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Supplementary Materials for

TMEM16F phospholipid scramblase mediates trophoblast fusion and placental development

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

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Supplementary Figures

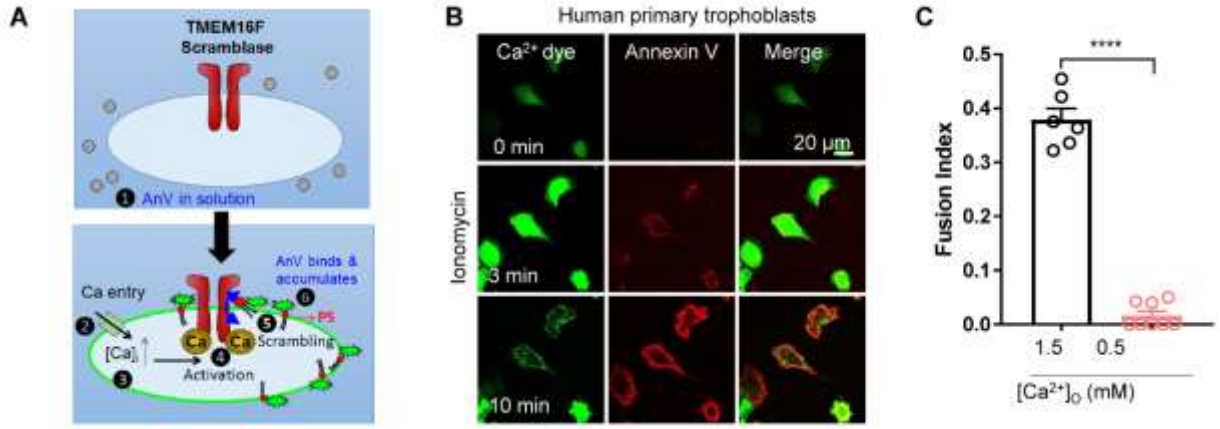

Fig S1. Human trophoblasts exhibit active Ca^{2+}-activated phospholipid scramblase activity and Ca^{2+}-dependent cell fusion. (A) An optimized microscope-based, fluorescent imaging assay to monitor Ca^{2+}-activated phospholipid scramblase (CaPLSase) activities. In this assay, Ca^{2+} ionophores or agonists trigger intracellular Ca^{2+} elevation, which subsequently activates CaPLSases, resulting in PS surface exposure and cell surface accumulation of fluorescently tagged PS binding protein, Annexin V (AnV). **(B)** Primary human placental trophoblasts exhibit robust CaPLSase activities when triggered with 1 μM ionomycin. Ca^{2+} dye (Calbryte 520) and fluorescently tagged AnV proteins (AnV-CF594) were used to measure the dynamics of intracellular Ca^{2+} and cell surface PS, respectively. **(C)** Reducing extracellular Ca^{2+} concentration ($[Ca^{2+}]_O$) in the cell culture medium suppressed forskolin-induced BeWo cell fusion. Each dot represents the average of fusion indexes of six random fields from one coverslip. Unpaired two-sided Student's *t*-test. ****: $p < 0.0001$. Error bars indicate ±SEM. All fluorescence images are the representatives of at least three biological replicates.

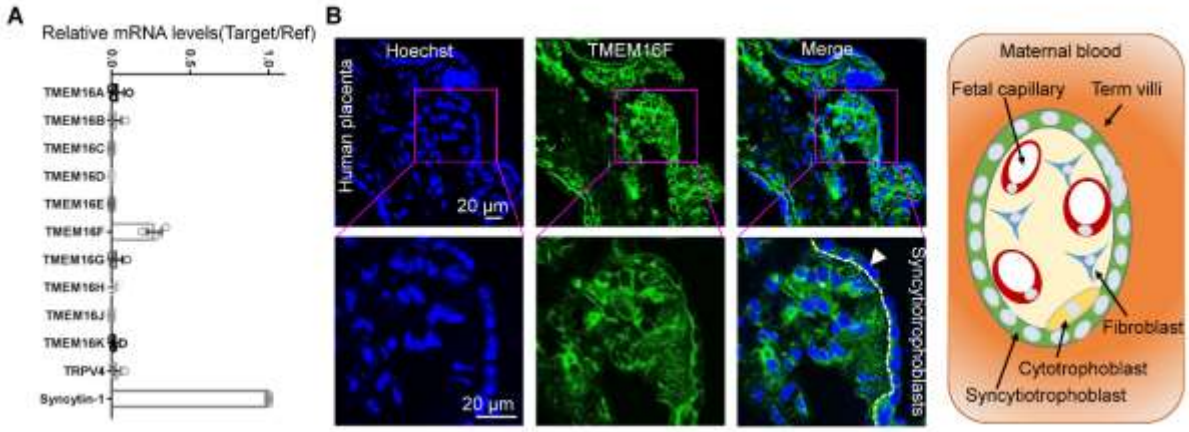

Fig. S2. TMEM16F is highly expressed in human placental trophoblasts. (A) qRT-PCR of TMEM16 family members in primary human trophoblast shows that TMEM16F mRNA has the highest express level over other family members. All genes were normalized to GAPDH and then normalized to Syncytin-1. **(B)** Immunofluorescence of TMEM16F (anti-TMEM16F, green) and nuclei (Hoechst, blue) in a human term placenta sample (upper panels). Higher magnification is shown in in the lower panels. TMEM16F protein is highly expressed in the basal membrane of the syncytiotrophoblasts (arrowhead). The white dotted line demarcates the basal membrane of the syncytiotrophoblast. Schematic of the maternal-fetal interface in term placental villi is shown on the right. Images and diagram are shown in cross-sections of the villi. All fluorescence images are the representatives of at least three biological replicates.

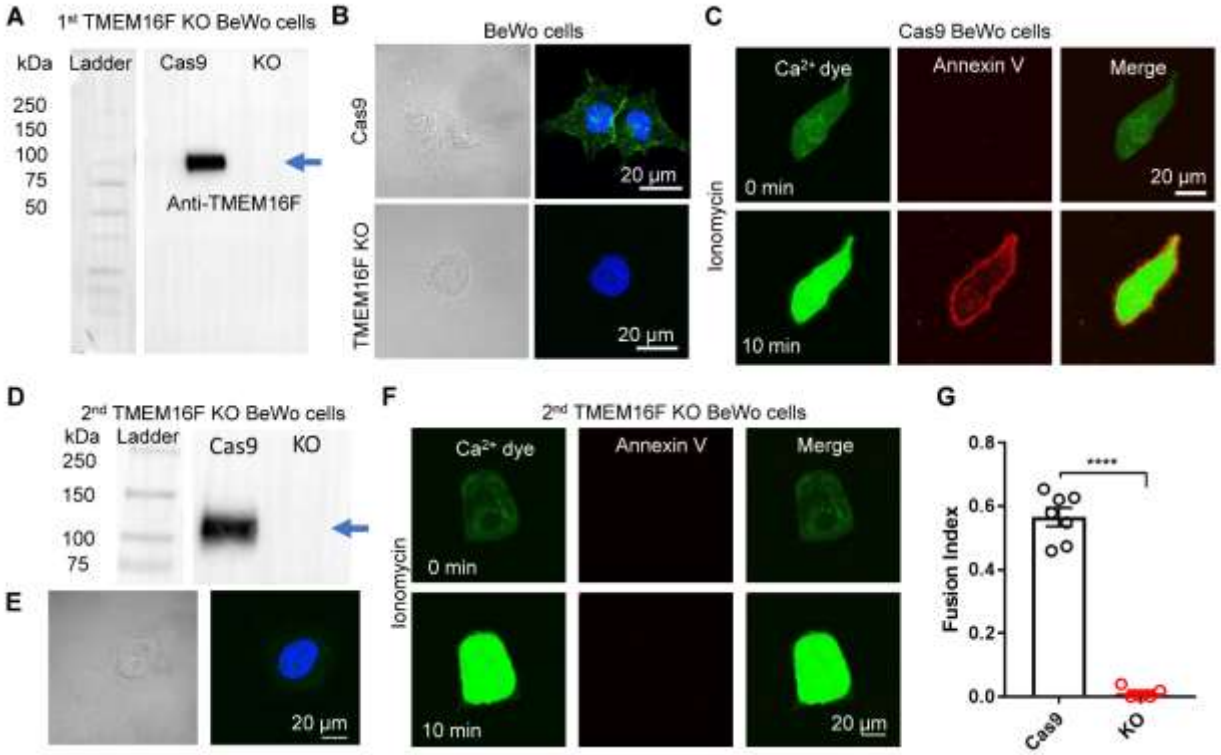

Fig. S3. TMEM16F is responsible for the CaPLSase activity in BeWo cells. (A) Western blotting of the Cas9 control and the TMEM16F knockout (KO) BeWo cells. The arrow labels the expected TMEM16F band. **(B)** Immunofluorescence of TMEM16F (green) in the Cas9 control BeWo cells (upper) and the TMEM16F knockout (KO) BeWo cells generated by CRISPR-Cas9 (lower). Cell nuclei are stained with Hoechst (blue). **(C)** The Cas9 BeWo cells exhibit robust CaPLSase activity triggered by 1 μM ionomycin. **(D)** Western blotting of the Cas9 control and an additional TMEM16F KO BeWo cells generated using a different sgRNA (upper). The arrow labels the expected TMEM16F band. **(E)** Immunofluorescence of TMEM16F (green) in the second TMEM16F knockout (KO) BeWo cells(lower). Cell nuclei are stained with Hoechst (blue). **(F, G)** The second knockout cell line behaves the same as the first BeWo TMEM16F KO cell line: lack of Ca^{2+}-activated lipid scrambling (E), and incapable to fuse (F). All fluorescence images are the representatives of at least three biological replicates.

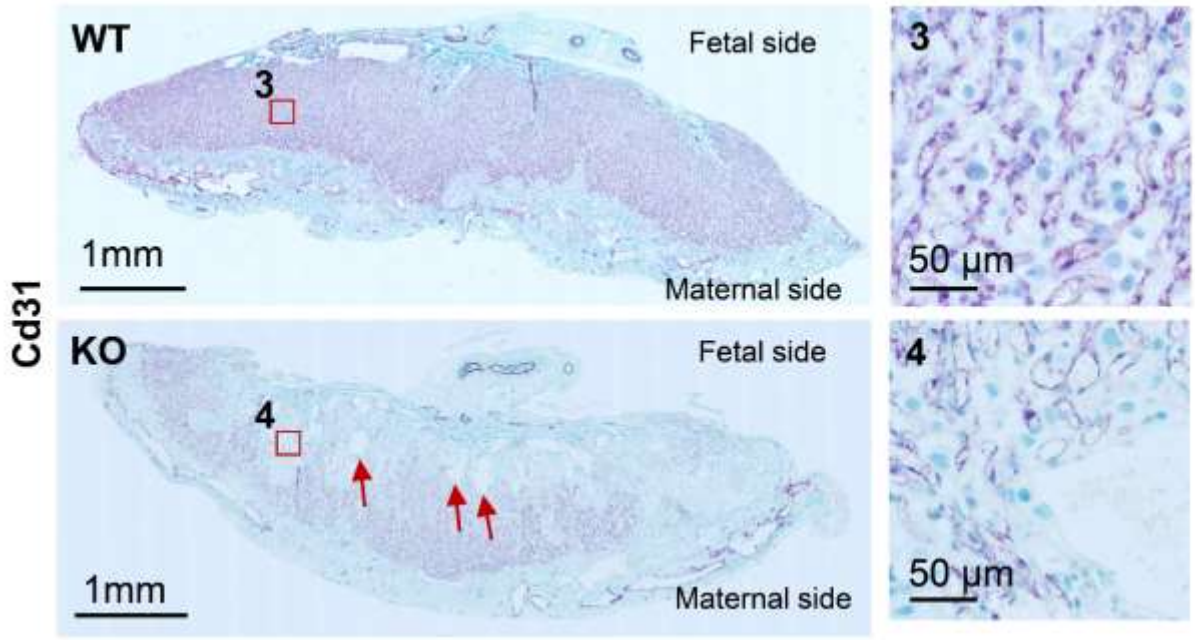

Fig. S4. Histological changes of the placentas from the TMEM16F deficient mice. Immunostaining against Cd31 on TMEM16F WT and KO placentas. The right panels show higher magnifications of the labyrinth layer of the placentas. Cd31 labels fetal blood vessels and its staining is greatly diminished at the fetal interface of the KO labyrinth layer. Red arrows point to the enlarged maternal blood spaces of the KO placenta. Images are representative of at least three independent mice per genotype.

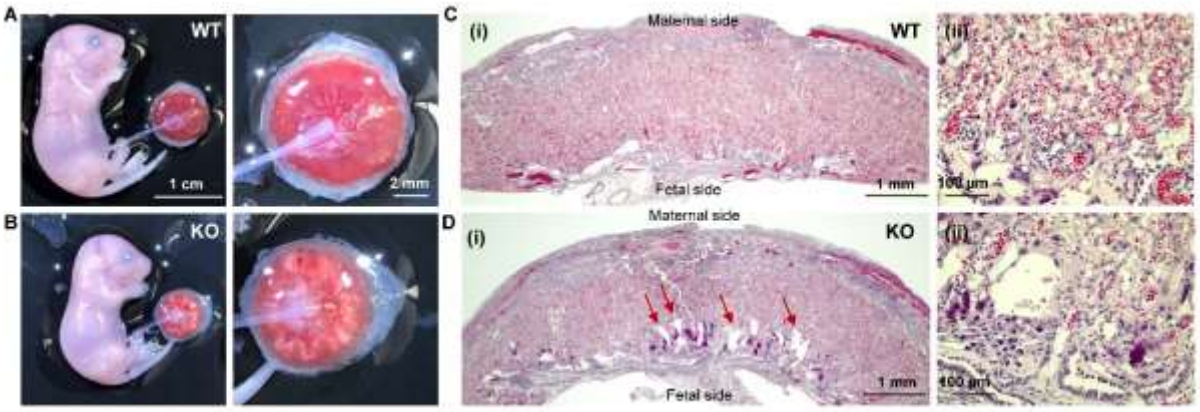

Fig. S5. The 'Gene Trap' TMEM16F knockout mice exhibit similar defects on placenta morphology and development. **(A-B)** Representative embryos and placentas of the wild-type (WT, A) and TMEM16F deficient (KO, B) mice from the same litter at E18.5. The right panels show higher magnifications of the placentas. **(C)** Hematoxylin and eosin (H&E) staining of the TMEM16F WT placenta (E18.5) at low magnification (i) and the higher magnification of the labyrinth layer (ii). **(D)** Hematoxylin and eosin (H&E) staining of the TMEM16F KO placenta (E18.5) at low magnification (i) and the higher magnification of the labyrinth layer (ii). Red arrows point to the enlarged maternal blood spaces of the KO placenta. Images are representative of at least three independent mice per genotype.

Supplementary Tables

Table S1. Genotype distribution of TMEM16F knockout mice.

	Total	WT	Heterozygous	Homozygous
Targeted Deletion*	115	34 (29.6%)	64 (55.7%)	17 (14.7%)
Gene Trap****	104	31 (29.8%)	68 (65.4%)	5 (4.8%)

[#]: het x het breeding scheme was used to generate TMEM16F knockout mice. Statistical analysis was performed using the Chi-quare test. ****: $p < 0.0001$. *: $p < 0.05$.

Table S2. Primers used for RT-PCR.

Gene	Forward Primer	Reverse Primer
TMEM16A	CTGATGCCGAGTGCAAGTATG	AGGGCCTCTTGTGATGGTACA
TMEM16B	TATGTACTTGCCTACCACTACCG	TTGGAGACGATAGCCAGCGA
TMEM16C	ACCATTCAGGCTCCATTCAGT	CGAGACGGTTTTAACGAAGTTTC
TMEM16D	CTGAATCGTTTGCTTACCAATGG	GTCGGTGGTTTTCTGCTCCA
TMEM16E	GCGGCGGCTTATGTTTCAAAA	CGCCTTTAACTCTGCGTCTTTC
TMEM16G	GACCTGGCGGGAGACTTTTC	CCCAGCCGCACGAAGATTAT
TMEM16H	TTCCCAGACACGACCGATGA	TACGTGGCGGTGACAAAGAAG
TMEM16J	GTCACCACGAGTCTCAGAATCC	AGATCCTCGAAGGTCTCACCA
TMEM16K	CGTCTGGCATCGTGATTCAG	GCAAACCGAGTGTACCAGGT
Syncytin-1	ATGCCCCGCAACTGCTATC	AGACAGTGACTCCAAGTCCTC

Legends for Supplementary Movies.

Movie S1. BeWo cells exhibited robust Ca^{2+}-activated phospholipid scramblase (CaPLSase) activity when triggered with 1 μM ionomycin. This video shows spontaneous imaging of Ca^{2+} (Calbryte 520, green) and fluorescently tagged Annexin V (AnV-CF594, red) in BeWo cells upon ionomycin stimulation. The overlap between green and red fluorescence signals on cell surface may appear as yellow. The time-lapse covers a period of ~ 10 minutes after ionomycin addition.

Movie S2. Ca^{2+}-induced PS exposure was eliminated in TMEM16F KO BeWo cells when triggered with 1 μM ionomycin. This video shows spontaneous imaging of Ca^{2+} (Calbryte 520, green) and fluorescently tagged Annexin V (AnV-CF594, red) in TMEM16F KO BeWo cells upon ionomycin stimulation. Ca^{2+}-induced PS exposure was eliminated yet intracellular Ca^{2+} elevation was normal. The time-lapse covers a period of ~ 10 minutes after ionomycin addition.

Movie S3. Reintroducing murine TMEM16F (mTMEM16F) to the TMEM16F deficient BeWo cells rescued CaPLSase activity. This video shows spontaneous imaging of Ca^{2+} (Calbryte 520, green) and fluorescently tagged Annexin V (AnV-CF594, red) in the TMEM16F deficient BeWo cells that was infected with murine TMEM16F (mTMEM16F, magenta). Ionomycin was used to stimulate TMEM16F activation. The overlap between magenta, green and red fluorescence signals on cell surface may appear as white. The time-lapse covers a period of ~ 10 minutes after ionomycin addition.