Deletion of the Polycomb-Group Protein EZH2 Leads to Compromised Self-Renewal and Differentiation Defects in Human Embryonic Stem Cells

Graphical Abstract

Highlights
- Comprehensive examination of EZH2 function in human ESC regulation
- EZH2 deficiency causes lineage-restricted derepression of developmental regulators
- More severe self-renewal and growth defects in EZH2-deficient hESCs than in mESCs
- EZH2-deficient hESCs can differentiate to early lineages but cannot form mature tissues

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In Brief
Collinson et al. use EZH2-deficient human ESCs to demonstrate the broad conservation of Polycomb-group protein function in controlling cell-fate decisions and transcriptional programs during early human development. The authors also uncover unexpected human-specific differences that result in a more severe self-renewal and proliferation phenotype than that of PRC2-deficient mouse ESCs.

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Deletion of the Polycomb-Group Protein EZH2 Leads to Compromised Self-Renewal and Differentiation Defects in Human Embryonic Stem Cells

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SUMMARY

Through the histone methyltransferase EZH2, the Polycomb complex PRC2 mediates H3K27me3 and is associated with transcriptional repression. PRC2 regulates cell-fate decisions in model organisms; however, its role in regulating cell differentiation during human embryogenesis is unknown. Here, we report the characterization of EZH2-deficient human embryonic stem cells (hESCs). H3K27me3 was lost upon EZH2 deletion, identifying an essential requirement for EZH2 in methylation of H3K27 in hESCs, in contrast to its non-essential role in mouse ESCs. Developmental regulators were derepressed in EZH2-deficient hESCs, and single-cell analysis revealed an unexpected acquisition of lineage-restricted transcriptional programs. EZH2-deficient hESCs show strongly reduced self-renewal and proliferation, thereby identifying a more severe phenotype compared to mouse ESCs. EZH2-deficient hESCs can initiate differentiation toward developmental lineages; however, they cannot fully differentiate into mature specialized tissues. Thus, EZH2 is required for stable ESC self-renewal, regulation of transcriptional programs, and for late-stage differentiation in this model of early human development.

INTRODUCTION

Polycomb-group (PcG) proteins are epigenetic repressors of transcriptional programs and maintain cellular identity during development, differentiation, and disease (Di Croce and Helin, 2013; Pasini and Di Croce, 2016; Pietersen and van Lohuizen, 2008; Schuettengruber and Cavalli, 2009; Surface et al., 2010). PcG proteins form two well-characterized and biochemically distinct chromatin-modifying complexes that are termed Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). PRC1 catalyzes histone H2A lysine 11 ubiquitination through the activity of the E3 ligases RING1A and RING1B (Müller and Verrijzer, 2009; Wang et al., 2004). PRC2 is composed of the core proteins EZH2, EED, and SUZ12, together with RBAP46/48 and several other accessory subunits, and is responsible for catalyzing di- and trimethylation on histone H3 lysine 27 (H3K27me2/3) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Margueron and Reinberg, 2011; Müller et al., 2002). EZH2 is a SET-domain containing histone methyltransferase and is the catalytic subunit of PRC2. EED and SUZ12 are required for substrate recognition, complex stability and for promoting the enzymatic activity of EZH2 (Cao and Zhang, 2004; Nekrasov et al., 2005; Pasini et al., 2004; Tie et al., 2007).

Genome-wide studies in mouse and human embryonic stem cells (ESCs) have shown that PRC2 and H3K27me3 occupy the promoters of many developmental regulators that are important for cell differentiation and lineage specification (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). This distribution of chromatin marks led to the concept that PRC2 may contribute to the maintenance of pluripotency by keeping developmental regulators transcriptionally repressed, while enabling the genes to be rapidly activated upon suitable differentiation cues and stimuli. Despite a central position within the regulatory framework, however, PRC2 is dispensable for the maintenance of undifferentiated mouse ESCs, as the deletion of PRC2 components, including Ezh2, has little effect on their morphology, self-renewal, or proliferation, although a subset of PRC2 target genes are modestly derepressed (Chamberlain et al., 2008; Leeb et al., 2010; Pasini et al., 2007; Rilling et al., 2014; Shen et al., 2008). H3K27me3 levels are globally reduced in Ezh2-deficient mouse ESCs; however, developmental regulators retain H3K27me3 at their gene promoters and are transcriptionally repressed (Shen et al., 2008). In this context, the Ezh2 homolog, Ezh1, forms a noncanonical PRC2 complex that is able to trimethylate H3K27 at target gene promoters and maintains transcriptional repression through methylation-dependent and potentially methylation-independent pathways (Margueron et al., 2008; Shen et al., 2008).
Figure 1. Targeted Deletion of EZH2 in hESCs

(A) Overview of EZH2 structure and targeting strategy. Exons encoding CXC and SET domains are indicated. The gRNA sequence is underlined and protospacer adjacent motif highlighted in red. DNA sequence of the deletions in one EZH2+/− ESC line and one EZH2−/− ESC line is shown for both alleles. Mutation causes frameshift and premature stop codon. An additional line is shown in Figures S1 and S2.
PRC2 deficiency has a more significant impact on mouse ESCs upon their differentiation, with defects in the repression of pluripotency networks and in the failure to fully activate differentiation transcriptional programs. This aberrant gene regulation results in impaired differentiation and proliferation (Chamberlain et al., 2008; Pasini et al., 2007; Shen et al., 2008). Further underscoring the critical role of PRC2 in directing differentiation programs, all three PRC2 core components (Ezh2, Eed, and Suz12) are essential for early mouse development, as loss-of-function mutant embryos initiate but fail to complete gastrulation and die between embryonic days 7 and 9 (Faust et al., 1995; O’Carroll et al., 2001; Pasini et al., 2004). The mutant phenotype is associated with mis-expression of lineage-specifying genes, decreased cell proliferation and an increased level of apoptosis (Pasini et al., 2004).

The well-conserved binding profiles of PRC2 components and H3K27me3 in human ESCs (hESCs) at the promoters of developmental regulators raises the possibility that PRC2 may also have an important role in controlling hESC pluripotency and differentiation (Gifford et al., 2013; Ku et al., 2008; Pan et al., 2007; Zhao et al., 2007). Moreover, coordinated changes to the epigenome, including H3K27me3 localization, occur upon differentiation of hESCs and are thought to be essential for lineage specification and memory of cellular identity, as they are in Drosophila and the mouse (Gifford et al., 2013; Xie et al., 2013). However, no functional studies of PRC2 in hESC regulation and early-stage differentiation have been reported to date. Human pluripotent cells represent a unique model in which to study human development and provide a platform for producing a source of differentiated cells relevant for basic and applied research. In addition, mouse and human ESCs are known to represent different pluripotent states and may therefore rely on different epigenetic pathways to confer their ability to self-renew and differentiate (Nichols and Smith, 2009; Rossant, 2015). Understanding the key epigenetic mechanisms that underpin hESCs is therefore a priority.

Here, we report the generation and characterization of EZH2-deficient hESCs. Our findings demonstrate that EZH2 is required to maintain the transcriptional repression of developmental regulators and for cells to undergo late-stage cell differentiation, thereby revealing the broad conservation of PRC2 function in this model of early human development. We also identify unexpected human-specific differences such as the essential requirement in hESCs for EZH2 to maintain PRC2 stability and retain memory of cellular identity, as they are in Drosophila and human ESCs (Pasini et al., 2004).

RESULTS

Targeted Deletion of EZH2 in hESCs

To investigate the role of EZH2 in human pluripotency and differentiation, we used CRISPR/Cas9 to disrupt EZH2 in hESCs. A guide RNA (gRNA) designed to target an early exon within all known EZH2 isoforms was nucleofected with Cas9 into the H9 hESC line (Figures 1A and S1A). Individual colonies were isolated, expanded, and analyzed by Sanger DNA sequencing. The efficiency of disrupting the target sequence within the EZH2 coding region was high, with ~35% clonal lines containing a mutation on one allele (EZH2+/−). However, no homozygous cell lines were obtained out of 110 screened lines. This result provided a first indication that EZH2-deficient hESCs may be compromised relative to EZH2-containing cells when plated as single cells at clonal density. To overcome this apparent defect, we introduced a doxycycline (DOX)-inducible EZH2 transgene using piggyBac transposition into an EZH2+/− line and re-targeted the cells with EZH2 gRNA and Cas9 in the presence of DOX. Using this strategy, we obtained several EZH2 homozygous lines (EZH2−/−; Figures 1A, S1B, and S1C). Once the EZH2−/− lines were isolated and established, they could be maintained without DOX-induced EZH2 expression. Although we did not detect any indication that the DOX-inducible plasmid was leaky in the absence of DOX, to rule out the possibility of low-level EZH2 expression, we transiently transfected EZH2−/− ESCs with piggyBac transposase and obtained stable EZH2−/− lines with all copies of the EZH2 transgene removed (Figures S2A and S2B).

RNA expression analysis confirmed that EZH2 transcripts were lower in EZH2−/− ESCs compared to parental EZH2+/− and EZH2−/+ lines (Figures 1B and S2C). Moreover, EZH2 protein was undetectable by western blot and by immunofluorescent microscopy using two different antibodies raised against N- and C-terminal epitopes of EZH2 (Figures 1C, 1D, and S2D–S2F). The disruption of EZH2 was accompanied by the loss of other PRC2 proteins, SUZ12 and EED, despite the presence of unchanged levels of SUZ12 and EED transcripts in EZH2−/− ESCs (Figures 1B and 1D). This finding unexpectedly contrasts with Ezh2-deficient mouse ESCs where Suz12 and Eed levels are unchanged due to the ability of Ezh1 to form non-canonical PRC2 (Shen et al., 2008) but is consistent with Suz12-deficient and Eed-deficient mouse ESCs in which PRC2 components are unstable outside of the complex (Pasini et al., 2004).
In hESCs, therefore, EZH1 is unable to form noncanonical PRC2 despite being present (Figure S2 G). Immunofluorescent microscopy revealed that the loss of EZH2 led to the reduction of H3K27me3 and H3K27me2 to background levels, and to the partial reduction of H3K27me1 (Figure 1 E). Applying DOX to induce ectopic EZH2 expression in EZH2−/− ESCs restored EZH2 and led to the stabilization of SUZ12 and EED proteins, and to the re-establishment of global H3K27 methylation (cells designated herein as EZH2−/− +EZH2; Figures 1B–1E).

**Loss of Promoter-Localized H3K27me3 in EZH2-Deficient hESCs**

To characterize the molecular phenotype of EZH2-deficient hESCs, we profiled genome-wide histone methylation by native chromatin immunoprecipitation combined with high-throughput sequencing (chromatin immunoprecipitation sequencing [ChiP-seq]). Quantitative trend plots of normalized ChiP-seq reads revealed a complete loss of H3K27me3 at all gene promoters in EZH2−/− ESCs (Figure 2 A). This finding contrasts with the retention of H3K27me3 in Ezh2−/− ESCs in mouse ESCs (Shen et al., 2008).

**Figure 2. EZH2 Deficiency in hESCs Results in Loss of H3K27me3**

(A) Quantitative trend plot of H3K27me3 normalized ChiP-seq reads over gene body ± 5 kb. High CpG (HCP), intermediate CpG (ICP), and low CpG (LCP) promoters are shown separately.

(B) Scatterplot of H3K27me3 (x axis) and H3K4me3 (y axis) normalized ChiP-seq reads in EZH2−/− relative to EZH2+/− (left) and relative to EZH2−/− +EZH2 (center), and EZH2−/− +EZH2 versus EZH2+/− (right). All transcriptional start sites (TSS) shown in gray; TSS that are positive for H3K27me3 in EZH2+/− ESCs highlighted in blue. Disruption of EZH2 leads to a strong reduction in H3K27me3 levels at TSS, with little effect on H3K4me3 levels. Expression of a DOX-mediated EZH2 transgene in the EZH2-deficient cells causes restoration of H3K27me3 levels to levels equivalent to EZH2+/−.

(C) ChiP-seq tracks of HOXB (left) and HOXD (right) loci illustrate the loss of H3K27me3 in EZH2−/− ESCs compared to control ESCs. H3K4me3 is relatively unaffected. All ChiP-seq data represent the average of three biological replicates for each cell line. These results were confirmed independently by qPCR analysis of ChiP DNA at several gene promoters (Figure S3 C).

2004, 2007). In hESCs, therefore, EZH1 is unable to form noncanonical PRC2 despite being present (Figure S2 G). EZH1 transcript and protein levels were largely unchanged upon EZH2 deletion (Figure S2 G). Immunofluorescent microscopy revealed that the loss of EZH2 led to the reduction of H3K27me3 and H3K27me2 to background levels, and to the partial reduction of H3K27me1 (Figure 1E). Applying DOX to induce ectopic EZH2 expression in EZH2−/− ESCs restored EZH2 and led to the stabilization of SUZ12 and EED proteins, and to the re-establishment of global H3K27 methylation (cells designated herein as EZH2−/− +EZH2; Figures 1B–1E).
Figure 3. Genes Encoding Developmental Regulators Are Transcriptionally Derepressed in EZH2-Deficient hESCs

(A) RNA-seq heatmap for EZH2^−/− ESCs and control ESCs (three biological replicates per line). Shown are all differentially expressed genes between EZH2^−/− and EZH2^−/− + EZH2 ESCs.

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Confirming the result in hESCs, scatterplot analysis of ~2,000 promoters that have high levels of H3K27me3 in EZH2+/+ ESCs (H3K27me3WT) revealed a loss of H3K27me3 in EZH2−/− ESCs (Figure 2B). The majority of H3K27me3WT promoters have histone H3 lysine 27 acetylation (H3K4me3) in hESCs (Pan et al., 2007; Zhao et al., 2007), and H3K4me3 levels were largely unaffected by EZH2 disruption (Figure 2B). ChIP-seq tracks for two example loci, HOXB and HOXD, illustrate the loss of H3K27me3 across the domains in EZH2−/− ESCs (Figure 2C). Comparison of H3K27me3 between EZH2+/+ and EZH2−/− cells revealed highly similar profiles, demonstrating that histone patterns are appropriately re-established upon EZH2 restoration (Figures 2A–2C). Interestingly, there was a modest increase in histone H3 lysine 27 acetylation (H3K27ac) levels at H3K27me3WT promoters in EZH2−/− ESCs, supporting a potential antagonism between H3K27 acetylation and trimethylation that has also been observed in other contexts (Ferrari et al., 2014; Gehani et al., 2010; Jung et al., 2010; Pasini et al., 2010; Schoenfelder et al., 2015) (Figure S3A). Last, an alternative ChIP-seq analysis strategy of genome binning confirmed a loss of H3K27me3 sequencing reads across the genome of EZH2−/− ESCs, further reinforcing the key finding that H3K27me3 levels are depleted upon deletion of EZH2 in hESCs (Figure S3B). Together, these results demonstrate that EZH2 is the main functional H3K27me2/3 methyltransferase in hESCs.

EZH2 Deficiency Causes Transcriptional Derepression of Key Developmental Genes

We next performed RNA sequencing (RNA-seq) to investigate the impact of loss of EZH2 and associated H3K27me3 on gene expression. The assays were carried out on samples that were flow-sorted using the hESCs cell-surface marker SSEA4 to ensure that we compared between equivalent cell populations (Figure S4A). The majority of genes were not altered transcriptionally by EZH2 disruption, but 911 genes were significantly upregulated and 282 genes were significantly downregulated in EZH2−/− ESCs compared to EZH2+/− +EZHE2 ESCs (p < 0.05; Figures 3A and S4B). Gene ontology (GO) analysis of the upregulated gene set identified categories associated with developmental and cellular differentiation, including pattern specification, embryonic morphogenesis, and tissue formation (Figure 3B). The upregulated group was significantly enriched for genes with EZH2 and H3K27me3 occupancy in EZH2+/+ ESCs and are thus expected to be sensitive to EZH2 disruption (Figures 3C and 3D). Notably, not all genes with EZH2-bound promoters were mis-regulated, suggesting that secondary events may be required for this group of genes to initiate transcriptional change in response to the loss of EZH2. In addition, a subset of upregulated genes (enriched for signaling and adhesion GO terms) are likely to be regulated indirectly as they are not PRC2 targets in hESCs. No GO categories were significantly enriched in the downregulated gene set, although of note, the top category was associated with the regulation of M-phase and indicate the decreased transcription of genes typically expressed in mitosis (Figure 3B).

We examined transcriptional changes in a set of ~100 classical developmental regulators that have strong EZH2 promoter occupancy, including genes within the FOX, GATA, LHX, T-box, and SOX families (Lee et al., 2006). A clear pattern emerges from this analysis: nearly all genes within this class of developmental regulator showed transcriptional derepression in the absence of EZH2 (Figures 3E and S4C). Gene derepression did not become more prevalent upon continued passaging, and therefore the EZH2−/− ESCs retained a similar transcriptional profile over time. ChIP-seq and RNA-seq tracks for several example genes, including SOX17, GATA4, T, and TBX3, illustrate the absence of promoter H3K27me3 and an associated increased transcript level in EZH2−/− ESCs (Figure 3F). We further showed that small hairpin RNA (shRNA)-mediated depletion of EZH2 causes derepression of developmental regulators in two additional human pluripotent stem cell lines (WIBR3 and FIPS; Figure S4D). We conclude that the deletion of EZH2 in hESCs leads to the loss of H3K27me3 and to the transcriptional derepression of genes that encode developmental regulators, thereby positioning EZH2 as a key factor in controlling the transcriptome of human cell types during early development.

Single-Cell Transcriptional Analysis Reveals Gene Mis-regulation Profiles

To investigate more precisely the transcriptional mis-regulation and cell-to-cell variability in response to EZH2 deficiency, we performed single-cell RNA-seq on individual SSEA4-positive, flow-sorted EZH2+/+ and EZH2−/− ESCs. The results show that a subset of EZH2−/− ESCs strongly upregulated EZH2-target genes, but individual genes are not robustly derepressed in most cells examined (Figure 4A). Unexpectedly, clustering of the data suggested that gene derepression occurs predominately within discrete transcriptional programs, such that...
Figure 4. Transcriptional Derepression Occurs Predominantly within Discrete Lineage-Specific Programs

(A) Single-cell RNA-seq expression levels for six example PRC2-target genes in EZH2−/− ESCs and EZH2+/+ ESCs, where each dot represent the results from a single cell. A pluripotency gene (LIN28) and housekeeping gene (HMBS) are shown for comparison. Robust upregulation of PRC2-target genes occurs in a subset of EZH2−/− ESCs.

(B) Heatmap of single-cell RNA-seq expression for EZH2−/− ESCs (right) and EZH2+/+ ESCs (left). Each column represents an individual cell. Each row represents an individual gene, grouped into three clusters corresponding to endoderm, mesoderm, and ectoderm cell lineages. Shown are PRC2-target genes from within the hESC scorecard assay, which is an assay that can classify differentiated cell lineages (Bock et al., 2011). Subsets of cells (boxed in purple) tend to mis-express many genes from within one lineage but rarely mis-express multiple genes derived from more than one lineage.
individual $EZH2^{-/-}$ ESCs have upregulated multiple genes associated with a particular cell lineage but rarely show strong signatures derived from several cell lineages (Figure 4B). In particular, $EZH2^{-/-}$ ESCs show lineage biases toward endoderm and mesoderm, but not to ectoderm. This response may be constrained by the hESC culture environment due to the activities of FGF and Activin/Nodal signaling within the media, which are known to promote endoderm and mesoderm specification and suppress ectoderm differentiation (Pauklin and Vallier, 2015). Together, these results reveal that the depletion of EZH2 does not cause global EZH2-target gene derepression in all ESCs, as might be predicted from cell population studies. Rather, its loss leads to the mis-regulation of subsets of genes and to the acquisition of lineage-restricted transcriptional programs.

### EZH2 Disruption Causes Self-Renewal and Proliferation Defects in hESCs

Undifferentiated EZH2-deficient ESCs could be maintained in culture for >50 passages; however, their growth and morphology were severely compromised compared to control lines. $EZH2^{-/-}$ colonies were highly variable in appearance with an increased prevalence of flatter cells that are characteristic of spontaneous differentiation (Figure 5A). To compare directly the ability of each hESC line to self-renew, we plated an equal number of...
SSEA4-positive flow sorted cells at clonal density, and after 7 days we counted the number of colonies that were positive for the undifferentiated hESC marker alkaline phosphatase (AP). We observed an ~40% reduction in colony number in EZH2−/− ESCs compared to control ESCs (Figure 5B).

Diminished colony formation was due to both compromised self-renewal and impaired proliferation. Categorizing EZH2−/− ESC colonies based on AP activity patterns revealed an ~50% reduction in the proportion of undifferentiated colonies in EZH2−/− ESCs compared to control ESCs, and an associated increase in the proportion of colonies with a mixed or fully differentiated phenotype (Figure 5C). This was accompanied by a decrease in the proportion of EZH2−/− ESC colonies that are entirely OCT4 positive, with an associated increase in the proportion of colonies that are positive for SOX17, a marker of early differentiated cells (Figure 5D). The proportion of undifferentiated and differentiated cells within the EZH2−/− ESC cultures was unchanged over passage and was re-established after plating of purified undifferentiated EZH2−/− ESCs, further highlighting the unstable nature of these cells.

Cell counts over four passages revealed an ~50% reduction in cell number in EZH2−/− ESCs, revealing that proliferation is significantly reduced in the absence of EZH2 (Figure 5E). The mitotic index, as determined by the proportion of histone H3 serine 10 phosphorylation (H3S10ph) positive cells, was significantly lower in EZH2−/− ESCs compared to control ESCs (Figure 5F). The reduction in mitotic cells within EZH2−/− cultures is in agreement with our RNA-seq results, which identify the transcriptional downregulation of genes associated with M-phase (Figure 3B). Of further relevance to this defect is that several negative regulators of the cell cycle, such as CDKN2A (encoding p16INK4A and p14ARF) and CDKN2B (encoding p15INK4B), were transcriptionally derepressed in EZH2-deficient ESCs (Figure S3). These findings are consistent with previous studies in other cell types that have identified a role for EZH2 in controlling the transcription of cell-cycle regulators (Bracken et al., 2003, 2007; Pasini et al., 2004; Sauvageau and Sauvageau, 2010; Varambally et al., 2002). Together, the results demonstrate that EZH2-deficient hESCs are strongly compromised in their ability to self-renew and proliferate, thereby identifying a more severe phenotype compared to mouse ESCs that are deficient for Ezh2 and other PRC2 proteins.

**Differentiation Defects in EZH2-Deficient hESCs**

We next investigated the impact of EZH2 deletion on the ability of hESCs to differentiate correctly. We injected each hESC line into the kidney capsule of three immunocompromised mice to test for teratoma formation. Control hESC lines produced teratomas consisting of mature cell types derived from all three germ lineages. By contrast, EZH2−/− ESCs failed to produce teratomas in two mice and generated a very small mass in one mouse, which consisted of a restricted set of cell types, including immature adipocytes and epithelial cells (Figures 6A, S6A, and S6B). Although the DOX-inducible EZH2 transgene could partially rescue the EZH2−/− phenotype, we noticed that the teratomas formed from the EZH2+/− × EZH2−/− ESCs were smaller and displayed different morphology compared to the EZH2+/+ and EZH2−/− ESC teratomas (Figures 6A, S6A, and S6B). The difference is likely to be because the cells were not provided with DOX once they were injected in situ, and therefore EZH2 levels would be lost gradually over several days. Further investigation of EZH2 function in late-stage cell differentiation revealed that very few EZH2−/− ESCs survived after 5 days of retinoic-acid-mediated differentiation in vitro, compared to EZH2+/+ and EZH2−/− ESCs (Figure 6B). Restoration of EZH2 with the DOX-inducible transgene partially rescued the defect, although we noticed that the transgene was silenced at the later stages of cell differentiation, thereby hindering a full rescue. Together, these results lead us to conclude that hESCs require EZH2 to form late-stage differentiated cell types.

We next studied the early stages of ESC differentiation. PRC2 loss-of-function mutant embryos initiate but fail to complete gastrulation (Faust et al., 1995; O’Carroll et al., 2001; Pasini et al., 2004); however, a detailed examination of PRC2-deficient mouse or human ESC differentiation toward early developmental progenitors using defined conditions has not been reported. To investigate these developmental events, we initiated directed and separate differentiation toward endoderm, mesoderm, and...
ectoderm progenitors, using defined conditions and lineage-specific markers (Supplemental Experimental Procedures). We observed that cell number declined sharply during endoderm and mesoderm differentiation of \( \textit{EZH2}^{-/-} \) ESCs, such that very few cells remained by the end of their differentiation protocols (Figures 6 C and 6D). In contrast, cell number was maintained in \( \textit{EZH2}^{+/+} \) ESCs during differentiation. We examined the cells at a mid-stage time point as this allowed us to obtain sufficient cells for analysis. Quantitative analysis of lineage-specific, cell-surface markers using flow cytometry showed that \( \textit{EZH2}^{-/-} \) ESCs were capable of differentiating into early-stage endoderm (defined as KIT\(^+\)/CXCR4\(^+\))(Nostro et al., 2011) and mesoderm (defined as PDGFR\(^a\)/KDR\(^+\))(Kattman et al., 2011) and, surprisingly, formed cell populations at these time points that were more uniform in marker expression than achieved upon differentiation of \( \textit{EZH2}^{+/+} \) ESCs (Figures 6 C and 6D). To ascertain whether a pre-existing subset of endoderm progenitors were responsible for generating endoderm cells in \( \textit{EZH2}^{-/-} \) cultures, we used flow cytometry to separate KIT\(^+\)/CXCR4\(^+\) (endoderm primed) and KIT\(^-\)/CXCR4\(^-\) (not endoderm primed) \( \textit{EZH2}^{-/-} \) populations and subjected the cells to endoderm differentiation. Flow cytometry analysis showed that KIT\(^-\)/CXCR4\(^-\) were highly efficient in generating endoderm, thereby demonstrating the ability of \( \textit{EZH2}^{-/-} \) ESCs to respond to appropriate differentiation cues and initiate early-stage differentiation (Figure S6C).

Upon cell differentiation, pluripotency factors POU5F1, NANOG, and SOX2 were downregulated to a similar extent in \( \textit{EZH2}^{-/-} \) ESCs compared to \( \textit{EZH2}^{+/+} \) ESCs (Figures 6E and 6F). This finding is in contrast to PRC2-deficient mouse ESCs, which exhibit a defect in silencing pluripotency networks during cell differentiation (Pasini et al., 2007; Shen et al., 2008). Consistent with our RNA-seq data, genes associated with endoderm and mesoderm differentiation were detected at higher levels at day 0 in \( \textit{EZH2}^{-/-} \) ESCs compared to \( \textit{EZH2}^{+/+} \) ESCs, and the transcript level of these genes increased further during differentiation, confirming their ability to undergo early-stage differentiation (Figures 6E and 6F). Ectopic expression of lineage-restricted genes occurred during \( \textit{EZH2}^{-/-} \) ESC differentiation, suggesting a failure to repress alternate transcriptional programs (Figures 6E and 6F).

Taken together, these results demonstrate that \( \textit{EZH2} \) is not required for the initial phase of hESC differentiation but is required for the robust generation of mature cell types that are produced in the later stages of differentiation in vitro or in teratoma assays.

**DISCUSSION**

PcG-proteins are essential regulators of cell-fate decisions and transcriptional programs during the development of several species, including \textit{Drosophila} and the mouse. Here, we show that this important function is also required in humans during the establishment of early developmental cell types that arise upon ESC differentiation (Figure 7). Furthermore, loss of \( \textit{EZH2} \) function in undifferentiated hESCs led to the transcriptional derepression of \( -900 \) genes including many important developmental regulators, thereby positioning \( \textit{EZH2} \) as a key factor in controlling the transcriptome of human cell types during early development. Interestingly, not all PRC2-target genes were mis-regulated, suggesting that redundant modes of transcriptional repression are in place, or that additional cues (such as transcription factor binding) are required to fully activate those genes. In addition to
developmental factors, we also detected an increased expression of cell-cycle regulators in the absence of EZH2, including CDKN2A (encoding p16INK4A and p14ARF) and CDKN2B (encoding p15INK4B). Given the close association between cell-cycle control and cell differentiation in hESCs (Pauklin and Vallier, 2013; Gonzales et al., 2015; Pauklin et al., 2016), it is likely that both processes contribute to the phenotype of EZH2-deficient cells. For example, an upregulation of p16INK4A would inhibit CDK4/6, which, in turn, would lead to an increase in Activin/ Nodal activity (Pauklin and Vallier, 2013). This signaling change would promote the transcriptional programs of endoderm and mesoderm lineages and suppress ectoderm differentiation. We speculate, therefore, that the increased expression of endoderm and mesoderm genes in EZH2-deficient hESCs is caused jointly by the removal of repressive H3K27me3 marks, by altered signaling activities that are mediated by cell-cycle machinery, and by the cell-culture environment. Notably, our transcriptional results are consistent with a recent study that reported derepression of a subset of PRC2-target genes in Ezh2-deficient mouse epiblast tissue, thereby underscoring the relevance of our observations to pluripotent cells in vivo (Zylicz et al., 2015). Importantly, our analysis of individual cells further revealed that misregulation of genes tended to occur in a coordinated manner within lineage-restricted transcriptional programs, rather than a haphazard derepression of all PRC2-target genes as might be predicted from global cell population analysis. These results suggest the presence of feedback mechanisms that are able to promote or repress alternative cell fates during the early phases of differentiation. An exciting set of future studies will be to model and investigate the mechanisms responsible for this feedback. Our findings also raise broader questions about how cells are committed to a particular lineage during differentiation. Purifying live hESC populations that are in different transcriptional states and challenging the cells to functional assays should begin to unravel the complexities of cell-fate commitment during human development.

Despite extensive conservation in their functions, differences exist between mouse and human ESCs that lack EZH2; self-renewal, morphology, and proliferation are seemingly perturbed to a greater extent in human EZH2−/− ESCs compared to mouse Ezh2−/− ESCs (Shen et al., 2008). One potential explanation is that differences in PRC2 protein stability or function could contribute to the distinct mouse and human ESC phenotypes. For example, Eed and Suz12 levels are unaffected by the loss of Ezh2 in mouse ESCs, potentially due to a partial compensation by Ezh1 (Shen et al., 2008). In contrast, we show here that depletion of EZH2 in hESC results in loss of EED and SUZ12, despite the presence of EZH1. Interestingly, Ezh1 cannot compensate for the absence of Ezh2 during mouse ESC differentiation or embryogastulation (O’Carroll et al., 2001; Shen et al., 2008). We speculate there is a context-dependent role for Ezh1 and that the compensatory function diminishes as cells enter the post-implantation phase of development, which could partially explain the apparent inability of EZH1 to fulfill a compensatory role in EZH2-deficient hESCs. In addition, Ezh1 is able to repress gene transcription through methylation-independent mechanisms in somatic cells, potentially via chromatin compaction (Margueron et al., 2008). It will therefore be interesting in future studies to more precisely define the functional interplay between EZH1 and EZH2 in early human developmental cell types.

A second potential explanation for the distinct phenotypes is that mouse and human ESCs are known to represent different pluripotent states, with hESCs considered to be primed for differentiation (Nichols and Smith, 2009; Rossant, 2015). The EZH2-deficient phenotype may therefore manifest differently depending on cell state, a concept recently proposed for DNMT1-depleted ESCs (Liao et al., 2015). It will be important in future studies to test this hypothesis by investigating the role of PRC2 in human “naïve” pluripotent cells, which are reported to be more similar to mouse ESCs (Manor et al., 2015). Furthermore, it is interesting to consider that PRC2 may contribute to the balance required for primed-state pluripotency by enabling low-level expression of lineage-specifying developmental regulators while constraining their levels so that they do not overwhelm the maintenance of the undifferentiated state. Given that the ectopic expression of several EZH2 target genes, such as SOX17 and GATA6, can induce the differentiation of hESC (Séguin et al., 2008; Wamaitha et al., 2015), it is plausible that derepression of these and other developmental regulators in the absence of EZH2 results in a shift toward an increased level of spontaneous differentiation that is observed in EZH2-deficient hESCs. Thus, our analysis of PcG function in hESCs should lead to a better understanding of the processes that regulate lineage priming and cell-fate commitment and inform similar events that occur in other species and cell types.

Genome-wide mapping has revealed that dynamic changes in epigenetic marks, including H3K27me3 localization, occur upon hESC differentiation (Gifford et al., 2013; Xie et al., 2013). A prevailing model proposes that this epigenetic reconfiguration is required to coordinate transcriptional programs and provide a memory of cell identity. We have now tested this model, and we show that EZH2 is not required for the initial phase of hESC differentiation as ectoderm, mesoderm, and endoderm germ lineages can form in the absence of EZH2; however, the mutant cells mis-express lineage-specific genes are unstable and are gradually lost over the differentiation time course. Interestingly, and in contrast to PRC2-deficient mouse ESCs (Pasini et al., 2007; Shen et al., 2008), pluripotency genes were downregulated appropriately upon differentiation of EZH2-deficient hESCs, suggesting that these genes are silenced by PRC2-independent pathways. The observed differentiation defects and reduction in cell survival are therefore unlikely to be caused by aberrant expression of pluripotency factors, but rather by misexpression of lineage-specifying genes and cell-cycle regulators. Finally, although the EZH2-deficient hESCs were unable to form mature cell types, the rescue of early differentiation defects by conditionally restoring EZH2 levels should enable the role of PRC2 to be investigated during late-stage differentiation. As the EZH2−/− Ezh2−/− ESCs could not fully recapitulate the parental wild-type cells in the teratoma and the RA-differentiation experiments, alternative conditional systems might be better suited for the investigation of EZH2 in late-stage cell differentiation. Interestingly, the results from our teratoma experiments suggest that once the EZH2−/− ESCs have overcome the initial early-stage differentiation barrier (enabled by residual EZH2), they are able to specialize along certain tissue lineages.
The differences in morphology and tissue composition of the teratomas are presumably, to some extent, a reflection of what differentiation pathways are accessible to EZH2-deficient cells. Future studies using alternative conditional strategies and more precise differentiation systems should lead to a better understanding of epigenetic modifiers in the generation of specialized cell types. Artificially controlling EZH2 levels may also have useful practical applications in producing desired cell types, as has been demonstrated recently to boost production of beta cell progenitors (Xu et al., 2014).

Taken together, our study provides a comprehensive examination of EZH2 function in HESC pluripotency and differentiation. Of note is that PRC2 mediates the self-renewal and differentiation of adult stem cells and cancer stem cells (Sauvageau and Sauvageau, 2010). Our findings therefore not only reveal the role of epigenetic modifiers and associated histone marks in regulating the genome during early human development, but also establish general principles that can be applicable to stem cells involved in homeostasis and disease.

EXPERIMENTAL PROCEDURES

Cell Culture
hESCs (H9/WA09, obtained from WiCell; WIBR3, kindly provided by Rudolph Jaenisch; FIPS, kindly provided by Austin Smith) were cultured at 37°C in 5% CO2 in air on CP-1 irradiated mouse embryonic fibroblasts (MEFs) in Advanced DMEM containing 20% knockout serum replacement supplemented with 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 mM penicillin/streptomycin, 1 µM non-essential amino acids (all from Thermo Fisher Scientific) and 4 ng/ml FGF2 (WT-MRC Cambridge Stem Cell Institute). Where indicated, DOX was added at 1 µg/ml. For feeder-free culture, ESCs were trypsined using Vironectin matrix in TeSR-E8 media (STEMCELL Technologies). Authentication of the hESCs was achieved by confirmation of expression of pluripotency gene and protein markers. Cells were routinely verified as mycoplasma-free using a PCR-based assay. Teratoma formation assays were performed in a designated facility under licenses granted by the UK Home Office. Additional cell-culture materials and methods are detailed in Supplemental Experimental Procedures.

Targeted Deletion of EZH2
EZH2 gRNA (CCGCTTCTGTCGTCCTTATG) was designed using http://crispr.mit.edu (Hsu et al., 2013). The gRNA sequence was incorporated into the U6 target gRNA expression vector (Mali et al., 2013) and synthesized as a gBlock by Integrated DNA Technologies. The EZH2 gRNA gBlock was sub-cloned into pCR2.1-TOPO (Thermo Fisher Scientific) and verified by sequencing. hESCs were dissociated into single cells using Accutase (Thermo Fisher Scientific). H9 ESCs (2 million) were nucleofected with 5 μg pCas99_GFP (Addgene plasmid # 44719) and 5 μg EZH2 gRNA expression vector. After 48 hr, 10,000 GFP-positive single cells were isolated by FACS and seeded onto MEF in a 10-cm tissue culture dish in ESC media supplemented with 10 μM Rho Kinase inhibitor (Cell Guidance Systems) for the first 24 hr. Individual clones were picked and expanded in 24-well plates. Mutations were validated by DNA sequencing of TOPO cloned PCR products. As a check for specificity, ten predicted off-target gRNA sites within genes were tested and verified to contain unmodified sequences.

Plasmid Constructs
To construct PB-TET-EZH2-ires-mCherry plasmid, the EZH2 coding sequence was amplified using primers EZH2_attb_F and EZH2_attb_R and sub-cloned into PB-TET-ires-mCherry plasmid. To generate EZH2-/-; +/-; +/+ ESCs, EZH2-/- ESCs were lipofected with 1 μg PB-TET-EZH2-ires-mCherry, 1 μg pCAG-rTA-Puro, and 2 μg pOyL43 (Wang et al., 2008) followed by selection with 1 μg/ml Puromycin.

To remove PB-TET-EZH2-ires-mCherry, DOX-induced EZH2-/-; +/-; +/+ ESC were nucleofected with 5 μg pCMV-hyPBase (Yusa et al., 2011) and 1 μg Turbo-GFP (Lonza). After 48 hr, 10,000 GFP/mCherry double-positive single cells were isolated by FACS and seeded onto MEFs in a 10-cm tissue culture dish in ESC media supplemented with 10 μM Rho Kinase inhibitor for the first 24 hr. Individual clones were picked and expanded in 24-well plates. DNA was genotyped using mCherry_Geno and TET-Prom_Geno primers to confirm removal of PB-TET-EZH2-ires-mCherry.

Statistics
For Figure 3D, the data are significantly departed from normality (p < 0.05; D’Agostino-Pearson omnibus normality test) and the variance is different between the groups (p < 0.05; Brown-Forsythe test); therefore, a non-parametric test was used. For statistical analysis of data within Figures 5 and 6, the scatter of the data lead us to assume that the samples come from a normally distributed population and that the variability between the groups is about the same; therefore, parametric tests were used.

ACCESSION NUMBERS
The accession number for the sequencing data reported in this paper is GEO: GSE76626.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.11.032.

AUTHOR CONTRIBUTIONS
A.C. and P.J.R.-G. designed the study, interpreted the results, and wrote the manuscript. A.C. generated all cell lines and performed all experiments. A.J.C. carried out the ectoderm differentiation experiments. N.P.M. generated several ChIP-seq libraries. A.R.S. performed cell-line characterization. T.C. assisted with generating the single-cell RNA-seq libraries. S.A. analyzed single-cell RNA-seq data. P.J.R.-G. conceived and supervised the project, performed experiments, and analyzed data. We consider A.J.C. and N.P.M. to have contributed equally.

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