Characterization of Calcium Homeostasis Parameters in TRPV3 and CaV3.2 Double Null Mice

Aujan Mehregan

University of Massachusetts Amherst

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CHARACTERIZATION OF CALCIUM HOMEOSTASIS PARAMETERS IN TRPV3 AND CAv3.2 DOUBLE NULL MICE

A Thesis Presented

by

AUJAN MEHREGAN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Molecular & Cellular Biology
CHARACTERIZATION OF CALCIUM HOMEOSTASIS PARAMETERS IN TRPV3 AND CAV3.2 DOUBLE NULL MICE

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AUJAN MEHREGAN

Approved as to style and content by:

Rafael A. Fissore, Chair

Dominique Alfandari, Member

Craig Albertson, Member

Dominique Alfandari, Program Leader, Molecular & Cellular Biology
DEDICATION

I dedicate this thesis to my dear friend, Patrick Jones, for his unwavering charisma, loyalty, and unsurpassable ambition. I will be forever grateful for your friendship.
ACKNOWLEDGMENTS

I would like to thank my advisor, Rafael Fissore, for his thoughtful and ambitious ideas and support. Thanks are also due to Dominique Alfandari for his undeviating leadership and to Craig Albertson, whose character and charisma create a persona that I aim to emulate in my career. Together, these advisors have provided invaluable guidance to my scientific career and professional development.

I want to thank our selfless lab manager, Changli He, for her endless contribution and support in the lab, and to my fellow teammate, Goli Ardestani, who trained and helped me extensively in this project.

I want to express my appreciation to the Xu lab for their donation of the TRPV3 knockout mice, and Jackson Laboratories for the CaV3.2 knockout mice.

A special thanks to my friends and family whose support, love, and patience kept me motivated and on track.
ABSTRACT

CHARACTERIZATION OF CALCIUM HOMEOSTASIS PARAMETERS IN
TRPV3 AND CA\textsubscript{v}3.2 DOUBLE NULL MICE

SEPTEMBER 2017

AUJAN MEHREGAN, B.S., UNIVERSITY OF MASSACHUSETTS, AMHERST
M.S., UNIVERSITY OF MASSACHUSETTS, AMHERST

Directed by: Professor Rafael A. Fissore

In mammals, calcium influx is required for oocyte maturation and egg activation, as it supports the persistent calcium oscillations induced by fertilization. These oscillations are required for the initiation of embryo development. The molecular identities of the plasma membrane calcium-permeant channels that underlie calcium influx are not established. Among these channels, Transient Receptor Potential Vanilloid, member 3 (TRPV3) allows divalent cations, namely strontium (Sr\textsuperscript{2+}) and calcium (Ca\textsuperscript{2+}) with high permeability, into cells, and its expression pattern seems to predict an essential role in the initiation of development. Another channel that was identified to be expressed in oocytes/eggs is the low-voltage-activated T-type channel, Ca\textsubscript{v}3.2. However, the ability to accurately probe the expression and function of these channels on Ca\textsuperscript{2+} homeostasis in mouse eggs is hindered by the lack of specific and known pharmacological agents and antibodies for these channels.

Here, we simultaneously knock out these two Ca\textsuperscript{2+} influx channels in the mouse to explore the effects on Ca\textsuperscript{2+} homeostasis. We examined fertility rates, development, and morphological defects that arose from the double null pups. Next, we investigated the consequences on Ca\textsuperscript{2+} store content in immature and mature oocytes and eggs. We also
examined the effects on fertilization-induced Ca\(^{2+}\) oscillations in response to *in vitro* fertilization and \(\text{PLC}\zeta\) cRNA microinjection. We found that female mice null for these channels display drastic subfertility compared to the single knockout mice for these channels. Additionally, the Ca\(^{2+}\) store content is significantly diminished in double knockout eggs versus controls, as was the frequency of the fertilization-induced Ca\(^{2+}\) oscillations. These results suggest that these channels play a crucial role in Ca\(^{2+}\) influx during maturation and contribute to maintain Ca\(^{2+}\) oscillations post-fertilization. These null oocytes and eggs will be an important tool to perform electrophysiological studies to accurately measure the native current(s) of a specific channel(s) in eggs, and to identify the channel(s) that mediate Ca\(^{2+}\) during fertilization.
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CHAPTER 1

INTRODUCTION

Mammalian egg activation is a widely-researched field, as it is the first stage of embryo development. During this event, the egg requires energy to undergo changes that will prepare it for fertilization, such as resuming meiosis, remodeling its outer cortex to block polyspermy, reorganizing the cytoskeleton and meiotic spindle, and translating, storing, and changing maternal mRNA and protein levels (Horner and Wolfner, 2008). The focus of the Fissore laboratory is to discover the mechanism by which sperm induces activation and subsequently triggers embryo development in mammals.

In this species, fertilization is induced when the sperm fuses to a mature oocyte (egg), and initiates changes in the intracellular concentration of free calcium ([Ca^{2+}]). Ca^{2+} homeostasis is regulated and optimized in the oocyte in preparation for fertilization (reviewed in Berridge et al., 2000; Wakai et al., 2011; Whitaker, 2006). At this critical moment, the sperm induces a series of precise Ca^{2+} rises, known as oscillations, that are ultimately responsible for triggering embryonic development via regulation of the stability of proteins that regulate cell cycle progression, and resumption and completion of meiosis (Miyazaki and Igusa, 1981; Ducibella et al., 2002; Ozil et al., 2005). Ca^{2+} oscillations rely on Ca^{2+} influx from the extracellular media to replenish the stores, and a goal of the laboratory is to identify the molecule(s)/channel(s) responsible for this influx, as this has not yet been established.

The channels that mediate Ca^{2+} influx are thought to reside in the plasma membrane (PM) of the egg. Research shows that the oocyte, during maturation – the process initiated prior to ovulation and fertilization and following the surge of luteinizing hormone – undergoes a plethora of changes including the increase of Ca^{2+} store content ([Ca^{2+}]_{ER}), which requires
Ca$^{2+}$ influx (reviewed in Wakai et al., 2011). Remarkably, previous studies have also demonstrated that during maturation, Ca$^{2+}$ influx progressively decreases as [Ca$^{2+}$]$_{ER}$ increases (reviewed in Wakai et al., 2011). Baseline Ca$^{2+}$ and [Ca$^{2+}$]$_{ER}$ are strictly controlled because an excess of Ca$^{2+}$ could cause parthenogenetic activation, fragmentation and/or apoptosis (Gordo et al., 2002; Ozil et al., 2005), whereas a deficit will impede many cellular functions, including protein synthesis, completion of maturation and initiation of embryonic development. Oocytes and eggs have several mechanisms to regulate elevated intracellular Ca$^{2+}$, including pumps and exchangers such as the PM Ca$^{2+}$-ATPase (PMCA) and Na$^{+}$/Ca$^{2+}$ exchangers that extrude excess Ca$^{2+}$, while the sarco-endoplasmic reticulum Ca$^{2+}$-ATPases reuptake Ca$^{2+}$ into the ER thereby refilling its stores (Berridge et al., 2000; Bootman et al., 2001). This entire phenomenon is known as the Ca$^{2+}$ toolkit, one that every cell type possesses to regulate Ca$^{2+}$ and trigger crucial processes such as muscle contraction, exocytosis, and metabolism, among others (Berridge et al., 2003).

The identification of the molecular mechanisms and channels responsible for Ca$^{2+}$ homeostasis in mammalian oocytes and eggs is largely incomplete. Among the PM channels, the transient receptor potential (TRP) family of channels play an essential role in detecting changes in the environment via stimuli ranging from changes in temperature to changes in voltage. With seven subfamilies and nearly 30 human members (Clapham et al., 2003), TRP channels are expressed in multiple cell types and tissues. Preliminary studies in the laboratory show that members of the TRP family of channels are expressed in mouse oocytes; specifically, using RT-PCR, we have demonstrated the presence of TRP Vanilloid, member 3 (TRPV3). TRPV3 allows divalent cations, namely strontium (Sr$^{2+}$) and Ca$^{2+}$ with high permeability, into cells, and its expression pattern seems to predict an essential role in the initiation of
development. Importantly, it is essential for triggering parthenogenetic embryonic development using Sr$^{2+}$ stimulation; however, it is not required for normal fertility, as null females are fertile (Carvacho et al., 2013). Another channel involved in Ca$^{2+}$ homeostasis is the T-type voltage-gated channel, Ca$_V$3.2. In a study illustrating the effects of Ca$_V$3.2 in oocytes and eggs, Bernhardt et al. show that a voltage-activated Ca$^{2+}$ current that is normally present in mouse eggs, is absent in Ca$_{cna1h}^{-/-}$ eggs (2015). Nevertheless, these females are mildly subfertile, which is consistent with the knowledge that changes in membrane potential during mouse fertilization are minor and the holding membrane potential of mouse oocytes and eggs is such that only a minor number of Ca$_V$ channels should be active (Igusa et al., 1983; Jaffe and Cross, 1984).

Furthermore, these channels are only differentially expressed in the maturing oocyte. TRPV3 expression is nearly absent in the early germinal vesicle (GV) stage of the oocyte, but rises steadily with its maximal expression being at a fertilization-competent MII egg (Carvacho et al., 2013). On the other hand, the expression levels of Ca$_V$ channels during oocyte maturation and in eggs are unknown. Remarkably, these channels were one of the first channels to be recorded via electrophysiology in oocytes/eggs (Peres, 1986; Peres; 1987). Why some of these channels are differentially expressed and/or regulated during oocyte maturation requires further investigation.

Thus, despite identification of some channels in mammalian oocytes and eggs, the complete set of channels responsible for filling the internal Ca$^{2+}$ stores and supporting oscillations has not been found. Furthermore, the ability to accurately probe the effects of channel inhibition on Ca$^{2+}$ homeostasis in mouse eggs is hindered by the lack of specific and known pharmacological agents. Therefore, evaluation of Ca$^{2+}$ store content, Ca$^{2+}$ changes in
response to agonists, and fertilization in oocytes and eggs null for specific channel(s) is one of the approaches used to identify the channel(s) responsible for supporting Ca\(^{2+}\) oscillations required for embryo development. In addition, these oocytes and eggs null for specific Ca\(^{2+}\) channel(s) will be an important tool to perform electrophysiological studies to accurately measure the native current(s) of a specific channel(s).

To these ends, we hypothesize that mice lacking both \textit{Trpv3} and \textit{Cacna1h} will 1) be subfertile and/or infertile and 2) exhibit altered Ca\(^{2+}\) homeostasis and possibly decreased developmental competence. The implications of this study will aid in the development of conditions to enhance developmental competence, especially of oocytes matured \textit{in vitro}. Moreover, since Ca\(^{2+}\) is required for egg activation, the elucidation of the channels that mediate influx at fertilization as well as the development of specific channel blockers, could become a novel method of contraception to be used in humans, or to prevent the uncontrolled population growth of wild life species.
CHAPTER 2

RESULTS

Double null mice lacking *Trpv3* and *Cacna1h* genes are subfertile.

Our first goal was to generate a double null mouse line lacking the *Trpv3* and *Cacna1h* genes. The rationale for this stemmed from the establishment of the single knockout lines for these genes displaying little effect on Ca$^{2+}$ homeostasis or influx in oocytes (Carvacho et al., 2013; Bernhardt et al., 2015). Our objectives were to determine the degree to which the double null mouse line will show fertility defects, and at the same eliminate two important channels that are present in oocytes/eggs that support Ca$^{2+}$ influx and display electrophysiological currents. Our ultimate goal is to pinpoint the channel(s) responsible for Ca$^{2+}$ influx during fertilization and egg activation. The double null mouse line was obtained following breeding of single knockout mice to generate the initial pool of double heterozygotes. Males and females of this generation were bred to generate the parent generation of double knockout and double heterozygous mice that were used in the following studies.

![Figure 1. PCR confirmation of targeted deletions of *Trpv3* and *Cacna1h* genes.](image)

PCR Genotyping of wild-type (WT), heterozygous (Het), and knockout (KO) mice. Two sets of primers were used to amplify *Trpv3* and *Cacna1h* as described in Methods. Products yielded from Primer Set A: WT 800 bp, Het 800 & 300 bp, KO 300 bp. Products yielded from Primer Set B: WT 480 bp, Het 480 & 330 bp, KO 330 bp. Germline deletion of the *Trpv3* and *Cacna1h* alleles was confirmed via PCR analysis using ear tissue DNA prepared from 21 day-old mice (Fig. 1).
We next evaluated the fertility of the males and females lacking the *Trpv3* and *Cacna1h* genes. The single knockout lines for *Trpv3*<sup>−/−</sup> and *Cacna1h*<sup>−/−</sup> have previously been shown to be viable and fertile (Cheng et al., 2010; Chen et al., 2003). Double heterozygous mice were used as controls. Five females from each group, double knockout (dKO) and double heterozygous (dHET) were bred with five males of the same genotype for 36 weeks. Data from the first seven litters was used for analysis. The time to first litter was significantly different, as double knockout females delivered pups after a mean of 41.2 ± 2.31 days post-introduction of the male compared to double heterozygous females who delivered pups after 26.6 ± 2.38 days (p ≤ 0.05).

The interval between double knockout litters increased dramatically by the fourth parturition;
68 days in the double knockout line versus 34.5 days in the double heterozygous line (Fig. 2A).

The average litter size from double knockout females decreased by about 50% to a mean of 4.56 ± 0.39 versus 8.47 ± 0.39 (p ≤ 0.0001), while the number of litters produced between the two groups was comparable (Table 1). Remarkably, in the double knockout line, the number of pups per litter decreased significantly by, or after, the third parturition (dKO: 4.9 ± 0.59 versus dHET: 8.47 ± 0.39; p ≤ 0.0001) (Fig. 2B). It is also worth noting that after the third parturition, and with each successive parturition, the number of neonatal deaths became prominent, with about 40-80% of pups dying per litter (data not shown). The total number of pups born from all females in each group varied significantly with a total of 115 pups yielded from double knockout females versus 223 pups yielded from the controls (p ≤ 0.0001) (Fig. 2C). These results demonstrate that these channels are not required for fertilization or to support embryo development to term; although, they seem necessary for full fertility.

Next, we investigated the possibility of obvious differences in ovarian size, ovulation rates, and on the rates of in vitro maturation and in vitro fertilization. First, we examined ovarian weight and number of eggs ovulated post hormone injection, and found that there was no significant difference in either category (Fig. 3A-B). Ovarian shape and size were also similar between the two groups (Fig. 3C). It is worth noting that these evaluations were

Table 1. Double knockout mice display substantial subfertility.

<table>
<thead>
<tr>
<th>Male</th>
<th>Crosses x</th>
<th>Female</th>
<th>Litters Born (35 week period)</th>
<th>Litter Size (Mean ± S.E.M.)</th>
<th>Pups/Female (35 week period)</th>
<th>Total Pups (35 week period)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dHET (n = 5)</td>
<td>x</td>
<td>dHET (n = 5)</td>
<td>24</td>
<td>8.36 ± 0.49</td>
<td>84 ± 4.0</td>
<td>204</td>
</tr>
<tr>
<td>dKO (n = 5)</td>
<td>x</td>
<td>dKO (n = 5)</td>
<td>22</td>
<td>4.56 ± 0.39***</td>
<td>24.3 ± 1.45***</td>
<td>78†</td>
</tr>
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Comparison of mating and population data from five individual mating pairs of double heterozygous (dHET) and double knockout (dKO) mice after 36 weeks of uninterrupted breeding. Litter size and pups/female were analyzed for statistical significance using two-way analysis of variants (ANOVA); whereas total pups was analyzed using the chi square test. Results are presented as mean ± S.E.M. of all five animals in each group. Statistical significance is indicated by *** superscript for ANOVA, or † superscript for chi square. p ≤ 0.0001 in all categories.
performed in young animals, which, as shown in the previous figures, were not expected to show defects in fertility. Next, we evaluated whether GV oocytes from the double heterozygous and double knockout mice were able to mature completely under in vitro conditions. We used Chatot, Ziomek, and Bavister (CZB) medium supplemented with 4 mg/mL bovine serum albumin (BSA) for 12 hours under 5% CO₂ and 37 °C. There was no delay in any stage of maturation in the double knockout oocytes compared to rates observed in control oocytes (Fig. 3D). Finally, in vivo matured MII eggs from each line were also fertilized in vitro, and development to blastocyst was examined; we were unable to observe delays or morphological differences between embryos of the two groups (Fig. 3E). This data reinforces the notion that TRPV3 and Cav3.2 channels are functionally present in

**Figure 3. Double knockout females display no morphological differences.**

A: Mean mass of superovulated ovaries taken after MII egg collection. Each pair of ovaries was weighed individually. Mean ± S.E.M. for each group was calculated from n = 28 ovaries. Statistical significance was calculated using two-tail t-test. B: Number of MII eggs ovulated 14 hours post-hCG injection. Mean ± S.E.M. for each group was calculated from n = 14 females. Statistical significance was calculated using two-tail t-test. C: Ovaries were prepared for imaging by removing excess fat tissue and oviduct. Images were taken on a Nikon dissection microscope outfitted with a 1X shutter. Scale bar represents 50 µM. D-E: DIC image of MII egg and 2-cell stage embryo. Images were taken on a Nikon Diaphot microscope outfitted with an 8 megapixel camera. Scale bars represent 10 µM.
mouse oocytes and eggs, but are not required for oocyte maturation or egg activation, at least in young female mice.

Functional evidence that double knockout females lack TRPV3 and Cav3.2 channels.

Strontium (Sr\(^{2+}\)), though toxic to cells after long exposures, is a useful method to induce parthenogenesis in rodent eggs. In MII eggs, Carvacho et al. demonstrated that Sr\(^{2+}\) influx is mediated via TRPV3 channels (2013). We therefore tested if exposing double knockout and double heterozygous oocytes and eggs to 10 mM SrCl\(_2\) for two hours induced oscillations. As expected, when TRPV3 and Cav3.2 channels were absent, double knockout GV oocytes showed significantly diminished spontaneous oscillations compared to double heterozygous oocytes that showed responses.

Figure 4. Functional evidence that double knockout eggs lack TRPV3 and Cav3.2 channels.

A: Spontaneous oscillations in GV oocytes induced by exposure to 10 mM SrCl\(_2\). Representative double heterozygous oocyte (black trace), have 2-3 oscillations in 60 minutes versus (n = 6/8). B: Spontaneous oscillations in GV oocytes induced by exposure to 10 mM SrCl\(_2\). Representative double knockout oocyte (red trace), have 0-1 oscillation in 60 minutes (n = 3/8). C-D: Oscillations induced in MII eggs by exposure to 10 mM SrCl\(_2\). C, black trace shows representative double heterozygous egg, showing 3-4 oscillations in 60 minutes (n = 16/20) versus D, red trace, which shows representative double knockout egg (no response; n = 0/20). 200 \(\mu\)M 2-APB was applied to media at the end of the experiment.
(Fig. 4A-B). At MII, double knockout eggs failed to show any Ca\(^{2+}\) oscillations due to Sr\(^{2+}\) influx, whereas the double heterozygous eggs showed responses (Fig. 4C-D). Further, when eggs were incubated in 10 mM SrCl\(_2\)-containing media for two hours then washed into culture media, double knockout eggs did not show any signs of activation, such as extrusion of the 2\(^{nd}\) polar body, pronucleus formation or cleavage, whereas controls showed complete egg activation (Fig 4E). Another way to test for the absence of TRPV3 is by examining the response to 2-Aminoethoxydiphenyl borate (2-APB), which was first identified as a blocker of IP\(_3\)R1 (Maruyama et al., 1997). Remarkably, 2-APB potentiates TRP Vanilloid channels, and is the most used activator of TRPV3 (Chung et al., 2004; Hu et al., 2004; Hu et al., 2009). Here, we show that at 200 µM, 2-APB does not induce a Ca\(^{2+}\) rise in the double knockout eggs, but it does in control eggs (Fig. 4C-D). This data confirms and reinforces the finding that 2-APB induces a Ca\(^{2+}\) rise in eggs through the TRPV3 channel and that our double knockout mice lack TRPV3.

The absence of Ca\(_V\)3.2 is harder to test without electrophysiology; although, recent evidence from our laboratory has shown that Ca\(_V\)3.2 may be an important mediator of Sr\(^{2+}\) influx at the GV stage. In wildtype (WT) mice, 10 mM SrCl\(_2\) exposure at the GV stage elicits spontaneous and irregular Ca\(^{2+}\) rises. We tested this effect in the double knockout oocytes, and observed that there was a diminished effect of SrCl\(_2\) exposure (Fig. 4B), suggesting that Ca\(_V\)3.2 channels may underlie most of the Ca\(^{2+}\) influx at the GV stage. Together, our results show that our double knockout mice lack functional expression of TRPV3 and Ca\(_V\)3.2 channels.

Ca\(^{2+}\) stores are affected in double knockout females.

The subfertility of the double knockout mice suggested that the oocytes may have some impaired Ca\(^{2+}\) homeostasis parameters. It has been previously documented that the Ca\(^{2+}\)
content within the ER increases ([Ca\textsuperscript{2+}]\textsubscript{ER}) throughout oocyte maturation, which effectively

![Graph A](image1)

![Graph B](image2)

**Figure 5. Ca\textsuperscript{2+} stores are affected in double knockout eggs.**

A-B: Total Ca\textsuperscript{2+} store content was approximated by the addition of 2.5 μM Ionomycin under nominal Ca\textsuperscript{2+} conditions. Black trace (A; n = 20) represents double heterozygous mean. Red trace (B; n = 20) represents double knockout mean. Ionomycin was added after 7 minutes of recording baseline values.

C-D: Summary of parameters measured. Area under the curve and relative max amplitude were calculated using Area Under the Curve analysis in Prism software after addition of IO. Baseline was calculated from mean of y values from x = 0 to x = 5 min. (AUC of dHET: 9.69 ± 0.86, n = 8; dKO: 5.13 ± 0.46, n = 10; p ≤ 0.0001).

E-F: [Ca\textsuperscript{2+}]\textsubscript{ER} was measured by the addition of 10 μM thapsigargin (TG) under nominal Ca\textsuperscript{2+} conditions. Black trace (E; n = 20) represents double heterozygous mean. Red trace (F; n = 20) represents double knockout mean. TG was added after 6 minutes of recording baseline values.

G-H: Summary of parameters measured from time of TG addition until return to baseline values. Area under the curve and relative max amplitude were calculated using Area Under the Curve analysis in Prism software after addition of TG. Baseline was calculated from mean of y values from x = 0 to x = 5 min. (AUC of dHET: 2.03 ± 0.095, n = 20; dKO: 1.33 ± 0.085, n = 20; p ≤ 0.0001).

plays a role in the preparation of the oocyte for fertilization (Jones et al., 1995; Wakai et al., 2011; Wakai et al., 2013). Little is known about the mechanism by which oocytes accumulate
Ca$^{2+}$ in the stores; though results from our laboratory suggest that the main source of increased $[\text{Ca}^{2+}]_{\text{ER}}$ is due to influx of external Ca$^{2+}$. Since TRPV3 and CaV3.2 are important Ca$^{2+}$ influx channels, we hypothesized that the $[\text{Ca}^{2+}]_{\text{ER}}$ would be diminished.

To test this hypothesis, we used a Ca$^{2+}$ ionophore, Ionomycin (IO), to empty all Ca$^{2+}$ stores in the cell (Fig. 5A-B). By analyzing the resulting area under the curve and relative maximum amplitude (Fig. 5C-D), we observed that the total Ca$^{2+}$ content, measured by area under the curve, was significantly decreased in the dKO mice (5.13 ± 0.46) compared to the control mice (9.69 ± 0.86; $p \leq 0.0001$). Next, we directly examined the impact on $[\text{Ca}^{2+}]_{\text{ER}}$ by using Thapsigargin (TG), a sarcoendoplasmic reticulum calcium ATPase (SERCA) inhibitor, which is the pump that fills the ER, the major Ca$^{2+}$ reservoir in the cell (Fig. 5E-F) (Jones et al., 1995; Kline and Kline, 1992; reviewed in Berridge et al., 2002). In the absence of extracellular Ca$^{2+}$, TG causes a transient increase in intracellular Ca$^{2+}$ levels by emptying the ER, and the empty stores can now support Ca$^{2+}$ influx to refill the stores. Such a mechanism can be visualized if, after returning to baseline, extracellular Ca$^{2+}$ is added back to the media. We tested the eggs’ ability to influx Ca$^{2+}$ after TG by adding 2 mM CaCl$_2$. We observed a significant difference in $[\text{Ca}^{2+}]_{\text{ER}}$ levels between the double knockout (mean area of 1.33 ± 0.085 AU) and double heterozygous oocytes (mean area of 2.03 ± 0.095 AU; $p \leq 0.0001$) (Fig. 5G-H). Notably, there was no significant difference in Ca$^{2+}$ influx capability between the two groups (data not shown). Collectively, these results suggest that oocytes null for two Ca$^{2+}$ influx channels can still maintain, but to a lesser degree, $[\text{Ca}^{2+}]_{\text{ER}}$ levels during maturation and in MII eggs, and therefore that TRPV3 and CaV3.2 channels are required to obtain a full amount of $[\text{Ca}^{2+}]_{\text{ER}}$. 
Ca$^{2+}$ influx is diminished post-fertilization in double knockout eggs.

Previous studies have suggested that the introduction of PLCζ following sperm-egg fusion underlies egg activation and fertilization-associated [Ca$^{2+}$]$_i$ oscillations (Saunders et al., 2002). This mechanism occurs via the activation of the egg’s phosphoinositide pathway to generate inositol 1,4,5-triphosphate (IP$_3$), which in turn triggers IP$_3$-receptor mediated Ca$^{2+}$ release from the internal stores (Saunders et al., 2002; Miyazaki et al., 1993; Miyazaki and Ito, 2006; Jellerette et al., 2000). TRPV3 has been previously shown to be unnecessary for the maintenance of fertilization-induced Ca$^{2+}$ oscillations (Carvacho et al., 2013). To examine how

**Figure 6. Ca$^{2+}$ influx is diminished post-fertilization.**

Oscillation pattern is affected following egg fertilization. A-B: Oscillations induced by *in vitro* fertilization in double heterozygous eggs (A, black trace; n = 10/20) have 10.7 ± 0.88 oscillations in 60 minutes versus double knockout eggs (B, red trace; n = 12/20) who have 4.8 ± 0.37 oscillations in 60 minutes. C: Summary of parameters measured. D-E: Oscillations induced by 0.01 µg/µL mouse PLCζ in double heterozygous (D, black trace; n = 18/20) have 3.06 ± 0.17 oscillations in 60 minutes versus double knockout eggs (E, red trace; n = 15/20) who have 1.54 ± 0.22 oscillations in 60 minutes. 10 mM CaCl$_2$ was applied at the end of the experiment. F: Summary of parameters measured. Statistical significance was calculated using two-tail t-test, significance in both cases had $p \leq 0.0001$. 

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double knockout eggs mounted oscillations, we performed *in vitro* fertilization in double knockout and double heterozygous eggs, and examined the Ca\(^{2+}\) responses (Fig. 6A-B). Although all mammalian studies to date mount oscillations in response to fertilization, the Ca\(^{2+}\) responses display certain degrees of species-specificity (Ozil et al., 2006; Ducibella and Fissore, 2008). We observed that while double heterozygous eggs showed the normal frequency of Ca\(^{2+}\) oscillations, the frequency of oscillations in double knockout eggs was substantially lower (Fig. 6C) with mean frequencies of 10.7 ± 0.88 oscillations per hour versus 4.8 ± 0.37 oscillations per hour, respectively (\(p \leq 0.0001\)). The total number of Ca\(^{2+}\) transients was also decreased in the double knockout eggs compared to the double heterozygous eggs (data not shown), suggesting that these channels are not required for the initiation of fertilization-induced oscillations, but remarkably affect the periodicity of such.

As a surrogate of fertilization, we tested the ability of the double knockout eggs to mount Ca\(^{2+}\) oscillations following PLCζ cRNA microinjection, which is the sperm’s component responsible for inducing Ca\(^{2+}\) oscillations (Parrington et al., 1999; reviewed in Swann et al., 2006; Parrington et al., 2007) As shown, the time to initiation was longer, and the mean number of Ca\(^{2+}\) transients in one hour after the first transient was lower for double knockout eggs (1.54 ± 0.22) versus control eggs (3.06 ± 0.17; \(p \leq 0.0001\)) (Fig. 6D-F); however, when 10 mM Ca\(^{2+}\) was added to the extracellular media, double knockout eggs still showed rapid oscillations, suggesting that these channels, while not required to maintain oscillations, play a role in Ca\(^{2+}\) influx during fertilization.
TRPM7 is expressed in eggs of double knockout mice.

The fact that the deletions of Trpv3 and Cacna1h did not fully prevent the filling of $[Ca^{2+}]_{ER}$ suggests the presence of $Ca^{2+}$ influx by other channels. TRP Melastatin 7 (TRPM7) presence has been identified via electrophysiology (Carvacho et al., 2016), and is functionally expressed in oocytes. This unique chanzyme is significantly modulated by free magnesium ($Mg^{2+}$) at the plasma membrane-associated domain, by $Mg^{2+}$-ATP at the intracellular kinase domain, as well as by high extracellular concentrations of $Mg^{2+}$ (Bates-Withers et al., 2011).

Remarkably, physiological concentrations of $Mg^{2+}$ in the cell and concentrations of $Mg^{2+}$ in commonly used culture media, like HEPES-buffered Tyrode’s lactate solution (TL HEPES), are great enough to partially obstruct TRPM7 current.

Recently, it has been shown that fertilization-induced embryo development in several species is increased in media with lower concentrations of $Mg^{2+}$ (Herrick et al., 2015). To determine if indeed high extracellular $Mg^{2+}$ was affecting oscillations, we first induced spontaneous oscillations in GV oocytes, and monitored oscillations in $Mg^{2+}$-containing and

**Figure 7. TRPM7 is expressed in double knockout females.**
A-B: Oscillations induced by 10 mM SrCl$_2$ in double heterozygous GV oocytes (A, black trace; n = 30/30) and double knockout GV oocytes (B, red trace; n = 18/20). Monitoring was continuous throughout changes in $Mg^{2+}$ concentrations.
Mg$^{2+}$-free environments (Fig. 7A-B). By adding Mg$^{2+}$ back to the media in the latter scenario, oscillation frequency slowed and, in some cases, ceased to continue. We observed the same effects in double knockout eggs, which suggest for the first time, that TRPM7 is expressed in eggs of double KO mice, and that its presence may explain, at least in part, the initiation and persistence of oscillations in these eggs.
CHAPTER 3
DISCUSSION & CONCLUSIONS

Here, we extended studies on the functions of two main plasma membrane channels responsible for Ca\(^{2+}\) influx in mouse oocytes and eggs, TRPV3 and Ca\(_V\)3.2. These channels were one of the first channels identified via molecular biology and electrophysiology (Peres, 1986; Peres, 1987; Peier et al., 2002; Xu et al., 2002). They are expressed in maturing oocytes, although definitive characterization of their expression, and function of Ca\(_V\)3.2 channels, during this process requires further investigation. Nonetheless, we aimed to study the extent to which these channels are responsible for maintaining and increasing \([\text{Ca}^{2+}]_{\text{ER}}\) during maturation as well as their role in fertilization. Previously, it has been shown that mouse oocytes null for only TRPV3 (Carvacho et al., 2013) or only Ca\(_v\)3.2 (Bernhardt et al., 2015) do not display any effect in developmental or fertilization competency and are neither necessary nor sufficient for Ca\(^{2+}\) oscillations. Nevertheless, given their prominent expression in oocytes and distinct expression pattern, at least in the case of TRPV3, we speculated their simultaneous elimination might have consequences in the regulation of Ca\(^{2+}\) homeostasis and/or fertility.

Mouse oocytes and eggs contain a host of other potential sources of Ca\(^{2+}\) influx via the PM. Notably, another TRP family member, TRPM7, has been reported to be imperative for embryonic development (Jin et al., 2008), suggesting that, in addition to magnesium and zinc, \([\text{Ca}^{2+}]\), might also be ferried through this channel. We recently demonstrated expression of this channel in GV oocytes and MII eggs (Carvacho et al., 2016), though further experiments are required to clarify the function of TRPM7 during oocyte maturation. Interestingly, we found that Ca\(^{2+}\) influx and oscillations post-fertilization are markedly impacted by the concentration
of extracellular Mg$^{2+}$ ([Mg$^{2+}$]$_o$). Magnesium homeostasis in the cell is largely mediated by TRPM7 (Bates-Withers et al., 2011). However, [Mg$^{2+}$]$_o$ also acts as an antagonist of TRPV3 (Luo et al., 2012). With this in mind, our results show that double knockout mice will serve as an invaluable tool to evaluate the effect of [Mg$^{2+}$]$_o$ on TRPM7 and ultimately on Ca$^{2+}$ influx.

An additional complication when attempting to characterize the function of these channels in mouse oocytes and eggs is that antibodies to determine protein expression and distribution of these channels, as well as specific inhibitors, are not commercially available. The only way to definitively determine the role of these channels is to perform calcium imaging experiments with the use of (non)-specific pharmacological agonists and antagonists, such as 2-APB, Mibefradil, and Sr$^{2+}$, as well as electrophysiology. To this end, we generated a double knockout line lacking these channels to directly measure the presence of other fundamental channels responsible for Ca$^{2+}$ influx. By knocking out these channels from the oocyte and egg, we can eliminate the use of non-specific agents that may have adverse effects on other PM channels and/or organelles.

**Double Knockout Fertility**

Ca$_v$3.2 channel function is not required for oocyte maturation, as a normal number of oocytes seem to complete maturation and reach the MII stage (Bernhardt et al., 2015). Similarly, TRPV3 channel function loss does not show an effect on maturation and ovulation rates (Carvacho et al., 2013). Remarkably, here we show a substantial decline in double knockout fertility rates, especially after the fourth litter, which also coincides in parturitions occurring at greater, though inconsistent, intervals. It is presently unclear what the underlying cellular or molecular reasons that progressively compromise fertility could be, as these defects are not observed in single KO lines. Reduced coronary function has been noted in mice lacking
CaV3.2 channels (Chen et al., 2003); although it is unclear how this might impact fertility, since as noted, this line has only a very mild and consistent reduced litter size (Bernhardt et al., 2015). The examination of histological sections of the ovaries, oviduct, and uterus following timed mating, which will be pursued in future studies, might allow us to reach more definitive conclusions concerning the factor(s) compromising fertility in this model.

**Ca$^{2+}$ Store Content in Double Knockout Mice**

Using calcium-imaging measurements of double heterozygous and double knockout mouse oocytes after calcium ionophore addition and/or SERCA inhibition, we found that double knockout eggs are still capable of Ca$^{2+}$ influx both at GV and at MII stages, although eggs of dKO mice showed a reduced [Ca$^{2+}$]$_{ER}$ store content, as assessed by addition of TG. While we presently do not know the channel(s) underlying the influx required to fill the internal stores, TRPM7, as previously noted, is a candidate. Regardless, our data demonstrate that these channels are not the sole channels required for Ca$^{2+}$ influx during maturation. Notably, we have generated a tool that can be used to more directly measure the main channel(s) responsible for these events during oocyte maturation and egg activation.

**Sr$^{2+}$ Response in Double Knockout Oocytes and Eggs**

Sr$^{2+}$ is the main agent used in parthenogenesis of rodent eggs. This ion influx at the MII stage in mouse MII eggs is mediated by TRPV3 (Carvacho et al., 2013), and is thought to sensitize IP$_3$R1 receptors, thereby facilitating Ca$^{2+}$ oscillations (Zhang et al., 2005). While TRPV3 is the main channel for Sr$^{2+}$ influx at MII, it is unlikely to be such at the GV stage, as at this stage, TRPV3 expression is not detected. Nevertheless, there is evidence in the literature that CaV channels may mediate divalent cation influx at the GV stage (Hirano et al., 1989a; Hirano et al., 1989b). The generation of double knockout mice seems to confirm these results,
as is shown that while WT or dHET GV oocytes displayed Sr\(^{2+}\) oscillations; oscillations were greatly reduced in GVs of dKO mice. Nevertheless, we found a \([\text{Mg}^{2+}]_o\)-regulated channel that allows Sr\(^{2+}\) influx in GV oocytes of the dKO line, although additional studies are required to characterize the nature of this influx, as its consequence in MII eggs remains unknown. Given that TRPM7 is also permeable to divalent cations, the channel responsible for this influx may be TRPM7, but further investigation using inhibitors and conditional knockout models is needed to confirm this.

**Ca\(^{2+}\) Measurements Post-Activation and -Fertilization**

To test the effects of the double channel deletion on fertilization-induced oscillations, we performed *in vitro* fertilization and monitored \([\text{Ca}^{2+}]_i\) responses. Double knockout eggs showed greatly decreased fertilization-induced Ca\(^{2+}\) oscillations, with oscillations showing increased intervals right from the initiation of the oscillations. Similar results were observed following injection of PLC\(\zeta\) cRNA. In addition, in both IVF and after PLC\(\zeta\) cRNA injection, the persistence of the oscillations seemed shortened. The mechanism whereby the absence of these channels precludes the mounting of a robust \([\text{Ca}^{2+}]_i\) response is unknown, but it appears that they contribute to support this influx, which is required to maintain the oscillations.

**Functional Role of TRPV3 and Ca\(_{\text{V}3.2}\) in Mouse Oocytes and Eggs**

Given that oscillations persist in eggs of the dKO mice, a question that arises is why these channels are present in oocytes and eggs. In the case of Ca\(_{\text{V}3.2}\) channels, which are voltage-gated channels, the question is very relevant, as the mammalian egg, a non-excitatory cell and in contrast to invertebrate species, experiences only a small change in membrane potential (Jaffé et al., 1983; Igusa et al., 1983). Nevertheless, as mentioned, Ca\(_{\text{V}3.2}\) currents have been measured in oocytes and eggs (Peres, 1986; Day et al., 1998); although at the
reigning resting membrane potential in eggs between -30 to -40 mV (Peres, 1986), they are largely inactive. Nevertheless, a portion of the channels may display persistent inward currents at low voltages, referred to as “window currents” (Backs et al., 2010; Igusa and Miyazaki, 1983). These currents have been detected in several cell types at or near the resting membrane potential, which was comparable to the resting potential of unfertilized mouse oocytes and eggs (Bernhardt et al., 2015). Thus, it is possible that these channels, in oocytes and eggs, may play a role in maintaining Ca\(^{2+}\) homeostasis and a part in establishing appropriate \([\text{Ca}^{2+}]_{\text{ER}}\) levels, as suggested by others (Bernhardt et al., 2015).

The functional expression of TRPV3 is also in agreement with this hypothesis. Single knockout, \(\text{Trpv3}^{-/-}\) eggs do not show a change in oscillation frequency post-fertilization or activation; thus, it is plausible that eggs may have a redundant, compensatory system that sustains a normal oscillation pattern. It is also possible that there are undetermined endogenous modulators of TRPV3 and Cav3.2 in the oocyte and egg. Future studies will examine this possibility, along with the expression and direct activation of these channels in humans. Nevertheless, we have provided evidence here that these channels, though their exact function remains unknown, contribute to maintaining Ca\(^{2+}\) homeostasis pre- and post-fertilization, as in their absence, the \([\text{Ca}^{2+}]_{\text{ER}}\) is less filled, and the oscillations after fertilization are more spaced out. More importantly, we have generated a tool that can be implemented in laboratory procedures to more accurately determine the channel(s) required and responsible for fertilization, both at a molecular level and at an electrophysiological level.

In conclusion, the eggs of this double knockout line have showed that these channels are not essential for the initiation and maintenance of oscillations, but contribute to Ca\(^{2+}\) homeostasis. Furthermore, since there appears to be a marked decline in fertility with time, a
full appreciation of their role on fertility requires additional studies. In addition, this line of double knockout mice and eggs will aid to: a) establish the specificity of commonly used agonists and antagonists for these channels; and b) perform electrophysiological procedures to identify remaining Ca\(^{2+}\) channels, as two of the main channels have been eliminated. Given that we still do not know most of the channels that mediate Ca\(^{2+}\) influx in oocytes and/or eggs, this strategy may allow identifying those active channels. Gaining insight into the mechanism of Ca\(^{2+}\) influx during maturation and fertilization will aid in the development of conditions that improve developmental competence, especially of oocytes matured \textit{in vitro}. Moreover, since Ca\(^{2+}\) is required for egg activation, the identification of the channels that mediate influx at fertilization as well as the development of specific channel blockers could become a novel, non-hormonal method of contraception to be used in humans, or to prevent the uncontrolled population growth of wild life species.
CHAPTER 4
MATERIALS & METHODS

Animal Husbandry

Double knockout mice and heterozygous mice were generated by breeding a female $TRPV3^{-/-}$ mouse (Cheng et al., 2010) of mixed C57BL/6J and 129/SvEvTac background to a male $Cacna1h^{-/-}$ mouse (Jackson Laboratories, Bar Harbor, ME) with a B6;129-Caca1h$^{tm1Kcam}$/J background to generate F1 offspring heterozygous for TRPV3 and Cacna1h (dHET; +/-). Initial double knockout (dKO; -/-) and dHET mice were obtained by intercross of heterozygotes and maintained on a mixed C57BL/6 and 129/SvEvTac background. Ear clips from offspring were collected prior to weaning.

Genotyping/PCR Analysis

Mice were identified and genotyped using tissue from an ear clip, which was collected and lysed using tail lysis buffer (Tris pH 8.8 [50mM], EDTA pH 8 [1mM], Tween 20 [0.5%], proteinase K [0.3 mg/mL]). Genomic DNA was then stored at -20°C for later use in PCR analysis. Mouse genotyping was routinely performed using PCR followed by separation of the amplified DNA fragments on a 1.2% agarose gel. For TRPV3, F7622, 5’-GACATGCCATGCAAAAAACTACA-3’ and R28432, 5’-GTCTGTTATATGTACAGGCATGG-3’ primers were used. The Trpv3 wildtype (WT) and mutant alleles yielded products of 300 bp and 800 bp, respectively. For Caca1h, 11395, 5’-ATTCAAGGGCTTCCACAGGGTA-3’, 11396, 5’-CATCTCAGGGCCTCTGGACCAC-3’, and oIMR2063, 5’-GCTAAAGCGCATGCTCCAGACTG-3’ primers were used. The Caca1h WT and mutant alleles yielded products of 480 bp and 330 bp, respectively. All primers were purchased from IDT Technologies (Coralville, IA). Primers and procedures were
in the original manuscripts reporting the generation of these KO lines (Cheng et al., 2010; Chen et al., 2003).

**Oocyte Collection**

Six-to-ten-week-old females were superovulated by intraperitoneal (i.p.) injection of 5 IU pregnant mare serum gonadotropin (PMSG, Calbiochem, EMD Biosciences), followed by i.p. injection of 5 IU human chorionic gonadotropin (hCG, Calbiochem, EMD Biosciences) 46-48 hours post-PMSG stimulation. Ovulated, metaphase-II (MII) arrested eggs were obtained by rupturing the oviducts with fine forceps in HEPES-buffered Tyrode’s Lactate (TL-HEPES) solution supplemented with 5% heat-treated fetal calf serum (FCS, Gibco) 12-14 hours post hCG stimulation. Cumulus cells were removed using 0.1% bovine testes hyaluronidase (Sigma, St. Louis, MO) and gentle aspiration through a pipette. For GV oocyte collection, ovaries of females were collected after 46-48 hours post-PMSG stimulation. Ovaries were gently macerated and cumulus-intact GV oocytes were recovered into a TL-HEPES solution supplemented with 5% FCS and 100 µM 3-isobutyl-1-methylxanthine (IBMX, Sigma) to block spontaneous progression of meiosis. All procedures were performed according to research animal protocols approved by the University of Massachusetts Institutional Animal Care and Use Committee.

**Calcium [Ca\(^{2+}\)] Imaging and Reagents**

[Ca\(^{2+}\)] monitoring was performed as previously reported by our laboratory (Kurokawa et al., 2007). Briefly, eggs were loaded with the Ca\(^{2+}\) sensitive dye Fura-2-acetoxymethyl ester (Fura-2AM, Molecular Probes; Invitrogen). Eggs were loaded with 1.25µM Fura-2AM supplemented with 0.02% pluronic acid (Molecular Probes) for 20 minutes at room temperature. To estimate [Ca\(^{2+}\)], eggs were thoroughly washed and immobilized on a glass
bottom monitoring dish (Mat-Tek Corp., Ashland, MA) submersed in FCS-free TL-HEPES under mineral oil. Eggs were monitored under a Nikon Diaphot microscope outfitted for fluorescence measurements. The objective used was a 20X Nikon Fluor. The excitation lamp was a 75 W Xenon lamp, and emitted light >510 nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, AZ) using NIS-Elements software (Nikon, Melville, NY). Eggs were alternatively illuminated with 340 nm and 380 nm light by a MAC5000 filter wheel/shutter control box (Ludl Electronic Productions Ltd.), and fluorescence was captured every 20 s.

To examine the role of Ca\(^{2+}\) influx in refilling [Ca\(^{2+}\)]\(_{ER}\), we monitored eggs in nominal Ca\(^{2+}\)-free, FCS-free TL HEPES. After a 5-8-minute baseline recording, [Ca\(^{2+}\)]\(_{ER}\) levels were assessed by the addition of 10\(\mu\)M Thapsigargin (TG; Calbiochem, San Diego, CA), an inhibitor of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump, which induced a Ca\(^{2+}\) leak via an unknown mechanism. The magnitude of the TG-induced Ca\(^{2+}\) rises were regarded as an estimate of the [Ca\(^{2+}\)]\(_{ER}\) content, which was calculated from the area under the curve of the [Ca\(^{2+}\)]\(_i\) rise using Prism (GraphPad Software, La Jolla, CA). When [Ca\(^{2+}\)]\(_i\) returned to near baseline values, ∼35 min after TG addition, 5 mM CaCl\(_2\) was added to the medium, and the amplitude of the [Ca\(^{2+}\)]\(_i\) rise caused by the addition was used to estimate Ca\(^{2+}\) influx. In other experiments, the addition of 2.5\(\mu\)M Ionomycin (IO), a Ca\(^{2+}\) ionophore, was used to assess total store content of the egg. IO-induced Ca\(^{2+}\) rises were regarded as the total [Ca\(^{2+}\)]\(_i\) that could be estimated from the area under the curve of the [Ca\(^{2+}\)]\(_i\) rise using Prism.

**Parthenogenetic Activation**

For TRPV3-mediated egg activation, eggs were collected as described above in TL-HEPES supplemented with 5% FCS. For Ca\(^{2+}\) monitoring, eggs were loaded with Fura-2AM,
then immobilized to a glass-bottom monitoring dish (Mat-Tek Corp) under nominal Ca\(^{2+}\)- and FCS-free TL-HEPES supplemented with 10mM SrCl\(_2\) submersed in mineral oil. For Sr\(^{2+}\) activation, eggs were incubated in 5% CO\(_2\) at 37\(^\circ\) C for 2 h in Ca\(^{2+}\)-free Chatot, Ziomek, or Bavister (CZB; Chatot et al., 1989) medium supplemented with either 3 mg/mL BSA or 0.1% polyvinyl alcohol (PVA), and 10mM SrCl\(_2\). Eggs were then washed and transferred to potassium-supplemented simplex optimized medium with amino acids (KSOM\(^{AA}\)), and cultured to the 2-cell stage. Eggs were evaluated at 5-6 h and 22-24 h post treatment under phase contrast microscopy. Activated eggs were classified according to the following criteria: (1) PN group, consisted of zygotes forming a single PN with first and second polar bodies (5 h post-treatment); (2) cleaved group; eggs undergoing immediate cleavage after 24 h. Eggs without 2\(^{nd}\) polar bodies, PN formation, or those failing to cleave were considered as non-activated (MII egg). Fragmented eggs were excluded from analysis.

**Microinjections**

Eggs were microinjected as described previously by our lab (Kurokawa et al., 2005). cRNA was heat denatured and centrifuged. The top 2\(\mu\)L was used to prepare microdrops from which glass micropipettes were loaded by aspiration. 7-12 pL (1-3% of the total volume of the egg) cRNA was delivered into eggs by pneumatic pressure (PLI-100 picoinjector, Harvard Apparatus). The full-length of mouse PLC\(\zeta\), cDNA, a kind gift from Dr. K. Fukami (Tokyo University of Pharmacy and Life Science, Japan) was subcloned into pcDNA6/ myc-His (Invitrogen, Carlsbad, CA) for in vitro transcription. Plasmids were linearized and the cDNA was in vitro transcribed using the T7 mMESSAGEmMACHINEKit (Ambion, Invitrogen).
Sperm Isolation

Sperm isolation and IVF sperm cells were obtained from 10-16 week-old male CD1 mice. The cauda epididymis of the sacrificed male was collected and sliced with scissors in 500μL of TL-HEPES or Toyoda, Yokoyama, Hosi (TYH) medium supplemented with 4 mg/mL bovine serum albumin (BSA; Sigma). The epididymis was incubated for 10-15 min at 37˚ C and 5% CO₂ then then removed, and sperm were incubated for an additional 1 h under the same conditions. Procedures were as described by Navarrete et al., 2016.

IVF

For standard IVF, expanded cumulus-oocyte-complexes were released from the oviduct and directly transferred to 90μL drops of TYH medium supplemented with 4 mg/mL BSA that was equilibrated overnight in 5% CO₂ at 37˚ C, and 0.1-0.3 x 10⁶ sperm/mL were added. Complexes were incubated for 1 h, washed of excess sperm, and loaded with Fura-2AM for Ca²⁺ monitoring as described above. IVF procedures were performed as described by the Visconti Lab (Navarrete et al., 2016).

ICSI

ICSI was performed according to the methods described by Yoshida and Perry, 2007. Isolated sperm were sonicated for 5 s and sperm tails were washed away through serial bench centrifugation and supernatant removal and dilution. Sperm heads were then mixed 1:1 with 6% polyvinylpyrrolidone (PVP; Sigma). Eggs were injected using a piezo micropipette-driven unit (PiezoDrill; Burleigh Instruments Inc., Rochester, NY). ICSI was performed in TL-HEPES supplemented with 5% FCS and 2% sucrose (Sigma).
Statistical Analysis

Values from three or more experiments performed on different batches of eggs were used for evaluation of statistical significance. Prism (GraphPad Software) was used to perform the Student’s $t$-test, one-way ANOVA, and graph productions. All data are presented as mean ± SEM. Differences were considered significant at $p < 0.05$ and denoted in bar graphs by the presence of asterisks.

Chemical Reagents

Ionomycin, thapsigargin, PMSG, and hCG were purchased from Calbiochem (San Diego, CA). Fura-2AM and pluronic acid were purchased from Invitrogen (Carlsbad, CA). All other chemicals were from Sigma (St Louis, MO), unless otherwise specified.
BIBLIOGRAPHY


