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Supplemental information

Essential role of Mg^{2+} in mouse preimplantation embryo development revealed by TRPM7 chanzyme-deficient gametes

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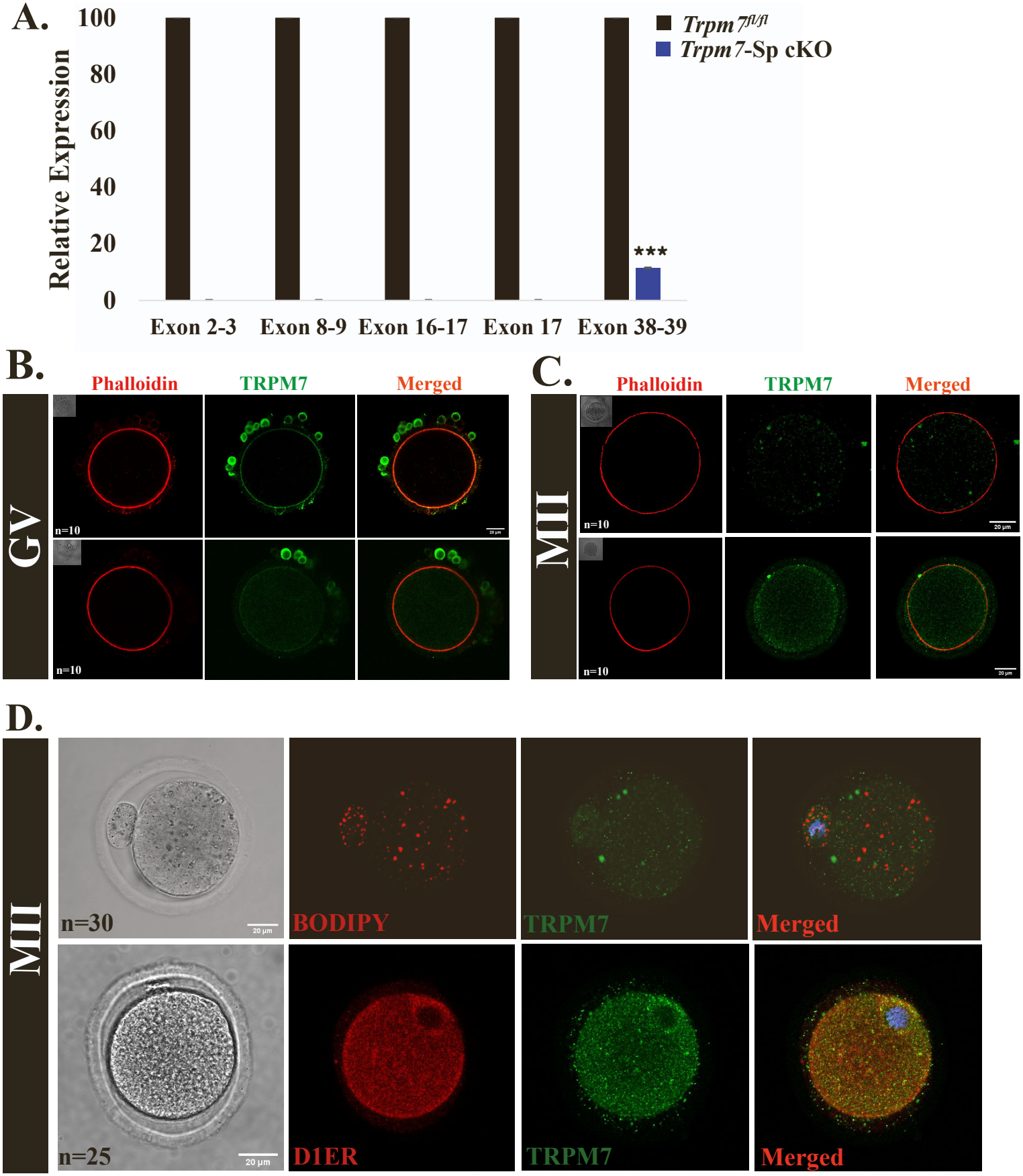
A.
Relative Expression
100
80
60
40
20
0
Trpm7fl/fl
Trpm7-Sp cKO
***
Exon 2-3
Exon 8-9
Exon 16-17
Exon 17
Exon 38-39
B.
Phalloidin
TRPM7
Merged
GV
n=10
n=10
C.
Phalloidin
TRPM7
Merged
MII
n=10
n=10
D.
MII
n=30
BODIPY
TRPM7
Merged
n=25
D1ER
TRPM7
Merged
20 μm

Figure S1. *The conditional deletion of Trpm7 prevents expression of Trpm7 mRNA in spermatozoa, while* TRPM7 *intensity and distribution changes during oocyte maturation with minimal colocalization with two intracellular organelles.* **A.** Sperm-specific deletion of *Trpm7, Trpm7*-Sp cKO, was performed in mice carrying *loxP* sites flanking exon 17, $Trpm7^{fl/fl}$, followed by mating with transgenic males expressing a *Hspa2-Cre* recombinase. Primers to detect the gene mutation bound to exons 2-3, 8-9, 16-17, and 38-39 of *Trpm7*. Real-time PCR using total RNA extracted from $Trpm7^{fl/fl}$ sperm (controls) or sperm of Sp-cKO males showed absence or reduced *Trpm7* expression in KOs vs. controls. **B.** IF images of $Trpm7^{fl/fl}$ oocytes and **(C)** eggs displaying phalloidin (actin-red- left panels) and TRPM7 signals (green-center panels); merged images are shown in the right panels. The upper panels were captured with the threshold intensity used for GV oocytes, whereas the lower panels were captured using the threshold intensity used for MII eggs. **D.** IF image of $Trpm7^{fl/fl}$ eggs displaying staining of lipid vesicles (BODIPY, red-upper panels) and ER (endoplasmic reticulum) staining (D1ER, red-lower panels). The right panels are the merged images showing minimal colocalization of TRPM7 with the identified organelles. Bright-field images are shown as insets in the left columns. Data are presented as control mean ± SEM; ***P<0.001 (*Student's t-test*). n= oocytes and eggs examined for each condition. *Related to Figure 1.*

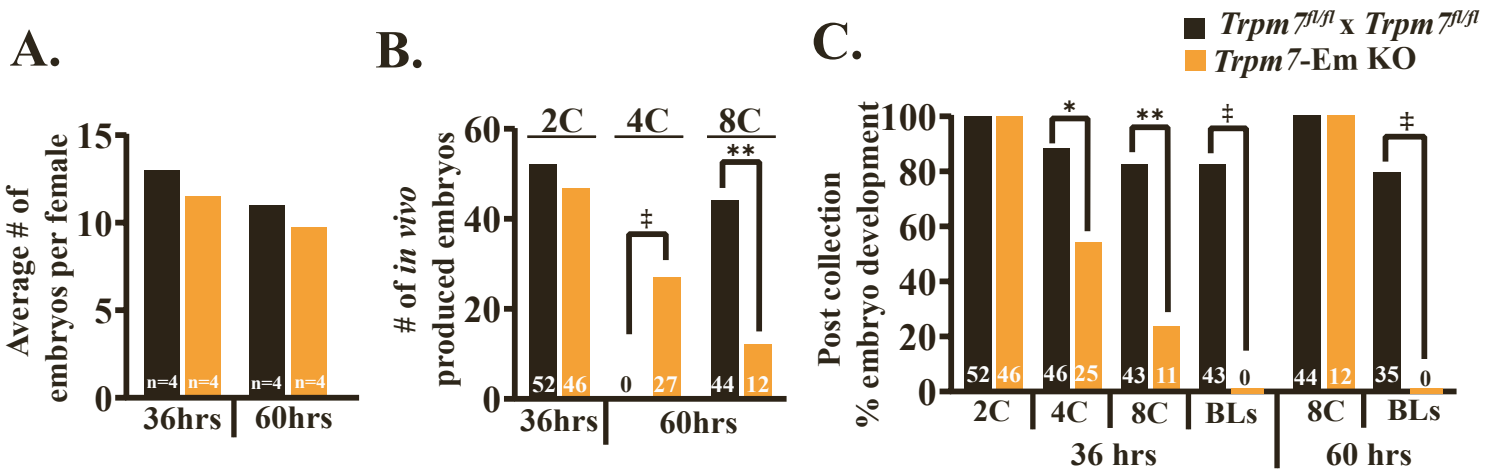

Figure S2. *Trpm7*-Em KO *embryos in vivo undergo development arrest between the 4C and 8C stages.* **A-C** Bar graphs depicting the average number of embryos collected per female **(A)**, number of *in vivo* produced embryos collected 36 h post-mating (2C) or after 60 h (8C) **(B),** and % of *in vitro* development post-collection of embryos collected 36 and 60h post-mating from *Trpm7*$^{fl/fl}$ and *Trpm7*-Em KO pairing. *P<0.05; **P<0.01 (*Student's t-test*) (**C**). n= embryos examined for each condition. *Related to Figure 2.*

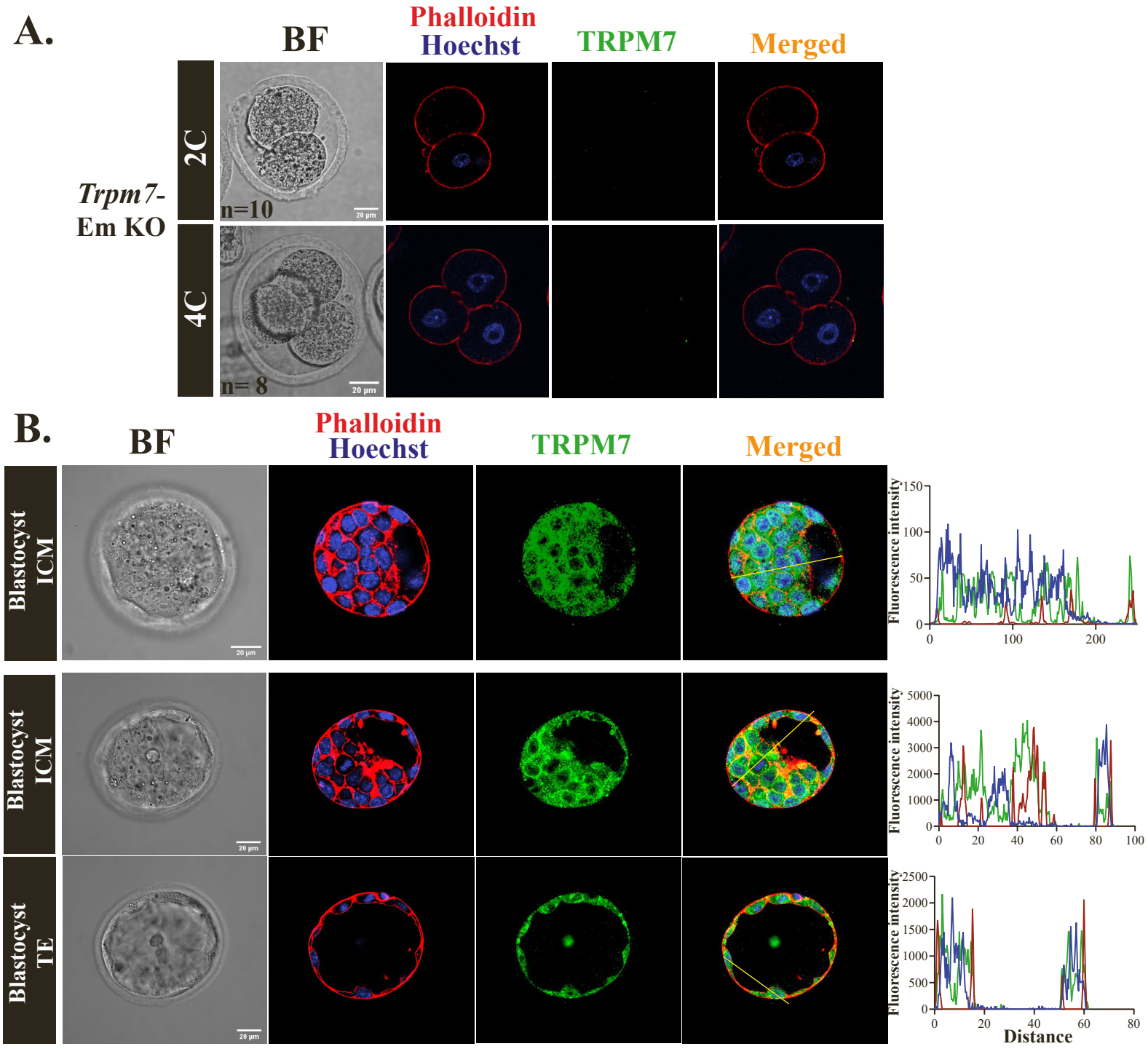

Figure S3. *Conditional deletion of Trpm7 abolishes expression of the chanzyme in 2C and 4C embryos and expression in inner cell mass (ICM) and trophectoderm (TE) of BLs.* **A.** Bright-field (left column) and IF images, Phalloidin (actin-red), Hoechst (DNA-blue) (center left column), TRPM7 (green) (center right column), and Merged (right column), are shown. **B.** Representative confocal images of a BL section that includes ICM and TE cells; two BLs assessed and ICM in the top row corresponds to the BL in **Figure 3**. Left column, BF, bright-field images, first center column, IF images of Phalloidin (red-actin) and Hoechst (blue-DNA). The center-right column shows TRPM7 (green). The rightmost column shows the merged images. A yellow trace in the merged column denotes the region of interest where the fluorescent signals were assessed and displayed in line plots to the right. The white bars represent distance in µm. n= represents the number of embryos examined. *Related to Figure 3.*

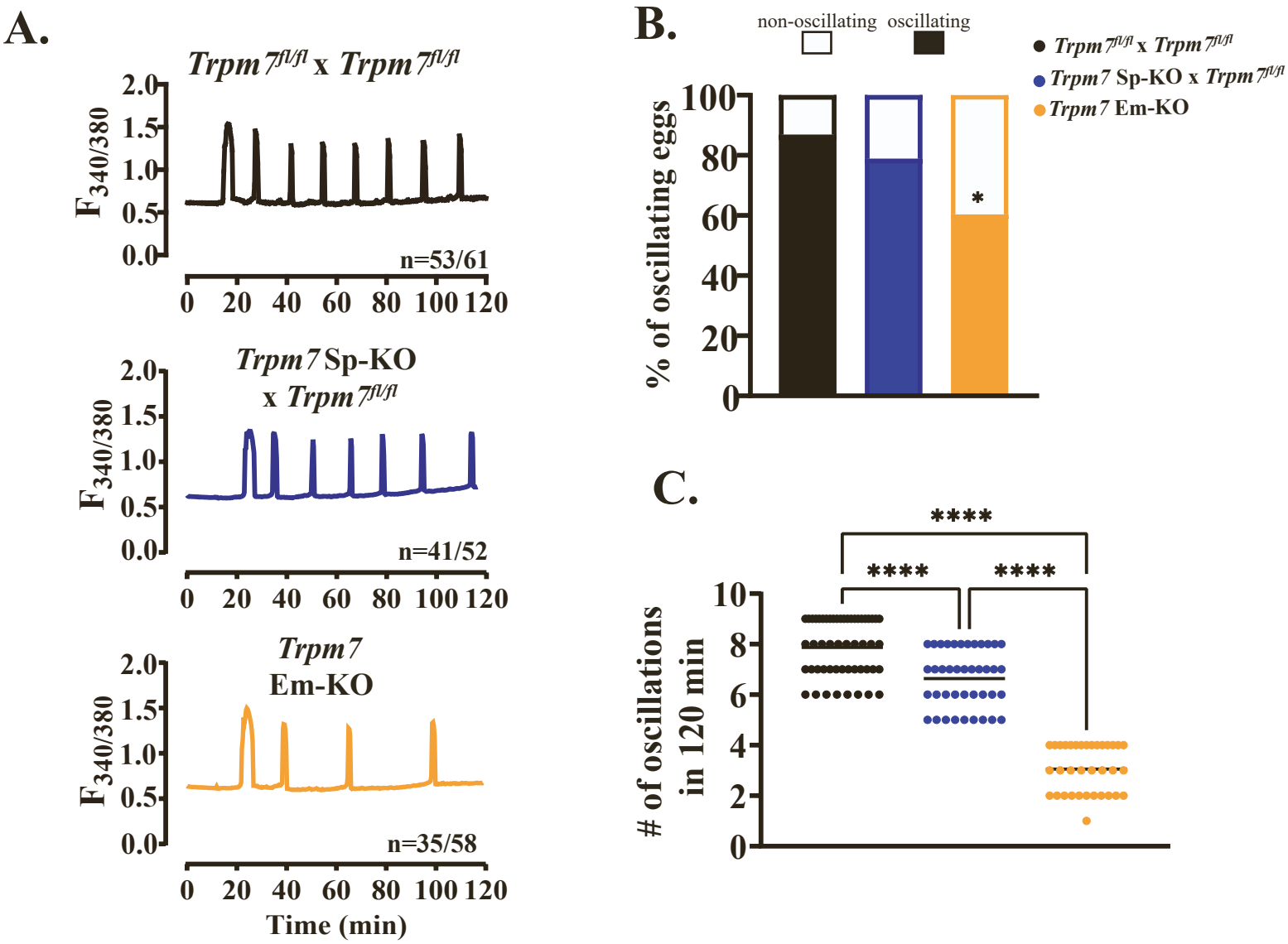

Figure S4. *Trpm7 null sperm initiates lower frequency but persistent fertilization Ca^{2+} oscillations.* **A**. Traces shown correspond, top to bottom, to *Trpm7*$^{fl/fl}$, *Trpm7*-Sp cKO x *Trpm7*$^{fl/fl}$, *Trpm7*-Sp cKO x *Trpm7*-Oo cKO zygotes. Oscillations were monitored for 120 min for all gamete combinations and parameters quantified. N= represents the number of oscillating zygotes over the total number of monitored eggs for each strain combination. **B**. Stacked bar graph depicting the number of oscillating eggs in all groups (*P<0.05). **C**. Dot plot showing mean and distribution of the number of Ca^{2+} oscillations in 120 min in groups examined (ANOVA followed by Tukey's posthoc test; ****P<0.0001). *Related to Figure 4.*

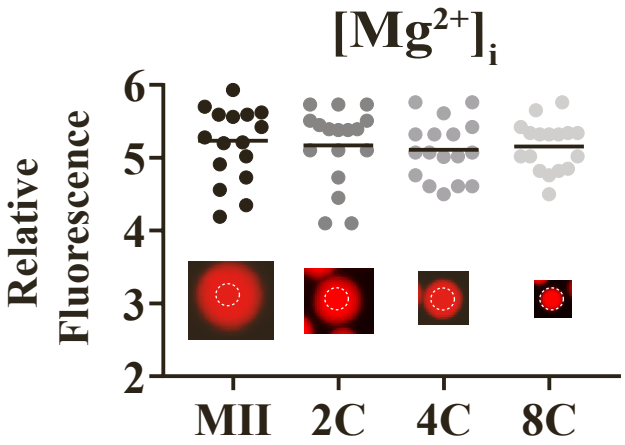

Figure S5. *Intracellular Mg^{2+} concentration remains consistent from eggs and through the 8C stage. A* dot plot graph showing normalized intracellular Mg^{2+} concentrations in *Trpm7*$^{fl/fl}$ eggs and early embryo stages until the 8C stage, where cells from these embryos could be assessed individually. Representative images are shown below the dot plots displaying fluorescence intensities. Intracellular Mg^{2+} levels remained steady, and the broken circles within cells or blastomeres represent the ROI used to assess fluorescence. (ANOVA; *P>0.05). *Related Figure 4.*

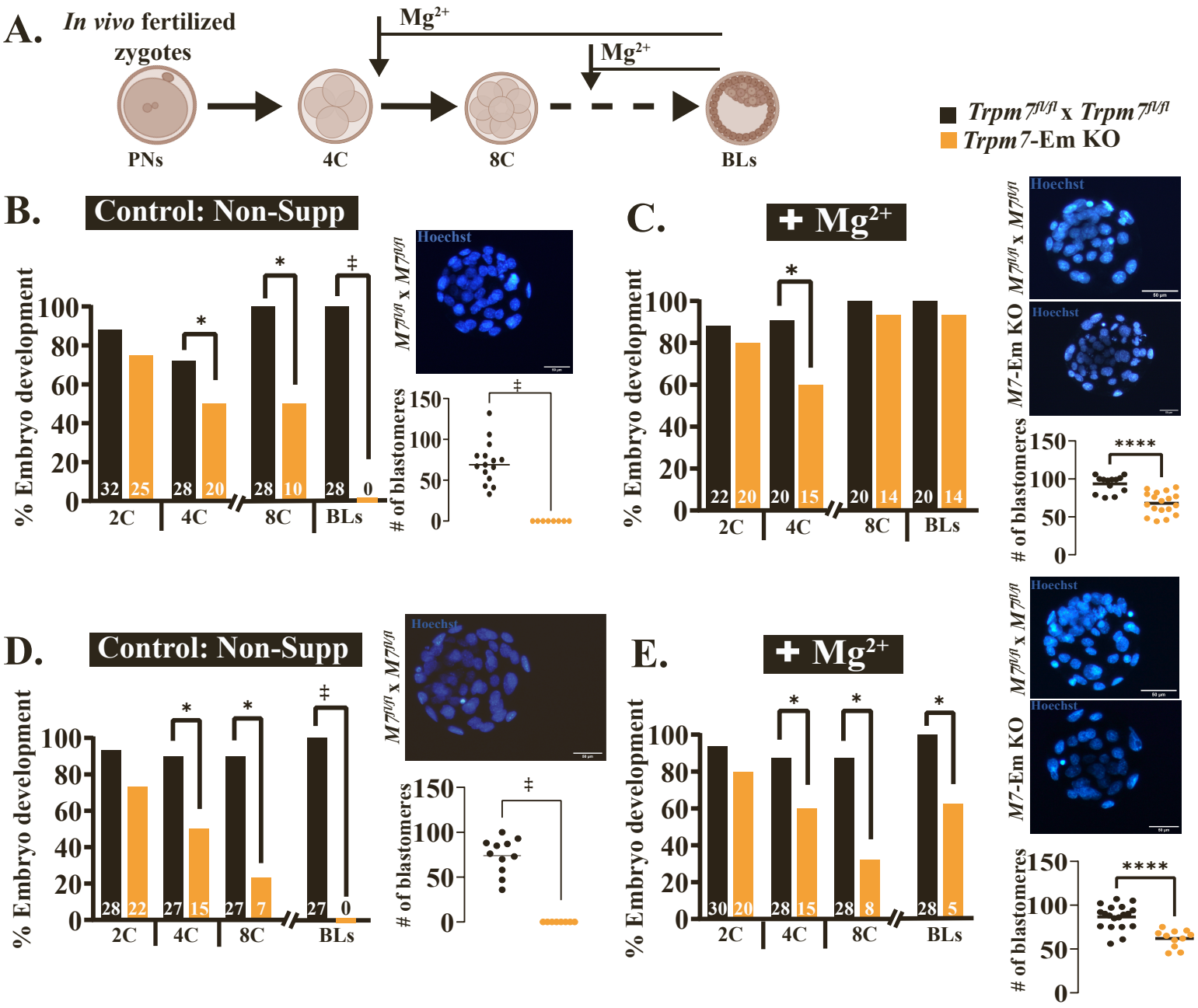

Figure S6. *Mg^{2+} supplementation of 4C- or 8-C embryos differentially rescues their development.* **A.** Schematic showing supplementation of Mg^{2+} at 4C- or 8C embryos followed by culture to the BL stage in supplemented media. **B-E.** Bar graphs depicting development rates of *in vivo* fertilized zygotes from *Trpm7*$^{fl/fl}$ (controls) and *Trpm7*-Em KO mice. **B** and **C**, data for 2C- and 4C embryos are % of embryos reaching each stage relative to the number of PNs that was 100% ($P<0.05$), whereas, for development to the 8C and BL stages, marked by a break in the X-axis, the %s reflect the numbers of embryos that reached each stage relative to the number of 4C embryos at the time of supplementation. **D** and **E**, as B and C, but rates of BL development were calculated from the number of 8C embryos, which was the stage of supplementation. Representative images of BLs derived from 4C- or 8C *Trpm7*$^{fl/fl}$ and *Trpm7*-Em KO embryos supplemented with Mg^{2+} and stained with Hoechst are displayed to the right of each bar graph. Dot plots comparing BL mean cell counts and distribution between strains and treatments are below the DNA images for all groups. (Student's *t-test*; **P <0.01 or ****P<0.0001). *Related to Figure 5.*

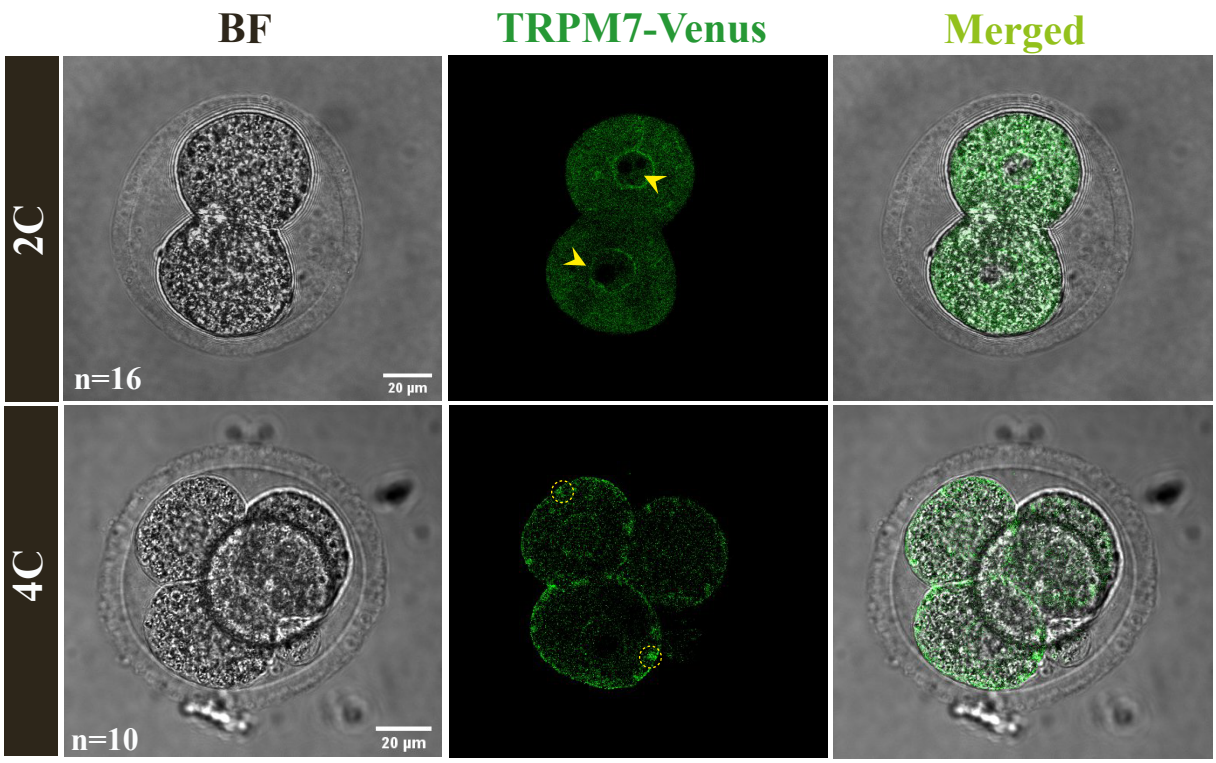

Figure S7. Heterologous e*xpression of Trpm7-Venus mRNA attains distribution of TRPM7 analogous to the endogenous protein in 2C and 4C embryos*. *In vivo*-produced zygotes were injected with *Trpm7-Venus* mRNA, and live-confocal imaging was performed 24- and 48-h later. The left column portrays bright-field (BF) images, the center column displays their fluorescent signals, and the right column shows the merged images. The yellow arrowheads point at the nuclear presence of TRPM7-Venus in 2C embryos, which also showed distinct accumulation in the nuclear membrane. The broken circles in the 4C images denote the presence of cortical/PM accumulations of TRPM7-Venus. *Related to Figure 6.*

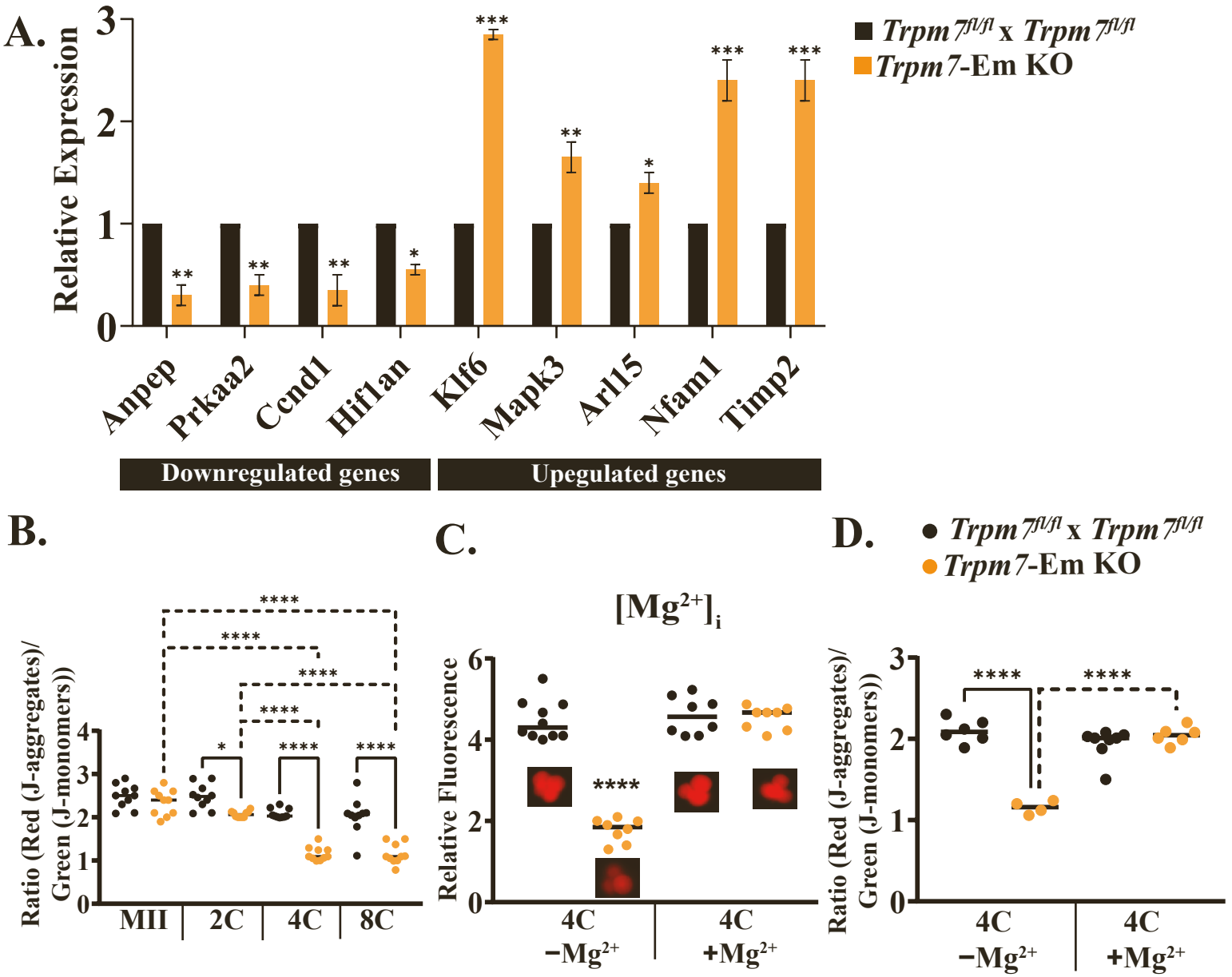

Figure S8. *Reversal of the increased cellular oxidation and decreased JC-1 ratios in Trpm7-Em KO embryos by Mg^{2+} supplementation, which restored intracellular Mg^{2+} levels in these embryos.* **A.** Relative expression of genes shown to be upregulated or downregulated in 4C *Trpm7*-Em KO relative to controls *Trpm7*$^{fl/fl}$ embryos; the selected genes are involved in functions associated with or affected by the absence of TRPM7 and/or Mg^{2+}, which includes oxidative stress pathways (*Anpep, Klf6, Mapk3, Nfam, Prkaa2, Timp2*), gene regulation (*Cnnd1, Hif1an*) and channel function (*Arl15*). The examined genes were selected from the differential expression in two Datasets (Supplementary Tables 3 and 5). Following total RNA extraction, gene expression comparisons were done using *qPCR* and *Actb* as the reference transcript (n=25 embryos per replicate and strain, two replicates). The results were normalized to the C_t values of the reference transcript and plotted in bar graphs. (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; *Student's t tests*). **B, C.** Quantification and mean comparisons of JC-1 ratios performed in MII eggs and embryos examined in **Fig. 7E** from *Trpm7*$^{fl/fl}$ and *Trpm7*-Em KO lines following *in vitro* culture without (**B**) or with Mg^{2+} supplementation (**C**). Following staining with JC-1 and acquiring of equivalent red and green images in eggs and embryos, ratios of red (JC-1 aggregates)/green (JC-monomers) fluorescence intensities were calculated and displayed in a dot chart. The solid connecting lines denote comparisons within stages between the two strains, whereas broken lines show comparisons within a strain (ANOVA followed by Tukey's post hoc test; *P<0.05-****P<0.0001). **(D)** *In vivo* produced *Trpm7*$^{fl/fl}$ and *Trpm7*-Em KO zygotes were cultured to the 2C stage, supplemented or not with 10-mM Mg^{2+}, and cultured to the 4C. At this point, the levels of Mg^{2+} were quantified, normalized using the fluorescent dye Mag-Fura 2, and values were displayed in dot plots for all embryos examined; representative images of the relative fluorescent intensity of each cohort are also shown. Non-supplemented *Trpm7*-Em KO embryos displayed lower values than all other groups (ANOVA followed by Tukey's post hoc test; P<0.0001). *Related to Figure 7.*

Table S1. *Trpm7*$^{fl/fl}$ and *Trpm7*-Sp cKO lines have comparable sperm counts and morphology.

Male genotype	Concentration (x 10^6 spermatozoa/ml)	Normal morphology (%)
***Trpm7*$^{fl/fl}$**	13.7±1.9	93.7±0.7
***Trpm7*-Sp cKO**	17.1±3.9$^{n.s.}$	92.7±0.9$^{n.s.}$

Statistical comparisons were done using t-test (middle column) and chi-square ($p>0.05$)

Table S2. *Trpm7*$^{fl/fl}$ and *Trpm7*-Sp cKO lines display normal and equivalent acrosome status and sperm motility parameters before and after capacitation.

	Male genotype	Intact acrosome (%)	Mot (%)	ProMot (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)
NCAP	***Trpm7*$^{fl/fl}$**	79.6±2.3	14.9±3.4	12.2±3.1	187.1±8.0	70.4±3.8	90.1±3.9	36.7±1.8
	***Trpm7*-Sp cKO**	75.1±1.6$^{n.s.}$	12.2±3.9$^{n.s.}$	9.4±3.3$^{n.s.}$	181.2±10.0$^{n.s.}$	72.1±5.9$^{n.s.}$	92.3±5.7$^{n.s.}$	38.9±3.1$^{n.s.}$
CAP	***Trpm7*$^{fl/fl}$**	59.7±2.5	19.7±2.1	14.0±2.3	189.4±10.7	87.9±5.0	109.1±5.0	46.1±3.3
	***Trpm7*-Sp cKO**	59.4±0.8$^{n.s.}$	20.5±2.9$^{n.s.}$	14.8±2.0$^{n.s.}$	192.1±9.5$^{n.s.}$	95.5±2.4$^{n.s.}$	114.6±2.8$^{n.s.}$	49.3±2.4$^{n.s.}$

NCAP-Non-Capacitated, CAP-Capacitated

Mot-Motility, ProMot-Progressive motility

VCL-Curvilinear velocity, VSL-Straight line velocity, VAP-Average path velocity, LIN-Linearity

Statistical comparisons were done using t-test and chi-square, as indicated by the type of data analyzed ($p>0.05$)

Table S6. The sequence of all oligonucleotide primers used in the study.

Genotyping Primers	Source
Gdf9-Cre F: 5′-CAGGTTTTGGTGCACAGTCA-3′ R: 5′-GGCATGCTTGAGGTCTGATTAC-3′	IDT Technologies (Coralville, IA)
Hspa2-Cre F: 5’ GCGGTCTGGCAGTAAAAACTATC-3’ R: 5’-GTGAAACAGCATTGCTGTCACTT-3’	IDT Technologies (Coralville, IA)
***Trpm7*$^{fl/fl}$** F**:** 5′-TTTCTCCAATTAGCCCTGTAG-3′ R: 5′-CTTGCCATTTTACCCAAATC-3′	IDT Technologies (Coralville, IA)
RT-qPCR primers	**Source**

Actb F: 5'-CGGTTCCGATGCCTGAGGCTCTT-3' R: 5'-CGTCACACTTCATGATGGAATTGA-3'	IDT Technologies (Coralville, IA)
Anpep F: 5'GCGTGCAGTCCTAGGTAGATT3' R: 5'CACCCGGTAGGAGTCAGGTA3'	IDT Technologies (Coralville, IA)
Arl15 F**:** 5' TCGCTGTTGCTGGCTTTTTC3' R: 5' GGAGATCAGACATCCGGCAG3'	IDT Technologies (Coralville, IA)
Ccnd1 F: 5'GCTAAACAAGGACCCCCTCC3' R: 5'GCTCCCTACTCTCAGGGTGA3'	IDT Technologies (Coralville, IA)
Hif1an F: 5'GTCCTTGTGCCTTAGGGTCC3' R: 5'GAGGCATGGAGAGTCATCGG3'	IDT Technologies (Coralville, IA)
Klf6 F: 5'GGGAACAGTTTCTGCTCGGA3' R: 5' AGCCTACAGGATTCGTCCCT3'	IDT Technologies (Coralville, IA)
Mapk3 F: 5'ACACTGGCTTTCTGACGGAG R: 5'TGATGCGCTTGTTTGGGTTG3'	IDT Technologies (Coralville, IA)
Nfam1 F: 5' GCCTTAGTCTGCAGTGACCTG R: 5' GTTTGCCAGGGACACCATGA3'	IDT Technologies (Coralville, IA)
Prkaa2 F: 5'GGCAAAGTGAAGACTACCAGG3' R: 5'TCGTAGGAGGGGTCTTCAGG3'	IDT Technologies (Coralville, IA)
Timp2 F: 5'CTCGGAGCGCAATAAAACGG3' R: 5'CCTCTTGATGGGGTTGCCAT3'	IDT Technologies (Coralville, IA)
Trpm7 2F: 5'-GGATAGAGAGCACTTTGACC-3' 3R: 5'- CTGACAAGTTGCTGACAAATC-3' 8F: 5'- GGAGTACCTTCAGGAAAG-3' 9R: 5'- CATACACTCCATCATTGTTTG-3' 16F: 5'-AGAGTGACCTGGTAGATGATACT3' 17F: 5'-AGGATGAAACGATGGCTATGAA-3' 17R: 5'-AGCCGTCCCAT--CCACATATC-3' 38F: 5'-GCCCTGCCAATCTAGGAGAA-3' 39R: 5'-TGCTTCTGATTCTTTGGTGGA-3'	IDT Technologies (Coralville, IA)