Bach1 regulates self-renewal and impedes mesendodermal differentiation of human embryonic stem cells

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The transcription factor BTB and CNC homology 1 (Bach1) is expressed in the embryos of mice, but whether Bach1 regulates the self-renewal and early differentiation of human embryonic stem cells (hESCs) is unknown. We report that the deubiquitinase ubiquitin-specific processing protease 7 (Usp7) is a direct target of Bach1, that Bach1 interacts with Nanog, Sox2, and Oct4, and that Bach1 facilitates their deubiquitination and stabilization via the recruitment of Usp7, thereby maintaining stem cell identity and self-renewal. Bach1 also interacts with polycomb repressive complex 2 (PRC2) and represses mesendodermal gene expression by recruiting PRC2 to the genes’ promoters. The loss of Bach1 in hESCs promotes differentiation toward the mesendodermal germ layers by reducing the occupancy of EZH2 and H3K27me3 in mesendodermal gene promoters and by activating the Wnt/β-catenin and Nodal/Smad2/3 signaling pathways. Our study shows that Bach1 is a key determinant of pluripotency, self-renewal, and lineage specification in hESCs.

INTRODUCTION

Stem cell identity, differentiation, and development are regulated, in large part, by histone modifications and chromatin remodeling, and the polycomb group (PcG) is a set of chromatin modifiers that maintain cellular identity and control differentiation by suppressing critical developmental genes (1). In mammals, PcG proteins are classified into two primary categories, polycomb repressive complexes 1 (PRC1) and 2 (PRC2), and whole-genome studies have shown that PRC2 occupies the promoters of many developmental genes in both human and mouse embryonic stem cells (hESCs and mESCs, respectively) (2). PRC2 is composed of three core subunits embryonic ectoderm development (EED), SUZ12 polycomb repressive complex 2 subunit (SUZ12), and enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) (2), and mutations in any one of these subunits lead to gastrulation defects and early lethality in mice (3). EZH2 suppresses gene expression by catalyzing the di- and trimethylation of histone H3 on lysine 27 (H3K27) (producing H3K27me3) and by serving as a recruitment platform for DNA methyltransferases (4); thus, the loss of EZH2 leads to severe defects in the self-renewal and differentiation of hESCs (5), but the mechanisms by which PRC2 is recruited to its target genes have yet to be identified.

Stem cell pluripotency and self-renewal are also modulated via the activity of ubiquitin ligases and deubiquitinases (DUBs). DUBs maintain stemness by stabilizing stem cell pluripotency factors (6); for example, ubiquitin-specific processing protease 7 (Usp7) deubiquitinates tumor suppressors such as p53, phosphatase and tensin homolog (PTEN), and MDM2 proto-oncogene (HDM2) (7), and the inactivation of Usp7 during early embryonic development causes embryonic lethality via an increase in p53 activity (8). Notably, Usp7 also appears to be involved in the ubiquitination of histone H2B in mESCs (9) and is required for the osteogenic differentiation of human adipose-derived stem cells (10).

The early stages of embryonic development are regulated by the coordinated activities of a variety of signaling molecules, including Wnt and the transforming growth factor–β (TGF-β) family member Nodal, which function cooperatively to trigger mesendodermal differentiation (11). The demethylases JMJD3 and UTX induce Wnt3 expression by demethylating lysine 27 (K27) in histone 3 (H3) at the Wnt3 promoter region (12), and Wnt3 binds frizzled (Fzd) receptors to activate β-catenin, which binds upstream regulatory regions in most mesendodermal genes (13). Nodal drives mesendodermal differentiation during gastrulation (14) and binds activin receptors to promote the phosphorylation of the Smad2 and Smad3 transcription factors; then, phosphorylated Smad2/Smad3 form a complex with Smad4, translocate into the nucleus, and induce mesendodermal differentiation (15). β-catenin and Smad2/3 transcription factors collectively activate genes that induce mesendodermal differentiation (16); however, Wnt/β-catenin signaling is suppressed by BTB and Cap ‘n’ Collar (CNC) homology 1 (Bach1), a ubiquitously expressed member of the CNC and basic region leucine zipper (CNC-bZip) family of transcription factors (17) that plays a key role in the regulation of oxidative stress, heme oxidation, and cell cycle progression (18, 19). Bach1 is highly expressed in mouse embryos (20, 21), and mice with homozygous deletions of all Bach1 coding exons are subviable and produced in low numbers. Bach1 also inhibits the differentiation of
erythroid cells, macrophages, and adipocytes (22) and impairs both developmental angiogenesis in zebrafish embryos (23) and the angiogenic response to peripheral ischemic injury in adult mice (24), but whether Bach1 participates in the self-renewal and the early differentiation of hESCs remains unknown.

The studies described in this report begin to investigate the role of Bach1 in the self-renewal and early differentiation of hESCs. Experiments were conducted with wild-type hESCs (WT hESCs), with Bach1-knockout hESCs (Bach1-KO hESCs) that had been created via CRISPR-Cas9 genome editing and with WT and Bach1-KO hESCs that had been transfected with a vector coding for doxycycline-inducible Bach1 (DoxBach1) expression. Our results suggest that Bach1 interacts with the pluripotency factors Nanog, Sox2, and Oct4 and stabilizes them by recruiting Usp7. Bach1 also interacted with PRC2, and Bach1 deficiency in hESCs induced mesendodermal differentiation by reducing the occupancy of EZH2 and H3K27me3 in mesendodermal gene promoters and by activating the Wnt/β-catenin and Nodal/Smad2/3 signaling pathways.

RESULTS

Bach1 preserves stem cell identity in hESCs

Previously published results from protein mass spectrometry (MS) analyses found that in preimplantation mouse embryos, Bach1 and Oct4 are similarly up-regulated during the morula and blastocyst stages (fig. S1A, left) (20), and the publicly available RNA sequencing (RNA-seq) data analyses in mouse embryos indicated that Bach1 is down-regulated from embryonic day (E) 3.5 to 7.5, while mesodermal genes (T and Mesp1) are up-regulated from E5.5 to E7.5 (fig. S1A, right) (25). Our observations identified Bach1 expression in the inner cell mass (ICM) and trophectoderm cells and suggested that Bach1 colocalized with Oct4 in the ICM cells of mouse blastocysts (fig. S1B, upper panels). Bach1 was also broadly expressed in the endoderm, mesoderm, and ectoderm of E7.5 embryos, while T was mainly expressed in the primitive streak region (fig. S1B, lower panels).

Bach1 expression was observed in both the nucleus and cytoplasm of undifferentiated hESCs (fig. S1C). Bach1-KO hESCs were generated via CRISPR-Cas9 genome editing (fig. S1D), and the DoxBach1 was generated with the PiggyBac transposon system (fig. S1E); subsequent analyses confirmed that Bach1 was not expressed in Bach1-KO hESCs (Fig. 1A), while Bach2 expression was unchanged (fig. S1F), and that Bach1 expression was restored in DoxBach1-transfected Bach1-KO hESCs after treatment with Dox (Fig. 1A).

Colonies of Bach1-KO hESCs were more flattened than those of WT hESCs, and alkaline phosphatase (AP) activity was lower in Bach1-KO hESCs than in WT hESCs but restored to near WT levels in Bach1-KO hESCs after Dox treatment (Fig. 1A). A greater proportion of Bach1-KO than WT hESC colonies was composed primarily of differentiated or mixed hESC populations (Fig. 1B), and Bach1-KO hESCs were less proliferative (Fig. 1C) and expressed lower protein levels of the pluripotency factors Sox2, Oct4, and Nanog (Fig. 1, D and E); notably, the levels of Nanog, Sox2, and Oct4 transcripts in Bach1-KO and WT hESCs were similar (fig. S1F), indicating that the role of Bach1 in maintaining the protein levels of these pluripotency factors occurs after transcription.

In DoxBach1-transfected Bach1-KO hESCs, Dox treatment restored WT-like colony morphology and increased both proliferation and expression of pluripotency factors (Fig. 1, A, C, and D). The expression of pluripotency factors also increased in DoxBach1-transfected WT hESCs after treatment with Dox (fig. S1G), and cell cycle analyses indicated that a greater proportion of Bach1-KO than WT hESCs was in G1 (fig. S1H), which is consistent with the lower proliferation rates observed in Bach1-KO cells, while the loss of Bach1 expression was not associated with substantial changes in apoptosis (fig. S1I). Furthermore, observations in hESCs that had been transfected with lentiviral Bach1–short hairpin RNAs (shRNAs) (Fig. 1, F to H) were similar to those observed in Bach1-KO hESCs, and when Bach1 was overexpressed in human fibroblasts that had been transduced with the transcription factors Oct4, Klf4, Sox2, and c-Myc, the number of hESC-like (i.e., AP-positive) clones increased (Fig. 1I). Collectively, these results suggest that Bach1 contributes to the maintenance of stem cell identity and stem cell self-renewal, as well as the reprogramming of cells to pluripotency.

Bach1 interacts with and stabilizes Nanog, Sox2, and Oct4 by recruiting the DUB Usp7

Because the loss of Bach1 in hESCs was associated with declines in Nanog, Sox2, and Oct4 protein levels, but not mRNA levels, we investigated whether Bach1 contributed to the maintenance of stem cell identity by impeding the rate at which pluripotency factors are degraded. When DoxBach1 WT hESCs were treated with cycloheximide (CHX) to block the synthesis of new proteins, Dox-induced Bach1 overexpression significantly prolonged the half-life and delayed the degradation of Nanog, Sox2, and Oct4 protein (Fig. 2A), while treatment with the proteasome inhibitor MG132 restored Nanog, Sox2, and Oct4 protein levels in Bach1-KO hESCs (Fig. 2B). Bach1 overexpression also significantly decreased the ubiquitination of Nanog, Sox2, and Oct4 (Fig. 2C and fig. S2A), which suggests that Bach1 promotes the stability of these pluripotency factors by disrupting ubiquitin-dependent proteasomal degradation. Furthermore, evidence of the interaction between Bach1 and Usp7 was observed in human embryonic kidney (HEK) 293T cells that expressed Flag-Bach1 via immunoprecipitation (IP) and MS (fig. S2B) and in hESCs via IP and Western blot (Fig. 2D). Bach1 also interacted with Nanog, Sox2, and Oct4, but not c-Myc, in hESCs (Fig. 2D), while both mRNA and protein levels of Usp7 were significantly lower in Bach1-KO hESCs than in WT hESCs (Fig. 2, E and F) and significantly greater in Bach1-overexpressing hESCs than in hESCs with normal levels of Bach1 expression (fig. S2, C and D). Chromatin IP (ChIP)–quantitative polymerase chain reaction (qPCR) assays confirmed that Bach1 occupied the Usp7 promoter (Fig. 2G), indicating that Usp7 is a direct target of Bach1, and when Usp7 activity was down-regulated via transfection with small interfering RNAs (siRNAs) and/or treatment with the Usp7 inhibitor P5091, Nanog ubiquitination increased (fig. S2E), pluripotency protein levels declined (fig. S2F) in WT hESCs, and the increase in pluripotency protein levels associated with Bach1 overexpression was abolished (Fig. 2H and fig. S2G). Furthermore, the decline in pluripotency protein levels observed in Bach1-KO hESCs was partially rescued by higher levels of Usp7 expression (fig. S2H), and the decline in Nanog polyubiquitylation observed in Bach1-overexpressing hESCs was partially restored via Usp7 inhibition (Fig. 2I). Together, these results indicate that the Bach1-induced stabilization of pluripotency proteins is mediated via the deubiquitination activity of Usp7.

Bach1 knockout promotes mesendodermal differentiation in hESCs

RNA-seq analysis of WT hESCs and Bach1-KO hESCs suggested that Bach1 deficiency increased the expression of 1562 genes and reduced the expression of 2311 genes (fold change > 1.5, P < 0.05; fig. S3, A and B, and table S1I). Many of the up-regulated genes were associated
Fig. 1. Bach1 is required for maintaining hESCs identity. (A) Immunoblot analysis of Bach1 protein level and alkaline phosphatase (AP) staining of colonies in WT hESCs and DoxBach1-transfected Bach1-KO hESCs that had been treated with Dox (Bach1-KO + Dox) or without Dox (Bach1-KO − Dox). Scale bars, 500 μm. (B) Bars show mean percentages of differentiated (Diff.) AP-negative, mixed (some cells AP-positive, some-negative), and undifferentiated (Undiff.) uniformly AP-positive embryonic stem cell colonies in WT hESCs and Bach1-KO hESCs ($n = 3$). *$P < 0.05$; **$P < 0.01$ compared with WT, t-test. (C) WT, Bach1-KO − Dox, and Bach1-KO + Dox hESCs were seeded into Matrigel-coated wells (3 × 10⁴ cells per well), and proliferation was evaluated by monitoring cell counts over the ensuing 4-day culture period ($n = 3$). *$P < 0.05$; **$P < 0.01$ compared with WT; $\#$ $P < 0.05$, $\##$ $P < 0.01$ compared with Bach1-KO − Dox; one-way analysis of variance. (D) Nanog, Sox2, Oct4, and Bach1 protein levels in WT, Bach1-KO − Dox, and Bach1-KO + Dox hESCs were evaluated via Western blot (left panel) and quantified (right panel); β-Actin levels were also evaluated to confirm equal loading ($n = 3$). **$P < 0.01$ compared with WT; $\##$ $P < 0.01$ compared with Bach1-KO − Dox; one-way analysis of variance. (E) WT and Bach1-KO hESCs were immunofluorescently stained for Sox2 or Oct4 expression, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars, 100 μm. (F) AP staining of colonies and mean percentages of differentiated, mixed, and undifferentiated cell colonies in hESCs treated with lentivirus control shRNA (Lv-Con) or lentivirus Bach1-shRNAs (Lv-shBach1). Scale bars, 500 μm. *$P < 0.05$; **$P < 0.01$ compared with Lv-Con; t-test. (G and H) Western blot analysis of pluripotent factors and quantification of cell numbers for 4 days in hESCs treated with Lv-Con or Lv-shBach1 ($n = 3$). *$P < 0.05$; **$P < 0.01$ compared with Lv-Con; t-test. (I) Overexpression of Bach1 enhanced reprogramming of human dermal fibroblasts to pluripotency. Left: AP staining of reprogramming colonies. Right: Quantitative and statistical analysis of AP-positive colonies ($n = 4$). *$P < 0.05$ compared with adenovirus green fluorescent protein (AdGFP). Data were collected from three or four independent replicates and are shown as means ± SD.
Fig. 2. Bach1 interacts with and stabilizes Nanog, Sox2, and Oct4 by recruiting DUB Usp7. (A) DoxBach1-transfected WT hESCs that were treated with or without Dox were treated with CHX (10 μg/ml) for the indicated times. The levels of Nanog, Sox2, and Oct4 proteins were determined by immunoblotting (IB). The band intensities from immunoblots were quantified (n = 3). *P < 0.05; **P < 0.01 compared with Dox−; t test. (B) WT and Bach1-KO hESCs were treated with or without proteasome inhibitor MG132 (10 μM) for 6 hours, and the levels of Nanog, Sox2, and Oct4 proteins were determined by immunoblotting and quantified (n = 3). *P < 0.05 compared with WT, dimethyl sulfoxide (DMSO); #P < 0.05; ##P < 0.01 compared with Bach1-KO, DMSO; one-way analysis of variance. (C) DoxBach1-transfected WT hESCs that were treated with or without Dox were treated with MG132 (10 μM) for 6 hours. The ubiquitinated proteins were pulled down using an anti-ubiquitin antibody, and the ubiquitination of Nanog was detected by Western blotting using anti-Nanog antibody. Ub, ubiquitination. (D) Bach1 was immunoprecipitated from WT hESCs; then, the amount of Usp7, Nanog, Sox2, Oct4, and c-Myc present in the precipitate was evaluated via Western blot. (E and F) Usp7 mRNA (E) and protein (F) levels in WT hESCs and Bach1-KO hESCs were evaluated via qRT-PCR or Western blot and quantified; β-Actin levels were also evaluated to confirm equal loading (n = 3). *P < 0.05; **P < 0.01 compared with WT; t test. (G) Bach1 signal track for representative locus Usp7 in hESCs from published ChIP-seq data, and the binding of promoter sequences for Usp7 to Bach1 was evaluated in WT hESCs via ChIP-qPCR, and the immunoglobulin G (IgG) served as a negative control (n = 3). *P < 0.05 compared with IgG; t test. (H) Western blot analysis of pluripotent factors in DoxBach1-transfected WT hESCs that had been treated with (Dox+) or without (Dox−) Dox and transfected with or without a pool of Usp7 siRNAs (n = 3). *P < 0.05; **P < 0.01 compared with Dox−, Control siRNA (Consi); ##P < 0.01 compared with Dox+, Consi; one-way analysis of variance. (I) Ubiquitylation of Nanog in DoxBach1-transfected WT hESCs after transfection of either nonspecific or Usp7-specific siRNAs in the presence and absence of Dox. IP was performed using anti-ubiquitin antibody, and the level of Nanog was determined by immunoblotting. Data were collected from three independent replicates and are shown as means ± SD.
with developmental processes, including embryonic organ development, blood vessel morphogenesis, heart development, and skeletal system development (fig. S3C). In general, mRNA levels for mesodermal and endodermal developmental regulators [e.g., Brachyury (T), mesoderm posterior 1 (Mesp1), Mso2, Eomes, Wnt3, Nodal, Gata4, Gata6, Foxa2, and Sox17] were greater in Bach1-KO than in WT hESCs (Fig. 3A), while many of the down-regulated genes were associated with neuro-transmitter transport, synapse organization, and transmembrane transport (fig. S3D). The expression of neuroectodermal markers (e.g., Sox1, Olig3, Pax6, and Otx2) was also lower in Bach1-KO hESCs than in WT hESCs (Fig. 3A), so we investigated whether the loss or overexpression of Bach1 in hESCs altered germ layer marker expression in hESCs, as the cells spontaneously differentiated into embryoid bodies (EBs).

The expression of most of the genes associated with each of the three germ layers (mesoderm, endoderm, and ectoderm) peaked at day 4 or 6 of EB differentiation, which is consistent with a previous report (26); thus, since the goal of these experiments was to investigate the role of Bach1 during the early stages of hESC differentiation, we conducted our analyses from days 0 to 6 of hESC-EB differentiation. In WT hESCs, mRNA levels of mesoderm markers (T and Mso2), endoderm markers (Gata4 and Gata6), and neuroectoderm markers (Otx2 and Pax6) gradually increased during the first 4 days of spontaneous EB differentiation (Fig. 3B), and although the time dependence of marker expression in WT and Bach1-KO hESCs was largely similar, mesendoderm marker expression was consistently higher, and neuroectoderm marker expression was consistently lower, in Bach1-KO hESCs than in WT hESCs (Fig. 3B). Gata6 and T expression also appeared to be much higher, and Sox2 and Oct4 expression appeared to be much lower, in Bach1-KO hESCs than in WT hESCs when evaluated via immunofluorescence (Fig. 3C) and/or Western blot (Fig. 3D), while Bach1 expression was up-regulated at days 2 and 4 of hESC differentiation and declined at day 6 (Fig. 3D). We observed similar changes in germ layer marker expression on day 3 of differentiation in hESCs when Bach1 was knocked down via transfection with lentiviral Bach1-shRNAs (fig. S3E), and qRT-PCR measurements performed on day 3 of differentiation indicated that the changes in marker expression associated with the loss of Bach1 were completely rescued in DoxBach1-transfected Bach1-KO hESCs after treatment with Dox (Fig. 3E).

The expression of markers for cardiac progenitor cells (Isl1), chondroprogenitor cells (Sox9 and Col2a1), erythroid cells (β-globin), and pancreas-like cells (Pdx1) was also higher in Bach1-KO hESCs than in WT hESCs at day 12 of EB differentiation, while the expression of a hepatic marker gene (Aldh) was unchanged (fig. S3F), and although both WT and Bach1-KO hESCs formed teratomas that contained all three germ layers (endoderm, mesoderm, and ectoderm) when injected into adult severe combined immunodeficient (SCID) mice, mesendodermal marker expression was higher, and neuroectoderm marker expression was lower, in teratomas formed from Bach1-KO hESCs (Fig. 3F). Mesendodermal gene mRNA levels also increased significantly in both WT and Bach1-KO hESCs when the cells were transfected with Usp7 siRNA (fig. S4A), while the overexpression of Usp7 partially abolished the up-regulation of most mesendodermal genes in Bach-KO hESCs (fig. S4B).

**Bach1 represses mesendodermal gene expression in hESCs via the EZH2-catalyzed trimethylation of H3K27 in mesendodermal gene promoters**

Analysis of a published set of Bach1 ChIP sequencing (ChIP-seq) data in hESCs (27) identified Bach1 binding sites in both intergenic and intragenic regions, with a significant portion of the intragenic peaks located at promoters and introns (fig. S5A), and the genes associated with Bach1 peaks were involved in protein and nucleic acid binding, metabolic and developmental processes, cell cycle regulation, and ubiquitin-mediated proteolysis (fig. S5, B to D). Furthermore, heatmaps produced from the hESC ChiP-seq data indicated that Bach1, H3K27me3, and EZH2 co-localized broadly across the genome (Fig. 4A), and that all three proteins bound the genomic loci of mesendodermal developmental regulators (T, Gata6, Wnt3, and Nodal) (Fig. 4B). Bach1, H3K27me3, and EZH2 peaks also tended to be located near transcription start sites (TSSs) (fig. S6A), and ChIP-qPCR assays confirmed that Bach1 occupied the genes’ promoter regions (fig. S6B). Thus, we investigated whether interactions among Bach1, EZH2, and H3K27me3 contribute to the regulation of genes involved in hESC differentiation.

For the differentiation genes that were up-regulated by Bach1 deficiency, the occupancy of both H3K27me3 and EZH2 within ±5 kilobase (kb) of the TSS was lower in Bach1-KO hESCs than in WT hESCs (Fig. 4C and table S1.2). The occupancy of both proteins in the promoter regions of mesendodermal developmental regulators (T, Gata6, Wnt3, Nodal, Mesp1, Mso2, and Gata4) also declined in response to Bach1 deficiency (Fig. 4D and fig. S6C), and ChIP-qPCR analyses confirmed that the binding of H3K27me3 and EZH2 to the promoter regions of T, Gata6, and a number of other mesendodermal genes was lower in Bach1-KO hESCs than in WT hESCs (Fig. 4E). Notably, the binding of H3K4me3 to these promoters was elevated in Bach1-KO hESCs (Fig. 4E), while the levels of H3K27me3, EZH2, H3K4me3, H3K9me2, H3K9ac, and H3K27ac protein in WT and Bach1-KO hESCs were similar (fig. S6D).

Mesendodermal gene mRNA levels declined in DoxBach1-transfected WT hESCs after treatment with Dox, but the decline was partially abolished by the EZH2 inhibitor DZNep (Fig. 4F), indicating that EZH2 is involved in the inhibition of mesendodermal gene expression by Bach1. We identified regions of Bach1 localization in T and Gata6 (Fig. 4B), which contain Bach1 binding sites in their promoter regions, so we conducted experiments with a set of luciferase reporter constructs containing versions of the T or Gata6 promoter sequences. Dox–induced Bach1 overexpression significantly reduced luciferase activity in WT hESCs that had been transfected with luciferase reporter constructs containing the endogenous T or Gata6 promoter sequences (Fig. 4G), but not when the Bach1 binding sites in the T promoter sequence were mutated (Fig. 4H). Bach1 also colocalized with EZH2 in the nucleus (Fig. 5A) and coimmunoprecipitated with EZH2, as well as two other PRC2 subunits, EED and SUZ12 (Fig. 5, B and C), in WT hESCs. Notably, the binding between Bach1 and EZH2 persisted in the presence of deoxyribonuclease (DNase) or ribonuclease (RNase) A (Fig. 5D), which suggests that the interaction between Bach1 and EZH2 does not require a DNA or RNA molecule. Bach1 and EZH2 also coprecipitated from the lysate of HEK293 cells that had been engineered to express Flag-tagged Bach1 and hemagglutinin (HA)–tagged EZH2 (Fig. 5E), and when glutathione-S-transferase–tagged Bach1 constructs containing the full Bach1 sequence (Bach1-Full–GST) or sequences lacking the C-terminal bZIP domain (Bach1-N–GST) or the N-terminal BTD domain (Bach1-C–GST) were incubated with His-tagged EZH2 and passed over glutathione–Sepharose beads, EZH2 coprecipitated most strongly with the full-length sequence (Fig. 5F). Collectively, these observations suggest that the N- and C-terminal regions of Bach1 interact directly with EZH2 and that Bach1 recruits EZH2 to Bach1-binding sites in the promoter regions of several mesendodermal genes, which promotes the trimethylation of H3K27 and represses mesendodermal gene expression.
Fig. 3. Bach1-KO promotes mesendodermal gene expression in hESCs. (A) Heatmap illustrating the RNA expression in WT and Bach1-KO hESCs of RNA-seq analysis for selected genes of different lineages. FPKM, fragments per kilobase of transcript per million mapped reads. (B) mRNA levels of mesoderm, endoderm, and neuroectoderm markers in WT and Bach1-KO hESCs were measured via qRT-PCR at the indicated time points, as the cells spontaneously differentiated into embryoid bodies (EBs); results were normalized to measurements for WT cells at the beginning of the differentiation period (n = 3). *P < 0.05; **P < 0.01 compared with WT; t test. (C) WT and KO hESCs were immunofluorescently stained for Gata6 (red) and T (green) expression, and nuclei were counterstained with DAPI (blue). Scale bars, 100 μm. (D) Gata6, T, Sox2, Oct4, and Bach1 protein levels were evaluated in WT and Bach1-KO hESCs via Western blot. β-Actin levels were also evaluated to confirm equal loading. (E) mRNA levels of mesendodermal and neuroectodermal genes were measured in WT, Bach1-KO – Dox, and Bach1-KO + Dox hESCs on day 3 of EB differentiation via qRT-PCR; results were normalized to measurements in WT cells (n = 3). *P < 0.05; **P < 0.01 compared with WT; #P < 0.05; ##P < 0.01 compared with Bach1-KO; one-way analysis of variance. (F) WT or Bach1-KO hESCs were subcutaneously injected into SCID mice; 8 weeks later, teratomas were harvested, sectioned, and stained with hematoxylin and eosin for histological analysis (left). Scale bars, 100 μm. mRNA levels of lineage marker genes in teratomas from mice that had been injected with WT, or Bach1-KO hESCs were measured via qRT-PCR (right panel; n = 3). *P < 0.05; **P < 0.01 compared with WT; t test. Data were collected from three independent replicates and are shown as means ± SD.
Fig. 4. Bach1 suppresses mesendodermal gene expression in hESCs via the EZH2-catalyzed trimethylation of H3K27 in mesendodermal gene promoters. (A) The heatmap of Bach1, EZH2, and H3K27me3 at genomic regions surrounding (±5 kb) TSSs (transcription start sites) in hESCs from published ChIP-seq data. (B) Bach1, EZH2, and H3K27me3 signal tracks for representative loci T, Gata6, Wnt3, and Nodal in hESCs from published ChIP-seq data. (C) H3K27me3 and EZH2 ChIP-seq signals at differentiation genes up-regulated by loss of Bach1 within ±5 kb of TSSs in WT and Bach1-KO hESCs. (D) H3K27me3 and EZH2 signal tracks for representative loci T and Gata6 in WT and Bach1-KO hESCs. (E) The binding of promoter sequences for mesendodermal genes, Nodal, and Wnt3 to H3K27me3, EZH2, and H3K4me3 was evaluated in WT and Bach1-KO hESCs via ChIP-qPCR (n = 3). *P < 0.05; **P < 0.01 compared with WT; t test. (F) Mesendodermal gene mRNA levels were measured via qRT-PCR in DoxBach1-transfected WT hESCs that had been treated with (Dox+) or without (Dox−) Dox and with (+DZNep) or without (−DZNep) the EZH2 inhibitor DZNep (20 ng/ml) at 12 hours after EB differentiation (n = 3). *P < 0.05; **P < 0.01 compared with Dox−, DMSO; ##P < 0.01 compared with Dox+, DMSO; one-way analysis of variance. (G and H) DoxBach1-transfected WT hESCs were transfected with plasmids containing luciferase reporter constructs controlled by (G) the T or Gata6 promoter sequence or (H) WT and mutated versions of the T promoter, then, luciferase activity was measured in cells that had been treated with (Dox+) or without Dox (Dox−), and results were normalized to measurements in Dox− hESCs (n = 3). *P < 0.05; **P < 0.01 compared with Dox−; t test. Data were collected from three independent replicates and are shown as means ± SD.
Although the occupancy of some mesendodermal genes by H3K27me3 declined slightly when WT hESCs were transduced with Usp7 siRNA (fig. S4C), the decline in H3K27me3 mesendodermal gene promoter occupancy that was observed in Bach1-KO hESCs was not rescued by Usp7 overexpression (fig. S4D). Furthermore, despite evidence that PRC1 is recruited to mesodermal genes (28), the occupancy of mesendodermal genes by the E3 ligase Ring1B, a component of PRC1 that monoubiquitinates histone H2A (forming H2AK119ub) and H2AK119ub, was not affected by Bach1 deficiency (fig. S6, E and F).

**Bach1 knockout promotes mesendodermal differentiation by activating Wnt/β-catenin signaling**

The mesendodermal specification of hESCs is mediated, at least in part, by the activation of Wnt/β-catenin signaling (29), and RNA-seq analysis...
Analysis of the Bach1 ChIP data identified a Bach1 binding site near the whether the role of Bach1 in the suppression of mesendodermal differ-
in WT hESCs (fig. S7, A and B), and receptors (Fzd-1, Fzd-2, and Fzd-10), as well as a downstream target of Wnt lymphoid enhancer binding factor 1 (LEF1) (Fig. 6, A and B); (ii) in the amount of nonphosphorylated (active) β-catenin and total β-catenin (Fig. 6, B and C); and (iii) in the activity of the TOPFlash luciferase reporter (Fig. 6D), which contains eight copies of the Tcf/lef binding site. However, TOPFlash activity in WT and Bach1-KO hESCs was similar when the cells were treated with IWP2, an inhibitor of Wnt production (Fig. 6D), or IWR1-e which inhibits β-catenin (fig. S7C), and when Wnt signaling was stimulated by culturing the cells in Wnt3a, TOPflash activity and mesendodermal gene expression were significantly higher in Bach1-KO cells than in WT cells (fig. S7, C and D). ChIP-qPCR analyses indicated that binding between mesendodermal gene promoters and the active form of β-catenin was greater in Bach1-KO hESCs than in WT hESCs and declined when DoxBach1-transfected WT hESCs were treated with Dox (Fig. 6F). Mesendodermal gene expression increased on day 3 of EB differentiation in Bach1-KO hESCs and decreased significantly when WT or Bach1-KO hESCs were transfected with Ad-β-catenin shRNA or treated with IWP2 (Fig. 6, G and E), while analysis of Bach1 ChIP-seq data in hESCs identified a Bach1 binding site in the promoter of Wnt3 (Fig. 4B). ChIP-qPCR assays confirmed that Bach1 occupied the Wnt3 promoter (fig. S6B), and both Wnt3 mRNA levels (Fig. 6H) and the activity of a Wnt3 luciferase reporter construct, but not a Wnt3 luciferase reporter containing a mutation in the Bach1 binding site, declined in response to Dox-induced Bach1 overexpression (Fig. 6I). Thus, the relationship between Bach1 deficiency and the mesendodermal specification of hESCs appears to be at least partially mediated by the Wnt/β-catenin pathway.

**Bach1 knockout promotes mesendodermal differentiation by activating Nodal signaling**

Wnt works cooperatively with Nodal, a member of the TGF-β superfamily, during mesendoderm specification (29), and the results from our RNA-seq analysis of day 3 EBs indicated that the expression of Nodal and TGF-β signaling genes was greater in Bach1-KO hESCs than in WT hESCs (fig. S7, A and B, and table S1.3); thus, we investigated (i) increases in mRNA and/or protein levels of several Wnt ligands (Wnt3, Wnt2b, and Wnt5b) and receptors (Fzd-1, Fzd-2, and Fzd-10), as well as a downstream target of Wnt lymphoid enhancer binding factor 1 (LEF1) (Fig. 6, A and B); (ii) in the amount of nonphosphorylated (active) β-catenin and total β-catenin (Fig. 6, B and C); and (iii) in the activity of the TOPFlash luciferase reporter (Fig. 6D), which contains eight copies of the Tcf/lef binding site. However, TOPFlash activity in WT and Bach1-KO hESCs was similar when the cells were treated with IWP2, an inhibitor of Wnt production (Fig. 6D), or IWR1-e which inhibits β-catenin (fig. S7C), and when Wnt signaling was stimulated by culturing the cells in Wnt3a, TOPflash activity and mesendodermal gene expression were significantly higher in Bach1-KO cells than in WT cells (fig. S7, C and D). ChIP-qPCR analyses indicated that binding between mesendodermal gene promoters and the active form of β-catenin was greater in Bach1-KO hESCs than in WT hESCs and declined when DoxBach1-transfected WT hESCs were treated with Dox (Fig. 6F). Mesendodermal gene expression increased on day 3 of EB differentiation in Bach1-KO hESCs and decreased significantly when WT or Bach1-KO hESCs were transfected with Ad-β-catenin shRNA or treated with IWP2 (Fig. 6, G and E), while analysis of Bach1 ChIP-seq data in hESCs identified a Bach1 binding site in the promoter of Wnt3 (Fig. 4B). ChIP-qPCR assays confirmed that Bach1 occupied the Wnt3 promoter (fig. S6B), and both Wnt3 mRNA levels (Fig. 6H) and the activity of a Wnt3 luciferase reporter construct, but not a Wnt3 luciferase reporter containing a mutation in the Bach1 binding site, declined in response to Dox-induced Bach1 overexpression (Fig. 6I). Thus, the relationship between Bach1 deficiency and the mesendodermal specification of hESCs appears to be at least partially mediated by the Wnt/β-catenin pathway.

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ificant in Bach1-KO hESCs than in WT hESCs but declined significantly when Bach1-KO hESCs were cultured with SB-431542, an inhibitor of the Nodal receptor (Fig. 7F), and were reduced even more when SB-431542 treatment was combined with β-catenin inhibition (Fig. 7G). Thus, the increased mesendodermal specification of Bach-deficient hESCs appears to be mediated by both Nodal and Wnt/β-catenin signaling.

**DISCUSSION**

Bach1 is highly expressed during the blastocyst stage of mouse development, and mice with homozygous deletions of all Bach1 coding exons are subviable, but the molecular mechanisms underlying their subviability have not been fully elucidated. Here, our data demonstrated that Bach1 maintains the stemness of hESCs by stabilizing pluripotency factors and repressing the expression of lineage-specific genes (Fig. 7H). We also show that the loss of Bach1 in hESCs led to the activation of mesendodermal gene expression and differentiation; thus, Bach1 activity appears to be a key determinant of cell fate and lineage specification in hESCs.

The activity of pluripotency factors in ESCs is controlled, in part, via protein ubiquitination and deubiquitination. For example, Oct4 and Sox2 protein levels are regulated by the E3 ligase WW2P2 (30, 31), and ubiquitin-specific-processing protease 21 (Usp21) deubiquitinates and stabilizes Nanog, which maintains pluripotency (32), while FBXW8, an E3 ligase, ubiquitiniates and destabilizes Nanog, thereby promoting ESC differentiation (33). However, although Usp7 maintains the pluripotency of neural progenitor/stem cells by deubiquitinating and stabilizing repressor element 1-silencing transcription factor (REST) and c-Myc (6, 34), whether Usp7 activity contributes to the role of Bach1 in hESC stemness was largely unknown. In this study, we demonstrated that Bach1 directly targets and increases the expression of Usp7 in hESCs and that Bach1 interacts with Nanog, Sox2, and Oct4 and recruits Usp7, which facilitates pluripotency protein deubiquitination and maintains hESC stemness. We also found that the increase in mesendodermal gene expression associated with Bach1 deficiency can be partially abolished by up-regulating Usp7 expression, which suggests that Usp7 may be a component of the mechanism by which the loss of Bach1 promotes hESC differentiation, although its role may be a secondary consequence of the concomitant decline in pluripotency protein levels.

Previous reports have shown that Bach1 regulates the expression of many genes that are involved in the cell cycle and apoptosis (18) and promotes apoptosis while inhibiting proliferation in human umbilical vein endothelial cells (35); however, apoptosis was not altered by Bach1 deficiency in hESCs, and proliferation rates were lower in Bach1-KO hESCs than in WT hESCs. Collectively, these observations demonstrate that the effect of Bach1 on apoptosis and cell proliferation can differ profoundly among cell types.

The majority of genes that are differentially expressed (DE) in hESCs and hESC-derived cells are GC-rich and transcriptionally repressed by the deposition of H3K27me3 (36). Notably, although Bach1 is known to regulate cancer metastasis, as well as oxidative stress, heme oxidation, and angiogenesis, and Bach1 depletion reduce H3K27me3 levels at specific gene promoters in breast cancer cells (37), the decline in mesendodermal gene occupancy by H3K27me3 in Bach1-KO hESCs cannot be solely attributed to a decline in Usp7 expression because it was not restored by Usp7 overexpression. PRC2 is also essential for repressive H3K27 methylation and has been linked to stem cell pluripotency and cell lineage specification during development (38, 39). The EZH2
Fig. 6. Bach1-KO promotes mesendodermal differentiation by activating Wnt/β-catenin signaling. (A) mRNA levels of components of the Wnt signaling pathway were measured in WT and Bach1-KO hESCs on day 3 of differentiation into EBs via qRT-PCR and normalized to measurements in WT cells (n = 3). *P < 0.05; **P < 0.01 compared with WT; t test. (B) Protein levels of Wnt3, Fzd1, active β-catenin, and total β-catenin were evaluated in WT and Bach1-KO hESCs on day 3 of differentiation via Western blot. (C) Total β-catenin and histone 3 protein levels were evaluated in the nucleus and cytoplasm of WT and Bach1-KO hESCs on day 3 of differentiation via Western blot. (D) WT and Bach1-KO hESCs were transfected with the TOPflash luciferase reporter, which contains eight copies of the binding site for Tcf/lef, a downstream target of Wnt signaling; then, the cells were treated with or without the Wnt inhibitor IWP2 (10 μM), and luciferase activity was evaluated on day 3 of differentiation and normalized to measurements in WT cells (n = 3). *P < 0.01 compared with WT (IWP2−); ##P < 0.01 compared with Bach1-KO (IWP2−); one-way analysis of variance. (E) mRNA levels of mesendodermal genes were evaluated in WT and Bach1-KO hESCs that had been treated with or without IWP2 (10 μM) on day 3 of differentiation (n = 3). *P < 0.01 compared with WT, DMSO; #P < 0.05; ##P < 0.01 compared with Bach1-KO, DMSO; one-way analysis of variance. (F) The binding of promoter sequences for mesendodermal genes and for the signaling molecules Wnt3 and Nodal to activated β-catenin was evaluated in WT and Bach1-KO hESCs (left) and in DoxBach1-transfected WT hESCs that had (Dox+) or had not (Dox−) been treated with Dox (right) on day 3 of differentiation via ChIP-qPCR; results were normalized to WT (left) or Dox− (right) cells (n = 3). *P < 0.05; **P < 0.01 compared with WT or Dox−; t test. (G) mRNA levels of mesendodermal genes and protein level of β-catenin were evaluated in WT and Bach1-KO hESCs that had been transfected with adenoviruses coding for β-catenin shRNA (Ad-shβ-catenin) or a control shRNA sequence (Ad-shCtrl) on day 3 of differentiation (n = 3). **P < 0.01 compared with WT, Ad-shCtrl; #P < 0.01 compared with KO, Ad-shCtrl; one-way analysis of variance. (H) mRNA levels of Wnt3, Nodal, and Bach1 were evaluated in DoxBach1-transfected WT hESCs that had (Dox+) or had not (Dox−) been treated with Dox on day 3 of differentiation via qPCR (n = 3). *P < 0.05 compared with Dox−; t test. (I) DoxBach1-transfected WT hESCs were transfected with plasmids containing luciferase reporter constructs controlled by WT or mutated versions of the Wnt3 promoter; then, luciferase activity was measured in cells that had been treated with (Dox+) or without Dox (Dox−) on day 3 of differentiation, and results were normalized to measurements in Dox− cells (n = 3). *P < 0.05 compared with Dox−; t test. Data were collected from three independent replicates and are shown as means ± SD.
Bach1-KO promotes mesendodermal differentiation by activating Nodal signaling. (A) mRNA levels of Nodal and the Nodal receptors ALK4 and ACVRiIB were evaluated in WT and Bach1-KO hESCs on day 3 of differentiation via qRT-PCR, and the results were normalized to measurements in WT cells (n = 3). **P < 0.01 compared with WT; t test. (B) Protein levels of Nodal, ACVRiIB, phosphorylated Smad2 and Smad3 (p-Smad2 and p-Smad3, respectively), and total Smad2/3 were evaluated in WT and Bach1-KO hESCs on day 3 of differentiation via Western blot. (C) DoxBach1-transfected WT hESCs were transfected with plasmids containing luciferase reporter constructs controlled by WT or mutated versions of the Nodal promoter; then, luciferase activity was measured in cells that had been treated with (Dox+) or without Dox (Dox−) on day 3 of differentiation, and results were normalized to measurements in Dox− cells (n = 3). *P < 0.05 compared with Dox−; t test. (D) p-Smad2, total Smad2/3, and histone 3 protein levels were evaluated in the nucleus and cytoplasm of WT and Bach1-KO hESCs on day 3 of differentiation via Western blot. (E) The binding of promoter sequences for mesendodermal genes and for the signaling molecules Wnt3 and Nodal was evaluated in WT and Bach1-KO hESCs and in DoxBach1-transfected WT hESCs that had (Dox+) or had not (Dox−) been treated with Dox on day 3 of differentiation via ChiP-qPCR; results were normalized to measurements in WT or Dox− cells (n = 3). *P < 0.05; **P < 0.01 compared with WT or Dox−; t test. (F and G) mRNA levels of mesendodermal genes were evaluated in WT and Bach1-KO hESCs and in WT and Bach1-KO hESCs that had been treated with SB-431542 (20 μM), which inhibits TGF-β receptors (F), or transfected with adenoviruses coding for β-catenin shRNA and treated with SB-431542 (G), and assessments were performed on day 3 of differentiation via qRT-PCR; results were normalized to measurements in WT cells (n = 3). **P < 0.01 compared with WT, DMSO (F) or WT, DMSO, Ad-shCtrl (G); ###P < 0.01 compared with KO, DMSO (F) or KO, DMSO, Ad-shβ-catenin (G); one-way analysis of variance. Data were collected from three independent replicates and are shown as means ± SD. (H) Model showing that Bach1 plays an important role in the maintenance of hESC self-renewal and suppression of mesendodermal differentiation. Bach1 interacts with Nanog, Sox2, and Oct4 and facilitates deubiquitylation of these pluripotency factors by recruiting DUB Usp7 and therefore stabilizes pluripotency factors and maintains the stem cell self-renewal. Bach1 also impedes mesendodermal differentiation via recruitment of PRC2, which leads to the deposition of H3K27me3 and gene silencing, as well as inhibiting Wnt/β-catenin and Nodal/Smad2/3 signaling.
subunit of PRC2 catalyzes the di- and trimethylation of H3K27 (4, 5), and deficiencies in EZH2 reduce pluripotency and promote the spontaneous differentiation of hESCs toward a mesendodermal fate (5); however, the mechanism by which EZH2 transcriptionally represses mesendodermal genes was unclear. Here, we show that EZH2 interacts directly with Bach1 and colocalizes with Bach1 and H3K27me3 in the promoters of a number of mesendodermal genes. Furthermore, Bach1 knockdown reduced EZH2 and H3K27me3 promoter occupancy, up-regulated mesendodermal gene expression, and induced the mesendodermal specification of hESCs, while mesendodermal gene mRNA levels declined significantly in response to Bach1 overexpression but not when the cells were treated with the EZH2 inhibitor DZNep. Bach1 also cooperates with EZH2 to suppress the transcription of Raf kinase inhibitory protein (37). Collectively, our observations suggest that Bach1 represses differentiation in hESCs through at least two independent pathways: (i) via the recruitment of Usp7 and (ii) by functioning as a scaffold for PRC2 recruitment.

Our results also indicated that the increased expression of endoderm and mesoderm genes in Bach1-deficient hESCs was accompanied by increases in Wnt/β-catenin and Nodal/Smad2/3 signaling and was repressed by the inhibition of either Wnt production or the Nodal receptor. This observation is consistent with previous reports that activin/Nodal and Wnt signaling promoted the transcriptional programs of endoderm and mesoderm lineages while repressing ectoderm differentiation (11, 16, 29) and that direct interactions between β-catenin and Smad2/Smad3 are required to activate mesendodermal gene transcription (29). Furthermore, the repressive H3K27me3 marker must be removed for the combined effects of activin/Nodal and Wnt signaling to promote mesendoderm differentiation (16). Thus, the increase in Wnt/β-catenin and Nodal/Smad2/3 pathway activity associated with Bach1 deficiency in hESCs appears to prime the cells to differentiate more efficiently toward the endoderm and mesoderm lineages.

In summary, the results presented here show that Bach1 maintains stem cell identity in hESCs by functioning at the apex of multiple distinct mechanisms, including the stabilization of pluripotency factors via recruitment of the deubiquitinating factor Usp7, the silencing of mesendodermal gene expression via the PRC2-induced deposition of H3K27me3 at the genes’ promoters, and the inhibition of Wnt3 and Nodal signaling. These observations provide new insights into the role of Bach1 in the cell fate determination and lineage specification of hESCs, but more work is needed to fully characterize the role of Bach1 in early mouse development.

**MATERIALS AND METHODS**

**Cell culture**

hESCs (H7; WiCell Research Institute, Madison, WI) were maintained in mTeSR1 medium (no. 05851; STEMCELL Technologies, Vancouver, Canada) on plates coated with a 1:80 dilution of Matrigel (no. 354277; BD Biosciences, San Diego, CA) in DMEM-F12 (no. 11320-033; Life Technologies, Carlsbad, CA). For expansion, colonies were split (1:5 ratio) after Accutase (no. A6964; Sigma-Aldrich, St. Louis, MO) treatment, the medium was replaced daily, and the cells were passaged by mechanical dissociation every 5 to 6 days for 30 to 60 passages. HEK293 T cells were originally from the American Type Culture Collection and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (no. 12800082; Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (no. 10099-141; Life Technologies, Carlsbad, CA) and antibiotics.

**Genetic manipulation of hESCs**

Bach1-KO hESCs were generated with CRISPR-Cas9 genome editing technology (40). Single guide RNAs (sgRNAs) targeting the genomic regions of interest were designed with the CRISPR Design Tool (http://crispr.mit.edu/) and synthesized by the Shanghai Sunny Biotechnology Company. Annealed sgRNA oligos were cloned into LentiCRISPR V2 (no. 52961; Addgene, Watertown, MA) and transiently transduced into hESCs via electroporation (no. MPK5000; Life Technologies, Carlsbad, CA) and selected with puromycin (no. ant-pr-5b; InvivoGen, San Diego, CA). Positive clones were identified by sequencing the gene of interest and confirmed by analyzing protein levels. Methods to detect off-target were performed as described previously (41). The cloned sgRNA sequences and off-target primer sequences are listed in table S2.1. DoxBach1 hESCs, including DoxBach1-transected Bach1-KO hESCs and DoxBach1-transfected WT hESCs, were generated with the PiggyBac transposon system (42). Bach1 complementary DNA (cDNA) was cloned into the vector (pPB-TRE-EGFP-PGK-neol2) and cotransfected into WT or Bach1-KO hESCs with transactivator and transposase-encoding vectors (pCAG-T7-mPB and pPB-CAG-rtTA-Adv-IRES-Puro); then, transfected cells were isolated with G418 (no. ant-gn-1; InvivoGen, San Diego, CA) and puromycin selection, and clones were chosen for use in subsequent experiments. The PiggyBac transposon system plasmids were provided by J. Na (Tsinghua University, China). Primers for cloning human Bach1 cDNA are listed in table S2.2.

**Animals**

All animal studies were approved by the Ethics Committee of Experimental Research at Fudan University Shanghai Medical College and were in accordance with the Institutional Animal Care and Use Committee Guidelines. Mice were housed under a 12-hour light/dark cycle under pathogen-free conditions at 22° ± 2°C with food and water available ad libitum.

**Mouse embryos**

C57BL/6 female mice (7 to 8 weeks old) were superovulated by intraperitoneally injecting with pregnant mares serum gonadotropin and human chorionic gonadotrophin and then mated to BDF1 male mice. Midday the following day was designated E0.5. The fertilized embryos (zygote) could be collected from oviducts. Then, E4.5 blastocyst embryos were obtained by culturing zygotes in G1 plus medium (no. 10128; Vitrolife, Göteborg, Sweden). E7.5 embryos were dissected from the pregnant mice, fixed for 30 min in 4% paraformaldehyde, and dehydrated in 30% sugar, and then, embryos were embedded in optimal cutting temperature compound (OCT), frozen, and sectioned for immunofluorescence.

**Teratoma formation in SCID mice**

Undifferentiated hESCs (1 × 10^7) were harvested, washed twice with DMEM-F12, suspended in DMEM-F12 with 30% Matrigel, and subcutaneously injected into the upper limbs of SCID mice. Teratomas were harvested from anaesthetized mice approximately 8 weeks after cell injection. For histological analysis, tissues were fixed with 4% paraformaldehyde, dehydrated with a graded ethanol series, embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin. For qRT-PCR assay, the teratomas were dissected into small pieces, which were immediately stored in RNA TRIzol reagent (no. 15596018; Life Technologies, Carlsbad, CA).
**Construction of vectors**

Human Bach1 gene was generated and cloned into pcDNA3.1 as described previously (43). Briefly, the full-length cDNA of human Bach1 was generated by PCR and verified by DNA sequencing. Flag-epitope tags were introduced to the C terminus of Bach1 gene. The EZH2-HA plasmid was provided by J. Xu (Fudan University, China). GST-Bach1 full or GST-Bach1-N terminal or GST-Bach1-C terminal fusion protein was constructed by inserting PCR-generated DNA fragments encoding regions of Bach1 full (1 to 736 amino acids [aa]) or Bach1 N-terminus (1 to 560 aa) or Bach1 C-terminus (127 to 736 aa) into PGEX-6P-1 vector.

His-EZH2 fusion protein was constructed by inserting PCR-generated DNA fragments encoding regions of EZH2 into pET-28 (a) + plasmid.

The TOPflash reporter vector was purchased from Millipore (Billerica, MA). The pGL3-Basic vector was provided by J. Xu (Fudan University, China). GST-Bach1 was constructed by inserting PCR-generated DNA fragments encoding regions of Bach1 full [1 to 560 amino acids (aa)] or Bach1 N-terminus (1 to 560 aa) or Bach1 C-terminus (127 to 736 aa) into PGEX-6P-1 vector. This construct was introduced in the forward primer of these gene promoter construct. PCR products were cloned into the luciferase vector pGL3-basic. Primers for reporter and mutation are listed in table S2.2. All constructs were verified by sequencing.

**Reprogramming**

For reprogramming experiment, we used the CytoTune-iPS 2.0 Sendai Reprogramming Kit (no. A16517; Life Technologies, Carlsbad, CA). The human dermal fibroblast (HDF) cells were provided by Q. Jing (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China). The HDF cells were maintained in fibroblast medium (no. 2301; ScienCell, Carlsbad, CA), and the medium was replaced every other day. The HDF cells were infected with Sendai virus containing Yamanaka factors and cultured in fibroblast medium for 7 days. The cells were then detached using TrypLE reagent (no. 12605010; Life Technologies, Carlsbad, CA), seeded onto Matrigel-coated 12-well plates (5 × 10⁴ cells per well), and cultured in Essentia 8 medium (no. 05990; STEMCELL Technologies, Vancouver, Canada). Seven days later, the recombiant adenoviruses encoding human Bach1 gene (Ad-GFP-Bach1) or GFP control (Ad-GFP) were used to infect cells at a multiplicity of infection of 10. Thirty days after reprogramming, the cells were washed once with phosphate-buffered saline (PBS) and stained for the activity of AP with AP staining of hESCs.

**AP staining of hESCs**

hESC colonies were cultured on Matrigel-coated six-well plates for 3 to 4 days, and AP staining was performed with the Alkaline Phosphatase Kit (no. 86R-1KT; Sigma-Aldrich, St. Louis, MO). The AP-positive colonies were counted under a microscope.

**In vitro differentiation of hESCs**

For spontaneous differentiation (44), hESCs were dissociated into single cells with Accutase at 37°C for 3 min and plated (1 × 10⁵ cells/ml) on Ultra Low Cluster Plates (no. 191-3001; Crystalgen, Commack, NY) in mTesR1 with 10 μM rock inhibitor Y27632 (no. S1049; Selleckchem, Houston, TX) for 1 day; then, EBs were suspended and cultured for 2 days in DMEM, supplemented with 20% fetal bovine serum, 1% non-essential amino acids (no. 11140-050; Life Technologies, Carlsbad, CA), 1 mM l-glutamine (no. 35050038; Life Technologies, Carlsbad, CA), and 0.1 mM β-mercaptoethanol (no. M3148; Sigma-Aldrich, St. Louis, MO). EBs were transferred to cell culture plates and cultured with differentiation medium until the indicated time points.

**RNA extraction, real-time PCR, and genome-wide gene expression**

Total RNA was extracted using TRIzol reagent, and cDNA was made by using the ReverTra Ace qPCR RT Kit (no. FSQ-101; TOYOBO, Osaka, Japan). Real-time qPCR was performed with the qPCR SYBR Green Master Mix (no. 11201ES03; Yeason, Shanghai, China), and the primer sequences are listed in table S2.3. The mixture was heated to 95°C for 5 min, cycled 40 times (95°C for 30 s, 58°C for 30 s, and 72°C for 30 s), and held at 72°C for 7 min. Melting curves were generated by increasing the temperature from 55° to 95°C in 0.5°C increments at 10-s intervals and then visually to ensure that a single peak was present for each primer. Threshold amplification values (Ct) were assigned by the CFX Manager analysis software (Bio-Rad). For genome-wide expression analysis, nonribosomal RNA was isolated from 1 μg of total RNA by using a TrueLib Poly (A) mRNA Magnetic Isolation Module (no. NGS00-4011; ExCell Bio, Shanghai, China). The libraries were prepared by using the TrueLib mRNA Library Prep Kit for Illumina (no. NGS00-2012; ExCell Bio, Shanghai, China). Libraries were sequenced on the HiSeq X Ten sequencer (Illumina Inc., San Diego, CA) by the Annoroad Company (Beijing, China).

**Viral transduction of hESCs**

The corresponding three shRNAs for Bach1 or control shRNA was cloned into the pLKO.1 constructs (no. 8453; Addgene, Watertown, MA). Lentiviral particles of three Bach1-shRNAs or control shRNA was generated by transfecting HEK293T cells with a pLKO.1 shRNA vector and packaging vectors psPAX2 and pMD2.G. hESCs were transduced with the concentrated lentivirus for 48 hours; then, the cells were sorted with puromycin (2 μg/ml) selection, and clones were chosen for use in subsequent experiments. The sequences of Bach1 shRNAs are shown in table S2.4.

**RNA interference**

hESCs were transfected with the Usp7 siRNAs or control siRNAs by Lipofectamine RNAiMAX Transfection Reagent (no. 13778150; Life Technologies, Carlsbad, CA). The transfection was performed according to the manufacturer’s instructions. siRNA sequences are listed in table S2.5.

**Cell proliferation assay**

hESCs were seeded on Matrigel-coated 12-well plates (3 × 10⁴ cells/ml) and cultured for the indicated time periods; then, the cells were dissociated with Accutase and counted with a cell counting chamber. Each experiment was performed independently three times.

**Cell apoptosis assay**

Annexin V-FITC Apoptosis Detection Kit (no. APOAF-50TST; Sigma-Aldrich, St. Louis, MO) was used for cell apoptosis assay. hESCs were harvested and incubated with propidium iodide and annexin V-fluorescein isothiocyanate for 15 min at 37°C. The samples were then analyzed by using a flow cytometer (Beckman Coulter, Brea, CA). The percentage of annexin V–positive cells (earlier apoptotic cells) of total cells was calculated. Each experiment was repeated three independent times.
Cell cycle profile

Cell Cycle Analysis Kit (no. C1052; Beyotime, Shanghai, China) was used in cell cycle assay. hESCs were harvested and fixed in 70% ethanol for 2 hours at 4°C and then stained with a solution containing propidium iodide (0.05 mg/ml), RNase A (1 mg/ml), and 0.3% Triton X-100 in the dark for 30 min. The percentage of cells in different phases of the cell cycle was examined by measuring the DNA content (propidium iodide intensity) with a flow cytometer (Beckman Coulter, Brea, CA), and populations of G1, S, and G2/M phase cells were determined with the ModFIT software. Each experiment was repeated three independent times.

Luciferase assay

The luciferase assay was performed as described previously (45). Briefly, the cells were transfected with β-galactosidase (β-gal) plasmid and the indicated DNA reporter plasmid or the pGL3-basic luciferase reporter plasmid. Transfection was performed with Lipofectamine 2000, and the transfected cells were cultured for 36 hours; then, the cells were harvested, and luciferase activity was measured with the Luciferase Assay Kit (Promega, Madison, WI). β-Gal activity was measured. Relative Luciferase activity was calculated as the ratio of Luc/β-gal activity. Each experiment was performed independently three times.

Immunoblotting and IP

The cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 0.1 mM phenylmethylsulfonyl fluoride (no. ST505; Beyotime, Shanghai, China) and 1 mM protease inhibitor cocktail (no. B14001; Bimake, Houston, TX). The cytoplasmic and nuclear protein extraction was performed according to the instructions described in the Nuclear and Cytoplasmic Protein Extraction Kit (no. P0027; Beyotime, Shanghai, China). For IP, 1 mg of total protein was incubated overnight with 3 to 5 μg of antibody at 4°C and then with Protein A/G PLUS-Agarose (no. sc-2003; Santa Cruz Biotechnology, Santa Cruz, CA), at 4°C for an additional 4 hours; then, the precipitates were washed once with RIPA and three times with PBS and resuspended in 40 μl of 2× SDS loading buffer, and immunoblotting was performed via standard protocols. The following antibodies were used for immunoblotting and/or IP: Nanog (1E6C4) (no. sc-293121; Santa Cruz Biotechnology, Santa Cruz, CA), Brachyury (T) (no. AF2085; R&D Systems; Minneapolis, MN), Gata6 (no. 5851; Cell Signaling Technology, Danvers, MA), SUZ12 (D39F6) XP (no. 3737; Cell Signaling Technology, Danvers, MA), EED (no. 16818-1-AP; Proteintech, Rosemont, IL), Bach1 (no. sc-271211; Santa Cruz Biotechnology, Santa Cruz, CA), Bach1 (no. AF5776; R&D Systems, Minneapolis, MN), Sox2 (no. sc-17320; Santa Cruz Biotechnology, Santa Cruz, CA), Oct4 (no. sc-5279; Santa Cruz Biotechnology, Santa Cruz, CA), Usp7 (no. A300-033A-M; Bethyl Laboratories, Montgomery, TX), β-actin (actin) (no. sc-7477; Santa Cruz Biotechnology, Santa Cruz, CA), H3K27me3 (no. 07-449; Millipore, Billerica, MA), H3K9me2 (no. 07-212; Millipore, Billerica, MA), H3K27ac (no. MABE670; Millipore, Billerica, MA), H3K9ac (no. 06-942; Millipore, Billerica, MA), EZH2 (no. 5246; Cell Signaling Technology, Danvers, MA), H3 (no. 46208; Cell Signaling Technology, Danvers, MA), Nodal (no. ab109317; Abcam, Cambridge, UK), Wnt3 (no. ab172612; Abcam, Cambridge, UK), HA (no. sc-7392; Santa Cruz Biotechnology, Santa Cruz, CA), Flag (no. F1804; Sigma-Aldrich, St. Louis, MO), FzD1 (no. sc-398802; Santa Cruz Biotechnology, Santa Cruz, CA), active β-catenin (no. 8814S; Cell Signaling Technology, Danvers, MA), total β-catenin (no. sc-7963; Santa Cruz Biotechnology, Santa Cruz, CA), ACVR1IB (no. sc-376593; Santa Cruz Biotechnology, Santa Cruz, CA), p-Smad2 (no. CY5859; Abwys, Shanghi, China), p-Smad3 (no. CY5140; Abwys, Shanghi, China), and Smad2/3 (no. 8685S; Cell Signaling Technology, Danvers, MA).

Immunofluorescence

The cells were seeded onto Matrigel-coated 24-well plates, fixed with 4% paraformaldehyde for 20 min at room temperature, washed three times for 5 min with PBS, permeabilized with 0.3% Triton X-100 (no. T9284; Sigma-Aldrich, St. Louis, MO) and 5% donkey serum in PBS, incubated overnight with primary antibodies at 4°C, washed with PBS, and then stained with 4′,6-diamidino-2-phenylindole (no. D9542; Sigma-Aldrich, St. Louis, MO) for 5 min at room temperature. Images were captured with a Leica DMi6000B microscope (Leica Microsystems GmbH). The following antibodies were used for immunofluorescence staining: Bach1 (no. sc-271211; Santa Cruz Biotechnology, Santa Cruz, CA), Sox2, Oct4, T, Gata6, and EZH2.

GST pull down

GST-fused proteins were expressed in Escherichia coli and purified by glutathione-Sepharose 4B beads. The proteins were incubated with glutathione-Sepharose 4B beads for 4 hours at 4°C. The beads were washed and incubated with target proteins for 12 hours at 4°C. The beads were washed three times and resuspended in 40 μl of SDS loading buffer. SDS–polyacrylamide gel electrophoresis was performed, and proteins were detected using their respective antibodies. GST (no. 66001; Proteintech, Rosemont, IL) and His (no. 66005; Proteintech, Rosemont, IL) antibodies were used for this assay. Each experiment was repeated three independent times.

ChIP and ChIP-seq

The cells were cross-linked with 1% formaldehyde (no. F8775; Sigma-Aldrich, St. Louis, MO) at 37°C for 10 to 15 min; then, the reaction was quenched with 0.125 M glycine for 5 min at room temperature, and the chromatin was sonicated for 30 min in 30-s intervals to shear chromatin into 200–1000–base pair lengths. ChIP was performed as described previously (24). Briefly, samples were incubated overnight at 4°C with 3 to 5 μg of antibody bound to 60 μl of Protein A/G PLUS-Agarose; 1 to 2% of precleared chromatin was reserved for use as input DNA before incubation with the antibody. The beads were washed, and the chromatin was eluted; then, the reverse cross-linked ChIP DNA was dissolved in 10 mM tris buffer (pH 8.0). For ChIP–qPCR, immunoprecipitated DNA was analyzed by qRT–PCR; then, the amplification product was expressed as percentage of the input and normalized to the control experiment for each condition. EZH2, H3K27me3, H3K4me3, Smad 2/3, and active β-catenin antibodies were used; ChIP–qPCR primers are listed in table S2.6. DNA libraries from EZH2-ChIP and H3k27me3-ChIP, corresponding to the input DNA samples were prepared and sequenced by BasePair BioTechnology Company (Suzhou, China) with an Illumina Genome Analyzer, as directed by the manufacturer’s protocol.

Analysis of RNA-seq data

Raw sequencing reads were first processed to remove adaptors and low-quality bases using Trimomatic (v. 0.33). Then, clean reads were mapped to human genome GRCh38 (from ENSEMBL) using STAR (020201) with default parameters. Raw counts generated by STAR were mapped to human genome GRCh38 (from ENSEMBL) using STAR (020201) with default parameters. Raw counts generated by STAR were mapped to human genome GRCh38 (from ENSEMBL) using STAR (020201) with default parameters.
ontology enrichment analysis was performed using GOEAST. All plots were generated using www.ehbio.com/ImageGP.

**Analysis of ChIP-seq data**

Raw sequencing reads were first estimated, and no preprocessing was performed because of high reads quality. Then, reads were mapped to human genome GRCh38 using BWA (0.7.15-r1140) with default parameters. Low-quality mapped reads and PCR duplicates were removed using SAMTools (v1.2) and PICARD (1.138) (http://broadinstitute.github.io/picard). Promoters were defined as upstream 1 kb and downstream 0.5 kb of TSS. Meta gene profile and TSS profile were generated using deepTools2 (3.0.1). Normalized reads distribution profile were produced using deepTools2 and visualized using the University of California, Santa Cruz (UCSC) genome browser. MACS2 was used to identify enrichment peaks for Bach1, EZH2 (with default parameters for “sharp” peaks), and H3K27me3 (with default parameters for “broad” peaks). The accession number for the RNA-seq and ChIP-seq data of WT and Bach1-KO hESCs reported in this paper is GEO: GSE113817. The accession numbers for published ChIP-seq data of Bach1, EZH2, and H3K27me3 are GEO: GSE31477, GEO: GSE29611, and GEO: GSM466734, respectively.

**Statistical analysis**

Data are expressed as means ± SD in the figure legends. Differences among groups were determined with one-way analysis of variance, followed by Tukey’s test for multiple comparisons. Differences between two groups were assayed by two-tailed Student’s t test. The 0.05 level of probability was used as the criterion of significance. Analyses were conducted with GraphPad Prism software.

**SUPPLEMENTARY MATERIAL**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/3/eaau7887/DC1

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