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Supplementary Information for

Placental trophoblast syncytialization potentiates macropinocytosis via mTOR signaling to adapt to reduced amino acid supply

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Supplementary experimental procedures

Study participants and samples

Pregnancy outcome was determined according to the definition in Williams Obstetrics (23rd edition) and the American College of Obstetricians and Gynecologists (ACOG) guidelines (1). In brief, normal pregnancy was defined as gestation in a healthy woman who did not exhibit any complications during pregnancy and delivered a healthy neonate at a gestational age of longer than 37 weeks (2). FGR was defined as the patient bearing a fetus with birth weight below the 10th percentile for the gestational age. The FGR patients enrolled in this study exhibited higher S/D index of umbilical Doppler flow. They did not have other obstetrical complications, and the fetuses had no chromosomal or congenital abnormalities. Because most of the FGR patients delivered at gestational age earlier than 37 weeks, gestational-age-matched placentas from unexplained PTL without FGR were collected and used as the gestational-age-matched controls (3). PTL was defined as birth at earlier than 37 weeks for unknown cause. Pregnancies complicated by gestational diabetes, hypertensive disorders, renal or cardiovascular disease, intrauterine fetal death, fetal chromosomal or congenital abnormalities, or pregnancies that were conceived with the assistance of reproductive technologies were excluded from this study.

Culture of PHT cells from human term placenta and trophoblast cell line

PHT isolation and culture: The minced placenta tissues were digested with trypsin (Sigma Aldrich, MO, USA) and DNase I (Sigma Aldrich). The supernatant was centrifuged, and the cell suspension were separated by Percoll (GE Healthcare BioSciences AB, Uppsala, Sweden) density gradient. CTB cells were collected from the density layer at 30-50% and cultured in Ham's F12: DMEM media (HyClone, CT, USA) with 10% fetal bovine serum (FBS; GIBCO, MA, USA) and antibiotics in a 5% CO_2 air incubator at 37°C. Cells were then maintained for 72 h after plating.

BeWo cells were thawed and maintained in F-12K: DMEM media (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and sodium pyruvate. JEG-3 and HepG2 cells were cultured in DMEM media (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and sodium pyruvate. HTR8/SVneo was a kind gift from Dr. CH Graham at Queen's University, Canada (4), and were cultured in RPMI1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and sodium pyruvate.

Western blot analysis

Whole lysates from cells or tissues were extracted with RIPA buffer containing protease inhibitor cocktail (Sigma Aldrich). Protein concentrations were determined using the BCA™ Protein Assay Kit (Pierce, MA, USA). The proteins were resolved on 10% SDS-PAGE gels under reducing conditions and electro-transferred to nitrocellulose membranes (GE Healthcare, CT, USA). The

membranes were further incubated with the indicated antibodies (*SI Appendix*, Table S2), followed by the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Promega, Madison, WI). The signals were visualized using an ECL kit (Thermo Scientific, MA, USA), followed by exposure in GeneGenome XRQ Chemiluminescence imaging system (Syngene, Cambridge, UK) and analyzed using Quantity One® 1-D Analysis Software, version 4.4 (Bio-Rad, CA, USA). The relative density of each specific molecule was measured by densitometry, normalized to β-actin expression from the corresponding blot.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from tissues or cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and stored at -80°C. 2 µg of total RNA were reverse-transcribed into cDNA in a total volume of 20 µl using 0.5 µg of oligo(dT) primer (GE Healthcare, CT, USA) and 500 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Ontario, Canada). The reaction was carried out in a buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol) containing 0.5 mM dNTPs for 2 h at 42°C. An aliquot of each cDNA sample (2 µl) was subjected to qPCR using a Lightcycler (Roche, Basel, Switzerland) in the presence of reaction mixture contained SYBR Green PCR mix (Takara, Shiga, Japan), and 10 pmol of primers. All primer sequences are listed in *SI Appendix*, Table S3. All PCR reactions were performed in triplicates, and the relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method (5) with normalization by ActB.

Immunohistochemistry

The tissues were fixed in 4% paraformaldehyde (PFA), gradually dehydrated in ethanol and embedded in paraffin. 5 µm-thick paraffin sections were subjected to routine rehydration and antigen retrieval before incubation with the primary antibodies (*SI Appendix*, Table S2). Negative controls were performed by replacing the specific antibody with rabbit or mouse IgG. The sections were further incubated with HRP-conjugated secondary antibody and were visualized with a DAB (Dako Cytomation, Glostrup, Denmark) solution containing 0.03% H_2O_2. The sections were counterstained with hematoxylin and mounted.

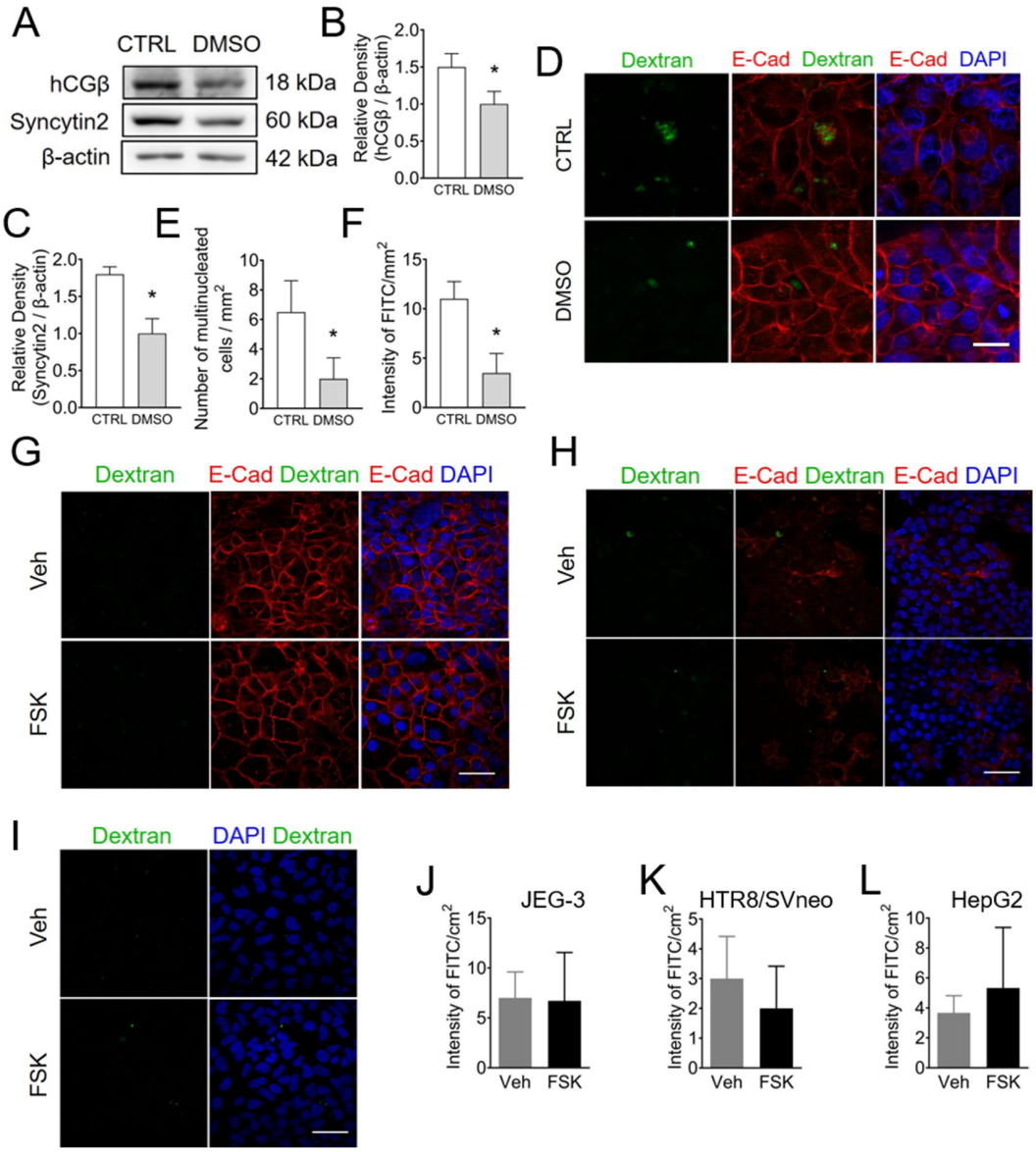

Fig. S1. Macropinocytosis is identified in trophoblast cells committed to cell-cell fusion, while human trophoblast and non-trophoblast cell lines with low context of syncytialization exhibit a very low level of macropinocytosis. (*A-C*) *A*, Western blots (*A*) and corresponding quantification (*B&C*) of hCGβ and syncytin2 in PHT cells cultured in medium with or without 1.5% DMSO. *B&C*, Semi-quantification of hCGβ and Syncytin2. (*D-F*) *D*, Representative immunostaining of Dextran (Green) and E-cadherin (red) in PHT cells cultured in the indicated conditions. *E&F*, Quantification of multinucleated cell and FITC intensity. (*G-I*) Representative confocal images of FITC-Dextran uptake measuring macropinocytosis activity upon 20 μM FSK exposure in JEG-3 (*G*), HTR8/SVneo (*H*), and HepG2 (*I*) cells. (*J-L*), Quantification of FITC-Dextran puncta in the corresponding experiments in *G-I*. The data are shown as mean ± SD, and the statistical analysis was carried out by two-tailed t test based on the results from at least three independent experiments. Scale bars in all panels, 20 μm.

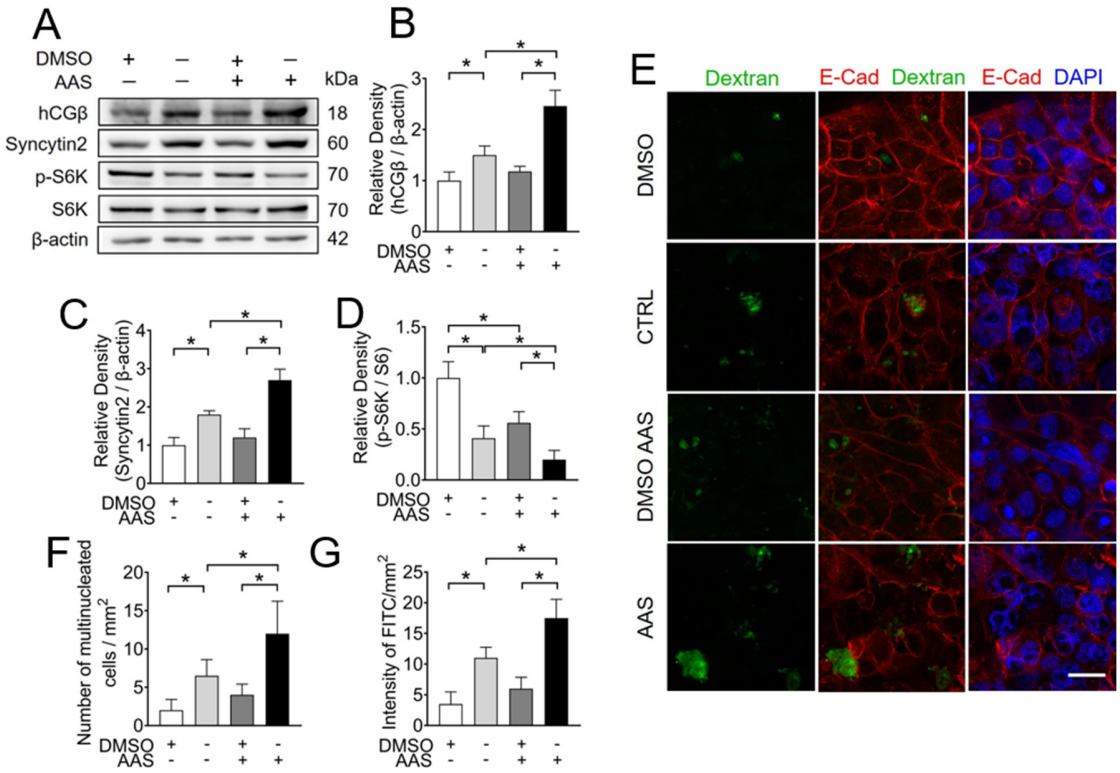

Fig. S2. Amino acids shortage (AAS) simultaneously promotes syncytialization and macropinocytosis in PHT cells. (*A-D*) *A*, Western blots (*A*) and corresponding quantification (*B-D*) of hCGβ, syncytin2, p-S6K and S6K in PHT cells cultured in amino acids shortage media (AAS, concentrations of Lys, Glu and Arg are 1/8 of the normal levels) with or without 1.5% DMSO. *B-D*, Semi-quantification of hCGβ, Syncytin2 and p-S6K/S6K. (*E-G*) *E*, Representative immunostaining of Dextran (Green) and E-cadherin (red) in PHT cells cultured in indicated conditions. *F&G*, Quantification of multinucleated cell and FITC intensity. Data were shown as mean ± SD and analyzed by one-way ANOVA test and Tukey-Kramer multiple comparison test based on at least three independent experiments. *, $p<0.05$. Scale bar, 40 μm.

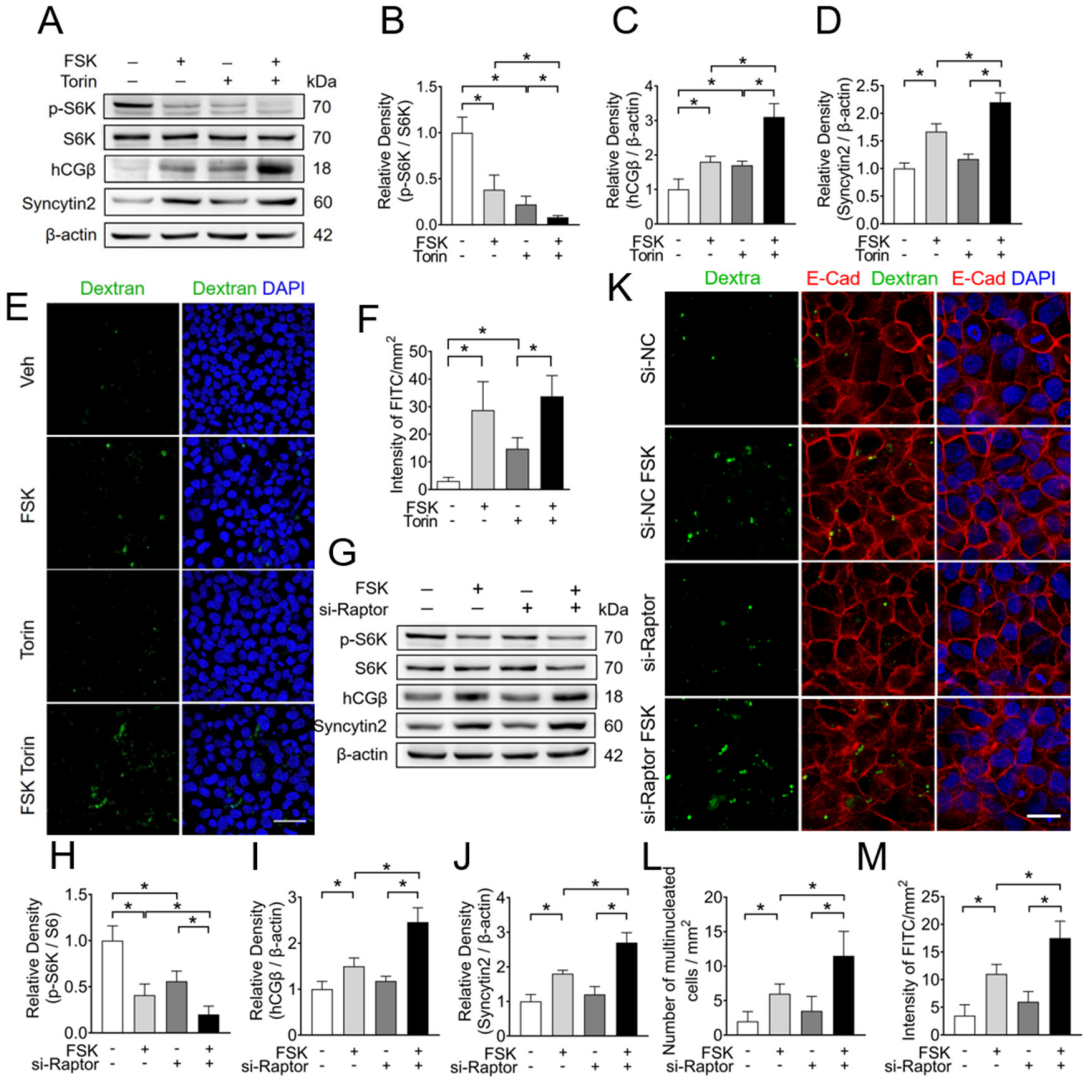

Fig. S3. mTOR inhibition is necessary for the induction of syncytialization and macropinocytosis in BeWo cells. (*A-D*) Western blots (*A*) and corresponding quantification (*B-D*) of p-S6K, S6K, hCGβ and syncytin2 in BeWo cells exposed to 20 μM FSK together with or without 250 nM Torin. *B-D*, Semi-quantification of the relative density of p-S6K/S6K, hCGβ and syncytin2. (*E, F*) Representative FITC-Dextran (green) uptake experiment in BeWo cells cultured in the indicated conditions (*E*). *F*, Quantification of FITC intensity. (*G-J*) Western blots (*G*) and corresponding quantification (*H-J*) of p-S6K, S6K, hCGβ and syncytin2 in BeWo cells transfected with 100 nM Raptor-specific siRNA or nonspecific control (NC) siRNA together with or without 20 μM FSK exposure. *H-J*, Semi-quantification of the relative density of p-S6K/S6K, hCGβ and syncytin2. (*K-M*) *K*, Representative immunostaining of Dextran (Green) and E-cadherin (red) in BeWo cells cultured in indicated conditions. *L&M*, Quantification of multinucleated cell and FITC intensity. Scale bars, 20 μm. The statistical data are shown as mean ± SD, and the statistical analysis is carried out by one-way ANOVA test and Turkey-Kramer multiple comparison test based on the results from at least three independent experiments. *, P<0.05.

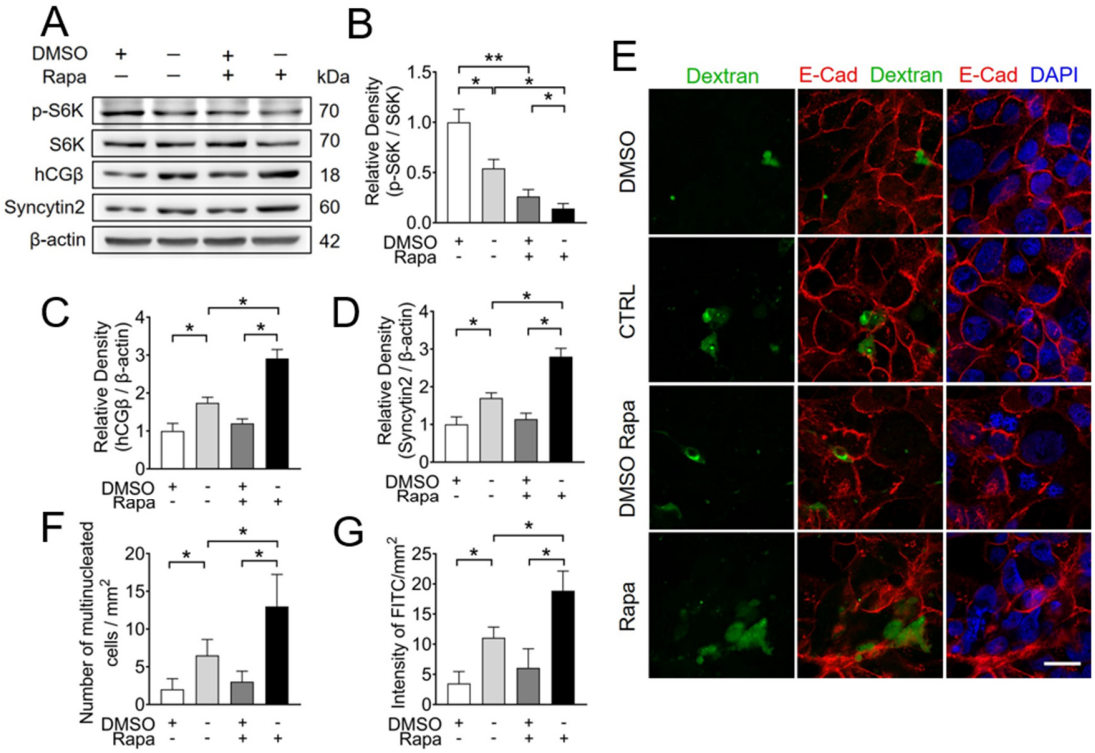

Fig. S4. mTOR inhibition is necessary for the induction of syncytialization and macropinocytosis in PHT cells. (A-*D*) Western blots (*A*) and corresponding quantification (*B-D*) of p-S6K, S6K, hCGβ and syncytin2 in PHT cells exposed to 100 nM Rapamycin (Rapa) together with or without 1.5% DMSO. *B-D*, Semi-quantification of the relative density of p-S6K/S6K, hCGβ and syncytin2. (*E-G*) *E*, Representative immunostaining of Dextran (Green) and E-cadherin (red) in PHT cells cultured in indicated conditions. *F&G*, Quantification of multinucleated cell and FITC intensity. Scale bars, 20 μm. The statistical data are shown as mean ± SD, and the statistical analysis is carried out by one-way ANOVA test and Turkey-Kramer multiple comparison test based on the results from at least three independent experiments. *, P<0.05.

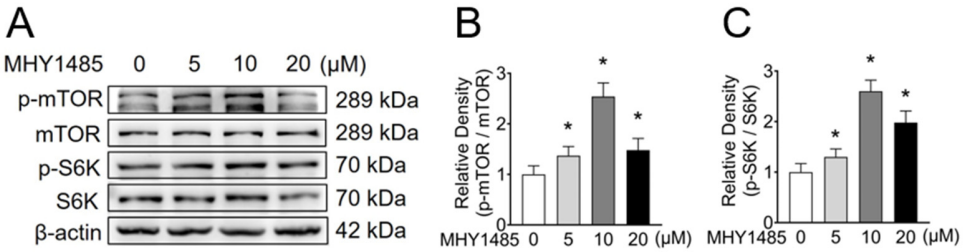

Fig. S5. Exposure to MHY1485 activates mTOR signaling in BeWo cells. (*A*-*C*) Western blots (*A*) and corresponding quantification (*B&C*) of p-mTOR, mTOR, p-S6K and S6K in BeWo cells exposed to indicated concentration of mTOR specific activator MHY1485. *B&C*, Semi-quantification of the relative density of p-mTOR/mTOR and p-S6K/S6K. The statistical data are shown as mean ± SD, and the statistical analysis is carried out by one-way ANOVA test and Turkey-Kramer multiple comparison test based on at least three independent experiments. *, P<0.05.

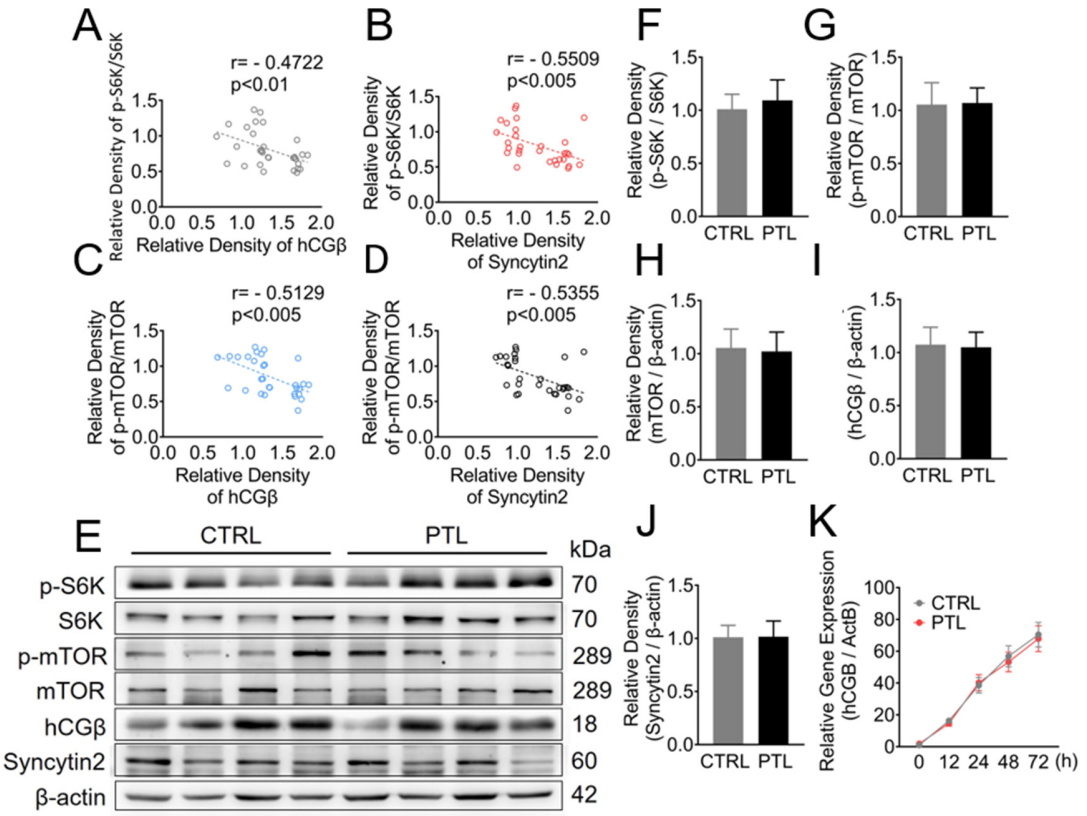

Fig. S6. Trophoblast syncytialization and mTOR signaling in the placentas from normal term pregnancy (CTRL, n=16) and unexplained preterm labor (PTL, n=10). (*A-D*) The correlation analysis between the relative density of p-S6K/S6K and hCGβ (*A*), p-S6K/S6K and Syncytin2 (*B*), p-mTOR/mTOR and hCGβ (*C*), p-mTOR/mTOR and Syncytin2 (*D*) in placentas from normal pregnancy (CTRL, n=16) or FGR (n=14). (*E-J*) Western blots (*E*) and corresponding quantification (*F-J*) of p-S6K, S6K, p-mTOR, mTOR, hCGβ and Syncytin2 in the placenta of CTRL and PTL. *F-J*, Semi-quantification of the relative density of p-S6K/S6K, p-mTOR/mTOR, mTOR, hCGβ and Syncytin2. (*K*) mRNA levels of *hCGB* during the syncytialization process of PHT cells derived from CTRL or PTL placentas. The data are shown as mean ± SD, and the statistical analysis is carried out by nonparametric Spearman correlation test (*A-D*) or two-tailed t test (*F-K*).

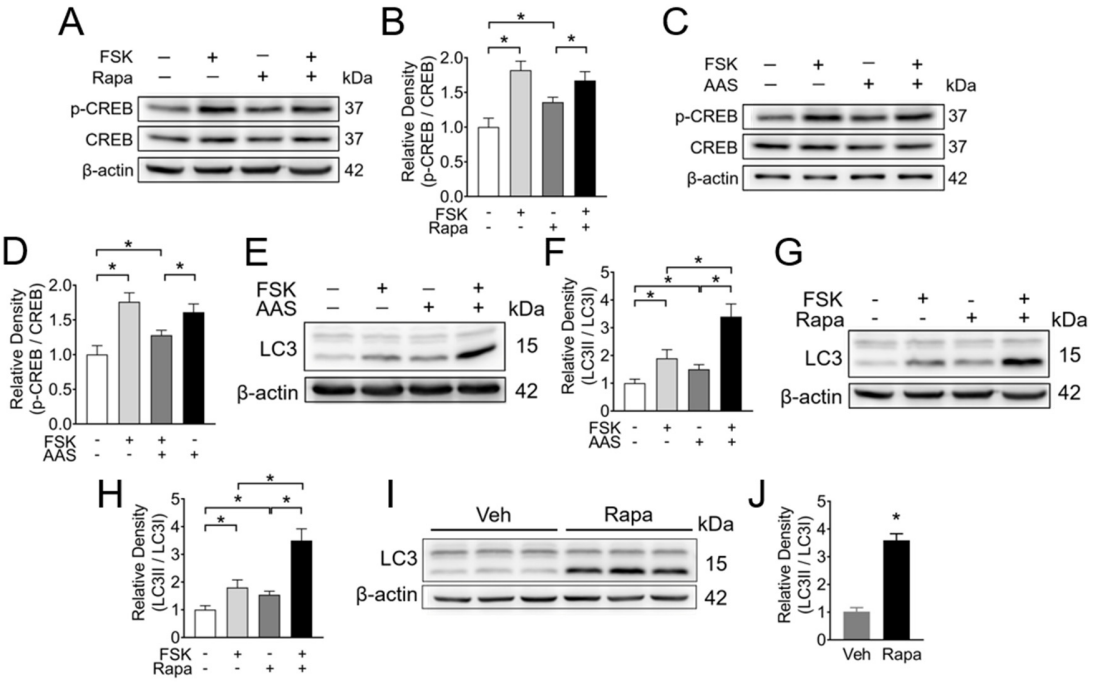

Fig. S7. Rapa exposure or AAS medium culture activates PKA signaling and autophagy in BeWo cells and in mouse placenta. (*A, B*) Western blots (*A*) and corresponding quantification (*B*) of p-CREB and CREB in BeWo cells exposed to 20 μM FSK and/or 100 nM Rapa. *B*, Semi-quantification of the relative density of p-CREB/CREB. (*C, D*) Western blots (*C*) and corresponding quantification (*D*) of p-CREB and CREB in BeWo cells cultured in AAS medium together with or without 20 μM FSK exposure. *D*, Semi-quantification of the relative density of p-CREB/CREB. (*E, F*) Western blots (*E*) and corresponding quantification (*F*) of LC3II to LC3I in BeWo cells cultured in AAS media with or without 20 μM FSK. (*G, H*) Western blots (*G*) and corresponding quantification (*H*) of LC3II to LC3I in BeWo cells exposed to 20 μM FSK and/or 100 nM Rapa. (*I, J*) I, Western blots (I) and the corresponding quantification (J) of LC3II to LC3I in the mouse placentas treated by vehicle (n=10) or Rapa (n=10). J, Semi-quantification of LC3II to LC3I. The statistical data are shown as mean ± SD, and the statistical analysis is carried out by one-way ANOVA test and Turkey-Kramer multiple comparison test (B, D, F and H) or two-tailed t test (J) based on the results from at least three independent experiments. *, P<0.05.

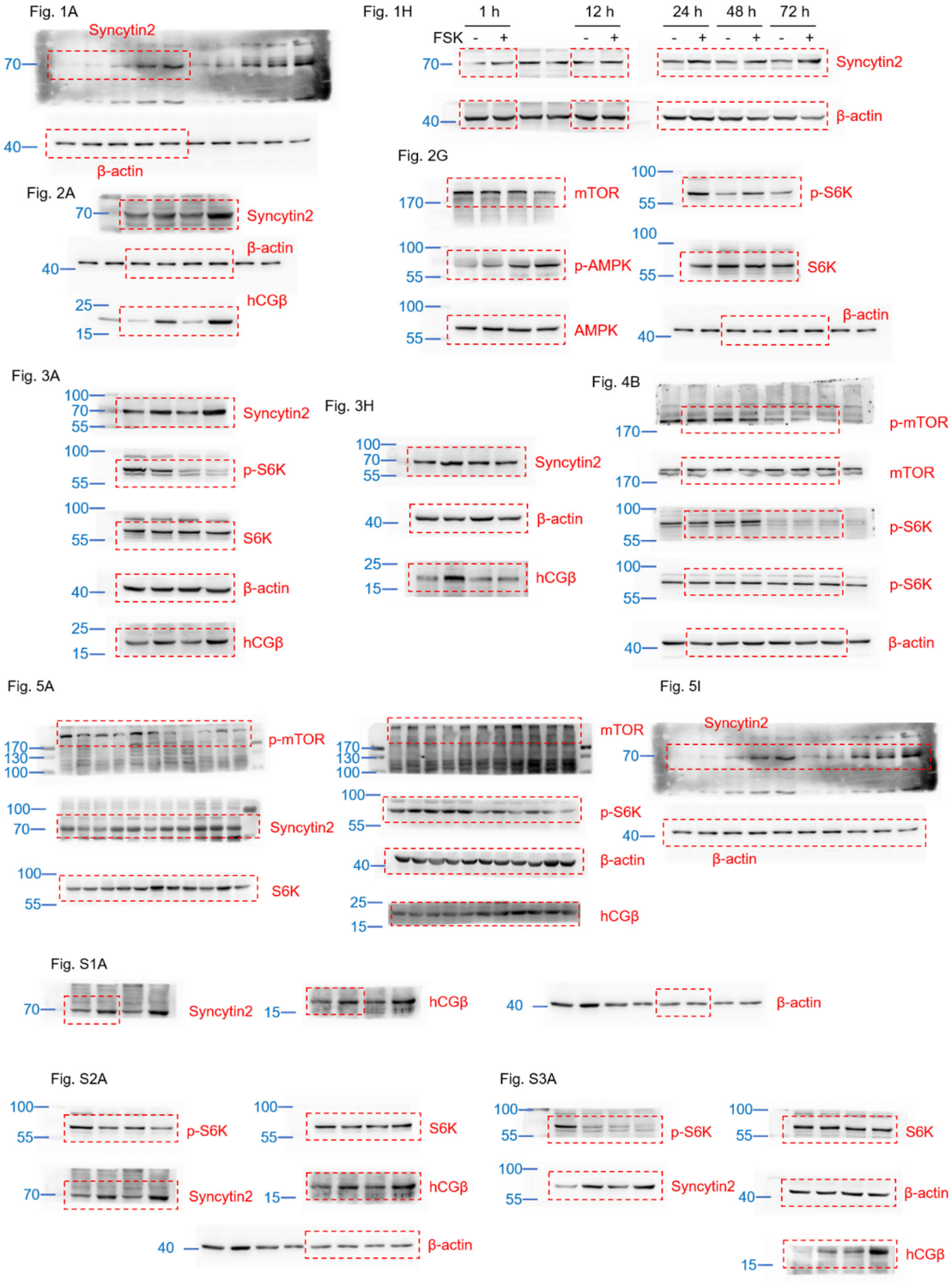

Fig. S8. Unedited gel scan.

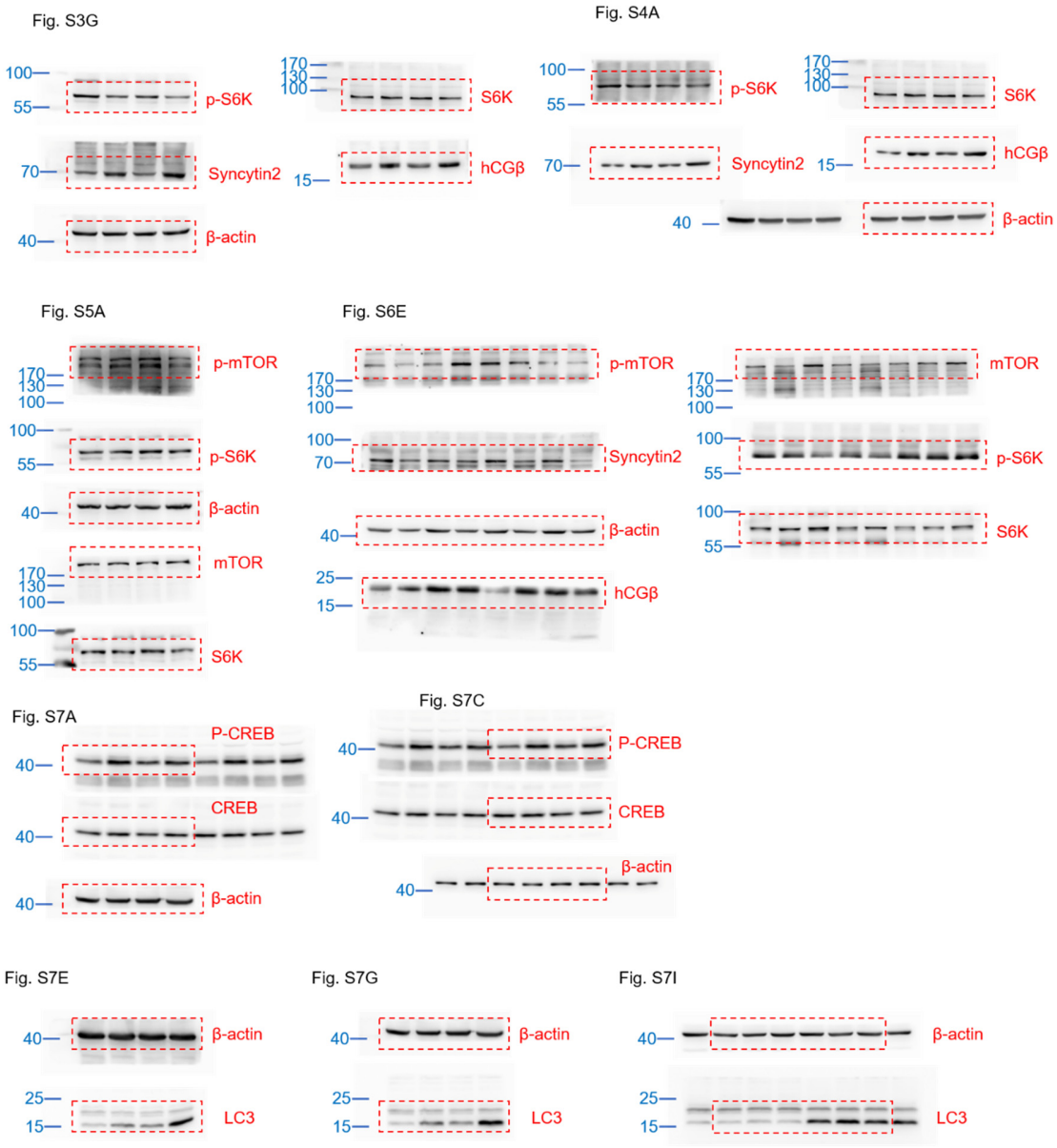

Fig. S8. Unedited gel scan (continued).

Table S1. Clinical characteristics of the pregnant women enrolled in this study[a].

	Normal Pregnancy (Control, n=16)	Unexplained Preterm Labor (n=10)	Fetal Growth Restriction (n=14)
Age (yr)	28.6±4.6	28.7±4.9	28.3±4.9
Body-Mass Index[b]	23.7±5.1	22.1±4.7	24.2±5.3
50g Glucose Challenge Test (mmol/L)	6.4±1.2	6.4±1.9	6.3±2.3
Systolic Blood Pressure (mmHg)	106.6±23.7	107.2±10.8	106.9±9.6
Diastolic Blood Pressure (mmHg)	79.8±8.7	80.4±9.1	81.1±7.9
Gestational Age at delivery (d)	270.3±7.1	234.9±8.6 (P<0.001)[c]	239.5±5.1 (P<0.001)[c]
Infant Birth Weight (g)	3431±442	2273±392 (P<0.001)[c]	1873±346 (P<0.001)[c, d]
Umbilical artery S/D ratio	2.47±0.42	3.86±1.35 [c]	4.43±1.68 [c, d]

[a] Plus-minus values are mean ± SD; P values are only shown for the significant differences between groups.

[b] Body-mass index, the weight (kg) divided by the square of the height (m).

[c] Compared with Normal Pregnancy (Control) group.

[d] Compared with Unexplained Preterm Labor group.

Table S2. Antibodies used in this study.

Antibody	Host species	Catalogue number	Manufacturer	Final concentration
β-actin	Mouse	#3700	CST	1:5000
E-Cadherin	Mouse	#3195	CST	1:2000
hCGβ	Mouse	ab9582	Abcam	1:1000
HERV-FRD	Rabbit	AP13018A	Abgent	1:1000
mTOR	Rabbit	#2972	CST	1:1000
p-AMPK (T172)	Rabbit	#2535	CST	1:1000
AMPK	Rabbit	#5831	CST	1:1000
p-S6K (S371)	Rabbit	#9208	CST	1:1000
S6K	Rabbit	#9202	CST	1:1000
p-mTOR (S2448)	Rabbit	BS4706	Bioworld	1:1000
MCT1	Chicken	AB1286	Sigma	1:200
MCT4	Rabbit	AB3314P	Sigma	1:200
Cytokeratin 7	Rabbit	ab181598	Abcam	1:8000
p-CREB (S133)	Rabbit	Ab32096	Abcam	1:1000
CREB	Rabbit	#9197	CST	1:1000
LC3B	Rabbit	L7543	Sigma	1:1000

CST: Cell signaling technology, Danvers, MA. Abcam: Abcam, Cambridge, UK. Abgent: Abgent, San Diego, CA. Bioworld: Bioworld, St. Louis Park, MN. Sigma: Sigma-Aldrich, St. Louis, MO. HERV-FRD: Syncytin2. MCT1: Monocarboxylate Transporter 1. MCT4: Monocarboxylate Transporter 4.

Table S3. Sequence of the primers for real-time qPCR used in this study.

	Forward Primer	Reverse Primer
Homo Sapiens		
ActB	CGAGCACAGAGCCTCGCCTT	TGCACATGCCGGAGCCGTTG
hCGB	GAGCTCACCCCAGCATCCTATCACC	TTGATGGGGCGGCACCGTGG
Mus musculus		
Actb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Gcm1	GATACTGAGCTGGGACATTAACG	CTGTCGTCCGAGCTGTAGATG
Syna	ATGGTTCGTCCTTGGGTTTTC	GTGTTGAGTGAGGTTTACCAGG
Synb	ATGACAGGCTTTTGGGTCCTC	GTTGGTATCACGTAGGATGTGG

Reference Cited in *SI Appendix*

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