Supplementary Information

Atypical Protein Kinase C iota (PKCλ/ι) Ensures Mammalian Development by Establishing the Maternal-Fetal Exchange Interface

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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 2**	
Figure S3	**Related to Figure 2**	
Figure S4	**Related to Figure 3**	
Figure S5	**Related to Figure 6**	
Figure S6	**Related to Figure 7**	
Dataset S1	**Related to Figure 5**	Describes significantly downregulated (≤2 fold) genes in *Prkci* KD mouse TSCs
Dataset S2	**Related to Figure 5**	Describes significantly upregulated (≥2 fold) genes in *Prkci* KD mouse TSCs
Dataset S3	**Related to Figure 5**	Describes both down and up regulated genes with a stringent FDR cutoff.

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SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Mouse TSC culture: Mouse TSCs were cultured using RPMI-1640 (Sigma) supplemented with 20% Fetal Bovine Serum (FBS), sodium pyruvate, β-mercaptoethanol and primocin (to avoid any mycoplasma contamination). Proliferative cells were maintained using mouse embryonic fibroblast (MEF)-conditioned media (CM) and basal media (70:30 ratio), FGF4 (25ng/ml) and heparin (1µg/ml). For differentiation assays, cells were allowed to differentiate by removing CM, FGF4 and heparin from the media. Cells were harvested at different time points and total RNA and protein were extracted for RT-PCR and western blot analyses.

Human placental tissue sample analyses: Fresh and Formaldehyde fixed, de-identified first trimester placental tissues were obtained from Mount-Sinai hospital, Toronto. Term Placental tissues were obtained at the University of Kansas Medical Center with consent from patients. All collections and studies were approved by the University of Kansas IRB and the IRB at Mount Sinai Hospital. Fresh term placental tissues were embedded in OCT and cryo-sectioned. Formaldehyde fixed and frozen sections were analyzed by immunohistochemistry.

Laser Capture Micro dissection and analyses of micro-dissected tissues: Tumors generated from transplantation of control and *PRKCI* KD HTS cells were stained for cytokeratin 7 and dissected out using laser capture microdissection (ZEISS Palm Microbeam). Term CTBs/SynTs were also dissected out using the same protocol. RNA was prepared using Arcturus Picopure RNA Isolation kit (Applied Biosystems-12204-01). cDNA was prepared using the entire RNA and amplification of the cDNA was performed using GenomePlex Complete Whole Genome Amplification (WGA) kit (Sigma-WGA2). The sample was purified using QIAquick PCR purification kit (Qiagen- 28104). Concentration of the DNA was measured using Nanodrop ND1000 Spectrophotometer.

mRNA expression analyses: Total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen-74104) using manufacturer's protocol. cDNA was prepared from total RNA (1000ng). Primer cocktail comprising of 200ng/µl oligo dT and 50ng/µl random hexamer was annealed to the RNA at 68^{O} for 10 minutes, followed by incubation with the master mix comprising of 5X first strand buffer, 10mM dNTPs, 0.1M DTT, RNase Inhibitor and M-MLV transcriptase (200U/µl) at 42^{O} for 1 hour. The cDNA solution was diluted to 10ng/µl and heat inactivated at 95^{O} for 5 minutes. Real-time PCR was performed using oligonucleotides (listed below). 20ng equivalent of cDNA was used for amplification reaction using Power SYBR Green PCR master mix (Applied Biosystems-4367659).

Western blot analyses: Cell pellets were washed once with 1X PBS followed by addition of 1X Laemmli SDS-PAGE buffer for protein extract preparation and western blot analyses were performed following earlier described protocol (1). Antibodies used for the study are mentioned below.

RNA interference with mouse and human TSCs: Lentiviral-mediated shRNA delivery approach was used for RNAi. For mouse cells, shRNA were designed to target the 3' UTR sequence 5'-GTCGCTCTCGGTATCCTGTC-3' of the mouse *Prkci* gene. For control, a scramble shRNA (Addgene-1864) targeting a random sequence 5'-CCTAAGGTTAAGTCGCCCTCGC-3' was used. For human cells, short-hairpin RNA against *PRKCI* target sequence 5'-AGTACTGTTGGTTCGATTAAACTCGAGTTTAATCGAACCAACAGTACT-3' was used to generate lentiviral particles (Sigma Mission-TRCN0000219727). Lentiviral particles were generated by transfecting HEK293T cells. Earlier described protocols (2) were followed to collect and concentrate viral particles. Mouse and human TSCs were transduced using viral particles with equal MOI at 60-70% confluency. The cells were treated with 8µg/ml polybrene

prior to transduction. Cells were selected in the presence of puromycin (1.5-2µg/ml). Selected cells were tested for knockdown efficiency and used for further analyses. Freshly knocked-down cells were used for each individual experimental set to avoid any silencing of shRNA expression due to DNA-methylation at LTR. To generate data at least four individual experiments were done to get statistically significant results.

Immunofluorescence and Immunohistochemistry analyses: Immunofluorescence was performed using 10µm embryo cryosections. The sections were fixed using 4% paraformaldehyde in 1X PBS, permeabilized using 0.25% Triton X-100 in 1X PBS and blocked for 1 hour using 10% Normal Goat serum (Thermo Fisher scientific- 50062Z). The details of antibodies used are listed below. Immunohistochemistry was performed using paraffin sections of human placenta. The slides were deparaffinized by histoclear and subsequently with 100%, 90%, 80% and 70% ethanol. Antigen retrieval was done using Decloaking chamber at 80^{o}C for 15minutes. The slides were washed with 1X PBS and treated with 3% H_2O_2 to remove endogenous peroxidase followed by 3 times wash with 1X PBS. 10% goat serum was used as a blocking reagent for 1hour at RT followed by overnight incubation with 1:100 dilution of primary antibody or IgG at 4^{o}C. The slides were washed with 1X PBS and 1:200 dilution of secondary antibody was used for 1 hour at RT. The slides were washed again with 1X PBS followed by treatment with horseradish peroxidase streptavidin for 20 minutes at RT. The slides were washed again and proceeded to color development using DACO 1ml buffer and 1 drop of chromogen. The reaction was stopped in distilled water after sufficient color developed. The slides were counterstained with Mayer's hematoxylin for 5minutes and washed with warm tap water until sufficient bluish coloration observed. The slides were then dehydrated by sequential treatment using 70%, 80%, 90%, 100% ethanol and histoclear. The sections were completely dried and mounted using Toluene as mountant and imaged using Nikon TE2000 microscope.

Cell proliferation assay: Mouse and Human TSCs were seeded (30,000cells/well of 12 well plate) and cultured for 24, 48, 72, 96 hours to assess cell proliferation. Cell proliferation was assessed using both BrDU labeling assay and detection kit (Roche Ref#11296736001) in live cells and with MTT assay kit (Catalog# Sigma CGD1) after harvesting cells at distinct time intervals. We followed manufacturers' protocol.

RNA-In Situ hybridization (RNA-ISH): RNA-ISH was performed using ACDBio RNASCOPE kit (Catalog # 320851 and 322310) and following manufacturer's protocol. The probes for mouse *Prkci* (Catalog#403191), *Dlx3* (Catalog#425191), *Syna* (Catalog#446701), *Gcm1* (Catalog#429661), *Tpbpa* (Catalog#405511) and human *GATA2* (Catalog#414361) and *GCM1* (Catalog#429711) were custom designed by ACDbio.

Testing HCG secretion from Human TSCs: Cell supernatants were collected from control and *PRKCI* KD HTS at Day5 of differentiation towards syncytiotrophoblast. ELISA kit was used from Sigma (RAB0092-1KT) and manufacturer's protocol was followed.

RNA-seq analyses: Total RNA was used to construct RNA-seq libraries using the Illumina TruSeq Stranded Total RNA Sample Preparation Kit according to manufacturer's instructions. RNA seq was performed using Illumina HiSeq 2500 platform. Raw sequence reads in fastq format were mapped to the *Mus musculus* GRCm38 genome with STAR-2.5.2b (3). Gene expression levels were estimated with StringTie-1.3.0 (4) and cuffmerge (5) from the cufflinks2 (6) package. Gene counts were obtained with htseq-0.6.1p1 followed by differential gene expression analysis with EdgeR (7). The data has been deposited and is available in the GEO database (Accession number- GSE100285).

Oligomers Used for The Study

Genotyping primers:

Gene	Forward	Reverse
Prkci wildtype	*AACACCAGGGAGAGTGG*	*GCCTAGAAAGTAACCCAC*
Prkci knockout	*AACACCAGGGAGAGTGG*	*GACGAGTTCTTCTGAGGGG*

RT-PCR mouse primers

Gene	Forward	Reverse
Prkci Ex9	*CTAGGTCTGCAGGATTTCG*	*TTGACGAGCTCTTTCTTCAC*
Prkci (Ex1/2)	*TCCGGGTGAAAGCCTACTAC*	*CAAAGGAGATGGAAGGCTCA*
Prkcz	*GGACAACCCTGACATGAACAC*	*GGCCTTGACAGACAGGAAAC*
18SrRNA	*AGTTCCAGCACATTTTGCGAG*	*TCATCCTCCGTGAGTTCTCCA*
Cdx2	*GGACGTGAGCATGTATCCTAGCT*	*TAACCACCGTAGTCCGGGTACT*
Eomes	*ACCAATAACAAAGGTGCAAACAAC*	*TGGTATTTGTGCAGAGACTGCAA*
Esrrb	*AGTACAAGCGACGGCTGG*	*CCTAGTAGATTCGAGACGATCTTAGTCA*
Elf5	*ATGTTGGACTCCGTAACCCAT*	*GCAGGGTAGTAGTCTTCATTGCT*
Gata3	*CGGGTTCGGATGTAAGTCGA*	*GTAGAGGTTGCCCCGCAGT*
Gcm1	*AGAGATACTGAGCTGGGACATT*	*CTGTCGTCCGAGCTGTAGATG*
Dlx3	*CACTGACCTGGGCTATTACAGC*	*GAGATTGAACTGGTGGTGGTAG*
Tead4	*ATCCTGACGGAGGAAGGCA*	*GCTTGATATGGCGTGCGAT*
Gata2	*GGAAGATGTCCAGCAAATCC*	*TGGAGAGCTCCTCGAAACAT*
Pparg	*AGCTGTCATTATTCTCAGTGGAG*	*ATGTCCTCGATGGGCTTCAC*
Ascl2	*AAGCACACCTTGACTGGTACG*	*AAGTGGACGTTTGCACCTTCA*
Hand1	*CTACCAGTTACATCGCCTACTTG*	*ACCACCATCCGTCTTTTTGAG*
Cx31	*TCTGGCTGTCAGTAGTGTTCG*	*GCCTGGTGTTACAGTCAAAGTC*
Tpbpa	*TCCGGTCAGCTAACTGATGA*	*TCCTCTTCAAACATTGGGTGT*
Prl3d1	*ACATTTATCTTGGCCGCAGATGTGT*	*TTTAGTTTCGTGGACTTCCTCTCGAT*

RT-PCR human primers:

Gene	Forward	Reverse
PRKCI	*AGGTCCGGGTGAAAGCCTA*	*TGAAGAGCTGTTCGTTGTCAAA*
PRKCZ	*ATGACGAGGATATTGACTGGGT*	*CAGGAGTGTAATCCGACCAGG*
GATA2	*CCAGCTTCACCCCTAAGCAG*	*CCACAGTTGACACACTCCCG*
PPARG	*ACCAAAGTGCAATCAAAGTGGA*	*ATGAGGGAGTTGGAAGGCTCT*
GCM1	*GGCGCAAGATCTACCTGAGA*	*CACAGTTGGGACAGCGTTT*
ERVW-1	*CTACCCCAACTGCGGTTAAA*	*GGTTCCTTTGGCAGTATCCA*
CGA	*TCTGGTCACATTGTCGGTGT*	*TTCCTGTAGCGTGCATTCTG*
CGB	*GTGTGCATCACCGTCAACAC*	*GGTAGTTGCACACCACCTGA*
PSG4	*CGATGGGACTGGAGGAGTAA*	*AGTTGCTGCTGGAGATGGAG*
HPRT1	*ACCCTTTCCAAATCCTCAGC*	*GTTATGGCGACCCGCAG*
18SrRNA	*AACCCGTTGAACCCCATT*	*CCATCCAATCGGTAGTAGCG*
CDX2	*CTGGTTTCAGAACCGCAGAG*	*TGCTGCTGCAACTTCTTCTT*
ELF5	*CTGCCTTTGAGCATCAGACA*	*TCCAGTATTCAGGGTGGACTG*
TEAD4	*ACGGCCTTCCACAGTAGCAT*	*CTTGCCAAAACCCTGAGACT*

Antibodies Used for the Study

List of primary antibodies:

Name	Company	Catalog#
PKCiota	*BD Transduction Laboratories*	*610175*
Pan-cytokeratin	*Abcam*	*ab9377*
CDX2	*Abcam*	*ab76541*
β-actin	*Sigma*	*A5441*
HCGβ	*Abcam*	*ab53087*
Cytokeratin 7	*Dako*	*M7018*
E-cadherin	*Abcam*	*ab1416*
PPARγ (mouse)	*Proteintech*	*16643-1-AP*
PPARγ (human)	*SantaCruz*	*sc81152*
GATA2	*Abcam*	*ab109241*
MCT1	*EMD Millipore*	*AB1286-I*
MCT4	*EMD Millipore*	*AB3314P*
Proliferin	*Santa Cruz*	*sc-47347*

List of secondary antibodies:

Name	Company	Catalog#
Alexa fluor 488 goat anti-rabbit IgG	*Invitrogen*	*A11008*
Alexa fluor 568 goat anti-mouse IgG	*Invitrogen*	*A11031*
Alexa fluor 488 donkey anti-mouse IgG	*Invitrogen*	*A21202*
Alexa fluor 568 donkey anti-rabbit IgG	*Invitrogen*	*A10042*
Alexa fluor 568 donkey anti-goat IgG	*Invitrogen*	*A11057*
Alexa fluor 488 goat anti-chicken IgY	*Invitrogen*	*A32931*
Goat anti-mouse IgG-HRP	*Santa Cruz*	*sc2005*
Goat anti-rabbit IgG-HRP	*Santa Cruz*	*sc2004*

References mentioned in the Supplementary Methods:

1. 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.

2. Home P*, et al.* (2009) GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. *J Biol Chem* 284(42):28729-28737.

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1. (A) Immunofluorescence of mouse blastocyst using anti-PKCλ/ι antibody (red), anti-CDX2 (green) and DAPI. Images show that PKCλ/ι is expressed in both inner cell mass and in trophectoderm. (B) RNA in situ hybridization was performed using fluorescent probes against *Prkci* mRNA in E7.5 mouse implantation site. Images show expression of *Prkc*i mRNA (red punctate dots marked by red arrows) in the embryonic cells as well in the TSPCs within placenta primordium. (C) RNA in situ hybridization was performed in E9.5 mouse implantation site showing prominent *Prkc*i mRNA expression in the labyrinth zone and maternal decidua. (D) Immunofluorescence of E12.5 mouse embryo using anti-pan-cytokeratin (PanCK, trophoblast marker) antibody (left panel) and anti-PKCλ/ι antibody (right panel). Nuclei were stained with DAPI. At this developmental stage, PKCλ/ι protein expression is prominent in both placenta and in the embryo proper. (E) The image shows a region of a term placenta after part of the SynT layer was captured via LCM. (F) Quantitative RT-PCR analyses using isolated CTBs from first-trimester and term placentae and LCM-captured SynTs (mean ± SE; n = 4, p≤0.001). The plot shows more abundant *PRKCI* mRNA level in first-trimester CTBs compared to that in term CTBs and strong repression of *PRKCI* mRNA expression in SynTs.

Figure S2. (A) Tables showing total number of embryos analyzed at E7.5 and E9.5 stages. (B) Quantitative RT-PCR analyses using placental tissue from control and PKCλ/ι KO embryos. *Prkcz* mRNA level remained unaltered in KO placentae. PKCλ/ι KO placentae showed near complete loss of *Gcm1*, a SynT-specific gene.

Figure S3. (A) Developing control and PKCλ/ι KO embryos were isolated at ~E9.5 and representative images are shown. The control embryo shows an organized layer of PLF-expressing (Green) parietal TGC. In contrast, the PKCλ/ι KO placenta contains multiple layers

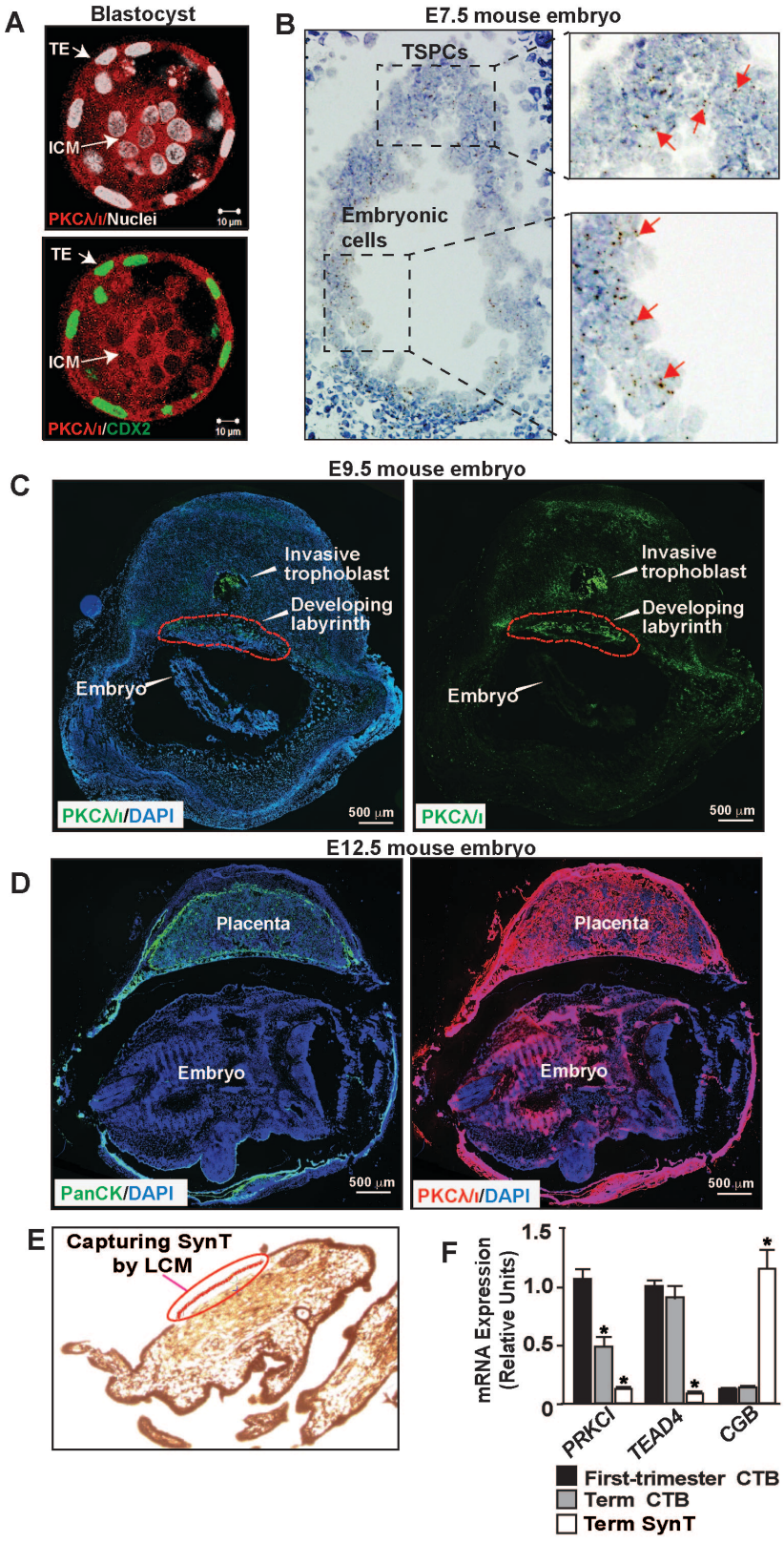
of TGCs. The PKCλ/ι KO placenta is one of the few examples, where other trophoblast cells (within red border) can be visible besides TGCs. The relative sizes of the control and PKCλ/ι KO embryo and placentae are also highlighted. (B) Control and PKCλ/ι KO placentae (Shown in A above) were analyzed via ISH to test expression of marker genes that are representative of different trophoblast cell types. The red borders indicate the developing labyrinth region. Both control and PKCλ/ι KO placentae show presence of *Tpbpa*-expressing and *SynA* expressing (yellow arrows) cells. However, *Gcm1*-expressing cells (green arrows) were not detected in PKCλ/ι KO placenta. (C) Control and PKCλ/ι KO placentae were immunostained for MCT1 (green) and MCT4 (red) expression pattern to test formation of SynT-I and SynT-II populations, respectively. The fluorescence image panel for the control placenta shows formation of two continuous SynT layers. In contrast, the PKCλ/ι KO placenta lacks any MCT4-expressing cells but contains few MCT1 expressing cells. However, unlike in control embryos, the MCT1 expressing cells in PKCλ/ι KO placenta are dispersed, indicating lack of formation of a matured SynT-I population, which arise via cell-cell fusion.

Figure S4. (A) Table shows total number of *Prkci KD* embryos, analyzed for the study. (B) Control and *Prkci* KD mouse embryos were immunostained with anti-PKCλ/ι antibody (red) and DAPI. PKCλ/ι expression was significantly reduced in the TGCs of *Prkci* KD placenta (marked by white arrows). In contrast high level of PKCλ/ι expression was maintained in trophoblast cells of control placenta (marked by yellow arrows). (C) Consecutive sections of *Prkci* KD mouse embryo were immunostained with anti-Pan Cytokeratin (Pan CK, left panel, (red)] and anti-PLF (right panel, green) antibodies. Images show that almost all of the Pan-CK expressing cells are PLF-expressing TGCs.

Figure S5. (A) Micrographs show maintenance of stem state colony morphology of both control and *PRKCI* KD human TSCs for multiple passages. (B) Immunofluorescence images show incorporation of BrdU in both control and *PRKCI* KD human TSCs, indicating maintenance of cell proliferation (C) Quantitative Assessment of cell proliferation rate of control and *PRKCI* KD human TSCs by MTT assay. (D) Quantitative RT-PCR analyses (mean ± SE; n = 4, $p \leq 0.01$) showing relative mRNA expression of trophoblast stem state genes in control and *PRKCI* KD human TSCS.

Figure S6. (A) Regions of trophoblastic lesion, obtained after transplantation of control and *PRKCI KD* human TSCs in immunocompromised mice were captured via LCM for quantitative mRNA expression analyses. Images show example of such a region before and after cell capturing. (B) Quantitative RT-PCR analyses using LCM-captured cell from trophoblastic lesions (mean ± SE; n = 3, $p \leq 0.01$). The plot shows significant downregulation of both *GATA2* and *GCM1* mRNA expressions in lesions, derived from *PRKCI* KD human TSCs. The *PPARG* mRNA level was also reduced but the data was not statistically significant. (C) The reduced levels of *GATA2* and *GCM1* mRNA expression were also confirmed by ISH.

Supplementary Figure1



Supplementary Figure 2

A

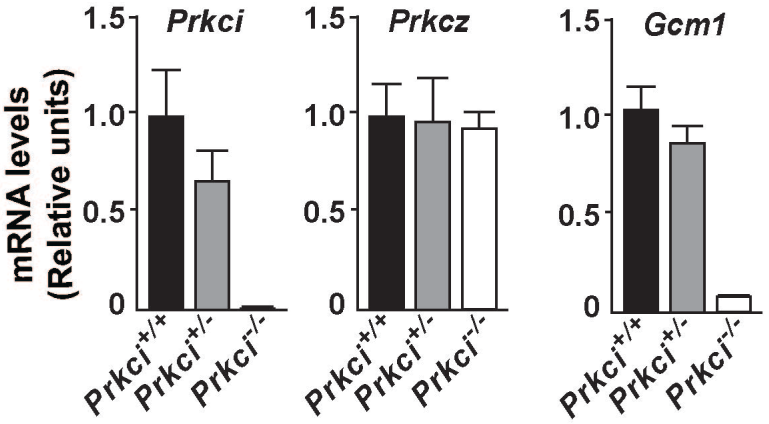
Analyses at E7.5

	Total No of embryos Analyzed (86) (From 12 litters)	Average length of embryo
WT	16	1 ± 0.1 mm
HET	50	1 ± 0.1 mm
KO	20	1 ± 0.1 mm

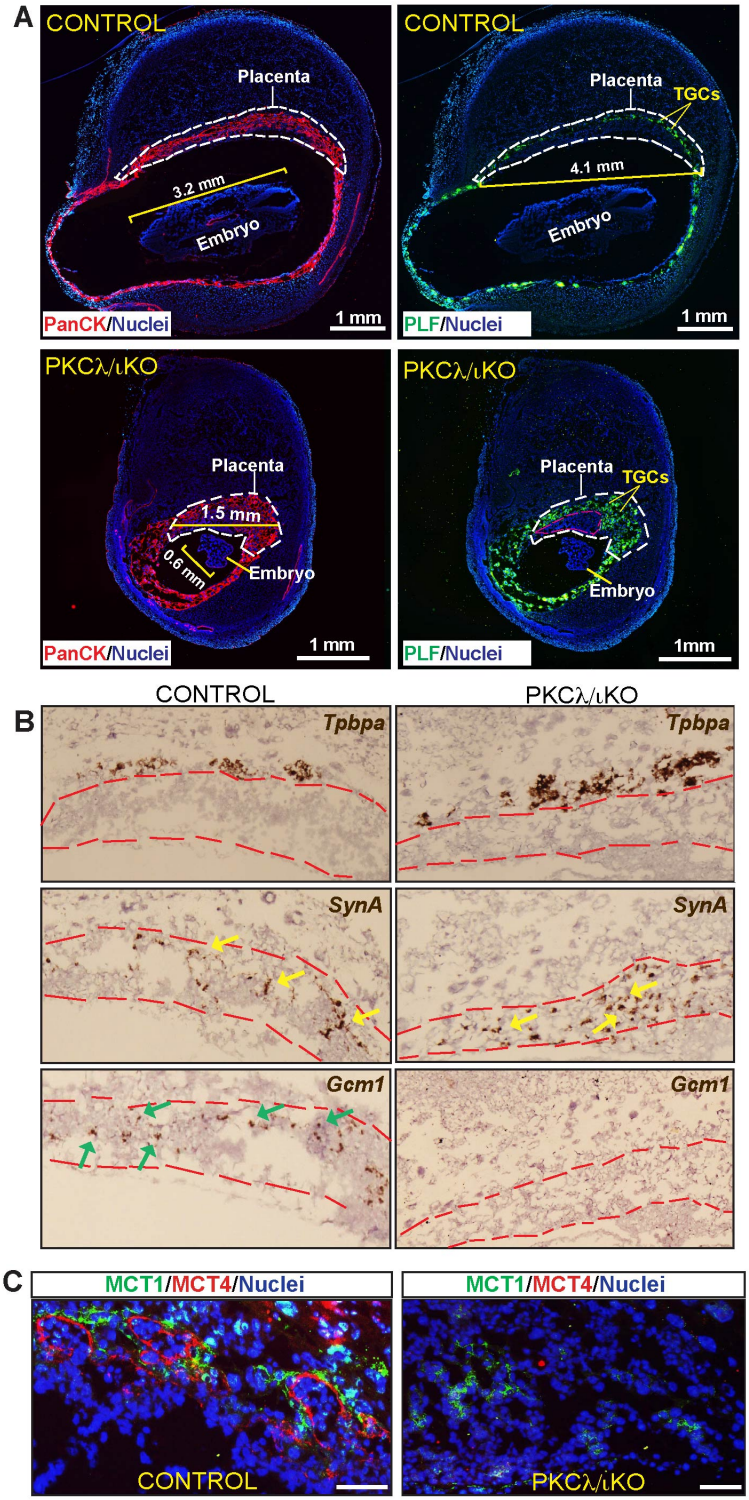
Analyses at E9.5

	No of embryos dissected (66 from 8 litters)	No of embryo sections analyzed (101 from 11 litters)	Average length of embryo	Average length of developing placenta with labyrinth
WT	14	29	3 ± 0.4 mm	3.1 ± 1 mm
HET	37	40	3 ± 0.4 mm	3.1 ± 1 mm
KO	15	32	0.9 ± 0.3 mm	1.5 ± 0.7mm

B Gene expression analyses in E9.5 placentae



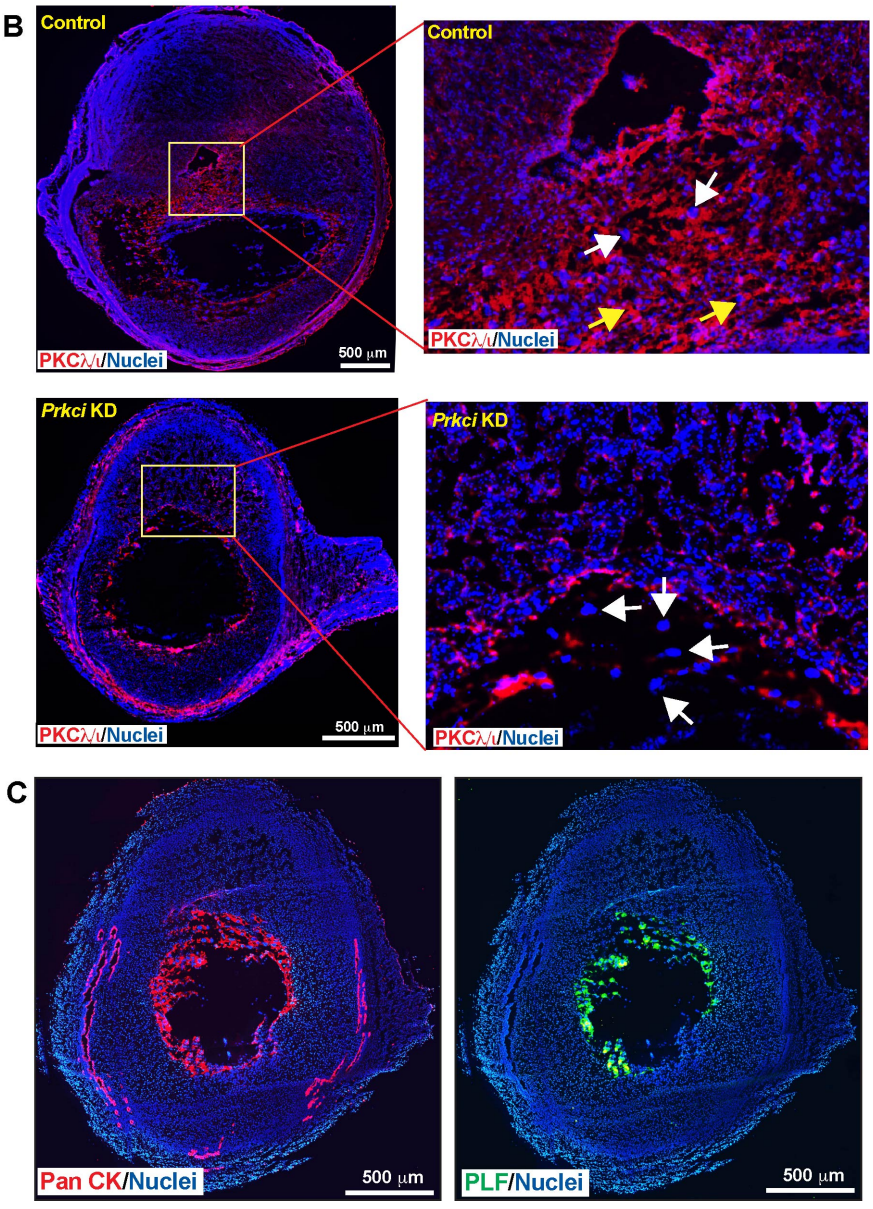
Supplementary Figure 3



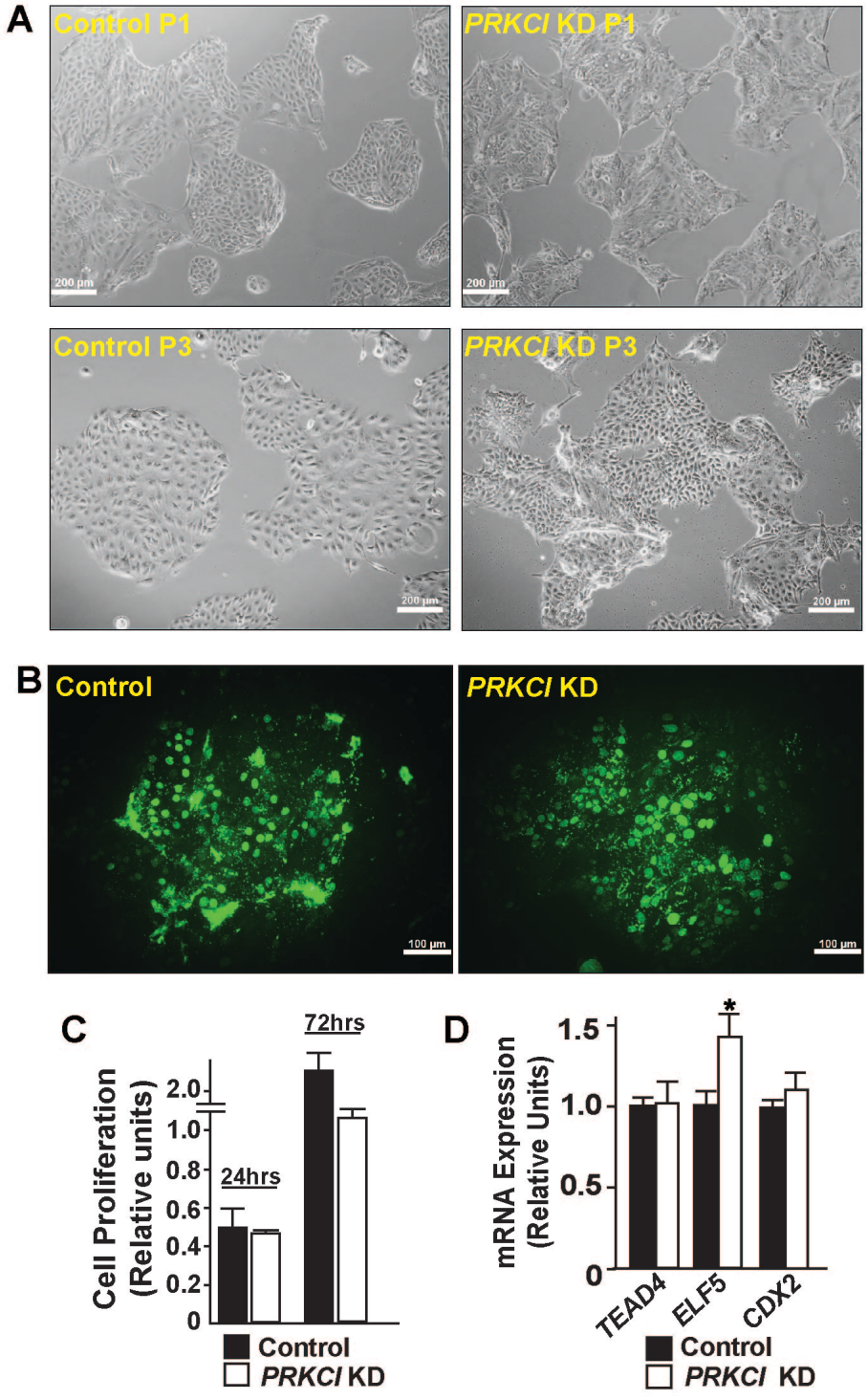
Supplementary Figure 4

A

Total No of embryos analyzed (71 from 8 litters)	No of embryos dissected	No of embryos immunostained
Control	18	17
Prkci TE KD	21	15



Supplementary Figure 5



Supplementary Figure 6