Elf5-centered transcription factor hub controls trophoblast stem cell self-renewal and differentiation through stoichiometry-sensitive shifts in target gene networks

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While the regulatory nature of TF stoichiometry has been explored in ESCs and iPSCs, it is not known whether similar mechanisms of control over self-renewal and differentiation underpin the behavior of stem cells of the trophoblast lineage. Trophoblast stem cells (TSCs) are a stem cell type representative of the extraembryonic placental lineage most akin to cells in the extraembryonic ectoderm (ExE), a structure that eventually gives rise to specialized placental cell types (Tanaka et al. 1998; Adachi et al. 2013). TSCs can self-renew and remain multipotent in

Figure 1.

overexpression at these early stages, we compared global expression profiles (RNA sequencing [RNA-seq]) of chorionic trophoblast tissue recovered from these E8.5 paraffin-embedded sections, thus allowing us to directly correlate histological appearance and transcriptomic changes. This analysis confirmed up-regulation of *Elf5* and down-regulation of *Tpbpa* in transgenic trophoblasts, as expected (Fig. 1E). It also revealed misregulation of a number of genes involved in cytoskeletal and extracellular matrix organization (e.g., *Actg2*, *Col1a2*, *Col3a1*, *Col6a1*, *Col6a3*, and *Myh11*), growth factor availability (*Chrdl2* and *Pappa2*), and angiogenesis (*Rgs5*), as reflected by gene ontology enrichments (Supplemental Fig. S1E). These expression changes underpin the cellular dysmorphology phenotype at the molecular level.

At E10.5, transgenic implantation sites exhibited a vast overabundance of trophoblast giant cells and were often infiltrated by leukocytes, indicative of a starting resorption process (Fig. 1F). When embryos were dissected at E13.5, only control conceptuses exhibiting placental GFP signals were retrieved (Table 1). Taken together, these data demonstrated that *Elf5* overexpression causes precocious trophoblast differentiation, leading to embryonic lethality around mid-gestation.

Elf5 protein interactome

Having shown that Elf5 levels are critical for establishing a proliferative TSC compartment in vivo, we aimed to identify the protein interaction partners of Elf5 that

Figure 2. Identification of the Elf5 protein interactome in TSCs. (A) Summary of high-confidence Elf5 interactors as identified in three independent immunoprecipitation (IP) and mass spectrometry experiments. Note the interaction of Elf5 with the two other TSC TFs: Eomes and Tfap2c. The full list of interactors is provided in Supplemental Table S1. (B) Independent validation of the interaction between Elf5 and Eomes by anti-Flag immunoprecipitation of the 3xFlag-tagged (3xF) Elf5 protein followed by Western blotting. (C) Independent validation of the interaction between Elf5 and Tfap2c by immunoprecipitation followed by Western blotting. The asterisk highlights the Elf5-3xFlag band in the input sample. Additional validation of the Elf5–Tfap2c interaction is provided in Figure 3F and Supplemental Figure S2, D and E. (D)actia-19iiiiiiiii

were capable of forming colonies and could be propagated long-term (Fig. 4E; Supplemental Fig. S3D,G). These data were entirely in line with the Elf5 overexpression phenotype that we observed in vivo (Fig. 1).

In contrast, Eomes-overexpressing cells exhibited an undifferentiated, epithelial morphology and a TSC-like gene expression pattern (Fig. 4C,D; Supplemental Fig. S3H,I). Significantly, the Elf5- and Tfap2c-induced

Figure 3. Dynamics of Elf5, Eomes, and Tfap2c expression. (A) Expression dynamics of *Eomes*, *Elf5*, and *Tfap2c* by RT-qPCR in short-term (*top* row) and longer-term (*bottom* row) differentiation time-course experiments of TSCs. (B) Western blots confirming the dynamic regulation of Eomes, Elf5, and Tfap2c upon trophoblast differentiation. (C) Immunofluorescence stainings of TSCs grown in stem cell conditions (TSC) and after 1 d of differentiation (1 d diff.) for Eomes, Elf5, and Tfap2c. (D) Immunofluorescence stainings of E6.5 conceptuses for Eomes, Elf5, and Tfap2c. All three TFs are expressed in the ExE region that harbors trophoblast cells with stem cell potential (bracket). Elf5 and Tfap2c expression is retained in trophoblast cells outside this compartment that start to differentiate. (E) Schematic representation of the comparative expression patterns and relative protein levels (darker shading indicates higher expression levels) of Eomes, Elf5 and Tfap2c in the trophoblast compartment of early post-implantation conceptuses. (F) Immunoprecipitation (IP) of 3x-Flag-tagged (3xF) Elf5 and empty vector (vec) control from TSCs and 1-d (24-h)-differentiated TSCs followed by Western blotting for the factors indicated. Note the higher enrichment of Eomes in Elf5 immunoprecipitations from TSCs versus differentiated cells. Conversely, Tfap2c is more abundant in Elf5 immunoprecipitations from differentiated trophoblasts. The Western blot for Elf5 confirms the presence of the tagged Elf5-3xFlag protein in the corresponding cell lines that is of higher molecular weight than the endogenous Elf5 present in the input samples. (G) Mass spectrometric quantification of Eomes and Tfap2c peptide enrichment in Elf5-3xFlag immunoprecipitations. Values were normalized against Hspa8 that did not change enrichment with differentiation. Results show the significant decrease in Eomes and in-

differentiation-promoting effect could be quenched by concomitant overexpression of Eomes. This was evident in TSC lines carrying the Eomes_Elf5 construct but also upon cotransfection of Eomes with Elf5 and/or Tfap2c (Fig. 4E). Eomes expression reduced differentiation rates even in established Elf5 and Tfap2c_Elf5 cell lines (Fig. 4F).

To further assess this apparent dependence on protein stoichiometry, we performed knockdown experiments of Eomes in TSCs (Supplemental Fig. S4). Mirroring the relative shift in TF balance induced by Elf5 and Tfap2c overexpression, reduced Eomes levels also promoted TSC

that (1) Eomes-overexpressing cells are most similar to TSCs, (2) down-regulation of Eomes and Elf5 rapidly triggers significant differentiation, and (3) overexpression of Elf5 globally induces a state of early-onset differentiation, which corroborates the critical role of Elf5 levels precisely at the tipping point between TSC self-renewal and exit from the stem cell state.

Collectively, this careful dissection of TF levels revealed that a balanced expression of Eomes and Elf5 promotes the stem cell state, while proportionally higher Elf5 and Tfap2c levels trigger TSC differentiation (Fig. 4G).

Dissecting the transcriptional networks established by Eomes, Elf5, and Tfap2c

To identify genomic loci occupied by the various binary and ternary combinations between Eomes, Elf5, and Tfap2c, we performed ChIP-seq for Tfap2c and integrated these data with previously published Eomes and Elf5 ChIP-seq profiles in TSCs (Chuong et al. 2013). In total, we identified 1254 loci cobound by all three TFs, and 4254, 2035, and 766 sites cobound only by Elf5–Tfap2c, Elf5–Eomes, and Eomes–Tfap2c, respectively (Fig. 5A). These were distributed across the genome in a largely similar pattern (Supplemental Fig. S5A). Importantly, the numbers of observed combinatorial binding sites were highly statistically significant over co-occupancy expected at random, in particular for the triple, the Elf5–Tfap2c, and the Elf5–Eomes combinations (Fig. 5B). Interestingly, co-occupancy of Tfap2c with Sox2, another TF known to interact with Tfap2c in TSCs (Adachi et al. 2013), was confined to a set of sites largely distinct from those bound by Tfap2c and Elf5 (Fig. 5C). Overlap with Cdx2 was marginal (Supplemental Fig. S5B). These chromatin-binding patterns strongly support our functional and protein–protein interaction data of a concerted action of Eomes, Elf5, and Tfap2c in TSCs.

Eomes–Elf5–Tfap2c triple occupancy demarcates active TSC genes

Analysis of genes associated with the various co-occupied regions revealed that triply bound elements were highly enriched for TSC genes, including Eomes, Elf5, and Tfap2c themselves as well as others such as Bmp4, Cdh1, Fgfr2, Gata3, Sox2, Spry4, and Zic3 (Fig. 5D; Supplemental Table S2). This observation was corroborated by functional annotation (Genomic Regions Enrichment of Annotations Tool [GREAT]) analyses (McLean et al. 2010) in which triply bound genes were tightly linked with blastocyst formation and very early stages of trophoblast development (Fig. 5E; Supplemental Fig. S5C). In contrast, genes associated with Elf5–Tfap2c doubly bound loci such as Cdh1

intricate detail the stoichiometry-driven changes of the transcriptional networks regulating the onset of trophoblast differentiation.

Differential TF-binding motif distribution underlies the shift toward a differentiation-promoting program

To reveal the mechanism underlying these shifts in relative genome occupancy, we searched for TF motifs within the triple, double, and single ChIP-seq peaks. Globally, Tfap2c ChIP-seq elements were very tightly correlated with the Tfap2c consensus binding sequence, with 76% of peaks overlapping the cognate Tfap2c motif. In stark contrast, the Tfap2c motif was significantly underrepresented at the 1254 triply bound sites, as only 405 (32%) of them contained one (Fig. 6E). In comparison, the frequency of Elf5 motif recognition was similar or even enriched at triply bound sites over all Elf5 peaks. The Eomes motif did not lend itself to this same type of analysis, as it is too poorly defined. These findings implied that Tfap2c binding to triple elements is mediated through its physical association with Elf5 and Eomes. In contrast, Tfap2c peaks that increased with differentiation harbored the cognate Tfap2c motif.

Overall, these findings provide a molecular mechanism for how the same TFs can orchestrate both self-renewal and differentiation-promoting transcriptional programs through a stoichiometry-sensitive shift in function of a

Figure 6. Elevated Elf5 and Tfap2c levels trigger TSC differentiation. (A) Differential ChIP-seq binding enrichment of Tfap2c in TSCs and upon 1 d of differentiation. The 1254 triply bound elements predominantly have a higher Tfap2c abundance in TSCs, indicated by the blue shading of elements. By comparison, a significantly larger proportion of the 4254 Elf5–Tfap2c double and 28,473 (intergenic) Tfap2c single peaks exhibit a higher Tfap2c abundance upon differentiation, indicated by the red-shaded elements (highlighted by the dashed lines). Please note that, since this analysis is based on the differential enrichment of Tfap2c between TSCs and 1-d-differentiated cells, only peak combinations with Tfap2c can be analyzed. (B) Example browser views of the differential Tfap2c peak enrichment in TSCs grown in stem cell conditions and upon 1 d of differentiation. Tfap2c binding is higher in TSC conditions at triply bound sites at genes more highly expressed in TSCs such as Spred1; conversely, Tfap2c binding is enriched at Tfap2c singly and Elf5–Tfap2c doubly occupied sites in differentiation conditions surrounding genes up-regulated upon differentiation, such as Id3. (C) Correlation of differential Tfap2c ChIP-seq peak binding intensity at Elf5–Tfap2c elements with expression levels of the associated genes. Higher enrichment of Tfap2c correlates with elevated gene expression both for TSC genes in stem cell conditions (left of vertical dotted line) and at genes up-regulated with differentiation in differentiation-promoting conditions (right of vertical dotted line). (D) ChIP of Elf5 followed by qPCR for Elf5–Tfap2c target loci in TSCs that harbor a doxycycline (dox)-inducible Elf5 expression construct. Elf5 recruitment to these sites is increased upon Elf5 overexpression (Elf5 TSC + dox). ( ) $P < 0.05$. (E) Differential distribution of Tfap2c motifs: Globally, Tfap2c ChIP-seq peaks overlap extremely closely with the Tfap2c consensus binding sequence, with 76% of peaks positioned over the cognate motif. In contrast, triply bound sites are significantly depleted for the cognate motif, with only 32% of peaks overlapping the consensus sequence. Significance was calculated with a ^{2} test with Yates' correction; the odds ratio is 6.7. The consensus motif for Elf5 is less tightly defined, with, globally, 41% of peaks overlapping the cognate sequence; this is increased to 52% at triply bound elements. (F) Model of the stoichiometry-depen-

small group of interacting TFs centered on Elf5 acting as a cell fate switch (Fig. 6F).

Discussion

Insights into the transcriptional networks required to maintain the self-renewal state of TSCs have largely relied on the analysis of mouse mutants and the inability to derive TSCs from them. These studies have revealed a number of TFs that are essential to establish and maintain TSCs, including Eomes, Elf5, and Tfap2c (Russ et al. 2000; Donnison et al. 2005; Kuckenberg et al. 2010). Here, we show that these same three TFs physically bind to each other to form multimeric TF complexes and co-occupy trophoblast genes, including themselves, driving their expression. In this sense, the self-renewal network of TSCs shares important mechanistic features with pluripotency circuits in ESCs not only in terms of mutual self-reinforcement but also as far as protein– protein interactions and genomic cobinding are concerned.

In addition to the identification of these core trophoblast TF interactomes and the networks that they establish, our findings add another important dimension to the understanding of TSC regulation: Not only are certain TFs essential for TSC self-renewal, but their proportional abundance is equally critical to maintain the stem cell state. We show that precise levels of Elf5 determine the balance between TSC self-renewal and onset of differentiation. Loss but also overexpression of Elf5 result in depletion of the proliferative stem cell compartment in vitro and in vivo and embryonic lethality around mid-gestation (Donnison et al. 2005). These data bear a strong resemblance to Oct4 as a key node of the pluripotency network in ESCs, whose fine-tuned expression levels control both the entry into and the exit from naïve pluripotency (Radzisheuskaya et al. 2013). Complete depletion of Oct4 causes differentiation toward trophoblast-like cells, but, conversely, high Oct4 levels are also required for differentiation into embryonic lineages and the germline, albeit the mechanism underlying this sensitivity to expression levels remains unknown (Niwa et al. 2000; Radzisheuskaya et al. 2013). Our insights reveal an analogous finely tuned balance for Elf5 in TSCs and place Elf5 at the center of the transcriptional networks governing self-renewal as well as differentiation in TSCs.

To gain a better molecular understanding of Elf5's function in this circuit, we analyzed its interacting partners in TSCs using mass spectrometry. In addition to Eomes and Tfap2c, we identified other Elf5 interactors with known roles in trophoblast and placental development, including Grhl2 (Walentin et al. 2015) and Bptf (Goller et al. 2008). Our data showed that Elf5 associates with components of both activating (e.g., Bptf and Chd7) and repressive (Sin3 and Polycomb) chromatin complexes, indicating its role in both genebothl2 7nd repre228 To3t compleWnv (matin(as)b296((tha1t)-2ee364(ste(5218(of)280(d54035t-299(tn)1))-bl2nJ /T1

(pCAG-EGFP or p|CAG-Elf5; 1000 ng of p24 per mL) for 5 h. Transduced blastocysts were implanted into the uteri of E2.5 pseudopregnant females. Litters were dissected at the indicated days of development.

Stem cell culture

TSC lines EGFP, Rs26, and " CTRL WT " (a kind gift of the Rossant [Toronto, Canada] and Okano [Kobe, Japan] laboratories) were cultured as described previously (Tanaka et al. 1998). Further manipulations were performed with piggyBac vectors containing the complete ORFs of TFs as indicated. TFs were cloned and sequence-verified. All transformations to yield stable cell lines were performed in the TS-EGFP cell line, which exhibits proven placental contribution competence (Tanaka et al. 1998; Cambuli et al. 2014).

Histological analyses

Standard immunohistochemistry was performed on 7-μm sections as detailed in the Supplemental Material. Images were taken on an Olympus BX41 or BX61 epifluorescence microscope or a Zeiss LSM 780 confocal microscope.

For RNA in situ hybridizations, linearized plasmids containing cDNA inserts from Plf and Tpbpa cDNAs were used to generate digoxigenin (DIG)-labeled riboprobes according to the manufacturer's instructions (Roche). Hybridizations were carried out overnight at 52°C using standard procedures. Signals were detected with anti-DIG-alkaline phosphatase-conjugated antibody (Roche), and staining was performed overnight using NBT and BCIP reagents (Promega). Sections were counterstained with nuclear Fast Red (Sigma).

Coimmunoprecipitation

TS-EGFP cells were transfected with a piggyBac-CAG-Avi-Elf5-3xFlag-Ires-Neo, piggyBac-CAG-Eomes-3xFlag-Ires-Bsd, or pig-

quantitation pipeline in SeqMonk software (http://www.bioinformatics.babraham.ac.uk).

Bioinformatic analysis

Raw reads were aligned to mouse genome build mm9 using Bowtie, and peak calling was performed with MACS2 with default parameters (Zhang et al. 2008). To identify overlapping elements from different ChIP experiments, BED files of ChIP elements were compared using the BedTools (Quinlan and Hall 2010) function multiinter with the cluster option to reduce redundant interactions to the highest-order interactions. Overlapping regions required a 50% overlap to be included. To model the all by all possible interactions, we used the shuffle function in the R library ChIPseeker (Yu et al. 2015) to make randomized BED files of each ChIP experiment. We then used the multiinter function of BedTools to generate a table of expected interactions of the randomized data. This was repeated 10,000 times to build average

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