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PDILT is required for quality control of ADAM3 and male fertility

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Abstract

A disintegrin and metalloproteinase 3 (ADAM3) is a sperm membrane protein critical for both sperm migration from uterus into oviduct and sperm primary binding to zona pellucida (ZP). Here we show that the testis specific protein disulfide isomerase homologue (PDILT) cooperates with the testis specific calreticulin-like chaperone, calsperin (CALR3), in the endoplasmic reticulum and plays an indispensable role in the disulfide-bond formation and folding of ADAM3. *Pdilt*^{-/-} mice were male infertile because ADAM3 could not be folded properly and transported to the sperm surface without the PDILT/CALR3 complex. Peculiarly we find that not only *Pdilt*^{-/-}, but also *Adam3*^{-/-}, spermatozoa effectively fertilize eggs when the eggs are surrounded in cumulus oophorus. These findings reveal that ADAM3 requires testis specific private chaperones to be folded properly and that the principle role of ADAM3 is for sperm migration into oviduct but not for the fertilization event. Moreover the importance of primary sperm ZP binding that has been thought to be a critical step in mammalian fertilization should be reconsidered.

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Introduction

In mammals, ejaculated spermatozoa are not able to fertilize eggs immediately, but acquire their fertilizing ability after spending some time in the female reproductive tract. The phenomenon underlying this acquisition of fertilizing ability is called sperm capacitation, and the discovery made it possible to perform mammalian *in vitro* fertilization (IVF) by mixing capacitated sperm with ovulated eggs (1, 2). Although various sperm factors have been reported to be important using the IVF system and inhibitory assays, recent gene-knockout studies have revealed that many of the factors are not essential for the fertilization process *in vivo* (including acrosin for ZP binding and penetration, hyaluronidase for cumulus oophorus penetration, zonadhesin for ZP binding and fertilin for sperm egg fusion) ((3) and refs therein).

In contrast, gene knockout studies unexpectedly discovered the fertilization related molecules (3).

ADAM3, one of these essential factors, was first reported as a sperm egg fusion protein candidate but later was proven to be dispensable in fusion reaction (4, 5). However, *Adam3* knockout male mice are infertile because the mutant spermatozoa cannot migrate through the uterotubal junction (UTJ) *in vivo* and are unable to bind to ZP *in vitro* (6). It was also reported that ADAM3 has affinity to ZP (7). There are at least 7 lines of knockout mice showing similar male infertility phenotypes: (*Ace* (8), *Adam1a* (9), *Adam2* (10), *Adam3* (5), *Calr3* (11), *Clgn* (12), and *Tpst2* (13)). ADAM3 was lost from the sperm or was defective in all these mutant mice. Since ADAM3 is involved in so many infertility phenotypes, elucidating its maturation and function is important in understanding the process of mammalian fertilization.

ADAM3 is a cysteine-rich, glycosylated membrane protein that is co-translationally translocated into the endoplasmic reticulum (ER) of spermatids, where numerous molecular chaperones and catalysts promote glycoprotein folding as well as the disposal of misfolded proteins. Whereas homologous lectin chaperones, calnexin/calreticulin (CANX/CALR), chiefly mediate nascent glycoprotein folding in the somatic cells

(14), testicular germ cells contain additional homologues, called calmeglin (CLGN) and calsperin (CALR3), respectively (15, 16). We have previously demonstrated that the disruption of either *Clgn* or *Calr3* genes resulted in male infertility concurrent with the absence of ADAM3 from mature spermatozoa. Whereas CLGN mediates ADAM1A/ADAM2 heterodimerization that is required for subsequent ADAM3 maturation (6, 17), CALR3 directly binds to and regulates ADAM3 maturation(11).

Appropriate intra- and inter- molecular disulfide bond formation also promotes protein folding in the ER. For disulfide bond formation in the ER, more than 20 protein disulfide isomerase (PDI) family proteins have been implicated in the process of oxidation, reduction, and isomerization (18, 19). Among the family members, PDIA3 (ERP57) is notable because it associates with CANX/CALR and contributes to the quality control cycle of newly synthesized glycoproteins in the ER (20, 21). In addition to ubiquitous PDIs including PDIA3, male germ cells also express a testis specific PDI-like protein, PDILT (22). Although PDILT does not have consensus CXXC motifs and is not a classical oxido-reductase, it does interact with CLGN and with model proteins *in vitro*, suggesting that it may have a role in the maturation of spermatid proteins and consequently, male infertility *in vivo* (23).

In the present study, we showed that PDILT interacts with CALR3 in the testicular germ cells and plays an indispensable role in the disulfide formation in the ADAM3. The PDILT knockout mice were male infertile, consistent with the previous observations that spermatozoa lacking ADAM3 cannot migrate through the UTJ and cannot bind to ZP. However, we found that the spermatozoa lacking ADAM3 do fertilize eggs effectively *in vivo* when capacitated spermatozoa were directly deposited into the oviduct. Our data demonstrate the importance of a testis-specific, ER-quality control system for sperm fertilizing ability, and calls into question the importance of primary sperm ZP binding that has been thought to be a critical step in mammalian fertilization.

Results

PDILT forms a chaperone complex and interacts with ADAM3

We first examined the expression of PDILT in adult mouse organs and in developing testes by western blotting analysis (Fig. S1 A and B). PDILT was specifically detected in testis, consistent with previous observations in rat and human (23, 24). In developing mouse testes, while PDIA3 (ERP57) was continuously detected throughout development, PDILT was not detectable until the mice became 3-wks old, indicating post-meiotic expression. As we have shown previously (11), CALR3 and ADAM3 were also expressed from 3-wks old, whereas CLGN expression starts from 2-wks after birth. When we immunostained testicular germ cells (Fig. S1C), CLGN was detected from spermatocytes to spermatids, whereas CALR3 and PDILT were only expressed in haploid spermatids. These data confirmed that mouse PDILT is a male haploid germ cell-specific protein co-expressed with CLGN and CALR3.

We next examined the interaction of PDILT with ER chaperones and ADAM proteins by co-immunoprecipitation analysis (Fig. 1 A and B), because CLGN- and CALR3-deficient mice had infertile phenotypes attributed to failures in ADAM1/ADAM2 heterodimerization and ADAM3 maturation, respectively (11). PDILT interacted with neither CANX nor CALR, but did bind to CLGN in wild type mice, consistent with the data from rat (23). We further showed that PDILT interacted with CALR3 and that the signal was stronger than with CLGN (Fig. 1A). PDILT interacted with neither ADAM1B nor ADAM2, but did interact with ADAM3 (Fig. 1B). The signal was weak, possibly because PDI family:client interactions are known to be difficult to visualize, given that only a small proportion of most client proteins will be in the process of oxidative protein folding (25).

Generation of *Pdilt* KO mice

To analyze the physiological function of PDILT *in vivo*, we generated *Pdilt* gene-disrupted mice. The targeting vector was designed by substituting exon 2 with a neomycin resistance gene cassette in reverse orientation relative to the *Pdilt* transcriptional unit (Fig. S2A). The correct targeting event in embryonic

stem cells and the germ-line transmission were confirmed by PCR analysis (Fig. S2B). Mating between heterozygous F1 mice yielded the expected mendelian ratios of offspring: 22 wild-type (+/+), 48 heterozygous (+/-), and 24 homozygous (-/-) mutants. No overt developmental abnormalities were observed in *Pdilt* -/- mice. In the *Pdilt* -/- testis, as expected, *Pdilt* mRNA and PDILT protein was not detected (Fig. S2 C and D). There were no significant differences in the protein amount of CLGN, CALR3, PDI and PDIA3 (Fig. S2E). When we looked at testicular sections by optical microscopy, disruption of PDILT caused no deleterious effect on spermatogenesis and sperm produced by the mutants were morphologically normal (Fig. S2F).

Disappearance of ADAM3 from Pdilt -/- spermatozoa

The CLGN protein is involved in the heterodimerization of ADAM1A/ADAM2 (only found in the ER) and ADAM1B/ADAM2 (Fertilin, found in both ER and at the cell surface) (17, 26). Although PDILT interacts with CLGN, normal ADAM1/2 heterodimerization was observed in *Pdilt* -/- testis (Fig. 1C) and the ADAM1B/ADAM2 protein was successfully presented on mature spermatozoa (Fig. 1 D and E). However, ADAM3 disappeared selectively from mature *Pdilt* -/- spermatozoa while various sperm fertilizing proteins were normally presented, as observed in *Calr3* -/- mice (11).

To trace the fate of ADAM3 in *Pdilt* -/- mice, we analyzed spermatozoa from testis and epididymis by western blotting. ADAM3 is known to be processed from an uncleaved ~90 kDa form to a functional ~30 kDa form in the epididymis (27). In *Pdilt* -/- mice, ADAM3 was expressed normally in testis, but was absent from caput epididymis, an early segment of the epididymis (Fig. 2A). This experiment indicated that ADAM3 disappeared from testicular spermatozoa before entering the epididymis.

We then performed a limited trypsin digest of testicular spermatogenic proteins to determine whether the ADAM proteins are presented on the cell surface (Fig. 2B). When we trypsinized live cells, extracellular trypsin could digest ADAM2 but not ADAM3 on the *Calr3* -/- and *Pdilt* -/- cells, implying that the

mutant cells present ADAM2 but not ADAM3 on their surfaces. In contrast, the opposite finding was obtained in *Clgn* *-/-* cells (11). When whole cell lysates were trypsinized, both ADAM2 and ADAM3 were digested similarly in all wild-type, *Clgn* *-/-*, *Calr3* *-/-*, and *Pdilt* *-/-* mice (Fig. S3). These data indicated that ADAM3 remains in the secretory pathway in the absence of PDILT or CALR3, whereas it is transported onto the sperm surface in the absence of CLGN.

PDILT dependent disulfide bond formation in ADAM3

To investigate whether PDILT, CALR3, and CLGN participate in the proper folding of ADAM proteins, spermatogenic cell membrane proteins were separated into detergent depleted or enriched phases by Triton X-114 (28) and subjected to SDS-PAGE under reducing and non-reducing conditions (Fig. 2C). In the detergent depleted fraction, ADAM3 was detected as a doublet under non-reducing conditions, with the more compact, oxidised form being the most prominent in wild-type mice. The compact, oxidised form was weaker in *Pdilt* *-/-* and *Calr3* *-/-* compared to wild-type and *Clgn* *-/-*. In the detergent enriched fraction, ADAM3 was mostly recovered in the oxidised form, and the ADAM3 signals were very faint in the *Pdilt* *-/-* and *Calr3* *-/-* mice. Since the ADAM3 signals under reducing conditions were comparable among all mutants, the data suggests that both PDILT and CALR3 promote intra- molecular disulfide bond formation in ADAM3 and subsequent folding, thus altering ADAM3 antigenicity. It should be noted that the ADAM2 signal was weaker in the *Clgn* *-/-* mice when analysed under non-reducing conditions, emphasizing the importance of CLGN in supporting oxidative protein folding of ADAM2.

To further determine whether the interaction of PDILT with ADAM3 is chaperone dependent, we immunoprecipitated PDILT complexes, subjected them to SDS-PAGE and western blotting, then probed them with ADAM3 antibodies. PDILT associated normally with ADAM3 in the absence of CLGN. However, the interaction was almost abolished when CALR3 was not present (Fig. 2D). When we immunoprecipitated CLGN and CALR3 complexes in the absence of PDILT, CLGN normally associated with ADAM3. The CALR3/ADAM3 association was perturbed but not abolished (Fig. 2E). These data

indicate that the CALR3 recognizes ADAM3 with the aid of PDILT and the complex promotes ADAM3 maturation.

Pdilt* ^{-/-} sperm fertilizing ability *in vivo

To examine *Pdilt* ^{-/-} mouse fertility, adult *Pdilt* ^{-/-} females and males were mated with wild-type males and females, respectively, for a month. Whereas *Pdilt* ^{-/-} females were fertile, *Pdilt* ^{-/-} males were infertile, despite normal mating behavior with successful sperm ejaculation and vaginal plug formation (Fig. 3A). Therefore, we observed the behavior of ejaculated spermatozoa in female reproductive tracts. For this experiment, we introduced a transgene which labels spermatozoa with a GFP-tagged acrosome and DsRed2-tagged mitochondria (29) into the *Pdilt* ^{-/-} genetic background to visualize the spermatozoa inside the female reproductive tract (Fig. S2F). Although many *Pdilt* ^{+/-} spermatozoa were observed in both uterus and oviduct 2 hours after coitus, *Pdilt* ^{-/-} spermatozoa were observed only in uterus but not in oviduct (Fig. 3B). In addition, there were no differences in the sperm motility parameters assessed by the CEROS computer assisted sperm analysis system in the spermatozoa collected from uterus at 2 hours after coitus (Fig. S4). Taken together, the data indicated that the defective migration of sperm from uterus into oviduct was not due to impaired sperm motility.

To further investigate sperm fertilizing ability *in vivo*, we directly deposited capacitated spermatozoa into the oviduct to circumvent sperm migration from uterus into oviduct (Fig. 3C). When we observed the females 24 hours after the artificial insemination with wild-type spermatozoa, $69.7 \pm 19.3\%$ of eggs were fertilized (Fig. 3D). Not only *Pdilt* ^{-/-}, but also *Adam3* ^{-/-}, spermatozoa were able to fertilize eggs as effectively as wild-type ($47.8 \pm 9.8\%$ and $64.1 \pm 2.9\%$, respectively) (Fig. 3D). Healthy and fertile offspring developed from these fertilized eggs, indicating the genomic integrity of the mutant spermatozoa (Fig. 3E).

Pdilt* ^{-/-} sperm fertilizing ability *in vitro

We next examined the sperm fertilizing ability *in vitro*. When cumulus-free ZP-intact eggs were inseminated with spermatozoa, the *Pdilt* *-/-* spermatozoa seldom bound to ZP (+/-: 33.7 ± 15.3 , -/-: 1.4 ± 2.6 per egg, Fig. 4A and Movie S1). Under this condition, *Pdilt* *-/-* spermatozoa rarely fertilized eggs (+/-: $63.7 \pm 22.3\%$, -/-: $3.9 \pm 5.4\%$, Fig. 4B). These defects were also reported in *Adam3* *-/-* spermatozoa (5). Why were mutant spermatozoa able to fertilize eggs in the oviduct but not *in vitro*? To address this question, we performed IVF with cumulus-intact eggs to mimic the physiological fertilization process. Although the eggs were surrounded by intact ZP under the cumulus layer, both *Pdilt* *-/-* and *Adam3* *-/-* spermatozoa fertilized eggs as effectively as wild-type (+/+ : $75.2 \pm 10.1\%$, *Pdilt* *-/-*: $63.9 \pm 28.0\%$, *Adam3* *-/-*: $76.6 \pm 13.9\%$, Fig. 4C). When we performed cumulus free IVF with cumulus conditioned media, *Adam3* *-/-* sperm fertilizing ability was not fully but partially recovered (cumulus intact: $95.8 \pm 3.8\%$, cumulus free: $21.3 \pm 6.9\%$, and cumulus free with conditioned media: $45.9 \pm 22.6\%$). The data suggested that in addition to cumulus cells, hyaluronan and other soluble factors maintained in these layers may be required to help spermatozoa that lack ADAM3 to fertilize eggs.

Discussion

Proper glycoprotein folding in the ER is chiefly achieved by CANX/CALR lectin chaperones that also recruit PDIA3 for native protein disulfide bond formation (20, 21, 30). In the present study, we showed that appropriate disulfide formation in ADAM3 relies on a CALR3/PDILT partnership. Thus, we propose that CALR3/PDILT is a new quality control hub unique to the male germ cells, alongside the CANX/PDIA3 and BIP/PDI partnerships found in somatic cells (31). Although PDILT lacks a redox-active CXXC motif, the fact that PDILT can be recovered in disulfide dependent complexes (23) suggests that the monocysteine thioredoxin motif may trap cysteine in the substrate to facilitate protein folding. PDILT is not active as a reductase against the model substrate insulin *in vitro* (23); however, it remains possible that PDILT contributes to oxidative protein folding in the context of other folding factors in the spermatid. The rescue experiment with mutated PDILT and ADAM3 as a model substrate would be informative in future studies. Another interesting possibility is that, by competing with PDIA3 or other protein disulfide oxidoreductases, PDILT might prevent and/or slow down the rate of disulfide bond formation for a particular substrate. This might be needed in specialized circumstances, such as low temperature in testis, to promote appropriate disulfide bond formation in ADAM3.

The CALR3/PDILT complex is not the only unusual quality control system required for ADAM3 maturation. CLGN is required for the heterodimerization of ADAM1A/ADAM2 and this ER resident heterodimer is essential for ADAM3 maturation (9, 17). Angiotensin converting enzyme (ACE) is a well known factor for blood pressure maintenance and has a testis specific promoter in intron 12. The male germ cell specific variant (tACE) controls ADAM3 distribution on the membrane (6). The GPIase activity of tACE (i.e. the release of glycosyl-phosphatidylinositol anchored proteins from the membrane) is implicated in ADAM3 modification during epididymal maturation (32). In addition to tissue specific factors, recent studies indicated that the ubiquitously expressed proteins, TPST2 (Protein-tyrosine sulfotransferase 2) and INPP5B (inositol polyphosphate-5-phosphatase B), are involved in ADAM3 quality control (13, 33). Although aberrant folding and modification in cells can be associated with

pathological conditions, including amyloid diseases such as Parkinson's and Alzheimer's diseases (34, 35), the complex relationships between different quality control systems *in vivo* is not fully understood (36). Therefore, further studies of the ADAM3 maturation mechanism will help us to understand the requirements for different factors in divergent protein quality control systems and protein misfolding diseases.

Defective sperm-ZP binding has been thought to be the critical cause of male infertility in mice lacking sperm ADAM3 (5, 6, 9-13). In general, it is believed that acrosome intact spermatozoa bind to ZP and release their acrosomal contents (acrosome reaction), then penetrate ZP prior to fusing with eggs. In the present study, we show that spermatozoa lacking ADAM3 were able to fertilize freshly ovulated eggs as effectively as wild type spermatozoa when capacitated spermatozoa were directly deposited into the oviduct. Moreover, *Adam3* *-/-* spermatozoa successfully fertilized ZP intact eggs *in vitro* if they were surrounded in cumulus oophorus cells. It was also reported that the *Adam1a* *-/-* spermatozoa are not able to bind to ZP but are able to fertilize cumulus invested eggs *in vitro* (9). These data suggest that the presence of numerous acrosome intact sperm binding to the ZP surface of cumulus free eggs *in vitro* (called primary ZP binding) could have been a "red herring" and less important than previously supposed. Recent reports that most fertilizing mouse spermatozoa begin their acrosome reaction during cumulus penetration before contact with the ZP (37) and that acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs (38) also support the idea.

Whereas primary sperm binding to ZP could be bypassed entirely, acrosome reacted sperm binding to ZP (called secondary binding) through acrosomal components and/or by the inner acrosomal membrane could play important role during fertilization (39). In the present study, cumulus conditioned media partially (but not fully) restored *Adam3* *-/-* sperm fertilizing ability to cumulus free zona intact eggs. Therefore, in physiological circumstances, we suggest that the layers of cumulus oophorus may help spermatozoa to bind/about ZP not only by structurally but also by inducing an acrosome reaction that leads

to secondary binding. This is consistent with the previous observations on the beneficial roles of the cumulus oophorus cells (40). Nongenomic activation of spermatozoa by steroid hormones should also be considered as a possible mechanism (41, 42).

Since defective sperm ZP binding is not the cause of male infertility in physiological circumstances, we should pay more attention to the defective sperm migration through the UTJ. The fact that mutant mice lacking sperm ADAM3 have defects in both UTJ migration and ZP binding implies that both UTJ and ZP present the same sperm associating factor(s). Since genes coding the three major ZP glycoproteins (ZP1, ZP2, and ZP3) are not expressed in the UTJ region, other (minor) components of the ZP extracellular matrix and also protein modifications such as glycosylation should be considered (43).

It should be noted that Adam3 is a pseudogene in human (44). Since UTJ morphology is divergent among species and is a rather simple funnel shape in human females (45), human spermatozoa may no longer need ADAM3 for UTJ migration. However, human spermatozoa are able to bind to cumulus free ZP and thus ZP binding ability is well correlated with male fertility (46, 47). There are at least 21 ADAM family members found in human and 7 are testis specific, so other ADAM protein(s) may substitute for the ADAM3 function. All the testis specific ER chaperones (*Clgn*, *Calr3*, and *Pdilt*) are widely conserved in placental mammals including human, indicating that not only ADAM3, but also other sperm fertilizing proteins, must require this unique quality control system for their maturation. We conclude that testis specific quality control for nascent proteins in the ER is crucial in equipping spermatozoa with their fertilizing ability and in understanding how to develop new strategies for both contraception and treatment for male infertility.

Materials and Methods

Animal experimentation

All animal experiments were approved by the Animal Care and Use committee of the Research Institute for Microbial Diseases, Osaka University.

Antibodies

Rabbit antisera against two PDILT C-terminal peptides (IRKPEEPERRKETA and QPKPEQPKPERKLEV) were raised for this study. Rabbit antisera against CAXN, CALR, CLGN, CALR3 and ADAM3 were described previously (9, 11). The monoclonal antibodies were described previously [TRA369 for CLGN (48), 1D5 for ACE (6) and KS130-158 for ADAM1b, KS107-190 for ADAM2, and KS64-125 for IZUMO1 (11) and purchased from suppliers [7C1 for ADAM3 (Chemicon), 9D for ADAM2 (Chemicon), SC53029 for α -TUBULIN (Santa Cruz Biotechnology) and Map for PDIA3 (Abcam)]. Anti ZPBP1 polyclonal antibody was kindly provided by Dr. Martin M. Matzuk (49).

Immunoblot

Immunoblot analysis was performed as described previously (6). Briefly, sperm from the epididymis and vas deferens were collected and incubated in lysis buffer containing 1% Triton X-100 for 20 min on ice. The various tissues were excised and homogenized in lysis buffer and then placed on ice for 1 hr. These extracts were centrifuged, and the supernatants were collected. Proteins were separated by SDS-PAGE under reducing or non-reducing conditions and transferred to PVDF membranes (Millipore). After blocking, blots were incubated with primary antibodies overnight at 4°C, and then incubated with horseradish-peroxidase conjugated secondary antibodies. The detection was performed using an ECL plus Western blotting detection kit (GE Healthcare).

Immunoprecipitation

Immunoprecipitation was performed by magnetic bead separation (MACS separation; Miltenyi Biotec) according to the manufacturer's protocol with minor modifications. Briefly, testes were solubilized with 1 ml of ice-cold lysis buffer [10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1% Triton X-100, and 1% Protease Inhibitor Cocktail (Nacalai Tesque)] and thus prepared lysates were centrifuged at 15,300 x g for 10 min at 4°C. One-mg of testis lysate was incubated with 2 µl of each antiserum and 50 µl of protein A-conjugated microbeads (MACS Protein A Microbeads; Miltenyi Biotec) for 1 hr at 4°C. The samples were applied to columns in the magnetic field of the micro-MACS separator, and the columns were rinsed four times with 200 µl of the wash buffer (1 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0) and once with 100 µl of low salt wash buffer (50 mM Tris-HCl pH 8.0 containing 1% Nonidet P-40). Finally, 70 µl of preheated (96°C) 1 x SDS sample buffer was applied to the columns, and eluate fractions were collected for analysis by SDS-PAGE.

Immunohistochemistry

Fresh sperm collected from the epididymis of wild type and mutant mice were mounted on glass slides and dried. For ADAM3 immunostaining, sperm were fixed in 4% paraformaldehyde for 30 min. After washing with PBS, slides were blocked with 10% Goat Serum /PBS for 2 hr and incubated with primary antibodies in 10% New Born Calf Serum (NBCS)/PBS at 4°C overnight. After washing with PBS, the slides were incubated with Alexa Fluor 546 conjugated goat anti-rat IgG or anti-rabbit IgG antibodies (Invitrogen) in 10% NBCS/PBS for 2 hr. After washing with PBS, the slides were observed under an Olympus IX-70 fluorescence microscope.

Trypsin treatment

For cell surface proteins, live germ cells were collected from testes. The seminiferous tubules were minced with a razor blade to release germ cells. The cell suspension in PBS was filtered through a nylon mesh, the cells were collected by centrifugation at 2000 rpm for 5 min and the cell pellet was resuspended in PBS. The germ cells (5×10^7 cells/ml) were incubated with 0, 20 or 40 µg/ml L-1-tosylamido-2-

phenylethyl chloromethyl ketone-treated trypsin (Sigma) for 5 min at 20°C. Trypsin digestion was terminated by the addition of soybean trypsin inhibitor (Sigma). Samples were analyzed by reducing SDS-PAGE and immunoblotted with anti ADAM2 or ADAM3 antibodies.

Phase separation of Triton X-114 extracts of sperm

Phase separation of Triton X-114 extracts of testes were performed as described Yamaguchi et al. (6).

Fertility test and sperm migration assay

B6D2F1 mice were used as wild-type controls. Three females were caged with each male for 1 month and kept another 3 weeks to observe pregnancy. The copulation was confirmed by observing the plug every morning. Sperm migration analysis was performed as described previously (29, 50).

Sperm deposition into oviduct

Sperm deposition into oviducts was performed as described previously (51). After 24-27 hr of sperm deposition, eggs were collected by flushing out the oviducts and fertilized eggs were implanted into the fallopian tubes of a pseudopregnant female mouse.

In vitro fertilization

Eggs collected from superovulated females 14 hr after hCG injection were placed in a 100 µl TYH medium drop covered with paraffin oil. Conditioned media were made by culturing cumulus intact eggs collected from 4 female mice in 100 µl TYH medium for 2 hours. To remove cumulus cells, eggs were treated with hyaluronidase (1 mg/ml) for 5 min. Epididymal sperm were collected from 3 month old male mice and incubated in TYH medium (52) for 2 hr for capacitation. Capacitated sperm were added to the drop containing eggs at a final concentration of 2×10^5 sperm/ml. After 8 hr of coincubation, the formation of pronuclei was observed under a Hoffman modulation contrast microscope. To assess the

zona pellucida binding ability, cumulus free eggs were incubated with capacitated sperm for 20 min prior to observation.

Statistical analysis

All the values were the means \pm standard deviation of at least three independent experiments. Statistical analyses were performed using student's t-test. Differences were considered significant at $P < 0.05$.

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Figure legends

Fig. 1. PDILT associates with testis specific chaperones and ADAM3.

(A) Immunoprecipitates (IP) with anti-PDILT antibody from testis lysates (100 μ g) were probed with antibodies against CANX, CALR, CLGN, and CALR3. Testis lysates (10 μ g) were loaded as the input control. PDILT associated with CLGN and CALR3, but not with CANX and CALR. Asterisk indicates an IgG signal recognized by the secondary antibodies.

(B) Immunoprecipitates (IP) with anti-PDILT antibody from testis lysates (100 μ g) were probed with antibodies against ADAM1B, ADAM2, ADAM3, and PDILT. PDILT only associated with ADAM3.

(C) Heterodimerizations of ADAM1A/ADAM2 and ADAM1B/ADAM2 were probed by anti-ADAM2 antibody under nonreducing conditions (17). Both complexes were absent in *Clgn*^{-/-} testis but were not impaired in *Pdilt*^{-/-} testis.

(D) Western blot analysis of sperm lysates. ADAM3 was not detected in *Pdilt*^{-/-} sperm, whereas other sperm fertilizing proteins were present.

(E) Immunofluorescence staining of sperm with antibodies against ADAM2 and ADAM3. ADAM3 was not detected in *Pdilt*^{-/-} sperm, whereas ADAM2 remained.

Fig. 2. ADAM3 requires PDILT/CALR3 to be folded properly and transported onto the sperm membrane.

(A) Western blot analysis of testis and epididymal sperm lysates. ADAM3 was not observed in caput epididymal sperm of *Pdilt*^{-/-} mice. Since testis and caput epididymis contain fewer sperm, 20 μ g of extracts were loaded, whereas 6 μ g were loaded for corpus and cauda epididymis. Asterisk indicates an IgG signal recognized by the secondary antibodies.

(B) Live germ cells collected from testes of the different mutant mice were trypsinized and then analyzed by western blotting. The lysate prepared from 5×10^5 cells was loaded in each lane. ADAM2 was not digested in the *Clgn*^{-/-} germ cells, whereas ADAM3 was not digested in the *Calr3*^{-/-} and *Pdilt*^{-/-} germ cells.

(C) Membrane proteins from testicular germ cells were separated into detergent depleted or enriched fractions with Triton X-114. The proteins were separated by SDS-PAGE under reducing (R) or non-reducing (NR) conditions, then subjected to western blotting analysis. The control proteins ZPBP1 (that localizes to the acrosomal matrix) and IZUMO1 (that contains a transmembrane domain) were distributed in detergent depleted and enriched phases, respectively.

(D, E) Immunoprecipitates (IP) with anti-CLGN, CALR3, or PDILT antibodies from testis lysates of wild type or each mutant mouse (100 µg) were examined by western blotting with the anti-ADAM3 antibody.

Fig. 3. Impaired sperm migration from the uterus into the oviduct in *Pdilt* ^{-/-} mice.

(A) Pregnancy rate (pregnancy/vaginal plug formation) presented as a percentage. *Pdilt* ^{-/-} males copulated normally but failed to induce pregnancy. Four *Pdilt* ^{-/-} males were examined. The number of males used and the total number of plugs observed are indicated in parentheses.

(B) Uterus and oviduct collected from females mated with *Pdilt* ^{+/-} and *Pdilt* ^{-/-} males carrying Acr3-EGFP and CAG/su9-DsRed2 tagged sperm 2 hr after coitus (29). Although heterozygous mutant sperm were observed in the oviduct and the UTJ, *Pdilt* ^{-/-} sperm were not observed in the UTJ.

(C-D) Artificial sperm deposition into oviduct. Capacitated spermatozoa were directly deposited into the oviduct with a fine glass needle under a stereomicroscope. The eggs were collected 24-27 hr after the sperm deposition and the 2-cell stage embryos were judged as fertilized.

(E) New born pups developed from the eggs fertilized with *Adam3* ^{-/-} sperm after artificial sperm deposition into oviduct.

Fig. 4. Sperm unable to bind to ZP can fertilize cumulus intact eggs.

(A, B) *Pdilt* ^{-/-} sperm were not able to fertilize eggs *in vitro*. Heterozygous-type sperm successfully bound to the egg ZP, but *Pdilt* ^{-/-} sperm failed to adhere despite frequent collisions (supplemental Movies S1). The average fertilization rate from three independent experiments is presented.

(C) *Pdilt* ^{-/-} sperm can fertilize cumulus-intact and ZP-intact eggs *in vitro*.

Fig. 1

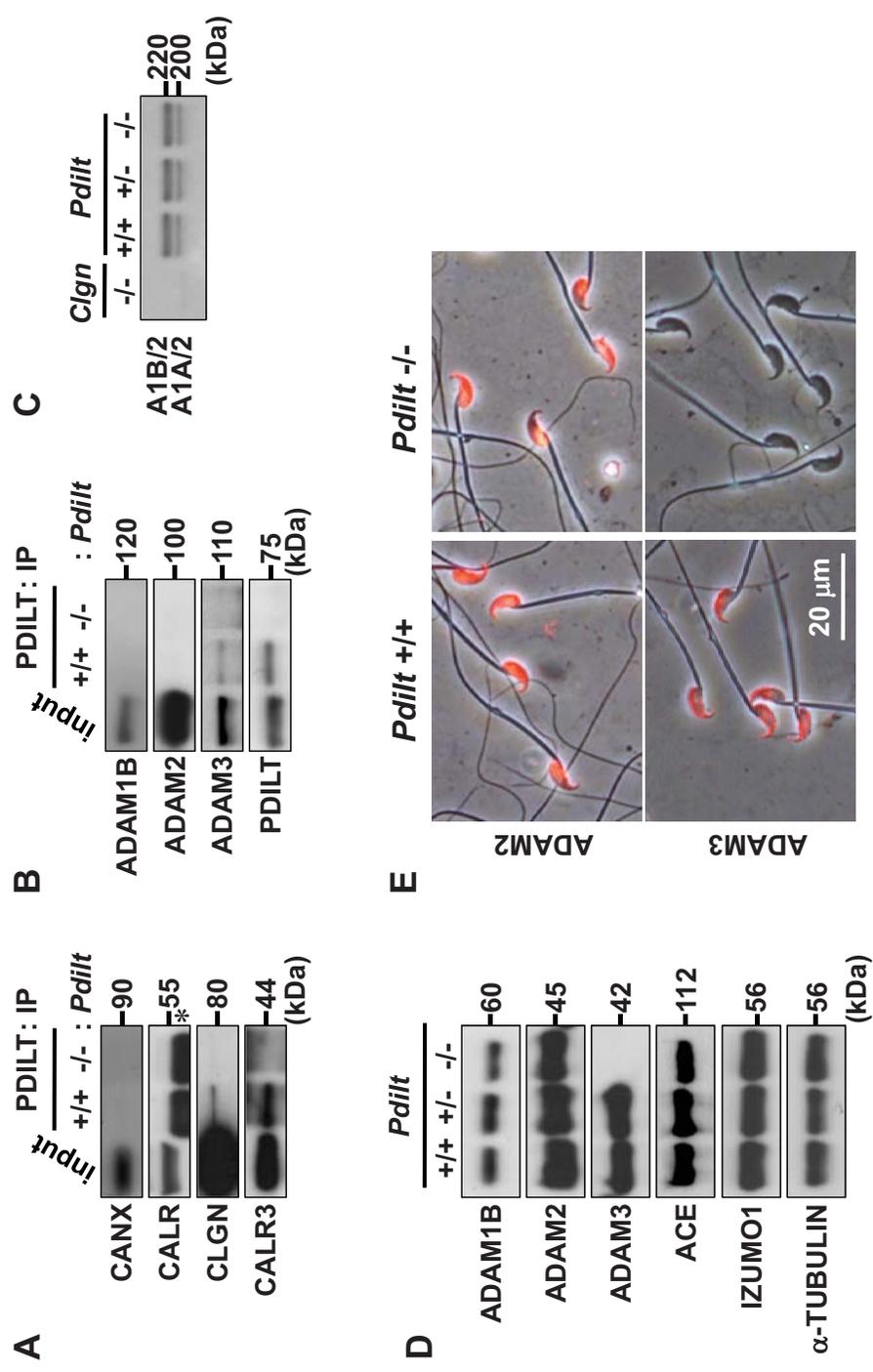


Fig. 2

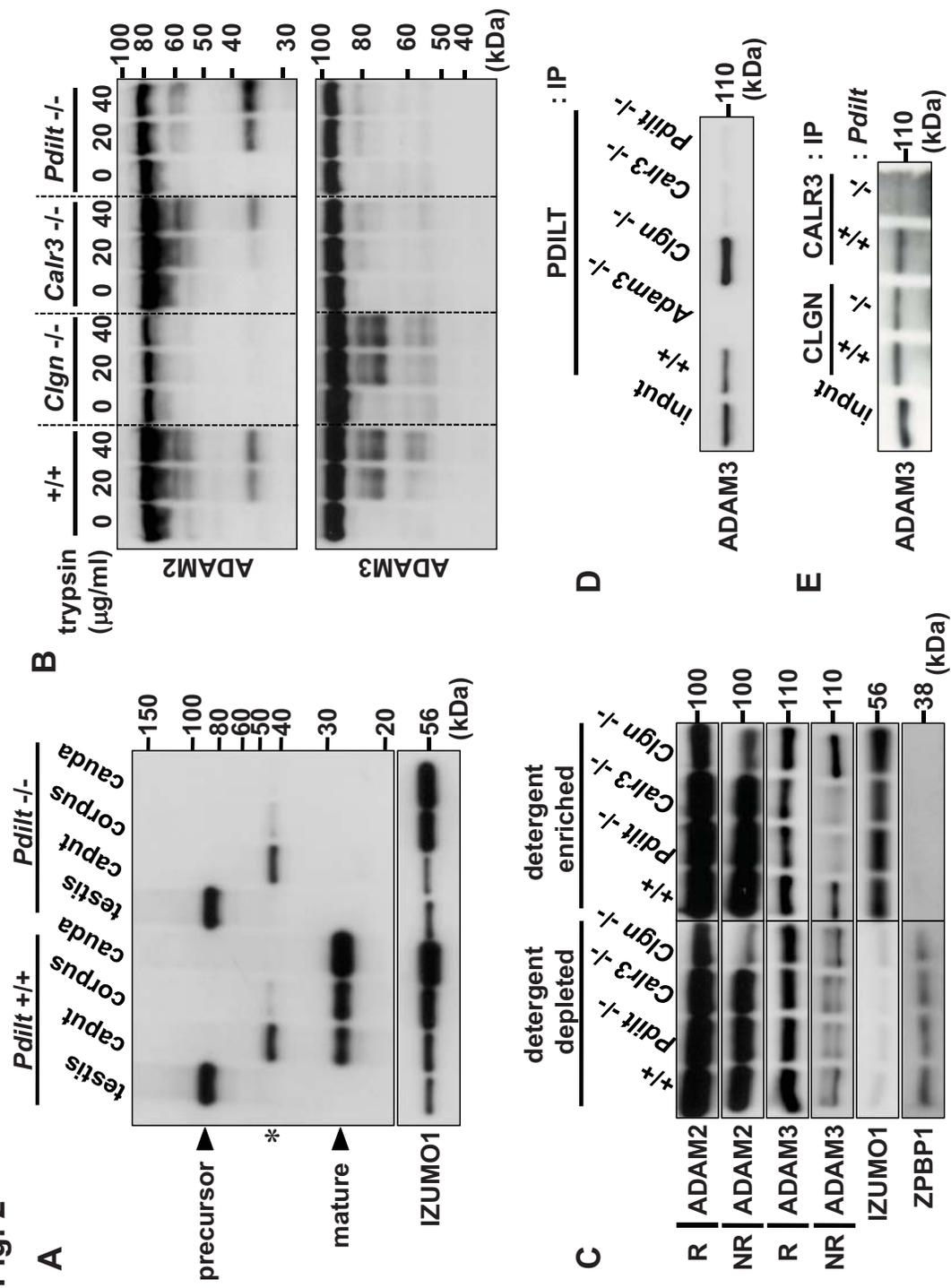


Fig. 3

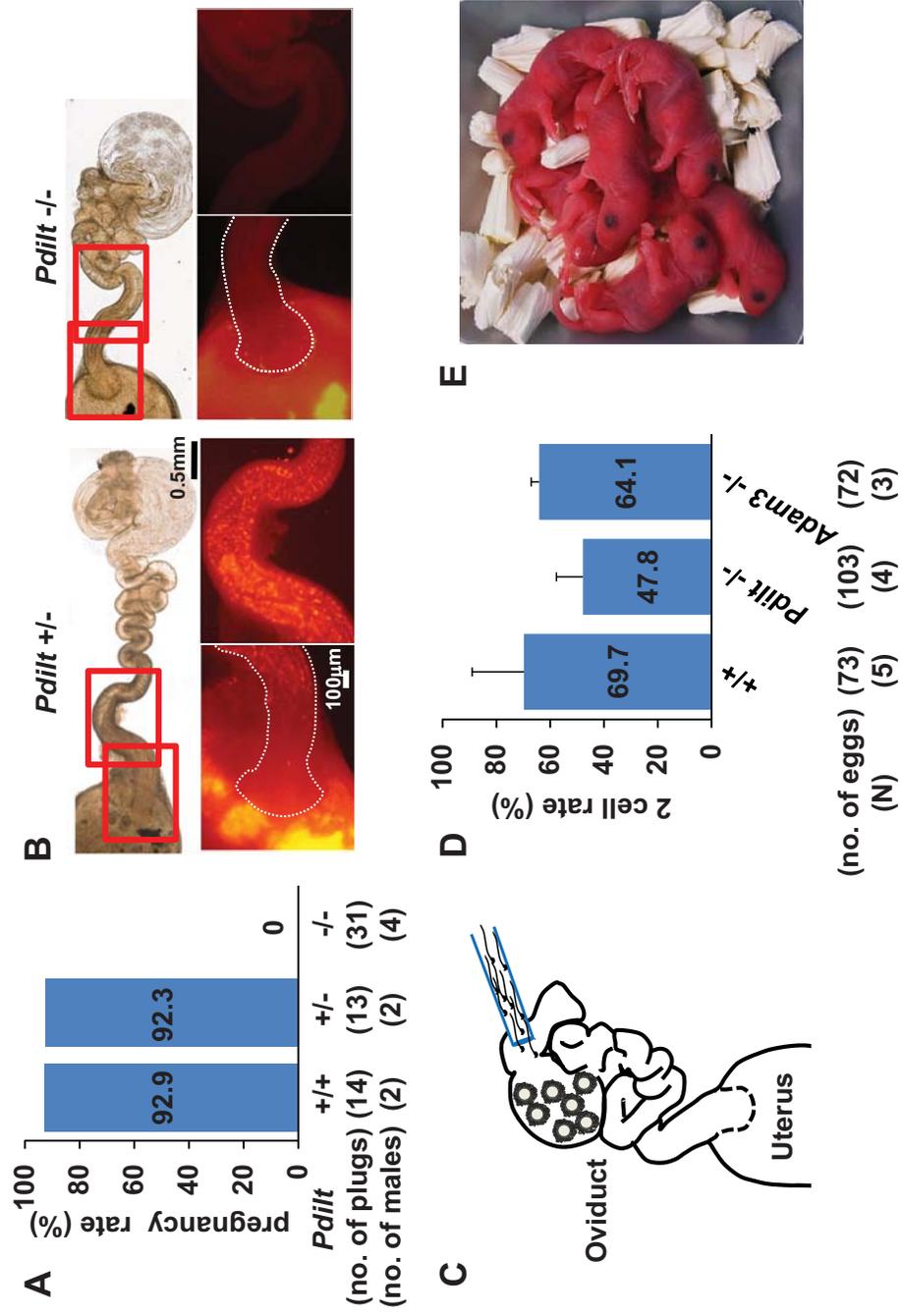


Fig. 4

