

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

1 **Title:** Characterization of BRD4 during mammalian post-meiotic sperm development

2

3 **Authors:** Jessica M. Bryant^{1,2,3}, Greg Donahue¹, Xiaoshi Wang⁴, Mirella Meyer-Ficca⁵, Lacey
4 J. Luense¹, Angela H. Weller¹, Marisa S. Bartolomei¹, Gerd A. Blobel⁶, Ralph G. Meyer⁵,
5 Benjamin A. Garcia⁴, and Shelley L. Berger^{1,#}

6

7 ¹Epigenetics Program, Department of Cell and Developmental Biology, University of

8 Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104, USA

9 ²Biomedical Graduate Studies, University of Pennsylvania ³Current address: Institut Pasteur,

10 Paris, France 75724 ⁴Epigenetics Program, Department of Biochemistry and Biophysics,

11 University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104,

12 USA ⁵Department of Animal Biology, School of Veterinary Medicine, University of

13 Pennsylvania, Pennsylvania 19104, current address: Department of Animal, Dairy and

14 Veterinary Sciences, School of Veterinary Medicine, College of Agriculture and Applied

15 Sciences, Utah State University, Logan, Utah, 84322, USA ⁶Division of Hematology, The

16 Children's Hospital of Philadelphia, The Perelman School of Medicine at the University of

17 Pennsylvania, Philadelphia, PA 19104, USA [#]Correspondence should be addressed to S.L.B.

18 (e-mail: bergers@mail.med.upenn.edu)

19

20 **Running title:** Characterization of BRD4 in mammalian spermiogenesis

21

22 Materials and Methods word count: 2,011

23 Introduction, Results, and Discussion word count: 4,969

24

25 **ABSTRACT**

26 During spermiogenesis, the post-meiotic phase of mammalian spermatogenesis, transcription is
27 progressively repressed as nuclei of haploid spermatids are compacted through a dramatic
28 chromatin reorganization involving hyper-acetylation and replacement of most histones with
29 protamines. Although BRDT functions in transcription and histone removal in spermatids, it is
30 unknown whether other BET family proteins play a role. Immunofluorescence of
31 spermatogenic cells revealed BRD4 in a ring around the nuclei of spermatids containing hyper-
32 acetylated histones. The ring lies directly adjacent to the acroplaxome, the cytoskeletal base of
33 the acrosome, previously linked to chromatin reorganization. The BRD4 ring does not form in
34 acrosomal mutant mice. ChIP sequencing in spermatids revealed enrichment of BRD4 and
35 acetylated histones at the promoters of active genes. BRD4 and BRDT show distinct and
36 synergistic binding patterns, with a pronounced enrichment of BRD4 at spermatogenesis-
37 specific genes. Direct association of BRD4 with acetylated H4 decreases in late spermatids as
38 acetylated histones are removed from the condensing nucleus in a wave following the
39 progressing acrosome. These data provide evidence for a prominent transcriptional role of
40 BRD4 and suggest a possible removal mechanism for chromatin components from the genome
41 via the progressing acrosome as transcription is repressed in response to chromatin condensation
42 during spermiogenesis.

43

44

45

46

47

48 INTRODUCTION

49 Mammalian spermatogenesis has emerged as a focus of epigenetic study, as this
50 conserved process requires vast changes in transcription and chromatin organization (1).
51 Spermatogenesis, or the formation of the mature male gamete, takes place in the seminiferous
52 tubules of the testes and begins with the stem-like spermatogonia. Diploid spermatogonia can
53 differentiate into spermatocytes, which enter meiosis to produce four genetically unique haploid
54 round spermatids (Fig. 1A). During the post-meiotic process of spermiogenesis, a spermatid
55 differentiates into a motile spermatozoon by shedding most cytoplasm, forming a flagellum, and
56 compacting the nucleus.

57 In mice, nuclear morphology changes dramatically during spermiogenesis: the nucleus is
58 initially round (in round spermatids), then elongates (in elongating spermatids), and finally
59 condenses into a small hook-like shape (in condensing/condensed spermatids) (Fig. 1A). This
60 process is necessary for the formation of fertile sperm and involves chromatin compaction and
61 consequent vast transcriptional repression (1). Nuclear compaction is accomplished via near-
62 complete replacement of canonical histones, some with testes-specific histone variants; but most
63 histones are initially replaced with transition proteins and then with protamines (2). Although
64 several groups have shown that the small percentage of histones that remain associated with the
65 genome in mature sperm are specifically post-translationally modified and enriched at
66 developmentally important loci (3, 4) and gene regulatory sequences (5), recent studies have
67 provided contrasting evidence that in mouse sperm, histone retention occurs preferentially in
68 large gene-poor genomic regions (6-8). The mechanism by which almost all histones are
69 removed and degraded has yet to be elucidated, but several important factors in this process
70 have been discovered (9).

71 Chromatin reorganization during spermiogenesis begins concurrently with acrosome
72 formation and histone hyper-acetylation. The acrosome is a cap-like, membrane-bound
73 organelle derived from the Golgi apparatus that covers the apical part of the mature sperm
74 nucleus. This organelle contains digestive enzymes that are released upon contact with the egg
75 to facilitate fertilization. Acrosome biogenesis begins after meiosis is complete and is
76 accomplished via fusion of fragments of the Golgi apparatus at the acroplaxome, or the
77 cytoskeletal base of the forming acrosome (10). The acroplaxome consists of actin and keratin
78 and anchors the acrosome to the adjacent nuclear membrane of the spermatid. Recently, several
79 studies have linked acrosome biogenesis to the dramatic chromatin reorganization that takes
80 place during spermiogenesis. Mouse mutants with defective acrosome formation produce
81 abnormal, round-headed sperm that show defective nuclear compaction (11-15). Moreover,
82 histone removal in human spermatids takes place adjacent to the acroplaxome as the acrosome
83 progressively caps the nucleus (16). These studies suggest that acrosome biogenesis plays a role
84 in sperm head shaping and nuclear compaction, but the mechanisms by which this may happen
85 are unknown.

86 After meiosis is complete and acrosome formation has begun, histones become hyper-
87 acetylated in the spermatid nucleus (17-19). Histone hyper-acetylation is believed to facilitate
88 histone removal either through a direct loosening of the chromatin or via binding of
89 bromodomain-containing proteins such as PA200, the activator of the “spermatoproteasome”,
90 and BRDT, the testes-specific BET (Bromo- and Extra-Terminal domain) family protein (20-
91 23). Like all BET family members, BRDT contains two bromodomains at its N-terminus and an
92 extra-terminal (ET) domain at its C-terminus. Recently, BRDT has been shown to play a dual
93 role during spermatogenesis (24). First, BRDT plays a transcriptional role, binding to

94 acetylated histones and P-TEFb at the promoters of meiotic and post-meiotic genes that are
95 aberrantly repressed in its absence (25). In fact, *Brd1* knockout or treatment of male mice with
96 JQ1 – a small-molecule inhibitor of BET family proteins – results in meiotic arrest and a
97 significant decrease in fertility (25, 26). Second, BRDT may play a structural role in chromatin
98 dynamics during spermiogenesis. Mice expressing BRDT lacking the first bromodomain show
99 defects in fertility caused by abnormal nuclear compaction and chromatin organization during
100 spermiogenesis (20, 27). However, it is unclear whether these later defects are again due to
101 transcriptional de-regulation or rather to decreased binding of BRDT to hyper-acetylated
102 histones.

103 It is also unclear whether other members of the BET family are integral to the process of
104 spermatogenesis, as *Brd2* and *Brd4* null mouse mutants show embryonic lethality (28, 29).
105 However, these genes are expressed during spermatogenesis at the mRNA and protein level
106 (30). Interestingly, *Brd4* heterozygous null mice show defects in spermatogenesis, although
107 this phenotype has not been well characterized (29). BRD4 has been shown to bind to the
108 acetylated tails of histones H3 and H4 and is generally associated with active gene transcription
109 (31). However, BRD4 also plays non-transcriptional roles such as tethering the human
110 papilloma virus genome to host chromatin during mitosis (32).

111 In this study, we investigate BRD4 during spermiogenesis. We show that BRD4 is
112 found in post-meiotic cells and investigate a novel BRD4 ring structure in spermatid nuclei that
113 is closely associated with the acrosome. Moreover, we show with CHIP sequencing that BRD4
114 has an unanticipated prominent association with genes expressed in post-meiotic cells. Taken
115 together, our results suggest an interesting mechanism for nuclear protein removal by linking
116 BRD4 and transcription shutdown to acrosome formation during spermiogenesis.

117 **RESULTS**118 **BRD4 is expressed in meiotic cells and spermatids, but not in mature sperm**

119 Although a transcriptional role of BRDT has been demonstrated during meiosis and
120 spermiogenesis, it is unclear if other BET family proteins play a role in spermatogenesis (25).
121 As mentioned above, *Brd4* heterozygous null male mice show spermatogenic defects,
122 suggesting that BRD4 may play a role in spermatogenesis (28, 29). To investigate the
123 expression pattern of BRD4 over the course of spermatogenesis, we analyzed protein levels in
124 different cell types obtained by STA-PUT velocity sedimentation from mature mouse testes
125 (33). With this method, we collected four cell populations (see Fig. 1A): 1) a mixture of meiotic
126 cells (spermatocytes: Sc), 2) early post-meiotic spermatids (round spermatids: RSp), 3) later
127 post-meiotic spermatids (elongating and condensing spermatids: E/CSp), and 4) a mixture of
128 early and later spermatids (R/E/CSp). Mature sperm were isolated from the cauda epididymis of
129 wild-type mice.

130 In addition to microscopic verification of purity via cellular and nuclear morphology
131 [see (33) for our methods], we used western blot analysis of lysates from these cells to confirm
132 relative purity. Although H4 protein is depleted in late spermatids (E/CSp), H4K5,8,12,16ac,
133 but not H3K9ac, was relatively enriched in these elongating and condensing spermatids (Fig.
134 1B). This analysis also revealed the presence of the long isoform of BRD4 protein (indicated
135 with an asterisk Fig. 1B) in meiotic cells (“Sc”), round spermatids (“RSp”), and
136 elongating/condensing spermatids (“E/CSp”), but not in mature sperm (“Sperm”). A second
137 smaller BRD4 isoform or degradation product was also detected in later spermatids (arrow in
138 Fig. 1B, upper panel). The specificity of the BRD4 antibody for the long and shorter forms was
139 confirmed by peptide competition (second panel, Fig. 1B). The canonical BRD4 short isoform

140 (~723aa) was not detected in any spermatogenic cells in this analysis. In addition to BRD4,
141 BRDT and BRD2 are expressed over the course of spermatogenesis. While BRDT is expressed
142 most highly in spermatocytes, BRD2 and BRD4 protein levels increase over the course of
143 spermiogenesis.

144

145 **BRD4 forms a ring around the nucleus of spermatids as histones become hyper-acetylated**

146 To gain insight into a possible function for BRD4 during spermatogenesis, we
147 determined its subcellular localization by performing indirect immunofluorescence (IF) on
148 tissue sections from the testes of adult wild-type male mice. Using intact tissue allows for the
149 identification of specific steps of spermatogenesis within the seminiferous tubules (34).
150 Interestingly, we detected BRD4 in a distinct, complete ring around the nucleus beginning in
151 approximately stage 7-8 spermatids (Sp) (Fig. 2A). The BRD4 ring is not present in
152 spermatogonia or spermatocytes (Sc) (Fig. 2A) and appears concurrently with the post-meiotic
153 hyper-acetylation of histones in the nucleus, not seen in spermatocytes (compare Sp to Sc in Fig.
154 2B). We confirmed that the apparently distinct “ring” is not associated with the entire nuclear
155 periphery with pseudo-3D images created with z-stacked individual confocal images of early
156 and late elongating spermatid nuclei (Movies A and B, resp.). We found the BRD4 structure
157 closely changes shape along with the condensing nucleus of the spermatid: from round to
158 oblong, always at the periphery of the DAPI-stained nucleus (Fig. 2A,B and Movies A,B). This
159 ring structure is specific to BRD4, as IF analysis of a mixed population of spermatogenic cells
160 showed BRD2 and BRDT in a diffuse nuclear staining pattern in spermatocytes and round
161 spermatids (Fig. 2C,D).

162

163 **BRD4 forms a ring within the nuclear envelope at the base of the acrosome**

164 To further investigate the sub-cellular location of BRD4, we performed indirect IF for
165 BRD4 and Lamin B1, a key component of the nuclear membrane. Co-detection of these
166 proteins revealed that BRD4 is located at an important transitional region of the nuclear
167 membrane in spermatids (Fig. 3A). More specifically, Lamin B1 and nuclear pores become
168 polarized to the posterior end of the spermatid nuclear membrane, clearly distinct from the
169 anterior end, which becomes closely covered by the acrosome (Fig. 3B, acrosome location
170 indicated by an asterisk in Fig. 3A) (35-37). The acrosome is partly anchored to the nuclear
171 envelope by a cytoskeletal plate called the acroplaxome, which forms a ring-like structure very
172 similar to the BRD4 ring in the region where the Lamin B1-associated nuclear envelope meets
173 the acrosome-associated nuclear envelope (Fig. 7D) (10). Therefore, we hypothesized that
174 BRD4 may be associated with the acroplaxome.

175 To determine whether the BRD4 ring is linked to the acrosome/acroplaxome, we
176 performed IF on spermatogenic cells, probing with fluorophore-conjugated peanut agglutinin
177 (PNA) to detect the acrosome, or with phalloidin to detect actin in the acroplaxome (Fig. 3C and
178 Movie C, respectively). Indeed, the BRD4 ring appears directly at the base of the acrosome
179 during capping in late round spermatids (top panel) and persists in elongating (middle panel)
180 and condensing spermatids (bottom panel) (Fig. 3C). Using a confocal microscope to create a
181 pseudo-3D image, we discovered that the BRD4 ring lies just adjacent to the actin ring of the
182 acroplaxome, but closer to the DAPI-stained nucleus (Movie C).

183 To provide additional evidence of the acrosome-associated BRD4 ring, we incubated
184 spermatogenic cells with JQ1-biotin followed by fluorophore-conjugated streptavidin. JQ1 is a
185 small molecule that binds to the bromodomains of all BET family proteins, which are expressed

186 in the nucleus of multiple spermatogenic cell types [Fig. 2C,D and (30, 38, 39)]. Thus, JQ1-
187 biotin shows a diffuse nuclear staining pattern in most spermatogenic cells types (Fig. 3D, top
188 panel); however, this staining is not a random artifact of streptavidin binding (Fig. 3D, bottom
189 panel). Importantly, in addition to a diffuse nuclear staining, JQ1-biotin is enriched in a ring
190 structure that overlaps with the BRD4 ring in spermatids [late round (top panel) and elongating
191 (bottom panel) spermatids in Fig. 3E]. Moreover, this JQ1-biotin enrichment is found at the
192 base of the acrosome, providing additional evidence for the existence of the BRD4 ring (Fig.
193 3F).

194 Because the BRD4 ring and the acroplaxome are remarkably similar in shape and
195 location, we hypothesized that acrosome formation is needed for BRD4 ring formation. To test
196 this hypothesis, we analyzed *Hrb* (also known as *Agfg1*) null mice, which produce infertile
197 sperm that lack acrosomes and have round, poorly compacted nuclei (11). In wild-type male
198 mice, the acrosome is formed by the fusion at the acroplaxome of proacrosomic vesicles derived
199 from the Golgi apparatus (Fig. 4A, top panels) (40). In *Hrb*^{-/-} male mice, the proacrosomic
200 vesicles begin to form in round spermatids, but they are unable to fuse properly to form a
201 mature acrosome (Fig. 4A, bottom panels). *Hrb*^{-/-} mice also show defects in nuclear elongation
202 and compaction later in condensing spermatids when the acrosome is completely absent (Fig.
203 4A, bottom right panel).

204 To determine if acrosome development is required for BRD4 ring formation, we
205 performed indirect IF on cryosectioned testes tissue from adult *Hrb*^{+/-} (which have normal
206 spermatogenesis) and *Hrb*^{-/-} mice (11). Strikingly, the BRD4 ring does not form properly in
207 *Hrb*^{-/-} mice (Fig. 4B, right panel). A small amount of BRD4 can be seen around spermatid
208 nuclei in *Hrb*^{-/-} mice, but the conspicuous ring structure never develops and exists only in small

209 fragments, if at all, in late stage spermatids (Fig. 4B, right panel). Because the acroplaxome
210 forms partially, although aberrantly, in *Hrb*^{-/-} acrosomal mutant mice, it is possible that the
211 BRD4 ring is able to form partially due to an association with certain components of the
212 acroplaxome that may be able to assemble in *Hrb*^{-/-} mice despite the lack of a functional
213 acrosome (41).

214

215 **BRD4 is enriched at the promoters of active genes in spermatids**

216 It is possible that BRD4 is present diffusely throughout the nucleus in round spermatids,
217 undetectable by IF until it is present in a higher concentration in the ring structure in very late
218 round spermatids. To determine if BRD4 interacts with the genome in spermatids, we analyzed
219 the genome-wide enrichment of BRD4 with chromatin immunoprecipitation followed by
220 sequencing (ChIP-seq). We performed analysis in round spermatids to characterize BRD4
221 binding in the context of the distinct post-meiotic gene expression program, as a previous study
222 has done for BRDT (25). We also determined the genome-wide localization of various other
223 histone post-translational modifications (PTMs) to assess whether BRD4 shows a binding
224 preference for any of these PTMs (all of which were normalized to input). All ChIP-seq data
225 alignment information can be found in Table 1.

226 Upon initial examination of the ChIP-seq data, it is evident that BRD4 and H3 and H4
227 acetylation (H3/H4ac) are enriched in genic regions of the genome as opposed to an established
228 heterochromatin PTM, H3K9me3, which is enriched in large intergenic regions (Fig. 5A).
229 Indeed, bioinformatic analysis revealed that the majority of peaks of BRD4, H3ac, and H4ac are
230 located within promoters [1 kilobasepair (kb) upstream of the transcriptional start site (TSS)] or
231 genes (introns or exons), unlike H3K9me3, which is located primarily in intergenic regions (Fig.

232 5B). Upon closer examination, BRD4 is enriched at the TSS of active housekeeping genes such
233 as *Actb* and active spermatogenesis-specific genes such as *Tnp1* (Fig. 5C). Conversely, BRD4
234 is not found at repressed housekeeping genes such as *Myc* (Fig. 5C). BRD4 is not bound to all
235 active genes even though some, such as *Vps45*, may be enriched for H3 or H4 acetylation (Fig.
236 5C).

237 In addition, we did not observe significant differences in enrichment patterns among
238 acetylation at different H3 and H4 residues. In general, H3K9ac and H4K5, 8, 12, and 16ac
239 appear to be enriched surprisingly similarly at the TSS of active genes (Fig. 5A,C). Although
240 BRD4 is not present at the TSS of all active genes, heat map analysis revealed that levels of
241 BRD4, H3K9ac, and H4ac at gene promoters (green in Fig. 6A) show a strong correlation with
242 levels of transcriptional activity of those genes in round spermatids [red in Fig. 6A, transcription
243 data from (42, 43)].

244 We compared our BRD4 ChIP-seq data in round spermatids to previously published
245 BRDT ChIP-seq data from the same cell type (25). Although this previous study showed that
246 BRDT binds to the TSS of active genes in round spermatids, we found the majority of BRDT
247 peaks (~64%) to be present in intergenic regions of the genome and only 3% of peaks within
248 gene promoters (Fig. 5B). However, BRDT enrichment at gene promoters does correlate with
249 the transcriptional activity of those genes (Fig. 6A). These data suggest that while most BRDT
250 is found in intergenic regions, the small percentage located at gene promoters correlates well
251 with transcriptional activity.

252 Because both BRD4 and BRDT were found to bind to the promoters of active genes in
253 round spermatids, we sought to investigate possible differences between the roles of these BET
254 family proteins. We defined a list of genes bound by BRD4 based on the presence of peaks 1kb

255 upstream of the TSS. We then intersected this list with a list of approximately 1,544 genes
256 previously shown to be bound by BRDT in round spermatids (25). Thus, we generated three
257 categories of genes: BRD4-bound (approximately 2,093 genes), BRDT-bound (approximately
258 1,347 genes), and co-bound (approximately 197 genes). Genes bound only by BRD4 showed an
259 average expression level that is slightly higher than genes bound only by BRDT (Fig. 6B).
260 However, the average expression levels of BRD4- or BRDT-bound genes were approximately
261 two-fold higher than the average expression level of all genes and similar to the average
262 expression level of spermatogenesis-specific genes [$p < 2.2e^{-16}$ for BRD4- and BRDT-bound in
263 (Fig. 6B)]. Interestingly, genes that were co-bound by BRD4 and BRDT show the highest
264 average transcription level [$p < 2.2e^{-16}$ in (Fig. 6B)]. These same trends can be seen with levels
265 of H3 and H4 acetylation at genes bound by BRD4, BRDT, or both (Fig. 6C). Histone H3/H4
266 acetylation levels are higher at the promoters of genes that are only bound by BRD4, but are
267 highest at the promoters of genes that are co-bound by BRD4 and BRDT [see Table 2 for p-
268 values in (Fig. 6B,C)]. As a control, H3K9me3 enrichment is extremely low at the promoters of
269 genes bound by BRD4 and BRDT.

270 Next, we performed Gene Ontology (GO) analysis of BRD4-, BRDT-, or co-bound
271 genes to determine if BRD4 and BRDT could possibly regulate different categories of genes.
272 Categories of housekeeping genes such as “RNA processing” or “protein folding” can be found
273 in genes bound by BRD4 or BRDT only (Fig. 6D,E left two panels). However,
274 spermatogenesis-specific genes are enriched in the gene set bound by BRD4 only or co-bound
275 by BRD4 and BRDT (Fig. 6D,E left and right panels). Indeed, heatmap analysis of BRD4 and
276 BRDT enrichment at the TSS of all spermatogenesis-specific genes reveals a strong positioning
277 of BRD4 (Fig. 6F). These data suggest that BRD4 and BRDT both play a role in the activation

278 of transcription in post-meiotic spermatids, but that BRD4 may play a particularly strong role in
279 activation of spermatogenesis-specific genes.

280 The necessity of BRD4 in transcriptional activation during spermiogenesis is difficult to
281 determine in the current absence of a conditional knockout mouse or reliable spermatogenic cell
282 culture system. Knockdowns in the germline are extremely difficult to generate, and staged cell
283 populations are not easily obtainable or manipulated in cell culture. Moreover, treatment of
284 male mice with JQ1 results in a meiotic arrest, before spermiogenesis (26). Thus, currently we
285 are unable to directly test the function of BRD4 during spermiogenesis.

286

287 **BRD4 association with poly-acetylated histone H4 diminishes in late spermatids as**
288 **acetylated histones are removed from the condensing nucleus**

289 Although our ChIP-sequencing data provides correlative evidence of BRD4 binding to
290 both acetylated histone H3 and H4, it does not demonstrate direct binding to these histone
291 PTMs. To investigate the composition of BRD4-associated chromatin, we performed
292 immunoprecipitation (IP) of BRD4 in round spermatids (Fig. 7A). Total cell lysate and BRD4-
293 immunoprecipitated proteins were separated on an SDS-PAGE gel and analyzed with mass
294 spectrometry. We quantified the fraction of peptides bearing different combinations of acetyl
295 and methyl PTMs for histones H3 and H4 in total chromatin and BRD4-immunoprecipitated
296 chromatin (Table 3). When the values of immunoprecipitated peptides were normalized to the
297 values for total chromatin in round spermatids, we noticed an approximate 4- and 10-fold
298 enrichment of tri- and tetra-acetylated H4 peptide, respectively (log-transformed ratios in Fig.
299 7A bottom left panel; see Table 3 for raw values). Specifically, various combinations of tri- and
300 tetra-acetylated H4 peptides (K5, 8, 12, and 16ac) were highly enriched in immunoprecipitated

301 chromatin, but all these most highly enriched combinations include H4K5ac or H4K8ac (Fig.
302 7A right panel and Table 3). In general, H3K9ac is not enriched in BRD4-immunoprecipitated
303 chromatin and H3K14ac is only slightly enriched when combined with different degrees of
304 H3K9 methylation (Fig. 7A upper left panel). The reason for the relative absence of H3
305 acetylation and higher H3K9me3 is not clear.

306 We then quantified the change in total and BRD4-immunoprecipitated H4 acetylation
307 over the course of spermatogenesis (raw values in Table 4). We generated ratios of H4
308 acetylation in round spermatids versus spermatocytes and compared these to the ratio of H4
309 acetylation in elongating/condensing spermatids versus round spermatids. First, tri- and tetra-
310 acetylated H4 peptide levels increase significantly in total chromatin over the course of
311 spermiogenesis (from spermatocytes to round spermatids to elongating/condensing spermatids)
312 (Fig. 7B left graph). Although levels of BRD4-immunoprecipitated mono-, tri-, and tetra-
313 acetylated H4 increase from spermatocytes to round spermatids, the levels of tetra-acetylated H4
314 decrease significantly from round spermatids to elongating/condensing spermatids (Fig. 7B right
315 graph). Interestingly, the immunofluorescent signal of hyper-acetylated histones is depleted
316 from the nuclear region underlying the acrosome (Movie D), as was shown in human spermatids
317 (16), and adjacent to the BRD4 ring (Fig. 7C). Taken together, these data suggest a localization
318 of BRD4 from the genome to the ring structure followed by removal of hyper-acetylated
319 histones from the genome as the acrosome caps the nucleus (see model in Fig. 7D).

320

321 **DISCUSSION**

322 Mammalian spermatogenesis results in a specialized sperm cell with a highly compacted
323 nucleus. A hallmark of this nuclear compaction is the removal of almost all histones from the

324 genome, with a small percentage retained at developmentally important loci (3, 4) and repetitive
325 DNA sequences (7, 8). While the mechanism of mass histone removal and degradation remains
326 unclear, it is believed that histone hyper-acetylation and thus, bromodomain-containing proteins,
327 especially BRDT, are integral to this process (2, 20, 21, 25, 27). Surprisingly little is known
328 about the involvement of the other BET family members – BRD2, BRD3, and BRD4 – during
329 spermiogenesis. A previous study used immunohistochemistry of testes tissue to show BRD4
330 expression specifically in spermatogonia (30). In this study, however, we found that BRD4 is
331 expressed during meiotic and post-meiotic phases of mouse spermatogenesis using several
332 approaches. First, we detect BRD4 gene expression in meiotic and post-meiotic cells with
333 western blotting and RT-qPCR (Fig. 1B and data not shown). Second, we immunoprecipitated
334 BRD4 from meiotic and post-meiotic cells and detect its association with expressed genes and
335 with acetylated histones [(44) and Figs. 5, 6, and 7]. Thus, BRD4 protein is present during the
336 post-meiotic phase of spermatogenesis.

337 Our characterization of BRD4 in spermatids provides evidence for an interesting
338 mechanism by which transcription is attenuated by the progressive removal of BRD4 itself and
339 acetylated histones via the acrosome. It is possible that this might be a general mechanism for
340 removal of transcriptionally relevant proteins. We initially observed BRD4 in a novel ring-like
341 structure that is closely associated, both spatially and functionally, with the
342 acrosome/acroplaxome. However, our unprecedented combination of IP/mass spectrometry and
343 ChIP-seq analysis of BRD4 with endogenous antibodies demonstrate the *in vivo* binding of
344 BRD4 throughout the chromatin of post-meiotic cells. Importantly, we performed these
345 analyses in round spermatids, just before the BRD4 ring first appears and an increase in histone
346 H4 acetylation can be detected by western blot, IF, and mass spectrometry (Figs. 1B, 2A,B and

347 7B). Also, in this cell type, we were able to capture the chromatin state before the initiation of
348 histone replacement, vast compaction, and transcriptional shutdown.

349 BRD4 has traditionally been associated with euchromatin, active transcription, or mitotic
350 bookmarking (45-51). Our CHIP-sequencing data suggest that BRD4 plays a similar role in
351 transcriptional activation in round spermatids. BRD4, H3K9ac, and H4K5,8,12, and 16ac are
352 present at the TSS of active genes in round spermatids, and their enrichment correlates with
353 transcription levels (Figs. 5 and 6A). While BRD4 is known to bind to poly-acetylated histone
354 H4 *in vitro*, our study is the first to confirm this preference for acetylated H4 (especially poly-
355 acetylated H4 modified at K5 and K8) over H3 by *in vivo* IP/mass spectrometry using an
356 antibody against endogenous BRD4 [Fig. 7A and (49, 52, 53)].

357 When we compared our CHIP-sequencing data to that published in a recent study of
358 BRDT in the same cell type (25), we found that BRD4 and BRDT enrichment at gene promoters
359 correlates with the transcriptional activity of those genes (Fig. 6A). BRD4 is especially
360 enriched at spermatogenesis-specific genes and shows a very robust positioning around their
361 TSS, suggesting that this BET family protein may play a strong role in their activation (Fig. 6F).
362 Interestingly, genes that were co-bound by BRD4 and BRDT showed higher average
363 transcription and histone acetylation levels than genes bound only by BRD4 or BRDT (Fig.
364 6B,C). It is unclear how BRD4, BRDT, or both would be recruited to specific subsets of genes,
365 but these data suggest that synergistic binding of these two BET family proteins could lead to
366 higher histone acetylation and expression levels via increased recruitment of the P-TEFb
367 complex, as has been shown for both BRD4 and BRDT (25, 50, 51).

368 Although BRDT binds to a considerable number of gene promoters, we found that the
369 majority of BRDT peaks occur in intergenic regions of the genome (Fig. 5B). Interestingly, a

370 recent study demonstrated that nucleosomes that are retained in the mature mouse sperm
371 genome show a ten-fold overrepresentation at promoter regions (54). Because BRDT has also
372 been implicated in the histone-to-protamine transition via removal of acetylated histones, we
373 propose that BRDT may show a binding preference for regions where histones are largely
374 evicted from the mature sperm genome, perhaps even as early as the round spermatid stage (20,
375 23, 25). In the future, it will be interesting to investigate the genome-wide binding patterns of
376 other BET family proteins over the course of spermatogenesis.

377 Because transcription is largely de-activated as the genome is highly compacted during
378 spermiogenesis, it is reasonable to postulate that BRD4 and other transcriptional co-activators
379 must be removed from the genome during this process. Indeed, western blot analysis shows that
380 BRDT, BRD2, and BRD4 are largely absent from mature sperm (Fig. 1B). It is currently
381 unclear, however, how histones and so many other chromatin components may be removed
382 and/or degraded during this nuclear condensation. In approximately stage 7-8 spermatids, when
383 histone hyperacetylation and nuclear elongating/compaction begin, the BRD4 ring appears just
384 adjacent to the acroplaxome (Figs. 1B, 2B, 3C and Movies A-C). At the same stage of
385 spermiogenesis, we observed by western blot a BRD4 peptide of a slightly lower molecular
386 weight than the full length BRD4 isoform (Fig. 1B). It is possible that the BRD4 ring may
387 contain a spermatid-specific isoform that binds to acetylated histones or other acetylated
388 chromatin-associated proteins in order to provide a tethering force (via the acroplaxome) for
389 chromatin compaction and reorganization. Indeed, a growing body of evidence implicates
390 BRD4 in structural roles such as tethering, insulating, and maintaining higher order chromatin
391 structure (55-58). Alternatively, because BRD4 is undetectable in mature sperm by IF and

392 western blot, this smaller BRD4 peptide may simply be a degradation product that is created
393 during the removal of BRD4 from the genome via the acroplaxome-associated ring.

394 Interestingly, the BRD4 ring does not form in acrosomal mutant mice, which show
395 nuclear compaction and fertility defects that are highly similar to human globozoospermia, a
396 condition in which the acrosome is malformed or absent, the sperm head is round, and
397 chromatin compaction is abnormal [Fig. 4 and (11-15)]. Chromatin compaction during
398 spermiogenesis may be incomplete in these mutant mice due to abnormal retention of
399 chromatin-associated proteins and histones. Future ChIP-sequencing studies in acrosomal
400 mutant mice will elucidate potential defects in the chromatin signature characteristic of
401 spermiogenesis.

402 Our observations strongly support an increasing body of evidence that acrosome
403 formation plays a key role in nuclear compaction and chromatin remodeling during
404 spermiogenesis. Our IF data in mouse (Fig. 7C and Movie D) and that of a recent study in
405 human spermatids shows that acetylated histones in the nucleus are depleted first in the region
406 directly adjacent to the acrosome, where initial DNA compaction occurs (16). Moreover,
407 various chromatin-associated proteins known to be involved in chromatin compaction, such as
408 H1 T2, are found in the nucleus adjacent to the acrosome (59). Finally, it has been suggested that
409 the acroplaxome is able to provide contractile force to the compacting nucleus (10). It is unclear
410 whether histones are degraded within the nucleus or shuttled out of the nucleus first during
411 spermiogenesis; however, recent studies have shown that histone removal, while delayed, still
412 occurs in mice lacking PA200, the acetylated histone-binding activator of the spermatogenesis-
413 specific proteasome (21).

414 As histone removal followed by degradation appears to be the most supported model, the
415 acroplaxome is in an ideal location to facilitate this potential shuttling (22). It has been shown
416 that the nuclear membrane underlying the expanding acrosomal cap is devoid of any nuclear
417 pores and may be impenetrable to exiting nuclear components (37). If the acrosome is coupled
418 to the extensive removal of histones and other chromatin components from the genome, the
419 force-providing acroplaxome could act to facilitate shuttling at the base of the acrosome where
420 nuclear pores and lamins B1 and B3 are still present. Future studies, such as mass spectrometry
421 analysis, will provide critical insight into potential binding partners or post-translational
422 modifications of BRD4 that may localize this protein to the acroplaxome in spermatids.

423 Regardless, this striking BRD4 localization suggests an interesting link between a chromatin
424 component known to bind to acetylated histones and extra-nuclear spermatogenic structures.

425 Our combined approaches of immunofluorescence, biochemistry, mass spectrometry,
426 and ChIP-sequencing suggest that BRD4 may play a fundamental role in transcription of
427 spermatogenesis-specific genes and then in the transition from the transcriptionally active
428 genome of early post-meiotic spermatids to the highly compact, transcriptionally silent genome
429 of mature sperm. Importantly, our characterization of BRD4 contributes to the growing body of
430 evidence that dramatic chromatin events taking place in the nucleus during spermiogenesis may
431 be directly affected by extra-nuclear changes in cell structure and composition. Further
432 investigation should reveal other proteins that are also involved in this process and elucidate the
433 mechanism by which histones and other chromatin components are removed from the genome
434 in an apparently acrosome-dependent manner. Finally, it would be interesting to further
435 investigate how BET family proteins function during spermiogenesis to first activate
436 transcription and then to repress it by potentially removing acetylated histones from the

437 spermatid genome. The future study of various steps in this intricate process of transcriptional
 438 repression and nuclear compaction will lead to a better understanding of chromatin dynamics
 439 during spermatogenesis, epigenetic signatures in sperm, and mammalian fertility.

440

441 MATERIALS AND METHODS

442 Antibodies/Reagents

Application	Antibodies/Reagents
Immunofluorescence	BRD2 (Abcam ab111078), BRD4 (60), BRDT (Abcam ab5157), H3K9Ac (Active Motif AM39137), H4K5,8,12,16ac (Millipore 05-1355), JQ1-PEG2-biotin (39), Lamin B1 (Abcam ab16048), Lamin B1 (Santa Cruz sc-373918), phalloidin-488 (Invitrogen A12379), and PNA-488 (Invitrogen L21409)
Western blot	β -actin (Cell Signalling 4970), BRD2 (Lifespan Biosciences (aa524-573) IHC-plus LS-B923), BRD4 (60), BRDT (Abcam ab5157), Cyclin T1 (Santa Cruz H245 sc-10750), GAPDH (Fitzgerald 10R-G109a), H3K9Ac (Active Motif AM39137), H4 (Abcam ab10158), H4K5ac (Millipore 07-327), H4K8Ac (Millipore 07-328), H4K12Ac (Millipore 07-595), H4K16Ac (Active Motif AM39167), H4K5,8,12,16ac (Millipore 05-1355)
Immunoprecipitation/ ChIP	H3 (Abcam ab1791), H3K9ac (Active Motif AM39137), H3K9me3 (Abcam ab8898), H4K5ac (Millipore 07-327), H4K8ac (Millipore 07-328), H4K12ac (Millipore 07-595), H4K16ac (Active Motif AM39167), H4K5,8,12,16ac (Millipore 06-866), BRD4 (Bethyl A301-985A50)

443

444

445 Mouse Models

446 Male 129S6/SvEvTac mice (Taconics, Germantown, NY) as well as *Hrb*^{-/-} gene-disrupted mice
 447 (11) were maintained and humanely euthanized according to the guidelines of the University of
 448 Pennsylvania Institutional Animal Care and Use Committee.

449

450 Mouse spermatogenic cell fractionation

451 Spermatogenic cell fractionation was performed by sedimentation of cells prepared from adult
452 mouse testes through a BSA gradient as previously described (33). Each fractionation
453 experiment used approximately 22 testes. Fractions were analyzed for purity based on cell and
454 nuclear morphology (via DAPI staining) and pooled. Mature spermatozoa were obtained from
455 epididymides of adult mice, and contaminating cell types were eliminated by incubating in
456 somatic cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in DEPC H₂O) on ice for 20 minutes.

457

458 **Western blot analysis**

459 Cells were resuspended in buffer [20mM Tris pH 7.5, 1mM MgCl₂, 1mM CaCl₂, 137mM NaCl,
460 10%Glycerol, 1% NP-40, Complete protease inhibitor EDTA-free (Roche), 300nM trichostatin
461 A (Sigma T1952)], and rotated at 4°C for one hour in the presence of 12.5 U/mL Benzonase
462 (Novagen 70746). Protein content was measured with Bradford dye and cell lysate containing
463 25µg of protein or sperm lysate containing 5µg of protein was added to each well of a 4-12%
464 1mm Bis-Tris NuPAGE protein gel. Protein was transferred to a PVDF membrane and blocked
465 for one hour in 5% BSA-TBST. Membranes were incubated with primary antibody (in 1%
466 BSA-TBST) for one hour, washed, incubated with secondary HRP-conjugated antibody (in 1%
467 BSA-TBST) for 30 minutes, washed, and detected using enhanced chemiluminescence. For
468 peptide competition, primary antibody was incubated with 5µg/mL immunizing peptide for one
469 hour at room temperature before application to the membrane.

470

471 **Immunofluorescence**

472 Immunofluorescence was performed on 8µm cryosections of mouse testes from ten-week-old
473 mice or cell suspensions obtained using the spermatogenic cell separation method described

474 above (33). Testes were dissected and immediately placed in 4% PFA (in PBS) or snap frozen
475 in liquid nitrogen. The PFA-fixed testes were incubated in 15%, then 30% sucrose (in PBS) and
476 embedded in TissueTek OCT compound. Fixed or frozen tissue was cryosectioned onto
477 Superfrost Plus slides (Fisher). Cell suspensions in PBS were allowed to adhere to Superfrost
478 Plus slides for 1hr at 33°C. Snap frozen sections and cell suspensions were fixed for 15min in
479 4% paraformaldehyde at room temperature. Sections/cells were washed 3 x 5min in PBS and
480 incubated with 125mM glycine (in PBS) for one minute at room temperature. Sections/cells
481 were washed 3 x 5 min in PBS, then permeabilized with 0.1% Triton-X100 for two minutes at
482 4°C. Sections/cells were washed 3 x 5 min in PBS, then blocked with 3% donkey serum in PBS
483 for one hour at 37°C. Sections/cells were incubated with primary antibody or 40µM JQ1-biotin
484 (in 3% donkey serum in PBS) for one hour at 37°C and washed 3 x 5 min in PBS.
485 Sections/cells were incubated with 5µg/mL (in 3% donkey serum in PBS) Alexafluor secondary
486 antibody (Invitrogen) for 30min at 37°C, then washed 3 x 5min in PBS. Sections/cells were
487 then incubated with DAPI (Invitrogen, 5µg/mL in 3% donkey serum in PBS) and embedded in
488 Prolong Gold Anti-fade reagent (Invitrogen). Sections/cells were imaged with a Leica TCS SP8
489 confocal microscope and pseudo-3D images were created using Velocity 6 software. Minimal
490 changes to immunofluorescence images (contrast and pseudo-coloring) were made using
491 ImageJ.

492

493 **Chromatin immunoprecipitation and sequencing**

494 ChIP-seq for BRD4 and histone post-translational modifications was carried out as previously
495 described with minor modifications (61). Cells were cross-linked in 1% formaldehyde in PBS
496 for 10 minutes at room temperature. The reaction was quenched with 125mM glycine in PBS

497 for five minutes at room temperature. After cell lysis, lysates were sonicated for 20 minutes
498 with a Covaris S220 sonicator (5% duty cycle, 140 watts peak incident power, 200 cycles per
499 burst). For each IP, 500 μ g of protein (measured with BCA assay) from the cell lysate, 30 μ L
500 protein G Dynabeads (Life Technologies), and 5 μ g-10 μ g of antibody or IgG (Pierce 31235)
501 were used. CHIP libraries for sequencing were prepared using 5ng DNA and the NEBNext
502 Ultra DNA library prep kit for Illumina. Size selection was performed using AMPure XP beads
503 (Beckman Coulter, Inc. #A63881). Libraries were sequenced using a NextSeq 500 machine
504 (Illumina) as per manufacturer's protocols.

505

506 **ChIP-seq data analysis**

507 ChIP-seq data generated using a NextSeq 500 were demultiplexed using the bcl2fastq utility
508 (02.14.01.07). Data were then aligned using bowtie 0.12.7 (parameters -m 1 --best) to mouse
509 genomic assembly mm9.

510 *UCSC Genome Browser Tracks*

511 Visual tracks of ChIP-seq data were generated in the following way. For each sample, the
512 aligned data file for PCR duplicated reads was filtered (i.e., any set of aligned reads with the
513 same chromosome, start, and stop coordinates was reduced to a single representative). Coverage
514 maps were then created using the BEDTools utility genomeCoverageBed. Resulting bedGraphs
515 were scaled using the RPKM coefficient, a measure of the number of billions of bases
516 sequenced per sample, to correct for sequencing efficiency biases. Finally, an input coverage
517 map was subtracted for the BRD4 and each histone PTM coverage map. The BRDT data from
518 (25) were treated similarly.

519 *Genome Compartment Plot*

520 The genomic compartment table was generated by first calling peaks for each histone PTM or
521 BRD4 using SICER (peaks were called against input as the background; window, fragment, and
522 gap size parameters were fixed at 200bp; and the FDR was controlled at 0.1%). Previously
523 published peak locations from (25) were used for BRDT. Peak locations were overlapped using
524 BEDTools' intersect utility with RefSeq promoters, exons, and introns, in an exclusive way: if a
525 peak overlapped a promoter, it was removed from consideration for overlap with exons or
526 introns, and if it overlapped an exon it was removed from consideration for overlap with introns.

527 *Expression versus ChIP Enrichment Heatmap*

528 RefSeq transcripts were assessed for expression in round spermatids by loading two previously
529 published replicate data sets (GSM95950 and GSM95951 in GEO series GSE4193) into the
530 Partek Genomics Suite software package. Data were background-corrected with GC-RMA,
531 quantile-normalized, and median polished along with all other data sets in the GSE4193 series.
532 Promoters (1kb upstream regions) associated with these expression-scored transcripts were
533 assessed for numbers of aligned tags in each indicated ChIP. Tag counts were then normalized
534 to the number of millions of tags sequenced as well as input. Brightness for each track is scaled
535 to the maximum ChIP value in that track; tracks are sorted in order of least expressed to most
536 expressed genes in GSM95950.

537 *Expression Boxplots*

538 BRDT target genes are as described in (25). BRD4 target genes were those with a SICER-
539 determined BRD4 peak in the promoter where the peak enrichment was in the 90th percentile of
540 all BRD4 peaks. For each of the three gene sets, expression enrichments were determined using
541 GSM95950 (data processing described above) and ChIP-seq enrichments were determined using
542 normalized tag counts at the promoter (data processing described above). Table 2 p-values and

543 W statistics (reflecting expression distribution and promoter acetylation distribution differences
544 between genes bound by BRD4, BRDT, both, or neither) were estimated using the one-sided
545 Mann-Whitney test in R.

546 *Gene Ontology Pie Charts*

547 Gene Ontology enrichment analysis of BRD4 target genes, BRDT target genes, and co-bound
548 genes was done using DAVID (62). GO terms in the "Biological Process" hierarchy were
549 collapsed to a single representative term if they shared the same gene; if one GO term's
550 associated target genes were a subset of another's, that GO term was dropped in favor of the
551 other. GO terms were further combined if they shared more than 15 transcripts. GO terms are
552 represented by their overall gene "real estate." Each gene is given a vote inversely proportional
553 to the number of terms it appears in. Each term's weight is the sum of the votes of its genes.
554 Finally, pie charts were simplified by visual inspection.

555 *Promoter Heatmap*

556 Genes enriched in the Gene Ontology category "Spermatogenesis" (GO:0007283) were
557 associated with RefSeq transcripts using UniProt and DAVID. For each transcript, a vector
558 describing the ChIP-seq enrichment profile around the TSS (2.5kb upstream and 2.5kb
559 downstream) was assessed for BRD4 and BRDT (ChIP-seq enrichment data were normalized to
560 input, length, and number of millions of reads, as above). Profiles were sorted top-to-bottom by
561 overall BRD4 enrichment intensity. The maximum green in each plot was determined by the
562 enrichment value at the 90th percentile.

563

564 **Immunoprecipitation and mass spectrometry**

565 Antibody-coupled beads were prepared by incubating 30 μ L/IP protein G Dynabeads (Life
566 Technologies) with 10 μ g primary antibody or rabbit IgG (Pierce 31235) in 0.5% BSA-PBS for
567 six hours at 4°C with rotation. Cells were resuspended in lysis buffer (20mM Tris pH 7.5, 1mM
568 MgCl₂, 1mM CaCl₂, 137mM NaCl, 10% glycerol, 1% NP-40, Complete Protease inhibitor
569 EDTA-free (Roche), 10mM NaB, 300nM Trichostatin A), after which 12.5 U/mL Benzoylase
570 was added. Lysates were incubated for one hour at 4°C with rotation and then cleared by
571 centrifugation at 14,000 rpm for 10 minutes. Supernatant was removed and protein
572 concentration was measured with Bradford dye. After incubating with antibody, beads were
573 washed three times with 1mL buffer. One mg protein from the lysate was added to the beads
574 and incubated overnight at 4°C with rotation. Beads were washed five times with 1mL buffer.
575 Beads were resuspended in 30 μ L sample buffer and incubated for five minutes at 90°C. Eluate
576 was separated from the beads, separated on a 4-12% 1mm Bis-Tris NuPAGE protein gel, and
577 analyzed for enriched histone modifications compared to input samples with mass spectrometry
578 (MS).

579 In-gel histone proteins were derivatized twice with a mixture of propionic anhydride and
580 100 mM ammonium bicarbonate (1:1) for 15 min under vigorous vortexing and then digested
581 with 12.5 ng/ μ L of trypsin at room temperature overnight. Resultant histone peptides were
582 extracted from the gel, re-propionylated twice, and then desalted using C18-based homemade
583 stage-tips before MS analysis. Desalted peptides were separated by reverse phase nanospray
584 liquid chromatography with the Thermo Scientific Easy-nLC 1000 system and an in-house
585 packed C₁₈ resin column (15cm in length and 3 μ m in particle size). Buffer A is water with 0.1%
586 formic acid. Buffer B is acetonitrile with 0.1% formic acid. Histone peptides were eluted by a
587 gradient from 2% to 30% of buffer B for 35 min, 30% to 98% of buffer B for 20 min followed

588 by a wash at 98% of buffer B for 15min with a flow rate of 200 nL/min. Mass spectrometry was
589 performed on a Thermo Scientific Orbitrap Velos Pro hybrid ion trap-Orbitrap mass
590 spectrometer. Each cycle includes one full MS scan (m/z 290 to 1400, resolution of 60,000,
591 AGC target value of 1×10^6), followed by seven data-dependent MS2 scans of the most intense
592 peptide ions using CID (normalized collision energy of 35%, isolation width of 3 m/z, AGC
593 target value of 1×10^4). In the section between 23 min and 45 min, MS2 scans targeting precursor
594 ions with m/z of 528.30, 570.84, 768.95, 761.94 and 754.93 (isolation width of 1 m/z) were
595 performed for the determination of acetylation sites on histone peptides with multiple lysines.
596 Dynamic exclusion of 25 s was used to prevent repeated analysis of the same components. Ions
597 with a charge state of one or more than four and a rejection list of common contaminant ions
598 were excluded from the analysis. Histone peptides were identified based on retention times and
599 tandem MS. Abundance of histone peptides were quantified by integrating the area under each
600 peak in the MS chromatogram using Thermo Scientific Xcalibur Qual Browser. The LC-
601 MS/MS data sets were also analyzed using in-house developed software as previously described
602 (49).

603

604 ACKNOWLEDGEMENTS

605 We thank the members of the Berger lab, especially Dr. Parisha Shah, for all their
606 support and advice. We thank Dr. Jérôme Govin for his guidance. We thank Dr. Saadi
607 Khochbin for his advice and sharing his BRDT CHIP-seq data. We thank Dr. Jan Van Deursen
608 for sharing his *Hrb*^{-/-} mice. We thank Dr. James Bradner for sharing JQ1-biotin. We thank
609 Andrea Stout of the Cell and Developmental microscopy core for her help with the

610 immunofluorescence imaging. We thank Joseph Grubb and Jonathan Schug of the University of
611 Pennsylvania Functional Genomics Core for their help with ChIP sequencing.

612 Support to JMB was from the T32 Genetics Training Grant at the University of
613 Pennsylvania (GM008216). Support to SLB was from NIH grants GM055360 and U54-
614 HD068157. BAG acknowledges funding from NIH grant GM110174 and Innovator grant
615 (DP2OD007447) from the Office of the Director. RGM was supported by NIH grants
616 R01HD048837 and U54HD068157.

617

618 **DISCLOSURES**

619 The authors declare that they have no competing financial interests. All experiments requiring
620 the use of laboratory mice were executed in compliance with all relevant guidelines, regulations
621 and regulatory agencies. Mouse experiments were conducted under the guidance and approval
622 of the University of Pennsylvania institutional animal care and use committee.

623

624 **REFERENCES**

625

- 626 1. **Rajender S, Avery K, Agarwal A.** 2011. Epigenetics, spermatogenesis and male
627 infertility. *Mutation Research/Reviews in Mutation Research* **727**:62-71.
- 628 2. **Govin J, Caron C, Lestrat C, Rousseaux S, Khochbin S.** 2004. The role of histones in
629 chromatin remodelling during mammalian spermiogenesis. *European Journal of*
630 *Biochemistry* **271**:3459-3469.
- 631 3. **Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley EJ, Roloff TC, Beisel C,**
632 **Schubeler D, Stadler MB, Peters AH.** 2010. Repressive and active histone methylation

- 633 mark distinct promoters in human and mouse spermatozoa. *Nat Struct Mol Biol* **17**:679-
634 687.
- 635 4. **Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR.** 2009.
636 Distinctive chromatin in human sperm packages genes for embryo development. *Nature*
637 **460**:473-478.
- 638 5. **Arpanahi A, Brinkworth M, Iles D, Krawetz SA, Paradowska A, Platts AE, Saida**
639 **M, Steger K, Tedder P, Miller D.** 2009. Endonuclease-sensitive regions of human
640 spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences.
641 *Genome Research* **19**:1338-1349.
- 642 6. **Carone BR, Hung JH, Hainer SJ, Chou MT, Carone DM, Weng Z, Fazio TG,**
643 **Rando OJ.** 2014. High-resolution mapping of chromatin packaging in mouse embryonic
644 stem cells and sperm. *Dev Cell* **30**:11-22.
- 645 7. **Meyer-Ficca ML, Lonchar JD, Ihara M, Bader JJ, Meyer RG.** 2013. Alteration of
646 poly(ADP-ribose) metabolism affects murine sperm nuclear architecture by impairing
647 pericentric heterochromatin condensation. *Chromosoma* **122**:319-335.
- 648 8. **Samans B, Yang Y, Krebs S, Sarode GV, Blum H, Reichenbach M, Wolf E, Steger**
649 **K, Dansranjavin T, Schagdarsurengin U.** 2014. Uniformity of nucleosome
650 preservation pattern in Mammalian sperm and its connection to repetitive DNA
651 elements. *Dev Cell* **30**:23-35.
- 652 9. **Gaucher J, Reynoird N, Montellier E, Boussoar F, Rousseaux S, Khochbin S.**
653 2010. From meiosis to postmeiotic events: The secrets of histone disappearance. *FEBS*
654 *Journal* **277**:599-604.

- 655 10. **Kierszenbaum AL, Tres LL.** 2004. The acrosome-acroplaxome-manchette complex
656 and the shaping of the spermatid head. *Arch Histol Cytol* **67**:271-284.
- 657 11. **Kang-Decker N, Mantchev GT, Juneja SC, McNiven MA, van Deursen JM.** 2001.
658 Lack of acrosome formation in Hrb-deficient mice. *Science* **294**:1531-1533.
- 659 12. **Fujihara Y, Satouh Y, Inoue N, Isotani A, Ikawa M, Okabe M.** 2012. SPACA1-
660 deficient male mice are infertile with abnormally shaped sperm heads reminiscent of
661 globozoospermia. *Development* **139**:3583-3589.
- 662 13. **Lin YN, Roy A, Yan W, Burns KH, Matzuk MM.** 2007. Loss of zona pellucida
663 binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm
664 morphogenesis. *Molecular and Cellular Biology* **27**:6794-6805.
- 665 14. **Xiao N, Kam C, Shen C, Jin W, Wang J, Lee KM, Jiang L, Xia J.** 2009. PICK1
666 deficiency causes male infertility in mice by disrupting acrosome formation. *J Clin*
667 *Invest* **119**:802-812.
- 668 15. **Yao R, Ito C, Natsume Y, Sugitani Y, Yamanaka H, Kuretake S, Yanagida K, Sato**
669 **A, Toshimori K, Noda T.** 2002. Lack of acrosome formation in mice lacking a Golgi
670 protein, GOPC. *Proc Natl Acad Sci U S A* **99**:11211-11216.
- 671 16. **De Vries M, Ramos L, Housein Z, De Boer P.** 2012. Chromatin remodelling initiation
672 during human spermiogenesis. *Biology Open* **1**:446-457.
- 673 17. **Grimes SR, Jr., Henderson N.** 1984. Hyperacetylation of histone H4 in rat testis
674 spermatids. *Exp Cell Res* **152**:91-97.
- 675 18. **Meistrich ML, Trostle-Weige PK, Lin R, Bhatnagar YM, Allis CD.** 1992. Highly
676 acetylated H4 is associated with histone displacement in rat spermatids. *Mol Reprod Dev*
677 **31**:170-181.

- 678 19. **Govin J, Escoffier E, Rousseaux S, Kuhn L, Ferro M, Thevenon J, Catena R,**
679 **Davidson I, Garin J, Khochbin S, Caron C.** 2007. Pericentric heterochromatin
680 reprogramming by new histone variants during mouse spermiogenesis. *The Journal of*
681 *Cell Biology* **176**:283-294.
- 682 20. **Shang E, Nickerson HD, Wen D, Wang X, Wolgemuth DJ.** 2007. The first
683 bromodomain of Brdt, a testis-specific member of the BET sub-family of double-
684 bromodomain-containing proteins, is essential for male germ cell differentiation.
685 *Development* **134**:3507-3515.
- 686 21. **Qian MX, Pang Y, Liu CH, Haratake K, Du BY, Ji DY, Wang GF, Zhu QQ, Song**
687 **W, Yu Y, Zhang XX, Huang HT, Miao S, Chen LB, Zhang ZH, Liang YN, Liu S,**
688 **Cha H, Yang D, Zhai Y, Komatsu T, Tsuruta F, Li H, Cao C, Li W, Li GH, Cheng**
689 **Y, Chiba T, Wang L, Goldberg AL, Shen Y, Qiu XB.** 2013. Acetylation-mediated
690 proteasomal degradation of core histones during DNA repair and spermatogenesis. *Cell*
691 **153**:1012-1024.
- 692 22. **Goudarzi A, Shiota H, Rousseaux S, Khochbin S.** 2014. Genome-Scale Acetylation-
693 Dependent Histone Eviction during Spermatogenesis. *J Mol Biol.*
- 694 23. **Pivot-Pajot C, Caron C, Govin J, Vion A, Rousseaux S, Khochbin S.** 2003.
695 Acetylation-Dependent Chromatin Reorganization by BRDT, a Testis-Specific
696 Bromodomain-Containing Protein. *Molecular and Cellular Biology* **23**:5354-5365.
- 697 24. **Bryant JM, Berger SL.** 2012. Low-hanging fruit: targeting Brdt in the testes. *EMBO J*
698 **31**:3788-3789.
- 699 25. **Gaucher J, Boussouar F, Montellier E, Curtet S, Buchou T, Bertrand S, Hery P,**
700 **Jounier S, Depaux A, Vitte A-L, Guardiola P, Pernet K, Debernardi A, Lopez F,**

- 701 **Holota H, Imbert J, Wolgemuth DJ, Gérard M, Rousseaux S, Khochbin S.** 2012.
702 Bromodomain-dependent stage-specific male genome programming by Brdt. The
703 EMBO Journal **31**:3809-3820.
- 704 26. **Matzuk Martin M, McKeown Michael R, Filippakopoulos P, Li Q, Ma L, Agno**
705 **Julio E, Lemieux Madeleine E, Picaud S, Yu Richard N, Qi J, Knapp S, Bradner**
706 **James E.** 2012. Small-Molecule Inhibition of BRDT for Male Contraception. Cell
707 **150**:673-684.
- 708 27. **Berkovits BD, Wolgemuth DJ.** 2011. The first bromodomain of the testis-specific
709 double bromodomain protein Brdt is required for chromocenter organization that is
710 modulated by genetic background. Developmental Biology **360**:358-368.
- 711 28. **Shang E, Wang X, Wen D, Greenberg DA, Wolgemuth DJ.** 2009. Double
712 bromodomain-containing gene Brd2 is essential for embryonic development in mouse.
713 Developmental Dynamics **238**:908-917.
- 714 29. **Houzelstein D, Bullock SL, Lynch DE, Grigorieva EF, Wilson VA, Beddington**
715 **RSP.** 2002. Growth and Early Postimplantation Defects in Mice Deficient for the
716 Bromodomain-Containing Protein Brd4 Molecular and Cellular Biology **22**:3794-3802.
- 717 30. **Shang E, Salazar G, Crowley TE, Wang X, Lopez RA, Wang X, Wolgemuth DJ.**
718 2004. Identification of unique, differentiation stage-specific patterns of expression of the
719 bromodomain-containing genes Brd2, Brd3, Brd4, and Brdt in the mouse testis. Gene
720 Expression Patterns **4**:513-519.
- 721 31. **Voigt P, Reinberg D.** 2011. BRD4 jump-starts transcription after mitotic silencing.
722 Genome Biology **12**:133.

- 723 32. **McBride AA, Jang MK.** 2013. Current understanding of the role of the Brd4 protein in
724 the papillomavirus lifecycle. *Viruses* **5**:1374-1394.
- 725 33. **Bryant JM, Meyer-Ficca ML, Dang VM, Berger SL, Meyer RG.** 2013. Separation of
726 spermatogenic cell types using STA-PUT velocity sedimentation. *J Vis Exp*.
- 727 34. **Meistrich ML, Hess RA.** 2013. Assessment of spermatogenesis through staging of
728 seminiferous tubules. *Methods Mol Biol* **927**:299-307.
- 729 35. **Schutz W, Alsheimer M, Ollinger R, Benavente R.** 2005. Nuclear envelope
730 remodeling during mouse spermiogenesis: postmeiotic expression and redistribution of
731 germline lamin B3. *Exp Cell Res* **307**:285-291.
- 732 36. **Vester B, Smith A, Krohne G, Benavente R.** 1993. Presence of a nuclear lamina in
733 pachytene spermatocytes of the rat. *J Cell Sci* **104 (Pt 2)**:557-563.
- 734 37. **Ho HC.** 2010. Redistribution of nuclear pores during formation of the redundant nuclear
735 envelope in mouse spermatids. *J Anat* **216**:525-532.
- 736 38. **Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM,**
737 **Keates T, Hickman TT, Felletar I, Philpott M, Munro S, McKeown MR, Wang Y,**
738 **Christie AL, West N, Cameron MJ, Schwartz B, Heightman TD, La Thangue N,**
739 **French CA, Wiest O, Kung AL, Knapp S, Bradner JE.** 2010. Selective inhibition of
740 BET bromodomains. *Nature* **468**:1067-1073.
- 741 39. **Anders L, Guenther MG, Qi J, Fan ZP, Marineau JJ, Rahl PB, Loven J, Sigova**
742 **AA, Smith WB, Lee TI, Bradner JE, Young RA.** 2014. Genome-wide localization of
743 small molecules. *Nat Biotechnol* **32**:92-96.
- 744 40. **Abou-Haila A, Tulsiani DR.** 2000. Mammalian sperm acrosome: formation, contents,
745 and function. *Arch Biochem Biophys* **379**:173-182.

- 746 41. **Kierszenbaum AL, Tres LL, Rivkin E, Kang-Decker N, van Deursen JM.** 2004. The
747 acroplaxome is the docking site of Golgi-derived myosin Va/Rab27a/b- containing
748 proacrosomal vesicles in wild-type and Hrb mutant mouse spermatids. *Biol Reprod*
749 **70**:1400-1410.
- 750 42. **Mulugeta Achame E, Wassenaar E, Hoogerbrugge JW, Sleddens-Linkels E, Ooms**
751 **M, Sun ZW, van IWF, Grootegoed JA, Baarends WM.** 2010. The ubiquitin-
752 conjugating enzyme HR6B is required for maintenance of X chromosome silencing in
753 mouse spermatocytes and spermatids. *BMC Genomics* **11**:367.
- 754 43. **Namekawa SH, Park PJ, Zhang LF, Shima JE, McCarrey JR, Griswold MD, Lee**
755 **JT.** 2006. Postmeiotic sex chromatin in the male germline of mice. *Curr Biol* **16**:660-
756 667.
- 757 44. **Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, Bradner JE, Lee TI,**
758 **Young RA.** 2013. Selective inhibition of tumor oncogenes by disruption of super-
759 enhancers. *Cell* **153**:320-334.
- 760 45. **Dey A, Nishiyama A, Karpova T, McNally J, Ozato K.** 2009. Brd4 Marks Select
761 Genes on Mitotic Chromatin and Directs Postmitotic Transcription. *Molecular Biology*
762 *of the Cell* **20**:4899-4909.
- 763 46. **Mochizuki K, Nishiyama A, Jang MK, Dey A, Ghosh A, Tamura T, Natsume H,**
764 **Yao H, Ozato K.** 2008. The Bromodomain Protein Brd4 Stimulates G1 Gene
765 Transcription and Promotes Progression to S Phase. *Journal of Biological Chemistry*
766 **283**:9040-9048.

- 767 47. **Yang Z, He N, Zhou Q.** 2007. Brd4 Recruits P-TEFb to Chromosomes at Late Mitosis
768 To Promote G1 Gene Expression and Cell Cycle Progression. *Molecular and Cellular*
769 *Biology* **28**:967-976.
- 770 48. **Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, Magoon D, Qi J,**
771 **Blatt K, Wunderlich M, Taylor MJ, Johns C, Chicas A, Mulloy JC, Kogan SC,**
772 **Brown P, Valent P, Bradner JE, Lowe SW, Vakoc CR.** 2011. RNAi screen identifies
773 Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* **478**:524-528.
- 774 49. **Leroy G, Chepelev I, Dimaggio PA, Blanco MA, Zee BM, Zhao K, Garcia BA.**
775 2012. Proteogenomic characterization and mapping of nucleosomes decoded by Brd and
776 HP1 proteins. *Genome Biology* **13**:R68.
- 777 50. **Jang MK, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K.** 2005. The
778 bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates
779 RNA polymerase II-dependent transcription. *Molecular Cell* **19**:523-534.
- 780 51. **Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, Zhou Q.** 2005. Recruitment of
781 P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4.
782 *Molecular Cell* **19**:535-545.
- 783 52. **Liu Y, Wang X, Zhang J, Huang H, Ding B, Wu J, Shi Y.** 2008. Structural basis and
784 binding properties of the second bromodomain of Brd4 with acetylated histone tails.
785 *Biochemistry* **47**:6403-6417.
- 786 53. **Dey A, Chitsaz F, Abbasi A, Misteli T, Ozato K.** 2003. The double bromodomain
787 protein Brd4 binds to acetylated chromatin during interphase and mitosis. *Proc Natl*
788 *Acad Sci U S A* **100**:8758-8763.

- 789 54. **Erkek S, Hisano M, Liang CY, Gill M, Murr R, Dieker J, Schubeler D, van der**
790 **Vlag J, Stadler MB, Peters AH.** 2013. Molecular determinants of nucleosome retention
791 at CpG-rich sequences in mouse spermatozoa. *Nat Struct Mol Biol* **20**:868-875.
- 792 55. **You J, Croyle JL, Nishimura A, Ozato K, Howley PM.** 2004. Interaction of the
793 bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic
794 chromosomes. *Cell* **117**:349-360.
- 795 56. **Baxter MK, McPhillips MG, Ozato K, McBride AA.** 2005. The mitotic chromosome
796 binding activity of the papillomavirus E2 protein correlates with interaction with the
797 cellular chromosomal protein, Brd4. *Journal of Virology* **79**:4806-4818.
- 798 57. **Floyd SR, Pacold ME, Huang Q, Clarke SM, Lam FC, Cannell IG, Bryson BD,**
799 **Rameseder J, Lee MJ, Blake EJ, Fydrych A, Ho R, Greenberger BA, Chen GC,**
800 **Maffa A, Del Rosario AM, Root DE, Carpenter AE, Hahn WC, Sabatini DM, Chen**
801 **CC, White FM, Bradner JE, Yaffe MB.** 2013. The bromodomain protein Brd4
802 insulates chromatin from DNA damage signalling. *Nature* **498**:246-250.
- 803 58. **Wang R, Li Q, Helfer CM, Jiao J, You J.** 2012. Bromodomain Protein Brd4
804 Associated with Acetylated Chromatin Is Important for Maintenance of Higher-order
805 Chromatin Structure. *Journal of Biological Chemistry* **287**:10738-10752.
- 806 59. **Martianov I, Brancorsini S, Catena R, Gansmuller A, Kotaja N, Parvinen M,**
807 **Sassone-Corsi P, Davidson I.** 2005. Polar nuclear localization of H1T2, a histone H1
808 variant, required for spermatid elongation and DNA condensation during
809 spermiogenesis. *Proc Natl Acad Sci U S A* **102**:2808-2813.
- 810 60. **Lamonica JM, Deng W, Kadauke S, Campbell AE, Gamsjaeger R, Wang H, Cheng**
811 **Y, Billin AN, Hardison RC, Mackay JP, Blobel GA.** 2011. Bromodomain protein

812 Brd3 associates with acetylated GATA1 to promote its chromatin occupancy at erythroid
813 target genes. Proc Natl Acad Sci U S A **108**:E159-168.

814 61. **Shah PP, Donahue G, Otte GL, Capell BC, Nelson DM, Cao K, Aggarwala V,**
815 **Cruikshanks HA, Rai TS, McBryan T, Gregory BD, Adams PD, Berger SL.** 2013.

816 Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression
817 and the chromatin landscape. Genes & Development **27**:1787-1799.

818 62. **Huang da W, Sherman BT, Lempicki RA.** 2009. Systematic and integrative analysis
819 of large gene lists using DAVID bioinformatics resources. Nat Protoc **4**:44-57.

820

821 TABLES

822 Table 1: ChIP-seq data alignment information

823 Total aligned reads compared to uniquely-mapped reads for each ChIP-seq sample

ChIP	Total Reads	Aligned Reads	Unique Reads	% Aligned	% Unique	% Genome Coverage
Input	54990464	35623672	32940684	64.78	92.47	90.64
H3	71828757	37798741	30151960	52.62	79.77	82.96
H3K9me3	84339867	39799707	27591619	47.19	69.33	75.92
H3K9ac	67856707	48595679	34042406	71.62	70.05	93.67
H4K5ac	66596815	46239262	34065138	69.43	73.67	93.73
H4K8ac	79795471	48838021	29541448	61.20	60.49	81.28
H4K12ac	71252246	46341541	33485169	65.04	72.26	92.14
H4K16ac	77287145	49326598	28973938	63.82	58.74	79.72
H4ac	70049830	48662721	32799658	69.47	67.40	90.25
BRD4	84730635	47671985	24864246	56.26	52.16	68.41

824

825 Table 2: Calculation of significant differences for Figures 6B and C

Figure 6B

Comparison of BRD4-, BRDT-, or co-bound gene expression

Test	W Statistic	p-value
BRD4 vs. All Genes	W = 2683292	p < 2.2e-16
BRDT vs. All Genes	W = 1729944	p < 2.2e-16

Co-bound vs. All Genes	W = 378363	p < 2.2e-16
Co-bound vs. BRD4	W = 181821	p = 2.6e-08
Co-bound vs. BRDT	W = 127587	p = 6.8e-11

Figure 6C

Comparison of BRD4-, BRDT-, or co-bound gene promoter acetylation level

Test	Histone PTM	W Statistic	p-value
BRD4 vs. BRDT	H3K9ac	W = 1403278	p < 2.2e-16
BRD4 vs. BRDT	H4K5ac	W = 1484792	p < 2.2e-16
BRD4 vs. BRDT	H4K8ac	W = 1466692	p < 2.2e-16
BRD4 vs. BRDT	H4K12ac	W = 1468998	p < 2.2e-16
BRD4 vs. BRDT	H4K16ac	W = 1444708	p < 2.2e-16
BRD4 vs. BRDT	H4Kac	W = 1479768	p < 2.2e-16
Co-bound vs. BRD4	H3K9ac	W = 276948.5	p = 7.411e-16
Co-bound vs. BRDT	H3K9ac	W = 158058	p < 2.2e-16
Co-bound vs. BRD4	H4K5ac	W = 273017.5	p = 2.435e-14
Co-bound vs. BRDT	H4K5ac	W = 162835.5	p < 2.2e-16
Co-bound vs. BRD4	H4K8ac	W = 277942.5	p = 2.972e-16
Co-bound vs. BRDT	H4K8ac	W = 162476.5	p < 2.2e-16
Co-bound vs. BRD4	H4K12ac	W = 272689	p = 3.232e-14
Co-bound vs. BRDT	H4K12ac	W = 161513.5	p < 2.2e-16
Co-bound vs. BRD4	H4K16ac	W = 271330.5	p = 1.027e-13
Co-bound vs. BRDT	H4K16ac	W = 158641.5	p < 2.2e-16
Co-bound vs. BRD4	H4Kac	W = 277562	p = 4.223e-16
Co-bound vs. BRDT	H4Kac	W = 162543	p < 2.2e-16

826

827 **Table 3: Mass spectrometry analysis of BRD4-immunoprecipitated histones from round**
 828 **spermatids**

829 Quantification of the degree of acetylation or methylation of histone H3 (amino acids 9 to 17,
 830 KSTGGKAPR) or H4 peptide (amino acids 4 to 17, GKGGKGLGKGGAKR). Shown are
 831 average ratios of the percentage of peptide of H3 or H4 in BRD4-immunoprecipitated chromatin
 832 to total chromatin. Standard deviation is calculated for two and four biological replicates for H3
 833 and H4, respectively.

Peptide	AVG IP/Total	St Dev (±)
H3 9-17		
Unmodified	1.02	0.18

H3K9me1	0.65	0.28
H3K9me2	1.26	0.73
H3K9me3	4.24	1.51
H3K9ac	1.11	1.58
H3K14ac	1.09	0.08
H3K9me1K14ac	0.09	0.13
H3K9me2K14ac	1.35	1.09
H3K9me3K14ac	3.04	2.97
H3K9,14ac	0.00	0.00
H4 4-17		
Unmodified	1.14	0.20
H4Kac1	0.61	0.12
H4Kac2	0.98	0.08
H4Kac3	3.72	1.04
H4Kac4	10.07	5.17
Unmodified	1.17	0.21
H4K5ac	3.31	2.85
H4K8ac	2.67	4.89
H4K12ac	0.48	0.51
H4K16ac	0.87	0.42
H4K5,8ac	2.54	0.96
H4K5,12ac	1.36	0.87
H4K5,16ac	0.99	0.33
H4K8,12ac	0.83	0.35
H4K8,16ac	0.85	0.24
H4K12,16ac	0.80	0.13
H4K5,8,12ac	3.73	2.69
H4K5,8,16ac	4.18	1.51
H4K5,12,16ac	3.30	2.68
H4K8,12,16ac	2.82	1.54
H4K5,8,12,16ac	10.93	6.10

834

835 **Table 4: Mass spectrometry analysis of BRD4-immunoprecipitated H4 during**836 **spermatogenesis**

837 Quantification of the degree of acetylation (% total peptide) of histone H4 peptide (amino acids

838 4 to 17, GKGGKGLGKGGAKR) in total and BRD4-immunoprecipitated chromatin from

839 spermatocytes (Sc), round spermatids (RSp) and elongating/condensing spermatids (E/CSp).

840 Standard deviation is calculated for two biological replicates.

	Avg Total	St Dev Total	Avg BRD4 IP	St Dev BRD4 IP
Sc				
Unmodified	48.75%	0.89%	53.01%	18.06%
1ac	39.41%	1.37%	27.42%	2.80%
2ac	10.45%	2.52%	9.88%	8.31%
3ac	0.94%	0.15%	3.60%	2.42%
4ac	0.45%	0.10%	6.09%	4.52%
RSp				
Unmodified	30.39%	0.46%	37.40%	4.57%
1ac	57.27%	1.28%	40.78%	2.51%
2ac	8.67%	1.13%	7.70%	0.49%
3ac	2.26%	0.01%	4.27%	1.52%
4ac	1.42%	0.32%	9.85%	3.08%
E/CSp				
Unmodified	35.91%	0.72%	42.74%	7.08%
1ac	34.90%	3.29%	35.69%	3.77%
2ac	12.51%	0.63%	11.43%	1.50%
3ac	8.55%	0.76%	5.78%	1.22%
4ac	8.12%	1.18%	4.35%	0.59%

841

842

843 **FIGURE LEGENDS**

844 **Figure 1: BRD4 is expressed in meiotic cells and spermatids, but not in mature sperm**

845 (A) Schematic of the progression of spermatogenesis beginning with meiotic cells

846 (spermatocytes) and progressing through spermiogenesis from round to elongating to

847 condensing spermatids. Changes in cell (tan) and nucleus (blue) size and shape are

848 shown for reference in figures to follow.

849 (B) Western blot analysis of whole cell extracts from spermatocytes (Sc), round spermatids

850 (RSp), elongating/condensing spermatids (E/CSp), a mixture of round, elongating, and

851 condensing spermatids (R/E/CSp) obtained with STA-PUT velocity sedimentation, and

852 mature sperm (Sperm). Asterisks mark the full length BRD4 isoform and the arrow
853 indicates a novel shorter BRD4 peptide in spermatids. Peptide competition of BRD4
854 antibody shows specificity of the BRD4 antibody.

855

856 **Figure 2: BRD4 forms a ring around the nucleus of spermatids as histones become hyper-**
857 **acetylated**

858 (A) Indirect immunofluorescence of cryosectioned mouse testes tissue shows that BRD4
859 (green) forms a ring around the nucleus (DAPI-stained DNA shown in blue) of early
860 (top panel) to late (bottom panel) elongating spermatids. The ring is absent in all non-
861 spermatid cell types such as spermatocytes (Sc).

862 (B) Indirect immunofluorescence of cryosectioned mouse testes tissue shows that the BRD4
863 ring (green) forms at the onset of histone H4 hyper-acetylation (red) in the nucleus
864 (DAPI in blue) of early (top panel) to late (bottom panel) elongating spermatids.
865 Stage of spermatogenesis shown in upper left hand corner of each panel. Separation of
866 spermatocytes (Sc) and spermatids (Sp) within the seminiferous tubule is indicated with a
867 grey dotted line. Inset shows 3x magnification of the spermatids outlined with a dotted
868 square.

869 (C) and (D) Indirect immunofluorescence of a mixed population of spermatogenic cells
870 shows that BRD2 and BRDT (red) are diffusely localized in the nuclei (DAPI in blue) of
871 spermatocytes (Sc) and round spermatids (RSp), but not condensing spermatids (CSp).

872 Scale bar represents 10 μ m.

873

874 **Figure 3: BRD4 forms a ring within the nuclear envelope at the base of the acrosome**

- 875 (A) Indirect immunofluorescence of BRD4 (green), Lamin B1 (red), and DAPI-stained DNA
876 (blue) in a round spermatid. Asterisk indicates location of the acrosome.
- 877 (B) Indirect immunofluorescence of Lamin B1 (green), the acrosome (detected with PNA in
878 red), and DAPI-stained DNA (blue) in a round spermatid.
- 879 (C) Indirect immunofluorescence of BRD4 (green), acrosome (detected with PNA in red), and
880 DAPI-stained DNA (blue) in a round spermatid (top), early elongating spermatid (middle),
881 and condensing spermatid (bottom).
- 882 (D) Indirect immunofluorescence of fluorophore-conjugated streptavidin (red) with (top) or
883 without (bottom) JQ1-biotin and DAPI-stained DNA (blue) in a mixed population of
884 spermatogenic cells.
- 885 (E) Indirect immunofluorescence of BRD4 (green), JQ1-biotin (red), and DAPI-stained DNA
886 (blue) in a late round (top panel) and elongating (bottom panel) spermatid.
- 887 (F) Indirect immunofluorescence of the acrosome (detected with PNA in green), JQ1-biotin
888 (red), and DAPI-stained DNA (blue) in a condensing spermatid.
- 889 Scale bar represents 5 μ m (A-C,E,F) and 10 μ m (D).

890

891 **Figure 4: BRD4 does not form a ring in *Hrb*^{-/-} acrosomal mutant mice**

- 892 (A) Indirect immunofluorescence of the acrosome (detected with PNA in green) and DNA
893 (DAPI in blue) in testes tissue sections from wild-type and *Hrb*^{-/-} mice. The stage of
894 spermatogenesis is shown in the upper left hand corner of each panel.
- 895 (B) Indirect immunofluorescence of BRD4 (green) and DNA (DAPI in blue) in elongating
896 spermatids of cryosectioned testes tissue from *Hrb*^{+/-} (left) and *Hrb*^{-/-} (right) mice.

897 Inset shows 3x magnification of the spermatids outlined with a dotted square. Scale bar
898 represents 10 μ m.

899

900 **Figure 5: BRD4 is enriched at the promoters of active genes in round spermatids**

901 (A) ChIP sequencing of BRD4 and various histone PTMs reveals that BRD4 and histone H3
902 and H4 acetylation are associated with gene-rich regions of the genome while H3K9me3 is
903 enriched in intergenic, gene poor regions. UCSC-defined genes are shown at the bottom in
904 dark blue. Genomic location is indicated at the top. The y axis is ChIP enrichment
905 (normalized to input). The x axis is DNA sequence.

906 (B) Percentage of defined ChIP-seq peaks of BRD4, BRDT, and various histone PTMs in
907 promoter (1kb upstream of the TSS), gene (intron versus exon), or intergenic regions in
908 round spermatids.

909 (C) ChIP sequencing of BRD4 and various histone PTMs at housekeeping (*Actb*) and
910 spermatogenesis-specific (*Tnp1*) transcriptionally active genes and an inactive gene, *Myc*.
911 BRD4 is not present at all gene promoters that are enriched for H3/H4 acetylation (*Vps45*).
912 UCSC-defined genes are shown at the bottom in dark blue. The y axis is ChIP enrichment
913 (normalized to input). The x axis is DNA sequence.

914

915 **Figure 6: BRD4 and BRDT bind to different subsets of transcriptionally active genes in**
916 **round spermatids**

917 (A) Heat map representation of ChIP-sequencing enrichment at promoters of all genes (green)
918 compared to the level of transcription of these genes (red) in round spermatids. Brightness
919 indicates higher levels of enrichment or transcription.

- 920 (B) Box and whisker plot of transcription levels of genes that are bound by BRD4, BRDT, or
921 both. Overall transcription levels are shown for spermatogenesis-specific genes (“Sperm.”)
922 and all genes, as a reference.
- 923 (C) Box and whisker plot of enrichment levels of different histone PTMs at the gene promoters
924 (1kb upstream of the TSS) bound by BRD4, BRDT, or both (presence or absence of
925 binding indicated with “+” or “-“, respectively, at bottom).
- 926 (D) Pie chart representations of GO Terms of genes enriched for BRD4 (left), BRDT (middle),
927 or both (right) in round spermatids.
- 928 (E) ChIP sequencing of BRD4, BRDT, and various histone PTMs at promoters of genes
929 present in the top-most represented GO Term category from the corresponding pie chart in
930 (D) above. UCSC-defined genes are shown at the bottom in blue. The y axis is ChIP
931 enrichment (normalized to input). The x axis is DNA sequence.
- 932 (F) Heat map representation of BRD4 and BRDT enrichment around the TSS (± 2.5 kb) of
933 spermatogenesis-specific genes in round spermatids. Brightness indicates higher levels of
934 enrichment.

935

936 **Figure 7: BRD4 association with poly-acetylated histone H4 diminishes in late spermatids**
937 **as acetylated histones are removed from the condensing nucleus**

- 938 (A) Mass spectrometry analysis of BRD4-immunoprecipitated histones from round spermatids.
939 Heatmap representation of enrichment of acetylation or methylation of histone H3 (amino
940 acids 9 to 17, KSTGGKAPR) or H4 peptide (amino acids 4 to 17,
941 GKGGKGLGKGGAKR) in BRD4-immunoprecipitated chromatin normalized to total

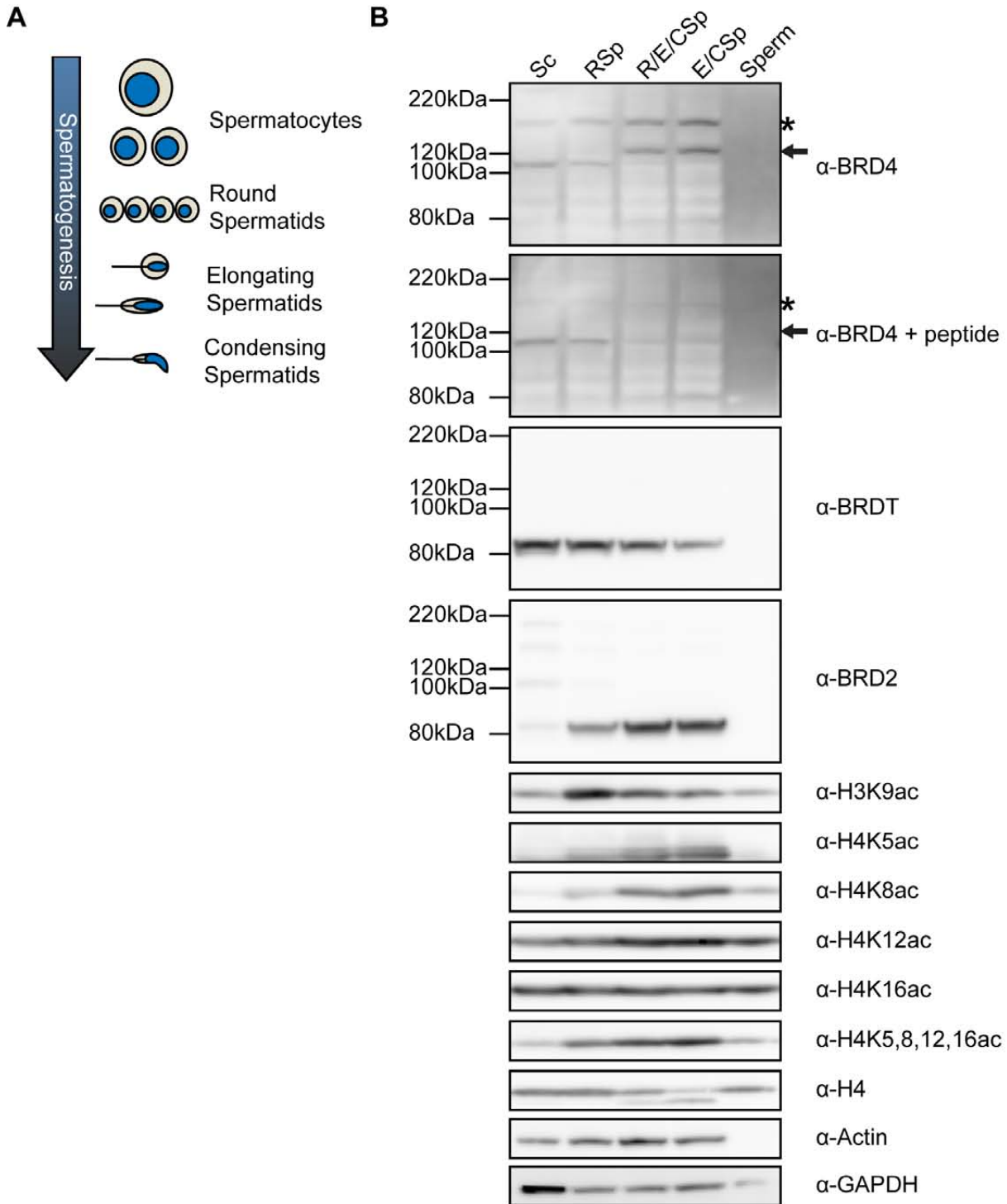
942 chromatin (log transformed). Raw values used to generate the heatmaps are found in Table
943 3.

944 (B) Comparison of degree of H4 peptide acetylation (amino acids 4 to 17,
945 GKGGKGLGKGGAKR) in total and BRD4-immunoprecipitated (IP) chromatin over the
946 course of spermatogenesis. Black and grey bars indicate changes in H4 acetylation from
947 spermatocytes (Sc) to round spermatids (RSp) and from round spermatids to
948 elongating/condensing spermatids (ECSp), respectively, via log-transformed ratio of
949 percentage acetylated H4 peptide. Raw values used to generate the graphs are found in
950 Table 4.

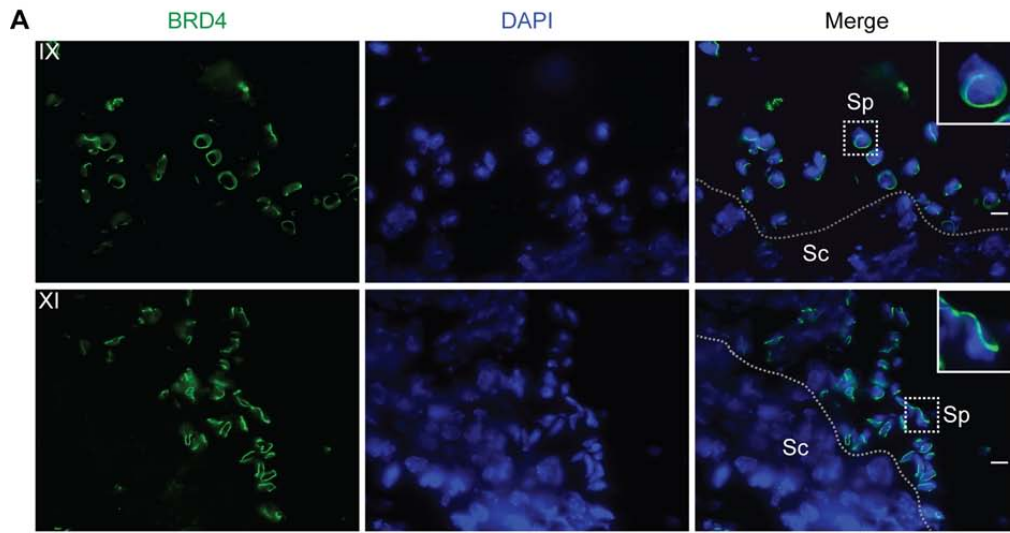
951 (C) Indirect immunofluorescence of BRD4 (green), H4K5,8,12,16ac (red), and DAPI-stained
952 DNA (blue) in a condensing spermatid. Scale bar represents 5 μ m.

953 (D) Model showing the locations of different cellular features associated with the spermatid
954 nucleus. BRD4 (green) forms a ring structure between the nucleus (blue) and the
955 acroplaxome (red) in a key region of the nuclear membrane where the Lamin B1- and
956 nuclear pore-enriched posterior portion meets the acrosome-associated, nuclear pore-
957 depleted anterior portion.

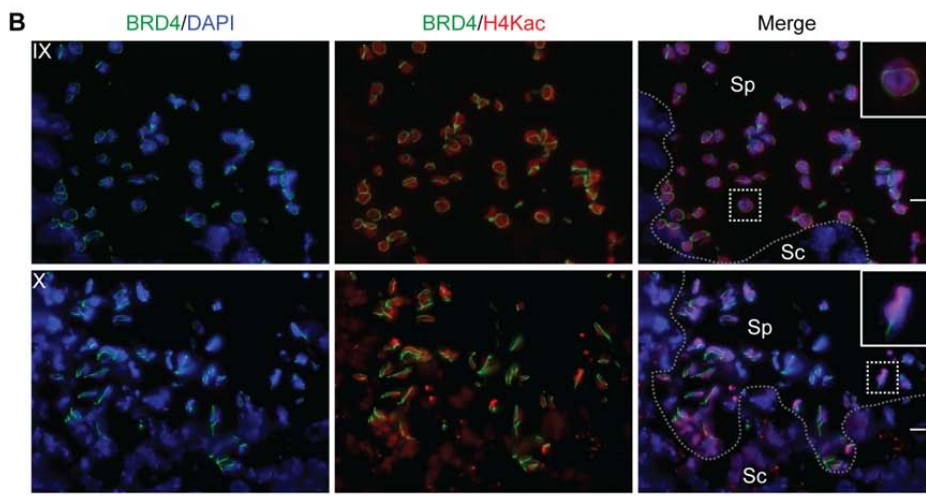
Bryant_Fig1



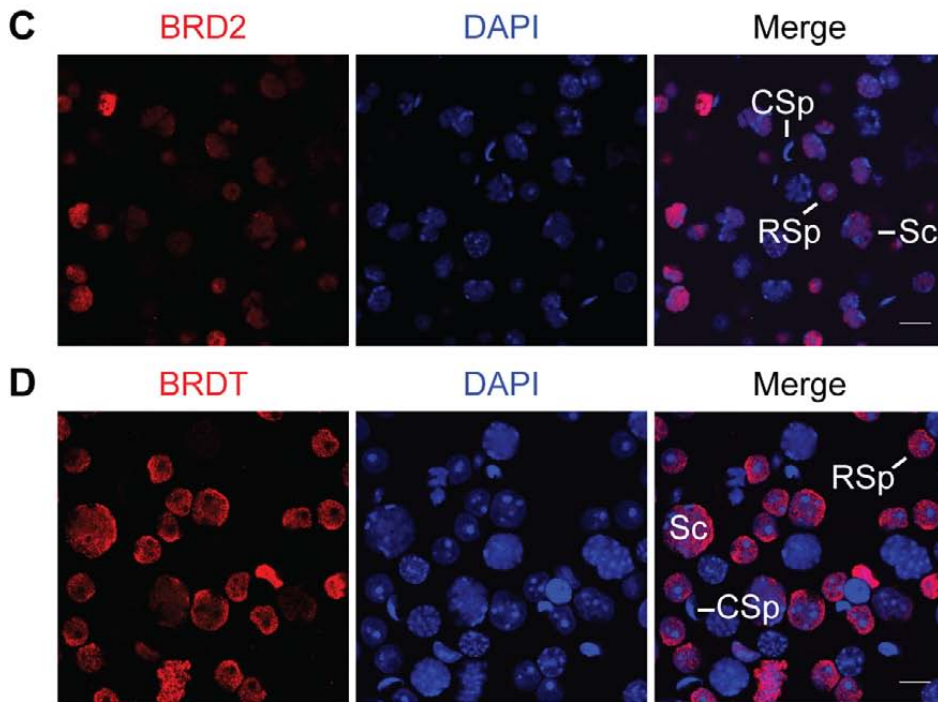
Bryant_Fig2



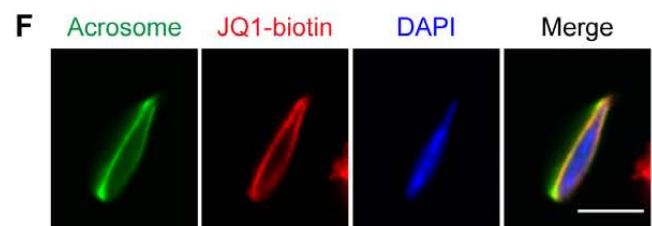
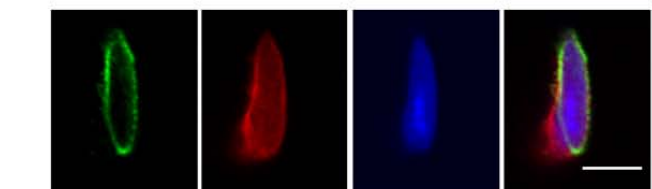
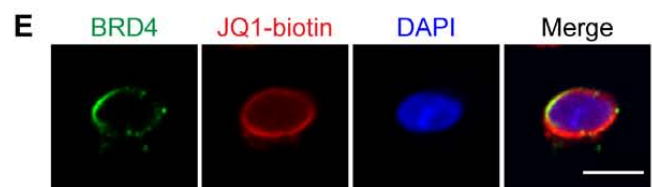
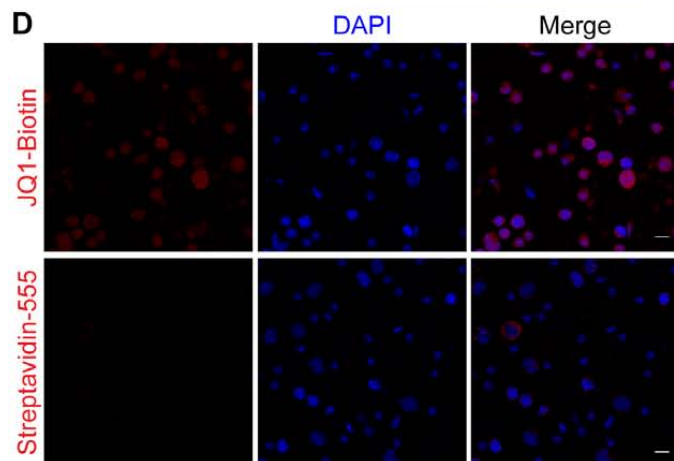
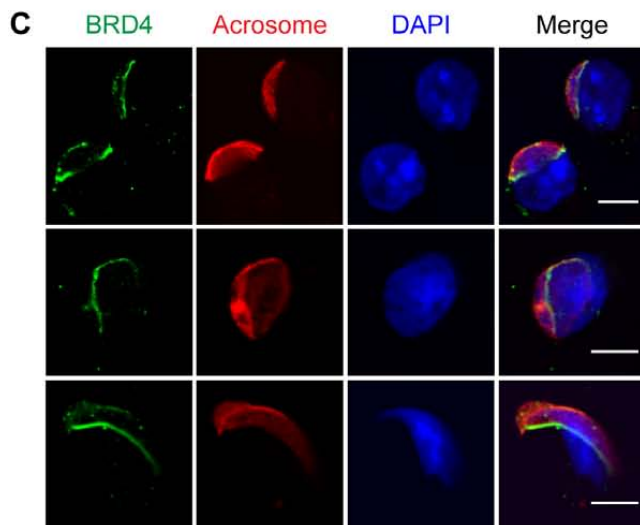
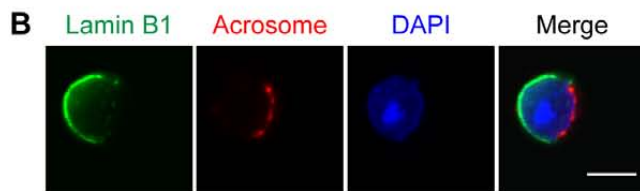
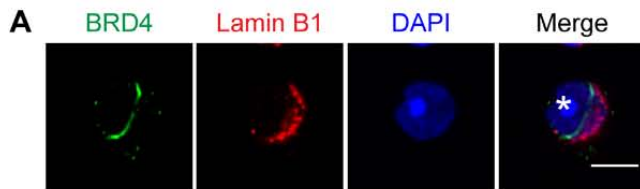
Bryant_Fig2



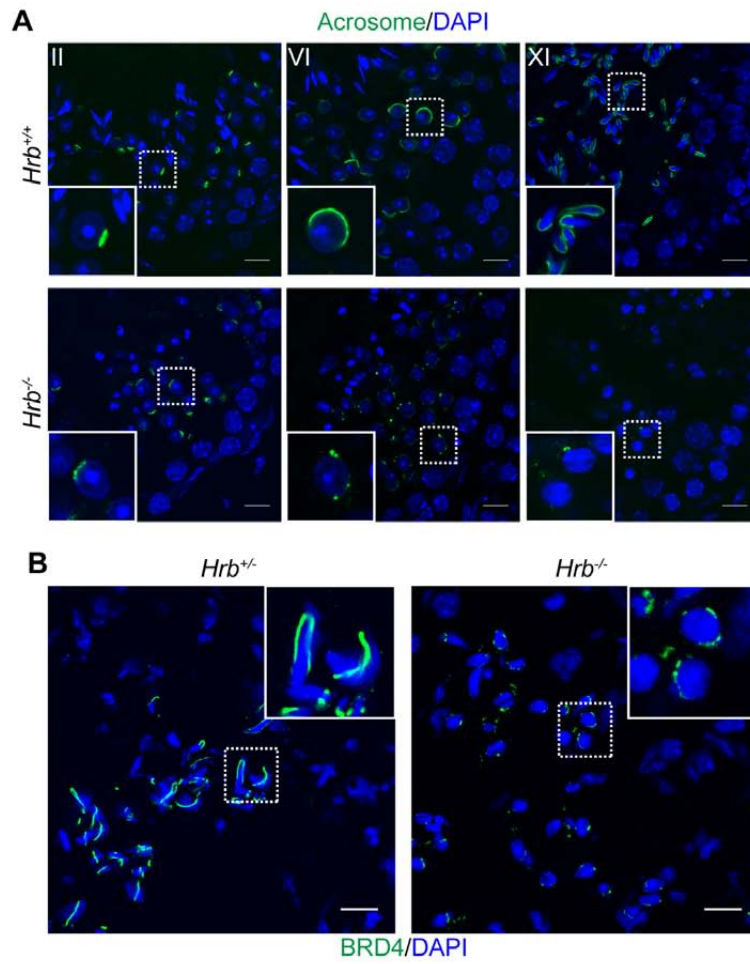
Bryant_Fig2



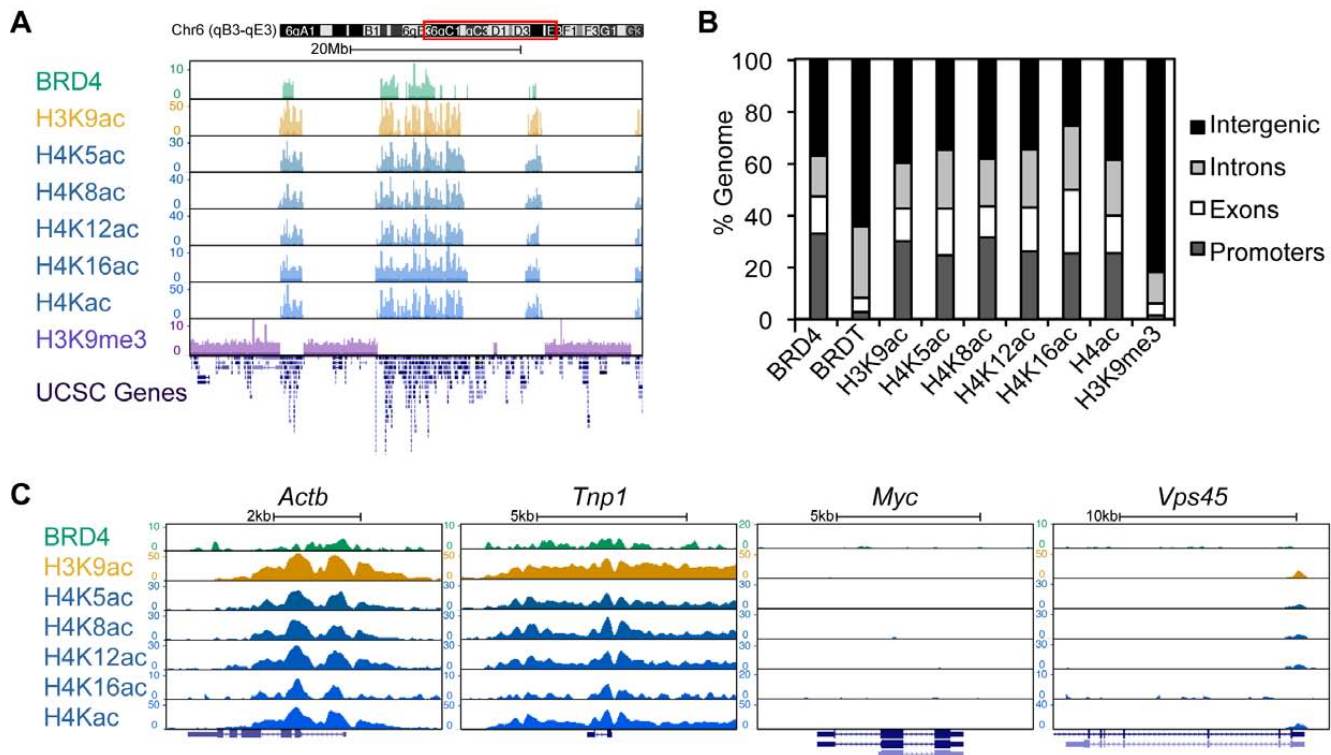
Bryant_Fig3



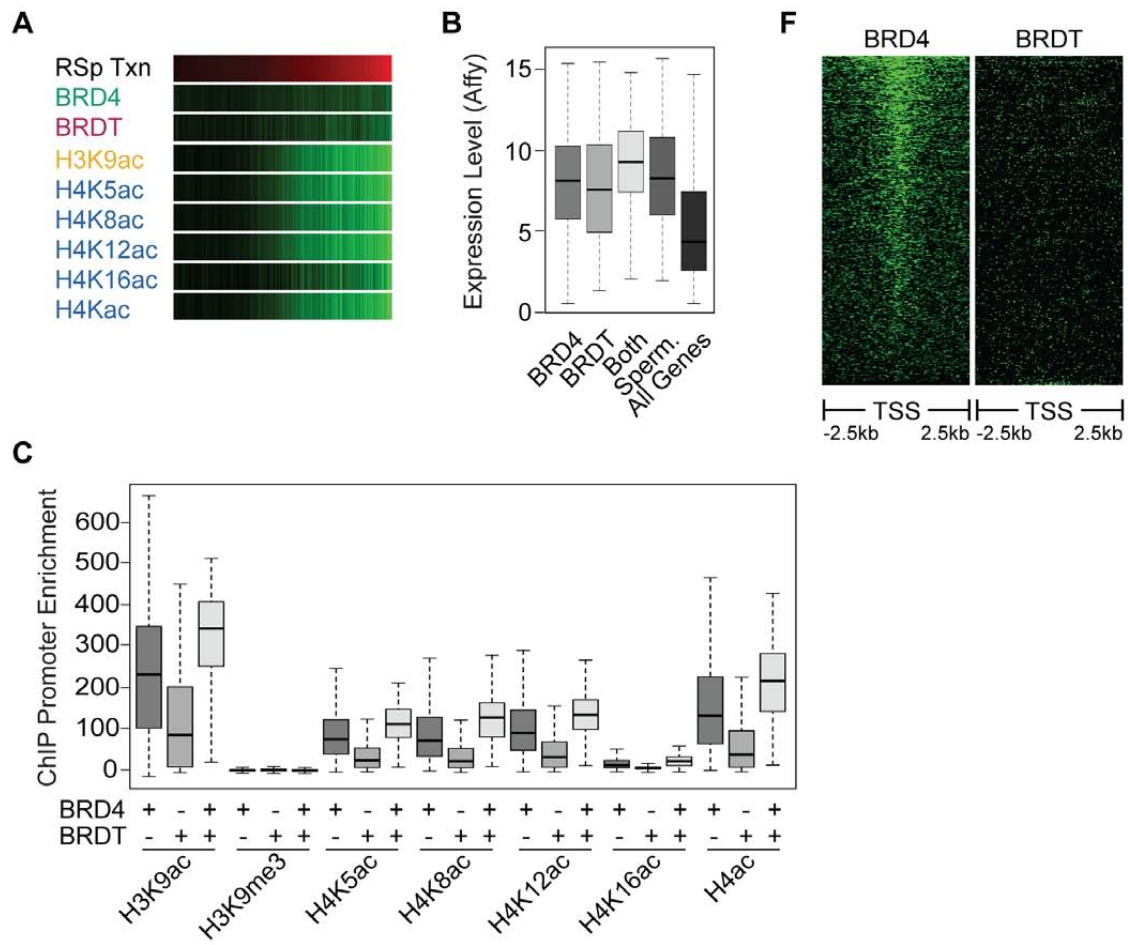
Bryant_Fig4



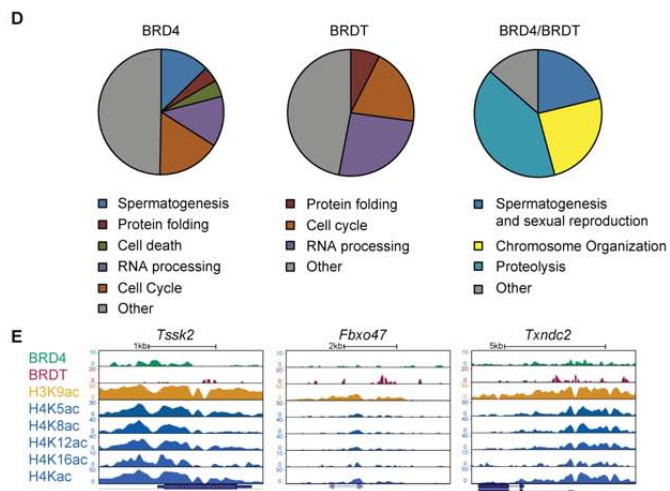
Bryant_Fig5

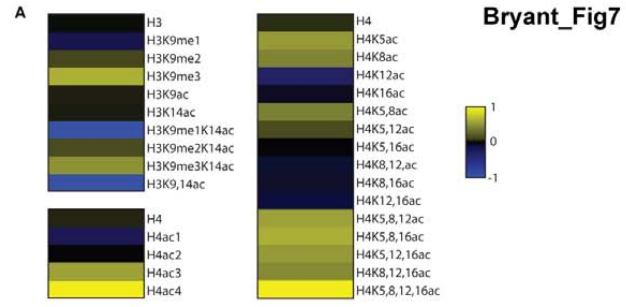


Bryant_Fig6



Bryant_Fig6





Bryant_Fig7

