Pluripotency transcription factors and Tet1/2 maintain Brd4-independent stem cell identity

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A robust network of transcription factors and an open chromatin landscape are hallmarks of the naive pluripotent state. Recently, the acetyllysine reader Brd4 has been implicated in stem cell maintenance, but the relative contribution of Brd4 to pluripotency remains unclear. Here, we show that Brd4 is dispensable for self-renewal and pluripotency of embryonic stem cells (ESCs). When maintained in their ground state, ESCs retain transcription factor binding and chromatin accessibility independent of Brd4 function or expression. In metastable ESCs, Brd4 independence can be achieved by increased expression of pluripotency transcription factors, including STAT3, Nanog or Klf4, so long as the DNA methylcytosine oxidases Tet1 and Tet2 are present. These data reveal that Brd4 is not essential for ESC self-renewal. Rather, the levels of pluripotency transcription factor abundance and Tet1/2 function determine the extent to which bromodomain recognition of protein acetylation contributes to the maintenance of gene expression and cell identity.

The interplay between transcription factors and the chromatin landscape is a critical determinant of lineage-specific gene expression programmes that define cell identity. In embryonic stem cells (ESCs), a network of transcription factors, including Oct4, Sox2 and Nanog, contributes to self-renewal and pluripotency. The ability of transcription factors to control gene expression can be amplified or repressed by histone and DNA modifications; in turn, transcription factors influence the expression and localization of chromatin-modifying proteins. Repressive chromatin modifications, such as methylation of DNA and certain histone lysine residues, have been reported to occlude transcription factor binding and block the ability of transcription factors to maintain transcriptional networks. By contrast, histone acetylation can promote the recruitment of transcription factors and bromodomain-containing proteins that are required for pluripotency.

Mouse ESCs cultured in conventional medium containing serum and leukaemia inhibitory factor (LIF; hereafter S/L) exhibit heterogeneous expression of pluripotency-associated transcription factors and levels of DNA methylation comparable to that observed in somatic cells. The addition of kinase inhibitors against MAP-kinase kinase (MEK) and glycosyn synthase kinase 3β (GSK-3β; ‘2i’) drives murine ESCs into a naive ‘ground state’ of pluripotency marked by homogenous expression of pluripotency-associated transcription factors and global DNA hypomethylation. Whereas a fraction of S/L-cultured ESCs can be considered naive, the majority is ‘metastable’ and prone to spontaneous differentiation. By contrast, 2i-cultured ESCs are homogenously naive and continuously self-renew in culture. Histone and DNA demethylation have been implicated in the establishment of the naive ground state, but the role of acetylation of either histones or transcription factors in maintaining naive pluripotency has been less clear.

Histone acetylation promotes gene expression in cis, by modulating charge-driven nucleosomal contacts, and in trans, by recruiting transcriptional co-activator complexes. Bromodomain and extraterminal (BET) domain-containing proteins, such as Brd4, bind to acetylated histone tails and acetylated transcription factors and recruit complexes, including the Mediator complex, that promote gene transcription. Inhibitors against BET family members inhibit gene expression and proliferation in cancer cells and ESCs. Brd4, in particular, has been reported to be essential for mouse post-implantation development and the culture of ESCs in vitro, suggesting that the reading of acetylation by BET family members is essential for the maintenance of embryonic pluripotency. However, whether Brd4 is essential for the maintenance of the naive pluripotent state remains an open question.

Results

Brd4 is dispensable in naive ground-state ESCs. Acetylation of histone residues such as H3K9 and H3K27 (H3K9ac and H3K27ac, respectively) decorates promoters of active genes and is associated with transcription. Analyses of chromatin immunoprecipitation followed by sequencing (ChIP–seq) data sets of H3K9ac and H3K27ac in metastable ESCs revealed that marks mediated by high promoter histone acetylation are enriched for pluripotency-associated gene signatures. We asked whether these marks are modulated in the ground state of pluripotency. As serum is lipid laden, traditional serum-free naive culture conditions (2i/L) impose a substantial burden for de novo lipid biosynthesis. To exclude possible confounding effects of serum on histone acetylation, which competes with lipid biosynthesis for cytosolic acetyl-coenzyme A, we compared histone acetylation in ESCs cultured in S/L with or without 2i, as 2i is sufficient to drive many of the developmental programmes that define cell identity.
The addition of 2i increased both H3K9ac and H3K27ac levels at key cis-regulatory loci including enhancers and promoters of pluripotency genes (Fig. 1b), prompting us to examine the role of Brd4 in the ground state of pluripotency. Genome-wide analyses of Brd4 binding confirmed that Brd4 binding correlated with H3K9ac and H3K27ac levels and revealed that 2i increased Brd4 binding to chromatin genome wide (Fig. 1c and Supplementary Fig. 1a,b). The genes associated with the largest Brd4 peaks were enriched for pluripotency gene signatures (Supplementary Fig. 1c) and included key pluripotency loci, such as the Nanog enhancer and the Pou5f1 promoter (Fig. 1d).

Treating ESCs with small-molecule inhibitors of Brd4, such as JQ1, has been reported to block stem cell self-renewal by displacing Brd4 from key stem cell genes and reducing their expression. Consistent with these findings, JQ1 treatment inhibited Brd4 binding to pluripotency genes in cells cultured in either S/L or S/L + 2i (Fig. 1d). Surprisingly, whereas S/L-cultured cells ceased proliferation when treated with JQ1 (Fig. 2a), S/L + 2i (right) treated with DMSO (vehicle) or 500 nM JQ1 for 24 h. Bars represent the mean of n = 3 technical replicates from one immunoprecipitation (b,d).

Although each inhibitor (CHIR and PD) provided partial resistance to JQ1, the combined effect of both inhibitors (2i) was required for full JQ1 resistance (Fig. 2g and Supplementary Fig. 2f,g). This result suggests that the ground state of pluripotency, rather than MEK or GSK-3β inhibition individually, induces JQ1 resistance. Naïve ESCs, but not metastable ESCs, were capable of giving rise to AP-positive (AP+) colonies even in the ongoing presence of JQ1 (Supplementary Fig. 2k). To determine whether ESCs remain bona fide pluripotent cells after JQ1 treatment, ESCs exposed to JQ1 were injected into blastocysts to assay for differentiation competence in vivo. After treatment with JQ1, only ESCs cultured in S/L + 2i contributed to live-born chimaeras (Fig. 2i,j).

Although BET inhibitors, such as JQ1 and ibET, target the bromodomain of BET family proteins, inhibition of Brd4 drives the majority of the published effects of BET inhibitors. To probe the role of Brd4 specifically, we performed clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9)-mediated editing of Brd4 using single guide RNA (sgRNA) against Brd4 bromodomain 1 (BD1). Expression of sgRNA against Brd4 severely compromised the number of viable cells, resulting in the addition of S/L + 2i to the 2i protocol. This addition restored the viability of Brd4-deleted ESCs (Fig. 2c). An alternative BET inhibitor, iBET, similarly caused metastable ESCs to arrest and enlarge, whereas naïve ESCs remained resistant (Supplementary Fig. 2d,e). 2i-induced resistance to BET inhibitors did not require an embryonic cell of origin: pro-B cells reprogrammed to pluripotency maintained proliferation, volume and AP staining when treated with JQ1 (Fig. 2h and Supplementary Fig. 2f,g).
colony formation in S/L-cultured ESCs but had no effect on colony formation in S/L + 2i-cultured ESCs (Fig. 3a and Supplementary Fig. 3a,b). Just as 2i preserved Nanog expression in response to JQ1 treatment, genetic editing of Brd4 did not alter Nanog expression in S/L + 2i-cultured ESCs (Supplementary Fig. 3a). Accordingly, each Brd4–edited clone retained normal morphology and proliferation when cultured in the presence of 2i (Supplementary Fig. 3c,d). This resistance to the effect of Brd4 editing required 2i: within 48 h of 2i removal, Brd4-deficient clones exhibited severe growth defects (Supplementary Fig. 3d). When seeded at single-cell density, only clones cultured with 2i were able to form AP+ colonies (Fig. 3b,c).

We next manipulated Brd4 expression using inducible short hairpin RNA (shRNA) against Brd4 (Fig. 3d). Brd4 knockout recapitulated the effects of BET inhibition, flattening colonies and blocking proliferation of S/L-cultured ESCs while having no obvious effect on the morphology or proliferation of S/L + 2i-cultured ESCs (Fig. 3e,f). Although Brd4 was absolutely required for the self-renewal of metastable ESCs, naive ESCs were capable of forming AP+ colonies even in the presence of continuous Brd4 depletion (Fig. 3g). Together, these results suggested that ground-state ESCs tolerate pharmacological and genetic inhibition of Brd4.

**Naive ESCs maintain transcriptional networks independently of Brd4 function.** The ability of ESCs grown in 2i to maintain pluripotency in the presence of BET inhibitors suggests that these cells are capable of maintaining pluripotency-associated transcription networks independently of BET protein recognition of acetylated lysines. Indeed, although expression of pluripotency transcription factors required Brd4 activity in S/L-cultured ESCs as previously reported20,22,23, 2i-cultured ESCs maintained high levels of transcription factor expression even in the presence of pharmacological or genetic inhibition of Brd4 activity (Fig. 4a,b). To determine how ESCs sustain transcriptional networks without the help of BET protein co-activators, we analysed gene expression in ESCs treated with JQ1 for 72 h. In metastable ESCs, most genes comprising the pluripotency-associated gene network31 lost Brd4-binding ability and were dramatically downregulated by prolonged exposure to JQ1 (Fig. 4c and Supplementary Fig. 4a). By contrast, the level of expression of these genes remained high in naive ESCs regardless of JQ1 treatment (Fig. 4c), suggesting that these cells can sustain transcription in the absence of Brd4 bromodomain function.

To assess the activity of pluripotency-associated transcriptional networks, we looked at the expression of genes identified as direct targets of Oct4, Sox2 and Nanog (OSN)31. In the absence of Brd4 function, OSN targets were preferentially downregulated in cells cultured in S/L medium (Supplementary Fig. 4b). Similarly, genes that were directly bound by pluripotency factors, such as Oct4, Sox2, Nanog, Klf4 and Rex1 (ref. 32), were repressed by JQ1 treatment of cells growing in S/L medium (Supplementary Fig. 4c). In both cases, the addition of 2i preserved the expression of these gene sets during JQ1 treatment (Supplementary Fig. 4d,e). Thus, whereas JQ1 abrogated pluripotency-associated transcription factor binding to key regulatory loci in metastable ESCs, transcription factor binding to these same loci was higher and relatively resistant to JQ1 in naive ESCs (Fig. 4d).

**Fig. 2** Naive ESCs are resistant to Brd4 inhibitors. a–b, Growth curve of ESCs cultured in S/L (a) or S/L + 2i (b) with DMSO (vehicle) or 500 nM JQ1. c–d, GFP intensity of Nanog-GFP reporter ESCs cultured in S/L (c) or S/L + 2i (d) treated with DMSO or 500 nM JQ1 for 24 h as measured by FACS. The light-grey-shaded peak represents the negative control. e–f, Representative images of ESCs grown in S/L (e) or S/L + 2i (f) treated with DMSO or 500 nM JQ1 for 24 h. AP, AP staining; BF, brightfield. Scale bars, 100 μm. g, Population doublings of ESCs grown in the indicated medium and cultured with 500 nM JQ1. CH, GSK-3β inhibitor; PD, MEK inhibitor. h, Population doublings of induced pluriotent stem cells (iPSCs) generated from haematopoietic CD19+ pro-B cells grown in S/L or S/L + 2i with DMSO or 500 nM JQ1. iPSCs generated from injection of ESCs cultured in S/L or S/L + 2i with or without 500 nM JQ1 for 48 h. Black coat colour indicates ESC contribution to live offspring. For growth curves (a–g), n = 3 independent samples are shown and the connecting line joins the mean values of each time point (a,b,g); all experiments (a–h) were repeated independently at least two times with similar results.
Chromatin accessibility can be used to infer recruitment of transcriptional activators and co-activators to regulatory elements associated with gene expression\(^1\). The above results suggested that naive ESCs grown in 2i-containing medium might maintain chromatin accessibility independently of Brd4. Consistent with this hypothesis, assay for transposase-accessible chromatin using sequencing (ATAC-seq) analyses revealed that, although JQ1 induced profound loss of chromatin accessibility in ESCs grown in S/L medium, ESCs cultured in medium containing 2i exhibited minimal changes in chromatin accessibility in the presence of JQ1 (Fig. 4e and Supplementary Fig. 4f). Over 60% of the ATAC-seq peaks that were lost in S/L-cultured cells as a result of JQ1 treatment and preserved in S/L + 2i-cultured cells were OSN sites (Fig. 4f).

In addition to maintaining chromatin accessibility\(^1\), Brd4 can potentiate gene expression by associating with the Mediator complex that bridges transcription factor binding and RNA polymerase II recruitment to regulatory elements\(^1\). Displacement of the Med1 subunit of the Mediator complex following JQ1 treatment correlates with loss of gene expression\(^8\,18\). Consistent with these previous findings, S/L-grown cells exhibited a profound reduction in genome-wide Med1 recruitment when treated with JQ1. By contrast, ESCs grown in S/L + 2i medium retained high genome-wide Med1 recruitment despite JQ1 treatment (Fig. 4g and Supplementary Fig. 4g). Although JQ1 reduced both chromatin accessibility and Med1 binding at pluripotency genes in S/L-cultured ESCs, it had no consistent effect on either parameter at these genes in S/L + 2i-cultured ESCs (Supplementary Fig. 4h,i).

The above results suggested that persistent Mediator recruitment might sustain Brd4-independent gene expression in S/L + 2i-cultured cells.
Fig. 4 | Naive ESCs maintain transcriptional networks in the presence of BET inhibition. a, qRT-PCR on key pluripotency genes in ESCs cultured in S/L or S/L + 2i treated with DMSO (vehicle) or 500 nM JQ1 for 24 h, b, qRT-PCR on key pluripotency genes in S/L or S/L + 2i-cultured ESCs expressing doxycycline-inducible shRen or shBrd4 and treated with doxycycline for 48 h. Shown are the levels of Nanog and Esrrb in cells with doxycycline relative to control S/L shRen cells without doxycycline. c, Heatmap showing the expression of pluripotency-associated genes measured by RNA-seq in ESCs cultured in S/L or S/L + 2i with vehicle (veh; DMSO) or 500 nM JQ1 for 72 h. The colour scale represents log2 relative to the mean expression level. d, Nanog binding to key pluripotency loci in ESCs cultured in S/L + JQ1 treated with 500 nM JQ1. ESCs cultured in S/L or S/L + 2i treated with DMSO or 500 nM JQ1. Data from two independent replicates are shown. f, At baseline, 82% of OSN-binding sites have an ATAC-seq peak above background. OSN sites make up 60% (4,250 out of 7,084) of the peaks that maintain accessibility despite JQ1 treatment in S/L + 2i-cultured ESCs but only 17% (93 out of 545) of peaks lost in both conditions. g, ChIP-seq metaprofile for Med1 binding in ESCs cultured in S/L or S/L + 2i treated with DMSO or 500 nM JQ1 for 24 h, assessed by ChIP-qPCR. Bars represent the mean of n = 3 technical replicates from one immunoprecipitation (IP). pr, promoter. e, ATAC-seq metaprofile of chromatin accessibility in ESCs cultured in S/L (left) or S/L + 2i (right) with DMSO or 500 nM JQ1. Data from two independent replicates are shown. h, ATAC-seq metaprofile for Med1 ChIP–seq sites. j, ATAC-seq sites. k, ChIP–seq sites. l, ChIP–seq sites. m, ChIP–seq sites. n, ChIP–seq sites. o, ChIP–seq sites. p, ChIP–seq sites. q, ChIP–seq sites. r, ChIP–seq sites. s, ChIP–seq sites. t, ChIP–seq sites. u, ChIP–seq sites. v, ChIP–seq sites. w, ChIP–seq sites. x, ChIP–seq sites. y, ChIP–seq sites. z, ChIP–seq sites. **P < 0.01; ***P < 0.001; ****P < 0.0001, Fisher’s exact test. © 2018 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
tured ESCs. In support of this model, genes that were sensitive to JQ1 for 24 h. Vec, vector. Data are presented as the mean ± s.e.m. (±) or mean ± s.e.m. (±) of n = 3 independent samples. Statistics were calculated by unpaired, two-tailed Student’s t-test (a,f) or one-way ANOVA with Tukey’s post-test (h).

Fig. 5 | Increased transcription factor activity provides resistance to BET inhibition in the absence of 2i. a. Population doublings of cells expressing G-CSF-activated LIF receptor transgene (GY118F) cultured with or without G-CSF and treated for 48 h with 500 nM JQ1 relative to vehicle (DMSO)-treated controls. b. Heatmap depicting the relative gene expression in cells expressing G-CSF-activated LIF receptor transgene (GY118F) treated for 24 h with vehicle (DMSO) or 500 nM JQ1. c. Growth curve of ESCs expressing empty vector or Nanog and cultured with 500 nM JQ1 (left) or 500 nM iBET (right). n = 3 independent samples are shown and the connecting line joins the mean values of each time point. d. Heatmap depicting the relative gene expression in cells expressing empty vector or Nanog and treated with vehicle (DMSO) or 500 nM JQ1. e. AP staining of colony formation assays in which vector or Nanog-expressing cells are treated for the final 48 h with vehicle (DMSO), JQ1 or iBET. The experiment was performed at least two times with similar results. f. Population doublings of cells expressing empty vector or Klf4 treated for 48 h with 500 nM JQ1 relative to vehicle (DMSO)-treated controls. g. Heatmap depicting the relative gene expression in cells expressing empty vector or Klf4 and treated with vehicle (DMSO) or 500 nM JQ1 for 24 h. h. Quantification of the number of AP+ colonies formed in the presence of continuous JQ1 in cells expressing empty vector, Klf4, Nanog or Klf4 + Nanog. Values above the bars indicate the P values. i. Heatmap depicting the relative gene expression in cells shown in panel h treated with 500 nM JQ1 for 24 h. Vec, vector. Data are presented as the mean ± s.d. (a) or mean ± s.e.m. (f,h) of n = 3 independent samples. Statistics were calculated by unpaired, two-tailed Student’s t-test (a,f) or one-way ANOVA with Tukey’s post-test (h).

In contrast to Brd4 inhibition, both CDK7 and CDK9 inhibition triggered loss of Nanog expression and impaired proliferation, regardless of the presence of 2i (Supplementary Fig. 4j,k).

Pluripotency-associated transcription factors enable resistance to BET inhibitors. Sites associated with high Med1 binding were highly enriched (96%) in known OSN-binding sites (Fig. 4i), suggesting that the pluripotency transcription factor network enabled resistance to BET inhibition by promoting Brd4-independent Mediator recruitment. To further examine the
Fig. 6 | JQ1 affects Nanog and Tet target genes. a. Relative chromatin accessibility at peaks associated with key pluripotency genes in ESCs expressing empty vector or Nanog and treated with DMSO or 500 nM JQ1 for 72 h. See also Supplementary Table 5. b. GSEA showing genes ranked by fold change in total accessibility with Nanog overexpression as measured by ATAC-seq of duplicate samples. Genes identified as direct targets of Nanog, Oct4 and Sox2 are enriched among genes whose accessibility is increased by Nanog overexpression. c. GSEAs of RNA-seq data from triplicate samples showing the enrichment of genes identified as bound and activated by Tet1. Dotted line represents y = 0. For GSEA, P values are calculated based on 1,000 permutations by the GSEA algorithm and were not adjusted for multiple comparisons. NES, normalized enrichment score. d. Colony formation assay of Nanog-overexpressing cells cultured in the presence of 500 nM JQ1 and the indicated dose of CoCl2 for 6 days. e. Colony formation assay of Nanog-overexpressing cells cultured in the presence of DMSO or 200 nM JQ1 and 100 μg ml−1 vitamin C (VitC) for 6 days. Data are presented as the mean ± s.e.m. of n = 3 independent samples (d,e).

hypothesis that a strengthened pluripotency network bestows Brd4 independence, we generated ESCs expressing a granulocyte colony-stimulating factor (G-CSF)-inducible LIF receptor transgene harbouring a mutation at tyrosine 118 that results in hyperactivation of the Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3) signalling pathway (Supplementary Fig. 5a) and drives ESCs into the ground state of pluripotency41. Induction of STAT3 signalling rendered cells resistant to JQ1: G-CSF-treated ESCs were able to maintain proliferation, cell volume and expression of pluripotency-associated genes even in the presence of JQ1 (Fig. 5a,b and Supplementary Fig. 5b).

These results suggested that direct reinforcement of the pluripotency transcription network might provide JQ1 resistance. Brd4 is recruited to many of the sites as key pluripotency transcription factors, such as Nanog, Oct4, Sox2, Klf4 and Esrrb42 (Supplementary Fig. 5c). Brd4-bound sites form three major clusters: Nanog-bound, Oct4/Sox2-bound and Klf4-bound clusters (Supplementary Fig. 5d). Thus, we tested whether increased expression of transcription factors representing distinct clusters might be sufficient to enable resistance to BET inhibition. ESCs with transgenic Nanog expression (Supplementary Fig. 5e) maintained proliferation, cell volume and AP activity in the presence of either JQ1 or iBET (Fig. 5c,e and Supplementary Fig. 5f,g). Moreover, Nanog overexpression sustained the expression of pluripotency genes in JQ1-treated cells (Fig. 5d). Similarly, transgenic expression of Klf4, which binds to a different set of Brd4 targets than Nanog (Supplementary Fig. 5d,h), improved cell proliferation, prevented increases in cell volume and blunted decreases in pluripotent gene expression in response to JQ1 treatment (Fig. 5f,g and Supplementary Fig. 5i). Dual expression of both Nanog and Klf4 further enhanced resistance to JQ1: expression of pluripotency-associated genes was highest in JQ1-treated Nanog + Klf4-expressing cells (Fig. 5i), and continuous JQ1 had no significant effect on the formation of AP+ colonies in Nanog + Klf4 lines (Fig. 5h and Supplementary Fig. 5j). Thus, resistance to BET inhibition correlates with the strength of the pluripotency transcriptional network.

Tet1/2 cooperate with transcription factors to maintain Brd4-independent pluripotency. To determine the mechanism through which enhanced transcription factor expression rendered metastable ESCs resistant to BET inhibitors, we performed ATAC-seq on Nanog transgenic cells in the presence and absence of JQ1. At baseline, Nanog overexpression induced chromatin opening at loci associated with key pluripotency genes (Fig. 6a). Moreover, Nanog overexpression blunted the JQ1-induced loss of chromatin accessibility at these regions (Fig. 6a). More generally, Nanog overexpression increased accessibility across a wide range of direct targets of OSN pluripotency factors in JQ1-treated cells (Fig. 6b).

Binding by OSN transcription factors during the transition to the naive ground state of pluripotency is associated with local loss of DNA methylation43. Tet1 (ten–eleven translocation 1) and Tet2, which are methylcytosine oxidases that hydroxylate DNA 5-methylcytosine, are reported to contribute to DNA hypomethylation at a subset of regulatory elements in naive ESCs12,44,45. In addition, during reprogramming, Nanog cooperates with Tet1 and Tet2 to activate the expression of key reprogramming genes45. Tet1 binds to 70% of Brd4-bound sites46 (Supplementary Fig. 6a,b), and 73.3% of these sites are also bound by OSN factors (P < 2.2 × 10−16, Fisher’s exact test).

Consequently, we tested whether the ability of transcription factors to maintain gene expression in JQ1-treated cells might involve Tet1/2. As preliminary evidence, we found that genes that were reported to be both bound and activated by Tet1 in mouse ESCs46 are significantly enriched among genes that were downregulated by JQ1 treatment in S/L-cultured ESCs (Fig. 6c). However, in
2i-cultured ESCs, the expression of Tet1-target genes was maintained despite JQ1 treatment (Fig. 6c). Furthermore, treatment of Nanog-overexpressing ESCs with cobalt chloride (CoCl2), which inhibits iron-dependent dioxygenases including Tet1/2, induced a dose-dependent loss of resistance to JQ1 (Fig. 6d). Conversely, vitamin C, a cofactor that promotes Tet1/2 activity in ESCs44, significantly enhanced the ability of Nanog-overexpressing cells, but not control cells, to resist JQ1 treatment (Fig. 6c and Supplementary Fig. 6c). These results suggested that transcription factors, in cooperation with Tet1/2 and potentially other iron-dependent demethylases, drive JQ1 resistance.

As both CoCl2 and vitamin C can affect multiple cellular processes, we generated ESCs with CRISPR–Cas9-derived mutations in both Tet1 and Tet2 (Supplementary Fig. 7a). Consistent with previous studies demonstrating that Tet1 and Tet2 are dispensable for pluripotency45, Tet1/2 double-mutant ESCs had no obvious defect in chromatin accessibility, Brd4 expression, growth or morphology under basal conditions (Supplementary Fig. 7b–d). However, loss of Tet1/2 function clearly sensitized cells to JQ1, indicating that, in the absence of Tet1/2, cells were more dependent on Brd4 to maintain gene expression (Supplementary Fig. 7e). We next tested whether Tet enzymes contributed to the BET inhibitor resistance induced by Nanog or 2i. Overexpression of Nanog in Tet1/2 double-
mutant ESCs (Supplementary Fig. 7f) revealed that Tet1/2 mutation severely compromised the ability of Nanog to maintain proliferation and generate AP+ colonies in the presence of JQ1 (Fig. 7a,b and Supplementary Fig. 7g). Similarly, 2i-treated ESCs required Tet1 and Tet2 to sustain JQ1 resistance (Fig. 7c–f).

Both 2i treatment and Nanog overexpression promoted chromatin accessibility at pluripotency genes in JQ1-treated ESCs (Fig. 7g). Tet1/2 were required for Nanog-induced increases in accessibility at peaks associated with pluripotency genes (Supplementary Fig. 7h). Although Nanog expression was sufficient to preserve accessibility at a subset of JQ1-sensitive peaks, this ability was compromised in the absence of Tet1/2 (Supplementary Fig. 7i). In particular, a subset of the peaks whose accessibility was lost with JQ1 treatment but preserved by Nanog overexpression required Tet1/2 in order to be maintained by Nanog (Supplementary Fig. 7i). Motif analyses revealed that pluripotency-associated motifs are strongly enriched in all clusters, including Tet1/2-dependent sites (Supplementary Fig. 7i). These results suggested that Tet1/2 are required for maintenance of accessibility at a subset of pluripotency-associated transcription factor targets, and loss of Tet1/2 therefore weakens the pluripotency transcriptional network and compromises JQ1 resistance. Indeed, the ability of both Nanog overexpression and 2i treatment to maintain the expression of pluripotency-associated transcription factors during JQ1 treatment was blunted in Tet1/2 double-mutant ESCs (Fig. 7h,i).

We next asked whether the reported ability of 2i treatment to enhance glutamine-dependent production of α-ketoglutarate, a co-substrate for DNA and histone-modifying enzymes including Tet1/2, contributed to the maintenance of pluripotent gene expression during JQ1 treatment. Culture with low levels of glutamine did not affect Brd4 expression (Supplementary Fig. 7j). However, low glutamine levels impaired the ability of 2i to maintain Nanog expression in the presence of JQ1 (Fig. 7j). Although glutamine withdrawal probably has pleiotropic effects, these data indicate that 2i-induced production of Tet substrates may also contribute to the resistance to BET inhibitors exhibited by naive ESCs.

Discussion

BET inhibitors have gained wide attention for their ability to block proliferation and self-renewal of stem cells and cancer cells. The efficacy of these inhibitors is tied to their ability to inhibit the expression of key stem cell maintenance genes and oncogenic transcription factors, such as Myc. Here, we demonstrate that naive ESCs exhibit inherent resistance to BET inhibition. When grown in 2i-containing medium or provided enhanced JAK–STAT3 signaling, ESCs maintain proliferation, self-renewal and pluripotency in the presence of BET inhibitors. The ability of naive ESCs to maintain Brd4-independent proliferation and self-renewal is mediated by both a strengthened transcription factor network and the Tet1/2 DNA methylation oxidases, which converge to maintain transcriptionally permissive chromatin environment. Naive ESCs represent a highly optimized transcriptional state; under less-optimized conditions, such as with metastable ESCs, the reading of histone acetylation remains essential to the maintenance of cellular identity. The present data indicate that the dependency on reading of histone acetylation to consolidate gene networks is most critical when transcription factor binding and chromatin accessibility are limiting. However, the data do not preclude contributions from additional chromatin modifications, transcriptional activators or co-activators to Brd4-independent maintenance of cell identity, and future work may uncover additional proteins that compensate for BET family inhibition. We favour the general view that when transcription factor networks and chromatin methylation states are collectively optimized, the recognition of lysine acetylation by bromodomain-containing proteins has a relatively minor role in modulating gene expression. In support of this model, loss of DNA (cytosine-5)-methyltransferase 3A (DNMT3A) and polycomb repressive complex 2 (PRC2), which deposit repressive DNA methylation and histone H3 lysine 27 trimethylation (H3K27me3), can lead to BET inhibitor resistance in AML cell lines. Similarly, AML cells expressing mutant forms of isocitrate dehydrogenase 2 exhibit hypermethylation and enhanced sensitivity to Brd4 inhibition. Taken together, these data reveal that a combination of transcription factor networks and permissive chromatin modifications can render Brd4 dispensable to the maintenance of cell identity and illustrate that chromatin modification-based transcriptional regulation is context dependent.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-018-0086-3.

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Author contributions

L.W.S.F., S.A.V. and B.W.C. designed, performed and analysed all experiments under the guidance of C.D.A. and C.B.T. R.K. and S.A.V. provided technical assistance. L.W.S.F., S.A.V. and B.W.C. assisted with the RNA-seq analyses. D.A.-C. and S.W.L. assisted with the generation of microarray and genome-wide chromatin immunoprecipitation data. C.D.A. and C.B.T. wrote the manuscript.

Competing interests

C.B.T. is a founder of Agios Pharmaceuticals and a member of its scientific advisory board. He also serves on the board of directors of Merck and Charles River Laboratories.

Additional information

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Methods

Cell culture. Mouse ESC lines (ESC1) were generated from C57BL/6 x Sylae F1 male embryos as previously described.10 D34 ESCs were previously described.10 Nanog green fluorescent protein (GFP) reporter ESCs and induced pluripotent stem cells generated from haematopoietic CD19+ cells were a gift from R. Jaenisch (Massachusetts Institute of Technology, Cambridge, MA, USA). ESC1 cells were used for all experiments unless otherwise noted. ESCs were maintained on gelatin-coated plates in S/L medium containing Knockout DMEM ( Gibco) supplemented with 10% ESC-qualified FBS (Gemini), penicillin/streptomycin (Life Technologies), 0.1 mM 2-mercaptoethanol, 2 mM l-glutamine (Life Technologies) and 1.000 μM LIF (Millipore). For culture in 2i (S+L+2i), S/L medium was supplemented with 2i; 3 μM CHIR90021 (Stemgent) and 1 μM PD0325901 (Stemgent). Cells were adapted to S/L+2i for at least three passages prior to experiments and were maintained in S/L+2i for no more than nine passages. Serum-free 2i/L consisted of a 1:1 mix of DMEM/F12 (13100-033, Life Technologies) and Neurobasal medium (21103-049, Life Technologies) containing N2 and B27 supplements (17502-048 and 17504-044, respectively, Life Technologies, 1:100 dilutions), 0.1 mM 2-mercaptoethanol, 0.1 mM L-glutamine, LIF, CHIR90021 at 3 μM (Stemgent) and PD0325901 at 1 μM (Stemgent). For glucose and glutamine deprivation experiments, cells were cultured in medium containing a 1/1 mix of glutamine-free and glucose-free DMEM (A14430-01, Invitrogen) and glutamine-free and glucose-free Neurobasal medium (005012BD, Invitrogen) including 10% dialysed FBS and all supplements as described above and containing glucose (Sigma) at either 20 mM or 2 mM or glutamine at either 2 mM or 0.2 mM. pQ1 and pQET (Sigma) were dissolved in dimethylsulfoxide (DMSO) and used at indicated concentrations (0.1–10 μM) and were dissolved in water for later and used at indicated doses. The Leukocyte Alkaline Phosphatase Kit (Sigma) was used to stain cells with AP reagent according to the manufacturer’s instructions. Images of cells were taken using a Leica microscope.

Generation of ESC lines. Generation of transgenic cell lines. Nanog and Kif4 mouse genomic DNA were amplified from piggybac (pCAGGS-Neo) plasmids gifted to H. Niwa, Institute of Molecular Embryology and Genetics, Kumamoto, Japan) and pFeHa-1RES-GFP (System Biosciences) plasmids. Approximately 5 × 10^4 ESC1 cells cultured in S/L medium were electroporated with piggybac plus transposase (pBase) in 3/1 ratio using Amaxa ESC Nucleofector Kit (VPH-1001, H. Niwa, Institute of Molecular Embryology and Genetics, Kumamoto, Japan) and pFeHa-1RES-GFP (System Biosciences) plasmids. Approximately 5 × 10^4 ESC1 cells cultured in S/L medium were electroporated with piggybac plus transposase (pBase) in 3/1 ratio using Amaxa ESC Nucleofector Kit (VPH-1001, program A-023, Lonza) and plated onto gelatin-coated plates. About 36–48 h after electroporation, the medium was changed to S/L+G418 at 300 μg/ml for at least 2–3 passages. Nanog + Kif4 double-transgenic lines were generated by electroporating Nanog-expressing lines with pFeHa-Kif4-GFP and then sorting the top 10% GFP-positive cells. Hyperactivated STAT3 ESCs were generated in Nanog- GFP ESCs using GV118 chimeric LIF receptor piggybac plasmid as described above and selected using 200 μg/ml hygromycin for at least three passages.

Generation of Tet1/2 knockout cell lines. Tet1/2 double-receptor ESCs were obtained using CRISPR-Cas9–derived mutation of Tet1 and Tet2. Tet1 and Tet2 guide RNA sequences (gRNAs; Tet1, 5′-ggcgcagctgacaggtc-3′; Tet2, 5′-tgaatgagcaactatctgc-3′) were cloned into px459 v1 (Addgene plasmid number 48139) containing 2A-PURO selection cassette (Addgene, Massachusetts, USA). The PCR product was amplified, cloned into plasmid containing tet-on promoter (Amazc, Lonza), and micrococcal nuclease to recover mono- to tri-nucleosomes. Nuclei were lysed by subjecting to sonication and dialysed into N-ChIP buffer (10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 1% SDS, 0.1% sodium deoxycholate and 1% Triton X-100) for 2 h at 4 °C. Soluble chromatin was incubated overnight at 4 °C after the addition of 0.5–1 μg H3K9ac antibody (9649, Cell Signaling Technologies) or H3K27 antibody (39133, Active Motif) bound to 25 μl protein A Dynal magnetic beads (Invitrogen), with 5% kept as input DNA. Magnetic beads were washed, chemically eluted, and ChIP DNA was dissolved in 10 mM Tris pH 8.0 for quantitative PCR (qPCR) reactions (see Supplementary Table 3 for primer pairs). Three independent ChIP experiments were performed. The data shown are averaged qRT–PCR values of technical replicates (n = 3) from one representative immunoprecipitation.

Brd4, Nanog and Med1 ChiP was carried out as previously described ES cells (10^6) grown under either SL or S/L+2i for at least three passages as described above and selected using 200 μg/ml hygromycin for at least three passages. Nanog + Kif4 double-transgenic lines were generated by electroporating Nanog-expressing lines with pFeHa-Kif4-GFP and then sorting the top 10% GFP-positive cells. Hyperactivated STAT3 ESCs were generated in Nanog-GFP ESCs using GV118 chimeric LIF receptor piggybac plasmid as described above and selected using 200 μg/ml hygromycin for at least three passages.

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a single immunoprecipitation. Each immunoprecipitation was performed at least two independent times. For both Brd4 and Med1 ChIP–seq, libraries for Illumina sequencing were prepared using Nextflex Barcoding Adapters (Bioo Scientific) and a KAPA library amplification kit according to the manufacturer’s instructions. Samples were sequenced using Illumina HiSeq generating 50-bp paired-end reads. Reads were trimmed for quality and Illumina adapter sequences using ‘trim_galore’ before aligning to mouse assembly mm9 with bowtie2 using the default parameters. Aligned reads with the same start site and orientation were removed using the Picard Tools MarkReadGroups (http://picard.sourceforge.net/). Denoising profiles were created by extending each read to the average library fragment size and then computing the density using the BEDTools suite (http://bedtools.readthedocs.io). Enriched regions were discovered using MACS 1.4 with default parameters and scored against matched input libraries. All genome browser tracks and read density tables were normalized to sequencing depth. Promoter-based comparisons were used to identify the denoised read density profile start site, whereas non-promoter promoters were also normalized to peak length. Dynamic regions between two conditions were scored using MACS with the second ChIP library replacing the input, and only sites with an absolute fold change greater than two were scored as a gain/loss. For gene-based analyses, all peaks within 30 kb of a gene were assigned to the relevant gene (or genes). For ChIP–seq scatter plots, read density was calculated using featureCounts (http://subread.sourceforge.net/) in paired-end mode with a minimum quality threshold of ten. Read density was subsequently normalized to a sequencing depth of 10 million mapped reads.

ATAC-seq. ESCs were seeded at 150,000 cells per well of a six-well plate and treated the following day with DMSO or 500 nM JQ1. Cells were passaged into fresh six-well plates after 24 h of treatment and harvested after a total of 72 h of treatment. Duplicate samples from replicate wells were prepped for each condition. For each sample, 100,000 cells were harvested and washed with ATAC buffer (Tris 10 mM pH 7.4, 10 mM NaCl and 3 mM MgCl2). Samples were spun at 1,000 g for 5 min at 4 °C. Pellets were resuspended in 10 mM pH 7.4, 10 mM NaCl, 3 mM MgCl2, and 0.1% NP-40 (or Igepal-Ca630). Nuclei were collected after centrifugation at 1,000 g for 10 min at 4 °C. After discarding supernatant, tagmentation was performed at 37 °C for 30 min (FC-121-1030, Illumina Nexxta DNA Sample Preparation Kit). DNA was collected using a magnetic particle kit. PCRs were performed according to the manufacturer’s instructions and eluted in 10 µl elution buffer (10 mM Tris pH, 8.0). Eluted DNA was mixed with NEBNext Quick Start Hot Start HiFi PCR Master Mix (M0543L, NEB) and primer mix (Nexxta). DNA was amplified by PCR (65 °C, 5 min; 98 °C, 30 s; 98 °C, 10 s, 65 °C, 1 min 15 s (12x)) and purified using AMPure XP beads (ratio of 1:5). Library quality control was performed using a Quant-IT PicoGreen dsDNA Assay Kit (P7589, Life Technologies) and the median fragment size using an Agilent DNA1000 screentape (5067-5582). Samples were pooled for multiplexing and sequenced using paired-end sequencing technology on the Illumina NexxtSeq 500 platform. Sequencing data were processed and identity was checked to ChIP–seq, except in the density profile creation step, in which reads were not extended. Enriched regions were discovered using MACS and scored against matched input libraries (fold change > 2 and P < 0.005), and peaks within 500 bp were merged. All genome browser tracks and read density tables were normalized to sequencing depth. For gene-based analyses, each peak was assigned to the most proximal transcription start site based on the linear genomic distance. K-means clustering of ATAC-seq data was performed using the software package ‘gaga’ (http://gaga.readthedocs.io) according to the manufacturer’s instructions. Chimaera assays. Mice and embryos. Animal experiments comply with all relevant ethical regulations. All animal experiments were carried out according to the protocol approved by the Institutional Animal Care and Use Committee of Wellcome Trust Sanger Institute (numbers: 2014-0037, ICR female). Females were purchased from Taconic Farms. Females were superovulated at 6–8 weeks with 10 IU PMSG (pregnant mare serum gonadotrophin, Sigma-Aldrich) and 10 IU hCG (human chorionic gonadotrophin, Sigma-Aldrich) at intervals of 48 h. The females were mated individually to males and checked for the presence of a vaginal plug the following morning. Males were killed in a CO2 chamber 1.5 days after hCG injection for collection of 2-cell embryos. These embryos were flushed from the oviducts with KSOM + amino acids (speciality media) and cultured in KSOM for 2.5 days in vitro at 37 °C under 5% CO2, in an air to the blastocyst stage.

ChiP-seq data was generated using Illumina NexxtSeq 500, generating 75-bp single-end reads. Fastq files were aligned to genome build mm9 using TopHat with default parameters. Aligned features were counted with htseq-count, and differential expression was determined using the edgeR package in Bioconductor as previously described8. Colony formation assays. Cells were seeded at the indicated density in six-well plates. For experiments with continuous JQ1, 200 cells were seeded per well. The next day, cells were washed with PBS and put into fresh medium with DMSO or 500 nM JQ1 and medium was changed daily thereafter. For experiments with acute JQ1 treatment, 200 cells were seeded per well and medium was refreshed every 2–3 days. Forty-eight hours prior to fixation, cells were given fresh medium containing 0% DMSO or 500 nM JQ1. ATAC-lysis and DNA shearing in ATAC lytic buffer for ChIP-seq data was performed on all peaks whose accessibility was maintained and/or increased with Nanog overexpression and whose accessibility also decreased with JQ1 treatment in control cells. Dynamic peaks were annotated with Homer v4.5 (http://homer.salk.edu/homer/ngs/annotation.html) using a distance threshold of 10 kb. Values and statistical tests are reported in the figure analyses. Error bars, P values and statistical tests are reported in the figure legend. Statistical tests include paired or unpaired two-tailed Student’s t-test, one-way analysis of variance (ANOVA) and Bonferroni’s post hoc test. All experiments were performed independently at least two times.

Chimaera assays. Mice and embryos. Animal experiments comply with all relevant ethical regulations. All animal experiments were carried out according to the protocol approved by the Institutional Animal Care and Use Committee of Wellcome Trust Sanger Institute (numbers: 2014-0037, ICR female). Females were purchased from Taconic Farms. Females were superovulated at 6–8 weeks with 10 IU PMSG (pregnant mare serum gonadotrophin, Sigma-Aldrich) and 10 IU hCG (human chorionic gonadotrophin, Sigma-Aldrich) at intervals of 48 h. The females were mated individually to males and checked for the presence of a vaginal plug the following morning. Males were killed in a CO2 chamber 1.5 days after hCG injection for collection of 2-cell embryos. These embryos were flushed from the oviducts with KSOM + amino acids (speciality media) and cultured in KSOM for 2.5 days in vitro at 37 °C under 5% CO2, in an air to the blastocyst stage.

Blastocyst injection and embryo transfer. ESCs were treated with DMSO or JQ1 as described above and kept on ice until injection. A flat-tip micropipette was used for ESC injection using the Piezo Micromanipulator (Prime Tech) to break the embryos. ESCs were picked up in the end of the injection pipette and 15–20 ESCs were injected into each blastocyst. The injection pipette was also used to collect ESCs as a clump and to place them close to the inner cell mass of the blastocyst. The injected blastocysts were kept in KSOM + amino acids until embryo transfer. Ten injected blastocysts were transferred into each uterine horn of 2.5d.p.c. pseudopregnant ICR females.

FACS of Nanog reporter lines. Nanog-GFP ESCs were seeded at a concentration of 40,000 cells per well of a 12-well plate. The next day, cells were washed with PBS and put in fresh medium with indicated inhibitors or relevant vehicle control. Cells were counted on the indicated days thereafter using a Beckman Multisizer 3 with a cell volume gate of 400–10,000 fl. Medium was changed every day. For growth curves using cells expressing shRNA, cells were pre-treated with or without doxycycline for 48 h prior to seeding for curve and subsequent cell counts were normalized to seeding density. All curves were performed at least two independent times.

Quantification of gene expression. RNA was isolated from six-well plates using TRIzol (Invitrogen) according to the manufacturer’s instructions and 200 ng RNA was used for cDNA synthesis using Script (Bio-Rad). Quantitative real-time PCR analysis was performed in technical triplicate using QuantStudio 7 Flex (Applied Biosystems) with Power SYBR Green (Life Technologies). All data were generated using cDNA from triplicate wells for each condition. Actin was used as an endogenous control for all samples. PCR analysis was performed in technical triplicate using QuantStudio 7 Flex (Applied Biosystems) and the median fragment size using a distance threshold of 10 kb. Values and statistical tests are reported in the figure analyses. Error bars, P values and statistical tests are reported in the figure legend. Statistical tests include paired or unpaired two-tailed Student’s t-test, one-way analysis of variance (ANOVA) and Bonferroni’s post hoc test. All experiments were performed independently at least two times.
Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. RNA-seq, ChIP–seq and ATAC-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus under the accession codes GSE88760 and GSE88769. Previously published sequencing data that were re-analysed here are available under the accession codes GSE49847, GSE26833, GSE90895 and GSE56312. Source data for Figs. 4i and 6a and Supplementary Fig. 4a,b have been provided as Supplementary Table 5. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Software and code

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Data collection

No software was used for data collection.

Data analysis

Statistical tests were performed using GraphPad Prism v7.0a (for all bar graphs). For GSEA studies, statistical results are included as part of the default parameters of the GenePattern GSEAPreranked v6.0.10 module. ImageJ v 1.43u was used for colony formation assays.

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- A description of any restrictions on data availability

RNA-seq, ChIP-seq and ATAC-seq data that support the findings of this study have
been deposited in the Gene Expression Omnibus (GEO) under the accession codes GSE88760 and GSE88769. Previously published sequencing data that were reanalysed here are available under accession codes GSE49847, GSE26833, GSE90895 and GSE56312. Source data for Fig. 4h, Fig. 6a and Supplementary Fig. 4a,h,i have been provided as Supplementary Table 5. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Cell biology experiments were performed in triplicate, which extensive experience has shown to be sufficient to determine reproducible results from cultured cells. |
| Data exclusions | No data were excluded. |
| Replication | All experiments were reliably reproduced. Each experiment (with the exception of large sequencing experiments) was performed independently at least twice, but usually many more times. |
| Randomization | Samples were randomly distributed into groups. |
| Blinding | Blind analysis was used for manual quantification of colony formation assays. In all other cases, analysis was objective and did not require blinding. |

Materials & experimental systems

Policy information about availability of materials

n/a Involved in the study

- Unique materials
- Antibodies
- Eukaryotic cell lines
- Research animals
- Human research participants

Unique materials

Obtaining unique materials Unique materials are available from corresponding authors upon reasonable request.

Antibodies

Antibodies used Antibodies used for Western blots (at 1:1,000 unless otherwise noted) were pSTAT3 (Cell Signaling Technologies (CST), 9138), STAT3 (CST, 9139) pERK (CST, 4377), ERK (CST, 9102), Tubulin (Sigma, T9026 at 1:10,000), Brd4 (Bethyl Laboratories, A301-985A100 at 1:2,000), Nucleolin (Abcam, ab22758, 1:5000), Nanog (Ebioscience, 14-5761-80) and Actin (Sigma, A3854 at 1:20,000). Antibodies used for ChIP were as follows: H3K9ac (Cell Signaling Technologies, 9649) or H3K27 antibody (Active Motif, 39133) at 0.5–1 μg antibody per ChIP; Brd4 (Bethyl A301-985A50), Med1 (CRSP1/TRAP220, Bethyl A300-793A) and Nanog (Cosmo Bio REC-RCA002P-F) at 10 μg/mL (5.5 μg in 550 μL for 10 million cells).

Validation Antibodies were validated as noted by suppliers.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) Mouse ESC lines (ESC1) were generated by B.C. from C57BL/6 x 129S6/SvEvTac F1 male embryos as previously described (Carey et al., Nature 2015). Nanog-GFP reporter ESCs and iPSCs generated from hematopoietic CD19+ pro-B cells were a gift from R. Jaenisch (MIT). D34 ESCs were generated in the Lowe laboratory as described by Dow et al., PloS One 2014.

Authentication Cell lines were not externally authenticated.
Mycoplasma contamination

Cells were routinely tested for mycoplasma.

Commonly misidentified lines

(See ICLAC register)

No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample.

Research animals

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Animals/animal-derived materials

8 week old outbred ICR female mice from Taconic Farms were used for chimera assays. All animal experiments were carried out according to the protocol approved by the IACUC of Weill Cornell Medical College.

Method-specific reporting

n/a | Involved in the study

ChiP-seq

Flow cytometry

Magnetic resonance imaging

ChIP-seq

Data deposition

☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☑ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication.

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE95642

Files in database submission

Raw ChIP:
ChIP_SLI-Brd4_1.fastq.gz
ChIP_SLI-Brd4_2.fastq.gz
ChIP_SLI-Brd4-Input_1.fastq.gz
ChIP_SLI-Brd4-Input_2.fastq.gz
ChIP_SLIJQ-Med1_1.fastq.gz
ChIP_SLIJQ-Med1_2.fastq.gz
ChIP_SLIJQ-Med1-Input_1.fastq.gz
ChIP_SLIJQ-Med1-Input_2.fastq.gz
ChIP_SL-Brd4_1.fastq.gz
ChIP_SL-Brd4_2.fastq.gz
ChIP_SL-Brd4-Input_1.fastq.gz
ChIP_SL-Brd4-Input_2.fastq.gz
ChIP_SLJQ-Brd4_1.fastq.gz
ChIP_SLJQ-Brd4_2.fastq.gz
ChIP_SLJQ-Brd4-Input_1.fastq.gz
ChIP_SLJQ-Brd4-Input_2.fastq.gz
ChIP_SLJQ-Med1_1.fastq.gz
ChIP_SLJQ-Med1_2.fastq.gz
ChIP_SLJQ-Med1-Input_1.fastq.gz
ChIP_SLJQ-Med1-Input_2.fastq.gz
ChIP_SL-Med1_1.fastq.gz
ChIP_SL-Med1_2.fastq.gz
ChIP_SL-Med1-Input_1.fastq.gz
ChIP_SL-Med1-Input_2.fastq.gz

Processed ChIP:
ChIP_SLI-Brd4_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SLI-Brd4-Input_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SLIJQ-Med1_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SLIJQ-Med1-Input_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SL-Brd4_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SL-Brd4-Input_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SLJQ-Brd4_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SLJQ-Brd4-Input_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SLJQ-Med1_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SLJQ-Med1-Input_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SL-Med1_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SL-Med1-Input_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SL-JQ-Med1_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SL-Med1-Input_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SLQ-JMed1-Input_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SL-Med1_1_val.mm9.sorted.RmDup.10mNorm.bw
ChIP_SL-Med1-Input_1_val.mm9.sorted.RmDup.10mNorm.bw

Raw ATAC:
1A-5-DMSO-1_S1_R1_001.fastq.gz
1A-5-DMSO-1_S1_R2_001.fastq.gz
1A-5-DMSO-2_S2_R1_001.fastq.gz
1A-5-DMSO-2_S2_R2_001.fastq.gz
1A-5-JQ1-1_S3_R1_001.fastq.gz
1A-5-JQ1-1_S3_R2_001.fastq.gz
1A-5-JQ1-2_S4_R1_001.fastq.gz
1A-5-JQ1-2_S4_R2_001.fastq.gz
1A_SL_2i_DMSO-1_S7_R1_001_copy2.fastq.gz
1A_SL_2i_DMSO-1_S7_R2_001_copy2.fastq.gz
1A_SL_2i_DMSO-3_S9_R1_001_copy2.fastq.gz
1A_SL_2i_DMSO-3_S9_R2_001_copy2.fastq.gz
1A_SL_JQ1-1_S10_R1_001_copy2.fastq.gz
1A_SL_JQ1-1_S10_R2_001_copy2.fastq.gz
1A_SL_JQ1-2_S11_R1_001_copy2.fastq.gz
1A_SL_JQ1-2_S11_R2_001_copy2.fastq.gz
1A_SL_DMSO-2_S2_R1_001_copy2.fastq.gz
1A_SL_DMSO-2_S2_R2_001_copy2.fastq.gz
1A_SL_DMSO-3_S3_R1_001_copy2.fastq.gz
1A_SL_DMSO-3_S3_R2_001_copy2.fastq.gz
1A_SL_JQ1-1_S4_R1_001_copy2.fastq.gz
1A_SL_JQ1-1_S4_R2_001_copy2.fastq.gz
1A_SL_JQ1-3_S6_R1_001_copy2.fastq.gz
5-14-DMSO-1_S9_R1_001.fastq.gz
5-14-DMSO-1_S9_R2_001.fastq.gz
5-14-DMSO-2_S10_R1_001.fastq.gz
5-14-DMSO-2_S10_R2_001.fastq.gz
5-14-JQ1-1_S11_R1_001.fastq.gz
5-14-JQ1-1_S11_R2_001.fastq.gz
5-14-JQ1-2_S12_R1_001.fastq.gz
5-14-JQ1-2_S12_R2_001.fastq.gz
5-14-Nanog-Tg-DMSO-2_S5_R1_001.fastq.gz
5-14-Nanog-Tg-DMSO-2_S5_R2_001.fastq.gz
5-14-Nanog_Tg-DMSO-3_S6_R1_001.fastq.gz
5-14-Nanog_Tg-DMSO-3_S6_R2_001.fastq.gz
5-14-Nanog_Tg-JQ1-1_S7_R1_001.fastq.gz
5-14-Nanog_Tg-JQ1-1_S7_R2_001.fastq.gz
5-14-Nanog_Tg-JQ1-3_S8_R1_001.fastq.gz
5-14-Nanog_Tg-JQ1-3_S8_R2_001.fastq.gz
5-9-DMSO-1_S5_R1_001.fastq.gz
5-9-DMSO-1_S5_R2_001.fastq.gz
5-9-DMSO-2_S6_R1_001.fastq.gz
5-9-DMSO-2_S6_R2_001.fastq.gz
5-9-JQ1-1_S7_R1_001.fastq.gz
5-9-JQ1-1_S7_R2_001.fastq.gz
5-9-JQ1-2_S8_R1_001.fastq.gz
5-9-JQ1-2_S8_R2_001.fastq.gz
5-9-Nanog_Tg-DMSO-1_S5_R1_001.fastq.gz
5-9-Nanog_Tg-DMSO-1_S5_R2_001.fastq.gz
5-9-Nanog_Tg-DMSO-2_S6_R1_001.fastq.gz
5-9-Nanog_Tg-DMSO-2_S6_R2_001.fastq.gz
5-9-Nanog_Tg-JQ1-1_S7_R1_001.fastq.gz
5-9-Nanog_Tg-JQ1-1_S7_R2_001.fastq.gz
5-9-Nanog_Tg-JQ1-2_S8_R1_001.fastq.gz
5-9-Nanog_Tg-JQ1-2_S8_R2_001.fastq.gz

Processed ATAC:
1A-5-DMSO-1_S1_R1_001_val.mm9.sorted.RmDup.10mNorm.bw
1A-5-DMSO-2_S2_R1_001_val.mm9.sorted.RmDup.10mNorm.bw
1A-5-JQ1-1_S3_R1_001_val.mm9.sorted.RmDup.10mNorm.bw
1A-5-JQ1-2_S4_R1_001_val.mm9.sorted.RmDup.10mNorm.bw
### Genome browser session

(e.g. UCSC)

No longer applicable.

### Methodology

#### Replicates

ChIP-seq was performed with a singlicate for each condition, but validation of signal was done by ChIP-qPCR in triplicate, with high concordance. ATAC-seq was performed in duplicate, with two different Tet1/2 double knockouts, hence four ATAC-seq profiles per condition for the knockout experiments.

#### Sequencing depth

For ChIP-seq, the sequencing depth varied between 18.96 million reads (9.48 mil fragments; ChIP_SL2i-Med1) at the minimum and 73.6 million reads (36.8 mil fragments; ChIP_SL2i-Brd4) at the maximum.

For ATAC-seq, the sequencing depth varied between 33 million reads (16.5 mil fragments; ATACseq_JQ1_2) at the minimum and 215 million reads (107.5 mil fragments; ATAC_5-14-Nanog-Tg-JQ1-1) at the maximum. Paired-end sequencing was used for both ChIP and ATAC.

#### Antibodies

ChIP: Brd4 (Bethyl A301-985A50, Lot A301-985A50-4 and A301-985A50-5)

Med1 (CRSP1/TRAP220, Bethyl A300-793A, Lot A300-793A-3)

Nanog (Cosmo Bio REC-RCAB002P-F, lot A01OA06)

at 10 µg/mL (5.5 µg in 550 µL for 10 million cells).

#### Peak calling parameters

Enriched regions were discovered using MACS and scored against matched input libraries (fold change > 2 and p-value < 0.005), filtered for ENCODE black-listed regions, and peaks within 500 bp were merged.

#### Data quality

Signal-to-noise ratio was measured in 3 ways: 1) cross-correlation coefficient plot, 2) ratio of peak height to background levels in a composite plot over all significant peaks, 3) number of peaks called relative to matched input sample. Counts for peaks meeting fold change and FDR threshold ranged from 897 (inhibitor treatment) to 30,569 (control).

#### Software

ChIP-seq data was analyzed using samtools, bowtie, Bedtools, bwttools, ngsplot, DESeq2, and R.

### Flow Cytometry

#### Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

#### Methodology

Sample preparation: ESC cell lines were removed from gelatin coated plates using Accutase cell detachment solution (Thermo Fisher A1110501) for 5
Sample preparation

minutes at 37°C. They were centrifuged, washed 3x in FACS buffer and resuspended in FACS buffer containing DAPI. They were analyzed on a flow cytometer within 30 minutes.

Instrument

BD LSR Fortessa (for analysis), BD FACS Aria III (for sorting)

Software

FACSDiva was used for data acquisition. FlowJo 9.0 was used for data analysis.

Cell population abundance

Clonal cell lines were used for all experiments.

Gating strategy

The gating strategy was initially FSC-A by SSC-A, followed by doublet exclusion using FSC-H and SSC-H, and finally DAPI exclusion using FSC-A by DAPI-A in which DAPI-negative cells were in the lowest quartile of DAPI-A expression. In all cases, DAPI positive cells retained 100x more DAPI than negative cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.