Epigenetic restriction of embryonic cell lineage fate by methylation of *Elf5*

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Abstract

Mouse ES cells can differentiate into all three germ layers of the embryo but are generally excluded from the trophoblast lineage. Here we show that ES cells deficient in DNA methylation can differentiate efficiently into trophoblast derivatives. In a genome-wide screen we identify the transcription factor *Elf5* as methylated and repressed in ES cells, and hypomethylated and expressed in TS and methylation-deficient ES cells. *Elf5* creates a positive feedback loop with TS cell determinants *Cdx2* and *Eomes* that is restricted to the trophoblast lineage by epigenetic regulation of *Elf5*. Importantly, the late-acting function of *Elf5* allows initial plasticity and regulation in the early blastocyst. Thus, Elf5 acts downstream of initial lineage determination as a gatekeeper to reinforce commitment to the trophoblast lineage, or to abort this pathway in epiblast cells. This epigenetic restriction of cell lineage fate provides a molecular mechanism for Waddington's concept of canalization of developmental pathways.

Formation of the first two cell populations of the blastocyst, the trophectoderm (TE) and the inner cell mass (ICM), represents the earliest differentiation event in mammalian development. TE cells are restricted in their differentiation potential towards the trophoblast lineage that is essential for formation of the placenta. By contrast, cells of the inner cell mass (ICM) lose their capacity to differentiate into trophoblast derivatives, but remain pluripotent and give rise to all cell types of the embryo proper as well as to extraembryonic endoderm and mesoderm1-4. Embryonic stem (ES) cells are derived from the ICM of blastocyst-stage embryos5. Like ICM cells, they are extremely efficient in colonising all germ layers of the embryo6, but are generally excluded from the trophoblast lineage7. Conversely, trophoblast stem (TS) cells, derived from the TE, differentiate into all trophoblast subtypes of the placenta, but are excluded from embryonic tissues8,9. Thus, the stem cell populations derived from the blastocyst stably retain the cell lineage restrictions that are imposed by this stage.

The initial cell lineage specification is established by key transcription factors such as Oct4, Nanog, Sox2, as well as Cdx2 and Eomes, that are capable of defining embryonic and trophoblast lineage identity, respectively10. Once fixed, lineage determination must be accompanied by epigenetic modifications that ensure the stable inheritance of cell fate11. It

is particularly important to determine how lineage-specific transcription factor networks interact with different epigenetic marking systems in order to achieve this stability. One of the earliest global epigenetic marks that distinguishes the ICM and TE lineages is DNA methylation: ICM cells become *de novo* methylated in early blastocysts while TE cells remain hypomethylated[12], and this global difference in methylation between embryonic and extraembryonic tissues is maintained throughout development[13,14]. Indicating a functional role of this differential methylation pattern, expression of few markers of differentiated trophoblast has been observed in embryoid bodies derived from *Dnmt*-deficient (and therefore hypomethylated) ES cells[15]. Here, we have examined in detail the role of DNA methylation in the stability of embryonic lineage determination. We show that ES cells and cells of the embryo proper that are deficient in methylation can differentiate into trophoblast derivatives, progressing in an orderly fashion from stem cells towards giant cells. DNA methylation-mediated silencing of a trophoblast-specific transcription factor, *Elf5*, normally restricts this differentiation pathway. The identification of this epigenetically regulated gatekeeper provides an explanation for the early, definitive nature of embryonic and trophoblast cell lineage separation.

Results

Trophoblast from *Dnmt1-/-* embryonic cells

To evaluate whether DNA methylation is essential for the stability of embryonic cell lineage determination, we examined whether trophoblast differentiation occurs from ES cells deficient in DNA methylation due to deletion of the maintenance DNA methyltransferase, *Dnmt1*. Formation of trophoblast giant cells, a morphologically distinct placental cell type, was observed only sporadically when *Dnmt1-/-* ES cells were grown in standard ES cell medium. In sharp contrast, ~ 25% of all *Dnmt1-/-* ES cells differentiated into trophoblast giant cells upon culture in TS cell conditions as assessed by morphological criteria and by marker gene expression (Fig. 1a, b). In a time course experiment where *Dnmt1-/-* ES cells were subjected to TS cell conditions for 2-12 days, trophoblast stem cell markers *Eomes* and *Cdx2* were the first to be detected, followed by ectoplacental cone and spongiotrophoblast markers *Ascl2* and *Tpbpa*, and subsequently giant cell-expressed genes *Pl1 (Prl3d1)* and *Pl2 (Prl3b1;* Fig. 1b). This sequence of expression was comparable to that observed when TS cells undergo differentiation[8]. No significant trophoblast differentiation or marker expression was observed with wildtype ES cells grown under TS cell conditions. Trophoblast differentiation was not restricted to methylation-deficient ES cells, but also occurred in *Dnmt1-/-* embryos *in vivo*. Quantitative RT-PCR analysis showed a striking up-regulation of markers for intermediate diploid trophoblast (*Ascl2, Tpbpa*) and trophoblast giant cells (*Pl1, Pl2*) in E9.5 *Dnmt1*-deficient embryos (Fig. 1c), thus reflecting a mid-to-late stage in the time course of trophoblast transdifferentiation from embryonic cells. The spatial distribution and morphology of these ectopically differentiating trophoblast cells was determined by *in situ* hybridization where cells positive for *Tpbpa, Plf* (*Prl2c2*), and *Pl1* were detected in *Dnmt1-/-* but not wildtype embryos, with giant cell marker-expressing cells exhibiting an enlarged cell size compared to surrounding embryonic cells (Fig. 1d, e). These data clearly demonstrate the ectopic differentiation of trophoblast derivatives in *Dnmt1*-depleted embryos and ES cells, showing that appropriate DNA methylation levels are crucial for maintenance of the embryonic lineage.

The major difference between ES and TS cell culture conditions is the absence of LIF and presence of FGF4 in TS cell medium. Therefore, we investigated whether the FGF signalling receptor in trophoblast stem cells, Fgfr2c[8,16,17], was expressed concomitantly with trophoblast differentiation. Like *Cdx2, Fgfr2c* was activated in *Dnmt1-/-*, but not wildtype, ES cells upon growth in TS cell medium. Importantly, wildtype ES cells could be induced to express trophoblast genes at levels similar to those observed in *Dnmt1-/-* ES cells

by addition of the methylation inhibitor 5-azacytidine (Fig. 2a, b). As in $Dnmt1^{-/-}$ ES cells, activation of trophoblast markers coincided with the appearance of giant cells (Fig. 2c). Interestingly, azacytidine-treatment of $Dnmt1^{-/-}$ ES cells did not lead to a further increase in trophoblast stem cell-specific $Cdx2$ and $Fgfr2c$ expression but instead caused an up-regulation of markers of differentiated trophoblast (Fig. 2b), suggesting critical methylation threshold levels for trophoblast stem cell maintenance.

We next asked if trophoblast differentiation was triggered by down-regulation of Oct4, since loss of Oct4 expression in ES cells leads to transdifferentiation into trophoblast[18]. Interestingly, even after six days of culture in TS cell medium most cells co-expressed Cdx2 and Oct4 (Fig. 2d). This pattern suggests that demethylation of ES cells has expanded their developmental plasticity to include both extraembryonic and embryonic fate, rather than switching fate to an exclusive extraembryonic one. It may also reflect the pattern in compacting morulae where Oct4 levels remain high in both $Cdx2^+$ and $Cdx2^-$ cells[19]. A more reciprocal pattern was observed with Cdx2 and Nanog (Fig. 2d).

To correlate the temporal progression and spatial distribution of transdifferentiating trophoblast derivatives *in situ, Dnmt1$^{-/-}$* ES cells were co-stained for Cdx2 and *Plf* 2-6 days after culture in TS cell medium. Cdx2-positive cells were somewhat enlarged compared to surrounding ES cells and typically emerged at the margins of ES cell colonies (Fig. 3a, i). At the earliest time points of giant cell differentiation, all *Plf*-positive giant cells were located directly adjacent or in very close spatial proximity to Cdx2-positive cell groups (Fig. 3a, ii). In addition, some cells were observed in a transition state of differentiation, staining weakly positive for both Cdx2 and *Plf* (Fig. 3a, iii). The non-random appearance of giant cells indicates a clonal origin of trophoblast derivatives in $Dnmt1^{-/-}$ ES cells and suggests that they recapitulate a differentiation pathway comparable to that of the trophoblast lineage proper (Fig. 3a).

$Dnmt1^{-/-}$ ES cells contribute to the TE

Since hypomethylation caused loss of embryonic lineage restriction in embryos and ES cells, we examined whether $Dnmt1^{-/-}$ ES cells could directly contribute to the TE in aggregation chimeras. For this experiment, histone H2B-GFP labelled $Dnmt1^{+/+}$ and $Dnmt1^{-/-}$ ES cells were 'pre-conditioned' for transdifferentiation by culture in TS cell medium for 1-3 days prior to aggregation with 8-cell embryos. Comparable to original studies[7], control ES cells contributed extremely rarely to the TE. $Dnmt1^{-/-}$ ES cells, however, were observed in the TE of two-thirds of all blastocysts and exhibited a 5-fold increase in contribution to the TE compared to their wildtype counterparts (Fig. 3b).

$Elf5$ is differentially methylated

Our finding that DNA methylation prevents trophoblast differentiation in ES cells and embryos suggested that cell lineage stability is achieved by methylating trophoblast determinants in the embryonic lineage. Hence, we analyzed the key determinants of the trophoblast lineage for promoter methylation in ES cells. Although *Cdx2, Eomes* and *Fgfr2* were induced in differentiating $Dnmt1^{-/-}$ ES cells, we found that these genes were unmethylated in both ES and TS cells (Supplementary Information, Fig. S1). We thus carried out a genome-wide screen for promoter methylation by meDIP array hybridization[20], comparing ES with TS cells. We screened for promoters that exhibited higher methylation levels in ES cells compared to TS cells. A very small number of candidate promoters of this type were identified (see Methods); intriguingly, however, after validation by Sequenom® mass array technology only one gene with a robust difference in methylation was confirmed, *Elf5* (Fig. 4a; Supplementary Information, Fig. S2). *Elf5* encodes a transcription factor of the Ets family that plays a pivotal role in the trophoblast

lineage21. Upon ablation of *Elf5*, the TE lineage can be specified and blastocysts implant into the uterus, but the proliferative capacity of trophoblast stem cells is not maintained and conceptuses die shortly after implantation due to a lack of extraembryonic ectoderm21.

Detailed characterization of the *Elf5* promoter methylation profile by bisulphite sequencing analysis revealed that the vast majority of CpG residues in the 1 kb upstream region were methylated in ES cells but unmethylated in TS cells (Fig. 4b). Importantly, *Dnmt1*-deficient ES cells exhibited hypomethylation of the *Elf5* promoter compared to wildtype ES cells, especially closer to the transcriptional start site. This methylation pattern was stable as it did not change over a 10-day time course of culture in TS cell conditions (Supplementary Information, Fig. S2). The mosaic methylation pattern of *Elf5* in *Dnmt1*$^{-/-}$ ES cells provides a likely explanation why not all, but typically a total of 40-60% of all *Dnmt1*$^{-/-}$ cells were found to undergo transdifferentiation into trophoblast derivatives (i.e. stem, intermediate, spongio- and giant trophoblast cells) during this time period.

Elf5 marks diploid trophoblast

Consistent with the trophoblast-specific expression of *Elf5* in early mouse embryos21, *Elf5* was detected in undifferentiated TS cells but was absent from ES cells (Fig. 5a, b). Upon differentiation of TS cells, *Elf5* expression was strongly down-regulated (Fig. 5b). Elf5 staining mostly overlapped with the TS cell marker Cdx2, but was also observed in some additional diploid trophoblast cells that were Cdx2-negative (Fig. 5c). Analysis of the temporal regulation *in vivo* showed that *Elf5* expression was initiated later than *Cdx2*. In contrast to *Cdx2*, only marginal amounts of *Elf5* transcripts and no protein were detected in blastocysts (data not shown). However, Elf5 was readily detected in blastocyst outgrowths where a central core of Cdx2$^+$ cells was overlaid by Cdx2$^+$ and Elf5$^+$ double-positive and then Cdx2-negative, Elf5-positive diploid, proliferative trophoblast (Fig. 5d). Similarly, Elf5 marked the extraembryonic ectoderm of conceptuses immediately after implantation from E5.5 onwards (Fig. 5e). These data show that *Cdx2* precedes *Elf5* expression in the emerging TE, but that *Elf5* expression is sustained longer in diploid proliferating trophoblast of the extraembryonic ectoderm (Fig. 5f).

Elf5 activates *Cdx2* and *Eomes*

To analyze whether hypomethylation of the *Elf5* promoter was correlated with its expression in cells of the embryonic lineage, we first assessed *Dnmt1*$^{-/-}$ embryos and ES cells for ectopic activation of *Elf5* and found that they expressed high levels of this transcription factor. In fact, *Elf5* was the earliest and most significantly induced trophoblast marker in the time course of ES-to-trophoblast transdifferentiation and was expressed in derivatives of every single cell (Fig. 6a-c; Supplementary Information, Fig. S4). *Elf5* was also strongly activated upon treatment of wildtype ES cells with 5-azacytidine (Supplementary Information, Fig. S4). To further test the direct relationship between hypomethylation and expression of *Elf5* and the activation of trophoblast markers, we examined other hypomethylated ES cell models null for *Np95, Dnmt3a/b* or *Cxxc1*22-24. In all three cell lines, the *Elf5* promoter was extremely hypomethylated resulting in high expression of *Elf5* and up-regulation of other trophoblast markers (Supplementary Information, Fig. S3). Thus, *Elf5* expression is associated with a loss of embryonic cell lineage determination irrespective of how promoter demethylation is achieved. Consistent with the observations of Cdx2 and Oct4 co-expression shown above, activation of *Elf5* at earliest stages in the transdifferentiation cascade was independent of Oct4 repression (Fig. 6d).

If expression of hypomethylated *Elf5* was responsible for trophoblast differentiation from embryonic cells, *Elf5* might directly activate the trophoblast stem cell determinants *Cdx2* and *Eomes*. Indeed both the *Cdx2* and *Eomes* promoters contain the core binding motif for

the transcription factor Elf525. Consistent with this hypothesis, we found that forced expression of *Elf5* in wildtype ES cells resulted in a strong induction of *Eomes* as early as 24h after transfection. Likewise, *Cdx2* expression was significantly (>3-fold) activated after 24h but peaked somewhat later after 2-3 days of transfection with *Elf5* (Fig. 6e). We confirmed that Elf5 was able to activate the *Cdx2* and *Eomes* promoters in co-transfection experiments of *Elf5* with reporter constructs, and that conversely, Cdx2 (and likely Eomes) could also activate the *Elf5* promoter (Fig. 6f, Supplementary Information, Fig. S3). Chromatin immunoprecipitation (ChIP) assays demonstrated that Elf5 directly interacted with the *Cdx2* and *Eomes* promoters and that this interaction occurred exclusively in TS cells and in *Dnmt1*^{-/-} ES cells, but not in wildtype ES cells (Fig. 6g). Hence, ectopic expression of *Elf5* directly induces trophoblast stem cell determinants and thereby initiates the trophoblast differentiation cascade in cells of the embryonic lineage. Importantly, this differentiation pathway depends on Elf5 since trophoblast marker activation is significantly decreased in hypomethylated ES cells in which *Elf5* expression is diminished (Fig. 7a).

These data establish that Elf5 is essential to reinforce the expression of critical trophoblast stem cell factors, which provides an explanation for the observed loss of *Cdx2* expression in *Elf5*-mutant conceptuses21. As indicated by the immunostaining patterns of blastocyst outgrowths and early conceptuses, however, this transcriptional activator function of Elf5 is limited to a relatively narrow spatio-temporal window since *Elf5* expression is sustained in diploid trophoblast cells that have ceased to express *Cdx2* (Fig. 5). Consequently, stable expression of *Elf5* in wildtype ES cells, as well as continued culture of *Dnmt1*^{-/-} ES cells in TS cell conditions, resulted in the widespread differentiation of cells with trophoblast (giant) cell morphology, but did not allow the establishment of self-renewing TS cell lines (Fig. 7b, c; Supplementary Information, Fig. S4). Thus, Elf5 creates a cell lineage-restricting niche at the critical interface between promotion and loss of the self-renewing capacity of trophoblast stem cells.

Discussion

In this study we have made three significant observations: First, ES cells deficient in DNA methylation can differentiate efficiently into the trophoblast lineage. We demonstrate that the trophoblast differentiation programme occurs *in vivo* as well as *in vitro* and involves expression of TS cell transcription factors *Cdx2, Eomes*, and *Elf5* and progression in an orderly fashion from stem cells and intermediate trophoblast to giant cells. Second, the transcription factor Elf5 binds to and positively regulates the *Cdx2* and *Eomes* promoters, establishing a positive feedback loop which is critical for the maintenance of the trophoblast stem cell population. Third, the *Elf5* gene is hypomethylated in the trophoblast lineage, so that the feedback loop is sustained and leads to expansion of the stem cell compartment and subsequent differentiation. By contrast, *Elf5* is stably repressed by DNA methylation in the embryonic lineage; as a result any induction of trophoblast cell fate mediated by stochastic expression of *Cdx2* or *Eomes* cannot be sustained in this compartment.

Several studies have demonstrated that epigenetic regulation through Polycomb group proteins is essential to maintain the pluripotent state within the embryonic lineage in ES cells26-28. Here, we show that DNA methylation functions as a lineage barrier between the embryonic and trophoblast lineage compartments (Fig. 7d). Consequently, hypomethylated ES cells can adopt a trophoblast cell fate. It is important to note, however, that methylation-deficient ES cells have not simply switched from an ES to a trophoblast fate, but have broadened their potency to include both an embryonic and extraembryonic fate. Thus, they adopt a state comparable to an earlier developmental stage prior to lineage commitment, and this interpretation is consistent with the observed overlapping staining patterns of lineage markers. While methylation-deficient ES cells are able to express trophoblast determinants

Cdx2 and *Eomes* (as well as *Fgfr2*), the promoters of none of these genes are methylated in wildtype ES cells. Therefore, we performed a genome-wide screen for promoter methylation differences between ES and TS cells. This analysis revealed that the global methylation asymmetry observed between the embryonic and trophoblast lineage is not reflected by the methylation profile of gene promoters on a large scale. The major differences in methylation between the two lineages are therefore likely to be located in non-genic regions such as, for example, centromeric heterochromatin. These observations are similar to the X chromosome where global hypermethylation but promoter-specific hypomethylation is observed on the active X[29]. However, by specifically screening for gene promoters with higher methylation levels in ES cells, we identified *Elf5* as the gatekeeper gene that is methylated in ES cells but unmethylated in TS cells. *Elf5* is activated through Fgf-Fgfr signalling[30] and in turn activates *Eomes* and *Cdx2*. When the *Elf5* promoter is methylated, low levels of expression of *Eomes* and/or *Cdx2* (Fig. 1a) remain inconsequential because the transcriptional loop is interrupted at the level of *Elf5*. The robust positive feedback loop created by Elf5 in the trophoblast stem cell niche allows this compartment to expand in a temporally defined window. Consequently, this cell population is lost and *Cdx2* expression cannot be sustained in *Elf5*-mutants[21]. This compartment can also be established by overexpression of *Cdx2* or *Eomes* in ES cells[31] which we show is independent of *Elf5* demethylation at early stages, but over prolonged periods leads to *Elf5* demethylation and expression thereby maintaining the feedback loop and allowing progression of the trophoblast differentiation pathway (Supplementary Information, Fig. S4). Normally, the feedback loop established by Elf5 is temporally restricted to a few cell divisions, thereby positioning Elf5 at the interface between reinforcing trophoblast fate and onset of differentiation. This may be due to the gradual loss of other regulatory factors required for *Cdx2* (or *Eomes*) expression. Consequently, Elf5 can induce trophoblast differentiation from ES cells but cannot, unlike Cdx2, convert them into continuously self-renewing TS cell lines. We cannot exclude that there may be additional gatekeeper genes such as *Elf5* which are methylated in ES cells and the epiblast, but our screen was exhaustive and validation studies show that robust methylation differences are detected reliably[32].

In the absence of silencing by DNA methylation or in a force-expression system, Elf5 has trophoblast-determining functions due its capacity to activate *Cdx2* and *Eomes*. Within the normal developmental context, however, Elf5 acts downstream of the trophoblast determinants *Cdx2* and *Eomes* as well as the recently identified *Tead4* which has been shown to induce *Cdx2*, making it the gene currently at the top of the extraembryonic fate cascade[33-35]. A gatekeeper function for such a relatively late-acting gene may at first appear counterintuitive. However, the temporal position of Elf5 in the trophoblast determination cascade correlates perfectly with the developmental fixation of lineage restriction and specifically allows the plasticity and regulation that is characteristic of early mammalian development[3,36,37]. Thus, individual blastomeres are still able to cross lineages and expression of lineage 'markers' remains mosaic until the mid-blastocyst stage[19]. Only slightly later, *Elf5* expression in the extraembryonic ectoderm reinforces extraembryonic cell fate, while the inability to activate *Elf5* in the epiblast due to promoter methylation restricts its descendants to the embryonic cell fate. The late-acting function of Elf5 is in line with its position downstream of lineage-determining transcription factors at the interface of stem cell self-renewal and onset of differentiation.

To what extent blastomeres of early mouse cleavage-stage embryos are pre-determined towards their future fate has been a matter of some debate[38]. It is now well accepted that position, cell polarization and relative threshold levels of a few interacting transcription factors bias a blastomere's fate towards the embryonic or extraembryonic cell lineage[39]. Irreversible fixation of cell fate only occurs at the late blastocyst stage, however, and our findings provide, for the first time, a molecular mechanism for this transition from initial

plasticity to lineage-restricted potency in development. This regulative view of development was most famously expressed by C. H. Waddington, who likened the path of a cell lineage towards terminal differentiation to a ball travelling downwards along branching valleys; once it has entered its final valley it cannot easily cross the mountain into the neighbouring one or return to the beginning. This creates canalization of developmental pathways so that they become stable and potentially irreversible[40]. Our observations give rise to a molecular model of regulation and canalization in early mammalian development. Manipulation of gatekeeper genes such as *Elf5* or their epigenetic regulation may help with strategies in regenerative medicine that aim to generate appropriate cell types by transdifferentiation or by reprogramming of somatic cells.

Methods

Animals and tissue preparation

Mice heterozugous for the *Dnmt1*^{c} allele[41] were maintained on a C57BL/6 background. Homozygous null *Dnmt1*$^{c/c}$ conceptuses were obtained by heterozygous matings, counting the day of the vaginal plug as E0.5. For histology, E8.5 conceptuses were fixed with 4% paraformaldehyde and processed for routine paraffin histology. For RNA preparation, embryos free of any trophoblast tissues were carefully dissected and snap frozen.

Tissue culture

ES cell lines used were: wildtype J1 and R1, ES cells derived from 129Sv x (*M. cast.* x 129 Sv) blastocysts either wildtype (*Dnmt1*$^{+/+}$) or homozygous for the mutant *s* allele (*Dnmt1*$^{-/-}$) at the DNA methyltransferase 1 locus[41], *Np95*$^{-/-}$, *Dnmt3a/b* double mutant and *Cxxc1*$^{-/-}$. For normal growth and expansion, ES cells were grown on gelatinized dishes on embryonic feeder cell layers in standard ES cell medium. For transdifferentiation experiments, 1×10^5 cells were plated in gelatin-coated 6-well dishes and grown in standard TS cell conditions (20% fetal bovine serum, 1 mM Na-pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-mercaproethanol, 25 ng/ml bFGF (Sigma), and 1 µg/ml heparin in RPMI1640 (Invitrogen), with 70% of the medium pre-conditioned on embryonic feeder cells[8]). TS cell differentiation medium consisted of the unconditioned medium without bFGF and heparin. Culture was for the indicated time periods, with media changes every two days. The TS-GFP line[8] was used as control. For *Elf5* overexpression and generation of stable cell lines, the full sequence-verified open reading frame was cloned into the hrGFP1a-IRES-EGFP (Stratagene), pcDNA3.1 (Stratagene) and pCALL42 vectors. Promoter constructs consisted of the 2 kb upstream regions of *Cdx2, Eomes* and *Elf5* in the pTimer-1 (Clontech) vector. Transfections were carried out with Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's instructions. FACS sorting of GFP-positive cells was carried out on a FACSAria cell sorter.

Aggregation chimeras and blastocyst outgrowths

For aggregation experiments, *Dnmt1*$^{+/+}$ and *Dnmt1*$^{-/-}$ ES cells stably transfected with an H2B-GFP expression vector were cultured for 1-3 days in TS cell medium and then aggregated with 8-cell C57BL/6 embryos. Aggregated embryos were cultured in KSOM or DMEM containing 10% FBS for 2-3 days. Blastocysts were embedded in fibrin clots and analyzed on a Zeiss 510 Meta confocal microscope. The duration of preconditioning of ES cells in TS medium did not affect the rate of TE contribution. Blastocyst outgrowths were obtained by culturing C57BL/6 blastocysts in DMEM with 10% FBS for 3-5 days.

Staining of histological sections and cultured ES cells

In situ hybridizations on paraffin sections were carried out according to a standard protocol43 using digoxigenin-labelled riboprobes. For detection of ectopic giant cells, serial sections 42-70 μm apart were hybridized with the pan-trophoblast giant cell marker *Plf*. Counterstaining was performed with nuclear fast red. For *in situ* hybridization on cultured cells, ES cells plated on gelatinized coverslips were fixed for 20 min with 4% paraformaldehyde and processed using a standard protocol44. Immunostainings were performed after blocking in 0.1% serum, 0.5% BSA for 15 min using the following antibodies and dilutions: anti-Cdx2 (BioGenex) at 1:300-1:400; anti-Eomes (Abcam) 1:200; anti-Elf5 (Santa Cruz Biotechnology) 1:250; anti-Oct3/4 (Santa Cruz Biotechnology) 1:400; anti-Nanog (Abcam) 1:250. Secondary antibodies were Alexa fluorophores from Molecular Probes. Photographs were taken on an Olympus BX41 epifluorescence microscope and a Zeiss 510 Meta confocal microscope.

Quantitative gene expression analysis

Total RNAs were isolated from cultured cells or embryos using Trizol reagent and digested with 20U DNase for 30 min at room temperature. cDNA synthesis was performed with Powerscript reverse transcriptase (BD Biosciences) according to the manufacturer's protocol. 1:10 diluted cDNAs were used for quantitative PCR on an ABI PRISM 7700 Sequence Detector and Stratagene Mx3005P lightcycler using SYBR Green I mastermix (Applied Biosystems) and intron-spanning primer pairs. Ct values were normalized against 3 housekeeping genes. All samples were analyzed at least in triplicate.

Northern and Southern blotting

For Northern blots, 20 μg total RNA was electrophoresed and blotted onto Nytran Supercharge Nylon membrane (Schleicher & Schuell). For Southern blots, 20 μg DNA was digested with *Nsi*I or *Bgl*II at 37°C, precipitated and digested with *Msp*I or *Hpa*II. Digests were electrophoresed on 0.8-2% agarose gels and blotted in 0.4M NaOH onto Nytran Supercharge Nylon membrane. Random-primed DNA labelling was carried out with 25-40 μCi ^{32}P-dCTP, followed by hybridization at 65°C according to standard protocols45.

Methyl-DIP chip

Genomic DNA from ES and TS cells was sheared to an average size of 300-1000 bp and immunoprecipitated with an anti-5-methylcytosine specific antibody (Eurogentec). The quality of the immunoprecipitation was determined by analysing enrichment of standard genes with known methylation patterns. 4 μg of immunoprecipitated DNA and 3 μg of sonicated input DNA were hybridized to NimblGen oligonucleotide promoter tiling arrays (2005-03-31_MM5) containing 35497 mouse promoter fragments. The hybridizations were carried out in triplicate with one dye swap using three independent biological samples. The microarray raw data were analyzed using the in-house developed software ChipMonk. Microarray data comparison was performed using an empirically determined algorithm32 where candidate regions containing 5 or more oligonucleotide probes were filtered to give a CpG proportion of 2-9% and to ensure that the differences were statistically significant (p<0.01). We confirmed that this algorithm was a robust predictor of methylation state with 88% accuracy (22/25 calls correct).

Sequenom analysis

Two regions were identified as candidates for higher methylation in ES versus TS cells: *Elf5* and *Sca10*. The methylation pattern of these loci was analyzed by Sequenom® mass array technology (Sequenom, San Diego, CA). 1 μg of genomic DNA from ES and TS cells was bisulphite treated using the Zymo EZ DNA methylation kit (Zymo Research). Promoter

regions were selected based on the position of the oligonucleotides on the NimbleGen promoter array and primer pairs were designed using the MethPrimer program (http://www.urogene.org/methprimer/index1.html). Amplification of the bisulphite converted DNA and preparation of the PCR product for quantitative analysis of the promoter methylation detected by the Mass Array system is according to the protocol provided by the manufacturer. The number of differentially methylated CpG dinucleotides for these promoters was: 9/10 for *Elf5* (methylated CpG's in ES cells: 45.5%; methylated CpG's in TS cells: 5.36%) and 2/3 for *Sca10* (ES 24.33%; TS 7.67%). We then examined correlation of methylation status with gene expression levels. *Sca10* exhibited equal expression levels in ES and TS cells; thus, the very limited number of differentially methylated CpG's was non-functional and did not affect transcriptional activity. Only *Elf5* was expressed at significantly higher levels in TS cells as predicted from the methylation pattern. Thus, *Elf5* was the only differentially methylated gene with functional relevance of the modification in the ES versus TS cell screen.

Bisulphite genomic sequencing

1 µg of DNA was processed for bisulphite sequencing analysis using the EpiTect Bisulphite kit (Qiagen) according to the manufacturer's protocol. Nested PCR reactions were performed for all genes analyzed. Primer sequences are available upon request. PCR products were eluted, sub-cloned and sequenced.

Chromatin immunoprecipitation

Cells from 4 92mm dishes were formaldehyde-crosslinked according to a standard protocol46. 150 µg chromatin was pre-cleared and incubated with 2 µg goat anti-Elf5 antibody (Santa Cruz Biotechnology) overnight at 4°C, and then precipitated with Protein G sepharose. Incubation with goat IgG or with protein G sepharose only was used as mock control. Bound, unbound and input fractions were analyzed by standard and qPCR for *Cdx2* and *Eomes* promoter regions, and normalized against mock control. ChIP was performed in triplicate from independent samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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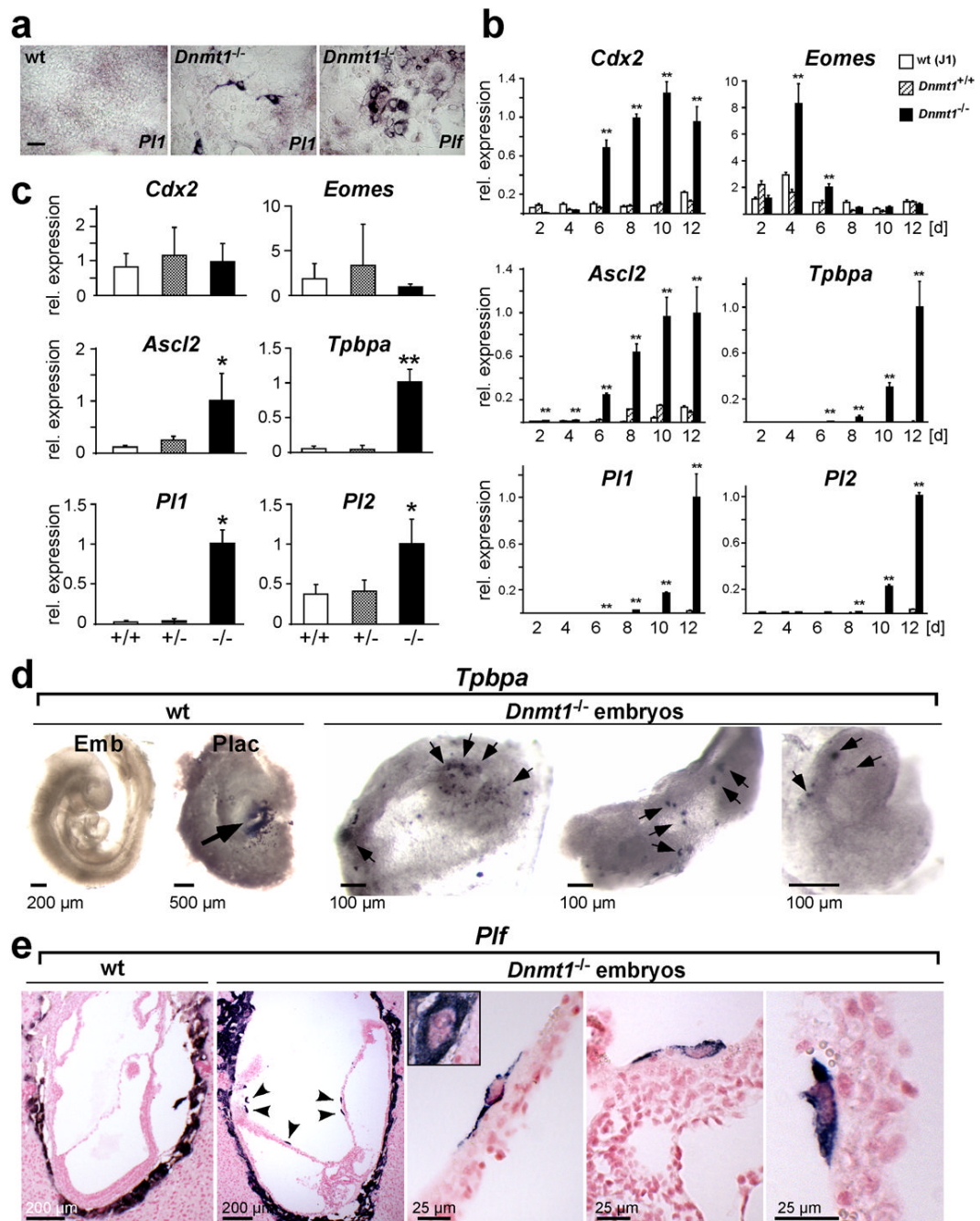
Fig. 1.

Dnmt1-deficiency enables trophoblast differentiation from cells committed to the embryonic cell lineage.

(a) Trophoblast giant cells differentiate from *Dnmt1⁻/⁻* ES cells, but not wildtype (wt) ES cells, *in vitro* when cultured in TS cell conditions. *In situ* hybridization with trophoblast giant cell-specific markers *Pl1* (after 12d) and *Plf* (after 8d). **(b)** Time course of trophoblast marker activation in ES cells upon culture in TS cell medium for 2-12 days. Significant expression levels are only observed in *Dnmt1*-deficient ES cells (data are mean ± s.d., **P<0.005; n=3). Trophoblast stem cell markers (*Cdx2, Eomes*) are the first to be up-

regulated, followed by intermediate diploid trophoblast markers *Ascl2* and *Tpbpa*, and trophoblast giant cell markers *Pl1* and *Pl2*. **(c)** Quantitative RT-PCR analysis of trophoblast markers on E9.5 embryos from *Dnmt1*^{+/-} intercrosses. Trophoblast-restricted genes *Ascl2, Tpbpa, Pl1* and *Pl2* are detected at significant levels only in *Dnmt1*^{-/-} embryos (data are mean ± s.d., *P<0.05, **P<0.005; n=7). **(d)** Whole mount *in situ* hybridization with the trophoblast marker *Tpbpa* on E9.5 *Dnmt1*^{-/-} embryos and littermate control (wt) embryos and placentas. Positive staining (dark purple-black) is seen in the ectoplacental cone/ spongiotrophoblast area (arrow) of the placenta (Plac). No staining is observed in the wt embryo (Emb). In *Dnmt1*^{-/-} embryos, numerous *Tpbpa*-positive cells are present (arrows). **(e)** *In situ* hybridization with the giant cell marker *Plf* on E8.5 wildtype (wt) and *Dnmt1*-deficient conceptuses. Arrowheads point towards *Plf*-positive cells (blue-purple) that are observed within embryonic structures in *Dnmt1*^{-/-} conceptuses only. Higher magnification views show the morphology of these cells; they are larger than surrounding cells but do not reach the size of parietal trophoblast giant cells (shown in inset). Scale bar in **a** represents 100 μm, scale bars in **d** and **e** are as indicated.
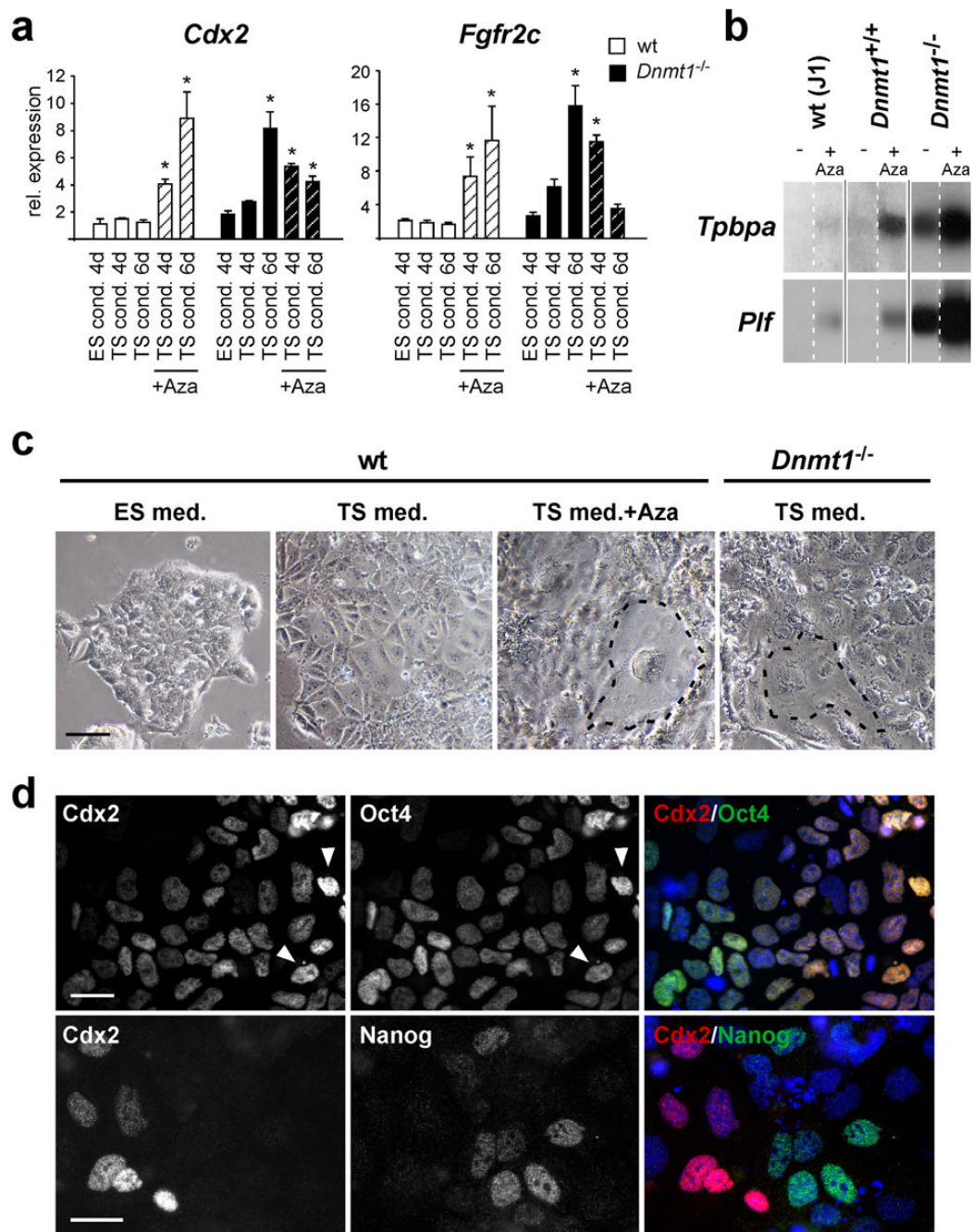
Fig. 2.
Trophoblast differentiation is induced in wildtype ES cells upon inhibition of DNA methylation and is independent of Oct4 down-regulation.
**(a)** Expression levels of *Cdx2* and *Fgfr2c* in wildtype and *Dnmt1*^{-/-} ES cells cultured in ES-, TS- and TS cell medium with 5-azacytidine over the indicated time periods. Culture in TS cell medium alone is sufficient to induce expression of *Cdx2* and *Fgfr2c* from *Dnmt1*^{-/-} ES cells, but expression levels of these trophoblast stem cell markers cannot be further enhanced by 5-azacytidine treatment. In wildtype ES cells, *Cdx2* and *Fgfr2c* expression can be induced by inhibition of DNA methylation with 5-azacytidine. Data are mean ± s.d.,

*P<0.05; n=3. **(b)** Northern blot hybridization with *Tpbpa* and *Plf* on wildtype (J1 and *Dnmt1*^{+/+}) and *Dnmt1*^{-/-} ES cells after 12d culture in TS cell medium in the presence (+ Aza) or absence (-) of 5-azacytidine. 5-azacytidine treatment induces differentiation of trophoblast derivatives in wildtype ES cells and further enhances the amount of differentiated trophoblast subtypes from *Dnmt1*^{-/-} ES cells. The separated lanes are parts of the same original blot; full scans of blots are provided in Supplemental Information, Fig. S5. **(c)** Wildtype and *Dnmt1*^{-/-} ES cells grown for 4-6 days under the indicated conditions. Wildtype ES cells cultured in TS cell conditions undergo differentiation into embryonic lineage derivatives, but rarely form trophoblast giant cells. Trophoblast-like morphology and the appearance of giant cells (encircled) are evident in wildtype ES cells treated with 5-azacytidine and in *Dnmt1*^{-/-} ES cells cultured in TS cell conditions. **(d)** Double immunofluorescence labelling of *Dnmt1*^{-/-} ES cells grown for 6 days in TS cell conditions for Oct4 (*Pou5f1*), Nanog and Cdx2. Trophoblast differentiation is not triggered by down-regulation of Oct4. Arrows point to cells expressing high amounts of both Cdx2 and Oct4. A more reciprocal expression pattern is observed for Nanog and Cdx2. Scale bar in **c** represents 100 μm, scale bars in **d** represent 25 μm.
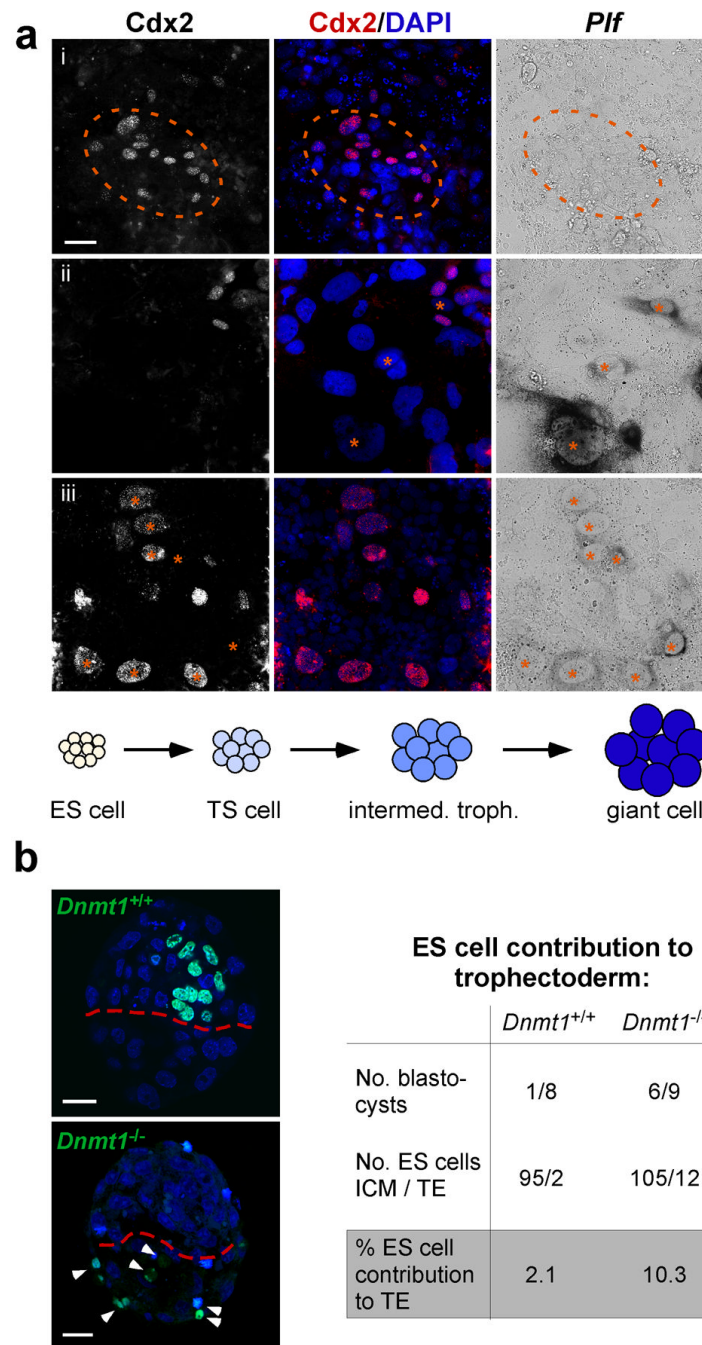
Fig. 3.
Dnmt1^{-/-} ES cells transdifferentiate into functional trophoblast derivatives.
(a) Double labelling of *Dnmt1*^{-/-} ES cells grown in TS cell conditions for 6 days and stained by *in situ* hybridization for the giant cell marker *Plf* and by immunofluorescence for the TS cell marker Cdx2. Cdx2-positive cells are observed in small groups consisting on average of 5-15 cells. At this early time point of transdifferentiation, giant cells (*Plf*) are either absent (i) or emerging in direct vicinity to Cdx2-positive TS cell clusters (ii). On rare occasions, cells can be captured at an intermediate stage of concomitant low Cdx2 and *Plf* expression (iii; marked with asterisks), demonstrating that giant cells differentiate from pre-existing
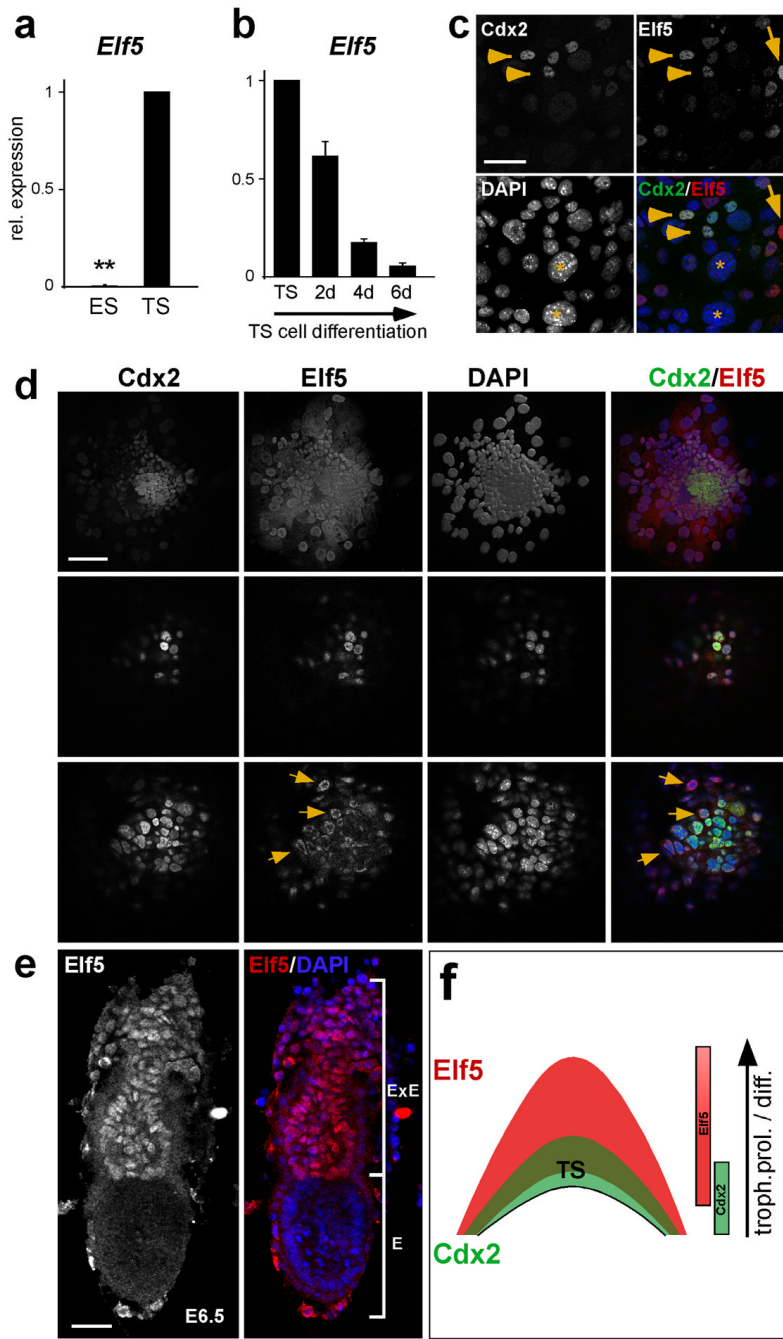
trophoblast stem cells. Thus, trophoblast differentiation from *Dnmt1*$^{-/-}$ ES cells proceeds via a trophoblast stem cell stage from which other trophoblast subtypes differentiate sequentially, recapitulating the differentiation steps within the trophoblast lineage proper (graph). **(b)** Blastocysts derived from aggregation of wildtype embryos with H2B-GFP labelled *Dnmt1*$^{+/+}$ or *Dnmt1*$^{-/-}$ ES cells that were pre-conditioned in TS cell medium. *Dnmt1*$^{-/-}$ ES cells differentiate into functional trophoblast and exhibit a 5-fold increased efficacy to contribute to trophectoderm. Arrowheads point to individual GFP-positive *Dnmt1*$^{-/-}$ cells within the mural trophoectoderm. Scale bar in **a** represents 20 µm, scale bars in **b** represent 25 µm.
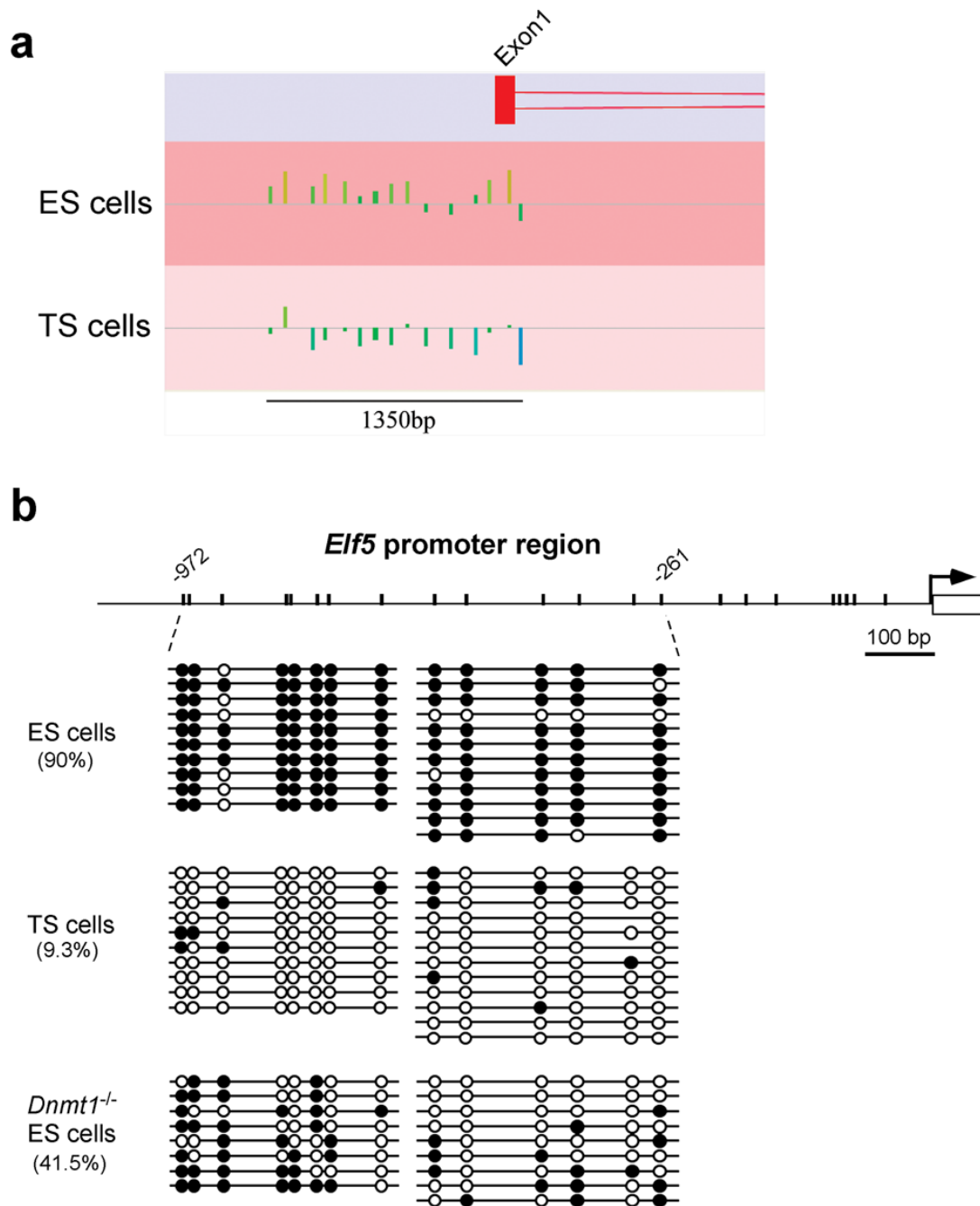
Fig. 4.
Global promoter methylation screen identifies *Elf5* as the key gene that is methylated in ES cells and unmethylated in TS cells.

(a) meDIP chip screen reveals strikingly differential methylation at the *Elf5* promoter. ChipMonk profile of the *Elf5* upstream region in ES and TS cells. The line represents the median signal intensity of the array; vertical bars above indicate relative hypermethylation and vertical bars below hypomethylation at individual oligonucleotide probes. **(b)** Bisulphite analysis of the 1 kb promoter region of *Elf5* in ES, TS, and *Dnmt1*^{-/-} ES cells. The promoter is almost fully DNA methylated (filled circles) in ES cells, but largely hypomethylated

(open circles) in TS cells. *Dnmt1*$^{-/-}$ ES cells exhibit intermediate DNA methylation levels. Note that the CpG site at position -355 bp is polymorphic.

Fig.5.
Elf5 is a trophoblast stem cell transcription factor specific to proliferative trophoblast.
(a) *Elf5* is not expressed in ES cells but exhibits high expression levels in TS cells (Data are mean ± s.d., **P=1.12E-12; n=3). **(b)** *Elf5* expression is specific to the stem cell state of TS cells and is down-regulated upon differentiation of TS cells. **(c)** Double immunofluorescence staining for Elf5 and Cdx2 on cultured TS cells shows co-localization of both transcription factors in most stem cells (arrowheads). Some diploid trophoblast cells only express Elf5 (arrow), while giant cell nuclei are mostly negative for both factors (asterisks). **(d)** Characterization of Elf5 expression in blastocyst outgrowths. Selected

confocal section planes are shown. Compared to Cdx2, Elf5 marks a larger population of trophoblast cells (top row). A few central cells only express Cdx2, followed by a core of double positive trophoblast stem cells (middle row). Elf5 staining extends beyond this core into surrounding trophoblast cells (bottom row). Nuclear localization adopts a cortical distribution where diploid trophoblast starts to differentiate into giant cells (arrows). Giant cells are devoid of nuclear Elf5. **(e)** Whole mount staining of an E6.5 embryo for Elf5. Elf5 expression is strictly trophoblast lineage-specific and is confined to the extraembryonic ectoderm (where trophoblast stem cells are located) and to the ectoplacental cone. E=embryonic portion, ExE=extraembryonic portion. **(f)** Schematic diagram representing the temporal and spatial distribution of Cdx2 and Elf5. A small Cdx2$^+$ population is overlaid by a Cdx2$^+$ Elf$^+$ trophoblast stem cell core. Elf5 extends from there into the surrounding diploid trophoblast population that undergoes proliferation (prol.) and differentiation (diff.) at the margins of the ectoplacental cone. Scale bar in **c** represents 50 µm, scale bars in **d** and **e** represent 100 µm.
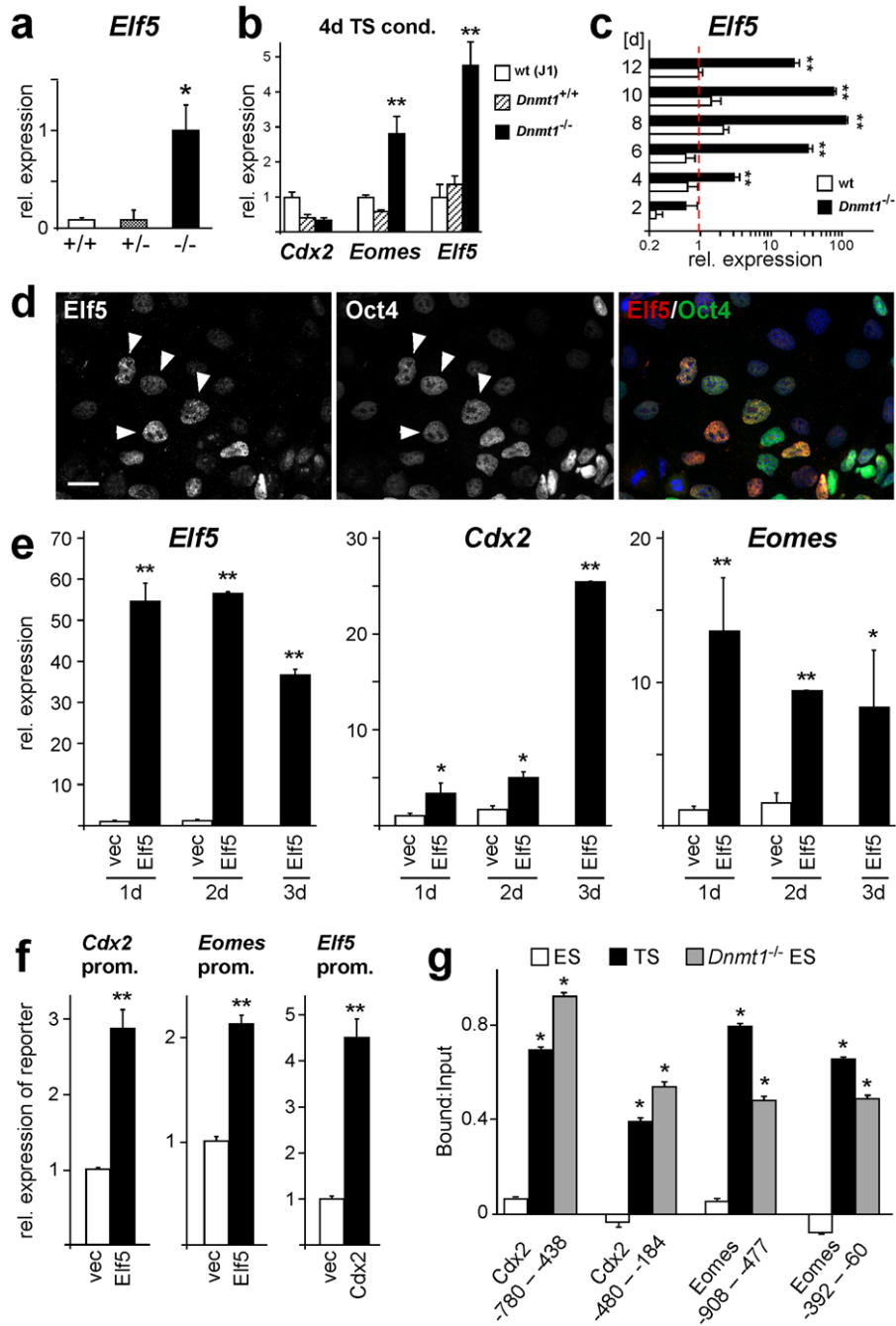
Fig. 6.
Epigenetically controlled *Elf5* expression acts in a positive feedback loop to reinforce trophoblast identity.

(a) *Elf5* expression is strongly induced in *Dnmt1*^{-/-} embryos, but not in wildtype and heterozygous littermate embryos (data are mean ± s.d., *P=0.026; n ≥ 6). **(b)** *Elf5* is the earliest, most strongly activated trophoblast marker in *Dnmt1*^{-/-} ES cells cultured for 4 days in TS cell conditions (data are mean ± s.d., **P=0.00185 [*Eomes*] and P=0.00051 [*Elf5*]; n=3). **(c)** *Elf5* expression is further enhanced in *Dnmt1*-deficient ES cells during the following days of culture in TS cell conditions (data are mean ± s.d., **P<0.005; n=3). **(d)**

Double labelling of *Dnmt1*$^{-/-}$ ES cells grown for 4 days in TS cell conditions for Elf5 and Oct4. Oct4 repression is not required for activation of Elf5. **(e)** *Elf5* expression in wildtype ES cells causes activation of TS cell markers *Cdx2* and *Eomes*. Wildtype ES cells were transfected with an *Elf5*-GFP expression construct, FACS sorted and assessed by qRT-PCR. The construct confers high levels of *Elf5* expression to transfected cells (Elf5) compared to control transfected cells (vec) that do not express *Elf5*. Elf5 induces high expression of *Eomes*. *Cdx2* expression is significantly elevated after 1 and 2 days (>3-fold), and reaches highest activation levels after 3 days of *Elf5* transfection (data are mean ± s.d., *P<0.05, **P<0.005; n=3). **(f)** *Elf5, Cdx2*, or empty vector control, were co-transfected with 2 kb *Cdx2, Eomes* or *Elf5* promoter-reporter constructs. Expression of the reporter gene was analyzed by qRT-PCR 24 hours after transfection. Elf5 can induce both *Cdx2* and *Eomes* promoters. Cdx2 can activate the *Elf5* promoter (data are mean ± s.d., **P<0.005; n=3). **(g)** Chromatin immunoprecipitation assays with an anti-Elf5 antibody. Elf5 binds directly to the *Cdx2* and *Eomes* promoters in TS cells and 3-day transdifferentiated *Dnmt1*$^{-/-}$ ES cells, but not in wildtype ES cells. Values are represented as Bound:Input and normalized against mock control (data are mean ± s.d., *P<0.05; n=3). Scale bar in **d** represents 25 μm.
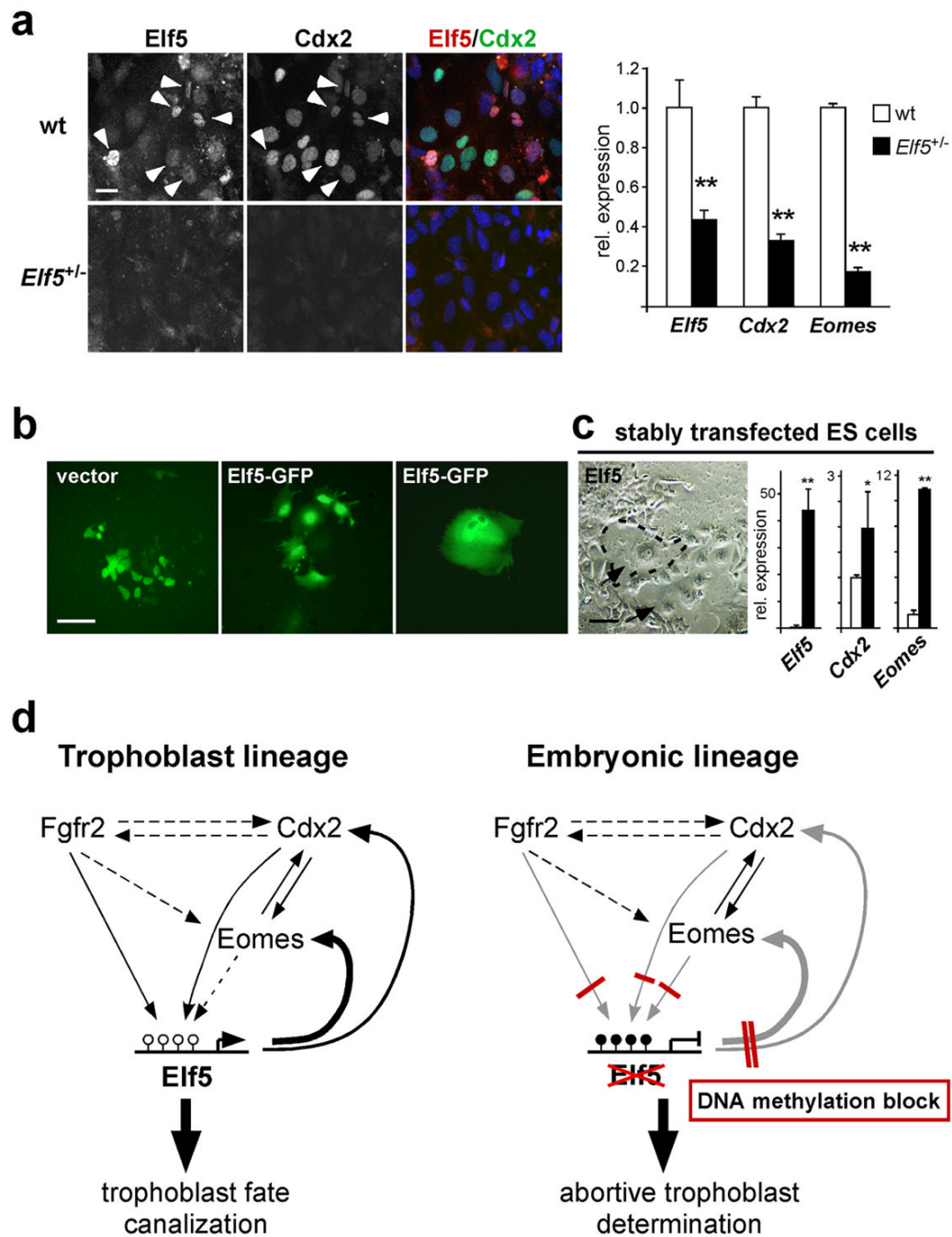
Fig. 7.
Gatekeeper function of Elf5 in cell lineage specification.
(a) Initiation of the trophoblast differentiation cascade depends on Elf5. ES cells heterozygous for a gene-trap insertion at the *Elf5* locus grown for 5 days in TS medium with 5-azacytidine and assessed for *Elf5, Cdx2* and *Eomes* expression. The gene-trap insertion reduces *Elf5* mRNA levels to 44% and further reduces protein levels. Elf5 and Cdx2 staining is abundant in wild-type ES cells (arrowheads); almost no Elf5 immunostaining is detected in *Elf5*^{+/-} cells. Low Elf5 expression is associated with a dramatic decrease in Cdx2 protein and *Cdx2* and *Eomes* mRNA levels. Images were taken at identical exposure

settings; qRT-PCR data are mean ± s.d., **P<0.005; n=3. **(b)** Wildtype ES cells transfected with *Elf5*-GFP or empty vector were FACS sorted and re-plated in TS cell medium for 4 days. Control cells proliferate and form colonies; *Elf5*-transfected cells cease to proliferate and differentiate into trophoblast giant cells. **(c)** Wildtype ES cells stably transfected with a Cre-inducible *Elf5* expression construct were transfected with Cre-GFP, FACS-sorted, and re-plated in TS cell medium for 5 days. *Elf5* expression leads to trophoblast differentiation and appearance of trophoblast giant cell clusters (arrows, examples encircled). White and black bars represent cells before and after Cre-mediated *Elf5* induction, respectively (data are mean ± s.d., *P<0.05, **P<0.005; n=3). **(d)** Model of Elf5 function in lineage canalization. Developmental onset of expression and genetic data place *Elf5* downstream of *Cdx2, Eomes* and *Fgfr2*. The developmental position of *Elf5* coincides with the definitive fixation of cell lineage fate at the late blastocyst stage. *Elf5* is regulated by DNA methylation, but the upstream players *Cdx2, Eomes* and *Fgfr2* are not. *Elf5* creates an essential feedback loop to reinforce *Cdx2* and *Eomes* expression in trophoblast stem cells. Epigenetic silencing of *Elf5* by DNA methylation interrupts this trophoblast-specific reinforcement loop in the embryonic lineage, and thereby safeguards embryonic cells from differentiating into trophoblast derivatives. Scale bar in **a** represents 25 μm, scale bar in **b** and **c** represents 100 μm.