Supplemental Information

Metal Ion Fluxes Controlling Amphibian Fertilization

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Materials and Methods:

Confocal Imaging Parameters: All confocal imaging was done on a Leica SP5 laser scanning confocal using the Leica Application Suite AF software in the Biological Imaging Facility at Northwestern University (RRID: SCR_017767). Images were taken with a 2.5x0.07 NA objective with an open pinhole (600 μm = 7.91 airy units). The excitation laser used was an argon laser (488 nm) coupled with a DD 488/561 beamsplitter. A Leica Hybrid (HyD) detector was set to collect emission wavelengths of 505-575 nm, using gain of 100%. Transmitted light images were taken with a transmitted light PMT using the 488 nm laser as an illumination source. For the fertilization timeseries, images were taken every 2.573 seconds for a total time of 19 minutes. Using FIJI, brightness/contrast were adjusted to 12/72. For the ionomycin timeseries, images were taken every 1.29 seconds for a total time of 9.5 minutes. Using FIJI, brightness/contrast were adjusted to 35/131. For clarity, brightness and contrast were adjusted to 19/46 in Supplemental Figure 1A.

Determination of the Rate of the Zinc Spark: The stack of confocal images of the zinc spark was analyzed using ImageJ.\(^1\) The egg was modeled as a sphere. Using a custom macro, 18 areas (circles with radii of 50 μM) were placed around the periphery of the egg. The circles were placed 20° apart, with 0° at the approximate beginning of the zinc release (Supplemental Figure 1A). The software measured the average white value (i.e. fluorescence) of each circle in every frame. The data were then graphed (Supplemental Figure 1B and 1C). The zinc spark travels approximately linearly in time around most of the circumference of the egg, the linear portions were analyzed.

The circumference of the egg was calculated from its diameter and every degree on the circle could therefore be given a distance. The time at which each circle reached half-maximal fluorescence was determined. To determine the rate of zinc release, the distance of each circle along the circumference from the origin was plotted against the time at which it reached half-maximal fluorescence. A linear fit was then taken and the calculated velocities from the zinc release traveling clockwise and counterclockwise from the origin were averaged for a final value.

Evaluating the Ability of Intracellular Chelators to Induce Parthenogenetic Activation of Xenopus Eggs: Xenopus eggs were obtained via established methods. The jelly coat of the eggs was removed through soaking in a 3% w/v solution of cysteine-HCl, pH 8 for 10 minutes. ~20-30 dejellied eggs were put into a well of a 12-well dish. A 20-minute video was taken with an Olympus SZX12 microscope using a Q-Color 3 Camera. To quantify the data, each individual egg was followed over the course of the video and assigned one of three conditions: activated, not activated, or dead. None of the following treatment conditions showed signs of toxicity. Activated eggs were those which showed shrinkage/darkening of the pigment in their animal pole and shrinkage of the white activation spot. The ratio of activated eggs to activated + not activated was then calculated. Eggs tested in all four conditions came from the same frog. For replication, eggs from 3 frogs were tested. The ratios from the 3 frogs were averaged and plotted with standard deviations.

Four milliliters of standard media for Xenopus eggs (0.1X Marc’s Modified Ringer’s [MMR] buffer, – 10 mM NaCl, 200 μM KCl, 100 μM MgSO\(_4\), 200 μM CaCl\(_2\), 500 μM HEPES, 10 μM EDTA, pH 7.4) were placed in the wells of a 12-well dish along with the following:

1. 1% DMSO (control)
2. 10 mM 1,10-phenanthroline monohydrate (Sigma) in 1% DMSO
3. 20 μM ionomycin calcium salt (Sigma) dissolved in 1% DMSO
4. 10 mM ammonium tetrathiomolybdate (Sigma) in 1% DMSO

Egg activation was scored for the untreated group (buffer alone) as a negative control and eggs treated with 20 μM ionomycin as a positive control (Supplemental Figure 2, Videos 3-6). Ionomycin led to complete activation. Treatment with 10 mM 1,10-phenanthroline (OP - KD Mn [II] = 5 x 10^{-11} M, KD Fe [II] = 10^{-21} M, KD Cu [II] = 1.0 x 10^{-21} M, KD Zn [II] = 8 x 10^{-18} M)^{2,3} activates the same percentage of eggs as ionomycin (within a standard deviation). To test whether OP causes activation through copper chelation, we treated the eggs with 10 mM ammonium tetrathiomolybdate (TTM), which has a high selectivity and specificity for copper (KD Cu [I] = 2.32 x 10^{-20} M)^{4} TTM did not activate the eggs, suggesting that fertilization is linked to zinc depletion.

Treatment of eggs with 3 mM of N,N,N’,N’-tetrakis -(2-pyridylmethyl)-ethylenediamine (TPEN), a heavy metal chelator (KD Cu [II] = 10^{-20}, KD Zn [II] = 10^{-16})^{5} did not lead to activation. Due to its low aqueous solubility and modest DMSO solubility (25 mM) we were not able to test significantly higher concentrations of TPEN. EtOH at a concentration as low as 2% can parthenogenically activate eggs, so it could not be used as a solvent.

**Inductively Coupled Plasma Mass Spectrometry (ICP-MS):** Dejellied *Xenopus* eggs were prepared as described above. The eggs were then rinsed in 0.1X MMR. To prepare samples for ICP-MS, 20 eggs were placed in a metal-free conical tube (#89049-170, VWR, Radnor, PA) with a transfer pipet and rinsed 3X with 15 ml MilliQ H2O. 20 eggs per tube were analyzed in order to be within the standard curve and improve the signal-to-noise for copper and manganese, which are the elements with the lowest measured concentrations.

Embryos were obtained through mincing ¼ testis in a small amount of 0.1X MMR. The solution was mixed with eggs in a Petri dish and allowed to sit for 10 minutes. The dish was then flooded with 0.1X MMR. After 30 minutes, the jelly was removed from the embryos and samples were prepared as above.

Following sample collection, the eggs/embryos were dried in a heat block at 70 C for at least 5 hours. They were then dissolved in 450 μl 67-70% trace-metal free nitric acid (#NX0407, Supelco, Burlington, MA) overnight at 70 C. The solution was then diluted to 15 ml using MilliQ H2O. ICP elemental standards were obtained from Inorganic Ventures (Christiansburg, Virginia). The elemental concentrations were measured using a Thermo iCAP Q ICP-MS and a Thermo iCAP 7600 ICP-OES with Qtogra software (Quantitative Bio-element Imaging Center, Northwestern University). Eggs and embryos from 4 frogs were analyzed, and for each frog 3-5 tubes of eggs/embryos were measured. In order to calculate the molarity of the eggs/embryos, their average volume was assumed to be 1 μl.\(^6\) Two-tailed, heteroscedastic T-tests were run between eggs and embryos in order to determine if there was a significant difference in metal content. No adjustments were made for multiple comparisons.

In order to determine the metal content of the frog tank water, HNO\(_3\) was added to the water in metal-free tubes to obtain a final concentration of 2%. In order to determine the concentrations of highly concentrated elements, dilutions of the samples were made with MilliQ H\(_2\)O/HNO\(_3\). Elemental concentrations were measured using a Thermo iCAP Q ICP-MS and a Thermo Element2 High Resolution Magnetic Sector Field ICP-MS (Quantitative Bio-element Imaging Center, Northwestern University).
**EPR/ENDOR/ESEEM Spectroscopy:** Frog eggs and embryos were loaded into a quartz EPR sample tube, flash-frozen in liquid nitrogen, and stored at 77 K until analysis.

**EPR Spectroscopy:** 35 GHz continuous-wave (CW) EPR spectra were recorded using a lab-built 35 GHz EPR spectrometer with LabVIEW 2016.7 Absorption-display EPR spectra were collected from eggs/embryos and Mn-metabolites by using CW “rapid passage” methods at 34.9 GHz and 2 K as previously described.8,9 The spectra of Mn2+ are characteristic of an S = 5/2 ion with small zero-field splitting (ZFS), with the principal ZFS parameter, D, much less than the microwave quantum (hν). Such spectra show a central 55Mn (I = 5/2) sextet arising from hyperfine interactions, A ∼ 90 G, that is associated with transitions between the ms = ±1/2 and ±1/2 electron-spin substrates. These features “ride on” and are flanked by significantly broader signals from the four “satellite” transitions involving the other electron-spin substates (ms ±5/2 ⇔ ±3/2; ±3/2 ⇔ ±1/2). The net absorption spectrum is the sum of the five envelopes of these five transitions among substrates.

The Mn2+ EPR spectra of both frozen eggs and embryos show a resolved six-line 55Mn hyperfine pattern centered at g-2 (~12 kG) riding on “wings” extending to both high and low magnetic field with a total field span of 4 kG, features which are suppressed in the derivative-mode CW EPR spectra. The wings are relatively narrow in the high-symmetry Mn complexes discussed here (Figure 3); broad wings are seen in low-symmetry complexes with chelating ligands and proteins. The sextet is accentuated in the derivative spectra similar to those in the commonly used “derivative-mode” EPR spectra (Figure 3A inset); however, the full absorption-display spectra show this central feature.

EPR experiments were performed on three batches of egg and embryo samples. The shapes of the EPR spectra of eggs and embryos are the same (Supplemental Figure 3) so the amplitude of their EPR spectra is an appropriate indicator of total Mn2+. The embryos contain on average about one-third the amount of Mn2+ of the eggs (~30 ± 10% remaining) across the three batches of samples. As noted in the main text, the decrease in the Mn2+ EPR amplitude upon fertilization agrees with the loss of Mn2+ measured by ICP-MS (Figure 2) within the mutual uncertainties of the two measurements.

**ENDOR/ESEEM Spectroscopies:** Pulsed ENDOR/ESEEM spectra were recorded using a lab-built 35 GHz pulsed EPR spectrometer with Femi Instruments SpecMan4EPR.10 All spectra were recorded at 2 K using an immersion helium cryostat. 31P, 1H Davies ENDOR spectra were recorded using the pulse sequence π − T − π/2 − τ − π − τ − echo, where T is the time interval for the radio-frequency (RF) pulse, which is randomly hopped.11

The frozen-solution spectrum of an 1 =1/2 nucleus, such as 31P and 1H, coupled to Mn2+ comprises a set of doublet features centered at the nuclear larmor frequency and split by multiples of the electron-nuclear hyperfine coupling (A). The primary doublet is associated with the ms = ±1/2 electron spin sublevels of Mn2+ and is split by A; weaker satellite doublets associated with the ms = ±3/2 and ±5/2 sublevels are split by 3A and 5A. All spectra in this study display 1H signals that can be assigned to the protons of bound water. In addition, all the spectra, except for the aqueous solution, show a sharp ms = ±1/2 31P doublet from a phosphate moiety bound to Mn2+ center. The intensities of 31P and 1H signals differ significantly among the spectra, and analysis of these intensities provide a means of assessing cellular Mn2+ speciation.12 ENDOR experiments were performed using the following conditions: MW frequency 34.8 GHz, temperature 2K, magnetic field ~ 12.5 kG, tπ/2 = 60 ns, τ = 400 ns, Trf = 160 µs, repetition time 10 ms.
3-Pulse ESEEM spectra were recorded using the pulse sequence, $\pi/2 - \tau - \pi/2 - T - \pi/2 - \tau$ – echo where T is the time varied between second and third microwave pulses, with four-step phase cycling to suppress unwanted Hahn and refocused echoes. A $^{14}$N nucleus (I = 1) directly coordinated with Mn$^{2+}$ creates modulation in the electron spin echo decay, which is dominated by $^{14}$N hyperfine interaction. To quantitate $^{14}$N ESEEM responses from cellular Mn$^{2+}$, we chose as a standard the $^{14}$N response from the Mn-imidazole complex, which binds one imidazole and (presumably) five waters. ESEEM experiments were performed using the following conditions: MW frequency 34.9 GHz, temperature 2K, magnetic field ~ 12.5 kG, $t_{\pi/2} = 50$ ns, $\tau = 400$ ns, repetition time 10 ms.

**Speciation Calculations:** Experiments were performed on three sets of samples and the average $^{31}$P%, $^1$H% were used to calculate the fractional Mn$^{2+}$ speciation. The $^{31}$P%, $^1$H% ENDOR values vary somewhat among the triplicate samples and to make sure that this variation is not attributed to spectrometric variability, ENDOR spectra of Mn-Pi were recorded along with frog eggs/embryos in every case (Figure 3B) in every sample measurement, confirming that small yet significant variation in $^{31}$P%, $^1$H% ENDOR response for frog eggs/embryos across the triplicate experiments is independent of the experimental conditions and purely a physiological issue. The fractional populations of Mn-L, L = H$_2$O, Pi, polyP, and ENDOR-silent complexes reported as percentages were obtained using a heuristic binding model as described.

**Frozen Eggs/Lysate Controls to Test for Possible Freeze-Lysis Artifacts:** By conducting EPR/ENDOR studies on frozen intact eggs, we are able to circumvent a hurdle in understanding Mn$^{2+}$ speciation inside cells. When cells are lysed, any substitutionally-labile Mn$^{2+}$ complexes are at risk of undergoing rapid ligand exchange reactions. In such cases the spectra of Mn$^{2+}$ species in the lysate may not reflect the chemical state of the manganese complexes present in intracellular compartments. The same problem can arise upon rapid freezing of large cells such as frog eggs: ice crystal formation can inadvertently rupture and mix the contents of the intracellular compartments. We have directly examined this premise by “scrambling” the eggs, gently rupturing them by ~30 seconds agitation with a Vortex™ mixer, which disrupted egg membranes and created a liquid suspension. It was anticipated that even such a mild treatment would expose Mn$^{2+}$ sequestered in the vicinity of the plasma membrane to millimolar concentrations of small molecule ligands in the cytosol such as cysteine, histidine, NADP, NTPs, etc. As shown in Supplemental Figure 3, this expectation is realized. In comparison to intact eggs, ENDOR spectra of crushed eggs show three-time higher $^{31}$P% and two-time higher $^1$H% response. This corresponds to an increase of the Mn-P population to ~20% and reduction of the ENDOR-silent population to ~70%, with the remainder present as aquo-Mn$^{2+}$. To test whether these Mn-P species arise from complex ion formation with buffer constituents, $^{31}$P% ENDOR spectra were acquired on a frozen solution of 0.1X MMR spiked with 200 μM MnSO$_4$·H$_2$O. No Mn-P species were observed, leading us to conclude that the lysis-induced emergence of a significant population of Mn-P complexes likely arise from components of the egg cytoplasm, organelles etc. These controls suggest that freezing the eggs did not disturb the vesicular compartmentalization of Mn$^{2+}$ and provide support the conclusion that the most abundant manganese species in the cortical granules are Mn$^{2+}$-carboxylate complexes.

**Sulfide Fixation of Eggs and Embryos:** To chemically fix the eggs/embryos, the supernatant was removed from a tube containing jelly-striped eggs/embryos and 10 ml of fixation solution (2.5% glutaraldehyde, 2% paraformaldehyde, 20 mM NaSH, in 0.1 M PIPES buffer, pH 7.4) were added. Eggs/embryos were fixed for 2 hours at RT. We used 16% paraformaldehyde (#15710), 25%
glutaraldehyde (#16220) and 0.3 M PIPES stock solutions (#11610) from EMS, Hatfield, PA. NaSH (#161527-5G, Sigma-Aldrich, St. Louis, MO) from a 1 M stock solution was added before the pH was adjusted. After fixation, the eggs were rinsed 10 minutes in 0.1 M PIPES and 3 x 5 minutes in ddH₂O, followed by dehydration in 30%, 50%, 70%, and 90% ethanol for 15 minutes each, and finally in pure ethanol for 2 x 10 minutes.

After dehydration, eggs were infiltrated with a Durcupan resin: ethanol dilution of 1:1 overnight and in pure resin for 4 hours before transfer into fresh resin in silicon molds, and polymerization at 65°C for 24 hours. Sections of 400 and 2 μm thickness were obtained with a Ultracut-S ultramicrotome (Leica, Buffalo Grove, IL) using a diamond knife (Diatome/EMS Hatfield, PA).

**X-ray Fluorescence Microscopy:** Egg and embryo sections were mounted on 200 nm-thick silicon nitride windows (#NX5150D, Norcada, Edmonton, AB, Canada) and were glued to aluminum sample holders using clear nail polish. The sections were analyzed using scanning X-ray fluorescence microcopy at Beamline 2-ID-D at the Advanced Photon Source at Argonne National Laboratory. The source was a 3.3-cm-period undulator, and X-rays were monochromatized by a double multilayers monochromator to provide 20-25 times higher intensity than conventional crystal monochromators. An X-ray energy of 10.3 keV was selected to excite the Kα emission lines of elements from Si to Zn. A Fresnel zone plate focused the X-ray beam onto the sample with a spot size of ~0.3 μm. The specimen was placed in a helium environment and mounted on an X-Y translation stage at 75° to the incident beam. An energy-dispersive silicon drift detector (Vortex-EM, Hitachi High-Technologies Science America, Northridge, CA) was used to collect X-ray fluorescence spectra while the sample was being scanned across the focus spot. Because of their large diameters, a fly scan of each egg/embryo was first taken with a 20 μm step size and a 40 ms/pixel dwell time. An area near the cortex would be selected, and then a moderate-resolution scan with a 2 μm step size and a 40 ms/pixel dwell time would be taken. The sample would be checked for damage and then a final, high resolution scan of the cortex would be imaged with a 300 nm step size and a 500 ms/pixel dwell time. Using the program MAPS written in IDL, XRF spectrum at each pixel were individually fitted to remove background and overlaps between adjacent emission lines. Conversion of fluorescence intensities to areal densities in μg/cm² was performed by comparing X-ray fluorescence intensities with those from thin film standards NBS-1832 and NBS-1833 (NIST, Gaithersburg, MD).

**Metal Compartment Concentrations:** 400 nm-thick slices from 3 frogs were used to determine the approximate metal concentrations of the small metal-dense compartments in the egg animal pole. 8-bit grayscale images of each element were taken from the program MAPS and analyzed with ImageJ. A grayscale value of 55/255 was determined to best capture the distribution of zinc-containing compartments and was then used for all the other elements in order to maintain consistency. The images were thresholded and ROIs of at least 2 pixels in size (300 x 600 nm) were selected. The grayscale values of each ROI were converted back into μg/cm² as well as mM by extrapolating for the volume of each voxel (300 x 300 x 400 nm). The same procedure was used to determine the metal concentrations of the compartments in the embryo animal pole. The voxel molar concentrations should be considered to be minimal values for two reasons. First, because XRF analyzes slices of *Xenopus* eggs, it is possible that a slice contains part of a compartment and part of the cytoplasm. Second, the smaller class of compartment was only several
pixels in area, meaning that the compartment areas selected may contain some of the cytoplasm near their circumferences.

In order to determine the elemental content of yolk platelets, 400 nm-thick slices from 3 frogs were used for the egg animal pole. The cluster analysis function of MAPS was used to select for areas of high phosphorous. ROIs were then drawn for each platelet – however the corresponding area of the grayscale image was checked for each to make sure that it was not an agglomeration of multiple vesicles. Clusters of four or more pixels (≥ 0.36 μm²) were selected. Elemental concentrations were then acquired for each ROI and then averaged for each frog.

**Pearson Coefficients:** Grayscale images of each element were taken from MAPS. To avoid bias, coefficients were determined for the entire scanned slice areas, rather than focusing on areas rich in metal-containing clusters. Using ImageJ, a mask of the egg/embryo area was drawn for each element using the sulfur image as a base. This allowed analysis of only the egg/embryo area and not the blank space outside the plasma membrane, as well as excluding any microscopic holes in the slice. The white value at each X/Y coordinate was then saved. For each pair of elements, linear regressions were run in order to obtain Pearson coefficients. Means and standard deviations were calculated from the Pearson coefficients averaged for slices from 3-4 frogs.

**Cortical Electron-Dense Vesicle Distribution:** TEM images of the entire cortex of ~150 nm-thick slices of *Xenopus* eggs and embryos were obtained using ThermoFisher Talos F20 and the prototype ANL PicoProbe Analytical Electron Microscopes. In order to determine the distribution of small electron-rich vesicles, four images at a time were stitched together using the MosaicJ plugin for ImageJ. Brightness and contrast were adjusted, and the images were then imported into Adobe Illustrator. The plasma membrane was approximately traced, and boxes were drawn with marks at 2, 5, and 10 μm from the membrane (Supplemental Figure 10B). The number of small electron-rich vesicles was counted in each box. It should be noted that these are probably under-estimations, as some vesicles fall out of the fixed slice during sectioning. In order to determine the vesicle density per 100 μm², the number of vesicles was divided by the cortical area analyzed.

**Xenopus Fertilization Experiments:** Approximately 50-100 *Xenopus* eggs were placed in small Petri dishes. The control dish was filled with 0.1X Marc’s Modified Ringers without EDTA (MMR: 10 mM NaCl, 200 μM KCl, 100 μM MgSO₄, 200 μM CaCl₂, 500 μM HEPES, pH 7.4) while experimental dishes were filled with 0.1X MMR containing the following metal concentrations: 50 μM ZnSO₄, 100 μM ZnSO₄, 250 μM ZnSO₄, or 500 μM ZnSO₄; 0.5 mM MnCl₂, 1 mM MnCl₂, 3 mM MnCl₂, or 5 mM MnCl₂. The eggs were soaked in the solutions for 15 minutes. The solutions were removed, and the eggs were fertilized using a sperm solution created by grinding a portion of a testis in 0.1X MMR. The sperm solutions for the metal-containing dishes were spiked with metal at their corresponding concentrations. 15 minutes after fertilization, the dishes were flooded with their corresponding buffers. After cleavage became apparent (90-120 minutes), the contents of the dishes were scored according to three categories: unfertilized, properly cleaved, and failed cleavage. There were no signs of toxicity to the eggs. Because egg quality can vary, the percent proper cleavage in each experimental dish was normalized to that of its corresponding control dish. The metal content of the buffer in the control dishes was measured via ICP-MS. IC₅₀ values were calculated using Graphpad.
Calculations:

Estimated Manganese Release Concentration: Via ICP-MS, *Xenopus* eggs contain on average 8.8 x 10^{13} atoms of Mn while embryos contain 4.1 x 10^{13} atoms. The amount of Mn released following fertilization is:

\[
\frac{(8.8 \times 10^{13} \text{ atoms} - 4.1 \times 10^{13} \text{ atoms})}{6.022 \times 10^{23} \text{ atoms/mol}} = 7.8 \times 10^{-11} \text{ mol}
\]

The average radius of a *Xenopus* egg is 625 μm. If we model Mn release into a 50 μm “shell” around the embryo following fertilization, the volume of the “shell” is:

\[
\frac{4\pi}{3} \times ((6.75 \times 10^{-4} \text{ m})^3 - (6.25 \times 10^{-4} \text{ m})^3) = 2.7 \times 10^{-10} \text{ m}^3 = 2.7 \times 10^{-7} \text{ L}
\]

The concentration of Mn released into the 50 μm “shell” would therefore be:

\[
\frac{7.8 \times 10^{-11} \text{ mol}}{2.7 \times 10^{-7} \text{ L}} = 290 \mu M
\]
References:

1. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature methods* **9**, 671-675 (2012).
2. Martell, A. E. & Smith, R. M. *Critical Stability Constants: Volume 2: Amines.* (Springer US, 1975).
3. Martell, A. E. & Smith, R. M. *Critical Stability Constants: First Supplement.* (Springer US, 1982).
4. Smirnova, J. et al. Copper(I)-binding properties of de-coppering drugs for the treatment of Wilson disease. α-Lipoic acid as a potential anti-copper agent. *Sci. Rep.* **8**, 1463 (2018).
5. Arslan, P., Di Virgilio, F., Beltrame, M., Tsien, R. Y. & Pozzan, T. Cytosolic Ca2+ homeostasis in Ehrlich and Yoshida carcinomas. A new, membrane-permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free Ca2+. *J. Biol. Chem.* **260**, 2719-2727 (1985).
6. Hausen, P. & Riebesell, M. *The Early Development of Xenopus Laevis: An Atlas of the Histology.* (Verlag der Zeitschrift für Naturforschung, 1991).
7. Werst, M. M., Davoust, C. E. & Hoffman, B. M. Ligand spin densities in blue copper proteins by q-band proton and nitrogen-14 ENDOR spectroscopy. *J. Am. Chem. Soc.* **113**, 1533-1538 (1991).
8. Sharma, A. et al. Across the tree of life, radiation resistance is governed by antioxidant Mn(2+), gauged by paramagnetic resonance. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E9253-e9260 (2017).
9. Tsednee, M. et al. Manganese co-localizes with calcium and phosphorus in Chlamydomonas acidocalcisomes and is mobilized in manganese-deficient conditions. *J. Biol. Chem.* **294**, 17626-17641 (2019).
10. Davoust, C. E., Doan, P. E. & Hoffman, B. M. Q-Band Pulsed Electron Spin-Echo Spectrometer and Its Application to ENDOR and ESEEM. *Journal of Magnetic Resonance, Series A* **119**, 38-44 (1996).
11. Schweiger, A. J., Gunnar. *Principles of Pulse Electron Paramagnetic Resonance.* (Oxford University Press, 2001).
12. McNaughton, R. L. et al. Probing in vivo Mn2+ speciation and oxidative stress resistance in yeast cells with electron-nuclear double resonance spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 15335-15339 (2010).
13. Salikhov, K. M. Schweiger, A., Jeschke, G.:Principles of pulse electron paramagnetic resonance. *Applied Magnetic Resonance* **22**, 319 (2002).
14. Vogt, S. MAPS : A set of software tools for analysis and visualization of 3D X-ray fluorescence data sets. *J. Phys. IV France* **104**, 635-638 (2003).
15. Thevenaz, P. & Unser, M. User-friendly semiautomated assembly of accurate image mosaics in microscopy. *Microsc. Res. Tech.* **70**, 135-146 (2007).
Supplemental Figures:

Supplemental Figure 1: Procedure and data used for determining the rate of the zinc release. **a.** Representative image of the procedure used to measure the half-maximal fluorescence around the circumference of the egg. Scale bar = 500 μm. **b.** Graph charting fluorescence changes in sections of the egg circumference following fertilization. The graph represents Fluozin-3 fluorescence traveling clockwise from the origin of the zinc spark. **c.** Graph charting fluorescence changes in sections of the egg circumference following fertilization. The graph represents Fluozin-3 fluorescence traveling counter-clockwise from the origin of the zinc spark. **a., b.,** and **c.** are representative images/graphs of n = 3 biologically independent eggs examined in a single experiment.
Supplemental Figure 2: The metal chelator 1,10-Phenanthroline parthenogenetically activates *Xenopus* eggs whereas ammonium tetrathiomolybdate does not, indicating that activation is driven by a decrease in accessible zinc. **a.** Graph of the percentage of eggs to activate 20 minutes after chemical treatment (measured by darkening and contraction of the animal pole and contraction of the activation spot). Iono: Ionomycin, OP: 1,10-Phenanthroline, TTM: Ammonium tetrathiomolybdate. Data are presented as mean ± SD, n = 3 frogs examined over 3 independent experiments (15 – 35 eggs used per datapoint). **b.** Representative images of dejellied eggs 0, 10, and 20 minutes after the addition of chemical. Scale bar = 1 mm. n = eggs from 3 separate frogs examined over 3 independent experiments.
Supplemental Figure 3: Manganese has similar coordination environments in both eggs and embryos; freezing *Xenopus* eggs does not alter manganese coordination. **a.** Overlay of the EPR spectra of eggs (black) and embryos (red); the shape of the embryo signal is unchanged from that of the egg. This is a representative experiment of \( n = 3 \) biologically independent samples examined over 3 independent experiments. **b.** Intentionally destroying *Xenopus* eggs significantly changes their ENDOR spectra, demonstrating that freezing the eggs does not affect the vesicular compartmentalization of manganese. \( ^{31}\text{P}, ^{1}\text{H} \) Davies pulsed ENDOR spectra of intact (black), crushed (red) frog eggs and frozen solution of Mn-spiked buffer (purple). Experimental conditions as in Figure 3B. \( n = 1 \) biologically independent sample.
Supplemental Figure 4: X-ray fluorescence microscopy images of the animal pole of fixed *Xenopus* egg/embryo slices showing metal localization in small compartments. Scale bar = 20 µm. Images acquired at Beamline 2-ID-D at Argonne National Laboratory. Pixel size: 300 x 300 nm, slice thickness: 2 µm, scan time: 500 ms/pixel. These are representative images of slices of eggs/embryos from 7 different frogs imaged over 3 experiments.
Supplemental Figure 5: X-ray fluorescence microscopy images of the vegetal pole of fixed *Xenopus* egg/embryo slices. The yolk platelets show high phosphorous and sulfur content. Metals are localized to small compartments, however there are far fewer than in the animal pole. Scale bar = 20 µm. Images acquired at Beamline 2-ID-D at Argonne National Laboratory. Pixel size: 300 x 300 nm, slice thickness: 2 µm, scan time: 500 ms/pixel. These are representative images of slices of eggs/embryos from 7 different frogs imaged over 3 experiments.
Supplemental Figure 6: Metals cluster in the small cortical compartments. Cobalt, copper, and nickel group together as well as calcium, manganese and zinc. **a.** Pearson coefficients of elemental overlap in the egg animal pole. Data are presented as mean ± SD. n = 4 (for Mn, Co, Cu, and Zn) and n = 3 (for Ca and Ni because of contamination in a sample) biologically independent samples examined over 2 independent experiments. **b.** Representative X-ray fluorescence microscopy images of fixed *Xenopus* egg/embryo slices showing elemental overlap. Scale bar = 10 µm. Images acquired at Beamline 2-ID-D at Argonne National Laboratory. Pixel size: 300 x 300 nm, slice thickness: 400 nm, scan time: 500 ms/pixel.
Supplemental Figure 7: The cortex of the animal pole embryo has small zinc-containing vesicles as well as yolk platelets. **a.** HAADF-STEM images of the cortex of the animal pole of a fixed *Xenopus* embryo. **b.** Hyperspectral elemental intensity distribution maps, background corrected, gaussian blur at 3 pixels, no normalization, no quantification. Same conditions as in **a.** Scale bar = 2 µm in both **a.** and **b.** These are representative images of 2 separate scans.
Supplemental Figure 8: The egg vegetal pole mostly contains yolk platelets. 

a. HAADF-STEM images of the cortex of the vegetal pole of a fixed *Xenopus* egg. 
b. Hyperspectral elemental intensity distribution maps, background corrected, gaussian blur at 3 pixels, no normalization, no quantification. Same conditions as in a. Scale bar = 2 µm in both a. and b. These are results from a single AEM experiment.
Supplemental Figure 9: The embryo vegetal pole mostly contains yolk platelets. a. HAADF-STEM images of the cortex of the vegetal pole of a fixed *Xenopus* embryo. b. Hyperspectral elemental intensity distribution maps, background corrected, gaussian blur at 3 pixels, no normalization, no quantification. Same conditions as in a. Scale bar = 2 µm in both a. and b. These are results from a single AEM experiment.
Supplemental Figure 10: TEM data of eggs and embryos demonstrate that the majority of electron-dense vesicles disappear following fertilization. **a.** Table showing the differences in small, electron-dense vesicle content between the cortices of animal pole eggs and embryos. 8,263 µm² and 6,767 µm² of the egg and embryo cortices, respectively, were analyzed. **b.** Representative image showing the methodology of counting. TEM image of a ~150 nm thick slice of the animal pole egg. 47 images of the egg cortex and 47 images of the embryo cortex were analyzed.
Supplemental Figure 11: Raw counts of the number of unfertilized eggs, properly dividing embryos, and failed cleavage for eggs fertilized in the presence of extracellular ZnSO₄. n = number of eggs used per frog for each data point.
Supplemental Figure 12: Raw counts of the number of unfertilized eggs, properly dividing embryos, and failed cleavage for eggs fertilized in the presence of extracellular MnCl₂. n = number of eggs used per frog for each data point.
Supplemental Tables:

**Elemental Content of *Xenopus* Eggs and Embryos from ICP**

|       | Egg (Atoms)       | Embryo (Atoms)      |
|-------|-------------------|---------------------|
| Na    | 3.7 ± 0.12 x 10^{16} | 3.7 ± 0.13 x 10^{16} |
| Mg    | 1.3 ± 0.065 x 10^{16} | 1.3 ± 0.077 x 10^{16} |
| P     | 1.5 ± 0.033 x 10^{17} | 1.5 ± 0.036 x 10^{17} |
| S     | 7.1 ± 0.19 x 10^{16} | 7.0 ± 0.20 x 10^{16} |
| K     | 5.0 ± 0.065 x 10^{16} | 5.0 ± 0.094 x 10^{16} |
| Ca    | 7.8 ± 0.22 x 10^{14} | 7.4 ± 0.51 x 10^{14} |
| Mn    | 8.8 ± 0.94 x 10^{13} | 4.1 ± 0.60 x 10^{13} |
| Fe    | 6.8 ± 0.66 x 10^{14} | 7.3 ± 0.95 x 10^{14} |
| Cu    | 4.2 ± 0.39 x 10^{13} | 3.9 ± 0.21 x 10^{13} |
| Zn    | 1.4 ± 0.11 x 10^{15} | 1.3 ± 0.061 x 10^{15} |

Mean ± SEM. n = 4 frogs

**Supplemental Table 1:** Elemental contents of *Xenopus* eggs and embryos (1 hour post-fertilization) in atoms/cell. Data obtained from ICP-MS and ICP-OES. 20 egg/embryos per tube, values reported as mean ± SEM per egg/embryo. n = samples from 4 separate frogs analyzed in a single ICP experiment.
## Yolk Platelet Elemental Content

|       | Mean μg/cm² | Voxel Concentration (mM) |
|-------|-------------|--------------------------|
| P*    | 0.20 ± 0.025 | 2 ± 0.2 x 10²            |
| Ca    | 1.2 ± 0.24 x 10⁻² | 7 ± 1                  |
| Mn    | 9.8 ± 3.5 x 10⁻⁴ | 0.4 ± 0.2              |
| Co    | 3.6 ± 3.5 x 10⁻⁴ | 0.2 ± 0.1              |
| Ni    | 2.0 ± 0.17 x 10⁻⁴ | 8 ± 0.7 x 10⁻²         |
| Cu    | 4.7 ± 2.8 x 10⁻³ | 2 ± 1                  |
| Zn    | 1.6 ± 0.20 x 10⁻² | 6 ± 1                  |

Data from egg animal pole  
Mean ± SD, n = 3 frogs

*: Note this is a minimum value, as there is phosphorous loss during fixation (Seeler et al., in preparation)

### Supplemental Table 2:
Elemental concentrations of larger elemental compartments in the egg animal pole. Voxel size 0.3 x 0.3 x 2 μm. Data acquired at Beamline 2-ID-D, Argonne National Laboratory. Values reported as mean ± SD, n = 3 biologically independent samples examined over 2 independent experiments.
### Elemental Content of Frog Tank Water

| Element | Concentration (M) | n  |
|---------|------------------|----|
| Na      | 0.20 ± 0.024     | 3  |
| Mg      | 2.9 ± 0.025 x 10^{-2} | 3  |
| P       | 2.4 ± 0.061 x 10^{-3} | 3  |
| K       | 6.4 ± 0.046 x 10^{-3} | 3  |
| Ca      | 7.6 ± 0.027 x 10^{-3} | 3  |
| V       | 1.8 ± 0.076 x 10^{-9} | 6  |
| Cr      | 4.6 ± 3.4 x 10^{-10} | 6  |
| Mn      | 1.3 ± 0.21 x 10^{-9} | 6  |
| Fe      | 3.8 ± 0.39 x 10^{-8} | 6  |
| Co      | 3.5 ± 0.12 x 10^{-10} | 6  |
| Ni      | <1.1 x 10^{-9}    | 6  |
| Cu      | 8.5 ± 0.16 x 10^{-9} | 6  |
| Zn      | 8.2 ± 0.092 x 10^{-8} | 6  |
| As      | 1.1 ± 0.11 x 10^{-9} | 6  |
| Se      | 7.8 ± 0.45 x 10^{-10} | 6  |
| Mo      | 2.4 ± 0.15 x 10^{-9} | 6  |

**Supplemental Table 3:** Elemental content of frog tank water measured by ICP-MS. Values are in M and presented as mean ± SD. n is the number of tubes of water analyzed by ICP (in order to take into account variation in sample preparation/detection).
Supplemental Table 4: 

**a.** The percent of *Xenopus* embryos that properly cleaved in buffer containing ZnSO₄. The percentages were normalized to control fertilizations. n = 4 frogs (39 – 102 eggs per datapoint).

| ZnSO₄ Concentration | % Properly Cleaved |
|---------------------|---------------------|
| 50 µM               | 58 ± 10             |
| 100 µM              | 12 ± 5.7            |
| 250 µM              | 5.1 ± 2.4           |
| 500 µM              | 2.5 ± 1.6           |

*n = 4*

**b.** The percent of *Xenopus* embryos that properly cleaved in buffer containing MnCl₂. The percentages were normalized to control fertilizations. n = 4 frogs (36 – 100 eggs per datapoint). For both **a.** and **b.** data are presented as mean ± SEM.

| MnCl₂ Concentration | % Properly Cleaved |
|---------------------|---------------------|
| 0.5 mM              | 86 ± 11             |
| 1 mM                | 40 ± 6.3            |
| 3 mM                | 5.3 ± 4.5           |
| 5 mM                | 0                   |

*n = 4*
Supplemental Video Captions:

Seeler Supplemental Video 1: Zinc spark following fertilization of a *Xenopus* egg (time as mm:ss). Brightness and contrast were adjusted.
Seeler Supplemental Video 2: Zinc spark following parthenogenic activation of a *Xenopus* egg by ionomycin (time as mm:ss). Brightness and contrast were adjusted.
Seeler Supplemental Video 3: Control eggs in 0.1X MMR buffer with 1% DMSO (time as mm:ss).
Seeler Supplemental Video 4: Eggs treated with 20 μM ionomycin (time as mm:ss).
Seeler Supplemental Video 5: Eggs treated with 10 mM 1,10-phenanthroline (time as mm:ss).
Seeler Supplemental Video 6: Eggs treated with 10 mM ammonium tetrathiomolybdate (time as mm:ss).