Genetic Redundancy of GATA Factors in Extraembryonic Trophoblast Lineage Ensures Progression of both Pre and Postimplantation Mammalian Development

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Abstract

GATA transcription factors are implicated in establishing cell fate during mammalian development. In early mammalian embryos, GATA3 is selectively expressed in the extraembryonic trophoblast lineage and regulates gene expression to promote trophoblast fate. However, trophoblast-specific GATA3 function is dispensable for early mammalian development. Here, using dual conditional knockout mice, we show that genetic redundancy of GATA3 with paralog GATA2 in trophoblast progenitors ensures the successful progression of both pre and postimplantation mammalian development. Stage-specific gene deletion in trophoblasts reveals that loss of both GATA genes, but not either one alone, leads to embryonic lethality prior to the onset of their expression within the embryo proper. Using ChIP-seq and RNA-seq analyses, we define the global targets of GATA2/GATA3 and show that they directly regulate a large number of common genes to orchestrate stem vs. differentiated trophoblast fate. Also, in trophoblast progenitors GATA factors directly regulate BMP4, Nodal and Wnt signaling components that promote embryonic-extraembryonic signaling cross-talk, essential for the development of the embryo proper. Our study provides genetic evidence that impairment of trophoblast-specific GATA2/GATA3 function could lead to early pregnancy failure.

Introduction

Extra-embryonic trophoblast cell lineage is unique to mammals and is essential for successful progression of mammalian reproduction. Trophoblast cells only exist during embryonic development and originate during the first cell fate decision in preimplantation embryos (Cockburn and Rossant, 2010; Pfeffer and Pearton, 2012; Roberts and Fisher, 2011; Rossant and Cross, 2001). Subsequently, trophoblast cells mediate implantation of the developing embryo to the maternal uterus and establish a maternal-fetal interface for vascular connection with the mother for nutrient and gas transport to the embryo (Rossant and Cross, 2001). Failure in the determination of the trophoblast lineage during preimplantation development leads to defective embryo implantation (Cockburn and Rossant, 2010; Pfeffer and Pearton, 2012; Roberts and Fisher, 2011; Rossant and Cross, 2001), which is a leading cause of infertility. After implantation, defective development and function of trophoblast progenitors lead to either early pregnancy failure or pregnancy-associated complications like intrauterine growth retardation (IUGR), preeclampsia (Myatt, 2006; Pfeffer and Pearton, 2012; Redman and Sargent, 2005; Rossant and Cross, 2001), or causes postnatal or adult diseases (Gluckman et al., 2008).

Development of the trophoblast cell lineage is a multi-step process (Fig. S1) and begins with the establishment of the trophectoderm (TE) in blastocysts. The TE mediates blastocyst implantation and is the source of trophoblast stem and progenitor cells (TSPCs). In an early postimplantation mouse embryo, TSPCs proliferate and differentiate to develop the extra-embryonic ectoderm (ExE). Later, ~embryonic day (E)7.0-E8.0, the ectoplacental

cone (EPC) and chorion are developed. Subsequently, lineage-specific trophoblast progenitors arise from TSPCs, which differentiate to specialized trophoblast subtypes leading to successful placentation. Thus, trophoblast lineage development relies upon proper spatial and temporal regulation of gene expression during (i) TE-development in preimplantation embryos; (ii) maintenance of self-renewal within TSPCs of an early postimplantation embryo; and (iii) subsequent differentiation of trophoblast progenitors to specialized trophoblast subtypes of a matured placenta.

Studies with gene knockout mice and mouse trophoblast stem cells (TSCs) implicated several transcription factors including GATA3, in regulation of the trophoblast lineage development (Barak et al., 1999; Hemberger et al., 2010; Home et al., 2009; Keramari et al., 2010; Nishioka et al., 2008; Ralston and Rossant, 2008; Russ et al., 2000; Strumpf, 2005; Yagi et al., 2007). Earlier, other laboratories and we reported that GATA3 is selectively expressed in extra-embryonic TE and TSPCs during early mouse development and is involved in TE-specific gene regulation (Home et al., 2009; Ralston et al., 2010). Also, ectopic expression of *Gata3* in mouse embryonic stem cells (ESCs) or mouse fibroblasts could instigate trophoblast fate (Benchetrit et al., 2015; Kubaczka et al., 2015; Ralston et al., 2010). However, gene knockout studies in mice revealed that *Gata3*-null mouse embryos die at ~E11.5 due to defective neuroendocrine system development (Lim et al., 2000; Pandolfi et al., 1995) indicating that trophoblast-specific GATA3 function is not essential for mammalian development.

Like GATA3, GATA2 is also implicated in the regulation of a few trophoblast genes in the mouse placenta (Bai et al., 2011; Ma et al., 1997; Ray et al., 2009). Also, both GATA2 and GATA3 are selectively expressed in the TE of a preimplantation human embryo (Assou et al., 2012; Blakeley et al., 2015). However, *Gata2*-null mouse embryos die at ~E10.5 due to defective hematopoiesis (Tsai and Orkin, 1997), indicating that, like GATA3, trophoblast-specific GATA2 function is not essential for early mammalian development. Thus, although both GATA2 and GATA3 are implicated in gene regulation at different stages of trophoblast lineage development, individual functions of GATA2 or GATA3 is dispensable for this process.

As GATA factors often show functional redundancy in other tissue developments (Fujiwara et al., 2004; Peterkin et al., 2007), we hypothesized that GATA2 and GATA3 have functional redundancy in the developing trophoblast lineage and one GATA factor compensates for the loss of the other. To test this hypothesis, we established inducible gene knockout mice, in which *Gata2* and *Gata3* could be conditionally deleted individually or in combination. We discovered that combinatorial functions of GATA2 and GATA3 are important to establish trophoblast lineage development in both pre and postimplantation embryos. We found that both GATA2 and GATA3 target transcriptionally active and silent genes to orchestrate developmental stage-specific gene expression program in TSPCs, which in turn ensure both pre and early postimplantation mammalian development. Due to the lack of an early trophoblast phenotype in these gene knockout studies, it is still unknown whether trophoblast-specific functions of GATA2 and GATA3 are essential to assure the early development of mammalian embryos.

Results

GATA2 and GATA3 expression are confined within extraembryonic trophoblast cells during early mouse development

During preimplantation mouse development, *Gata3* mRNA expression is induced at the 4-cell stage, and GATA3 protein expression is detectable during the 8- to 16-cell transition (Home et al., 2009; Ralston et al., 2010). However, in a matured blastocyst, GATA3 mRNA and protein expression becomes restricted only to the TE lineage (Home et al., 2009; Ralston et al., 2010). Recently, other studies showed that both *Gata2* and *Gata3* mRNAs are selectively expressed within the TE lineage of a human preimplantation embryo (Assou et al., 2012; Blakeley et al., 2015). However, GATA2 protein expression is not well documented during preimplantation development. So, we tested GATA2 protein expression at different stages of mouse preimplantation development. We found low levels of GATA2 protein expression in blastomeres of 2-16 cell embryos. However, GATA2 expression is upregulated in outer TE-lineage cells and is repressed in the inner cell mass during blastocyst maturation (Fig. 1A). In matured blastocysts, both GATA2 and GATA3 are only expressed within the TE lineage (Fig. 1B).

We also tested GATA2 and GATA3 protein expression in early postimplantation embryos. We found that up to Theiler stage 10c (~E7.25), expression of GATA2 and GATA3 are mostly confined in the extraembryonic trophoblast cells, including TSPCs within the EPC (Fig. 1C). Around E7.25-E7.5, a few cells of the extraembryonic yolk sac mesoderm also begin to express GATA2 protein (Fig. 1C). However, GATA2 and GATA3 proteins are not expressed in the embryonic cells prior to E7.5. Subsequently,

GATA2 and GATA3 expression are induced in the embryo proper and also maintained in trophoblast cells (Fig. 1D). Thus, our study confirmed a trophoblast-restricted expression pattern of GATA2 and GATA3 during blastocyst maturation and early postimplantation development in the mouse. We also tested expression of GATA2 and GATA3 within trophoblast progenitors of developing first-trimester human placenta and found that simultaneous expression of GATA2 and GATA3 in cytotrophoblast progenitors is a conserved event during early human development (Fig. 1E).

GATA factors are essential to establish functional TE lineage during preimplantation mouse development

To test the functional importance of GATA2 and GATA3 during early mouse development, we studied conditional knockout mice, in which *Gata2* and *Gata3* could be efficiently deleted individually (*Gata2*-KO or *Gata3*-KO) or in combination (*Gata*-DKO), by inducing the activity of a Cre-ERT2 recombinant protein with tamoxifen (Fig. S2). Given the fact that expression of both GATA factors are restricted within the developing trophoblast lineage of an early mouse embryo, this inducible gene knockout system allowed us to study the importance of trophoblast-specific GATA2/GATA3 functions at distinct stages of early mouse development.

Earlier, using RNAi strategy, we showed that GATA3 depletion in preimplantation mouse embryos partially impairs blastocyst maturation (Home et al., 2009). However, preimplantation mouse development in the absence of both GATA2 and GATA3 was never tested. Therefore, we began our study by testing the importance of individual as well as

combinatorial GATA2/3 function during preimplantation mouse development. We isolated fertilized embryos at E0.5, induced *Gata* deletion with tamoxifen and monitored preimplantation development *ex-vivo* (Fig. 2A-D). We found that GATA2 is dispensable for blastocyst maturation (Fig. 2B, C) and, similar to RNAi experiment, conditional deletion of *Gata3* partially affected blastocyst maturation (Fig. S3A). Interestingly, combinatorial loss of both GATA factors also resulted in a mixed preimplantation phenotype. A large number of *Gata*-DKO embryos failed to form blastocysts. However, several of the *Gata2^f/f;Gata3^f/f;UBC-cre/ERT2* embryos matured to the blastocyst stage (Fig. 2B, C) despite the fact that Cre-mediated gene excision resulted in the loss of both GATA proteins in those embryos (Fig. 2D).

Next, we tested whether *Gata*-DKO blastocysts have altered expression of TE-specific genes. Our analysis confirmed that mRNA expression of several TE-specific genes, including *Cdx2* (GATA target), *Eomes* (GATA target)*, and *Elf5* (GATA target) were strongly downregulated (Fig. 2E) in *Gata*-DKO preimplantation embryos. In contrast, mRNA expression of *Prl3b1* and *Ascl2*, which are predominantly expressed in differentiated trophoblast cells, were highly induced in *Gata*-DKO preimplantation embryos (Fig. 2E). Interestingly, except *Cdx2* mRNA expression in *Gata3*-KO embryos, expression of all these genes were not significantly altered in either *Gata2*-KO or *Gata3*-KO embryos (Fig. 2E). *Cdx2* expression was repressed by ~40% in *Gata3*-KO embryos. However, *Cdx2* expression was reduced by >80% in *Gata*-DKO embryos. These results indicated that although a few *Gata*-DKO preimplantation embryos could mature to the blastocyst stage, the TE-specific gene expression is altered in those embryos.

Therefore, we next tested in-utero implantation efficiency of *Gata*-DKO blastocysts.

As continuous tamoxifen exposure could negatively affect implantation efficiency of a blastocyst (Dao et al., 1996), we used two different experimental strategies to test implantation efficiency of *Gata*-DKO blastocysts. First, we ectopically expressed CRE-recombinase in *Gata2*^*f/f*^*;Gata3*^*f/f*^ preimplantation embryos via lentiviral transduction (Fig. S3B). We found that ectopic CRE-mediated excision of *Gata* genes also resulted in a mixed phenotype and several *Gata*-DKO embryos matured to the blastocyst stage (Fig. S3B). However, those *Gata*-DKO blastocysts failed to implant when they were transferred to the uterine horns of pseudopregnant surrogate female mice (Fig. 2F).

In the second approach, we transiently cultured both wild type and *Gata2*^*f/f*^*;Gata3*^*f/f*^*;UBC-cre/ERT2* preimplantation embryos with tamoxifen (Fig. S4). The transient tamoxifen exposure ensured *Gata*-genes deletion and defective blastocyst maturation in the majority of the *Gata2*^*f/f*^*;Gata3*^*f/f*^*;UBC-cre/ERT2* embryos (Data not shown). We transferred transiently tamoxifen-exposed *Gata2*^*f/f*^*;Gata3*^*f/f*^*;UBC-cre/ERT2* and wild type embryos, which matured to the blastocyst stage, to the uterine horns of pseudo-pregnant mice. We found that wild-type blastocysts with tamoxifen exposure readily implanted (Fig. S4), indicating that transient exposure to tamoxifen does not affect blastocyst implantation efficiency. However, blastocysts that were developed from *Gata2*^*f/f*^*;Gata3*^*f/f*^*;UBC-cre/ERT2* (GATA-DKO blastocysts) after transient exposure to tamoxifen failed to implant (Fig. S4). Collectively, these results indicated that, although GATA2 and GATA3 functions are not essential for blastocoel cavitation, they are

required to maintain proper gene expression balance and implantation efficiency within the developing TE-lineage.

GATA2/GATA3 functions in trophoblast lineage are essential for postimplantation mammalian development

As GATA2 and GATA3 are selectively expressed in TSPCs of an early postimplantation mouse embryo (Fig. 1), we also tested the importance of TSPC-specific GATA2/GATA3 function during early postimplantation development. For this study, we started tamoxifen treatment at ~E5.5, as the presence of tamoxifen on or before E4.5 affects the implantation process (Bloxham and Pugh, 1977; Dao et al., 1996; Pugh and Sumano, 1982). Also, we crossed $Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2$ males with $Gata2^{f/f};Gata3^{f/f}$ females to confine $Gata$ genes deletion only within developing embryos.

Individual deletion of $Gata2$ and $Gata3$ induces mouse embryonic lethality after E10.5 (Pandolfi et al., 1995; Tsai et al., 1994). Therefore, after inducing $Gata2/Gata3$ deletion at E5.5, we monitored embryonic development on or before E9.5 (Fig. 3A). As expected, individual loss of GATA2 and GATA3 did not induce embryonic lethality by E9.5 (Table 1). However, combinatorial deletion of GATA2 and GATA3 at E5.5 prevented developments of most of the embryos, resulting in embryonic death/ loss at implantation sites before E7.5 (Fig. 3B). Although a few embryos developed, they died at ~E7.5-E8.0 and none of them developed beyond Theiler stage 12a (~E8) (Fig. 3C). Furthermore, analysis of surviving $Gata$-DKO conceptuses revealed impaired placentation (Fig. 3C, D, E). ExE/EPC regions were not properly developed in $Gata$-DKO conceptuses and were

characterized by near complete loss of CDX2-expressing TSPCs (Fig. 3D). Similarly, when analyzed at E9.5, the *Gata*-DKO conceptuses revealed defective embryonic-extraembryonic attachment and were characterized by near-complete loss of trophoblast progenitors (Fig. 3E) at the maternal-fetal interface.

Next, we asked whether combinatorial functions of GATA2 and GATA3 are essential for the development of differentiated trophoblast subtypes. In a developing mouse embryo, progenitors for differentiated trophoblast subtypes arise within the EPC and the chorionic ectoderm at ~E8.0-E8.5 (Fig. S1). Therefore, to test the importance of GATA factors during trophoblast progenitor differentiation, we induced *Gata2/Gata3* deletion at E7.5. At E7.5, GATA3 is not expressed in embryonic cells, and we were unable to determine GATA2 protein expression in the embryo proper before E7.5. Individual knockout of *Gata2* or *Gata3* induces mouse embryonic death on or after E10.5. So, we analyzed embryonic development on or before E10.5 (Fig. 4A). We found that deletion of both GATA factors at E7.5 induced embryonic death at an earlier stage (~E9.5) compared to individual knockouts (Fig. 4B). Placenta development was not overtly affected in either *Gata2*-KO or *Gata3*-KO embryos (Fig. 4B). In contrast, placentae in *Gata*-DKO embryos were significantly smaller with severely reduced labyrinth zones and significantly smaller junctional zones (Fig. 4C, D). Furthermore, junctional zones of *Gata*-DKO placentae were characterized by significant reduction of spongiotrophoblast (SpT) cells (Fig. 4E) without any significant loss in TGC population. Interestingly, complete loss of blood development (a more severe phenotype than *Gata2*-KO embryo) was also observed in *Gata*-DKO embryos and placentae (Fig.

4B). This complete loss of hematopoiesis is being studied in detail and will be reported elsewhere.

Collectively, conditional gene deletions at distinct developmental stages revealed that GATA2 and GATA3 functions in extraembryonic trophoblast lineage are essential for both pre and postimplantation embryonic development.

GATA factors fine-tune gene expression to maintain trophoblast stem state

Trophoblast genes that are directly regulated by GATA2 and/or GATA3 are incompletely understood. An earlier study (Kidder and Palmer, 2010) used chromatin immunoprecipitation with DNA microarray hybridization analysis to investigate GATA3 binding at 28,000 promoter regions in mouse TSCs. However, global targets of GATA2, as well as GATA3 targets beyond the gene promoters, are not defined in trophoblast stem cells. Also, how GATA2 and GATA3 orchestrate different stages of trophoblast development is not well characterized. We hypothesized that being pioneer transcription factors (Chen and Dent, 2014; Zaret and Carroll, 2011), GATA2 and GATA3 could target both open and silent chromatins in trophoblast cells to instigate developmental stage-specific gene expression programs, thereby establishing stem/progenitors vs. differentiated cell fate. To test this hypothesis, we established TSCs, in which *Gata2* and *Gata3* could be conditionally deleted individually (*Gata2*-KO and *Gata3*-KO TSCs) or in combination (*Gata*-DKO TSCs) (Fig. 5A and Fig. S5A and B) and asked whether GATA factor-dependent transcriptional program is important to balance TSC self-renewal vs. differentiation. Our *in vitro* cell culture studies in TSC undifferentiated culture condition with Fibroblast Growth

Factor 4 (FGF4) and heparin showed that loss of GATA2 and GATA3 induced TSC differentiation leading to loss of stem state colony morphology (Fig. 5A). Furthermore, the *Gata*-DKO TSCs failed to form chimera with the developing TE lineage when they were injected into developing preimplantation mouse embryos (Fig. 5B, C). In contrast, TSCs with individual deletion of either *Gata2* (*Gata2*-KO TSC) or *Gata3* genes (*Gata3*-KO TSC) maintained their self-renewal ability, although they showed a relatively higher propensity for spontaneous differentiation compared to wild type control TSCs in standard TSC culture condition (Fig. S5A). These studies indicated that individual loss of either GATA2 or GATA3 is dispensable to maintain TSC stem-state. However, TSCs with the combinatorial loss of both GATA factors are unable to maintain trophoblast stem-state.

To validate that GATA factors directly regulate key trophoblast genes we performed ChIP-seq analysis in wild type control TSCs. We identified 12949 GATA2 binding and 5638 GATA3 binding regions at the mouse TSC genome (Tables S1A and S1B). We followed that with RNA-seq analysis in control vs. *Gata*-DKO TSCs and showed that loss of both GATA factors altered expression of 9775 genes by ≥1.5 fold (Table S2). A comparative analysis of ChIP-seq and RNA-seq data revealed that, ~68% among those 9775 genes are direct targets of either GATA2 (6667 target genes) or GATA3 (4746 target genes) and ~43% genes (4243 genes) have both GATA2 and GATA3 occupancy at their chromatin domains (Table S3, Fig. 5D). Thus, our global genomics analysis revealed that ~90% of GATA3 target genes (4243 genes out of 4746 genes) are also targets of GATA2 in TSCs, strongly supporting functional redundancy of these two GATA factors in gene regulation during early trophoblast development.

Our analyses showed altered expression of a large number of genes that are targeted by both GATA2 and GATA3 in TSCs (Fig. 5E) and revealed multi-modal bio-functions of dual GATA-regulated genes (Fig. 5F). Several of those GATA target genes are implicated in trophoblast and placenta development. For example, mRNA expression of *Elf5, Esrrb, and Bmp4,* which are direct targets of both GATA2 and GATA3 (Fig. 5G) and are implicated in TSC self-renewal, were strongly repressed in *Gata*-DKO TSCs (Fig. 5E). Our quantitative RT-PCR analyses also validated the RNA-seq data (Fig. S5C). In contrast, mRNA expression of *Prl3d1* (GATA target), *Prl2a1* (GATA target) that are only expressed in terminally differentiated TGCs and *Ascl2* (GATA target), which is induced in SpTs, were upregulated in *Gata*-DKO TSCs (Fig. 5G). Furthermore, ChIP-seq analyses confirmed that all these genes are direct targets of either GATA2 or GATA3 in TSCs (Fig. 5G). Thus, our ChIP-seq and RNA-seq analyses indicated that GATA2 and GATA3 mediate two important functions in undifferentiated TSCs; (i) maintain transcription of key genes that promote trophoblast stem state and (ii) suppress transcription of genes that promote TSC differentiation.

To further confirm GATA-mediated regulation of stem-state genes, we studied gene expression in primary TSPCs. We established *ex-vivo* explant cultures with ExEs/EPCs from early postimplantation mouse embryos (Fig. 6A). These explant cultures contain nearly pure (≥97%) primary trophoblast cells (Fig. 6B) and could be maintained in stem/progenitor states in the presence of FGF4 and heparin containing

TSC culture condition (Fig. 6A). Also, in the absence of FGF4/Heparin, TSPCs in the explant culture undergo differentiation (Fig. 6A).

We performed gene expression analysis with these primary TSPCs after maintaining them in TSC culture condition. We found that loss of both GATA factors often impairs expansion of primary TSPCs (Fig. 6C) and represses mRNA expression of several TSC/TSPC-specific genes, including *Esrrb, Elf5,* and *Cdx2* (Fig. 6D). Also, expression of *FoxD3* (GATA target), which is important for TSPC self-renewal (Tompers et al., 2005), was strongly down-regulated in *Gata*-DKO TSPCs. In contrast, expression of these genes either not altered or only marginally altered in the *Gata2*-KO or *Gata3*-KO TSPCs (Fig. 6D). Interestingly, we noticed that mRNA expression of *Ets2* (GATA target) *and Tfap2c,* which are also implicated in the maintenance of TSPCs in an early postimplantation embryo (Choi et al., 2012; Georgiades and Rossant, 2006; Kuckenberg et al., 2012), were not significantly altered in *Gata*-DKO TSPCs (Fig. 6D). Collectively, our studies in *Gata*-DKO TSCs and primary TSPCs strongly indicated that functional redundancy of GATA2 and GATA3 ensures gene expression balance to promote self-renewal and expansion of TSPCs during early postimplantation mammalian development.

In TSPCs GATA factors regulate key signaling components that mediate embryonic-extraembryonic signaling cross-talk

How does the loss of GATA2 and GATA3 in extraembryonic trophoblast lineage impair embryonic development at an early postimplantation stage? Postimplantation

embryonic development depends on BMP4 and Nodal signaling cross-talk between TSPCs and cells of the embryo proper (Beppu et al., 2000; Brennan et al., 2001; Kimura et al., 2000; Mishina et al., 1995; Rodriguez et al., 2005; Soares et al., 2005; Soares et al., 2008; Tam and Loebel, 2007; Winnier et al., 1995). TSPCs produce BMP4, which is required for primitive streak development (Murohashi et al., 2010; Streit et al., 1998). TSPCs also express convertase enzymes, PCSK3, and PCSK6, which process Nodal precursors to ensure proper embryo patterning (Guzman-Ayala et al., 2004). In addition to Nodal and BMP4, TSPCs could produce other factors that regulate the Wnt signaling pathway to control postimplantation development. For example, secretory protein Dickkopf-1 (*Dkk1*), which negatively regulates the Wnt/βcatenin pathway, is required for gastrulation (Peng et al., 2008). Also, TSPCs express porcupine homolog (*Porcn*), which is necessary for palmitoylation and secretion of functional Wnt molecules (Biechele et al., 2013). RNA-seq analysis confirmed that along with *Bmp4* (GATA target), expression of *Pcsk6* (GATA target) and *Dkk1* (GATA target) are repressed in *Gata*-DKO TSCs (Table S2), whereas, *Porcn* (GATA target) is very lowly expressed in both control and *Gata*-DKO TSCs. As the loss of GATA factors strongly down-regulated *Bmp4*, *Pcsk6* and *Dkk1* expression in TSCs, we tested whether loss of GATA factors also impairs their expression in primary TSPCs of a postimplantation embryo. Our gene expression analysis confirmed that loss of GATA factors strongly represses *Bmp4, Pcsk6* and *Dkk1* expression in TSPCs (Fig. 6E). In contrast, *Porcn* expression is induced in *Gata*-DKO TSPCs (Fig. 6E). These results strongly indicate that GATA2 and GATA3 regulate expression of BMP4, Nodal and Wnt signaling components in TSPCs, thereby facilitating embryonic-extraembryonic signaling cross-talk during early

postimplantation development.

GATA factors promote trophoblast differentiation by activating differentiation-specific genes.

In mice, attachment of chorion to the allantois gives rise to GCM1 (Basyuk et al., 1999) and DLX3 (Morasso et al., 1999) expressing labyrinth trophoblast progenitors (LTPs), which differentiate to syncytiotrophoblasts within the labyrinth zone (Fig. S1). On the other hand, in the EPC, ASCL2$^+$, PRDM1$^+$ and TPBPA$^+$ progenitors (Mould et al., 2012; Simmons et al., 2007; Tanaka et al., 1997) arise. These progenitors subsequently differentiate to specialized trophoblast subtypes of the junctional zone, which contains trophoblast giant cells (TGCs), Spongiotrophoblast cells (SpT) and Glycogen trophoblasts (GlyT) (Fig. S1). As *Gata2/Gata3* deletion in E7.5 embryos leads to defective development of both the labyrinth and the junctional zones, we asked whether GATA factors mediate differentiation stage-specific function by promoting transcription of key genes during trophoblast progenitor differentiation.

Global transcriptome profile in TSCs revealed that GATA factors occupy chromatin domains of key trophoblast genes that are transcriptionally repressed in TSCs but are critical to induce trophoblast lineage differentiation (Table S4). For example, we identified GATA factor occupancy at the chromatin domains of *Gcm1* (GATA target)*, Dlx3* (GATA target) and *Prdm1* (GATA target), which are implicated in the development of syncytiotrophoblasts and TGCs (Basyuk et al., 1999; Berghorn et al., 2005; Hughes et al., 2004; Morasso et al., 1999; Mould et al., 2012). These genes

are transcriptionally silent in TSCs (Table S4). However, their expression are induced in differentiated cultures of TSCs (Fig. 7A and Fig. S5D).

We asked whether *Gcm1*, *Dlx3*, *Prdm1,* and other trophoblast differentiation markers are induced in *Gata*-DKO TSCs in FGF4-free differentiating culture condition. In particular, we wanted to test whether genes like *Ascl2, Prl3d1,* and *Prl3b1*, which are expressed at higher level in *Gata*-DKO TSCs in standard TSC culture condition, changes in differentiating culture condition. Intriguingly, gene expression analyses revealed that differentiation potential is impaired in *Gata*-DKO TSCs (Fig. 7A and Fig. S5D). *Gcm1*, *Dlx3,* and *Prdm1* remained suppressed in *Gata*-DKO TSCs, when cultured in differentiating condition for multiple days (Fig. 7A and Fig. S5D). Also, mRNA expression of *Ascl2, Prl3d1,* and *Prl3b1* were not further induced. However, *Hand1* (GATA target), a gene that promotes TGC differentiation (Hemberger et al., 2004), were upregulated in *Gata*-DKO TSCs. Collectively, these results indicated that GATA2 and GATA3 promote trophoblast differentiation by directly regulating expression of key differentiation genes.

To further validate the importance of GATA-mediated gene regulation during differentiation of trophoblast progenitors to specialized trophoblast cells, we tested gene expression in *Gata*-DKO TSPC explants. For gene expression analysis in the differentiating TSPCs, we isolated ExE/EPC explants from E7.5 embryos. We cultured them in differentiating culture condition and induced *Gata* gene deletion with tamoxifen (Fig. 7B). Our gene expression analyses confirmed that the presence of either GATA2 or

GATA3 is sufficient for induction of key trophoblast differentiation genes, namely *Ascl2, Prdm1, Gcm1,* and *Dlx*3 (Fig. 7B). However, the loss of both GATA2 and GATA3 impaired induction of these genes during differentiation of TSPCs to specialized trophoblast cells (Fig. 7B). Furthermore, analysis of their chromatin domains in TSPCs revealed that loss of both GATA factors impaired RNA Polymerase-II recruitment and maintained repressive histone marks at those gene loci (Fig. 7C, D).

We also assessed gene expression in differentiated trophoblast cells of *Gata*-DKO placentae (Fig. 7E). Similar to the TSPC explant cultures, we noticed strong repression of *Gcm1*, *Prdm1*, *Dlx3*, and *Ascl2* in *Gata*-DKO placentae (Fig. 7E). We also noticed that loss of GATA factors strongly inhibits expression of other TGC-specific genes *Prl3b1*, *Prl3d1* and *Prl2c2* (Fig. 7E), an observation earlier reported with individual *Gata*-knockout placentae (Ma et al., 1997). Expression of syncytin-A (*Syna*) and syncytin-B (*Synb*), which are essential for labyrinth trophoblast syncytialization (Dupressoir et al., 2005; Dupressoir et al., 2009; Dupressoir et al., 2011), were also strongly repressed in *Gata*-DKO placentae. However, similar to *Gata*-DKO TSCs, expression of *Hand1* was not significantly altered in *Gata*-DKO placentae.

In summary, gene expression analysis in the TE, TSCs, and primary trophoblast populations provided developmental snapshots of gene regulatory mechanisms by GATA2 and GATA3 during trophoblast lineage development. The loss of function analysis in *Gata*-DKO TSCs, TSPCs and placentae showed that functional redundancy of GATA2 and GATA3 not only maintain trophoblast stem state genes in TSPCs, they are also

important to induce expression of key trophoblast genes that initiate trophoblast progenitor differentiation to specialized trophoblast cells during placentation.

Discussion

Recently, multiple studies implicated GATA2 and GATA3 in orchestrating gene expression patterns during trophoblast development (Bai et al., 2011; Home et al., 2009; Ma and Linzer, 2000; Ma et al., 1997; Ralston et al., 2010; Ray et al., 2009). However, due to lack of an overt phenotype in gene knockout studies, the importance of trophoblast cell-specific GATA function during early mammalian development was hard to understand. Here, by studying a dual gene knockout model, we show that trophoblast-specific GATA2 and GATA3 functions are essential at multiple stages of early embryonic development. Our analyses also revealed that both GATA2 and GATA3 directly regulate a large number of trophoblast genes. These findings strongly support a complementary role of GATA2 and GATA3 during trophoblast development and explain the lack of overt trophoblast phenotype in individual knockout studies.

Unlike *Gata2*-KO preimplantation embryos, *Gata3*-KO preimplantation embryos show a partial defect in blastocyst maturation, which is surprising as ChIP-seq analyses revealed that ~90% of the GATA3 target genes in TSCs are also GATA2 targets. However, ChIP-seq studies in TSCs provided snapshots of GATA factor binding at their chromatin targets in a large cell population. Thus, it is possible that during blastocyst maturation GATA3 and GATA2 have dynamic chromatin occupancy with more genes

being bound by GATA3. Alternatively, a few genes that are selectively regulated by GATA3 may have more importance for blastocyst maturation. Nevertheless, blastocyst formation in most of the *Gata2*-KO and majority of *Gata3*-KO preimplantation embryos supports functional redundancy of GATA2 and GATA3 during blastocyst maturation.

Developmental snapshots of gene expression in *Gata*-DKO TSCs and TSPCs showed that temporal fine-tuning of gene expression by GATA2/GATA3 regulates distinct stages of trophoblast development. For example, expression of *Prl3b1* and *Ascl2* are induced in *Gata*-DKO TE. In contrast, these genes are repressed in *Gata*-DKO differentiated trophoblast cells. These findings imply that GATA2 and GATA3 orchestrate trophoblast lineage development by ensuring developmental stage-specific gene expression patterns. How GATA factors fine-tune temporal gene expression in trophoblast cells is a subject of further study. One hypothesis is, in response to different cellular signaling, GATA2/GATA3 form distinct protein-protein complexes at different chromatin domains, leading to the alternative transcriptional outcome. Also, pioneer transcription factors are known for Pol II recruitment at both poised and transcribed genes (Hsu et al., 2015). Thus, it will be interesting to identify how GATA-dependent mechanisms regulate trophoblast chromatin at different stages of development and whether those mechanisms are conserved in multiple mammalian species, including human.

Interestingly, unlike the labyrinth trophoblast and SpTs, development of TGCs, including parietal TGCs that separate the developing placenta from the maternal

decidua, was not overtly affected in *Gata*-DKO placentae. Studies with mouse TSCs indicated that TGC development might be a default pathway as withdrawal of FGF4 and other TSC self-renewal factors promote spontaneous differentiation of TSCs to TGCc (Tanaka et al., 1998). Also, expression of HAND1, a factor implicated in TGC development (Hemberger et al., 2004), is not dependent upon GATA factors. Thus, TGC development during mouse placentation is not absolutely dependent on GATA factor function. However, GATA2 and GATA3 are expressed in TGCs and regulate expression of TGC-specific genes *Prl2c2*, *Prl3d1* and *Prl3b1* (Ma et al., 1997). Thus, GATA factors might be important to maintain proper TGC functions that include the production of placental hormones and other secretory molecules to ensure the progression of pregnancy. Also, GATA2/GATA3 function might be important to develop other TGC subtypes, including the invasive trophoblast population. Thus, future studies with TGC-specific GATA2/GATA3 deletion will provide more in-depth information regarding their importance in TGCs.

Another interesting finding is a complete lack of blood development in *Gata*-DKO placenta and embryo when gene deletion is induced at E7.5. The placenta is a major site of hematopoiesis and the placental hematopoiesis in mice begins after E9.0, when definitive multilineage progenitors appear (Alvarez-Silva et al., 2003). In contrast, mature hematopoietic stem cells in the embryo proper are found around E10.5 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Although previous studies (Minegishi et al., 1998; Shi et al., 2014) reported that *Gata2* mRNA and protein are expressed in the lateral mesoderm of a ~E7.5-E8.0 mouse embryo, we found that ~E7.5 both GATA2 and GATA3 proteins

are mainly expressed in trophoblast cells (Fig. 1C). At this stage, no hematopoietic cell exists in the embryo proper or in the placenta. Also, the *Gata*-DKO embryos under that experimental condition do not mature beyond E9.5, a developmental stage before the augmentation of definitive hematopoiesis in the embryo proper. Thus, it is possible that trophoblast cell-specific GATA function is required for proper hematopoiesis during embryonic development. Only future studies with GATA gene deletion in specific trophoblast cell types will address the importance of trophoblast cell-specific GATA factor function in hematopoietic development.

Materials and Methods

Derivation of mouse TSC lines

Gata2$^{f/f}$;Gata3$^{f/f}$;UBC-cre/ERT2$^{/+}$ TSCs were established from 3.5 days post coitum (E3.5) blastocysts according to the protocol (Tanaka et al., 1998) and culture in the condition described above. *Gata2* and *Gata3* floxed alleles were efficiently excised from *Gata2$^{f/f}$;Gata3$^{f/f}$;UBC-cre/ERT2* TSCs by culturing the cells in the presence of tamoxifen (1 μg/ml).

Gata2$^{f/f}$ and *Gata3$^{f/f}$* TSCs were established in a similar fashion. Gene deletions were induced in these cell lines by transient transfections with Puro.Cre empty vector (Addgene Plasmid #17408) (Kumar et al., 2008) according to a protocol described earlier (Home et al., 2009).

All cell lines were tested negative for contamination and were validated using experiments described.

Generation of conditional knockout mice strains

All procedures were performed after obtaining IACUC approvals at the Univ. of Kansas Medical Center. Female *Gata2flox/flox* (*Gata2$^{f/f}$*) mice (Charles et al., 2006) were mated with B6;129S-Tg(*UBC-cre/ERT2*)1Ejb/J male (JAX Lab, Stock 007001) (Ruzankina et al., 2007) in order to generate *Gata2$^{f/+}$;UBC-cre/ERT2*. In the next step, *Gata2$^{f/+}$;UBC-cre/ERT2*female mice were bred with *Gata2$^{f/+}$;UBC-cre/ERT2* males to generate *Gata2$^{f/f}$;UBC-cre/ERT2*. Similarly female *Gata3flox/flox* (*Gata3$^{f/f}$*) mice (Zhu et al., 2004) were used to generate *Gata3$^{f/f}$;UBC-cre/ERT2*. Again, *Gata2$^{f/f}$;UBC-cre/ERT2* and Gata3$^{f/f}$;*UBC-cre/ERT2* mice were crossed to generate Gata2$^{f/+}$;Gata3$^{f/+}$;*UBC-cre/ERT2*.

Later *Gata2^{f/+};Gata3^{f/+};UBC-cre/ERT2* males and females were crossed to generate *Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* strain.

Gata gene deletion in postimplantation embryos

Mating were set between *Gata2^{f/f};UBC-cre/ERT2* male with *Gata2^{f/f}* female, *Gata3^{f/f};UBC-cre/ERT2* male with *Gata3^{f/f}* female and *Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* male and *Gata2^{f/f};Gata3^{f/f}* female. Once copulation plugs were confirmed, intraperitoneal injections of 200 μl Tamoxifen solution (10mg/ml) in corn oil were administered at desired day points in each of the females.

Collection and culture of preimplantation embryos

All procedures were performed with IACUC approvals at Univ. of Kansas Medical Center. One-cell stage mouse embryos were harvested according to the protocol (Home et al., 2012; Saha et al., 2013) and were cultured in KSOM (Millipore) in presence or absence of 1µg/ml tamoxifen at 37°C in a humidified chamber, maintained at 5% CO_2 and 5% Oxygen level.

Additional materials and methods are described in the Supplementary Information.

Competing interests

The authors declare no competing interests.

Author contributions

PH and SP planned, performed experiments, analyzed data and wrote the manuscript. RP, AG, BS, JMF, BB, SR performed experiments. SG, AP analyzed data. SC, PF provided reagents.

Data availability

Datasets will be uploaded on a public database upon the acceptance of the manuscript.

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Figures
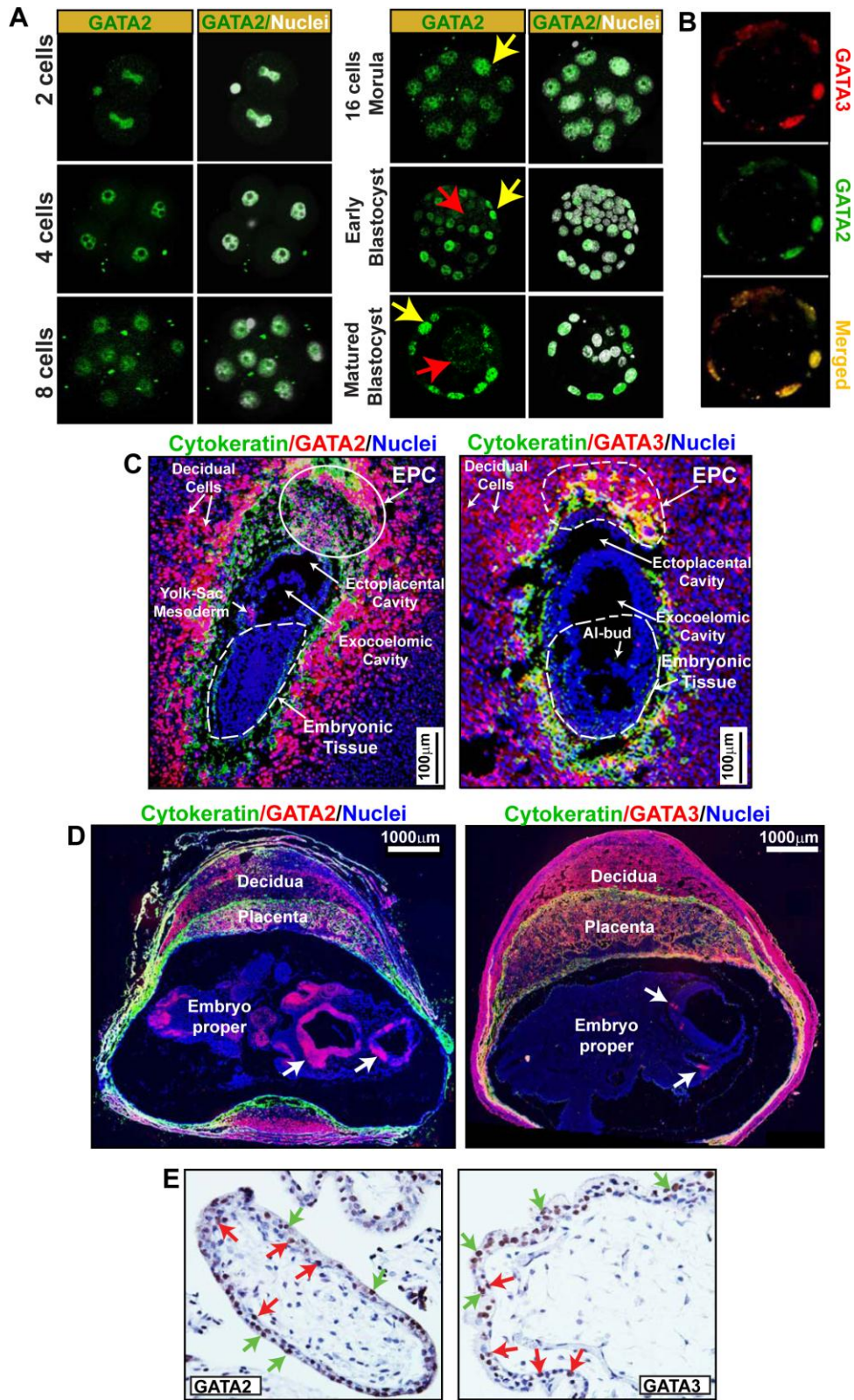
Fig. 1. GATA2 and GATA3 are selectively expressed in trophoblast cells of preimplantation and early postimplantation mammalian embryos.
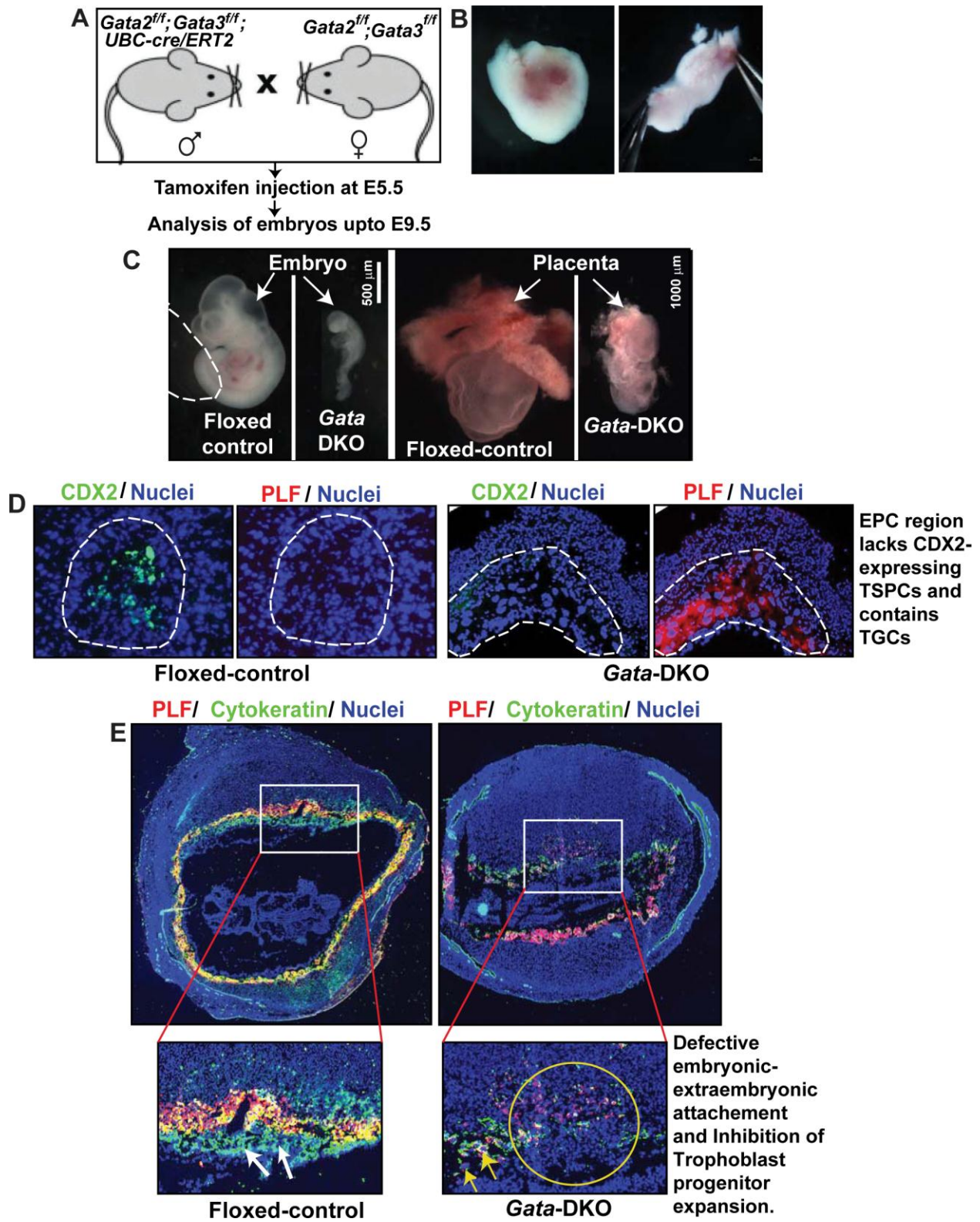
(A) Immunofluorescence images showing GATA2 expression at different stages of preimplantation mouse development. During blastulation, GATA2 is induced in outer TE lineage (yellow arrows) but is repressed in the inner cell mass lineage (red arrows). (B) A mouse blastocyst showing that both GATA2 and GATA3 are selectively expressed within the TE lineage. (C) ~E7.5 mouse implantation sites showing pan-Cytokeratin, GATA2, (left), GATA3 (right) and nuclei (DAPI). White areas show EPC and embryonic tissue; other structures are marked with white arrows. (D) E10.5 mouse implantation sites showing pan-Cytokeratin, GATA2 (left), GATA3 (right) and nuclei (DAPI). At this stage, GATA2 and GATA3 are expressed in both embryonic cells (white arrows) and extraembryonic tissues. (E) Immunohistochemistry showing both GATA2 (left) and GATA3 (right) are selectively expressed within trophoblast cells [both cytotrophoblast progenitors (red arrows) and syncytiotrophoblasts (green arrows)] of a first-trimester (8 weeks) human placenta.
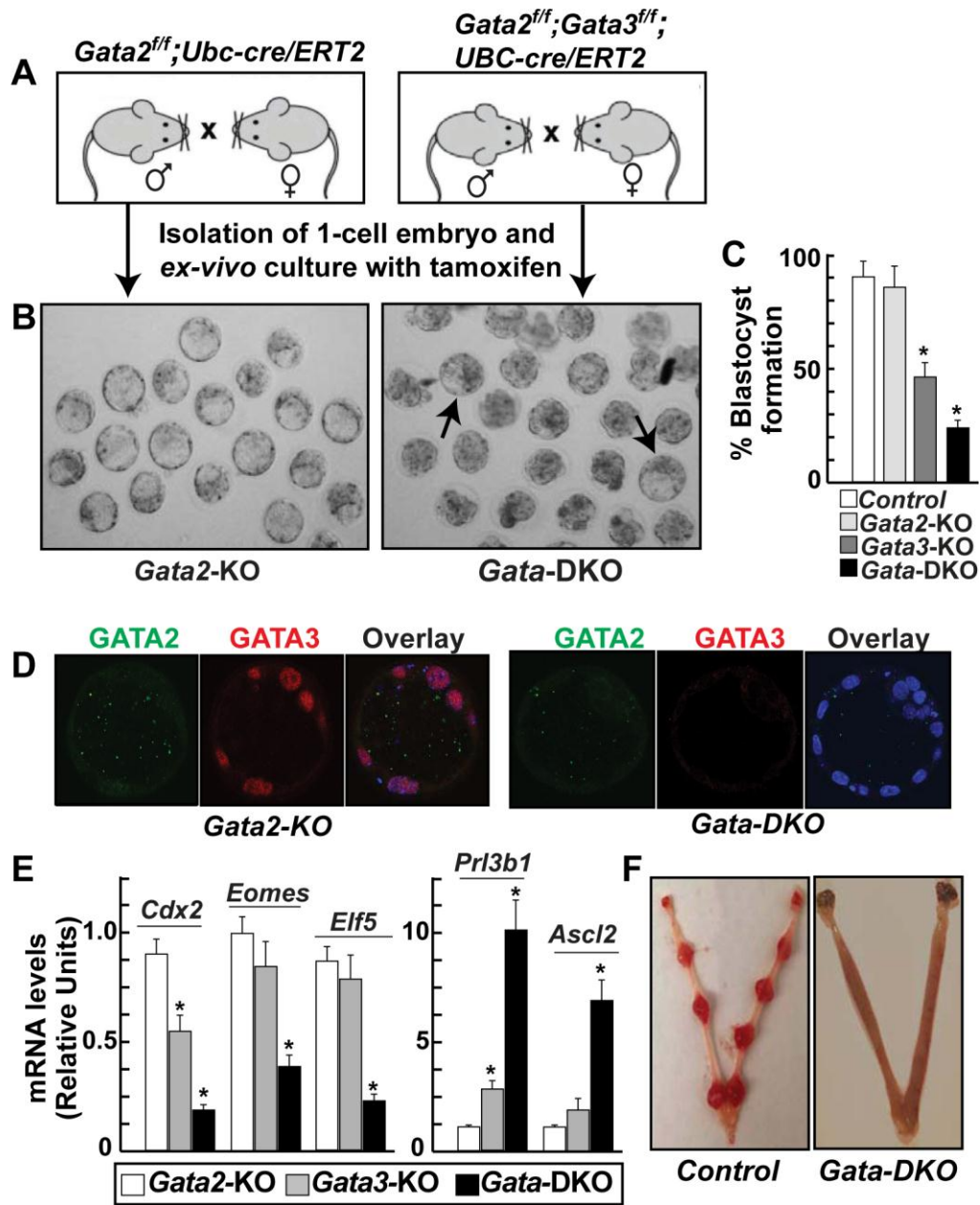
Fig. 2. Combinatorial loss of GATA2 and GATA3 impairs functional TE development.

(A) Experimental strategy to define the importance of GATA factors during mouse preimplantation development. (B) Micrographs show that the loss of GATA2 is dispensable for blastocyst maturation, whereas loss of both GATA2 and GATA3 result

in a partial defect in blastocyst formation. (C) The plot shows the percentage of preimplantation embryos that matured to the blastocyst stage upon loss of GATA factors (mean ± SE; n = 3, p≤0.01). (D) Immunofluorescence confirmed the loss of GATA2 expression in Gata2-KO blastocysts and loss of both GATA2 and GATA3 expression in Gata-DKO blastocysts. (E) Analysis of mRNA expression showing significant changes in TE-specific genes in *Gata*-DKO embryos compared to *Gata2*-KO or *Gata3*-KO embryos (Expression level of a gene in control embryos were considered 1, mean ± SE; n = 3, p≤0.001). (F) Images of uterine horns from E7.5 pseudopregnant female mice transferred with either control (left) or *Gata*-DKO (right) blastocysts. *Gata*-DKO blastocysts showed implantation failure.

A Gata2^f/f;Gata3^f/f; UBC-cre/ERT2 ♂ X Gata2^f/f;Gata3^f/f ♀

Tamoxifen injection at E5.5

Analysis of embryos upto E9.5

B

C
Embryo — Placenta

Floxed control | *Gata* DKO | Floxed-control | *Gata*-DKO

500 μm | 1000 μm

D
CDX2/Nuclei | PLF/Nuclei | CDX2/Nuclei | PLF/Nuclei

Floxed-control | *Gata*-DKO

EPC region lacks CDX2-expressing TSPCs and contains TGCs

E
PLF/Cytokeratin/Nuclei | PLF/Cytokeratin/Nuclei

Floxed-control | *Gata*-DKO

Defective embryonic-extraembryonic attachement and Inhibition of Trophoblast progenitor expansion.

Fig. 3. Concurrent loss of GATA2 and GATA3 impairs early postimplantation development.

(A) Mating strategy to define the importance of GATA2 and GATA3 during early postimplantation mouse development. (B) An E7.5 *Gata*-DKO conceptus without the developing embryo inside. (C) Control and *Gata*-DKO conceptuses were isolated at ~E9.5 and tested for embryonic morphology (left) and placentation (right). The image of the *Gata*-DKO embryo is the representative of a few embryos that developed to Theiler stage 12a. None of the *Gata*-DKO embryos developed beyond this stage. (D) Fluorescence images showing loss of CDX2 (green) expressing TSPCs but the presence of Proliferin (PLF also known as PRL2C2, red)-expressing TGCs within the prospective EPC region of an E7.5 *Gata*-DKO conceptus. (E) Placentation at the control and *Gata*-DKO implantation sites were analyzed ~E9.5 (images are not in the same scale). Sections were immunostained with pan-Cytokeratin (green) and TGC marker PLF (red). The maternal-fetal interface in *Gata*-DKO embryo lacks the presence of trophoblast progenitors (insets, white arrows in control) but contain the primary TGC layer (yellow arrows). Also, unlike control, the developmentally arrested *Gata*-DKO embryo proper is attached to the placentation site.

A

Gata2^{f/f};Gata3^{f/f}; UBC-cre/ERT2 ♂ X Gata2^{f/f};Gata3^{f/f} ♀

Tamoxifen injection at E7.5
and
analysis of embryo at E10.5

B

| Floxed-control | Gata2-KO | Gata3-KO | Gata-DKO |

Embryo

Placenta

C

Cytokeratin/Vimentin

Floxed-control

Junctional zone

Decidua

Labyrinth

Defined Junctional and Labyrinth Zones

Decidua

TGCs

SpTs

Junctional zone

Cytokeratin/Vimentin

Gata-DKO

Junctional Zone

Decidua

Significantly Reduced Labyrinth

Decidua

TGCs

SpTs

Junctional zone with fewer SpTs

D

*p<0.001
*p<0.02

Thickness (μm)

Labyrinth zone | Junctional zone

E

*p<0.01

Cell density (relative %)

■ Floxed-control
□ Gata-DKO

TGCs | SpTs

Fig. 4. GATA2 and GATA3 ablations in differentiating trophoblast cells impair placental development and induce early embryonic lethality.

(A) Experimental strategy to define the importance of GATA factors during differentiation of trophoblast progenitors to specialized trophoblast cells. (B) E10.5 embryos along with placentae showing severe developmental defects in *Gata*-DKO compared to individual *Gata*-KO and control embryos. (C) Immunofluorescence analyses showing a severe reduction of the labyrinth zone in the *Gata*-DKO placenta compared to the control. Pan-cytokeratin was used to mark the trophoblast layers, while Vimentin was used to differentiate the junctional zone from the labyrinth zone and the uterine tissue. Insets show magnified regions of junctional zones with the presence of TGCs (white arrows). (D) Quantitative plots showing the width of the labyrinth and junctional zones in control and *Gata*-DKO placentae. (E) Quantitative plot showing junctional zone in the *Gata*-DKO placentae contain a similar number of TGCs but is associated with significantly less number of spongiotrophoblast cells (SpTs).

A

Wild type control TSC
(+) tamoxifen

Gata-floxed TSC
(−) tamoxifen
(Floxed-control)

Gata-floxed TSC
(+) tamoxifen
(*Gata*-DKO)

B

EGFP inserted *Gata*-floxed TSC

↓

culture with or without tamoxifen

↓

microinjection into preimplantation embryo

Floxed-control

Gata-DKO

ICM

TE

Blastocoel cavity

C

% of blastocysts showing TE-integration of injecetd TSCs

■ Floxed-Control
□ *Gata*-DKO

D

6667 GATA2 target genes

4243 common target genes

4746 GATA3 target genes

E

- ● Not Significant
- ● 1.5 ≤ |FC| < 2
- ● 2 ≤ |FC| < 3
- ● 3 ≤ |FC|

Log^2 FPKM (*Gata*-DKO TSC)

Log_2 FPKM (control TSC)

Elf5

Esrrb

BMP4

F

Significant Biofunctions of GATA-Regulated Genes

(−)log10(p-value)

Cell Migration
Cell Proliferation
Cell Differentiation
Angiogenesis
Cell Invasion
Vasculogenesis
Hypoxia

0.00E+00   0.50E+00   1.00E+00   1.50E+00   2.00E+00

G

| Gene	Fold Change (RNA-seq)	ChIP-seq	
		GATA2 Target	GATA3 Target
Esrrb	(−) 2.5	YES	YES
Elf5	(−) 1.9	YES	YES
Bmp4	(−) 3.5	YES	YES
Prl3d1	(+) 32.9	YES	NO
Prl2a1	(+) 18	YES	NO
Ascl2	(+) 9.0	NO	Yes

Fig. 5. Loss of GATA factors impair stem state gene expression program in TSCs and primary TSPCs.

(A) Micrographs of control and *Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* (*Gata*-Floxed) TSC colonies in standard TSC culture condition. Unlike wild type TSCs (left), *Gata*-Floxed TSCs that were cultured with tamoxifen to delete *Gata* genes (*Gata*-DKO, right) lost stem-state colony morphology. *Gata*-Floxed TSCs that were cultured without tamoxifen (*Floxed*-control, middle) maintained stem-state colony morphology. (B) TE-Chimerism analyses of TSCs. Micrographs show that unlike *Floxed*-control TSCs, *Gata*-DKO TSCs failed to integrate into the TE and stayed within the blastocoel cavity. (C) Quantitative plot of TE-chimerism analyses (mean ± SE; n = 3, p≤0.001). (D) Venn diagrams showing a number of genes that are direct targets of GATA2/GATA3 and also showed significant changes in their mRNA expression in *Gata*-DKO TSC. (E) The scatter plot shows fold change in mRNA expression of common GATA2 and GATA3 target genes, including TSC-specific genes, *Bmp4*, *Esrrb,* and *Elf5,* in control vs. *Gata*-DKO TSCs. (F) Ingenuity Pathway Analysis showing major biofunctions associated with GATA2/GATA3 regulated genes in TSCs. (G) The table shows downregulation of TSC-specific genes and upregulation of differentiated trophoblast markers in *Gata*-DKO TSCs. These representative genes are also direct targets of GATA2 and/or GATA3.
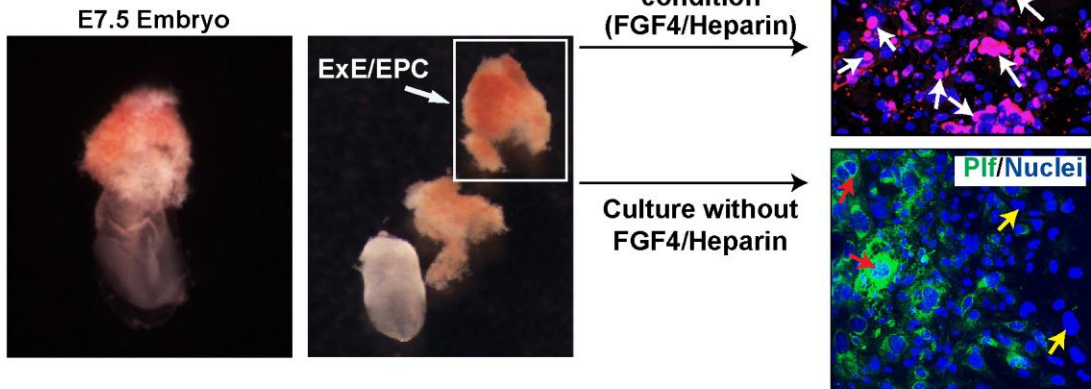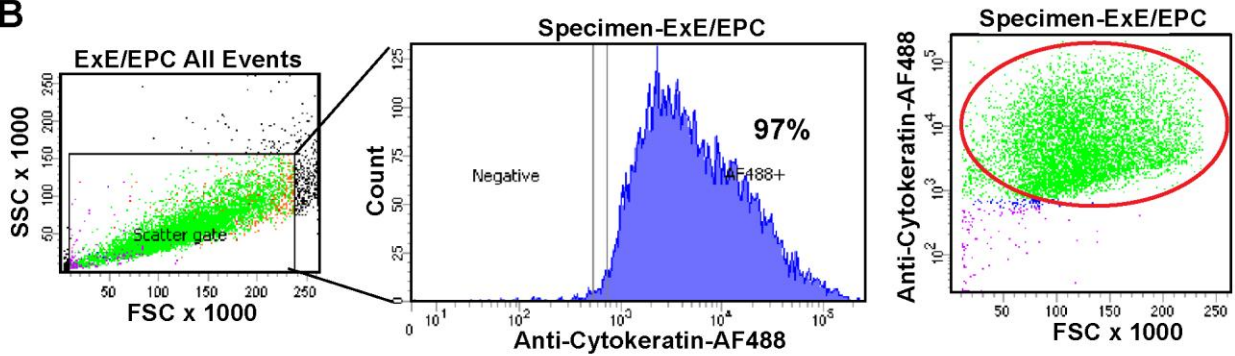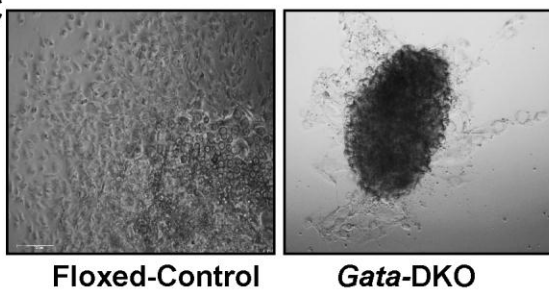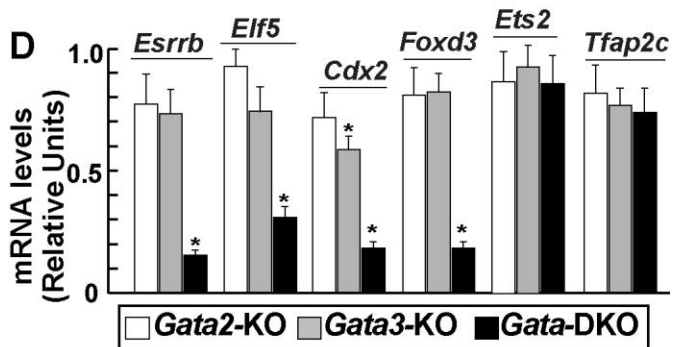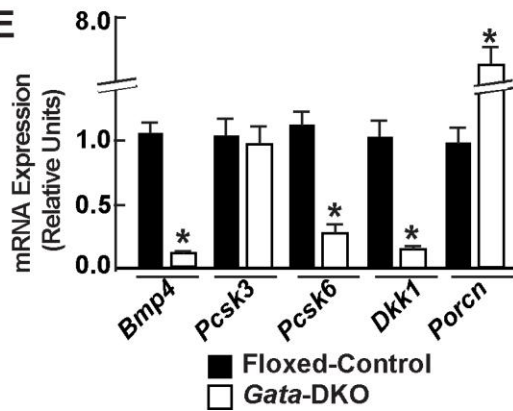
Fig. 6. GATA factors are required to activate developmental stage-specific gene expression in trophoblast progenitors to ensure differentiation and embryonic-extraembryonic crosstalk.

(A) ExE/EPC explant cultures, maintained in the presence of FGF4 and Heparin, showed the presence of CDX2 expressing TSPCs (white arrows). Explants, cultured in the absence of FGF4, undergo differentiation. In this condition, TSPCs differentiate into multiple cell types, including TGCs with (red arrows) or without (yellow arrows) expression of proliferin (Plf/ Prl2c2), a marker of parietal TGCs. (B) Fluorescence-activated cell sorting (FACS) using intracellular Cytokeratin labeling shows ExE/EPC explant culture consists of a high percentage of trophoblast cells. (C) Micrographs show *ex vivo* primary TSPC culture from floxed-control and *Gata*-DKO embryos with tamoxifen in the absence of FGF4. The *Gata*-DKO samples often showed reduced cell number indicating abnormal proliferation of TSPCs. (D) Analysis of mRNA expression showing significant reduction of several trophoblast stem and progenitor specific genes in the *Gata*-DKO TSPCs compared to *Gata2*-KO and *Gata3*-KO (Expression level of a gene in control TSPCs were considered as 1, mean ± SE; n = 3, p≤0.001). (E) Analysis of mRNA expression in control and *Gata*-DKO TSPCs showing altered expression of BMP4, Nodal and Wnt signaling genes that are implicated in successful gastrulation (mean ± SE; n = 3, p≤0.01).
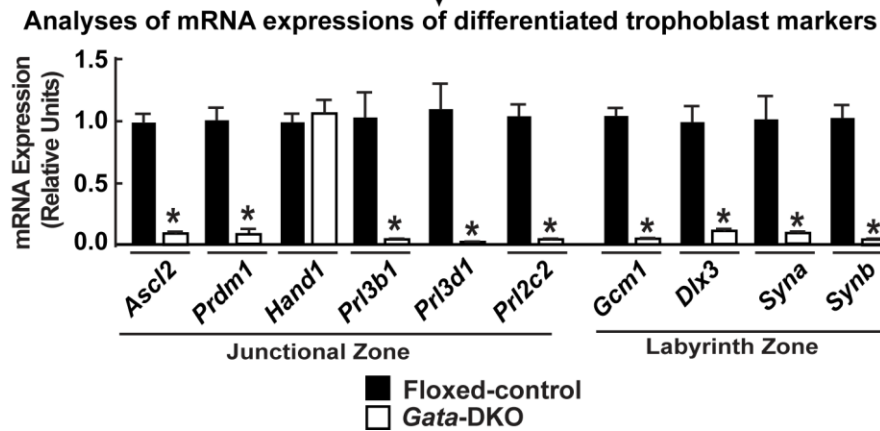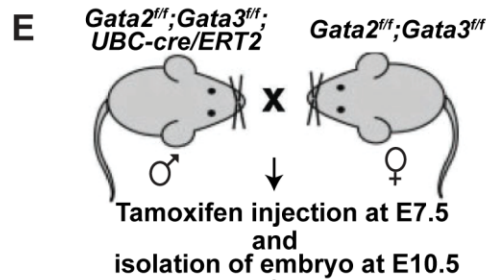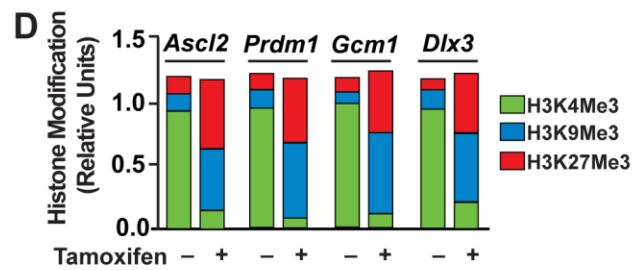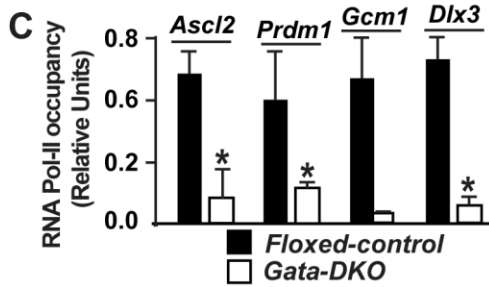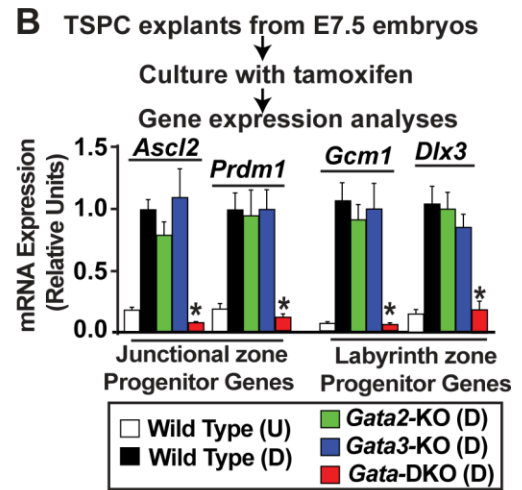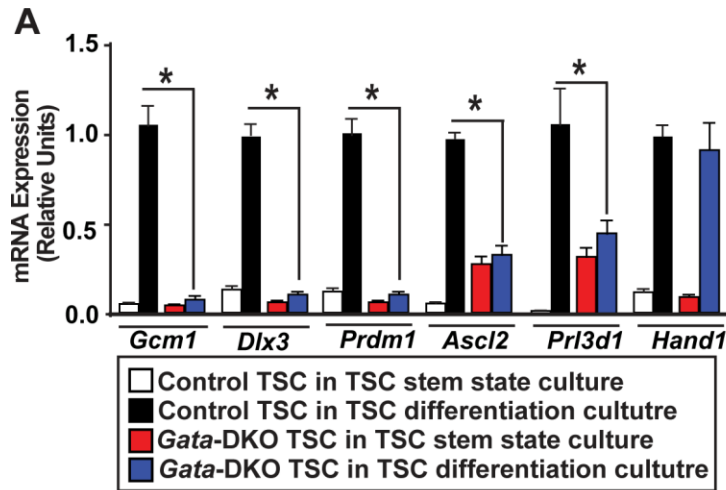
Fig. 7. Functional redundancy of GATA2 and GATA3 ensure proper gene expression during trophoblast progenitor differentiation.

(A) Control and *Gata*-DKO TSCs were cultured for 4 days in TSC differentiation culture condition, and mRNA expression of trophoblast differentiation genes were tested by qRT-PCR (mean ± SE; n = 3, p≤0.001). The plot shows that except *Hand1*, mRNA induction of trophoblast differentiation markers were impaired in *Gata*-DKO TSCs. (B) Schema of gene expression analyses in differentiating TSPCs. The plot shows impaired induction of key differentiation genes in *Gata*-DKO TSPCs. (C) RNA Pol II recruitment at promoters of key trophoblast genes in differentiating control and *Gata*-DKO TSPCs (mean ± SE; n = 3, p≤0.01). (D) The plot shows maintenance of repressive histone marks at the promoter regions of key trophoblast genes in differentiating *Gata*-DKO TSPCs. (E) Schema of gene expression analyses in *Gata*-DKO placentae. The plot shows relative mRNA expression of different trophoblast genes in control and *Gata*-DKO placentae (mean ± SE; n = 3 individual experiments, *, p≤0.01).

Contents

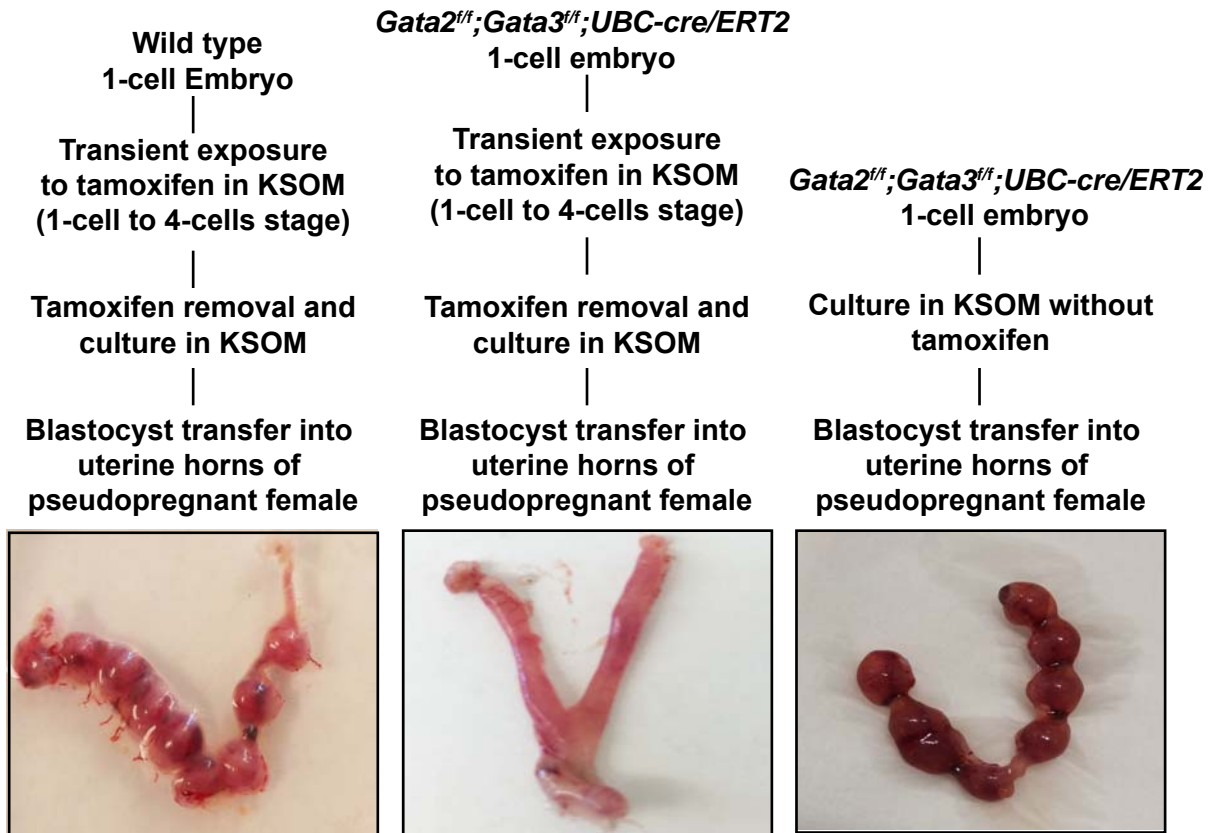| Items	General Description
Fig. S1	Schematic diagram of different stages of mouse trophoblast lineage development along with stage-specific markers.
Fig. S2	Genotyping data confirming *Gata* gene deletion upon tamoxifen treatment.
Fig. S3	Experiments showing preimplantation embryonic development in *Gata3*-KO and *Gata*-DKO mice.
Fig. S4	Experiments showing the importance of GATA2 and GATA3 functions in embryo implantation.
Fig. S5	Experiments showing imporatnce of GATA2 and GATA3 functions in differentiation of TSCs.
Supplementary Materials and Methods	Describes additional materials and methods, details of bioinformatics analysis of ChIP-seq and RNA-seq data.
Supplementary References	Lists references mentioned in the Supplementary Materials and Methods.
Table S5	Primer List: Oligonucleotides used in the study.
Table S6	Antibodies used in the study.
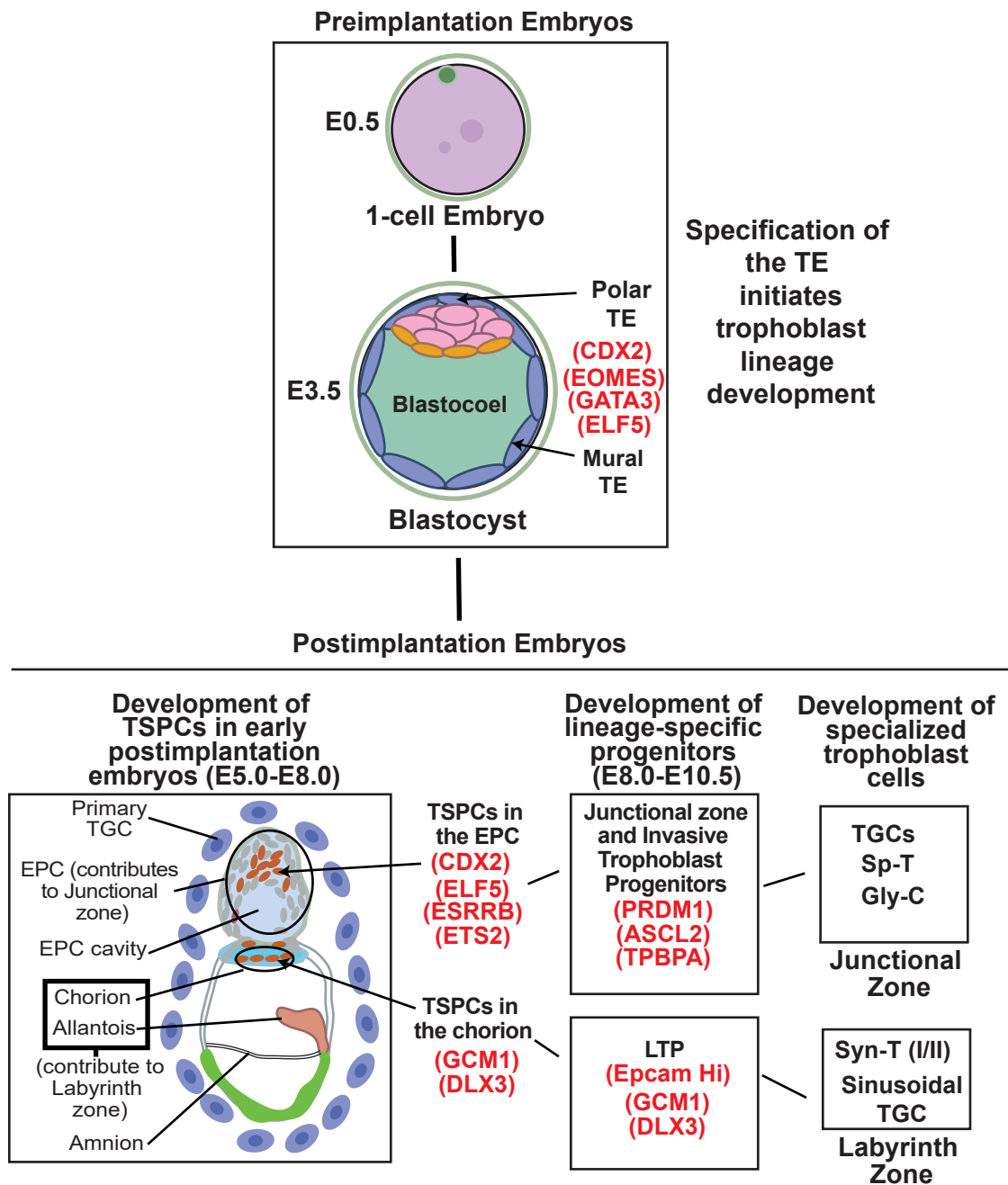
Fig. S1. Schematic presentation of different stages of trophoblast lineage development in mouse. Developmental stage-specific markers are indicated in red. In the preimplantation embryo, trophoblast development starts with the specification of TE-lineage, which mediates embryo implantation. In an early postimplantation embryo, trophoblast stem and progenitor cells (TSPCs) arise. Both TE cells and TSPCs express stem-state markers, like CDX2, EOMES and ELF5. GATA3 is also implicated in gene regulation within the TE. Later, TSPCs within the EPC and chorion differentiate into the specific trophoblast progenitor populations. PRDM1+, ASCL2+ and TPBPA+ junctional zone progenitors arise in the EPC, whereas GCM1+ and DLX3+ labyrinth trophoblast progenitors (LTPs) arise upon chorio-allantoic attachment. Finally, specialized trophoblast subtypes like trophoblast giant cells (TGCs), Spongiotrophoblast cells (Sp-T), Glycogen cells (Gly-C), and Synciotrophoblasts I. and II (SynTI, SynTII) arise to form a mature placenta
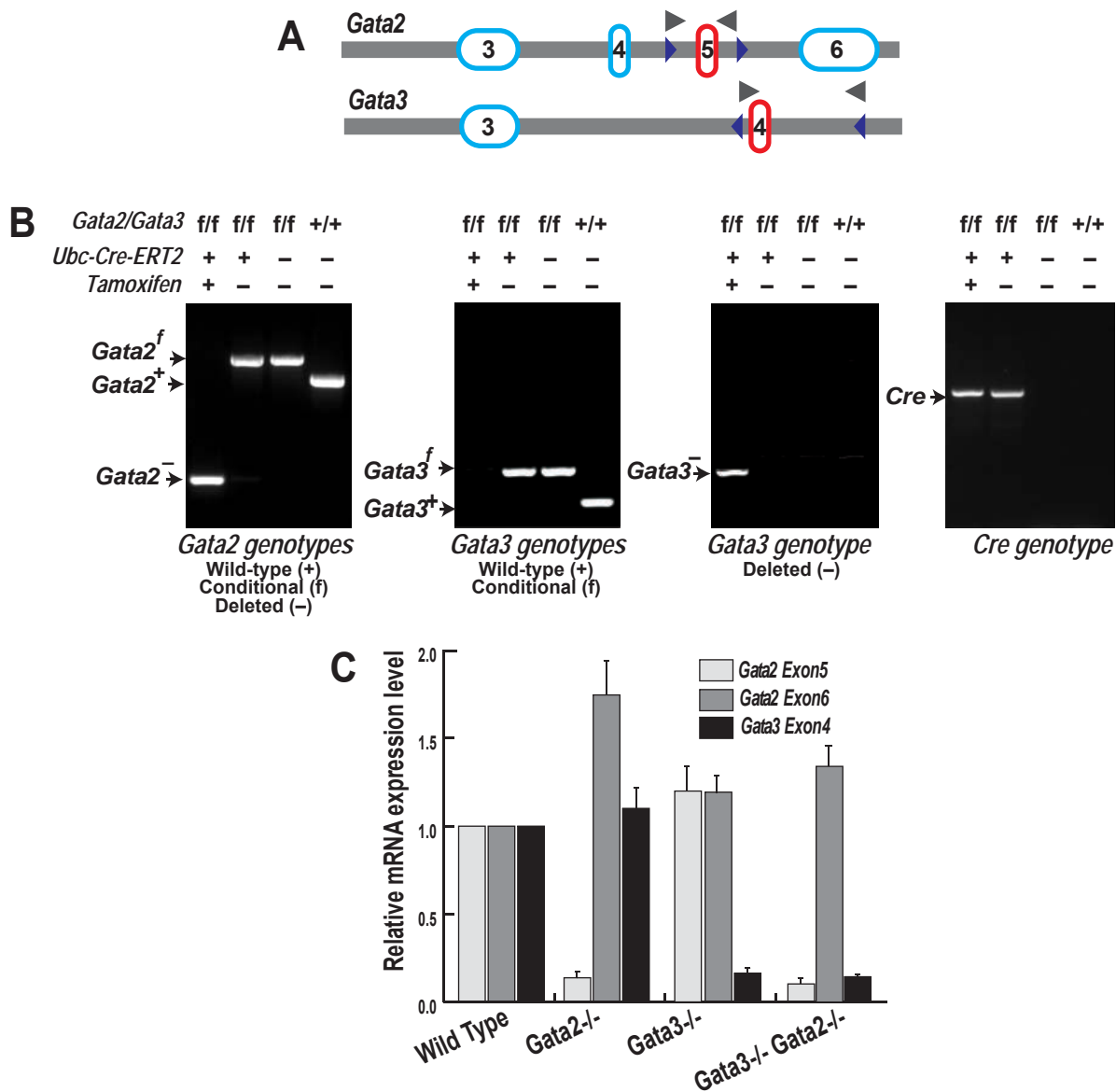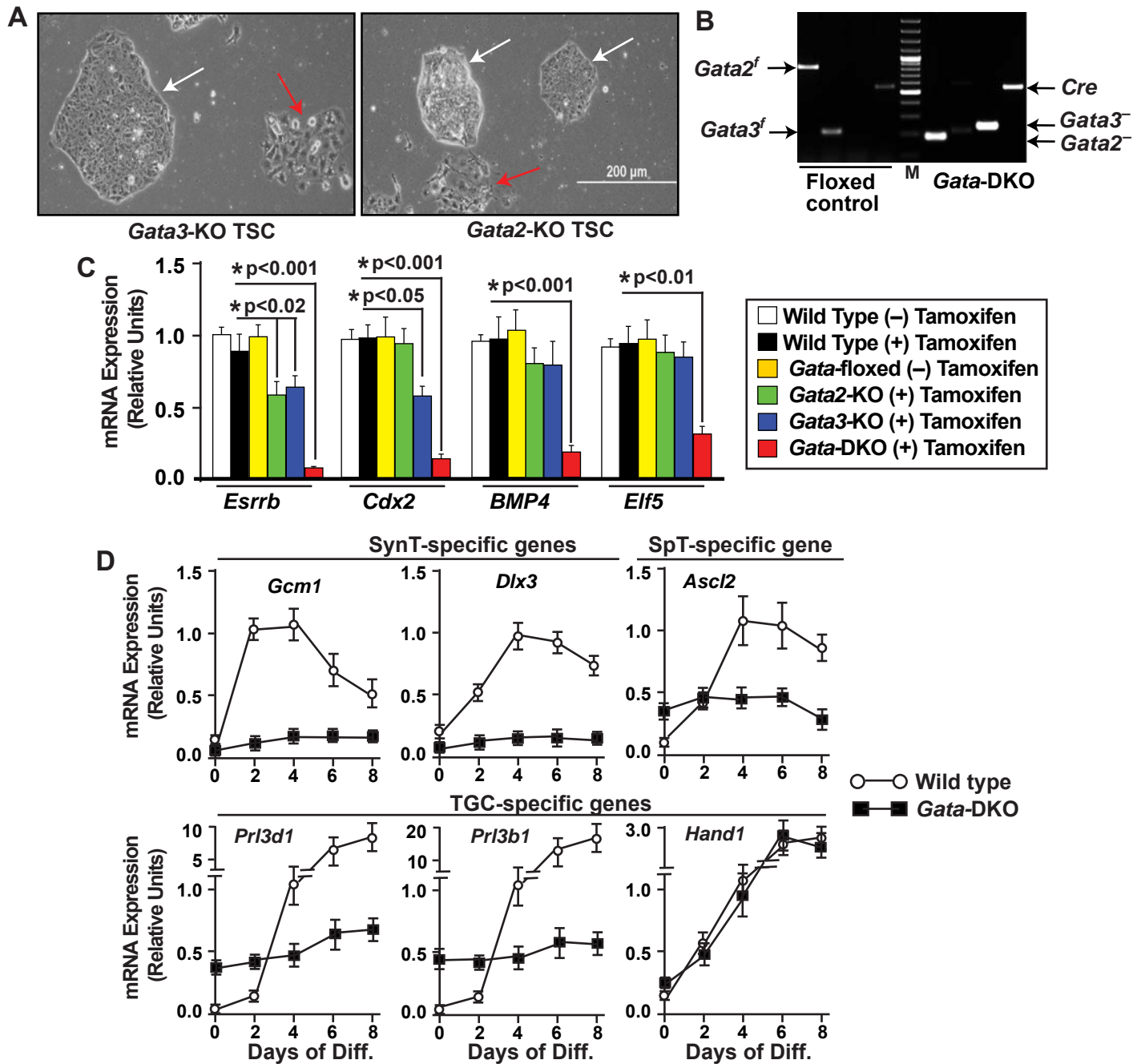
Fig. S2. (A) Schematic diagram showing part of the *Gata2* and *Gata3* loci on chromosome 6 and chromosome 2 respectively. *Gata2* exons 3, 4, 5 and 6 are shown on the diagram where exon 5 is flanked by loxP sites (blue triangle). Similarly, *Gata3* exons 3 and 4 are shown on the diagram where exon 4 is flanked by loxP sites (blue triangle). (B) Genotyping data confirming tamoxifen treatment induces deletion of floxed *Gata2* and *Gata3* alleles in the Cre expressing mouse embryos. PCR products corresponding to *Gata2 +, Gata3 +, Gata2 flox, Gata3 flox, Gata2 -, Gata3 -* are indicated. (C) Exon-specific RT-PCR analyses of the embryonic tissues showing efficient Cre-mediated excision of *Gata2* and *Gata3* floxed alleles.
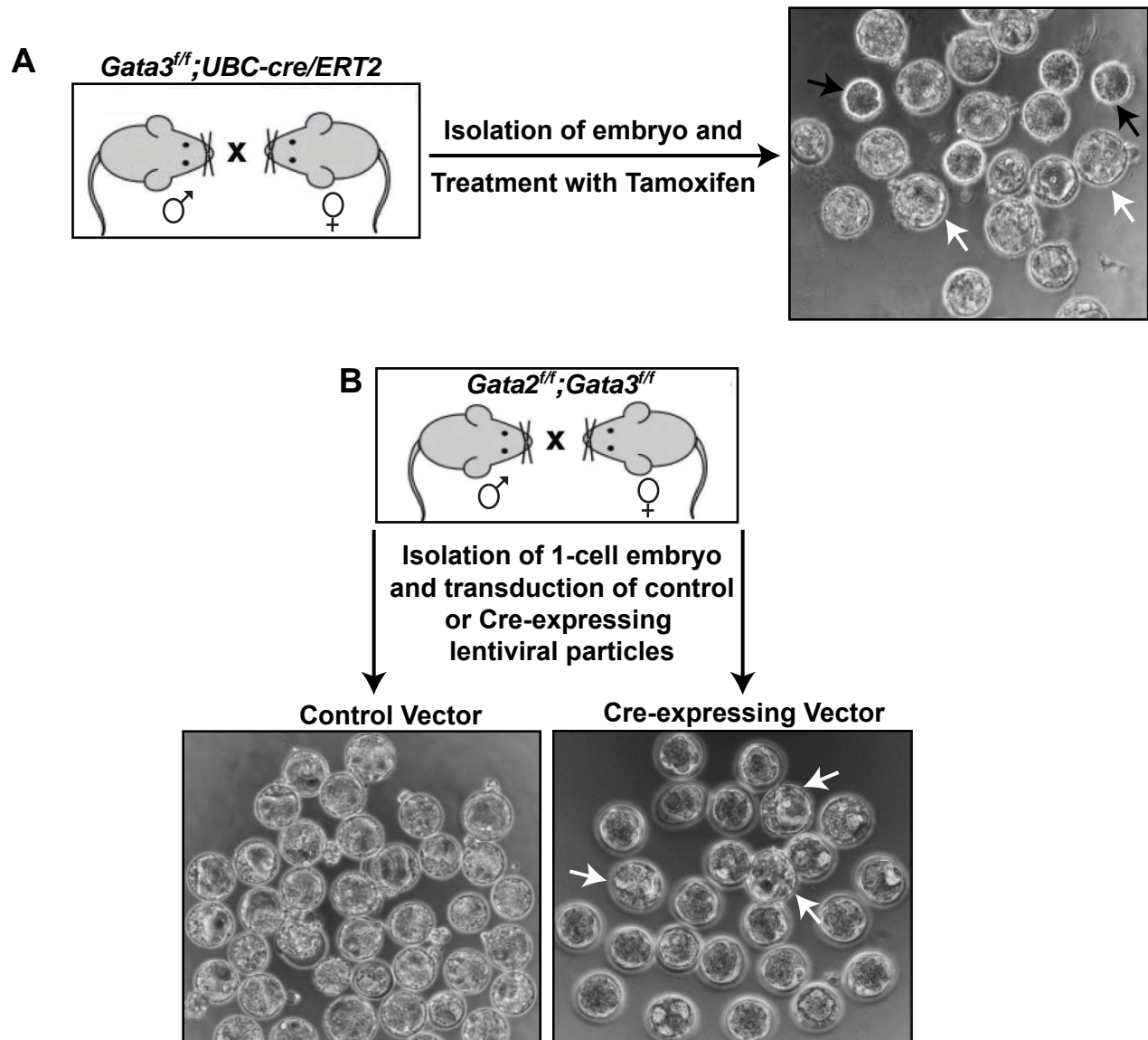
Fig. S3. (A) Schematic presentation of isolation and culture of one-cell stage *Gata3^{f/f};UBC-cre/ERT2* mouse zygotes in the presence of tamoxifen results in the partial impairment of trophectoderm development. While several embryos did not develop beyond morula (black arrow), other embryos matured to the blastocyst stage (white arrows). (B) Transduction of *Gata2^{f/f};Gata3^{f/f}* one-cell stage mouse embryos with Cre recombinase expressing lentiviral particle results in the partial impairment of trophecto-derm development but several of them matured to the blastocyst stage (white arrows). Almost all control embryos, transduced with lentiviral particles containing empty vector, matured to the blastocyst stage.

Wild type 1-cell Embryo	*Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* 1-cell embryo	
|	|	
Transient exposure to tamoxifen in KSOM (1-cell to 4-cells stage)	Transient exposure to tamoxifen in KSOM (1-cell to 4-cells stage)	*Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* 1-cell embryo
|	|	|
Tamoxifen removal and culture in KSOM	Tamoxifen removal and culture in KSOM	Culture in KSOM without tamoxifen
|	|	|
Blastocyst transfer into uterine horns of pseudopregnant female	Blastocyst transfer into uterine horns of pseudopregnant female	Blastocyst transfer into uterine horns of pseudopregnant female

Fig. S4. Experimental strategy showing the importance of GATA2 and GATA3 function in blastocyst implantation. Wild type and *Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* 1-cell embryos were treated with tamoxifen. At 4-cells stage, embryos were transferred to KSOM medium without tamoxifen and allowed to develop *ex vivo for* blastocyst maturation. In addition, *Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* embryos without tamoxifen exposure were used as control. Although, transient exposure to tamoxifen impaired blastocyst development in the majority of *Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* embryos, several of them developed to the blastocyst stage. Blastocyst developments were unaffected in wild type embryos upon transient tamoxifen exposure. Finally, matured blastocysts from each experimental condition were transferred into uterine horns of pseudopregnant mice. Images show impaired implantation efficiency of *Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* blastocysts that were developed after tamoxifen exposure. In contrast, blastocysts from tamoxifen exposed wild type embryos and *Gata2^{f/f};Gata3^{f/f};UBC-cre/ERT2* embryos without tamoxifen exposure readily implanted.

Fig. S5. (A) Micrographs show colony morphologies of *Gata2*-KO and *Gata3*-KO TSCs that were maintained in standard TSC culture condition. Both *Gata2*-KO and *Gata3*-KO TSCs largely maintains stem-state colony morphology (white arrows). However, both have higher propensities for spontaneous differentiation as revealed by the presence of colonies with differentiated cells (red arrows). (B) Genotyping shows *Gata* genes deletion in TSCs. (C) qRT-PCR analyses shows that mRNA expression of trophoblast stem state genes are strongly repressed in *Gata*-DKO TSCs. The plot shows that tamoxifen treatment does not alter gene expression in wild type control TSCs (black bars). The starting TSC population for *Gata*-DKO TSCs [*Gata* floxed TSCs (–) tamoxifen (yellow bars)] and wild type TSCs (white bars) have similar levels of gene expression. mRNA expression of *Esrrb*, which is a target gene of both GATA2 and GATA3, are repressed by ~50% in both *Gata2*-KO (green bars) and *Gata3*-KO (blue bars) TSCs. However, *Esrrb,* as well as other stem state genes were more strongly repressed in *Gata*-DKO TSCs (red bars). The expression level of a gene in wild type TSCs was considered as 1 (mean ± SE; n = 3). (D) qRT-PCR analyses showing mRNA expressions of trophoblast differentiation markers in wild-type and *Gata*-DKO TSCs when they were cultured in TSC differentiation culture condition different time interval. Representative genes of syncytiotrophoblast (SynT), spongiotrophoblast (SpT) and trophoblast giant cells (TGC) were analyzed. The expression level of a gene in wild type TSCs at day 4 of differentiation was considered as 1 (mean ± SE; n = 3).

Supplementary Materials and Methods

Cell culture and reagents

Mouse trophoblast stem cells (TSCs) were cultured with FGF4, Heparin and MEF-conditioned medium (CM) according to the protocol (Tanaka et al., 1998). To induce differentiation, Fibroblast Growth Factor 4 (FGF4), Heparin, as well as CM, were withdrawn from the culture and cells were allowed to grow. ExE/ EPCs were harvested from ~E7.0-E7.5 pregnant female mouse and were grown in the presence of FGF4, Heparin, and CM (to maintain undifferentiated state) or in mouse TSC media only (for differentiation) at the normoxic condition. For time course analysis differentiation was induced, and cells were harvested at different day point for RNA analysis.

Gene deletions in preimplantation embryos

For lentiviral-mediated Cre expression, 1-cell stage embryos were harvested from superovulated females. These embryos were subjected to perivitelline space microinjection, (Home et al., 2012), with concentrated lentiviral particles prepared from Puro.Cre empty vector expressing Cre recombinase. Injected embryos were allowed to grow in KSOM as described above. PCR confirmed successful expression of Cre recombinase and subsequent gene deletions.

For implantation efficiency experiments, wild type and *Gata2$^{f/+}$;Gata3$^{f/+}$;UBC-cre/ERT2* 1-cell embryos were treated with tamoxifen. At 4-cells stage, embryos were transferred to KSOM medium without tamoxifen and allowed to develop ex vivo for blastocyst maturation. In addition, *Gata2$^{f/+}$;Gata3$^{f/+}$;UBC-cre/ERT2* embryos without tamoxifen exposure were used as control. Matured blastocysts were transferred to surrogate females following earlier described procedures (Saha et al., 2013)

Tissue collection from postimplantation embryos

Injected animals were euthanized on at desired day points as indicated in the main text. Uterine horn and conceptuses were photographed. Conceptuses were dissected to isolate embryos, yolk sacs, and placentae. All embryos, yolk sacs, and placentae were photographed at equal magnification for comparison purposes. Uteri containing placentation sites were dissected from pregnant female mice on E7.5, E9.5, E11.5, E13.5, and E18.5 and frozen in dry ice-cooled heptane and stored at −80°C until used for histological analysis. Tissues were subsequently embedded in optimum cutting temperature (OCT) (Tissue-Tek) and were cryosectioned (10μm thick) for immunohistochemistry (IHC) studies using Leica CM-3050-S cryostat.

Flowcytometry

96 hours explant cultures of mouse ectoplacental cones were trypsinized. Single cell suspension was formaldehyde fixed and permeabilized using BD Cytofix/Cytoperm Fixation and Permeabilization solution (BD Biosciences, #554722) according to the manufacturer's protocol. All washings were done using saponin containing wash buffer. A standard protocol for doing FACS staining & analysis was followed using anti-wide spectrum Cytokeratin antibody (Abcam) to analyze Cytokeratin-positive placental trophoblast populations in a BD LSR II flow cytometer.

Genotyping

Genomic DNA was prepared using tail tissue from mouse using REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich). Genotyping was done using REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich) and respective primers. Genomic DNA from individual blastocysts was prepared

by the following technique using REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich). Each blastocyst was collected into separate PCR tubes and was lysed with 4 μl of Extraction buffer and 1μl of Tissue Prep buffer. Briefly, they were incubated at 42°C for 10 mins followed by heat inactivation at 98°C for 3 mins. and neutralization with 4μl of Neutralization buffer. 4μl of this genomic DNA was used for a 20μl PCR reaction. For genotyping in embryos, part of the yolk sac or embryo proper from each conceptus was used to prepare genomic DNA as described above. Respective primers are listed in the Table S5.

Quantitative RT-PCR

Total RNA from cells was extracted with RNeasy Mini Kit (Qiagen) with on-column DNaseI digestion. Purified RNA was used to prepare cDNA using cDNA preparation kit. All these samples were analyzed by qRT-PCR following procedures described earlier (Dutta et al., 2008). For expression analysis in preimplantation embryos, total RNA was isolated from embryos using PicoPure RNA isolation kit (Thermo Fisher Scientific) and processed as described earlier (Home et al., 2012). Primers, used for qRT-PCR analysis, are listed in the Table S5.

Immunofluorescence

For immunostaining, preimplantation embryos were fixed with 4% paraformaldehyde, permeabilized in 0.25% Triton X-100, and blocked with 10% fetal bovine serum and 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 1 h at room temperature. Embryos were incubated with antibodies (1:100 dilution) overnight at 4 °C, washed with 0.1% Triton X-100 in PBS. After incubation (1:400, 1 hour, room temperature) with conjugated secondary antibodies, embryos were washed and mounted using anti-fade mounting medium (Thermo Fisher Scientific) containing DAPI and viewed in LSM 5 Laser Scanning Microscope (Carl Zeiss Microimaging). For IHC with mouse tissues, slides containing cryosections were thawed and fixed with 4% PFA

followed by permeabilization with 0.25% Triton X-100 and blocking with 10% fetal bovine serum and 0.1% Triton X-100 in PBS. Sections were incubated with primary antibodies overnight at 4 °C, washed with 0.1% Triton X-100 in PBS. After incubation (1:400, 1 h, room temperature) with conjugated secondary antibodies, sections were washed, mounted using anti-fade mounting medium (Thermo Fisher Scientific) containing DAPI and visualized using Nikon Eclipse 80i fluorescent microscope. To test expression of GATA3 in postimplantation embryos, *Gata3-LacZ* knock-in mice (Pandolfi et al., 1995) were used. Staining was done using placentation sites from pregnant *Gata3$^{-/+}$* female mice (*lacZ* knock-in). Anti-β Galactosidase antibody was used to stain β Galactosidase in the GATA3 expressing cells. Antibodies, used for immunofluorescence analyses are mentioned in Table S6.

Immunohistochemistry

Paraffinized placental sections were processed for immunostaining according to the protocol described by Holets et al. (Holets et al., 2006). Briefly, 10-µm tissue sections were cut from paraffinized first-trimester placentas. Sections (10µm thick) were placed onto slides, rehydrated and were subjected to heat mediated antigen retrieval using citrate based Reveal buffer (BioCare Medical). Non-specific immunoglobulin binding was blocked with 10% normal goat serum (Thermo Fisher Scientific). The primary antibody or its isotype-specific control (IgG1) was incubated with the tissue sections for 4°C overnight. Secondary antibody (biotinylated goat anti-mouse/ rabbit IgG) (Vector Laboratories) incubation was followed by endogenous peroxidase depletion using 3% H_2O_2. Reactivity was detected using the streptavidin-peroxidase (Thermo Fisher Scientific) and DAB reagent kit (Dako) and tissues were counterstained with Mayer's hematoxylin (Sigma-Aldrich). Positive staining was confirmed as a brown coloration under the microscope.

Quantitation of trophoblast cell population

Tissue sections from three individual placentation sites were used to quantitate trophoblast giant cell (TGC) and spongiotrophoblast (SpT) numbers in *Floxed*-control and *Gata*-DKO embryos. Cell populations at three different areas of equal size within the junctional zone of each tissue sections were counted for TGCs and SpTs. The data was plotted as a relative percentage considering the average cell number/area in control embryos as 100%.

Statistical analyses

We used at least 3 independent cultures for the experiments with single KO or double KO analyses and indicated those numbers with "n" in the legends. Similarly, at least 3 biological replicates were used for the analyses with blastocysts, ExE/ EPC explant cultures, placentae and conceptuses.

ChIP and ChIP-Seq

Quantitative ChIP analysis was performed following published protocols (Home et al., 2009; Home et al., 2012). TSCs, homogenized EPCs, and homogenized placentae cells were cross-linked by with 0.4% formaldehyde (Sigma) for 10 mins at room temperature with gentle rotation. Chromatin crosslinking were stopped with glycine (125mM). These samples were sonicated. Chromatin fragments were immunoprecipitated with different antibodies. Quantification of the precipitated DNA was performed using qPCR amplification. A list of the primers used for ChIP analysis and the antibodies used for ChIP analysis are mentioned in the Table S5 and S6. For ChIP-seq in TSCs, immunoprecipitated chromatin fragments from three independent

experiments were pooled. Libraries were sequenced in Illumina Genome Analyzer II using TruSeq SBS kit v5-GA chemistry and in Illumina HiSeq using TruSeq SBS v2-HS chemistry (Illumina, San Diego, CA) to generate 35 bp single-end reads. Binding of the nonspecific immunoglobulin G (IgG) antibody was used as the negative control for eliminating false positive peaks.

Sequences were aligned using ELANDv2 (CASAVA 1.7) to the mouse reference genome (NCBI37/mm9) using default parameters. Peak detection was performed using the Model-based Analysis of ChIP-Seq (MACS) software (Zhang et al., 2008). MACS was run with the peak detection p-value cutoff set at 1e-5 (default). Highly enriched peaks were selected from the two experiments based on a false discovery rate (FDR) cutoff of 1% for GATA2 sites and 15% for GATA3 sites. We further searched for the GATA2 and GATA3 consensus sequence in their respective ChIP-Seq targets within a 250 bp region from either side of the peak center using a weight-matrix match with at least 80% similarity. The weight matrices were obtained from the JASPAR database (Sandelin et al., 2004). A substantial proportion of the highly enriched GATA2 and GATA3 ChIP-Seq binding sites consisted of at least one instance of the consensus motif. All raw data for ChIP-seq analyses are submitted to the GEO database (http://www.ncbi.nlm.nih.gov/gds), with accession number GSE92295.

RNA-Seq analysis

The changes in gene expression as a result of the double knockout of GATA2 and GATA3 was measured by whole transcriptome sequencing (RNA-Seq) of control and *Gata*-DKO TSCs. Sequencing was performed on an Illumina HiSeq 2000 sequencing machine (*Illumina, San Diego, CA*) at a 50 bp single-end resolution. Sequence reads were mapped to the mouse reference genome (GRCm38) using STAR (Dobin et al., 2013) with default parameters. Transcript abundance estimates were generated using Cufflinks (Trapnell et al., 2010) and

differential gene expression calculated using Cuffdiff (Trapnell et al., 2013) with default parameters. Approximately, 44.3 and 41.1 million reads were generated of which around 96.7% and 96.6% were mapped to the genome for the control and *Gata*-DKO TSC samples respectively. In the absence of replicate samples, Cuffdiff uses a heuristic approach to generate a significance p-value (adjusted for false discovery by the Benjamini and Hochberg method (Benjamini and Hochberg, 1995) giving a q-value) where the variance is measured across conditions under the assumption that most transcripts are not differentially expressed. While these p-values do not substitute for a p-value derived with biological replicates, they form a reasonable statistic to filter the gene list. Genes with an absolute fold change ≥ 1.5 fold and a q-value ≤ 0.05 were deemed significant for further analysis. All raw data for RNA-seq analyses are submitted to the GEO database (http://www.ncbi.nlm.nih.gov/gds), with accession number GSE92295.

Combining ChIP-Seq and RNA-Seq data

Genes with a highly enriched GATA2 and GATA3 binding site within 50,000 bp upstream from the 5 prime end or downstream from the 3 prime end or overlapping the gene were selected. These genes were further filtered to include genes that were significantly differentially expressed or remained silent in the *Gata*-DKO sample. Ingenuity Systems Pathway Analysis software (IPA, Ingenuity Systems, www.ingenuity.com) was used to identify the biological functions that are associated with significantly differentially expressed genes (from the *Gata*-DKO samples) that contained both an enriched GATA2 and GATA3 site in its vicinity. IPA performs this task with the aid of its knowledge base which has curated information from the literature of genes and gene products that interact with each other. IPA use the right-tailed Fisher's exact test to calculate a significance p-value of the overlap between these genes and genes associated with a particular biological function. A *p-value* less than or equal to 0.05 is considered significant. The IPA database contains information from the literature on the relative

direction of a gene's expression in relation to a biological function. Using this information, IPA calculates an activation z-score for a biological function in relation to a set of genes (with expression information) indicating whether the function is activated of inhibited, based on the directionality of expression of the genes (Kramer et al., 2014). A positive activation z-score signifies an increase in the biological function's activity and a negative score signifies a decrease in its activity. Biological functions with an absolute activation z-score greater than or equal to 2 were considered significant.

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Table S1A

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Table S1B

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Table S2

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Table S3

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Table S4

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Table S5. Primer list

Primers used for genotyping			
Mouse	Gata2-floxed and Gata2-null	GCCTGCGTCCTCCAACACCTCTAA	TCCGTGGGACCTGTTTCCTTAC
Mouse	Gata3-flox	CAGTCTCTGGTATTGATCTGCTTCTT	GTGCAGCAGAGCAGGAAACTCTCAC
Mouse	Gata3-null	TCAGGGCACTAAGGGTTGTTAACTT	GTGCAGCAGAGCAGGAAACTCTCAC
	Cre	AAAATTTGCCTGCATTACCG	ATTCTCCCACCGTCAGTACG

Primers used for quantitative RT-PCR analysis			
Species	Gene	Forward 5'	Reverse 3'
Mouse	*Gata3 Ex3/4*	CGGGTTCGGATGTAAGTCGA	GTAGAGGTTGCCCCGCAGT
Mouse	*Gata2 Ex5*	CAGACGACAACCACCACCTT	GCTTGTAGTAGAGGCCACAGG
Mouse	*Gata2 Ex6*	GGAAGATGTCCAGCAAATCC	TGGAGAGCTCCTCGAAACAT
Mouse	*Cdx2*	GCAGTCCCTAGGAAGCCAAGTGA	CTCTCGGAGAGCCCGAGTGTG
Mouse	*Hand1*	GGCAGCTACGCACATCATCA	CCTGGCATCGGGACCATAG
Mouse	*Prl3d1*	ACATTTATCTTGGCCGCAGATGTGT	TTTAGTTTCGTGGACTTCCTCTCGAT
Mouse	*Prl3b1*	GGGGCACTCCTGTTGCTGGCA	GGACTTGCTCGCTGTTTTCTGGA
Mouse	*Elf5*	ATGTTGGACTCCGTAACCCAT	GCAGGGTAGTAGTCTTCATTGCT
Mouse	*Ascl2*	AAGTGGACGTTTGCACCTTCA	AAGCACACCTTGACTGGTACG
Mouse	*Gcm1*	CTGACTGGTTCCAGGAGTGG	TGTCGTCCGAGCTGTAGATG
Mouse	*Prl2c2*	GACCATTCCTCATTGCACACA	TCCTGGATACTGCTCCTACTACT
Mouse	*Prdm1*	TCAAGCCGAGGCATCCTTAC	AGCGTGTTCCCTTCGGTATG
Mouse	*Esrrb*	AGTACAAGCGACGGCTGG	CCTAGTAGATTCGAGACGATCTTAGTCA

Mouse	Tfap2c	ATCCCTCACCTCTCCTCTCC	CCAGATGCGAGTAATGGTCGG
Mouse	Dlx3	CACTGACCTGGGCTATTACAGC	GAGATTGAACTGGTGGTGGTAG
Mouse	Foxp1	GGTCTGAGACAAAAAGTAACGGA	CGCACTCTAGTAAGTGGTTGC
Mouse	Bmp4	GACTTCGAGGCGACACTTCTA	GAATGACGGCGCTCTTGCTA
Mouse	Foxd3	ACCACGTCGCTCATCAAGTC	GCGCCTATGATGTTCTCGAT
Mouse	Ets2	ACGGGCCTGGATTCTGTCT	TGAGCAAAGGCAGCTCGC
Mouse	Pcsk3	TCGGTGACTATTACCACTTCTGG	CTCCTGATACACGTCCCTCTT
Mouse	Pcsk6	CAGGCGCGAAGTGACTCTC	GACCGACAGCGACTGTTCTT
Mouse	Porcn	GCATGCTTCAGGTAAGACGG	CCATCTGCTTCGCCTGCC
Mouse	18s rRNA	AGTTCCAGCACATTTTGCGAG	TCATCCTCCGTGAGTTCTCCA
Mouse	Gapdh	TGCCCCATGTTTGTGATG	TGTGGTCATGAGCCCTTCC
Primers used for quantitative ChIP analyses			
Mouse	Prdm1 promoter	CGAAGTACGTCGGATCCTGT	GGGGACTCCTCCTCAAAAGA
Mouse	Gcm1 promoter	TGATTGGACAGTTGCCAGAG	AAGTGGTCGCTGTTCCCTAA
Mouse	Dlx3 promoter	TCCTTCCACAAACACCCAAT	GGTGGGCTTAGGTGAGATGA
Mouse	Ascl2 promoter	GGAGAGCTGGCTGTAAGGTG	TTGCCCTGACCTGAGAGAAT

Table S6. Antibody list

Primary antibodies	Species raised in	Vendor	Catalog number	Batch/ Lot number	Dilutions used
anti-GATA2	Rabbit	Abcam	ab109241	GR143635-2	1:100 (IF/IHC), 6μg/10 million cells for ChIP
anti-GATA3	Mouse	BD Biosciences	558686	5288632	1:100 (IF/IHC), 6μg/10 million cells for ChIP
anti- β-Galactosidase	Mouse	Promega	Z3781	0000125058	1:200 (IF)
anti-CDX2	Rabbit	Abcam	EPR2764Y	GR133702-8	1:100 (IF)
anti-Oct-3/4	Mouse	Santa Cruz Biotechnology	sc-5279	G1610	1:100 (IF)
anti-wide spectrum Cytokeratin	Rabbit	Abcam	ab9377	GR218349-5	1:100 (IF)
anti-pan Cytokeratin	Mouse	Abcam	ab7753	GR185314-12	1:100 (IF)
anti-Vimentin	Mouse	Santa Cruz Biotechnology	sc-373717	K2713	1:100 (IF)
anti-Proliferin	Goat	Santa Cruz Biotechnology	sc-47347	K1212	1:75 (IF)
anti-trimethyl Histone H3 (Lys9)	Mouse	Millipore	05-1242	NG1698976	6μg/10 million cells for ChIP
anti-trimethyl Histone H3 (Lys27)	Rabbit	Millipore	CS200603	1987188	6μg/10 million cells for ChIP
anti-trimethyl Histone H3 (Lys4)	Rabbit	Millipore	CS200580	DAM1612220	6μg/10 million cells for ChIP
Anti- RNA Pol II (H5)	Mouse	BioLegend	MMS-129R	14862302	6μg/10 million cells for ChIP

Purified IgG1 k isotype control	Mouse	BD Biosciences	554121	4324640	6μg/10 million cells for ChIP
Purified IgG	Rabbit	BD Biosciences	550875	2139944	6μg/10 million cells for ChIP
Secondary antibodies	**Species raised in**	**Vendor**	**Catalog number**	**Batch/ Lot number**	**Dilutions used**
Alexa Fluor 568 anti-rabbit IgG	Donkey	Thermo Fisher Scientific	A10042	1606268	1:400 (IF)
Alexa Fluor 488 anti-mouse IgG	Donkey	Thermo Fisher Scientific	A21202	1562298	1:400 (IF)
Alexa Fluor 647 anti-goat IgG	Donkey	Thermo Fisher Scientific	A21447	1661244	1:400 (IF)
Biotinylated anti-mouse IgG	Goat	Vector Labs	BA-9200	W0206	10μg/ml (IHC)
Biotinylated anti-rabbit IgG	Goat	Vector Labs	BA-1000	X0212	10μg/ml (IHC)