-Catenin safeguards the ground state of mouse pluripotency by strengthening the robustness of the transcriptional apparatus

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MOLECULAR BIOLOGY

β-Catenin safeguards the ground state of mouse pluripotency by strengthening the robustness of the transcriptional apparatus

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Mouse embryonic stem cells cultured with MEK (mitogen-activated protein kinase kinase) and GSK3 (glycogen synthase kinase 3) inhibitors (2i) more closely resemble the inner cell mass of preimplantation blastocysts than those cultured with SL [serum/leukemia inhibitory factor (LIF)]. The transcriptional mechanisms governing this pluripotent ground state are unresolved. Release of promoter-proximal paused RNA polymerase II (Pol2) is a multistep process necessary for pluripotency and cell cycle gene transcription in SL. We show that β-catenin, stabilized by GSK3 inhibition in medium with 2i, supplies transcriptional coregulators at pluripotency loci. This selectively strengthens pluripotency loci and renders them addicted to transcription initiation for productive gene body elongation in detriment to Pol2 pause release. By contrast, cell cycle genes are not bound by β-catenin, and proliferation/self-renewal remains tightly controlled by Pol2 pause release under 2i conditions. Our findings explain how pluripotency is reinforced in the ground state and also provide a general model for transcriptional resilience/adaptation upon network perturbation in other contexts.

INTRODUCTION

Pluripotency can be sustained in vitro through culture in specific conditions. Mouse embryonic stem cells (ESCs) in conventional serum/leukemia inhibitory factor (LIF) (SL) medium are considered to exhibit naïve, preimplantation-like pluripotency because they contribute to chimeras with relative high efficiency upon blastocyst complementation. Yet, only a proportion of ESCs in SL are truly naïve at a given time, and the entire population is highly metastable,
RESULTS

Pluripotency maintenance in the ground state requires a residual BRD4 level

To investigate distinctive transcriptional features of mouse ESCs in 2iL, we identified two long-hairpin RNA (shRNA) clones targeting BRD4 in 2iL (Fig. S1A). These clones included known regulators of Pol2 pause release, histone methyltransferases, histone acetyltransferases/deacetylases, histone acetylation readers, and splicing regulators, many of which are known to be necessary for ESC maintenance in SL (table S1). For example, the knockdown was determined by measuring the expression of the core pluripotency markers Oct4 (Pou5f1), Nanog, and Klf2 by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Promoting pluripotency genes in SL but not in 2iL compared to SL (Fig. 1B and fig. S1C). In contrast to SL, ESC colonies in 2iL transduced with Brd4 shRNA remained domed and compact, as well as alkaline phosphatase (AP) positive, even after 10 to 14 days of single cells (fig. S1, G and H). We then validated the differential effects of Brd4 knockdown in both conditions (Fig. 1B and fig. S1C). These results demonstrated that Brd4 is less required for preserving pluripotency in 2iL than SL but remains necessary for self-renewal (i.e., robust proliferative expansion in vitro) under both conditions.

To further verify the differential sensitivity of pluripotency characteristics to BRD4 suppression in ESCs cultured in SL and 2iL, we used JQ1, a well-known BET inhibitor that binds to the two BRD4 bromodomains to prevent their interaction with acetylated histones (17). At lower doses (100 and 200 nM) for 60 hours, JQ1 notably impaired pluripotency in SL and 2iL compared to SL (Fig. 1B and fig. S1F), but we observed reduced proliferation in 2iL using two additional ESC lines and two more batches of ESC-qualified serum from different vendors (fig. S2, A to E). These results demonstrated that BRD4 is less required for preserving pluripotency in 2iL than SL but remains necessary for self-renewal (i.e., robust proliferative expansion in vitro) under both conditions.

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Fig. 1. Differential requirement for BRD4 in SL- and 2iL-cultured ESCs. (A) Heat map showing the relative expression of Pou5f1, Nanog, and Klf2 in ESCs in SL or 2iL transduced with shRNA for the indicated genes. (B) RT-qPCR for the indicated genes in ESCs in SL or 2iL transduced with shRNA for Luciferase (shLuc) or two shRNAs for Brd4 (shBrd4#1 and shBrd4#2). Data are the mean values ± SEM with the indicated significance (P value was calculated using two-tailed unpaired Student’s t test, also for all subsequent experiments unless otherwise noted). n = 3. (C) Growth curve of ESCs in 2iL transduced with shLuc, shBrd4#1, or shBrd4#2 measured by cell counting in triplicate at passage 1 after transduction. n = 2. A representative experiment is shown. (D) Percentage of cells in different cell cycle phases in ESCs in 2iL transduced with shLuc, shBrd4#1, or shBrd4#2 measured by flow cytometry at passage 1 after transduction (mean values ± SEM, n = 3). (E) Phase contrast and alkaline phosphatase (AP) activity of ESCs in SL or 2iL treated with vehicle [dimethyl sulfoxide (DMSO)] or JQ1 at the indicated doses. Scale bar, 50 μm. (F) As in (E) but shows RT-qPCR result for the indicated genes (mean values ± SEM, n = 3). (G) RT-qPCR for the indicated genes in ESCs in 2iL treated with DMSO or JQ1 at the indicated doses (mean values ± SEM, n = 3). (H) As in (C) but ESCs were treated with DMSO or JQ1 at the indicated doses for passage 0 (P0) or passage 1 (P1). n = 2. A representative experiment is shown. (I) As in (D) but ESCs were treated with DMSO or JQ1 at the indicated doses (mean values ± SEM, n = 3). (J) Heat map showing the fold change of pluripotency genes and cell cycle genes measured by RNA sequencing (RNA-seq) in ESCs in SL or 2iL treated with DMSO or 100 nM JQ1. *P < 0.05, **P < 0.01, ***P < 0.001.
less and differentiated when changed to SL culture conditions (fig. S4, E and F). We also noticed that, in contrast to wild-type clones, low doses of JQ1 could effectively reduce pluripotency gene expression in heterozygous Brd4 knockout ESCs in 2iL. (fig. S4G). We concluded that pluripotency maintenance is more resistant to BRD4 suppression in ESCs in 2iL than in SL, but reducing BRD4 beyond a threshold also affects pluripotency in 2iL.

Suppressing Pol2 pause release at pluripotency loci is better tolerated in the ground state

Pol2 pausing is mediated by pausing factors including DRB sensitivity–inducing factor (DSIF) and negative elongation factor (NELF), whereas pause release is triggered through phosphorylation of Pol2 on serine-2 (Ser2P) by CDK9. A major role of BRD4 is to induce Pol2 pause release by activating CDK9 (13), a target that was also identified as less necessary for 2iL in our shRNA screen (see above Fig. 1A). Consistently, analysis of CDK9 ChHiP-seq in SL showed notable overlap with BRD4 ChIP-seq in SL or 2iL. (Fig. 2A). Likewise, a sizeable proportion of genes down-regulated by JQ1 in SL or 2iL were bound by BRD4 and CDK9 (Fig. 2B), including many pluripotency (in SL) and cell cycle genes (in SL and 2iL) (Fig. 2, B and C, and fig. S5A). To confirm the differential CDK9 dependence in SL and 2iL, we repeated the knockdown experiments and also used a specific CDK9 inhibitor [LDCC000067; (18)]. As with Brd4 knockdown, Cdka9 knockdown severely affected colony morphology, AP activity, and pluripotency gene expression in SL but had no obvious effect in 2iL (Fig. 2, D and E), and this persisted for several passages (fig. S5, B and C). Proliferation and the cell cycle were significantly affected by Cdka9 knockdown in 2iL too (Fig. 2, F and G), although to a lesser extent than in SL (Fig. 2D). These effects were validated using an additional E2F line (fig. S5, D and E). Similarly, 10 μM LDCC000067 impaired colony morphology, AP activity, and pluripotency gene expression in SL but not in 2iL (even after multiple passages as single cells), but a higher dose had severe consequences in both conditions (Fig. 2, H and I, and fig. S5, F and G). Likewise, LDCC000067 reduced cell growth in SL and 2iL, impaired the cell cycle, and enhanced apoptosis significantly (Fig. 2, H and J to L). The consistent phenotypes of suppressing Brd4 and Cdka9 implied that reducing Pol2 pause release at pluripotency genes is better tolerated in 2iL than SL, suggesting a major change in transcriptional control in the two culture conditions.

β-Catenin induces resistance to Pol2 pause release suppression in the ground state

BET inhibitors including JQ1 are a promising therapeutic avenue for cancer, but recent reports have described resistance to BET inhibitors through activation of Wnt/β-catenin signaling (19, 20). In this pathway, Wnt ligands trigger stabilization and nuclear translocation of β-catenin, which then binds to and transactivates T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to switch on gene expression (21). We envisaged that β-catenin could also confer resistance to BRD4 suppression in ESCs cultured in 2iL, as, similarly to Wnt ligands, CHIR stabilizes β-catenin through Gsk3 inhibition (3). Moreover, β-catenin has been proposed to promote ground-state pluripotency by alleviating the repressor function of TCF3, which associates with pluripotency transcription factors at target loci (21–23). Yet, the specific mechanisms are not well understood. Accordingly, Tcf3 or Gsk3 depletion allow expansion of ESCs in serum-free medium with PD alone (3, 21, 23), whereas β-catenin is strictly required for expansion in 2iL medium without LIF (21, 22).

We first studied whether PD alone, CHIR alone, or the 2i added to ESCs in SL could rescue the negative effects of 100 nM JQ1 on colony morphology, AP activity, and pluripotency gene expression, which we tested using two ESC lines. PD alone had some rescue effect on Nanog expression but not on the other genes tested. CHIR was more effective in restoring pluripotency characteristics, but only the combined effect of PD and CHIR achieved a complete rescue (Fig. 3, A and B, and fig. S6A). As for cell proliferation, we observed that the moderate rescue effect of adding 2i to ESCs in SL treated with JQ1 was mostly mediated by PD (Fig. 3C).

To systematically dissect the role of specific components of the Wnt/β-catenin pathway, we treated wild-type ESCs in SL with WNT3A or used several knockout ESC lines lacking either Gsk3 (24), Ctnnb1 (encoding β-catenin) (22), or Tcf3 (23). In addition, we used wild-type ESCs overexpressing a mutant form of β-catenin (S33Y β-catenin) resistant to GSK3-mediated degradation (25). The authentication of Gsk3 and Ctnnb1 knockout ESC lines, and ESCs overexpressing S33Y β-catenin, was performed with a β-catenin/TCF reporter (fig. S6B), whereas Tcf3 knockout cells were validated by PCR amplification and sequencing (fig. S6C). WNT3A treatment significantly reversed the effects of 100 nM JQ1 on pluripotency characteristics in SL, and Gsk3 knockout achieved a stronger rescue (Fig. 3, D and E, and fig. S6, D and E). The latter was also confirmed by RNA-seq (fig. S3F and fig. S6F). The stronger effect of Gsk3 knockout compared to WNT3A and CHIR alone is possibly related to the extent and length of GSK3 suppression. Likewise, Tcf3 knockout and S33Y β-catenin overexpression induced significant resistance to JQ1 in SL (Fig. 3, G to J). Moreover, Ctnnb1 knockout became sensitive to 200 nM JQ1 in 2iL, but the effect on pluripotency gene expression was not as strong as for wild-type ESCs in SL (Fig. 3, K and L). We also validated the resistance to the CDK9 inhibitor LDCC000067 in Gsk3 or Tcf3 knockout ESCs (fig. S6, G and H). Therefore, GSK3 inhibition is the main mediator of the resistance of pluripotency genes to suppression of pause release in 2iL, of which β-catenin stabilization is a major component.

β-Catenin increases transcription initiation at pluripotency loci in the ground state

To understand how β-catenin mediates resistance to suppression of Pol2 pause release at pluripotency loci, we compared β-catenin bound sites (table S3) in ChIP-seq (from a study using SL + CHIR) (26) with BRD4 bound sites in 2iL. There was a good genome-wide overlap (Fig. 4A), mostly at distal enhancers but also at promoters (fig. S7A), although the binding of BRD4 was more widespread. Moreover, we noticed that most β-catenin/BRD4 cobound genes were not down-regulated by JQ1 in 2iL. We then named β-catenin/BRD4 cobound genes that are down-regulated by JQ1 in SL but not 2iL as group 1 genes (Fig. 4B). By contrast, group 2 genes were defined as genes down-regulated by JQ1 in 2iL that are bound by BRD4 but not β-catenin. Group 2 included many cell cycle genes, whereas group 1 included many pluripotency regulators (Fig. 4B and table S4). ChIP-qPCR confirmed enhanced β-catenin binding at selected group 1 pluripotency loci in 2iL compared to SL, whereas at group 2 cell cycle–related loci did not change (fig. S7B). ChIP-seq analysis also showed that TCF3 binds to a notable proportion of group 1 genes, whereas most of the group 2 genes were negative (fig. S7C and table S4). These findings suggested that β-catenin promotes resistance to Pol2 pause release suppression through cobinding with BRD4/CDK9 at target loci, including pluripotency loci.
Fig. 2. Differential requirement for CDK9 in SL- and 2iL-cultured ESCs. (A) Venn diagrams showing the overlap between BRD4 bound sites in ESCs in SL or 2iL and CDK9 bound sites. (B) Venn diagrams showing the overlap between genes down-regulated by 100 nM JQ1 in ESCs in SL or 2iL and BRD4/CDK9 cobound genes. (C) Genome views for a BRD4/CDK9 cobound pluripotency gene (Nanog) and a cell cycle gene (Mdm4) in ESCs cultured as indicated. (D) Phase contrast and AP activity of ESCs in SL or 2iL transduced with shLuc or two shRNAs for Cdk9 (shCdk9#1 and shCdk9#2). Scale bar, 50 μm. (E) As in (D) but shows RT-qPCR result for the indicated genes (mean values ± SEM, n = 5). (F) Growth curve of ESCs in 2iL transduced with shLuc, shCdk9#1, or shCdk9#2 measured by cell counting in triplicate at passage 1 after transduction. n = 2. A representative experiment is shown. (G) Percentage of cells in different cell cycle phases in ESCs in 2iL transduced with shLuc, shCdk9#1, or shCdk9#2 measured by flow cytometry at passage 1 after transduction (mean values ± SEM, n = 3). (H) Phase contrast and AP activity of ESCs in SL or 2iL treated with DMSO or LDC000067 (CDK9i) at the indicated doses. Scale bar, 50 μm. (I) As in (H) but shows RT-qPCR result for the indicated genes (mean values ± SEM, n = 4). (J) As in (F) but ESCs were treated with DMSO or LDC000067. n = 2. A representative experiment is shown. (K) As in (G) but ESCs were treated with DMSO or LDC000067 (mean values ± SEM, n = 3). (L) Percentage of apoptotic cells in ESCs in 2iL treated with DMSO or LDC000067 (mean values ± SEM, n = 4). *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 3. Wnt/β-catenin signaling endows resistance to BRD4 suppression in 2iL–cultured ESCs. (A) Phase contrast and AP activity of ESCs cultured in SL with PD, CHIR, or 2i and treated with DMSO or JQ1. Scale bar, 50 μm. (B) As in (A) but shows RT-qPCR result for the indicated genes (mean values ± SEM, n = 3). P value was calculated using two-way ANOVA with Tukey’s multiple comparison posttest. (C) Population doublings of ESCs in SL with PD, CHIR, or 2i, and treated with 500 nM JQ1 for 4 days relative to controls treated with DMSO (mean values ± SEM, n = 4). (D) Phase contrast and AP activity of wild-type (WT) and Gsk3 knockout (KO) ESCs in SL treated with DMSO or JQ1. Scale bar, 50 μm. (E) As in (D) but shows RT-qPCR result for the indicated genes (mean values ± SEM, n = 3). P value was calculated using two-way ANOVA with Sadik’s multiple comparison posttest, also for (H), (J), and (L). (F) Heat map showing the fold change of pluripotency (left) and cell cycle genes (right) measured in RNA-seq of wild-type or Gsk3 knockout ESCs in SL treated with DMSO or 100 nM JQ1. (G) Phase contrast and AP activity of wild-type and Tcf3 knockout ESCs in SL treated with DMSO or JQ1. Scale bar, 50 μm. (H) As in (G) but shows RT-qPCR result for the indicated genes (mean values ± SEM, n = 3). (I) Phase contrast and AP activity of wild-type and S33Y β-catenin–overexpressing ESCs in SL treated with DMSO or JQ1. Scale bar, 50 μm. (J) As in (I) but shows RT-qPCR result for the indicated genes (mean values ± SEM, n = 3). (K) Phase contrast and AP activity of wild-type and Ctnnb1 knockout ESCs in 2iL treated with DMSO or 200 nM JQ1. Scale bar, 50 μm. (L) As in (K) but shows RT-qPCR result for the indicated genes (mean values ± SEM, n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Fig. 4. β-Catenin increases transcription initiation at pluripotency loci in 2iL-cultured ESCs. (A) Venn diagram showing the overlap between BRD4 bound sites in ESCs in 2iL and β-catenin bound sites. (B) Venn diagram showing the overlap between BRD4 bound genes down-regulated by 100 nM JQ1 in ESCs in SL or 2iL and β-catenin bound genes. (C) Occupancy plots for genome-wide nuclear run-on sequencing (GRO-seq) signal around the TSS of group 1 and 2 genes in ESCs in 2iL and SL. (D) Violin plots showing the corresponding normalized read counts of GRO-seq at the proximal promoter or gene body for group 1 and 2 genes. RPM, reads per kilobase per million mapped reads. Y axis was added for the indicated times before sample collection. *P < 0.05, **P < 0.01, ***P < 0.001.
Next, we sought to elucidate the molecular mechanism underlying the above observations. To rule out the possibility that β-catenin compensates for the negative effect of JQ1 on pluripotency genes by enhancing mRNA stability in 2iL (27), we measured a panel of pluripotency mRNAs after actinomycin D treatment, which blocks transcription. Their stability was similar or lower in 2iL compared to SL (fig. S7D). This hinted to β-catenin maximizing transcriptional flux at target genes in 2iL as a way to counteract a reduction in Pol2 pause release. So, we turned our attention to potential differences in transcriptional dynamics between ESCs cultured in SL and 2iL. In this regard, a recent Pol2 ChIP-seq study (6) showed a global increase of promoter-proximal signal in 2iL that was not matched in the gene body, concluding that Pol2 pausing is more prevalent in 2iL than in SL. This was attributed to low expression of c-MYC in ESCs in 2iL, as c-MYC induces Pol2 pause release via CDK9 (28). Our reanalysis of this dataset showed a strong increase of Pol2 signal at the proximal promoter in 2iL for both group 1 and group 2 genes (fig. S7, E to G), but this could represent either more Pol2 pausing or more transcriptional initiation. As opposed to Pol2 pausing, more transcriptional initiation implies more gene body elongation if the degree of pausing remains constant and, hence, often associates with increased gene expression. Consistent with the former possibility, the Pol2 signal along the gene body only increased moderately at both groups of genes in 2iL, especially at group 1 (fig. S7, E to G). To define the extent of pausing at these loci more accurately, we used the Pol2 traveling ratio (TR), which compares the ratio in the signal of the proximal promoter and the gene body (12, 28, 29). The TR of both group 1 and group 2 genes was higher in 2iL (fig. S7H), supporting the idea that there is indeed more Pol2 pausing in both groups of genes in 2iL. Yet, it is difficult to reconcile the resistance of group 1 genes to BRD4/CDK9 suppression in 2iL with an increased Pol2 pausing that, in principle, would reduce gene expression. In summary, the recruitment of β-catenin to BRD4 bound sites in 2iL changes the mode of transcriptional regulation at target loci including pluripotency loci, which then rely more on transcription initiation for gene body elongation in detriment to Pol2 pause release.

β-Catenin supplies coregulators to maximize transcriptional flux at pluripotency loci

We searched β-catenin protein interaction networks looking for partners whose recruitment or reinforcement at group 1 genes in 2iL could explain the above phenomena. In addition to chromatin remodeling complexes (35), we observed two modules corresponding to transcription initiation and elongation (Fig. 5, A and B). Among other β-catenin interacting proteins in these modules, we noticed Pol2, TATA-binding protein–associated factors (TAF5/6/7), cohesin components (SMC1A and SMC3), and, interestingly, BRD4 and CDK9 as well. Pol2 and TAFs are critical for transcription initiation (36), whereas cohesin regulates transcription by forming ring-like structures that allow enhancer-promoter looping (36). We also noticed previous reports describing the interaction of β-catenin with mediator (37, 38) and p300 (39) in other cell contexts. Mediator was immediately interesting because it is a well-known partner of BRD4 that controls transcription initiation through both cross-talk with TFIH and enhancer-promoter looping (40). Immunoprecipitation of β-catenin followed by Western blotting confirmed the interaction with mediator (MED1 and MED12), cohesin (SMC1A), and BRD4 in ESCs in 2iL (Fig. 5C). Likewise, ChIP-seq analysis showed genome-wide colocalization of β-catenin, MED1, SMC1A, and BRD4 in 2iL at many pluripotency genes belonging to group 1 (Fig. 5D and fig. S9, A and B). To see whether β-catenin is actually promoting the recruitment of these coregulators at target loci, we compared ChIP-seq datasets for MED1, SMC1A, and BRD4 in 2iL and SL. We observed higher levels of the three coregulators at β-catenin binding sites in 2iL (Fig. 5, E to G). BRD4 also showed increased signal outside β-catenin
Fig. 5. β-Catenin supplies coregulators to reinforce the transcriptional apparatus in 2iL-cultured ESCs. (A) Gene Ontology (GO) analysis of β-catenin protein-interactome data based on a previous report (35). GO terms associated with transcriptional regulation and ESC identity are shown (Benjamini-Hochberg corrected P value). (B) Functional network of β-catenin interacting proteins related to transcriptional regulation based on STRING protein interaction database (60) as visualized by Cytoscape. β-Catenin partners found in both STRING database and the above protein interactome data are highlighted in gray. SMC1A and SMC3 also interact with β-catenin but belong to the GO term stem cell population maintenance. (C) Western blotting following immunoprecipitation (IP) of β-catenin but belong to the β-catenin interacting proteins related to transcriptional regulation based on STRING protein interaction database (60). (D) Genome views of ATAC-seq and H3K27ac, DNA-templated transcription, elongation, and initiation. (E) Occupancy plot (top) and boxplot (bottom) showing the normalized read counts for MED1 ChIP-seq signal in ESCs in SL or SL plus 2i (S2iL). (F) Occupancy plot (top) and boxplot (bottom) showing the normalized read counts for SMAC1A ChIP-seq signal in ESCs in SL or SL plus 2i (S2iL). (G) Occupancy plot (top) and boxplot (bottom) showing the normalized read counts for BRD4 ChIP-seq signal in ESCs in SL or SL plus 2i (S2iL). (H) RT-qPCR for the indicated genes in wild-type (WT) compared to β-catenin knockout (β-Ctnnb1 KO) ESCs. (I) RT-qPCR for SMC1A at β-catenin knockout (β-Ctnnb1 KO) compared to wild-type (WT) ESCs. (J) RT-qPCR for BRD4 at β-catenin knockout (β-Ctnnb1 KO) compared to wild-type (WT) ESCs. (K) ChIP-qPCR for β-catenin at β-catenin knockout (β-Ctnnb1 KO) compared to wild-type (WT) ESCs. (L) ChIP-qPCR for β-catenin at β-catenin knockout (β-Ctnnb1 KO) compared to wild-type (WT) ESCs.
of splicing factors (48). We did not observe any notable difference in the number of alternatively spliced genes regulated by GSK3 between group 1 and group 2 genes (fig. S9I). Yet, we noticed that β-catenin interacts with multiple splicing regulators including SRSF3 and TRA2B (fig. S9J) (35), both of which also appeared in our screen as differentially required in SL and 2iL (see above Fig. 1A). We validated that Srsf3 and Tra2b knockdown is better tolerated in 2iL compared to SL (fig. S9K). This observation suggests that β-catenin helps stabilize splicing regulators at pluripotency genes to render ESCs more resistant to a splicing reduction in 2iL. Although a potential role in modifying the speed of gene body elongation would need to be investigated, these results support the model depicting β-catenin as a scaffold that strengthens transcription at pluripotency loci in 2iL.

Permissive chromatin features help maximize transcriptional flux at pluripotency loci in the ground state

We also investigated chromatin features that could further contribute to maximizing transcriptional flux at group 1 genes in 2iL compared to SL. We focused on histone acetylation and DNA hypomethylation because these epigenetic marks associate with chromatin opening, transcription activation, and reduced Pol2 pausing (42, 43). H3K27 acetylation (H3K27ac) around β-catenin binding sites was higher in 2iL than in SL (Figs. 5D and 6A), consistent with the recruitment of histone acetyltransferases (e.g., p300) by β-catenin (39). Similarly, we observed an increase in H3K27ac in 2iL when comparing the −2-kb to +2-kb region around the TSS of group 1 genes. By contrast, H3K27ac did not increase at group 2 genes in 2iL compared to SL, and group 2 genes in 2iL had lower H3K27ac than group 1 genes (Fig. 6B). Consistent with the changes in H3K27ac, we noticed a clear increase in open chromatin with an assay for transposase-accessible chromatin sequencing (ATAC-seq) at β-catenin binding sites in 2iL compared to SL and more moderately also at group 1 genes, whereas, at group 2 genes, it was slightly reduced in 2iL (Figs. 5D and 6, C and D). Notably, DNA methylation at β-catenin binding sites was lower in 2iL than in SL (Fig. 6E). Yet, this effect extended to the entire locus of not only group 1 but also group 2 genes (Fig. 6F), indicating that it is not directly mediated by β-catenin. The latter is in agreement with the existence of global DNA hypomethylation in 2iL, which is mostly driven passively through the suppression of UHRF1 protein stability induced by PD (44). In this regard, the limited number of sites actively demethylated by the ten-eleven translocation (TET) enzymes in the conversion of ESCs from SL to 2iL (44) included few β-catenin binding sites (fig. S9E). Accordingly, TET1/2 double and TET1/2/3 triple knockout ESCs (45) did not show increased sensitivity of pluripotency genes to JQ1 in 2iL compared to the control (fig. S9, F to H). We concluded that permissive chromatin features, some of which are induced by β-catenin, likely contribute to strengthening pluripotency gene transcription in 2iL by facilitating the assembly of multiprotein complexes (see schematic in Fig. 7).

In addition to the recruitment of coregulators and the chromatin changes, other mechanisms may participate in inducing transcriptional resilience at pluripotency loci in 2iL. For example, alternative RNA splicing is a cotranscriptional event that can influence the speed with which Pol2 moves along the gene body (46), and it has also been shown that specific splicing regulators participate in Pol2 pause release (47). Likewise, Gsk3 knockout in ESCs in SL reduces the amount of alternative splicing due to impaired GSK3-mediated phosphorylation

DISCUSSION

Mouse ESC pluripotency can be viewed as a continuum of hierarchical interconvertible states on the road to a somatic phenotype. The more naïve or closer to inner cell mass characteristics, the more pluripotency is consolidated, but the underlying mechanisms are poorly understood. We have shown here that β-catenin stabilized by CHIR selectively reinforces the pluripotency gene network in 2iL by potentiating the recruitment of BRD4, CDK9, mediator, cohesin, p300, and other transcriptional coregulators to pluripotency loci. This selectively heightens transcription initiation at pluripotency loci, enhancing gene body elongation in 2iL and making it more—albeit not completely—dependent of Pol2 pause release by BRD4/CDK9 than in SL. The enhanced transcriptional elongation in 2iL likely explains why expression of multiple pluripotency genes is higher than in SL and potentially also why there is less oscillation in gene expression (an underlying cause of metastability) (7). The removal of TCF3 from pluripotency loci causes a similar transcriptional consequence to β-catenin stabilization, conceivably by allowing closer interactions between coregulators and the pluripotency transcription factors or by removing detrimental epigenetic activities [e.g., histone deacetylases (49)]. PD also contributes to inducing resistance to suppression of Pol2 pause release in 2iL possibly by inducing Nanog mRNA and stabilizing NANOG protein (50). The former effect might be caused by preventing extracellular signal–regulated kinase–mediated phosphorylation and dissociation of coregulators including MED24 from Pol2-containing complexes at the Nanog locus (51). As opposed to pluripotency genes, proliferation genes are not bound by β-catenin and, thus, remain very sensitive to suppression of Pol2 pause release in 2iL.

In recent years, it has become evident that phase-separated biomolecular condensates compartmentalize biochemical reactions within cells, including transcription (52). This is caused by multivalent interactions between proteins, many of which have intrinsically disordered regions (IDRs) that confer the physicochemical properties of the condensate. In this regard, it was recently proposed that, thanks to its two disorganized domains at the N-terminal (amino acids 1 to 141) and C-terminal (amino acids 727 to 781) ends, β-catenin is attracted to stable chromatin phase-separated condensates formed by mediator and BRD4 to execute its signaling role in ESCs in 2iL (53). Our findings suggest that β-catenin might be a priming event for the stabilization of these condensates in ESCs in 2iL by enhancing the cooperative and multivalent interactions between coregulators at pluripotency loci (Fig. 7). This is consistent with our observation...
that the C-terminal domain of β-catenin containing one of its IDRs is not necessary for the resilience of ESCs in 2iL to JQ1 and the fact that β-catenin IDRs are much shorter than those of BRD4 and MED1 (53, 54). The physicochemical forces created within these condensates and the interaction with β-catenin could cause a remnant of BRD4, CDK9, and other coregulators to tend to localize to pluripotency loci despite genome-wide depletion induced by shRNAs or chemical inhibitors.

Finley et al. recently reported that BRD4 is dispensable for pluripotency and self-renewal in the ground state (41). A reduced rather than abolished requirement for BRD4 in the early embryo is perhaps easier to understand from a developmental point of view, as it is supported by the observation that Brd4 null mouse embryos cannot maintain the inner cell mass (15, 55). Finley et al. also proposed that a strengthened network of pluripotency transcription factors and the recruitment of TET enzymes partially contribute to the resistance to BRD4 suppression in 2iL. The former mechanism fits well with our observations, as transcription factors can recruit coregulators and enhance transcription initiation (11). Yet, we did not observe any evidence for TET involvement, which may be related to variations among ESC lines or in the culture conditions. Despite the differences, both studies are relevant and highlight the striking similarities in transcriptional adaptation upon network perturbation between ESCs in the ground state and cancer cells. Further mechanistic knowledge will mutually contribute to understand ground-state pluripotency and cancer cell resistance to drugs. For example, ESCs in 2iL may prove to be a useful model to identify either more effective anticancer drugs or synergistic combinations. In this regard, our findings with ESCs in 2iL suggest that treatment of BRD4-addicted cancers with a combination of JQ1 and inhibitors of transcription initiation might be a more robust and applicable anticancer therapy for a general patient base than JQ1 alone.

Fig. 6. β-Catenin induces permissive chromatin features to maximize transcriptional flux at pluripotency loci. (A) Occupancy plot (left) and boxplot (right) showing the normalized read counts for H3K27ac ChIP-seq signal in ESCs in SL or 2iL around β-catenin bound sites. (B) Occupancy plot (left and middle) and boxplot (right) showing the normalized read counts for H3K27ac ChIP-seq signal in ESCs in SL or 2iL around the TSS of group 1 and 2 genes. (C) Occupancy plot (left) and boxplot (right) showing the normalized read counts for ATAC-seq signal in ESCs in SL or 2iL around β-catenin bound sites. (D) Occupancy plot (left and middle) and boxplot (right) showing the normalized read counts for ATAC-seq signal in ESCs in SL or 2iL around the TSS of group 1 and 2 genes in ESCs in SL and 2iL. (E) Occupancy plot (left) and boxplot (right) showing the normalized read counts for DNA methylation in ESCs in SL or 2iL around β-catenin bound sites. (F) Occupancy plot (left and middle) and boxplot (right) showing the normalized read counts for DNA methylation in ESCs in SL or 2iL around the TSS of group 1 and 2 genes.
In the future, it will be important to study whether the molecular interface regulating the interaction between β-catenin and transcriptional coregulators can be used to develop specialized anticancer drugs. It will also be interesting to test whether the principles presented here can yield optimized methods for sustaining ground-state pluripotency in vitro in a broad spectrum of mammals.

**MATERIALS AND METHODS**

**Cell lines and culture conditions**

Human embryonic kidney–293T (HEK293T) cells were purchased from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM)/high glucose (Corning, 10-017-CVR) containing 10% fetal bovine serum (FBS; Biowest). THP1 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium (Thermo Fisher Scientific, C11875500CP) supplemented with 10% FBS (Biowest), GlutaMAX (Gibco, 35050079), penicillin/streptomycin (Hyclone, SV30010), and β-mercaptoethanol (Gibco, 2198503). ESCs in SL medium were cultured in DMEM/high glucose containing 15% FBS (Biological Industries; unless otherwise specified), GlutaMAX, penicillin/streptomycin, nonessential amino acids (Gibco, 11140050), sodium pyruvate (Corning, 25-000-CI), β-mercaptoethanol, and LIF (1000 U/ml) on mitomycin-C–treated mouse embryonic fibroblasts (as feeders); they were split onto 0.2% gelatin-precoated plates before each experiment. ESCs in 2iL medium were cultured in a 1:1 mix of DMEM/F12 (Hyclone, SH30023.01) and Neurobasal medium (Gibco, 21103049) with N2 (Gibco, 17502048) and B27 (Gibco, 17504044) supplements, GlutaMAX, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, β-mercaptoethanol, LIF (1000 U/ml), 3 μM CHIR99021 (StemRD, CHIR-50), and 1 μM PD0325901 (StemRD, PD-50) on 0.2% gelatin-precoated plates. SL and 2iL media were changed daily. ESCs cultured in SL medium were cryopreserved in CELLBANKER 2 (Amsbio, 11891). After cell thawing, the same vial was used for culture in SL or 2iL. For the latter, ESCs cultured in SL were adapted to 2iL for three passages before each experiment. ESCs in SL or 2iL were passaged as single cells using 0.05% trypsin (Gibco, 25300054) every 3 days. The other two types of serum for SL medium were purchased from Fisher Scientific and Biowest; both were tested for ESC maintenance beforehand in the Esteban laboratory. Other inhibitors including JQ1 (BPS Bioscience, 27402), LDC000067 (Selleck Chemicals LLC, S7461), THZ1 (MedChemExpress, HY-80013), and actinomycin D (Sigma-Aldrich, A1410) were dissolved in dimethyl sulfoxide and added into the medium at the indicated concentrations. JQ1 and
LDC000067 were added for 60 hours unless otherwise specified. E14gt2a (E14) ESCs were provided by I. Samokhvalov (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, China); they were used for all experiments unless otherwise specified. 129 and OG2 ESCs were provided by J. Liu (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, China). Tcf3 knockout ESCs (23) were provided by B. Merrill (University of Illinois at Chicago, USA). Gsk3 knockout ESCs, S33Y β-catenin–overexpressing ESCs, Ctnnb1 knockout ESCs, Ctnnb1 knockout ESCs rescued by either a wild-type or a C-terminal truncated format of β-catenin, Tet1/2 double knockout ESCs, and Tet1/2/3 triple knockout ESCs were previously reported (21, 24, 25, 45).

shRNA transduction, RNA isolation, RT-qPCR, and RNA-seq

For shRNA experiments, ESCs cultured in SL or 2iL medium were infected with lentiviruses generated from HEK293T cells. Samples were extracted 96 hours after infection unless otherwise specified. shRNA inserts were cloned into pLKO.1 lentiviral vectors. All shRNA target sequences and RT-qPCR primers are listed in table S5. RNA samples were isolated using TRIzol reagent (Thermo Fisher Scientific, 15596026). RT-qPCR was performed using the SYBR Premix Ex Taq Kit (Takara, RR420A) with an ABI 7500 real-time PCR machine. Data were analyzed in triplicate and normalized on the basis of Actb values. RNA-seq was performed by Ribobio Co. Ltd., China.

Animal study

Animal experiments were compliant with all relevant ethical regulations regarding animal research and were conducted under the approval of the Animal Care and Use Committee of the Guangzhou Institutes of Biomedicine and Health under license number 2016012. For teratomas, ESCs were trypsinized, and 2 × 10^6 cells were injected into the flanks of immunocompromised nude mice. Mice were euthanized when the tumor diameter reached 1.5 cm, and the teratomas were processed for histological analysis. Chimeras were produced by injecting ESCs into blastocysts followed by implantation into a pseudopregnant C57BL/6j mouse.

Proliferation, cell cycle, apoptosis, and AP activity assays

For proliferation assays, 60,000 ESCs were seeded, unless otherwise specified, per well of a six-well plate (three wells per time point). ESCs were counted at the indicated time points with a Bright-Line hemacytometer (Marienfeld). Cell cycle experiments were performed with propidium iodide staining (Beyotime, C1052) followed by flow cytometry analysis. Apoptosis experiments were performed with the Annexin V–FITC (fluorescein isothiocyanate) Apoptosis Detection Kit (Vazyme Biotech, A211) followed by flow cytometry analysis. Flow cytometry data were analyzed with FlowJo (v10.4) software. AP activity was detected with the BCIP-NBT Alkaline Phosphatase Color Development Kit (Roche, 11681451001).

Brd4 conditional knockout

Plasmid construction

Dual single guide RNAs (sgRNAs) were designed to target upstream and downstream intron of exon 5, respectively. Two sets of sgRNAs were designed, and the more efficient set was used for the experiments. sgRNAs were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene, 42230). PKD-EF1α–CreER with a puromycin resistance gene was obtained by subcloning pCAG-CreERT2 (Addgene, 14797) into a PKD-EF1α lentiviral backbone plasmid. The left and right homologous arms of the mouse genome and a fragment containing LoxP-exon5–FRT-PKG-Neo–FRT-LoxP were cloned into pMD-19 T donor plasmid (Takara, 6013).

Generation of Brd4(fl/fl) clones

ESCs cultured in 2iL medium were transduced with the donor and PX330-CAS9-sgRNA plasmids using Lipofectamine 3000 (Invitrogen, L3000015). G418 (Merk, 108321-42-2) was added 24 hours after transduction for selection. After selection, the remaining cells were seeded into a 96-well plate for genotyping. To obtain Brd4(fl/fl) clones, the remaining cells were again transfected with pCAG-FlpeGFP plasmid (Addgene, 13788) and the green fluorescent protein (GFP)–positive cells were sorted out to remove the selective marker Neo that was already integrated. Cells were then transduced with the donor and PX330-CAS9-sgRNA plasmids for a second round. After selection with G418, all the remaining cells were seeded again into a 96-well plate for genotyping. For Brd4(fl/fl) clones, the left-LoxP-exon5–containing fragment and right-LoxP–containing fragment were amplified for Sanger sequencing to make sure that the sequence and position of the LoxPs and exon 5 were correctly modified.

Generation of Brd4(fl/fl) and Brd4(fl−/−) clones

Brd4(fl/fl) clones were transduced with the PKD-EF1α–CreER plasmid and selected with puromycin (InvivoGen, ant-pr-1) for 2 days. The expression level of CreER was tested by RT-qPCR. Cells were seeded into a 96-well plate, and 4-hydroxytamoxifen was added to induce deletion of the floxed alleles. Genotyping was performed to obtain Brd4(fl/fl) and Brd4(fl−/−) clones. Brd4(fl−/−) clones were transduced with pCAG-CreGFP plasmid (Addgene, 13776), and GFP-positive cells were sorted 72 hours later. The sorted GFP-positive cells were seeded into a 96-well plate for genotyping to get Brd4(fl−/−) clones. All primers are listed in table S5.

Chlp-qPCR

Ten million cells were cross-linked in freshly prepared formaldehyde solution (1% final concentration for 10 min at room temperature) and then quenched with 125 mM glycine (for 5 min at room temperature). Fixed cells were washed with cold phosphate-buffered saline (PBS), harvested, flash-frozen in liquid nitrogen, and stored at −80°C for further use. For β-catenin, Pol2 Ser5P, and Pol2 Ser2P Chlp-qPCR, immunoprecipitation was performed as reported by Ward et al. (56). For MED1, SMC1A, and BRD4 Chlp-qPCR, immunoprecipitation was performed as reported by Finley et al. (41). After elution of antibody-bound complexes from the beads, cross-linking was reversed by overnight incubation at 65°C. Samples were diluted in TE (Tris-EDTA) buffer and then treated with ribonuclease A (Sigma-Aldrich, R6513) for 1 hour at 37°C, followed by incubation with proteinase K (Thermo Fisher Scientific, 25530049) for 2 hours at 55°C. DNA was purified using the QIAquick PCR Purification Kit (Qiagen, 28106). Antibodies used for Chlp-qPCR were immunoglobulin G (Abcam, ab172730), anti–β-catenin (Abcam, ab32572), anti–Pol2 Ser5P (Abcam, ab5131), anti–Pol2 Ser2P (Abcam, ab5095), anti-MED1 (Bethyl, A300-793), anti-SMC1A (Bethyl, A300-055), and anti-BRD4 (Bethyl, A301-985A). Primers for Chlp-qPCR are listed in table S5.

GRO-seq

GRO-seq was performed as previously described (57). Briefly, nuclei from 107 ESCs were extracted and run-on-transcribed with BrUTP (Sigma-Aldrich, B7166) and other nucleoside 5’-triphosphates at 30°C for 5 min. Nascent RNA was enriched by agarose-coated anti-BrUTP
ChIP-seq, binding sites were called using MACS2 (v2.1.0) with the `-q 30.` Duplicated reads were collapsed using Picard (v1.9.0). For using Bowtie2 (v2.2.5) with the settings `--very-sensitive.` Low-performed by ClusterProfiler (v3.6.0) (58).

Functional annotation was further determined by DESeq2 (v1.18.1) and were defined as absolute fold change of >2 and value of <0.1. For ChIP-seq and ATAC-seq libraries, data were aligned to the mm10 mouse genome assembly using Bowtie2. For quantification of Pol2 ChIP-seq and GRO-seq signals, the proximal promoter was considered as the −100- to +300-bp region around the annotated TSS, and the gene body was considered as the +300-bp to +2-kb region downstream of the annotated TSS. Reads were first normalized as reads per million mapped reads or reads per kilobase per million mapped reads using deepTools (v 3.3.1) and further assigned to the corresponding regions, while the top 1% of the values were trimmed. For whole-genome bisulfite sequencing analysis, data were aligned to the mm10 mouse genome assembly using BSMAP with the settings `"-v 0.1 -g 1 -p 8 -R -u"` and further assigned to corresponding regions. Occupancy plots were generated by deepTools. Cumulative plots, violin plots, and boxplots were generated by ggplot2 (v2.2.1); the black central line of boxplots is the median, the boxes indicate the upper and lower quartiles, and the whiskers indicate the 1.5 interquartile range.

Wnt reporter activity assay
ESCs were seeded 12 hours before transfection on gelatin-precoated 24-well plates at a density of 30,000 cells per well. TOPflash or FOPflash report plasmids (Millipore, 17-285) and Renilla luciferase plasmids were transduced using Lipofectamine 3000. Twenty-four hours after transfection, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, E1910).

Cell viability assay for THP1 cells
CCK-8 Cell Counting Kit (Vazyme Biotech, A311-02) was used to evaluate the cell viability of THP1 cells. THP1 cells (8,000 per well) were seeded in a 96-well plate. For measurements, 10 μl of CCK-8 solution was added to each well, and the plates were incubated for 1 to 4 hours at 37°C before the absorbance was measured at 450 nm using an Epoch 2 microplate spectrophotometer from BioTek.

Supplementary materials are available at http://advances.sciencemag.org/cgi/content/full/6/29/eaba1593/DC1 view/request a protocol for this paper from Bio-protocol.

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β-Catenin safeguards the ground state of mouse pluripotency by strengthening the robustness of the transcriptional apparatus

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