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The Role of the Double Bromodomain-Containing BET Genes During Mammalian Spermatogenesis

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Abstract

The double bromodomain-containing BET (bromodomain and *extra terminal*) family of proteins is highly conserved from yeast to humans and consists not just of transcriptional regulators but also histone-interacting chromatin remodelers. The four mammalian BET genes are each expressed at unique times during spermatogenesis, and the testis-specific gene *Brd1* is essential for spermatogenesis. Loss of the first bromodomain of BRDT results in improper/incomplete spermatid elongation and severely morphologically defective sperm. The elongation defects observed in mutant spermatids can be directly tied to altered postmeiotic chromatin architecture. BRDT is required for creation/ maintenance of the chromocenter of round spermatids, a structure that forms just after completion of meiosis. The chromocenter creates a defined topology in spermatids, and the presence of multiple chromocenters rather than a single intact chromocenter correlates with loss of spermatid polarity, loss of heterochromatin foci at the nuclear envelope, and loss of proper spermatid elongation. BRDT is not only essential for proper chromatin organization but also involved in regulation of transcription and in cotranscriptional processing. That is, transcription and alternative splicing are altered in spermatocytes and spermatids that lack full-length BRDT. Additionally, the transcription of mRNAs with short 3' UTRs, which is characteristic of round spermatids, is also altered. Examination of the genes regulated by BRDT yields many possible targets that could in part explain the morphologically abnormal sperm produced by the BRDT mutant testes. Thus, BRDT and possibly the other BET genes are required for proper spermatogenesis, which opens up the possibility that the recently discovered small molecule inhibitors of the BET family could be useful as reversible male contraceptives.

1. INTRODUCTION

1.1. The bromodomain and bromodomain-containing proteins

The bromodomain is a conserved protein motif found in all eukaryotes and is the only known protein module that binds acetylated lysine residues (Mujtaba, Zeng, & Zhou, 2007; Sanchez & Zhou, 2009). This motif was first discovered in the *Drosophila* gene *brhma* (*brm*) from which it derives its name (Haynes et al., 1992; Tamkun et al., 1992), and it has subsequently been identified in genes from yeast to humans. There are 42 mammalian

bromodomain-containing genes, 12 of which contain multiple bromodomains (Arrowsmith, Bountra, Fish, Lee, & Schapira, 2012; Yang, 2004).

In light of the prevalence of the acetyl posttranslational modification, proteins that can “read” this mark have a wide variety of functions. Not surprisingly, bromodomains are found in proteins with diverse functions, including histone acetyltransferases (Jacobson, Ladurner, King, & Tjian, 2000; Mishima et al., 2011; Ohshima, Suganuma, & Ikeda, 2001; Wang et al., 1997), histone methyltransferases (Gregory et al., 2007; Hess, 2004), histone kinases (Xiao et al., 2009), transcriptional coactivators (Denis, 2010; Ogryzko, Schiltz, Russanova, Howard, & Nakatani, 1996; Tamkun, 1995), transcriptional corepressors (Sankar et al., 2008; Tae et al., 2011; Zeng et al., 2008; Zhou & Grummt, 2005), ubiquitin ligases (Agricola, Randall, Gaarenstroom, Dupont, & Hill, 2011; Allton et al., 2009), sumo ligases (Liang et al., 2011), and chromatin remodelers (Liu, Mulholland, Fu, & Zhao, 2006; Racki et al., 2009; Thompson, 2009; Wilson & Roberts, 2011). The ~110 aa-long bromodomain motif folds into two loops, each containing two alpha helices, thereby creating a hydrophobic pocket between them that interacts with acetylated lysine (Florence & Faller, 2008; Owen et al., 2000). Bromodomains have been shown to bind to acetylated lysines both in histone tails (Jacobson et al., 2000; Jeanmougin, Wurtz, Le Douarin, Chambon, & Losson, 1997) and in nonhistone proteins (Barlev et al., 2001; Gamsjaeger et al., 2011).

1.2. The BET subfamily of bromodomain-containing proteins

The BET (bromodomain and extra terminal) family of bromodomain-containing proteins is so-called because each member contains an N-terminal bromodomain and a unique C-terminal *extra terminal* (ET) domain that is found only in this family (Florence & Faller, 2001). In plant BETs, there is only a single bromodomain, but all other BET members contain a pair of tandem bromodomains near the N-terminus. The ET domain is a protein–protein interacting domain (Matangkasombut, Buratowski, Swilling, & Buratowski, 2000; Platt, Simpson, Mitnacht, & Schulz, 1999) composed of two alpha helices followed by a loop and then a beta sheet and another alpha helix. At the very C-terminus of the ET domain is often found a SEED motif, which comprises serine, aspartic acid, and glutamic acid residues. The SEED domain is found in some but not all plant BET proteins but is conserved from yeast to humans. There are four mammalian BET genes, *Brd2*, *Brd3*, *Brd4*, and *Brdt*, two in yeast, *BDF1* and *BDF2*, and the founding member of the BET family, the *Drosophila female sterile (1) homeotic (fs(1)h)* gene, is the only fly BET gene (Fig. 11.1).

The presence of a single bromodomain in BET proteins in plants, versus the tandem domains in other eukaryotes, has led to the hypothesis that the tandem bromodomains arose from duplication within an ancestral BET gene. Bromodomain 1 (BD1) and bromodomain 2 (BD2) of the BET family genes have since diverged sufficiently such that there is greater similarity between BD1 or BD2 from different mammalian BET proteins than there is between BD1 and BD2 in a single BET protein. For instance, BD1 and BD2 of mouse *Brdt* are 73% and 76% identical to BD1 and BD2 of mouse *Brd2*, respectively, but BD1 and BD2 of mouse *Brdt* are only 40% identical to each other (Fig. 11.1). Interestingly, the ET domain of mammalian BET proteins is even more highly conserved than either bromodomain. For example, the ET domains of mouse *Brdt* and mouse *Brd2* are 85% identical (Fig. 11.1). Two other minor motifs, named the A and B motifs, are present in all four mammalian BETs and *Drosophila fs(1)h*; however, motif A is absent from the yeast BET genes (Paillisson et al., 2007).

1.2.1 *Drosophila female sterile (1) homeotic*—*Drosophila fs(1)h* was the first BET gene identified and was discovered in a screen for genes with a maternal effect phenotype

(Gans, Audit, & Masson, 1975; Gans, Forquignon, & Masson, 1980). Although *fs(1)h* is the only *Drosophila* BET gene, it encodes two distinct protein isoforms: fs(1)hS and fs(1)hL (Haynes, Mozer, Bhatia-Dey, & Dawid, 1989). The fs(1)hS protein contains both bromodomains and the ET domain. The fs(1)hL protein contains the entire short isoform (except for 4 aa near the C-terminus) but also has an ~1000 aa-long tail (Fig. 11.1). Numerous mutant alleles of *fs(1)h* have been characterized at the molecular level, revealing modular functions for the fs(1)h protein. The first such mutations were temperature-sensitive mutant alleles; however, it is important to note that even at high nonpermissive temperatures, fs(1)h protein was present, albeit at low levels, suggesting that these mutations do not reflect complete loss of function (Florence & Faller, 2008; Gans et al., 1975, 1980). When females were shifted to high (26.5–29° C) temperatures, a reduced number of nonproductive eggs were laid such that the flies were considered to be infertile. Loss of fs(1)h function resulted in alteration of *Ultrabithorax* (*Ubx*) and *trithorax* (*trx*) expression, which in turn caused late segmentation defects (Huang & Dawid, 1990). In a study in which a highly conserved aspartic acid residue in BD1 was mutated to valine, a different mutant phenotype was observed (Florence & Faller, 2008). These mutant flies were also sterile, but the maternal effects resulted in head and gut defects caused by loss of repression of *tailless* (*tll*) and *hückerbein* (*hkb*) at the initiation of transcription during embryogenesis. A mutant allele that contained both intact bromodomains but lacked the ET domain showed a phenotype that was similar to the temperature-sensitive mutants, with altered transcription of segment genes, but no head and gut defects (Haynes et al., 1989). The reciprocal nature of the two phenotypes suggests separate functions for the different regions of the fs(1)h protein.

1.2.2 Yeast BDF1 and BDF2—In the yeast *Saccharomyces cerevisiae*, there are two BET genes, *BDF1* and *BDF2*, of which *BDF1* is far more extensively studied. *BDF1* was discovered in a screen for genes affecting snRNA transcription (Lygerou et al., 1994) and along with *BDF2* has been shown to be a general transcription factor (Durant & Pugh, 2007; Matangkasombut et al., 2000). Mutational studies of *BDF1* have also identified a number of other functions beyond its role as a component of the basal transcriptional machinery. Early work on *BDF1* found that although *BDF1* null cells were viable, there were defects in metabolism of certain sugars and meiosis was abnormal (Chua & Roeder, 1995). Interestingly, mutations in BD1 and the SEED domain had no effect on meiosis, suggesting that BDF1, like fs(1)h, has modular functions. *BDF1* has also been shown to affect transcription by delineating heterochromatin–euchromatin boundaries (Ladurner, Inouye, Jain, & Tjian, 2003). That is, BDF1 binds to acetylated H4 found in euchromatin adjacent to constitutive heterochromatin. Its physical presence on chromatin competes with the SIR2 and SIR3 histone deacetylases and stops them from spreading beyond the normally transcriptionally silenced chromatin. Mutation of both bromodomains abolished BDF1's histone-binding and resulted in transcriptional downregulation of genes located near constitutive heterochromatin, reflecting aberrant spreading of heterochromatin.

2. MAMMALIAN BET GENE EXPRESSION DURING SPERMATOGENESIS

All four mammalian BET genes are expressed in the adult testis and the timing of that expression is highly regulated (Shang et al., 2004). BRD4 is strongly expressed and BRD2 more weakly expressed in spermatogonia; however, exactly which types of spermatogonia expresses these two proteins has not yet been determined. BRD2 protein is most strongly expressed in late spermatocyte stages and round spermatids. *Brd3* mRNA is strongly expressed in round spermatids, and interestingly, two distinct mRNA species exist in the testis, the full-length 6.2 kb transcript, which is found in somatic cells, and a testis-specific 3.1 kb transcript (Shang et al., 2004). The significance of this transcript is unknown and nothing is known about BRD3 protein in the testis. There may also be two *Brdt* mRNA

transcripts expressed in the murine testis, but the data to date are conflicting. The initial finding was that a short 1.9 kb transcript and a long 3.8 kb transcript were equally abundant in the murine testis (Taniguchi et al., 2001). Another group subsequently reported an abundant 3.5 kb transcript and a less abundantly expressed 4.0 kb transcript (Pivot-Pajot et al., 2003). A third group found only a highly abundant 3.8 kb transcript (Shang et al., 2004). *Brd1* mRNA is most highly expressed in spermatocytes and only to a lesser extent in round spermatids (Shang et al., 2004). BRDT protein expression is present throughout the pachytene stage into diplotene spermatocytes (Shang, Nickerson, Wen, Wang, & Wolgemuth, 2007). However, there are conflicting observations as to whether the protein is present in round spermatids and elongating/compacting spermatids. The first report on BRDT protein expression observed no protein present in round spermatids but strong expression in elongating spermatids that perfectly colocalized with hyperacetylated histone H4 (Govin et al., 2006). This was in direct contradiction to a later report that found BRDT protein highly expressed in round spermatids but absent from elongating spermatids (Shang et al., 2007). Both of these reports examined murine BRDT; a subsequent study reported that rat BRDT was expressed in round spermatids and also in elongating spermatids (Dhar, Thota, & Rao, 2012). However, the expression in elongating spermatids did not extensively overlap with hyperacetylated H4 (only 20%), in contrast to the perfect colocalization that had been observed by Govin et al. (2006). Differences in the specificity and/or sensitivity of the antibodies used in these studies may be responsible for these discrepancies. In this light, it should be noted that the antibody used in Shang et al. (2007) was capable of specifically detecting the truncated BRDT protein as well, thus confirming that it does specifically recognize BRDT.

All three reports do seem to agree that BRDT protein is absent from the nucleus after the removal of the majority of canonical histones is complete. However, this is in contrast to what has been reported in human testis: BRDT protein has been reported in spermatocytes, round and elongated spermatids, and fully mature spermatozoa (Barda et al., 2012; Zheng, Yuan, Zhou, Xu, & Sha, 2005). It should also be noted that there are seven different *BRDT* mRNA transcripts in human testes annotated in the RefSeq database, ranging in length from 3.0 to 3.7 kb. Further, a BRDT splice variant containing seven extra amino acids, termed BRDT-NY, is the isoform found in mature sperm (Zheng et al., 2005). A summary of murine BET testicular expression, including probable BRDT expression, is depicted in Fig. 11.2.

2.1. Mammalian BRD2

Human *BRD2* (originally known as *RING3*) and mouse *Brd2* (originally known as *Fsrg1*) were the first mammalian BET genes discovered (Beck, Hanson, Kelly, Pappin, & Trowsdale, 1992; Rhee, Brunori, Besset, Trowsdale, & Wolgemuth, 1998). Among the murine BETs, *Brd2* is similar to *fs(1)hS* as it has no long C-terminal tail (Fig. 11.1). Recent studies have revealed that the regulation of *BRD2/Brd2* expression is complex (Shang et al., 2011). *BRD2* can be transcribed from multiple promoters which leads to transcripts with both long and short 5' UTRs, and an alternate splice form exists in which a region of intron 2 is included in transcripts as exon 2A. Only the transcripts with a short 5' UTR and lacking exon 2A yielded translation of full-length protein *in vitro* and *in vivo* in cultured cells (Shang et al., 2011). Mouse *Brd2* is ubiquitously expressed and long and short transcripts are found in all tissues (Taniguchi et al., 1998), although the ratio of long to short differed. *Brd2* has been reported to be highly expressed in hormonally modulated epithelia, including the mammary gland, ovary, kidney, and uterus (Rhee et al., 1998; Trowsdale & Wolgemuth, 2004), as well as in the developing brain and the adult cerebellum and cerebral cortex (Shang et al., 2011).

Like BDF1/2 and fs(1)h, BRD2 has been shown to modulate transcription, in particular, in cell cycle-induced transcriptional activation. For example, BRD2 was found to translocate from cytoplasm to nucleus upon serum stimulation (Denis, Vaziri, Guo, & Faller, 2000; Guo, Faller, & Denis, 2000) and was shown to be nuclear in proliferating cells but cytoplasmic in nondividing, more differentiated cells (Crowley, Brunori, Rhee, Wang, & Wolgemuth, 2004). In the nucleus, BRD2 can act as a scaffold, interacting with TATA-binding protein (TBP) through its BD1 (Peng et al., 2007) and with E2F through the ET domain (Denis et al., 2000; Guo et al., 2000) to activate cell cycle regulatory genes that contain E2F-binding sites in their promoters. BD1 of BRD2 has been shown to bind H4K12ac as a dimer (Kanno et al., 2004) and BD2 binds H4 diacetylated on K5 and K12 (Huang et al., 2007). Although acetylated H4 is often present in the promoters of transcriptionally active genes, BRD2 does not directly interact with the promoters but rather through its association with E2F (Sinha, Faller, & Denis, 2005). It has also been reported that BRD2 binds to the acetylated chromatin in the body of transcriptionally active genes and aids in polymerase progression through the gene (LeRoy, Rickards, & Flint, 2008).

2.2. Mammalian BRD3

Brd3, which also lacks a long C-terminal tail, is thus more closely homologous to *Brd2* than to other mammalian BET genes (Fig. 11.1) and is also ubiquitously expressed (Thorpe et al., 1997) but is the least studied of the mammalian BET genes. BRD3 has been reported to bind via BD1 to GATA1 that was either diacetylated on residues K312 and K315 or tetraacetylated on residues K312, K314, K315, and K316 both *in vitro* and *in vivo* in an erythroid cell line (Gamsjaeger et al., 2011; Lamonica et al., 2011). One study showed that the binding only occurred in the presence of CREB-binding protein, and this binding was required for complete occupancy of GATA1 at its target promoters in erythroid cells (Lamonica et al., 2011). Thus, direct binding of BRD3 to the promoter region of a gene has also yet to be shown, but like BRD2, BRD3 has been reported to bind the acetylated lysines in transcription factors and in chromatin in the body of transcriptionally active genes (LeRoy et al., 2008).

2.3. Mammalian BRD4

Brd4, unlike *Brd2* and *Brd3*, encodes a long C-terminal region after the ET domain and is thus most homologous structurally with *fs(1)h* (Fig. 11.1). Like *fs(1)h*, *Brd4* also produces a short and a long protein variant (Dey et al., 2000; Florence & Faller, 2001). The short variant of BRD4 contains both bromodomains and the ET domain, but due to a unique splicing event, the last 3 aa of the short isoform are different from the amino acid sequence of the long isoform (similar to what occurs in *fs(1)h*). The C-terminal region is over 750 aa long, but the only known motif present is at the very 3' end and has been named the C-terminal domain (CTD) (Wu & Chiang, 2007). This domain is also present in fs(1)hL.

BRD4 has been well studied as a transcriptional coactivator. BRD4 binds to the positive transcription elongation factor b (P-TEFb) and liberates it from inhibitory binding by HEXIM1. Via BRD4's binding to acetylated histones, it recruits the complex to the promoters of genes poised to be transcribed (Jang et al., 2005; Yang, He, & Zhou, 2008; Yang et al., 2005). However, the mechanism of binding of BRD4 to P-TEFb is somewhat in dispute. The initial report found that BRD4 bound to cyclin T1 (CCNT1), a subunit of P-TEFb, via either of the bromodomains but that an amino acid change in the other subunit of P-TEFb, cyclin-dependent kinase 9 (CDK9), abolished binding (Yang et al., 2005). A later report found that it was the CTD, not the bromodomains, that bound P-TEFb and that the CTDs of BRD2 and fs(1)hL were also capable of this interaction (Bisgrove, Mahmoudi, Henklein, & Verdin, 2007). A third report found that it was the CTD that bound P-TEFb and that both subunits, CDK9 and CCNT1, were bound (Rahman et al., 2011). This last report

also found that the ET domain of BRD4 (and BRD2 and BRD3 as well) binds to transcriptional activators GLTSCR1, NSD3, and JMJD6 and carries out P-TEFb-independent transcriptional activation (Rahman et al., 2011). Finally, a very recent report found that both observations may be correct, as it was observed that the BD2 in short BRD4 isoforms (lacking the C-terminal tail) bound to a triacetylated form of CCNT1 (Schroder et al., 2012). However, this binding did not alleviate inhibitory binding of HEXIM1, and indeed, binding of BRD4 to P-TEFb through its CTD was required to relieve this inhibition.

BRD4 is also of great interest because it has been reported to be retained on mitotic chromosomes when most chromatin interacting proteins are removed (Dey, Nishiyama, Karpova, McNally, & Ozato, 2009; Dey et al., 2000; Nishiyama, Dey, Miyazaki, & Ozato, 2006). It has been postulated that this “epigenetic memory” would allow for rapid BRD4-dependent transcription of M-G(1) cell cycle genes via P-TEFb recruitment (Ai et al., 2011; Yang et al., 2008). Additionally, it was found that BRD4 plays a role in overall chromatin architecture as, via its CTD, it maintains higher order chromatin structure (Wang, Li, Helfer, Jiao, & You, 2012).

2.4. Mammalian BRDT

Brdt is unique among other BET proteins in that it is nearly exclusively expressed in the testis (Jones, Numata, & Shimane, 1997; Pivot-Pajot et al., 2003; Shang et al., 2004; Taniguchi et al., 2001). Although one report showed mRNA expression in oocytes (Paillisson et al., 2007), this is not confirmed by three other reports (Jones et al., 1997; Pivot-Pajot et al., 2003; Shang et al., 2004). Very low levels of expression in the brain were reported by one group as well (Shang et al., 2004). It is possible that *Brdt* is expressed only in very specific regions of the brain, as there are reports in the databases of its being detected in olfactory neurons [GEO series accession GSE26373 (Clowney et al., 2011)]. Like *Brd4*, *Brdt* has a long C-terminal region after the ET domain which contains a CTD at the very C-terminus, making it most similar structurally to *Brd4* and *fs(1)h* (Florence & Faller, 2001; Wu & Chiang, 2007). Whether a short *Brdt* mRNA, comparable to *fs(1)hS* or short *Brd4*, exists is not clear. No such isoform has been reported in humans (Jones et al., 1997) and no short BRDT protein isoform has been observed to date in mouse or human.

3. BRDT IS REQUIRED FOR PROPER SPERMATOGENESIS AND MALE FERTILITY

The first genetic approach to elucidate BRDT's function generated mice with a 5' deletion in the endogenous *Brdt* gene that includes the first bromodomain (*Brdt*^{ΔBD1}) (Shang et al., 2007). Consistent with the expression pattern of *Brdt*, homozygous mutants were viable and females were fully fertile. However, while truncated mutant BRDT protein (BRDT^{ΔBD1}) was produced in this mouse at levels comparable to control, and in the same cell types, the homozygous *Brdt*^{ΔBD1/BD1} mice were completely sterile. Additionally, a *BRDT* allele with a point mutation in between BD2 and the B motif that converts an asparagine residue to lysine was found to associate with male infertility in humans (Plaseski, Noveski, Popeska, Efremov, & Plaseska-Karanfilska, 2011). Thus, complete BRDT function, and specifically the first bromodomain, is required for male fertility.

3.1. Chromocenter formation and maintenance

The first visible morphological defect in *Brdt*^{ΔBD1/ΔBD1} mutant testes was observed in step 1 round spermatids (Berkovits & Wolgemuth, 2011). In these cells, the pericentromeric heterochromatin of each spermatid is normally coalesced together in a chromatin structure known as the chromocenter (Meyer-Ficca, Muller-Navia, & Scherthan, 1998; Zalensky et al., 1995). This structure persists throughout spermatid differentiation and can still be found

in mature spermatozoa (Hoyer-Fender, Singh, & Motzkus, 2000). In *Brd^{ΔBD1/ΔBD1}* spermatids, instead of a single focus of heterochromatin in the chromocenter, multiple heterochromatic foci were observed in the majority of spermatids (Berkovits & Wolgemuth, 2011). This multiple chromocenter defect was observed at about the same frequency in all stages of round spermatid differentiation and indeed was observed at the earliest stage of round spermatid differentiation, just after completion of meiosis. Whether the defect in chromatin organization occurred during meiosis in these mutant cells but was only observable postmeiotically or whether the defect originated after the completion of MI remains to be determined.

The mechanisms that result in the coalescence of the centromeric heterochromatin into the chromocenter are not well understood. TATA box-binding protein-like 1 (TBPL1) is required for chromocenter formation but is not itself present in early round spermatids (Martianov et al., 2002). It has been suggested that, in spermatocytes, TBPL1 might regulate the transcription of factors required for chromocenter formation. Loss of high mobility group box 2 (HMGB2) leads to reduced male fertility (Ronfani et al., 2001) and chromocenter defects (Catena, Ronfani, Sassone-Corsi, & Davidson, 2006). This protein associates with euchromatin in spermatocytes and may also regulate transcription of factors involved in chromocenter formation. Interestingly, HMGB family members have been shown to interact with TATA box-binding proteins (Das&Scovell, 2001), suggesting a possible link with TBPL1. Even more interestingly, careful inspection of the data in Ronfani et al. (2001) suggests that HMGB2 appears to be localized within the chromocenter of round spermatids, although the authors do not discuss this. Thus, HMGB2 could play a role in creating and/or maintaining the chromocenter.

BRDT, in contrast, is excluded from the heterochromatin of the chromocenter but exhibits an increased localization directly adjacent to that heterochromatin (Berkovits & Wolgemuth, 2011). As mentioned briefly earlier, yeast BDF1 is known to localize at constitutive heterochromatin–euchromatin boundaries, where it maintains the boundary by halting the spreading of SIR2/3 into euchromatin (Ladurner et al., 2003). The SIR deacetylases in conjunction with the SU(VAR)3-9 methyltransferase are responsible for generating trimethylated H3K9, which is the hallmark of heterochromatin (Schotta, Ebert, & Reuter, 2003; Shankaranarayana, Motamedi, Moazed, & Grewal, 2003). In round spermatids, the mammalian SU(VAR)3-9 homologs SUV39H1/2 are localized in the chromocenter (O'Carroll et al., 2000), but the SIR2 homolog Sirtuin 1 (SIRT1) is found in euchromatin, except in the region of high BRDT expression directly around the chromocenter (Berkovits & Wolgemuth, 2011). In this way, BRDT appears to create a “buffer” region between the deacetylase and the methyltransferases and thus halts ectopic spreading of pericentromeric heterochromatin. Indeed in the *Brd^{ΔBD1}* mutant, SIRT1 is present directly adjacent to the chromocenter (see cartoon in Fig. 11.3), and the level of heterochromatin in the testis is increased by greater than threefold (Berkovits & Wolgemuth, 2011). It may be that an expanded region of heterochromatin keeps the centromeres from coalescing or creates instability in the chromocenter that leads to its fragmentation. It is also possible, and not mutually exclusive, that BRDT modulates gene expression in spermatocytes, as has been hypothesized for TBPL1 and HMGB2. Or as previously mentioned, BRDT may alter the chromatin architecture of spermatocytes in such a way that meiosis can be completed, but the chromocenter cannot form or be maintained properly.

3.2. BRDT and spermatid elongation

The failure to form a singular intact chromocenter correlates with many of the later defects observed in *Brd^{ΔBD1/ΔBD1}* spermatids. At step 9, when spermatid elongation begins in control testes, *Brd^{ΔBD1/ΔBD1}* spermatids fail to elongate properly, with some spermatids

exhibiting stunted elongation and some not elongating at all (Berkovits & Wolgemuth, 2011; Shang et al., 2007). Although chromatin compaction appears to occur in condensing spermatids, without proper elongation condensed but still round spermatid nuclei result. A model proposing a direct connection between the multiple chromocenter defect and loss of nuclear shaping is presented in Fig 11.4. Whole chromosome domains are arranged around the chromocenter, suggesting that the chromocenter is a central organizing structure that helps create a defined nuclear topology in spermatids (Haaf & Ward, 1995; Meyer-Ficca et al., 1998). This hypothesis has been supported by later findings in three different mutant models, which showed that loss of a singular intact chromocenter can lead to mislocalization of chromatin domains (Berkovits & Wolgemuth, 2011; Catena et al., 2006). Specifically, the presence of multiple chromocenters correlates with a loss of localization of the testis-specific histone variant H1FNT to the nuclear region adjacent to the acrosome (Martianov et al., 2005). In these cells, H1FNT is eventually distributed around the periphery of the nucleus instead of being restricted to the postacrosomal region. H1FNT localization has been implicated in blocking the formation of heterochromatin foci around the nuclear periphery of spermatids (Martianov et al., 2005). Indeed, in *Brdt*^{ΔBD1/ΔBD1} spermatids, these foci are not localized to the nuclear periphery (Shang et al., 2007). Heterochromatin foci are hypothesized to connect spermatid chromatin to the actin network in the sperm head and thus coordinate sperm head shaping. In their absence, the sperm nucleus has no tether by which it can be elongated. Although the Sertoli cell's contractile ring may still exert a force on the nucleus to narrow it, the disconnect between the nucleus and the cytoskeleton leads to incomplete or absent elongation.

The link between the chromocenter and proper spermatid nuclear elongation was most evident when the *Brdt*^{ΔBD1} mutation was examined on two different pure genetic backgrounds (Berkovits & Wolgemuth, 2011). On a pure 129 Sv/Ev background, *Brdt*^{ΔBD1/ΔBD1} mice exhibited highly penetrant chromocenter and elongation defects. By contrast, although *Brdt*^{ΔBD1/ΔBD1} mutant mice on a C57Bl/6 background were infertile, the chromocenter defect was far less pronounced as was the elongation defect. That these two aspects of the *Brdt*^{ΔBD1} phenotype should so closely correlate in severity with respect to the specific genetic background strongly supports the link between the chromocenter and spermatid elongation.

3.3. BD1 of BRDT is dispensable for completion of meiosis

As BRDT is expressed from the pachytene through diplotene stages of spermatocytes, we predicted that it would have an important function during meiosis. However, no overt defects were observed in ΔBD1 spermatocytes, and both meiosis I and meiosis II were successfully completed with abundant numbers of round spermatids forming (Shang et al., 2007). Loss of the first bromodomain did lead to an increased expression of the testis-specific histone variant HIST1H1T in spermatocytes and BRDT was shown to bind to a distal region of the *Hist1H1t* promoter (Shang et al., 2007). However, HIST1H1T is dispensable for meiosis as *Hist1h1t* homozygous mutant mice are fertile (Fantz, Hatfield, Horvath, Kistler, & Kistler, 2001; Lin, Sirotkin, & Skoultschi, 2000). Additionally, BRDT was shown to interact with several components of the spliceosome in the testis, and alteration of splicing of at least one target gene was observed in spermatocytes (Berkovits, Wang, Guarnieri, & Wolgemuth, 2012).

We hypothesize that loss of the BD1 does not represent a complete loss of BRDT function, as BET proteins are known to be highly modular. It is therefore possible, even probable, that BRDT is indispensable for meiosis, but that other domains (BD2, the ET domain, the CTD) carryout this function. Such a case is observed in the yeast BET gene *BDF1*. *BDF1* is essential for sporulation and *BDF1* null yeast has defects in both meiosis I and meiosis II

and fail to complete meiosis (Chua & Roeder, 1995). However, when mutations were made in specific regions of BDF1, only mutations in BD2 led to failed meiosis. Mutations in BD1 or the ET domain had no overt effect on the completion of meiosis. Thus, although the BD1 of BRDT is not essential for meiotic completion, the BD2 of BRDT may well be involved.

4. BRDT AND REGULATION OF TRANSCRIPTION AND POSTTRANSCRIPTIONAL PROCESSING DURING SPERMATOGENESIS

The BET family of proteins does not directly bind to DNA but rather binds acetylated histone tails and other acetylated proteins and, via these interactions, have been shown to be transcriptional modulators (Florence & Faller, 2001; Lamonica et al., 2011; Peng et al., 2007; Wu & Chiang, 2007). BD1 of BRDT has been shown to bind histone H4 diacetylated on K5 and K8 (Moriniere et al., 2009). Acetylation of H4 tails is well known to be associated with euchromatin (Suka, Suka, Carmen, Wu, & Grunstein, 2001) and active transcription (Grunstein, 1997). It is also known that BRD4, as part of the P-TEFb complex, regulates the active transcription of a large number of genes via BRD4's interaction with acetylated H3 and H4 (Yang et al., 2005). BRD4's binding to the components of the P-TEFb complex (cyclin T and CDK9) is mediated by the CTD of BRD4, and the CTD of BRDT was also found capable of this interaction (Bisgrove et al., 2007). The CTD domain is not present in BRD2 or BRD3; thus BRDT is the only BET protein that would be capable of carrying out P-TEFb-mediated transcriptional regulation in spermatocytes or spermatids (as BRD4 is not expressed in these cells). As previously mentioned, the first documented transcriptional regulation mediated by BRDT was repression of *Hist1h1t* expression and not activation (Shang et al., 2007).

4.1. BRDT and transcriptional regulation

The *Brd^tΔBD1/ΔBD1* phenotype also revealed acrosome detachment, excess cytoplasm, malformed tails and heads, and reduced motility (Berkovits & Wolgemuth, 2011; Shang et al., 2007), defects that cannot all be easily explained by alterations in the chromocenter. The simplest explanation for these defects would be that BRDT is indeed a transcriptional modulator, like other BET family members, and in spermatids, BD1 is required for proper transcription of essential mRNAs, some of which are translated later in spermiogenesis. The phenomenon of translational delay is well characterized in spermatids, as transcription is shutoff after step 8, mRNAs for proteins required for later stages of differentiation must be transcribed prior to step 9 of spermiogenesis and sequestered until later translation (Steger, 2001).

Microarray analysis of control and *Brd^tΔBD1/ΔBD1* mutant round spermatids has indeed demonstrated that a significant alteration in transcription occurs in the absence of BD1 of BRDT (Berkovits et al., 2012). Over 1000 genes were upregulated and >400 were repressed in mutant spermatids. Many of these genes were involved in many diverse functions that have putative roles in spermiogenesis (Table 11.1). Some of the more interesting genes with regard to the *Brd^tΔBD1/ΔBD1* phenotype include those involved in intracellular protein transport and protein localization, as the highly asymmetric nature of spermatozoa requires extensive cellular restructuring and protein localization (Kierszenbaum, Rivkin, & Tres, 2011). For example, defective Golgi vesicle transport could contribute to the observed acrosome defects. The enrichment of genes involved in cytoskeleton organization could also explain some of the sperm structural defects, especially in the tail. As glycolysis is the main source of ATP for flagellar motion (Miki et al., 2004), the inclusion of genes involved in glucose metabolism might also contribute to poor motility. It should be noted that direct binding of BRDT to the promoters of these genes has not been investigated, so it is not known whether BRDT directly regulates them, regulates them as part of a complex (possibly

P-TEFb), or whether BRDT may modulate transcription of an intermediate factor. That the transcription of only 400 genes (some of which may not be directly bound by BRDT) are normally activated by BRDT suggests that if BRDT does have a P-TEFb-dependent function, it may not be abrogated in the absence of the first bromodomain. Indeed, studies have shown that the BD2 of BRD4 in conjunction with the CTD is required for P-TEFb function (Schroder et al., 2012).

4.2. BRDT, the spliceosome, and 3' end formation

Protein-protein interaction studies from our lab suggested that another possible mechanism by which BRDT may regulate gene expression during spermatogenesis is via its interaction with the spliceosome (Berkovits et al., 2012). Mass spectrometry of proteins pulled down in 293T cells ectopically expressing full-length BRDT revealed that BRDT can complex with a variety of spliceosome components. The interaction of BRDT in testicular extracts with four such splicing proteins, SRSF2, DDX5, HNRNPK, and TARDBP, was confirmed, and interestingly, BD1 was not required for this interaction. However, TARDBP, a transcriptional corepressor as well as a splicing modulator, was aberrantly spliced in the mutant testis, demonstrating that while BRDT^{ΔBD1} may still be capable of complexing with the spliceosome, alternative splicing did not proceed normally. It is interesting to note that alternative splicing is more prevalent in the testis than in any organ other than the brain (Elliott & Grellscheid, 2006; Yeo, Holste, Kreiman, & Burge, 2004).

There was also a striking alteration in the processing of the 3' UTR of a large number of mRNAs in *Brdt*^{ΔBD1/ΔBD1} round spermatids (Berkovits et al., 2012). A preference for mRNA isoforms that use a more proximal polyadenylation site and thus have a shorter 3' UTR is a common feature of round spermatids (Liu et al., 2007). The 3' UTR is a target for RNA-binding proteins (Idler & Yan, 2012) as well as microRNAs (He, Kokkinaki, Pant, Gallicano, & Dym, 2009) and may be involved in the timing and rate of translation of the corresponding mRNA (Yu, Raabe, & Hecht, 2005). In mutant round spermatids, the presence of longer 3' UTRs correlated with markedly reduced protein levels for the three genes tested. The full extent of alteration in 3' UTR processing is not known due to the limited number of microarray probes for the majority of genes; however, at least 250 genes, for which there was a microarray probe both in the coding region or proximal 3' UTR and in the distal 3' UTR, are predicted to have extended 3' UTRs and reduced protein levels. Thus, 3' UTR processing, in addition to transcription and splicing, is another mechanism by which BRDT regulates postmeiotic gene function.

While nothing is known about how BRDT interacts with the spliceosome and the 3' end formation machinery, there are some observations as to how BRD4, as part of P-TEFb, interacts with these complexes. SRSF2, a major splicing factor and component of the spliceosome, was shown to have an active role in transcriptional elongation mediated by P-TEFb in cultured mouse embryonic fibroblasts (Lin, Coutinho-Mansfield, Wang, Pandit, & Fu, 2008). Reciprocally, ectopic P-TEFb function led to accumulation of SRSF2 and alterations in splicing in HeLa cells (Barboric et al., 2009). Although P-TEFb and the spliceosome appear to be linked in their control of transcriptional elongation and splicing, the prevailing hypothesis has been that both complexes interact with the CTD of Pol II, but not with each other (Lenasi & Barboric, 2010). It has been demonstrated that some genes with paused Pol II can proceed with low levels of transcription without phosphorylation of the CTD by P-TEFb, but these nascent transcripts lack cotranscriptional processing (Hargreaves, Horng, & Medzhitov, 2009). This suggests that P-TEFb is essential for not just splicing, but mRNA capping and 3' end maturation. Indeed, the cap-binding protein complex (CBC), which is essential for mRNA 5' capping, was also found in the complex with P-TEFb, SRSF2, and Pol II in HeLa cells (Lenasi, Peterlin, & Barboric, 2011). There is

no direct evidence that P-TEFb interacts with 3' end maturation proteins, but the CTD of Pol II (Licatalosi et al., 2002) and the spliceosome do (Martinson, 2011). As mentioned previously, we suggest that BRDT might replace BRD4 as part of P-TEFb in spermatocytes and spermatids and thus modulate transcription, splicing, and UTR processing.

5. BRDT AND LATER STAGES OF SPERMOGENESIS

5.1. BRDT and chromatin compaction

It had been previously reported that BRDT ectopically expressed in cultured cells causes an acetylation-dependent reorganization of chromatin (Pivot-Pajot et al., 2003). This reorganization of chromatin only occurred after the cells were treated with the deacetylase inhibitor trichostatin A (TSA), and both bromodomains and motif B were required. The significance of the ET domain, however, is less clear. The initial report found the ET domain to be dispensable, and in fact, a BRDT fragment lacking the ET domain exhibited enhanced chromatin reorganization (Pivot-Pajot et al., 2003). However, a later study found that a fragment with just the bromodomains and a fragment with the bromodomains and the ET domain had similar reorganization capabilities (Dhar et al., 2012). Whether these different results are due to the different cultured cells used (Cos-7 vs. RAG) is not known, and the general significance of this purported chromatin reorganizing ability is not at all clear.

Hyperacetylation of H4 and H2A beginning in step IX elongating spermatids is a well-documented component of chromatin remodeling during mammalian spermatid differentiation (Meistrich, Trostle-Weige, Lin, Bhatnagar, & Allis, 1992). Species that replace their canonical histones with transition proteins and then protamine all feature this hyperacetylation (Christensen & Dixon, 1982; Gatewood, Cook, Balhorn, Schmid, & Bradbury, 1990; Wolf, Jeppesen, & Mitchell, 1993), while species that do not replace their histones (Kennedy & Davies, 1980). This hyperacetylation is hypothesized to weaken the association of DNA to the histone octamer (Shogren-Knaak et al., 2006) and thus facilitate removal of canonical histones and replacement with first transition proteins and then protamines. Indeed, male mice lacking RNF8, a histone ubiquitin E3 ligase, are infertile due to a failure to deposit the transition proteins and protamines into chromatin (Lu et al., 2010). There is a loss of histone H2A ubiquitination and a loss of histone H4K16 acetylation in the elongating spermatids of these mice. Thus, ubiquitination of H2A is required for complete H4 hyperacetylation and loss of this hyperacetylation was concomitant with retention of canonical histones. The mechanism by which the histone to transition protein exchange is carried out is as yet unknown, and the possibility that BRDT plays a role is enticing. However, for BRDT to carry out such a function *in vivo* it would have to be present in hyperacetylated spermatids and colocalize with hyperacetylated H4. This was indeed reported by one group that examined BRDT and acetylated H4 expression in the mouse testis (Govin et al., 2006). As mentioned previously, while we clearly detected BRDT in murine round spermatids through step 8, we do not detect it in elongating spermatids (Shang et al., 2007) and a third study reported that the expression in elongating rat spermatids was restricted to the anterior region of the spermatid and only minimally overlapped with the nucleus (Dhar et al., 2012). It is difficult to understand how BRDT could be involved in the histone to transition protein exchange if it is not present when this event occurs or if it does not physically colocalize with the majority of nuclear chromatin.

The chromatin remodeling assay that was carried out in nonspermatogenic cells (Dhar et al., 2012; Pivot-Pajot et al., 2003) was extended to cultured round spermatids (Dhar et al., 2012). Round spermatids are normally hypoacetylated due to expression of histone deacetylases, and TSA is capable of inducing hyperacetylation in these cells (Hazzouri et al., 2000). However, when these cultured round spermatids were treated with TSA, there was no

observable chromatin remodeling (as had been seen in TSA-treated BRDT-transfected Cos-7 and RAG cells), despite the fact that round spermatids strongly express BRDT. Only upon overexpression by transfection with a BRDT-expressing plasmid was acetylation-dependent chromatin remodeling observed. Ectopic expression by plasmid transfection, in cells that already express BRDT, likely results in protein levels well beyond physiological relevance. However, there is the possibility that BRDT might normally require another factor for efficient chromatin reorganization and that this factor is only expressed after the round spermatid stage. Further, in the absence of this factor, abnormally high levels of BRDT might be required for reorganization.

It has been suggested that such a factor could be SMARCE1, a Swi/Snf chromatin remodeling factor (Dhar et al., 2012). SMARCE1 and BRDT were found to coimmunoprecipitate and the two proteins partially colocalize in spermatids. Additionally, colocalization was enhanced by H4 hyperacetylation. However, colocalization of BRDT and SMARCE1 was never greater than 20% of their total distribution of expression. While they may indeed interact, neither protein was abundant in elongating spermatid chromatin, and without localization throughout the hyperacetylated spermatid nucleus, it is difficult to envision these proteins facilitating either histone eviction or transition protein depositing.

Last, the phenotype of the *Brd*^{ABD1/ABD1} mutant is not consistent with a BD1-dependent role in chromatin compaction. The report of BRDT colocalizing with hyperacetylated H4 in elongating spermatids resulted in the suggestion that BRDT may itself physically compact chromatin (Govin et al., 2006). In the *Brd*^{ABD1/ABD1} mutant spermatids, severe morphological abnormalities were initially observed at step IX, just when hyperacetylation occurs (Shang et al., 2007), seemingly supporting this hypothesis. However, the suggestion that BRDT physically compacts acetylated chromatin is difficult to understand, as compaction is not characteristic of elongating hyperacetylated spermatids but rather only begins when transition proteins are integrated into chromatin at step XII (Meistrich, Mohapatra, Shirley, & Zhao, 2003). Thus, a defect at the onset of hyperacetylation temporally correlates with defective elongation and not condensation. Further, highly condensed but nonelongated spermatids are a prominent feature of the mutant testes on all genetic backgrounds (Berkovits & Wolgemuth, 2011; Shang et al., 2007), strongly suggesting that in mutant spermatids, condensation *per se* is not defective. Many other chromatin remodeling events occur during spermatogenesis (Govin, Caron, Lestrat, Rousseaux, & Khochbin, 2004; Lewis, Abbott, & Ausio, 2003), and theoretically may be regulated by BRDT; however, the canonical histone to transition protein to protamine exchange does not appear to be one of them.

5.2. BRDT in human spermatozoa

Although BRDT protein has not been observed after the removal of 85–95% of the canonical histones in step XII compacting rodent spermatids, BRDT protein has been reported to be present in human spermatozoa (Barda et al., 2012; Zheng et al., 2005). BRDT protein was detected throughout the sperm nucleus, although there did appear to be regions of more intense staining (Barda et al., 2012). The distribution of BRDT did not appear to colocalize with regions containing the retained hyperacetylated canonical histones, suggesting that it did not remain associated with hyperacetylated chromatin after the elongation stage of these cells. A number of transcription factors have been observed in mature spermatozoa and are suggested to have a role in early embryogenesis (Pittoggi et al., 2001). It has also been reported that in sperm nuclei, canonical histones are retained at the promoters of some genes whose transcription is required in the early embryo (Hammoud et al., 2009). Thus, it is possible that during early decompaction of the sperm nucleus, residual

BRDT interacts with these retained histones and is involved with modulating transcription of these early paternal genes.

6. BET INHIBITION AS A POSSIBLE CONTRACEPTIVE

6.1. BET family inhibition

There has been a recent explosion in the discovery of small molecules that specifically antagonize the interaction of BET bromodomains with their biological targets. At least five such molecules have been identified, all of which bind in the acetyl-lysine-binding pocket and thus block BET function (Dawson et al., 2011; Filippakopoulos et al., 2010; Hewings et al., 2011; Nicodeme et al., 2010; Zhang et al., 2012). Although BET bromodomains are structurally similar, they do bind very distinct targets and thus may have different binding affinities for these various inhibitory molecules. Compound JQ1 bound to the first bromodomains of the BET proteins with the following order of affinity: BRD4>BRD3>BRD2>BRDT, with very strong binding to the BD1 of BRD4, and very weak binding to the BD1 of BRDT (Filippakopoulos et al., 2010). The second bromodomains all bound JQ1 relatively equally, although the BD2 of BRDT was not investigated. The I-BET compound was found to bind in a ratio of two molecules to one BET protein, and it bound the tandem bromodomains of BRD2, BRD3, and BRD4 relatively equally (Chung, Coste, et al., 2011). I-BET binding to BRDT was not reported.

Inhibition of BRD4 was shown to downregulate transcription of the oncogene *c-Myc* in a variety of leukemia and lymphoma cell lines (Dawson et al., 2011; Mertz et al., 2011). In mouse models of multiple myeloma, lowering levels of c-MYC resulted in cellular senescence and tumor regression (Delmore et al., 2011). A cell line derived from a rare cancer known as NUT midline carcinoma, in which a translocation of the N-terminus of BRD4 or BRD3 is fused to the testis-specific gene NUT (French, 2010), showed squamous differentiation and loss of proliferation upon treatment with a BET inhibitor (Filippakopoulos et al., 2010). The inflammatory response can also be attenuated by BET inhibitors. One such molecule was shown to repress the transcription of inflammation genes in response to endotoxic shock both in cell lines and in mice (Nicodeme et al., 2010), and another molecule inhibited inflammatory transcription in response to HIV infection in kidney cells (Zhang et al., 2012).

Considering that all four BET proteins are expressed at various stages of male germ cell development, it seems likely that this class of small molecules would have effects on spermatogenesis. A molecule that specifically inhibited BRDT function would be the obvious choice for a male contraceptive. As BRDT bromodomains are the most divergent of any mammalian BET (Fig. 11.1), it would seem likely that a compound could be created that had the highest affinity for BRDT. This would alleviate a possible deleterious affect by a BET inhibitor in any other tissue. Further BRDT is not expressed in mitotically dividing spermatogonia, and thus a BRDT-specific inhibitor would not affect the spermatogonial stem cell population, theoretically making any spermatogenic impairment reversible.

As no BRDT-specific inhibitor has yet been reported, the question arises as to whether inhibition of the other three BET genes might also perturb spermatogenesis. BRD4 and, at lower levels, BRD2 are present in mitotically dividing spermatogonia. Homozygous null mutations of BRD4 and BRD2 are embryonic lethal (Houzelstein et al., 2002; Shang, Wang, Wen, Greenberg, & Wolgemuth, 2009), and as both genes regulate expression of cell cycle genes, it seems likely that their inhibition would disrupt spermatogonial division. Thus, while inhibition of BRD4 and BRD2 function would likely be an effective contraceptive, it is unclear whether such a disruption of spermatogenesis would be reversible. Maintenance of spermatogonial stem cells is required for continued fertility (Singh, Burnicka-Turek,

Chauhan, & Hou, 2011) and their number is the main determinant of spermatogenic recovery after insult (Meistrich, 1986, 1993). If the BET genes were inhibited in these cells permanent sterility might result. BRD3, however, did not appear to be expressed in spermatogonia, although its mRNA was abundantly detected in spermatids (Shang et al., 2004). Thus, inhibition of BRD3 might indeed be a useful and reversible method of contraception. However, as BRD3 is expressed in many adult tissue (Thorpe et al., 1997), the question of possible toxicity to other organs would need to be investigated.

Lastly, the possibility exists that even though the BET genes are expressed in other tissues, their inhibition may still function as an effective and reversible contraceptive. The testis is an acutely sensitive organ to damage (Bonde, 2010), and there are numerous examples wherein low level doses of a damaging agent resulted in sterility and no other phenotype (Boekelheide & Schoenfeld, 2001; Petrelli & Figa-Talamanca, 2001; Potashnik, Ben-Aderet, Israeli, Yanai-Inbar, & Sober, 1978). Further, inhibition of a widely expressed transcriptional regulator has been shown to have a dramatic and reversible effect on spermatogenesis but no abnormalities in any other tissue/organ (Chung, Wang, et al., 2011). Specifically, low doses of a retinoic acid receptor inhibitor (BMS 189453) over extended dosing periods caused reversible sterility in the mouse model. Thus, low doses of the current inhibitors might have a specific effect on spermatogenesis even if their targets are expressed in other tissues as well. Alternatively, even if the inhibitors only bind weakly to BRDT, this disruption might be sufficient to impair spermatogenesis. An in-depth study of the effect of BET inhibition on spermatogenesis is certainly needed, and a BET inhibitor with higher affinity for BRDT than the other BET proteins could be an ideal reversible male contraceptive.

7. CONCLUSIONS

The members of the BET family are generally thought of as transcriptional regulators, but their role as modulators of chromatin architecture is conserved from yeast to mammals. Both aspects of BET function are at play in the mammalian testis, where full-length BRDT is required both for proper meiotic and postmeiotic transcription and postmeiotic chromosome organization. BRDT is also required in the testis for cotranscriptional processing. This is hypothetically due to BRDT replacing BRD4 in the P-TEFb complex in spermatocytes and spermatids, and this hypothesis must be further investigated to fully understand both BRDT and BRD4 function. Given the distinct patterns of expression of all four BET family members in the testis, it is likely that they each have distinct roles—testing this hypothesis will require the use of conditional knockout models as both *Brd2* and *Brd4* mutant mice are embryonic lethal. The possibility of using BET bromodomain inhibitors as male contraceptives is promising, but much work is still needed to characterize these molecules potential targets, as well as their mechanisms of action, in the testis.

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Figure 11.1.

Structural comparison of the BET family members in *S. cerevisiae*, *D. melanogaster*, and *M. musculus*. The percentages under each bromodomain and the ET domain indicate the percentage of identical amino acid residues in the domain as compared to the corresponding domain in mBRD2, the founding member of the mouse BET family. The percentages under the CTD are compared to mBRD4.

Figure 11.2.

Expression patterns of the BET family in the adult mouse testis. Expression depicted for BRD2, BRD4, and BRDT is at the protein level, and for *Brd3*, it is at the mRNA level. Solid bars represent high levels of expression and dashed bars represent less abundant but detectable expression. *The figure is adapted from Shang et al. (2004).*

Figure 11.3.

Cartoon depicting chromocenter formation and function in control and *Brdt*^{ABD1} spermatids. BRDT protein is found throughout the nucleus (dark red) but is excluded from the chromocenter (black). In control spermatids, BRDT is more densely localized around the chromocenter (bright red), but this localization is lost in mutant spermatids. SUV39H1/2 (green) is located exclusively in the heterochromatin of the chromocenter both in control and mutant spermatids. SIRT1 (blue) is located throughout the nucleus except in the chromocenter and in the region of high BRDT localization. In control spermatids, the high localization of BRDT around the chromocenter might function to separate SIRT1 and SUV39H1/2. In mutant spermatids, the loss of BRDT localization allows overlap of SIRT1 and SUV39H1/2 which may cause ectopic heterochromatin.

Figure 11.4.

Model for how the presence of multiple chromocenters correlates with aberrant localization of H1FNT, loss of heterochromatin foci lining the nuclear membrane, and defective spermatid elongation. In control spermatids, an intact chromocenter (black circle) is present starting in step 1 spermatids. Starting in step 4 spermatids, H1FNT expression (red) begins and is restricted to a subacrosomal chromatin domain. In step 7 spermatids, heterochromatin foci (small black dots) begin to appear at the nuclear membrane in regions where H1FNT is not present. Nuclear elongation occurs in steps 9–11 spermatids, and chromatin condensation begins in step 12 spermatids and is completed in step 13 spermatids. In the *Brd1*^{ΔBD1} mutant, the majority of spermatids do not have a single intact chromocenter (large black spots). Although H1FNT is initially localized in the subacrosomal region, ectopic localization appears in late mutant round spermatids. In step 9 spermatids, H1FNT is abnormally localized just inside the entire nuclear membrane and blocks the localization of heterochromatin foci in this region. Mutant spermatid nuclei do not fully elongate or take on a normal hook shape. However, chromatin condensation still occurs in steps 12 and 13 spermatids. Heterochromatin is shown in black.

Table 11.1Biological processes enriched in genes upregulated in *Brdt^{ABD1}* round spermatids

Biological process	No. of genes	Possible genes of interest
mRNA processing	45	<i>Cnot6l</i> (RNA suppression), <i>Cstf2</i> (3' UTR), <i>Tardbp</i> (transcriptional repression), <i>Srsf2</i> (alternative splicing)
Intracellular transport	57	<i>Dynll2</i> (microtubule motor), <i>Hook1</i> (acrosome, manchette, microtubules), <i>Mtx2</i> (mitochondrial transport), <i>Kpna3</i> (nuclear import), <i>Cdc42</i> (acrosome, capacitation)
Macromolecular complex assembly	41	<i>Capza2</i> (F-actin cap), <i>Cdk5</i> (tail structure), <i>Tuba1a</i> and <i>Tuba1b</i> (microtubules), <i>Tubb3</i> and <i>Tubb5</i> (microtubules)
Protein localization	67	<i>Rab11a</i> (junction remodeling), <i>Rab18</i> (trafficking), <i>Rab7</i> (junction remodeling), <i>Mcfld2</i> (ER-Golgi transport), <i>Tmsb4x</i> (cytoskeletal capping)
RNA localization	14	<i>Thoc4</i> (RNA transport, protein interaction), <i>Fmr1</i> (translation, tail microtubules), <i>Srsf13a</i> (Crem splicing), <i>Qk</i> (mRNA stability, translation)
Modification-dependent protein catabolic process	49	<i>Cdap</i> (cytoskeletal polarity), <i>Ranbp2</i> and <i>Cbx4</i> (sumo ligases), 37 genes in the ubiquitination pathway
Chromosome organization	41	<i>Hp1bp3</i> (heterochromatin structure), <i>Sgol2</i> (protects centromeres), <i>Rbl1</i> (heterochromatin formation), <i>Cbx1</i> (Hp1 β)
Regulation of cytoskeleton organization	16	<i>Acp3</i> (actin polymerization), <i>Rac1</i> (sperm head), <i>Espn</i> (ectoplasmic specilization), <i>Plk4</i> (centriole, flagella)
Golgi vesicle transport	11	<i>Sec23a</i> (acrosome), <i>Lman1</i> (ER-Golgi transport), <i>Golga7</i> (Golgi to cell surface transport)
Glucose catabolic process	11	<i>Ldhh</i> (glycolosis), <i>Pgk1</i> (glycolosis first step), <i>Gapdh</i> (glycolosis), <i>Rpia</i> (carbohydrate metabolism), <i>Taldo1</i> (sperm mitochondria)
Negative regulation of gene expression	40	<i>Cttnb1</i> (acrosome, junctions), <i>Smarca5</i> (fertilization), <i>Wt1</i> (represses Cttnb1 signaling), <i>Khdrbs1</i> (transcription, mRNA sequestering)
Protein complex biogenesis and assembly	26	<i>Prk2b</i> (focal adhesion), <i>Tmem70</i> (mitochondrial ATP synthesis), <i>Gsn</i> (actin capping)