

# Bromodomain-dependent stage-specific male genome programming by Brdt

Jonathan Gaucher<sup>1</sup>, Fayçal Boussouar<sup>1</sup>,  
Emilie Montellier<sup>1</sup>, Sandrine Curtet<sup>1</sup>,  
Thierry Buchou<sup>1</sup>, Sarah Bertrand<sup>1</sup>,  
Patrick Hery<sup>2</sup>, Sylvie Jounier<sup>2</sup>,  
Arnaud Depaux<sup>2</sup>, Anne-Laure Vitte<sup>1</sup>,  
Philippe Guardiola<sup>3</sup>, Karin Pernet<sup>4</sup>,  
Alexandra Debernardi<sup>1</sup>, Fabrice Lopez<sup>5</sup>,  
Hélène Holota<sup>5</sup>, Jean Imbert<sup>5</sup>,  
Debra J Wolgemuth<sup>6</sup>, Matthieu Gérard<sup>2</sup>,  
Sophie Rousseaux<sup>1,\*</sup> and Saadi Khochbin<sup>1,\*</sup>

<sup>1</sup>INSERM, U823, Université Joseph Fourier—Grenoble 1, Institut Albert Bonniot, Grenoble, France, <sup>2</sup>CEA, iBiTec-S, Gif-sur-Yvette, France, <sup>3</sup>INSERM, U892, Centre de Recherche sur le Cancer Nantes Angers et UMR\_S 892, Université d'Angers, Plateforme SNP, Transcriptome and Epigénomique, Centre Hospitalier Universitaire d'Angers, Angers, France, <sup>4</sup>INSERM U836, Université Joseph Fourier, Grenoble Institute of Neuroscience, Grenoble, France, <sup>5</sup>INSERM UMR\_S 1090, TGML/TAGC, Aix-Marseille Université, Marseille, France and <sup>6</sup>Department of Genetics and Development and Obstetrics and Gynecology, Columbia University Medical Center, New York, NY, USA

**Male germ cell differentiation is a highly regulated multi-step process initiated by the commitment of progenitor cells into meiosis and characterized by major chromatin reorganizations in haploid spermatids. We report here that a single member of the double bromodomain BET factors, Brdt, is a master regulator of both meiotic divisions and post-meiotic genome repackaging. Upon its activation at the onset of meiosis, Brdt drives and determines the developmental timing of a testis-specific gene expression program. In meiotic and post-meiotic cells, Brdt initiates a genuine histone acetylation-guided programming of the genome by activating essential genes and repressing a 'progenitor cells' gene expression program. At post-meiotic stages, a global chromatin hyperacetylation gives the signal for Brdt's first bromodomain to direct the genome-wide replacement of histones by transition proteins. Brdt is therefore a unique and essential regulator of male germ cell differentiation, which, by using various domains in a developmentally controlled manner, first drives a specific spermatogenic gene expression program, and later controls the tight packaging of the male genome.**  
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\*Corresponding authors. S Rousseaux or S Khochbin, INSERM, U823, Université Joseph Fourier, Grenoble 1, Institut Albert Bonniot, Grenoble F-38700, France. Tel.: +33 4 76 54 95 83; Fax: +33 4 76 54 95 95; E-mail: sophie.rousseau@ujf-grenoble.fr or Khochbin@ujf-grenoble.fr

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## Introduction

With the recent availability of specific bromodomain inhibitors, the highly conserved double bromodomain factors of the BET family (Florence and Faller, 2001; Sanchez and Zhou, 2009) have become promising targets, especially in anticancer therapies (Muller *et al.*, 2011). A precise understanding of their diverse activities is essential for a rational targeting of their functions. In this regard, *Brdt*, a BET member with a restricted expression in male germ cells, is a prototype of choice, since spermatogenesis involves very specific gene expression patterns (Sassone-Corsi, 2002), as well as major reorganization of chromatin (Gaucher *et al.*, 2010). Indeed, one of the most dramatic examples of chromatin remodelling takes place during the post-meiotic phases, when an almost genome-wide histone removal occurs, associated with the assembly of new DNA-packaging structures formed by non-histone proteins (Rousseaux *et al.*, 2008). Since a correlation between histone hyperacetylation and histone removal had been observed in many organisms (Govin *et al.*, 2004), we looked for bromodomain-containing proteins specifically expressed in male germ cells as potentially interesting candidate factors capable of establishing a functional link between these two events. An *in silico* search led to the identification of the double bromodomain protein, Brdt, with a testis-specific pattern of expression, as a potentially important factor acting on acetylated chromatin in post-meiotic cells (Pivot-Pajot *et al.*, 2003). This idea received strong support from our subsequent structural studies, which revealed the unusual capacity of its bromodomains to recognize a defined combination of acetylated histones. In particular, Brdt's first bromodomain, BD1, binds histone H4 only if a simultaneous acetylation of H4K5 and H5K8 occurs (Moriniere *et al.*, 2009). Since acetylation of both H4K5 and H4K8 is a characteristic of hyperacetylated H4 (Zhang *et al.*, 2002; Garcia *et al.*, 2007), Brdt was also identified as the first factor specific for hyperacetylated H4. Interestingly, this property of Brdt to recognize hyperacetylated H4 was confirmed *in vivo* independently, in living cells, in studies using a particular Brdt-H4 FRET probe (Sasaki *et al.*, 2009). This hyperacetylation-dependent activity of Brdt allowed us to predict its action precisely when histone hyperacetylation occurs, at the time of histone replacement by transition proteins (TPs) (Moriniere *et al.*, 2009). Another observation supporting this prediction was that male mice expressing Brdt deleted of its BD1 showed defective spermatogenesis (Shang *et al.*, 2007), with abnormalities appearing in post-meiotic cells exactly when spermatids begin their elongation and exchange their histones for TPs.

To unravel the functions of Brdt, we investigated spermatogenesis in three mouse models, including *Brdt* KO mice, where the protein is totally absent, and mice expressing a non-functional or a partially functional protein. This work enabled us to demonstrate that Brdt is one of the most critical

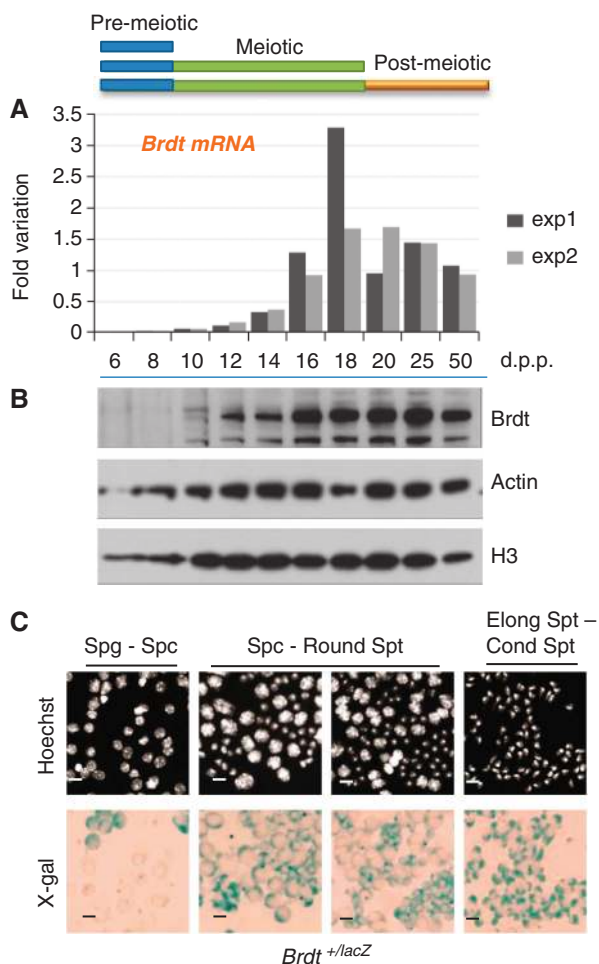
factors involved in male genome programming in a domain-specific manner, both in meiotic and in haploid cells.

Altogether, we provide here the first thorough molecular analysis of the activity of a BET factor in its physiological context and shed light on its context and domain-dependent activity.

## Results

### *Brdt* gene activation at the onset of meiosis

As a first step towards the understanding of *Brdt*'s function, its expression during spermatogenic differentiation was determined at the mRNA and protein levels during the first wave of spermatogenesis, as well as in adult mouse testis. First, RNA extracted from pre-pubertal and adult mouse testes at time points corresponding to defined steps of spermatogenesis was used to monitor the presence of *Brdt* mRNA. Figure 1A shows that the first detectable accumulation of *Brdt* mRNA corresponds to a particular period when



**Figure 1** *Brdt* is activated at the onset of meiosis. The expression of *Brdt*/*Brdt* was analysed either by RT-qPCR (A) or by western blots (B). The histograms represent the values of biological duplicates (normalized with respect to *Actin* as a control gene and to *Brdt* mean expression in 50-day-old wt testes).  $\beta$ -Galactosidase activity, driven by *Brdt* gene promoter, was assessed in seminiferous tubules sections (C, lower panels: 'X-gal'). The corresponding sections were stained by Hoechst (C, upper panels: 'Hoechst'). Scale bars, 10  $\mu$ m. Figure source data can be found with the Supplementary data.

type B spermatogonia give rise to early meiotic cells (preleptotene, leptotene and zygotene) at 10–12 days post partum (d.p.p.), producing a clearly detectable protein at 12 d.p.p. (Figure 1B). The activity of the *Brdt* promoter was visualized using a mouse strain driving the expression of the *lacZ* gene under *Brdt* gene promoter (*Brdt*<sup>+/lacZ</sup>). Sections of seminiferous tubules of adult mouse testes were stained for  $\beta$ -galactosidase activity and the results confirmed both the absence of *Brdt* expression in spermatogonia and its expression in spermatogenic cells at later stages (Figure 1C; Supplementary Figure S1). Hence, according to the timing of expression of its gene, *Brdt* should first act in spermatogenic cells during meiotic prophase.

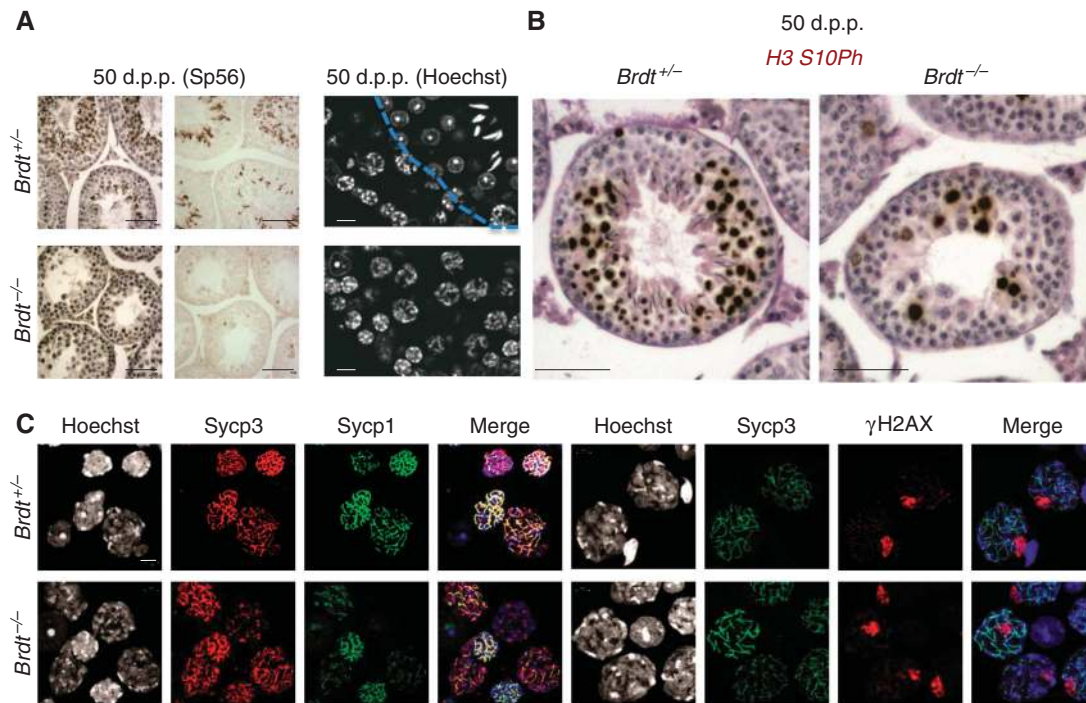
### *Brdt* ensures critical functions before the first meiotic division

To understand *Brdt*'s function in spermatocytes, testes from *Brdt*<sup>-/-</sup> mice were analysed morphologically for the occurrence of the first detectable defects. Figure 2A shows a total absence of post-meiotic cells in adult mice testes, since no cells expressed the acrosomal protein, Sp56, in the seminiferous tubules sections, and no round spermatids could be detected in DNA stained tubule sections. The staining of histological sections of these testes with an antibody specific for H3 phosphorylated at serine 10 (H3S10Ph), showed a dramatic decrease in cells bearing this histone mark (Figure 2B), suggesting that a blockage occurs at the end of the meiotic prophase when chromosomes should undergo compaction in preparation for the first meiotic division. A detailed analysis of spermatocytes in seminiferous tubule sections showed that earlier meiotic events occur normally, including chromosome pairing, synaptonemal complexes and sex body formation (Figure 2C). Therefore, *Brdt*'s action becomes specific and indispensable in late pachytene spermatocytes, but its absence does not significantly alter the total number of pachytene cells or cells at earlier stages. A comparable number of spermatocytes up to diplotene stage could be observed in testes from wild-type and *Brdt* KO mice (Supplementary Figure S2).

### *Brdt* is a major determinant in the establishment of a testis-specific gene expression program

To investigate the molecular basis of *Brdt*'s action in spermatocytes, transcriptomic analyses of *Brdt*<sup>-/-</sup> spermatogenic cells at 17 and 20 d.p.p. were carried out. These timings were chosen since they correspond to an accumulation of pachytene spermatocytes (17 d.p.p.) and to the pachytene/diplotene transition (20 d.p.p.), respectively, and are stages before the appearance of a visible phenotype in the *Brdt*<sup>-/-</sup> animals. The results at 20 d.p.p. show that *Brdt* controls the expression levels of >3000 genes, with approximately two-thirds of the genes being downregulated and one-third upregulated in the absence of *Brdt* (corresponding to *Brdt*-activated and *Brdt*-repressed genes, respectively). At 17 d.p.p., 10% of these genes are already affected, but, in contrast to 20 d.p.p., mostly *Brdt*-activated genes are observed (Figure 3A).

We decided to exploit our transcriptomic data to uncover the characteristics of *Brdt*-regulated genes. The list of *Brdt*-regulated genes at 20 d.p.p. was subdivided into two groups: genes requiring *Brdt* for their activation (*Brdt*-activated genes) and genes repressed in the presence of *Brdt*



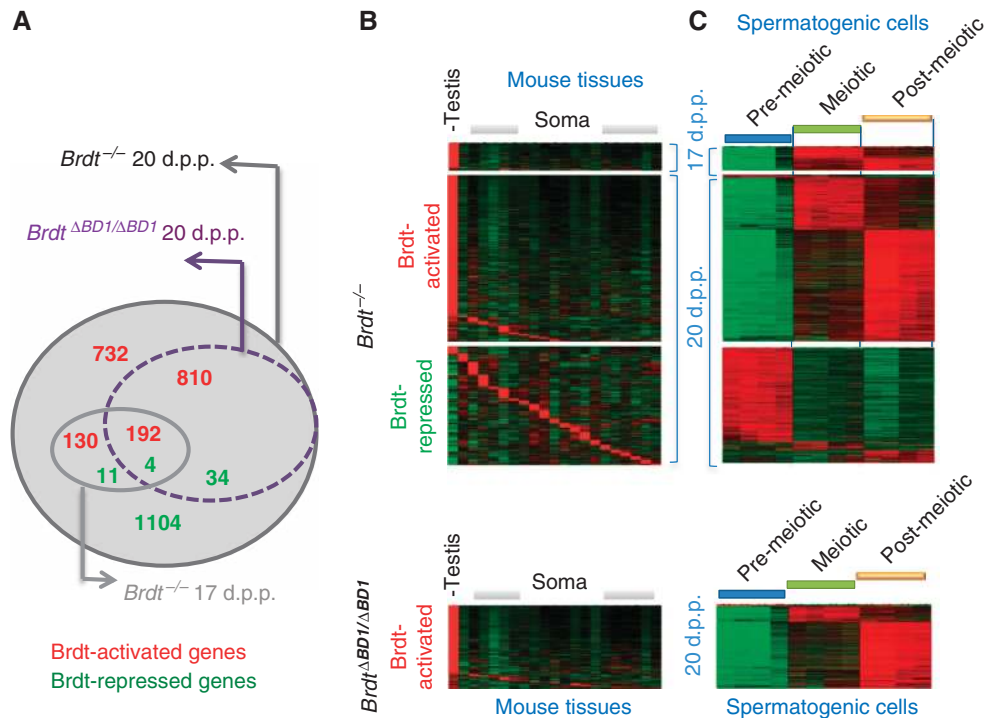
**Figure 2** Brdt is required at the late stages of male meiotic prophase. (A) Testis histological sections, from 50 d.p.p. *Brdt*<sup>+/-</sup> and *Brdt*<sup>-/-</sup> animals, either stained by PAS/HE (Periodic Acid Schiff/Haematoxyline Eosine), or analysed by immunohistochemistry (IH) of the post-meiotic specific acrosome antigen, Sp56, without counter staining (left and middle panels, respectively). Scale bars, 50  $\mu$ m. A higher magnification of adult testis sections at 50 d.p.p., stained with Hoechst is represented (right panels). A dotted line separates meiotic cells (lower left side of the panel) from post-meiotic cells (upper right side of the panel). Scale bars, 10  $\mu$ m. (B) Testis histological sections, from 50 d.p.p. *Brdt*<sup>+/-</sup> and *Brdt*<sup>-/-</sup> animals stained by IH with an antibody against S10 phosphorylated histone H3. Scale bars, 50  $\mu$ m. (C) Synaptonemal complex components, Sycp1 and Sycp3, and sex body marker  $\gamma$ H2AX, were co-detected as indicated, in seminiferous tubule sections of *Brdt*<sup>+/-</sup> and *Brdt*<sup>-/-</sup> mice. Scale bars, 5  $\mu$ m.

(Brdt-repressed genes). First, we asked whether these Brdt-regulated genes are normally specifically or predominantly expressed in a particular mouse tissue. Mouse tissue transcriptomic data (available from the GEO website: GSE10744, GSE12950 and GSE9954) were downloaded and the expression pattern of our lists of Brdt-activated and Brdt-repressed genes in different tissues was established. As shown in Figure 3B (upper panels), almost all Brdt-activated genes are predominantly expressed in the testis, while the Brdt-repressed genes show no particular tissue specificity of expression. Interestingly, this profile is also visible with the Brdt-activated genes at 17 d.p.p. (Figure 3B). We then investigated whether these Brdt-regulated genes, testis-specific or not, would show any particular patterns of expression in spermatogenic cells. To this end, we used available transcriptomic data corresponding to the staged mouse spermatogenic cells: spermatogonia, spermatocytes and post-meiotic cells (GSE21749 and GSE4193). Figure 3C shows that the Brdt-activated testis-specific genes are all switched on in spermatocytes (meiotic cells), and are either fully active in these cells or have their maximum expression level in post-meiotic cells. None of these genes are expressed in spermatogonia, in agreement with the absence of Brdt expression in these cells (Figure 1). The complete opposite occurs with Brdt-repressed genes, which are nearly all predominantly expressed in spermatogonia (Figure 3C, pre-meiotic, Brdt-repressed genes). Together, these data show that Brdt is a major determinant of spermatocyte cell identity, turning on a spermatocyte-specific gene expression program and directly or indirectly repressing pre-meiotic genes.

To explore the specific contribution of BD1 in Brdt's transcriptional activity, we also performed a transcriptomic analysis of 20 d.p.p. testes from *Brdt*<sup>ABD1/ABD1</sup> mice. The results show first that all of the genes affected by the absence of BD1 are included in the *Brdt*<sup>-/-</sup> differentially expressed genes (Figure 3A), giving high confidence in the robustness and reliability of the transcriptomic data. These data show that BD1 is required for the transcriptional activation of more than half of the genes whose activation is also affected by the total absence of Brdt (downregulated in *Brdt*<sup>-/-</sup> testis). Interestingly, within the affected genes, almost none correspond to the upregulated in the *Brdt*<sup>-/-</sup> testes, demonstrating that BD1 has no role in gene repression (Figure 3A). To better characterize the BD1-dependent genes, we examined their expression patterns in various mouse tissues and in spermatogenic cells (using the same approach as described above). Figure 3B and C (lower panels) shows first that nearly all these genes are testis-specific, and second that a large majority of them are active in spermatocytes and/or in post-meiotic cells.

#### ***Brdt* specifically activates promoter-acetylated testis-specific genes**

In order to have a deeper insight into the molecular basis of Brdt transcriptional regulatory activities, Brdt-bound genomic regions were mapped in both meiotic and post-meiotic cells. We took advantage of our recent mapping of nucleosomes bearing acetylated histones in the same cell fractions that showed the presence of histone acetylation at the transcriptional start sites (TSSs) of a group of testis-specific genes



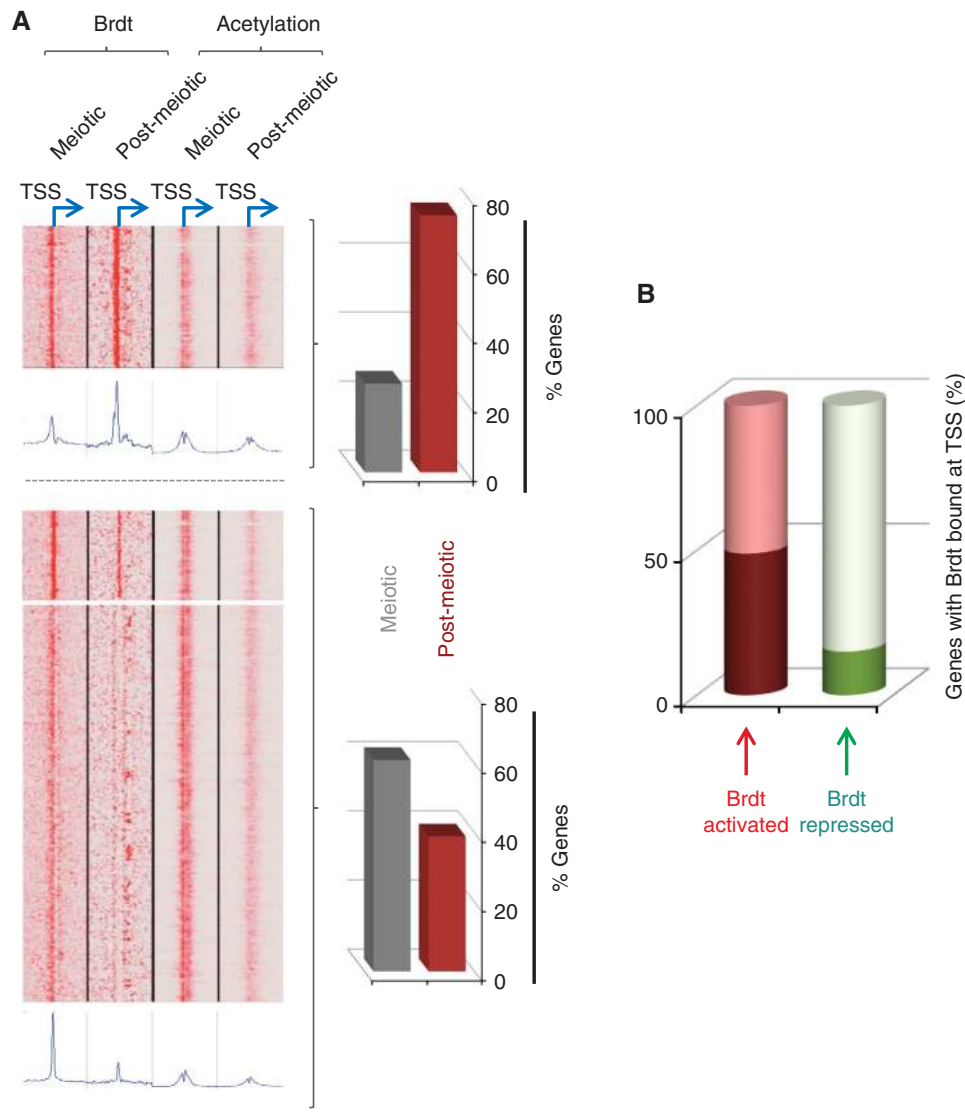
**Figure 3** Brdt is a major testis-specific transcriptional regulator. (A) The transcriptome of the *Brdt*<sup>-/-</sup> testes at 20 d.p.p. shows a total of 3017 genes affected by the absence of Brdt, of which 1153 genes were repressed by Brdt (upregulated in *Brdt*<sup>-/-</sup> testes compared to heterozygous testes) and 1864 genes activated by Brdt (downregulated in *Brdt*<sup>-/-</sup> testes compared to heterozygous testes). The genes differentially expressed in the testes of *Brdt*<sup>-/-</sup> mice at 17 d.p.p. ( $n=337$ ) were all included in those downregulated in the *Brdt*<sup>-/-</sup> testes at 20 d.p.p. The genes affected by the absence of the first bromodomain BD1 ( $\Delta BD1/\Delta BD1$ ) ( $n=1040$ ) nearly all corresponded to a subset of downregulated genes in the *Brdt*<sup>-/-</sup> testes. (B, C) RNA extracted from testes at 17 and 20 d.p.p. from *Brdt*<sup>+/-</sup> (Ctrl,  $n=6$ ) or *Brdt*<sup>-/-</sup> ( $n=6$ ), as well as from 20 d.p.p. testes from *Brdt*<sup>+/+</sup> ( $n=5$ ) and *Brdt*<sup>ΔBD1/ΔBD1</sup> ( $n=5$ ) was used for a transcriptomic analysis. The differentially expressed genes (fold change threshold of 1.5), between *Brdt*<sup>+/-</sup> and *Brdt*<sup>-/-</sup> mice at 17 d.p.p. and at 20 d.p.p., as well as between *Brdt*<sup>ΔBD1/ΔBD1</sup> and wild-type tests at 20 d.p.p., were respectively classified as ‘Brdt-activated genes’ (genes repressed in the absence of Brdt or in the presence of  $\Delta BD1$  Brdt), or as ‘Brdt-repressed genes’ (genes activated in the absence of Brdt). Their respective expression status was then retrieved from mouse tissue transcriptomes (B) and staged spermatogenic cell transcriptomes (C) and shown as heatmaps. The low versus high expression levels are represented on a green to red scale. ‘Soma’ represents the following mouse tissues from left to right: ovary, embryonic stem cells, placenta, foetus, brain, eye, pituitary, adipose tissue, adrenal gland, bone marrow, heart, kidney, liver, lung, muscle, salivary gland, seminal gland, small intestine, spleen and thymus.

(Tan *et al*, 2011). Here, we used the SeqMiner software capable of ChIP-Seq peak visualization in defined genomic regions (Ye *et al*, 2010) to look at Brdt binding in the TSS region of these genes (10 kb, centred on the TSS), with respect to histone acetylation. Interestingly, this approach identified a group of >3700 genes showing a precise colocalization of Brdt on the TSSs marked by histone acetylation (Figure 4A). These genes could be divided into two broad categories with respect to Brdt binding in meiotic cells and in round spermatids (post-meiotic). In the first category, an increased Brdt binding was observed after the completion of meiosis while in the second group, in contrast to the first group, a decrease or loss of Brdt was observed in the post-meiotic cells. After analysing the expression profile of the genes from these two categories, using the approach presented above (i.e., by using these gene lists to interrogate available transcriptomic data corresponding to the staged mouse spermatogenic cells, spermatogonia, spermatocytes and post-meiotic cells, from GSE21749 and GSE4193), we discovered that the first group is remarkably enriched in genes, which are highly expressed in round spermatids (Figure 4A, histogram, upper panel), while the expression of most genes of the second category culminates in meiotic cells (Figure 4A, histogram, lower panel). It is of note that, due to the inheritance of stable RNAs

expressed in spermatocytes by the haploid cells, the post-meiotic genes listed in this category are not necessarily active.

We then confronted the list of genes affected by the absence of Brdt in our transcriptomic data (Figure 3) to the list of genes bearing Brdt at their TSS found here. This analysis showed that about half of the genes requiring Brdt for their activation in 20 d.p.p. spermatogenic cells are bound by Brdt at their TSS (Figure 4B, Brdt activated), suggesting a direct role of Brdt in their activation. However, only a small fraction (about 15%) of genes repressed by Brdt in the same spermatogenic cells (Figure 4B, Brdt repressed) are bound by Brdt at their TSS, indicating that the observed Brdt-mediated repression of pre-meiotic genes is rather an indirect phenomenon.

Overall, these analyses showed that the presence of Brdt on TSS-bearing acetylated histones is an important determinant in Brdt-dependent meiotic gene activation in spermatogenic cells, whereas Brdt-repressive action on pre-meiotic genes should be mostly indirect, probably due to the activation of critical repressors. We also interrogated our ChIP-Seq data on the general distribution of Brdt over the genome in spermatocytes and round spermatids. This analysis shows that only a fraction of Brdt-bound regions are in fact gene TSS and that



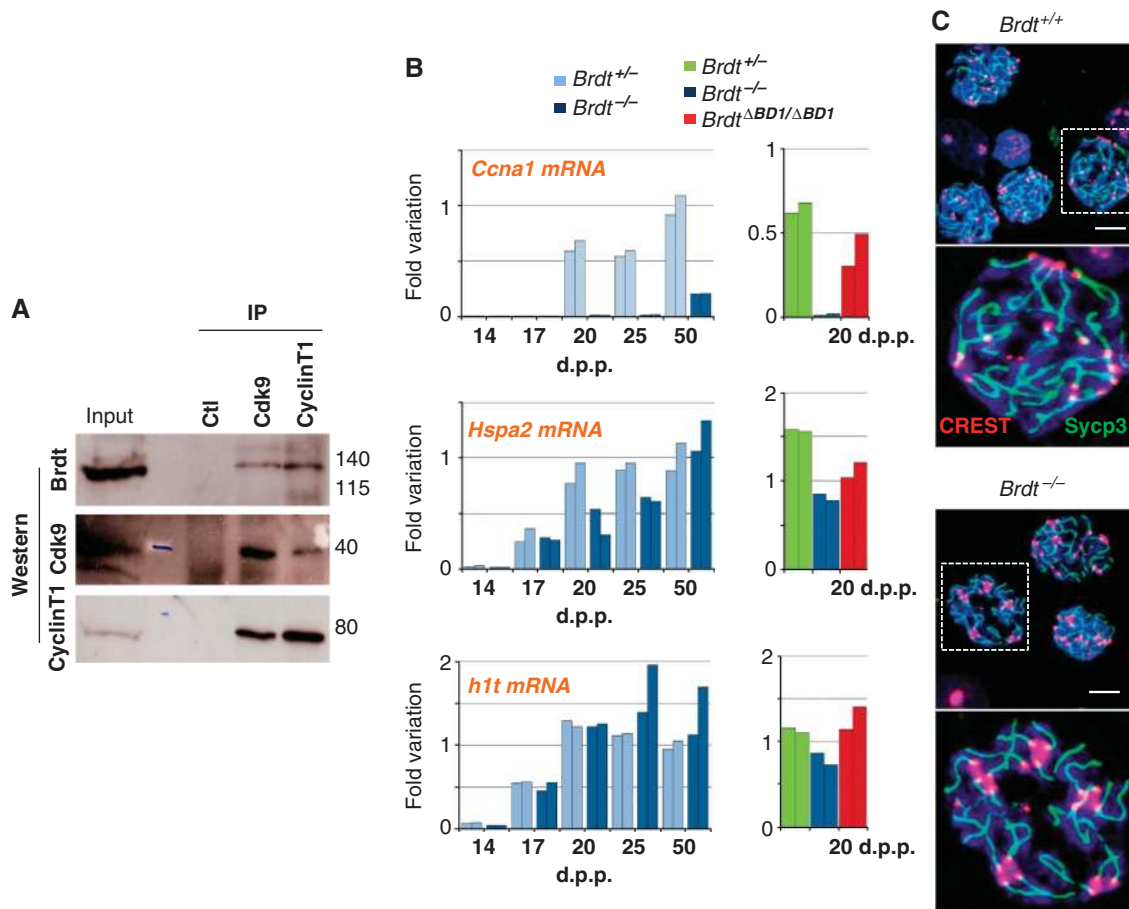
**Figure 4** Brdt binding and histone acetylation directed stage-specific gene expression. **(A)** Chromatin-bound Brdt was immunoprecipitated from fractionated spermatocyte populations and round spermatids and the associated DNA sequenced and position and the intensity of Brdt peaks determined. The SeqMiner software (Ye *et al*, 2010) was used to illustrate Brdt (this work) and histone acetylation peaks, which we recently reported from the same cell populations (Tan *et al*, 2011), at  $\pm 5$  kb around gene TSSs. Left panels: heatmaps and quantification profiles of Brdt or histone acetylation peaks in TSS regions in meiotic and post-meiotic male germ cells corresponding to two categories of Brdt-bound TSSs: the upper panels show TSS associated with an increase of Brdt binding in post-meiotic cells as compared to meiotic cells, whereas the lower panels show TSS with a decrease or no Brdt binding in post-meiotic cells; The histograms (right) indicate the proportion of genes with meiotic and post-meiotic profile of expression in each of these two gene categories. The gene expression profiles were established following the strategy described for Figure 3C (but only genes with fold changes  $> 1.2$  between the meiotic and post-meiotic cells expression levels were taken into account). **(B)** Respective proportions of Brdt-activated and Brdt-repressed genes (see Figure 3) whose TSS is associated with a Brdt peak and enriched in acetylated histones: nearly half of the Brdt-activated genes show colocalized Brdt and histone acetylation peaks in TSS, whereas this is the case of only a minority of Brdt-repressed genes.

the majority of the peaks are intergenic (66 and 60%, respectively, in spermatocytes and round spermatids), suggesting that Brdt may also have a global genome organizing role (Supplementary Figure S3).

#### ***Brdt* is essential to the completion of meiosis I**

The observation of a transcriptional regulatory role of Brdt suggested that it could be a tissue-specific paralogue of Brd4, with a 'Brd4-like' function. Indeed, a previous report demonstrated a striking sequence similarity between the C-terminal region of Brd4 with Brdt among all BET members (Bisgrove *et al*, 2007). This report also showed that this domain in

Brd4 and in ectopically expressed Brdt is required for the recruitment of positive transcription elongation factor b (P-TEFb), suggesting that Brdt's transcriptional function could involve the same mechanism as Brd4. One prediction of this hypothesis is that in spermatogenic cells, at least a fraction of Brdt should be found in a complex with P-TEFb components, cyclin T1 and Cdk9. Extracts from adult mouse testes were therefore subjected to immunoprecipitation by anti-Cdk9 and anti-cyclin T1 antibodies to test their interaction with Brdt (Figure 5A). The same experiment was carried out in parallel with extracts from *Brdt*<sup>-/-</sup> testes, which gave an indication of the specificity of the experiment



**Figure 5** *Brdt* is a testis-specific ‘Brd4-like’ factor and a major driver of meiosis I. (A) Extracts from *Brdt*<sup>+/+</sup> testes were used for an immunoprecipitation using an anti-Cdk9, an anti-cyclin T1 antibody or an irrelevant antibody (pre-immune rabbit serum). Immunoprecipitated proteins were detected with the indicated antibodies. (B) RNA was extracted from testes at the indicated postnatal ages and the expression of three meiotic genes *Ccna*, *H1t* and *Hspa2* (Dix *et al*, 1996) was monitored by RT-qPCR. The right panels show the expression of the same genes in testis from the three indicated genotypes, at 20 d.p.p. The histograms represent the values of biological duplicates, normalized with respect to *Actin* as a control gene, and to *Brdt* mean expression in 50-day-old wt testes. (C) The synaptonemal complex component, Sycp3 (green), and centromeric proteins CREST (red), were co-detected as indicated, in seminiferous tubule sections of *Brdt*<sup>+/+</sup> and *Brdt*<sup>-/-</sup> mice. The panels show higher magnifications of two representative spermatocytes either wild type or *Brdt*<sup>-/-</sup>. A general view of the spermatocytes is also shown. Scale bars, 5 μm. Figure source data can be found with the Supplementary data.

(Supplementary Figure S4). These figures show that significant amounts of Brdt could be co-immunoprecipitated with both Cdk9 and cyclin T1, only from wild-type testes and that no cyclin T1, Cdk9 or Brdt could be detected when an irrelevant antibody was used in the immunoprecipitation of a wild-type testis extract (Figure 5A). These data confirmed that Brdt is a true functional tissue-specific paralogue of Brd4.

We then addressed the question of whether some of the Brdt-regulated genes could give a molecular explanation for the meiotic arrest occurring in the absence of Brdt. Within the list of genes repressed by the absence of Brdt, we noticed a critical meiotic gene, *Ccna1*, which was strongly downregulated (14-fold compared to control according to our 20 d.p.p. transcriptomic analysis). *Ccna1* is a testis-specific A-type cyclin gene, known to be expressed in spermatocytes and to be absolutely essential for spermatocytes to enter into the first meiotic division (Liu *et al*, 1998; Nickerson *et al*, 2007). We confirmed the specific downregulation of *Ccna1* gene in the absence of Brdt by RT-qPCR approach (Figure 5B). Pachytene spermatocytes appear at 14 d.p.p. and accumulate up to 20 d.p.p. when diplotene spermatocytes become visible.

Using testes from aged-matched wild-type and *Brdt*<sup>-/-</sup> mice at different times of pachytene spermatocytes differentiation, we monitored the expression of *Ccna1*, as well as of two other genes, *Hspa2* and *H1t*, known to be activated in pachytene cells but whose expression was not significantly affected by the absence of Brdt (according to our transcriptomic analysis). Figure 5B shows that in wild-type testes, the appearance of pachytene spermatocytes is associated with the activation of the three genes, but that *Ccna1* is expressed after the other two, at late pachytene/diplotene stage (appearing in 20 d.p.p. testes). These experiments confirm first that, in the absence of Brdt, pachytene spermatocytes are present and second that Brdt specifically controls the expression of *Ccna1* as opposed to other pachytene-specific meiotic genes.

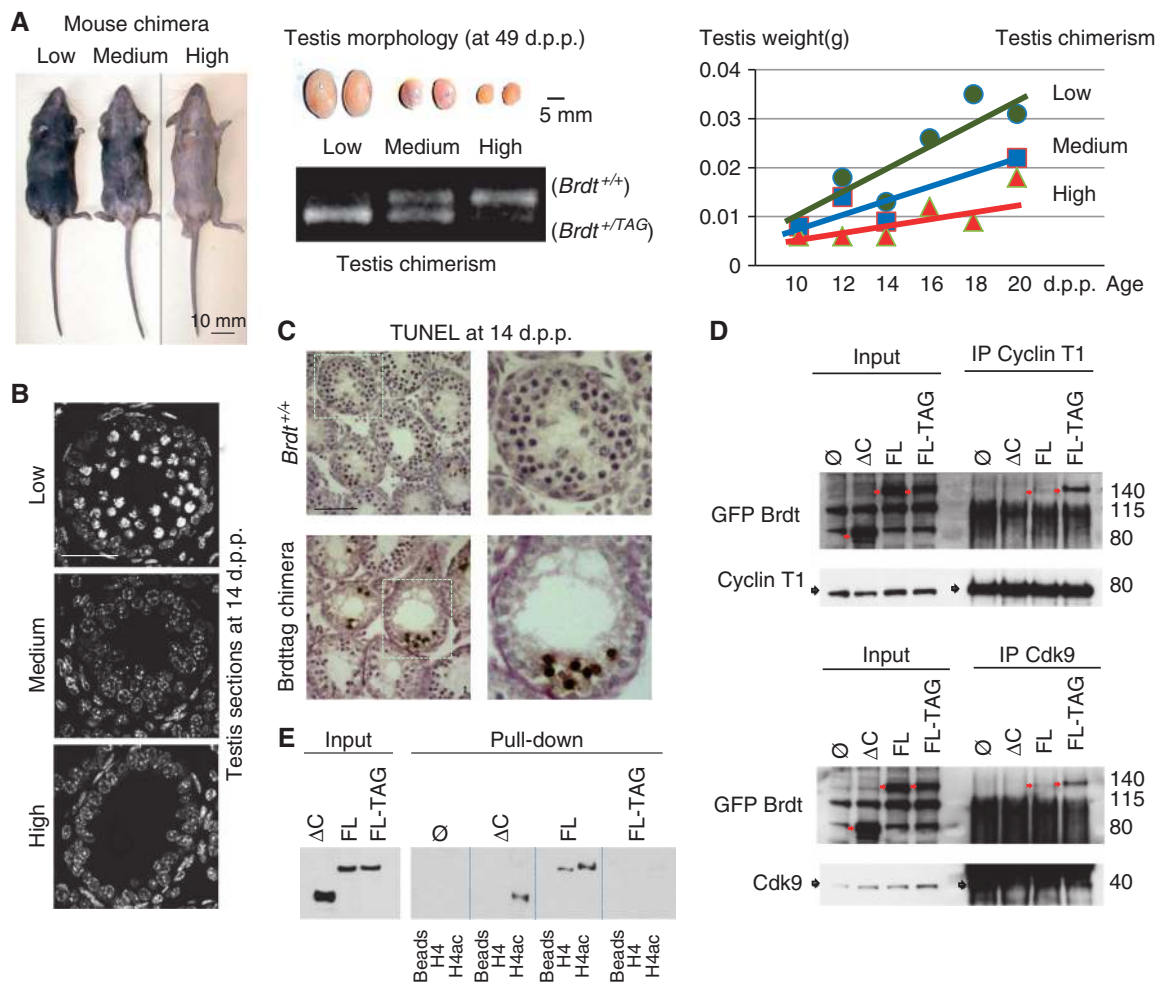
Finally, the published phenotype of *Ccna1* KO mice (Liu *et al*, 1998) is very similar to that exhibited here in the total absence of *Brdt*, in particular the absence of post-meiotic cells with a significant decrease of H3S10Ph-positive meiotic cells (Figure 2B) and an abnormal centromere clustering (Nickerson *et al*, 2007; Figure 5C; Supplementary Figure S5), suggesting that the absence of *Ccna1* expression

in *Brdt*<sup>-/-</sup> spermatocytes could by itself explain the phenotype observed in *Brdt* KO animals. We also checked our ChIP-Seq data to know whether Brdt could directly regulate *Ccna1* expression and binding to its TSS. Our data showed no significant Brdt binding to this gene promoter suggesting that either Brdt-dependent activation of *Ccna1* is indirect or that for some reasons Brdt bound to this gene was not accessible to our antibody.

***Brdt*'s function is critical for spermatocyte survival**

The hypothesis of a critical role of the C-terminal domain of Brdt as being responsible for P-TEFb recruitment during male germ cell differentiation received unexpected support from another mouse model: chimeric mice with male germ cells expressing a C-terminal tagged *Brdt*.

In order to tag the endogenous Brdt, three epitopes (tap tag) were introduced at the 3' end of *Brdt*'s last exon in mouse embryonic stem (ES) cells (Supplementary Figure S6A). Two clones (AT1 agouti, *Brdt*<sup>+ /tag</sup>) were injected into blastocysts from C57BL/6 (black *Brdt*<sup>+ /+</sup>) mice. Unexpectedly, in chimeric mice, whenever heterozygous *Brdt*<sup>+ /tag</sup> ES cells significantly contributed to testis formation, testes were of smaller volume and reduced weight as compared to wild-type testes (Figure 6A), and seminiferous tubules were devoid of meiotic and post-meiotic cells (Figure 6B). The same knock-in, in an independent ES cell line, 46C, resulted in chimeric mice displaying exactly the same phenotype (unpublished work). In addition, AT1 ES cells were used to tap tag another testis-specific gene, *th2b* encoding the testis-specific histone H2B variant, TH2B, known to be expressed in



**Figure 6** C-terminal-tagged Brdt severely interferes with spermatogenic cell differentiation. (A) The degree of the contribution of AT1 *Brdt*<sup>+ /tag</sup> ES cells (129) to embryogenesis was evaluated by coat colour (left panel) and more specifically to testis formation by genome type-specific PCR on testis DNA thanks to the strain-specific polymorphism of the *D2Mit94* locus (middle lower panel). Chimeric mice testes were grouped into three classes according to the PCR-based estimation of their level of chimerism and their respective weights were plotted as a function of postnatal age (right panel). Representative testes from 49-day-old mice belonging to the three indicated classes are shown on the middle upper panel. (B) Testes from the three classes of 14-day-old chimeric mice were fixed and stained by Hoechst (scale bars, 50 µm). (C) TUNEL assay was carried out on age-matched wild-type or chimeric mouse testes harvested at 14 d.p.p. Scale bars, 50 µm. (D) HeLa cells were transfected with the indicated GFP-Brdt constructs. ΔC corresponds to GFP-Brdt deleted of its C-terminal region (Pivot-Pajot *et al*, 2003). FL and FL TAG correspond to the full-length GFP-Brdt without or with the C-terminal tag, respectively (using the same tag as in the knock-in approach). After immunoprecipitation using an anti-cyclin T1 (upper panels) or an anti-Cdk9 antibody (lower panels), the presence of co-immunoprecipitated GFP-Brdt was monitored using an anti-GFP antibody. The positions of the GFP-Brdt fusion proteins in the input and immunoprecipitated materials are indicated by red arrows. (E) Cos7 cells were transfected with the same constructs as above but the extracts were used to test Brdt H4 histone tail binding. Extracts from cells expressing the indicated constructs were divided into three and incubated with streptavidin beads or streptavidin beads coupled with biotinylated H4 or tetra-acetylated H4 peptides (indicated). Bound proteins were then revealed by an anti-GFP antibody. ∅ represents extracts used in the pull down from non-transfected cells. Figure source data can be found with the Supplementary data.

spermatocytes (Supplementary Figure S6B), and, not only was germline transmission successful and a mouse strain established, but also the tagged protein was expressed, with no apparent toxicity in spermatocytes (Supplementary Figure S7), demonstrating the specific effect of the C-terminal tagged Brdt in these cells and not of the tag itself.

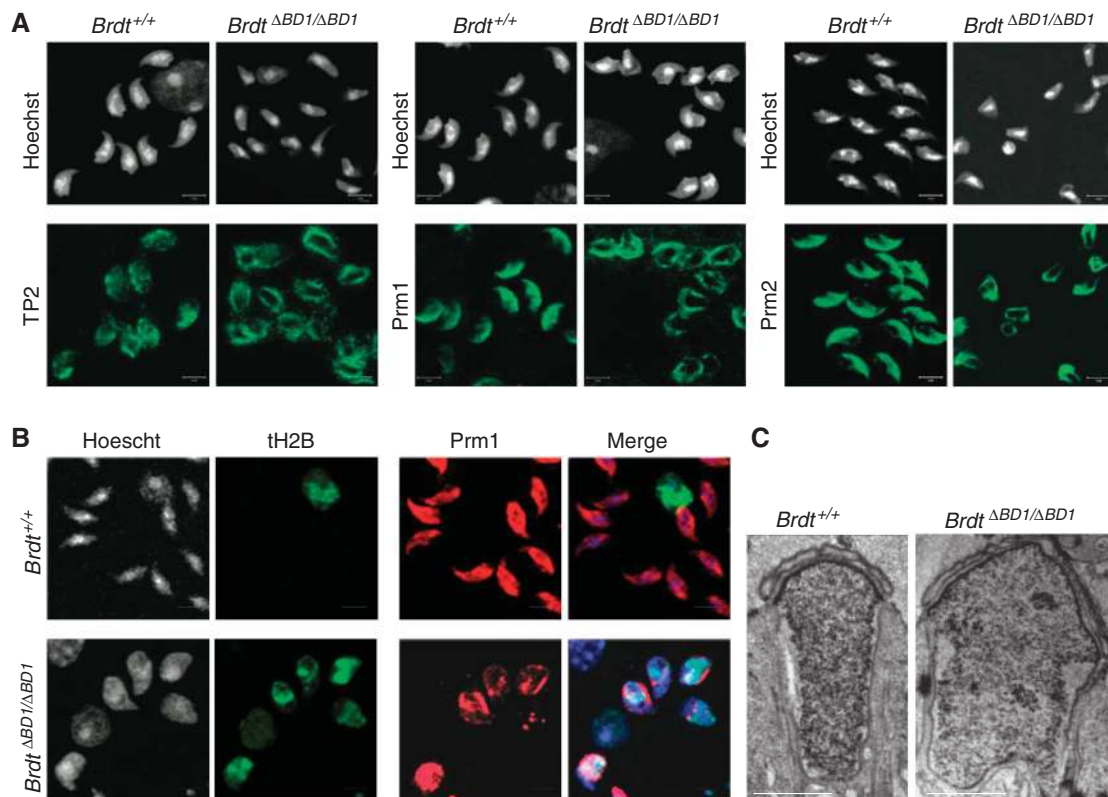
These experiments indicate that the tag alters critical functions of Brdt, as soon as the protein is expressed in early spermatocytes. In line with this conclusion, in testes from 14-day-old chimeric mice, only a few days after the activation of the *Brdt* gene, spermatocytes massively disappear and most of the remaining cells are apoptotic (Figure 6C).

Due to its C-terminal position, we hypothesized that the tag may interfere with the capacity of Brdt's 'Brd4-like' C-terminal domain to recruit P-TEFb complex to the acetylated chromatin (Bisgrove *et al*, 2007). Accordingly, using the ectopic expression of different Brdt constructs, we explored P-TEFb recruitment by the C-terminal domain of Brdt. Unexpectedly, we found that the presence of the tag in its vicinity leads to a remarkable enhancement of P-TEFb recruitment (Figure 6D, FL TAG). We also observed in a peptide pull-down experiment that the C-terminal tag strongly interferes with the capacity of Brdt's bromodomains to bind an acetylated histone H4 peptide (Figure 6E, FL TAG). This result is actually in good agreement with the recent model for Brd4-P-TEFb complex proposing a conformational change bringing the CTD at the vicinity of the Brd4 bromodomains (Schroder *et al*, 2012). These results suggest that the

enhancement of Brdt-P-TEFb complex formation in the presence of the C-terminal tag could reflect the inability of the protein to bind chromatin, which would remain bound to P-TEFb in the nucleosolic fraction. It is important to note that these experiments could not be performed in spermatogenic cells from chimeric mice because of the rapid disappearance of cells from the testis of these animals (Figure 6C).

***Brdt's* BD1 is essential to link histone removal to TPs assembly**

To test our original hypothesis of a potential role of Brdt in the replacement of hyperacetylated histones in elongating spermatids, we focused our attention on post-meiotic cells, where the global and genome-wide histone hyperacetylation occurs. We first questioned whether the wave of histone hyperacetylation itself normally occurs in the absence of a functional Brdt BD1. Supplementary Figure S8 shows that elongating spermatids from *Brdt*<sup>ΔBD1/ΔBD1</sup> testes contain hyperacetylated histones, as normal elongating spermatids. Because of the capacity of Brdt's BD1 to specifically act on hyperacetylated histones, we questioned whether the replacement of histones is maintained in the absence of BD1. The analysis of elongating spermatids from *Brdt*<sup>ΔBD1/ΔBD1</sup> mice showed that, although TPs and protamines (Prms) are synthesized, they remain in the cytoplasm (Figure 7A) and histone replacement does not occur, as judged by the co-detection of TH2B and protamine 1 (Figure 7B). This situation is never observed in wild-type spermatids, where Prm1 accumulates in the nucleus after the disappearance of



**Figure 7** Post-meiotic functions of Brdt's first bromodomain. (A) An immunofluorescence detection of TP2 and of Prm1 and Prm2 (B) as well as a co-detection of the histone TH2B (green) and of Prm1 (red) were performed; Scale bars, 5 μm. (C) A comparative observation by electronic microscopy (EM) of two representative elongating spermatids at the same stage (as evaluated by the percentage of coverage of the spermatids' heads by the acrosome) from wild-type (+/+) and *Brdt*<sup>ΔBD1/ΔBD1</sup> mice is shown. Scale bars, 1 μm.



histones (Figure 7B). The absence of genome compaction in elongating cells expressing a BD1-less Brdt is also clearly visible by electron microscopy, comparing elongating spermatids at the same stage of acrosome development (Figure 7C).

These data suggest that BD1 is required for the post-meiotic re-organization of the male genome and directly and/or indirectly mediates the replacement of acetylated histones. Additionally, we observed that, when TPs and Prms accumulate in the cytoplasm of elongating spermatids of *Brdt*<sup>ΔBD1/ΔBD1</sup> mice, BD1-less Brdt is also in the cytoplasm while at earlier stages, in the absence of TP synthesis, the protein is found in the nucleus of spermatids (Supplementary Figure S9A). In fact, the ability of Brdt to interact with acetylated chromatin, essentially through its first bromodomain, and to 'squeeze' acetylated nucleosomes could be important for the protein to mediate the histone-TP exchange (Supplementary Figure S9B). Indeed, we previously observed that Brdt is capable of inducing a dramatic compaction of chromatin in an acetylation-dependent manner (Pivot-Pajot *et al*, 2003). This activity of Brdt is enhanced when Brdt lacks its C-terminal part, which is close to a splice variant of Brdt, also produced in spermatogenic cells (Pivot-Pajot *et al*, 2003). Therefore, in elongating spermatids, histone hyperacetylation in the presence of Brdt could lead to a similar phenomenon, which would be coupled to histone eviction and TP assembly. Interestingly, the capacity of Brdt in inducing an acetylation-dependent chromatin compaction has recently been shown in the rat germ cells (Dhar *et al*, 2012). Here, our experimental data suggest that this action requires a bromodomain-dependent protein 'polymerization', as shown by the occurrence of FRET between a N-terminal GFP of a Brdt molecule and a C-terminal RFP of another Brdt molecule (Supplementary Figures S9C). Based on this observation, we propose a working model, according to which, in elongating spermatids, Brdt uses the histone hyperacetylation signal to bind chromatin and induce a 'chromatin squeezing' process through a Brdt-Brdt interaction facilitating histone eviction and their replacement by TPs.

## Discussion

The functional dissection of Brdt's activity reported here opens the door to important conceptual developments and brings to light crucial and so far unknown aspects of mammalian spermatogenesis. First, *Brdt* is activated at the onset of meiosis and becomes critical for the activation of hundreds of testis-specific genes, which are fully active either in spermatocytes or in post-meiotic spermatids. Brdt acts therefore as a transcriptional activator whose action becomes visible at the time when pachytene spermatocytes appear and culminates at the late pachytene/diplotene transition. We also found that Brdt's binding to the TSS of a gene is a strong predictor of its stage-specific pattern of expression. Indeed, although during meiosis both histone acetylation and Brdt are observed at the TSS of many testis-specific genes, a change in the level of Brdt binding between meiotic and post-meiotic cells is an indicator of their meiotic or post-meiotic nature. An enhancement of Brdt recruitment after the completion of meiosis clearly distinguishes a set of genes, which are predominantly expressed in round spermatids. Interestingly, this marking of the TSS of post-meiotic genes

in spermatocytes by both histone acetylation and Brdt is probably facilitating gene activation in round spermatids, similar to the action of Brd4 on bookmarked genes described in somatic cells (Zhao *et al*, 2011).

In contrast, a loss of Brdt's binding at a gene's TSS after meiosis is associated with a decrease of its expression in haploid cells. Remarkably, nearly half of the genes whose meiotic activation is dependent on Brdt (with a decreased expression in *Brdt*<sup>-/-</sup> spermatocytes) are genes whose TSS is enriched in acetylated histones and bound by Brdt, indicating a direct role for Brdt in their activation. In contrast, only a small fraction of Brdt-repressed genes contained histone acetylation and Brdt at their TSS, suggesting that the repression of pre-meiotic genes in spermatocytes is predominantly indirectly controlled by Brdt.

It is also of note that many genes with Brdt-bound TSS (represented in Figure 4A) are not affected by the absence of Brdt (their expression was not changed in *Brdt*<sup>-/-</sup> spermatocytes). It is likely that, in the absence of Brdt, some of these genes could be rescued by other members of the BET family, Brd2, Brd3 and Brd4, all expressed in male germ cells (Shang *et al*, 2004).

The partial functional redundancy of Brdt and other BET members is also suggested by the phenotype of tap-tagged Brdt-expressing cells, which die soon after the activation of *Brdt* expression in early spermatocytes, while these early meiotic cells appear healthy in the total absence of Brdt in *Brdt*<sup>-/-</sup> mice. Indeed, in the absence of Brdt other BET proteins may ensure some Brdt-like functions, but the non-functional tagged Brdt could block this compensation by other BET members and hence become rapidly deleterious.

The confrontation of our transcriptomic and ChIP-Seq data also revealed important indirect transcriptional roles of Brdt, both as repressors and as activators. These functions could be relayed by a direct Brdt-dependent activation of transcriptional repressors and activators in spermatocytes.

This work also highlights a 'Brd4-like' nature of Brdt, by demonstrating the presence of Brdt in the P-TEFb complex in spermatogenic cells. This observation is perfectly in line with the presence in Brdt of a C-terminal domain similar to that of Brd4, shown to be involved in the recruitment of P-TEFb (Bisgrove *et al*, 2007; Schroder *et al*, 2012). It is also in agreement with the deleterious effect of a tag placed adjacent to this domain, as shown here. We unexpectedly found that the C-terminal tag not only strongly enhanced the Brdt-P-TEFb interaction but also prevented its binding to an acetylated histone H4 peptide. A recent work from Melanie Ott's laboratory proposes a simultaneous binding of Brd4 BD2 and of its C-terminal Brdt-like domain with acetylated cyclin T1 leading to a structural change bringing the C-terminal to the vicinity of the bromodomains (Schroder *et al*, 2012). In the case of Brdt, the presence of the tag next to the C-terminal domain may interfere with the required structural change and accessibility of the bromodomains for chromatin/histone binding. The tagged Brdt-p-TEFb complex could therefore accumulate in cells due to the inability of Brdt to transfer the kinase complex onto the paused polII bound promoters.

All the findings reported here also point to Brdt as a particularly attractive target in pharmacological approaches to male fertility. Indeed, the recent discovery of bromodomain inhibitors makes it possible to design Brdt-specific

bromodomains inhibitors to disrupt spermatogenesis. Accordingly, a small molecule inhibitor targeting the bromodomains of the BET members, JQ1, has very recently been shown to severely interfere with spermatocytes and round spermatids development in a reversible manner (Matzuk *et al*, 2012).

Finally, from our previous structural studies demonstrating that Brdt's BD1 is the first bromodomain known to specifically recognize hyperacetylated histone H4 (Moriniere *et al*, 2009), we anticipated that BD1 should mainly act in elongating spermatids, during the genome-wide hyperacetylation of histones, which occurs before their replacement. In the absence of a functional BD1, the predicted defects in the removal of hyperacetylated histone were confirmed here. Indeed, in *Brdt*<sup>ABD1/ABD1</sup> mice, while histone replacing proteins, TPs and Prms, were synthesized and histone hyperacetylation occurred normally, histone removal and the assembly of TPs and Prms did not happen. TPs and Prms accumulated in the cytoplasmic and perinuclear regions of these elongating spermatids, where the BD1-less Brdt was also observed. This latter observation suggests that Brdt may provide a link between hyperacetylated nucleosomes and TPs and mediate their exchange. Accordingly, in the absence of BD1, the mutated protein unable to bind acetylated chromatin, although nuclear in round spermatids, becomes trapped in the cytoplasmic and perinuclear regions, as soon as TPs are synthesized in elongating spermatids.

We previously showed that Brdt, in the presence of hyperacetylated nucleosomes, could 'squeeze' the chromatin fibre (Pivot-Pajot *et al*, 2003), and this observation has recently been confirmed even in the rat haploid male germ cells (Dhar *et al*, 2012). Our data also suggest that Brdt polymerizes on chromatin fibres, explaining its 'squeezing' effects. The presence of TPs and appropriate chaperones around Brdt could mediate the final eviction of histones and their replacement. It is however important to note that we cannot exclude an indirect effect of Brdt in histone replacement. Indeed, as shown here, a significant number of post-meiotic genes require Brdt BD1 to be fully active, and among these, there are specific factors, including those involved in splicing, which may affect the function of other genes, as recently shown (Berkovits *et al*, 2012).

In summary, we present here a comprehensive functional study of a member of the BET family, demonstrating for the first time in a physiological setting that the bromodomains have specific roles and act in a stage-dependent manner.

## Materials and methods

### Mouse models

*Brdt*<sup>ABD1/ABD1</sup> mice were previously described (Shang *et al*, 2007). *Brdt* KO mice were obtained from the International KnockOut Mouse Consortium, Welcome Trust Sanger Institute, UK, and their genotype confirmed. The 'KnockOut-first' allele (tm1a) contains an IRES:*lacZ* trapping cassette and floxed promoter-driven *neo* cassette inserted into the 4th intron of the *Brdt* gene, disrupting *Brdt* function. The construction of the targeting vector, electroporation of C57BL/6N ES cells and generation of mice bearing a null mutation of *Brdt* was described in Skarnes *et al* (2011). *Brdt* and *th2b* tap tagging was accomplished by adapting the gap-repair recombineering technique from Liu *et al* (2003) to construct our *Brdt* and *th2b* tap-tag targeting vectors for homologous recombination in ES cells (AT1 and 46C lines). Animal experiments were approved by *ad hoc* committees and all the investigators directly involved, have an official animal-handling authorization obtained after a 2-week intensive training

and a final exam. Knock-in through homologous recombination in ES cells is described in detail in Supplementary Materials and methods.

### Analyses of testis sections and spermatogenic cells

Spermatogenic cells were analysed either on sections of paraffin-embedded AFA-fixed testes or seminiferous tubules or testis 'imprints' on slides, as previously described (Hazzouri *et al*, 2000; Govin *et al*, 2007, 2010).

### Spermatogenic cell preparations for in situ analysis

Staged seminiferous tubules: Mice were sacrificed by cervical dislocation. Testis and epididymis were biopsied, washed in PBS. Albuginea was removed and seminiferous tubules were dissected at 4°C in DMEM with 100 mM sucrose. Microdissection and isolation of staged tubules was carried out under a transillumination microscope, as previously reported (Kotaja *et al*, 2004). Tubules were cut into 2-mm segments and each segment was 'squashed' by pressure between a coverslip and the slide, prefixed 20 s in liquid nitrogen, 10 min in 90% ethanol, air dried, and processed for immunofluorescence or  $\beta$ -galactosidase activity staining.

Testis 'imprints' were prepared as follows. Testes were frozen in liquid nitrogen, cut in two parts and directly applied on slides by apposition. Each slide was fixed in 90% ethanol, air dried, and processed for immunofluorescence staining. X gal staining for Brdt expression is detailed in Supplementary Materials and methods.

### Immunofluorescence, immunohistochemistry and microscopy

Protein visualization by immunofluorescence was carried out as previously described (Govin *et al*, 2007). Protein detection by immunohistochemistry was performed exactly as described in Hazzouri *et al* (2000). The antibodies used were as follows: goat polyclonal anti-TP2 antibody 1/250 (Santa Cruz biotechnology), rabbit polyclonal anti-TP1 antibody 1/500 (gift from Dr Kistler, University of South Carolina), rabbit polyclonal anti-H4ac4 antibody 1/500 (Millipore), mouse monoclonal anti-Prm1 and Prm2 antibodies 1/250 (Shaltech), rabbit polyclonal anti-acrosomal protein (Sp56) antibody 1/200, rabbit polyclonal anti-H3S10Ph antibody 1/500 (Millipore), mouse monoclonal anti-Sycp3 antibody 1/500 (Abcam), rabbit polyclonal anti-Sycp1 antibody 1/500 (Abcam), rabbit monoclonal anti-phosphoH2Ax 1/500 (Epitomics). Anti-Brdt antibodies were generated either as described in Govin *et al* (2006) or in Shang *et al* (2007). The secondary antibodies were Alexa 488 or Alexa 546 fluor conjugates (Molecular Probes, Invitrogen). Apoptotic cells were detected by TUNEL assay, using a Millipore kit following the furnished instructions.

Electron Microscopy morphological analysis is described in Supplementary Materials and methods.

### FRET experiments

FRET experiments are described in Supplementary Materials and methods.

### PCR-based analyses

The degree of AT1 ES cell (129 strain) contribution to C57BL/6 mice germline was evaluated by PCR on testis DNA as described in Cho *et al* (2001). Gene expression analysis was carried out on purified RNA using appropriate sets of primers, listed in Supplementary Materials and methods. Chimeric mice testis DNA genotyping is also detailed in Supplementary Materials and methods.

### Transcriptomic analyses

The transcriptomes of age-matched testes from control (wild type) and mutant mice as well as fractionated spermatogenic cells were obtained using the Illumina mouse WG-6 V2.0 gene expression array. For testes, three independent experiments were performed using two mouse models: *Brdt* KO at 17 d.p.p. or at 20 d.p.p., or *Brdt*<sup>ABD1/ABD1</sup> mice at 20 d.p.p. and age-matched wild-type testes (5 or 6 replicates of the mutated and of the corresponding controls for each model). Testis and fractionated cells RNA was harvested with TRI Reagent (Sigma), and RNA was isolated following manufacturer's instructions.

The detailed experimental procedure is described in Supplementary Materials and methods.

### Analyses of expression patterns using online expression data

The patterns of expression of the genes, found differentially expressed in *Brdt* KO and *Brdt<sup>ABD1/ABD1</sup>* mice, were analysed in normal mouse tissues and during spermatogenesis using transcriptomic data available online as described in Tan *et al* (2011). The raw expression data (.CEL files) of four studies (available from the GEO website: <http://www.ncbi.nlm.nih.gov/geo/>; GSE10744, GSE12950, GSE9954, GSE21749 and GSE4193) were downloaded from the GEO website and RMA normalized using the GeneSpring GX software (Agilent Technologies). The analysis was carried out as described in Tan *et al* (2011). The expression patterns are represented as heatmaps using the Permutmatrix software (<http://www.lirmm.fr/~caraux/PermutMatrix/index.html>).

### Immunoprecipitation, pull-down and ChIP analyses

An anti-Brdt ChIP was performed on micrococcal digested chromatin as described by Tan *et al* (2011). Details of sequencing and data analyses are described in Supplementary Materials and methods. Immunoprecipitation of Cdk9 and cyclin T1 was as follows. Testis or HeLa cell extracts were used for immunoprecipitations with anti-Cdk9 and anti-cyclin T1 rabbit polyclonal antibodies (Santa Cruz Biotechnology). Lysis, immunoprecipitation and washes were performed in LSDB500 buffer (HEPES 50 mM pH 8, MgCl<sub>2</sub> 3 mM, KCl 250 mM, Glycerol 20%, NP-40 1%, Complete Protease inhibitor EDTA-free Roche). Bound proteins were migrated on 4–12% Bis-Tris SDS PAGE (NuPAGE Precast gel, Invitrogen), transferred onto a nitrocellulose membrane (Hybond C+, GE), and probed with an anti-Brdt rabbit polyclonal antibody (produced against Brdt C-terminal peptide), as well as with anti-Cdk9 and anti-cyclin T1. Brdt pull-down tests were performed as described in Huang *et al* (2010).

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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**Author contributions:** SK and SR initiated and coordinated the entire project, specific author's contributions and wrote the manuscript. SR directed the bioinformatics analyses. JG carried out all the characterizations of the three mouse models used. FB generated vectors for recombineering and established *Brdt<sup>+ /tag</sup>* ES cells. PH, SJ and ADepaux carried out the generation of chimeric mice. The recombineering, ES cell and chimeric mice generation were performed in MG laboratory under his supervision. ALV and TB ensured mouse genotyping and animal handling. EM and TB performed spermatogenic cell fractionation and ChIP experiments. SC prepared vectors and performed the FRET experiments. SC and SB performed the peptide pull-down experiments. PG managed the illumina transcriptomic platform and analysed the raw data. KP performed the EM analysis. ADebernardi performed bioinformatics analyses under the supervision of SR. HH and FL performed ChIP sequencing and raw data analyses, respectively, and JI supervised these analyses and ensured the management of the TGML/TAGC sequencing platform. DJW provided the Brdt  $\Delta$ BD1 mice, discussed the experiments and helped writing the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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