Characterization of mouse sperm TMEM190, a small transmembrane protein with the trefoil domain: evidence for co-localization with IZUMO1 and complex formation with other sperm proteins

Hitoshi Nishimura¹, Surabhi Gupta², Diana G Myles² and Paul Primakoff¹

Departments of ¹Cell Biology and Human Anatomy, School of Medicine and ²Molecular and Cellular Biology, University of California Davis, Davis, California 95616, USA

Correspondence should be addressed to H Nishimura who is now at Department of Biology, Emory University, Atlanta, Georgia 30322, USA; Email: hnishim@emory.edu

Abstract

TMEM190, a small transmembrane protein containing the trefoil domain, was previously identified by our proteomic analysis of mouse sperm. Two structural features of TMEM190, 'trefoil domain' and 'small transmembrane protein', led us to hypothesize that this protein forms a protein–protein complex required during fertilization, and we characterized TMEM190 by biochemical, cytological, and genetic approaches. We showed in this study that the mouse *Tmem190* gene exhibits testis-specific mRNA expression and that the encoded RNA is translated into a 19-kDa protein found in both testicular germ cells and cauda epididymal sperm. Treatment of the cell surface with proteinase K, subcellular fractionation, and immunofluorescence assay all revealed that mouse TMEM190 is an inner-acrosomal membrane protein of cauda epididymal sperm. During the acrosome reaction, TMEM190 partly relocated onto the surface of the equatorial segment, on which sperm–oocyte fusion occurs. Moreover, TMEM190 and IZUMO1, which is an immunoglobulin-like protein required for gamete fusion, co-localized in mouse sperm both before and after the acrosome reaction. However, immunoprecipitates of TMEM190 contained several sperm proteins, but did not include IZUMO1. These findings suggest that a mouse sperm protein complex(es) including TMEM190 plays an indirect role(s) in sperm–oocyte fusion. The role(s), if any, is probably dispensable since *Tmem190*-null male mice were normally fertile.

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Introduction

Fertilization is a reproductive strategy to create a diploid zygote from two haploid gametes, which are usually a sperm and an oocyte. In mammals (Yanagimachi 1994), fertilization is initiated by sperm binding to the zona pellucida (ZP), an extracellular glycoprotein matrix surrounding the oocyte cell. The ZP-bound sperm penetrates through the ZP, reaches the perivitelline space between the ZP and the oocyte, and binds to and fuses with the oocyte plasma membrane. To complete this process, the sperm acrosome reaction plays two major roles: to release acrosomal contents into the extracellular space, which possibly facilitate sperm penetration through the ZP, and to remodel the sperm surface so that it can participate in sperm–oocyte fusion.

We do not currently understand mammalian fertilization at the molecular level. For example, ZP3, which is a component glycoprotein of the oocyte ZP, had been long believed to act as a sperm receptor that triggers the acrosome reaction (Litscher *et al.* 2009,

Wassarman & Litscher 2009). However, there are recent data that contradict this interpretation (Baibakov *et al.* 2007, Dean 2007, Gahlay *et al.* 2010). As another example, both the sperm immunoglobulin-like protein IZUMO1 (Inoue *et al.* 2005) and the oocyte tetraspanin protein CD9 (Kaji *et al.* 2000, Le Naour *et al.* 2000, Miyado *et al.* 2000) appear to be required for sperm–oocyte fusion in mice, yet the fusogenic mechanism including how sperm IZUMO1 and oocyte CD9 are involved is still mysterious.

Currently, there are too many gaps in the available data to allow a comprehensive and coherent model of fertilization in animals. A fundamental problem is that sperm and oocytes are specialized, terminally differentiated cell types that are haploid. Consequently, there are few useful tools, such as RNA-mediated interference using tissue-cultured cells, which can be applied to explore gene functions *in vitro*. Analysis of genetically manipulated animals is currently the only rigorous way to investigate the functions of fertilization-related genes.

Indeed, much recent progress in fertilization research has resulted from studies of gene-targeted mice (Inoue *et al.* 2007, Okabe & Cummins 2007, Ikawa *et al.* 2008).

We have recently evaluated the mouse sperm proteome (Stein *et al.* 2006), in order to identify candidate genes that would be investigated by gene targeting. Among the identified mouse sperm proteins, there was a single-pass transmembrane protein (166 amino acids) encoded by the *Tmem190* gene (also known as 4930572D21Rik or UniGene Ms.160043). *Tmem190* contains five exons (Fig. 1A, upper) and is a single-copy gene located on the chromosome 7. This gene is likely testis-specific, since mouse expressed sequence tag (EST) clones of *Tmem190* were isolated exclusively from the testis. As for the human ortholog gene *TMEM190* (also known as *MDAC1* or UniGene Hs.590943), its EST clones were isolated from the pharynx and the ovary. However, it is not yet clear whether *TMEM190* is expressed in the human testis.

Mouse and human *TMEM190* proteins (68% identity to each other) possess a single trefoil domain (also called the P-domain or TFF domain) in the extracellular region (Fig. 1A, lower), although the entire sequences of these

two proteins show no significant homology to those of other proteins. The trefoil domain consists of six conserved Cys residues that form three disulfide linkages in the pattern of Cys1–Cys5, Cys2–Cys4, and Cys3–Cys6 (Fig. 1A, lower). This domain has been implicated in binding, probably to carbohydrates and/or proteins (Hoffmann & Hauser 1993, Otto & Wright 1994, Kjellev 2009). Indeed, the trefoil domain is contained in a variety of proteins that participate in protein–protein and/or protein–carbohydrate interactions, such as sucrase-isomaltase (Alfalah *et al.* 1999, Spodsborg *et al.* 2001), the ZP-glycoprotein ZP1 (Conner *et al.* 2005), the human ZP-glycoprotein ZP4 (Conner *et al.* 2005), and trefoil factors 1–3 (Hoffmann & Hauser 1993, Otto & Wright 1994, Kjellev 2009).

Moreover, small single-pass transmembrane proteins from various species and tissues have been known to associate with self and/or non-self proteins to play roles in regulation of Na/K-ATPase (Rivard *et al.* 2005), mitochondrial oxidative phosphorylation (Zickermann *et al.* 2010), ligand-independent activation of receptors (Freeman-Cook & Dimaio 2005, Lai *et al.* 2005), polarization of epithelial cells (Makarova *et al.* 2003,

Figure 1 The mouse *Tmem190* gene encodes a 19-kDa sperm-specific transmembrane protein containing a single trefoil domain. (A) Structures of the mouse *Tmem190* gene and its predicted protein. (Upper) Protein-coding and non-coding regions in each exon (Ex.) are shown by black and white boxes respectively, while intervening horizontal lines represent introns. Broken lines indicate boundaries of the protein regions that each exon encodes. (Lower) *Tmem190* codes for a protein that consists of a signal sequence followed by the trefoil, transmembrane (TM), and cytoplasmic tail domains. The prediction of each domain was based on the Ensemble (<http://uswest.ensembl.org>) and/or SMART (<http://smart.embl-heidelberg.de>) databases. In this protein, six out of nine Cys residues (C) are presumed to form three disulfide bonds in the pattern of C1–C5, C2–C4, and C3–C6. AA, amino acid. (B) RT-PCR analysis for the *Tmem190* gene. Gene expression of *Tmem190* was examined by RT-PCR in various mouse tissues including testis and TGCs. The mouse testis-specific genes *Adam2* and *Adam3* were also tested as positive controls. RT-PCR for the mouse *Gapdh* gene, which is ubiquitously expressed, was used as an internal positive control. Calculated sizes of each PCR product are 435 bp (*Tmem190*), 411 bp (*Adam2*), 398 bp (*Adam3*), and 983 bp (*Gapdh*). The data shown here are representatives of three independent experiments. Sk., skeletal. (C) Immunoblot analysis for TMEM190 protein. Cell lysates of TGCs (0.5×10^6 TGCs/lane) or cauda epididymal sperm (1×10^6 sperm/lane) were separated by 14% (w/v) SDS-PAGE in the presence or absence of 2-mercaptoethanol (2ME), followed by immunoblotting using α -TMEM190. Arrowheads indicate immunoreactive signals derived from TMEM190, since they were not detected with rblgG (data not shown). Three independent experiments confirmed these data.

Roh *et al.* 2003), synthesis of poly-phosphate chains (Fang *et al.* 2007), and organization of the Golgi apparatus (Rojo *et al.* 2000).

Based on these lines of evidence, we hypothesized that TMEM190 acts during fertilization by creating a protein complex with itself and/or other mouse sperm proteins. In this study, mouse TMEM190 was characterized by biochemical and cytological approaches, and a mouse line that carries a null-mutated allele of the *Tmem190* gene was created. Our data demonstrated that TMEM190 is co-localized with IZUMO1 in mouse sperm before and after the acrosome reaction. TMEM190 was also found to associate with several sperm proteins, but not with IZUMO1. Since male mice lacking TMEM190 were normally fertile, the function(s) of this mouse sperm protein is probably dispensable during fertilization.

Results

The mouse *Tmem190* gene shows testis-specific transcription and encodes a 19-kDa protein found in male germ cells and gametes

The only EST clones of mouse *Tmem190* were isolated from testis, suggesting that this mouse gene has testis-specific transcription. To confirm this possibility, we examined expression of the *Tmem190* gene in various mouse tissues by RT-PCR. As shown in Fig. 1B, a PCR product for *Tmem190* was found only in first-strand cDNA samples created from mRNA of mouse testis or testicular germ cells (TGCs, a mixed population of spermatogenic cells at various stages). This pattern of testis-specific expression was also observed for the *Adam2* or *Adam3* genes, but the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase gene *Gapdh* was amplified in all tested samples. These data show that the mouse *Tmem190* gene is expressed specifically in the testis.

Next, we investigated expression of the *Tmem190*-encoded protein during spermatogenesis. Protein extracts of TGCs or sperm were examined by immunoblotting using affinity-purified rabbit anti-mouse TMEM190 antibody (α -TMEM190; Fig. 1C). In TGC lysates, a prominent 19-kDa protein was detected with α -TMEM190 under the non-reducing and reducing conditions. A 14-kDa immunoreactive signal was detected in non-reduced TGC proteins, but not in reduced samples. These results indicate that TGCs contain TMEM190, which exists mostly as a 19-kDa single-chain protein. The 14-kDa variant is probably a multi-chain form of the TMEM190 protein where two or more chains are linked together by disulfide bonds.

Non-reduced sperm extracts contained 19-, 15-, and 14-kDa proteins that reacted strongly and 37- and 32-kDa proteins that reacted weakly with α -TMEM190. Sperm extracts after reduction lacked the two largest

variants but still contained prominent 19-, 15-, and faint 14-kDa proteins. These data suggest that most of the 19- and 15-kDa bands represent single-chain forms, whereas the 14-kDa band corresponds to a multi-chain protein. The 37- and 32-kDa proteins observed under non-reducing conditions are likely to be multimeric forms consisting of some combination of the 19-, 15-, and 14-kDa TMEM190 variants.

TMEM190 mostly resides within TGCs, although this protein is partly present on the cell surface

To gain clues about the TMEM190 function(s) during fertilization, we determined the localization of this protein in mouse TGCs, acrosome-intact sperm (AI-sperm), and acrosome-reacted sperm (AR-sperm). Firstly, TGCs were prepared from the mouse line B6FJ51, in which soluble green fluorescent protein (GFP) accumulates exclusively in the sperm acrosomes (Kim & Gerton 2003). Therefore, TGCs, which are progenitor cells of sperm, contain GFP as an intracellular protein. Cell suspensions (Fig. 2A, left upper panel) and lysates (Fig. 2A, right upper panel) of TGCs were digested with proteinase K, followed by immunoblotting for the TGC-surface protein SPAM1 (formerly PH-20; Nishimura *et al.* 2007). This protein almost or completely disappeared in cell suspensions and lysates at any tested concentration of proteinase K, demonstrating that most SPAM1 resides on the TGC surface. On the other hand, both 29- and 27-kDa proteins of GFP, which presumably correspond to precursor and mature forms respectively, were not affected by up to 100 μ g/ml proteinase K in intact TGCs (Fig. 2A, left middle panel). In TGC lysates (Fig. 2A, right middle panel), proteinase K degraded 29-kDa GFP at all tested concentrations and 27-kDa GFP at 20 μ g/ml or higher concentrations. These results expectedly show that GFP is intracellularly localized in TGCs.

By treatment of intact TGCs with 10–100 μ g/ml proteinase K, small but significant amounts of 19-kDa TMEM190 were degraded into a 15-kDa protein (Fig. 2A, left lower panel). As lysed TGCs were used (Fig. 2A, right lower panel), the 19-kDa immunoreactive signal was completely missing even at the lowest concentration (10 μ g/ml) of proteinase K, and the 15-kDa signal was mostly erased by the addition of 100 μ g/ml proteinase K. Therefore, testicular TMEM190 seems to be an intracellular protein, although a small fraction of this protein is present on the cell surface.

We also examined the TMEM190 localization in mouse TGCs by immunofluorescence (Fig. 2B). Methanol-fixed TGCs were treated with mock or 0.1% (v/v) Triton X-100 (TX100) for permeabilization, followed by staining with α -TMEM190. To monitor the acrosome formation during spermatogenesis, we counter-stained the same TGCs with peanut agglutinin (PNA), which is a probe to detect the sperm acrosomes. Among mock-permeabilized TGCs,

Figure 2 Testicular TMEM190 is mostly an intracellular protein. (A) Proteinase K digestion of TGC-surface proteins. TGCs were isolated from the transgenic mouse line B6SJF1, in which GFP is present within the sperm acrosomes. Therefore, GFP is intracellularly present in TGCs. Cell suspensions and lysates were prepared from the GFP-carrying TGCs, digested with various concentrations of proteinase K, and analyzed by immunoblotting (0.5×10^6 TGCs/lane) for SPAM1, GFP, or TMEM190. SPAM1 and GFP were examined as positive controls for TGC-surface and intracellular proteins respectively. This result was confirmed by three independent experiments. Prot. K, proteinase K. (B) Immunofluorescence of mouse TGCs for TMEM190. After methanol-fixed TGCs of CD1 mice were treated with mock (–TX100) or TX100 (+TX100) for permeabilization, these TGC samples were incubated with rblgG or α -TMEM190. The same cells were counter-stained with DAPI for nuclei and PNA for monitoring the acrosome formation. We did not detect any significant immunoreactive signal with rblgG (data not shown). Three independent experiments confirmed the data shown here. DIC, differential interference contrast.

round spermatids contained TMEM190 on the cell surface (Fig. 2Bd and Be), whereas this protein was barely found on the testicular sperm surface (Fig. 2Bi and Bj). As TGCs were permeabilized with TX100, immunoreactive signals of TMEM190 were unevenly distributed within the intracellular space of primary spermatocytes (Fig. 2Bn and Bo). In round spermatids, TMEM190 was localized both on the cell surface and in the ring-shaped organelle, which probably corresponds to an intermediate structure of the acrosome whose formation processes during spermatogenesis (Fig. 2Br and Bt). We carried out similar experiments using normal rabbit IgG (rblgG) instead of α -TMEM190, but no significant immunoreactive signal was detected in any tested sample (data not shown).

These findings indicate that primary spermatocytes pre-meiotically contain intracellular TMEM190 protein.

It was also shown that, in post-meiotic round spermatids, TMEM190 resides mostly in the intracellular space and partly on the cell surface, whereas the surface of testicular sperm barely contains TMEM190. Moreover, these immunofluorescence-based data (Fig. 2B) were in good agreement with the results obtained by the proteinase K digestion assay of TGCs (Fig. 2A).

TMEM190 relocates from the inner-acrosomal membrane onto the equatorial segment surface during the acrosome reaction

We next determined the localization of mouse TMEM190 in AI- and AR-sperm. For the proteinase K digestion assay, AI-sperm (cauda epididymal sperm) were prepared from B6FJS1 mice. Intact (Fig. 3A, left upper panel) and lysed (Fig. 3A, right upper panel)

Figure 3 TMEM190 is present in the intracellular head region of mature sperm. (A) Proteinase K digestion of sperm-surface proteins. Cauda epididymal sperm carrying acrosomal GFP were isolated from B6SJF1 mice. Cell suspensions and lysates were prepared from the sperm, digested with various concentrations of proteinase K, and analyzed by immunoblotting (1×10^6 sperm/lane) for ADAM2, GFP, or TMEM190. ADAM2 and GFP were examined as positive controls for sperm-surface and intracellular proteins respectively. This result was confirmed by three independent experiments. Prot. K, proteinase K. (B) Subcellular localization of TMEM190 in mouse sperm. (Left) Subcellular fractionation of mouse sperm that were subjected to the A23187-induced acrosome reaction. OAM, outer-acrosomal membrane; PM, plasma membrane; SACs, soluble acrosomal contents; ACR, acrosomal cap region; N, nucleus; MP, midpiece; IAM, inner-acrosomal membrane; EQR, equatorial region; and PHR, posterior head region. (Right) Immunoblotting of subcellular components for TMEM190. Proteins contained in each subcellular fraction (equivalent to 1×10^6 sperm/lane) were separated by 14% (w/v) SDS-PAGE and then analyzed by immunoblotting for ADAM2, GFP, or TMEM190. ADAM2 and GFP represent typical cell-surface and soluble acrosomal proteins respectively, and they served as controls. These data are representatives of three independent experiments.

Al-sperm were treated with proteinase K and then analyzed by immunoblotting for the sperm-surface protein ADAM2 (Kim *et al.* 2003, 2004). The amounts of this protein were reduced as a function of the proteinase K concentration, and similar proteolytic rates were observed irrespective of whether or not the Al-sperm were lysed prior to proteinase K addition; as expected, our results confirmed that ADAM2 is a sperm-surface protein. In contrast, intracellular GFP was not obviously affected by up to 100 µg/ml proteinase K (Fig. 3A, left middle panel), but definite proteolysis of GFP was observed in Al-sperm lysates at either 50 or 100 µg/ml proteinase K (Fig. 3A, right middle panel).

The 19-, 15-, and 14-kDa TMEM190-derived immunoreactive signals were detected in intact sperm at all tested concentrations of proteinase K (Fig. 3A, left lower panel). However, in sperm lysates, proteinase K digested the 19-kDa TMEM190 at any tested concentration and the 15- and 14-kDa proteins at

50 or 100 µg/ml (Fig. 3A, right lower panel). These data indicate that most TMEM190 is intracellular in Al-sperm. However, TMEM190 might be partly located on the surface of intact sperm, since 19-kDa TMEM190 seemed to be slightly degraded into a 15- or a 14-kDa protein by increasing the proteinase K concentration (Fig. 3A, left lower panel).

We also determined the subcellular fraction(s) of mouse sperm that contained TMEM190 (Fig. 3B). Cauda epididymal sperm were prepared from B6FJS1 mice, incubated with the calcium ionophore A23187 to induce the acrosome reaction, and fractionated into subcellular components (Fig. 3B, left). We prepared two fractions that were analyzed by immunoblotting (Fig. 3B, right): the soluble acrosomal contents plus the membrane proteins released during the acrosome reaction (RPA fraction) and mixed vesicles of the plasma and outer-acrosomal membranes (PM/OAM fraction). As previously reported (Kim *et al.* 2003, 2004), ADAM2

Figure 4 Intracellular TMEM190 protein partly relocates onto the equatorial segment surface of mouse sperm during the A23187-induced acrosome reaction. After AI- or AR-sperm of CD1 mice were treated with mock (– TX100) or TX100 (+ TX100) for permeabilization, these sperm samples were incubated with rblgG or α -TMEM190. The same cells were counter-stained with DAPI and PNA for nuclei and acrosomes respectively. White arrowheads show the localization of TMEM190 in AI- or AR-sperm. No obvious signal was detectable with rblgG (data not shown). Three independent experiments confirmed the data shown here. Bar = 10 μ m.

was found in PM/OAM fraction and unfractionated AR-sperm (Fig. 3B, right upper panel). GFP, a marker protein of the sperm acrosome, was exclusively present in RPA fraction after the acrosome reaction (Fig. 3B, right middle panel). Therefore, previously characterized subcellular components of mouse sperm were fractionated in the expected manner. We found that the TMEM190 protein is present in AI- and AR-sperm, but not in RPA and PM/OAM fractions (Fig. 3B, right lower panel). Furthermore, TMEM190 was contained in the acrosome-reacted sperm head (ARH) fraction (data not shown). These results show that most TMEM190 stays at the sperm heads even after the acrosome reaction.

Immunofluorescence was then conducted to further examine the subcellular location of TMEM190 in mouse sperm (Fig. 4). In mock-permeabilized AI-sperm, any significant immunoreactive signal was not detected with rblgG (data not shown) or α -TMEM190. However, AI-sperm permeabilized with 0.1% (v/v) TX100 showed an immunoreactive signal with α -TMEM190, but not with rblgG (data not shown), around the rim of the acrosomes. These data indicate that TMEM190 is mostly localized in the inner-acrosomal membrane of AI-sperm. In contrast, regardless of whether or not AR-sperm were permeabilized, TMEM190 seemed to partly relocate during the acrosome reaction from the inner-acrosomal membrane onto the equatorial segment surface. Interestingly, this relocation pattern was similar to that of

mouse IZUMO1 (Kawai *et al.* 1989, Yamashita *et al.* 2007, Fujihara *et al.* 2010), an essential protein for sperm–oocyte fusion (Inoue *et al.* 2005).

TMEM190 is co-localized with IZUMO1 in mouse sperm before and after the acrosome reaction

To our knowledge, IZUMO1 is the only protein to satisfy both of that it resides in sperm similarly to TMEM190 and that its role during fertilization is clear. Therefore, we examined whether TMEM190 and IZUMO1 are co-localized together in permeabilized AI- or AR-sperm (Fig. 5), to access the TMEM190 function(s). TMEM190 was found to co-localize with IZUMO1 at the inner-acrosomal membrane of AI-sperm. In AR-sperm, both TMEM190 and IZUMO1 were present at the inner-acrosomal membrane and partly at the equatorial segment. These data demonstrate that TMEM190 and IZUMO1 co-localize in mouse sperm both before and after the acrosome reaction.

TMEM190 forms a protein complex(es) with other sperm proteins, but not with IZUMO1

We immunoprecipitated TMEM190 to investigate whether this protein associates with IZUMO1 (Fig. 6A). In all tested cell lysates (total), immunoprecipitates with neither rblgG- nor α -TMEM190-beads included

Figure 5 Mouse sperm TMEM190 is co-localized with IZUMO1. TX100-permeabilized AI- or AR-sperm were stained with molgM or α -IZUMO1 and rblgG or α -TMEM190. All sperm samples were also stained with DAPI to visualize each nucleus. White arrowheads indicate that IZUMO1 and TMEM190 are co-localized together in AI- or AR-sperm. These data are representatives of three independent experiments. DIC, differential interference contrast. Bar=10 μ m.

IZUMO1 (Fig. 6A, left panel), whereas TMEM190 clearly formed an immune complex specifically with α -TMEM190 (Fig. 6A, right panel). These data reveal that TMEM190 is physically independent of IZUMO1 in mouse TGCs, AI-sperm, and AR-sperm under immunoprecipitating conditions. However, it remains possible that TMEM190 and IZUMO1 are loosely associated and do not withstand the methods used for immunoprecipitation.

We also explored the possibility that TMEM190 associates with other sperm proteins (Fig. 6B). Cell-surface proteins on AR-sperm were biotinylated, bound to monomeric avidin-beads, washed, and eluted to prepare a fraction containing sperm-surface proteins

(surface). To check whether biotinylation is restricted to cell-surface proteins, 'total' and 'surface' fractions of AR-sperm before or after biotinylation were examined by immunoblotting for the sperm-surface protein ADAM2 (Fig. 6B, left upper panel) or the intracellular protein α -tubulin (Fig. 6B, left lower panel). ADAM2 exhibited a slower migration in biotinylated 'total' fractions than control samples, indicating that ADAM2 was modified with biotin. Indeed, this protein was found in 'surface' fractions of biotinylated, but not mock-biotinylated, AR-sperm. On the other hand, α -tubulin was indistinguishable in size between mock- and actually biotinylated 'total' samples. Moreover, 'surface' samples of biotinylated AR-sperm did not contain this protein.

Figure 6 TMEM190 is not directly associated with IZUMO1 but forms a complex(es) with other sperm proteins. (A) Complex formation of TMEM190 with IZUMO1. For immunoprecipitation, protein extracts (total) of TGCs, AI-sperm, or AR-sperm were incubated with rIgG- or α-TMEM190-immobilized Sepharose beads. The precipitated protein fractions (0.5×10^7 TGCs/lane or 1×10^6 sperm/lane) and 'total' samples prior to immunoprecipitation (input, 0.5×10^6 TGCs/lane or 1×10^6 sperm/lane) were then examined by immunoblot analysis using α-IZUMO1 or α-TMEM190. We confirmed this result by three independent experiments. (B) Complex formation of TMEM190 with other sperm proteins. (Left) Surface protein fractions (surface) were prepared from biotinylated AR-sperm. To confirm that cell-surface proteins, but not intracellular proteins, were biotinylated, 'total' and 'surface' fractions (equivalent to 1×10^6 sperm/lane) of non-biotinylated (-) or biotinylated (+) AR-sperm were analyzed by immunoblotting for ADAM2 (cell-surface protein) or α-tubulin (intracellular protein). Asterisks show that ADAM2 is size-shifted due to modification with biotin. (Right) 'Surface' fractions of AR-sperm were immunoprecipitated with rIgG- or α-TMEM190-beads. The precipitated biotinylated proteins (equivalent to 1×10^7 sperm/lane) were analyzed by blotting using NeutrAvidin or α-TMEM190 as probes. Molecular sizes (kDa) of the co-precipitated proteins are expressed as 'p+ number' (for example, p65 represents a 65-kDa protein). The data shown here are representatives of three independent experiments.

Therefore, 'surface' fractions of AR-sperm were appropriately prepared.

TMEM190 was then immunoprecipitated with α-TMEM190-beads in 'surface' fractions. After co-precipitated proteins were separated by SDS-PAGE, they were transferred onto membranes and captured by incubation of the blotted membranes with NeutrAvidin (Fig. 6B, middle panel) or α-TMEM190 (Fig. 6B, right panel). TMEM190 and its co-precipitated proteins were contained only in the immunoprecipitates with α-TMEM190-beads. Particularly, 65-, 43-, 40-, and 19-kDa proteins showed clear association with TMEM190. It is not yet clear whether the biotinylated 19-kDa protein is TMEM190, since a 15-kDa surface protein was not found in the immune complexes including both 19- and 15-kDa TMEM190. Efficiency of biotinylation might be different between these two TMEM190-derived proteins. At any rate, these combined results demonstrate that TMEM190 forms a protein complex(es) on the AR-sperm surface with, at least, 65-, 43-, and 40-kDa proteins.

TMEM190-null mice are fertile

Finally, we created mice lacking TMEM190 to elucidate the *in vivo* function(s) of this protein. The entire region

of the *Tmem190* gene was replaced by the neomycin-resistance gene cassette in the mouse genome by homologous recombination (Fig. 7A). This gene targeting was confirmed by PCR genotyping of tail DNA samples that had been prepared from *Tmem190*^{+/+}, *Tmem190*^{+/-}, or *Tmem190*^{-/-} mice (Fig. 7B). Moreover, TMEM190 protein was completely missing in TGCs and cauda epididymal sperm of *Tmem190*-null mice, whereas IZUMO1, a co-localized protein with TMEM190, was of normal amounts in the same TGC or sperm samples (Fig. 7C). Protein levels of ADAM2, ADAM3, and SPAM1, of which functions are presumably independent of the TMEM190 function(s), were similar between *Tmem190*^{+/+} and *Tmem190*^{-/-} cell lysates of TGCs or cauda epididymal sperm (data not shown). These data show that a mouse line lacking TMEM190 was established.

By crossing of *Tmem190*^{+/-} male and female mice, *Tmem190*^{-/-} mice were produced according to the expected Mendelian frequency (*Tmem190*^{+/+}:*Tmem190*^{+/-}:*Tmem190*^{-/-} = 32 (24%):68 (50%):36 (26%)), and a ratio of *Tmem190*^{-/-} male and female progeny was also as expected (male:female = 17 (47%):19 (53%)). There was no significant abnormality affecting the health, body size, and behavior in *Tmem190*^{-/-} mice.

Figure 7 *Tmem190*-null mice are fertile. (A) The targeting strategy used for the mouse *Tmem190* gene. Exons and introns of *Tmem190* are shown by black boxes and intervening horizontal lines respectively. White boxes represent the neomycin-resistance gene (*neo*) cassette. A targeting vector was linearized at the KpnI site (not shown) before electroporation into mouse ES cells. FW, WTRV, and NeoRV were PCR primers used for genotyping of ES cell clones or mouse tails. WT, wild-type; KO, knock-out; Sc, SacI; X, XhoI; and SI, SalI. (B) PCR genotyping of mice carrying the targeted mutation in the *Tmem190* gene. Genomic DNA was prepared from tails of *Tmem190*^{+/+} (*+/+*), *Tmem190*^{+/-} (*+/-*), or *Tmem190*^{-/-} (*-/-*) mice and used for PCR genotyping. This result is a representative of three independent experiments. (C) Immunoblotting of *Tmem190*-null TGCs and sperm. Protein extracts were prepared from TGCs or cauda epididymal sperm of *Tmem190*^{+/+} (*+/+*) or *Tmem190*^{-/-} (*-/-*) mice and then analyzed by immunoblotting (0.5 × 10⁶ TGCs/lane and 1 × 10⁶ sperm/lane). Arrowheads indicate immunoreactive signals for 19-kDa TMEM190 or 56-kDa IZUMO1. These data were confirmed by three independent experiments. (D) *In vivo* fertility test of *Tmem190*-null mice. *Tmem190*^{+/+} (*+/+*) or *Tmem190*^{-/-} (*-/-*) male mice were individually placed with two C57BL/6 (B6) female mice in a cage. Each female that had a vaginal plug was transferred into a new cage, and the numbers of pups born were counted. The same experiments were also conducted in mating pairs of B6 males and *+/+* or *-/-* females. Data are expressed as 'mean ± s.d.'.

Figure 7D showed the data on *in vivo* fertility of *Tmem190*-null mice. As male mice lacking TMEM190 were crossed to C57BL/6 female mice, an average litter size (mean ± s.d.) of the males (8.7 ± 0.90, *n* = 6) was similar to that of *Tmem190*^{+/+} males (8.5 ± 0.65, *n* = 7). Breeding of C57BL/6 males and tested females resulted in similar average litter sizes between *Tmem190*^{+/+} and *Tmem190*^{-/-} females (8.0 ± 0.65, *n* = 6 vs 8.5 ± 0.71, *n* = 6). Hence, the mouse *Tmem190* gene is not essential for *in vivo* fertilization.

Discussion

We previously identified TMEM190 in the mouse sperm proteome as a small transmembrane protein containing the trefoil domain (Stein *et al.* 2006). From two keywords, 'trefoil domain' and 'small transmembrane protein', we hypothesized that TMEM190 acts during fertilization through the association with self and/or non-self proteins. We found in this study that mouse TMEM190 resides at the inner-acrosomal membrane of AI-sperm and relocates onto the plasma membrane of

the equatorial segment during the acrosome reaction. We also found that mouse sperm contain a protein complex(es) including TMEM190. Lastly, mice lacking TMEM190 were normally fertile.

We observed 19-, 15-, and 14-kDa proteins in both non-reduced and reduced sperm extracts by immunoblotting using α -TMEM190. Therefore, the 19-kDa protein probably corresponds to intact sperm TMEM190 and is degraded into the 15- or the 14-kDa proteins. A calculated size (16 476 kDa) of the predicted TMEM190 polypeptide lacking a 21-mer signal sequence suggests that the 19-kDa TMEM190 possesses a post-translational modification(s). N-glycans might be the case, because of the Asn⁵³-Leu⁵⁴-Cys⁵⁵ sequence in the extracellular region of TMEM190. An atypical Asn-X-Cys sequence has been demonstrated to act as an attachment site for N-glycosylation in a variety of proteins, such as human protein C (Gil *et al.* 2009), epidermal growth factor receptor (Sato *et al.* 2000), and CD69 (Vance *et al.* 1999). Another possible modification is phosphorylation, since the cytoplasmic tail of TMEM190 contains seven Ser (residues 124, 129, 131,

136, 140, 146, and 158) and one Thr (residue 162) residues, which are potential substrates for protein kinases (<http://www.cbs.dtu.dk/services/NetPhos/>). On the other hand, most TMEM190 is intracellular in AI-sperm so that we cannot exclude the possibility that the 19-kDa TMEM190 variant corresponds to the predicted full-length polypeptide (calculated size: 18 491 kDa).

Currently, the biological significance of the 15- and 14-kDa TMEM190 remains elusive. Even when we used sperm lysates that had been treated with various protease inhibitors during preparation (data not shown), 19-, 15-, and 14-kDa proteins were detected with α -TMEM190 in both non-reduced and reduced samples. Moreover, ratios of these TMEM190-derived proteins showed the batch-to-batch variation, regardless of whether or not sperm proteins were extracted in the presence of protease inhibitors. The degradation of the 19-kDa TMEM190 might occur during the sperm lysate preparation due to unknown sperm protease(s) resistant to our protease inhibitor treatment.

As we determined the TMEM190 localization in mouse TGCs by immunofluorescence, this protein was present partly on the surface of round spermatids that were contained in TGC samples (Fig. 2Bd and Bs). However, testicular sperm, which are a spermatogenic cell population at the later spermatogenesis stage than round spermatids, contained no TMEM190 protein on the surface (Fig. 2Bi). Moreover, by immunofluorescence of mock-permeabilized AI-sperm, we did not obtain evidence for the surface localization of TMEM190 (Fig. 4). It is yet unclear whether TMEM190 is partly localized on the surface of testicular and AI-sperm. Surface TMEM190 on round spermatids, whose ratio to total TMEM190 is low (Fig. 2Bs), might be distributed onto the whole surface of the testicular sperm heads during spermatogenesis, so that immunoreactive signals of the surface TMEM190 would be too diffused to be detected. As another possibility, the surface form of testicular TMEM190 might be proteolytically degraded at later spermatogenesis stage(s), since TMEM190 seems to be highly susceptible to protease digestion as discussed above.

Certain aspects of TMEM190 biology imply that it might be involved in fertilization. One highly suggestive clue is that TMEM190 co-localizes with IZUMO1 (Kawai *et al.* 1989, Yamashita *et al.* 2007, Fujihara *et al.* 2010), which is an essential protein for sperm–oocyte fusion (Inoue *et al.* 2005). The relocation of both TMEM190 and IZUMO1 during the acrosome reaction is intriguing, since the final destination of these two proteins is the equatorial segment, where the gamete fusion occurs (Yanagimachi 1994). Indeed, when either of the testis-specific serine kinase 6 gene *Tssk6* (Sosnik *et al.* 2009) or the sperm equatorial segment protein 1 gene *Spesp1* (Fujihara *et al.* 2010) is disrupted in mice, their mutant sperm are defective in both the relocation of IZUMO1

and sperm–oocyte fusion. Since we were not able to show that TMEM190 and IZUMO1 interact with each other and *Tmem190*-null mice are fertile, the fusogenic role(s) of TMEM190, if any, is probably indirect and dispensable.

The second clue is a recent study on sperm CD9 (Ito *et al.* 2010); this protein also exhibits essentially the same subcellular localization pattern in mouse sperm as those of TMEM190 and IZUMO1. While oocytes from mice lacking CD9 are incapable of being fused with wild-type sperm, CD9-deficient sperm are normal in all tested sperm functions that include the ability to fertilize oocytes (Kaji *et al.* 2000, Le Naour *et al.* 2000, Miyado *et al.* 2000). This shows that the function(s) of mouse sperm CD9 is dispensable during fertilization, like mouse sperm TMEM190 (Fig. 7D). Therefore, CD9 might be functionally related to TMEM190 in mouse sperm with both indirectly regulating sperm–oocyte fusion.

There are two facts about TMEM190 that are puzzling. First, the human *TMEM190* gene seems to be expressed predominantly in the pharynx and the ovary unlike the testis-specific expression of the mouse *Tmem190* gene. One currently unexplored possibility is that the human gene might be expressed in the testis at low but enough levels to produce the functional TMEM190 protein. If human sperm do not contain TMEM190, another protein(s) might play the same role(s) as that of mouse TMEM190. This possibility is based on the following findings: the mouse testis-specific genes *Adam1a* (Nishimura *et al.* 2004) and *Adam3* (Shamsadin *et al.* 1999, Nishimura *et al.* 2001) are essentially required for male fertility, even though these two genes are both pseudogenes in the human genome (Jury *et al.* 1997, Grzmil *et al.* 2001). Currently, there is no follow-up evidence yet in this case, but other human testis/sperm-specific ADAM proteins presumably compensate for the functions of mouse ADAM1a and ADAM3.

Second, *Tmem190*-null mice show normal fertility. *Tmem190* might be a mouse gene that requires a two or more multiple-gene knockout within the same animal to cause a profound defect(s) (Shastri 1995). Mouse TMEM190 is found in a multi-protein complex(es) and perhaps if one or more of other components for the sperm protein complex(es) are targeted, the mutant males would be sterile or subfertile.

As a summary, we propose an indirect role(s) of mouse TMEM190 in gamete fusion by comprising a sperm protein complex(es). Alternatively, the protein complex(es) is possibly involved in regulation of the acrosome reaction, since the protein dynamics of TMEM190 is clearly linked to the acrosome reaction. At any rate, the TMEM190 role(s) within the mouse sperm protein complex(es) requires to be examined further. Particularly, TMEM190 and CD9 should be investigated to determine whether they are functionally linked to each other in mouse sperm. It would also

be worthwhile to clarify whether human sperm contain a protein complex(es) that is functionally equivalent to the TMEM190-bearing mouse sperm protein complex(es). This would lead to understanding the molecular basis of human male infertility.

Materials and Methods

Animals

CD1 and C57BL/6 mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA) and the Jackson Laboratory (Bar Harbor, ME, USA) respectively. The transgenic mouse line B6SJF1 (Kim & Gerton 2003), in which GFP is localized in the sperm acrosomes, was a kind gift from Dr G L Gerton (University of Pennsylvania, Philadelphia, PA, USA). *Tmem190*-null mice were created in this study (for details, see 'Creation of *Tmem190*-null mice'). All these mouse lines were maintained in an animal facility at the University of California Davis (UCD). Rabbits used for immunization to raise α -TMEM190 were maintained in the UCD Comparative Pathology Laboratory. All experiments using animals were carried out under approval by the UCD Institutional Animals Care and Use Committee.

Isolation of TGCs and mature sperm

TGCs (Phelps *et al.* 1990, Nishimura *et al.* 2007) and mature sperm (Claassens *et al.* 1998, Nishimura *et al.* 2007) were isolated from ~16-week-old male mice. Briefly, for TGC preparation, cell suspensions containing all testicular cell types were obtained by thoroughly mincing testicles with razor blades, and TGCs were separated from the testicular cell suspensions on a 52% (v/v) gradient of Percoll (GE Healthcare Life Sciences, Piscataway, NJ, USA). Mature sperm were released from the cauda epididymis and subsequently purified by using OptiPrep Density Gradient Medium (Sigma-Aldrich). To prepare protein extracts of TGCs or mature sperm, cells (1×10^8 cells/ml) were suspended in PBS containing 1% (w/v) *n*-octyl β -D-pyranoglucoside (PBS/OG) and incubated for 30 min on ice. After centrifugation of the suspensions at 20 000 *g* for 10 min at 4 °C, the supernatants were used for experiments.

We also used PBS/OG containing the EDTA-free Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science) for extraction of TGC or sperm proteins. However, the protease inhibitor cocktail showed no significant effect on blocking the degradation of TMEM190 protein, which presumably occurred during the cellular protein extractions (data not shown). Therefore, all cell lysates used in this study were prepared in PBS/OG containing no protease inhibitors.

RT-PCR

Gene expression of *Tmem190* was examined in various mouse tissues by RT-PCR, in which first-strand cDNAs of the tested tissues were used as templates for conventional PCR. Except for TGCs, all tested mouse tissues could be evaluated using various first-strand cDNAs that were contained in the Mouse Multiple Tissue cDNA Panel I Kit (Clontech Laboratories, Inc.).

To prepare first-strand cDNA of mouse TGCs, poly(A)⁺ RNA was purified from 1×10^8 TGCs with the FastTrack 2.0 mRNA Isolation Kit (Invitrogen Life Technologies). First-strand cDNA was then synthesized from 100 ng of the mouse TGC poly(A)⁺ RNA with random primers in 20- μ l reaction mixtures by using the StrataScript First-Strand cDNA Synthesis Kit (Agilent Technology, Inc., formerly Stratagene, Santa Clara, CA, USA), according to the manufacturer's protocol.

PCR was carried out in 10- μ l reaction mixtures composed of $1 \times$ PCR buffer, 200 μ mol/l dNTP mixture, 0.2 μ mol/l forward primer, 0.2 μ mol/l reverse primer, 1 μ l of first-strand cDNA, and $1 \times$ Advantage cDNA Polymerase Mix (Clontech). Primer sets used were 5'-AATGGAATCCAGGGATTTTCTAC-3' (forward) and 5'-CTATTCTTCACCTCCTGTGTCC-3' (reverse) for *Tmem190* (435 bp), 5'-ATCGTCTCTCACTATTGGAACAT-3' (forward) and 5'-TAAAATAATTGCAAGTGATCCAGAGT-3' (reverse) for *Adam2* (411 bp) (Kim *et al.* 2006), 5'-TAACAAAGAGAATTCTGAGGATAAAGA-3' (forward) and 5'-AAAGCCCTCCACAGCTAACGTCTTTGG-3' (reverse) for *Adam3* (398 bp) (Kim *et al.* 2006), and 5'-TGAAGGTGCGAGTCAACGGATTGGT-3' (forward) and 5'-CATGTGGGCGATGAGGTCCACCAC-3' (reverse) for *Gapdh* (983 bp) (Kim *et al.* 2006). Target cDNAs were amplified by one cycle of 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min, and one cycle of 72 °C for 10 min. The resulting PCR products were separated by 0.8% (w/v) agarose gel electrophoresis.

Antibodies

Mouse MAB against mouse IZUMO1 (α -IZUMO1; IgM, clone OBF13; Okabe *et al.* 1987) was a gift of Dr M Okabe (Osaka University, Osaka, Japan). Affinity-purified mouse polyclonal antibody against mouse SPAM1/PH-20 was produced in our laboratory (Nishimura *et al.* 2001). Mouse MABs against mouse ADAM2 (IgG2b, clone 9D2.2) and ADAM3 (IgG1, clone 7C1.2) were provided by Chemicon/Millipore Corporation (Temecula, CA, USA). A mixture of two mouse MABs against GFP (IgG1, clones 7.1 and 13.1) was purchased from Roche, mouse MAB against α -tubulin (IgG1, clone AA13) was from Sigma-Aldrich, HRP-conjugated goat anti-mouse IgM (molgM) (μ chain) antibody was from Thermo Scientific (formerly Pierce Biotechnology; Rockford, IL, USA), and rblgG, normal molgM, and HRP-conjugated goat antibodies against rblgG and mouse IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor 488-conjugated goat anti-molgM (μ chain) antibody and Alexa Fluor 568-conjugated donkey anti-rblgG antibody, which were the secondary antibodies used for immunofluorescence, were purchased from Molecular Probes/Invitrogen (San Diego, CA, USA).

We prepared α -TMEM190 as follows: a DNA fragment encoding the N-terminal region of mouse TMEM190 (residues 22–80, N-TMEM190) was ligated into a pGEX-4T-1 vector (GE Healthcare). By using this expression vector, a glutathione S-transferase (GST)-tagged recombinant protein of N-TMEM190 (GST/N-TMEM190) was produced in the *Escherichia coli* strain BL21. GST/N-TMEM190 was purified on a column of GST-Bind Resin (Novagen/EMD Chemicals, Inc., Gibbstown, NJ, USA) and used for immunization

of rabbits. For affinity purification of α -TMEM190, the DNA fragment coding for N-TMEM190 was ligated into pET-23d (Novagen), and this vector was used to produce a recombinant protein of N-TMEM190 that is tagged at its C-terminal 6 $\times$ His (N-TMEM190/His6) in the *E. coli* strain BL21 (DE3). The N-TMEM190/His6 protein was immobilized onto Sepharose 4B (GE Healthcare), incubated with raw α -TMEM190 sera, and washed with PBS containing 500 mmol/l NaCl. α -TMEM190 was eluted from the beads with 0.1 mol/l glycine-HCl, pH 2.5, and immediately neutralized with one-ninth volume of 2 mol/l Tris-HCl, pH 9.0. The purified antibody was dialyzed against PBS prior to using in experiments.

Immunoblotting

SDS-PAGE using 14% (w/v) Novex Tris-Glycine Gels (Invitrogen) was carried out under the reducing conditions, unless otherwise noted. Separated proteins were transferred onto Immobilon-P Transfer Membranes (Millipore, Bedford, MA, USA), followed by incubation of the membranes at room temperature for 1.5 h with 1 μ g/ml primary antibodies and subsequently for 30 min with 50 ng/ml HRP-conjugated secondary antibodies. To capture biotinylated proteins, blots were incubated for 1.5 h at room temperature with 0.5 μ g/ml High Sensitivity NeutrAvidin-HRP (Thermo Scientific). Immune or biotin-NeutrAvidin complexes were then detected by using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) according to the manufacturer's instruction.

Proteinase K digestion of cell-surface proteins

TGCs or cauda epididymal sperm of B6SJF1 mice were suspended in PBS (1×10^8 cells/ml), and 90- μ l aliquots from each cell suspension were incubated with 10 μ l of various concentrations of proteinase K for 30 min at 37 °C. To terminate the proteinase K digestion, 2 μ l of 100 mmol/l phenylmethanesulfonyl fluoride (PMSF) was added, followed by additional incubation for 15 min at room temperature. The digested cell samples were washed three times with PBS by centrifugation at 800 *g* for 5 min at 4 °C, suspended in 2 μ l of 100 mmol/l PMSF plus 100 μ l of PBS/OG, and incubated for 30 min on ice. After centrifugation at 20 000 *g* for 10 min at 4 °C, the supernatants were used as 'cell suspension'. Similar experiments were also carried out using TGC or sperm protein extracts derived from B6SJF1 mice (equivalent to 1×10^8 cells/ml, see 'Isolation of TGCs and mature sperm' for preparation), instead of cell suspensions. In this case, reaction mixtures after the PMSF treatment to terminate proteinase K digestion were used as 'cell lysate'. Proteins that were contained in aliquots (5.7 μ l for TGCs and 11.3 μ l for sperm) from 'cell suspension' or 'cell lysate' samples were analyzed by immunoblotting for SPAM1, ADAM2, GFP, or TMEM190.

Subcellular fractionation of mature sperm

Subcellular components of mature sperm were prepared according to the published methods (Honda *et al.* 2002, Kim *et al.* 2003, 2004) with slight modifications. Briefly, AI-sperm of B6SJF1 mice (1×10^8 sperm/ml) were incubated for 1 h at 37 °C

with 10 μ mol/l calcium ionophore A23187 in Human Tubal Fluid (HTF) medium (without BSA, Irvine Scientific, Santa Ana, CA, USA). The sperm suspensions after the acrosome reaction were centrifuged at 800 *g* for 5 min at room temperature to remove AR-sperm, and the supernatants were again centrifuged at 20 000 *g* for 5 min at room temperature. The low-speed supernatants were subjected to ultracentrifugation at 100 000 *g* for 90 min at 4 °C, and the resulting high-speed supernatants included proteins released during the acrosome reaction plus soluble acrosomal contents (RPA). The pellets obtained by ultracentrifugation were washed once with PBS and suspended in PBS/OG to prepare protein extracts (for preparation, see 'Isolation of TGCs and mature sperm'). The extracts were then used as a fraction of the PM and OAM that had been fused to each other during the acrosome reaction (PM/OAM). AR-sperm, obtained by centrifugation after the incubation of AI-sperm with A23187, were washed three times with PBS, resuspended in PBS (1×10^8 cells/ml), and sonicated to detach their tails from the sperm heads. Fractions of the AR-sperm heads (ARH) and tails (ART) were subsequently separated on a sucrose density gradient. Proteins in each subcellular fraction (equivalent to 1×10^6 sperm) were analyzed by immunoblotting for ADAM2, GFP, or TMEM190.

Immunofluorescence

TGCs were isolated from CD1 mice and suspended (0.5×10^7 cells/ml) in HEPES-buffered saline containing Mg^{2+} (Phelps *et al.* 1990, Nishimura *et al.* 2007). Ten- μ l aliquots from the TGC suspensions were placed as $\sim$ 1-cm spots onto coverslips that had been coated by poly-L-lysine and allowed to stand at room temperature for $\sim$ 10 min until the edge of each spot became dried. The cell samples were then fixed in methanol for 15 min at -20 °C, followed by washing three times with PBS. As for sperm samples, AI- or AR-sperm (for preparation, see 'Subcellular fractionation of mature sperm') of CD1 mice were washed three times with PBS at 800 *g* for 5 min at room temperature, suspended (1×10^7 sperm/ml) in PBS containing 4% (w/v) paraformaldehyde (PBS/PFA), and incubated for 30 min on ice to allow these sperm samples to be fixed. After washing three times with PBS, sperm were resuspended in PBS (0.5×10^7 sperm/ml), placed onto poly-L-lysine-coated coverslips (5- μ l sperm suspension/spot), and kept for $\sim$ 15 min at room temperature until the sperm became completely dried. The sperm spots were then washed three times with PBS. The cell spots of TGCs, AI-sperm, or AR-sperm that had been fixed followed by washing with PBS were incubated for 30 min at room temperature in 100 μ l of PBS containing 3% (w/v) BSA (PBSA) or PBSA containing 0.1% (v/v) TX100 (PBSAT) and then washed once with PBS. The cells were subsequently incubated for 1 h at room temperature in 100 μ l of PBSA containing 10 μ g/ml Alexa Fluor 488-conjugated PNA (Molecular Probes) and/or 5 μ g/ml rblgG or α -TMEM190 and/or 5 μ g/ml molgM or α -IZUMO1. After washing three times with PBS containing 0.1% (v/v) TX100 (PBST), the cells were incubated for 30 min at room temperature in 100 μ l of PBSAT containing 5 μ g/ml 4',6-diamino-2-phenylindole (DAPI) and 10 μ g/ml Alexa

Fluor 568-labeled donkey anti-rbIgG antibody and/or 10 µg/ml Alexa Fluor 488-labeled goat anti-molIgM antibody. After the cell-bearing coverslips were washed three times with PBST and twice with PBS, one drop of the ProLong antifade reagent (Invitrogen) was put onto each cell spot. The cell samples were then observed under the FluoView FV1000 Confocal Microscope (Olympus America, Inc., Center Valley, PA, USA).

Immunoprecipitation

Protein extracts of TGCs, AI-sperm, and AR-sperm from CD1 mice (total) were prepared as described in 'Isolation of TGCs and mature sperm'. Cell-surface protein fractions of AR-sperm (surface) were prepared as previously reported (Nishimura *et al.* 2007). Briefly, AR-sperm (0.5×10^8 sperm/ml) were incubated for 30 min at room temperature with 1 mmol/l EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) in 2 ml of HTF medium. After washing three times with PBS, the biotinylated AR-sperm were suspended in 1 ml of PBS/OG to prepare protein extracts. To isolate biotinylated proteins, the cell lysates (equivalent to 1×10^8 sperm) were incubated for 30 min at room temperature with 100 µl of 50% (v/v) slurry of Monomeric Avidin Agarose (Thermo Scientific) in 2 ml of PBS/OG containing 500 mmol/l NaCl (Binding Buffer). The beads were washed three times with Binding Buffer, suspended in 200 µl of 5 mmol/l D-biotin in the same buffer, and incubated for 30 min at room temperature to allow biotinylated proteins to be eluted from the monomeric avidin-beads. Following centrifugation at 500 *g* for 5 min at room temperature, the supernatants containing AR-sperm surface proteins were used as 'surface' fraction. To confirm that only cell-surface proteins are biotinylated, 'total' and 'surface' fractions of AR-sperm with or without biotinylation were subjected to immunoblotting for ADAM2 or α -tubulin.

For immunoprecipitation, 'total' or 'surface' samples (equivalent to 2.5×10^7 cells) were incubated for 2 h on ice in 1 ml of Binding Buffer containing 25 µl of 50% (v/v) slurry of Sepharose 4B-beads where rblgG or α -TMEM190 was immobilized (~ 2 mg IgG/ml beads). After washing three times with Binding Buffer, the beads were suspended in 62.5 µl of 0.2 mol/l glycine-HCl, pH 2.5, containing 1% (w/v) OG, and incubated for 15 min at room temperature. The suspensions were then centrifuged at 500 *g* for 5 min at room temperature, and their supernatants containing mouse TMEM190 and its associated proteins were mixed with one-fourth volume of 2 mol/l Tris-HCl, pH 9.0, to neutralize the supernatant solutions. Proteins in the immunoprecipitated fractions were analyzed by immunoblotting for TMEM190, IZUMO1, or biotinylated proteins.

Creation of *Tmem190*-null mice

The mouse genomic clone bMQ-202C5, including the *Tmem190* gene, was obtained from the Wellcome Trust Sanger Institute (Cambridge, UK). To construct a targeting vector, 2.2- and 6.3-kbp DNA fragments that correspond to 5'- and 3'-flanking regions of *Tmem190* respectively were excised from the bMQ-202C5 DNA. These two fragments

and an expression cassette of the neomycin-resistance gene (*neo*) were then ligated together into a pBluescript II SK(+) vector (Agilent Technology). After the targeting vector that had been linearized by KpnI digestion was electroporated into mouse embryonic stem (ES) cells, homologous recombinants were selected by using G418. By PCR screening (for details, see 'PCR screening'), seven ES cell clones carrying the targeted mutation in the *Tmem190* gene were identified among 96 G418-resistant clones and subsequently injected into C57BL/6 mouse blastocysts. The resulting chimeric male mice were crossed to C57BL/6 female mice to obtain heterozygous mutant progeny for the *Tmem190* gene. Homozygous mutant mice were obtained by mating of heterozygous males and females.

PCR screening

Tmem190 genotypes of ES cell clones or mice were determined by PCR. Primer sets used were as follows: 5'-TTCCCCTCCCCATCTTTCACCACT-3' (FW, forward) and 5'-TCTGCTTTAGGGAACACAGACCAG-3' (WTRV, reverse) for the wild-type locus, and the FW primer (forward) and 5'-GACCGCTTCCTCGTGCTTACGGT-3' (NeoRV, reverse) for the mutant locus. Template genomic DNA samples were prepared from ES cells or mouse tails, according to the published method (Aljanabi & Martinez 1997). PCR mixtures (20 µl) were composed of 1× PCR buffer, 200 µmol/l dNTP mixture, 0.2 µmol/l forward primer, 0.2 µmol/l reverse primer, ~ 100 ng of genomic DNA, and 1× Advantage cDNA Polymerase Mix. The PCRs were subjected to one cycle of 94 °C for 3 min, followed by 32 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 3 min, and one cycle of 72 °C for 10 min. Amplified DNAs were then subjected to 0.8% (w/v) agarose gel electrophoresis.

In vivo fertility test

Each tested male (8–16 weeks old) was placed with two C57BL/6 females (6–8 weeks old) in a cage, while two tested females were crossed to one C57BL/6 male. Once a day, every female was checked for the vaginal plug formation, and plug-positive females were individually transferred into new cages. After ~ 20 days, the numbers of pups born were counted to compare *in vivo* fertility of *Tmem190*-null mice with that of wild-type mice.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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