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-cell 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 non-coding 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; Merscher 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
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 confirming in vivo BRD4-OCT4 interaction (qChIP) (Figure 1E). Using a stringent statistical criteria (p < 0.05) we identified significant enrichment of ChIP-seq peaks (Figure 1F). 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
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 DxDs34, the epigenetic switch/control region for XCI. Deletion of DxDs34 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 female ESCs is essential for maintaining pluripotency.
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 IncRNAs 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 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...
Figure 2. BRD4 Occupies Regulatory Regions within the Pluripotency Genes and the IncRNAs 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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OCT4 can reverse the BET inhibition. Interestingly, over-expression 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 stemness-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 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, Neuro2, and Mso1 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 4D and 4E).

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...

(B) qChIP 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, and as Sox2 promoter sites in 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, and as 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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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 (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.

| Gene   | Control | JQ1 | JQ1 | JQ1 |
|--------|---------|-----|-----|-----|
| Oct4   | 10      | 5   | 2   | 1   |
| c-Myc  | 15      | 10  | 5   | 2   |
| ACTIN  | 20      | 15  | 10  | 5   |

Graphs indicate three independent biological replicates. Error bars represent 1 SD from the mean.
cells. Global X chromosome silencing emanates from the Xic during female ESC differentiation with the process nearly complete at d7. As shown in Figure 5E, 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
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 full-length Myc-BRD4 is expressed. 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
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 showed 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 control (Figure 6C, left). Brd4, Cyclin D1 (CycD1), cMyc, Tci1 (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 male ESCs. A panel of genes was tested for expression following Brd4 versus control siRNA treatment. Brd4, Cyclin D1 (CycD1), cMyc, Tci1 (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, Tci1, 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 bookmarks 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-Sanjuán 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 IncRNAs 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 IncRNAs 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 at the Well Cornell Medical College Epigenomics 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/GRCh38) 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’-CCTGATTACTATAAGATT-3’ and 5’-CA GACAAACCACAAGTCCAAT-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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The BET Family Member BRD4 Interacts with OCT4 and Regulates Pluripotency Gene Expression

Tao Wu, Hugo Borges Pinto, Yasunao F. Kamikawa, and Mary E. Donohoe
Supplemental Figure 3, Wu, et al
**Brd2/Brd4 KD in undifferentiated ESCs**

- **A**
  - siRNA: Scr, Brd2
  - α BRD2
  - α ACTIN

- **B**
  - Normalized Brd4 Protein Levels
    - ESC d6
    - siRNA Ctl
    - siRNA Brd4

- **C**
  - Normalized Brd4 Protein Levels
    - ESC d6
    - siRNA Ctl
    - siRNA Brd4

Supplemental Figure 6, Wu, et al
SUPPLEMENTAL FIGURE LEGENDS

**SUPPLEMENTAL Figure 1. Validation of chromatin immunoprecipitation of BRD4 in undifferentiated male ESCs subjected to ChIP-Seq.** (A) Quantitative chromatin immunoprecipitation (qChIP) analysis of BRD4 and IgG at the Oct4 enhancer and Nanog in day 0 (d0) male ESCs. Graphs indicate three independent biological replicates. Error bars represent one standard deviation from the mean.

(B) Chromosomal enrichment of BRD4 in ChIP-Seq.

**SUPPLEMENTAL Figure 2. Inhibition with JQ1 displaces BRD4 and CDK9 occupancy but does not alter histone 4 acetylation at pluripotent genes.** (A) Quantitative chromatin immunoprecipitation (qChIP) analysis of BRD4 and IgG at the Xite enhancer, Tsix Site D, Oct4 enhancer, Sox2 promoter, Xist intron 1B (int1B), Nanog promoter, and the Xist promoter transcriptional start site (TSS) following JQ1 or control (DMSO) treatment on day 4 (d4) male ESCs. (B) Quantitative chromatin immunoprecipitation (qChIP) analysis of CDK9 and IgG at the Xite enhancer, Tsix Site D, Oct4 enhancer, Sox2 promoter, Xist intron 1B (int1B), Nanog promoter, and the Xist promoter transcriptional start site (TSS) following JQ1 or control (DMSO) treatment on day 4 (d4) male ESCs. (C) Quantitative chromatin immunoprecipitation (qChIP) analysis of acetylated Histone 4 (H4Ac) and IgG at the Xite enhancer, Tsix Site D, Oct4 enhancer, Sox2 promoter, Xist intron 1B (int1B), and the Nanog promoter following JQ1 or control (DMSO) treatment on day 4 (d4) male ESCs. The background is
calculated compared to control IgG. Graphs indicate three independent biological replicates. Error bars represent one standard deviation from the mean.

**SUPPLEMENTAL Figure 3. BET inhibition by JQ1 reduces BRD4 binding with OCT4.** (A) HEK cells were transfected (Tf) with Flag alone or Flag-OCT4 following treatment with vehicle (DMSO) versus JQ1 BET inhibitor. Whole cell extracts were immunoprecipitated with anti-Flag antibodies and western blot analysis with anti-BRD4 antibodies. The top panel shows a diminution of BRD4 co-immunoprecipitating with Flag-OCT4. Lower panels are controls of input. (B) Pluripotent gene expression is diminished in undifferentiated male ESCs following JQ1 treatment. (C) Over expression of OCT4 does not rescue Xist levels following JQ1 treatment. ESCs were transfected (Tf) with an epitope-tagged full-length OCT4 (Myc-OCT4) or Myc-empty vector (EV) in the presence of DMSO vehicle or the BET inhibitor JQ1. Western blot using anti-Myc antibodies shows that Myc-OCT4 is expressed. (D) RT-qPCR shows that the total levels of Oct4 mRNA levels are elevated after ectopic expression of Myc-Oct4. Nanog, Tsix, and Xist levels are not restored after ectopic OCT4 expression. Graphs indicate three independent biological replicates. Error bars represent one standard deviation from the mean.

**SUPPLEMENTAL Figure 4. Flavipiridol (FP) treatment does not decrease BRD4 protein levels.** (A) Western blots from ESCs using anti-BRD4, anti-RNA polymerase II phosphor serine 2, and anti-ACTIN antibodies following DMSO vehicle or FP treatment. (B) Many pluripotent genes are P-TEFb dependent in female ESCs.
Western analysis using α-pSer2 RNAP II, α-OCT4, and α-ACTIN on WCE prepared from wild type d4 female ESCs following exposure to FP or DMSO control. (C) RT-qPCR of the Xist, Tsix, Oct4, c-Myc, Nanog, and Sox2 genes prepared from the d4 female ESCs shown in (E). Graphs indicate three independent biological replicates. Error bars represent one standard deviation from the mean. (D) JQ1 effects on Retinoic Acid (RA) induced ESC differentiation. BET inhibition dramatically changes the cell morphology in RA induced ESC differentiation. upper left: morphology of cells which were treated with vehicle (DMSO) for 5 days. upper right: morphology of cells which were treated with 1 uM RA alone for 5 days. lower left: cells were first treated with 1 uM RA for 3 days and RA was washed out, then cells were treated with 2 uM JQ1 for another 2 days and the morphology of cells were then imaged. lower right: cells were treated for 3 days with 1uM RA and then 2uM JQ1 was added to treat the cells together with RA for another 2 days. Note the morphological changes of the cells between JQ1 treated samples (lower panel) and DMSO or RA alone treated samples (upper panel). Cells demonstrate neuronal like morphology after JQ1 treatment. (E): qRT-PCR showing the expression level of Oct4 and neuronal lineage markers (Neuro D1, Pax6, and Calbindin 1) after RA and/or BET inhibition. Consistent with the neuronal like morphological changes, inhibition of BET family can greatly enhance the expression of neuronal lineage marker.

SUPPLEMENTAL Figure 5. Quantification of Xist RNA-FISH and H3K27me3 levels following JQ1 treatment. LF2 female ESCs were differentiated by forming EBs and removing LIF. EBs were treated with JQ1 for 24 hr (day6 to day7). For both
of DMSO and JQ1, n=60. (A) Box plot of H3K27me3 immunostaining  (B) Scatter plot, Y axis= Xist RNA-FISH signal; X axis= H3K27me3 signal.

SUPPLEMENTAL Figure 6. (A) Knockdown of Brd4 but not Brd2 reduces pluripotent gene expression in undifferentiated ESCs. (B) Normalized BRD4 protein levels as compared to ACTIN protein loading controls in siRNA control (ctl) versus Brd4 in day 6 (d6) male ESCs. The control siRNA was set at 100%. Quantification was performed using LiCor. This figure panel corresponds to Figure 6C. (C) Normalized BRD4 protein levels as compared to ACTIN protein loading controls in siRNA control (ctl) versus Brd4 in day 6 (d6) female ESCs. The control siRNA was set at 100%. Quantification was performed using the LiCor machine. This figure panel corresponds to the data shown in Figure 6E.
Supplemental Table 1
Real time RT-PCR PRIMERS:
Ogt F-TGCAGCTGGCAACAAACCTG
Ogt R-TTCCCCACAAGGTTGAGGC

Kdm5c F-ACAAACCAAGATGCCGGTG
Kdm5c R-TACCGCTGCTCCCCACTAC

Eif2s3x F-GAGGTTGAGTGACTCTCGG
Eif2s3x R-AGCCTTCACCACAGTGAGCTT

Kdm6a F-GACAGCGGAGGAGGGAG
Kdm6a R-CGAACAGCCCTTGCCCAGC
Rnf12 F-TGGATCGCTTGGATCGGGAA
Rnf12 R-TTTTATCATGGGCTCTGTTGT
Zfx F-CCGCTTTTTGTCAGCTCCGA
Zfx R-GGGCACAGTTATACGCACCG
Sesn3 F-CAT GCCTT CCT CAC TCA GA
Sesn3 R-GCC AAA GTC TTC GTA CCC AA
Hexim1 F-GAGCCACTCTTGAGACAGAATC
Hexim1 R-TGCAAGGGCCTCTCGATTG
Brd4 F-CCATGGACATGAGCACAATC
Brd4 R-TGGAGACATCAATCGGACA
Zfp521 F-GAGGGAAGGAGGATTGGG
Zfp521 R-AGTTCCAAGGTGAGGTCTAC
Sox1 F-CCTCGGATCTCTGGTCAAGT
Sox1 R-GCAGGTACATGCTGATCATC
N-cadherin F-CAGGTTGGACGTATTTCTAG
N-cadherin R-AGGTCTCCACCACTGATTCT
Pax6 F-AGTGAATGGGCGGATTTG
Pax6 R-ACCTGGAGGGAAGCTAC
Gapdh F-CTTCACCACCATGGAGAGGC
Gapdh R-CCCTTTTGCGCTCCACCCT
CycD1 F - GCTGGAGGACTGTTGAGGA
CycD1 R - CTCTCCGGCCGGATAGAGTT

c-Myc F - TCCTGTACCTGTCCCTGATTC
c-Myc R - GGTTTCTGCTCTCTCCACAG

Tcl1 F - CAGTGGGTCTGTGCTTCCTCTG
Tcl1 R - AGGTCTTGTCCTCCCATGGTG

Oct4 F - CAGAAGGAGCTAGAAACAGTTTGCC
Oct4 R - AGATGGGTGCTGGCTGAACCA
**Primers for ChIP:**
- **Tsix site D F:** CCGTAGAGTTGCTAAATGTTAC;
- **Tsix site D R:** CCGAGCTGAGTTGCTAAATGTTAC;
- **Xist intron1 F:** AACCCCTTTTAAGGCCAATGTTAC;
- **Xist intron1 R:** CCGAGCTGAGTTGCTAAATGTTAC;
- **Oct4 enhancer F:** GGAACCTGAGTTGCTAAATGTTAC;
- **Oct4 enhancer R:** CCGAGCTGAGTTGCTAAATGTTAC;
- **Nanog F:** CCGAGCTGAGTTGCTAAATGTTAC;
- **Nanog R:** CCGAGCTGAGTTGCTAAATGTTAC;
- **Sox2 F:** CCGAGCTGAGTTGCTAAATGTTAC;
- **Sox2 R:** CCGAGCTGAGTTGCTAAATGTTAC;
- **Brd4 F:** CCGAGCTGAGTTGCTAAATGTTAC;
- **Brd4 R:** CCGAGCTGAGTTGCTAAATGTTAC;
**Supplemental Experimental Procedures**

**Antibodies**
The following antibodies were used: RNA PolII Phospho serine 2 (AbCam, ab5095), BRD4 (Bethyl, A301-985A), ACTIN (Sigma, A5316), OCT4 (Santa Cruz, sc-9081 and Millipore, MABD76), Histone 3 (AbCam, ab1791), Histone 4 acetyl (H4Ac) (Active Motif, 39925), MYC (Sigma, C3956), Flag (Sigma, F1804), IgG (Santa Cruz, sc-2027), SOX1 (AbCam, ab87775), N-CADHERIN (AbCam, ab12221), H3K27me3 (Active Motif), and CDK9 (Santa Cruz, sc-8338).

**Protein co-immunoprecipitations.** The full-length Brd4 was produced using AddGene #14441 (pcDNA4cBrd4) (produced in Dr. Peter Howley's lab (You et al., 2004)) as a template and cloned into a Myc-tag containing vector (Clontech). Cells were co-transfected with Flag-Oct4 and harvested as described previously (Donohoe et al., 2009). Endogenous co-immunoprecipitations (co-IPs) were performed as described (Donohoe et al., 2009) using 1-2 mM of DSP (ThermoScientific) cross-linker and quenched with 25 mM Tris pH 7.5.

**RNA-FISH**
The Stellaris Xist RNA-FISH probe with Quasar 570 dye was purchased from Biosearch Technologies. The RNA-FISH was performed as described (Donohoe et al., 2007).