Zinc sparks are triggered by fertilization and facilitate cell cycle resumption in mammalian eggs

Alison M. Kim[1,2], Miranda L. Bernhardt[1], Betty Y. Kong[1], Richard W. Ahn[2,3], Stefan Vogt[4], Teresa K. Woodruff[1,2,5]*, Thomas V. O'Halloran[2,3,5]*

[1]Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA, [2]The Chemistry of Life Processes Institute, [3]Department of Chemistry, Northwestern University, Evanston, IL 60208, USA, [4]X-ray Science Division, Argonne National Laboratory, Argonne, IL 60439, [5]Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208, USA.

*To whom correspondence should be addressed. Email: tkw@northwestern.edu and t-ohalloran@northwestern.edu

SUPPORTING MATERIALS

Supplemental Figures

Figure S1. Zinc sparks are an evolutionarily conserved event during egg activation. Zinc sparks were observed in eggs from two different non-human primate species, *Macaca mulatta* (**a**, **b**) and *Macaca fascicularis* (**c**, **d**). In both cases, a single calcium transient was induced by ionomycin. Intracellular calcium was monitored with Calcium Green-1 AM, and extracellular zinc with FluoZin-3. The first panel begins at 00:04:35 in **c** and 00:06:37 in **d**. Each subsequent panel represents an image acquired 4 s following the previous panel. Time is expressed as hh:mm:ss, wherein 00:00:00 represents the start of image acquisition. Scale bar = 40 μm in **c** and **d**.

Figure S2. Zinc sparks do not occur in the absence of calcium transients. The membrane permeable derivative of FluoZin-3 (FluoZin-3 AM) did not detect intracellular oscillations in

zinc (**a**), suggesting that those detected by Calcium Green-1 AM were specific to calcium. Insets are brightfield images taken at the beginning (left, first polar body denoted as PB1) and end (right) of the acquisition period. In each case, the presence of the second polar body (PB2) at the conclusion of imaging was observed, confirming successful egg activation and development. Zinc sparks were absent in the absence of an activating agent such as strontium chloride (**b**), or in eggs treated with the calcium-selective chelator BAPTA AM (**c**). Representative images illustrate that BAPTA AM-treated eggs neither extrude a second polar body (**b**, 2 hrs post-activation, or hpf) or form a pronucleus (**c**, 6 hpf). Intracellular calcium was detected by Calcium Green-1 AM (black line) and extracellular zinc was detected by FluoZin-3 (green line) in **b** and **c**. Scale bar = 75 μm in **d** and **e**.

Figure S3. Total zinc content of the egg gradually decreases upon fertilization. Eggs and embryos were analyzed by XFM for their transition metal content. The mean zinc quota was highest in the *in vivo* ovulated (IVO) MII egg and trended towards a decrease following fertilization (see 2 and 6 hpf), reaching its lowest mean abundance in the two-cell embryo. The iron and copper quotas were an order of magnitude less than zinc at all timepoints examined. Red lines indicate mean values and symbols indicate individual replicates for the corresponding timepoint. Asterisks indicate previously published data[S1], which was included with permission for clarity.

Figure S4. Intracellular zinc decreases in the activated egg. Corresponding to the onset of the zinc sparks (FluoZin-3, green line), there is a gradual decrease in intracellular labile zinc that is detectable with a membrane-permeable zinc fluorophore (FluoZin-3 AM, black line).

Successful egg activation and development was confirmed using simultaneous brightfield imaging, which revealed extrusion of a second polar body (PB2). The first (0 min, left) and final (30 min, right) brightfield images are shown.

Figure S5. Confirmation of the cortical localization of zinc-enriched compartments. Optical sections from areas near the meiotic spindle (as detected by DNA markers Syto 64 and Hoechst 33342) were selected from the complete confocal Z-series and projected to show the cortical localization of zinc-enriched vesicles as detected by two independent fluorophores, zinquin ethyl ester and FluoZin-3 AM. Merged images are also shown for clarity. We note that the zinc-enriched compartments detected by these two fluorophores, while preferentially localized to the cortex, differ in the extent of cytoplasmic localization and size. These differences likely arise from two factors: a) the chemistry of the AM ester function and b) the weaker fluorescence intensity of the zinquin probe. It is clear that AM esters must undergo cleavage via intracellular esterases before they can bind the target ion. Cleavage of one mole of FluoZin-3 releases three moles of formaldehyde and acetic acid. As with the widely used calcium probes including Fura-2, this can lead to a number of well-known artifacts including changes in intravesicular pH and vesicular appearance. Probes in the zinquin family do not need to undergo hydrolysis before binding their target ion and are membrane permeable until they bind zinc; however, zinquin is a significantly less sensitive fluorphore than FluoZin-3. The brightness factor for Zn-FluoZin-3 (i.e., the product of the extinction coefficient times the quantum yield, or 35×10^3) is 22-times higher than that of Zn-Zinquin (1.6×10^3). Given these trade-offs, it is rare to see exactly the same image with two probes for the same metal ion; however, in general the distribution of zinquin and FluoZin-3 AM fluorescence appear to

corroborate. These images correspond to those shown as full projections in Fig. 3. Scale bar = 25 μm.

Figure S6. Zinc sparks occur through an exocytic mechanism. Exocytosis was inhibited by treating eggs with cytochalasin B prior to activation with strontium chloride. Cytochalasin B did not affect the calcium oscillations (**a**) but blocked all but the first zinc spark in most cases (**b**). Black line represents Calcium Green-1 AM (intracellular calcium) fluorescence; green line represents FluoZin-3 (extracellular zinc) fluorescence.

Figure S7. Dose response of unfertilized eggs to increasing concentrations of zinc pyrithione (ZnPT). Unfertilized eggs were treated with 10, 20, or 50 μM ZnPT for 15 min. A clear cytotoxic phenotype was only seen at 50 μM concentration. Thus, the effects of pyrithione that we reported in Fig. 4 are significantly below the threshold concentration (i.e., 500% lower) and do not involve cytotoxicity, as pyrithione was used at a minimum concentration (10 μM) and for a shorter duration (10 min). Scale bar = 80 μm.

Figure S8. Perturbation of intracellular zinc availability causes activation of the egg. Following 8 hours of culture, control eggs maintain a metaphase II spindle (**a**, sp) with individual chromosomes aligned at the metaphase plate (**b**). In contrast, eggs exposed to 10 μM TPEN for the same period artificially activate as indicated by the formation of an intact pronucleus (**c**, pn) with decondensed chromatin surrounding a nucleolus (**d**). The number of eggs displaying a pronucleus is significantly higher in the TPEN-treated group (**e**). We confirmed that 10 μM TPEN was a nontoxic concentration in a previous report[S1]. Furthermore, others have exposed

mouse eggs to much higher concentrations (100 μM) and achieved live mouse births[S2], which could not have occurred if TPEN was cytotoxic to the cell.

Supplemental Videos

Video S1. Eggs release zinc into the extracellular environment through short but intense "sparks" upon activation. Changes in extracellular zinc were monitored with FluoZin-3 during parthenogenesis of mouse eggs. These zinc sparks occur in response to an activating stimulus (such as strontium chloride, used here) and the pattern of zinc release is unique to each egg. Note the polarized distribution of zinc release. The video is excerpted from a longer time-lapse series.

Video S2. Zinc sparks are an evolutionarily conserved phenomenon and occur in higher mammals such as non-human primates. Activated non-human primate eggs exocytose zinc through the zinc sparks upon the induction of a calcium transient. *Macaca fascicularis* is shown here as a representative example. The video is excerpted from a longer time-lapse series; the imaging time is shown in the upper left corner. Scale bar = 100 μm.

Video S3. The exocytosis of zinc is immediately preceded by an intracellular calcium transient in activated mouse eggs. Mouse eggs were monitored for changes in intracellular calcium and extracellular zinc upon activation with strontium chloride. Following a series of calcium transients (detected by Calcium Green-1 AM), the zinc sparks initiate and continue asynchronously in each egg throughout the imaging period. The video is excerpted from a longer time-lapse series.

Video S4. Zinc insufficient eggs are unable to initiate the zinc sparks. Eggs matured *in vitro* in the absence (C) or presence (T) of the heavy metal chelator TPEN were pooled and simultaneously activated with strontium chloride. While control (C) eggs initiated zinc sparks shortly after activation, zinc-insufficient eggs (T) did not exhibit any zinc sparks throughout the imaging period. The video is excerpted from a longer time-lapse series; the imaging time is shown in the upper left corner. Scale bar = 50 μm.

Supplementary References

S1. Kim, A. M., Vogt, S., O'Halloran, T. V., and Woodruff, T. K. (2010) Zinc availability regulates exit from meiosis in maturing mammalian oocytes. *Nat. Chem. Biol. 6*, 674-681.

S2. Suzuki, T., Yoshida, N., Suzuki, E., Okuda, E., Perry, A.C. (2010). Full-term mouse development by abolishing Zn^{2+}-dependent metaphase II arrest without Ca^{2+} release. *Development 137*, 2659-2669.