Spring 5-2005

Chromatin Remodeling in Mice

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May 2005

ABSTRACT

Controlling gene regulation is an important aspect in the life of cells that provides them the ability to carry out their functional roles within an organism. Unregulated or misregulated gene expression can lead to cell immortalization or death. Chromatin remodeling functions as a regulator for many important DNA functions including transcription, the first step of gene expression in cells. The *C*hromodomain-*H*elicase-*D*NA binding domain gene family (CHD) is evolutionarily conserved and has distinct structural motifs that indicate a role in chromatin remodeling and DNA repair. The CHD proteins have both helicase activity, allowing the winding and unwinding of DNA, and an effect on histone acetylation through their role of the Nucleosome Remodeling and Histone Deacetylation (NuRD) complex. The NuRD complex participates in the deacetylation of chromatin histones, in addition to orchestrating ATP-dependent remodeling of the the chromatin structure.. Histones are, in their native state, positively charged, interacting tightly with the negatively charged DNA that wraps around them. Deacetylating previously acetylated histones returns them to this state and forces them to have greater attraction to the DNA, causing low levels of transcription and gene expression. Chd4 is the largest protein in the NuRD complex and can carry out many functions of the complex on its own. We are using Chd4 knockout mice and cell lines to look further into the function of the protein. Preliminary data shows that homozygous null embryos are lethal, but the definite day of lethality has yet to be determined. In addition, cell line experiments show that cells heterozygous for Chd4 grow faster than wild type cells. Analysis of gene expression in mouse embryos shows gene expression

in brain precursors, dermal precursors, and the dorsal aorta. Future experiments will address organismal cancer susceptibility and the transformation potential of cell lines.

CHROMATIN REMODELING

Though there are many ways to regulate genes in the nucleus of any given cell, one of the most basic ways is through the relative state of chromatin. Cells use chromatin to store vast amounts of DNA in a relatively compact space through the formation of a structure that looks like "beads on a string." The basic unit of chromatin, the bead, consists of 146 or 147 base pairs of DNA wrapped tightly around an octamer core that consists of two of each of the following proteins: H2A, H2B, H3, and H4 (2,3). These proteins are all highly positively charged, allowing for a strong interaction between them and the negatively charged backbone of DNA. Many factors contribute to how tightly the "beads" are wrapped; these differing levels of tightness are an important contributing factor in determining the level of gene transcription. Additionally, nucleosomes are

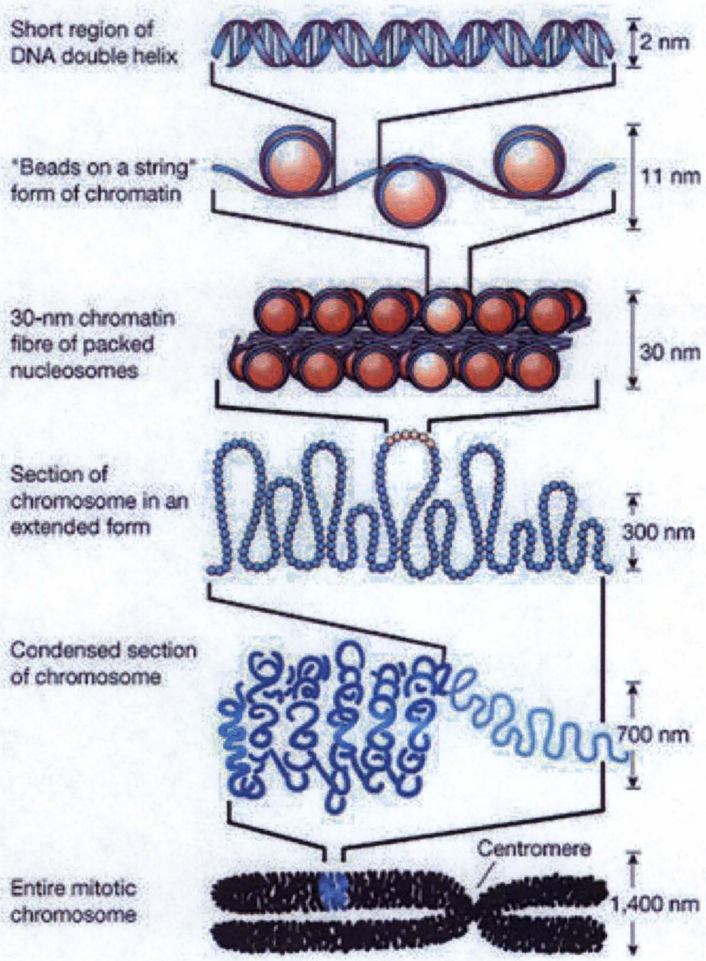

Figure 1. Chromatin structure from the DNA strand to the entire chromosome. From (1).

folded into higher order structures, as shown in Figure 1 (3). One important factor in this folding of histones into larger structures is the histone tail. These tails, usually on the amino end of the protein, protrude from the central octamer and are frequent targets of modification (4). These modifications either disrupt or enhance the formation of higher order structures, making them extremely important in chromatin maintenance and gene expression.

There are two major classes of chromatin remodeling complexes: those whose action on chromatin is dependent of the use of ATP to disturb histone-DNA interactions and those which covalently modify the histone proteins themselves, resulting in differential histone-DNA interaction (5). The ATP-dependent remodeling enzymes can range from a single polypeptide to complexes greater than 1 MDa in mass (4). For ease of reference, both the single peptides and the complexes will be referred to as complexes. At the core of each complex is a helicase-like subunit that belongs to the SWI2/SNF2 family of proteins. Based on the relative homologies of these subunits, three major subfamilies have emerged: the SWI2/SNF2 family, the ISWI family, and the Mi-2/CHD family (4,5). The SWI2/SNF2 family was first discovered in yeast using genetic screens that identified *swi*tch and *s*ucrose *n*on*f*ermenting mutations. After characterization, investigators discovered that both mutations affected the same complex, one which had the ability to make nucleosomal DNA more easily accessible dependent on the presence of ATP (6). The ATPase ISWI, first discovered in *Drosophila melanogaster*, can remodel nucleosomes either by itself or in its full complex. Further characterization of ISWI family members showed that they act by the induction of nucleosome sliding to different DNA segments, effectively freeing up the

portion of DNA for access by various factors (6). In addition to the ATPase domain, the Mi-2/CHD family members all have chromodomains and a DNA-binding domain (5). These family members show efficient nucelosome remodeling activity alone and in complex (7). All three of these complexes have members represented in humans, showing that while they might perform some similar functions, evolutionarily, they most likely have some unique roles as well (4).

In addition to ATP-Dependent chromatin remodeling, chromatin is also regulated through covalent modification of histones. One common method of chromatin modification, and perhaps the most investigated, is acetylation. In this method, histone acetyltransferase (HAT) and histone deacetlyase (HD) complexes either add or remove, respectively, acetyl groups from the lysine residues on histone tails (5). These lysine residues are positively charged. After acetylation by the HAT complexes, they lose this positive charge. This causes a decrease in the electrostatic interactions between the previously positively charged histones and the negatively charged DNA, allowing the DNA to be more accessible to the binding of various transcription factors.

In addition to acetylation, histones are also modified by phosphorylation, methylation, and ubiquitination (8). There is building evidence that the combination of modifications on histone tails can lead to distinct downstream events with regard to transcription or silencing of genes (9). Phosphorylation seems to be evident in both transcriptional activation and chromosome condensation, activities that upon first glance would seem to be dichotomous. Although there is not currently a conclusive explanation, this data seems to point to the modifications altering the binding surface of the histone as opposed to directly altering the chromatin (8). Methylation is involved in

both gene activation and suppression. Though this also seems counterintuitive, it is possible because varying substrates allow for different outcomes. Upon arginine methylation, for instance, genes seem to be activated, whereas upon lysine methylation, they are silenced (8). Ubiquitination is important to both meiotic and mitotic growth, in addition to possible roles in transcription. Though all of these upon first glance seem to paint a rather blurry picture, the "histone-code" hypothesis attempts to include all of the tail modifications in a unified theory. All of these modifications are on either specific residues or at least on a subset of them, indicating perhaps that "every amino acid in histone tails has specific meaning and is part of the vocabulary of the overall code" (9). This theory points to the specific combination of modifications on a nucleosome determining unique outcomes and downstream effects. It is only beginning to be investigated and has vast implications if it is proved to be correct.

Mi-2/NuRD COMPLEX

As discussed previously, the Mi-2/NuRD complex, also known as the NURD or NRD, hereafter referred to as the NuRD complex for simplicity, is an ATP-Dependent chromatin remodeling complex (10). In addition to the ATPase subunit CHD4, the NuRD complex also contains the histone deacetylases HDAC1 and HDAC2, the histone binding proteins RbAp46 and RbAp48, a methyl-CpG-binding domain-containing protein, MBD3, and a member of the metastasis-associated protein family, possibly leading to specialization of the complex (10,11). The proteins HDAC1, HDAC2, RbAp46, and RbAp48 form a fundamental core that is shared between the Sin3/HDAC complex and the NuRD complex. The Sin3 complex also contains 3 additional peptides

outside of the core, but, unlike the NuRD, does not show nucleosome remodeling activity in addition to the histone deacetylase activity of the complex (11).

The largest component of the NuRD, CHD4, will be discussed in great detail in the next section, but is a multi-domain protein with two PHD Zn-finger domains, two chromodomains, the SWI2/SNF2-related ATPase domain, an additional helicase domain, and other domains whose functions are as yet unknown (12). Another large component of the NuRD is the histone deacetylases. These enzymes lead to the deacetylation of previously acetylated histones, causing the histone-bound DNA to become less accessible for transcription (13). HDAC1 and HDAC2 are homologous to a great extent, sharing 84% identity. It is currently unclear whether they have unique functions, but they seem to function similarly in the repression of transcription (14). They have been implicated in both short and long term patterns of gene activity, usually in a repressive capacity (15). There are two major HDAC1/2 complexes that have been identified in human cell lines, cI and cII, with cII being similar to the NuRD (14). HDAC1 and HDAC2 can each be found either alone or together in these complexes, and can be dissociated from one another using a mild agent. This data led Humphrey et al. to present a model through which these two histone deacetylases interact via dimerization. This dimerization also led them to propose a method for targeting the HDAC complexes. In this model, MBD2, which was found in the HDAC1 cII complex, specifically binds to methylated DNA. HDAC2 might be colocalized with HDAC1 via this dimerization to allow the complex to work to silence methylated DNA (14).

The RbAp46/48 proteins are histone chaperones that are involved in many complexes associated with chromatin functions including the NuRD and Sin3

complexes as previously mentioned. Additionally, they are also found in Hat1, a histone acetyl transferase; NURF, a *Drosophila* transcription factor; CAF-1, a factor key in the assembly of chromatin; and PRC2, a histone methylating complex linked to transcriptional repression. Generally, researchers view RbAp46/48 as centrally involved in all of these complexes through their histone interactions, allowing the complexes to be localized and have their respective effects on the histones (16).

Another protein found in the NuRD is MBD3, which contains a methyl-CpG binding domain. In Xenopus, this MBD3 specifically binds to methylated DNA, but this is not the case in mammalian cells (10,17). Upon examination, it appears that MBD3 is not involved with the localization of mammalian NuRD to methylated DNA, but instead acts as a mediator between MTA2 and the fundamental histone deacetylase core of the NuRD (11). This is further substantiated by the fact that it seems to be inaccessible to antibodies while in the NuRD complex. Additionally, there are two forms of MBD3 in the complex, one with an entire methyl binding domain and the other with it lacking the amino terminal half; these differences are apparently due to an alternative splice acceptor site in the middle of the DNA sequence for the MBD (17). Though Hendrich and Bird found that "the shorter message makes up a significant portion of total Mbd3 message", Zhang et al. showed that "the major form" is the shorter version with the truncated MBD (11,17). This accounts for why the MBD3 seems to lack specificity for binding methylated DNA even though it has an MBD sequence. As a side note, though the majority of the NuRD complex does not coassociate with MBD2, part of it does. This addition of MBD2 creates a larger complex that shows specific interaction with methylated DNA (18).

Though the last component of the NuRD complex originally described by Zhang et al. is MTA2, recent information has shown that isoforms of MTA1 and MTA3 can also associate with the complex, possibly leading to functional differences between unique forms of the complex (10,11,19). MTA2, so named because of its homology to MTA1, a metastasis associated protein, was originally shown to promote the activity of the histone deacetylase components without the presence of either MBD3 or Mi2 to a level comparable to that of the native complex (11). There has been considerable confusion regarding the identity of the MTA family member in the complex until it was recently postulated that there could be functional specialization based on the specific member involved (10). To begin this, Tong et al. found that their version of the complex, NRD, contained MTA1, not MTA2 (20). When Zhang et al. published the following year, they discounted this based on the fact that the regions used to identify the family member were shared between MTA1 and MTA2 (11). Later, Fujita et al. showed that MTA3 could be a member of the NuRD in a manner dependent on the level of estrogen present in the cell environment (21). These results provide an important link between breast cancer and the MTA family of proteins. To further complicate matters, both MTA1 and MTA3 exist in alternatively spliced forms, present in different levels in cancer cell lines of different types of tumors (10,21). In sum, there is growing evidence that the particular MTA family member involved in the NuRD dictates functional specificity, though this has not yet been conclusively shown. Additionally, the presence of a metastasis-associated protein in the NuRD implicates it in having a role in cancer processes.

ROLE OF CHD4

CHD4, also know as Mi-2β, was found to be the largest subunit in the NuRD complex (13). It was originally identified as an autoantigen in dermatomyositis, an acquired muscle disease characterized by a bluish-purple rash along with muscle weakness, often leading patients to have difficultly sitting up or lifting things (22,23). Though Zhang et al. only reported finding CHD4 in the NuRD, Tong et al. reported finding both CHD3 and CHD4, though the CHD4 in greater amounts (13,20). CHD3 and CHD4 belong to a subfamily of the CHD proteins; the domain structure is similar between the two, with the main difference being that the CHD3 and CHD4 carboxy-terminal DNA binding domains vary significantly (24). The CHD proteins were so named because of their *C*hromodomain-*H*elicase-*D*NA-binding domains (25). CHD4 is a 230-240 kD protein which has several domains including a Nuclear Localization Signal, 2 PHD Zn-fingers, 2 chromodomains, a SWI2/SNF2-related ATPase domain, and an additional carboxy terminal helicase (12,13,20). The nuclear localization signal allows this protein to be guided back to the nucleus once it has been translated in the cytoplasm; this is necessary because all of its roles are in the nucleus.

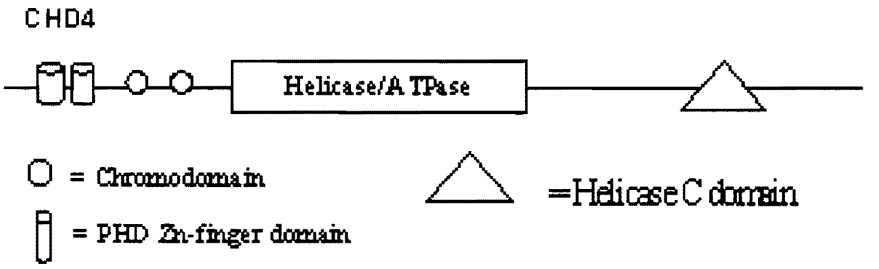

Figure 2. Protein motifs in CHD4.

The PHD Zn-finger domains are formed of conserved cysteine residues that bind to Zinc ions; it is postulated that CHD4 interacts with HDAC1 in the NuRD via the PHD Zn-fingers (24). Chromodomains, named for their *chr*omatin *o*rganization *mo*difier activity, are conserved protein folds that influence chromosome structure, leading to

changes in function. These domains are found in organisms from protists to mammals, indicating strong evolutionary conservation (26). The SWI2/SNF2-related ATPase is a DNA-dependent helicase (13). This activity indicates a role in nucleosome remodeling, even leading to enzyme classification as nucleosome remodeling enzymes (6). Not much research has been performed regarding the C-terminal helicase domain, but it likely assists the SWI2/SNF2 helicase, though it could possibly be involved in DNA damage repair pathways as well (27).

CHD4, the main component of the NuRD complex, contains ATPase activity stimulated by histones and dependent on DNA (7). Wang et al. characterized the biochemical activity of CHD4 outside of the NuRD, finding that recombinant CHD4 had nucleosome remodeling activity comparable to the activity of the NuRD complex as a whole. In their experiments, they found that both CHD4 and the NuRD complex required the presence of nucleosomes rather than just naked DNA, indicating a role for the histones (7). Additionally, Shimono et al. showed that the amino-terminal region of CHD4 has a binding site for BRG1, the major component of the SWI/SNF complex, a chromatin remodeling complex having known activation ability (28, 29). They also found that the carboxy-terminal region of CHD4 binds RET finger protein (RFP), increasing its repressive capability in a manner dependent on the amount of CHD4 present. Together, these results show that association of chromatin remodeling proteins could lead to "supercomplexes" with the ability to effect epigenetic repression through cooperation of methylation, deacetylation, and other methods of gene silencing (28). In addition, von Zelewsky showed that the Mi-2 (CHD3 and CHD4) homologues in *C. elegans* played essential roles in development. Particularly, both homologues played

a role in the determination of vulval cell fate during development (30). As a result of these studies, it is now known that CHD4 has roles outside of solely being the key component of the NuRD complex (thought to be only repressive), including possible roles during development and influences on transcriptional activation.

RELEVANCE TO CANCER

Though not yet shown to directly involve CHD4, chromatin remodeling has links to human disease, notably cancer. It is significant that the MTA genes are part of the NuRD complex because they were so named because of their correlation with metastasis (31). Specifically, Toh et al. found that mRNA expression of *mta1* was four times as high in highly metastatic cell lines than in nonmetastatic cell lines. Additionally, it is particularly notable that even in isogenic model organism populations, different phenotypes are still evident. One possible cause for this is chromatin remodeling in addition or in response to DNA methylation (32). Mutations in either the function or targeting abilities of chromatin remodeling complexes usually have multi-system effects or lead to cancer. This can be explained by loss of regulation by the particular complex at multiple loci (32). One specific example of a chromatin remodeling enzyme related to cancer is BRG1, a component of the SWI/SNF complex. It seems to act as a tumor suppressor, an effect shown by its high mutation rate in a variety of cancer cell lines (33). Perhaps even more interestingly, reintroduction of wild type BRG1 into cells lacking BRG1 expression allowed the cells to revert from their transformed phenotype and induce senescence (32, 33). BRG1 also regulates cell cycle control through its interactions with Rb and BRCA1 (32 and references therein).

On a different, but related, note, global histone hypoacetylation has been shown in gastrointestinal tumor cells and is correlated with increased tumor invasion and metastasis (34). Histone deacetylase inhibitors such as Trichostatin A (TSA) induce growth arrest and apoptosis in tumor cell lines, providing a possible treatment solution for cancer with a great deal of potential. In conclusion, the chromatin structure inherent to DNA storage can be modified to make the expression of some genes more or less likely, and these modifications can have a vast influence on processes from cell cycle regulation to chromosome stability, even to the point of aberrant regulation leading to disease phenotypes such as cancer (35).

GENERATION OF MOUSE MODELS

Cells which had a genetrap inserted into the *Chd4* gene were obtained from the Baygenomics Embryonic Stem cell library. The location of the trap between exons 6 and 7 was confirmed by PCR analysis using the primers shown in Figure 3. Once confirmed, the ES cells were used for blastocyst injections and the embryos were then implanted into pseudo-pregnant females. The result of these efforts was the generation of chimeric mice which were later mated with wild type mice to determine which of the chimeras had germ line mutations in *Chd4*. Offspring were tested to ascertain their genotype using the PCR analysis described in Figure 3 and heterozygous mice were further bred to expand the colony.

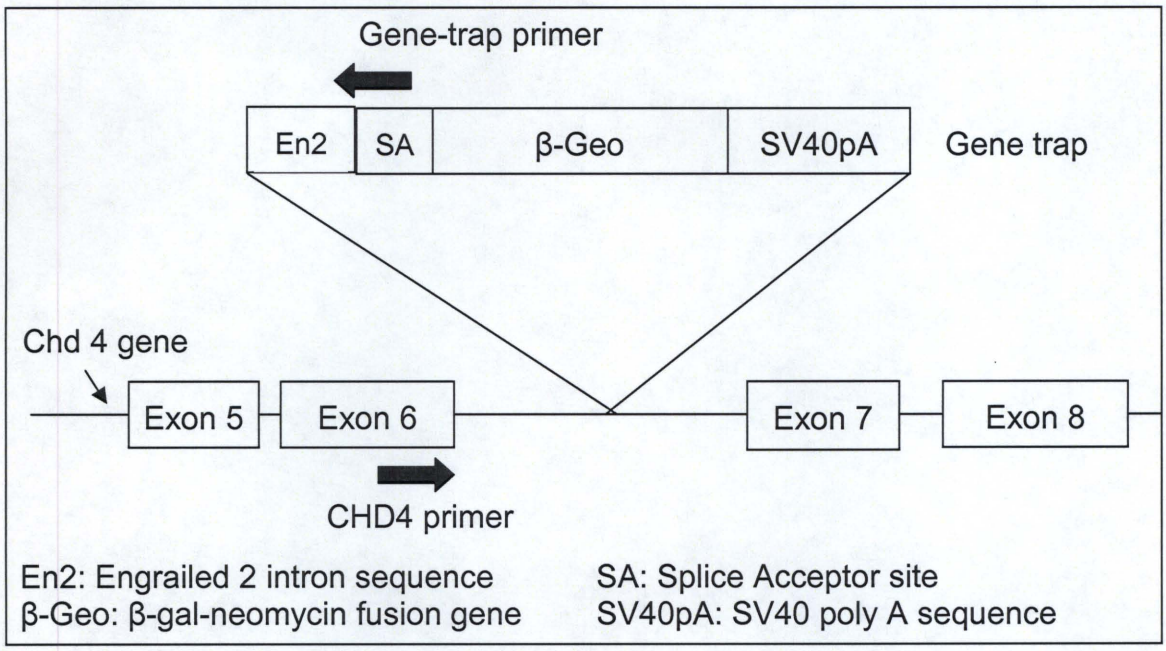

Figure 3. Schematic representation of the *Chd4* disruption in ES cells.

RESULTS AND DISCUSSION

To systematically analyze the role of CHD4 and determine tissue specificity in both young and old mice, we have analyzed tissue specific expression patterns using RT-PCR analysis with total RNA isolated from different tissues. In order to allow us to determine differential expression, we used a 28 cycle PCR reaction that allows semi-quantitative analysis. As shown in Figure 4, *Chd4* is expressed at different levels in a tissue specific manner in young and old mice. One specific difference is that though three month old mice show expression in the liver,

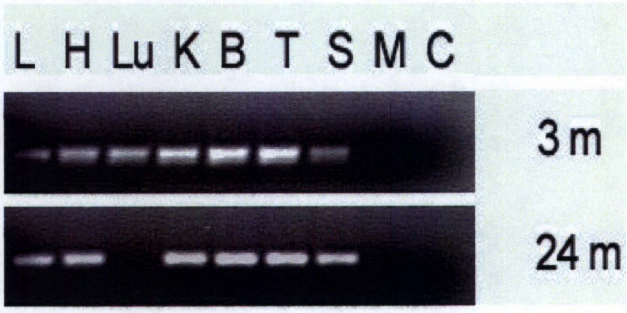

Figure 4. RT-PCR analysis of *Chd4* expression. Total RNA (5 μg) isolated from various tissues (L=liver, H=heart, Lu=lung, K=kidney, B=brain, T=testis, S=spleen, M=skeletal muscle) was reverse transcribed and used in PCR reactions with primers specific for *Chd4* exons. C refers to control reaction using brain RNA without reverse transcriptase.

heart, lung, kidney, brain, testes, and spleen, twenty-four month old mice show expression in all of these except the lung. Additionally, there was conspicuous absence of transcript from the skeletal muscle. It remains to be seen what these differences signify. This data also needs to be further confirmed with protein assays. After procurement of an appropriate CHD4 antibody, we will check actual protein expression levels in various tissues of both young and old mice to verify that different levels of mRNA expression in the cells actually lead to different levels of protein production.

Since *Chd4* homologues have been found to play a role in development in *C. elegans* (30), we decided to investigate possible roles and expression patterns for *Chd4* in mouse embryos. We were able to do this because of the nature of the trap inserted to disrupt the gene. Using X-gal and the promoter-less β-galactosidase-neomycin fusion construct, we were able to determine gene expression patterns in 10.5 days post coitum mating of a heterozygotic male to a wild type female. As shown in Figure 5, expression was evident in the brain precursors, the dorsal aorta, and dermal precursors. Interestingly, the expression in the dermal precursors was significant enough to mask the internal expression in the whole mount embryo (Figure 5B). Upon slicing, though, it became clear that there was not ubiquitous expression throughout the embryo, only in the previously mentioned areas (Figure 5C). This expression pattern indicates distinct, tissue specific expression pointing to organ specific functions of CHD4. We plan to examine embryonic expression data for *Chd4* at 8.5, 12.5, and 14.5 dpc to allow for a more comprehensive perspective of its role in development.

Figure 5. Expression analysis of *Chd4* in 10.5 d.p.c. embryos. Whole embryos were stained with X-gal overnight to measure β-galactosidase activity and photographed. Panel A is a control wildtype embryo obtained from a *Chd4* +/- cross to a wildtype mouse. Please note that the whole mounts of the controls was taken on a black background for visualization and better contrast. Panel B is a stained whole mount embryo from a *Chd4* heterozygous embryo, while Panel C is a cross section obtained from the same heterozygous embryo.

Upon expansion of the colony and observation of the genotypes of the offspring of heterozygous intercrosses, it became evident at first look that there were not any pups nullizygous for *Chd4*. Though we have not yet had enough offspring for the data to be statistically significant, the lack of any nullizygous offspring is at the least conspicuous (expected and actual numbers of offspring are shown in Table 1). This absence of nullizygous mice underlines the importance of CHD4 and its ability to affect downstream targets. In addition to embryonic lethality showing that CHD4 is essential for development, it would also show that there are no compensatory mechanisms that can offset its absence. To further investigate this, we plan to continue expanding the colony and determine the day and mechanism of embryonic lethality, if future results confirm our preliminary data. Upon colony expansion, we will also investigate organismal cancer susceptibility and relative life span.

Results of heterozygous intercrosses			
	Wild Type	Heterozygous	**Null**
Actual	4	21	**0**
Expected*	6	13	**6**
*based on a Mendelian distribution of the total number of progeny			

Table 1. Heterozygous intercross results. Genomic DNA samples obtained from either tail clippings in the case of pups or processing of the whole cell line in the case of MEF cell lines were analyzed by PCR to determine their genotype. These data support our preliminary conclusion that *Chd4* is necessary for embryonic development.

Lastly, mouse embryonic fibroblasts (MEFs) were obtained from timed matings between a heterozygous male and a wild type female. We characterized these cell lines for growth kinetics and observed that the heterozygous line grew faster than did the wild type (Figure 6). This enhanced growth rate gives us preliminary information that CHD4 might be involved in cell growth suppression pathways, and its loss might lead to cancerous phenotypes. In addition, it leads us to believe that CHD4 mutant mice are likely to be more susceptible to organismal cancer growth. According to the multi-step model of carcinogenesis, though, additional downstream mutations will be necessary for a cancer phenotype to ensue. We eventually hope to obtain nullizygous cell lines, even if the nullizygous pups display embryonic lethality. These cell lines will enhance the growth curves and will also allow us to run microarray experiments to determine which genes CHD4 affects downstream. In addition to the growth kinetic experiments on the various cell lines, we will also do colony formation and DNA damage repair assays.

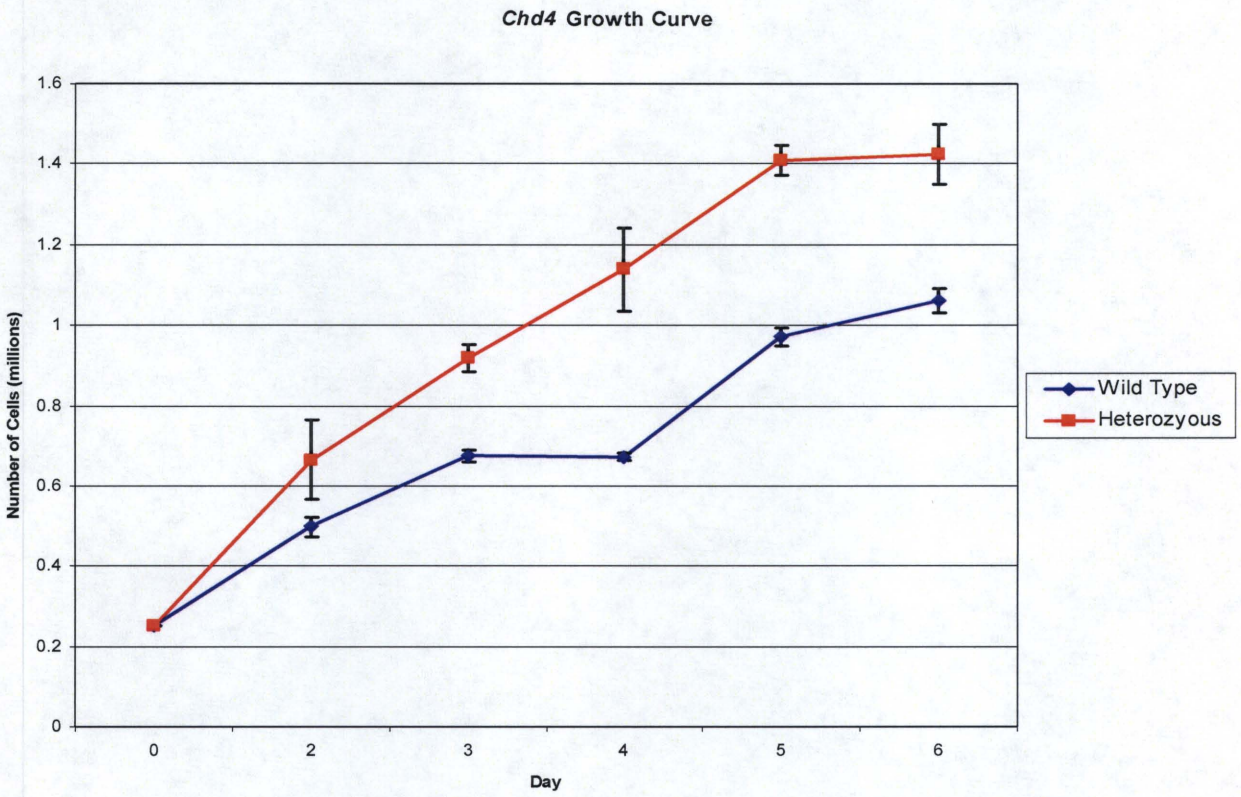

Figure 6. *Chd4* Growth Curve. Cell lines heterozygous for *Chd4* grow more rapidly than do their sibling wild type counterparts. These experiments were conducted in duplicate on two heterozygous and two wild type cell lines. Error bars represent standard error.

METHODS

For the RT-PCR experiment, total RNA was isolated from the indicated tissues using Trizol reagent (Invitrogen) from 3 and 24 month old mice. Expression of *Chd4* mRNA determined using reverse transcriptase PCR analysis followed by agarose gel electrophoresis.

To determine the embryonic expression pattern, a wild type female was mated with a male heterozygous for *Chd4*. The female was sacrificed at 10.5 dpc. The gene trap inserted into the Chd4 gene (see Figure 3) contains a promoter-less β-galactosidase-neomycin fusion gene along with a splice acceptor site. This promoter-

less β-galactosidase gene allows visualization using X-gal staining in heterozygotic embryos. It represents the expression pattern of CHD4 in the embryos due to its presence after the splice acceptor site. When the gene is activated, the mRNA is made normally until the location of the trap. The strong splice acceptor located in the trap prevents alternative splicing with the appropriate exon and allows expression of the β-galactosidase protein in the cells. Upon introduction of X-gal into the embryos, portions of the embryo expressing β-galactosidase will turn the usually transparent X-gal blue, allowing for differentiation between regions expressing β-galactosidase and those that do not.

Mouse embryonic fibroblast (MEF) cell lines were obtained from a cross between a male heterozygous for *Chd4* and a wild type female. Briefly, 13.5 days post coitum (dpc) embryos were harvested from pregnant females and disaggregated using a syringe. The embryonic tissues were then plated onto 100 mm tissue culture plates and passaged upon confluency. These cell lines were maintained at 37°C using humidified air supplemented with 5% CO_2 in DMEM with 15% fetal bovine serum and Penstrep. For the cell growth kinetic assays, 250,000 cells were plated on sterile 100 mm plates. This was considered day 0. The plates were counted on days 2, 3, 4, 5, and 6 using a Hemacytometer. Cell lines were labeled with nondescript names by a third party to prevent any possible bias.

ACKNOWLEDGEMENTS

I would like to thank Dr. Sundaresan Venkatachalam, my advisor, for all of his input and guidance throughout this entire process. Additionally, I am greatly indebted to

Amanda Gentry and Dr. Prabakaran Nagarajan for their continued help whenever I needed it.

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