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The BET Family Member BRD4 Interacts with OCT4 and Regulates Pluripotency Gene Expression

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SUMMARY

Embryonic stem cell (ESC) pluripotency is controlled by defined transcription factors. During cellular differentiation, ESCs undergo a global epigenetic reprogramming. Female ESCs exemplify this process as one of the two X-chromosomes is globally silenced during X chromosome inactivation (XCI) to balance the X-linked gene disparity with XY males. The pluripotent factor OCT4 regulates XCI by triggering X chromosome pairing and counting. OCT4 directly binds *Xite* and *Tsix,* which encode two long noncoding RNAs (lncRNAs) that suppress the silencer lncRNA, *Xist*. To control its activity as a master regulator in pluripotency and XCI, OCT4 must have chromatin protein partners. Here we show that BRD4, a member of the BET protein subfamily, interacts with OCT4. BRD4 occupies the regulatory regions of pluripotent genes and the lncRNAs of XCI. BET inhibition or depletion of BRD4 reduces the expression of many pluripotent genes and shifts cellular fate showing that BRD4 is pivotal for transcription in ESCs.

INTRODUCTION

Master transcription regulators control the pluripotent gene expression in embryonic stem cells (ESCs) (Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998). X chromosome inactivation (XCI) is a crucial epigenetic process that silences one of the two female X chromosomes to ensure equal X-linked gene expression with XY males (Payer and Lee, 2008; Lee and Bartolomei, 2013). XCI is tightly linked with pluripotency, as this epigenetic silencing occurs upon cellular differentiation and conversion of female somatic cells to the induced stem-ness state is accompanied by a global epigenetic reprogramming and reactivation of the silenced X (Maherali et al., 2007; Navarro et al., 2008).

The transcription factor OCT4 lies at the top of the XCI hierarchy regulating the pluripotent-associated long noncoding RNAs (lncRNAs): *Xite* (the enhancer for *Tsix*) and *Tsix (*the anti-sense repressor of *Xist)* (Donohoe et al., 2009). Together *Xite* and *Tsix* mediate X-X homologous pairing and inhibit the silencer *Xist* prior to X chromosome choice (Xu et al., 2006). In addition to their roles in the study of pluripotency and cellular differentiation, mouse ESCs are established as ex vivo models of XCI, faithfully recapitulating XCI in the embryo (Clerc and Avner, 1998; Lee and Jaenisch, 1997; Lee and Lu, 1999; Penny et al., 1996; Rastan and Robertson, 1985). In undifferentiated ESCs, the single male X and both female X chromosomes are active. The lncRNAs *Xite*, *Tsix*, and *Xist* are all expressed on these active X chromosomes in the pluripotent state. ESCs can be differentiated by suspension culture for 4 days without leukemia inhibitory factor (LIF) and maintained thereafter under adherent conditions (Martin and Evans, 1975). Following differentiation, the male X chromosome loses expression of these lncRNAs to retain activity of the single X, whereas the female ESCs have a choice of active versus inactive X. On the future active X, *Xite* and *Tsix* expression persists to keep *Xist* levels low. In contrast, on the future inactive X, *Xite* and *Tsix* are extinguished, and *Xist* levels are greatly upregulated. OCT4 partners with the chromatin insulator CTCF, specifying the early decisions of XCI (counting, X-X pairing, and choice) (Xu et al., 2006, 2007; Donohoe et al., 2009).

During differentiation, ESC chromatin shifts from a transcriptionally permission, euchromatic, to a more heterochromatic state (Azuara et al., 2006; Meshorer and Misteli, 2006; Niwa, 2007). These changes in chromatin packaging are accompanied by alterations in histone post-translational modifications (PTMs) crucial for modulation of chromatin structure and gene expression (Bernstein et al., 2006). Histone PTM writers such as the Polycomb group proteins (Boyer et al., 2006) and erasers such as the demethylases (Adamo et al., 2011; Loh et al., 2007; Mansour et al., 2012; Wang et al., 2011) play important roles in early development. We postulate that histone readers together with OCT4 play a role in the transcriptional control of the XCI lncRNAs as well as pluripotent genes. One candidate is the chromatin reader, BRD4.

BRD4 is a member of the BET (bromodomain and extra-terminal domain) family of tandem bromodomain-containing proteins that can bind acetylated histones H3 and H4 and influence transcription (Chiang, 2009). BRD4 is an epigenetic reader originally identified as a mitotic chromosome-binding protein that remains associated with

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acetylated chromatin throughout the entire cell cycle and is thought to provide epigenetic bookmarking after cell division (Dey et al., 2000, 2003). BRD4 has a direct role in transcription as it associates with positive transcription elongation factor b (P-TEFb) to enhance RNA polymerase II (RNAP II) and control productive mRNA synthesis (Yang et al., 2008). At many developmental genes RNAP II stalls or pauses after transcribing a nascent transcript about 20–65 nucleotides in length (Adelman and Lis, 2012). Nearly 30% of the genes in human ESCs commence transcription initiation but do not undergo transcriptional elongation (Guenther et al., 2007). This suggests that transcriptional pausing is an additional checkpoint control during development (Levine, 2011). The release from transcriptional pausing is associated with P-TEFb recruitment, the eviction of pause factors, the phosphorylation at serine 2 of the carboxyl-terminal domain (CTD) in RNAP II, and the production of elongated mRNAs.

Although BRD4 is known to play crucial roles in the oncogenic and viral programs, very little is known about its function in early normal development. The loss of *Brd4* in the mouse results in peri-implantation lethality, with an ablation of the inner cell mass the source for ESCs (Houzelstein et al., 2002), suggesting a role for this gene in the cell differentiation-linked processes of XCI and pluripotency. Here we investigate BRD4's function in these crucial developmental processes. Our studies show that Brd4 interacts with the pluripotent factor OCT4 and is important for maintaining stem cell fate and the expression of the lncRNAs controlling XCI.

RESULTS

The Epigenetic Reader BRD4 Is Expressed during ESC Differentiation and Binds the Pluripotent Factor OCT4

We postulate that a co-activator such as BRD4 might play a role in epigenetic memory for binary cell fate ("stem-ness" versus differentiation) and XCI (active versus inactive X chromosome) status in ESCs. To explore this possibility, we examined the developmental expression pattern for the BRD4 protein in differentiating female and male ESCs. To differentiate the ESCs, we removed LIF and mouse embryonic feeders on nonadherent plates as previously described (Donohoe et al., 2007). Our results show that the BRD4 protein is expressed at similar levels during differentiation day 0 (d0) (pre-XCI), day 4 (d4) (time of the establishment of XCI), and day 8 (d8) (post-XCI) in both female and male ESCs (Figure 1A). In contrast, the OCT4 protein is present at d0 and d4 and is greatly reduced by d8 in these cells. Because the loss of mouse *Brd4* has a peri-implantation phenotype (at the time random XCI takes place in the epiblast) and given the tight linkage between XCI and differentiation, we questioned whether BRD4 might interact with pluripotent factors. Using a candidate approach, we tested BRD4 for partnering with "stem-ness"-associated transcription factors. Full-length Myc-tagged BRD4 and Flag-OCT4 were co-transfected into human embryonic kidney (HEK) cells and tested for their interaction. We observed a specific OCT4-BRD4 interaction following co-immunoprecipitation (co-IP) (Figure 1B).

Next, we queried whether an endogenous partnering of BRD4 and OCT4 occurs in undifferentiated male and female ESCs. BRD4 co-IPs with a specific OCT4 antibody in female ESCs confirming in vivo BRD4-OCT4 interaction (Figure 1C). Reciprocal co-IP confirms the endogenous OCT4-BRD4 complex (Figure 1D). Similar results were observed in male ESCs (Figures 1E and 1F). Although there are three BRD4 isoforms, the antibody used here recognizes an epitope present only in the longest isoform, suggesting that this is the isoform that partners with Oct4. Taken together, the chromatin reader BRD4 is expressed throughout cellular differentiation in both male and female ESCs and partners in vitro and in vivo with OCT4, a key regulator of pluripotency and XCI.

BRD4 Occupies Genes Controlling Pluripotency and the lncRNAs Regulating X-Chromosome Inactivation

We determined that BRD4 interacts with OCT4. To further investigate the genes that it targets, we examined BRD4 occupancy in undifferentiated male ESCs using chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq). First, we validated the BRD4 and OCT4 occupancy at *Oct4* and *Nanog* regulatory regions in d0 male ESCs using quantitative chromatin immunoprecipitation (qChIP) (Figure S1A). Using a stringent statistical criteria ($p < 0.05$) we identified significant enrichment of ChIP-seq peaks (Figure S1B). As shown in Figure 2A, BRD4 binds the gene body and regulatory regions of *Nanog*, *Pou5f1/Oct4*, *Sox2*, *c-Myc*, *Fgf4*, and the lncRNAs *Tsix* and *Xist*. An enhanced peak of BRD4 binding is situated over *Sox2*.

To investigate whether BRD4 is involved in XCI, we examined BRD4 occupancy in male and female day 4 of differentiation (d4) ESCs using qChIP. We used the OCT4-binding sites within the pluripotency-associated *Xite* and *Tsix* lncRNAs (*Xite* enhancer, *Tsix site D*, and *Xist intron 1B*) as guides for potential BRD4 in vivo binding in the XCI locus (Donohoe et al., 2009; Navarro et al., 2008, 2010), as well as several known OCT4-regulated promoters (*Nanog* and *Sox2*) (Chew et al., 2005; Rodda et al., 2005) and enhancers (*Oct4*) (Chew et al., 2005). In addition to BRD4, these loci were tested for OCT4 binding and the acetylated histone 4 (H4Ac), a PTM associated with gene activation and a mark that bromodomain-containing proteins can bind and read (Chiang, 2009). Among the BET family

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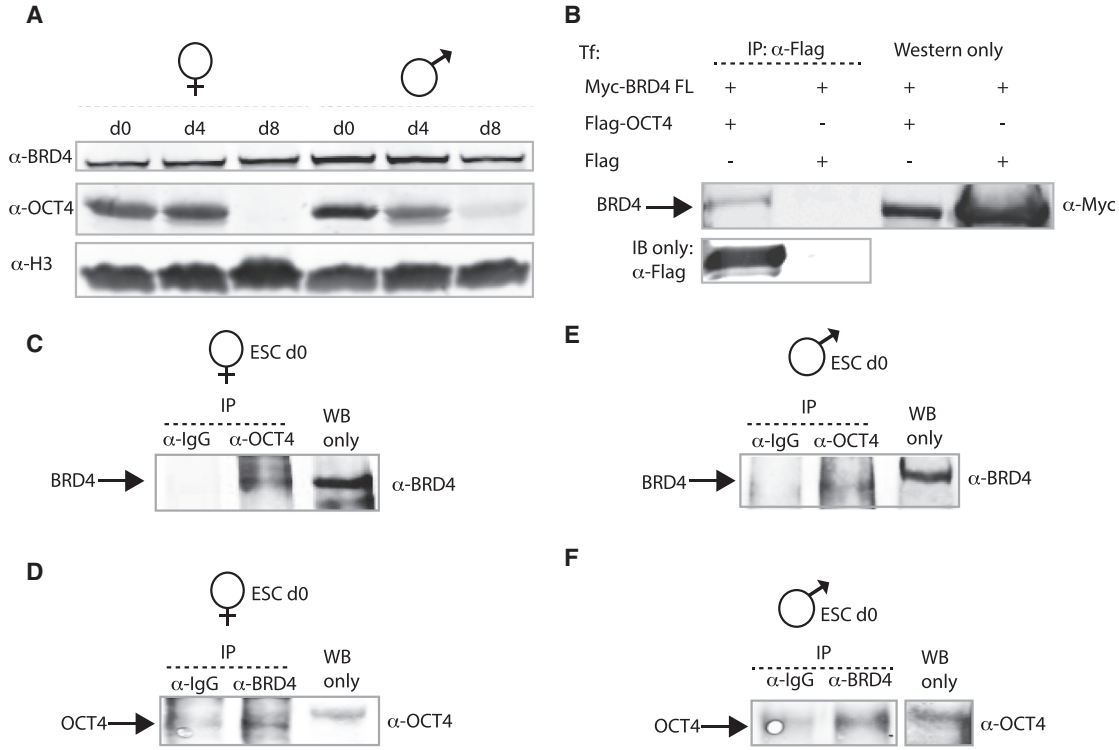

Figure 1. BRD4 Is Expressed in Differentiating Female and Male ESCs, Interacts with OCT4, and the Expression of Many Pluripotent Genes Rely on the BRD4 Protein Partner P-TEFb

(A) Western blot analysis of BRD4 (top) and OCT4 (middle) in WT female and male ESCs whole cell extracts (WCEs) on differentiation d0, d4, and d8. Histone 3 (H3) (bottom) is used as a load control for protein expression.

(B) HEK cells were co-transfected (Tf) with full-length Myc-tagged BRD4 and Flag-tagged OCT4. WCEs were immunoprecipitated (IP) with anti-Flag antibodies and western blot analysis with anti-Myc antibodies. The arrow denotes BRD4 binding OCT4 in the upper panel. The lower shows Flag-OCT4 expression detected by western blot.

(C) Co-IP of BRD4 and OCT4 in ESCs. Immunoprecipitation with anti-OCT4 or control antibodies to test interaction with endogenous BRD4 in d0 female ESCs. Arrow marks BRD4 detected by anti-BRD4 western.

(D) Reciprocal immunoprecipitation using anti-BRD4 or control antibodies to test interaction with OCT4 in female ESCs. Arrow marks OCT4 detected by anti-OCT4 western.

(E) Endogenous BRD4 co-immunoprecipitates with endogenous OCT4 in d0 male ESCs. Arrow marks BRD4 following western with anti-BRD4.

(F) Reciprocal immunoprecipitation using anti-BRD4 or control antibodies to test binding to OCT4 in male ESCs. Arrow marks OCT4 following anti-OCT4 western.

members, BRD4's bromodomains have the highest affinity for H4Ac (Jung et al., 2014).

Chromatin was prepared from d4 male ESCs and subjected to qChIP. BRD4 is enriched nearly 16-fold at *Tsix site D* chromatin (Figure 2B). *Tsix site D* resides in the repeat region *DxPas34*, the epigenetic switch/control region for XCI. Deletion of *DxPas34* on one of the two female Xs nearly always results in this X chromosome to be chosen for inactivation (Lee and Lu, 1999). *Xite* and *Xist intron 1B*, a known strong OCT4 binding domain chromatin shows enhanced BRD4 and OCT4 occupancy over control at d4 in male ESCs. In contrast the immunity gene *Toll-like receptor 9* (*TLR9*), a negative control for OCT4 occupancy (Mochizuki et al., 2008), is not enriched for either OCT4 or BRD4. The *Oct4* enhancer chromatin shows a 10- and 25-fold enrichment of BRD4 and OCT4 binding, respectively. At d4 H4Ac PTM shows high levels at all pluripotency-associated regulatory chromatin. We next examined BRD4, OCT4, and H4Ac in vivo occupancy in differentiating d4 female ESCs. Here we observe similar dynamic changes. At d4, the time of XCI establishment in female ESCs, we see BRD4 and OCT4 enrichment compared with background at *Xite*, *Xist intron 1B*, *Oct4*, *Nanog*, and *Sox2* (Figure 2D). The *TLR9* regulatory region is an exception, showing a lack of BRD4 or OCT4 occupancy. All of these chromatin sites tested show enrichment of H4Ac PTM. Collectively, our data confirm that BRD4 binding to the OCT4-associated regulatory regions in both male and

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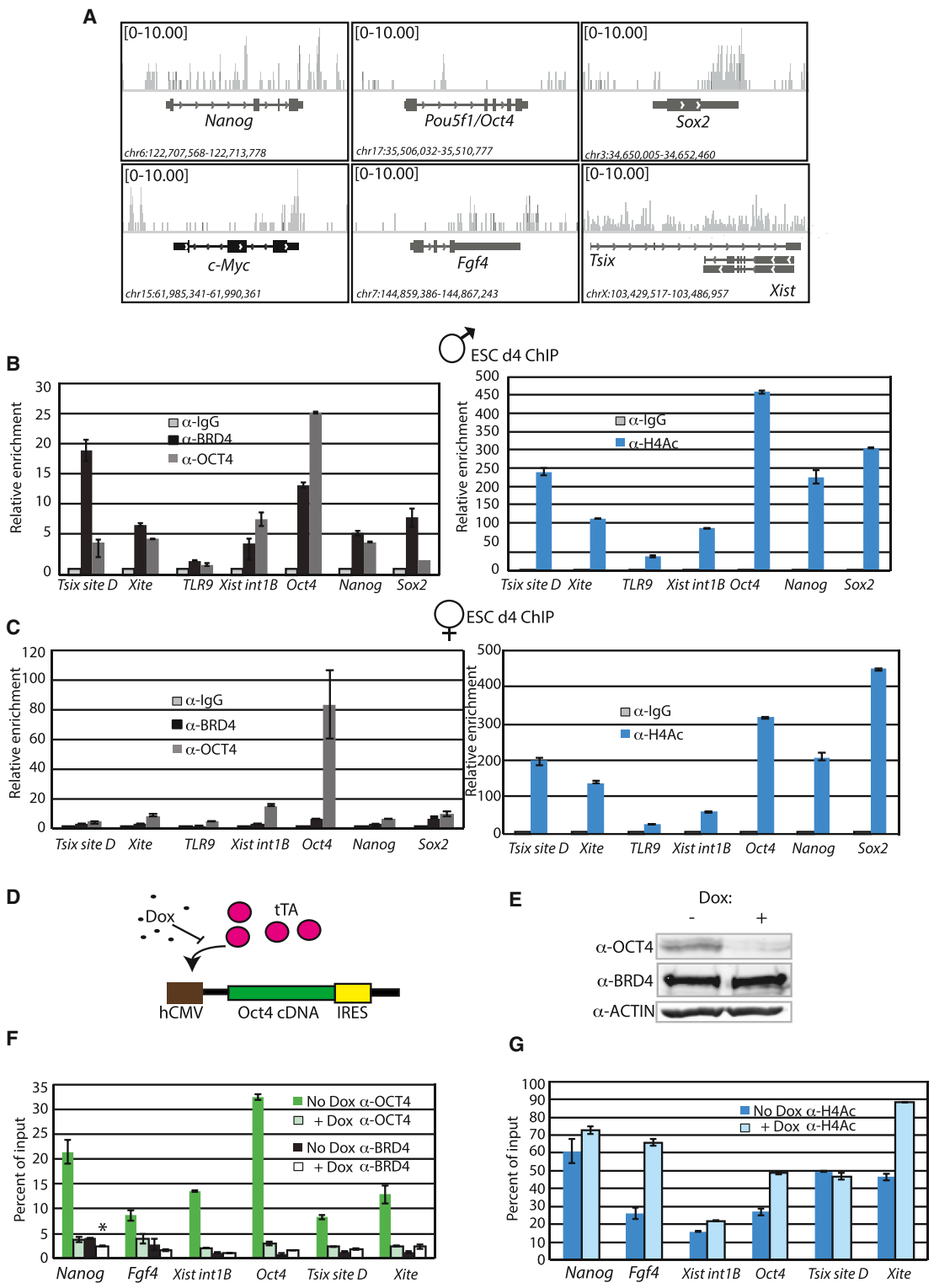

Figure 2. BRD4 Occupies Regulatory Regions within the Pluripotency Genes and the lncRNAs Involved in XCI

(A) ChIP-seq binding profiles for BRD4 at the *Nanog*, *Pou5f1/Oct4*, *c-Myc*, *Fgf4*, and *Tsix/Xist* loci in undifferentiated male ESCs. The y axis denotes peak reads of BRD4 binding.

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female ESCs. These results suggest that both BRD4 and OCT4 can occupy the same regulatory elements in XCI and pluripotency.

OCT4 Recruits BRD4 to Selective Regulatory Regions

Although BRD4 can directly recognize and bind acetylated histones, recent studies show that its interaction partners can stabilize its binding to chromatin (Chiang, 2009; Stewart et al., 2013). Therefore, we asked whether OCT4 could tether and enhance BRD4 binding to pluripotent regulatory regions facilitating active transcription. To address this, we utilized the OCT4-regulated ESC line, ZHBTc4 (Niwa et al., 2000), which depletes OCT4 protein expression upon addition of doxycycline (Dox) (Figure 2D). Following exposure to Dox, ZHBTc4 ESCs ablate OCT4 protein, whereas BRD4 and ACTIN levels do not change (Figure 2E). We performed qChIP for OCT4 and BRD4 on these OCT4-regulated sites in the absence and presence of Dox. As shown in Figure 2F, we observe a reduction of OCT4 occupancy on all regulatory regions following OCT4 protein depletion. After OCT4 removal, the *Nanog* promoter shows a statistically significant reduction (~50%) in BRD4 binding, as does the *Tcl1* promoter (data not shown). In contrast, the *Fgf4* enhancer, the *Xist intron 1B*, the *Oct4* enhancer, *Tsix* site D, and the *Xite* enhancer do not show a significant change in BRD4 occupancy. Ablation of OCT4 protein reveals preferential changes in H4Ac levels at the *Fgf4*, *Oct4*, and *Xite* enhancers (Figure 2G). Our qChIP results suggest that BRD4 binding is dynamic and that OCT4 can recruit or stabilize BRD4 to particular pluripotent regulatory regions.

Inhibition of the BET Domain Diminishes Pluripotent Gene Expression and Enhances the P-TEFb Inhibitor Complex Genes *Hexim1* and *Sesn3*

The BET domain proteins can be inhibited by the small molecule JQ1, which selectively binds the tandem bromodomains displacing BRD4 and P-TEFb from acetylated chromatin leading to a decrease in RNAP II elongation at active genes (Filippakopoulos et al., 2010). Because BRD4 can associate with the regulatory regions of the pluripotent genes, we hypothesize that BET inhibition alters the expression of pluripotent-associated genes. To test this, we first performed a dose curve using JQ1 and DMSO control in both male and female d6 ESCs. We found that JQ1 treatment of male ESCs can inhibit the c-MYC protein expression in a dose-dependent manner without the accompaniment of cell death as reported (Filippakopoulos et al., 2010) (Figure 3A). Intriguingly, the pluripotency *trans*-factor OCT4 is decreased as compared with BRD4 and ACTIN levels following BET inhibition in male ESCs (Figure 3A).

We queried BRD4 and P-TEFb occupancy by qChIP following JQ1 BET inhibition. BRD4 and CDK9 occupancy are lost at the *Oct4 enhancer*, *Sox2 promoter*, and the *Nanog promoter* as well as *Xite*, *Tsix Site D*, *Xist intron 1B*, and the *Xist promoter* transcriptional start site (TSS) (Figures S2A and S2B). In contrast, the histone 4 acetylation (H4Ac) levels at these sites did not consistently show a diminution after JQ1 BET inhibition (Figure S2C). Next, we asked whether JQ1 treatment might alter BRD4's ability to partner with OCT4. Indeed, BET inhibition diminishes BRD4 binding to OCT4 (Figure S3A). These results suggest that JQ1 exposure specifically displaces BRD4 binding to the pluripotent and XCI regulatory regions without altering the H4Ac levels and BET inhibition alters its partnering with OCT4.

The XCI lncRNAs *Xist* and *Tsix* were greatly depressed after BET inhibition (Figure 3B). BRD4 recruits P-TEFb to chromosomes to promote G1-phase gene transcription and progression to S phase in fibroblast cell cycles (Yang et al., 2008). Consistent with this, we find that the cell cycle regulator *p21* (Figure 3B) and *Cyclin D1* (Figure 4B) are diminished in male ESCs following BET treatment. Next we examined a panel of pluripotency genes levels to see whether they were altered by BET displacement. Undifferentiated ESCs show a great reduction of *Oct4*, *Nanog*, *Tsix*, and *Xist* after JQ1 exposure (Figure S3B). Consistent with

(B) qChIP analysis of BRD4, OCT4, and IgG (left) and acetylated histone 4 (AcH4) and IgG (right) at *Tsix Site D*, *Xite*, *Toll-like receptor* (*TLR9*), *Xist intron 1B*, *Oct4 enhancer*, *Nanog promoter*, and the *Sox2 promoter* in differentiating d4 male ESCs.

(C) qChIP analysis of BRD4, OCT4, and AcH4 at *Tsix Site D*, *Xite*, *Toll-like receptor* (*TLR9*), *Xist intron 1B*, *Oct4 enhancer*, *Nanog promoter*, and the *Sox2 promoter* sites in d4 female ESCs.

(D) Schematic of the Dox-regulated OCT4 transgenic ESC. The addition of Dox interferes with the tetracycline activation (tTA) of the OCT4-transgene and depletes OCT4 levels.

(E) Western blot analysis of OCT4-inducible ESCs following vehicle (No Dox) versus Dox. Anti-OCT4, anti-BRD4, and anti-ACTIN antibodies were tested on WCEs following treatment.

(F) qChIP of OCT4 and BRD4 on chromatin prepared from ESCs in (E) at the *Nanog* promoter, *Fgf4* enhancer, *Xist intron 1B*, the *Oct4* enhancer, *Tsix site D*, and the *Xite* enhancer.

(G) qChIP of total acetylated histone H4 (H4Ac) on the chromatin prepared from ESCs in (E). The background is calculated compared with control IgG. Graphs indicate three independent biological replicates. Error bars represent 1 SD from the mean. *$p < 0.05$, as determined by Student's t test analysis.

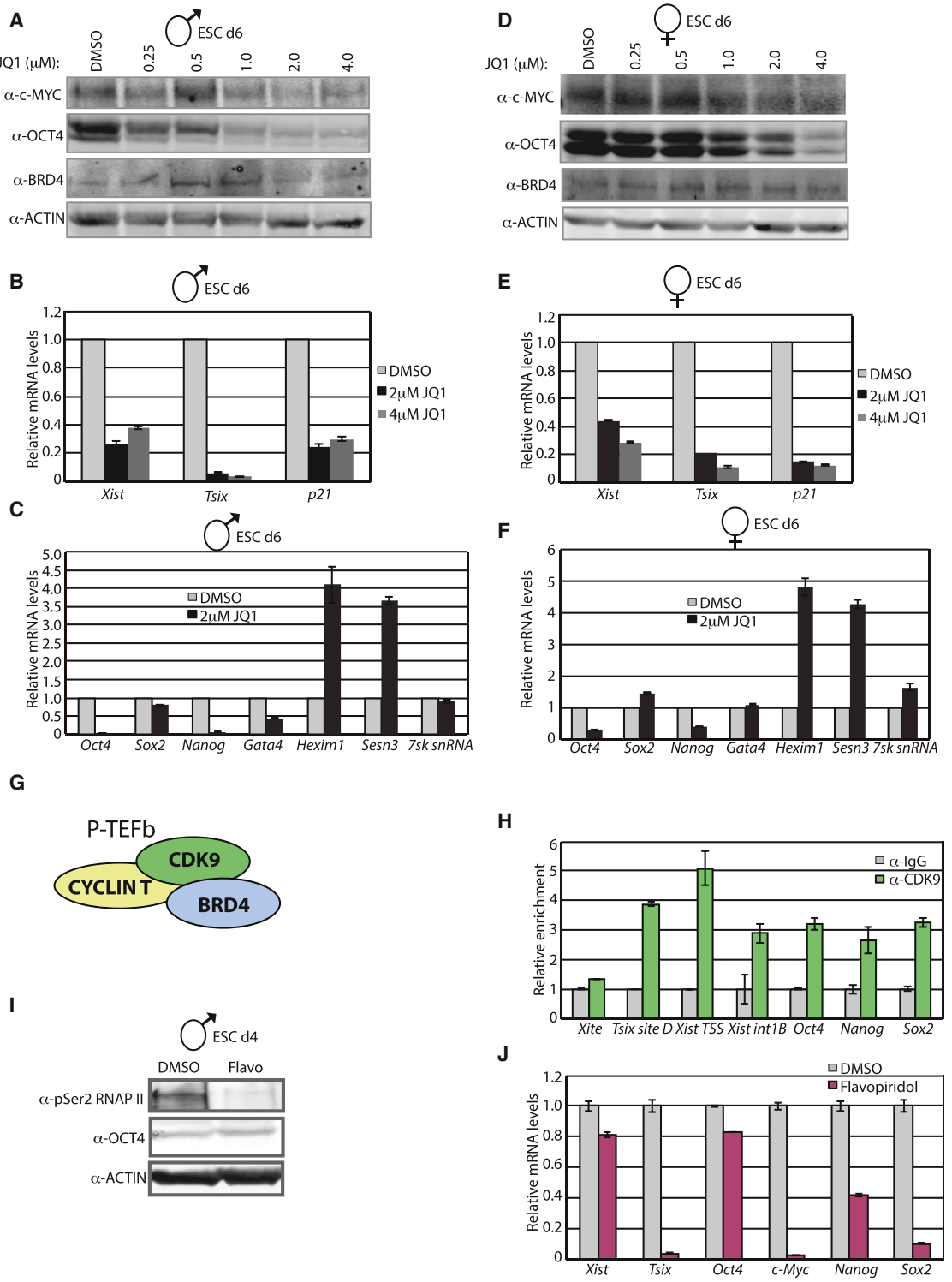

Figure 3. Inhibition of BET Domain Diminishes Pluripotent Gene Expression

(A) Western blot analysis of d6 male ESCs following 48-hr treatment with DMSO control and JQ1 BET inhibitor dose curve. Anti-cMYC, anti-OCT4, anti-BRD4, and anti-ACTIN antibodies were tested on WCEs following BET inhibition.

(B) *Xist*, *Tsix*, and *p21* mRNAs levels in male ESCs after two different doses of JQ1 treatment.

(C) RT-qPCT of the *Oct4*, *Sox2*, *Nanog*, *Gata4*, *Hexim1*, *Sesn3*, and *7sk snRNA* genes following JQ1 or DMSO treatment in male ESCs.

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a diminished protein level, *Oct4* mRNA levels were vastly depressed following JQ1 treatment as well as the *Nanog* gene (Figure 3C). The endodermal marker *Gata4* was repressed in male ESCs (Figure 3C). *Sox2*, another pluripotent factor, was not depressed. Nor was *7sk snRNA*, a P-TEFb inhibitor (Yang et al., 2001; Nguyen et al., 2001). Interestingly the *Hexim1*, another component of the P-TEFb inhibitor complex (Li et al., 2005; Hong et al., 2012), and *Sesn3* (Chen et al., 2010) genes were greatly enhanced following JQ1 exposure (Figure 3C).

Next we tested female ESCs with various doses of JQ1. Consistent with the male ESC BET inhibition, the female ESCs also show a dramatic diminution of c-MYC and OCT4 protein levels as compared with BRD4 and ACTIN (Figure 3D). We observed a loss of *Xist*, *Tsix*, and *p21* mRNA levels after BET inhibition (Figure 3E). Next, we asked whether BET inhibition in the female ESCs altered the pluripotency genes. Similar to what we observed with male cells, the female ESCs show a loss of *Oct4* and *Nanog* expression upon JQ1 exposure (Figure 3F). In contrast to what we saw with BET treatment of male ESCs, *Sox2* levels were slightly enhanced, and *Gata4* levels did not exhibit a change in the female ESCs (Figure 3F). *Hexim1* and *Sesn3* levels were upregulated following JQ1 treatment, and the P-TEFb inhibitor *7sk snRNA* was enhanced in female ESCs. Considering the role of OCT4 in ESC pluripotency, we asked whether providing ectopic OCT4 can reverse the BET inhibition. Interestingly, overexpression of OCT4 in ESCs is not sufficient to rescue the effect of JQ1 treatment on pluripotent gene expression (Figures S3C and S3D).

Inhibition of the BRD4 Protein Partner P-TEFb Blocks Most Pluripotent Gene Transcription In Vivo

BRD4 is unique among the BET family proteins in that it can bind and recruit the kinase active form of P-TEFb to gene promoters (Jang et al., 2005; Yang et al., 2005). The active P-TEFb complex consists of two proteins, CYCLIN T (CYCLIN T1, 2, or 3) and CDK9 (Zhou et al., 2012) (Figure 3G). CDK9 kinase phosphorylates RNAP II and pause control factors (Larochelle et al., 2012). This allows active transcriptional elongation by increasing the number of RNAP II molecules that can synthesize full-length mRNAs (Zhou et al., 2012). We asked whether productive pluripotency and XCI mRNA transcription relies on BRD4's ability to recruit P-TEFb. First, we tested the genes occupied by BRD4 for endogenous CDK9 binding. CDK9 binding is enriched at *Tsix site D*, the *Xist* TSS, *Xist intron 1*, the *Oct4 enhancer*, *Nanog*, and *Sox2* promoter chromatin as compared with the background IgG negative control in d4 male ESCs (Figure 3H). In contrast, the *Xite* enhancer does not show CDK9 occupancy. To further investigate whether P-TEFb is involved in their regulation, we treated male ESCs with flavopiridol (FP). FP is a cyclin-dependent kinase inhibitor that can specifically inactivate P-TEFb and block most RNAP II transcription in vivo, as evident by a loss in phosphorylated serine 2 RNAP II (pSer2 RNAP II) (Chao et al., 2000; Chao and Price, 2001). As shown in Figure 3I, FP treatment (1 μM for 3 hr) results in ablation of pSer2 RNAP II levels as compared with actin protein levels. Interestingly, the OCT4 protein does not decrease following FP treatment (Figure 3I), nor does the BRD4 protein (Figure S4A). We examined the stem-ness-associated gene mRNA levels in these male ESCs after FP exposure. As compared with control, *Oct4* and *Xist* levels show a 20% reduction, whereas *Tsix*, *c-Myc*, *Nanog*, and as *Sox2* expression levels are greatly decreased after FP exposure (Figure 3J).

In addition, we tested the effects of FP in d4 (the differentiation time for XCI establishment) female ESCs and observe similar results as male ESCs (Figure S4B). Collectively, our studies show that many pluripotency-associated genes rely on the BRD4 interacting complex P-TEFb for productive transcriptional elongation.

BET Domain Inhibition Shifts ESC Fate from Pluripotency to Neuroectoderm

Because BET inhibition can dramatically repress pluripotent genes, we next asked what was the consequence of the loss of pluripotency and whether the ESCs differentiated to a particular lineage. Following BET inhibition, male ESCs lost stem-ness and exhibited a gain of

(D) Western blot analysis of d6 female ESCs following JQ1 inhibitor dose curve c-MYC, -OCT4, -BRD4, and -ACTIN antibodies were tested on WCEs following JQ1 exposure.
(E) *Xist*, *Tsix*, and *p21* gene levels following two different doses of JQ1.
(F) Expression of the *Oct4*, *Sox2*, *Nanog*, *Gata4*, *Hexim1*, *Sesn3*, and *7sk snRNA* genes in female ESCs.
(G) The active P-TEFb complex consists of CDK9, CYCLIN T, and BRD4.
(H) qChIP analysis of CDK9 and IgG at the *Xite*, *Tsix Site D*, *Xist TSS*, *Xist intron1B*, *Oct4 enhancer*, *Nanog promoter*, and *Sox2 promoter* sites in d4 male ESCs.
(I) Western analysis using α-phosphorylated serine 2 RNAP II (pSer2 RNAP II), α-OCT4, and α-ACTIN antibodies on WCE prepared from WT d4 male ESCs treated with Flavo or DMSO control.
(J) RT-qPCR of the *Xist*, *Tsix*, *Oct4*, *c-Myc*, *Nanog*, and *Sox2* genes prepared from the d4 male ESCs shown in (I).
Graphs indicate three independent biological replicates. Error bars represent 1 SD from the mean.

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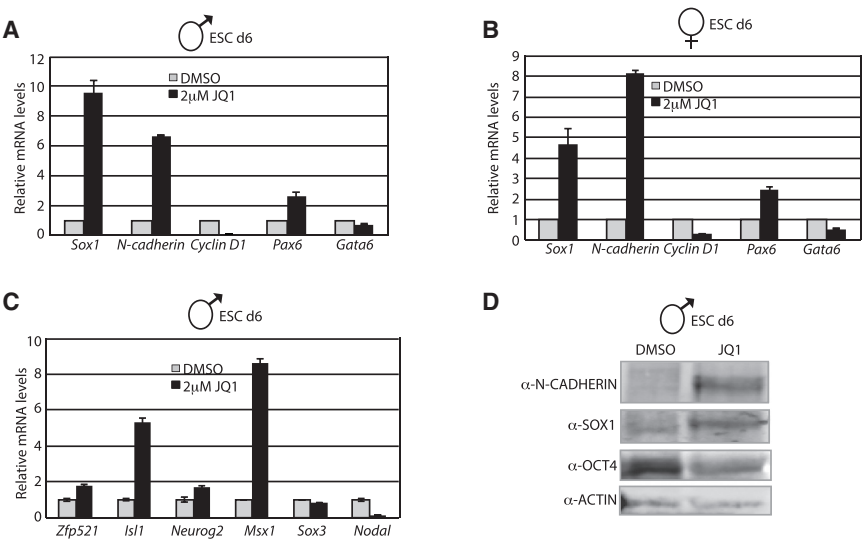

Figure 4. Interference with the BET Domain Strongly Promotes Neural Differentiation in ESC Culture

(A) Expression in male ESCs of the *Sox1*, *N-Cadherin*, and *Pax6* neuroectoderm markers as well as *Cyclin D1* and *Gata6* (endodermal marker) after vehicle (DMSO) or JQ1 BET inhibitor treatment.

(B) Expression of the *Sox1*, *N-Cadherin*, and *Pax6* (neuroectoderm genes) with *Cyclin D1* and *Gata6* mRNA levels in female ESCs upon DMSO versus JQ1 exposure.

(C) qRT-PCT of *Zfp521*, *Isl1*, *Neurog2*, *Msx1*, *Sox3*, and *Nodal* following vehicle (DMSO) or JQ1 BET inhibitor in male ESCs.

(D) Anti-N-CADHERIN, -SOX1, -OCT4, and -ACTIN antibodies were tested on WCEs prepared from male ESCs following vehicle (DMSO) or BET inhibitor JQ1.

Graphs indicate three independent biological replicates. Error bars represent 1 SD from the mean.

neuroectoderm fate with the upregulation of *Sox1*, *N-cadherin*, and *Pax6* neural genes (Figure 4A). In contrast, *Gata6* (endodermal marker) was decreased upon JQ1 exposure. The female ESCs show a loss of stem-ness fate with a concomitant enrichment of *Sox1*, *N-cadherin*, and *Pax6* and a decrease in *Cyclin D1* and *Gata6* (Figure 4B). Additional neuroepithelial mRNAs such as *Zfp521*, *Isl1*, *Neurog2*, and *Msx1* are elevated, whereas *Nodal* (a mesodermal marker) was depleted in response to BET inhibition (Figure 4C). The N-CADHERIN and SOX1 proteins are induced providing further evidence for ESC conversion into neural progenitors following JQ1 treatment (Figure 4D). We also investigated the JQ1 effects on retinoic acid-induced (RA) ESC differentiation and found that the presence of BET inhibitor can facilitate the process of neuroectoderm fate as compared with RA alone (Figures S4D and S4E).

Together, these data implicate BET bromodomains with the pluripotent state in both male and female ESCs. We observe a loss of the XCI status with JQ1 exposure in female cells with a strong reduction in *Xist* and *Tsix* expression. Our results indicate that BET family members repress the RNAP II inhibitors *Hexim1 and Sesn3*. Together, these findings show that inhibition of BET domain proteins results in a loss of the pluripotent fate and a shift toward neuroectoderm identity.

Loss of *Xist* by BET Inhibition Does Not Reactivate Genes along the Silenced Female X Chromosome

Next we queried whether the loss of *Xist* expression following BET inhibition correlates with an increase or reactivation of the genes on the silenced female X chromosome. To test this, we treated WT d6 female ESCs (post-XCI) with the BET inhibitor JQ1 versus control and examined protein and mRNA levels 24 hr later (day 7 [d7] harvest). As shown in Figure 5A, the levels of OCT4 protein are greatly diminished following JQ1 as compared with control (DMSO) treatment. Consistent with what we observed at earlier and later days of cellular differentiation, *Xist* expression is greatly reduced in the female ESCs following JQ1 treatment, with relative mRNA levels less than 10% that of control treated cells. The *Tsix*, *Oct4*, *c-Myc*, and *Nanog* pluripotency genes show decreased mRNA levels following treatment with the BET inhibitor (Figure 5B).

Next we examined *Xist* RNA levels in these female ESCs by fluorescent in situ hybridization (FISH) together with immunostaining for the inactive X-chromosome-associated histone PTM, histone 3 lysine 27 trimethylation (H3K27me3). Consistent with a diminution of *Xist* mRNA levels by quantitative RT-PCR (RT-qPCR), we observe a dramatic reduction in *Xist* and H3K27me3 foci using immuno-FISH following JQ1 treatment (Figure 5C). Following JQ1 exposure, the *Xist* RNA is reduced to approximately half that of control (Figure 5D), as is the H3K27me3 intensity (Figure S5).

A number of genes along the female mouse X chromosome were investigated including genes subject to XCI: *Kdm6a*, *Mecp2*, *Zfx*, *Ogt*, and *Rnf12*, as well as genes that escape XCI, *Eif2s3x*, and *Kdm5c* (Carrel and Willard, 2005) (Figure 5E). We examined genes harbored upstream and downstream of the X *chromosome inactivation center* (*Xic*), the region that controls dosage compensation in female cells. Global X chromosome silencing emanates

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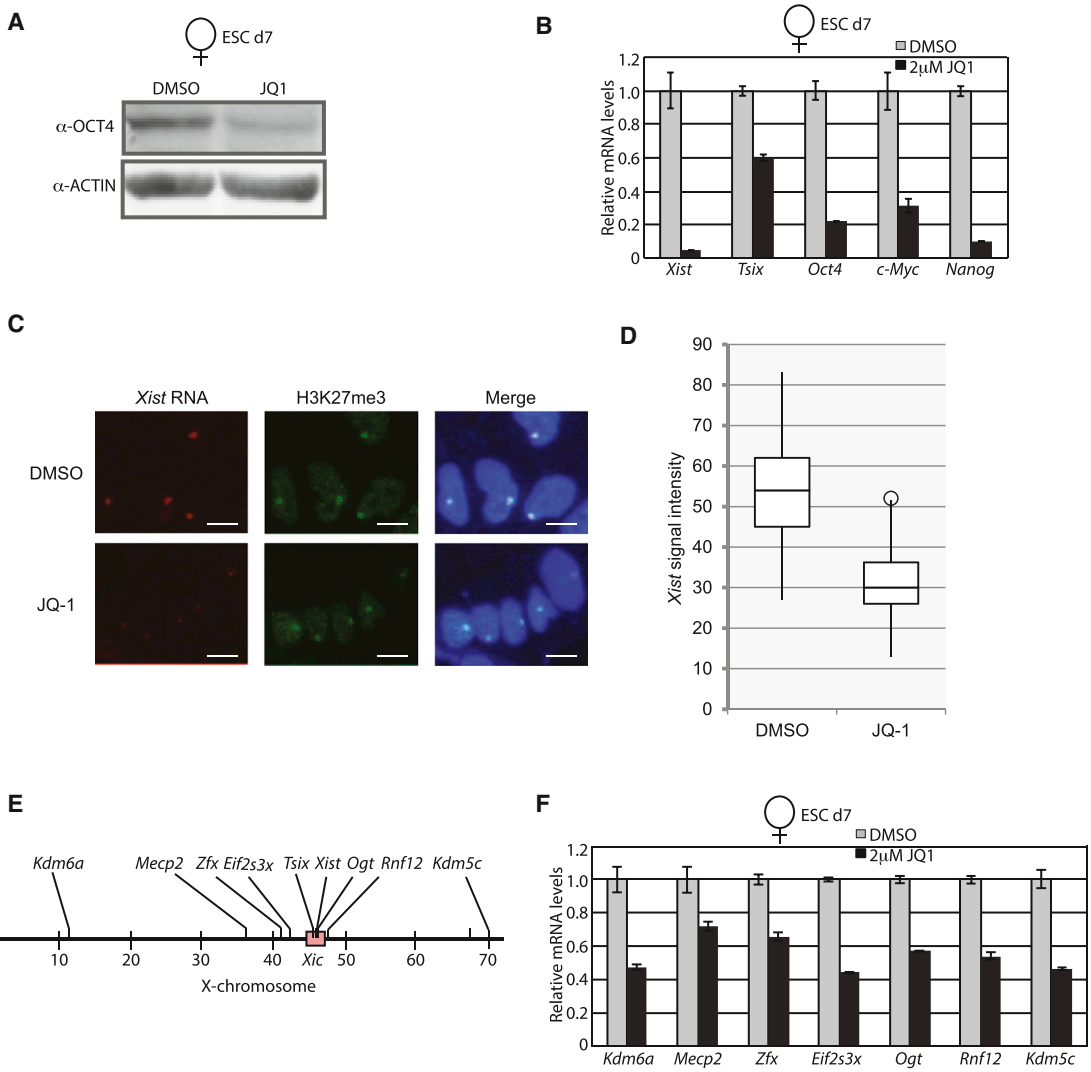

Figure 5. BET Inhibition Dramatically Decreases *Xist* Levels but Does Not Reactivate X-Linked Genes

(A) Western blot analysis for OCT4 and ACTIN from d7 female ESCs following JQ1 or control treatment.

(B) Real-time RT-qPCR for designated sites after BET inhibition and DMSO control treatment.

(C) Immunostaining and RNA-FISH (Immuno-FISH) on vehicle DMSO (top row) versus JQ1 (bottom row) treated d7 female ESCs. *Xist* RNA-FISH (red) (left), H3K27me3 immunostaining (green) (middle), and merged image (right). Scale bar represents 20 μM.

(D) *Xist* signal intensity was quantified by counting 60 cells following DMSO or JQ1 treatment and displayed as a Box plot. $p < 5 \times 10^{-15}$.

(E) Map of the mouse X chromosome with genes tested for expression. The X *chromosome inactivation center* (*Xic*) is shown as a pink box. The X chromosome scale is in centimorgans (cM) with the centromere depicted as a circle.

(F) Treatment with the BET inhibitor, JQ1 and the expression of the X-linked genes shown in (E): *Kdm6a*, *Mecp2*, *Zfx*, *Eif2s3x*, *Ogt*, *Rnf12*, and *Kdm5c*.

Graphs indicate three independent biological replicates. Error bars represent 1 SD from the mean.

from the *Xic* during female ESC differentiation with the process nearly complete at d7. As shown in Figure 5F, X-linked genes show a reduction in expression following BET inhibition. Overall, we conclude that BET inhibition greatly diminishes *Xist* expression in differentiated female cells but does not result in a chromosomal-wide reactivation of the X.

Overexpression of BRD4 Protein in ESCs Enhances Pluripotent Genes

Approximately 50% of cellular P-TEFb is sequestered in an inactive complex consisting of HEXIM1 and 7sk snRNA (Yang et al., 2005). The active P-TEFb complex is associated with BRD4. Because inhibition of BRD4's BET domain markedly reduced pluripotent mRNAs, we asked whether

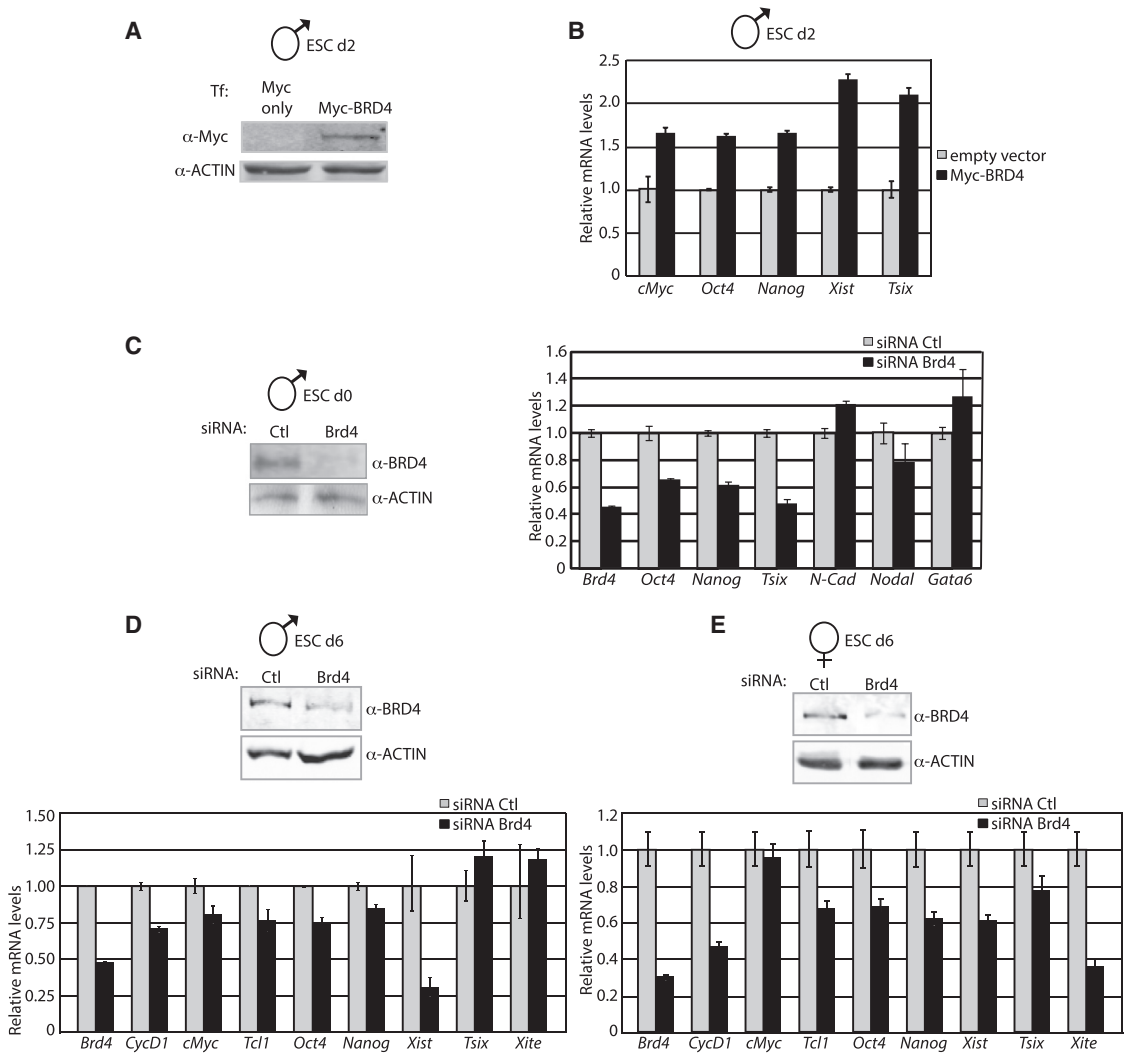

Figure 6. *Brd4* Overexpression Induces while *Brd4* Depletion Causes a Reduction in Pluripotent Gene Expression

(A) Undifferentiated (d0) male ESCs were transfected with Myc-tagged BRD4 or Myc-only control and harvested at d2. Western blot analysis shows that the full-length Myc-BRD4 is expressed.

(B) qRT-PCR of the *c-Myc*, *Oct4*, *Nanog*, *Xist*, and *Tsix* genes following the overexpression of myc-BRD4 versus myc-only empty vector.

(C) Western blot analysis confirms knockdown in WT d0 male ESCs with scramble (Ctl) and *Brd4* siRNA. RT-qPCR shows the expression of *Brd4*, *Oct4*, *Nanog*, *Tsix*, *N-cadherin (N-cad)*, *Nodal*, and *Gata6* genes.

(D) Western blot confirms BRD4-knockdown in d6 WT male ESCs. RT-qPCR shows expression levels of the *Brd4*, *Cyclin D1 (CycD1)*, *cMyc*, *Tcl1*, *Oct4*, *Nanog*, *Xist*, *Tsix*, and *Xite* genes.

(E) *Brd4* knockdown is confirmed in WT d6 female ESCs. Gene expression analysis (qRT-PCR) of the *Brd4*, *Cyclin D1 (CycD1)*, *cMyc*, *Tcl1*, *Oct4*, *Nanog*, *Xist*, *Tsix*, and *Xite* genes.

Graphs indicate three independent biological replicates. Error bars represent 1 SD from the mean.

forced expression of BRD4 would alter P-TEFb's stoichiometry and drive the expression of stem-ness genes. Full-length Myc-tagged BRD4 was transfected into d0 male ESCs. Whole-cell extracts (WCEs) and mRNA were harvested after 48 hr (d2). Western blot shows that the Myc-fused BRD4 protein (180 kDa) is overexpressed as compared with the Myc-only control (Figure 6A). Forced expression of BRD4 induced *cMyc*, *Oct4*, *Nanog*, *Xist*, and *Tsix* expression (Figure 6B). These results suggest that BRD4 induction can activate the pluripotent and XCI genes in ESCs.

Depletion of *Brd4* Reduces Pluripotent Gene Expression

Targeted disruption of mouse *Brd4* results in an early lethality that precludes the analysis of XCI and

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pluripotency (Houzelstein et al., 2002). Therefore, to examine the mechanistic in vivo function for BRD4 in male and female ESCs, we used small interfering RNAs (siRNAs). Two different regions of *Brd4* were targeted for knockdown (a second siRNA targeted to a different region of Brd4 shows a similar result; data not shown). First, we examined depletion of BRD4 in undifferentiated ESCs by transfection with *Brd4* siRNA and harvested for protein and RNA analysis. Efficacy of the *Brd4* knockdown was confirmed by western blot analysis of WCEs from siRNA control (scramble) versus *Brd4* (Figure 6C, left). *Brd4*, *Oct4*, *Nanog*, and *Tsix* show decreased expression following *Brd4* knockdown. In contrast, the *N-Cadherin* gene shows a reproducible upregulation. In contrast, the *Nodal* and *Gata6* genes do not show repression. Interestingly, *Brd4* but not another BET family member, *Brd2*, knockdown in undifferentiated ESCs mimics the effect of JQ1 BET inhibition, suggesting the importance of Brd4 in pluripotency (Figure S6A).

Day 4 male ESCs were transfected with *Brd4* siRNA and harvested for protein and RNA analysis 48 hr later. As shown in Figure 6D, BRD4 protein is depleted (approximately 60%; Figure S6B) following *Brd4* siRNA compared with the actin protein expression in male ESCs. A panel of genes was tested for expression following *Brd4* versus control siRNA treatment. *Brd4*, *Cyclin D1* (*CycD1*), *cMyc*, *Tcl1* (the T cell leukemia oncogene, an OCT4 target, and pluripotent gene) (Matoba et al., 2006), *Oct4*, *Nanog*, and *Xist* mRNA levels were decreased following knockdown of *Brd4* in the male ESCs (Figure 6D, lower). In contrast, *Tsix* and *Xite* did not show a diminution of expression in knockdown versus control in the male ESCs. To investigate XCI, we examined *Brd4* knockdown in female ESCs. A dramatic decrease (~70%; Figure S6C) in Brd4 protein was observed following *Brd4* siRNA (Figure 6E, upper). RT-qPCR shows that the *Brd4*, *CycD1*, *Tcl1*, *Oct4*, *Nanog*, *Xist*, *Tsix*, and *Xite* expression levels all decrease in the *Brd4* knockdown as compared with the control (Figure 6E, lower). Taken together, we show that the specific removal of *Brd4* in both male and female ESCs corroborates the BET inhibition with a decrease in XCI- and pluripotency-associated genes.

DISCUSSION

Stem cells have a fundamental feature in that they have molecular determinants to specify cell fate after mitosis: retain pluripotency versus differentiate. Histone PTMs and epigenetic bookmarkers may facilitate genes regulation in ESCs.

Histone PTMs play a crucial role in modulating chromatin structure and transcription (Sterner and Berger, 2000; Zeng and Zhou, 2002). Acetylation of histones is used as a cellular signaling mechanism. The BET proteins bind and read acetylated histones, but to date, it is unknown how they function in early developmental processes. Data presented here reveal a crucial role for the histone reader BRD4 in maintaining pluripotency and XCI status in stem cells. Inhibition or loss of *Brd4* results in a shift away from stem-ness to the neural fate. Neural fate is postulated to be a default state as it is the intrinsic direction of ESCs when the exogenous signals are minimized in differentiation culture (Kamiya et al., 2011; Muñoz-San-juán and Brivanlou, 2002). Our results reveal that BRD4 fortifies this decision to retain pluripotent fate by binding OCT4 at pluripotent regulator regions. OCT4 is one of the core pluripotent factors that occupies super-enhancers; regulatory region clusters in genes that control the pluripotent state, suggesting transcriptional control of cell state (Hnisz et al., 2013; Whyte et al., 2013). As a transcriptional facilitator, BRD4 at these sites could enhance these pluripotent genes transcription either by recruiting P-TEFb or by other P-TEFb-independent mechanisms. Although BRD4 can directly recognize and bind acetylated histones, our results show that BRD4 binding can be enhanced or stabilized by OCT4 at particular regulatory regions such as the *Nanog* promoter, supporting the hypothesis that BRD4 may be stabilized by other transcription factors.

The X *chromosome inactivation center* has a plethora of lncRNAs that are transcribed in the sense and antisense orientation (Payer and Lee, 2008; Lee and Bartolomei, 2013). This suggests that tight control of RNAP II is necessary for their regulation. OCT4 can partner with the chromatin insulator CTCF, and both proteins mediate the X-X homologous pairing in XCI (Xu et al., 2007). Active transcription is required for pairing (Xu et al., 2007). Data here suggest that both BRD4 and P-TEFb are additional factors for the transcription of the lncRNAs in mouse XCI. Interestingly, human XCI does not correlate with the pluripotent state as in the mouse (reviewed in Lessing et al., 2013); therefore, future studies are necessary to ascribe roles for BRD4 and P-TEFb for XCI in human ESCs. While this manuscript was in revision, the Hernando lab reported a role for BRD4-dependent transcriptional elongation in human ESCs at super-enhancers of pluripotency genes (Di Micco et al., 2014).

JQ1 small-molecule inhibition can displace the other BET bromodomain family members Brd2, 3, and 4 from binding acetylated histones (Delmore et al., 2011). The Young lab recently investigated genome-wide JQ1 binding using ligand-affinity capture followed by massive parallel DNA sequencing (Chem-Seq) and showed that the pattern of JQ1 occupancy is best associated with Brd4 occupancy (Anders et al., 2014). We believe that BRD4 is the most important BET-containing protein for ESC pluripotency and XCI. First, deletion of mouse *Brd4* is a peri-implantation lethal

(Houzelstein et al., 2002) during the time of lineage segregation and XCI establishment in female cells. Second, BRD4 was identified in an RNAi screen of chromatin regulators necessary for maintaining pluripotency (Fazzio et al., 2008). Third, the BET family member BRDT is specifically expressed in male germ cells (Shang et al., 2004). Fourth, the *Brd2* mouse mutation results in embryonic lethality by day 11.5 with proliferative and neural tube closure defects, suggesting that it plays later roles in development (Shang et al., 2009). Although there is a possibility of redundancy with the other BET proteins, our *Brd4* knockdown data confirm the JQ1 inhibition data establishing its function for pluripotency. Taken together, BRD4 binds with OCT4 at many stem-ness gene regulatory regions, including the lncRNAs involved in XCI. BRD4 and its recruitment of P-TEFb exert crucial roles in stem cell identity and the transcription of lncRNAs in XCI.

EXPERIMENTAL PROCEDURES

Cell Lines, Chemicals, and Chromatin Immunoprecipitations

The ESC lines, fibroblast, and cell culture conditions have been described (Lee and Lu, 1999). For these studies, we used three different male (J1, R1, and ZHBTc4) and two different female (EL16.7 and LF2) ESCs. The Dox-regulated Oct4 transgenic ESC line ZHBTc was treated with 1 μg/ml Dox for 24 hr (Niwa et al., 2000). JQ1 (BPS Biosciences) was diluted in DMSO with various dosages. Flavipiridol (Santa Cruz) was diluted in DMSO and used at 1 μM for a 3-hr treatment. Chromatin immunoprecipitations were performed as previously published (Donohoe et al., 2009) with the PCR primers described in Table S1. A total of 40 ng of amplified DNA was used to prepare sequencing libraries using the Illumina ChIP-seq HT Sequencing Library preparation protocol. Sequencing was performed performed at the Weill Cornell Medical College Epigenemics Core using the Illumina HiSeq2000 system (Illumina) with each library sequenced in a 50 bases single-read run. Reads were aligned to the mouse genome (mm10/GRCm38) using SeqMonk (www.bioinformatics.babraham.ac.uk). Peak calling was normalized to input DNA sequencing data. Tracks were viewed using the Integrative Genomics Viewer (Broad Institute). The accession number for the BRD4 ChIP-seq data in this paper is SAMN0332740.

Gene Silencing by RNA Interference and Expression Analysis

Murine *Brd4* and MISSION Universal Negative Control siRNAs were purchased from Sigma-Aldrich. Brd4 siRNA1 and siRNA2 targeted the sequences 5′- CCTGATTACTATAAGATTA-3′ and 5′-CAGACAAACCAACTGCAAT-3′, respectively. ESCs were transfected using Lipofectamine 2000 (Invitrogen) with 200 pmol of siRNA and the WCEs and RNA were collected 48 hr later. Western analysis of WCEs confirmed protein knockdown. RNA was prepared using Trizol (Life Technology) and reverse transcribed to cDNA as described (Donohoe et al., 2009) using the Applied Biosystems 7500 Fast Real-Time PCR System. PCR was performed using the primers described in Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.01.012.

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