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The first bromodomain of Brdt, a testis-specific member of the BET sub-family of double-bromodomain-containing proteins, is essential for male germ cell differentiation

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Brdt is a testis-specific member of the distinctive BET sub-family of bromodomain motif-containing proteins, a motif that binds acetylated lysines and is implicated in chromatin remodeling. Its expression is restricted to the germ line, specifically to pachytene and diplotene spermatocytes and early spermatids. Targeted mutagenesis was used to generate mice carrying a mutant allele of *Brdt*, $Brdt^{\Delta BD1}$, which lacks only the first of the two bromodomains that uniquely characterize BET proteins. Homozygous $Brdt^{\Delta BD1/\Delta BD1}$ mice were viable but males were sterile, producing fewer and morphologically abnormal sperm. Aberrant morphogenesis was first detected in step 9 elongating spermatids, and those elongated spermatids that were formed lacked the distinctive foci of heterochromatin at the peri-nuclear envelope. Quantitative reverse transcription (RT)-PCR showed threefold increased levels of histone *H1t* (*Hist1h1t*) in $Brdt^{\Delta BD1/\Delta BD1}$ testes and chromatin immunoprecipitation revealed that Brdt protein, but not $Brdt^{\Delta BD1}$ protein, was associated with the promoter of *H1t*. Intracytoplasmic sperm injection suggested that the DNA in the $Brdt^{\Delta BD1}$ mutant sperm could support early embryonic development and yield functional embryonic stem cells. This is the first demonstration that deletion of just one of the two bromodomains in members of the BET sub-family of bromodomain-containing proteins has profound effects on in vivo differentiation.

KEY WORDS: Brdt, Bromodomain, Spermatogenesis, Mouse

INTRODUCTION

The bromodomain is an evolutionarily conserved motif first identified in the *Drosophila* genes *brahma* and *female sterile (1) homeotic* [*fs(1)h*] (Haynes et al., 1992; Tamkun et al., 1992). More than thirty genes encoding bromodomain-containing proteins have now been documented in mouse and humans, and these proteins have a wide variety of functions, including replication, transcription, splicing, silencing and chromatin remodeling (Yang, 2004). Although the in vivo functions of most bromodomain-containing proteins are still not well understood, the histone acetyl-lysine binding activity of the bromodomain has been demonstrated unequivocally (Cairns et al., 1999). In addition to histones, the bromodomain has also been demonstrated to bind acetyl lysines of non-histone proteins, for example, p53 (Barlev et al., 2001), HIV Tat (Mujtaba et al., 2002) and c-Myb (Sano and Ishii, 2001). Structural studies of several bromodomain-containing proteins have provided some insight into the specificity of the interaction between the bromodomain and acetylated lysines (Dhalluin et al., 1999; Jacobson et al., 2000; Rojas et al., 1999).

The BET proteins constitute a sub-family of bromodomain-containing proteins uniquely characterized by the presence of two bromodomains and a region of homology in the C-terminal region, designated the extra terminal (ET) motif (Florence and Faller, 2001). The original members of this sub-class include the *Drosophila* gene *fs(1)h*, the yeast *Saccharomyces cerevisiae* gene *BDF1*, and the human gene *BRD2* (*RING3*). *fs(1)h* was initially identified as a maternal effect gene required later in development (Gans et al., 1975; Gans et al., 1980), interacting synergistically with loci controlling patterning and the production of homeotic transformations during development, such as *Ultrabithorax* and *trithorax* (Digan et al., 1986). *BDF1* was identified in a screen for genes that affect the synthesis of small nuclear RNA (snRNA) (Lygerou et al., 1994) and shown to be required for meiotic division during sporulation (Chua and Roeder, 1995). Bdf1 was subsequently shown to associate with TFIID and to be recruited to TATA-containing promoters (Matangkasombut et al., 2000). Bdf1 interacts with acetylated histone H4, competing with the Sir2 deacetylase, and is required for acetyl-H4-mediated anti-silencing function at heterochromatin boundaries (Ladurner et al., 2003; Matangkasombut and Buratowski, 2003; Pamblanco et al., 2001). Bdf1 has also been detected as a component of SWR1 nucleosome remodeling complex involved in the deposition of a minority histone variant H2A.Z (Krogan et al., 2003; Mizuguchi et al., 2004; Raisner et al., 2005; Zhang et al., 2005).

There are four BET sub-family members in the mouse and human genomes: *Brd2*, *Brd3*, *Brd4* and *Brdt* (Florence and Faller, 2001). The human *BRD2* gene was identified during a chromosome walk in the region of the class II major histocompatibility complex on chromosome 6 (Beck et al., 1992). We have recently generated a null mutation for the *Brd2* gene in the mouse model, which results in embryonic lethality (E.S. and D.J.W., unpublished results). Gene-trap mutagenesis has revealed that *Brd4* is also an essential gene: Brd4-deficient mouse embryos die shortly after implantation (Houzelstein et al., 2002). $Brd4^{+/-}$ embryonic stem (ES) cells were recently reported to be impaired in their ability to recover from nocodazole-induced mitotic arrest (Nishiyama et al., 2006).

The embryonic lethality both of *Brd4*-deficient and *Brd2*-deficient mice complicates understanding the in vivo function of BET family genes at a mechanistic level. By contrast, the apparent testis-specific

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expression of *Brdt* in mouse (Shang et al., 2004) and humans (Jones et al., 1997) affords the possibility of generating in vivo mutations of the mouse *Brdt* gene, because interfering with its expression would be predicted to not compromise embryonic development and overall viability. Furthermore, spermatogenesis, and spermiogenesis in particular, represents a unique model system in which to investigate the role of specific genes in the process of chromatin remodeling, one of the purported functions for bromodomain-containing proteins. During spermiogenesis, the post-meiotic spermatids undergo global chromatin remodeling, in which the haploid genome is compacted with the highly basic, sperm-specific nuclear proteins known as the protamines. There is also a wave of hyperacetylation of histones in the elongating spermatids, which has been proposed to be involved with the sequential removal and replacement of sperm chromatin components, ultimately yielding the highly condensed sperm nucleus. Interestingly, ectopic expression of human BRDT protein in somatic cells has been shown to have the unique property of specifically compacting chromatin that is acetylated (Pivot-Pajot et al., 2003). It was suggested that Brdt could play a similar role in spermiogenesis, functioning in elongating spermatids by binding to acetylated histones or other acetylated nuclear proteins and compacting the chromatin (Govin et al., 2006). We have therefore disrupted the function of mouse *Brdt* by gene targeting and demonstrate unambiguously that *Brdt* is essential for normal spermatogenesis. Importantly, loss of just one of the two bromodomains in Brdt resulted in strikingly abnormal spermatids and complete sterility.

MATERIALS AND METHODS

Gene targeting

A targeting vector was constructed by PCR-amplifying two homologous flanking fragments from mouse 129/SvEv genomic DNA and insertion into the vector pPNT (Tybulewicz et al., 1991). The right arm was the 3.2 kb *Bam*HI-*Bam*HI fragment including exon 5 and part of intron 4 and 5, and was directly inserted into the *Bam*HI site of pPNT between PGK-neo and HSV-TK. The left arm was the 5 kb fragment from the middle of intron 1 to the middle of exon 2 with synthetic restriction sites *Not*I and *Xho*I, inserted into the *Not*I and *Xho*I sites of pPNT. The construct was linearized at the *Not*I site, electroporated into 129/SvEv ES cells and screened for positive neo^r colonies using G418. Selected clones were analyzed by Southern blot using a 5′ diagnostic probe. Targeted cell clones were expanded and injected into C57BL/6J blastocysts as described previously (Mombaerts et al., 1996; Shang et al., 2002). One injected clone successfully contributed to the mouse germline in the resulting chimeras, which were then mated to develop mice heterozygous for the $Brdt^{\Delta BD1}$ allele. Heterozygous mice were then intercrossed to generate homozygous $Brdt^{\Delta BD1/\Delta BD1}$ mice on a mixed C57BL/6J:129Sv background. Mice were genotyped with PCR using primers located in exon 4 (forward), exon 6 (backward) and Neo (forward).

Northern blot hybridization, immunoblot, RT-PCR and real-time PCR analyses

For northern blot analysis, total RNA was isolated from testes, electrophoresed and transferred onto nitrocellulose membrane using standard procedures (Chomczynski and Sacchi, 1987). The blot was hybridized with ^{32}P-labeled RNA probe transcribed from a full-length *Brdt* cDNA as described (Shang et al., 2004). For immunoblot, mouse testicular lysates were separated by SDS-PAGE, transferred to PVDF membranes and the blots were incubated with anti-Brdt antibodies (α-CT and α-interbromo). For immunoblots of protamine 1 (Prm1), testes were homogenized in modified RIPA buffer [50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and protease inhibitor cocktail (Roche)], incubated for 30 minutes at 4°C, and centrifuged at 12,000 ***g*** for 10 minutes at 4°C (Iida et al., 2001). The supernatant was mixed with 2× SDS sample buffer, the pellet, which contains chromatin, was suspended in 1× SDS sample buffer, boiled for 5 minutes, and immunoblot analysis proceeded as described (Zhang et al., 1997). Reverse transcription (RT)-PCR was performed by using the ONE-Step RT-PCR kit from Invitrogen using total testicular RNA. Real-time RT-PCR was performed on Smart Cycler II (Cepheid, Sunnyvale, CA) with OmniMix HS lyophilized PCR master mix kit and SYBR green following the manufacturer's protocol. The primers were designed with Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Sigma-Genosys.

Histological analysis, immunohistochemistry and ultrastructural studies

Testis and epididymis were dissected and fixed in Bouin's fixative or 4% paraformaldehyde at 4°C overnight. The tissues were dehydrated in ethanol, embedded in paraffin and sectioned at 6 μm. For immunostaining with chromogenic detection, staining with antibody and detection of signal were performed as described previously (Zhang et al., 1999). The anti-Brdt antibody (α-CT) was used at a dilution of 1:1000, anti-Prm1 antibody (Santa Cruz, CA) at a 1:200 dilution, and anti-H1t antibody (gift of Peter Moens, Department of Biology, York University, Toronto, Canada) at a 1:2000 dilution. The orange-brown signal was generated with the peroxidase/diaminobenzidine system (Vector Laboratories, Burlingame, CA). The blue nuclear counterstain was hematoxylin.

For electron microscopy study of epididymal sperm, the cauda epididymis was dissected, the sperm were released, fixed in 2.5% glutaraldehyde/100 mM phosphate buffer (pH 7.4), and centrifuged at 1000 ***g*** to pellet the sperm. The sperm pellet was post-fixed in 1% osmium tetroxide, dehydrated and processed for routine transmission electron microscopy (Kissel et al., 2005). For testes, anesthetized animals were perfused with 2.5% glutaraldehyde/100 mM phosphate buffer (pH 7.4), the testes were dissected, cut into 1 mm pieces, post-fixed in glutaraldehyde for 1 hour and processed as above.

Collection of oocytes and spermatozoa, and in vitro fertilization

Females of the B6D2F1 were injected with 5 units of pregnant mare's serum gonadotrophin followed by injection of 5 units of human chorionic gonadotropin (hCG) 48 hours later. Oocytes were collected from oviducts about 15 hours after hCG injection. The oocytes were used directly for in vitro fertilization (IVF) following a protocol from The Jackson Laboratory (www.jax.org), whereas oocytes for intracytoplasmic sperm injection (ICSI) were freed from cumulus cells by a 5- to 10-minute treatment with 0.1% bovine testicular hyaluronidase (300 units/mg; ICN) in HEPES-CZB (Chatot et al., 1990). Spermatozoa were collected from the cauda epididymis and suspended in HTF (IVF) or CZB (ICSI) medium.

ICSI and derivation of ES cells

ICSI was carried out as described previously (Kimura and Yanagimachi, 1995) except that operations were performed at room temperature (25°C) rather than at 17°C. The ICSI was performed using Eppendorf Micromanipulators (Micromanipulator TransferMan; Eppendorf, Germany) with a piezo-electric actuator (PMM Controller, model PMAS-CT150; Prima Tech, Tsukuba, Japan). A single spermatozoon was drawn, tail first, into the injection pipette and injected into an oocyte. After sperm injection, the oocytes were transferred to CZB medium under mineral oil and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

The derivation of ES cell lines was carried out as described previously (Tesar, 2005). ES cells were cultured in DMEM with 15% FCS containing 1000 units/ml leukocyte-inhibiting factor on gamma-irradiated primary feeder fibroblasts. For blastocyst injection, ES cells were trypsinized, resuspended in DMEM, and first pre-plated on a standard 10 cm tissue culture dish for 30 minutes to remove feeder cells and debris.

Preparation of tetraploid embryos by electrofusion and injection of ES cells

B6D2F1 females were superovulated and mated with B6D2F1 males. Two-cell embryos were harvested by flushing the oviducts with FHM (Specialty Media, Phillipsburg, New Jersey) and electrofused to produce one-cell tetraploid embryos as described (Eakin et al., 2005; James et al., 1992). Briefly, two-cell embryos were transferred to 0.3 M D-mannitol and 0.3% BSA and fused with a GSS-250 electrode attached to a CF-150 electrofusion device (BLS, Budapest, Hungary). After the electrofusion, the embryos were

transferred to KSOM+AA media (Specialty Media). Embryos that fused were considered to be tetraploid and were cultured in microdrops under silicon oil to the blastocyst stage under 5% CO_2 at 37°C. Tetraploid blastocysts were injected with ten ES cells upon expansion of the blastocyst cavity.

Generation of anti-Brdt antibodies

One antibody (α-CT) was generated using a peptide representing the 14 C-terminal amino acids of Brdt. These procedures were performed by Sigma-Genosys. The second antibody (α-interbromo) was generated with a ~80 amino acid His-tagged fusion protein corresponding to the region between the two bromodomains. The protein was sent to Covance to generate the antibody. The α-CT antibody was affinity-purified with the peptide coupled to Sulfolink Coupling Gel following the manufacturer's protocol (Pierce).

Chromatin immunoprecipitation (ChIP)

For chromatin immunoprecipitation, a protocol from Upstate was followed. Briefly, four testes were decapsulated and resuspended in 8 ml RPMI 1640 medium containing 5 mg/ml collagenase and incubated at 34°C for 12 minutes. The cells were centrifuged and resuspended in 8 ml DMEM medium containing 5 mg/ml trypsin and incubated at 34°C for 12 minutes. The cells were crosslinked with 1% formaldehyde in PBS at 34°C for 20 minutes and the DNA was sheared to 300-500 bp with sonication. The debris was removed by centrifugation at 16,000 ***g*** for 10 minutes at 4°C and the supernatant was incubated with the affinity-purified α-CT antibody and protein A/G beads at 4°C overnight. The beads were washed and the chromatin was eluted and reverse cross-linked by incubation at 65°C. The DNA was purified and used as template for PCR detection.

RESULTS

Targeting of the mouse *Brdt* locus

The mouse *Brdt* gene, located on the distal region of chromosome 5, consists of 19 exons and spans ~55 kb. The first intron is ~10 kb long and the exact transcription initiation site has not been determined (Ensembl.org). Our targeting strategy deleted exons 3 and 4, and part of exon 2, of the *Brdt* gene, a deletion that included the translation start codon (ATG) and most of the first bromodomain (BD1; Fig. 1A). ES cells were electroporated with this construct and successful homologous recombination confirmed by southern blot and PCR analysis (data not shown). These cells were then used for blastocyst injection to generate chimeric animals carrying one recombined and one wild-type *Brdt* allele, according to standard procedures (see Materials and methods). The resulting chimeric male mice were then used to produce heterozygous progeny, which were then interbred. Heterozygous mice carrying one normal and one recombined *Brdt* allele were viable and bred normally. The offspring of heterozygous matings exhibited the three predicted genotypes at Mendelian frequencies and the ratio of male to female mice was approximately 1:1.

To confirm that the mutant *Brdt* allele was indeed not expressed, we examined the *Brdt* transcripts in testicular RNAs (Shang et al., 2004). Northern blot analysis of RNA from the three genotypes revealed the unexpected observation that the mutant allele was still expressed, albeit as a slightly shorter transcript (Fig. 1B).

To determine whether this shorter transcript actually produced a protein, immunoblotting was performed with our two different anti-Brdt antibodies, α-CT and α-interbromo (see Materials and methods). The shorter transcript expressed from the mutant allele was translated into a correspondingly truncated protein (Fig. 1C). $Brdt^{+/+}$ testes contained only the longer Brdt protein, the heterozygous testes expressed both, and the homozygous testes had only the shorter protein. Both antibodies recognized the truncated protein (data not shown), suggesting that the truncation was not the result of a premature termination and loss of the carboxyl end of the protein.

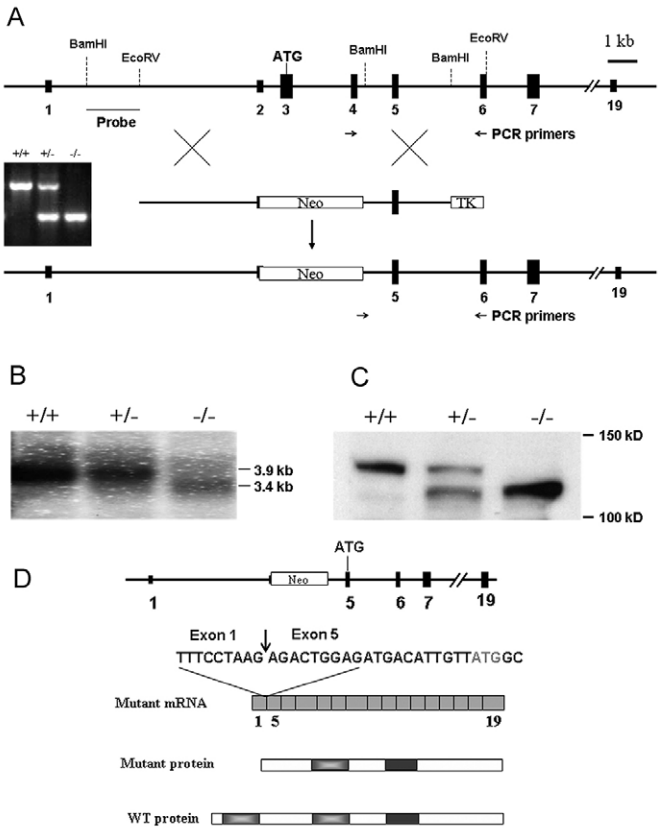

Fig. 1. Targeting of the mouse *Brdt* locus. (**A**) Targeting construct used to generate *Brdt* mutant mice. The top line depicts the genomic region of the *Brdt* gene, along with the PCR primers and probes used for genotyping targeted embryonic stem (ES) cells and subsequent progeny; the middle line depicts the targeting construct with a Neo and TK cassette; the bottom line indicates the organization of the resulting recombined allele. (**B**) Northern hybridization revealed that the mutant allele is still transcribed but produces an mRNA that is smaller in size (~3.4 kb). (**C**) Western blot showing that the shorter mRNA is translated in frame to produce a protein that is recognized by our α-CT antibody. (**D**) Splicing of the mutant *Brdt* mRNA and the predicted corresponding protein product. Reverse transcription (RT)-PCR of the mutant testicular mRNA was used to define the exact nature of the mRNA and the putative truncated protein. The mRNA results from splicing from exon 1 to exon 5; there is an in-frame ATG at the beginning of exon 5. The original translation start codon (ATG) is located in exon 3, which has been deleted in the mutant allele. The allele that produces this altered mRNA is referred to as $Brdt^{\Delta BD1}$.

To further understand the nature of the smaller transcript in the mutant testis and the truncated protein that would be predicted to be produced from it, we performed reverse transcription (RT)-PCR using primers in exon 1 and exon 5. We found that the smaller transcript is a product of alternative splicing from exon 1 to exon 5, which removes the Neo cassette. At the beginning of exon 5, there are in-frame ATGs that could serve as alternative translation start codons (Fig. 1D). Based on these sequence data and the relative molecular weight on immunoblot analysis, we concluded that the truncated protein lacks the first bromodomain but contains the inter-bromodomain region, the second bromodomain and the entire C-terminal region. We have thus generated a *Brdt* mutant allele which results in a shorter, mutant protein that lacks the first bromodomain. We designated and refer to this mutant allele as $Brdt^{\Delta BD1}$.

Brdt protein is expressed in pachytene and diplotene spermatocytes, and in round spermatids

Previously, we reported the cell type-specific expression of mouse *Brdt* mRNA by northern blot and in situ hybridization (Shang et al., 2004). With our newly generated anti-Brdt antibodies, we tested the cellular specificity of expression of Brdt protein. Immunostaining of histological sections of testes revealed that Brdt protein was highly expressed in the nuclei of pachytene and diplotene spermatocytes (Fig. 2A,B). During the meiotic divisions, Brdt protein was distributed throughout the cell, but after the meiotic divisions, Brdt protein was again predominantly nuclear in the round spermatids (Fig. 2C,E). The observed staining of Brdt decreased with the progression of spermatid differentiation and disappeared in the elongating stage of spermiogenesis (Fig. 2E). The shortened Brdt protein showed a similar pattern of expression and sub-cellular distribution in the homozygous testes (Fig. 2B,D,F). The staining in Fig. 2 used the α-CT antibody, but both antibodies gave a similar staining distribution, which was inhibited by pre-incubation with the corresponding immunogen (data not shown).

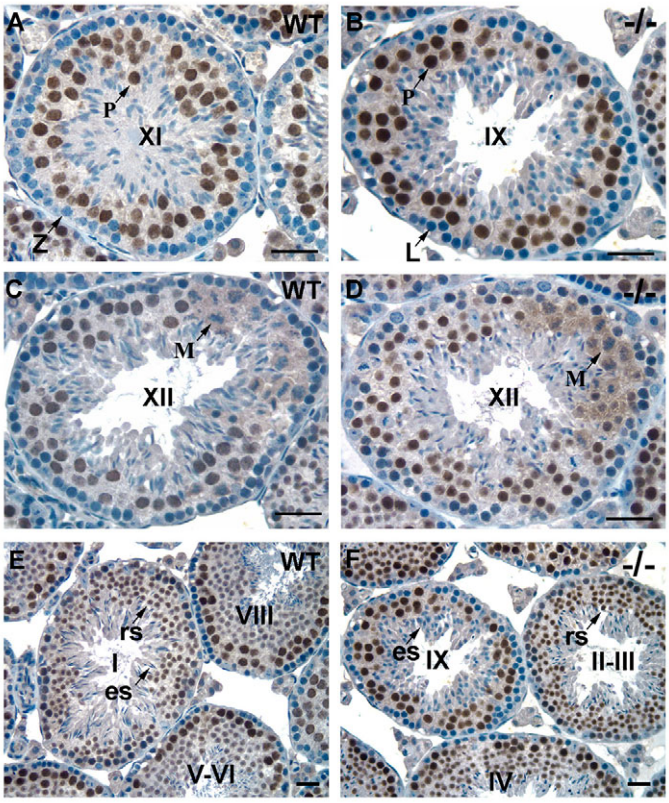

Fig. 2. Immunostaining of testicular sections with anti-Brdt antibodies. DAB staining produces a brownish color at the site of antibody localization. The α-CT antibody was used in the staining. Roman numerals within each tubule denote the stage of the tubule as defined previously (Russell et al., 1990). (**A**) A wild-type stage XI tubule showing the expression of Brdt protein in the nucleus of pachytene (P) but not zygotene (Z) spermatocytes. (**B**) A mutant stage IX tubule showing the expression of $Brdt^{\Delta BD1}$ protein in the nucleus of pachytene (P) but not leptotene (L) spermatocytes. (**C**) A stage XII wild-type tubule showing that, during the meiotic divisions (M), Brdt protein was distributed throughout the cell, but after the meiotic divisions, Brdt protein was predominantly found in the nucleus. (**D**) A stage XII mutant tubule showing a similar expression pattern and sub-cellular distribution of the truncated Brdt protein in the $Brdt^{\Delta BD1/\Delta BD1}$ testes. (**E**) Stage I, V-VI and VIII wild-type tubules showing the expression of Brdt protein in round spermatids (rs) but not in elongating spermatids (es). (**F**) Stage II-III, IV and IX mutant tubules showing the expression of the truncated Brdt protein in round spermatids (rs) but not in elongating spermatids (es). L, leptotene spermatocytes; Z, zygotene spermatocyte; P, pachytene spermatocyte; M, meiotic M phase; rs, round spermatid; es, elongating spermatid. Scale bar: 40 μm.

Homozygous-mutant male mice are infertile

$Brdt^{\Delta BD1/\Delta BD1}$ homozygous mice were viable and overtly normal, confirming our prediction that mutations in the apparently testis-specific BET family member *Brdt* should not compromise embryonic development. Homozygous females were fertile but homozygous males were sterile. The homozygous males copulated with equal frequency as control mice, as assessed by presence of seminal plugs, but mating of 20 homozygous males with control females for 3 months failed to yield any offspring.

Epididymal sperm of homozygous-mutant male mice are reduced in number and are misshapen

Histological sections of the epididymides were prepared from mice of all three genotypes and at different ages. The epididymides from young adult control and heterozygous animals appeared overtly similar, but there were clearly fewer epididymal sperm in the homozygotes (Fig. 3A). Light microscopic analysis of epididymal sperm revealed that those sperm that were present exhibited defective motility, moving slowly or barely moving (data not shown). Light and electron microscopic analyses showed that those sperm that were present were highly abnormal in appearance, with misshapen heads and aberrant morphology in the head and tail structures (Fig. 3B,C). Such a phenotype, in which one observes severe reduction in the number, reduced motility and a high percentage of abnormalities in the sperm in the semen, can be designated as oligo-astheno-teratozoospermia (Kastner et al., 1996). We never obtained progeny from mating studies on over 20 homozygous-mutant males, although we noted that there were differences in the severity of the oligo-astheno-teratozoospermia from animal to animal. In fact, in order to perform the ultrastructural and intracytoplasm sperm-injection experiments described below, it was necessary to sacrifice several mice in order to find epididymides that contained enough sperm or epididymal sperm from several mice were combined to obtain enough sperm for analysis.

Defects in the differentiation of male germ cells from homozygous-mutant mice were apparent at the elongating spermatid stage

To further investigate the primary defect in spermatogenesis of $Brdt^{\Delta BD1/\Delta BD1}$ mice, we examined the mutant testes by light and electron microscopy. In histological sections of the testicular tubules of young adult mice, abnormalities were first detectable in step 9 elongating spermatids (Fig. 4A,B). At step 12, aberrant spermatids were obvious, with a shortened shape and lacking any sign of the characteristic sharp hook present in control spermatids at this stage. At later stages of differentiation, some spermatids appeared to collapse into highly condensed, small ball-like structures (Fig. 2D, Fig. 4D).

During late stages of spermiogenesis, protamine replaces histones to further pack DNA into a more condensed conformation. It has been shown that deletion of only one allele of either *protamine 1* (*Prm1*) or *protamine 2* (*Prm2*), or precocious

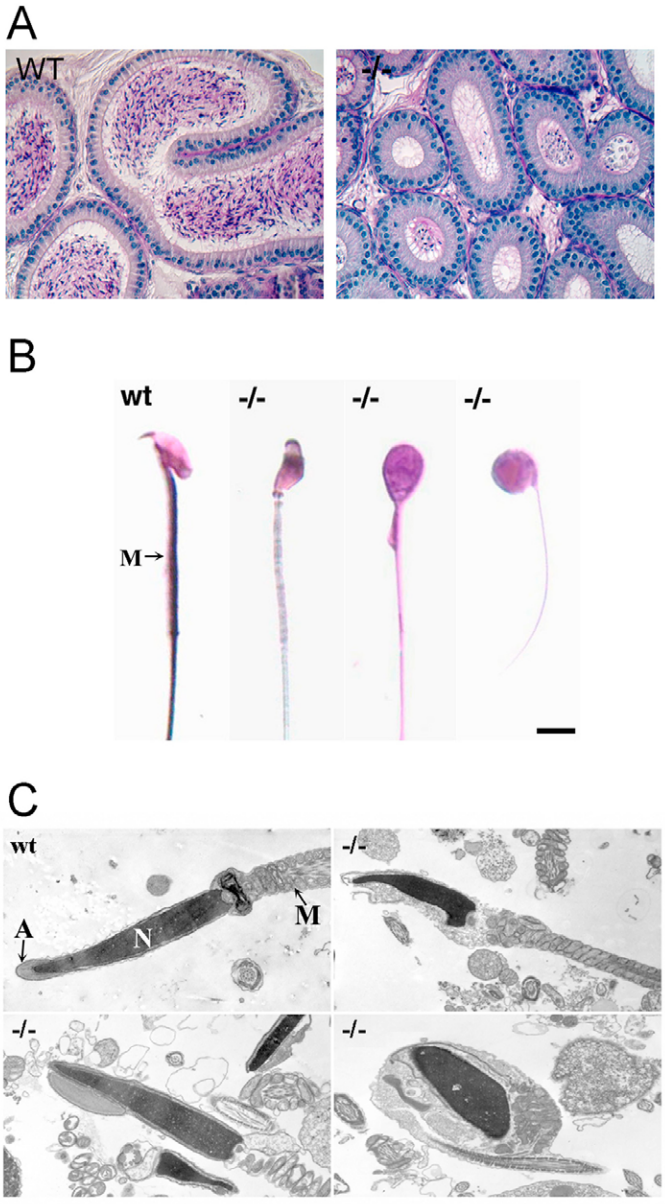

Fig. 3. *Brdt* mutant epididymal sperm are abnormal. (**A**) Morphology of epididymis from controls and homozygous mutants showing that very few sperm are present in the epididymis of the $Brdt^{\Delta BD1/\Delta BD1}$ mutant mice. (**B**) Light microscopy showing epididymal sperm of wild-type and $Brdt^{\Delta BD1/\Delta BD1}$ mutant mice. The photomicrographs show the variations of the abnormalities in the mutants, which occur throughout the sperm, including in the sperm head, mid-piece (M) and tail. (**C**) Electron microscopy of sperm reveals variation in the extent of nuclear condensation, as reflected by regions of reduced electron density and also by a failure of the acrosome to form normally. Residual cytoplasm, which should have been discarded as the residual body, is seen and it contains aberrant mitochondria. Magnifications in C: WT, ×13,000; mutant, upper right, ×8,300; lower left, ×10,000; lower right, ×8,300. A, acrosome; N, nucleus; M, midpiece. Scale bar: 5 μm.

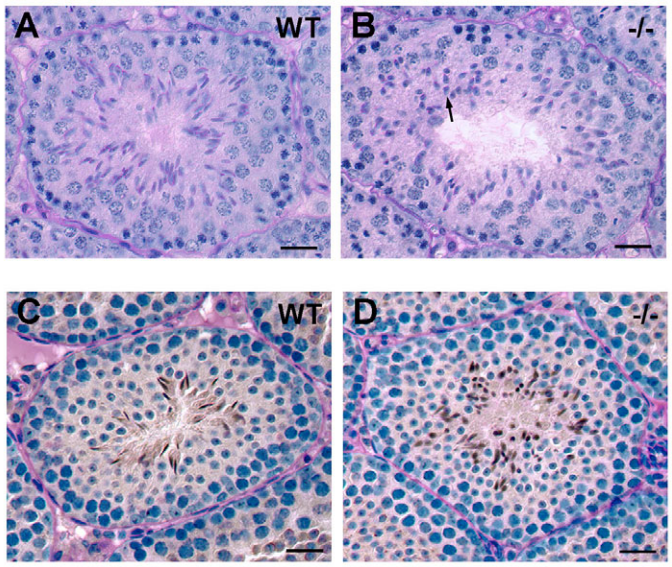

Fig. 4. The abnormalities of spermatogenesis in $Brdt^{\Delta BD1/\Delta BD1}$ mice were apparent at the elongating spermatid stage. (**A**,**B**) PAS staining of histological sections of testes from wild-type (A) and *Brdt* mutant (B) mice showing the abnormal shape of the elongating spermatids (B; arrow). (**C**,**D**) Immunostaining of histological sections of testes from wild-type (C) and *Brdt* mutant (D) mice with anti-Prm1 antibody, showing that the expression of Prm1 appeared on schedule, although the morphogenesis of the spermatids was abnormal in the mutants. Scale bar: 40 μm.

expression of *Prm1*, causes male infertility in mice (Cho et al., 2001; Lee et al., 1995). To test whether abnormal protamine expression was involved in the development of the aberrant head morphology of $Brdt^{\Delta BD1/\Delta BD1}$ spermatids, we performed immunostaining of histological sections with α-Prm1 antibodies. The expression of Prm1 appeared to be on schedule, being detectable in elongating spermatids in the mutant tubules (Fig. 4C,D). Immunoblot analysis of control and mutant testicular lysates further revealed that Prm1 protein was associated with nuclear chromatin in the mutant testis (data not shown).

Electron microscopic analysis of elongating spermatids revealed that the polarized localization of foci of heterochromatin, which are normally observed at the nuclear envelope (Fig. 5A) (Martianov et al., 2005), was absent in $Brdt^{\Delta BD1/\Delta BD1}$ elongating spermatids and, concurrently, an increase in heterochromatin-like structures was observed in the interior of the developing sperm head (Fig. 5B). These foci of heterochromatin have been postulated to represent anchorage points of heterochromatin on the nuclear envelope that might be involved in the coupling of the intrinsic force of chromatin condensation and the external structures such as microtubules and actin (Martianov et al., 2005). Such coupling could be important for the process of shaping sperm heads.

Given that the spermatids were so aberrant in morphology and the numbers of sperm so reduced, we were interested to know whether the elongating spermatids were undergoing apoptosis. A robust apoptotic response has been reported in a number of mutant strains of mice that exhibit impaired fertility or sterility (Salazar et al., 2003). We therefore performed in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assays on histological sections of control and mutant testes of different ages (3 weeks, 2 months, 6 months and 1 year of age) to detect DNA fragmentation. We did not observe a wave of apoptosis among the elongating spermatids, nor was there a detectable increase in the number of apoptotic cells at any stage of spermatogenesis in the mutant testes (data not shown).

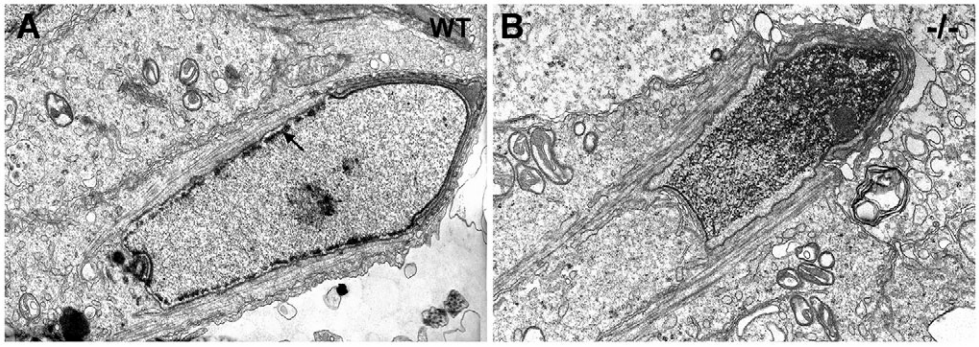

Fig. 5. Ultrastructural analysis of the elongating spermatids. Electron microscopy revealed that the normally occurring foci of heterochromatin (**A**, arrow) at the nuclear envelope are absent in *Brdt* mutant elongating spermatids (**B**). Magnifications: WT, ×8,300; mutant, ×13,000.

Gene expression is altered in *Brdt*$^{\Delta BD1/\Delta BD1}$ mutant testes

To begin to understand whether Brdt plays a role in modulating the expression of genes that are crucial for spermatogenesis, we examined the expression of 20 selected genes in mutant and control testes using real-time RT-PCR. These genes were selected either because their mutations have been shown to cause abnormalities in spermatogenesis, because they are testis-specific, and/or because they are highly or specifically expressed at appropriate stages relative to the expression *Brdt*. Among the genes examined was *Brdt* itself, using primers specifically designed such that they would not recognize the alternatively spliced transcripts. As predicted, no normal *Brdt* transcripts were detected (Fig. 6A, first bar). Although there were some slight changes in expression among the genes examined (Fig. 6A), only histone *H1t* levels were noticeably altered, being increased by approximately threefold in the mutant testes (Fig. 6A, second bar). Immunoblot and immunostaining analyses showed that H1t protein levels increased correspondingly (Fig. 6B,C).

Brdt protein binds the histone *H1t* promoter

In view of the purported role of bromodomain-containing proteins in transcription-regulating complexes and the altered expression of *H1t* in *Brdt*$^{\Delta BD1/\Delta BD1}$ mutant testes, we investigated whether Brdt protein was in fact part of a protein complex that binds the

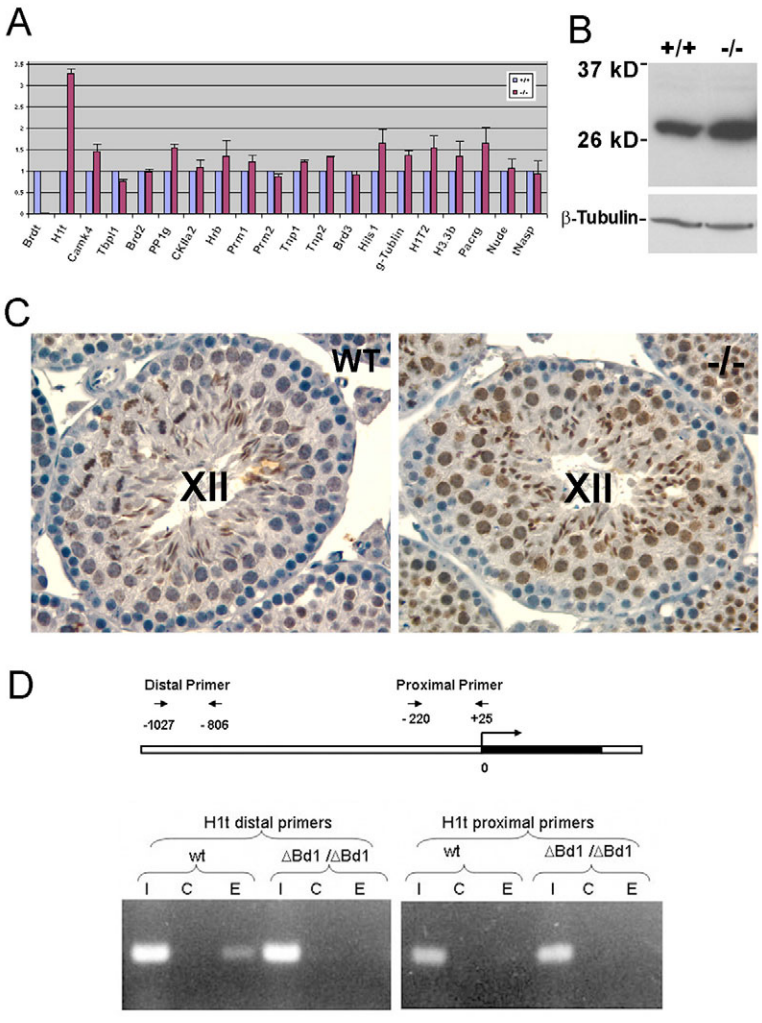

Fig. 6. Brdt protein is involved in the regulation of expression of histone *H1t*. (**A**) Histone *H1t* expression is upregulated in *Brdt*$^{\Delta BD1/\Delta BD1}$ mutant testes. Real-time reverse transcription (RT)-PCR was used to quantify changes in the levels of expression of selected genes from control and *Brdt*$^{\Delta BD1/\Delta BD1}$ mutant testes. Among the genes examined for expression was *Brdt* itself, using primers specifically designed such that they would not recognize the mutant transcripts. As predicted, no normal *Brdt* mRNA was detected (first bar). The expression levels are presented as relative levels of the wild-type expression and the wild-type expression levels are set as one. The results, corrected with GAPDH expression, are means±s.e.m. of at least three experiments in three pairs of animals. (**B**) Immunoblot of H1t protein in wild-type and *Brdt*$^{\Delta BD1/\Delta BD1}$ mutant testes. H1t protein level was elevated in the mutant testis as compared with control. β-tubulin was used as a loading control. (**C**) Immunostaining of testicular sections with anti-H1t antibody, showing that H1t was expressed in pachytene spermatocytes and spermatids and that H1t protein level was elevated in the mutant testis. (**D**) Chromatin immunoprecipitation (ChIP) assay showing that Brdt protein binds the histone *H1t* promoter. Two pairs of PCR primers corresponding to the histone *H1t* promoter proximal and distal regions were used. Total testicular cells were prepared from wild-type and *Brdt*$^{\Delta BD1/\Delta BD1}$ mutant testis and used for ChIP experiments with anti-Brdt C-terminal antibody. The cartoon in the upper panel shows the positions of the primers in the *H1t* promoter. I, input chromatin; C, Brdt peptide blocked control αBrdt antibody; E, αBrdt unblocked antibody experimental.

histone *H1t* promoter. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) experiments using affinity-purified α-CT antibody and primers against the *H1t* proximal and distal promoter region. The primers were selected based on previous studies showing that the proximal and distal regions are involved in the activation and repression of H1t expression, respectively (vanWert et al., 1998; Wolfe et al., 1999). The results revealed that immunoprecipitated full-length Brdt protein (from control testes) was found in association with the distal region of the histone *H1t* promoter (–806 to –1027 from the transcription initiation site), but not to the more proximal region of the promoter (Fig. 6D). However, the truncated Brdt protein from the homozygous-mutant testes did not immunoprecipitate with the *H1t* promoter (Fig. 6D). Negative controls, which included rabbit IgG alone and anti-Brdt antibody neutralized with the immunogenic peptide, resulted in no PCR-amplifiable template DNA in the reaction (Fig. 6D).

The DNA of *Brdt*$^{\Delta BD1/\Delta BD1}$ mutant sperm is capable of supporting embryonic development

As noted above, examination of epididymal sperm from the mutant mice revealed that there were greatly reduced numbers of sperm, and those that were present were aberrantly shaped and barely moved. As expected from such severe oligo-astheno-teratozoospermia, in vitro fertilization with these epididymal sperm confirmed that the mutant sperm were unable to fertilize meiotically mature oocytes, as compared with controls (data not shown). However, to test whether the mutant sperm nucleus could still support embryonic development, we performed ICSI using cauda epididymal sperm from mutant and control animals. In total, 107 mutant sperm were individually injected into control oocytes, of which 64 survived. Among the surviving fertilized eggs, 11 developed to the blastocyst stage. Considering the small number of blastocysts, we did not transplant them into foster mothers. Instead, we derived ES cells from the blastocysts and then injected these *Brdt*$^{+/\Delta BD1}$ ES cells into control tetraploid blastocysts. Because tetraploid cells can form functional extraembryonic tissue but cannot incorporate into the embryo proper, the resulting progeny are derived solely from the injected ES cells. We obtained *Brdt*$^{+/\Delta BD1}$ heterozygous mice from the ES cell injection into tetraploid blastocyst experiments; these mice, as expected, appeared normal in phenotype. Furthermore, these mice were found to breed normally and to transmit the mutant *Brdt*$^{\Delta BD1}$ allele. We therefore concluded that the DNA in the *Brdt*$^{\Delta BD1}$ mutant sperm is functional.

DISCUSSION

These studies report the first in vivo functional insight into the role of the *Brdt* gene in mammalian development and, to the best of our knowledge, present the first data among the studies on mammalian bromodomain-containing proteins in which a specific deletion of a single bromodomain rather than a null mutation has been analyzed. That is, we have generated a mutant allele of *Brdt* in mouse that expresses a truncated Brdt protein lacking the first bromodomain. The expression levels and sub-cellular distribution of the truncated protein were comparable with the normal Brdt protein, and the heterozygous mice were viable and fertile, demonstrating that the mutant protein did not function as a dominant-negative.

Both normal and mutant Brdt$^{\Delta BD1}$ proteins were observed to be highly expressed in pachytene and diplotene spermatocytes as well as in round spermatids. We therefore expected that we would observe defects in spermatocytes late in meiotic prophase or perhaps during the meiotic divisions. However, no obvious defects were detected in spermatocytes and the cells underwent the meiotic divisions to yield spermatids. There are several possible explanations for these interesting results. It is possible that, although Brdt protein is produced in spermatocytes, its role in spermiogenesis is manifested by downstream targets of its function. For example, mutant Brdt$^{\Delta BD1}$ protein might yield mis-expression of genes in spermatocytes whose protein products function in later stages, such as in step 9 elongating spermatids in which we first observe overt abnormalities. Alternatively, it might be that the mutant protein that we engineered, lacking the first bromodomain, can function appropriately during meiotic prophase but cannot function correctly in round spermatids, in which it is required for subsequent stages of spermiogenesis to proceed.

In our previous in situ hybridization study of *Brdt* expression, we found that *Brdt* mRNA is highly expressed in pachytene and diplotene spermatocytes, and that its levels dropped in early round spermatids (Shang et al., 2004). The continued presence of Brdt protein in round spermatids in spite of decreased levels of its mRNA might be explained by the Brdt protein being relatively stable. Also, the low levels of *Brdt* mRNA remaining in the round spermatids could still produce Brdt protein. However, it is clear that, at least at the limit of sensitivity of immunohistochemical staining, Brdt protein was not present in the elongating spermatids, the stage at which the defects are grossly apparent. In addition to the alternatives discussed above to explain the gap between when we know that the protein is most abundant and the stage when we first observe defects, it is possible that there are in fact subtle aberrations present at the earlier stages that are manifested in the more overt abnormalities we see in elongating spermatids. Such detailed analyses will be the subject of future studies.

It has previously been reported that, in yeast, different mutant alleles of the BET homolog *BDF1* show distinct phenotypes (Chua and Roeder, 1995). Mutants with transposon insertions anywhere in the *BDF1* coding region display a reduced rate of vegetative growth and sensitivity to a DNA-damaging agent. Interestingly, only null mutants and mutants with insertions specifically in the second bromodomain yield defects in meiosis. By contrast, mutants with insertions in the first bromodomain do not show meiotic defects. It was proposed and indirectly confirmed that, for mutants with insertions in the first bromodomain, transcription and subsequent translation of *BDF1* reinitiated in either the transposon or in the *BDF1* coding region. By analogy to these studies in yeast, we suggest that the mutants that we have generated, in which Brdt lacks the first bromodomain, represent a hypomorphic allele of *Brdt*. By further analogy, one might predict that a complete null mutation of *Brdt* would show a phenotype in meiotic prophase cells.

Very recently, the crystal structure of the first bromodomain (BD1) of the human BRD2 protein was solved and revealed that the BRD2-BD1 protein can form an intact homodimer (Nakamura et al., 2007). If the first bromodomain of Brdt is also involved in dimerization, and if the dimer and monomer have distinct functions, then we may speculate that the Brdt$^{\Delta BD1}$ mutant protein has lost the dimeric function but might still have some function as a monomer. Such partial function could result in a potentially stage-specific hypomorphic phenotype, just as we might be observing in the *Brdt*$^{\Delta BD1/\Delta BD1}$ mutant sperm.

One striking aspect of the abnormal morphology of the mutant elongating spermatids was the absence of foci of heterochromatin that are usually found in a polarized distribution in the region of the posterior peri-nuclear envelope (Martianov et al., 2005). Recently, it was reported that the lack of mouse histone *H1t2* function causes a loss of polarity in the distribution of the peri-nuclear

heterochromatin foci but not the complete absence of such structures (Martianov et al., 2005). That is, in normal elongating spermatids, the peri-nuclear foci of heterochromatin are distributed along the posterior region of the nuclear envelope. By contrast, in spermatids lacking *H1t2*, the foci of heterochromatin were located randomly, including in the region beneath the acrosome. In $Brdt^{\Delta BD1/\Delta BD1}$ mutant spermatids, we found that the peri-nuclear foci of heterochromatin were missing but that there was an increase in the appearance of similar-appearing foci randomly distributed in the interior of the nucleus. Such foci of heterochromatin have been speculated to be involved in anchoring chromatin on the nuclear envelope and in coupling the intrinsic action of chromatin condensation with structures external to the nucleus, such as microtubules and actin. Such coupling could be important for the process of shaping the sperm heads, and the absence of such structures might result in an aberrant morphology of the sperm heads, such as was seen in our $Brdt^{\Delta BD1/\Delta BD1}$ mutant mice.

In our quantitative RT-PCR of 20 selected genes in $Brdt^{\Delta BD1/\Delta BD1}$ mutant and wild-type testes, we found that the expression of *H1t* was elevated. Furthermore, we confirmed that wild-type Brdt protein, but not the truncated Brdt protein, binds the *H1t* promoter. H1t is a testis-specific histone H1 variant expressed in pachytene spermatocytes to round and elongating spermatids. $H1t^{-/-}$ mice are fertile and apparently normal (Drabent et al., 2000; Lin et al., 2000). Although the expression of *H1t* is elevated in $Brdt^{\Delta BD1/\Delta BD1}$ mutant mice, we do not propose that this alteration in *H1t* expression alone resulted in the aberrant spermatogenesis and consequent sterility of $Brdt^{\Delta BD1/\Delta BD1}$ mutant mice. This is particularly relevant because mice lacking H1t protein are fully fertile (Drabent et al., 2000; Lin et al., 2000). Rather, we hypothesize that Brdt regulates chromatin remodeling in a manner such that mutation of Brdt would alter the expression of multiple genes, and that the phenotype in $Brdt^{\Delta BD1/\Delta BD1}$ mutant mice is the result of a combination of these alterations.

The highly abnormal $Brdt^{\Delta BD1}$ mutant sperm that were produced were also quite reduced in number, suggesting that, at some point during spermiogenesis, significant numbers of cells were lost. It was therefore interesting to note that examination of the mutant testes for apoptosis, as detected by the TUNEL assay, did not reveal increased numbers of TUNEL-positive cells. The apoptosis machinery has been speculated to function in a sub-cellular compartment-specific manner during the differentiation of highly specialized cells that lack certain organelles (Jacobson et al., 1997). It has further been reported that, in differentiating spermatids, the apoptosis machinery is different from that found in most somatic cells; that is, the DNA fragmentation machinery in the nucleus is either inactive or uncoupled from the machinery in the cytoplasmic compartment (Arama et al., 2003; Arama et al., 2006; Blanco-Rodriguez and Martinez-Garcia, 1999). Steller and colleagues found that essential components of the apoptosis machinery, caspases and cytochrome c, are required in *Drosophila* sperm cell differentiation, but are regulated in a subcellular manner: they work in the cytoplasmic compartment to form the 'waste bag' but leave the nucleus intact (Arama et al., 2003; Arama et al., 2006). That is, during the process of late elongated spermatid differentiation, apoptotic activity is restricted to the cytoplasm and the nuclear DNA is not fragmented. This could explain why we did not detect DNA fragmentation in the defective elongating spermatids in $Brdt^{\Delta BD1/\Delta BD1}$ mutant testes. In fact, caspase-activated DNase (CAD), the endonuclease responsible for the apoptotic DNA fragmentation, is not essential in mice (Kawane et al., 2003). CAD-knockout mice are grossly normal and cells from CAD-deficient mice could undergo apoptosis, but without DNA fragmentation. When apoptotic cells were phagocytosed by macrophages, their DNA was degraded by DNase II (Kawane et al., 2003).

Finally, it is of great interest to note that there are concurrent studies that suggest a role for *BRDT* during human spermatogenesis as well. It should be recalled that this member of the BET sub-family was actually first identified in humans and so designated *BRDT* because it was testis-specific (Jones et al., 1997). Khochbin and colleagues (S. Rousseaux and S. Khochbin, personal communication) are studying the molecular basis of male infertility, in particular those individuals who are otherwise healthy but have defective spermatogenesis. They have recently observed four polymorphisms in the DNA from infertile men that could result in specific amino acid changes in the human BRDT protein. One of these putative mutations is of particular interest because it represents a non-conservative change in a highly conserved amino acid within the first bromodomain of BRDT.

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