Development 137, 2483-2492 (2010) doi:10.1242/dev.048363

Ring1B and Suv39h1 delineate distinct chromatin states at bivalent genes during early mouse lineage commitment

Olivia Alder[1,*], Fabrice Lavial[1,*], Anne Helness[1,*], Emily Brookes[2], Sandra Pinho[1], Anil Chandrashekran[1], Philippe Arnaud[3], Ana Pombo[2], Laura O'Neill[4] and Véronique Azuara[1,†]

SUMMARY

Pluripotent cells develop within the inner cell mass of blastocysts, a mosaic of cells surrounded by an extra-embryonic layer, the trophectoderm. We show that a set of somatic lineage regulators (including Hox, Gata and Sox factors) that carry bivalent chromatin enriched in H3K27me3 and H3K4me2 are selectively targeted by Suv39h1-mediated H3K9me3 and de novo DNA methylation in extra-embryonic versus embryonic (pluripotent) lineages, as assessed both in blastocyst-derived stem cells and in vivo. This stably repressed state is linked with a loss of gene priming for transcription through the exclusion of PRC1 (Ring1B) and RNA polymerase II complexes at bivalent, lineage-inappropriate genes upon trophoblast lineage commitment. Collectively, our results suggest a mutually exclusive role for Ring1B and Suv39h1 in regulating distinct chromatin states at key developmental genes and propose a novel mechanism by which lineage specification can be reinforced during early development.

KEY WORDS: Bivalent chromatin, Early development, Histone methylation, Mouse, Silencing, Stem cells

INTRODUCTION

Gene expression programmes must be tightly controlled to govern cell identity and lineage choice. An inherent challenge for developing organisms is to maintain a critical balance between stable and flexible gene regulation (Reik, 2007). This is most obvious in pluripotent embryonic stem (ES) cells, which are functionally characterised by their ability to self-renew and to generate all somatic lineages when induced. Accordingly, ES cells express genes that encode self-renewal factors, while repressing many lineage-specific regulators that are ultimately required during development (Jaenisch and Young, 2008). A series of recent reports have indicated how Polycomb-mediated gene repression might provide short-term, and therefore flexible, epigenetic silencing of such developmental genes in pluripotent cell lines (Azuara et al., 2006; Bernstein et al., 2006a; Boyer et al., 2006; Lee et al., 2006; Pan et al., 2007; Zhao et al., 2007). This contrasts with long-term repression mechanisms as conferred, for example, by DNA methylation at transposons, imprinted and pluripotency-associated genes in somatic cells (Bird, 2002; Delaval and Feil, 2004; Epsztejn-Litman et al., 2008; Farthing et al., 2008; Weber and Schubeler, 2007).

Two distinct Polycomb Repressive Complexes (PRC), PRC1 and PRC2, are known to be important for the function of ES cells. PRC2 contains Ezh2, which catalyses histone H3 lysine 27 tri-methylation (H3K27me3), as well as Eed and Suz12. PRC1 contains the E3 ubiquitin ligases Ring1A (also known as Ring1) and Ring1B (Rnf2 – Mouse Genome Informatics) that mono-ubiquitylate histone H2A lysine 119. Other PRC1 components include Bmi1, Mel18 (Pcgf6 – Mouse Genome Informatics) and proteins of the Cbx family with H3K27 methylation affinity (Bantignies and Cavalli, 2006; Pietersen and van Lohuizen, 2008; Ringrose and Paro, 2007; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007; Simon and Kingston, 2009). Candidate-based (Azuara et al., 2006) and genome-wide (Bernstein et al., 2006a) studies of histone methylation in ES cells led to the remarkable finding that many PRC2-target genes not only carry the repressive H3K27me3 mark, but are also enriched for conventional indicators of active chromatin, including methylated H3K4. These so-called bivalent chromatin domains are thought to silence key developmental regulators while keeping them primed for future activation (or repression), and thus generally resolve to monovalent configurations upon differentiation, in accordance with gene expression changes (Bernstein et al., 2006a; Mikkelsen et al., 2007; Pan et al., 2007). Further work showed that multipotent stem cells and some differentiated cells also possess bivalent domains, albeit perhaps fewer than in ES cells (Barski et al., 2007; Bernstein et al., 2006a; Bracken et al., 2006; Cui et al., 2009; Mikkelsen et al., 2007; Pan et al., 2007; Roh et al., 2006; Sanz et al., 2008; Wei et al., 2009), indicating that plasticity might be maintained at loci that are required for the function and differentiation of lineage-committed cells. Consistent with gene priming, bivalent genes assemble RNA polymerase II complexes preferentially phosphorylated on Ser5 residues (poised RNAP) and are transcribed at low levels (Brookes and Pombo, 2009; Guenther et al., 2007; Stock et al., 2007). Productive expression is, however, prevented by the action of PRC1 with conditional deletion of Ring1A/B, resulting in an upregulation of target gene expression in ES cells (from primed to overt transcription) (Endoh et al., 2008; Guenther et al., 2007; Jorgensen et al., 2006; Stock et al., 2007).

Clearly, Polycomb repressors are functionally required to prevent premature expression of primed genes and thus to stably maintain a pluripotent ES cell identity in culture (Azuara et al., 2006; Boyer et al., 2006; Chamberlain et al., 2008; Endoh et al., 2008; Leeb and Wutz, 2007; van der Stoop et al., 2008). Whether ES cell epigenetic signatures can also be seen in the developing

[1]Institute of Reproductive and Developmental Biology, Imperial College School of Medicine, Hammersmith Campus, Du Cane Road, London W12 0NN, UK. [2]MRC-Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Campus, Du Cane Road, London W12 0NN, UK. [3]Institute of Molecular Genetics, CNRS UMR-5535, University of Montpellier II, Montpellier 34293, France. [4]Institute of Biomedical Research, University of Birmingham Medical School, Birmingham B15 2TT, UK.

*These authors contributed equally to this work
†Author for correspondence (v.azuara@imperial.ac.uk)

Accepted 25 May 2010

embryo and when (and how) bivalent domains are established and subsequently resolved upon lineage commitment in vivo remains to be elucidated. In this study, we have focused on the earliest stages of mouse development to address whether poised chromatin structures are unique hallmarks of the founder (pluripotent) cells of the early embryo, and to investigate the kinetics of appearance and resolution of bivalent domains during the first lineage decision event (trophectoderm formation). We provide in vivo evidence that bivalent histone marking operates in the early mouse embryo from the eight-cell up to the blastocyst stage. Unexpectedly, we show that bivalent domains are retained at a subset of genes encoding key somatic lineage regulators in extra-embryonic restricted cells, as assessed both in vitro and in vivo. However, and in contrast to pluripotent cells, PRC1 (Ring1B) and poised RNAP are not engaged at these PRC2 (Suz12)-bound genes, consistent with a loss of gene priming. Instead, bivalent genes become selectively targeted by Suv39h1-mediated repression upon trophoblast lineage commitment. Collectively, our results suggest a mutually exclusive role for Ring1B and Suv39h1 in specifying the fate of bivalent genes in a lineage-specific manner upon blastocyst formation.

MATERIALS AND METHODS

Cell lines

The B1-TS cell line was established and maintained as described (Mak et al., 2004). ZHBTc4-ES cells were cultured on gelatinised plates with LIF 1000 U/μl (ESGRO-LIF; Millipore) (Azuara et al., 2006). *Pou5f1* repression in ZHBtc4-ES was induced by addition of doxycycline (Sigma) at 1 μg/ml. TS-like (TSL) cells were derived from Dox-treated ZHBTc4-ES cells plated at low density on irradiated feeders in the presence of 37 ng/ml FGF4 (R&D Systems) and 1 μg/ml heparin (Sigma) (Niwa et al., 2000). Note that Dox treatment was sustained for approximately two weeks in these conditions; until the time at which positive staining for the trophectoderm stem cell marker Cdx2 was seen in the same proportion of ZHBTc4-derived TSL cells as B1-TS cells, while Oct4 was undetectable in either culture. TSL cells could then be serially passaged and propagated in culture for at least 6 weeks.

RNA interference

Lentivirus vector containing a short-hairpin RNA (shRNA) targeting mouse *Suv39h1* mRNA was cloned in pLKO.1 (Sigma). The sequence used to successfully knockdown expression of *Suv39h1* is as follows: 5′-GCCTTTGTACTCAGGAAAG-3′ (TRCN97439). 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 after 24 and 48 hours of transfection, pooled, concentrated and used for infection. Control and *Suv39h1*-knockdown TS cells were collected at 5 days post-infection for subsequent expression and chromatin analysis.

Embryo collection and dissection

Pre-pubescent CBAB6 F1 females were super-ovulated by intrauterine injections of pregnant mare serum gonadotropin (PMSG; 5U Folligon; Intervet, UK) followed 42 hours later by human chorionic gonadotropin (HCG; 5U Chorulon; Intervet, UK) and mating overnight with CBAB6 F1 males. At 2.5 days post-coitum, compacted morula were flushed from the oviduct in EmbryoMax M-2 (Millipore), snap frozen or cultured overnight up to the blastocyst stage in 30 μl droplets of equilibrated EmbryoMax M-16 (Millipore) under mineral oil. Early-stage blastocysts were selected based on morphology; TE samples were acquired using Leica manual manipulators and micro-dissection blades (Bioniche) on a Nikon Diaphot with DIC optics, as previously described (O'Neill et al., 2006). Individual ICMs were isolated by immunosurgery on a Nikon SMZ dissection microscope, using rabbit anti-mouse serum, guinea-pig complement and acidified Tyrode's solutions (Sigma) (Solter and Knowles, 1975). Eight-cell embryos were collected at 2 days post-coitum and snap frozen for subsequent expression and chromatin analyses.

Antibodies

Antibodies used were as follows. For ChIP: anti-H3 (ab1791; Abcam), anti-H3K4me2 (07-030; Millipore), anti-H3K4me3 (ab8580; Abcam), anti-H3K27me3 (07-449; Millipore), anti-H3K9me3 (07-442; Millipore), anti-IgG (20259; Dako), anti-Ring1B (a kind gift from H. Koseki) (Atsuta et al., 2001), anti-Suz12 (Pab-029-050; Diagenode); anti-Suv39h1 (Abcam; ab12405); anti-hypophosphorylated (8WG16; Covance) and anti-Ser5-phosphorylated RBP1 CTD (4H8; Millipore) (Stock et al., 2007). For MeDIP: anti-5mC (BI-MECY-1000; Eurogentec). For western blotting: anti-Suz12 (07-379; Millipore), anti-Ring1B (Atsuta et al., 2001), anti-tubulin (ab6046; Abcam) and anti-mouse or anti-rabbit-HRP (Santa Cruz). For immunofluorescence: anti-Suz12 (07-379; Millipore), anti-Ring1B (Atsuta et al., 2001), anti-mouse IgG or anti-rabbit IgG conjugated with Alexa Fluor 488/568 (Molecular Probes).

Epigenetic profiling analysis

Histone ChIP on cross-linked chromatin from cells (Azuara et al., 2006) and sequential ChIP (Bernstein et al., 2006a); histone ChIP on native chromatin from tissues (Sanz et al., 2008); RNAP, PRC1 and PRC2 ChIP (Stock et al., 2007) and methylated DNA immunoprecipitation (MeDIP) analysis (Mohn et al., 2008) were carried out as previously described. Carrier ChIP followed by quantitative PCR (qPCR) was performed as described with minor modifications (O'Neill et al., 2006). S2 *Drosophila* cells (5×10^7 cells/ml) were mixed with eight-cell embryos (40n), compacted morula (20n), ICM or TE (10-12n) for immunoprecipitations. Native chromatin fragments were prepared by digestion with 5U of micrococcal nuclease (GE Healthcare) per 2.5 μg of chromatin, and were subsequently incubated with 15 μl of commercially available antibodies against modified histones or control IgG antibodies. Protein A sepharose (GE Healthcare) was added to immobilise chromatin-antibody complexes; unbound chromatin fractions were recovered upon centrifugation and bound complexes further washed in a series of stringent buffers. Elution was carried in 1% SDS prior to proteinase K (0.2 mg/ml; Sigma) treatment of both unbound and bound fractions. DNA was subsequently purified on gel extraction columns (Qiagen) into a final volume of 50-60 μl TE buffer. DNA concentrations were determined by PicoGreen fluorimetry (Invitrogen) and unbound fractions were diluted to match bound concentrations, before qPCR analysis using mouse-specific primers. Real-time qPCR analysis was carried out on a Chromo4 DNA engine (Bio-Rad, Hercules, CA), using SYBR green jumpstart PCR master mix (Sigma) and 300 nM primers. Each qPCR reaction was performed in triplicate. Primer sequences are listed in Table S1 in the supplementary material.

Expression analysis

Total RNA was isolated using the RNeasy Mini (cell line samples) or Micro (embryo samples) kits and DNase I treatment (Qiagen). Samples were reverse transcribed using Superscript II following the manufacturer's recommendations (Invitrogen) and analysed by qPCR. Primer sequences are listed in Table S2 in the supplementary material.

Western blot analysis

Protein concentrations of whole cell extracts were measured using the Bradford assay (Thermo Fisher Scientific). Samples (20 μg) were loaded onto 10% acrylamide gels and blotted onto methanol-activated polyvinylidene fluoride membranes using a semi-dry transfer system. After incubation and washing, membranes were treated with enhanced chemiluminescent substrate (Thermo Fisher Scientific).

Immunofluorescence analysis

Cells were seeded on gelatinised glass coverslips and fixed in PBS with 4% paraformaldehyde. Samples were permeabilised and blocked at room temperature before incubation with antibodies. Coverslips were mounted on Vectashield with DAPI (Vector Laboratories) and examined using a Leica SP5 confocal microscope (×40 or ×63 lenses).

RESULTS

Bivalent domains mark silent, lineage-inappropriate genes in trophoblast stem cells

In pre-implantation development the transition from morula to blastocyst is the starting point for lineage segregation into the inner cell mass (ICM) and trophectoderm (TE; Fig. 1A). ES cells are derived from the ICM and are pluripotent. By contrast, TE-restricted trophoblast stem (TS) cells are multipotent and contribute solely to placenta formation in vivo (Rossant and Tam, 2009). To investigate whether bivalent signatures are unique attributes of pluripotent cells in the early embryo, we directly compared the chromatin environment of key developmental genes in ES and TS cells. In particular, we focused on previously identified bivalent, CpG-rich promoters in ES cells (Azuara et al., 2006; Bernstein et al., 2006a; Mikkelsen et al., 2007) that were described as PRC2 and PRC1 joint targets (Fig. 1) (Ku et al., 2008). We have included in this study a panel of transcriptional regulators (*Atoh1*, *Gata4*, *Hoxa7*, *Kdr*, *Mixl1*, *Pax3*, *Sox1* and *Sox7*) covering different embryonic lineages, as well as TS-associated markers (*Cdx2*, *Fgfr2* and *Hand1*). Note that the genetic inactivation of most of these genes results in embryonic lethality (see Table S3 in the supplementary material).

Chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR) were performed to initially evaluate the abundance of repressive H3K27me3 and permissive H3K4me2 histone marks at selected promoters in ES and TS cells (Fig. 1B). For these analyses, qPCR primers were designed to amplify up to 600 bp upstream of the transcriptional start site of each gene. Modified histone levels were normalised against detected H3, and expressed as relative enrichment (% over H3). As anticipated, significant enrichment for H3K27me3 and H3K4me2 marks was detected at the promoters of silent genes in ES cells (Fig. 1B, left panel). Actively transcribed actin showed, by contrast, low H3K27me3 and high H3K4me2 levels, whereas an intergenic region used as negative control was not enriched for either histone modification. In TS cells, all of the silent promoters analysed (including *Atoh1*, *Sox1*, *Hoxa7*, *Mixl1*, *Gata4* and *Sox7*) also showed high levels of H3K27me3 together with H3K4me2, suggesting these genes remain bivalent despite the fact that they are not normally expressed upon placental development (see Fig. S1 in the supplementary material). By contrast, the active *Cdx2*, *Fgfr2*, *Hand1*, *Kdr* and *Pax3* promoters were only marked by H3K4me2 in TS cells, as expected (Fig. 1B, right panel).

The retention of bivalent domains in trophoblast-restricted cells at genes that are not required in this lineage was unexpected. To verify that methylated H3K27 and H3K4 co-exist on the same allele in TS cells, we performed a series of sequential ChIP experiments in which chromatin was immunoprecipitated firstly with H3K27me3 and secondly with H3K4me2 antibodies (Fig. 1C), and vice versa (data not shown). Results confirmed that all genes with bivalent domains were simultaneously enriched for both marks in TS cells, as well as in ES cells, in comparison with expressed genes and controls. Remarkably, substantial enrichment for both H3K4me2 and H3K4me3 was still detected at bivalently marked genes when TS cells were induced to differentiate into spongiotrophoblasts and trophoblast giant cells, even though these genes were not activated (see Fig. S2 in the supplementary material). Taken together, these data show that the restriction of developmental potency in TE-derived stem cells and differentiated progeny is not necessarily accompanied by a loss of activating marks at silent, lineage-inappropriate genes.

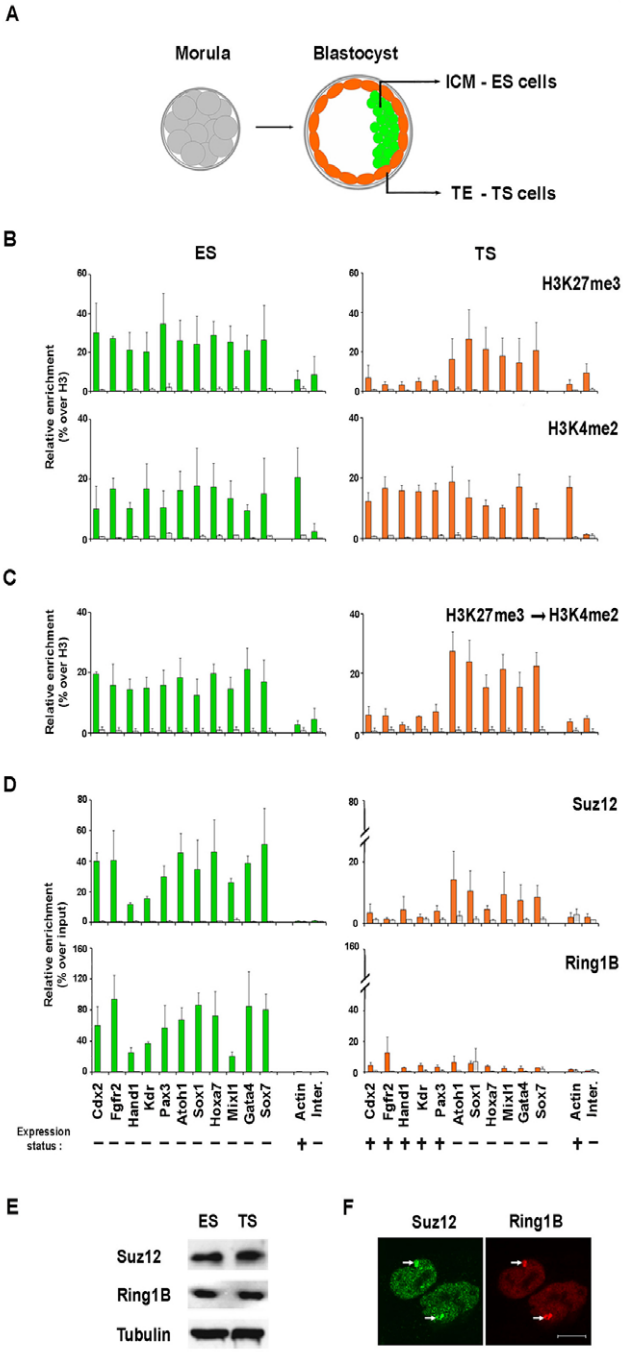

Fig. 1. Comparison of epigenetic features in blastocyst-derived stem cells. (**A**) Schematic of morula and blastocyst embryos, depicting lineage segregation of the inner cell mass (ICM) and trophectoderm (TE). ES and TS cells can be derived from the ICM and TE, respectively. (**B**) Relative enrichment of H3K27me3 and H3K4me2 at a panel of selected promoters and controls in ZHBTc4-ES (green bars) and B1-TS (orange bars) cells. Background levels (from control IgG antibodies) are shown as grey bars. (**C**) Sequential ChIP using anti-H3K27me3 and anti-H3K4me2 antibodies. Enrichment is expressed relative to H3. (**D**) Binding of PRC2 and PRC1 components Suz12 and Ring1B, respectively, was assessed by ChIP and qPCR. Enrichment is expressed relative to input. Error bars represent s.d. of three independent experiments. (**E**) Western blot analysis using anti-Suz12, anti-Ring1B and anti-tubulin (loading control) antibodies. (**F**) Immunofluorescence analysis in female B1-TS cells, showing colocalisation of Suz12 and Ring1B on inactive X chromosomes (white arrows). Scale bar: 10 μm.

Bivalent histone marking operates in vivo at silent genes in the early mouse embryo

To address whether bivalent domains are also present in vivo, we examined the histone profile of key developmental genes in the ICM and TE of newly formed blastocysts as well as in early eight-cell and morula-stage embryos. Individual ICMs were isolated by immunosurgery of early-stage blastocysts, whereas mural TE samples were prepared by manual dissection (Fig. 2A). Typically, 10-12 ICM or TE preparations were combined per immunoprecipitation. Eight-cell embryos (40n) and compacted morula (20n) were freshly collected and pooled for subsequent analyses. Histone modifications were analysed using carrier ChIP (C-ChIP) followed by qPCR (O'Neill et al., 2006; VerMilyea et al., 2009). C-ChIP allows small pools of ex vivo populations to be analysed by mixing with excess carrier *Drosophila* S2 cells, before preparation of nuclei and chromatin (see protocol outlined in Fig. 2B). Using this approach, we assessed the relative enrichment for H3K27me3 and H3K4me2 at the promoters of *Sox1* and *Gata4* – two examples of bivalently marked genes in both ES and TS cells – as well as of *Pou5f1* and *Cdx2*, which show differential gene expression patterns upon pre-implantation development (Dietrich and Hiiragi, 2007; Jedrusik et al., 2008). *Pou5f1* transcripts were detected in eight-cell and morula embryos and remained present in both ICM and TE tissues of newly formed blastocysts, whereas *Cdx2* was predominantly expressed in the TE, as expected (see Fig. S3 in the supplementary material).

Sox1 and *Gata4* were undetectable in eight-cell embryos onwards (see Fig. S3 in the supplementary material). In agreement with the lack of expression, these promoters displayed high levels of the repressive H3K27me3 marks in all four populations analysed, in contrast to actively transcribed *Pou5f1*, which shows low H3K27me3 and high H3K4me2 levels (Fig. 2C). H3K4me2 was also detected alongside H3K27me3 at *Sox1* and *Gata4*, demonstrating the occurrence of bivalent domains in vivo. These domains were seen from the eight-cell embryo up to the blastocyst stage at silent loci, suggesting that they are established early during pre-implantation development and are stably retained upon blastocyst formation in both ICM and TE lineages. *Cdx2* showed, by contrast, a more dynamic histone profile, being enriched for both marks in early eight-cell embryos and ICM cells where this gene is expressed at low or variable levels, but depleted of H3K27me3 marks in TE-committed cells (Fig. 2C, bottom panel). Together, these results corroborate our in vitro data in ES and TS cells, and further reveal that bivalent histone signatures associated with gene silencing exist in the early mouse embryo, prior to blastocyst formation and during the blastocyst stage.

Ring1B and poised RNAP are not recruited to genes that retain bivalent signatures in TE-derived stem cells

To further explore the chromatin status of bivalent, lineage-inappropriate genes in trophoblast-committed cells, we assessed the patterns of PRC2 and PRC1 binding at selected promoters in TS cells as compared with ES cells. As anticipated from the presence of H3K27me3, the PRC2 component Suz12 was found at all bivalent promoters analysed in both populations, although relatively lower levels were seen in TS cells (Fig. 1D). However, in striking contrast to ES cells, silent, Suz12-bound genes showed only low (or undetectable) enrichment for the PRC1 component Ring1B in TS cells, as seen at their active counterparts. These altered profiles of Suz12 and Ring1B binding did not correlate with overall changes in protein levels in the two cell types (Fig. 1E). The presence of Ring1B proteins in TS cells was also verified by immunofluorescence, confirming the colocalisation of Ring1B and Suz12 components on the inactive X chromosome of female trophoblast stem cells, as expected (Fig. 1F) (de Napoles et al., 2004; Fang et al., 2004).

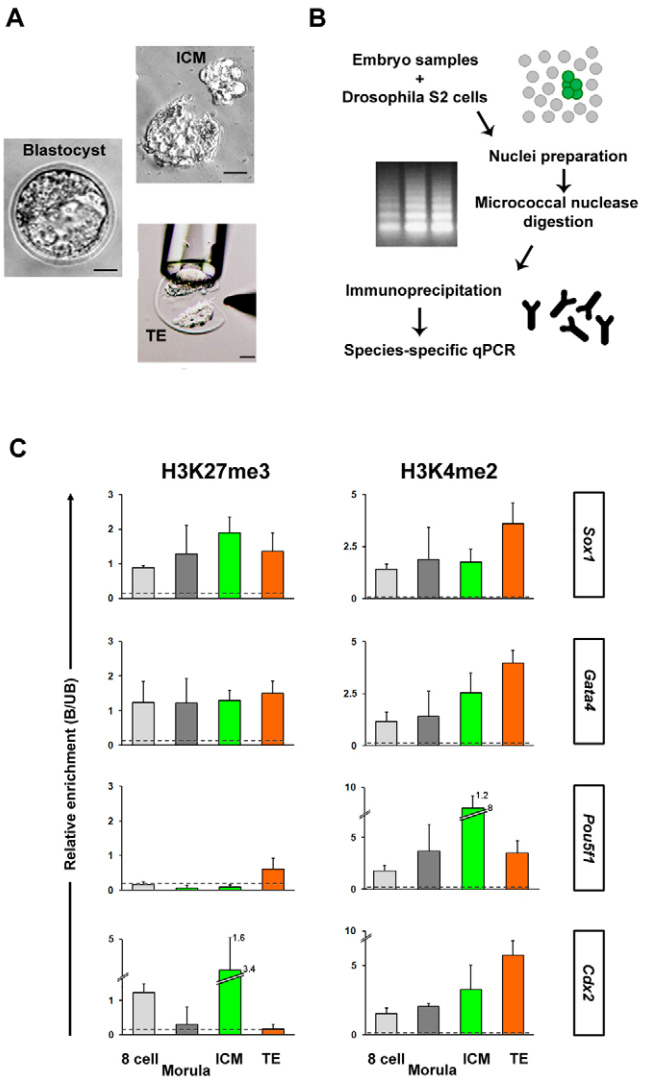

Fig. 2. In vivo analysis of bivalent domains at selected promoters in early mouse embryos prior to and after blastocyst lineage segregation. (**A**) Typical early-stage blastocyst selected for these experiments. ICM samples were isolated by immunosurgery based on selective, complement-induced lysis of trophoblast cells, which can then be washed away to leave a relatively pure ICM preparation. Mural TE samples were prepared by manual dissection; blastocysts were retained by a holding pipette and part of TE cut away to ensure no contamination with ICM. (**B**) Diagrammatic summary of the carrier ChIP (C-ChiP) protocol. (**C**) Quantification by C-ChIP of H3K27me3 and H3K4me2 levels (bound/unbound ratios) at the promoters of *Sox1*, *Gata4*, *Pou5f1* and *Cdx2* in eight-cell (light grey), compacted morula (grey), ICM (green) and TE (orange). Antibody-bound and unbound chromatin fractions were amplified in parallel by qPCR using the same quantity of DNA. Unspecific precipitation was monitored by control IgG antibodies; background levels are denoted by dotted lines. Error bars represent s.d. of three independent experiments.

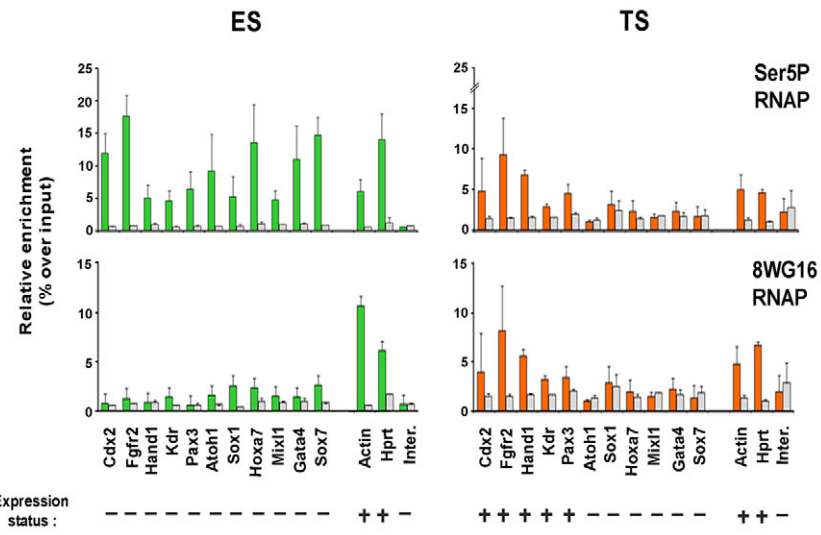

Fig. 3. RNA polymerase II occupancy and conformation at bivalent genes in ES and TS cells. Abundance of Ser5-phosphorylated (Ser5P) or hypophosphorylated (8WG16) RNAP at bivalent, active promoters, and intergenic control, in ZHBTc4-ES (green bars) and B1-TS (orange bars) cells. Background levels (from control antibodies) are shown as grey bars. Enrichment was assessed by ChIP and qPCR and is expressed relative to input. Error bars represent s.d. of three independent experiments.

PRC1 and RNAP act together in ES cells to maintain a poised chromatin state at bivalent genes. To investigate whether bivalently marked genes in TS cells are also primed by RNAP, we next examined the recruitment and conformation of these complexes by ChIP assay using previously validated antibodies against hypophosphorylated (8WG16) or Ser5-phosphorylated (Ser5P; 4H8) RNAP (Stock et al., 2007). As shown in Fig. 3, bivalent genes and active (actin and *Hprt*) controls were found to be highly enriched for Ser5P RNAP at their promoter regions in ES cells. A poised RNAP conformation was discriminated by measuring the level of 8WG16 binding, which was low at bivalent promoters but abundant at actively transcribed controls, as previously reported (Guenther et al., 2007; Stock et al., 2007). In TS cells, Ser5P and 8WG16 RNAP both marked expressed *Cdx2*, *Fgfr2*, *Hand1*, *Kdr* and *Pax3* genes and active controls, as expected (Fig. 3, right panel). RNAP complexes were, by contrast, virtually absent from the promoters of *Atoh1*, *Sox1*, *Hoxa7*, *Mixl1*, *Gata4* and *Sox7*, which is consistent with a loss of gene priming for transcription at these loci in TS cells.

Selective induction of the H3K9 tri-methyltransferase Suv39h1 upon trophoblast lineage commitment

The loss of Ring1B and RNAP recruitment at bivalently marked promoters in TS cells, as compared with ES cells, suggests that alternative repressive pathways might operate to regulate these loci in extra-embryonic and embryonic tissues. To further investigate the relationship between lineage identity and targeted chromatin changes, we exploited the potential to convert ES cells into TS-like cells, using a previously established ES cell line that carries a Doxycycline (Dox)-regulated *Pou5f1* transgene (ZHBTc4) (Niwa et al., 2000) (Fig. 4A). In this system, Dox-treated ES cell colonies gradually acquire a TS-like (TSL) morphology upon *Pou5f1* shutdown and provision of permissive conditions (Fig. 4B), whereas established TSL cell cultures show uniform staining for the TE-associated marker *Cdx2*, as seen in TS cells (Fig. 4C). Here, we surveyed by qRT-PCR the relative mRNA levels for a cohort of chromatin regulators, including PRC components, histone lysine methylases (KMTs) and demethylases (KDMs) as well as DNA methyltransferases (DNMTs), in ES and TS cells, and looked at dynamic gene expression alterations upon *Pou5f1* shutdown and TSL cell derivation (Fig. 4D).

In agreement with previous reports (Mak et al., 2004; Matoba et al., 2006), we verified that *Eed* expression is rapidly and significantly reduced, in contrast to that of its partners *Ezh2* and *Suz12*, in Dox-treated ES, TSL and TS cells, as compared with ES cells. Surprisingly, we identified a pronounced imbalance in mRNA levels for several H3K9 KMTs [*Ehmt1*, *G9a* (*Ehmt2*), *Suv39h1* and *Suv39h2*] and H3K9 KDMs (*Jmjd1a* and *Jmjd2c*; also known as *Kdm3a* and *Kdm4c*, respectively) between ES and TS cell populations. In particular, H3K9 tri-/di-demethylase *Jmjd2c* (Cloos et al., 2006; Wissmann et al., 2007), which is highly expressed in undifferentiated ES cells (Loh et al., 2008) was promptly downregulated upon *Pou5f1* shutdown (Fig. 4E). Conversely, H3K9 tri-methyltransferase *Suv39h1* was dramatically upregulated upon TSL cell derivation and *Cdx2* induction with a 30-fold increase in TSL and TS cells relative to ES cells (Fig. 4E). By comparison, *Suv39h2*, *G9a* or *Ehmt1* showed a relatively modest induction (from 4- to 6-fold) upon ES cell differentiation, whereas *Eset* (also known as *Setdb1*) remained expressed at similar levels in all populations analysed (Fig. 4E, bottom panel). These results point to a key role for Suv39h1 in the establishment or maintenance of a TSL cell fate; an observation that is consistent with the selective induction of *Suv39h1* (in contrast to *Suv39h2*, *G9a*, *Ehmt1* or *Eset*) in TE relative to ICM or morula cells in vivo (Fig. 4F).

H3K9 tri-methylation specifies epigenetic asymmetry at bivalent genes between ICM and TE lineages

To address whether Suv39h1 could directly target bivalent genes upon trophoblast lineage commitment and to assess its functional implication, we compared the levels of Suv39h1 occupancy, H3K9 tri-methylation and associated DNA methylation (Fuks et al., 2003) at the same panel of developmental regulators and control (actin and γ-*satellite*) loci in ES, Dox-treated ES (ES^{Dox}48h), TSL and TS cells (Fig. 5; see also Fig. S4 in the supplementary material). As anticipated, *Cdx2*, *Fgfr2*, *Hand1*, *Flk1* and *Pax3* expression was upregulated to levels seen in TS cells upon Dox treatment and TSL cell derivation (see Fig. S5 in the supplementary material). Thus, little H3K9me3 (Fig. 5A) or DNA methylation (Fig. 5B) associated with gene silencing was detected at these induced promoters that were marked by H3K4me2 only in TSL cells, as previously seen in TS cells (see Fig. S4 in the supplementary material). By contrast,

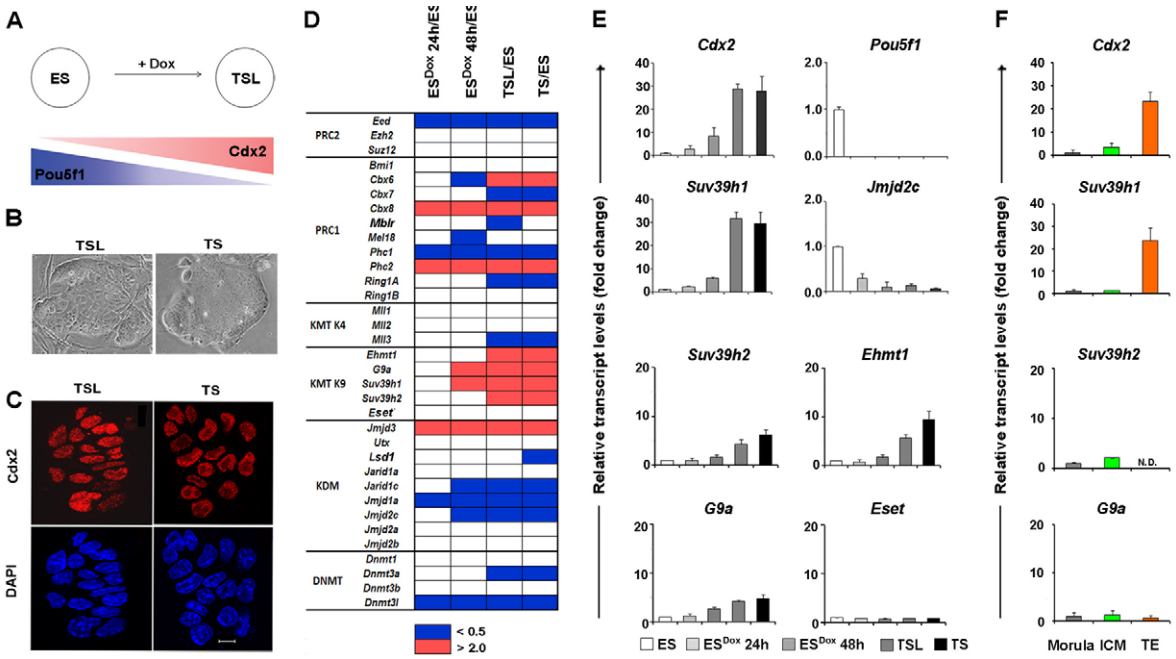

Fig. 4. Developmentally regulated changes in gene expression profiles of chromatin regulators upon *Pou5f1* repression and TS-like cell derivation. (**A**) Schematic of *Pou5f1* shutdown and *Cdx2* activation induced upon Doxycyline (Dox) treatment of ZHBTc4-ES cells. Differentiation into TS-like (TSL) cells was accomplished by culture on inactivated primary embryonic fibroblasts and in the presence of FGF4 and heparin. (**B**) Light microscope images comparing the morphology of ZHBTc4-derived TS-like (TSL) and B1-TS (TS) colonies. (**C**) Immunofluorescence analysis showing homogenous expression of the TS-associated marker Cdx2 in TSL and TS cells. Note that no Cdx2 signal was detected in untreated ZHBTc4-ES cells, as expected (data not shown). (**D**) The expression of selected chromatin regulators was compared in ZHBTc4-ES cells after 24 and 48 hours of Dox treatment (ES^{Dox}) in the presence of LIF, and in ZHBTc4-derived TSL (TSL) cells and B1-TS (TS) cells, using quantitative RT-PCR (qRT-PCR). Values shown are normalised to actin and *L19* and are expressed relative to untreated ZHBTc4-ES cells (ES=1). Upregulation (mean±s.d.>2) and downregulation (mean±s.d.<0.5) are coloured in red and blue, respectively. (**E**) Histograms showing the expression pattern of *Cdx2*, *Pou5f1*, *Suv39h1*, *Jmjd2c*, *Suv39h2, Ehmt1*, *G9a* and *Eset* upon *Pou5f1* shutdown and TSL cell derivation. Error bars represent s.d. of three independent experiments. (**F**) Expression levels of *Cdx2*, *Suv39h1*, *Suv39h2* and *G9a*, as assessed by qRT-PCR, in morula (grey), dissected ICM (green) and TE (orange) sample preparations. Values shown are normalised to *S17* and *L19* mRNA levels and are expressed relative to morula samples (morula=1). Error bars represent s.d. of three independent experiments. Note that *Ehmt1* and *Eset* transcript levels were below detection in all three samples under the same experimental conditions (data not shown). N.D., not detectable.

Atoh1, *Sox1*, *Hoxa7*, *Mixl1*, *Gata4* and *Sox7* genes remained inactive (see Fig. S5 in the supplementary material) and bivalently marked (see Fig. S4 in the supplementary material) in Dox-treated ES and TSL cells. These genes, however, became selectively targeted by Suv39h1 and rapidly acquired substantial levels of H3K9me3 upon loss of pluripotency and trophoblast lineage commitment (Fig. 5A; see also Fig. S4 in the supplementary material). Conversely, *Suv39h1* knockdown by RNAi in TS cells (Fig. 5C) led to a reduction of H3K9me3 levels at bivalently marked genes, thereby confirming Suv39h1-dependent H3K9 methylation (Fig. 5D; see also Fig. S6 in the supplementary material). This establishes an unusual trivalent (H3K9me3-H3K27me3-H3K4me2) histone profile at silent, lineage-inappropriate genes in TS cells, as further verified by sequential ChIP assays (Fig. 5E). Interestingly, PRC1 (Ring1B) together with Ser5P RNAP forms were transiently retained at un-induced loci, as opposed to at activated genes in Dox-treated ES cells, yet were released in TSL and TS cells, suggesting that the loss of gene priming and the acquisition of Suv39h1-mediated H3K9me3 at bivalent genes are likely to be coordinated (see Fig. S4 in the supplementary material). Note, however, that Suv39h1 is not required to prevent transcriptional activation of *Atoh1*, *Sox1*, *Hoxa7*, *Mixl1*, *Gata4* and *Sox7* in Dox-treated ES cells (data not shown) or TS cells (see Fig. S6 in the supplementary material), but might rather stabilise a repressed state at silent, bivalent genes in synergy with other epigenetic marks, including H3K27me3. Consistently, chromatin changes from primed to repressed states at Suv39h1-bound genes were reinforced by de novo DNA methylation associated with stable silencing (Lande-Diner and Cedar, 2005) in TSL and TS cells in the absence of Ring1B (Fig. 5B; see also Fig. S4 in the supplementary material).

To further verify that bivalent, lineage-inappropriate genes are selectively targeted by Suv39h1-mediated H3K9 methylation upon TE formation in vivo, we examined the levels of H3K9me3 at *Sox1*, *Gata4* and *Cdx2* promoters prior to (morula) and after (ICM and TE) blastocyst formation, by C-ChIP and qPCR (Fig. 5F). The results showed that *Sox1* and *Gata4* genes harboured high levels of H3K9me3 in addition to H3K27me3 and H3K4me2 in the TE, but not in the ICM; an epigenetic signature also maintained in the developing placenta in vivo (data not shown). Moreover, H3K9me3 levels were low in compacted morula, which further suggested that the acquisition of the repressive H3K9me3 marks might be a late event linked with trophoblast cell fate consolidation and loss of lineage plasticity. By contrast, actively transcribed *Cdx2* remained depleted of H3K9me3 in the TE, as expected (Fig. 5F; bottom panel). Together, our results indicate that alternative silencing strategies operate in a lineage-specific manner at key developmental regulators in the early embryo.

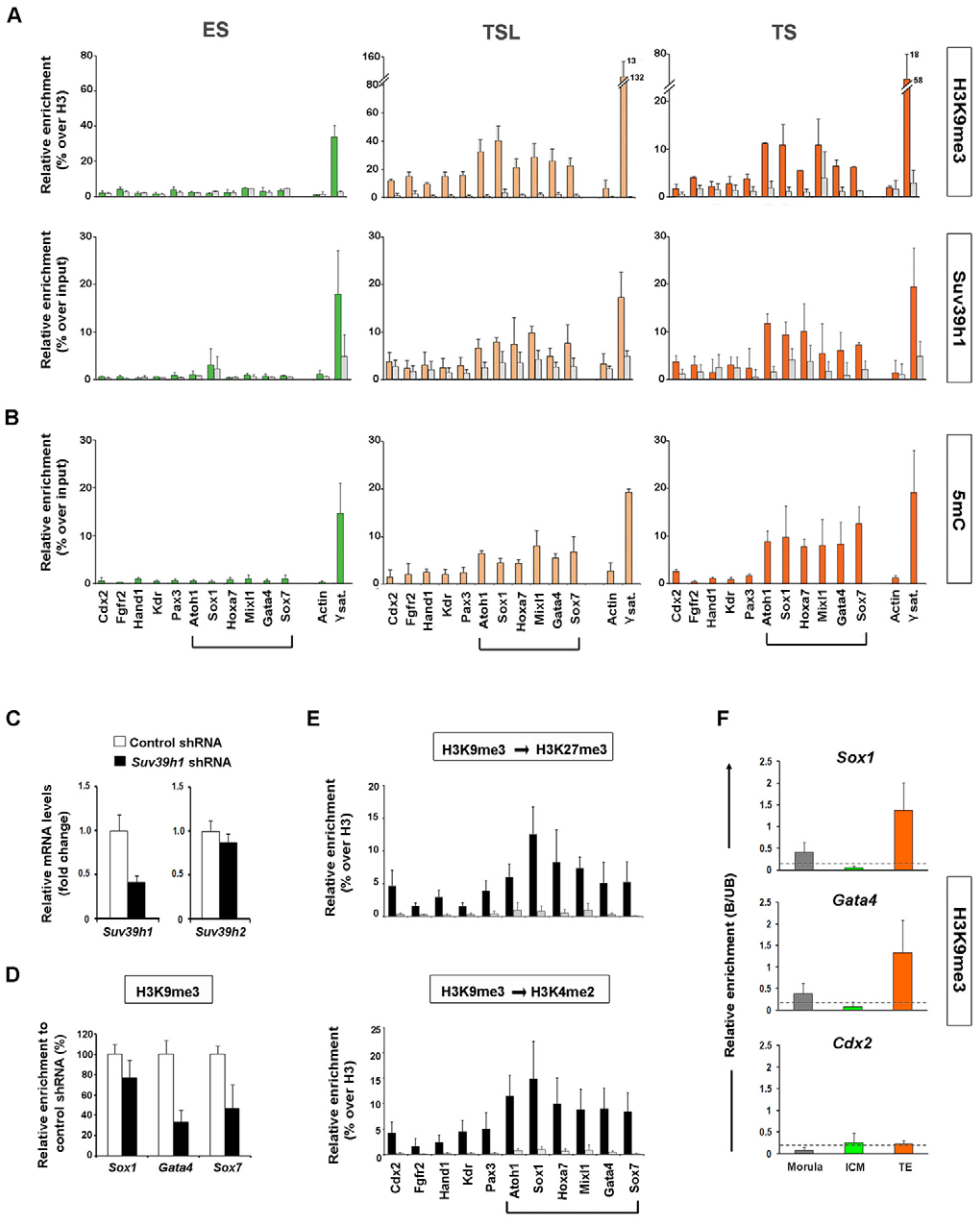

Fig. 5. Bivalent genes are targeted by Suv39h1-mediated H3K9me3 upon trophectoderm lineage commitment in vitro and in vivo. (**A**,**B**) The abundance of H3K9me3 and Suv39h1 was assessed by ChIP (A) and DNA methylation (5mC) by MeDIP (B) at candidate gene promoters and controls in ZHBTc4-ES (green; ES), ZHBTc4-derived TS-like (light orange; TSL) and B1-TS (orange; TS) cells. Background levels (from control antibodies) are shown as grey bars. Genes that remain inactive and bivalently marked in TSL and TS cells are highlighted. Enrichment is expressed relative to H3 or input. Error bars represent s.d. of three independent experiments. (**C**) Expression levels of *Suv39h1* and *Suv39h2*, as assessed by qRT-PCR, in TS cells after knockdown using *Suv39h1* shRNA construct. Values shown are normalised to *S17* and *L19* mRNA levels and are expressed relative to control TS cells. Error bars represent s.d. of three independent experiments. (**D**) Quantification by C-ChIP and qPCR of H3K9me3 levels at the promoters of *Sox1*, *Gata4* and *Sox7* in *Suv39h1*-knockdown TS cells relative to control cells after normalising against their respective unbound fractions and IgG controls. Error bars represent s.d. of three independent experiments. (**E**) Sequential ChIP using anti-H3K9me3 and anti-H3K27me3 (upper panel), or anti-H3K9me3 and anti-H3K4me2 (bottom panel) antibodies in B1-TS cells. Enrichment is expressed relative to H3. Error bars represent s.d. of three independent experiments. (**F**) Quantification by C-ChIP and qPCR of H3K9me3 levels at the promoters of *Sox1*, *Gata4* and *Cdx2* in compacted morula (grey), ICM (green) and TE (orange). Unspecific precipitation was monitored by control IgG antibodies; background levels are denoted by dotted lines. Error bars represent s.d. of three independent experiments.

DISCUSSION

In eukaryotes, H3K9 tri-methyltransferases Suv39h1 and Suv39h2 are commonly associated with constitutive heterochromatin formation and function. In the zygote, however, paternal constitutive heterochromatin lacks Suv39h-mediated H3K9me3, and is instead targeted by PRC1 components, including Ring1B (Arney et al., 2002; Probst et al., 2007; Puschendorf et al., 2008; Santos et al., 2005). This differential loading of Ring1B and Suv39h onto heterochromatin underlies parental genome asymmetry in the zygote and is subsequently maintained through cell divisions up to the eight-cell stage (Puschendorf et al., 2008). In this study, we find that Ring1B and Suv39h1 selectively target

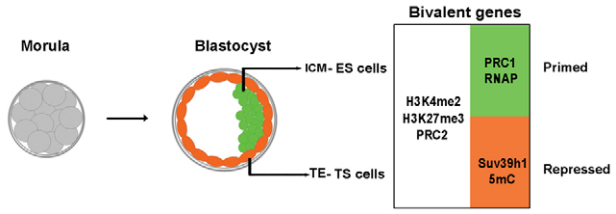

Fig. 6. Model for the establishment of distinct chromatin states at bivalent genes in vivo upon blastocyst lineage segregation. RNA polymerase II (RNAP) and Ring1B (PRC1) depletion at Suz12 (PRC2)-bound bivalent (H3K4me2 and H3K27me3) loci is associated with the acquisition of an extra-embryonic fate and transcriptional repression by Suv39h1-mediated H3K9me3 and DNA methylation (5mC).

key developmental genes in a lineage-specific manner at the blastocyst stage, suggesting that Ring1B and Suv39h1 mutual exclusion could occur throughout pre-implantation development.

In particular, we propose that PRC1 (Ring1B) and poised RNAP – two hallmarks of gene priming in pluripotent cells, are dynamically removed from the promoters of bivalent genes upon TE cell fate acquisition and Suv39h1 recruitment (see model in Fig. 6). In this model, H3K9me3 and downstream repressive activities (e.g. DNA methylation) would reinforce the repressed state of Suv39h1-bound genes upon loss of Ring1B. Absence of Ring1B enrichment at these loci does not, however, exclude the possible recruitment of other PRC1-like complexes in TE-restricted cells, as compared with pluripotent cells, owing to the dynamic composition of Polycomb complexes during development (Otte and Kwaks, 2003; Schuettengruber and Cavalli, 2009). This is exemplified by the reverse expression pattern of *Phc1* and *Phc2* in ES and TS cells (Fig. 4D) and the differential recruitment of these proteins on the inactive X chromosome in the two cell types (de Napoles et al., 2004). The distribution and density of methylated H3K27 and H3K9 marks in the vicinity of bivalent promoters could also alter the affinity of Cbx members, as has been previously suggested (Bernstein et al., 2006b).

An important aspect of our findings is the addition of the key repressive marks, H3K9me3 alongside DNA hypermethylation at promoters that retain high levels of methylated H3K4; an epigenetic state also reported in embryonic carcinoma cells (Ohm et al., 2007). This contrasts with prevailing views that loss of H3K4 methylation is a prerequisite for 'locking-in' a repressed state at bivalent genes that are not activated upon differentiation (Bernstein et al., 2006a; Meissner et al., 2008; Mohn et al., 2008). Our study reveals a novel mechanism by which 'bivalency' (and gene priming) can be resolved in extra-embryonic restricted cells through Suv39h1 recruitment and the formation of a trivalent (H3K9me3-H3K27me3-H3K4me2) histone conformation at silent, somatic-associated genes. Interestingly, trivalent histone configurations were recently uncovered using a genome-wide approach at a limited set of genes encoding trophoblast and early differentiation markers in ES cells (Bilodeau et al., 2009; Yuan et al., 2009). This indicates that 'trivalency' might not be particular to the trophoblast lineage. In pluripotent cells, however, many of these genes are occupied by a different member of the H3K9 KMT family, *Eset* (also known as *Setdb1*), highlighting the distinct roles and/or target specificity of these chromatin regulators in early development and stem cells. However, whether Ring1B and poised RNAP are also excluded from *Eset*-bound promoters remains to be elucidated.

Importantly, we provide in vivo evidence that bivalent chromatin domains exist at silent developmental regulators in the early mouse embryo. These domains are present in eight-cell embryos and persist throughout pre-implantation development, providing a platform for further protein recruitment that would, in turn, dictate the fate of bivalent genes (repressed versus primed) upon lineage commitment. Although trophoblast lineage 'fixation' is shown to involve Suv39h1 recruitment at bivalent, lineage-inappropriate genes, it is equally possible that transcriptional priming and pluripotency is retained through active protection from H3K9 and/or DNA methylation. Interestingly, and in keeping with this idea, *Suv39h1* and *Jmjd2c*, which mediate H3K9 tri-methylation and demethylation, respectively, display dynamic and opposite gene expression patterns in vivo. Whereas *Jmjd2c* is highly expressed in early cleavage-stage embryos (Wang et al., 2009), *Suv39h1* mRNA levels gradually increase during pre-implantation development with a peak at the transition from morula to blastocyst (Puschendorf et al., 2008). In our study, these two epigenetic factors are also shown to be differentially expressed in blastocyst-derived stem cells (Fig. 4D). As previously reported, *Jmjd2c* is instrumental in stably maintaining ES cell identity in vitro (Loh et al., 2008). Similarly, we find that RNAi depletion of *Suv39h1* in TS cells causes a loss of TS cell markers (*Cdx2*, *Eomes*, *Fgfr2* and *Gata3*) and induction of differentiation-associated markers (*Pl1* and *Tpbpa*), suggesting that *Suv39h1* might also be required for TS cell self-renewal (see Fig. S7 in the supplementary material).

In summary, we show that Ring1B and Suv39h1 delineate distinct chromatin states at bivalent genes in a lineage-specific manner during early mouse lineage commitment. A key function for Ring1B in the epigenetic gene regulation of early embryos is consistent with the embryonic lethality of Ring1B-mutant mice (Voncken et al., 2003). By contrast, Suv39h1/h2 mutant mice show a relatively mild phenotype, which probably reflects the synergy of repressive marks (Peters et al., 2001). A possible role for Suv39h1 at early-stages of development is, however, strongly supported by its selective induction (in contrast to other H3K9 KMTs) in the newly formed trophectoderm. To address whether Ring1B loss and/or Suv39h function directly impacts on early cell fate decisions will require further in vivo lineage-tracing investigations.

Acknowledgements
We thank N. Brockdorff for the B1-TS cell line, H. Koseki for anti-Ring1B antibodies, H. Niwa for the ZHBTc4-ES cell line, and C. Williamson and B. Owen for placenta samples. J. Godwin, T. Rodriguez, M. D. VerMilyea and Z. Webster are thanked for help and advice on embryo collection and manipulation. N. Dillon, H. Jorgensen, M. Parker, A. Peters and T. Rodriguez are acknowledged for discussion and critical reading of the manuscript. This work was supported by the IOGT, BBSRC, MRC, FCT-Portugal and Imperial College London. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.048363/-/DC1

References
Arney, K. L., Bao, S., Bannister, A. J., Kouzarides, T. and Surani, M. A. (2002). Histone methylation defines epigenetic asymmetry in the mouse zygote. *Int. J. Dev. Biol.* **46**, 317-320.
Atsuta, T., Fujimura, S., Moriya, H., Vidal, M., Akasaka, T. and Koseki, H. (2001). Production of monoclonal antibodies against mammalian Ring1B proteins. *Hybridoma* **20**, 43-46.
Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jorgensen, H. F., John, R. M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M. et al. (2006). Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* **8**, 532-538.

Bantignies, F. and Cavalli, G. (2006). Cellular memory and dynamic regulation of polycomb group proteins. *Curr. Opin. Cell Biol.* **18**, 275-283.
Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Schones, D. E., Wang, Z., Wei, G., Chepelev, I. and Zhao, K. (2007). High-resolution profiling of histone methylations in the human genome. *Cell* **129**, 823-837.
Bernstein, B. E., Mikkelsen, T. S., Xie, X., Kamal, M., Huebert, D. J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K. et al. (2006a). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315-326.
Bernstein, E., Duncan, E. M., Masui, O., Gil, J., Heard, E. and Allis, C. D. (2006b). Mouse polycomb proteins bind differentially to methylated histone H3 and RNA and are enriched in facultative heterochromatin. *Mol. Cell. Biol.* **26**, 2560-2569.
Bilodeau, S., Kagey, M. H., Frampton, G. M., Rahl, P. B. and Young, R. A. (2009). SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. *Genes Dev.* **23**, 2484-2489.
Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev.* **16**, 6-21.
Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K. et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**, 349-353.
Bracken, A. P., Dietrich, N., Pasini, D., Hansen, K. H. and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes Dev.* **20**, 1123-1136.
Brookes, E. and Pombo, A. (2009). Modifications of RNA polymerase II are pivotal in regulating gene expression states. *EMBO Rep.* **10**, 1213-1219.
Chamberlain, S. J., Yee, D. and Magnuson, T. (2008). Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. *Stem Cells* **26**, 1496-1505.
Cloos, P. A., Christensen, J., Agger, K., Maiolica, A., Rappsilber, J., Antal, T., Hansen, K. H. and Helin, K. (2006). The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature* **442**, 307-311.
Cui, K., Zang, C., Roh, T. Y., Schones, D. E., Childs, R. W., Peng, W. and Zhao, K. (2009). Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. *Cell Stem Cell* **4**, 80-93.
de Napoles, M., Mermoud, J. E., Wakao, R., Tang, Y. A., Endoh, M., Appanah, R., Nesterova, T. B., Silva, J., Otte, A. P., Vidal, M. et al. (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev. Cell* **7**, 663-676.
Delaval, K. and Feil, R. (2004). Epigenetic regulation of mammalian genomic imprinting. *Curr. Opin. Genet. Dev.* **14**, 188-195.
Dietrich, J. E. and Hiiragi, T. (2007). Stochastic patterning in the mouse pre-implantation embryo. *Development* **134**, 4219-4231.
Endoh, M., Endo, T. A., Endoh, T., Fujimura, Y., Ohara, O., Toyoda, T., Otte, A. P., 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.
Epsztejn-Litman, S., Feldman, N., Abu-Remaileh, M., Shufaro, Y., Gerson, A., Ueda, J., Deplus, R., Fuks, F., Shinkai, Y., Cedar, H. et al. (2008). De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. *Nat. Struct. Mol. Biol.* **15**, 1176-1183.
Fang, J., Chen, T., Chadwick, B., Li, E. and Zhang, Y. (2004). Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X inactivation. *J. Biol. Chem.* **279**, 52812-52815.
Farthing, C. R., Ficz, G., Ng, R. K., Chan, C. F., Andrews, S., Dean, W., Hemberger, M. and Reik, W. (2008). Global mapping of DNA methylation in mouse promoters reveals epigenetic reprogramming of pluripotency genes. *PLoS Genet.* **4**, e1000116.
Fuks, F., Hurd, P. J., Deplus, R. and Kouzarides, T. (2003). The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res.* **31**, 2305-2312.
Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R. and Young, R. A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* **130**, 77-88.
Jaenisch, R. and Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* **132**, 567-582.
Jedrusik, A., Parfitt, D. E., Guo, G., Skamagki, M., Grabarek, J. B., Johnson, M. H., Robson, P. and 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, H. F., Giadrossi, S., Casanova, M., Endoh, M., Koseki, H., Brockdorff, N. and Fisher, A. G. (2006). Stem cells primed for action: polycomb repressive complexes restrain the expression of lineage-specific regulators in embryonic stem cells. *Cell Cycle* **5**, 1411-1414.
Ku, M., Koche, R. P., Rheinbay, E., Mendenhall, E. M., Endoh, M., Mikkelsen, T. S., Presser, A., Nusbaum, C., Xie, X., Chi, A. S. et al. (2008). Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. *PLoS Genet.* **4**, e1000242.
Kutner, R. H., Zhang, X. Y. and Reiser, J. (2009). Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. *Nat. Protoc.* **4**, 495-505.
Lande-Diner, L. and Cedar, H. (2005). Silence of the genes-mechanisms of long-term repression. *Nat. Rev. Genet.* **6**, 648-654.
Lee, T. I., Jenner, R. G., Boyer, L. A., Guenther, M. G., Levine, S. S., Kumar, R. M., Chevalier, B., Johnstone, S. E., Cole, M. F., Isono, K. et al. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* **125**, 301-313.
Leeb, M. and Wutz, A. (2007). Ring1B is crucial for the regulation of developmental control genes and PRC1 proteins but not X inactivation in embryonic cells. *J. Cell Biol.* **178**, 219-229.
Loh, Y.-H., Zhang, W., Chen, X., George, J. and Ng, H.-H. (2008). Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. *Genes & Development* **21**, 2545-2547.
Mak, W., Nesterova, T. B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A. P. and Brockdorff, N. (2004). Reactivation of the paternal X chromosome in early mouse embryos. *Science* **303**, 666-669.
Matoba, R., Niwa, H., Masui, S., Ohtsuka, S., Carter, M. G., Sharov, A. A. and Ko, M. S. (2006). Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. *PLoS ONE* **1**, e26.
Meissner, A., Mikkelsen, T. S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B. E., Nusbaum, C., Jaffe, D. B. et al. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* **454**, 766-770.
Mikkelsen, T. S., Ku, M., Jaffe, D. B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T. K., Koche, R. P. et al. (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**, 553-560.
Mohn, F., Weber, M., Rebhan, M., Roloff, T. C., Richter, J., Stadler, M. B., Bibel, M. and Schubeler, D. (2008). Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. *Mol. Cell* **30**, 755-766.
Niwa, H., Miyazaki, J. and Smith, A. G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24**, 372-376.
Ohm, J. E., McGarvey, K. M., Yu, X., Cheng, L., Schuebel, K. E., Cope, L., Mohammad, H. P., Chen, W., Daniel, V. C., Yu, W. et al. (2007). A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat. Genet.* **39**, 237-242.
O'Neill, L. P., VerMilyea, M. D. and Turner, B. M. (2006). Epigenetic characterization of the early embryo with a chromatin immunoprecipitation protocol applicable to small cell populations. *Nat. Genet.* **38**, 835-841.
Otte, A. P. and Kwaks, T. H. (2003). Gene repression by Polycomb group protein complexes: a distinct complex for every occasion? *Curr. Opin. Genet. Dev.* **13**, 448-454.
Pan, G., Tian, S., Nie, J., Yang, C., Ruotti, V., Wei, H., Jonsdottir, G. A., Stewart, R. and Thomson, J. A. (2007). Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. *Cell Stem Cell* **1**, 299-312.
Peters, A. H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A. et al. (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107**, 323-337.
Pietersen, A. M. and van Lohuizen, M. (2008). Stem cell regulation by polycomb repressors: postponing commitment. *Curr. Opin. Cell Biol.* **20**, 201-207.
Probst, A. V., Santos, F., Reik, W., Almouzni, G. and Dean, W. (2007). Structural differences in centromeric heterochromatin are spatially reconciled on fertilisation in the mouse zygote. *Chromosoma* **116**, 403-415.
Puschendorf, M., Terranova, R., Boutsma, E., Mao, X., Isono, K., Brykczynska, U., Kolb, C., Otte, A. P., Koseki, H., Orkin, S. H. et al. (2008). PRC1 and Suv39h specify parental asymmetry at constitutive heterochromatin in early mouse embryos. *Nat. Genet.* **40**, 411-420.
Reik, W. (2007). Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* **447**, 425-432.
Ringrose, L. and Paro, R. (2007). Polycomb/Trithorax response elements and epigenetic memory of cell identity. *Development* **134**, 223-232.
Roh, T. Y., Cuddapah, S., Cui, K. and Zhao, K. (2006). The genomic landscape of histone modifications in human T cells. *Proc. Natl. Acad. Sci. USA* **103**, 15782-15787.
Rossant, J. and Tam, P. P. (2009). Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. *Development* **136**, 701-713.
Santos, F., Peters, A. H., Otte, A. P., Reik, W. and Dean, W. (2005). Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. *Dev. Biol.* **280**, 225-236.
Sanz, L. A., Chamberlain, S., Sabourin, J. C., Henckel, A., Magnuson, T., Hugnot, J. P., Feil, R. and Arnaud, P. (2008). A mono-allelic bivalent chromatin domain controls tissue-specific imprinting at Grb10. *EMBO J.* **27**, 2523-2532.

Schuettengruber, B. and Cavalli, G. (2009). Recruitment of polycomb group complexes and their role in the dynamic regulation of cell fate choice. *Development* **136**, 3531-3542.
Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B. and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. *Cell* **128**, 735-745.
Schwartz, Y. B. and Pirrotta, V. (2007). Polycomb silencing mechanisms and the management of genomic programmes. *Nat. Rev. Genet.* **8**, 9-22.
Simon, J. A. and Kingston, R. E. (2009). Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat. Rev. Mol. Cell Biol.* **10**, 697-708.
Solter, D. and Knowles, B. B. (1975). Immunosurgery of mouse blastocyst. *Proc. Natl. Acad. Sci. USA* **72**, 5099-5102.
Stock, J. K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A. G. and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat. Cell Biol.* **9**, 1428-1435.
van der Stoop, P., Boutsma, E. A., Hulsman, D., Noback, S., Heimerikx, M., Kerkhoven, R. M., Voncken, J. W., Wessels, L. F. and 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.
VerMilyea, M. D., O'Neill, L. P. and Turner, B. M. (2009). Transcription-independent heritability of induced histone modifications in the mouse preimplantation embryo. *PLoS ONE* **4**, e6086.
Voncken, J. W., Roelen, B. A., Roefs, M., de Vries, S., Verhoeven, E., Marino, S., Deschamps, J. and van Lohuizen, M. (2003). Rnf2 (Ring1b) deficiency causes gastrulation arrest and cell cycle inhibition. *Proc. Natl. Acad. Sci. USA* **100**, 2468-2473.
Wang, J., Zhang, M., Zhang, Y., Kou, Z., Han, Z., Chen, D. Y., Sun, Q. Y. and Gao, S. (2009). The histone demethylase JMJD2C is stage-specifically expressed in preimplantation mouse embryos and is required for embryonic development. *Biol. Reprod.* **82**, 101-111.
Weber, M. and Schubeler, D. (2007). Genomic patterns of DNA methylation: targets and function of an epigenetic mark. *Curr. Opin. Cell Biol.* **19**, 273-280.
Wei, G., Wei, L., Zhu, J., Zang, C., Hu-Li, J., Yao, Z., Cui, K., Kanno, Y., Roh, T. Y., Watford, W. T. et al. (2009). Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* **30**, 155-167.
Wissmann, M., Yin, N., Muller, J. M., Greschik, H., Fodor, B. D., Jenuwein, T., Vogler, C., Schneider, R., Gunther, T., Buettner, R. et al. (2007). Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. *Nat. Cell Biol.* **9**, 347-353.
Yuan, P., Han, J., Guo, G., Orlov, Y. L., Huss, M., Loh, Y. H., Yaw, L. P., Robson, P., Lim, B. and Ng, H. H. (2009). Eset partners with Oct4 to restrict extraembryonic trophoblast lineage potential in embryonic stem cells. *Genes Dev.* **23**, 2507-2520.
Zhao, X. D., Han, X., Chew, J. L., Liu, J., Chiu, K. P., Choo, A., Orlov, Y. L., Sung, W. K., Shahab, A., Kuznetsov, V. A. et al. (2007). Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. *Cell Stem Cell* **1**, 286-298.