RESEARCH ARTICLE

tBRD-1 and tBRD-2 regulate expression of genes necessary for spermatid differentiation

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ABSTRACT

Male germ cell differentiation proceeds to a large extent in the absence of active gene transcription. In Drosophila, hundreds of genes whose proteins are required during post-meiotic spermatid differentiation (spermiogenesis) are transcribed in primary spermatocytes. Transcription of these genes depends on the sequential action of the testis meiotic arrest complex (tMAC), Mediator complex, and testis-specific TFIID (tTFIID) complex. How the action of these protein complexes is coordinated and which other factors are involved in the regulation of transcription in spermatocytes is not well understood. Here, we show that the bromodomain proteins tBRD-1 and tBRD-2 regulate gene expression in primary spermatocytes and share a subset of target genes. The function of tBRD-1 was essential for the subcellular localization of endogenous tBRD-2 but dispensable for its protein stability. Our comparison of different microarray data sets showed that in primary spermatocytes, the expression of a defined number of genes depends on the function of the bromodomain proteins tBRD-1 and tBRD-2, the tMAC component Aly, the Mediator component Med22, and the tTAF Sa.

KEY WORDS: Testis-specific transcription, tTAFs, tMAC, Mediator complex, BET proteins

INTRODUCTION

In Drosophila melanogaster and mammals, the post-meiotic phase of spermatogenesis (spermiogenesis) is characterized by extensive morphological cell changes (Rathke et al., 2014). In flies, transcription almost ceases as the cells enter meiotic division; therefore, these changes mainly rely on proteins arising from translationally repressed and stored mRNAs synthesized in primary spermatocytes (Olivieri and Olivieri, 1965; White-Cooper et al., 1998). Hence, a tightly regulated gene transcription program is required to ensure proper spermiogenesis and male fertility.

In primary spermatocytes, numerous transcripts are synthesized and translationally repressed (Fuller, 1993; White-Cooper et al., 1998). Transcription of the corresponding genes (spermiogenesis-relevant genes) depends on two testis-specific transcription complexes: the testis meiotic arrest complex (tMAC), and the testis-specific TFIID complex, which consists of testis-specific TATA box binding protein-associated factors (tTAFs) (Beall et al., 2007; Hiller et al., 2004, 2001). Recruitment of tTAFs to chromatin requires the coactivator complex Mediator, and localization of Mediator subunits to chromatin depends on tMAC (Lu and Fuller, 2015). Based on these data, it has been suggested that Mediator acts as a key factor in a tTAF- and tMAC-dependent gene regulatory cascade that leads to transcriptional activation of spermiogenesis-relevant genes (Lu and Fuller, 2015).

Acetylated lysines of histone play an important role in gene transcription (Sanchez and Zhou, 2009). These histone modifications are recognized by bromodomain-containing proteins (Dhalluin et al., 1999). The bromodomain forms a well-conserved structure within functionally distinct proteins, such as histone acetyltransferases, chromatin-remodeling factors, transcriptional co-activators and mediators, and members of the bromodomain and extra-terminal (BET) family (Josling et al., 2012). Members of the BET family are characterized by having one (in plants) or two (in animals) N-terminal bromodomains and an conserved extra-terminal domain that is necessary for protein–protein interactions (Florence and Faller, 2001; Matangkasombut et al., 2000; Platt et al., 1999). BET proteins contribute to transcription mainly by recruiting protein complexes, e.g. transcription factors and chromatin remodelers (Josling et al., 2012; Krogan et al., 2003; Matangkasombut et al., 2000). In mammals, the BET proteins BRD2, BRD3, BRD4, and BRDT are expressed in male germ cells (Klaus et al., 2016; Shang et al., 2004). BRDT is involved in gene expression during spermatogenesis, among other roles (Berkovits et al., 2012; Gaucher et al., 2012), but the functions of BRD2, BRD3, and BRD4 in male germ cells are not well understood.

In Drosophila, three testis-specific bromodomain proteins (tBRDs) have been described (Leser et al., 2012; Theofel et al., 2014). tBRD-1 contains two bromodomains, is essential for male fertility, and partially co-localizes with tTAFs in primary spermatocytes (Leser et al., 2012). Likewise, the BET family members tBRD-2 and tBRD-3 partially co-localize with tBRD-1 and tTAFs in primary spermatocytes (Theofel et al., 2014). In addition, subcellular localization of the three tBRDs depends on both tTAF function and the level of acetylation within the cell (Leser et al., 2012; Theofel et al., 2014). Loss of tBRD-1 function leads to an altered distribution of tBRD-2 and tBRD-3 and to a significant down-regulation of a subset of tTAF target genes (Theofel et al., 2014). Protein–protein interaction studies have revealed that tBRD-1 forms homodimers and also heterodimers with tBRD-2, tBRD-3, and tTAFs (Theofel et al., 2014). The loss of tBRD-1 or tBRD-2 leads to similar post-meiotic phenotypes, e.g. nuclear elongation...
defects (Kimura and Loppin, 2015; Leser et al., 2012). It has been postulated that in primary spermatocytes, tBRDs cooperate with tTAFs to regulate expression of selected spermiogenesis-relevant genes (Theofel et al., 2014).

Here, we show that a tbrd-1-eGFP transgene restores not only male fertility of tbrd-1 mutants but also localization of tBRD-2 to chromosomal regions. Protein–protein interaction studies demonstrated that both bromodomains are dispensable for tBRD-1 homodimer formation and that the extra-terminal domain of tBRD-2 interacts with the C-terminal region of tBRD-1. Peptide pull-down experiments indicated that tBRD-1 but not tBRD-2 preferentially recognizes acetylated histones H3 and H4. Microarray analyses revealed that several genes are significantly down-regulated in tbrd-2-deficient testes. A comparison of different microarray data sets demonstrated that tBRD-1, tBRD-2, the tMAC component Aly, the Mediator component Med22, and the tTAF Sa share a subset of target genes. Finally, immunofluorescence stainings showed that the sub-cellular localization of tBRD-1 and tBRD-2 requires Aly function.

**RESULTS**

**Expression of tBRD-1-eGFP reconstitutes proper sub-cellular localization of tBRD-2 in tbrd-1 mutant spermatocytes**

Recently, we have shown that the tbrd-1 mutant phenotype is rescued by a tbrd-1-eGFP transgene, which contains the tbrd-1 open reading frame together with upstream and downstream sequences (Theofel et al., 2014). Here, we aim at mapping the interaction domains required for dimerization using a series of tBRD-1 and tBRD-2 truncation mutants in the yeast two-hybrid assay (Fig. 2; Figs S2 and S3). tBRD-1 and tBRD-2 contain several conserved domains, namely the bromodomains and an extra-terminal domain, which consists of a NET domain and a SEED domain and is predicted to mediate protein–protein interactions (Florence and Faller, 2001; Matangkasombut et al., 2000; Platt et al., 1999). Accordingly, we focused our analysis on these domains. Full-length tBRD-1 formed homodimers with tBRD-1AN, which lacks the first bromodomain (BD1) (Fig. 2A; Fig. S2B) and with tBRD-1ΔC, which lacks both bromodomains and consists only of the spacer region that connects these two domains (Fig. 2A; Fig. S2B). No interaction was observed between full-length tBRD-1 and tBRD-2ΔC, which contains the first bromodomain but an incomplete spacer region (Fig. 2A; Fig. S2B). These results indicated that the spacer region between the bromodomains (amino acids 165–336) is essential for tBRD-1 homodimer formation (Fig. 2C). Next, we sought to determine which tBRD-2 sequences mediate binding to tBRD-1. We analyzed the interaction of several tBRD-2 deletion

![Fig. 1. The tBRD-1-eGFP fusion protein restores the localization of tBRD-2 to chromosomes in spermatocytes of tbrd-1 mutants.](http://bio.biologists.org/Downloaded from http://bio.biologists.org/)
mutants with full-length tBRD-1 (Fig. 2B; Fig. S3A-D,F,H). We first mapped the binding to a C-terminal region containing the NET and SEED domains. Further analysis revealed that neither of these two domains was essential for tBRD-1 binding. Instead, tBRD-1 interaction required the region connecting the NET and SEED domains (amino acids 444–580). Finally, we showed that the C-terminus (amino acids 410–514) of tBRD-1 is required for heterodimerization with tBRD-2 (Fig. 2A; Fig. S3E,G). In summary, our results showed that the spacer region between the two bromodomains mediates tBRD-1 homodimerization (Fig. 2C) and indicated that tBRD-1 and tBRD-2 interact via the C-terminus of tBRD-1 with the extra-terminal domain of tBRD-2 (Fig. 2D).

**tBRD-1 recognizes acetylated histones H3 and H4 in vitro**

Previously, we have shown that localization of tBRD-1 and tBRD-2 to the chromosomal regions in spermatocytes is acetylation dependent (Leser et al., 2012; Theofel et al., 2014). This finding implied that tBRD-1 and tBRD-2 might directly interact with acetylated histone tails. To test this hypothesis, we purified recombinant tBRD-1 and tBRD-2 using the baculovirus system and performed peptide pull-down assays with histone H3 and histone H4 peptides that were unmodified or acetylated at specific residues. Immobilized peptides were incubated with recombinant tBRD-1 or tBRD-2, and bound proteins were analyzed in western blots using tBRD-1- or tBRD-2-specific antibodies (Fig. 3A). tBRD-1 bound to all unmodified or acetylated histone H3 and H4 peptides analyzed, in keeping with the idea that histone interactions might contribute to chromatin binding of tBRD-1, but tBRD-1 preferentially bound to acetylated histone tails (Fig. 3A). Likewise, tBRD-2 bound to all unmodified or acetylated histone peptides tested. In contrast to tBRD-1, however, tBRD-2 did not preferentially bind acetylated peptides, and acetylation instead appeared to reduce binding affinity. We concluded that tBRD-1 and tBRD-2 both interact with histone tails in vitro and that this binding reaction is sensitive to histone acetylation. To investigate whether these acetylated histones are present in spermatocytes, we stained them with immunofluorescent antibodies raised against different histone H3 and H4 acetylation marks (Fig. 3B–J). H3K9ac (Fig. 3B), H3K18ac (Fig. 3D), H3K23ac (Fig. 3E), H3K27ac (Fig. 3F), H4K5ac (Fig. 3H), H4K8ac (Fig. 3I), and H4K12ac (Fig. 3J) signals were detected at the chromosomal regions in primary spermatocytes (arrows) and acetylated

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**Fig. 2. The region between the two bromodomains is required for tBRD-1 homodimerization, and the C-terminus interacts with the extra-terminal domain of tBRD-2.**

(A,B) Schematic overview of the full-length and mutated (A) tBRD-1 and (B) tBRD-2 proteins analyzed in yeast two-hybrid experiments for interaction with (A,B) full-length tBRD-1 and (A) tBRD-2 proteins. +, interaction observed; –, no interaction observed. (C) Schematic representation of the observed interaction between tBRD-1 and tBRD-1 via the region between the two bromodomains. (D) Schematic representation of the observed interaction between the C-terminus of tBRD-1 with the extra-terminal domain of tBRD-2. BD, bromodomain; NET, NET domain; SEED, SEED domain.
fig. 3. tBRD-1 associates with acetylated histones. (A) Association of recombinant tBRD-1-Flag and tBRD-2-Flag proteins with non-acetylated and acetylated histone H3 and H4 peptides [H3(1–22), H3(14–33), H3(23–42)]. H4 (1–20)] was examined in peptide pull-down assays. Bound proteins were analyzed in western blots using tBRD-1- or tBRD-2-specific antibodies. Acetylated proteins are indicated, e.g., H3K9ac, K14ac: H3 acetylated on Lys9 and Lys14. (B–J) Single primary spermatocyte nuclei of w^{1118} flies stained with specific antibodies against histone H3 acetylated on (B) Lys9, (C) Lys14, and (J) Lys12. Arrows, chromosomes; arrowheads, nucleoli. Scale bar: 5 μm.

histones H3K14ac and H3K36ac were barely detected at the chromosome regions in primary spermatocytes (Fig. 3C,G, arrows).

tBRD-2 and tBRD-1 share a subset of target genes
In microarray experiments, we analyzed the impact of tBRD-2 on gene expression in the testis using RNA of bam\(\triangleright tbrd-2^{RNAi}\) testes with testes RNA of tbrd-2\(^{RNAi}\) and bam-Gal4 males as controls. Depletion of tbrd-2 in testes was validated by quantitative PCR (qPCR), western blot analyses, and immunofluorescence microscopy (Fig. 4A–C). Knockdown of tbrd-2 led to a significant reduction of tbrd-2 transcripts compared to control testes (Fig. 4A). Likewise, tBRD-2 was not detected in bam\(\triangleright tbrd-2^{RNAi}\) testes in western blots (Fig. 4B) and immunofluorescence analyses (Fig. 4C–C'; Fig. S1). By contrast, transcript and protein levels of tbrd-1 and tbrd-3 were not altered in bam\(\triangleright tbrd-2^{RNAi}\) testes (Fig. S4A–D'). Further analyses revealed that bam\(\triangleright tbrd-2^{RNAi}\) males were sterile (Fig. S5) and exhibited spermatid differentiation defects, e.g., altered Nebenkern formation (Fig. 4D, arrow) and lack of nuclear elongation (Fig. 4E; Fig. S1B, arrowheads). In both controls (Fig. 4D, D'), the phase-dark, round Nebenkern was nearly the same size as the nucleus. In bam\(\triangleright tbrd-2^{RNAi}\) spermatids, the Nebenkern seemed to be fused together (Fig. 4D'). Mst77F-positive spermatid nuclei of bam-Gal4 (Fig. 4E, arrow) and tbrd-2\(^{RNAi}\) (Fig. 4E; arrow) were elongated and started to develop the typical needle-like structure of mature sperm nuclei, whereas Mst77F-positive spermatid nuclei of bam\(\triangleright tbrd-2^{RNAi}\) did not elongate and remained round (Fig. 4E, arrow).

For microarray experiments, Affymetrix Drosophila Genome 2.0 arrays were used, and three independent hybridizations per genotype were performed. The expression values for each probe set from the three arrays of the same genotype were averaged, and the log2-fold change between tbrd-2 knockdown and one of the controls (undriven tbrd-2\(^{RNAi}\) or bam-Gal4) was calculated. Knockdown of tbrd-2 led to a significant down-regulation of 73 probe sets, reflecting 69 protein-coding genes (log2-fold change\(\leq-1\); corrPVal \(\leq0.05\)) compared to both controls (Fig. 5A); 104 probe sets, reflecting 99 protein-coding genes, were significantly up-regulated (log2-fold change\(\geq1\); corrPVal \(\leq0.05\)) (Fig. 5B). As expected, tbrd-2 was one of the most down-regulated genes in bam\(\triangleright tbrd-2^{RNAi}\) testes. In agreement with qPCR results (Fig. S4A), tbrd-1 and tbrd-3 were not affected.

In order to identify common target genes of tBRD-2 and tBRD-1, the transcriptomes of bam\(\triangleright tbrd-2^{RNAi}\) and tbrd-1 mutant testes (Theofel et al., 2014) were compared. Among the 69 down-regulated protein coding genes in bam\(\triangleright tbrd-2^{RNAi}\), 38 protein-coding genes were also significantly down-regulated in tbrd-1 mutants (data not shown). Hence, 55% of the protein-coding genes that were positively regulated by tBRD-2 likewise require tBRD-1. Among the 99 up-regulated protein-coding genes, only 25 were affected in the two transcriptomes (data not shown). In a previous study, we have shown that transcripts of CG13946, CG17917, CG18673, CG42827, CG42828, and Yp3 are significantly down-regulated in tbrd-1 mutant testes, whereas TwdIV, CG1441, CG31750 and cutlet are significantly up-regulated (Theofel et al., 2014). According to our microarray data presented here, CG13946, CG17917, CG18673, CG42827, CG42828 and TwdIV depended on tBRD-2 function, but Yp3, CG1441, CG31750, and cutlet did not. Therefore, qPCRs using cDNA of bam\(\triangleright tbrd-2^{RNAi}\) and control testes were carried out to validate common and specific tBRD-2 and tBRD-1 target genes (Fig. 5C,D). Indeed, transcript levels of CG13946, CG17917, CG18673, CG42827, CG42828 and TwdIV were significantly reduced in bam\(\triangleright tbrd-2^{RNAi}\) testes compared to controls, but transcript levels of Yp3 were not (Fig. 5C). Likewise, only transcript levels of TwdIV were significantly up-regulated in bam\(\triangleright tbrd-2^{RNAi}\) testes (Fig. 5D). Our results demonstrated that tBRD-2 directly or indirectly regulates gene expression in the testis and shares a subset of target genes with tBRD-1.

tBRD-1, tBRD-2, the tMAC component Aly, the Mediator complex subunit Med22, and the tTAF 5a share a defined set of target genes
We compared the transcriptomes of bam\(\triangleright tbrd-2^{RNAi}\) (relative to that of undriven tbrd-2\(^{RNAi}\) control testes), tbrd-1 (Theofel et al., 2014), aly, Med22 and sa mutant testes (Lu and Fuller, 2015) (Fig. 6A–C). We focused on the role of tBRD-1 and tBRD-2 in activating transcription. Numerous probe sets significantly down-regulated in bam\(\triangleright tbrd-2^{RNAi}\) testes, in tbrd-1 mutant testes, or in
both were likewise down-regulated in aly (Fig. 6A), Med22 (Fig. 6B), and sa (Fig. 6C) mutant testes. Of the 447 probe sets that were down-regulated in tbrd-1 mutants (Tables S1 and S3), 60 were likewise down-regulated in tbrd-2 knockdown testes (Table S3). Of the 387 probe sets affected in tbrd-1 but not in tbrd-2 mutants (Table S1), 71 were likewise down-regulated in all three (aly, Med22, and sa) mutant testes, whereas 231 were unaffected in all of these mutant testes (Table S1). Of the 141 down-regulated probe sets in tbrd-2 mutants (Tables S2 and S3), 60 were likewise down-regulated in tbrd-1 mutant testes (Table S3). Of the 81 probe sets affected in tbrd-2 but not in tbrd-1 mutant testes (Table S2), 27 were likewise down-regulated in all three (aly, Med22, and sa) mutant testes, whereas 35 were unaffected. Of the 60 down-regulated probe sets in both tbrd-1 and tbrd-2 mutants, 39 were likewise down-regulated in all three (aly, Med22, and sa) mutant testes, whereas 13 were not dependent on Aly, Med22, and Sa function (Table S3). In all three situations (tbrd-1 with aly, Med22, and sa mutants; bam->tbrd-2RNAi with aly, Med22, and sa mutants; tbrd-1/bam->tbrd-2RNAi with aly, Med22, and sa mutants) the observed overlap between down-regulated genes was much stronger than expected in a random distribution (tbrd-1: hypergeometric $P<6.6\times10^{-11}$; bam->tbrd-2RNAi: hypergeometric $P<9.8\times10^{-11}$; tbrd-1/bam->tbrd-2RNAi: hypergeometric $P<3.2\times10^{-20}$). By contrast, up-regulated genes only showed minor overlaps that were not significant (Tables S1–S3). In total, 39 probe sets representing 31 protein-coding genes were significantly down-regulated in bam->tbrd-2RNAi, tbrd-1, aly, Med22 and sa mutant testes (Table S3). A comparison of this defined set of genes with the *Drosophila* Spermatogenesis Expression Database (http://mnlab.uchicago.edu/spress/; Vibriovski et al., 2009) revealed that the corresponding transcripts are enriched mainly in post-meiotic male germ cells (Table S4). This led us to postulate that transcription of these genes gives rise to translationally repressed mRNAs coding for spermiogenesis-relevant proteins. In addition, according to FlyAtlas (Chintapalli et al., 2007), most of the transcripts are enriched in the testes (Table S4). Hence, we assume that expression of a precise number of genes, relevant for post-meiotic spermatogenesis, are regulated by all five proteins, namely tBRD-1, tBRD-2, the tTAF Sa, and the tMAC component Aly, the Mediator complex subunit Med22, and the tTAF Sa.

**The tMAC component Aly is required for proper sub-cellular localization of tBRD-1 and tBRD-2**

Previously, we have shown that subcellular localization of tBRD-1 and tBRD-2 depends on tTAF function (Leser et al., 2012; Theofel et al., 2014). Here, we analyzed the localization of tBRD-1 and tBRD-2 in heterozygous and homozygous aly mutants (Fig. 7). Immunofluorescence staining showed that correct localization of tBRD-1 (Fig. 7A–B″) and tBRD-2 (Fig. 7C–D″) required wild-type Aly function. The localization of tBRD-1 and tBRD-2 to the chromosomal regions was strongly reduced in homozygous aly mutant spermatocytes (Fig. 7B,D, arrows). Likewise, the localization of tBRD-1 and tBRD-2 to the nucleoli was clearly reduced (Fig. 7B,D, arrowheads). In addition, tBRD-1- and tBRD-2-positive nuclear speckles were larger and reduced in number in aly mutant spermatocytes (Fig. 7B,D).
DISCUSSION

In Drosophila, spermatocytes execute a highly active and strictly regulated transcription program to provide transcripts necessary for post-meiotic spermiogenesis. Transcription of spermiogenesis-relevant genes is based on the cooperation among tTAFs, tMAC components, and Mediator complex components (Beall et al., 2007; Chen et al., 2011; Hiller et al., 2004; Lu and Fuller, 2015). Recently, we have postulated that the testis-specific bromodomain proteins tBRD-1, tBRD-2, and tBRD-3 cooperate with the testis-specific TFIID complex in regulating transcription of a subset of spermiogenesis-relevant genes (Theofel et al., 2014). Here, we uncovered additional potential links between tBRD proteins, Mediator, and tMAC.

The function of tBRD-1 is essential for proper sub-cellular localization of endogenous tBRD-2

Previously, we have shown that in testes of transgenic flies, endogenous tBRD-1 interacts with tBRD-2-eGFP (Theofel et al., 2014). Here, we further focused on the interaction between tBRD-1 and tBRD-2 and showed that expression of tBRD-1 can restore sub-cellular localization of tBRD-2 in primary spermatocytes in a tbrd-1 mutant background. These results indicated that tBRD-1 and tBRD-2 indeed interact in Drosophila spermatocytes. The structure of tBRD-1 and tBRD-2 proteins differ from that of classical BET family members in animals, which are mainly characterized by two N-terminal bromodomains and a C-terminal extra-terminal domain consisting of a NET motif and a SEED motif (Florence and Faller, 2001). tBRD-1 contains two bromodomains but no extra-terminal domain, and tBRD-2 contains only one bromodomain but does contain a C-terminal extra-terminal domain (Theofel et al., 2014). The extra-terminal domain has been described as necessary for protein–protein interactions (Florence and Faller, 2001).
and Faller, 2001; Matangkasombut et al., 2000; Platt et al., 1999). However, it has been shown that human BRD2 requires the first N-terminal bromodomain for dimerization (Nakamura et al., 2007). More recent results have shown that homodimer and heterodimer formation of BET proteins is mediated by a conserved motif, termed motif B, between the second bromodomain and the extra-terminal domain (Garcia-Gutierrez et al., 2012). We showed in yeast two-hybrid experiments that the C-terminal part of tBRD-1 and the extra-terminal domain of tBRD-2 are essential for interaction of the two proteins. By contrast, homodimer formation of tBRD-1 proteins required the region between the two bromodomains.

Recently, it has been suggested that the interaction of tBRD-1 and tBRD-2 is required for their protein stability (Kimura and Loppin, 2015). However, we did not observe lower amounts of tBRD-2 protein in tbred-1 mutant spermatocyte nuclei. However, also in this case, we did not observe a reduced tBRD-2 signal in tbred-1 mutant spermatocyte nuclei. Hence, the loss of tBRD-1 seems to affect the sub-cellular localization of tBRD-2. Our results showed that the function of tBRD-1 is required for proper sub-cellular localization of tBRD-2 but not vice versa. In addition, the function of tBRD-1 seems to be dispensable for tBRD-2 protein stability. Whether this dependency is based upon direct interaction of the two proteins still has to be clarified.

tBRD-1 binds to acetylated histones independently of tBRD-2

Previously, we have shown that an increased acetylation level in spermatocytes enhances the localization of tBRD-1 and tBRD-2 to the chromosomal regions (Leser et al., 2012; Theofel et al., 2014).
However, it was unclear whether both proteins directly bind to acetylated histone tails. In the current study, *in vitro* experiments demonstrated that the double bromodomain protein tBRD-1 bind to H3 peptides acetylated at lysines 9 and 14 and to H4 peptides acetylated at lysines 5, 8, and 12. By contrast, tBRD-2 exhibited a higher affinity for non-acetylated histone peptides under the same conditions. Acetylation of N-terminal histone tails of H3 and H4 is a typical feature of transcriptional active chromatin and serves as a binding platform for epigenetic regulators, such as BET proteins (Davie and Candido, 1978; Dhalluin et al., 1999; Hebbes et al., 1988). It has been previously shown that the acetylation marks tested in this study are recognized by BET proteins (Marchand and Caflisch, 2015) and are involved in active gene expression (Morris et al., 2007; Wang et al., 2008). In addition, all tested acetylation marks except those of H3K14ac and H3K36ac were detected in spermatocyte nuclei, which indicated that tBRD-1 recognizes acetylated H3 at lysine 9 and/or 14 and acetylated H4 at lysine 5, 8, and/or 12 also *in vivo*.

In murine round spermatids, acetylated H3 and H4 are enriched at the transcription start sites of spermiogenesis-relevant genes and are recognized by the BET proteins BRD4 and BRDT (Bryant et al., 2015). Recently, it has been suggested that the interaction of tBRD-1 and tBRD-2 allows the two proteins to function together as a single BRDT-like BET protein (Kimura and Loppin, 2015). Therefore, it is conceivable that tBRD-2 requires tBRD-1 for efficient binding to chromatin. However, it is also possible that tBRD-2 recognizes other, not yet tested acetylation marks independently of tBRD-1. As tBRD-1 and tBRD-2 regulate both common and different sets of target genes, both scenarios could occur in spermatocytes. Our data suggest that in *Drosophila*, as in mice, bromodomain proteins act together to efficiently support the activation of spermiogenesis-relevant genes by binding to acetylated lysine residues.

### tBRD-1 and tBRD-2 co-regulate a subset of target genes

Our microarray analyses showed that tBRD-2, like tBRD-1, is involved in gene activation and repression. The comparison of transcriptome data of a *tbrd-1* mutant with that of a *tbrd-2* knockdown clearly indicated that the two bromodomain proteins share a subset of target genes. However, we observed that the expression of some genes was altered in *tbrd-1* mutant tests but not in *tbrd-2* knockdown testes and vice versa, which suggested that some genes are regulated specifically by either tBRD-1 or tBRD-2. In mice, the BET proteins BRDT and BRD4 cooperate to regulate transcription of spermiogenesis-relevant genes, although they can also act independently. Importantly, it has been demonstrated that genes co-bound by BRDT and BRD4 show a higher transcriptional activity than genes bound only by BRD4 or BRDT (Bryant et al., 2015). Further experiments are required to examine whether tBRD-1 and tBRD-2 directly bind to their target genes and whether the binding of both enhances transcription.

### An overlapping set of spermiogenesis-relevant genes is regulated by tBRD-1, tBRD-2, the tMAC complex, Mediator complex, and tTAFs

It has been proposed that the activation of spermiogenesis-relevant genes in *Drosophila* spermatocytes requires the sequential action of the tMAC complex, Mediator complex, and testis-specific TFIID (tTFIID) complex (Chen et al., 2011; Lu and Fuller, 2015). The tMAC component Topi interacts with the Mediator component Med22, but no direct interaction has been observed between Mediator and tTFIID components. However, when Med22 is knocked down, the tTAF 5a fails to localize to chromatin, which suggests that tTAFs depend on Mediator to be recruited to chromatin or stabilized there (Lu and Fuller, 2015). Previously, we have shown that the proper localization of tBRD-1 and tBRD-2 depends on tTAF function (Leser et al., 2012; Theofel et al., 2014). In addition, we have demonstrated that tBRD-1 and the tTAF 5a share a subset of target genes (Theofel et al., 2014). In our current study, immunofluorescence analyses revealed a dramatically reduced localization of tBRD-1 and tBRD-2 to chromosomal regions in homozygous *aly* mutant spermatocytes. We hypothesized that also tBRD-1 and tBRD-2 are involved in the gene regulatory cascade in spermatocytes recently proposed by Lu and Fuller (2015).

Therefore, we compared our *tbrd-1* and *tbrd-2* mutant transcriptome data with that of *sa*, *aly*, and *med22* mutants (Lu and Fuller, 2015; Theofel et al., 2014). Indeed, a defined subset of 31 genes were regulated by all five factors. The transcripts of most of these genes are enriched in the testes and accumulate in post-meiotic germ cells (Chintapalli et al., 2007; Vibranovski et al., 2009), which suggests that these transcripts are among the translationally repressed mRNAs required for spermatid differentiation. In contrast to Sa, Aly, and Med22, tBRD-1 and tBRD-2 are involved in the regulation of only a small number of genes. Expression of known tTAF-, tMAC-, and Mediator-dependent spermiogenesis-relevant genes, e.g., *fzo, janB, gdl* and *CG9173*, is not affected in *tbrd-1* and *tbrd-2* mutants. Nevertheless, our data showed that tBRD-1, tBRD-2, Sa, Aly, and Med22 regulate a common set of genes. We hypothesize that tBRD-1 and tBRD-2 act at the end of a gene regulatory cascade involving tMAC, Mediator, and tTAF functions to regulate expression of spermiogenesis-relevant genes.

### MATERIALS AND METHODS

#### Fly strains and RNAi experiments

Flies were maintained under standard conditions at 25°C. *w*^118^ was used as the wild-type strain. To knockdown *tbrd-2*, homozygous males of the RNAi line v37671 (Vienna *Drosophila* Resource Center; Dietzl et al., 2007) were crossed against virgins of *bam-Gal4*/*bam-Gal4*; *Sp/CyO; bam-Gal4*VP16/ *MKRS* (Caporilli et al., 2013; Chen and McKearin, 2003). *bam-Gal4*/*tbrd-2* RNAi males analyzed in this study carried two copies of *bam-Gal4* (on chromosomes X and III) and one copy of *tbrd-2* RNAi (on chromosome III). Undriven *tbrd-2* RNAi control males carried one copy of *tbrd-2* RNAi, and *bam-Gal4* control males carried two copies of *bam-Gal4* (on chromosomes X and III). RNAi crossings (including control flies) were maintained at 30°C. The transgenic line *tbrd-1-GFP* and the *tbrd-1* mutant strain *tbrd-1* ^-1^ have been recently described (Leser et al., 2012). *aly*^5^ mutants were kindly provided by Helen White-Cooper (Cardiff University, School of Biosciences).

#### Fertility tests

Batches of 20 flies were tested for fertility. Adult males were crossed individually against two wild-type virgin females in separate vials at 25°C. After 6 days, the parental generation was removed. The number of offspring was counted after two weeks.

#### Immunofluorescence stainings

Testes squash preparations were immunofluorescently stained essentially as described in Hime et al. (1996) and Rathke et al. (2007). A peptide antibody was raised against tBRD-2 (amino acids 436–457) in rabbit (Pineda-Antibody-Service; http://www.pineda-abservice.de). The affinity-purified antibody was used at a dilution of 1:1000. Anti-tBRD-1 was applied at a dilution of 1:5000 (Leser et al., 2012). Anti-tBRD-3 (Theofel et al., 2014) and anti-Mst77F (Rathke et al., 2010) were used at a dilution of 1:1000. Anti-histone (Millipore; clone F152.C25.WJ1) was used at a dilution of 1:1200. To detect acetylated histones, the following antibodies were used: anti-H3K9ac (Sigma-Aldrich, H9286; 1:500), anti-H3K14ac (Active Motif, Carlsbad, CA, USA, 39698; 1:500), anti-H3K18ac (Active Motif, 39756;
1:500), anti-H3K23ac (Active Motif, 39132; 1:600), anti-H3K27ac (Active Motif, 39136; 1:500) and anti-H3K36ac (Active Motif, 39380; 1:250). H4K5ac, H4K8ac, and H4K12ac were detected using the acetyl-histone H4 antibody set (17-211) from Millipore. DNA was visualized via Hoechst staining. As secondary antibodies, Cy3-conjugated anti-rabbit (Dianova; 1:100), Cy2-conjugated anti-rabbit (Dianova; 1:100), and Cy5-conjugated anti-mouse (Dianova; 1:100) were used. Immunofluorescence stainings were examined using a Zeiss microscope (AxioPlan2). Figures were designed using Adobe Photoshop CS2.

**Western blot experiments**

Western blot experiments were performed as recently described (Lester et al., 2012). For each protein extract, 20 testes of heterozygous or homozygous tbrd-1Δ mutants or tbrd-2Δ mutants, undriven tbrd-2ΔRNAi, or tbrd-2ΔRNAi were used. Anti-tBRD-1, anti-tBRD-2, and anti-Pan-Actin (Cell Signaling Technologies, 4968) were used at a dilution of 1:1000.

**Yeast two-hybrid assays**

Yeast two-hybrid interaction tests were performed using the Matchmaker™ GAL4 Two-Hybrid System 3 from Clontech according to the manufacturer’s manual. tBRD-1 and tBRD-2 full-length yeast constructs are described in Theofel et al. (2014). Mutated tbrd-1 and tbrd-2 ORFs were PCR amplified using specific primers with linked restriction sites (Table S5) and ligated into pGADT7 (bait vector) and pGBKT7 (prey vector). Translational start and stop codons were introduced via the specific primers. To amplify tbrd-1-LAC (base pairs 1–492) the primer pair tbrd-1-LAC-fw/tbrd-1-LAC-rv was used. tbrd-1-LAC2 (base pairs 1–1227) was amplified using tbrd-1-LAC-fw/tbrd-1-LAC2-rv. tbrd-1AN (base pairs 493–1542) was amplified using tbrd-1AN-fw/tbrd-1AN-rv. tbrd-1A (base pairs 382–1008) was amplified using tbrd-1A-fw/tbrd-1A-rv. To amplify tbrd-2LAC (base pairs 1–1062), the primer pair tbrd-2LAC-fw/tbrd-2LAC-rv was used. tbrd-2AN (base pairs 1060–2025) was amplified using tbrd-2AN-fw/tbrd-2AN-rv. The tbrd-2ABD consists of two parts. Base pairs 1–150 were amplified using the primer pair tbrd-2ABD-fw1/tbrd-2ABD-rv1, and base pairs 358–2025 were amplified using the primer pair tbrd-2ABD-fw2/tbrd-2ABD-rv2. The two PCR products were ligated together into the TOPO-TA vector (Life Technologies) using Xmal and Xhol. The constructed final tbrd-2ABD containing the required restriction sites to ligate the PCR product into pGADT7 or pGBK7 was amplified using the primers tbrd-2-Y2H-Ndel-fw and tbrd-2-Y2H-EcoRI-rv. The first part of tbrd-2ANET (base pairs 1–1086) was amplified using tbrd-2ANET-fw1/tbrd-2ANET-rv1; the second part (base pairs 1330–2025) was amplified using tbrd-2ANET-fw2/tbrd-2ANET-rv2. The two parts were ligated together into the TOPO-TA vector (Life Technologies) using Xmal and Xhol. The constructed final tbrd-2ANET was amplified using tbrd-2-Y2H-Ndel-fw/tbrd-2-Y2H-EcoRI-rv. To amplify tbrd-2ΔSEED (base pairs 1–1740) the primer pair tbrd-2ΔSEED-fw/tbrd-2ΔSEED-rv was used. tbrd-2ΔSEED was cloned into pVL1392 (vector pVL1392). Transfection of Sf9 cells, recombinant baculovirus production, and recombinant protein expression and purification essentially followed the methods described in Brehm et al. (2000).

**Peptide pull-down experiments**

H3 and H4 peptides were synthesized (PSL Peptide Specialty Laboratories) and coupled to SulfoLink™ coupling resin (Thermo Scientific) according to the manufacturer’s instructions. One microgram of each peptide was added to 1 μl beads: 2.5 μl of the coupled beads were mixed with 17.5 μl uncoupled beads and washed in pull-down buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 1 mM DTT, protease inhibitors) for 5 min twice. After blocking for 1 h at 4°C in blocking buffer [1 mg/ml BSA, 1% fish skin gelatin (Sigma) in pull-down buffer], beads were incubated with 0.25 μg recombinant proteins for 2 h at 4°C. Beads were washed four times in pull-down buffer. Bound proteins were analyzed by SDS-PAGE and western blotting using tBRD-1 and tBRD-2-specific antibodies; 20% of the input was loaded on the gel.

**Data availability**

The microarray data are available at the NCBI gene expression omnibus (GEO) under the accession number GSE81019.

Total RNA from 100 bamΔ/tbrd-2ΔRNAi testes, undriven tbrd-2ΔRNAi testes, and bam-Gal4 testes was extracted using TRIzol (Invitrogen). RNA was treated with RNase-Free DNase (Promega). For cDNA synthesis, 1 μg of DNase-digested RNA and the Transcriptor First Strand cDNA Synthesis Kit (Roche) were used. qPCR reactions contained 7.5 μl iQ™ Universal SYBR® Green Supermix (Bio-Rad), 5.2 μl ddH2O, 2 μl diluted cDNA, 0.3 μl (10 μM) gene-specific primer 1, and 0.3 μl (10 μM) gene-specific primer 2. qPCR (three technical replicates) was performed with a SybrGreen platform on a Bio-Rad CFX Cycler. Data were analyzed using Bio-Rad CFX Manager™ software. Values were normalized to the mRNA expression level of Rp49. Differences between groups were determined with analyses of variance. Delta Ct values were analyzed for ANOVA using the aov function of R. For the differences between individual groups post hoc tests were calculated by Tukey’s honest significant difference test (TukeyHSD function). Two groups were compared using one-way ANOVA. The corresponding P-values indicated in the figures are **P<0.05, ***P<0.01, and ****P<0.001. Primers are given in Table S6.

**Author contributions**

Conceived the project and designed the experiments: I.T. and C.R. Performed the experiments: I.T., T.B., S.M.K.G., C.R., and J.K. Analyzed and interpreted the data: I.T., M.B., A.B., and C.R. Wrote the paper: I.T. and C.R.

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