The Arf GAP SMAP2 is necessary for organized vesicle budding from the trans-Golgi network and subsequent acrosome formation in spermiogenesis

Tomo Funaki\textsuperscript{a,*}, Shunsuke Kon\textsuperscript{a,*}, Kenji Tanabe\textsuperscript{b}, Waka Natsume\textsuperscript{a}, Sayaka Sato\textsuperscript{a}, Tadafumi Shimizu\textsuperscript{a}, Naomi Yoshida\textsuperscript{a}, Won Fen Wong\textsuperscript{a}, Atsuo Ogura\textsuperscript{a}, Takehiko Ogawa\textsuperscript{a}, Kimiko Inoue\textsuperscript{c}, Narumi Ogonuki\textsuperscript{d}, Hiromi Miki\textsuperscript{d}, Keiji Mochida\textsuperscript{d}, Keisuke Endoh\textsuperscript{d}, Kentarou Yomogida\textsuperscript{a}, Manabu Fukumoto\textsuperscript{a}, Reiko Horai\textsuperscript{f}, Yoichiro Iwakura\textsuperscript{f}, Chizuru Ito\textsuperscript{g}, Kiyotaka Toshimori\textsuperscript{g}, Tosho Watanabe\textsuperscript{a}, and Masanobu Satake\textsuperscript{a}

\textsuperscript{a}Department of Molecular Immunology, Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan; \textsuperscript{b}Medical Research Institute, Tokyo Women’s Medical University, Tokyo 162-8666, Japan; \textsuperscript{c}RIKEN Bio Resource Center, Tsukuba 305-0074, Japan; \textsuperscript{d}Department of Urology, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan; \textsuperscript{e}Department of Food Science and Nutrition, School of Human Environmental Science, Mukogawa Women’s University, Nishinomiya 663-8558, Japan; \textsuperscript{f}Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; \textsuperscript{g}Department of Anatomy and Developmental Biology, Chiba University Graduate School of Medicine, Inohana 260-8670, Japan; \textsuperscript{h}Department of Biological Sciences, Nara Women’s University, Nara 630-8506, Japan

ABSTRACT The trans-Golgi network (TGN) functions as a hub organelle in the exocytosis of clathrin-coated membrane vesicles, and SMAP2 is an Arf GTPase-activating protein that binds to both clathrin and the clathrin assembly protein (CALM). In the present study, SMAP2 is detected on the TGN in the pachytene spermatocyte to the round spermatid stages of spermatogenesis. Gene targeting reveals that \textit{SMAP2}-deficient male mice are healthy and survive to adulthood but are infertile and exhibit globozoospermia. In \textit{SMAP2}-deficient spermatids, the diameter of proacrosomal vesicles budding from TGN increases, TGN structures are distorted, acrosome formation is severely impaired, and reorganization of the nucleus does not proceed properly. CALM functions to regulate vesicle sizes, and this study shows that CALM is not recruited to the TGN in the absence of SMAP2. Furthermore, syntaxin2, a component of the soluble \textit{N}-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex, is not properly concentrated at the site of acrosome formation. Thus this study reveals a link between SMAP2 and CALM/syntaxin2 in clathrin-coated vesicle formation from the TGN and subsequent acrosome formation. \textit{SMAP2}-deficient mice provide a model for globozoospermia in humans.
INTRODUCTION

The cells of all eukaryotic organisms possess a trafficking system of membrane vesicles that is vital to maintaining cellular homeostasis. It was suggested that the dysregulation of this trafficking is involved in the genesis of tumors as well as in neurodegenerative diseases in humans (McMahon and Boucrot, 2011). As a caveat, in most cases, this hypothesis merely reflects the fact that the gene discovered in one study as responsible for disease onset or progression is known from another study to be involved in vesicle trafficking. Therefore it is not always clear how the possible dysregulation of vesicle trafficking inside the cells might lead to the pathologies seen in particular diseases. One of the reasons for this lack of knowledge is the relatively scant number of studies targeting genes involved in vesicle trafficking.

Clathrin-coated vesicles represent one type of membrane vesicle and are used in a broad range of trafficking pathways, including endocytosis and exocytosis of membrane proteins. The formation of clathrin-coated vesicles is regulated in a coordinated manner by many molecules and Arf proteins, which belong to a family of small GTPases and play key roles in the process (D’Souza-Schorey and Chavrier, 2006; Gillingham and Munro, 2007; Kahn, 2009; Donaldson and Jackson, 2011). The GTPase activity of Arf is activated by an Arf GTPase-activating protein (Arf GAP). Of interest, Arf GAP not only functions as a GAP for Arf but also possesses an Arf-independent function that contributes to the formation of clathrin-coated vesicles (Randazzo and Hirsch, 2004; Spang et al., 2010; Kahn, 2011; Kon et al., 2011).

There are 31 Arf GAP family proteins, and SMAP1 and SMAP2 constitute a small subgroup. SMAPs harbor a clathrin-binding motif and a domain capable of binding to the clathrin assembly protein (CALM; Tanabe et al., 2005, 2006; Natsume et al., 2006). Because of these characteristics, SMAP may exert unique functions in the formation of clathrin-coated vesicles that are not seen in the other Arf GAPs. For example, SMAP1 functions as an Arf6-specific GAP in the endocytosis of the transferrin receptor and is also involved in sorting of endocytosed c-Kit from multivesicular bodies to lysosomes (Tanabe et al., 2005; Kon et al., 2008, 2013). SMAP2 exhibits a similar extent of GAP activity for both Arf1 and Arf6 in vitro. SMAP2 most likely behaves as an Arf1-prefering GAP in cells, however, since the introduction of a GAP-negative mutant of SMAP2 renders cells resistant to brefeldin A, an inhibitor of Arf1 guanine nucleotide exchange factor (GEF) but not Arf6 GEF (Natsume et al., 2006). Moreover, SMAP2 is localized on early endosomes and the trans-Golgi network (TGN) and appears to function in the early endosome–to-TGN transport of TGN46/38 and in the TGN–to-plasma membrane transport of the vesicular stomatitis virus G protein (Natsume et al., 2006; Funaki et al., 2011). Furthermore, SMAP2 can compensate for the lack of SMAP1 in endocytosis of the transferrin receptor (Kon et al., 2013). Therefore SMAP2 may play multiple roles in various intracellular trafficking pathways.

All of the foregoing findings regarding SMAP1 and SMAP2 were obtained using cell cultures in vitro (Tanabe et al., 2005; Natsume et al., 2006; Kon et al., 2008; Funaki et al., 2011). Recently we reported that SMAP1-targeted cells have defects in the trafficking of the transferrin receptor as well as c-Kit, and targeted mice are predisposed to develop myelodysplastic syndrome and, eventually, acute myeloid leukemia (Kon et al., 2013). These results demonstrate a possible link between the dysregulated endocytosis in cells and oncogenesis. The TGN functions as a hub organelle in exocytosis by sorting either secreted molecules to the cell membrane or hydrolytic enzymes to lysosomes (Bonifacio and Rojas, 2006). How the functions of tissues/organs would be impaired if the TGN machinery is disrupted is unclear. In this study, to examine the physiological roles of SMAP2 in vivo with respect to TGN transport, we targeted SMAP2 and found that SMAP2-deficient male mice were infertile and exhibited globozoospermia. Vesicle budding from the TGN, as well as acrosome formation, was disorganized in SMAP2-deficient spermatogenesis.

RESULTS

Generation of SMAP2-deficient mice

We generated SMAP2-deficient mice using a gene-targeting method. A SMAP2-targeting construct was designed to replace the Metneo-containing exon 1 of SMAP2 with LacZ and neomycin-resistance (neo) gene sequences (Figure 1A). Homologous recombination should give rise to a protein consisting of the amino-terminal 12 amino acids of SMAP2 fused in-frame with LacZ. We obtained two independent founder mice possessing the germline-transmitted, targeted SMAP2 allele. Because essentially similar results were obtained for both lines of mice, representative results from one of the lines are described here. To generate SMAP2(−/−) mice, SMAP2(+/−) F1 mice were mated, and the F2 offspring were genotyped by Southern blot and PCR analysis (Figure 1B). SMAP2(+/−) and (−/−) mice were present in the expected Mendelian ratios.

SMAP2 expression was examined in tissues of wild-type mice by Northern blot analyses (Figure 1C). Two transcripts, 2.9 and 1.3 kb, which contain the same open reading frame but differ in their 3′-untranslated regions, were detected in the tissues examined (Figure 1C, top). Because the major phenotype of SMAP2(−/−) mice was defective spermatogenesis, as described later, SMAP2 expression at various stages of spermatogenesis was examined using wild-type testicular cells. SMAP2 transcripts were detected in pachytenepachytestocytes to the round spermatid stages (Figure 1C, bottom). Immunoblot analyses using lysates from brain and testis detected SMAP2 at 47 kDa in the wild-type tissue but not in the SMAP2(−/) tissue (Figure 1D). This result verified the SMAP2 deficiency in the SMAP2-targeted mice.

Infertility and globozoospermia seen in SMAP2-targeted male mice

SMAP2(−/−) mice grew to adulthood and were apparently healthy, but mating outcomes were not normal. When we conducted cross-breeding experiments using SMAP2(−/−) mice (Table 1), we found that female SMAP2(−/−) mice were fertile when mated to SMAP2(+/+) male mice. Male SMAP2(−/−) mice, however, were infertile regardless of the SMAP2 genotype of the female. Thus male infertility was detected as a phenotype of SMAP2-targeted mice.

Epididymides of SMAP2(−/−) male mice were examined, and the numbers of sperm present in the epididymides were similar in SMAP2(+/−) and (−/−) mice (Table 2). When epididymal sperm were observed by differential interference contrast (DIC) microscopy (Figure 2A), however, a normal hook-shaped head was observed for the SMAP2(+/+) sperm, whereas a cylindrical or round head was observed for the SMAP2(−/−) sperm. The percentages of sperm possessing hook-shaped and round heads were quantified (Table 2). A majority of SMAP2(+/−) sperm possessed the hook-shaped head (94%), whereas most SMAP2(−/−) sperm were round headed (79%). Thus the morphology of SMAP2(−/−) sperm is typical of globozoospermia (Dam et al., 2007).

Testes were sectioned, stained with hematoxylin–eosin, and examined by light microscopy (Figure 2B). In the seminiferous tubules of SMAP2(+/+) testes, many elongated spermatids possessing hook-shaped heads were detected. By contrast, most spermatids in the seminiferous tubules from SMAP2(−/−) testes possessed round heads. Similar observations were obtained for epididymal sperm.
Mitochondria in sperm were examined by staining cells lack of an acrosome (Suzuki-Toyota et al., 2004). The distribution pattern of mitochondria in sperm was most likely due to defects in spermatogenesis. Moreover, localization of sp56, a marker for acrosome formation, was very irregular or absent in SMAP2(−/−) sperm (Figure 2D). Thus the morphology of SMAP2(−/−) sperm indeed corresponds to globozoospermia in humans.

In mouse sperm, energy necessary for sperm motility is supplied from mitochondrial respiration according to some reports (Narisawa et al., 2002; Ford, 2006) or from anaerobic glycolysis according to others (Miki et al., 2004; Mukai and Okuno, 2004; Mukai and Travis, 2012). Structural changes in the mid piece, which contains the mitochondria, are closely associated with sperm motility (Wilton et al., 1992; Mundy et al., 1995; Piomboni et al., 2012). In addition to proper motility, the acrosome reaction is also required for successful fertilization. With this background in mind, we examined the motility and fertilizing capacity of SMAP2(−/−) sperm in vitro. Measurements of various motility parameters revealed that the motility of SMAP2(−/−) sperm was significantly (p < 0.05) reduced compared with that of SMAP2(+/+), (−/−), and (−/−) sperm (59 and 58%, respectively). The decreases included percentage of motile sperm, average path velocity, straight-line velocity, curvilinear velocity, amplitude of lateral head displacement, and beat frequency. In vitro fertilization by SMAP2(−/−) sperm was evaluated using wild-type oocytes (Supplemental Table S2). The percentage of two-cell embryos derived from SMAP2(−/−) sperm was 3.9%, whereas the fertilization rate by SMAP2(+/+) sperm was 38%. When intracytoplasmic sperm injection experiments were performed (Supplemental Table S1), however, pups were produced at similar levels by the SMAP2(−/−) and (+/+) sperm (59 and 58%, respectively). Taken together, these results indicate that SMAP2(−/−) sperm possess a developmentally competent haploid genome but are defective in motility and ability to penetrate the oocyte.

**Globozoospermia is intrinsic to SMAP2-targeted germ cells**

SMAP2 transcripts were detected by Northern blot analyses in testes of Sl/Sld mice defective in motility and ability to penetrate the oocyte.
and injected into the seminiferous tubules of testes of W/W<sup>−</sup> mice that lack germ cells. Three months later, differentiated spermatids were readily detected, but most exhibited the same distinctive round heads (Supplemental Figure S1, left). In a reciprocal transplantation experiment, germ stem cells were prepared from the testes of pCXN-EGFP transgenic mice and implanted into the seminiferous tubules of busulfan-treated MAP2<sup>(−/−)</sup> animals whose testes lack germ cells. Spermatids from these transplants possessed hook-shaped heads and appeared properly differentiated (Supplemental Figure S1, right). These results suggest that the defects causing globozoospermia are likely intrinsic to the MAP2-targeted germ cells and not the supporting cells.

**Stage-dependent expression and localization of MAP2 at the TGN during spermatogenesis**

MAP2 expression during spermatogenesis was examined by immunofluorescence using a testicular cell suspension that was spun down onto microscope slides (Figure 3A). 4',6-Diamidino-2-phenylindole (DAPI) staining showed the nuclear morphology, as well as chromatin condensation, whereas γH2AX staining detected sites of DNA double-strand breaks. Combined use of DAPI and γH2AX allowed the identification of the stages of spermatogenesis (Xu, 2003; Figure 3A). MAP2 expression was not detected in spermatogonia or in leptotene spermatocytes but could first be detected in the cytoplasm of zygotene spermatocytes as multiple punctae. In pachytenes spermatocytes and round spermatids, MAP2 fluorescence appeared not only as multiple punctae but also in a single, large focus. MAP2 fluorescence was absent in elongated spermatids and sperm. Thus MAP2 expression was stage dependent. Immunofluorescence localization of MAP2 is consistent with the detection by Northern blot (Figure 1C).

We previously reported that MAP2 was distributed on early endosomes (as multiple punctae) and on the TGN (as a single large focus) in tissue culture cells (Natsume et al., 2006; Funaki et al., 2011). Therefore we examined whether MAP2 is similarly localized in spermatogenesis. Double-labeling immunofluorescence was performed on pachytenes spermatocytes and round spermatids (Figure 3B). In the single large focus of MAP2 staining, fluorescence from MAP2 overlapped with that from TGN38, syntaxin6, and clathrin. Pearson’s r was significant (r > 0.5) for each combination. In tissue culture cells, clathrin is located on various organelles but is particularly concentrated on the TGN (Huang et al., 2007; Burgess et al., 2011).

| MAP2 genotype | Number of mice examined | Number of sperm in epididymis (x10<sup>6</sup>) | Number of sperm examined per mouse | Hook-shaped head (%) | Cylindrical or round head (%) |
|---------------|-------------------------|-----------------------------------------------|----------------------------------|---------------------|-------------------------------|
| (+/-)         | 7                       | 3.27 ± 0.68                                   | 57                              | 21 ± 16             | 79 ± 16                       |
| (+/-)         | 4                       | 3.27 ± 0.57                                   | 60                              | 94 ± 6.8            | 6 ± 6.8                       |

**TABLE 2: Number of sperm and their morphology.**

To correlate the abnormalities in the acrosome to the phases of acrosome formation, we frozen sectioned testis tissues and stained them with peanut agglutinin (PNA), a lectin that reacts with polysaccharides in the acrosome. Supplemental Figure S2A shows the Golgi

**TABLE 1:MAP2 genotype and fertility.**
Acrosomal defects in SMAP2 deficiency

Acrosome formation is initiated by the budding of proacrosomal vesicles from the TGN. To examine possible abnormalities in this step, we processed testes sections for analysis by transmission electron microscopy (TEM; Figure 5A). In the Golgi phase of acrosome formation, wild-type spermatids (Figure 5A, I) possess an umbrella-shaped TGN, and multiple proacrosomal vesicles of uniform size are present between the TGN and nuclear membrane. By contrast, in SMAP2-targeted spermatids (Figure 5A, II), proacrosomal vesicles were present but were not uniform in size and appeared to be larger than those in wild-type cells. In other SMAP2-targeted spermatids (Figure 5A, III), the lamellar structure of the TGN formed loose whorls. These observations suggest that there are abnormalities in the budding of vesicles from the TGN in SMAP2-targeted cells.

To evaluate the foregoing observations more quantitatively, we measured the diameter of proacrosomal vesicles on the TEM sections. Approximately 20 vesicles per cell and a total of 15 cells were measured for each genotype. Figure 5B (also see Supplemental Figure S3) shows the range of proacrosomal vesicle sizes and confirms that the SMAP2-targeted cells tended to produce larger-diameter vesicles than do wild-type cells. Therefore abnormal acrosome formation in SMAP2-targeted cells appears to involve budding of proacrosomal vesicles from the TGN.

Formation of the acrosome from proacrosomal vesicles is disrupted in SMAP2-targeted spermiogenesis

The later phases of acrosome formation were examined by TEM (Figure 6). In the cap phase in wild-type cells (Figure 6, I), proacrosomal vesicles that had budded from the TGN fused with each other and formed a large acrosome (Ac) located at the acroplaxome (Apx), a unique substructure composed of keratin and actin that serves as a site for anchoring the acrosome to the nuclear membrane (Kierszenbaum et al., 2003). By contrast, in a cap-phase SMAP2-targeted cell (Figure 6, II), acrosomes of intermediate size were formed but never fused to form one large acrosome and remained as multiple pseudoacrosomes (indicated by the arrows). These pseudoacrosomes attached to multiple sites on the nuclear envelope. In another SMAP2-targeted cell (Figure 6, III), one acrosome appeared to be formed but was greatly enlarged in size (the TGN was also disorganized and partly distended; arrows). These observations indicate that acrosome formation from proacrosomal vesicles in SMAP2-deficient cells is disrupted, even though the acroplaxome structures are well organized on the nuclear lamina. Therefore it is unlikely that a defect in the acroplaxome caused the aberrant acrosome formation.

Secondary abnormalities in spermiogenesis in SMAP2-deficient cells

In the acrosome phase in wild-type cells (Figure 6, IV), the acrosome was detected as a long and continuous layer along the nuclear membrane. In the enlargement of marginal rings (arrowhead), ectoplasmic specializations (two white arrows) derived from Sertoli cells cover the surface of the acrosome. A manchette structure is also

FIGURE 2: Morphology of sperm and spermatids in the epididymis and testis. (A) Sperm were collected from the epididymis of SMAP2(+/+) and (−/−) mice and observed using DIC microscopy. (B, C) Testis and epididymis specimens from SMAP2(+/+) and (−/−) mice were sectioned, stained with hematoxylin–eosin, and viewed using a light microscope. (D) Sperm from SMAP2(+/+) and (−/−) mice were stained with anti-sp56, DAPI, and MitoRed. Bars, 5 μm (A, D), 50 μm (B, C).
observed (asterisk). In a SMAP2(−/−) cell (Figure 6, V), however, the ectoplasmic specializations were interrupted (arrow), and the acrosome appeared swollen near the marginal ring (arrowhead).

Finally, in the maturation phase in wild-type cells (Figure 6, VII), the ectoplasmic specialization (two white arrows) and the manchette (asterisk) were observed, but in a SMAP2(−/−) cell (Figure 6, VIII), the nucleus appeared fragmented, the acrosome was dispersed (arrow), and the manchette was located ectopically (asterisk). An invagination of the manchette into the nucleus was observed in an another SMAP2(−/−) cell (Figure 6, IX). Finally, in SMAP2(−/−) sperm of the epididymis, the shedding of cytoplasmic content appeared to be incomplete, since many mitochondria were detected near the nucleus (Figure 6, X). These alterations in the acrosome and maturation phases in SMAP2-deficient sperm may be secondary events that result from the defects in proacrosomal vesicle budding and acrosome formation.

Molecules involved in the formation of proacrosomal vesicles

The proacrosomal vesicles budding from the TGN in SMAP2(−/−) spermatids had larger diameters than those in the wild-type cells (Figure 5). CALM is a protein that affects the size of clathrin-coated vesicles, since vesicles become enlarged in cells lacking CALM/AP180 (Zhang et al., 1998; Nonet et al., 1999; Meyerholz et al., 2005). In addition, we previously reported a physical association between SMAP2 and CALM (Natsume et al., 2006). Therefore, to explore a possible link between SMAP2 and the vesicle size, we performed double immunofluorescence of SMAP2 and CALM using a germ cell suspension (Figure 7A). In wild-type spermatocytes and round spermatids, SMAP2 was concentrated at the TGN, as described earlier, whereas CALM fluorescence was detected diffusely in the cytoplasm but also colocalized with SMAP2. In the SMAP2(−/−) cells, CALM was no longer localized to the TGN (the overall amount of CALM was not affected by SMAP2 deficiency; data not shown). When the wild-type and SMAP2(−/−) germ cells were mixed at a 1:1 ratio, CALM was colocalized with SMAP2 at the TGN in the wild-type cells but homogenously distributed in the SMAP2(−/−) cells. Thus mobilization of CALM to the TGN appears to be dependent on SMAP2.

We also used coimmunoprecipitation to confirm a physical association between SMAP2 and CALM (Figure 7B). Lysates were prepared from wild-type testes and immunoprecipitated with an anti-CALM antibody. Immunoblotting of the precipitate with an anti-SMAP2 antibody revealed the presence of SMAP2, indicating an association between CALM and SMAP2.

An important implication of the observations of acrosome formation (Figure 6) is that some process in the fusion of proacrosomal vesicles was impaired in SMAP2(−/−) cells. Syntaxin2 is a component of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which serves as the fusion machinery for proacrosomal vesicles (Rizo and Sudhof, 2002; Roquet-Rivera et al., 2011). In Figure 7C, cells were double stained for syntaxin2 and sp56. In wild-type spermatocytes, syntaxin2 was concentrated at foci in the cytoplasm. In round spermatids, syntaxin2 was detected adjacent to the sp56 fluorescence. In contrast, in the SMAP2(−/−) cells, although syntaxin2 was concentrated at foci during the spermatocyte stage, during the round spermatid stage...
DISCUSSION

In this study we showed that SMAP2-deficient male mice are infertile. Sperm in the testes and epididymides in these mice exhibit morphological abnormalities indicative of globozoospermia, a phenotype seen also in infertile men. Formation of the acrosome is disrupted in SMAP2-deficient sperm, and several reports suggested that defects in acrosome formation cause globozoospermia (Holstein et al., 1973; Ito et al., 2004; Lin et al., 2007; Roqueta-Rivera et al., 2011). Therefore the globozoospermia seen in the SMAP2 deficiency is likely due to defects in acrosome formation.

During spermiogenesis, a large number of proacrosomal vesicles bud from the TGN, fuse with each other, anchor to the nuclear
SMAP2(+/+)

Cap phase

Acrosome phase

Maturation phase

Epididymis

SMAP2(-/-)

membrane, and eventually form a large organelle known as the acrosome (Abou-Haila and Tulsiani, 2000; Moreno and Alvarado, 2006). We reported previously that SMAP2 is located on the TGN and functions in vesicle transport from the TGN in tissue culture cells such as HeLa and Cos7 cells (Funaki et al., 2011). These results suggested that SMAP2 might be directly involved in the budding of proacrosomal vesicles from the TGN in spermatogenesis. This hypothesis is supported by the following observations. 1) SMAP2 protein is detected in wild-type germ cells and in the pachytene spermatocyte to the round spermatid stages. Acrosomal proteins are actively synthesized at the pachytene stage (Anakwe and Gerton, 1990), and enormous amounts of proacrosomal vesicles are supplied from the TGN until the round spermatid stage. Thus the observed pattern of SMAP2 expression coincides with the period of acrosome formation. 2) In wild-type spermatogenic cells, SMAP2 is located exclusively on the TGN. 3) In SMAP2-deficient germ cells, the TGN tend to lose structural integrity, appearing as loose whorls, and the diameters of proacrosomal vesicles become significantly larger than in wild-type cells. The primary alteration in spermiogenesis in SMAP2-deficient mice was the enlargement of the proacrosomal vesicles, suggesting that this change may ultimately cause the globozoospermic morphology. In other words, SMAP2 may function to standardize

FIGURE 6: Electron microscopic observations of acrosome formation and subsequent events in wild-type and SMAP2-targeted germ cells. Cells at the cap, acrosome, and maturation phases of acrosome formation are shown. Sections of epididymides are also shown. See the text for the description of structural abnormalities. A, axoneme; Ac, acrosome; Apx, acroplaxome; G, Golgi apparatus; M, mitochondria; N, nucleus; Sc, Sertoli cell. Arrowheads in IV and V are marginal rings. Asterisks in IV and VII–IX are manchettes. Double white arrows in IV and VII are ectoplasmic specializations derived from Sertoli cells. White arrows indicate pseudoacrosomes (II), a disorganized TGN structure (III), interrupted ectoplasmic specializations (V), invagination of a Sertoli cell into the germ cell (VI), and fragmented acrosomes (VIII). Bars, 1 μm (I–IX), 2 μm (X, XI).
with this hypothesis, CALM is detected at the TGN in wild-type but not in SMAP2(-/-) cells.

In addition to the morphological abnormalities, SMAP2(-/-) proacrosomal vesicles may have other functional defects. Vesicle-associated membrane proteins (VAMPs) are components of the SNARE complex, and CALM can function to recruit some VAMPs to clathrin-coated vesicles through physical association with the VAMPs (Harel et al., 2008; Koo et al., 2011; Miller et al., 2011). Therefore recruitment of SNARE complex components may be defective in SMAP2-deficient proacrosomal vesicles, since CALM was not

the size of TGN-derived vesicles. Thus a principal issue is how SMAP2 might function to regulate vesicle size during vesicle formation. CALM and its homologue, AP180, control the size of clathrin-coated vesicles. When CALM/AP180 is abolished, vesicles become enlarged (Zhang et al., 1998; Nonet et al., 1999; Meyerholz et al., 2005), similar to the effects of SMAP2 deficiency. We reported previously that SMAP2 binds to CALM directly (Natsume et al., 2006). One possible scenario for SMAP2 deficiency is that CALM is not recruited to the site of vesicle formation, thus causing the vesicle size to be unregulated. Consistent with this hypothesis, CALM is detected at the TGN in wild-type but not in SMAP2(-/-) cells.

In addition to the morphological abnormalities, SMAP2(-/-) proacrosomal vesicles may have other functional defects. Vesicle-associated membrane proteins (VAMPs) are components of the SNARE complex, and CALM can function to recruit some VAMPs to clathrin-coated vesicles through physical association with the VAMPs (Harel et al., 2008; Koo et al., 2011; Miller et al., 2011). Therefore recruitment of SNARE complex components may be defective in SMAP2-deficient proacrosomal vesicles, since CALM was not
concentrated at the TGN. Although the association of CALM and syntaxin2 was not detected in lysates of wild-type cells, we did observe a rather irregular localization of syntaxin2 in SMAP2(−/−) cells. Such alterations in the composition of proacrosomal vesicles might be related to the apparent defect in fusion of proacrosomal vesicles into an acrosomal structure that resulted in the generation of non-fused, segmented acrosomes. The present study therefore suggests a scenario in which the budding and fusion of proacrosomal vesicles are linked by the recruitment of specific proteins to the vesicles.

Malformation of the acrosome causes secondary effects on the manchette (Kierszenbaum et al., 2004; Pierre et al., 2012). In particular, the manchette cannot be localized to the perinuclear ring; thus the manchette–acroplasm connection becomes disorganized, and spermatid–Sertoli cell adhesion is perturbed (Kierszenbaum et al., 2007). In SMAP2-deficient spermatids, the manchette was sometimes mislocalized, and the ectoplasmic specializations from the Sertoli cells were not formed properly, or the anchoring of the acrosome to the nucleus appeared weak, thus allowing invagination of the Sertoli cells into the spermatid. Thus, in SMAP2-deficient spermatogenesis, both primary defects in proacrosomal vesicle formation and secondary defects in manchette attachment likely contribute to the abnormal globozoospermic morphology (Kierszenbaum and Tres, 2004; Kierszenbaum et al., 2011; Fujihara et al., 2012).

Endocytosis of cell membrane proteins also contributes to the formation of the acrosome (Moreno and Alvarado, 2006; Berruti et al., 2010; Rainey et al., 2010; Paiardi et al., 2011). Our previous study using cell lines showed that SMAP2 is located on early endosomes (Natsume et al., 2006). Therefore we cannot exclude the possibility that some defects in retrograde traffic from early endosomes to the TGN might also be involved in the genesis of the globozoospermia seen in SMAP2(−/−) mice.

There are several mouse models reported to exhibit abnormalities in proacrosomal vesicle formation from the TGN. In gene-targeted mice with knockouts of HIV-Rev-binding protein (Hrb), Golgi-associated PDZ and coiled-coil motif-containing protein (GOPC), Hook homologue 1 (Hook1), or protein interacting with C kinase 1 (Pick1), acrosome formation is defective. All of these molecules are involved in the transport of proacrosomal vesicles from the TGN (Kang-Decker et al., 2001; Yao et al., 2002; Moreno et al., 2006; Xiao et al., 2009; Kierszenbaum et al., 2011). Among these knockouts, Hrb belongs to an Arf GAP family, as does SMAP2. Hrb-deficient male mice exhibit globozoospermia, but the details of the phenotype appear distinct from SMAP2-deficient spermatogenesis. In Hrb-deficient mice, the number of mature sperm is extremely low, some sperm possess multiple nuclei due to a defect in meiosis, and other sperm possess multiple flagella due to an increased number of centrioles (Juneja and van Deursen, 2005). These features were not observed in SMAP2-deficient spermogenesis. Furthermore, Hrb plays a role distinct from SMAP2, in that the Hrb protein is located on the cytoplasmic side of the proacrosomal vesicles and functions in their fusion. Thus, although both SMAP2 and Hrb are Arf GAPs and their deficiencies cause globozoospermia, the two proteins appear to exert nonoverlapping, unique functions during spermatogenesis.

MATERIALS AND METHODS

Generation of SMAP2-targeted mice

A lambda phage clone encompassing exon 1 of SMAP2, which contains the methionine start codon, was isolated from a 129/SvJ genomic library (Stratagene, Santa Clara, CA). A targeting vector was constructed in which a fragment containing the LacZ and PGK-neoR sequences was inserted into the KpnI site of exon 1, and a diphtheria toxin-A--encoding fragment was ligated at the 5′ end. The vector was linearized by NotI digestion and electroporated into E14 ES cells. Of 610 G418-resistant clones, five were judged to have undergone proper homologous recombination based on PCR and Southern blot analysis of genomic DNA. Procedures for PCR and Southern blotting were as previously described (Wong et al., 2010). Cells with a normal karyotype were selected using a Colcemid treatment method. Three ES cell clones were injected into blastocysts, and the chimeric males that were produced were mated with C57BL/6J female mice. Finally, two lines of SMAP2(+/+) founder mice were established that possessed the SMAP2-targeted allele in the germline. PCR primers used for genotyping were as follows. To detect the wild-type allele, the forward and reverse primers were 5′-CACTCG-GGTCAAGTGTGCG-3′ and 5′-CCAGAACCCCCCTCCCCACTC-3′, respectively. To detect the targeted allele, the forward and reverse primers were 5′-CGCCCTTCTATGCCCTTCTGACG-3′ and 5′-CTTTCCGCCTCAGAACCCCATAG-3′, respectively.

In vitro fertilization and intracytoplasmic sperm injection

For in vitro fertilization, mature oocytes were collected from oviducts of B6D2F1 female mice that had been superovulated by the injection of 5 IU each of equine and human chorionic gonadotrophin. Sperm were collected from the epididymides of SMAP2(+/+) or (−/−) male mice. In each experiment, oocytes from two females were incubated with sperm from one male at a concentration of 200 sperm/μl. Fertilization was confirmed by development to the two-cell stage by 24 h after insemination.

For intracytoplasmic sperm injection, germ cells were collected from seminiferous tubules of SMAP2(+/+) or (−/−) testes. Elongated spermatids that were at differentiation steps 9–11 were picked up and injected into oocytes using a piezo-driven micromanipulator (PrimeTech, Tsuchiura, Japan). Oocytes that survived injection were cultured in a potassium simplex optimized medium, and those that developed to the two-cell stage were transplanted into oviducts of pseudopregnant ICR female mice. The offspring were counted at term, namely, on day 19.5, by caesarian section or after natural delivery.

Transplantation of spermatogonial stem cells

Four-week-old WBB6F1-W/W+ mice that were devoid of spermatogenic cells were purchased from Japan SLC (Hamamatsu, Japan) and used as recipient animals. Spermatogonial stem cells were prepared from SMAP2(−/−) testes by a two-step enzymatic digestion technique as described previously (Ogawa et al., 1997). A volume of 10 μl of spermatogenic cells, (2–3) × 107 cells/ml, was introduced into the seminiferous tubules of the WBB6F1-W/W+ mouse testis. As a control experiment, spermatogonial stem cells of SMAP2(+/+) mice were transplanted into SMAP2(−/−) mice. For recipient preparation, busulfan (44 mg/kg body weight) was injected intraperitoneally into 3-wk-old SMAP2(−/−) male mice. After 6 wk, the busulfan-treated testes were devoid of endogenous spermatogenesis. Spermatogonial stem cells were prepared from the testes of pCXN-EGFP transgenic, SMAP2(+/+) male mice (Okabe et al., 1997) and introduced into the seminiferous tubules of the busulfan-treated SMAP2(−/−) mice.

At 3 mo posttransplantation, mice were killed and testes were removed. The seminiferous tubules were dissected out of the testes, and sperm released from the tubules was observed with an Olympus BX50 microscope (Olympus, Tokyo, Japan) using DIC optics. The tubules were transferred into a fixative containing 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and processed for microscopic observation.
Histology and immunofluorescence detection

Testes were fixed in Bouin’s solution, embedded in paraffin, and sectioned. Deparaffinized sections were stained with hematoxylin and eosin, toluidine blue, or PAS, according to standard procedures. Some testes were fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS), immersed in 20% (wt/vol) sucrose in PBS, frozen in an optimal cutting temperature compound, cryo-ostat sectioned, and processed for immunofluorescence. For some samples, germ cells were liberated as single-cell suspensions from testes or epididymides, centrifuged onto slide glasses, fixed, and processed for immunofluorescence. The following antibodies were used: anti-SMAP2 (Sigma-Aldrich, St. Louis, MO), anti-β2AX (Trevigen, Gaithersburg, MD), anti-TGN38 (AbD Serotec, Raleigh, NC), anti-clathrin heavy chain (Affinity BioReagents, Golden, CO), anti-syntaxin6 (BD Transduction Labs, Lexington, KY), anti-GM130 (BD Transduction Labs), anti-SP56 (QED Bioscience, San Diego, CA), anti-syntaxin2 (Abcam, Cambridge, MA), anti-CALM (Santa Cruz Biotechnology, Santa Cruz, CA), and appropriate fluorophore-conjugated secondary antibodies. Rhodamine-conjugated PNA and MitoRed were obtained from Vector Laboratories (Burlingame, CA) and Djoindo (Rockville, MD), respectively. Fluorescence was observed using a B29000 fluorescence microscope (Keyence, Osaka, Japan). The Pearson’s r quantifying the colocalization between images was calculated using the JACoP plug-in in ImageJ (National Institutes of Health, Bethesda, MD).

Electron microscopic observations

Mice were killed and perfused with a fixative containing 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Testes and epididymides were removed and treated with the same fixative at 4°C overnight. Tissues were cut into small pieces, rinsed, and postfixed in 1% (wt/vol) OsO4 in distilled water for 1 h. The samples were dehydrated using increasing concentrations of ethanol and embedded in Epon 812. The plastic-embedded tissues were sectioned using an ultramicrotome (model Ultracut E; Reihert-Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. The samples were observed using a JEOL 1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

Northern blot, immunoblot, and immunoprecipitation analyses

RNA was isolated from cells/tissues of C57BL/6J mice using TRIzol (Life Technologies, Carlsbad, CA). When necessary, germ cells from testes were fractionated into various differentiation stages by centrifugal elutriation (Barchi et al., 2009). For Northern blots, 10 μg of total RNA was separated in a 1% (wt/vol) formaldehyde/agarose gel and transferred to a Hybond membrane (Amersham, Piscataway, NJ), according to the manufacturer’s instructions. The filter was hybridized with a radioabeled Spf fragment of SMAP2 cDNA. Protein lysates of cells/tissues were prepared with a Polytron homogenizer using a buffer containing 9 M urea, 2% (vol/vol) Triton X-100, and 1% (wt/vol) dithiothreitol. The lysates were centrifuged at 13,000 rpm for 15 min, and the supernatant was collected. Lithium dodecyl sulfate was added to a final concentration of 2% (wt/vol), and the solution was sonicated. The protein concentration was determined using a Bio-Rad (Hercules, CA) protein assay kit. Protein samples (20 μg) were separated on an 8% SDS–PAGE gel and electrottransferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Immunoblotting was performed using anti-β actin (Sigma-Aldrich), anti-SMAP2, anti-CALM, or anti-syntaxin2 antibodies and an ECL reagent (Amersham). Some lysates were first immunoprecipitated, as described previously (Natsume et al., 2006), and the precipitates were then blotted using one of the antibodies described.

Statistical analyses

Statistical significance was evaluated using a two-tailed Student’s t test, and differences of p < 0.05 were considered statistically significant.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, Culture and Technology, Japan. M.S. is a member of the Global COE “Network Medicine” at Tohoku University.

REFERENCES

Abou-Haila A, Tulisani DR (2000). Mammalian sperm acrosome: formation, contents, and function. Arch Biochem Biophys 379, 173–182.
Anakwe OO, Gerton GL (1990). Acrosome biogenesis begins during meiosis: evidence from the synthesis and distribution of an acrosomal glycoprotein, acrogranin, during guinea pig spermatogenesis. Biol Reprod 42, 317–328.
Barchi M, Geremia R, Magliozzi R, Bianchi E (2009). Isolation and analyses of enriched populations of male mouse germ cells by sedimentation velocity: the centrifugal elutriation. Methods Mol Biol 558, 299–321.
Berruti G, Ripolone M, Ceriani M (2010). USP8, a regulator of endosomal sorting, is involved in mouse acrosome biogenesis through interaction with the spermatid ESCRT-0 complex and microtubules. Biol Reprod 82, 930–939.
Bock JB, Klumperman J, Davanger S, Scheller RH (1997). Syntaxin 6 functions in trans-Golgi network vesicle trafficking. Mol Biol Cell 8, 1261–1271.
Bonifacino JS, Rojas R (2006). Retrograde transport from endosomes to the trans-Golgi network. Nat Rev Mol Cell Biol 7, 568–579.
Burgess J et al. (2011). AP-1 and clathrin are essential for secretory granule biogenesis in Drosophila. Mol Biol Cell 22, 2094–2105.
Dam AH, Feenstra I, Westphal JR, Ramos L, van Golde RJ, Kremer JA (2007). Globozoospermia revisited. Hum Reprod Update 13, 63–75.
Donaldson JG, Jackson CL (2011). ARF family G proteins and their regulators: roles in membrane transport, development and disease. Nat Rev Mol Cell Biol 12, 362–375.
D’Souza-Schorey C, Chavrier P (2006). ARF proteins: roles in membrane trafficking and beyond. Nat Rev Mol Cell Biol 7, 345–357.
Ford WC (2006). Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go round? Hum Reprod Update 12, 269–274.
Fujihara Y, Satoh M, Inoue N, Isotani A, Ikawa M, Okabe M (2012). SPACA1-deficient male mice are infertile with abnormally shaped sperm heads reminiscent of globozoospermia. Development 139, 3583–3589.
Funaki T, Kon S, Ronn RE, Hemmi Y, Kobayashi Y, Watanabe T, Nakayama K, Tanabe K, Satake M (2011). Localization of SMAP2 to the TGN and its function in the regulation of TGN protein transport. Cell Struct Funct 36, 83–95.
Gillingham AK, Munro S (2007). The small G proteins of the Arf family and their regulators. Annu Rev Cell Dev Biol 23, 579–611.
Harel A, Wu F, Mattson MP, Morris CM, Yoo PJ (2008). Evidence for CALM in directing VAMP2 trafficking. Traffic 9, 417–429.
Hermo L, Rambourg A, Clermont Y (1980). Three-dimensional architecture of the cortical region of the Golgi apparatus in rat spermatids. Am J Anat 157, 357–373.
Holstein AF, Schirren C, Schirren CG (1973). Human spermatids and spermatozoa lacking acrosomes. J Reprod Fertil 35, 489–491.
Huang C, Chang SC, Yu IC, Tsay YG, Chang MF (2007). Large hepatitis delta antigen is a novel clathrin adaptor-like protein. J Virol 81, 5985–5994.
Humphrey JS, Peters PJ, Yuan LC, Bonifacino JS (2011). Localization of TGN38 to the trans-Golgi network: involvement of a cytoplasmic tyrosine-containing sequence. J Cell Biol 190, 1123–1135.
Ito C, Suzuki-Toyota F, Maekawa M, Toyama Y, Yao R, Noda T, Toshimori K (2004). Failure to assemble the peri-nuclear structures in GOPC deficient spermatids as found in round-headed spermatzoa. Arch Histol Cytol 67, 349–360.
Juneya SC, van Deursen JM (2005). A mouse model of familial oligoasthenoteratozoospermia. Hum Reprod 20, 881–893.
Narisawa S, Hecht NB, Goldberg E, Boatright KM, Reed JC, Millan JL, Nakamura N, Rabouille C, Watson R, Nilsson T, Hui N, Slusarewicz P, Kreis Mundy AJ, Ryder TA, Edmonds DK (1995). Asthenozoospermia and the Mukai C, Travis AJ (2012). What sperm can teach us about energy produc Moreno RD, Palomino J, Schatten G (2006). Assembly of spermatid Miller SE, Sahlender DA, Graham SC, Honing S, Robinson MS, Peden AA, Meyerholz A, Hinrichsen L, Groos S, Esk PC, Brandes G, Ungewickell EJ McMahon HT, Boucrot E (2011). Molecular mechanism and physiological Lin YN, Roy A, Yan W, Burns KH, Matzuk MM (2007). Loss of zona pellucida Kierszenbaum AL, Rivkin E, Tres LL (2011). Cytoskeletal track selection dur Kierszenbaum AL, Rivkin E, Tres LL (2007). Molecular biology of sperm head shapeing. Soc Reprod Fertil Suppl 65, 33–43. Kierszenbaum AL, Rivkin E, Tres LL (2011). Cytoskeletal track selection dur Kierszenbaum AL, Rivkin E, Tres LL (2004). The acrosome-acroplaxome-manchette complex and the shaping of the spermatic head. Arch Histol Cytot 67, 271–284. Kierszenbaum AL, Tres LL, Rivkin E, Kang-Decker N, Mantchev GT, Juneja SC, McNiven MA, van Deursen JM (2002). Testis-specific cytochrome c-null mice produce functional sperm. Reprod 71, 540–547. TE, Warren G (1995). Characterization of a new inner nuclear membrane protein, causes globozoosperma in mice by preventing the anchoring of the acrosome to the nucleus. Development 139, 2955–2965. Piomboni R, Focarelli R, Stendardi A, Ferramosca A, Zara V (2012). The role of mitochondria in energy production for human sperm motility. Int J Androl 35, 109–124. Rainey MA et al. (2010). The endocytic recycling regulator EHD1 is essential for spermatogenesis and male fertility in mice. BMC Dev Biol 10, 37. Randazzo PA, Hirsch DS (2004). Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodelling. Cell Signal 16, 401–413. Rino J, Sudhof TC (2002). Snares and Munc18 in synaptic vesicle fusion. Nat Rev Neurosci 3, 641–653. Roqueru-Rivera M, Abbott TL, Sivaguru M, Hess RA, Nakamura MT (2011). Deficiency in the omega-3 fatty acid pathway results in failure of acrosome biogenesis in mice. Biol Reprod 85, 721–732. Spang A, Shibuya R, Randazzo PA (2010). Arf GAPs: gatekeepers of vesicle generation. FEBS Lett 584, 2646–2651. Susi FR, Leblond CP, Clermont Y (1971). Changes in the Golgi apparatus during spermatogenesis in the rat. Am J Anat 130, 251–267. Suzuki-Toyota F, Ito C, Toyama Y, Maekawa M, Yoo R, Noda T, Toshimori K (2004). The coiled tail of the round-headed spermatozoon appears during epididymal passage in GPCD-deficient mice. Arch Histol Cytot 67, 361–371. Tanabe K, Kon S, Natsume W, Torii T, Watanabe T, Satake (2006). Arf GAPs: gatekeepers of vesicle trafficking: implications in cancer cell biology. Cancer Sci 97, 801–806. Tanabe K, Torii T, Natsume W, Braesch-Andersen S, Watanabe T, Satake M (2005). A novel GTPase-activating protein for ARF6 directly interacts with clathrin and regulates clathrin-dependent endocytosis. Mol Cell Biol 15, 1617–1628. Wilton LJ, Temple-Smith PD, de Kretser DM (1992). Quantitative ultrastructural analysis of sperm tails reveals flagellar defects associated with persistent asthenozoospermia. Hum Reprod 7, 510–514. Wong WF et al. (2010). Over-expression of Runx1 transcription factor impairs the development of thymocytes from the double-negative to double-positive stages. Immunology 130, 243–253. Xiao N, Kam C, Chen C, Jin W, Wang J, Lee KM, Jiang L, Xia J (2009). PICK1 deficiency causes male infertility in mice by disrupting acrosome forma- tion. J Clin Invest 119, 802–812. Xu X (2003). Impaired meiotic DNA-damage repair and lack of crossing-over during spermatogenesis in BRCA1 full-length isoform deficient mice. Development 130, 2001–2012. Yao R, Ito C, Natsune Y, Sugitani Y, Yamamaka H, Kuretake S, Yanagida K, Sato A, Toshimori K, Noda T (2002). Lack of acrosome formation in mice lacking a Golgi protein, GOPC. Proc Natl Acad Sci USA 99, 11211–1122. Zhang B, Koh YH, Beckstead RB, Budnik V, Gantiezky B, Bellen HJ (1998). Synaptic vesicle size and number are regulated by a clathrin adapter protein required for endocytosis. Neuron 21, 1465–1475.