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## Gamete Recognition in Mice Depends on the Cleavage Status of an Egg's Zona Pellucida Protein

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At fertilization, mouse sperm bind to the zona pellucida (which consists of glycoproteins ZP1, ZP2, and ZP3) that surrounds eggs. A ZP2 cleavage model of gamete recognition requires intact ZP2, and a glycan release model postulates that zona glycans are ligands for sperm. These two models were tested by replacing endogenous protein with ZP2 that cannot be cleaved ( $Zp2^{Mut}$ ) or with ZP3 lacking implicated O glycans ( $Zp3^{Mut}$ ). Sperm bound to two-cell  $Zp2^{Mut}$  embryos despite fertilization and cortical granule exocytosis. Contrary to prediction, sperm fertilized  $Zp3^{Mut}$  eggs. Sperm at the surface of the zona pellucida remained acrosome-intact for more than 2 hours and were displaced by additional sperm. These data indicate that sperm-egg recognition depends on the cleavage status of ZP2 and that binding at the surface of the zona is not sufficient to induce sperm acrosome exocytosis.

Most models of gamete recognition postulate that a single ligand in the extracellular zona pellucida (ZPI, ZP2, or ZP3) surrounding eggs interacts with a sperm surface receptor. Although individ-

Fig. 1. Expression of mutant zona proteins. (A) Immunoblot of eggs (lanes 1 to 3) or embryos (lanes 4 to 6) from normal (lanes 1 and 4), Zp2<sup>mut</sup> transgenic (lanes 2 and 5), and Zp2<sup>Mut</sup> (lanes 3 and 6) mice using ZP2 antibodies (3). Molecular mass is at left. (B) Eggs and embryos from normal (1 to 4) or Zp2<sup>Mut</sup> (5 to 8) mice were stained with rhodamine-conjugated LCA to image cortical granules, which were present in ovulated (1 and 5) but not in fertilized (3 and 7) eggs. The metaphase spindle (arrows) was stained with a fluoresceinconjugated antibody to  $\alpha$ -tubulin, and pronuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) (asterisks). (C) Same as (A) but with eggs and embryos from normal (lanes 1 and 3) and Zp3<sup>Mut</sup> (lanes 2 and 4) mice using ZP3 antibodies (19).

ual zona proteins were initially considered as possible ligands, the absence of ZP1 or the replacement of ZP2 and ZP3 with human homologs does not affect the specificity of sperm-egg recognition (1-3). Carbohydrate side chains on mouse ZP2 and ZP3 that are released after fertilization have attracted greater investigative attention. Both N and O glycans have been implicated in sperm-egg recognition (4), but a particularly precise and widely embraced glycan release model proposes that O glycans attached at Ser<sup>334</sup> and Ser<sup>334</sup> on ZP3 act as ligands for a sperm-surface receptor (5, 6).

A more-recent ZP2 cleavage model proposes that rather than sperm binding a single ligand, sperm binding is supported by a three-dimensional zona structure. This model is predicated on the cleavage status of ZP2, rendering the zona pellucida either permissive (uncleaved ZP2) or nonpermissive (cleaved ZP2) to account for sperm binding to the zona pellucida surrounding eggs, but not to that surrounding two-cell embryos (*3*). Both models remain controversial—the first over the presence and identity of the carbohydrate ligand (7–13) and the second for validation with human ZP2 that was fortuitously not cleaved in transgenic mice (14). To test the two models (fig. S1), transgenic mouse lines mutated to either pre-

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Normal

Zp2<sup>Mut</sup>

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vent cleavage of mouse ZP2 or the attachment of implicated O glycans on ZP3 (figs. S2 and S3) were back-crossed into appropriate null backgrounds (3, 15) to establish  $Zp2^{Mut}$  ( $Zp1^{+/+}$ ,  $Zp2^{tn/tm:mut/mut}$ ,  $Zp3^{+/+}$ ) and  $Zp3^{Mut}$  ( $Zp1^{+/+}$ ,  $Zp2^{+/+}$ ,  $Zp3^{tm/tm:mut}$ ) lines, where *tm* indicates a null allele.

In ovulated eggs, ZP2 with a molecular mass of 120 kD is detected, reflecting uncleaved protein. After fertilization, normal ZP2 is cleaved, but mutant ZP2 is not. Thus, ZP2 was detected as a 90-kD (cleaved) protein in normal embryos and as a 120-kD (uncleaved) protein in  $Zp2^{Mut}$  two-cell embryos.

Both cleaved (endogenous) and uncleaved (mutant) ZP2 were present in mutant Zp2 transgenic mice  $(Zp1^{+,+}, Zp2^{+,+;mut}, Zp3^{+,+})$  (Fig. 1A). The inability to cleave ZP2 after fertilization could reflect the absence of cortical granule exocytosis. Therefore, ovulated and fertilized eggs were isolated from normal or  $Zp2^{Mut}$  females and stained with *Lens culinaris* agglutinin (LCA) to detect cortical granules. In both normal and  $Zp2^{Mut}$  mice, cortical granules were present at the periphery of ovulated eggs. Fertilization in the one-cell zygotes was confirmed by the presence of two pronuclei, and in each geno-

type, LCA staining was absent, reflecting postfertilization cortical granule exocytosis (Fig. 1B). Thus, the inability to cleave ZP2 in embryos derived from  $Zp2^{Mut}$  mice was independent of fertilization and cortical granule exocytosis.

No change in molecular mass was detected before and after fertilization in zonae from either normal or  $Zp3^{Mut}$  mice. Although only Ser<sup>332</sup> and Ser<sup>334</sup> have been proposed as the binding sites for O glycan ligands that are required for sperm binding, the adjacent Ser<sup>329</sup>, Ser<sup>331</sup>, and Ser<sup>333</sup> were also mutated to validate comparisons with



**Fig. 2.** Sperm binding to eggs and embryos. (**A**) Sperm binding to  $Zp2^{Mut}$  eggs (1 and 2) and two-cell embryos (3 and 4) was assayed after 1 hour of incubation with normal capacitated sperm using normal two-cell embryos

Fig. 3. Reversible sperm binding. (A) Normal (1), Zp2<sup>Mut</sup> (2), and  $Zp3^{Mut}$  (3) eggs and  $Zp2^{Mut}$  embryos (4) were incubated with capacitated sperm for 1 hour and stained with Hoechst before imaging by confocal microscopy. After a brief rinse, capacitated Acr3-EGFP sperm  $[5 \times 10^5 \text{ ml}^{-1} \text{ of human tubal fluid (HTF)}]$  were added and incubated for an additional 1 hour. After washing with normal two-cell embryo controls (insets) to remove nonadherent sperm, eggs and embryos were stained with Alexa 568-SBTI before imaging by confocal microscopy (5 to 8). Acrosomereacted and -intact sperm were labeled with Alexa 568 and EGFP, respectively. Images were modified in Adobe Photoshop to remove nuclear staining from EGFP-positive sperm; thus, Hoechst-positive, EGFP-negative sperm reflect those that were not displaced by EGFP sperm. (B) Quantification of the number of sperm bound to eggs and two-cell embryos before (blue bars labeled original) and after (green bars labeled displaced) the addition of Acr3-EGFP sperm. Residual sperm (blue bars) reflect those not displaced by Acr3-EGFP after 1 hour of incubation and removal of nonadherent sperm.





earlier investigations (6). The loss of Ser<sup>329</sup> and Ser<sup>332</sup> disrupted two attachment sites [ $^{327}NCS^{329} \rightarrow ^{327}NCA^{329}$  (where Ser<sup>329</sup> is changed to Ala);  $^{330}NSS^{332} \rightarrow ^{330}NVG^{332}$  (where Ser<sup>331</sup> is changed to Val and Ser<sup>332</sup> is changed to Gly)] for N glycans that are occupied in native mouse ZP3 (10, 16). Thus, ZP3<sup>Mut</sup> zonae lacking two of five N glycans had a lower average molecular mass (~75 kD) than was normal (~85 kD) (Fig. 1C).

To test sperm-egg recognition, eggs and two-cell embryos were isolated from normal,  $Zp2^{Mut}$ , and  $Zp3^{Mut}$  mice. After insemination, sperm bound avidly to  $Zp2^{Mut}$  eggs (67.2 ± 9.5, n = 12 eggs), as compared with normal two-cell embryos used as wash controls (7.0 ± 0.8, n = 13 embryos). However, sperm also bound (49.3 ± 5.5, n = 19 embryos) to two-cell embryos isolated from  $Zp2^{Mut}$  females, in which ZP2 remained uncleaved (Fig. 2A). Contrary to prediction, sperm bound to  $Zp3^{Mut}$  eggs (96.1 ± 1.9, n = 7 eggs) in assays using normal twocell embryos as negative wash controls (Fig. 2B). Thus, sperm binding was unaffected by the ZP3 mutations (Ser<sup>332</sup>  $\rightarrow$  Ala; Ser<sup>334</sup>  $\rightarrow$  Ala), which preclude the attachment of O glycans at those sites. Rather, sperm binding to the surface of the zona pellucida required uncleaved ZP2, in a process that was independent of fertilization and cortical granule exocytosis.

To determine the reversibility of gamete interactions on the surface of the zona pellucida, eggs and embryos were inseminated for 1 hour with normal sperm labeled with Hoechst stain. The fertilized eggs and embryos were then rinsed to remove loosely adherent sperm and challenged with capacitated Acr3-enhanced green fluorescent protein (EGFP) sperm (1 hour) followed by washing, using normal two-cell embryos as controls (Fig. 3A). Although comparable numbers of total sperm bound normal,  $Zp2^{Mut}$ , and  $Zp3^{Mut}$ eggs as well as  $Zp2^{Mut}$  embryos, 91 to 97% of the initially bound sperm had been replaced with Acr3-EGFP sperm (Fig. 3B). These results indicate that the reversibility of sperm adherence to the zona pellucida for eggs (normal,  $Zp2^{Mut}$ , and

 $Zp3^{Mut}$ ) is also observed in  $Zp2^{Mut}$  two-cell embryos in which ZP2 remains uncleaved.

Integral to current glycan release models of sperm-egg recognition is that a zona glycan also induces exocytosis of the acrosome, which is a subcellular organelle at the head of sperm (6, 17). Using Acr-EGFP sperm to monitor acrosome status,  $Zp2^{Mut}$  and  $Zp3^{Mut}$  eggs were fertilized in vitro. Sperm were present at the surface of the zona pellucida for >2 hours after insemination. The number of sperm adherent to normal,  $Zp2^{Mut}$ , and  $Zp3^{Mut}$ eggs was comparable among the genotypes at 1 hour (80.8  $\pm$  5.6, 76.7  $\pm$  4.5, and 77.6  $\pm$  13.5, respectively) and 2 hours (39.8  $\pm$  3.6, 45.8  $\pm$  3.3, and  $43.5 \pm 7.5$ , respectively). Sperm also remained adherent to the surface of the zona matrix surrounding  $Zp2^{Mut}$  embryos at 1 (98.4 ± 4.1) and 2  $(25.7 \pm 0.9)$  hours (Fig. 4A). During this time period, virtually all adherent sperm remained acrosomeintact. Thus, binding to the surface of the zona pellucida is not sufficient to induce acrosome exocytosis. However, only acrosome-reacted sperm



**Fig. 4.** Sperm binding to  $Zp2^{Mut}$  and  $Zp3^{Mut}$  eggs and embryos. (**A**) Normal (1 to 6),  $Zp2^{Mut}$  (7 to 9), and  $Zp3^{Mut}$  (10 to 12) eggs or  $Zp2^{Mut}$  two-cell embryos (13 to 15) were inseminated with capacitated *Acr*-EGFP sperm (1 × 10<sup>5</sup> ml<sup>-1</sup> of HTF), and fertilized eggs and embryos were imaged at 1, 2, and 4 hours by differential interference contrast and confocal microscopy. (**B**) Capacitated *Acr3*-EGFP sperm were incubated in the same media as in (A) and assayed by computer-assisted sperm analysis after 1, 2, 4, 6, and 24 hours. Each data point, which is an average of three independent biological samples ± SEM, reflects the percent of sperm meeting the specific criteria. (**C**) Ovulated eggs in cumulus from normal,  $Zp2^{Mut}$ , and  $Zp3^{Mut}$  mice were inseminated with capacitated sperm (1 × 10<sup>5</sup> sperm ml<sup>-1</sup> of HTF), and fertilization was assayed by the presence of two-cell embryos after overnight incubation. The data reflect the average of five separate experiments for each genotype (a total of 66 to 92 eggs) ± SEM.



are present in the perivitelline space, which suggests that acrosome exocytosis is initiated either before arrival at the surface of the zona pellucida or during penetration of the zona matrix (18).

By 4 hours after insemination, few Acr-EGFP sperm remained on the zona surface of normal,  $Zp2^{Mut}$ , and  $Zp3^{Mut}$  eggs (1.8 ± 0.4, 12.0 ± 1.8, and 1.1 ± 0.3, respectively) or  $Zp2^{Mut}$  embryos (10.9 ± 2.1). To determine whether release was secondary to acrosome exocytosis, sperm were stained with Alexa 568-conjugated soybean trypsin inhibitor (SBTI), which binds to the inner acrosomal membrane after the acrosome reaction. With rare exceptions (2 of 1929 sperm observed), no acrosome-reacted sperm were detected on the surface of the zona matrix. The disappearance of sperm from the zona surface of  $Zp2^{Mut}$  eggs and embryos (which must be independent of ZP2 cleavage) as well as from normal and  $Zp3^{Mut}$  eggs correlated with a pronounced decrease in progressive sperm motility (Fig. 4B), although causality has not been established.

In vitro fertilization was determined by the addition of capacitated sperm to ovulated eggs in cumulus obtained from normal,  $Zp2^{Mut}$ , and  $Zp3^{Mut}$  female mice. Both rescue lines had fertilization rates that were comparable to those observed with normal controls (Fig. 4C). To assess in vivo fertilization,  $Zp2^{Mut}$  or  $Zp3^{Mut}$  females were paired with corresponding transgenic females as controls and mated with normal male mice that were proven to be fertile. The size of  $Zp3^{Mut}$  litters was comparable to those of co-caged control female mice (table S1), indicating that  $Zp3^{Mut}$  mice have normal fertility both in vitro and in vivo.

However, only half (3 out of 6) of the  $Zp2^{Mut}$ females produced pups in vivo, with 36% as many litters, the size of which were significantly smaller than those of co-caged controls. Similar numbers of eggs and one-cell embryos were recovered from the oviducts of  $Zp2^{Mut}$  and control females after gonadotrophin stimulation or in vivo fertilization, respectively (tables S1 and S2). There was no evidence of supernumerary sperm in the perivitelline space  $(0.05 \pm 0.03 \text{ sperm per embryo}, n = 40 \text{ em-}$ bryos), indicating that an effective postfertilization block to polyspermy was imposed independently of ZP2 cleavage. After flushing oviducts at embryonic day 3.5 (E3.5), significantly fewer blastocysts were recovered from mutant as compared with control female mice, of which mating was confirmed by the presence of a copulatory plug (table S2). Thus, early embryonic loss rather than defects in fertility appears as the major contributor to the smaller litter sizes observed in  $Zp2^{Mut}$  females.

The normal fertility of  $Zp3^{Mut}$  mice is not consistent with glycan release models in which O glycans attached to ZP3 Ser<sup>332</sup> or Ser<sup>334</sup> play an essential role in sperm-egg recognition. More generally, the ability of sperm to bind to  $Zp2^{Mut}$  embryos after cortical granule exocytosis does not support any zona ligand in a glycan release model. Mutant mouse ZP2, which differs in only three amino acids from the native protein, has the same extent of posttranslational modifications and reconstitutes a zona pellucida in Zp2 null mice. The observed binding of sperm to  $Zp2^{Mat}$  two-cell embryos is not consistent with glycan release models, in which a cortical granule glycosidase cleaves off a zona glycan to account for the inability of sperm to bind after fertilization. For sperm to bind to the zona pellucida after cortical granule exocytosis, the candidate glycan would have to remain accessible to sperm and yet have been inaccessible for cleavage by a cortical granule glycosidase. This inconsistency applies both to N and O glycan candidate ligands. We thus conclude that glycan release models, as currently formulated, do not offer an adequate explanation of sperm-egg recognition.

Rather, recent and accumulating data support a ZP2 cleavage model for sperm-egg recognition, in which sperm adhere to the surface of the zona pellucida if ZP2 is intact, independent of fertilization and cortical granule exocytosis. Thus, sperm bind to normal eggs but not to two-cell embryos in which ZP2 has been cleaved by a protease that is released during cortical granule exocytosis. However, mutant ZP2 protein cannot be cleaved, and sperm bind to two-cell embryos derived from  $Zp2^{Mut}$ mice. A direct effect of ZP2 cleavage on the threedimensional matrix provides a parsimonious explanation of these results, rendering the zona pellucida either permissive (intact ZP2) or nonpermissive (cleaved ZP2) for sperm-egg recognition.

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## Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5988/216/DC1 Materials and Methods Figs. S1 to S3 Tables S1 and S2 References

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## Ku70 Corrupts DNA Repair in the Absence of the Fanconi Anemia Pathway

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A conserved DNA repair response is defective in the human genetic illness Fanconi anemia (FA). Mutation of some FA genes impairs homologous recombination and error-prone DNA repair, rendering FA cells sensitive to DNA cross-linking agents. We found a genetic interaction between the FA gene *FANCC* and the nonhomologous end joining (NHEJ) factor *Ku70*. Disruption of both *FANCC* and *Ku70* suppresses sensitivity to cross-linking agents, diminishes chromosome breaks, and reverses defective homologous recombination. Ku70 binds directly to free DNA ends, committing them to NHEJ repair. We show that purified FANCD2, a downstream effector of the FA pathway, might antagonize Ku70 activity by modifying such DNA substrates. These results reveal a function for the FA pathway in processing DNA ends, thereby diverting double-strand break repair away from abortive NHEJ and toward homologous recombination.

**P** anconi anemia (FA) proteins are components of a conserved metazoan DNA repair pathway that facilitates homologous recombination (HR) and DNA translesion synthesis (TLS) (1, 2). Genetic and biochemical evidence point to an upstream role for the FA pathway in interstrand cross-link repair during replication (3, 4). Consequently, FA knockout cell lines accumulate large numbers of chromatid breaks after exposure to cross-linking agents, indicating a crucial role for the FA proteins in resolving DNA double-strand breaks (DSBs) created at

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