Supporting Information

Backs et al. 10.1073/pnas.0912658106

SI Materials and Methods

Generation of CaMKIIγ$^{-/-}$ Mice. Genomic regions of the mouse CaMKIIγ locus were isolated from 129SvEv genomic DNA by *TaKaRa LA Taq* polymerase (Takara Bio Inc., Shiga, Japan) and cloned into pGKF2L2DTA. A 4.6-kb genomic sequence (5′ targeting arm) upstream of CaMKIIγ exon 1 was cloned upstream of the 5′ loxP sequence in the NotI site of pGKneoF2L2DTA. A 1.9-kb genomic sequence (3′ targeting arm) downstream of CaMKIIγ exon 2 was cloned downstream of the 3′ loxP sequence between NheI and HindIII. A 2.9-kb region (conditional knockout arm) was cloned with blunt ends between the 5′ loxP sequence and the 5′ FRT sequence into the SmaI site. The resulting vector was verified by restriction mapping and DNA sequencing. The vector was linearized at a newly introduced SalI site upstream of the 5′ targeting arm and electroporated into 129SvEv-derived embryonic stem cells. Cells were then treated with G418, and negative selection was accomplished by the diphtheria toxin A cassette in the pGKneoF2L2DTA vector. Southern blot analysis was performed by using probes located 5′ of the 5′ targeting arm, 3′ of the 3′ targeting arm and within the neomycin resistance cassette. Almost 6% of the selected embryonic stem cell clones were successfully targeted for CaMKIIγ (26 of 465). Three clones were expanded and injected into C57BL/6 blastocysts that were transferred to pseudopregnant females. High-percentage chimeric male mice (CaMKIIγ$^{neo\text{-}loxP/+}$) were bred into a C57BL/6 background to obtain germ-line transmission. To obtain mice with a CaMKIIγ null allele (CaMKIIγ$^{-/+}$), an intercross to CAG-Cre transgenic mice was performed. By intercrossing CaMKIIγ$^{-/+}$ to each other, we generated homozygous CaMKIIγ null mice (CaMKIIγ$^{-/-}$). All experiments were conducted in a 129SvEv/C57Bl6/CD1 mixed background. Animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center and the University of Pennsylvania, and were consistent with National Institutes of Health guidelines.

Oocyte, Egg, and Embryo Collection and Microinjection. Fully grown, germinal vesicle–intact oocytes were obtained from primed mice as described previously (1). Germinal vesicle breakdown was inhibited by adding 2.5 μM milrinone to isolation and culture media (2). Unfertilized and fertilized eggs were isolated from superovulated females as described (3). Oocytes were microinjected with 5 pl of a 0.5 mg/mL cRNA solution (GFP cRNA, CaMKIIγ cRNA, or CaMKIIδ cRNA); the injections were performed as previously described (3). At 1 or 2 h after microinjection, oocytes were washed and cultured in milrinone-free medium for 16 h to allow meiotic maturation.

Parthenogenetic Activation, in Vitro Fertilization, and Ca^{2+} Imaging. Metaphase II eggs were cultured for 4 h in calcium/magnesium-free CZB medium containing 10 mM $SrCl_2$. After activation, the eggs were transferred to CZB medium for further culture (4). Roscovitine was used in some experiments to inhibit MPF and induce egg activation. In these experiments, a group of eggs was cultured with 50 μM roscovitine in CZB for 6 h; another group was cultured with 50 μM roscovitine and 10 mM $SrCl_2$ in calcium/magnesium-free CZB for 6 h. Control, untreated eggs were cultured in CZB containing 0.01% DMSO (vehicle of roscovitine). All of these cultures were done in 750-μL drops of medium without oil. In vitro fertilization and Ca^{2+} imaging were performed as described before (5), except that ZP-intact MII eggs were inseminated with 5×10^5/mL sperm.

Immunoblotting. Lysates containing equal numbers of oocytes or eggs from mice of different genotypes were electrophoresed in a 10% gel and transferred to a PVDF membrane using wet transfer. The membrane was blocked with ECL Advance Blocking Agent (3% wt/vol) dissolved in PBS containing 0.1% Tween 20 (PBST). The membrane was then probed with the primary antibody. For CaMKII detection, the membrane was blocked for 1 h at room temperature and incubated with mouse anti-CaM Kinase II antibody (catalog no. 611292; BD Biosciences; 1:5,000) for 16 h at 4°C. For ZP2 detection, the membrane was blocked overnight at 4°C and then incubated with rat anti-mouse ZP2 antibody (M2c.2; kindly provided by Jurrien Dean (NIDDK, NIH, Bethesda, MD); 1:10,000) for 1 h at room temperature. After washing four times in PBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) at a 1:200,000 dilution. The signal was detected using ECL Advance Western Blotting System (GE Healthcare).

Histochemistry and Immunofluorescence. Ovaries prepared for histochemistry were fixed overnight in Bouin's fixative, embedded in paraffin, sliced to 10-μm sections, and stained with H&E and periodic acid-Schiff. Immunofluorescence was performed as previously described (6). The primary antibody was anti–β-tubulin (Sigma, T4026) and goat anti-mouse IgG1 fluorescein (FITC)–conjugated (SouthernBiotech, 1070-02) was used as secondary antibody. DAPI (Sigma, 1.5 μg/mL) was added to the mounting medium (Vectashield, Vector Laboratories).

In Vitro Synthesis of cRNA. cDNAs encoding full-length and wild-type CaMKIIγ and CaMKIIδ were subcloned into pT7TS (a gift from O. Cleaver, UT Southwestern Medical Center, Dallas, TX). These plasmids were linearized and transcribed in vitro using the T7 mMESSAGE mMACHINE kit (Ambion), according to the manufacturer's instructions. The cRNA was purified using RNeasy kit (Qiagen), eluted in RNase-free water, and stored at −80°C until use.

[^{35}S]-Methionine Metabolic Radiolabeling and SDS/PAGE. Twenty unfertilized or fertilized metaphase II eggs were radiolabeled for 3 h in 50-μl drops of CZB medium containing 1 mCi/mL of [^{35}S]-methionine (1,500 Ci/mmol; Amersham). After labeling, the eggs/embryos were washed free of unincorporated radiolabel and then placed into 20 μL of Laemmli sample buffer. The samples were resolved by 10% polyacrylamide gels containing SDS and proteins separated by electrophoresis. The gels were dried and exposed to storage phosphor screens for 24 h. Gel images were collected using a Typhoon 9410 Variable Mode Imager (GE Healthcare).

RNA Isolation and Quantitative Real-Time RT-PCR. Total RNA was extracted from 20 fully-grown oocytes using the RNAqueous-micro kit (Ambion) according to the manufacturer 's instructions. Reverse transcription and real-time PCR were performed as described (3). Quantification was normalized to the endogenous upstream binding factor (Ubtf). The TaqMan gene expression assays used were as follows: Mm00437967_m1 (CaMKIIα), Mm00432296_m1 (CaMKIIβ), Mm00618054_m1 (CaMKIIγ), Mm00499266_m1 (CaMKIIδ), Mm00456972_m1 (Ubtf).

1. Svoboda P, Stein P, Hayashi H, Schultz RM (2000) Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development* 127:4147–4156.
2. Tsafriri A, Chun SY, Zhang R, Hsueh AJ, Conti M (1996) Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: Studies using selective phosphodiesterase inhibitors. *Dev Biol* 178:393–402.
3. Knott JG, *et al.* (2006) Calmodulin-dependent protein kinase II triggers mouse egg activation and embryo development in the absence of Ca^{2+}oscillations. *Dev Biol* 296:388–395.
4. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I (1989) An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J Reprod Fertil* 86:679–688.
5. Igarashi H, Knott JG, Schultz RM, Williams CJ (2007) Alterations of PLCβ1 in mouse eggs change calcium oscillatory behavior following fertilization. *Dev Biol* 312:321–330.
6. Anger M, Stein P, Schultz RM (2005) CDC6 requirement for spindle formation during maturation of mouse oocytes. *Biol Reprod* 72:188–194.

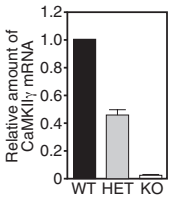

Fig. S1. Relative levels of CaMKIIγ mRNA in oocytes of different CaMKIIγ genotypes. Graph depicts quantification of results of three independent experiments, normalized using upstream binding factor (Ubtf) as internal control. Fold-change was calculated using the comparative C_T method. Data are expressed as mean ± SEM.

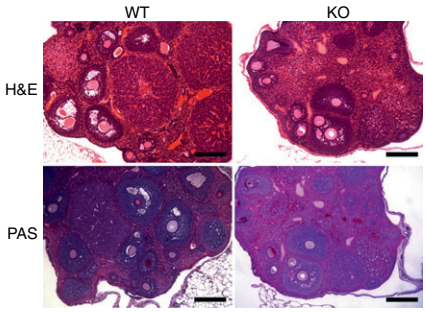

Fig. S2. Histological sections of ovaries from CaMKIIγ wild-type (WT) and CaMKII$\gamma^{-/-}$ (KO) mice were stained with H&E and periodic acid-Schiff (scale bar, 200 μm). No morphological abnormalities were observed.

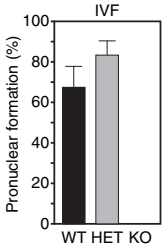

Fig. S3. In vitro fertilization of metaphase II eggs collected from WT, HET, and KO mice. The fertilization rate was determined by PN formation 7 h post-insemination. The experiment was performed three times. Data are expressed as mean ± SEM.

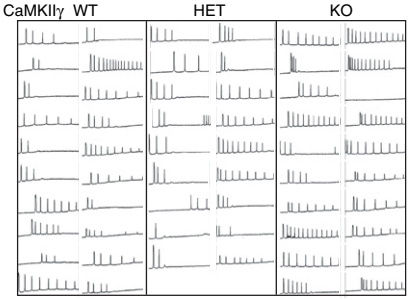

Fig. S4. Sperm-induced Ca^{2+} oscillations in CaMKII$\gamma^{-/-}$ eggs. Ca^{2+} oscillatory patterns are shown for wild-type (WT), CaMKII$\gamma^{+/-}$ (HET) and CaMKII$\gamma^{-/-}$ (KO) eggs after insemination in vitro. The *y* axis represents 340/380 ratio (from 0 to 1.6), and the *x* axis is time (from 0 to 180 min, with 0 being the time of insemination). The experiment was performed three times. KO eggs had significantly more Ca^{2+} rises than WT or HET eggs (WT, 5.15 ± 0.71; HET, 4.28 ± 0.57; KO, 7.95 ± 0.69*; *$P < 0.05$).