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Supplementary Information for

TRPM7 and $Ca_V3.2$ channels mediate Ca^{2+} influx required for egg activation at fertilization

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Supplementary Information

SI Materials and Methods

Mice

Trpm7$^{f/f}$ mice ((1); stock #018784), *Cacna1h*$^{-/-}$ mice ((2); stock #013770), *Gdf9-cre* mice ((3); stock #011062), C57BL/6J females, and B6SJLF1/J males were obtained from The Jackson Laboratory. *Trpm7*$^{f/f}$ females were crossed to *Gdf9-cre* males to generate oocyte-specific *Trpm7* conditional knockout (cKO) mice. *Trpm7*$^{f/f}$ mice were also crossed to *Cacna1h*$^{-/-}$ mice to generate *Trpm7*$^{f/f}$;*Cacna1h*$^{-/-}$ mice. The resulting female *Trpm7*$^{f/f}$;*Cacna1h*$^{-/-}$ mice were then crossed to *Gdf9-cre* males to generate females null for *Cacna1h* and with an oocyte-specific deletion of *Trpm7* (dKO). Some *Trpm7*$^{f/f}$;*Cacna1h*$^{-/-}$ and dKO mice had respiratory distress and reduced viability, likely due to defects in tracheal development previously described for *Cacna1h*$^{-/-}$ mice (4). To determine fertility, seven *Trpm7*$^{f/f}$, seven *Trpm7*$^{f/f}$;*Gdf9-cre*, seven *Trpm7*$^{f/f}$;*Cacna1h*$^{-/-}$, and seven *Trpm7*$^{f/f}$;*Cacna1h*$^{-/-}$;*Gdf9-cre* female mice were bred with B6SJLF1/J males of proven fertility for a period of 6 months. Numbers of litters, time between litters, number and sex of pups were determined. The first litter from each female was used for weight measurements. Pups were weighed weekly starting at one week of age and for 7 more weeks. At 3 weeks of age, pups were weaned and separated by sex. All animal work was performed in accordance with National Institutes of Health and National Institute of Environmental Health Sciences guidelines under approved animal care and use protocols.

Gamete and embryo collection and culture

Six- to ten-week-old female mice were primed by intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG) and sacrificed by CO_2 inhalation 44–48 h later for collection of fully-grown, GV-intact oocytes. For MII egg collection, mice were injected with 5 IU human chorionic gonadotropin (hCG) 46–48 h after eCG and sacrificed 13–15 h later. For generation of one-cell embryos, after eCG and hCG administration, females were mated with B6SJLF1/J males and sacrificed 20 h after hCG. Oocytes

and eggs were collected as previously described (5). One-cell embryos were collected in the same way as MII eggs. Oocytes were cultured in Minimal Essential Medium Alpha (Life Technologies) containing 5% fetal calf serum and 10 μM milrinone, and eggs were cultured in KSOM medium (EMD Millipore, cat# MR-106-D), both in a humidified atmosphere of 5% CO_2 in air at 37°C. One-cell embryos were cultured in KSOM in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 37°C.

RNA isolation and real-time RT-PCR

Total RNA was isolated from 20 eggs from individual *Trpm7*$^{f/f}$ and *Trpm7*$^{f/f}$;*Gdf9-cre* females (5 *Trpm7*$^{f/f}$, 3 *Trpm7*$^{f/f}$;*Gdf9-cre*) using the PicoPure™ RNA Isolation Kit (Thermo Fisher) according to the manufacturer's instructions. Reverse transcription was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Thermo Fisher) using random hexamers. Real-time PCR was done using one egg equivalent per reaction and the *Power* SYBR™ Green PCR Master Mix (Thermo Fisher). β-actin served as an internal control for normalization. Primer sequences were: Trpm7.16F: 5'-AGAGTGACCTGGTAGATGATACT-3'; Trpm7.17F: 5'- AGGATGAAACGATGGCTATGAA-3'; Trpm7.17R: 5'-AGCCGTCCCATCCACATATC-3'; Trpm7.38F: 5'-GCCCTGCCAATCTAGGAGAA-3'; Trpm7.39R: 5'-TGCTTCTGATTCTTTGGTGGA-3'; actin.F: 5'-CGGTTCCGATGCCCTGAGGCTCTT-3'; actin.R: 5'-CGTCACACTTCATGATGGAATTGA-3'. Products were amplified using Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher). Quantification was done after normalizing to β-actin using the comparative C_T method (6).

Electrophysiology

Whole-cell currents were measured at 22-24°C using an Axoptach200B amplifier digitized at 10 kHz (Digidata 1440A) and filtered at 5 kHz. Electrophysiology recordings were performed on the same day of egg isolation up to 8 hours post-collection. Eggs were maintained in human tubal fluid medium (HTF, EMD Millipore) at 37ºC and 5% CO_2. Pipettes of 1-3 MΩ resistance were made from glass capillaries (593600, A-M systems). The intracellular solution contained (in mM): 142 Cs-methanesulfonate, 10

HEPES, 3 NaATP, 0.3 NaGTP, 5 EGTA, 3 $CaCl_2$ (free 100 nM), pH: 7.3-7.4. Concentration of Ca^{2+} was calculated using WincMax Chelator. The external solution for giga seal formation contained (in mM): 125 NaCl, 6 KCl, 20 $CaCl_2$, 20 HEPES-NaOH, pH: 7.3-7.4. TRPM7 basal responses to NS8593 were measured in an external solution containing (in mM): 140 NaCl, 10 HEPES, 10 glucose, 4 KCl, 1 $MgCl_2$, and 2 $CaCl_2$. All voltages were corrected for calculated junction potentials present between the internal and external solution before seal formation. TRPM7 currents were activated by voltage ramps from 100 mV to -100 mV (600 ms, every 2 s) in the presence or absence of NS8593. The holding potential was zero.

In vitro fertilization and Ca^{2+} *imaging*

In vitro fertilization (IVF) was performed as previously described (7) with the following modifications: Fura-2-loaded, zona pellucida-free eggs were adhered to Cell-Tak-treated glass-bottom dishes in 75 µl of BSA-free KSOM. Twenty µl of HTF containing 4 mg/ml BSA (HTF-BSA) was then added, and imaging was started. Three to five minutes later, 5 µl of sperm were added in HTF containing 4 mg/ml BSA to a final concentration of 10^5 sperm/ml, and a final concentration of BSA of 1 mg/ml. Both KSOM and HTF contain 0.2 mM $MgSO_4$. Different parameters, such as duration of the first Ca^{2+} transient, frequency and persistence of oscillations, were calculated as described (7). In some experiments, $CaCl_2$ to a final concentration of 10 mM was added 90 minutes after sperm addition, followed by $MgCl_2$ addition (10 mM final concentration) 20 minutes later. To assess the effects of changes in Mg^{2+} concentration on Ca^{2+} oscillations, imaging was started in a 200-µl drop of BSA-free KSOM/HTF-BSA (3:1). After 35-45 minutes, 20 µl of 20 mM $MgCl_2$ were added to reach a final Mg^{2+} concentration of 1.8 mM. After eggs had at least 3 oscillations in this high Mg medium, 1980 µl of BSA-free KSOM/HTF-BSA (3:1) were added to bring the final concentration of Mg to 0.36 mM, and imaging was performed for another 30-50 minutes. To assess preimplantation development of IVF-generated embryos, zona pellucida-intact eggs were inseminated for 3 h in a 100-µl drop of BSA-free KSOM/HTF-BSA (3:1) with 5 x 10^5 sperm/ml in a

humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 37°C. Fertilized eggs were washed and cultured to the blastocyst stage in KSOM in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 37°C.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) was performed as described (8) with slight modifications. Briefly, a B6SJLF1/J male mouse was sacrificed, epididymides and a portion of vas deferens were removed, and placed in a Petri dish containing a 900-µl drop of HTF/4 mg/ml BSA covered with mineral oil. After making several cuts in the tissue, the dish was returned to the incubator for 10 min to allow sperm to swim out. The sperm suspension was collected into a 1.5-ml microcentrifuge tube, and centrifuged at 700 x g for 5 min at 4°C. After removing the supernatant, 500 µl ice-cold nuclear isolation medium (NIM; (9)) containing 1% PVA (NIM/PVA) was added, the tube was placed in a sonicator water bath, and sonicated for 15 seconds at 4°C to clip sperm heads from tails. The tube was centrifuged at 700 x g for 5 min at 4°C and further washed twice with 500 µl NIM/PVA. The final pellet was resuspended in NIM/PVA containing 50% glycerol and stored at -20°C until use. On the day of injection, 10 µl of sperm suspension was washed twice with NIM/PVA to remove the glycerol and resuspended in 25 µl of NIM/PVA. A small group of eggs (pre-loaded with Fura-2 AM) from control and dKO females were subjected to ICSI in parallel, in two different microinjection rigs for about 5 minutes, and then eggs from both groups were placed side by side in a glass-bottom dish containing 3 parts of BSA-free KSOM and 1-part of HTF + 4 mg/ml BSA. Imaging was done as previously described for IVF (7) and was started ~7-10 minutes after the first egg in each group was injected; therefore, for some eggs the first Ca^{2+} transient was missed.

ER Ca^{2+} stores, store-operated Ca^{2+} entry, and spontaneous Ca^{2+} influx assays

Zona pellucida-intact Fura-2-AM-loaded oocytes or eggs were adhered to glass-bottom dishes in 1.85 ml of Ca^{2+}/ Mg^{2+}/BSA-free CZB. Baseline ratiometric imaging was performed for 3 min, followed by

addition of different reagents diluted in 150 µl of medium. For assays of ER Ca^{2+} stores, thapsigargin was added to a final concentration of 10 µM, except for MII eggs from $Trpm7^{f/f}$ and $Trpm7^{-/-}$ females, for which a thapsigargin concentration of 500 nM was used. Thirty minutes later, $CaCl_2$ was added to a final concentration of 5 mM, and imaging continued for at least 15 minutes. For spontaneous Ca^{2+} influx assays, $CaCl_2$ to a final concentration of 5 mM was added. In experiments using naltriben or mibefradil, oocytes were adhered to glass-bottom dishes in 1.85 ml of Mg^{2+}/BSA-free CZB (containing 2 mM $CaCl_2$). Naltriben was added at 3 minutes (40 µM final concentration), and at 33 minutes (80 µM final concentration). Similarly, mibefradil was added at 3 minutes (10 µM final concentration), and at 33 minutes (50 µM final concentration). The area under the curve was measured for 10 minutes after addition of the different reagents, as described previously (7).

DNA methylation analysis

Female mice were sacrificed by CO_2 asphyxiation, then liver samples were excised, snap frozen on dry ice, and stored at -80ºC until DNA isolation. DNA methylation was measured at the imprinting control regions (ICR) of multiple imprinted genes using bisulfite pyrosequencing. Bisulfite mutagenesis was performed on 1 µg of isolated genomic DNA from tissues using the Epitect Bisulfite Kit (Qiagen). Bisulfite pyrosequencing was carried out as described previously (10). The primer sequences for *H19* ICR, IG-DMR and *Kcnq1ot1* are in (10) and for *Igf2* DMR1 are in (11). Methylation profiles at repetitive elements throughout the genome were assessed using LUMA (12). This assay utilizes *HpaII* and *MspI* restriction cut sites on 1 µg of genomic DNA followed by polymerase extension on the overhangs using pyrosequencing technology to calculate global methylation levels.

Statistical analysis

Statistical analyses (except for mouse weight determinations, see below) were performed using GraphPad Prism software, version 7.0d. Data were tested for normal distribution using the D'Agostino and Pearson

normality test and were analyzed using Student's t-test, Mann–Whitney test, Fisher's exact test, one-way, two-way, or mixed model ANOVA, Kruskal-Wallis test, chi-square test, and appropriate post-hoc tests for multiple comparisons, as indicated in the figure legends. For all graphs, error bars indicate s.e.m.

Mouse weight data were analyzed with mixed-model ANOVA using SAS software, version 9.3. Because the variance structure differed between the offspring from the *Trpm7* cKO (*Trpm7*$^{f/f}$;*Gdf9-cre* vs. *Trpm7*$^{f/f}$) and dKO (*Cacna1h*$^{-/-}$;*Trpm7*$^{f/f}$;*Gdf9-cre* vs. *Cacna1h*$^{-/-}$;*Trpm7*$^{f/f}$) breeding trials, we fit data from these trials in separate models. The model for the mean weight was the same for both and included terms for genotype (control, knockout), sex (female, male) and week (1 through 8) together with their two- and three-way interactions; it also included a regression adjustment for differing litter sizes. We tested hypotheses about sex- and week-specific genotype comparisons using t-tests. The variance models included random effects for litter and week, and they allowed heterogeneous residual variances across weeks with autoregressive temporal correlations. For both *Trpm7* cKO and dKO trials, the litter variance component differed by genotype (control vs. knockout); for the dKO trial, residual variance components also differed by genotype. For the *Trpm7* cKO trial, residual variance components differed between knockout males and the other three sex-genotype categories. We used the model selection criterion AIC to choose these variance models from several candidates and used chi-squared tests to probe whether separate variance components by genotype (dKO trial) or for *Trpm7* cKO males enhanced fit. A detailed description of the mixed-model analyses of both breeding trials is included below.

Full details of mixed models analyses

Analysis of *Trpm7* cKO breeding trial

This section outlines of the results of mixed-model ANOVA fitted to data from the *Trpm7* cKO breeding trial. It has three sections: General, Female, and Male. Data from both sexes were included in this analysis, but most results are reported separately for the two sexes in the appropriate sections.

General

Table S1 illustrates how the variance-covariance matrix of the observations was modeled. The "intercept" parameters estimate a litter-to-litter component of variance; because pups are within litters, it also serves as an estimate of an intra-class "covariance" between data from pups in the same litter (pups from different litters are independent). The "week" parameter is mainly to increase the covariance for measurements from two pups in the same litter in the same week (the idea is that the correlation between pups is likely a bit higher for measurements at the same age than at different ages). The sets of "Var(wk 1) … Var(wk 8)" parameters estimate a between-pups variance component separately for each week (notice that these generally increase across weeks as expected). The "ARH" parameters model temporal correlations between successive measurements for a pup in one particular way; the correlation decays in a prescribed manner the farther apart the measurements are in time. A number of different models were checked for the variance-covariance structure and this one provided a good balance of parsimony and fit as indicated by model selection indices.

Table S1: Covariance Parameter Estimates

Cov Parm	Subject	Group	Estimate	Standard Error	Z Value	Pr Z	Alpha	Lower	Upper
Intercept	litter	trt Con	0.1212	0.1194	1.01	0.1552	0.05	0.03326	4.3756
Intercept	litter	trt Exp	0.3771	0.2693	1.40	0.0807	0.05	0.1344	3.2135
week	litter		0.08992	0.02356	3.82	<.0001	0.05	0.05708	0.1622
Var(1)	pupid(litter)	MES 0	0.2725	0.05373	5.07	<.0001	0.05	0.1917	0.4182
Var(2)	pupid(litter)	MES 0	0.6721	0.1394	4.82	<.0001	0.05	0.4649	1.0575
Var(3)	pupid(litter)	MES 0	1.0293	0.1846	5.58	<.0001	0.05	0.7454	1.5139
Var(4)	pupid(litter)	MES 0	1.4144	0.2247	6.29	<.0001	0.05	1.0598	1.9833
Var(5)	pupid(litter)	MES 0	1.4600	0.2130	6.85	<.0001	0.05	1.1183	1.9871
Var(6)	pupid(litter)	MES 0	1.1701	0.1612	7.26	<.0001	0.05	0.9087	1.5636
Var(7)	pupid(litter)	MES 0	1.0912	0.1433	7.62	<.0001	0.05	0.8570	1.4370
Var(8)	pupid(litter)	MES 0	1.0870	0.1493	7.28	<.0001	0.05	0.8449	1.4512
ARH(1)	pupid(litter)	MES 0	0.7625	0.02789	27.34	<.0001	0.05	0.7078	0.8171
Var(1)	pupid(litter)	MES 1	0.5872	0.1909	3.08	0.0010	0.05	0.3393	1.2550
Var(2)	pupid(litter)	MES 1	1.6330	0.5663	2.88	0.0020	0.05	0.9146	3.7098
Var(3)	pupid(litter)	MES 1	1.9886	0.5893	3.37	0.0004	0.05	1.1987	3.9282
Var(4)	pupid(litter)	MES 1	2.8173	0.7256	3.88	<.0001	0.05	1.8009	5.0251
Var(5)	pupid(litter)	MES 1	1.9550	0.4573	4.27	<.0001	0.05	1.2964	3.2840
Var(6)	pupid(litter)	MES 1	2.0493	0.4511	4.54	<.0001	0.05	1.3890	3.3263
Var(7)	pupid(litter)	MES 1	2.1403	0.4470	4.79	<.0001	0.05	1.4771	3.3790
Var(8)	pupid(litter)	MES 1	2.4631	0.5301	4.65	<.0001	0.05	1.6826	3.9499
ARH(1)	pupid(litter)	MES 1	0.8306	0.03562	23.32	<.0001	0.05	0.7608	0.9005

This variance structure is different from the one used for the dKO breeding trial. Because TRT is applied to litters as units, TRT was used as the grouping variable when allowing different size litter-to-litter variance components. For the week-specific between-pups variance components, however, different grouping variables were chosen because the preliminary plots of standard errors vs. week showed that the male exp group (MES=1) had a different pattern across weeks than the male con, female exp, or female con groups (MES=0).

Table S2 illustrates a comparison between the covariance model in Table S1 and a related model that does NOT have separate parameters for the two levels of MES (0 and 1). The bottom three rows of the table show that two of the three model-selection criteria prefer the model in Table S1 and the third is virtually a toss-up. One can use the first row of Table S2 to construct a likelihood ratio test of whether including the

separate parameters improves the model. This test is a Chi-squared test with 9 df, p=0.006 – evidence that separate variance components by MES improve the model. (Note: this test does not take account of the separate litter variance components by TRT.) Because in Table S2, each variance component for MES=0 is smaller than the corresponding variance component for MES=1, it is fair to conclude that for the *Trpm7* cKO trial, variability is larger for MES=1 than for MES=0.

Table S2: Fit Statistics

Index	Separate variance components by MES	Common variance components across MES
-2 Res Log Likelihood	2080.0	2103.2
AIC (smaller is better)	2122.0	2127.2
AICC (smaller is better)	2123.7	2127.5
BIC (smaller is better)	2135.4	2134.8

Table S3 is the usual ANOVA table. Effects that do not explicitly include "sex" are averaged across the two sexes. The Table foreshadows that several effects involving TRT are statistically significant (p<0.05). Many of the highly significant effects (p<.0001) are completely expected (trt*week is an exception). Note that the litter-size adjustment is non-significant because litter sizes are nearly the same.

Table S3: Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	11.5	21.49	0.0006
sex	1	103	335.60	<.0001
trt*sex	1	103	6.10	0.0151
c_root_litsiz	1	5.84	0.79	0.4091
week	7	67.7	2395.55	<.0001
trt*week	7	67.7	5.54	<.0001
sex*week	7	211	128.87	<.0001
trt*sex*week	7	211	2.25	0.0318

Females

Table S4 shows the data plotted as Least-Squares Means for Females in the *Trpm7* cKO trial (Fig. 3*H*). (Note: the fractional degrees of freedom [DF] may seem odd; they are used to improve p-value estimation when fitting mixed models to unbalanced data – meaning the number of pups differs among litters and between sexes across litters.)

Table S4: Model-based estimates of Weekly Means by TRT for Females in *Trpm7* cKO trial

trt	sex	week	Estimate	Standard Error	DF	t Value	Pr>|t|	Lower	Upper
Con	F	1	4.6112	0.2201	9.78	20.95	<.0001	4.1194	5.1031
Con	F	2	7.2134	0.2559	16.9	28.19	<.0001	6.6733	7.7535
Con	F	3	9.9943	0.2839	24.1	35.20	<.0001	9.4084	10.5801
Con	F	4	14.1239	0.3113	34.4	45.38	<.0001	13.4915	14.7562
Con	F	5	17.2697	0.3143	37.1	54.94	<.0001	16.6329	17.9065
Con	F	6	18.0988	0.2941	30.3	61.54	<.0001	17.4984	18.6992
Con	F	7	18.7705	0.2884	28.6	65.08	<.0001	18.1803	19.3607
Con	F	8	19.0738	0.2884	28.4	66.13	<.0001	18.4834	19.6642
Exp	F	1	4.2734	0.2785	7.96	15.35	<.0001	3.6307	4.9160
Exp	F	2	6.2222	0.3019	10.8	20.61	<.0001	5.5565	6.8880
Exp	F	3	8.6761	0.3205	13.7	27.07	<.0001	7.9873	9.3649
Exp	F	4	12.8166	0.3394	17.2	37.76	<.0001	12.1011	13.5321
Exp	F	5	16.1765	0.3411	17.6	47.43	<.0001	15.4588	16.8941
Exp	F	6	16.7007	0.3272	15.1	51.03	<.0001	16.0035	17.3980
Exp	F	7	17.2083	0.3234	14.4	53.22	<.0001	16.5166	17.9001
Exp	F	8	17.9623	0.3233	14.4	55.56	<.0001	17.2707	18.6539

Table S5 shows estimates and tests for the week-by-week differences in estimated means (based on the fitted model) between Con and Exp for females in the *Trpm7* cKO trial. All these differences are statistically significant except week 1.

Table 5: Estimates of Week-by-Week Con – Exp differences

Effect	Week	Estimate	Standard Error	DF	t Value	Pr > |t|
Con - Exp	**1**	0.3379	0.3626	14.86	0.93	0.3663
Con - Exp	**2**	0.9911	0.4024	21.72	2.46	0.0222
Con - Exp	**3**	1.3182	0.4341	28.65	3.04	0.0051
Con - Exp	**4**	1.3073	0.4660	37.89	2.81	0.0079
Con - Exp	**5**	1.0932	0.4693	39.88	2.33	0.0250
Con - Exp	**6**	1.3981	0.4458	33.65	3.14	0.0035
Con - Exp	**7**	1.5622	0.4392	32.01	3.56	0.0012
Con - Exp	**8**	1.1115	0.4392	31.94	2.53	0.0165

Table S6 summarizes tests of interest for females in the *Trpm7* cKO trial. The test in the row labeled "Overall" asks whether any of the eight Con – Exp differences across the eight weeks are non-zero. The row labeled "Interaction" asks whether the Con-Exp differences change in magnitude across the eight weeks. (These tests with multiple numerator DF have no corresponding estimates and standard errors.) The test labeled "Main" asks whether, averaged across the eight weeks, the Con – Exp average difference is non-zero. The test in the last row asks whether the average Con – Exp difference over the first three weeks (pre-weaning) is different from the average Con – Exp difference over the last five weeks (post-weaning). All of these tests except the pre-post weaning test were statistically significant.

Table S6: Tests of interest for Females in *Trpm7* cKO trial

Effect	Estimate	Standard Error	Num DF	Den DF	F Value	Pr > F
Overall	_	_	8	55.76	2.70	0.0140
Interaction	_	_	7	102.2	2.35	0.0286
Main	1.1399	0.3609	1	15.27	9.99	0.0064
Interaction: Pre- vs. post-weaning	-0.4120	0.2498	1	325	2.72	0.1000

Males

Table S7 shows the data plotted as Least-Squares Means for Males in the *Trpm7* cKO Trial (Fig. 3*H*).

Table S7: Model-based estimates of Weekly Means by TRT for Males in *Trpm7* cKO trial

trt	sex	week	Estimate	Standard Error	DF	t Value	Pr>|t|	Lower	Upper
Con	M	1	4.8445	0.2161	9.12	22.42	<.0001	4.3566	5.3324
Con	M	2	7.3223	0.2472	15	29.62	<.0001	6.7954	7.8492
Con	M	3	10.5629	0.2720	21	38.83	<.0001	9.9972	11.1285
Con	M	4	17.0486	0.2964	29.5	57.51	<.0001	16.4428	17.6543
Con	M	5	21.7973	0.2992	31.6	72.86	<.0001	21.1876	22.4069
Con	M	6	23.6390	0.2811	25.8	84.09	<.0001	23.0609	24.2170
Con	M	7	25.3871	0.2761	24.4	91.96	<.0001	24.8178	25.9564
Con	M	8	26.5161	0.2761	24.3	96.05	<.0001	25.9466	27.0855
Exp	M	1	4.2862	0.2979	10.1	14.39	<.0001	3.6236	4.9489
Exp	M	2	6.3856	0.3534	16.3	18.07	<.0001	5.6374	7.1338
Exp	M	3	8.8844	0.3705	20.8	23.98	<.0001	8.1135	9.6554
Exp	M	4	14.5544	0.4067	27.5	35.79	<.0001	13.7206	15.3883
Exp	M	5	19.5588	0.3693	21.1	52.96	<.0001	18.7910	20.3265
Exp	M	6	20.8090	0.3732	23	55.76	<.0001	20.0369	21.5811
Exp	M	7	22.7889	0.3771	24.1	60.43	<.0001	22.0107	23.5671
Exp	M	8	24.0857	0.3912	27.1	61.57	<.0001	23.2831	24.8883

Table S8 contains estimates and tests for the week-by-week differences in estimated means (based on the fitted model) between Con and Exp for males in the *Trpm7* cKO Trial. All of these differences are statistically significant except week 1.

Table S8: Estimates of Week-by-Week Con – Exp differences

Effect	Week	Estimate	Standard Error	DF	t Value	Pr > |t|
Con - Exp	1	0.5583	0.3756	16.8	1.49	0.1557
Con - Exp	2	0.9367	0.4376	26.59	2.14	0.0416
Con - Exp	3	1.6784	0.4654	35.3	3.61	0.0010
Con - Exp	4	2.4941	0.5085	47.51	4.90	<.0001
Con - Exp	5	2.2385	0.4808	41.64	4.66	<.0001
Con - Exp	6	2.8300	0.4729	40.37	5.98	<.0001
Con - Exp	7	2.5983	0.4731	40.64	5.49	<.0001
Con - Exp	8	2.4304	0.4845	43.91	5.02	<.0001

Table S9 summarizes tests of interest for males in the *Trpm7* cKO trial. The test in the row labeled "Overall" asks whether any of the eight Con – Exp differences across the eight weeks are non-zero. The row labeled "Interaction" asks whether the Con-Exp differences change in magnitude across the eight weeks. (These tests with multiple numerator DF have no corresponding estimates and standard errors.) The test labeled "Main" asks whether, averaged across the eight weeks, the Con – Exp average difference is non-zero. The test in the last row asks whether the average Con – Exp difference over the first three weeks (pre-weaning) is different from the average Con – Exp difference over the last five weeks (post-weaning). All these tests were highly statistically significant.

Table S9: Tests of interest for Males in *Trpm7* cKO trial

Effect	Estimate	Standard Error	Num DF	Den DF	F Value	Pr > F
Overall	_	_	8	66.1	6.50	<.0001
Interaction	_	_	7	109.1	6.00	<.0001
Main	1.9706	0.3890	1	19.88	25.70	<.0001
Interaction: Pre- vs. post-weaning	-1.4604	0.2719	1	363	28.84	<.0001

Analysis of dKO breeding trial

This document outlines of the results of mixed-model ANOVA fitted to data from the dKO breeding trial. It has three sections: General, Female, and Male. Data from both sexes are included in this analysis, but most results are reported separately for the two sexes in the appropriate sections.

General

Table S10 illustrates how the variance-covariance matrix of the observations was modeled. The "intercept" parameters estimate a litter-to-litter component of variance; because pups are within litters, it also serves as an estimate of an intra-class "covariance" between data from pups in the same litter (pups from different litters are independent). The "week" parameter is mainly to increase the covariance for measurements from two pups in the same litter in the same week (the idea is that the correlation between pups is likely a bit higher for measurements at the same age than at different ages). The sets of "Var(wk 1) … Var(wk 8)" parameters estimate a between-pups variance component separately for each week (notice that these generally increase across weeks as expected). The "ARH" parameters model temporal correlations between successive measurements for a pup in one particular way; the correlation decays in a prescribed manner the farther apart the measurements are in time. A number of different models were checked for the variance-covariance structure and this one provided a good balance of parsimony and fit as indicated by model selection indices.

Table S10: Covariance Parameter Estimates

Cov Parm	Subject	Trt	Estimate	Standard Error	Z Value	Pr Z	Alpha	Lower	Upper
Intercept	**litter**	**Con**	0.1574	0.1181	1.33	0.0914	0.05	0.05396	1.5853
Intercept	**litter**	**Exp**	0.7506	0.5179	1.45	0.0736	0.05	0.2744	5.7494
week	**litter**		0.1351	0.03072	4.40	<.0001	0.05	0.09054	0.2233
Var(wk 1)	**pupid(litter)**	**Con**	0.03608	0.008659	4.17	<.0001	0.05	0.02370	0.06154
Var(wk 2)	**pupid(litter)**	**Con**	0.07646	0.01968	3.89	<.0001	0.05	0.04889	0.1363
Var(wk 3)	**pupid(litter)**	**Con**	0.2935	0.07550	3.89	<.0001	0.05	0.1877	0.5231
Var(wk 4)	**pupid(litter)**	**Con**	0.5161	0.1162	4.44	<.0001	0.05	0.3471	0.8482
Var(wk 5)	**pupid(litter)**	**Con**	0.6678	0.1380	4.84	<.0001	0.05	0.4625	1.0488
Var(wk 6)	**pupid(litter)**	**Con**	0.7002	0.1344	5.21	<.0001	0.05	0.4966	1.0613
Var(wk 7)	**pupid(litter)**	**Con**	0.8195	0.1524	5.38	<.0001	0.05	0.5870	1.2247
Var(wk 8)	**pupid(litter)**	**Con**	1.0058	0.2010	5.00	<.0001	0.05	0.7044	1.5535
ARH(1)	**pupid(litter)**	**Con**	0.5626	0.05294	10.63	<.0001	0.05	0.4589	0.6664
Var(wk 1)	**pupid(litter)**	**Exp**	0.1536	0.05210	2.95	0.0016	0.05	0.08698	0.3415
Var(wk 2)	**pupid(litter)**	**Exp**	0.4796	0.1706	2.81	0.0025	0.05	0.2652	1.1180
Var(wk 3)	**pupid(litter)**	**Exp**	1.3384	0.4289	3.12	0.0009	0.05	0.7786	2.8235
Var(wk 4)	**pupid(litter)**	**Exp**	2.3686	0.7366	3.22	0.0007	0.05	1.3971	4.8686
Var(wk 5)	**pupid(litter)**	**Exp**	1.2540	0.3227	3.89	<.0001	0.05	0.8018	2.2355
Var(wk 6)	**pupid(litter)**	**Exp**	1.3098	0.3121	4.20	<.0001	0.05	0.8627	2.2246
Var(wk 7)	**pupid(litter)**	**Exp**	1.2466	0.2870	4.34	<.0001	0.05	0.8315	2.0749
Var(wk 8)	**pupid(litter)**	**Exp**	1.4756	0.3469	4.25	<.0001	0.05	0.9768	2.4860
ARH(1)	**pupid(litter)**	**Exp**	0.7322	0.05499	13.32	<.0001	0.05	0.6245	0.8400

Table S11 illustrates a comparison between the covariance model in Table 1 and a related model that does NOT have separate parameters for the two levels of Trt (Con and Exp). The bottom three rows of the table show that the three model-selection criteria prefer the model in Table S10. One can use the first row of Table S11 to construct a likelihood ratio test of whether including the separate parameters improves the model. This test is a Chi-squared test with 10 df, p=1.2E-07 – strong evidence that separate variance components by TRT improve the model. Because in Table S11, each variance component for Con is smaller than the corresponding variance component for Exp, it is fair to conclude that for the dKO breeding trial, variability is larger for TRT=Exp than for TRT=Con.

Table S11: Fit Statistics

Index	Separate variance components by TRT	Common variance components across TRT
-2 Res Log Likelihood	1205.0	1254.8
AIC (smaller is better)	1247.0	1276.8
AICC (smaller is better)	1248.7	1277.3
BIC (smaller is better)	1260.4	1283.9

Table S12 is the usual ANOVA table. Effects that do not explicitly include "sex" are averaged across the two sexes. The Table foreshadows that any effects involving TRT are non-significant. The highly significant effects (p<.0001) are completely expected. Notice that the litter-size adjustment is statistically significant for the dKO breeding trial where litter sizes cover a broad range.

Table S12: Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	9.88	0.71	0.4199
sex	1	36.8	276.69	<.0001
trt*sex	1	36.8	0.68	0.4156
c_root_litsiz	1	9	7.94	0.0201
week	7	81.5	1896.10	<.0001
trt*week	7	81.6	1.38	0.2239
sex*week	7	80	85.62	<.0001
trt*sex*week	7	80	0.85	0.5510

Females

Table S13 shows the data plotted as Least-Squares Means for Females in the dKO trial (Fig. 5*E*). (Note: the fractional degrees of freedom [DF] may seem odd; they are used to improve p-value estimation when fitting mixed models to unbalanced data – meaning the number of pups differs among litters and between sexes across litters.)

Table S13: Model-based estimates of Weekly Means by TRT for Females in dKO trial

trt	sex	week	Estimate	Standard Error	DF	t Value	Pr>|t|	Lower	Upper
Con	**F**	**1**	4.5604	0.2119	12.5	21.52	<.0001	4.1006	5.0201
Con	**F**	**2**	6.7948	0.2166	13.6	31.37	<.0001	6.3290	7.2605
Con	**F**	**3**	9.4172	0.2396	20.1	39.30	<.0001	8.9175	9.9169
Con	**F**	**4**	14.1128	0.2607	27.1	54.13	<.0001	13.5780	14.6476
Con	**F**	**5**	17.2622	0.2740	31.3	63.01	<.0001	16.7036	17.8207
Con	**F**	**6**	17.9078	0.2766	32.9	64.74	<.0001	17.3449	18.4706
Con	**F**	**7**	18.6895	0.2864	36.4	65.26	<.0001	18.1088	19.2701
Con	**F**	**8**	19.2014	0.3010	40.5	63.79	<.0001	18.5932	19.8096
Exp	**F**	**1**	4.6201	0.4069	8.49	11.35	<.0001	3.6912	5.5490
Exp	**F**	**2**	7.3388	0.4360	10.8	16.83	<.0001	6.3774	8.3002
Exp	**F**	**3**	10.1458	0.5014	17.8	20.23	<.0001	9.0914	11.2001
Exp	**F**	**4**	14.6275	0.5680	24.6	25.75	<.0001	13.4568	15.7982
Exp	**F**	**5**	17.2610	0.4947	17.6	34.89	<.0001	16.2201	18.3018
Exp	**F**	**6**	17.9096	0.4990	18.3	35.89	<.0001	16.8625	18.9567
Exp	**F**	**7**	18.5619	0.4944	17.9	37.55	<.0001	17.5226	19.6012
Exp	**F**	**8**	19.4029	0.5107	19.7	38.00	<.0001	18.3367	20.4691

Table S14 shows estimates and tests for the week-by-week differences in estimated means (based on the fitted model) between Con and Exp for females in the dKO trial. None of these differences are statistically significant.

Table S14: Estimates of Week-by-Week Con – Exp differences

Effect	Week	Estimate	Standard Error	DF	t Value	Pr > |t|
Con - Exp	1	-0.05976	0.4718	12.27	-0.13	0.9012
Con - Exp	2	-0.5440	0.4992	14.99	-1.09	0.2930
Con - Exp	3	-0.7286	0.5671	23.74	-1.28	0.2113
Con - Exp	4	-0.5147	0.6353	32.59	-0.81	0.4237
Con - Exp	5	0.001214	0.5772	26.23	0.00	0.9983
Con - Exp	6	-0.00185	0.5821	27.21	-0.00	0.9975
Con - Exp	7	0.1276	0.5830	27.56	0.22	0.8284
Con - Exp	8	-0.2015	0.6039	30.84	-0.33	0.7409

Table S15 summarizes tests of interest for females in the dKO trial. The test in the row labeled "Overall" asks whether any of the eight Con – Exp differences across the eight weeks are non-zero. The row labeled "Interaction" asks whether the Con-Exp differences change in magnitude across the eight weeks. (These tests with multiple numerator DF have no corresponding estimates and standard errors.) The test labeled "Main" asks whether, averaged across the eight weeks, the Con – Exp average difference is non-zero. The test in the last row asks whether the average Con – Exp difference over the first three weeks (pre-weaning) is different from the average Con – Exp difference over the last five weeks (post-weaning). None of these tests were statistically significant.

Table S15: Tests of interest for Females in dKO trial

Effect	Estimate	Standard Error	Num DF	Den DF	F Value	Pr > F
Overall	_	_	8	55.31	0.74	0.6574
Interaction	_	_	7	124.5	0.82	0.5706
Main	-0.2402	0.4774	1	12.94	0.25	0.6233
Interaction: Pre- vs. post-weaning	-0.3263	0.2977	1	209.4	1.21	0.2744

Males

Table S16 shows the data plotted as Least-Squares Means for Males in the dKO Trial (Fig. 5*E*).

Table S16: Model-based estimates of Weekly Means by TRT for Males in dKO trial

trt	sex	week	Estimate	Standard Error	DF	t Value	Pr>|t|	Lower	Upper
Con	M	1	4.5646	0.2117	12.4	21.56	<.0001	4.1051	5.0241
Con	M	2	6.8689	0.2162	13.5	31.77	<.0001	6.4037	7.3341
Con	M	3	9.4917	0.2386	19.8	39.78	<.0001	8.9937	9.9898
Con	M	4	15.8844	0.2594	26.6	61.24	<.0001	15.3518	16.4170
Con	M	5	20.3665	0.2726	30.7	74.71	<.0001	19.8104	20.9227
Con	M	6	22.2593	0.2753	32.3	80.86	<.0001	21.6988	22.8198
Con	M	7	24.0194	0.2851	35.8	84.26	<.0001	23.4411	24.5977
Con	M	8	25.2304	0.2997	39.9	84.19	<.0001	24.6247	25.8362
Exp	M	1	4.6420	0.4056	8.36	11.44	<.0001	3.7136	5.5704
Exp	M	2	7.5051	0.4325	10.5	17.35	<.0001	6.5474	8.4628
Exp	M	3	10.3287	0.4933	16.9	20.94	<.0001	9.2874	11.3699
Exp	M	4	17.2584	0.5559	23.5	31.04	<.0001	16.1097	18.4071
Exp	M	5	21.2561	0.4872	16.7	43.63	<.0001	20.2270	22.2853
Exp	M	6	22.5290	0.4911	17.3	45.87	<.0001	21.4942	23.5638
Exp	M	7	24.0159	0.4868	16.9	49.33	<.0001	22.9882	25.0436
Exp	M	8	25.2319	0.5019	18.6	50.27	<.0001	24.1796	26.2841

Table S17 contains estimates and tests for the week-by-week differences in estimated means (based on the fitted model) between Con and Exp for males in the dKO trial. Only one (Week 4) of these differences is statistically significant.

Table S17: Estimates of Week-by-Week Con – Exp differences

Effect	Week	Estimate	Standard Error	DF	t Value	Pr > |t|
Con - Exp	1	-0.07740	0.4703	12.1	-0.16	0.8720
Con - Exp	2	-0.6362	0.4956	14.58	-1.28	0.2192
Con - Exp	3	-0.8369	0.5589	22.72	-1.50	0.1480
Con - Exp	4	-1.3740	0.6234	31.27	-2.20	0.0350
Con - Exp	5	-0.8896	0.5693	25.08	-1.56	0.1307
Con - Exp	6	-0.2697	0.5740	25.96	-0.47	0.6424
Con - Exp	7	0.003540	0.5751	26.32	0.01	0.9951
Con - Exp	8	-0.00146	0.5951	29.41	-0.00	0.9981

Table S18 summarizes tests of interest for males in the dKO trial. The test in the row labeled "Overall" asks whether any of the eight Con – Exp differences across the eight weeks are non-zero. (Note: this result may appear to contradict Table 8 where the week-4 difference was significant; the multiple degree-of-freedom test has some protection against multiple testing issues so there is no real contradiction.) The row labeled "Interaction" asks whether the Con-Exp differences change in magnitude across the eight weeks. (These tests with multiple numerator DF have no corresponding estimates and standard errors.) The test labeled "Main" asks whether, averaged across the eight weeks, the Con – Exp average difference is non-zero. The test in the last row asks whether the average Con – Exp difference over the first three weeks (pre-weaning) is different from the average Con – Exp difference over the last five weeks (post-weaning). None of these tests were statistically significant.

Table S18: Tests of interest for Males in the dKO Trial

Effect	Estimate	Standard Error	Num DF	Den DF	F Value	Pr > F
Overall	_	_	8	53.26	1.42	0.2080
Interaction	_	_	7	123.3	1.58	0.1465
Main	-0.5102	0.4726	1	12.45	1.17	0.3008
Interaction: Pre- vs. post-weaning	-0.01062	0.2902	1	211.8	0.002	0.9708

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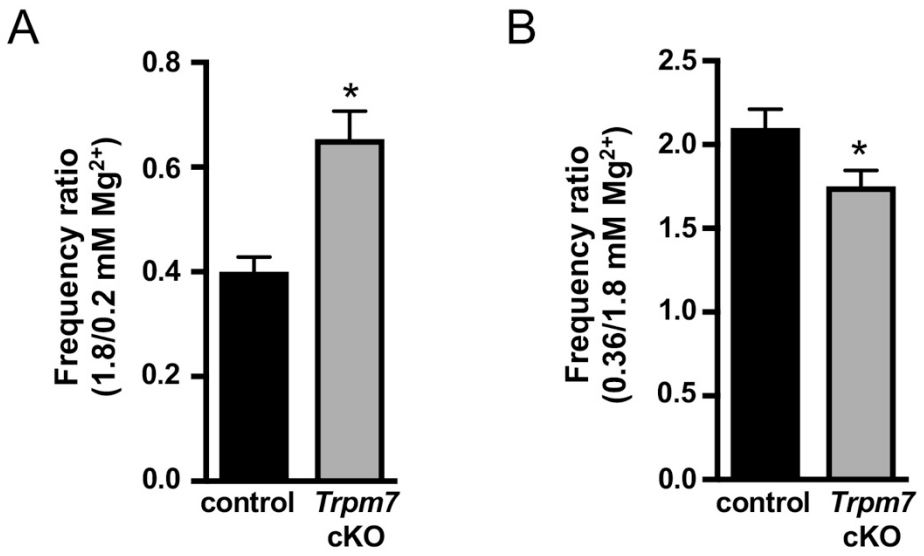

Figure S1. Ratios of Ca^{2+} oscillation frequencies in response to altered Mg^{2+} concentrations.
(A) Ratio of oscillation frequency in control and *Trpm7* cKO eggs at 1.8 mM Mg^{2+} relative to 0.2 mM Mg^{2+}. (B) Ratio of oscillation frequency in control and *Trpm7* cKO eggs at 0.36 mM Mg^{2+} relative to 1.8 mM Mg^{2+}. *$p<0.05$, Mann-Whitney test.

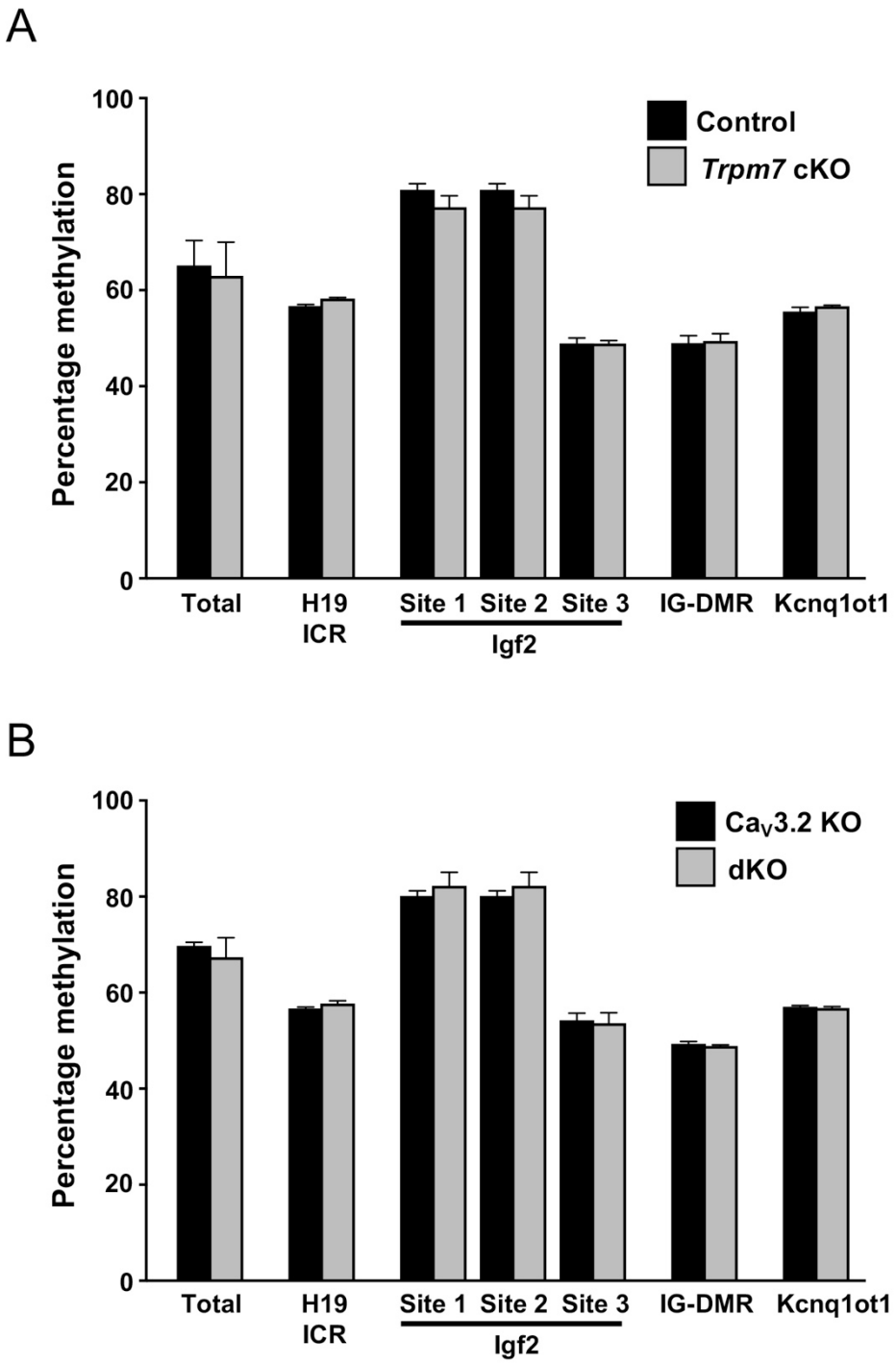

Figure S2. DNA methylation analysis. DNA methylation at repetitive elements (LUMA) and average DNA methylation at the indicated imprinted loci in liver tissue of offspring derived from (A) Control and *Trpm7* cKO and (B) $Ca_V3.2$ KO and dKO oocytes. N=4 for each dam genotype (1 female and 1 male per litter from 2 different litters were assayed).