REGULATION OF ANDROGEN-RESPONSIVE TRANSCRIPTION BY THE CHROMATIN REMODELING ENZYME CHD8

by

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To Nisha, Amma and Achan

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Abstract

Eukaryotic DNA is packaged into a highly condensed chromatin state, which inherently serves as a barrier to critical cellular processes such as DNA replication, repair and transcription. The modulation of chromatin structure to allow access to the underlying DNA is vital for the appropriate regulation of these processes. One class of enzymes responsible for modulation of chromatin structure is the SNF2 superfamily of ATP-dependent chromatin remodeling enzymes. These enzymes use the energy from hydrolysis of ATP to mobilize, disrupt and modulate nucleosomes. These enzymes are classified into several different families based on their domain architecture, the largest of which is the CHD (Chromodomain Helicase DNA-binding) family. This family is comprised of nine enzymes, CHD1 through CHD9, which are further divided into three subfamilies, CHD1-2, CHD3-5, and CHD6-9. Relatively little is known about the third subfamily, and the research described here is directed towards one particular member of this subfamily, CHD8.

Previous studies have established a functional association between the CHD6-9 subfamily and nuclear receptor-mediated transcriptional regulation. One such nuclear receptor, the androgen receptor (AR), mediates the effect of androgens through its transcriptional function during both normal prostate development and in the emergence and progression of prostate cancer. AR is known to assemble coactivator complexes at target promoters to facilitate transcriptional activation in response to androgens. Here we

identify CHD8 as a novel coregulator of androgen-responsive transcription. We show that CHD8 directly associates with AR and that CHD8 and AR simultaneously localize to the enhancers of androgen-responsive genes following androgen treatment. In the LNCaP prostate cancer cell line, reduction of CHD8 levels by siRNA treatment severely diminishes androgen-dependent activation of these genes. We demonstrate that the recruitment of AR to target promoters in response to androgen treatment requires CHD8. CHD8 also facilitates the androgen-stimulated proliferation of LNCaP cells, emphasizing the physiological importance of CHD8 in prostate cancer.

Further studies were conducted to examine the mechanism by which chromatin remodeling by CHD8 is involved in androgen-responsive transcription. We found that CHD8 can remodel nucleosomes without histone tails and prefers substrates that are methylated at H3K4. The association of CHD8 with H3K4 methylation was supported by our findings that CHD8 interacts with the MLL1-WAR histone methyltransferase complex. The interactions of CHD8 with this complex and their effect on its remodeling activity were further characterized.

These studies collectively implicate CHD8 in the regulation of androgen-responsive gene expression and as a novel coregulator of AR-mediated transcription. We also establish potential mechanistic details of transcriptional regulation by CHD8. Our results present CHD8 as a novel diagnostic, preventative, or therapeutic target in prostate cancer.

CHAPTER I

General Introduction

Chromatin Structure

The hereditary information that specifies the structural composition and functional characteristics of all living organisms is contained within discrete sub-cellular elements called genes. These genes are encoded within the DNA, which is a macromolecule comprised of four different polymerized bases. The specific sequence of these different bases is where all the genetic information required for the development and survival of the organism is stored. This information is so complex and vast for even the simplest organisms that a huge number of genes, and consequently an immense amount of DNA, is required to store all of it. The human genome contains an estimated 30,000 genes carried on over 3 billion base pairs of DNA (1). The DNA from one single human cell would stretch up to 2 meters in length and all of the DNA in the human body would be enough to reach from the earth to the sun and back 610 times (2). This enormous amount of DNA needs to be packed into the nucleus of a eukaryotic cell, which is only about 6 μm in diameter. This poses a daunting challenge to the cell and requires extremely efficient packaging of the DNA into the nucleus.

In eukaryotic cells, the DNA is packaged into a highly condensed structure called chromatin. Chromatin is made up of a fundamental repeating unit called the nucleosome

(3). Each nucleosome consists of ~146 bp of DNA wrapped around a histone octamer, which is comprised of two units each of the four histone proteins H2A, H2B, H3 and H4 (4). The H3 and H4 histones associate as a tetramer consisting of two units of each, and this in turn interacts with two dimers of H2A-H2B to form the histone octamer. The 146 bp of DNA then wraps 1.65 turns around this histone octamer, with 14 points of contact between the DNA and the histone core (5). This makes the nucleosome structure a uniquely stable DNA-protein complex, which is suitable for its packaging function. Several of these nucleosomes are strung together into linear arrays so that they give a "beads on a string" appearance under the microscope (6). These linear arrays of nucleosomes can be tightly packed together by twisting into three-dimensional coils and irregular, interdigitating structures, resulting in the formation of condensed chromatin fibers. These chromatin fibers then fold into looped domains by attachment of the DNA to a network of non-histone proteins in the nuclear matrix which form a chromosomal scaffold. The more loosely packed, diffuse regions of chromatin, known as the euchromatin, represent the part of the genome that is being actively transcribed. Transcriptionally inactive regions of the chromatin exist in a more condensed form called heterochromatin, which appears as dark spots under a microscope upon staining the DNA. Most of the chromatin in metabolically active cells exists as diffuse euchromatin as the genome is constantly being transcribed. However, during cell division all the chromatin in the cell condenses into heterochromatin and this helps to compact the DNA into larger subcellular structures which comprise the visible form of the chromosomes. The metaphase chromosomes, which are microscopically distinguishable as distinct,

highly condensed structures, are thus a result of several degrees of compaction of the chromatin (7).

Chromatin and Transcription

The function of chromatin extends beyond merely that of DNA compaction. Due to the extensive packaging of DNA, the chromatin structure inherently prevents access to the underlying DNA due to steric hindrance and thus interferes with critical cellular processes like transcription, replication and DNA repair. Indeed, it has been well established that incorporation of nucleosomes impedes the transcription of DNA templates *in vitro* (8). It has been shown that the elimination of histones at promoter regions results in changes in expression of downstream genes *in vivo* further indicating the importance of chromatin in the transcriptional process (9).

The stereotypical RNA polymerase II (Pol II) transcription cycle begins with the binding of transcriptional activators, which are sequence-specific DNA-binding proteins, to the promoter region upstream of the transcription start site of the gene being transcribed (10). This results in the recruitment of adaptor complexes like SAGA (Spt-Ada-Gcn5 acetyltransferase) and mediator, which in turn recruit the general transcription factors (GTFs) (11, 12). A combination of these GTFs (TFIID, TFIIA and TFIIB) then positions Pol II and TFIIF at the core promoter, forming the closed preinitiation complex (PIC). TFIIH then assists in the opening of double-stranded DNA so that the single-stranded DNA template can be positioned at the active site of the open Pol II complex to initiate RNA synthesis (13). The carboxy-terminal domain (CTD) of Pol II is phosphorylated by TFIIH and dissociates from the GTFs before progressing into the

elongation stage of the transcription cycle. The phosphorylated CTD then recruits factors involved in transcriptional elongation and mRNA processing (14).

All of these processes require the factors involved to be able to access the DNA being transcribed, but the highly condensed packaging of this DNA into chromatin serves as a physical barrier to many of these steps in the transcriptional cycle. It prevents the binding of activators to their specific target DNA sequences. It also prevents the recruitment of the massive general transcriptional machinery to target promoters and their subsequent assembly into the PIC. It presents further impediment to the melting of the DNA and establishment of the DNA-Pol II holoenzyme-RNA complex. Chromatin is also a physical barrier to transcriptional elongation by Pol II, as well as to the recruitment of elongation, mRNA processing and splicing factors.

Transcriptional Regulation by Chromatin Remodeling Enzymes

The regulation of chromatin structure is essential for allowing the factors involved in the transcriptional process adequate access to the nucleosomal DNA, and thus serves as an important regulatory point in transcriptional control. This regulation of chromatin structure is achieved by two classes of chromatin remodeling enzymes: one that is involved in the covalent modifications of histones, and the other that uses energy derived from ATP-hydrolysis to modulate the contacts between histones and DNA within nucleosomes. Covalent modifications of histones include acetylation, methylation, phosphorylation and ubiquitination besides others of specific histone residues. Each of these modifications is catalyzed by a distinct category of enzymes and can serve as molecular signals which are recognized and bound by various factors that influence

chromatin structure and general transcription. The ATP-dependent remodeling enzymes usually occur in large complexes that alter chromatin structure by disrupting or mobilizing histones to regulate access to the nucleosomal DNA.

Transcription factors (TFs) or activators recognize specific DNA sequences in their target promoters and can bind them in the context of free DNA (15). When these target sequences are buried within chromatin, eukaryotic TFs have to employ different strategies to facilitate optimal binding to these recognition sites. There are numerous examples of chromatin remodeling enzymes being involved in stimulating the binding of TFs to their target chromatin elements (16, 17). After binding the promoter, activators recruit a number of coactivators, many of which are also involved in chromatin remodeling. This is not surprising since extensive modification of the chromatin structure is required for enhanced binding of the activators themselves, as well as for the docking of the large PIC and its many auxiliary factors on the DNA. Histone acetylation has been linked with the promoters of actively transcribed genes (16). Acetylation of the histone tails is known to be associated with a more "open" conformation of chromatin, allowing access of the transcriptional machinery to the underlying DNA. Activators thus recruit histone acetyltransferases (HATs) which are capable of acetylating histones, thus maintaining the target chromatin in a transcriptionally active state (18). Specific histone methylation marks are also known to be associated with transcriptional activation and these marks are deposited at initiation sites by the corresponding histone methyltransferases (HMTs). In addition, promoter-bound activators also recruit ATP-dependent chromatin remodeling enzymes to make the DNA more accessible (19).

After PIC assembly, Pol II-mediated transcription is further hindered by nucleosomal barriers, which may cause pausing of the polymerase at the transcriptional start site (20). This pausing can also lead to backtracking of the Pol II. The transcription elongation factor TFIIS reactivates Pol II transcription through such nucleosome barriers (21, 22). Both classes of chromatin remodeling enzymes have been implicated in alleviating this kind of transcriptional pausing due to nucleosomal barriers (23, 24).

Once Pol II has entered the elongation phase, two major phosphorylation sites in its CTD are targeted by either TFIIH at Ser 5 during early elongation, or by Ctk (CTD kinase) at Ser 2 later in the elongation phase (14). These phosphorylation sites are responsible for the association of the elongating polymerase with various elongation factors and chromatin remodelers. Chromatin remodeling enzymes of both classes are required to appropriately modify and mobilize the oncoming nucleosomes as Pol II transcribes through a gene. As Pol II advances it also displaces histones, and chromatin remodeling factors are required to catalyze the redeposition of these histones as well as the reassembly of free histones onto the DNA behind the elongating polymerase. Depending on the phosphorylation status of the Pol II CTD, different chromatin remodeling enzymes are recruited to facilitate the process of transcriptional elongation by carrying out the above functions (13).

Covalent Modification of Histones

The histones that form the protein core of the nucleosome are largely globular, except for their N-terminal tails which are unstructured and protrude away from the core bodies. A large number of histone residues, with a majority of them being on the N-

terminal tails, are known to be covalently modified. There are eight known histone modifications, including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation. A further level of complexity is introduced by that fact that methylation can be of varying degrees, as histone lysine residues can be mono-, di- or tri-methylated (25). The deposition and removal of each of these different histone modifications is catalyzed by distinct histone modifying enzymes like HATs, histone deacetylases (HDACs), HMTs or histone demethylases.

Histone modifications function through two different mechanisms. The first is the alteration of chromatin structure by disrupting histone-DNA contacts via the covalent modifications. Histone lysine acetylation, for example, is capable of unfolding chromatin by neutralizing the charged lysine residue. Since inter-nucleosomal interactions have been seen to be necessary for maintaining chromatin structure, any changes in histone charge would affect the chromatin architecture (25). In fact, by chemically modifying histones and reconstituting them into nucleosomes, it has been shown that H4K16 acetylation prevents formation of the 30 nm chromatin fiber and of higher order chromatin structures (26).

The other mechanism by which histone modifications function is by the recruitment of non-histone proteins to the modified sites. These proteins recognize specific histone modifications through certain specialized domains. For example, bromodomains bind to histone acetylation marks, chromodomains and PHD (Plant Homeodomain) domains bind methylation marks and certain domains found in 14-3-3 proteins bind phosphorylation marks (25). Thus these histone marks can be used to tether various enzymatic activities to the chromatin, further affecting regulation of its structure.

There are several examples of different chromatin remodeling activities being targeted to chromatin via interaction with modified histones. These interactions are mediated either by specific recognition domains within the chromatin remodeling enzymes themselves or through such domains present in other intermediary proteins that are in complex with these enzymes.

The ATP-dependent chromatin remodeling activity of SWI/SNF (mating type switching/sucrose non-fermenting) is targeted to chromatin by the recognition of acetylated histones by its bromodomains (27). The histone demethylase JMJD2A (Jumonji domain-containing) contains Tudor domains which bind methylated H3 lysine 4 (H3K4) residues (28). This same methyl mark tethers the chromatin remodeling activity of CHD1 (Chromodomain Helicase DNA-binding) through its chromodomains (29, 30). The PHD domains of ING2 (Inhibitor of growth) and BPTF (Bromodomain PHD finger Transcription Factor) bind methyl-H3K4 marks and mediate the recruitment of other proteins containing different chromatin remodeling activities, like HDAC1 and the ATP-dependent remodeling NURF (Nucleosome Remodeling Factor) complex, respectively (31, 32). The chromodomains of HP1 (Heterochromatin Protein) tethers HDAC and HMTase activities to H3K9 methylated target sites (33-35). Recruitment of these different chromatin remodeling activities to target sites facilitates further regulation of chromatin structure and consequently of gene expression. In fact, the extensive modification of histones on a larger scale in the cell results in the establishment of distinct global chromatin environments. The genome can thus be generally divided based on the modification and architecture of the chromatin into the transcriptionally active, accessible euchromatin and the transcriptionally inactive, inaccessible heterochromatin.

ATP-Dependent Chromatin Remodeling Enzymes

The chromatin-mediated regulation of transcription requires the coordination of the covalent modification of histones with the appropriate mobilization and positioning of nucleosomes on genomic DNA. The latter function is achieved by the action of ATP-dependent chromatin remodeling enzymes, which can use the energy from ATP hydrolysis to slide nucleosomes along a DNA template, disrupt DNA-histone contacts or eject nucleosomes entirely from the underlying DNA (Fig. 1.1). These enzymes are also known to reassemble nucleosomes on DNA by incorporating free histones and to replace histones within nucleosomes with variant forms. By affecting these changes in nucleosomal organization, these enzymes are able to regulate accessibility to the genomic DNA.

All the identified enzymes in this class contain an ATPase domain called the Snf2 helicase domain which is the characteristic feature of the SNF2 superfamily of proteins. The SNF2 superfamily of ATP-dependent remodeling enzymes is classified into different families according to the presence of other characteristic domains (Fig. 1.2). All eukaryotes contain at least five families of SNF2 helicase-containing chromatin remodelers: SWI/SNF, ISWI (Imitation Switch), CHD, INO80 (Inositol requiring) and SWR1 (SWI/SNF Related) (36). The Rad54 family of proteins can also be included with these since they can alter nucleosomes *in vitro* and may remodel nucleosomes to regulate access during DNA repair (37).

SWI/SNF Family

The SWI/SNF family, which includes yeast SNF2 and STH1 as well as mammalian BRM (Brahma) and BRG1 (Brahma Related Gene), is characterized by the presence of bromodomains, which recognize acetylated residues on histone tails (27). Yeast SWI/SNF is targeted to acetylated chromatin by its bromodomain and the tandem bromodomains in the RSC (Remodels the Structure of Chromatin) complex recognize acetylated H3 K14 residues, thus targeting their chromatin remodeling activities to specific loci (27, 38). These remodelers tend to reorganize nucleosomes into more disordered arrays (39, 40). Hence they are thought to participate in transcriptional regulation by disrupting chromatin to allow the binding of transcriptional activators or repressors (36, 41, 42).

ISWI Family

The ISWI family includes yeast Isw1 and Isw2 and their mammalian homologs SNF2H (SNF2 homolog) and SNF2L (SNF2-like). They are distinguished by the presence of SANT (SWI3, ADA2, NCOR, TFIIB) and SLIDE (SANT-like domain) histone binding modules. The SANT domain is believed to be involved in binding to histone tails, while the SLIDE domain may bind to the linker DNA between adjacent nucleosomes (43, 44). ISWI proteins primarily organize and position nucleosomes into ordered arrays (45, 46). This family of remodelers is generally thought to have a role in chromatin assembly and maintenance and in the translational phasing of nucleosomes following DNA replication (47, 48). Since their function is critical in maintaining heterochromatin, ISWI proteins can play a repressive role during transcriptional

regulation (46). However, there are also examples where ISWI can promote transcriptional elongation (49).

INO80 and SWR1 Families

The INO80 and SWR1 families are characterized by the presence of a spacer region which splits the conserved ATPase domain within these proteins (50). The INO80 proteins are the only SNF2 proteins known to have DNA helicase activity and are involved in DNA repair (51). SWR1 proteins are known to be capable of exchanging H2A for the H2A.Z variant form of histones (52). Thus the SNF2 superfamily of ATP-dependent remodeling enzymes is a diverse family of proteins, with varying specificities and functions in the context of chromatin.

CHD Family

The family of chromatin remodeling enzymes we are interested in studying is the CHD (Chromodomain Helicase DNA-binding) family. While there is only one CHD protein found in yeast (CHD1), there are nine identified human enzymes belonging to this family, namely CHD1 thru CHD9. These proteins are characterized by the presence of tandem chromodomains toward their N-terminus along with the Snf2 helicase domain located in the central part of these proteins (53, 54). The chromodomain is an evolutionarily conserved sequence motif associated with many proteins involved in chromatin remodeling and transcriptional regulation. Chromodomains are believed to mediate the contact of these proteins with chromatin by binding methylated residues of histones (55).

The CHD family of proteins can be further subdivided into three subfamilies based upon the presence of additional domains (55). The CHD1-CHD2 subfamily contains a DNA-binding domain in their C-terminal region which binds AT-rich DNA sequences (54, 56). The CHD3-CHD5 subfamily is characterized by the presence of a pair of N-terminal PHD Zn-finger-like domains. PHD domains are protein-protein interaction domains found in several nuclear proteins associated with chromatin remodeling and transcriptional regulation and have been implicated in binding to methylated histones (31, 57-59). The third subfamily includes the proteins CHD6 to CHD9 that contain other domains in their C-terminal region like paired BRK (Brahma and Kismet) domains, SANT domains and DNA-binding domains. The BRK domain is conserved in a number of higher eukaryotic chromatin remodeling enzymes but not in their yeast homologs, indicating the possibility of them being involved in a function or interaction of chromatin components specific for higher eukaryotes (55). SANT domains and DNA-binding domains are similar to those found in the other SNF2 families mentioned above.

CHD1-2 Subfamily

CHD1 is the only member of this subfamily found in yeast, but higher eukaryotes also have a CHD2 protein, that is highly homologous to CHD1 in all its conserved domains. However, they are significantly divergent in their C-terminal regions, suggesting that these two proteins could have different functions. The majority of previous reports about CHD1 suggest that it is involved in transcriptional elongation due to associations with various elongation factors and with the elongating form of Pol II (60-62). Therefore this subfamily of CHD proteins appears to play a role in general

transcription downstream of the involvement of SWI/SNF remodelers which are more involved with transcription factor binding and transcriptional initiation (36, 61). The nucleosomal remodeling properties of CHD1 have been shown to be quite distinct from those of SWI/SNF remodelers (63) and more similar mechanistically to certain members of the ISWI family (64). In fact, a recent report has shown that CHD1 is involved in deposition of the histone variant H3.3 and plays a role in early developmental, replication-independent nucleosomal assembly (65), exhibiting further functional similarities to the ISWI family. The limited studies on CHD2 report that it is involved in DNA-damage response (66) and in maintaining kidney function (67), both of which could be in connection with some of the general roles ascribed to this subfamily above. These proteins, therefore, appear to display a fair amount of functional diversity.

The chromodomains of human, but not yeast, CHD1 have been shown to recognize and bind methylated H3K4 tails from *in vitro* experiments and structural data (30). Interestingly, due to variation in the length of a linker sequence within the chromodomains of CHD1 and CHD2, CHD2 shows weaker binding to H3K4me3 (68). This interaction has been shown to target human CHD1 to sites of active transcription and mediate the recruitment of the spliceosome, which is required for the proper splicing and maturation of the transcribed pre-mRNA (69). This suggests a mechanism for the involvement of the CHD1 subfamily proteins in general transcription.

Both CHD1 and CHD2 are ubiquitously expressed in all human tissues, further supporting a role in universal transcriptional elongation for these proteins. In yeast, however, CHD1 has been shown to interact with H3K4me *in vivo* and to recruit the SAGA and SLIK (SAGA-like) multi-subunit HAT complexes to chromatin (29). These

complexes are known coactivator complexes that are involved with transcriptional initiation at promoter regions rather than the elongation events occurring in the gene body (70). Furthermore, H3K4 methylation is known to be associated with transcriptional start sites and CHD1 has been shown to be required for this methylation in yeast (71), indicating a point of action early in the transcriptional cycle. While the binding of the CHD1 chromodomains to methylated histones appears somewhat ambiguous, it is clear that this subfamily is likely to have evolved in function from yeast to higher eukaryotes. It is to be noted that since CHD1 is the only CHD family member found in yeast, its function may have been more diverse in that model system. Indeed, there are evidences of yeast CHD1 being involved in transcriptional elongation and mRNA processing as well (60, 72). This supports the hypothesis of a more functionally redundant CHD1 in yeast evolving into a more specialized elongation factor in mammals. This is consistent with the fact that a single CHD protein in yeast has evolved into nine CHD proteins in mammals. Thus it is likely that there has been a trend towards the specialization of function of this family of proteins over the course of evolution.

CHD3-5 Subfamily

The second subfamily of CHD proteins includes CHD3 and CHD4, also known as Mi-2α and Mi-2β, and CHD5. These proteins lack a C-terminal DNA binding domain and instead have a pair of PHD domains in their N-terminal region. This family has a single homolog in *Drosophila* called dMi-2. The best known function of CHD3 and CHD4 is as the ATPase components of the NURD (Nucleosome Remodeling and Deacetylation) complex which is involved in transcriptional repression (73, 74). This complex also contains HDAC1/2 subunits and is involved in the active deacetylation and

remodeling of chromatin, resulting in the establishment of transcriptional repressed loci (75, 76).

CHD3 and CHD4 appear to be largely redundant in their functions in relation to this repressive complex, except for the evidence that CHD3-containing complexes, with similar composition as NURD, are specifically recruited by the corepressor KAP1. This interaction is mediated by a C-terminal region that is unique to CHD3 and is responsible for recruiting these complexes to specific genes targeted for repression by KAP1 (77). Thus CHD 3 and CHD4 are both involved in NURD-mediated transcriptional repression, with CHD3 having some involvement in corepressor-specific repression of certain loci. On a more global scale, NURD has been reported to be targeted to sites of DNA methylation by its MBD (Methyl CpG-binding Domain) subunits, and this could be the mechanism of recruitment of the majority of NURD complexes to sites marked for transcriptional silencing by CpG methylation (78). In fact, CHD4 has been reported to be more abundant in purified NURD complexes and is suspected to be the major, or perhaps the sole Mi-2 isoform present in these complexes (73, 76). Thus, it may be possible that CHD4 is involved in these more generally recruited NURD complex involved in DNA methylation-mediated silencing, while the less abundant, more specialized, CHD3-containing NURD complexes may be recruited by KRAB (Kruppel-associated box) domain proteins like KAP1 to specific target sites. It is clear, however, that this subfamily has a repressive role in transcription and thus has evolved distinctly in function from the CHD1-2 subfamily, which is involved in transcriptional elongation.

Further differences in CHD3-5 and CHD1-2 subfamilies are seen in the binding characteristics of their chromodomains. Structural analyses of the CHD3/4

chromodomains predict that they would not bind to methylated H3K4 residues (30). Consistent with this prediction, methylation of H3K4 has been found to preclude the binding of NURD to H3 tails (79, 80). NMR studies of the CHD4 PHD domains have revealed that they preferentially bind methylated H3K9 and that H3K4 methylation reduces binding to unmodified H3 tails, further strengthening the hypothesis that CHD3-4 bind to chromatin with a specificity and mechanism distinct from CHD1-2 (81). H3K4 methylation is associated with transcriptionally active chromatin and H3K9 is a mark of repressive chromatin. Thus these observations about the binding properties of CHD3-4 are consistent with their functional role in transcriptional repression. The nucleosomal remodeling properties of Mi-2, however, have been shown to be similar to those of CHD1, with a preference to mobilize nucleosomes towards the center of a DNA template (82). Thus it appears that while the catalytic properties of these subfamilies of CHD proteins have been conserved by maintaining the sequence homology in their SNF2 domains, they have evolved considerably in function. Their specificity for chromatin has been changed by the divergence in their chromodomains and by the presence of distinct histone-binding modules. Their association with other factors, mediated through their non-conserved sequences, is likely to be responsible for the modulation of their catalytic activity so they can be adapted for distinct functional roles.

Unlike CHD3 and CHD4, very little is known about the cellular function of CHD5. It has been shown to be expressed in neural tissue (83) and was found to be a tumor suppressor gene that was deleted in brain tumors (84). More recent reports have shown that the CHD5 gene is silenced by hypermethylation of CpG islands in various cancers (85, 86). From evolutionary analysis of this subfamily it appears CHD5 is the

product of a late gene duplication event of CHD4. Thus it is possible it may be functionally similar to CHD4, playing a role in transcriptional repression, which would explain its potential as a tumor suppressor. It is equally likely that its specificity and function would have changed some, since its expression pattern and role in human disease appear to be distinct from CHD3/4.

CHD6-9 Subfamily

The last subfamily of CHD proteins is the CHD6-9 subfamily, characterized by the presence of C-terminal domains like SANT and BRK domains in addition to the chromodomains and the conserved SNF2 domain. This subfamily also has a single *Drosophila* homolog, Kismet, which also contains a BRK domain in its C-terminal region. While the CHD1-2 and CHD3-5 subfamilies have been extensively studied in terms of their functional roles, relatively little is known about the CHD6-9 subfamily.

Kismet was identified as a trithorax group protein which acted as an extragenic suppressor of Polycomb mutations (87). The Polycomb (Pc) group of repressors and the trithorax group of activators act in concert to regulate the expression of the homeotic (Hox) genes, which are transcription factors responsible for body segmentation and segment identity during *Drosophila* development (88). Since Kismet acts antagonistically to Pc, it appears to have a role in transcriptional activation. One report confirms this theory by elucidating a role for Kismet during an early step in transcriptional elongation (61). By looking at the localization patterns of Kismet and other factors involved in transcriptional elongation on polytene chromosomes, this study found that Kismet localized to sites of transcriptionally active chromatin and was

required for the recruitment of elongating Pol II, dCHD1 and other elongation factors to these sites. Kismet was thus found to be involved upstream of CHD1 in the transcriptional cycle, but downstream of BRM action in transcriptional initiation. Thus Kismet was predicted to be required for the transition from early to late stages of transcription, perhaps in relieving the phenomenon of transcriptional pausing and facilitating promoter clearance by the polymerase, as discussed earlier in this chapter.

It has also been observed that Kismet colocalized with the trithorax group proteins ASH1 and TRX, which are involved in the methylation of H3K4 (89). The loss of Kismet resulted in a reduction in the level of these two proteins associated with chromatin and an increase in H3K27 methylation, while global H3K4 methylation levels did not change. Since H3K27 methylation is a mark of Pc group silencing, Kismet appears to counteract this silencing by perhaps recruiting a H3K27 demethylase. Thus Kismet is involved not only with the basic mechanism of transcriptional elongation, but also with gene-specific coactivator-mediated transcriptional response.

Consistent with the potential general and locus-specific transcriptional roles of Kismet, the human CHD6-9 members have been found to have varying cellular functions. CHD6, for example is associated with both hyper- and hypo-acetylated Pol II and localizes to sites of mRNA synthesis, suggesting a role in general transcriptional elongation (90). It is also known to be involved in the transcriptional activation of specific genes involved in cellular redox homeostasis by the Nrf2 transcription factor (91).

CHD7 mutations are known to be associated with a complex human disorder called CHARGE syndrome, which is characterized by the following symptoms: ocular Coboloma, Heart defects, Atresia of chonae, Retardation of growth and development, Genital hypoplasia and Ear anomalies (92, 93). CHD7 has also been implicated in early neural development (94). CHD7 has also been shown to be important in the process of osteoblastogenesis from bone marrow mesenchymal stem cells by attenuating PPAR-γ-(peroxisome proliferation activated receptor)-mediated adipogenesis in response to Wnt signaling in these cells (95). CHD7 was found to be a component of a H3K9 HMTase-containing complex which interacts with PPAR-γ in response to Wnt signaling at response elements of adipogenic target genes, attenuates recruitment of PPAR coactivators at these sites and facilitates the transcriptional repression of these genes driving the stem cells towards an alternative osteogenic cellular fate. In the same study, the chromodomains of CHD7 were found to selectively interact with H3K4me3 and K3K9me3, but not with acetylated histones, showing the further specialization of these chromodomains of the CHD family proteins. The recognition of both activating and repressive histone marks by CHD7 shows that the CHD6-9 subfamily may have contrasting roles in both these forms of transcriptional regulation unlike the other subfamilies.

CHD9, also known as CReMM (Chromatin-Related Mesenchymal Modulator), has been shown to be differentially expressed in marrow mesenchymal cells during bone development (96) and to localize to promoters of genes involved in osteogenic cell function (97). Thus CHD9 and CHD7 have parallel lines of evidence suggesting their involvement in developmental regulation of gene expression of skeletal tissue.

Furthermore, it was seen that CHD9, like CHD7, participates in osteogenic gene regulation via associations with nuclear receptors. CHD9 was found to mediate the transcriptional response of hormones like dexamethasone (Dex) and 17β-estradiol by the glucocorticoid receptor (GR) and the estrogen receptor (ER) respectively during osteogenic stem cell differentiation (98, 99). In another study CHD9 was shown to act as a transcriptional coactivator of PPAR-α and also interact *in vitro* with other nuclear receptors like constitutive androstane receptor (CAR), retinoid X receptor (RXR) and ER. CHD9 does not, however, interact with PPAR-γ like CHD7, exhibiting mutually exclusive associations with different isoforms of PPAR and indicating the divergence in specificity between different subfamily members. Also, CHD9 has been reported to be a coactivator of nuclear receptors whereas CHD7 was shown to act in the repression of PPAR-γ signaling, once again showing distinguishing functionalities within this subfamily. The above examples from literature suggest a role for CHD6-9 in nuclear receptor-specific transcriptional regulation and perhaps in a more general capacity in the developmental regulation of different tissues.

CHD8

The focus of my thesis research is the fourth member of the CHD6-9 subfamily, CHD8. Previous reports from our group have identified CHD8 as a *bona fide* ATP-dependent chromatin remodeling enzyme and ascribed a transcriptional role for this protein. In fact, among all the members of the CHD6-9 subfamily, CHD8 is the best understood enzyme from a functional aspect. Reports from previous literature alluding to the cellular function of CHD8 indicate that this protein, like others member of this subfamily, is a functionally diverse molecule, with predicted roles in both locus-specific

and general transcriptional processes. CHD8 has been shown to interact with elongating RNA Pol II and regulate the expression of the cyclin E2 cell cycle regulatory gene (100). It has also been reported to be involved in RNA Pol III-mediated transcription in association with human Selenocysteine tRNA Activating Factor (hStaf), in addition to its role in Pol II transcription (101). Another report suggests that CHD8 recruits the linker histone H1 to p53 target genes and inhibits apoptosis during embryogenesis (102). All these examples suggest that CHD8 could have an activating role in general transcriptional elongation, like the CHD1-2 subfamily, as well as in histone deposition and assembly of repressive chromatin structure like CHD3-5 proteins.

CHD8 also interacts with CTCF through its BRK domains and cooperates with CTCF in facilitating transcriptional repression through its insulator function at specific genomic loci (103). Previous studies from our lab have also shown a role for CHD8 in the negative regulation of β-catenin responsive genes (104). The remodeling properties of CHD8 are similar to those of CHD1 and ISWI, with a propensity to rearrange a random array of nucleosomes into a couple of ordered conformations, with a preference for positioning nucleosomes towards the center of a DNA template (104). A couple of the studies mentioned above have also reported that the chromodomains of CHD8 can bind methylated H3K4 (100, 101), another similarity to both CHD1 and CHD7. CHD8 has also been recently reported to be involved in the ER-responsive activation of the cyclin E2 gene, once again highlighting the variety of roles that the CHD6-9 subfamily members are capable of exhibiting (105).

All these examples from literature indicate that CHD8, like its CHD6-9 subfamily relatives, can be involved in both transcriptional activation and repression, in both

general and in specific contexts. We also see the continued involvement of this subfamily in nuclear receptor-mediated transcriptional regulation making this an attractive field for further studies into the functional relevance of these proteins. Thus we see that CHD8, and the other members of the CHD6-9 subfamily are chromatin remodeling enzymes with diverse cellular functions, and appear to have specialized evolutionarily to develop distinct functions in various transcriptional processes, both in different specific systems like those mediated by various nuclear receptors as well as in different steps in basic transcription.

Research Aims

This dissertation is a description of my research work which was designed to address the following objectives:

1. To investigate the transcriptional function of CHD8 by studying its role in the regulation of androgen-responsive transcription.
2. To elucidate further mechanistic details of chromatin remodeling by CHD8 by characterizing its substrate specificity to determine how this aspect of its activity may affect its transcriptional function.
3. To characterize the novel interaction of CHD8 with the MLL1-WAR histone methyltransferase complex and investigate how this may affect its functional role.

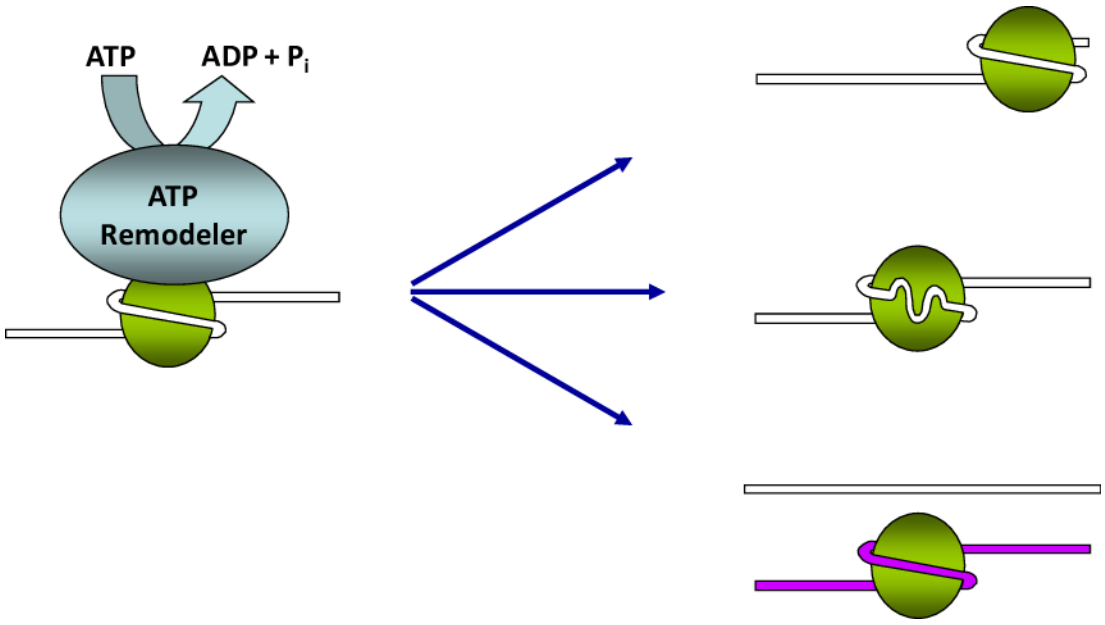

Figure 1.1: ATP-dependent chromatin remodeling. ATP-dependent chromatin remodeling enzymes contain a characteristic Snf2 domain, which is capable of binding and hydrolyzing ATP. These enzymes can utilize the energy derived from ATP hydrolysis to mobilize nucleosomes along a DNA template, disrupt histone-DNA contacts within a nucleosome or displace histone octamers from one DNA template to another, as shown above.

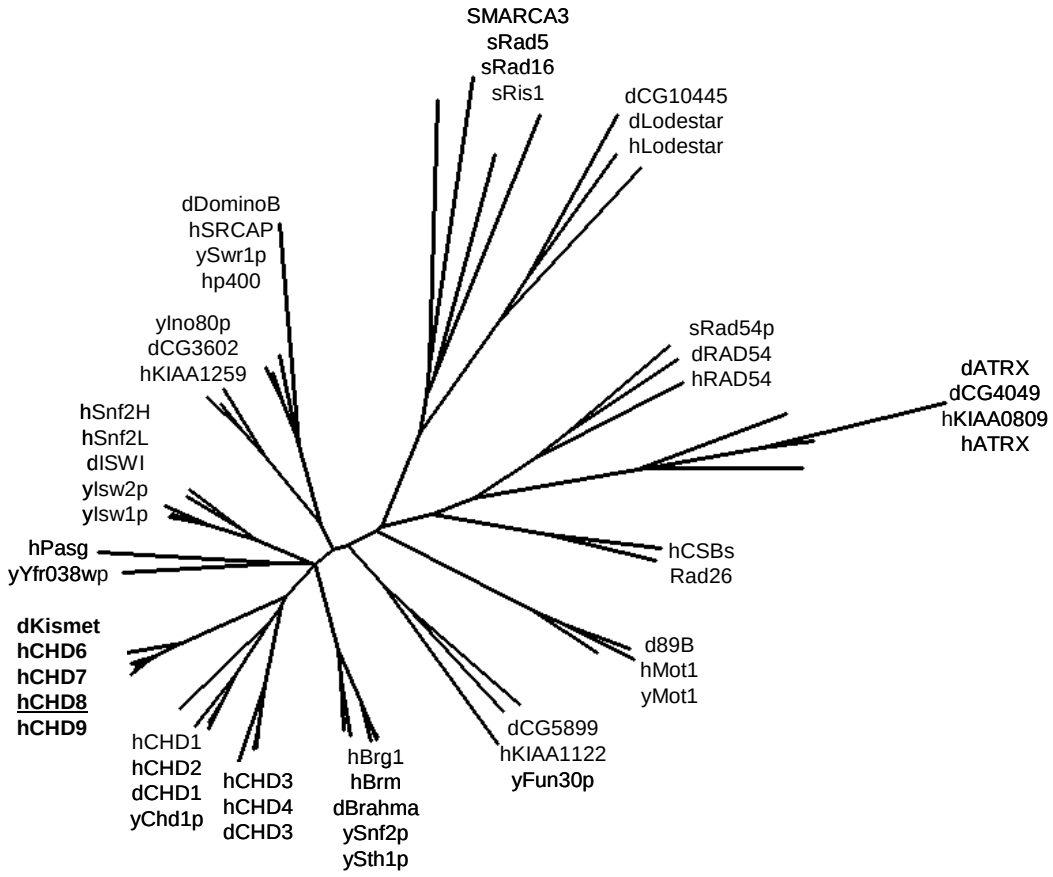

Figure 1.2: Evolutionary analysis of the SNF2 family of proteins. Alignments for analysis were generated with representative sequences from humans (h), Drosophila (d), and *S. cerevisiae* (y/s) and included only the SNF2 domain. Neighbor-joining distance analysis employed the PHYLIP 3.6 software package. SEQBOOT and the aligned sequences were used to generate 500 bootstrap replicates. Pairwise distances were estimated using PROTDIST with the "Dayoff" option invoked. Neighbor-joining trees were generated using these pairwise distances in NEIGHBOR. CONSENSE was used to derive the consensus tree. Branch lengths on the consensus tree were generated using FITCH, the pairwise distance output from PROTDIST using the aligned sequences, and the neighbor-joining consensus tree as a user tree. Figure courtesy of D.A. Bochar.

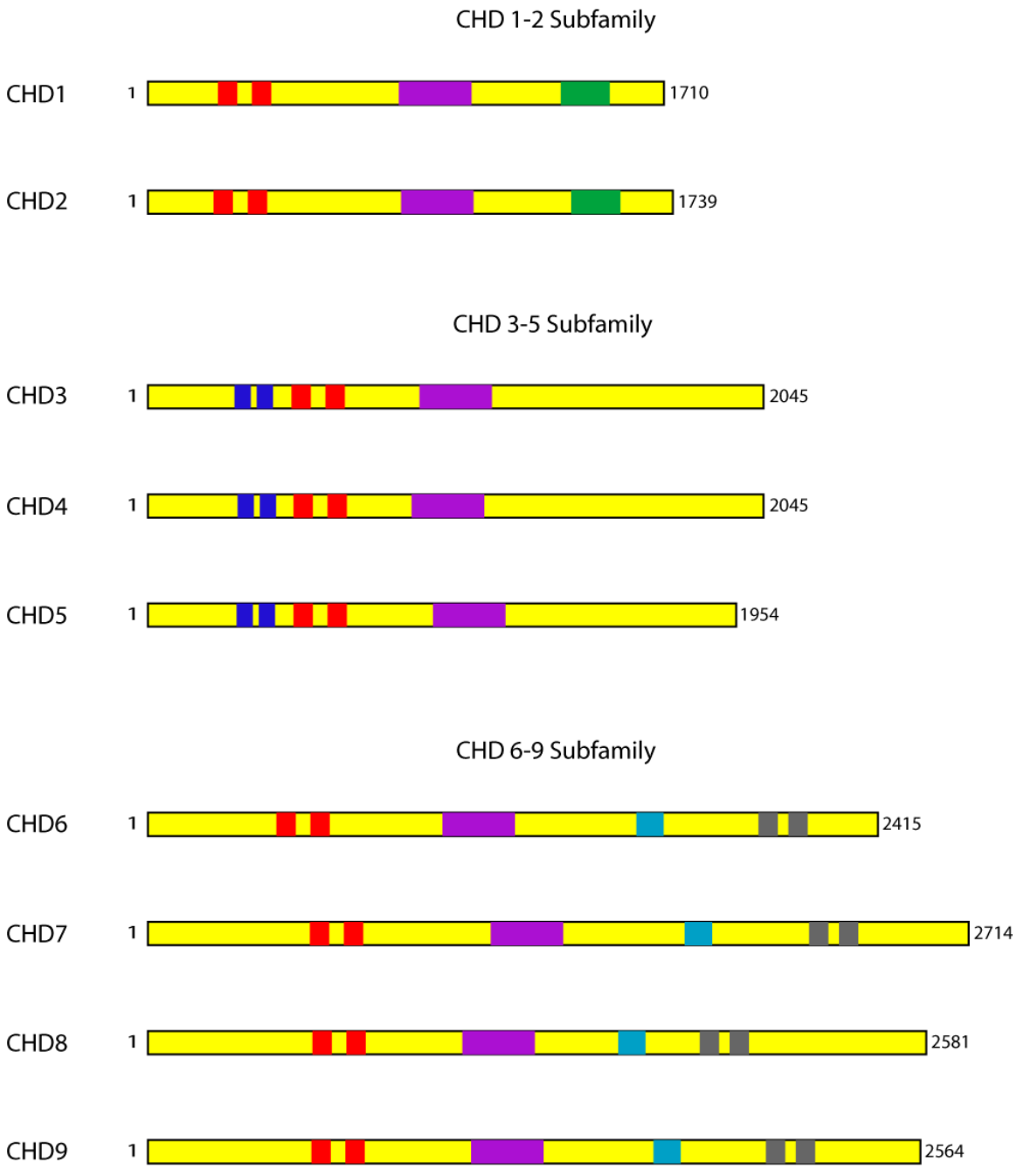

Figure 1.3: Domain architecture of the CHD family of chromatin remodeling enzymes. The CHD family consists of nine proteins, all of which contain the characteristic N-terminal tandem chromodomains (red) and the central catalytic Snf2 domains (purple). The three subfamilies within this group are distinguished by the presence of additional domains, like the C-terminal DNA-binding domains (green) of the CHD1-2 subfamily, the N-terminal double PHD fingers (blue) of the CHD3-5 subfamily and the C-terminal SANT domains (light blue) and paired BRK domains (grey) of the CHD6-9 subfamily. Domains identified using the SMART database (106, 107).

CHAPTER II

Regulation of Androgen-Responsive Transcription by CHD8

Introduction

Androgens and Prostate Development

The prostate is a male glandular organ located at the base of the urinary bladder comprised of glandular and muscular tissue (108). The prostatic secretions of the glandular tissue, which are responsible for semen coagulation and liquefaction, are dispelled into the urethra by muscular contractions of the prostate upon ejaculation (109). The action of testicular androgens, like testosterone or dihydrotestosterone (DHT), mediated by the transcriptional activity of the androgen receptor (AR), plays a critical role in the development, growth and normal function of the prostate (110). The prostate normally ceases to grow upon reaching maturity, but in some men androgen-dependent prostate growth may resume, resulting in benign prostate hyperplasia (BPH), pre-malignant prostatic intraepithelial neoplasia (PIN) or prostate cancer (PCa), depending on the degree of malignancy of this continued growth (111).

Prostate Cancer

Prostate cancer is currently the most commonly diagnosed form of cancer and the second leading cause of cancer deaths among males in the United States (112). The dysregulation of androgen-responsive AR signaling has been implicated in the development and progression of prostate cancer. Over 80% of prostate cancers are androgen-dependent at initial diagnosis, and thus most common therapeutic approaches are directed towards androgen ablation or inhibition of AR (113). These methods prove to be effective initially in causing the regression of androgen-dependent tumors, thus highlighting the role of AR activity in early prostate tumorigenesis. However, these treatments often ultimately fail due to progression of the prostate cancer to a hormone refractory state (114). While it is known that the androgen receptor and its transcriptional coregulators play a key role in the progression of prostate cancer, the precise mechanism of their involvement are still not fully understood.

The Androgen Receptor

The androgen receptor is a member of the nuclear receptor superfamily (115). It functions as a ligand-dependent transcription factor, mediating the effects of androgens on cellular responses. The domain architecture of AR (Fig. 2.1) is similar to that of other nuclear receptors, and is comprised of an N-terminal transactivation domain, a central DNA-binding domain, and a C-terminal ligand-binding domain (116). In the absence of ligand, AR is sequestered in an inactive state by a protein-chaperone complex in the cytoplasm (Fig. 2.2). Upon exposure to androgens, AR dissociates from these complexes and binds the ligand, whereupon it dimerizes and translocates to the nucleus, where it can

then bind to specific DNA sequences, called androgen response elements (AREs), located in the target genes (116, 117).

Two activation function (AF) domains, AF1 and AF2, then serve to recruit coactivators that can alter the chromatin structure to facilitate transcription, stabilize AR/DNA interactions, and recruit the general transcriptional machinery (118-120). Some of the proposed mechanisms for the transition of androgen-dependent prostate cancer to an androgen-independent state include the increased expression of AR or its associated factors, mutations of AR that make it responsive to a broader spectrum of ligands, activation of the receptor through alternate pathways, and altered function of the AR coregulators (117, 121, 122).

Androgen Receptor Target Genes

The androgen receptor binds to and regulates the expression of a subset of genes which are important for prostate growth and function. The Prostate-Specific Antigen (PSA) gene is one of the best characterized AR target genes. PSA belongs to the human tissue kallikrein gene family and it is expressed in the prostate epithelium as well as in prostate cancer and is thus the most commonly used bio-marker for prostate cancer (123). It is an androgen-regulated serine protease that gets secreted by the glandular epithelium of the prostate into the lumen, where it is responsible for cleaving specific protein components of the semen coagulum (124). The transcription of PSA is positively regulated by AR and it has been extensively studied as the model AR target gene (125). PSA contains a consensus ARE site located between -156 to -170 base pairs (bp) from its transcriptional start site (126) and a non-consensus ARE between -365 and -400 bp

upstream of the start site (127). These are displayed as ARE I and ARE II in the schematic representation of the PSA gene in Fig. 2.3. Further studies have mapped the region responsible for androgen-responsive PSA expression to a fragment of about 450 base pairs, located approximately 4 kb upstream of the transcriptional start site (128). This locus contains one strong consensus ARE as well as multiple non-consensus AREs and is called the PSA distal enhancer or ARE III (Fig. 2.3).

Another well-characterized AR target gene is the Transmembrane Protease, Serine 2 (TMPRSS2) gene. This is another androgen-regulated serine protease produced in basal prostate cells as well as in primary prostate cancer cells (129). It has been reported that the TMPRSS2 gene is found to be fused with the ETS family oncogenes, ERG or ETV, in 80% of prostate tumors (130) making it also an attractive bio-marker for cancer. The enhancer region of the TMPRSS2 gene, around 13.5 kb upstream from the start site (Fig. 2.3), has been characterized as an ARE to which AR binds in an androgen-responsive fashion and upregulates the expression of this gene (131). Other AR target genes implicated in prostate cancer include and FKBP5 (132), FGF8 (133), Cdk1 and Cdk2 (134).

Androgen Receptor Coactivators

The best characterized coactivators for nuclear receptors are members of the p160 steroid receptor coactivator (SRC) family; SRC-1 (NCoA-1), SRC-2 (GRIP1, TIF2, or NCoA-2) and SRC-3 (p/CIP, RAC3, ACTR, AIB1, or TRAM-1) (135). These primary coactivators can facilitate AR-mediated transcriptional activation either directly through their own intrinsic histone acetyltransferase activity (136), or indirectly through the

recruitment of secondary coactivators, such as CBP, p300, p/CAF, CARM-1, and PRMT1 (137). These in turn catalyze site specific acetylation and methylation events at the target promoter that modify the chromatin structure (135, 138). Chromatin remodeling events are not just limited to covalent modifications, as ATP dependent chromatin remodeling enzymes also play a key role in activation by AR. These include the human SWI/SNF chromatin remodeling complex (139-143), SRCAP (144), and ARIP4 (145-147).

Coactivators and Prostate Cancer

Several of these coactivators have been implicated in prostate cancer, specifically in AR-mediated control of primary prostate cancer tumorigenesis and progression (148). Furthermore, alterations in AR-coregulator levels and function have also been proposed to contribute to the emergence of the hormone-refractory disease (149). Members of the SRC family of coactivators have been found to be expressed at higher levels in prostate cancers and to have a role in tumor growth, progression and recurrence (150-152). In addition to these primary coactivators, certain chromatin remodeling coregulators have also been implicated in prostate cancer. The histone acetyltranferase p300 has been shown to promote prostate cancer progression and aggressiveness through the modulation of nuclear morphology (153, 154). The histone demethylases LSD1 and JMJD2C have also been shown to coactivate AR and affect prostate cancer cell proliferation (155, 156). Finally, the ATP-dependent chromatin remodeling complex, SWI/SNF, coactivates AR-mediated transcription and is targeted by its BAF57 (Brahma-associated factor) subunit which is aberrantly expressed in prostate cancer (140, 157). In order to better understand the mechanisms of prostate cancer and develop more effective therapies against it, it is

desirable to study the association and interplay of AR with its many coregulators, both novel and those previously identified.

ATP-dependent Chromatin Remodeling

The regulation of transcription is contingent upon the coordination of the recruitment of the transcriptional machinery with the appropriate modification and remodeling of chromatin to allow access to nucleosomal DNA. This regulation of chromatin structure is achieved by two classes of chromatin remodeling enzymes: one that is involved in the covalent modifications of histones, and the other that uses energy derived from ATP-hydrolysis to modulate the contacts between histones and DNA within the nucleosome. The ATP-dependent remodeling enzymes usually occur in large complexes that alter chromatin structure by disrupting or mobilizing histones to regulate access to the nucleosomal DNA. All the identified enzymes in this class contain an ATPase domain, called the Snf2 helicase domain, which is the characteristic feature of the SNF2 superfamily of proteins.

CHD Family of Chromatin Remodelers

The SNF2 superfamily of ATP-dependent remodeling enzymes is further classified into different families, like the SWI/SNF, ISWI and CHD families, based upon the presence of other characteristic domains in their domain architecture. The CHD (Chromodomain Helicase DNA-binding) family includes CHD1 thru CHD9, and they are characterized by the presence of tandem chromodomains toward their N-terminus along with the Snf2 helicase domain located in the central part of these proteins. The CHD family of proteins can be further subdivided into three subfamilies based upon the

presence of additional domains. The CHD1-CHD2, the CHD3-CHD5 and the CHD6-CHD9 subfamilies are each characterized by the presence of distinct domains in their C-terminal region. While the CHD1-2 and CHD3-5 subfamily proteins have been well studied in the context of their chromatin remodeling activity and their functional role in transcriptional regulation, relatively little is known about the CHD6-9 subfamily.

CHD8

Previous work from our group has shown that CHD8 is an ATP-dependent chromatin remodeling enzyme involved in transcriptional regulation of β-catenin responsive genes (104). Other studies have linked CHD8 to CTCF-mediated chromatin insulator function (103), to RNA polymerase III (RNAP III) transcription in association with hStaf (101), to control of p53-mediated apoptosis (102) and to RNAP II-associated transcription of the cyclin E2 gene (100). Thus CHD8 displays a fairly versatile portfolio of functions in transcriptional regulation. A recent report shows that CHD8 is required for optimal estrogen-responsive induction of the cyclin E2 gene (158). There is additional evidence that the very closely related CHD family member CHD9, also known as PRIC320 or CReMM, acts as a coactivator of the nuclear receptor peroxisome proliferator activated receptor (PPARα) (159) and also interacts with the glucocorticoid receptor (98) and estrogen receptor (99). These observations from previous studies, raise the possibility that CHD8 may be involved n transcriptional regulation by other nuclear receptors.

Hypothesis and Summary of Results

The implication of CHD8, and its homologous subfamily member CHD9, functioning as coactivators for other nuclear receptors prompted us to further investigate connections between CHD8 and nuclear receptor-mediated transcriptional regulation. In order to do this, publicly available gene expression sets were examined using the ONCOMINE database (160). This investigation revealed that CHD8 was found to be significantly upregulated in several prostate cancer versus normal tissue data sets (161-165). We have previously identified and characterized CHD8 as an ATP-dependent chromatin remodeling enzyme involved in the regulation of gene transcription (104). Given the fact that many AR coactivators are upregulated in prostate cancer (147), the finding that CHD8 is upregulated in several studies suggests that CHD8 may function in the regulation of AR mediated transcription.

In these studies we show that endogenous CHD8 associates with AR in prostate cancer cells by performing co-immunoprecipitations from nuclear extracts. We further demonstrate that this association is due to a direct physical interaction, by co-expressing the proteins in insect cells using a baculovirus expression system and then performing co-immunoprecipitations. We then showed by chromatin immunoprecipitation (ChIP) that CHD8 is present at the AR target promoters PSA and TMPRSS2, and that it localizes there even prior to the recruitment of AR to this site upon DHT induction. Also, we show that AR and CHD8 co-localize to the TMPRSS2 promoter at the same time by conducting re-ChIP experiments. We then examined a functional role for CHD8 in AR-mediated transcriptional activation of androgen responsive genes by depleting CHD8 using shRNA treatment. Upon CHD8 depletion in androgen-dependent LNCaP cells, the AR-dependent activation of TMPRSS2 and PSA in response to DHT induction was

severely curtailed. However, knocking down CHD8 in androgen-independent cells did not affect the expression of the target genes. Thus, CHD8 is required for optimal androgen-responsive transcriptional activation of AR target genes in an androgen-dependent context. ChIPs were repeated under conditions of CHD8 depletion and we found that the recruitment of AR to the TMPRSS2 promoter in response to DHT treatment was abrogated. Taken together with the previous result, this suggests a mechanism by which CHD8, via the facilitation of androgen-responsive recruitment of AR, is involved in the transcriptional activation of AR target genes. Finally we show that CHD8 depletion adversely affects androgen-responsive cell proliferation of LNCaPs. In summary, we present evidence of a functional, and possible mechanistic role for CHD8 in AR-mediated transcriptional activation of it target genes.

Materials and Methods

Cell Culture

LNCaP, 22RV1, PC-3 and DU-145 cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in RPMI Medium 1640 (Invitrogen) containing 10% fetal bovine serum (FBS: Hyclone) and 1X penicillin-streptomycin-glutamine (Invitrogen). All human cell lines were maintained at 37°C in 5% CO_2. Androgen treatments were done using dihydrotestosterone (DHT) dissolved in ethanol and used at a final concentration of either 10 nM or 50 nM, as specified. Prior to androgen treatment, cells were switched to phenol red-free RPMI Medium 1640 (Invitrogen) supplemented with Dextran/Charcoal-stripped FBS (Hyclone) and 1X

penicillin-streptomycin-glutamine (Invitrogen) for at least 24 hours. SF9 insect cells were cultured at 24°C in 1X Grace's Insect medium (Invitrogen) containing 10% FBS (Hyclone) and 1X penicillin-streptomycin-glutamine (Invitrogen).

Antibodies and Reagents

CHD8 rabbit polyclonal antibodies used were previously described (104). Rabbit polyclonal (N-20) and mouse monoclonal (441) AR antibodies were obtained from Santa Cruz Biotechnology. Rabbit IgG and Actin control antibodies were from Sigma. Anti-rabbit IgG AP conjugate secondary antibodies were from Promega. All oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IN). Primer sequences are listed in Table 2.1.

Recombinant Protein Production

Recombinant baculoviruses were used to express AR and CHD8 in SF9 insect cells. The AR baculovirus was a kind gift from James Dalton (Ohio State University, Columbus, OH). The Flag-CHD8 baculovirus was obtained by cloning the full-length protein in the pBlueBac-Flag vector and generating viral stocks using the Bac-N-Blue Baculovirus Expression System (Invitrogen). For protein interaction studies, 5×10^6 SF9 cells were co-infected with 1 mL each of AR and Flag-CHD8 baculovirus and incubated at 25°C for 2 days. Cells were then collected by centrifugation at 500×g for 2 minutes at room temperature, washed with cold phosphate buffered saline (PBS) and resuspended in 500 μL of IP buffer (20mM Tris-HCl [pH=7.9], 0.2 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) with 150 mM KCl and 0.1% NP-40. Lysates

were cleared by centrifugation at 20,800×g for 10 minutes at 4°C and used for co-immunoprecipitations as described below.

Co-immunoprecipitations

For the *in vivo* interaction studies between endogenous CHD8 and AR, nuclear extracts were prepared from the various cell lines as described by Dignam et al. (166). These extracts were incubated overnight at 4°C with protein A-agarose beads (Repligen) cross-linked to the specified antibodies. The beads were then washed sequentially with 1 mL each of 150 mM KCl in IP buffer, 150 mM KCl in IP buffer with 0.1 % NP-40 and then in 150 mM KCl IP buffer again. Samples were eluted in SDS-loading buffer (125 mM Tris-HCl [pH=6.8], 0.5% SDS, 10% glycerol, 0.175 M BME) and then subjected to SDS-PAGE followed by Western blot analysis with the indicated antibodies.

Co-immunoprecipitations of recombinant proteins from baculovirus infected SF9 cells were performed by incubating lysates prepared as described above with 20 μL of anti-Flag M2 agarose beads (Sigma) at 4°C. Beads were washed and eluted as above and subjected to SDS-PAGE and Western blot analysis with the indicated antibodies.

Chromatin Immunoprecipitations

ChIP experiments were performed essentially as described in the ChIP assay kit (Upstate). Briefly, ~ 1×10^6 cells per immunoprecipitation were fixed with 2.5% formaldehyde for 10 minutes at 37°C. Cells were washed with cold PBS and lysed with ChIP lysis buffer. The chromatin was sheared by sonication (~ 200 – 1000 bp fragments) and cleared by centrifugation at 20,000×g for 10 minutes at 4°C. Samples were diluted

10-fold in ChIP dilution buffer and then pre-cleared with protein A agarose beads (Repligen) blocked with salmon sperm DNA (Invitrogen). Chromatin was immunoprecipitated by incubating with the indicated antibodies overnight at 4°C. Antibody-chromatin complexes were precipitated by incubation with protein A agarose beads blocked with salmon sperm DNA. Samples were then washed and eluted as described in the instructions, except washes were done for 15 minutes each.

For re-ChIPs, the first ChIP was done as described above, except the washed chromatin-antibody complexes were eluted in 50 μL TE with 10 mM dithiothreitol (DTT) and then diluted 20-fold in Re-ChIP buffer (150 mM NaCl, 0.5 mM DTT, 1% Triton X-100, 20 mM Tris-HCl [pH=8.1] 2mM EDTA). These were then immunoprecipitated with the second specified antibody and washed and eluted again as above.

RT-PCR and Quantitative PCR.

cDNA was prepared by extracting total RNA from the indicated cells using the RNeasy kit (Qiagen) following the manufacturer's instructions. Reverse transcription reactions employed total RNA, random decamers (Ambion), and Superscript II reverse transcriptase (Invitrogen) following the manufacturers' instructions. Real-time quantitative analysis employed the indicated primers, iQ SYBR Green 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 expression analysis, threshold cycle (C_t) values were normalized to levels of RNA polymerase III-transcribed human H1 RNA. For ChIP experiments, DNA quantities were expressed relative to input levels. The following primers were used for ChIP experiments. TMPRSS2 -13.5 kb: TGGTCCTGGATGATAAAAAAAGTTT and

GACATACGCCCCACAACAGA. TMPRSS2 -7 kb: ACGCCTTCGCTGTCCTACCT and TGCAATGAAGTTCCCTGCAA. The following primers were used for quantitative RT-PCR analysis. TMPRSS2: GGACAGTGTGCACCTCAAAGA and TTGCTGCCCATGAACTTCC. H1 control: ACTCCACTCCCATGTCCCTTG and CCGTTCTCTGGGAACTCACCT.

RNAi Experiments

CHD8 expression was knocked down in the various prostate cancer cells using RNAi technology utilizing the UI2-Puro SIBR shRNA vectors (167). CHD8 was knocked down using two shRNA cassettes, 493 and 6410, cloned into the SIBR vector. UI2-Puro SIBR GFP-479, containing a shRNA cassette targeting the GFP mRNA, was used as a control shRNA vector. 2 or 10 μg of each of these constructs was transfected into cells which were at ~80% confluency in either 6-well plates (for expression studies) or in 10 cm dishes (for ChIPs), respectively, using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Transfections were done in phenol red-free RPMI 1640 medium containing 10% dextran/charcoal-stripped FBS. 16 hours post-transfection, the cells were selected with 10 μg/mL puromycin for 48 hours more. Cells were then treated with DHT or ethanol for 6 hours. For expression studies shRNA transfections were done in duplicate and one set was harvested for RNA extraction while the other was lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH=7.4], 1 mM EDTA, 0.2 mM PMSF) and Western blotted with the indicated antibodies to verify the knockdown of protein expression. For ChIPs, the cells were fixed with 2.5% formaldehyde after treatment and harvested for the ChIP protocol.

Cell Proliferation Assays

LNCaP cells were transfected with the indicated shRNAs and selected as described above. The cells were then PBS-washed, trypsinized and counted in suspension in media. The cells were then plated in 96-well plates at 5000 cells per well in duplicate for the control and the CHD8 shRNA-transfected cells. Cells were plated in sets of eight in four separate plates, one for each time point, and treated with either 50 nM DHT or an equal volume of ethanol. Cells were harvested at the specified time points and the cell viability was measured based on quantitation of ATP from metabolically active cells present using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's instructions. Data was expressed as fold DHT-induced proliferation, which was calculated as the ratio of the luminescent signal from the induced cells to that obtained from the uninduced cells for each shRNA treatment at each indicated time point.

Results

Analysis of Microarray Data

In order to explore the possibility of the CHD6-9 family of chromatin remodeling enzymes functioning as novel cofactors for AR in the progression of prostate cancer, publicly available gene expression sets were examined using the ONCOMINE database (160). One factor that was found to be significantly upregulated in several prostate cancer samples was CHD8, which we have previously identified and characterized as a

novel ATP-dependent chromatin remodeling enzyme involved in regulation of gene transcription (104). Four distinct microarray data sets were examined and the expression levels of CHD8 were compared between normal prostate tissue, primary prostate cancer tumors and metastatic tumors. It was observed that in three out of the four data sets there was a highly statistically significant ($p = < 0.01$) increase in the expression of CHD8 in metastatic tumors, but not in primary tumors, when compared to normal prostate tissue (data available at www.oncomine.org – search gene:CHD8). This increase in expression was greater than two-fold the average expression level of CHD8 in the normal prostate tissue samples in 15, 19 and 17 samples respectively out of 25 total samples of metastatic tumors included in the studies. This increase of expression was comparable in statistical significance and in magnitude to that of Enhancer of Zeste Homolog 2 (Ezh2) expression between normal and metastatic prostate samples from the same expression data set. This was used as the basis of the study by Verambally et al. linking Ezh2 to prostate cancer progression (168). However, the expression of CHD9 was not increased in tumors compared to normal prostate tissue, suggesting that CHD8 specifically is involved prostate tumorigenesis. Given the fact that several AR coregulators that are known to be upregulated in prostate cancer, this observation gives rise to the possibility of CHD8 functioning as a coregulator of AR.

CHD8 Interacts with AR

To verify whether this possible connection between AR and CHD8 was due to a physical interaction of the endogenous proteins, co-immunoprecipitations were performed from different prostate cancer cells. Nuclear extracts were prepared from the androgen-dependent LNCaP prostate cancer cell line, as well as from the androgen-

independent prostate cancer cell lines, 22RV1, PC-3 and DU-145 (169-171). Androgen-dependent cells require androgens for continued cell growth and proliferation, while androgen-independent cell lines are capable of growth independent of androgens. Immunoprecipitations were performed from these extracts using anti-CHD8 polyclonal antibodies. When the co-IPs were washed, subjected to SDS-PAGE and Western blotted with anti-CHD8 polyclonal and anti-AR monoclonal antibodies, it was observed that endogenous AR and CHD8 do indeed interact in androgen-dependent LNCaP cells but not in the androgen-independent cell lines (Fig. 2.4), indicating that AR and CHD8 associate with each other in an androgen-dependent context.

To further investigate this interaction between AR and CHD8, co-immunoprecipitations of the recombinant proteins expressed using a baculovirus expression system in SF9 insect cells were also carried out. SF9 cells were infected with either AR alone, or co-infected with AR and Flag-CHD8 and then cell extracts were immunoprecipitated using anti-Flag antibodies. AR immunoprecipitated with the Flag beads along with the Flag-tagged, recombinant CHD8, but not by itself (Fig. 2.5), thus verifying a direct physical interaction between these two proteins.

CHD8 and AR Co-localize to the Promoters of Androgen-Responsive Genes

Having established the interaction between AR and CHD8, it was then examined whether this association was evident at endogenous AR target promoters, with CHD8 being localized to these sites along with AR. We have previously shown that CHD8 localizes to β-catenin target promoters (104) and to the Hox locus (unpublished data) by chromatin immunoprecipitation. To examine CHD8 occupancy at AR target promoters,

LNCaP cells were either mock-treated with ethanol or induced with DHT for 6 hours and ChIP assays were done using antibodies against AR, CHD8 or with normal rabbit IgG as a control. The immunoprecipitated chromatin was analyzed by quantitative RT-PCR using primers directed against the previously defined enhancer regions in the promoters of PSA and TMPRSS2 (126, 131). The ChIPs for AR and CHD8 were at considerably higher levels than those for IgG under each of the conditions and therefore the IgG ChIP data is not shown. As expected, AR was recruited to the TMPRSS2 ARE upon induction by DHT (Fig. 2.6). It was also observed that CHD8 localized to this ARE both with and without induction by DHT, thus indicating that CHD8 is targeted to this promoter in an AR-independent manner. CHD8 was not present at the control site located about 7 kb upstream of the start site in the TMPRSS2 promoter, thus CHD8 is not non-specifically binding to this promoter. Similar androgen-independent localization of CHD8 was also seen on the ARE I/II region of the PSA promoter (Fig. 2.7). although CHD8 levels are reduced upon androgen treatment.

In order to confirm that AR and CHD8 are present at the same target sites simultaneously, re-ChIP assays were carried out in similarly treated LNCaP cells. These were done by immunoprecipitating the cross-linked chromatin-protein complexes using first CHD8 and then AR antibodies in two successive chromatin immunoprecipitations. Control re-ChIPs were done using either two consecutive immunoprecipitations with IgG antibodies or with IgG followed by AR antibody. Analysis of the immunoprecipitated chromatin using the same primers as for the TMPRSS2 ChIPs above indicated that indeed AR and CHD8 did co-localize to the TMPRSS2 ARE site upon DHT induction at the same time (Fig 2.8).

CHD8 Coactivates AR-Mediated Transcription

Having established the direct association between CHD8 and AR and their co-localization at the TMPRSS2 promoter, we then examined whether CHD8 has a functional role in the regulation of TMPRSS2 gene expression by using an shRNA-based approach to deplete endogenous CHD8. LNCaP cells were transfected with either control shRNA constructs or with shRNA constructs directed against CHD8. These vectors were designed so they also co-expressed the puromycin resistance gene along with the shRNAs. Thus cells successfully transfected with the shRNA constructs should also be puromycin-resistant and this feature was used to select for transfected cells by treating the transfected cells with puromycin for 48 hours. After selection, the cells were treated with either ethanol or DHT for 6 hours and then total RNA was extracted from these cells and reverse transcribed into cDNA. These cDNA samples were used as template for quantitative RT-PCR analysis to measure TMPRSS2 and PSA expression levels.

We found that, as expected, TMPRSS2 expression was induced almost 6-fold upon DHT treatment in the cells treated with control shRNA. However, under conditions of CHD8 depletion we see that the transcriptional activation of the TMPRSS2 gene upon DHT induction was almost completely abrogated (Fig. 2.9). This suggests that CHD8 is required for the optimal androgen-induced transcriptional activation of the AR target gene, TMPRSS2, in LNCaP cells. Similar, albeit less dramatic, effects of CHD8 knockdown were also observed on the expression of PSA in these cells (Fig. 2.10). The effectiveness of the depletion of CHD8 by our shRNA treatment and selection strategy is

shown in Fig. 2.11, where we see significantly reduced levels of CHD8 protein by Western blotting.

CHD8 is an Androgen-Dependent AR Coactivator

The same CHD8 RNAi experiment was carried out in androgen-independent PC-3, DU-145 and 22RV1 cells. These cells do not respond to androgen treatment due to absence of functional AR (PC-3 and DU-145) or due to transition into an androgen-independent state (22RV1) (150, 172). In all three of these cell lines we found that induction by DHT treatment did not activate TMPRSS2 expression and CHD8 depletion did not significantly affect the expression of this gene either (Fig. 2.12). Similar results were obtained with PSA expression levels in each of these cell lines (2.13). While there were minimal changes in TMPRSS2 and PSA expression in upon CHD8 depletion in PC-3s and 22RV1s, these changes were insignificant compared to the drastic reduction of expression level seen in androgen-dependent LNCaP cells. The knockdown of CHD8 expression in each of these cell lines was verified by Western blot, as was done for the LNCaP cells, and a similar depletion of CHD8 was observed in each case (data not shown). These data support the hypothesis that CHD8 plays an important role in transcriptional activation of the TMPRSS2 gene by AR in an androgen-dependent context.

CHD8 Facilitates AR Binding to Target Promoters

Next, we attempted to observe the effects of CHD8 depletion on the localization of AR and CHD8 at the TMPRSS2 ARE site. CHD8 expression was knocked down in LNCaP as above and the transfected, puromycin-selected cells were treated with DHT for

6 hours. Since AR only binds to the TMPRSS2 ARE upon DHT induction the experiment was done only under induced conditions. ChIPs were carried out as before using antibodies against AR and CHD8 as well as IgG as a control. Quantitative RT-PCR analysis was carried out to observe the localization of AR and CHD8 at the target site using the same ChIP primers as in Fig. 2.6. It was observed that, as expected, the level of CHD8 at the ARE was significantly reduced in the cells where CHD8 had been knocked down (Fig. 2.14). Interestingly, while AR was appropriately recruited to the promoter upon DHT induction in control cells, this androgen-induced recruitment of AR was abrogated in the CHD8 depleted cells. This indicates that CHD8 is required for the recruitment and binding of AR to target promoters in response to androgen treatment. The fact that CHD8 is present at target promoters prior to AR recruitment to these sites upon DHT treatment, validates the possibility that CHD8 may be involved in the recruitment of AR to the TMPRSS2 ARE upon induction by androgens.

CHD8 is Involved in Androgen-Dependent Cell Proliferation

We have shown that CHD8 is required for the optimal transcriptional activation of the TMPRSS2 gene upon induction by DHT and that it may be required for the appropriate androgen-responsive recruitment of AR to target promoters. In order to examine whether this functional role for CHD8 in transcriptional regulation of AR-responsive genes has any physiological relevance, proliferation assays were conducted in LNCaP ells. Cells were transfected with either control or CHD8-targeting shRNA and selected as before. They were then induced with 50 nM DHT or mock-treated with ethanol. Proliferation assays were carried out at the indicated time points using the CellTiter-Glo kit from Promega in 96-well plates, using eight replicate sets for each

condition. Data was expressed as a ratio of the luminescent signal from the induced cells to that obtained from the uninduced cells for each shRNA treatment at each given time point, and displayed as fold DHT-induced proliferation. We saw that, while DHT treatment resulted in a significant increase in proliferation of control cells, the CHD8-depleted cells did not show similar increases in proliferation upon DHT induction after 2 or 4 days (Fig. 2.15). Thus it appears that CHD8 is required for androgen-dependent expression and also for androgen-induced cell proliferation of LNCaP cells. CHD8 could therefore be an important and novel regulator of androgen-dependent prostate tumor growth.

Discussion

A Model for Coactivation of AR-Mediated Transcription by CHD8

The analysis of microarray data from the ONCOMINE database comparing expression profiles of normal prostate tissue to prostate tumors led to the discovery that CHD8 is upregulated in prostate cancer. Several AR coactivators are known to be similarly upregulated in prostate cancer. Given the background of CHD8 as a chromatin remodeling enzyme and the abundance of chromatin-related coactivators known to associate with AR in the course of its transcriptional regulatory function, this led us to hypothesize that CHD8 is a coactivator of AR.

We verified this hypothesis by showing a direct physical interaction between both endogenous and recombinant AR and CHD8 (Fig. 2.4 and Fig. 2.5). The interaction between the endogenous proteins was only evident in androgen-dependent cells and not in cells that are unresponsive to androgens, indicating this interaction is mediated in some

way by androgens (Fig. 2.4). This is typical of other AR coactivators which are known to physically associate with ligand-bound AR in an androgen-dependent manner (118, 135). These coactivators contain one or more of the conserved signature NR box motifs, LXXLL, which are both necessary and sufficient for interaction with the AF-2 region in the C-terminal ligand-binding domain (LBD) of nuclear receptors (173). This interaction, in turn, serves to recruit these coactivators to target promoters. A novel variant to this signature NR box motif, defined by the sequence FXXLL, has also been found to be capable of mediating interactions of coactivators with nuclear receptor LBDs (174). It has also been reported that, compared to other nuclear receptors, the LBD of AR has a unique coactivator binding groove in its LBD that binds FXXLF motifs with greater affinity than the traditional NR box motif (175-177). In fact, one such motif is found in the NTD of AR and is known to strongly bind to the coactivator groove in the LBD (178, 179), facilitating optimal transactivation of the receptor (180-182). This interaction between the N- and C- terminal domains takes place only in the mobile fraction of AR and the interaction is relieved upon the binding of the receptor to DNA, allowing the coactivator groove to become accessible by other cofactors (183).

Protein sequence analysis of CHD8 revealed the presence of similar consensus motifs within its helicase domain. The traditional NR box in CHD8 is a LFSLL motif located at 996–1000 aa within the SNF2 helicase domain of the protein. This motif is conserved only in members of the CHD 6-9 family and is not found within the helicase domains of other SNF2 family proteins and thus could be the candidate binding motif for its specific interaction with AR. Closely homologous to this signature motif is the FXXLL sequence, which in CHD8 is a FVFLL motif located at 1212–1216 aa in the

helicase domain. Alignments of the evolutionarily conserved helicase domains of members of the SNF2 superfamily, this motif was found to be conserved within several other SNF2 family helicases including CHD 3-5, INO80, KIAA 1122 and Brg1, besides CHD 6-9. A closely homologous sequence, FVFML, was found in ISWI. This indicates that this motif may not be responsible for AR binding since it is a widely conserved motif in all SNF2 helicases and may be of functional importance for the activity of the helicase domain. None of the variations of the AR-specific interaction motif, FXXLF, were found in the CHD8 protein sequence.

The AR-specific FXXLF motif is capable of stimulating stronger binding with the coactivator groove of AR than the general LXXLL NR box motif, due to the fact that the bulkier F residues make better hydrophobic contacts with the amino acids lining the coactivator groove than L residues (176, 177). However, LXXLL motif interactions are still made possible by an induced fit mechanism which allows the coactivator groove to alternatively allow L or F residues at the terminal positions of the binding motif (184-186). Thus it is possible for there to be differential strength of binding of coactivators to AR, based on the kind of binding motif present. In the case of CHD8, it may not be desirable to have a strong interaction with AR, and thus this interaction may be mediated by the less potent LXXLL motif. In fact, common nuclear receptor coactivators, like SRC1 and TIF2, interact with AR through traditional NR box motifs (176, 184, 187). This may be to enable the more general transcriptional coactivators to selectively bind with AR during transcriptional activation and then easily dissociate from the receptor once their role is completed, to carry on their other cellular functions. CHD8 has also been shown to be an enzyme with diverse functions and thus its interaction with AR may

be mediated by an NR box motif by design, so as to allow this association to be more transient. In fact, since most FXXLF-mediated interactions appear to be those of more AR-specific coactivators, like ARA54 and ARA70 (188), it makes sense that CHD8 does not have one of these motifs, as it is clearly more diverse in function and it would not be ideal for it to be tethered to AR. Furthermore, the fact that CHD8 interacts with AR through an LXXLL motif may indicate that it could be involved in coactivation of other nuclear receptors. This theory is supported by the fact that the closely homologous subfamily member, CHD9, is known to interact with several other nuclear receptors, like the peroxisome proliferator activated receptor (PPARα), glucocorticoid receptor (GR), constitutive androstane receptor (CAR), retinoid X receptor (RXR) and the estrogen receptor (ER) through its LXXLL motifs (159). Further work is required to more accurately map the interaction of AR with CHD8 to verify these speculations.

We observed that CHD8 co-localized with AR to target AREs in the PSA and TMPRSS2 promoters. However, unlike other coactivators, CHD8 was associated with these promoters independent of androgen induction (Fig.2.6 and Fig. 2.7). In this respect CHD8 is distinct from most common AR coactivators which get recruited to the target promoters by AR in response to androgen treatment. This raises the possibility that perhaps CHD8 is functioning differently than other typical coactivators in its role in AR-mediated transcriptional regulation.

When CHD8 was depleted in androgen-dependent cells, the androgen-stimulated transcriptional activation of TMPRSS2 and PSA was abrogated (Fig. 2.9 and Fig. 2.10). Since this activation is mediated by the action of AR, we can conclude that CHD8 is a required coactivator for the optimal activation of androgen-responsive genes. Depletion

of CHD8, however, had no effect on the expression of these genes in androgen-independent cells (Fig. 2.12 and Fig. 2.13). Thus we have established that CHD8 is a coactivator of AR and functions in an androgen-responsive manner to regulate the expression of target genes upon induction by androgens.

While we have shown that the transcriptional role of CHD8 in regulating the expression of androgen-responsive genes is similar to other coactivators of AR, its mechanistic function is still unclear. How can a chromatin remodeling enzyme that is present at target promoters in an androgen-independent fashion function as an androgen-responsive AR coactivator? This is partly addressed by our observation that depletion of CHD8 prevented AR from binding AREs in the target promoters (Fig. 2.14). We know that CHD8 is a nucleosome remodeling enzyme that is present at these promoters even prior to androgen treatment and that it interacts with AR in an androgen-dependent fashion. Hence, we can postulate that ligand-bound AR homodimers bind to CHD8 at the target promoters, modulating its remodeling activity to reposition nucleosomes in a manner that facilitates the binding of AR to the underlying DNA. Thus our model is that CHD8 is present at AR target promoters, where it may have some basal activity in maintaining the chromatin in a transcriptionally inactive state. Upon induction by androgens, ligand bound AR homodimers translocate from the cytoplasm to the nucleus, where they interact with CHD8 at the AREs of target promoters. They then modulate the nucleosomal remodeling activity of CHD8 in a way that facilitates nucleosome repositioning to allow AR to bind to the ARE DNA sequences. The promoter-bound AR subsequently regulates the transcriptional activation of the target gene by recruiting other coactivators and the transcriptional machinery to these sites. Recent studies from our

group have indicated a similar role for CHD8 in the recruitment of the histone methyltransferase complex, WAR, to Hox promoters (unpublished data).

While this is an attractive model based on the results described above, it needs to be examined in greater detail to verify the precise mechanistic role of CHD8 in AR-mediated transcriptional regulation. The role of CHD8 is, however, physiologically relevant in terms of the growth and proliferation of prostate cancer cells, as evidenced by our observation that CHD8 depletion adversely affects the androgen-responsive proliferation of LNCaP cells (Fig. 2.15). This validates our hypothesis of CHD8 as an androgen-dependent AR coactivator and also supports a model in which CHD8 is required for optimal recruitment and subsequent transcriptional activation of AR target genes. This result also suggests that CHD8 is involved in the regulation of a larger subset of AR target genes, due to its effect on the overall growth of these prostate cancer cells in response to androgens.

Comparisons to SWI/SNF, another ATP-dependent Chromatin Remodeling Coactivator of AR

SWI/SNF has been well established as an ATP-dependent chromatin remodeling complex known to function as an AR coactivator. It is recruited by AR to target promoters in response to androgen and plays a role in the transcriptional activation of these genes through its nucleosome remodeling activity (189). The catalytic activity of human SWI/SNF is conferred by one of two core ATPases, Brg1 or hBRM. Of these alternative SWI/SNF complexes, hBRM-containing complexes were preferentially recruited by AR as a coactivator in the course of its transcriptional regulatory function

(143). The interaction of the SWI/SNF complex to AR has been shown to be mediated through a direct interaction with the BAF57 subunit of the complex, which is responsible for the specificity of this association (140). In contrast, CHD8 appears to directly associate with AR as there were no additional components bridging the interaction demonstrated between the recombinant proteins in the baculovirus co-infection experiment (Fig. 2.5). Also BAF57 reportedly interacts with AR in an androgen-independent manner (140), whereas CHD8 does not interact with AR in androgen-independent cell lines. Further experiments mapping the AR-CHD8 interaction are necessary to fully understand their association, possibly through the unique LFSLL motif found in CHD8.

Another prominent difference in these two coactivators is the pattern of their recruitment. SWI/SNF gets recruited by AR in response to androgens to target promoters. On the other hand CHD8 is present at these promoters even in the absence of AR. Upon androgen treatment, it interacts with the nuclear, ligand-bound AR in a way that stimulates its nucleosome remodeling activity to facilitate AR binding to these sites. Thus CHD8 may be involved in the AR-mediated transactivation process upstream of the involvement of SWI/SNF. Indeed it may be responsible for the subsequent recruitment of SWI/SNF via AR. There is apparently a temporal separation between the points of action of these two chromatin remodeling coactivators of AR in androgen-responsive transcriptional regulation. This in itself is not an unprecedented observation since it is known that during RNA polymerase II-mediated transcription in *Drosophila*, BRM facilitates transcriptional initiation at a step prior to polymerase recruitment to promoters, while the CHD8 homolog Kismet, is implicated at a later stage involving promoter

clearance by the polymerase complex and the transition from early to late stages of elongation (61).

The CHD6-9 Subfamily and Nuclear Receptors

There are a few lines of evidence from previous studies connecting CHD6-9 subfamily members with nuclear receptors and other similar transcription factors. CHD9 is known to interact with the glucocorticoid receptor and the estrogen receptor (98, 99) and another report demonstrates its role as a coactivator of PPARα (159). This same study also reported the interaction of CHD9 with other nuclear receptors like constitutive androstane receptor (CAR), retinoid X receptor (RXR) and the estrogen receptor (ER). CHD7 has been shown to interact with PPAR-γ as part of a complex and repress the expression of its target genes in conjunction with an HMTase component of this complex (95). All these reports point towards a role for this subfamily of chromatin remodeling enzymes in nuclear receptor-mediated transcriptional regulation. Our results have revealed CHD8 to be a coactivator of AR and thus have further expanded this nuclear receptor-associated role of the CHD6-9 subfamily.

There are, however, subtle yet significant differences between the role of CHD8 and its other subfamily members in their capacity as nuclear receptor coregulators. CHD9 appears to interact with PPARα through one or more of five LXXLL motifs located within its protein sequence, since different fragments of this protein were pulled down with GST- PPARα, with the greatest affinity appearing to be of a C-terminal fragment containing two of these motifs (159). On the other hand CHD8 only has one LXXLL and one FXXLL motif, both located within in SNF2 helicase domain. There are

no homologous C-terminal NR box motifs in CHD8 and thus the basis of its interaction is likely different from that of CHD9. Also, CHD9 interacts with PPARα bound to DNA. In the case of CHD8, it appears that CHD8 is bound to the target DNA elements prior to AR binding, and, in fact, it appears to facilitate the binding of AR to these sites. This model of CHD8 function does indeed seem unique among the previously reported transcriptional regulatory roles of all other CHD family members, even though CHD6 and CHD9 have been shown to have a similar coactivating effect in association with other nuclear receptors and similar transcription factors.

Several other reports help validate our findings about CHD8 and AR. CHD8 has also been linked to ER and has been reported to be required for the ER-dependent upregulation of the cyclin E2 gene (105). Another study also reported that CHD8 upregulates cyclin E2 through interaction with elongating RNA polymerase II (100). This report also demonstrates that CHD8 is constitutively bound to the CCNE2 promoter, in the same way we see it bound to AR target promoters. Also, its regulation of cell cycle genes would be consistent with a role for CHD8 in promoting androgen-responsive cell proliferation. A similar constitutive recruitment of CHD9 has been observed at the ER-responsive osteocalcin promoters during bone development (99). It has also been reported that CHD8 suppresses p53-mediated apoptosis (102), once again supporting the tumorigenic role for this protein in our model. Finally there are reports connecting the inappropriate activation of AR by the β-catenin pathway, suggesting that β-catenin interacts with AR, translocates with it to the nucleus and binds target promoters as a complex (190). This again links CHD8 to AR since CHD8 has been previously shown by our group to interact with β-catenin (104). The transcriptional role of CHD8 appears to

be contradictory in these two systems, with CHD8 acting as a coactivator of AR and as a negative regulator of β-catenin-mediated transcription. However, there are examples both of CHD8 (100, 102-105, 190) and of other chromatin remodeling enzymes (191) having differential transcriptional activities depending on its interaction with different cofactors.

CHD8 as a Novel Therapeutic and Diagnostic Target for Prostate Cancer

An interesting observation that emerged from the microarray data analysis was that CHD8 levels were significantly upregulated in metastatic prostate tumors compared to normal tissue but not in primary prostate tumors. This may indicate a role for CHD8 in the transition to hormone refractory prostate cancer and not in primary tumorigenesis. This may seem counter-intuitive since we show that CHD8 is not involved in AR-mediated transcription in the androgen-independent cell lines studied here, but PC-3 and DU-145 are AR-negative and perhaps aberrant AR function in the hormone refractory 22RV1 cells precludes CHD8 involvement. However, if we consider the observation that CHD8 depletion results in abrogation of AR recruitment to target genes, and the fact that CHD8 is upregulated only in metastatic tumors, one can envision a scenario where overexpression of CHD8 may be responsible for the aberrant recruitment of AR to target genes thus enabling the androgen-independent transactivation of these genes. Since CHD8 is in fact constitutively present at these target promoters even in the absence of androgens, it is possible that its overexpression would result in a constitutive, androgen-independent recruitment of AR to these sites, giving rise to aberrant transcription of these genes and leading to a hormone refractory state. While we know that androgen-signaling is typically required for AR translocation to the nucleus, this requirement is clearly

circumvented in the hormone refractory phase due to the dysregulation of AR by various mechanisms including AR mutations changing its ligand-specificity, overexpression of AR or its activation by alternate pathways. In addition, AR that is targeted to the nucleus during the early androgen-dependent phase of primary tumors may not have been expelled from the nucleus. Upon the overexpression of CHD8 in tumors that have progressed to a metastatic state, both aberrantly targeted AR and remnant nuclear AR from the androgen-dependent state would get constitutively recruited to target promoters, hence establishing and maintaining tumors in a hormone refractory state.

These observations indicate that CHD8 could be critical in the transition of tumors from androgen-dependence to an androgen-independent state and in the subsequent maintenance of this state. This makes it a novel therapeutic target for the treatment of androgen-independent prostate cancer. Most current therapies like androgen-ablation and targeting AR, are not effective against androgen-independent prostate cancer. CHD8 is a particularly intriguing prospective target because of its potential role in the transition of tumors to a hormone refractory state, and thus by targeting it one could prevent this transition. Due to the fact that overexpressed CHD8 in metastatic tumors could constitutively recruit aberrantly translocated AR to its target genes, it may also be involved in maintaining these tumors in an androgen-independent state. Thus targeting CHD8 should abolish this recruitment pattern and would effectively ablate AR transactivation of these genes and could also be used to treat androgen-independent malignancies. In addition, due to its upregulation in metastatic tumors, CHD8 could also be used as a diagnostic marker for prostate cancer. Future studies will have to be directed towards understanding the specifics of CHD8 expression in prostate

cancer and the precise mechanism of its coactivation of AR, before such therapeutic and diagnostic strategies can be pursued.

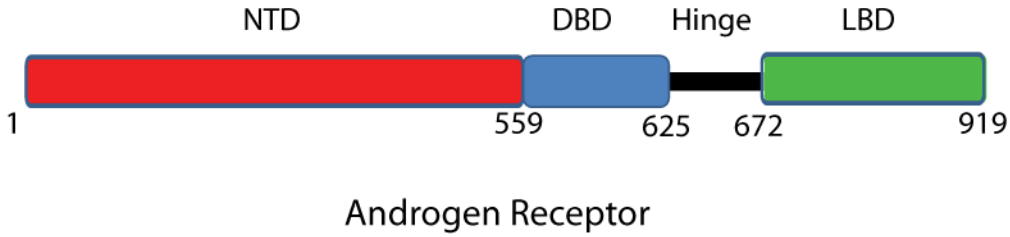

Figure 2.1: Domain structure of the androgen receptor. The androgen receptor is comprised of an N-terminal transactivation domain (red), a central DNA-binding domain (blue) and a C-terminal ligand-binding domain (green). The hinge region between the DNA-binding and ligand-binding domains serves as a structurally flexible element that allows the protein undergo a conformational change upon transactivation to allow interaction of the N-terminal domain with the ligand-binding domain.

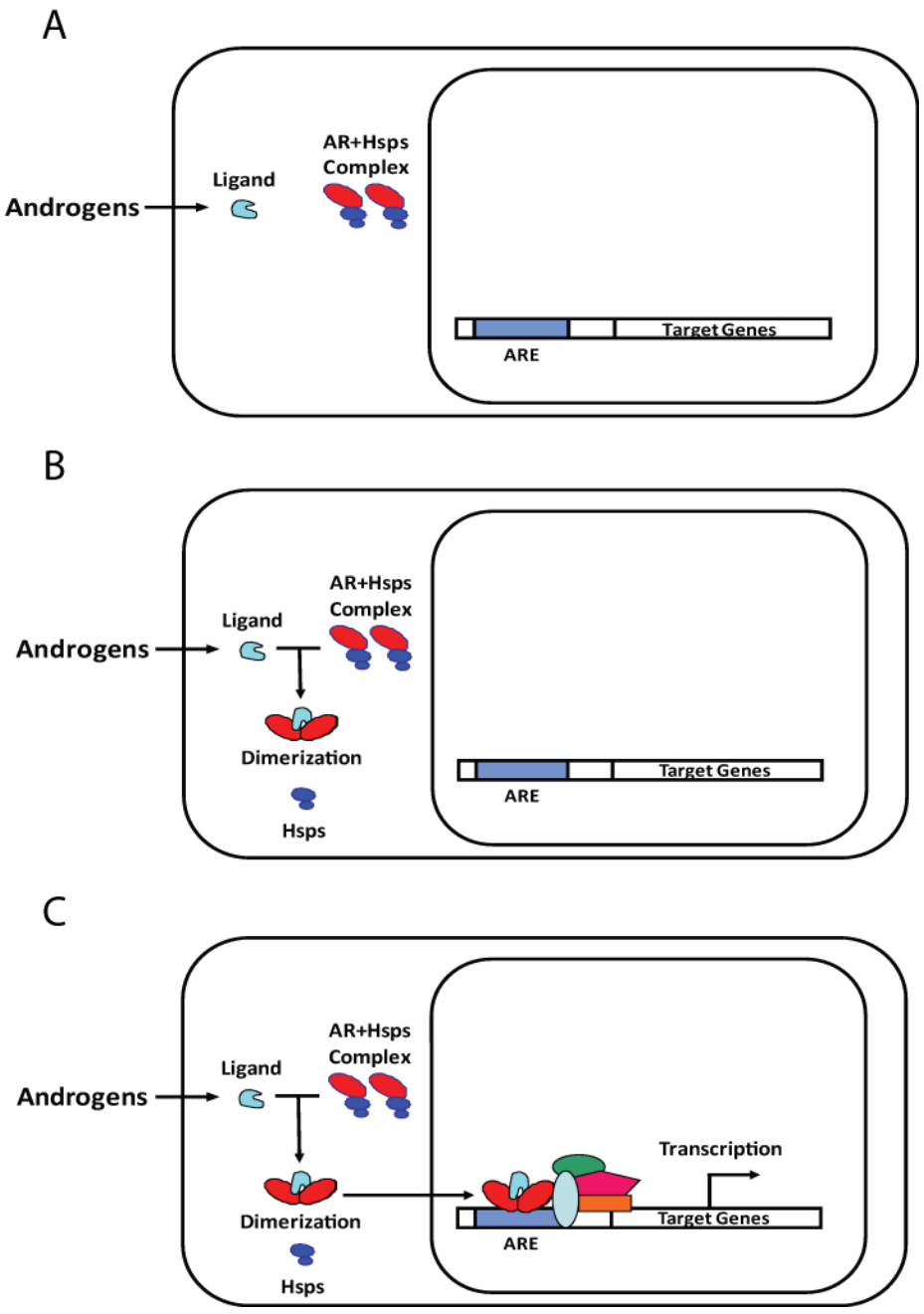

Figure 2.2: The androgen receptor signaling pathway. Under uninduced conditions the androgen receptor (AR) remains sequestered in the cytoplasm in a protein chaperone complex (A). Upon induction by androgens, AR dissociates from this complex and binds the ligand, whereupon it dimerizes (B) and translocates to the nucleus where it can bind to androgen response elements (AREs) on the promoters of target genes (C). Here, AR recruits a complex of coactivators which facilitate the transcriptional activation of the target androgen-responsive genes.

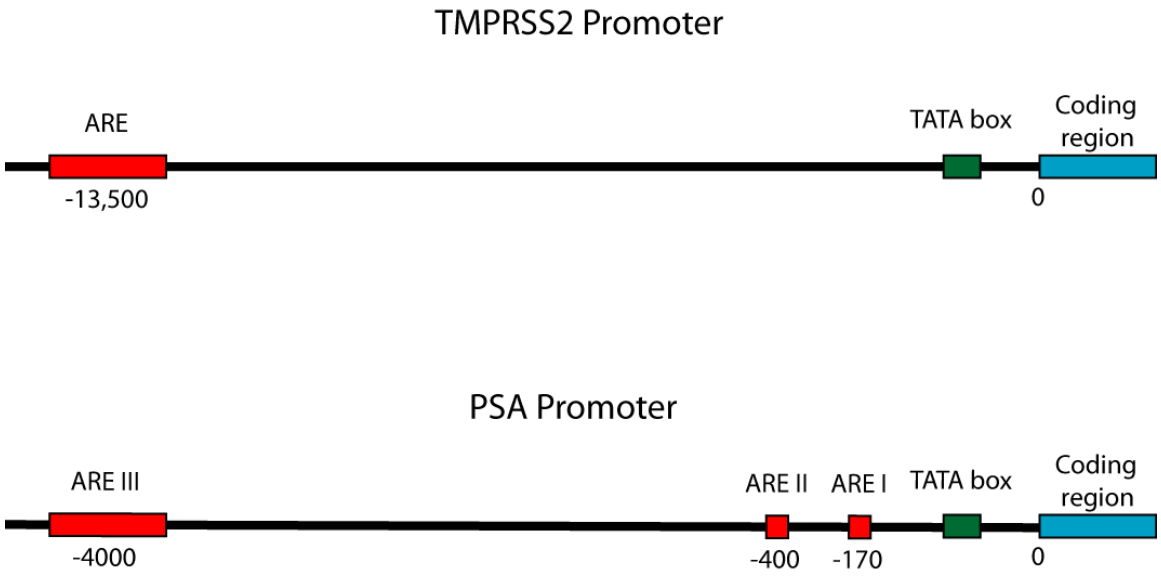

Figure 2.3: Schematic representation of the TMPRSS2 and PSA promoters. The TMPRSS2 promoter (above) has a single characterized ARE located 13.5 kb upstream of the transcriptional start site. The PSA promoter (below) has three characterized androgen response elements (AREs), which are shown in red. The consensus ARE I is located between -156 to -170 bp from the transcriptional start site and the non-consensus ARE II lies between -365 to -400 bp upstream of the start site. In addition, the ARE III region spanning about 450 bp and located approximately 4 kb upstream of the transcriptional start site contains one consensus and multiple non-consensus sites.

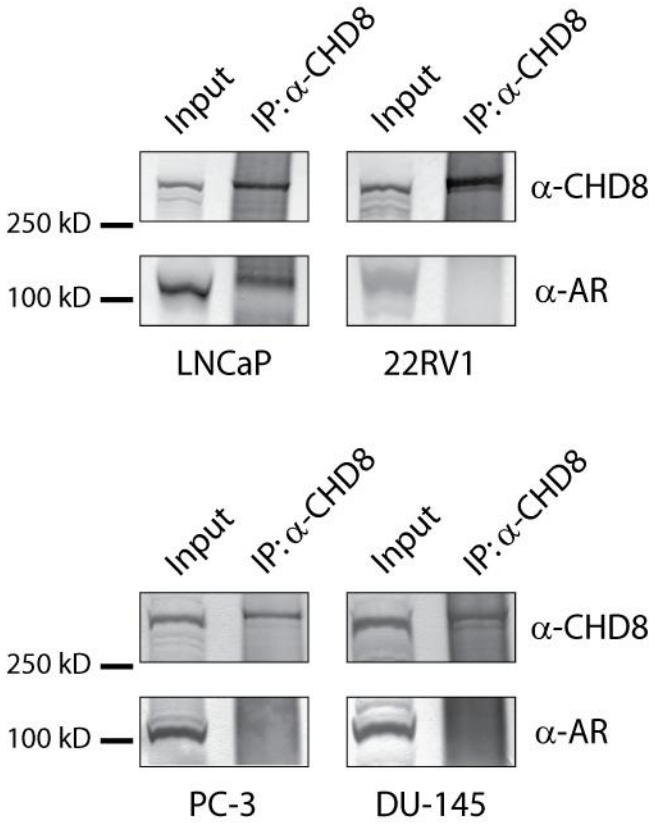

Figure 2.4: Androgen-dependent interaction of endogenous CHD8 and AR. Nuclear extracts were prepared from the indicated cell lines and immunoprecipitated with α-CHD8 antibodies. After washing, the input and immunoprecipitated samples (IP) were subjected to Western blot analysis using the indicated antibodies.

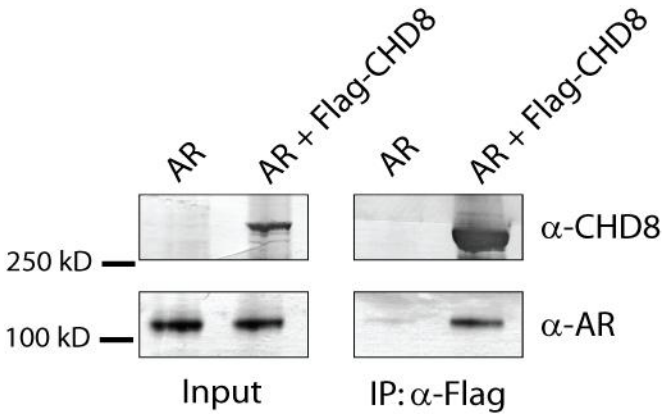

Figure 2.5: Direct association of recombinant CHD8 and AR. Cellular extracts were prepared from SF9 cells following co-infection with the indicated viruses. Immunoprecipitations were performed with anti-Flag antibody-linked M2 agarose beads. Immunoprecipitated samples were subjected to Western blot analysis using the indicated antibodies.

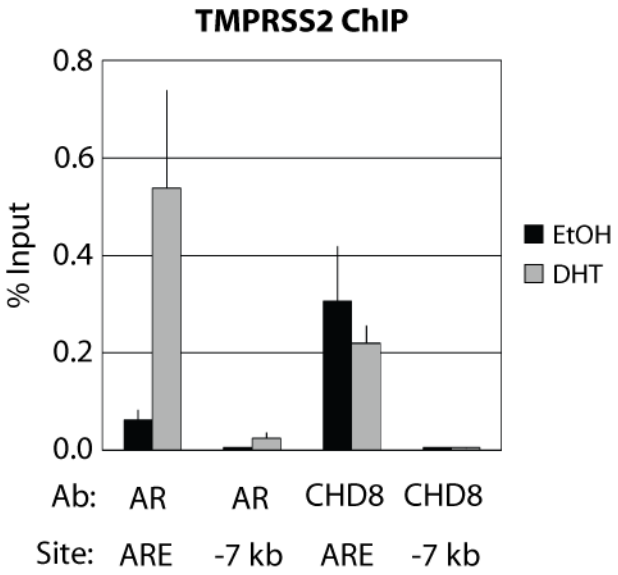

Figure 2.6: Co-localization of CHD8 and AR to the ARE of the TMPRSS2 promoter. LNCaP cells were treated with ethanol or 10 nM DHT for 6 hours. Chromatin was crosslinked *in vivo* with formaldehyde. Cells were lysed and chromatin immunoprecipitations were performed with the indicated antibodies. Bound DNA was detected by quantitative PCR using primers to the ARE of TMPRSS2 (ARE) or a control TMPRSS2 promoter region (-7 kb). Control IgG-precipitated samples were less than 0.005% of input and therefore are not shown. Data is representative of multiple experiments.

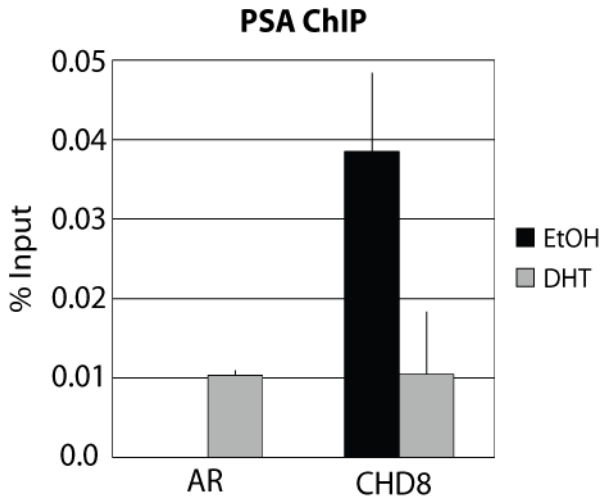

Figure 2.7: Co-localization of CHD8 and AR to the ARE of the PSA promoter. LNCaP cells were treated with ethanol or 10 nM DHT for 6 hours and harvested for ChIP using antibodies to AR and CHD8. Immunoprecipitated chromatin was analyzed by qPCR using primers targeting the PSA ARE I/II region. Control ChIPs done with IgG antibodies were less than 0.005% of input and were therefore not shown. Data is representative of multiple experiments.

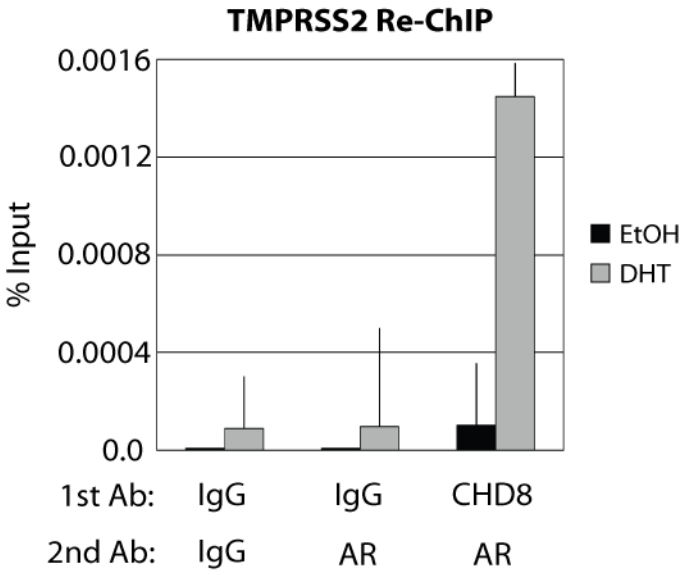

Figure 2.8: Simultaneous co-localization of CHD8 and AR to the TMPRSS2 ARE. LNCaP cells were treated as in Fig. 2.6. Re-ChIP experiments were performed by successively immunoprecipitating the cross-linked chromatin with the indicated antibodies. Bound DNA was detected by quantitative PCR using primers to the TMPRSS2 ARE. Data is representative of multiple experiments.

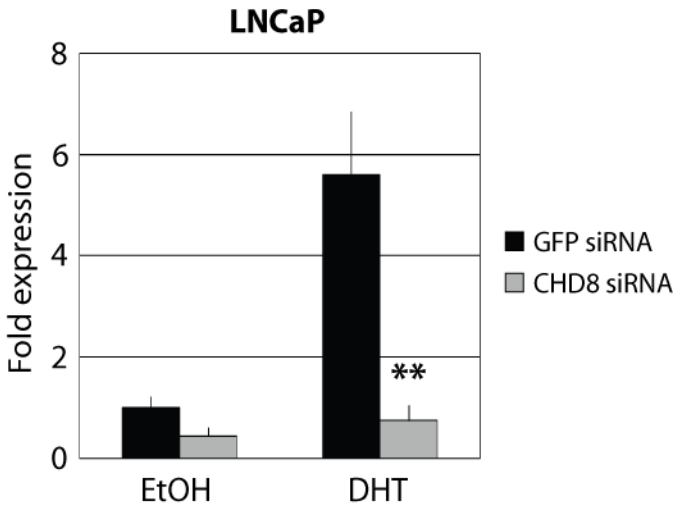

Figure 2.9: Coactivation of AR-mediated transcription of the TMPRSS2 gene by CHD8. LNCaP cells were transfected with the indicated siRNA constructs. Following selection of the transfected cells, cultures were treated with ethanol or 10 nM DHT for 6 hours. Total RNA was isolated and TMPRSS2 expression was analyzed by quantitative RT-PCR. Data is representative of multiple experiments. ** = $p<0.01$ by Student's t-test.

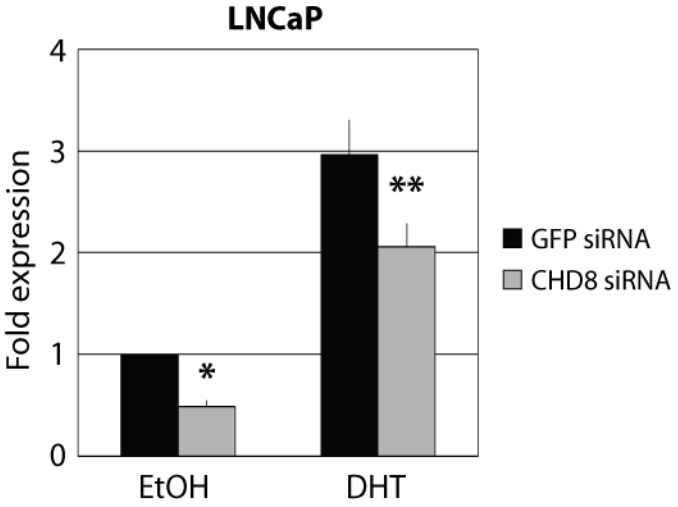

Figure 2.10: Coactivation of AR-mediated transcription of the PSA gene by CHD8. LNCaP cells were transfected with the indicated siRNA constructs, selected and treated with ethanol or 10 nM DHT for 6 h. Total RNA was isolated and PSA expression was analyzed by RT-qPCR. Expression of PSA was normalized to a reference RNA. Data is representative of multiple experiments. * = $p<0.05$ and ** = $p<0.01$ by Student's t-test.

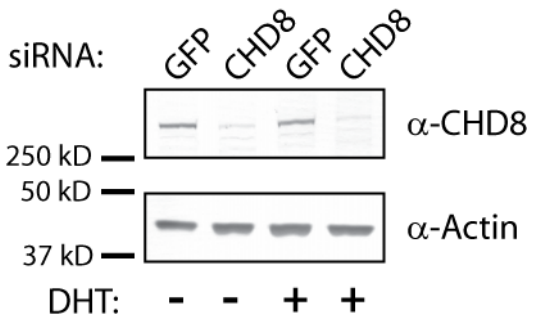

Figure 2.11: Efficacy of CHD8 knockdown in LNCaP cells. Efficiency of CHD8 depletion by siRNA treatment in Fig. 2.9 and Fig. 2.10 was determined by Western blot analysis with CHD8 antibodies. Actin is blotted as a loading control.

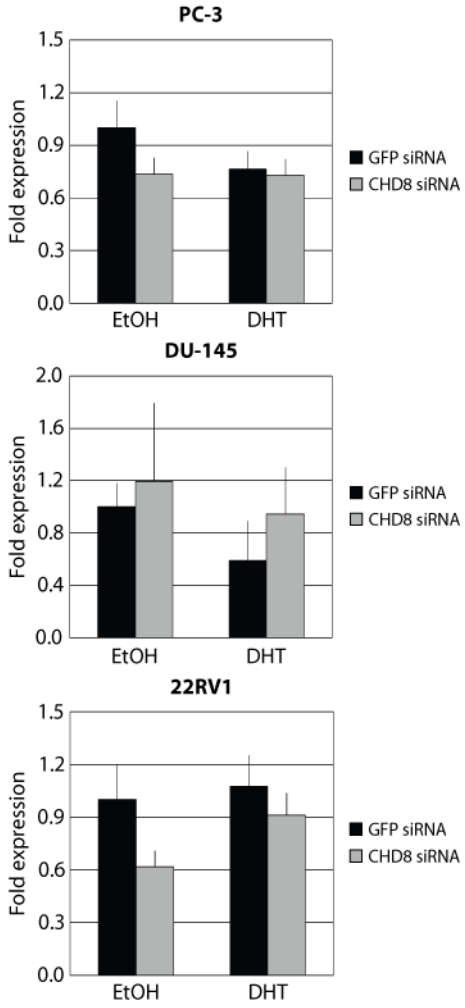

Figure 2.12: Effect of CHD8 depletion on TMPRSS2 expression in androgen-independent cell lines. The indicated androgen-independent cell lines were transfected with the specified siRNA constructs. Following selection of the transfected cells, cultures were treated with ethanol or 10 nM DHT for 6 hours. Total RNA was isolated and TMPRSS2 expression was analyzed by quantitative RT-PCR. Data is representative of multiple experiments.

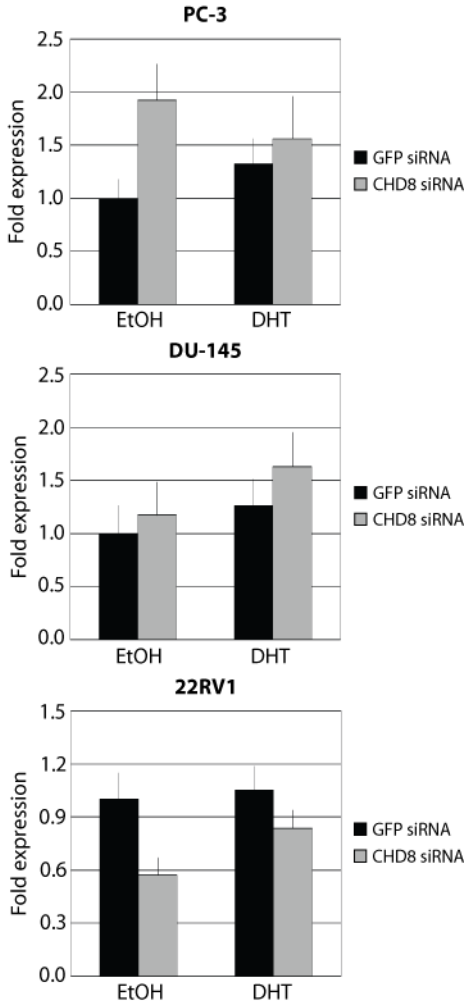

Figure 2.13: Effect of CHD8 depletion on PSA expression in androgen-independent cell lines. The indicated androgen-independent cell lines were transfected with the specified siRNA constructs. Following selection of the transfected cells, cultures were treated with ethanol or 10 nM DHT for 6 hours. Total RNA was isolated and PSA expression was analyzed by quantitative RT-PCR. Data is representative of multiple experiments.

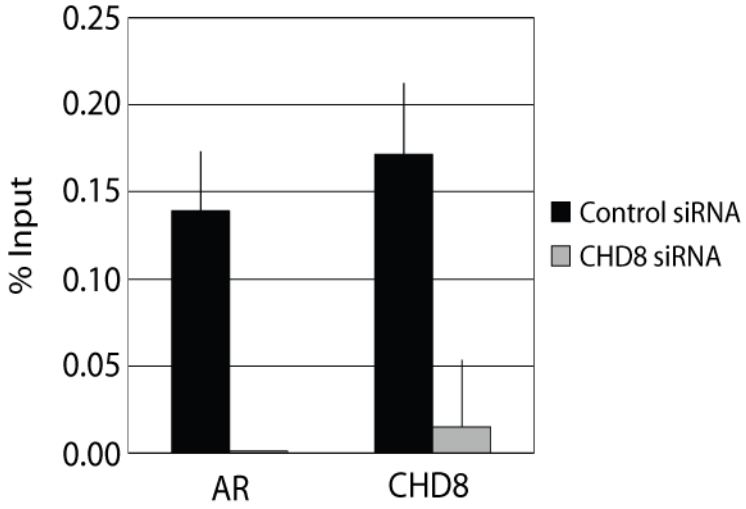

Figure 2.14: Abrogation of androgen-responsive recruitment of AR to the TMPRSS2 ARE upon CHD8 depletion. LNCaP cells were transfected with the indicated siRNA constructs. Following selection of the transfected cells, cultures were treated with ethanol or 10 nM DHT for 6 hours. Chromatin was crosslinked *in vivo* with formaldehyde. Cells were lysed and chromatin immunoprecipitations were performed with the indicated antibodies. Bound DNA was detected by quantitative PCR using primers to the TMPRSS2 ARE. Control IgG-precipitated samples were less than 0.005% of input and therefore are not shown. Shown is a typical result from multiple experiments.

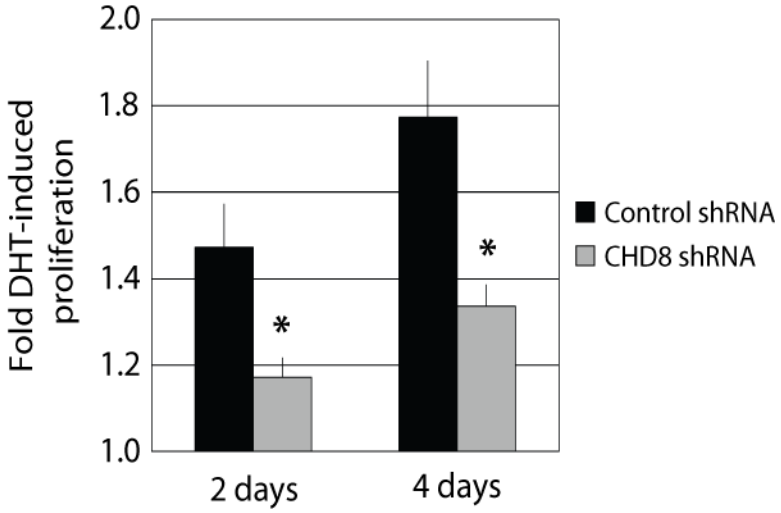

Figure 2.15: Adverse effect of CHD8 depletion on androgen-dependent cell proliferation. LNCaP cells were transfected with the indicated siRNA constructs. Following selection of the transfected cells, cultures were treated with ethanol or 50 nM DHT. At the indicated time points, proliferation was determined using a luminescent-based assay of metabolically active cells. Data is expressed as fold DHT-induced proliferation, which is calculated as the ratio of the luminescent signals from the induced to the uninduced cells for each siRNA treatment at each indicated time point. *=$p<0.02$ by student's t-test.

CHAPTER III

Substrate Specificity and Requirements for Remodeling by CHD8

Introduction

Interplay of Histone Modifications and Nucleosome Remodeling

As discussed previously, eukaryotes manipulate chromatin structure by employing diverse strategies. These include covalent modification of histones, as well as ATP-driven nucleosome remodeling. The interplay between the factors responsible for these two classes of chromatin remodeling can exist at different levels. The proteins involved in these two processes can interact by direct association with one another. They can also interact via the histone marks deposited by chromatin modifying enzymes, which can then be recognized and bound by various domains found in nucleosome remodeling factors. Both of these mechanisms are discussed further in Chapter IV. In this chapter, we will address another form of interplay between histone modification and nucleosome remodeling; namely the modulation of the substrate specificity of ATP-dependent chromatin remodeling enzymes for nucleosomal substrates, due to the modification and alteration of histone structure.

Histone Tails and Chromatin Structure

The majority of covalent modifications of histones occur in the N-terminal tail regions, which can account for up to 30% by mass of the histone proteins (192). These tails extend away from the globular core of the nucleosome and are thought to be largely unstructured, allowing for maximal structural flexibility and accessibility for covalent modification (5). Modifications of the histone tails generate epitopes for recognition by other chromatin remodeling factors, which can then affect changes in chromatin structure. Histone tails can also affect the chromatin structure by altering the dynamic properties of nucleosomes. Nucleosomes are highly dynamic structures that can be modulated in different ways. Nucleosomes can form arrays that condense into chromatin fibers, their positioning can be altered by nucleosomal sliding and their structures can be disrupted to dissociate the DNA from the histone octamers (39, 193-195). These processes occur both spontaneously as well as in an ATP-dependent fashion by the action of chromatin remodeling enzymes. Nucleosome dynamics are dependent on their inherent structure, which in turn is influenced by the presence and nature of the histone tails. Therefore it is not surprising that histone tails and their covalent modifications have been shown to directly affect these dynamic properties of nucleosomes (196-199). A recent study examined the effects of histone tail deletions on the intrinsic stability and mobility of nucleosomes (200). Deletion of H2A tails increased the intrinsic mobility of nucleosomes, while H4 and H2B tail deletions reduced the same, showing these tails assist in nucleosome sliding. Deletion of H3 caused striking structural changes of the nucleosome, disrupting DNA wrapping and destabilization of dimers. Thus histone tails,

being critical in inherent nucleosome structure and dynamics, would also be expected to be important for ATP-dependent remodeling.

Histone Tails and ATP-dependent Chromatin Remodeling

The histone tails provide ideal contact points between the nucleosome and the chromatin remodeling enzymes, being the only part of the nucleosome structure that extends away from the otherwise compact nucleosomal core. There are several examples from previous literature that suggest that histone tails are important for ATP-dependent nucleosome remodeling. The genetic link between histone tails and chromatin remodeling was originally elucidated by deletion studies in yeast. *In vivo*, the deletion of histone tails in yeast was found to profoundly affect chromatin structure and gene regulation on a genome-wide scale (201-203). Deletion of H3 or H4 histone tails eliminates heterochromatin-based transcriptional repression of several genes (204, 205). Histone tails have also been linked to more specific effects on chromatin remodeling. For example, deletion of H2A or H2B tails affected the chromatin remodeling activity of SWI/SNF at specific subsets of genes (206, 207).

Several studies have examined the effect of histone tail deletions on nucleosome remodeling *in vitro* as well. The histone tails are required for nucleosomal remodeling by ISWI, SWI/SNF and RSC complexes (208, 209). Mi-2 containing complexes, on the other hand do not require the presence of histone tails for efficient nucleosomal remodeling (210, 211). In fact, the specificity of certain chromatin remodelers for histone tails extends even further. ISWI specifically requires H4 tails to remodel nucleosomes, by recognizing a critical epitope consisting of a DNA bound N-terminal

sequence of H4 (212, 213). H3 tail deletion or mutation reduced nucleosome remodeling by RSC and SWI/SNF but did not significantly affect remodeling by CHD1 (214).

Histone Modifications and Nucleosome Remodeling

The importance of histone tails in chromatin remodeling is further emphasized by evidence that histone tail modifications can also affect nucleosome remodeling. Acetylation of H3K14 has been shown to enhance remodeling by RSC (215), while it inhibits remodeling by ACF, (26) showing that specific modifications can have differential effects on different remodeling enzymes. Indeed RSC has been found to interact genetically with H3K14 but not with H3K9 (38). Acetylation of multiple lysines on the H4 tail, on the other hand, has no effect on RSC-catalyzed sliding of nucleosomes, but it inhibits remodeling by Isw2 (215). Furthermore, it was seen that CHD1 also required H4 tails to efficiently catalyze nucleosome sliding and acetylation of the H4 tail reduced its rate of remodeling as well (215). As mentioned before, however, another CHD family protein, Mi-2, did not require the presence of histone tails to catalyze nucleosome remodeling (211) Thus it is possible that even within the same family of remodelers one may find that particular histone modifications can have differential effects on remodeling activity. Indeed, the ISWI-containing Remodeling and Spacing Factor (RSF) was also found to assemble nucleosome arrays more efficiently upon p300-mediated acetylation of the H4 tails of its nucleosomal substrates (216), while remodeling by Isw2 is adversely affected by the same modification. The acetylation of H4K8 has been reported to facilitate remodeling by INO80 and SWR1 complexes (217).

The effect of H3K14 acetylation on remodeling by RSC acetylation can be attributed to the increased binding capacity of RSC for the acetylated nucleosomes (24, 27, 215). In this case, the rate of catalysis is being affected by tighter binding of the enzyme to the substrate. On the other hand, acetylation of H4 does not have any effect on the binding of either Isw2 or CHD1 to nucleosomes and appears to affect nucleosome remodeling by both these enzymes by decreasing the turnover rate of ATP hydrolysis (215). This shows that histone modification can affect nucleosome remodeling at different stages of catalysis and by different mechanisms. While acetylation of H4 was shown to not affect nucleosome sliding by RSC, it appears to increase the rate of octamer transfer by RSC, probably by destabilizing the nucleosome. Thus the same modification can also control different aspects of chromatin remodeling by the same enzyme in different ways.

Substrate Specificity of Chromatin Remodeling Enzymes

Several chromatin remodeling enzymes have different specificities towards preferred nucleosomal substrates based on the presence and modifications of histone tails. Studying the substrate specificities and requirements for the remodeling activities of these enzymes could provide valuable insights into their cellular functions. It is logical that RSC, which is typically associated with transcriptional activation, prefers acetylated nucleosomal substrates for its remodeling activity, since this modification is usually enriched at sites of active transcription. The SWI/SNF family of chromatin remodelers, to which RSC belongs, contains bromodomains that bind histone acetyl marks and this reinforces the possibility that these enzymes recognize nucleosome substrates bearing acetylation marks. Conversely, remodeling by Isw2, which is known to have a

transcriptionally repressive role, is adversely affected by histone acetylation. The ISWI family appears to recognize an epitope in the unmodified H4 tail in the course of catalysis. This epitope may be masked by acetylation of H4 causing the observed inhibition of remodeling by these enzymes.

The CHD subfamilies appear to be regulated differentially by histone tails and their modifications, and this too is not unexpected given the functional variation among these proteins. CHD1 of the CHD1-2 subfamily requires the H4 but not the H3 tail for optimal remodeling activity, and this activity is inhibited by H4 acetylation, much like ISWI. This may seem counter-intuitive since CHD1 is known to be involved in active transcription via association with elongating RNA Pol II, and is known to interact with the HAT complexes, SAGA and SLIK (29). However, CHD1 contains methylation-specific chromodomains and not the acetyl-recognizing bromodomains. Thus it is possible that the mode of substrate recognition by the CHD1-2 subfamily is through the H4 tail, like ISWI remodelers, and acetylation of this tail prevents the necessary enzyme-substrate contacts for optimal remodeling activity. Mi-2 of the CHD3-5 subfamily, on the other hand, appears to not require histone tails at all for its remodeling activity. However, the binding of CHD3/4-containing NURD complexes to histones is abrogated by H3K4 methylation, indicating that these proteins require the presence of unmodified H3 tails for binding to the substrate (80). We can reconcile this contradiction by proposing that the CHD3-5 proteins require an epitope in the histone core bodies, which may be modified or unmodified, and that this recognition is affected by the methylation of H3K4. The CHD6-9 subfamily has been relatively poorly studied from the aspect their remodeling activity. This will be examined further in this chapter.

Nucleosome Remodeling by CHD8

While it has been previously shown that CHD6 and CHD9 have DNA-stimulated ATPase activity (90, 96), nucleosome remodeling by other CHD6-9 subfamily members has not previously been characterized. Studies from our lab were the first to identify and characterize CHD8 as a *bona fide* ATP-dependent chromatin remodeling enzyme (104). It was demonstrated that CHD8, like other typical chromatin remodelers, has intrinsic ATPase activity that was stimulated in the presence of nucleosomes. It also has a conserved lysine residue within the ATP-binding site of its catalytic Snf2 helicase domain, which is conserved among Snf2-containing chromatin remodeling enzymes and is required for their catalytic activity. Mutation of this cognate lysine severely impaired the nucleosome-stimulated ATPase activity of CHD8, showing that it has canonical Snf2-domain driven catalytic activity. It was also shown that CHD8 was able to remodel mononucleosomes in an ATP-dependent manner. Results obtained from nucleosome sliding assays revealed that CHD8, like CHD1 and CHD3, repositions nucleosomes toward the center of the DNA template upon which they are assembled. However, the substrate specificity of CHD8 has not been examined, to date. Thus an investigation into the substrate preference or requirement of CHD8 for remodeling nucleosomes, would further elucidate new aspects of the remodeling activity and cellular functions of the CHD family of remodelers.

CHD8, like other members of the CHD family, has a pair of tandem chromodomains. Since these domains are known to recognize and bind histone methylation marks, it is possible that those of CHD8 could be involved in the recognition of specific methylated histone tails on its nucleosomal substrates. The chromodomains of

CHD1 are known to bind dimethylated H3K4 (30), while this same modification precludes the binding of CHD3/4 to the H3 tail (79, 80). Thus the chromodomains of CHD6-9 may also specify or prevent binding to specifically methylated histone tails, defining its substrate preference for remodeling. Indeed the chromodomains of CHD8 have been shown to bind dimethylated H3K4 (100). It should be noted that for other CHD family proteins, the binding specificity of their chromodomains is independent of their requirements for remodeling, since despite their binding properties to H3 tails, both CHD1/2 and CHD3/4 do not require the H3 tail for their remodeling activity. In these cases the chromodomains may be involved in the appropriate targeting of their remodeling activity and not in substrate recognition. In the case of RSC, however, the interaction of its bromodomains with acetylated H3K14 is responsible for the substrate specificity of this enzyme for remodeling (215). It would thus be intriguing to examine whether the substrate requirements for remodeling by CHD8 are defined by its chromodomains or not. In addition, while the effects of histone tail acetylation have been widely demonstrated on nucleosome remodeling, histone methylation has never been well-studied in this context. It would thus be interesting to narrow down the requirements for remodeling and the substrate specificity of CHD8, by examining the effect of tail deletions and histone methylation on its remodeling activity.

Hypothesis and Summary of Results

Based on the evidence that the chromodomains of CHD8 binds methylated H3K4, we hypothesized that CHD8 would preferentially remodel nucleosomes containing H3K4 methylation marks. To determine this we examined the effects of various histone alterations on the remodeling activity of CHD8. We began by examining the remodeling

of CHD8 on nucleosomes reconstituted using recombinant histones, which are presumably unmodified, and comparing this to nucleosomes assembled using core histones extracted from HeLa cells containing a plethora of histone modifications, to see whether these modifications affect remodeling by CHD8. We found that CHD8 could remodel unmodified, recombinant nucleosomes, and from a competitive remodeling assay where both nucleosome types were present in the same reactions we saw that CHD8 prefers the core nucleosomes as a substrate. Next we attempted to narrow in on the histone tail upon which these preferred modifications were located by doing remodeling assays on nucleosomes containing truncated histone tails. We found that deletion of the H3-H4 tails did not prevent nucleosome sliding of these nucleosomes on a DNA template. In fact, the deletion of these tails appeared to enhance nucleosome remodeling by CHD8 when compared to nucleosomes containing wild-type, full-length recombinant histones using a competitive nucleosome sliding assay. Finally we looked at the effect of H3K4 methylation on remodeling by CHD8. Histone H3 was synthetically modified so that its H3K4 residue was replaced by either unmodified or dimethylated lysine analogs. Remodeling of nucleosomes reconstituted using these synthetically modified histones was compared in a competitive nucleosome sliding assay. It was observed that CHD8 did preferentially remodel the dimethylated nucleosomes over the unmodified ones. Thus we concluded that CHD8 does not require the H3-H4 tails for its remodeling activity, but prefers nucleosomal substrates bearing H3K4 methylation.

Materials and Methods

Recombinant Protein Production

Recombinant baculovirus containing Flag-tagged CHD8 was created using the Bac-N-Blue baculovirus expression system (Invitrogen). This was used to express recombinant, full-length CHD8 for use in the enzymatic assays described below. For protein expression, Sf9 cells at 1×10^6 cells per mL were infected with the recombinant Flag-CHD8 virus and grown for 4 days. Cells were harvested, washed with PBS and resuspended in IP buffer (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF) containing 500 mM KCl and 1% NP-40 as well as 1 µg/mL of each of the protease inhibitors, aprotinin, leupeptin and pepstatin. The cells were then lysed using a Dounce homogenizer and lysates were cleared by centrifugation at 30,000×*g* at 4°C for 30 minutes. Samples were then dialyzed against IP buffer with 50 mM KCl and inverted overnight at 4°C with 250 µL of agarose beads conjugated with anti-Flag antibody M2 (Sigma). The beads were then washed sequentially with 10 column volumes each of 150 mM KCl in IP buffer, 350 mM KCl in IP buffer and again in 150 mM KCl in IP buffer. The protein was eluted with 400 µg/mL Flag peptide (Sigma) in 150 mM KCl IP buffer with 1 µg/mL each of aprotinin, leupeptin and pepstatin.

Recombinant histones were expressed and extracted from BL21 (DE3) *E. coli* cells essentially as described by Luger et al (218). Briefly, 1 L cultures of cells expressing recombinant histones were grown up to an OD_{600} of 0.4 to 0.6, induced by the addition of 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown for another 3 hours at 37°C. The cells were harvested by centrifugation at 4,500×*g* for 10 minutes at

4°C and then resuspended in 20 mL IP buffer with 100 mM KCl and 5 mM BME (β-mercaptoethanol). The cell suspension was lysed by two passages through a French pressure cell and the lysate was centrifuged at 12,000×*g* for 20 minutes at 4°C to pellet the inclusion bodies. The inclusion bodies were then washed once in IP buffer with 100 mM KCl and 1% Triton X-100 and then once in IP buffer with 100 mM KCl. Inclusion bodies were pelleted again by centrifugation at 12,000×*g* for 20 minutes at 4°C and extracted in unfolding buffer (7 M guanidine hydrochloride, 20 mM Tris-HCl [pH 7.5], 10 mM DTT [dithiothreitol]) at room temperature for 1 hour. The extracted inclusion bodies were centrifuged again at 12,000×*g* for 20 minutes at 4°C. The supernatant containing the extracted histone protein was removed and saved and the pellet was extracted once again in the same way as before. The supernatant from the second extraction was combined with that from the first extraction and this sample was dialyzed against 4 L of low-salt urea buffer (7M urea, 20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 5mM BME) overnight at 4 °C. The dialysate was centrifuged at 23,000×*g* for 20 minutes at 4°C and the supernatant was injected onto tandem Q Sepharose and SP Sepharose (Sigma-Aldrich) columns, arranged in that order, pre-equilibrated in low-salt urea buffer. The Q Sepharose column was then removed and the SP Sepharose column was eluted over a salt gradient set up using low-salt urea buffer and high-salt urea buffer (7M urea, 20 mM Tris-HCl [pH 7.5], 600 mM NaCl, 1 mM EDTA, 5mM BME), while collecting fractions over the entire gradient. The fractions were analyzed by Bradford assay and SDS-PAGE and the peak fractions were pooled and dialyzed overnight against 4 L of water at 4°C. The dialyzed histones were then aliquoted, lyophilized and saved.

Chemical Modification of Histones

Recombinant histones produced as described above were chemically modified so that their H3K4 residue was replaced with either a synthetic lysine analog or methyl-lysine analogs which functionally mimic their natural counterparts. This was done essentially as described by Simon et al (219). Briefly, a double point mutant histone H3 K4C/C110A was expressed and purified as described above. The chemistry to generate these analogs utilized the ability to alkylate cysteines using electrophilic ethylamines to yield aminoethylcysteine, a lysine analog. In addition, alkylation of target cysteines by (2-halo-ethyl) amines would yield the desired methyl-lysine analogs as well. Since the only natural cysteine residue in H3 is C110, this was mutated to an alanine, so that we could then generate lysine analogs at a specific position by introducing mutant cysteines at the desired site and alkylating that site with the appropriate reagent. To achieve this 6 mg of lyophilized H3 K4C/C110A histones were dissolved in 900 µL of alkylation buffer (1 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] [pH 7.8], 4 M guanidine hydrochloride, 10 mM L-methionine, 10 mM TCEP [tris(2-carboxyethyl)phosphine] and incubated at 37°C for 1 hour. 1 M solutions of 2-bromoethylamine and 2-(dimethylamino) ethyl chloride were prepared in alkylation buffer and 50 µL of each of these were added to the dissolved histones. Alkylation reactions were allowed to proceed in the dark at room temperature for 2 hours before renewing the reactions by adding another 50 µL and allowing them to proceed for another 2 hours. Reactions were stopped by adding 50 µL of 14.3 M BME and were dialyzed into water overnight at 4°C. Generation of the desired lysine and dimethyl-lysine analog-bearing histones was verified by mass spectrometric analysis.

Nucleosome Reconstitution

The lyophilized histones were resuspended in unfolding buffer to a concentration of 2 mg/mL and the unfolding reaction was allowed to proceed by inverting the samples for 2-3 hours at 4°C. The unfolded histones were then combined in equimolar ratios and the final concentration was adjusted to 1 mg/mL with unfolding buffer. This was dialyzed overnight against 4 L of refolding buffer (2M NaCl, 10 mM Tris-HCl [pH 7.5], 1mM EDTA, 5mM BME) at 4°C. The refolded octamers were then concentrated to 1 mL using an Amicon Ultra Ultracel-10K (Millipore) and the concentrated sample was centrifuged at 20,000×*g* for 20 minutes at 4°C. This sample was injected onto a Hi-Prep 16/60 Sephacryl S-200 column (GE Healthcare), equilibrated with refolding buffer, and run at 0.5 mL/min while collecting 2.5 mL fractions. The fractions were analyzed by SDS-PAGE. The fractions containing the histone octamer peak were pooled and concentrated to 1 mL using an Amicon Ultra Ultracel-10K.

Fluorescently labeled 277 bp DNA templates for nucleosome reconstitution were generated using standard PCR techniques with pGEM3z-601 as a template (220). The 601 forward (CGGGATCCTAATGACCAAGGAAAGCA) and 601 reverse (CTCGGAACACTATCCGACTGGCA) primers were used to generate the DNA templates for restriction enzyme accessibility assays and the 601slid forward (GTGATGGACCCTATACGCG) and 601slid reverse (ACTCACTATAGGGCGAATTC) primers were used for the nucleosome sliding assays. These primers generated DNA templates upon which the nucleosome would either be positioned in the middle or the end respectively of the template. A 0.1/0.9 ratio of the fluorescent primers (either 5'-Cy5 or 5'-AlexaFluor 488-*N*-hydroxysuccinimide ester) to

the non-fluorescent primers was used in the PCR reactions. The PCR product was analyzed on a 2% agarose gel, ethanol precipitated and resuspended in TE.

The appropriate combination of histone octamers and fluorescent DNA templates prepared as described above were combined in a range of ratios into reconstitution reactions. Multiple reactions were set up using a mean ratio of 1:1 between the histones and DNA and varying the amount of histones by a fraction of 0.125 to cover a range of ratios between 0.5:1 to 1.25:1. 100 µL reactions were set up in Slide-A-Lizer Mini Dialysis Units (MWCO = 3,500) (Pierce) with 2M NaCl and 0.5 mg/mL BSA (Bovine Serum Albumin) (New England Biolabs) along with the appropriate amounts of histone octamers and DNA. The reconstitution reactions were then dialyzed over a gradual salt gradient going from high-salt TE buffer (2M NaCl, 10 mM Tris-HCl [pH 8.0], 1mM EDTA, 0.2 mM PMSF] to low-salt TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.2 mM PMSF). The dialysis units were placed in a beaker containing 500 mL of high-salt TE buffer and the buffer was pumped out from this beaker at the same rate (1.6 mL/min) as 4 L of low-salt TE buffer was pumped into it over a period of 42 hours. Nucleosomes were analyzed on a 5% non-denaturing acrylamide/bisacrylamide (49:1) 0.2X Tris-Borate-EDTA (TBE) gel. Concentrations of reconstituted nucleosomes were calculated based on the final volumes of the reactions after dialysis.

Restriction Enzyme Accessibility Assay

Accessibility assays were performed essentially as described by Smith and Peterson (221). Nucleosomes containing the regular 601 sequence as the DNA template were generated as described above. They were then subjected to nucleosome digest tests

to determine which samples contained nucleosomes that were suitable for restriction enzyme accessibility experiments based on the level of protection of their intrinsic restriction sites. Assays were conducted in triplicate using 50 nM of the selected nucleosomes. Competitive assays contained 25 nM of each species of alternatively labeled nucleosomes. Each 15 µL reactions contained 20 nM Flag-CHD8 in the presence of 1 mM ATP in DNase buffer (20 mM HEPES [pH 8.0]3 mM $MgCl_2$, 50 mM NaCl, 2 mM DTT, 0.1 mg/mL BSA). Reactions also contained saturating amounts of HhaI restriction enzyme (New England Biolabs). They were incubated at 30°C for the indicated times and were terminated by adding 2X stop solution (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. Samples were analyzed on a 3% agarose gel and bands were quantified using a Typhoon Trio+ Imager and the ImageQuant TL software (GE Healthcare). Data presented is representative of the average value of each triplicate.

Nucleosome Sliding Assay

Nucleosome sliding assays were performed similar to the restriction enzyme accessibility assays above, without including restriction enzyme. Suitable nucleosomes assembled on 601slid DNA templates were selected for these assays based on the gel analysis of the reconstitutions. Each 15 µL reaction contained a total of 50 nM of reconstituted nucleosomes, 1 mM ATP and 20 nM Flag-CHD8 in DNase buffer. For competitive sliding assays, 50 nM each of two alternatively labeled nucleosome species were used. Reactions were incubated at 30°C for the indicated times and then terminated by adding 3 µL of 6X stop solution (30% glycerol, 10 mM Tris [pH 7.8], 1 mM EDTA, 334 µg/mL HeLa core nucleosomes, 334 µg/mL salmon sperm DNA [Invitrogen]) and

incubating for a further 15 minutes at 30°C. Samples were then analyzed on a non-denaturing 5% acrylamide/bisacrylamide (49:1) 0.2X TBE gel and fluorescent bands were detected using a Typhoon Trio+ Imager and ImageQuant TL software.

Results

CHD8 Preferentially Remodels Core Nucleosomes over Recombinant Nucleosomes

We began examining the substrate preferences for CHD8 by reconstituting nucleosomes from recombinant wild-type histones. These histones would presumably lack any covalent modifications, since they were expressed in *E. coli* where there is no known pathway for the post-translational modficiation of histones, and we could thus examine whether CHD8 is capable of remodeling unmodified nucleosomes. Mononucleosomes were reconstituted by incorporating recombinant histone octamers into a DNA template containing the 601 nucleosome positioning sequence with a unique HhaI restriction site located near its dyad axis (220). This restriction site is protected from access by the restriction enzyme when the template is reconstituted into a nucleosome by salt dialysis with histone octamers. We used this feature to carry out restriction enzyme accessibility assays to determine the remodeling activity of CHD8 on recombinant nucleosomes, as was done previously for HeLa core nucleosomes (104). Recombinant nucleosomes were incubated with either no CHD8 or with 10 nM or 20 nM CHD8 in the presence of restriction enzyme. We found that in the presence of CHD8 there was increased cutting of the DNA template as compared to the control reactions (Fig. 3.1). The fraction of template that was cut in the presence of CHD8 was

comparable to but slightly lower than that seen with HeLa core nucleosomes (104). This demonstrates that CHD8 is capable of remodeling unmodified, recombinant nucleosomes, albeit to a slightly lesser degree than modified core nucleosomes.

While this assay confirmed the remodeling activity of CHD8 on recombinant nucleosomes, we cannot quantitatively compare the extent of CHD8 remodeling between core and recombinant nucleosomes. To do this we performed competitive restriction enzyme accessibility assays in which equimolar amounts of core and recombinant nucleosomes were incubated in the same reactions and remodeling on each of these templates was then directly compared. To do this recombinant and core octamers were incorporated into 601 DNA templates that were differentially labeled with the Cy5 and Alexa488 fluorophores respectively. This allows us to distinguish between each of the nucleosomal species on a gel based on the differing fluorescent signals. Equimolar amounts of each of these nucleosomes were then incubated in reactions along with 20 nM CHD8 with excess restriction enzyme and restriction enzyme accessibility assays were performed as before. A time course of remodeling was conducted to determine whether either nucleosomal substrate was preferentially remodeled over the other. We found that, while CHD8 remodeled both species fairly robustly, it did so with a preference for the core nucleosomes (Fig. 3.2). The core nucleosomes were remodeled at both a faster initial rate, which implies that CHD8 has a substrate preference for one or more of the modifications that are present on the core nucleosomes but not on the recombinant species. The following experiments were designed to determine which modifications on which of the histone tails specifies the substrate requirement or preference of CHD8.

CHD8 does not Require the H3-H4 Tails for Remodeling Activity

The restriction enzyme accessibility assays described above allowed us to quantitate the capacity of CHD8 to remodel wild-type recombinant nucleosomes by exposure of the protected restriction site within the nucleosomal DNA template. This is an important result because it allows us to use and manipulate recombinant nucleosomes by introducing various mutations and modifications to examine the substrate preference of CHD8. While the restriction enzyme assay was quantitative, it does not yield any information on the qualitative nature of CHD8-remodeled nucleosomes as pertaining to nucleosome repositioning. Our previous studies on CHD8 remodeling on experimental templates lacking a nucleosome positioning sequence revealed that CHD8 prefers to reposition nucleosomes towards the center of a DNA template as determined by nucleosome sliding assays (104). Thus we employed similar sliding assays to look at the remodeling activity of CHD8 on mutated or modified nucleosomes assembled on 601slid DNA templates where the nucleosome positioning sequence was located at one end of the DNA template. These templates should be targeted by CHD8 in a manner that would reposition these nucleosomes from the DNA ends towards the center of the DNA template.

Wild-type recombinant octamers or octamers with their H3-H4 tails deleted were reconstituted into nucleosomes on the 601slid template. These were then subjected to nucleosome sliding assays to observe whether CHD8 is capable of repositioning nucleosomes lacking H3-H4 tails. As mentioned before, the requirement for histone tails is a key determinant of remodeling activity for several chromatin remodeling enzymes. ISWI and CHD1 require H4 tails for their remodeling activity, while nucleosome

remodeling by CHD3/4 does not require the presence of histone tails. We found that CHD8 was able to shift both the wild-type and the H3-H4 tailless nucleosomes away from the DNA ends and towards the center of the DNA template (Fig. 3.3), as seen before with core nucleosomes on unpositioned templates (104). We observed a shift in the fluorescently-labeled nucleosomes towards higher bands on the gel upon remodeling by CHD8. It has been previously observed that centrally positioned nucleosomes migrate slower than end-positioned nucleosomes (104). Since we observe a slower migrating species in Fig. 3.3, we concluded that CHD8 repositions both tailed and H3-H4 tailless nucleosomes towards the center of the DNA template. This may indicate that deletion of H3-H4 tails causes the nucleosomes to reconstitute into just one predominant species upon assembly, while wild-type nucleosomes form a couple of different species. Both remodeling reactions appeared to occur at comparably robust rates, but we cannot make a true comparison between the two from this experiment. We can, however, conclude that CHD8 does not require either the H3 or the H4 tail to catalyze nucleosome remodeling.

In order to directly compare CHD8 remodeling on wild-type and H3-H4 tailless recombinant nucleosomes, competitive nucleosome sliding assays were carried out. These were set up similar to the sliding assays described above, except 50 nM each of Alexa488-labeled wild-type and Cy5-labeled H3-H4 tailless nucleosomes were added to the same reactions. A time course of remodeling was then carried out as described in the presence of 20 nM CHD8 and the simultaneous remodeling of both nucleosome species was examined by detection of the alternately labeled fluorescent bands on the gel. CHD8 would be expected to shift the fluorescent bands corresponding to the preferred substrate at an earlier time point than the less preferred substrate and this way we could determine

whether CHD8 preferred substrates with or without H3-H4 tails. We observed that CHD8 remodeled both tailed and tailless nucleosomes at similar rates (Fig. 3.4). Interestingly, each of these nucleosome species appeared to migrate to slightly different positions on the gel, indicating that they were being repositioned by CHD8 to different locations on the DNA template. Therefore, we concluded that while CHD8 did not exhibit any particular preference for substrates with or without H3-H4 tails, it appeared to shift nucleosomes to different positions based on the presence or absence of these tails. In this respect, CHD8 appears to be more akin to CHD3/4 in its lack of dependence on histone tails for remodeling activity, however it does seem to exhibit unique specificity for repositioning nucleosomes based on the presence of the H3-H4 tails.

CHD8 Preferentially Remodels H3K4 Methylated Nucleosomes

The chromodomains of CHD8 have been implicated in binding dimethylated H3K4 marks on chromatin (100). In order to examine whether this binding determines the substrate specificity of the remodeling activity of CHD8, synthetically modified nucleosomes were generated to contain either unmodified or H3K4 dimethylated lysine analogs replacing their natural H3K4 residues. These nucleosomes were also assembled on to 601slid DNA templates and were utilized in competitive nucleosome sliding assays as described above. 20 nM each of the control H3K4C/C110A unmodified lysine analog-bearing nucleosomes and the corresponding dimethylated nucleosomes were added together in the same sliding reactions. We observed that in a time course of simultaneous remodeling by CHD8 of the unmodified and H3K4 methylated nucleosomes, the methylated substrate was remodeled earlier than the unmodified control (Fig. 3.5). This indicates that CHD8 preferentially remodels nucleosomes that are dimethylated at H3K4,

suggesting that the recognition of this mark by its chromodomains may influence its remodeling activity.

Discussion

The experiments described above were intended to elucidate the remodeling activity of CHD8 at several different levels. We first tried to address the basic substrate requirements for remodeling by CHD8. Does CHD8 require histone tails for remodeling nucleosomes? What, if any, are the required epitopes for efficient nucleosomal remodeling by CHD8? Next, we attempted to observe the effects of histone mutations and modifications on the substrate specificity of CHD8 remodeling by examining the differences between the various resultant remodeled nucleosome species. Lastly, we address the preference of CHD8 for certain nucleosomal substrates, by examining whether it preferentially remodels substrates bearing specific modifications.

Remodeling by CHD8: Substrate Requirements

The histone N-terminal tails extend well beyond the globular histone core and could serve as important points of contact with both the histone proteins of adjacent nucleosomes as well as non-histone proteins like chromatin remodeling enzymes. Internucleosomal contacts mediated by histone tails are important for the stabilization of higher order chromatin structure and the contacts of nucleosomes with chromatin remodelers via these tails are critical for maintenance and regulation of chromatin structure as well (222, 223). Thus it is not surprising that the deletion of histone tails has

been shown to affect the nucleosome remodeling activity of a number of ATP-dependent chromatin remodelers. We therefore examined the effect of histone tail deletions on CHD8 remodeling activity.

The experimental results from Fig. 3.1 and Fig. 3.3 show the basic requirements of CHD8 for remodeling nucleosomes. In Fig. 3.1 we see that CHD8 can remodel unmodified recombinant nucleosomes, indicating that histone modifications of any kind are not a necessity for CHD8 remodeling and that these recombinant nucleosomes can be used as a suitable substrate upon which to study the remodeling activity of CHD8. Fig. 3.3 shows that CHD8 can also remodel nucleosomes with their H3-H4 tails deleted. Since histone tails are convenient points of contact between chromatin remodeling enzymes and nucleosomes, several chromatin remodeling enzymes show very stringent requirements for specific histone tails in the course of catalysis. RSC and SWI/SNF appear to require all four of the histone tails for efficient remodeling. Genetic deletion of the H2A and H2B tails eliminates optimal chromatin remodeling and transcriptional regulation of target genes by SWI/SNF (206, 207). In addition deletion of the all four histone tails or even of the H3 tail alone *in vitro* adversely affects remodeling by RSC and SWI/SNF (208, 214). Thus the SWI/SNF family remodelers appear to utilize the histone tails to remodel nucleosomes. ISWI and CHD1 have been shown to require the H4 tails for remodeling (212, 213, 215), while CHD3/4 do not require the presence of histone tails (210, 211). Our observation that CHD8 does not require the presence of histone tails emphasizes the similarities between the CHD3-5 and CHD6-9 subfamilies in the context of their requirements for remodeling activity. This indicates that CHD8, like

CHD3/4, probably remodels nucleosomes by making contacts with the globular histone core bodies rather than their tails in the course of catalysis.

The similarity in mechanism of chromatin remodeling may also extend to the functional role of these proteins, since CHD8, like CHD3-5 has been implicated in transcriptional repression at select loci. However, the CHD8 has also been shown to play a role in transcriptional activation in several instances. Therefore, the mechanism of nucleosome remodeling is unlikely to be the sole determinant of the functional role of these enzymes. Their association with other factors, as well as their spatial and temporal regulation, is likely to be important in modulating their functional role at different target genes and in various cellular systems. The slightly lower values for the fraction of DNA template cut by restriction enzyme due to remodeling by CHD8 for the recombinant nucleosomes compared to core nucleosomes (Fig. 3.1) is indicative of a possible enhancement of its activity due to one or more of the modifications present in the core nucleosomes. This possibility was investigated in further experiments and is an attractive model for determining the identity of the chromatin sites which CHD8 would remodel *in vivo*. The capability of chromatin remodeling enzymes to target chromatin bearing particular histone modifications is a well-established paradigm. While the deletion of histone tails is not a naturally relevant physiological phenomenon, it does give us the opportunity to restrict the list of possible preferred modifications to particular histone tails. It also helps us to understand the mechanism by which CHD8 may operate even at the cellular level, using contacts with the histone core bodies to leverage nucleosomal repositioning, as suggested by the results in Fig. 3.3. These contacts would be distinct from those allowing other histone tail-dependent chromatin remodelers like ISWI,

SWI/SNF or CHD1 to remodel nucleosomes, permitting multiple such remodelers to be acting on nucleosomes at the same time. This mode of contact of CHD8 with its substrate would also be conducive to allowing histone modifying enzymes which predominantly associate with the histone tails to interact with the same nucleosome at the same time. Given the examples presented in Chapter IV of associations between CHD8 and other chromatin remodeling factors, this would be a useful feature of its remodeling activity, allowing simultaneous modification of the histone tails and nucleosome mobilization via the histone core of the same nucleosome. Thus this distinct mechanism of CHD8 remodeling may also allow it to cooperate with other remodeling factors, by contacting different parts of the nucleosome in the course of their action.

Remodeling by CHD8: Substrate Specificity

The second aspect of nucleosome remodeling by CHD8 that we studied was how the specificity of CHD8 towards its substrate influenced the generation of remodeled products by its catalytic activity. This pertains to the differential mobilization by CHD8 of nucleosomes that have been variously modified. We examined this effect in Fig. 3.4, when we compared the remodeling of nucleosomes lacking H3-H4 tails to that of intact nucleosomes in a competitive sliding assay. This assay allowed us to look at the generated products of CHD8 remodeling activity and detect the presence of different species via their differential migration on a non-denaturing gel. We found that indeed CHD8 did remodel tailless nucleosomes differently from intact ones, as demonstrated by the detection of distinct fluorescent bands for each of the remodeled species. The non-overlapping products of CHD8 remodeling on these distinct nucleosome forms showed that in the absence of H3-H4 tails CHD8 repositions nucleosomes to slightly different

positions on a gel than in the presence of them. It did, however, mobilize both nucleosomal species towards the center of the DNA template as evidenced by the net upward shift of the bands containing both fluorescent labels, indicating the generation of slower migrating, more centrally positioned nucleosomes. This was verified by running a control centrally-positioned nucleosome alongside the remodeled ones, to compare their migration through the gel. Upon close examination of the bands, it appears that CHD8 remodeling on the intact nucleosomes yielded a single centrally positioned product along with the original unremodeled, end-positioned substrate. Remodeling of the tailless nucleosomes, however, generated two distinct product species, both shifted towards the center compared to the substrate nucleosomes. Thus while CHD8 can adequately contact and mobilize nucleosomes lacking tails by interacting with the globular core, it might use the tails to stabilize remodeled products into a single centrally positioned form.

The directionality of CHD8 remodeling is consistent with that of both CHD1 and CHD3, which have also been shown to reposition nucleosomes towards the center of a DNA template (224). Whether this directionality of remodeling has any physiological relevance remains to be seen, but given the fact that CHD8 is able to remodel a nucleosome positioned at one end of a DNA template raises the possibility that it does not require interaction with DNA segments on both sides of the nucleosome to catalyze remodeling. This distinguishes it from ISWI which has been predicted to remodel substrates with DNA segments on either side of a central nucleosome, since ISWI cannot remodel end-positioned nucleosomal templates (211). Thus remodeling by ISWI was predicted to involve two molecules of ISWI interacting with one central nucleosome. In this case then, it would appear that monomeric CHD8 would be capable of remodeling

nucleosomes. The ability of CHD8 to reposition nucleosomes to specific positions is evidence that chromatin remodelers like CHD8 are complex organizers of chromatin and are capable of determining the genomic distribution of nucleosomes by their action. The nucleosome-remodeler interactions are likely determinants of these positions based on the binding affinity of these interactions. One can imagine that nucleosome positioning is the net result of transient interactions of the remodeler with the nucleosome, allowing it to associate and mobilize the nucleosome to certain thermodynamically favored positions, and then disassociate at these preferred sites. For CHD8, it appears that the histone tails foster some such interactions which alter the dynamics of nucleosome-remodeler interactions so that the remodeling reaction yields nucleosomes positioned at different positions when the tails are eliminated. Thus while contacts with the histone core are sufficient for remodeling by CHD8, histone tail contacts may facilitate the process by defining the association-dissociation dynamics of the substrate and the enzyme so as to determine the positioning of end products.

Remodeling by CHD8: Substrate Preference

The last aspect of chromatin remodeling by CHD8 that we investigated was the preference of CHD8 for substrates containing specific features. In this case we looked at histone methylation as a possible preferred modification. The connection between ATP-dependent chromatin remodeling enzymes and histone methylation has been well established. This association can occur at different levels. Histone methylation could serve as a recognition mark for the recruitment of ATP-dependent remodelers. Alternatively HMTs could directly associate with nucleosome remodelers bringing together these two chromatin–related activities within the same complex. Both of these

scenarios are revisited in Chapter IV. Finally, the interplay between these two processes could exist at the level of modulation of chromatin remodeling activity, where histone methylation could affect the activity of nucleosome remodelers, or vice versa, ATP-dependent repositioning of nucleosomes could modulate histone methylation.

Based on the result in Fig. 3.2, where we saw that core nucleosomes were remodeled at a faster initial rate and to a greater final extent by competitive restriction enzyme accessibility assays, we concluded that CHD8 preferentially remodeled core nucleosomes over recombinant ones. Thus the presence of certain histone modifications appears to enhance the enzyme-substrate interactions so that remodeling proceeds with greater efficiency. The higher initial rate of modification could be due to a specifically remodeled nucleosome species being preferentially remodeled by CHD8 ahead of an unmodified substrate. The greater final extent of remodeling is also indicative of a particular substrate bearing a preferred modification being remodeled in sustained manner over the period of the reaction at the expense of unmodified substrates. We initially attempted to identify potential preferred modifications by eliminating histone tails to determine which ones were required for remodeling, which would subsequently help us narrow down the range of candidate modifications to the ones on the required tails. However the result from Fig. 3.3 and 3.4 revealed that CHD8 did not require histone tails for its remodeling activity. Thus while a particular tail or its modified residues may not be required for CHD8 remodeling, one or more histone modifications could enhance its activity. Indeed, it has been observed that H3K14 acetylation is responsible for enhanced remodeling by RSC (215). We attempted to examine whether CHD8 similarly preferred any particular histone modification for catalysis.

We took a candidate approach towards exploring histone modifications that could be preferred by CHD8 in the course of its remodeling activity. Histone methyl marks have been shown to be critical for the recruitment and activity of several other ATP-dependent chromatin remodelers, which is discussed further in Chapter IV. Characteristic domains found in many nucleosome remodelers, like PHD and chromodomains have been shown to recognize and bind histone methylation in the course of action of these remodeling enzymes. The chromodomains of CHD8, like those of CHD1, have been reported to bind to dimethylated H3K4 (100). This makes this histone mark a promising candidate for the substrate preference of CHD8 remodeling activity.

We examined this possibility in Fig. 3.5, where synthetic analogs of nucleosomes containing unmodified or dimethylated H3K4 were used in competitive sliding assays. We found that the H3K4 dimethylated nucleosomes were preferentially remodeled by CHD8. Given the evidence of CHD8 binding to this histone mark through its chromodomains, this effect on remodeling is not unexpected. Indeed the enhanced activity of RSC due to H3K14 acetylation, was found to be due to the increased binding of RSC to the substrate through its bromodomains (38, 215). A similar mechanism could be responsible for the preference that CHD8 shows for remodeling nucleosomes bearing the dimethyl-H3K4 mark. However, this binding-based mechanism is not the only way chromatin remodelers may show substrate preference. Remodeling by ISWI and CHD1 were adversely affected by H3K14 acetylation, but not due to their binding properties to this mark, rather by reducing the rate of ATP turnover. Thus it is possible that other histone modifications could also effect CHD8 remodeling by either of these mechanisms. However, H3K4 dimethylation does provide an attractive target modification which,

through recognition by the chromodomains of CHD8, could be involved in both the recruitment and enhancement of chromatin remodeling activity of CHD8. Indeed, methylation of H3K4 has been shown to be required for AR binding to target enhancers in LNCaP cells (225) and it is tempting to speculate that this recruitment is mediated by the recognition and remodeling of H3K4-methylated nucleosomes by CHD8, which then facilitates AR binding to these sites. Connections between CHD8 and this histone modification are further explored in Chapter IV.

While the effect of histone acetylation on the chromatin remodeling activities of a number of nucleosome remodelers has been well studied (215-217), histone methylation has not been previously known to exert such an influence on nucleosome remodeling. Here, we show that CHD8 preferentially remodels H3K4 dimethylated nucleosomes. The possibility exists that mono- and trimethylation of this mark may also influence CHD8 remodeling. Also, this modification could affect remodeling by other CHD family remodelers which are known to have binding specificity for this mark. These possibilities need to be verified by further experiments. The work described here, however, lays the foundation for understanding the intricacies of the chromatin remodeling activity of CHD8 and consequently of the CHD6-9 subfamily.

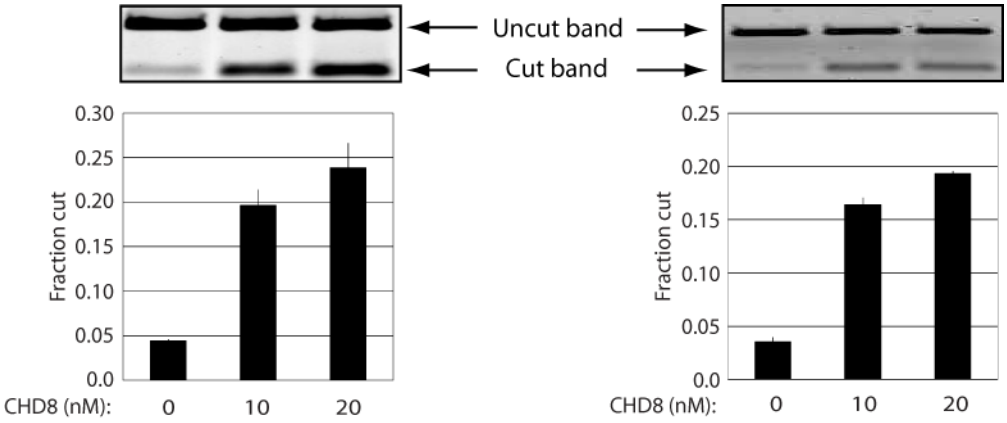

Figure 3.1: Comparable chromatin remodeling of core and recombinant nucleosomes by CHD8. Recombinantly produced histone octamers were incorporated into fluorescently labeled DNA templates to form mononucleosomes. 50 nM of these nucleosomes were assayed for restriction enzyme accessibility by incubating with the indicated concentrations of CHD8 along with ATP and the HhaI restriction enzyme for 30 minutes at 30 °C. Reactions were done in triplicate and were analyzed by running on an agarose gel. Gels were imaged on a Typhoon Trio+ Imager and the indicated bands were quantified using the ImageQuant TL software. The average fraction cut was calculated from this and is plotted in the panel to the right with an image of samples from the gel above it. The results from the restriction enzyme accessibility assays performed with core mononucleosomes by Thompson et al.(104) is included in the left panel for comparison.

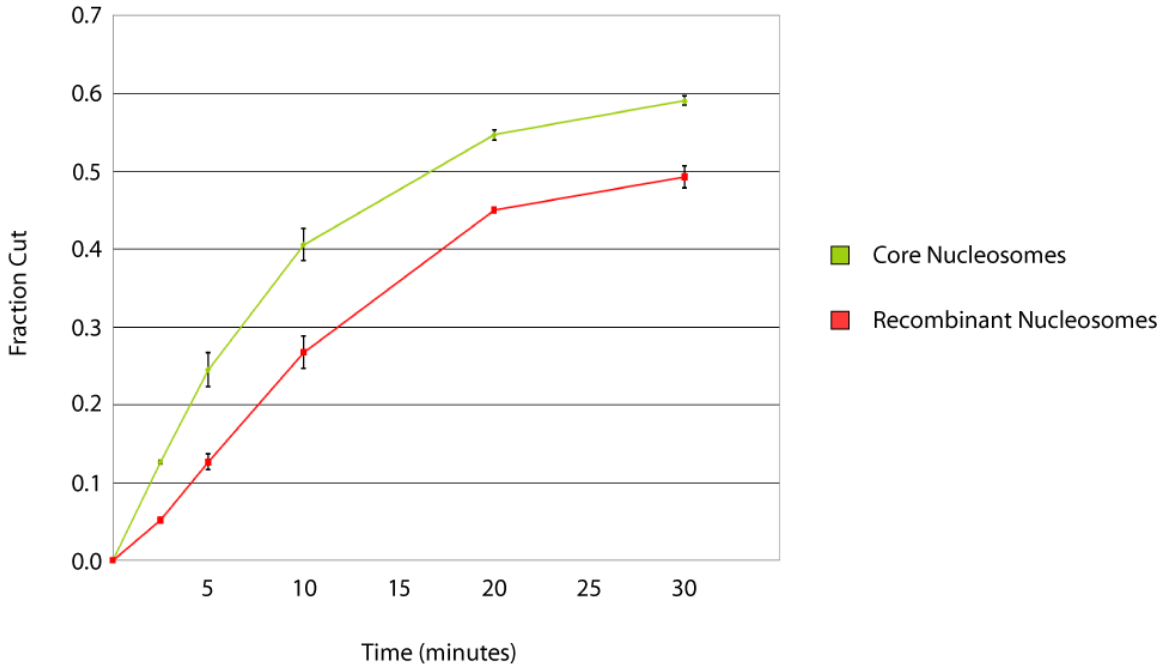

Figure 3.2: Preferential remodeling of core nucleosomes over recombinant nucleosomes by CHD8. Competitive restriction enzymes accessibility assays were set up using equimolar amounts (25 nM) of alternately labeled core (Alexa488) or recombinant (Cy5) nucleosomes. These mixes were incubated with 20 nM CHD8 in the presence of HhaI restriction enzyme for the indicated time. The fraction of DNA template cut due to nucleosome remodeling at each time point for each nucleosomal substrate was measured and plotted.

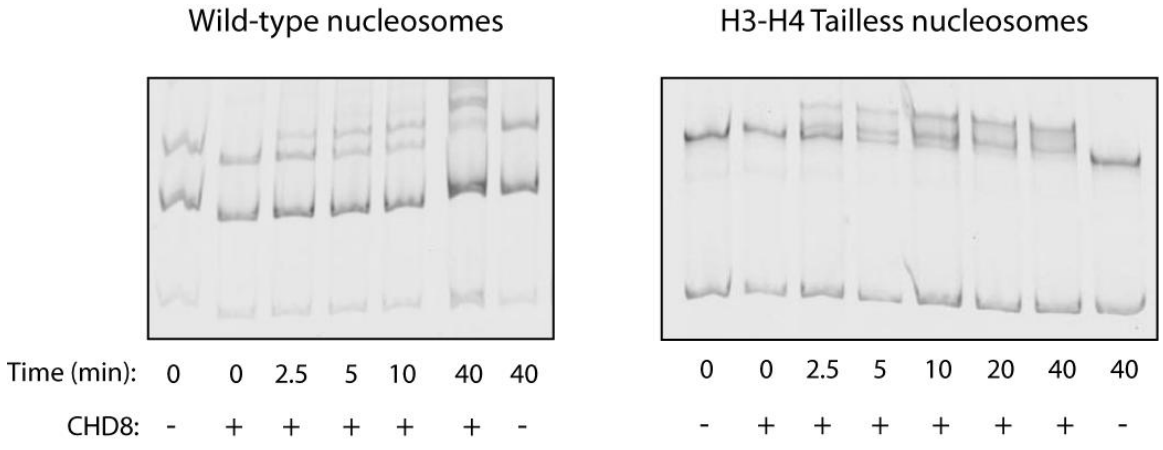

Figure 3.3: Remodeling Activity of CHD8 on Nucleosomes lacking H3-H4 Tails. Nucleosome sliding assays were conducted using either wild-type or H3-H4 tailless histone octamers incorporated into a fluorescent DNA template designed so that the nucleosomes are positioned at one end of the template. 50 nM of each of these nucleosomes was incubated along with 20 nM CHD8 for the indicated time. Reactions were subjected to electrophoresis on a non-denaturing gel and the resultant bands of the remodeled products were analyzed using fluorescence imaging.

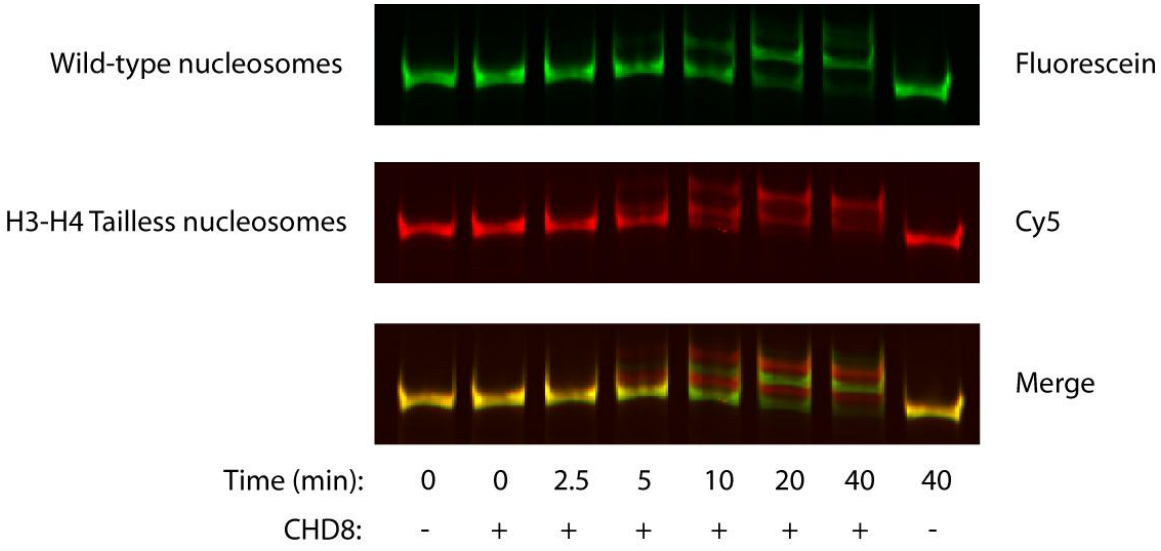

Figure 3.4: Differential Remodeling of Wild-Type and H3-H4 Tailless Nucleosomes by CHD8. Competitive nucleosome sliding assays were conducted by incubating equimolar amounts (50 nM each) of alternately labeled wild-type (fluorescein) and H3-H4 tailless (Cy5) nucleosomes for the indicated time points with 20 nM CHD8. Remodeled products were analyzed on a non-denaturing gel by fluorescence imaging. Differentially generated products of the remodeling reaction were analyzed by merging the two fluorescent signals.

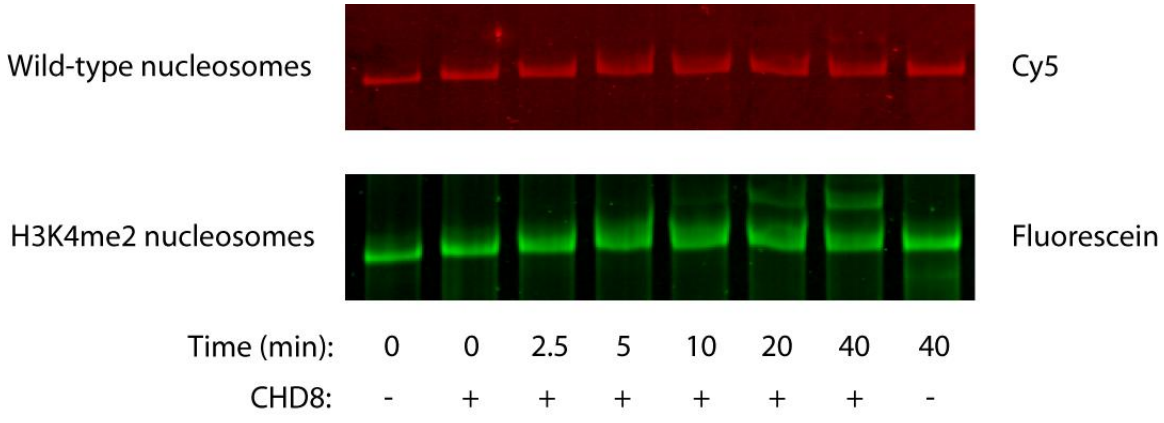

Figure 3.5: Preferential Remodeling of H3K4-Dimethylated Nucleosomes by CHD8. H3K4C/C110A double mutant histones were expressed and were then chemically modified to give synthetic analogs that mimic either an unmodified lysine or a dimethylated lysine at the H3K4 position. These chemically modified histones were incorporated into octamers and then reconstituted into nucleosomes on a DNA template with a nucleosome positioning sequence located at one end. These were then included in competitive sliding assays as in Fig. 3.4, using 20 nM of each nucleosomal substrate and 10 nM of CHD8. Remodeled products were analyzed on a non-denaturing gel by fluorescence imaging.

CHAPTER IV

CHD8 and the MLL1-WAR Complex

Introduction

ATP-dependent Chromatin Remodeling Complexes

As discussed in Chapter I, control of DNA accessibility is an important regulatory point for several cellular processes, like replication, repair and transcription. Due to the packaging of DNA into chromatin, eukaryotic cells have evolved different mechanisms to regulate chromatin structure. These include DNA methylation, covalent modification of histones and ATP-dependent chromatin remodeling which are catalyzed by different classes of enzymes. ATP-dependent remodeling enzymes utilize the energy from ATP-hydrolysis to modulate nucleosome structure and positioning. The catalytic ATPase subunit is usually present within large, multiprotein complexes containing several other components. SWI/SNF was originally identified as an ~11 subunit complex in yeast (226). RSC is another SWI/SNF family remodeling complex found in yeast and has 15 subunits (227). These complexes are very large and can be up to 2 MDa in size. On the other hand, ISWI family ATPase-containing complexes like ACF (ATP-utilizing Chromatin Factor), NURF (Nucleosome Remodeling Factor) and CHRAC (Chromatin

Accessibility Complex) are much smaller; only about 300-800 kDa in size containing only 2 to 4 subunits (226). CHD family proteins have been reported to function as monomers (CHD1) as well as in complexes (CHD3-4/Mi-2) such as NURD (228, 229). INO80 and SWR1 are also large multiprotein complexes consisting of 15 and 14 subunits respectively (226).

The non-ATPase subunits of ATP-dependent chromatin remodeling complexes are involved in the direct regulation of the nucleosome remodeling activities of the ATPases (230). The BAF155, BAF170 and INI1/SNF5 subunits form a core functional complex along with BRM and BRG1 and stimulate the nucleosome remodeling activities of human SWI/SNF complexes (231, 232). As mentioned in Chapter II, the BAF57 subunit of SWI/SNF is responsible for targeting its remodeling activity in AR-mediated transcriptional regulation (140). The ACF1 subunit found in ISWI-containing ACF and CHRAC complexes and the NURF301 subunit of the NURF complex, have been shown to enhance nucleosome remodeling by ISWI and to affect the specific targeting of these complexes (233-236). While the monomeric ISWI ATPase subunit by itself requires the presence of all four histone tails in order to efficiently remodel nucleosomes, in the presence of ACF1 only the H4 tail is required for its remodeling activity on nucleosomes (212). Both the ACF1 and NURF301 subunits appear to contribute to ISWI remodeling by providing additional points of contact with the nucleosome through their PHD fingers (32, 58). Thus the protein subunits of chromatin remodeling complexes can contribute to the activity of their ATPase components by stimulating the efficiency of their nucleosome remodeling activity, targeting them to specific chromatin sites, altering their substrate requirements or enhancing their binding to nucleosomes.

Based on the subunit composition of ATP-dependent chromatin remodeling complexes, their cellular functions can be modulated in many different ways. The RSC and SWI/SNF complexes in yeast are differentiated by the presence of unique subunits within each of these complexes that distinguish them apart both functionally and compositionally. Yeast RSC complexes contain an Sth1 catalytic subunit and additional Rsc1-Rsc15 subunits, while SWI/SNF contains a SWI2/SNF2 ATPase as well as the additional subunits, Swi1 –Swi11. RSC and SWI/SNF are found to regulate entirely different subsets of genes due to the specificity imparted to them by their unique subunits (230). Furthermore, RSC complexes also exist in two functionally distinct forms based upon the mutually exclusive presence of either of the subunits Rsc1 or Rsc2 within the complex (237). This divergence in SWI/SNF remodeling complexes is conserved from yeast to higher eukaryotes as well. Just as in yeast, there are two compositionally diverse SWI/SNF complexes in both *Drosophila* and mammals. These are the RSC-homologous dBAP/BAF complex, which is characterized by the presence of the dOSA/BAF250 subunits, and dPBAP/PBAF, which contains unique dPolybromo/BAF180 subunits and is homologous to yeast SWI/SNF. Furthermore, in humans, BAF complexes may contain either one of the two SWI/SNF-homologous catalytic units, hBRM or BRG1, whereas PBAF complexes only contain BRG1 as their catalytic component. These two functionally distinct human SWI/SNF complexes have been shown to associate with different promoters and regulate their expression by interacting with different transcription factors (238, 239). BRG1 interacts with Zn-finger proteins like GATA1 through a unique N-terminal domain not present in BRM, while BRM interacts with ankyrin-repeat proteins which are critical components of the Notch signaling pathway

(239). There is also considerable tissue-specific variation in the subunit composition of human SWI/SNF, where different factors like BRCA1 and components of the HDAC complex Sin3 differentially associate with the remodeling components (227). From these instances we see that the activity of ATP-dependent chromatin remodeling enzymes is significantly regulated by their association with different subunits found within their naturally occurring complexes.

Histone Modifications and Chromatin Remodeling

ATP-dependent chromatin remodeling complexes often including various chromatin modifying enzymes like histone acetylases, deacetylases, methyltransferases and demethylases. The coordination of these two different kinds of chromatin remodeling events is necessary for the establishment of the appropriate chromatin state. This is achieved by functional interactions between these two classes of chromatin remodeling factors within complexes. Histone modifications can alter chromatin structure either directly by sterically altering the chromatin structure, or indirectly by recruiting trans-acting factors like chromatin remodeling enzymes (240). These trans-acting factors often have specialized domains which are capable of recognizing and binding specific histone modifications. Some of the characteristic domains involved in recognition of histone modifications that are found in ATP-dependent chromatin remodeling enzymes have been discussed in Chapter I.

The association of histone modifying and chromatin remodeling enzymes is demonstrated in the NURD complex which contains the Mi-2 ATPase subunit as well as the deacetylases HDAC1 and HDAC2 and is involved in transcriptional repression.

CHD1 has been reported to associate with the SAGA complex, which contains Gcn5-mediated HAT activity as well as the deubiquitylation activity of its Ubp8 subunit (241). Components of the repressive mSin3 HDAC complex were found to co-purify with both varieties of human SWI/SNF complexes, those containing hBRM as well as those with BRG1, which usually regulate the activation of transcription (242). Further work revealed that the HMTase PRMT5 was also found in a complex containing mSin3 and BRG1 that was involved in transcriptional repression of target genes (243). Since BRM and BRG1 are usually involved in transcriptional activation, these examples reveal that the association of different histone modifying activities with chromatin remodelers is capable of modulating the function of these complexes from transcriptional activation to repression and vice versa.

The MLL Methyltransferase Complex

Histone methylation can be either an activating or repressive chromatin mark for transcription, depending on the specific residue being methylated (244). Di- and tri-methylation of H3K4 is associated with transcriptional activation, while H3K9 and H3K27 methylation is implicated in repression. All of the identified HMTases involved in the methylation of histone tails contain the conserved catalytic SET (Su(var), Enhancer of Zeste, Trithorax) domain. In humans, four SET domain-containing proteins have been identified as H3K4 HMTases: Set1A, Set1B and MLL1-4 (245). MLL1, which is a homolog of the *Drosophila* protein Trithorax, is involved in certain oncogenic chromosomal translocations found in a number of acute lymphoid and myeloid leukemias (246). These chromosomal rearrangements result in the fusion of the genomic sequence encoding the N-terminus of the MLL1 protein with the C-terminal encoding sequences of

other translocation partners, generating oncogenic fusion proteins (247). The MLL1 protein contains a central PHD domain, which along with the characteristic SET domain at its C-terminus, is homologous to Trithorax. The N-terminal AT-hook and DNA methyltransferase (DNMT) homology regions are retained in MLL1 rearrangements and are thought to bind to specific chromosomal regions (247). Full-length MLL1 is comprised of heterodimers of MLL-N (including the AT-hooks, DNMT domain and PHDs) and MLL-C (containing the SET domain). MLL-N by itself appears to be repressive in function due to interactions with Pc group repressors and the recruitment of HDACs by its DNMT region (248). However, heterodimerization with MLL-C results in transcriptional activation by the complex as evidenced by its interactions with a coactivator, CREB-binding protein (CBP), and its recruitment of HATs and SWI/SNF, chromatin remodeling complexes (249, 250). Finally, the SET domain of MLL-C has been shown to have intrinsic HMTase activity specific for H3K4, a mark of active chromatin (250, 251). Therefore MLL1 acts as a platform for bringing together various chromatin remodeling activities into a complex with alternative transcriptional functions, depending on its dimerization status.

A common element to the hSet1 and MLL family members is a core complex comprised of WDR5 (WD-40 Repeat protein), Ash2L (Absent Small or Homeotic-like) and RbBP5 (Retinoblastoma Binding Protein) (252-256). This core complex, hereafter referred to as WAR, was found to be required for optimal HMTase activity of MLL1, both *in vivo* and *in vitro* (252, 257, 258). The WAR complex also appears to associate independent of MLL1, providing a structural platform for association with other H3K4 HMTases of the hSet1/MLL families (257). While there has been considerable debate

about the binding specificity of WAR with histones, structural studies have shown that WDR5 binds the H3 tail, raising the possibility that WAR may be involved in targeting MLL1 activity to catalyze H3K4 methylation (259, 260). More recent studies have suggested that WDR5 binds MLL through the same binding pocket with which it interacts with H3 and that this interaction is essential for complex formation and consequently for catalytic activity (261-263). Additionally, the WAR complex by itself has been recently shown to have H3K4-specific HMTase activity, and its association with MLL1 results in greatly enhanced methylation of H3K4 (264). All these examples lead to the conclusion that WAR is essential for targeting MLL1 to H3K4 sites, where it plays an important role in catalysis of H3K4 methylation by MLL1.

Hox Gene Regulation by the MLL-WAR Complex

In vertebrates, the homeobox (Hox) gene clusters encode a group transcription factors that play a highly conserved role in the control of cell fate during embryonic development (88, 265). Their *Drosophila* counterparts are known to specify body segment identity by regulating the transcription of a number of downstream target genes (87). Mutations or dysregulation of the Hox genes result in homeotic transformations involving either duplication or loss of body structures (266). Therefore, it is very important for the transcription of these genes to be precisely regulated so that specific combinations of Hox transcription factors are expressed in a spatially and temporally coordinated manner.

The Hox gene clusters are organized as discrete groups named HoxA, HoxB, HoxC etc. The Hox genes towards the 3' end of these clusters tend to be expressed in

tissues towards the anterior end of the developing embryo earlier on in development, while the genes located progressively towards the 5' end of the clusters are expressed subsequently in the posterior tissues (267). Treatment with a*ll trans* retinoic acid (ATRA) is known to induce such a transcriptional cascade of Hox gene expression from the 3' to the 5' end of each cluster. Retinoic Acid Response Elements (RARE) have been identified near the 3' end genes of each cluster, like HoxA1 and HoxB1, and removal of these RAREs or ATRA depletion results in delayed Hox gene activation (268). Once established, the expression pattern of these Hox genes has to be faithfully maintained for the duration of their function in development.

This is achieved by the opposing but coordinated action of the Trithorax group (TrxG) of activators and the Polycomb group (PcG) of repressor proteins. Both these groups of transcriptional regulators contain both HMTase activity and proteins that bind histone lysine methylation marks. PcG mutations are known to result in ectopic expression of the Hox genes giving rise to posterior homeotic transformations (266). Polycomb Repressive Complex 2 (PRC2) maintains the long-term repression of specific Hox genes by depositing repressive H3K27 methyl marks (269). PRC1, which gets recruited by methylated H3K27, inhibits chromatin remodeling and promotes chromatin condensation into a transcriptionally repressive form (270). Conversely, TrxG proteins activate Hox genes by regulating the methylation of H3K4, which is generally associated with active transcription. Trx genes were identified in screens for extragenic suppressors of the homeotic phenotype displayed by PcG mutations (271). Thus the TrxG and PcG proteins counteract one another by regulating histone methylation of alternative sites.

MLL1 is one such TrxG activator known to methylate H3K4 at target Hox genes and positively regulate their transcription (251, 272). Knockout studies in mice have shown that Hox gene expression patterns are appropriately initiated but not maintained during development in the absence of MLL1 (272). While the deletion of MLL1 ($MLL^{-/-}$) is embryonic lethal, mice expressing MLL1 with a deleted SET domain (MLLΔSET) were both viable and fertile, showing that functions of MLL1 outside those of its SET domain are necessary for embryonic development (273). However, Hox gene expression levels were found to be lower in MLLΔSET mice when compared to wild type mice, showing that methylation of H3K4 by MLL1 is important in Hox gene expression (274). Mouse embryo fibroblasts (MEFs) derived from $MLL^{-/-}$ mice were found to have specifically downregulated expression levels of the 5' genes of the HoxA and HoxC clusters (275). In addition, ChIP analyses have shown that MLL1 localizes to broad transcriptionally active regions of the HoxA cluster, particularly at the late 5' genes HoxA9-HoxA13, and also to the promoter of the HoxC8 gene (251, 276). Thus MLL1 plays a critical role in the regulation of Hox gene expression.

Given the functional role of the WAR complex in the regulation of HMTase activity of MLL1, as discussed above, one would expect WAR to also be involved in Hox gene regulation. Indeed, when each of the components of WAR was knocked down individually using siRNAs, the expression levels of the well-characterized MLL1 target genes HoxA9 and HoxC8 were reduced (257). Methylation levels of H3K4 were also reduced upon knockdown of each WAR component at both of these loci, highlighting the importance of WAR in MLL1-mediated methylation (245, 257). The recruitment of MLL1 to these target genes was not affected by knocking down WAR components,

showing that the involvement of WAR in Hox gene regulation is due to its regulation of the HMTase activity of MLL1 (245, 257). Other studies have also highlighted the role of individual components of WAR, like WDR5 (252) and Ash2L (258), in the regulation of H3K4 methylation, and subsequently expression, of MLL1 targeted Hox genes. Thus the WAR complex acts in conjunction with MLL1 in regulating Hox gene expression via H3K4 methylation.

CHD8 and WAR

As discussed above, most ATP-dependent chromatin remodeling enzymes are found in large molecular weight multiprotein complexes. In order to examine whether CHD8 is also present in such a complex a partial purification of CHD8 from HeLa cell nuclear extract was performed in a previous study from our group (104). The analysis of the purification revealed that CHD8 was a component of a complex of about 900 kDa. Since Chd8 itself is only about 290 kDa in size, this result suggests that CHD8 associates with other proteins in a higher molecular weight complex. Affinity purification of CHD8 followed by mass spectrometry of the predominant CHD8-associated polypeptides identified a number of chromatin-related proteins. These included components of the WAR and CoREST chromatin modifying complexes and of the SWI/SNF and NURD remodeling complexes among others. Due to the previous identification of CHD8 as a component of an MLL HMTase complex (256), the association of CHD8 with components of the WAR complex was further examined. CHD8 co-purified with WDR5, Ash2L and Rbbp5, but not with MLL1 (104), indicating that CHD8 may associate with WAR in an MLL-independent manner. Indeed, WAR has been reported to form a subcomplex independent of MLL (257, 258). WDR5 has been reported as a component

of ATAC and MOF HAT complexes (277-279). The WAR complex has also been shown to associate with nuclear receptor coregulator interacting factor (NIF-1) and enhance transcriptional activation of nuclear receptor responsive genes (280). Thus to verify an association between WAR and CHD8, coimmunoprecipitation experiments were performed in HEK293 cells transfected with Flag-WDR5. Immunoprecipitation of cellular extracts followed by Western blot analysis revealed that WDR5 interacts with endogenous CHD8. This interaction was verified using coimmunoprecipitation of recombinant GST-WDR5 and CHD8 proteins. Thus we have evidence of a direct interaction between both endogenous and recombinant CHD8 and WDR5.

Hypothesis and Summary of Results

Given the evidence of interaction between WDR5 and CHD8, the examples of WAR associating with factors other than MLL, and the propensity of ATP-dependent chromatin remodelers to form complexes, we hypothesized that CHD8 and WAR could form a discrete complex, either with or without MLL1. To show this, co-infection experiments were done using baculovirus to express the different component of WAR, as well as CHD8 and MLL1, in insect cells. Complexes were purified utilizing epitope tags on either CHD8 or WDR5 and analyzed by Western blot with antibodies specific for each complex component. It was seen that CHD8 forms a complex with WAR, both with and without MLL1, indicating that CHD8 can form a complex with WAR independent of MLL1. Also, loss of any one component of the CHD8-WAR-MLL1 complex did not preclude complex formation by the other components, showing that CHD8-WAR-MLL1 complex formation is not dependent on any one component. The pairwise binding of each component of the complex with CHD8 was then examined. This was done by

coexpressing each individual component in insect cells along with Flag-CHD8 and performing co-immunoprecipitations using anti-Flag antibodies. It was seen that CHD8 interacted directly with each component of MLL1-WAR. This, along with the previous co-infection experiments emphasizes the fact that there are extensive contacts within the CHD8-WAR-MLL1 complex holding it together. We then purified extracts from co-infected insect cells by size exclusion chromatography to see whether CHD8 co-elutes with WAR and MLL1. We found that CHD8 co-eluted in the same fractions as components of WAR, as well as MLL1, indicating their presence in the same complexes. Finally, we tested the effect of the association of CHD8 with these complexes on its chromatin remodeling activity. We found that association with WAR or with MLL1-WAR had no effect on CHD8 remodeling activity on core nucleosomes. In summary, we characterize distinct complexes of CHD8 with WAR alone and with WAR-MLL1, define the nature of the interactions within these complexes and the examine effect on remodeling of these complexes.

Materials and Methods

Cell Culture and Reagents

SF9 cells were cultured at 24°C in 1X Grace's Insect medium (Invitrogen) containing an additional 10% fetal bovine serum and 1X penicillin-streptomycin-glutamine. CHD8 rabbit polyclonal antibodies were previously described (104). The anti-Flag M2 (F3165) antibody and rabbit normal IgG immunoglobulin (I8140) were purchased from Sigma. The anti-RbBP5 (A300-109A) anti-Ash2L (A300-489A) and

anti-MLL1 (A300-086A) antibodies were purchased from Bethyl. The anti-WDR5 (22512-100) antibody was purchased from Abcam. .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 baculovirus containing MLL-C, WDR5, Ash2L, and RbBP5. The WDR5 construct was a kind gift of Y. Dou. MLL-C, Ash2L and RbBP5 constructs were the generous gift of J. F. Couture. The Flag-tagged CHD8 baculovirus used was previously described (104). 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 PBS and resuspended in 500 μl of IP lysis buffer (0.2 mM EDTA, 1% NP-40, 10% glycerol, 0.2 mM 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.

For chromatin remodeling assays, recombinant Flag-CHD8 alone or Flag-CHD8 in complex with WAR or MLL1-WAR was prepared by infecting 1×10^8 SF9 cells with the appropriate recombinant baculoviruses. Cells were harvested 3 days post-infection, washed with PBS, and suspended in IP lysis buffer with 150 mM KCl, 1 μg/ml aprotinin,

1 μg/ml leupeptin, and 1 μg/ml pepstatin. The lysates were centrifuged at 20,800×*g* for 15 minutes at 4°C. Cleared lysates were incubated with 80 μl anti-Flag M2 conjugated agarose beads overnight at 4°C with rotation. Flag-IPs were washed with 10 column volumes of each of the following buffers: IP lysis buffer with 150 mM KCl, IP lysis buffer with 350 mM KCl, and IP lysis buffer with 150 mM KCl. Flag-IPs were eluted in buffer A (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF) containing 500 μg/ml Flag peptide (Sigma), 150 mM KCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin.

Fractionation of Complexes by Size Exclusion Chromatography

SF9 cells were co-infected with combinations of baculovirus to co-express either the core components of the WAR complex, with Flag-CHD8 and WAR, or with Flag-CHD8, MLL-C and WAR, as described above. Cells were lysed in IP lysis buffer with 150 mM KCl as above and the lysates were centrifuged at 20,000×*g* for 20 minutes at 4°C. Cleared lysates were fractionated by size exclusion chromatography over a Superose 6 HR 10/30 column (GE Healthcare) equilibrated and run in IP lysis buffer with 350 mM KCl. Samples of the eluted fractions containing the protein peak were subjected to SDS-PAGE followed by Western blot analysis using the specified antibodies.

Chromatin Remodeling Assay

A restriction enzyme accessibility assay was employed to measure chromatin remodeling. This assay was adapted from methods outlined by Smith and Peterson (221) and was performed as described in Chapter III. Samples were analyzed on a 3% agarose gel and bands were quantified using a Typhoon Trio+ Imager and ImageQuant TL

software (GE Healthcare). Data points represent the average value of triplicate experiments. Quantitative Western blotting was used to ensure that the final concentration of CHD8 used in the restriction enzyme accessibility assays was 20 nM, allowing for direct comparison between CHD8 alone and CHD8 in complex.

Results

CHD8 forms a Complex with WAR and with MLL1-WAR

Previous work from our lab has identified several CHD8 associated polypeptides (104). We previously reported that CHD8 can interact with WDR5 both *in vivo* and *in vitro*. Initially we focused in on WDR5 as this polypeptide has been reported to be part of an MLL histone methyltransferase complex (252-256, 281-283), possibly linking chromatin remodeling and chromatin modification in a single complex. Further evidence for this link was provided by the discovery of CHD8 in a WDR5-containing complex (256). As WAR can associate as an independent core complex in the absence of MLL (257, 258), we asked whether CHD8 could also associate with WDR5, both in the context of the WAR core complex as well as in association with MLL1-WAR. This 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, Ash2L, and RbBP5 results in the formation of a stable complex, and this complex can also form with the C-terminal fragment of MLL1 (MLL-C) (257). Co-infection of SF9 cells with WDR5, Ash2L, RbBP5, MLL-C and Flag-CHD8, followed by affinity purification demonstrated that indeed CHD8 can associate with the MLL1-WAR

complex (Fig. 4.1). When the same experiment was repeated excluding MLL-C, it was seen that CHD8 can also form a complex with the WAR core complex by itself.

While these results demonstrate that CHD8 can associate with WDR5 in the context of the complex, they do not address whether other interactions also exist in this complex. To systematically test the assembly of the WAR/CHD8 complex, further baculovirus co-infection experiments were performed, each time omitting one component. As shown in Fig. 4.1, the removal of any one of the subunits does not preclude a stable association of the remaining subunits. These results demonstrate that CHD8 can interact with subunits other than WDR5. To further explore these interactions, baculovirus co-infection experiments using pairwise combinations with CHD8 were performed. As shown in Figure 4.2, CHD8 is capable of interacting directly with each component of the MLL1-WAR complex. These results demonstrate that CHD8 has extensive contacts with the MLL1-WAR complex, unlike the interactions of WAR with MLL that are dependent solely on WDR5 (257, 258, 261, 262, 284, 285).

CHD8 Co-fractionates with Components of the MLL1-WAR Complex

SF9 cells were co-infected with baculovirus encoding the following combinations of proteins: the components of the WAR core complex alone, CHD8 and the WAR complex or CHD8 and MLL1-WAR. As a control, cells were mock-infected. Each of these cell lysates were fractionated over a Superose 6 size exclusion chromatography column. Fractions containing the protein peak from each of these runs were subjected to SDS-PAGE followed by Western blot analysis with the indicated antibodies. Co-elution

of proteins in the same fractions, is suggestive of a physical interaction between them, causing them to migrate together through the size exclusion column.

It was observed that the components of the WAR core complex co-eluted in the same range of fractions from the size exclusion column, indicating they form a discrete complex in these cells (Fig. 4.3A). When the lysates of cells co-infected with CHD8 and WAR were fractionated, we found that the elution profile of CHD8 was largely coincident with those of WDR5, Ash2L and RbBP5 (Fig. 4.3B). Interestingly, the fractions containing the major CHD8 peak did not coincide with the elution peak for WAR. This indicates that while CHD8 can associate with the WAR complex, the majority of CHD8 is found independent of the WAR complex. Finally, the elution profiles of lysates containing CHD8 and MLL1-WAR were examined (Fig. 4.3C). Once again, CHD8 co-eluted with the components of the WAR core complex and with MLL1 as well. These results demonstrate that CHD8 can form a complex with both WAR and MLL1-WAR, confirming the results of the co-immunoprecipitation experiments shown in Fig. 4.1 and Fig. 4.2.

Association with WAR or MLL1-WAR does not Affect Chromatin Remodeling by CHD8

To gain insight into the significance of the association CHD8 with the WAR and MLL1-WAR complexes, we performed *in vitro* chromatin remodeling assays to test the consequence of this association. SF9 cells were co-infected to express either CHD8 alone, CHD8 and the WAR complex or CHD8 and the MLL1-WAR complex. CHD8 and its associated complexes were affinity purified. The amount of CHD8 in each of

these samples was determined by quantitative Western blots. Chromatin remodeling was measured for each of these complexes by using equal amounts of CHD8 enzyme in restriction enzyme accessibility assays as described in Chapter III. As shown in Figure 4.4, the presence of either of the WAR complexes with CHD8 neither enhances nor hinders CHD8 remodeling activity in the context of this assay. This result indicates a possible role for the WAR and MLL1-WAR complexes outside of direct involvement in the enzymatic function of CHD8, perhaps in targeting or binding of CHD8 to promoters.

Discussion

The CHD8-WAR-MLL1 Complex

We have shown that the ATP-dependent chromatin remodeling enzyme CHD8 is capable of interacting with both the WAR core complex alone as well as with the MLL1-WAR HMTase complex. We have demonstrated that both the CHD8 complexes are formed by extensive contacts between the component proteins and elimination of any one component does not preclude the formation of a stable complex. This indicates extensive contacts between CHD8 and the the components of the MLL1-WAR complex. This is confirmed by the observation that CHD8 also interacts individually and directly with each component of the MLL1-WAR complex. In this respect, the interaction of WAR with CHD8 is different from that between WAR and MLL1, which is mediated by WDR5 alone (257, 258). In fact, recent reports have shown that a single 6-13 amino acid motif in MLL1, called the WDR-interaction (*Win*) motif, is responsible for the interaction with WDR5, which stabilizes the complex with WAR (261, 262, 285). However, another

recent report suggests that there are more extensive interactions of MLL1 with WAR, as the interaction of RbBP5 and Ash2L with MLL1, even in the absence of WDR5, have been shown to stimulate the catalytic activity of the MLL1-SET domain (286). CHD8 does not possess the conserved motif by which WDR5 is known to bind SET domain proteins, further supporting a different mechanism of association of CHD8 with the MLL1-WAR complex. Further work is required to elucidate the exact nature of the interactions of CHD8 within this complex.

CHD8, as well as MLL1 and WAR, have been reported to exist in complex with other factors related to chromatin. CHD8 has been isolated in association with complexes containing hStaf, involved in transcriptional elongation by RNA Pol III, as well as in a complex containing the elongating form of RNA Pol II (100, 101). In addition, CHD8 has been found to be associated with various chromatin-related proteins like the chromatin insulator CTCF, the transcription factor β-catenin and the nuclear receptor ER (103-105). It has also been found to promote association between p53 and the H1 histone to form a trimeric complex on chromatin that is involved in suppressing the transcription of genes involved in p53-mediated apoptosis (102). Indeed, CHD8 was identified as a component of an MLL1-WAR complex isolated from HeLa cells overexpressing Flag-WDR5 (256).

MLL1 is inherently found in a multi-subunit protein complex as discussed earlier in this chapter. In addition, it can be aberrantly associated with other chromatin factors during cancer. An MLL-ENL (eleven-nineteen leukemia) fusion protein is known to be associated with a SWI/SNF chromatin remodeling complex that is involved in the oncogenic activation of Hox A7 (287). This MLL-ENL fusion protein is also known to

be associated with various other complexes containing other factors like the RNA Pol II CTD, positive transcriptional elongation factor (P-TEFb), the H3K79 HMTase Distribution of Organized Tributaries 1-like (DOT1L) and multiple PcG proteins (288). Another fusion partner of MLL, AF4, also interacts with P-TEFb and DOT1 to activate transcriptional elongation by Pol II (289). Finally, WAR has also been implicated in complexes other than with MLL HMTases, like with the HAT complexes ATAC and MOF and the nuclear receptor coactivator NIF-1, as discussed in the Introduction to this chapter (277-280). WDR5 has also been found to be associated with the ISWI-containing ATP-dependent chromatin remodeling complex, NURF, providing further evidence of the components of MLL-WAR being associated with nucleosome remodelers (32). Thus there is ample evidence of each of these component proteins being present in complexes other than the ones they are traditionally found in.

Hypothetical Model 1: Methylation by MLL1-WAR Targets CHD8 Binding to Histones

Based on the extensive interactions of CHD8 with the MLL1-WAR complex, one could hypothesize that CHD8 gets recruited by this complex to function in gene regulation at its target sites. The purification of CHD8 in complex with WAR and MLL1-WAR by size exclusion chromatography supports the hypothesis that these proteins form a stable complex that could be involved in the regulation of MLL target genes. Indeed, recent experiments in the lab have shown that CHD8 can bind and regulate the expression of the MLL-regulated HoxA genes (unpublished data). The fact that the overlap in coelution profiles of CHD8 in complex with MLL1-WAR was greater than that for the CHD8-WAR complex alone could indicate a preferred specificity of

CHD8 for MLL1-WAR. Thus it is possible that CHD8 and MLL1, through interactions enhanced by the WAR complex, are working in coordination to regulate the expression of the Hox genes. This possibility is made even more likely given the fact that the *Drosophila* homolog of CHD8, Kismet, is a TrxG protein known to be involved in the regulation of Hox genes. Methylation of the H3K4 mark at target promoters by MLL1 might reinforce the localization of CHD8 to these sites. This possibility is supported by recent work that suggests that the chromodomains of CHD8 can specifically bind dimethylated H3K4 (100).

Chromatin remodeling assays performed using each of the indicated CHD8-containing complexes (Fig. 4.4) indicated that the interaction with WAR or MLL1-WAR does not affect the chromatin remodeling activity of CHD8. This suggests that the association with these complexes regulates some aspect of CHD8 other than its enzymatic activity, like the binding or targeting of CHD8 to promoters, as mentioned above. Thus the recognition of H3K4 methylation by the chromodomains of CHD8 seems to be involved in histone binding by CHD8 in the context of its recruitment to chromatin rather than the substrate specificity of its nucleosome remodeling activity. It is also a possibility that, once recruited to target sites, chromatin remodeling by CHD8 could affect the HMTase activity of the MLL1-WAR complex. A recent study revealed that the SET domains of MLL1 and SET7 specifically recognize nucleosomes remodeled by ISWI and subsequently catalyze methylation of these nucleosomes (290). More experiments are needed to verify the precise role of CHD8 with regards to its association with this complex. However, the data presented here can be used to propose a model where CHD8 is targeted via its chromodomains to sites of H3K4 methylation by MLL1-

WAR, whereupon it associates with this complex and rearranges nucleosomes in a way that allows for optimal transcriptional regulation of the target genes, either by enhancing further methylation by MLL1-WAR, or by allowing the binding of other factors.

WAR has been shown to exist in complexes independent of MLL or SET proteins and also that CHD8 can bind WAR independent of MLL1. This gives rise to an interesting variation of the proposed model. The binding of WAR to histones and to MLL appear to be mutually exclusive, since both H3 and MLL bind WDR5 through the same motif (262). Thus it is possible that WAR can foster the interaction of CHD8 with MLL1, or recruit CHD8 to chromatin by binding to H3. It is also possible that WDR5 could competitively bind to methylated H3K4, dissociating in the process from MLL1 after the methylation event, as suggested by Song et al (262). In this case, CHD8 could be tethered on to H3K4 methylated chromatin via its extensive interactions with WAR after prior methylation of these sites by MLL1. Thus there are several modes by MLL1-WAR could be recruiting CHD8 to target promoters, the precise mechanism of which needs to be explored by further studies. Given the fact that AR binding to its target promoters has been shown to be dependent on H3K4 methylation (225), this model could be elaborated upon further. Methylation of H3K4 by MLL1-WAR could be involved in targeting the associated CHD8 to target promoters, where its enhanced remodeling of nucleosomes bearing H3K4 dimethylation marks would then serve to facilitate the binding of AR

Hypothetical Model 2: Remodeling by CHD8 Recruits MLL1-WAR

An alternative hypothesis for the role of CHD8 is that it is involved with the proper establishment of histone methylation patterns at target genes by recruiting the MLL1-WAR HMTase complex. Depletion of its *Drosophila* homolog, Kismet, has been shown to result in elevated global levels of H3K27 methylation (89). The action of Kismet appears to counteract the H3K27 methyltransferase E(Z) and promote the association of H3K4 HMTases, including the MLL homolog TRX and ASH1 with chromatin (89). Thus it is possible that CHD8 interacts with MLL1 in a similar capacity to facilitate its binding to target chromatin sites via its remodeling activity. However, it is to be noted that CHD6-9 in humans have a single ortholog in *Drosophila*, namely Kismet. Thus it is also possible that the CHD6-9 proteins have diverged evolutionarily and developed different, more specific functions within the cell. Given the evolutionary distance between flies and humans, it would not be entirely unexpected if CHD8 and Kismet do not have the exact same mechanisms of action in transcriptional regulation in association with MLL.

A variation of this hypothesis of CHD8 recruiting the H3K4 HMTase activity to target sites could be based on our observation that CHD8 can associate with the WAR core complex independent of MLL1. This gives rise to another possibility that CHD8 could be directly involved in recruiting WAR to MLL target sites, bringing it in contact with MLL1 and thus enhancing H3K4 methylation. Several lines of evidence support this possibility as well. Previous studies have demonstrated that WAR is required for complete methylation of target promoters by MLL1, but loss of WAR does not prevent proper recruitment of MLL1 to these promoters (257, 258). Taken together with our

data, this would suggest a model where chromatin remodeling by CHD8 is involved in recruiting WAR to allow the proper methylation of target promoters via association with MLL1. It has also been reported recently that the WAR complex itself is a H3K4 HMTase, even in the absence of MLL (285). This raises the possibility that the CHD8/WAR complex is also a HMTase complex, and the coordinated function of nucleosomal remodeling and histone methylation by this complex is capable of establishing appropriate chromatin modifications at target promoters independent of MLL1. Future experiments will need to be performed to verify which of these proposed models is actually operational during transcriptional regulation by this novel CHD8-containing complex. However, in the context of androgen-responsive transcriptional regulation by CHD8, we can speculate that the targeting of MLL1-WAR or WAR alone by CHD8 remodeling at AREs allows the establishment of the H3K4 methylation pattern that is subsequently responsible for the recruitment of AR (225) to these genomic loci.

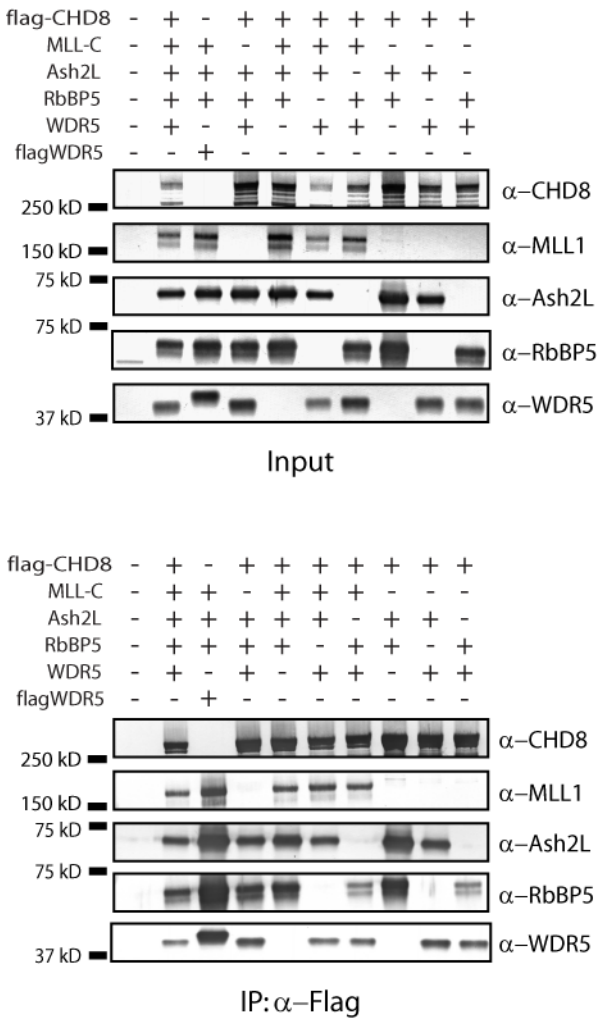

Figure 4.1: Direct Interaction of CHD8 with the MLL1-WAR Complex. Cellular extracts were prepared from SF9 cells following co-infection with the indicated viruses. Immunoprecipitations were performed with anti-Flag-M2 antibodies. After extensive washing, purified samples were subjected to SDS-PAGE followed by Western blotting analysis using the indicated antibodies.

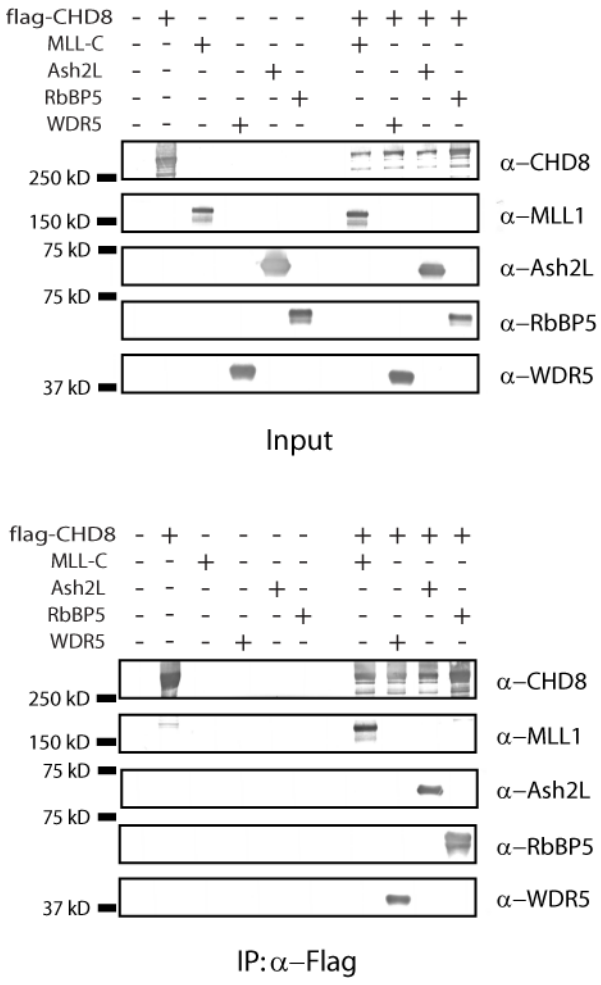

Figure 4.2: Direct Interaction of CHD8 with each Component of the MLL1-WAR Complex. Pairwise coinfections were performed using the indicated baculoviruses and immunoprecipitated as in Fig. 3.1. Inputs (top panel) and IPs (bottom panel) were analyzed by SDS-PAGE followed by Western blotting with the indicated antibodies.

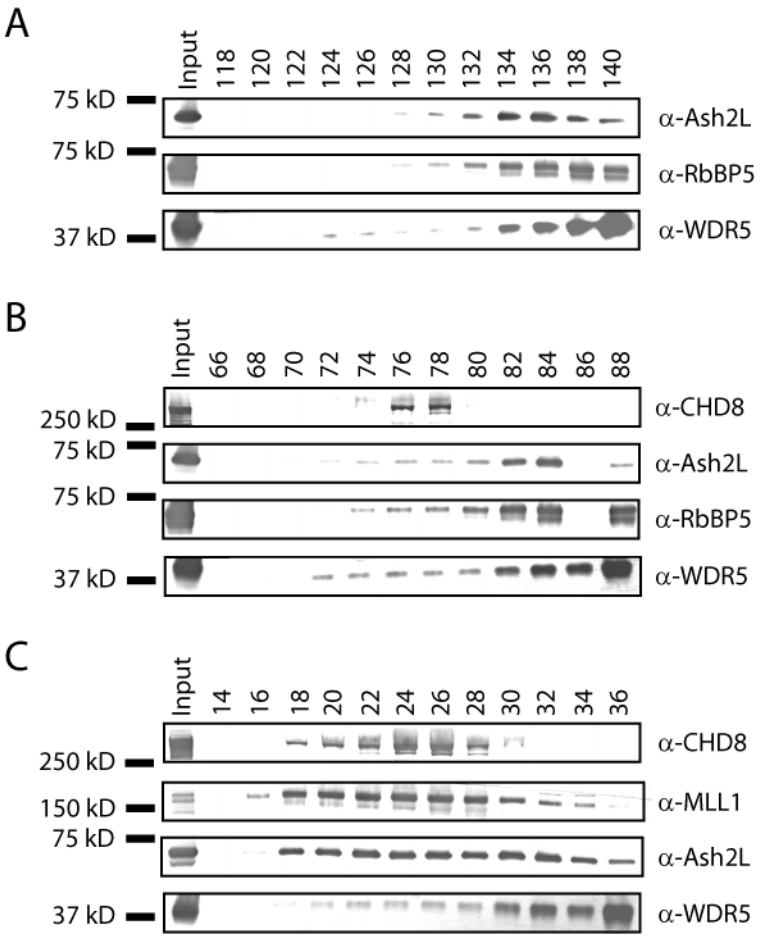

Figure 4.3: Size Exclusion Chromatography of CHD8-Associated Complexes. SF9 cells were coinfected with baculovirus encoding the components of either the WAR subcomplex alone (A), CHD8 and WAR (B) or CHD8 and the MLL1-WAR complex (C). Cellular extracts were prepared and fractionated by size exclusion chromatography over a Superose 6 column. Peak fractions were analyzed by SDS-PAGE followed by Western blotting with the indicated antibodies.

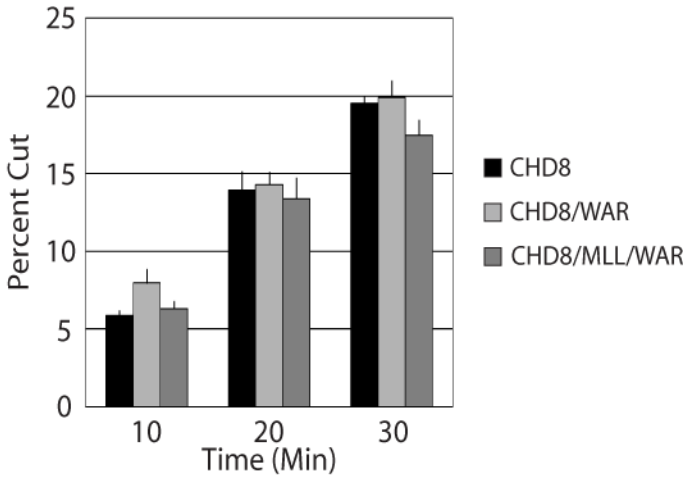

Figure 4.4: Remodeling Activity of CHD8 in Association with MLL1/WAR Complexes. Recombinant CHD8 or CHD8 in complex with either WAR or MLL1-WAR was assayed for chromatin remodeling as measured by increased restriction enzyme accessibility on mononucleosomes. Three separate experiments were performed in triplicate and representative data is shown.

CHAPTER V

Conclusion

Background

The DNA within eukaryotic cells is packaged into highly condensed chromatin, which inherently serves as a physical barrier to critical cellular processes like DNA replication, repair and transcription. The class of enzymes that modulate the chromatin structure to allow access to the underlying DNA are called the ATP-dependent chromatin remodeling enzymes. These enzymes belong to the SNF2 superfamily which is characterized by the presence of a characteristic SNF2 domain that is capable of hydrolyzing ATP to derive energy that is utilized by the enzyme to remodel chromatin in different ways. There are several families of SNF2 ATP-dependent chromatin remodelers, one of which is the CHD family. This family is comprised of nine proteins, named CHD1 through CHD9, that are further divided into subfamilies based on domain architecture. While the remodeling activity and cellular functions of the CHD1-2 and CHD3-5 subfamilies have been well studied, relatively little is known about the third subfamily, CHD6-9.

The studies described in this thesis were designed to elucidate the function of one of the members of this subfamily, CHD8. Specifically, the role of CHD8 in androgen-responsive transcription was studied to expand upon the previously known functional association between the CHD6-9 subfamily and nuclear receptors. These studies also highlight the potential role of CHD8, not only in the regulation of transcription, but also in prostate cancer tumorigenesis and the progression of diseased states. In addition to these functional studies, the substrate specificity for chromatin remodeling by CHD8 was also studied so as to derive further mechanistic insights into its transcriptional function. Finally, its association with a known histone methyltransferase complex, MLL1-WAR, was characterized in order to understand its interplay with other chromatin-related factors and processes involved in transcriptional regulation. This additional knowledge can then be applied to the role of CHD8 in androgen receptor-mediated transcription and to understand how CHD8 may be involved in the regulation of transcription and cancer.

Functional Studies of CHD8 in Androgen-Responsive Transcription

Evidence from previous literature suggests that the CHD6-9 subfamily proteins are involved in transcriptional regulation by nuclear receptors and are implicated in several diseases. CHD8 specifically has been shown to have a diverse role in transcriptional regulation and in a variety of pathways related to human disease. We investigated the potential role of CHD8 in nuclear receptor-mediated transcription in Chapter II. Our preliminary analysis of publicly available microarray data from the ONCOMINE database revealed that CHD8 was upregulated in several prostate cancer samples, implicating it in androgen receptor signaling, which is commonly dysregulated

in prostate cancer. Thus we focused in on the androgen receptor pathway to study the cellular function of CHD8 and investigate its implied role in cancer.

We investigated the possible association between AR and CHD8 by doing interaction studies, where we found a direct interaction between both the recombinant and endogenous proteins. Interestingly, endogenous CHD8 only interacted with AR in androgen-dependent cell lines, indicating that this association was dependent on androgen induction. The fact that these two proteins interact directly was demonstrated by co-immunoprecipitation if the recombinant proteins by co-expression in a baculovirus expression system. While the interaction studies reveal a direct physical and physiologically relevant association of these proteins, further studies are required to elucidate the specifics of this interaction. The interaction could be mapped further by using recombinantly expressed, epitope-tagged fragments of either protein and conducting pull down studies similar to those described in Chapter II. It would be interesting to map this interaction to see whether the consensus LFSLL nuclear receptor interaction motif in CHD8, located at 996-1000 aa within its SNF2 domain, is responsible for the binding to AR as predicted in Chapter II. Conversely, we could map the interaction site on AR to see whether it lies within the C-terminal LBD as is typical for most interactions between nuclear receptors and their coactivators. It would also be interesting to examine the association between endogenous AR and CHD8 in more prostate cancer cell lines to verify that this interaction is indeed androgen-dependent.

ChIP and re-ChIP experiments were done to see whether this interaction fostered co-localization of these proteins at endogenous AR-mediated promoters. It was found that CHD8 did indeed occupy AREs on the androgen-responsive TMPRSS2 and PSA

genes at the same time as AR. The constitutive binding of CHD8 to these promoters independent of androgen induction distinguishes it from other typical AR coregulators. The precise localization pattern of CHD8, and concurrently of AR, on these target promoters could be determined by performing ChIP experiments tiling over the promoter sequence to see where these proteins localize to before and after androgen treatment. Also, ChIPseq or re-ChIPseq experiments could be designed to obtain the sequences from ChIPs of AR and CHD8, done either separately or sequentially, which can then be analyzed to identify new target genes that are regulated by AR and CHD8.

Based on the co-localization patterns of CHD8 with AR on the AREs of androgen-responsive target promoters, we hypothesized that CHD8 is involved in the transcriptional activation of these genes upon induction by androgens. This was confirmed by showing that CHD8 depletion significantly diminished the transcriptional activation of AR target genes in response to androgen induction in androgen-dependent LNCaP cells. It was further demonstrated that this transcriptional role of CHD8 in AR-mediated gene activation was androgen-dependent, by showing that the effect of CHD8 depletion observed in LNCaP cells was not replicated in several androgen-independent cell lines. Our results indicate that CHD8 is involved in the transcriptional activation of two well-characterized androgen-responsive genes, TMPRSS2 and PSA, which are aberrantly activated in prostate cancer. This implicates CHD8 in the aberrant gene expression observed in prostate cancer. It would be interesting to identify other genes that are regulated by CHD8 in an androgen-dependent manner by generating a stable knockdown of CHD8 in a prostate cancer cell line like LNCaPs. We could then observe by microarray analysis which particular genes' androgen-responsive activation is affected

by CHD8 depletion upon induction by androgens, as compared to a control cell line. In addition, we could individually examine novel target genes identified from this microarray analysis or from the ChIPseq experiments proposed above to identify common targets of CHD8 and AR, and look for effects on androgen-responsive activation by CHD8 by the same siRNA-based strategy employed in Chapter II. Thus the identification of CHD8 as a novel AR coactivator presents us a unique opportunity to investigate novel AR-mediated transcriptional targets in prostate cancer.

While we have verified the functional role of CHD8 in AR-mediated transcriptional activation, the mechanism behind how chromatin remodeling by CHD8 contributes to this process is not clear. Our ChIP experiments under conditions of CHD8 depletion revealed that CHD8 was required for optimal recruitment of AR to the target promoter. This role for its remodeling activity resembles that of other chromatin remodelers which have been found to be involved in the binding of transcriptional activators to promoters (19). There is a possibility since CHD8 directly interacts with AR that it is this interaction and not the remodeling of nucleosomes by its catalytic activity that is responsible for recruitment of AR by CHD8. This could be tested by doing an experiment where the K842R point mutant of CHD8, which has been shown to be a catalytically dead mutant (104), could be overexpressed as a dominant-negative form of CHD8. This mutant CHD8 is unable to remodel nucleosomes due to the mutation of the conserved lysine in its SNF2 domain that is required for ATPase activity and hence for remodeling activity. The loss of AR targeting upon expression of this inactive form of CHD8 would verify that it is the remodeling of nucleosomes by CHD8 and not its interaction with AR that is responsible for its recruitment of AR to target sites. Indeed

we have shown that CHD8 is capable of remodeling nucleosomes assembled on a DNA template sequence encoding the ARE of TMPRSS2 *in vitro* in the presence of AR. This verifies aspects of our model that CHD8 remodels nucleosomes at target loci to allow AR to bind. Further experiments could be done using these nucleosomal templates to examine whether ARE nucleosomes that have been remodeled by CHD8 show enhanced binding with AR by gel shift assays. The loss of AR binding upon CHD8 depletion could be verified at other novel or known AR target promoters by ChIP and also in different androgen-dependent and androgen-independent cell lines to verify whether this recruitment is hormone dependent. The genome-wide targeting of AR could be evaluated by performing AR ChIPs under normal and CHD8-depleted conditions from LNCaP cells and analyzing by a ChIPseq or ChIP-on-chip approach whether the recruitment pattern of AR is disrupted at a subset of target genes. These experiments will expand upon this novel and unique mode of coactivation by CHD8.

Finally we verified the physiological importance of CHD8's role in the AR signaling pathway, by showing that it is required for androgen-dependent cell proliferation of LNCaP cells. This result shows how CHD8 might be important for prostate cancer tumorigenesis and progression. Similar proliferation assays as described in Chapter II could be performed in androgen-independent cell lines to verify the hormone dependency of this growth effect upon CHD8 depletion. Alternative physiological assays like tumor invasion assays could be performed to determine the effects of CHD8 depletion on different aspects of tumor growth. Doing cell growth or proliferation assays on stable knockdowns of CHD8 would provide a better indication of its physiological role as well by eliminating variability due to transfection or knockdown

efficiency. Taken together, however, the results presented in Chapter II provide evidence of a novel functional role for CHD8 in a prostate cancer model system.

The role of CHD8 in nuclear hormone signaling is supported by other lines of evidence. CHD8 is a member of the highly related CHD6-9 subfamily of proteins. Several members of this family have been shown to functionally associate with nuclear hormone receptors. CHD9 (CReMM/PRIC320) has been shown to interact with PPARα, CAR, ERα, RXR, and GR, and has also been shown to function as a coactivator for PPARα (98, 159). CHD7 has also been isolated as a component of a corepressor complex that inhibits PPARγ mediated transcription (95). Recently, CHD8 has also been reported to be required for the estrogen mediated upregulation of the cyclin E2 gene (105). Taken together with our studies on CHD8 and AR, these results suggest that the CHD6-9 family is an important regulator of nuclear hormone signaling.

Deciphering the mechanistic role of CHD8 in transcriptional regulation is complicated by the numerous functions reported for CHD8. Previous work from our group has shown that CHD8 is an ATP-dependent chromatin remodeling enzyme involved in transcriptional regulation of β-catenin responsive genes (104). However, CHD8 was found to act in the negative regulation of activated β-catenin responsive genes, unlike our current report here of a role for CHD8 in the activation of TMPRSS2 in response to androgens. This suggests that CHD8 can differentially regulate numerous target genes. Indeed, expression profiling of control and CHD8-depleted cells identified transcripts both positively and negatively regulated by CHD8 (100).

Further insight into the function of CHD8 can be found by examining the reported functions of Kismet, the Drosophila ortholog of CHD8. Kismet was originally identified as an extragenic suppressor of Polycomb and therefore assigned as a member of the trxG of activators (291). Further studies revealed that Kismet assists in an early step in transcriptional elongation (61, 89). This reported data is consistent with CHD8 regulating the cyclin E2 gene via interactions with the elongating polymerase (100). AR not only plays a role in transcriptional initiation but also transcriptional elongation. AR has been reported to interact with COBRA1 (NELF-B), a subunit of negative elongation factor (NELF), and depletion of endogenous NELF-B enhances DHT-mediated transcriptional activation (292). AR also interacts with the positive elongation factor P-TEFb, and this interaction serves to enhance transcriptional elongation (293). In addition, AR has been shown to regulate transcriptional initiation as well as elongation via interactions with the general transcription factors TFIIF and TFIIH (294, 295). Taken together with our current studies, these reports suggest CHD8 could possibly be regulating AR mediated transcription by modulating transcriptional elongation.

In this study, we have identified the binding of CHD8 to the TMPRSS2 enhancer region located approximately 13.5 kb upstream from the start site. This data initially seems to be at odds with the model proposed above. However, the investigation of various nuclear receptors binding to DNA at both proximal and distal sites accompanied with reports of RNA polymerase II localization to these sites suggests that enhancer/promoter looping may play an important role in the regulation of nuclear hormone regulated transcription (296). Indeed, a direct interaction is reported between the TMPRSS2 -13.5 kb enhancer and the promoter region (131). Therefore, the

recruitment of CHD8 to the TMPRSS2 distal enhancer does not preclude CHD8 functioning in transcriptional elongation. More experiments need to be performed to determine the precise point of action for CHD8 in the transcriptional cycle.

In summary, the activity of AR is critical for normal prostate development and function, but also plays a major role in the development and progression of prostate cancer. Understanding the mechanisms of transcriptional regulation by AR and AR-associated cofactors is critical to the development of new therapies for prostate cancer. Here we report the characterization of a novel AR-associated cofactor required for the proper regulation of the androgen responsive gene TMPRSS2. These results highlight the potential of CHD8 as a novel diagnostic, preventative, or therapeutic target in prostate cancer.

Mechanistic Studies of Substrate Specificity of CHD8 Chromatin Remodeling

To better understand the functional implications of chromatin remodeling by CHD8, the substrate specificity of its catalytic activity was determined. This would provide further insights into the nature of the chromatin substrates that CHD8 preferentially remodels and thus allow us to build upon the model of CHD8 function in androgen-responsive transcriptional regulation. We found that CHD8 can remodel recombinant, unmodified nucleosomes at a similar rate to core nucleosomes. This result indicates that histone modifications are not necessary for CHD8 remodeling on substrates and also that we could further utilize recombinant nucleosomes for experiments to determine its substrate preference. Competitive assays using core and recombinant nucleosomes revealed that CHD8 preferentially remodeled core nucleosomes, indicating

that while histone modifications were not a requirement *per se* for CHD8 remodeling activity, one or more particular modifications that were present on the core nucleosomes and not on the recombinant defines the preferred substrates of CHD8 activity.

In order to narrow down on the preferred nucleosomal substrate for CHD8 remodeling, we reconstituted nucleosomes from histones with their H3-H4 tails deleted and performed remodeling assays on these. It was seen that CHD8 can remodel H3-H4 tailless nucleosomes, indicating that CHD8, similar to CHD3/4 does not require histone tails for remodeling, unlike CHD1. Competitive nucleosome sliding assays using wild-type and tailless recombinant nucleosomes showed that while CHD8 can remodel both substrates, it appears to remodel nucleosomes lacking H3-H4 tails less effectively as indicated by the alternate migration of the remodeled species on a gel, indicating differential repositioning of these substrates based on the presence or absence of histone tails. This is an interesting result from the perspective of which histone contacts CHD8 utilizes during catalysis and how these contacts affect the remodeled product as discussed in Chapter III. We determined from these results that while CHD8 can reposition nucleosomes by contacts mediated by the globular core of the histone octamer, specific contacts with histone tails may be responsible for additional substrate specificity with regards to the positioning of the remodeled products by CHD8.

Finally, we examined the effect of a specific histone modification on the chromatin remodeling activity of CHD8. We chose to examine the H3K4 dimethyl mark for its effect on CHD8 remodeling, due to the previously established binding of the chromodomains of CHD8 to this mark (100). We generated synthetic analogs that mimicked the K4-dimethylated histone H3 or unmodified H3 by the strategy devised by

Simon et al (219). When the unmodified control and H3K4 dimethylated nucleosomes, produced as described in Chapter III, were subjected to competitive sliding assays, we found that CHD8 preferentially remodeled the dimethylated species. This observation, along with the results of the competitive assays done using core vs. recombinant nucleosomes, led to the conclusion that CHD8 preferentially remodels substrates bearing the dimethyl H3K4 mark. This preference may be due to the enhanced binding to the substrate due to the chromodomains which recognize this histone mark and hence would improve catalysis by lowering the K_m of binding.

Further experiments need to be performed to verify the substrate preferences of CHD8 for nucleosome remodeling. The same experiments described above could be performed upon nucleosomes incorporated into a DNA template encoding the sequence of an ARE targeted by CHD8, to examine these remodeling results in an androgen-responsive context by doing them in the presence of androgens and AR. Deletion of the individual histone tails may elucidate further aspects of the substrate specificity and nature of remodeling by CHD8. We might be able to see differentially remodeled products when particular histone tails are eliminated and thus we could infer the specifics of particular histone tail contacts in the remodeling activity of CHD8. Finally, the effect of other histone modifications on CHD8 activity could be determined by introducing different histone methylation marks as described here or by using enzyme-modified histone substrates bearing other candidate modifications like acetylation, sumoylation or ubiquitination, and assaying remodeling activity on these nucleosomal substrates.

Loci such as PSA and TMPRSS2, which are specifically activated in prostate cancer have been found to have constitutively elevated levels of H3K4 di- and

trimethylation in prostate cancer cell lines like LNCaP, C4-2B and PC-3, independent of androgen stimulation (297). This methylation pattern of H3K4 could be responsible for the androgen-independent constitutive localization of CHD8 that we observe at these sites, via recruitment mediated by its chromodomains. In addition, the presence of H3K4 dimethylated nucleosomes at CHD8 target sites may enhance its remodeling activity at these genomic locations. Indeed a recent study has shown that H3K4 dimethylation is required for the binding of AR to target enhancers, as the overexpression of the H3K4 demethylase KDM1, also known as LSD1, completely abolished AR binding to these enhancers (225). We can thus speculate that the deposition of H3K4 dimethyl marks is important for CHD8 recruitment to target sites via its chromodomains, whereupon AR is the recruited to these sites by the enhanced nucleosome remodeling by CHD8 at these H3K4-methylated loci.

Interaction Studies of CHD8 with the MLL1-WAR Complex

The association between CHD8 and H3K4 methylation is further supported by previous findings from our group that indicate CHD8 interacts with components of the WDR5/Ash2L/Rbbp5 (WAR) complex which is common to the MLL family of histone methyltransferases. MLL1 in association with the WAR complex is known to specifically methylate H3K4 (250, 251). It has also been recently shown that H3K4 methylation is required for AR recruitment to its target promoters (225). This raises the possibility that if CHD8 interacts with the MLL1-WAR complex, it would establish the missing link between H3K4 methylation and the remodeling by CHD8 in androgen-responsive transcriptional regulation.

The studies outlined in Chapter IV were therefore designed to characterize the interactions of CHD8 with the MLL1-WAR complex, and determine how these interactions may affect its chromatin remodeling activity. We showed that CHD8 interacts with MLL1-WAR through extensive contacts. Elimination of any one component does not preclude complex formation and CHD8 also interacts individually with each component of this complex. The precise interactions could be mapped further to specific interacting domains or epitopes by using smaller recombinant fragments of the complex component proteins and doing pull-down experiments. Size exclusion chromatography of purified complexes showed that CHD8 had more extensive contacts with MLL1-WAR than with WAR alone. This could be verified by doing competitive binding studies to derive binding constants between the exact fragment of CHD8 involved in these interactions and either the MLL1-WAR complex or the WAR subcomplex alone. We also showed that association of CHD8 with either of these complexes did not affect its remodeling activity. This analysis was done using the restriction enzyme assay which allows us to quantitate the remodeling activity of CHD8 by itself or in each of these complexes. While these activities were not distinguishable by this assay, we could compare the remodeled products by nucleosome sliding assays to detect changes in the remodeling activity of CHD8 due to association with either complex as compared to its monomeric form. Alternatively, the converse hypothesis that nucleosome remodeling by CHD8 affects histone methylation by MLL1-WAR complex could also be tested by doing HMTase assays of either the complex alone or the complex in association with CHD8. These additional assays will help to determine the mechanistic details of the interplay between CHD8 and MLL1-WAR and consequently

provide a more complete picture of how these enzymes participate in androgen-responsive gene activation by AR. In fact, a recent report has established global details of this kind of interplay between histone modifying and nucleosome remodeling enzymes on a genome-wide scale (298). Similar genome-wide studies of loci where MLL1-WAR and CHD8 interact will yield further opportunities to study their cooperative effect on transcriptional regulation.

Taken together, these studies delineate a novel pathway for AR-mediated transcriptional activation of androgen-responsive genes. We propose a model where MLL1-WAR is recruited to AR target genes and methylates H3K4 marks at the AREs on their promoters. This mark is recognized by the chromodomains of CHD8 which facilitates its binding to target AREs and also enhances its remodeling activity on nucleosomes bearing this modification at these sites. Nucleosome remodeling by CHD8 then facilitates the recruitment of AR to the AREs of target promoters upon induction by androgens which subsequently leads to the optimal androgen-responsive transcriptional activation of these genes. This model is somewhat complicated by reports that H3K4 methylation is drastically increased at AR target promoters upon androgen induction (299). Thus if MLL1-WAR is involved in maintenance of constitutive H3K4 methylation of target promoters allowing for the constant recruitment of CHD8 to these sites, this androgen-responsive increase in H3K4 methylation may be due to another HMTase. In fact, this spike in H3K4 methylation upon induction was found to be highly coincident with the recruitment pattern of AR (299), unlike the androgen-independent basal level of methylation which was found to be necessary to be already established for AR recruitment (225). Thus this later, androgen-responsive methylation may be due to a

different HMTase which is recruited by AR as part of its coactivation complex to activate transcription, while the constitutive, basal levels of H3K4 methylation may be maintained by MLL1-WAR to establish proper recruitment of CHD8. Thus basal H3K4 methylation levels may maintain constitutive CHD8 localization to target genes which is required for the proper recruitment of AR upon reception of androgen signaling. This may then trigger higher levels of methylation of the promoter in the course of transcriptional activation by AR. This novel pathway of AR regulation is an important subject for future studies which could then help us understand its potential as a target for the prevention and therapy of prostate cancer.

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