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Bmi1 facilitates primitive endoderm formation by stabilizing Gata6 during early mouse development

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The transcription factors Nanog and Gata6 are critical to specify the epiblast versus primitive endoderm (PrE) lineages. However, little is known about the mechanisms that regulate the protein stability and activity of these factors in the developing embryo. Here we uncover an early developmental function for the Polycomb group member Bmi1 in supporting PrE lineage formation through Gata6 protein stabilization. We show that Bmi1 is enriched in the extraembryonic (endoderm [XEN] and trophectodermal stem [TS]) compartment and repressed by Nanog in pluripotent embryonic stem (ES) cells. In vivo, Bmi1 overlaps with the nascent Gata6 and Nanog protein from the eight-cell stage onward before it preferentially cosegregates with Gata6 in PrE progenitors. Mechanistically, we demonstrate that Bmi1 interacts with Gata6 in a Ring finger-dependent manner to confer protection against Gata6 ubiquitination and proteasomal degradation. A direct role for Bmi1 in cell fate allocation is established by loss-of-function experiments in chimeric embryoid bodies. We thus propose a novel regulatory pathway by which Bmi1 action on Gata6 stability could alter the balance between Gata6 and Nanog protein levels to introduce a bias toward a PrE identity in a cell-autonomous manner.

[Keywords: Bmi1; Nanog; Gata6; cell fate; early mouse embryo; stem cells]

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During early mouse development, the transition from morula to blastocyst around embryonic day 3.5 [E3.5] marks the onset of differentiation into the inner cell mass (ICM) and trophectoderm [TE] [Jedrusik et al. 2008; Rossant 2008]. At this stage, the ICM is heterogeneous and composed of pluripotent epiblast and extraembryonic primitive endoderm [PrE] progenitors, as revealed by a “salt-and-pepper” distribution of the key epiblast [Nanog] and PrE [Gata6] markers at E3.75 [Koutsourakis et al. 1999; Chambers et al. 2003, 2007; Mitsui et al. 2003; Chazaud et al. 2006; Plusa et al. 2008; Silva et al. 2009]. Compartmentalization of two distinct expression domains for Nanog and Gata6 is then achieved by cell sorting and apoptosis and strictly delineates the newly formed epiblast and PrE lineages [E4.5] [Plusa et al. 2008; Meilhac et al. 2009]. These confined expression patterns are stably maintained in blastocyst-derived embryonic [ES] and extraembryonic endoderm [XEN] stem cells—two cell populations that retain the properties of the epiblast and PrE, respectively [Evans and Kaufman 1981; Martin 1981; Kunath et al. 2005; Rossant 2008]. Prior to blastocyst formation, however, Nanog and Gata6 are seen to overlap in most cells from the eight-cell up to the morula stage. This early expression pattern is also characterized by highly dynamic and variable protein levels among blastomeres [Dietrich and Hiiragi 2007; Plusa et al. 2008]. How Nanog and Gata6 segregation is triggered and how their expression is stabilized in the epiblast and PrE progenitors remain largely unknown. Epigenetic factors have emerged as key regulators of cell fate decisions during early development [Torres-Padilla et al. 2007]. Among them, the Polycomb-repressive complexes PRC1 [Ring1A, Ring1B, Bmi1, and MeI18] and PRC2
that Nanog negatively controls in RCN control cells, uniquely identified as being expressed inversely to (Fig. 1A). Among the PRC members analyzed, 1446 GENES RT–PCR (qRT–PCR) analysis was performed in control sion levels (Chambers et al. 2003, 2007). Quantitative Nanog and PRC members, we took advantage of geneti-

Results

Bmi1 is a direct target of Nanog in pluripotent stem cells

To investigate a possible transcriptional link between Nanog and PRC members, we took advantage of genetically modified ES cell lines with distinct Nanog expression levels (Chambers et al. 2003, 2007). Quantitative RT–PCR [qRT–PCR] analysis was performed in control RCN[t], Nanog\(^{-}\) RCN\(^{+}\)[t], and Nanog-overexpressing EF4 ES cells maintained in self-renewing conditions (Fig. 1A). Among the PRC members analyzed, Bmi1 was uniquely identified as being expressed inversely to Nanog (Fig. 1A; data not shown). While detected at low levels in control cells, Bmi1 transcript was markedly up-regulated in RCN\(^{+}\)[t] cells and repressed in EF4 cells, suggesting that Nanog negatively controls Bmi1 expression in ES cells.

Eight putative Nanog-binding sites (BS) were identified across the Bmi1 locus based on the Nanog consensus se-

Bmi1 expression is mosaic among undifferentiated ES cells

Nanog is heterogeneously expressed within Oct3/4-pos-

Bmi1 is an early marker of extraembryonic endoderm cell commitment

Remarkably, however, Bmi1 was not up-regulated in all Nanog\(^{-}\) RCN\(^{+}\)[t] ES cells [\(n = 61/265\)] (data not shown), but instead was selectively detected in a subset of cells that coimmunostained for Gata6 [\(n = 58/61\), \(P < 0.01\), Wilcoxon test] (Fig. 1G). This confined expression pattern was confirmed in tamoxifen-inducible Nanog\(^{-}\) RCN\(^{+}\)HB ES cells, where Bmi1 and Gata6 were promptly and simultaneously induced upon Nanog depletion, followed by Gata4 and Dab2—two late markers of the PrE lineage [Supplemen-

Colocalization of Bmi1, Gata6, and Gata4 protein in
Nanog-depleted cells was verified by immunostaining (Supplemental Fig. S2B), further pointing to a close association between Bmi1 up-regulation and the acquisition of an extraembryonic cell identity. Consistently, we found that Bmi1 was highly expressed in XEN cells as well as in TS cells—two stem cell populations derived from the PrE and TE lineages that lack Nanog, in contrast to ES cells [Supplemental Fig. S3]. Taken together, these data demonstrate that Bmi1 is rapidly up-regulated in PrE-like cells upon Nanog depletion and suggest a role for Bmi1 in extraembryonic lineages.

Bmi1 is dynamically expressed alongside Nanog and Gata6 in vivo

To explore this function, we investigated Bmi1 expression profile alongside Nanog and Gata6 in the early developing embryo. Bmi1 is a maternally inherited factor that is highly expressed in cleavage stage embryos (Puschendorf et al. 2008). Consistently, Bmi1 protein was homogeneously detected in all blastomeres of four-cell stage embryos (Fig. 2A). From eight-cell up to the early morula stage (20 cells, E3.0), Bmi1 overlapped in most cells with the nascent...
Nanog and Gata6 protein (Fig. 2B; data not shown). This pattern was dynamically altered around cavitation (E3.25), when cell heterogeneity arose among blastomeres. In particular, we observed the emergence of a subpopulation of cells (14.6%) that coexpress Bmi1 and Gata6 but not Nanog (29- to 43-cell embryos; \( P < 0.0001, \) Wilcoxon test) (Fig. 2B, C). Bmi1 protein staining became noticeably weaker in the developing blastocyst (E3.5–E4.5) (data not shown) despite Bmi1 transcript being detected throughout (see below), possibly reflecting a change in Bmi1 post-translational modifications (Voncken et al. 2005). These data reveal a dynamic protein expression pattern for Bmi1 and confirm its close association with Gata6 in vivo.

**Bmi1 preferentially cosegregates with Gata6 in PrE progenitors in the developing blastocyst**

Single-cell PCR analysis was exploited to dissect RNA segregation events and further examine the relationship between Bmi1 and Gata6 expression during epiblast/PrE lineage specification. ICMs were isolated from blastocysts by immunosurgery and dissociated into single blastomeres. Embryos analyzed in these experiments were staged based on the average cell number scored among littermates. Bmi1 expression was then examined by qRT–PCR in each individual blastomere, alongside Gapdh, Gata6, Gata4, Nanog, and Ring1B (Fig. 3). In the early blastocyst (49- to 50-cell stage; E3.25), Gata6 and Nanog were expressed in most, if not all, ICM cells, with little variability between blastomeres (Fig. 3A, top panel). Mutually exclusive expression of Gata6 and Nanog emerged at the 75- to 91-cell stage (E3.5) (Fig. 3A, middle panel) and became more prominent at the 163- to 227-cell stage (E4.5) (Fig. 3A, bottom panel) \( P < 0.05 \) at E3.5 and \( P < 0.01 \) at E4.5, Spearman test) (Fig. 3B), as previously reported (Kurimoto et al. 2006; Guo et al. 2010). At these developmental stages, Gata6 expression was correlated with Gata4 \( P < 0.05 \) at E3.25 and \( P < 0.01 \) at E3.5 and E4.5, Spearman test), denoting PrE lineage emergence and establishment within the ICM. Bmi1 expression was similarly detected in almost all ICM cells of the early blastocyst (E3.25), and its expression was gradually restricted to Gata6-positive/Nanog-negative, presumptive PrE cells (Fig. 3A). Remarkably, at E3.5 and E4.5, Bmi1 expression exhibited a significant correlation with Gata6 \( P < 0.01, \) Spearman test) (Fig. 3B). In contrast, the expression of another PRC1 component, Ring1B, did not correlate with Gata6 in the late blastocyst (E4.5). These results establish that Bmi1 preferentially cosegregates with Gata6 at the transcript level in nascent PrE progenitors during blastocyst development.

**Bmi1 is physically associated with Gata6 in extraembryonic XEN cells**

The observed association between Bmi1 and Gata6 prompted us to investigate a possible transcriptional cross-regulation between the two factors. Ectopically expressing Bmi1 in ES cells did not, however, impact on Gata6 expression. Conversely, Gata6 overexpression, carried out as previously described (Fujikura et al. 2002; Shimosato et al. 2007), only led to a slight increase in Bmi1 mRNA levels (data not shown), suggesting that no direct transcriptional cross-regulation operates between Bmi1 and Gata6. To test whether Bmi1 and Gata6 could be part of a same protein complex, Cos-7 cells were cotransfected with Gata6 and Bmi1, and cell lysates were subjected...
to anti-Gata6 immunoprecipitation. Bmi1 was strongly coimmunoprecipitated with Gata6, as revealed by anti-Bmi1 immunoblotting, thus demonstrating that Bmi1 and Gata6 are indeed physically associated (Supplemental Fig. S4A). Importantly, we confirmed that endogenous Bmi1 and Gata6 protein can also be successfully coimmunoprecipitated with anti-Bmi1 (Fig. 4A, top panel) or anti-Gata6 (Fig. 4A, bottom panel) antibodies in XEN cells and furthermore found this association to be DNA-independent, as indicated by Benzonase treatment of the protein extracts (data not shown). In contrast, the Bmi1 paralog Mel18 failed to interact with Gata6 in parallel experiments, highlighting the specificity of Bmi1/Gata6 association (Supplemental Fig. S4B). As expected, Bmi1 could readily interact with other core PRC1 members expressed in XEN cells, including Ring1B and Chx8 (Supplemental Fig. S4C, left panels). However, Gata6 was not detected in the same complex (Supplemental Fig. S4C, right panels), further suggesting that Bmi1/Gata6 interaction might not take place in a canonical PRC1 complex.

Bmi1 stabilizes Gata6 protein levels and enhances its transcriptional activity

Critically, we next demonstrated that Bmi1/Gata6 association directly impacts on Gata6 stability and degradation in PrE derivatives. In this analysis, XEN cells were stably transfected with two shRNA vectors targeting Bmi1 [Supplemental Fig. S4D,E]. Gata6 protein levels were assessed in control [XEN_Control] and Bmi1 knockdown [XEN_Bmi1KD] XEN cells cultured for 0, 1, 2, and 4 h in the presence of cycloheximide (CHX). Without protein syn-
thesis, Gata6 protein levels gradually decreased in control cells, with little or no effect on Gata6 transcription [Fig. 4B; data not shown]. This trend was dramatically accelerated in the absence of Bmi1, suggesting that the Bmi1/Gata6 interaction protects Gata6 from degradation. In contrast, no such difference was detected in Gata4 protein decay (Fig. 4B), a factor that was not found to interact with Bmi1 by coimmunoprecipitation in XEN cells (data not shown). The addition of Gata6 and Gata4 protein was quantified using ImageJ software and normalized to Actin as shown in graphs. Error bars represent the SD of four biological replicates. Similar results were obtained using two independent shRNA vectors targeting Bmi1 in XEN cells [data not shown]. (C,D) Same experiment as in B in the presence of the proteasome inhibitor MG132 (1 μM) and E1 ubiquitin ligase inhibitor PYR41 (1 μM), respectively. (E) Gata6 and Gata4 ubiquitination levels in XENControl and XENBmi1KD cells. XENControl and XENBmi1KD cells were cultured for 7 h in the presence of MG132. Protein extracts were subjected to immunoprecipitation with anti-Gata6 (left panel) or anti-Gata4 (right panel) antibodies, and the levels of ubiquitination [Ub] were revealed by immunoblotting with anti-ubiquitin antibodies. Anti-Gata6 and anti-Gata4 immunoblots confirm the uniform recovery of Gata or Gata4 protein by immunoprecipitation across cell samples. (F) Gata6 reporter assay in XENControl and XENBmi1KD cells. Both cell populations were transiently transfected with Gata6-dependent Hnf4 reporter, and luciferase activity was assessed 48 h post-transfection. Data were normalized to Renilla. Error bars represent the SD of three biological replicates. The F-value was calculated using the Student’s t-test.

Figure 4. Bmi1 interacts with Gata6 and regulates its stability and activity in XEN cells. (A) Coimmunoprecipitation of Gata6 and Bmi1 proteins in XEN cells. Protein extracts were immunoprecipitated (IP) with control anti-IgG, anti-Bmi1 (top panel), or anti-Gata6 (bottom panel) antibodies and subjected to immunoblotting (IB) with anti-Gata6 and anti-Bmi1 antibodies, respectively. Three independent experiments were performed with similar results. (B) Gata6 and Gata4 stability assay in control (XENControl) and Bmi1 knockdown (XENBmi1KD) XEN cells. Bmi1, Gata6, and Gata4 protein levels were assessed by immunoblotting in XENControl and XENBmi1KD cells cultured with CHX for the indicated times. The amount of Gata6 and Gata4 protein was quantified using ImageJ software and normalized to Actin as shown in graphs. Error bars represent the SD of four biological replicates. Similar results were obtained using two independent shRNA vectors targeting Bmi1 in XEN cells [data not shown]. (C,D) Same experiment as in B in the presence of the proteasome inhibitor MG132 (1 μM) and E1 ubiquitin ligase inhibitor PYR41 (1 μM), respectively. (E) Gata6 and Gata4 ubiquitination levels in XENControl and XENBmi1KD cells. XENControl and XENBmi1KD cells were cultured for 7 h in the presence of MG132. Protein extracts were subjected to immunoprecipitation with anti-Gata6 (left panel) or anti-Gata4 (right panel) antibodies, and the levels of ubiquitination [Ub] were revealed by immunoblotting with anti-ubiquitin antibodies. Anti-Gata6 and anti-Gata4 immunoblots confirm the uniform recovery of Gata or Gata4 protein by immunoprecipitation across cell samples. (F) Gata6 reporter assay in XENControl and XENBmi1KD cells. Both cell populations were transiently transfected with Gata6-dependent Hnf4 reporter, and luciferase activity was assessed 48 h post-transfection. Data were normalized to Renilla. Error bars represent the SD of three biological replicates. The F-value was calculated using the Student’s t-test.
The C-terminal domain of Gata6 mediates its interaction with Bmi1 and ubiquitin-dependent degradation

In an attempt to establish which domain of Gata6 was critical for ubiquitin-dependent proteasome degradation, we next generated truncated Gata6 mutants lacking the C-terminal domain alone (∆CT) or including its zinc finger region (∆CTZF), where putative lysine ubiquitination sites are preferentially mapped [highlighted by asterisks in Fig. 5A]. Flag-tagged wild-type, ∆CT, or ∆CTZF Gata6 constructs were transfected into Cos-7 cells, and Gata6 protein decay and ubiquitination status were assessed as previously performed [Fig. 4]. Both deletions resulted in an increased Gata6 protein stability [Fig. 5B] and a reduced sensitivity to ubiquitination [Fig. 5C], identifying the C-terminal domain as being critical for Gata6 degradation via ubiquitination. Moreover, this domain was found to be equally important for Gata6 interaction with Bmi1, as demonstrated by coimmunoprecipitation assays [Fig. 5D]. Conversely, using a similar mutagenesis approach for Bmi1, we validated that Bmi1/Gata6 interaction is mediated via the Bmi1 Ring domain [Fig. 5E,F; Hosokawa et al. 2006] and furthermore demonstrated that an intact Bmi1 is required for enhancing Gata6 transcriptional activity [Fig. 5G]. Taken together, these results reiterate the functional importance of Bmi1/Gata6 interaction and further suggest how Bmi1 binding can confer protection against Gata6 ubiquitination and degradation, most likely by masking lysine residues in the Gata6 C-terminal domain from ubiquitin-conjugating enzymes.

Bmi1 promotes the emergence of PrE-like cells upon EB formation

The experiments described thus far show that Bmi1 cosegregates with Gata6 in PrE derivatives, where it interacts with and stabilizes Gata6 protein levels. This suggests an early developmental function for Bmi1 in regulating extraembryonic endoderm lineage formation that we investigated in ES-derived EBs. In this system, ES cells are induced to form aggregates in hanging drops, and differentiation is allowed to proceed over 5 d. During this time window, PrE- and epiblast-like cells first emerge in a salt-and-pepper manner [Rula et al. 2007]. They then segregate with the formation of an organized, outer PrE-like layer that contains for Bmi1, Gata6, and Gata4, as visualized by immunofluorescence on day 5 EBs [Fig. 6A]. As previously reported, Nanog-overexpressing EBs were not capable of forming a proper outer layer [Chambers et al. 2003; Niakan et al. 2010], and this phenotype was associated with a loss of Bmi1 induction alongside Gata6 and Gata4 [data not shown].

To directly assess the effect of Bmi1 depletion on this process, Bmi1 knockdown ES cells were established by stable transfection with different Bmi1 shRNA vectors. These cells showed no increased incidence of differentiation when grown in self-renewing conditions [data not shown]. Importantly, Bmi1 knockdown was efficiently maintained upon EB formation, as assessed at the mRNA and protein levels [Fig. 6B, data not shown]. Here, we observed a pronounced defect on PrE-like cell differentiation in the absence of Bmi1. While Oct3/4 and Nanog were down-regulated in both control and Bmi1 knockdown EBs, the induction of the PrE markers Gata6 and Sox17 was impaired [Fig. 6B], with no proper outer layer organization [Fig. 6C]. This phenotype most closely resembles that of Gata6−/− EBs but differs from that of Sox17−/− EBs, in accord with Bmi1 action on Gata6 stability [Fig. 4; Koutsourakis et al. 1999; Fujikura et al. 2002; Niakan et al. 2010, Artus et al. 2011]. Moreover, and as shown in Figure 6C, only very few PrE-like, Bmi1-depleted cells emerged, and these cells expressed Gata4 protein alongside Gata6, further highlighting that the emergence of PrE-like progenitors, rather than their maturation, might be directly affected by Bmi1 depletion.

Bmi1 biases cell fate toward a PrE identity in a cell-autonomous manner

To assess whether the observed defect was cell-autonomous, we repeated these experiments and mixed control [ESControl] cells with Bmi1 knockdown [ESBmi1KD] ES cells to form chimeric EBs. Cells were first labeled by stable Gfp transfection followed by FACS sorting, and the GFP-labeled ESControl or ESBmi1KD cells aggregated with unlabeled ESControl cells upon EB formation [Fig. 6D]. The fate of labeled cells was assessed based on their position within the EB structure [inner/outer], and the emergence of PrE-like cells was monitored by looking at Gata6 expression [Fig. 6E, Supplemental Fig. S5]. Remarkably, GFP-ESΔSmi1KD cells were preferentially located within the inner part of EBs [P < 0.05, Student’s t-test] [Supplemental Fig. S5A], in contrast to GFP-ESControl cells, which appeared to be evenly distributed. This observation was consistent with a lower frequency of Gata6-positive, GFP-ESΔSmi1KD cells detected in day 5 EBs as compared with controls [P < 0.005, Student’s t-test] [Supplemental Fig. S5B]. Taken together, these results demonstrate a direct role for Bmi1 in cell allocation between a PrE- and an epiblast-like fate upon EB formation.

Discussion

In this study, we identify a novel role for the Polycomb group member Bmi1 in regulating cell fate choice between extraembryonic endoderm and pluripotent lineages [Fig. 7]. We show that Bmi1 is readily detected in vivo in all blastomeres of cleavage stage embryos and overlaps with Nanog and Gata6 from the eight-cell stage onward. This pattern dynamically changes upon blastocyst formation, when Bmi1 becomes mosaic among ICM cells, preferentially cosegregating with Gata6 in nascent PrE progenitors. Critically, we demonstrate that Bmi1 controls Gata6 protein stability and its resultant activity by conferring protection against ubiquitination and proteasome-dependent degradation, as confirmed by Bmi1 knockdown in XEN cells. This effect is thought to be mediated through Bmi1/Gata6 interaction via the Bmi1 Ring domain, which could, in turn, alter Gata6 protein conformation and/or mask lysine residues in the Gata6 C-terminal domain from...
Figure 5. The C-terminal domain of Gata6 is critical to trigger its ubiquitin-dependent degradation and interacts with the Bmi1 Ring domain. (A) Scheme depicting Flag-tagged Gata6 wild-type and truncated forms. Wild-type Gata6 (Wt) mouse cDNA was flagged, and mutants lacking either the C-terminal domain (ΔCT) alone or including its zinc finger region (ΔCTZF) were generated by PCR-based mutagenesis. Asterisks highlight the location of putative ubiquitination lysine sites in Gata6 protein. (B) Comparative protein stability assay using wild-type [Wt] and mutant Gata6 forms. Cos-7 cells were transiently transfected with wild-type, ΔCT, or ΔCTZF Gata6. Flagged Gata6 protein levels were assessed by immunoblotting following CHX treatment for the indicated times. The amount of Gata6 protein was quantified using ImageJ software and normalized to Actin as shown in graphs. Error bars represent the SD of three biological replicates. (C) Ubiquitination status of Gata6 mutant forms. Cos-7 cells were transiently transfected with Flagged wild-type, ΔCT, or ΔCTZF Gata6 and cultured for 7 h in the presence of MG132. (Top panel) Protein extracts were subjected to immunoprecipitation with anti-Flag antibodies, and the levels of Gata6 multiubiquitination (Ub) were revealed by immunoblotting with anti-ubiquitin antibodies. Anti-Flag immunoblots confirmed the efficient recovery of Gata6 protein following immunoprecipitation across cell samples. (D) Coimmunoprecipitation of Bmi1 and Gata6 mutant forms. (Bottom panel) Cos-7 cells were transiently cotransfected with Flag-tagged wild-type, ΔCT, or ΔCTZF Gata6 and Myc-tagged Bmi1, and protein extracts were immunoprecipitated (IP) with anti-Flag antibodies and subsequently subjected to immunoblotting (IB) with anti-Flag (recovery control) or anti-Myc antibodies to detect Bmi1 protein. (E) Scheme depicting Myc-tagged wild-type and truncated Bmi1 forms. Bmi1 mouse cDNA was Myc-tagged (wild-type [Wt]), and a mutant lacking the Ring domain (ΔRing) was generated. (F) Coimmunoprecipitation of Gata6 and wild-type or ΔRing Bmi1. Cos-7 cells were transiently cotransfected with Gata6 and Myc-tagged wild-type or ΔRing Bmi1, and protein extracts were subjected to immunoprecipitation (IP) with anti-Myc antibodies (top panel) and immunoblotting (IB) with anti-Gata6 antibodies (bottom panel). Inputs and anti-Myc immunoblots confirmed homogeneous levels of different transfected forms. Three independent experiments were performed with similar results. (G) Gata6 reporter assay using wild-type and ΔRing Bmi1 forms. HEK293 cells were transiently cotransfected with Gata6-dependent Hnf4 reporter and wild-type versus ΔRing Bmi1 constructs, and luciferase activity was assessed 48 h post-transfection. Data were normalized to Renilla. Error bars represent the SD of three biological replicates. The P-value was calculated using the Student’s t-test.
ubitiquin-conjugating enzymes (Clurman et al. 1996). Importantly, we establish that Bmi1 plays a cell-autonomous role in promoting the induction of the PrE lineage, as assessed in vitro in chimeric EBs. In the context of the early embryo, Gata6 and Nanog expression is first stochastic (Dietrich and Hiiragi 2007), and cell fate is thought to remain flexible (Yamanaka et al. 2010). We propose here that Bmi1 action on Gata6 stability could directly alter the balance between Gata6 and Nanog protein levels in individual blastomeres and thus impact on cell fate.

Figure 6. Bmi1 promotes PrE emergence in a cell-autonomous manner. (A) Coimmunostainings for Bmi1, Gata6, and Gata4 were performed on agarose-embedded and microsectioned EBs cultured for 5 d. (B) Relative transcript levels for Bmi1, Oct3/4, Nanog, Gata6, Gata4, and Sox17 as assessed by qRT–PCR in control [ESControl] and Bmi1 knockdown [ESBmi1KD] ES cells upon EB formation for 5 d. Data were normalized to S17 and L19 and expressed relative to undifferentiated ESControl cells. Error bars represent the SD of two biological replicates. (C) Coimmunostainings for Gata6 and Gata4 performed on EBs cultured for 5 d in the presence (ESControl; top panel) or absence (ESBmi1KD; bottom panel) of Bmi1. Bars, 20 μM. (D) Schematic of chimeric EB formation. GFP-labeled ESControl or ESBmi1KD cells were mixed with unlabeled ESControl cells [ratio 1/1–1/3] and allowed to differentiate for 5 d upon EB formation. (E) Coimmunostainings for GFP and Gata6 performed on chimeric EBs formed as described in D. The outer layer of the EB structure is denoted by dotted lines. Bars, 20 μM.
decisions by introducing a bias toward a PrE identity. Interestingly, Gata4 does not interact with Bmi1, and this together with the late onset of Gata4 protein expression in vivo further suggests that Gata4 and Sox17 could, in turn, reinforce Gata6 function in a Bmi1-independent manner.

Interestingly, we provide novel evidence that Nanog represses Bmi1 in ES cells, as for Gata6 (Fig. 7; Singh et al. 2007), further supporting the view that Nanog actively suppresses the PrE identity in pluripotent cells while being required for proper PrE differentiation in a non-cell-autonomous manner (Silva et al. 2009; Messerschmidt and Kemler 2010; Frankenberg et al. 2011). We demonstrate that Bmi1 expression mirrors Nanog fluctuations within ES cell cultures and constitutes an early hallmark of extraembryonic differentiation upon Nanog depletion. Interestingly, while Bmi1 protein expression is mutually exclusive with Nanog, the Bmi1 transcript remained detected, although at variable levels, in Nanog-high and Nanog-low ES cell populations (Fig. 1), suggesting that Bmi1 itself may be post-transcriptionally regulated in this context. Several microRNAs were previously shown to target the Bmi1 3′ untranslated region (UTR) in various cancer cell lines (Shimono et al. 2009). Here we identified miR-200c as being uniquely expressed in ES cells, as opposed to XEN and TS cells (Supplemental Fig. S6A), and positively regulated by Nanog (Supplemental Fig. S6A,B). Moreover, inhibiting miR-200c was found sufficient to release Bmi1 protein expression in ES cells (Supplemental Fig. S6C,D), adding another level of Bmi1 expression control mediated by Nanog (Fig. 7). Conversely, we found that ectopic expression of Bmi1 in ES cells reduces Nanog mRNA levels without altering Oct4, Sox2, or Klf4, yet is not capable of driving PrE differentiation alone (data not shown). Similar cross-regulatory events between Bmi1 and Nanog might also take place in vivo as Bmi1/Gata6 and Nanog transcripts segregate upon epiblast/PrE lineage specification, a pattern that is stably established in the late blastocyst by E4.5. By comparison, Bmi1 and Gata6 protein were seen to cosegregate in a subset of blastomeres in the early blastocyst around E3.25, suggesting that the Bmi1/Gata6 protein–protein interaction could be an early event in PrE emergence, while Nanog-mediated transcriptional repression of Bmi1, Gata6, and other factors would “lock in” or reinforce the epiblast/pluripotent identity both in vivo and in vitro. Additional mechanisms, such as the timing of cell internalization and signaling cascades, have also been shown to act in concert to dictate or consolidate PrE lineage specification (Nichols et al. 2009; Morris et al. 2010; Yamanaka et al. 2010).

Bmi1 is a pleiotropic factor with roles linked to cell cycle regulation and cancer (Bruggeman et al. 2007; Grinstein and Mahotka 2009) as well as to the homeostasis of adult stem cells (van der Lugt et al. 1994; Molofsky et al. 2003; Park et al. 2003; Bruggeman et al. 2007). Our study unveils a previously unrecognized developmental function for Bmi1 (Puschendorf et al. 2008), acting as a key post-transcriptional regulator of Gata6, a factor essential for extraembryonic endoderm development both in vitro and in vivo (Koutsourakis et al. 1999; Lim et al. 2008). Of interest, Bmi1 was also found to interact with Gata3 in TE-derived TS cells (Supplemental Fig. S7A; Home et al. 2009; Ralston et al. 2010). Bmi1 depletion in TS cells notably led to a loss of stem cell identity accompanied by a rapid and drastic reduction in Gata3 protein levels (Supplemental Fig. S7B–E). This indicates that Bmi1 might regulate Gata3 protein expression in TS cells and suggests a broader role for Bmi1 in the formation and/or maintenance of extraembryonic lineages during early mouse development.

Materials and methods

Cell culture

Mouse ES, TS, and XEN cell lines were grown as previously described (Tanaka et al. 1998; Kunath et al. 2005; Alder et al. 2010). For Nanog depletion, the RCN68B cell line was treated with 1 μg/mL 4′-OH-tamoxifen (Chambers et al. 2007). Transfections were carried out using Lipofectamine 2000 (Invitrogen, 11668) following the manufacturer’s recommendations. For stable clone derivation, cells were treated 24 h post-transfection with puromycin (Sigma, p8833) at 1 μg/mL for 8–10 d. Clones were then pooled or picked individually, depending on the experimental design. EB formation was induced in hanging drops as previously described (Lavial et al. 2007).

Antibodies

Anti-Bmi1 (Millipore, F6), anti-Gata6 [R&D Systems, AF1700], anti-Gata4 (Santa Cruz Biotechnology, sc-9053), anti-Gata3

Figure 7. Model of Bmi1 function in extraembryonic endoderm lineage emergence. Nanog transcription factor protects the pluripotent state by repressing Bmi1 and Gata6 PrE-associated genes (depicted as black solid lines) and activating miR-200c, a microRNA also shown to modulate Bmi1 protein expression in ES cells (depicted as a black dotted line, indicating no evidence that this regulation is direct). (BS) Location of the Nanog-binding site upstream of the Bmi1 and miR-200c transcription start sites (arrows). Bmi1 physically interacts with and stabilizes Gata6 protein levels by protecting it from ubiquitination and proteasomal degradation (depicted as red triangles and dashed box). Formation of this complex enhances Gata6 transcriptional activity on target genes and promotes PrE emergence. Gata6 lies upstream of Gata4 and Sox17 in differentiation cascade (depicted as red dotted lines, indicating no evidence that these regulations are direct). Bmi1 expression also leads to a reduction in Nanog levels by an unknown mechanism (depicted as a black dotted line).
[Santa Cruz Biotechnology, sc-268], anti-Cdx2 [Biogenex, MUS92A-UC], anti-Nanog [Cosmobio, RCA 8000 2P-F], anti-ubiquitin [Biomol, FK2], anti-Ring1B [Active Motif, 39663], anti-Cbx8 [Bethyl Laboratories, A300-882A], anti-Mel18 [Abcam, ab5267], anti-GFP [Abcam, AB290], anti-Myc [Santa Cruz Biotechnology, sc-40], anti-Flag [Sigma, M2], and anti-Actin [Abcam, AB8227] were used. For immunoprecipitation experiments, anti-Gata6 [Santa Cruz Biotechnology, sc-9055] was used. For immunofluorescence, Alexa secondary antibodies were used [Invitrogen]. For immunoblotting and coimmunoprecipitation experiments, mouse [Santa Cruz Biotechnology], rabbit [Santa Cruz Biotechnology], and goat [Dako] secondary antibodies were used.

**RNA expression analysis**

Total RNA was isolated using the Qiagen RNeasy minikit and DNase I-treated. Samples were oligo(dT) reverse-transcribed using Invitrogen SuperScript III or M-MLV following the manufacturer’s recommendations and analyzed by qRT-PCR using Sigma Jumpstart SYBR Green. Primer sequences are available on request.

**Vector construction**

pLKO.1 vectors containing hairpins directed against Bmi1 cDNA were purchased from Sigma: shRNA vector 1 [CCGCCCACGCAA GTATTTGCTATTCTTCGAGAAATGGACAATCTTCTGCT GGTITTTTT] and shRNA vector 2 [CGCCGCCTAAAGATGTACG AGTCAAGATACCTGGAGCTATGTTAGCTTCGTTT]. The Bmi1 2.4-kb promoter was PCR-amplified on mouse ES cell genomic DNA using long Taq [Roche Biomedicals, 1168134001] and Bmi1prom-F [5'-TCCCTGGACACGT TTTCATT-3'] and Bmi1prom-R [5'-CGTAAATGACCACGG GATT-3'] primers. Taq polymerase (Invitrogen 10342-020) was used to add adenines and clone the fragment into pGEMEasy (Promega, TM042). The Bmi1 1.9-kb promoter fragment was then subcloned into the pGL3 promoter [Promega, E1761] using MluI and BglII restriction enzymes [New England Biolabs]. Mutations in the Nanog-binding site BS1 were inserted by using PfuTurbo polymerase [Stratagene 60250], DpnI restriction enzyme [New England Biolabs], and Bmi1mut-F [5'-TAAATGCTCTGGCG AGACTCGAATTTGTCAAGGCTTTAAGGCTATCTTTAA GACAAATCATTT-3'] and Bmi1mut-R [5'-AAGTGGATTTGTCT TAAATACTCTTTAATCAGGCTCAATGCTGTCTG CGACACCAGACATT-3'] primers. Gata6 cDNA was subcloned in the BglIII site of pSG5-Flag [Stratagene] using primers G6-F [5'- ATAGATCTAGCGCTGACGACCCGGG-3'] and G6-R [5'-AT AGATCTATCGACGGCCGAGG-3']. Gata6 truncated forms were generated with primers ΔCTZF-F [5'-CTCTGTTCG GAGAGCGCTGATAGCTTGAGAC-3'], ΔCTZF-R [5'-GGT ACCAGATCTATCGGCGTCGCTGAGACAGC-3'], ΔDCTF-F [5'-GG AATCTAACCAGAAACAAATGATAGATCTGGTACC ACTA-3'], and ΔDCT-R [5'-TAGTGATCCAGATCAGATTCT GTCCAACCGTGATCCAGATCTGCC-3']. Recombinant lentiviruses were generated using a three-plasmid system in 293T cells as previously described [Kutner et al. 2009]. Virus-containing culture supernatants were collected 24 and 48 h after transfection, pooled, concentrated, and used for infection. Control and Bmi1KD-infected TS cells were collected 4 d post-infection as previously described [Alder et al. 2010]. The Gata6-dependent Hnf4 promoter was cloned into the pGL3 promoter as previously described [Morrissey et al. 1998]. miR-200c inhibitor was purchased from Exiqon and transfected following the manufacturer’s recommendations.

**Luciferase reporter assay**

Luciferase assays were carried out using 2.5 × 10^5 HEK293T cells in 96-well plates. Transfections with pGL3 promoter vectors and control pEGFP-C1 [Clontech] or pRenilla-Tk [Promega] were performed using Lipofectamine 2000 [Invitrogen, 11668-019]. Luciferase activity was assessed 48 h post-transfection using a Steadylite kit [PerkinElmer, 6016756] following the manufacturer’s recommendations. Transfection efficiency was corrected using GFP or Renilla levels.

**Immunoblotting analysis**

Cells were lysed in RIPA buffer (50 mM Tris at pH 8, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl) supplemented with a protease inhibitor tablet [Roche Diagnostics, 11836153001]. Protein fractionation was performed using NE-PER kit [Thermo Fisher Scientific, 78833]. Protein concentrations of whole-cell extracts were measured using a Bradford assay [Thermo Fisher Scientific, 23225]. Thirty-microgram samples were loaded onto 10% acrylicamide gels and blotted onto methanol-activated polyvinylidene fluoride membranes [Millipore, IPVLO010] using a semidy or wet transfer system. Membranes were treated with enhanced chemiluminescent substrate [Thermo Fisher Scientific, 32106].

**ChIP and coimmunoprecipitation**

ChIP was carried out as previously described [Alder et al. 2010]. For coimmunoprecipitation experiments, 800 μg of cell protein extracts was precleared with protein A Sepharose beads [Sigma, P3391] for 2 h at 4°C and then incubated overnight at 4°C with the indicated antibodies. Protein A beads were then added for 5 h, washed with RIPA and TSE buffer (2 mM EDTA, 20 mM Tris at pH 8, 150 mM NaCl) four times, and loaded on either 7% or 14% acrylamide gels. Protein extracts were treated for 2 h at 4°C with 50 U of Benzonase (Merck, 71205) where indicated.

**Protein stability assay**

Cells were split, and 3 × 10^6 cells were plated back into 10-cm plates. On the following day, cells were treated with 100 μM CHX [Sigma], CHX plus 1 μM MG132 [Calbiochem, 474790], or CHX plus 1 μM PYR41 [Calbiochem, 662105] for the indicated times. Protein amounts were quantified using ImageJ software and normalized to Actin. Protein samples were run on SDS-10% PAGE gels and transferred to nitrocellulose membranes (Whatman, 32015). Membranes were blocked in 5% milk in TBST at room temperature, incubated with primary antibodies (Table 2), and visualized using chemiluminescent substrate (Thermo Fisher Scientific, 32106).

**Ubiquitination assay**

Cells were treated for 7 h with 1–5 μM MG132, lysed in the presence of deubiquitination inhibitor NEM [Sigma, E3876], sonicated, and subjected to overnight immunoprecipitation with control anti-IgG, anti-Gata6, anti-Gata4, or anti-Flag antibodies. Protein G beads [GE Healthcare, 17-0618-01] were added for 4 h at 4°C, and the levels of ubiquitination were subsequently revealed by immunoblotting with anti-ubiquitin antibody.

**Immunofluorescence analysis**

Cells were seeded on gelatinized glass coverslips and fixed in PBS with 4% paraformaldehyde. Samples were permeabilized and blocked at room temperature before incubation with the indicated antibodies. Coverslips were mounted on VectaShield with DAPI [Vector Laboratories, H-1200] and examined using a Leica SP5 confocal microscope [40× or 63× lens]. Embryo immunostainings were performed as previously described [Chazaud et al. 2006]. EBs were fixed overnight in formalin at 4°C and embedded in agarose and wax. Five micromolar sections were used for immunochemistry and observations on a Leica SP5 confocal microscope.
Embryo collection and staging for single-cell PCR analysis

BL/6xC3H F1 mice were bred naturally, and the embryos were recovered at E3.25, E3.5, or E4.5 by flushing either the oviduct or uterus. ICMs were isolated from blastocystos by immunosurgery and further dissociated into single blastomeres by pipetting in a solution of 1 mM EDTA dissolved in HBS in order to treat with 1% trypsin (Sigma, T-4549) and 1 mM EDTA in HBS. Staging of embryos subjected to single-cell PCR analysis was defined as follows. Upon recovery, average-size embryos were selected for subsequent analysis, and the remaining littermates were fixed in PBS with 4% paraformaldehyde (Electron Microscopy Sciences, 19208) and stained in PBS with 10 μM DAPI (Molecular Probes, D3571) and 5 U/mL Alexa Fluor 633 or Alexa Fluor 564 phallloidin (Molecular Probes, A22284 or A22283, respectively). Images were acquired on a Zeiss LSM 510 META or 710 microscope and analyzed using IMARIS software (Bitplane). The total cell number of each embryo was counted, and an average cell number of littermates (excluding those with maximum and minimum cell numbers) was used to define the developmental stage of each embryo processed for single-cell PCR analysis. Experiments were performed in accordance with European Union guidelines for the care and use of laboratory animals.

Single-cell cDNA amplification

Single-cell cDNA amplification from each blastomere was performed as previously reported (Kurimoto et al. 2006). Briefly, single blastomeres were lysed in individual tubes without purification, and first strand cDNAs were synthesized using a modified poly(dT)-tailed primer. The unincorporated primer was specifically digested by exonuclease, and the second strands were generated with a second poly(dT)-tailed primer after poly[dA] tailing of the first strand cDNAs. The cDNAs were amplified by PCR first with poly[dT]-tailed primers and subsequently with primers bearing the T7 promoter sequence. The resultant cDNA products were used for further real-time PCR analysis. Primer sequences are available on request. Note that “spike” RNAs that consist of poly(A)-tailed RNAs artificially designed from Bacillus subtilis genes were added to each sample as amplification control to estimate the copy number of gene “spike” RNAs that were estimated using IMARIS software (Bitplane). Images were acquired on a Zeiss LSM 510 META or 710 microscope and analyzed using IMARIS software (Bitplane). The total cell number of each embryo was counted, and an average cell number of littermates (excluding those with maximum and minimum cell numbers) was used to define the developmental stage of each embryo processed for single-cell PCR analysis. Experiments were performed in accordance with European Union guidelines for the care and use of laboratory animals.

Statistics analysis

Statistical analyses were performed using GraphPad Prism 5. For single-cell PCR analysis, the Spearman’s rank correlation coefficient test was performed using R software to evaluate gene expression correlations with Gata6.

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References

Alder O, Laval F, Helness A, Brookes E, Pinho S, Chandrashekaran A, Arnaud P, Pombo A, O’Neill L, Azzurra V. 2010. Ring1B and Suv39H1 delineate distinct chromatin states at bivalent genes during early mouse lineage commitment. Development 137: 2483–2492.

Arney KL, Erhardt S, Drewell RA, Surani MA. 2001. Epigenetic reprogramming of the genome—from the germ line to the embryo and back again. Int J Dev Biol 45: 533–540.

Artus J, Piliszsek A, Hadjantonakis AK. 2011. The primitive endoderm lineage of the mouse blastocyst: Sequential transcription factor activation and regulation of differentiation by Sox17. Dev Biol 350: 393–404.

Azzurra V, Perry P, Sauer S, Spivakov M, Jorgensen HE, John RM, Gouti M, Casanova M, Warres G, Merkenschlager M, et al. 2006. Chromatin signatures of pluripotent cell lines. Nat Cell Biol 8: 532–538.

Bayer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, et al. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441: 349–353.

Bruggeman SW, Hulsman D, Tanger E, Buckle T, Blom M, Zevenhoven J, van Tellingen O, van Lohuizen M. 2007. Bmi1 controls tumor development in an Ink4a/Art-independent manner in a mouse model for glioma. Cancer Cell 12: 328–341.

Cape-Chichi CD, Rula ME, Smedberg JL, Vanderweer L, Parmacek MS, Morrisey EE, Godwin AK, Xu X. 2005. Perception of differentiation cues by GATA factors in primitive endoderm lineage determination of mouse embryonic stem cells. Dev Biol 286: 574–586.

Chamberlain SJ, Yee D, Magnuson T. 2008. Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. Stem Cells 26: 1496–1505.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113: 643–655.

Chambers I, Silva J, Colby D, Nichols J, Nijmeijer B, Robertson M, Vrana J, Jones K, Grotewold L, Smith A. 2007. Nanog safeguards pluripotency and mediates germline development. Nature 450: 1230–1234.

Chazaud C, Yamanaka Y, Pawson T, Rossant J. 2006. Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grib2–MAPK pathway. Dev Cell 10: 615–624.

Clurman BE, Sheaff RJ, Thress K, Groudine M, Roberts JM. 1996. Turnover of cyclin E by the ubiquitin–proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. Genes Dev 10: 1979–1990.

Dietrich JE, Hiraig T. 2007. Stochastic patterning in the mouse pre-implantation embryo. Development 134: 4219–4231.

Endoh M, Endo TA, Endoh T, Fujimura Y, Ohara O, Toyota T, Otte AP, Okano M, Brockdorff N, Vidal M, et al. 2008. Polycomb group proteins Ring1A/B are functionally linked to the core transcriptional regulatory circuitry to maintain ES cell identity. Development 135: 1513–1524.

Evans MJ, Kaufman MH. 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154–156.

Frankenberg S, Gerbe F, Bessonard S, Belville C, Pouchin P, Bardot O, Chazaud C. 2011. Primitive endoderm differentiation via a three-step mechanism involving Nanog and RTK signaling. Dev Cell 21: 1005–1013.

Fujikura J, Yamato E, Yonemura S, Hosoda K, Masui S, Nakao K, Miyazaki Ji J, Niwa H. 2002. Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev 16: 784–789.
Grinstein E, Mahotka C. 2009. Stem cell divisions controlled by the proto-oncogene BMI-1. J Stem Cells 4: 141–146.

Guo G, Huss M, Tong GQ, Wang C, Li Sun L, Clarke ND, Robson P. 2010. Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. Dev Cell 18: 675–685.

Home P, Ray S, Dutta D, Bronshcteyn I, Larson M, Paul S. 2009. GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. J Biol Chem 284: 28729–28737.

Hosokawa K, Kimura MY, Shinnakasu R, Suzuki A, Miki T, Kosoki H, van Lohuizen M, Yamasita M, Nakayama T. 2006. Regulation of TH2 cell development by Polycybm group gene bmi-1 through the stabilization of GATA3. J Immunol 177: 7656–7664.

Jedrusik A, Farritt DE, Guo G, Skamagki M, Grabarek JB, Koutsourakis M, Langeveld A, Patient R, Beddington R, Grosveld F. 2009. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. Nat Protoc 4: 495–505.

Kuttner RH, Zhang XY, Reiser J. 2009. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. Nat Protoc 4: 495–505.

Lavial F, Acloque H, Bertocchini F, Macleod DJ, Boast S, Johnson MH, Robson P, Zernicka-Goetz M. 2008. Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. Genes Dev 22: 2692–2706.

Kousoulakis M, Laneveld A, Patient R, Beddington R, Grosveld F. 1999. The transcription factor GATA6 is essential for early extraembryonic development. Development 126: 723–732.

Kunath T, Arnaud D, Uy GD, Okamoto I, Chureau C, Yamazaki Y, Heard E, Gardner RL, Avner P, Rossant J. 2005. Imprinted X-inactivation in extra-embryonic embryonic cell lines from mouse blastocysts. Development 132: 1649–1661.

Kurimoto K, Yabuta Y, Ohinata Y, Ono Y, Uno KD, Yamada RG, Ueda HR, Saitou M. 2006. An improved single-cell cDNA amplification method for efficient high-density oligonucleotide microarray analysis. Nucleic Acids Res 34: e42. doi: 10.1093/nar/gkl050.

Lavial F, Acloque H, Bertocchini F, Macleod DJ, Boast S, Bachelard E, Montillet G, Thenot S, Sang HM, Stern CD, Jablonski SA, Howe PH, Smith ER, Xu XX. 2007. Cell lineage-specific regulators in embryonic stem cells. Cell Cycle 5: 1411–1414.

Liang J, Silva J, Roode M, Smith A. 2009. Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. Development 136: 3215–3222.

Luu PH, Acloque H, Bertocchini F, Macleod DJ, Boast S, Johnson MH, Robson P, Zernicka-Goetz M. 2008. Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. Genes Dev 22: 2692–2706.

Jorgensen HE, Giadrossi S, Casanova M, Endoh M, Koseki H, Brockdorff N, Fisher AG. 2006. Stem cells primed for action: Polycybm repressive complexes restrain the expression of lineage-specific regulators in embryonic stem cells. Cell Cycle 5: 302–305.

Koski H, van Lohuizen M, Yamashita M, Nakayama T, Koseki H, van Lohuizen M, Yamashita M, Nakayama T. 2006. Regulation of TH2 cell development by Polycybm group gene bmi-1 through the stabilization of GATA3. J Immunol 177: 7656–7664.

Koutoukakis M, Laneveld A, Patient R, Beddington R, Grosveld F. 1999. The transcription factor GATA6 is essential for early extraembryonic development. Development 126: 723–732.

Johnson MH, Robson P, Zernicka-Goetz M. 2008. Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. Genes Dev 22: 2692–2706.

Jorgensen HE, Giadrossi S, Casanova M, Endoh M, Koseki H, Brockdorff N, Fisher AG. 2006. Stem cells primed for action: Polycybm repressive complexes restrain the expression of lineage-specific regulators in embryonic stem cells. Cell Cycle 5: 1411–1414.

Koutoukakis M, Laneveld A, Patient R, Beddington R, Grosveld F. 1999. The transcription factor GATA6 is essential for early extraembryonic development. Development 126: 723–732.

Kunath T, Arnaud D, Uy GD, Okamoto I, Chureau C, Yamazaki Y, Heard E, Gardner RL, Avner P, Rossant J. 2005. Imprinted X-inactivation in extra-embryonic embryonic cell lines from mouse blastocysts. Development 132: 1649–1661.

Kurimoto K, Yabuta Y, Ohinata Y, Ono Y, Uno KD, Yamada RG, Ueda HR, Saitou M. 2006. An improved single-cell cDNA amplification method for efficient high-density oligonucleotide microarray analysis. Nucleic Acids Res 34: e42. doi: 10.1093/nar/gkl050.

Kuttner RH, Zhang XY, Reiser J. 2009. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. Nat Protoc 4: 495–505.

Lavial F, Acloque H, Bertocchini F, Macleod DJ, Boast S, Bachelard E, Montillet G, Thénost S, Sang HM, Stern CD, et al. 2007. The Oct4 homologue PouV and Nanog regulate pluripotency in chicken embryonic stem cells. Dev Biol 301: 146–159.

Lim CY, Tam WL, Zhang J, Ang HS, Jia H, Lipovich L, Ng HH, Wei CL, Sung WK, Robson P, et al. 2008. Sall4 regulates distinct transcription circuits in different blastocyst-derived stem cell lineages. Cell Stem Cell 3: 543–554.

Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci 78: 7634–7638.

Meilhac SM, Adams RJ, Morris SA, Danckaert A, Le Garrec JE, Zernicka-Goetz M. 2009. Active cell movements coupled to positional induction are involved in lineage segregation in the mouse blastocyst. Dev Biol 331: 210–221.

Messerschmidt DM, Kemler R. 2010. Nanog is required for primitive endoderm formation through a non-cell autonomous mechanism. Dev Biol 344: 129–137.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. 2003. The homeo-protein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113: 631–642.
Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J. 1998. Promotion of trophoblast stem cell proliferation by FGF4. Science 282: 2072–2075.

Torres-Padilla ME, Parfitt DE, Kouzarides T, Zernicka-Goetz M. 2007. Histone arginine methylation regulates pluripotency in the early mouse embryo. Nature 445: 214–218.

van der Lugt NM, Domen J, Linders K, van Roon M, Robanus-Maandag E, te Riele H, van der Valk M, Deschamps J, Sofroniew M, van Lohuizen M, et al. 1994. Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. Genes Dev 8: 757–769.

van der Stoop P, Boutsma EA, Hulsman D, Noback S, Heimerikx M, Kerkhoven RM, Voncken JW, Wessels LE, van Lohuizen M. 2008. Ubiquitin E3 ligase Ring1b/Rnf2 of polycomb repressive complex 1 contributes to stable maintenance of mouse embryonic stem cells. PLoS ONE 3: e2235. doi: 10.1371/journal.pone.0002235.

van Lohuizen M. 1999. The trithorax-group and polycomb-group chromatin modifiers: Implications for disease. Curr Opin Genet Dev 9: 355–361.

Voncken JW, Niessen H, Neufeld B, Rennefahr U, Dahlmans V, Kubben N, Holzer B, Ludwig S, Rapp UR. 2005. MAPKAP kinase 3pK phosphorylates and regulates chromatin association of the Polycomb group protein Bmi1. J Biol Chem 280: 5178–5187.

Yamanaka Y, Lanner F, Rossant J. 2010. FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. Development 137: 715–724.

Yang DH, Smith ER, Roland IH, Sheng Z, He J, Martin WD, Hamilton TC, Lambeth JD, Xu XX. 2002. Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis. Dev Biol 251: 27–44.