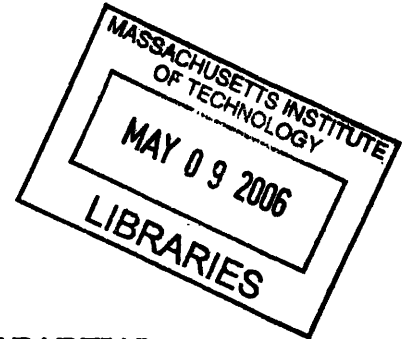# The Role of *Cdyl* and *CDY* in Mammalian Spermatogenesis

by

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ABSTRACT

Mouse *Cdyl* was originally identified based on homology to the human gene *CDY* (Lahn and Page, 1999), which is found in four copies on the human Y chromosome (Kuroda-Kawaguchi et al., 2001). Because *CDY* is expressed specifically in the testis (Lahn and Page, 1999) and is found in Y chromosomal regions that are deleted in some infertile men (Kuroda-Kawaguchi et al., 2001), *CDY* is thought to have an important role in male fertility and spermatogenesis. However, human studies have not yet been able to directly implicate *CDY* in male infertility. Even though mouse *Cdyl* is not located on the Y chromosome, it is the closest known mouse homologue of human *CDY* and is expressed highly in the testes (Lahn and Page, 1999), which suggests that mouse *Cdyl* provides a suitable model for the study of human *CDY* function. However, unlike human *CDY*, mouse *Cdyl* is expressed in tissues other than the testis (Lahn and Page, 1999).

We have generated mice deficient for *Cdyl* to study its role in spermatogenesis and characterized their phenotype on a pure BALB/c background. Nearly 2/3 of *Cdyl* knockout mice die shortly after birth, but those that survive to adulthood appear healthy except for spermatogenic defects. Mice lacking *Cdyl* produce spermatozoa with misshapen heads and exhibit substantial germ cell death. The loss of germ cells is evident in some knockout mice as early as 3.5 weeks of age, affects spermatogonia, spermatocytes, and spermatids, and is so severe that at 5 months of age the seminiferous tubules of *Cdyl* knockout mice appear nearly empty. These results demonstrate that *Cdyl* plays a crucial role in spermatogenesis and suggest that the homologous human gene *CDY* does as well. However, our results do not support a previously suggested hypothesis that *Cdyl* participates in the global acetylation of histone H4 in spermatid nuclei (Lahn et al., 2002), as hyperacetylated histone H4 was detected in both wildtype and knockout spermatids.

To directly study the role of the human gene *CDY* in spermatogenesis, we have attempted to rescue the *Cdyl*-/- spermatogenic phenotype by constructing a transgenic mouse that expresses human *CDY* and breeding the *CDY* transgene onto a *Cdyl*-/- background. Preliminary data from these crosses suggests that human *CDY* can not rescue the spermatogenic phenotype observed in *Cdyl*-/- mice, as *Cdyl*-/- Tg*CDY* mice still exhibited germ cell loss. However, we currently possess Tg*CDY* mice only on a C57BL/6 background, and in the course of these rescue experiments we observed that the spermatogenic phenotype of *Cdyl*-/- mice is not as severe on a C57BL/6 background, on a mixed C57BL/6x129 background, or on a mixed C57BL/6xBALB/c background as it is on a pure BALB/c background. We believe that future studies, performed on a pure BALB/c background, will be able to better address whether human *CDY* can rescue the *Cdyl* knockout mouse spermatogenic phenotype.

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INTRODUCTION

Almost every cell in the human body, from neurons in the brain to muscles in the leg, represents a differentiated cell type. These specialized cells are generated when precursor cells become altered through a combination of structural and physiological changes. However, understanding the molecular processes responsible for generating differentiated cell types remains a fundamental challenge facing biologists. In recent years one program of cellular differentiation that has been intensely studied is the transformation of an undifferentiated spermatogonial stem cell into a mature spermatozoan, a process called spermatogenesis. One need only briefly observe these two disparate cell types to begin to appreciate the sweeping changes required to convert the one into the other (Figure 1, below). Perhaps the most important concern motivating study of this particular differentiation pathway is the recognition that defects in this pathway are one of the chief causes of male infertility.
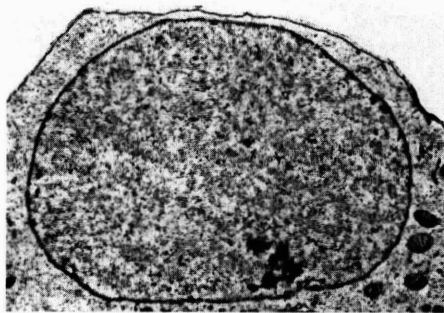
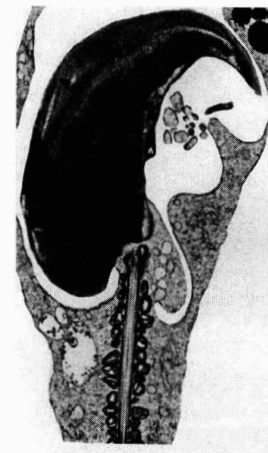SPERMATOGONIUM   SPERMATOZOAN

Figure 1 Electron micrographs of a rat spermatogonium and a rat spermatozoan (from Russell et al., 1990).

SPERMATOGENESIS

In mammals an entire organ, the testis, is dedicated to producing spermatozoa. The testis is divided into many smaller compartments, called seminiferous tubules, which is where the spermatogonial stem cells differentiate into sperm. The seminiferous tubules are a highly organized environment, with less differentiated cells residing closer to the basal membrane of the tubule and more differentiated cells residing away from the membrane in the lumen of the tubule (shown in Figure 2, following page). Three phases of spermatogenesis can be identified: spermatogonial proliferation, meiosis, and spermiogenesis.

SPERMATOGENESIS: SPERMATOGONIAL PROLIFERATION

Spermatogonial proliferation lasts approximately 9 days in mice and consists of 9-11 mitotic divisions (reviewed in de Rooij, 2001). This phase of spermatogenesis is important because it ensures that a large quantity of sperm will be produced, as each cell present at the end of these numerous divisions becomes an individual sperm. In the first division, a spermatogonial stem cell divides to form two A_{paired} spermatogonia. A_{paired} spermatogonia then divide from one to three times to form $A_{aligned}$ spermatogonia (reviewed in de Rooij, 2001). It should be noted that cytokinesis is incomplete during these mitotic divisions and the germ cells therefore remain connected to each other by cytoplasmic bridges, a condition which persists until spermatogenesis is nearly complete. The $A_{aligned}$ spermatogonia then differentiate into A1 spermatogonia, which divide 5 more times, forming in turn A2, A3, A4, intermediate, and type B spermatogonia

Figure 2 Light micrograph (inset) and an artist's rendition of the cross section of a mouse seminiferous tubule.

Cytology of the Seminiferous Tubule (Modified from Christiansen, 1996, *Endocrine Reviews*)

BL = Basal lamina; ML = Myoid cell layer; Ca = Capillary; JC = Junctional complex, tight junction;
IS = Intercellular spaces; Se = Sertoli cell; Sg = Spermatogonia; Sc = Spermatocyte; Sd = Spermatid

(reviewed in de Rooij, 2001). Finally, the type B spermatogonia divide to form spermatocytes.

SPERMATOGENESIS: MEIOSIS

The second phase, meiosis, begins when spermatocytes are formed and lasts about 13 days in mice (Nebel et al., 1961). This phase of spermatogenesis helps generate the diversity on which natural selection acts by promoting the mixture of alleles into new combinations. This is accomplished in two independent ways during meiosis. First, meiosis is comprised of only one round of DNA replication, but two rounds of cell division, which ultimately leads to the production of haploid cells. When the haploid gametes from males combine with the haploid gametes produced by females, the resulting offspring contain a mixture of genetic material from the male and the female. Second, during the prophase that precedes the first meiotic division, homologous chromosomes pair and exchange DNA, which leads to novel combinations of alleles on the chromosomes. This prophase is divided into several stages: (1) leptotene, when chromosomes begin to condense; (2) zygotene, when homologous chromosomes pair and the synaptonemal complex forms; (3) pachytene, when DNA recombination occurs; and (4) diplotene, when recombination is complete and the synaptonemal complex breaks downs (reviewed in Cooke and Saunders, 2002).

SPERMATOGENESIS: SPERMIOGENESIS

The third phase of spermatogenesis, called spermiogenesis, begins when haploid cells, called spermatids, are generated by the meiotic divisions; this phase

lasts 13 days in mice and culminates in the production of spermatozoa (Nebel et al., 1961). During this phase spermatids undergo many structural changes that are needed in order for spermatozoa, once released, to reach and fertilize an oocyte. There are 16 types of spermatids in mice, and these are named step 1 spermatids through step 16 spermatids (Russell et al., 1990). Step 1 through step 8 spermatids are round, but beginning at step 9 their nucleus begins changing shape, elongating and eventually forming a hook at the anterior end (Russell et al., 1990). This dynamic morphological remodeling is thought to be controlled by a structure called the microtubule manchette, because drugs that affect microtubule assembly have been shown to cause defects in sperm head morphogenesis, and mutation of several proteins associated with the manchette, such as HOOK1 and E-MAP-115, cause defects in sperm head morphogenesis (Mendoza-Lujambio et al., 2002; Komada et al., 2000). From steps 12-14 the spermatid chromatin becomes highly condensed, a process that is thought to protect the DNA and further streamline the spermatozoa. This chromatin condensation is achieved through the removal of histones from the DNA and the repackaging of DNA with small basic proteins called protamines (reviewed in Braun, 2001). Because of these morphological alterations, step 9-11 spermatids are called elongating, step 12-14 spermatids are called condensing, and step 15-16 spermatids are condensed (Roest et al., 1996).

Other important structural changes that occur during spermiogenesis include the formation of the acrosome, a cap on the sperm head that forms from the golgi apparatus and which contains enzymes needed for fertilizing the egg, the formation of the flagellum, an axoneme-containing structure needed for movement, and the expulsion of almost all cytoplasm from the germ cell (Russell et al, 1990). Additionally, important accessory structures develop that are

associated with the flagellum: the mitochondrial sheath, the fibrous sheath, and the outer dense fibers. The fibrous sheath and the outer dense fibers are cytoskeletal structures that play a mechanical role in the flagellum, by helping to maintain the structure and define the shape of the flagellum (Fawcett, 1975). The mitochondrial sheath contains a helical arrangement of mitochondria that are important for generating the energy needed for sperm motility (Russell et al., 1990).

SPERMATOGENESIS: CYCLING

Spermatogenesis is a lengthy process, and takes 70 days in the human and 35 days in the mouse (Russell et al., 1990). Spermatogensis is also a cycling process, as the spermatogonial stem cell population of the testis continually proliferates and produces differentiated cells. Each time spermatogonia differentiate to produce spermatozoa, a new "wave" of spermatogenesis is considered to be occurring. To reflect this cycling, seminiferous tubules can be identified based on stages, which are descriptions of the types of germ cells found in a seminiferous tubule at a particular point in time (Russell et al., 1990). 12 stages of the seminiferous cycle have been identified in the mouse. Germ cells exhibiting highly varying degrees of differentiation are found adjacent to each other in tubules of a particular stage, because they originate from different spermatogenic waves. For example, a stage IV tubule contains step 15 spermatids, step 4 spermatids, pachytene spermatocytes, and intermediate spermatogonia (Russell et al., 1990).

SPERMATOGENESIS: SOMATIC CELLS

The differentiation of spermatogonial stem cells into mature sperm is further complicated by the fact that this is not a cell autonomous process. Additional somatic cells that reside in the testis, most importantly the Sertoli cells and the Leydig cells, play a crucial role in the differentiation of the germ cells. Sertoli cells are intermixed among the germ cells within the seminiferous tubules. Due to the large size of Sertoli cells, they are in contact with all the germ cells in a tubule. As such, the Sertoli cells are important in providing overall structure and organization of the seminiferous tubule. They assist in the movement of germ cells toward the lumen through the formation of different cellular junctions, and they also assist in spermiation, the release of sperm into the lumen (reviewed in Mruk and Cheng, 2004). When germ cells undergo apoptosis, they clear these dying cells from the tubule through phagocytosis. Sertoli cells also form a blood-testis barrier that separates the seminiferous tubule into two compartments (reviewed in Mruk and Cheng, 2004). One compartment contains spermatogonia and leptotene spermatocytes, and the other compartment contains all other types of germ cells. This barrier provides a specialized environment where spermiogenesis and most of meiosis occurs, and shields developing germ cells from the immune system. Because of this barrier, another critical function of Sertoli cells is to provide nutrients to spermatocytes and spermatids. Overall, Sertoli cells are critical regulators of the entire spermatogenic process (reviewed in Mruk and Cheng, 2004). They secrete many factors, such as growth factors, hormones, and proteases that influence the proliferation, differentiation, and movement of germ cells. Leydig cells reside in

the interstitial space outside of the seminiferous tubules. They regulate

spermatogenesis chiefly through the production of the sex hormone testosterone.

MOUSE MUTANTS

Much of our understanding regarding the molecular mechanisms

underlying spermatogenesis comes from the study of knockout mice or mice with

spontaneously arising mutations. Knockout mice especially have proven to be an

extremely valuable tool for identifying genes that participate in spermatogenesis

and for determining what role those genes play. To date well over 100 mutant

mouse models exist that display a spermatogenic phenotype. (For clarity

"mutants" here refers to loss of function mutants, and they are generally assumed

to be functional nulls.) Therefore, I have developed a classification scheme to

provide a comprehensive framework for describing spermatogenesis mutants.

Within this scheme the vast majority of mutant mouse spermatogenic phenotypes

can be classified into one of three general categories: Category I-Spermatogenesis

is arrested at a particular stage such that very little or no mature sperm is made;

Category II-Spermatogenesis proceeds to completion, such that mature sperm are

made, but these sperm are defective; Category III-Spermatogenesis can proceed to

completion, but due to substantial germ cell loss the process can't be sustained.

MOUSE MUTANTS: CATEGORY I (ARREST)

Category I (arrest) mutant mice exist for each of the three phases of

spermatogenesis (spermatogonial proliferation, meiosis, and spermiogenesis).

These mutants are usually highly informative about the function of the defective

gene product, as they can be used to precisely pinpoint when during spermatogenesis it is required. Mutants in this class could be misleading, though, if a mutant phenotype only manifests itself far after when the knocked-out protein normally acts. Relatively few knockout mice exist in whom an arrest during spermatogonial proliferation is observed; this likely reflects the relatively small number of spermatogonially expressed genes that have been knocked out, when compared to the large number of spermatocyte and spermatid genes that have been knocked out. A large number of mouse mutants exhibiting an arrest during meiotic prophase or meiosis have been generated. This is probably owing to the extensive identification and characterization of meiosis genes in lower organisms, especially yeast. Finally, several mutants exhibiting a spermiogenesis arrest have been generated, but most of these mutants arrest during the early phases of spermiogenesis. Apparently, mutation of genes required for the later stages of spermiogenesis usually does not result in an arrest, but rather in the production of defective spermatozoa (these mutants will be discussed later).

Very few mutants exhibiting a clear arrest during spermatogonial proliferation exist. One classic example of this type of mutant is the *c-Kit* mutant mouse. *c-Kit* is a tyrosine kinase receptor expressed in spermatogonia and known to be involved in cell cycle regulation (reviewed in Rossi et al., 2003). Mice carrying mutations in *c-Kit* contain very few spermatogonia, and these appear to undergo arrest shortly after differentiation into A1 spermatogonia (Ohta et al., 2003). Mice mutant for *Dmrt1*, a gene that contains a DNA binding domain, also exhibit a spermatogonial arrest, shortly before the onset of meiosis (Raymond et al., 2000).

A wide spectrum of mutant mice exists that undergo arrest during the meiosis phase of spermatogenesis. In many of these mouse models, an arrest in

the first meiotic prophase is seen, and apoptosis of spermatocytes follows the initial arrest. *Spo11* knockout mice arrest in zygotene with mostly unsynapsed chromosomes (Romanienko and Camerini-Otero, 2000); in yeast, *Spo11* was shown to generate double stranded DNA breaks during meiosis (Keeney et al., 1997). Mutation of *Msh5*, a DNA mismatch repair gene, results as well in zygotene arrest and the failure of homologous chromosomes to pair (Edelmann et al., 1999). Mutation of another mismatch repair gene, *Mlh1*, results in pachytene arrest (Edelmann et al., 1996). In this mutant, chromosome pairing is observed, but no chiasmata (points of crossing-over between chromatids) are formed. A knockout mouse for the thiamin transporter *Slc19a2* also exhibits pachytene arrest (Oishi et al., 2004). Spermatocytes in mice mutant for cyclin A1 progress through most of meiotic prophase, but appear to arrest in early diplotene, as chromosome desynapsis is not observed (Liu et al., 1998). Other genes that result in meiotic arrest when mutated include *Rec8*, a meiotic cohesin required for sister chromatid cohesin (Bannister et al., 2004), *Hsp70-2*, a heat shock protein (Mori et al., 1999), and *Mili*, which may be involved in RNAi (Kuramochi-Miyagawa et. al., 2004). Occasionally mutants present an incomplete arrest phenotype: in knockout mice for the zinc-finger transcription factor *Egr4*, most spermatocytes arrest in pachytene, but a few spermatocytes are able to progress beyond this arrest and form mature spermatozoa (albeit abnormal ones) (Tourtellotte et al., 1999).

Many mutants exist in which arrest occurs in haploid germ cells during the spermiogenesis phase. Mutants for the RNA helicase *Ddx25* arrest at step 8 of spermiogenesis, just prior to the onset of spermatid elongation (Tsai-Morris et al., 2004). *Ddx25* is thought to function in translation, and the authors demonstrated that angiotensin converting enzyme (*Ace*) isn't translated properly

in *Ddx25*-/- testes. However, *Ace*-/- mice don't exhibit a spermiogenic arrest
(Hagaman et al., 1998), so other as yet unidentified targets are likely responsible
for the observed phenotype of *Ddx25* mutant mice. An arrest during step 7 of
spermiogenesis was observed for mutants in the poly(A) polymerase *Tpap*
(Kashiwabara et al., 2002). Even though the mRNA transcripts of several genes
were found to have shorter poly(A) tails in *Tpap*-/- testes, surprisingly most of
these genes were still translated at normal levels. Mice in which the
transcription factor *Trf2* is knocked out exhibit an arrest at the round spermatid
stage (Zhang et al., 2001). Even though the authors demonstrated that many
spermatid genes are expressed at lower levels in *Trf2* knockout testes, it is
difficult to ascertain whether this is directly due to reduced transcriptional
activity of these genes or merely a reduction in the spermatids where these genes
are normally expressed.

Some genes have been identified which lead to an arrest in elongating or
late stage spermatids when knocked out: *Ae2*, an anion exchanger that moves
anions such as bicarbonate across cell membranes (Medina et al., 2003); *Camk4*,
a kinase that has been demonstrated to phosphorylate protamine 2 *in-vitro* (Wu
et al., 2000); and *Hsl*, an enzyme that hydrolyzes many types of molecules,
including triglycerides and cholesterol (Chung et al., 2001). While the precise
role of *Hsl* in spermatids is unclear, the phenotype of *Hsl* mutants is particularly
striking: Even though the cytoplasmic bridges connecting cohorts of
spermatogonia and spermatocytes are unaffected, these bridges appear to break
down in spermatids so as to generate giant spermatids with multiple nuclei
sharing one cytoplasm.

Most of the genes discussed thus far that result in a Category I (arrest)
phenotype when knocked out are expressed in the germ cells but not the somatic

cells of the testes. An exception is lutenizing hormone, which interacts with a receptor found on Leydig cells and is important for testosterone production (reviewed in Pierce and Parsons, 1981). In a mouse knockout for the beta subunit of LH, spermatogenesis was arrested at the round spermatid stage, presumably due to a reduction in intratesticular testosterone (Ma et al., 2004).

MOUSE MUTANTS: CATEGORY II (DEFECTIVE SPERM)

A large number of genes expressed in late stage spermatids do not result in spermatogenic arrest when knocked out, but rather in the production of abnormal spermatozoa. This class of mutants represents the second major category of spermatogenic mutants. Even though these mutants produce sperm, they are either infertile or subfertile (one exception are the transition protein mutants; see below). Spermatozoa from Category II (defective sperm) mutants often exhibit defects in one or more of the following: acrosome formation, nuclear condensation, sperm head morphology, and motility. In addition, these mutants may produce reduced numbers of spermatozoa.

Mutations that result in sperm motility defects often affect genes that are necessary for proper functioning of the flagellum. The flagellum of a spermatozoan requires large amounts of ATP to generate movement. Mutation of *Gapds*, an enzyme in the glycolytic pathway that is expressed specifically in sperm, results in a significant reduction of ATP levels in spermatozoa and very poor sperm motility (Welch et al., 2000; Miki et al., 2004). The gene *Spag6* is associated with the axoneme, a structure in the flagellum containing two central microtubules and nine surrounding microtubule doublets (Neilson et al., 1999).

Sperm from *Spag6* knockout mice are immotile and have severely disorganized flagella (Sapiro et al., 2002). In addition, some *Spag6* knockout spermatozoa had detached heads.

Several genes have been identified that impair formation of the acrosome. Acrosomes are formed early in spermiogenesis from vesicles that originate from the Golgi body. Knockouts deficient for GOPC, a protein that is associated with the Golgi body, and mice deficient for HRB, a protein that is associated with the budded vesicles that give rise to the acrosome, both produce round-headed spermatozoa that lack an acrosome (Yao et al., 2002; Kang-Decker et al., 2001). Mice that are mutant for the kinase *Csnk2a2* also produce round-headed sperm that have highly defective or absent acrosomes (Xu et al., 1999). However, it is not clear what the relevant targets of *Csnk2a2* are in germ cells.

Chromatin condensation occurs in spermatid nuclei when the histones that normally package the chromatin are removed and the chromatin is repackaged transiently with transition proteins and then with protamines. Knockout mice for both protamines (Cho et al., 2001) and transition proteins (Yu et al., 2000; Zhao et al., 2001) have been made, and both mouse models produce sperm that exhibit chromatin condensation defects. The phenotype of protamine mutants was more severe than that of transition protein mutants, as haploinsufficiency for either protamine 1 or 2 resulted in complete loss of fertility, whereas mutants for either transition protein 1 or 2 were still fertile. Also, mice mutant for the Sertoli cell expressed FSH receptor produced sperm with inadequate DNA compaction (Krishnamurthy et al., 2000).

Many Category II (defective sperm) mutants have been identified that produce spermatozoa with misshapen heads. Often a spectrum of abnormal head shapes is observed among the spermatozoa from an individual mutant. *Hook1* is

associated with the microtubule manchette (Mendoza-Lujambio et al., 2002), a structure thought to play a critical role in the shaping of the spermatid head. *Hook1-/-* mice have misshapen heads due to improper positioning of the microtubule manchette (Mendoza-Lujambio et al., 2002). Mutation in the transcriptional coactivator *Act* leads to spermatozoa with abnormally shaped heads, as well as defective, folded tails (Kotaja et al., 2004). However, the transcriptional targets of *Act* remain unclear. Whereas most knockout mice that produce defective sperm are mutant for genes normally found in haploid germ cells but not in earlier types of germ cells, *Mgc1* knockout mice present an exception. *Mgc1* is predominantly expressed in pachytene and diplotene spermatocytes, and is a component of the nuclear envelope (Leatherman et al., 2000). *Mgc1-/-* germ cells first exhibit abnormalities in the nuclear envelope at the spermatocyte stage, but continue to develop into spermatozoa that have defects in head shape, chromatin condensation, and the acrosome (Kimura et al., 2003).

Some Category II (defective sperm) mutants produce normal quantities of what appear to be normal spermatozoa. However, these mutants are still infertile, due to the inability of their sperm to bind to eggs. One mutant mouse exhibiting this phenotype is the fertilin beta knockout mouse (Cho et al., 1998). Fertilin is a sperm surface protein that belongs to the ADAM family, and likely mediates binding between the sperm and receptors on the egg. Interestingly, proper heterodimerization of fertilin subunits with each other was shown to require calmegin (Ikawa et al., 2001), a chaperone found in the endoplasmic reticulum, and when calmegin knockout mice were made, they displayed the same phenotype as fertilin knockout mice (Ikawa et al., 1997).

MOUSE MUTANTS: CATEGORY III (GERM CELL LOSS)

The final category of spermatogenic mutants and the most difficult to interpret are Category III (germ cell loss) mutants. These mutants are characterized by the ability to produce spermatozoa when they are young and a progressive loss of germ cells as they age, such that eventually many seminiferous tubules appear greatly depleted of germ cells. Because it is not feasible to monitor the germ cell content of a testis in real time as a mouse ages, one must in part infer this phenotype by examining several individual mice at various ages and comparing them. In some cases Category III (germ cell loss) mutants become infertile as they age, but other mutants in this category manufacture defective sperm and never are fertile.

Likely owing in part to the complexity of the Category III (germ cell loss) phenotype, there are a number of descriptions of knockout mice in the literature which are consistent with this phenotype but which do not provide an adequate level of detail to unambiguously assign them to this category. Often times authors will describe the histology of the mutant testes as containing some empty seminiferous tubules, some degenerating seminiferous tubules, and some normal seminiferous tubules, but do not provide key information such as the number of individual mice examined and the ages of the mice. Other times, the authors simply don't present enough data (e.g., only describing the phenotype of knockout mice at one age). Descriptions of likely Category III (germ cells loss) mutants that are problematic include those for *Sox3* (a transcription factor) (Weiss et al., 2003), *Apaf* (an activator of apoptosis) (Honarpour et al., 2000), *Pf20* (an axonemal protein) (Zhang et al., 2004), and *Cnot7* (a transcriptional cofactor)

(Nakamura et al., 2004). It is certainly worth revisiting these mutants and performing a more thorough characterization of their spermatogenic phenotypes, so that the field has a clearer understanding of whether they truly are Category III (germ cell loss) mutants.

Nonetheless, many Category III (germ cell loss) mutants have been more fully characterized. *Plzf*, a transcriptional repressor, is expressed in the testis exclusively in undifferentiated spermatogonia. Mutation of *Plzf* results in progressive germ cell loss that is likely due to an impairment in stem cell self renewal (Costoya et al., 2004; Buaas et al., 2004). Unlike many Category III (germ cell loss) mutants, increased apoptosis was not observed in *Plzf*/- testes. *Tif2* is a nuclear receptor coactivator expressed in the Sertoli cells (Gehin et al., 2002). *Tif2*/- mice exhibit progressive germ cell loss and produce defective sperm (Gehin et al., 2002). Enigmatically, the extent of germ cell loss varied considerably among individual *Tif2*/- mice. Only 2 out of 14 mutants aged 3 months and 1 out of 4 mutants aged 6 months exhibited substantial germ cell loss, but 2 out of 2 mutants aged 9 months exhibited severe germ cell loss. Variability in the severity of the phenotype was also observed for mutants in the transcription factor *Nmp4* (Nakamoto et al., 2004) and the ubiquitin conjugating enzyme *Hr6b* (Roest et al., 1996), both of which are expressed in multiple testicular cell types. Some *Nmp4* mutants exhibited testicular degeneration at eight weeks of age, while others had apparently normal testes; in *Hr6b* mutants germ cell loss was first observed in 4-5 week old mice, and 10- 20% of adult mice were completely devoid of germ cells. *Bmp8a* is an intercellular signaling factor expressed predominantly in step 6-8 spermatids based on *in situ* hybridization (although up to 3.5 weeks transcripts are also found in spermatogonia and spermatocytes) (Zhao and Hogan, 1996). Until 6 weeks of age testes from

Bmp8a⁻ mice appear normal, but 47% of testes from mutants aged 12 to 30 weeks show varying amounts of degeneration (Zhao et al., 1998). Knockout mice for the microtubule associated protein *E-map-115* (Komada et al., 2000) the cyclin dependent kinase *Cdk4* (Rane et al., 1999), and the estrogen receptor (Eddy et al., 1996) also exhibit a progressive loss of male germ cells.

Currently we have a poor understanding of the underlying molecular defects in Category III (germ cell loss) mutants, beyond the observation that many of these mutants have increased numbers of apoptotic germ cells or problems in spermatogonial proliferation. Perturbations in the complex signaling that occurs between germ cells and somatic cells are frequently blamed for giving rise to this phenotype, but researchers continue to struggle to more fully characterize these mutants. Our failure to understand these knockout models points to one of the major challenges facing the spermatogenesis field. While much work has gone into elucidating roles that individual genes play in spermatogenesis, little progress has been made in defining the molecular pathways and transcriptional networks that regulate the process as a whole. Only when we achieve a more global understanding of spermatogenesis will the Category III (germ cell loss) phenotypes become more comprehensible.

MOUSE MUTANTS: UNIQUE CASES

Of course, in most classification schemes, there exists a small number of cases that do not fit cleanly into any of the established categories. This is true too of the above classification system for spermatogenic mutants; several outliers

exist which cannot be described as Class I (arrest) mutants, Class II (defective sperm) mutants, or Class III (germ cell loss) mutants.

Many of these exceptional cases represent subtle spermatogenic phenotypes that do not greatly affect fertility. For example, mutants for *Nkd* have a reduction in the numbers of late stage spermatids and sperm, but still produce normal sperm and are fertile (Li et al., 2004). The normal function of *Nkd* is to inhibit the Wnt/ß-Catenin signaling pathway (Yan et al., 2001). Mutants for *Ink4d*, an inhibitor of cyclin-dependent kinases, had reduced testis sizes and increased apoptosis of germ cells, but still produced normal sperm and had fertility similar to wildtype mice (Zindy et al., 2000). In contrast, mutants for the cyclin-dependent kinase inhibitor p27^{kip1} had increased testis weights due to an over abundance of type A spermatogonia (Beumer et al., 1999). Additionally, a small number of preleptotene spermatocytes seemed unable to enter meiosis in p27$^{kip1-/-}$ mice, but these knockout mice too were fertile.

Some mouse mutants have been generated which are similar to the Category III (germ cell loss) mutants, but have unique features that disqualify them from being placed in that category. Mutants for the testicular nuclear orphan receptor 4 undergo a progressive loss of germ cells, but when spermatogenesis is observed in these mutants it proceeds at a slower rate, due to a delay at the end of meiotic prophase (Mu et al., 2004). *Man2a2*$^{-/-}$ mice can continue to produce sperm even as they become older, but they have reduced numbers of germ cells of all stages (Akama et al., 2002). *Man2a2* was shown to be important for germ cell adhesion to Sertoli cells, and many germ cells in this mutant become detached into the lumen before spermatogenesis completes. Mice in which the transcription factor *Six5* is knocked out undergo arrest in spermiogenesis, such that sperm is never produced; however, significant germ

cell apoptosis of earlier staged germ cells (i.e., spermatocytes and spermatogonia) is observed (Sarkar et al., 2004).

MOUSE MUTANTS: SPECIAL PROBLEMS

Analyzing spermatogenic defects in knockout mice is an extremely arduous task. It should be noted that the above knockout mouse descriptions are intended to be but brief summaries of the mutant phenotypes and are provided to showcase the variety of spermatogenic mutants that exist. Much painstaking work, such as analysis of testis histology, spermatozoa morphology, gene expression differences, etc., went into providing a characterization for these mutant mice. Furthermore, there are several additional issues which researchers investigating mice with spermatogenic failure must consider: strain background effects, functional redundancy among genes, expression of a gene in multiple testicular cell types, and expression of a gene in multiple tissues.

Many different mouse strains are used today for the production of knockout mice; some common strains include C57BL/6, BALB/c, and 129. Importantly, the phenotype of a knockout mouse can differ from one strain background to another. In some cases a spermatogenic phenotype for a mouse mutant may be apparent on one strain background but not on another. Unfortunately, it is not possible to determine *a priori* which strain background is most suited to the study of a particular knockout mouse. For example, *Bmp4*+/- mice on a mixed (129Sv/Ev x Swiss Black) background did not show any spermatogenic defects, but *Bmp4*+/- mice on a C57BL/6 background displayed a phenotype characteristic of Category III (germ cell loss) mutants (Hu et al.,

2004). Importantly, strain background affects are not limited to spermatogenesis genes, as mouse knockouts of many genes not involved in spermatogenesis also have strain background-dependent phenotypes. In addition, it is often advisable to backcross knockout mice onto a pure strain background, as mixed background knockout mice may display phenotypic variability. For example, the Category III (germ cell loss) mutant *Hr6b* exhibited phenotypic variability among individual mutant mice, but these studies were conducted on a mixed genetic background (Roest et al., 1996). Therefore, it is unclear whether the variability with regard to germ cell loss results from strain background variation among the individual mice.

Some genes that are expressed in the testis are also expressed in other tissues. Mouse knockouts of such genes often display phenotypes in addition to spermatogenic failure. These phenotypes are especially problematic for investigators studying spermatogenesis if they result in decreased viability. For example, about half of *Spag6*-/- mice had reduced weights, and these mice died by 8 weeks of age due to hydrocephalus (a condition marked by fluid accumulation in the brain) (Sapiro et al., 2002). Mice mutant for the transcriptional co-repressor *Tif1ß* die embryonically, so to study the role of *Tif1ß* in spermatogenesis a conditional germ-line specific knockout allele of *Tif1ß* needed to be constructed (Weber et al., 2002). Using this approach it was determined that loss of *Tif1ß* results in a Category III (germ cell loss) phenotype.

Some genes show expression in more than one cell type of the testis. When these genes are knocked out, this can lead to confusion as to whether all the cell types in which the gene is normally expressed actually require function of the gene. For example, Leydig cells, Sertoli cells, haploid germ cells, and peritubular myoid cells (cells that line the basement membrane of seminiferous tubules) have

all been shown to express the androgen receptor (Shan et al., 1997; Zhou et al., 1996). To establish whether androgen receptor is required in Sertoli cells for normal spermatogenesis, a conditional Sertoli cell specific knockout for androgen receptor was generated (Chang et al., 2003; De Gendt et al., 2004). This conditional knockout mouse demonstrated that loss of androgen receptor function in Sertoli cells leads to a Category I (arrest) phenotype, with male germ cells arresting in meiosis. The transcriptional cofactor *Cnot7* is expressed at low levels in germ cells and at high levels in somatic cells of the testis (Nakamura et al., 2004). To establish whether *Cnot7* is required in germ cells, a spermatogonial transplantation experiment was performed: spermatogonia from *Cnot7*$^{-/-}$ mice (these mice appear to exhibit a Category III (germ cell loss) phenotype and produce abnormal spermatozoa) were injected into testes which had been chemically depleted of germ cells but still contained functional somatic cells (Nakamura et al., 2004). Normal spermatogenesis was observed after the *Cnot7*$^{-/-}$ spermatogonia were transplanted and colonized the recipient testes, meaning that *Cnot7* function is not required in germ cells for normal spermatogenesis.

Some genes needed for spermatogenesis have closely conserved homologues in the genome. If homologous genes have redundant or overlapping functions, then knocking out just one of the genes may result in only a mild phenotype or no phenotype at all, due to the activity of the other gene. In these cases it may be necessary to construct a double knockout mouse in order to fully reveal the function of the homologous genes. For example, even though mice with mutations in either transition protein 1 or transition protein 2 were fertile, double knockout mice for both transition proteins were infertile and produced spermatozoa with considerably more severe chromatin condensation defects than either of the single mutants (Zhao et al., 2004; Shirley et al., 2004).

TRANSGENIC MICE

Whereas knockout mice are missing the function of one gene normally found in their genome, transgenic mice have an additional gene inserted, usually in a random location, into their genome. Although not as widely used to study spermatogenesis as knockout mice, transgenic mice have been used to make some important contributions to the field.

For example, it is known that the protamine genes are first transcribed in round spermatids but not translated until several days later in elongating spermatids (Balhorn et al., 1984; Kleene et al., 1984; Yelick et al., 1989). To determine whether the translational repression of protamine has an important functional role, transgenic mice were generated which expressed a protamine 1 gene lacking a 3' UTR region required for translational repression (Lee et al., 1995). These transgenic mice were infertile, and exhibited premature chromatin condensation in spermatids and spermiogenic arrest, thereby demonstrating that translational repression of protamines in essential.

As discussed earlier, *Hsl*-/- mice exhibit an arrest in spermiogenesis. However, *Hsl* is known to be expressed in several testicular cell types, including spermatogonia, spermatocytes, and spermatids (Blaise et al., 1999; Blaise et al., 2001). To assess whether *Hsl* deficiency in spermatids is responsible for the arrest, a transgenic mouse was constructed that expresses *Hsl* specifically in spermatids (Wang et al., 2004). When this transgenic mouse was crossed with the *Hsl* knockout mouse, it was found that *Hsl* expression in spermatids is sufficient to restore fertility to the knockout mouse.

Some human genes do not have a mouse orthologue, so it is difficult to directly study the function of these genes in mice using a conventional knockout approach. However, if there exists in mouse a similar gene (perhaps a paralogue), it may be possible to study the function of the human gene using a transgenic mouse. Humans contain a Y-chromosomal gene called *DAZ*, while mice don't have a *DAZ* orthologue, they contain *Dazl*, a homologous gene. To study the function of human *DAZ*, transgenic mice were made expressing human *DAZ*, and these mice were crossed to *Dazl* knockout mice (Slee et al., 1999). It was found that *Dazl*$^{-/-}$ mice expressing human *DAZ* had greatly increased numbers of spermatogonia and spermatocytes compared to *Dazl*$^{-/-}$ mice, suggesting that human *DAZ* can partially rescue the early spermatogenic arrest of *Dazl*$^{-/-}$ mice.

Cdyl GENE FAMILY

Even though the spermatogenic function of many genes has already been investigated through knockout mouse analysis, there are certainly many additional genes that are important for this process. The literature is rife with accounts of genes that show testis expression, but many of these candidate spermatogenesis genes await functional validation. One particularly interesting candidate gene is *Cdyl*.

Cdyl GENE FAMILY: EXPRESSION

Cdyl is a nine exon gene located on mouse chromosome 13. Northern analysis and cloning demonstrated that two transcripts were produced from the

Cdyl locus (Lahn and Page, 1999). These transcripts appeared to code for identical proteins, but differed in length due to alternate polyadenylation sites in the 3' UTR. The larger 3.6 kb transcript was detected in all tissues tested, including heart, brain, liver lung, spleen and testis (Lahn and Page, 1999). This transcript was postulated to provide a general housekeeping function. The shorter 2.8 kb *Cdyl* transcript was expressed abundantly in testes, but was not detected in any other tissues (Lahn and Page, 1999). This transcript was postulated to play an important role in spermatogenesis. Additional Northern analysis showed that the smaller testis specific transcript was not expressed in the mouse testis until 25-30 days after birth, which suggests that it is expressed only in haploid spermatids (Lahn et al., 2002). The larger *Cdyl* transcript is expressed in the testis from birth and is even present in largely germ cell depleted W^v mutant testis, which suggests that this transcript is found in somatic cells of the testis (Lahn et al., 2002). Immunohistochemistry experiments confirmed that CDYL is expressed in haploid spermatids of the testis; specifically, strong staining was observed in step 9-12 spermatids (Lahn et al., 2002). However, no staining was observed in other testicular cell types, including somatic cells. This result was somewhat puzzling, and draws into question which testicular cell types besides spermatids express CDYL protein.

Cdyl is found in all mammals studied and has also been cloned from Xenopus (Lahn and Page, 1999; Caron et al., 2003), but it is not found in lower eukaryotes such as yeast and flies. Human *CDYL* contains 9 exons and is found on chromosome six. However, human *CDYL* has a distinctly different expression pattern from mouse *Cdyl*. Human *CDYL* produces only one transcript, which is widely expressed in many tissues (Lahn and Page, 1999).

Humans also contain 4 intact copies of a related gene called *CDY*, which is located on the Y chromosome. Human *CDY* has 73% nucleotide identity in the coding sequence to human *CDYL*, but it lacks the introns of *CDYL*; this observation strongly suggests that *CDY* arose from a retroposition of *CDYL* (Lahn and Page, 1999). (Some *CDY* transcripts do contain one intron in their 3' UTR, which apparently arose subsequent to the retroposition.) In general, the retroposition of genes in mammals occurs when reverse transcriptase and other enzymatic machinery of mobile genetic elements such as LINES acts on a processed RNA transcript, causing it to be reverse transcribed and reinserted into the genome (Esnault et al., 2000). Because the four copies of *CDY* all have extremely high similarity to each other (about 99% nucleotide identity in the coding sequence), it is likely that they arose from genomic amplifications that followed the original retroposition. *CDY* is not found in mice, and southern blotting demonstrated that *CDY* is only present in primates (Lahn and Page, 1999). However, sequence analysis suggests that *CDY* actually retroposed over 100 million years ago, and was subsequently lost in many mammalian lineages (Dorus et al., 2003).

Interest was drawn to the *CDY* genes when it was found that some copies of *CDY* are removed in large Y-chromosomal deletions known to cause male infertility (e.g., the AZFc deletion) (Kuroda-Kawaguchi et al., 2001). However, several other genes are removed in addition to *CDY* when these deletions occur, so it is not possible to pinpoint which gene(s) are responsible for the resulting phenotype. Importantly, *CDY* expression appears to be restricted to the testis (Lahn and Page, 1999). Additionally, CDY is expressed in the same testicular cell type as is mouse CDYL, elongating spermatids (Lahn and Page, 2002). The expression patterns of the *Cdyl* family genes led Lahn and Page to hypothesize

that the function of Mouse *Cdyl* was split into two genes in the human: *CDYL*, which performs housekeeping functions, and *CDY*, which has an important role in spermatogenesis (Lahn and Page, 1999).

Mice and humans also have an autosomal paralogue to *Cdyl* called *Cdyl2*, which is found on chromosome 8 in mice and chromosome 16 in humans (Dorus et al., 2003). The genomic duplication event which created *Cdyl2* likely predates the retroposition of *CDY* onto the Y chromosome. Mouse *Cdyl2* has an expression pattern similar to mouse *Cdyl*, in that it produces a widely expressed transcript and a testis specific transcript, and human *CDYL2* has an expression pattern to similar to human *CDYL*, in that it produces only one widely expressed transcript (Dorus et al., 2003). The evolutionary relationships among the *Cdyl* family genes are illustrated in Figure 3 (following page).

Figure 3 Evolution of *Cdyl* family genes. Based on sequence analysis, *Cdyl* and *Cdyl2* diverged prior to the retroposition of *CDY*.

Human *CDY*

Human *CDYL*

Mouse *Cdyl*

Human *CDYL2*

Mouse *Cdyl2*

Adapted from Dorus et al., 2003
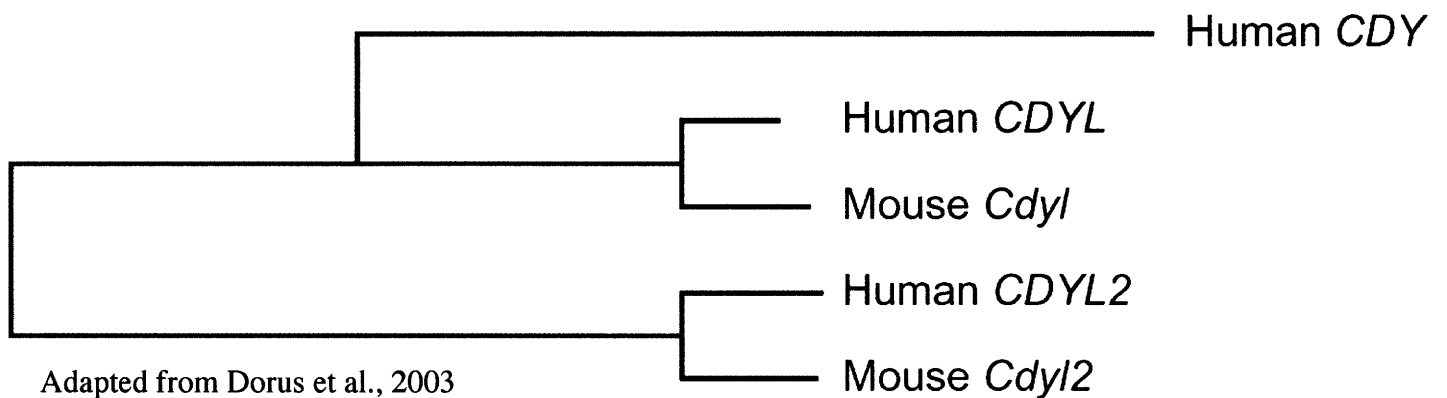
Cdyl GENE FAMILY: PROTEIN FUNCTION

All CDYL family proteins have two conserved functional domains: a chromodomain (the name *CDY* comes from **C**hromo**D**omain on the **Y** chromosome) and an enoyl CoA hydratase (ECH) domain.

Chromodomains function most commonly as chromatin interaction domains. Recently, a sequence-based analysis was performed on all known chromodomain-containing proteins and 11 classes of chromodomains were established (Brehm et al., 2004). In this analysis the chromodomain of CDYL was placed in the same class as that of HP1 (heterochromatin protein-1) and Polycomb, which is involved in silencing. The chromodomain of HP1 has been shown to specifically bind to methylated lysine 9 of histone H3 (Bannister et al., 2001; Lachner et al., 2001), and the Polycomb chromodomain recognizes methylated lysine 27 of histone H3 (Czermin et al., 2002). Consistent with a role in chromatin binding, HA-tagged CDYL was shown to localize to the nucleus when expressed in HeLa cells (Caron et al., 2003), and immunohistochemical staining for CDYL in spermatids appears nuclear (Lahn et al., 2002).

Proteins containing an ECH domain belong to the functionally divergent crotonase superfamily (reviewed in Gerlt and Babbitt, 1998). Over 100 proteins contain an ECH domain, and most of these proteins are still uncharacterized. However, there is great diversity in the catalytic function of the ECH domain in those proteins that have been studied. ECH domain containing proteins have been identified which function as isomerases (3,2-*trans*-enoyl CoA isomerase), dehalogenases (4-chlorobenzoyl CoA dehalogenase), hydratases (Enoyl CoA hydratase), or decarboxylases (Methylmalonyl CoA decarboxylase). The crystal

structures of several different ECH-containing proteins have been characterized, and despite their varied biochemical functions, ECH-containing proteins appear to share a common catalytic mechanism: the use of an oxyanion hole to stabilize Coenzyme A containing intermediates (reviewed in Gerlt and Babbitt, 1998). However, as the structure of CDYL has not been solved, it may function through a completely different biochemical mechanism.

No other proteins in the medline database besides CDY, CDYL, and CDYL2 contain both a chromodomain and an ECH domain. As CDYL family proteins represent a novel combination of domains, and because it is unclear what reaction is catalyzed by the ECH domain in CDYL family proteins, the cellular function of CDYL is not immediately evident. However, additional biochemical and cell biological work has led to the formation of two independent and somewhat contradictory models for the cellular function of CDYL family proteins. One model is that they function as histone acetyltransferases (HATs), and the other model is that they function as transcriptional co-repressors.

In-vitro biochemical experiments largely serve as the basis for the HAT model. In these experiments recombinant CDY, mouse CDYL, or human CDYL proteins were incubated with free histones (histones H2A, H2B, H3, and H4) and [³H]acetyl-CoA (Lahn et al., 2002). The histones were then run on a gel and checked for incorporation of the [³H]acetyl label. It was found that CDY, mouse CDYL, and Human CDYL could each acetylate histones and did so with the same specificity. Histone H4 was highly acetylated by the CDYL family proteins, and histone H2A was weakly acetylated.

Importantly, in step 9-12 spermatids, where CDYL is expressed, histone H4 is known to become highly acetylated (based on experiments performed with antisera specific to highly acetylated forms of histone H4) (Hazzouri et al., 2000;

Meistrich et al., 1992). The hyperacetylation of histone H4 in spermatids is thought to facilitate its removal from chromatin, in preparation for the repackaging of chromatin with protamines. The observation that CDYL expression is coincident with histone H4 hyperacetylation in spermatids, along with the evidence that CDYL can function as a HAT *in vitro*, led Lahn et al. to speculate that CDYL may participate in the hyperacetylation of histone H4 during spermiogenesis

The model of *Cdyl* family genes as transcriptional co-repressors is predicated upon two lines of evidence. First, CDYL was found to be a member of the CtBP repressor complex. CtBP is a transcriptional co-repressor known to associate with over a dozen transcription factors, including BKLF, FOG1, SOX6, and BRCA-1 (reviewed in Chinnadurai, 2002). CtBP has been shown to directly repress expression of the *E-cadherin* gene in U2OS cells (Shi et al., 2003), but the role of CtBP in the testis is unknown. Recently the CtBP complex was biochemically purified from HeLa cells using antisera against CtBP, and the proteins in the complex were subjected to mass spectroscopy (Shi et al., 2003). CDYL was found in the complex, along with other proteins normally associated with repression, such as histone deacetylases (HDACs) and histone methyltransferases. Another line of evidence arguing for the model of CDYL as a transcriptional co-repressor is the finding that Gal4DB-CDYL (a fusion protein in which the Gal4 DNA binding domain is attached to CDYL) can repress expression of a luciferase reporter gene that is downstream of Gal4 binding sites (Caron et al., 2003). In this same study it was also shown that CDYL can bind directly to HDAC1 and HDAC2.

In most instances proteins that function as HATs act as activators of transcription, not repressors; therefore, the specific biochemical function of CDYL

remains elusive. However, there are a couple observations that may help reconcile these two models for CDYL function. First, even though they are rare, some HATs which function as transcriptional co-repressors have been described; for example, the histone acetyltransferase Tip60 has been shown to mediate repression when interacting with the transcriptional repressor TEL (Nordentoft and Jorgensen, 2003). Interestingly, Tip60 has a chromodomain, just as CDYL does. Second, it may be possible that CDYL has dual functions, and the presence or absence of HDACs serves to switch between these functions. It was demonstrated that CDYL loses the ability to bind Coenzyme A when bound to HDACs, so CDYL could potentially function as a HAT in the absence of HDACs, but as a transcriptional co-repressor when HDACs are present (Caron et al., 2003).

Cdyl GENE FAMILY: EXPERIMENTAL OVERVIEW

This work seeks primarily to directly demonstrate whether *Cdyl* family genes function in spermatogenesis. The first approach we are taking is analysis of a *Cdyl* knockout mouse. By analyzing spermatogenesis in this mouse, we seek (1) to establish whether mouse *Cdyl* is required for normal spermatogenesis, and if it is, (2) to further characterize the spermatogenic defect(s) that arise due to loss of *Cdyl*. In the second set of experiments, we seek to directly demonstrate a role for human *CDY* in spermatogenesis by attempting to rescue any spermatogenic defects observed in the *Cdyl* knockout mouse with a transgenic mouse expressing human *CDY*. In addition to these experiments, we provide further characterization of the mouse *Cdyl* transcripts.

II

Characterization of Mouse *Cdyl* Transcripts and KO Phenotype

Perhaps the most compelling evidence that mouse *Cdyl* functions in spermatogenesis has come from the analysis of expression data. When *Cdyl* was originally cloned from a mouse testis cDNA library, two distinct transcripts were reported (Lahn and Page, 1999). This finding was corroborated by Northern analysis, which revealed that the mouse testis contains a long 3.6 kb transcript of *Cdyl* that is also found in many other tissues, as well as a shorter 2.8 kb transcript found only in the testis (Lahn and Page, 1999). This shorter transcript is thought to have an important role in spermatogenesis. Additional experiments showed that the longer transcript is expressed in somatic cells of the testis, but that the shorter transcript is germ cell specific (Lahn et al., 2002). One of these transcripts (presumably the longer, widely expressed transcript) has a longer 3' UTR due to an alternate polyadenylation site, but they were otherwise thought to be identical.

CHARACTERIZATION OF *Cdyl* TRANSCRIPTS

Using a probe specific to this extended 3' UTR, we verified with Northern analysis that the shorter testis specific variant of *Cdyl* doesn't contain the extended 3' UTR (Fig 1C, see left lane). Using 3' RACE we also found that the 3' UTR of the longer transcript is 440 bases longer than previously reported. We also found by performing Northern analysis using a probe for exons 1-3 of *Cdyl* that the shorter testis specific variant of *Cdyl* but not the longer variant contains these exons (Fig 1A, see left lane). This result suggested that the 3.6 kb transcript does not have the same 5' end as the 2.8 kb transcript. We have now identified a full length clone in GenBank (BC062123) that contains an

Figure 1 Northern analysis of *Cdyl* transcripts using RNA samples from wildtype or *Cdyl* mutant testes (see Figure 2 for details on *Cdyl* mutant allele). (A) RNA extracts from wildtype or *Cdyl*+/- testes were probed with a DNA fragment corresponding to the 5' end of the previously reported *Cdyl* transcript. The larger 3.6 kb transcript was not detected. (B) RNA extracts from wildtype or *Cdyl*+/- testes were probed with a DNA fragment corresponding to exons 5-7 of *Cdyl*. In the knockout testis both transcripts are present but reduced in size. (C) RNA extracts from wildtype or *Cdyl*+/- testis were probed with a DNA fragment corresponding to the 3' UTR of *Cdyl*. Only the longer 3.6 kb fragment was detected. (D) Structure of the two *Cdyl* transcripts. Shaded regions correspond to open reading frames.

Figure 1
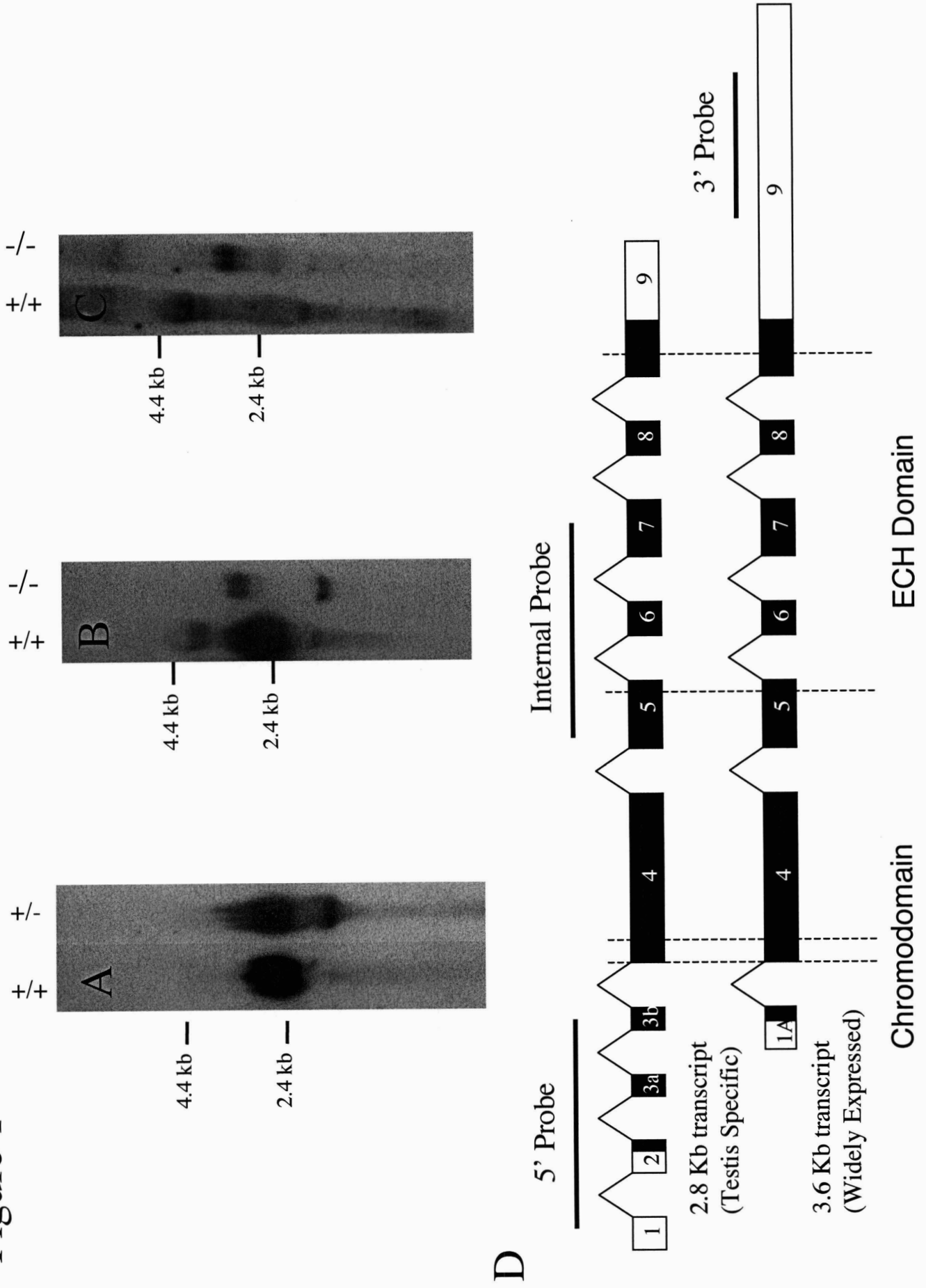
alternatively spliced 5' exon of *Cdyl* (Exon 1A). We expect that the correct 5' end of the longer transcript was not identified during the initial cloning of *Cdyl* because overlapping partial cDNA sequences were used to construct the full length *Cdyl* sequence. Exon 1A is located in a large intron of the previously reported *Cdyl* gene (Fig 2A). Sequencing of RT-PCR products verified that exon 1A is spliced to exon 4 in testis and brain RNA extracts. The longer *Cdyl* transcript containing exon 1A encodes a protein of 545 AA, whereas the previously identified testis specific transcript of *Cdyl* encodes a 594 AA protein (Fig 1D). The newly discovered coding sequence in exon 1A is very short (MASEELYE), and does not have any homology to the previously identified CDYL protein or to any known domains.

One final error was found in the previously reported structure of *Cdyl*: Blasting exon three of the testis specific *Cdyl* transcript against the mouse genomic sequence revealed that it is actually two individual exons, which we denote as exon 3a and exon 3b. This finding was confirmed by the observation that a PCR assay whose forward primer was in exon 3a and whose reverse primer was in exon 3b gave a product with cDNA but not with genomic DNA (data not shown). No product was observed for genomic DNA because the intron because exon 3a and 3b is large (Fig 2A).

CHARACTERIZATION OF *Cdyl* KNOCKOUT MICE

To directly address whether *Cdyl* is needed for spermatogenesis, we generated a mutant allele of mouse *Cdyl*. A targeting construct containing a Neo cassette was used to remove exon 4 of *Cdyl* in 129 ES cells (Fig 2A). Correctly

Figure 2 Generation of *Cdyl* knockout mice. (A) A construct containing a Neo cassette was used to target the *Cdyl* locus and remove exon 4, which contains a chromodomain (X=XbaI). (B) Southern blot of XbaI digested genomic DNA from WT and targeted ES cells. Probe used is shown in (A). Wt band is about 8 kb, targeted band is about 7kb. (C) PCR genotyping assay using genomic DNA. In *Cdyl*-/- mice the Neo cassette but not exon 4 of *Cdyl* is detected. (D) Western analysis using anti-CDYL #1. Proteins corresponding to the expected sizes of 65 kd (from the 2.8 kb transcript) and 60 kd (from the 3.6 kb transcript) are present in wildtype but not mutant testis. The amount of CAMKIV, which is found in both spermatogonia and spermatids, and the amount of S-AKAP84, a spermatid-specific protein, were similar between wildtype and mutant testis.

Figure 2
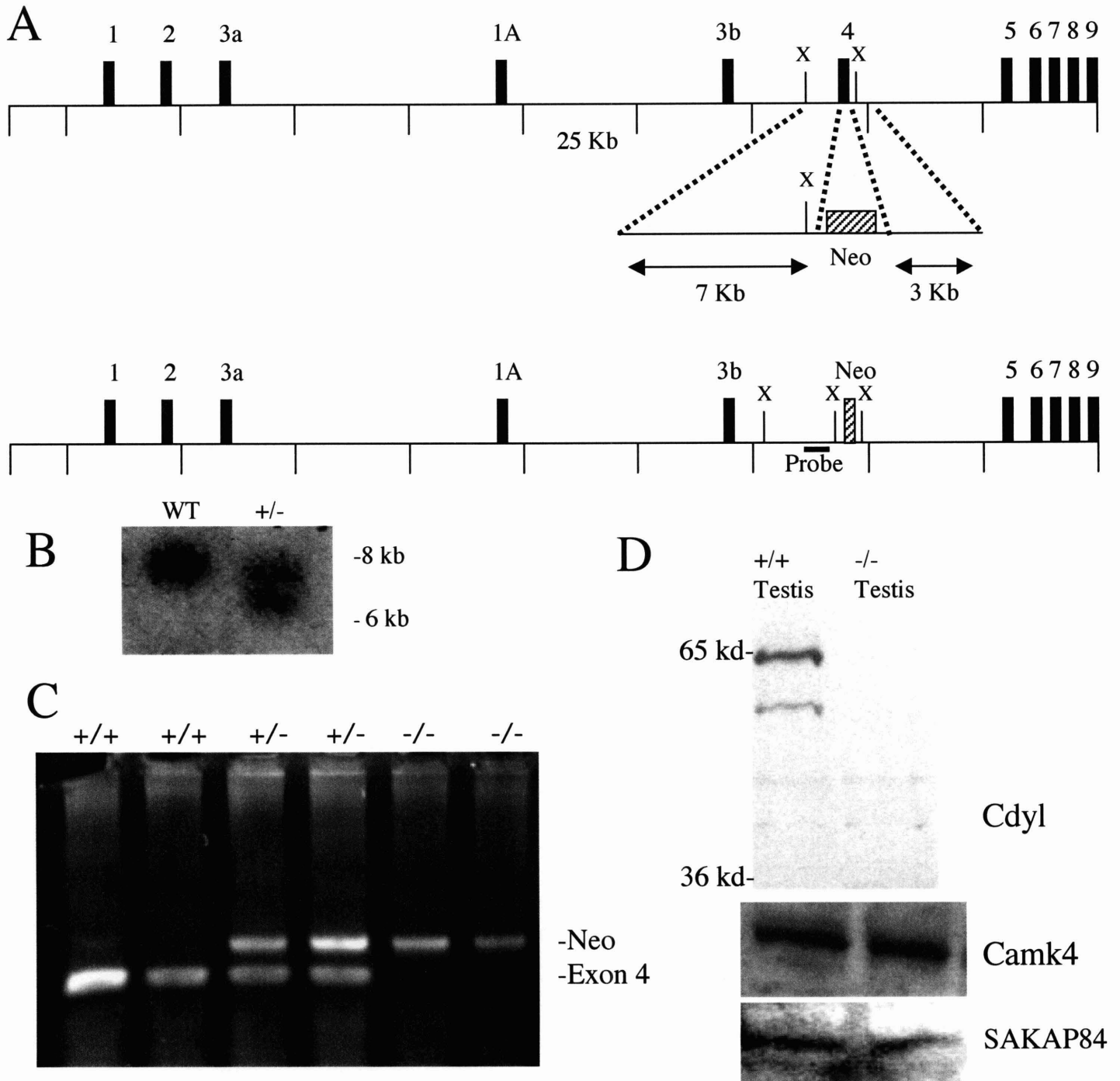
targeted ES cells were identified by Southern blotting (Fig 2B). Two individually targeted ES cell clones of the same mutant allele were used for injection into blastocysts. Chimeras that gave germline transmission were crossed with BALB/c mice, and each of the two individually targeted *Cdyl* alleles was backcrossed for 10 generations onto the BALB/c background. (As expected, the two backcrossed lines displayed the same phenotype; see below for description.) Additionally, chimeras produced from one of the ES cell clones were used to backcross the *Cdyl* mutation for 10 generations onto the C57BL/6 background

A PCR assay on genomic DNA was used to confirm the absence of exon 4 in the mutant mice (Fig 2C). However, Northern analysis demonstrated that truncated *Cdyl* transcripts are still present in the mutant mice (Fig 1B). Sequence analysis of an RT-PCR product showed that one of these truncated transcripts was produced by the splicing of exon 3b directly to exon 5. We expect that the other truncated transcript was produced by the splicing of exon 1A to exon 5, but were unable to confirm this using RT-PCR. In both of these cases, the removal of exon 4 results in severe disruption of the *Cdyl* ORF. In accordance, Western blotting using an antiserum raised against a C-terminal peptide of CDYL (anti-CDYL #1) demonstrated that both CDYL protein isoforms are absent in the knockout testes (Fig 2D). We therefore conclude that a functional null allele of *Cdyl* has been generated.

When litters from *Cdyl*+/- x *Cdyl*+/- matings were genotyped at 2 weeks of age, only 9% of the progeny were *Cdyl*-/-, which suggests that *Cdyl*-/- mice have partial lethality (Table 1). *Cdyl*-/- mice alive at 2 weeks were still in good health when terminated at 1 year of age, though. Interestingly, the penetrance of the lethality phenotype varied when examined on strain backgrounds other than BALB/c. On the C57BL/6 background, approximately the same percentage of

Table 1 Ratios of progeny obtained from heterozygous intercrosses at 2 weeks of age (first three rows) or at E 18.5 (fourth row).

Table 1

Background	Age	Total # pups	% +/+	% +/-	% -/-
129	2 Weeks	149	39%	58%	3%
BALB/c	2 Weeks	202	31%	58%	10%
C57BL/6	2 Weeks	291	32%	59%	9%
129	E 18.5	36	28%	53%	19%

Cdyl-/- mice were still alive at 2 weeks of age as on the BALB/c background, but
almost all of the C57BL/6 knockout mice died by 5 months of age (data not
shown). On the 129 background, only 3% of the offspring genotyped at 2 weeks
were *Cdyl*-/-. When E 18.5 embryos were genotyped, though, 19% of the offspring
were *Cdyl*-/-, which suggests that most of the *Cdyl*-/- mice die at or shortly after
birth (Table 1). Because of the higher inviability of *Cdyl*-/- mice on the C57BL/6
and 129 backgrounds, all further phenotypic analysis was performed only on the
BALB/c background.

The BALB/c *Cdyl*-/- mice alive at two weeks of age were generally healthy,
although they weighed on average two grams less than their littermates (Fig 3A).
However, this discrepancy in weight disappeared by nine weeks (Fig 3B).
Because we are particularly interested in the role of *Cdyl* in spermatogenesis, we
examined the testis weights of *Cdyl*-/- mice. The testis weights of *Cdyl*-/- mice were
until about four weeks the same as their heterozygous and wildtype littermates.
However, starting at 4-5 weeks of age, the testis weights of *Cdyl*-/- mice were
decreased substantially (by up to 50%) compared to that of littermate controls,
which suggested that spermatogenesis in *Cdyl*-/- mice is impaired (Fig 3C).

Examination of testis histology from *Cdyl*-/- mice revealed that their testes
appear normal at 2-3 weeks of age (N=4 mice), but starting at 3-4 weeks, which is
when haploid spermatids first appear in the testis, a decrease in germ cell
number was observed in *Cdyl*-/- testes (Fig 4). In those testes displaying germ cell
loss, the normal complements of spermatogonia, spermatocytes, and especially
spermatids were reduced. Surprisingly, the extent of germ cell loss in *Cdyl*-/- mice
was highly variable in two respects: two individual mice of the same age often
had different amounts of germ cell loss, and two individual testes in the same
mouse could vary greatly in the number of germ cells still present. Of 8 *Cdyl*-/-

Figure 3 Mouse body weights (A,B) and testis weights (C). Male knockout

mice at 3 weeks (A) weigh slightly less than their same sex littermates, but

at 9 weeks (B) weigh about the same. Each column (1-9) represents an

individual litter, and each data point represents the body weight of an

individual mouse. Horizontal lines represent average weights for knockout

mice (red) and controls (blue). (C) Testis weights. Each point shown is

the average of the two testes in an individual mouse.

Figure 3
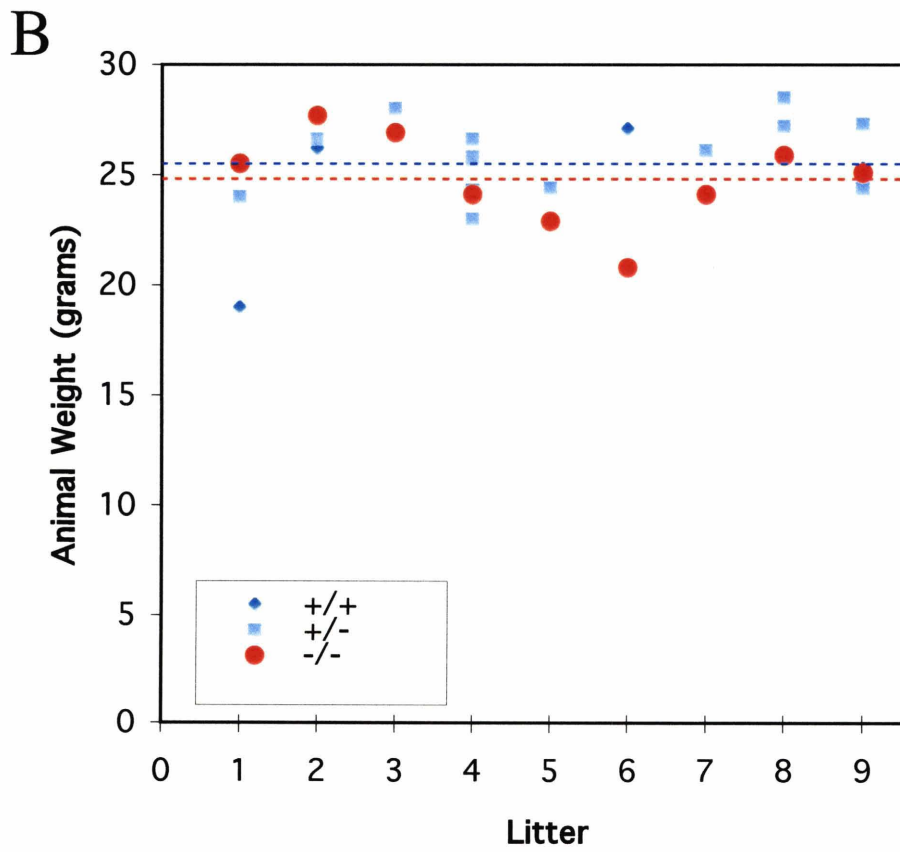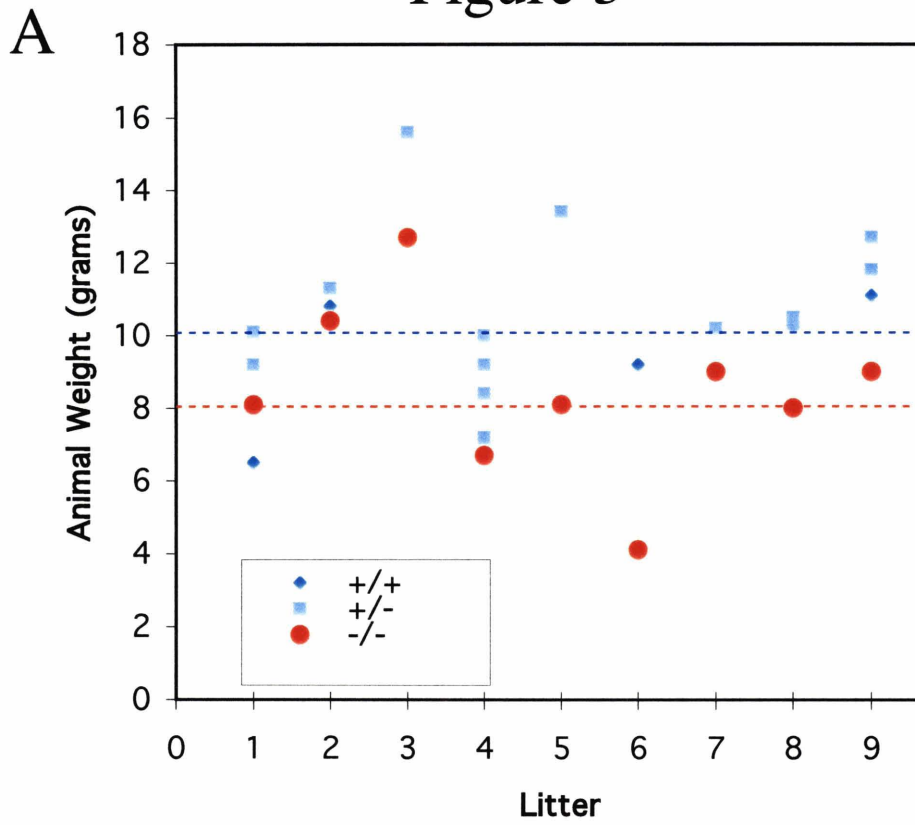
Figure 3

C
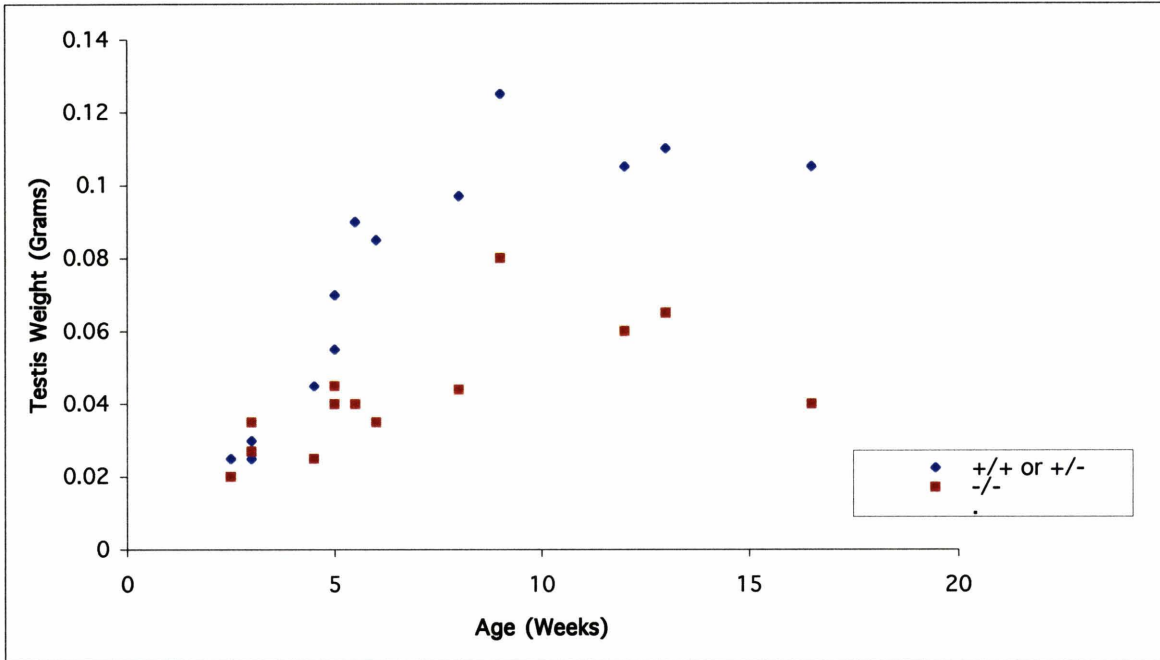
Figure 4 Histology from testes of 19 day old (A,B) and 25 day old (C,D)

mice. At 19 days no germ cell loss is observed in $Cdyl^{-/-}$ testis (A,B), but by

25 days some $Cdyl^{-/-}$ testes exhibit germ cell loss (C,D).

Figure 4
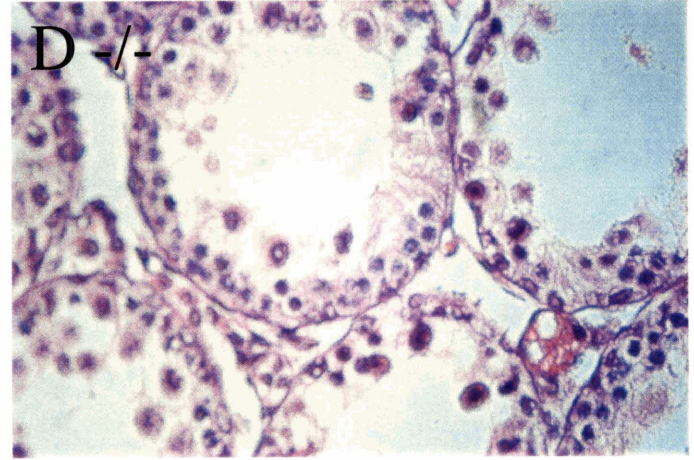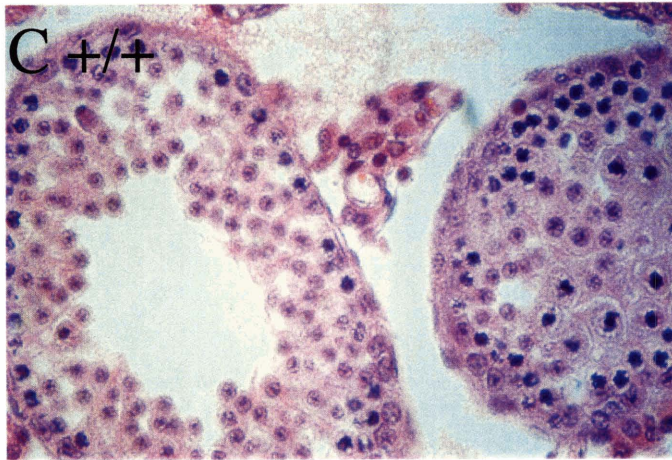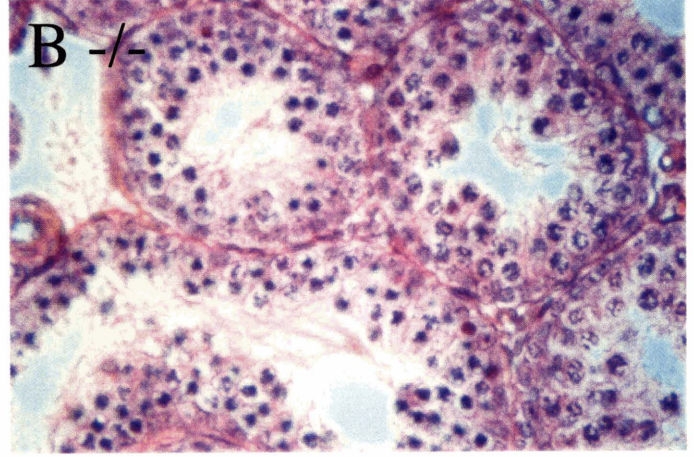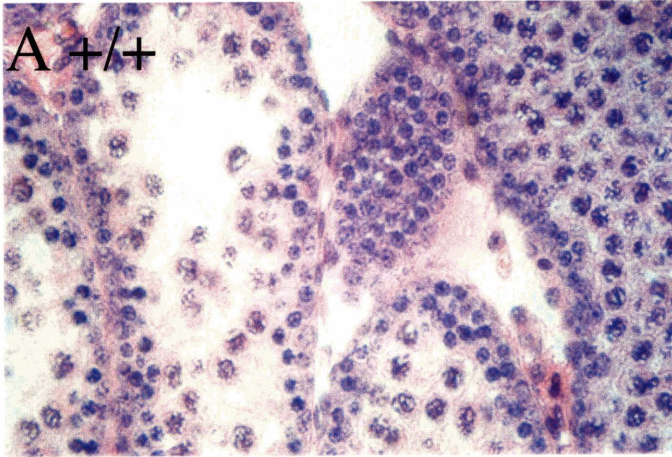
mice aged 5-6 weeks that were examined, 3 had overtly normal spermatogenesis in both testes, 4 had normal spermatogenesis in one testis but greatly reduced germ cell numbers in the other testis, and 1 had significant germ cell loss in both testes.

This variability was also observed in the adult Cdyl-/- mice aged 1.5-5 months that were examined, almost all of which exhibited differing degrees of germ cell loss in one or both testes (Fig 5A-D). In these adult knockout mice, a comparable amount of germ cell loss was observed from one seminiferous tubule cross section to another in 75% of testes examined, but in 25% of the cases there was considerable heterogeneity in germ cell loss among adjacent tubules (Fig 6). Several features commonly associated with germ cell loss, including reduction of seminiferous tubule diameter, disorganization of germ cells inside of the tubules, and the presence of large multinucleated cells and vacuoles within the tubules, were observed in the testes of Cdyl-/- mice. Finally, in all 7 Cdyl-/- mice examined that were 5-10 months old, both testes appeared uniformly highly degenerate, with very few germ cells (mostly spermatocytes and spermatogonia) remaining (Fig 5E,F). However, testicular somatic cell lineages, such as Sertoli cells and Leydig cells, were still present in highly degenerate mutant testes. Neither Cdyl+/+ nor Cdyl+/- mice 5-10 months of age appeared to have any spermatogenic defects.

To assess whether testes from Cdyl-/- mice had increased numbers of apoptotic cells, immunohistochemical staining with an antibody for cleaved Caspase-3, a marker of apoptosis, was performed. The testes from Cdyl-/- mice that still resembled wildtype testes histologically did not have increased numbers of apoptotic cells, but the testes from Cdyl-/- mice that already appeared to have lost significant numbers of germ cells had a 3 fold increase over wildtype testes in the number of apoptotic cells (Fig 7).

Figure 5 Histology from adult testes. (A-D) Comparison of germ cell loss

between the left and right testes from 2 individual knockout mice both 13

weeks old. The right testis of mouse #1 appears similar to a wildtype

testis, whereas the left testis of mouse #1 has undergone a moderate

amount of germ cell loss, as judged by the large empty lumens in the

tubules. By contrast, the right testis of mouse #2 appears highly depleted

of germ cells and contains no late stage spermatids or sperm; the left testis

of mouse #2 appears moderately depleted of germ cells, similar to the left

testis of mouse #1. (E,F) Testes from 7 month old mice. Tubules in

knockout mice are consistently nearly empty at this age.

Figure 5
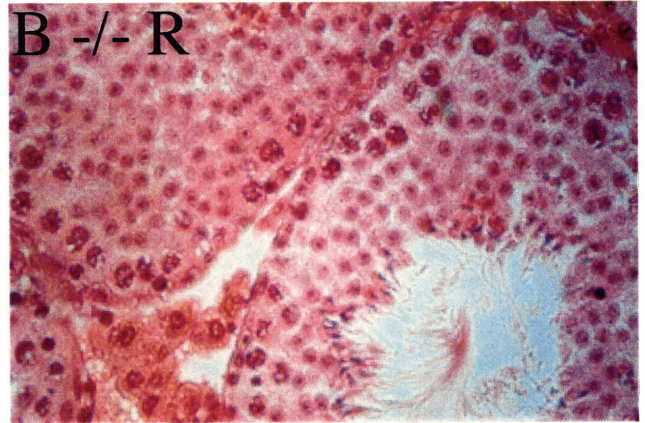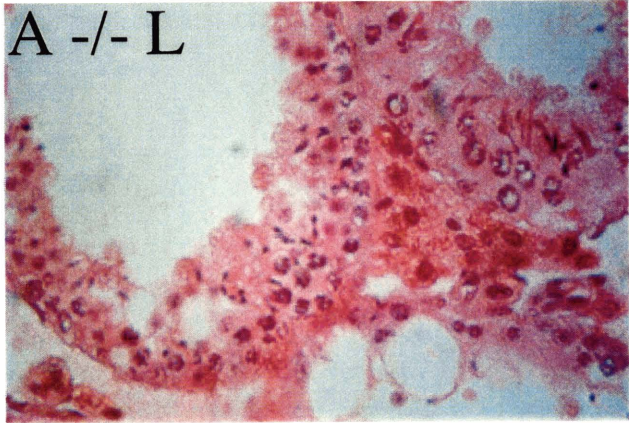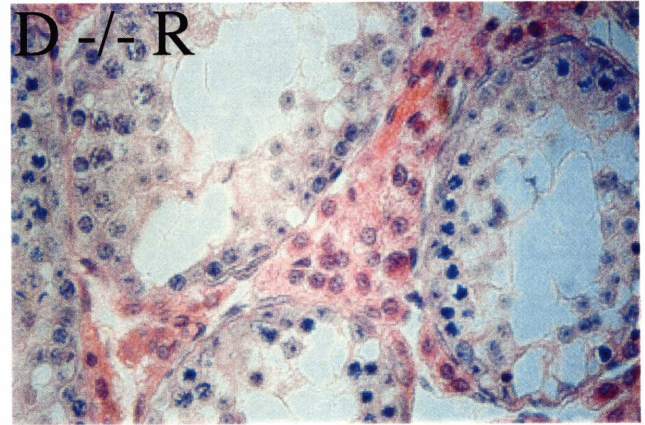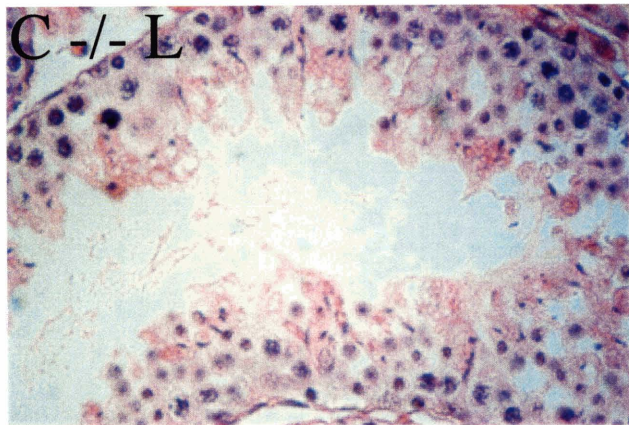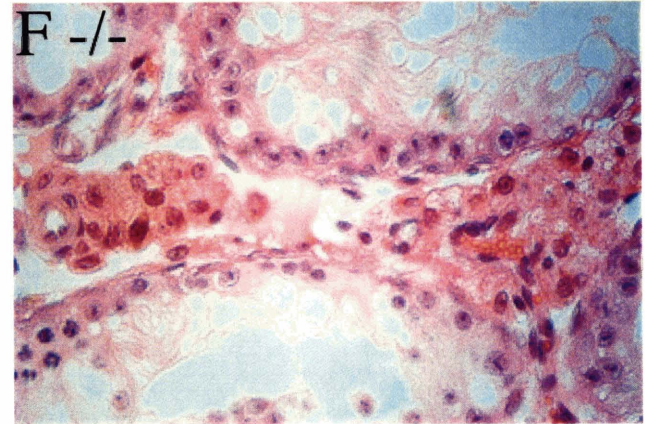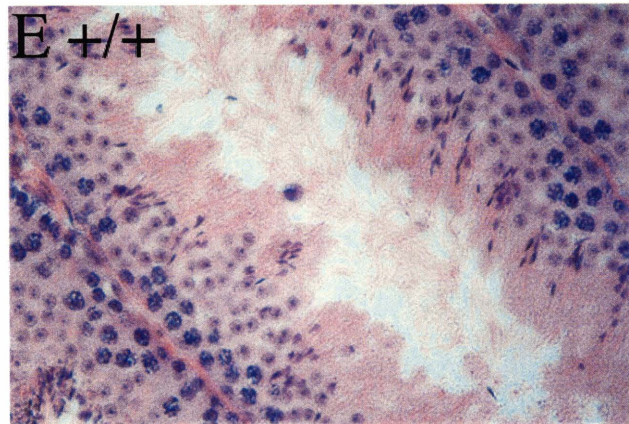
Figure 6 Knockout mouse testes displaying moderate germ cell loss (B) and substantial germ cell loss (C) are compared to a wildtype testis (A). Occasionally mutant testes were seen in which adjacent tubules had dramatically different numbers of germ cells (D).

Figure 6
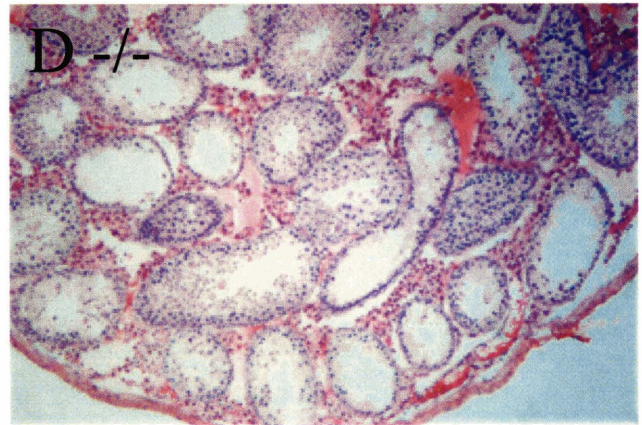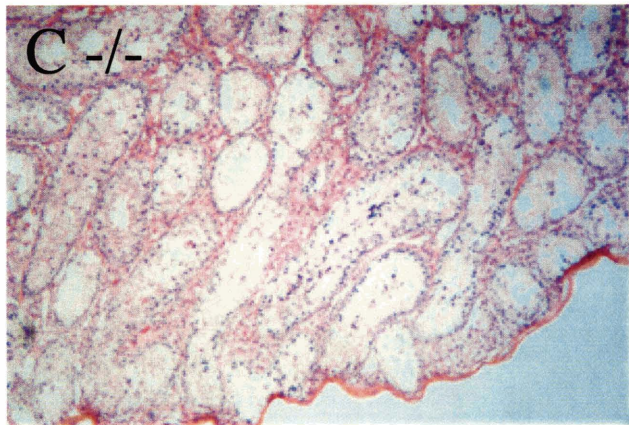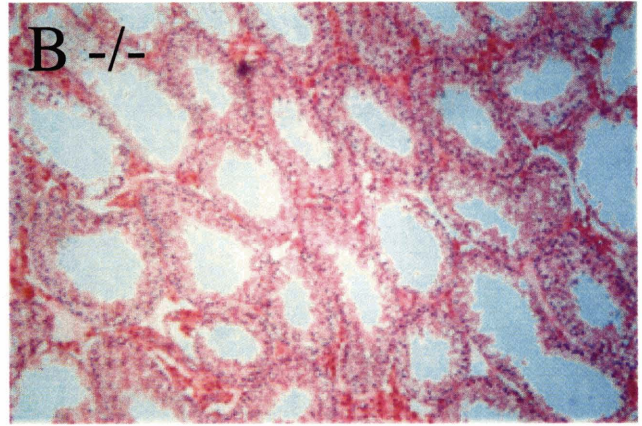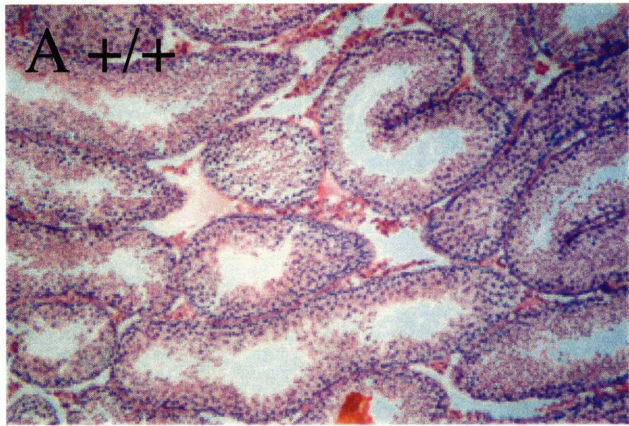
Figure 7 Detection of apoptotic cells with an antibody for cleaved Caspase-

3. Degenerating tubules from knockout mice (B,C) contain increased

numbers of apoptotic cells when compared with tubules from wildtype

mice (A). Based on the size and position of the dying cells, spermatogonia,

spermatocytes and round spermatids all appeared affected in mutant

tubules.

Figure 7
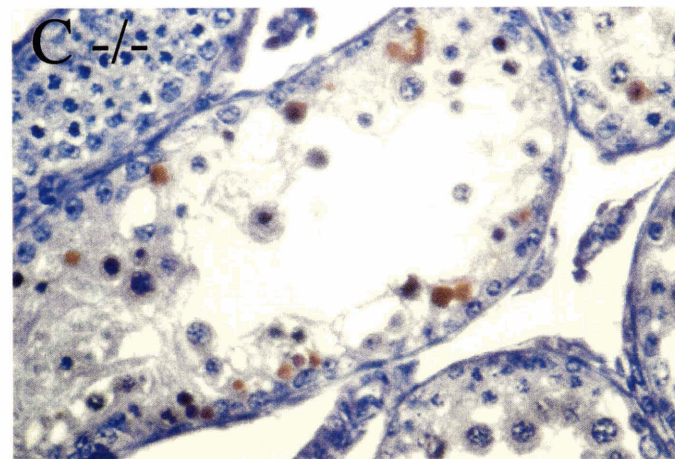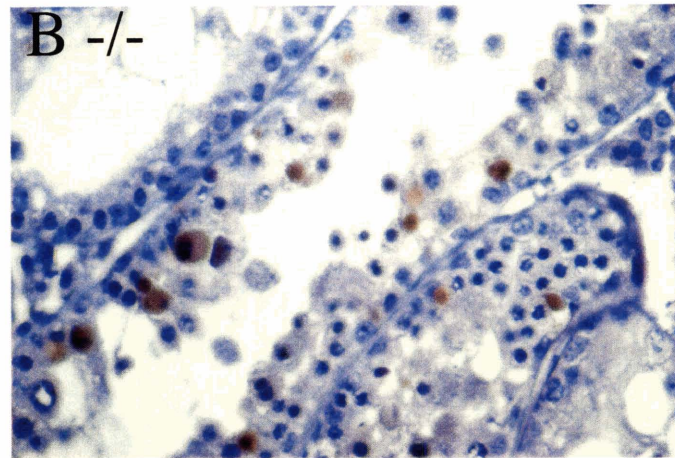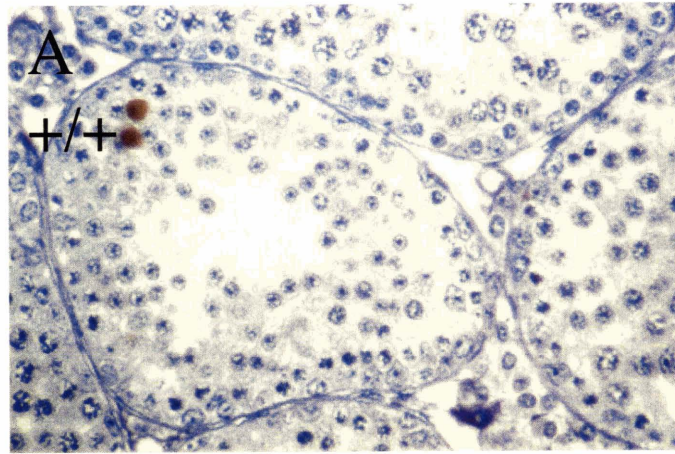
Even though *Cdyl⁻/⁻* males formed plugs when mated with wildtype females,

only 4 of 22 *Cdyl⁻/⁻* males were able to sire progeny, and these males produced

only 1 small litter each. By contrast, *Cdyl⁺/⁻* males and *Cdyl⁻/⁻* females were fertile.

The germ cell loss in *Cdyl⁻/⁻* males certainly played a role in their reduced fertility,

but we expected more *Cdyl⁻/⁻* males to be able to sire offspring, because most *Cdyl⁻/⁻*

males appeared from histological analysis to produce spermatozoa (in at least one

testis) when they were young. We therefore examined the epididymal sperm of

Cdyl⁻/⁻ males between 6-8 weeks of age. In *Cdyl⁻/⁻* males from which epididymal

sperm was recovered, 70%-90% of the sperm exhibited a variety of morphological

head defects, including narrow, rounded, and otherwise misshapen heads (Fig 8).

These sperm were motile when resuspended in PBS, but further study using

computer assisted analysis of sperm motility is needed to establish whether the

knockout sperm have minor reductions in motility. The abnormal morphology of

their spermatozoa likely contributes to the reduced fertility of *Cdyl⁻/⁻* males.

To investigate the hypothesis that CDYL participates in the

hyperacetylation of histone H4 in spermatids, we compared the acetylation status

of histone H4 in spermatids of wildtype mice to that of spermatids in knockout

mice. Immunohistochemical staining using an antibody against hyperacetylated

histone H4 resulted in a similar intensity of staining between wildtype and

mutant spermatids (Fig 9). As a control, immunohistochemistry using a CDYL

antiserum (anti-CDYL #2) demonstrated that CDYL is expressed in the elongating

spermatids of wildtype mice, but not in the spermatids of *Cdyl* knockout mice

(Fig 9). Additionally, antibodies against four different mono-acetylated forms of

histone H4 (H4 K5, H4 K8, H4 K12, H4 K16) stained both wildtype and

Figure 8 Observation of epididymal sperm. (A) A normal wildtype sperm. (B-F) A wide range of abnormalities, including rounded or narrow heads, was observed among the knockout sperm.
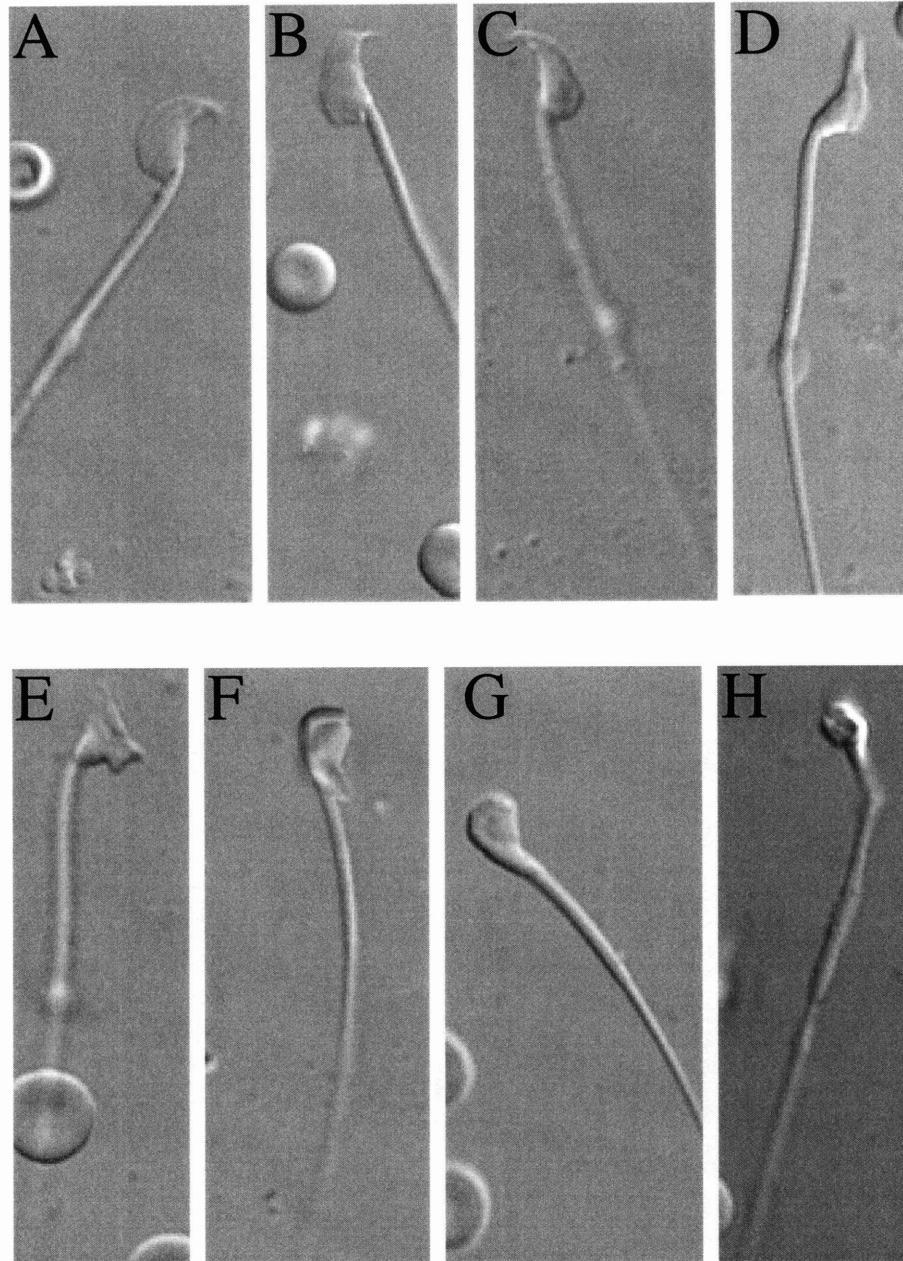
Figure 8

Figure 9 Immunohistochemical staining of testis sections with an

antibody against hyperacetylated histone H4 (A,B) or anti-CDYL #2 (C,D).

No differences in staining of spermatids was seen between wildtype and

mutant sections for hyperacetylated histone H4. However, CDYL staining

was observed in wildtype but not mutant sections. Presence of

hyperacetylated histone H4 or CDYL is revealed by a brown stain. The

spermatids that stain brown are the cell types near the middle of the

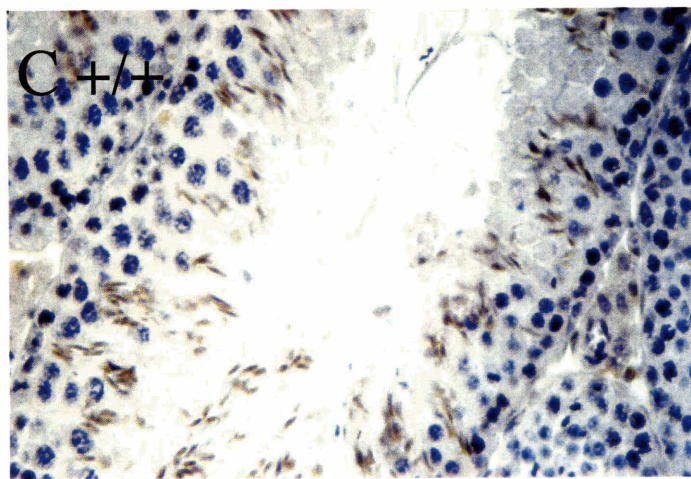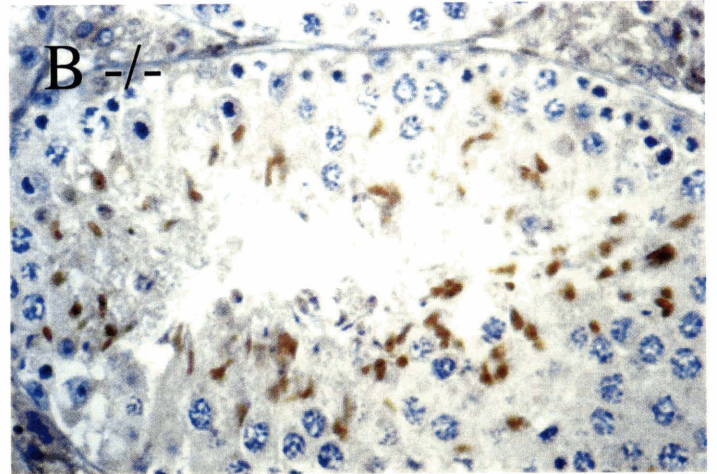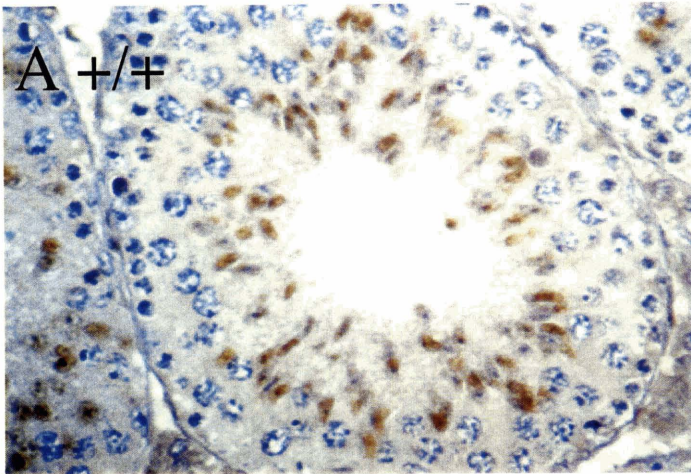tubules, closest to the lumen.

Figure 9
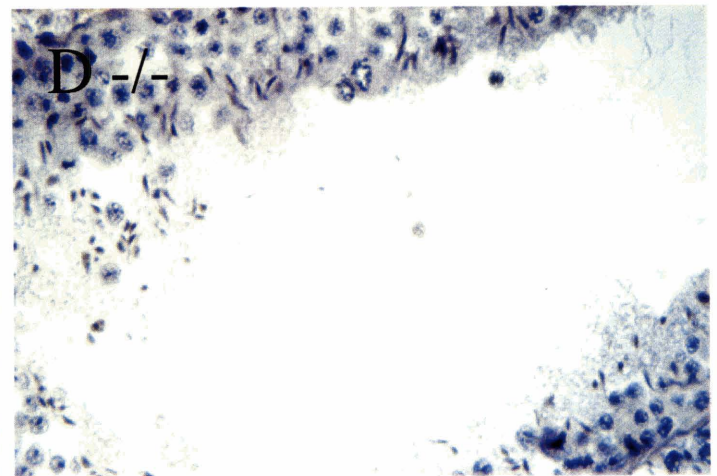
Figure 10 Immunohistochemical staining of testis sections with antisera against histone H4 acetylated on lysine 5 (A,B), lysine 8 (C,D), lysine 12 (E,F) or lysine 16 (G,H). Spermatids from knockout mice and wildtype mice stained for all four acetylated histone H4 residues. Spermatids staining brown for the acetylated lysine residues are found in the middle of the tubules.

Figure 10
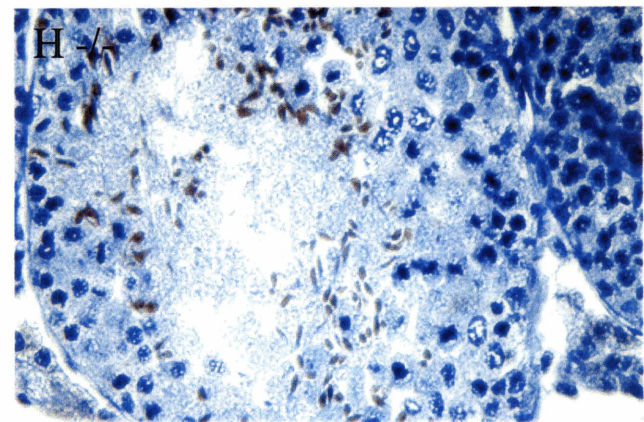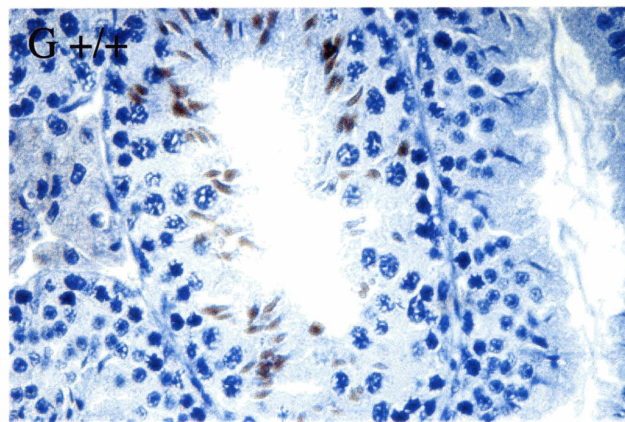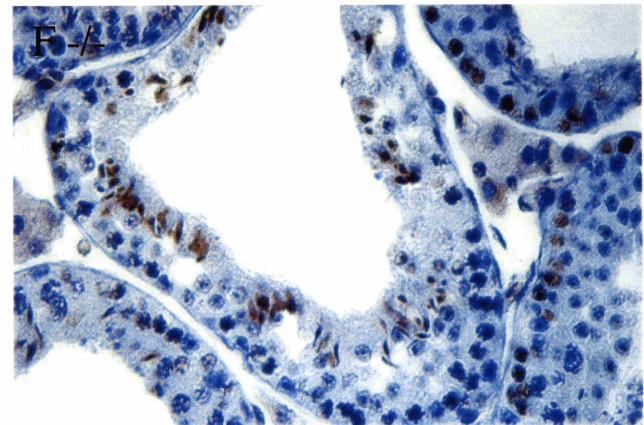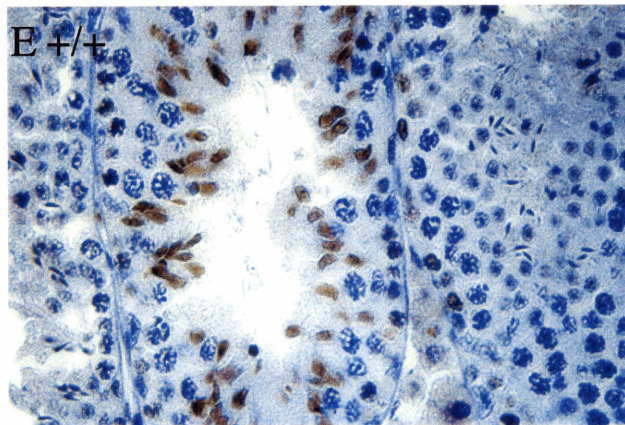
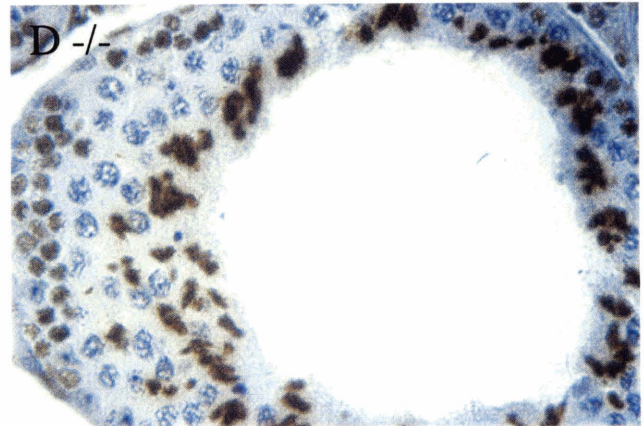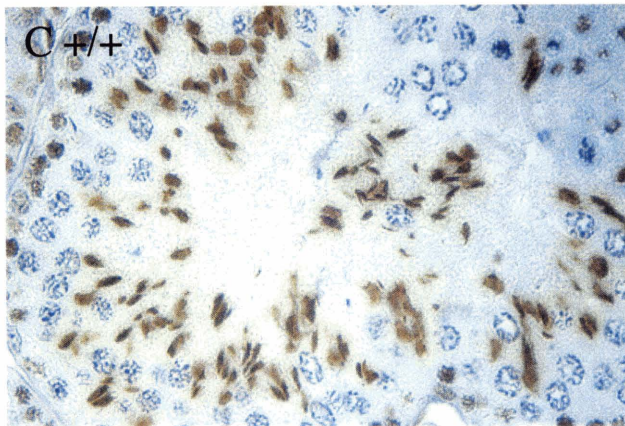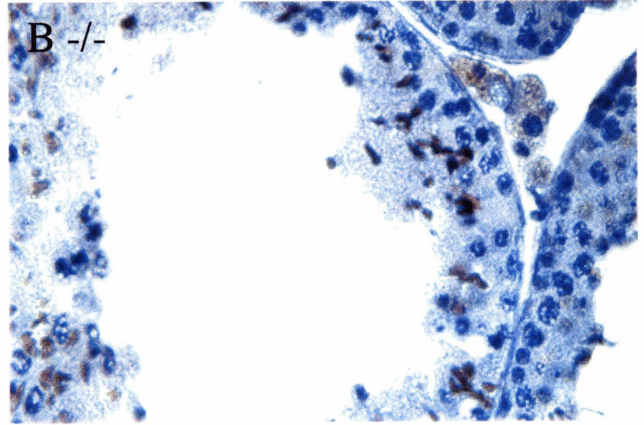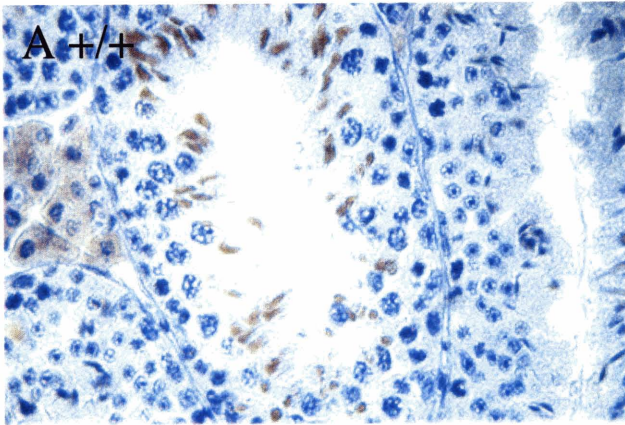knockout spermatids with similar intensities (Fig 10). We therefore conclude that the global acetylation status of histone H4 is not significantly altered in *Cdyl*−/− spermatids.

To address whether *Cdyl* is required for chromatin condensation during spermiogenesis, we performed electron microscopy on spermatids from wildtype and mutant mice. In accordance with the observation that many mature sperm from *Cdyl*−/− mice had abnormal head morphology, most spermatids from the mutant mice appeared misshapen (Fig 11). Electron microscopy also revealed that most *Cdyl* knockout spermatids had acrosomes. However, testes from both wildtype and mutant mice contained spermatids with highly condensed chromatin, which suggests that there is no requirement for *Cdyl* in this process (Fig 11). Consistent with this finding, late stage spermatids in *Cdyl* knockout mice stained with antisera for protamine 1 and protamine 2 (Fig 12).

Evidence that *Cdyl* regulates transcription led us to assess the expression status of some known spermatogenesis genes in *Cdyl*−/− testes (Caron et al., 2003). Because it is unclear which genes *Cdyl* may regulate in the testes, genes on this panel were chosen for a variety of reasons, including their having an expression pattern similar to *Cdyl* or their producing a phenotype similar to *Cdyl* when knocked out. However, none of the genes that we tested appeared to be significantly upregulated or downregulated in *Cdyl* knockout testis (Fig 13). Although some genes, such as protamine 1, appeared to be expressed at lower levels in *Cdyl*−/− testis, we believe this reflects a reduction in the haploid germ cell content of the mutant testis rather than a difference in transcriptional regulation.

In conclusion, *Cdyl*−/− mice displayed partial lethality, and had modest reductions in body weight when young. These findings reflect the observation that *Cdyl* is expressed in many mouse tissues and demonstrate that *Cdyl* has an

important role in development. In addition, spermatogenesis was severely disrupted in the *Cdy1*-/- mice. When younger, most *Cdy1*-/- mice did produce mature sperm, but these sperm had aberrant head morphology. However, extensive degeneration of spermatogenesis and death of germ cells occurred in *Cdy1*-/- mice as they grew older, such that by about 5 months of age the seminiferous tubules contained few germ cells and essentially no mature sperm. The severe reduction in fertility observed in the *Cdy1*-/- mice suggests that loss of the homologous human gene CDY is a contributing factor to infertility in human males. However, because mouse *Cdy1* and human *CDY* are not orthologous, this work does not conclusively demonstrate a role for human *CDY* in spermatogenesis.

Figure 11 Transmission Electron microscopy of spermatids from wildtype

(A) and knockout (B,C) mice. Even though the mutant spermatid heads

have a deformed shape, the chromatin appears highly condensed as judged

by the smooth, homogenous texture of the heads.

Figure 11
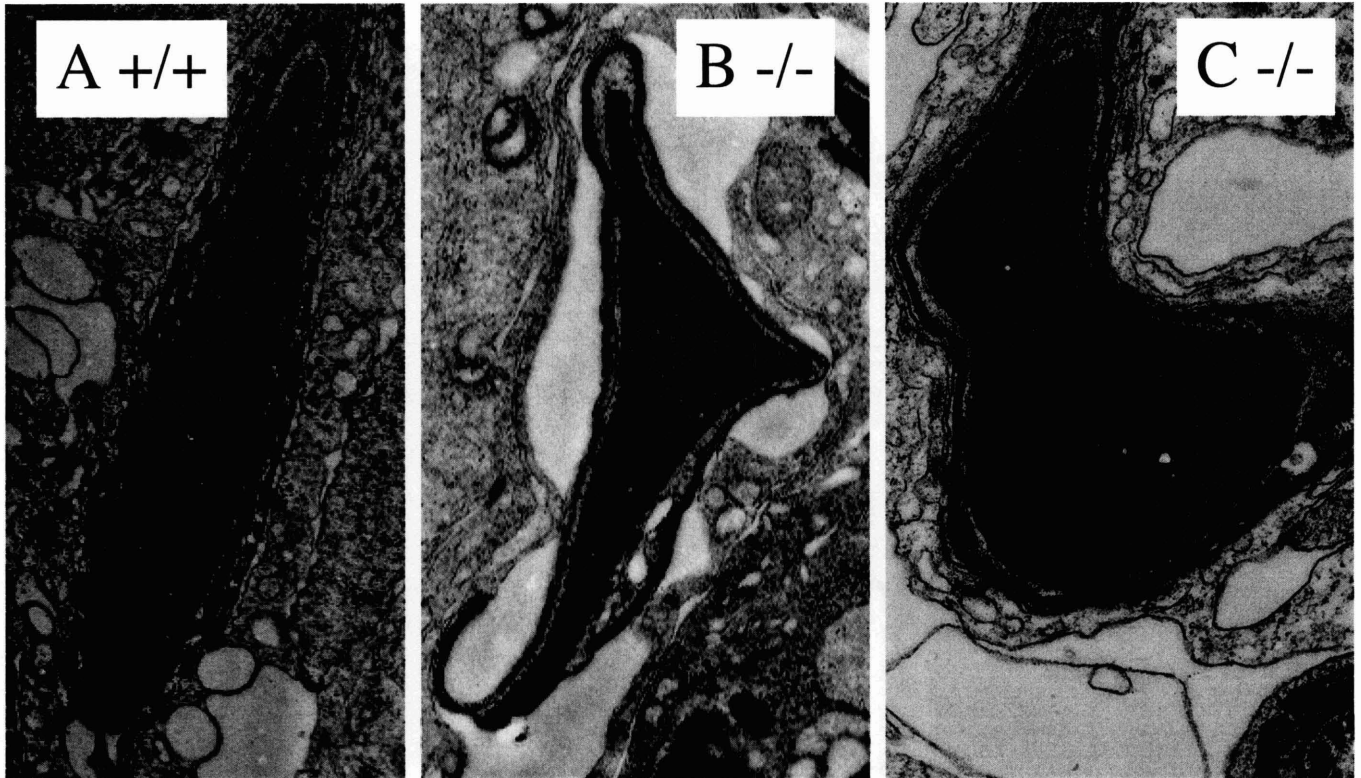
Figure 12 Immunohistochemical staining of testis sections with antisera

against protamine 1 (A,B) or protamine 2 (C,D). Protamine 1 and

Protamine 2 are expressed in wildtype and mutant testis. Spermatids

expressing the protamines stain brown and are the cells found closest to

the lumen of the tubule.

Figure 12
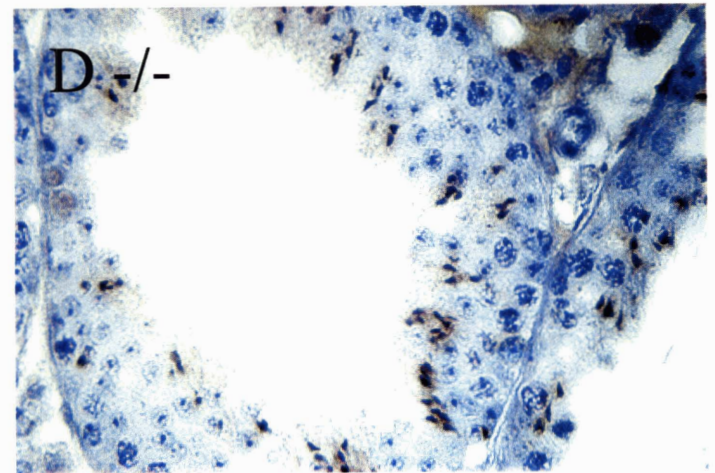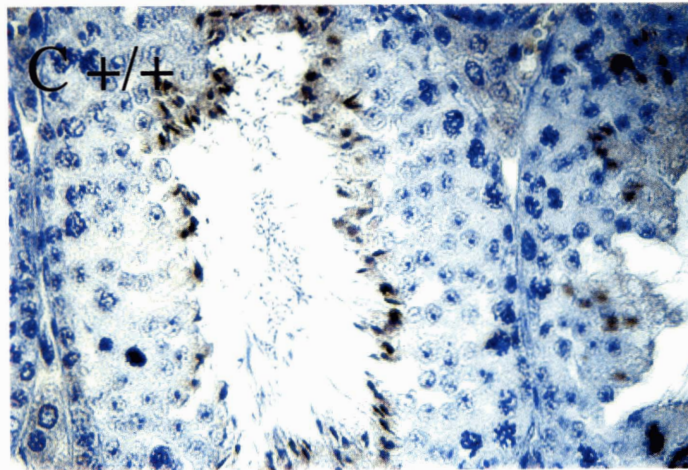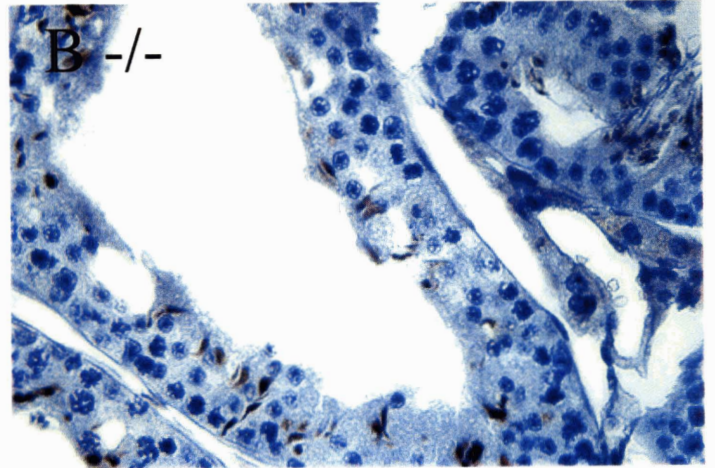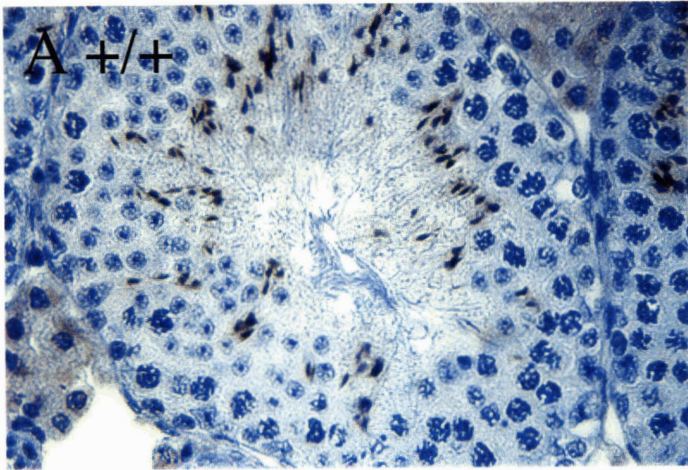
Figure 13 RT-PCR panel of testis-expressed genes. No significant

differences in expression were observed between wildtype and mutant

testes. Some genes, such as protamine 1 and E-map-115, are likely

expressed at lower levels in knockout testis because they are found

exclusively in spermatids, and many knockout testes have reduced

numbers of spermatids.

Figure 13
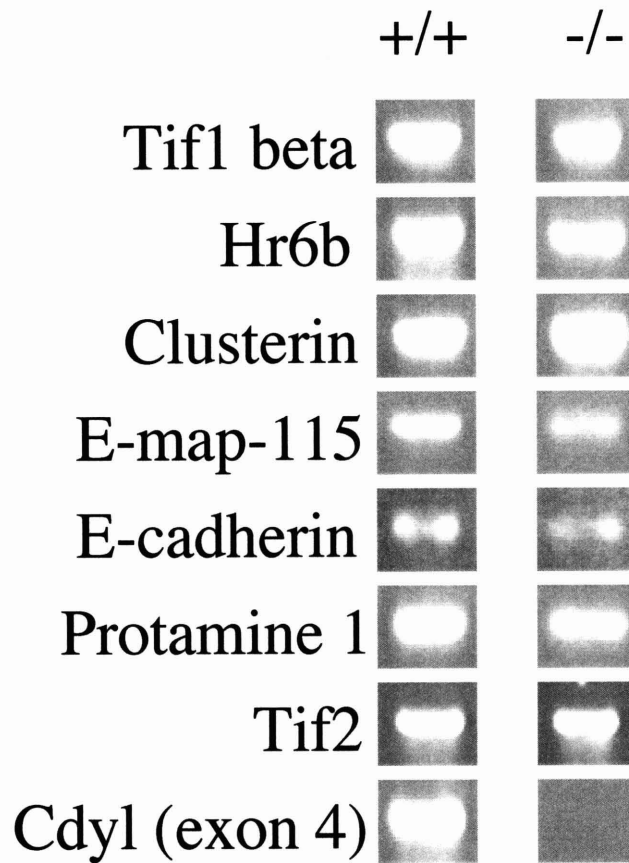
III

Rescue of *Cdyl* Knockout Mouse

With Human CDY

As *Cdyl‑/‑* mice displayed a marked spermatogenic phenotype, we sought to directly demonstrate a function for human *CDY* by rescuing this phenotype with a human *CDY* transgene. Several observations provide the rationale for this experiment: (1) of all known mouse genes, *Cdyl* is the mouse gene that has the highest homology to human *CDY* (AA identity = 61%); (2) both CDY and CDYL possess *in vitro* HAT activity that is specific for histones H4 and H2A; and (3) both CDY and CDYL are expressed in elongating spermatids.

Mice transgenic for human CDY were produced through male pronuclear injection of a human Y chromosomal BAC containing *CDY*. Pronuclear injections were performed on a (C57BL/6xSJL)F2 background. Of 32 founder mice, 8 tested positive for human CDY using a PCR assay on tail DNA. Tg*CDY* founders were backcrossed onto the C57BL/6 background, and we present here the characterization of one line that was backcrossed 10 generations.

Using PCR assays specific to the human Y chromosome, we determined that a 33-40 kb fragment of human genomic DNA is present in the Tg*CDY* mice (Fig 1). *CDY* is the only known gene contained in this fragment of DNA. However, when the BAC used for injection was mapped using these assays, we found that it is approximately 140 kb in size, suggesting that only a fraction of the BAC became integrated into the Tg*CDY* mice.

Northern blotting with a human *CDY* probe demonstrated that a *CDY* transcript approximately 2.5 kb in size is expressed in the testis of Tg*CDY* mice, but not in somatic tissues, including the brain, kidney, and liver (Fig 2A). The size of the CDY transcript found in the transgenic mouse testis agrees with the

Figure 1 STS mapping of the transgene. To estimate the size of the

transgene in Tg*CDY* mice, PCR assays (#1-15) that detect the human Y

chromosome were performed. Based on these assays, a 33kb-40kb piece of

the human Y chromosome that contains *CDY* is present in Tg*CDY* mice. As

a positive control for these assays, human male genomic DNA was used,

and as a negative control, wildtype mouse genomic DNA was used.

Additionally, the BAC that was injected to produce the transgenic mice was

estimated to be about 140kb using these assays, meaning only part of the

BAC became integrated in the transgenic mice.

Figure 1
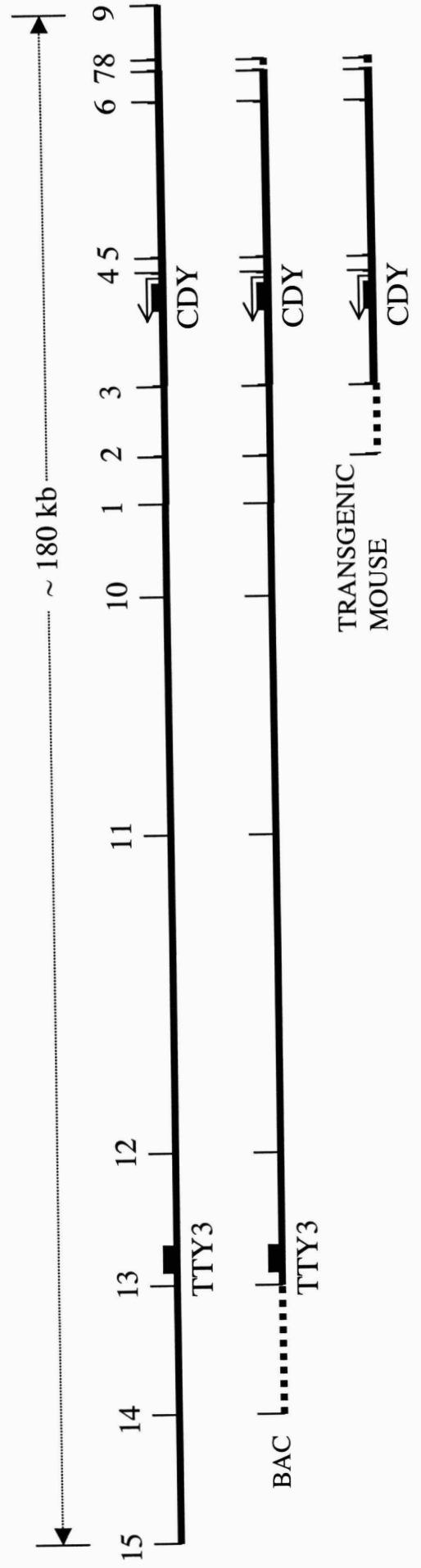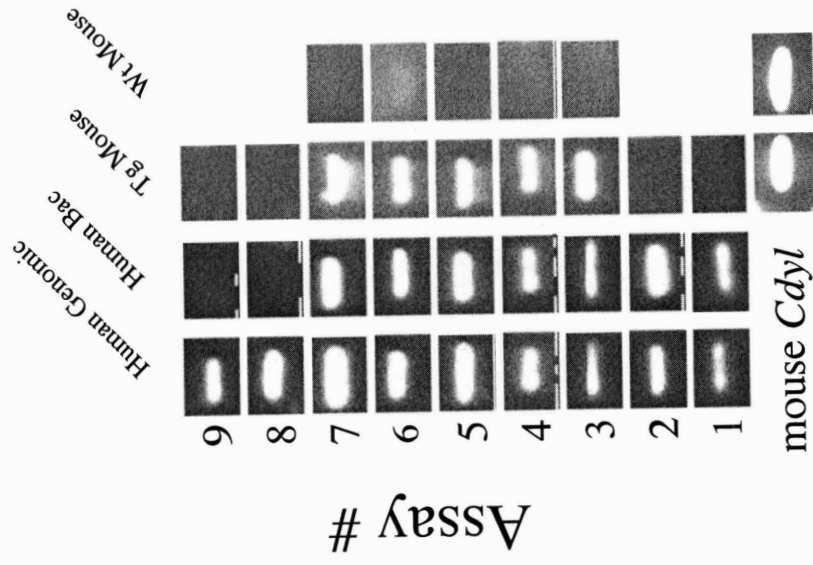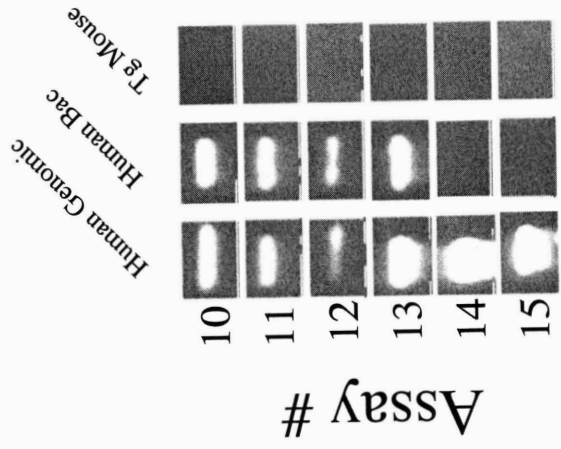
Figure 2 Expression of human *CDY* (A) Northern analysis with a probe

for human *CDY.* A 2.5 kb *CDY* transcript is expressed highly in the testis

of Tg*CDY* mice, but no expression was detected in several somatic tissues.

Additionally, this *CDY* probe does not cross-hybridize with mouse *Cdyl*, as

no signal was detected in wildtype testis. (B) Western blotting with

antisera for CDY. CDY protein of the expected size, 62 kd, is present in

adult Tg*CDY* testes and human testis extract, but not in adult wildtype

testis. Additionally, CDY is not detected in 21 day old Tg*CDY* testes,

suggesting that expression of CDY is restricted to haploid germ cells.

Figure 2

A

WT   Transgenic

T   T T B H K L

T=Testis
B=Brain
H=Heart
K=Kidney
L=Liver

— 4.4 Kb

— 1.8 Kb

Beta Actin

B

Tg (21 day)   WT (Adult)   Tg (Adult)   Human

— 80 kd

— 38 kd
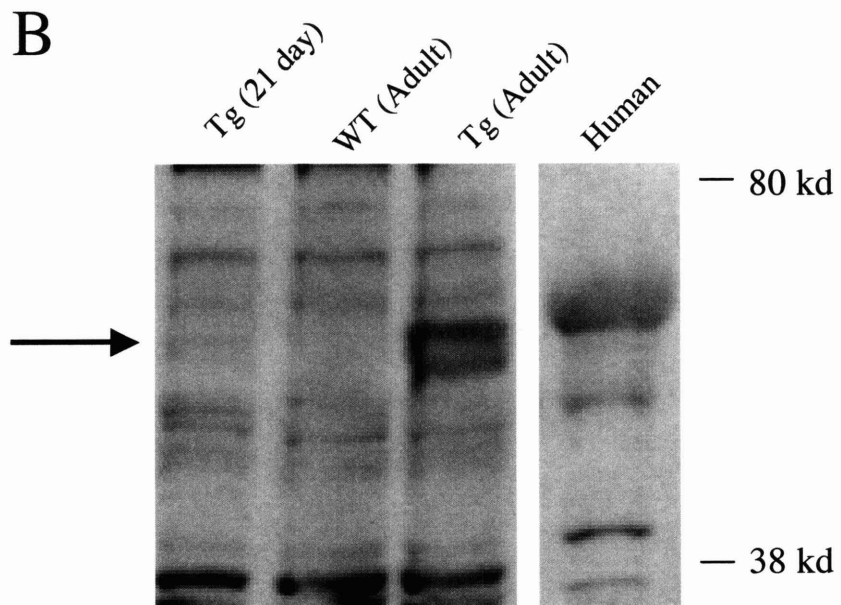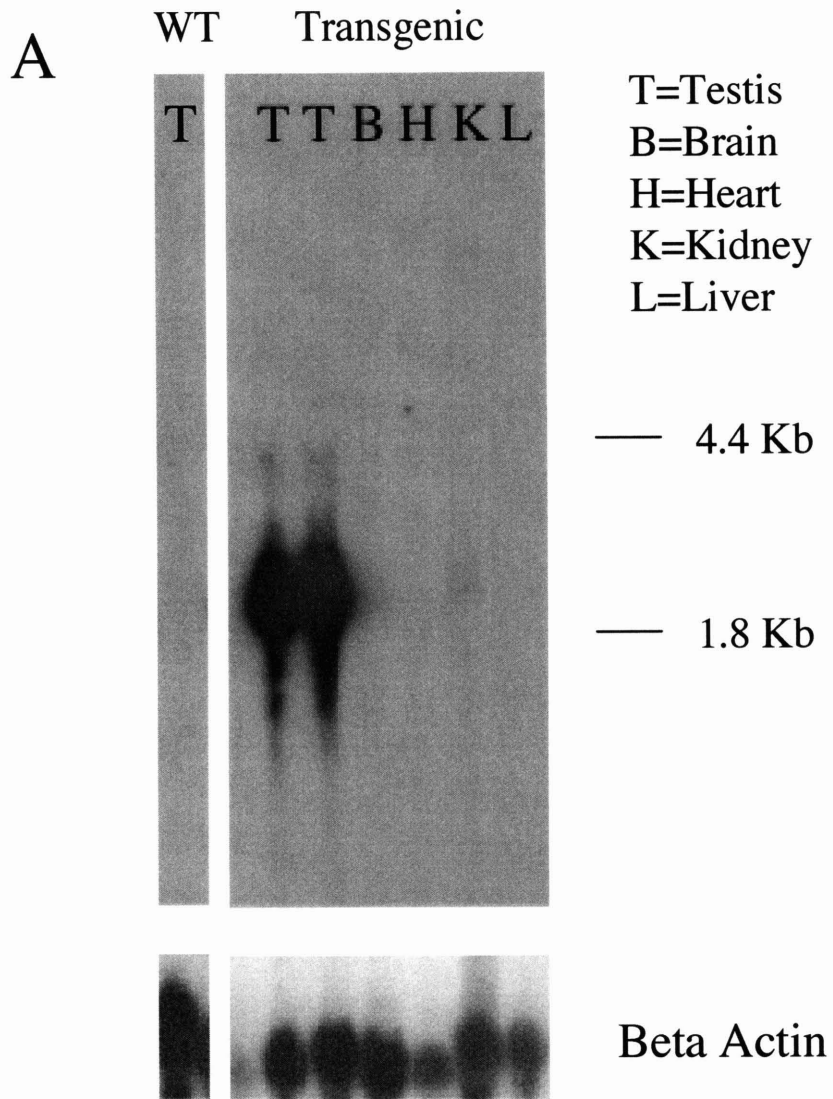
previously reported size of CDY transcripts from the human testis (Lahn and

Page, 1999). As an additional control, no *CDY* expression was detected in

wildtype testes. RT-PCR products for the open reading frame of *CDY* were

generated from the testis of a Tg*CDY* mouse and sequenced, confirming the

absence of point mutations in the coding region of *CDY*. Furthermore, western

blotting with antisera raised against a peptide from CDY demonstrated that CDY

protein of the predicted size, 62 kd, is expressed in the testis of Tg*CDY* mice but

not WT mice (Fig 2B). The level of CDY expression in the transgenic mouse

appears similar to the level of endogenous CDYL expression (see Chapter 2, Fig

2), suggesting that the expression level of CDY should be adequate to rescue a loss

of CDYL.

To move the *CDY* transgene onto a *Cdyl*-/- background, C57BL/6 *Cdyl*+/+

Tg*CDY* mice were mated with C57BL/6 *Cdyl*+/- mice to generate C57BL/6 *Cdyl*+/-

Tg*CDY* mice (Fig 3). The C57BL/6 *Cdyl*+/- Tg*CDY* mice were then mated with

Cdyl+/- mice on one of three backgrounds-C57BL/6, BALB/c, or 129-to generate

Cdyl-/- Tg*CDY* mice. Even though the original characterization of *Cdyl*-/- mice was

performed on a pure BALB/c background, we were hindered from using this

strain background for the rescue experiments because the transgene was only

present on a pure C57BL/6 background.

It was found when progeny from *Cdyl*+/-x*Cdyl*+/- Tg*CDY* crosses were

genotyped at 2 weeks of age that *Cdyl*-/- mice on a C57BL/6, C57BL/6x129, or

C57BL/6xBALB/c background exhibit partial inviability. On these backgrounds,

only 3.7%, 6.2%, and 3.6% of mice, respectively, were *Cdyl*-/-, instead of the

expected 12.5% (Table 1). Not surprisingly, as the expression of the transgene

appears to be restricted to the testis, the inviability phenotype was not rescued in

Cdyl-/- Tg*CDY* mice; on the three above strain backgrounds, only 1.9%, 3.1%, and

Figure 3 (A) Breeding scheme for the rescue of $Cdyl^{-/-}$ mice with TgCDY

mice. (Legend: B6=C57BL/6; Tg=TgCDY; +/+, +/-, -/- refer to genotype at the

$Cdyl$ locus.) (B) Examples of the multiplex PCR that was used to genotype

mice generated during this breeding; from left to right, the four example

genotypes are $Cdyl^{+/-}$ TgCDY, $Cdyl^{+/+}$ TgCDY, $Cdyl^{+/+}$, and $Cdyl^{-/-}$ TgCDY.

Figure 3

A

B6 +/+ TG x B6 +/-

B6 +/+   B6 +/+ TG   B6 +/-   B6 +/- TG x +/- (129, BALB/c, or B6)

-/- TG, -/-, +/+, +/+ TG, +/-, +/- TG

B

Gentoyping

-Human *CDY*

-Neo Cassette
-*Cdyl* exon 4
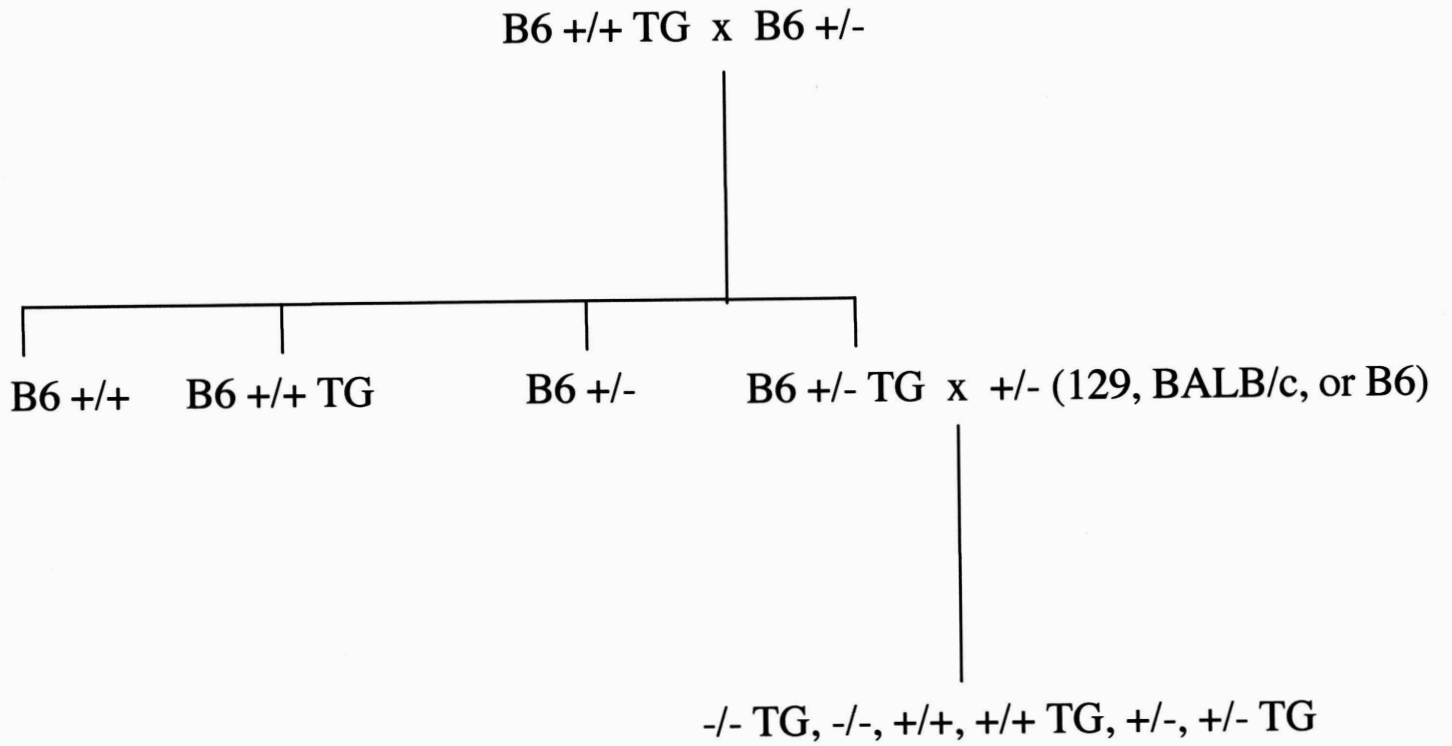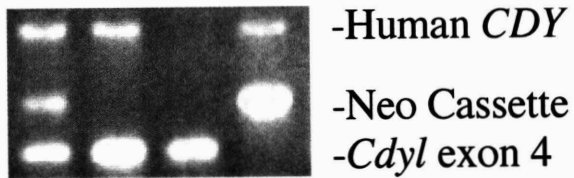
Table 1 Progeny ratios obtained at two weeks of age from $Cdyl^{+/-}$ x $Cdyl^{+/-}$ TgCDY crosses on a C57BL/6xBALB/c background (A), a C57BL/6x129 background (B), and a C57BL/6 background (C). Only data from males is presented. (C57BL/6xBALB/c $Cdyl^{-/-}$ TgCDY mice were from a second transgenic line produced from an independent insertion event).

Table 1

A

	Expected	Observed
+/+	12.5%	17.2%
+/+ TG	12.5%	11.7%
+/-	25%	29.7%
+/- TG	25%	32.4%
-/-	12.5%	3.6%
-/- TG	12.5%	5.4%

N=111 male mice

B

	Expected	Observed
+/+	12.5%	19.4%
+/+ TG	12.5%	16.3%
+/-	25%	24.0%
+/- TG	25%	31.0%
-/-	12.5%	6.2%
-/- TG	12.5%	3.1%

N=129 male mice

C

	Expected	Observed
+/+	12.5%	16.8%
+/+ TG	12.5%	19.3%
+/-	25%	29.8%
+/- TG	25%	28.6%
-/-	12.5%	3.7%
-/- TG	12.5%	1.9%

N=161 male mice

5.4% mice, respectively, were *Cdyl-/-* Tg*CDY* (Table 1). Additionally, almost all knockout mice still alive at 2 weeks on each of these three backgrounds had reduced bodyweights as adults and died by 5 months of age. Even though BALB/c *Cdyl-/-* mice also exhibited partial inviability shortly after birth, BALB/c *Cdyl-/-* mice still alive at 2 weeks of age were healthy as adults and had normal lifespans.

We next studied spermatogenesis in *Cdyl-/-* mice on each of the three strain backgrounds available for the rescue experiment. Examination of testis histology from 2-4 month old *Cdyl-/-* mice on a C57BL/6 background (N=3 mice), a C57BL/6x129 background (N=3 mice), or a C57BL/6xBALB/c background (N=3 mice) revealed only mild defects when compared to the severe germ cells loss we observed in BALB/c *Cdyl* knockout mice (Fig 4). On the C57BL/6xBALB/c background and the C57BL/6x129 background, both testes from all mice examined appeared only slightly abnormal, with between four and eight germ cell-depleted seminiferous tubules present in an entire testicular cross section. As these empty seminiferous tubules were not observed in age matched *Cdyl+/+* controls, we believe this germ cell loss represents a subtle yet reproducible phenotype. *Cdyl-/-* mice on a C57BL/6x129 background or on a C57BL/6xBALB/c background also produced normal spermatozoa and sired normal sized litters (data not shown). On the C57BL/6 background, 2 month old *Cdyl-/-* mice also exhibited the same subtle spermatogenic phenotype, with only a small number of empty tubules, but older mice (>3 months old) exhibited slightly higher numbers of empty tubules (Fig 4). However, C57BL/6 *Cdyl-/-* also produced normal spermatozoa and sired normal sized litters (data not shown).

We next examined the testis histology in a small number of *Cdyl-/-* Tg*CDY* mice. Both testes from a 2 month old C57BL/6 *Cdyl-/-* Tg*CDY* mouse and a 3 month old C57BL/6 *Cdyl-/-* Tg*CDY* mouse contained several germ cell depleted

seminiferous tubules and appeared similar to age matched C57BL/6 *Cdyl-/-* testes

(Fig 5). Similarly, both testes from a two month old C57BL/6x129 *Cdyl-/-* Tg*CDY*

mouse exhibited several defective tubules. In addition, a second line of CDY

transgenic mice produced from an independent insertion event did not rescue the

germ cell loss phenotype when the transgene was present on a C57BL/6xBALB/c

Cdyl-/- background (data not shown). This preliminary data suggests that

the *CDY* transgene is not able to rescue the spermatogenic phenotype in *Cdyl-/-*

mice.

In conclusion, we were successful in generating a transgenic mouse that

highly expressed the human gene *CDY* in its testis. The level of *CDY* transgene

expression appeared comparable to that of mouse *Cdyl* (See Fig 1 and Fig 2,

Chapter 2). However, when this *CDY* transgene was moved onto a *Cdyl-/-*

background, it was not capable of rescuing the germ cell loss that occurs in *Cdyl-/-*

testes. Also, in the course of these experiments, we found that the germ cell loss

phenotype in *Cdyl-/-* mice is highly dependent on strain background:

Spermatogenesis in *Cdyl-/-* mice on the BALB/c background is much more severely

affected than is spermatogenesis in *Cdyl-/-* mice on a C57BL/6 background, a

C57BL/6x129 background, or a C57BL/6xBALB/c background.

Figure 4 Testis histology from *Cdyl*$^{+/+}$ and *Cdyl*$^{-/-}$ mice. Shown are testis sections from 3 month old *Cdyl*$^{+/+}$ and *Cdyl*$^{-/-}$ C57BL/6xBALB/c mice (A,B); 3 month old *Cdyl*$^{+/+}$ and *Cdyl*$^{-/-}$ C57BL/6x129mice (C,D); 2 month old *Cdyl*$^{+/+}$ and *Cdyl*$^{-/-}$ C57BL/6 mice (E,F); and 3 and 6 month old C57BL/6 *Cdyl*$^{-/-}$ mice (G,H).
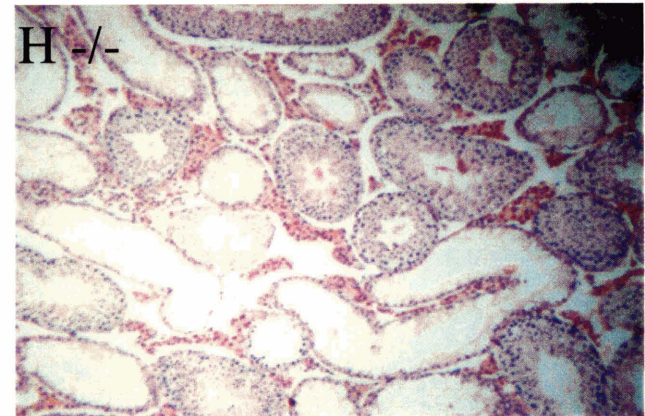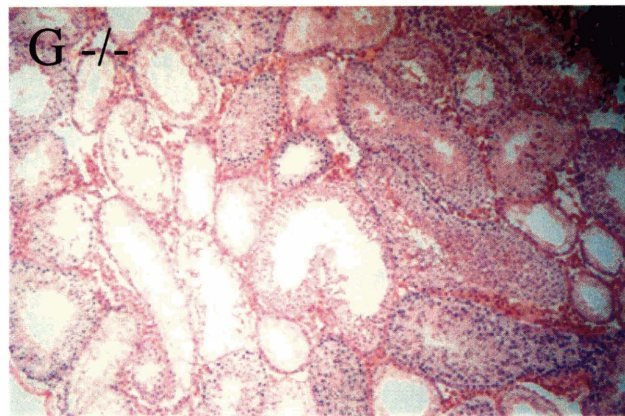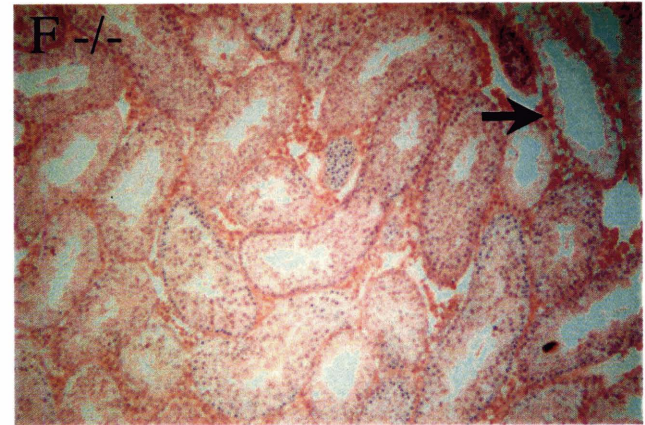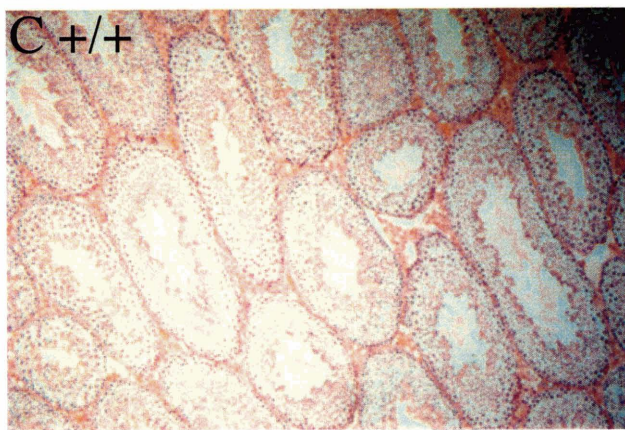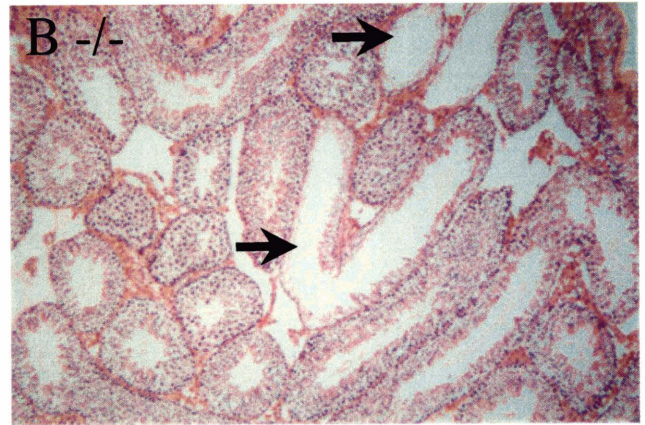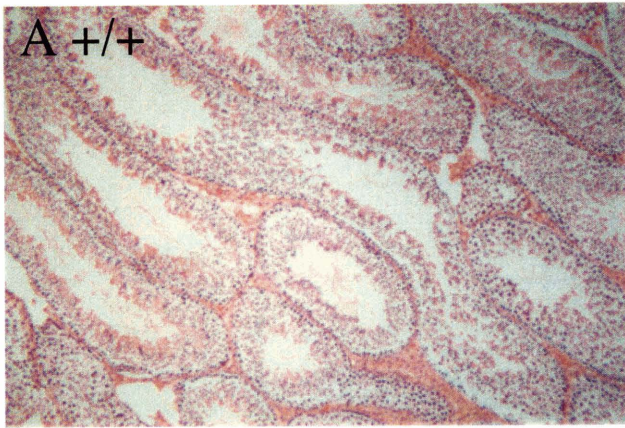
Figure 4

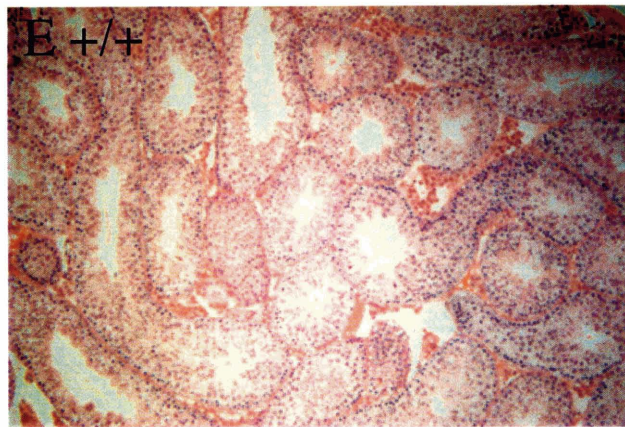**Figure 5** Testis histology of *Cdyl*^{-/-} Tg*CDY* mice. Shown are the left (A) and

right (B) testes of a 2 month old mouse on the C57BL/6 background and

the left (C) and right (D) testes of a 3 month old mouse on the

C57BL/6x129 background.

Figure 5
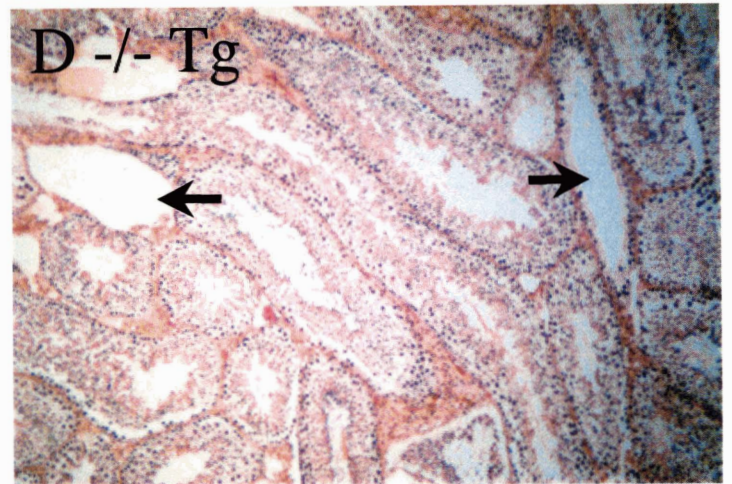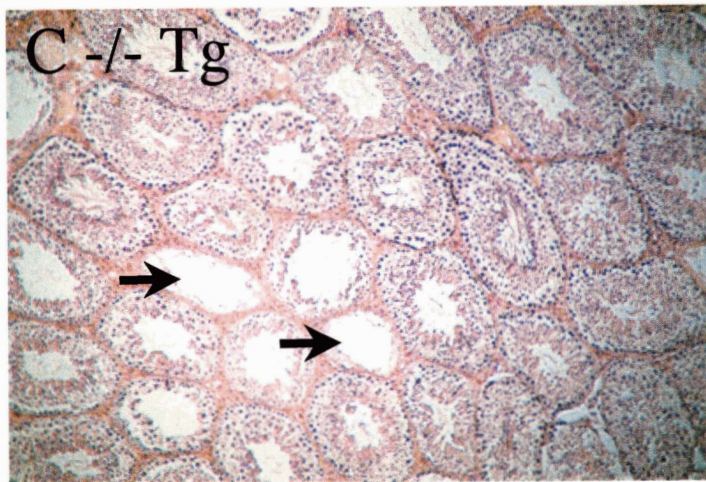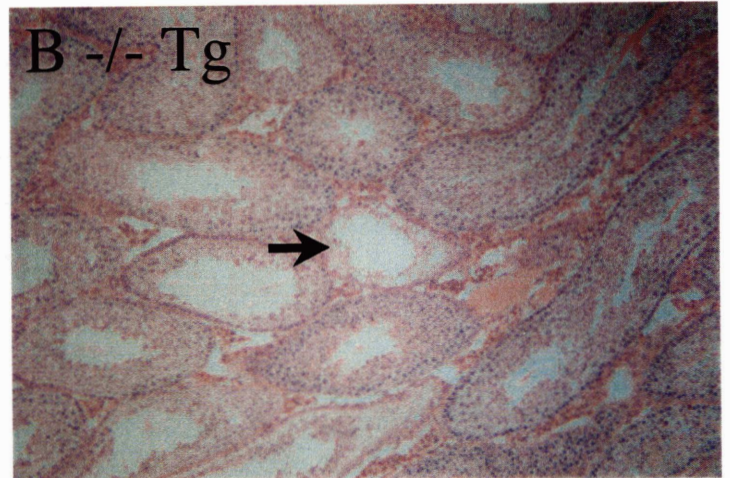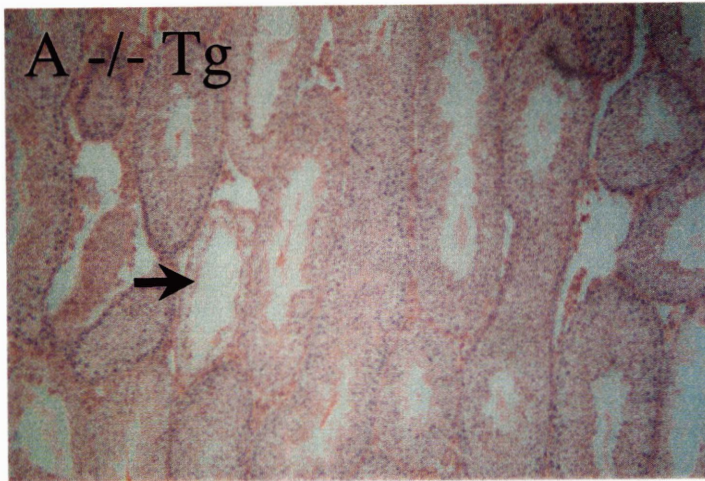
IV

Discussion

Further characterization of the two mouse *Cdyl* transcripts revealed that they actually code for two distinct protein isoforms of CDYL. These two isoforms both contain a conserved chromodomain and a conserved ECH domain, but they diverge in sequence at the amino terminus. While I do not rule out the possibility that the two protein isoforms are functionally diverged, I believe it is more likely that the two distinct *Cdyl* transcripts are produced as a result of transcriptional regulation of *Cdyl* within specific cell populations of the testis: The long transcript is found in somatic cells of the testis, whereas the short transcript appears to be restricted to haploid germ cells (Lahn et al., 2002). Even though immunohistochemical staining only detected the presence of CDYL in haploid germ cells, western blotting revealed that both protein isoforms are expressed in the testis; however, the 594 AA isoform coded for by the short transcript is much more abundant than the 545 AA protein isoform coded for by the long transcript. Therefore, I expect that some testicular somatic cells do express CDYL, but perhaps at low levels.

I have demonstrated that the mouse gene *Cdyl* is needed for normal spermatogenesis. *Cdyl* knockout mice on a BALB/c background exhibit a Category III (germ cell loss) phenotype and produce sperm with misshapen heads. As a result, *Cdyl* knockout mice have severely reduced fertility. Even though variability in germ cell loss was observed among individual mutant mice, by 5 months of age all BALB/c *Cdyl* knockout mice had an extremely low number of germ cells in their tubules. Because a pure BALB/c strain background was used for analysis, strain background variation was not responsible for the phenotypic variability. However, the primary cause of the germ cell loss in *Cdyl*-/- mice is not known. I have preliminary evidence that testosterone levels may be reduced in *Cdyl*-/- mice, as adult *Cdyl*-/- mice appeared to have smaller seminal vesicles than

controls. I believe further work comparing the levels of testosterone between

Cdyl-/- and wildtype mice is warranted. However, if testosterone levels are

affected in *Cdyl*-/- mice, it is not clear whether this is a cause of or a consequence

of the germ cell loss. Also, I do not rule out the possibility that a defect in the

proliferation of spermatogonial stem cells is responsible for the germ cell loss. I

feel this is unlikely, though, because previously described mutant mice with

defects in spermatogonial proliferation, such as *Plzf* and *Taf4b* mutants (Buass et

al., 2004; Falender et al., 2005), do not have increased numbers of apoptotic germ

cells (as *Cdyl* mutant mice do), and germ cell loss in *Cdyl*-/- mice is first observed

during the spermiogenesis phase of the first wave of spermatogenesis, which is

not consistent with a defect in the spermatogonial proliferation phase.

Additionally, we have no evidence that *Cdyl* is expressed in spermatogonia.

Most of the spermatozoa produced by BALB/c *Cdyl*-/- mice before

spermatogenesis becomes derailed have abnormal head morphology. While the

mechanisms involved in reshaping the spermatid nucleus are poorly understood,

a structure called the microtubule manchette is thought to be integral to the

process (Russell et al., 1991). A microtubule manchette was observed in the

spermatids of BALB/c *Cdyl*-/- mice using EM, but it is possible that this structure

does not function properly in *Cdyl*-/- mice. More extensive ultrastructural and

biochemical analysis (such as immunohistochemistry against tubulin and other

manchette-associated proteins) may reveal abnormalities of the microtubule

manchette in *Cdyl*-/- mice.

As mouse *Cdyl* is widely expressed and found in many tissues other than

testis, it was not surprising that BALB/c *Cdyl*-/- mice had other phenotypes in

addition to spermatogenic failure. BALB/c *Cdyl*-/- mice displayed partial lethality,

and had minor reductions in body weight when young. Because I was primarily

interested in spermatogenesis, I did not thoroughly investigate these defects. However, *Cdyl* clearly has an important role outside of spermatogenesis. Histological analysis of different tissues from newborn *Cdyl*-/- mice may help to elucidate additional functions of *Cdyl*. Additionally, I can not rule out the possibility that defects in non-testicular cells are responsible for impairing spermatogenesis in *Cdyl*-/- mice. Because BALB/c *Cdyl*-/- mice are healthy as adults, this is unlikely to be the case for knockout mice on the BALB/c background. However, *Cdyl* knockout mice on other strain backgrounds were not healthy as adults (see below), so the possibility of non-testicular cells influencing spermatogenesis in *Cdyl*-/- mice on other strain backgrounds is greater.

Assuming the spermatogenic failure in *Cdyl* knockout mice is testicular in origin, I do not know whether this failure is due to defects in the germ cells themselves, or rather due to defects in testicular somatic cells, such as Sertoli or Leydig cells. Performing spermatogonial transplantation experiments would allow me to determine whether *Cdyl* is required in somatic cells of the testes, germ cells, or both. In such an experiment two reciprocal transfers would be performed: *Cdyl*-/- spermatogonia would be transplanted into wildtype testes that had been chemically depleted of germ cells, and wildtype spermatogonia would be transplanted into *Cdyl*-/- testes. If abnormal spermatogenesis is observed in the former transfer, I could conclude that germ cells require *Cdyl* function, whereas if abnormal spermatogenesis is observed in the latter transfer, I could conclude that somatic cells of the testis require *Cdyl* function.

A related question is whether both *Cdyl* transcripts are required for normal spermatogenesis. This point could be addressed by generating two additional *Cdyl* knockout mice, one in which only the longer widely expressed transcript is disrupted, and one in which only the shorter germ cell specific

transcript is disrupted. It should be possible to disrupt one transcript while leaving the other intact, as the transcripts have different 5' ends. If both transcripts are required for normal spermatogenesis, this experimental approach might allow us to finely dissect the spermatogenic functions of *Cdyl*. For example, a mouse in which only the longer transcript is disrupted may produce normal spermatozoa but exhibit germ cell loss, while a mouse in which only the shorter transcript is disrupted may exhibit no germ cell loss, but produce spermatozoa with misshapen heads. Alternatively, I may find that one transcript but not the other functions in spermatogenesis.

Previously, Lahn et al. hypothesized that CDYL participates in the hyperacetylation of histone H4 during spermiogenesis. However, hyperacetylated histone H4 was still observed in the spermatid nuclei of BALB/c *Cdyl* knockout mice. Additionally, chromatin in the spermatids of BALB/c *Cdyl*-/- mice appeared to condense normally. These results show that *Cdyl* is not required for global H4 histone acetylation or for chromatin condensation in spermatid nuclei. Additionally, antisera for four mono-acetylated forms of Histone H4 stained similarly between wildtype and knockout sections, suggesting that a reduction in the acetylation status of one particular lysine residue does not occur in *Cdyl* knockout spermatids. It remains possible histone H4 hyperacetylation levels in *Cdyl*-/- spermatids underwent a small reduction that we were not able to detect. Western blotting may provide a more quantitative means of assessing whether small reductions in global histone H4 hyperacetylation occurred in *Cdyl*-/- testes. It is also possible that changes in the acetylation status of histone H4 in *Cdyl*-/- testes were not global, but were confined to specific genetic loci and therefore were not detected. Also, even though histone H4 hyperacetylation appeared to occur in the elongating spermatids of *Cdyl*-/- testis, which is the same

developmental time point it occurs at in wildtype testis, there may be subtle perturbations of the timing of acetylation in the mutant testis.

A mouse paralogue of *Cdyl*, called *Cdyl2*, was recently cloned (Dorus et al., 2003). *Cdyl2*, like *Cdyl*, is expressed in the testis. It is not known whether CDYL2 is expressed in spermatids. Further expression studies of CDYL2, including assessing whether it is upregulated in the *Cdyl* knockout testis, will be useful in interpreting the *Cdyl* knockout mouse phenotype. Perhaps CDYL and CDYL2 both function to acetylate histones in spermatid nuclei, and in *Cdyl* knockout mice, CDYL2 can compensate for the missing CDYL function. Study of *Cdyl2* mutant mice and mice carrying mutations in both *Cdyl* genes will be necessary to address this possibility. Also, in *Cdyl/Cdyl2* double knockout mice, I may find that other phenotypes of the *Cdyl*−/− mouse, such as germ cell loss, are exacerbated; this would further demonstrate functional redundancy between the two genes.

Recently, CDYL was reported to function as a transcriptional co-repressor and to be a component of the CtBP repressor complex. (Caron et al., 2003 and Shi et al., 2003) Even though the biochemical mechanism by which CDYL contributes to repression is unclear, these findings raise the possibility that the defects observed in *Cdyl*−/− mice are due to the misexpression of genes whose regulation is normally controlled by complexes containing CDYL. However, the *E-cadherin* gene, which is a known target of CtBP mediated repression (Shi et al., 2003), was not upregulated in *Cdyl*−/− testes. Identification of potential CDYL target genes in the mouse testis will likely require a systematic approach, such as microarray analysis. Transcriptional profiling has already been carried out on *Ctbp*−/− mouse embryonic fibroblasts (Grooteclaes et al., 2003). Perhaps this data could be of assistance when analyzing transcriptional differences between *Cdyl*+/+

and $Cdyl^{-/-}$ testes, as genes that are significantly misexpressed in both $Cdyl$

mutants and $Ctbp$ mutants could be highly relevant target genes.

Apropos of the model that CDYL contributes to transcriptional repression,

I would like to suggest another possible role for CDYL in spermatogenesis. It has

been demonstrated by measuring [^{3}H]uridine incorporation that step 8-10

spermatids undergo a global repression of transcription that results in a complete

cessation of transcriptional activity for the duration of spermatogenesis

(Kierszenbaum and Tres, 1974; Zhao et al., 2004). As CDYL is known to become

dramatically upregulated during this time, I propose that CDYL may participate

in this global repression. Additional work examining transcriptional activity in

$Cdyl^{-/-}$ spermatids is needed to address this possibility.

I observed marked differences in the $Cdyl$ knockout mouse phenotype

when it was moved to different strain backgrounds. In contrast to the BALB/c

background, $Cdyl$ knockout mice had poor viability as adults on the C57BL/6

background and on C57BL/6 mixed backgrounds (C57BL/6xBALB/c and

C57BL/6x129); almost all $Cdyl^{-/-}$ mice on these backgrounds died by 5 months of

age. However, $Cdyl^{-/-}$ mice had much less pronounced spermatogenic failure on

these backgrounds than on a BALB/c ground. $Cdyl^{-/-}$ mice on a C57BL/6

background or a C57BL/6 mixed background produced normal looking

spermatozoa, and exhibited only mild germ cell loss compared to BALB/c $Cdyl^{-/-}$

mice.

As I was initially unaware of the effects that strain background has on the

$Cdyl^{-/-}$ phenotype, I regrettably backcrossed the transgenic CDY mice to the

C57BL/6 background rather than to the BALB/c background. Because $Cdyl^{-/-}$ mice

have poorer viability and less pronounced spermatogenic failure on a C57BL/6

background or a C57BL/6 mixed background than on a BALB/c background, it

was difficult to establish whether human *CDY* can rescue spermatogenesis in *Cdyl⁻/⁻* mice using these C57BL/6 and C57BL/6 hybrid mouse strains. Ideally, these rescue experiments would be performed on a BALB/c background, where the phenotypic signal is much greater and it may be possible to detect partial rescue. Nonetheless, I collected preliminary data that suggests human *CDY* is not able to rescue the relatively mild loss of germ cells observed on these backgrounds.

If these preliminary observations are substantiated with further work on a BALB/c background, one of the following possibilities may explain the failure to rescue: (1) CDY has diverged from CDYL, such that it can no longer properly function in the mouse (e.g., CDY can't interact with the mouse orthologue of a human protein it normally binds to); (2) CDY has evolved to perform a completely new function in primates that is not required in the mouse; or (3) the *CDY* transgene is not expressed in the testicular cell type(s) responsible for the germ cell loss. Although the first two possibilities are difficult to distinguish between, additional work carefully analyzing protein expression of CDYL and the CDY transgene within the testis, as well as spermatogonial transplantation experiments (described above) may be able to address the third. If this third possibility is true, perhaps a human *Cdyl* transgene would be more successful in rescuing the germ cell loss in *Cdyl⁻/⁻* mice. A human *Cdyl* transgene would also be expected to rescue the partial lethality of *Cdyl⁻/⁻* mice, as human *Cdyl* is expressed widely in somatic tissues. Also, because *Cdyl⁻/⁻* mice on a C57BL/6, a C57BL/6xBALB/c, or a C57BL/6x129 background do not produce spermatozoa with abnormal head morphology, I could not address in my preliminary experiments whether the human *CDY* transgene can rescue this phenotype. It is

possible that rescue experiments on a BALB/c background will reveal a role for

human CDY in sperm head morphogenesis.

V

Materials and Methods

GENOMIC DNA PCR ANALYSIS Mouse tail samples were incubated overnight at 55 C in 100 ul lysis buffer (100 mM Tris ph 8.5, 5 mM EDTA, .2% SDS, 200mM NaCl, .3 mg/ml proteinase K). Samples were then diluted 1:10 in H_2O, and 5 ul was used in a 20 ul reaction. PCR cycling conditions are as follows: 96 for 4', 95 for 1', 60 for 1', 72 for 30", repeat steps 2-4 34 times, 72 for 5'. Primers used for genotyping and transgene mapping are given in Table 1.

RNA Analysis. RNA was extracted from mouse tissue using TRIZOL Reagent (Invitrogen). For RT-PCR, samples were incubated at room temperature for 15' with DNAase (Invitrogen) and then at 65 C for 15' with 25 mM EDTA. Reverse transcription was carried out at 42 C for 2 hours using Stratascript RT (Stratagene) and random hexamer primers. PCR was performed using the same cycling conditions listed above. For RT-PCR primers, see Table 2. 3' RACE was performed using Marathon-Ready liver cDNA (Clontech) with the primer AAGCAGGTGGTCCAGTGTCTGTGAGTCA. For northern analysis RNA was separated on a glyoxal gel (Ambion) and transferred to a Brightstar nylon membrane (Ambion). Probes were generated with PCR using the following primers: *Cdyl* 5' probe F:AGCATCCCACTTGTTCCTTG and R:GCTGGCAGGTGTTATCATCA; *Cdyl* internal probe F: GATGGCTTCACCCACATCTT and R:GCTGGGCCATTAACAGCTAC; *Cdyl* 3' probe F: GAGCACTAGGCTGCTCTTGG and R:CACAGACACTGGACCACCTG; human *CDY* probe: F: ACACCACAGGTCCAAAAACC and R: GCTGGCACTTTCTGTTAGCC. Probes were labeled with ^{32}P-CTP using the Megaprime DNA Labeling System (Amersham). Hybridizations were carried out according to manufacturer's directions using ExpressHyb Hybridization Solution (Clontech), and blots were exposed to film.

Mouse Construction A genomic clone containing exon 4 of Cdyl was obtained by screening the mCIT library (Research Genetics). The knockout mouse targeting construct was generated by ligating a 7kb genomic fragment located 5' of *Cdyl* exon 4 and a 5 kb genomic fragment located 3' of exon 4 into the Aat II and Sal I sites, respectively, of pPGKRN (a gift from R. Jaenisch), such that the two arms flank the Neo selection cassette. 129 ES cells were electroporated with the construct, DNA isolated from G418 selected cells was digested with Xba I, and southern blotting with a probe 5' of exon 4 was used to identify correctly targeted cells. Chimeric mice were generated with these ES cells by DNX Transgenic Sciences through blastocyst injection. *CDY* transgenic mice were generating by male pronuclear injection of the Research Genetics Library B BAC 16L1 into (C57BL/6xSJL)F2 fertilized eggs; microinjections were performed by DNX Transgenic Sciences.

Antibody Production and Western Blotting Antisera against mouse CDYL (anti-CDYL #1) was generated by injecting rabbits with the synthetic peptide EKAEQPTDDNTC conjugated to KLH (Covance). Anti-CDYL #1 antisera was affinity purified using the Sulfolink Kit (Pierce Biotechnology). CDY antisera was generated by injecting rabbits with the synthetic peptide VKHLRNNRNTASLC conjugated to KLH (Covance). Mouse proteins for western blotting were extracted by homogenizing mouse tissue in ice cold elution buffer (1% Triton-X, 5mM EDTA, 20mM tris-HCL ph 8, 150 mM NaCl). The protein lysates were mixed with Laemlli loading buffer and heated for 5' at 95 C. Proteins were separated by SDS-PAGE on a 10% tris-HCL gel running at 170 V. Proteins were transferred

onto a .45 micron nitrocellulose membrane for 1 hour at 250 milliamps. The

membrane was blocked overnight at 4 C in 5% dry milk, rinsed 3X with PBS,

incubated with primary antibody for one hour (CDYL antisera at 1:50, CDY

antisera at 1:300, AKAP antisera (from Santa Cruz Biotechnology) at 1:100, or

CamKIV antisera (from Santa Cruz Biotechnology) at 1:50), rinsed 3X with PBS,

incubated with HRP Conjugated secondary antibody at 1:10,000 for 40 minutes

(Amersham), rinsed 3X with PBS, incubated for 1' with ECL reagent

(Amersham), and exposed to film.

Immunohistochemistry and Histology Mouse testes were fixed in 4%

paraformaldehyde or Bouins fixative for up to 1 week and washed into 70%

ethanol. Samples were embedded in paraffin, sectioned, and then stained with

hematoxylin and eosin for histology or left unstained for immunohistochemistry.

Unstained slides were heated for 10' at 55 C, rinsed 2x5' in xylene, 2x5' in 100%

ethanol, 2x5' in 90% ethanol, 2x5' in 80% ethanol, 2x5' in 70% ethanol, and 2x2'

in dH$_2$O. Antigen retrieval was performed by immersing samples in 10 mM

sodium citrate buffer (pH=6) and heating for 2x10' at HIGH in a microwave.

Samples were allowed to cool, rinsed 2x2' with dH$_2$O, incubated for 10' in 3%

hydrogen peroxide, rinsed 2x2' with PBS, blocked for 2 hours at 4 C in 3% goat

serum, and rinsed 2x2' with PBS. Incubation with primary antibody was

performed overnight at 4 C using the following dilutions: hyperacetylated H4

(1:500), H4 Ac K5 (1:1000), H4 Ac K8 (1:120), H4 Ac K12 (1:200), H4 Ac K16

(1:500), H2A Ac K5 (1:5000), cleaved caspase-3 (1:1000), protamine 1 (1:1500),

protamine 2 (1:2000), and anti-CDYL #2 (1:200). All antisera for acetylated

histones were from Upstate Cell Signaling Solutions; Caspase-3 antisera was from

Cell Signaling Technology; anti-CDYL #2 antisera was from Abcam; protamine antisera was a gift from R. Balhorn. After primary antibody incubation, samples were rinsed 2x2' with PBS, incubated for 30' with biotinylated secondary antibody (Vector Laboratories), rinsed 2x2' with PBS, incubated for 30' with ABC reagent (Vector Laboratories), rinsed 2x2' with PBS, and incubated with DAB substrate (Vector Laboratories) for 2-6 minutes. Samples were then rinsed in tap water, stained with hematoxylin, and dehydrated.

Microscopy Epididymal sperm were collected by mincing the epididymis in PBS or Ham's F-10 Media and observed at 600x magnification using a Nikon Eclipse TE300 microscope. Testes for EM analysis were fixed in 2.5% gluteraldehyde, 3% paraformaldehyde with 5% sucrose in 0.1M sodium cacodylate buffer (pH 7.4). Tissue was then post fixed in 1% OsO_4 in veronal-acetate buffer. The tissue was stained in block overnight with 0.5% uranyl acetate in veronal-acetate buffer (pH6.0), then dehydrated and embedded in epon-812 resin. Sections were cut on a Leica ultra cut UCT at a thickness of 70nm using a diamond knife, stained with 2.0% uranyl acetate followed by 0.1% lead citrate and examined using a Philips EM410.

Table 1

ASSAY	FORWARD	REVERSE
Cdyl (exon4) (genotyping)	CTCCAAGACCAACTCCAAAG	GTCCATGTTCTTGCGGTTTG
Human CDY (genotyping)	ACACCACAGGTCCAAAAACC	GCTGGCACTTTCTGTTAGCC
Neo Cassette (genotyping)	AGACAATCGGCTGCTCTGAT	ATACTTTCTCGGCAGGAGCA
Transgene Assay 1	ATGGCAGAATGTAAATGGGG	TGTGTCAGAAATTTTAACCTAGCC
Transgene Assay 2	CAGGAAGCCCATCACAATCT	CCAGGTCGAACTACGTCACA
Transgene Assay 3	TTTTGGACAACCTTACTTTGACTG	CAACAGTGAGTTTTGGTTTTCC
Transgene Assay 4	TTTTCACTACACCAGTGGTGACA	TGGCAGCAAATAACTTGCG
Transgene Assay 5	AAGACACGGGATTGGAACAG	TCGTCATAGCAACCACAATTAAA
Transgene Assay 6	CAAGCCCTCAGAAATAATCTTA	CTTTGTCAGATGAGTAGGTTGTG
Transgene Assay 7	CCACTTGACCCCTGTCACATC	GTCCATCAGCCTAGAACCTAGACT
Transgene Assay 8	AGGAGATGTCAGGACTATCAGC	TCCATCCAGCTGGTCATATT
Transgene Assay 9	TGGTTTGATTGTCTATTATGCCA	CCATCTTGGCCTCATTAAGTGA
Transgene Assay 10	TGGGCATTTAGTGCTATCCG	ACAAAGAGCCTTCCAGACCAC
Transgene Assay 11	CGAATGTCCTAGTGGGGAAA	GGCCTTCAGCTGATTCACA
Transgene Assay 12	TTTCGCTCTGCTGGAATTTT	AGTGCTACCCTCTTAGGGAACT
Transgene Assay 13	TTGTCATCACAGCCATTTTGA	TTTACGTTTTTCTCTGGGCG
Transgene Assay 14	TCCTGCAAAGCCAAAGAGTT	ATCCCTTGAACCTGGGAGTT
Transgene Assay 15	GTCGTGGTATTGCGTTCCTT	TTCTATGGCCCACCTGTCTC

Table 2

ASSAY	FORWARD	REVERSE
Tif1 beta	GCCTTTGCCAGATTCAGAAG	CGAGAGTGCCATCAGACAAA
Tif2	ATAGACCAGGCATCGTTTGC	GTTGGTTTGGCTGGTTCTGT
Hr6b	AGCTGCGGAGCATGTCG	AAGGATGAGCAGACCAGG
Clusterin	AGAAAAAACCAACGCAGAGC	ATGATGCCAGATGCCCGAGC
E-cadherin	TTCGGAAGACTCCCGATTCA	CCAGGTTCTTTGGGAATTCA
E-Map-115	AAAGAAGAGCAGGAACGGCTGG	CATTGGTGACATCTAATCTGGACG
Protamine1	AGGTTGGCTGGCTCGACC	GCAGGAGTTTTGATGGAC
Cdyl (exon4)	CTCCAAGACCAACTCCAAAG	GTCCATGTTCTTGCGGTTTG
Cdyl (exon3a/3b)	AGCCAGCCTAATTCACAGGA	GCTTGATCGGGTTCTGAGAC
Cdyl (exon1A/exon4)	GCAGGGACCCAACTGGAAC	CGCACCAGATATTCTGTCTTCC

VI

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