Fgf and Esrrb integrate epigenetic and transcriptional networks that regulate self-renewal of trophoblast stem cells

Paulina A. Latos¹,²,*, Angela Goncalves³, David Oxley⁴, Hisham Mohammed¹, Ernest Turro⁵,⁶ & Myriam Hemberger¹,²,*

Esrrb (oestrogen-related receptor beta) is a transcription factor implicated in embryonic stem (ES) cell self-renewal, yet its knockout causes intrauterine lethality due to defects in trophoblast development. Here we show that in trophoblast stem (TS) cells, Esrrb is a downstream target of fibroblast growth factor (Fgf) signalling and is critical to drive TS cell self-renewal. In contrast to its occupancy of pluripotency-associated loci in ES cells, Esrrb sustains the stemness of TS cells by direct binding and regulation of TS cell-specific transcription factors including Elf5 and Eomes. To elucidate the mechanisms whereby Esrrb controls the expression of its targets, we characterized its TS cell-specific interactome using mass spectrometry. Unlike in ES cells, Esrrb interacts in TS cells with the histone demethylase Lsd1 and with the RNA Polymerase II-associated Integrator complex. Our findings provide new insights into both the general and context-dependent wiring of transcription factor networks in stem cells by master transcription factors.
The placenta is an essential organ that ensures the exchange of nutrients, oxygen, hormones, metabolic by-products and other molecules between the maternal and fetal bloodstream. Essential insights into the molecular pathways controlling placental development have been gained by using trophoblast stem (TS) cells that can self-renew and differentiate into the various placental trophoblast cell types in vitro. Mouse TS cells are derived from the trophectoderm of blastocysts and represent the developmental counterpart to embryonic stem (ES) cells derived from the preimplantation epiblast. Unlike ES cells, TS cells can also be derived from the extraembryonic ectoderm of early post-implantation conceptuses. Derivation and maintenance of TS cells depends on fibroblast growth factor (Fgf) and Nodal/Activin signalling. Consequently, the withdrawal of both components leads to the differentiation of TS cells into various trophoblast cell types of the chorioallantoic placenta including spongiotrophoblast, syncytiotrophoblast and giant cells.

In TS cells, Fgf signalling predominantly stimulates the Mek/Erk pathway leading to the expression of essential TS cell-specific transcription factors (TFs) such as Cdx2 (refs 2, 8, 9). In addition to Cdx2, other key TFs that are critical to maintain the stem cell state of TS cells include Eomes, Esrrb, Elf5, Sox2 and Tafap2c (refs 10–15). Interestingly, some of these, such as Eomes, Elf5 and Tafap2c, have seemingly TS cell-specific functions during this developmental window, whereas others, notably Sox2 and Esrrb, have pivotal roles also in regulating pluripotency of ES cells.

Recent findings suggest that the requirement for Fgf (Fgf4) signalling in TS cells cannot be replaced by the ectopic expression of a single one of these TFs (that is, Elf5, Eomes, Cdx2, Tafap2c, Sox2 or Esrrb). However, the combined ectopic expression of Sox2 and Esrrb has been shown to be capable of sustaining TS cell self-renewal in the absence of Fgf (ref. 18). While Sox2 functions by interacting with Tafap2c, which in turn recruits Sox2 to Fgf-regulated genes, the critical interactors of Esrrb in TS cells remain unknown.

Esrrb (oestrogen-related receptor beta) plays a key role in trophoblast development as embryos deficient for Esrrb die before E10.5 because of severely impaired placental formation, characterized by an abnormal chorion layer and overabundance of giant cells. In line with a pivotal role in trophoblast development, TS cells cannot be derived from Esrrb mutants. Tetraploid aggregation experiments proved that the embryonic lethality can be rescued by wild-type (wt) trophoblast cells, thus demonstrating that the essential function of Esrrb during early development resides in the trophoblast compartment.

Although Esrrb is dispensable for development of the embryo proper, it is required for self-renewal of mouse ES cells in ground-state conditions. In this context, Esrrb cooperates with a range of TFs (e.g., Oct4, Sall4 and Ncoa3), chromatin-remodelling complexes and with components of the transcriptional machinery including the Mediator complex and RNA Polymerase II (RNAPII) to regulate self-renewal. Thus, similar to Sox2, Esrrb is a key TF in both ES and TS cells, raising questions about its specificity in different developmental contexts and whether it acts as a more general determinant of stemness irrespective of stem cell type.

Here we address the function of Esrrb in TS cells. We show that the regulation and target gene network differ profoundly between ES and TS cells. Unlike in ES cells, Esrrb is the most prominent early-response gene to Mek inhibition in TS cells, the main downstream effector of Fgf signalling in the trophoblast compartment. We demonstrate that Esrrb depletion results in downregulation of the key TS cell-specific TFs, consequently causing TS cell differentiation. This function of Esrrb is exerted by directly binding, and activating, a core set of TS cell-specific target genes including Elf5, Eomes, Bmp4 and Sox2, with little overlap to its chromatin occupancy in ES cells. Finally, by characterizing the Esrrb protein interactome we discovered a number of novel, TS cell-specific interactions. Unlike in ES cells, Esrrb interacts in TS cells with the histone demethylase Lsd1 and with the RNAPII-associated Integrator complex. Taken together, our data reveal that Esrrb regulates highly stem cell-type-specific networks due to distinct interaction partners that are essential to maintain the self-renewal state of TS cells.

**Results**

**Esrrb is an early target of Fgf/Erk signalling in TS cells.** Derivation and maintenance of TS cells depend on the presence of Fgf signalling. Numerous gene knockout experiments identified the mitogen-activated kinase Mek/Erk branch of the Fgf signalling pathway as predominantly active in both TS cells and extraembryonic ectoderm. Therefore, we first tested changes in expression of key TS cell TFs on Mek/Erk inhibition using the Mek inhibitor PD0325901 (‘PD03’; Fig. 1a). Among the candidate TFs we examined after 3–48 h of treatment, Esrrb was the fastest and most profoundly downregulated gene, followed closely by Sox2, in line with a recent report (Fig. 1b). Some TFs implicated in TS cell maintenance including Eomes, Elf5 and Cdx2 were also downregulated on Mek inhibition albeit at a slower pace, whereas the expression of others such as Ets2 or Tafap2c remained unchanged. These data were confirmed by immunostaining for some of the most prominent TS cell TFs, namely Cdx2, Elf5, Eomes and Tafap2c (Fig. 1c; Supplementary Fig. 1a). To further refine this analysis and to obtain an unbiased genome-wide coverage of the immediate-early-response genes of Mek inhibition in TS cells, we performed RNA sequencing (RNA-seq) analysis after 3 and 24 h of PD03 treatment. This global expression analysis identified in total 399 genes that were deregulated after 3 and 24 h by Fgf signalling (Fig. 1d; Supplementary Data 1). The majority of these genes were induced by Erk activation as 240 of them were downregulated on Mek inhibition, while only 159 genes were upregulated using stringent confidence parameters (Fig. 1d,e; Supplementary Data 1). Functional gene annotation analysis using MouseMine confirmed that affected genes were specifically enriched for extraembryonic (trophoblast) tissue development, as well as for embryonic lethality and transcriptional control in particular for the downregulated genes (Supplementary Fig. 1b,c). Of particular note were the dynamics of downregulation on Mek inhibition; thus, we identified 38 early responders that were downregulated, but only 10 that were upregulated (Fig. 1d). Notably, of the known TS cell TFs, this analysis confirmed Esrrb as the earliest, most rapidly silenced gene on PD03 treatment (Fig. 1e). These results provided a comprehensive overview of Fgf-regulated genes in TS cells and identified many potential candidates with a role in trophoblast development.

The finding that Esrrb was the most rapidly downregulated gene after 3 h of PD03 exposure suggested that it may be a direct target of Mek/Erk signalling. Next, we asked whether in addition to Fgf either Nodal/Activin or Bmp4 signalling can also regulate Esrrb expression in standard TS cell culture conditions. Because levels of Esrrb were not affected by either SB431542 (a Nodal/Activin signalling inhibitor) or LDN (a Bmp signalling inhibitor) treatment, we concluded that, unlike Fgf/Mek signalling, Nodal/Activin and Bmp4 signalling did not directly regulate Esrrb expression in TS cells (Supplementary Fig. 1d). Notably, the Esrrb sensitivity to Fgf pathway inhibition is TS cell-specific, as PD03 treatment of ES cells does not affect Esrrb levels. Instead, in ES cells Esrrb expression is strongly induced by the Gsk3-beta inhibitor and Wnt agonist CHIR99021 (CH). To examine...
whether Gsk3-beta and Wnt signalling are involved in regulation of Esrrb in TS cells, we treated them with either CH or the canonical Wnt inhibitor IWR-1. After 72 h of treatment, we found that Esrrb levels were unaffected by either of these compounds (Supplementary Fig. 1e). Hence, the regulation of Esrrb diverges profoundly in ES and TS cells, as it is mediated by Gsk3-beta and Erk1/2 signalling, respectively. Taken together, these insights prompted us to investigate the specific function of Esrrb in TS cells in greater detail.

Esrrb is pivotal to maintain the TS cell state. To gain first insights into which genes may be primary targets of Esrrb, we...
treated TS cells with the synthetic nonsteroidal oestrogen diethylstilbestrol (DES), an oestrogen-related receptor (ERR) antagonist, for 24 h and 4 days. This compound interacts with all three ERR isoforms Esrra, Esrrb and Esrrg but mainly acts through Esrrb in early development. It blocks co-activator binding and thus prevents transcriptional activity, and in vitro leads to TS cell differentiation. Indeed, we observed morphological changes on DES treatment, indicative of TS cell differentiation. To obtain unbiased genome-wide coverage of transcriptional changes on short (24 h) and prolonged (4d) DES treatment, we performed RNA-seq and identified 654 differentially expressed genes. Numerous differentiation markers were upregulated including the family of placental lactogen genes characteristic for giant cells (Supplementary Data 2). Importantly, we found that transcripts of TS cell Tfs Nrr0b1, Zip3, Sox2, Eomes, Elf5 and Id2 were downregulated after 24 h of DES treatment, suggesting that they may be direct targets of Esrrb (Fig. 2a). We confirmed these findings by reverse transcriptase–quantitative polymerase chain reaction (RT–QPCR) and at the protein level by immunostaining for Eomes and Elf5 (Fig. 2b,c). Interestingly, when specifically examining the trajectories between control and 24 h DES treatment, other prominent TS cell regulators such as Cdx2 were less influenced during this immediate-response window (Fig. 2a). To further examine Esrrb as a primary mediator of TF induction by Fgf signalling in TS cells, we analysed the overlap of affected genes between the DES and PD03 RNA-seq data sets (Fig. 2d,e). Strikingly, we found that both DES and PD03 treatments had an impact on the same set of prominent stem cell genes Nrr0b1, Zip3, Sox2, Id2, Cdx2, Eomes and Elf5 (Fig. 2d,e). Taken together, these data indicated that Fgf-Mek signalling regulates, via Esrrb, essential Tfs such as Sox2, Cdx2, Eomes and Elf5 that sustain TS cell self-renewal.

To account for possible off-target effects of DES treatment, for example, on Esrra and Esrrg, we also performed knockdown (KD) experiments using three short-hairpin RNAs (shRNAs) directed against Esrrb (KD-1, KD-2 and KD-3) and two scrambled shRNAs as controls (scr-1 and scr-2). Esrrb transcript levels were reduced in the KD-1, KD-2 and KD-3 lines by up to 90% compared with control lines, and these results were confirmed on the protein level (Fig. 2f,g). We found that depletion of Esrrb triggered differentiation despite the presence of Fgf as indicated by the morphological appearance of trophoblast giant cells and loss of proliferative capacity (Fig. 2h). Expression analysis revealed the rapid loss of stem cell markers including Cdx2, Eomes, Elf5, Nrr0b1 and Bmp4, and concomitant upregulation of genes associated with trophoblast differentiation including Syna, Gcm1, Cdkn1c, Prl2c2 (also known as Proliferin = Plf) and Prl3d1 (placental lactogen 1 = Pl1; Fig. 2g). We confirmed these results at the protein level by using western blot analysis (Fig. 2f). Moreover, this effect was specific to Esrrb depletion as cotransfecting the KD 1 shRNA targeted against the 3′-untranslated region with an Esrrb-coding region expression construct fully rescued the KD phenotype (Supplementary Fig. 2a,b). These data demonstrate that Esrrb is required for TS cell gene expression and self-renewal.

To gain further insights into the cohort of genes regulated by Esrrb, we performed an RNA-seq analysis on Esrrb KD-1 and KD-2 TS cells 5 days after transfection. Global expression analysis identified 59 genes that were affected by Esrrb KD in TS cells (Supplementary Fig. 2c; Supplementary Data 3). Gene ontology (GO) term analysis revealed overrepresentation of processes related to placental development and trophoblast morphology among genes affected by the Esrrb KD (Supplementary Fig. 2d,e).

In addition, on the global level, downregulated genes contained known TS cell markers including Eomes, Cdx2, Nrr0b1, Id2 and Sox2; whereas upregulated genes were highly enriched for factors associated with trophoblast differentiation. These results confirmed that Esrrb presides over a network of genes involved in extraembryonic development and specifically in maintenance of the stem cell state within the trophoblast niche.

**Esrrb forms stem cell-type-specific transcriptional networks.** To explore whether Esrrb directly regulates the key TS cell genes, we performed chromatin immunoprecipitation (ChiP) followed by QPCR and found extensive binding on putative transcriptional regulatory regions of Elf5, Eomes, Esrrb, Sox2, Bmp4, Cdx2 and Tjp2c (Fig. 3a). To obtain a comprehensive global overview of the binding sites of Esrrb in TS cells, we carried out ChiP followed by high-throughput sequencing (ChiP-seq) and compared these data to the binding profile of Esrrb in ES cells where it plays a well-appreciated role in maintaining pluripotency. We identified 14507 Esrrb-binding sites in TS cells (Fig. 3b; Supplementary Data 4). Globally, these sites were predominantly found at intronic and intergenic regions (Fig. 3c), similar in feature distribution to that observed in ES cells. However, their precise location exhibited only a partial (3,027) overlap with those in ES cells (Fig. 3b; Supplementary Data 3). The markedly different Esrrb-binding profile between ES and TS cells was exemplified by a significant enrichment of genes involved in trophodermal differentiation and placental development among the TS cell-specific peaks compared with the ES cell-specific peaks (Fig. 3d; Supplementary Fig. 3a). These results suggest that context-dependent binding of Esrrb is linked to specific developmental processes. Notably, we identified Esrrb binding at principally all known core TS cell genes, including itself, implying that Esrrb has a self-reinforcing function similar to that ascribed to many pluripotency genes in ES cells (Fig. 3e; Supplementary Fig. 3b). We tested the functionality of the Esrrb-binding sites at Eomes and Elf5, that is, two of the important TS cell genes we had identified as primary targets of Esrrb by ChiP–QPCR and ChiP-seq, in luciferase assays. Selected regions of both genes stimulated reporter activity (Fig. 3f), and this effect was abolished by either mutating Esrrb-binding sites or by DES treatment.

---

**Figure 2** | **Esrrb depletion results in TS cell differentiation.** (a) Temporal expression dynamics of a number of selected TS cell genes as identified using RNA-seq analysis after 24 h and 4 days of treatment with the oestrogen-related receptor antagonist DES compared with untreated controls. (b) RT–QPCR showing expression of TS cell genes in TS cells treated for 24 h with DES compared with untreated controls. (c) Immunostaining showing downregulation of TS cell markers Eomes and Elf5 in TS cells treated for 24 h with Esrrb antagonist DES. Magnification bars, 100 μm. (d) Plot of differentially expressed genes identified using RNA-seq analysis after 3 and 24 h of PD03 exposure and 4 h and 4-day DES treatment. (e) Venn diagram showing overlap of genes deregulated on PD03 and DES treatments. (f) Western blot analysis showing depletion of Esrrb, Cdx2 and Eomes in Esrrb KD TS cell lines (KD-1, KD-2 and KD-3) compared with controls (scr-1 and scr-2; Supplementary Fig. 10b). (g) RT–QPCR analysis of Esrrb KD (Kim, Park et al.) and control (scr-1 and scr-2) TS cells. TS cell markers (Cdx2, Elf5, Eomes and Nrr0b1) were downregulated in Esrrb-depleted cells, whereas differentiation markers (Syna, Cdkn1c, Prl2c2 and Prl3d1) were upregulated. Bars indicate the mean of three biological replicates ± s.e.m. (h) Phase contrast microscopic images of TS cells 5 days after transfection with Esrrb KD (KD-2) or scrambled control (scr-1) constructs. Esrrb KD lines were severely differentiated despite the presence of Fgf, whereas control lines formed tight, epithelial colonies. These images are representative for KD-1 and KD-2 Esrrb KD lines; KD-3 showed less severe phenotype in line with the reduced KD levels (Fig. 2f,g). Experiments were performed in biological triplicates. Magnification bar, 50 μm.
These results further confirmed that Esrrb directly binds to and regulates Eomes and Elf5 in TS cells. On a more global level, the majority of genes deregulated either on Esrrb KD or 24 h DES treatment were directly bound by Esrrb (Fig. 3g; Supplementary Fig. 3d).

To gain better insights into the context-dependent Esrrb binding, we performed de novo motif analysis using MEME/DREME followed by Tomtom suits. In TS cells, similar to ES cells, Esrrb peaks (defined here as ±200 bp around peak summit) were highly enriched in the canonical Esrrb/Esrra-binding motifs,
putative Eomes overrepresented in the Esrrb peaks. (Esrrb on pGL3-promoter). Bars show an average of four replicates peaks of Esrrb. (1) promoters, (2) exons (3), introns, (4) downstream and (5) intergenic. (genomic features in TS and ES cells. Peaks overlapping more than one type of genomic region were assigned to regions with the following priority: context-dependent activity of Esrrb in TS versus ES cells.

recruit Esrrb to TS cell-specific sites and thereby mediate the findings raised the question of whether Cdx2 could potentially secondary motif enriched in a number Esrrb peaks (Fig. 3h). These Space motif analysis (SpaMo) identified, among others, Cdx2 as a on other TFs (Fig. 3h). Central motif enrichment analysis32 showed suggesting that the context-dependent binding specificity may rely on other TFs (Fig. 3h). Central motif enrichment analysis32 showed centred and symmetrical Esrrb/Esrra motif distribution (Fig. 3i). Space motif analysis (SpaMo) identified, among others, Cdx2 as a secondary motif enriched in a number Esrrb peaks (Fig. 3h). These findings raised the question of whether Cdx2 could potentially recruit Esrrb to TS cell-specific sites and thereby mediate the context-dependent activity of Esrrb in TS versus ES cells.

To examine the functional overlap of genes regulated by Cdx2 and Esrrb, we depleted Cdx2 in TS cells by shRNA-mediated KD. Expression analysis showed that similar to the Esrrb KD, key TS cell markers were downregulated (Esrrb, Eomes and Elf5), whereas differentiation markers were upregulated (Supplementary Fig. 4a). However, when we compared ChIP-seq data sets of Esrrb (this study) and Cdx2 (published by Chuong et al.33), we identified only a small (4.1%) subset of Esrrb peaks that were

Figure 3 | ChIP-seq analysis to identify Esrrb genome-wide occupancy in TS cells. (a) Anti-Esrrb ChIP followed by QPCR showing Esrrb binding to TS cell marker genes; the Ensra region serves as a negative control. Bars indicate average of three biological replicates ± s.e.m. Statistical test: unpaired t-test with Welch’s correction. (b) A Venn diagram showing the number of high-confidence Esrrb TS cell-specific peaks identified in five independent biological replicates of ChIP-seq experiments, ES cell-specific peaks29 and those overlapping in both stem lines. (c) Proportion of Esrrb ChIP-seq peaks overlapping genomic features in TS and ES cells. Peaks overlapping more than one type of genomic region were assigned to regions with the following priority: (1) promoters, (2) exons (3), introns, (4) downstream and (5) intergenic. (d) Twenty top terms of the GREAT ontology enrichments for TS cell-specific peaks of Esrrb. (e) Examples of Esrrb-binding profiles at the Eomes and Elf5 loci. (f) Luciferase reporter analysis of TS cells transiently transfected with putative Eomes (Eomes: pGL3-promoter-Eomes) or Elf5 (Elf5: pGL3-promoter-Elf5) enhancer constructs and controls (basic: pGL3-basic and promoter: pGL3-promoter). Bars show an average of four replicates ± s.d., statistical test: unpaired t-test with Welch’s correction. (g) Pie chart of genes deregulated on Esrrb knockdown (posterior probability > 0.6) that are also bound by Esrrb. (h) Motifs found by MEME and/or DREME and SpaMO to be overrepresented in the Esrrb peaks. (i) CentriMO plot of the positional distribution of the best-matched motifs.
co-bound by Cdx2 when using the previously published list of 11462 Cdx2-specific peaks (Supplementary Fig. 4b; Supplementary Data 5) and even fewer (<1%) when applying the identical analysis criteria used in our study on the Cdx2 ChIP-seq data set for peak calling (Supplementary Fig. 4c). This small subset of co-bound loci did not contain any prominent known TS cell genes. To further examine the potential cooperation between Esrrb and Cdx2, we performed co-immunoprecipitation experiments followed by either western blot or mass spectrometry analysis. While we identified a number of Cdx2 interactors including Tead4, Eomes and Tfcp2, we were unable to detect Esrrb (Supplementary Fig. 5a–d). Thus, despite the fact that Esrrb and Cdx2 depletion interferes with TS cell maintenance, ultimately by affecting a similar subset of genes, we found neither that Cdx2 accompanied Esrrb binding at the key TS cell loci nor that they interacted at the protein level. Thus, in line with the evidence that Cdx2 is not among the early responders on 24 h DES treatment, it is likely that Cdx2 and Esrrb function in parallel pathways to regulate the stem cell state of TS cells.

Epigenetic protein interaction network of Esrrb in TS cells. Esrrb is part of a large protein network in ES cells that is required to maintain pluripotency22,23. Two main classes of interactors dominate this network: (i) epigenetic protein complexes that remodel or modify nucleosomes (for example, SWI/SNF, NuRD, p400) and (ii) TFs/cofactors that can directly stimulate RNAPII recruitment and activation (Mediator complex, components of transcriptional machinery, TFs)22,23. We thus set out to explore which of these distinct mechanisms of Esrrb-mediated control of gene expression were predominant in TS cells.

To identify the interaction partners of Esrrb that are specific to TS cells, we established a TS cell line expressing modest levels of C-terminally 3xFlag-tagged Esrrb. RT–QPCR and western blot

**Figure 4 | Lsd1 interacts with Esrrb in TS cells.** (a) Venn diagram showing total numbers and highlighted examples of TS- and ES-cell-specific, as well as shared, Esrrb interactors. Esrrb-interacting proteins as identified using mass spectrometry analysis. High-confidence (that is, present in both Esrrb samples and absent or showing negligible amount in vector control samples) hits are shown. (b) Esrrb-3xFlag immunoprecipitates analysed by western blot probed with anti-Lsd1 antibody independently confirms the interaction between Esrrb and Lsd1. (c) ChIP-QPCR showing co-occupancy of Esrrb and Lsd1 at the key TS cell marker genes. Ensa serves as a negative control. Bars represent average of three biological replicates ± s.e.m. (note that Esrrb data are the same as in Fig. 3a). (d) Venn diagram depicting the overlap between Esrrb and Lsd1 ChIP-seq peaks in TS cells. (e) Proportion of Esrrb and Lsd1 ChIP-seq peaks overlapping genomic features in TS cells. Peaks overlapping more than one type of genomic region were assigned to regions with the following priority: (1) promoters, (2) exons, (3) introns, (4) downstream and (5) intergenic. (f) Esrrb- and Lsd1-binding profiles at the Elf5 and Eomes loci. (g) RT-QPCR expression analysis of TS cell markers on 48 h treatment with Lsd1 inhibitor GSK-Lsd1 in TS cells grown in stem cell conditions. Ovol2 and Zic3 serve as positive controls as they were reported to be upregulated on Lsd1 depletion16.
analysis showed that the Esrrb-Flag TS cell line was indistinguishable from the vector control (Supplementary Fig. 6a,b). Next, we purified Esrrb-bound proteins in mild conditions, identical to those employed in ES cells.23 Using an unbiased protein identification approach using liquid chromatography-tandem mass spectrometry (LC-MS/MS), we found Esrrb (29 and 30 unique peptides, protein annotated as ‘ERR2’) in addition to numerous high-confidence interaction partners in several independent experiments (Table 1, Supplementary Data 6). Among these, we detected a number of epigenetic complexes that were previously identified as parts of the Esrrb interactome in ES cells including multiple subunits of NuRD, p400/Trrap and Mll/Trrap (Fig. 4a).23 Interestingly, we never detected any component of the SWI/SNF complex, another prominent interactor in ES cells compared this with the Esrrb occupancy profiles. Importantly, Lsd1 bound to the core set of Esrrb targets including Elf5, Eomes, Bmp4 and Sox2 (Fig. 4c); globally 60% of Esrrb peaks were co-occupied by Lsd1 (Fig. 4d–f; Supplementary Data 8) and co-bound loci were associated with a significant proportion of genes deregulated on Esrrb inhibition or KD (Supplementary Fig. 7a). However, when we specifically inhibited Lsd1, genes involved in onset of differentiation were upregulated (including Ovol2 and Zic3) but expression of the key TFs controlling TS cell self-renewal was not, or only mildly, affected (Fig. 4g). This result is in line with previous reports suggesting a role of Lsd1 primarily in regulating differentiation genes,36 as also supported by Lsd1’s broad expression pattern within the entire trophoblast compartment (Supplementary Fig. 7b).

We then sought to investigate in more detail the cooperative function between Lsd1 and Esrrb. For this purpose, we performed Lsd1 ChIP–QPCR and ChIP-seq analyses in TS cells and compared this with the Esrrb occupancy profiles. Importantly, Lsd1 bound to the core set of Esrrb targets including Elf5, Eomes, Bmp4 and Sox2 (Fig. 4c); globally 60% of Esrrb peaks were co-occupied by Lsd1 (Fig. 4d–f; Supplementary Data 8) and co-bound loci were associated with a significant proportion of genes deregulated on Esrrb inhibition or KD (Supplementary Fig. 7a). However, when we specifically inhibited Lsd1, genes involved in onset of differentiation were upregulated (including Ovol2 and Zic3) but expression of the key TFs controlling TS cell self-renewal was not, or only mildly, affected (Fig. 4g). This result is in line with previous reports suggesting a role of Lsd1 primarily in regulating differentiation genes,36 as also supported by Lsd1’s broad expression pattern within the entire trophoblast compartment (Supplementary Fig. 7b).
Table 1 | TS cell-specific Esrrb interactome.

| Identified Proteins | Accession | Score | Number of unique peptides | % Coverage |
|---------------------|-----------|-------|---------------------------|-----------|
| Steroid hormone receptor ERR2 | ERR2_MOUSE | 1362 | 30 | 65 |
| LSD1 complex | | | | | |
| Lysine-specific histone demethylase 1A | KDM1A_MOUSE | 291 | 27 | 48 |
| REST co-repressor 1 | RCOR1_MOUSE | 202 | 7 | 20 |
| Histone-lysine N-methyltransferase EHMT1 | EHMT1_MOUSE | 79 | 15 | 13 |
| C-terminal-binding protein 1 | CTBP1_MOUSE | 53 | 5 | 17 |
| Histone-lysine N-methyltransferase EHMT2 | A2CG76_MOUSE | 51 | 15 | 17 |
| MII complex | | | | | |
| Sentrin-specific protease 3 | SENP3_MOUSE | 508 | 21 | 42 |
| Ribosomal biogenesis protein LAS1L | LAS1L_MOUSE | 442 | 26 | 34 |
| Host cell factor 1 | HCFC1_MOUSE | 123 | 7 | 9 |
| Set1/ASH2 histone methyltransferase complex subunit ASH2 | ASH2L_MOUSE | 110 | 7 | 17 |
| Integrator complex | | | | | |
| Integrator complex subunit 7 | INT7_MOUSE | 322 | 14 | 21 |
| Integrator complex subunit 6 | INT6_MOUSE | 312 | 16 | 23 |
| Integrator complex subunit 10 | INT10_MOUSE | 91 | 5 | 9 |
| Integrator complex subunit 9 | INT9_MOUSE | 62 | 5 | 13 |
| p400 complex | | | | | |
| EIA-binding protein p400 | EP400_MOUSE | 236 | 2 | 12 |
| DNA methyltransferase 1-associated protein 1 | DMAP1_MOUSE | 100 | 3 | 10 |
| NuRD complex | | | | | |
| Transcriptional repressor p66-beta | P66B_MOUSE | 113 | 9 | 22 |
| Methyl-CpG-binding domain protein 3 | MBD3_MOUSE | 77 | 11 | 38 |
| Transcription factors | | | | | |
| Steroid hormone receptor ERR1 | ERR1_MOUSE | 584 | 20 | 69 |
| LINE-1 type transposase domain-containing protein 1 | G3UYN0_MOUSE | 516 | 27 | 37 |
| Upstream-binding protein 1 | UBP1_MOUSE | 277 | 15 | 40 |
| Zinc-finger protein 281 | ZN281_MOUSE | 256 | 13 | 19 |
| Ras-responsive element-binding protein 1 | RREB1_MOUSE | 235 | 11 | 16 |
| Zinc-finger protein 462 | A2SW42_MOUSE | 229 | 9 | 15 |
| Nuclear receptor subfamily 0 group B member 1 | NR0B1_MOUSE | 195 | 9 | 26 |
| Transcription factor CP2-like protein 1 | TF2L1_MOUSE | 160 | 8 | 33 |
| Zinc-finger protein 687 | ZN687_MOUSE | 159 | 12 | 14 |
| Runt-related transcription factor 1 | RUNX1_MOUSE | 149 | 10 | 41 |
| Alpha-globin transcription factor CP2 | TFCP2_MOUSE | 124 | 3 | 26 |
| Protein Prdm2 | A2A7B5_MOUSE | 105 | 8 | 7 |
| Transcription factor jun-B | JUNB_MOUSE | 97 | 3 | 16 |
| Undifferentiated embryonic cell transcription factor 1 | UTF1_MOUSE | 85 | 6 | 31 |
| Transcription factor E8 | TFEB_MOUSE | 63 | 2 | 6 |
| Zinc-finger protein 592 | ZN92_MOUSE | 62 | 7 | 8 |
| MAX gene-associated protein | MGAP_MOUSE | 59 | 3 | 3 |
| Zinc-finger protein 655 | Q6P9P9_MOUSE | 48 | 5 | 9 |
| Zinc-finger protein 143 | ZN143_MOUSE | 32 | 2 | 7 |

Transcriptional protein interactome of Esrrb in TS cells. Besides interactors involved in epigenetic regulation of transcription, we identified also TFs and cofactor complexes that directly interact with RNAPII (Table 1; Fig. 4a). Similar to some shared epigenetic complexes, we found that the TFs Nr0b1, Esrra, Tfl2l1, Zfp462 and others overlapped with the Esrrb interactome in ES cells, thereby further validating our immunoprecipitation (IP) LC-MS/MS analysis (Fig. 4a). Since Nr0b1 has been found to have an important role in ES cell self-renewal, we confirmed by co-immunoprecipitation that it also interacts with Esrrb in TS cells (Fig. 5a). ChiP-seq analysis for Nr0b1 in TS cells showed binding overlap with Esrrb on a subset of essential TS cell-specific (for example, Cdx2 and Tfl2l1) and general developmental loci (Lin28a and Cdh1; Fig. 5b–d; Supplementary Fig. 8a; Supplementary Data 8). As with Esrrb before, we observed that Nr0b1 binding in TS and ES cells showed a small overlap, with only 52 Esrrb/Nr0b1 co-bound regions shared between ES and TS cells (Supplementary Fig. 8b, Supplementary Data 8). These detailed novel data on the context-specific wiring of transcriptional networks are supported also by the limited overlap of Tfcp2l1, another TF that complexes with Esrrb in both TS and ES cells, with Esrrb TS cell peaks (Supplementary Fig. 8c; Supplementary Data 8).

Intriguingly, in contrast to the Esrrb interactome in ES cells23, we never detected components of the prominent RNAPII-associated complex Mediator as an Esrrb interactor in TS cells. This finding prompted us to search for alternative explanations of Esrrb-mediated RNAPII recruitment and activation at its target

---

NATURE COMMUNICATIONS | DOI: 10.1038/ncomms8776

© 2015 Macmillan Publishers Limited. All rights reserved.
genes involved in TS cell self-renewal. Strikingly, instead of components of the Mediator complex we identified four subunits of another key RNAPII cofactor complex named Integrator (Table 1). We validated expression of some Integrator complex components as well as other identified Esrrb interactors in ES and TS cells and observed similar levels despite context-specific interactions (Supplementary Fig. 9). We also confirmed the interaction of Integrator components with Esrrb by co-immunoprecipitation (Fig. 5e,f). Until recently, the Integrator complex was implicated in small nuclear RNA transcription but a recent study found that it also functions in Fgf-mediated transcriptional activation of immediate-early-response genes [8,39]. This important finding may explain how Esrrb attracts the transcriptional machinery in the absence of the interaction with Mediator in TS cells (Fig. 5g).

In summary, our results provide comprehensive insights into the stem cell-type-specific regulation and function of Esrrb, suggesting an exciting mechanism of how Fgf via Esrrb can rapidly and specifically impact on the transcription of key genes controlling self-renewal of TS cells (Fig. 5h).

**Discussion**

Esrrb is known to play a central role in maintaining pluripotency of ES cells by acting in concert with various other key pluripotency genes. Despite this, mouse mutants deficient for Esrrb die of a trophoblast defect that can be rescued by tetraploid ES cells by acting in concert with various other key regulators such as Elf5 [2015 Macmillan Publishers Limited. All rights reserved.]

Esrrb is known to play a central role in maintaining pluripotency of ES cells by acting in concert with various other key pluripotency genes. Despite this, mouse mutants deficient for Esrrb die of a trophoblast defect that can be rescued by tetraploid ES cells by acting in concert with various other key regulators such as Elf5 [2015 Macmillan Publishers Limited. All rights reserved.]

Esrrb is known to play a central role in maintaining pluripotency of ES cells by acting in concert with various other key pluripotency genes. Despite this, mouse mutants deficient for Esrrb die of a trophoblast defect that can be rescued by tetraploid ES cells by acting in concert with various other key regulators such as Elf5 [2015 Macmillan Publishers Limited. All rights reserved.]

Esrrb is known to play a central role in maintaining pluripotency of ES cells by acting in concert with various other key pluripotency genes. Despite this, mouse mutants deficient for Esrrb die of a trophoblast defect that can be rescued by tetraploid ES cells by acting in concert with various other key regulators such as Elf5 [2015 Macmillan Publishers Limited. All rights reserved.]

Esrrb is known to play a central role in maintaining pluripotency of ES cells by acting in concert with various other key pluripotency genes. Despite this, mouse mutants deficient for Esrrb die of a trophoblast defect that can be rescued by tetraploid ES cells by acting in concert with various other key regulators such as Elf5 [2015 Macmillan Publishers Limited. All rights reserved.]

On differentiation, Lsd1 decommissions these enhancers ensuring the shutdown of the pluripotency programme [40]. In contrast, in TS cells, it has been shown that the transcription of stem cell marker genes Cdx2 and Eomes is reduced considerably faster in the absence of Lsd1 than in controls on induction of differentiation, in line with the observation that Lsd1-depleted TS cells exhibit a lowered threshold for differentiation onset [56]. Thus, although depletion [56] or inhibition of Lsd1 has no clear-cut effect on TS cell marker silencing in stem cell conditions (Fig. 4g), it appears that Esrrb and Lsd1 cooperatively promote the ‘naïve’ TS cell state to maintain a fine-tuned balance of gene transcription at joint TS cell target genes.

Besides epigenetic regulators, we identified numerous TFs that interact with Esrrb in TS cells. One of these factors is Nr0b1 (=Dax1), which associates with Esrrb also in ES cells [23,41,42]. Nr0b1 is part of the ES cell self-renewal network where it interacts with Oct4 and gets recruited to Oct4/Sox2-binding sites [23,42]. However, we discovered that similar to Esrrb, Nr0b1 does not show an extensive binding overlap between TS and ES cells, again underpinning the finding that, although both TFs are shared between ES and TS cells, they exert largely divergent functions depending on stem cell type. This raises the question about how the context-dependent recruitment of Esrrb and Nr0b1 to distinct sites is achieved in different stem cells. Regarding Esrrb, Cdx2, as a key TS cell regulator, is an obvious candidate for this role. This notion is further supported by our findings that similar genes are downregulated on Esrrb and Cdx2 depletion. However, we could not detect an extensive overlap between published Cdx2 (ref. 33) and our Esrrb ChIP-seq-binding profiles, and neither did we observe a direct interaction between these two factors at the protein level. We did, however, identify other prominent Cdx2 interactors including Tead4 and Eomes, thus strongly validating our approach. Although Esrrb and Cdx2 ultimately co-regulate, directly or indirectly, a similar set of target genes, it is therefore likely that both TFs function in parallel pathways to regulate the stem cell state of TS cells. Taken together, these findings provide new and comprehensive insights into the TF interaction network that governs TS cell self-renewal and identity. It will be important to elucidate in the future how this network exerts specificity in TS cells with partially shared components present also in ES cells.

In fact, our comprehensive identification of interaction partners may provide first leads into how this context-dependent wiring of transcriptional networks is achieved, by revealing
association with distinct components of the core transcriptional machinery depending on stem cell type. In ES cells, Esrbr was identified as being uniquely associated with the RNAPII complex and numerous subunits of the Mediator complex, indicating a critical role for Esrbr in transcriptional activation. The Mediator complex is a multifunctional RNAPII-associated scaffold that is required for mRNA transcription at different stages of the process. The interaction with TFs is crucial for recruitment and specificity in response to signalling. In TS cells we did not detect an interaction between Esrbr and the Mediator complex raising the question of an alternative way to stimulate transcription. Instead, we identified numerous subunits of the Integrator complex interacting with Esrbr. Although the Integrator complex has been implicated mostly in the transcription of small nuclear RNAs, a recent study demonstrated its involvement in both initiation and release from pausing of transcription of some key genes, notably mechanisms was demonstrated for early-response genes that are specific role of Esrbr and provide key insights into mechanisms of association with the Integrator complex and release of RNAPII from pausing (Fig. 5g). This would suggest that not only specific signals and TFs shape self-renewal and identity of different stem cell types but that general mechanisms of transcriptional control also contribute to confer stem cell specificity.

Taken together, we demonstrate here an essential TS cell-specific role of Esrbr and provide key insights into mechanisms of Fgf-Erk-mediated self-renewal in TS cells.

Methods

Tissue culture and transfections. Mouse TS cells (blastocyst-derived TS EGFP line, a kind gift of Dr Janet Rossant, Toronto, Canada), proven to exhibit full developmental competence as they colonize all trophoblast layers in chimeras, were cultured as described previously. Briefly, TS cells were grown in a standard TS medium (RPMI 1640 supplemented with 20% fetal calf serum, 2 mM L-Glutamine, 2 mM sodium pyruvate and 100 mM 2-mercaptoethanol) containing 70% mouse embryonic fibroblast -conditioned medium and 25 ng/ml FGF2 and 1 μg/ml heparin. Cells were split every third day using trypsin. Transfections were performed for 6 h in OptiMEM media supplemented with FGF2 and heparin using 1% Lipofectamine 2000 (Life Technologies) on nonadherent dishes. After 24 h, cells were selected with 300 μg/ml G418. Since FGF has also a very rapid impact on transcription of some key genes, notably Esrbr, in TS cells, this raises the exciting possibility that Esrbr activates transcription by association with the Integrator complex and release of RNAPII from pausing (Fig. 5g). This would suggest that not only specific signals and TFs shape self-renewal and identity of different stem cell types but that general mechanisms of transcriptional control also contribute to confer stem cell specificity.

Taken together, we demonstrate here an essential TS cell-specific role of Esrbr and provide key insights into mechanisms of Fgf-Erk-mediated self-renewal in TS cells.

Chromatin immunoprecipitation. Immunoprecipitations were carried out as described. Briefly, cells (1–2 × 10^6) were fixed in 2 mM Di(N-succinimidyl) glutarate (DSG) (80424, Sigma) in PBS at room temperature (RT) for 45 min. After washing in PBS, cells were fixed again in 1% formaldehyde in TS base media at RT for 10 min. Fixation was stopped by adding glycine to a final concentration of 0.125 M. Cells were washed twice in PBS and resuspended in wash buffer 1 (10 mM Hepes pH 7.5, 0.5 mM EDTA and 0.75% Triton X-100) and incubated at 4°C for 10 min. After pelleting, cells were resuspended in wash buffer 2 (10 mM Hepes pH 7.5, 100 mM NaCl, 0.1% FBS, 0.5 mM EDTA and 0.5% sodium deoxycholate) and incubated at 4°C for 10 min. After pelleting, cells were lysed in the lysis/sonication buffer (150 mM NaCl, 25 mM Tris pH 7.5, 5 mM EDTA, 0.1% Triton X-100, 1% SDS and 0.5% sodium deoxycholate) containing complete protease inhibitors (Roche) on ice for 30 min. Chromatin was sonicated 30× on/30 off for 25–30 cycles using the BioRuptor (Diagenode) to the average size 500 bp. Chromatin was pelleted 1/10 with the dilution buffer (150 mM NaCl, 25 mM Tris pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate) containing complete protease inhibitors. Protein G magnetic Dynabeads (10004D, Invitrogen) were blocked with 1 μg/ml BSA and incubated at 4°C for 1 h and washed with buffer A (150 mM NaCl, 25 mM Tris pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate). Chromatin was pre-cleared with pre-blocked beads at 4°C for 1 h. Three hundred and fifty micrograms of chromatin and ten micrograms of antibody (mouse anti-Esrbr (Perseus Proteomics PP-H6705-00), mouse normal IgG (Santa Cruz sc-2025), rabbit anti-Nrnb1 (Santa Cruz sc-841X) and rabbit normal IgG (Santa Cruz sc-2027X) were used per each IP. IP was performed overnight at 4°C with rotation. Pre-blocked magnetic beads were added next morning for 7–8 h. Beads were washed at 4°C with buffer A (150 mM NaCl, 25 mM Tris pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate) three times, buffer B (50 mM Tris pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 and 1 mM EDTA) buffer C (50 mM Tris pH 8.0, 250 mM LiCl, 0.5% sodium deoxycholate, 1% NP-40 and 1 mM EDTA) and rinsed with TE buffer. DNA was eluted from beads in the elution buffer (1% SDS, 0.1 M NaHCO3). Samples were treated with RNase A and Proteinase K and reverse-crosslinked overnight at 55°C. DNA was phenol–chloroform-extracted, ethanol precipitated and extracted with ETOH/Glyo (glycerol). Extracted DNA (PCR purified or purified on the PCR purification columns (Qagen; for ChIP-seq libraries). To generate a library, DNA from four IPs was pooled and the NEB Next DNA Library Prep Master Mix (NEB E6040u) was used according to the manufacturer’s instructions. Libraries were amplified using 18 PCR cycles, purified with Agencourt AMPure XP SPRI beads (Beckman Coulter, A68381) and size-selected on an agarose gel. The DNA was extracted using a Qiaquick gel extraction kit (Qiagen) and its concentration determined using the KAPA Illumina SYBR Universal Ligid Q Kit (KAPA Biosystems KK8424) and Bioanalyzer 2100 system (Agilent). Libraries were sequenced on Illumina HiSeq1000 sequencer.

RT-QPCR. RNA was isolated using the RNeasy kit (Qiagen) and DNA-treated with the TURBO DNA-free kit (Life Technologies AM1907) according to the manufacturer’s instructions. cDNA was synthesized using 3 μg RNA primed with random hexamers according to the RevertAid H Minus M-MulV Reverse Transcriptase protocol (Thermo Scientific). PCR reactions were performed using SYBR Green Jump Start Taq Ready Mix (Sigma S4438), on a Bio-Rad CFX96 thermocycler. Primer pairs are provided in Supplementary Table 2.

RNA KD. RNA KD experiments were performed using the Dicer-neo system. Oligos (see Supplementary Table 3 for shRNA sequences) were cloned into BglII/XhoI sites. TS cells were transfected with 4.5 μg of plasmid and selected after 24 h with 400 μg/ml G418.

Western blot analysis. Whole-cell extracts were prepared with TG buffer (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol and 1.5 mM MgCl2) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitors (50 mM NaF and 1 mM Na3VO4). Nuclear extracts were prepared with hypotonic buffer 10 mM Hepes pH 7.9, 1.5 mM MgCl2, supplemented with protease inhibitor cocktail (Roche). After centrifugation at 10,000g for 10 min, nuclear pellets were extracted with 10 mM Hepes pH 7.9, 400 mM NaCl, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA and 12.5% Glycerol supplemented with protease inhibitor cocktail (Roche). Protein lysates were resolved using SDS–PAGE and transferred using a Bio-Rad Mini Trans Blot system 170–3,930 on poly(vinylidene difluoride) membrane (Immobilon-P, Millipore). Membranes were blocked with 5% milk powder and incubated with specific primary antibodies overnight at 4°C (1:1000 anti-Cdx2 (Biogenex MUX932-UA–C), 1:500 anti-Eifs (Santa Cruz sc-9645), 1:750 anti-Eomes (Abcam ab23345), 1:1000 anti-anti-Esrbr (Perseus Proteomics PP-H6705-00), 1:500 anti-tubulin (Abcam ab61660), 1:1000 anti-Int1 (Bethyl Laboratories A300-361A), 1:1000 anti-Int9 (Bethyl Laboratories A300-412A), 1:2000 anti-Flag (SIGMA F1804), 1:1000 anti-Ld1 (Abcam ab17721), 1:2000 anti-Nrbr1 (Santa Cruz sc-841X), 1:750 mouse, anti-phospho Erk1/2 (Cell Signal. 9106), 1:1000 mouse anti-Erk1/2 (BD 610031), 1:1000 mouse...
Supplementary Table 5.

Detection was carried out with enhanced chemiluminescence reaction (GE anti-Oct4 (Santa Cruz sc-5279), followed by horseradish peroxidise-conjugated

Immunostaining. Cells were fixed in 4% paraformaldehyde/PBS for 20 min at 4 °C, permeabilized and blocked for 30 min in 0.5% bovine serum albumin and 0.1% Triton X-100. In the following primary antibodies with given dilutions used were: anti-Cdx2 1:500 (Biogenex MU929-UC), anti-EisF-1 1:200 (Santa Cruz sc-9645) and anti-Eomes 1:400 (Abcam ab23345). Alexa Fluor-conjugated secondary antibodies (Life Technologies) were applied at 1:1,000 in 0.5% bovine serum albumin and 0.1% Tween-20 in PBS (PBT-BSA) blocking solution. Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and imaged using a Zeiss LSM700 confocal microscope with the ZEN software.

RNA-seq. Total RNA was prepared using the RNeasy kit (Qiagen 74104) followed by DNase treatment using the TURBO DNA-free kit (Life Technologies AM1907) according to the manufacturer’s instructions. Libraries were quantified indexed using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epiconcept SSV21106) according to the manufacturers’ instructions. Libraries were quantified assessed using both the KAPA Library Quantification Kit (KAPA Biosystems) and the NeoSpective Assay System (Life Technologies) and with the relevant project and personal licenses in place. Sections (7 μm) were deparaffinized, boiled for 30 min in 10 mM sodium citrate pH 6.0 or 1 mM EDTA pH 7.5, 0.05% Tween-20 and blocked with PBT-BSA. Primary antibodies and dilutions used were as follows: rabbit anti-Oct4 1:500 (Santa Cruz sc-5279), anti-rabbit anti-Nr0b1/Dax1 1:200 (Santa Cruz sc-841), anti-rabbit anti-Sox2 1:100 (R&D Systems AF2081). Primary antibodies were detected with appropriate secondary AlexaFluor 488, 568 or 647 antibodies, counterstained with DAPI and observed using an Olympus BX41 or BX61 epifluorescence microscope. All antibodies used are listed in Supplementary Table 5.

Co-immunoprecipitation. Esrr-b coding sequence (PiggyBac-Esrrb-ires-Neo, a kind gift from Austin Smith, CSIR, Cambridge, UK) was cloned to result in PiggyBac-CAG-Avi-Esrrb-3xFlag-ires-Neo constructs. TS EGFP cells were transfected with the construct along with the empty vector control using Lipofectamine 2000 (Invitrogen), selected with G418 and expanded in 10-15 cm dishes. Co-immunoprecipitation was performed as described before9. Cells were washed in PBS, harvested and resuspended in Buffer A (10 mM Hepes pH 7.6, 1.5 mM MgCl₂, and 10 mM KCl) and disrupted by 10 strokes in dounce homogenizer. Extracts were spun down and the pellet resuspended in Buffer C (20 mM Hepes pH 7.6, 25% Glycerol, 420 mM NaCl, 1.5 mM MgCl₂ and 0.2 mM EDTA) using dilauryl cysteine (Fishier Scientific). Anti-FLAG M2 agarose beads (120 μl; Sigma) equilibrated in buffer D were added to 1.5 ml of nuclear extract in No Stick microcentrifuge tubes (Alpha Laboratories) and incubated for 3 h at 4 °C in the presence of Benzonase (Novagen). Beads were washed five times for 5 min with buffer D containing 0.5% NP-40 (C0%) and bound proteins were eluted four times for 15 min at 4 °C with buffer C-100 containing 0.2 mg/ml • 1-FLAG-tripptide (Sigma). Eluates were pooled and analysed using mass spectrometry or western blot.

Mass spectrometry. Immunoprecipitated proteins from two biological replicates of each Esrrb- and vector-transfected TS cells were run a short distance (~5 mm) into an SDS-PAGE gel, which was then stained with colloidal Coomassie stain (Imperial). The fluorescent stained gel pieces were excised, stained, reduced, carbamidomethylated and digested overnight with trypsin (Promega sequencing grade, 10 ng/ml in 25 mM ammonium bicarbonate) as previously described1. The resulting tryptic digests were analysed using LC/MS/MS on a system comprising a nanoLC (Proxeon) coupled to a LTQ Orbitrap Velos Pro mass spectrometer (Thermo Scientific). LC separation was achieved on a reversed-phase column (Reprosil C18AQ, 0.075 × 150 mm, 3 μm particle size), with an acetonitrile gradient (0–35% over 180 min, containing 0.1% formic acid, at a flow rate of 300 nl min−1). The mass spectrometer was operated in a data-dependent acquisition mode, with an acquisition cycle consisting of a high-resolution precursor ion scanning range 350–1,500, followed by up to 20 CID spectra (with a 30+ dynamic exclusion of former target ions). Mass spectrometric data were processed using Protome Discoverer v1.4 (Thermo Scientific) and searched against the mouse entries in Uniprot 2013.09, and against a database of common contaminants, using Mascot v2.3.5 (Matrix Science). Quantitative values were calculated with Proteome Discoverer for each identified protein as the average of the three highest peptide ion peak areas. The search results and quantitative values were imported into Scaffold v3.6 (Proteome Software Inc.), which reported a total of 1,249 proteins across the four samples, with a calculated protein false discovery rate of 0.2%. After applying further filters (minimum of two unique peptides per protein with at least one in both biological replicates, ratio of quantitative values >2 for both Esrrb/vector pair) 90 proteins remained, as shown in Supplementary Data 6.

RIME. RIME was carried out as described37. Briefly, cells were crosslinked in media containing 1% formaldehyde (EM grade; tebbu bio) for 8 min. Crosslinking was quenched by adding Glycine to a final concentration of 0.2 M. The cells were washed with and harvested in ice-cold PBS. The pellet was resuspended in 10 ml of LBI buffer (30 mM HEPEs-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 1% viscous agar, and 0.25% Triton X-100) for 10 min at 4 °C. Cells were pelleted, resuspended in 10 ml of LBI buffer (10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA), and mixed at 4 °C for 5 min. Cells were pelleted and resuspended in 300 μl of LBI buffer (10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate and 0.5% N-laurolecithin) and sonicated in a microtip bath sonicator (Diagenode Bioruptor). A total of 30 μl of Triton X-100 was added, and the lysate was centrifuged for 10 min at 20,000 r.c.f. The supernatant was then incubated with 100 μl of magnetic beads (Dynal) prebound with 20 μg of either anti-Isld1 (ab 17721 Aabcam) or anti-lgg (sc-2027 Santa Cruz) antibody, and IP was conducted overnight at 4 °C. The beads were washed 10 times in 1 ml of RIPA buffer and twice in 100 mM ammonium hydrogen carbonate solution. Detailed results including peptide sequences, peptide scores, ion scores, expect values and Mascot scores have been included in Supplementary Data 7.

Luciferase assays. Putative wt or mutated EisF and Esrrb enhancers were cloned into the BamH I site of the pGL3-promoter vector (Promega) and co-transfected with Renilla plasmid into the TS EGFP line. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer’s instructions. Control lines were generated by co-transfection of Renilla with either pGL3 promoter or pGL3-basic vectors (Promega). Cells were harvested 48 h after transfection and luciferase activity was determined using a Promega GloMax 96-well luminometer.
References

1. Cross, J. C., Werb, Z. & Fisher, S. J. Implantation and the placenta: key pieces of the development puzzle. Science 266, 1508–1518 (1994).

2. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A. & Rossant, J. Promotion of trophoblast stem cell proliferation by FGFR4. Science 282, 2072–2075 (1998).

3. Latos, P. A. & Hemberger, M. Review: the transcriptional and signalling networks of mouse trophoblast stem cells. Placenta 32(Suppl A): S81–S85 (2014).

4. Rossant, J. Stem cells and early lineage development. Cell 132, 527–531 (2008).

5. Erlebacher, A., Price, K. A. & Glimcher, L. H. Maintenance of mouse trophoblast stem cell proliferation by TGF-beta/activin. Dev. Biol. 275, 158–169 (2004).

6. Natale, D. R., Hemberger, M., Hughes, M. & Cross, J. C. Activin promotes differentiation of cultured mouse trophoblast stem cells towards a labyrinth cell fate. Dev. Biol. 335, 120–131 (2009).

7. Kubaczkai, C. et al. Derivation and maintenance of murine trophoblast stem cells under defined conditions. Stem Cell Rep. 2, 232–242 (2014).

8. Kunath, T., Strumpf, D. & Rossant, J. Early trophoblast determination and stem cell maintenance in the mouse: a review. Placenta 25(Suppl A): S32–S38 (2004).

9. Krueger, F. et al. Enhancer decomposition by LSD1 during embryonic development. Nature 510, 300–304 (2013).

10. Kubaczkai, C. et al. Derivation and maintenance of trophoblast stem cells in a defined medium. PLoS One 7, e36140 (2012).

11. Strumpf, D. et al. Cdx2 is required for correct cell fate specification and differentiation of trophodermid in the mouse blastocyst. Development 132, 2093–2102 (2005).

12. Luo, J. et al. Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERp-beta. Nature 388, 778–782 (1997).

13. Domnison, M. et al. Loss of the extraembryonic ectoderm in Eif5s mutants leads to defects in embryonic patterning. Development 132, 2299–2308 (2005).

14. Avilion, A. A. et al. Multipotent cell lineages in early mouse development depend on Sox2 function. Genes Dev. 17, 126–140 (2003).

15. Auman, H. J. et al. Sox2: a transcription factor that crucially regulates mesoderm formation. Nature 404, 95–99 (2000).

16. Martello, G. et al. Sox2 is a pivotal target of the Gsk3/Tcf3 axis regulating trophoblast stem cell proliferation by TGF-beta/activin. Mol. Cell Biol. 33, 2056–2066 (2013).

17. Kim, J., Chu, J., Shen, X., Wang, J. & Orkin, S. H. An extended transcriptional network for pluripotency of embryonic stem cells. Cell 132, 1049–1061 (2008).

18. Malik, S. & Roeder, R. G. The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat. Rev. Genet. 11, 761–772 (2010).

19. Himeono, E., Tanaka, S. & Kunath, T. Isolation and manipulation of mouse trophoblast stem cells. Curr. Protoc. Stem Cell Biol. Chapter 1, Unit 4E 4 (2008).

20. Tavares, L. et al. RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. Cell 148, 664–678 (2012).

21. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transformer. Bioinformatics 25, 1754–1760 (2009).

22. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 9, R137 (2008).

23. Li, Q., Brown, J. B., Huang, H. & Buckler, J. P. Measuring reproducibility of high-throughput experiments. Ann. Appl. Stat. 5, 1752–1779 (2011).

24. Machanic, P. K., Esteller, M. & Wann, M. RNA-seq: a lens through which to view the epigenome. Trends Genet. 30, 505–514 (2014).

25. Turro, E., Astle, W. J. & Tavare, S. Flexible analysis of RNA-seq data using mixed effects models. Ann. Appl. Stat. 8, 981–986 (2014).

26. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transformer. Bioinformatics 25, 1754–1760 (2009).

27. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 9, R137 (2008).

28. Li, Q., Brown, J. B., Huang, H. & Buckler, J. P. Measuring reproducibility of high-throughput experiments. Ann. Appl. Stat. 5, 1752–1779 (2011).

29. Machanic, P. K., Esteller, M. & Wann, M. RNA-seq: a lens through which to view the epigenome. Trends Genet. 30, 505–514 (2014).

30. Turro, E., Astle, W. J. & Tavare, S. Flexible analysis of RNA-seq data using mixed effects models. Bioinformatics 30, 180–188 (2014).

Acknowledgements

We thank Sasha Mendjan for helpful comments and fruitful discussions. We are grateful to Kristina Tabbada for Illumina high-throughput sequencing, Dominika Dudzinska for help with generating sequencing libraries, to Simon Andrews and Felix Krueger for help with bioinformatic analysis, to Judith Webster for assistance with Mass Spectrometry experiments, to Wojciech Latos for technical assistance and to Professor Wolf Reik for support. This work was supported by a Next Generation Fellowship awarded to P.A.L. by the Centre for Trophoblast Research, University of Cambridge by the Biotechnology and Biological Sciences research Council (BBSRC) and by the Wellcome Trust.

Author contributions

P.A.L., D.O. and H.M. conducted experiments, A.G. and E.T. performed the bioinformatic analyses and P.A.L. and M.H. designed the study, interpreted data and wrote the manuscript.

Additional information

Accession codes.

The RNA-seq and ChIP-seq data have been deposited in the ArrayExpress database with accession code E-MTAB-3565. The mass spectrometry proteome data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD002183.

Supplementary Information

accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.
Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Latos, P. A. et al. Fgf and Esrrb integrate epigenetic and transcriptional networks that regulate self-renewal of trophoblast stem cells. Nat. Commun. 6:7776 doi: 10.1038/ncomms8776 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/