scribed in (*1*). Transformation of all three *clb5Δ* strains with the WT *CLB5* gene on a CEN plasmid [Ycplac111::CLB5 (LEU2); D74] restored sporulation.

20. For fluorescence-activated cell sorter (FACS) analysis, 1-ml aliquots of cultures were fixed in 70% ethanol, treated overnight with ribonuclease (RNase) A (300 μg/ml) at 37°C, rinsed in phosphate-buffered saline (PBS), and stained with propidium iodide (40 μg/ml in PBS); 10,000 cells per sample were analyzed on a Becton-Dickinson FACSscan. The same propidium iodide–stained cells were used to record chromosome segregation by fluorescence microscopy. RNA extraction and RNA blotting conditions were as described in (*2*).
21. L. Dirick and L. Goetsch, unpublished data.
22. S. Chu and I. Herskowitz, *Mol. Cell* **1**, 685 (1998).
23. Protein immunoblot analysis of Sic1 was done as described [U. Surana *et al.*, *EMBO J.* **12**, 1969 (1993); A. Amon *et al.*, *Cell* **77**, 1037 (1994)] with a 1:2000 dilution of polyclonal antibody to Sic1 (M. Tyers). Proteins were detected with the enhanced chemiluminescence detection system (ECL; Amersham). Kinase assays were done as described in [U. Surana *et al.*, *EMBO J.* **12**, 1969 (1993)] starting from 140 μg of protein, except for *ime2Δ* in YPD where 70 μg was used. The monoclonal antibody to hemagglutinin (HA), 12CA5, was used at a 1:100 dilution to immunoprecipitate the HA3-tagged Clb5 proteins (*8*). Specificity was confirmed on an SK1 WT strain carrying untagged *CLB5* (L111).
24. Y. Iino, Y. Hiramine, M. Yamamoto, *Genetics* **140**, 1235 (1995).
25. C. J. Sherr, *Cell* **79**, 551 (1994).
26. A. P. Mitchell, *Microbiol. Rev.* **58**, 56 (1994).
27. T. T. Nugroho and M. D. Mendenhall, *Mol. Cell Biol.* **14**, 3320 (1994).
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Fertilization Defects in Sperm from Mice Lacking Fertilin β

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Fertilin, a member of the ADAM family, is found on the plasma membrane of mammalian sperm. Sperm from mice lacking fertilin β were shown to be deficient in sperm-egg membrane adhesion, sperm-egg fusion, migration from the uterus into the oviduct, and binding to the egg zona pellucida. Egg activation was unaffected. The results are consistent with a direct role of fertilin in sperm-egg plasma membrane interaction. Fertilin could also have a direct role in sperm-zona binding or oviduct migration; alternatively, the effects on these functions could result from the absence of fertilin activity during spermatogenesis.

The sperm surface protein fertilin, a member of the ADAM family, is a heterodimer composed of α and β subunits (*1*–*4*). The ADAM family is comprised of structurally related cell surface proteins proposed to have cell adhesion activity, protease activity, or both (*5*). Fertilin α and β are made as precursors in spermatogenic cells and are processed before sperm maturation is complete (*2*, *6*). Both precursors are composed of the multiple domains found in all ADAM family membrane proteins: pro-, metalloprotease, disintegrin, cysteine-rich, transmembrane, and cytoplasmic domains (*4*, *7*). Proteolytic processing removes the pro- and metalloprotease domains, leaving an NH_2-terminal disintegrin domain on mature forms of the α and β subunits (*3*, *8*). Processed fertilin may promote sperm-egg binding, fusion, and egg activation (*9*–*11*).

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We disrupted the fertilin β gene, which shows testis-specific expression, by deleting exon 14. This exon encodes a region of the disintegrin domain that has been predicted to include the active binding site of fertilin β (*9*, *12*) (Fig. 1A). Fertilin β, using this active site, apparently binds to an egg integrin $\alpha_6\beta_1$ (*13*). Homozygous (fertilin $\beta^{-/-}$) mutant mice were identified by polymerase chain reaction (PCR) and Southern (DNA) blot analyses of genomic DNA (Fig. 1B). Fertilin $\beta^{-/-}$ mice (both male and female) are viable and develop normally. Both precursor and processed fertilin β proteins were absent from spermatogenic cells and mature sperm, respectively, from fertilin $\beta^{-/-}$ male mice (Fig. 1C). In mice lacking fertilin β, the level of fertilin α precursor was reduced (Fig. 1C). Fertilin α may be degraded when unable to form a heterodimer with β. Similar loss of one subunit of a plasma membrane heterodimer has been observed in other cases when the gene

Table 1. Phenotypic analysis in fertilin $\beta^{+/+}$ and fertilin $\beta^{-/-}$ mice. Data in sperm analysis (*20*) represent the mean ± SEM of *n* individual measurements indicated in parentheses. Cauda epididymal sperm were used for motility analysis. Motility parameters were measured by computer-assisted sperm analysis (*21*). Additional motility parameters measured, including amplitude of lateral head displacement, beat cross frequency, mean angle of deviation, average path velocity, and straight line velocity, were not different between +/+ and −/−. Also, there was no time-dependent difference during capacitation in all the motility parameters between +/+ and −/−. The values shown for acrosome reaction are from sperm incubated in medium with 3% BSA. At a lower concentration of BSA (0.5%), the values for acrosome reaction were also not different between +/+ and −/−. The numbers of eggs observed in egg activation (*20*) are shown in parentheses.

Parameter	Genotype	
	+/+	−/−*
Sperm analysis		
Number of sperm		
Epididymal (×10^7)	3.8 ± 1.0 (4)	4.4 ± 0.7 (4)
Ejaculated (×10^6)	3.2 ± 0.9 (7)	3.7 ± 0.3 (8)
Motility		
Motile sperm (%)	67 ± 8 (16)	62 ± 5 (17)
Curvilinear velocity (μm/s)	266.0 ± 7.1 (4)	245.8 ± 20.4 (3)
Linearity (%)	21.5 ± 2.9 (4)	20.6 ± 4.4 (3)
Straightness (%)	62.9 ± 5.4 (4)	62.9 ± 7.5 (3)
Acrosome reaction (%)		
Incubation time (min)		
0	14 ± 4 (2)	15 ± 4 (2)
120	39 ± 7 (2)	38 ± 2 (2)
270	57 ± 6 (4)	60 ± 3 (4)
Egg activation		
Fused eggs with Ca^{2+} oscillations (%)	100 (219)	98.2 (56)
Fused eggs with polar body formation (%)	100 (29)	82.4 (17)

* Values for all parameters are not significantly different from +/+ ($P > 0.3$, Student's *t* test).

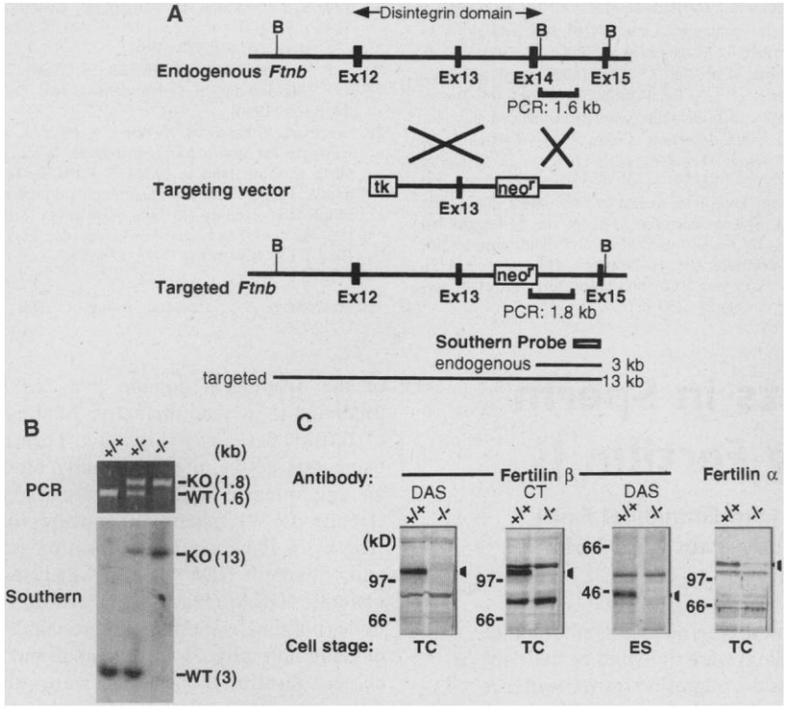

Fig. 1. Generation of mice carrying a mutation in the fertilin β gene (*23*). (**A**) Gene-targeting strategy. Exons and introns are represented by vertical bars and intervening horizontal lines, respectively. A neomycin-resistance gene driven by a PGK promoter (*neo*^{r}) and herpes simplex virus thymidine kinase gene (tk) are indicated as white boxes. A region of the fertilin β gene, containing exon 14 that encodes the predicted disintegrin domain active site, was replaced by the *neo*^{r} cassette by homologous recombination. This caused the loss of a diagnostic Bgl II (B) restriction site near exon 14. (**B**) Polymerase chain reaction (PCR) and Southern blot analysis of tail DNA from fertilin $\beta^{+/+}$, fertilin $\beta^{+/-}$, and fertilin $\beta^{-/-}$ mice. (**C**) Protein immunoblot analysis of spermatogenic cells and sperm. Fertilin β was identified with anti-peptide antibodies that recognize the putative disintegrin domain active site (DAS) or a cytoplasmic tail domain (CT). Antibody against the fertilin α metalloprotease domain was used to probe fertilin α. Cell stage: TC, testicular spermatogenic cell; ES, cauda epididymal sperm.

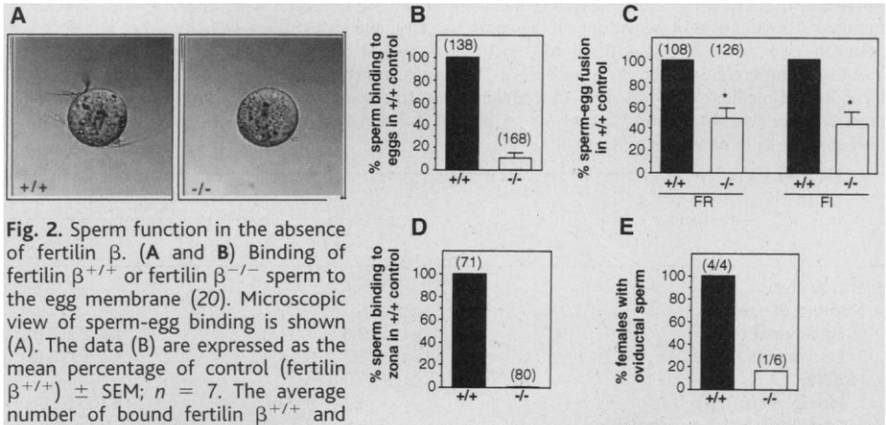

Fig. 2. Sperm function in the absence of fertilin β. (**A** and **B**) Binding of fertilin $\beta^{+/+}$ or fertilin $\beta^{-/-}$ sperm to the egg membrane (*20*). Microscopic view of sperm-egg binding is shown (A). The data (B) are expressed as the mean percentage of control (fertilin $\beta^{+/+}$) ± SEM; $n = 7$. The average number of bound fertilin $\beta^{+/+}$ and fertilin $\beta^{-/-}$ sperm per egg was 8.8 ± 2.4 and 1.1 ± 0.5, respectively. (**C**) Fusion of fertilin $\beta^{+/+}$ or fertilin $\beta^{-/-}$ sperm with the egg membrane (*20*), expressed as the mean percentage of control ± SEM; $n = 6$. Two measures of fusion were used: fertilization rate (FR, percentage of eggs fused with at least one sperm) and fertilization index (FI, mean number of fused sperm per egg). The average values of FR and FI in controls were 66.6 ± 8.3 and 0.74 ± 0.11, respectively. $^*P < 0.005$ compared to +/+. (**D**) Binding of fertilin $\beta^{+/+}$ or fertilin $\beta^{-/-}$ sperm to zona pellucida (*20*), expressed as the mean percentage of control ± SEM; $n = 3$. The average number of bound fertilin $\beta^{+/+}$ and fertilin $\beta^{-/-}$ sperm per zona-intact egg was 15.6 ± 2.4 and 0.1 ± 0.1, respectively. (**E**) Presence of fertilin $\beta^{+/+}$ or fertilin $\beta^{-/-}$ sperm in the oviduct (*24*). In the single case where fertilin $\beta^{-/-}$ sperm were found in the oviduct, their number was about one-tenth of that found for fertilin $\beta^{+/+}$ sperm. The numbers in parentheses indicate the numbers of eggs (A to D) and the numbers of animals (E) observed.

for the second subunit was knocked out (*14*).

Microscopic analysis indicated that the morphology of fertilin $\beta^{-/-}$ sperm was normal. The number and motility of ejaculated as well as epididymal sperm were not affected by the fertilin β knockout (Table 1). Fertilin $\beta^{-/-}$ sperm underwent normal spontaneous acrosome reaction during capacitation (Table 1).

We analyzed sperm functions in fertilin $\beta^{-/-}$ sperm using in vitro sperm-egg adhesion and fusion assays. We tested interaction of sperm and egg plasma membranes using eggs from which the zonae pellucidae had been removed. Binding of fertilin $\beta^{-/-}$ sperm to the egg plasma membrane was reduced to one-eighth the original value (Fig. 2, A and B). Microscopic observation during sperm-egg incubation revealed that the mutant sperm made contact with the egg surface as frequently as wild-type (WT) sperm, but most of them failed to attach to the egg membrane.

The rate of fertilin $\beta^{-/-}$ sperm fusion with the egg plasma membrane was 45 to 50% of the rate of WT sperm fusion (Fig. 2C). Thus, the reduction in mutant sperm binding is coupled with a corresponding, but less extreme, reduction in the rate of mutant sperm fusion.

It has been proposed that interaction between fertilin β and an egg receptor (integrin) during sperm-egg adhesion leads to egg activation (*9*, *10*). To test this hypothesis, we monitored egg-activation events in the eggs fused with fertilin $\beta^{-/-}$ sperm. The pattern and frequency of intracellular Ca^{2+} oscillations, an early sign of egg activation, were the same in WT eggs fused with mutant sperm or WT sperm (Table 1). Additionally, WT eggs, fused with fertilin $\beta^{-/-}$ sperm, completed meiosis and formed a second polar body at the same incidence as those fused with fertilin $\beta^{+/+}$ sperm (Table 1). These results suggest that fertilin β is not required for egg activation.

Unexpectedly, fertilin $\beta^{-/-}$ sperm were severely impaired in other steps of fertili-

Table 2. Fertility of fertilin $\beta^{+/+}$, fertilin $\beta^{+/-}$, and fertilin $\beta^{-/-}$ males (*22*).

Parameter	Genotype of males		
	+/+	+/−	−/−
Number of males mated	9	6	17
Number of females mated	40	27	74
Males producing plugs (%)	89	100	100
Pregnant females (%)	50	48	4
Average litter size	5.3	4.0	0.1
Fertility rate*	100	75	2

* Defined as the percentage of average litter size in +/+.

zation. Results from in vitro sperm-zona binding assays in which fertilin $\beta^{-/-}$ sperm were incubated with zona-intact eggs indicate that mutant sperm were unable to adhere to the zona pellucida (Fig. 2D). Also, fertilin $\beta^{-/-}$ sperm were rarely found in the oviduct of WT females after mating with mutant males although normal numbers of sperm, with normal motility, were found in the uterus (Fig. 2E and Table 1). This suggests that mutant sperm are defective in migrating into the oviduct, a process in which sperm may bind transiently to the epithelium of the uterotubal junction (*15*).

The in vivo fertility rate of fertilin $\beta^{-/-}$ males was reduced 98% relative to WT (Table 2). Fertilin $\beta^{-/-}$ females showed normal fertility when mated with WT males (*16*). Mating experiments showed no significant difference in the frequency of copulation plugs between females mated with fertilin $\beta^{+/+}$ and fertilin $\beta^{-/-}$ males (Table 2). It is likely that infertility of the knockout males results from the combined effect of multiple defects in sperm function, in particular, absence of sperm from the oviduct and inability to bind to the zona pellucida.

Relevant to previous hypotheses about fertilin function, the finding that fertilin $\beta^{-/-}$ sperm are defective in sperm-egg plasma membrane binding (Fig. 2, A and B) indicates that fertilin β has a key role in plasma membrane adhesion. Other sperm surface molecules, such as cyritestin (*17*), may also be active in plasma membrane adhesion. Fertilin β and cyritestin may act either sequentially or concurrently, and the activity of both may be required for successful adhesion of a motile sperm to the egg plasma membrane.

Although fertilin β binding is important in sperm-egg plasma membrane adhesion, it is not required for egg activation. However, binding by fertilin β seems essential for the normal rate of sperm-egg fusion. The fusion observed in our in vitro assays with mutant sperm indicates that mutant sperm in vitro may enter the fusion pathway downstream from fertilin β binding or that a second, fertilin β–independent path to fusion exists.

Sperm lacking fertilin β are defective in binding to the zona pellucida. The time-dependent changes in the spontaneous acrosome reactions between WT and mutant sperm were similar. Also, there was no difference during capacitation in the motility parameters between WT and mutant sperm (Table 1). Thus, impairment of binding to the zona pellucida is not due to altered acrosome reaction or motility in mutant sperm. We do not know the mechanism by which fertilin might function in sperm-zona binding. Several different sperm surface proteins have been proposed to function as zona adhesion molecules (*10*). Although fertilin could have a direct role in sperm-zona binding, neither fertilin α nor β shares sequence homology with any of the previously identified candidate proteins for zona binding.

The result that fertilin $\beta^{-/-}$ sperm are rarely found in the oviduct indicates that a sperm function besides motility is required for sperm to enter the oviduct. Sperm are stored temporarily at the uterotubal junction and adjacent isthmus in the oviduct before they migrate toward the ampulla (*15*). Sperm undergo transient adhesion to the epithelium at this storage site, but it is unknown whether this adhesive interaction is required for further progression into the oviduct. A required adhesion step, involving fertilin, could occur at this site.

Poor adhesion of sperm to the zona or poor sperm progression into the oviduct could also be due to altered activity of fertilin during spermatogenesis, in particular, the partial absence of fertilin α (Fig. 1C). Like the ADAM protein kuzbanian, which has a metalloprotease activity required in development of the nervous system (*18*), fertilin α (but not β) has the correct amino acids in its metalloprotease domain active site to be a functional protease. During spermatogenesis, the fertilin α metalloprotease might process and thus regulate sperm molecules involved in zona binding and sperm transport. However, sperm surface proteins (galactosyl transferase, PH-20, and cyritestin) potentially involved in these sperm-binding processes are unaltered on fertilin $\beta^{-/-}$ sperm (*19*). Continued effort to understand ADAM function in this system should provide more information about the versatile capabilities of this protein family and the molecular mechanisms underlying mammalian fertilization.

References and Notes

1. P. Primakoff, H. Hyatt, J. Tredick-Kline, *J. Cell Biol.* **104**, 141 (1987).
2. C. P. Blobel, D. G. Myles, P. Primakoff, J. M. White, *ibid.* **111**, 69 (1990).
3. C. P. Blobel *et al.*, *Nature* **356**, 248 (1992).
4. T. G. Wolfsberg *et al.*, *Dev. Biol.* **169**, 378 (1995).
5. C. P. Blobel, *Cell* **90**, 589 (1997).
6. B. M. Phelps, D. E. Koppel, P. Primakoff, D. G. Myles, *J. Cell Biol.* **111**, 1839 (1990).
7. T. G. Wolfsberg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10783 (1993).
8. L. Lum and C. P. Blobel, *Dev. Biol.* **191**, 131 (1997).
9. D. G. Myles, *ibid.* **158**, 35 (1993).
10. W. J. Snell and J. M. White, *Cell* **85**, 629 (1996).
11. D. G. Myles and P. Primakoff, *Biol. Reprod.* **56**, 320 (1997); D. Bigler, M. Chen, S. Waters, J. M. White, *Trends Cell Biol.* **7**, 220 (1997).
12. D. G. Myles, L H. Kimmel, C. P. Blobel, J. M. White, P. Primakoff, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4195 (1994); C. Cho, L. Turner, P. Primakoff, D. G. Myles, *Dev. Genet.* **20**, 320 (1997).
13. E. A. C. Almeida *et al.*, *Cell* **81**, 1095 (1995).
14. R. O. Hynes, *Dev. Biol.* **180**, 402 (1996); M. Zijlstra *et al.*, *Nature* **344**, 742 (1990).
15. R. Yanagimachi, in *The Physiology of Reproduction*, E. Knobil and J. D. Neill, Eds (Raven, New York, 1994), pp. 189–317; S. S. Suarez, *Biol. Reprod.* **36**, 203 (1987); T. T. Smith and R. Yanagimachi, *ibid.* **42**, 450 (1990).
16. C. Cho *et al.*, data not shown.
17. R. Yuan, P. Primakoff, D. G. Myles, *J. Cell Biol.* **137**, 105 (1997); B. Linder and U. A. O. Heinlein, *Dev. Growth Differ.* **39**, 243 (1997).
18. J. Rooke, D. Pan, T. Xu, G. M. Rubin, *Science* **273**, 1227 (1996); D. Pan and G. M. Rubin, *Cell* **90**, 271 (1997).
19. C. Cho *et al.*, unpublished observation.
20. Sperm were either directly isolated from males or obtained from females mated with males [P. Olds-Clarke, *Biol. Reprod.* **34**, 453 (1986)]. Epididymal sperm or uterine sperm (flushed from the uterus after mating) were incubated in Whittingham's medium, containing 3% bovine serum albumen (BSA) for capacitation, for 2 to 4 hours (for sperm-egg fusion assay) or 1 to 3 hours (for zona-binding assay). Eggs were obtained from C57BL6/N females superovulated by pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) as described (*17*). Eggs were incubated with sperm in 100-μl drops of Whittingham's medium with 3% BSA under mineral oil at a concentration of 10^5 to 10^6 sperm per milliliter for 30 to 50 min (zona-free eggs) and 20 to 50 min (zona-intact eggs). Ca^{2+} oscillations in fertilized eggs were detected [D. Kline and J. T. Kline, *Dev. Biol.* **149**, 80 (1992)] by preloading the DAPI (4′,6′-diamidino-2-phenylindole)–labeled eggs with the Ca^{2+} indicator Oregon Green 488 BAPTA-1 AM.
21. J. Seed *et al.*, *Reprod. Toxicol.* **10**, 237 (1996).
22. Each male (2 to 4 months old) was placed with one to two females (C57BL6/J or C57BL6/N) for 5 days to 2 months. One group of mated females was killed to count the number of fetuses at 10 to 15 days after the first day of mating. From the other group of females, the number of pups born was counted.
23. Two cloned DNA fragments from mouse strain 129 fertilin β genomic DNA (*12*) were used to construct a targeting vector: a 4.1-kb Eco RV–Eco RV fragment (containing exon 13) and a 1.3-kb Bgl II–Bam HI fragment (containing a portion of the intron between exons 14 and 15) as 5′ and 3′ homologous arms, respectively. E14TG2a/BK4 embryonic stem cells, derived from the 129/Ola strain, were electroporated with Sal I–linearized targeting vector. Chimeric mice were bred to C57BL6/N females to produce heterozygous F_1 progeny carrying the targeted fertilin β gene.
24. Female mice (C57BL6/J or C57BL6/N) were primed with hormones and mated with males as described above. At 1.5 to 7 hours after a plug was found, the oviduct was dissected out of the females and flushed or diced in Whittingham's medium to release sperm and cumulus masses with eggs. The presence of sperm was determined by counting sperm that were fused with eggs, bound to the egg surface, found with cumulus cells, and found free in the oviduct.
25. Animal care was in accordance with U.S. Public Health Service guidelines for use of animals, and all procedures were approved in advance by the Institutional Animal Care and Use Committee of the University of California, Davis, and the National Institute of Environmental Health Science.

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