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Zonadhesin Is Essential for Species Specificity of Sperm Adhesion to the Egg Zona Pellucida*

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Interaction of rapidly evolving molecules imparts species specificity to sperm-egg recognition in marine invertebrates, but it is unclear whether comparable interactions occur during fertilization in any vertebrate species. In mammals, the sperm acrosomal protein zonadhesin is a rapidly evolving molecule with species-specific binding activity for the egg zona pellucida (ZP). Here we show using null mice produced by targeted disruption of *Zan* that zonadhesin confers species specificity to sperm-ZP adhesion. Sperm capacitation selectively exposed a partial von Willebrand D domain of mouse zonadhesin on the surface of living, motile cells. Antibodies to the exposed domain inhibited adhesion of wild-type spermatozoa to the mouse ZP but did not inhibit adhesion of spermatozoa lacking zonadhesin. *Zan*$^{-/-}$ males were fertile, and their spermatozoa readily fertilized mouse eggs *in vitro*. Remarkably, however, loss of zonadhesin increased adhesion of mouse spermatozoa to pig, cow, and rabbit ZP but not mouse ZP. We conclude that zonadhesin mediates species-specific ZP adhesion, and *Zan*$^{-/-}$ males are fertile because their spermatozoa retain adhesion capability that is not species-specific. Mammalian sperm-ZP adhesion is therefore molecularly robust, and species-specific egg recognition by a protein in the sperm acrosome is conserved between invertebrates and vertebrates, even though the adhesion molecules themselves are unrelated.

Sperm adhesion to the egg extracellular coat is a key cell recognition event in animal reproduction. This essential interaction represents the first direct, physical contact between the gametes, and in diverse animals confers species specificity to fertilization (1). Sperm-egg recognition in externally fertilizing marine invertebrates such as sea urchin (phylum Echinodermata, class Echinoidea) and abalone (phylum Mollusca, class Gastropoda) occurs by species-specific binding of single proteins in the acrosome, a secretory organelle on the apical head of spermatozoa, to complementary molecules on the egg (2). Although species-specific gamete recognition in these two taxa is mediated by a conserved subcellular interaction (the sperm acrosome with the egg vitelline layer), the abalone and sea urchin gamete recognition proteins are structurally unrelated to each other (2). Nevertheless, in both taxa rapid molecular evolution by positive selection produced the structural diversity underlying the recognition proteins' species-specific binding activities (2), and the species specificity of fertilization conferred by these proteins contributes to the reproductive isolation of their constituent species (2).

Decades of effort have not produced a comprehensive understanding of mammalian sperm-ZP[4] adhesion. One challenge is that the mammalian fertilization environment *in vivo* cannot easily be reproduced *in vitro*. In addition, mammalian sperm populations are functionally heterogeneous because the cells undergo progressive, asynchronous physiological changes during transport and capacitation in the female tract that alter their adhesion activity prior to interaction with the egg at the site of fertilization (1). Despite such complications, however, numerous candidate ZP adhesion molecules have been identified, including β-1,4-galactosyltransferase (3), SED-1(4), proacrosin/acrosin (5), ZP3R/sp56 (6, 7), zonadhesin (8, 9), and others (10). Current evidence collectively suggests that mammalian gamete adhesion occurs by binding of multiple sperm proteins to the glycoprotein components of the ZP (10, 11), but no studies have defined the sequence of those interactions or their relative contributions to the overall specificity of sperm-egg recognition.

Among several suspected ZP adhesion molecules in mammalian spermatozoa (10), zonadhesin is unique in its ability to bind directly and in a species-specific manner to native ZP (8).

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We dedicate this paper to the memory of David L. Garbers.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4, Table 1, and Refs. 1–3.

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[4] The abbreviations used are: ZP, zona pellucida; ES, embryonic stem; GST, glutathione *S*-transferase; ANOVA, analysis of variance; LSD, least significant difference; VWD, von Willebrand D; MAM, meprin/A5 antigen/*mu* receptor tyrosine phosphatase; PNA, peanut agglutinin.

Like species-specific egg recognition proteins of sea urchin and abalone spermatozoa, zonadhesin is an acrosomal protein (12, 13) that becomes exposed by induced exocytosis during fertilization. Zonadhesin is homologous to von Willebrand factor and other adhesion molecules (9, 14) and differs dramatically among species not only as a consequence of rapid evolution by positive selection (2, 15, 16) but also because of domain duplication (14, 17), mRNA splice variation,[5] and processing heterogeneity during the functional maturation of the protein (12, 18, 19). Although the binding activity, localization, and other properties of zonadhesin suggest that it mediates species-specific ZP adhesion at the onset of acrosomal exocytosis (12), its function in fertilization has not been tested directly. Here we report results of *in vitro* and *in vivo* loss-of-function experiments showing that zonadhesin is a ZP adhesion molecule that confers species specificity to sperm-egg adhesion.

EXPERIMENTAL PROCEDURES

Zan Targeting—A null *Zan* allele was produced by replacing the proximal promoter and first six exons of the gene with a *Neo* cassette using the vector PGKneolox2DTA and 129S1/Sv-derived R1 embryonic stem (ES) cells. Details of the targeting procedure are provided in the legend for supplemental Fig. S1.

Null Animal Production and Colony Expansion—Cleavage stage Crl:(CD-1) ICR embryos (Charles River Laboratory) were aggregated with correctly targeted ES clones (20). Germ line-transmitting chimeras for ES cell clones ZV1-B9 ("B9") and ZV1-E5 ("E5") identified by agouti coat color and black eyes of offspring from crosses with CD-1 mice were then bred to 129S2/SvPasCrl (Charles River Laboratory) to establish mouse lines on a relatively uniform genetic background similar to that of the 129S1/Sv ES cells used for *Zan* targeting. Natural mating studies were performed with 129S2/SvPas mouse lines derived from both ES cell clones and also with 129S2/SvPas lines intercrossed with C57BL/6Crl mice to examine fertility on a more fecund background. Zonadhesin function *in vitro* was characterized using spermatozoa from littermates bred 5–6 generations to the C57BL/6Crl background.

Genotyping—The short arm Southern blot probe (336 bp) was amplified by PCR using Zan_202574F and Zan_202940R (primer sequences listed in supplemental Table S1). The long arm Southern probe (333 bp) was generated using primers Zan_long_F3 and Zan_long_R1. PCR genotyping of DNA from proteinase K-digested ear punches using primers Zan_195666F and Zan_195891 amplified a 225-bp band that is absent in the recombined *Zan* locus. Zan_199946R and the vector-derived primer ZV1_inloxF amplified a 357-bp band to detect the modified *Zan* locus.

Detection of Zonadhesin mRNA—RNA (2 μg) isolated using TRIzol (Invitrogen) from mouse testes flash-frozen in $N_2(l)$ was reverse-transcribed with 200 units of murine-Maloney leukemia virus reverse transcriptase (Invitrogen) and 25 ng/ml oligo$(dT)_{15}$. Two PCR primers sets (11F/13R and D3F/D3R2) amplified a 372-bp product encoded by exons 11–13 (5 kb downstream of the deletion) and a 329-bp product encoded by exons 33–35 (37 kb downstream of the deletion) using the first strand cDNA template (25 μg RNA equivalent). A third primer set (MAMF/R) amplified a 310-bp product in the region deleted by gene targeting and served as a negative control for cDNA from null animals. Positive control primer sets amplified 222-bp β2-microglobulin and 983-bp *G3pdh* products.

Zonadhesin Antibodies—Domain-specific antibodies to mouse zonadhesin and control antibodies to glutathione *S*-transferase (GST) were affinity-purified from rabbit antisera as for preparation of antibodies to pig zonadhesin (18). Antigenic regions (see Fig. 1*A*) of von Willebrand D3 (Ile^{2168}–Thr^{2270}) and D3p18 (Cys^{4502}–Lys^{4621}) domains (accession number AAL04416) encoded by nucleotides 6680–6989 and 13,669–14,050, respectively, of the mouse zonadhesin transcript (accession number U97068) were expressed in *Escherichia coli* strain BL21 as recombinant GST fusion proteins using pGEX-4T-1 (Amersham Biosciences).

Western Blots—Zonadhesin polypeptides were detected (18) using anti-D3 or anti-D3p18 (80 or 40 ng/ml overnight, respectively) on blots of disulfide-reduced (25 mM dithiothreitol) proteins extracted from cauda epididymal spermatozoa (1×10^6 sperm cells/lane) (21, 22) and resolved on 4–10% gradient gels.

Immunofluorescence—Sperm antigens were detected in methanol-fixed and permeabilized mouse spermatozoa (18) using anti-D3 (2 μg/ml) or anti-D3p18 (1 μg/ml) affinity-purified antibodies or anti-hyaluronidase antiserum (1:400). Bound antibody was visualized with Alexa Fluor 594®-conjugated goat anti-rabbit IgG (3 μg/ml; Molecular Probes, Eugene, OR). Acrosomes were then labeled with biotinylated lectin from peanut (*Arachis hypogaea*) agglutinin (PNA; 0.1 mg/ml; Sigma) detected with Alexa Fluor 488®-conjugated streptavidin (3 μg/ml; Molecular Probes). Cells were viewed by epifluorescence and phase contrast microscopy at 600× magnification. Zonadhesin on living, motile spermatozoa was detected by incubating cells in suspension with anti-D3 (2 μg/ml) or anti-D3p18 (1 μg/ml) antibodies (37 °C, 30 min). After centrifugation (500 × *g*, 3 min), bound antibody was detected either on cells in suspension or on cells smeared and dried on slides as for immunofluorescence on fixed cells, with simultaneous detection of acrosomes using PNA.

Sperm Capacitation and Mouse Fertilization in Vitro—Capacitation was inferred by assessing sperm cells' exocytotic response to 10 μM calcium ionophore A23187 (Sigma), as only capacitated spermatozoa undergo acrosomal exocytosis induced by the ZP or by subthreshold concentrations of A23187 (23). Spermatozoa were incubated 15–135 min in modified-Tyrode's medium (24) and then an additional 15 min with or without ionophore. Acrosomes were detected with PNA, and the percentage of capacitation was calculated as the difference in the number of cells lacking acrosomes with and without ionophore addition per 100 cells total (23). Mouse fertilization *in vitro* was also performed in modified-Tyrode's medium (24) using 5000 capacitated spermatozoa incubated 3 h with 15–20 cumulus-intact oocytes per 50-μl drop. Fertilization was scored by detection of Hoechst 33258-stained sperm nuclei in the egg cytoplasm and by expulsion of the second polar body. Effects of antibodies on fertilization *in vitro* were tested by including anti-D3, anti-D3p18, or anti-GST (1.5 μg/ml) during capacitation

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[5] T. L. Cheung, M. D. Wassler, S. Tardif, G. A. Cornwall, J. A. Harris, and D. M. Hardy, unpublished data.

and transferring the diluted antibodies with spermatozoa when oocytes were inseminated.

Sperm-ZP Adhesion Assays—Mouse sperm-ZP adhesion was assessed by gamete co-incubation as for fertilization *in vitro*, except that cumulus cells were first removed from oocytes with hyaluronidase (0.25 mg/ml, ≤10 min), 10–15 oocytes were incubated 30 min with 2500 spermatozoa per 50-μl drop, and sperm-oocyte complexes were washed twice by pipetting (65–75-mm mouth diameter). Two-cell mouse embryos recovered 28 h after overnight mating were included with oocytes, and oocytes were pipette-washed until all loosely attached spermatozoa were removed from the embryos. Spermatozoa were counted on ZP viewed by differential interference contrast microscopy. Antibody inhibition of mouse sperm-ZP adhesion was determined as for testing antibody effects on fertilization *in vitro*. Pig, rabbit, and bovine oocytes were aspirated from mature follicles of fresh ovaries, cumulus cells were removed with hyaluronidase, and sperm adhesion to their ZP was assessed the same as for mouse oocytes. Pig sperm capacitation was induced by a 3-h incubation in a Krebs-Ringer bicarbonate medium (22). In co-insemination experiments comparing species specificity of $Zan^{+/+}$ and $Zan^{-/-}$ sperm adhesion to the ZP simultaneously on the same oocytes, spermatozoa from the two genotypes were separately capacitated 75 min at 1×10^7 cells/ml and labeled with Hoechst 33258 or MitoTracker (Invitrogen) for the final 15 min of capacitation. The differentially labeled cells were mixed in defined ratios (25:75 or 50:50% null:wild type), and adhesion of the mixed cells to ZP of mouse, pig, rabbit, or bovine oocytes was tested as for ZP adhesion using unlabeled cells. Neither dye affected sperm motility, and both were used alternately on the two *Zan* genotypes in the ZP adhesion assays. 2500 sperm cells were added to each drop containing 10–15 eggs, representing a 400-fold total dilution of the sperm suspension.

Statistical Analyses—Differences in litter sizes, surface exposure of zonadhesin, antibody inhibition of fertilization, and sperm-ZP adhesion were tested for significance by analysis of variance (ANOVA) using general linear model procedures (SAS software). The Fisher's protected least significant difference (LSD) test was conducted when the main effect was significant ($p < 0.05$). The *Z*-test was used to compare the ratio of inseminated spermatozoa with the ratio of ZP-adherent cells in co-insemination ZP adhesion assays.

RESULTS

Production of Zonadhesin-null Mice—To produce spermatozoa lacking zonadhesin, we disrupted the mouse zonadhesin gene by homologous recombination (Figs. 1 and 2 and supplemental Fig. S1). Of seven targeted ES cell clones verified by Southern blotting (Fig. 1), two (B9 and E5) were used to make aggregation chimeras with Crl:(CD-1) ICR embryos. Germ line-transmitting, chimeric male founders for both lines were crossed to wild-type mice to produce $Zan^{+/-}$ and $Zan^{-/-}$ males and females (Fig. 1). Loss of expression from the targeted allele was confirmed by reverse transcription-PCR (Fig. 2*A*) to detect zonadhesin mRNA in testes and by Western blotting (Fig. 2*B*) and indirect immunofluorescence to detect zonadhesin protein in spermatozoa. On permeabilized spermatozoa from $Zan^{+/+}$ and $Zan^{+/-}$ males, affinity-purified antibodies specific for the zonadhesin von Willebrand D3 and D3p18 domains detected zonadhesin in 100% of cells by immunofluorescence, but no zonadhesin immunoreactivity was detected in spermatozoa from $Zan^{-/-}$ males (more than 20 individuals tested; not shown). Both antibodies also recognized an M_r 340,000 zonadhesin polypeptide (p340) in extracts of cauda epididymal spermatozoa from $Zan^{+/+}$ and $Zan^{+/-}$ males (Fig. 2*B*), but not in sperm proteins from $Zan^{-/-}$ males (more than 100 individuals tested), thereby confirming that the targeting strategy produced a null allele and that zonadhesin was absent from spermatozoa of null males.

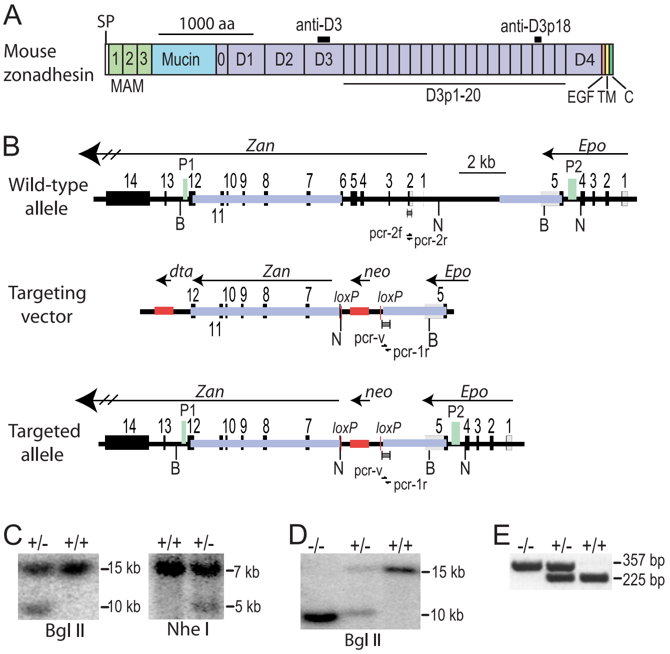

FIGURE 1. **Targeted disruption of *Zan*.** *A*, mouse zonadhesin is a mosaic protein comprising four extracellular domain types, including meprin/A5 antigen/mu receptor tyrosine phosphatase (MAM) and von Willebrand D (VWD) domains. The tandemly repeated VWD domains of the protein include an expansion of 20 partial domains (D3p1–20) related to the D3 domain that is absent from zonadhesin in spermatozoa of non-rodent species. Affinity-purified antibodies were directed toward 100–120-amino acid segments of the D3 and D3p18 domains (*black bars*). (*SP*, signal peptide). *aa*, amino acids; *EGF*, epidermal growth factor; *TM*, transmembrane; *C*, C terminus. *B*, homologous recombination of the targeting construct with the *Zan* locus replaced exons 1–6 and the presumptive promoter of *Zan* with a *PGK-neo* cassette and altered BglII and NheI restriction fragments. Southern blot probes P1 and P2 flank their respective arms in the wild-type and targeted alleles. Primer locations for PCR genotyping are also shown. *B*, Bg/II site; *N*, NheI site. *C*, on Southern blots of BglII-digested DNA from R1 ES cells (+/+) and targeted ES cell clones (+/−; one of seven shown), hybridization of probe P1 to predicted 15,393-bp (wild-type) and 9924-bp (mutant) fragments confirmed correct recombination of the long arm (*left panel*). On Southern blots of NheI-digested DNA from seven expanded ES clones, hybridization of probe P2 to predicted 7047-bp (wild-type) and 5363-bp (mutant) fragments confirmed correct recombination within the short arm (*right panel*). *D*, hybridization of probe P1 to the predicted wild-type and mutant BglII fragments on Southern blots confirmed production of $Zan^{-/-}$ offspring from an intercross of $Zan^{+/-}$ mice. *E*, predicted wild-type (357-bp) and mutant (225-bp) bands were amplified in PCR genotyping of offspring from $Zan^{+/-}$ intercrosses.

Zonadhesin-null Males Are Fertile—To test fertility of null mice on a relatively uniform genetic background related to the 129S1/Sv ES cells used for *Zan* targeting, we initially bred founders to 129S2/SvPasCrl. In brother-sister intercrosses on the 129S2/SvPasCrl background, null males from the two independent ES cell lines were equally fertile (B9 line: mean = 7.4

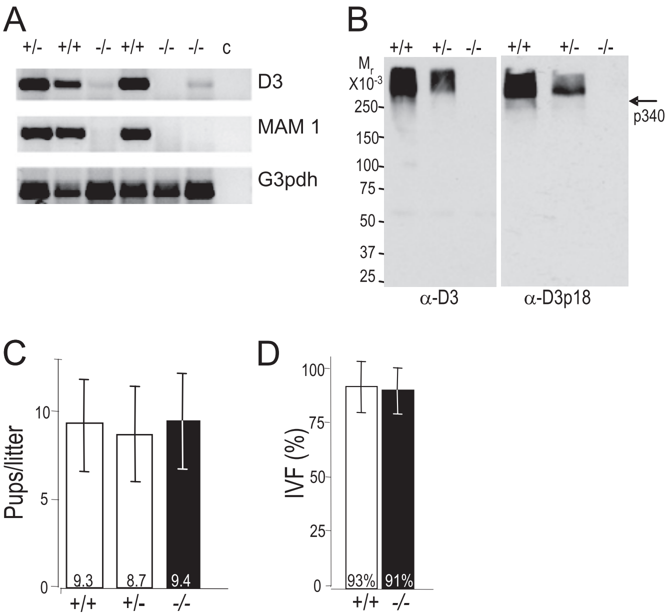

FIGURE 2. **Males lacking zonadhesin are fertile.** *A*, loss of zonadhesin mRNA was determined by reverse transcription-PCR in testes of littermates from intercrosses of *Zan*$^{+/-}$ mice. Primer sets amplified products from three regions of the zonadhesin transcript corresponding to the MAM1 (encoded by exons 3–5 deleted by the targeting strategy) and VWD3 domains (encoded by exons 33–35, 37 kb downstream of the deletion) of the protein. Strong products were amplified from *Zan*$^{+/+}$ and *Zan*$^{+/-}$ testicular RNA templates in all regions examined (including the MAM3 domain encoded by exons 11–13, not shown). No MAM1 region product (from exons replaced with *neo*) was amplified from *Zan*$^{-/-}$ testes, nor was a MAM3 region product (not shown). A faint VWD3 region product was rarely amplified from testes of 3 out of 11 *Zan*$^{-/-}$ males examined, possibly reflecting marginal synthesis of a truncated transcript from a cryptic promoter located among the 82 exons not deleted by the targeting strategy. A *G3pdh* gene-derived product was amplified from each template RNA. Animals' genotypes are indicated above each lane. Reactions in lane (*c*) contained no template RNA. *B*, affinity-purified antibodies to the mouse zonadhesin von Willebrand D3 and D3p18 domains bound an M_r 340,000 zonadhesin polypeptide (p340) on Western blots of sperm proteins (10^6 cells/lane, protein disulfides reduced) but detected no p340 in sperm proteins from *Zan*$^{-/-}$ males. *C* and *D*, regardless of zonadhesin genotype, on a C57BL/6 genetic background males sired ~9 offspring per litter (p = 0.92, n = 23), and their spermatozoa fertilized more than 90% of CD-1 mouse oocytes *in vitro* (p = 0.75, n = 5). *Error bars* indicate S.E. *IVF*, *in vitro* fertilization.

pups/litter, S.D. ±1.2, n = 8; E5 line: mean = 6.0 pups/litter, S.D. ±2.5, n = 13; p = 0.16). Overall, fertility of *Zan*$^{-/-}$ males from the two lines on the 129S2/SvPasCrl background (mean = 6.5 pups/litter, S.D. ±2.2, n = 21) did not differ (p = 0.17) from that of wild-type males (mean = 5.5 pups/litter, S.D. ±2.8, n = 23). To examine fertility in a more fecund strain and generate animals for *in vitro* studies, we bred 129S2/SvPasCrl mice from both ES cell lines to C57/BL6Crl. In brother-sister intercrosses on the C57BL/6Crl background, null males from the two lines were equally fertile (B9: mean = 9.6 pups/litter, S.D. ±2.0, n = 19; E5: mean = 8.6 pups/litter, S.D. ±2.3, n = 5; p = 0.6), and litter sizes produced by *Zan*$^{-/-}$ males were the same as those of heterozygous null and wild-type males in this more fecund mouse strain (Fig. 2*C*). The *Zan* genotype of females did not affect fertility on either genetic background. *In vitro*, spermatozoa from *Zan*$^{+/+}$ and *Zan*$^{-/-}$ males possessed identical ability to fertilize mouse oocytes (Fig. 2*D*). Consistent with the equivalent fertility of wild-type and zonadhesin-null spermatozoa, *Zan* genotype did not affect total or progressive motility (supplemental Fig. S2).

Progeny from heterozygote crosses showed no deviation (χ^2 (2, n = 135) = 4.29, p = 0.25) from the expected 1:2:1 Mendelian ratio. Likewise, male offspring from backcrosses between *Zan*$^{+/-}$ males and wild-type females (129S2/SvPasCrl and C57BL/6Crl data combined) revealed no deviation from the expected 1:1 heterozygote to wild-type ratio (χ^2 (1, n = 59) = 2.05, p = 0.25), indicating that the ability of an individual sperm cell to fertilize the egg was not affected by its *Zan* allele genotype. This finding could not be attributed to a lack of competition between sperm genotypes because immunofluorescence localization revealed that zonadhesin was present in all spermatozoa from *Zan*$^{+/-}$ males, although *Zan* is not transcribed until developing male germ cells become haploid spermatids (8, 12, 13).

Cell Surface Exposure of Zonadhesin during Sperm Capacitation—Because zonadhesin is an acrosomal protein, we determined whether it is exposed on living, motile cells capable of interacting productively with the ZP. In immunofluorescence, affinity-purified antibodies to the D3p18 or D3 domains of mouse zonadhesin (Fig. 1*A*) labeled 100% of fixed, permeabilized spermatozoa from wild-type males (Fig. 3, *A*, *B*, and *D*). Neither antibody labeled significant numbers of living, noncapacitated spermatozoa from *Zan*$^{+/+}$ males, nor did they label any spermatozoa from *Zan*$^{-/-}$ males (Fig. 3, *B* and *C*). However, after optimal sperm capacitation *in vitro* (Fig. 3*D*), anti-D3p18 labeled the apical heads of some but not all wild-type cells (Fig. 3, *A* and *D*). Surface exposure of the D3p18 domain closely tracked the kinetics of sperm capacitation (Fig. 3*D*). In contrast, anti-D3 immunoreactivity did not increase with sperm capacitation (Fig. 3*B*). Loss of zonadhesin did not affect PH-20/hyaluronidase immunoreactivity (Fig. 3*C* and supplemental Fig. S3), nor did it substantially affect the profiles of other gamete recognition proteins of spermatozoa, including SED-1, β-1,4-galactosyltransferase, proacrosin, ZP3R/sp56, ADAM 2, or ADAM 3, or alter the pattern of sperm phosphoproteins or total protein (supplemental Fig. S3), consistent with the fertility of *Zan*$^{-/-}$ males. Exposure of the D3p18 domain on living, motile spermatozoa was dependent on incubation in capacitating medium (Fig. 3*E*). No comparable capacitation-dependent increase in surface exposure of the D3 domain occurred (Fig. 3*E*), suggesting that this zonadhesin domain remains masked, while the D3p18 domain becomes exposed.

Zonadhesin Antibody Inhibits ZP Adhesion—To examine the gamete adhesion activity of surface-exposed zonadhesin, we tested the effects of the D3 and D3p18 antibodies on fertilization and ZP interaction. The D3p18 antibody partially inhibited fertilization of cumulus intact mouse eggs by wild-type spermatozoa *in vitro* (Fig. 4*A*). The partial inhibition was consistent with the surface exposure of zonadhesin on only 25–35% of cells and was significant in comparison with the effect of affinity-purified control antibody to GST (anti-D3p18: 14.3% inhibition; anti-GST: 3.2%; S.E. ±2.8%; $p < 0.05$). The D3p18 antibody did not inhibit fertilization by spermatozoa from *Zan*$^{-/-}$ males (1.9% inhibition *cf.* 2.7% by anti-GST). The anti-D3p18 antibody also inhibited ZP adhesion. Under conditions where no spermatozoa adhere to the ZP of two-cell embryos (Fig. 4*B*), adhesion of wild-type spermatozoa to the ZP of cumulus-free mouse eggs averaged 15–16 spermatozoa/oocyte either in the

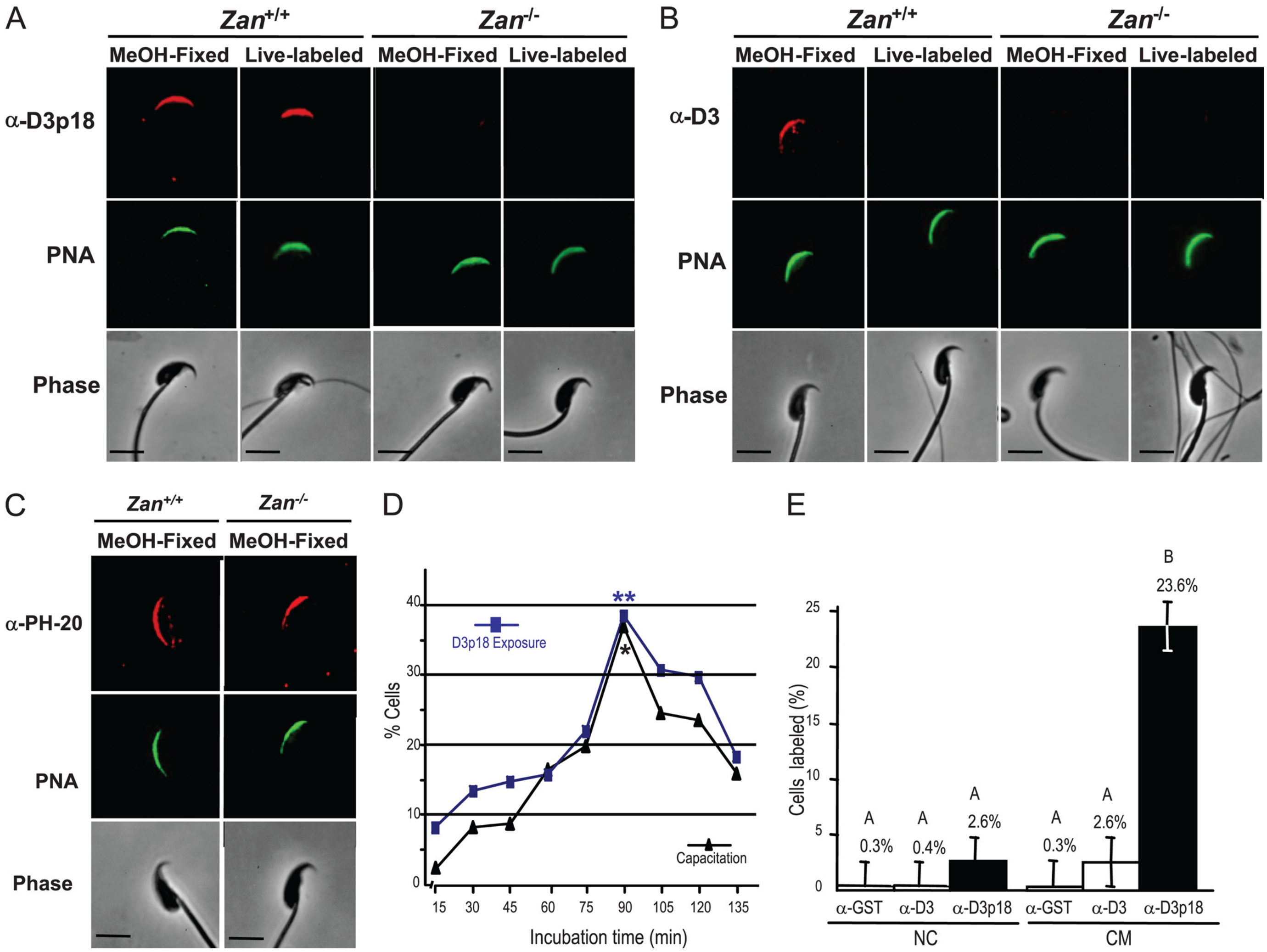

FIGURE 3. **Zonadhesin D3p18 domain is exposed during sperm capacitation.** *A* and *B*, by immunofluorescence, affinity-purified antibody to the zonadhesin D3p18 domain (α-D3p18; *A*) labeled the apical head of both living, capacitated spermatozoa (*Live-labeled*) and fixed, permeabilized spermatozoa (*MeOH-Fixed*), but anti-D3 (α-D3; *B*) detected zonadhesin only in fixed cells. The presence of the acrosome was confirmed by labeling with PNA. *Bar* = 10 μm. *C*, antibody to sperm hyaluronidase (α-PH-20) labeled the apical head of fixed, permeabilized spermatozoa from both $Zan^{+/+}$ and $Zan^{-/-}$ males. *D*, mouse sperm capacitation assessed by inducing acrosomal exocytosis with 10 μM A23187 (*black triangles*, $n = 4$) closely tracked exposure of the zonadhesin D3p18 domain (*blue squares*, $n = 4$; correlation = 1.0; $R^2 = 0.94$; * and **, significantly different from other time points). The proportion of responsive cells declined after 90 min because of increased spontaneous exocytosis. *E*, zonadhesin exposure on spermatozoa (determined by immunofluorescence with D3p18 antibody; *solid black bars*) increased on living, wild-type spermatozoa incubated in capacitating medium (*CM*) but not on cells incubated in non-capacitating medium (*NC*). D3 domain immunoreactivity in either capacitated or non-capacitated cells was not different from labeling by control antibody to GST (α-GST). ANOVA: S.E. ±2.2; LSD *A* and *B*: $p < 0.01$. $n = 5$.

absence of added antibody or in the presence of anti-GST or anti-D3 (Fig. 4*C*) but was inhibited by 50% to 7.5 spermatozoa/oocyte by anti-D3p18 (S.E. ±2.30 spermatozoa/oocyte; $p < 0.05$). The D3p18 antibody did not inhibit ZP adhesion by spermatozoa from $Zan^{-/-}$ males (20 spermatozoa/oocyte; Fig. 4*C*). None of the antibodies affected sperm motility (supplemental Fig. S4).

Loss of Zonadhesin Decreases Species Specificity of ZP Adhesion—To determine whether zonadhesin contributes to species specificity of ZP adhesion, we compared the ability of spermatozoa from wild-type or *Zan*-null males to recognize heterologous ZP. *Zan* genotype did not affect the relatively low adhesion (4–6 cells/ZP) of non-capacitated spermatozoa to pig or rabbit ZP (Fig. 5, *A* and *B*). With capacitation, adhesion of wild-type spermatozoa to the pig ZP did not increase, but adhesion of *Zan*-null spermatozoa increased 3-fold, to more than 15 cells/ZP (Fig. 5*A*). This capacitation-dependent adhesion of *Zan*-null mouse spermatozoa to pig ZP was comparable to the adhesion of homologous, capacitated pig spermatozoa (Fig. 5*A*). Loss of zonadhesin also increased sperm adhesion to rabbit ZP (Fig. 5*B*); with sperm capacitation, adhesion of *Zan*-null spermatozoa increased by more than 4-fold, to nearly 25 per rabbit ZP, but adhesion of wild-type spermatozoa did not increase significantly (Fig. 5*B*). Furthermore, when defined mixtures of sperm cells ranging from 100% wild-type to 100% *Zan*-null cells were incubated with pig or rabbit oocytes, ZP adhesion progressively increased with increasing proportion of *Zan*-null spermatozoa in the mixture (not shown).

We also directly compared adhesion of wild-type and *Zan*-null spermatozoa to ZP by co-inseminating oocytes with sperm cells from the two genotypes. Capacitated spermatozoa from $Zan^{-/-}$ and $Zan^{+/+}$ males were alternately labeled with differ-

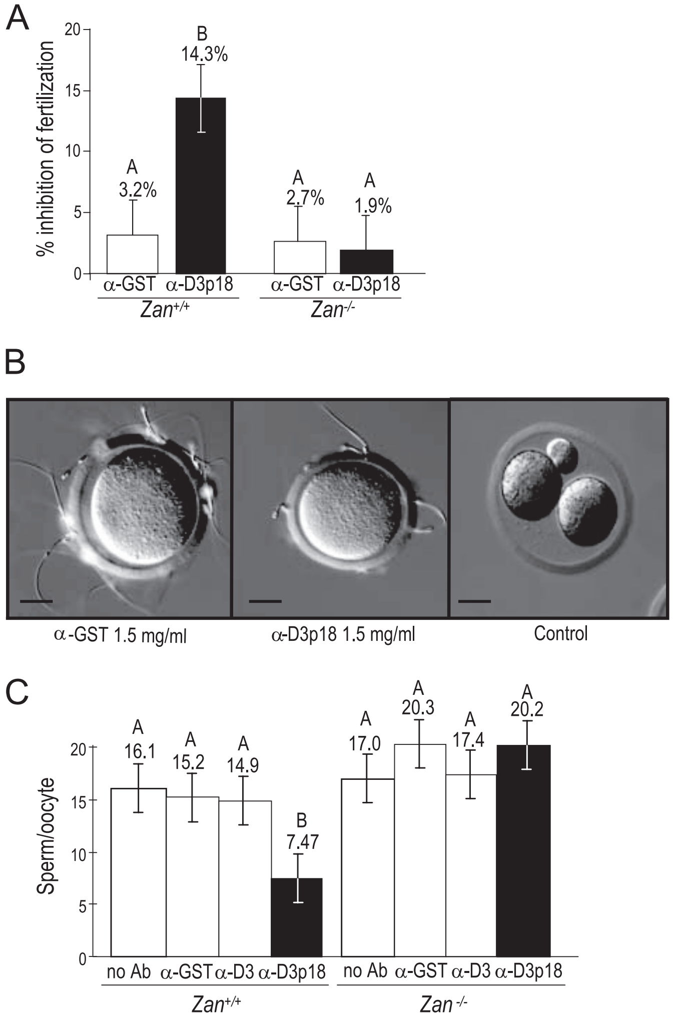

FIGURE 4. **Zonadhesin D3p18 domain antibodies inhibit fertilization and ZP adhesion.** *A*, antibody to the zonadhesin D3p18 domain inhibited *in vitro* fertilization of mouse oocytes by spermatozoa from wild-type but not zonadhesin-null males. Control antibody to GST did not affect fertilization rate regardless of *Zan* genotype. ANOVA: S.E. ±2.80; LSD *A* and *B*: $p < 0.01$; three independent experiments with >200 oocytes scored per antibody. *B*, antibody to the D3p18 domain of mouse zonadhesin (*center panel*) inhibited adhesion of capacitated, wild-type spermatozoa to ZP surrounding cumulus-free oocytes (viewed by differential interference contrast microscopy), as compared to effects of anti-GST control antibody (*left panel*), under conditions where no spermatozoa adhere to ZP surrounding two-cell embryos (*right panel*). *Bar* = 20 μm. *C*, antibody to the zonadhesin D3p18 domain (*solid black bars*) inhibited ZP adhesion by spermatozoa from wild-type but not zonadhesin-null males. Antibody to the zonadhesin D3 domain did not inhibit ZP adhesion in comparison to no antibody (*no Ab*) and anti-GST controls. ANOVA: S.E. = ±2.30; LSD *A* and *B*: $p < 0.01$; more than four independent experiments with >200 oocytes scored per antibody.

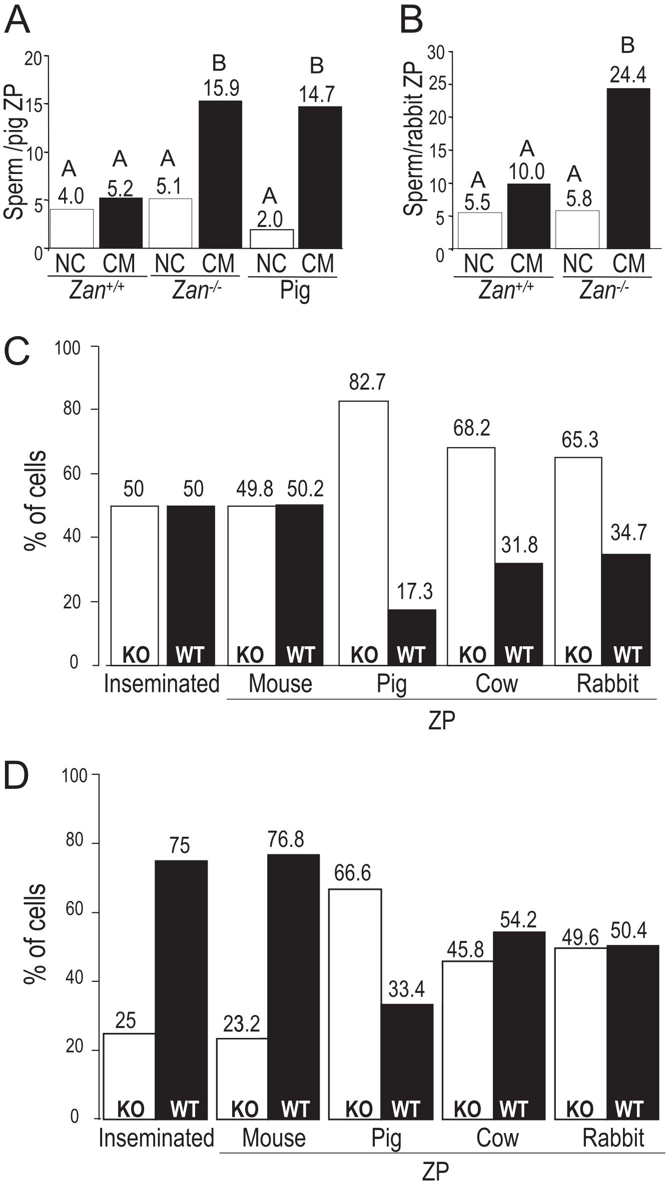

FIGURE 5. **Loss of zonadhesin decreases species specificity of ZP adhesion.** *A*, adhesion of wild-type mouse, zonadhesin-null mouse, or pig spermatozoa to ZP of pig oocytes was assessed after sperm incubation in capacitating (*CM*) or non-capacitating (*NC*) medium. Increased adhesion of spermatozoa from zonadhesin-null males to pig ZP was capacitation-dependent. ANOVA: *S.E.* ±1.0; LSD *A* and *B*: $p < 0.01$; four independent experiments with >150 oocytes scored/group. *B*, adhesion of wild-type or zonadhesin-null mouse spermatozoa to ZP of rabbit oocytes was examined as for *panel A*. ANOVA: S.E. ±5.5; LSD *A* and *B*: $p < 0.01$; four independent experiments with >100 oocytes scored/group. *C*, mouse, pig, cow, and rabbit oocytes were inseminated with equal numbers (50:50 ratio) of differentially labeled (Hoechst and MitoTracker) spermatozoa from wild-type (*WT*) and *Zan*-null (*KO*) males. On mouse oocytes, the ratio of ZP-adherent wild-type to zonadhesin-null cells did not differ from the 50:50 insemination ratio ($p > 0.05$; *Z*-test), but on pig, cow, and rabbit oocytes, the proportion of ZP-adherent, zonadhesin-null spermatozoa was significantly higher than the insemination ratio ($p < 0.001$; *Z*-test). *D*, oocytes were co-inseminated as in *panel C* but with a 25:75 ratio of zonadhesin-null to wild-type spermatozoa. The proportion of ZP-adherent zonadhesin-null spermatozoa was again significantly higher than the 25:75 insemination ratio on pig, cow, and rabbit oocytes ($p < 0.001$; *Z*-test) but not mouse oocytes.

ent dyes (MitoTracker or Hoechst 33258), mixed in defined ratios, and then incubated with cumulus-free oocytes. When mouse oocytes were co-inseminated with equal numbers of mouse spermatozoa from wild-type and *Zan*-null males, ZP adhesion by the two cell types was identical (Fig. 5*C*). In contrast, co-insemination of pig, rabbit, or bovine oocytes resulted in preferential adhesion of *Zan*-null spermatozoa to the heterologous ZP (Fig. 5*C*). This loss of species specificity was most dramatic in interactions with pig ZP, where adhesion of spermatozoa lacking zonadhesin was nearly 5-fold higher than adhesion of wild-type spermatozoa on co-insemination with equal numbers of the two cell types. Even when pig oocytes were co-inseminated with three times more wild-type than *Zan*-null spermatozoa, two-thirds of the ZP-adherent cells were *Zan*-null (Fig. 5*D*). At all tested ratios, adhesion of *Zan*-null spermatozoa to heterologous ZP was disproportionately high in comparison with the fraction of those cells in the insemination mixture (Z-test $p < 0.001$).

DISCUSSION

The fertility of *Zan*-null mice is reminiscent of the fully or partially fertile phenotypes observed on ablation of other ZP adhesion molecules (5, 25, 26). However, null alleles of other sperm proteins do not appear to result in decreased species specificity of sperm-ZP recognition. Our loss-of-specificity phenotype was not a consequence of generally increased adhesiveness of *Zan*-null spermatozoa because adhesion to mouse ZP did not increase. The detection of zonadhesin protein on the surface of some but not all live, capacitated spermatozoa and commensurate inhibition of ZP adhesion by zonadhesin antibodies support prior results implicating zonadhesin as a ZP adhesion molecule that functions during the initial stages of acrosomal exocytosis (12). From these findings, we conclude that zonadhesin in the acrosome becomes exposed on the apical head of spermatozoa, probably coincident with membrane fusion at the onset of acrosomal exocytosis, whereupon it mediates species-specific adhesion of mouse spermatozoa to the mouse ZP. We further conclude that loss of zonadhesin does not result in sterility because null spermatozoa retain capacity for ZP adhesion that is not species-specific.

Mammalian fertilization is not strictly species-specific, as shown vividly by the existence of natural (27) and anthropogenic (19) hybrids between closely related species. Some studies have raised questions about the extent and significance of species specificity in sperm-ZP adhesion (28, 29), and discrepancies in existing evidence cannot be resolved easily because of species differences in conditions for optimal capacitation and fertilization *in vitro* (23, 30). Nevertheless, here we overcame the inherent difficulties of such studies by directly comparing adhesion of *Zan*-null and wild-type mouse spermatozoa simultaneously on the same oocytes. Species specificity of ZP adhesion was readily observed when oocytes were co-inseminated with relatively low numbers of optimally capacitated spermatozoa that included cells with surface-exposed zonadhesin. The loss of ZP adhesion specificity in *Zan*-null mouse spermatozoa not only shows unequivocally that mammalian gamete interactions occur with some degree of species specificity, but together with results on species variation of zonadhesin (15, 16, 19), also suggests that variation in zonadhesin is a primary determinant of species specificity during egg recognition.

The robustness that multiple adhesion molecules confers to sperm-ZP recognition (31) should perhaps be expected in a process such as fertilization that is vital to species survival. Furthermore, transient, sequential binding of multiple adhesion molecules during ZP interactions might also be expected because spermatozoa must sustain adhesion as they shed membranes and acrosomal contents during acrosomal exocytosis and then penetrate through the ZP (10). Our results support a model of sperm-ZP interactions (Fig. 6) that includes degenerate/overlapping activities of multiple ZP adhesion molecules, including β-1,4-galactosyltransferase and SED-1 on the sperm cell surface and zonadhesin, proacrosin, and ZP3R/sp56 in the acrosome. In *Zan*-null cells, the two likely acrosomal mediators of ZP adhesion are proacrosin and ZP3R/sp56. Both of these proteins are prominent components of the sperm acrosomal matrix (32). Proacrosin ZP binding activity is well documented (33) and promiscuous (8). ZP3R/sp56 possesses ZP3 binding activity (6) and can mediate ZP adhesion (7), although the species specificity of its binding activity has not been reported. Because zonadhesin-null males are fertile, it should be feasible to breed animals with multiple null alleles and thereby identify the other proteins that individually or collectively compensate for its loss.

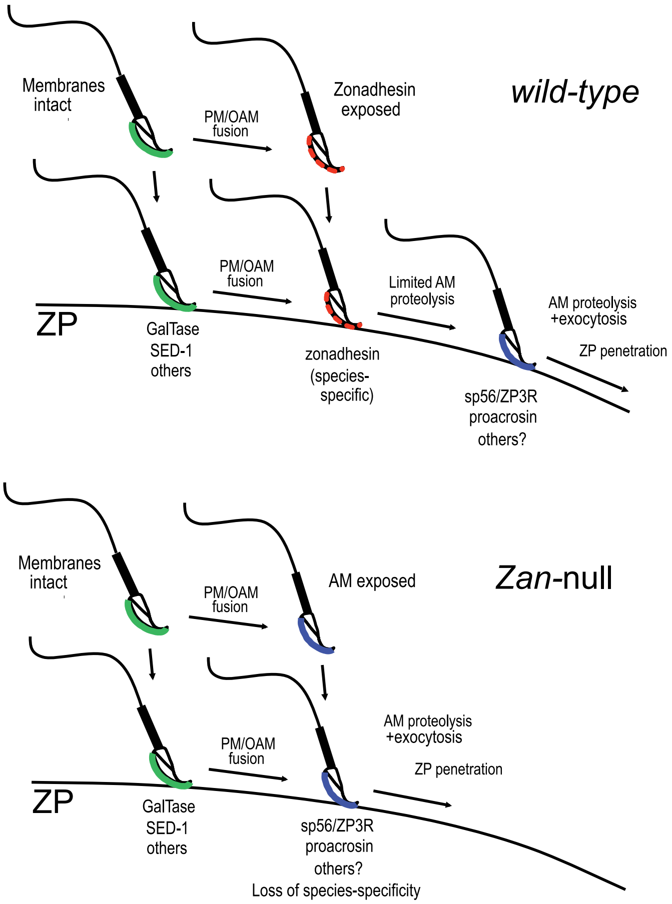

FIGURE 6. **Model of mammalian gamete adhesion.** Sperm-ZP adhesion occurs by successive, transient interaction of multiple sperm proteins with the ZP. Sperm plasma membrane proteins including β-1,4-galactosyltransferase (*GalTase*) SED-1, and possibly others mediate adhesion of acrosome-intact spermatozoa, resulting in induction of acrosomal exocytosis. Alternatively, acrosomal exocytosis may commence prior to ZP contact, bypassing the sperm plasma membrane interactions. Regardless of upstream pathway, zonadhesin exposed at the onset of exocytosis transiently mediates species-specific ZP adhesion (*upper panel*). During subsequent degranulation of the acrosome, ZP adhesion is sustained by the ZP binding activities of ZP3R/sp56 and proacrosin/acrosin associated with the acrosomal matrix. Zonadhesin antibodies inhibit adhesion of wild-type cells by interrupting the progression of the sequential interactions (*upper panel*), whereas in the absence of zonadhesin, ZP adhesion proceeds with no effect on downstream interactions mediated by ZP3R/sp56 and proacrosin. The combination of alternate exocytosis pathways and the degenerate/overlapping activities of multiple ZP adhesion molecules increases the robustness of the interaction. *PM*, plasma membrane; *OAM*, outer acrosomal membrane; *AM*, acrosomal matrix.

Reproductive processes are extraordinarily diverse among animal species. Although exocytosis of the acrosome must occur before a sperm cell can penetrate the egg (1), the content of the acrosome varies dramatically between marine invertebrates and mammals. The egg recognition molecules bindin and lysin are predominant components of the sea urchin (34) and abalone (35) acrosome, respectively. In these organisms, the primary function of the acrosome is to mediate species-specific gamete recognition when exocytosis is induced by female-derived factors surrounding the egg, and thereby prevent formation of sterile hybrids during external fertilization (2). Mammalian sperm cells also release and expose acrosomal components in response to egg factors (1), but the mammalian acrosome contains hundreds of proteins, including hydrolases (36) and relatively abundant matrix components (32, 37) that presumably support a more complex function of the acrosome during internal fertilization in mammals. Our finding that zonadhesin mediates species-specific ZP recognition shows that at least one function of the acrosome in fertilization is conserved between mammals and marine invertebrates. This conservation of acrosome function seems remarkable considering that the egg recognition proteins (lysin, bindin, and zonadhesin) are evolutionarily unrelated in these diverse species. Because mating barriers limit cross-species fertilization in animals that fertilize internally, potentially relieving the need for species-specific gamete recognition to prevent formation of sterile hybrids (28), the function of zonadhesin in species-specific ZP adhesion may reflect a contribution in ancestral mammals to the speciation process itself (2).

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