CSL-
Mastermind interaction is composed of the C-terminal helix of Mastermind binding across a
concave surface formed by the NTD β-sheet (Figure 2B). Located at one end of the NTD β-
sheet is the NTD-loop, which is formed by a β-hairpin motif and is in an open conformation in
both worm and human ternary complex structures and in a closed conformation in the
coregulator-free structure of worm CSL (Figure 5A and 5B). Opening of the NTD-loop is a
necessary prerequisite for Mastermind binding, in order to remove steric hindrances with the
NTD-loop in the closed conformation (Figure 5). The magnitude of this conformational change,
as measured by the shift in Cα positions going from the closed to open conformation, ranges
from approximately 3-7 Å. For our current structural studies, we wanted to determine the
conformation of the NTD-loop in the context of our CSL-RAM structures and whether the
isolated RAM domain of NotchIC is the trigger for the conformational change in the NTD-loop.
As shown in Figure 5C and 5D, both of our worm RAM-CSL complexes, which are in
completely different crystal lattices, reveal that the NTD-loop adopts the open conformation.
Moreover, for our mouse CSL-DNA complex, which does not contain RAM, the NTD-loop is in
a closed conformation (Figure 5A). All of the NTD-loop structures are well resolved and their
respective conformations are supported by compelling omit map electron density over this
region, as shown in Figure S2. In addition, it is unlikely that effects from the crystal lattice
influence the conformation of the NTD-loop, as none of the NTD-loop regions participate in
crystal lattice contacts. It should also be mentioned that the corresponding NTD-loop movements
Figure 5. Analysis of NTD-loop conformations. (continued next page)
Figure 5. Analysis of NTD-loop conformations. Figure shows detailed structural comparisons of the NTD-loop from CSL proteins in the coregulator-free, complexed with RAM, and ternary complex forms represented in cross-eyed stereo pairs, and emphasizes the steric clash of Mastermind with the closed conformation of the NTD-loop. The NTD of CSL and the C-terminal helix of Mastermind are depicted as ribbon diagrams and colored cyan and black, respectively. (A) Comparison of NTD-loop conformations from the coregulator-free structure of mouse CSL (cyan loop) and human CSL from the ternary complex (grey loop). Residues I131-Q139 and I91-Q99 in the NTD-loop for mouse and human CSL, respectively, are represented as sticks and colored by atom type. The side chains of Q136 and L59 from mouse CSL and human Mastermind, respectively, are colored red to represent the putative steric clash between the NTD-loop in the closed conformation with Mastermind. For panels B, C, and D, worm CSL residues I292-Q300, which correspond to the NTD-loop, are drawn as sticks and colored by atom. The side chains of R299 and L99 from worm CSL and Mastermind, respectively, are colored red to indicate potential steric clashes. (B) Comparison of NTD-loop conformations for coregulator-free structure of worm CSL (cyan loop) with worm CSL from the ternary complex (grey loop). (C) Comparison of NTD-loop conformations for coregulator-free structure of worm CSL (cyan loop) with worm CSL from RAM complex (magenta loop). (D) Comparison of NTD-loop conformations for worm CSL-RAM complex (magenta loop) with worm CSL from the ternary complex (grey loop).
Figure S2. NTD-loop electron density. Figure shows representative electron density for NTD-loops from (A) wCSL-RAM-DNA in P2₁2₁2₁ crystal, (B) wCSL-RAM-DNA in C222₁ crystal, and (C) mCSL-DNA structure. For all panels, *left*, 2Fo-Fc map (grey) contoured at 1σ; *middle*, composite omit map (magenta) contoured at 1σ; *right*, simulated annealing omit map (green) contoured at 3σ. For simulated annealing omit map, residues 292-300 and 131-139 were omitted for worm and mouse CSL, respectively.
between worm and mouse CSL are analogous, but not identical, which is likely due to the conservative changes in sequence over this region (Figure S3). Taken together, our comparison of the CSL complexes determined here with previously determined CSL structures reveals a striking result - in the absence of RAM, the NTD-loop of CSL is in a closed conformation, which sterically prevents Mastermind interacting with CSL; upon RAM binding to the BTD of CSL, this interaction triggers a long range conformational change in CSL, in which the NTD-loop changes from a closed to an open conformation, providing a docking site for loading the C-terminal helix of Mastermind.

*Role of RAM in Ternary Complex Assembly.* We next sought to examine the functional significance of the RAM induced conformational changes observed in our structural studies. Using electrophoretic mobility shift assays (EMSA) and our recombinantly purified components, we analyzed the contribution of RAM to assembly of the CSL-NotchIC-Mastermind ternary complex on a DNA substrate. As shown in Figure 6, for both mouse and worm components, a RAM peptide added *in trans* facilitates formation of a ternary complex composed of CSL, ANK, and Mastermind. It was previously shown for reconstitution of the human CSL-NotchIC-Mastermind ternary complex (27) that the ternary complex can be formed in the absence of RAM, but does so inefficiently and requires ANK in large excess. We observe similar results for our mouse components, but show that in the presence of an exogenous RAM peptide, the ternary complex forms more efficiently than complexes formed only in the presence of ANK (Figure 6C and 6D). Interestingly, at all concentrations that we were able to analyze binding, assembly of the worm ternary complex does not occur without RAM; however, addition of the RAM peptide with ANK allows for ternary complex formation (Figure 6A). Taken together, these results suggest that RAM has an additional role in ternary complex formation independent of targeting
**Figure S3.** NTD-loop sequence alignment.
Sequence alignment of CSL orthologs over the NTD-loop region. Residue numbering corresponds to worm CSL (Lag-1). Arrows denote NTD β-strands. Red brackets denote NTD-loop residues. Absolutely and highly conserved residues are highlighted in cyan.
Figure 6. EMSA analysis of ternary complex assembly. (continued next page)
**Figure 6. EMSA analysis of ternary complex assembly.** Figure shows contribution of RAM peptide to formation of the CSL-NotchIC-Mastermind ternary complex. Components of each binding reaction are denoted above each gel. EMSAs showing additional controls are included in the supplementary data (Figure S4). (A) Assembly of worm ternary complex. The concentration of CSL is 1µM and the concentrations of all other components (RAMANK, RAM, ANK, Mastermind, and DNA) are 10µM. For lanes 7, 8, and 9, increasing concentrations of ANK 10, 20, and 30µM, respectively were utilized in the binding reaction. The worm ternary complex does not form without RAM, lanes 7-9; however, addition of an exogenous RAM peptide allows for the ternary complex to form, lane 4. (B) Assembly of mouse ternary complex. The concentration of DNA is 1.5µM; the concentration of CSL, RAMANK, and ANK are 1.0µM, except for lanes 7, 8 and 9, in which the concentrations of ANK are 0, 2.5 and 5.0µM, respectively. The concentration of Mastermind and RAM are 10µM. The mouse ternary complex forms with or without RAM, lanes 4, 6, 8, and 9. (C) Efficiency of mouse ternary complex formation with and without exogenous RAM peptide. For all lanes, the concentration of CSL, Mastermind, RAM, and DNA are 1µM, 10µM, 10µM, and 1.5µM, respectively. Increasing concentrations of ANK (0.025, 0.1, 0.5, and 2.0µM) are included in lanes 2-5 and 7-10. Addition of the RAM peptide (lanes 2-5) increases the efficiency of ternary complex formation, as compared to ternary complex formation without RAM peptide (lanes 7-10). (D) Quantitation of mouse ternary complex formation. Data points were generated from integration of band intensities in (C) from three independent experiments. Bar graph shows percent ternary complex formation (y-axis) as a function of ANK concentration (x-axis). A control peptide, consisting of a scrambled RAM sequence, had no effect on ternary complex formation (data not shown).
Figure S4. EMSA controls.
Figure shows EMSA control experiments for mouse (A) and worm (B) Notch pathway components. For both gels, lanes correspond to 1 DNA; 2 CSL+DNA; 3 RAMANK+DNA; 4 RAM+DNA; 5 ANK+DNA; 6 MM+DNA; 7 CSL+RAMANK+DNA; 8 CSL+RAM+DNA; 9 CSL+ANK+DNA; 10 CSL+MM+DNA. For the mouse EMSA (A), the final concentration of CSL in the binding reaction is 1.0 µM with the other components at 1.5 µM final concentration. For the worm EMSA (B), CSL is at 1.0 µM and all of the other components are at 10 µM final concentration.
NotchIC to CSL in the nucleus, and that RAM binding to CSL actively facilitates ternary complex formation.

**Thermodynamics of the CSL-RAM Interaction.** Subsequently, we sought to determine the thermodynamic parameters that underlie CSL-RAM complex formation. We performed ITC (isothermal titration calorimetry) binding studies with our purified recombinant constructs of CSL and RAM, in which CSL was titrated with RAM peptide at 25°C. As shown in Table 1, the interaction of core CSL with RAM is an enthalpically driven reaction with a 1:1 stoichiometry and dissociation constants for mouse and worm CSL of ~30nM and ~2µM, respectively (Figure S5). Moreover, to assess any potential differences in the binding reaction when CSL is bound to DNA, we performed additional calorimetry experiments, in which CSL was bound to a 19mer of duplex DNA containing a single CSL binding site. Overall, the affinity and free energy of binding for the CSL-RAM interaction is unchanged whether CSL is free in solution or bound to a cognate DNA (Table 1). Interestingly, closer examination of the mouse CSL-RAM thermodynamic parameters in the presence and absence of DNA reveals different enthalpic/entropic contributions to the reaction, but similar free energies of binding (Table 1). The approximate 2 kcal/mol enthalpy/entropy compensation between the two reactions may reflect a favorable preordering of mouse CSL when bound to DNA prior to interacting with RAM. A similar compensation may also occur with the worm components, but the compensatory effect is not as prominent and may reflect fundamental thermodynamic differences between worm and mammalian CSL.

We then pursued a molecular explanation for the observed differences in binding for mouse and worm CSL-RAM complexes. Due to the high degree of sequence conservation and essentially identical tertiary structures for worm and mammalian CSL, the greater than 50 fold
Table 1: Calorimetric data for RAM and RAMANK binding to CSL

<table>
<thead>
<tr>
<th>CSL</th>
<th>Ligand</th>
<th>$K$ (M$^{-1}$)</th>
<th>$K_d$ (µM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$-T\Delta S^\circ$ (kcal/mol)</th>
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<tr>
<td>mBTD</td>
<td>mRAM</td>
<td>5.15(±0.11) x 10$^7$</td>
<td>0.032</td>
<td>-10.2 ± 0.05</td>
<td>-15.5 ± 0.2</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>mCSL</td>
<td>mRAM</td>
<td>5.15(±2.1) x 10$^7$</td>
<td>0.022</td>
<td>-10.4 ± 0.3</td>
<td>-17.1 ± 0.3</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>mCSL + DNA</td>
<td>mRAM</td>
<td>2.94(±0.13) x 10$^7$</td>
<td>0.034</td>
<td>-10.1 ± 0.002</td>
<td>-15.0 ± 0.3</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>mCSL + DNA</td>
<td>wRAM (Lin12)</td>
<td>3.35(±1.1) x 10$^7$</td>
<td>0.032</td>
<td>-10.2 ± 0.2</td>
<td>-15.2 ± 1.6</td>
<td>5.0 ± 1.8</td>
</tr>
<tr>
<td>mCSL + DNA</td>
<td>wRAM (Glp1)</td>
<td>1.32(±0.20) x 10$^7$</td>
<td>0.077</td>
<td>-9.7 ± 0.09</td>
<td>-15.1 ± 0.19</td>
<td>5.4 ± 0.10</td>
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<tr>
<td>mCSL + DNA</td>
<td>mRAMANK</td>
<td>7.47(±2.5) x 10$^7$</td>
<td>0.014</td>
<td>-10.7 ± 0.2</td>
<td>-19.7 ± 2.3</td>
<td>9.02 ± 2.5</td>
</tr>
<tr>
<td>wCSL</td>
<td>wRAM (Lin12)</td>
<td>3.20(±0.22) x 10$^5$</td>
<td>3.13</td>
<td>-7.4 ± 0.02</td>
<td>-15.6 ± 0.7</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>wCSL + DNA</td>
<td>wRAM (Lin12)</td>
<td>4.87(±1.6) x 10$^5$</td>
<td>2.05</td>
<td>-7.8 ± 0.1</td>
<td>-15.5 ± 3.5</td>
<td>7.8 ± 3.4</td>
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<tr>
<td>wCSL + DNA</td>
<td>wRAM (Glp1)</td>
<td>6.43(±1.0) x 10$^5$</td>
<td>1.58</td>
<td>-7.9 ± 0.1</td>
<td>-16.9 ± 0.02</td>
<td>8.9 ± 0.12</td>
</tr>
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</table>

Values are the mean of at least three independent experiments and errors represent the standard deviations of multiple experiments.
Figure S5. ITC binding data for CSL-RAM. 
Figure shows representative isothermal titration calorimetry data of the CSL-RAM complex interaction for mouse (left) and worm (right) Notch components. Both raw heat signals and integrated data from RAM titrations of CSL are shown. The titrations consisted of 40, 7µl injections, spaced 120 seconds apart.
difference in affinity for RAM was unexpected. Initially, we examined, as the source of the difference, the interaction of worm CSL with the RAM domain from Glp-1, the other Notch receptor paralog in *C. elegans*; however, the affinity and thermodynamic parameters of the worm CSL interaction with Glp-1 RAM were essentially identical to those measured for Lin-12 RAM (Table 1). Next, we performed cross-species calorimetric binding studies, in which mouse CSL was titrated with worm RAM and vice-versa. While mouse CSL binds to both worm RAM peptides with essentially identical thermodynamic parameters (Table 1), titration of worm CSL with mouse RAM resulted in no measurable heat changes during the reaction. Additionally, we tried calorimetry experiments at different temperatures and buffers; however, in all cases a change in heat upon worm CSL – mouse RAM complexation was unmeasurable, which is not necessarily indicative that the binding reaction does not occur. We therefore turned to EMSA to characterize the worm CSL – mouse RAM interaction. As shown in Figure S6A, worm CSL titrated with either worm or mouse RAM results in an apparent K\(_d\) of ~1µM, which is comparable to the ~2µM disassociation constant measured by ITC. Additionally, we performed EMSAs for mouse CSL with mouse RAM and worm RAM (Figure S6B), as well as for mouse CSL and worm CSL with mouse and worm RAMANK (described below), in order to validate the complementarity of the two approaches for measuring binding constants. As expected, the binding constants measured by ITC and EMSA are equivalent within experimental error (Table 1 and Figure S6). Taken together, these results suggest that the difference in binding affinity of mouse CSL versus worm CSL for RAM is not due to the RAM paralog or ortholog used in the reaction, but rather the difference in affinity is intrinsic to the species-specific CSL protein.

Previous experiments by Lubman *et al.* used biochemical methods and ITC to characterize the interaction of the isolated BTD of CSL with NotchIC from mouse (93). From
Figure S6. EMSA analysis of RAM and RAMANK binding to CSL. 
Figure shows titration of CSL-DNA complexes with decreasing concentrations of RAM and RAMANK. For all panels, CSL and DNA are at concentrations of 0.1µM and 1µM, respectively. RAM or RAMANK concentrations are indicated in micromolar above each lane. For each experiment, a red box above a gel lane highlights a concentration of RAM or RAMANK that corresponds to approximately 50% complex formation (~K_d). (A) Worm CSL-DNA is titrated with worm (left) or mouse (right) RAM. (B) Mouse CSL-DNA is titrated with mouse (left) or worm (right) RAM. (C) Worm CSL-DNA is titrated with worm (left) or mouse (right) RAMANK. (D) Mouse CSL-DNA is titrated with mouse (left) or worm (right) RAMANK.
their studies, they concluded that RAM is necessary and sufficient for the interaction of NotchIC with the BTD of CSL, and that other domains of NotchIC, such as ANK and PPD, do not participate in the interaction with BTD. Overall, the results from Lubman et al. are very consistent with our studies of core CSL with RAM; however, as Lubman et al. focused only on the BTD of CSL, we were keen to determine if other domains of NotchIC participate with RAM for binding to core CSL, since ANK makes extensive interactions with the CTD of CSL in the CSL-NotchIC-Mastermind ternary complex structures. For mouse, the free energy of binding and the disassociation constant for the interaction of mouse RAMANK with mouse CSL is, within error, only marginally distinguishable from the complex formed by mouse RAM with mouse CSL (Table 1). An additional ITC experiment, in which mouse CSL was titrated with mouse ANK, produced no measurable heat (data not shown). Due to buffer restraints for ITC, which were incompatible with our worm RAMANK construct, we again turned to EMSA to analyze and compare the interaction of worm CSL with worm RAM and RAMANK. As shown in Figure S6C, worm CSL forms a complex with worm RAMANK, as well as mouse RAMANK, with an apparent $K_d$ of $\sim 1\mu M$, which is comparable to the disassociation constants measured by ITC and EMSA for worm CSL with worm RAM or mouse RAM. For completeness, we also performed EMSAs for mouse CSL with mouse RAMANK and worm RAMANK (Figure S6D), and as expected, the disassociation constants estimated by EMSA were very consistent with those determined by ITC. Thus, as similarly observed by Lubman et al. for the isolated BTD of CSL, within our experimental system of NotchIC and core CSL bound to DNA, the RAM domain of NotchIC solely facilitates the initial interaction with CSL, with ANK interacting very weakly or not at all with CSL in the absence of Mastermind.
Discussion

Canonical Notch signaling ultimately results in changes in gene expression, which initiate transcriptional programs that are manifested at the cellular level. The widespread role of Notch signaling in development and adult homeostasis, as well as its pathogenic role when misregulated in human disease, underscores the importance of a molecular understanding of transcriptional regulation in the Notch pathway. The transcription factor CSL plays a central role in transducing Notch signals into transcriptional outputs, and formation of the CSL-NotchIC-Mastermind ternary complex is essential to upregulating transcription from Notch target genes. While the previously determined structures of the human and worm CSL-NotchIC-Mastermind ternary complex structures illuminated the molecular details of an active Notch pathway transcription complex, our knowledge of the exact sequence of molecular events leading to the formation of the active ternary complex is incomplete. In particular, the role of the conserved RAM domain of NotchIC, which likely targets and forms the initial interaction of NotchIC with CSL in the nucleus, remains poorly understood. In order to bridge this gap in our understanding, our study focused on elucidating the function of RAM in ternary complex assembly.

Our analyses of Notch pathway components from disparate organisms afford us the opportunity to identify elements of function that are conserved through evolution from those aspects of function that are organism specific. Our structural studies of worm CSL-RAM complexes reveal that these structures share similarities with both the coregulator-free and ternary complex forms of CSL, lending support to the notion that these CSL-RAM structures are intermediary complexes towards formation of the active ternary complex structure. While the RAM interaction with the BTD of CSL is equivalent between the CSL-RAM complexes
determined here and the CSL-RAM interaction described in the worm ternary complex structure (Figure 3C), the overall relative domain arrangements of NTD, BTD, and CTD are different between these complex structures (Figure 4D). The disposition of domains for the CSL-RAM complex more closely corresponds to the coregulator-free form of worm CSL (Figure 4C). Thus, RAM is not the trigger for the large interdomain conformational changes observed for CSL in the CSL-NotchIC-Mastermind ternary complex structure. In spite of this, the conformation of the NTD-loop in the CSL-RAM structures is more similar to the open conformation observed in the worm ternary complex structure and not the closed conformation observed in the coregulator-free worm CSL structure (Figure 5B-D).

The opening and closing of the NTD-loop in response to bound coregulators appears to be conserved in mammals (Figure 5A). The mouse structure of CSL determined here, which does not contain RAM, is overall very similar to the structure of CSL from the human ternary complex (Figure 4B), with one important difference – the conformation of the NTD-loop is in a closed conformation, similar to the coregulator-free worm CSL structure (Figure 5, compare panels A and B). In addition, our EMSA analysis of ternary complex assembly from both mouse and worm demonstrate that RAM can act in trans, facilitating formation of the ternary complex (Figure 6). Taken together, our studies reveal a provocative new role for the RAM domain of NotchIC – the binding of RAM to the BTD of CSL induces a long-range allosteric change in the NTD, the function of which is to create a binding surface for the C-terminal helix of Mastermind.

Two other points should also be addressed; first, the RAM induced change in the NTD-loop creates approximately one-half of the Mastermind binding site in the CSL-NotchIC-Mastermind ternary complex. The molecular events that create the second-half of the Mastermind binding site, which is formed between the CTD of CSL and the ANK domain of
NotchIC, are not understood at the molecular level and beyond the scope of the current study. Second, while our structural analyses strongly suggest a long-range conformational change in the NTD-loop triggered by RAM, our structures do not definitively point to an explanation for how this allosteric change is transmitted through the protein. However, an interesting area of future study will be the contribution of the absolutely conserved charged residue pair Asp336 - Arg338 in worm CSL (Asp160 - Arg162 in mouse), which participates in polar interactions with the NTD-loop, and at least in the worm CSL structures, makes substantially different interactions with the NTD-loop when it is either in the open or closed conformation.

Our binding studies of CSL-RAM complexes from both worm and mouse reveal that CSL forms a high affinity complex with RAM and a nanomolar disassociation constant (Table 1). However, our binding studies revealed one important difference between CSL orthologs, i.e. there is an approximate 50 fold (2 kcal/mol) difference in the affinity for binding to RAM; the affinity of mouse CSL for RAM is greater than the affinity of worm CSL for RAM. Moreover, this difference is not due to the RAM ortholog or paralog, but rather intrinsic to the species-specific CSL molecule. Our EMSA experiments reveal additional differences between mouse and worm CSL in assembly of the ternary complex, i.e. the mouse ternary complex can be formed in the absence of RAM, albeit inefficiently, but formation of the worm ternary complex, at least in vitro, is absolutely dependent on RAM (Figure 6). Interestingly, the mouse NTD-loop structure is less well ordered, as judged by its corresponding electron density (Figure S2), than for the worm NTD-loops. We speculate that the mammalian NTD-loop is more dynamic, which could account for the formation of the mammalian ternary complex in vitro in the absence of RAM. While the biological ramifications of these differences are unclear, these data suggest that
at the thermodynamic level there is a fundamental difference between mouse and worm CSL with regards to how these molecules interact with RAM.

Our binding studies of CSL-RAM complexes additionally demonstrate that the affinity of CSL for RAM is unchanged whether CSL is free in solution or bound to DNA (Table 1). Due to the properties of linked equilibria (94), this implies that the affinity for DNA by CSL or CSL-RAM is equivalent. While a detailed binding study of the interaction of CSL with DNA is not available, these results do suggest that there is no energetic preference for CSL interacting with NotchIC in the nucleoplasm or while CSL is bound to DNA. Moreover, this observation lends support to emergent models of CSL-mediated transcription complexes forming prior to binding at Notch target genes (95,96), which challenge the dogmatic view of CSL statically bound to DNA. Certainly, it will be of interest in future studies to characterize the thermodynamic parameters of CSL-DNA and CSL-corepressor complexes, which will bear significantly upon these new mechanisms.

In conclusion, we envision a revised model describing the molecular events that assemble the active CSL-NotchIC-Mastermind transcription complex (Figure 7). Upon pathway activation, RAM targets and initiates the interaction of NotchIC with CSL both free in the nucleoplasm and bound to target gene DNA. Our results here show that RAM binding to the BTD of CSL produces an allosteric change in the NTD, the function of which is to create one-half of the docking site required for Mastermind to bind to the complex. Results from others have shown that the requirement for RAM can be obviated when ANK is overexpressed (15-18). As the Notch signal is not amplified and limiting concentrations of NotchIC must compete with abundant corepressors in the nucleus for CSL, we speculate that RAM makes NotchIC a more effective competitor for CSL binding under physiological conditions. Following RAM binding,
Figure 7. Revised Model of ternary complex assembly. Figure diagrams sequence of events leading to formation of transcriptionally active ternary complex. CSL is drawn bound to DNA with all three functional domains - NTD (N), BTD (B), and CTD (C), which are colored cyan, green, and orange, respectively. The RAM and ANK domains of NotchIC are colored red and yellow, respectively. Mastermind (Mm) is colored grey. (A) Upon pathway activation, RAM binding to CSL both targets NotchIC to CSL and triggers an allosteric change in the NTD of CSL, which is denoted by a red asterisk. (B) Two possibilities exist: top, Mastermind (Mm) interacts with the complex to direct ANK binding to CSL; or bottom, ANK interacts with the CTD of CSL, creating the complete Mastermind docking site. The second scenario is more likely, due to the tethering of ANK to CSL through RAM, which would dramatically increase the local concentration of ANK (30). (C) Either case leads to formation of CSL-NotchIC-Mastermind ternary complexes, occupying sites at Notch target genes. We favor the second possibility, as tethering of ANK to CSL through the RAM-BTD interaction would substantially increase the local concentration of ANK (97).
the subsequent steps required for complete assembly of the CSL-NotchIC-Mastermind ternary complex are still unresolved. We put forward two possibilities (Figure 7): first, RAM binding to CSL allows for the C-terminal helix of Mastermind to interact with the complex followed by the interaction of ANK and the N-terminal helix of Mastermind with CTD; and second, RAM binding to CSL triggers the NTD conformational change, and subsequently ANK binds to the CTD, forming a complete docking site for Mastermind to interact with the complex. We favor the second possibility, as tethering of ANK to CSL through the RAM-BTD interaction would substantially increase the local concentration of ANK (97). While we were not able to observe an interaction between ANK and CSL, the CSL-ANK interaction may be very weak and not detectable under our experimental conditions for ITC. Consistent with this hypothesis, using FRET the Blacklow laboratory has recently reported a very weak but measurable affinity between CSL and ANK (98).
Chapter III

Thermodynamic and structural insights into CSL-DNA complexes

Abstract

The Notch pathway is an intercellular signaling mechanism that plays important roles in cell fates decisions throughout the developing and adult organism. Extracellular complexation of Notch receptors with ligands ultimately results in changes in gene expression, which is regulated by the nuclear effector of the pathway, CSL (CBF-1, Su(H), Lag-1). CSL is a DNA binding protein that is involved in both repression and activation of transcription from genes that are responsive to Notch signaling. One well characterized Notch target gene is HES-1 (Hairy and Enhancer of Split-1), which is regulated by a promoter element consisting of two CSL binding sites oriented in a head-to-head arrangement. While previous studies have identified in vivo and consensus binding sites for CSL, and crystal structures of these complexes have been determined, to date, a quantitative description of the energetics that underlie CSL-DNA binding is unknown. Here we provide a thermodynamic and structural analysis of the interaction between CSL and the two individual sites that comprise the HES-1 promoter element. Our comprehensive studies that analyze binding as a function of temperature, salt, and pH reveal moderate, but distinct, differences in the affinities of CSL for the two HES-1 binding sites. Similarly, our structural results indicate that overall CSL binds both DNA sites in a similar manner; however, minor changes are observed in both the conformation of CSL and DNA. Taken together, our results provide a quantitative and biophysical basis for understanding how CSL interacts with DNA sites in vivo.
Introduction

Notch signaling is an evolutionarily conserved cell-to-cell signaling pathway in metazoans that has indispensable roles during embryonic development and postnatal tissue homeostasis, such as during organogenesis and lymphopoesis, respectively (2,3). Improper signaling results in congenital defects, cardiovascular disorders, and cancer (4,6,99). Signaling is initiated when the ligand, termed DSL (Delta, Serrate, Lag-2), on the surface of one cell binds to the extracellular region of the receptor Notch on a neighboring cell (100). This interaction results in two proteolytic cleavage events of Notch, culminating in the release of the intracellular domain of the Notch receptor (NotchIC) from the membrane and translocation of NotchIC to the nucleus. Once inside the nucleus, NotchIC binds the transcription factor CSL (CBF-1, Su(H), Lag-1), which results in recruitment of the transcriptional coactivator Mastermind and conversion of CSL from a repressor to an activator of transcription from Notch responsive genes (78).

Previous studies of CSL orthologs from Homo sapiens (CBF-1), Mus musculus (RBP-Jk), Drosophila melanogaster (Su(H)), and Caenorhabditis elegans (Lag-1) revealed that CSL is a sequence specific DNA binding protein, which binds the consensus sequence –C/tGTGGGAA– (36,39,101,102). DNA sequences similar to the consensus have been identified within the promoter regions of Notch target genes in organisms ranging from flies and worms to humans, and even in the genomes of the herpesviruses EBV and KSHV (66,103-105). However, more complicated arrangements of CSL binding sites have also been observed, for example the Enhancer of split gene complex [E(spl)-C] in flies (103). Certain genes of the E(spl)-C contain a unique
promoter architecture, termed SPS [Su(H) paired site], consisting of two CSL binding sites arranged in a head-to-head manner with an approximately 16 base pair A/T rich spacer sequence (Figure 1A). The SPS architecture has also been identified in the HES-1 (Hairy and Enhancer of Split-1) genes found in mammals (25). Interestingly, one of the binding sites of the HES-1 SPS conforms precisely to the consensus binding site determined for CSL (-TGTGGGAA-), while the second site deviates from the consensus (-CGTG\text{T}GAA-); that is the T/A base step in the fifth position of the binding site was not observed in one of the consensus binding studies and infrequently observed in the other (39,101). Moreover, the sequence corresponding to the nonconsensus site for the HES-1 SPS is conserved in mammals, and conserved in some, but not all SPS sites found in Drosophila, Zebrafish, and Xenopus. The -TGTGGGAA- and -CGTG\text{T}GAA- sites that comprise the HES-1 SPS are hereafter referred to as consensus and nonconsensus sites, respectively.

More recently, high resolution crystal structures for CSL-DNA and CSL-coregulator-DNA complexes have been determined by our group, as well as by Nam et al., for CSL orthologs from worm, mouse, and human (Figure 1B) (71,73,74,106). All of these complex structures used oligomeric DNA duplexes that corresponded to the HES-1 consensus (Figure 1A), and to date, no CSL-DNA structure has been determined using any other known CSL binding site. These structures elucidated the molecular interactions that underlie specific DNA binding by CSL, and overall, despite different bound coregulators, are remarkably similar. The N-terminal (NTD) and beta-trefoil (BTD) domains of CSL cooperate to specifically recognize base pairs in the major and minor grooves, respectively, as well as form a positively charged surface to make
Figure 1. HES-1 SPS and CSL structure. (a) Figure shows nucleotide sequence and schematic representation of the mammalian HES-1 SPS (top) and the oligomeric DNA duplexes corresponding to the consensus and nonconsensus sites of the HES-1 SPS (bottom) that were utilized in the herein described binding studies. CSL binding sites are in bold text and arrows denote directionality of binding sites. The T/A base pair that deviates from the consensus is colored red. (b) Ribbon diagram for mouse CSL-DNA structure (3BRG). The NTD, BTD, and CTD are colored cyan, green, and orange, respectively. The DNA is in a stick representation with carbon, oxygen, nitrogen, and phosphorous atoms colored grey, red, blue, and orange, respectively. The DNA from this complex structure corresponds to the HES-1 consensus binding site.
nonspecific interactions with the phosphodiester backbone of DNA. The protein residues involved in DNA binding are absolutely conserved amongst all CSL orthologs. Moreover, crystal structures of worm and human CSL-NotchIC-Mastermind ternary complexes displayed protein-DNA interactions very similar to CSL-DNA structures, suggesting that formation of an active transcription complex does not alter how CSL binds to DNA (82). Following the determination of the human CSL-NotchIC-Mastermind ternary complex structure, Nam et al. described the cooperative assembly of two ternary complexes on the HES-1 SPS, in which interactions between the ankyrin repeats of NotchIC mediated the observed cooperativity (96). Moreover, the cooperative binding was dependent on the orientation and spacing of the two CSL binding sites within the SPS (96).

Despite these advances in the field, there remains a significant gap in our understanding of the energetics that underlie CSL binding to DNA, and whether any structural or affinity differences arise when CSL binds to DNA sites other than the HES-1 consensus site. The goal of this study is to address this gap in our understanding by (1) thermodynamically characterizing the binding of CSL to the two individual sites that compose the HES-1 SPS and (2) determining the X-ray structure for CSL bound to the HES-1 nonconsensus site. Our isothermal titration calorimetry (ITC) data show moderate differences for the affinity of CSL with the consensus and nonconsensus sites of the HES-1 SPS. A thorough analysis of binding as a function of temperature, salt, and pH allowed us to construct thermodynamic profiles of CSL binding to each individual site, which reveals distinct thermodynamic modes of binding. Our determination of a structure of CSL bound to the HES-1 nonconsensus site reveals, when compared to
previous structures, alternative modes of binding utilized by CSL to interact with DNA. Taken together, our thermodynamic and structural studies provide for a more thorough understanding of CSL-DNA interactions; data that is essential for developing and interpreting models of cooperative assembly of CSL mediated transcription complexes binding at the HES-1 promoter element.
Materials and Methods

Cloning, Expression, and Protein Purification

The cloning, expression, and purification of *C. elegans* and *M. musculus* CSL recombinant proteins from bacteria were described previously (71,106).

Oligomeric DNA duplexes for ITC binding Studies

The following 19-mer oligomeric DNA duplexes that correspond to the 5’ consensus and 3’ nonconsensus sites of the HES-1 SPS were purchased from Operon Biotechnologies – consensus: 5’-CGGCCCTGTGGGAAACTTCC-3’, 5’-GGAAGTTTCCCACAGGCCG-3’, nonconsensus; 5’-CGGCTCGTGTGAACTTCC-3’, 5’-GGAAGTTTCACACGAGCCG-3’. Single-stranded DNA oligos were resuspended in a buffer containing 10 mM Tris 8.0, 500 mM NaCl, and 1 mM MgCl₂, quantitated spectroscopically at A₂₆₀, mixed equimolar with the complimentary oligo, boiled for ten minutes, and allowed to slow cool at room temperature to ensure optimal duplex annealing. Size exclusion chromatography was used to purify 19-mer DNA duplexes and buffer match DNA samples for ITC binding experiments. Samples were validated for CSL binding via EMSA and were quantified by UV absorbance measurements at 260 nm.

Isothermal Titration Calorimetry

ITC experiments were performed using a Microcal VP-ITC micocalorimeter. For all binding reactions approximately 100 µM DNA was loaded into the syringe and titrated into 10 µM CSL in the cell. The c value (c=Kₘ[M]N) for all experiments ranged between
ITC binding experiments were performed in 50 mM sodium phosphate pH 6.5, 150 mM NaCl at 5º, 10º, or 15ºC; experiments performed at 20º, 25º, 37º, or 45ºC yielded no measurable heat associated with binding. The salt dependence of binding experiments were performed in 50 mM sodium phosphate pH 6.5, and 50, 100, 125, 150, 175, or 200 mM NaCl. pH dependent experiments were performed in 50 mM sodium phosphate, pH 6.0, 6.5, 7.0, 7.5, or 8.0, and 150 mM NaCl. The collected data were analyzed using the ORIGIN software and fit to a one site binding model with an average N (ligand/macromolecule) value of 0.93 and 0.92 for CSL binding the consensus and nonconsensus DNA, respectively.

**Crystallization and Data Collection**

A 13-mer DNA duplex with single-stranded TT/AA overhangs corresponding to the 3’ nonconsensus site from the HES-1 SPS was cocrystallized with mouse CSL. CSL-DNA complexes were set up in a 1:1.1 molar ratio and screened for crystallization conditions using the Hampton Research Index Screen and an Art Robbins Phoenix Crystallization Robot. The final optimized crystallization conditions were in a mother liquor containing 100 mM magnesium formate and 19% polyethylene glycol 3350 at 4ºC. Crystals were cryoprotected in mother liquor solutions containing 20% xylitol and flash frozen in LN2. The diffraction data was collected at the Advanced Photon Source (APS), beamline 22-ID. The crystals diffracted to 2.0 Å and belong to the orthorhombic space group P2₁2₁2, with unit cell dimensions a= 63.45Å, b=93.14Å, and c=112.50Å (Supplemental Table S1).
Table S1. Diffraction data and structure refinement statistics

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<tr>
<td>I/sigma(I)</td>
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<td>Rsym</td>
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<td>R_free</td>
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<td>RMSD bonds</td>
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<td>B RMSD bonds (Å²) Side Chain</td>
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<tr>
<td>Additional allowed regions (%)</td>
<td>2.9%</td>
</tr>
<tr>
<td>Disallowed regions (%)</td>
<td>0.5%</td>
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</table>
Structure Determination, Model Building, and Refinement

The structure of mouse CSL bound to DNA (3BRG), corresponding to the 5’ consensus site of the HES-1 SPS, was used with Phaser to obtain a molecular replacement solution for our mouse CSL-nonconsensus DNA diffraction data (87). Prime-and-switch phasing from RESOLVE was used to decrease model bias and Coot was used to rebuild missing parts of the model (88,89). TLS parameters were generated and used for refinement in Refmac (90,107). The structure was validated with PROCHECK and Molprobity (108,109). The final model of CSL consisted of amino acids 53-474, as well as the entire DNA duplex. The structure has been refined to an \( R_{\text{work}} = 20.0\% \) and \( R_{\text{free}} = 24.4\% \) with good geometry (Supplemental Table S1) and deposited in the Protein Data Bank. Pymol was used to align structures and to create Figure 5 (pymol.sourceforge.net). The PISA server was used to analyze protein-DNA interfaces (110). Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 3IAG.
Results

Thermodynamics of CSL-DNA interactions

In order to address questions regarding the affinity of CSL for DNA, we used ITC to characterize the binding of recombinantly purified murine CSL protein with chemically synthesized oligomeric DNA duplexes that correspond to the consensus and nonconsensus sites of the HES-1 SPS (Figure 2). All ITC experiments were performed by titrating the DNA duplex from the syringe into the cell containing CSL. Initial experiments at 25°C displayed little or no measurable heat signal upon binding, precluding analysis; however, experiments performed at the following temperatures (5°, 10°, and 15°C) allowed for determination of the thermodynamics of CSL-DNA binding. As shown in Table I, CSL binds the consensus and nonconsensus DNA sites with similar energetics. The stoichiometries of the complexes are 1:1 with ~150nM and ~300nM dissociation constants (K_d) observed for CSL binding to the consensus and nonconsensus sites, respectively, i.e. a modest two-fold tighter and ~0.3 kcal/mol greater free energy of binding observed for the consensus DNA across the temperature range tested. Both consensus and nonconsensus binding reactions are endothermic at temperatures between 5° and 15°C, and as typical for many DNA-binding proteins, the binding reaction is entropically driven (111). As shown in Figure 3, plotting the thermodynamic data as a function of temperature highlights the enthalpy/entropy compensation that is observed, as the overall free energy of binding (ΔG°) is virtually temperature independent. Again, this binding phenomenon has been observed for other DNA-binding proteins (112). Figure 3
Figure 2. CSL-DNA ITC binding assays. Figure shows representative thermograms (raw heat signal and nonlinear least squares fit to the integrated data) for CSL binding to DNA corresponding to the HES-1 5’ consensus site (top) and the 3’ nonconsensus site (bottom). Data were measured at 5˚, 10˚, and 15˚C in a phosphate buffer at pH 6.5 with 150mM NaCl. Forty titrations were performed per experiment, consisting of 7μl injections of DNA that were spaced 120 seconds apart.
<table>
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<tr>
<th>T (°C)</th>
<th>K (M⁻¹)</th>
<th>Kd (nM)</th>
<th>∆G° kcal/mol</th>
<th>∆H° kcal/mol</th>
<th>-T∆S° kcal/mol</th>
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<td>5</td>
<td>7.78 (±0.80) x 10⁶</td>
<td>129</td>
<td>-8.76 ± 0.06</td>
<td>9.18 ± 0.31</td>
<td>-17.9 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>5.05 (±1.54) x 10⁶</td>
<td>209</td>
<td>-8.66 ± 0.16</td>
<td>7.69 ± 0.18</td>
<td>-16.3 ± 0.3</td>
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<tr>
<td>15</td>
<td>5.56 (±0.72) x 10⁶</td>
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<td>-8.88 ± 0.08</td>
<td>6.08 ± 0.54</td>
<td>-14.7 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>3.45 (±0.91) x 10⁶</td>
<td>307</td>
<td>-8.30 ± 0.16</td>
<td>6.54 ± 0.80</td>
<td>-14.8 ± 0.6</td>
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<tr>
<td>10</td>
<td>3.97 (±0.14) x 10⁶</td>
<td>252</td>
<td>-8.54 ± 0.02</td>
<td>5.67 ± 0.35</td>
<td>-14.2 ± 0.4</td>
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<tr>
<td>15</td>
<td>3.04 (±0.42) x 10⁶</td>
<td>333</td>
<td>-8.54 ± 0.08</td>
<td>3.60 ± 0.05</td>
<td>-11.9 ± 0.04</td>
</tr>
</tbody>
</table>

Table I. Temperature dependence of CSL binding to consensus and nonconsensus sites of the HES-1 SPS. Consensus DNA -GTTAC\text{GTGGGA}AAAGAAAG-; Nonconsensus DNA -CGGCT\text{CGTGGA}A\text{ACTTCC}-. Values are the mean of at least three independent experiments and the errors represent the standard deviation of multiple experiments.
Figure 3. Thermodynamic profiles for CSL binding to the consensus and nonconsensus HES-1 DNA sites. Figure shows plots of thermodynamic parameters for CSL binding to the consensus site (a) and nonconsensus site (b) that comprise the HES-1 SPS. A straight line was fit to data collected at 5°C, 10°C, and 15°C, highlighting the compensatory changes in enthalpy ($\Delta H^\circ$) and entropy ($-T\Delta S^\circ$) as a function of temperature, which maintain a relatively temperature independent free energy of binding ($\Delta G^\circ$). The heat capacity change ($\Delta C_p$) was calculated from the slope of the line fit to the enthalpic data measured at 5°C, 10°C, and 15°C. The $\Delta C_p$ values for CSL binding to the consensus and nonconsensus sites are -0.31 kcal/mol/K and -0.30 kcal/mol/K, respectively.
also illustrates why we were unable to measure binding at 25°C, as the enthalpic contribution to binding approaches zero near 25°C. We were also unable to measure binding at 37° and 45°C, which we attribute to a lack of measurable heat upon complex formation at these temperatures.

We next determined the change in heat capacity (ΔCp) associated with the binding of CSL to the consensus and nonconsensus DNA sites, by analyzing the enthalpy of binding as a function of temperature (Figure 3 and S1). It has been suggested that a large and negative ΔCp value associated with a protein-DNA binding reaction correlates with burying a sizeable amount of nonpolar surface area and/or a conformational change upon complex formation (113-115). The ΔCp values determined from our ITC binding experiments are -0.31 kcal mol⁻¹ K⁻¹ and -0.30 kcal mol⁻¹ K⁻¹ for CSL interacting with the consensus and nonconsensus DNA sites, respectively. Comparison of the ΔCp values suggest no significant thermodynamic differences in the amount of buried surface area (BSA) or conformational changes for the two complexes.

We attempted to determine the ΔCp of binding based on the amount of buried surface area calculated from the structures of CSL-DNA complexes; however, as similarly reported by others, we found a large discrepancy between the observed and calculated ΔCp values, regardless of which method we utilized (116,117). Our calculated values tended to underestimate the ΔCp of binding by as much as 0.1 kcal mol⁻¹ K⁻¹. Since there are no structures of CSL in the absence of DNA, our calculations cannot account for folding of CSL coupled to DNA binding, which may account for the discrepancy in the experimentally determined and calculated values of ΔCp. Interestingly, in a previous study we observed a 2 kcal/mol enthalpy/entropy
Figure S1. ΔCp calculation for CSL binding to the consensus and nonconsensus HES-1 DNA sites. Figure shows the plot of the enthalpy of binding (ΔH°) as a function of temperature (T) for CSL binding the HES-1 consensus DNA site (■) and nonconsensus DNA site (▲). The heat capacity change (ΔCp) was calculated from the slope of the line fit to the enthalpic data measured at 5°C, 10°C, and 15°C. The ΔCp values for CSL binding to the consensus and nonconsensus sites are -0.31 kcal/mol/K and -0.30 kcal/mol/K, respectively.
compensation for the binding of CSL to the RAM domain of NotchIC in the presence and absence of DNA, which may suggest that regions of CSL undergo folding coupled to DNA binding (106).

CSL orthologs binding to DNA

In order to address whether the affinity of CSL for DNA is conserved, we performed similar ITC experiments using the worm and fly orthologs of CSL, Lag-1 and Su(H), respectively. As shown in Table II, binding of both Lag-1 and Su(H) to DNA is similar to what we observed for our murine CSL binding experiments, that is the affinity of CSL orthologs for the consensus DNA site was approximately 2-fold stronger than binding to the HES-1 nonconsensus site with $K_d$ values of ~200nM and ~400nM, respectively. Moreover, the free energy of binding was similar – approximately -8 kcal/mol. Due to the similarity of DNA binding observed for the three CSL orthologs (mouse, worm, and fly) and the high degree of sequence identity between orthologs, we conclude that the affinity of CSL for DNA has been highly conserved through evolution, and for the remainder of our analysis, we focused our binding studies exclusively on murine CSL with the HES-1 consensus and nonconsensus sites.

Effect of Salt Concentration on DNA Binding

To investigate the contribution of ionic interactions to complexes formed between CSL and DNA, we performed a series of ITC binding experiments over a range of NaCl concentrations. As shown in Table III and plotted in Figure 4, the binding of CSL to DNA is sensitive to increasing concentrations of salt, displaying the same trend for both
<table>
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<th>CSL</th>
<th>DNA</th>
<th>$K_{app}$ (M$^{-1}$)</th>
<th>$K_d$ (nM)</th>
<th>$\Delta G_{obs}$ kcal/mol</th>
<th>$\Delta H_{obs}$ kcal/mol</th>
<th>$-T\Delta S_{obs}$ kcal/mol</th>
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<tbody>
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<td>worm CSL (Lag-1)</td>
<td>HES-1 consensus</td>
<td>5.20 ($\pm$1.8) x 10$^6$</td>
<td>192</td>
<td>-8.79</td>
<td>6.11 ($\pm$0.2)</td>
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<tr>
<td>worm CSL (Lag-1)</td>
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<td>2.34 ($\pm$0.5) x 10$^6$</td>
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<td>6.49 ($\pm$0.2)</td>
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<td>fly CSL Su(H)</td>
<td>HES-1 consensus</td>
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<td>9.6 ($\pm$0.1)</td>
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<td>fly CSL Su(H)</td>
<td>HES-1 nonconsensus</td>
<td>3.49 ($\pm$0.4) x 10$^6$</td>
<td>286</td>
<td>-8.3</td>
<td>6.9 ($\pm$0.1)</td>
<td>-15.2</td>
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</table>

**Table II. CSL orthologs binding to DNA.** All experiments performed at 5°C. Consensus DNA -GTTACTGTGGGAAAGAAAG-; Nonconsensus DNA -CGGCTCGTGGAACCTCC-. The errors represent the standard deviation of the non-linear least squares fit of the data to the titration curves.
<table>
<thead>
<tr>
<th>Salt – NaCl (mM)</th>
<th>$K_{app}$ (M$^{-1}$)</th>
<th>$K_d$ (nM)</th>
<th>$\Delta G_{obs}$ kcal/mol</th>
<th>$\Delta H_{obs}$ kcal/mol</th>
<th>$-T\Delta S_{obs}$ kcal/mol</th>
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<tr>
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<td>129</td>
<td>-8.76 ($\pm$0.1)</td>
<td>9.18 ($\pm$0.3)</td>
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<td></td>
<td></td>
<td></td>
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<td>-7.00</td>
<td>4.70 ($\pm$0.3)</td>
<td>-11.7</td>
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**Table III.** Salt dependence of CSL binding to consensus and nonconsensus HES-1 sites. All experiments performed at 5ºC. For all experiments the errors represent the standard deviation of the non-linear least squares fit of the data to the titration curves, except for binding experiments performed at 150mM, where the data values and errors are taken from Table I.
Figure 4. Salt dependence of CSL binding to DNA. Figure shows the plot of log $K_a$ (association constant) versus the log of NaCl concentration for CSL binding to the HES-1 consensus DNA site (■) and nonconsensus DNA site (▲). Binding experiments were done at 50, 100, 125, 150, 175, and 200mM NaCl.
consensus and nonconsensus sites-as the concentration of salt increases, correspondingly the association constant (K) and free energy of binding ($\Delta G_{\text{obs}}$) decrease; however, binding displayed a nonlinear dependence on concentrations of salt below 125mM (Figure 4). While the molecular basis for this nonlinearity is outside the scope of this study, it should be mentioned that a similar nonlinear dependence of binding has been observed for the structurally related transcription factor NF-kappaB (118), as well as for other DNA binding proteins (119). A closer examination of the enthalpic and entropic contributions to binding at 150mM and 200mM salt reveals a modest change in the enthalpy of binding (less than 1.0 kcal/mol), but a large entropic penalty of ~3.0 kcal/mol for both reactions. These data demonstrate that increasing the salt concentration affects the entropy of binding to a greater extent than the enthalpy, which has been observed for other DNA binding proteins, whereby binding is driven by the entropically favorable release of counterions from DNA (120,121).

Effect of pH on CSL-DNA interaction

In order to address the influence solution pH has on CSL-DNA complexes and potentially identify any ionizable groups involved in binding, a series of experiments were performed in buffers ranging from pH 6.0 to 8.0 (Table IV and Figure S2). CSL binding to both consensus and nonconsensus DNA sites is nearly linearly dependent on pH between 6.0 and 8.0, with significantly stronger binding occurring at pH 6.0. Comparison of binding at pH 6.0 and pH 8.0 reveals that there is approximately a 15-fold and 1.5 kcal/mol difference in the association constant and free energy of binding, respectively (Table IV). While the overall free energies of binding for both the
Table IV. pH dependence of CSL binding to consensus and nonconsensus HES-1 sites. All experiments performed at 5ºC. The errors represent the standard deviation of the non-linear least squares fit of the data to the titration curves.
Figure S2. pH dependence of CSL binding to DNA. Figures shows the plot of log $K_a$ (association constant) versus pH for CSL binding to the HES-1 consensus DNA site (■) and nonconsensus DNA site (▲).
consensus and nonconsensus sites are similar over the pH range, the enthalpic and entropic contributions to binding are strikingly different. There is approximately a 3 kcal/mol more favorable enthalpic contribution to binding for CSL complexes formed with the nonconsensus DNA, and conversely, a 3 kcal/mol more favorable entropic contribution to binding for CSL complexes formed with the consensus DNA site. As shown in Figure S2, the slope of the line, resulting from plotting the log of the association constant (K) as a function of pH, reveals the number of ionizable residues that participate in complex formation (94). The slope of these lines for CSL binding to both consensus and nonconsensus DNA sites, over the pH range tested, is approximately one (Figure S2).

**CSL binding other nonconsensus sites**

We next sought to address the functional significance of the conserved T/A base step in the 3’ nonconsensus site of the HES-1 SPS (−CGTGTAAGAA−) by performing ITC binding experiments with DNA duplexes that have either an Adenine (−CGTGAGAA−) or a Cytosine (−CGTGCGAA−) base substituted at this position. Experiments performed with the T→A substitution revealed binding affinities and energetics similar to those observed with the HES-1 consensus DNA site (Kₐ = ~150nM and ΔGₐₒbs = -8.6 kcal/mol, see Table V). These data are consistent with previous consensus binding site studies for CSL, in which an A/T base step was observed at this position, albeit with considerable lower frequency than a G/C base step (39,101). However, experiments performed with the T→C substitution revealed a strikingly lower affinity of CSL for this DNA with a Kₐ of ~1µM and ΔGₐₒbs = -7.6 kcal/mol (Table V). This represents an approximately 7- and 3-
Table V. Thermodynamic data for CSL binding to the HES-1 nonconsensus site with either T→A or T→C base pair substitutions. T→A mutation DNA -CGGCTCGTGAGAACTTCC-; T→C mutation DNA -CGGCTCGTGCGAACTTCC-. All experiments performed at 5ºC. Values are the mean of at least two independent experiments and the errors represent the standard deviation of multiple experiments.

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<th>$\Delta H^\circ$ kcal/mol</th>
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fold difference in affinity when compared to CSL binding to the consensus and nonconsensus sites, respectively, of the HES-1 SPS.

**Structure of murine CSL bound to nonconsensus DNA**

In order to identify any structural differences for CSL binding the nonconsensus site, as well as potentially identify a structural basis for the differences observed in the thermodynamics of binding, we determined the X-ray structure of murine CSL bound to the nonconsensus site at 2.0 Å resolution. This structure was compared with our previously determined structure of murine CSL bound to the consensus site of the HES-1 SPS (PDB ID: 3BRG) (106). The CSL-nonconsensus DNA complex crystallizes with similar unit cell dimensions, belongs to the same space group symmetry, and forms comparable crystal lattice contacts as the previously determined CSL-consensus DNA complex. Structural alignment of the Cα atoms for the two structures reveals that overall the two protein-DNA complexes are similar – 408 corresponding Cα atoms overlay with an RMSD of 1.1 Å. Alignments of individual domains of CSL (NTD, BTD, and CTD) display higher degrees of correspondence, with RMSDs of 0.27, 0.29, and 0.20 Å for overlays of NTD, BTD, and CTD, respectively; however, there are moderate differences in the relative arrangement of domains, as overlaying the NTD from the consensus and nonconsensus structures reveals shifts of 4-5 Å in the corresponding positions for the Cα atoms in the BTD and CTD.

Comparison of the protein-DNA interfaces between the two complex structures also reveals a high degree of similarity. The interface formed between CSL and the consensus and nonconsensus DNA sites buries 1,070 Å² and 1,046 Å² of surface area,
respectively (110). The similarity in buried surface area between the two complexes is entirely consistent with the nearly identical $\Delta$Cp values determined above. Moreover, essentially all of the nonspecific and specific protein-DNA contacts that are observed in the CSL-consensus DNA complex are maintained in the CSL-nonconsensus DNA complex. In addition, the number of water molecules bound at the protein-DNA interface is similar for the two complexes. Overall, the backbone conformations of the two DNA duplexes are also similar with moderate differences observed for the DNA base parameters. Overlaying the corresponding phosphorus atoms from the phosphodiester backbone of the two DNA duplexes results in a 0.77 Å RMSD, with greater structural correspondence observed between aligning the top single DNA strands (-ACTGTGGGAAAGA- vs. -ATCGTGGAAGA-) over the bottom single strands (-TCTTCCCACAGT- vs. –TCTTTACACGAT-). Analysis of the T/A base step in the nonconsensus duplex (-CGTG\textsc{T}GAA-) reveals neither any large perturbations in the conformation of the DNA bases or backbone nor any changes in the protein. However, the nonconsensus DNA structure displays a much higher degree of propeller twist than the consensus DNA duplex (Table S2). The average propeller twist for base steps of typical B-DNA is +/- 10°; (122) the consensus and nonconsensus DNA sites have average propeller twists of -11 and -14, respectively. Moreover, base pairs upstream and downstream of the T/A base step in the nonconsensus DNA duplex have elevated propeller twists values, ranging from -17° to -20°. In addition, a modest 2 Å decrease in the width of the minor groove upstream of the T/A base step is observed for the nonconsensus site, as compared to the consensus site, with a compensatory increase of the major groove width downstream of the T/A base step.
|   | con     | noncon | Propeller Twist (con) | Propeller Twist (noncon) | | Δ | |
|---|---------|--------|----------------------|--------------------------|---|---|
| 1 | T - A   | T - A  | -5.14                | -8.52                    | 3.38 |
| 2 | C - G   | C - G  | -8.22                | -16.38                   | 8.16 |
| 3 | T - A   | T - A  | -9.77                | -20.09                   | 10.32 |
| 4 | T - A   | T - A  | -19.19               | -20.95                   | 1.76 |
| 5 | T - A   | T - A  | -13.62               | -17.70                   | 4.08 |
| 6 | C - G   | C - G  | -12.40               | -10.23                   | 2.17 |
| 7 | C - G   | A - T  | -2.93                | -5.57                    | 2.64 |
| 8 | C - G   | C - G  | -6.83                | -6.74                    | 0.09 |
| 9 | A - T   | A - T  | -11.80               | -17.47                   | 5.67 |
| 10| C - G   | C - G  | -9.90                | -10.39                   | 0.49 |
| 11| A - T   | G - C  | -11.94               | -17.45                   | 5.51 |
| 12| G - C   | A - T  | -18.00               | -18.06                   | 0.06 |
|   | avg     |        | -10.56               | -13.97                   | 3.41 |

**Table S2.** Propeller twist values determined from CSL-consensus (3BRG) and CSL-nonconsensus (3IAG) DNA complex structures. DNA analyzed with 3DNA software package.
We next examined and compared other regions of the consensus and nonconsensus DNA complexes with CSL, which revealed three other notable structural differences between the complexes: *first*, a β-hairpin loop in the BTD, which makes contacts in the minor groove of DNA, assumes an alternate conformation in the consensus complex, but not the nonconsensus DNA; *second*, a large loop structure in the BTD, which binds the RAM domain of NotchIC, is completely ordered in the nonconsensus complex, but largely disordered in the consensus complex; and *third*, a loop in the NTD (termed the NTD-loop), which adopts an open conformation when CSL binds Mastermind, is in an open conformation in the nonconsensus complex, but in a closed conformation in the consensus complex.

In previous CSL structures it was shown that the BTD recognizes the first two base steps of the consensus binding site (–TGTTGGGAA–) via a β-hairpin loop structure that inserts into the minor groove, making nonspecific and specific contacts with the DNA (Figure 5A) (71). These structures revealed that the side chain of an absolutely conserved glutamine residue and the backbone carbonyl of an absolutely conserved serine residue make hydrogen bonding interactions with the purine bases of the T/A and G/C base steps, respectively (Figure 5B). As shown in Figure 5C, despite the T/A to C/G base pair change at this position, similar interactions are maintained for CSL binding to the nonconsensus site (–CGTGTTGAA–). However, for the mouse CSL consensus structure, the conformation of the β-hairpin loop substantially deviates from previous structures (Figure 5A and 5D). In this structure, the glutamine side chain (Gln222) repositions itself into an orientation that points away from the DNA, eliminating any
Figure 5. BTD-DNA interactions. (continued next page)
**Figure 5. BTD-DNA interactions.** Figure shows the structural details of the BTD interaction with DNA and highlights the conformational differences in a β-hairpin loop motif between structures. Wall-eye stereo pairs are depicted. (a) Simplified overview of interactions between the BTD of CSL and DNA, showing the insertion of the β-hairpin loop motif into the minor groove of DNA. The cox trace for the NTD and BTD of CSL are colored cyan and green, respectively. The DNA backbone is represented as a ribbon and colored orange. The β-hairpin loop motifs from the murine CSL consensus DNA structure (3BRG), the human ternary complex structure (2F8X), and the murine CSL nonconsensus DNA structure (3IAG) are colored green, red, and blue, respectively. Note the high degree of structural correspondence between 2F8X (red) and 3IAG (blue), but the large structural change in the β-hairpin loop motif for 3BRG (green). (b) Zoom view of the BTD interaction with DNA from the human CSL-NotchIC-Mastermind ternary complex structure (2F8X). The β-hairpin loop is in a stick representation with standard coloring for the atoms (yellow, red, and blue for carbon, oxygen, and nitrogen atoms, respectively). The DNA is also in a stick representation and colored grey, red, blue, and orange for carbon, oxygen, nitrogen, and phosphorous atoms, respectively. For clarity only three base pairs of the DNA are shown (TGTGGGAA). Hydrogen bonds are indicated with black dashed lines. As also observed in previous CSL structures (e.g. 1TTU and 2FO1), Q182 makes hydrogen-bonding interactions with the adenine in the first T/A base step and the backbone carbonyl of S181 (denoted with a cyan asterisk) makes hydrogen-bonding interactions with the guanine at the following G/C base step. Nonspecific interactions with the DNA backbone mediated by residues R178, R180, and T183 are also shown, and the backbone carbonyl of R180 is indicated with a magenta asterisk. (c) Corresponding zoom view of murine CSL nonconsensus DNA complex structure determined here (3IAG). Orthologous residues and hydrogen bonding pattern are depicted, demonstrating identical interactions as described above. (d) Zoom view of murine CSL consensus DNA complex structure (3BRG), highlighting structural rearrangement of β-hairpin loop. In this conformation of the BTD loop the side chains of Q222 and T223 and the backbone carbonyl of S221 no longer form interactions with the DNA; however, the rearrangement allows the side chain of S221 to make hydrogen-bonding interactions with the adenine in the T/A base step (TGTGGGAA) and the backbone carbonyl of R220 to make hydrogen-bonding interactions with the guanine in the G/C base step (TGTGGGAA). Thus, despite the rearrangement, equivalent interactions with the DNA are maintained.
interactions with the T/A base step (Figure 5D). Interestingly, new BTD-DNA contacts are made in the minor groove that maintain specificity – the side chain of Ser221 moves into the space vacated by Gln222, making equivalent hydrogen bonding interactions with the T/A base step, and the backbone carbonyl of Arg220 makes equivalent interactions with the G/C base step (Figure 5D). Thus, despite the dramatic structural rearrangement, equivalent specific and nonspecific interactions with the DNA are maintained.

The second notable structural difference between the consensus and nonconsensus CSL-DNA structures occurs within a large loop structure of the BTD, which functions in binding the RAM domain of NotchIC. Previous structures have shown that in the absence of RAM or other bound coregulators this BTD loop is largely disordered (71,106). This is the case for the previously determined mouse CSL-consensus DNA structure – residues 256-261 were not modeled in the structure due to a lack of interpretable electron density for this region; however, in the mouse CSL-nonconsensus DNA structure determined here this RAM binding loop of the BTD is completely resolved, albeit with elevated B-factors, forming a comparable structure to what is observed for CSL-RAM structures and CSL-NotchIC-Mastermind ternary complex structures. We attempted to remodel and refine the disordered BTD loop from the CSL-consensus DNA complex, using the ordered BTD loop structure from the nonconsensus CSL-DNA complex. While the refinement was successful, no appreciable or significant additional electron density was observed for the BTD loop in the CSL-consensus DNA complex. Moreover, the side chain of Arg264, which is ordered in both structures and makes protein contacts at the C-terminal end of the BTD loop, is in two different, but well-defined conformations. Taken together, these data suggest that the BTD loop forms
two distinct structural elements in the two complexes and supports our original decision not to model this loop in the CSL-consensus DNA structure.

The final structural difference of note is observed within the NTD of CSL, regarding a β-hairpin loop structure that is involved in Mastermind binding and termed the NTD-loop. In CSL structures complexed with NotchIC and Mastermind, i.e. a transcriptionally active complex, the NTD-loop is an open conformation in order to accommodate binding of the C-terminal helical region of Mastermind (73,74). In structures of CSL without bound coregulators the NTD-loop is in a closed conformation (71,106); in structures of CSL bound to the RAM domain of NotchIC, the loop is also in an open conformation (106). These data have in part led to an allosteric model, in which RAM binding to the BTD triggers opening of the NTD-loop, in order to facilitate Mastermind binding and ternary complex formation. In the previously determined mouse CSL-consensus DNA structure the NTD-loop was observed in a closed conformation. In the CSL-nonconsensus DNA structure determined here the NTD-loop adopts a more open conformation, with the caveat that the B-factors are greatly elevated for the main chain atoms in this region – over the residue range Ile131 to Glu137 the average Cα B-factor is 54 and 37 for the nonconsensus and consensus DNA complexes with CSL, respectively. The potentially correlative conformations of the BTD-RAM binding loop and the NTD-loop are discussed below.
Discussion

The ability of CSL to specifically recognize and bind DNA sites within the promoter regions of Notch responsive genes *in vivo* is one of the fundamental aspects to understanding how transcription is regulated in the pathway. Despite this simple axiom, very little is understood at the quantitative level regarding the affinity of CSL for DNA. In fact, to our knowledge, only one early study in the field has attempted to determine the dissociation constant (K\(_d\)) for CSL binding to DNA. In this previous study, a K\(_d\) of 1nM was estimated from Scatchard plot analyses of CSL-DNA EMSAs, using CSL (*aka* RBP-jk) protein that was partially purified from nuclear extracts (123). Despite the caveats associated with these types of experiments and data analysis, the apparent high affinity of CSL for DNA, in conjunction with subsequent studies that demonstrated CSL functions as both an activator and repressor, has led to current models in the field that suggest CSL is statically bound to DNA while regulating transcription from Notch target genes. However, more recent studies suggest that CSL binding to DNA is a dynamic rather than static process and that recruitment of CSL, via cooperative interactions, to target genes is an important mechanism of regulation (95,96,124). Taken together, these studies imply our understanding of CSL-DNA interactions and the role these play in transcriptional regulation are incomplete.

The focus of our studies were twofold – *first*, we wanted to provide a quantitative and comprehensive characterization of the energetics that underlie CSL-DNA binding, thereby providing a baseline for understanding the assembly of Notch transcription complexes at more complicated DNA elements, such as the HES-1 SPS. For these studies, we used highly purified recombinant preparations of CSL and chose to analyze
individually the two CSL binding sites that compose the HES-1 SPS, as this would provide binding data for CSL interacting with both a consensus and nonconsensus binding site. And second, we wanted to provide additional structural information for CSL interacting with DNA sites other than the 5’ consensus site from the HES-1 SPS, as all CSL-DNA complex structures determined to date have used the same DNA binding site.

Our binding studies demonstrate that CSL has only moderate affinity for DNA with dissociation constants of at least 100nM and 250nM for the HES-1 consensus and nonconsensus DNA sites, respectively (Table I). This represents at least 100-fold weaker affinity for DNA than what was previously reported. Moreover, the observed trends in binding were conserved in worm and fly CSL orthologs (Table II), suggesting that the moderate affinity of CSL for DNA is a universal aspect of Notch signaling in all organisms. In contrast, under identical experimental conditions, we previously determined that the RAM domain of NotchIC from mouse has an approximately 3-8 fold higher affinity for CSL than what was determined here for CSL-DNA complexes. For comparison, we also determined the DNA affinity for a structurally related transcription factor - the p50 homodimer, a repressor in the NF-kappaB signaling pathway - binding to the Ig-κB DNA site (Figure S3). In contrast to CSL, p50 binding to DNA was very sensitive to temperature with >30 fold higher affinity observed at 30°C than binding experiments performed at 10°C; nonetheless, over the temperature range tested, p50 in general, has a similarly modest affinity for DNA, suggesting that both CSL and p50 binding to DNA may be modulated by interactions with neighboring transcription factors.

Two other points should also be mentioned, first, our experimentally determined values
Figure S3: Thermodynamic analysis of p50 homodimer binding to Ig-κB DNA

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Temperature dependence of p50 homodimer binding to Ig-κB DNA site. *n.b.d.* = *no binding detected*. Buffer: 50mM sodium phosphate pH 6.5 and 150mM NaCl. For all binding reactions, the average value of N=1.23, *i.e.* DNA:p50 homodimer stoichiometry.

Thermodynamic profile and ΔCp calculation for p50 homodimer binding to Ig-κB DNA site (-TCTGA\textbf{GGGACTTTCC}TGATC-).
for the affinity of p50 for DNA by ITC are very similar to the values previously reported using gel-shift and fluorescence anisotropy binding assays (118); and second, the moderate affinity of CSL for DNA we determined here is not an artifact of our experimental setup – as shown in Table S3, a number of transcription factors with low nanomolar dissociation constants for DNA have been characterized using ITC. Taken together, the modest affinity of CSL for DNA suggests that not all CSL binding sites in vivo are occupied at all times and that CSL-coregulator complexes are likely forming/exchanging in the nucleoplasm as well as on DNA – which in our mind, places much more emphasis on cooperative mechanisms that recruit CSL to sites on the DNA than previously appreciated.

Our comparative analyses of CSL binding the 5’ consensus and 3’ nonconsensus sites of the HES-1 SPS revealed only minor differences in the affinity and overall free energy for the two complexes. In both cases the binding reaction is entropically driven, relatively insensitive to temperature, and endothermic at the temperatures tested (5°C - 15°C); however, the differences in binding are significantly larger than the error in the measurements and the entropic/enthalpic contributions to the overall free energy of binding are distinctly different for the two complexes (Table I). This suggests that the two DNA binding sites, at least at the thermodynamic level, are not identical in the complexes they form with CSL. Additional comparisons, as a function of temperature, salt, and pH, did not reveal any further differences between the consensus and nonconsensus sites. In both cases, binding was very sensitive to the concentration of salt (Table III), suggesting that ionic interactions contribute largely to binding; this is consistent with the entropically favorable release of counterions underlying complex
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Table S3. Summary and comparison of ITC binding data for CSL and other DNA binding proteins.
formation. Interestingly, a similar strong dependence on salt concentration is observed for the p50/p65 NF-κB heterodimer (118). Under the experimental conditions tested, CSL binding to both sites was linearly dependent upon pH, with the tightest binding observed at pH 6.0. While a binding study over a broader pH range would be more definitive, nevertheless these data potentially indicate that one ionizable protein residue with a pKa in the range of 6.0 to 8.0, possibly a cysteine or histidine residue, plays a role in CSL-DNA binding (94). However, if this were the case, there is neither a histidine nor a cysteine residue located at the protein-DNA interface that would readily explain the pH dependence of binding.

Given the small differences in affinity that we observed for CSL binding the 5’ consensus and 3’ nonconsensus sites of the HES1 SPS, we were curious as to why previous studies that identified the consensus binding site for CSL revealed a strong preference for a G/C base step at this position (–C/tGTG\textit{GGAA}–), as opposed to A/T, C/G, and T/A base steps. Previous structures have shown that in some, but not all CSL-DNA complexes, the side chain of an absolutely conserved glutamine residue makes a water mediated contact with the guanine base in the major groove, providing some explanation for the specificity and tolerance for purine bases at this position (71). In spite of these structural results, there is relatively strong conservation for a T/A base step, \textit{i.e.} pyrimidine base, at this position in the HES-1 SPS found in mammals, Xenopus, and Zebrafish. While the T\rightarrow A mutation of the nonconsensus site (-CGTG\textit{TGAA}-) actually enhanced binding similar to the consensus site, strikingly, the T\rightarrow C mutation had a profound reduction in binding. Taken together, these results suggest that the identity of this base step is important for the affinity and specificity of CSL binding; however, a
satisfactory molecular explanation for the observed differences in binding are still lacking.

With the exception of a $\beta$-hairpin loop structure in the BTD that participates in DNA binding, which will be discussed in greater detail below, our comparison of the CSL-nonconsensus DNA structure determined here with the previously determined CSL-consensus DNA structure revealed no overtly conspicuous structural differences between the protein-DNA contacts between the two complexes; the amounts of buried surface area at the protein-DNA interface were similar; the number of specific and nonspecific DNA interactions were similar; and, the number of water molecules bound at the protein-DNA interface were also similar. Thus, overall our structural studies are consistent with our binding studies. However, upon closer examination, we noticed that the base pairs in the nonconsensus DNA duplex had a much higher degree of propeller twist than for the corresponding regions in the consensus DNA duplex (Table S2). In fact, certain base steps in the nonconsensus DNA duplex had propeller twists that were far outside the values typically observed for B-DNA. We believe that the higher degree of propeller twist observed in the CSL-nonconsensus DNA complex may account for two observations from our binding data: (1) If the binding of CSL to the nonconsensus DNA induces the unfavorable propeller twist observed in this complex, then this would likely decrease the overall free energy ($\Delta G^o$) of complex formation, which is consistent with our binding data (Table I); and (2) If the greater propeller twist observed in the nonconsensus DNA allows for increased hydrogen bonding interactions between consecutive base steps in duplex (122) then this could account for the more favorable enthalpic contribution to binding observed for the CSL-nonconsensus DNA complex (Table I).
Additional comparisons of the two complexes did reveal a striking conformational difference in the CSL-consensus DNA structure for a β-hairpin loop located within the BTD of CSL (Figure 5). This β-hairpin loop contributes to DNA binding via nonspecific and specific contacts mediated through the minor groove of DNA. Despite the dramatic rearrangement of a glutamine residue within this loop, which contributes specific DNA contacts in all other CSL-DNA structures, the new side chain and backbone interactions that are formed maintain equivalent nonspecific and specific interactions with the DNA. Thus, we do not expect that these structural changes account for any of the differences in affinity we observed in our consensus and nonconsensus binding studies with CSL. Interestingly, an analogous structural rearrangement occurs within one of the previous CSL-RAM-DNA complex structures we determined with Notch components from C. elegans (PDB ID: 3BRF) (106). We believe these structures reveal a novel mode of DNA binding mediated by the BTD of CSL, highlighting an additional level of plasticity in DNA recognition, which likely accounts for the less stringent requirements for base pairs in this region of the consensus binding site.

Finally, structural comparisons of the two CSL-DNA complexes revealed additional molecular details regarding the dynamic interplay between a large loop structural element in the BTD, which is critical for binding the RAM domain of NotchIC, and the NTD-loop, which is important for binding the C-terminal helix of Mastermind. In the previous CSL-consensus DNA structure the BTD-RAM binding loop is disordered, with minimal interpretable electron density associated with it, and the NTD-loop is in a closed conformation (106); in the CSL-nonconsensus DNA structure determined here the BTD-RAM binding loop is completely ordered, with continuous electron density, and the
NTD-loop is in more of an open conformation, but with significantly elevated B-factors. It is interesting to note that in all previous CSL-coregulator structures (human and worm CSL-NotchIC-Mastermind ternary complexes, and worm CSL-RAM complexes) when the BTD-RAM binding loop forms an ordered structure the NTD-loop is in an open conformation. These data suggest that opening and closing of the BTD-RAM binding loop affects whether the NTD-loop is an open or closed conformation, *i.e.* ordering of the BTD-RAM binding loop either intrinsically or through binding the RAM domain of NotchIC allosterically induces the opening of the NTD-loop, which is required for Mastermind binding and formation of the active ternary complex. Intriguingly, Arg226, which resides at the C-terminal end of the BTD-RAM binding loop, may be serving as a hinge or switch residue, as its side chain assumes two distinct conformations in the two complexes, seemingly dependent on whether the BTD-RAM binding loop forms an ordered or disordered structural element.
Chapter IV

Thermodynamic characterization of the MINT-CSL interaction
Abstract

The Notch pathway provides an essential cellular signaling mechanism, which ultimately modulates gene expression. A fundamental component of the pathway is the DNA binding transcription factor CSL. CSL plays an indispensable role during signaling, operating both as a repressor and activator of transcription; and while several studies have addressed the biochemical and structural properties of CSL in an activation complex, many questions still remain regarding the molecular details of CSL mediated repression complexes. Several proteins have been implicated as functional components of the CSL repression complex, but little evidence has been shown of a direct repressor-CSL interaction or how these proteins affect the formation of activation complexes. Here we provide a quantitative analysis of the interaction between the corepressor protein, MINT, and CSL. Our biophysical analysis reveals structural and thermodynamic similarities between MINT and the RAM domain from the coactivator Notch. These data are crucial, as they help us further understand the model of competition between coactivators and corepressors for binding to CSL, which dictates whether CSL functions as an activator or repressor of transcription.
Introduction

Embryogenesis, cellular development, and the maintenance of differentiated cells require cellular communication for the coordinated expression and repression of specific genes. Cell signaling pathways, such as the Notch pathway, facilitate this communication through receptor-ligand interactions on neighboring cells, which culminate in changes in gene expression (78). The necessity of this evolutionarily conserved pathway is self-evident, as mutations or deletions of components of the signaling cascade have been shown to result in developmental defects, cardiac abnormalities, cancer, and death (5,135). While organisms contain different numbers of ligands and receptors involved in the Notch pathway, such as two in C. elegans and four in humans, all organisms signal through one transcription factor, CSL (CBF-1, Su(H), Lag-1).

CSL, which functions as both an activator and repressor of transcription, plays an indispensable role in the signaling mechanism, as deletion of CSL is embryonic lethal (37). In the absence of a Notch signal, CSL along with corepressors are bound to the promoters of Notch responsive genes keeping gene expression repressed. Interaction of the extracellular domain of Notch with a ligand on a neighboring cell results in the cleavage and translocation of the Notch intracellular domain (NotchIC) to the nucleus. The binding of NotchIC to CSL is believed to displace corepressors from CSL, recruit the coactivator Mastermind, and form a transcriptionally active complex (43). Extensive biochemical and genetic studies have identified crucial domains of NotchIC and Mastermind necessary for complex formation and activation of transcription (24,49). Furthermore, high-resolution crystal structures of CSL and the CSL-NotchIC-
Mastermind ternary complex have greatly increased our knowledge and understanding of the molecular details underlying the formation of this complex (73,74).

The identification of genetic abnormalities in the Notch receptor, which ultimately lead to disease states, has provided the context for many studies investigating the Notch activation complex. But while studies have proposed the involvement of several proteins in the CSL mediated repression complex, the composition and essential components of this complex are not well understood. Biochemical experiments first suggested that CSL operated as a repressor through its interaction with the transcription factor TFIID, resulting in “sub-optimal” conformational changes in the TFIID complex, ultimately leading to decreased levels of transcription (136). Yeast two-hybrid studies identified two different CSL corepressor binding partners, both of which associate with HDAC-1. Kao et al. showed that CSL interacted with the SMRT (Silencing mediator of retinoid and thyroid hormone)/HDAC-1 (histone deacetylase-1) complex, and that the deacetylase activity of this complex was responsible for modulating transcription (44). Furthermore, it was shown that CSL mutants defective in repression, first identified by the Hayward Lab, did not interact with this SMRT complex and failed to repress transcription (137). The second yeast two-hybrid study isolated a different HDAC-1 containing complex, which suggested the link to CSL mediated repression was facilitated by a different protein, CIR (CBF-1 interacting corepressor) (45). In yet other work, Zhou and colleagues identified another protein component of the SMRT/HDAC-1 corepressor complex, SKIP (Ski-interacting protein) (46). Interestingly, this work showed SKIP to interact with both the repression and activation complexes mediated by CSL. Subsequent work integrated all of these findings, proposing a model of CSL
mediated repression that suggests: 1) corepressors interact with CSL to repress transcription from Notch responsive genes, and 2) nuclear localization of Notch displaces corepressors from CSL, thus activating transcription (137,138).

While the previously mentioned works have contributed to our understanding of CSL regulated transcriptional repression, attempts by our group and others to biochemically, biophysically, and structurally characterize the interaction between SMRT, SKIP, or CIR with CSL have yielded little evidence of a direct interaction (data not shown and personal correspondence with Franz Oswald). This is in contrast to our studies with the co-activator Notch that shows a strong affinity for CSL in the absence of other binding partners (106). This raises the question as to whether these proteins directly interact with CSL, or if not, what factor is facilitating the interaction of CSL with these protein complexes? A potential link could be the protein MINT/SHARP. Originally identified in two independent studies, MINT (Msx2-interacting nuclear target protein) or SHARP (SMRT/HADC1 Associated Repressor Protein) are orthologs of mouse and human origin, respectively, that were first observed to have negative regulatory effects on a homeodomain protein and a nuclear receptor, respectively (139,140). Subsequent studies provided compelling evidence of a direct interaction between SHARP and CSL, and demonstrated a role for SHARP in the HDAC repression complex that is vital for regulation of Notch activated genes (47). Oswald and colleagues later showed that a CSL/SHARP complex is capable of recruiting the corepressors CtIP/CtBP to silence Notch target genes (141). Similarly, domain mapping of the mouse homolog, MINT, determined similar regulatory regions important for CSL and RNA recognition, and provided strong in vivo evidence of MINT’s negative regulation of
Notch signaling through its ability to block differentiation of precursor B cells into marginal zone B cells (See Figure 1) (142,143).

While several proteins have been suggested to be components of CSL mediated repression complexes, the exact composition or structure of any of these complexes is yet to be resolved. Current understanding of the CSL mediated “switch” between a repressed and activated transcription complex proposes that corepressors are displaced by Notch, which then masks the repression domains of CSL. This hypothesis seems to indicate that Notch needs to disrupt and out-compete an avidly interacting corepressor. With compelling evidence of a direct MINT-CSL interaction, the goals of this study are to biochemically and biophysically characterize the MINT-CSL interaction. The data presented here shows that MINT, like the Notch RAM domain, interacts with a high affinity for CSL. Circular dichroim analysis shows that both MINT and RAM are disordered. Furthermore, our isothermal titration calorimetry experiments indicate that unlike the RAM-CSL interaction, which only occurs in the beta-trefoil domain (BTD) of CSL, MINT interacts with the BTD as well as another domain of CSL. Our thorough calorimetric analysis of the MINT-CSL binding, in conjunction with our previous analysis of RAM-CSL binding, provides unique insight into how these two proteins may compete for CSL. This work presents the first quantitative study of a corepressor-CSL interaction, information that will provide greater insight into how CSL mediated repression complexes are formed and how they may compete with activators for CSL binding.
Figure 1: Domain schematic of MINT. Diagram represents the characterized domains of MINT, most of which are conserved in the human homolog, SHARP. RRM, RNA recognition motif; NLS, nuclear localization signal; MSXB, Msx2 binding domain; CID, CSL interacting domain; SPOC, Spen paralog and ortholog C-terminal domain (shown to bind SMRT). Small yellow rectangles below the CID represent the portions of MINT previously identified to be crucial for mediating repression via CSL, as well as the peptides used in this study to investigate MINT binding to CSL. 76-33, amino acids 2776-2833; 76-00, amino acids 2776-2800; 01-33, amino acids 2801-2833.
Materials and Methods

Protein, Expression, and Protein Purification

The cloning, expression, and purification of *M. musculus* CSL, core domain and BTD, NotchIC, RAM, RAMANK, ANK, and Mastermind were described previously (106). The MINT peptides were generated by cloning MINT residues 2776-2833, 2776-2800, or 2801-2833 into a modified pET 28b(+) vector (generous gift of Dr. Christopher Lima). This vector encodes a fragment of SMT3, producing a His-SMT3-MINT fusion protein. The fusion protein was overexpressed using a published autoinduction protocol (144). The cells were harvested, resuspended in binding buffer, flash frozen, thawed and lysed. The lysate was run over a nickel affinity column. The column was washed, and fusion protein eluted with imidazole to isolate the MINT fusion protein. The fusion protein was then cleaved, to remove His-SMT3 from the MINT moiety using the Ulp1 protease (gift of Dr. Christopher Lima), which leaves only an N-terminal serine residue attached to MINT following cleavage. The His-SMT3-MINT fusion proteins or the cleaved MINT proteins were further purified using a combination of affinity, ion exchange, and size exclusion chromatography to ensure proper buffer matching for subsequent ITC experiments. Purity was assessed by SDS-PAGE, and determined to be > 98%. Samples were subjected to mass spectrometry for verification of sample homogeneity and purity.

Isothermal titration calorimetry (ITC)

ITC experiments were performed using a Microcal VP-ITC microcalorimeter. The majority of the ITC experiments were performed at 25°C, unless otherwise noted, in a buffer containing 50mM sodium phosphate pH 6.5, 150mM NaCl, and 1mM TCEP. All
MINT peptides and CSL components were buffer-matched by size exclusion chromatography. For experiments that contained CSL bound to DNA, two DNA oligos were annealed resulting in a 19mer blunt-ended DNA duplex containing one CSL binding site and purified by size exclusion chromatography in a matched buffer for ITC. The experiments were performed with either core CSL or the BTD of CSL in the microcalorimeter cell (10-30 µM), with or without the 19mer DNA (11 µM and 33 µM), and titrated with MINT or His-SMT3-MINT proteins (100-300 µM). Protein concentrations were calculated using spectrophotometry at UV280, as well as BCA assay (Pierce). Data were analyzed using the ORIGIN software, and fitted to a one-site binding model. Data reported is the average of at least three individual experiments. The c-value (c= $K_a[M]N$) for all experiments was between 5 and 300.

**Circular Dichroism (CD)**

Proteins were dialyzed into CD compatible buffer, 10 mM sodium phosphate pH 6.5, 150mM sodium fluoride and analyzed using an Aviv spectrophotometer. Data were collected in triplicate at 25ºC, in 1nm steps, with a 2 second averaging time, from 260 to 190 nm. Buffer blank results were subtracted from the experimental data.
Results

*MINT-CSL Interaction*

Based on previous studies that roughly mapped the CSL interacting domains of SHARP/MINT, we identified a highly conserved stretch of 60 amino acids that likely serves as the core of the interaction between SHARP/MINT and CSL, and served as the starting construct for our analysis. In light of our previous work with mouse CSL, RBP-Jκ, our analysis of the SHARP/MINT-CSL interaction was carried out using the mouse orthologous components – MINT and RBP-Jκ. In order to quantify the affinity of the MINT-CSL interaction, isothermal titration calorimetry (ITC) experiments were performed using purified recombinant proteins. Initial studies utilized the peptide fragment of MINT corresponding to amino acids 2776-2833, hereafter referred to as MINT CID (CSL interacting domain), and the core domain of mouse CSL, amino acids 53-474. Experiments were performed at 25°C by titrating MINT CID into the cell containing CSL in the presence or absence on DNA. The interaction between MINT CID and CSL was observed to be an enthalpically driven, entropically unfavorable reaction with an approximate 12 nM $K_d$ (Table 1). The presence of DNA did not seem to alter the binding of MINT CID to CSL, as experiments with CSL prebound to DNA yielded almost identical thermodynamic parameters.

The affinity of the MINT CID-CSL interaction appeared very similar to the Notch RAM domain-CSL interaction we had determined previously, 14 nM and 34 nM, respectively (Table I) (106). This observation, along with the proposed displacement mechanism hypothesis where Notch competes for CSL binding interfaces with
Table I: Calorimetric data of the MINT CID-CSL interaction. mCSL, mouse CSL (residues 53-474); MINT CID, MINT CSL interacting domain; DNA, 19mer consensus CSL binding site; mBTD, mouse BTD (residues 204-352); mRAM, mouse RAM domain (residues 1774-1801). Values are the mean of at least three independent experiments and the errors represent the standard deviation of multiple experiments.

<table>
<thead>
<tr>
<th>CSL</th>
<th>Ligand</th>
<th>$K (M^{-1})$</th>
<th>$K_d$ (µM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$-T\Delta S^\circ$ (kcal/mol)</th>
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<td>mCSL</td>
<td>MINT CID</td>
<td>8.00 (±1.1) x 10$^7$</td>
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<td>-14.8 ± 0.1</td>
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<td>7.28 (±1.2) x 10$^7$</td>
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<td>MINT CID</td>
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<td>-14.8 ± 3.5</td>
<td>8.8 ± 3.5</td>
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<td>-10.1 ±0.002</td>
<td>-15.0 ± 0.3</td>
<td>4.8 ± 0.3</td>
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<tr>
<td>mBTD</td>
<td>RAM</td>
<td>5.15 (±0.11) x 10$^7$</td>
<td>0.032</td>
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<td>-15.5 ± 0.2</td>
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corepressors and the knowledge that the RAM domain binds only to the BTD, led us to pursue ITC studies using MINT CID and the BTD of CSL. Experiments were performed in a similar manner with MINT CID being titrated into the cell containing the BTD of CSL at 25°C. Unlike RAM binding to the BTD, whose binding was almost identical to the binding observed with full length CSL, the MINT CID-BTD interaction did not occur with the same affinity or thermodynamics as observed with full length CSL (Table 1). The dissociation constant ($K_d$) of the MINT CID-BTD interaction was several thousand fold weaker, 41µM; and the free energy ($\Delta G^\circ$) of the MINT CID-BTD interaction was also much less favorable, -5.9 kcal/mol, than the MINT CID-CSL interaction, -10.7 kcal/mol. This 5 kcal/mol difference in free energy seems to be attributed to the much larger entropic penalty (-$T\Delta S^\circ$) associated with the MINT CID-BTD interaction, 8.8 kcal/mol, compared to 4.1 kcal/mol for the CSL interaction. Taken together, these results suggest that while MINT does bind the BTD, the BTD of CSL alone does not provide the total binding interface for the MINT CID-CSL interaction, as it does for the RAM-CSL interaction (106).

*Dissecting the MINT CSL interacting domain*

While our initial thermodynamic results provided quantitative data regarding the MINT CID-CSL interaction, we sought to further characterize the interaction through the use of smaller MINT constructs. Analysis of the MINT CID (residues 2776-2833), through the use of the SABLE server for secondary structure prediction, revealed no alpha-helical or beta-strand structure that would suggest a specific location for division of the peptide (145). However, it was decided to subdivide MINT-CID into two equally smaller
peptides corresponding to residues 2776-2800 and 2801-2833. Fusion protein constructs encoding these two different peptides were generated and expressed in *E. coli*. Upon cleavage of the SMT3 tag from the MINT 2776-2800 construct, hereafter referred to as MINT (76-00), the peptide irreversibly precipitated. Attempts to resuspend the peptide in alternate buffers, as well as DMSO, failed to solubilize the peptide, thus preventing further analysis of this peptide and CSL. The C-terminal fragment of the MINT CID (2801-2833) was readily expressed and purified to homogeneity for use in binding studies. Once again ITC was employed to characterize the interaction for comparison of this fragment to full-length MINT CID. MINT 2801-2833, hereafter referred to as MINT (01-33), was titrated into the cell containing either CSL or CSL pre-bound to DNA. Similar to experiments using MINT CID, the interaction was enthalpically driven but the dissociation constant and free energy of the interaction were quite different. The MINT (01-33) *K*_d was determined to be approximately 8 µM in the presence or absence on DNA, roughly 600 fold weaker than MINT CID, and the ∆G° was -6.9 kcal/mol, with or without DNA present, a decrease of almost 4 kcal/mol of free energy when compared to MINT CID (Table 2). When ITC experiments were performed by titrating MINT (01-33) into the cell containing BTD, the dissociation constant, 12µM, and free energy (∆G°), -6.6 kcal/mol, were nearly identical as when full length CSL was titrated with MINT (01-33) (Table II). These results provide us two important details of the MINT-CSL interaction: 1) MINT (01-33) does not provide the entire binding interface for the MINT CID-CSL interaction, and 2) thermodynamic data suggests that MINT (01-33) binds the BTD of CSL.
<table>
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<tr>
<th>CSL</th>
<th>Ligand</th>
<th>$K (M^{-1})$</th>
<th>$K_d$ (µM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
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<td>$-6.6$</td>
<td>$-8.83$</td>
<td>$2.2$</td>
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**Table II: Calorimetric data of the MINT (01-33)-CSL interaction.** MINT (01-33), MINT peptide encoding C-terminal residues of MINT CID, residues 2801-2833. mCSL, DNA, and mBTD same is in Table 1. Values are the mean of at least three independent experiments and the errors represent the standard deviation of multiple experiments.
**SMT-fusion ITC experiments**

While the insolubility of the MINT (76-00) peptide precluded a calorimetric analysis of MINT (76-00) binding to CSL, we were able to perform ITC experiments with the uncleaved SMT3 fusion protein corresponding to the MINT (76-00) peptide. These experiments were performed by titrating SMT3-MINT (76-00) into the cell containing CSL. Analysis of the titration experiment of SMT3-MINT (76-00) into CSL revealed an exothermic reaction that was both enthalpically and entropically favorable, occurring with a free energy ($\Delta G^\circ$) of -7.3 kcal/mol and a modest $K_d$ of 4.1μM (Table III). While the free energy and affinity of this interaction appear similar to the MINT (01-33)-CSL interaction, unlike MINT (01-33), ITC experiments performed by titrating SMT3-MINT (76-00) into the cell containing only the BTD of CSL yielded no detectable heat spikes, suggesting that unlike MINT (01-33), MINT (76-00) does not bind the BTD. For completeness similar experiments were performed using both MINT (01-33) and the Notch RAM domain fused to SMT3 to account for any effects the fusion tag would have on the interaction (Table III). Comparison of these experiments to those performed without the SMT3 tag (Table II) yielded very similar association constants and free energies of binding, with slightly different enthalpic and entropic contributions. Overall, these experiments allow us to conclude that MINT (76-00), like MINT (01-33), binds CSL with a modest affinity and this interaction must occur with another domain of CSL outside of the BTD.
Table III: Calorimetric data of MINT and RAM fusion protein-CSL interaction.
SMT3-MINT (76-00), MINT peptide encoding MINT CID N-terminal residues 2776-2800 fused to SMT3 tag. SMT3-MINT (01-33), MINT peptide encoding MINT CID C-terminal residues 2801-2833 fused to SMT3 tag. SMT3-RAM, RAM peptide encoding RAM residues 1774-1801 fused to SMT3 tag. mCSL and mBTD same as in Table 1. Values are the mean of at least three independent experiments and the errors represent the standard deviation of multiple experiments. N.B.D.= No Binding Detected
Heat Capacity ($\Delta C_p$) Determination

We next sought to determine the change in heat capacity ($\Delta C_p$) associated with formation of the MINT-CSL and RAM-CSL complexes, in order to compare the interactions. A series of experiments were performed at 5º, 15º, 25º and 35º C, by titrating either MINT-CID or RAM into the cell containing CSL. The enthalpies observed at each of the different temperatures were plotted versus the experimental temperature, as shown in Figure 2 and Table IV, and the slope of the line fit to the data is the $\Delta C_p$. A large negative $\Delta C_p$ has been correlated with the burial of surface area upon complex formation or conformational change (113-115). This same type of analysis was performed for the mouse Notch1 RAM domain–mouse CSL interaction as well as for worm Lin-12 RAM domain–worm CSL interaction, the results of which are plotted in Figure 2 for comparison to MINT. The $\Delta C_p$ of the MINT-CSL interaction was determined to be $-0.47$ kcal mol$^{-1}$ K$^{-1}$; the $\Delta C_p$ for the mouse and worm RAM domain interaction were determined to be $-0.61$ kcal mol$^{-1}$ K$^{-1}$ and $-0.39$ kcal mol$^{-1}$ K$^{-1}$, respectively.

The lack of a MINT-CSL structure prevents us from attempting to calculate a $\Delta C_p$ value for comparison to our experimentally derived value; however our knowledge of worm CSL structures in RAM bound and unbound states allows for this type of in silico analysis. Nonetheless, attempts to calculate the $\Delta C_p$ based solely on burial of solvent exposed surface area alone fail to reproduce the experimentally derived value, regardless of what method is used to calculate surface area or algorithm to compute the $\Delta C_p$ (115,146,147).
Figure 2: ΔCp calculation for MINT and RAM binding CSL. Figure shows the plot of the enthalpy of binding (ΔH°) as a function of temperature (K, degrees Kelvin) for MINT CID binding CSL (■), mouse RAM, mRAM, binding mouse CSL, mCSL, (▲), and worm RAM, wRAM, binding worm CSL, wCSL, (●). The change in heat capacity (ΔCp) was calculated from the slope of the line fit to the data measured at 5°, 15°, 25°, and 35° C, and shown in Table 4. The ΔCp values of binding are as follows: MINT-CSL, -0.47 kcal mol⁻¹ K⁻¹; mouse RAM-mouse CSL, -0.61 kcal mol⁻¹ K⁻¹; and worm RAM-worm CSL, -0.39 kcal mol⁻¹ K⁻¹
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<th>$\Delta G^\circ$ (kcal/mol)</th>
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Table IV: Calorimetric data of the temperature dependence of MINT and RAM binding to CSL. wCSL, worm CSL; wRAM, worm RAM. mCSL, MINT, mRAM same as in Table 1. Values are the mean of at least three independent experiments and the errors represent the standard deviation of multiple experiments.
Circular Dichroism

To determine if the MINT-CID contained secondary structure in the absence of a binding partner, circular dichroism (CD) experiments were performed. While the use of computer algorithms such as SABLE and SMART did not predict the MINT-CID to contain any secondary structure or conserved domains, CD experiments were carried out on our MINT-CID peptide to support and validate the in silico predictions (145,148). Samples were prepared and data was collected as detailed in the Materials and Methods. For comparison, CD spectra were collected for other protein components of the Notch pathway: the mouse Notch1 RAM peptide (RAM), the mouse Notch1 ankyrin repeats (ANK) and the mouse Mastermind-like 1 coregulator (MAML1). Both the MINT-CID and RAM peptide CD spectra are characterized by a minima around 200 nm, which has been shown to be the typical CD spectra of a protein devoid of secondary structure (Figure 3) (149). This is in sharp contrast to the spectra of ANK or MAML1. X-ray crystallographic studies, as well as previous CD analysis, have shown both of these proteins to be highly alpha-helical (68,73). Both proteins yield spectra with minima around 222 nm and 208 nm, an observation indicative of alpha helix containing proteins. Taken together, these results suggest that MINT-CID, like the mouse Notch1 RAM peptide, is an unstructured peptide with little to no secondary structure.
Figure 3: CD spectra of Notch signaling components. Proteins were purified, dialyzed into a CD compatible buffer, and a wavelength scan was collected as described in the materials and methods. The data represent proteins at the following micromolar concentrations: MINT (■), 468 µM; ANK (▲), 8.9 µM; RAM (▲), 333 µM; MAML (○), 152 µM.

Discussion:
Our understanding of Notch regulated transcription places CSL at the center of this cellular mechanism. CSL has been shown to be the protein component upon which a Notch transcriptional activation complex is built and numerous studies have investigated the crucial NotchIC-CSL interaction that facilitates this complex formation (25,93). Models also suggest CSL to play a similar role in the repression of transcription, but while many components of this complex have been identified, which factors directly bind CSL is unknown. It would seem logical that in a manner analogous to the activation complex, the formation of the repression complex would be initiated by an avid protein-CSL interaction. Our thermodynamic analysis of the MINT-CSL interaction reveals a binding event that is similar to the NotchIC-CSL interaction and our CD data highlights structural similarities between MINT and the RAM domain of NotchIC. These results, in conjunction with our previous studies of RAM-CSL binding, provide us with a more thorough understanding of the complexities behind the CSL mediated transcriptional switch.

The current model of CSL regulated transcription posits that in the absence of a Notch signal, corepressors bind CSL and together this complex represses the transcription from Notch responsive genes. The nuclear localization of NotchIC is thought to displace these corepressors from CSL and subsequently activate transcription. While the mechanism of displacement of corepressors is currently unknown, it has been suggested that this occurs because NotchIC interacts with a higher affinity for CSL than any corepressor. This suggestion has most likely persisted due to the lack of evidence regarding a high affinity interaction between a corepressor and CSL. Moreover, the uncertainty about the components of a repression complex that make direct interactions
with CSL has prevented this quantitative data from being obtained. Our ITC analysis of the MINT CID-CSL interaction presented here shows that MINT CID interacts directly with CSL with a high affinity $K_d$ of 14 nM, and furthermore this binding is nearly identical in the presence or absence of DNA (Table I). When comparing the MINT CID-CSL interaction to that of NotchIC RAM-CSL interaction, not only are the affinities strikingly similar, but so are the other thermodynamic parameters. This provides the first quantitative evidence of a repressor binding to CSL with a high affinity; and would seem to imply that in the absence of other factors, NotchIC RAM or MINT CID could interact equally well with CSL. We must however be mindful that MINT is a much larger protein than the construct used in these studies and the thermodynamics of that interaction are currently unknown. Our previous comparison between RAM and RAMANK binding to CSL demonstrated an enthalpy-entropy compensation in order to maintain the favorable free energy and nanomolar dissociation constant (106). It is possible that a similar compensation may occur when full-length MINT interacts with CSL, or the thermodynamics and affinity could be altered drastically. Regardless, it seems likely that the displacement of corepressors from CSL would not be based solely on affinity alone, and that other factors would likely contribute.

Mutational analysis of CSL performed in the absence of structural knowledge revealed crucial residues necessary for proper repression of transcription (65). Structural elucidation of CSL showed that many of these residues, including a very essential phenylalanine, lie within the BTD (71). Interestingly this domain was also shown to be vital for activation complex formation, and the location of the high affinity RAM domain interaction (106). These results seem to imply that the BTD may be serving as a dynamic
region for coactivator and corepressor binding and displacement. To investigate if the BTD had a similar role in MINT binding as it did in NotchIC binding, ITC experiments using only the BTD of CSL were performed. Interestingly, unlike the NotchIC interaction that bound to CSL or the BTD with nearly the same affinity, MINT binding to the BTD alone could not recover all of the binding observed with full length CSL. MINT CID binding to BTD displayed a reduced affinity as well as a substantial decrease in the free energy of the interaction (Table I). These two parameters were almost identical for the RAM-CSL and RAM-BTD interaction. It is of interest to note that regardless of the reduced affinity, the enthalpic contribution remains almost identical for MINT CID binding to CSL or the BTD alone (Table I). Despite a clear explanation for the thermodynamics of this interaction, it is obvious that the BTD is utilized by MINT for binding and seems likely that another domain of CSL may be necessary to facilitate proper complex formation. However, it is unknown whether it is the NTD or CTD that may be bound by MINT, which emphasizes the need to investigate the potential interaction of either of these domains with MINT CID.

By generating smaller peptides that encoded portions of MINT CID, it was our hope that we could elucidate which portion of MINT CID bound to the BTD, as well as determine if both N and C terminal fragments of MINT are required to interact with CSL. Our results show that the C-terminal portion of MINT, amino acids 2801-2833, binds with approximately the same affinity and thermodynamics to CSL or the BTD (Table II). Additionally, this binding seems very similar to the MINT CID-BTD interaction (Table I), which together support our claim that the C-terminal portion of MINT CID binds the BTD. Our fusion ITC experiments further validate our assertion that MINT (01-33)
binds the BTD, as no binding was observed when SMT3 fused MINT (76-00) was titrated into the BTD. The weak binding that was observed when the SMT3-MINT (76-00) ligand was titrated into CSL also substantiates our hypothesis that MINT binds a domain other than the BTD as well. It must also be noted that our choice of MINT CID division appears to have divided the high affinity interacting molecule in half, with each peptide binding CSL with low micromolar affinity. The MINT CID construct that we had initially synthesized was chosen based on previous domain mapping experiments and sequence similarity between the mouse and human MINT homologs. It was not known exactly which part of this construct bound CSL, and the results show that neither the N- nor C-terminus alone can recover the binding observed with the full-length construct. The development of additional MINT constructs that contain N- and C-terminal truncations will reveal the residues necessary and sufficient to achieve high affinity binding. If shorter constructs are shown to contain all of the binding for interacting with CSL, removal of these unnecessary residues from either end could also potentially aid in the crystallization of a MINT-CSL complex, which to date, our attempts at crystallization of this complex have been unsuccessful.

Our initial binding studies seem to show that there is little difference in affinity between RAM and MINT binding to CSL, which complicates our understanding as to how these two molecules may compete for the same binding interface. Secondary structural analysis by circular dichroism highlights another similarity of these two molecules in that they both seem unstructured in the absence of a binding partner (Figure 3). The structure of the RAM domain has been resolved in complex with CSL, but even then only a few residues of RAM form a short beta-strand with CSL (106). Thus far it is
unclear if MINT binds in a similar orientation as RAM or across another interface of the BTD. With the structure of the RAM-CSL complex in hand, our ITC temperature dependence experiments allowed us to calculate a $\Delta C_p$ value for each of these interactions, and correlate this value to the known structure (Figure 2 and Table 4). This value is believed to be associated with the amount of surface area buried upon complex formation (113,114). The $\Delta C_p$ calculated for the MINT-CSL interaction, $-0.47$ kcal mol$^{-1}$ K$^{-1}$, was determined to be less than the $\Delta C_p$ for the RAM-CSL interaction, $-0.61$ kcal mol$^{-1}$ K$^{-1}$ (Figure 2). If these values do correlate to the amount of surface area buried upon complex formation, it would appear that RAM buries more surface area than MINT. With the MINT construct used being twice as big as the RAM construct, this may suggest that a large portion of MINT CID is not making contacts with CSL. If this is the case, in combination with our previous results and those presented here, we can propose a thermodynamic model for the displacement mechanism of corepressors by NotchIC. In the absence of NotchIC, MINT CID could bind the BTD and another domain of CSL burying a small surface area, reflected by our $\Delta C_p$ calculation. The presence of RAM, which binds as tight as MINT, but seems to bury a greater amount of surface area and all of it in the BTD, could displace the weakly interacting MINT (01-33) residues from the BTD. This initial displacement could beget the overall disruption of the repression complex, which subsequently results in activation complex formation. It must be mentioned that this model does not account for how other corepressors may be influencing the overall repression complex or how conformational changes may influence the mechanism, but does provide a model that could be tested using established gel shift techniques and competition ITC experiments.
In sum, our thermodynamic and biophysical analysis of the MINT CID-CSL interaction reveals the first quantitative evidence of a high affinity corepressor-CSL interaction. Our studies identified similarities as well as differences between this interaction and that of RAM-CSL. These data are essential to the Notch field as it uncovers the subtle differences between corepressor and coactivator binding to CSL.
Chapter V

Notch pathway transcription complexes – defining coactivator and corepressor binding surfaces on CSL
Abstract

Activation and repression of Notch responsive genes relies on the ability of CSL to form protein complexes with either coactivators or corepressors, respectively. Genetic screens have identified various protein components of these complexes and biochemical experiments have begun to elucidate how they interact with each other and CSL; however, many questions still remain regarding the molecular details of CSL mediated repression and activation complexes. Recently, we have determined the crystal structure of a Notch pathway active transcription complex bound to DNA, consisting of CSL, the intracellular domain of the receptor Notch (NotchIC), and the transcriptional coactivator Mastermind. Based on this ternary complex structure, we have designed mutations in CSL that would likely perturb formation of the ternary complex and inhibit transcription. We have tested these CSL mutants using biochemical, biophysical, and cellular assays to assess the effect these mutations have on transcription. Our studies have identified four residues in the BTD (beta-trefoil domain) of CSL at the NotchIC interface that are critical for both activation and repressive functions of CSL. When mutated, these residues drastically alter transcription in vivo. Furthermore, our ITC binding studies reveal how these mutations affect the thermodynamics of CSL interactions with NotchIC and the corepressor MINT. Taken together, these results provide molecular insights into how CSL functions as both a repressor and activator for genes that are responsive to Notch signaling.
Introduction

The Notch signaling pathway is a cellular communication system that has been conserved amongst metazoa and provides an indispensable function for cellular development and homeostasis (150). The pathway is activated when the Notch receptor interacts with a ligand on a neighboring cell. This interaction results in the cleavage and release of the intracellular domain of the receptor (NotchIC) from the membrane, which is then translocated to the nucleus. Once inside the nucleus, NotchIC forms a ternary activation complex with the transcription factor of the pathway CSL (CBF-1, Su(H), Lag-1) and the coactivator Mastermind, which together activate the expression of Notch responsive genes (27,80). This ternary activation complex has been shown to be critical for proper signaling, and defects in these core components have been shown to be extremely deleterious to the cell. With the importance of the CSL-NotchIC-Matermind complex established, it seems evident that a more molecular understanding of this complex could result in therapeutics designed to modulate only Notch signaling and limit off target side effects.

Our extensive knowledge of the activation complex components, steps involved in its formation, and means of affecting its cellular function provide us with useful data about CSL mediated transcriptional activation complexes, but provide few details regarding CSL mediated repression complexes. Early biochemical characterization of CSL described its role as a transcriptional repressor necessary for proper gene expression patterns in B-cells. These studies described the interaction of Epstein Barr Virus nuclear antigen 2 (EBNA2) with CSL, and detailed how this interaction was significant for EBV
infected B-cells. In uninfected resting B-cells, B-cell activation genes such as CD21 and CD23, are expressed at low levels (151). Upon infection, EBV, more specifically EBNA2, activates the expression of these genes. Analysis of these genes promoters revealed CSL binding sites, suggesting that activation is regulated by CSL. Furthermore, a mutational analysis of CSL proposed that EBNA2 binding results in the masking of a CSL “repression domain” through the displacement of corepressors, which allows the activation of genes necessary for viral latency and B-cell immortalization (65). A similar mechanism has also been put forth as the way in which NotchIC functions in a normal cellular context, that is to displace corepressors in order to activate transcription. Subsequently, several groups have characterized some of these corepressors (SMRT, Skip, SHARP/MINT, and KyoT2), and shown them to be necessary for CSL mediated repression (44,46,47,152). In sum, it is evident that the role of CSL as a transcriptional repressor is equally as important as its role as an activator. Moreover, understanding the molecular nature of CSL-corepressor complexes will not only provide details about the homeostatic function of CSL in Notch signaling, but also could aid in the development of drugs to combat Epstein Barr viral infections.

Altogether, our structural descriptions of various CSL complexes have detailed the interfaces of CSL that are important for activation complex formation, but how perturbation of these interfaces affects overall transcription levels has yet to be examined. To address this question, we have developed a series of CSL BTD mutants that we believe will alter ternary complex formation and transcriptional outputs through the disruption of the high affinity RAM-CSL interaction (93,106). Through the use of a retroviral transduction system, the mutants were expressed in a CSL null mouse
embryonic fibroblast (MEF) cell line and assayed for their ability to effectively activate and repress transcription relative to wild-type. Using recombinantly expressed and purified mutant proteins we were also able to: 1) assay the ability of the mutant proteins to form ternary complex by EMSA analysis, and 2) quantify the mutants capability to bind the coactivator NotchIC RAM domain and the repressor MINT by ITC. Our results indicate that mutations in the BTD-RAM interface alter the ability to activate transcription. Additionally, mutations made in the BTD designed to disrupt activation drastically affected repression. Our biophysical analysis of the BTD mutations provides a quantitative comparison of the mutant’s ability to interact with coregulators, highlighting the importance of certain residues over others for binding coactivators or corepressors. Overall, the results presented here help to confirm the necessity of certain interfaces for transcriptional activation complex formation, as well as reveal how these same interfaces play an extremely important functional role in transcriptional repression.
Materials and Methods

Cell lines

Wild type CSL mouse embryonic fibroblasts (MEFs), OT13, and CSL knockout MEFs, OT11, were a generous gift of Dr. Honjo and were maintained at 37°C in 5% CO₂ in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin. All CSL expressing MEFs were generated by retroviral transduction (explained below) and maintained in an identical manner.

Plasmids for tissue culture and reporter constructs

DNA encoding amino acids 1-526 of mouse CSL, as well as a C-terminal FLAG tag, was cloned from cDNA and ligated into the BglII and EcoRI sites of the MigR1 vector, generating the MigR1-CSL construct, which was used for retroviral transduction. Quickchange Mutagenesis was used following manufacturer’s protocol to generate the following BTD mutants: M-1, F261R; M-2, V263R; M-3, A284R; M-4, Q333R; M-16, V263R and A284R; M-17, V263R and Q333R; M-18, A284R and Q333R; and M-22, V263R, A284R, and Q333R. The 3X FLAG tagged mouse Notch1 construct used was obtained from the Kopan Lab and was described previously (153).

The 4XCBS_LUC reporter was a generous gift from the Kopan Lab and was described previously (154). phRL was used in the dual luciferase reporter assay to express Renilla luciferase, and pBluescript was used to control for transfection efficiency. The 4XCBS_TK reporter was generated by excising the TK promoter from phRG-TK
with the restriction enzymes BglII and HindIII. This fragment was then ligated into the pGL4.13 vector that had been digested with BglII and HindIII, generating the pGL-TK construct. Two complimentary oligos were ordered that contained the four CSL binding sites with identical spacers and SacI and EcoRV sticky ends. These oligos were annealed and ligated into the pGL-TK construct that had been digested with SacI and EcoRV, resulting in the 4XCBS_TK reporter.

*Retroviral production and transduction*

Wild type as well as mutant CSL-MigR1 constructs were developed as described above. The MigR1 construct contains an internal ribosome entry site downstream of the multiple cloning sequence which controls the expression of green fluorescent protein (GFP) (155). MigR1, wt CSL-MigR1, or mutant CSL-MigR1 vectors were co-transfected with pVSV-G into the packaging cell line HEK GP2-293. Supernatants containing the retroviruses were collected 96 hours post transfection. Retroviral transduction of OT11 MEFs (CSL-null) was accomplished by plating 1.1 x 10^5 cells in a 12 well plate along with adding 0.5ml of the appropriate supernatant containing the retrovirus. Cells were maintained in media and sorted by GFP fluorescence to select for CSL expressing cells. CSL expression was further verified by western blotting and compared to MigR1 mock transduced OT11 cells.

*Luciferase reporter assays*

OT11 cells expressing CSL and CSL mutants were maintained as described above. Cells were plated at less than 50% confluence in 6-well plates. At approximately 80%
confluence, cells were transfected with the experimentally specified constructs using the Satisfaction reagent and following the manufacturers protocol, with the amount of DNA transfected normalized using pBluescript (Stratagene). 48 hours post-transfection, the cells were harvested and assayed for luciferase activity using the Promega Dual Luciferase Kit. Firefly luciferase expression was normalized to Renilla luciferase expression and an average of two individual readings was determined. Graphically presented values correspond to the average of three individual experiments.

**Protein expression and purification**

Mouse CSL, RAMANK, Mastermind, RAM domain, and MINT CID were expressed and purified as described previously (106). Mouse CSL mutants used in EMSA and ITC experiments were generated by Quickchange mutagenesis (Invitrogen), using our previously described GST-mouse CSL fusion construct (106). Point mutations were verified through DNA sequencing and proteins were expressed and purified in an identical manner as the wild type CSL.

**Electrophoretic mobility shift assays (EMSA)**

Experiments were performed as described previously using the following binding buffer: 10 mM Tris 8, 150 mM KCl, 5mM MgCl, .1% Triton X-100, 100 ug/ml BSA, 1 mM DTT, and 5% Glycerol (106). All experiments were performed using the following concentration of components: 0.75 µM DNA, 1 µM CSL, 1 µM RAMANK, and 10 µM mastermind.
Isothermal Titration Calorimetry (ITC)

Experiments were performed as described previously (106).
Results

*Development of wild type CSL and mutant CSL expressing MEFs using retroviral transduction*

Our structural studies as well as several biochemical and cellular analyses have highlighted the importance of the RAM domain and its interaction with CSL. Therefore, due to the significance of this interface we began our mutational studies in the BTD of CSL. Based on the worm ternary complex structure, residues in the BTD were identified that appeared to be making direct contacts with the RAM domain (73). While structures of mammalian CSL bound by RAM are currently unknown, the residues chosen based on the worm structure are absolutely conserved in mouse and human CSL proteins, and therefore, would likely make similar contacts with RAM. To address the function of these mutations in a cellular context, we performed experiments using mutants of the mouse homolog of CSL, RBP-Jκ, that were retrovirally transduced into CSL null MEFs. Using site-directed mutagenesis, four different single site mutations were made into our wild-type MIG-CSL-FLAG tagged construct. These mutations introduced drastic amino acid changes that would likely disrupt the CSL-RAM interaction. The four single mutations are as follows and will hereafter be referred to as: F261R, M-1; V263R, M-2; A284R, M-3; and Q333R, M-4 (Figure 1). Using established protocols, retrovirus corresponding to the empty MIG vector, wild type MIG-CSL-FLAG, and mutant MIG-CSL\textsuperscript{Mut}-FLAG were made in a packaging cell line and then retrovirally transduced into CSL null mouse embryonic fibroblast cells (155). Transduction and expression of the construct was monitored by flow cytometry that monitored expression of the green
**Figure 1: BTD-RAM interface mutants.** Image is a surface and cartoon representation of the worm ternary complex structure (2FO1). CSL surface representation is colored by domain with the NTD, cyan; BTD, green; CTD, orange. The ankyrin repeats of NotchIC are in cartoon representation in yellow and the RAM domain is shown in sticks with carbons colored yellow, oxygens colored red, and nitrogens colored blue. Mastermind is in cartoon representation colored black, and DNA is in cartoon representation. Red surfaces on the BTD represent the location of mutations made and are identified with black arrow. All of the mutations made and used are listed on the right. Figure made with Pymol.
fluorescent protein (GFP) from an IRES downstream of the CSL coding region. Western blot analysis of CSL-FLAG confirmed the expression of CSL for the wild type and mutant transduced cells, as well as the lack of CSL expression in the empty MIG vector transduced cells. (Data not shown)

**BTD mutants – transcriptional activation**

To assess the ability of the CSL BTD mutants to activate transcription, a dual luciferase assay was performed using the 4X CBS_LUC reporter. This reporter contains four consensus CSL (CBS) binding sites oriented in the same direction upstream of the SV40 promoter, and is diagramed in Figure 2. By placing this CSL response element upstream of the weak SV40 promoter, we are able to evaluate the activation function of our CSL mutants in comparison to activation from wild-type CSL. As shown in Figure 3, using wild type CSL expressing cells we observed an increase in luciferase expression from the reporter, in a dose dependent manner. Subsequent transfection experiments all used 150 ng of NotchIC to activate the luciferase reporter. As shown in Figure 4, all data were graphed as a ratio normalized to the wild-type CSL. Cells expressing mutant CSL displayed a significant decrease in expression from the 4X CBS reporter as compared to wild type (Figure 4). M-1 exhibited an approximate five-fold decrease in luciferase expression, and M-2, M-3, and M-4 all had a decrease in expression of 2-3 fold as compared to wild type. Taken together, these data suggest that these residues at the BTD-RAM interface are important for formation of the ternary complex and for activation of transcription from the 4X CBS reporter. Since M-1 was observed to be more deleterious to transcriptional activation than the other mutants, we wanted to
**Figure 2:** Schematic diagram of constructs used in transfection assays. Diagrams represent the different constructs used in dual luciferase assays. NotchIC encodes the intracellular domain of mouse Notch1, amino acids 1744-2531, with an N-terminal 3X FLAG tag. pCMV, promoter CMV. 4X CBS_LUC and 4X CBS_TK are the two different firefly luciferase reporters responsive to CSL utilized in these studies. pSV40, promoter SV40. pTK, promoter thymidine kinase. pRL, renilla luciferase expressing reporter used for normalization of firefly luciferase.
Figure 3: **Notch activation of 4X CBS_LUC reporter.** Luciferase assay showing the response of the reporter to increasing amounts of transfected NotchIC in OT11 cells that have been retrovirally transduced with wild type CSL.
Figure 4: Transcriptional activation by CSL mutants. Luciferase assay to assess the ability of OT11 cells expressing wild type, WT, or mutant CSL to activate transcription and express luciferase form the 4X CBS_LUC reporter. Cells were transfected with 150 ng of NotchIC and graphed values represent a ratio of luciferase expression with and without NotchIC transfection and are normalized to wild type. Mutants are listed on the x-axis. MIG-Ctrl, OT11 cells transduced with empty MigR1 vector showing the lack of activation by OT11 cells.
investigate if there was any combination of the other mutants that could achieve the same weak activation as M-1. Three double mutants and one triple mutant CSL (See Fig. 1) were made using site-directed mutagenesis and were retrovirally transduced into CSL null MEFs as described previously; similarly these mutants were assayed for their ability to activate transcription from the 4X CBS_LUC reporter, the levels of which are shown in Figure 4. M-17 and M-18 showed a slight decrease in expression compared to the single mutants, but neither achieved the same reduction as M-1. Interestingly, M-16 increased expression compared to its single mutants. The triple mutant, M-23, however, showed decreased expression to levels similar to those shown by M-1.

**BTD mutants – transcriptional repression**

The luciferase reporter assays performed thus far have only allowed us to characterize the effects mutant CSL proteins have on activation of transcription relative to wild type, but not assay the repressive function of CSL. This is due to the weak basal level of expression from the SV40 promoter even in cells lacking CSL. In order to determine the effects these mutants have on transcriptional repression, a reporter construct was developed that contained the 4X CBS response element upstream of the constitutively active thymidine kinase (TK) promoter, termed 4X CBS_TK and is diagramed in Figure 2. The ability of CSL to repress luciferase expression was assessed relative to the TK reporter (pGLTK) that does not contain the 4X CBS sequence, and it was determined that inclusion of the 4X CBS response element resulted in ~50% less transcription (Figure 5). This reporter was then assayed using the single, double, and triple BTD mutants (Figure 6). These experiments showed that while the wild type
Figure 5: CSL represses activity of 4X CBS_TK reporter. Luciferase assay to test the ability of OT11 cells expressing wild type CSL to repress the expression of luciferase from the 4X CBS_TK reporter. Graphed values represent the ratio of luciferase expression from wild type CSL cells to MIG control cells normalized to the TK reporter without the 4X CBS insert, pGLTK. Two different amounts of reporter were tested: 10 ng, dashed bar; and 50 ng, black bar.
Figure 6: Repression of transcription by CSL mutants. Luciferase assay to assess the ability of OT11 cells expressing wild type, WT, or mutant CSL to repress transcription and expression of luciferase from the 4X CBS_TK reporter. Graphed values represent luciferase expression from 25 ng of the 4X CBS_TK reporter in the absence of Notch normalized to MIG control cells. Mutants are listed on the x-axis. MIG, OT11 cells transduced with MigR1 vector alone.
expressing cells were able to repress expression of luciferase, all of the mutants tested had defects in repression. Moreover, the CSL mutants had higher luciferase levels than measured in the CSL-null cells transduced with the empty MIG vector (Figure 6). It should be mentioned that all of the mutants demonstrated an increase in luciferase expression in the absence of activated Notch. Compared to the MIG control cells, the increase in expression varied for the different mutants – mutants 1 and 3 changed 2-fold, mutants 2 and 4 changed 1.5-fold, and mutant 18 changed 3-fold. This result would seem to indicate that mutations made in the BTD not only perturb the ability of CSL to activate transcription, but also disrupt the ability of CSL to properly repress transcription.

**CSL mutants and defects in ternary complex formation**

Our reporter assays revealed that mutations in the BTD affect both transcriptional activation and repression in cultured cells. As mentioned previously, western blot analysis showed that mutant CSL expression was similar to that of wild-type CSL; however, this evaluates neither the DNA binding nor protein-protein interaction capabilities of these mutants. To this end, we generated GST-CSL fusion constructs that correspond to our BTD mutants and expressed these proteins in bacteria and purified them to homogeneity for use in subsequent binding studies. We used EMSA to test the capability of the mutants to bind DNA, interact with RAMANK, and form ternary complex with RAMANK and Mastermind. As shown in Figure 7, compared to wild-type, all of the mutants bound DNA similarly (first lane in each gel); however, when RAMANK was added to the CSL-DNA complexes, none of the mutant CSL-DNA complexes displayed shifts similar to the wild-type CSL (compare second lane of each
Figure 7: EMSA analysis of DNA binding, RAMANK binding, and ternary complex formation by CSL mutants. EMSA showing the ability of each CSL mutant to bind DNA, RAMANK, and form a ternary complex with RAMANK and Mastermind. CSL used in each experiment is listed below each gel. First lane of each gel is CSL and DNA only. Second lane has the addition of RAMANK, RA. Third lane has the addition of RAMANK, RA, and Mastermind, MM. Shifted positions of CSL complexes are shown to the right of the three gels that used wild type CSL. Concentrations used are detailed in the text. Note the inability of the mutants to form the same complex as wild type with RAMANK, but their ability to form ternary complex with RAMANK and Mastermind.
gel), which suggests defects in RAMANK binding by the mutant CSLs. While all of the mutants formed CSL-RAMANK-MM ternary complexes comparable to wild-type (compare third lane of each gel), Mastermind was present in excess in the binding reactions. In should also be mentioned that CSL-Notch1-MM ternary complexes can be formed in the complete absence of RAM, albeit less efficiently (27).

**Thermodynamics of RAM and MINT interactions with mutant CSL**

The EMSA analysis of mutant CSL proteins indicated that the mutants could bind DNA similar to wild-type, but were defective in RAMANK binding. To quantify this binding deficiency we used isothermal titration calorimetry (ITC) to analyze the binding of the NotchIC RAM domain with our mutants. We used peptides that corresponded to the RAM domain of NotchIC and not constructs of RAMANK, because previous studies have shown that RAM and RAMANK bind the BTD of CSL with nearly identical affinities and thermodynamics (106). ITC binding experiments were performed as detailed in the Materials and Methods and the results are shown in Table I. As judged by the dissociation constants ($K_d$), the binding affinity of RAM for the mutant CSL proteins was from 17 to 1,000 fold weaker relative to wild-type. This was associated with a decrease in free energy ($\Delta G^\circ$) between 2 and 4 kcal/mol, relative to wild type. A closer examination of the enthalpic and entropic contributions to the overall free energy of the interactions indicates that for mutants 2, 3, and 4, the decrease in free energy is attributed to a greater entropic penalty associated with RAM binding (Table I); these interactions appear to maintain similar enthalpy as wild type, $\sim$ -17 kcal/mol. However, the binding of RAM to M-1 occurs with almost 2 kcal/mol less of an enthalpic contribution (-15.5
<table>
<thead>
<tr>
<th>CSL</th>
<th>Ligand</th>
<th>$K \left(M^{-1}\right)$</th>
<th>$K_d$ (µM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$-T\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1 mCSL</td>
<td>RAM</td>
<td>$4.85 \pm 2.1 \times 10^5$</td>
<td>23</td>
<td>$-6.3 \pm 0.2$</td>
<td>$-15.5 \pm 6.1$</td>
<td>$8.9 \pm 6.6$</td>
</tr>
<tr>
<td>M-2 mCSL</td>
<td>RAM</td>
<td>$2.43 \pm 0.5 \times 10^6$</td>
<td>0.43</td>
<td>$-8.6 \pm 0.1$</td>
<td>$-17.0 \pm 1.0$</td>
<td>$8.3 \pm 1.0$</td>
</tr>
<tr>
<td>M-3 mCSL</td>
<td>RAM</td>
<td>$1.37 \pm 0.3 \times 10^6$</td>
<td>0.74</td>
<td>$-7.9 \pm 0.2$</td>
<td>$-17.9 \pm 0.3$</td>
<td>$9.1 \pm 0.3$</td>
</tr>
<tr>
<td>M-4 mCSL</td>
<td>RAM</td>
<td>$2.73 \pm 0.6 \times 10^6$</td>
<td>0.38</td>
<td>$-8.7 \pm 0.1$</td>
<td>$-16.7 \pm 0.6$</td>
<td>$7.6 \pm 0.8$</td>
</tr>
<tr>
<td>WT mCSL</td>
<td>RAM</td>
<td>$5.15 \pm 2.1 \times 10^7$</td>
<td>0.022</td>
<td>$-10.4 \pm 0.3$</td>
<td>$-17.1 \pm 0.3$</td>
<td>$6.6 \pm 0.1$</td>
</tr>
</tbody>
</table>

**Table I: Calorimetric data of the RAM-mutant CSL interaction.** mCSL, mouse CSL (residues 53-474); mRAM, mouse RAM domain (residues 1774-1801). Mutants defined in Figure 1. Values are the mean of at least three independent experiments and the errors represent the standard deviation of multiple experiments.
kcal/mol vs. -17.1 kcal/mol) as well as a 2 kcal/mol entropic penalty. These results indicate that while all of these residues play an important role in RAM binding and can alter binding efficiency, certain mutations are more detrimental than others.

While these mutations were designed based on the structure of an active ternary complex, and as expected, these mutants disrupted transcriptional activation and RAM binding in reporter and binding assays respectively, we were also curious as to whether these mutations had an effect on the repressive function of CSL. Previous studies have implicated the BTD in corepressor binding, but it is currently unclear from a structural standpoint how corepressors interact with CSL. Certainly our ITC experiments have shown that MINT binds CSL, and more specifically, we showed that part of this binding occurs in the BTD. Therefore, we sought to determine whether these BTD mutants also affect MINT binding, which may suggest Notch and MINT compete for similar binding surfaces on CSL. ITC experiments were performed as described in the Materials and Methods and the results are shown in Table II. Similar to the RAM mutant-1 interaction, the MINT mutant-1 binding displayed the largest decrease in affinity compared to wild type, ~300 fold. This mutation caused a 6 kcal/mol decrease to the enthalpic contribution, which resulted in a 4 kcal/mol decrease in the overall free energy (-6.8 kcal/mol for M-1 vs. -10.7 kcal/mol for wild type). Mutants 2 and 4 caused relatively minor changes in the enthalpy and entropy contributions to binding, relative to wild type, but overall did not seem to affect the CSL-MINT interaction. Interestingly, M-3 also decreased the binding affinity of MINT 130-fold, which was a much greater effect than M-3 had on RAM binding. Similar to the MINT mutant-1 interaction, the MINT mutant-3 binding is characterized by a loss of enthalpic contribution. In the absence of a MINT-
### Table II: Calorimetric data of the MINT CID-mutant CSL interaction.

<table>
<thead>
<tr>
<th>CSL</th>
<th>Ligand</th>
<th>$K (M^{-1})$ (µM)</th>
<th>$K_d$ (µM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$-T \Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1 mCSL</td>
<td>MINT CID</td>
<td>2.27 (±0.2) x 10³</td>
<td>4.2</td>
<td>-6.8 ± 0.04</td>
<td>-9.0 ± 0.5</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>M-2 mCSL</td>
<td>MINT CID</td>
<td>6.43 (±1.7) x 10⁴</td>
<td>0.016</td>
<td>-9.9 ± 0.2</td>
<td>-11.8 ± 1.0</td>
<td>1.2 ± 1.0</td>
</tr>
<tr>
<td>M-3 mCSL</td>
<td>MINT CID</td>
<td>7.19 (±2.7) x 10³</td>
<td>1.6</td>
<td>-7.4 ± 0.2</td>
<td>-8.9 ± 0.4</td>
<td>1.0 ± 0.09</td>
</tr>
<tr>
<td>M-4 mCSL</td>
<td>MINT CID</td>
<td>7.28 (±0.7) x 10⁷</td>
<td>0.013</td>
<td>-9.9 ± 0.05</td>
<td>-12.6 ± 1.1</td>
<td>1.9 ± 1.0</td>
</tr>
<tr>
<td>WT mCSL</td>
<td>MINT CID</td>
<td>8.00 (±0.1) x 10⁷</td>
<td>0.012</td>
<td>-10.7 ± 0.09</td>
<td>-14.8 ± 0.1</td>
<td>4.0 ± 0.1</td>
</tr>
</tbody>
</table>

mCSL, mouse CSL (residues 53-474); MINT CID, MINT CSL interacting domain (residues 2776-2933). Mutants defined in Figure 1. Values are the mean of at least three independent experiments and the errors represent the standard deviation of multiple experiments.
CSL structure these, results suggest that the CSL residues phenylalanine 261 (M-1) and alanine 284 (M-3) are critical for binding MINT and mediating repression.
Discussion

In a very general sense the role of CSL in transcriptional regulation has been known and understood for some time now. It is an essential component of the Notch signaling pathway and is an activator as well as a repressor of transcription. But how this protein is able to interact with both activators and repressors and differentially regulate transcription is poorly understood. High resolution crystal structures of transcriptional activation complexes provided the groundwork for the current study, which was to define key binding interfaces necessary for complex formation and activation of transcription (73,74). Our previous analysis and characterization of the high affinity RAM-BTD interaction suggested that mutations of this interface might severely inhibit transcriptional activation (106). Interestingly, our mutational analysis of the BTD demonstrated that these sites were not only important for activation, but were also important for mediating repression complexes with the corepressor MINT.

The identification of the BTD as a “repression domain” and the proposed model of the BTD as the site of competition between activators and repressors have been made previously; however, we are currently still lacking a molecular understanding for how corepressors bind and compete with coactivators for interfaces and specific residues CSL (65,151). The amino acid, Phe 261, was previously shown to be vital for CSL mediated repression and was shown to disrupt activation, as mutation of this residue disrupted these functions (156). However, many of these studies utilized transiently transfected CSL and others used CSL-GAL4 fusions and GAL4 responsive reporters (45,51). These experimental caveats require us to be cautious in our interpretation of the results, as wild-type CSL is present in all cell types and tissues. We feel that our system of retrovirally
transducing CSL null MEFs has several advantages over previous studies. Our tissue culture assays, using the 4X CBS_LUC and 4X CBS_TK reporter, provide cellular data that support the hypothesis that coactivators and corepressors utilize similar, but not completely overlapping, CSL interfaces to impart function. Of the single site mutations made, M-1 was found to be the most deleterious in both the cellular activation and repression assays. In the structure of RAM bound CSL, Phe-261, is located at the BTD-RAM interface (73). Interestingly though, it is not located in the hydrophobic pocket of the BTD and it is not making contacts with the conserved hydrophobic tetrapeptide motif of RAM. Instead it appears to be making contacts with the semi-conserved region of RAM between the hydrophobic tetrapeptide and the N-terminal positively charged region. Taken together these data suggest: 1) while quantitative studies have focused on the conserved hydrophobic motif of RAM and its binding to CSL, this interaction N-terminal to the hydrophobic core seems equally important; and 2), while it is structurally unknown which corepressors may bind CSL at this residue (ITC experiments indicate MINT binding here), it is apparent that both repressors and activators utilize phenylalanine 261 to carry out their respective functions.

Of the other single mutants developed, M-2 and M-4 altered transcriptional activation and repression to a lesser extent, with M-3 being slightly more potent, but not to the level of M-1. The results of the double and triple mutants developed are somewhat confusing and display differential effects. Double mutants that include M-4 seem to suppress activation, and double mutants that include M-3 seem to suppress repression. Even without a clear understanding of the combinatorial effects of these mutations, it seems evident that these residues play a role in repression, as well as activation.
The mutational analysis performed here was guided by the structural knowledge of the ternary activation complex. While the effect of these mutations was previously unknown, it seemed likely, due to the importance of the RAM interaction, that these mutations would greatly influence activation. With the experimental observation being just that, a model could be proposed where transcriptional activation is regulated mainly by the BTD-RAM interaction and fails to incorporate how other components of the ternary complex or DNA promoter structure may change transcriptional activation or repression. With this in mind, two important elements must be considered when interpreting these results. First, the reporter used in these studies contains four consensus CSL binding sites in the same orientation, equally spaced, upstream of the promoter. This structure has not been identified in the promoters of Notch responsive genes, and represents an artificial element. However, genes have been identified with multiple CSL binding sites arranged in a similar head to tail fashion as well as head to head fashion with one site deviating from the consensus sequence (157). It has been proposed that this promoter arrangement, which is found in the HES-1 gene, facilitates cooperative assembly of ternary complexes on the consensus site and nonconsensus site and is necessary for proper activation of transcription (96). To that end, it would be useful to perform reporter assays using our mutants and a reporter that contained a biologically relevant CSL promoter. Secondly, our focus has been on how perturbation of the high affinity RAM-BTD interaction modulates transcription, but studies have shown that transcription can be activated by high levels of the ankyrin repeats alone in the absence of RAM (85). Furthermore, it has been suggested that the cooperative formation of ternary complexes on the previously mentioned HES-1 promoter and the subsequent activation of
the associated gene is facilitated by interactions between the ankyrin repeats of the two different complexes (96). These results would indicate that activation of transcription may be affected by mutation of the CSL-ankyrin repeat interface, but it is unclear exactly which residues to mutate in this rather large interface. In sum, our transcriptional reporter assays have shown that mutations at the BTD-RAM interface affect both activation and repression of transcription. This observation further validates the BTD’s central role in Notch regulated transcription and emphasizes the need to determine structures of corepressors bound to CSL.

Our cellular based transcription assays revealed the effects of BTD mutations on transcriptional outputs. They are able to suggest to us that certain mutations alter activation, while other mutations influence repression more; but these experiments provide few details about how these mutants change the protein interactions necessary for proper activation and repression to occur. The biochemical and biophysical analysis performed here using the single BTD mutants affords us this insight, and in conjunction with our structures of CSL complexes, offers a truly molecular explanation for the changes observed in transcriptional yields.

The ability of CSL to recognize and bind DNA is absolutely essential for activation of Notch responsive genes. Our gel shift analysis showed that all of the mutants tested in our tissue culture assay retained the ability to bind DNA (Figure 7). While these experiments were qualitative in nature, they seem to indicate the DNA binding is not affected by the mutations. Our recent and thorough thermodynamic dissection of CSL binding to DNA gives us confidence in our conclusions drawn about the mutant CSL-DNA interactions (158). However, a similar quantitative study could be
performed using the mutants to ensure that our observations in our tissue culture assays were not solely based on the inability of the mutants to bind the reporters.

The observation that RAMANK was unable to shift a mutant CSL-DNA complex to the same extent as a wild-type CSL-DNA complex was reminiscent of an earlier published result. Quantitative gel-shift experiments to investigate RAMANK-CSL binding showed that at low concentrations of RAMANK the CSL-DNA complex was only shifted slightly, but saturating concentrations of RAMANK shifted the complex to a higher position in the gel (106). The $K_d$ extrapolated for the RAMANK-CSL interaction from these experiments mirrored the $K_d$ determined from ITC binding experiments. The inability of an equal concentration of RAMANK to shift a mutant CSL-DNA complex suggests that these mutations have perturbed the affinity of the RAMANK-CSL interaction, which we verified by ITC (Table I). These experiments provide a molecular explanation for the results of our cellular activation assays, i.e. alteration of residues at the RAM-BTD interface changes the affinity of Notch for CSL. Therefore, a higher nuclear concentration of NotchIC needs to be present in order to achieve the same level of activation observed with wild type. Since Notch signals are not amplified once they are activated, it is critical that Notch is able to bind CSL and activate Notch responsive genes to ensure the appropriate cellular function is carried out. With limiting amounts of Notch in the nucleus, the evolutionary maintenance of these CSL residues begins to make sense. The system seems to have “tuned” itself to effectively and avidly bind what little amount of Notch is present, therefore guaranteeing robust gene expression.

Our analogous thermodynamic characterization of MINT CID-mutant CSL interactions reveals a similar parallel between the affinity of MINT for the various
mutants (Table II) and the mutant’s ability to repress transcription (Figure 6). Mutants 1 and 3 cause the affinity of MINT for CSL to decrease drastically from low nanomolar to micromolar $K_d$s, but mutations 2 and 4 have almost no effect on the affinity of the MINT-CSL interaction (Table II). These same changes relative to wild type are also seen in our cellular repression assays (Figure 6). Mutants 1 and 3 are most impaired for repressing transcription, with mutants 2 and 4 derepressed to a lesser extent. It is unclear exactly as to why mutants 2 and 4 do not alter MINT binding (Table II), but do change the ability of CSL to repress transcription (Figure 6). This could indicate that these mutations disrupt an interaction with another corepressor other than MINT or another region of MINT outside of our construct. Additionally, while our results indicate that certain mutations affect MINT CID binding, it is unknown as to what role MINT CID plays in the assembly of the much larger repression complex. Nonetheless, these results indicate that these BTD mutations alter the ability of CSL to properly repress transcription and decrease the affinity of the MINT CID-CSL interaction.

In conclusion, our mutational analysis has offered us new information about the role of the BTD in transcriptional activation and repression. While it has been suggested that the BTD is the location where coactivators and corepressors compete for binding to CSL, the exact interfacial residues for which the competition occurs have been unclear. The results presented here allow us to construct a residue priority map of the BTD. That is, using our cellular and biochemical data, we can map the relative importance of a particular residue to the activation or repression of transcription. In spite of this advance, this data only represents how CSL mutations affect one protein interface and emphasize
the need to perform a similar analysis on other interfaces of CSL known to interact with coactivators and corepressors.
Chapter VI

A model of MINT regulated transcriptional repression
Proper gene expression requires the coordinated recruitment of a multitude of protein factors to proximal and distal gene promoter elements. Likewise, transcriptional repression requires the localization of several different protein factors to many of these same promoter elements to silence gene expression. This genomic regulation is facilitated by a number of basal transcriptional activators and repressors, but many, if not all genes, utilize specific activating and repressing transcription factors to precisely control their expression. The activation and repression of Notch target genes by the transcription factor CSL has been well characterized; however, new data presented here describing a corepressor-CSL interaction allows us to speculate how CSL repression complexes are formed, as well as how CSL functions as the centerpiece of a transcriptional switch.

In the following text, we will discuss some of our most recent results that have been instrumental in our understanding of CSL repression complexes. Initial competition EMSA and ITC experiments have begun and future experiments will be proposed. A key structural comparison will be given, highlighting residues we believe may be mediating MINT interaction. And finally, we will discuss some future tissue culture and biochemical experiments that could be performed in an attempt to validate our proposed model of CSL mediated repression, as well as provide a greater understanding of how CSL functions in concert with larger transcription complexes.

*Competition ITC and EMSA*

The model of CSL’s mechanism of action, which has been developed from a multitude of different experimental approaches, suggests that CSL is bound to the
promoters of Notch responsive genes at all times. The activation of the Notch receptor is believed to displace corepressors from CSL, allowing for the activation of transcription. The subsequent degradation of the Notch signal is believed to be accompanied by the return of corepressors to CSL, thus repressing transcription (outlined in Figure 1 Chapter II). While this model has proved useful, recent work performed in our lab has revealed additional molecular details of this mechanism that should be integrated into the model, in order to provide a more quantitative explanation for how CSL regulates transcription. Our thermodynamic analysis of the MINT-CSL interaction presented in Chapter IV detailed the high affinity interaction between these two molecules. However, a large question remains as to how this tight binding is out-competed by the equally tight binding RAM-CSL interaction (Chapter II), as the current model in the field suggests. Recently we have performed an EMSA analysis of this competition reaction for CSL by Notch and MINT. These competition experiments suggest that in the absence of other factors MINT can out-compete RAMANK for binding to CSL. The details of this competition are still preliminary and we should use caution in interpreting these results. These types of experiments do not indicate if the competition is for the same site on CSL or if binding induces an allosteric change that precludes binding of the other molecule. To address these questions it would be useful to perform displacement ITC experiments (159). This type of analysis has proved useful in understanding the competition between RAM and EBNA2 and could be very insightful in the absence of a MINT-CSL structure (personal correspondence with Barrick Laboratory).

We have also used a gel shift analysis to investigate what effect MINT has on formation of the CSL-NotchIC-MM ternary complex. A majority of our efforts thus far
have focused on the interaction of MINT and RAMANK with the BTD and trying to understand how this interface is crucial for mediating interactions that define the transcriptional switch. But our thermodynamic analysis of the MINT-CSL interaction clearly indicated that this interaction is not solely mediated by the BTD, and that either the NTD or CTD participate in MINT binding (Chapter IV). With these domains being the interface for both the ankyrin repeats of Notch and Mastermind, we wanted to characterize the effect these components would have on the ability of MINT to bind CSL. Initial experiments seem to suggest that the presence of Mastermind greatly enhance the formation of ternary complex at the expense of the MINT-CSL complex. Furthermore, experiments performed with RAMANK separated into its two individual domains, RAM and ANK, appear to show that RAM is necessary for the displacement of MINT, and ANK and Mastermind alone cannot displace MINT from CSL (Chapter II). Further experiments are needed to confirm these results with careful consideration being given to concentrations and the order of addition of the various components. In sum, these initial competition experiments suggest that the displacement of MINT by RAMANK and subsequent ternary complex formation may not be as simple as the current models suggest. The displacement mechanism cannot be explained by differences in affinity alone. More likely, it involves a complex interplay between RAMANK, Mastermind, and MINT for CSL binding.

*Potential MINT binding surface*

All of the binding experiments discussed thus far have utilized mouse CSL and MINT, but work from others in our lab using the *D. melanogaster* and *C. elegans* CSL homologs, Su(H) and Lag-1 respectively, has provided additional insights into MINT
binding and helped us refine our model of MINT mediated repression. Since it has been shown that RAM molecules have the ability to bind CSL proteins from different organisms, we wanted to test the ability of MINT to bind CSL from different organisms as well (106). Interestingly MINT was able to bind fly CSL (Su(H)), but was not able to bind worm CSL (Lag-1) (data not shown). It has been suggested that worms encode a homolog of MINT, but the comparison has been based on the C-terminal SPOC domain, 26% identical to mouse SPOC domain, and it was noted that sequence conservation in other portions of MINT was very divergent (160). Furthermore, alignment of the proposed *C. elegans* MINT homolog to the mouse and human MINT proteins does not reveal the conserved CSL interacting domain (CID). Regardless, the protein sequence and tertiary structure of the mouse and worm CSL proteins are highly conserved and an explanation for the lack of MINT binding to worm CSL is not immediately clear. To date, a high-resolution structural characterization of a MINT-CSL complex has yet to be resolved, preventing a molecular explanation for how co-repressors such as MINT can bind CSL and repress transcription. In the absence of this structural data, but with the knowledge that: 1) the BTD provides a critical interface for MINT, 2) mutation of a conserved phenylalanine in the BTD affects MINT binding and repression, and 3) MINT does not bind *C. elegans* CSL; a closer examination of the BTD from different organisms may suggest where MINT binds to CSL (65,161).

A sequence alignment of the human, mouse, fly, and worm CSL molecules reveals some BTD residues that are absolutely conserved in human, mouse, and fly, but that are not conserved in worms. Furthermore, these are surface exposed residues that could potentially mediate protein-protein interactions (71). These residues lie on a
different face of the BTD, adjacent to where the RAM domain is known to interact, and could very well represent a unique binding interface for the C-terminal portion of MINT. Our CD data indicates that MINT is a random coil (Chapter IV) and a model could be envisioned where the C-terminus of MINT lies across these BTD residues, crossing over the conserved phenylalanine in the “repression domain” (Chapter IV and V) and with the N-terminus of MINT making contacts with the CTD (Figure 1). While there is no direct evidence for MINT making contacts with the CTD, there are surface exposed residues in the CTD that could potentially make contacts with the N-terminus of MINT. Another possibility is placing the N-terminus of MINT so that it interacts with the NTD of CSL, more specifically the NTD loop that is suggested to be allosterically regulated by RAM binding and provides an interface for Mastermind interaction (Figure 1) (106). Our preliminary EMSAs show that Mastermind facilitates displacement of MINT from CSL, and this may be the site of competition between these two molecules. However, the role the conserved phenylalanine would play in this model is not as clear. Correlating either of these multi-domain binding models with our thermodynamic ∆Cp calculations observed for MINT and RAM binding does raise some potential questions (Chapter IV). If MINT were binding two domains of CSL, it would seem likely that it buries a larger surface area, compared to RAM, but the ∆Cp’s calculated would suggest just the opposite. This, however, is only relevant if we adhere to the assumption that burial of solvent exposed surface area is the only contributing factor to ∆Cp. Clearly, studies have shown discrepancies in this thinking and work by Linda Jen-Jacobsen has proposed other contributing factors to ∆Cp for protein-DNA interactions that could be applicable to
Figure 1: Sequence alignment of the BTD and surface representation of possible modes of MINT binding CSL. (continued next page)
Figure 1: Sequence alignment of the BTD and surface representation of possible modes of MINT binding CSL. (A) Protein sequence alignment of the BTD from mouse (mBTD), human (hBTD), Drosophila (dmBTD), and worm (wBTD). Residues highlighted in black are conserved in all four species, and those with white background deviate in one or more species. Red stars indicate residues conserved in mouse, human, and drosophila, but not worms. These surface exposed residues that differ in worm could explain why MINT does not bind wCSL in our ITC studies. Black star indicates absolutely conserved phenylalanine important for CSL function (1). Numbers generated during alignment and do not represent amino acid position of any particular CSL. (B) Surface representation of CSL highlighting possible modes of MINT CID binding. CSL is that of mouse CSL bound to HES-1 nonconsensus DNA (3IAG) and is colored as follows: NTD, cyan; BTD, green; strand, magenta; CTD, orange. DNA represented as cartoon. The worm RAM domain (3BRD) is modeled onto the structure and represented as sticks. The BTD residues marked with a red star in panel A are highlighted red here as well. The conserved BTD phenylalanine stared black in panel A is highlighted black here and its position is pointed to by the black arrow. The blue residues highlighted in the CTD are those residues flagged by the SPPIDER server as potential protein-protein interaction residues. The transparent yellow ovals represent MINT CID. It was shown that residues 01-33 bound the BTD, could potentially make contact with this face of the BTD. It is unclear where residues 76-00 bind, but two possibilities are shown here (see text for details).
protein-protein interactions as well (162). Regardless, more data are needed to verify either of these models and until that time, both models should be considered.

In an attempt to validate the BTD residues highlighted in Figure 1, as MINT interfacial residues, we have begun to mutate these residues in the mouse CSL molecule to the residues found in worm CSL. These mutants were assayed by ITC to assess their ability to bind MINT. Thus far only single mutants have been generated, but there does seem to be a modest effect. Mutating isoleucine 289 to an arginine (I289R) and lysine 317 to an isoleucine (K317I) results in nearly a three fold and five-fold decrease in the affinity of MINT binding, respectively. Other single mutants are in the process of being generated, as well as double, triple, and quadruple mutants. These mutants should also be assayed with RAM to ensure that they specifically alter MINT binding and not RAM. Likewise, it could be advantageous to mutate the worm CSL residues to those found in the mouse homolog to see if this is capable of enabling MINT binding by worm CSL. These experiments will definitely enhance our understanding of how MINT interacts with CSL, and will hopefully further our knowledge of the displacement mechanism in the transcriptional switch.

_A larger MINT repression complex_

The work detailing the MINT-CSL interaction presented here is the first quantitative description of a corepressor-CSL interaction. However, several other proteins have been implicated in the CSL repression complex, but there has been little quantitative evidence for how all of these factors interact to form a repression complex.
While the work presented here does not address this larger complex, it does provide the impetus for further studies using MINT as the scaffold on which the complex is built. MINT/SHARP domain mapping has identified RNA recognition motifs, Msx2 binding domains, and the well characterized C-terminal SPOC domain (142). The crystal structure of the human SPOC domain has been solved and shown to bind a highly conserved acidic motif at the C-terminus of SMRT (160). In addition to this interaction, the SPOC domain binds the N and C-terminus of CtIP, which recruit CtBP and is essential for repression (141). The MINT CID may provide the link for the localization of all these proteins to a CSL mediated repression complex. We envision a detailed model of repression where in the absence of a Notch signal the corepressors SMRT, CtIP, and CtBP are tethered to the SPOC domain and localized to CSL by the MINT CID to keep transcription repressed. Activated Notch, localized to the nucleus, could begin to displace MINT CID from the BTD, but full displacement of a repression complex and formation of an activation complex would not be achieved until Mastermind is localized to CSL. Once the activation complex falls apart it would be advantageous for the system to quickly and effectively repress transcription. MINT, more specifically the MINT CID, could provide this quick silencing of transcription through its avid interaction with CSL (Chapter IV), and along with it bring the return of the larger repression complex (Figure 2). This model has yet to be tested on a biochemical or cellular level, but provides a testable hypothesis. Protein expression constructs are currently being developed for the MINT SPOC domain, as well as for the SMRT CSL interacting domain and the SMRT SPOC interacting domain. Previous attempts to characterize the SMRT-CSL interaction by EMSA failed to identify a complex between these two proteins. This observation
Figure 2: Revised model of CSL mediated transcriptional switch. Transcription of Notch responsive genes is repressed by a multi-protein complex that is formed upon CSL. The recruitment of this complex to CSL is facilitated by the high affinity interaction of MINT CID with CSL (shown here). The SPOC domain at the C-terminus of MINT forms interactions with CtIP, CtBP, and SMRT, all of which have all been shown to be important for repression. Furthermore, SMRT recruitment brings with it the corepressors SKIP, CIR, and HDACs. Nuclear localization of NotchIC and recruitment of Mastermind displace this complex and activate transcription. The repression complex remains nearby, and once the activation complex is phosphorylated and marked for degradation, the MINT CID can facilitate a quick return to the repressed state.
could be due to the lack of MINT CID and SPOC domain. MINT could be necessary to facilitate the interaction between SMRT and CSL by forming an interface for SMRT to bind or through an allosteric change induced by MINT. It must also be noted that attempts to form this complex with the separate domains of MINT (CID and SPOC) could fail, indicating that the two domains need to be tethered in some way or that the intervening segment is necessary. This portion of MINT is roughly 500 amino acids with little known structure or function, and the feasibility of expressing and purifying a protein that encompasses the CID through the SPOC would be technically challenging. If purifying this protein proves to be intractable, it may be useful to generate a MINT CID-SPOC construct that lacks the intervening sequence. The development of these reagents will help us piece together how this larger repression complex is formed and establish a more complete picture of the transcriptional switch mechanism. Furthermore, understanding the high affinity CSL interactions that facilitate transcriptional activation or repression could aid in the development of more targeted and potent therapeutics. Recent work using small synthetic peptides corresponding to a portion of Mastermind has been shown to be effective in inhibiting leukemia cell growth and disease in mouse models (163). Likewise, it has been suggested that therapeutics designed to target the MINT-CSL interaction may be a useful treatment for those with Alagille syndrome (164).

**Future Directions**

_Tissue culture and MINT mediated repression_

Our tissue culture studies indicated that CSL mutants designed to disrupt RAM interactions and transcriptional activation also affected transcriptional repression.
Moreover, as measured by ITC, some of these mutants exhibited a decreased affinity for binding MINT (Chapter V). While it is currently unclear if this observation is due to the inability of MINT to facilitate repression, it does provide a reasonable explanation that could be tested in many ways. RT-PCR analysis of the OT11 transduced cells indicates that these cells do in fact express MINT as well as the corepressor SMRT (data not shown). Studies have shown that transcription complexes can be purified through the use of co-immunoprecipitation techniques allowing for the identification of the proteins in the complex (165). Nuclear lysates isolated from our different CSL expressing cells lines could be assayed using this technique to determine if MINT is present in CSL repression complexes. The absence of MINT from CSL complexes immunoprecipitated from BTD mutant cell lines would suggest that the loss of repression might indeed be attributed to the lack of MINT recruitment. This technique could also allow for identification of other corepressors that are essential for proper repression. As shown in Chapter V, BTD M-2 and M-4 both were defective in their ability to properly repress transcription; however, these mutants still retained the ability to bind MINT as well as wild type in our ITC studies. Immunoprecipitation of CSL complexes from these cell lines could identify which corepressors are no longer capable of binding CSL due to these mutations, allowing for the identification of other corepressor binding interfaces.

The tissue culture experiments described thus far have all been performed using cell lines that express CSL containing BTD mutations, but our mutational analysis has not been limited to this one domain of CSL. Guided by our structure of the ternary activation complex (2FO1) we have made CSL mutations at the CTD-ankyrin repeat interface, the CTD-Mastermind interface, and the NTD-Mastermind interface. These
cells have been assayed using the same reporters described in Chapter V, but the results are still being interpreted. Characterization of CSL complex components by the immunoprecipitation technique mentioned above could help to explain these experimental results.

*MINT interface mutants and MINT retrovirus*

Using our established retroviral transduction system it would be useful to generate CSL mutants that we have shown to affect MINT binding. If the I289R and K317I mutations showed both a defect in repression, but retained the ability to activate transcription, it would then seem likely that these residues serve as an interface for MINT interaction, and/or other corepressors are necessary for proper transcriptional regulation. These mutations could also be made in tandem with the previously mentioned BTD mutations to see if the combination further exacerbates the defects in repression or if the phenotype is rescued. Work is also in progress to generate retrovirally expressed MINT proteins encoding the CSL interacting domain (CID). Technical consideration must be given to the development of these constructs and the addition of a nuclear localization signal may need to be added to ensure the small protein is trafficked properly to the nucleus. This protein could act as a dominant negative, binding CSL and preventing endogenous MINT from interacting, which could in turn either derepress transcription or prevent the activation complex from forming in the presence of Notch. However, it is not known if this small construct will have any function in our cellular assays; thus larger MINT constructs encoding other functional domains may need to be generated.
Do coactivators or corepressors affect CSL's ability to bind DNA in vivo?

Our ITC studies of RAM-CSL and MINT-CSL interactions show that the presence of DNA does not alter the affinity of these interactions. Because of the property of linked equilibrium, this implies that the affinity of DNA for CSL, MINT-CSL, or RAM-CSL is the same (94). Models of CSL regulated transcription suggest that CSL is statically bound to DNA where it operates as a hub for protein-protein interactions. However, results from our DNA-CSL studies show that compared to some other protein-DNA interactions, the CSL-DNA interaction is relatively weak (Chapter III), and CHIP experiments performed previously seem to indicate an increase in CSL localization to the promoter upon Notch activation (52). With this being said, it seems likely that the CSL-DNA interaction is more dynamic than once thought and may be largely influenced by other proteins. To test this hypothesis we could perform CHIP using antibodies to CSL, MINT, SMRT, RAMANK, and Mastermind for our initial analysis. These experiments will provide a snapshot of the CSL promoter before and after Notch activation, as well as highlight how CSL mutations are affecting the localization of different proteins to the promoter regions. Furthermore, different promoters can be assessed using this technique to see if promoter architecture plays a role in coactivator/corepressor recruitment to CSL regulated promoters. Preliminary results from our tissue culture experiments have shown differential expression patterns for mutant CSL’s when the HES-1 reporter is used. This may indicate that certain promoters utilize different repressors, and a mutation in CSL, which ablates a particular repressor or activator from binding, could have promoter specific effects. While this new view of CSL-DNA interactions may change our model
of the transcriptional switch rather drastically, this concept of an accessory protein affecting a transcription factor-DNA interaction would be in support of recent ideas regarding transcriptional regulation. Pan et al. propose a model in which activator or repressor binding induces allosteric changes in the transcription factor, which subsequently dictate which site the transcription factor binds. This may present a way in which limiting concentrations of a transcription factor can perform multiple functions according to the fluctuation in cellular conditions (166).

*CSL transcription complexes and their interactions with other protein-DNA complexes*

The Notch signaling pathway is an extremely complex system of extracellular interactions, protein trafficking, and several protein-protein and protein DNA interactions. At the core of this biological mechanism is the transcription factor CSL, which ultimately converts Notch signals into gene expression necessary for developmental and cellular maintenance processes. In an attempt to understand the mechanism of CSL transcriptional regulation we have reduced this process to a rather simple model, and often times do not give much consideration to the other protein-DNA complexes that may be working in conjunction with CSL. One of the first functions attributed to CSL was its repressive role in transcription through interactions with TFIID and TFIIA (136). This observation seems to indicate that CSL may be directly interacting with the general transcription machinery, but the specifics of this interaction have never been expanded upon. Likewise, models of CSL mediated repression identify histone deacetylases to be components of these complexes. However, it is unclear if
these enzymes are interacting with CSL alone or if they are present as part of a larger DNA remodeling complex. In light of our knowledge regarding eukaryotic transcriptional regulation and the DNA modifications and remodeling that occur during this process, it would seem likely that CSL complexes may interact with these factors in some way. While evidence of these interactions are currently unknown, it will be important to keep the greater context of transcription in mind as our knowledge of CSL regulated transcription continues to grow.
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