

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 \pm 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 \pm 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 μ m, scale bar in **b** represents 100 μ m.

Supplementary Information, Fig. S1 *Cdx2*, *Fgfr2* and *Eomes* are not regulated by DNA methylation.

Bisulphite analysis of the promoters and upstream regions of **(a)** *Cdx2*, **(b)** *Fgfr2* and **(c)** *Eomes*. The *Cdx2* and *Fgfr2* promoters and first exon–intron regions contain CpG islands. All (or the vast majority of) CpG dinucleotides of the 5'-regions and 5'-CpG

islands of *Cdx2* and *Fgfr2* were analyzed spanning basepairs -1537 – +809 and -308 – +1405, respectively. Filled circles represent methylated cytosine residues. *Eomes* is a non-CpG island promoter. No differential DNA methylation was observed for any of these genes between ES and TS cells in the analyzed regions. Methylation was also analyzed by Southern blotting with the indicated probes (blue bars) yielding the same result (data not shown).

Supplementary Information, Fig. S2 Gene promoters are not globally hypomethylated in trophoblast cells.

(a) Gene promoters are not globally hypomethylated in trophoblast cells. Only *Elf5* was identified to be more highly methylated in ES cells than in TS cells. The Sequenom profile of *Elf5* is shown which confirmed differential methylation at the sites identified by the meDIP array. Dark green-blue circles indicate full methylation, yellow circles indicate no methylation at individual CpG's. Mass array spectra for individual CpG's on which the colour coding is based are also shown. **(b)** DNA methylation levels of *Elf5* remain largely unchanged over continued culture of *Dnmt1*^{-/-} ES cells in TS cell conditions. *Dnmt1*^{-/-} ES cells were grown for the indicated time points in TS cell medium and then analyzed by bisulphite genomic sequencing. The hypomethylated state of the *Elf5* promoter and the distribution of unmethylated CpG residues do not change significantly over prolonged periods of culture.

Supplementary Information, Fig. S3 Demethylation-mediated expression of *Elf5* is correlated with trophoblast marker activation in different hypomethylated ES cell models.

(a) Similar to the methylation pattern in TS cells, the *Elf5* promoter is drastically hypomethylated in ES cells that lack *Dnmt1*, *Np95*, *Dnmt3a/b* and *Cxxc1* (formerly called CGBP). The Sequenom profile and mass array spectra of two selected CpG residues are shown. **(b)** Bisulphite sequencing of the *Elf5* promoter confirms the Sequenom data. The corresponding bisulphite data for ES, TS and *Dnmt1*^{-/-} ES cells are shown in Figure 4. **(c)** ES cells express *Elf5* when hypomethylated at its promoter and this correlates with the induction of trophoblast cell markers. The kinetics of induction of *Elf5*, *Cdx2*, and *Eomes* vary slightly between the different cell lines. Immunostainings of *Dnmt1*^{-/-} and *Np95*^{-/-} ES cells after 4d in TS conditions are shown. Both cell lines exhibit very similar, trophoblast-like morphology and

widespread expression of *Elf5*. *Cdx2* is also up-regulated and detected within *Elf5*-positive cell groups. Up-regulation of *Elf5* and *Eomes* compared to wildtype ES cells is additionally shown by qRT-PCR depicted in the graphs below. Very high expression levels of *Elf5* are also observed in *Dnmt3a/b*^{-/-} and *Cxxc1*^{-/-} ES cells, corresponding to their very low methylation status at the *Elf5* promoter. Induction of *Cdx2* and *Eomes* over wildtype levels is shown (data are mean ± s.d., **P*<0.05, ***P*<0.005; n≥3). Additionally, the trophoblast giant cell marker *Pl1* was detected in all cell lines at later stages of transdifferentiation (not shown). **(d)** Wildtype ES cells transfected with an *Eomes* expression construct analyzed 24h post-transfection. *Eomes* can induce *Elf5* expression; however, since the DNA binding motif of *Eomes* is not well defined, the reporter assay did not reveal whether *Elf5* activation was a direct or an indirect effect of *Eomes* expression. Scale bar in **c** represents 25 μm.

Supplementary Information, Fig. S4 *Elf5* function in maintaining the trophoblast stem cell niche can be overridden by continued force-expression of *Cdx2*.

(a) Single cell-derived colonies of wildtype and *Dnmt1*^{-/-} ES cells grown for 5 days in TS cell conditions. *Elf5* expression is drastically up-regulated in every *Dnmt1*^{-/-} ES cell colony compared to wildtype (mean value of 2 wildtype colonies is shown), albeit at variable absolute levels. This demonstrates that every *Dnmt1*^{-/-} ES cell has the potential to induce *Elf5* and that cross-feeding does not have a major effect. **(b)** *Elf5* expression in wildtype and *Dnmt1*^{-/-} ES cells grown in ES and TS cell medium and TS cell medium with 5-azacytidine over the indicated time periods. *Elf5* can be activated from wildtype ES cells when treated with 5-azacytidine. *Elf5* expression peaks at similar levels upon 5-azacytidine treatment of wildtype and *Dnmt1*^{-/-} ES cells indicating maximal activation. Data are mean ± s.d., **P*<0.05; n=3. **(c)** Wildtype ES cells stably transfected with the *Elf5*-pCALL vector, a system where *Elf5* expression can be activated with Cre recombinase⁴². Cells were transfected with Cre-GFP, FACS-sorted and re-plated in TS cell medium for 2 days (first panel) and 5 days (second panel). Immunostaining shows *Elf5* expression in almost every cell. Note the enlarged, differentiated cell sizes in the second panel after 5d culture indicative of trophoblast differentiation. *Elf5* expression levels after Cre activation (ES-*Elf5* +Cre) were 43-fold higher than in the parental ES cells (ES-*Elf5* -Cre). **(d)** Wildtype ES cells stably transfected with a tamoxifen-inducible *Cdx2* expression construct³¹ 6 days after plating in TS cell medium containing tamoxifen (Tx). The cells clearly adopt

trophoblast morphology as shown in the phase contrast image and by expression of trophoblast markers *Ascl2* and *P11* (data are mean \pm s.d., * P <0.05, ** P <0.005; n=3). *Cdx2* is expressed in every cell. *Elf5* is not expressed, correlating with the promoter remaining fully methylated as shown by bisulphite sequencing in (e). This demonstrates that initially, trophoblast differentiation mediated by forced expression of *Cdx2* is independent of *Elf5* and can override the *Elf5*-dependent feedback loop. Immunofluorescent images were taken at identical settings to positive controls. (e) *Elf5* becomes progressively demethylated and activated upon continued force-expression of *Cdx2* for 8 and 10 days to allow further progression of the trophoblast differentiation pathway. The promoter region analyzed by bisulphite sequencing is identical to that shown in Figure 4. Scale bar in **c** represents 25 μ m; scale bars in **d** represent 100 μ m (phase contrast) and 25 μ m (immunofluorescence).

Supplementary Information, Fig. S5 Full size scan of original blots shown in Fig. 2.