The bromodomain-containing protein tBRD-1 is specifically expressed in spermatocytes and is essential for male fertility

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Summary

By a conserved cellular differentiation process, spermatogenesis leads to formation of haploid sperm for successful reproduction. In *Drosophila* and in mammals, post-meiotic spermatid differentiation depends on several translationally repressed and stored mRNAs that are often expressed exclusively in the testis through a cell type specific transcriptional program. In *Drosophila*, the mRNAs of proteins required for post-meiotic chromatin reorganisation, like ProtB and Mst77F, are transcribed in meiotic spermatocytes and subjected to translational repression for days. Transcription of many of these translationally repressed mRNAs depends on testis-specific homologs of TATA box binding protein-associated factors (tTAFs). Here, we identified the testis-specific bromodomain protein, tBRD-1, that is only expressed in primary spermatocytes. Bromodomain proteins are able to recognise and bind acetylated histones and non-histone proteins. We generated *tbrd-1* mutant flies and observed that function of tBRD-1 is required for male fertility. tBRD-1 partially colocalised with tTAFs, TAF1 and Polycomb to a Fibrillarin-deficient region within the spermatocyte nucleolus. The nucleolar localisation of tBRD-1 depended on tTAF function but not the other way round. Further, we could show that ectopically expressed tBRD-1-eGFP is able to bind to the interbands of polytene chromosomes. By inhibitor treatment of cultured testis we observed that sub-cellular localisation of tBRD-1 may depend on the acetylation status of primary spermatocytes.

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Key words: *Drosophila*, Acetylation, Bromodomain proteins, Polycomb, Spermatogenesis, tTAF

Introduction

Cell type specific transcription programs are a major feature of metazoans prerequisite for the specialisation of different cell types during development and tissue maintenance. In addition, translational control programs play important roles in events like oocyte maturation, very early embryogenesis, and spermatogenesis, where a series of temporally controlled events must take place in the absence of transcription. In *Drosophila* spermatogenesis, most transcription ceases with entry into meiotic divisions. Therefore, many genes encoding proteins required for spermatid differentiation are transcribed in primary spermatocytes but translationally repressed until the appropriate time later in gamete development (reviewed by Fuller, 1993; Renkawitz-Pohl et al., 2005; White-Cooper, 2009). In primary spermatocytes, more than 2000 testis-specific or enriched transcripts are synthesised (Doggett et al., 2011; reviewed by White-Cooper, 2010). Gene transcription in primary spermatocytes depends on a group of genes collectively named "meiotic arrest" loci (Ayyar et al., 2003; Doggett et al., 2011; Jiang et al., 2007; Jiang and White-Cooper, 2003; Lin et al., 1996; Perezgasga et al., 2004; Wang and Mann, 2003; White-Cooper et al., 2000; White-Cooper et al., 1998). Two types of meiotic arrest genes are described: the *aly*-class and the *can*-class (White-Cooper et al., 1998). The *aly*-class genes (*aly*, *comr*, *tomb*, *topi* and *achi/vis*) are required for expression of a broader range of target genes than the *can*-class genes (*can*, *mia*, *nht*, *rye* and *sa*). Proteins of the *aly*-class together with other proteins form the testis meiotic arrest complex (tMAC) (Beall et al., 2007). The *can*-class proteins are homologs of TATA box binding protein-associated factors (TAFs) and are expressed only in testis: Cannonball (Can; dTAF5 homolog), No hitter (Nht; dTAF4 homolog), Meiosis I arrest (Mia; dTAF6 homolog), Spermatocyte arrest (Sa; dTAF8 homolog) and Ryan express (Rye; dTAF12 homolog) (Hiller et al., 2001; Hiller et al., 2004). In males mutant for any of these tTAF genes transcription of several spermatid differentiation relevant genes, such as *Mst87F*, *dj* or *dj like*, and *fzo*, is greatly reduced (Hempel et al., 2006; Hiller et al., 2004; White-Cooper et al., 1998). In addition, tTAFs are required for cell cycle progression and mutant males show a meiotic arrest phenotype (reviewed by White-Cooper, 2010). Previously, it was shown that tTAFs are concentrated in a Fibrillarin-deficient sub-compartment within the spermatocyte nucleolus, along with components of the Polycomb Repression Complex 1 (PRC1), such as Polycomb (Pc) (Chen et al., 2005).

Localisation of Pc to the spermatocyte nucleolus depended on tTAF activity and it has been proposed that tTAFs promote displacement of PRC1 from promoters of tTAF target genes to allow robust transcription (Chen et al., 2005). Beside tTAFs also a TAF1 isoform, presumably TAF1-2, localises to the nucleolus in primary spermatocytes in a tTAF dependent manner (Metcalf and Wassarman, 2007). Thus far, it is not known how tTAFs are recruited to their target genes and also hints for tTAF effector molecules are missing.

The activation of transcription in eukaryotes requires modifications to open the chromatin-packaged DNA (Jenuwein and Allis, 2001). Covalent modifications of N-terminal histone tails, such as acetylation, methylation, phosphorylation and ubiquitination can control patterns of gene expression (Strahl and Allis, 2000). Acetylation of histone tails is connected to gene activation and these residues can be recognised by bromodomain containing proteins (Dhalluin et al., 1999; Jacobson et al., 2000; Owen et al., 2000). Bromodomains were first discovered in the *Drosophila* protein Brahma which is required for activation of many homeotic genes (Kennison and Tamkun, 1988; Tamkun et al., 1992). The sequence of bromodomains is highly conserved between yeast, *Drosophila* and humans (Haynes et al., 1992).

Here, we identify a novel bromodomain-containing protein tBRD-1, expressed specifically in primary spermatocytes. tBRD-1 partially colocalises with tTAFs, TAF1 and Polycomb to a Fibrillarin-deficient region within the nucleolus. Nucleolar localisation of tBRD-1 depends on tTAF function as well as on the acetylation status of primary spermatocytes. In addition, ectopically expressed tBRD-1 is able to bind euchromatic interbands on polytene chromosomes. Function of tBRD-1 is required for proper differentiation of spermatids: *tbrd-1* mutants are male sterile, although tBRD-1 function is not required for progression into the meiotic divisions or for transcription of the three thus far known direct tTAF target genes. Here, with tBRD-1 we propose for the first time a promising candidate who could act as a cofactor and/or effector of tTAFs.

Results

The bromodomain protein tBRD-1 is expressed specifically in testis and is required for male fertility

The *Drosophila CG13597* gene encodes a 513 amino acid protein of 59.2 kDa (FlyBase) (Tweedie et al., 2009) with two widely spaced bromodomains (amino acid 55 to 127 and 336 to 409) (PROSITE database) (Sigrist et al., 2010) (Fig. 1C). RT-PCR

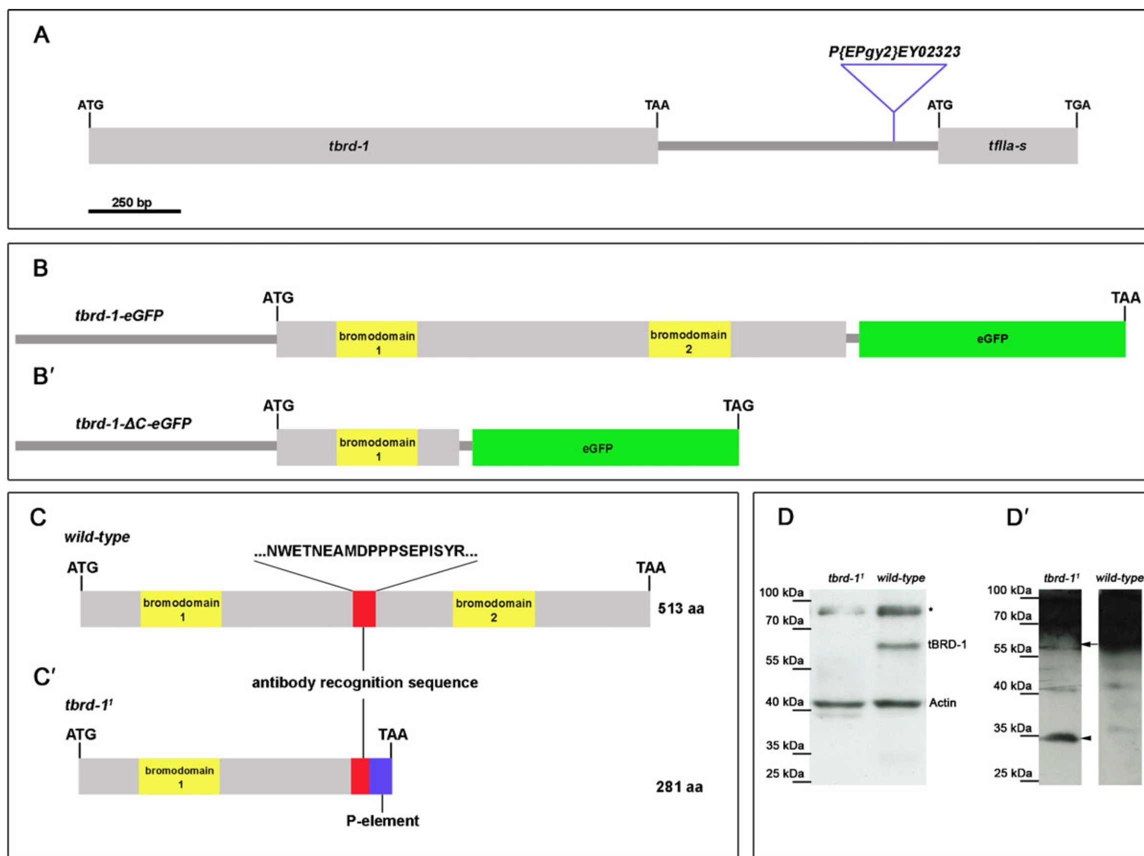

Fig. 1. The *tbrd-1* gene encodes a 59.2 kDa protein with two bromodomains. (**A**) Scheme of the genomic region of *tbrd-1*. *P(EPgy2)*^{EY02323} used to generate the *tbrd-1* mutant allele is inserted 635 bp downstream of the translational stop codon of *tbrd-1*. (**B**,**B′**) Scheme of the *tbrd-1-eGFP* and *tbrd-1Δc-eGFP* constructs. (**C**,**C′**) Scheme of the full length tBRD-1 protein and the situation in *tbrd-1* mutant flies. The two bromodomains are indicated in yellow (amino acid 55 to 127 and 336 to 409, respectively). The sequence used for generating the peptide antibody is indicated in red (amino acid 249 to 267). (**D**,**D′**) Western blot experiments using testis protein extracts of homozygous *tbrd-1*^{1} mutants and *wild-type* flies. (D) The anti-tBRD-1 antibody detected a protein at about 60 kDa (tBRD-1 predicted molecular mass: 59.2 kDa) in extracts of *wild-type* testes, while this protein was not detectable in extracts of homozygous *tbrd-1*^{1} mutants (exposure time: 5 minutes). An unspecific protein at about 90 kDa was visible in both extracts (asterisk). As control anti-Actin antibody was used. The 42 kDa Actin protein was visible in both extracts. (D′) After an exposure time of 3 hours a protein at about 35 kDa (arrowhead) was detected in extracts of homozygous *tbrd-1*^{1} mutants, which might represent a truncated protein (predicted molecular mass: 32.0 kDa). The full length tBRD-1 protein was not detectable (arrow).

experiments revealed that transcripts are specific to male gonads (data not shown), consistent with Affymetrix expression data also indicating a strong enrichment of *CG13597* transcripts in the testis (FlyAtlas: the *Drosophila* gene expression atlas) (Chintapalli et al., 2007). Very low levels of *tbrd-1* expression in other tissues were detected by RNA-seq (modENCODE Project led by Sue Celniker, visible on FlyBase via GBrowse). Accordingly, we named CG13597 tBRD-1 (testis-specifically expressed bromodomain containing protein-1) and the corresponding gene *tbrd-1*. A *tbrd-1* mutant allele (*tbrd-1*^{1}) was generated by remobilisation and subsequent integration of *P(EPgy2)*^{EY02323} into the *tbrd-1* gene (Fig. 1A,C′; Materials and Methods). This led to a premature translational stop codon after amino acid 281 (262 amino acids of tBRD-1 and 19 amino acids encoded by the P-element) (Fig. 1C′). We raised a peptide antibody (aa 249 to aa 267) against tBRD-1 (Fig. 1C). By Western blot analysis neither the full length nor a truncated tBRD-1 protein could be detected in protein extracts of homozygous *tbrd-1*^{1} mutant testes (Fig. 1D). After strong overexposure a protein is visible at about 35 kDa, which might represent a truncated tBRD-1 protein (predicted molecular mass: 32 kDa) (Fig. 1D′, arrowhead). Analysis of *tbrd-1* mutants revealed that tBRD-1 is required for male but not for female fertility. Fertility tests with both *tbrd-1*^{1} homozygotes and *tbrd-1*^{1}/*Df(3R)ED10893* or *tbrd-1*^{1}/*Df(3R)Exel9014* trans-heterozygous males demonstrated that mutation of *tbrd-1* leads to complete male sterility. Seminal vesicles of 5 days old *tbrd-1* mutant males were devoid of sperm also when males were kept isolated from females. A *tbrd-1-eGFP* transgene made from the genomic region (Fig. 1B; Materials and Methods) rescued the sterility of homozygous *tbrd-1*^{1} males demonstrating that the male infertility was due to the compromised activity of tBRD-1. Whole mount testis and squashed preparations of homozygous *tbrd-1*^{1} (Fig. 2A,D) and *tbrd-1*^{1}/*Df(3R)ED10893* or *tbrd-1*^{1}/*Df(3R)Exel9014* transheterozygous (data not shown) males demonstrated testis tubes filled with elongated spermatids (Fig. 2A,D, arrows) indicating substantial differentiation of post-meiotic stages. Nevertheless, first effects were already detectable in early round spermatids (Fig. 2B). In *wild-type* males the phase-dark Nebenkern formed by the two mitochondrial derivatives and the phase-light nucleus have nearly the same size and are arranged side by side in early round spermatids. During spermatid differentiation the derivatives elongate beside the growing flagellar axoneme (reviewed by Fuller, 1993). In homozygous *tbrd-1*^{1} mutants (Fig. 2B) the Nebenkern (double arrow) was larger than the nucleus (arrowhead) and was surrounded by several nuclei varying in size. Introduction of the *tbrd-1-eGFP* genomic transgene restored normal morphology of round spermatids (Fig. 2C). Spermatid differentiation is accompanied by an extensive reorganisation of the nucleus. The nuclei develop from a round shape in early spermatids (Fig. 2C, arrowhead) to a very thin needle shape in mature sperm. Visualising DNA by Hoechst revealed that spermatid nuclei in homozygous *tbrd-1*^{1} mutant testis become very small, however, the nuclei remained round

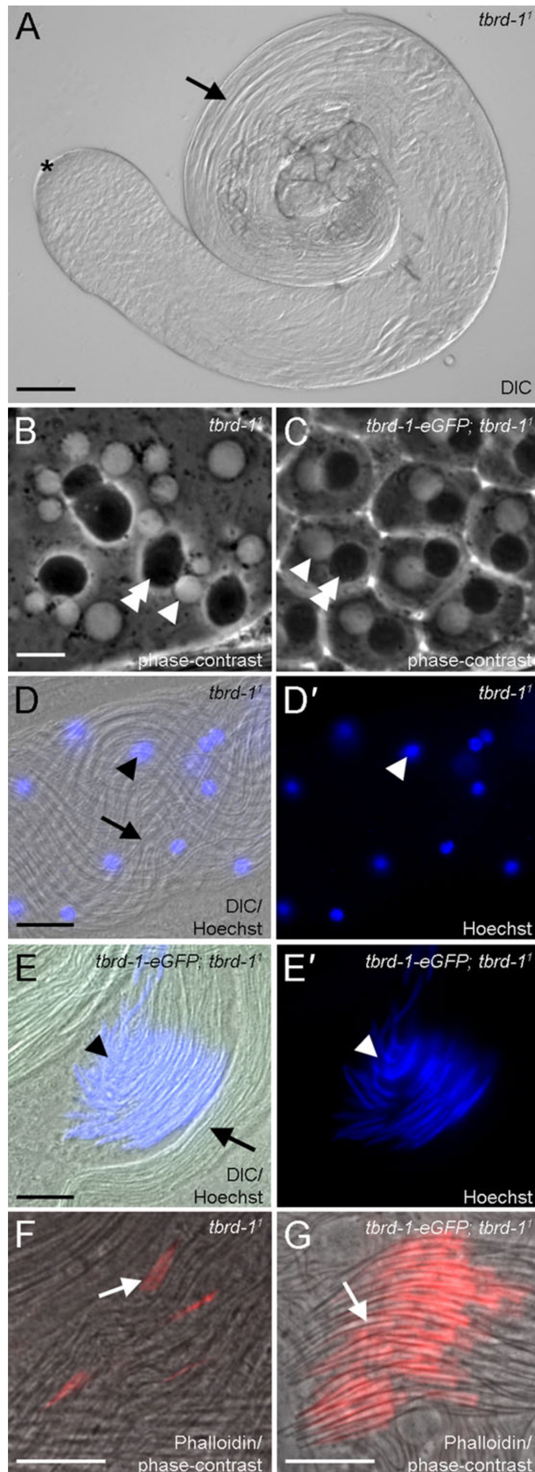

Fig. 2. Expression of tBRD-1 is essential for male fertility. (**A**) Whole mount testis of a homozygous *tbrd-1*^{1} mutant. Post-meiotic stages were clearly visible due to well-elongated flagella (arrow). Asterisk marks the testis tip. Scale bar: 100 µm. (**B**,**C**) Phase contrast images of homozygous *tbrd-1*^{1} mutant (B) and rescued (C) early round spermatids. (C) The phase-dark Nebenkern (double arrow) and the phase-light nucleus (arrowhead) had nearly the same size and were arranged side by side in early round spermatids of rescued *tbrd-1*^{1} mutants. (B) In homozygous *tbrd-1*^{1} mutants the Nebenkern (double arrow) was larger than the nucleus (arrowhead) and one Nebenkern was surrounded by several nuclei varying in size. Scale bars: 10 µm. (**D-G**) Squash preparations of homozygous *tbrd-1*^{1} mutant (D,D′,F) and rescued (E,E′,G) testis stained with Hoechst (D-E′) or Phalloidin (F,G) to visualise DNA or F-actin. (D,E,F) Merged fluorescence and phase-contrast/DIC images showing the positions of nuclei and flagella (D,E) or actin cones and flagella. (D′,E′) Corresponding DNA stainings. Post-meiotic nuclei of homozygous *tbrd-1*^{1} mutants (D, arrowhead) were small and round and did not show the typical elongated shape of nuclei in rescued testis (E, arrowhead). In contrast to the rescued sample (E) in homozygous *tbrd-1*^{1} mutants (D) the nuclei were randomly distributed within one cyst. Scale bars: 10 µm.

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(Fig. 2D,D′, arrowheads). In addition, spermatid nuclei were scattered throughout the elongated bundles of flagella (Fig. 2D, arrowhead) instead of being clustered at one end as in the *wild-type*. At the end of spermatid differentiation mature sperm become individualised by the individualisation complex. This complex is a coordinated array of discrete investment cones which can be visualised by phalloidin staining. Each investment cone individualises a single spermatid (Fabrizio et al., 1998). In homozygous *tbrd-1*[1] mutants, investment cones are formed (Fig. 2F, arrow) but individualisation complexes were never observed. Introduction of the *tbrd-1-eGFP* genomic transgene restored normal nuclear shaping and clustering as well as individualisation complex formation (Fig. 2E,E′, arrowheads, Fig. 2G, arrow).

tBRD-1 is dispensable for histone to protamine transition

The extensive reorganisation of the nucleus during spermatid differentiation is accompanied by a compaction of the chromatin. In mammals, the testis-specific bromodomain protein BRDT is involved in chromatin reorganisation and is essential for male germ cell differentiation (Pivot-Pajot et al., 2003; Shang et al., 2007). A common feature of mammalian and *Drosophila* spermatid differentiation is the dramatic reorganisation of chromatin due to replacement of histones by protamines. In *Drosophila*, Protamine A (Mst35Ba, ProtA), Protamine B (Mst35Bb, ProtB) and Mst77F are major chromatin components of the mature sperm (Jayaramaiah Raja and Renkawitz-Pohl, 2005; Rathke et al., 2010). *In situ* hybridisations as well as immunofluorescence stainings demonstrated that expression of *Mst77F* and *protB* was unaffected in homozygous *tbrd-1*[1] mutant testis (Fig. 3A,C,E,G). In addition, the histone to protamine transition is accompanied by the occurrence of many DNA breaks and it was shown that hyper-acetylation of histone H4 is essential for chromatin reorganisation (Awe and Renkawitz-Pohl, 2010; Rathke et al., 2007). In homozygous *tbrd-1*[1] mutants, hyper-acetylation of histone H4, disappearance of histones as well as the occurrence of DNA breaks was not obviously altered (data not shown). Apparently, tBRD-1 is not required for main features of post-meiotic chromatin reorganisation. Nevertheless, many other reorganisations, besides chromatin remodelling, accompany post-meiotic spermatid differentiation.

The bromodomain protein tBRD-1 localises to Fibrillarin-deficient regions within the nucleolus in primary spermatocytes

In adult testis, different staged germ cells are arranged in a spatially ordered manner from germline stem cells at the apical tip to mature sperm in the seminal vesicles at the basal end (reviewed by Fuller, 1993). Analysis of whole mount testis of flies bearing the *tbrd-1-eGFP* genomic rescue transgene showed expression of tBRD-1-eGFP starting with the onset of the spermatocyte stage (Fig. 4A, arrowhead). Stem cells and spermatogonia in the testis tip as well as post-meiotic stages were free of eGFP signal (Fig. 4A, arrow, double arrow). This is in agreement with *in situ* hybridisation analyses showing that *tbrd-1* transcripts were restricted to spermatocytes (Fig. 5A). Examination of mature spermatocytes expressing tBRD-1-eGFP or immunostained with an anti-tBRD-1 antibody at higher magnification revealed strong localisation of tBRD-1 to the spermatocyte nucleolus, with lower intensity signal distributed over the partially condensed chromosomes (Fig. 4A′,A″, arrowheads, double arrows). In addition, within the nucleoplasm tBRD-1 localises in a set of nuclear speckles (Fig. 4A′,A″, arrows). All tBRD-1 expression vanished with the breakdown of the nucleolus during the G_2/M transition of meiosis I (data not shown). Thus, expression of tBRD-1 and first spermatogenesis defects in homozygous *tbrd-1*[1] mutants do not coincident in time. This indicates that mutant testis show secondary effects in post-meiotic stages due to loss of tBRD-1 expression in pre-meiotic and meiotic stages. In young spermatocytes tBRD-1 became visible first at the periphery of the nucleolus (Fig. 4B). When spermatocytes grew concurrently the tBRD-1 signal accumulated at the nucleolus and speckles within the nucleoplasm appeared (compare Fig. 4B, Fig. 4E, Fig. 4H). Analysis of testis of flies bearing a further transgene, *tbrd-1Δc-eGFP*, made from the genomic region but lacking the C-terminal part of the gene (Fig. 1B′; Materials and Methods) showed exactly the same expression pattern like testis from *tbrd-1-eGFP* (data not shown). The missing C-terminal part encodes amino acid 349-513 containing the second bromodomain. Obviously, the second bromodomain is not essential for proper localisation of tBRD-1.

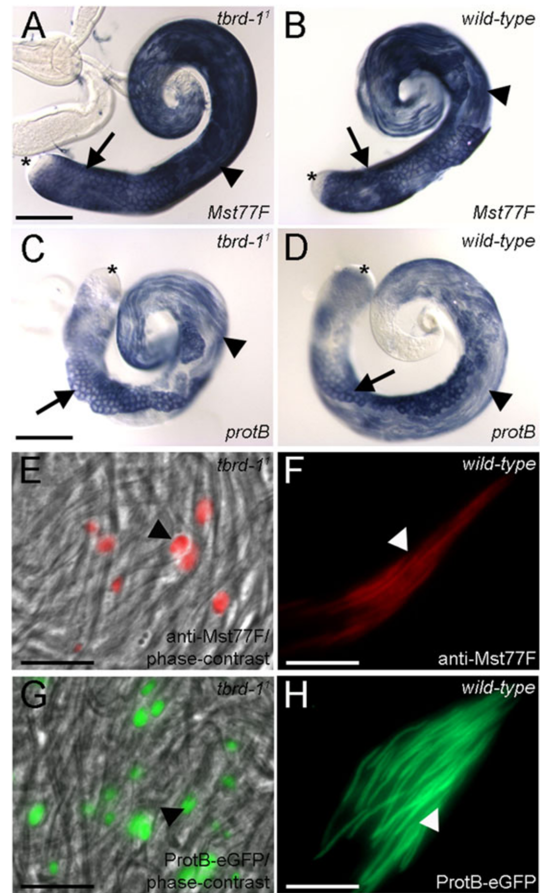

Fig. 3. Function of tBRD-1 is dispensable for expression of Mst77F and ProtB. (**A–D**) *In situ* hybridisation to whole mount homozygous *tbrd-1*[1] mutant (A,C) and *wild-type* (B,D) testis using an *Mst77F*- and *protB*-specific probe. Asterisks mark the testis tip. Scale bars: 200 µm. (A,C) *Mst77F* and *protB* mRNAs were clearly detected in spermatocytes from homozygous *tbrd-1*[1] mutant testis (arrows) and lasted until post-meiotic stages (arrowheads). (**E**,**G**) Strong expression of Mst77F and ProtB-eGFP could be observed in post-meiotic nuclei of homozygous *tbrd-1*[1] mutant testis (arrowheads). (**F**,**H**) Mst77F and ProtB-eGFP expression in *wild-type* testis. Scale bars in E–H: 10 µm.

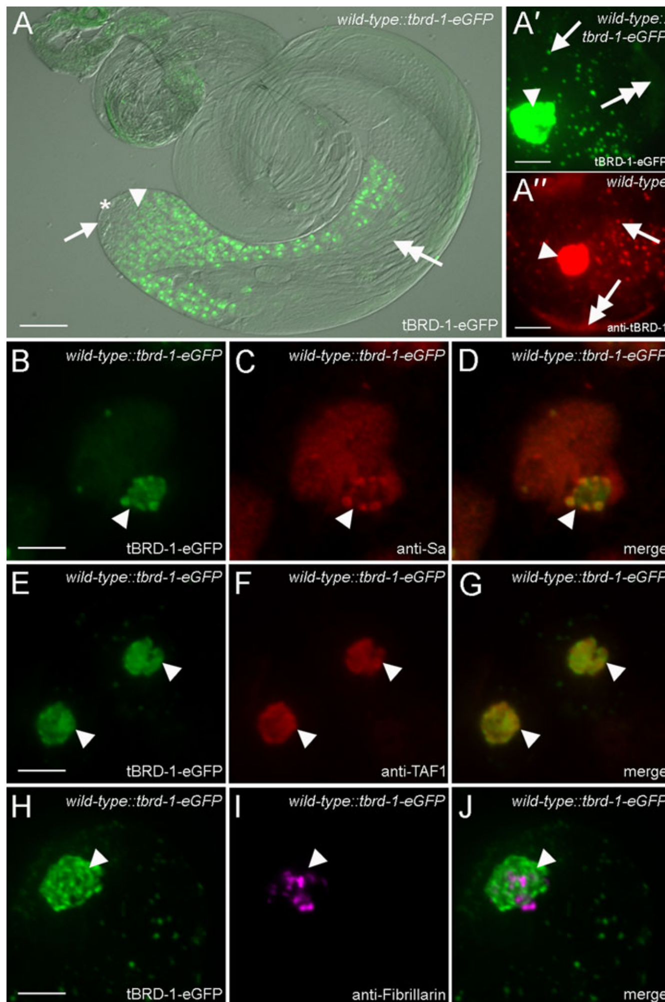

Fig. 4. High amounts of tBRD-1 localise to Fibrillarin-deficient regions within the nucleolus of spermatocytes. (**A**) A whole testis of a transgenic fly expressing tBRD-1-eGFP, showing high levels of tBRD-1-eGFP expression in spermatocytes (arrowhead), but no visible expression in very young germ cells at the tip of the testis (arrow) or in post-meiotic stages (double arrow). Asterisk indicates the tip of the testis. Scale bar: 100 µm. (**A′,A″**) Single primary spermatocyte nuclei of flies expressing tBRD-1-eGFP (A′) or *wild-type* stained for anti-tBRD-1 (A″). Scale bars: 5 µm. The highest amounts of tBRD-1 were detected in nucleoli (arrowheads), while only low amounts were visible over the chromosome territories (double arrows). Within the nucleoplasm many tBRD-1 positive speckles could be observed (arrows). (**B–J**) Single primary spermatocytes from testis squash preparations of tBRD-1-eGFP expressing flies stained with anti-Sa (B–D), anti-TAF1 (E–G) or anti-Fibrillarin (H-J) antibody. tBRD-1-eGFP partially colocalised with Sa (D) and TAF1 (G) within the nucleolus (arrowheads). Concentration of tBRD-1-eGFP in the nucleolus (arrowhead) was restricted to Fibrillarin-deficient regions (J). Scale bars: 5 µm.

In addition, flies expressing two copies of *tbrd-1Δc-eGFP* showed normal spermatogenesis and were fertile. The *tbrd-1Δc-eGFP* transgene was not able to rescue the sterility of homozygous *tbrd-1^1* males (data not shown). Immunofluorescence staining of testis of flies bearing *tbrd-1-eGFP* showed that tBRD-1-eGFP partially colocalises with the tTAF Sa, the general TAF TAF1 and Polycomb in the nucleolus of primary spermatocytes (Fig. 4B–D,E–G, arrowheads; data not shown). Like tBRD-1 also Sa, TAF1 and Polycomb showed a stepwise accumulation at the nucleolus. The tBRD-1 localised to the Fibrillarin-deficient regions of the nucleolus (Fig. 4H–J). Fibrillarin is a component of the nucleolus involved in ribosomal RNA processing (Girard et al., 1993; Jansen et al., 1991). As tBRD-1 and Sa showed a highly similar expression pattern we analysed the three thus far known direct tTAF target genes *fzo*, *dj* and *Mst87F* in homozygous *tbrd-1^1* mutant testis. By RT-PCR we were able to detect all three transcripts in homozygous *tbrd-1^1* mutant testis (data not shown).

Localisation of tBRD-1 to the nucleolus requires tTAFs

In situ hybridisation and Western blot experiments using homozygous *can^{12}* mutant testis revealed that expression of tBRD-1 was independent of tTAFs (Fig. 5B; data not shown). Immunofluorescence staining of homozygous *can^{12}* and *sa^2* mutant testis showed that localisation of tBRD-1 to the nucleolus required *wild-type* function of tTAFs (Fig. 5E,F; data not shown). The prominent localisation of tBRD-1 to the nucleolus in *wild-type* (Fig. 5C, arrow) was strongly reduced in homozygous *can^{12}* and *sa^2* mutant spermatocytes (Fig. 5E, arrow; data not shown). A reduced tBRD-1 localisation to the nucleolus was also observed in homozygous *can^{12}* and *sa^2* mutants, which express the tBRD-1-eGFP fusion protein (data not shown). In addition, an increased tBRD-1 signal was visible within chromosome territories in homozygous *can^{12}* and *sa^2* mutants while hardly any nuclear speckles were detectable (Fig. 5E; data not shown). Analyses of homozygous *tbrd-1^1* mutant spermatocytes demonstrated that localisation of tTAFs, TAF1 and Polycomb was independent of tBRD-1. Localisation of tTAFs, TAF1 and Polycomb to the nucleolus could still be detected in homozygous *tbrd-1^1* mutant spermatocytes (Fig. 5G,I, arrows; data not shown).

Sub-cellular localisation of tBRD-1 depends on the acetylation status of primary spermatocytes

Pupal testis of tBRD-1-eGFP transgenic flies dissected 24 hours after puparium formation (APF) were treated with 50 µM of the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) or 150 µM of the histone acetyltransferase (HAT) inhibitor anacardic acid (AA) for about 24 hours in culture (for establishment of culture conditions, see Awe and Renkawitz-Pohl, 2010). This affected the localisation of tBRD-1 within spermatocyte nuclei (Fig. 6). Immunofluorescence staining with an antibody against acetylated histone H4 revealed a strong increase in H4 acetylation upon TSA-treatment (Fig. 6F), while AA-treatment blocked acetylation nearly completely (Fig. 6J). In contrast to the untreated control (Fig. 6A–D), in TSA-treated testis (Fig. 6E–H) an increased tBRD-1-eGFP signal was visible within chromosome territories (Fig. 6E, arrow). No obvious consequence of TSA-treatment was observed for tBRD-1-eGFP concentrations in the nucleolus (Fig. 6E, arrowhead). Conversely, testis treated with AA showed strong reduction of tBRD-1-eGFP within the nucleolus (Fig. 6I, arrowhead). Similar results were obtained using anti-tBRD-1 to stain for endogenous tBRD-1 in non-transgenic flies (data not shown).

tBRD-1 directly binds to polytene chromosomes

To investigate if tBRD-1 is able to bind chromatin we isolated salivary glands of larvae and prepared polytene squashes. As tBRD-1 is normally not expressed in salivary glands *UAS-tbrd-1-eGFP* was driven by Sgs58AB for ectopic expression. Immunofluorescence staining using anti-GFP antibody and Hoechst showed that ectopically expressed tBRD-1-eGFP binds at multiple sites along polytene chromosomes (Fig. 7A). While

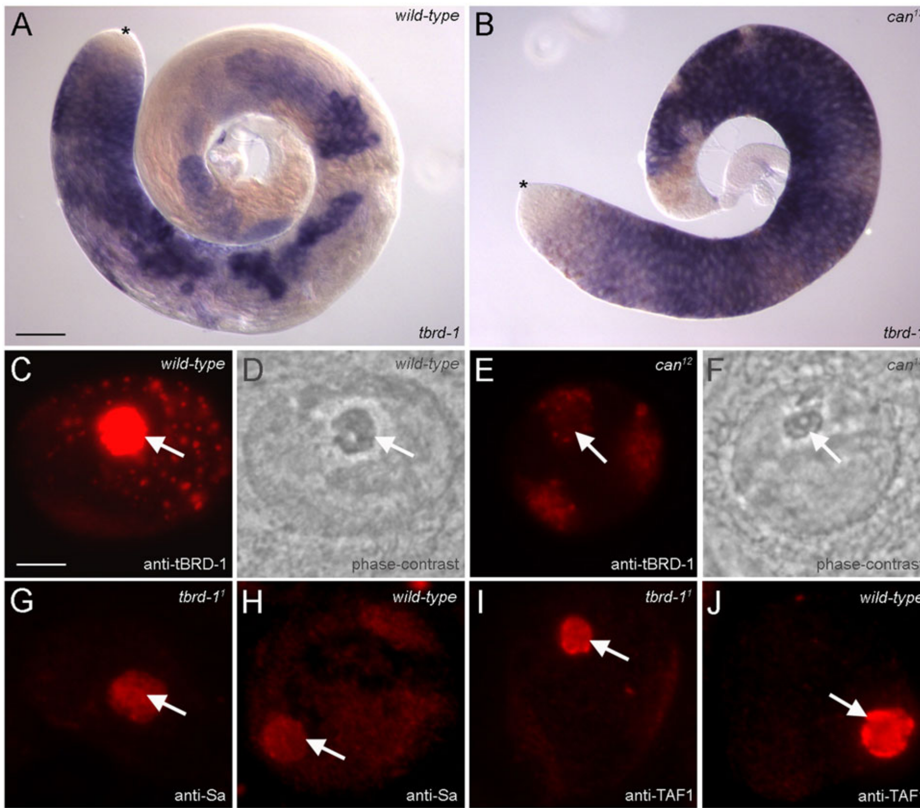

Fig. 5. Recruitment of tBRD-1 to the nucleoli requires tTAFs. (**A**,**B**) *In situ* hybridisation to whole mount *wild-type* (A) and homozygous can^{12} (B) testis using *tbrd-1*- specific probes. After the same time of staining reaction, *tbrd-1* mRNA was abundant in *wild-type* testis (A) and homozygous can^{12} mutant testis (B). Asterisks indicate the tips of testis, where germline stem cells reside. Scale bar: 100 µm. (**C–F**) Single primary spermatocytes from testis squash preparations of *wild-type* (C,D) and homozygous can^{12} (E–F) flies stained with anti-tBRD-1 antibody. (D,F) Phase-contrast images to visualise nucleoli (arrows). In homozygous can^{12} mutant testis (E) the amount of tBRD-1 in the nucleoli was severely reduced in comparison to the *wild-type* situation (C) (arrows). (**G–J**) Single primary spermatocytes from testis squash preparations of homozygous $tbrd\text{-}1^{1}$ mutants (G,I) and *wild-type* (H,J) stained with anti-Sa (G,H) or anti-TAF1 (I,J) antibody. Localisation of Sa and TAF1 within spermatocyte nuclei was not obviously altered in homozygous $tbrd\text{-}1^{1}$ mutants (G,I, arrows). The scale bar (5 µm) in C corresponds to panels C–J.

the chromocenter showed no significant binding of tBRD-1 the puffs are highly stained (Fig. 7A, double arrow, arrows). Deeper analysis showed colocalisation of tBRD-1-eGFP with the interbands of polytene chromosomes (Fig. 7B–D, arrowheads show one interband as example).

Discussion

With tBRD-1 we have identified here a bromodomain-containing protein specifically expressed in the testis in the nuclei of primary spermatocytes. Bromodomain modules are part of many chromatin-associated proteins including histone acetyltransferases (HATs), ATP-dependent chromatin-remodelling factors and the BET family of nuclear proteins, such as Brd2, Brd4 and Bdf1 (Jeanmougin et al., 1997). Moreover, bromodomains can also bind acetylated lysines of non-histone proteins like p53 or c-Myb (Barlev et al., 2001; Sano and Ishii, 2001). tBRD-1 shows similarity to proteins of the BET family (predicted by Ensembl) (Flicek et al., 2011). Function of tBRD-1 is required for spermatid differentiation as mutant males are sterile and exhibit partially disturbed spermatid differentiation. Spermatid nuclei become

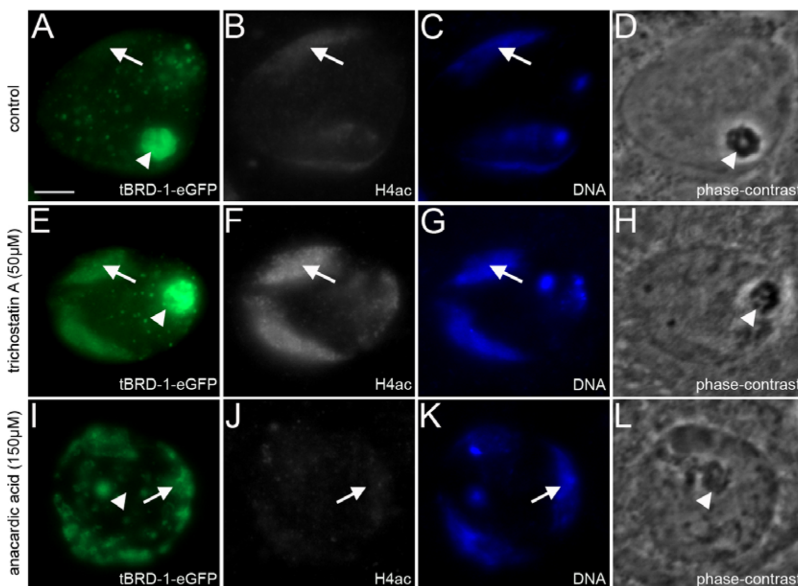

Fig. 6. Acetylation status in primary spermatocytes influences tBRD-1 localisation. (**A–L**) Pupal testis of tBRD-1-eGFP expressing flies were treated with TSA (E–H) or anacardic acid (I–L) for 24 hours in culture and afterwards spermatoyctes were analysed by immunofluorescence using an antibody against acetylated histone H4. (A–D) Untreated control. (C,G,K) Hoechst DNA staining. (D,H,L) Phase-contrast images to visualise the nucleoli (arrowheads). (E) TSA treatment led to an increased localisation of tBRD-1-eGFP to the chromosomes (arrow) while localisation to the nucleolus (arrowhead) was not obviously altered in comparison to the control (A, arrowhead). (F) Strong increase of histone H4 acetylation due to TSA treatment.
(I) Incubation of testis with anacardic acid led to a strong decrease of tBRD-1-eGFP localisation to the nucleolus (arrowhead), while chromosome territories showed a spotted pattern of tBRD-1-eGFP (arrow) in comparison to the control (A, arrow). (J) Only faint amounts of histone H4 acetylation are left upon anacardic acid treatment. Scale bar: 5 µm.

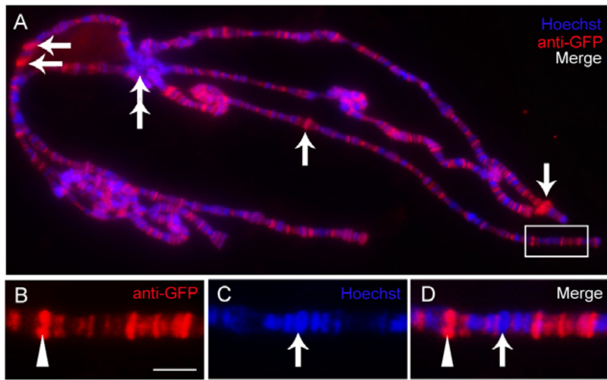

Fig. 7. Ectopically expressed tBRD-1-eGFP localises to interbands of polytene chromosomes. (**A–D**) Polytene chromosomes of larvae expressing UAS-tBRD-1-eGFP driven by Sgs58AB. tBRD-1-eGFP is visualised by anti-GFP antibody in red. DNA is counterstained with Hoechst in blue. (A) An overall view of the polytene spread. tBRD-1-eGFP binds at multiple sites along the polytene chromosomes and on the puffs (arrows). The chromocenter is not significantly stained (double arrow). (B–D) One part of a chromosome arm in a higher magnification. Localisation of tBRD-1-eGFP is prominent at euchromatic interbands (arrowheads show one interband as example) while heterochromatic bands are free of staining (arrows show one heterochromatic band as example). Scale bar is 5 µm in B and corresponds to B–D; the degree of zoom is 3.3.

condensed but remain round and are randomly distributed within spermatid bundles. The typical features of chromatin remodelling during spermatid differentiation are not disturbed in homozygous *tbrd-1^1* mutants. Nevertheless, many other processes and different genes are necessary for this dramatic reorganisation of germ cells. About 350 protein components of mature sperm were identified in whole sperm proteomics (Dorus et al., 2006; reviewed by White-Cooper, 2010). A testis-specifically expressed member of the BET-family in mammals is BRDT which is also required for spermatogenesis. Lacking of the first bromodomain of BRDT in mice leads to production of malformed sperm and male sterility despite unaffected protamine expression (Shang et al., 2007). Previously, in *Drosophila*, the bromodomain-related protein Mtsh was identified. In *mtsh* mutant males meiosis and spermiogenesis proceed though with lack of proper coordination. Mtsh is proposed to participate in transcriptional regulation of spermatogenesis-specific genes (Bergner et al., 2010).

When we analysed the sub-cellular localisation of tBRD-1 within the testis, we observed that expression is restricted to primary spermatocytes. This is in clear contrast to the situation in homozygous *tbrd-1^1* mutants which show first defects of spermatogenesis in post-meiotic stages when expression of tBRD-1 has already vanished. Within primary spermatocytes, we observed a strong localisation of tBRD-1 to the nucleolus as well as localisation over the chromosomes and to several nuclear speckles. In 2009 it was published that CG13597 (tBRD-1) was found as putative component of spliceosomal complexes formed in Kc cell nuclear extracts. However, the authors note that this might simply be a contamination (Herold et al., 2009). As the nuclear speckles tBRD-1 localises to where devoid of active chromatin marks as active RNA-Polymerase II or acetylated histones (data not shown) we focused on the nucleolar and chromosomal localisation of tBRD-1. Fibrillarin is involved in processing of ribosomal RNA (Girard et al., 1993; Jansen et al., 1991). As tBRD-1 proteins largely localised to Fibrillarin-deficient regions within the nucleolus this might argue against a role of tBRD-1 for ribosomal RNA processing. At the same time also tTAFs, TAF1 and Polycomb localised in a Fibrillarin-deficient region within the nucleolus and immunofluorescence stainings showed a partial overlap of tBRD-1 with the tTAF Sa, TAF1 and Polycomb. Only low amounts of tBRD-1 were detectable within chromosome territories, which is also true for tTAFs. Two functions for tTAFs in primary spermatocytes have been described so far: tTAFs directly bind to promoters of several spermatid differentiation genes and they also recruit PRC1 components to the nucleolus (Chen et al., 2005). Thus far, it is not known how tTAFs are recruited to their target genes and also hints for tTAF effector molecules are missing. Analyses of tTAF mutants revealed that tBRD-1 required tTAF function for nucleolar localisation. This was also true for Polycomb and TAF1 (Chen et al., 2005; Metcalf and Wassarman, 2007). In addition, tTAF mutants showed an increased tBRD-1 signal within chromosome territories when compared to the *wild-type* situation. While tBRD-1 required tTAF function for nucleolar localisation, localisation of tTAFs, TAF1 and Polycomb to the nucleolus was not visibly altered in homozygous *tbrd-1^1* mutants when analysed by immunofluorescence. Function of tBRD-1 is required for spermatid differentiation and protein expression is limited to spermatocytes. This holds also true for tTAFs. However, unlike tTAF mutants, which arrest spermatocytes at the G2/M transition and show a complete absence of spermatid differentiation (Hiller et al., 2001; Hiller et al., 2004; Lin et al., 1996), homozygous *tbrd-1^1* mutants do not show a meiotic arrest phenotype. Also expression of spermatid differentiation relevant and tTAF dependent genes *fzo*, *dj* and *Mst87F* was not obviously changed in homozygous *tbrd-1^1* mutant testis. Nevertheless, thus far, only these three direct target genes of tTAFs are known (Chen et al., 2005) and all three gene products fall into three different classes of proteins. The GTPase Fzo, required for mitochondrial fusion, is expressed early after meiosis (Hales and Fuller, 1997). Thus, the mRNA is under translational repression only for a very short time in comparison to that of Mst87F, which encode a protein of the sperm tail, expressed very late during spermatogenesis (Kuhn et al., 1988). DJ shows a dual expression as a chromatin component until the time of histone degradation and as flagellar protein in later stages of spermiogenesis and in mature sperm (Rathke et al., 2007; Santel et al., 1998). All three proteins have different functions and their mRNAs are released from repression at completely different stages. Considering the high amount of gene products required for proper spermatid differentiation many different mRNAs, encode for many different classes of proteins, have to be synthesised in primary spermatocytes. We propose that a set of tTAF target genes exist which is dependent on tBRD-1 function while other tTAF target genes, like *fzo*, *dj* and *Mst87F*, are independent on tBRD-1 function. In humans, for instance, the bromodomain protein BRD7 is required for efficient p53-mediated transcription of a subset of target genes (Drost et al., 2010; Mantovani et al., 2010).

Inhibitor treatment of cultured testis showed that the acetylation status of primary spermatocytes influenced tBRD-1 localisation within the cell. Reduced acetylation disturbed the normal sub-cellular localisation of tBRD-1 and localisation to the nucleolus was strongly reduced, while induced hyperacetylation led to an enhanced localisation of tBRD-1 to the chromosome territories. Obviously, like other bromodomain proteins, also tBRD-1 is able to recognise and bind acetylated histones and/or non-histone proteins. Ectopically expressed tBRD-1 is per se able

to bind polytene chromosomes and localises to euchromatic interbands. This supports the idea that tBRD-1 might regulate transcription.

We hypothesise, that tBRD-1 acts as a reader of acetylated residues of histones and/or non-histone proteins at the promoters of a special set of yet unknown tTAF target genes. Thereby, tBRD-1 may facilitate binding of tTAFs to the promoters of certain genes relevant for spermatid differentiation. In addition, tBRD-1 may support detachment of Polycomb from these promoters. The fact that tTAFs and Polycomb are still detectable within the nucleolus in *tbrd-1* mutants is not mutually exclusive with this theory. Indeed it strengthens this idea because transcription of some tTAF target genes, like *fzo*, *dj* and *Mst87F*, is independent of tBRD-1 function and Polycomb is recruited from the promoters of these genes to the nucleolus.

Materials and Methods

Fly strains and culture

Drosophila melanogaster strains were maintained on standard medium at 25℃. w^{1} (Klemenz et al., 1987) and w^{1118} were used as *wild-type* strains. $P(EPgy2)^{EY02323}$ (BL15415), *Df(3R)ED10893* (BL28827), *Df(3R)Exel9014* (BL7992), *ZH-86Fb* (BL24749) and BL25709 were obtained from the Bloomington Stock Center. *Pc-GFP* flies were kindly provided by R. Paro (Dietzel et al., 1999). can^{12} and sa^{2} mutants (Hiller et al., 2001) were kindly provided by M.T. Fuller (Palo Alto). can^{12} were used for *in situ* hybridisations, immunofluorescence stainings and Western blot analyses. The sa^{2} mutant strain (Hiller et al., 2004) was used for immunofluorescence stainings. *Sgs58AB* (GAL4 strain under control of the regulatory regions of *sgs4*) was kindly provided by A. Hofmann and M. Lehmann (Berlin; unpublished).

In situ hybridisation

Whole mount *in situ* hybridisation of adult testis was performed with modifications according to White-Cooper et al. (White-Cooper et al., 1998). DIG labelled RNA probes were generated using 500 to 800 bp fragments of the corresponding ORFs amplified by PCR on genomic DNA and cloned into pCR®II-TOPO® Vector (Invitrogen).

Remobilisation of $P(EPgy2)^{EY02323}$

The insertion in $P(EPgy2)^{EY02323}$ lies 635 bp downstream of the translational stop codon of *tbrd-1*, 115 bp downstream of the poly(A) signal (Bellen et al., 2004). This strain is homozygous viable and fertile. Before remobilisation the P insertion site was proven by PCR strategy. Genomic DNA of $P(EPgy2)^{EY02323}$ was isolated and used as template DNA in standard PCR reactions. One primer was chosen out of the insertion element and the secondary primer out of the neighbouring genomic region. The insertion was analysed from the 5′ as well as from 3′ end. PCR products were analysed by sequencing. $P(EPgy2)^{EY02323}$ was remobilised by using the transposase source of line *w; Δ2-3 Ki/TM3, Sb* (C. Klämbt, Münster). 80 single jumpstarter males were crossed with females of the balancer line *w; Dr/TM3, Sb Dfd-lacZ*. The P-element was followed by monitoring eye colour. Jump-out of the P-element was indicated by loss of white eye marker. Jump-in of the P-element at a new position was indicated by altered red eye colour in comparison to the red eye colour of the original $P(EPgy2)^{EY02323}$ insertion. Individual white-eyed $P(EPgy2)^{EY02323\text{-}jo}$ jump-out lines as well as $P(EPgy2)^{EY02323\text{-}ji}$ jump-in lines were isogenised and analysed with regards to lethality and male sterility. Loss of tBRD-1 full length expression was proven by immunofluorescence staining and Western blot analyses using anti-tBRD-1. The molecular analysis of $tbrd\text{-}1^{1}$ was done using standard PCR experiments and subsequent sequencing.

Sterility test

20 young adult males (wild-type or tbrd-1-eGFP/+; tbrd-1^{1}/tbrd-1^{1} or tbrd-1-eGFP/tbrd-1-eGFP; tbrd-1^{1}/tbrd-1^{1} or tbrd-1Δc-eGFP/tbrd-1Δc-eGFP; tbrd-1^{1}/tbrd-1^{1}) were placed individually with three wild-type virgin females in separate vials at 25℃. After 5 days the parental generation was removed. The number of offspring in every vial was counted after two weeks.

RT-PCR of *tbrd-1*

Total RNA was extracted from *wild-type* testis, carcass males (testis were removed by dissection), embryos (0–24 hours), larvae (mixture of male and female) and whole bodies of females by using TRIzol® (Invitrogen). We used the OneStep RT-PCR Kit (Qiagen) to amplify a 332 bp cDNA fragment from the open reading frame (ORF) of *tbrd-1*. The chosen primer pair spans an intron of 61 bp to distinguish between PCR products based on cDNA template and those from genomic DNA contamination.

Cloning of the *tbrd-1-eGFP* rescue construct

To generate a *tbrd-1* rescue construct, the open reading frame (ORF) of *tbrd-1* gene together with a 531 bp sequence upstream of the ATG translational start was PCR amplified using genomic DNA and primers with linked *EcoRI* and *SpeI* restriction sites. The PCR fragment was inserted into *p*BSIIKS^{+}*eGFP*, which was opened with *EcoRI* and *XbaI*, in frame with the *eGFP*. This clone was digested with *EcoRI* and *NotI* and the resulting *tbrd-1-eGFP* fragment was cloned into the germline transformation vector *p*ChabΔsal (Thummel et al., 1988) (*lacZ* sequences were removed). Transgenic fly strains were established by injection into w^{1} as described by Michiels et al. (Michiels et al., 1993).

Cloning of the deletion construct *tbrd-1Δc-eGFP*

To generate *tbrd-1Δc-eGFP* the N-terminal part of *tbrd-1* gene together with a 531 bp sequence upstream of the AUG translational start was PCR amplified using genomic DNA and the primer pair: 5′CACCACTGGGACTCCGCCTTATA-GCC3′ and 5′GGAAAAGCGCAAGAGAGAAAGGCTACT3′. The PCR fragment *tbrd-1Δc* was inserted into *p*ENTR™/D-TOPO® (Invitrogen™). *tbrd-1Δc* was subsequently inserted into the transformation vector *pUAST* containing the attR cassettes for Gateway® recombination cloning technology (Invitrogen™), the attB recognition site for phiC31 mediated integration at attP destination sites in the genome as well as the coding sequence for the C-terminal tag eGFP (pUAST-attB-rfa-eGFP; kindly provided by S. Bogdan, Münster; unpublished). Recombination reaction was catalysed by using the Gateway® LR Clonase® II enzyme mix (Invitrogen™). Transgenic fly strains were established by injection into *ZH-86Fb* (BL24749) and BL25709 (Bischof et al., 2007; Markstein et al., 2008).

Cloning of the UAS-*tbrd-1-eGFP* construct

To generate UAS-*tbrd-1-eGFP* the ORF of *tbrd-1* gene was PCR amplified using genomic DNA and primers with linked *EcoRI* and *SacII* restriction sites. The PCR fragment was inserted into *pUASTgreen*, which was opened with *EcoRI* and *SacII*, in frame with the *eGFP*. Transgenic fly strains were established by injection into w^{1118} as described by Michiels et al. (Michiels et al., 1993). *pUASTgreen* was generated by transferring the *eGFP* and the MCS of *pEGFP-N1* (Clontech) into *pUAST* (Brand and Perrimon, 1993) using *BglII* and *XbaI* restriction sites (kindly provided by M. Schäfer, Kassel; unpublished).

Antibodies and immunofluorescence staining

Hoechst staining was used to visualise chromatin. All antibodies were used in immunofluorescence stainings of squashed testis carried out essentially as described in Hime et al. (Hime et al., 1996) and Rathke et al. (Rathke et al., 2007). We raised a peptide antibody (aa 249 to aa 267) against tBRD-1 in rabbit and applied the affinity-purified antibody in a dilution 1:5000 (Pineda- Antibody-Service; http://www.pineda-abservice.de). Other antibodies were used at the following dilutions: anti-Sa 1:500 (guinea pig) and anti-Fibrillarin straight; from M.T. Fuller (Palo Alto) (Chen et al., 2005), anti-TAF1-C 1:800 (rabbit; kindly provided by D. Wassarman (Madison) (Maile et al., 2004), anti-acetyl-histone H4 1:300 (rabbit; Upstate, Cat#06-598), anti-Mst77F 1:500 (Rathke et al., 2010). Cy3-conjugated anti-rabbit (Dianova; 1:100), Cy3-conjugated anti-guinea pig (Dianova; 1:100) and Cy5-conjugated anti-mouse (Dianova; 1:100) were used as secondary antibodies. TUNEL staining was done essentially as described in Rathke et al., 2007. Polytene chromosomes were prepared and stained as described in (Murawska and Brehm, 2012). The GFP-antibody (rabbit, Rockland Inc.) was applied in a 1:500 dilution. Immunofluorescence, eGFP and Hoechst signals were examined using a Zeiss microscope (AxioPlan2) equipped with appropriate fluorescence filters. Images were individually recorded and processed with Adobe Photoshop 7.0.

Immunoblotting

Western blots were performed using standard methods. Protein extracts were made from *wild-type*, homozygous can^{12} and homozygous $tbrd\text{-}1^{1}$ mutant testis. We used 20 testis per protein extract. Dissected testis were homogenised in 20 µl 2×SDS sample buffer by sonication for 30 minutes and incubated afterwards for 5 minutes at 37℃. The whole testis extract was applied to a 10% SDS-gel. Anti-tBRD-1 was used at 1:1000 in 5% dry milk in 1×TBS. Anti-Actin (Biomeda) was used at 1:1000. POD-conjugated anti-rabbit and anti-mouse antibodies were subsequently applied at 1:5000 (Jackson Immunology). ECL reagents (Amersham Pharmacia) were used according to manufacturers recommendation to detect the signals.

Culture of pupal testis and inhibitor treatment

Pupal testis were dissected, cultured and treated with inhibitors as previously described (Awe and Renkawitz-Pohl, 2010). Pupal testis (one day after puparium formation) were dissected in Shields and Sang M3 insect culture medium (Sigma-Aldrich Cat#S8398) supplemented with 10% fetal bovine serum (heat inactivated,

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insect culture tested, Sigma-Aldrich Cat#F3018), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco-Invitrogen Cat#15140-148). Cultures were incubated at 25℃ and treated with inhibitors for about 24 hours before fixation. Anacardic acid (Merck Biosciences Cat#172050) was dissolved in DMSO to obtain a 28.69 mM stock solution. Trichostatin A (Cell Signalling Tech. Cat#9950) was dissolved in ethanol to obtain a 4 mM stock solution. For treatment, inhibitors were diluted appropriately in culture medium and added to the culture chambers. Immunofluorescence staining procedure is the same as described above.

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Competing Interests

The authors declare that there are no competing interests.

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