

Zn²⁺ is Essential for Ca²⁺ Oscillations in Mouse Eggs

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Abstract

Changes in the intracellular concentration of free calcium (Ca²⁺) underpin egg activation and initiation of development in animals and plants. In mammals, the Ca²⁺ release is periodical, known as Ca²⁺ oscillations, and mediated by the type 1 inositol 1,4,5-trisphosphate receptor (IP₃R1). Another divalent cation, zinc (Zn²⁺), 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 Zn²⁺ are indispensable for sperm-initiated Ca²⁺ oscillations because Zn²⁺-deficient conditions induced by cell-permeable chelators abrogated Ca²⁺ responses evoked by fertilization and other physiological and pharmacological agonists. We also found that chemically- or genetically generated Zn²⁺-deficient eggs displayed reduced IP₃R1 sensitivity and diminished ER Ca²⁺ leak despite the stable content of the stores and IP₃R1 mass. Resupplying Zn²⁺ restarted Ca²⁺ oscillations, but excessive Zn²⁺ prevented and terminated them, hindering IP₃R1 responsiveness. The findings suggest that a permissive window of Zn²⁺ concentrations is required for Ca²⁺ responses and IP₃R1 function in eggs, ensuring optimal response to fertilization and egg activation.

eLife assessment

This manuscript describes a **compelling** study demonstrating an **important** mechanistic role for zinc fluctuations during fertilization. The results show strong evidence that zinc availability modulates inositol triphosphate receptor and that the cytosolic zinc concentration is critical for calcium oscillations that are required for egg activation and fertilization.

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 (PLC ζ) 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), $\text{IP}_3\text{R1}$ 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 $\text{IP}_3\text{R1}$ 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 (Kim et al., 2011; Que et al., 2019; 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_3Rs 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_3R , the most widespread isoform (Fissore et al., 1999; Parrington et al., 1998). $\text{IP}_3\text{R1}$ 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 $\text{IP}_3\text{R1}$ (Taylor and Tovey, 2010). For example, following fertilization, $\text{IP}_3\text{R1}$ undergoes ligand-induced degradation caused by the sperm-initiated long-lasting production of IP_3 that effectively reduces the $\text{IP}_3\text{R1}$ mass (Brind et al., 2000; Jellerette et al., 2000). Another regulatory mechanism is Ca^{2+} , a universal cofactor, which biphasically regulates IP_3Rs ' channel opening (Iino, 1990; Jean and Klee, 1986), congruent with several Ca^{2+} and calmodulin binding sites on the channel's sequence (Sienaert et al., 1997; Sipma et al., 1999). Notably, Zn^{2+} may also participate in $\text{IP}_3\text{R1}$ regulation. Recent studies using

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; Suzuki et al., 2010b). Studies in mouse eggs also showed that the addition of μM 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 induces a sharp drop in basal Fura-2 F340/ F380 ratios, consistent with Fura-2's high affinity for Zn²⁺ (Snitsarev et al., 1996).

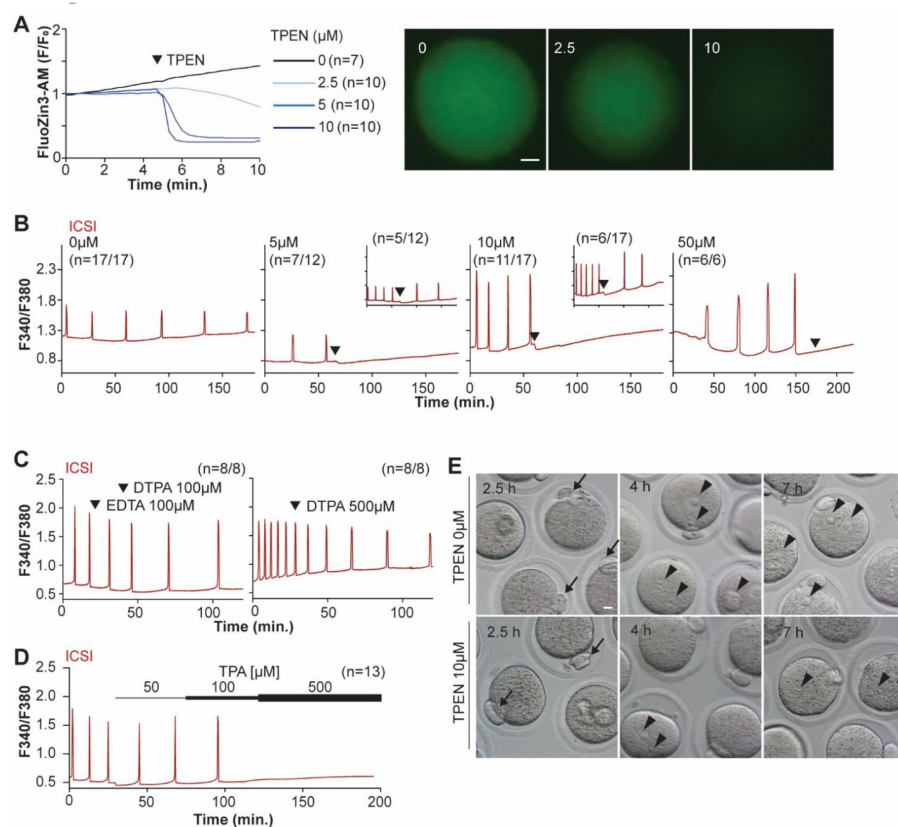


Figure 1.

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-3AM supplemented with 0, 2.5, and 10 μM of TPEN. Scale bar: 10 μm . (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 hr. after sperm injection. Arrows and arrowheads denote the second polar body and PN formation, respectively. Scale bar: 10 μm .

We next used membrane-permeable and -impermeable chelators to assess whether TPEN inhibited Ca^{2+} oscillations by chelating Zn^{2+} from intracellular or extracellular compartments. The addition of the high-affinity but cell-impermeable Zn^{2+} chelators DTPA and EDTA neither terminated nor temporarily interrupted ICSI-induced Ca^{2+} oscillations (Fig. 1C). Protractedly, Ca^{2+} oscillations slowed down, possibly because of chelation and lowering of external Ca^{2+} (Fig. 1C). Conversely, TPA, a permeable Zn^{2+} chelator, blocked the ICSI-initiated oscillations but required higher concentrations than TPEN (Fig. 1D). Collectively, the data suggest that basal levels of labile Zn^{2+} are essential to sustain the fertilization-initiated Ca^{2+} oscillations in eggs.

We next evaluated whether TPEN supplementation prevented the completion of meiosis and pronuclear (PN) formation following fertilization (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-hr. 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.

Table 1.

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

Group*	No. of zygotes	2 nd polar body (2.5h)	PN	
			4h	7h
Untreated	26	25 (96.1%)	23 (88.5%)	23 (88.5%)
TPEN (10μM)	27	24 (88.9%)	1 (3.7%)*	2 (7.4%)*

*** $P < 0.001$

*Data from three different replicates for each group.

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 ζ stimulate the phosphoinositide pathway leading to the production of 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 $\text{IP}_3\text{R1}$ without producing IP_3 . The mechanism(s) of SrCl_2 is unclear, although its actions are reportedly directly on the $\text{IP}_3\text{R1}$ (Hajnóczky and Thomas, 1997; Hamada et al., 2003; Nomikos et al., 2015, 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. *mPlcz* mRNA injection, like fertilization, induces persistent Ca^{2+} oscillations, although *mPlcz*'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 $\text{IP}_3\text{R1}$ was responsible for the slow-down or termination of the oscillations by TPEN. To accomplish this, we examined the $\text{IP}_3\text{R1}$ mass in eggs (Jellerette et al., 2004) with and without TPEN supplementation and injection of *mPlcz* mRNA. By 4-h post-injection, the mRNA induced the expected down-regulation of $\text{IP}_3\text{R1}$ reactivity vs. uninjected eggs, but in TPEN-treated and *Plcz* mRNA-injected eggs, the decrease was insignificant (Fig. 2F). These findings together show that Zn^{2+} deficiency inhibits the $\text{IP}_3\text{R1}$ -mediated Ca^{2+} oscillations independently of IP_3 production or loss of receptor, suggesting a role of Zn^{2+} on $\text{IP}_3\text{R1}$ function (Fig. 2E).

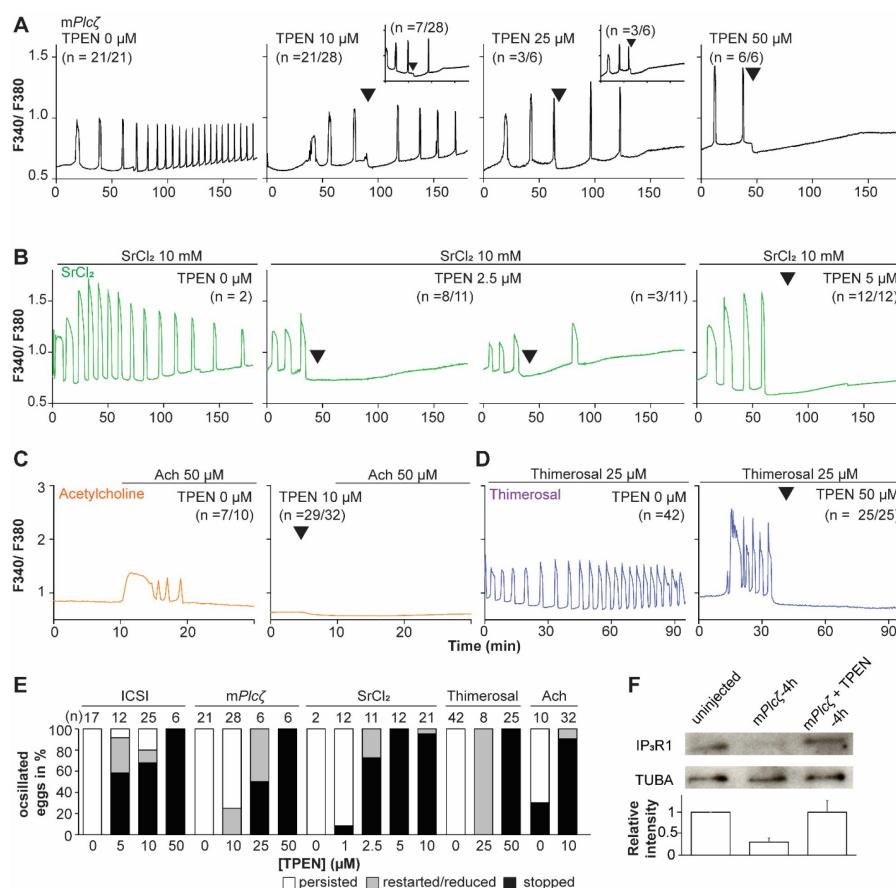


Figure 2.

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

(A-D) Representative Ca^{2+} responses induced by (A) *mP/cζ* mRNA microinjection (0.01 $\mu\text{g}/\mu\text{l}$, 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 denote 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 ago-

nists in A-D. (F) Western blot showing the intensities of $\text{IP}_3\text{R1}$ and alpha-tubulin bands in MII eggs or in eggs injected with *mP/cζ* 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.

Zn^{2+} depletion reduces $\text{IP}_3\text{R1}$ -mediated Ca^{2+} release

To directly assess the inhibitory effects of TPEN on $\text{IP}_3\text{R1}$ function, we used caged IP_3 (cIP_3) that, after short UV pulses, releases IP_3 into the ooplasm (Wakai et al., 2012; Walker et al., 1987). To exclude the possible contribution of external Ca^{2+} to the responses, we performed the experiments in Ca^{2+} -free media. In response to sequential cIP_3 release 5 min. apart, control eggs displayed corresponding Ca^{2+} rises that occasionally transitioned into short-lived oscillations (Fig. 3A). The addition of TPEN after the third cIP_3 release prevented the subsequent Ca^{2+} response and prematurely terminated the in-progress Ca^{2+} rises (Fig. 3B and inset). Pre-incubation of eggs with TPEN precluded cIP_3 -induced Ca^{2+} release, even after 5 sec. of UV exposure (Fig. 3C). The addition of excess ZnSO_4 (100 μM) overcame TPEN's inhibitory effects, but only if added before (Fig. 3E) and not after the addition of TPEN (Fig. 3D). Similar concentrations of MgCl_2 or CaCl_2 failed to reverse TPEN effects (Fig. 3F, G). Together, the results show that Zn^{2+} is required for $\text{IP}_3\text{R1}$ -mediated Ca^{2+} release downstream of IP_3 production, appearing to interfere with receptor gating, as suggested by TPEN's rapid termination of in-progress Ca^{2+} rises and ongoing oscillations.

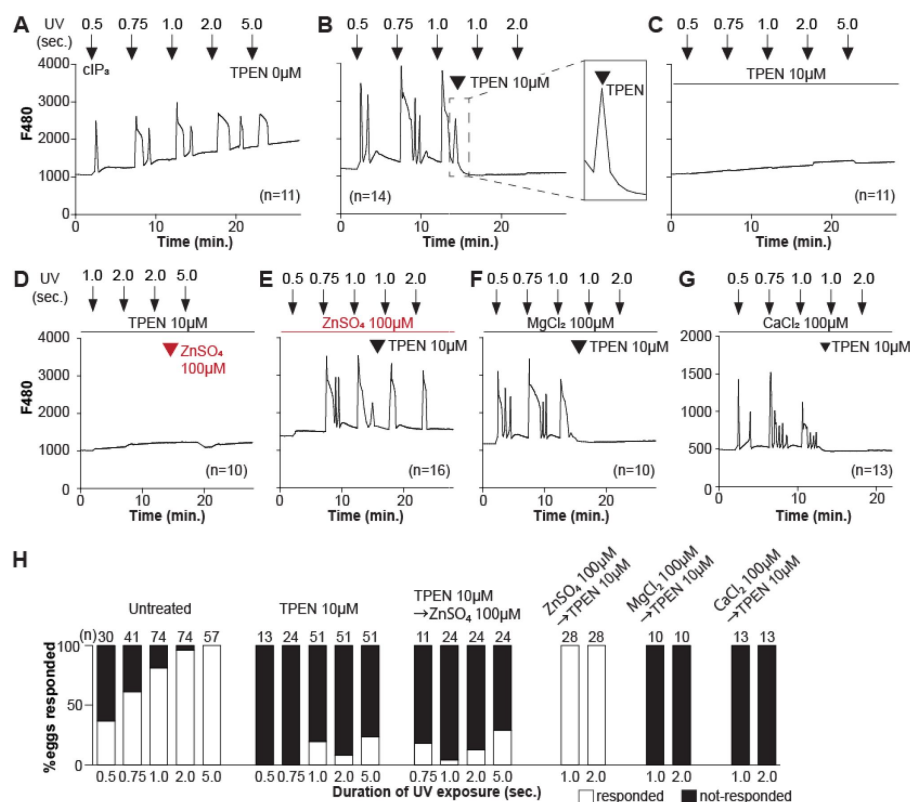


Figure 3.

TPEN inhibition of cIP₃-induced Ca²⁺ release is precluded by ZnSO₄ supplementation before TPEN exposure.

(A-G) Representative Ca²⁺ responses in MII eggs triggered by the release of caged IP₃ (cIP₃) induced by UV light pulses of increasing duration (arrows). (A) A representative control trace without TPEN, and (B) following the addition of 10 μ M 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 μ M TPEN but in (D) 100 μ M ZnSO₄ was added between the second and the third pulses. (E)

Recording started in the presence of 100 μ M ZnSO₄ followed by the addition of 10 μ M TPEN between the third and the fourth pulses. (F, G) Recording started in the presence of 100 μ M MgSO₄ (F) or 100 μ M CaCl₂ (G) and 10 μ M 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.

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. 1A), and overexpression of Erp44 modified neither the Ca²⁺ oscillations induced by agonists (Supplementary Fig. 1B) 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 Ca²⁺ release and interrupted in-progress Ca²⁺ 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 Zn²⁺-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 Zn²⁺ deficiency may be disturbing Ca²⁺ release to the cytosol and out of the ER. To probe this possibility, we queried if pre-treatment with TPEN inhibited Ca²⁺ release through IP₃R1. We first used Thapsigargin (Tg), a Sarcoplasmic/ER Ca²⁺ ATPase pump inhibitor (Thastrup et al., 1990) that unmasks a constitutive Ca²⁺ 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 Ca²⁺ leak into the cytosol, resulting in delayed and lowered amplitude Ca²⁺ responses (Fig. 4A; *P* < 0.05). To test whether the reduced response to Tg means that TPEN left a temporarily increased Ca²⁺ content in the ER after it, we added the Ca²⁺ ionophore ionomycin (Io), which empties all stores independently of IP₃Rs. Io-induced Ca²⁺ responses were 3.3-fold greater in TPEN-treated cells, supporting the view that TPEN interferes with the ER Ca²⁺ leak (Fig. 4A; *P* < 0.05). We further evaluated this concept using *in vitro* aged eggs that often display reduced Ca²⁺ store content than freshly collected counterparts (Abbott et al., 1998). After culturing eggs in the presence or absence of TPEN for 2-hr., we added Io during Ca²⁺ monitoring, which in TPEN-treated eggs induced bigger Ca²⁺ 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).

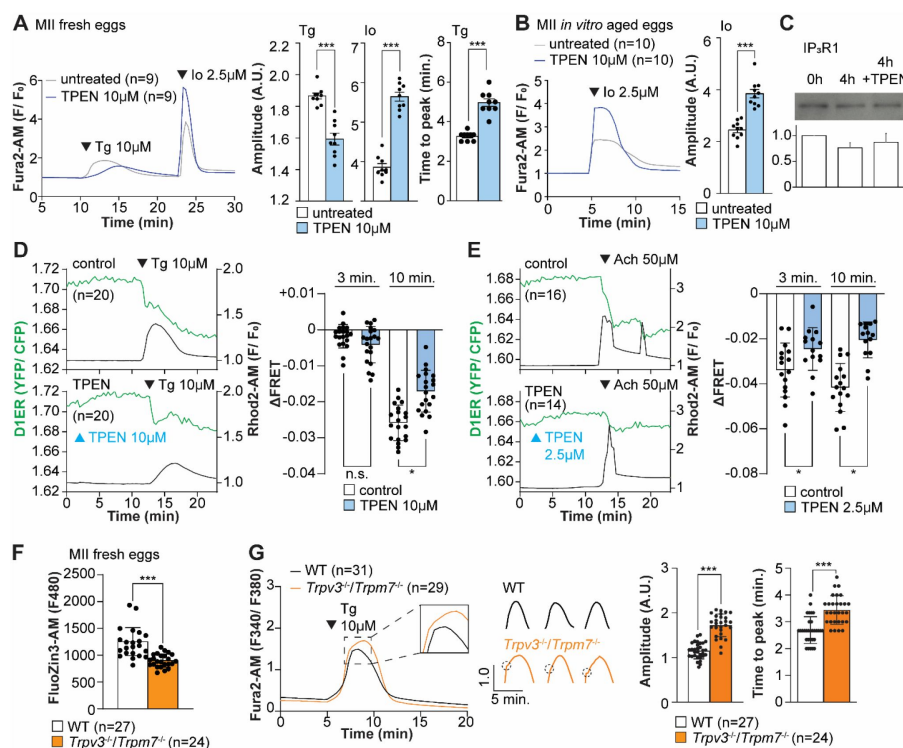


Figure 4.

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 2-hr. 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 4-hr. 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 Zn^{2+} level comparison in WT (open bar) and *Trpv3^{-/-}/Trpm7^{-/-}* (dKO, orange bar) MII eggs. Each plot represents the FluoZin3 measurement at 5 min. after starting monitoring. **(G)** (Left panel) Representative Ca^{2+} traces of WT (black trace) and dKO (orange trace) MII eggs after adding Tg. Insets represent the magnified traces at the peak of Ca^{2+} spike from different sets of eggs. (Middle panel) Individual traces of WT and dKO eggs after Tg addition. Dashed circles represent the inflection 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 ($P < 0.001$)

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^{2+} levels changes following the additions of Tg or Ach. TPEN was added 10 min. before 10 μM Tg or 50 μM Ach, and we simultaneously monitored changes in cytosolic and intra-ER Ca^{2+} (Fig. 4D, E). For the first three min., the Tg-induced decrease in Ca^{2+} -ER was similar between groups. However, while the drop in Ca^{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^{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^{2+} occurred without pharmacology. To this end, we examined a genetic model where the two non-selective plasma membrane channels that could influx Zn^{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 labile Zn^{2+} levels (Fig. 4F), and the addition of Tg revealed an expanded Ca^{2+} store content in these eggs vs. control WT eggs (Fig. 4G). Remarkably, in dKO eggs, the Ca^{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^{2+} release, suggesting that Zn^{2+} deficient levels modify ER Ca^{2+} release independently of chelators.

Ca^{2+} oscillations in eggs occur within a window of Zn^{2+} concentrations

We next examined if resupplying Zn^{2+} could restart the Ca^{2+} oscillations terminated by Zn^{2+} depletion. Zn pyrithione (ZnPT) rapidly increases cellular Zn^{2+} upon extracellular addition (Barnett et al., 1977; Robinson, 1964). Dose titration studies and imaging fluorimetry revealed that 0.01 μM ZnPT caused subtle and protracted increases in Zn^{2+} levels, whereas 0.1 μM ZnPT caused rapid increases in eggs' Zn^{2+} baseline (Fig. 5A). We induced detectable Ca^{2+} oscillations by injection of *mPlcz* mRNA followed by 50 μM TPEN (Fig. 5B), which terminated them. After 30 min, we added 0.1 μM 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 μM ZnPT did not restore the Ca^{2+} oscillations retrained by TPEN, but 0.5 μM ZnPT did so (Fig. 5E). These results demonstrate that Zn^{2+} plays a pivotal, enabling role in the generation of Ca^{2+} oscillations in mouse eggs.

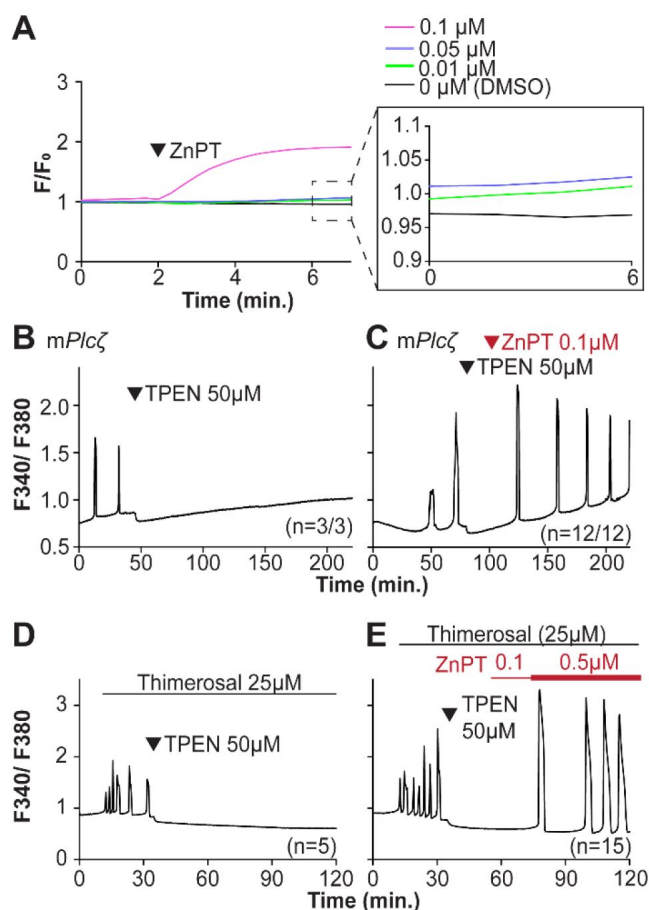


Figure 5.

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 μM 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 ζ* mRNA (0.01 $\mu\text{g}/\mu\text{l}$)-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 μM) 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.

Excessive intracellular Zn^{2+} inhibits Ca^{2+} oscillations

Zn^{2+} is necessary for diverse cellular functions, consistent with numerous amino acids and proteins capable of binding Zn^{2+} within specific and physiological ranges (Pace and Weerapana, 2014). Excessive Zn^{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^{2+} , a previous study showed that high concentrations of ZnPT, ~50 μM , prevented SrCl_2 -induced egg activation and initiation of development (Bernhardt et al., 2012; Kim et al., 2011). We examined how ZnPT and excessive Zn^{2+} levels influence Ca^{2+} oscillations. Our conditions revealed that pre-incubation or continuous exposure to 0.1 μM or 1.0 μM ZnPT delayed or prevented egg activation induced by *mPlc ζ* mRNA injection (Supplementary Fig. 2). We used these ZnPT concentrations to add it into ongoing oscillations induced by ICSI and monitored the succeeding Ca^{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^{2+} oscillations (Fig. 6A-D). *mPlc ζ* mRNA-initiated Ca^{2+} responses were also interrupted by adding 0.1 μM ZnPT, whereas untreated eggs continued oscillating (Fig. 6E, F). ZnPT also inhibited $\text{IP}_3\text{R1}$ -mediated Ca^{2+} release triggered by cIP_3 , suggesting that excessive Zn^{2+} directly inhibits $\text{IP}_3\text{R1}$ function (Fig. 6G).

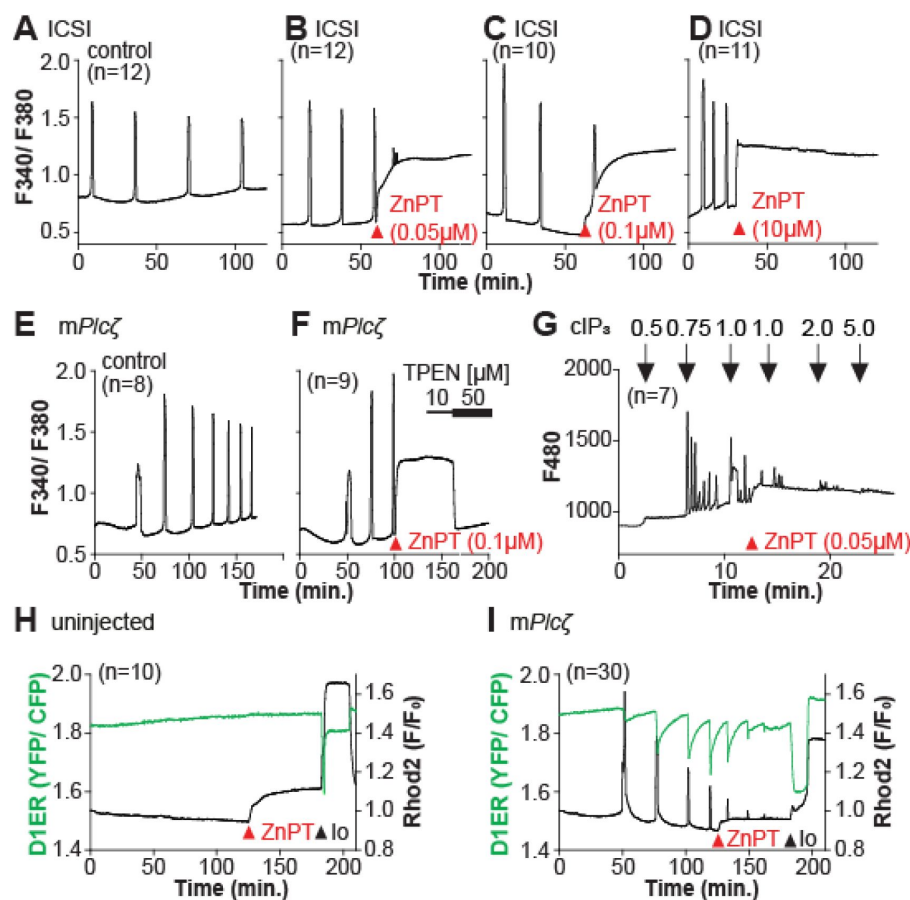


Figure 6.

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 $0.01 \mu\text{g}/\mu\text{l}$ *mPlc ζ* mRNA in untreated eggs (E) or in eggs treated with $0.1 \mu\text{M}$ ZnPT followed by $10 \mu\text{M}$ TPEN first and then $50 \mu\text{M}$ (F). (G) cIP_3 -induced Ca^{2+} release as expected when the UV pulses in the absence but not in the presence of $0.05 \mu\text{M}$ 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 ($1 \mu\text{g}/\mu\text{l}$ mRNA). Uninjected and $0.01 \mu\text{g}/\mu\text{l}$ *mPlc ζ* mRNA-injected eggs were monitored. After initiation and estab-

lishment of the oscillations, $0.1 \mu\text{M}$ ZnPT was added into the media followed 30 min later by $2.5 \mu\text{M}$ Io. Experiments were repeated two different times. Red and black arrowheads indicate the time of addition of ZnPT and Io, respectively.

The increased basal ratios of Fura-2 caused by the addition of ZnPT are unlikely to represent changes in basal Ca^{2+} levels because they would have likely caused some 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^{2+} levels, which was the case because the addition of TPEN lowered the Fura-2 ratios without restarting the Ca^{2+} oscillations (Fig. 6F). To ensure the impact of ZnPT abolishing Ca^{2+} oscillations was not an imaging artifact obscuring ongoing rises, we simultaneously monitored cytoplasmic and ER Ca^{2+} levels with Rhod-2 and D1ER, respectively. This approach allowed synchronously observing opposite Ca^{2+} changes in the two compartments. In uninjected eggs, the fluorescent values remained unchanged, and in *mPlc ζ* mRNA-injected eggs, the reporters' signals displayed the expected opposite changes (Fig. 6H, I). The addition of ZnPT rapidly increased Rhod-2 signals in uninjected and oscillating eggs, unlike D1ER's, suggesting it cannot detect changes in Zn^{2+} levels to this extent despite faithfully reporting Ca^{2+} changes. In oscillating eggs following ZnPT addition, D1ER progressively showed fewer and lower amplitude changes, consistent with the diminishing and eventual termination of the Ca^{2+} oscillations. Noteworthy, the basal fluorescent ratio of D1ER in these eggs remained unchanged after ZnPT, further demonstrating its unresponsiveness to Zn^{2+} changes of this magnitude. We confirmed that both reporters were still in working order, as the addition of Io triggered Ca^{2+} changes detected by both reporters (Fig. 6H, I).

Discussion

The present study demonstrates that appropriate levels of labile Zn^{2+} are essential for initiating and maintaining IP_3R1 -mediated Ca^{2+} oscillations in mouse eggs regardless of the initiating stimuli. Both deficient and excessive Zn^{2+} compromise IP_3R1 sensitivity, diminishing and mostly terminating Ca^{2+} oscillations. The results demonstrate that IP_3R1 and Zn^{2+} act in concert to modulate Ca^{2+} signals revealing previously unexplored crosstalk between these ions at fertilization (Fig. 7).

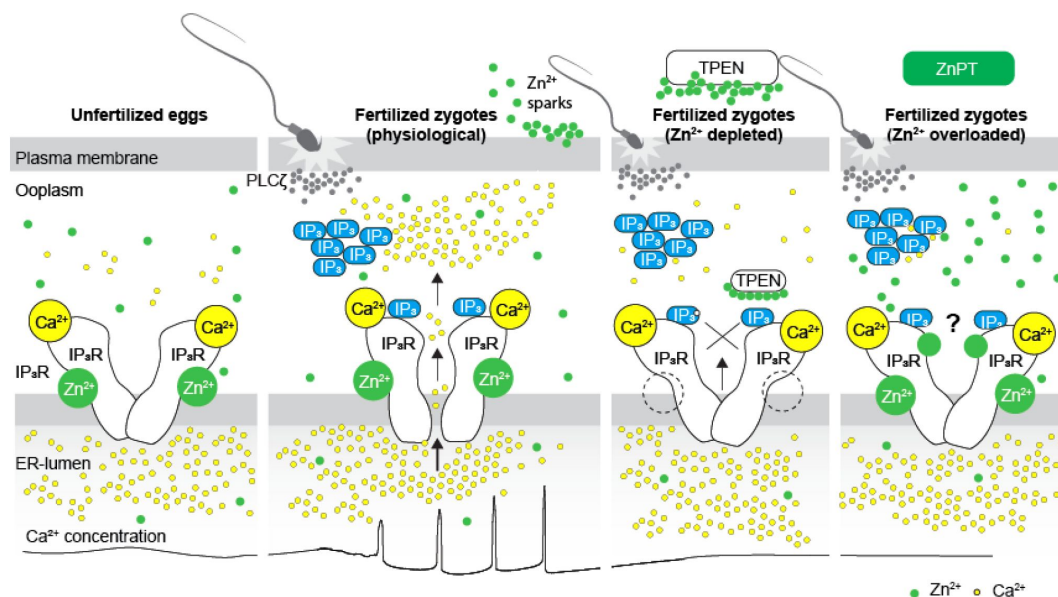


Figure 7.

Schematic of proposed regulation of IP_3R1 function by Zn^{2+} in eggs and fertilized zygotes.

In MII eggs, left panel, IP_3R1 s 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_3R1 . 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_3R1 function. Zn^{2+} deficiency caused by TPEN or other permeable Zn^{2+} chelators, right center panel, dose-dependently impairs IP_3R1 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_3R1 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_3R1 -mediate Ca^{2+} release, possibly by non-specific binding of thiol groups present in cysteine residues throughout the receptor (denoted by ?). We submit that optimal Ca^{2+} oscillations in mouse eggs unfold in the presence of a permissive range of Zn^{2+} concentration.

Zn^{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^{2+} chelators have uncovered what appears to be a cell-specific, narrow window of Zn^{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^{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^{2+} oscillations (Lawrence et al., 1998) or inducing egg activation of mouse eggs (Suzuki et al., 2010b). Our data demonstrating that $\sim 2.5 \mu\text{M}$ is the threshold concentration of TPEN in eggs that first causes noticeable changes in basal Zn^{2+} , as revealed by FluoZin, is consistent with the ~ 2 to $5 \mu\text{M}$ Zn^{2+} concentrations in most culture media without serum supplementation (Lo et al., 2020), and with the $\sim 100 \text{ pM}$ basal Zn^{2+} in cells (Qin et al., 2011). Lastly, the effects on Ca^{2+} release observed here with TPEN and other chelators were due to the chelation of Zn^{2+} , as pretreatment with ZnSO_4 but not with equal or greater concentrations of MgCl_2 or CaCl_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^{2+} deficiency inhibits Ca^{2+} release in eggs, we induced Ca^{2+} oscillations using various stimuli and tested the effectiveness of membrane-permeable and impermeable chelators to abrogate them. Chelation of extracellular Zn^{2+} failed to terminate the Ca^{2+} responses, whereas membrane-permeable chelators did, pointing to intracellular labile Zn^{2+} levels as essential for Ca^{2+} release. All agonists used here were susceptible to inhibition by TPEN, whether their activities depended on IP_3 production or allosterically induced receptor function, although the effective TPEN concentrations varied across stimuli. Some agents, such as *mPlcz* mRNA or thimerosal, required higher concentrations than SrCl_2 , Ach, or cIP_3 . The reason underlying the different agonists' sensitivities to TPEN will require additional research, but the persistence of IP_3 production or change in $\text{IP}_3\text{R1}$ 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 $\text{IP}_3\text{R1}$ models that signaling molecules can allosterically induce channel gating from different starting positions in the channel by mechanically coupling the binding effect to the ion-conducting pore in the C-terminal end of IP_3R (Fan et al., 2015). The cytosolic C-terminal domain of each $\text{IP}_3\text{R1}$ 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 $\text{IP}_3\text{R1}$ 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 $\text{IP}_3\text{R1}$ channel (Bhanumathy et al., 2012; Uchida et al., 2003). These results are consistent with the view that the C-terminal end of IP_3Rs 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

of IP₃R1 diminishes the leak, suggesting it occurs through IP₃R1 (Wakai and Fissore, 2019). Consistent with this view, TPEN pre-treatment delayed the Ca²⁺ response induced by Tg, implying that Zn²⁺ deficiency hinders Ca²⁺ release through IP₃R1. An expected consequence would be increased Ca²⁺ content in the ER after Tg. To that mobilizes Ca²⁺ independently of IP₃Rs (Toeplitz et al., 1979) induced enhanced responses in TPEN-treated eggs vs. controls, confirming the accumulation of Ca²⁺-ER in Zn²⁺ deficient conditions. We demonstrated that this accumulation is due to hindered emptying of the Ca²⁺ ER evoked by agonists in Zn²⁺-deficient environments resulting in reduced cytosolic Ca²⁺ increases, as IP₃R1 is the pivotal intermediary channel between these compartments. Noteworthy, the initial phase of the Tg-induced Ca²⁺ release out of the ER did not appear modified by TPEN, as if it was mediated by a Zn²⁺-insensitive Ca²⁺ channel(s)/transporter, contrasting with Ach-induced emptying that was abrogated from the outset. Remarkably, independently of Zn²⁺ chelators, emptying of Ca²⁺ ER was modified in a genetic model of Zn²⁺-deficient oocytes lacking two TRP channels, confirming the impact of Zn²⁺ on Ca²⁺ release. It is worth noting that TPEN did not reduce but increased the mass of IP₃R1, which might result in the inhibition of Zn²⁺-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 IP₃R1 degradation, thereby favoring the accumulation of IP₃R1.

Despite accruing Zn²⁺ during oocyte maturation, fertilization witnesses a necessary Zn²⁺ release into the external milieu, known as “Zn²⁺ sparks” (Kim et al., 2011; Que et al., 2019, 2015). This release of Zn²⁺ 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 Zn²⁺ 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 Zn²⁺ levels prevent egg activation (Kim et al., 2011), whereas lowering Zn²⁺ with chelators leads to egg activation without Ca²⁺ 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 Zn²⁺ (Kim et al., 2011, 2010). Here, we extend this concept and show that IP₃R1 function and the Ca²⁺ oscillations in mouse eggs require this optimal level of labile Zn²⁺ because the Ca²⁺ responses interrupted by TPEN-induced Zn²⁺-insufficiency are rescued by restoring Zn²⁺ levels with ZnPT. Furthermore, unopposed increases in Zn²⁺ by exposure to ZnPT abrogated fertilization-initiated Ca²⁺ oscillations and prevented the expected egg activation events. It is unclear how excess Zn²⁺ disturbs the function of IP₃R1. Nevertheless, IP₃R1s have multiple cysteines whose oxidation enhances the receptor sensitivity to IP₃ (Joseph et al., 2018), and it is possible that excessive Zn²⁺ aberrantly modifies them, disturbing IP₃R1 structure and function. These results reveal a close association between the Zn²⁺ levels controlling meiotic transitions and the Ca²⁺ release necessary for egg activation, placing the IP₃R1 at the center of the crosstalk of these two divalent cations.

Abrupt Zn²⁺ 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 Ca²⁺ rises and oscillations, and Zn²⁺ sparks are an egg activation event downstream of this Ca²⁺ 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 Ca²⁺ oscillations rely on optimized IP₃R1 function underpinned by ideal Zn²⁺ levels set during oocyte maturation. Future studies should explore if artificial alteration of Zn²⁺ levels can extend the fertile lifespan of eggs, improve developmental competence, or for developing non-hormonal methods of contraception.

Materials and Methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (<i>Mus musculus</i>)	CD1	Charles River	022	
Genetic reagent (<i>Mus musculus</i>)	C57BL/6J	JAX	JAX: 000664	
Genetic reagent (<i>Mus musculus</i>)	<i>Trpm7</i> -floxed	A generous gift from Dr. Carmen P. Williams (NIEHS) (PMID: 30322909)		C57BL/6J and 129s4/SvJae mixed background
Genetic reagent (<i>Mus musculus</i>)	<i>Gdf9-cre</i>	JAX	JAX: 011062	
Genetic reagent (<i>Mus musculus</i>)	<i>Trpv3</i> ^{-/-}	A generous gift from Dr H. Xu (PMID: 20403327)		C57BL/6J and 129/SvEv mixed background
Biological sample (mouse oocyte)	<i>Mus musculus</i>	this paper		Eggs at the metaphase of the second meiosis
Biological sample (mouse sperm)	<i>Mus musculus</i>	this paper		Matured sperm from cauda epididymis
Recombinant DNA reagent	pcDNA6-mouse <i>Plcz1-venus</i> (plasmid used as a template for mRNA synthesis)	Published in previous Fissore lab paper (PMID: 34313315. Mouse <i>Plcz1</i>		mouse <i>Plcz1</i> mRNA was fused with Venus and

		sequence was a generous gift from Dr. Kiyoko Fukami (PMID:18028898)		inserted in pcDNA6 vector
Recombinant DNA reagent	pcDNA6-CALR-D1ER-KDEL (plasmid used as a template for mRNA synthesis)	Published in previous Fissore lab paper PMID: 24101727. Original D1ER vector was a generous gift from Dr. Roger Y Tsien (PMID: 15585581)		FRET construct D1ER was inserted between ER-targeting sequence of calreticulin and KDEL ER retention signal in pcDNA6 vector
Recombinant DNA reagent	pcDNA6-human <i>ERp44-HA</i> (plasmid used as a template for mRNA synthesis)	This paper. Original human ERp44 sequence was a generous gift from Dr. Roberto Sitia (PMID: 11847130)		human <i>ERp44</i> mRNA fused with HA in pcDNA6/Myc-His B vector
Antibody	Monoclonal HA (Mouse monoclonal)	Roche	11581816001	Dilution: 1:200
Antibody	Polyclonal IP ₃ R1 (Rabbit polyclonal)	(Parys et al., 1995)		Dilution: 1:1000
Antibody	Monoclonal α -tubulin (Mouse monoclonal)	Sigma-Aldrich	T-9026	Dilution: 1:1000
Antibody	Alexa Fluor 488 (goat anti mouse)	Invitrogen	Invitrogen: A32723	Dilution: 1:400
Commercial assay or kit	T7 mMESSAGE mMACHINE Kit	Invitrogen	Invitrogen: AM1344	Used for <i>in vitro</i> mRNA synthesis
Commercial assay or kit	Poly(A) Tailing Kit	Invitrogen	Invitrogen: AM1350	Used for poly (A)

				tailing of synthesiz ed mRNA
Chemical compound, drug	Hyaluronidase from bovine testes	Sigma-Aldrich	H3506	
Chemical compound, drug	3-Isobutyl-1- methylxanthine (IBMX)	Sigma-Aldrich	I5879	
Chemical compound, drug	Polyvinylpyrrolidone (PVP) (average molecular weight: 360,000)	Sigma-Aldrich	PVP360	Used for mRNA microinje ction and ICSI
Chemical compound, drug	N,N, N',N'-Tetrakis (2- pyridylmethyl) ethylenediamine (TPEN)	Sigma-Aldrich	P4413	Prepared in DMSO and kept at -20 °C until use
Chemical compound, drug	Zinc Pyrithione (ZnPT)	Sigma-Aldrich	PHR1401	Prepared in DMSO and kept at -20 °C until use
Chemical compound, drug	Strontium chloride hexahydrate (SrCl ₂)	Sigma-Aldrich	255521	Freshly dissolved in water on the day of experime nt
Chemical compound, drug	Calcium chloride dihydrate (CaCl ₂)	Sigma-Aldrich	C3881	Freshly dissolved in water on the day of experime nt
Chemical compound, drug	Magnesium chloride hexahydrate (MgCl ₂)	Sigma-Aldrich	M2393	Freshly dissolved in water on the day of experime nt
Chemical compound, drug	Zinc sulfate monohydrate (ZnSO ₄)	Acros Organics	389802500	Freshly dissolved

drug				in water on the day of experiment
Chemical compound, drug	Ethylenediaminetetraacetic acid sodium dihydrate (EDTA)	LabChem	LC137501	Prepared as 0.5M aqueous solution with pH 8.0 adjusted by NaOH
Chemical compound, drug	Diethylenetriaminepentaacetic acid (DTPA)	Sigma-Aldrich	D6518	
Chemical compound, drug	Tris (2-pyridylmethyl) amine (TPA)	Santa Cruz	sc-477037	
Chemical compound, drug	Dimethyl sulphoxide (DMSO)	Sigma-Aldrich	D8418	Used as a solvent
Chemical compound, drug	Acetylcholine chloride	Sigma-Aldrich	A6625	
Chemical compound, drug	Thimerosal	Sigma-Aldrich	T5125	Freshly dissolved in water on the day of experiment and kept on ice until use
Chemical compound, drug	Ionomycin calcium salt	Tocris	1704	Working concentration: 2.5 μ M
Chemical compound, drug	Thapsigargin	Calbiochem	#586500	Working concentration: 10 μ M
Other	Pluronic F-127 (20% solution in DMSO) (Pluronic acid)	Invitrogen	P3000MP	
Other	Fura-2 AM	Invitrogen	F1221	Used at

				1.25 μ M in TL- HEPES containin g 0.02% Pluronic acid
Other	FluoZin-3 AM	Invitrogen	F24195	Used at 1.25 μ M in TL- HEPES containin g 0.02% Pluronic acid
Other	Fluo-4 AM	Invitrogen	F14201	Used at 1.25 μ M in TL- HEPES containin g 0.02% Pluronic acid
Other	Rhod2-AM	Invitrogen	R1244	Used at 2.2 μ M in TL- HEPES containin g 0.02% Pluronic acid.
Other	ci-IP3/ PM	Tocris	6210	Dissolved in DMSO and kept at -20°C . Before use, the stock was diluted with water to make a final concentra tion of 0.25 mM.
Other	Pme1	New England	R0560S	Used to

		BioLabs	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. SrCl_2 , CaCl_2 , ZnSO_4 , and MgCl_2 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. Acetylcholine chloride and Thimerosal were dissolved in double-sterile water at 550 mM and 100 mM, respectively. Acetylcholine 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*-floxed (*Trpm7^{fl/fl}*) *Gdf9-Cre* and *Trpv3^{-/-}* mice were bred at our facility. *Trpm7^{fl/fl}* mice were crossed with *Trpv3^{-/-}* to generate *Trpm7^{fl/fl}; Trpv3^{-/-}* mouse line. Female *Trpm7^{fl/fl}; Trpv3^{-/-}* mice were crossed with *Trpm7^{fl/fl}; Trpv3^{-/-}; 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 Ca^{2+}

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-m*Plc*ζ-*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, m*Plc*ζ, 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⁺ imaging

Before Ca²⁺ imaging, eggs were incubated in TL-HEPES containing 1.25 µM Fura2-AM, 1.25 µM FluoZin3-AM, or 2.2 µM 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 Ca²⁺-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 Ca²⁺-ER and cytosolic Ca²⁺ simultaneously, eggs that had been injected with D1ER were loaded with Rhod-2AM, and CFP, YFP, and Rhod-2 intensities were collected every 20 seconds.

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 µM 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 (Millipore, Bedford, MA). After blocking with 5% fat-free milk + TBS, membranes were probed with the rabbit polyclonal antibody specific to $\text{IP}_3\text{R1}$ (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- β -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) TritonX-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 $\times$, 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 $\pm$ s.d. Differences were considered significant at $P < 0.05$.

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Competing interests

The authors declare no competing or financial interests.

Additional information

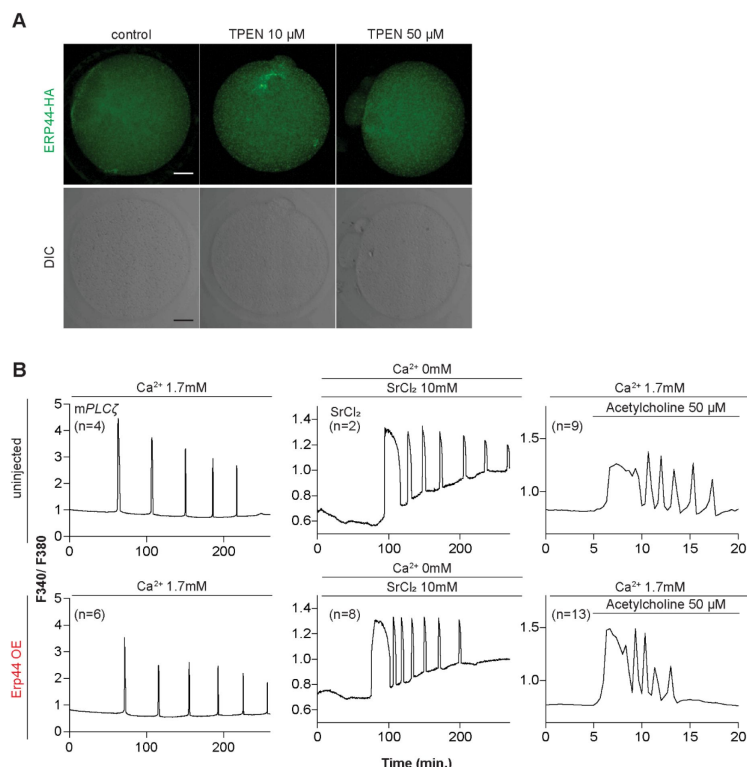
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Author contributions

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

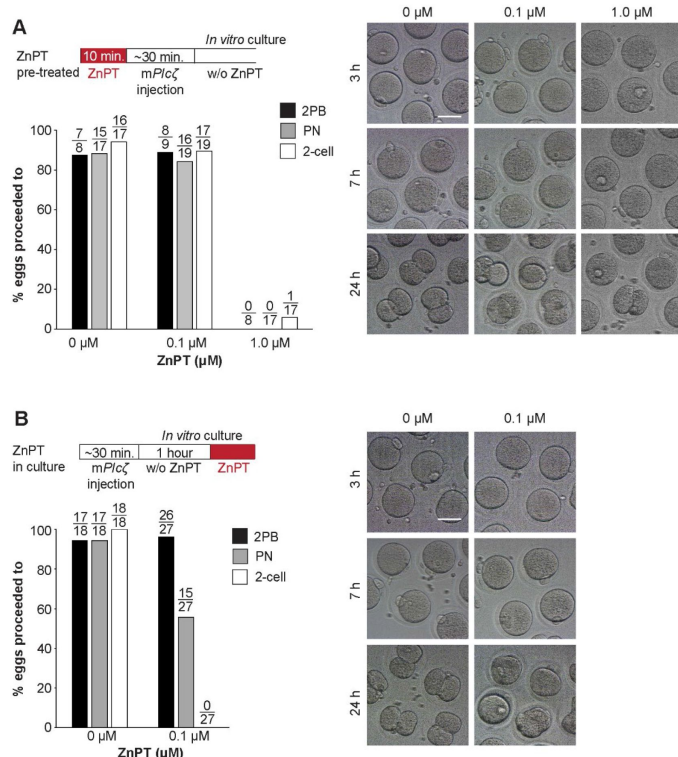
Figures and Legends



Supplementary Figure 1.

Overexpression of ER accessory protein ERp44 did not change the Ca²⁺ responses initiated by mPlcζ mRNA microinjection, Acetylcholine, or SrCl₂.

(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 Ca²⁺ responses induced by mPlcζ mRNA microinjection (0.01 μg/ μl-left column), SrCl₂ (10 mM-center column), and acetylcholine (50 μM-right column) in eggs with (top panels) or without (bottom panels) ERp44 overexpression.



Supplementary Figure 2.

Elevated Zn²⁺ 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 24 hr. PN formation and 2-cell development were checked at 7- and 24-hr 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 24 hr. The second polar body extrusion, PN formation, and 2-cell development were checked at 2.5-, 7- and 24-hr. post-microinjection. Bars represent the percentages of injected eggs that reached the PN and the 2-cell stage. Scale bar: 50 μm.

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Reviewer #1 (Public Review):

The study utilizes a variety of methods, chemical and expressed probes, caged release of IP3, as well as oocytes with mutations that alter zinc availability, that provide an elegant

examination of how zinc deficiency and zinc excess modulate the transient and cyclic release of calcium during egg activation. In this manuscript, the authors sought to determine if there is any interplay between zinc and calcium, two divalent cations that have been demonstrated to have important roles during fertilization. They employ agents that disrupt normal zinc homeostasis and then monitor the resulting calcium oscillations during egg activation. If zinc was made unavailable via chelation with TPEN, then the calcium oscillations halted. This occurred regardless of the activation method, which included ICSI, PLC ζ , Acetylcholine, strontium chloride, and thimerosal. This phenotype could be rescued by introducing zinc back into the egg via an ionophore, such as zinc pyrithione; however, too much zinc pyrithione also halted calcium oscillations. Taken together, these two results demonstrate that there is a threshold level of zinc that is required for proper calcium oscillations to occur.

Furthermore, the authors sought to understand how zinc affects the IP3 receptor, IP3R1. IP3R1 is the receptor that modulates the release of calcium from the endoplasmic reticulum. The authors cited a previous study that identified zinc binding sites on IP3R1. The authors highlight that there exist no studies regarding the regulation of IP3R1 by zinc; however, such studies were cited for a similar calcium channel, the RyRs. The authors use thapsigargin to inhibit the SERCA pump, leading to calcium leak from the IP3R1. TPEN blunted the amount of calcium leaked from the ER following treatment, suggesting that zinc occupancy is necessary for IP3R1 function.

The results of these experiments support the authors conclusions that zinc is essential for the IP3R1-mediated release of calcium in an oscillatory manner during egg activation. These results provide further insight into signals necessary for proper egg activation and the ultimate success of the resulting embryo.

Reviewer #2 (Public Review):

The manuscript describes more fully the relationship between zinc fluxes and calcium oscillations during egg activation by directly manipulating the level of zinc ions inside and outside the cell with chelators and ionophores and then measuring resulting changes in Ca⁺⁺ oscillations. The authors have provided solid evidence consistent with their hypothesis that zinc ions regulate Ca⁺⁺ oscillations by directly modulating the gating of the IP3-R which is the main calcium channel responsible for calcium release into the cytoplasm. The authors employ well established methods of calcium measurement along with various chelators, ionophores and a wide variety of methods that cause egg activation to demonstrate that an optimal level of zinc ions are required for Ca⁺⁺ oscillations to occur.

Helpfully, the authors provide a model to explain their observations in Figure 7. In the model, the increase in zinc during maturation is permissive for later IP3-R gating in response to IP3 generated at fertilization. The experiments with TPEN solidly demonstrate that Zn is required because lowering free zinc, as indicated by FluoZin staining), abrogates Ca⁺⁺ oscillations. This is true regardless of the method of activation. What is not clearly described in the model or in the manuscript is whether the levels of zinc at MII are simply permissive for IP3-R gating or whether there is a more acute relationship between zinc fluxes and Ca⁺⁺ oscillations. In the original paper describing the zinc spark (Kim et al., ACS Chem Biol 6:716-723), the authors show that zinc efflux very closely mirrors Ca⁺⁺ oscillations suggesting a more active relationship such that zinc efflux associate with each calcium spike could be necessary for terminating the Ca spike by depleting cytoplasmic Zn. There is some evidence in the present manuscript to support this. For example, in figure 3B, TPEN appears to acutely terminate a Ca spike. Whether this is repeatable is not known. Conversely, in Figure 5C and 5E, PyT leads to a rapid restoration of Ca oscillations within minutes

demonstrating that changes in free Zn can cause rapid changes in Ca^{++} oscillations. Perhaps, rather than simply a permissive role, the normal Zn fluxes during activation may be acutely changing IP3-R gating sensitivity.

The role of TRPV3 and Trpm7 in Zn homeostasis during egg activation seems to be minor and the results in the dKO oocytes compared to TPEN are a bit confusing. In the dKO oocytes, zinc acquisition was sufficient to make it to MII suggesting Zn transport through these channels is dispensable for maturation. During activation, however, the response to Tg in dKO eggs was opposite that of TPEN, higher cytosolic Ca and increase amplitude (Figure 4G) vs lower cytoplasmic Ca and frequency for TPEN (Figure 4A). Perhaps loss of these two channels changes Ca gating independent of Zinc.

The effect of PyT on the apparent rise in cytoplasmic Ca^{++} in figure 6 is interpreted as caused by an artifact of high Zn concentrations. However, it is not clear that 0.05 μM PyT would necessarily increase cytoplasmic Zn to the point where FURA-2 fluorescence would increase. A simpler interpretation is that PyT allows sufficient Zn to enter the cell and keeps the IP3-R channels open causing a sustained rise in cytoplasmic Ca and preventing oscillations in Ca^{++} . This interpretation would also preclude inhibitory effects of high Zn concentrations as shown in figure 7 which may or may not be present but are simply speculation.

Overall, this study significantly advances our understanding of egg activation and could lead to better ways of in vitro egg activation in women undergoing assisted reproduction.

Reviewer #3 (Public Review):

This study investigated the role of Zn^{2+} on the maintenance of Ca^{2+} oscillation upon fertilization. TPEN was used to reduce the level of available Zn^{2+} in fertilized oocytes and different inhibitors were used to pinpoint the mechanistic involvement of intracellular Zn^{2+} on the maintenance of Ca^{2+} oscillation. As also stated in the manuscript, previous studies have demonstrated the role of Zn^{2+} for the successful completion of meiosis/fertilization. However, the mechanistic actions of Zn^{2+} on the hallmark of fertilization processes such as Ca^{2+} oscillation has not been elucidated. A previous publication used TPEN to cease Ca^{2+} oscillation, but the study was not focused on the involvement of Zn^{2+} signal. The manuscript expands our understanding of fertilization process by describing how the level of Zn^{2+} regulates Ca^{2+} channels and stores. The manuscript is well-organized and the topic is important in early embryo development fields.