

BRD4 regulates *Nanog* expression in mouse embryonic stem cells and preimplantation embryos

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Bromodomain-containing protein 4 (BRD4) is an important epigenetic reader implicated in the pathogenesis of a number of different cancers and other diseases. *Brd4*-null mouse embryos die shortly after implantation and are compromised in their ability to maintain the inner cell mass, which gives rise to embryonic stem cells (ESCs). Here we report that BRD4 regulates expression of the pluripotency factor *Nanog* in mouse ESCs and preimplantation embryos, as well as in human ESCs and embryonic cancer stem cells. Inhibition of BRD4 function using a chemical inhibitor, small interfering RNAs, or a dominant-negative approach suppresses *Nanog* expression, and abolishes the self-renewal ability of ESCs. We also find that BRD4 associates with BRG1 (brahma-related gene 1, aka Smarca4 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4)), a key regulator of ESC self-renewal and pluripotency, in the *Nanog* regulatory regions to regulate *Nanog* expression. Our study identifies *Nanog* as a novel BRD4 target gene, providing new insights for the biological function of BRD4 in stem cells and mouse embryos. Knowledge gained from these non-cancerous systems will facilitate future investigations of how *Brd4* dysfunction leads to cancers.

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Bromodomain-containing protein 4 (BRD4) belongs to the bromodomain and extraterminal (BET) protein family.¹ BRD4 functions as an epigenetic reader by binding to acetylated histones on chromatin through its two bromodomains, and has a central role in transcriptional regulation, cellular growth control and cell cycle progression.² BRD4 supports transcriptional activation by actively recruiting the positive transcription elongation factor b, mediators and several other transcriptional activators.^{2,3}

BRD4 is implicated in the pathogenesis of a number of cancers and other diseases.^{3–8} In some cancers, BRD4 regulates expression of *c-Myc* and other oncogenes.^{3,4} It also selectively binds to the ‘super-enhancers’ of tumor oncogenes, which are large clusters of enhancers that control expression of these genes.^{3,9} Although these recent studies have shed light on the gene-specific activity of BRD4, how alterations in BRD4 function contribute to the development of cancers and other diseases is not well understood. This lack of knowledge reflects the need to better understand the normal function of BRD4 in noncancerous cells, as most of the previous studies of BRD4 function were performed in cancer cells.

In *Brd4* knockout mice, the homozygous embryos die shortly after implantation.¹⁰ Cells derived from the inner cell

mass (ICM) of these homozygous embryos are completely degenerated, although the rest of the embryo appears morphologically normal.¹⁰ These observations suggest that *Brd4* is required for the development and/or maintenance of the ICM,¹⁰ which gives rise to embryonic stem cells (ESCs) in culture. ESCs with homozygous *Brd4* deletion are nonviable,¹¹ further supporting the idea that *Brd4* is important for ESC proliferation and maintenance. We therefore examined *Brd4* function in ESCs and preimplantation embryos.

ESCs derived from the ICM of day 3.5 mouse blastocysts are characterized by their pluripotency and self-renewal capacity. The transcription factors NANOG, POU5F1 (OCT4) and SOX2 are the core regulatory factors of self-renewal and pluripotency that maintain ESC propagation in an undifferentiated state.¹² Expression of these pluripotency regulators is tightly controlled through a transcriptional circuitry consisting of auto-regulatory feedback loops.¹³ *Nanog* has a particularly important role in establishing ESC ground state pluripotency.^{14,15} *Nanog* null ESCs are prone to differentiate,¹⁶ and knockdown of *Nanog* in mouse ESCs causes loss of self-renewal and induction of trophectoderm and primitive endoderm differentiation.¹⁷ *Pou5f1* and *Nanog* are also key regulators of early mouse embryo development.^{14,15,18,19} Although these studies have shed

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Abbreviations: Brd4, bromodomain-containing protein 4; ICM, inner cell mass; ESCs, embryonic stem cells; BET, bromodomain and extraterminal protein; LIF, leukemia inhibitory factor; BMP4, bone morphogenetic protein 4; AP, alkaline phosphatase; siRNAs, small interfering RNAs; BDI/II, double bromodomains; ChIP, chromatin immunoprecipitation; BRG1, brahma-related gene 1, aka Smarca4 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4); ChIP-reChIP, sequential ChIP analysis; H3K27me3, trimethylation of Lys27 of histone 3; FBS, fetal calf serum; GV, germinal vesicle; MII, metaphase II; PVP, polyvinylpyrrolidone; KSOM + AA, KSOM medium containing amino acids; RA, retinoic acid

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light on how transcription factor networks regulate ESC pluripotency, further studies are necessary to understand fully the molecular mechanisms that regulate these core factors in preimplantation embryos and ESCs.

We report that BRD4 regulates *Nanog* expression in ESCs and preimplantation embryos. Inhibiting BRD4 function abolishes *Nanog* expression and abrogates ESC maintenance. Furthermore, we find that BRD4 associates with the chromatin-remodeling protein BRG1 (brahma-related gene 1, aka Smarca4 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4)) and binds to the *Nanog* regulatory regions, a finding that likely underlies the ability of BRD4 to regulate *Nanog* expression in ESCs.

Results

Brd4 is downregulated during mouse ESC differentiation.

To explore *Brd4* function in ESCs, we analyzed BRD4 protein levels in two different mouse ESC lines, TL1 and R1, before and after differentiation induced by withdrawal of leukemia inhibitory factor (LIF)/bone morphogenetic protein 4 (BMP4) and addition of retinoic acid (RA). Interestingly, *Brd4* protein levels decreased markedly in both ESC lines when they were induced to differentiate (Figure 1). The reduction in the amount of BRD4 protein paralleled the decrease in the amount of NANOG, POU5F1 and SOX2 protein during ESC differentiation. This result suggests that BRD4 may have a more important role in undifferentiated ESCs than in differentiated cells.

BRD4 inhibitor JQ1(+) impairs ESC maintenance. To examine further BRD4 function in undifferentiated ESCs, we tested how inhibiting BRD4 affected ESC growth and maintenance. JQ1(+), which is a BET protein inhibitor, displays the highest affinity for BRD4, and prevents BRD4 binding to acetylated histones.^{4,6} TL1 and R1 ESCs were treated with JQ1(+) and the inactive stereoisomer, JQ1(-),

to test their effect on ESC maintenance. Compared with untreated or JQ1(-)-treated cells, JQ1(+)-treated ESCs displayed an abnormal morphology, and also generated very few ESC colonies with high alkaline phosphatase (AP) activity, a marker for undifferentiated ESCs (Figures 2a and b). This observation suggests that JQ1(+) inhibits ESC maintenance and induces cell differentiation.

We then analyzed the effect of JQ1(+) on expression of the key stem cell pluripotency regulators, *Nanog*, *Pou5f1* and *Sox2*. Upon JQ1(+) treatment, the NANOG protein level was reduced to 25% ± 6% in TL1 cells and 20% ± 3% in R1 cells when compared with untreated cells, whereas the level in JQ1(-)-treated cells remained similar to untreated cells (Figure 2c and Supplementary Figure S1A). On the other hand, a very moderate reduction was observed for POU5F1 (87% ± 7% in TL1, 86% ± 8% in R1) and SOX2 (74% ± 13% in TL1, 76% ± 8% in R1) in JQ1(+)-treated cells when compared with untreated (Figure 2c and Supplementary Figure S1A). The protein level of c-MYC, a known BRD4 target in cancer cells, was also not markedly reduced by JQ1(+) treatment (72% ± 3% in TL1, 85% ± 5% in R1). *c-Myc* is dispensable in ESCs and in mouse embryos before E10.5,²⁰ suggesting that BRD4 regulates genes other than *c-Myc* in ESCs and during early mouse development. We therefore focused on the core ESC pluripotency regulators, *Nanog*, *Pou5f1* and *Sox2*. Importantly, JQ1(+) treatment of human ESCs also led to a clear reduction of NANOG protein level (Supplementary Figure S1B).

JQ1(+) inhibition of *Nanog* expression was confirmed by quantitative reverse transcription and real-time PCR (qRT-PCR) in both TL1 and R1 cells, in which JQ1(+) had no marked effect on *Pou5f1* and *Sox2* expression (Figure 2d and Supplementary Figure S1C). In addition, JQ1(-) treatment did not cause clear changes in the expression of any of these genes (Figure 2d and Supplementary Figure S1C).

To confirm that JQ1(+) promotes ESC differentiation, qRT-PCR was performed to analyze expression of several early differentiation markers in the untreated cells and cells treated with either JQ1(+) or JQ1(-). JQ1(+) treatment resulted in a selective upregulation of trophodermal makers *Cdx2* and *Hand1* in both TL1 and R1 cells (Figure 2e and Supplementary Figure S1D), a finding consistent with the observation that *Nanog* knockdown leads to mouse ESCs differentiating into the extraembryonic endoderm/trophoderm lineages.^{15,17} Induction of these differentiation markers by JQ1(+) also indicated that BRD4 inhibition was targeting expression of specific genes, that is, it did not result in a global inhibition of transcription. On the other hand, it is important to note that the induction of the differentiation markers shown in Figure 2e and Supplementary Figure S1D could also result from the JQ1(+) inhibition of other BET proteins, which is addressed in the small interfering RNA (siRNA) experiments (see below). Collectively, these data demonstrate that JQ1(+) impairs ESC maintenance, leading to ES cell differentiation.

***Brd4* knockdown and a BRD4 dominant-negative inhibitor abolish *Nanog* expression and ESC maintenance.** To rule out effects of JQ1(+) on other BET proteins and to confirm the functional requirement for *Brd4* in ESCs, we

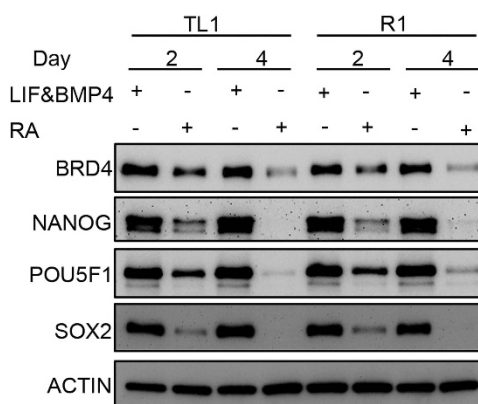


Figure 1 BRD4 is downregulated during ESC differentiation. TL1 and R1 ESCs were cultured in the presence of 1000 U/ml LIF and 10 ng/ml human BMP4 or in the presence of 1 μ M RA without LIF and BMP4 for 48 or 96 h to induce differentiation. Whole-cell lysates were immunoblotted using the indicated antibodies. ACTIN was used as a loading control. This experiment was performed at least three times with similar results

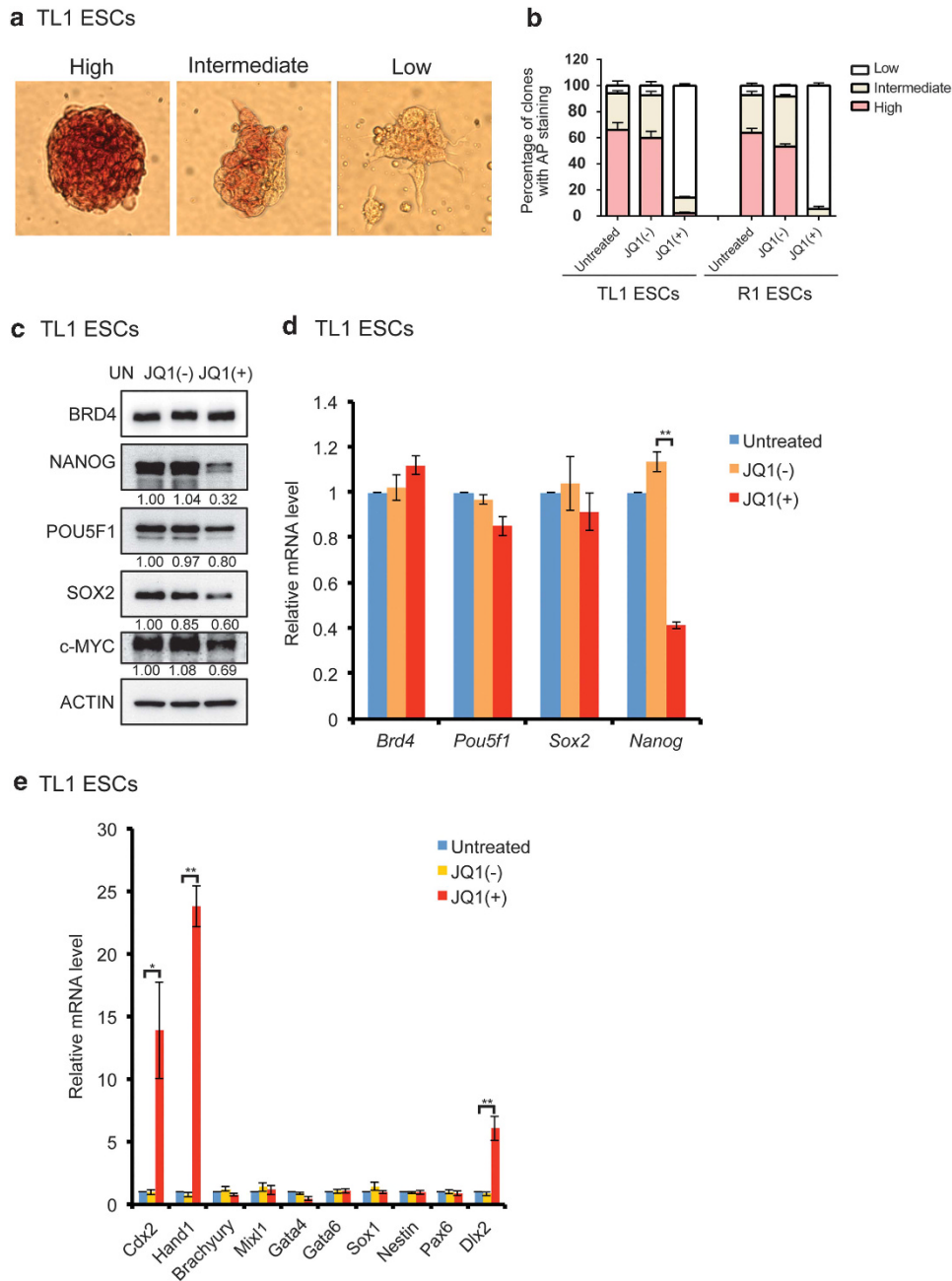


Figure 2 JQ1 (+) impairs ESC maintenance. (a) Representative images of ESC colonies showing high, intermediate or low AP activity. (b) TL1 and R1 ESCs were either untreated, treated with 300 nM of JQ1 (-) or JQ1 (+) for 48 h. The percentage of ESC colonies with high, intermediate and low AP activity was quantified from > 100 colonies. (c) TL1 ESCs were prepared as in b. Whole-cell lysates were immunoblotted with the indicated antibodies. ACTIN was used as a loading control. UN, untreated cells. This experiment was performed at least three times with similar results. (d and e) TL1 ESCs were treated as in b for 24 h (in d) or 48 h (in e). The mRNA levels were measured by RT-qPCR and normalized to *Gapdh* mRNA levels. The mRNA levels in untreated cells were set as 1. All error bars represent S.E.M. from three independent experiments. * $P < 0.05$, ** $P < 0.01$

knocked down *Brd4* transcripts using two different siRNAs. As complete loss of *Brd4* prevents ESC proliferation,¹¹ we suppressed *Brd4* expression using a siRNA concentration that partially knocked down *Brd4* transcripts and proteins in TL1 and R1 cells (Figures 3a–c, Supplementary Figures S2A–S2C). We also confirmed that partial *Brd4* knockdown induced by these siRNAs only moderately inhibited proliferation of either TL1 or R1 cells (Supplementary Figure S3).

Consistent with the observation in JQ1 (+)-treated cells, *Brd4* knockdown greatly reduced NANOG protein (41% ± 6% in TL1, 65% ± 3% in R1) or transcripts in ESCs, but there was very little decrease in the amount of POU5F1 protein (93% ± 4% in TL1, 94% ± 2% in R1) and SOX2 protein (99% ± 2% in TL1, 94% ± 7% in R1) or these transcripts in both ESC lines (Figures 3a and b, Supplementary Figures S2A and S2B). In addition, *Brd4* knockdown in TL1 and R1

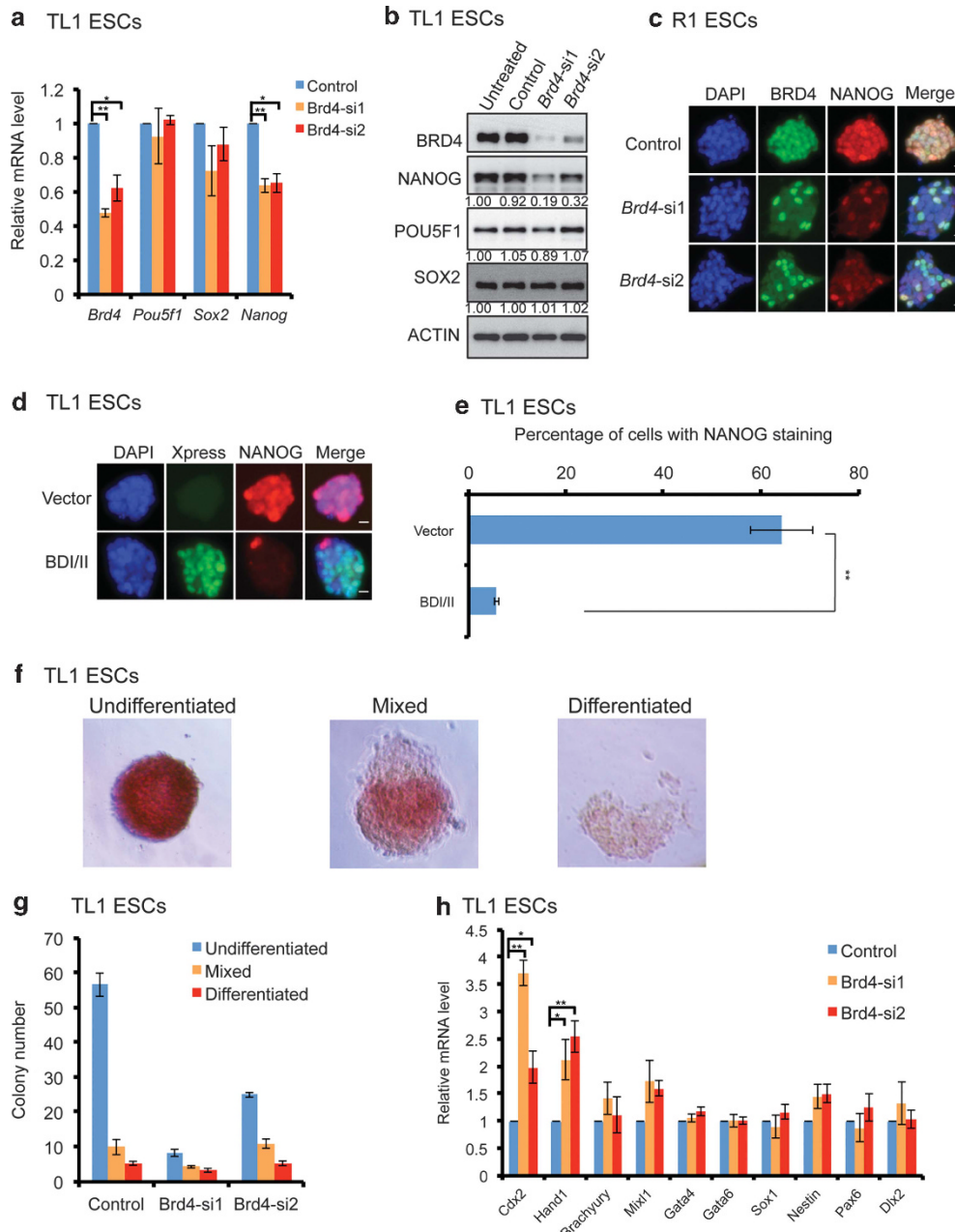


Figure 3 BRD4 inhibition abolishes *Nanog* expression in ESCs. (a) TL1 ESCs were transfected with control siRNA, *Brd4* siRNA 1 or *Brd4* siRNA 2. At 48-h post-transfection, the mRNA levels were measured by RT-qPCR and normalized to *Gapdh* mRNA levels. The mRNA levels in control cells were set as 1. (b) TL1 ESCs were either untreated or transfected as in a. At 48-h post-transfection, whole-cell lysates were extracted and immunoblotted with the indicated antibodies. Protein signal intensity was quantified using ImageJ. ACTIN was used as a loading control. This experiment was performed at least three times with similar results. (c) R1 ESCs were transfected as in (a). At 48-h post-transfection, the cells were co-stained with BRD4 and NANOG antibodies and counterstained with DAPI. Bar, 10 μ m. (d and e) TL1 ESCs carrying a BDI/II-inducible expression cassette or an empty vector were treated with 0.1 μ g/ml doxycycline for 24 h to induce BDI/II expression. The cells were co-stained with Xpress and Nanog antibodies and counterstained with DAPI. Bar, 10 μ m. (e) The percentage of cells with positive Nanog signal was quantified from > 100 cells. (f) Representative images of undifferentiated, mixed and differentiated TL1 ESC colonies. (g) TL1 ESCs were transfected as in a. The number of colonies generated from 500 seeded cells was quantified at day 6 post-transfection. (h) TL1 ESCs were transfected as in a. At 72-h post-transfection, the mRNA levels were measured by RT-qPCR and normalized to *Gapdh* mRNA levels. The mRNA levels in control cells were set as 1. Values in (a), (e), (g) and (h) represent the average of three independent experiments with error bars indicating S.E.M. * $P < 0.05$, ** $P < 0.01$

cells grown in 2i/LIF condition also led to a clear reduction of NANOG protein (Supplementary Figure S2D).

We also performed microarray analysis to examine the global gene expression changes induced by *Brd4* knockdown in mESCs (GEO accession number: GSE59142). As anticipated,

we identified a large number of genes differentially regulated by *Brd4* knockdown (Supplementary Figure S4). Among the core regulators of ESC pluripotency, *Nanog* was the only one inhibited by *Brd4* knockdown whereas expression of *Sox2* and *Pou5f1* was unaltered by *Brd4* knockdown. We also

compared the differentially expressed genes identified in the *Brd4* knockdown microarray analysis with the *Nanog* knockdown data (GEO accession number: GSE26520)²¹ and found a striking overlap between the lists of genes downregulated by *Brd4* knockdown and *Nanog* knockdown (P -value = 5×10^{-11}) (Supplementary Figure S5 and Supplementary Table S1).

To detect specifically the reduction of NANOG protein in *Brd4* knocked-down cells, we performed NANOG immunostaining in cells treated with either control or *Brd4*-targeting siRNAs. This approach showed that *Nanog* expression was specifically inhibited in *Brd4*-depleted TL1 and R1 cells, whereas neighboring BRD4-positive cells showed a normal level of NANOG protein (Figure 3c and Supplementary Figure S2C). In addition, *Brd4* knockdown in these cells did not cause much of a decrease in SOX2 protein (Supplementary Figure S6), confirming that *Brd4* knockdown cells were viable and that *Brd4* knockdown caused specific inhibition of *Nanog* expression.

We also used a BRD4 dominant-negative inhibitor encoding the BRD4 double bromodomains (BDI/II) to confirm further BRD4's role in controlling *Nanog* expression in ESCs. We previously demonstrated that the BDI/II inhibitor binds to acetylated histones and competitively dissociates BRD4 from chromatin without affecting the closely related BRD2.²² Immunostaining analysis showed that *Nanog* expression was specifically inhibited in cells expressing the BDI/II molecule (Figures 3d and e).

As NANOG is important for maintaining the propagation of ESCs in an undifferentiated pluripotent state,^{12–17} we examined how *Brd4* knockdown-induced *Nanog* repression affected ESC proliferation. For both TL1 and R1, the large majority of cells grew into undifferentiated ES colonies, in which all cells displayed a high level of AP activity (Figure 3f, undifferentiated). There was also a small number of ESC colonies that either contained a mixture of AP-positive and AP-negative cells (Figure 3f, mixed) or cells that were completely negative for AP activity (Figure 3f, differentiated). Compared with the control siRNA-treated cells, cells treated with either *Brd4* siRNA showed a markedly reduced number of 'undifferentiated' colonies without drastically affecting the 'mixed' and 'differentiated' population (Figures 3f and g, and Supplementary Figure S2E), indicating that *Brd4* is crucial for maintaining the growth and/or seeding efficiency of undifferentiated ESC colonies. In addition, qRT-PCR analysis revealed that *Brd4* knockdown in ESCs also resulted in a selective upregulation of *Cdx2* and *Hand1* (Figure 3h and Supplementary Figure S2F). Collectively, the data show that *Brd4* knockdown inhibited *Nanog* expression and prevented ESC proliferation in the undifferentiated stage. In line with the observation that BRD4 was markedly reduced during ES differentiation (Figure 1), the specific inhibition of undifferentiated cells by *Brd4* knockdown suggested an important role of Brd4 in the maintenance of undifferentiated ESCs. Similar to JQ1(+) treatment, *Brd4* knockdown showed little inhibitory effect on *Pou5f1* and *Sox2*, consistent with BRD4 specifically regulating *Nanog* expression and that *Brd4* inhibition does not result in a global inhibition of transcription.

***Brd4* knockdown downregulates *Nanog* in preimplantation mouse embryos.** During preimplantation development, *Nanog* mRNA is first detected at the four-cell stage,²³ whereas NANOG protein is first detected at the morula stage.¹⁵ As JQ1(+) and *Brd4* siRNAs decrease *Nanog* expression in ESCs, we asked whether BRD4 regulates *Nanog* expression during preimplantation development. We first analyzed the expression profile of *Brd4* in different stages of preimplantation embryos (Supplementary Figure S7A). BRD4 was observed at all stages of preimplantation development. A similar pattern of *Brd4* expression in oocytes to two-cell stage embryos was observed in a previous study.²⁴

To determine whether BRD4 regulates *Nanog* expression during preimplantation development, we performed *Brd4* knockdown by microinjecting control or *Brd4* siRNA into two-cell mouse embryos. The embryos were then cultured to the late morula or blastocyst stages and stained for both BRD4 and NANOG proteins. In *Brd4* siRNA-treated embryos, the amount of BRD4 protein was efficiently reduced in all blastomeres (Figure 4). Although the embryos microinjected with control siRNA showed a normal level of NANOG protein, nearly no NANOG signal was detected in blastomeres in which BRD4 was substantially reduced (Figure 4). In contrast, robust POU5F1 signals were detected in *Brd4* siRNA-treated late morula stage embryos (Supplementary Figure S7B), confirming that these embryos are viable. Compared with control embryos, a slight reduction of POU5F1 signal was observed in *Brd4* siRNA-treated embryos (Supplementary Figure S7B). This finding may reflect the marked repression of *Nanog* expression induced by *Brd4* knockdown as *Nanog* null ICMs also demonstrate reduced level of POU5F1 staining.¹⁴ Interestingly, all the embryos, including those treated with *Brd4* siRNA, developed to the blastocyst stage and appeared morphologically normal (Supplementary Figure S7C); there was, however, a delay in development of *Brd4* knockdown embryos, which were smaller and had fewer ICM cells (Figure 4 and Supplementary Figures S7B and C). These results are consistent with a previously published study showing that *Brd4* homozygous null embryos isolated at E3 were morphologically indistinguishable from wild-type blastocysts.¹⁰ After *in vitro* culture for 2 days, explants grown from *Brd4* homozygous null embryos appeared smaller than wild-type embryos but otherwise showed normal morphology.¹⁰ Similarly, E4.5 *Nanog* null mouse embryos carried blastocysts containing reduced ICM cells than wild-type embryos.^{14,15} The inhibition of *Nanog* expression by *Brd4* knockdown during early mouse embryo development was consistent with the observations made in ESCs (Figure 3 and Supplementary Figure S2). Collectively, these studies provide evidence that *Brd4* regulates *Nanog* expression not only in ESCs but also in mouse embryos.

BRD4 occupies regulatory regions of the *Nanog* gene in ESCs. Both JQ1(+) and *Brd4* siRNA inhibition of *Brd4* expression reduced *Nanog* expression in ESCs, suggesting that BRD4 contributes to *Nanog* transcriptional regulation. To understand the molecular mechanism underlying BRD4's function, we investigated whether *Nanog* is a direct BRD4 target by performing chromatin immunoprecipitation (ChIP)

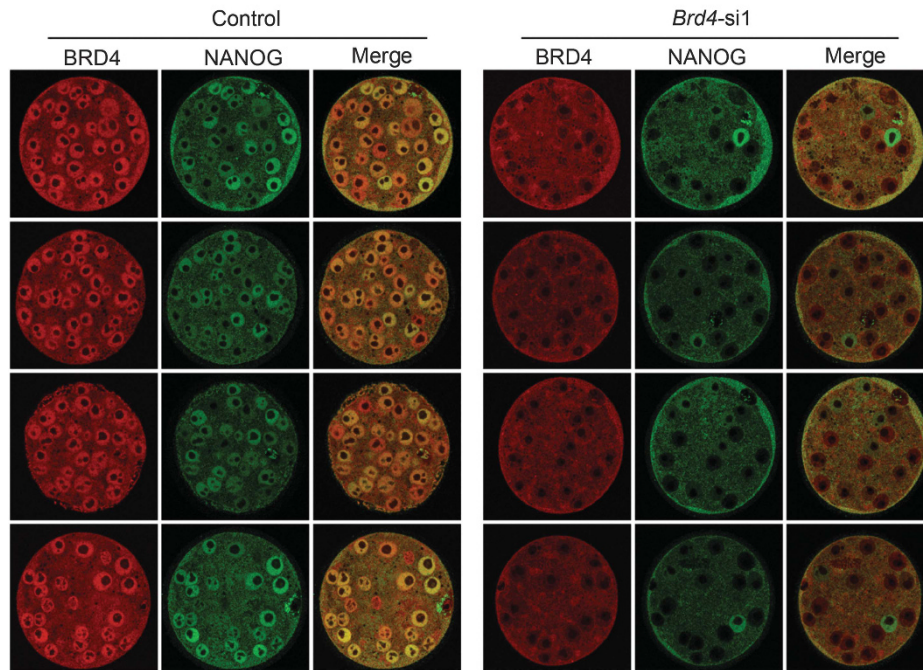
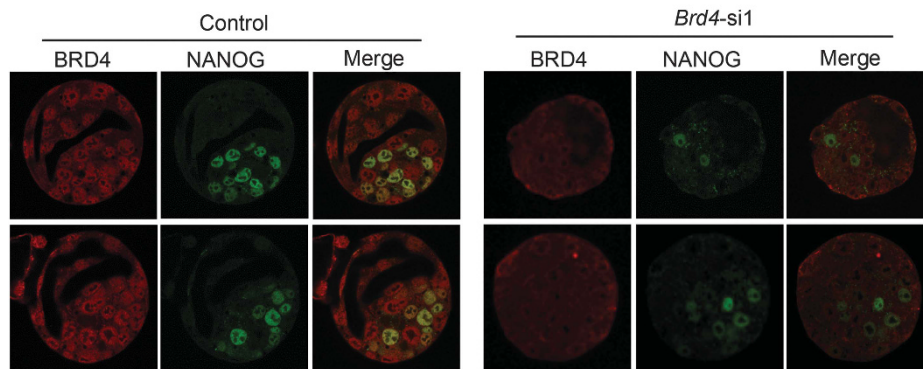
a Late morula stage**b** Blastocyst stage

Figure 4 *Brd4* knockdown reduces NANOG levels in preimplantation embryos. Two-cell embryos were microinjected with control siRNA or *Brd4* siRNA 1 and cultured to the late morula (**a**) or blastocyst (**b**) stage. Immunofluorescence was performed for BRD4 (red) and NANOG (green). The experiment was performed three times using at least 20 embryos per group. Representative images are shown

using an affinity-purified Brd4 antibody.²⁵ The proximal promoter (−188) and distal enhancer (−4546) regions of *Nanog* gene have been defined previously.^{26–28} Primers spanning these regions were used in ChIP-qPCR assays to detect the binding of BRD4 (Figure 5a). Based on a published study,²⁹ we also designed primer pairs for two additional *Nanog* regulatory regions (−951 and −3329). In addition, a pair of primers targeting a region ~6000-bp downstream of *Nanog* transcription start site was used to show Brd4 binding outside the *Nanog* gene (Figure 5a, +6001).

As JQ1(+) promotes BRD4 dissociation from chromatin, we tested how it affects BRD4 binding to the *Nanog* locus. BRD4 ChIPs were performed for both TL1 and R1 cells treated with 500 nM of either JQ1(+) or JQ1(−) for 6 h, at

which time JQ1(+) does not have a significant effect on cell proliferation (Supplementary Figure S8). In JQ1(−)-treated cells, BRD4 was specifically enriched at the previously established *Nanog* core proximal promoter and distal enhancer regions (Figure 5b and Supplementary Figure S8A). In contrast, BRD4 binding at the +6001 region was much lower in both TL1 and R1 cells (Figure 5b and Supplementary Figure S9). JQ1(+) treatment efficiently inhibited BRD4 binding to the *Nanog* promoter and enhancer regions (Figure 5b and Supplementary Figure S9). These data support the observation that JQ1(+) inhibits *Nanog* expression in ESCs (Figure 2 and Supplementary Figure S1) and further demonstrate that Brd4 is recruited to the *Nanog* promoter and enhancer regions to support *Nanog* expression, thereby identifying *Nanog* as a novel BRD4 target gene in ESCs.

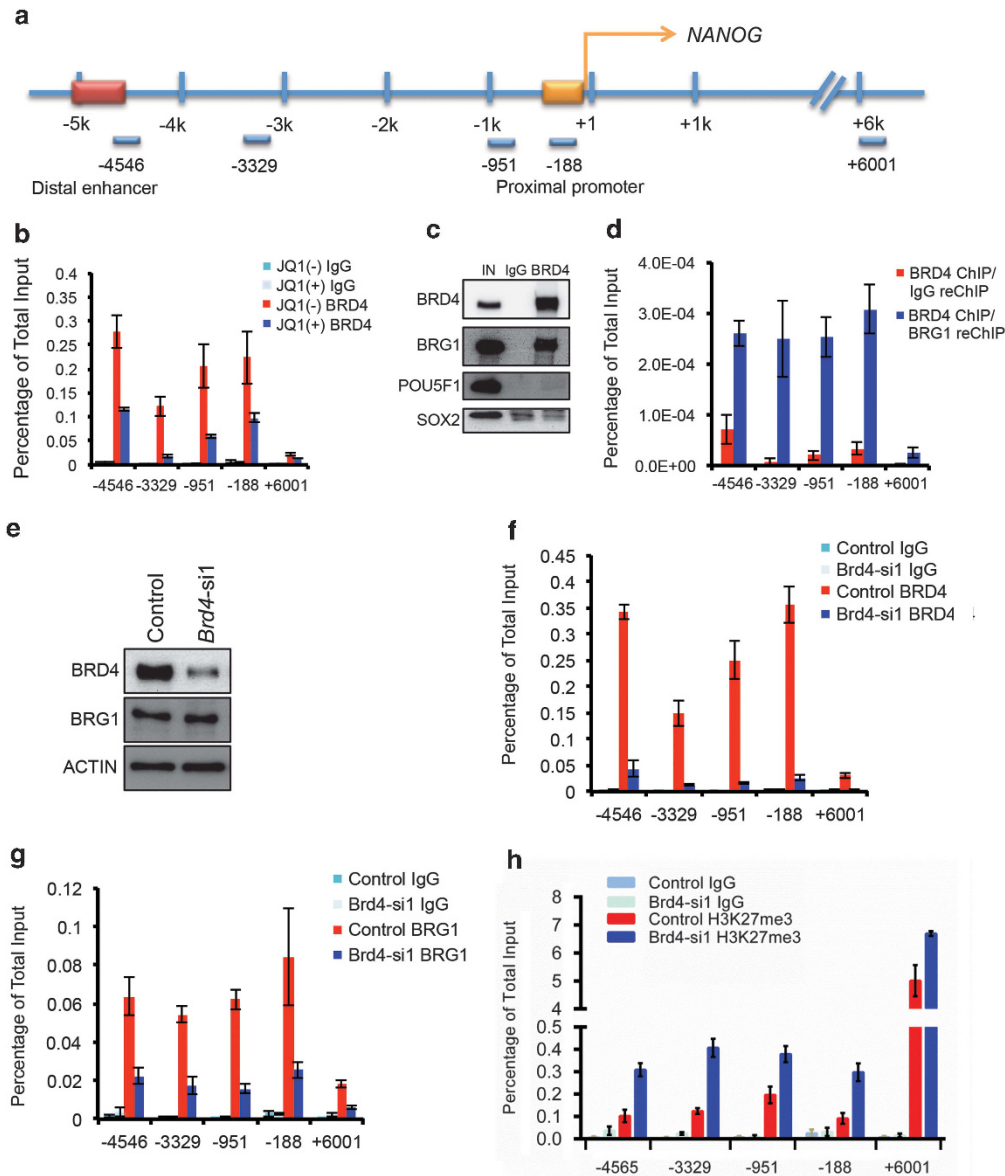


Figure 5 BRD4 interacts with BRG1 in the *Nanog* regulatory regions to regulate its expression in ESCs. (a) Schematic diagram of the *Nanog* promoter showing regions analyzed in ChIP. (b) R1 cells were treated with 500 nM JQ1(–) or JQ1(+) for 6 h and subjected to the ChIP assay with normal rabbit IgG or BRD4 antibody. ChIP samples were analyzed by qPCR using primers targeting the regions indicated in a. (c) R1 nuclear extracts immunoprecipitated with normal rabbit IgG or the BRD4 antibody were immunoblotted with the indicated antibodies. IN, input. (d) For the ChIP-reChIP assays using R1 ESCs, the primary ChIP with BRD4C antibody was followed by a secondary ChIP (reChIP) using either IgG or a BRG1 antibody. (e) R1 ESCs were transfected with control siRNA or *Brd4* siRNA 1. At 48-h post-transfection, whole-cell extracts were extracted and immunoblotted with the indicated antibodies. ACTIN was used as a loading control. (f–h) R1 ESCs were transfected as in e. At 48-h post-transfection, the cells were subjected to ChIP assay with normal rabbit IgG, BRD4C antibody (f), BRG1 antibody (g) or H3K27me3 antibody (h). All ChIP values represent the average of three independent experiments with error bars indicating S.E.M.

BRD4 regulates *Nanog* expression through association with BRG1. We further examined the molecular mechanism by which BRD4 regulates *Nanog* expression. In mouse ESCs, BRG1, which is the ATPase subunit of an ESC-specific chromatin-remodeling complex,³⁰ has critical roles in transcriptional regulation of key factors in ESC self-renewal and pluripotency.^{30,31} We identified BRG1 as a BRD4-interacting protein by mass spectrometry (Supplementary Figure S10A). As BRG1 binds to the *Nanog* promoter to support gene expression,^{30,31} we speculated that BRD4 may

regulate *Nanog* expression through interaction with BRG1. We then performed co-immunoprecipitation to confirm the interaction between BRD4 and BRG1 in ESCs (Figure 5c). The interaction between BRD4 and BRG1 was also validated in an embryonic cancer stem cell line (Supplementary Figure S10B).

BRD4 binds to the same regions in the *Nanog* locus as those published for BRG1 (Figure 5b, Supplementary Figure S9 and Kidder *et al.*³¹). Accordingly, we tested if BRD4 and BRG1 co-occupy the *Nanog* regulatory regions in ESCs.

Sequential ChIP (ChIP-reChIP) showed that BRG1 was specifically enriched with BRD4 in the *Nanog* regulatory regions (Figure 5d). We also tested if BRD4 supported BRG1 recruitment to the *Nanog* regulatory regions. ChIP assays were performed using ESCs transfected with control or *Brd4* siRNA to determine how *Brd4* knockdown affected BRG1 binding to the *Nanog* regulatory regions. Western blotting showed that *Brd4* knockdown did not affect BRG1 expression (Figure 5e). As expected, binding of BRD4 to the *Nanog* regulatory regions was greatly reduced following *Brd4* knockdown (Figure 5f). More importantly, BRG1 binding to the *Nanog* regulatory regions was markedly reduced when *Brd4* was knocked down (Figure 5g). This result suggests that BRD4 is important for recruiting BRG1 to their common binding sites at the *Nanog* locus. It is also possible that inhibition of *Nanog* expression by *Brd4* knockdown reduces BRG1 recruitment to the *Nanog* regulatory regions through other mechanism(s).

BRG1 activates pluripotent gene expression by binding to the target genes and preventing localized trimethylation of Lys27 of histone 3 (H3K27me3), which is commonly associated with gene repression.³⁰ This BRG1 function prompted us to test how *Brd4* knockdown-mediated inhibition of BRG1 recruitment to the *Nanog* regulatory regions affects H3K27me3 modifications at this site. ChIP assays showed that in control cells the repressive H3K27me3 modification was present at the *Nanog* regulatory regions at relatively low levels compared with the +6001 region (Figure 5h). Interestingly, the higher level of H3K27me3 at the +6001 region was inversely correlated with the low level of BRD4 and BRG1 detected in this region. *Brd4* knockdown led to an increase of H3K27me3 at the *Nanog* regulatory regions (Figure 5h). As BRG1 has been shown to repress H3K27me3 silencing modifications at its target genes genome wide,³⁰ our observations suggest that when BRG1 recruitment was inhibited by *Brd4* knockdown, H3K27me3 expanded into the *Nanog* regulatory regions, leading to formation of repressive chromatin and transcriptional silencing. Collectively, these studies suggest that BRD4 associates with BRG1 chromatin-remodeling protein in the *Nanog* regulatory regions to inhibit H3K27me3 silencing modifications, thereby promoting *Nanog* expression in ESCs.

Discussion

We report here that the amount of BRD4 protein is markedly decreased during mouse ESC differentiation, suggesting that BRD4 has an important role in undifferentiated ESCs. We further demonstrate that BRD4 specifically regulates the pluripotency transcription factor *Nanog* in ESCs. BRD4 inhibitors and *Brd4* knockdown cause great inhibition of *Nanog* expression, leading to impaired ESC maintenance. More importantly, *Brd4* knockdown markedly inhibits *Nanog* expression in the late morula and blastocyst stage embryos. Together, these studies demonstrate that BRD4 is critical for regulating *Nanog* expression both in ESCs and during preimplantation development.

In mouse embryos, NANOG functions primarily during formation and expansion of the ICM.¹⁶ *Nanog*-null embryos are not able to establish a pluripotent ground state because

Nanog-deficient ICMs do not generate pluripotent cells.¹⁴ BRD4's role in *Nanog* gene expression unmasked in this study therefore provides an explanation for ICM degeneration in *Brd4* knockout mice,¹⁰ revealing a novel function for Brd4 in preimplantation embryo development.

In addition to maintaining pluripotency in ESCs, *Nanog* overexpression is observed in a large number of cancers, where it not only correlates with a poor clinical outcome but also regulates cancer stem cell sub-populations.³² We observe BRD4 binding to *Nanog* regulatory regions and regulation of *Nanog* expression in two embryonic cancer stem cell lines, P19 and NTERA-2 (Supplementary Figure S11), suggesting that this function of BRD4 could be preserved during cancer development. This finding is important in light of recent studies revealing that stem cell regulatory networks frequently contribute to the ESC-like phenotype displayed by many aggressive tumors.^{33,34}

We also find that BRD4 is recruited to *Nanog* promoter and enhancer regions and this occupancy is inhibited by either JQ1(+) or *Brd4* knockdown. These results are consistent with recent studies showing that Brd4 binds to the 'super-enhancer' regions of key cell identity genes.^{3,35} We find that BRD4 complexes with BRG1 at the *Nanog* locus and inhibits H3K27me3 silencing modifications to promote *Nanog* expression. Currently, very little is known about how BRD4 function is coupled to chromatin remodeling besides the fact that it binds to acetylated histones. The interplay between BRD4 and the epigenetic reprogramming factor BRG1 in regulating *Nanog* expression described here provides new insights for understanding the epigenetic event that modulates BRD4 function in ESCs. In line with our finding, BRD4 and BRG1 were recently reported to be enriched in the same regions of super-enhancers in ESCs.⁹ As a result the BRG1-associated SWI/SNF chromatin-remodeling complex is the most frequently mutated chromatin-regulatory complex in human cancer,³⁶ it will be important to determine if the tumor suppression function of the BRG1 complex is abrogated in BRD4-associated cancers.

In summary, this study identifies *Nanog* as a novel target gene regulated by BRD4. The functional interaction between BRD4 and BRG1 provides novel insights for the mechanism by which BRD4 functions in transcription regulation as well as the molecular event that regulates *Nanog* expression in ESCs. Many epigenetic readers such as BRD4 recognize various histone modifications, and translate the 'histone codes' into gene expression/repression, providing a tight control for cell proliferation. Dysfunction of this process frequently leads to cancers and other human diseases, underscoring the biological significance of epigenetic signaling. By elucidating the biological function of the important cancer target BRD4, our findings lay the groundwork for understanding how dysregulation of BRD4 function could contribute to cancer development.

Materials and Methods

Cell culture. Mouse TL1 and R1 ESCs (kindly provided by Dr. Mitchell J Weiss, the Children's Hospital of Philadelphia, Philadelphia, PA, USA) were maintained feeder-free on gelatin-coated tissue culture dishes in Knockout DMEM/F12 medium (Invitrogen, Life Technologies, Grand Island, NY, USA) with 0.5% N-2 supplement (Invitrogen), 1% B27 supplement without vitamin A (Invitrogen),

0.05% BSA (Invitrogen), 1% GlutaMAX (Invitrogen), 10 ng/ml human BMP4 (R&D, Minneapolis, MN, USA), 1000 U/ml LIF (Millipore, Bedford, MA, USA), 1.5×10^{-4} M 1-thioglycerol (Sigma, St. Louis, MO, USA), 100 nM FGF receptor inhibitor PD173074 (Sigma) and 1% Pen-Strep as described previously.^{15,37,38} For the 2i/LIF condition, ESCs were also maintained feeder-free on gelatin-coated tissue culture dishes in Knockout DMEM/F12 medium (Invitrogen) with 0.5% N-2 supplement (Invitrogen), 1% B27 supplement without vitamin A (Invitrogen), 0.05% BSA (Invitrogen), 1% GlutaMAX (Invitrogen), 1.5×10^{-4} M 1-thioglycerol (Sigma), 1000 U/ml LIF (Millipore), 1 μ M PD0325901 and 3 μ M CHIR99021 as described previously.³⁹ The ESCs were cultured on gelatin-coated plates and propagated by trypsinization and re-plating at a ratio of 1 to 10 every 2 or 3 days. All cell lines were depleted of feeder cells and transitioned to serum-free medium over several passages before experiments as described.⁴⁰ HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal calf serum (FBS) (Hyclone, Logan, UT, USA) or 10% Tet system approved FBS (Clontech, Mountain View, CA, USA). P19 Cells (kindly provided by Dr. M Celeste Simon, University of Pennsylvania, Philadelphia, PA, USA) were maintained in α MEM medium (Invitrogen) with 7.5% newborn calf serum (Hyclone) and 2.5% FBS (Hyclone).

siRNA knockdown. The non-targeting control siRNA (D-001210-01), *Brd4* siRNA 1 (D-004937-02) and *Brd4* siRNA 2 (D-004937-04) were purchased from Dharmacon (Thermo Scientific, Hudson, NH, USA). For siRNA-mediated knockdown, ESCs were transfected during subculture using DharmaFECT 2 (Thermo Scientific) following the manufacturer's instructions. At 18- to 24-h post-transfection, the medium was changed to ESC medium without FGF receptor inhibitor PD173074 (Sigma). P19 cells were transfected during subculture using DharmaFECT 3 (Thermo Scientific). All transfections were performed following the manufacturer's instructions.

Retinoic-acid-induced differentiation. ESCs were trypsinized and plated on gelatin-coated plates in ESC medium. The next day, the medium was replaced with ESC medium without LIF and BMP4 but with 1 μ M RA (Sigma). Cells were cultured for an additional 48 or 96 h with one medium change.

Colony formation assays. For JQ1(+) experiment, ESCs were trypsinized to obtain a single-cell suspension and plated at 150 000 cells per well in six-well plate using Knockout DMEM/F12 medium (Invitrogen) with 0.5% N-2 supplement (Invitrogen), 1% B27 supplement without vitamin A (Invitrogen), 0.05% BSA (Invitrogen), 1% GlutaMAX (Invitrogen), 10 ng/ml human BMP4 (R&D), 1000 U/ml LIF (Millipore) and 1.5×10^{-4} M 1-thioglycerol (Sigma). After 2 days of culture, plates were stained for AP using the Alkaline Phosphatase Staining Kit (Sigma) following the manufacturer's recommendation. For *Brd4* knockdown experiments, siRNA-transfected cells were seeded at 500 cells per well in 12-well plates. The AP staining was performed after 6 days of culture.

Immunoprecipitation and western blot analysis. These analyses were performed using standard protocols. Additional experimental details are provided in Supplementary Information Materials and Methods.

ESC immunofluorescent staining. Cells cultured on chamber slides were fixed with 3% paraformaldehyde in PBS for 20 min. Immunofluorescent staining was performed as previously described.⁴¹ The following primary antibodies were used: anti-BRD4C (recognizes BRD4 aa 1313–1362, 1:20 000), anti-SOX2 (1:1000, MAB2018, R&D), anti-NANOG (1:1000, ab80892, Abcam, Cambridge, UK), anti-Nanog (1:1000, 560259, BD Pharmingen, San Diego, CA, USA) and anti-Xpress (1:1000, R910–25, Invitrogen). The secondary antibodies used were Alexa Fluor 594 goat anti-rabbit IgG (1:1000, A11012, Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG (1:500, A11001, Invitrogen). All immunofluorescent images were captured using an inverted fluorescence microscope (Olympus, IX81, Tokyo, Japan) as described previously.²² The scale bars were added using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Quantitative reverse transcription and real-time PCR. Total RNA was isolated using NucleoSpin RNA II Kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's instruction. Reverse transcription was performed using a 20 μ l reaction mixture containing 350 ng of total RNA, oligo(dT) primer (Invitrogen), dNTP (Invitrogen) and M-MLV reverse transcriptase (Invitrogen).

Quantitative real-time PCR was performed using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with IQ SYBR Green supermix (Bio-Rad). mRNA level of each gene was normalized to *Gapdh* mRNA level. Primer sequences are shown in Supplementary Table S2.

ChIP and ChIP-reChIP. ChIP assay using TL1, R1 or P19 cells were performed based on published methods with the modifications indicated below.^{42,43} Formaldehyde was added directly to cell culture media at a final concentration of 1%. Fixation was completed after incubation for 10 min at room temperature and stopped by adding glycine to a final concentration of 0.125 M. Cells were scraped, collected, centrifuged and swelled in cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 1% NP-40, 0.1 mM PMSF and 1 μ g/ml leupeptin, aprotinin and pepstatin A). After 1-h incubation on ice, nuclei were collected by centrifugation at 3200 g for 10 min in 4 °C, resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA, 0.1 mM PMSF, and 1 μ g/ml leupeptin, aprotinin and pepstatin A), and incubated on ice for 10 min. Samples were sonicated on ice to an average of 500-bp DNA length and centrifuged at 20 000 g. The chromatin solution was pre-cleared with Staph A cells (pre-blocked with 1 mg/ml sheared herring sperm DNA and 1 mg/ml BSA at 4 °C overnight) for 15 min at 4 °C. Chromatin from about 10⁷ cells was incubated with 3 μ g of normal rabbit IgG (Upstate, Millipore), an affinity-purified rabbit polyclonal antibody generated in our lab for BRD4C (recognizes BRD4 aa 1313–1362), an anti-Brg1 antibody (J1, kindly provided by Dr. Weidong Wang, National Institute on Aging, NIH, Baltimore, MD, USA) or H3K27me3 antibody (07–449, Millipore). After rotating at 4 °C overnight, chromatin and antibody complexes were immunoprecipitated by mixing with pre-blocked Staph A cells at 4 °C for 15 min. Immunoprecipitates were centrifuged at 20 000g for 5 min. The supernatant from the normal rabbit IgG antibody sample was saved as total chromatin input. Staph A immuno-complexes were washed twice with dialysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.2% Sarkosyl and 0.1 mM PMSF) and four times with ChIP wash buffer (100 mM Tris, pH 9.0, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid and 0.1 mM PMSF). Immunocomplexes were eluted from the Staph A cells using elution buffer (50 mM NaHCO₃ and 1% SDS). Crosslinks were reversed by adding NaCl to the eluted supernatants to a final concentration of 300 mM and incubating at 67 °C overnight. RNA was removed by incubation with RNase A at 37 °C for 30 min. DNA samples were purified using PCR purification kit (Qiagen, Gaithersburg, MD, USA) and eluted in 50 μ l EB buffer. Real-time PCR was performed as described above using primers listed in Supplementary Table S2. Two micro-liters of IPed DNA or 1/50 of total input chromatin was used as templates for PCR reaction.

For ChIP-reChIP assay, the first ChIP was performed using BRD4C antibody as described above. For the reChIP experiments, protein–DNA complexes isolated from the first ChIP were washed with dialysis buffer and washing buffer described in the ChIP protocol. Complexes were eluted by incubation with elution buffer (50 mM Tris-HCl pH 8.0 and 50 mM EDTA supplemented with 1% SDS and protease inhibitors) for 15 min at 65 °C. After centrifugation, the supernatants were diluted 10 times with ChIP dilution buffer supplemented with 20 μ g GST-BRD4CTD described above, 50 μ g of BSA and protease inhibitors. After rotating at 4 °C for 2 h, the chromatin solution was pre-cleared with Staph A cells for 15 min at 4 °C and centrifuged at 20 000 g for 5 min at 4 °C. The supernatants were then subjected to the second ChIP procedure.

Oocyte and embryo collection, culture and microinjection. Full-grown germinal vesicle (GV)-intact oocytes, metaphase II (MII)-arrested eggs and preimplantation embryos were collected from 6- to 8-week-old CF1 female mice (Harlan, Indianapolis, IN, USA). GV oocytes were collected from mice 46–48 h after injection with 5 IU eCG. For MII-egg and embryo collection, female mice were injected with 5 IU eCG followed by 5 IU hCG 46–48 h later. MII eggs were obtained from the oviducts 13–15 h after hCG injection. For embryo collection, after hCG administration, females were mated to B6D2F1/J males (Jackson Laboratory, Bar Harbor, ME, USA). One-cell, two-cell, four-cell, eight-cell and blastocyst stage embryos were flushed from the oviducts/uteri of superovulated and mated mice 20, 44, 61, 68 and 96-h post-hCG, respectively. The collection medium was bicarbonate-free minimal essential medium (Earle's salt) supplemented with 3 mg/ml of polyvinylpyrrolidone (PVP) and 25 mM Hepes (MEM/PVP), pH 7.3. For oocyte collection, 2.5 μ M milrinone (Sigma-Aldrich, St. Louis, MO, USA) was added to the collection medium to prevent spontaneous resumption of meiosis. For the microinjection experiments, two-cell embryos were collected and cultured in KSOM medium containing amino acids

(KSOM + AA)⁴⁴ at 37 °C in a humidified atmosphere containing 5% CO₂ in air. One of the two blastomeres of a two-cell embryo was microinjected in MEM/PVP with ~5 μl of 20 μM non-target control siRNA (D-001210-01) or *Brd4* siRNA1 (D-004937-02). The injections were performed as previously described.⁴⁵ Microinjected embryos were returned to KSOM + AA and cultured for 2 days (morula) or 3 days (blastocyst) at 37 °C.

Embryo immunofluorescent staining and laser-scanning confocal microscopy. Immunofluorescence was performed as previously described,⁴⁶ except that the *zona pellucida* was not removed. Both anti-BRD4C (recognizes BRD4 aa 1313–1362) and anti-Nanog (560259, BD Pharmingen) antibodies were used at a 1:100 dilution. Anti-Pou5f1 antibody (sc-8628, Santa Cruz, Santa Cruz, CA, USA) was used at a 1:50 dilution. FITC-conjugated goat anti-mouse IgG1 (SouthernBiotech, Birmingham, AL, USA), Cy5-conjugated donkey anti-rabbit IgG and anti-goat IgG (both from Jackson ImmunoResearch, West Grove, PA, USA), all at a 1:100 dilution, were used as secondary antibodies. DNA was stained with 1 μM SYTOX Green (Life Technologies). Fluorescence was detected on a Leica TCS SP laser-scanning confocal microscope (Wetzlar, Germany). For each experiment, all images were taken at the same laser power and single confocal sections were collected.

JQ1 treatment of human ESCs. The H9 human ESC line was obtained from WiCell Research Institute (Madison, WI, USA) and was cultured feeder-free under standardized conditions in a humidified incubator at 37 °C/5% CO₂/5% O₂. Cells were plated onto Matrigel (BD Biosciences, San Diego, CA, USA) coated plates and grown in mTeSR 1 medium (Stemcell Technologies, Vancouver, BC, Canada). Cells were passaged every 4–6 days using Accutase (Innovative Cell Technologies, San Diego, CA, USA). For JQ1 treatment, H9 cells were passaged into six-well plates and grown for 2–3 days until cells were exponentially growing and small colonies were visible. The cells were then treated with 300 nM JQ1(+) or JQ1(-) in mTeSRTM1 media for 48 h. Untreated cells were used as a control. Media containing fresh compounds were replaced daily. After 48 h, cells were washed once in mTeSRTM1 media and harvested for western blot analysis.

Ethics statement. All procedures involving animals have been conducted as approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Conflict of Interest

The authors declare no conflict of interest.

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