Two bromodomain proteins functionally interact to recapitulate an essential BRDT-like function in Drosophila spermatocytes

Shuhei Kimura and Benjamin Loppin

Centre de Génétique et de Physiologie Moléculaire et Cellulaire, CNRS UMR5334, Université Claude Bernard Lyon 1, 69622 Villeurbanne cedex, France

1. Summary

In mammals, the testis-specific bromodomain and extra terminal (BET) protein BRDT is essential for spermatogenesis. In Drosophila, it was recently reported that the tBRD-1 protein is similarly required for male fertility. Interestingly, however, tBRD-1 has two conserved bromodomains in its N-terminus but it lacks an extra terminal (ET) domain characteristic of BET proteins. Here, using proteomics approaches to search for tBRD-1 interactors, we identified tBRD-2 as a novel testis-specific bromodomain protein. In contrast to tBRD-1, tBRD-2 contains a single bromodomain, but which is associated with an ET domain in its C-terminus. Strikingly, we show that tbrd-2 knock-out males are sterile and display aberrant meiosis in a way highly similar to tbrd-1 mutants. Furthermore, these two factors co-localize and are interdependent in spermatocytes. We propose that Drosophila tBRD-1 and tBRD-2 associate into a functional BET complex in spermatocytes, which recapitulates the activity of the single mammalian BRDT-like protein.

2. Introduction

Bromodomains are highly conserved domains known to recognize acetylated lysine residues of histones and other proteins [1]. Members of the BET family of nuclear proteins typically have two bromodomains in their N-terminus associated with one extra terminal (ET) domain in their C-terminus [2]. The ET domain is considered as a protein–protein interacting domain [3] although its function remains unknown. Members of the BET family of proteins are generally well conserved in many species from yeast to human [2]. In mammals, there are four BET proteins, BRD2, BRD3, BRD4 and BRDT, which are well studied for their important roles as transcriptional co-regulators and cell-cycle regulators [4]. Recently, BET proteins were also identified as promising drug targets for cancer treatment [5], and BRDT was notably proposed as a potential target for male contraception [6]. Mammalian BRDT is a testis-specific factor essential for spermatogenesis [7]. Functional studies in mice have revealed that BRDT plays widespread roles at different stages of spermatogenesis, including transcriptional regulation, meiosis, chromocentre organization, mRNA splicing and 3'UTR-truncation [8–11]. On the other hand, it has been shown that BRDT has the capacity to remodel chromatin in an acetylation-dependent manner [12], and crystal structure analysis revealed that bromodomain 1 of BRDT specifically recognizes mono-acetylated histone H4 tails [13]. These data raised the attractive hypothesis that BRDT recognizes and removes hyper-acetylated histones before protamine incorporation and genome compaction during spermiogenesis [11].

The founding member of the BET family of proteins is encoded by the female sterile (1) homeotic (fs(1)h) gene identified in Drosophila [14,15]. The Fs(1)h protein, which is ubiquitously expressed, has been shown to activate homeotic genes during embryogenesis [16,17]. In Drosophila, two testis-specific genes, testis-specifically expressed bromodomain-containing protein-1 (tbrd-1) [18] and mitoshell (mtsh) [19] were reported to express bromodomain proteins essential...
for spermatogenesis. In contrast to Mtsl that has one bromodomain-related region in which the ligand-binding residues are not conserved, tBRD-1 contains two bromodomains related to BET protein bromodomains; bromodomain 1 of tBRD-1 is the closest to bromodomain 1 of Fs(1)h using BLASTP analysis (data not shown). These features open the possibility that tBRD-1 could represent the functional equivalent of mammalian BRDT in flies. However, the tBRD-1 protein, which is shorter than Fs(1)h, lacks an ET domain in its C-terminus and hence cannot be definitively classified into the BET family of proteins.

Here, in an attempt to clarify the role of tBRD-1 during spermatogenesis, we searched for tBRD-1 protein partners using biochemical approaches and identified a novel testis-specific protein, CG7229/tBRD-2. We show that tBRD-2 is also a bromodomain protein, but which additionally contains an ET domain. tBRD-1 and tBRD-2 are both essential for male fertility, and their respective mutants display remarkably similar meiotic defects. Finally, we demonstrate that tBRD-1 and tBRD-2 co-localize in spermatocyte nuclei and are interdependent, suggesting that these two factors collectively behave as a single BET protein, in a way reminiscent of BRDT in mammalian male germ cells.

3. Results and discussion

3.1. Generation of a tbrd-1 null mutant

The tBRD-1 protein has two bromodomains, which are similar to the bromodomains of the Fs(1)h protein (figure 1a). Expression of tbrd-1 mRNA in adult flies is essentially restricted to the testis according to the FlyAtlas database [20]. The previously reported tbrd-11 allele is a deletion of the C-terminal region but the truncated protein is apparently still expressed at low levels in the mutant [18]. Here, we recovered another tbrd-1 allele (tbrd-12) by imprecise excision [21] of a P-element inserted 137 bp upstream of the putative transcrip-tional start site (TSS) of the tbrd-1 gene (figure 1b). The tbrd-12 allele removes a 1940 bp genomic region containing the TSS. Accordingly, tbrd-12 homozygous males showed complete sterility, although females were fertile (data not shown). Male sterility was rescued by two types of transgenic constructs; one expresses tBRD-1 fused in frame with the monomeric red fluorescent protein1 (mRFP1) and another expresses tBRD-1 fused in frame with the monomeric red fluorescent protein1 (mRFP1) and 1 × Flag tag (tbrd-1::mRFP1-1 × Flag rescue). We also generated a rabbit polyclonal antibody against a mixture of two

Figure 1. Generation of tbrd-1 null mutant. (a) Representations of Fs(1)hS (short isoform of Fs(1)h) and tBRD-1 proteins. Bromodomain 1 (BD1) of tBRD-1 showed 44% identity with BD1 of Fs(1)h and 40% identity with bromodomain 2 (BD2) of Fs(1)h. BD2 of tBRD-1 showed 23% identity with BD2 of Fs(1)h, but no significant homology (--) with BD1 of Fs(1)h. Sequence identity was analysed using ClustalW. BD, bromodomain; ET, ET domain; SEED, seed motif. Blue boxes showed the peptides used as antigens for anti-tBRD-1 antibody generation. (b) A map of the tbrd-1 gene locus. The deletion range in tbrd-1 genomic rescue and tbrd-1::mRFP1-1 × Flag rescue is shown. Triangle indicates the position of the P(GSV6)GS13976 insertion mobilized to generate the tbrd-12 allele. (c) Western blotting analysis of tbrd-1 protein. Anti-α-tubulin antibody was used as control. Wild-type and tbrd-1 genomic rescue lanes showed endogenous tBRD-1 protein at about 65 kDa (black arrowhead), which is not detected in tbrd-12 extracts. In the tbrd-1::mRFP1-1 × Flag rescue lane, a higher molecular weight band (white arrowhead) was observed, corresponding to mRFP1-1 × Flag fused tBRD-1. (d) Phase contrast view of spermatids at onion stage. In wild-type (left panel), haploid nuclei (white circles) and mitochondrial derivatives, so-called Nebenkern (black circles), are all at the same size. By contrast, spermatids from tbrd-12 testes (right panel) contain nuclei of various sizes and irregularly shaped mitochondrial derivatives. Note that in these preparations, spermatids occasionally fuse during the course of live observation. Scale bar, 10 μm.
peptides from the very N- and C-terminal regions of tBRD-1, respectively. In western blotting (WB) experiments, this antibody recognized a band corresponding to tBRD-1 at about 65 kDa (predicted molecular weight is 59.2 kDa) in wild-type testicular extracts but not in tbrd-1Δ extracts. Furthermore, WB analysis of protein extracts from transgenic tests confirmed the specificity of this band (figure 1c). We conclude that tbrd-1Δ is a null or at least a strong loss of function allele. Phase contrast observation of mutant tests revealed that post-meiotic spermatid nuclei at the onion stage contained nuclei of various sizes associated with irregularly shaped mitochondria derivatives (figure 1d). This phenotype is indicative of abnormal chromosomal segregation and cytokinesis in meiosis [22]. After meiosis, although some spermatid cysts started to elongate, they appeared abnormal in shape and contained aberrant nuclei scattered throughout their length, in contrast to wild-type (data not shown). These phenotypes appeared very similar to what was previously reported for tbrd-1Δ by Leser et al. [18].

3.2. tBRD-1 interacts with a novel extra terminal domain-containing protein, CG7229/tBRD-2

To gain insight about how tBRD-1 could exert its function in the male germline, we searched for tBRD-1 interacting proteins using immunoprecipitation followed by proteomic analyses. We conducted two different strategies for the immunoprecipitation procedures. First, we used wild-type tests and tbrd-1/mRFP1-1×Flag rescue tests (which express mRFP1 and 1×Flag-tagged tBRD-1 without endogenous tBRD-1; figure 2a). This recombinant protein is functional as it rescues the sterility of mutant males (data not shown). Crude testicular lysates were incubated into an anti-Flag M2 agarose affinity gel. Finally, total elution products were analysed by liquid chromatography–mass spectrometry (LC-MS). Interestingly, this first approach identified a remarkable abundance of peptides derived from the putative protein encoded by the CG7229 gene, along with tBRD-1 peptides (electronic supplementary material, table S1). In a second approach, we directly used our anti-tBRD-1 antibody for immunoprecipitation using wild-type testicular lysates (figure 2b). Elution products were separated by electrophoresis and analysed by LC-MS. Again, this independent approach identified CG7229 as a potential tBRD-1 interacting protein (figure 2b; electronic supplementary material, table S2). As these independent immunoprecipitation strategies both identified CG7229 reproducibly as a putative binding partner of tBRD-1, we then focused on its characterization. Remarkably, CG7229 is also a bromodomain-containing protein, and like tBRD-1, is also specifically expressed in the adult testis, according to FlyAtlas [20]. However, CG7229 only has a single predicted bromodomain which shows 40% and 36% identity with the first and second bromodomains of Fs(1)h, respectively (figure 2c). Interestingly, the CG7229 protein has an ET domain characteristic of BET proteins (InterPro site annotated ‘NET domain (N-terminus of ET domain); N-terminal part of ET domain well-conserved in BET protein; figure 2d). Finally, this protein has a series of serine, aspartic acid and glutamic acid residues in the C-terminal, corresponding to the SEED motif as often seen in BET family of proteins (figure 2c) [7]. We also found that the CG7229 coding region contained one polymorphism in the sequence (T1627C) compared with the reference sequence in Flybase. This substitution induces the replacement of serine 543 with a proline between the ET and SEED domains. While preparing this report, another group also identified CG7229 as an interacting partner of tBRD-1, and named this gene tbrd-2 (testis-specifically expressed bromodomain-containing protein-2) [23].

3.3. tBRD-2 is a spermatocyte-specific nuclear protein essential for male fertility

To examine the in vivo role of the tbrd-2 gene, we generated tbrd-2 mutant flies. As there was no available P-element insertion in or near the tbrd-2 locus, we selected the homologous recombination method in order to obtain a precise deletion of the gene [24]. We indeed obtained a tbrd-2 mutant allele, tbrd-2ΔR1, in which the tbrd-2 gene was replaced by the white marker cassette from the recombination vector (figure 3a,b). tbrd-2ΔR1 homozygous males were completely sterile (data not shown). This sterility was fully rescued by a transgene expressing tBRD-2 fused with enhanced green fluorescent protein (eGFP) and 6×Histidine tag (tbrd-2::eGFP-6×His rescue) under the control of its endogenous putative promoter. We also used this rescue line to study the localization of tBRD-2 protein during spermatogenesis. tBRD2::eGFP-6×His protein initially appeared in young primary spermatocytes, and its level increased during primary spermatocyte growth (figure 3c). Interestingly, tBRD2::eGFP-6×His was highly enriched in the nucleolus and additionally showed a weaker staining in the main chromatin masses of spermatocyte nuclei (figure 3d). This protein localization pattern was very similar to the distribution of tBRD-1 [18]. In fact, immunostaining of tBRD-1 in tbrd-2::eGFP-6×His rescue flies showed almost complete merge of the eGFP and tBRD-1 signals, supporting our previous proteomics data. tBRD2::eGFP-6×His then rapidly disappeared during the G2–M transition when two prominent asters appeared at the onset of chromosome condensation (figure 3e).

To investigate the cause of male sterility, tbrd-2ΔR1 mutant tests were observed under phase contrast microscopy. tbrd-2ΔR1 mutant tests showed the same phenotype as tbrd-1Δ [18] and tbrd-1Δ’ (figure 1c) mutant tests. Spermatids were produced but contained nuclei of various sizes and irregular mitochondria, and displayed aberrant cytokinesis at the onion stage (figure 3f(i)). By contrast, spermatogenesis exhibited normal appearance in rescued tests (figure 3f(ii)). Furthermore, the spermatid phenotype of tbrd-2ΔR1/tbrd-1Δ double mutants was not different form the phenotype of each tbrd-1Δ or tbrd-2ΔR1 single mutant, suggesting that these two factors genetically function in the same pathway (figure 3f(iii)). In addition, the double mutant did not affect primary spermatocyte morphology at earlier stages (figure 3g). Taken together, these genetic and cytological data strongly suggest that tBRD-1 and t-BRD2 have highly related functions in spermatocyte nuclei.

3.4. Meiotic defects in the tbrd-2 mutant

To dissect the details of meiosis in the tbrd-2 mutant, we observed squashed testes under the confocal microscope. To distinguish the stages of meiosis, α-tubulin staining was used as a marker, and the chromosome morphology was also analysed [25]. In G2 phase, we were not able to find clear
differences between wild-type and tbrd-2HR1 mutant (figure 3h–j). However, at prophase I when chromatin started to condense into the typical three main chromosome masses, the two prominent asters of microtubules present in wild-type spermatocytes (figure 3i) were either not formed (figure 3k), aberrant in aspect (figure 3l) or present in excessive numbers in the tbrd-2HR1 mutant (figure 3m). Subsequently, in metaphase I, anaphase I and telophase I in tbrd-2HR1 mutant spermatocytes, sister chromatid segregation appeared abnormal (data not shown), as a likely consequence of aberrant positioning or number of asters in prophase I. Although tbrd-2 mutant spermatocytes display severe meiotic defects in microtubule organization and chromosome behaviour, these phenotypes are likely to be indirect consequences of earlier defects as tBRD-2 is not detected during meiosis.

3.5. tBRD-1 and tBRD-2 proteins are interdependent
Since tBRD-1 and tBRD-2 appeared functionally related in our previous experiments, we wondered whether tBRD-1 and tBRD-2 proteins were dependent upon one another for their stability or distribution. First, we analysed the cellular localization of tBRD-1::mRFP1 in the tbrd-2 mutant testes. In tbrd-2 homozygous mutants, the tBRD-1::mRFP1 signal was strongly...
decreased compared to the tbrd-2 heterozygous control (figure 4a). We also confirmed this result using the anti-tBRD-1 antibody (electronic supplementary material, figure S1). Conversely, we analysed the signal of tBRD-2::eGFP in tbrd-1 homozygous mutant testes. Like the decrease of tBRD-1::mRFP1 signal, tBRD-2::eGFP was barely detected in tbrd-1 homozygous mutant testes in contrast to the tbrd-1 heterozygous control (figure 4b). Next, we tested whether they affected
each other at the mRNA expression level. RT-PCR analysis indicated that tbrd-1 and tbrd-2 mRNA levels were not decreased in tbrd-2 and tbrd-1 mutants, respectively (figure 4c). In conclusion, these results suggest that tBRD-1 and tBRD-2 mutually depend on each other for their stability in growing primary spermatocytes (figure 4d,e).

These biochemical and genetic data support a functional partnership of tBRD-1 and tBRD-2 to ensure normal spermatogenesis in Drosophila. Gene fission is an evolutionary process in which an ancestral gene encoding a multi-domain protein splits into two complementary genes. It can occur through gene duplication followed by partial degeneration of protein domains in each duplicate [26]. For instance, such a scenario has been proposed for the evolution of the cara mitad/Lost PHDs of trr and trithorax-related (trr) genes, which are, respectively, orthologous to the N- and C-terminus of the mammalian transcriptional regulator MLL3 [27,28]. In the case of tBRD-1 and t-BRD2, we hypothesize that these genes could have arisen after the evolutionary split of an ancestor BRDT-like gene. Indeed, tBRD-1 protein
has two bromodomains characteristic of BET proteins but lacks an ET domain. On the other hand, tBRD-2 has a clear ET domain and SEED motif, but only a single bromodomain. Interestingly, with the exception of plant BET proteins that harbour a single bromodomain, members of this protein family always contain two bromodomains, from yeast to human. We thus propose that the cooperation of these two factors could restore the functional equivalent of an ancestor BET protein, such as Fs(1)h5, for instance. Although the tBRD-1/2 association cumulates three bromodomains, nothing is known about the actual functionality of each individual domain and this point will require additional analyses. We cannot exclude that one of these three potential bromodomains is actually in the process of degeneration. In addition, a recent report identified CG30417/tBRD-3 (testis-specific bromodomain-containing protein-3) as another testis-specific bromodomain and ET domain-containing protein [23]. Although tBRD-3 was shown to interact with tBRD-1 in a yeast two-hybrid assay, we could not identify any tBRD-3 peptides in our LC-MS analysis of tBRD-1::mRFP1-1 Flag interactors (electronic supplementary material, table S1). In any case, a functional analysis of tBRD-3 will be interesting to clarify the role of this factor during spermatogenesis.

In the case of mammalian BRDT, two types of functions were mainly reported [11,12]. One is a transcriptional regulator type of activity which relies on the recruitment of P-TEFb on target genes, in a way similar to BRD4. The other one is a chromatin remodeler type of activity based on the recognition of acetylated chromatin. These two types of activities seem to be exerted at different stages of spermatogenesis. In the case of Drosophila tBRD-1 and tBRD-2, their localization are restricted to primary spermatocytes. In these cells, tBRD-1 could function as a cofactor of testis-specific basic transcriptional factors (TAFs), such as spermatocyte arrest (sa) [23,29]. On the other hand, our proteomic analyses of tBRD-1 interactors also identified PSI (RNA splicing protein) and Rb97D (mRNA binding protein; figure 2b). Interestingly, orthologues of these proteins (KH-type splicing regulatory protein (KHRSRP) and heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), respectively) were previously identified as BRDT interacting proteins in mouse, among other RNA splicing factors [10]. Furthermore, a previous study aimed at identifying spliceosomesome associated factors in S2 cells listed up tBRD-1 itself [30]. It thus raises the intriguing possibility that tBRD-1 and tBRD-2 could be similarly involved in the splicing of meiosis-related mRNAs, which further supports the hypothesis that mammalian BRDT and Drosophila tBRD-1/2 could be functionally related.

Finally, our finding that two bromodomain factors essential for spermatogenesis probably reconstitute the functional equivalent of a BET protein supports the conserved need for a BRDT-like activity in animals.

4. Material and methods

4.1. Fly stocks

w1118 and yw67c stocks were used as wild-type strains. P(GSV6)GS13976 stock was obtained from Drosophila Genetic Resource Center at Kyoto Institute of Technology. Other stocks were obtained from Bloomington Drosophila Stock Center. Flies were maintained on standard medium at 25°C.

4.2. Generation of mutant flies

4.2.1. tbrd-1

P(GSV6)GS13976 is a P-element insertion 137 bp upstream of the putative tbrd-1 TSS. After its mobilization with the P transposase expressing Δ2–3 stock, we established 382 stocks for individual imprecise excision events. We identified three stocks with a genomic deletion covering the tbrd-1 TSS site by genomic PCR and sequencing. All of three mutants showed the same phenotype, and we characterized in detail only one allele (tbrd-1Δ) in this report. The deletion range of tbrd-1Δ extends from 650 bp upstream to 1290 bp downstream of the putative TSS of tbrd-1 gene.

4.2.2. tbrd-2

Approximately 3.0 kbp upstream (Arm#1) and downstream (Arm#2) of the tbrd-2 gene locus were respectively amplified using tbrd-2-arm#1-BsiWI-f: CGCTTCGATCCGCTTTAATT and tbrd-2-arm#1-Acc65I-r: TAATCCGGCG CGCCTTGTACGAGGATCCAAG. tbrd-2-arm#2-Acc65I-f: TAATCCGGTACCGATCTAGCCG and tbrd-2-arm#2-NotI-r: TAATCCGGCGCCGCGATCGATGACCTTC GAG primers, and cloned into the pW25 vector [31] with BsiWI, Acc65I, and NotI restriction sites, respectively. pW25-tbrd-2 transgenic flies were crossed with y w (Dpt2;Y); P( hs-hid)Y; P(70FLP); 23 P(70LM; Scel)4A/TM3, P(hs-hid)14, Sb stock [32] to perform Ends-out targeting. Among 303 individual F1 stocks, three lines showed the desired recombination event at the tbrd-2 locus. The loss of tbrd-2 in these alleles was confirmed by genomic PCR. All three tbrd-2 alleles showed the same cytological defects in phase contrast microscopy, so we randomly selected one allele, tbrd-2Δ70I, for further characterization.

4.3. Transgenic flies

4.3.1. Genomic tbrd-1

The genomic region from approximately 1.0 kbp upstream to approximately 0.5 kbp downstream of the tbrd-1 gene locus was amplified with tbrd-1-g1-SalI-r: GGGGTACCCCACAA and tbrd-1-g2-NotI-f: CGCTTCGATCCGCTTTAATT. This construct was inserted into the pW8 vector using KpnI and NotI restriction sites.

4.3.2. Genomic tbrd-1::mRFP1-1 × Flag

Approximately 1.0 kbp upstream and ORF of the tbrd-1 gene was amplified using tbrd-1-g1-KpnI-f: GGGGTACCCCACAA and tbrd-1-g2-SalI-r: ATGCGCGGCC GCACCAGGTGGCCACCTTG primers. PCR fragment was inserted into the pW8 vector using KpnI and NotI restriction sites.
4.3.3. Genomic tbrd-2:eGFP-6 x His

Approximately, 1.0 kbp upstream and ORF of the tbrd-2 gene was amplified with tbrd-2-g1-Sall-f: ATGTCGACATTGTAATCACTTTTACCTAGATGG and tbrd-2-g1-XhoI-r: ATGCTCGAGGCTCTACGTGTCCTCCTG primers. Primarily, 0.5 kbp downstream of the tbrd-2 gene including tbrd-2 3'UTR was amplified with tbrd-2-g2-Spel-f: ATGACTAGTATCAATCAAAGAAGTCTTATG and tbrd-2-g2-NotI-r: ATGCAGGCGCGCTTATCAGAGAGTGC primers.

To make the eGFP-6 x His sequence inserted into the C-terminus of the tbrd-2 ORF immediately upstream of the stop codon, these two fragments were linked upstream and downstream of the eGFP-6 x His sequence using Sall, and SpeI/NotI sites. This construct was subcloned into the pW8 vector with Xhol and NotI sites.

Additional plasmid construction details are available on request.

4.4. Fertility test

Five 0- to 1-day-old males were placed with five 0- to 1-day-old virgin females at 25°C. After 7 days, F0 flies were discarded, and the number of F1 larvae was counted.

4.5. Antibody generation

Two peptides from tBRD-1 (most N-terminus: MNEIQLSNSNQPF+C, most C-terminus: C+EFSPGTEPLDQTYDSD) were synthesized, and cysteine residues were conjugated with KLH (keyhole limpet haemocyanin). The mixture of these two peptides was used for the immunization of two rabbits. Crude sera were purified by immunoaffinity using the same peptides.

4.6. Immunoprecipitation

Preparation of crude protein lysate from testes. For each immunoprecipitation (IP), 200 pairs of testes were dissected in cold PBS and centrifuged at 3000 rpm for 5 min at 4°C. After discarding the supernatant, 1 × TNE buffer (20 M Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40) was added to the protease inhibitor cocktail (Roche) was added, before sonication and left for 10 min on ice. After centrifugation, samples were loaded on SDS-PAGE. WB was performed using standard procedures. Anti-α-tubulin (Sigma #T9026, mouse monoclonal, DM1A) was used at a 1/2000 dilution. Protein A (Invitrogen) and cross-linked using dimethyl pimelic acid were frozen in liquid nitrogen and stored at –80°C.

4.7. Liquid chromatography–mass spectrometry

Total eluted proteins were precipitated with methanol–chloroform, trypsinized and peptides and were then analysed in LC-MS/MS using an LTQ Velos Orbitrap instrument (Thermo Fisher Scientific, Pittsburgh, PA, USA). In-gel digested peptides were also analysed by LC-MS/MS. To identify the proteins, spectra were analysed by PROTEOME DISCOVERER v. 1.2 (Thermo Scientific) against SEQUEST using a 5% false discovery rate (FDR) cutoff. Additional details are as previously described [33].

4.8. Western blotting

For each genotype, five pairs of testes were dissected in cold PBS and centrifuged at 3000 rpm for 5 min at 4°C. After discarding the supernatant, 1 × SDS sample buffer was added, and the samples were boiled for 10 min at 95°C. After brief centrifugation, samples were loaded on SDS-PAGE. WB Detection System (GE Healthcare).

4.9. Immunostaining

The testes squash and methanol–acetone fixation method was slightly adapted from Cenci et al. [25]. Testes were dissected in testis buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris–HCl pH 6.8, 1 mM EDTA), and then opened from the tip using a fine needle. Dissected testes were placed into a drop of the testis buffer on poly-l-lysine-coated slides. Non-siliconized coverslips were put on the samples, the slides were inverted, squashed with moderate pressure and immediately placed in liquid nitrogen. After removing the coverslips, slides were immersed into methanol for 5 min at –20°C and subsequently in acetone for 2 min at –20°C. Next, samples were washed once with 1 × PBST (1 × PBS, 0.1% Triton X-100) buffer and incubated with primary antibodies in 1 × PBST buffer overnight at 4°C. After three 1 × PBST washes, secondary antibodies in 1 × PBST were added overnight at 4°C. Finally, samples were washed three times, and propidium iodide (PI) or DRAQ5 containing DAKO mounting medium was added. For the PI staining, samples were incubated in 2 mg ml⁻¹ RNase A in 1 × PBST for 1 h at 37°C before mounting. Anti-α-tubulin (Sigma #T9026, mouse monoclonal, DM1A) was used at a 1/100 dilution, anti-tBRD-1 was used at a 1/100 dilution, DyLight 488 or 550-conjugated anti-mouse or rabbit antibody (Thermo Scientific) were used at a 1/300 dilution. PI (Sigma #P4170) and DRAQ5 (Cell Signalling Technology #4084L) were used to visualize DNA. Pictures were taken with an LSM 510 inverted confocal microscope (Zeiss). Figures were edited using the IMAGEJ software and Adobe PHOTOSHOP.
4.10. Phase contrast microscopy

Testes were dissected in testis buffer and opened using a fine needle. Testes were then observed on a slide with a coverslip placed without pressure. Live testes were observed using a Zeiss Axio inverted 2000 and MetaView v. 2 software. Figures were edited using the ImageJ software and Adobe Photoshop.

4.11. Genomic PCR and RT-PCR

Genomic DNA was extracted from a single fly using standard protocol. For cDNA preparation, 20 pairs of testes for each genotype were dissected, and total RNA was extracted using TRIzol reagent (Invitrogen) by standard protocol. From 70 ng total RNA, cDNA was produced using Super Script II reverse transcriptase (Invitrogen) by standard protocol. PCR for both genome and cDNA was produced using Taq DNA polymerase (5 PRIME) as follows: denaturation at 95°C for 1 min; annealing at 52°C for 1 min; elongation at 72°C for 1 min, 35 cycles. PCR primers were used as below: 

- rp49-f: AAGATCGTAGAGAAGGCAC
- rp49-r: ACTGTTCCTCGAAGAGCG
- tbrd-1-f: TGAACAGAGTGGCAC
- tbrd-1-r: ACTGCTGTTCCTGCAAACAGG
- tbrd-2-f: TAGGACAAATGCTTCTAG
- tbrd-2-r: TGAGGATCATGCTAAC

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