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Store-operated Ca^{2+} entry is not required for fertilization-induced Ca^{2+} signaling in mouse eggs

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Abstract

Repetitive oscillations in cytoplasmic Ca^{2+} due to periodic Ca^{2+} release from the endoplasmic reticulum (ER) drive mammalian embryo development following fertilization. Influx of extracellular Ca^{2+} to support the refilling of ER stores is required for sustained Ca^{2+} oscillations, but the mechanisms underlying this Ca^{2+} influx are controversial. Although store-operated Ca^{2+} entry (SOCE) is an appealing candidate mechanism, several groups have arrived at contradictory conclusions regarding the importance of SOCE in oocytes and eggs. To definitively address this question, Ca^{2+} influx was assessed in oocytes and eggs lacking the major components of SOCE, the ER Ca^{2+} sensor STIM proteins, and the plasma membrane Ca^{2+} channel ORAI1. We generated oocyte-specific conditional knockout (cKO) mice for *Stim1* and *Stim2,* and also generated *Stim1/2* double cKO mice. Females lacking one or both STIM proteins were fertile and their ovulated eggs displayed normal patterns of Ca^{2+} oscillations following fertilization. In addition, no impairment was observed in ER Ca^{2+} stores or Ca^{2+} influx following store depletion. Similar studies were performed on eggs from mice globally lacking ORAI1; no abnormalities were observed. Furthermore, spontaneous Ca^{2+} influx was normal in oocytes from *Stim1/2* cKO and ORAI1-null mice. Finally, we tested if TRPM7-like channels could support spontaneous Ca^{2+} influx, and found that it was largely prevented by NS8593, a TRPM7-specific inhibitor. Fertilization-induced Ca^{2+} oscillations were also impaired by NS8593. Combined, these data robustly show that SOCE is not required to support appropriate Ca^{2+} signaling in mouse oocytes and eggs, and that TRPM7-like channels may contribute to Ca^{2+} influx that was previously attributed to SOCE.

Graphical Abstract

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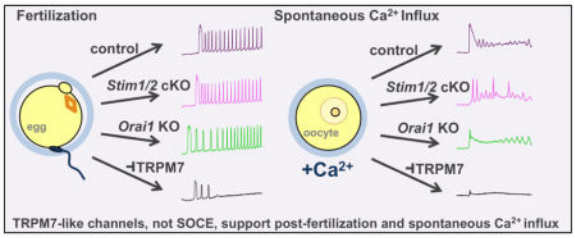
AUTHOR CONTRIBUTIONS

MLB and CJW designed the experiments. MLB, EPB, and PS performed the experiments. MLB, PS, and YZ analyzed the data. MLB and CJW wrote the paper, and all authors edited the paper.



Keywords

oocyte; calcium; store-operated calcium entry; fertilization; egg activation

1. INTRODUCTION

A large increase in the level of cytoplasmic Ca^{2+} is the key trigger for activation of development at fertilization in all animal species. In preparation for fertilization, mammalian germinal vesicle (GV)-intact, prophase I-arrested oocytes take up Ca^{2+} from the extracellular space and suppress Ca^{2+} leak from the endoplasmic reticulum (ER), resulting in a significant increase in ER Ca^{2+} stores as they resume meiosis and become arrested at metaphase II (MII eggs) [1–3]. In addition, they increase expression of the inositol 1,4,5-trisphosphate (IP_3) receptor and alter ER localization such that it becomes enriched in the cortical region below the plasma membrane [4–6]. Together, these and other maturation-associated changes result in MII eggs that efficiently release Ca^{2+} in response to the fertilizing sperm. The initial Ca^{2+} release event at fertilization is induced by the activity of the sperm-specific phospholipase C zeta (PLCζ), which enters the egg's cytoplasm following sperm-egg plasma membrane fusion [7, 8]. PLCζ catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and IP_3; IP_3 then activates the IP_3 receptor to cause Ca^{2+} release from ER stores.

In mammals, periodic Ca^{2+} release from ER stores, known as Ca^{2+} oscillations, continue for several hours after fertilization. The persistence of these oscillations appears to be a critical driver of the success of embryo development [9]. In the absence of extracellular Ca^{2+}, reductions in intraluminal ER Ca^{2+} levels that occur with each release event lead to cessation of the oscillations over time [10–12]. Hence, it is apparent that Ca^{2+} influx from the extracellular space, followed by accumulation into the ER, must occur during both oocyte maturation and following fertilization to support effective initial Ca^{2+} release, continued Ca^{2+} oscillations and optimal embryo development.

A common mechanism to replenish depleted ER Ca^{2+} stores via influx from extracellular sources is known as store-operated Ca^{2+} entry (SOCE) [13, 14]. The main molecular mediators of SOCE are STIM proteins, which sense ER Ca^{2+} levels, and ORAI proteins, which serve as plasma membrane Ca^{2+} channels. In mammals there are two STIM proteins, STIM1 and STIM2, and three ORAI proteins, ORAI1, ORAI2, and ORAI3. In response to a reduction in ER Ca^{2+}, STIM proteins oligomerize and undergo redistribution within the ER to regions closely apposed to the plasma membrane (PM), known as ER-PM junctions. There, the STIM proteins interact directly with ORAI channels, inducing Ca^{2+} influx. This

Ca^{2+} is subsequently pumped back into the ER by the action of sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCA).

SOCE would be a logical mechanism to mediate Ca^{2+} entry following fertilization. Indeed, SOCE has been reported in both oocytes and MII eggs, though it appears more robust in oocytes [1, 15]. However, there is conflicting data in the literature regarding whether or not SOCE is an important physiological mediator of Ca^{2+} influx following fertilization. We and others demonstrated previously that chemical inhibitors of SOCE did not prevent Ca^{2+} influx following fertilization in the mouse [16, 17]. In contrast, several studies in both mouse and porcine oocytes suggest, instead, that SOCE is essential for this process [18–21].

To definitively determine whether SOCE is required to support Ca^{2+} influx at fertilization in the mouse, we generated and analyzed conditional knockout mouse lines in which the oocytes lacked the major SOCE components. Here we show that there is no requirement for either STIM1, STIM2, or ORAI1 to support Ca^{2+} influx during oocyte maturation or to support persistent Ca^{2+} oscillations following fertilization. Experiments to determine what alternative channels mediate Ca^{2+} influx in mouse oocytes uncovered compelling evidence that spontaneous Ca^{2+} influx in oocytes and post-fertilization Ca^{2+} influx, which were previously attributed to SOCE, are instead mediated by either TRPM7, the melastatin-related transient receptor potential channel, or a TRPM7-like channel.

2. MATERIALS AND METHODS

2.1. Mice

Stim1$^{flox/flox}$ and *Stim2*$^{flox/flox}$ mice [22] were crossed with a *Zp3*-cre transgenic line (Jackson Laboratory, Bar Harbor, ME, USA, Stock No. 003651) [23] to generate oocyte-specific conditional knockout (cKO) mice for *Stim1* and *Stim2*. These mice were further intercrossed to obtain *Stim1*-*Stim2* double cKO mice. All mice for these crosses were maintained on a predominantly C57Bl/6J genetic background, and the *Zp3*-cre transgene was bred through the male to avoid germline transmission of excised alleles. *Orai1* full knockout mice [24] outbred with Institute of Cancer Research (ICR) strain mice [25] were also used. CF-1 strain female mice were obtained from Charles River (Wilmington, MA, USA). B6SJLF1 males for sperm collection and C57Bl/6J male breeders were from Jackson Laboratory. For analysis of fertility, littermate *Stim1/2* cKO and *Zp3*-cre-negative *Stim1*$^{flox/flox}$;*Stim2*$^{flox/flox}$ control females were paired with adult C57Bl/6J males at 7–12 weeks of age and were housed together as breeding pairs for 6 months. Pups were counted on the day of birth and sexed at weaning 21 days later. All mice were maintained under a standard 12 h light/dark cycle and provided food and water ad libitum. All studies and procedures were performed under an animal study protocol approved by the National Institutes of Health Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals*.

2.2. Gamete Collection

For collection of GV stage oocytes, female mice 6–15 weeks of age were injected with 5 IU pregnant mare serum gonadotropin (PMSG, Calbiochem) and sacrificed by CO_2

asphyxiation 42–48 h later. Ovaries were placed in Leibovitz L-15 medium (L-15, Life Technologies, Grand Island, NY, USA) containing 1% calf serum (Atlanta Biologicals, Norcross, GA, USA) and 10 μM milrinone, and antral follicles were punctured using 27 G needles. Cumulus cells were removed by gentle pipetting through a narrow bore capillary. For collection of metaphase II (MII)-arrested eggs, PMSG-primed mice were injected with 5 IU human chorionic gonadotropin (hCG) 46–48 h after PMSG injection and sacrificed 13–14 h later. Cumulus-oocyte masses were removed from oviducts and cumulus cells were removed by brief treatment with 0.03% hyaluronidase in L-15. Prior to imaging or collection, oocytes and eggs were held in Minimal Essential Medium Alpha (aMEM, Life Technologies) containing 5% calf serum or potassium simplex optimized medium (KSOM) with amino acids (EMD Millipore, Billerica, MA, USA) at 37° C/5% CO_2 in drops of pre-equilibrated medium under light mineral oil.

2.3. Immunoblotting

Immunoblot analysis was carried out essentially as previously described [26]. LF PVDF membranes (BioRad, Atlanta, GA, USA) were blocked in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Primary antibodies directed against STIM1 and STIM2 were obtained from Cell Signaling (Danvers, MA, USA; AntibodyRegistry: AB_2271287 and AB_2198021) and were diluted 1:250 in blocking solution. Peroxidase-conjugated anti-rabbit secondary antibody from Jackson ImmunoResearch (West Grove, PA, USA; #711-035-152) was used at 1:10,000 dilution, and SuperSignal West Femto Chemiluminescent Substrate (ThermoFisher Scientific, Grand Island, NY, USA) was used for detection.

2.4. Real-time PCR

Quantitative real time PCR was performed essentially as previously described [27]. Samples were spiked with EGFP cRNA as an internal reference, as described in [16], and RNA was isolated from three groups of 30 GV oocytes each from *Stim2*$^{flox/flox}$ (control) and *Stim2*$^{flox/flox}$;*Zp3*-cre^{+} (cKO) mice using an Arcturus PicoPure RNA Isolation Kit (Life Technologies). Reverse transcription and amplification were performed as previously described [26]. Primer sequences for detection of *Stim2* were as follows: [Exon3–4 Forward: GGATTTGTGGAAACAGTGGAA; Reverse: GGGAGTGTTGTTCCCTTCAC], [Exon4–5 Forward: TGTGAAGGGAACAACACTCC; Reverse: GTCAGAGGCCCAAACAGAAC].

2.5. In vitro fertilization (IVF) and Ca^{2+} Imaging

Imaging of Ca^{2+} flux following fertilization was performed essentially as previously described [27]. Briefly, zona pellucida-free fura-2 AM (Life Technologies)-loaded eggs were adhered to Cell Tak (EMD Millipore)-treated glass-bottom dishes, and 510 nm fluorescence was imaged with excitation at 340 nm and 380 nm as 5×10^4 capacitated sperm/mL was added to the imaging drop. Change in F340/F380 ratio was measured 8 times per minute and is used to reflect changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$). For all experiments using genetic mouse models, control and KO eggs were placed side-by-side in the same media drop and were imaged simultaneously. For experiments using inhibitor treatment, eggs were pre-incubated in 20 uM NS8593 for 15–30 min prior to sperm addition; parallel control experiments were performed in the same manner immediately before or after inhibitor

studies using gametes from the same mice and vehicle (DMSO) treatment. Analysis of Ca^{2+} oscillation parameters was performed as previously described [27].

2.6. ER Ca^{2+} Store, Store-operated Ca^{2+} Entry, and Spontaneous Ca^{2+} Influx Assays

Measurement of Ca^{2+} release following addition of thapsigargin, reflecting ER Ca^{2+} store content, was performed essentially as described [27]. Briefly, zona pellucida-intact oocytes or eggs were adhered to glass-bottom dishes in 1.85 mL Ca^{2+}-, Mg^{2+}-, and bovine serum albumin (BSA)-free CZB medium [28] containing 2 mM EGTA. Solutions of thapsigargin, $CaCl_2$, or $MgCl_2$ diluted in 150 μL medium were added drop-wise to dishes during imaging. Cells were imaged for 3–5 min in Ca^{2+}-free medium before adding thapsigargin at a final concentration of 10 μM; 35–40 min later, 5 mM $CaCl_2$ was added and imaging continued for at least 15 min. For spontaneous Ca^{2+} influx assays, Ca^{2+} influx was measured for GV oocytes without prior depletion of ER Ca^{2+} using thapsigargin [1]. GV oocytes were placed in Ca^{2+}-, Mg^{2+}-, and BSA-free CZB and imaged as $CaCl_2$ was added to a final concentration of 2 mM or 5 mM. Ten μM milrinone was included in medium used for GV oocytes to maintain meiotic arrest. Area under the curve for 10 minutes following each addition was calculated for individual oocytes or eggs by computing trapezoidal area for each curve following subtraction of the average baseline measurement for 2–3 min prior to the response.

2.7. Additional Chemicals and Reagents

If not otherwise specified, all chemicals, reagents, and inhibitors were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.8. Statistical Analysis

GraphPad Prism, version 7.0a was used to perform all statistical tests. Student's t-test was performed for normally distributed data, as determined by D'Agostino & Pearson omnibus normality test. For datasets lacking normal distribution, the Mann-Whitney U-test was used. Contingency data was analyzed using Fisher's exact test. Asterisks indicate $p < 0.05$ and ns indicates non-significant differences in all graphs, and all error bars show SEM.

3. RESULTS

3.1. STIM1 and STIM2 are dispensable for Ca^{2+} influx in mouse oocytes and eggs

Oocyte-specific conditional knockout (cKO) mice for *Stim1* and *Stim2* were generated by crossing lines carrying loxP-flanked (*flox*) alleles with a *Zp3*-cre transgenic line [22, 23]. Eggs were collected from superovulated females for immunoblot analysis to confirm loss of STIM1 and STIM2 proteins. STIM1 was detected in a lysate of 200 eggs from $Stim1^{flox/flox}$ (control) mice but was absent from eggs of $Stim1^{flox/flox}$;*Zp3*-cre^{+} (cKO) mice, confirming that *Zp3*-cre causes efficient loss of STIM1 protein in *Stim1* cKO eggs (Fig. 1A). Although a specific band for STIM2 was observed at approximately 115 kD in a lysate of control neonatal lung tissue and was absent from *Stim2* knockout tissue, we were unable to detect STIM2 protein by immunoblot using 260 eggs (Fig. 1B). However, using real time PCR, we found that *Stim2* transcript levels were greatly reduced in *Stim2* cKO eggs, with transcripts containing the loxP-flanked exon 3 reduced by 68-fold, becoming nearly undetectable (Fig.

1C). We conclude that efficient loss of both STIM1 and STIM2 was achieved using this method.

To determine whether STIM1 is necessary for normal Ca^{2+} signaling following fertilization, we performed live-cell ratiometric Ca^{2+} imaging on control eggs from *Stim1*$^{flox/flox}$ females alongside cKO eggs lacking STIM1 from *Stim1*$^{flox/flox}$;*Zp3*-cre^{+} females during in vitro fertilization. Representative traces for 60 minutes of imaging are shown, along with analysis of data from 5 replicate experiments (Fig. 2A–E). None of the parameters measured, including first transient length, maximum amplitude, oscillation frequency, and persistence of oscillations to 60 minutes, differed between control and *Stim1* cKO eggs. Of note, the oscillation frequency we observe is slightly faster than that reported in some previous studies (E.g., [16, 29]), but is consistent with our previous data using the same method [27]. We attribute this variance primarily to differences in media composition, use of intracytoplasmic sperm injection vs. IVF, and genetic background of mice used in these studies. Based on our analysis of Ca^{2+} oscillation parameters, STIM1 is not required for appropriate Ca^{2+} signaling following fertilization.

We next investigated whether loss of STIM1 impacts Ca^{2+} stores or Ca^{2+} influx following store depletion. Ca^{2+} imaging was performed on control and *Stim1* cKO eggs placed side-by-side in Ca^{2+}-free medium, and thapsigargin was added to inhibit SERCA-mediated refilling of Ca^{2+} stores and allow leak of Ca^{2+} from the ER (Fig. 2F). Area under the curve and peak amplitude were measured as indicators of ER Ca^{2+} content. Peak amplitude was similar and area under the curve was slightly higher for *Stim1* cKO eggs compared to control (Fig. 2G,H), indicating that absence of STIM1 did not impair accumulation of ER Ca^{2+} in eggs. Thapsigargin treatment was continued for at least 30 min to deplete ER stores, then 5 mM $CaCl_2$ was added and the increase in $[Ca^{2+}]_i$ was measured for 10 minutes as an indication of Ca^{2+} influx following store depletion. Neither peak amplitude nor area under the curve was significantly different for *Stim1* cKO eggs compared to controls (Fig. 2I,J). Overall, these results indicate that STIM1 is dispensable for normal Ca^{2+} oscillations and homeostasis in mouse eggs.

Whereas STIM1 is activated by substantial release of Ca^{2+} from ER stores, STIM2 can be activated by alterations in ER Ca^{2+} closer to the resting level [30, 31]. We therefore also sought to determine whether STIM2 was necessary for Ca^{2+} signaling in mouse eggs. As for the *Stim1* cKO model, we performed Ca^{2+} imaging during in vitro fertilization for control and *Stim2* cKO eggs. Representative traces and summary analyses are shown in Fig. 3A–E. Ca^{2+} oscillations of *Stim2* cKO eggs appeared largely normal. Oscillation frequency and amplitude were unaltered, and only very subtle decreases were observed in length of the first Ca^{2+} transient and in the proportion of eggs with oscillations persisting throughout the 60 min imaging period. There was no significant difference in either ER Ca^{2+} store content or Ca^{2+} influx following store depletion (Fig. 3F–I). Based on these data, loss of STIM2 did not impair Ca^{2+} store accumulation or influx and had at most a very minor impact on fertilization-induced oscillations.

Although loss of STIM1 and STIM2 individually had very little impact on oocyte physiology and Ca^{2+} signaling, we also wanted to address the possibility of compensation

between STIM1 and STIM2. *Stim1* and *Stim2* single cKO lines were intercrossed to produce a *Stim1-Stim2* oocyte-specific double conditional knockout line (*Stim1/2* cKO). Fertilization-induced Ca^{2+} oscillations of *Stim1/2* cKO eggs were no different from controls (Fig. 4A–E) and ER Ca^{2+} stores measured by thapsigargin-mediated Ca^{2+} release were essentially identical to controls (Fig. 4F,G). Likewise, Ca^{2+} influx following store depletion was not significantly altered in *Stim1/2* cKO eggs (Fig. 4H,I). It was previously shown that Ca^{2+} influx following store depletion is greater in GV oocytes than MII eggs [1]; therefore, we also assayed ER Ca^{2+} stores and Ca^{2+} influx in *Stim1/2* cKO GV oocytes. Following thapsigargin addition, similar levels of Ca^{2+} release were observed in control and *Stim1/2* cKO GV oocytes (Fig. 4J,K), and Ca^{2+} influx following store depletion was also unaltered (Fig. 4L,M). Taken together, these data indicate that in mouse oocytes and eggs neither STIM1 nor STIM2 is required for Ca^{2+} influx following ER store depletion.

To determine whether oocyte-specific loss of STIM1 and STIM2 had any impact on fertility, we conducted a formal breeding study for *Stim1/2* double cKO females. *Stim1/2* cKO females were fertile, and fertility parameters including average litter size, days to first litter, and total live pups per breeding pair were indistinguishable from those of littermate controls (Fig. 4N–P). One *Stim1/2* cKO female did not produce pups during the study and was omitted from analysis because male infertility could not be excluded. We also housed several *Stim1* and *Stim2* single cKO females with males to confirm their fertility; all produced pups (4/4 females for each genotype), but a detailed breeding study was not carried out because this was done with the *Stim1/2* cKO. Altogether we conclude that STIM1 and STIM2 are dispensable for normal fertility in female mice.

3.2 ORAI1 is dispensable for Ca^{2+} signaling in oocytes and eggs

Female *Orai1* knockout mice are fertile [32]; however, Ca^{2+} signaling following fertilization has not been studied in eggs lacking ORAI1. As for *Stim1* and *Stim2* cKO mice, we performed Ca^{2+} imaging during in vitro fertilization and found no significant difference in the pattern of Ca^{2+} oscillations between *Orai1* KO and wild-type control eggs (Fig. 5A–E). Similarly, there was no difference in ER Ca^{2+} stores (Fig. 5F,G) or Ca^{2+} influx following store depletion (Fig. 5H,I) for *Orai1* KO MII eggs compared to controls. Likewise, ER Ca^{2+} stores in *Orai1* KO GV oocytes were not significantly different from controls (Fig. 5J,K). Ca^{2+} influx following store depletion was increased in *Orai1* KO eggs (Fig. 5L,M), in contrast to the expected decrease in influx that would be anticipated if ORAI1 channels were critical for mediating this influx. Thus, ORAI1 channels are also dispensable for normal Ca^{2+} signaling in oocytes and eggs.

3.3. TRPM7-like channels, not SOCE components, support spontaneous Ca^{2+} influx in mouse oocytes

When placed in nominal Ca^{2+}-free medium, GV oocytes respond to addition of 2–5 mM Ca^{2+} by showing a rise in $[Ca^{2+}]_i$, reflecting measurable spontaneous Ca^{2+} influx, while MII eggs do not show such a response [1]. To investigate whether SOCE mediators STIM1, STIM2, or ORAI1 contribute to this spontaneous Ca^{2+} influx in GV oocytes, we placed *Stim1/2* cKO and control oocytes in Ca^{2+}-free medium and added 2 mM $CaCl_2$ during Ca^{2+} imaging. Both control and *Stim1/2* cKO oocytes displayed increased $[Ca^{2+}]_i$ in response to

Ca^{2+} addition (Fig. 6A), and no significant difference was observed in their response when peak amplitude and area under the curve for 10 min following Ca^{2+} addition were measured (Fig. 6B,C). *Orai1* KO oocytes, as compared to wild-type controls, also had no difference in peak amplitude or area under the curve following Ca^{2+} addition (Fig. 6D,E). Based on these results, spontaneous Ca^{2+} influx in GV stage oocytes does not require STIM1, STIM2, or ORAI1.

We next addressed the question of what SOCE-independent mechanisms of Ca^{2+} influx are at work in mouse oocytes. We recently demonstrated that $Ca_V3.2$ channels contribute to the increase in ER Ca^{2+} stores during oocyte maturation and support Ca^{2+} oscillations following fertilization; however, it is evident that additional channels also support Ca^{2+} influx in oocytes and eggs [27]. We hypothesized that transient receptor potential (TRP) channels were likely candidates for this role because these cation conducting channels are expressed in all types of cells and can contribute to a wide array of cellular responses (reviewed in [33]). TRPV3 current has been demonstrated in mouse eggs, but this current was not detected in GV oocytes [34]; therefore, TRPV3 is unlikely to mediate spontaneous Ca^{2+} influx in GV oocytes. Mice lacking all seven TRPC channels have been generated and shown to be viable and fertile [35]. In a limited set of experiments, we also observed normal Ca^{2+} oscillations in eggs from *Trpc* hepta-KO mice (not shown), indicating that TRPC channels are unlikely to contribute significantly to Ca^{2+} influx. Through surveying publicly available microarray data ([36], GEO Accession GDS3295), we found that in mouse oocytes, *Trpm7* was highly expressed relative to other *Trp* channels, and there is electrophysiological evidence for the presence of functional TRPM7-like channels in oocytes [37]. To test whether TRPM7-like channels support spontaneous Ca^{2+} influx in mouse oocytes, we used two different TRPM7 inhibitors, Mg^{2+} and NS8593, which was initially characterized as a small conductance Ca^{2+} activated K^+ channel inhibitor [38–40]. GV oocytes from wild-type CF-1 females were placed in Ca^{2+}- and Mg^{2+}-free medium, and 5 mM $CaCl_2$ was added, causing an increase in $[Ca^{2+}]_i$ (Fig. 6F). After 15 minutes, 10 mM $MgCl_2$ was added to the medium; this treatment caused a substantial drop in $[Ca^{2+}]_i$ (Fig. 6F). In parallel experiments, GV oocytes were placed in Ca^{2+}- and Mg^{2+}-free medium containing 20 μM NS8593 for 15 min prior to $CaCl_2$ addition. In NS8593-treated oocytes, Ca^{2+} addition caused a substantially lower increase in $[Ca^{2+}]_i$ relative to controls, and subsequent Mg^{2+} addition did not further decrease $[Ca^{2+}]_i$ (Fig. 6G). Both area under the curve and peak amplitude following Ca^{2+} addition were significantly decreased in the presence of NS8593 (Fig. 6H,I). These results indicate that TRPM7-like channels contribute significantly to spontaneous Ca^{2+} influx in GV oocytes.

3.4. TRPM7-like channels contribute to Ca^{2+} influx following fertilization in mouse eggs

To test whether TRPM7-like channels also contribute to Ca^{2+} influx necessary to sustain post-fertilization Ca^{2+} oscillations, wild-type CF-1 eggs were treated with 20 μM NS8593 and fertilized in vitro (Fig. 6J). Compared to controls, NS8593-treated eggs had impaired Ca^{2+} oscillations, with significantly shorter first Ca^{2+} transients, reduced peak amplitude, lower frequency, and reduced persistence (Fig. 6K–N). These results suggest that TRPM7 or TRPM7-like channels also contribute to Ca^{2+} influx needed to maintain robust Ca^{2+} oscillations following fertilization.

4. DISCUSSION

A rise in intracellular Ca^{2+} is a universal activator of embryo development, and Ca^{2+} influx is required to sustain Ca^{2+} oscillations following mammalian fertilization [10, 11, 41]; however, the question of how this influx occurs is not fully resolved. We have recently shown that $Ca_V3.2$ channels contribute to Ca^{2+} influx in mouse oocytes and eggs, but additional Ca^{2+} influx mechanisms must also be functional [27]. Because Ca^{2+} content of the ER decreases and Ca^{2+} influx increases with each Ca^{2+} oscillation [12, 42], SOCE is a plausible candidate mechanism for mediating Ca^{2+} influx following fertilization. In this study we show that three SOCE mediators expressed in oocytes, STIM1, STIM2, and ORAI1, are dispensable for proper Ca^{2+} signaling and homeostasis in mouse oocytes and eggs. Our results are consistent with previous data showing that pharmacological inhibition of SOCE or expression of dominant negative SOCE components does not inhibit fertilization-induced oscillations in mouse eggs [16, 17].

Despite evidence to the contrary, there is a persistent view that SOCE is the primary mechanism of oocyte Ca^{2+}influx. The idea that SOCE is critical in oocyte physiology has even appeared in review literature [43–45]. This may not be surprising because SOCE participation in fertilization seems logical, and because roles for SOCE in mouse and pig oocytes have been suggested through experiments using overexpression, knockdown, and inhibitor strategies [18–21]. However, our current data show that critical mediators of SOCE are expendable for fertilization and Ca^{2+} signaling in mouse eggs. We show that the only two STIM proteins characterized to date, STIM1 and STIM2, are both dispensable in mouse oocytes and eggs. We also show that loss of ORAI1 does not impair Ca^{2+} signaling in mouse oocytes and eggs. Based on microarray data, *Orai1* is by far the most highly expressed transcript of the three *Orai* genes in mouse oocytes [36]; however, ORAI1 protein is internalized during meiosis so it is unlikely to function as a Ca^{2+} influx channel in eggs [15]. Results of prior studies supporting the actions of SOCE in mammalian eggs could have arisen in part from off-target effects of siRNA or morpholino oligonucleotides; however, it is likely that important differences between species also exist. For example, porcine oocytes may regulate ER Ca^{2+} differently than mouse oocytes and could therefore rely more heavily on SOCE. This interpretation is supported by experiments showing that the same SOCE inhibitors that halt Ca^{2+} oscillations in porcine eggs do not impair oscillations in mouse eggs [16, 17, 21]. Mouse eggs also have so-called “thapsigargin-insensitive” Ca^{2+} stores, capable of release by IP_3 or Ca^{2+} ionophore but not thapsigargin, whereas these stores are absent in porcine eggs [11, 21].

SOCE supports Ca^{2+} oscillations in somatic cells [46]; therefore, it seems somewhat unusual that eggs use alternate means to refill ER stores following fertilization. Meiotic cell cycle arrest is unique to oocytes, and Ca^{2+} is a critical signal that regulates meiosis, prompting the MII-arrested egg to reenter the cell cycle and switch to a mitotic program. Therefore, it is logical that mature eggs must control Ca^{2+} signaling very tightly and have multiple mechanisms to ensure that meiotic arrest is robust [47]. Perhaps down-regulating SOCE at this time serves as an additional means of insulating the egg from extraneous Ca^{2+} signals. SOCE is downregulated in *Xenopus* oocytes during meiosis [48, 49], and although we show it is not reliant on SOCE components, Ca^{2+} influx following store depletion decreases

during oocyte maturation in mice [1, 15]. This finding is also consistent with the down-regulation of SOCE that occurs as somatic cells enter mitotic M phase, which may help ensure that cell stage-specific needs for ion fluxes are met [50, 51].

Given that SOCE is dispensable for appropriate Ca^{2+} signaling in mouse oocytes, the question of what pathways support Ca^{2+} influx remains. The data reported here and that of others suggest that TRPM7-like channels contribute, at least in part, to Ca^{2+} influx in oocytes. Functional expression of TRPM7-like channels was recently shown in mouse oocytes [37]. Similar to our observation that TRPM7-like channels support spontaneous Ca^{2+} influx in GV oocytes, NS8593 reduces oocyte Ca^{2+} oscillations that occur in response to raising extracellular Ca^{2+} from 2 mM to 5mM [37]. Spontaneous influx in GV oocytes was previously attributed to SOCE because this response could be inhibited by 2-APB [1], but 2-APB is a notoriously non-specific inhibitor [33]. In addition to inhibiting Ca^{2+} influx mediated by SOCE [52], 2-APB also acts on a number of other channels and pathways, including acting as a TRPM7 inhibitor at concentrations below 1 mM [39]. Therefore, our hypothesis that spontaneous Ca^{2+} influx is mediated by TRPM7 is further supported by past experiments showing that 2-APB blocks Ca^{2+} influx in GV oocytes [1]. Furthermore, we show that inhibition of TRPM7-like channels during IVF impairs Ca^{2+} oscillations, suggesting that these channels also contribute to Ca^{2+} influx following fertilization. This conclusion is further supported by recent data demonstrating that NS8593 effectively blocks TRPM7-like current in mouse eggs [37]. TRPM7 is a promising candidate for mediating SOCE-like responses in mouse oocytes and eggs; however, as we have seen with SOCE, knowledge that can be gained using solely pharmacological inhibition is inherently limited. Therefore, a genetic approach will be needed to definitively determine the contribution of TRPM7.

We postulate that TRPM7 contributes to the Ca^{2+} influx that had previously been attributed to SOCE, but TRPM7 is unlikely to act alone in supporting Ca^{2+} influx in oocytes and eggs. We previously demonstrated that $Ca_V3.2$ contributes to Ca^{2+} influx to support ER store accrual and robust Ca^{2+} oscillations, but females lacking $Ca_V3.2$ are merely subfertile, indicating that this channel does not function alone either [27]. TRPV3 channels are present in mouse eggs and contribute to Sr^{2+}-mediated parthenogenesis; however, *Trpv3*$^{-/-}$ females are fertile and their eggs have normal Ca^{2+} oscillations following fertilization, indicating that these channels do not have a required physiological function in eggs [34]. It will be interesting to begin investigating the effects of knocking out these channels in combination to overcome inherent limitations of studies using pharmacological inhibitors and ultimately determine the mechanisms of Ca^{2+} influx that support egg activation.

We show that genetic knockout of SOCE components causes no measurable disruption of Ca^{2+} signaling in mouse oocytes and eggs; however, these cells display several behaviors reminiscent of SOCE, and explanations for these SOCE-like effects are still undetermined. Introduction of extracellular Ca^{2+} causes measurable Ca^{2+} influx in eggs that have depleted ER stores, but not in those with intact stores [1, 11, 15]. Membrane permeability to Ca^{2+} appears to rise following fertilization, and influx spikes occur concomitant with reduction in ER Ca^{2+} during initial Ca^{2+} oscillations [11, 42]. Overexpression of SOCE components in oocytes and eggs enhances the magnitude of SOCE-like responses and impacts Ca^{2+}

oscillations and influx, indicating that SOCE components can function in eggs when expressed at supra-physiological levels [1, 15]. Furthermore, while some effects of 2-APB on oocyte and egg responses can be explained by modulation of TRPM7 or TRPV3 channels, it is possible that this inhibitor also impacts SOCE-like responses in other ways in these cells [1, 15, 34, 37]. Thus, it is apparent that the status of ER Ca^{2+} stores influences Ca^{2+} influx regulation in mouse oocytes and eggs, but this effect is not mediated by STIM-ORAI interaction. Studies to define the pathways connecting ER Ca^{2+} stores to channels other than ORAI and TRPC in oocytes and eggs and may lead to discovery of a yet undescribed cellular mechanism linking Ca^{2+} store regulation and influx in these specialized cells.

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Highlights

- SOCE is not required for Ca^{2+} influx in mouse oocytes and eggs
- Eggs lacking STIM1, STIM2, or ORAI1 have normal Ca^{2+} oscillations after fertilization
- Oocyte spontaneous Ca^{2+} influx does not require STIM1, STIM2, or ORAI1
- Trpm7-like channels support Ca^{2+} influx in mouse oocytes and eggs

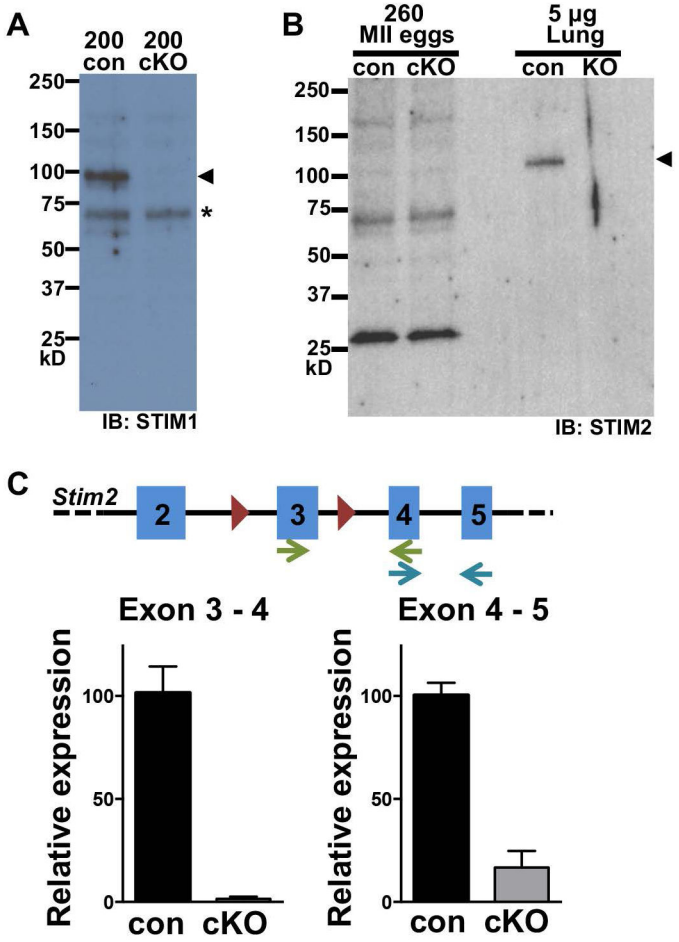

Figure 1. STIM1 and STIM2 expression in control and cKO eggs

(A) Representative immunoblot of STIM1 protein in *Stim1* cKO and control (con) eggs. 200 eggs per lane; blot is one of three independent replicates. Arrowhead indicates STIM1 protein band; asterisk indicates non-specific band. (B) Representative immunoblot of STIM2 protein in control and *Stim2* cKO eggs; 260 eggs/lane. Lung protein extract (5 µg) from either control or *Stim2*$^{-/-}$ postnatal day 1 pups was used for positive and negative controls. Arrowhead indicates STIM2 band. (C) Real time qPCR of *Stim2* mRNA in control and *Stim2* cKO eggs. Schematic shows *Stim2* exons (blue), loxP sites (red triangles), and primer pairs (arrows). Graphs show expression relative to that in control eggs for the indicated primer pair.

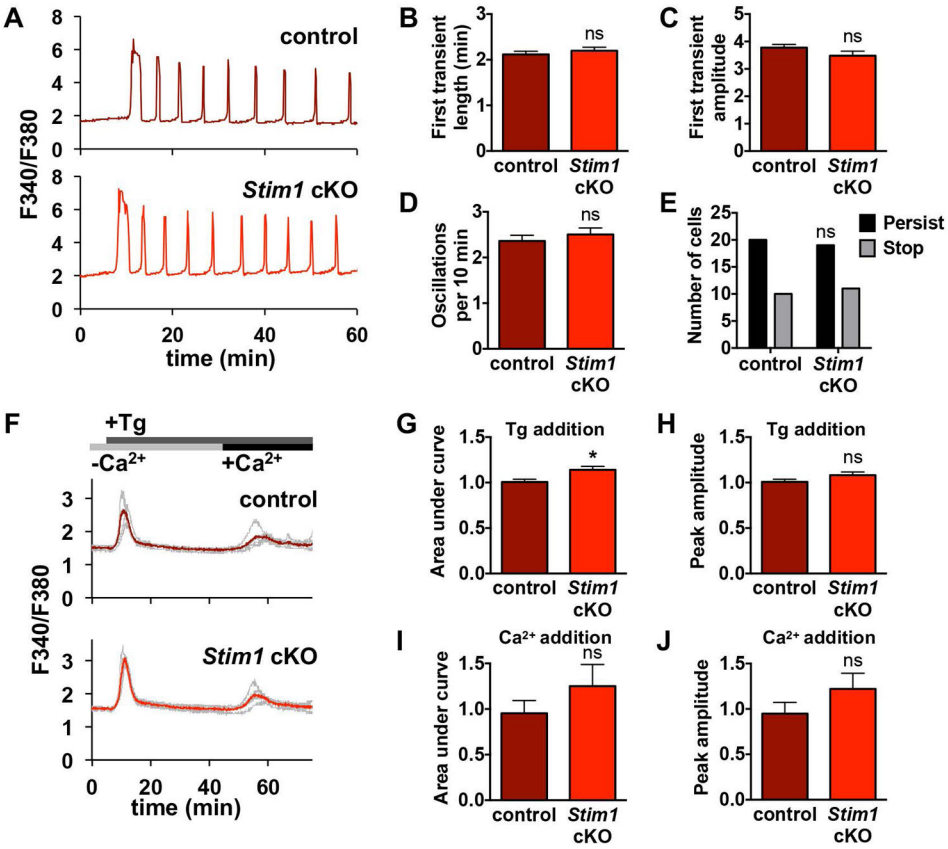

Figure 2. Ca^{2+} oscillation patterns following IVF and ER Ca^{2+} stores in eggs lacking STIM1

Ratiometric Ca^{2+} imaging of control and *Stim1* cKO eggs was performed during IVF. (A) Representative traces are shown. (B–E) Analysis of Ca^{2+} oscillation patterns, n=30 eggs per genotype over 5 replicate experiments. (B) Length of first transient. (C) Amplitude of first transient. (D) Oscillation frequency. (E) Persistence of oscillations to the end of the 60 min imaging period. (F–J) Measurements of ER Ca^{2+} stores and Ca^{2+} influx 35–40 min after thapsigargin (Tg)-induced ER store depletion in *Stim1* cKO and control eggs. (F) Representative traces (gray) and mean curve (red) are shown for one experiment. Timing of Tg and Ca^{2+} addition indicated in gray and black bars. (G–J) Analysis of Ca^{2+} stores and influx, n=24–33 eggs per genotype over 4 replicate experiments. (G,H) Graphs of ER Ca^{2+} stores indicated by area under the curve (G) and peak amplitude (H). * $p < 0.05$, unpaired t test. (I,J) Graphs of Ca^{2+} entry following store depletion indicated by area under the curve (I) and peak amplitude (J).

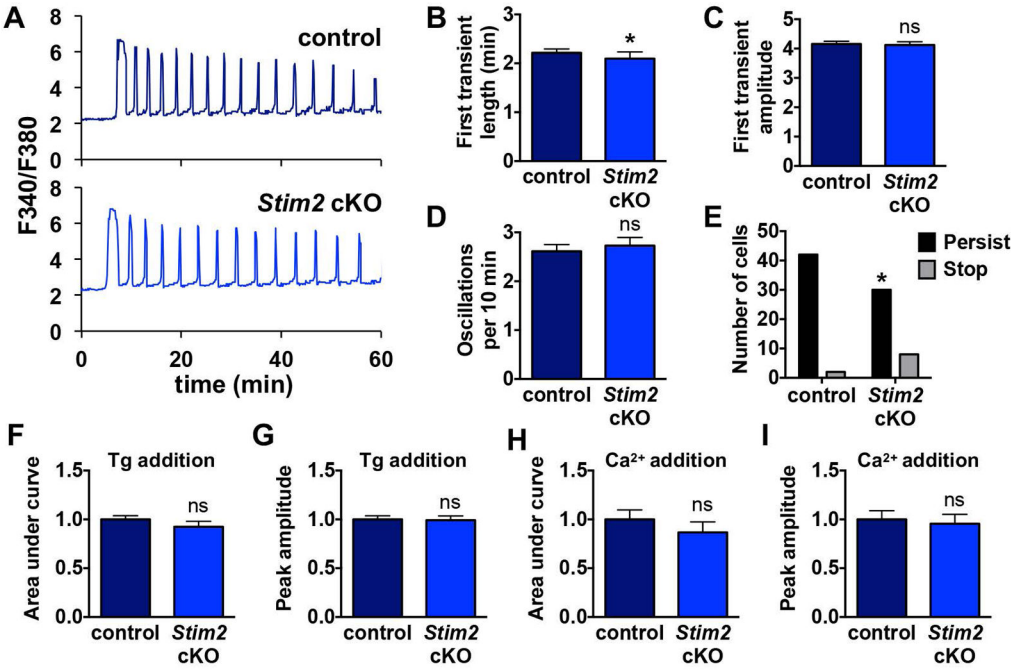

Figure 3. Ca^{2+} oscillation patterns following IVF and ER Ca^{2+} stores in eggs lacking STIM2

Ratiometric Ca^{2+} imaging of control and *Stim2* cKO eggs was performed during IVF. (A) Representative traces are shown. (B–E) Analysis of Ca^{2+} oscillation patterns, n=36–45 eggs per genotype over 6 replicate experiments. (B) Length of first transient. * $p < 0.05$, Mann Whitney. (C) Amplitude of first transient. (D) Oscillation frequency. (E) Persistence of oscillations to the end of the 60 min imaging period. * $p < 0.05$, Fisher's exact test. (F–I) Measurements of ER Ca^{2+} stores and Ca^{2+} influx 35–40 min after thapsigargin (Tg)-induced ER store depletion in *Stim2* cKO and control eggs, n=23 eggs per genotype over 4 replicate experiments. (F,G) Graphs of ER Ca^{2+} stores indicated by area under the curve (F) and peak amplitude (G). (H,I) Graphs of Ca^{2+} entry following store depletion indicated by area under the curve (I) and peak amplitude (J).

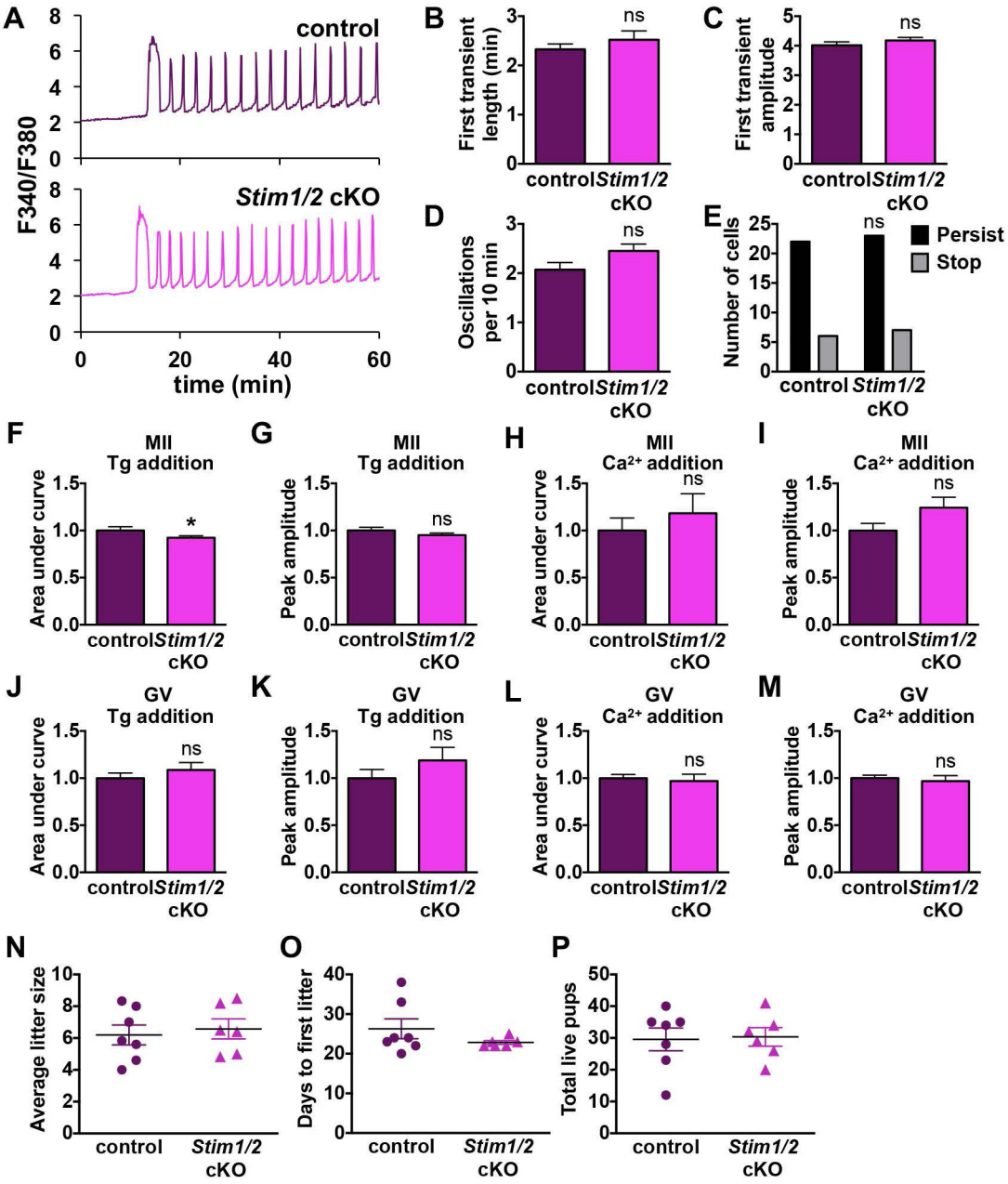

Figure 4. Analysis of MII eggs and GV oocytes lacking both STIM1 and STIM2

Ratiometric Ca^{2+} imaging of control and *Stim1/2* cKO eggs was performed during IVF. (A) Representative traces are shown. (B–E) Analysis of Ca^{2+} oscillation patterns, n=28–31 eggs per genotype over 4 replicate experiments. (B) Length of first transient. (C) Amplitude of first transient. (D) Oscillation frequency. (E) Persistence of oscillations to the end of the 60 min imaging period. (F–I) Measurements of ER Ca^{2+} stores and Ca^{2+} influx 35–40 min after thapsigargin (Tg)-induced ER store depletion in *Stim1/2* cKO and control eggs, n=23 eggs per genotype over 4 replicate experiments. (F,G) Graphs of ER Ca^{2+} stores indicated by area under the curve (F) and peak amplitude (G). * $p < 0.05$, Mann Whitney. (H,I) Graphs of Ca^{2+} entry following store depletion indicated by area under the curve (H) and peak

amplitude (I). (J–M) Measurements of ER Ca^{2+} stores and Ca^{2+} influx 35–40 min after thapsigargin (Tg)-induced ER store depletion in *Stim1/2* cKO and control GV oocytes, n=23–24 oocytes per genotype over 4 replicate experiments. (J,K) Graphs of ER Ca^{2+} stores indicated by area under the curve (J) and peak amplitude (K). (L,M) Graphs of Ca^{2+} entry following store depletion indicated by area under the curve (L) and peak amplitude (M). (N–P) Fertility of female *Stim1/2* cKO mice. (N) Average litter size. (O) Days to first litter. (P) Total number of live pups.

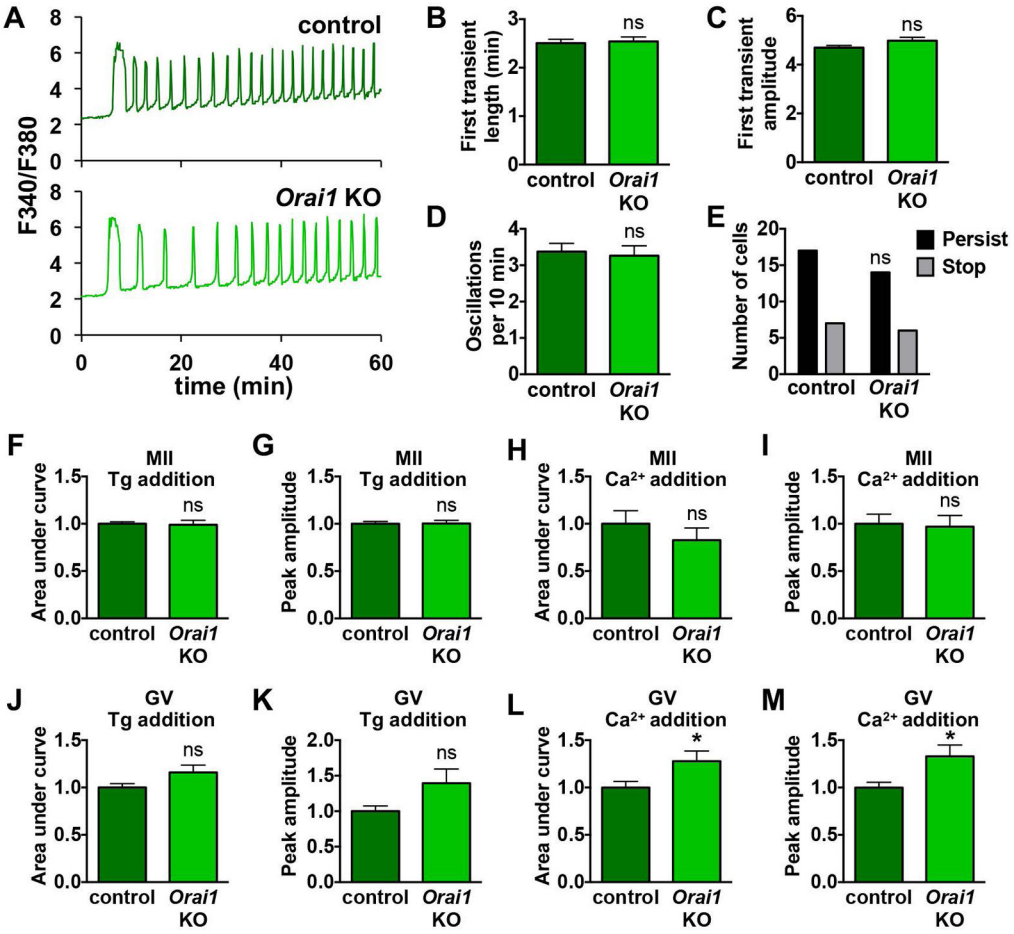

Figure 5. Analysis of MII eggs and GV oocytes lacking ORAI1

Ratiometric Ca^{2+} imaging of control and *Orai1* KO eggs was performed during IVF. (A) Representative traces are shown. (BE) Analysis of Ca^{2+} oscillation patterns, n=20–24 eggs per genotype over 4 replicate experiments. (B) Length of first transient. (C) Amplitude of first transient. (D) Oscillation frequency. (E) Persistence of oscillations to the end of the 60 min imaging period. (F–I) Measurements of ER Ca^{2+} stores and Ca^{2+} influx 35–40 min after thapsigargin (Tg)-induced ER store depletion in *Orai1* KO and control eggs, n=18–25 eggs per genotype over 3–4 replicate experiments. (F,G) Graphs of ER Ca^{2+} stores indicated by area under the curve (F) and peak amplitude (G). (H,I) Graphs of Ca^{2+} entry following store depletion indicated by area under the curve (H) and peak amplitude (I). (J–M) Measurements of ER Ca^{2+} stores and Ca^{2+} influx 40 min after thapsigargin (Tg)-induced ER store depletion in *Orai1* KO and control GV oocytes, n=22–23 oocytes per genotype over 4 replicate experiments. (J,K) Graphs of ER Ca^{2+} stores indicated by area under the curve (J) and peak amplitude (K). (L,M) Graphs of Ca^{2+} entry following store depletion indicated by area under the curve (L) and peak amplitude (M). * $p < 0.05$, unpaired t test.

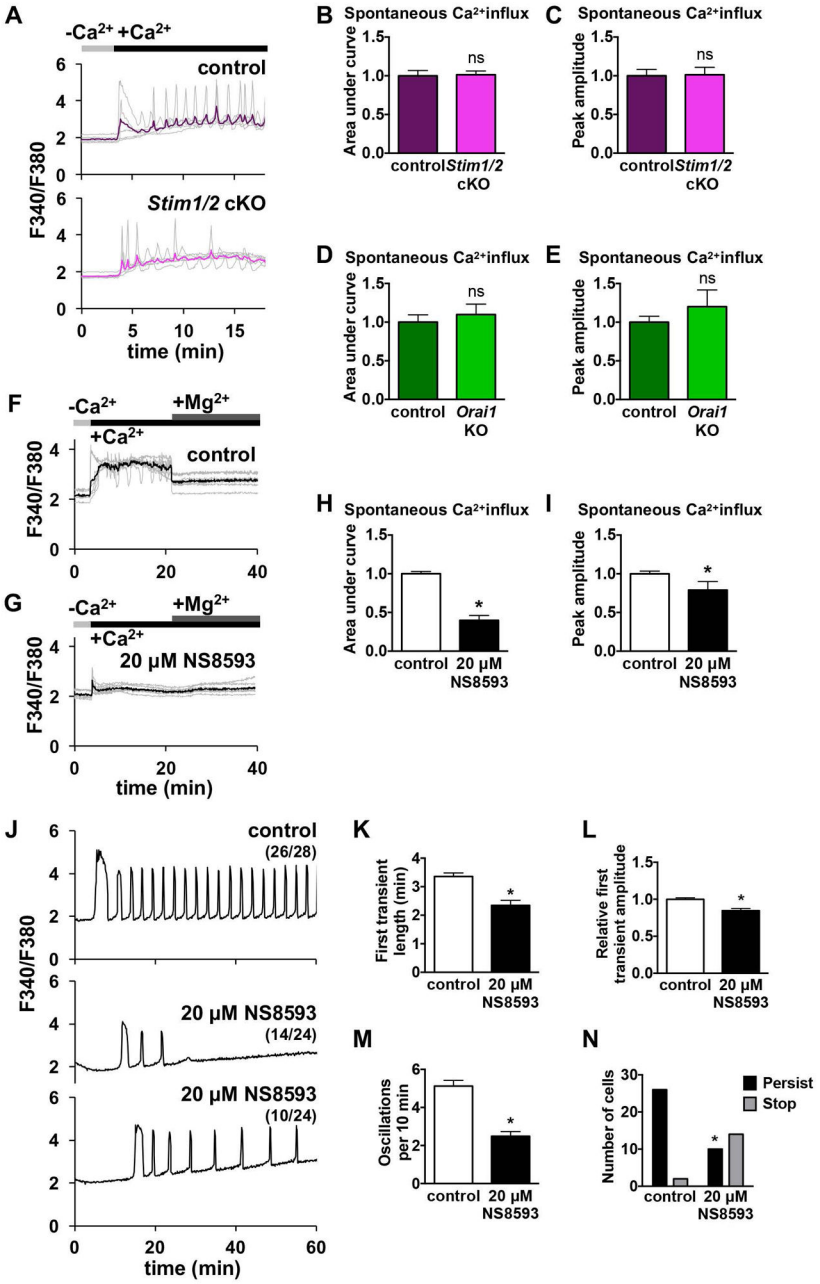

Figure 6. Spontaneous Ca^{2+} influx in mouse GV oocytes and post-fertilization Ca^{2+} influx in mouse eggs are mediated by TRPM7-like channels

(A–E) Ratiometric Ca^{2+} imaging of control and *Stim1/2* cKO or *Orai1* KO GV oocytes treated with 2 mM Ca^{2+} 3 min after placement into Ca^{2+}-free medium. (A) Representative traces (gray) and mean curve (red) are shown for one experiment. Timing of Ca^{2+} addition indicated by black bars. (B–E) Analysis of spontaneous Ca^{2+} influx in control and *Stim1/2* cKO or *Orai1* KO oocytes, n=18–26 oocytes per genotype over 3–4 replicate experiments. (B) *Stim1/2* cKO, area under the curve. (C) *Stim1/2* cKO, peak amplitude. (D) *Orai1* KO, area under the curve. (E) *Orai1* KO, peak amplitude. (F–I) Effect of TRPM7 channel inhibition on spontaneous Ca^{2+} influx in control GV oocytes. (F,G) Ratiometric Ca^{2+}

imaging of control oocytes treated with either vehicle (DMSO) or 20 μM NS8593 and then tested for spontaneous Ca^{2+} influx by addition of 5 mM Ca^{2+}. 10 mM Mg^{2+} was added 18 min following Ca^{2+} addition. (H,I) Graphs of spontaneous Ca^{2+} entry, n=39–46 oocytes per group over 4 replicate experiments. (H) Area under the curve. (I) Peak amplitude. * $p < 0.05$, Mann Whitney. (J–N) Ratiometric Ca^{2+} imaging of wild-type CF-1 eggs during IVF in medium containing vehicle (DMSO) or 20 μM NS8593. J) Representative traces are shown. (K–N) Analysis of Ca^{2+} oscillation patterns, n=24–28 eggs per genotype over 3 replicate experiments. (K) Length of first transient. * $p < 0.05$, Mann Whitney. (L) Amplitude of first transient. * $p < 0.05$, Mann Whitney. (M) Oscillation frequency. * $p < 0.05$, unpaired t test. (N) Persistence of oscillations to the end of the 60 min imaging period. * $p < 0.05$, Fisher's exact test.