Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy

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The mouse zona pellucida is composed of three glycoproteins (ZP1, ZP2, and ZP3), of which ZP2 is proteolytically cleaved after gamete fusion to prevent polyspermy. This cleavage is associated with exocytosis of cortical granules that are peripherally located subcellular organelles unique to ovulated eggs. Based on the cleavage site of ZP2, ovastacin was selected as a candidate protease. Encoded by the single-copy Astl gene, ovastacin is an oocyte-specific member of the astacin family of metalloendoproteases. Using specific antiserum, ovastacin was detected in cortical granules before, but not after, fertilization. Recombinant ovastacin cleaved ZP2 in native zona pellucidae, documenting that ZP2 was a direct substrate of this metalloendoprotease. Female mice lacking ovastacin did not cleave ZP2 after fertilization, and mouse sperm bound as well to Astl-null two-cell embryos as they did to normal eggs. Ovastacin is a pioneer component of mouse cortical granules and plays a definitive role in the postfertilization block to sperm binding that ensures monospermic fertilization and successful development.

Introduction

Because polyspermy is an embryonic lethal, at least three postfertilization blocks to gamete interactions have evolved in mice. The first two occur rapidly after fertilization and prevent additional sperm from fusing with the egg’s plasma membrane or penetrating the extracellular zona pellucida surrounding eggs and preimplantation embryos (Sato, 1979; Stewart-Savage and Bavister, 1988). The third and definitive block occurs over several hours and ensures that sperm do not bind to the surface of the zona pellucida (Inoue and Wolf, 1975; Baibakov et al., 2007). The molecular basis of the first two blocks remains largely unknown, and the third correlates with egg cortical granule exocytosis (Barros and Yanagimachi, 1971).

Cortical granules are Golgi apparatus–derived, membrane-bound vesicles (0.2–0.6 µm) that accumulate during oogenesis and form a uniform layer in the cortex of fully grown mouse eggs. The observed 15-fold increase in cortical granules during oocyte growth reflects both an increase in granule density and in the cortical area as oocytes increase in diameter from 40 to >80 µm (Zamboni, 1970; Nicosia et al., 1977; Ducibella et al., 1994). During meiotic maturation and germinal vesicle breakdown, cortical granules redistribute and are excluded from the region of the metaphase I spindle (Ducibella et al., 1988a; Deng et al., 2003). Cortical granules become competent to undergo exocytosis just before ovulation, and the ~8,000 cortical granules observed in fully grown oocytes decline to ~4,800 in ovulated eggs (Ducibella et al., 1994). Fertilization triggers cortical granule migration to the plasma membrane, where they fuse and exocytose their contents (Wessel et al., 2001; Ducibella et al., 2002).

Little is known about the contents of mouse cortical granules (Liu, 2011), and the only documented biological function is the postfertilization cleavage of ZP2 (Bleil et al., 1981), which, along with ZP1 and ZP3, forms a structured extracellular glyco-matrix that surrounds mouse eggs (Bleil and Wassarman, 1980). Cleavage of ZP2 is N terminal of a diacidic residue (Gahlay et al., 2010), a known cleavage site for the astacin family of metalloendoproteases. Ovastacin (Astl, the official gene name) is expressed in growing mouse oocytes and has a signal peptide to direct it into a secretory pathway but has no known function (Quesada et al., 2004). We now localize ovastacin as a pioneer component of mouse egg cortical granules and document its ability to modify the zona pellucida to prevent postfertilization sperm binding and provide a definitive block to polyspermy.

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Results and discussion

Ovastacin is present in mouse egg cortical granules

ZP1, ZP2, and ZP3 form the extracellular zona pellucida that surrounds mouse eggs and early embryos (Bleil and Wassarman, 1980). Sperm bind to eggs but not two-cell embryos, and the only documented biochemical change in the zona matrix is cleavage of ZP2 (Fig. 1 A; Bleil et al., 1981). This cleavage is associated with cortical granule exocytosis and is N terminal of a diacidic motif, TEDDE^{166} (Gahlay et al., 2010). The site is well conserved among mammals (Hasegawa et al., 1994; Tian et al., 1999; Lindsay and Hedrick, 2004), but the identity of the presumptive cortical granule protease has as of yet remained unknown (Fig. 1 B). Ovastacin (Fig. 1 C) is a member of the large astacin family of metalloendoproteases (Dumperth et al., 1999; Lindsay and Hedrick, 2004), but the identity of a candidate protease for the postfertilization cleavage of ZP2.

Taking advantage of the unique C-terminal extension of ovastacin, a peptide-specific (395PLALFPEARDKPAP408) rabbit antibody was used to image ovulated eggs by confocal microscopy. LCA-FITC (Lens culinaris agglutinin conjugated to FITC) is a marker of cortical granules (Ducibella et al., 1988b), and its colocalization in the periphery of ovulated eggs indicates the presence of ovastacin within these granules (Fig. 1 D). Disappearance of ovastacin after fertilization and cortical granule exocytosis was observed by confocal microscopy (Fig. 1 D) and confirmed by an immunoblot that detected the two known isoforms of the enzyme (Quesada et al., 2004) in eggs but not two-cell embryos (Fig. 1 E). From these observations, we conclude that ovastacin is expressed in eggs, where it localizes to peripheral cortical granules and is discharged during postfertilization cortical granule exocytosis.

In the absence of ovastacin, cortical granules persist in ovulated eggs

To determine its function, the single-copy Astl gene was successfully targeted for ablation in mouse embryonic stem (ES) cells using a neomycin cassette flanked 5' and 3' by 5.3 and 1.5 kbp of homology, respectively (Fig. 2 A). Colonies were initially screened by PCR, and 14 positive clones were confirmed by Southern blot analysis using 5' and 3' probes outside the regions of homology (Fig. 2 B). After blastocyst injection, two coat-color chimeric male mice were identified, and germline transmission of the null allele was confirmed by the genotype of tail DNA (Fig. 2 C). Mice, bred to homozygosity for the mutant Astl allele, were fertile. Although there was a modest decrease in fecundity, there was considerable overlap in the size of litters (Fig. 2 D), which may reflect effects of mixed genetic backgrounds.

To confirm the absence of ovastacin protein, eggs and two-cell embryos were stained with LCA-FITC or ovastacin antibodies and imaged by confocal microscopy. Colocalization in the periphery of eggs, but not two-cell embryos, was observed in normal mice (Fig. 3 A). Similar results were obtained for eggs and embryos from heterozygous null females, although the intensity of the signals was diminished. As anticipated, ovastacin was not detected in the homozygous null eggs, but, unexpectedly, LCA-FITC reactivity was lost as well (Fig. 3 A). Similar results were obtained for eggs and embryos from heterozygous null females, although the intensity of the signals was diminished. As anticipated, ovastacin was not detected in the homozygous null eggs, but, unexpectedly, LCA-FITC reactivity was lost as well (Fig. 3 A).
cortical granules with great confidence. However, using EM, cortical granules were readily detected in AstlHet and AstlNull but not in two-cell embryos. Native ovastacin immunoprecipitated from oocytes or recombinant ovastacin expressed in insect cells did not react with LCA lectin (Fig. 3 C) despite LCA binding to a higher–molecular mass protein (not depicted) that validates the assay (Liu et al., 2003a). Thus, the molecular identity of the LCA-positive molecule in cortical granules remains to be determined. From these observations, we conclude that cortical granules remain present in the periphery of AstlNull eggs (Fig. 3 B).

After fertilization, ZP2 remains uncleaved in the absence of ovastacin
Preimplantation development is a period of particular vulnerability for mammalian embryos. Biochemical or genetic removal of the protective zona pellucida causes resorption into the oviductal epithelium (Bronson and McLaren, 1970; Modliński, 1970; Rankin et al., 2001), and maternal effect genes that arrest or delay cleavage-stage development result in embryonic lethality (Li et al., 2010). Polyspermic aneuploidy is also a significant threat to embryonic survival, and mice have developed strategies to ensure monospermic fertilization. Prevention of sperm binding to the zona pellucida provides the ultimate postfertilization block to polyspermy. If sperm do not bind, they cannot penetrate the zona matrix, and they cannot fuse with the egg plasma membrane.

The postfertilization cleavage of ZP2 (120 kD) was initially reported to result in two fragments (30 and 90 kD) that remain disulfide bonded (Bleil et al., 1981). Using an mAb (M2c.2) that recognizes the 90-kD C-terminal fragment, this cleavage was detected by immunoblotting run under reducing conditions (Rankin et al., 2003) and occurs over 6–8 h after fertilization (Baibakov et al., 2007). ZP2 in AstlNull eggs had a normal mass of 120 kD but was not cleaved in embryos isolated from AstlNull females; ZP2, in two-cell embryos from normal and AstlHet females, was cleaved and served as positive controls (Fig. 4 A). Using a 30-min de novo binding assay, capacitated mouse sperm bound to AstlNull eggs (56.6 ± 2.8 SEM sperm/egg) but not normal two-cell embryos (3.8 ± 0.7 SEM sperm/egg), which served as positive and negative controls, respectively. In sharp contrast, mouse sperm bound to two-egg embryos from AstlNull mice (63.5 ± 3.0 SEM sperm/embryo) in a manner that was indistinguishable from the positive control (Fig. 4 B). Thus, the cleavage status of ZP2, independent of fertilization and cortical granule exocytosis, is the major determinant of sperm binding to the surface of the zona pellucida.

In reexamining the primary structure of mouse ZP2, two additional diacidic motifs were identified in the 30-kD N-terminal fragment (\textsuperscript{103-134}DD) and five were present in the 90-kD C-terminal fragment (Fig. 5 A). The detection of an intact 90-kD fragment on immunoblots of normal two-cell embryos probed with mAb M2c.2 (Fig. 4 A) suggests that additional C-terminal sites are not cleaved by ovastacin. To determine the cleavage status of the additional diacidic motifs in the 30-kD N terminus, immunoblots of eggs and two-cell embryos from normal and homozygous null Astl females were run under reducing conditions and probed with an mAb, IE-3, specific to ZP2\textsuperscript{103-134} (East and Dean, 1984; Sun et al., 1999). As anticipated, ZP2 in
embryos from AstlNull females was not cleaved (Fig. 5 B). However, rather than a single 30-kD ZP2 fragment in embryos from normal females, four peptides were detected on the immunoblot probed with IE-3 (Fig. 5 B, arrows). One N-glycan (Arg83), but no O-glycans, is present in the N terminus of ZP2, as determined by mass spectrometry (Boja et al., 2003). The largest glycopeptide observed on the immunoblot could represent the entire 30-kD N-terminal fragment, with the smaller peptides reflecting heterogeneity of cleavage at the three sites (DE55, DD127, and DE166), given the binding site of the mAb (Fig. 5 A). Of note, all of the N-terminal ZP2 peptides remain disulfide bonded to the larger C-terminal fragment (Greenhouse et al., 1999). Thus, rather than a single cut, it now appears that the 30-kD fragment is further degraded through proteolysis.
The $\text{Asth}^\text{Null}$ phenotype recapitulates mutation of the ZP2 cleavage site (\textsuperscript{16}\text{DE} \rightarrow \textsuperscript{16}\text{GS} \textsuperscript{16} \text{AA}, Gahlay et al., 2010), in which ZP2 remains intact in the zona pellucida surrounding two-cell embryos. Mutation of \textsuperscript{16}\text{DE} prevents any cleavage of ZP2 (Gahlay et al., 2010), which indicates that it must be cut first before the \textsuperscript{34}DE and \textsuperscript{127}DE sites. In both $\text{Zp2}^\text{Mut}$ and $\text{Asth}^\text{Null}$ mouse lines, capacitated sperm bind to the zona pellucida surrounding two-cell embryos from homozygous mutant females despite fertilization and cortical granule exocytosis. However, the significant decrease in fecundity observed in $\text{Zp2}^\text{Mut}$ females was not present in $\text{Asth}^\text{Null}$ mice. A notable difference between the two mutant lines is a thinner zona pellucida (4 µm vs. 7 µm in normal mice) present in the $\text{Zp2}^\text{Mut}$ mice, which could result in precocious hatching from the protective zona matrix and embryonic lethality (Bronson and McLaren, 1970; Modliński, 1970; Rankin et al., 2001).

ZP2 is a direct target of ovastacin

These results indicate that ovastacin is exocytosed from cortical granules after fertilization and that the subsequent cleavage of ZP2 prevents sperm binding to the zona pellucida surrounding two-cell embryos. However, it was not clear whether ZP2 was the direct substrate for ovastacin or whether ovastacin acted indirectly through a cascade of proteolytic activators. Therefore, recombinant mouse ovastacin was expressed in insect cells and partially purified by column chromatography. The addition of the enzyme to zonae pellucida isolated from normal eggs resulted in progressive cleavage of ZP2 over 8 h (Fig. 5 C), consistent with kinetics of cleavage observed in vivo (Baißakov et al., 2007). Similar cleavage of ZP2 was observed in the zona pellucida surrounding $\text{Asth}^\text{Null}$ as with normal eggs, and no cleavage was observed using cell supernatant from the same expression system not secreting ovastacin (Fig. 5 D). Thus, we conclude that ZP2 is the direct substrate for mouse ovastacin. Collectively, a simple explanation of these observations is that sperm bind to the N-terminal domain of ZP2 at the surface of the zona pellucida before penetration and fusion with the egg plasma membrane. After fertilization and cortical granule exocytosis, ovastacin diffuses through the zona matrix and fragments the N-terminal domain of ZP2. This proteolysis takes several hours and renders the zona pellucida unable to support sperm binding.

Although destruction of the sperm docking domain is definitive in preventing polyspermy, mice have additional strategies to ensure monospermic fertilization. Particularly striking is the ability to restrict the number of sperm that encounter ovulated eggs in the oviduct. Although millions of sperm are deposited in the female reproductive tract at coitus, only thousands traverse the utero–tubal junction, where they are initially confined to the oviductal isthmus by adherence to the epithelial lining (Suarez and Pacey, 2006). After capacitation and hyperactivation, individual sperm free themselves and ascend to fertilize ovulated eggs present in the oviductal ampulla. Gamete recognition occurs at low ratios in vivo, and the number of sperm does not exceed the number of eggs until 50% have been fertilized (Cummins and Yanagimachi, 1982). Nevertheless, the imperative of monospermy invokes additional defenses to polyspermy. The most immediate block prevents fusion of additional sperm in the perivitelline space with the egg plasma membrane. This block is independent of cortical granule exocytosis but requires fusion with sperm, as it can be bypassed by intracellular sperm injection (Horvath et al., 1993; Maleszewski et al., 1996) and does not depend on membrane depolarization (Jaffe et al., 1983). Within minutes of fertilization, a second block that is dependent on cortical granule exocytosis prevents additional sperm from penetrating through the zona pellucida. The rapidity...
of this block suggests diffusion of a small molecule or prompt propagation of a structural modification of the zona matrix. However, cleavage of ZP2 by ovastacin provides the most definitive block to polyspermy by destroying the sperm docking domain on the zona pellucida to ensure monospermic fertilization and successful development.

The Astl gene is well conserved in humans (Quesada et al., 2004) and presumably plays a similar role in the postfertilization cleavage of ZP2 (Bauskin et al., 1999). The modest decrease in fecundity observed with AstlNull mice lacking ovastacin could have a greater impact in humans with their preponderance of single births. If this leads to recurrent pregnancy loss, testing for mutations in the human ASTL gene could become relevant in the clinic. In this context, it will be important to determine whether the absence of ovastacin affects the incidence of polyspermy (rarely reported in humans) or the structural integrity of the zona pellucida that protects the embryo as it passes down the oviduct (Bronson and McLaren, 1970; Modliński, 1970; Rankin et al., 2001). It is also noteworthy that precocious release of ovastacin from cortical granules could prematurely cleave ZP2 to prevent sperm binding and fertilization. Thus, the mouse may provide a model in which to begin to test such contrapuntal strategies for possible future use in human biology.

Materials and methods

Antibodies
A rabbit PAb was generated against peptide 395-PLALFPARDFKPAQP of mouse ovastacin attached N terminal to a cysteine residue and conjugated to keyhole limpet hemocyanin (Sigma-Aldrich). mAbS IE-3 and M2c.2 that bind to the N- and C-terminal regions of ZP2, respectively, were previously described (East and Dean, 1984; Rankin et al., 2003), and the following antibodies and lectins were obtained commercially: LCA-FITC (Sigma-Aldrich), donkey anti–rabbit conjugated with Alexa Fluor 555 (Invitrogen), goat anti–rabbit DyLight 649 (Thermo Fisher Scientific), donkey anti–rabbit conjugated with HRP (Jackson Immunoresearch Laboratories, Inc.), and goat anti–rat conjugated with HRP (Jackson Immunoresearch Laboratories, Inc.).

Egg and embryo collection and culture
4–5 wk-old female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin followed by 5 IU of human chorionic gonadotropin 48 h later. Ovulated eggs and embryos were collected before and after mating, respectively, in M2 medium (Millipore) containing protease inhibitors (Roche). Embryos were subsequently cultured in potassium simplex optimized medium (Millipore) at 37°C in 5% CO2. All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health under the Division of Intramural Research, National Institute of Diabetes and Digestive and Kidney Diseases approved animal study protocols.

Immunoblot analysis
Eggs and two-cell embryos were lysed in 2 or 4x Tris-glycine SDS loading buffer with DTT, separated on 4–20% Tris-glycine gels by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Invitrogen), blocked in 3 or 5% nonfat milk in PBS, and probed with primary antibodies followed by secondary antibodies conjugated to HRP (Gahlay et al., 2010). Chemiluminescence was performed with ECL Plus (GE Healthcare), and signals were acquired by the Luminescent Image Analyzer LAS-3000 (Fujiﬁlm) or with BioMax XAR ﬁlm (Kodak).

For detection of ovastacin, blots were incubated with a 1:1,000 dilution of peptide-puriﬁed, rabbit anti-mouse ovastacin antibody (1.7 mg/ml) in 5% nonfat milk in TBS with 0.1% Tween 20 (TBST) at 4°C overnight. On the following day, blots were incubated with a 1:10,000 dilution of goat anti–rabbit HRP in TBST for 1 h at room temperature. For staining with LCA, blots were incubated with 10 µg/ml of the biotinylated LCA (JUS Biological) in 5% nonfat milk in TBST at 4°C overnight. Blots were then incubated with a 1:10,000 dilution of HRP-streptavidin (Thermo Fisher Scientiﬁc) in TBST and incubated for 1 h at room temperature.

Establishment of the Astl-null mouse line
Astl is a single-copy gene that encodes ovastacin. Mouse lines lacking ovastacin protein were established using DNA recombinineering (Liu et al., 2003b) and targeted ablation in ES cells (Zheng and Dean, 2009). The targeting construct contained positive (neomycin resistance) and negative (herpes simplex virus thymidine kinase) selectable markers and replaced exons 2 and 3 of the transcriptional and translational start sites. Correctly targeted ES cells were identiﬁed by Southern hybridization of SspI-digested genomic DNA using 32P-labeled probes 5′ (−6,766 to −6,234 bp of the transcriptional start site) and 3′ (2,688–3,056 bp) to the targeting vector. Heterozygous null ES cells were injected into mouse blastocysts to establish chimeric founder lines. Germline transmission of the null allele and subsequent genotyping were determined by allele-speciﬁc PCR products of tail DNA. Primers P1 (5′-AGGCCCTGCTCAACCGTGATG-3′) and P2 (5′-CCAGAGAAATGAGGAGGACG-3′) were used to detect the normal allele (797 bp), and primers P2 and P3 (forward 5′-GGGAGGATTGGGAAGGACAT-3′) were used to detect the null allele (399 bp) in PCR genotyping of tail DNA. The PCR condition consisted of one cycle at 94°C for 5 min, 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min and 30 s, and a full extension cycle at 72°C for 10 min.

Fertility
Astl+/− and Astl−/− females were caged with FVB males of proven fertility to determine the number and size of litters for a period of 8–10 mo.

EM
Oocytes and embryos from Astl+/− and Astl−/− females were ﬁxed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and incubated at 4°C for 2 h. After extensive washing in the cacodylate buffer, the oocytes and embryos were embedded in 2% agarose. The samples were then dehydrated through a graded series of ethanol and processed for embedding in London Resin white. Ultrathin sections were obtained with an ultramicrotome (MICROM International GmbH) and mounted on Formvar-coated nickel grids. For lectin cytochemistry (Jiménez-Movilla et al., 2004), grids were preincubated (for 10 min at room temperature) in PBS (1% BSA) and transferred to a drop of WGA-HRP lectin (Sigma-Aldrich) in PBS for 1 h. After rinsing in PBS, grids were ﬂoated on a drop of rabbit anti-HRP PAb (Sigma-Aldrich) diluted 1:500 in PBS for 1 h. Grids were then washed in PBS and ﬂoated on a drop of Protein A gold (15 nm)–conjugated antibody (1:60; Utrecht University) for 1 h. After washing in twice-distilled water, ultrathin sections were counterstained with uranyl acetate followed by lead citrate and imaged in a transmission electron microscope (Philips Tecnai 12; FEI). WGA-positive cortical granules were observed in five nonserial ultrathin sections (total; mean ± SEM/section) from three Astl+/− oocytes (180; 12.0 ± 0.7), Astl+/− (337; 22.5 ± 0.7) oocytes and two-cell embryos (no cortical granules) served as positive and negative controls, respectively.

Immunoprecipitation
Ovaries from 8-wk-old FVB mice were homogenized in cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol) with one tablet of protease inhibitor cocktail (Roche) added to every 10 ml of buffer. The sample was centrifuged (13,200 rpm at 4°C for 20 min), and supernatants were collected. 10 µg rabbit anti–mouse ovastacin antibody was added to the supernatant in a ﬁnal volume of 800 µl and rotated overnight at 4°C. 30 µl protein G Sepharose beads (GE Healthcare) was equilibrated with lysis buffer and added to the protein–antibody mixture. This was rotated for an additional 2 h at 4°C. After a brief centrifugation,
the supernatant was removed, and the beads were washed three times with 50 mM Tris, pH 8.0, 150 mM NaCl, and 1% NP-40 buffer. Two 30-µl aliquots of elution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and 1% SDS) were added to the beads for 10 min at 30°C. Eluates were separated on 12% SDS-PAGE gels before immunoblotting.

Sperm binding assay
Caudal epididymal sperm were isolated from wildtype FvB mice and placed under oil [Irvine Scientific] in human tubal fluid (Millipore) previously equilibrated with 90% N₂, 5% O₂, and 5% CO₂ and capacitated by an additional 30–60 min of incubation at 37°C [Baibakov et al., 2007]. Sperm binding to 10–20 ovulated eggs and two-cell embryos isolated from normal and Astfα mice was quantified using capacitated sperm and normal two-cell embryos as wash controls. Samples were fixed in 2% PFA for 30 min, stained with Hoechst 33342, and imaged by confocal microscopy.

Recombinant ovastacin
pDon253 is a Gateway Donor vector modified from pDon201 [Life Technologies]. pDon253 replaces the kanamycin resistance gene with a gene encoding spectinomycin resistance and contains several sequencing primer sites to aid in sequence verification of this clone. The following oligonucleotides (Eurofins MWG Operon) were used: 5'-GGGGAACAGCTGCG-3'; 5'-CGTTGCCCTTGTTTTTATGGTCGTATACATTTC-3'; 5'-GGGGACAACTTTGTACAAAAAAGTTGGCACCATGAAATTCTTAGTC-3'; 5'-CGGTGGCTCTTGTATTTATGTCGTTATACATTTC-3'; and 5'-CGTTGCCCTTGTTTTTATGGTCGTATACATTTC-3'.

Mouse ovastacin was cloned using PCR from cDNA templates for baculovirus expression. A honeybee melittin signal peptide leader sequence was added to the 5' end of each construct to enhance secretion of the protein in insect cells, and a C-terminal noncleavable Stop2 tag (GSWSHPQFEKG) was added for purification purposes. Initial PCR was performed using Phusion DNA polymerase (New England Biolabs, Inc.) under standard conditions using a 40s extension time and 200 nM of flanking primers. After five cycles of amplification, 200 nM of each adapter primer was added, and amplification was continued for 20 additional cycles. The final PCR products were flanked by Gateway recombinational sequence signals attB1 at the 5' end and attB2 at the 3' end. The PCR products were cleaned using the QIAquick PCR purification kit (QIAGEN) and recombined into pDon253 using the Gateway BP recombination reaction [Life Technologies] and the manufacturer's protocols. BP reactions were transformed into Escherichia coli DH10B cells, and colonies were isolated on lysogeny broth plates containing 50 µg/ml spectinomycin. Plasmid DNA was prepared and sequenced using a variety of internal and external sequencing primers to verify the sequence.

The sequence-verified Entry clones were subcloned by Gateway LR recombination [Life Technologies] into pDest-8 for insect cell expression. Final expression clones were verified by size and restriction digest pattern. The expression clones were then transformed into E. coli DH10Bac (Life Technologies) and plated on selective media containing gentamycin, kanamycin, tetracycline, IPTG, and X-gal as per the manufacturer's protocols. White colonies were selected from these plates, and bacmid DNA was generated by alkaline lysis plasmid preparation and verified by PCR amplification across the bacmid junctions.

The bacmid DNAs were complexed with XpressNOW transfection reagent (Lonza) and transfected into 100 ml of Sf9 insect cells at 1.5 × 10⁸ ml⁻¹ in SFX-Insect medium [HyClone; Thermo Fisher Scientific]. At 5 d after transfection, the cultures were centrifuged at 1,100 g, and the virus-containing supernatant was collected. For expression, one liter of High Five cells was set in SFX medium in a three-liter Erlenmeyer (Corning) at a cell concentration of ~1.5 × 10⁷ ml⁻¹ and infected (multiplicity of infection of three) with 40 ml of the recombinant baculovirus. The culture was grown in a shaker incubator at 21°C for 3 d, and the supernatant was collected after centrifugation at 1,100 g.

The conductivity of the supernatant was adjusted to 12.61 mS/cm with 20 mM Hepes, pH 7.3. The supernatant (41 ml) was applied to a 5-ml Q Sepharose column [GE Healthcare] equilibrated with 20 mM Hepes, pH 7.3, and 75 mM NaCl. A flow-through sample was collected, and then a 5-ml S Sepharose column (previously equilibrated in 20 mM Hepes, pH 7.3, and 75 mM NaCl) was added for subsequent purification on both columns. After completion of the load, the columns were washed to baseline in 20 mM Hepes, pH 7.3, and 75 mM NaCl. A 10-column volume elution from 75 mM to 1 M NaCl was completed by collecting 2.5-ml fractions across the gradient. Analysis was performed via SDS-PAGE/Coomassie staining and immunoblotting. The rabbit anti-ovastacin antibody was incubated overnight at 4°C, washed at room temperature with 1× TBS with Tween 20, incubated 1 h in 1× 1,000 anti-rabbit secondary antibody, and washed five times in 1× TBS with Tween 20. SuperSignal West Pico Chemiluminescent Substrate [Thermo Fisher Scientific] was used to develop the signal. The fractions with the highest concentration of ovastacin were used in subsequent assays.

In vitro cleavage assay
Zona pellucidae were isolated from 150 oocytes by freeze thawing four times in 100 µl PBS, pH 7.4, 0.1% IGEPAL CA-630 (Sigma-Aldrich), and 0.5 M NaCl. Isolated zonae were solubilized in 30 µl PBS, 0.4% PVP, and 0.1% SDS by heating at 60°C for 30 min. Solubilized zona samples were incubated with recombinant ovastacin at 37°C over time (0–8 h), and cleavage was analyzed by immunoblotting with mAb M2:2.

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Note added in proof.
While this manuscript was under review, Sachdev et al. (2012. Dev. Biol. doi:10.1016/j.ydbio.2011.12.021) reported on SAS1β, which is the same protein as ovastacin.

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