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# Sperm equatorial segment protein 1, SPESP1, is required for fully fertile sperm in mouse

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## Summary

Mammalian fertilization is a multistep process that culminates in the fusion of the sperm and egg plasma membrane. It is widely accepted that the equatorial segment of the acrosome-reacted sperm is important in initiating fusion with the egg plasma membrane during fertilization. There are various proteins known to be distributed only in the equatorial segment of sperm. The role of these proteins must be clarified to understand the membrane fusion process. We produced a mouse line that lacked SPESP1 (sperm equatorial segment protein 1) and analyzed the fertilizing ability of the sperm. The average number of pups that were fathered by *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ males was significantly lower than that of wild-type fathers. In these mouse lines, fewer sperm were found to migrate into oviducts and fewer eggs were fertilized. The *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ sperm showed a lower fusing ability compared with the wild-type sperm. The disruption of *Spesp1* was shown to cause an aberrant distribution of various sperm proteins. Moreover, scanning electron microscopy revealed that the membrane in the equatorial segment area, which usually forms an acrosomal sheath, disappears after acrosome reaction in *Spesp1*-deficient mice. It was demonstrated that SPESP1 is necessary to produce the fully 'fusion competent' sperm.

**Key words:** Equatorial segment, Fertilization, Knockout mouse, Sperm-egg fusion

## Introduction

After meiosis, terminally differentiated gametes are no longer able to divide and need to fuse with gametes from the other sex to survive and continue the species. Thus, cell-cell fusion is a very important process in species that adopt sexual reproduction. However, until recently, the precise mechanism of sperm-egg fusion has remained unclear. At the molecular level, the only factors that have been proved to be essential are IZUMO1 on the sperm (Inoue et al., 2005) and CD9 on the egg (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Electron microscopy indicated that the equatorial segment is the place where fusion initiates in mammals (Yanagimachi, 1994). This suggests that the fusion protein has to be localized in the equatorial segment. However, the fusion protein should not be available in the equatorial segment of fresh sperm because sperm are not able to fuse with eggs before acrosome reaction. IZUMO1 is not expressed on the sperm surface, and appears on the sperm surface only after acrosome reaction. However, it localizes not only in the equatorial segment, but also spreads into the post-acrosomal region (Kawai et al., 1989). Therefore, the localization of IZUMO1 does not perfectly match the fusion competent area observed using electron microscopy. This led us to speculate that there might be a protein that localizes only to the equatorial segment and thereby facilitates fusion.

In search of an equatorial segment-localized protein, SPESP1 (sperm equatorial segment protein 1) (Wolkowicz et al., 2003) caught our attention. It was reported that the anti-SPESP1 antibody inhibited sperm-egg fusion in the human sperm-hamster egg system (Wolkowicz et al., 2008) and also in the mouse in vitro fertilization system (Lv et al., 2009). In the work reported here, we produced, by homologous recombination, a mouse line that lacks *Spesp1* and we analyzed the fertilizing ability of their sperm. Here we describe a haplo-insufficient phenotype of *Spesp1*-disrupted sperm, probably resulting from the aberrant localization of membrane proteins, accompanied by a destabilization of the equatorial segment membrane.

## Results

### Expression and localization of mouse SPESP1

Examination of a number of different mouse tissues by RT-PCR showed that *Spesp1* was expressed specifically in testis (Fig. 1A). Haploid-specific expression was confirmed by the appearance of SPESP1 in tissue from mice aged between 2 and 3 weeks (faint and clear bands, respectively in supplementary material Fig. S1). The onset of *Spesp1* expression occurred along with the expression of the gene for the putative fusion protein IZUMO1. SPESP1 was detected by immunostaining over a range of developmental stages, from round spermatid to cauda epididymal sperm. The localization was noticeably different from that of IZUMO1 (Fig. 1B,C). Since SPESP1 resides in the acrosomal vesicle, the SPESP1 antigen became reactive to the antibody after membrane permeabilization. When sperm underwent the acrosome reaction (estimated by the spreading of IZUMO1 over the entire head), the SPESP1 protein was no longer detectable in the equatorial segment (Fig. 1C). When SPESP1 was extracted with Triton X-114 and subjected to a phase separation, the protein was distributed in the detergent-depleted phase, indicating that SPESP1 is a soluble protein (Fig. 1D). By contrast, the transmembrane protein IZUMO1 was distributed in the detergent-enriched phase.

### Fertilizing ability of *Spesp1*-deficient mice

The *Spesp1* gene was disrupted by homologous recombination as described in supplementary material Fig. S2. Adult males of the

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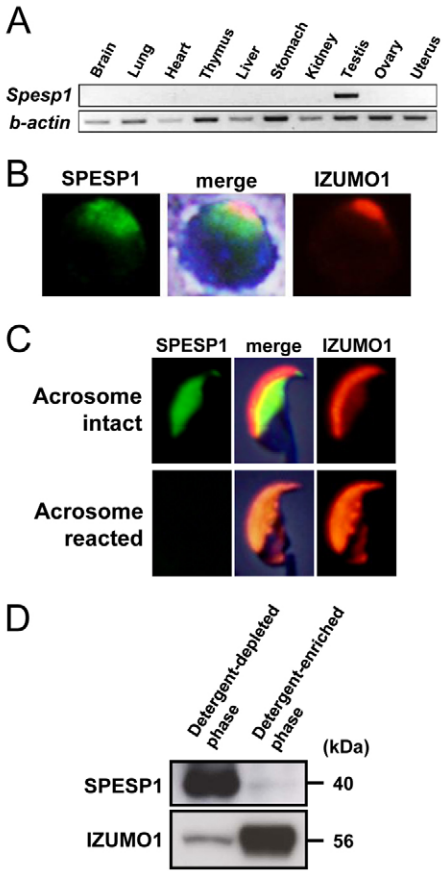


**Fig. 1. Characterization of mouse SPESP1.** (**A**) RT-PCR analysis of the mouse *Spesp1* gene. The expression of mouse *Spesp1* was examined by multi-tissue RT-PCR using RNA from various organs. *Spesp1* was expressed only in testis. The β-actin gene (b-actin) was used as an expression control. (**B**,**C**) Immunofluorescence staining of SPESP1and IZUMO1 in mouse spermatid (B) and acrosome-intact and acrosome-reacted sperm (C). SPESP1 was spread across the entire acrosome, unlike IZUMO1, which was localized in the primordial acrosomal cap area. SPESP1 was present in the equatorial segment in acrosome-intact sperm but no longer detectable in acrosome-reacted sperm. (**D**) Phase separation of Triton X-114 extracts of sperm. Sperm proteins were extracted with 1% Triton X-114 and were subjected to phase separation. SPESP1 was found only in the detergent-depleted fraction, whereas the membrane protein IZUMO1 was found in the detergent-enriched phase.

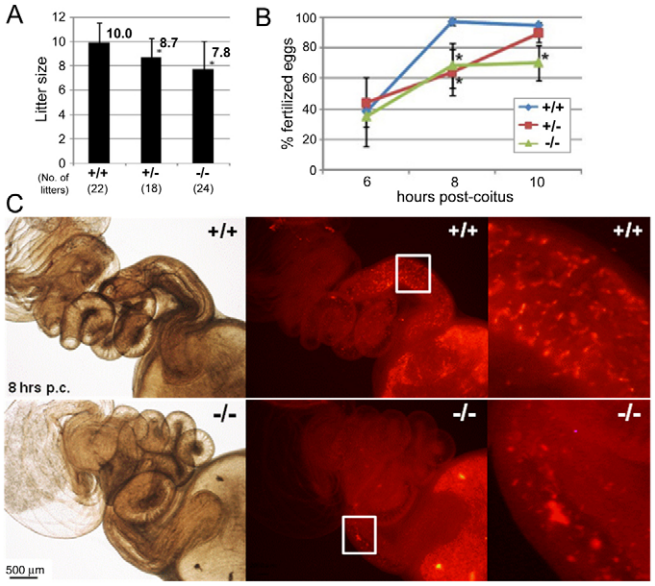


**Fig. 2. The fertilizing ability of *Spesp1*-deficient sperm in vivo.** (**A**) Sperm from *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ fathers produced lower litter sizes than sperm from wild-type males. (**B**) In vivo fertilization rate of eggs. The ratio of fertilized eggs was calculated for each time point: *Spesp1*$^{+/+}$: *Spesp1*$^{+/-}$: *Spesp1*$^{-/-}$, 38.3±22.7% (*n*=162): 44.1±3.3% (*n*=236): 35.1±6.5% (*n*=225) for 6 hours; 97.1±2.0% (*n*=241): 64.3±15.1%* (*n*=196): 68.7±14.6%* (*n*=246) for 8 hours; 94.8±2.0% (*n*=289): 89.8±6.0% (*n*=510): 70.6±11.5%* (*n*=445) for 10 hours (*significantly different from wild-type, $P<0.05$, Student *t*-test). Values in A and B are means ± standard deviations. (**C**) The sperm from *Spesp1*-deficient males, which migrated into the oviduct, were visualized using GFP-tagged acrosomes and RFP-tagged mitochondria 'RBGS sperm' (Hasuwa et al., 2010) 8 hours after of coitus. The number of sperm in oviducts of females mated with *Spesp1*-deficient males was significantly less compared to that of wild-type males.

three genotypes were bred with wild-type females for 3 months. Although *Spesp1*$^{-/-}$ females show normal fertility, both *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ males had statistically significant fewer offspring than wild-type males (Fig. 2A, *Spesp1*$^{+/+}$ 10.0±1.6, *Spesp1*$^{+/-}$ 8.7±1.6*, *Spesp1*$^{-/-}$ 7.8±2.3*; *significantly different from wild-type, $P<0.05$).

Next, we examined the fertilization in vivo and plotted the fertilization ratio against the time after coitus (Fig. 2B). Interestingly, sperm from *Spesp1*$^{-/-}$ males showed a delayed onset of fertilization ratio compared with wild-type males. Since many gene-disrupted mouse sperm have defects in migrating into the oviduct (Cho et al., 1998; Ikawa et al., 1997; Krege et al., 1995; Nishimura et al., 2004; Shamsadin et al., 1999; Yamagata et al., 2002; Yamaguchi et al., 2009; Yamaguchi et al., 2006), we examined the ability of sperm from *Spesp1*$^{-/-}$ males to migrate into the oviduct. For this purpose, we first introduced a transgene which produces sperm with a GFP-tagged acrosome and RFP-tagged mitochondria (Hasuwa et al., 2010) into the *Spesp1*$^{-/-}$ genetic background to visualize the sperm inside the uterus and oviduct (Yamaguchi et al., 2009). When females were mated with *Spesp1*$^{-/-}$ males and observed about 8 hours after coitus, significantly fewer sperm were found in the isthmus of the oviducts, in spite of an equal number of sperm observed in the uterus (Fig. 2C; supplementary material Fig. S3).

### In vitro sperm analysis in *Spesp1*-deficient mice

There was no difference observed in the number, motility and swimming patterns of sperm obtained from *Spesp1*-disrupted mice compared with those of wild-type mice, as shown in the supplementary material Movies 1 and 2. When added to the eggs, we found no defect in zona binding ability in *Spesp1*-disrupted mouse sperm (supplementary material Fig. S4). However, both *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ sperm were less fertile in the in vitro fertilization system compared with wild-type sperm (Fig. 3A: *Spesp1*$^{+/+}$ 90.9±5.1, *Spesp1*$^{+/-}$ 37.2±24.5*, *Spesp1*$^{-/-}$ 36.1±17.5*; *significantly different from wild-type, $P<0.005$). Moreover, when we examined the fusing ability using zona-free eggs, sperm from both *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ had significantly less fusion ability compared with wild-type sperm (Fig. 3B: *Spesp1*$^{+/+}$4.2±0.4,

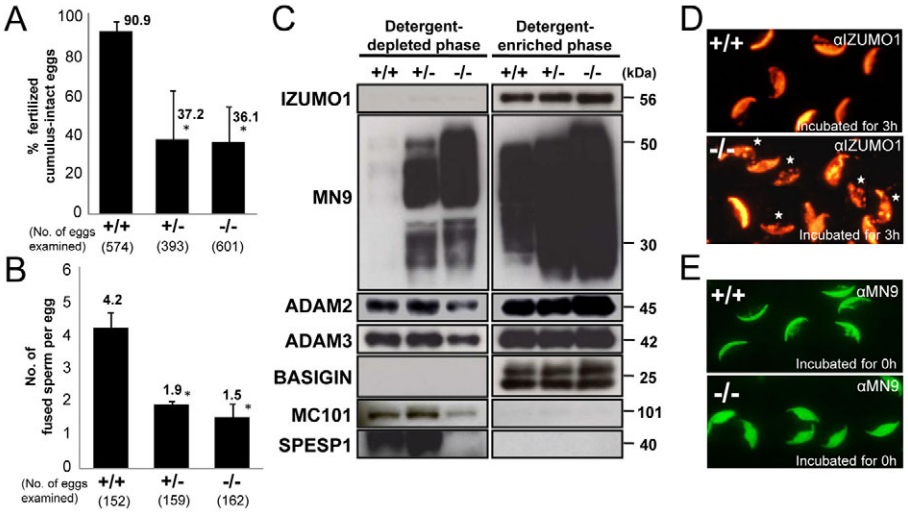


**Fig. 3. Fertilizing ability of *Spesp1*-deficient sperm in vitro and the western blot analysis of sperm proteins.** (**A**,**B**) The ability of both *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ sperm to fertilize eggs was reduced compared with wild-type sperm, using both cumulus-intact (A) and zona-free (B) eggs (*$P$<0.05, Student's $t$-test). (**C**) Sperm proteins were extracted from wild-type, *Spesp*$^{+/-}$ and *Spesp1*$^{-/-}$ animals with 1% Triton X-114 and analyzed by immunoblot using antibodies to IZUMO1, MN9 antigen, ADAM2, ADAM3, basigin, MC101 and SPESP1. The most prominent difference in the three genotypes was found in MN9 antigen, while basigin and IZUMO1 showed no difference. (**D**) Protein localization. Many of the *Spesp1*-disrupted sperm showed abnormal spreading of IZUMO1 after acrosome reaction (indicated by asterisks in the figure). The localization pattern of MN9 antigen was significantly affected by the disruption of SPESP1. (**E**) Normally, MN9 antigen localizes to the acrosomal cap area and spreads to the equatorial segment after acrosome reaction, but in *Spesp1* disrupted sperm, MN9 antigen showed an acrosome-reacted pattern before acrosome reaction .

*Spesp1*$^{+/-}$ 1.9±0.1*, *Spesp1*$^{-/-}$ 1.5±0.4*; *significantly different from wild-type, $P$<0.05).

### Analysis of membrane proteins from *Spesp1*-deficient sperm

The extraction of proteins using Triton X-114 and subsequent phase separation divides proteins into either a detergent-depleted or detergent-enriched phase, depending on their conformation (Bordier, 1981). An immunoglobulin superfamily protein basigin (BSG) (Igakura et al., 1998) was found exclusively in the detergent-enriched phase, and the amount was the same in *Spesp1*$^{-/-}$ and wild-type sperm (Fig. 3C). By contrast, the soluble acrosomal protein MC101 (Toshimori et al., 1995) was lower in *Spesp1*$^{-/-}$ sperm. ADAM family proteins implicated in sperm-zona binding were also affected by the disruption of *Spesp1* (less in the detergent-depleted phase). The amount of IZUMO1 (Inoue et al., 2005) in *Spesp1*$^{-/-}$ sperm was the same as in wild-type sperm (supplementary material Fig. S2E). Another potential candidate for a fusion protein, MN9 antigen (Toshimori et al., 1998), was much higher in the detergent-depleted phase from *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ sperm (Fig. 3C). As a whole, it was found that the disruption of the equatorial segment protein SPESP1 affected the amount and distribution of some proteins specifically.

Concerning the localization of IZUMO1, it was found that IZUMO1 spread in a punctate manner in many of the *Spesp1*$^{-/-}$ sperm (Fig. 3D). The most evident difference was in the staining of MN9 antigen. The normal characteristic pattern of MN9 antigen is to migrate to the plasma membrane covering the equatorial segment after acrosome reaction (Toshimori et al., 1992). However, in *Spesp1*$^{-/-}$ sperm, MN9 antigen was found in the equatorial segment before acrosome reaction (Fig. 3E).

### Electron microscopy of *Spesp1*-deficient sperm

The offspring of *Spesp1*$^{-/-}$ mice crossed with a transgenic mouse line producing sperm with GFP in the acrosome was subjected to fluorescence-activated cell sorting (supplementary material Fig. S5) to separate acrosome-intact and acrosome-reacted sperm, followed by fixation and observation by scanning electron microscopy (SEM). The acrosome-intact, *Spesp1*$^{-/-}$ sperm were morphologically similar to wild-type sperm (Fig. 4A,B). However, when acrosome-reacted sperm were examined, it was found that in almost all of the sperm, the equatorial segment membrane was completely lost in *Spesp1* null sperm (Fig. 4C,D). The electron microscopy results demonstrated that SPESP1 is required for the correct architecture of the equatorial segment.

## Discussion

We re-confirmed that the SPESP1 protein was localized in the equatorial segment of mouse sperm, using a newly available anti-SPESP1 peptide antibody (Fig. 1). No nonspecific reactivity was observed with our antibody because the immunostaining band in western blotting and the immunostaining of sperm was completely absent in *Spesp1*$^{-/-}$ sperm (supplementary material Fig. S2E). However, unlike the findings from previous studies (Lv et al., 2009; Wolkowicz et al., 2008), SPESP1 disappeared from sperm after the acrosome reaction (Fig. 1C). One of the reasons for this discrepancy could be the different recognition sites of the antibodies used. If so, there is a chance that a part of the SPESP1 fragment may remain in the equatorial segment even after the acrosome reaction.

The disruption of *Spesp1* resulted in a reduction in the fertilizing ability of sperm in a haplo-insufficient manner. One of the causes of this infertility was the impaired migration of sperm into the oviduct in *Spesp1* null (and *Spesp1*$^{+/-}$) sperm. It should be noted

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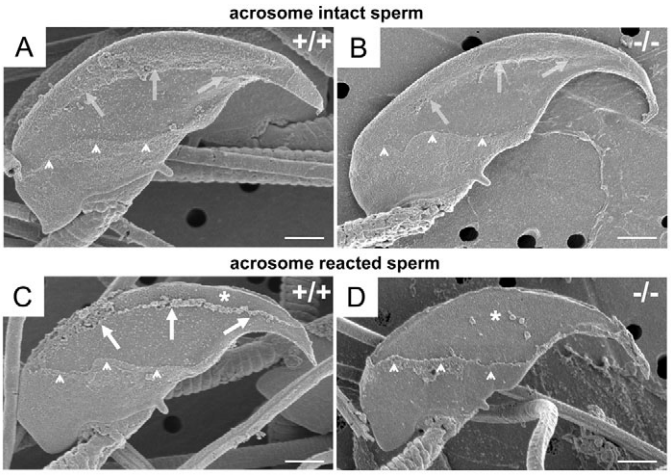


**Fig. 4. Scanning electron microscopy of *Spesp1*-deficient and wild-type sperm.** GFP-tagged 'green sperm' (Nakanishi et al., 1999) transgenic mice were mated with *Spesp1*-deficient mice. After preparation, sperm were incubated for 2 hours. After incubation, acrosome intact (upper panel) and acrosome-reacted sperm (lower panel) were separated by flow cytometry according to acrosomal GFP. (**A**,**B**) Acrosome-intact sperm from wild-type (A), and *Spesp1*-deficient (B) mice appeared similar. Both of them showed an apparent border (indicated by grey arrows) of the acrosomal cap area and the edge of the equatorial segment (arrowheads). (**C,D**) After acrosome reaction, in wild-type sperm (C) the membrane of the acrosome cap area disappeared along the acrosomal cap border. However, in *Spesp1*-deficient sperm (D), the plasma membrane disappeared up to the edge of the equatorial segment (indicated by arrowheads) exposing the inner acrosomal membrane (* in wide area). Scale bars: 1 μm.

that the migration was not impaired by a decrease in number of ejaculated sperm or by decreased sperm motility. A sufficient number of sperm were observed in the uterus of animals mated with *Spesp1*-disrupted males, and the epididymal sperm suspension obtained from *Spesp1*-disrupted mice showed no difference in concentration and in motility (supplementary material Movies 1 and 2). There are at least five genes known to affect sperm migration into the oviduct and all are reported to relate to each other, resulting in sperm with aberrant ADAM3 behavior (Cho et al., 1998; Ikawa et al., 1997; Krege et al., 1995; Nishimura et al., 2004; Shamsadin et al., 1999; Yamagata et al., 2002; Yamaguchi et al., 2009; Yamaguchi et al., 2006). Therefore, various sperm antigens, including ADAM3, have been examined in *Spesp1*-disrupted sperm. Although a slight difference was observed in ADAM3 from *Spesp1* disrupted sperm, it was not clear if this difference caused the impaired sperm migration into oviduct. However, we found a significant increase in the amount of MN9 antigen (Toshimori et al., 1998), which has been reported to function in sperm-egg fusion (Yoshinaga et al., 2001). Interestingly, when *Spesp1* was disrupted, not only the amount but also the localization of MN9 antigen was significantly affected. MN9 antigen is localized in the base of the acrosomal cap area and spreads to the equatorial segment after acrosome reaction. However, in *Spesp1*-deficient sperm, the MN9 antigen was found in the equatorial segment before acrosome reaction (Fig. 3E). One of the functions of SPESP1 may therefore be to limit the localization of MN9 antigen in the acrosomal cap area. The disappearance of SPESP1 after acrosome reaction may trigger the timing of migration of MN9 antigen into the equatorial segment in wild-type sperm (Toshimori et al., 1998).

As shown in the Results, the ability of *Spesp1*$^{-/-}$ sperm to fuse with eggs was diminished to less than half that of wild-type sperm (Fig. 3B). This might be caused by a combination of downstream effects on various sperm proteins. However, the infertility caused by the *Spesp1* disruption may derive not only from the aberrant placement of membrane proteins (Fig. 3C) but also by the altered physical nature of the sperm membrane, demonstrated by the loss of equatorial membrane after acrosome reaction using SEM in *Spesp1*-disrupted sperm (Fig. 4). This drastic change was observed in almost all of the *Spesp1* KO sperm examined. How SPESP1 maintains the integrity of the equatorial segment membrane will have to be clarified in the future.

As the past gene disruption research history indicates, it is very difficult to predict the role of certain factors in fertilization (Okabe and Cummins, 2007); however, defects of more than 100 genes are known to cause infertility (http://www.informatics.jax.org/searches/Phat.cgi?id=MP:0001925#top). This indicates that the best way to elucidate the mechanism of fertilization is to accumulate information on genes that relate to infertility by gene disruption experiments. In this context, the relationships between sperm proteins that we have clarified in the *Spesp1*$^{-/-}$ mouse are valuable for understanding the fertilization process. In turn, the information described here will assist the development of new treatments for infertility and/or new methods for contraception in the future.

## Materials and Methods

### RT-PCR

Mouse cDNA was prepared from various tissues of adult ICR, and testes from 1- to 5-week-old ICR males. RT-PCR was performed using 10 ng of cDNA and the following forward and reverse primers: 5′-GCTTCCGTCGTGAATGCTGA-3′ and 5′-TGTAAGGCCAGCTCTTCACAG-3′ for *Spesp1*. 5′-AAGTGTGACGTTGACATCCG-3′ and 5′-GATCCACATCTGCTGGAAGG-3′ for the β-actin gene.

*Izumo1*, calmegin, *Scp3*, and *Prm1* were amplified using the following primers. 5′-CTATTGTGGATGCTTCGTCA-3′ and 5′-GGTCTCAGAACTTTGCTCCCAAACCCTGTA-3′ for *Izumo1*. 5′-GGCCCCAGAGAAGCCAGA-3′ and 5′-AGAGTTTGTGCAGATGCGCAG-3′ for calmegin. 5′-ATGATGGAAACTCAGCAGCAAGAGA-3′ and 5′-TTGACACAATCGTGGAGAGAACAAC-3′ for *Scp3*. 5′-ACGAAGATGTCGCAGACGGAGGAG-3′ and 5′-CATCGGCGGTGGCATTTTTCAAGA-3′ for *Prm1*.

The amplification conditions were 1 minute at 96°C, followed by 36 cycles for the experiments shown in Fig. 1A and 27 cycles for the experiments shown in supplementary material Fig. S1. Cycles were 96°C for 15 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, with a final 1-minute extension at 72°C.

### Antibodies

Rabbit anti-mouse SPESP1 polyclonal antiserum was produced by immunization with mouse SPESP1 polypeptide (MYGSNVFPEGRTSD). Anti-IZUMO1 monoclonal antibody (mAb#125) was previously generated in our laboratory according to the standard method (Yamaguchi et al., 2006). Mouse monoclonal antibodies MN9 and MC101, were gifts from Dr Kiyotaka Toshimori (Chiba University, Chiba, Japan). Monoclonal antibodies against mouse ADAM2 (fertilin-b; 9D2.2) and ADAM3 (cyritestin; 7C1.2) were purchased from Chemicon International Inc. (Temecula, CA). Immunoglobulin superfamily protein basigin (CD147) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

### Immunofluorescent staining

Immunofluorescent staining was performed as described previously (Kotaja et al., 2004) for round spermatid, and for epididymal sperm (Yamaguchi et al., 2008).

### Phase separation of Triton X-114 extracts of sperm

Phase separation of Triton X-114 extracts of sperm were performed as described Yamaguchi et al. (Yamaguchi et al., 2006).

### Immunoblot analysis

Immunoblot analysis was performed as described previously (Inoue et al., 2008) All the samples were subjected to SDS-PAGE followed by western blotting with primary antibody under reducing conditions.

### Construction of the *Spesp1* gene disruption vector

A targeting vector was constructed using pNT1.1 containing the Neo-resistance gene (Neor) as a positive selection marker and a herpes simplex virus thymidine kinase as a negative selection marker. A 0.9-kb *Pme*I-*Not*I fragment as a short arm and a

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6.1-kb *Asc*I-*Avr*II fragment as a long arm were obtained by PCR using genomic DNA in D3 embryonic stem (ES) cells as a template. The PCR primers used were as follows: 5′-CCGTTTAAACCCGGTGAGCCAGTGTGCGTCTACTGTAGTG-3′ and 5′-CCGCGGCCGCCTATGCTAGCTCTTAAGTACAGAAGTCAAG-3′ for the short arm: 5′-CCGGCGCGCCAATGGTACACTTGAGGCTGATGTCGGAACT-3′ and 5′-CCCCTAGGTACAAGCGCGCCTCATCCAAATCCTCGCTT-3′ for the long arm.

These two fragments were inserted into a pNT1.1 vector and the targeting construct was linearized with *Pme*I digestion. ES cells were electroporated and colonies were screened.

#### Generation of *Spesp1*-disrupted mice

The mouse *Spesp1* gene consists of two exons and maps to chromosome 9. The targeting vector was designed to remove a part of the second exon of the *Spesp1* gene (supplementary material Fig. S2A) and was electroporated into D3 ES cells after linearization. Potentially targeted ES cell clones were separated by positive-negative selection with G418 and ganciclovir. Correct targeting of the *Spesp1* allele in ES cell clones was determined by PCR and Southern blot analysis for homologous recombination at both ends (supplementary material Fig. S2B,C). Mating between heterozygous mutant mice yielded the expected mendelian ratios: $Spesp1^{+/+}$, 24.6%; $Spesp1^{+/-}$, 50.8%; $Spesp1^{-/-}$ 24.6% of offspring ($n$=65). Northern and western blot analysis showed that *Spesp1* mRNA expression was undetectable in the $Spesp1^{-/-}$ testis (supplementary material Fig. S2D). The SPESP1 protein was also not detected in sperm from the cauda epididymis and vas deferens in $Spesp1^{-/-}$ mice (supplementary material Fig. S2E). $Spesp1^{-/-}$ mice were healthy and showed no overt developmental abnormalities. Mice used in this study were of B6; 129 mixed background and male mice of three genotypes, and $Spesp1^{+/+}$ littermates were used as controls.

#### Southern and northern blot and PCR analysis

*Sca*I-digested and *Afl*II-digested genomic DNA (20 μg) were electrophoresed though a 0.8% agarose gel, transferred to a Hybond N+ membrane (Amersham Bioscience), and hybridized with the $^{32}$P-labeled 5′ and 3′ probes. These probes amplified by the primers used were as follows: 5′-CTCTGAGAAGAGGAAGCTCCCTTAG-3′ and 5′-ATCCAACAGTGAGTTTAGAGTTTTCAGG-3′ for 5′-probe; 5′-GAGCTAA-GAGAGCTAAGTGCACAG-3′ and 5′-GCTCTGATAAGGACCACTCTTGC-3′ for 3′-probe.

Northern hybridization was performed using 10 μg of total RNA extracted from various tissues of adult ICR mice. RNAs were separated by electrophoresis on agarose gels, transferred to Hybond-N+ membranes (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and hybridized to $^{32}$P-labeled probes at 60°C overnight. Mouse *Spesp1* and glyceraldehyde 3-phosphatate dehydrogenase (*Gapdh*) cDNAs were used as probes. The *Spesp1* probe consisted of a cDNA fragment amplified from mouse testis total RNA by RT-PCR using 5′-ATGAAGCTGGTGGTCCTAGT-3′ and 5′-TCATGGGACATTAAGAATAT-3′ as primers.

Genomic DNA derived from tail biopsy specimens of $Spesp1^{+/+}$, $Spesp1^{+/-}$ and $Spesp1^{-/-}$ mice was used as the template for PCR analysis. The primers used were as follows: 5′-CGATGAAACGACAAATTCCCGTACTAGAGG-3′ and 5′-CTTTTC-TCTCAGCTCTGATGGTACACGCTT-3′ for the wild-type allele; 5′-GAGAACG-GTGGCCTGGTG-3′ and 5′-GCCTTCTATCGCCTTCTTGACGAGTTCTTC-3′ for the mutant allele.

#### Assessment of the fertilizing ability of *Spesp1*-deficient mice

Sexually mature male mice of $Spesp1^{+/+}$, $Spesp1^{+/-}$ and $Spesp1^{-/-}$ genotypes were caged with (C57BL/6J 3 DBA/2N)F1 (also known as B6D2F1) female mice (>2 months old; CLEA Japan, Inc., Tokyo, Japan) for 3 months, and the number of pups in each cage was counted within a week of birth. All experiments were performed with the consent of the Animal Care and Use Committee of Osaka University.

#### Time-dependent observation of in vivo fertilization

B6D2F1 females were superovulated by intraperitoneal injection of 5 IU equine chorionic gonadotropin (eCG), followed 48 hours later by 5 IU human CG (hCG). Superovulated females were caged with test males 12 hours after hCG injection, and the females were inspected every 30 minutes for the formation of the vaginal plug. Once plug formation was confirmed, the male was removed from the cage. At about 6, 8 and 10 hours after copulation, the pronucleus stage embryos were recovered from oviducts, and assessed for fertility.

#### In vitro fertilization assay

In vitro fertilization (IVF) and penetration assays of zona-free-egg by sperm were performed as described previously (Ikawa et al., 1997; Inoue et al., 2005). For IVF and sperm-egg fusion assays, both the cumulus-intact and zona-free eggs were incubated with $2\times10^5$ sperm/ml.

#### Sperm migration analysis

Sperm migration analysis was performed as described previously (Yamaguchi et al., 2009; Hasuwa et al., 2010).

#### Electron microscopy

Flow cytometry using *Acr-EGFP* sperm was performed as described by Nakanishi et al. (Nakanishi et al., 1999). Green fluorescent and non-fluorescent sperm were clearly distinguishable from each other (supplementary material Fig. S5). Both sorted acrosome-intact (green) and -reacted (non-green) sperm were captured on the SEMpore membrane (JEOL Datum, Ltd, Tokyo, Japan) and fixed with 1% glutaraldehyde, 100 mM NaCl, 2 mM $CaCl_2$, 30 mM HEPES-NaOH (pH 7.3) on ice, postfixed with 1% $OsO_4$ for 1 hour, 0.5% tannic acid for 1 hour and 0.5% $OsO_4$ for 1 hour, and dehydrated through a graded series of ethanol and *t*-butyl alcohol. Specimens were freeze-dried, coated with osmium and observed with an Hitachi S-4800 scanning electron microscope (Hitachi High-Technologies Co., Ibaraki, Japan).

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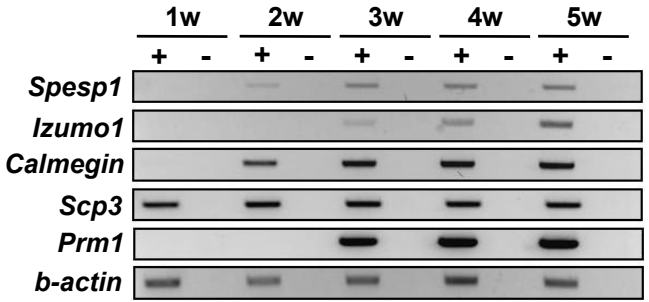


**Fig. S1.** *Spesp1* gene expression pattern during spermatogenesis.
RT-PCR was performed using 10 ng of total RNA extracted from testes from 1- to 5-week-old ICR males. *Scp3* was used as a marker for the meiotic spermatocyte stage, and *Prm1* was used as a marker for the spermatid stage.

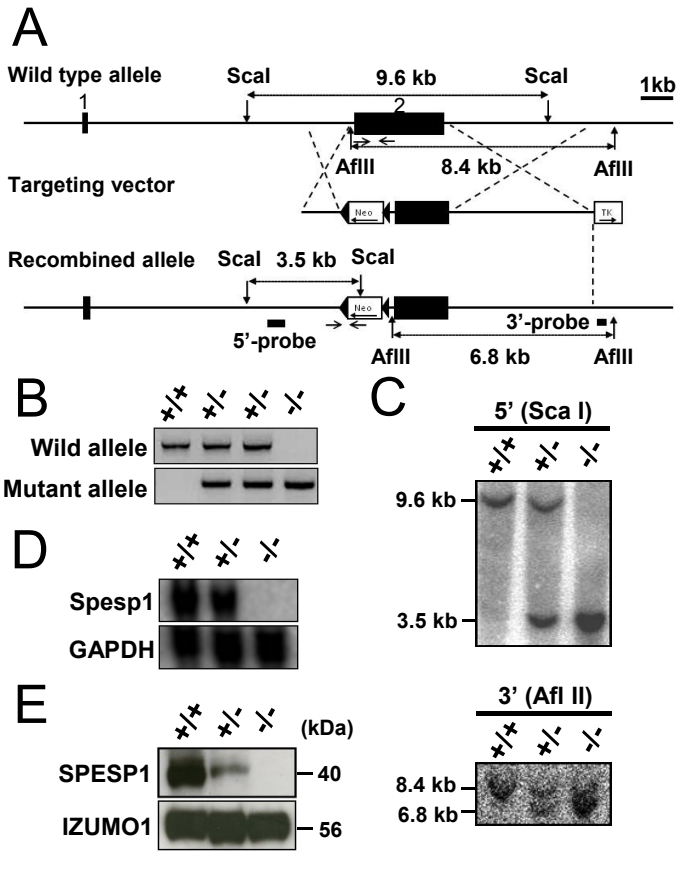


**Fig. S2.** Generation of *Spesp1* knockout mice.
Strategy of *Spesp1* gene disruption. (A) Confirmation of *Spesp1* knockout mice. (B-E) PCR analysis detected the mutant allele of *Spesp1* knockout mice (B). Southern blot results for wild-type and mutant alleles of the *Spesp1* gene (C). Northern blot analysis of total testis RNA confirmed the absence of gene-specific transcripts in KO testis (D). Western blot analysis also confirmed the absence of SPESP1 proteins in KO sperm (E).

# Fig.S4 (Y. Fujihara et al.)

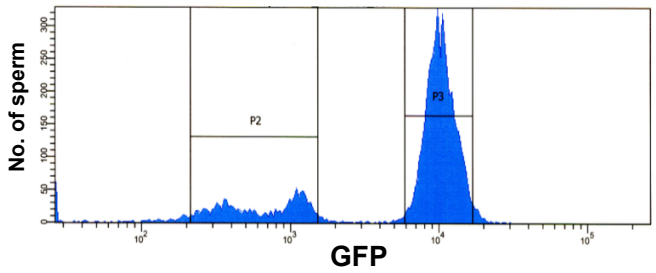


**Fig. S4.** A histogram of sperm GFP expression from *Spesp1*-deficient bearing *Acr-EGFP* transgenic mice. In the peaks on the left, acrosome-reacted (GFP negative) sperm were distributed, while acrosome-intact sperm were contained in peaks on the right.

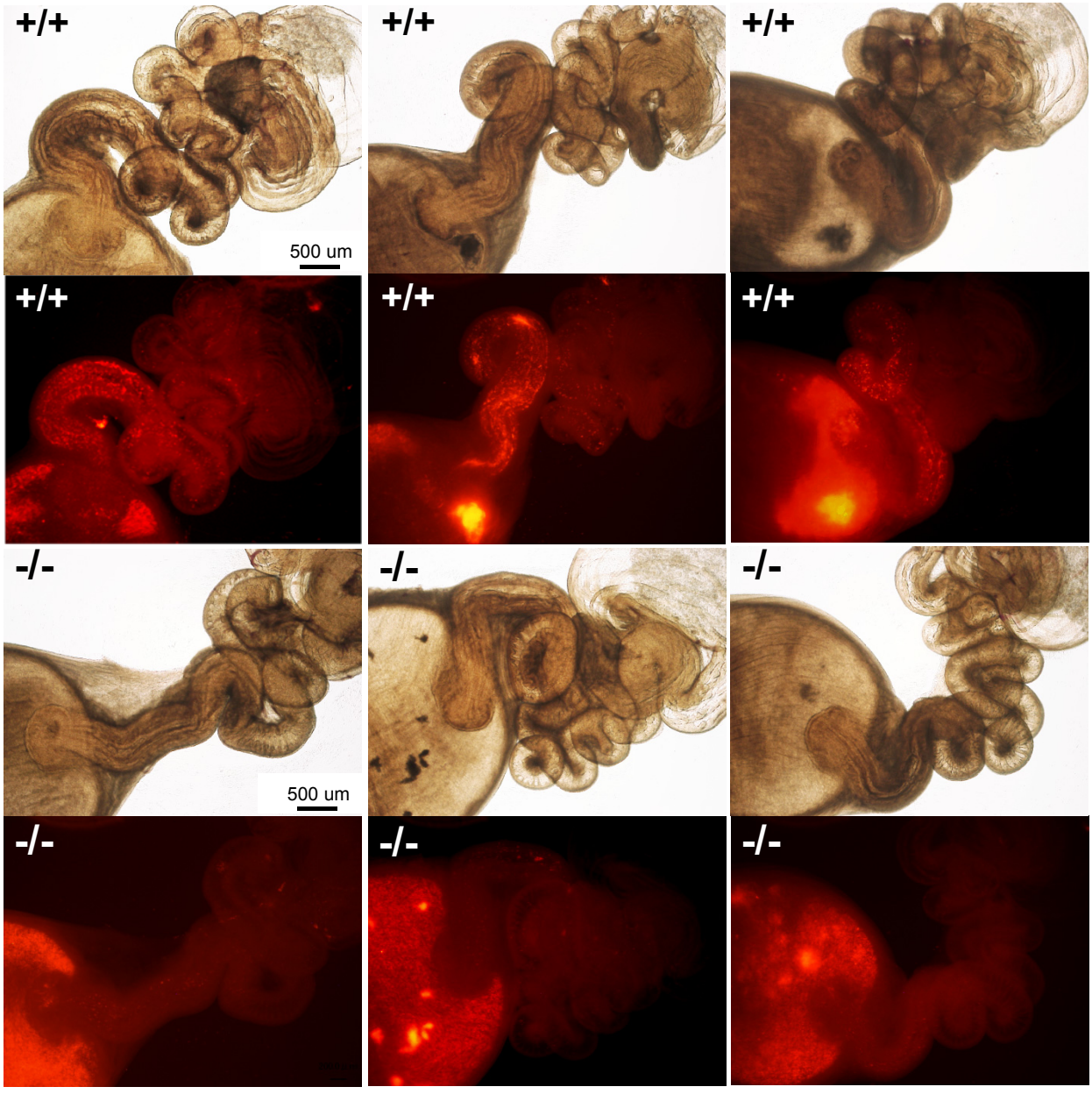


**Fig. S3.** Observation of oviducts from females mated with wild-type and *Spesp1* -/- males with mitochondria tagged RFP 8 hrs after coitus.
Red fluorescent illumination indicate the existence of sperm within the uterus and the oviductal isthmus. When *Spesp1* -/- males were mated, there were significantly lower number of migrated sperm into the oviductal isthmus compared to wild-type males.

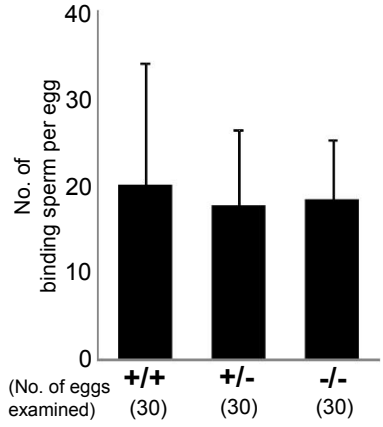


**Fig. S4.** Sperm-ZP binding assay in *Spesp1*-deficient sperm.
*Spesp1*-deficient sperm showed a normal ability to bind to the zona pellucida compared to wild-type sperm (A). A sperm-ZP binding assay was performed as described in (Yamaguchi et al., 2006)

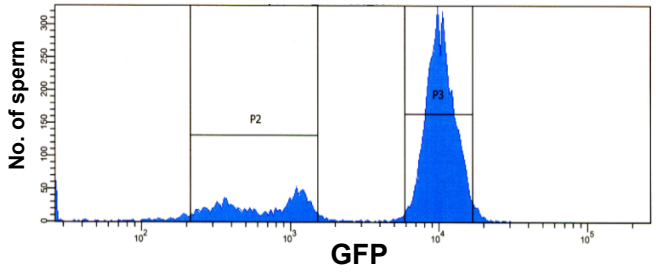


**Fig. S5.** A histogram of sperm GFP expression from *Spesp1*-deficient bearing *Acr-EGFP* transgenic mice. In the peaks on the left, acrosome-reacted (GFP negative) sperm were distributed, while acrosome-intact sperm were contained in peaks on the right.