SUPPLEMENTARY INFORMATION

Title: Hippo Signaling Cofactor, WWTR1, at the Crossroads of Human Trophoblast Progenitor Self-Renewal and Differentiation.

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Supplemental Information Inventory:

Items	Related to	General Description
Supplementary Experimental Procedures with References		Describes experimental procedures and lists oligonucleotides and antibodies that are used for the study.
Supplementary Figure Legends		
Figure S1	**Related to Figure 1**	
Figure S2	**Related to Figure 1**	
Figure S3	**Related to Figure 1**	
Figure S4	**Related to Figure 2**	
Figure S5	**Related to Figures 3,4,5**	
Figure S6	**Related to Figure 4**	
Figure S7	**Related to Figures 6**	
Figure S8	**Related to Figure 7**	
Figure S9	**Related to Figure 7**	
Figure S10	**Related to Figure 7**	
Dataset S1	**Related to Figure 3,4,5,6**	Gene Expression changes in *WWTR1*-KD human TSCs.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Bioinformatics Analyses of RNA-Seq data: Quality assessment has been done using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reads were aligned using the subread-2.0.3 (1), after that reads count file were obtained using featureCounts (2) of subread-2.0.3. After that differential expression was obtained using DESeq2 (3). The mean insert length and standard deviation calculated using picard 2.26.6 (https://broadinstitute.github.io/picard/). All the analysis has been done using UCSC hg38 from iGenomes illumine as reference data. The heatmap was generated using screening top 100 genes by using "genefilter" library followed by pheatmap v1.0.12. MA-plot were generated using ggmaplot function of ggpubr v0.4.0 (https://cran.r-project.org/web/packages/ggpubr/index.html) using the parameters fdr = 0.05, fc = 2. The volcano plot was done using ggplot2 v3.3.5 (https://ggplot2.tidyverse.org/) where intercept were provided at log2fold values at (-1,1) to get the better view. GSEA analysis was performed using GSEA_Linux_4.2.2 (4) after preparing the two files with extension '.gct' and '.cls' generated as per prescribed guideline of tool. Gene sets c5.go.mf.v7.5.1.symbols.gmt was selected for analyses for GO molecular Function, while c2.cp.v7.5.1.symbols.gmt was selected for analysis for Canonical Pathways.

Organoid generation from human trophoblast stem cells and cultivation: Human TSCs and first-trimester CTBs were used to generate the self-renewing organoids. For CTB organoid, we followed earlier described protocols (5, 6). To obtain organoids from human TSCs (control and *WWTR1*-KD human TSCs) we modified the protocol described previously (5). Human TSCs were harvested and re-suspended in ice-cold basic trophoblast organoid medium (b-TOM) containing advanced DMEM/F12 supplemented with 10mM HEPES (Sigma H3537), B27 (Gibco 17504-044), N2 (Gibco 17502-048) and 2mM glutamine (Gibco 25030081). The cells were then centrifuged for 3 minutes at 1200rpm following which the cells were re-suspended in ice-cold advanced trophoblast organoid medium (aTOM) which is b-TOM supplemented with 100ng/ml R-spondin (PeproTech 120-38-20UG), 1μM A83-01 (Sigma SML0788), 100ng/ml recombinant human epidermal growth factor (rhEGF, Sigma E9644), 50ng/ml recombinant murine hepatocyte growth factor (rmHGF, PeproTech 315-23-20UG), 2.5μM prostaglandin E2 (R&D System 2296/10), 3μM CHIR99021 (Sigma SML1046) and 100ng/ml Noggin (Invitrogen PHC 1506). Growth factor reduced matrigel (Corning) was added to the a-TOM cell suspension to reach a final concentration of 60%. 35μl of the viscous cell solution containing $2.5x10^4$ cells was plated in the center of a 24-well plate. The solution rests as a dome-shaped droplet in the center

of the well. The plates are then turned upside down and kept at 37°C for 10-15 minutes to ensure proper spreading of the cells in the solidifying matrigel domes. Finally, the plates are returned to their upward position and the domes are overlaid with 500µl of room temperature a-TOM medium. The organoids are allowed to form for 8-10 days with fresh media being changed every 2 days. Brightfield images were taken to observe the growth of the organoids.

Cell Proliferation Assay: Proliferations of control and *WWTR1*-KD human TSCs were determined via two different approaches. In the first approac**h,** $2x10^5$ number of cells were seeded on a 6 well plate and cell counts were taken for control and WWTR1-KD human TSCs at 24, 48, and 72 hours. Cells were imaged and compared using a brightfield microscope (Olympus IX71, Japan). In the second approach, 1.5x104 number of cells were seeded on a 12 well plate and the cell proliferation assay was performed by using the 5-Bromo-2′ deoxy-uridine labeling and detection kit I (Roche 12296736001) (according to the manufacturer's manual). The cells were cultured for 24, 48, 72 hours. Hoechst 33342 (5 µg/ml) prepared in slow fade (Invitrogen) was used to counter-stain the nuclei. The BrdU-positive cells were observed and calculated using a fluorescent microscope (Nicon Eclipse 80i).

Western blot analyses: Protein lysates and supernatants of cell cultures were separated on SDS/PAA gels, transferred to Hybond-P PVDF membranes (Amersham 10600023) and incubated with antibodies following an earlier described protocol (7).

Antibodies used for the Immunostaining and Western blot analyses: Following antibodies were used for the study (i) WWTR1 (Cell signaling E8E96), (ii) TEAD4 (Abcam ab56310), (iii) WNT7a, ab100792 (Abcam), (iv) P40 (Delta TP63), ACI3066A (BioCare Medical, CA), (v) CGB (hCGβ), ab53087 (Abcam), (vi) CDH1, ab1416 (Abcam), (vii) β-Actin, A5441 (Sigma, St.Louis, MO) and (viii) GAPDH: Cell Signaling 2118.

Chromatin Immuno-precipitation (ChIP) assay: Quantitative ChIP analyses were performed to determine WWTR1 occupancy at selected gene loci in Human TSCs. We followed a published protocol (8). Cells were crosslinked with 1% formaldehyde (Sigma) for 10 mins at room temperature with gentle rotation. Chromatin crosslinking was stopped with glycine (125mM). These samples were sonicated. Chromatin fragments were immunoprecipitated with 8 microgram of WWTR1 antibody (HPA007415 Sigma) per ChIP experiment.

Table S1: Oligonucleotides used for Real-Time PCR analysis

Primer Name	Forward	Reverse
TP63	GTCATTTGATTCGAGTAGAGG GG	CTGGGGTGGCTCATAAGG T
CGB	GTGTGCATCACCGTCAACAC	GGTAGTTGCACACCACCTGA
CGA	TCTGGTCACATTGTCGGTGT	TTCCTGTAGCGTGCATTCTG
PSG4	CGATGGGACTGGAGGAGTAA	AGTTGCTGCTGGAGATGGAG
ERVFRD-1	CCAAATTCCCTCCTCTCCTC	CGGGTGTTAGTTTGCTTGGT
MMP2	TCTCCTGACATTGACCTTGGC	CAGGGTGCTGGCTGAGTAGATC
Wnt7a	CTGTGGCTGCGACAAAGAGAA	GCCGTGGCACTTACATTCC
Wnt3	AGGGCACCTCCACCATTTG	GACACTAACACGCCGAAGTCA
Wnt4	GTACGCCATCTCTTCGGCAG	GCGATGTTGTCAGAGCATCCT
WWTR1	TCCCAGCCAAATCTCGTGATG	AGCGCATTGGGCATACTCAT
HPRT1	ACCCTTTCCAAATCCTCAGC	GTTATGGCGACCCGCAG
MMP2	TCTCCTGACATTGACCTTGGC	CAGGGTGCTGGCTGAGTAGATC
MMP11	CCGCAACCGACAGAAGAGG	ATCGTCCCATACCTTTAGGGC
MMP14	CGAGGTGCCCTATGCCTA	CTCGGCAGAGTCAAAGTGG
MMP15	AGGTCCATGCCGAGAACTG	GTCTCTTCGTCGAGCACACC
WWTR1	TCCCAGCCAAATCTCGTGATG	AGCGCATTGGGCATACTCAT
HPRT1	ACCCTTTCCAAATCCTCAGC	GTTATGGCGACCCGCAG
PLAC8	TGCTAGAAAAAGCCATGGAA	TCTGCCAGAGGGTCTTTAGG
YAP1	TCCACCAGTGCAGCAGAATA	TTCCCATCCATCAGGAAGAG
SMAD3	TGAGGCTTATTAAGTCCATTGC	TCATCATTTGTCATACTGCACAC

References mentioned in the Supplementary Methods:

1. Liao Y, Smyth GK, & Shi W (2013) The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* 41(10):e108.

2. Liao Y, Smyth GK, & Shi W (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30(7):923-930.

3. Love MI, Huber W, & Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15(12):550.

4. Subramanian A*, et al.* (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* 102(43):15545-15550.

5. Haider S*, et al.* (2018) Self-Renewing Trophoblast Organoids Recapitulate the Developmental Program of the Early Human Placenta. *Stem cell reports*.

6. Turco MY*, et al.* (2018) Trophoblast organoids as a model for maternal-fetal interactions during human placentation. *Nature* 564(7735):263-267.

7. Saha B*, et al.* (2013) EED and KDM6B coordinate the first mammalian cell lineage commitment to ensure embryo implantation. *Molecular and cellular biology* 33(14):2691-2705.

8. Home P*, et al.* (2012) Altered subcellular localization of transcription factor TEAD4 regulates first mammalian cell lineage commitment. *Proceedings of the National Academy of Sciences of the United States of America* 109(19):7362-7367.

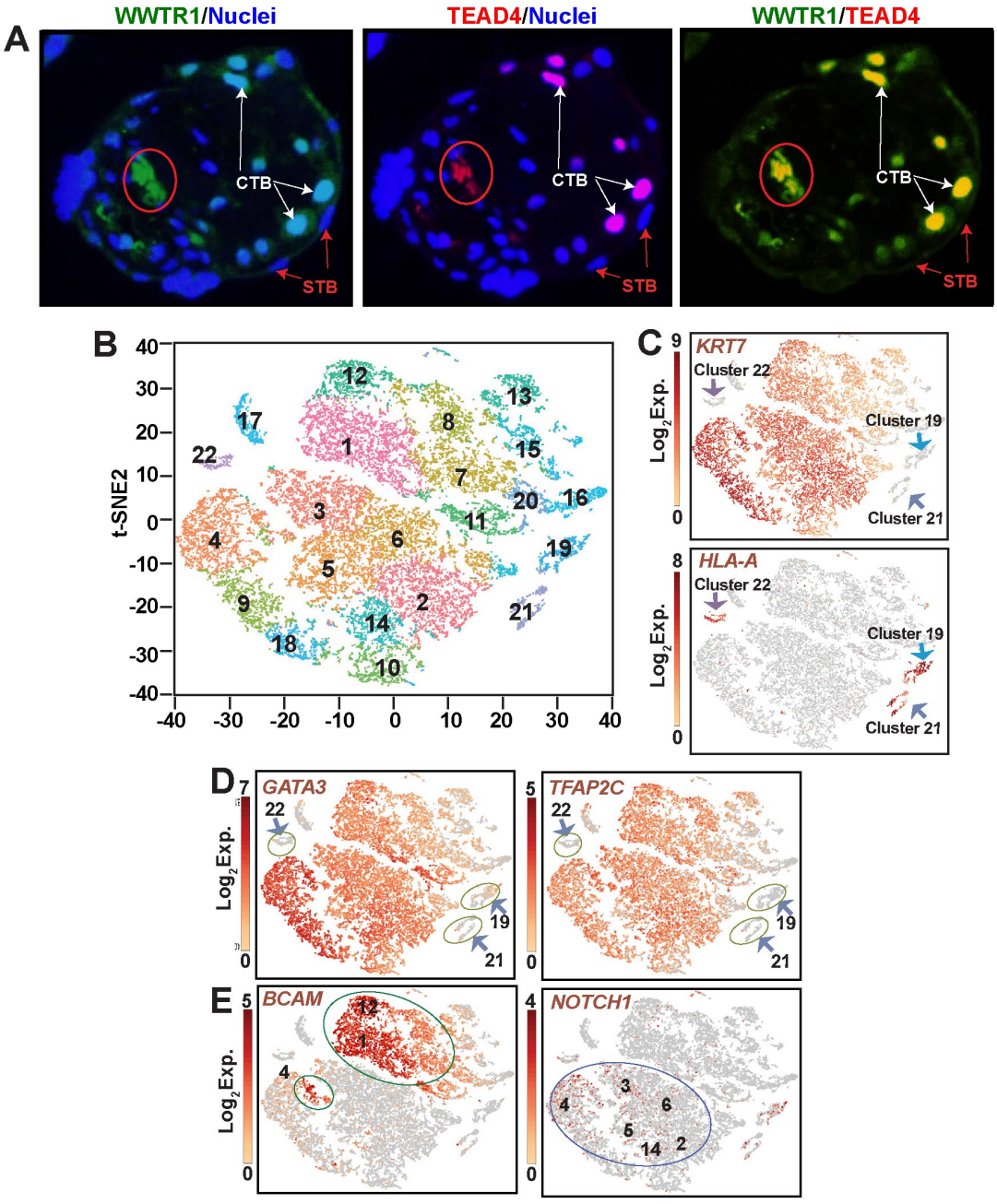

Fig. S1. (A) Term human placental sections were immunostained with antibodies against WWTR1 and TEAD4. Images show that both WWTR1 and TEAD4 are expressed in CTBs. In contrast STBs do not express either of these molecules. The red circle indicates blood cells which often show autofluorescence. (B) The t-SNE plot of the aggregate of the hierarchical clustering of 2 first-trimester human placental samples (one Week 7 and one Week 8) identified 22 different cell clusters. (C) Clusters of trophoblast and non-trophoblast cells are labeled by KRT7 and HLA-A expressions, respectively, on a t-SNE plot. Lack of KRT7 expression and induction of HLA-A expression identified clusters 19, 21 and 22 as non-trophoblast cells. (D) t-SNE plots show almost all cells within trophoblast cell clusters express GATA3 and TFAP2C, which are known to be expressed in all mononuclear trophoblast cell within a first-trimester human placenta. (E) t-SNE plots show expression patterns of BCAM and NOTCH1, which are predominantly expressed in stem-state CTBs and proximal column CTBs, respectively.

Fig. S2

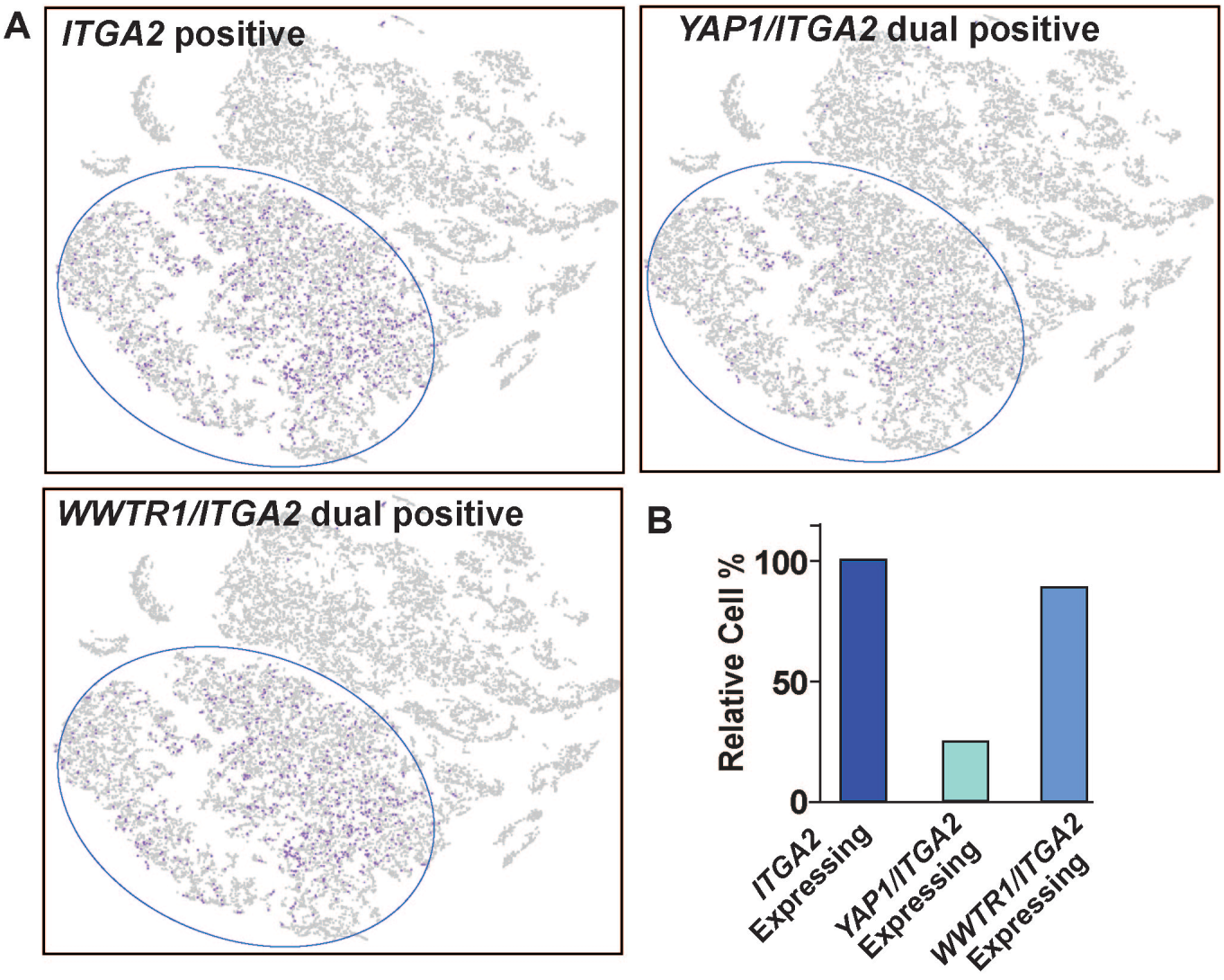

Fig. S2. WWTR1 and YAP1 expression in ITGA2 positive CTB progenitors. (A) and (B) *WWTR1* and *YAP1* expression was monitored and quantitated in *ITGA2*-expressing column CTB progenitors of a first-trimester human placenta via scRNA-seq analyses. Each blue dot represents CTB progenitors which are either *ITGA2* positive (A, upper left panel) and are also expressing *YAP1* (A, upper right panel) or *WWTR1* (A, Lower panel). Note that almost all *ITGA2*-expressing CTB progenitors also express *WWTR1*. However, only a fraction of *ITGA2* positive cells within a first-trimester placenta also express *YAP1*.

Fig. S3

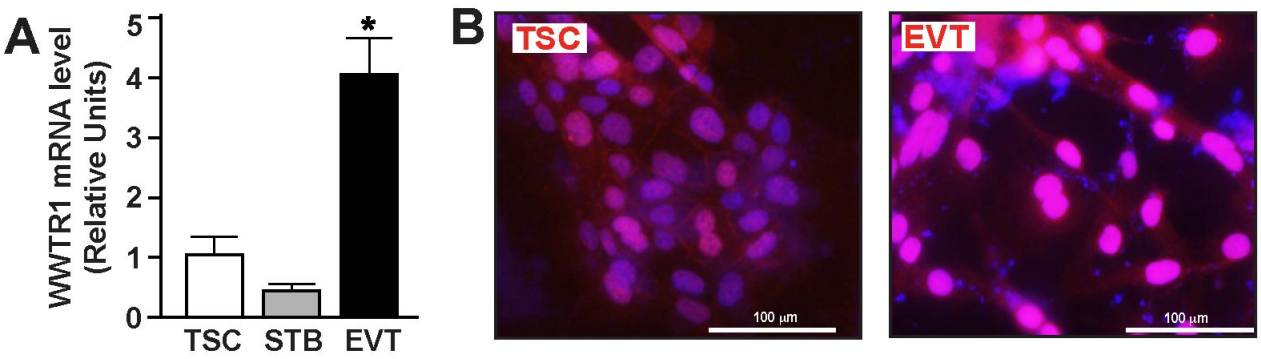

Fig. S3. WWTR1 expression in human TSCs. (A) RT-qPCR analyses showing relative expressions of *WWTR1* mRNA in undifferentiated human TSCs and after STB and EVT differentiation. (B) Immunofluorescence images show nuclear localization of WWTR1 in human TSCs when maintained at stem-state and after EVT differentiation.

Fig. S4

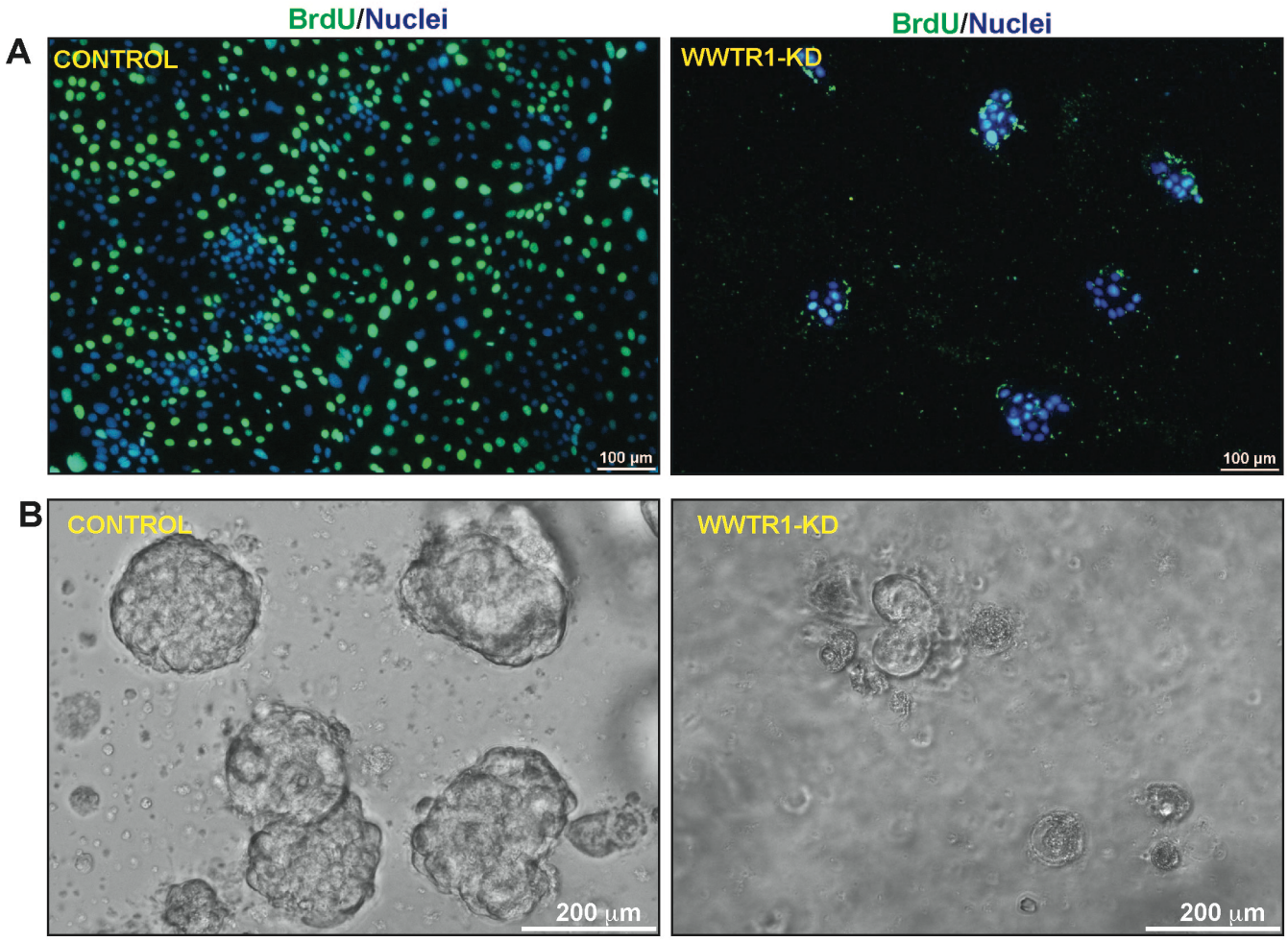

Fig. S4. Loss of WWTR1 expression affects human TSC self-renewal ability. (A) Fluorescence images showing BrdU incorporation in control and ***WWTR1***-KD human TSCs after 72 h culture in TSC medium. (B) Micrographs show passage 2 orgnoids with control and ***WWTR1***-KD human TSCs. Self-renewing organoids readily formed with control TSCs. In contrast, ***WWTR1***-KD TSCs could not maintain organoid structures.

Fig. S5

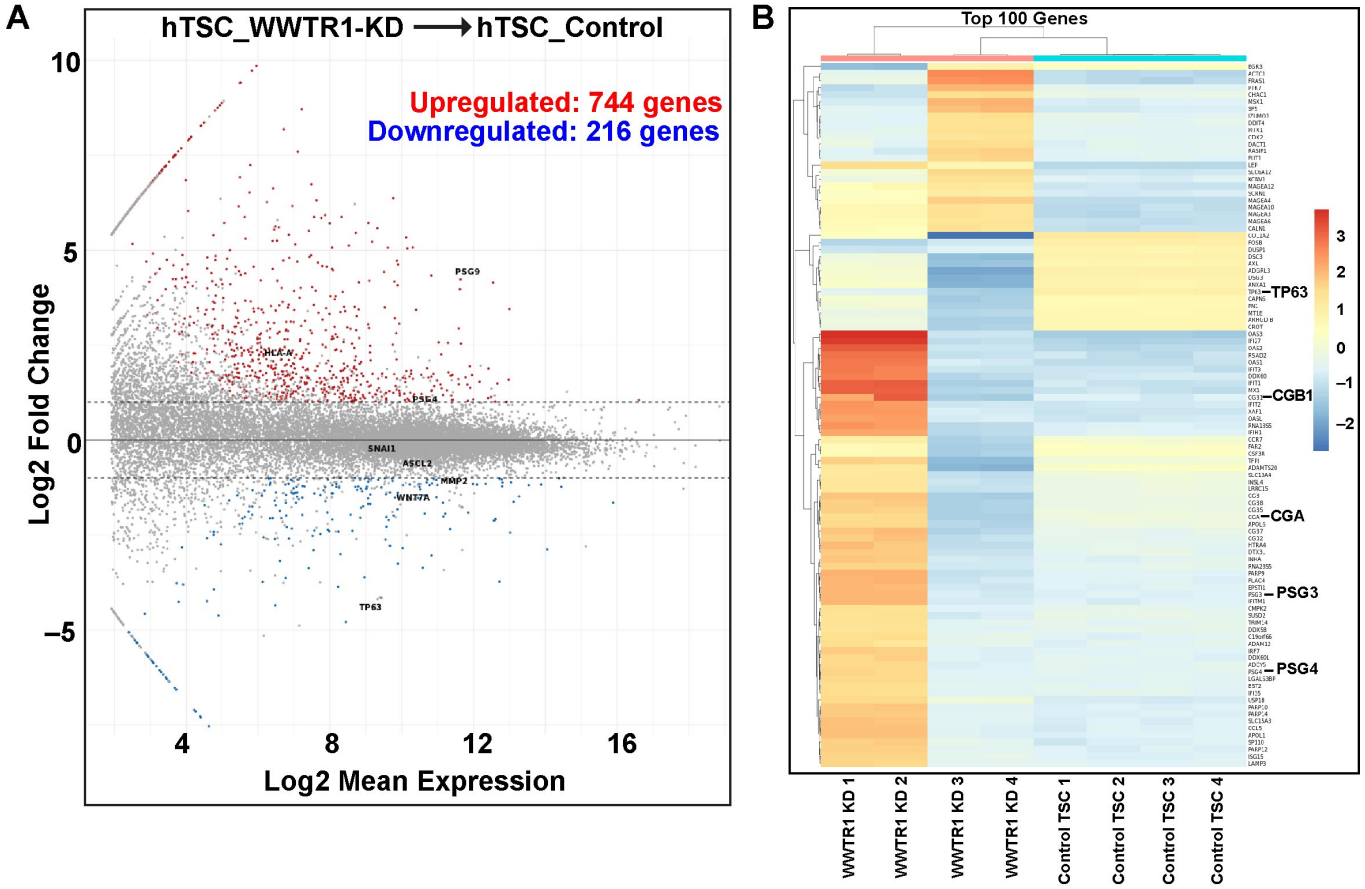

Fig. S5. Global Gene Expression in *WWTR1*-KD human TSCs. (A) Bland-Altman plot showing changes in global mRNA expression in human TSCs upon WWTR1 depletion. Colored dots indicate ≥2-fold change in mRNA expression with a false discovery rate of $P < 0.05$; red: up-regulated genes (total 744 genes), blue: down-regulated genes (total 216 genes). (B) The heat map shows top 100 differentially expressed genes in *WWTR1*-KD human TSCs.

Fig. S6

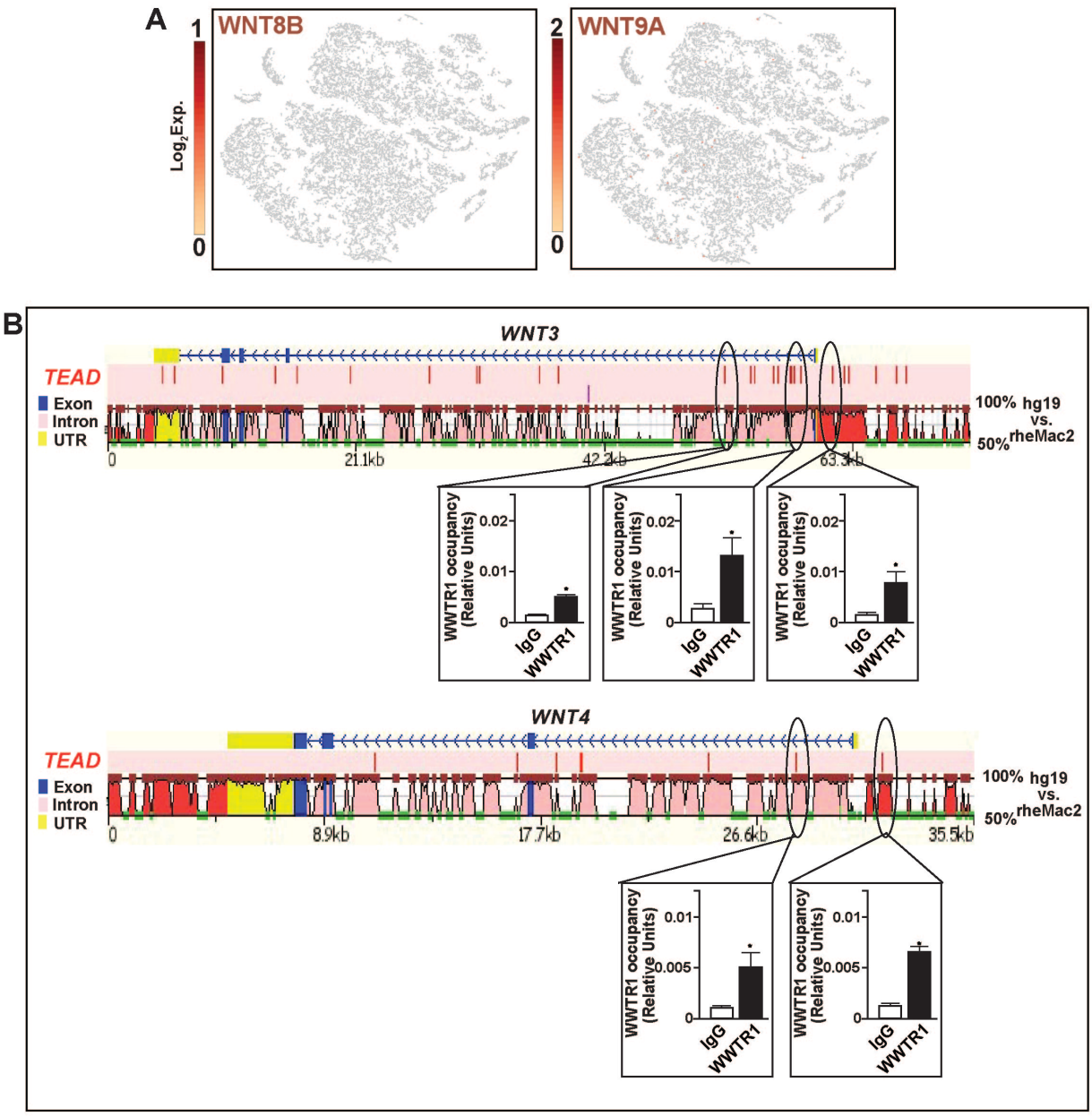

Fig. S6. (A) t-SNE plots show lack of WNT8B and WNT9A mRNA expression in single cell clusters from first-trimester human placenta. (B) Quantitative ChIP analysis showing WWTR1 occupancy at multiple conserved TEAD-motifs at the WNT3 and WNT4 loci in human TSCs (mean ± SE; n = 3, $p<0.01$).

Fig. S7

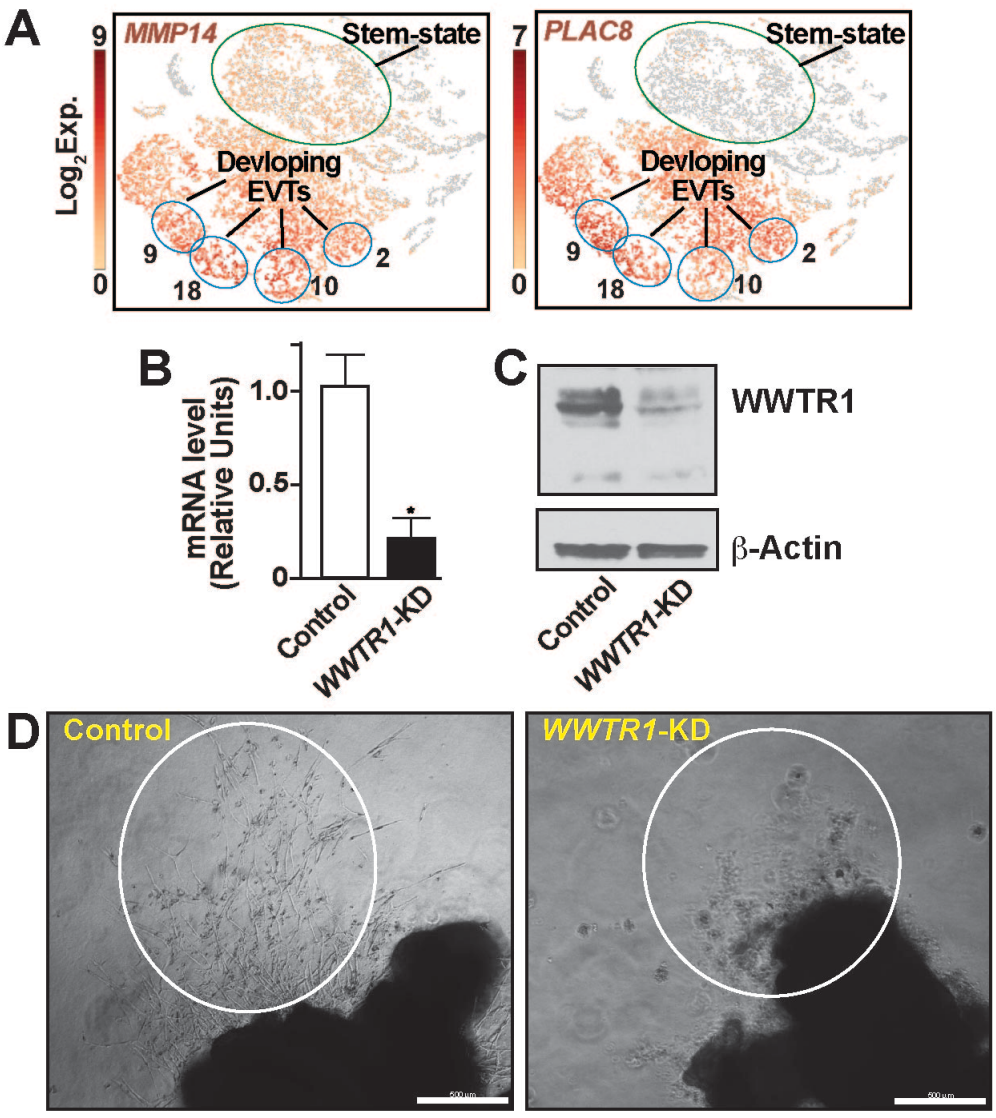

Fig. S7. (A) t-SNE plots showing mRNA induction of *MMP14*, and *PLAC8* in single cell clusters representing developing EVTs of first-trimester human placentae (B) and (C) RT-qPCR and Western Blot analysis, respectively, showing depletion of WWTR1 expression in first-trimester human plaecntal explants via shRNA-mediated RNAi. (D) Phase contrast images showing inhibition of EVT development (white elipses) from first-trimester human placental explants after RNAi-mediated depletion of WWTR1.

Fig. S8

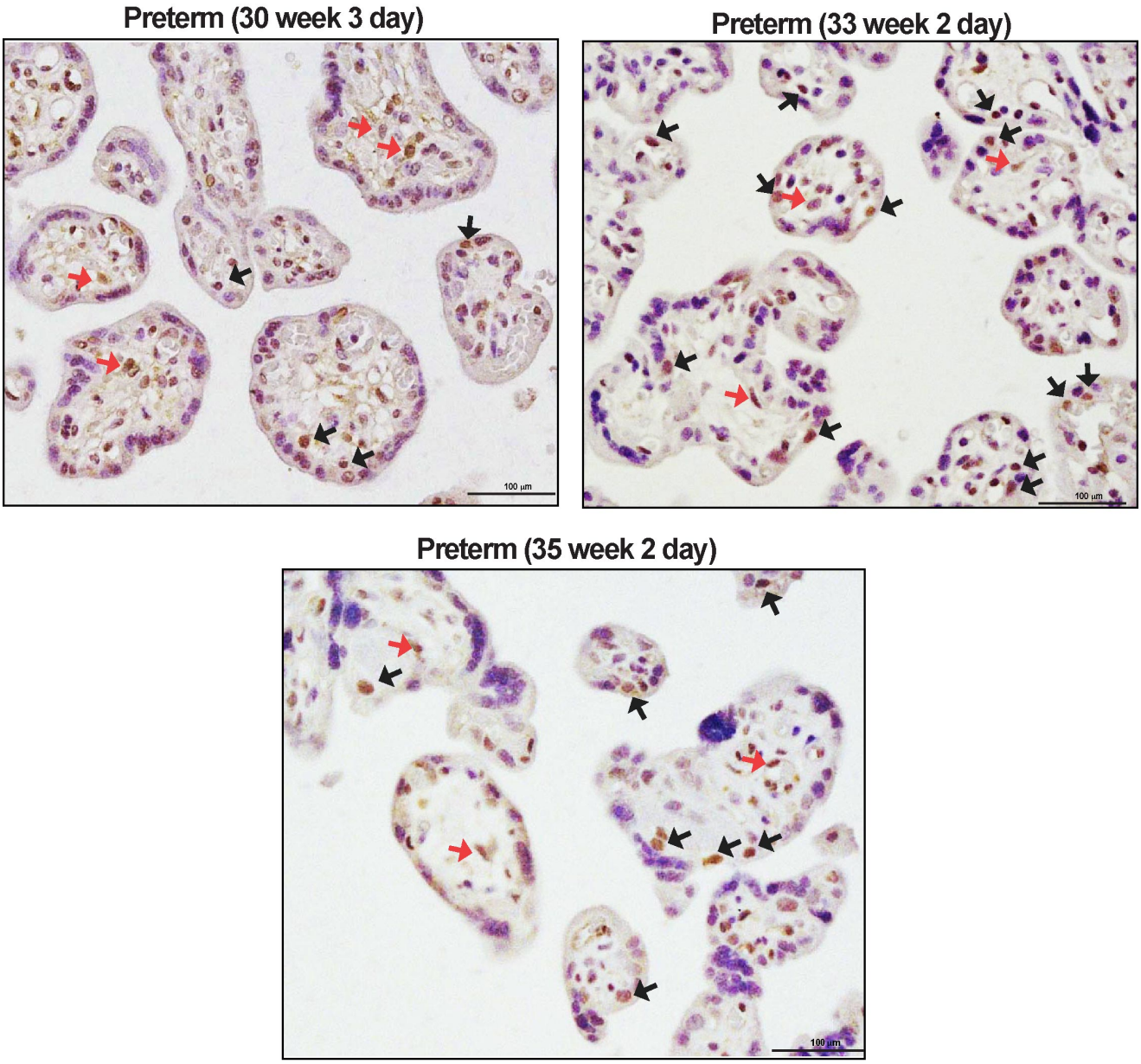

Fig. S8. WWTR1 expression in placentae from preterm pregnancies with various pregnancy duration. Representative immunostained images show WWTR1-expressing CTBs (Black arrows) in preterm placentae with various pregnancy duration. Compared to preterm placentae with more than 33 weeks of pregnancy duration, WWTR1-expressing CTBs were relatively less abundant in a preterm placenta with ~30 weeks of gestation. Red arrows indicate WWTR1-expressing non-trophoblast cells.

Fig. S9

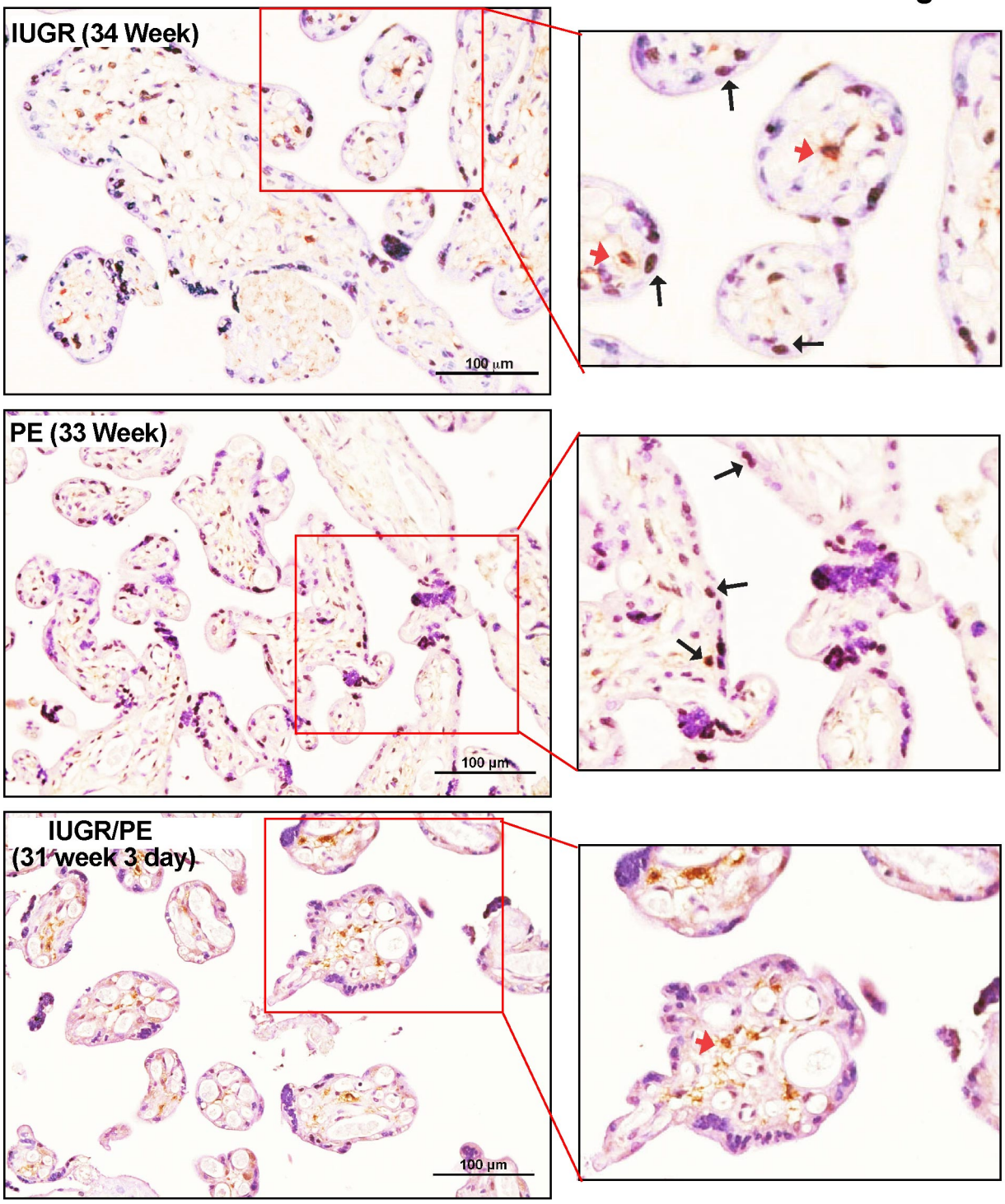

Fig. S9: WWTR1 expression in preterm pregnancies (≤34 weeks) associated with IUGR, PE or IUGR/PE. Representative immunostained images show WWTR1-expressing CTBs (Black arrows) in placentae from pregnancies that are associated with preterm birth (≤34 weeks of pregnancy duration) in association with IUGR, PE or IUGR/PE. Red arrows indicate WWTR1-expressing non-trophoblast cells.

Fig. S10

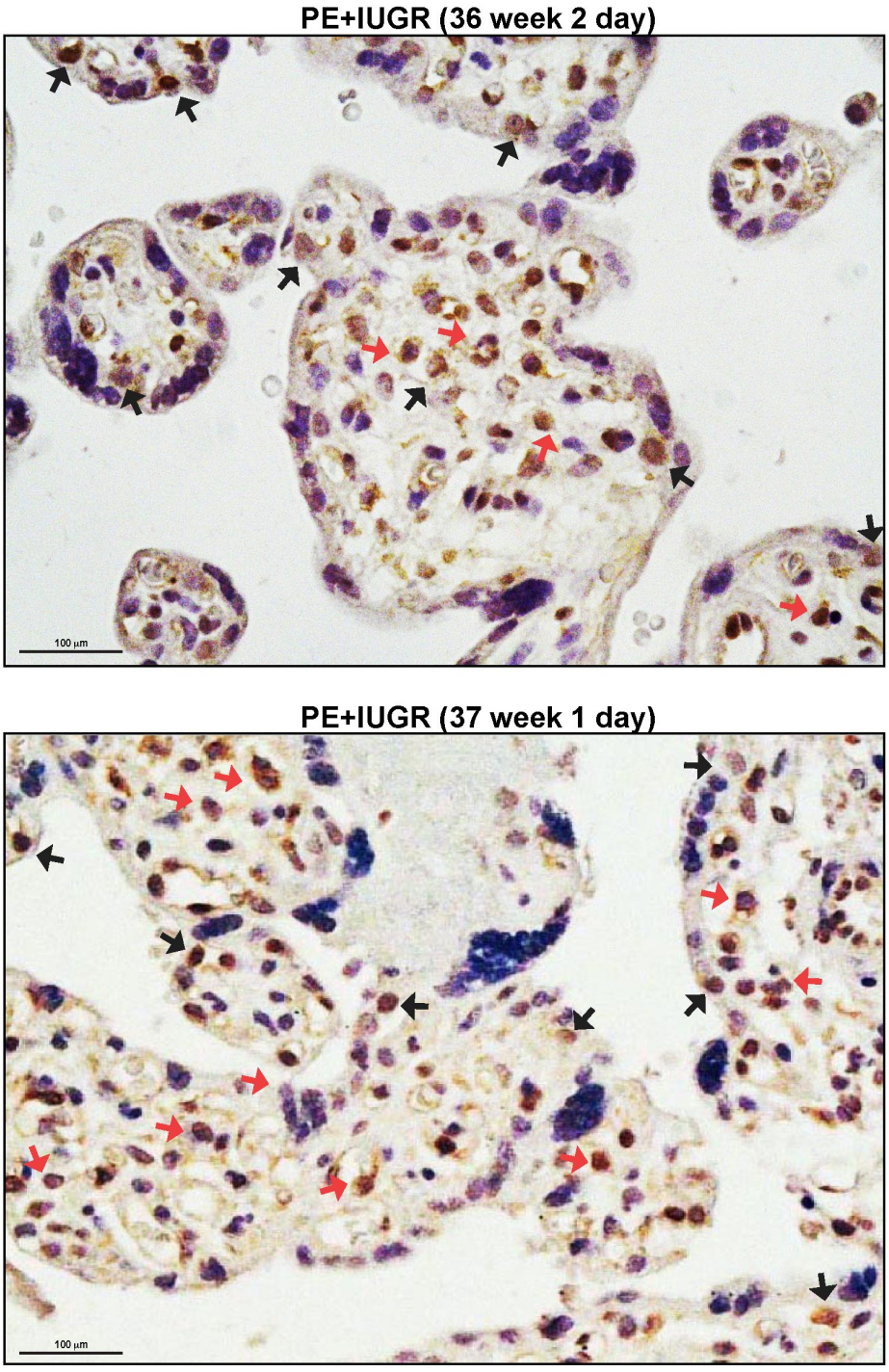

Fig. S10. WWTR1 expression in IUGR/PE placentae from pregnancies with more than 36 weeks of gestation. Representative immunostained images show WWTR1-expressing CTBs (Black arrows) in placentae from pregnancies that are associated with IUGR/PE. Red arrows indicate WWTR1-expressing non-trophoblast cells.