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 a conserved extra-terminal domain that is necessary for protein–protein interactions (Florence and Fuller, 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

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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 531 bp upstream of the open reading frame and 591 bp downstream together with 531 bp upstream of the open reading frame and 591 bp downstream of the open reading frame of the transgene, which contains the endogenous tbrd-1 (Leser et al., 2012). In addition, we have shown that tBRD-1 co-localizes with tBRD-2-eGFP, whose transgene contains the tbrd-2 open reading frame and 591 bp upstream of the translatable start fused in frame with eGFP. Furthermore, tBRD-1 function is required for proper tBRD-2-eGFP localization, and tBRD-1 interacts with tBRD-2-eGFP in vivo (Theofel et al., 2014). We have not been able to address whether localization of endogenous tBRD-2 protein is also dependent on tBRD-1 function. Towards this end, we raised a peptide antibody against tBRD-2 and tested its specificity in immunofluorescent stainings of tbrd-2 knockdown and control testes (Fig. S1). Flies carrying a UAS-tbrd-2RNAi transgene were crossed with a bam-Gal4 driver line (bam>UAS-tbrd-2RNAi) to down-regulate expression of tBRD-2 in the testis by RNAi. tBRD-2 was detected in spermatocyte nuclei of control testes (Fig. S1A), but almost no signal was observed in tbrd-2 knockdown testes (Fig. S1B). We then analyzed the localization of endogenous tBRD-2 in heterozygous and homozygous tbrd-1 mutants and in heterozygous and homozygous tbrd-1 mutants expressing a tBRD-1-eGFP fusion protein (Fig. 1). Western blot analyses revealed that endogenous tBRD-2 levels were not reduced in tbrd-1 mutant testes (Fig. 1A). In heterozygous tbrd-1 mutant spermatocyte nuclei, endogenous tBRD-2 localized to chromosomal regions, nucleolus, and nuclear speckles in the nucleoplasm (Fig. 1B). However, although tBRD-2 protein levels were not reduced in homozygous tbrd-1 mutant testes, only a faint tBRD-2 signal was visible in spermatocyte nuclei of homozygous tbrd-1 mutants (Fig. 1C). By contrast, expression of a full-length tBRD-1-eGFP fusion protein in the homozygous tbrd-1 mutant background reconstituted tBRD-2 localization to both the chromosomal regions and nucleolus (Fig. 1E'). These results extend our previous analysis and strengthen the idea that endogenous tBRD-1 and tBRD-2 interact and that tBRD-2 requires tBRD-1 for proper sub-cellular localization.

The bromodomains of tBRD-1 are dispensable for homodimer formation, and the very C-terminus of tBRD-1 interacts with the extra-terminal domain of tBRD-2

Recently, we have shown that tBRD-1 forms homodimers and also heterodimers with tBRD-2 (Theofel et al., 2014). Here, we aimed 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-1ΔC, 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 mutants with tBRD-1.
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 and the region between the NET and the SEED domains 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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**tBRD-2 and tBRD-1 share a subset of target genes**

In microarray experiments, we analyzed the impact of tBRD-2 and tBRD-1 on gene expression in the testis using RNA of *bam* > *tbrd-2* RNAi testes with testes RNA of *tbrd-2* RNAi and *gal4* controls (Theofel et al., 2014) were compared. Among the 69 down-regulated protein coding genes in *bam* > *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 positively regulated by tBRD-2. Among the 69 down-regulated protein-coding genes in *bam* > *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 positively regulated by tBRD-2. Among the 69 down-regulated protein-coding genes in *bam* > *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 positively regulated by tBRD-2.

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**tBRD-1, tBRD-2, the tMAC component Aly, the Mediator complex subunit Med22, and the tTAF Sa share a defined set of target genes**

We compared the transcriptomes of *bam* > *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* > *tbrd-2* RNAi testes, in *tbrd-1* mutant testes, or in...
In total, 39 probe sets representing 31 protein-coding genes were knocked down by RNAi-mediated knockdown of tbrd-2RNAi (Fig. 4). The detection of Actin with anti-Actin antibodies served as a loading control. Single primary spermatocyte nuclei of (C) bam-Gal4, (C’) tbrd-2RNAi, and (C’’) bam=tbrd-2RNAi flies stained with anti-tBRD-2 antibody. Photos had the same exposure time. Scale bar: 5 μm. (D) Replacement of histones by Mst77F in post-meiotic spermatid nuclei of (E) bam-Gal4, (E’) tbrd-2RNAi, and (E’’) bam=tbrd-2RNAi visualized by immunofluorescence staining using antibodies against histones (white) and Mst77F (green). Arrowheads: E.E’, needle-like structure of mature sperm nucleus; E”: unelongated, round sperm nuclei. Scale bars: 20 μm.

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-1RNAi 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-1RNAi mutants (Table S3). Of the 81 probe sets affected in tbrd-2RNAi 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 \(P<3.2\times10^{-28}\)). 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/; Vibranovski 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 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 aly2 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 aly2 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-eGFP 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 et al., 2001).

Fig. 5. tBRD-2 is required for gene expression. (A,B) Venn diagrams depicting the overlap of (A) 73 significantly down-regulated and (B) 104 significantly up-regulated probe sets in *bam>=>tbrd-2(RNAi)* tests compared to the controls (*bam-Gal4* and undriven *tbrd-2(RNAi)*). (C,D) qPCR using cDNA from 50 testes pairs of *bam>=>tbrd-2(RNAi)*, *tbrd-2(RNAi)*, and *bam-Gal4* tests. (C) Expression of genes CG13946, CG17917, CG18673, CG42827, CG42828 and Yp3. (D) Expression of TwdV, CG1441, CG31750 and cutlet. The values were normalized to the expression of Rpl32. ANOVA with post hoc Tukey’s honest significant difference test was used to evaluate statistical significance. *P*-values for significance: *P*≤0.05; ***P*≤0.001; NS, not significant.

Fig. 6. tBRD-1 and tBRD-2 share a subset of target genes with Aly, Med22, and Sa. (A–C) Scatter plots depicting transcript levels (log2-transformed gene expression values) in (A) *aly*, (B) *Med22*, and (C) *sa* mutant testes (y-axes) compared to wild-type control testes (x-axes). Green and blue dots represent significantly down-regulated genes in *tbrd-1* mutant testes in comparison to control testes. Red and blue dots represent transcripts of genes in *bam>=>tbrd-2(RNAi)* tests expressed significantly lower than in undriven *tbrd-2(RNAi)*. Blue dots represent transcripts that are affected by both tBRD-1 and tBRD-2.
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 protein stability (Kimura and Loppin, 2015). However, we did not observe an altered tBRD-1 protein distribution or changes in protein levels in \( tbrd-2 \) knockdown testes compared to controls. tBRD-2 proteins were barely detectable in \( tbrd-1 \) mutant spermatocyte nuclei. However, also in this case, we did not observe lower amounts of tBRD-2 protein in \( tbrd-1 \) mutant testes in western blots. 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 binds 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 testes 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 TFIID components. However, when Med22 is knocked down, the tTAF Sa 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 Sa 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.
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-1 mutants or barm>btd-2RNAI, undriven btd-2RNAI, or bam-Gal4 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 pGBK7T (prey vector). Translational start and stop codons were introduced via the specific primers. To amplify tbrd-1-AC (base pairs 1–492) the primer pair tbrd-1-AC-fw/tbrd-1-AC-rv was used. tbrd-1-AC was amplified using tbrd-1-AC-fw/tbrd-1-AC-rv. tbrd-1AN (base pairs 493–1542) was amplified using tbrd-1AN-fw/tbrd-1AN-rv. tbrd-1N (base pairs 382–1008) was amplified using tbrd-1A-fw/tbrd-1A-rv. To amplify tbrd-2AC (base pairs 1–1062), the primer pair tbrd-2AC-fw/tbrd-2AC-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 XmaI and XhoI. The constructed final tbrd-2ABD containing the required restriction sites to ligate the PCR product into pGADT7 or pGBK7T was amplified using the primers tbrd-2YH-Ndel-fw and tbrd-2YH-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 XmaI and XhoI. The constructed final tbrd-2ANET was amplified using tbrd-2YH-Ndel-fw/tbrd-2YH-EcoRI-rv. To amplify tbrd-2ASEED (base pairs 1–1740) the primer pair tbrd-2ASEED-fw/tbrd-2ASEED-rv was used, tbrd-2ASEED was amplified using specific primers and ligated into the baculovirus transfer expression vector pVL1392. Transfection of S9 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 Specialty Laboratory) 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, proteinase 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.

RNA isolation and microarray experiments

Total RNA was isolated from barm>btd-2RNAI, undriven btd-2RNAI, and bam-Gal4 testes using TRIzol (Invitrogen). RNA quality was monitored using the Agilent Bioanalyzer 2100 with the RNA 6000 Nano kit. Gene expression was analyzed using Affymetrix Drosophila Genome 2.0 arrays according to the manufacturer’s recommendations. For each array, independent RNA from whole testes pooled from 25 animals was used. Three independent replicates were prepared for each experimental condition. The data were analyzed in the R statistical environment using BioConductor packages (Huber et al., 2015). Scanned data were parsed as CEL files into R using the /affy/ package (Gautier et al., 2004). Expression estimates were extracted using RNA normalization with the /ma/ function. Differentially expressed genes were identified using Limma (Ritchie et al., 2015). Genes with log2 (fold expression change) >1 or –1 and an adjusted P-value <0.05 were selected as significantly up- or down-regulated, respectively.

For comparison with previously published data (Lu and Fuller, 2015), we downloaded CEL files from the GEO repository (GSE74784) and processed them as described above to obtain log2-transformed expression measures. To make the different data sets comparable, initial RNA was applied to the complete data set.

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

Quantitative real-time PCR

Total RNA from 100 barm>btd-2RNAI testes, undriven btd-2RNAI testes, and barm-Gal4 testes was extracted using TRIzol (Invitrogen). RNA was treated with RQ1 RNase-Free DNase (Promega). For cDNA synthesis, 1 µg DNase-digested RNA and the Transcriptor First Strand cDNA Synthesis Kit (Roche) were used. qPCR reactions contained 7.5 µl TaqMan 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 Sybyr green 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 Rpl32. Differences between groups were determined with analyses of variance. Delta Ct values were analyzed for ANOVA using the asv 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.

Expression and purification of recombinant tBRD-1 and tBRD-2

tbrd-1 and tbrd-2 cDNAs were FLAG tagged at the C-terminus by PCR using specific primers and ligated into the baculovirus transfer expression vector pVL1392. Transfection of S9 cells, recombinant baculovirus production, and recombinant protein expression and purification essentially followed the methods described in Brehm et al. (2000).

Competing interests

The authors declare no competing or financial interests.

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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Data availability

The microarray data are available at the NCBI gene expression omnibus (GEO); accession number GSE81019 at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE81019.
