

Supporting Information

Zinc sparks induce physiochemical changes in the egg zona pellucida that prevent polyspermy

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Explanation of zona pellucida zinc exposure estimates

Refer to Table S1 for inputted and calculated values of estimates. To estimate the concentration of zinc that the ZP could be exposed to during a zinc spark, we first determined the likely volume of exposure. This was calculated by subtracting the volume of a typical egg (assuming a spherical shape) from the volume of the space occupying 3 microns outside of the egg, representing the ZP region. In mouse, zinc sparks are hemispherically distributed and correspond to the regions in the egg that contain cortical granules (CGs), about 65% of the egg's surface. The ZP volume was multiplied by 0.65 to account for this.

Next, the number of zinc atoms was estimated. As Kim et al. reported in 2010 and 2011, the egg loses ~ 12 billion zinc atoms following fertilization and that zinc loss is largely mediated by zinc sparks.¹⁻³ Between one and five zinc sparks occur in the mouse egg when parthenogenically activated with SrCl₂.¹ To simplify the calculation, we assumed that equal number of zinc atoms are released during each spark. We then calculated the zinc content of a spark for scenarios where the egg sparks either once or five times to give a good idea of the potential range of zinc concentrations. Doing this, we estimate that the ZP can be exposed to between 0.1 and 0.5 mM zinc during a zinc spark. We used these values as a benchmark for our experiments.

Estimation of moles of protein in zona pellucida

The mouse ZP is comprised of three different proteins, mZP1 (200 kDa), mZP2 (120 kDa), and mZP3 (~83 kDa).⁴ The total protein mass in a mouse ZP is ~3.5 ng, and it is estimated that >80% of that mass comes from ZP2 and ZP3. Based on this number, we will assume that mZP1 comprises 20% of the ZP mass and mZP2 and mZP3 each comprise 40% of the ZP mass.

Thus, 0.7 ng of mZP1 is present in the ZP, or 4 fmol. Correspondingly, there are ~30 fmol of mZP2 and mZP3.

Materials and Methods

Immunofluorescence. Lens culinaris agglutinin (LCA) was used for cortical granule (CG) staining, rhodamine-phalloidin was used to stain f-Actin, and DAPI was used to label DNA. Protocol A was used for MII and MII + Zn samples. After cell treatments were complete, ZPs were removed using Acidic Tyrode's solution at 37 °C. ZP-free cells were then fixed in freshly prepared 3.8% PFA in PBS at 37 °C for 1h and then transferred to blocking solution (0.01% Tween 20, 0.01% NaN₃, 3 mg/mL BSA, PBS) and stored at 4 °C until all cells were ready for staining. Cells were permeabilized (0.1% TritonX-100, 0.005% NaN₃, 3 mg/mL BSA, PBS) for 15 min at room temperature. Cells were incubated in 10 µg/mL LCA-biotin overnight at 4 °C and then washed in blocking solution. They were then incubated in 5 µg/mL FITC-streptavidin and 2 U/mL rhodamine-phalloidin for 1h at room temperature. After washes in blocking solution, cells were mounted under coverslips in Vectashield + DAPI and stored at 4 °C until confocal analysis. Fluorescence imaging was performed using a TCS SP5 (Leica) confocal microscope. DAPI, FITC, and rhodamine fluorescence were detected using 405 nm, 488 nm, and 543 nm laser excitation respectively. Z-stacks were obtained for each cell.

ZP2 conversion assay. The conversion from ZP2 to ZP2f (the cleaved form of ZP2) was monitored in MII, MII + Zn, and Sr-activated cells using immunoblotting. Protocol A was used to obtain MII and MII + Zn samples. In 'whole cell' experiments, cells from different treatment groups were collected, 10 cells each were transferred to a small volume (1-2 µL) of L15/PVP (3

mg/mL polyvinyl pyrrolidone, a protein free medium) and snap frozen. In “isolated ZP” samples, ZPs were separated from the cell body using a 50 μ m bore Stripper micropipette. 10 ZPs for each group were then transferred to microcentrifuge tubes in minimal L15/PVP and snap frozen. Lysates were thawed and incubated in sample buffer containing beta-mercaptoethanol. Samples were electrophoresed in a 10% gel and transferred to a PVDF membrane using wet transfer. The membrane was blocked overnight at 4 °C. in 3% ECL Prime Blocking Agent in TBST. For ZP2 detection, the membrane was incubated in rat anti-mouse ZP2 antibody for 1h at room temperature (M2c.2, 1:1,000, kindly provided by Jurrien Dean (NIDDK, NIH, Bethesda, MD)). After washing 4x30 min in TBST, membranes were incubated in 1:10,000 anti-rat HRP for 1h at room temperature. The signal was detected using ECL Advance Western Blotting System. Sample protein bands were compared to Amersham ECL DualVue MI Markers (RPN810).

TEM image analysis. Image analysis was performed using Adobe Photoshop: 1) Original TEM image was binarized using a threshold that set background pixels to white and other pixels to black; 2) Regions outside the ZP were cropped; 3) Black and white pixels were quantified; 4) Pixel ratio was calculated by dividing black pixels by the total number of pixels. An example image analysis sequence is depicted in Figure S1A.

SEM image analysis. Image analysis was performed using ImageJ: 1) Original SEM image was cropped to remove bottom information bar; 2) Image was binarized to highlight surface ZP fibril bundles; 3) A Thickness analysis was performed using the BoneJ plug-in, resulting in an average thickness with standard deviation for each image. An example analysis sequence is depicted in Figure S1B.

Table S1. Estimation of transient zinc exposure in the zona pellucida during a zinc spark

Parameter	Value	
Radius of egg (μm)	39	
Radius of egg + ZP (μm)	42	
Volume of egg (μm^3)	2.5×10^5	
Volume of egg + ZP (μm^3)	3.1×10^5	
Difference volume (μm^3)*	6.2×10^4	
Difference volume (L)*	6.2×10^{-11}	
Cortical granule domain fraction	0.65	
Volume exposed to zinc spark (L) *	4.0×10^{-11}	
Total Zn atoms lost at fertilization	$1.2 \times 10^{10\dagger}$	
Number of sparks	1 [†]	5 [†]
Zn atoms/spark	1.2×10^{10}	2.4×10^9
Moles of Zn/spark	2.0×10^{-14}	4.0×10^{-15}
Approximate peak [Zn] in ZP during spark (μM)	~500	~100

* Difference volume includes portions of perivitelline space and zona pellucida

† Based on data from Kim et al. 2010 and 2011

Table S2. XFM elemental content in MII and Sr-activated zonae pellucidae

	Zn	Fe	Cu	Ca	S
MII (atoms x 10 ⁸)	3.1 ± 0.4	2.8 ± 1.1	2.0 ± 0.6	107 ± 18	7230 ± 555
Activated (atoms x 10 ⁸)	8.5 ± 3.8	1.2 ± 0.7	1.6 ± 0.4	120 ± 80	6700 ± 401
t-test	p = 0.0138	p = 0.0304	ns	ns	ns

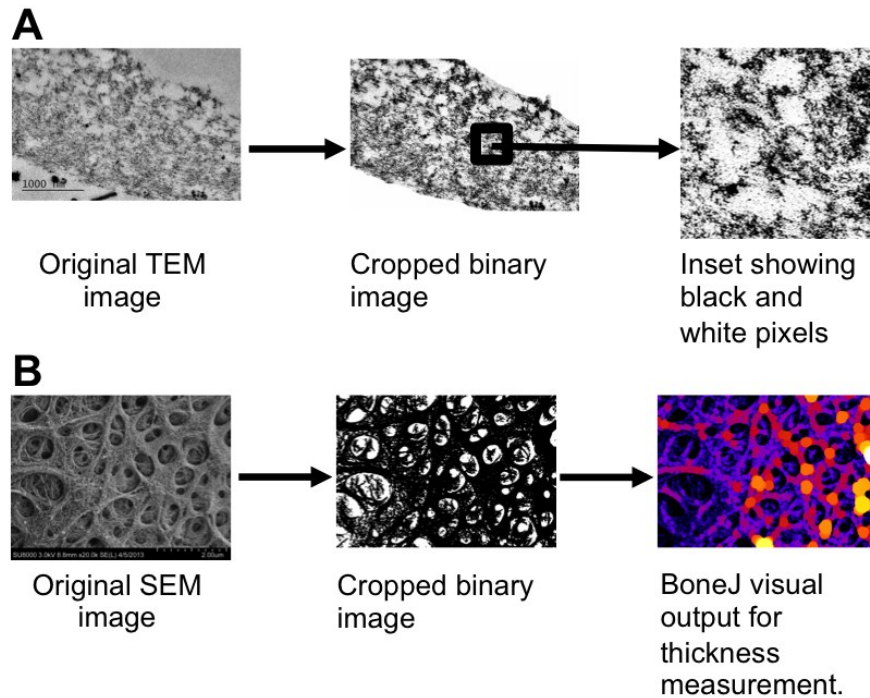
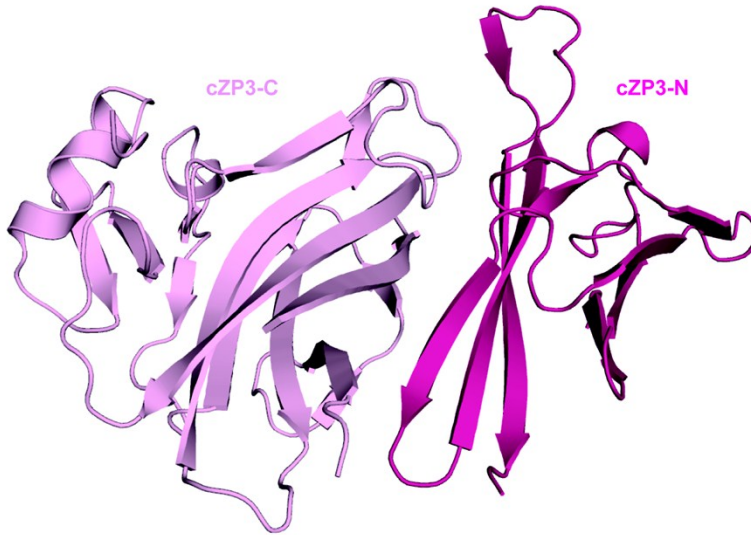


Figure S1. Electron microscopy image analysis protocols. A) TEM image analysis: The original TEM image was converted to a binary image and regions outside the ZP were cropped. Black and white pixels were quantified and the pixel ratio was calculated by dividing the number of black pixels by the number of total pixels in the binary image. B) SEM image analysis: The original SEM image was cropped and converted to a binary image. The BoneJ plug-in⁵ was then used to measure the mean bundle thickness and standard deviation in the image. The visual output uses a rainbow scale to depict regions of different thicknesses, with darker regions having smaller thickness values (black = 0), and brighter regions having larger thickness values (white = max thickness).

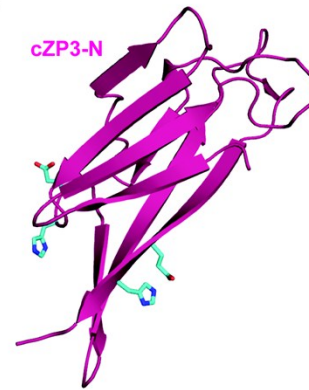
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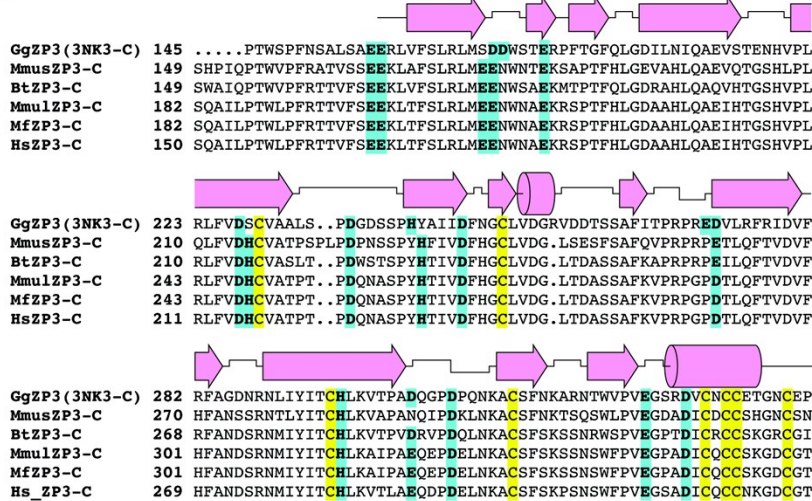
B



C



D



E

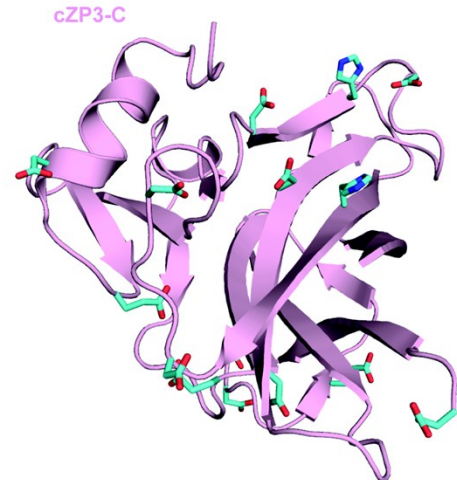


Figure S2. Structure-base multiple sequence alignment of ZP3 proteins from various species. A) Crystal structure of *Gallus gallus* (chicken) ZP3 (cZP3) dimer (PDB ID 3NK3).⁶ The structure is shown with the C-terminal (cZP3-C) and N-terminal (cZP3-N) domains colored light and dark pink, respectively. B) Structure-based multiple sequence alignment of the N-terminal domains of ZP3 proteins from various species (*Gallus gallus*, *Mus musculus*, *Bos taurus*, *Macaca mulatta*, *Macaca fascicularis* and *Homo sapiens*). The structure of cZP3 (3NK3) was used as a reference for alignments using STRAP.⁷ The secondary structure elements (arrows for β strands and cylinders for α helices) for cZP3 are shown above the sequences. Conserved surface His, Asp, Glu residues, are shown in bold and highlighted in cyan; the conserved Cys residues, most of which participate in disulfide bond formation and are thus unavailable to bind zinc until they are reduced, are highlighted in yellow. C) Structure of cZP3-N (shown as in A) with the possible zinc-binding residues shown as cyan sticks. D) Structure-based multiple sequence alignment of the C-terminal domains of ZP3 proteins from various species (performed as in B). E) Structure of cZP3-C (shown as in A) with possible zinc-binding residues shown as cyan sticks.

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