ESCs Require PRC2 to Direct the Successful Reprogramming of Differentiated Cells toward Pluripotency

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SUMMARY

Embryonic stem cells (ESCs) are pluripotent, self-renewing, and have the ability to reprogram differentiated cell types to pluripotency upon cellular fusion. Polycomb-group (PcG) proteins are important for restraining the inappropriate expression of lineage-specifying factors in ESCs. To investigate whether PcG proteins are required for establishing, rather than maintaining, the pluripotent state, we compared the ability of wild-type, PRC1-, and PRC2-depleted ESCs to reprogram human lymphocytes. We show that ESCs lacking either PRC1 or PRC2 are unable to successfully reprogram B cells toward pluripotency. This defect is a direct consequence of the lack of PcG activity because it could be efficiently rescued by reconstituting PRC2 activity in PRC2-deficient ESCs. Surprisingly, the failure of PRC2-deficient ESCs to reprogram somatic cells is functionally dominant, demonstrating a critical requirement for PcG proteins in the chromatin-remodeling events required for the direct conversion of differentiated cells toward pluripotency.

INTRODUCTION

PcG proteins were originally identified in Drosophila melanogaster where they comprise multiprotein complexes required for maintaining the transcriptional silencing of a subset of repressed genes (reviewed in Ringrose and Paro, 2004). In recent years the biochemical properties of different Polycomb repressor complexes (PRCs) have been clarified in mammals and invertebrates, as has their genome-wide distribution (Boyer et al., 2006; Lee et al., 2006; Schwartz et al., 2006). This provided valuable insights into the mechanisms by which they repress transcription and convey cellular memory. Two main repressor complexes, PRC1 and PRC2, with different catalytic properties and core components have been identified. In mammals PRC2 activity depends on three core subunits, embryonic ectoderm development (Eed), suppressor of Zeste 12 (Su z12) and the SET domain-containing protein Enhancer of Zeste homolog 2 (Ezh2) that catalyzes histone H3 methylation at lysine 27 (reviewed in Simon and Kingston, 2009). PRC2-mediated H3K27me3 provides a docking site for PRC1 (Min et al., 2003), a multiprotein complex thought to be a direct executor of silencing. It contains four generic core components (Ring1A/ Ring1B, Bmi1/Mel18, Ph1/2, and Pc1/2/3), and catalyzes histone H2A mono-ubiquitination of lysine 119 (reviewed in Simon and Kingston, 2009). PRC1-mediated silencing probably operates through a variety of mechanisms and there is evidence supporting PRC1 in the blocking of transcriptional elongation of RNA Polymerase II engaged at target sites (Stock et al., 2007), as well as in directing chromatin compaction (Francis et al., 2004). Studies in Drosophila melanogaster have also suggested that O-linked glycosylation, mediated by the PcG protein Sxc may also have a significant role in Polycomb repression (Gambetta et al., 2009; Sinclair et al., 2009).

We do not know the complete repertoire of roles played by PcG proteins in mammalian development. In human and mouse ESCs, PRC1 and PRC2 localize to the promoters of a subset of repressed genes encoding transcription factors required for specification later during development. These genes contain overlapping binding sites for the pluripotency-associated factors Oct4, Sox2, Nanog, and SalI4 within their promoters (Boyer et al., 2005, 2006; Lee et al., 2006; Yang et al., 2008) and are enriched for both histone H3K4me3 and H3K27me3 (Azuara et al., 2006; Bernstein et al., 2006). Withdrawal of PRC1 or PRC2 activity from mouse ESCs results in global gene derepression of these target genes (Azuara et al., 2006; Boyer et al., 2006; Jorgensen et al., 2006; Lee et al., 2006) and unscheduled differentiation (Boyer et al., 2006; Endoh et al., 2008), consistent with PRCs being important for maintaining pluripotency. Furthermore, Ezh2-deficient embryos die early in gestation and null blastocysts fail to generate ESC lines (O’Carroll et al., 2001), so PRC2 may also be critical for establishing pluripotency. To investigate this, we have taken advantage of the documented capacity of ESCs to directly reprogram differentiated somatic cells to pluripotency in experimental heterokaryons (Pereira and Fisher, 2009; Pereira et al., 2008). Previous studies with
this approach showed that the ability of ESCs to successfully reprogram somatic cells requires the expression of Oct4 (Pereira et al., 2008), a factor that is required for induced pluripotent stem cell (iPSC)-based reprogramming (Takahashi and Yamanaka, 2006; Yamanaka, 2009). Here, we show that mouse ESC lines conditionally depleted of PRC1 or PRC2 components fail to reprogram human B cells within heterokaryons, being unable to induce the successful remodeling of differentiated nuclei. This defective reprogramming is functionally dominant over wild-type, implying that misexpression of PRC target genes by ESCs actively interferes with their reprogramming capacity.

RESULTS

Human B Lymphocytes Are Reprogrammed by Mouse ESCs, ECCs, and EGCs

Heterokaryon assays in which human differentiated cells are reprogrammed by mouse ESCs have been used to study successful reprogramming and to define the factors required for this dominant conversion (Blau et al., 1985; Tada et al., 2001; Terranova et al., 2006). When fused with human B lymphocytes, mouse ESCs rapidly induce the expression of human pluripotency-associated genes (Pereira et al., 2008) and studies in hybrids suggest that ESCs share this property with other Oct4-expressing embryonic stem cells including embryonic carcinoma (EC, such as F9, P19) and embryonic carcinoma cells (ECCs, 19G and P19 cell lines, dark gray bars), and embryonic germ cells (EGCs, 19G and 23G cell lines, light gray bars) is shown. Error bars indicate the SD of 3 independent experiments.

Figure 1. Heterokaryon Reprogramming of Human B Lymphocytes to Pluripotency by Mouse ESCs, ECCs, and EGCs but Not ESCs Lacking either Eed or Suz12

(A) Schematic representation of the strategy for generating interspecies heterokaryons between human B lymphocytes and mouse ESCs. (B) Reprogrammation was evaluated by measuring the induction of human embryonic genes (OCT4, NANOG, CRIPTO, DNMT3B, SOX2, TLE1, TERT, and REX1) from human-derived nuclei, via qRT-PCR and human gene-specific primers, 3 days after cell fusion. The reprogramming potential of mouse embryonic stem cells (ESCs, OS25 and E14tg2A cell lines, black bars), embryonic carcinoma cells (ECCs, F9 and P19 cell lines, dark gray bars), and embryonic germ cells (EGCs, 19G and 23G cell lines, light gray bars) was monitored.

(C) qRT-PCR analysis of heterokaryons generated between human B lymphocytes and mouse ESCs knockdout for the PRC2 members Eed and Suz12 (white bars), Jarid2, Dnmt1, Dnmt3a/b, Mll, G9a, and Dicer (black bars). Data were normalized to human GAPDH expression. Error bars indicate the SD of 2–3 independent experiments.

(D) Alkaline phosphatase staining of hybrid colonies obtained from fusions of puromycin-resistant mouse B cells with either Eed-deficient (Eed<sup>−/−</sup>) or wild-type (Eed<sup>+/+</sup>) ESCs. Fused cells (+PEG) or unfused controls (−PEG) were plated on puromycin-resistant feeder cells in ESC medium supplemented with puromycin for 12 days and stained for alkaline phosphatase activity. Average number of colonies from three experiments: Eed<sup>−/−</sup> x mouse B (23 ± 11), Eed<sup>+/+</sup> x mouse B (1945 ± 998).

(E) Oct4 expression, detected by immunofluorescence labeling (green), is retained by hybrid clones generated from fusing Eed<sup>−/−</sup> ESCs with mouse B cells, but is not expressed by hybrid clones generated from fusing Eed<sup>+/+</sup> ESCs with mouse B cells. Actin (red) staining is shown as a control. Scale bars represent 50 μm. See also Figure S1 and Table S1.
levels of human SOX2 expression (and less REX1) than with F9, OS25, and E14tg2A cells in keeping with the idea that P19 preferentially differentiate toward neuronal lineages (Jones-Ville-neuve et al., 1983) and EGCs have a narrower range of lineage potential than ESCs (Chambers and Smith, 2004). Reprogramming by 19G and 23G EGCs (Tada et al., 1998) was generally less efficient (Figure 1B; Figure S1B) and induction of TERT and REX1, a gene that is downregulated upon mouse epiblast formation (Pelton et al., 2002), was not detected. No obvious correlation between the magnitude of gene expression by parental lines and the subsequent initiation of human genes in heterokaryons was evident (data not shown). These results suggested that although subtle differences in reprogramming reflect the developmental origin of individual lines, all pluripotent stem cells tested here shared the capacity to dominantly reprogram.

**ESCs that Lack PRC2 Activity Fail to Efficiently Reprogram**

To determine whether PRC2 activity is important for reprogramming, we fused human B cells to mouse ESC lines that lacked individual PRC2 components. Eed-deficient and Suz12-deficient ESC lines were unable to induce the expression of most human pluripotency-associated genes upon fusion with human lymphocytes (Figure 1C), in contrast to matched parental and heterozygous controls (Figures S1C–S1F). ESCs that lacked H3K27me2/me3 by virtue of loss of Eed (Figure S1C) or Suz12 (Figure S1E) induced the partial expression of human OCT4 (and either TLE1 or DNMT3B), but not other human pluripotency markers (Figure 1C; Figures S1D and S1F). To determine whether this failure of PRC2-deficient ESCs to efficiently reprogram human B cells reflects a specific requirement for PRC2-mediated repression, we tested the reprogramming capacity of ESCs deficient in either Jarid2 (a noncatalytic component of PRC2) (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009) or other chromatin-remodeling components (Figure 1C, middle and right panels; details in Table S1). ESCs genetically deficient for factors involved in DNA methylation (Dnmt1−/−, Dnmt3a/b−/−), histone 3 lysine 4 methylation (H3K4 −/−), histone 3 lysine 9 methylation (H3K9 −/−), or that lacked Jarid2 (Jarid2−/−), retained the ability to successfully reprogram human B cells at levels comparable with parental wild-type ESCs.

To confirm that Eed-deficient ESCs are unable to reprogram lymphocytes, we performed hybrid analysis. Mouse B cells (Puro+) were fused to either wild-type or Eed-deficient ESCs and hybrids were cultured for 12 days in the presence of puromycin. Reprogrammed colonies, visualized by alkaline phosphatase staining, were abundant in fusions performed with Eed+/− ESCs but very rare in fusions with Eed−/− ESCs (Figure 1D), and these colonies failed to express Oct4 protein (Figure 1E; Figure S1G). Reprogramming by wild-type ESCs was confirmed by the re-expression of GFP in hybrids formed with mouse B cells carrying an Oct4-GFP transgene (Figure S1H). To test whether this defect is ESC specific, we fused wild-type mouse ESCs (Hprt−/−) with either Ezh2flx/flx or Ezh2−/− T cells, and we cultured hybrids for 12 days in HAT-containing media. The number of reprogrammed colonies was similar with wild-type and Ezh2-deficient lymphocytes (26 versus 13, respectively) (Figure S1I), so our data suggest that ESCs selectively require PRC2 activity to induce pluripotent conversion of lymphocytes.

**Conditional Loss of PRC2 or PRC1 Ablates the Dominant Reprogramming Capacity of ESCs**

Because long-term growth of ESCs in the absence PRC2 results in inappropriate upregulation of many important transcription factors and may therefore result in additional (i.e., non-PRC2 targeted) changes, we confirmed these finding with ESC lines that are inducible null for the catalytic component of PRC2, Ezh2. ESCs were derived from conditional Ezh2 knockout mice (Su et al., 2003), Clone Ezh2−1.3 carries a tamoxifen-inducible conditional Ezh2 mutation on both endogenous alleles and is progressively depleted of Ezh2 protein after the addition of tamoxifen (Figure S2A). Loss of Ezh2 resulted in loss of H3K27me3 (and reduced H3K27me2/me1) at 96 hr after tamoxifen addition, without consequent changes in either ESC self-renew or the efficiency of PEG-mediated cell fusion (data not shown). The reprogramming capacity of Ezh2-depleted ESCs was severely impaired and several genes were either not induced (NANOG, REX1, TERT) or only partially induced (CRIPTO, OCT4) (Figure 2A, upper panel; Figure S2B). Long-term removal of Ezh2 (Δ/Δ) also resulted in compromised reprogramming, although low levels of human NANOG, CRIPITO, and OCT4 transcripts were detected, consistent with reports that Ezh1 can compensate for Ezh2 deficiency (Shen et al., 2008). Interestingly, silencing of human lymphoid-specific genes (CD19, CD45, and CD37) was unaffected by Ezh2 depletion (Figure 2A, lower panel), suggesting that ESCs require PRC2 selectively for activating pluripotency-associated genes during reprogramming.

To test whether the PRC1 activity was also required for reprogramming, we examined ESCs that were conditional null for Ring1A/B. Clone ES-ERT2 is homozygous null for the PRC1 component Ring1A and carries two tamoxifen-inducible conditional knockout Ring1B alleles. Accordingly, after tamoxifen addition, ES-ERT2 cells display a global reduction in H2A ubiquitylation and Ring1B expression, while overall levels of PRC2 proteins and trimethylated H3K27 remain largely unaffected (Endoh et al., 2008; Stock et al., 2007). ESCs lacking PRC1 did not successfully reprogram human B cells. As shown in Figure 2B and Figure S2C, tamoxifen pretreatment of ES-ERT2 cells resulted in the failure to induce REX1 and TERT and impaired induction of NANOG and CRIPITO, although silencing of human lymphoid genes was again unaffected.

**Reconstitution of Eed in Eed-Deficient ESCs Restores Reprogramming Activity**

To validate that the reprogramming capacity of ESCs was dependent on PRC1/2, we rescued Eed null ESCs (clone B1.3) by transfecting a 180 kb BAC clone carrying the entire Eed locus. As anticipated, wild-type ESCs and stable transfectants expressed Eed protein isoforms and H3K27me3 as detected by western blotting (clone B1.3BAC; Figure 3A). ESCs lacking Eed (B1.3Neo) or after Eed reconstitution (B1.3BAC) were separately fused to hB-lymphocytes and their reprogramming capacity compared. Restoration of Eed rescued the reprogramming capacity of ESCs (Figure 3B, compare solid and open bars; Figure S3).
Compromised Reprogramming by PRC2 Mutant ESCs Is Not due to a Deficit in Known Reprogramming Factors

To examine whether PRC2-deficient ESCs fail to efficiently reprogram human B cells because they misregulate endogenous factors that have previously shown to be critical for IPS-based reprogramming (Takahashi et al., 2007; Yu et al., 2007), we performed quantitative analyses of Oct4, Nanog, Sox2, c-Myc, Klf4, and Lin28 in different PRC2 mutant ESC lines (Figure 4A). No major differences were detected in expression of these transcripts in ESCs lacking Eed, Suz12, and Ezh2, relative to matching wild-type controls. Nevertheless, as even slight variations in Oct4 are known to impact on ESC self-renewal, differentiation (Niwa et al., 2000), and reprogramming (Pereira et al., 2008), we engineered Eed-deficient ESC lines to modestly overexpress Oct4 by stable transfection of a Flag-tagged mOct4 transgene (B1.3-Oct4, clones 1D3 and 2D1, Figure 4B).

Fusion of Oct4+Eed/C0/C0 ESC lines did not rescue their capacity to reprogram human B cells (Figure 4C), in marked contrast to ESC lines that had been reconstituted with Eed (B1.3BAC1, B1.3BAC2). Thus, defective reprogramming is unlikely to be mediated by insufficient iPSC-inducing factors.

**Dominant Reprogramming to Extraembryonic Lineages Is Not Induced by Eed-Deficient ESCs**

Several developmental regulators are derepressed in ESCs that lack PRC2 activity and H3K27 methylation (Figure 5A; Azuara et al., 2006; Boyer et al., 2006; Lee et al., 2006), including the transcription factors Cdx2 and Hand1, that are required for the specification of trophectoderm (Rossant and Tam, 2009; Scott et al., 2000) and Gata6 and Hnf4, which are required for the development of primitive endoderm (Duncan et al., 1997; Rossant and Tam, 2009). We asked whether the misexpression of these factors by PRC2-deficient ESCs (Jorgensen et al., 2006) might result in dominant reprogramming to extraembryonic fates as stem cells derived from early blastocyst stages are known to be highly related (Santos et al., 2010). To test this possibility, we fused Eed-deficient ESCs with human lymphocytes and analyzed the induction of Cdx2, Hand1, Gata6, and Hnf4 in the resulting heterokaryons. Whereas trophectoderm stem cells (TSCs) and extraembryonic endoderm (XEN) stem cells dominantly reprogrammed human B cells toward different extraembryonic lineages (inducing Cdx2/Hand1 or Gata6/Hnf4 expression,
respectively), PRC2-deficient ESCs failed to induce the correct expression of either set of markers (Figure 5B). These data suggest that PRC2-deficient ESCs do not reprogram human B cells toward an alternative, extraembryonic fate.

Eed-Deficient ESCs Display a Functionally Dominant Defect in Reprogramming

The compromised reprogramming capacity of Eed-deficient ESCs has at least two different interpretations. First, these ESCs could lack factors required for executing dominant conversion. Second, mutant ESCs may express factors that block the efficient reprogramming of differentiated cells. To distinguish between these possibilities, we generated heterokaryons that contained a human B cell nucleus, a wild-type mouse ESC nucleus, and an Eed-deficient ESC nucleus (Figure 5C) by prelabeling wild-type ESCs with DiI and Eed/C0/C0 ESCs with DiD. In brief, labeled cells were mixed in a 1:1 ratio with puromycin-resistant hB lymphocytes, fused with PEG, and cultured for

Figure 3. Reprogramming by Eed-Deficient ESCs Is Restored by Eed Rescue

(A) Eed-deficient B1.3 ESCs were rescued by insertion of a BAC clone that carried the Eed gene and neomycin resistance gene. Whole-cell lysates from these lines (B1.3BAC) and controls (B1.3Neo and WT ESCs) were analyzed by western blotting with antibodies to the Eed protein and anti-trimethylated histone 3 lysine 27 (H3K27me3). Equivalent protein loading is shown by Lamin B and total H4 detection.

(B) B1.3BAC and B1.3Neo ESCs were fused to human B lymphocytes and successful reprogramming was measured by activation of human embryonic genes 3 days after cell fusion. Data were normalized to human GAPDH expression. Error bars indicate the SD of 2–3 independent experiments. See also Figure S3.

Figure 4. PRC2-Deficient ESCs Express Normal Levels of Reprogramming Factors

(A) Expression of mouse Oct4, Nanog, Sox2, c-Myc, Klf4, and Lin28 transcripts by ESC lines deficient in PRC2 components (white); Eed (Eed/C0/C0), Suz12 (Suz12/C0/C0), Ezh2 (Tmx 96; Ezh2/D/D) versus their matched wild-type controls (black bars), analyzed by qRT-PCR. Data were normalized to mouse Gapdh expression and results shown are the mean and SD of three experiments.

(B) Western blotting of whole cell extracts of Eed-deficient ESCs (B1.3) and derived lines stably transfected with Oct4-Flag (clones 1D2, 1D3, 2C5, 2D1), with Oct4 or Flag antibodies. Transgene-derived Flag-Oct4 protein was detected in two clones (1D3 and 2D1) at levels similar to that of endogenous Oct4. Lamin B provides a control for protein loading. Approximate molecular sizes (in kD) are indicated.

(C) B1.3-Oct4 ESC clones were separately fused with human B cells and induction of human ESC-associated genes was monitored by qRT-PCR, 2 days after fusion. Eed-deficient ESCs transduced with an Eed containing BAC (clones B1.3BAC1 and B1.3BAC2) and Eed-deficient ESCs that do not express transgenic Oct4-Flag protein (clones B1.3-1D2 and B1.3-2C5) provided positive and negative controls for this analysis, respectively. Data were normalized to human GAPDH expression and results shown are the mean and SD of three independent experiments.
2 days in the presence of puromycin, and dual-labeled heterokaryons were purified by FACS sorting. Successful reprogramming was judged by the induction of human OCT4, NANOG, CRIPTO, DNMT3B, TLE1, and TERT transcripts via quantitative RT-PCR. Heterokaryons containing Eed-deficient nuclei (red-white histogram, Figure 5C) expressed low levels of each of these genes as compared with fusions that contained either a single (not shown) or a pair of (open histogram, Figure 5C) wild-type nuclei. Mouse Oct4, Nanog, Sox2, c-Myc, Klf4, Lin28 transcript expression was unaffected (Figure S4A) and served as a control in these experiments. These data argue that Eed-deficient ESCs do not simply lack factors required for reprogramming—because provision of a functionally competent (wild-type) nucleus did not restore reprogramming. Rather, Eed-deficient ESCs were functionally dominant over their wild-type counterparts, so PRC2 may be required to repress endogenous expression of genes that interfere with reprogramming. Attempts to define these PRC2 target genes examined 13 genes selected from transcriptome analyses of ESCs lacking Eed, Suz12, or Ezh2 (Figures S4B and S4C), but despite achieving efficient knockdown of candidate genes alone, or in combination, we were unable to fully restore reprogramming potential in Eed-deficient ESCs (Figure S4D).

Eed-Deficient ESCs Fail to Induce HP1α Redistribution and H3S10 Phosphorylation in Reprogrammed Lymphocytes

Previous studies have shown that reprogramming can result in profound changes in the nuclear volume and chromatin organization of differentiated nuclei (Pereira et al., 2008; Terranova et al., 2006). We examined whether PRC2-deficient ESCs induce similar changes in heterokaryons formed with human B cells. Mouse (ESC-derived) Oct4 protein was translocated into human nuclei with similar kinetics irrespective of whether wild-type or Eed-deficient ESCs were used as fusion partners (Figure 6A, arrow denotes human nuclei, and Table S2). We observed a rapid increase in nuclear size and changed architecture in human lymphocytes upon heterokaryon formation, to resemble human ESCs (Figures 6A and 6B). Eed-deficient ESCs induced similar changes in the volume (Figure 6B) and redistribution of nucleoli in lymphocyte-derived nuclei (Figure 6C; Figures S5A–S5C), as induced by wild-type controls. In contrast, Eed-deficient ESCs did not induce a redistribution of HP1α (from punctate to diffuse) and increased H3S10 phosphorylation, which are hallmark features of human ESCs and lymphocytes reprogrammed with wild-type ESCs (Figures 6D and 6E). Similar chromatin reorganization has recently been reported to occur upon reprogramming via nuclear transfer (Murata et al., 2010). HP1α recruitment is known to be modulated by Aurora B kinase-mediated phosphorylation of H3S10 (Fischle et al., 2003) and by JAK2 signaling (Dawson et al., 2009). Our data suggest a novel mechanistic link between these chromatin events and Eed-dependent reprogramming.

DISCUSSION

Here we show that ESCs, EGCS, and ECCs share the ability to efficiently reprogram human lymphocytes in heterokaryons and that this property is dependent on Polycomb repressor activity. The deletion of individual PRC1 or PRC2 members (Eed, Suz12, Ezh2, and Ring1A/B) that repress the expression of developmental regulators in ESCs abolishes the capacity of...
ESCs to induce reprogramming. Eed-deficient mouse ESCs are themselves self-renewing, pluripotent, and can contribute to the three germ layers in vivo and in vitro (Chamberlain et al., 2008), so our data show that pluripotency and reprogramming function can be dissociated.

We demonstrate that Polycomb-mediated gene repression is critical for ESCs to actively convert differentiated cells to pluripotency. Surprisingly, defective reprogramming by PRC2-null ESCs was not restored in heterokaryons supplemented with wild-type ESC nuclei, showing that the reprogramming defect is functionally dominant. A requirement for PRC2 in reprogramming is consistent with previous studies in cloned mouse embryos (Zhang et al., 2009) and iPSCs (Mikkelsen et al., 2008), showing that failed or partially reprogrammed cells lack PRC2-dependent H3K27me3. Furthermore, recent iPSC studies with mouse lymphocytes and fibroblasts have suggested that inappropriate expression of some lineage-specific genes can impede reprogramming. For example, successful reprogramming of mouse B cells requires silencing of the B cell-specific factor Pax5 (Hanna et al., 2008; Xie et al., 2004). Reactivation or incomplete repression of lineage-specifying factors is also postulated to interfere with the establishment of core pluripotency networks because transient silencing of interfering factors significantly improves reprogramming efficiency (Mikkelsen et al., 2008). In this respect, reports that ESCs contain at least two types of bivalent domains (targeted by PRC2 only or by both PRC1 and PRC2) may be informative because PRC1-positive domains are enriched for genes encoding transcription factors that specify developmental fate (Ku et al., 2008). It is conceivable that repression of these targets in ESCs is particularly critical for reprogramming.

Interestingly, we show here that Jarid2-null ESCs are fully competent in reprogramming assays. Although these ESCs display variable levels of H3K27me3 (Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009), they do not show a genome-wide upregulation of PRC2 target genes (characteristic of Eed, Suz12, Ezh2, or Ring1A/B-depleted ESCs) and there is evidence that Jarid2 is not required for PRC2-mediated repression (Landeira et al., 2010). Taken together, our studies therefore suggest that pluripotent cells require active PcG-mediated repression of lineage-specifying factors to efficiently convey multilineage potential during reprogramming. Whether additional properties of PRC2, including the recruitment of RBP2 (H3K4 demethylase), to target genes in ESCs (Pasini et al., 2008) is important for reprogramming, awaits investigation.

In Drosophila melanogaster, downregulation of several PcG proteins occurs in regenerating leg disc cells and transdifferentiation of leg-to-wing is enhanced in PcG heterozygous mutant ESCs was compared with human ESCs (hESC, H1 cell line). Nuclear volume was calculated as described in the Experimental Procedures, the volume of mouse nuclei before and after heterokaryon formation provides a control. Data shown are the mean and SD of 50–75 determinations per sample. (C–E) The number of nucleoli (C), distribution of HP1α (D), and presence of H3S10ph (and H3K9me3) (E) within human B nuclei before (hB) and 24 hr after fusion with Eed-deficient or wild-type mouse ESCs was assessed by immunofluorescence via B23 antibody (red, nucleoli arrowed), anti-HP1α (green), and anti-H3S10ph/H3K9me3 (red). Human nuclei were identified via DAPI staining (blue) and are marked by a dashed line. Human ESCs (cell line H1) are shown as controls. Scale bars represent 10 μm. See also Figure S5 and Table S2.
flies (Lee et al., 2005). Downregulation of PcG proteins is also observed in regenerating skin in the mouse (Shaw and Martin, 2009), suggesting that reduced PcG protein levels may encourage lineage plasticity and enhance transdifferentiation (Lee et al., 2005; McClure et al., 2008). In view of these findings, it is possible that Polycomb proteins have different roles in undifferentiated versus somatic cells. Alternatively, the high “transcriptional noise” that results from global PcG-mediated gene derepression in ESCs (Chi and Bernstein, 2009) could be contributing to the poor reprogramming capacity of PRC2-null ESCs. However, this is unlikely to be the sole cause of the reprogramming defect because ESCs that lack Dicer, a factor that is critical for the global regulation of small RNAs (Cobb et al., 2005; Murchison et al., 2005), reprogram human B cells efficiently. Likewise, although recent studies have shown that the induction of senescence is a critical roadblock for successful reprogramming via iP (reviewed in Krizhanovsky and Lowe, 2009), we show that levels of p53, p21, and p16 are not appreciably changed when comparing wild-type or Eed-deficient ESCs or heterokaryons derived from them (Figure SSD) and that overexpression of p16 (a PRC2-target) by ESCs does not inhibit reprogramming (Figures SSE and S5F). This suggests that senescence induction is unlikely to be the cause of PRC2-null reprogramming defects.

In summary, here we show that Polycomb-mediated repression is required by mouse ESC lines to direct the conversion of differentiated cells toward pluripotency. ESCs lacking Eed induced rapid changes in the volume and architecture of differentiated human nuclei, but failed to direct the redistribution of HP1α, increased phosphorylation of H3S10, and to induce pluripotent gene expression that underlies successful reprogramming. Our results support the view that reprogramming is multistep and define Polycomb-dependent as well as Polycomb-independent events associated with the acquisition of a lineage-plastic state. Collectively, these studies show that the pluripotent and reprogramming capacities of ESCs can be mechanistically “uncoupled” and provided compelling new evidence that Polycomb-mediated gene repression is critical for establishing pluripotency.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

EBV-transformed hB clones were maintained in RPMI supplemented with 10% fetal calf serum (FCS, PAA Laboratories GmbH, Pasching, Austria), 2 mM L-glutamine, and antibiotics (10 μg/ml penicillin and streptomycin). Abelson transformed mouse B cell lines were grown in RPMI supplemented with 20% FCS, nonessential amino acids, L-glutamine, 50 μg/ml 2-mercaptoethanol, antibiotics, and IL-7 (5 ng/ml; R&D systems, Minneapolis, MN). Mouse ESC, EGC, and ECC lines were maintained on either irradiated mouse embryonic fibroblast (MEFs) feeder layers or 0.1% gelatin-coated surfaces with KO-DMEM medium, 10% FCS, 5% knockout serum replacement (Invitrogen), nonessential amino acids, L-glutamine, 2-mercaptoethanol, antibiotics, and 1000 U/ml of leukemia inhibitory factor (ESGRO-LIF, Chemicon/Millipore). 4-hydroxy-tamoxifen (800 nM, Sigma) or puromycin (1 μg/ml, Sigma) was added to the cultures where indicated. Human ESC line H1 was cultured in medium conditioned by mitotically inactivated MEFs supplemented with 8 ng/ml bFGF (Peprotech, London, UK) on matrigel-coated plates, as previously described (Xu et al., 2001). Cells were routinely passaged at a 1:3 dilution by treatment with 200 U/ml collagenase IV (Invitrogen).

**Generation of Transgenic Cell Lines**

Eed-deficient ESCs (B1.3 clone) were transplanted with a bacterial artificial chromosome (BAC, RP23-370F10), containing approximately 185 kb (nucleotides 97076839-97260535) of the forward DNA strand of mouse chromosome 7. In this clone, the chloramphenicol acetyltransferase (CAT) gene that was replaced with a neomycin resistance cassette from plasmid pL452 (NCI, Frederick, MD). B1.3-Oct4 ESC lines were generated by stable transfection of Flag-tagged mouse Oct4 in Eed-deficient ESCs (B1.3 clone). Mouse Oct4 cDNA was cloned in the pDFLAG-cDNAIII vector (Invitrogen). The cDNA, including two flag sequences at the 5’ end, was excised and subcloned into a suitable vector for expression in ESCs (pCBa), with expression driven by the chicken β-actin promoter (Pereira et al., 2008). G418 selection (400 μg/ml; Invitrogen) was applied 48 hr after transfection and resistant clones were screened by western blot. Human and mouse B cells were transduced with a replication incompetent retroviral vector carrying a puromycin resistance gene and selected (1 μg/ml puromycin, Sigma) 48 hr after transfection.

**Experimental Heterokaryons**

Heterokaryons were generated by fusing mouse ESCs and human B lymphocytes with 50% polyethylene glycol (pH 7.4) (PEG 1500; Roche Diagnostics, Mannheim, Germany) as described previously (Pereira and Fisher, 2009). Mouse ESCs and hB lymphocytes were labeled with Vibrant 1,1'-dioctadecyl-3, 3', 3, 3'-tetramethylindocarbocyanine (DiD) and 1,1'-dioctadecyl-3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI) cell labeling solutions (Molecular Probes, Eugene, OR), respectively. Cells were resuspended at 1 × 10^6 cells/ml in DMEM and labeled with 5 μl/ml of dye at 37°C, 15 min. ESCs and hB were then mixed in a 1:1 ratio, washed, and fused with PEG (50%, at 37°C over 1 min before dilution). Cells were washed and cultured in ES media at 0.5 × 10^5 cells/cm². To eliminate unfused human B cells, Ouabain (10 μM; Sigma) was added to the medium 4 hr after cell fusion. Proliferating ESCs were eliminated by the addition of 10 μM Ara-C (Cytosine β-D arabinofuranoside; Sigma) 4-6 hr after fusion and then removed after 16 hr. Trikaryons were generated by labeling Eed-deficient ESCs and wild-type ESCs with DiD and Dil, respectively, and mixing with Puro<sub>0</sub> human B cells in 1:1:1 ratio before cell fusion. Puromycin and Ouabain were added 6 hr after cell fusion to select for heterokaryons. After 2 days of culture, DiD<sub>Dil</sub> cells were sorted and analyzed. For FACS analysis, a FACScalibur (BD Biosciences) with CellQuest software was used. FACS purification was performed with a FACSAria cell sorter.

**Quantitative RT-PCR Analysis**

RNA extraction was performed with RNA-BEE reagent (Tel-Test Inc., Friendswood, TX) and residual DNA was eliminated with the DNA-free kit (Ambion, Austin, TX). 3 μg of total RNA was then reverse transcribed with Superscript First-Strand Synthesis system (QIAGEN) with oligo (dT)12-18 (Invitrogen). cDNAs of interest were then quantified with real-time qPCR amplification. Real-time PCR analysis was carried out on a Opticon DNA engine with Opticon Monitor software (MJ Research Inc., Waltham, MA), running the following program: 95°C for 15 min, then 40 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 30 s, followed by plate-read. PCR reactions included 2× Sybr-Green PCR Mastermix (QIAGEN), 300 nM primers, and 2 μl of template in a 35 μl reaction volume. Assays were performed in triplicate and data normalized according to GAPDH expression. Primers were designed with Primer Express software (Applied Biosystems) and pretested for the selective detection of human transcripts (not mouse). Analysis of qPCR data was performed with the Opticon Monitor 3 software and the relative abundance of sequences was calculated with the ΔC(T) method (Pereira and Fisher, 2009).

**Immunofluorescence and Western Blot Analysis**

Immunofluorescence was performed as previously described (Terranova et al., 2006) with DAPI staining to distinguish mouse and human nuclei. Individual cells were delineated by F-actin staining (Phalloidin; A12380, Molecular Probes). Mouse monoclonal anti-Oct4 (611202; BD Biosciences, San Jose, CA), mouse monoclonal anti-HP1α antibody (MAB3584, Millipore, Lake Placid, NY), rabbit polyclonal anti-histone H3S10 phosphorylation (and H3K9me3) antibody (ab5819, Abcam, Cambridge, UK), and rabbit polyclonal anti-B23 (sc-5564; Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used at 1:100 dilution. Secondary antibodies conjugated with fluorochromes were
purchased from Molecular Probes and used at 1:400 dilution. Samples were analyzed on a Leica TCS SP5 confocal microscope and processed with Leica software and Adobe Photoshop. To estimate nuclear volumes, z-stacks (0.5 μm distance) spanning individual nuclei were acquired. Velocity image processing software was used for 3D reconstructions and to quantify the nuclear volume. Nucleoli were discriminated on the basis of B23 labeling and counted in reconstructed projections. Western blot analysis was performed as previously described (Azuara et al., 2006) with goat polyclonal anti-Oct3/4 (sc-8628; Santa Cruz Biotechnology), mouse monoclonal anti-Flag (F3165, Sigma), mouse monoclonal anti-Eed (kindly provided by A. Otte, University of Amsterdam, The Netherlands), rabbit polyclonal anti-Suz12 (CS-029-050; Diagenode SA, Liège, Belgium), rabbit polyclonal anti-Ezh2 (CS-039-050; Diagenode), rabbit polyclonal anti-histone 3 lysine 27 monomethylation (07-448; Millipore), rabbit polyclonal anti-histone 3 lysine 27 dimethylation (07-452; Millipore), rabbit polyclonal anti-histone 4 lysine 20 trimethylation (07-483; Millipore). As loading controls, blots were incubated with anti-Lamin B goat polyclonal (sc-6216; Santa Cruz Biotechnology), rabbit polyclonal anti-histone 4 C terminus (ab1791; Abcam), and/or mouse monoclonal anti-histone 4 C terminus antibody (ab31827, Abcam). Each lane contained 20 μg total protein. For alkaline phosphatase assays, hybrid colonies 12 days after cell fusion were stained with alkaline phosphatase assay kit.

Animal Procedures
Animals were housed and handled in accordance with the guidelines of the Imperial College subcommittee for animal research and the regulations set out by the British home office.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and two tables and can be found with this article online at doi:10.1016/j.stem.2010.04.013.

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