Involvement of a membrane skeletal protein, 4.1G, for Sertoli/germ cell interaction

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Abstract

We previously reported that a membrane skeletal protein, 4.1G (also known as EPB41L2), is immunolocalized in mouse seminiferous tubules. In this study, the 4.1G immunolocalization was precisely evaluated at various stages of the mouse seminiferous epithelial cycle with 'in vivo cryotechnique' and also with pre-embedding immunoelectron microscopy in testicular tissues whose ultrastructures were well preserved with glycerol treatment before cryosectioning. In addition, 4.1G-deficient mice were produced, and the morphology of their seminiferous tubules was also evaluated. The 4.1G immunolocalization was different among stages, indicating that it was not only along cell membranes of Sertoli cells, but also those of spermatogonia and early spermatocytes. To confirm the 4.1G immunolocalization in germ cells, *in vitro* culture of spermatogonial stem cells (SSCs) was used for immunocytochemistry and immunoblotting analysis. In the cultured SSCs, 4.1G was clearly expressed and immunolocalized along cell membranes, especially at mutual attaching regions. In testicular tissues, cell adhesion molecule-1 (CADM1), an intramembranous adhesion molecule, was colocalized on basal parts of the seminiferous tubules and immunoprecipitated with 4.1G in the tissue lysate. Interestingly, in the 4.1G-deficient mice, histological manifestation of the seminiferous tubules was not different from that in wild-type mice, and the CADM1 was also immunolocalized in the same pattern as that in the wild-type. Moreover, the 4.1G-deficient male mice were fertile. These results were probably due to functional redundancy of unknown membrane skeletal molecules in germ cells. Thus, a novel membrane skeletal protein, 4.1G, was found in germ cells, and considering its interaction with CADM family, it probably has roles in attachment of both Sertoli-germ and germ-germ cells.

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Introduction

Protein 4.1G (4.1G; also known as EPB41L2) is a membrane skeletal protein (Parra *et al.* 1998, Walensky *et al.* 1998), which is one of 4.1-family proteins originally identified in erythrocytes as 4.1R containing domains to attach spectrin–actin and intramembranous proteins (glycophorin C and band 3; Discher *et al.* 1995). In the past decade, some interacting intramembranous proteins of 4.1G have been documented, such as adenosine receptor (Lu *et al.* 2004a, 2004b), metabotropic glutamate receptor (Lu *et al.* 2004a, 2004b, Tateyama & Kubo 2007), platelet T-cell activating molecule (PTA1, CD226; Ralston *et al.* 2004), and parathyroid hormone receptor (Saito *et al.* 2005). Therefore, 4.1-family proteins are thought to have functions to connect various intramembranous proteins including ion channels and receptors to cytoplasmic membrane skeletal proteins as well as signal proteins.

We have reported that 4.1G is expressed in various cells and tissues including Schwann cells in the peripheral nervous system (Ohno *et al.* 2007a), microglia in the CNS (Ohno *et al.* 2005), and also in mouse seminiferous tubules (Terada *et al.* 2005); however, their functions and actual interacting proteins are still obscure. In the seminiferous tubules, as 4.1G was relatively immunolocalized as round and/or arch-shaped patterns in the basal compartment as well as honeycomb patterns in the adluminal compartment (Terada *et al.* 2005). In this study, we further evaluated the 4.1G immunolocalization at various stages of the mouse seminiferous cycle with 'in vivo cryotechnique (IVCT)' (Ohno *et al.* 1996) and found different 4.1G distributions among the various stages. In addition, with immunoelectron microscopy, 4.1G was found to be immunolocalized not only in Sertoli cells, but also in a subset of germ cells including spermatogonium.

Cell adhesion molecule-1 (CADM1), also designated as SynCAM/TSLOC1/SglSF/RA175/Necl-2, is a Ca^{2+} -independent immunoglobulin-like CADM which homophilically and heterophilically interacts with themselves or with other protein families (Sakisaka *et al.* 2007). In the seminiferous tubules, CADM1 was reported to function as an intramembranous molecule in germ cells (Wakayama *et al.* 2001, Wakayama & Koami 2003). In the testes of CADM1-deficient mice, dysfunction of germ cells has been reported, indicating its indispensable role in spermatogenesis (Surace *et al.* 2006, van der Weyden *et al.* 2006, Yamada *et al.* 2006, Fujita *et al.* 2007). As the CADM1 has a molecular domain to bind to the 4.1-family proteins (Wakayama & Iseki 2009), the 4.1G interaction to CADM1 was examined in the present study. Furthermore, to determine whether 4.1G is indispensable to form mouse seminiferous tubules, we produced genetically 4.1G-deficient mice and examined the morphology of their seminiferous tubules and immunolocalization of CADM1.

Results

Immunolocalization of 4.1G in the seminiferous epithelium

Using IVCT followed by freeze-substitution fixation (FS), structures reflecting native states were well preserved (hematoxylin–eosin (HE) staining in Fig. 1), and 4.1G

was immunolocalized as round and/or arch-shaped patterns in the basal compartments and weakly as honeycomb patterns in the adluminal compartment (Fig. 1). These patterns were consistent with our previous report (Terada *et al.* 2005), as shown with conventional perfusion fixation followed by cryosectioning with sucrose embedding. The present IVCT–FS method also reconfirmed the 4.1G expression and its similar immunolocalization in mouse seminiferous tubules.

The 4.1G immunolocalizations were different among the various stages of the seminiferous epithelial cycle. At stages I–II of the seminiferous epithelial cycle (Fig. 1a–d), the 4.1G was markedly immunostained in the basal part of the seminiferous tubule, where early pachytene spermatocytes and type B spermatocytes were present (P, B in Fig. 1b and d). Although the intensity of the 4.1G immunostaining appeared to be weak in the region where early spermatids (S1, S3 in Fig. 1b and d) were localized, another region where late spermatocytes (S13, S14 in Fig. 1b and d) were present was relatively strongly immunostained with 4.1G. At stage V (Fig. 1e and f), pachytene spermatocytes were progressively differentiating in lower parts of the adluminal compartment (P in Fig. 1e) where the 4.1G immunoreactivity was detected (P in Fig. 1f) as well as in the region where late spermatids (S15 in Fig. 1f) were present. Interestingly, with differentiation of spermatids from early to late steps,

Figure 1 Hematoxylin–eosin staining (a, c, e, g, i, k, and m) and 4.1G immunostaining (b, d, f, h, j, l, and n) in adjacent sections of seminiferous tubules of testicular tissues with *in vivo* cryotechnique followed by freeze-substitution fixation (IVCT–FS). Images of various stages (S), I (a and b), III (c and d), V (e and f), VIII (g and h), IX (i and j), X (k and l), XII (m and n), of the mouse seminiferous epithelial cycle are arranged in this panel. Note the line pattern of the 4.1G immunolocalization at the bottom of seminiferous tubules, including the basal compartment, and also around the top of the adluminal compartment. (o–r) Immunostaining with (o and p) or without (q and r) primary anti-4.1G antibody followed with secondary anti-rabbit IgG antibody. Both (p) and (r) are diffraction interference contrast (DIC) images corresponding to the immunostaining image of (o) and (q) respectively. B, type B spermatogonia; PI, preleptotene spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte; P, pachytene spermatocyte; M, meiotic division; S1–16, steps of spermiogenesis. Bars (a–n) 20 μm .

the 4.1G immunoreactivity appeared to be stronger (S8, S9 in Fig. 1h and j). At stage X (Fig. 1k and l), 4.1G was immunopositive in leptotene spermatocytes (L in Fig. 1l), whereas its immunostaining intensity was relatively weaker in later pachytene spermatocytes (P in Fig. 1l). At stage XII (Fig. 1m and n), 4.1G immunostaining was observed in a region where spermatocytes were at zygotene (Z in Fig. 1m and n), whereas its intensity appeared to be very weak in the region where spermatocytes differentiated into early spermatids through meiosis (M in Fig. 1m and n). In an adjacent section which was immunostained for 4.1G (Fig. 1o and p), the immunostaining was eliminated without the primary anti-4.1G antibody and only with the secondary antibody (Fig. 1q and r). These findings indicate the different expression of 4.1G at various stages of the seminiferous epithelial cycle.

Immunoelectron microscopy for 4.1G localization

Because the 4.1G immunostaining intensity varied at different seminiferous stages in testicular tissues with IVCT as described in the previous paragraph, it seemed that 4.1G was immunolocalized not only in Sertoli cells, but also in germ cells. To more precisely determine the 4.1G immunolocalization in the seminiferous tubules, pre-embedding immunoelectron microscopy was also

performed (Fig. 2). To well preserve their ultrastructures, infiltration of 30% sucrose/10% glycerol in phosphate buffer (PB) into tissues before freezing and cryosectioning was useful for its cryoprotectant effect. As shown in Fig. 2, because avidin–biotin complex (ABC) and diaminobenzidine (DAB) reaction products were mostly observed under cell membranes, but not in extracellular matrices, it was possible to distinguish between Sertoli and specific staged germ cells.

In addition to the 4.1G immunostaining along cell membranes of Sertoli cells (Se in Fig. 2), other immunoreaction products were also observed in the cytoplasm spermatogonium and some spermatocytes (Sg in Fig. 2). In the spermatogonium, the 4.1G immunostaining intensity appeared to be strong under the cell membranes facing Sertoli cells (white arrows in Fig. 2c, e–g and j). In some spermatocytes, the 4.1G immunostaining intensity was relatively weak (arrowheads in Fig. 2h and i). In the upper half of seminiferous tubules, where spermatocytes and spermatids were mainly localized, the immunoreaction products were not detected along cell membranes of the germ cells, but along those of Sertoli cells (data not shown), as reported before (Terada *et al.* 2005). These findings indicate that 4.1G was immunolocalized in both Sertoli cells and germ cells at the basal compartment and bottom parts of the adluminal compartment.

Figure 2 Immunoelectron micrographs of 4.1G in basal parts of mouse seminiferous tubules with the pre-embedding immunostaining method for mouse testis with perfusion fixation followed by sucrose/glycerol treatment and cryosectioning. Electron micrographs show Sertoli cells (Se) and germ cells, such as spermatogonia (Sg) and spermatocytes (Sc). Images (b), (c), (e, f, g, and j) and (i and j) are higher magnified views shown as black squares in (a), (b), (d), and (h) respectively. In the basal compartment, spermatogonia are attaching to Sertoli cells, and 4.1G immunodeposits are observed under cell membranes of both Sertoli cells (black arrows in c, f, and j) and spermatogonia (white arrows in c, e, f, g, and j) which are facing each other. 4.1G is immunolocalized along cell membranes of some spermatogonia facing spermatocytes (arrowheads in e) in addition to cytoplasmic processes of Sertoli cells (black arrow in e), whereas it is rarely immunolocalized under cell membranes attaching to basement membranes (BM in d and g). Intensity of the 4.1G immunodeposits between Sertoli cells and spermatogonia (arrows in h and i) appears to be higher than that between Sertoli cells and some spermatocytes (arrowheads in h and i). Bars (a–j) 1 μ m.

Immunolocalization of 4.1G in cultured spermatogonial stem cells

As described in the previous paragraphs, we have realized that 4.1G is immunolocalized not only in Sertoli cells, but also in the spermatogonium. To confirm the 4.1G production and immunolocalization in germ cells, spermatogonial stem cells (SSCs) were examined in a well-established culture system by proliferating them with glial cell line-derived neurotrophic factor (GDNF; Fig. 3). The SSCs were well proliferated over feeder cells (Fig. 3a–d), and clearly immunostained for 4.1G (Fig. 3a), whereas the feeder cells were rarely immunostained. The immunostaining pattern was seen to be like a honeycomb (asterisks in Fig. 3e), and such immunoreactivity was especially strong at attaching regions to the next SSCs (Fig. 3e). These findings were well recognized with confocal laser scanning microscopy (Fig. 3g–i), in which some spaces were observed between each nucleus and the 4.1G-immunostained region like a line pattern, indicating the 4.1G immunolocalization at the SSC–SSC attaching sites.

Furthermore, to determine whether the expressed 4.1G protein had a similar molecular size under the cell culture condition as that in the testicular tissue, additional immunoblotting analysis was performed (Fig. 3j). The molecular weight of the 4.1G-immunopositive line obtained from lysates of the cultured SSCs was the same as the molecular weight of that obtained from the tissue lysates, indicating that the 4.1G protein expressed in SSCs was the same isoform.

Double-immunostaining and immunoprecipitation study of 4.1G and CADM1

Next, the relationship between 4.1G and CADM1 was evaluated with double-immunofluorescence staining and also with an immunoprecipitation study (Fig. 4). The immunolocalization of CADM1 almost overlapped that of 4.1G in mouse testes (Fig. 4d). In addition, immunoprecipitates of CADM1 obtained from the mouse testicular tissue were immunoblotted with the anti-4.1G antibody (Fig. 4e). These findings indicate that 4.1G has a molecular complex with CADM1 in mouse seminiferous tubules.

Figure 3 Immunolocalization of 4.1G in cultured spermatogonial stem cells (SSCs). Images (a), (b), and (e) show 4.1G immunostaining, and images (c), (d), and (f) are corresponding DAPI-nuclear staining respectively. The SSCs form cell clumps on the feeder cell layer (arrows in c and d), and 4.1G is immunolocalized in the clumps (arrow in a). Without the primary anti-4.1G antibody, the 4.1G immunostaining is eliminated (arrow in b). At higher magnified views with fluorescence microscopy (e and f), the 4.1G immunostaining shows a honeycomb pattern (asterisks in e). With confocal laser scanning microscopy, the 4.1G immunolocalization is observed as line patterns (arrows in g), and the merged image (i) of 4.1G (g) and nuclei with TOPRO-3 (h; TOPRO) indicate the 4.1G immunolocalization at contact regions of SSCs. By immunoblotting analysis for 4.1G (j), the molecular size of 4.1G in SSCs was the same as that of the major blotted line in the mouse testicular tissue. The ratios show dilution of the cell lysates. Bars (a–d) 100 µm, (e–i) 20 µm.

Figure 4 Double-immunostaining of 4.1G (b) and CADM1 (c) in seminiferous tubules of the mouse testes prepared with IVCT-FS, and observed with a confocal laser scanning microscope. Image (a) shows nuclear staining with TOPRO-3 (TOPRO). Both 4.1G and CADM1 proteins show linear immunostaining patterns, and the merged image (d) indicates their similar immunolocalizations. Arrows indicate the basal portions of the seminiferous tubules. From an immunoprecipitation study (e), the 4.1G immunoreaction on the blotted membrane was obtained from the CADM1 immunoprecipitated testis lysate. IP (CADM1), immunoprecipitation without (–) or with (+) the anti-CADM1 antibody. Blot (4.1G), immunostaining of the blotted membrane with (+) or without (–) the anti-4.1G antibody. Bars (a–d) 20 μ m.

Morphology and immunohistochemistry in 4.1G-deficient mice

Next, morphology of seminiferous tubules in the 4.1G-deficient mouse testes was examined to clarify the development of germ cells without 4.1G. The 4.1G-deficient mice were born normal and developed for at least 8 months, and male mice were fertile. Lack of the 4.1G expression was confirmed with immunohistochemistry (Fig. 5c) and western blotting analysis (Fig. 5e). Even in the 4.1G-deficient mice, the 4.1G immunoreaction in Leydig cells remained, indicating a nonspecific immunoreaction of anti-4.1G antibody. Depletion of the 4.1G protein in the 4.1G-deficient mice was also confirmed in several other organs by immunohistochemistry, where we checked its

expression, such as the cerebrum and sciatic nerve tissues (data not shown).

Figure 5f–k shows representative images at each stage of the seminiferous epithelial cycle in the 4.1G-deficient mice; the timing of germ cell differentiation was not significantly disturbed as compared with that in wild-type mice shown in Fig. 1. In addition, the number of spermatozoa attaching to apical regions of Sertoli cells at stage VIII was statistically not different between the 4.1G-deficient mice and wild-type ones (Fig. 5l).

Finally, the CADM1 immunolocalization was also examined in the 4.1G-deficient mice (Fig. 6), which was almost the same as that at any stages of the seminiferous epithelial cycle in wild-type mice. Figure 6a and c shows representative images of the CADM1 immunolocalization at stages I–II. These findings indicate that other unknown proteins probably rescue a 4.1G function to locate CADM1 at the cell membranes of germ cells.

Discussion

In this study, 4.1G was found to be immunolocalized along cell membranes of germ cells, in addition to those of Sertoli cells. In germ cells of the mouse seminiferous tubules, 4.1G was immunolocalized in spermatogonia through to early pachytene spermatocytes, as schematically summarized in Fig. 7. The 4.1G expression in the germ cells was also confirmed in cultured SSCs. Moreover, it was shown to have a molecular complex with CADM1. We also produced 4.1G-deficient mice and confirmed no expression of 4.1G, but the morphology of their seminiferous tubules was unexpectedly well organized.

The 4.1G immunolocalization at the bottom parts of seminiferous tubules including basal compartments appeared mostly as round and/or arch-shaped patterns as shown in Fig. 1. On the other hand, we found that 4.1G was expressed in the cultured SSCs, and its immunolocalization was in the mutual attaching regions as shown in Fig. 3. These distribution patterns probably indicate a 4.1G function as attachment of both germ–germ and Sertoli–germ cells in the testis tissues. In the present culture system (Kubota *et al.* 2004), morphological interaction between SSCs and feeder cells was not so tight, resulting in SSCs tending to detach from the feeder cell layer, while such interaction among SSCs themselves was highly effective to form cell clumps. It is difficult to analyze a 4.1G disappearance during spermatogenesis after forming spermatocytes *in vitro* because of the lack of method for SSCs to differentiate into the subsequent stages such as meiosis and spermiogenesis.

Because we showed that 4.1G was immunolocalized in both germ and Sertoli cells, it may play a role in attachment of spermatogonia to each other in the basal compartment, and also in the sliding movement of spermatogonia and spermatocytes from the basal

Figure 5 Morphology of seminiferous tubules in 4.1G-deficient mouse testes obtained with IVCT-FS. The 4.1G immunostaining obtained in the wild-type mice (a) was eliminated in the 4.1G-deficient mice (c). Images (b) and (d) show DIC images corresponding to (a) and (c) respectively. The 4.1G immunoreaction in Leydig cells remains even in the 4.1G-deficient mouse section, indicating a nonspecific DAB reaction. Western blot analysis (e) shows deletion of the 4.1G protein of 140 and 110 kDa in the 4.1G-deficient mice (–/–), which are obtained in the wild-type mice (+/+). HE staining images in the 4.1G-deficient mouse seminiferous tubules show morphology at various stages (S), I (f), III (g), V (h), VIII (i), X (j), XII (k), which are not distorted compared with those of the wild-type mice (see morphology with HE staining in Fig. 2). B, type B spermatogonia; PL, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; M, meiotic division; S1–16, steps of spermiogenesis. (l) The bar graph depicts similar numbers of spermatozoa attaching to apical regions of Sertoli cells at stage VIII in wild-type mice (+/+) and the 4.1G-deficient mice (–/–). Bars (a–d) 100 µm, (f–k) 20 µm.

compartment into the adluminal one through the blood–testis barrier (BTB). Some new molecules have already been identified at adhesion junctions in the seminiferous tubules (Wong *et al.* 2008). Recently, at the site of Sertoli cells, the 14-3-3 protein family and focal adhesion kinase (FAK) were reported to function as regulators of BTB (Siu *et al.* 2009, Sun *et al.* 2009). These two proteins are thought to detach from tight junctions with their phosphorylation, resulting in the opening of BTB. It is possible that a balance of molecular interactions among FAK, 14-3-3, and 4.1-family proteins may have functional relationship to form the BTB structure. Indeed, the FAK protein has a similar molecular structure to the FERM domain (band 4.1, ezrin, radixin, and moesin homology; Lietha *et al.* 2007), and the 14-3-3 protein was reported to interact with another 4.1-family protein, 4.1B (Yu *et al.* 2002). Because 4.1G as well as 4.1B was not immunolocalized at the lower ectoplasmic specialization where Sertoli cells formed tight junctions (Terada *et al.* 2004, 2005), it may be interesting to examine

relative immunolocalizations of these proteins in junctional formation.

In this study, although 4.1G had a molecular complex with CADM1, as shown in Fig. 4, the CADM1 immunolocalization in the seminiferous tubules was not disturbed in the 4.1G-deficient mice (Fig. 6). This is probably one reason why the 4.1G-deficient mice were able to produce sperm and sire litters in the similar way of wild-type ones, although further comprehensive analyses of spermatogenesis, spermiogenesis, and sperm functions may be needed. Thus, it became clear that 4.1G does not seem to be the only protein to anchor CADM1 at cell membranes of germ cells, and the membrane skeletal complex in Sertoli and germ cells seems to be much larger than we expected. Membrane-associated guanylate kinase homolog (MAGUK) proteins, such as the p55 family identified in erythrocytes and renal proximal tubules of mice (Tseng *et al.* 2001, Terada *et al.* 2007), interacting with the 4.1-family proteins, may be another carrier of CADM1 into cell

Figure 6 Immunolocalization of CADM1 in the wild-type (a) or the 4.1G-deficient (c) mouse testes obtained with IVCT-FS. Images in (b) and (d) show DAPI-staining images corresponding to the immunostaining images in (a) and (c) respectively. Note that the CADM1 immunostaining pattern is similar even in the 4.1G-deficient mice. Bars (a–d) 20 μ m.

membranes because it has some domains attaching to the MAGUK proteins (Wakayama & Iseki 2009). Further examination to find such a molecular complex is necessary to determine functional relevance of membrane skeletons including 4.1-family proteins in seminiferous tubules, and also analyses of CADM1-deficient seminiferous tubules may help to understand the membrane skeletal complex. In addition, some 4.1-family proteins have been documented to have a tumor-suppression function (Wong *et al.* 2007). Another question about the 4.1G in the Sertoli and germ cells is whether it plays a role in tumor progression and

Figure 7 The 4.1G immunolocalization in germ cells during the mouse seminiferous epithelial cycle, which is restricted under cell membranes of spermatogonia through to early pachytene spermatocytes (pink lines). A and B, type A and B spermatogonia respectively; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; M, meiotic division; 1–16, steps of spermiogenesis.

appearance of phenotypes in testicular tumors without the 4.1G protein. In conclusion, we found the 4.1G localization and its molecular complex with CADM1 in germ cells.

Materials and Methods

Animals and anesthesia

All animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee in University of Yamanashi. Adult C57BL/6J mice and 4.1G-deficient mice described in the next paragraph, weighing 20–30 g, were anesthetized with pentobarbital (Terada *et al.* 2009b) and processed for the following preparation procedures.

Production and analysis of 4.1G-deficient mice

4.1G-deficient mice were generated by targeting exons 1 and 2, which contain the initiation codon of 4.1G (Parra *et al.* 2004), using a similar procedure for the generation of 4.1B-deficient mice (Ohno *et al.* 2009). A neomycin resistance cassette was inserted into the DNA fragments flanking exons 1 and 2 by using TaKaRa LA Taq (Takara Bio., Otsu, Shiga, Japan; Fig. 8a) and subcloning to a PGKneox2DTA vector. The targeting vector was introduced into AK7 embryonic stem (ES) cells with a 129/S4 background. Neomycin-resistant cell colonies were isolated and examined by Southern blotting analysis after XbaI digestion to confirm homologous recombination. The XbaI digestion generated a 4.9 kb band in the wild-type allele and a 2.9 kb band in the mutant allele following homologous recombination (Fig. 8b). Two independent clones of ES cells, where two predicted bands sized 2.9 and 4.9 kb were detected in the Southern blotting, were injected into blastocytes with the C57BL/6J background and several chimeric mice were generated. Mice heterozygous for the targeted 4.1G-mutant allele were mated for at least three generations, and then those carrying two mutant alleles and a mixed background of 129/S4 and C57BL/6J were obtained. The generated mice were first genotyped by PCR screening (Fig. 8c), and lack of 4.1G protein was confirmed in their testicular tissues by immunohistochemistry and western blot analysis.

IVCT and FS for living mouse testes

The IVCT-FS has some benefits for preserving biological molecules, including soluble proteins of animal tissues, as well as stopping dynamic morphology reflecting their living state, as reported before (Liao *et al.* 2006, Ohno *et al.* 2007b). Briefly, three wild-type mice or the 4.1G-deficient mice were anesthetized with pentobarbital, and IVCT was performed on their left testis by directly pouring 50 ml liquid isopentane-propane cryogen (-193°C), which was cooled in liquid nitrogen, as reported before (Liao *et al.* 2006, Zhou *et al.* 2008). The low temperature was maintained by additionally pouring liquid nitrogen (-196°C). The experimental mice were always frozen under normal blood circulation conditions with their

Figure 8 (a) Construction of the targeting vector (Vector) and organization of the 4.1G gene of wild-type (Wild) and mutant mice (Mutant). DTA, diphtheria toxin; Ex, exon; *Neo^R*, neo-resistance gene; ATG, the translation initiation codon of 4.1G; probe, a region of a digoxigenin-labeled probe for Southern blotting. DTA and *Neo^R* genes were inversely inserted. (b) In the Southern blotting, the genomic DNA of wild-type mice (+/+) digested with XbaI provides a 4.9 kb fragment, whereas that of heterozygous mice (+/-) provides 4.9 and 2.9 kb fragments. (c) The PCR for the wild-type allele (Wt) produces an ~1.0 kDa DNA fragment in wild-type (+/+) and heterozygous (+/-) mice, whereas that for the mutant allele (Mu) produces an ~1.5 kb DNA fragment in heterozygous (+/-) and homozygous (-/-) mice.

hearts beating. The frozen testicular tissues were removed with a dental electric drill in liquid nitrogen, and processed for routine FS in acetone containing 2% paraformaldehyde at -80°C for 24 h and then at -30 , -10 , 4°C , and room temperature (RT) for 2 h each as reported before [Liao *et al.* 2006]. They were washed in pure acetone and xylene, and routinely embedded in paraffin.

Immunostaining on paraffin sections

Paraffin-embedded tissues were cut at 4 μm thickness, routinely de-paraffinized with xylene, and infiltrated in a graded series of ethanol and PBS. Some sections were stained with HE for pure morphology at the light microscopic level. For quantifying the number of sperm cells in both wild-type mice and 4.1G-deficient mice, spermatozoa attaching to apical regions of Sertoli cells in 25 μm width of longitudinally cut seminiferous tubules at stage VIII were counted, and the numbers were statistically compared between the 4.1G-deficient mice and the wild-type ones (five regions in three mice each).

For the common immunostaining, other adjacent sections were pretreated with hydrogen peroxide and goat normal serum, followed by rabbit polyclonal anti-protein 4.1G antibody which reacts with the C-terminus region of 4.1G (ProteinExpress, Chiba, Japan) or chicken polyclonal anti-CADM1 antibody (clone 3E1; Medical and Biological Lab., Nagoya, Japan), as reported before [Ohno *et al.* 2007a]. They were treated with biotinylated anti-rabbit IgG

(Vector, Burlingame, CA, USA) or anti-chicken IgY antibody (Jackson ImmunoResearch, Cambridgeshire, UK), and then a HRP-labeled ABC (Pierce, Rockford, IL, USA), and visualized with metal-enhanced DAB method (Pierce). Finally, they were incubated in 0.04% osmium tetroxide solution for 30–60 s and observed under a light microscope. An immunocontrol was performed without the primary anti-4.1G antibody and only with the secondary anti-rabbit IgG antibody.

For double-immunofluorescence staining for 4.1G and CADM1, some sections were incubated with both primary antibodies at the same time, and they were treated with AlexaFluor488-conjugated anti-rabbit IgG and AlexaFluor 594-conjugated anti-chicken IgY antibodies (Invitrogen). After brief staining with TOPRO-3, they were observed under a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan).

The morphological evaluation with HE staining and the immunohistochemical study for 4.1G and CADM1 were performed at least in five sections from three different individuals of the wild-type mice or the 4.1G-deficient mice.

Pre-embedding immunoelectron microscopy

Conventional pre-embedding immunoelectron microscopy was performed for the mouse testes as reported before [Terada *et al.* 2009a]. Briefly, two anesthetized mice were perfused via their hearts with 2% paraformaldehyde in 0.1 M PB, pH 7.4. The testicular tissues were removed, cut into small pieces with razor blades, and immersed in the same fixative at 4°C for 2 h. After rinsing in PBS, they were then immersed in 30% sucrose/10% glycerol in PB at 4°C overnight, and frozen with isopentane cooled in dry ice. Six micrometer-thick cryosections were cut and treated with 0.3% hydrogen peroxide solution and 2% goat serum in PBS, and then immunostained with the same primary anti-4.1G antibody. They were subsequently incubated with biotinylated goat anti-rabbit IgG antibody. After rinsing in PBS, they were fixed again with 0.25% glutaraldehyde in PB for 10 min and visualized by the ABC–DAB method, as reported before [Terada *et al.* 2009a]. Then, they were additionally treated with 1% OsO_4 in PB for 20 min. The immunostained tissues were dehydrated with a graded series of ethanol and routinely embedded in epoxy resin by the inverted gelatin capsule method [Terada *et al.* 2005]. Ultrathin sections of 70 nm-thickness were routinely cut on an ultramicrotome and collected on copper grids. They were stained only with uranyl acetate, and observed in an electron microscope (H-7500; Hitachi) at an accelerating voltage of 75 kV.

Immunoprecipitation analysis

The immunoprecipitation analysis was performed for the adult C57BL/6J mouse testes, as reported before [Terada *et al.* 2007]. For the 4.1G–CADM1 interaction, tissue lysates were obtained from the tissue supernatant by homogenization with TENT buffer (20 mM Tris pH 7.4, 1 mM EDTA, 50 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma) and centrifugation at 10 000 g at 4°C for 30 min. The lysates were treated with protein G-Sepharose (GE Healthcare,

Piscataway, NJ, USA) and incubated with chicken anti-CADM1 antibody at 4 °C overnight. On the following day, goat anti-chicken IgY antibody was also added at 4 °C for 8 h, and then immunocomplexes were separated using protein G-Sepharose. All proteins were eluted from the sepharose beads by boiling in Laemmli sample buffer and subjected to SDS-PAGE and western blotting analyses with the anti-4.1G antibody. The blots were visualized using a chemiluminescent system (Pierce).

Cell culture

SSC cultures were established from C57BL/6N pup testes as described previously (Kubota & Brinster 2008). The SSCs provide a model to study stem cell biology because they undergo self-renewal and transmit genes to subsequent generations (Kubota *et al.* 2004). Germ cell clumps containing SSCs were maintained on mitotically inactivated STO cell feeders (SNL76/7; a kind gift from Dr Allan Bradley, Wellcome Trust Sanger Institute, Cambridge, UK) using a serum-free medium supplemented with 10 ng/ml human GDNF (R&D Systems, Minneapolis, MN, USA) and 1 ng/ml basic fibroblast growth factor (BD Biosciences, San Jose, CA, USA).

For the western blot analysis, SSCs, feeder cells, or testicular tissues were directly lysed with Laemmli sample buffer including β -mercaptoethanol at 95 °C, electrophoresed in 5–20% SDS-PAGE, blotted on PVDF membranes, and immunostained with the anti-4.1G antibody. For the fluorescence immunostaining, the cultured cells in dishes were fixed with 2% paraformaldehyde in 0.1 M PB for 15 min, and treated with 0.5% saponin in PBS at RT for 1 h. They were incubated with anti-4.1G antibody at RT for 2 h and then with AlexaFluor488-conjugated anti-rabbit IgG antibody (Invitrogen) at RT for 1 h. After brief counter-staining with DAPI (Vector) or TOPRO-3, they were observed under a fluorescence microscope or a confocal laser scanning microscope (FV1000; Olympus).

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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