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HLA-C expression in extravillous trophoblasts is determined by an ELF3–NLRP2/NLRP7 regulatory axis

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The distinct human leukocyte antigen (HLA) class I expression pattern of human extravillous trophoblasts (EVT) endows them with unique tolerogenic properties that enable successful pregnancy. Nevertheless, how this process is elaborately regulated remains elusive. Previously, E74 like ETS transcription factor 3 (ELF3) was identified to govern high-level HLA-C expression in EVT. In the present study, ELF3 is found to bind to the enhancer region of two adjacent NOD-like receptor (NLR) genes, NLR family pyrin domain-containing 2 and 7 (NLRP2, NLRP7). Notably, our analysis of ELF3-deficient JEG-3 cells, a human choriocarcinoma cell line widely used to study EVT biology, suggests that ELF3 transactivates *NLRP7* while suppressing the expression of NLRP2. Moreover, we find that NLRP2 and NLRP7 have opposing effects on HLA-C expression, thus implicating them in immune evasion at the maternal–fetal interface. We confirmed that NLRP2 suppresses HLA-C levels and described a unique role for NLRP7 in promoting HLA-C expression in JEG-3. These results suggest that these two NLR genes, which arose via gene duplication in primates, are fine-tuned by ELF3 yet have acquired divergent functions to enable proper expression levels of HLA-C in EVT, presumably through modulating the degradation kinetics of IkBα. Targeting the ELF3–NLRP2/NLRP7–HLA-C axis may hold therapeutic potential for managing pregnancy-related disorders, such as recurrent hydatidiform moles and fetal growth restriction, and thus improve placental development and pregnancy outcomes.

HLA-C | ELF3 | NLRP7 | immune tolerance | gene regulation

Significance

NOD-like receptors (NLRs) are well known for their roles in inflammasome-mediated immune responses. However, their functions in reproduction are poorly understood. We found that two NLR family members highly expressed during early human development, NLRP2 and NLRP7, are oppositely regulated by the transcription factor ELF3. Intriguingly, they also have opposite effects on HLA-C expression. While NLRP2 represses HLA-C, NLRP7 promotes its levels in the JEG-3 human choriocarcinoma cell line. Our data illustrate how NLRP7 may contribute to immune tolerance by up-regulating HLA-C levels. This ELF3–NLRP2/NLRP7–HLA-C regulatory axis provides unique insights into the establishment of immune tolerance during pregnancy and the mechanism of immune evasion in choriocarcinoma. It may also reveal new therapeutic targets for pregnancy complications.

During pregnancy, a fetus survives without rejection despite expressing paternally derived humanleukocyte antigen (HLA) molecules and other allogeneic antigens. Fetal extravillous trophoblasts (EVT) play a key role in establishing immune tolerance of maternal immune cells. Their enigmatic tolerogenic features underlie a unique combination of HLA, with limited expression of the polymorphic HLA-C, the expression of the nonclassical HLA-Ib molecules HLA-E and HLA-G, yet absence of the classical polymorphic HLA-A and HLA-B molecules which are normally found in any other nucleated cell (1–4). Despite extensive work delineating HLA transcriptional regulation, the regulatory mechanism underlying this unique HLA expression profile in trophoblast has not been fully elucidated. In somatic cells, NLRC5 and CIITA function as major histocompatibility complex (MHC) class I and MHC class II transactivators, both of which are NOD-like receptor (NLR) family proteins and promote MHC gene expression by assembling a nucleoprotein complex termed enhanceosome, together with the RFX complex, CREB/ATF1, and NF-Y factors (5–7).

Nucleotide-binding oligomerization domain (NOD) and leucine-rich repeat (LRR) containing receptors or NLRs have been widely studied as components of inflammasomes. Inflammasomes represent multimeric intracellular signaling platforms that link a breach in barrier function to a proinflammatory immune response (8, 9). Some NLRs such as NLRP1, NLRP3, and NLRC4 function as cytosolic pattern recognition receptors and elicit innate immune responses upon recognition of pathogen-associated molecular patterns or host-derived danger–associated molecular patterns. Upon inflammasome activation, procaspase 1 is cleaved and subsequently proteolytically activates the proinflammatory cytokines interleukin (IL)-1β and IL-18 (10). There are 22 NLR family members in humans (11), and some NLRs function as regulators of diverse immune responses independently of inflammasome assembly (12, 13). NLRC5 and class II major histocompatibility complex transactivator (CIITA), members of the NLRC clade, based on their N-terminal CARD domain, are two NLRs that shuttle to the nucleus and regulate class I and class II MHC gene expression, respectively (5–7). Since NLRC5 and CIITA are not expressed in trophoblasts, an EVT-specific transcriptional regulator of class I MHC expression is of great research interest.

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HLA-C is the most recently evolved HLA gene derived from the duplication of *HLA-B*, and it is assumed to have developed primarily as a ligand for the KIR2D family of receptors expressed by natural killer (NK) cells. Analyses of the *HLA-C* transcriptional start sites revealed a trophoblast-specific ETS/RFX binding site at the HLA-C promoter (14). We and others have shown that ELF3 can regulate HLA-C expression by binding to this promoter region (14, 15). However, it remains unclear whether other factors are involved in this process.

In this study, we find that ELF3 promotes HLA-C expression by up-regulating the expression of NLRP7, an understudied NLR family member uniquely expressed during early embryogenesis and in human EVT. By binding to a trophoblast-specific enhancer region of *NLRP7*, ELF3 drives *NLRP7* transcription while repressing the expression of the neighboring *NLRP2* gene which is transcribed in the opposite direction. *NLRP2* and *NLRP7* are the only two NLR genes highly expressed in trophoblasts. Moreover, we observed that NLRP2 and NLRP7 have opposite effects on HLA-C expression. Taken together, our findings reveal a unique mechanism of how ELF3 contributes to HLA-C expression by tuning the expression of two adjacent NLR genes.

Results

ELF3 Promotes HLA-C Expression in JEG-3. We previously reported that ELF3 contributes to the high-level HLA-C expression of trophoblasts through an autoregulatory feedback loop and direct binding to the HLA-C promoter region (15). To further validate the effect of ELF3 depletion on HLA-C expression, we generated ELF3 knockout (KO) JEG-3 cells by deleting the first coding exon of the *ELF3* gene (*SI Appendix*, Fig. S1*A*). Successful single-cell-derived knockout clones were validated by PCR and Western blot (Fig. 1*A* and *SI Appendix*, Fig. S1 *A* and *B*). While ELF3 knockout did not affect cell proliferation (*SI Appendix*, Fig. S1*C*), we observed a significant reduction of HLA-C levels in ELF3 knockout cells at both the transcriptional and protein level as detected by RT-qPCR (Fig. 1*B*) and flow cytometry (Fig. 1*C*).

Previous studies have uncovered a G/A single nucleotide polymorphism at an ETS/RFX binding site within the HLA-C proximal promoter, which provides a high-affinity binding site for ELF3 in trophoblasts rather than for RFX5 which binds to HLA-A and -B promoters (14, 16). Since RFX5 is expressed only at a low level in trophoblasts, we wondered whether RFX5 could substitute for ELF3 at the ETS/RFX site to regulate HLA-C expression. To this end, either ELF3 or RFX5 was overexpressed in ELF3 knockout JEG-3 cells and HLA-C expression was evaluated by flow cytometry. ELF3 but not RFX5 was able to restore HLA-C expression in ELF3$^{-/-}$ JEG-3 (Fig. 1*D*), indicating that ELF3, and not RFX5, is the dominant transcriptional regulator of HLA-C in trophoblasts.

ELF3 Tunes NLRP2 and NLRP7 Expression. To identify genes that are regulated by ELF3 in trophoblasts, we performed an RNAsequencing (RNA-seq) analysis on WT and ELF3 knockout JEG-3 cells (Fig. 2 *A*–*C*). Components of the "WNT signaling pathway" and "reproductive system development" were significantly enriched among the up-regulated genes by Gene Ontology analysis, while "negative regulation of viral processes" was enriched among the down-regulated genes (*SI Appendix*, Fig. S2 *A* and *B*). We also examined the effect of ELF3 depletion on the expression of all class I MHC genes. Similar to primary EVT, HLA-C, HLA-E, and HLA-G showed abundant expression in JEG-3, and HLA-C was remarkably decreased in the ELF3 knockout JEG-3 cells (Fig. 2*B*). Intriguingly, of the 552 significantly downregulated genes in the ELF3 knockout group (Padj < 0.05, |log2FoldChange| > 0.58), *NLRP7* was the most significantly down-regulated gene as illustrated in the volcano plot (Fig. 2*A*). *NLRP7* originated from a gene duplication of a *NLRP2* ancestor in primates (17, 18) and was also identified as the causative gene for recurrent hydatidiform moles (HM) and various forms of reproductive wastage (19–21).

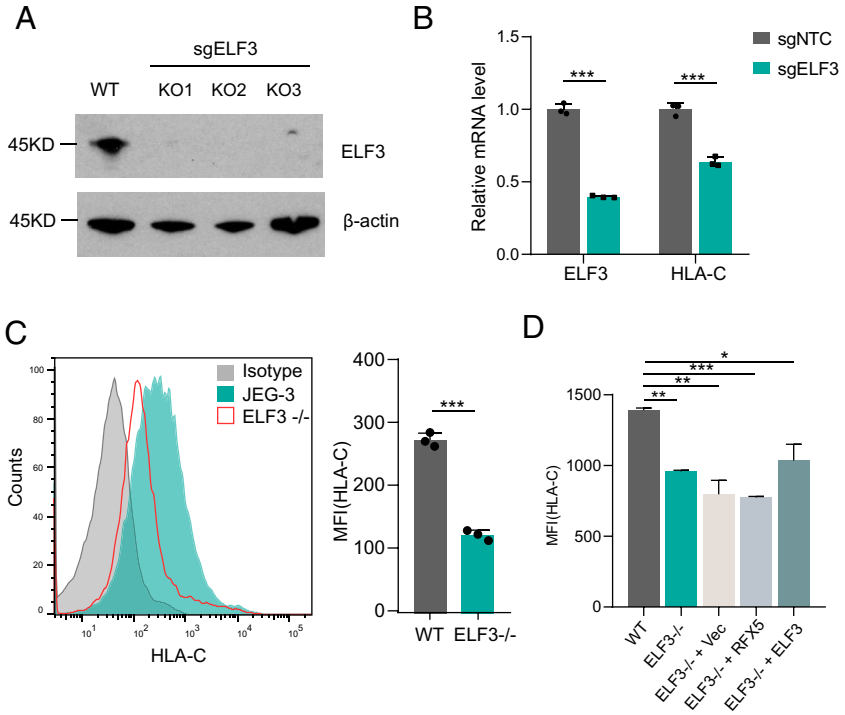

Fig. 1. Reduced HLA-C expression in ELF3-deficient JEG-3 cells. (*A*) Western blot of ELF3 protein expression in WT or ELF3$^{-/-}$ JEG-3 single cell–derived clones. β-actin was used as loading control. (*B*) Relative mRNA levels of ELF3 and HLA-C in cells targeted with short guide RNAs for ELF3 (sgELF3) or a nontargeting control sgRNA (sgNTC). (*C*) Representative flow cytometry data, histograms (*Left*) and HLA-C median fluorescence intensity (MFI, *Right*) of WT or ELF3$^{-/-}$ JEG-3. (*D*) HLA-C MFI of flow cytometry data from sgNTC, sgELF3, and rescue experiments including overexpressing of empty vector (Vec), RXF5, or ELF3 in JEG-3 cells treated with either nontargeting (sgNTC) or ELF3-specific sgRNAs (sgELF3).

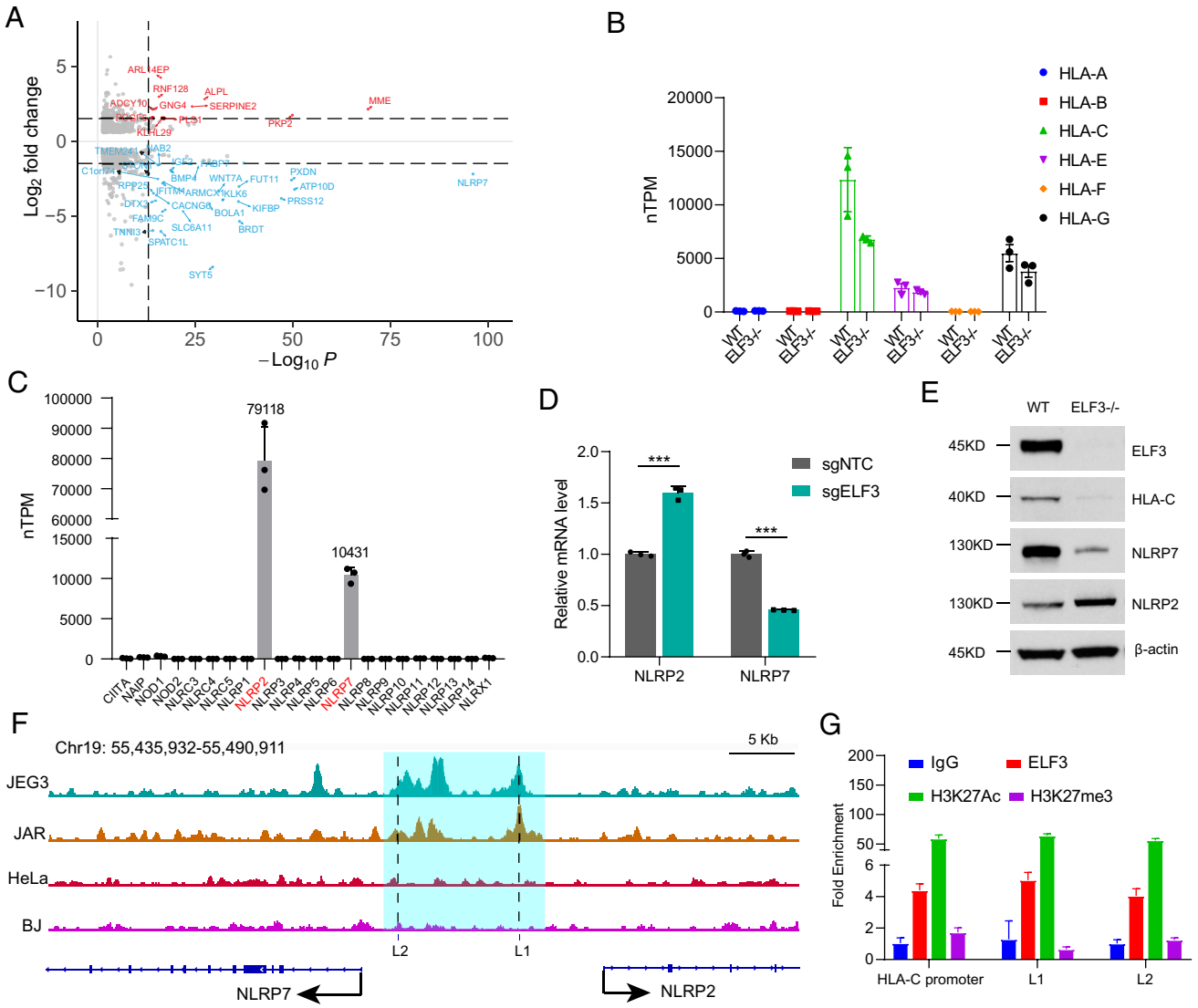

Fig. 2. ELF3 regulates NLRP2 and NLRP7 expressions by binding to their shared enhancer. (*A*) Volcano plot of ELF3$^{-/-}$ vs. WT JEG-3 RNA-seq data. (*B*) Normalized transcript per million (nTPM) of all MHC class I genes in WT or ELF3$^{-/-}$ JEG-3 (RNA-seq data). (*C*) nTPM of all 22 NLR family members in JEG-3 cells (RNA-seq data). (*D*) Relative mRNA expression of NLRP2 and NLRP7 in control or ELF3 knockout JEG-3. (*E*) Western blot analysis of ELF3, HLA-C, NLRP7, NLRP2, β-actin in WT, or ELF3$^{-/-}$ JEG-3. (*F*) Genome Browser view for ATAC-seq of JEG-3, JAR, HeLa, BJ fibroblasts at NLRP2, and NLRP7 genomic region. The shaded area highlights regions of active chromatin. (*G*) ChIP-qPCR of ELF3, H3K27Ac, H3K27me3, or IgG control at the HLA-C promoter, open chromatin locus 1 (L1), and open chromatin locus 2 (L2) at the NLRP2 and NLRP7 enhancer region.

According to our RNA-seq data, NLRP2 and NLRP7 are the only two NLR proteins expressed in JEG-3 cells above background levels (Fig. 2*C*). The genes encoding *NLRP2* and *NLRP7* are both located on chromosome 19 adjacent to each other yet are being transcribed in opposite directions. Next, we confirmed the expression levels of NLRP2 and NLRP7 in ELF3 knockout cells by RT-qPCR. NLRP7 expression decreased by half in the ELF3 knockout JEG-3, whereas, unexpectedly, NLRP2 expression increased by about 1.6-fold in the ELF3 knockout group (Fig. 2*D*). This trend was also observed by Western blot on the protein level by comparing WT and ELF3 knockout JEG-3 cells (Fig. 2*E*). Both HLA-C and NLRP7 protein levels were decreased in the ELF3 knockout group while NLRP2 protein levels were increased (Fig. 2*E*). The above observations suggest that ELF3 balances NLRP2 and NLRP7 transcription by repressing the former while promoting the expression of the latter.

ELF3 Regulates NLRP2 and NLRP7 Expression by Binding to Their Enhancer Region. To explore how ELF3 can impart such opposing effects on the expression of these two NLR genes, we first re-examined ATAC-seq (assay for transposase-accessible chromatin with high-throughput sequencing) data performed on trophoblast and somatic cell lines (15). Two trophoblast-specific open chromatin regions at around 5 Kb upstream of the NLRP2 and NLRP7 transcription start sites (TSS) were found (Fig. 2*F*). These two peaks are exclusively observed in JEG-3 and JAR trophoblast cells but not in the somatic cell line HeLa or in BJ fibroblasts (Fig. 2*F*). Similar open chromatin regions were observed by analyzing DNase-seq data (22) of the trophoblast cell lines JEG-3, BeWo, JAR, and BJ fibroblasts. As illustrated by DNase I hypersensitivity sites at the enhancer region of NLRP2 and NLRP7, again, trophoblast-specific open chromatin regions were found in JEG-3, BeWo, and JAR trophoblast cells but not in BJ fibroblasts (*SI Appendix*, Fig. S3*A*).

Motif analysis of the NLRP2/NLRP7 enhancer region identified two ETS-family transcription factor binding sites within the two open chromatin regions, named L1 and L2 thereafter (Fig. 2*F*). To investigate whether ELF3 regulates NLRP2 and NLRP7 gene expression by directly binding at these two loci, chromatin immunoprecipitation followed by qPCR analysis (ChIP-qPCR) was performed. Indeed, a fivefold enrichment and fourfold enrichment were detected at L1 and L2 loci, respectively, for ELF3 (Fig. 2*G*).

The proximal promoter region of HLA-C was included as the positive control for ELF3 binding. Acetylated histone H3 (Lys27) (H3KAc) and trimethylated histone H3 (Lys27) (H3K27me3) ChIP-qPCR for the same region were also included as open and condensed chromatin markers, respectively. As expected, the H3K27Ac mark showed a relatively high enrichment at all selected loci, while H3K27me3 was barely detected (Fig. 2*G*). These data imply that ELF3 regulates NLRP2 and NLRP7 expression directly by binding at the open chromatin region (enhancer region) about 5 kb upstream of the TSS of these two gene loci.

By reanalyzing another in vitro trophoblast differentiation study (23), we found that the transcriptional promoting marker H3K4me3 was found at the 5′ end of NLRP2 gene locus and was more enriched in trophoblast progenitors (represented as APA^+ cells) than undifferentiated stem cells (represented as $SSEA5^+$ cells) (*SI Appendix*, Fig. S3*B*). The transcriptional silencing marker H3K27me3 was absent at the NLRP2 and NLRP7 genomic regions in APA^+ trophoblast progenitors (*SI Appendix*, Fig. S3*B*). Interestingly, GATA3, a pivotal transcription factor during embryonic development, was found to be enriched at the enhancer region coinciding with the trophoblast-specific open chromatin region (*SI Appendix*, Fig. S3*B*). Those data suggest that besides ELF3, GATA3 might also be involved in regulating NLRP2 and NLRP7 expression during trophoblast differentiation. Moreover, H3K4me3 was specifically observed at the ELF3 promoter in APA^+ trophoblast progenitors, further supporting its role in trophoblast identity determination (*SI Appendix*, Fig. S3*C*).

NLRP7 Promotes HLA-C Expression in JEG-3 Cells. NLRP2 was reported as a suppressor of HLA-C expression in trophoblasts by modulating the NF-κB pathway (24). In line with this previous study, we observed increased HLA-C expression after knocking down NLRP2 in JEG-3 cells (*SI Appendix*, Fig. S4*A*). To examine whether NLRP7 can also impact HLA-C expression, we generated a NLRP7 knockout JEG-3 cell line using CRISPR-Cas9 and validated the KO by qRT-PCR and Western blot (*SI Appendix*, Fig. S4 *B–D*). NLRP7 knockout did not significantly increase cell proliferation of JEG-3 (*SI Appendix*, Fig. S4*E*). Interestingly, we found that HLA-C expression was decreased upon NLRP7 knockout on mRNA, protein, and cell surface protein levels, respectively (Fig. 3 *A–C*). In contrast, the expression levels of HLA-E and HLA-G were not affected by NLRP7 knockout (*SI Appendix*, Fig. S4 *F* and *G*). These data suggest that NLRP7—unlike NLRP2, which acts as a negative regulator—may act as an activator of HLA-C expression in EVT.

To further investigate the role of NLRP7 in promoting HLA-C expression, we overexpressed NLRP7 in JEG-3 cells. HLA-C levels were indeed up-regulated upon NLRP7 overexpression after 48 h (Fig. 3*D*). However, the expression levels of other HLA proteins found in trophoblasts, such as HLA-E and HLA-G, did not show an obvious increase after overexpressing NLRP7 (*SI Appendix*, Fig. S4 *H* and *I*). Interestingly, overexpression of NLRP7 in HEK293T cells did not impact HLA-C levels (Fig. 3*E*), suggesting a trophoblast-specific effect of NLRP7 on HLA-C expression. The above results further support the notion that NLRP7, in contrast to its ancestral paralogue NLRP2, is able to specifically promote HLA-C expression in trophoblasts.

Cytosolic NLRP7 Promotes NF-κB Signaling. The MHC class I transcriptional activator NLRC5 shuttles to the nucleus and promotes MHC class I gene expression in somatic cells (7, 25). To investigate whether NLRP7 can also enter the nucleus and thus directly contribute to HLA-C expression by forming an enhanceosome, we expressed a NLRP7–GFP fusion construct in JEG-3 cell. In

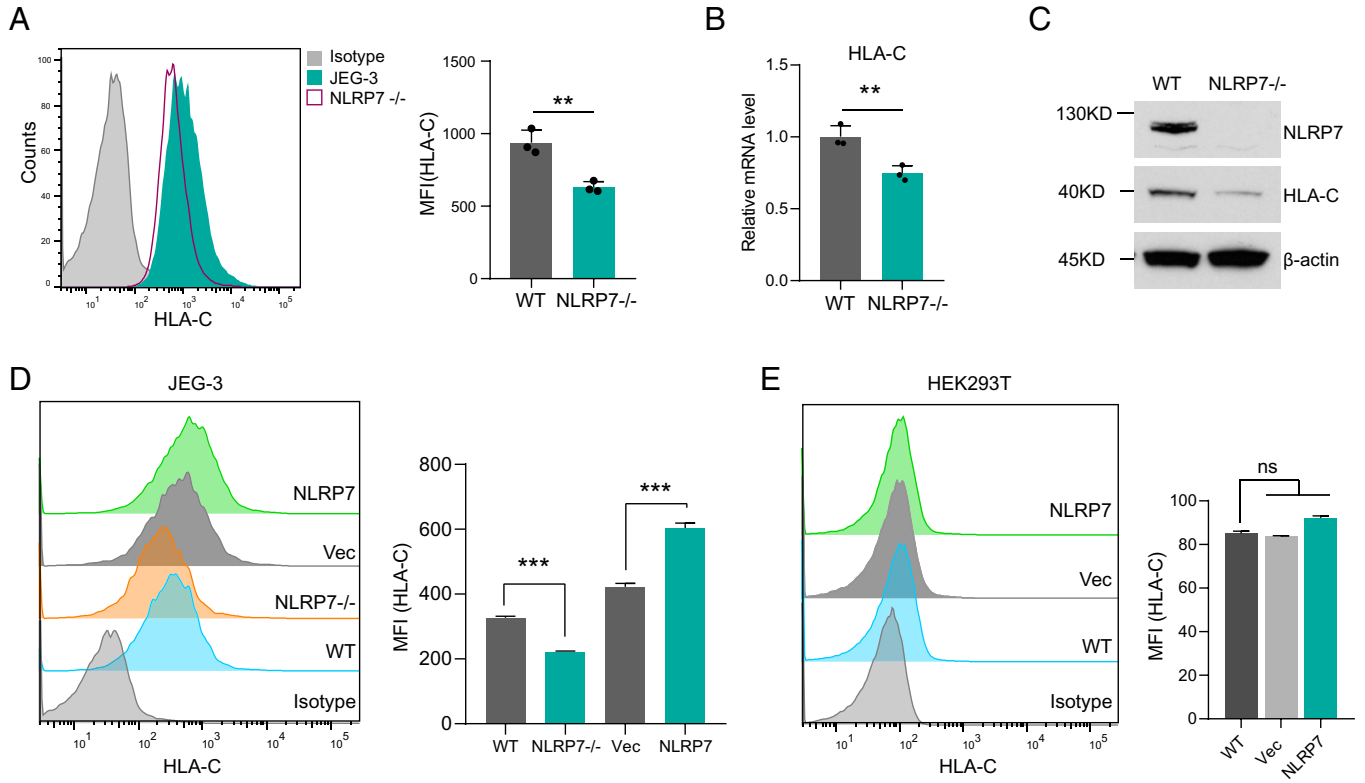

Fig. 3. NLRP7 promotes HLA-C expression in JEG-3. (*A*) Representative flow cytometry histograms (*Left*) and HLA-C MFI (*Right*) in WT or $NLRP7^{-/-}$ JEG-3 cells. (*B*) Relative mRNA levels of HLA-C in WT or $NLRP7^{-/-}$ JEG-3 cells. (*C*) Western blot analysis of NLRP7, HLA-C, β-actin in WT or $NLRP7^{-/-}$ cells. (*D*) Representative flow cytometry histograms (*Left*) and HLA-C MFI (*Right*) in WT, $NLRP7^{-/-}$, and JEG-3 transfected with either empty vector (Vec), or NLRP7. (*E*) Representative flow cytometry histograms (*Left*) and HLA-C MFI (*Right*) of HEK293T cells; untransfected (WT) or transfected with empty vector (Vec), or a NLRP7 overexpression plasmid.

agreement with other reports (26, 27), we found that NLRP7 is exclusively located in the cytosol under steady-state conditions. Moreover, blocking nuclear export with Leptomycin B did not result in a nuclear accumulation of NLRP7 (Fig. 4*A*). A similar cytosolic subcellular localization was observed by either looking at the endogenous distribution of NLRP7 by immunocytochemistry or by expressing a N-terminal GFP fusion NLRP7 in JEG-3 cells (*SI Appendix*, Fig. S5 *A* and *B*). Those results suggest that NLRP7 may not be involved in a nuclear MHC enhanceosome but rather function in the cytosol. Given that JEG-3 cells do not express the critical adapter protein ASC and Caspase-1 (28), two main components of the inflammasome, it is more likely that NLRP7 might function via an inflammasome-independent pathway.

To obtain insight as to how NLRP7 affects HLA-C expression, we performed RNA-seq analysis of NLRP7 knockout JEG3 cells and compared their global RNA levels to that of WT JEG-3. Differential gene expression analysis identified 767 genes that were significantly differentially expressed (*P*adj < 0.05, |log2FoldChange| > 0.58). "Ameboidal-type cell migration" and "response to peptide hormone" pathways were most significantly enriched among the down-regulated genes by Gene Ontology analysis, while genes involved in histone modification, and nuclear division were mostly enriched among the up-regulated genes (Fig. 4*B*). The EVT identity was barely changed as indicated by similar expression levels of a set of EVT markers (Fig. 4*C*) (29). Significant up- or down-regulated genes upon NLRP7 knockout are shown in a Volcano blot (Fig. 4*D*). Further gene set enrichment analysis (GSEA) revealed that NLRP7 predominantly affects genes implicated in TNF-α signaling via the NF-κB pathway, such as NR4A1, NR4A3, IL15RA, and FJX1 (Fig. 4*E*). NLRP2 has previously been shown to inhibit serine phosphorylation of the p65 subunit of NF-κB (Ser536) upon TNF-α stimulation (24, 30). To examine whether NLRP7 may act on the same targets, we stimulated WT or NLRP7$^{-/-}$ JEG-3 cells with TNF-α (20 ng/mL) and analyzed components of the NF-κB signaling pathway by Western blot at different time points. In agreement with our hypothesis that

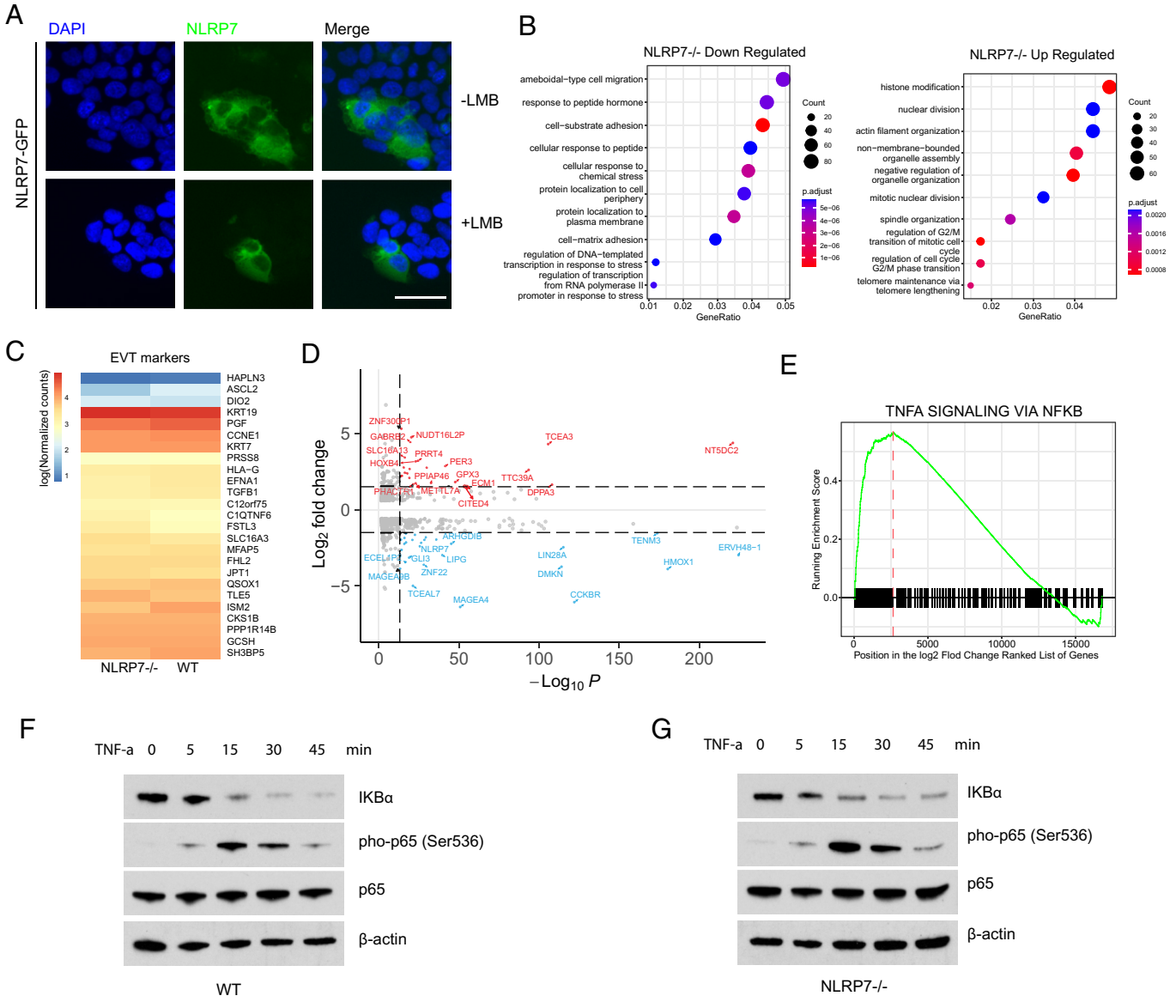

Fig. 4. NLRP7 regulates HLA-C via NF-κB signaling. (*A*) Cytosolic localization of NLRP7 as determined by overexpressing a NLRP7-GFP fusion protein in the presence or absence of Leptomycin B. (Scale bar, 20 μm.) (*B*) Gene Ontology analysis of up (*Right*) or downregulated (*Left*) genes in NLRP7$^{-/-}$ JEG-3 cells. (*C*) Heatmap of EVT marker gene expression obtained by RNA-seq of WT or NLRP7$^{-/-}$ JEG-3 cells. (*D*) Volcano plot of NLRP7$^{-/-}$ vs. WT JEG-3 RNA-seq data. (*E*) GSEA plot of the most significant pathways enriched by the significantly differentially expressed genes (DEGs) in NLRP7$^{-/-}$ cells. (*F* and *G*) Western bot of IκBα, NF-κB p65 Ser536 phosphorylation, NF-κB p65 in WT (*F*), and NLRP7$^{-/-}$ (*G*) JEG-3 cells stimulated with TNF-α (20 ng/mL) at indicated time points. β-actin was used as loading control.

NLRP7 acts as a positive regulator of HLA-C expression through the NF-kB signaling pathway, we observed an attenuated degradation of the inhibitor of NF-κB protein IκBα in NLRP7$^{-/-}$ compared to WT cells. In contrast, NF-κB p65 Ser536 phosphorylation was barely changed in the NLRP7 knockout cells (Fig. 4 *F* and *G*) and also NF-κB p65 levels did not change noticeably upon TNF-α stimulation of the NLRP7 knockout when compared to WT JEG-3 (Fig. 4 *F* and *G*). Those results suggest that NLRP7 up-regulates HLA-C expression via the canonical NF-κB signaling pathway by promoting IκBα degradation.

NLRP2 and NLRP7 Are Expressed during Embryogenesis and First-Trimester Pregnancy. To obtain a more detailed view of the regulatory network comprising ELF3, NLRP7, and NLRP2 during early human development, publicly available RNA-seq datasets generated from preimplantation embryos, human embryonic stem cell lines (31), and single-cell transcriptomics of the maternal–fetal interface from first-trimester pregnancy were analyzed (29). ELF3 is highly expressed in trophectoderm (TE) during early embryogenesis (Fig. 5*A*). NLRP2 expression was observed as early as the two-cell stage and showed the highest expression level in TE. NLRP7 transcript levels also peaked in TE, coinciding with ELF3 expression (Fig. 5*B*). Both ELF3 and NLRP2 were expressed in EVT and syncytiotrophoblast (SCT) cells, yet negatively correlated, according to the first trimester single-cell RNA-seq data (Fig. 5 *C*–*E*), further supporting the notion that NLRP2 is suppressed by ELF3. Interestingly, ELF3 expression was also observed in epiblasts other than the trophoblast populations (Fig. 5 *C* and *D*). In contrast, NLRP7 was only detected at a low level in those three types of trophoblasts (Fig. 5 *D* and *E*), most likely due to the low sensitivity, a known technical limitation, of single-cell RNA-seq data, given that NLRP7 protein was detected in human placenta during the early stages of first-trimester pregnancy (32). Thus, during normal embryogenesis, the expression levels of NLRP2 and NLRP7 are elaborately balanced by ELF3, to maintain an adequate HLA-C level in trophoblasts. In conclusion, our data suggest that beyond ELF3 regulating HLA-C expression directly, ELF3 also indirectly impacts HLA-C

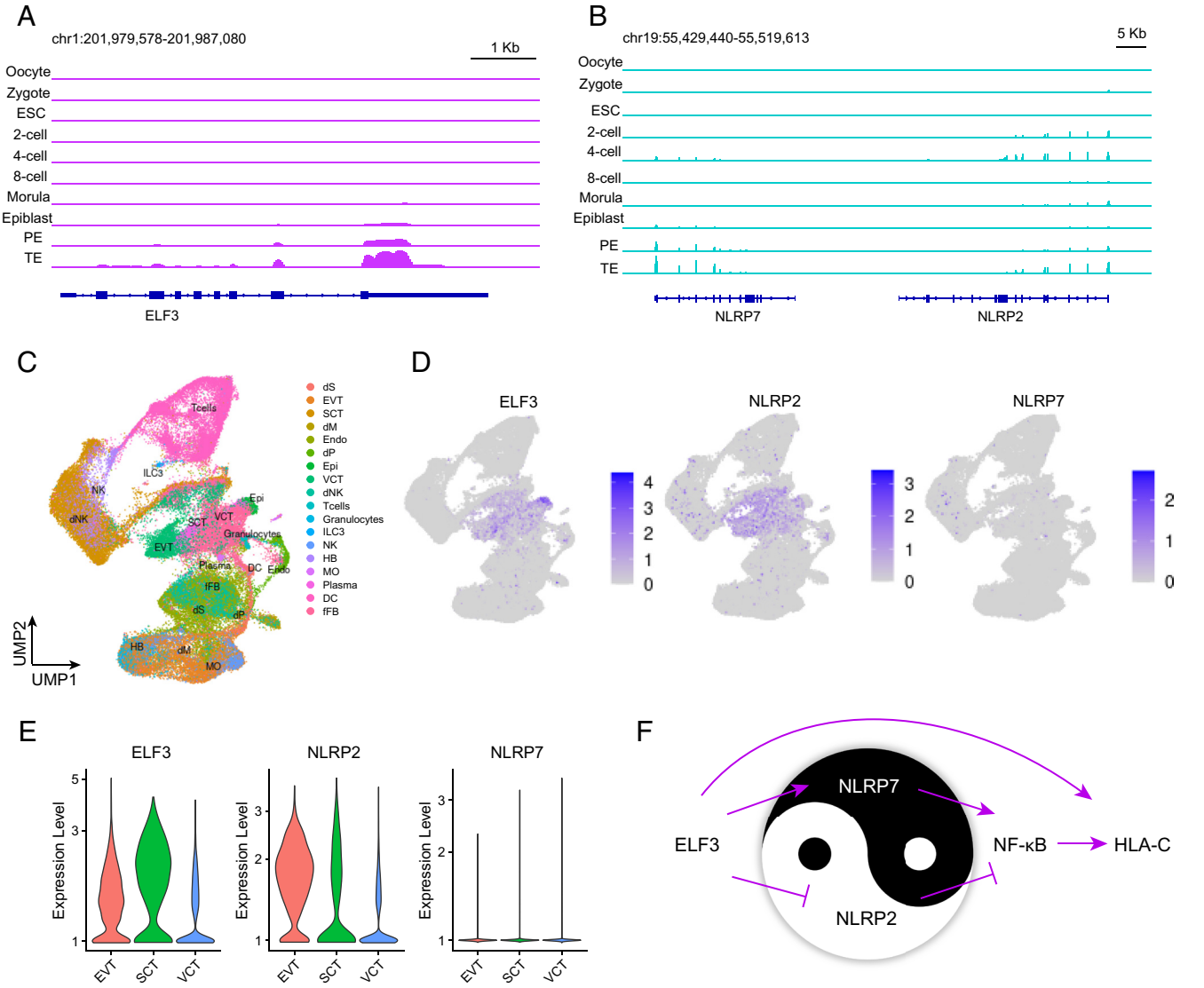

Fig. 5. ELF3, NLRP2, and NLRP7 expression during early human development and first trimester of pregnancy. (*A* and *B*) Transcriptional levels of ELF3, NLRP2, and NLRP7 at distinct stages of early embryogenesis as determined by RNA-seq (31). (*C*) Dimensional reduction plot of human maternal–fetal interface scRNA-seq dataset with cluster annotations (29). (*D*) Visualization of ELF3, NLRP2, and NLRP7 expression by dimensional reduction plot. TE, trophectoderm; PE, primitive endoderm EPI, epiblast; ESC, embryonic stem cell. (*E*) Expression of ELF3, NLRP2, and NLRP7 in EVT, SCT, and villous cytotrophoblast trophoblast subsets of first-trimester pregnancy single-cell RNA-seq dataset (29). (*F*) Working model of how HLA-C expression is regulated by the ELF3–NLRP2/NLRP7 axis.

expression through its antagonistic impact on NLRP2 and NLRP7 expression in EVT (Fig. 5*F*).

Discussion

As the only highly polymorphic HLA protein expressed in human trophoblasts, HLA-C plays a central role in both tolerance and immunity at the maternal–fetal interface by communicating with maternal NK and T cells (33, 34). This dual function of HLA-C requires tight transcriptional regulation, however, despite comprehensive knowledge of MHC class I transcriptional regulation in somatic cells, the distinct regulatory mechanisms in trophoblast are still poorly understood. In this study, we uncover that two members of the NLR family of cytosolic pathogen recognition receptors, NLRP2 and NLRP7, contribute to HLA-C expression in trophoblast with opposing effects. Expression of these two NLR genes was regulated by ELF3, a transcription factor highly expressed in trophoblasts.

Our findings are in agreement with previous studies that have shown ELF3 to be up-regulated in particular in invading trophoblasts (35). Moreover, ELF3 was shown to transactivate HLA-C expression directly by binding to its promoter (14, 15). In addition, ELF3 was reported to interact with p300 and CBP (36), providing a complementary mechanism to recruit the CBP/p300 transcriptional coactivators required for chromatin remodeling and activation of HLA-C expression in trophoblasts. In this study, we identified two unique binding sites for ELF3 in the NLRP2 and NLRP7 enhancer and unveiled NLRP7 promotes HLA-C expression in trophoblast. This surprising additional layer of regulation may have evolved to allow the integration of proinflammatory inputs that feed into HLA-C expression. By finetuning NF-κB activity through its action on the two antagonistic NLR proteins, NLRP2 and NLRP7, ELF3 may thus act as a rheostat adjusting appropriate HLA-C levels to the level of inflammation in the underlying tissue (Fig. 5*F*). As we did not observe an upregulation of HLA-C in the somatic cell line HEK293T, it would be intriguing to investigate whether there are other trophoblast-specific factors required assisting ELF3 in regulating NLRP2 and NLRP7, resulting in distinct transcriptional outcomes.

The NLRP subclade of NLR proteins is characterized by an N-terminal pyrin protein interaction domain. NLRP proteins have been shown to play pivotal roles in both the mammalian innate immune response as well as the reproductive system. Beyond their function as the core components of inflammasomes with important roles in innate immunity (37), a subset of NLRP genes is expressed in oocytes or early embryos as maternal effect genes during early mammalian development (38, 39). Human NLRP2 and NLRP7 have been shown to be maternal effect genes, regulating early embryonic development and establishment of maternal imprinting (40, 41). Taxonomic analyses revealed that the human NLRP7 gene may have originally evolved in Simiformes (i.e., Simians) nearly 43 million B.P. and may have acquired a divergent function in Homo sapiens (42). Further phylogenetic analyses showed that NLRP7 originated from a gene duplication of the NLRP2/7 ancestor in primates (17). Here, we report an opposing function of NLRP2 and NLRP7 in regulating the expression level of HLA-C in EVT, presumably through differentially modulating the degradation kinetics of IkBα in the cytosol. Sequence alignment of NLRP2 and NLRP7 revealed that the disordered regions following the Pyrin domain and LRR6 are not conserved in NLRP7 (*SI Appendix*, Fig. S6). Those elements might contribute to the functional differences between the two proteins.

It is well documented that NLRP7 plays a critical role in placental development, particularly in trophoblasts, which enable the supply of essential nutrients and oxygen to the embryo. NLRP7 was implicated in the regulation of trophoblast proliferation, differentiation, and invasion and was shown to modulate the inflammatory response in the placenta (43–45). Dysregulation of NLRP7 has been associated with several pregnancy-related disorders, including recurrent HM and fetal growth restriction (19–21, 32). Moreover, NLRP7 has been reported to contribute to choriocarcinoma tumorigenesis in an inflammasome-independent manner (28, 46). Here, we revealed that NLRP7 and NLRP2 expression are balanced by ELF3 in EVT, and we describe an unexpected role for NLRP7 in regulating high-level HLA-C expression level in EVT. Our findings implicate NLRP7 in promoting choriocarcinoma tumorigenesis and immune evasion by up-regulating HLA-C levels in EVT. Therefore, targeting NLRP7 could potentially benefit the above-mentioned pregnancy complications.

Materials and Methods

Details of cell culture and transfection, sequencing and cloning, knockout cell line construction, flow cytometry, RNA extraction and qPCR quantification, immunofluorescence staining and imaging, Western blot, bulk RNA-seq, ChIP-qPCR, cell clustering and visualization of single-cell RNA-seq data, and quantification and statistical analysis are given in *SI Appendix*.

Data, Materials, and Software Availability. RNA-seq data generated in this study are available under the GEO accession number GSE241792 (47). The ATAC-seq data from trophoblast or somatic cell lines were retrieved from the GEO database (accession no. GSE165511) (48). Single-cell RNA-seq data from early human embryonic development and placenta were retrieved from E-MTAB-6701 (49) and E-MTAB-6678 (50). The antibodies and oligonucleotides used in this study can be found in *SI Appendix*, Tables S1 and S2.

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