Zn\(^{2+}\) is Essential for Ca\(^{2+}\) Oscillations in Mouse Eggs

Hiroki Akizawa\(^1\), Emily Lopes\(^1,2\), Rafael A. Fissore\(^1\*)

\(^1\)Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, 661 North Pleasant Street, Amherst, Massachusetts, 01003, United States.
\(^2\)Molecular and Cellular Biology Graduate Program, University of Massachusetts, Amherst, Massachusetts, 01003, United States.

Running Title: Labile Zn\(^{2+}\) and Ca\(^{2+}\) release

Key Words: Fertilization, mammals, Ca\(^{2+}\), IP\(_3\)R1, oocytes, eggs, sperm, divalent cations, chelators

*Author for correspondence (rfissore@umass.edu)

*Rafael A. Fissore
661 North Pleasant Street
ISB-427A
Department of Veterinary and Animal Science
University of Massachusetts, Amherst, 01003
Phone:413-687-5773
Email: rfissore@umass.edu
Abstract

Changes in the intracellular concentration of free calcium (Ca$^{2+}$) underpin egg activation and initiation of development in animals and plants. In mammals, the Ca$^{2+}$ release is periodical, known as Ca$^{2+}$ oscillations, and mediated by the type 1 inositol 1,4,5-trisphosphate receptor (IP$_3$R1). Another divalent cation, zinc (Zn$^{2+}$), increases exponentially during oocyte maturation and is vital for meiotic transitions, arrests, and polyspermy prevention. It is unknown if these pivotal cations interplay during fertilization. Here, using mouse eggs, we showed that basal concentrations of labile Zn$^{2+}$ are indispensable for sperm-initiated Ca$^{2+}$ oscillations because Zn$^{2+}$-deficient conditions induced by cell-permeable chelators abrogated Ca$^{2+}$ responses evoked by fertilization and other physiological and pharmacological agonists. We also found that chemically- or genetically generated eggs with lower levels of labile Zn$^{2+}$ displayed reduced IP$_3$R1 sensitivity and diminished ER Ca$^{2+}$ leak despite the stable content of the stores and IP$_3$R1 mass. Resupplying Zn$^{2+}$ restarted Ca$^{2+}$ oscillations, but excessive Zn$^{2+}$ prevented and terminated them, hindering IP$_3$R1 responsiveness. The findings suggest that a window of Zn$^{2+}$ concentrations is required for Ca$^{2+}$ responses and IP$_3$R1 function in eggs, ensuring optimal response to fertilization and egg activation.

Introduction

Vertebrate eggs are arrested at the metaphase stage of the second meiosis (MII) when ovulated because they have an active Cdk1/cyclin B complex and inactive APC/C$^{Cdc20}$ (Heim et al., 2018). Release from MII initiates egg activation, the first hallmark of embryonic development (Ducibella et al., 2002; Schultz and Kopf, 1995). The universal signal of egg activation is an increase in the intracellular concentration of calcium (Ca$^{2+}$) (Ridgway et al., 1977; Stricker, 1999). Ca$^{2+}$ release causes the inactivation of the APC/C inhibitor Emi2, which enhances cyclin B degradation and induces meiotic exit (Lorca et al., 1993; Shoji et al., 2006; Suzuki et al., 2010a). In mammals, the stereotypical fertilization Ca$^{2+}$ signal, oscillations, consists of transient but periodical Ca$^{2+}$ increases that promote progression into interphase (Deguchi et al., 2000; Miyazaki et al., 1986). The sperm-borne Phospholipase C zeta1 (PLCζ) persistently stimulates the production of inositol 1,4,5-trisphosphate (IP$_3$) (Matsu-ura et al., 2019; Saunders et al., 2002; Wu et al., 2001) that binds its cognate receptor in the endoplasmic reticulum (ER), IP$_3$R1 and causes Ca$^{2+}$ release from the egg’s main Ca$^{2+}$ reservoir (Wakai et al., 2019). The intake of extracellular Ca$^{2+}$ via plasma membrane channels and transporters ensures the persistence of the oscillations (Miao et al., 2012; Stein et al., 2020; Wakai et al., 2019, 2013).
Before fertilization, maturing oocytes undergo cellular and biochemical modifications (see for review (Ajduk et al., 2008)). The nucleus of immature oocytes, known as the germinal vesicle (GV), undergoes the breakdown of its envelope marking the onset of maturation and setting in motion a series of cellular events that culminate with the release of the first polar body, the correct ploidy for fertilization, and re-arrest at MII (Eppig, 1996). Other organelles are also reorganized, such as cortical granules migrate to the cortex for exocytosis and polyspermy block, mitochondria undergo repositioning, and the cytoplasm’s redox state becomes progressively reduced to promote the exchange of the sperm’s protamine load (Liu, 2011; Perreault et al., 1988; Wakai et al., 2014). Wide-ranging adaptations also occur in the Ca\(^{2+}\) release machinery to produce timely and protracted Ca\(^{2+}\) oscillations following sperm entry (Fujiwara et al., 1993; Lawrence et al., 1998), including the increase in the content of the Ca\(^{2+}\) stores, ER reorganization with cortical cluster formation, and increased IP\(_3\)R1 sensitivity (Lee et al., 2006; Wakai et al., 2012). The total intracellular levels of zinc (Zn\(^{2+}\)) also remarkably increase during maturation, amounting to a 50% rise, which is necessary for oocytes to proceed to the telophase I of meiosis and beyond (Kim et al., 2010). Remarkably, after fertilization, Zn\(^{2+}\) levels need to decrease, as Emi2 is a Zn\(^{2+}\)-associated molecule, and high Zn\(^{2+}\) levels prevent MII exit (Bernhardt et al., 2012; Shoji et al., 2014; Suzuki et al., 2010b). Following the initiation of Ca\(^{2+}\) oscillations, approximately 10 to 20% of the Zn\(^{2+}\) accrued during maturation is ejected during the Zn\(^{2+}\) sparks, a conserved event in vertebrates and invertebrate species (Converse and Thomas, 2020; Kim et al., 2011; Mendoza et al., 2022; Que et al., 2019; Seeler et al., 2021; Tokuhiro and Dean, 2018; Wozniak et al., 2020; Zhang et al., 2016). The use of Zn\(^{2+}\) chelators such as N,N,N,N-tetrakis (2-pyridinylmethyl)-1,2-ethylenediamine (TPEN) to create Zn\(^{2+}\)-deficient conditions buttressed the importance of Zn\(^{2+}\) during meiotic transitions (Kim et al., 2010; Suzuki et al., 2010b). However, whether the analogous dynamics of Ca\(^{2+}\) and Zn\(^{2+}\) during maturation imply crosstalk and Zn\(^{2+}\) levels modulate Ca\(^{2+}\) release during fertilization is unknown.

IP\(_3\)Rs are the most abundant intracellular Ca\(^{2+}\) release channel in non-muscle cells (Berridge, 2016). They form a channel by assembling into tetramers with each subunit of ~270kDa MW (Taylor and Tovey, 2010). Mammalian eggs express the type I IP\(_3\)R, the most widespread isofrom (Fissore et al., 1999; Parrington et al., 1998). IP\(_3\)R1 is essential for egg activation because its inhibition precludes Ca\(^{2+}\) oscillations (Miyazaki and Ito, 2006; Miyazaki et al., 1992; Xu et al., 2003). Myriad and occasionally cell-specific factors influence Ca\(^{2+}\) release through the IP\(_3\)R1 (Taylor and Tovey, 2010). For example, following fertilization, IP\(_3\)R1 undergoes ligand-induced degradation caused by
the sperm-initiated long-lasting production of IP₃ that effectively reduces the IP₃R1 mass 
(Brind et al., 2000; Jellerette et al., 2000). Another regulatory mechanism is Ca²⁺, a 
universal cofactor, which biphasically regulates IP₃Rs’ channel opening (Iino, 1990; Jean 
and Klee, 1986), congruent with several Ca²⁺ and calmodulin binding sites on the 
channel’s sequence (Sienaert et al., 1997; Sipma et al., 1999). Notably, Zn²⁺ may also 
participate in IP₃R1 regulation. Recent studies using electron cryomicroscopy (cryoEM), 
a technique that allows peering into the structure of IP₃R1 with a near-atomic resolution, 
have revealed that a helical linker (LNK) domain near the C-terminus mediates the 
coupling between the N- and C-terminal ends necessary for channel opening (Fan et al., 
2015). The LNK domain contains a putative Zinc-finger motif proposed to be vital for 
IP₃R1 function (Fan et al., 2015; Paknejad and Hite, 2018). Therefore, the exponential 
increase in Zn²⁺ levels in maturing oocytes, besides its essential role in meiosis 
progression, may optimize the IP₃R1 function, revealing hitherto unknown cooperation 
between these cations during fertilization.

Here, we examined whether crosstalk between Ca²⁺ and Zn²⁺ is required to 
initiate and sustain Ca²⁺ oscillations and maintain Ca²⁺ store content in MII eggs. We 
found that Zn²⁺-deficient conditions inhibited Ca²⁺ release and oscillations without 
reducing Ca²⁺ stores, IP₃ production, IP₃R1 expression, or altering the viability of eggs or 
zygotes. We show instead that Zn²⁺ deficiency impaired IP₃R1 function and lessened the 
receptor’s ability to gate Ca²⁺ release out of the ER. Remarkably, resupplying Zn²⁺ re-
established the oscillations interrupted by low Zn²⁺, although persistent increases in 
intracellular Zn²⁺ were harmful, disrupting the Ca²⁺ responses and preventing egg 
activation. Together, the results show that besides contributing to oocyte maturation, Zn²⁺ 
has a central function in Ca²⁺ homeostasis such that optimal Zn²⁺ concentrations ensure 
IP₃R1 function and the Ca²⁺ oscillations required for initiating embryo development.

**Results**

**TPEN dose-dependently lowers intracellular Zn²⁺ and inhibits sperm-initiated Ca²⁺ 
oscillations.**

TPEN is a cell-permeable, non-specific chelator with a high affinity for transition 
metals widely used to study their function in cell physiology (Arslan et al., 1985; Lo et 
al., 2020). Mouse oocytes and eggs have exceedingly high intracellular concentrations of 
Zn²⁺ (Kim et al., 2011, 2010), and the TPEN-induced defects in the progression of meiosis 
have been ascribed to its chelation (Bernhardt et al., 2011; Kim et al., 2010). In support 
of this view, the Zn²⁺ levels of cells showed acute reduction after TPEN addition, as 
reported by indicators such as FluoZin-3 (Arslan et al., 1985; Gee et al., 2002; Suzuki et
al., 2010b). Studies in mouse eggs also showed that the addition of low µM (40-100) concentrations of TPEN disrupted Ca2+ oscillations initiated by fertilization or SrCl2 (Lawrence et al., 1998; Suzuki et al., 2010b), but the mechanism(s) and target(s) of the inhibition remained unknown. To gain insight into this phenomenon, we first performed dose-titration studies to determine the effectiveness of TPEN in lowering Zn2+ in eggs. The addition of 2.5 µM TPEN protractedly reduced Zn2+ levels, whereas 5 and 10 µM TPEN acutely and persistently reduced FluoZin-3 fluorescence (Fig. 1A). These concentrations of TPEN are higher than the reported free Zn2+ concentrations in cells, but within range of those of found in typical culture conditions (Lo et al., 2020; Qin et al., 2011). We next determined the concentrations of TPEN required to abrogate fertilization-initiated oscillations. Following intracytoplasmic sperm injection (ICSI), we monitored Ca2+ responses while increasing TPEN concentrations. As shown in Fig. 1B, 5 and 10 µM TPEN effectively blocked ICSI-induced Ca2+ oscillations in over half of the treated cells, and the remaining eggs, after a prolonged interval, resumed lower-frequency rises (Fig. 1B-center panels). Finally, 50 µM or greater concentrations of TPEN permanently blocked these oscillations (Fig. 1B-right panel). It is noteworthy that at the time of addition, TPEN concentrations of 5 µM or above induce a sharp drop in basal Fura-2 F340/ F380 ratios, consistent with Fura-2’s high affinity for Zn2+ (Snitsarev et al., 1996).

We next used membrane-permeable and -impermeable chelators to assess whether TPEN inhibited Ca2+ oscillations by chelating Zn2+ from intracellular or extracellular compartments. The addition of the high-affinity but cell-impermeable Zn2+ chelators DTPA and EDTA neither terminated nor temporarily interrupted ICSI-induced Ca2+ oscillations (Fig. 1C), although protractedly slowed them down, possibly because of chelation and lowering of external Ca2+ (Fig. 1C). These results suggest that chelation of external Zn2+ does not affect the continuation of oscillations. We cannot determine that EDTA successfully chelated all external Zn2+, but the evidence that the addition of EDTA to the monitoring media containing cell impermeable FluoZin-3 caused a marked reduction in fluorescence, suggests that a noticeable fraction of the available Zn2+ was sequestered (Supplementary Fig. 1A). Similarly, injection of mPlcζ mRNA in eggs incubated in Ca2+- and Mg2+-free media supplemented with EDTA, to maximize the chances of chelation of external Zn2+, initiated low-frequency but persistent oscillations, and addition of Ca2+ and Mg2+ restored the physiological periodicity (Supplementary Fig. 1B). Lastly, another Zn2+-permeable chelator, TPA, blocked the ICSI-initiated Ca2+ oscillations but required higher concentrations than TPEN (Fig. 1D). Collectively, the data suggest that basal levels of labile internal Zn2+ are essential to sustain the fertilization-initiated Ca2+ oscillations in eggs.
We next evaluated whether Zn\(^{2+}\) depletion prevented the completion of meiosis and pronuclear (PN) formation. To this end, ICSI-fertilized eggs were cultured in the presence of 10 µM TPEN for 8h, during which the events of egg activation were examined (Fig. 1E and Table 1). All fertilized eggs promptly extruded second polar bodies regardless of treatment (Fig. 1E). TPEN, however, impaired PN formation, and by 4- or 7-h post-ICSI, most treated eggs failed to show PNs, unlike controls (Fig. 1E and Table 1). Together, these results demonstrate that depletion of Zn\(^{2+}\) terminates Ca\(^{2+}\) oscillations and delays or prevents events of egg activation, including PN formation.

**TPEN is a universal inhibitor of Ca\(^{2+}\) oscillations in eggs.**

Mammalian eggs initiate Ca\(^{2+}\) oscillations in response to numerous stimuli and conditions (Miyazaki and Ito, 2006; Wakai and Fissore, 2013). Fertilization and its release of PLC\(\zeta\) stimulate the phosphoinositide pathway, producing IP\(_3\) and Ca\(^{2+}\) oscillations (Miyazaki, 1988; Saunders et al., 2002). Neurotransmitters such as acetylcholine (Ach) and other G-protein coupled receptor agonists engage a similar mechanism (Dupont et al., 1996; Kang et al., 2003), although in these cases, IP\(_3\) production occurs at the plasma membrane and is short-lived (Kang et al., 2003; Swann and Parrington, 1999). Agonists such as SrCl\(_2\) and thimerosal generate oscillations by sensitizing IP\(_3\)R1 without producing IP\(_3\). The mechanism(s) of SrCl\(_2\) is unclear, although its actions are reportedly directly on the IP\(_3\)R1 (Hajnóczky and Thomas, 1997; Hamada et al., 2003; Nomikos et al., 2011; Sanders et al., 2018). Thimerosal oxidizes dozens of thiol groups in the receptor, which enhances the receptor’s sensitivity and ability to release Ca\(^{2+}\) (Bootman et al., 1992; Evellin et al., 2002; Joseph et al., 2018). We took advantage of the varied points at which the mentioned agonists engage the phosphoinositide pathway to examine TPEN’s effectiveness in inhibiting their effects. mPlc\(\zeta\) mRNA injection, like fertilization, induces persistent Ca\(^{2+}\) oscillations, although mPlc\(\zeta\)’s tends to be more robust. Consistent with this, the addition of 10 and 25 µM TPEN transiently interrupted or belatedly terminated oscillations, whereas 50 µM acutely stopped all responses (Fig. 2A). By contrast, SrCl\(_2\)-initiated rises were the most sensitive to Zn\(^{2+}\)-deficient conditions, with 2.5 µM TPEN nearly terminating all oscillations that 5 µM did (Fig. 2B). TPEN was equally effective in ending the Ach-induced Ca\(^{2+}\) responses (Fig. 2C), but curbing thimerosal responses required higher concentrations (Fig. 2D). Lastly, we ruled out that downregulation of IP\(_3\)R1 was responsible for the slow-down or termination of the oscillations by TPEN. To accomplish this, we examined the IP\(_3\)R1 mass in eggs (Jellerette et al., 2004) with and without TPEN supplementation and injection of mPlc\(\zeta\) mRNA. By 4h post-injection, PLC\(\zeta\) induced the expected down-regulation of IP\(_3\)R1 reactivity, whereas was
insignificant in TPEN-treated and Plceζ mRNA-injected eggs, as it was in uninjected control eggs (Fig. 2F). These findings together show that Zn²⁺ deficiency inhibits the IP₃R1-mediated Ca²⁺ oscillations independently of IP₃ production or loss of receptor, suggesting a role of Zn²⁺ on IP₃R1 function (Fig. 2E).

**Zn²⁺ depletion reduces IP₃R1-mediated Ca²⁺ release.**

To directly assess the inhibitory effects of TPEN on IP₃R1 function, we used caged IP₃ (cIP₃) that, after short UV pulses, releases IP₃ into the ooplasm (Wakai et al., 2012; Walker et al., 1987). To exclude the possible contribution of external Ca²⁺ to the responses, we performed the experiments in Ca²⁺-free media. In response to sequential cIP₃ release 5 min apart, control eggs displayed corresponding Ca²⁺ rises that occasionally transitioned into short-lived oscillations (Fig. 3A). The addition of TPEN after the third cIP₃ release prevented the subsequent Ca²⁺ response and prematurely terminated the in-progress Ca²⁺ rises (Fig. 3B and inset). Pre-incubation of eggs with TPEN precluded cIP₃-induced Ca²⁺ release, even after 5 sec UV exposure (Fig. 3C). The addition of excess ZnSO₄ (100 µM) overcame TPEN’s inhibitory effects only if added before (Fig. 3E) and not after the addition of TPEN (Fig. 3D). Similar concentrations of MgCl₂ or CaCl₂ failed to reverse TPEN effects (Fig. 3F, G). Together, the results show that Zn²⁺ is required for IP₃R1-mediated Ca²⁺ release downstream of IP₃ production, appearing to interfere with receptor gating, as suggested by TPEN’s rapid termination of in-progress Ca²⁺ rises and ongoing oscillations.

ERp44 is an ER luminal protein of the thioredoxin family that interacts with the IP₃R1, reportedly inhibiting its ability to mediate Ca²⁺ release (Higo et al., 2005). The localization of ERp44 in the ER-Golgi intermediate compartment of somatic cells correlates with Zn²⁺’s availability and changes dramatically after TPEN treatment (Higo et al., 2005; Watanabe et al., 2019). To rule out the possibility that TPEN suppresses the function of IP₃R1 by modifying the subcellular distribution of ERp44, we overexpressed ERp44 by injecting HA tagged-Erp44 mRNA into MII eggs and monitored the effect on Ca²⁺ release. TPEN did not alter the localization of ERp44 (Supplementary Fig. 2A), and overexpression of ERp44 modified neither the Ca²⁺ oscillations induced by agonists (Supplementary Fig. 2B) nor the effectiveness of TPEN to block them (data not shown). Thus, TPEN and Zn²⁺ deficiency most likely inhibits Ca²⁺ release by directly interfering with IP₃R1 function rather than modifying this particular regulator.

**Zn²⁺ depletion diminishes the ER Ca²⁺ leak and increases Ca²⁺ store content.**
Our above cIP3 results that TPEN inhibited IP3R1-mediated Ca\textsuperscript{2+} release and interrupted in-progress Ca\textsuperscript{2+} rises despite the presence of high levels of environmental IP3 suggest its actions are probably independent of IP3 binding, agreeing with an earlier report showing that TPEN did not modify IP3’s affinity for the IP3R (Richardson and Taylor, 1993). Additionally, the presence of a Zn\textsuperscript{2+}-binding motif near the C-term cytoplasmic domain of the IP3R1’s channel, which is known to influence agonist-induced IP3R1 gating (Fan et al., 2015), led us to posit and examine that Zn\textsuperscript{2+} deficiency may be disturbing Ca\textsuperscript{2+} release to the cytosol and out of the ER. To probe this possibility, we queried if pre-treatment with TPEN inhibited Ca\textsuperscript{2+} release through IP3R1. We first used Thapsigargin (Tg), a Sarcoplasmic/ER Ca\textsuperscript{2+} ATPase pump inhibitor (Thastrup et al., 1990) that unmasks a constitutive Ca\textsuperscript{2+} leak out of the ER (Lemos et al., 2021); in eggs, we have demonstrated it is mediated at least in part by IP3R1 (Wakai et al., 2019). Treatment with TPEN for 15 min slowed the Tg-induced Ca\textsuperscript{2+} leak into the cytosol, resulting in delayed and lowered amplitude Ca\textsuperscript{2+} responses (Fig. 4A; \(P<0.05\)). To test whether the reduced response to Tg means that TPEN left a temporarily increased Ca\textsuperscript{2+} content in the ER after it, we added the Ca\textsuperscript{2+} ionophore ionomycin (Io), which empties all stores independently of IP3Rs. Iomycin-induced Ca\textsuperscript{2+} responses were 3.3-fold greater in TPEN-treated cells, supporting the view that TPEN interferes with the ER Ca\textsuperscript{2+} leak (Fig. 4A; \(P<0.05\)). We further evaluated this concept using in vitro aged eggs that often display reduced Ca\textsuperscript{2+} store content than freshly collected counterparts (Abbott et al., 1998). After culturing eggs in the presence or absence of TPEN for 2h, we added Io during Ca\textsuperscript{2+} monitoring, which in TPEN-treated eggs induced bigger Ca\textsuperscript{2+} rises than in control eggs (Fig. 4B; \(P<0.05\)). We confirmed that this effect was independent of IP3R1 degradation because TPEN did not change IP3R1 reactivity in unfertilized eggs (Fig. 4C; \(P<0.05\)).

Next, we used the genetically encoded FRET sensor D1ER (Palmer et al., 2004) to assess the TPEN’s effect on the ER’s relative Ca\textsuperscript{2+} levels changes following the additions of Tg or Ach. TPEN was added 10 min before 10 \(\mu\text{M}\) Tg or 50 \(\mu\text{M}\) Ach, and we simultaneously monitored changes in cytosolic and intra-ER Ca\textsuperscript{2+} (Fig. 4D, E). For the first three min, the Tg-induced decrease in Ca\textsuperscript{2+}-ER was similar between groups. However, while the drop in Ca\textsuperscript{2+} content continued in control eggs, in TPEN-treated eggs, it came to an abrupt halt, generating profound differences between the two groups (Fig. 4D; \(P<0.05\)). TPEN had even more pronounced effects following the addition of Ach, leading to a reduced- and prematurely terminated- Ca\textsuperscript{2+} release from the ER in treated eggs (Fig. 4E; \(P<0.05\)).

Lastly, we sought to use a cellular model where low labile Zn\textsuperscript{2+} occurred without pharmacology. To this end, we examined a genetic model where the two non-selective
plasma membrane channels that could influx Zn\textsuperscript{2+} in maturing oocytes have been deleted (Bernhardt et al., 2017; Carvacho et al., 2016, 2013), namely, the transient receptor potential melastatin-7 (TRPM7) and TRP vanilloid 3 (TRPV3), both members of the TRP superfamily of channels (Wu et al., 2010). We found that eggs from double knockout females (dKOs) had lower levels of labile Zn\textsuperscript{2+} (Fig. 4F), and the addition of Tg revealed an expanded Ca\textsuperscript{2+} store content in these eggs vs. control WT eggs (Fig. 4G). Remarkably, in dKO eggs, the Ca\textsuperscript{2+} rise induced by Tg showed a shoulder or inflection point before the peak delaying the time to peak (Fig. 4G, inset; \( P < 0.001 \)). These results in dKO eggs show a changed dynamic of the Tg-induced Ca\textsuperscript{2+} release, suggesting that lower levels of labile Zn\textsuperscript{2+} modify ER Ca\textsuperscript{2+} release independently of chelators.

\textit{Ca\textsuperscript{2+} oscillations in eggs occur within a window of Zn\textsuperscript{2+} concentrations.}

We next examined if resupplying Zn\textsuperscript{2+} could restart the Ca\textsuperscript{2+} oscillations terminated by Zn\textsuperscript{2+} depletion. Zn pyrithione (ZnPT) rapidly increases cellular Zn\textsuperscript{2+} upon extracellular addition (Barnett et al., 1977; Robinson, 1964). Dose titration studies and imaging fluorimetry revealed that 0.01 \( \mu \text{M} \) ZnPT caused subtle and protracted increases in Zn\textsuperscript{2+} levels, whereas 0.1 \( \mu \text{M} \) ZnPT caused rapid increases in eggs’ Zn\textsuperscript{2+} baseline (Fig. 5A). We induced detectable Ca\textsuperscript{2+} oscillations by injection of mPlc\( _{\zeta} \)mRNA followed by 50 \( \mu \text{M} \) TPEN (Fig. 5B), which terminated them. After 30 min, we added 0.1 \( \mu \text{M} \) ZnPT, and within 15 min the oscillations restarted in most TPEN-treated eggs (Fig. 5C). We repeated this approach using Thimerosal (Fig. 5D, E). Adding 0.1 \( \mu \text{M} \) ZnPT did not restore the Ca\textsuperscript{2+} oscillations retrained by TPEN, but 0.5 \( \mu \text{M} \) ZnPT did so (Fig. 5E). These results demonstrate that Zn\textsuperscript{2+} plays a pivotal, enabling role in the generation of Ca\textsuperscript{2+} oscillations in mouse eggs.

\textit{Excessive intracellular Zn\textsuperscript{2+} inhibits Ca\textsuperscript{2+} oscillations.}

Zn\textsuperscript{2+} is necessary for diverse cellular functions, consistent with numerous amino acids and proteins capable of binding Zn\textsuperscript{2+} within specific and physiological ranges (Pace and Weerapana, 2014). Excessive Zn\textsuperscript{2+}, however, can cause detrimental effects on cells and organisms (Broun et al., 1990; Hara et al., 2022; Sikora and Ouagazzal, 2021). Consistent with the deleterious effects of Zn\textsuperscript{2+}, a previous study showed that high concentrations of ZnPT, \( \sim 50 \mu \text{M} \), prevented SrCl\textsubscript{2}-induced egg activation and initiation of development (Bernhardt et al., 2012; Kim et al., 2011). We examined how ZnPT and excessive Zn\textsuperscript{2+} levels influence Ca\textsuperscript{2+} oscillations. Our conditions revealed that pre-incubation or continuous exposure to 0.1 \( \mu \text{M} \) or 1.0 \( \mu \text{M} \) ZnPT delayed or prevented egg activation induced by mPlc\( _{\zeta} \) mRNA injection (Supplementary Fig. 3). We used these
ZnPT concentrations to add it into ongoing oscillations induced by ICSI and monitored the succeeding Ca\textsuperscript{2+} responses. The addition of 0.05 to 10 µM ZnPT caused an immediate elevation of the basal levels of Fura-2 and termination of the Ca\textsuperscript{2+} oscillations (Fig. 6A-D). mPlcζ mRNA-initiated Ca\textsuperscript{2+} responses were also interrupted by adding 0.1 µM ZnPT, whereas untreated eggs continued oscillating (Fig. 6E, F). ZnPT also inhibited IP\textsubscript{3}R1-mediated Ca\textsuperscript{2+} release triggered by cIP\textsubscript{3}, suggesting that excessive Zn\textsuperscript{2+} directly inhibits IP\textsubscript{3}R1 function (Fig. 6G).

A noticeable feature of ZnPT is the increased basal ratios of Fura-2 AM. These changes could reflect enhanced IP\textsubscript{3}R1 function and increased basal Ca\textsuperscript{2+} concentrations caused by Zn\textsuperscript{2+} stimulation of IP\textsubscript{3}R1. This seems unlikely, however, because extended elevated cytosolic Ca\textsuperscript{2+} would probably induce cellular responses, such as the release of the second polar body, egg fragmentation, or cell death, neither of which happened. It might reflect, instead, Fura-2’s ability to report changes in Zn\textsuperscript{2+} levels, which seemed the case because the addition of TPEN lowered fluorescence without restarting the Ca\textsuperscript{2+} oscillations (Fig. 6F). To ensure the impact of ZnPT abolishing Ca\textsuperscript{2+} oscillations was not an imaging artifact obscuring ongoing rises, we simultaneously monitored cytoplasmic and ER Ca\textsuperscript{2+} levels with Rhod-2 and D1ER, respectively. This approach allowed synchronously observing Ca\textsuperscript{2+} changes in both compartments that should unfold in opposite directions. In control, uninjected eggs, the fluorescent values for both reporters remained unchanged during the monitoring period, whereas in mPlcζ mRNA-injected eggs, the reporters’ signals displayed simultaneous but opposite changes, as expected (Fig. 6H, I). The addition of ZnPT in uninjected eggs rapidly increased Rhod-2 signals but not D1ER’s, which was also the case in oscillating eggs, as the addition of ZnPT did not immediately alter the dynamics of the ER’s Ca\textsuperscript{2+} release, suggesting D1ER faithfully reports in Ca\textsuperscript{2+} changes but cannot detect changes in Zn\textsuperscript{2+} levels, at least to this extent; ZnPT progressively caused fewer and lower amplitude changes in D1ER fluorescence, consistent with the diminishing and eventual termination of the Ca\textsuperscript{2+} oscillations. Noteworthy, in these eggs, the basal D1ER fluorescent ratio remained unchanged after ZnPT, demonstrating its unresponsiveness to Zn\textsuperscript{2+} changes of this magnitude. The ZnPT-induced increases in Rhod-2 fluorescence without concomitant changes in D1ER values suggest that the changes in the dyes’ fluorescence do not represent an increase in basal Ca\textsuperscript{2+} and, more likely, signal an increase in intracellular Zn\textsuperscript{2+}. We confirmed that both reporters were still in working order, as the addition of Io triggered Ca\textsuperscript{2+} changes detected by both reporters (Fig. 6H, I).

Discussion
The present study demonstrates that appropriate levels of labile Zn\textsuperscript{2+} are essential for initiating and maintaining IP\textsubscript{3}R1-mediated Ca\textsuperscript{2+} oscillations in mouse eggs regardless of the initiating stimuli. Both deficient and excessive Zn\textsuperscript{2+} compromise IP\textsubscript{3}R1 sensitivity, diminishing and mostly terminating Ca\textsuperscript{2+} oscillations. The results demonstrate that IP\textsubscript{3}R1 and Zn\textsuperscript{2+} act in concert to modulate Ca\textsuperscript{2+} signals, revealing previously unexplored crosstalk between these ions at fertilization (Fig. 7).

Zn\textsuperscript{2+} is an essential micronutrient for living organisms (Kaur et al., 2014) and is required for various cellular functions, such as proliferation, transcription, and metabolism (Lo et al., 2020; Maret and Li, 2009; Yamasaki et al., 2007). Studies using Zn\textsuperscript{2+} chelators have uncovered what appears to be a cell-specific, narrow window of Zn\textsuperscript{2+} concentrations needed for cellular proliferation and survival (Carraway and Dobner, 2012; Lo et al., 2020). Further, TPEN appeared especially harmful, and in a few cell lines, even low doses provoked oxidative stress, DNA fragmentation, and apoptosis (Mendivil-Perez et al., 2012). We show here that none of the Zn\textsuperscript{2+} chelators, permeable or impermeable, affected cell viability within our experimental observations, confirming findings from previous studies that employed high concentrations of TPEN to interrupt the Ca\textsuperscript{2+} oscillations (Lawrence et al., 1998) or inducing egg activation of mouse eggs (Suzuki et al., 2010b). Our data demonstrating that \(~2.5\ \mu\text{M}\) is the threshold concentration of TPEN in eggs that first causes noticeable changes in basal Zn\textsuperscript{2+}, as revealed by FluoZin, is consistent with the \(~2\) to \(~5\) µM Zn\textsuperscript{2+} concentrations in most culture media without serum supplementation (Lo et al., 2020), and with the \(~100\) pM basal Zn\textsuperscript{2+} in cells (Qin et al., 2011). Lastly, the effects on Ca\textsuperscript{2+} release observed here with TPEN and other chelators were due to the chelation of Zn\textsuperscript{2+}, as pretreatment with ZnSO\textsubscript{4} but not with equal or greater concentrations of MgCl\textsubscript{2} or CaCl\textsubscript{2} rescued the inhibition of the responses, which is consistent with results by others (Kim et al., 2010; Lawrence et al., 1998).

To identify how Zn\textsuperscript{2+} deficiency inhibits Ca\textsuperscript{2+} release in eggs, we induced Ca\textsuperscript{2+} oscillations using various stimuli and tested the effectiveness of membrane-permeable and impermeable chelators to abrogate them. Chelation of extracellular Zn\textsuperscript{2+} failed to terminate the Ca\textsuperscript{2+} responses, whereas membrane-permeable chelators did, pointing to intracellular labile Zn\textsuperscript{2+} levels as essential for Ca\textsuperscript{2+} release. All agonists used here were susceptible to inhibition by TPEN, whether their activities depended on IP\textsubscript{3} production or allosterically induced receptor function, although the effective TPEN concentrations varied across stimuli. Some agents, such as mPlc\textgreek{z} mRNA or thimerosal, required higher concentrations than SrCl\textsubscript{2}, Ach, or cIP\textsubscript{3}. The reason underlying the different agonists’ sensitivities to TPEN will require additional research, but the persistence of IP\textsubscript{3} production or change in IP\textsubscript{3}R1 structure needed to induce channel gating might explain
it. However, the universal abrogation of Ca\(^{2+}\) oscillations by TPEN supports the view

drawn from cryo-EM-derived IP\(_3\)R1 models that signaling molecules can allosterically
induce channel gating from different starting positions in the receptor by mechanically
coupling the binding effect to the ion-conducting pore in the C-terminal end of IP\(_3\)R (Fan
et al., 2015). The cytosolic C-terminal domain of each IP\(_3\)R1 subunit is alongside the IP\(_3^{-}\)-
binding domain of another subunit and, therefore, well positioned to sense IP\(_3\) binding
and induce channel gating (Fan et al., 2015). Within each subunit, the LNK domain, which
contains a Zn\(^{2+}\)-finger motif (Fan et al., 2015), connects the opposite domains of the
molecule. Although there are no reports regarding the regulation of IP\(_3\)R1 sensitivity by
Zn\(^{2+}\), such evidence exists for RyRs (Woodier et al., 2015), which also display a conserved
Zn\(^{2+}\)-finger motif (des Georges et al., 2016). Lastly, mutations of the two Cys or two His
residues of this motif, without exception, resulted in inhibition or inactivation of the IP\(_3\)R1
channel (Bhanumathy et al., 2012; Uchida et al., 2003). These results are consistent with
the view that the C-terminal end of IP\(_3\)Rs plays a dominant role in channel gating
(Bhanumathy et al., 2012; Uchida et al., 2003). We propose that TPEN inhibits Ca\(^{2+}\)
oscillations in mouse eggs because chelating Zn\(^{2+}\) interferes with the function of the LNK
domain and its Zn\(^{2+}\)-finger motif proposed role on the mechanical coupling induced by
agonist binding to the receptor that propagates to the pore-forming region and required to
gate the channel’s ion-pore (Fan et al., 2022, 2015).

In support of this possibility, TPEN-induced Zn\(^{2+}\) deficient conditions altered the
Ca\(^{2+}\)-releasing kinetics in resting eggs or after fertilization. Tg increases intracellular Ca\(^{2+}\)
by inhibiting the SERCA pump (Thastrup et al., 1990) and preventing the reuptake into
the ER of the ebbing Ca\(^{2+}\) during the basal leak. Our previous studies showed that the
downregulation of IP\(_3\)R1 diminishes the leak, suggesting it occurs through IP\(_3\)R1 (Wakai
and Fissore, 2019). Consistent with this view, TPEN pre-treatment delayed the Ca\(^{2+}\)
response induced by Tg, implying that Zn\(^{2+}\) deficiency hinders Ca\(^{2+}\) release through IP\(_3\)R1.
An expected consequence would be increased Ca\(^{2+}\) content in the ER after Tg. Io that
mobilizes Ca\(^{2+}\) independently of IP\(_3\)Rs (Toeplitz et al., 1979) induced enhanced responses
in TPEN-treated eggs vs. controls, confirming the accumulation of Ca\(^{2+}\)-ER in Zn\(^{2+}\)
deficient conditions. We demonstrated that this accumulation is due to hindered emptying
of the Ca\(^{2+}\) ER evoked by agonists in Zn\(^{2+}\)-deficient environments, resulting in reduced
cytosolic Ca\(^{2+}\) increases, as IP\(_3\)R1 is the pivotal intermediary channel between these
compartments. Noteworthy, the initial phase of the Tg-induced Ca\(^{2+}\) release out of the ER
did not appear modified by TPEN, as if it was mediated by a Zn\(^{2+}\)-insensitive Ca\(^{2+}\)
channel(s)/transporter, contrasting with the abrogation of Ach-induced ER emptying from
the outset. Remarkably, independently of Zn\(^{2+}\) chelators, emptying of Ca\(^{2+}\) ER was
modified in a genetic model of Zn\textsuperscript{2+}-deficient oocytes lacking two TRP channels, confirming the impact of Zn\textsuperscript{2+} on Ca\textsuperscript{2+} release. It is worth noting that TPEN did not reduce but maintained or increased the mass of IP\textsubscript{3}R1, which might result in the inhibition of Zn\textsuperscript{2+}-dependent ubiquitin ligase Ubc7 by the Zn-deficient conditions (Webster et al., 2003). We cannot rule out that these conditions may undermine other conformational changes required to trigger IP\textsubscript{3}R1 degradation, thereby favoring the accumulation of IP\textsubscript{3}R1.

Despite accruing Zn\textsuperscript{2+} during oocyte maturation, fertilization witnesses a necessary Zn\textsuperscript{2+} release into the external milieu, known as “Zn\textsuperscript{2+} sparks” (Kim et al., 2011; Que et al., 2019, 2015). This release of Zn\textsuperscript{2+} is a conserved event in fertilization across species and is associated with several biological functions, including those related to fending off polyspermy (Kim et al., 2011; Que et al., 2019; Wozniak et al., 2020). The concomitant decrease in Zn\textsuperscript{2+} facilitates the resumption of the cell cycle and exit from the MII stage (Kim et al., 2011). Congruent with this observation, artificial manipulation that maintains high Zn\textsuperscript{2+} levels prevents egg activation (Kim et al., 2011), whereas lowering Zn\textsuperscript{2+} with chelators leads to egg activation without Ca\textsuperscript{2+} mobilization (Suzuki et al., 2010b). As posed by others, these results suggest that meiosis completion and the early stages of fertilization unfold within a narrow window of permissible Zn\textsuperscript{2+} (Kim et al., 2011, 2010). Here, we extend this concept and show that IP\textsubscript{3}R1 function and the Ca\textsuperscript{2+} oscillations in mouse eggs require this optimal level of labile Zn\textsuperscript{2+} because the Ca\textsuperscript{2+} responses interrupted by TPEN-induced Zn\textsuperscript{2+}-insufficiency are rescued by restoring Zn\textsuperscript{2+} levels with ZnPT. Furthermore, unopposed increases in Zn\textsuperscript{2+} by exposure to ZnPT abrogated fertilization-initiated Ca\textsuperscript{2+} oscillations and prevented the expected egg activation events. It is unclear how excess Zn\textsuperscript{2+} disturbs the function of IP\textsubscript{3}R1. Nevertheless, IP\textsubscript{3}R1s have multiple cysteines whose oxidation enhances the receptor sensitivity to IP\textsubscript{3} (Joseph et al., 2018), and it is possible that excessive Zn\textsuperscript{2+} aberrantly modifies them, disturbing IP\textsubscript{3}R1 structure and function or, alternatively, preventing their oxidation and sensitization of the receptor. Lastly, we cannot rule out that high Zn\textsuperscript{2+} levels directly inhibit the receptor’s channel. These results reveal a close association between the Zn\textsuperscript{2+} levels controlling meiotic transitions and the Ca\textsuperscript{2+} release necessary for egg activation, placing the IP\textsubscript{3}R1 at the center of the crosstalk of these two divalent cations.

Abrupt Zn\textsuperscript{2+} changes have emerged as critical signals for meiotic and mitotic transitions in oocytes, eggs, embryos, and somatic cells (Kim et al., 2011, 2010; Lo et al., 2020). Fertilization relies on prototypical Ca\textsuperscript{2+} rises and oscillations, and Zn\textsuperscript{2+} sparks are an egg activation event downstream of this Ca\textsuperscript{2+} release, establishing a functional association between these two divalent cations that continues to grow (Kim et al., 2011).
Here, we show that, in addition, these cations actively crosstalk during fertilization and that the fertilization-induced Ca\textsuperscript{2+} oscillations rely on optimized IP\textsubscript{3}R1 function underpinned by ideal Zn\textsuperscript{2+} levels set during oocyte maturation. Future studies should explore if artificial alteration of Zn\textsuperscript{2+} levels can extend the fertile lifespan of eggs, improve developmental competence, or develop methods of non-hormonal contraception.

**Materials and Methods**

**Key resources table**

<table>
<thead>
<tr>
<th>Reagent type (species) or resource</th>
<th>Designation</th>
<th>Source or reference</th>
<th>Identifiers</th>
<th>Additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic reagent (<em>Mus musculus</em>)</td>
<td>CD1</td>
<td>Charles River</td>
<td>022</td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (<em>Mus musculus</em>)</td>
<td>C57BL/6J</td>
<td>JAX</td>
<td>JAX: 000664</td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (<em>Mus musculus</em>)</td>
<td>Trpm7-floxed</td>
<td>A generous gift from Dr. Carmen P. Williams (NIEHS) (PMID: 30322909)</td>
<td>C57BL6/J and 129s4/SvJae mixed background</td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (<em>Mus musculus</em>)</td>
<td>Gdf9-cre</td>
<td>JAX</td>
<td>JAX: 011062</td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (<em>Mus musculus</em>)</td>
<td>Trpv3\textsuperscript{-/-}</td>
<td>A generous gift from Dr H. Xu (PMID: 20403327)</td>
<td>C57BL/6J and 129/SvEv mixed background</td>
<td></td>
</tr>
<tr>
<td>Biological sample (mouse oocyte)</td>
<td><em>Mus musculus</em></td>
<td>this paper</td>
<td>Eggs at the metaphase of the second meiosis</td>
<td></td>
</tr>
<tr>
<td>Biological sample (mouse sperm)</td>
<td><em>Mus musculus</em></td>
<td>this paper</td>
<td>Matured sperm from cauda</td>
<td></td>
</tr>
<tr>
<td>Recombinant DNA reagent</td>
<td>Published in previous Fissore lab paper (PMID: 34313315)</td>
<td>Mouse Plcz1 mRNA was fused with Venus and inserted in pcDNA6 vector.</td>
<td>Recombinant DNA reagent</td>
<td>Published in previous Fissore lab paper (PMID: 24101727)</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------------------------------------------</td>
<td>--------------------------------------------------------</td>
<td>-------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>pcDNA6-mouse Plcz1-venus (plasmid used as a template for mRNA synthesis)</td>
<td>Mouse Plcz1 sequence was a generous gift from Dr. Kiyoko Fukami (PMID: 18028898)</td>
<td></td>
<td>pcDNA6-CALR-D1ER-KDEL (plasmid used as a template for mRNA synthesis)</td>
<td>Original D1ER vector was a generous gift from Dr. Roger Y Tsien (PMID: 15585581)</td>
</tr>
<tr>
<td>pcDNA6-human ERp44-HA (plasmid used as a template for mRNA synthesis)</td>
<td>This paper. Original human ERp44 sequence was a generous gift from Dr. Roberto Sitia (PMID: 11847130)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Monoclonal HA (Mouse monoclonal)</th>
<th>Roche</th>
<th>115818160 01</th>
<th>Dilution: 1:200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Polyclonal IP&lt;sub&gt;3&lt;/sub&gt;R1 (Rabbit polyclonal)</td>
<td>(Parys et al., 1995)</td>
<td></td>
<td>Dilution: 1:1000</td>
</tr>
<tr>
<td>Antibody</td>
<td>Monoclonal α-tubulin (Mouse monoclonal)</td>
<td>Sigma-Aldrich</td>
<td>T-9026</td>
<td>Dilution: 1:1000</td>
</tr>
<tr>
<td>Antibody</td>
<td>Alexa Fluor 488 (goat anti mouse)</td>
<td>Invitrogen: A32723</td>
<td>Dilution: 1:400</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------------------------</td>
<td>--------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>Commercial assay or kit</td>
<td>T7 mMESSAGE mMACHINE Kit</td>
<td>Invitrogen: AM1344</td>
<td>Used for in vitro mRNA synthesis</td>
<td></td>
</tr>
<tr>
<td>Commercial assay or kit</td>
<td>Poly(A) Tailing Kit</td>
<td>Invitrogen: AM1350</td>
<td>Used for poly (A) tailing of synthesized mRNA</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>Hyaluronidase from bovine testes</td>
<td>Sigma-Aldrich: H3506</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>3-Isobutyl-1-methylxanthine (IBMX)</td>
<td>Sigma-Aldrich: I5879</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>Polyvinylpyrrolidone (PVP) (average molecular weight: 360,000)</td>
<td>Sigma-Aldrich: PVP360</td>
<td>Used for mRNA microinjection and ICSI</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN)</td>
<td>Sigma-Aldrich: P4413</td>
<td>Prepared in DMSO and kept at −20 °C until use</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>Zinc Pyrithione (ZnPT)</td>
<td>Sigma-Aldrich: PHR1401</td>
<td>Prepared in DMSO and kept at −20 °C until use</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>Strontium chloride hexahydrate (SrCl₂)</td>
<td>Sigma-Aldrich: 255521</td>
<td>Freshly dissolved in water on the day of experiment</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>Calcium chloride dihydrate (CaCl₂)</td>
<td>Sigma-Aldrich: C3881</td>
<td>Freshly dissolved in water on the day of experiment</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>Magnesium chloride</td>
<td>Sigma-Aldrich: M2393</td>
<td>Freshly dissolved in water on the day of experiment</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>Dissolved in water on the day of experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>--------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc sulfate monohydrate (ZnSO₄)</td>
<td>Aeros Organics 389802500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid sodium dihydrate (EDTA)</td>
<td>LabChem LC137501</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris (2-pyridylmethyl) amine (TPA)</td>
<td>Santa Cruz sc-477037</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulphoxide (DMSO)</td>
<td>Sigma-Aldrich D8418</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionomycin calcium salt</td>
<td>Tocris 1704</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Freshly dissolved in water on the day of experiment:
- Zinc sulfate monohydrate
- Ethylenediaminetetraacetic acid sodium dihydrate (EDTA)
- Tris (2-pyridylmethyl) amine (TPA)
- Dimethyl sulphoxide (DMSO)
- Ionomycin calcium salt

Prepared as 0.5M aqueous solution with pH 8.0 adjusted by NaOH:
- Diethylenetriaminepentacetic acid (DTPA)

Used as a solvent:
- Diethylenetriaminepentacetic acid (DTPA)

Freshly dissolved in water on the day of experiment and kept on ice until use:
- Dimethyl sulphoxide (DMSO)

Working concentration: 2.5 µM:
- Ionomycin calcium salt
<table>
<thead>
<tr>
<th>Chemical compound, drug</th>
<th>Other</th>
<th>Pluronic F-127 (20% solution in DMSO) (Pluronic acid)</th>
<th>Invitrogen</th>
<th>P3000MP</th>
<th>Used at 1.25 µM in TL-HEPES containing 0.02% Pluronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thapsigargin</td>
<td>Other</td>
<td>Fura-2 AM</td>
<td>Invitrogen</td>
<td>F1221</td>
<td>Used at 1.25 µM in TL-HEPES containing 0.02% Pluronic acid</td>
</tr>
<tr>
<td>Calbiochem #586500</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Working concentration: 10 µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Other</td>
<td>FluoZin-3 AM</td>
<td>Invitrogen</td>
<td>F24195</td>
<td>Used at 1.25 µM in TL-HEPES containing 0.02% Pluronic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Other</td>
<td>Fluo-4 AM</td>
<td>Invitrogen</td>
<td>F14201</td>
<td>Used at 1.25 µM in TL-HEPES containing 0.02% Pluronic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Other</td>
<td>Rhod2-AM</td>
<td>Invitrogen</td>
<td>R1244</td>
<td>Used at 2.2 µM in TL-HEPES containing 0.02% Pluronic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Other</td>
<td>ci-IP3/ PM</td>
<td>Tocris</td>
<td>6210</td>
<td>Dissolved in DMSO and kept at −20 °C. Before use, the stock was diluted</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
with water to make a final concentration of 0.25 mM.

Other | Pme1 | New England BioLabs | R0560S | Used to linearize pcDNA6 vectors for mRNA synthesis

| Software, algorithm | Prism | GraphPad Software | Version 5.01 |

N,N,N’,N’-tetrakis (2-pyridinylmethyl)-1,2-ethylenediamine (TPEN) and Zinc pyrithione (ZnPT) were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at -20°C until use. SrCl₂, CaCl₂, ZnSO₄, and MgCl₂ were freshly dissolved with double-sterile water at 1M and diluted with the monitoring media just before use. Ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) were reconstituted with double-sterile water at 0.5M and 10 mM, respectively, and the pH was adjusted to 8.0. Tris(2-pyridylmethyl) amine (TPA) was diluted in DMSO at 100 mM and stored at -20°C until use. Acetycholine chloride and Thimerosal were dissolved in double-sterile water at 550 mM and 100 mM, respectively. Acetycholine was stored at -20°C until use, whereas Thimerosal was made fresh in each experiment.

Mice
The University of Massachusetts Institutional Animal Care and Use Committee (IACUC) approved all animal experiments and protocols. Trpm7-flxed (Trpm7<sup>fl/fl</sup>) Gdf9-Cre and Trpv3<sup>−/−</sup> mice were bred at our facility. Trpm7<sup>fl/fl</sup> mice were crossed with Trpv3<sup>−/−</sup> to generate Trpm7<sup>fl/fl</sup>; Trpv3<sup>−/−</sup> mouse line. Female Trpm7<sup>fl/fl</sup>; Trpv3<sup>−/−</sup> mice were crossed with Trpm7<sup>fl/fl</sup>; Trpv3<sup>−/−</sup>; Gdf9-cre male to generate females null for Trpv3 and with oocyte-specific deletion for Trpm7. Ear clips from offspring were collected prior to weaning, and confirmation of genotype was performed after most experiments.

Egg Collection
All gamete handling procedures are as previously reported by us (Wakai and Fissore, 2019). MII eggs were collected from the ampulla of 6- to 8-week-old female mice.
Females were superovulated via intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma, St. Louis, MO) and 5 IU human chorionic gonadotropin (hCG, sigma) at 48hr. interval. Cumulus-oocyte-complexes (COCs) were obtained 13.5 hr. post-hCG injection by tearing the ampulla using forceps and needles in TL-HEPES medium. COCs were treated with 0.26% (w/v) of hyaluronidase at room temperature (RT) for 5 min to remove cumulus cells.

**Intracytoplasmic sperm injection (ICSI)**

ICSI was performed as previously reported by us (Kurokawa and Fissore, 2003) using described setup and micromanipulators (Narishige, Japan). Sperm from C57BL/6 or CD1 male mice (7-12 weeks old) were collected from the cauda epididymis in TL-HEPES medium, washed several times, heads separated from tails by sonication (XL2020; Heat Systems Inc., USA) for 5 s at 4°C. The sperm lysate was washed in TL-HEPES and diluted with 12% polyvinylpyrrolidone (PVP, MW = 360 kDa) to a final PVP concentration of 6%. A piezo micropipette-driving unit was used to deliver the sperm into the ooplasm (Primetech, Ibaraki, Japan); a few piezo-pulses were applied to puncture the eggs’ plasma membrane following penetration of the zona pellucida. After ICSI, eggs were either used for Ca²⁺ monitoring or cultured in KSOM to evaluate activation and development at 36.5°C in a humidified atmosphere containing 5% CO₂.

**Preparation and microinjection of mRNA**

pcDNA6-mPlcζ-mEGFP, pcDNA6-CALR-D1ER-KDEL, and pcDNA6-humanERp44-HA were linearized with the restriction enzyme PmeI and *in vitro* transcribed using the T7 mMESSAGE mMACHINE Kit following procedures previously used in our laboratory (Ardestani et al., 2020). A poly(A) tail was added to the *in vitro* synthesized RNA (mRNA) using Tailing Kit followed by quantification and dilution to 0.5 μg/μL in nuclease-free water and stored at -80°C until use. Before microinjection, mPlcζ, D1ER, and ERp44 mRNA were diluted to 0.01, 1.0, and 0.5 μg/μL, respectively, in nuclease-free water, heated at 95°C for 3 min followed by centrifugation at 13400×g for 10 min at 4°C. Cytoplasm injection of mRNA was performed under microscopy equipped with micromanipulators (Narishige, Japan). The zona pellucida and the plasma membrane of MII eggs were penetrated by applying small pulses generated by the piezo micromanipulator (Primetech, Ibaraki, Japan). The preparation of the injection pipette was as for ICSI (Kurokawa and Fissore, 2003), but the diameter of the tip was ~1 μm.

**Ca²⁺ and Zn²⁺ imaging**
Before Ca\(^{2+}\) imaging, eggs were incubated in TL-HEPES containing 1.25 \(\mu\)M Fura2-AM, 1.25 \(\mu\)M FluoZin3-AM, or 2.2 \(\mu\)M Rhod2-AM and 0.02% Pluronic acid for 20 min at room temperature and then washed. The fluorescent probe-loaded eggs were allowed to attach to the bottom of the glass dish (Mat-Tek Corp., Ashland, MA). Eggs were monitored simultaneously using an inverted microscope (Nikon, Melville, NY) outfitted for fluorescence measurements. Fura-2 AM, FluoZin3-AM, and Rhod2-AM fluorescence were excited with 340 nm and 380 nm, 480 nm, and 550 nm wavelengths, respectively, every 20 sec, for different intervals according to the experimental design and as previously performed in the laboratory. The illumination was provided by a 75-W Xenon arc lamp and controlled by a filter wheel (Ludl Electronic Products Ltd., Hawthorne, NY).

The emitted light above 510 nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, AZ). Nikon Element software coordinated the filter wheel and data acquisition. The acquired data were saved and analyzed using Microsoft Excel and GraphPad using Prism software (Ardestani et al., 2020). For Figures 1A, 4A-C, 5A, and 6H-I, values obtained from FluoZin3-AM, Fura2-AM, or Rhod2-AM recordings were divided by the average of the first five recordings for each treatment that was used as the F0.

To estimate relative changes in Ca\(^{2+}\)-ER, emission ratio imaging of the D1ER (YFP/CFP) was performed using a CFP excitation filter, dichroic beamsplitter, CFP and YFP emission filters (Chroma technology, Rockingham, VT; ET436/20X, 89007bs, ET480/40m, and ET535/30m). To measure Ca\(^{2+}\)-ER and cytosolic Ca\(^{2+}\) simultaneously, eggs that had been injected with D1ER were loaded with Rhod-2AM, and CFP, YFP, and Rhod-2 intensities were collected every 20 sec.

**Caged IP3**

Caged-IP3/PM (cIP3) was reconstituted in DMSO and stored at -20°C until use. Before injection, cIP3 stock was diluted to 0.25 mM with water and microinjected as above. After incubation in KSOM media at 37°C for 1-hr., the injected eggs were loaded with the fluorophore, 1.25 \(\mu\)M Fluo4-AM, and 0.02% Pluronic acid and handled as above for Fura-2 AM. The release of cIP3 was accomplished by photolysis using 0.5 to 5-sec pulses at 360 nm wavelengths. Ca\(^{2+}\) imaging was as above, but Fluo4 was excited at 488 nm wavelength and emitted light above 510 nm collected as above.

**Western blot analysis**

Cell lysates from 20-50 mouse eggs were prepared by adding 2X- Laemmli sample buffer. Proteins were separated on 5% SDS-PAGE gels and transferred to PVDF membranes.
After blocking with 5% fat-free milk + TBS, membranes were probed with the rabbit polyclonal antibody specific to IP₃R1 (1:1000; a generous gift from Dr. Jan Parys, Katholieke Universiteit, Leuven, Belgium; Parys et al., 1995). Goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) was used as a secondary antibody (1:5000; Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP; Invitrogen, Waltham, Ma). For detection of chemiluminescence, membranes were developed using ECL Prime (Sigma) and exposed for 1–3 min to maximum sensitivity film (VWR, Radnor, PA). Broad-range pre-stained SDS–PAGE molecular weight markers (Bio-Rad, Hercules, CA) were run in parallel to estimate the molecular weight of the immunoreactive bands. The same membranes were stripped at 50°C for 30 min (62.5 mM Tris, 2% SDS, and 100 mM 2-beta mercaptoethanol) and re-probed with anti-α-tubulin monoclonal antibody (1:1000).

**Immunostaining and confocal microscopy**

Immunostaining was performed according to our previous study (Akizawa et al., 2021). After incubation with or without TPEN, MII eggs were fixed with 4% (w/v) paraformaldehyde in house-made phosphate-buffered saline (PBS) for 20 min at room temperature and then permeabilized for 60 min with 0.2% (v/v) Triton X-100 in PBS. Next, the eggs were blocked for 45 min with a blocking buffer containing 0.2% (w/v) skim milk, 2% (v/v) fetal bovine serum, 1% (w/v) bovine serum albumin, 0.1% (v/v) Triton-X-100, 0.75% (w/v) glycine in PBS. Eggs were incubated overnight at 4°C with mouse anti-HA antibody (1:200) diluted in blocking buffer. Eggs were washed in blocking buffer 3X for 10 min, followed by incubation at room temperature for 30 min with a secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (H + L) (1:400) diluted in blocking buffer. Fluorescence signals were visualized using a laser-scanning confocal microscope (Nikon A1 Resonant Confocal with six-color TIRF) fitted with a 63×, 1.4 NA oil-immersion objective lens.

**Statistical analysis**

Comparisons for statistical significance of experimental values between treatments and experiments were performed in three or more experiments performed on different batches of eggs in most studies. Given the number of eggs needed, WB studies were repeated twice. Prism-GraphPad software was used to perform the statistical comparisons that include unpaired Student’s t-tests, Fisher’s exact test, and One-way ANOVA followed by Tukey’s multiple comparisons, as applicable, and the production of graphs to display the data. All data are presented as mean±s.d. Differences were considered significant at $P < 0.05$. 

---

(C) The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.
Acknowledgments

We thank Ms. Changli He for technical support and Dr. James Chambers for support with confocal microscopy support. We thank all members of the Fissore lab for useful discussions and suggestions. We thank Jan B. Parys, K.U. Leuven, Belgium, for initial discussions and advice.

Competing interests

The authors declare no competing or financial interests.

Additional information

Funding Sources

<table>
<thead>
<tr>
<th>Funder</th>
<th>Grant reference number</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>National Institute of Health</td>
<td>RO1 HD092499</td>
<td>Rafael A. Fissore</td>
</tr>
<tr>
<td>National Institute of Food and Agriculture</td>
<td>2021-06893</td>
<td>MG. Gervasi, R.A. Fissore, P.E. Visconti</td>
</tr>
<tr>
<td>Japan Society for the Promotion of Science Overseas Research Fellowship</td>
<td>Postdoctoral fellowship</td>
<td>Hiroki Akizawa</td>
</tr>
</tbody>
</table>

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

Hiroki Akizawa, Data curation, Formal analysis, Validation, Investigation, Visualization, Writing—original draft, Writing—review and editing; Emily Lopes, Data curation, Formal analysis, Validation; Rafael A Fissore, Conceptualization, Formal analysis, Supervision, Funding acquisition, Methodology, Writing—original draft, Project administration, Writing—review and editing

References


Ajduk A, Małagocki A, Maleszewski M. 2008. Cytoplasmic maturation of mammalian oocytes: Development of a mechanism responsible for sperm-induced Ca2+...


Lawrence Y, Ozil JP, Swann K. 1998. The effects of a Ca2+ chelator and heavy-metal ion chelators upon Ca2+ oscillations and activation at fertilization in mouse eggs suggest a role for repetitive Ca2+ increases. *Biochem J* **335**:335–342. doi:10.1042/bj3350335


Miyazaki SI, Yuzaki M, Nakada K, Shirakawa H, Nakanishi S, Nakade S, Mikoshiba K. 1992. Block of Ca2+ wave and Ca2+ oscillation by antibody to the inositol 1,4,5-
trisphosphate receptor in fertilized hamster eggs. *Science* (80-) **257**:251–255. doi:10.1126/science.1321497


Abbreviations
### Table 1. Addition of TPEN after ICSI does not prevent extrusion of the second polar body but precludes pronuclear (PN) formation.

<table>
<thead>
<tr>
<th>Group*</th>
<th>No. of zygotes</th>
<th>2nd polar body (2.5h)</th>
<th>PN 4h</th>
<th>PN 7h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>26</td>
<td>25 (96.1%)</td>
<td>23 (88.5%)</td>
<td>23 (88.5%)</td>
</tr>
<tr>
<td>TPEN (10µM)</td>
<td>27</td>
<td>24 (88.9%)</td>
<td>1 (3.7%)***</td>
<td>2 (7.4%)***</td>
</tr>
</tbody>
</table>

***P < 0.001

*Data from three different replicates for each group.
Figure 1. TPEN-induced Zn\(^{2+}\) deficiency inhibits fertilization-initiated Ca\(^{2+}\) oscillations in a dose-dependent manner.

(A) (Left panel) Representative normalized Zn\(^{2+}\) recordings of MII eggs loaded with FluoZin-3AM following the addition of increasing concentrations of TPEN (0 µM, DMSO, black trace; 2.5 µM, sky blue; 5 µM, blue; 10 µM, navy). TPEN was directly added to the monitoring media. (Right panel) Representative fluorescent images of MII eggs loaded FluoZin-3-AM supplemented with 0, 2.5, and 10 µM of TPEN. Scale bar: 10 µm. (B-D). (B) Representative Ca\(^{2+}\) oscillations following ICSI after the addition of 0, 5, 10, or 50 µM TPEN (arrowheads). Insets show representative traces for eggs that resumed Ca\(^{2+}\) oscillations after TPEN. (C) As above, but following the addition of 100 µM EDTA, 100 or 500 µM DTPA (time of addition denoted by arrowheads). (D) Ca\(^{2+}\) oscillations following ICSI after the addition of 50, 100, and 500 µM TPA (horizontal bars of increasing thickness). (E) Representative bright field images of ICSI fertilized eggs 2.5, 4, and 7 h after sperm injection. Arrows and arrowheads denote the second polar body and PN formation, respectively. Scale bar: 10 µm.

Figure 2. TPEN dose-dependently inhibits Ca\(^{2+}\) oscillations in eggs triggered by a broad-spectrum of agonists that stimulate the PI pathway or IP\(_3\)R1.

(A-D) Representative Ca\(^{2+}\) responses induced by (A) mPlc\(_{\zeta}\) mRNA microinjection (0.01 µg/µl, black traces), (B) strontium chloride (10 mM, green), (C) acetylcholine chloride (50 µM, orange), and (D) thimerosal (25 µM, purple) in MII eggs. Increasing concentrations of TPEN were added to the monitoring media (arrowheads above traces denotes the time of adding). Insets in the upper row show representative traces of eggs that stop oscillating despite others continuing to oscillate. (E) Each bar graph summarizes the TPEN effect on Ca\(^{2+}\) oscillations at the selected concentrations for each of the agonists in A-D. (F) Western blot showing the intensities of IP\(_3\)R1 and alpha-tubulin bands in MII eggs or in eggs injected with mPlc\(_{\zeta}\) mRNA and incubated or not with TPEN above (\(P < 0.01\)). Thirty eggs per lane in all cases. This experiment was repeated twice, and the mean relative intensity of each blot is shown in the bar graph below.

Figure 3. TPEN inhibition of cIP\(_3\)-induced Ca\(^{2+}\) release is precluded by ZnSO\(_4\) supplementation before TPEN exposure.

(A-G) Representative Ca\(^{2+}\) responses in MII eggs triggered by the release of caged IP\(_3\) (cIP\(_3\)) induced by UV light pulses of increasing duration (arrows). (A) A representative control trace without TPEN, and (B) following the addition of 10 µM TPEN between the
third and the fourth pulses. The broken line rectangle is magnified in the inset, farthest right side of the panel displaying the near immediate termination of an ongoing rise. (C, D) Recordings started in the presence of 10 µM TPEN but in (D) 100 µM ZnSO₄ was added between the second and the third pulses. (E) Recording started in the presence of 100 µM ZnSO₄ followed by the addition of 10 µM TPEN between the third and the fourth pulses. (F, G) Recording started in the presence of 100 µM MgSO₄ (F) or 100 µM CaCl₂ (G) and 10 µM TPEN added as above. Arrowheads above the different panels indicate the time of TPEN or divalent cation addition. (H) Bar graphs summarizing the number and percentages of eggs that responded to a given duration of UV pulses under each of the TPEN±divalent ions.

Figure 4. Zn²⁺ depletion alters Ca²⁺ homeostasis and increases Ca²⁺ store content independent of IP₃R1 mass.

(A, B) Representative Ca²⁺ traces of MII eggs after the addition of Tg and Io in the presence or absence of TPEN. Blue trace recordings represent TPEN-treated eggs whereas gray traces represent control, untreated eggs. (A) Io was added to fresh MII eggs once Ca²⁺ returned to baseline after treatment with Tg. Comparisons of mean peak amplitudes after Tg and Io are shown in the bar graphs in the right panel (P < 0.001). (B) MII eggs were aged by 2h. incubation supplemented or not with TPEN followed by Io addition and Ca²⁺ monitoring (P < 0.001). (C) Western blot showing the intensities of IP₃R1 bands in MII eggs freshly collected, aged by 4h. incubation without TPEN, and with TPEN. Thirty eggs per lane in all cases. This experiment was repeated three times.

(D, E) (Left panels) Representative traces of Ca²⁺ values in eggs loaded with the Ca²⁺-sensitive dye Rhod-2 AM and the ER Ca²⁺ reporter, D1ER (1 µg/µl mRNA). TPEN was added into the media followed 10 min later by (D) 10 µM Tg and (E) 50 µM Ach. (Right panel) Bars represent the difference of FRET value between at the time of Tg/ Ach addition and at 3 and 5 min later of the addition (P < 0.05). Experiments were repeated two different times for each treatment. Black and green traces represent cytosolic Ca²⁺ and Ca²⁺-ER, respectively. Blue and black arrowheads indicate the time of addition of TPEN and Tg/ Ach, respectively. (F) Basal Zn²⁺ level comparison in WT (open bar) and Trpv3⁻/⁻/Trpm7⁻/⁻ (dKO, orange bar) MII eggs. Each plot represents the Fluo3 measurement at 5 min after starting monitoring. (G) (Left panel) Representative Ca²⁺ traces of WT (black trace) and dKO (orange trace) MII eggs after adding Tg. Insets represent the magnified traces at the peak of Ca²⁺ spike from different sets of eggs. (Middle panel) Individual traces of WT and dKO eggs after Tg addition. Dashed circles represent the flection point in dKO traces. (Right panel) Comparisons of...
mean peak amplitudes after Tg and the time between Tg addition and the Ca\(^{2+}\) peak are shown in the bar graphs in the right panel (\(P < 0.001\)).

**Figure 5. Restoring Zn\(^{2+}\) levels with ZnPT rescues oscillations interrupted by TPEN-induced Zn\(^{2+}\) deficiency.**

(A) Representative traces of Zn\(^{2+}\) in MII eggs following the addition of 0.01 to 0.1 \(\mu\)M concentrations of ZnPT. The broken rectangular area is amplified in the next panel to appreciate the subtle increase in basal Zn\(^{2+}\) caused by the addition of ZnPT. (B, C) \(mPlc\_\zeta\) mRNA (0.01 \(\mu\)g/\(\mu\)l)-induced oscillations followed by the addition of TPEN (black arrowhead) (B), or after the addition of TPEN followed by ZnPT (red arrowhead) (C). (D, E) Thimerosal (25 \(\mu\)M) induced oscillations using the same sequence of TPEN (D) and ZnPT (E), but higher concentrations of ZnPT were required to rescue Thimerosal-initiated oscillations (E). These experiments were repeated at least two different times.

**Figure 6. Excess Zn\(^{2+}\) hinders Ca\(^{2+}\) oscillations.**

(A-D) ICSI-initiated Ca\(^{2+}\) response without (A) or following the addition of ZnPT (B, C) (the time of ZnPT addition and concentration are denoted above the tracing). (E, F) Representative Ca\(^{2+}\) responses induced by injection of 0.01 \(\mu\)g/\(\mu\)l \(mPlc\_\zeta\) mRNA in untreated eggs (E) or in eggs treated with 0.1 \(\mu\)M ZnPT followed by 10 \(\mu\)M TPEN first and then 50 \(\mu\)M (F). (G) cfIP\(_3\)-induced Ca\(^{2+}\) release as expected when the UV pulses in the absence but not in the presence of 0.05 \(\mu\)M ZnPT (the time of addition is denoted by a bar above the tracing). (H, I) Representative traces of Ca\(^{2+}\) values in eggs loaded with the Ca\(^{2+}\)-sensitive dye Rhod-2 AM and the ER Ca\(^{2+}\)-reporter, D1ER (1 \(\mu\)g/\(\mu\)l mRNA). Uninjected and 0.01 \(\mu\)g/\(\mu\)l \(mPlc\_\zeta\) mRNA-injected eggs were monitored. After initiation and establishment of the oscillations, 0.1 \(\mu\)M ZnPT was added into the media followed 30 min later by 2.5 \(\mu\)M Io. Experiments were repeated two different times. Red and black arrowheads indicate the time of addition of ZnPT and Io, respectively.

**Figure 7. Schematic of proposed regulation of IP\(_3\)R1 function by Zn\(^{2+}\) in eggs and fertilized zygotes.**

In MII eggs, left panel, IP\(_3\)R1s are in a Ca\(^{2+}\)-release permissive state with optimal levels of cytoplasmic Ca\(^{2+}\) and Zn\(^{2+}\) and maximum ER content, but Ca\(^{2+}\) is maintained at resting levels by the combined actions of pumps, ER Ca\(^{2+}\) leak, and reduced influx. Once fertilization takes place, left center panel, robust IP\(_3\) production induced by the sperm-borne PLC\(_\zeta\) leads to Ca\(^{2+}\) release through ligand-induced gating of IP\(_3\)R1. Continuous IP\(_3\) production and refilling of the stores via Ca\(^{2+}\) influx ensure the persistence of the
oscillations. Zn^{2+} release occurs in association with first few Ca^{2+} rises and cortical
granule exocytosis, Zn^{2+} sparks, lowering Zn^{2+} levels but not sufficiently to inhibit IP_{3}R1
function. Zn^{2+} deficiency caused by TPEN or other permeable Zn^{2+} chelators, right center
panel, dose-dependently impairs IP_{3}R1 function and limits Ca^{2+} release. We propose this
is accomplished by stripping the Zn^{2+} bound to the residues of the zinc-finger motif in the
LNK domain of IP_{3}R1 that prevents the allosteric modulation of the gating process
induced by IP_{3} or other agonists. We propose that excess Zn^{2+}, right panel, also inhibits
IP_{3}R1-mediate Ca^{2+} release, possibly by non-specific binding of thiol groups present in
cysteine residues throughout the receptor (denoted by a ?). We submit that optimal Ca^{2+}
oscillations in mouse eggs unfold in the presence of a permissive range of Zn^{2+}
concentration.
Supplementary Figure Legends

Supplementary Figure 1. Cell-impermeable chelators effectively reduce Zn\textsuperscript{2+} levels in external media but do prevent initiation or continuation of Ca\textsuperscript{2+} oscillations.

(A) A representative trace of FluoZin3 fluorescence in replete monitoring media (TL-HEPES). The media was supplemented with cell-impermeable FluoZin-3, and after initiation of monitoring, the addition of EDTA (100 μM) occurred at the designated point (triangle). (B) The left black trace represents Ca\textsuperscript{2+} oscillations initiation by injection of mPlcζ mRNA (0.01 μg/μl). The oscillations were monitored in Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free media and in the presence of EDTA (110 μM) to chelate residual divalent cations derived from the water source or reagents used to make the media. The right red trace represents the initiation of oscillations as above, but after a period indicated by the black and green bars, Ca\textsuperscript{2+} and Mg\textsuperscript{2+} were sequentially added back.

Supplementary Figure 2. Overexpression of ER accessory protein ERp44 did not change the Ca\textsuperscript{2+} responses initiated by mPlcζ mRNA microinjection, Acetylcholine, or SrCl\textsubscript{2}.

(A) Representative immunofluorescent images of MII eggs with overexpression of ERp44. At 5 hr. post microinjection, eggs were treated with 10 or 50 μM of TPEN and incubated for 1 hr, after which they were fixed and stained. An anti-HA antibody was used. Scale bar: 10 μm. (B) Representative Ca\textsuperscript{2+} responses induced by mPlcζ mRNA microinjection (0.01 μg/μl-left column), SrCl\textsubscript{2} (10 mM-center column), and acetylcholine (50 μM-right column) in eggs with (top panels) or without (bottom panels) ERp44 overexpression.

Supplementary Figure 3. Elevated Zn\textsuperscript{2+} impairs egg activation and the subsequent embryo development.

(A) MII eggs were incubated in TL-HEPES containing 0, 0.1, or 1.0 μM ZnPT at room temperature for 10 min and washed several times with fresh TL-HEPES and injected with mPlcζ mRNA. After it, eggs and zygotes were cultured in KSOM for 24h. PN formation and 2-cell development were checked at 7 and 24h post-microinjection. Bars represent the percentages of injected eggs that reached the PN and the 2-cell stage. Scale bar: 50 μm. (B) MII eggs injected with mPlcζ mRNA were incubated in KSOM without ZnPT for an hr. and then incubated in KSOM with 0 or 0.1 μM ZnPT for 24h. The second polar body extrusion, PN formation, and 2-cell development were checked at 2.5-, 7- and 24h. post-microinjection. Bars represent the percentages of injected eggs that reached the PN and the 2-cell stage. Scale bar: 50 μm.
Figure 1.

A. FluoZin3-AM (F/F) 

TPEN (μM) 
- 0 (n=7) 
- 2.5 (n=10) 
- 5 (n=10) 
- 10 (n=10)

B. ICSI 
- 0μM (n=17/17) 
- 5μM (n=7/12) 
- 10μM (n=11/17) 
- 50μM (n=6/17)

C. ICSI 
- DTPA 100μM (n=8/8) 
- EDTA 100μM 
- DTPA 500μM (n=8/8)

D. ICSI 
- TPA [μM] 
- 50 
- 100 
- 500 (n=13)

E. TPEN 0μM 
- 2.5h 
- 4h 
- 7h 
- TPEN 10μM 
- 2.5h 
- 4h 
- 7h
Figure 2.

A. mPlcζ
TPEN 0 µM
(n = 21/21)

TPEN 10 µM
(n = 21/28)

TPEN 25 µM
(n = 3/6)

TPEN 50 µM
(n = 6/6)

B. SrCl₂ 10 mM

SrCl₂ 10 mM
TPEN 0 µM
(n = 2)

TPEN 2.5 µM
(n = 8/11)

TPEN 5 µM
(n = 3/11)

C. Ach 50 µM

Ach 50 µM
TPEN 0 µM
(n = 7/10)

TPEN 10 µM
(n = 29/32)

D. Thimerosal 25 µM

Thimerosal 25 µM
TPEN 0 µM
(n = 42)

E. ICSI

mPlcζ
SrCl₂
Thimerosal
Ach

oscillated eggs in %

[TPEN] [µM]

0 5 10 25 50 100 150

0 5 10 25 50 100 150

0 5 10 25 50 100 150

0 5 10 25 50 100 150

0 5 10 25 50 100 150

F. uninjectd
mPlcζ
mPlcζ + TPEN

IP₃R1

TUBA

Relative intensity

0 0.5 1
Figure 3.

A. F480 (sec.)
- 0.5 0.75 1.0 2.0 5.0
- cIP3
- TPEN 0μM
- (n=11)

B. F480 (sec.)
- 0.5 0.75 1.0 2.0 5.0
- TPEN 10μM
- (n=14)

C. F480 (sec.)
- 0.5 0.75 1.0 2.0 5.0
- TPEN 10μM
- (n=11)

D. F480 (sec.)
- 1.0 2.0 2.0 5.0
- ZnSO4 100μM
- (n=10)

E. F480 (sec.)
- 0.5 0.75 1.0 2.0
- TPEN 10μM
- ZnSO4 100μM
- (n=16)

F. F480 (sec.)
- 0.5 0.75 1.0 2.0
- TPEN 10μM
- MgCl2 100μM
- (n=10)

G. F480 (sec.)
- 0.5 0.75 1.0 2.0
- CaCl2 100μM
- (n=13)

H. % eggs responded
- Duration of UV exposure (sec)
- Untreated
- TPEN 10μM
- TPEN 10μM → ZnSO4 100μM
- ZnSO4 100μM → TPEN 10μM
- MgCl2 100μM → TPEN 10μM
- CaCl2 100μM → TPEN 10μM
- responded
- not-responded
Figure 4.

A MII fresh eggs

B MII in vitro aged eggs

C

D

E

F MII fresh eggs

G

WT (n=31)  Trpv3<sup>−/−</sup>/Trpm7<sup>−/−</sup> (n=29)
Figure 5.

A

B mPlcζ

C mPlcζ

D

E Thimerosal (25μM)

ZnPT 0.1 0.5μM
Figure 6.

A  ICSI control (n=12)

B  ICSI (n=12)

C  ICSI (n=10)

D  ICSI (n=11)

E  mPlcζ control (n=8)

F  mPlcζ (n=9)

G  clP3 0.5 0.75 1.0 1.0 2.0 5.0 (n=7)

H  uninjected

I  mPlcζ
Supplementary Figure 1.

A  EDTA 100 μM
   ▼
  TL-HEPES (2 mM Ca²⁺, 0.5 mM Mg²⁺)

B  0 mM Ca²⁺, 0 mM Mg²⁺,
    110 μM EDTA
+0.5 mM Mg²⁺  +2 mM Ca²⁺

Fura-2AM (F340/380)

mPlcζ (n = 13)

mPlcζ (n = 16)
Supplementary Figure 2.

A

control  TPEN 10 µM  TPEN 50 µM

ERP44-HA

DIC

B

<table>
<thead>
<tr>
<th>Ca²⁺ 1.7mM</th>
<th>SrCl₂ 10mM</th>
<th>Acetylcholine 50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPLCζ (n=4)</td>
<td>SrCl₂ (n=2)</td>
<td>(n=9)</td>
</tr>
<tr>
<td>F340/ F380</td>
<td>Ca²⁺ 0mM</td>
<td>Ca²⁺ 1.7mM</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(n=8)</td>
<td>(n=13)</td>
</tr>
</tbody>
</table>

Time (min)
Supplementary Figure 3.

A

In vitro culture
ZnPT
pre-treated
ZnPT
mPlcζ
injection
w/o ZnPT

% eggs proceeded to

<table>
<thead>
<tr>
<th>ZnPT (μM)</th>
<th>0 μM</th>
<th>0.1 μM</th>
<th>1.0 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>7/8</td>
<td>15/17</td>
<td>16/17</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>8/9</td>
<td>16/19</td>
<td>17/19</td>
</tr>
<tr>
<td>1.0 μM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2PB  | PN  | 2-cell

B

In vitro culture
ZnPT
in culture
~30 min
1 hour
mPlcζ
injection
w/o ZnPT
ZnPT

% eggs proceeded to

<table>
<thead>
<tr>
<th>ZnPT (μM)</th>
<th>0 μM</th>
<th>0.1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>17/18/18</td>
<td>18/18</td>
</tr>
<tr>
<td>0.1 μM</td>
<td></td>
<td>15/27</td>
</tr>
</tbody>
</table>

2PB  | PN  | 2-cell