Supporting Online Material

Materials and Methods

Post-fertilization cleavage site of ZP2. Zonae pellucidae were isolated from 400 two-cell embryos by freezing and thawing (4X) in 500 µl of PBS, pH 7.4, 0.1% NP-40 (Roche Diagnostics GmbH, Mannheim, Germany), 0.5M NaCl followed by centrifugation (13,000 g x 5 min, 4^{o}C)(*S1,S2*). After reduction of disulfide bonds (5 mM DTT, 37^{o}C, 1 hr), 120 µl of Simply Blue (Invitrogen, Carlsbad, CA) was added to visualize the zona pellet which was twice washed by centrifugation (13,000 x g, 5 min, 4^{o}C) with 1.0 ml of PBS, 0.1% NP-40. The zonae pellucidae were solubilized in 50 µl of PBS, 0.4% PVP, 0.1% SDS by heating (60^{o}C,1 hr). Taking advantage of the observation that native ZP1 and ZP3 are blocked with amino terminal pyroglutamate (*S3*), the N-terminus of ZP2 was determined by Edman degradation (Research Technologies Branch, NIAID courtesy of Mark K. Garfield) of whole zonae pellucidae. Two ZP2 sequences were detected in approximately equal amounts. The first four amino acids (^{168}DENQ^{171}) at a predicted cleavage site (*S4*) were clearly observed in the chromatograph as well as the sequence from the previously defined (*S5*) N-terminal peptide (^{35}VXLPQ^{39}). These data define the cleavage site immediately N-terminal to the di-acidic motif ^{168}DE^{169} and suggest that even after reduction of the disulfide bond(s), the N-terminal peptide remained associated with the zona pellucida matrix of two-cell embryos.

Transgenic mouse lines. Using GalK DNA recombineering and site-directed mutagenesis, respectively, exon 6 of *Zp2* was mutated to ablate the cleavage site (^{165}LA$^{\downarrow}$DEN^{170} →

^{165}LGAAN^{170}) and exon 7 of *Zp3* was mutated to prevent O-glycosylation of Ser^{332} and Ser^{334} (^{329}SNSSSS^{334} → ^{329}ANVGAA^{334}). Five *Zp2*^{mut} (*Zp1*$^{+/+}$, *Zp2*$^{+/+;mut}$, *Zp3*$^{+/+}$) and three *Zp3*^{mut} (*Zp1*$^{+/+}$, *Zp2*$^{+/+}$, *Zp3*$^{+/+;mut}$) transgenic lines were established and two of each were crossed with *Zp2*$^{tm/tm}$ and *Zp3*$^{tm/tm}$ null mice (*S4,S6*) to establish *Zp2*^{Mut} (*Zp1*$^{+/+}$, *Zp2*$^{tm/tm;mut/mut}$, *Zp3*$^{+/+}$) and *Zp3*^{Mut} (*Zp1*$^{+/+}$, *Zp2*$^{+/+}$, *Zp3*$^{tm/tm;mut}$) lines, respectively (fig. S2). The zona matrix, absent in each null line, was reconstituted (fig. S3) and the two *Zp2*^{Mut} and the two *Zp3*^{Mut} rescue lines had similar phenotypes. Only one of each is described in detail.

To obtain the *Zp2*^{mut} transgene by GalK recombineering (*S7*), a cosmid clone (15.4 kb) of genomic *Zp2* encompassing 1.5 kb and 0.5 kb of the 5' and 3' flanking regions, respectively (*S8*) was transformed into SW102 bacterial cells, containing the λ prophage recombineering system. A PCR fragment (1331 bp) containing the *galK* operon flanked by 50 bp homology to *Zp2* gene 5' and 3' of the sequence encoding ^{166}LADEN^{170} was amplified (forward primer: 5'-CTTTTTACTTTTTTTTCCAGTTTTCTTTCCCACAACTTTTCTCTAGGCTT**CCTGTTGAC AATTAATCATCGGCA**-3'; reverse primer: 5'-AGGTGGGGGGAGGGACAGAGGAAGAAGACAGAACAAATTGTATTCTTACC**TCAGC ACTGTCCTGCTCCTT**-3', *galK* sequence in bold). After digestion with *DpnI* and purification, the PCR fragment was electroporated into the SW102 cells and recombinants were selected for by growth on minimal media with galactose (http://recombineering.ncifcrf.gov/Protocol.asp). Using a positive clone from this first step, the *galK* cassette was replaced by recombineering with a 100 bp double stranded oligonucleotide encoding ^{166}L**GAA**N^{170} with homology to *Zp2* on either side (5'-TTACTTTTTTTTCCAGTT TTCTTTCCCACAACTTTTCTCTAGGCTTG**G**TG**CG**G**CC**AACCAGGTAAGAATACAATT TGTTCTGTCTTCTTCCTCTGTCCCT-3' (mutations bold and underlined). Positive clones

containing the mutation were selected on minimal media with 2-deoxy-galactose and confirmed by digestion with *SpeI* and DNA sequence.

To obtain the *Zp3*^{mut} transgene, the serine residues implicated as attachment sites for O-glycans (*S9*) were modified by site-directed mutagenesis. First, two oligonucleotides, (5'-AATTGTGCCTGCAGGATTGGTACCATAGAATTCACCGCGGCCGCTGTAC-3' and 5'-AGCGGCCGCGGTGAATTCTATGGTACCAATCCTGCAGGCAC-3') were annealed (100mM Tris HCl pH7.5, 1M NaCl, 10 mM EDTA), and ligated into *EcoRI-KpnI* digested pBlueScript KS(+) to construct a plasmid (mpBKS) that contained *NotI-SbfI-EcoRI-KpnI-NotI* restriction sites. A 7.2 kb *EcoRI-KpnI* fragment of *Zp3* extending from the middle of exon 2 to ~1.3 kb 3' of exon 8 was gel purified from λ15.1 (*S10*) and ligated into mpBKS. Second, a 2.4 kb region of *Zp3* including exon 7 was PCR amplified (forward 5'-ATGAATTCTTCCTTAAGCACC-3' and reverse 5'- ATTGGTACCAATATCAGGGGC-3' primers). After cloning into the *EcoRI-KpnI* site of pCR2.1-Topo vector (Invitrogen), bases encoding serine residues 329, 331, 332, 333 and 334 were modified by site-directed mutagenesis (AGT-AAT-TCA-AGC-TCT-TCA to **GC**T-AAT-**GT**A-**G**GC-**G**CT-**G**CA) according to the manufacturer's instructions (Stratagene, La Jolla, CA). A correctly mutated clone identified by DNA sequence was digested with *ApaI-AflII* and the mutated fragment cloned into the aforementioned genomic *EcoRI-KpnI* fragment in mpBKS. Third, to complete the *Zp3*^{mut} transgene, a 4.4 kb *SbfI-EcoRI* genomic fragment (extending from 1.7 kb 5' of the transcription start site to the missing half of exon 2) was isolated from λ4.1 (*S10*), gel purified (Qiagen, Valencia, CA) and ligated into mpBKS.

Not I fragments containing either the *Zp2*^{mut} (15.4 kb) or the *Zp3*^{mut} (11.6 kb) transgene were gel purified and injected into the male pronucleus of fertilized FVB/N eggs (*S2*). *Zp2*^{mut}

mice were genotyped by PCR (forward 5'-TTCTCT**AGGCTTGGTGCGGCC**-3' and reverse 5'- GAGACACAGAGACACAGAGAA-3' primers, mutant sequence underlined in bold) and a 311 bp product was amplified from the mutant, but not the normal genomic allele. *Zp3*^{mut} mice also were genotyped by PCR (forward 5'- TGCACTGTAGTCCATGCTGGC-3' and reverse 5'- GGCAGGAGCTCTATCTACCAC-3' primers) and the 948 bp product from the mutant, but not the normal, allele was resolved into a two fragments (435 bp, 513 bp) after digestion with *HaeII*. All experiments using mice were conducted in compliance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health under a Division of Intramural Research, NIDDK-approved animal study protocol.

Expression of transgenes. Total RNA was isolated from 3 wk old mouse tissues using RNeasy Mini kit (Qiagen) and single strand cDNA, synthesized with Superscript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA), was used as a template for RT-PCR. *Zp2*^{mut} and *Zp3* transcripts were detected with gene specific primers (*Zp2*^{mut}, forward 5'-TTCTCTAGGCTTGGTGCGGCC-3' and reverse 5'-GTTCAGTGCGGGTTTTGTTT-3' primers; *Zp3*, forward 5'-ATGTGAGCAGCCACCCTATC-3' and reverse 5'-TGACACTTCCTGGTGACAGC-3' primers). To differentiate *Zp3*^{mut} transcript from normal, the 805 bp *Zp3* PCR fragment was digested with *HaeII* which resolved ZP3^{mut} into two fragments (562 bp, 243 bp) while normal ZP3 remained intact. To document integrity of the RNA, a 495 bp GAPDH transcript was amplified from each sample using forward (5'-ATGGTGAAGGTCGGTGTGAACG-3') and reverse (5'-GTTGTCATGGATGACCTTGGCC-3') primers.

In situ hybridization was performed with 4 μm ovarian sections (American Histolabs, Gaithersburg, MD) obtained from 3-wk old *Zp2*^{Mut} and *Zp3*^{Mut} females. To detect *Zp2* transcript, anti-sense (5'- TCAATTCCATTGGCATGCCATTGGTCCTCAGGGATGCTCCATTGTCCA-3') and sense (negative control) 48-mer DIG-labeled oligonucleotide probes were designed and synthesized by Genedetect (http://www.genedetect.com). To detect *Zp3* transcripts anti-sense (5'-TTCTGGAGCTGTTGGCAAAATGGAATACATCCACCGTGAACTGGAGAG-3') and sense (negative control) 48-mer DIG labeled oligonucleotide probes were designed and synthesized by GeneLink (http://www.genelink.com). Ovaries were fixed in 2% paraformaldehyde in PBS at 4°C O/N and dehydrate by serial washings in 30%, 50% and 70% ethanol. Ovarian sections were deparafinized, rehydrated, permeabilized with proteinase K and hybridized with probes at 37^{o}C O/N according to the manufacturer's protocol (http://genedetect.com/Merchant2/InsituParaffinDIGOXIGENIN.pdf). The hybridization signals were amplified with tyramide signal amplification and developed with diaminobenzidine tetrahydrochloride according to the manufacturer's protocol (Dako, Carpinteria, CA). Tissues were counterstained with hematoxylin, dehydrated through gradations of alcohol and xylene before being mounted and imaged on an Axioplan Zeiss microscope (Carl Zeiss, Thornwood, NY).

Light and confocal microscopy. Ovaries from 3 wk old normal, *Zp2*^{mut} transgenic, *Zp2*^{Mut} rescue, *Zp3*^{mut} transgenic and *Zp3*^{Mut} rescue female mice were fixed in glutaraldehyde and embedded in plastic to obtain 4 μm thick sections (American Histolabs, Gaithersburg, MD) for imaging on an Axioplan Zeiss microscope (*S11*). Differential interference contrast (DIC) and confocal laser scanning images of eggs and 2-cell embryos were obtained on a Zeiss LSM 510

microscope (*S4*). Cortical granule (*S4*), acrosome status (*S12*) and sperm binding to zonae pellucida (*S12*) were assayed as described.

Immunoblot analysis. After resolution by NuPAGE Novex Bis-Tris Gels (Invitrogen), proteins were transferred onto a PVDF membrane for immunoblot analysis using monoclonal antibodies to ZP2 (mC2.2, recognizes the C-terminus after cleavage)(*S4*) and ZP3 (IE-10)(*S13*) using 10-20 egg/embryos per lane (*S12*). Chemiluminescence signals were acquired by Luminescent Image Analyzer LAS-3000 (Fuji Film Medical Systems, Stanford, CT).

Sperm binding. Sperm binding to ovulated eggs and two-cell embryos isolated from normal, $Zp2^{Mut}$, and $Zp3^{Mut}$ mice was assayed using capacitated sperm and two-cell embryos as wash controls (*S2*). Alternatively, sperm binding was recorded by confocal microscopy after 1, 2 and 4 hr using capacitated *Acr3*-EGFP sperm and displacement of normal sperm with *Acr3*-EGFP sperm was performed as previously described (*S12*). The average number of sperm, ± s.e.m., was determined from 5-12 eggs or embryos.

Sperm analysis. *Acr*-EGFP cauda epididymides were placed in 700 μl of human tubal fluid (HTF, Millipore, Temecula, CA) previously equilibrated with 90% N_2, 5% O_2, 5% CO_2. After mincing the tissue, motile sperm were capacitated by an additional 1 hr of incubation. Sperm motility was evaluated on a HTM-IVOS (Version 12.3) motility analyzer (Hamilton Thorne Biosciences, Beverly, MA) with the following settings: phase contrast; frame rate, 60 Hz; minimum contrast, 30; min and max static size gates, 1.0-4.0; min and max intensity gates, 0.7-1.5; min and max static elongation gates, 10 and 100; default cell size, 13 pixels; default cell

intensity, 75; magnification, 0.78. Sperm (15 µl) were loaded into a pre-warmed, 80 µm 2X-CEL Dual Sided Sperm Analysis Chamber (Hamilton) and observed under 4X magnification. Data were collected and averaged from three independent biological samples each with 8 fields and a total of >500 sperm. Viability was determined by exclusion of viadent fluorescent dye and progressive motility was defined as the percentage of sperm with a path velocity >50 µm/sec; straightness >50%).

Fertility. Pairs (4-6 week old) of either *Zp2*$^{+/tm}$ (controls) and *Zp2*^{Mut} or *Zp3*$^{+/tm}$ (controls) and *Zp3*^{Mut} females were co-caged with a FVB male of proven fertility to determine the number and size of litters. Alternatively, females with the above genotype were stimulated with gonadotrophins, mated with FVB males and their oviducts were flushed at E0.5 and E3.5 to recover 1-cell embryos and blastocysts, respectively. To assess in vitro fertility (IVF) of *Zp2*^{Mut} and *Zp3*^{Mut} females, the rate of fertilization was calculated as the percentage of two-cell embryos observed in relation to the starting number of ovulated eggs in cumulus after overnight incubation with capacitated sperm (*S12*).

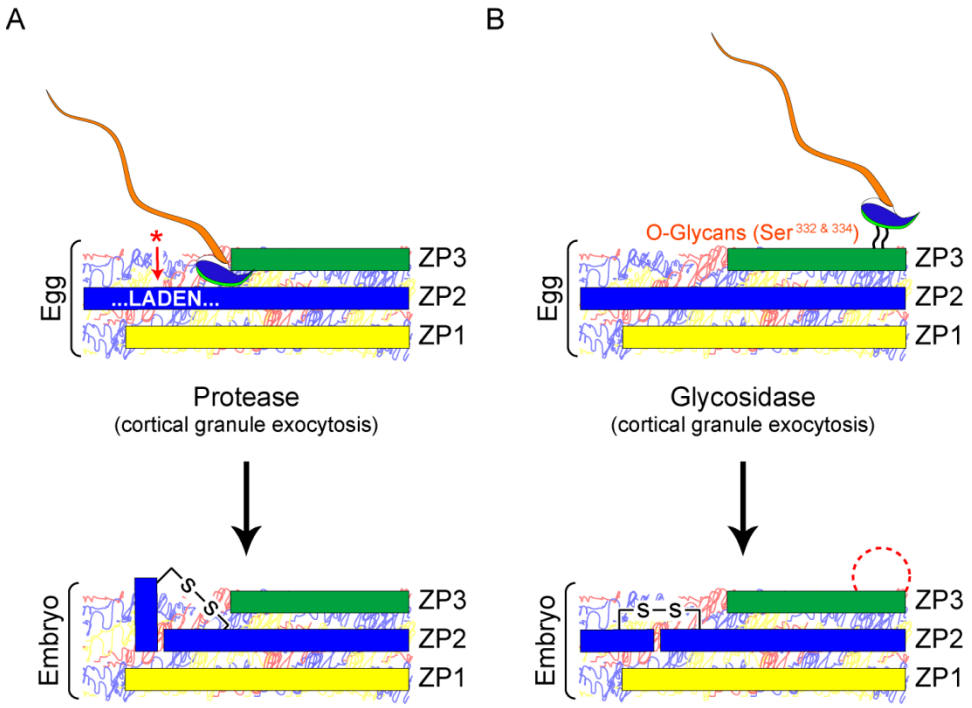

Fig. S1 Models of sperm-egg recognition. (**A**) The 'ZP2-cleavage' model in which intact ZP2 establishes a zona pellucida that is permissive for sperm-egg recognition. Following fertilization, a protease released from cortical granules cleaves ZP2 and renders the zona pellucida non-permissive for gamete recognition. ***Prediction:*** if ZP2 is mutated to prevent cleavage, sperm will bind to two-cell embryos despite fertilization and cortical granule exocytosis. (**B**) The 'glycan-release' model in which O-glycan ligands attached to ZP3 Ser^{332} and Ser^{334} interact with a sperm surface receptor to account for sperm-egg recognition. Following fertilization, a glycosidase released from cortical granules cleaves off the O-glycan and accounts for the inability of sperm to bind to the zona pellucida surrounding two-cell embryos. ***Prediction:*** if ZP3 is mutated to prevent attachment of O-glycans, sperm will not bind to the zona pellucida and female mice will be sterile.

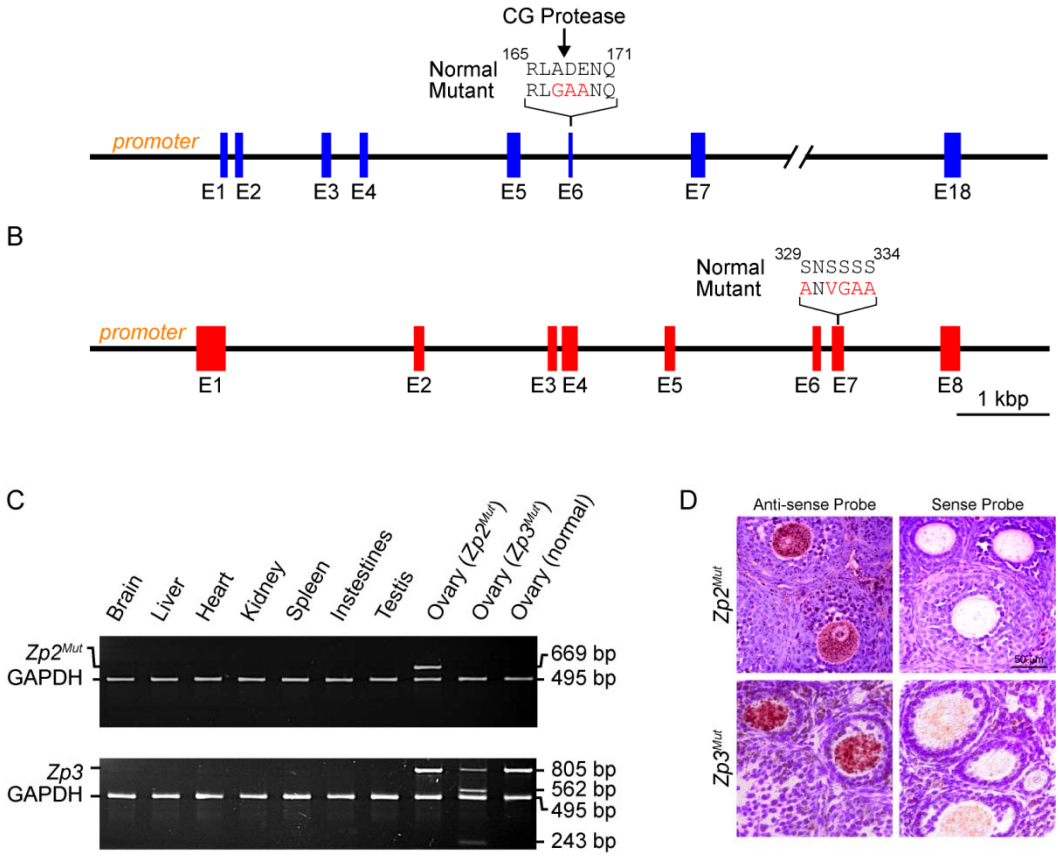

Fig. S2. *Zp2*^{Mut} and *Zp3*^{Mut} mice. (**A**) The *Zp2* locus (15.4 kb) was retrieved from a cosmid clone and exon 6 was mutated using a GalK shuttle cassette and DNA recombineering to modify the coding region from ^{165}LA$^{\downarrow}$DEN^{170} to ^{165}LGAAN^{170} and prevent post-fertilization cleavage of ZP2. (**B**) The *Zp3* locus (11.6 kb) was retrieved from two λ phage clones and exon 7 was mutated using site directed mutagenesis to modify the coding region from ^{329}SNSSSS^{334} to ^{329}ANVGAA^{334} to preclude O-glycosylation of Ser^{332} and Ser^{334}. (**C**) Total RNA was isolated from eight tissues of *Zp2*^{mut} and *Zp3*^{mut} transgenic mice for assay by RT-PCR using either primers specific for *Zp2*^{mut} transcripts (669 bp), upper panel, or for both normal *Zp3* and *Zp3*^{mut} transcripts (805 bp). After cleavage with *HaeII*, the 805 bp product from *Zp3*^{mut}, but not normal *Zp3*, was resolved into two bands (562 bp, 243 bp), lower panel. The presence of GAPDH

transcripts in each lane ensured the integrity of the sample RNA. (***D***) In situ hybridization of paraformaldehyde fixed, paraffin-embedded 4 μm ovarian sections from *Zp2*^{Mut} (*Zp1*, *Zp2*$^{tm/tm;mut/mut}$, *Zp3*) and *Zp3*^{Mut} (*Zp1*, *Zp2*, *Zp3*$^{tm/tm;mut}$) mice hybridized with DIG-labeled synthetic sense (negative controls) and anti-sense oligonucleotides specific for *Zp2* or *Zp3* transcripts, respectively.

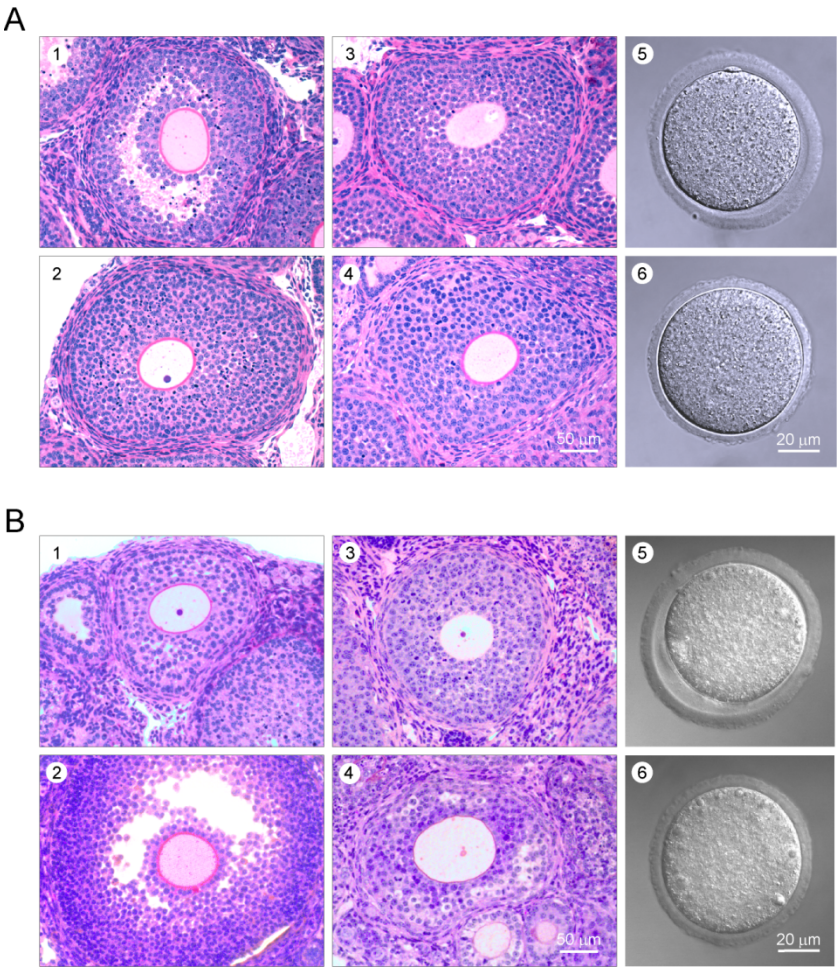

Fig. S3 Ovarian histology and ovulated eggs from transgenic mouse lines. (**A**) Glutaraldehyde fixed and plastic embedded ovarian sections from: normal (1), *Zp2*^{mut} transgenic (2), *Zp2*$^{tm/tm}$ null (3) and *Zp2*^{Mut} (*Zp1*, *Zp2*$^{tm/tm;mut/mut}$, *Zp3*)(4) mice were stained with periodic acid-Schiff reagent and counterstained with hematoxylin prior to photomicroscopy. Ovulated eggs after hormonal stimulation of: normal (5) and *Zp2*^{Mut} (6) mice. (**B**) As in (**A**), but with normal (1), *Zp3*^{mut} transgenic (2), *Zp3*$^{tm/tm}$ null (3) and *Zp3*^{Mut} (*Zp1*, *Zp2*, *Zp3*$^{tm/tm;mut}$)(4) mice. Ovulated eggs after hormonal stimulation of normal (5) and *Zp3*^{Mut} (6) mice.

Table S1 Fertility of *Zp2*^{Mut} and *Zp3*^{Mut} Female Mice

	Eggs/Animal[1]	**Pups/Litter**[2]
Control *Zp2* (*Zp1*$^{+/+}$, *Zp2*$^{+/tm}$, *Zp3*$^{+/+}$)	18.3 ± 7.2 (13)	8.3 ± 0.5 (25)
***Zp2*^{Mut}** (*Zp1*$^{+/+}$, *Zp2*$^{tm/tm;mut/mut}$, *Zp3*$^{+/+}$)	24.6 ± 6.8 (8)	3.0 ± 0.5 (6)
Control *Zp3* (*Zp1*$^{+/+}$, *Zp2*$^{+/+}$, *Zp3*$^{+/tm}$)	16.6 ± 3.1 (5)	7.1 ± 0.7 (7)
***Zp3*^{Mut}** (*Zp1*$^{+/+}$, *Zp2*$^{+/+}$, *Zp3*$^{tm/tm;mut}$)	16.4 ± 4.1 (5)	7.0 ± 0.8 (8)

[1]avg. ± s.e.m. (number of experiments) after stimulation with gonadotrophins
[2]avg. ± s.e.m. (number of litters) after mating 1:2 (♂:♀) with normal FVB males

Table S2 Preimplantation Development of Embryos from *Zp2*^{Mut} Female Mice

	1-Cell Embyro[1]	**Blastocysts**[2]
Control *Zp2* (*Zp1*$^{+/+}$, *Zp2*$^{+/tm}$, *Zp3*$^{+/+}$)	13.0 ± 6.2 (29)	6.9 ± 0.6 (7)
***Zp2*^{Mut}** (*Zp1*$^{+/+}$, *Zp2*$^{tm/tm;mut/mut}$, *Zp3*$^{+/+}$)	16.0 ± 4.8 (22)	1.0 ± 0.4 (5)

[1]avg. ± s.e.m./female (number of females) at E0.5 after stimulation with gonadotrophins
[2]avg. ± s.e.m./female (number of females) at E3.5 after stimulation with gonadotrophins

References

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