Characterization of the ATP-Dependent Chromatin Remodeling Enzyme CHD8

by

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To

Aimee

Leo and Charlotte

Mom and Dad

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Portions of Chapter Two have previously been published in FEBS Letters (221).

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Abstract

Chromodomain Helicase DNA-binding protein 8 (CHD8) is an ATP-dependent chromatin remodeling enzyme that has been implicated in a variety of cellular processes from establishment of chromatin insulator elements to control of β-catenin and androgen receptor-responsive transcription.

It is demonstrated here that CHD8 directly interacts with WDR5, RbBP5, and Ash2L both on an individual basis and in the context of the WDR5/RbBP5/Ash2L complex. CHD8 is localized to the promoters of the HOXA genes and is involved in the regulation of HOXA genes, specifically HOXA2. CHD8 is shown to be required for the proper recruitment of the WDR5/RbBP5/Ash2L complex to the HOXA2 promoter. CHD8 is also required for the proper establishment of histone methylation patterns at the HOXA2 locus upon activation of the gene. Additionally, the WDR5/RbBP5/Ash2L complex is demonstrated to be a histone H3 methyltransferase independently of the association with CHD8.

Chromodomains are generally recognized to bind to methylated histone tails, although the specific methyl mark bound varies depending on the chromodomain-containing protein. The tandem chromodomains of CHD8 are demonstrated here to bind to the core of histone H3 and H4 in a manner independent of methylation status. Each individual chromodomain of CHD8 also shows this same binding activity, discounting a cooperative fold mechanism seen in other CHD proteins. A mutant CHD8 construct lacking the N-terminus, including the chromodomains, loses nucleosome remodeling capabilities but displays enhanced ATPase activity, pointing towards a role for the chromodomains in the regulation of CHD8 enzymatic activity.

Initial studies have been performed on a second Snf2 domain-containing protein, helicase-like transcription factor (HLTF), which is related to the yeast DNA repair protein Rad5. This work has provided insight into the methylation status of the HLTF promoter in several cell lines. HLTF has been found in a complex approximately 150 kDa in size, which is likely indicative of HLTF existing primarily as a monomer. Finally, HLTF appeared not to function as an ATP-dependent nucleosome remodeling enzyme, providing evidence for HLTF involvement in an alternative cellular pathway, likely DNA repair.

Chapter One

Overall Introduction

DNA and Chromatin

Every living thing that man has so far discovered has its genetic blueprints encoded on one or more chemical polymers that consist of a series of linked deoxynucleotides. A deoxynucleotide is made up of a sugar (2-deoxyribose) which has attached to it a nitrogenous base as well as one or more phosphate moieties. The 'deoxy' in deoxynucleotide results from the absence of a hydroxyl group at the 2' carbon on the ribose sugar. The phosphate attached to the sugar of one deoxynucleotide can react with hydroxyl functional group on the sugar of a second deoxynucleotide, forming a phosphodiester bond and creating a dinucleotide. Successive additions of nucleotides to the chain result in a molecule of deoxyribonucleic acid, or DNA. Two chains of polynucleotides can come together via favorable interactions between their bases. There are four different bases, each one having a preferred binding partner. When two bases from opposing strands of DNA interact, a base pair is formed. It is the base sequence of the DNA that encodes the genetic information that organisms use to create functional molecules. One molecule of DNA can consist of several million base pairs of DNA. Human chromosome number one, for example, contains 223.8 million deoxynucleotides bound together in this fashion (59).

Discrete units of hereditary information encoded by DNA are called genes. A gene contains the specific nucleotide sequence that defines a protein or RNA product of some function to the cell. A chromosome is a single piece of double stranded DNA, along with its associated proteins, that is made up of genes and structurally important sequences of DNA. One cell can and often does contain multiple independent chromosomes containing unique genes.

As organisms began becoming more and more complex, the requirement for more genetic information was necessary. In order to accommodate a larger amount of DNA, the cell had to develop a means of compacting the DNA inside rather small cellular structures. This was

accomplished by wrapping ~146 bp of DNA around a core octamer of histone proteins. The histone octamer is made up of two dimers of histones H2A and H2B along with a tetramer of two molecules each of histones H3 and H4. DNA that is complexed with histones and other associated proteins is called chromatin. One histone octamer with DNA wrapped around it is called a nucleosome. A series of nucleosomes arranged on a single piece of DNA gives rise to a beads-on-a-string appearance under an electron microscope (94). Internucleosomal associations give rise to a 30 nm fiber. Continued compaction of these fibers enables the 224 million base pairs of DNA in human chromosome one mentioned above, along with 45 additional chromosomes, to fit into the space of a cell's nucleus, which averages six microns in diameter (2). This DNA, if stretched end-to-end, would measure approximately 6 feet in length.

Because there is so much genetic information in such a small space, cells had to evolve mechanisms to allow for access to the DNA when needed. This resulted in the evolution of several families of factors specifically tailored to tasks involving chromatin maintenance. For example, DNA methyltransferases can methylate the DNA, ultimately resulting in tighter compaction of the chromatin. Histone proteins can also be chemically modified, resulting in either an opening up or a compaction of the chromatin, depending on the location and specific chemical modification (192). There are also protein complexes that are able to cause local changes in the chromatin environment to allow for more discreet control of access to the genetic information (33). These mechanisms enable several cellular processes that require access to DNA, including replication, recombination, repair, and, of specific interest to this research, transcription.

Transcription

Transcription is the process by which a cell copies the contents of its DNA into RNA. There are three main types of RNA that can be generated, namely tRNA, rRNA, and mRNA. tRNA is a specialized RNA that is responsible for transport of amino acids to ribosomes and recognition of mRNA codons during protein translation. rRNA is the major component of the ribosome and is responsible for catalysis of the reaction that elongates polypeptide chains during translation. mRNA contains the necessary sequence information for the accurate production of polypeptides. Given the diversity of polypeptides produced in the cell that are

required for proper cellular identity and function, regulation of mRNA transcription is under tight control.

There are three classes of RNA polymerase enzymes that are utilized in eukaryotes to produce the different RNA molecules mentioned (27). RNA Polymerase I is primarily responsible for the production of the larger rRNA precursor molecules. RNA Polymerase II produces mRNA from protein coding genes. Finally, RNA Polymerase III transcribes the tRNAs and the smallest (5S) rRNA subunit precursor. Remaining discussion will focus on RNA Polymerase II (RNAP II) transcription due to the complexity of its regulatory mechanisms.

RNAP II is a large complex of proteins, consisting of twelve subunits (RPB1-RPB12) (27). RPB1-4 share sequence and functional characteristics with the core subunits of the prokaryotic RNA Polymerase, indicating that the catalytic mechanisms of the enzyme have remained relatively unchanged over evolutionary time. One important difference is the C-terminal domain (CTD) of RPB1, which contains 52 repeats of the consensus heptapeptide YSPTSPS (223). Phosphorylation of this CTD is vital to the regulation and processivity of RNAP II. RNAP II is also associated with numerous other factors that act in a cooperative manner at various points in the transcription cycle.

The canonical transcriptional cycle for the production of an mRNA molecule involves a specific order of events at the gene to be transcribed. There are four basic steps to the transcription of a gene, each of which is riddled with its own complexities. Transcription takes place via assembly the RNA polymerase complex at the gene promoter (preinitiation), and initiation, elongation, and termination of RNA polymerization.

Preinitiation of Transcription

The proteins and protein complexes that cooperate with RNAP II during the transcriptional cycle are referred to as general transcription factors (GTFs). GTFs serve to target, orient, and regulate the activity of the RNAP II holoenzyme. These GTFs can be relatively simple one subunit proteins, such as TFIIB, or quite large complexes, such as the 15 subunit TFIID (107). Additionally, there is another 24 subunit complex known as Mediator that is required for RNAP II function *in vivo* and is thus considered a general transcription factor as well as part of the RNAP II holoenzyme (195). Mediator is vital for the bridging of important regulatory signals to the polymerase (136). In order for a gene to be transcribed, the transcriptional machinery

needs to be localized to the promoter of interest. GTFs must bind to the target promoter in a specific order for transcription to occur, and it is this assembly of the transcriptional complex defines the preinitiation phase of transcription.

A promoter consists of DNA sequences that are used to specify the start site and transcriptional direction as well as to regulate the expression of a gene. Fundamental to the promoter is a core that encompasses the transcriptional start site (TSS) and contains all, some, or none of several promoter elements (180). These include a classical TATA box, a TFIIB recognition element (BRE), an initiator (Inr), and a downstream promoter element (DPE). A TATA box is a 6-10 base segment that is enriched for adenine and thymine and resides approximately 30 bp upstream of the TSS (-30 position) (180). TATA binding protein (TBP), which is a subunit of TFIID, is known to recognize and bind to the TATA box. The Inr and PDE core promoter elements are also recognized by subunits of TFIID. The Inr is a seven bp sequence located at -2 to +5 and is bound by TAF1 and TAF2 (180). The DPE is a consensus sequence typically located at +20 to +35 and is bound by TAF6. The BRE is the only core promoter element recognized by a GTF other than TFIID. This seven bp sequence is located immediately adjacent to the TATA box and is bound by the GTF TFIIB (180).

Conventional assembly of the preinitiation complex, or PIC, begins with the binding of the GTF TFIID to the promoter of the gene to be transcribed. This is followed by the sequential binding of TFIIA and TFIIB to stabilize the TFIID complex and create the necessary environment for recruitment of the RNA Polymerase II/TFIIF/mediator holoenzyme. The final factors to join the preinitiation complex are TFIIE and TFIIH (107).

Initiation of Transcription

Following the assembly of the PIC, initiation of transcription occurs. Initiation is considered to encompass melting of the promoter DNA surrounding the TSS through promoter clearance by RNAP II. Once the PIC is formed, the dsDNA surrounding the TSS is melted by the helicase activity of TFIIH. This allows for the addition of RNA nucleotides complementary to the template DNA strand to be inserted and added to the nascent RNA molecule. However, it is known that RNAP II will engage in multiple rounds of abortive initiation, where 2 to 3 nucleotide long RNA molecules will be released before RNAP II will backtrack to the TSS (49).

Successful transition to an elongating polymerase requires the modification of the protein-protein interactions that were made in the PIC. For example, although it is known that TFIIB has a supporting role for nascent RNA formation, it is also required to dissociate from RNAP II for the RNA to extend beyond four nucleotides long (95, 143). Additionally, hyperphosphorylation of serine 5 in the RNAP II C-terminal domain (CTD) by TFIIH is thought to be important for dissociation of RNAP II from the PIC to a complex capable of elongation.

Transcriptional Elongation

Once a nascent transcript gets to eight or nine nucleotides in length, RNAP II transitions into the elongation phase of transcription. It is at this length of RNA that each base from the melted DNA template is paired with an RNA counterpart, which provides stability to the complex and helps to prevent an abortive initiation (137). RNA polymerization continues via catalysis of phosphodiester bond formation by RNAP II. The 5' cap is added to the RNA molecule when the RNA is around 20 nucleotides in length. This cap is important for RNA stability and recognition by the cell as mRNA.

The association of Negative ELongation Factor (NELF), along with DRB Sensitivity-Inducing Factor (DSIF), with RNAP II occurs very early in elongation, although the presence of an RNA transcript is required (137, 154). Phosphorylation of serine 2 in the CTD of RNAP II is catalyzed by Positive Transcription Elongation Factor b (P-TEFb). P-TEFb recruitment is among the final steps in the transition from early elongation to a fully functioning elongating polymerase (137, 154). Following this last transition, RNAP II is capable of transcribing across hundreds of thousands of DNA bases without dissociation.

Termination of Transcription

Completion of the mRNA transcript and dissociation of the RNAP II machinery from the DNA comprise the major steps to transcription termination. Termination is closely tied to the 3' processing of the newly synthesized RNA (118). Current models suggest a mechanism whereby the RNAP II transcribes through the Poly(A) recognition site (conserved AAUAAA hexamer), and the RNA undergoes co-transcriptional cleavage, causing release of the transcript by RNAP II. The mRNA transcript is then polyadenylated, while the readthrough transcript is degraded. It is

possible that the degradation of the readthrough signals RNAP II release from the transcript and/or the DNA template ("torpedo" model) (118). RNAP II is then available to begin a new round of transcription.

Control of the Chromatin Environment

As there is a tremendous amount of genetic information packaged inside of the eukaryotic nucleus, there have evolved several mechanisms for controlling access to the DNA. Mechanisms used for control of transcription and other cellular processes include DNA methylation, the covalent modification of nucleosomal histones, and modification of chromatin compaction via ATP-dependent chromatin remodeling enzymes. Use of these mechanisms allows the cell to keep a euchromatic chromatin state at gene loci it uses on a constant basis, while loci that the cell will rarely or never use can be kept as heterochromatin.

DNA Methylation

Methylation of genomic DNA occurs on the 5 carbon of the cytosine ring in the context of CpG dinucleotides. Heterochromatin is generally hypermethylated, especially at structural chromosomal loci such as centromeres (29). The methylation of DNA can also occur at CpG islands, which are stretches of DNA enriched for cytosine and guanidine that are localized around the promoters of some genes. Methylation of the CpG dinucleotides at these promoters is used, in conjunction with other chromatin modifications, to prevent transcription of the gene. DNA methylation patterns are wiped during very early embryonic development, with re-establishment of DNA methylation occurring as development continues. DNA methylation is important for genomic imprinting as well (29). Genomic imprinting involves the establishment of one parental allele as the only one to be expressed in the offspring.

DNA methylation occurs via the action of four DNA methyltransferase enzymes: Dnmt1, Dnmt2, Dnmt3a, and Dnmt3b. Dnmt1 is expressed in proliferating somatic cells, functioning in maintaining methylation patterns by copying from parental strands to daughter strands during DNA replication. Dnmt2 is less well studied, but seems to be involved in non-canonical DNA methylation away from CpG dinucleotides (68, 97, 113, 197). Dnmt3a and Dnmt3b are highly expressed in very early embryogenesis; both enzymes are capable of *de novo* methylation,

suggesting these enzymes function in establishment of DNA methylation patterns during embryogenesis (29).

Covalent Histone Modification

As mentioned previously, a nucleosome is composed of DNA wrapped around a core octamer of histone proteins. Extending outward from a globular core, and through the encompassing DNA, are N- and C-terminal sections of the histone proteins. These 'tails' are the target of multiple covalent modification events, creating specific signals to the cellular machinery. Common modifications of histone tails include acetylation, methylation and ubiquitination of lysine residues, methylation of arginine residues, and phosphorylation of serine and threonine residues.

Histone Acetylation

Acetylation of histone tails is strongly associated with open chromatin and active transcription. Lysine residues are the target of acetylation (Figure 1.1). Specifically, the histone residues that are commonly acetylated are lysines 9, 14, and 18 of histone H3, lysines 5, 8, 12, and 16 of histone H4, lysine 5 on histone H2A, and lysines 12 and 15 of H2B (168). Acetylation of these lysine residues is catalyzed by various histone acetyltransferase (HAT) enzymes. Removal of the acetyl mark, thus causing down regulation of gene expression, is catalyzed by histone deacetylase (HDAC) enzymes. Because of the rather direct effect HATs and HDACs have on the transcriptional output of genes, they have become popular targets for drug design.

Histone Methylation

Histone tail methylation is functionally more complicated than histone acetylation. The specific residue that is methylated, as well as the degree to which it is methylated, signals for different transcriptional output. For example, trimethylation of lysine 4 on histone H3 (H3K4me3) is a known mark for transcriptional activation, while trimethylation of lysine 9 on histone H3 (H3K9me3) is a known repressive mark. Lysine residues can be mono-, di-, or trimethylated (Figure 1.2), while arginine residues can be monomethylated, symmetrically dimethylated, or asymmetrically dimethylated (Figure 1.3). Table 1.1 lists the common histone

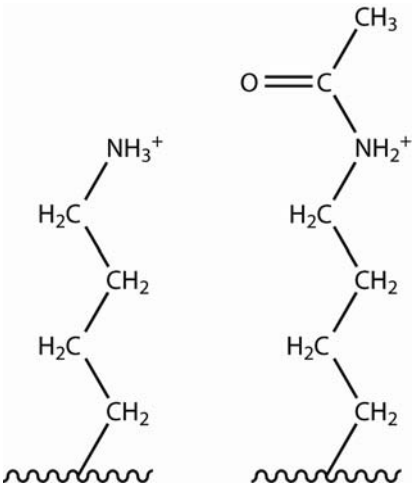

Figure 1.1 Molecular Geometry of Acetylated Lysine

Shown is the functional group of the amino acid lysine, which can be acetylated within N-terminal histone tails. Unmodified lysine (K) is on the left, and a lysine that has been acetylated (KAc) is on the right.

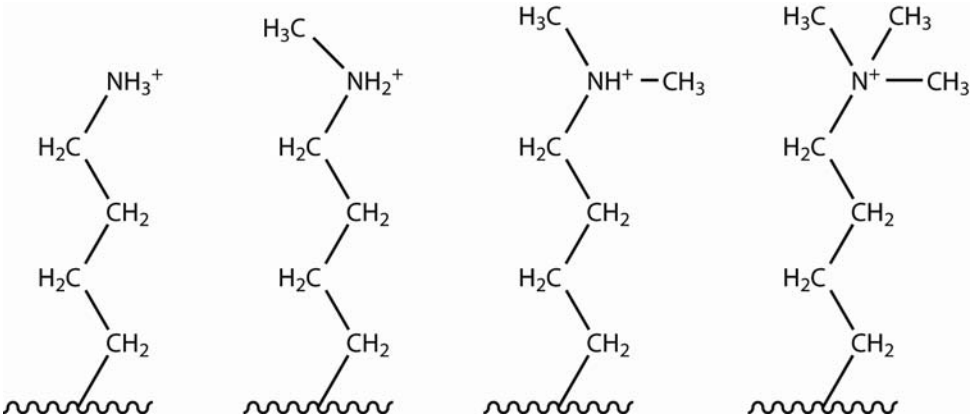

Figure 1.2 Molecular Geometry of Lysine with Varying Degrees of Methylation

Shown is the functional group of the amino acid lysine, which can be methylated to varying degrees, especially within the context of histone tails. From left to right are lysine (K), monomethylated lysine (Kme), dimethylated lysine (Kme2), and trimethylated lysine (Kme3).

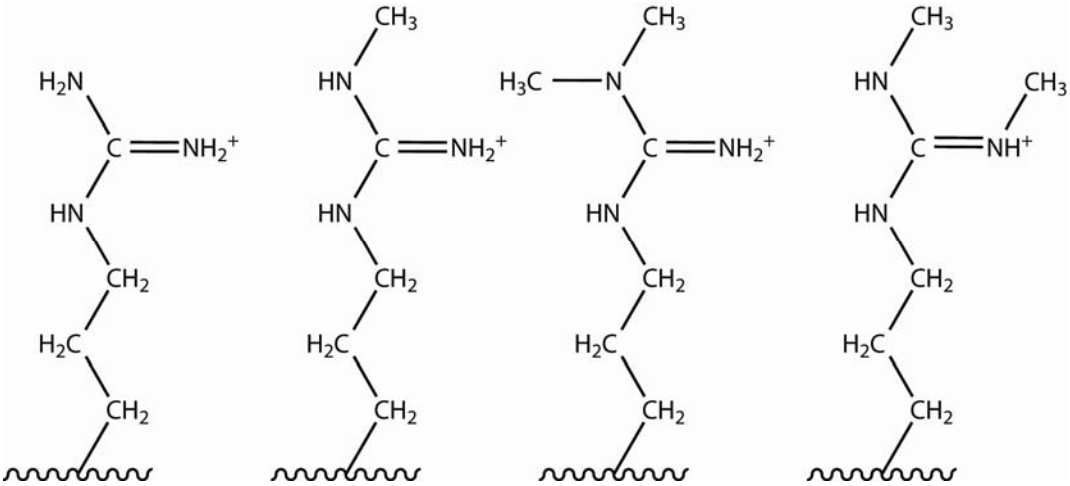

Figure 1.3 Molecular Geometry of Arginine with Varying Methylation States

Shown is the functional group of the amino acid arginine, which can be methylated in various ways. From left to right are arginine (R), monomethylated arginine (Rme), asymmetrically dimethylated arginine (Rme2a), and symmetrically dimethylated arginine (Rme2s).

Residue Methylated	Effect	HMTase
H3K4	Activating	MLL/COMPASS
H3K9	Repressing	Suv39, G9a
H3K27	Repressing	E(Z)
H3K36	Repressing	Set2
H3K79	Repressing	DOT1p
H4K20	Repressing	SET7/SET8
H3R2	Activating	CARM1
H3R17	Activating	CARM1
H3R26	Activating	CARM1
H4R3	Activating	PRMT1

Table 1.1 Methylated Histone Residues

Shown are amino acids of histones that are methylated, the effect on chromatin by the methylation, as well as the protein or protein complex that is primarily responsible for the methylation of the residue.

methylation events, along with their effects on the chromatin environment and the enzymes that are responsible for catalyzing the methylation.

Other Histone Modifications

In addition to histone acetylation and methylation, other modifications can be made to histone tails. Phosphorylation of histone H3 takes place on threonine 3, serine 10, threonine 11, and serine 28. All of these marks have been observed during cell division, and serine 10, threonine 11, and serine 28 phosphorylation have a role in transcriptional activation (153). This is suggestive of histone phosphorylation having a complex effect on the structure and regulation of chromatin. Ubiquitination of histone H2A at lysine 119, on its C-terminal tail, has involvement in chromatin silencing, specifically in X inactivation and Polycomb repression. Also, ubiquitination of histone H2B on lysine 120 has been shown to be required for methylation of histones by MLL/COMPASS and Dot1p (173).

ATP-dependent Chromatin Remodeling Enzymes

ATP-dependent chromatin remodeling enzymes use the energy from hydrolysis of ATP to cause local changes in the chromatin environment. These changes can include repositioning a nucleosome on a template, creating DNA bulges around nucleosomes by pumping DNA into the nucleosome, deposition of nucleosomes, and/or replacement of canonical histones with histone variants. The feature that is shared amongst all ATP-dependent chromatin remodeling enzymes is the presence of a Snf2 helicase domain. This domain is responsible for the hydrolysis of ATP to ADP, harnessing the energy necessary for the enzyme to carry out its task. In addition to a role in ATP-dependent chromatin remodeling, the Snf2 domain is seen in proteins that have other tasks within the cell, including roles in DNA repair (Rad5) and recombination (Rad54). The subset proteins within the proteome that contains a Snf2 domain is designated the SNF2 Family.

Besides the Snf2 domain, characteristics of ATP-dependent chromatin remodeling enzymes are quite diverse, and they have been grouped into subfamilies base upon sequence and domain architecture similarities. The most widely studied subfamilies of ATP-dependent remodelers are the SWI/SNF, ISWI, INO80, and CHD subfamilies.

SWI/SNF

The SWI/SNF complex was the first identified ATP-dependent chromatin remodeling factor discovered. Genes involved in the SWI/SNF complex were identified via genetic screens in yeast for mating type switching (SWI) or defects in growth using sucrose as a carbon source (sucrose non-fermenting, SNF) (138, 186). It has since been revealed that the SWI/SNF complex contains 11 subunits, with the catalytic protein identified as the DNA-dependent ATPase SWI2/SNF2 (22, 36, 102). Homologous complexes were identified in other organisms (45, 78, 98, 139, 196, 212, 213).

In *Drosophila*, the SNF2 homologue was identified as brahma (BRM) (50). BRM was originally discovered in a screen for rescue of the phenotype caused by mutation of the transcriptionally repressive Polycomb complex (87). Mutations in BRM caused homeotic transformations due to defects in expression of the Hox genes (196). BRM was shown to exist in a two MDa, eight subunit complex that contains proteins highly related to yeast SWI/SNF complex members (145). The BRM complex has since been suggested to play a role in global transcription, and loss of BRM function results in reduction of RNAP II association with chromatin (7).

Human homologues of SWI/SNF have been identified as hBRM (human brahma) and BRG1 (brahma related gene 1), which share a 74% identity at the amino acid level. However, unlike the yeast and *Drosophila* SWI/SNF complexes, which have static core compositions, human BRM/BRG1 complexes vary in both the identity of the catalytic subunit as well as the association of BRM/BRG1 Associated Factors (BAFs). The diversity in composition of BAF complexes likely explains the varying roles demonstrated for these enzymes. For example, BRM and BRG1 perform essential and distinct functions throughout neural development. These distinct functions appear to operate at different developmental stages, dictated by the combinatorial assembly of subunits in this ATP-dependent remodeling complex (222). Important transcriptional links include the role of SWI/SNF in nuclear receptor-mediated transcriptional activation. This function was first observed for the glucocorticoid receptor, but later established for most steroid hormone receptors where SWI/SNF is recruited to receptor binding sites and facilitates creation of an open chromatin structure (83). Outside of transcriptional regulation, the SWI/SNF chromatin-remodeling complex has been shown to play

an important role in DNA repair by facilitating nucleotide excision repair and DNA double-strand break repair (146, 231).

ISWI

Another class of ATP-dependent remodeling enzymes was discovered by the use of chromatin assembly assays in *Drosophila* embryo extracts (203). This newly identified NUcleosome Remodeling Factor (NURF), functioned in a much smaller complex (four subunits) than the SWI/SNF complex (eight to twelve subunits). Additionally, purified SWI/SNF did not produce the same results as NURF in both DNase I digestion and nucleosome disruption experiments, pointing toward NURF being a different class of enzymes. The catalytic subunit of this complex, as well as related complexes CHRomatin Accessibility Complex (CHRAC) (205) and ATP-utilizing Chromatin assembly and remodeling Factor (ACF) (80), was identified as imitation SWI (ISWI) (50). Complexes containing paralogues of the *Drosophila* ISWI protein have been identified in *S. cerevisiae* (125, 202, 206), *Xenopus* (62, 119), and humans (12, 15, 19, 63, 109, 110, 157, 191, 220).

Two human enzymes that are homologous to ISWI are Snf2H and Snf2L. Snf2H was originally identified in a two subunit complex with WCRF180/hACF1 (human ACF complex) (15). Snf2H also exists in a four subunit complex with WCRF180/hACF1, hCHRAC15, and hCHRAC17 (the human CHRAC complex) (92). Likewise, Snf2L has been found in a four subunit complex which includes BPTF, RbAp48, and RbAp46 (the human NURF complex) (12). These enzymes have been shown to have diverse functions in chromatin maintenance. For example, depletion of Snf2H results in a delay in the progression of DNA replication during S-phase (34). Snf2H also associates with the cohesion complex and functions in sister chromatid cohesion (63). Additionally, Snf2H functions in DNA repair, as this protein accumulates at sites of double stranded breaks and depletion of Snf2H results in a lower frequency of end joining at these sites (100).

INO80

A third class of ATP-dependent chromatin remodeling enzymes is the INO80 family. This family was also discovered in *S. cerevisiae*, and homologous proteins exist in *Drosophila* as well

as humans. INO80 is part of relatively large complexes of 10-14 subunits. This family is unique in that it is able to bind to and remodel more specialized DNA templates such as replication forks and Holliday junctions (170, 215). Also, INO80 family members are able to bind to variants of histone H2A, namely H2AZ and H2AX (65). Insertion of these histone variants into nucleosomes in place of the canonical histone is yet another mechanism used by the cell for control of the chromatin environment.

Following a DNA double stranded break (DSB) event, INO80 and another ION80 family member, SWR1, are localized to the break site, facilitating nucleosome eviction to allow for efficient repair (65). INO80 has also been found to relieve stalled replication forks, possibly by a similar mechanism (144). This family of ATP-dependent chromatin remodeling enzymes plays an important role in DNA repair and replication, as well as participating in histone variant exchange.

CHD

The first member of this family was discovered while cloning a non-related sequence-specific DNA binding protein (40). The CHD proteins make up the largest family of ATP-dependent remodeling enzymes, consisting of nine proteins that have a series of defining architectural domains. The defining feature of the CHD family is tandem chromodomains N-terminal to a SNF2 helicase domain. The CHD family is grouped into subfamilies CHD1-2, 3-5, and 6-9 based upon the presence of additional domains (Figure 1.4).

CHD1 and CHD2

CHD1 and CHD2 each contain the tandem chromodomains and the Snf2 domain that are the defining characteristics of the CHD family members. Additionally, a DNA binding motif has been observed near the C-terminus (188). The DNA binding domain is actually how the CHD family was 'accidentally' discovered, as mouse CHD1 was pulled out of an attempt to clone the cDNA of κY, which binds in a sequence-specific manner to immunoglobulin promoters (40).

Yeast only have one member of the CHD family, that being Chd1. *S. cerevisiae* Chd1 has been shown to function in RNAP II transcriptional elongation and termination (3, 96, 176). Chd1 was co-precipitated with known transcriptional elongation factors. Also, evidence points

Figure 1.4 Functional Domains of the CHD Family of ATP-Dependent Remodeling Enzymes

The nine proteins in the CHD family are grouped into the indicated subfamilies based upon the presence of functional domains. The defining domains of the CHD family are the tandem N-terminal chromodomains and the Snf2 ATPase domain. The CHD1-2 subfamily also possess a C-terminal DNA binding domain (188). Characteristic of the CHD3-5 subfamily are tandem PHD finger domains that are N-terminal of the chromodomains. The CHD6-9 subfamily have a SANT domain as well as tandem BRK domains C-terminal of the Snf2 domain.

towards a requirement for Chd1 to cooperate with ISWI to remodel the 3' end of a gene to allow for transcriptional termination. Chd1 has been shown via chromatin immunoprecipitation (ChIP) to localize to the 3' end of genes in yeast.

Roles for CHD1 have also been described in higher organisms. In *Drosophila*, CHD1 has been observed co-localizing with elongating RNAP II (184, 189) as well as being responsible for the incorporation of histone H3.3 during development (93). In mice, it has recently been shown that mCHD1 is plays a role in maintaining euchromatic DNA in embryonic stem cells (56). Loss of CHD1 expression leads to an accumulation of heterochromatin and a resulting loss of pluripotency. It should also be noted that CHD1 is found localized to the promoters of active genes in this study, which contrasts the localization of Chd1 in yeast (176).

CHD3-5

The defining domain structure of the CHD3-5 family of proteins is the presence of tandem PHD finger domains N-terminal to the chromodomains. The occurrence of these PHD fingers adjacent to the chromodomains is intriguing as the PHD fingers of CHD4 have been shown to function as histone H3-binding domains with a preference for methylated H3K9 and unmodified H3K4 (122, 135). The arrangement of these different histone binding domains in close proximity to one another may act as a single binding domain with a preference for a specific combination of histone marks.

This subfamily is absent in *S. cerevisiae*, one homologue exists in *Drosophila* (dCHD3) and three are present in humans (CHD3-5). In humans, CHD3 is also known as Mi-2α, and CHD4 is similarly known as Mi-2β. Members of this family have been identified as components of a multi-protein complex (NURD) that possess both nucleosome remodeling and histone deacetylase activities (200, 209, 218, 232). The histone deacetylase activity is due to the presence of a four subunit histone deacetylase core, HDAC1/2 and RbAp46/48. Another component of the NURD complex is the Methyl-CpG Binding Domain (MBD) protein MBD2 or MBD3 (67, 208, 233). Given the association with histone deacetylase activity and methyl-CpG DNA binding, it is suggested that this family functions in transcriptional repression of target genes. For example, the co-repressor KAP1 serves to target the NURD chromatin remodeling factors to sites bound by the KRAB-ZNF family of repressors (167).

CHD6-9

In addition to the tandem chromodomains and Snf2 helicase domain, the final subfamily of the CHD enzymes is defined by the presence of C-terminal SANT and tandem BRK domains. Interestingly enough, although it is these three domains that separate these CHD proteins from the others, little to nothing is known about the role of these domains as it relates to protein function. The SANT (SWI3, ADA2, N-COR, and TFIIB) domain is conserved among many regulators of transcription and chromatin structure (1). SANT domains have many described functions including acting as a DNA binding motif (89, 133), as a protein interaction domain (44, 86, 105, 106, 172, 219), and as a nucleosome/histone tail binding module (17, 18, 61, 106, 133, 172, 226). Functional studies with the ATP-dependent remodeling enzyme ISWI have shown that deletion of one of the SANT domains in ISWI can cause loss of catalytic function in this enzyme. The BRK (Brahma Kismet) domain was identified based on similarities observed in a 41 amino acid segment of the *Drosophila* proteins kismet and brahma as well as the human proteins hBRM and BRG1 (CHD6-9 were not yet characterized) (38). The BRK domain has been suggested to function in a reorganization of chromatin structure specific to higher eukaryotes (38); however, there has yet to be any functional role identified for this domain. The combined presence of these domains, along with the Snf2 helicase domain and the tandem chromodomains, suggest that the CHD6-9 proteins play a key role in the regulation of chromatin structure.

Like the CHD3-5 subfamily, this subfamily is absent in *S. cerevisiae*, and only one homologue exists in *Drosophila*. As numerous gene regulatory mechanisms have been initially identified through genetic analysis in *Drosophila*, it is highly significant that the CHD6-9 proteins appear to be human homologues of the protein encoded by the *Drosophila* kismet gene. kismet was identified in a genetic screen for dominant suppressors of polycomb (38). The polycomb group is made up of transcriptional repressors of homeotic genes. Loss of zygotic kismet results in homeotic transformations, suggesting that kismet is a member of the trithorax group of gene activators. These results are similar to the studies on brahma, the catalytic subunit of the *Drosophila* SWI/SNF complex, suggesting that kismet and brahma function by similar mechanisms (87, 196).

Loss of maternal kismet results in significant defects in larval body segmentation. Initial studies employing immunostaining of polytene chromosomes suggest that kismet is localized to

transcriptionally active chromatin overlapping RNAP II (184). Further studies demonstrated that loss of kismet results in reduced associated of the histone H3 lysine 4 methyltransferases TRX and ASH1 with chromatin. Concurrently, an increase in histone H3 lysine 27 methylation was also observed, suggesting that kismet functions in transcriptional elongation, counteracting the activity of the histone H3 lysine 27 methyltransferase E(Z) and promoting the association of the histone H3 lysine 4 methyltransferases TRX and ASH1 with chromatin (185). However, a biochemical characterization of kismet has not been performed.

The CHD6-9 subfamily is the largest CHD family, with four members in humans, and the importance of this family of proteins first came to light when it was reported that mutations or deletions in a gene encoding CHD7 were present in patients with CHARGE syndrome (207). CHARGE syndrome is an association of malformations occurring with choanal atresia, and it is estimated that this syndrome afflicts 1 in 10,000 births annually (64, 71). Common features of this syndrome include deafness, blindness, balance disorders, and congenital heart malformations (103). Further studies have confirmed that mutations in CHD7 as cause CHARGE syndrome, and mutations in the mouse CHD7 gene provide a model for this disease (6, 84, 85, 99, 165). Mechanistically little is known about CHD7, but several recent studies have added to our understanding of this enzyme. CHD7 has been identified as a component of a corepressor complex that inactivates PPARγ mediated transcription (194). Interestingly, this complex also contains SETDB1, a histone H3 lysine 9 methyltransferase (194), stressing the important interplay between ATP-dependent remodelers and histone methylation. Genome-wide localization studies on CHD7 have shown that this enzyme binds to both transcriptional start sites as well as to numerous distal regions believed to be enhancers (166). Most recently, it has been shown that CHD7 is required for the formation of migratory neural crest cells through activation of transcriptional regulators like Sox9, Twist, and Slug (11). It is interesting to note that this function of CHD7 requires the cooperation of another ATP-dependent chromatin remodeling factor, SWI/SNF (11).

CHD9 has also been shown to be an important regulator of gene transcription. CHD9 was first reported as a gene expressed in osteoprogenitors (123, 174) and later shown by ChIP to be localized to the promoters of genes involved in osteogenic differentiation (175). CHD9 was also recently isolated in a complex with liganded PPARα, was shown to interact with several other nuclear receptors, and was shown to coactivate PPARα dependent transcription *in vivo* and *in vitro* (193). However, the mechanisms behind this activity were not explored.

CHD8

While many questions remain about the CHD6-9 family, our laboratory focuses specifically on CHD8 for two main reasons. First, initial studies into the expression of CHD6-9 revealed CHD8 to have the broadest and highest expression profile across numerous tissues and cell lines (data not shown). Second and more importantly, the first study to implicate a member of the CHD6-9 family in transcriptional regulation identified a partial fragment of CHD8 (termed 'duplin') as a negative regulator of β-catenin mediated transcription (91, 164). This work on duplin inspired our initial studies on full length CHD8, revealing it to function as a negative regulator of β-catenin mediated transcription (199). Our laboratory demonstrated that CHD8 interacts directly with β-catenin and is also recruited specifically to the promoter regions of several β-catenin-responsive genes. Using short hairpin RNA (shRNA) techniques, we established that CHD8 can negatively regulate β-catenin-targeted gene expression, providing further insight into the importance of this association in the regulation of β-catenin targeted genes. Although sequence analysis predicted that CHD8 was an ATP-dependent remodeling enzyme, our laboratory was the first to demonstrate chromatin remodeling activity for any member of the CHD6-9 family of proteins. These studies were the first to highlight the importance of CHD8 in transcriptional regulation through the modulation of chromatin structure.

Our laboratory's continued studies on CHD8 also found that CHD8 is significantly upregulated in several prostate cancer versus normal tissue data sets (101, 117, 210, 214, 228). As androgen receptor (AR) coregulators commonly display altered expression in prostate cancer samples, this finding suggests that CHD8 may function in the regulation of AR mediated transcription. This observation was the basis for our recent report that CHD8 is a key transcriptional regulator of androgen-responsive gene transcription (127). In this study we were able to demonstrate that in the androgen-dependent prostate cancer cell line LNCaP, reduction of CHD8 levels by siRNA treatment severely diminishes DHT-dependent activation of the TMPRSS2 gene. Using ChIP experiments, we demonstrated that CHD8 is present at the TMPRSS2 ARE both before and after induction with DHT. This result was unexpected as most AR coactivators show hormone induced recruitment. Using re-ChIP experiments (in which the immunoprecipitate from a first antibody incubation is subjected to an immunoprecipitation with a second antibody), we demonstrated AR and CHD8 simultaneously localize to the TMPRSS2 ARE

following DHT treatment. These results illustrate that TMPRSS2 is a direct target of CHD8. To investigate the function of CHD8, we performed ChIP experiments for AR under conditions of CHD8 depletion. Using this approach, we found that the recruitment of AR to the TMPRSS2 promoter in response to DHT treatment requires the presence of CHD8. Finally, by utilizing siRNA against CHD8, we demonstrate that CHD8 facilitates DHT-stimulated proliferation of LNCaP cells. Taken together, our results indicate that CHD8 is directly required for optimal transcriptional activation and that CHD8 plays an important role in androgen-dependent cell growth of LNCaP cells.

Several other studies have also highlighted the importance of CHD8 in the regulation of gene transcription. CHD8 was identified as a CTCF binding protein and is required for CTCF-dependent insulator activity of the H19 differentially methylated region (79). CHD8 was also identified as a factor in HeLa nuclear extracts that can bind to recombinant hStaf and regulate both Pol II and Pol III snRNA promoters as well as the hStaf responsive IRF3 promoter (229). That same study also found that depletion of CHD8 by siRNA results in decreased expression of the Pol III-transcribed U6 gene and the Pol II-transcribed IRF3 gene. Further studies on CHD8 identified several apoptotic genes as additional targets of CHD8 (141). Both p21 and Noxa expression increased upon depletion of CHD8, and it was proposed that CHD8 functions by recruiting histone H1 to these target genes. A more recent study on CHD8 found this enzyme to regulate several genes involved in controlling cellular proliferation (161). In this study, microarray data analysis found ten genes significantly up regulated and 31 genes significantly down regulated. They also demonstrated that CHD8 can associate with the hyperphosphorylated form of Pol II and suggest that CHD8 functions in transcriptional elongation. The most recent study on CHD8 found a direct interaction between CHD8 and Serum Response Factor (SRF) (160). In this study, they found that CHD8 plays a role in smooth muscle cells by modulating SRF activity toward differentiation genes.

These studies in combination with our own work clearly demonstrate the breadth and importance of CHD8 in transcriptional regulation. This is ultimately highlighted by a report revealing that targeted deletion of first nine exons of mouse CHD8 results in death *in utero* between E5.5 and E7.5 (140). Further experiments probing the mechanisms of CHD8 targeting as well as *in vivo* enzymatic activity are needed to understand the cellular role of this important remodeling enzyme. This thesis aims to provide insight into the mechanism of CHD8 action,

and, by extension, into the broader mechanisms of general transcriptional regulation by the control of chromatin structure.

Chapter Two

CHD8 Association with WDR5, RbBP5, and Ash2L and Regulation of HOXA2 Expression

Introduction

MLL Proteins

The Mixed Lineage Leukemia proteins are a family of 5 proteins (MLL1-5) belonging to the trithorax group of transcriptional regulators. MLL proteins are SET domain-containing enzymes that have lysine methyltransferase activity. Within this protein family, MLL1 has been the most extensively studied due to its involvement in lymphoid and myeloid leukemias, wherein the chromosome containing the MLL1 gene is broken, and the MLL1 gene is fused to another chromosome (234). This ultimately results in mutant MLL1 fusion proteins that are key to the development of the leukemia.

MLL1 is translated as a 3969 amino acid polypeptide. It is cleaved by Taspase I into MLL-N and MLL-C, which dimerize via two domains, known as the FYRN and FYRC domains, to form the functional MLL1 enzyme (35). Additional functional regions include AT-Hooks, CXXC, PHD, TAD, and SET domains. Briefly, an AT-hook is a DNA binding domain that binds in the minor groove of an AT-rich segment of DNA (159). The CXXC domain has been shown to bind to nonmethylated CpG islands (9, 13) and is implicated in MLL1 targeting. A Plant HomeoDomain (PHD) is a zinc-finger motif that has been demonstrated to be involved in protein-protein interactions in the context of MLL1 (52). The TAD domain is implicated in CBP/p300 binding to MLL1 (39, 51). Finally, the SET domain of MLL is the catalytic domain responsible for lysine methyltransferase activity (183). A SET domain catalyzes the reaction which transfers a methyl group from the sulfur atom of S-Adenosyl Methionine (SAM) to the ε-amino group of the accepting lysine molecule, forming monomethyllysine. This process can then be repeated, where additional molecules of SAM donate additional methyl groups to the monomethyllysine, resulting in a dimethylated lysine and finally a trimethylated lysine (Figure 1.2).

MLL1 is known to be involved in the methylation of lysine four on histone H3 (H3K4), which is a histone mark that is strongly associated with the activation of target genes. The MLL1 complex has been shown to catalyze the mono-, di-, and trimethylation of H3K4 (46, 187), although recent evidence suggests a model whereby MLL1 is responsible for mono- and dimethylation and a second enzyme is responsible for trimethylation (35, 149). It should be noted, however, that these studies used different methodologies, one relying on the specificity of a trimethyl-specific antibody and the other relying on the use of dimethylated peptides as substrates. Although one can argue antibody specificity (and indeed, that is exactly the argument made against the MLL1 trimethylation model by those that favor the dimethylation model), one can also argue that an *in vitro* assay will be inferior to a cell-based assay in situations such as this because it is next to impossible to reconstitute a chromatin environment in a test tube. This is especially the case when the *in vitro* assay relies upon a completely artificial histone tail peptide, which varies considerably from the canonical nucleosomal substrate.

WDR5/RbBP5/Ash2L Bind to and Stimulate Activity of MLL1

The MLL1 protein is known to exist in a large molecular weight complex, and these associated proteins are required for MLL1 activity. Central and essential to this complex is a trimeric core that has been shown to interact with MLL1, MLL2, MLL3, and MLL4 (30, 46, 76). These three proteins, WDR5, RbBP5, and Ash2L, have also been shown to exist in complex on their own (46, 187). Importantly, although MLL1 itself does have intrinsic methyltransferase activity, this activity is enhanced dramatically when the MLL1 core complex members WDR5, RbBP5, and Ash2L are added to the reaction (8, 46, 183).

WDR5, RbBP5, and Ash2L are vital to MLL1 function. WDR5 is a protein that consists of seven WD40 repeats. These domains adopt a β-propeller confirmation with each domain folding into a series of four stranded β-sheets. The resulting structure resembles a donut, with a pocket that is used for binding in the center of the molecule. It has been demonstrated that WDR5 acts as a scaffold protein to bring other interacting proteins together (8, 142). WDR5 has been shown to bind MLL1, RbBP5, and histone H3 (8, 37, 142, 148, 163). RbBP5 is made up of a series of seven WD40 domains, a hinge domain, and a domain of unknown function near the C-terminus. RbBP5 has been demonstrated to bind to WDR5 opposite the MLL1 binding site (8,

142), as well as be necessary for bridging Ash2L to the complex (149). Ash2L has been demonstrated to be necessary for catalyzing the trimethylation of H3K4 by MLL1 (187).

The exact assembly of this MLL1 complex has been the focus of several recent publications (8, 46, 142, 148, 149). MLL1 has been demonstrated to bind to WDR5 by insertion of amino acids 3762–3773, dubbed the Win (WDR5 interacting) motif, into the center of the β-propeller fold of WDR5 (148). The binding of RbBP5 on the opposite side of WDR5 from MLL1's Win motif illustrates that the function of WDR5 is to position complex members in such a way that the enzymatic reaction can occur (8, 142). Following the binding of WDR5 to MLL1 and the binding of RbBP5 to WDR5, it is known that Ash2L then associates with the complex (8, 149). There is one additional protein whose function is currently unknown that is often associated with this complex and which binds last, DPY-30 (149).

WDR5, RbBP5, and Ash2L Methyltransferase Activity

In addition to their association with the histone methyltransferase MLL1, it has recently been reported by several groups that the WDR5, RbBP5, and Ash2L complex is the second known non-SET1 domain complex discovered to have histone lysine methyltransferase function (23, 149, 151). The WDR5, RbBP5, and Ash2L methyltransferase activity is surprising in that the other non-SET domain methyltransferase (Dot1) possesses an α/β fold structure that is employed by other SAM-dependent methyltransferases whose substrates are not histone lysines (131).

It was initially found that WDR5, RbBP5, Ash2L, and DPY-30 were capable of methylating an H3 peptide that was unmodified at the lysine 4 position. However, when the proteins were incubated with an H3 peptide that had been mono-, di-, or trimethylated on lysine 4, there was no observable methylation reaction (149). This same lab followed up with a report indicating that WDR5, RbBP5, and Ash2L are the minimal complex needed for methyltransferase activity, that DPY-30 adds increased specificity when included in the reactions, and that the WDR5/RbBP5/Ash2L/DPY-30 complex methyltransferase activity is zinc dependent. Additionally, it was demonstrated that this activity is limited to H3/H4 tetramer, as nucleosomal H3 is unable to be methylated without the addition of MLL1 (151).

Alternatively, it has been suggested that Ash2L in combination with a peptide from RbBP5 is the minimal required complex needed to observe histone methyltransferase

activity (23). Similar experiments have been published previously (even by the same author) that demonstrate Ash2L and RbBP5, both in combination with MLL1 and without MLL1, possess no histone H3 methyltransferase activity (46, 151). Thus, the intrinsic histone methyltransferase capability of a WDR5/RbBP5/Ash2L/DPY-30 complex, the minimal components to carry out the enzymatic reaction, any physiological relevance for methyltransferase activity by this complex, as well as the role this complex plays as part of the MLL1 methyltransferase complex are all lines of questioning that need further investigation.

MLL1 is a Regulator of the Hox Genes

There exist several clusters of Hox genes in the human genome that are involved in anterior to posterior embryonic development. These genes were discovered in *Drosophila* in 1978, and then in vertebrates in the mid to late 1980s (16, 26, 111, 126). It was proposed at that time by Lewis that the Hox genes were an example of colinearity, whereby each gene controlled one body segment (111). This model has been somewhat revised, mostly to include a timing aspect of expression. The finding that the Hox genes are also expressed temporally as they are arranged on the chromosome has led to the hypothesis of temporal colinearity (90).

Hox gene clusters have been a model system for evolutionary biologists, as it provides a compact system for the study of duplication and divergence (57). In *Drosophila*, there is one Hox gene cluster that is split among two genetic loci, where as humans have four distinct Hox clusters, each containing homologous genes. The duplications of, and additions to, the Hox clusters across species have provided a mechanism for the study of evolutionary time and mechanism. One mechanism proposed for the evolution of the Hox gene clusters is called unequal crossover. In this mechanism, a duplication event is followed by incorrect recombination events (i.e. gene 1 recombines with gene 2, giving rise to a chromosome with all three genes) (57). Successive rounds of these events will lead to a cluster of similar genes. How each new gene is then tasked with the development of a distinct body segment may provide an interesting line of work.

The regulation of the Hox cluster is of particular concern as the misexpression of a Hox gene spatially or temporally can lead to developmental defects or embryonic death. It has been discovered that the MLL1 complex is an important regulator of the Hox gene cluster (224, 225). MLL1 fusion proteins have been discovered to play roles in altered Hox gene expression in

mice (10). Knockdown of the MLL1 cleavage protein Taspase I caused a decrease in expression across the HoxA locus (74). Additionally, MLL1 has been shown to associate with specific active Hox genes (129, 130). Thus, it has been established that MLL1 is indeed involved in the proper expression of the Hox genes during development.

CHD8 Exists in Complex with WDR5

Through an affinity purification of CHD8 using anti-CHD8 antibodies, previous work in our lab identified WDR5 as a CHD8 binding protein (199). This interaction was confirmed via an affinity purification of Flag-WDR5 from transfected HEK293 cells followed by Western blotting for CHD8. A direct interaction was demonstrated by the use of a GST pulldown assay with GST-WDR5 incubated with recombinant CHD8 and Western blotting for the presence of CHD8 following extensive washing. It should be noted that in the immunoprecipitation and mass spectrometry of CHD8 associated complex, MLL1 was not among the identified components.

In an independent experiment from another lab, CHD8 was identified as a WDR5 complex component (47). A Flag-WDR5 stable HeLa cell line was generated with the goal of identifying MLL1 complex components. Immunoprecipitation of the Flag-WDR5 and mass spectrometry of the complex components was performed, and CHD8 was indeed identified as a member of a WDR5 complex. Taken together, these studies confirm a direct binding interaction between CHD8 and WDR5 and suggest that CHD8 and MLL1 may bind to WDR5 exclusive of one another.

Materials and Methods

Cell Culture and Reagents

Dulbecco's modified Eagle medium (DMEM) (Invitrogen) with 10% fetal bovine serum (Hyclone) and 1X penicillin-streptomycin-glutamine (Invitrogen) was used to culture NTERA2 cl. D1 (NT2/D1) cells. NT2/D1 cells were cultured at 37°C in 10% CO_2. For ATRA induction of NT2/D1 cells, 5×10^6 cells were placed into a 10 cm diameter dish. Cells were grown for 18 hours, at which point they were treated with 10 μM ATRA dissolved in DMSO while control cells were left untreated. Cells were grown an additional 24 hours before being harvested for RNA

isolation or chromatin immunoprecipitations. SF9 cells were cultured at 24°C in 1X Grace's Insect medium (Invitrogen) containing 10% fetal bovine serum and 1X penicillin-streptomycin-glutamine (Invitrogen).

CHD8 rabbit polyclonal antibodies were previously described (199). The anti-acetyl histone H4 (06-866) and anti-trimethyl histone H3 lysine 27 (07-449) antibodies were purchased from Millipore. The anti-Flag M2 (F3165) antibody and rabbit normal IgG immunoglobulin (I8140) were purchased from Sigma. The anti-RbBp5 (A300-109A) and anti-Ash2 (A300-489A) antibodies were purchased from Bethyl. The anti-WDR5 (22512-100) antibody was purchased from Abcam. The anti-histone H3 (39163) and the anti-trimethyl histone H3 lysine 4 (39159) antibodies were purchased from Active Motif. All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Production of Recombinant Proteins and Protein Interaction Studies

The Bac-N-Blue Baculovirus Expression System (Invitrogen) was used to prepare recombinant baculoviruses containing WDR5, RbBP5, and Ash2L. Primers for the creation of these constructs are listed in Table 2.1. Flag-tagged CHD8 was previously described (199). For protein interaction studies, co-infection experiments were performed utilizing these recombinant baculoviruses. SF9 cells (5×10^6) were infected with the indicated viruses and incubated at 24°C for 3 days before harvesting. Cells were collected by centrifugation at 500 × g for 2 minutes at room temperature. Cell pellets were washed once with cold phosphate buffered saline (PBS) and suspended in 500 µl of IP lysis buffer (0.2 mM EDTA, 1% NP-40, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 20 mM Tris-HCl [pH 7.9]) with 150 mM KCl. Lysates were centrifuged at 20,800 × g for 15 minutes at 4°C. Cleared lysates were then incubated overnight at 4°C with 20 µl of anti-Flag M2 agarose beads (Sigma). Beads were washed 3 times with IP lysis buffer prior to elution with 40 µl of 2X SDS loading dye. Samples were subjected to Western blot analysis using the indicated antibodies.

Chromatin Immunoprecipitation Assay

ChIP experiments were performed essentially as described in the ChIP Assay Kit (Upstate). Approximately 9×10^6 cells per immunoprecipitation were fixed with

Name	Sequence (5'→ 3')
HOXA1 Expression Forward	TCT CCA GCG CAG ACT TTT
HOXA1 Expression Reverse	GTA CTC TCC AAC TTT CCC TG
HOXA1 Expression Probe	/6-FAM/CTG GAT GAA AGT CAA AAG AAA CCC TCC C/IowaBlackFQ/
HOXA2 Expression Forward	GCC TCA GCC ACA AAG AAT
HOXA2 Expression Reverse	GGT CTG CAA AGG TAC TTG T
HOXA2 Expression Probe	/6-FAM/TGT TGG TGT AAG CAG TTC TCA GGC/IowaBlackFQ/
HOXA3 Expression Forward	GAT GGC CAA TCT GCT GAA CCT
HOXA3 Expression Reverse	TTA GCA TGC CCT TGC CCT TCT
HOXA3 Expression Probe	/6-FAM/TTC CAG AAT CGC CGC ATG AAG TAC AAA AA/IowaBlackFQ/
HOXA4 Expression Forward	CAT GTC AGC GCC GTT AA
HOXA4 Expression Reverse	GGG TCA GGT ATC GAT TGA AG
HOXA4 Expression Probe	/6-FAM/CTT AGG CTC CCC TCC GTT ATA ACT GG/IowaBlackFQ/
H1 RNA Expression Forward	GCT TGG AAC AGA CTC ACG
H1 RNA Expression Reverse	CTG AAT TGG GTT ATG AGG TCC
H1 RNA Expression Probe	/6-FAM/CGA AGT GAG TTC AAT GGC TGA GGT/IowaBlackFQ/
HOXA1 ChIP Forward	TTC CAG GAG GGT CTT CGA AAC
HOXA1 ChIP Reverse	GCC CCT CCA AGT CGA ATT ACA
HOXA2 ChIP Forward	CAG ACC GAG AGA GAT CAG TTT TGA
HOXA2 ChIP Reverse	AAG ATT TTG GTT GGG AAG GG
HOXA3 ChIP Forward	TGG GAA ACT CAC TTT TCT TGG T
HOXA3 ChIP Reverse	GGA ATT CGT TTG AGA AAC TTC G
HOXA4 ChIP Forward	GGA TCT GCG GTT GAG AAA ATG
HOXA4 ChIP Reverse	AGG CTA ACA GGC GAA AGG AAG
IL2 NheI Forward	GAC TGC TAG CGA AGA TGG ATT CAT ACC TGC
IL2 XbaI Reverse	AGC TCT AGA TTG TTC TTC TAC TCT TCC

Table 2.1 Primers Used in ChIP and Expression Experiments and in Cloning

2.5% formaldehyde for 10 minutes at 37°C. Cells were washed with phosphate-buffered saline and lysed in ChIP lysis buffer. The lysate was cleared by centrifugation, and the chromatin was sheared by sonication (~200-1000 bp fragments). Samples were precleared with protein A agarose (Repligen) blocked with salmon sperm DNA. The indicated antibodies or protein A purified normal rabbit serum were added and incubated overnight at 4°C. Antibody/chromatin complexes were precipitated by incubation with protein A agarose blocked with salmon sperm DNA. Samples were washed extensively washed for 30 minutes each in Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 0.2 mM PMSF, 150 mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 0.2 mM PMSF, 500 mM NaCl), LiCl Immune Complex Wash Buffer (250 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.1) one time each, followed by two 30 minute washes in TE Buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.2 mM PMSF). Samples were then eluted from the resin using 100 mM $NaHCO_3$ and 1% SDS. Crosslinking was reversed by overnight incubation at 65°C. Following Proteinase K treatment, DNA was recovered via Phenol/Chloroform extraction and ethanol precipitation.

RT-PCR and Quantitative PCR

For preparation of cDNA, total RNA was isolated from the indicated cells using the RNeasy Kit (Qiagen) following the manufacturer's instructions. Reverse transcriptase reactions employed random decamers (Ambion) and Superscript II (Invitrogen) following the manufacturers' protocols. Real time quantitative PCR analysis employed the indicated primers (Table 2.1), the iQ SYBR Green Supermix or iQ Supermix (Bio-Rad), and a MyiQ Single color Real-Time PCR Detection System (Bio-Rad). All real time PCR reactions were performed in triplicate. For RNA analysis, C_t values were normalized to the levels of the Pol III transcribed H1 RNA (114). For ChIP experiments, DNA quantities were expressed relative to input.

RNAi Knockdown Experiments

RNAi experiments in NT2/D1 cells utilized the UI2-SIBR shRNA expression vectors (32) modified to express the extracellular alpha subunit of the human interleukin 2 receptor as a selection marker (see Table 2.1 for primer sequences). CHD8 and control shRNA cassettes were

described previously (199). The indicated constructs (10 μg) were transfected into NT2/D1 cells in 10 cm dishes using lipofectamine 2000 (Invitrogen) as described by the manufacturer. Twenty four hours post-transfection, cells were either treated with ATRA or not (as indicated) and grown for an additional 24 hours before harvesting. Cells were trypsinized thoroughly and resuspended in DMEM. 20 μL of Dynabeads CD25 slurry (Invitrogen) were added to each tube and rotated at 4°C for 30 minutes. Following two 10 minute PBS washes, the cells were either lysed on the beads for RNA extraction or Western blotting. For ChIP experiments, the cells were cross linked on the beads in 2.5% formaldehyde for 10 minutes.

Methyltransferase Assay

In vitro methyltransferase experiments were performed using WDR5, RbBP5, and Ash2L that were purified as described above. Reactions were carried out in MTase Buffer consisting of 50mM Tris, pH 8.5, 150mM NaCl, 3 mM DTT, 5 mM $MgCl_2$, and 5% glycerol. Each reaction contained 2 μCi ^{3}H-S-Adenosyl Methionine (SAM), 2 μg of histone substrate, and 4 μg of WDR5/RbBP5/Ash2L complex. Reactions were carried out at 30°C for 2 hours and were stopped with 1X concentration of SDS Loading buffer. Half of each reaction was then loaded onto a 4-12% Bis-Tris gel (Invitrogen), Coomassie-stained, and imaged. Gels were then soaked in DMSO twice for 20 minutes. Gels were then soaked in a 1M solution of 2,5-diphenyloxazole (PPO, Sigma) in DMSO for one hour, at which point they were rinsed at least three times in water and dried under vacuum at 80°C for one hour then overnight at room temperature. Gels were exposed to film at -80°C for 18 days, at which point the film was developed.

Results

CHD8 Forms a Complex with the Core WDR5/RbBP5/Ash2L Complex

As described above, it was reported that CHD8 is found in a WDR5-containing complex (47), and WDR5 was also found in CHD8 containing complexes following an anti-CHD8 immunoprecipitation (199). WDR5 became a focus of that study as a result of its involvement and connection to the MLL family of proteins. It was hypothesized that CHD8 and MLL1, an

ATP-dependent remodeling enzyme and a histone methyltransferase enzyme, could cooperate to establish chromatin environments and control transcriptional output.

Indeed, an interaction between CHD8 and WDR5, both *in vivo* and *in vitro* was observed (199). As WDR5 is generally seen in complex with Ash2L and RbBP5, and as WDR5 is known to bridge the interaction between MLL1 and RbBP5, we asked whether CHD8 could also interact with RbBP5 and Ash2L within the context of the WDR5/RbBP5/Ash2L complex. This potential association was tested through the reconstitution of the complex using a baculovirus expression system. As previously reported, co-infection of SF9 cells with recombinant viruses that encode WDR5, RbBP5, and Ash2L results in the formation of a stable complex, and this complex can also form with the C-terminal fragment of MLL1 (46). Co-infection of SF9 cells with WDR5, RbBP5, Ash2L, and CHD8 followed by affinity purification demonstrated that indeed CHD8 can associate with the trimeric subcomplex (Figure 2.1).

As discussed previously, the assembly of the MLL1/WDR5/RbBP5/Ash2L complex occurs in a specific stepwise manner, with complex formation unable to occur in the absence of WDR5 (8, 46, 148, 182, 187). Because CHD8 is also able to form a complex with WDR5, RbBP5, and Ash2L, the requirements for each complex member for the assembly of the remaining complex was tested. Further baculovirus co-infection experiments were performed, each time omitting one component. As shown in Figure 2.1, the removal of any one of the subunits does not preclude a stable association of the remaining subunits, although omission of Ash2L results in a slightly diminished quantity of associated RbBP5.

Because the formation of the CHD8/WDR5/RbBP5/Ash2L complex assembles in a manner that is distinct from the assembly of the MLL1 complex, it was hypothesized that CHD8 would be able to interact with WDR5, RbBP5, and Ash2L each on an individual basis, and that the association of each complex member with CHD8 would not depend on the presence of another protein from the complex. To test these interactions, pairwise baculovirus co-infection experiments were performed. As shown in Figure 2.2, CHD8 is indeed capable of interacting directly with WDR5, RbBP5, and Ash2L. These results demonstrate that CHD8 has extensive contacts with the WDR5/RbBP5/Ash2L complex, unlike the interactions with MLL that are dependent on WDR5. The precise means by which these interactions occur, including interacting domains between each protein, remains to be elucidated.

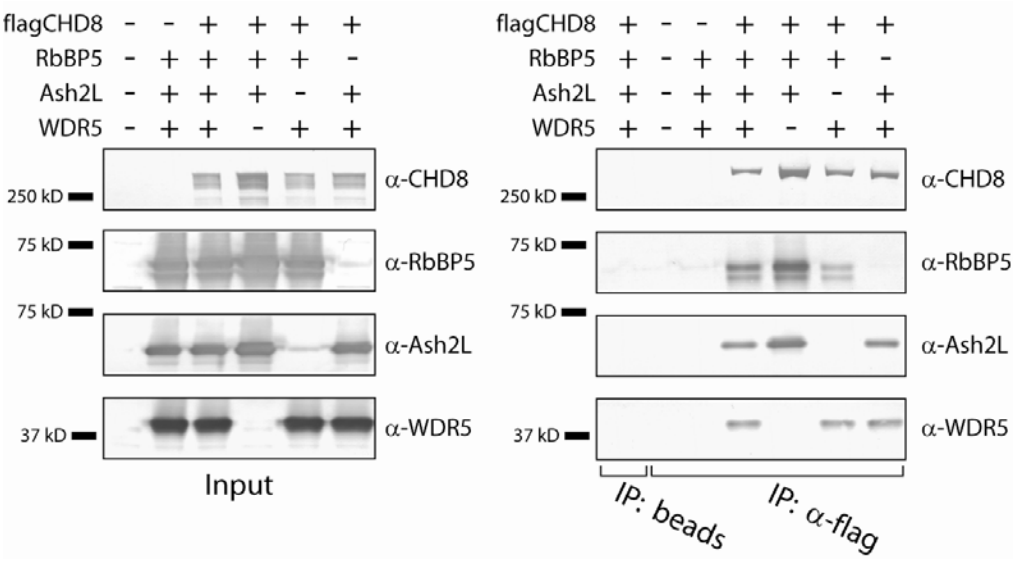

Figure 2.1 CHD8 Directly Interacts with the Core WDR5/Ash2L/RbBP5 Complex

Cellular extracts were prepared from SF9 cells following co-infection with the indicated viruses. Immunoprecipitations were performed with anti-Flag-M2 or control beads. After extensive washing, purified samples were subjected to SDS-PAGE followed by Western blotting analysis using the indicated antibodies.

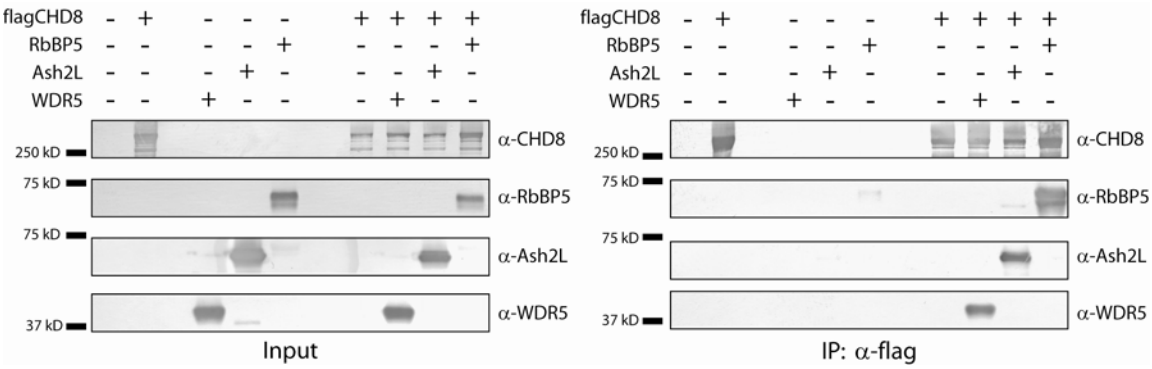

Figure 2.2 WDR5, Ash2L, and RbBP5 Interact with CHD8 Independently of Each Other

Cellular extracts were prepared from SF9 cells following pairwise co-infection with the indicated viruses. Immunoprecipitations were performed with anti-Flag-M2 or control beads. After extensive washing, purified samples were subjected to SDS-PAGE followed by Western blotting analysis using the indicated antibodies.

CHD8 is Bound to the Promoters of the HoxA Genes

Previous studies have demonstrated that WDR5 is recruited to the Hox locus and is an important regulator of Hox gene expression (46, 47, 187, 217). We therefore hypothesized that CHD8 is also recruited to the Hox locus where it could function in conjunction with WDR5, RbBP5, and Ash2L in the regulation of transcription. A relevant analysis would include conditions involving the activation of Hox gene expression, comparable to those encountered during cellular differentiation, when the Hox genes are most actively transcribed. We thus chose to study Hox gene regulation in the NT2/D1 embryonal carcinoma cell line. Treatment of NT2/D1 cells with all-trans retinoic acid (ATRA) induces the expression of many genes, including the Hox genes (16, 20, 73, 108, 124, 190), and ultimately induces cellular differentiation into neuronal-like lineages (4, 156, 158). As shown in Figure 2.3A, treatment of NT2/D1 cells with ATRA results in over 500 fold activation of HOXA1, and approximately 75, 10, and 4 fold activations of HOXA2, A3, and A4, respectively[1]. This system therefore presents an opportunity to study the transcriptional regulation of the Hox genes under highly activating conditions, including any potential roles of CHD8.

To test the hypothesis that CHD8 is indeed cooperating with WDR5, RbBP5, and Ash2L in the transcription regulation of these genes, we first tested whether or not CHD8 is bound to Hox promoters in NT2/D1 cells using chromatin immunoprecipitation (ChIP) assays. Due to the change in expression of these HoxA genes upon treatment with ATRA, it is reasonable to suspect that proteins involved in the regulation of these genes would also associate with these promoters in a dynamic manner following ATRA treatment. Prior to induction, we found CHD8 is indeed bound to the HOXA1 and HOXA4 promoters, and to a lesser extent, the HOXA2 and HOXA3 promoters (Figure 2.3B). We next compared the occupancy of CHD8 at the HOXA1 through HOXA4 promoters before and after ATRA treatment. As shown in Figure 2.3C, 24 hr treatment with ATRA causes a striking alteration in the localization of CHD8 at the HOXA1 through HOXA4 promoters, with the greatest change being the approximately 20 fold increase of CHD8 at the HOXA2 promoter.

[1] It is important to note that this particular experiment was done using the less stringent two primer SYBR green-based qPCR assay, as the more specific three primer Taqman-based qPCR assay is unable to detect appreciable levels of HoxA gene transcription in the absence of inducer.

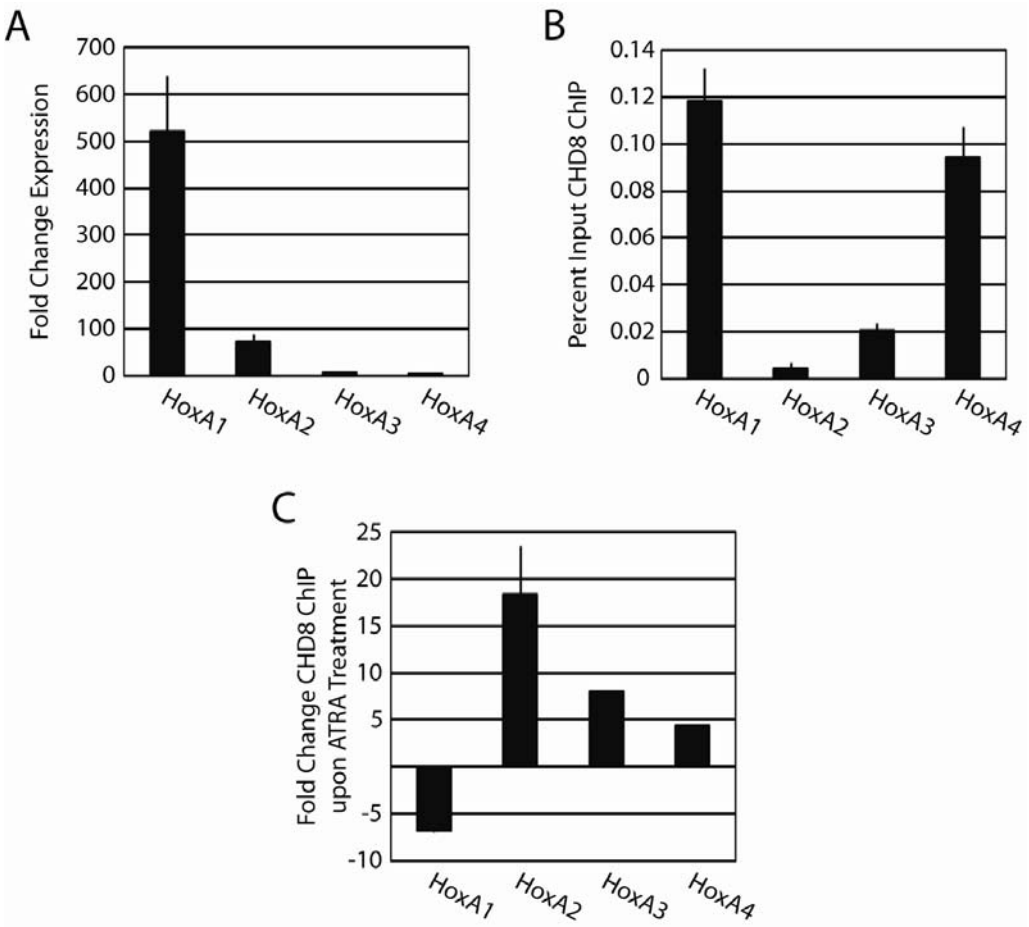

Figure 2.3 CHD8 is Bound to the HoxA Gene Cluster

(A) NT2/D1 cells were treated with ATRA for 36 h. Cells were harvested and RNA was isolated. Expression of the indicated genes was analyzed by RT-PCR. (B) Chromatin from NT2/D1 cells was crosslinked with formaldehyde. Cells were lysed, and ChIPs were performed with anti-CHD8 antibodies. Bound DNA was detected by quantitative PCR with primer pairs to the promoter region of the indicated gene. (C) ChIP experiments were performed using NT2/D1 cells that had been treated with ATRA or not. Results are expressed relative to untreated cells. Control IgG precipitated samples in all experiments were less than 0.001% of input and not shown.

Given the dynamic association of CHD8 with these promoters, this result suggests that CHD8 is indeed functioning in gene regulation at the HoxA locus. It is important to note that WDR5, RbBP5, and Ash2L have also been found to directly bind the HOXA2 promoter (77, 104). The finding that CHD8 also localizes to the HOXA2 promoter, in combination with the data demonstrating a direct interaction of CHD8 with the WDR5/RbBP5/Ash2L complex (Figure 2.1), lends credence to the hypothesis that CHD8 is interacting with the WDR5/RbBP5/Ash2L complex at this promoter to regulate Hox gene expression.

CHD8 Regulates HoxA Gene Expression

To further test this hypothesis, the effect of CHD8 upon the regulation of the HoxA locus was directly monitored using an shRNA strategy to deplete endogenous CHD8 from the cells. The transcriptional output of HOXA1-A4 were then measured and compared to control cells in which CHD8 was still present. NT2/D1 cells were transfected with control shRNA constructs or shRNA constructs targeting CHD8. Importantly, these vectors also co-express the extracellular portion of the IL2 receptor (IL2Rα/CD25 subunit). This allows for the efficient and precise selection of transfected cells using magnetic beads bound to an anti-IL2Rα monoclonal antibody. cDNA was produced from transfected cells for analysis by qPCR. Using this selection protocol, we were unable to detect measureable levels of HoxA gene expression in the absence of ATRA; therefore, all experiments were performed with ATRA treatment.

As shown in Figure 2.4A, depletion of endogenous CHD8 results in an increased expression of HOXA2, while the expression of HOXA1, 3, and 4 does not change in a statistically significant manner. The efficacy of CHD8 knockdown is demonstrated via Western blot in Figure 2.4B. It is important to compare the increase of HOXA2 transcription in Figure 2.4A to Figure 2.3C which illustrates that upon ATRA treatment, the HOXA2 promoter showed the greatest increase in CHD8 occupancy. Taken together, these data demonstrate that CHD8 indeed functions in the regulation of HOXA2 gene expression. Furthermore, under activating conditions at the HOXA2 promoter, the normal function of CHD8 appears to be the negative attenuation of HOXA2 gene transcription. This negative effect of CHD8 on HOXA2 expression is consistent with our prior studies of CHD8 and β-catenin mediated transcription where depletion of CHD8 results in an increase in target gene expression under activating conditions (199).

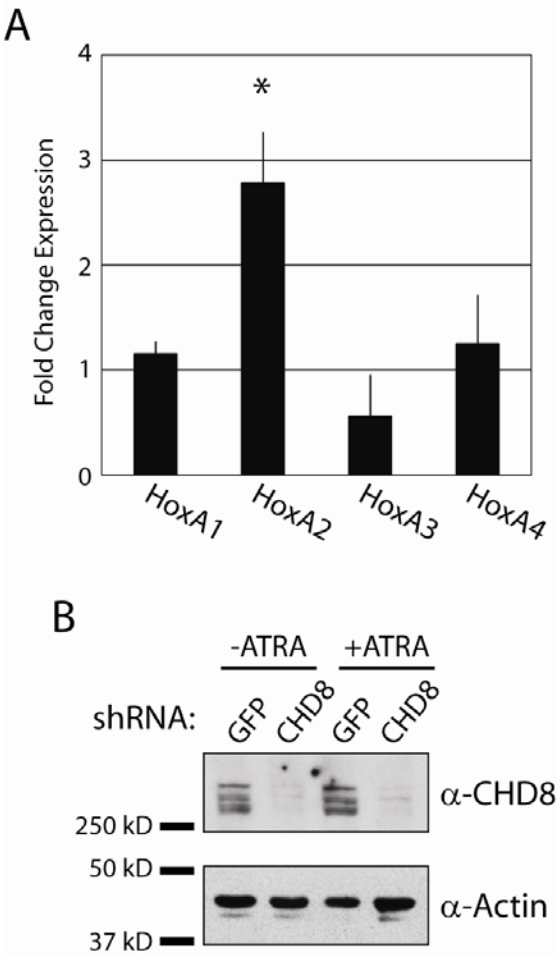

Figure 2.4 CHD8 Regulates Expression of the HoxA Gene Cluster

(A) NT2/D1 cells were transfected with control shRNA or CHD8 shRNA and induced with ATRA. Following selection, cells were harvested and total RNA was isolated. Expression of the indicated genes was analyzed by RT-qPCR. Results are normalized first to a reference RNA and then to the control shRNA transfection. *P < 0.03 by Student's t-test. (B) Western blot of control transfected shRNA (GFP) or CHD8 transfected shRNA cellular lysate. Actin was used as a loading control.

CHD8 is Required for Recruitment of the WDR5/RbBP5/Ash2L Complex

Having demonstrated that CHD8 interacts with WDR5, RbBP5, and Ash2L, and that CHD8 is bound to the promoter of and regulates HOXA2 gene expression, we hypothesized that CHD8 plays a cooperative role with WDR5, RbBP5, and Ash2L in the regulation of gene expression. Thus, it is possible that CHD8 is required for the proper recruitment of WDR5, RbBP5, and Ash2L to target genes.

We tested this hypothesis via knockdown of CHD8 in combination with chromatin immunoprecipitation. In our hands, antibodies to Ash2L were the most suitable for these ChIP experiments, as solidly reproducible ChIP signal was not obtainable with the WDR5 and RbBP5 antibodies that were available. As expected, upon treatment with control shRNA, we are able to detect Ash2L at the HOXA2 promoter. However, when CHD8 is depleted via shRNA treatment, Ash2L is lost from the HOXA2 promoter (Figure 2.5). Because Ash2L has been demonstrated to be vital for WDR5, RbBP5, and Ash2L complex function (46, 187), the absence of Ash2L is indicative of a lack of functional WDR5/RbBP5/Ash2L complex at that gene. This result is strong evidence for the requirement of CHD8 for proper WDR5, RbBP5, and Ash2L complex recruitment and function.

CHD8 Affects Methylation Patterns at the HOXA2 Promoter

As mentioned previously, the WDR5, RbBP5, and Ash2L complex is known to associate with many members of the SET1 family of histone H3 lysine 4 methyltransferases, playing an important regulatory role in gene expression, including transcription at the Hox loci (69). We demonstrate above that CHD8 is not only able to interact with the WDR5/RbBP5/Ash2L trimer (Figure 2.1), but it is also required for the proper recruitment of this complex to target genes (Figure 2.5). CHD8 is also recruited to and regulates expression of HOXA2 in NT2/D1 cells upon treatment with ATRA (Figures 2.4 and 2.5). These data together suggest that CHD8 may function by regulating histone methylation patterns at target genes.

In support of this hypothesis, kismet, the *Drosophila* orthologue of CHD8, has recently been demonstrated to have a dramatic effect upon global methlyation patterns in *Drosophila* salivary gland polytene chromosomes (185). Upon mutation of kismet, an approximately 7-fold increase in global lysine 27 trimethylation of histone H3 (H3K27me3) was demonstrated,

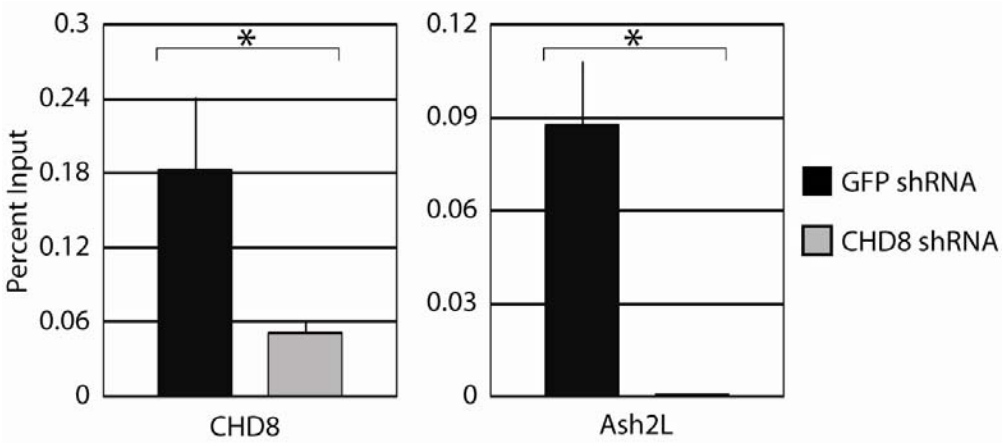

Figure 2.5 CHD8 is Required for the Recruitment of Ash2L to the HOXA2 Promoter

NT2/D1 cells were transfected with control shRNA or CHD8 shRNA and treated with ATRA. Following selection of the transfected cells, cells were cross linked using formaldehyde. Cells were then lysed, following which ChIP experiments were performed using the indicated antibodies. Bound DNA was detected by quantitative PCR with primer pairs to the promoter region of HOXA2. Control IgG precipitated samples were less than 0.001% of input and therefore are not shown. *$P < 0.05$ by Student's t-test.

without a significant change in lysine 4 trimethylation of histone H3 (H3K4me3). It was proposed that kismet is acting to counteract H3K27me3, reversing Polycomb group repression.

We performed chromatin immunoprecipitations with H3K4me3 and H3K27me3 antibodies to look at changes in methylation at the HOXA2 promoter upon treatment with ATRA (Figure 2.6). Trimethylation of H3K4 is typically associated with transcriptional activation while H3K27 trimethylation is associated with repression. Therefore, as expected upon activation of HOXA2 gene expression with ATRA, we observe the recruitment of CHD8 accompanied by a significant increase in H3K4me3 and a significant decrease in H3K27me3. Indeed, H3K4me3 levels at the HOXA2 promoter increased by nearly tenfold and H3K27me3 levels decreased over three fold at this time point with ATRA induction.

To test if these ATRA-induced changes in histone methylation at the HOXA2 promoter are dependent upon CHD8, we performed these same chromatin immunoprecipitation experiments after depletion of CHD8 by shRNA and treatment with ATRA. As shown in Figure 2.7A, depletion of CHD8 results in the expected loss of CHD8 associated with the HOXA2 promoter. Perhaps unexpectedly, we found a significant decrease in both H3K4me3 and H3K27me3 levels at the HOXA2 upon depletion of CHD8. Both H3K4me3 and H3K27me3 levels at the HOXA2 promoter decreased approximately three fold compared to the control shRNA treatment. These results are in contrast to the previously mentioned studies on *Drosophila* kismet, suggesting human CHD8 and *Drosophila* kismet function via differing mechanisms to regulate gene expression.

One possible explanation for the simultaneous decrease in both K4 and K27 trimethylation is that CHD8 functions in histone deposition at the HOXA2 promoter, i.e. the loss of observed methylation is due to loss of total H3 at this promoter. To address this, we again performed chromatin immunoprecipitation experiments after depletion of CHD8 by shRNA and treatment with ATRA. As shown in Figure 2.7B, depletion of CHD8 does not result in loss of total H3 at this promoter. Conversely, H3 occupancy at the HOXA2 promoter increases upon CHD8 depletion. This result suggests that the regulation of transcription by CHD8 occurs via multiple histone methylation/demethylation events at the promoters of target genes.

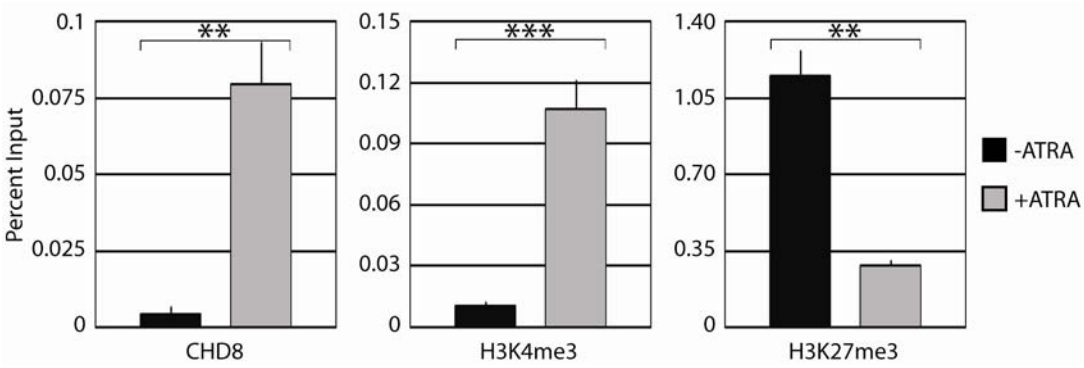

Figure 2.6 H3K4me3 and H3K27me3 Levels Change with ATRA Treatment

ChIP experiments using NT2/D1 cells that were either treated with ATRA or not were performed with the indicated antibodies. Bound DNA was detected by quantitative PCR with primer pairs to the promoter region of the HOXA2 gene. ** = $P<0.01$ by Student's t-test, *** = $P<0.001$ by Student's t-test.

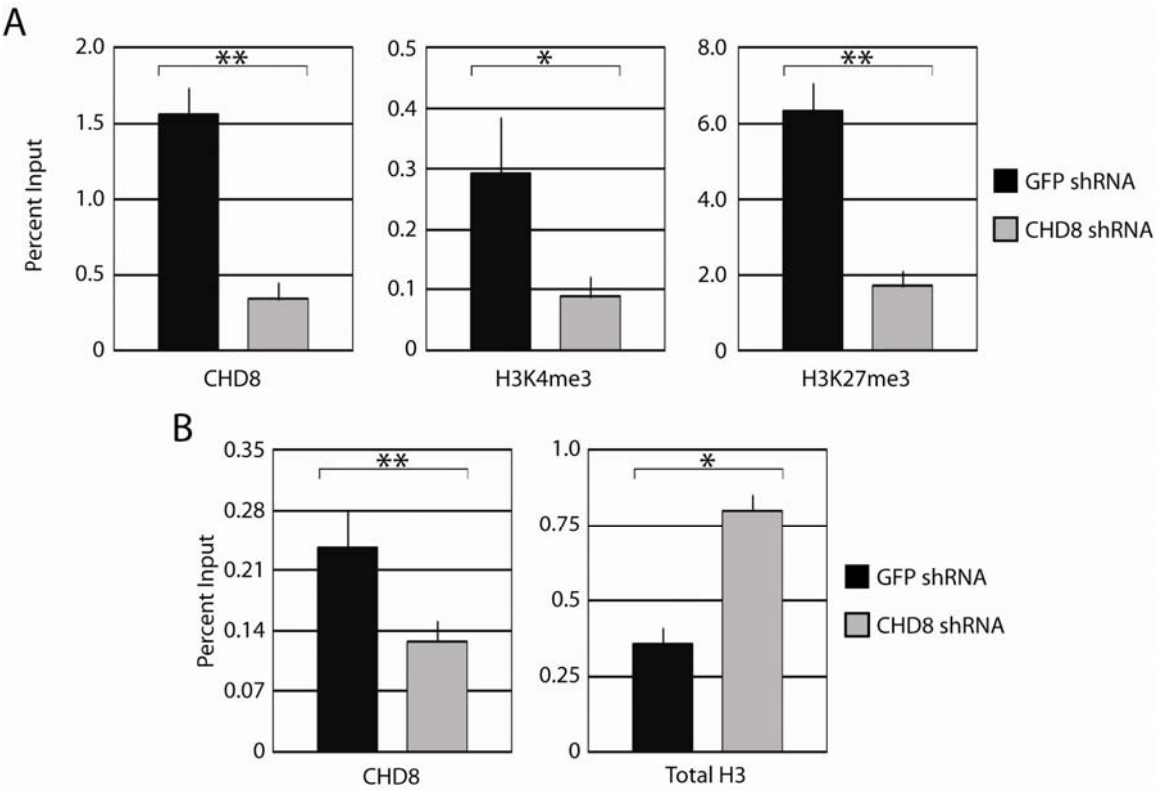

Figure 2.7 CHD8 Affects H3K4 Trimethylation at the HOXA2 Promoter

(A) NT2/D1 cells were transfected with control shRNA or CHD8 shRNA and treated with ATRA. Following selection of the transfected cells, cells were cross linked using formaldehyde. Cells were then lysed, following which ChIP experiments were performed using the indicated antibodies. Bound DNA was detected by quantitative PCR with primer pairs to the promoter region of HOXA2. (B) NT2/D1 cells were transfected, selected, and ChIPs were performed as in A using the indicated antibodies. Control IgG precipitated samples were less than 0.001% of input and therefore are not shown. * = $P<0.05$ by Student's t-test, ** = $P<0.01$ by Student's t-test.

WDR5/RbBP5/Ash2L Complex Methylates Histone H3 Tail

In light of several recent reports demonstrating a methyltransferase activity for the non-SET domain containing WDR5/RbBP5/Ash2L complex (23, 149, 151), we sought to test the activity of this complex on whole histone H3 substrates. In addition, we employed a series of H3 mutants wherein various lysine residues commonly modified had been mutated to cysteines (178). Using these histone H3 substrates, we sought to identify the specific residue that the WDR5/RbBP5/Ash2L complex is capable of methylating, as mutation from lysine to cysteine will abolish methyltransferase activity. It should be noted that at the time these experiments were performed, the WDR5/RbBP5/Ash2L methyltransferase activity had only been demonstrated on H3 tail peptides.

SF9 cells were coinfected with baculoviruses for flag-WDR5, RbBP5, and Ash2L followed by an affinity purification to isolate the complex. A mock purification was also done to control for any insect proteins that could be purified using the affinity beads. Purified PRMT1, an H4 methyltransferase (216), was used as a positive control. The reconstituted complex was then incubated in the presence of tritiated SAM with either HeLa core histones, H3, H3K4C, H3K9C, H3K27C, or H3Δ1-28. In addition, because of the demonstration of a CHD8/WDR5/RbBP5/Ash2L complex, we incubated CHD8 with the recombinant complex to test the possibility that the WDR5/RbBP5/Ash2L complex is able to modify other proteins with which it associates.

As shown in Figures 2.8 and 2.9, the recombinant WDR5/RbBP5/Ash2L complex is indeed an H3 specific methyltransferase, although it is unable to use CHD8 as a methyltransferase substrate. In addition, the histone methylation takes place on the N-terminal tail of histone H3, as removal of the tail abolishes all methyltransferase activity (Figure 2.8, ΔH3 lane). The individual lysine to cysteine mutation that had the largest effect on methylation activity was lysine 9, although mutation of lysine 4 affected activity to a lesser degree. Importantly, mutation of lysine 27 to cysteine resulted in methyltransferase activity equal to that of the wild type H3 substrate. This combination of results suggests that the WDR5/RbBP5/Ash2L complex primarily targets H3K9 for methylation, and to a lesser extent targets H3K4. Thus, WDR5, RbBP5, and Ash2L together form an H3K4 and H3K9 methyltransferase.

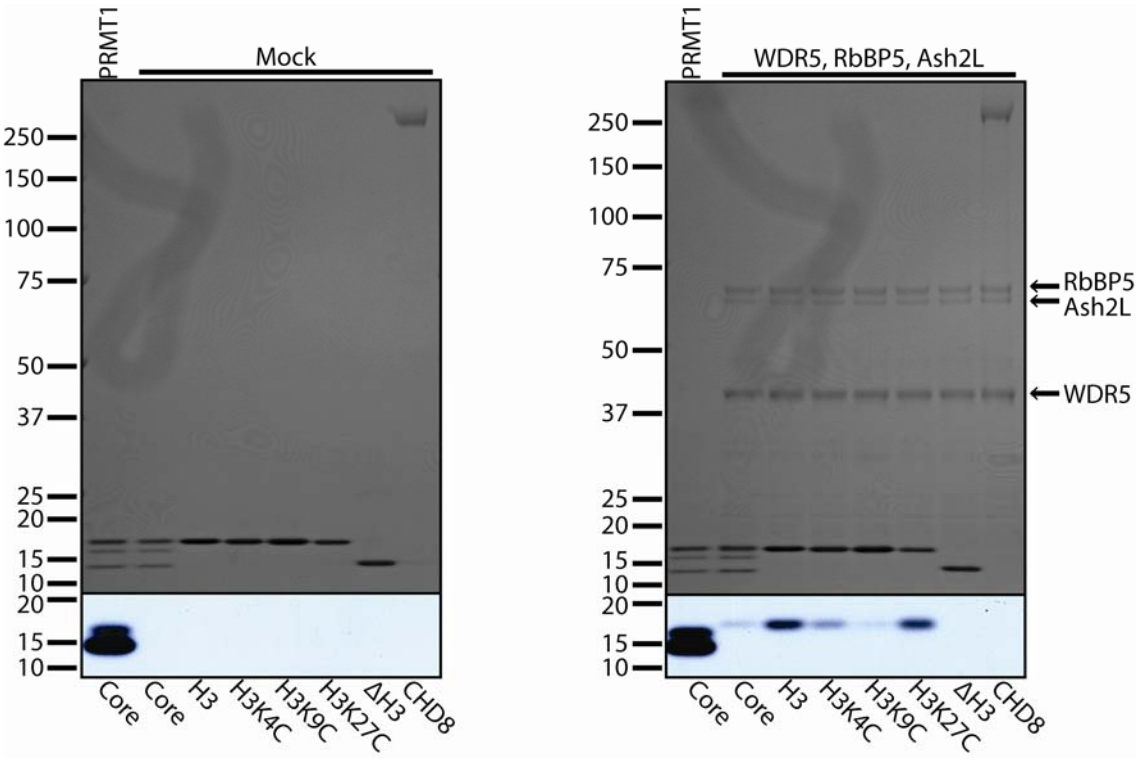

Figure 2.8 WDR5, RbBP5, and Ash2L Comprise an H3K4 and H3K9 Specific Methyltransferase

Methyltransferase assays were performed using the indicated substrates and either the affinity purified Flag-WDR5/RbBP5/Ash2L complex from baculovirus infected SF9 cells (right) or material purified from mock infected cells (left). Reactions were subjected to SDS-PAGE and Coomassie stained to visualize proteins (Top) followed by 18 day exposure for fluorography to visualize ^{3}H incorporation (Bottom). The ΔH3 histone is lacking the first 28 N-terminal amino acids. PRMT1 was used as a positive control for methyltransferase activity.

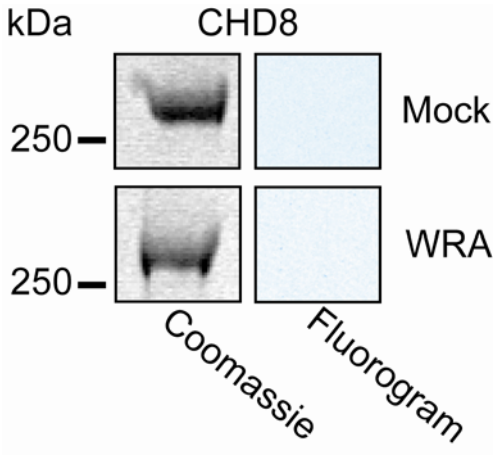

Figure 2.9 WDR5, RbBP5, and Ash2L Cannot Methylate CHD8

Methyltransferase assays from Figure 2.8 are again shown, with sections of the fluorograms containing CHD8 now displayed for visualization of ^{3}H incorportation.

Discussion

We have demonstrated that CHD8 is able to bind to the WDR5/RbBP5/Ash2L complex. Additionally, CHD8 is also able to bind each complex member individually. CHD8 is present at the promoters of the HOXA1-4 genes and relocalizes upon gene activation. We have shown a role for CHD8 not only the expression of HOXA2, but in the binding of the WDR5/RbBP5/Ash2L complex to, as well as the proper methylation of, the HOXA2 promoter. Finally, we demonstrate an H3K4 and K9-specific methyltransferase functionality for the WDR5/RbBP5/Ash2L complex.

Demonstration of an ATP-dependent chromatin remodeling enzyme binding to a complex that is involved in the histone methyltransferase activity of MLL1 is a novel and interesting finding. We have shown that CHD8 is able interact with the trimeric WDR5/RbBP5/Ash2L complex as well as with WDR5, RbBP5, and Ash2L individually (Figures 2.1 and 2.2). This is unlike the reported interaction between WDR5, RbBP5, Ash2L, and MLL1, which is mediated by WDR5 alone (46, 187). Indeed, recent reports demonstrate that a single 6-13 amino acid motif of MLL1 mediates the interaction of MLL with WDR5, suggesting the requirement of WDR5 for complex stability (148, 150, 182). It should be noted that a recent study suggests more extensive interactions may exist between the WDR5/RbBP5/Ash2L complex and MLL1 as the catalytic activity of the isolated MLL1 SET domain is stimulated by RbBP5 and Ash2L in the absence of WDR5 (183). In further support for a different mechanism of binding between CHD8 and WDR5, RbBP5, and Ash2L, CHD8 does not seem to possess the conserved *Win* peptide motif used by WDR5 to bind MLL1. The precise means by which CHD8/WDR5/RbBP5/Ash2L interactions occur, including interacting domains between each protein, remains to be elucidated.

It should also be noted that the evidence presented in Figures 2.1 and 2.2 could be made stronger by the inclusion of additional controls. Please note that the inclusion of flag-CHD8 in the experiment results in the pulldown of every other protein added throughout the series of experiments. The conclusions regarding the specificity of these interactions could be enhanced by the inclusion of a related protein that, when included in the infections, did not pulldown with CHD8. Proteins that could be used for this purpose include other WD40 domain-containing proteins or even other chromatin-related proteins such as DNA repair pathway proteins. Alternatively or additionally, demonstration of specificity between CHD8 and the WDR5, RbBP5, and Ash2L complex would be strengthened by the use of a Flag-tagged protein

that does not assemble the complex, or one that does not assemble the entire complex. For example, the catalytic subunit of the SWI/SNF complex, Brg1, or the MLL1-C construct that has been shown previously to depend on WDR5 for complex formation, could have been used.

Next we examined whether CHD8 is modulating chromatin structure at the same targets that MLL1 is known to covalently modify, specifically the Hox locus. We have shown here that CHD8 is indeed recruited to the HOXA2 promoter and regulates HOXA2 gene expression (Figures 2.3 and 2.4). The possibility exists that CHD8 and MLL1, bridged by WDR5, RbBP5, and Ash2L, are working together to regulate expression at the Hox locus. Alternatively, it is possible that modification of H3K4 by the MLL1 complex may serve as a mark to reinforce CHD8 localization. This hypothesis is supported by work that demonstrates the chromodomains of CHD8 can specifically bind to dimethylated H3K4 (161). For in depth discussion of the CHD8 chromodomains please refer to Chapter Three.

Although these studies focused on the role of CHD8 in the regulation of HOXA2, additional roles for CHD8 should be considered. The Hox genes are expressed in a temporal, collinear manner (57). All of these studies were carried out following 24 hours of induction with ATRA. It is quite possible that CHD8 plays a role in the expression of many or all of the Hox genes, but it does so as the genes are activated. Performing further studies using shorter and longer timepoints (perhaps 12, 24, 36, 48, and 60 hours, for example), would lead to an enhanced understanding of CHD8's role in the expression of the Hox genes.

There are a total of 39 Hox genes in mammals. These 39 genes are grouped into 13 homologous sets and are spread out across four Hox clusters with each Hox cluster containing between 9 and 11 Hox genes (48, 60). These studies examined HOXA1-A4 only. There is potentially much to be learned by combining an increased temporal study mentioned above with a more system-wide focus on the role of CHD8 in Hox gene expression.

The demonstration that CHD8 interacts with WDR5, RbBP5, and Ash2L suggested that CHD8 may be involved in the recruitment of the WDR5/RbBP5/Ash2L complex to the HOXA2 promoter. We show here that CHD8 is indeed required for the recruitment of Ash2L to the HOXA2 promoter (Figure 2.5). As Ash2L is known to be vital to the function of its complex, it is inferred that a lack of Ash2L also means a lack of WDR5 and RbBP5, or at the very least, the lack of a functioning WDR5/RbBP5/Ash2L complex. Obviously, the lack of ChIP-effective WDR5 and RbBP5 antibodies is a major limitation of this conclusion, and inclusion of these ChIPs would

complete the argument for the requirement of CHD8 for WDR5/RbBP5/Ash2L complex association with the promoter.

Previous studies have demonstrated that MLL1 requires WDR5, RbBP5, and Ash2L for complete methyltransferase activity at target promoters, but loss of WDR5, RbBP5, and Ash2L does not prevent the proper binding of MLL1 (46, 187). Taken together with our data, this suggests a model where CHD8 can function in chromatin remodeling and in the delivery of the WDR5/RbBP5/Ash2L complex to appropriate target genes, therefore allowing the establishment of normal histone methylation events via interaction with histone modifying enzymes, such as MLL1. Future experiments need to be performed to test whether the chromatin remodeling activity of CHD8 is required for the delivery of WDR5, RbBP5, and Ash2L, or whether these events are independently occurring. Also, the presence or absence of MLL1 following knockdown of CHD8 would be useful information in determining the reciprocal regulation of CHD8 and MLL1, if any.

We demonstrate a regulatory role for CHD8 at HOXA2, along with a role for CHD8 in recruitment of Ash2L to the HOXA2 promoter. Using this information, we hypothesized that CHD8 would be important for proper establishment of histone tail methylation patterns at target genes. In support of this hypothesis, depletion of the *Drosophila* orthologue of CHD8, kismet, has been demonstrated to result in an increase in global histone H3 lysine 27 methylation, counteracting the activity of the histone H3 lysine 27 methyltransferase E(Z), and promoting the association of the histone H3 lysine 4 methyltransferases TRX and ASH1 with chromatin (185). Interestingly, purification of the MLL2 and MLL3 complexes has identified UTX, a JmjC domain-containing histone H3 Lysine 27 demethylase, as a component of the complex (30, 104). This finding links WDR5, RbBP5, and Ash2L to histone methylation and demethylation, as well as to the CHD8 chromatin remodeling complex.

Indeed, we were able to demonstrate changes in the methylation pattern of the HOXA2 promoter via chromatin immunoprecipitation of CHD8 depleted cells (Figure 2.7). However, in contrast to the kismet studies, we note a significant decrease in H3K27me3, along with a decrease in H3K4me3. This discrepancy points toward differing mechanisms of action for CHD8 and kismet, which may not be entirely unexpected given the evolutionary distance between flies and humans. Because kismet is the homologue of the entire CHD6-9 subfamily of proteins, it is possible, even likely, that CHD6-9 have evolved differing mechanisms of action over time, perhaps leading to more specific roles within the cell. Additional studies of the CHD6-9

subfamily of proteins need to be conducted to gather a full picture of the regulation of expression of target genes. Are CHD6, CHD7, and CHD9 also in complex with WDR5, RbBP5, and Ash2L? Are they also capable of regulation of the Hox genes? The answers to these questions are of great interest.

It is vital to note that there is a discrepancy in the results of the CHD8 knockdown effect on expression data (Figure 2.4) and the effect observed on promoter methylation status (Figure 2.7). It would be expected that an increase in a gene's level of expression would be accompanied by an increase in the levels of H3K4 trimethylation. It is possible, however, that the promoter uses other histone marks, such as acetylation, as a mechanism to increase expression, and that in the absence of CHD8 and thus proper H3K4 trimethylation, promoter acetylation compensates. Obviously this hypothesis could be easily tested via ChIP for the acetylated histone H4, for example. Other activating histone marks could also be employed in a similar combinatorial or compensatory manner. It is also of interest to note that a gain in total H3 is seen at this promoter following the loss of CHD8. Perhaps remodeling events at the promoter do not occur in the absence of CHD8, resulting in the accumulation of nucleosomes.

Our data here appears to directly contradict that of Rodríguez-Paredes, et al., who have recently shown that depletion of CHD8 has no significant effect on H3K4me2 and H3K4me3 at the CCNE2 promoter (161). However, the CCNE2 gene is cell cycle dependent and would be expressed variably in an unsynchronized cell population. In fact, CCNE2 is only expressed during the G1/S transition (230), and so may only be actively transcribed in a small subset of cells within a population at any given time. Thus, it is possible that any effect of CHD8 on histone methylation at the CCNE2 promoter would be undetectable in this system. Due to the use of an inducible system in which every cell is activating expression of HOXA2, we are able to more clearly see the effects of CHD8 loss upon promoter methylation.

An alternative model arises based on recent work that demonstrates the WDR5/RbBP5/Ash2L complex itself has methyltransferase activity in the absence of MLL1 (23, 149, 151). As shown in Figure 2.8, we have demonstrated the WDR5/RbBP5/Ash2L is primarily an H3K9 and, to a lesser extent, an H3K4 methyltransferase. Also tested was the WDR5/RbBP5/Ash2L complex's ability to methylate CHD8, which it is unable to do (Figure 2.9). This could suggest the possibility that the CHD8/WDR5/RbBP5/Ash2L complex could also function as a histone methyltransferase. In this model, through the combined action of chromatin remodeling and histone modifications, CHD8/WDR5/RbBP5/Ash2L is required for the

proper establishment of histone modifications at target promoters. MLL1 ChIPs and/or knockdown experiments could test this hypothesis. Any effect of CHD8 on the methyltransferase activity of the WDR5/RbBP5/Ash2L complex needs to be tested in future studies. Additionally, WDR5, RbBP5, and Ash2L, in combination or individually, may alter the remodeling activity of CHD8 when in complex with it. These experiments would all provide useful mechanistic details regarding the cooperativity of these enzymes in establishing promoter environments.

The methyltransferase activity of the WDR5/RbBP5/Ash2L complex remains a topic of debate within the chromatin field, however. Several labs have indicated through personal communications that they have been unable to reconstitute histone methyltransferase activity from recombinant WDR5, RbBP5, and Ash2L. Nevertheless, our data support the methyltransferase activity of the complex. Evidence for activity coming from the reconstituted complex rather than a co-purified contaminant is convincing. First, in the Patel et al. follow-up paper (151), they note that each complex member was purified individually from *E. coli*. They also show that each individual component lacks methyltransferase activity on its own. However, when combined, activity is observed. If this activity were due to a co-purified contaminant, it is highly unlikely that the contaminant would only manifest activity in the presence of the reconstituted WDR5/RbBP5/Ash2L complex. Additionally, the WDR5/RbBP5/Ash2L complex that we used in these studies was co-purified from insect SF9 cells. It is unlikely that both groups co-purified H3 specific methyltransferase contaminants from two drastically different systems. The lack of a contaminant could be further strengthened via purification of the methylatransferase activity over a series of protein purification columns, followed by silver staining and mass spectrometry of the resulting complex.

It is interesting to note that the activity identified here for the WDR5/RbBP5/Ash2L complex primarily targets H3K9 for methylation, and to a lesser extent targets H3K4. The only other chromatin modifying enzyme known to target both H3K4 and H3K9 is Lysine-Specific Demethylase 1 (LSD1) (128). This enzyme alters its substrate specificity by association with the androgen receptor. The activity of the WDR5/RbBP5/Ash2L complex may also be directed to H3K4 or H3K9 based on the cofactors associated with the WDR5/RbBP5/Ash2L complex. Alternatively, the activity of this complex may be specified by the substrate presented. For example, the methylation specificity of the histone methyltransferase NSD2 changes from histone H4 to histone H3 upon incorporation of the histones into nucleosomes (112). Further

experiments into the specificity of this complex need to be performed to distinguish between these models.

CHD8 has been demonstrated to function within the cell in multiple contexts and with a variety of binding partners (79, 141, 161, 229). These studies in combination with the current work demonstrate the important, although cryptic, role of CHD8 within the cell. This is ultimately highlighted by a report revealing that the targeted deletion of the first nine exons of mouse CHD8 results in death *in utero* between E5.5 and E7.5 (140). In addition, if MLL1 and CHD8 are working to cooperatively regulate targets genes, the potential exists for erroneous regulation of CHD8 and leukemogenic MLL fusion proteins to exacerbate transcriptional misregulation in cancer. Thus, CHD8 may yet prove to be an effective target for novel therapeutics. Further experiments probing the mechanisms of CHD8 targeting as well as *in vivo* enzymatic activity are needed to understand the cellular role of this important remodeling enzyme.

Chapter Three

The Tandem Chromodomains of CHD8 are Required for Enzymatic Function

Introduction

Protein Domains

In general, a protein domain is a segment of a protein that folds into a compact independent structure and has a unique function in the context of the protein. Proteins are often made up of combinations of various protein domains that are arranged in such a way to create the overall function of the protein. Specific protein domains are often seen in multiple proteins in the cell, and will generally have similar functions from protein to protein (31, 152). This has led to the idea that protein domains are similar to protein building blocks, and that combining different domains in novel arrangements generates proteins with distinct functional characteristics (31).

There are two major types of protein domains: those with catalytic function and those without. Catalytic domains include any domain that catalyzes a chemical reaction in the cell. For example, the Snf2 domain in ATP-dependent remodeling enzymes catalyzes the hydrolysis of ATP to ADP, releasing the energy stored in that bond for use by the rest of the protein (25). A non-catalytic protein domain is one that serves a structural or other role, such as complex assembly, in the context of the protein. An example of this type of domain would be the WD40 domains of RbBP5 and WDR5, whose role is to create surfaces to which other proteins can bind, thus bringing together proteins with catalytic domains to combine functions in unique ways (8, 37).

Chromatin remodeling enzymes exist in large multi-subunit complexes that contain both catalytic and non-catalytic domains. Catalytic functions that are often seen in these complexes include Histone AcetylTransferases (HATs), Histone DeACetlyases (HDACs), SET and non-SET domain-containing Histone MethylTransferases (HMTases), and SNF2 helicases (33, 192). These

various catalytic activities are linked by non-catalytic domains such as bromodomains, PHD/Zn finger domains, SANT domains, and chromodomains. These non-catalytic domains are generally found to act in histone or DNA binding, and they are often thought to target catalytic activity to specific loci in the chromatin environment.

Domains of CHD8

The ATP-dependent chromatin remodeling enzyme CHD8 contains six defined protein domains of four different types. From N- to C-terminus, CHD8 contains two chromodomains, a Snf2 helicase domain, a SANT domain, and two BRK domains (Figure 1.4). The class-defining domain of enzyme is the Snf2 domain. This domain allows for the gathering of energy needed for the enzyme to perform its defined tasks. The SANT domain (Switching-defective protein 3 (Swi3), Adaptor 2 (Ada2), Nuclear receptor co-repressor (N-CoR), Transcription Factor IIIB (TFIIIB)) has been shown to recognize non-acetylated histone tails, and deletion of one of the SANT domains in ISWI can cause loss of catalytic function in this enzyme (18). The BRK (Brahma kismet) domain was identified based similarities observed in a 41 amino acid segment of the *Drosophila* proteins kismet and brahma as well as the human proteins hBRM and BRG1 (CHD6-9 were not yet characterized) (38). However, there has yet to be any functional role identified for the BRK domain. The chromodomain will be discussed in great detail below.

Chromodomains

The Chromo (CHRomatin Organization MOdifier) domain was first identified in sequence comparisons between the *Drosophila* proteins polycomb and HP1 (147). A chromodomain is a conserved region of around 60 amino acids in length and are interesting in that they are found in various arrangements with other domains within a protein structure. These arrangements can be divided into three classes of proteins: those that have a single chromodomain, proteins with an N-terminal chromodomain followed by a chromo shadow domain, and proteins with a tandem repeat of chromodomains.

An example of a protein that has a single chromodomain is Polycomb. Polycomb group proteins are involved in the silencing of HOX genes, in direct opposition to the Trithorax group of activators. Polycomb was found to be recruited to sites of H3K27 methylation, and the

chromodomain of polycomb was subsequently found to interact with methylated H3K27 peptides (24). Polycomb is a component of a large molecular weight complex termed PRC1 that contains both catalytic and non-catalytic functions. Although the mechanism is uncertain, it is generally accepted that PRC1 is directly responsible for the silencing of target genes (177).

An example of a protein that has a single chromodomain followed by a chromo shadow is the Heterochromatin Protein 1 (HP1) protein family. There is one HP1 protein in *Drosophila*, whereas there are three HP1 homologues in humans. The HP1 protein family is involved in gene-silencing and establishment of heterochromatin. The chromodomain of the HP1 family has been extensively studied. Replacement of the chromodomain of *S. pombe* HP1 with the mouse HP1β chromodomain resulted in a fully functioning protein (211), demonstrating a conserved utility for this domain. The chromodomain of HP1 has been shown to be vital to protein function by binding to methylated lysine nine of histone H3 (82, 155). C-terminal to the chromodomain is a closely related chromo shadow domain. This domain is known to dimerize and create a protein binding surface, which recruits other interacting proteins (115).

The final class of chromodomain-containing enzymes is those proteins that contain a set of tandem chromodomains, namely the CHD family of proteins. Elucidation of the roles for the tandem chromodomains of the CHD family, specifically those of yeast CHD1 and human CHD1, has been a target of several groups. It has been demonstrated that the chromodomains of human CHD1 are able to bind specifically to methylated H3K4, while the yeast chromodomains are unable to do so (54, 179). Importantly, recent work has provided a possible role for the yeast CHD1 chromodomains in catalytic regulation via conformational rearrangement of the chromodomains relative to the ATPase domain (66).

There have also been studies to discover the role for the CHD6-9 chromodomains and any potential histone mark that they may be binding. The chromodomains of CHD7 have been shown to interact with both trimethylated H3K4 and trimethylated H3K9 (194). There has been a report of CHD8 chromodomains interacting with H3K4me2 (161). In addition, a partially purified CHD8 containing complex has been shown to interact with a variety of methylated histone peptides, including H3K4 (229). However, these interactions cannot be attributed specifically to the chromodomains of CHD8, nor even directly to CHD8 itself.

The functional role of CHD8 chromodomains in the context of the full length enzyme has yet to be elucidated. As chromodomains have been shown to be histone-binding, the specific role of the CHD8 chromodomains could provide indispensable insight into the targeting,

localization, and/or binding of CHD8 to specific loci. This information will provide the basis for future functional studies of the enzyme.

Materials and Methods

Production of Recombinant Proteins

Constructs for the expression of glutathione S-transferase (GST) chromodomain fusion proteins were cloned into the GST-parallel vectors (169) using PCR full length human CHD1 or CHD8 as a template. GST and GST-chromodomain fusion proteins were expressed using C41(DE3) *E. coli* (132) containing the pRARE plasmid (Novagen). The pRARE plasmid contains tRNAs that are commonly used in human proteins but are less commonly expressed in *E. coli.* Following growth to OD_{600} of 0.4 to 0.6, protein production was induced via addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 16°C with vigorous shaking.

Cells were harvested and resuspended in BC buffer (20 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) plus 100 mM KCl (BC100). Cells were lysed by use of a French pressure cell, and lysates were cleared by centrifugation at 105,000 × *g* for 60 minutes at 4°C. Cleared lysates were flash frozen in liquid nitrogen and stored at -80°C until use. The concentration of GST or GST fusion proteins in the cellular lysates was determined by Coomassie staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). His-tagged chromodomain constructs were produced and expressed as GST-His proteins in *E. coli* as above, cleaving the GST tag with Tev protease following an initial purification step using glutathione agarose beads (Sigma). His-chromodomain proteins were then purified via TALON Superflow metal affinity resin (Clontech).

Recombinant baculoviruses for Flag-tagged CHD8 constructs were created by use of the Bac-N-Blue baculovirus expression system (Invitrogen). To express CHD8 protein constructs, SF9 cells were cultured to 1×10^6 cells per milliliter, infected with the desired baculovirus, and grown for three days. Cells were harvested by centrifugation, washed twice in Dulbecco's phosphate buffered saline (14190, Invitrogen), resuspended in IP-lysis buffer (20 mM Tris-HCl (pH 7.9), 500 mM KCl, 1% NP-40, 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF) plus 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin, and lysed by Dounce homogenization.

Lysates were cleared by centrifugation at 30,000 × *g* for 30 minutes at 4°C. Samples were then dialyzed against buffer BC50. Following dialysis, samples were centrifuged at 30,000 × *g* for 30 minutes at 4°C and incubated overnight with anti-Flag M2 agarose beads (Sigma). Beads were washed sequentially in ten column volumes each of BC100, BC350, BC350, and BC100. Proteins were eluted by the addition of 400 μg/mL of Flag peptide (Sigma) to BC100.

Generation of AEC Peptide and Histone Analogues

N-terminally FITC-labeled peptides were ordered from Chi Scientific encompassing histone lysine residues; however, the lysine residues were replaced with cysteines to allow for chemical alkylation of the peptides. Reactions were performed in alkylation buffer (1 M HEPES (pH 7.8), 10 mM L-methionine, 10 mM tris(2-carboxyethyl)phosphine (TCEP)). Peptide (1 mg) was diluted 8-fold with alkylation buffer to a final volume of 900 μL and incubated at room temperature for one hour in the dark. 50 μL of alkylation buffer, 1 M 2-bromoethylamine, or 2-(dimethylamino)ethylchloride were added to the mock, aminoethylcysteine (AEC), and dimethylaminoethylcysteine (AECme2) reactions, respectively. Reactions were incubated for two hours at room temperature in the dark. Another 50 μL of alkylation buffer, 1 M 2-bromoethylamine, or 2-(dimethylamino)ethylchloride were added to their respective reactions, and reactions were incubated for an additional three and half hours in the dark at room temperature. 50 μL of concentrated β-mercaptoethanol was added to stop each reaction.

Samples from the reactions were loaded onto a 250 mm × 4.6 mm 5 μm particle size Thermo C_{18} HPLC column (72105-254630). Buffers for binding and elution from the column were (A) water + 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile + 0.1% TFA. The column was run at 1 mL/min from 15% to 50% B over 25 minutes, 50% to 90% B over 2.5 minutes, and 90% B for an additional 2.5 minutes. Elution profiles were monitored at 220 nm and 480 nm.

Fluorescence Polarization Assay

Fluorescence polarization assays were performed essentially as described previously (54). Reactions were carried out in FP buffer (50 mM NaH_xPO_4 (pH 8.0), 25 mM NaCl, 100 μg/mL bovine serum albumin (BSA), and 5 mM TCEP) in flat-bottom black 96 well

plates (Costar). A constant concentration of 100 nM fluoroscein-labeled peptide was used in all reactions. A two-fold dilution series of His-Chromodomains from either CHD1 or CHD8 was used, giving final concentrations of approximately 0.1 μM to 100 μM. Prepared plates were scanned using a Tecan safire^2 plate reader using the fluorescence polarization program and the following settings: orbital shake for 30 seconds with medium intensity, settle for 10 seconds, excitation with 470 nm, emission measured at 525 nm, each well measured ten times with 0 ms between measurements, optimal gain, with 50 nM fluorescein used to calculate the G-factor.

Restriction Enzyme Accessibility Remodeling Assay

Restriction enzyme accessibility assays are based on previously published methods (181), although with significant modifications. Using pGEM3z-601 (116) as a template, 277 bp fluorescently labeled DNA was generated using primers 601 F (CGG GAT CCT AAT GAC CAA GGA AAG CA) and 601 R (CTC GGA ACA CTA TCC GAC TGG CA). A 0.1/0.9 ratio of 601 F fluorescent primer (5'-Alexa Fluor 488) to the non-fluorescent primer was used. Nucleosomes were reconstituted by salt dialysis with the generated DNA fragments with purified HeLa core histones from buffer TE (10 mM Tris (pH 8.0), 1 mM EDTA) with 2 M NaCl to buffer TE with no NaCl.

Assays were carried out in Chudate buffer (20 mM HEPES (pH 8.0), 3 mM $MgCl_2$, 50 mM NaCl, 1 μM $ZnCl_2$, 2 mM dithiothreitol, 100 μg/mL BSA) with 1 mM ATP, 50 nM nucleosomes, and 20 Units (1 μL) Hha I. 1X protein concentration is equal to 10 nM enzyme. Assays were performed in triplicate at 30°C for 30 minutes, at which point the reactions were stopped with 50% volume of stop buffer (10 mM Tris (pH 8.0), 0.6% SDS, 40 mM EDTA, 5% glycerol, 0.1 mg/mL Proteinase K) and incubating at 50°C for 20 minutes. Reactions were resolved on 3% agarose gel and visualized by the use of a Typhoon Trio+ imager. Images were loaded into ImageQuant TL software (GE Healthcare) for quantification.

ATPase Assay

ATPase reactions were also carried out in Chudate buffer in the presence of 7.5 μCi of γ^{32}P-ATP per reaction and 10 nM enzyme. HeLa dinucleosomes or DNA purified from HeLa dinucleosomes were added to a final concentration of 5 ng/μL as indicated. Reactions were

carried out at 30°C for 30 minutes, spotted onto polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) plates (Sigma), and resolved in 0.5 M LiCl in 1 M formic acid. Plates were dried and exposed to a phosphor screen for one minute. The phosphor screen was imaged using a Typhoon Trio+ imager, and bands were quantified using ImageQuant TL software.

Results

CHD8 Chromodomains Do Not Bind Methylated H3K4 or H3K27 Histone Tails

Fluorescence polarization (FP) is a technique that can be effectively employed to measure the binding constant between two molecules. It takes advantage of differences in rotational speed between a single smaller molecule which is fluorescently labeled, and that same molecule bound to a second, larger molecule. It is important to note that when a fluorophore is excited with plane-polarized light, the light emitted from the fluorophore will also be plane-polarized, assuming the fluorophore remains in a fixed position from the time of excitation to emission. When the small molecule is free in solution, however, its fluorescence emission is depolarized because it is rapidly tumbling. When the small molecule is bound by a larger molecule, the rate of rotation decreases such that the fluorescence emission remains polarized for a longer amount of time. By measuring the energy emitted from the fluorophore in a specific polarized plane, the rotation of the molecule can be quantified.

As previously mentioned, chromodomains from several other proteins have been observed to interact with specifically modified histone tails (24, 54, 82, 155). By using fluorescence polarization, the human CHD1 chromodomains have been shown to bind H3K4me3 (54). Incubation of fluorescently labeled modified histone tail peptides with recombinantly produced chromodomain constructs and measurement of fluorescence polarization allows for the calculation of the dissociation constant for the chromodomain/histone tail complex. The chromodomains ofCHD1 and CHD8 were cloned such that they had an N-terminal glutathione S-transferase (GST) tag followed by a Tev protease cleavage site and a six-His tag. This allowed for purification first by the use of the GST tag with glutathione agarose beads. Following cleavage with Tev protease, the GST was separated from the His-Chromodomains by the use of metal ion exchange chromatography.

One limitation to any technique whose goal is to identify a binging partner is the cost of producing the screening molecules. This is compounded when the binding partner is a histone tail peptide, as varying histone modification states have to be produced. However, one can chemically produce analogues of different modifications with the use of a single peptide by substitution of a cysteine residue in place of the normal amino acid where the modification-specific screen is desired (178). By alkylating the cysteine residue with a molecule containing the desired modification, a non-natural residue is created with a sulfur atom at the β position of the amino acid side chain rather than the canonical carbon (Figure 3.1).

Using this chemistry, the lysine analogue, aminoethylcysteine (AEC) was produced. Di- and trimethylated AEC lysine analogues were also synthesized (AECme2 and AECme3). The successful generation of these reagents was monitored via a shift in the elution profile of the peptide from a C_{18} HPLC column (Examples shown in Figure 3.2). As further evidence for the success of this chemistry strategy, modified whole histone H3 was also generated. *Xenopus* histone H3 only contains one cysteine residue, at position 110. This cysteine was mutated to an alanine (C110A), and cysteines were specifically incorporated into the histone at sites of interest (K4C, for example). The successful generation of modified histones was verified via MS/MS analysis (Figure 3.3), confirming further that site-specific chemical modification was indeed occurring.

Fluorescence polarization assays were carried out using a two-fold dilution series of His-Chromodomains from approximately 100 μM down to approximately 0.1 μM final concentration with a constant concentration of labeled histone peptide across all reactions. The initial modifications targeted were methylated H3K4AEC and H3K27AEC peptides. As mentioned previously, human CHD1 has been shown to bind H3K4me3, and the CHD8 *Drosophila* homologue kismet is thought to play a role in the regulation of H3K27me3 levels (54, 185). Following collection and analysis of data, it became apparent that the chromodomains of CHD8 were not binding with to these histone tail substrates (data not shown). As a control for the functionality of the assay, His-CHD1 chromodomains were incubated with H3K4AEC or H3K4AECme2 peptides, and binding constants were calculated (Figure 3.4). Although the observed binding constant for the CHD1 chromodomains to the dimethylated peptide was calculated to be 165 μM, or about 10 times higher than the expected value of 5-20 μM (54), this difference was attributed to using a histone tail analogue rather than the canonical synthesized peptide.

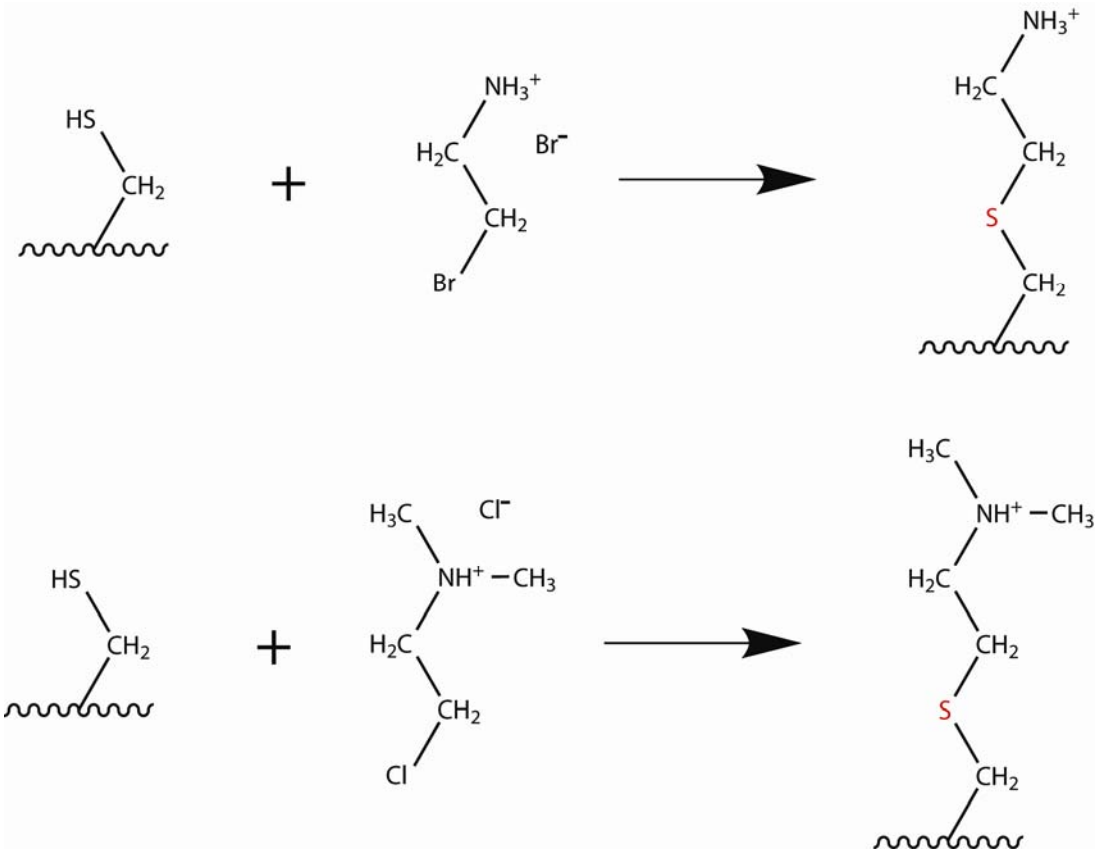

Figure 3.1 Chemical Modification of Cysteine Residues to Produce Methyl Lysine Analogues

The nucleophilic thiol side chain of cysteine is used to generate site-specific lysine analogues in the context of peptides or full length histones. Reaction of the cysteine thiol with the indicated molecules gives rise to the lysine (top) and methyl lysine (bottom) analogues shown, with the substituted sulfur atom highlighted in red.

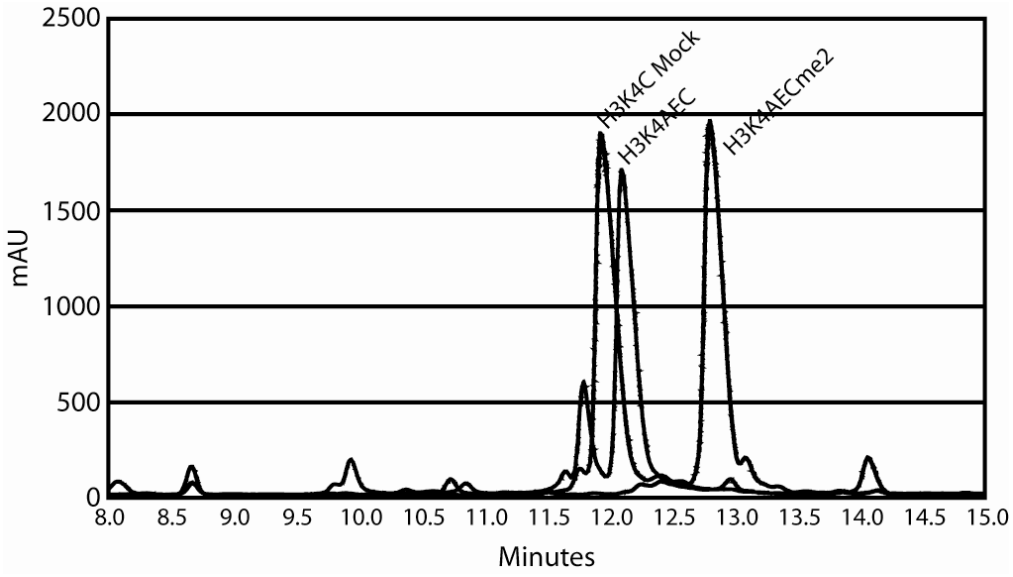

Figure 3.2 Histone Tail Peptides Can Be Alkylated to Create Methyl Lysine Analogues

Histone H3K4C peptides were subjected to alkylation reactions designed to create methyl lysine analogues. These peptides were then loaded and run across a C_{18} HPLC column with elution from the column monitored at 220 nm over time. Elution profiles demonstrate altered retention times between Mock, AEC, and AECme2 chemical modifications on the K4C peptide.

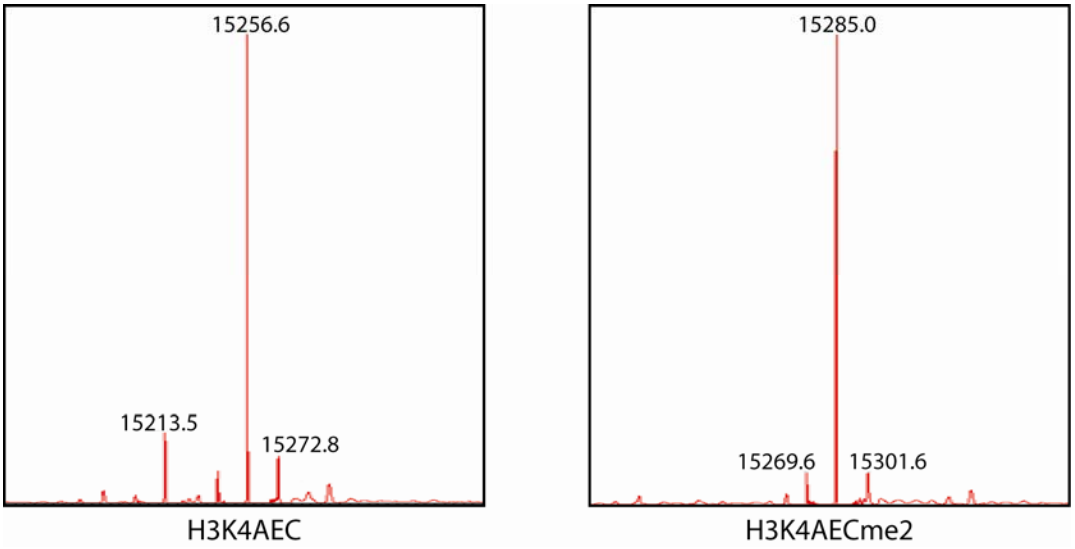

Figure 3.3 Full Length Histone H3 Can Be Alkylated to Create Methyl Lysine Analogue

Full length *Xenopus* histone H3 K4C,C110A was subjected to alkylation reactions designed to create a lysine (AEC) or dimethyl lysine (AECme2) analogue. Following the reactions, samples were sent for LC electrospray MS. Theoretical masses for histones are 15,258 Da for H3K4CAEC and 15,284 Da for H3K4AECme2. The accuracy of the MS results are ±0.01% of the molecular weight.

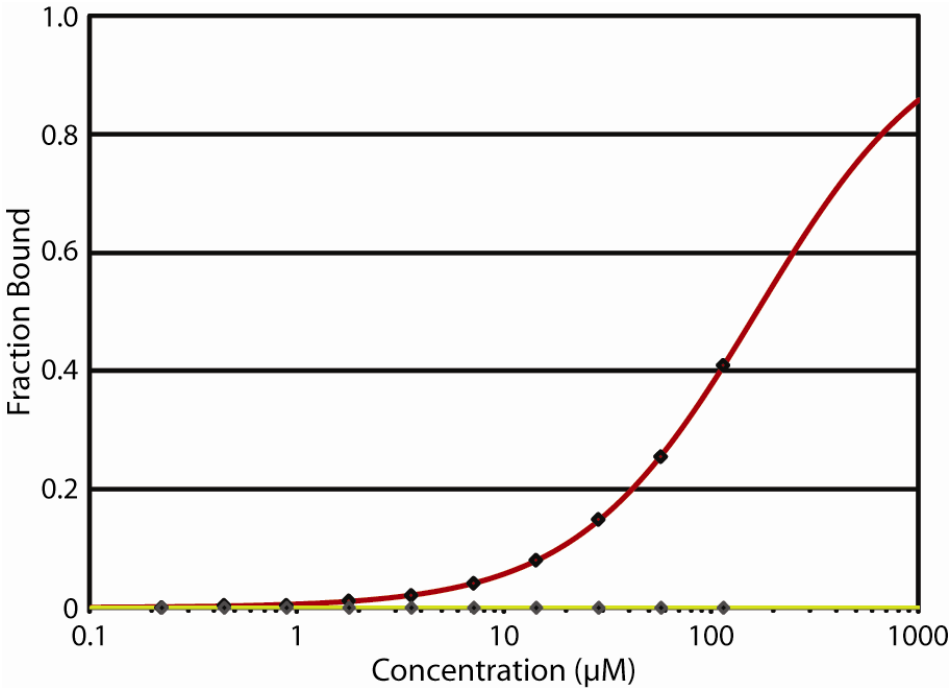

Figure 3.4 Human CHD1 Chromodomains Bind to H3K4AECme2 Peptide Analogue

Fluorescence polarization assays were used to determine binding between His-tagged human CHD1 Chromodomains and H3K4 lysine analogue peptides. Varying concentrations of His-CHD1 Chromos were incubated with a fixed concentration of fluorescent peptide. The CHD1 chromodomains were able to bind to the dimethylated H3K4 peptide analogue (red) while showing no binding to the unmodified lysine analogue (yellow).

The original experimental plan was to use the AEC analogues as a screen, which would then be followed up by ordering the actual modified peptides for the promising candidates. These native peptides would then be assayed to determine an accurate binding constant. However, the lack of quantifiable dissociation constants from the CHD8 chromodomains with AEC peptides led to the hypothesis that the chromodomains of CHD8 operate in a manner different than those of other chromodomain-containing proteins, perhaps not having histone tail methylation specificity at all.

CHD8 Chromodomains Bind to Histones H3 and H4

To test the hypothesis that the CHD8 chromodomains recognizes modifications or elements outside of the N-terminal tails, GST pulldown assays were utilized. Clarified lysate from *E. coli* expressing the CHD8 fragments GST-Chromos, GST-Chromo 1, or GST-Chromo 2 (Figure 3.5) was incubated with glutathione agarose beads. GST was used as a control. Following brief washing, the bead/protein complexes were incubated with purified HeLa core histones. After extensive washing, the protein complexes were eluted by boiling the beads, with the resulting eluate resolved via SDS-PAGE. As shown in Figure 3.6, histones H3 and H4 strongly associate with the CHD8 Chromos but not with the GST control.

To further investigate the mode of interaction between the CHD8 Chromodomains and histones H3 and H4, incubations were performed using recombinantly produced H3/H4 tetramer, recombinant H3 alone, and recombinant H4 alone. Because these histones were purified from *E. coli*, they are free of posttranslational modifications. This contrasts with the HeLa core histones, and a loss of binding would be indicative of a modification-specific binding mechanism. However, as shown in Figure 3.7, the chromodomains of CHD8 are able to interact with these unmodified histones, both as tetramers and individually. Additionally, these interactions persist when incubations were tested with each chromodomain individually. This result suggests that the chromodomains of CHD8 bind to the core of the histones, as the lack of modifications does not preclude binding.

In order to verify that this interaction was pertinent in the context of the full length CHD8 enzyme, a Flag immunoprecipitation experiment was performed. Similar to the GST pulldowns, clarified lysate from SF9 cells that were mock infected or infected with baculovirus to express Flag-tagged CHD8 was incubated with anti-Flag agarose beads. After washing, the

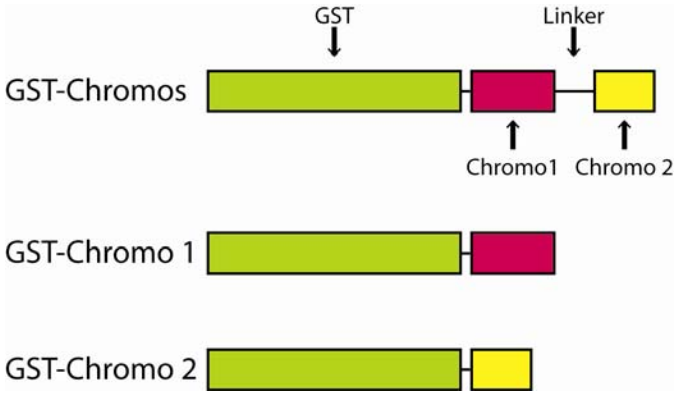

Figure 3.5 Schematic Representation of GST-CHD8 Chromodomain Constructs

To investigate the binding interactions of the CHD8 chromodomains, N-terminal GST-tagged constructs were created. The tandem chromodomains, with the encompassed linker, were cloned as GST-Chromos. Each individual chromodomain was then cloned individually, resulting in GST-Chromo 1 and GST-Chromo 2 as shown. Relative polypeptide chain lengths are drawn to scale.

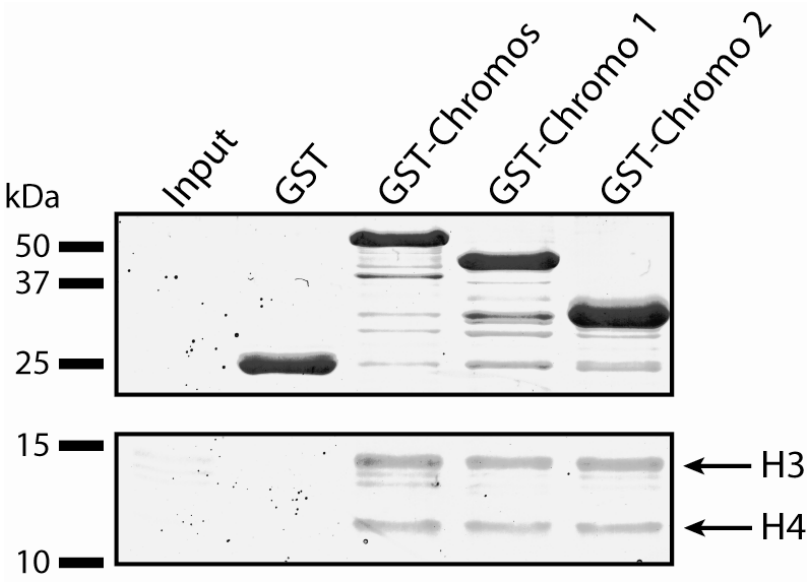

Figure 3.6 CHD8 Chromodomains Bind Histones H3 and H4 From HeLa Cells

Clarified *E. coli* lysate from cultures expressing the indicated GST construct was incubated with glutathione agarose beads and purified HeLa core histones. Following washing to remove non-specific interactions, bound complexes were eluted via addition of SDS-loading dye and boiling of the beads. Complexes were resolved by SDS-PAGE and visualized by Coomassie staining.

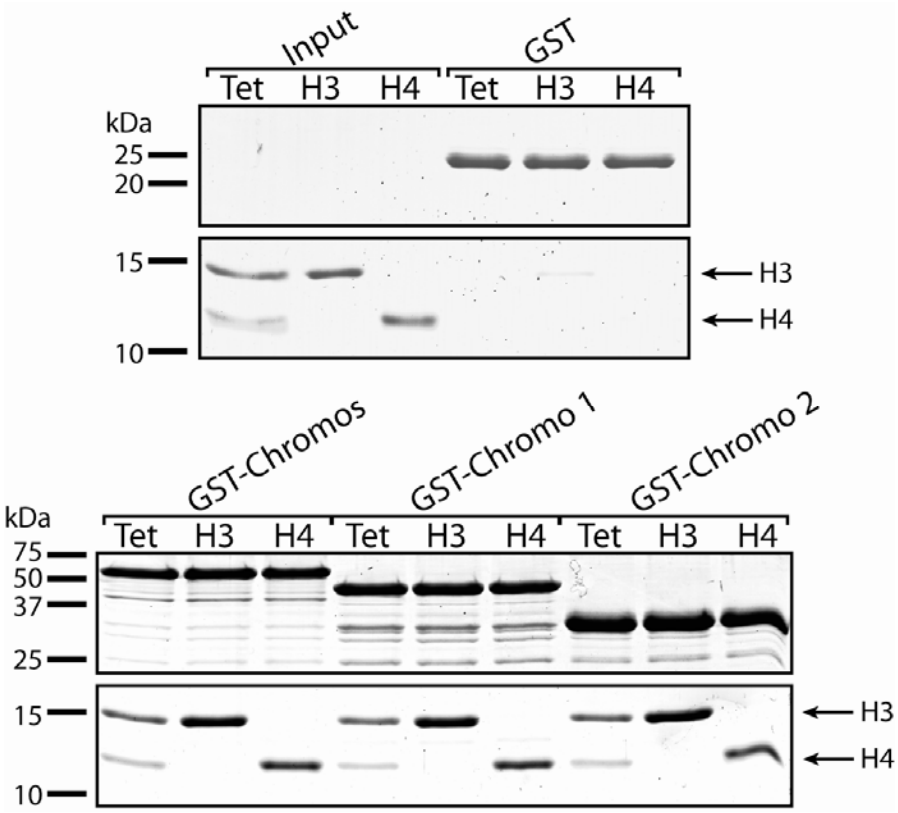

Figure 3.7 CHD8 Chromodomains Interact with Recombinant H3 and H4

Cleared cellular lysate from *E. coli* expressing GST, GST-Chromos, GST-Chromo 1, or GST-Chromo 2 was incubated with glutathione agarose beads and the indicated recombinant histones. Following washing, protein complexes were eluted by addition of SDS-loading buffer and boiling of the beads. Eluates were resolved by SDS-PAGE and were visualized by Coommassie staining.

protein-bead complexes were incubated with recombinant H3/H4 tetramer overnight. Following extensive washing and elution by boiling the beads, complexes were visualized via SDS-PAGE and Western blotting for CHD8 and histone H3. As shown in Figure 3.8, only Flag immunoprecipitants from CHD8 expressing cells specifically interacted with histone H3. This lends further evidence to the hypothesis that the chromodomains of CHD8 are indeed capable of binding to histone H3 in a manner independent of any posttranslational modifications.

CHD8$^{\Delta 788}$ Does Not Remodel *In vitro*

Due to a lack of histone mark binding by the chromodomains of CHD8, it was hypothesized that the chromodomains play a role in substrate recognition or binding in the context of the full length enzyme. To test this hypothesis, several CHD8 constructs were produced (Figure 3.9). In one, just the chromodomains were deleted (CHD8$^{\Delta 633\text{-}788}$). Two other constructs involved removal of the N-terminus of the enzyme, one leaving the chromodomains (CHD8$^{\Delta 636}$) and one removing them (CHD8$^{\Delta 788}$). Baculovirus constructs were created to produce these protein mutants, with all proteins being Flag-tagged for purification. Unfortunately, CHD8$^{\Delta 633\text{-}788}$ did not properly express and could not be used for the experiments described below.

Wild type CHD8, CHD8$^{\Delta 636}$, and CHD8$^{\Delta 788}$ were tested for nucleosome remodeling activity using a restriction enzyme accessibility assay. This assay is based on the fact that DNA in a nucleosome is largely protected from digestion by a restriction enzyme. A remodeling event can expose the restriction site to cleavage by a restriction enzyme. The product of this cleavage reaction is monitored as evidence for remodeling activity. To produce the nucleosomes for this assay, HeLa core histones were dialyzed with fluorescently-labeled 601 nucleosome positioning sequence (116) over a decreasing salt gradient, resulting in labeled mononucleosomes. The reactions were carried out by the addition of a CHD8 construct, ATP, and the restriction enzyme to the nucleosomes, and cleavage of the DNA was measured. Figure 3.10 shows that upon addition of WT CHD8 to these reactions, the restriction enzyme is able to digest a much larger fraction of the DNA as compared to the reaction with no remodeling enzyme. However, both CHD8 mutant proteins that were tested failed to result in DNA digestion over background levels, suggesting that their remodeling activity was abolished.

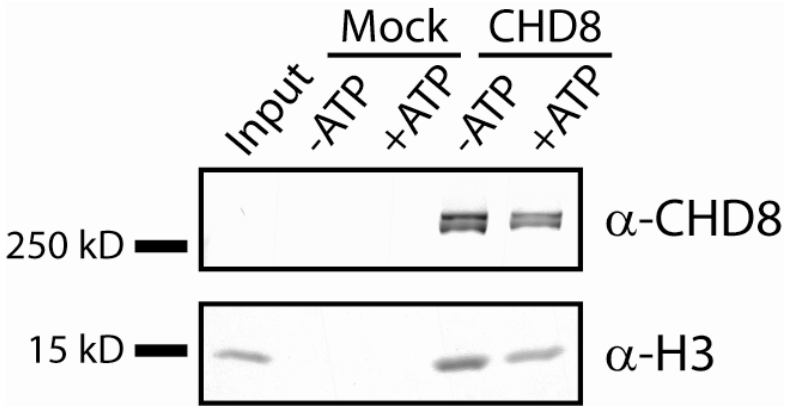

Figure 3.8 Full Length CHD8 Interacts with Recombinant Histone H3

Cellular extracts were prepared form SF9 cells following either mock or Flag-CHD8 baculovirus infection. Immunoprecipitations were performed using α-Flag agarose, which were then incubated with recombinant H3/H4 tetramers. Following extensive washing, protein complexes were eluted by addition of SDS-loading buffer and boiling of the beads. Eluates were resolved by SDS-PAGE and were visualized by Western blotting using the indicated antibodies.

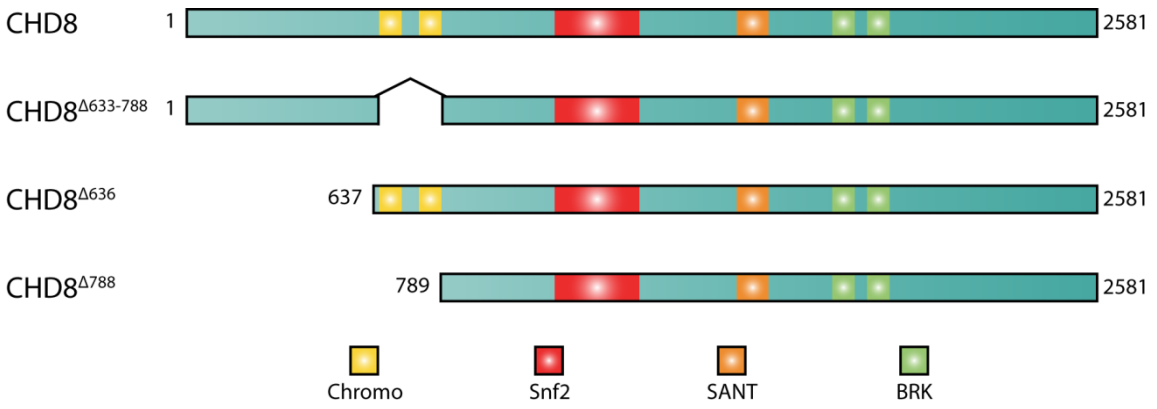

Figure 3.9 Schematic Representation of CHD8 Mutant Constructs

Baculoviruses were created for several CHD8 constructs to investigate the role of the tandem chromodomains in the context of the full length enzyme. $CHD8^{\Delta 633\text{-}788}$ is a deletion of the chromodomains, while leaving the rest of the protein intact. $CHD8^{\Delta 636}$ is a deletion of the N-terminus of CHD8 up to just before the start of the first chromodomain. $CHD8^{\Delta 788}$ is a deletion of the N-terminus of CHD8 through the end of the second chromodomain. All constructs were Flag-tagged for purification.

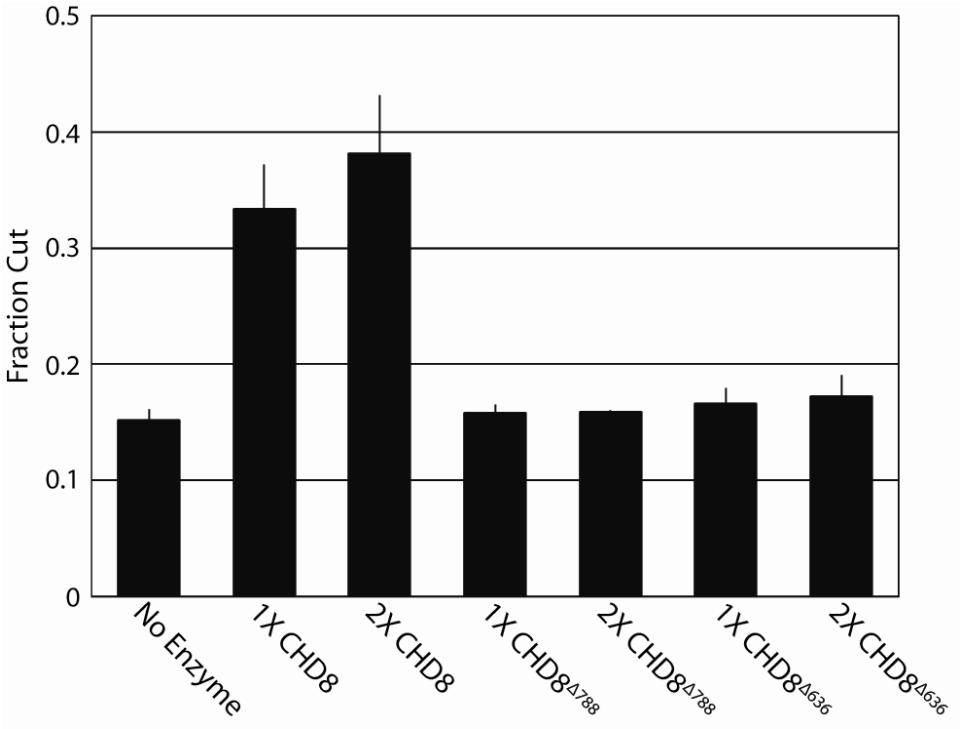

Figure 3.10 CHD8$^{\Delta 788}$ and CHD8$^{\Delta 633\text{-}788}$ Fail to Remodel Nucleosomes

Restriction enzyme accessibility assays were used to determine the remodeling capabilities of the indicated CHD8 constructs. Nucleosomes were generated by the use of HeLa core histones and DNA containing both a nucleosome positioning sequence and a restriction site that is buried upon incorporation into nucleosomes. The nucleosomes were incubated with the indicated CHD8 construct in the presence of restriction enzyme, and levels of DNA digestion were quantified.

This loss of activity could be the result of the necessity of the chromodomains for the catalysis of chromatin remodeling. Alternatively, deletion of a large portion of the N-terminus could simply result in the loss of function of the entire enzyme due to protein misfolding. All Snf2 helicase containing proteins have intrinsic ATPase activity that can be used to measure proper folding of the catalytic domain, allowing distinction between these scenarios.

CHD8$^{\Delta 788}$ Has Enhanced ATPase Activity

ATPase assays were used to determine if the deletion of the N-terminus of the protein eliminated the remodeling activity of the enzyme simply due to a misfolding of the catalytic domain. Because CHD8 is a known nucleosome-stimulated ATPase (199), ATPase assays were done in the presence of dinucleosomes purified from HeLa cells and compared to the ATPase activity in the presence of DNA alone. To test for the effect of chromodomain deletion on the ATPase activity of the enzyme, full length CHD8 and CHD8$^{\Delta 788}$ were assayed for ATPase activity. The activity of the enzymes was assessed by incubating them with γ^{32}P-ATP and monitoring the release of free γ^{32}P.

As shown in Figure 3.11, the ATPase activity of WT CHD8 increases over background levels in the presence of DNA, with a further increase in the presence of nucleosomes. However, CHD8$^{\Delta 788}$ demonstrated an increase in ATPase activity beyond that of the WT enzyme in the presence of both DNA and nucleosomes. The ATPase activity of CHD8$^{\Delta 788}$ appears to be DNA stimulated rather than nucleosome stimulated, as ATPase levels in the presence of free DNA was consistently higher than the levels with nucleosomes present. The retention of ATPase activity by this construct provides evidence that the loss in remodeling activity was not simply due to the misfolding of the catalytic Snf2 helicase domain. Deletion of the N-terminus of the enzyme results in a loss of remodeling activity with an aberrant loss of ATPase regulation, implying an important role for the chromodomains in the enzymatic activity of the enzyme.

Discussion

We have demonstrated that the chromodomains of CHD8 bind to histones H3 and H4 in a manner that is independent of any post-translational modifications to the N-terminal tails of the histones. Removal of the N-terminus, including the chromodomains, results in a loss of

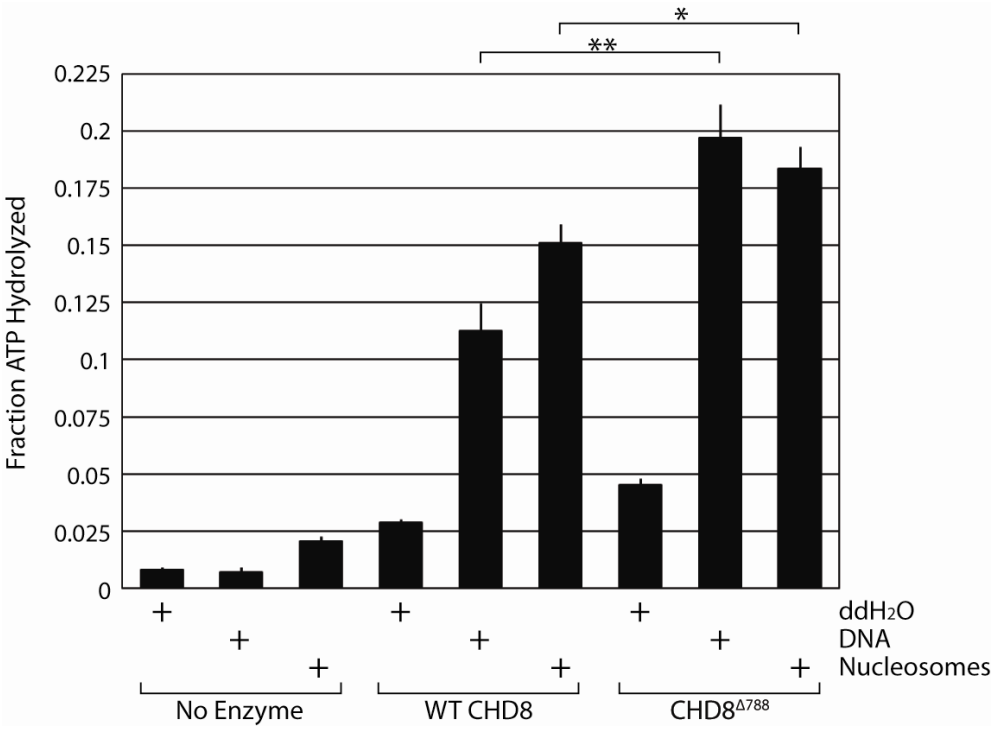

Figure 3.11 CHD8$^{\Delta 788}$ Displays Enhanced ATPase Activity

Full length (WT) CHD8 or CHD8$^{\Delta 788}$ was incubated with γ^{32}P-ATP in the presence or absence of DNA or purified HeLa cell dinucleosomes as indicated. $^{32}P_i$ release was measured by thin-layer chromatography, followed by exposure to a phosphorimager for analysis.

remodeling activity by CHD8. However, this deletion does not result in a loss of enzyme ATPase activity; in fact, the ATPase activity seems to proceed without regulation.

Establishment that the CHD8 chromodomains have significant affinity for the cores of histone H3 and H4 in the absence of any histone tail methylation is indeed a novel and important finding. It is important to note several key items regarding the fluorescence polarization assays. First and foremost, these assays were performed using CHD8 tandem chromodomains that were missing several amino acids on their amino terminus, resulting in the loss of the most N-terminal α helix. This was only discovered following consultation with a chromodomain expert (Sepideh Khorasanizadeh, personal communications) and FP has not been repeated using the appropriate constructs. However, the addition of these missing residues did not change any conclusions in the experiments that were repeated using the corrected construct (those being the GST pulldowns).

In addition to repeating the fluorescence polarization assays with the corrected chromodomain construct, using peptides that were synthesized to include specific chemical modifications should be used. As evidenced by the result using the chemically modified peptide and the CHD1 chromodomains, the peptide analogues do not appear to bind in a manner consistent with the synthesized peptide. This is likely due to the slightly different bond angle and bond length that the sulfur atom introduces into the molecule. Also, the sulfur-carbon bond has more rotational freedom than a saturated carbon-carbon bond, which could result in the side chain being out of proper binding position. Additionally, it should be noted that there has been very little use of this chemical modification strategy in the literature since its initial publication despite the obvious cost benefit. It is thus likely that other labs are also concluding that the analogous modification is, in fact, not a very good substitute.

Given the result observed in Figure 3.7 where the individually cloned chromodomains are able to interact with recombinant H3 and H4, it would also be important to test the individual chromodomains in the fluorescence polarization assays. These assays would be especially vital to the complete understanding of the CHD8 chromodomains in light of the published report showing that a functional second chromodomain is sufficient for binding to dimethylated histone H3K4 (161), although that study was done using mutations in the chromodomains rather than separately cloned and purified domains.

The result showing that full length CHD8 is able to interact with histone H3 in the context of an H3/H4 tetramer (Figure 3.8) is novel and lends credence to the interaction studies

that employ only the chromodomains. Extension of these full length interaction studies will provide further insight not only into the binding modes of CHD8, but also potentially into the overall mechanism of the enzyme. Obviously, verification of interaction between the full length CHD8 and histone H4 would be an important additional experiment to conduct. Additionally, full length CHD8 could be incubated with HeLa core histones and followed by a CHD8 IP. This would enrich any site-specific histone modification binding which could then be detected by the use of Western blotting with antibodies to various histone marks.

The structure of the tandem chromodomains of CHD1 revealed that they folded in such a way that both chromodomains were required to complete the binding pocket for the methylated lysine, with each domain contributing a portion of the binding surface (54). However, it has been shown for the CHD8 chromodomains that a functional second chromodomain is sufficient for binding to dimethylated H3K4 (161), suggesting that the chromodomains of CHD8 do not cooperate to bind the substrate like those of CHD1. However, the CHD8 chromodomain studies were done by mutation of the aromatic cage residues that are thought to make up the binding pocket of a chromodomain (21). If the chromodomains are indeed folding in a cooperative manner, with each domain contributing to the binding pocket, mutational analysis of a single domain's binding pocket would be intrinsically flawed. Thus, further work investigating the mechanism of binding by the CHD8 tandem chromodomains still has to be completed.

The finding that deletion of the N-terminus of CHD8 results in a loss of remodeling activity while at the same time leading to an increase in ATPase activity is quite intriguing. It is apparent from this work that there lies within the deleted segment of the protein a region that is vital to the proper regulation of CHD8. It is hypothesized that the chromodomains are that region that is needed for CHD8 to properly execute its function, as there are no other functional domains that have been identified in the N-terminus of the protein. This hypothesis could be strengthened by the completion of several different experiments. For example, remodeling assays could be performed with the *in trans* addition of the deleted protein segment as well as with the addition of just the chromodomains. A rescue of remodeling function when the chromodomains are added back would support a role for them in the regulation of the enzymatic activity of CHD8. Additional CHD8 constructs could be created such that individual chromodomains are deleted. These constructs would allow for the testing of the dependence of

CHD8 on each chromodomain independent of the other. Experiments such as these would also provide insight as to how the tandem chromodomains may interact.

It is worth noting that chromodomains, outside of those of human CHD1, have largely been shown to bind methylated lysine residues that are all associated with gene repression. Not only does this include the chromodomains of HP1 binding to methylated H3K9 (82, 155) and Polycomb binding to methylated H3 (24), but also the chromodomain of Male-Specific Lethal 3 (MSL3) binding to methylated H4K20 (88), and the chromodomain Y chromosome protein family members CDY and CDYL2 binding to H3K9me3 (53). The CHD6-9 homologue in *Drosophila* (kismet) has also been associated with H3K27 methylation (185). This combined with the result shown in Figure 2.4 as well as previously published work (199) demonstrating an upregulation of gene expression upon knockdown of CHD8 is evidence for further investigation into the role of the chromodomain in gene repression.

The conclusions from these studies have led to a model for the action of the chromodomains of CHD8 in the regulation of the enzymatic activity of the enzyme. This model hypothesizes that the chromodomains are responsible for binding of the enzyme to a nucleosomal substrate in a specific manner. This binding causes an activation of the ATPase function of CHD8, resulting in enzymatic catalysis of chromatin remodeling. Removal of the chromodomains prevents substrate binding, resulting in unrestricted ATPase activity. In support of this model, work was recently published on yeast Chd1 in which similar studies to these were performed. It was proposed that the chromodomains "gate" the ATPase motor, blocking function unless bound to a nucleosomal substrate (66).

Chapter Four

Initial Studies of Helicase-like Transcription Factor

Introduction

Helicase-like Transcription Factor

Helicase-like transcription factor (HLTF), also known as SMARCA3, is a DNA-dependent ATPase (171) that contains the seven conserved motifs of the Snf2 family of ATP-dependent DNA helicases. In addition to the Snf2 helicase domain, HLTF also contains a RING domain. A RING domain is a zinc-finger domain that has been shown to function in other proteins as an E3 ubiquitin ligase (28, 75, 121, 162). Finally, HLTF contains a HIRAN domain, an experimentally uncharacterized domain thought to be involved in DNA binding and recognizing stalled replication forks or damaged DNA (81).

It has been demonstrated that the HLTF promoter is hypermethlyated in 30-40% of colon cancers (70, 134). This level of transcriptional silencing could be indicative of HLTF acting as a tumor suppressor. Indeed, when HLTF is added back via transfection to colon cancer cell lines where the endogenous copies are silenced, a marked reduction in continued cell growth is seen (134). However, it should also be noted that in some non-gastrointestinal cancer cell lines, HLTF is overexpressed by as much as twenty-fold when compared to non-transformed cells (58). These findings make HLTF a very interesting target for controlling the formation, progression, and/or sustainability of several cancers.

HLTF is known to exist in two forms, differing only in their translational start site. The first is the full length version (HLTF Met1), while the other has a truncation of the N-terminus (HLTF Met123) (43). Interestingly, the shorter form has been implicated in transcriptional regulation, specifically upregulation of plasminogen activator inhibitor-1 (PAI-1) in cooperation with the transcription factors Sp1 or Sp3. This activity is not seen with the full length (Met1) form of HLTF (42). The Met123 form of HLTF results in the absence of roughly two-thirds of the HIRAN domain, providing the basis for a hypothesis implicating this domain in the function of

the full length form of the protein.

Within the Snf2 family of helicases, HLTF has been classified as a member of the Rad5/16-like subfamily based upon multiple sequence alignment of over 1300 Snf2 domains (55). Within the subfamily, further categorization yielded a grouping of Rad5, Rad16, and HLTF. Rad16 and Rad5 are yeast proteins which are involved in DNA repair. Rad16 is involved in nucleotide excision repair (NER) in the global genome repair (GGR) pathway (198, 227). Rad5 functions in reversal of stalled replication forks (14) and, in cooperation with Mms2 and Ubc13, polyubiquitination of proliferating cell nuclear antigen (PCNA) (72). Rad5 has been shown to be required for yeast error-free (template-switching) postreplication repair of damaged DNA (201). HLTF has been shown in recent work to polyubiquinate PCNA *in vitro* as well as in human cells when complexed withRad6-Rad18 and Mms2-Ubc13 (204).

Despite these interesting initial results for a cellular function, there remains very little biochemical characterization of HLTF. The goal of this project was to discover the biochemical function of the Snf2-domain containing protein HLTF, potentially offering a mechanism for its role in the proliferation of a large percentage of human colorectal carcinomas. Uncovering the mechanism by which HLTF acts as a tumor suppressor would provide potentially invaluable information in the understanding of colorectal carcinoma disease progression.

Materials and Methods

Cell Culture and Reagents

DLD1, HCT116, and SW480 colorectal carcinoma cell lines were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and 1X penicillin-streptomycin-glutamine (Invitrogen) at 37°C under 5% CO_2. Anti-SMARCA3 (HLTF) antibodies were purchased from Abcam (17984).

Recombinant baculoviruses for Flag-tagged HLTF and HLTF K300R constructs were created by use of the Bac-N-Blue baculovirus expression system (Invitrogen). To express HLTF constructs, SF9 cells were cultured to 1×10^6 cells per milliliter, infected with the desired baculovirus, and grown for three days. Cells were harvested by centrifugation, washed twice in Dulbecco's phosphate buffered saline (14190, Invitrogen), resuspended in IP-lysis buffer (20 mM Tris-HCl (pH 7.9), 500 mM KCl, 1% NP-40, 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF) plus

1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin, and lysed by Dounce homogenization.

Lysates were cleared by centrifugation at 30,000 × *g* for 30 minutes at 4°C. Samples were then dialyzed against buffer BC (20 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) plus 50mM KCl (BC50). Following dialysis, samples were centrifuged at 30,000 × *g* for 30 minutes at 4°C and incubated overnight with anti-Flag M2 agarose beads (Sigma). Beads were washed sequentially in ten column volumes each of BC100, BC350, BC350, and BC100. Proteins were eluted by the addition of 400 μg/mL of Flag peptide (Sigma) in BC100.

Methylation Specific PCR

Cells from one confluent 10 cm dish each of DLD1, HCT116, and SW480 cell lines were harvested and lysed in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1). Lysate was sonicated (output 4, duty cycle 30 for 10 seconds, Branson 250 Sonifier), phenol/chloroform extracted three times, and chloroform extracted once. Purified DNA was ethanol precipitated and resuspended in 10 mM Tris, pH 8.0, with 1 mM EDTA (TE). 1 μg of genomic DNA from each cell line was digested with either HpaII or MspI for one hour at 37°C. An enzyme-free reaction was performed to provide a positive control for the PCR. Heat inactivation of the restriction enzyme was done at 65°C for 20 min.

Two sets of primers were designed to amplify the CpG island at the HLTF promoter. Primer 87F (AAG GTC GTT TCC CTC CGT TT) was paired with Primer 438R (AGC GCA CGA CTG AAA GGT AAG) and Primer 203F (AAG AAC CCG GAT GGA ACC A) was paired with Primer 437R (GCG CAC GAC TGA AAG GTA AGT). PCR was performed by denaturing at 94°C for 2 min followed by 30 cycles of 94°C for 30 seconds, 58°C (decreasing by 0.3°C/cycle) for 30 seconds, and 72°C for 25 seconds. A final extension of 72°C for 10 minutes completed the reactions. Samples were resolved on a 3% agarose gel.

Colony Formation Assay

To test the effect of HLTF on the growth of cells, the DLD1 and HCT116 cell lines were transfected with a Flag-HLTF expressing vector using Lipofectamine 2000 (Invitrogen) following

the manufacturer's instructions. Following overnight incubation, cells were trypsonized and plated at a 1/3X concentration in media containing 2.5 μg/mL puromycin. Cells were allowed to grow for 16 (HCT116) or 17 (DLD1) days, changing media as needed, until colonies were visible.

Media was aspirated and 1 mL of 2% w/v methylene blue solution was added to each plate for 5 minutes with rocking. Excess dye was removed by vacuum, and the plates were submerged in tap water to remove remaining unbound dye. Colonies were counted by hand, and representative images were taken.

Partial Purification of Endogenous HLTF

Nuclear extract was prepared from HeLa cells using methods previously described and well established (41). Extract was run over a P11 phosphocellulose column (Whatman) and eluted with 100, 300, 500, or 1000 mM KCl in BC buffer. SDS-PAGE and Western blotting were done to identify the fraction containing the protein of interest, HLTF. The fraction containing HLTF was then loaded onto a Superose 12 HR 10/300 column (GE Healthcare) in BC350 and run at 0.5 mL/min collecting 0.5 mL fractions. Fractions were probed for HLTF by SDS-PAGE and Western blotting with anti-HLTF antibodies.

Restriction Enzyme Accessibility Remodeling Assay

Restriction enzyme accessibility assays are based on previously published methods (181), although with significant modifications. Using pGEM3z-601 (116) as a template, 277 bp fluorescently labeled DNA was generated using primers 601 F (CGG GAT CCT AAT GAC CAA GGA AAG CA) and 601 R (CTC GGA ACA CTA TCC GAC TGG CA). A 0.1/0.9 ratio of the fluorescent primer (5'-Alexa Fluor 488) to the non-fluorescent primer was used. Nucleosomes were reconstituted by salt dialysis with the generated DNA fragments with purified HeLa core histones from buffer TE (10 mM Tris (pH 8.0), 1 mM EDTA) with 2 M NaCl to buffer TE with no NaCl.

Assays were carried out in Chudate buffer (20 mM HEPES (pH 8.0), 3 mM $MgCl_2$, 50 mM NaCl, 1 μM $ZnCl_2$, 2 mM dithiothreitol, 100 μg/mL BSA) with 1 mM ATP, 50 nM nucleosomes, and 20 Units (1 μL) Hha I. 1X protein concentration is equal to 10 nM enzyme. Assays were performed in triplicate at 30°C for 30 minutes, at which point the reactions were stopped with

50% volume of stop buffer (10 mM Tris (pH 8.0), 0.6% SDS, 40 mM EDTA, 5% glycerol, 0.1 mg/mL Proteinase K) and incubating at 50°C for 20 minutes. Reactions were resolved on 3% agarose gel and visualized by the use of a Typhoon Trio+ imager. Images were loaded into ImageQuant TL software (GE Healthcare) for quantification.

Results

HLTF Promoter is Methylated in DLD1 and SW480 Cell Lines

Because HLTF is known to be silenced by promoter methylation in 30-40% of colon cancers (70, 134), we first sought to identify the promoter methylation status of the HLTF gene in the cell lines that were immediately available to us. This was done by the use of methylation-specific PCR. Methylation-specific PCR involves digestion of the template DNA with isoschizomers, which, strictly speaking, are restriction enzymes that recognize the same DNA sequence for cleavage. However, there is a subset of isoschizomers termed neoschizomers, which recognize the same DNA sequence yet cleave at different points in that sequence. Thus, the isoschizomers are generally understood to not only recognize the same sequence, but also cleave at the same site. Isoschizomers can differ in their ability to cut methylated DNA, which is useful in determining the methylation status of a DNA strand of interest.

Genomic DNA from three colorectal carcinoma cell lines was subjected to mock digestion or digestion with either the methylation-sensitive HpaII or the methylation-independent MspI. Following digestion the genomic DNA was used at a template for PCR. Primers were designed to amplify the CpG island in the promoter region of the HLTF gene. Should this promoter be methylated in a given cell line, the genomic DNA that was digested with HpaII will give rise to a PCR product while unmethylated promoters will not. As shown in Figure 4.1, HpaII-digested DNA gives rise to a PCR product in the DLD1 and SW480 cell lines but not in HCT116 cells. Thus, the HLTF promoter is methylated in DLD1 and SW480 cells, and HLTF expression should be silenced.

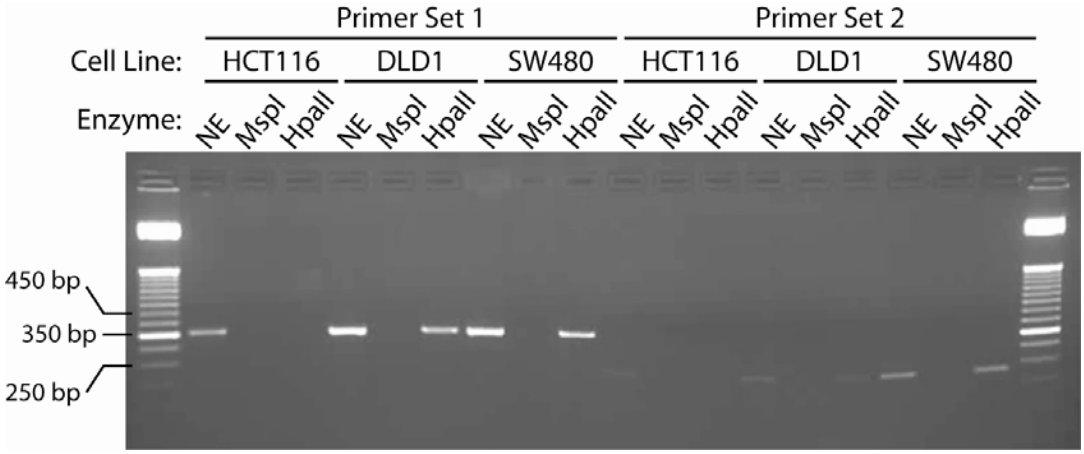

Figure 4.1 The Promoter of HLTF is Methylated in DLD1 and SW480 Cell Lines

Purified DNA from the indicated cell lines was mock digested (NE) or digested with either HpaII or MspI. Digestion with HpaII is sensitive to CpG methylation whereas digestion with MspI is methylation-independent. Two sets of primers were then used to detect undigested DNA. Samples were resolved on a 3% agarose gel.

Effect of HLTF on Cell Growth Inconclusive

As has been shown previously, addition of HLTF to colorectal carcinoma cell lines where it has been silenced can cause growth arrest in those cells (134). To test the effect of HLTF on the growth of the DLD1 and HCT116 cell lines, colony formation assays were used. As DLD1 cells have a methylated HLTF promoter while HCT116 cells do not, it is hypothesized that HLTF is silenced in DLD1 and expressed in HCT116. Thus, expressing HLTF will inhibit growth in the DLD1 cells and have little effect in the HCT116 cells. The transfection of a catalytically inactive HLTF should result in growth approximately equal to wild type levels, although a dominant negative effect could result in growth inhibition.

Empty pFlag vector, a vector encoding HLTF, or a vector encoding the supposed ATPase-defective HLTF K300R mutant along with puromycin resistance vector was transiently transfected into each cell line, and cells were grown in puromycin-containing media for approximately 2 weeks. This allowed time for the formation of colonies consisting of cells that contain the HLTF vector, and would thus be expressing HLTF. Should HLTF inhibit the growth of the DLD1 cells, the formation of visible colonies would be hindered, as the transfected cells would be unable to propagate. Colonies were visualized by methylene blue staining and counted.

As shown in Figure 4.2, the addition of both wild type and mutant HLTF to the control HCT116 cell line resulted in fewer colonies than the empty vector control. Somewhat interestingly, there was nowhere near the same hindrance in the DLD1 cell line which was hypothesized to have a greater reaction to the presence of HLTF. This data seems at odds with the methylation status of the HLTF promoters in these cell lines, although further experimental trials need to be done to confirm or deny this result.

HLTF Exists in an Approximately 150 kDa Complex

Snf2 domain-containing proteins are known to generally exist in large molecular weight complexes. Identification of complex components could prove vital to the characterization of the protein, as associating proteins generally provide insight into cellular roles or mechanisms of a protein. Thus, gel filtration chromatography of HeLa nuclear extract was used to determine the size of the potential HLTF-containing complex.

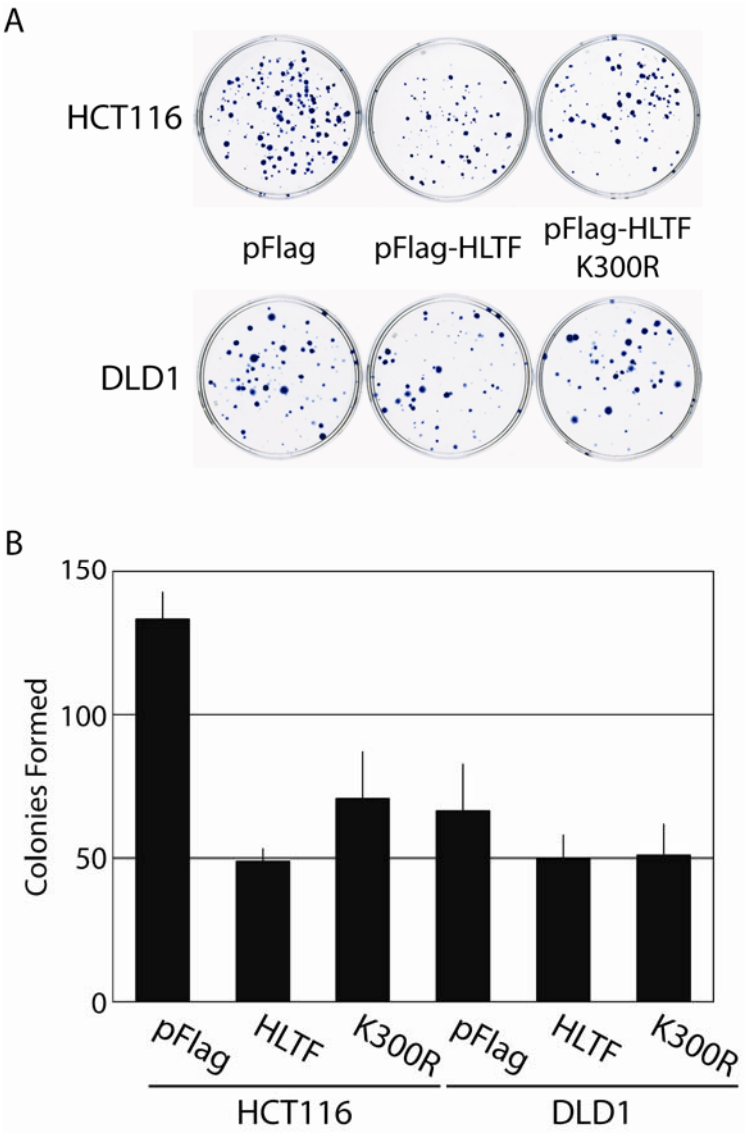

Figure 4.2 The Effect of HLTF on Colony Formation

The DLD1 and HCT116 cell lines were transfected with the indicated Flag-construct. After replating, cells were grown until colonies were visible. Cells were stained with methylene blue, and the colonies were counted. (A) A representative image is shown. (B) Colonies formed as in A were counted and the mean numbers of three independent experiments ± SD are shown.

Whole nuclear extract was bound to and fractionated from a P11 cellulose phosphate cation exchange column to partially purify the complex of interest. The fraction containing HLTF was then loaded onto a Superose 12 gel filtration column, with elution from the column monitored by Western blotting with anti-HLTF antibodies (Figure 4.3). This elution profile was compared to those of known sizing standards, and the elution profile of HLTF mirrored that of a 150 kDa standard. The molecular mass of HLTF was calculated to be 114 kDa. Thus, it is likely that HLTF exists as a monomer in a cellular context, although a more thorough conventional purification or an immunoprecipitation of HLTF would have to be completed to rule out additional lower weight complex members.

HLTF is Not a Nucleosome Remodeling Enzyme

Many Snf2-domain containing proteins are known to possess ATP-dependent nucleosome remodeling activity. Although HLTF is a member of the Rad5/16 family of Snf2 proteins (55) and thus may act in the process of DNA repair, the shorter transcript of HLTF has been implicated in transcriptional regulation (42). Unfortunately, HLTF Met123 is not yet available in the lab. Nucleosome remodeling assays were thus completed using full length HLTF as well as the ATPase defective mutant, HLTF K300R. Remodeling assays were carried out as in Chapter Three, using full length CHD8 as a positive control. As shown in Figure 4.4, both WT and ATPase mutant HLTF are unable to remodel nucleosomes in this assay, while CHD8 remodeled successfully. Although similar tests need to be completed with HLTF Met123, full length HLTF is not a nucleosome remodeling enzyme.

Discussion

These initial studies into the biochemical functions of HLTF have shown that the promoter HLTF is methylated in DLD1 and SW480 cell lines, presumably silencing expression of the protein. The effect of overexpression of HLTF in cell lines with endogenous silencing remains unclear, however. Additionally, HLTF likely exists in the cell as a monomer, although it is possible that it complexes with additional smaller molecular weight proteins. Finally, we have shown that HLTF is not able to remodel nucleosomes in an *in vitro* assay, indicating a function

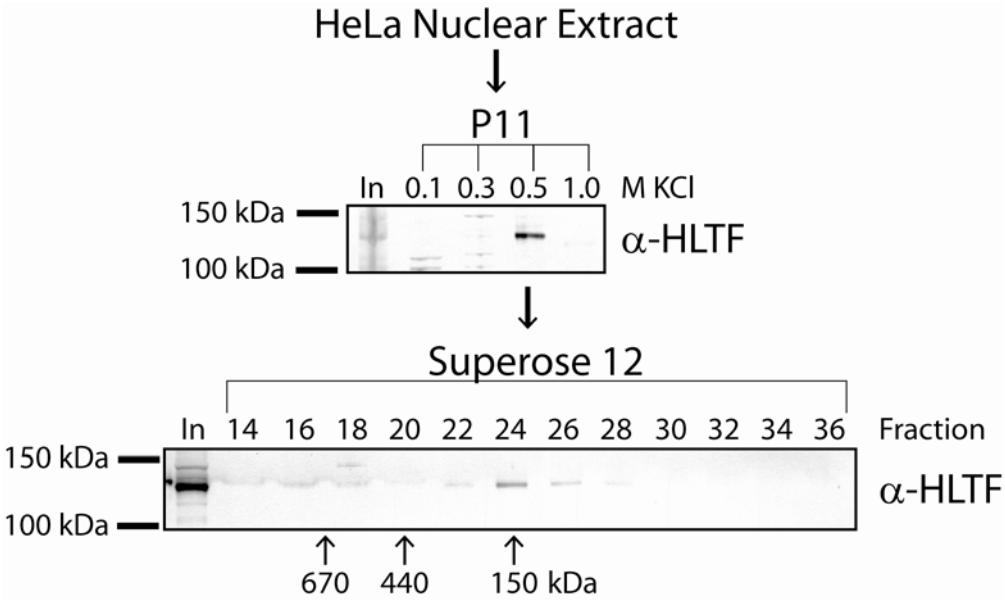

Figure 4.3 HLTF is Present in a 150 kDa Complex

HeLa nuclear extract was fractionated by phosphocellulose (P11) chromatography. Samples were eluted stepwise by the indicated KCl concentrations (0.1 M, 0.3 M, 0.5 M, 1.0 M) and analyzed by SDS-PAGE and Western blot using HLTF antibodies. The 0.5 M P11 fraction was further fractionated by chromatography on a Superose 12 HR 10/300 column. Fractions were analyzed by SDS-PAGE and Western blotting using HLTF antibodies. The arrows (bottom) denote the elution position of thyroglobulin (670 KDa), ferritin (440 KDa) and alcohol dehydrogenase (150 kDa).

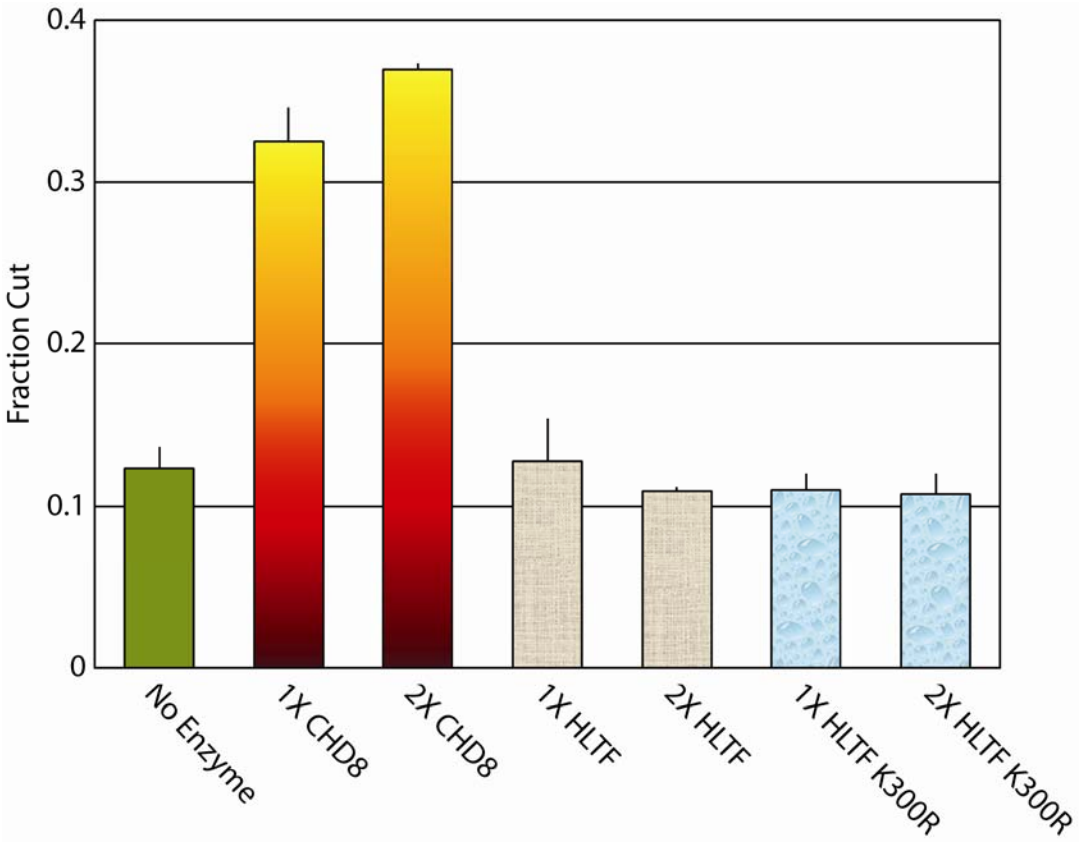

Figure 4.4 HLTF Does Not Remodel Nucleosomes

Restriction enzyme accessibility assays were used to determine the remodeling capabilities of HLTF. Nucleosomes were generated by the use of HeLa core histones and DNA containing both a nucleosome positioning sequence and a restriction site that is buried upon incorporation into nucleosomes. The nucleosomes were incubated in the presence of restriction enzyme with the purified wild-type or mutant HLTF protein. CHD8 was also used as a positive control for the assay. Levels of DNA digestion were quantified to determine remodeling activity.

for HLTF that is different than that of the ATP-dependent chromatin remodeling enzymes which share the catalytic Snf2 helicase domain.

The result indicating promoter methylation of HLTF in two of the three colorectal carcinoma cell lines that were tested provided a good starting point for the characterization of HLTF. The readily available reagents of cell lines with both methylated and unmethylated HLTF promoters would provide appropriate controls for any experiments that would be done. However, the silencing of the HLTF gene in these cell lines was assumed. Simply harvesting cells and testing for HLTF presence via Western blotting would provide adequate additional evidence regarding the expression of HLTF, although Western blotting from a nuclear extraction would provide more specific and cleaner data. Additionally, qRT-PCR could be performed to most accurately test the mRNA expression levels of HLTF in these cell lines. These lines of further evidence for HLTF loss would provide a more concrete characterization of HLTF levels in these cell lines.

The addition of HLTF to cell lines where endogenous expression is silenced was hypothesized to disrupt the growth of those cell lines. Although only preliminary experiments, it was shown here that addition of HLTF not only disrupted the growth of a cell line whose endogenous HLTF promoter is methylated (DLD1) but also the cell line without HLTF promoter methylation (HCT116) (Figure 4.2). It is likely that the expression levels of HLTF are tightly controlled and that the overexpression of the protein, even in cell lines where there are endogenous levels, causes disruption in the growth of the cells. It is also interesting to note that the same growth defect was observed with the addition of the ATPase-null mutant form of HLTF, perhaps indicating a dominant negative effect. However, if both active and inactive HLTF cause growth arrest, they would likely do so via differing mechanisms. Perhaps HLTF K300R blocks a transcription role of HLTF Met123, for example.

An extension of the colony formation assay would be to test the effect of HLTF loss on normal colorectal tissues. If HLTF is acting as a tumor suppressor in these tissues, the effect of HLTF loss in the colon may result in increased levels of tumor formation. This could be tested using a tissue-specific knockout mouse model, selectively eliminating HLTF expression in colorectal tissues. This could even be contrasted with HLTF loss in non-colorectal tissues, where HLTF has been found overexpressed in tumor cells (58). This type of *in vivo* study would provide valuable information as the effects of HLTF in tumor formation.

The results presented in Figure 4.3 are indicative of HLTF existing in the cell primarily as a monomer, although the possibility of an HLTF complex cannot be entirely discounted. Recent work has confirmed this conclusion (120), as a similar sizing experiment was done using HEK293 extracts. However, that group also performed immunoprecipitations from HEK293 cells that had been transfected with Flag-HLTF, identifying two potential HLTF binding proteins. The proteins identified were Pax Transactivation domain-Interacting Protein (PTIP) and the 70 kDa subunit of Replication Protein A (RPA70), both of which are involved in DNA damage response. HLTF has also been shown to interact with several members of the DNA replication repair pathway, namely Rad6, Rad18, Mms2, and Ubc13 by use of GST pulldown assays (204). This combination of results points towards HLTF probably existing in a cellular context as a monomer and only transiently binding to existing complexes to perform its enzymatic function.

The demonstration that HLTF does not remodel nucleosomes in Figure 4.4 provides evidence that HLTF is not functioning as an ATP-dependent chromatin remodeling enzyme and lends credit towards HLTF involvement in DNA repair pathways. However, the lack of a remodeling result really has to be complemented by a functional enzyme assay, such as an ATPase assay, to demonstrate enzyme viability. Although an ATPase assay was not performed in these studies, there was one published that demonstrated that HLTF has ATPase ability (120).

Continued study of HLTF will provide further insight into its role as a tumor suppressor in many colorectal carcinomas. Uncovering details regarding the mechanism of HLTF in the prevention of colorectal carcinoma could provide invaluable insight and novel therapeutic outcomes in fighting this common disease.

Chapter Five

Conclusion

Background

Eukaryotic cells package their DNA into a highly condensed state via formation of chromatin. The compaction of the DNA in this manner provides a barrier to cellular processes that require physical access to the DNA, such as DNA replication, DNA repair, and transcription. There are chromatin modifying enzymes and ATP-dependent chromatin remodeling enzymes that regulate the structure of chromatin. The ATP-dependent remodeling enzymes catalyze the hydrolysis of ATP to ADP and use the energy released to cause local changes to nucleosome positioning. There are several families of ATP-dependent chromatin remodeling enzymes which have varying roles in the cell. Proteins are grouped into these families based upon sequence and functional similarities. These families include the SWI/SNF, ISWI, INO80, and CHD families. The CHD family is defined by the presence of tandem chromodomains N-terminal to the catalytic Snf2 helicase domain. There are nine members of the CHD family, CHD1-CHD9. This family is further divided into three subfamilies, each with its own defining characteristics. CHD1-2 contain a C-terminal DNA binding domain, CHD3-5 possess tandem PHD domains that are N-terminal to the chromodomains, and CHD6-9 have a SANT domain and tandem BRK domains C-terminal to the Snf2 domain. Most research into the CHD family of proteins has focused on CHD1, while relatively little is known about CHD6-9.

The primary focus of this body of work has been the characterization of the CHD family member CHD8. The study sought to define a role for CHD8 in the transcriptional regulation of target genes. With this goal in mind, the study of CHD8 complex members allowed for the identification of the Hox gene locus as a model for regulation of expression by CHD8. It was demonstrated that CHD8 does indeed play a role in the proper control of HOXA2 gene expression, and that CHD8 seems to have an effect on the establishment of histone methylation patterns at the HOXA2 promoter. These studies have also provided insight into the functional roles of the chromodomains of CHD8, linking them not only to the binding of core histone H3

and H4, but also showing a role for the chromodomains in control of the enzymatic function of CHD8.

CHD8 Complex Function

As shown in Figure 2.1 and Figure 2.2, CHD8 is able to form a complex with WDR5, RbBP5, and Ash2L. These proteins have been studied in most detail as they relate to the histone methyltransferase MLL1, as they are core members of the MLL1 complex (8, 46). These shared complex components have led to the obvious hypothesis that both MLL1 and CHD8 exist in one complex together. This complex would thus possess both methyltransferase and nucleosome remodeling activity. This type of complex could be similar to the CHD3/4-containing NURD complex, where the chromatin remodeling function is complexed with the histone deacetylase activity of HDAC1/2 and RbAp46/48 (200, 209, 218, 232).

This type of bi-functional complex may indeed be the normal role for the CHD proteins in a cellular context, and the formation of multiple such complexes could provide explanation as to the functions of the nine CHD proteins. CHD1 is the only protein in the CHD family that is found in lower eukaryotes such as yeast. As organisms got more complex, there evolved more CHD-related proteins with more complicated domain architectures. Perhaps it was the evolution of these more complex domain structures that allowed for the integration of CHD proteins into specific chromatin modification complexes.

It is important to note, however, that CHD8 and MLL1 have not yet been observed in complex with one another in immunoprecipitation experiments of either protein from mammalian cell lines. The demonstration of a role for CHD8 in the recruitment of complex members to target promoters (Figure 2.5), in the establishment of proper methylation patters at those promoters (Figure 2.6), and in the regulation of transcription of a gene locus that MLL1 is known to be involved in as well (Figure 2.4) does seem to provide enough evidence of a link between CHD8 and MLL1 for continued investigation into a bi-functional complex. It is also worth noting that MLL2-4 also associate with WDR5, RbBP5, and Ash2L (30, 46, 76), and that, like the CHD family, the MLL proteins are thought to have redundant functions (5). Perhaps CHD8 preferentially associates with MLL2, MLL3, or MLL4.

Alternatively, it is possible that CHD8 and MLL1 do not exist in complex with one another, but work cooperatively or even antagonistically at target genes. MLL1 is involved in

production of activating histone methylation marks (5), although CHD8 seems to have a role in the attenuation of target gene expression (Figure 2.4). Perhaps it is the binding and conformational arrangement of WDR5, RbBP5, and Ash2L that determine that complex's additional binding partner, allowing for control of sequential CHD8 and MLL1 enzymatic activities at target promoters.

Role of CHD8 Chromodomains in Enzyme Function

In contrast to existing literature regarding the binding specificity of chromodomains, the vast majority of which indicates that a chromodomain is a methyl-histone binding domain, the chromodomains of CHD8 bind to the core of histones H3 and H4 (Figures 3.6 and 3.7). This is especially important to consider in contrast to the chromodomains of CHD1, which have been shown to interact with the trimethylated state of histone H3K4 (54, 179). Using x-ray crystallography, it has been shown that the tandem chromodomains of CHD1 fold together to create one binding pocket for the methylated histone tail (54). When the sequences of the CHD tandem chromodomains are aligned, however, it becomes apparent that the chromodomains of CHD1 and CHD2 are further removed from the chromodomains of the remaining CHD family members. The linker region between the tandem chromodomains of CHD1/2 is especially longer than that of the remaining CHD proteins. It is possible that that extended linker enables the cooperative fold that has been characterized, while the remaining CHD family members are perhaps unable to fold in such a manner.

The diversity of chromodomain binding targets that has been observed in the literature is rather large (24, 53, 54, 82, 88, 155, 161, 179, 185, 194). As mentioned previously, outside of the CHD1 chromodomains, chromodomains also seem to be involved in the binding of histone methylation marks that are involved in repression (24, 53, 82, 88, 155, 185). Thus, it appears that the chromodomains of CHD1 are not only apparent outliers within the CHD family, but in the context of the functional binding characteristics of a chromodomain. Additionally, it is important to note that the chromodomains of yeast Chd1 are incapable of recognition of the same histone H3K4me3 mark that the human enzyme's chromodomains are seen to bind to (179). Thus, it would appear that the chromodomains of human CHD1 (and likely CHD2) may be functionally distinct from the chromodomains in the rest of the CHD family.

The demonstration of the necessity for a functional N-terminus for successful remodeling activity (Figure 3.10) and proper regulation of CHD8 ATPase activity (Figure 3.11) may provide a key insight into functional roles for the chromodomains. However, completion of additional experiments to test the functionality of the CHD8 chromodomains that were addressed in Chapter Three need to be completed before more sound conclusions can be drawn. Worthy of further discussion are the conclusions drawn in recent work studying the yeast Chd1 chromodomains (66). Recall that these chromodomains are functionally distinct from their human CHD1 homologues (179). Perhaps the application of the model that was derived from these studies can be extended to the chromodomains of CHD8. This model dictates that the chromodomains fold over the Snf2 ATPase domain and physically block ATPase function, perhaps by blocking the binding pocket for ATP. When the chromodomains are bound to their preferred substrate, a nucleosome of some description, there is a conformational change in the Chd1 enzyme in which the chromodomains extend away from the Snf2 domain, allowing functional ATPase activity. This model does indeed fit the data thus collected for CHD8, as removal of the chromodomains from the enzyme results in unregulated hyperactivity of ATPase function.

Further characterization of CHD8 will be needed to understand this quite complex enzyme. There no doubt remains valuable understanding of the general mechanisms used in the regulation of transcription that will be discovered upon continued probing of the CHD family of ATP-dependent remodeling enzymes.

Helicase-like Transcription Factor

Helicase-like transcription factor (HLTF) is a very interesting protein that seems to act as a tumor suppressor in a large number of human colorectal carcinomas (134). Although only initial studies were conducted within this work, HLTF remains a potentially valuable target for the comprehensive understanding of colorectal carcinoma disease progression. Examination of HLTF effect on colorectal tissues in an *in vivo* mouse model could provide the basis for understanding HLTF mechanism of tumor suppression. These tissue-specific mouse models would likely provide more useful insights into the role of HLTF than the tissue culture models that have thus far been employed. Additionally, it might be valuable to attempt some HLTF growth inhibition experiments in primary cells. Because cell lines that are used in tissue culture

are generally far from the genetic norm, studies involving proteins thought to have a role in growth arrest may not always reveal information that would be gleaned from a genetic system or a primary cell culture model.

Also important to the understanding of HLTF mechanism is the further study of the alternately-spliced form of the protein, HLTFMet123, which has been implicated in transcriptional regulation of specific genes (42). It would be interesting to discover whether or not full length HLTF has a cellular role that is completely removed from that of HLTFMet123, or if the two variants perhaps interact to produce a common function. More extensive studies of the role for HLTFMet123 as a transcriptional regulator are needed. It would also be interesting to link the transcriptional activity of HLTFMet123 with the tumor suppressor activity of the full length HLTF.

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