Membrane-associated RING-CH 10 (MARCH10 Protein) Is a Microtubule-associated E3 Ubiquitin Ligase of the Spermatid Flagella*  

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Background: The mechanism underlying sperm flagellar development is unknown.  
Results: Mammalian MARCH10 is a microtubule-associated E3 ubiquitin ligase expressed in the tail of developing spermatids.  
Conclusion: MARCH10 is suggested to have a role in the organization and maintenance of the sperm flagella.  
Significance: This is additional evidence for the involvement of the ubiquitin-proteasome system in mammalian spermatogenesis.  

Spermiogenesis is a complex and dynamic process of the metamorphosis of spermatids into spermatozoa. There is a great deal that is still unknown regarding the regulatory mechanisms for the formation of the sperm flagellum. In this study, we determined that the membrane-associated RING-CH 10 (March10) gene is predominantly expressed in rat testes. We isolated two March10 isoforms encoding MARCH10a and MARCH10b, which are generated by alternative splicing. MARCH10a is a long RING finger protein, and MARCH10b is a short RING finger-less protein. Immunohistochemical staining revealed that the MARCH10 proteins are specifically expressed in elongating and elongated spermatids, and the expression is absent in epididymal spermatozoa. MARCH10 immunoreactivity was observed in the cytoplasmic lobes as well as the principal piece and annulus of the flagella. When overexpressed in COS7 cells, MARCH10a was localized along the microtubules, whereas MARCH10b was distributed throughout the cytoplasm. An in vitro microtubule cosedimentation assay showed that MARCH10a is directly associated with microtubules. An in vitro ubiquitination assay demonstrated that the RING finger domain of MARCH10a exhibits an E3 ubiquitin ligase activity along with the E2 ubiquitin-conjugating enzyme UBE2B. Moreover, MARCH10a undergoes proteasomal degradation by autoubiquitination in transfected COS7 cells, but this activity was abolished upon microtubule disassembly. These results suggest that MARCH10 is involved in spermiogenesis by regulating the formation and maintenance of the flagella in developing spermatids.

Mammalian spermatozoa are composed of two major parts, the head and the flagellum. Proper assembly of the flagellum is essential for sperm motility and fertilization. Morphologically, the flagellum is subdivided into the midpiece, the principal piece, and the end piece (1, 2). Common to the three parts is the central axoneme, which has a 9 + 2 arrangement of microtubules (9 doublets and 2 central singlets). The force for flagellar motility is driven by the active sliding of the doublet microtubules powered by dynein ATPase motors. In the midpiece, the axoneme is surrounded by nine longitudinal cytoskeletal structures, the outer dense fibers (ODF), on the outside of the doublet microtubules. The ODF are tightly wrapped, with helically arranged mitochondria (the mitochondrial sheath) that serve as the sites of the ATP production required for flagellar movement. In the principal piece, the ODF are extended, but two of them are replaced by the longitudinal columns of the fibrous sheath (FS) that are bridged by numerous circumferential ribs. The ODF and FS have been proposed to provide the necessary stiffness and elastic recoil for the flagellum, thereby modulating its beating pattern. The end piece is a very short terminal segment, in which the axoneme is surrounded only by the plasma membrane. Although a number of the components of the flagellum have been identified and characterized (3–5), the molecular mechanisms for the formation of the flagella remain to be established.

Ubiquitination is a post-translational modification in which the 76-amino acid polypeptide ubiquitin (Ub) is covalently attached to lysine residues in target proteins. Ubiquitination is catalyzed by the sequential actions of E1 Ub-activating, E2 Ub-conjugating, and E3 Ub ligase enzymes. The E3 enzymes are essential for specific substrate recognition. Ubiquitination is not only required for proteasomal degradation but is also involved in proteasome-independent functions such as protein localization and signaling (6). Recent investigations have suggested the involvement of the Ub-proteasome system in flagellar assembly and function as follows. 1) Sperm mitochondria

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB552845 and AB552846.  
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The abbreviations used are: ODF, outer dense fiber; FS, fibrous sheath; Ub, ubiquitin; EGFP, enhanced green fluorescent protein; HA-Ub, HA-tagged Ub; CHX, cycloheximide; SiM, structured illumination microscopy.
are highly ubiquitinated and thereby undergo degradation after fertilization, which enables maternal mitochondrial inheritance (7–9). 2) Spermatozoa in mice lacking the E2 enzyme Ube2b or the E3 Ub ligase Herc4 display morphological abnormalities and impaired flagellum motility (10–13). 3) PSMC3 (also known as TBP-1), a subunit of the 26 S proteasome, is present in the ODF of elongating spermatids and spermatozoa (14–16). Therefore, the identification and characterization of the flagellar Ub-proteasome system should provide novel insight into molecular mechanisms regulating the assembly and function of the mammalian sperm flagella.

The membrane-associated RING-CH (MARCH) family is a RING finger protein family of E3 Ub ligases, consisting of 11 members in mammals (17–19). Nine MARCH members (i.e. MARCH1–6, -8, -9, and -11) contain hydrophobic transmembrane spans and are localized to the plasma membrane and intracellular organelle membrane (20). Transmembrane MARCH proteins mediate the ubiquitination and subsequent down-regulation of cell-surface immune regulatory molecules, such as major histocompatibility complex class II and CD86 (17, 18, 21). Other proposed functions include endoplasmic reticulum-associated degradation (22), endosomal protein trafficking (23, 24), mitochondrial dynamics (25, 26), and spermatogenesis (19). MARCH7 (also known as Axotrophin) and MARCH10 are predicted to have no transmembrane spanning region. Indeed, MARCH7 has been shown to localize to the cytosol and nucleus in transfected cultured cells (27). Studies of March7-null mice have suggested that MARCH7 has important roles in T-cell proliferation and immune tolerance (28, 29). Therefore, the identification and characterization of MARCH10 proteins from the testis was performed as described previously (19). Polyclonal anti-MAR10N (808) and anti-MAR10M (809) antibodies were obtained by immunizing rabbits with GST-MAR10N and GST-MAR10M, respectively. Preparations of recombinant proteins and antibodies—Preparation of the recombinant proteins and the immunization protocol for the production of rabbit antisera were performed as described previously (19). Polyclonal anti-MAR10N (808) and anti-MAR10M (809) antibodies were obtained by immunizing rabbits with GST-MAR10N and GST-MAR10M, respectively.

**Northern Blot Analysis**—Northern blot analysis was carried out as described previously (30). The EcoRV-digested fragment of pBS-March10a was used as a probe, as shown in Fig. 1B. The cDNA fragments encompassing either exons 1–3, 4–5, 6–10, or exon 4 were amplified by PCR and then used as probes, as shown in Fig. 1C.

**Preparation of Recombinant Proteins and Antibodies**—Preparation of recombinant proteins and the immunization protocol for the production of rabbit antisera were performed as described previously (19). Polyclonal anti-MAR10N (808) and anti-MAR10M (809) antibodies were obtained by immunizing rabbits with GST-MAR10N and GST-MAR10M, respectively.

**In Vitro Ubiquitination Assay**—In vitro ubiquitination assays were performed with GST-RING or GST-RINGmut (1 μg) as described previously (19), with the exception that the reaction mixtures were incubated for 24 h.

**Cell Culture and Fluorescence Microscopy**—Maintenance of COS7 cells, transfection with plasmids, and immunofluorescence staining were performed as described previously (23).
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bound materials were eluted with 25 µl of 0.1 M glycine-HCl, pH 2.5, and 1% Nonidet P-40.

Cycloheximide (CHX) Chase Assay—COS7 cells transfected with either FLAG-MARCH10a or FLAG-RINGmut were treated for different times with 1 mg/ml CHX in the presence or absence of 5 µM MG132. Whole cell lysates (5 µg of proteins) were subjected to Western blotting with anti-FLAG antibody and with anti-β-tubulin. The band intensity was quantified using ImageJ software (rsbweb.nih.gov), and a graph was created using GraphPad Prism (GraphPad Software, San Diego). Student’s t test was performed to evaluate statistical differences.

Immunohistochemistry—Preparation and staining of cryosections (31) and smear samples (32) of rat testis and epididymis were performed according to the previously reported methods with the exception that rats were perfused with PBS containing 2% paraformaldehyde. Dilutions of anti-MARCH10 antisera and preimmune serum were 1:500. The testicular sections were observed under Axiostar 200 M inverted fluorescence microscope (Carl Zeiss, Thornwood, NY) or Leica TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany). For super resolution imaging, the immunostained smear samples were imaged with a DeltaVision Optical Microscope eXperimental (OMX; Applied Precision, Issaquah, WA). Three-dimensional reconstructions were performed from 23 z-stack images at intervals of 0.125 µm using softWoRx Explorer 2.0 software (Applied Precision).

Microtubule Cosedimentation Assay—MARCH10a was synthesized by in vitro transcription and translation using the TNT Quick-Coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer’s instructions. After the reaction, the lysate (20 µl) was added to 180 µl of PEM buffer (80 mM PIPES-KOH, pH 6.9, 1 mM EGTA, and 2 mM MgCl2) and then cleared of insoluble materials by centrifugation for 20 min at 50,000 rpm on a TLA100.3 rotor (Beckman Coulter, Fullerton, CA). Ninety microliters of the supernatant or PEM buffer containing 10 µg of bovine serum albumin (BSA) were supplemented with purified tubulin (Cytoskeleton, Denver, CO), GTP, Taxol (Sigma), and glycerol to final concentrations of 0.3 mg/ml, 1 mM, 30 µM, and 5%, respectively. The samples (100 µl) were layered on 900 µl of PEM buffer containing 0.1 mM GTP and 1 µM Taxol in a 1.5-ml microcentrifuge tube (Beckman Coulter), and were then incubated at 50,000 rpm on a TLA100.3 rotor for 30 min at 25 °C. The resulting supernatants (100 µl) and pellets (dissolved in 100 µl of Laemmli buffer) were subjected to Coomassie Brilliant Blue staining or Western blot analysis. The control experiments were performed in the absence of GTP and Taxol.

RESULTS

Expression of Two March10 Isoforms in the Rat Testis—The nucleotide sequences of mammalian March10 were found in the GenBankTM and Ensembl databases. Based on the sequence information, we designed a set of primers and obtained the entire open reading frame (ORF) of rat March10 by RT-PCR amplification from testis cDNA. The ORF encodes a protein of 790 amino acids with no characteristic structure other than the C-terminal RING finger domain (accession number AB552845; Fig. 1A). The overall structural features of March10 resemble those of MARCH7 (27), but sequence homology is only present in the RING finger domains (64% identity). Data base searches indicated that the March10 gene is limited to mammals, whereas the March7 gene is present in vertebrates ranging from fish to humans. These facts suggest that MARCH10 may have a distinct function from that of MARCH7. By utilizing the Ensemble exon definitions, there are at least two March10 isoforms in the rat (designated here as March10a and March10b). March10a is identical to the isolated March10 sequence that is comprised of 10 exons (Ensemble transcript ID ENSRNOT000009316; Fig. 1B). March10b is a shorter transcript that shares the first three exons but has a different exon between exons 3 and 4 (exon 4’; Ensemble transcript ID ENSRNOT000005136; Fig. 1B). This variant transcript encodes a 237-amino acid protein, in which the first 126 amino acid residues are identical to MARCH10a, but the following 111 residues are a nonrelated proline-rich (accession number, AB552846; Fig. 1A).

To determine the tissue expression pattern of rat March10 mRNA, Northern blot analysis of rat tissue total RNA was performed.
formed using the March10 ORF as a probe. Two mRNA species of 1.3 and 3.0 kb were detected predominantly in the testis and to a much lesser extent in the heart and lung (Fig. 1C). We expected that the upper and lower bands correspond to March10a and March10b, respectively. To verify the identity of the two bands, Northern blot analysis of rat testis was performed with four different March10 cDNA probes for exons 1–3, 4–5, 6–10, or 4'. As shown in Fig. 1D, the two bands were detected again with the common exon probe (exons 1–3; lane 1). The 3.0-kb band was detected with the March10a-specific probes (exons 4–5 and 6–10; lanes 2 and 3), whereas the 1.3-kb band was detected with the March10b-specific probe (exon 4'; lane 4). These results indicate that at least two March10 isoforms are generated by alternative splicing and are predominantly expressed in the rat testis.

**Protein Expression of MARCH10 in Testis**—To analyze MARCH10 protein expression in the testis, we prepared rabbit polyclonal antisera as follows: one against the N-terminal region common to both March10a and March10b (anti-MARCH10N) and the other against the middle portion of March10a (anti-MARCH10M). We were unable to produce antisera specific to March10b. To confirm their specificity, COS7 cells were transfected with either FLAG-tagged March10a or March10b and were then doubly stained with each of the anti-MARCH10 antisera and anti-FLAG antibodies. Anti-MARCH10N antisera detected both constructs, whereas anti-MARCH10M antisera recognized only FLAG-MARCH10a (supplemental Fig. S1). FLAG-MARCH10a staining showed a filamentous pattern within the cytoplasm in contrast to a diffuse cytoplasmic distribution of FLAG-MARCH10b, as described in detail below. To confirm the expression of March10a and March10b, the soluble fraction of rat testicular lysates was subjected to immunoprecipitation with anti-MARCH10N antisera or the preimmune serum. When the immunoprecipitates were analyzed by Western blotting with the anti-MARCH10N antisera, two immunoreactive bands were detected at 90 and 30 kDa, which were close to the estimated molecular masses of March10a and March10b, respectively (Fig. 1E, lane 1, arrowheads). Next, we determined the cell types expressing MARCH10 proteins by immunohistochemistry on rat testicular sections. As shown in Fig. 2, the anti-MARCH10N antisera reacted with the inner layers and luminal contents of a subset of seminiferous tubules where the spermatids were present. The signals were detected in the seminiferous tubules at stages I–III and XI–XIV. Negative control experiments with preimmune serum detected no such signal (supplemental Fig. S2).

**Localization of MARCH10 in Elongating Spermatids**—To further analyze the developmental expression profile in detail, we stained smear preparations of rat testis with anti-MARCH10N antisera. As shown in Fig. 3, A–D, the staining became detectable in the cytoplasmic lobe of spermatids at step 11 and was also steadily observed in the posterior portion of the tail. The staining was prominent at steps 13–15 and then gradually decreased. When the smear samples were stained with anti-MARCH10M antisera, signals were also detected in the cytoplasm and tail of elongating spermatids in steps 11–16, but the cytoplasmic staining was weaker than that with anti-MARCH10N antisera (Fig. 3, E and F). To analyze MARCH10 expression in mature spermatozoa, smear preparations of rat epididymis were stained with anti-MARCH10N antisera. No specific signal was observed in epididymal spermatozoa (Fig. 3G). Because the mammalian sperm tail consists of two different segments, the midpiece and the principal piece, the tail staining was most likely to be detected in the principal piece. To evaluate this, the smear samples were doubly stained with anti-MARCH10N antiserum and antibodies against either cytochrome c oxidase subunit 1 (COX-1; a midpiece marker), protein kinase A-anchor-
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FIGURE 4. Double immunofluorescence staining of MARCH10 in elongating spermatids. Rat elongating spermatids in smear preparations were stained with anti-MAR10N antiserum (green) followed by mouse monoclonal antibodies to AKAP4 (left panels, red), COX-1 (middle panels, red), or α- and β-tubulin (right panels, red). Nuclei were visualized using Hoechst 33342 (blue). Images were obtained by confocal microscopy. Bottom panels show high magnification images of the boundary region of the midpiece and the principal piece. Bars, 20 μm in the 3 top panels and 2 μm in bottom panel.

FIGURE 5. Super resolution imaging of MARCH10 staining in elongating spermatids. A–C, three-dimensional SIM images of the flagella of the rat elongating spermatids labeled with anti-MAR10N antiserum (green) and either anti-AKAP4 (red in A) or anti-COX-1 (red in B and C) antibody. Arrow indicates the ring-like staining corresponding to the annulus. Bars, 5 μm in A and B and 2 μm in C. D, schematic illustration of the principal piece of the flagellum. MARCH10 (green) is likely to be expressed in the inner parts of the longitudinal columns (LC) of the fibrous sheath (FS).

Microtubule Association of MARCH10a—As described above, exogenously expressed FLAG-MARCH10a was localized to cytoplasmic filamentous structures in COS7 cells, whereas FLAG-MARCH10b was distributed throughout the cytoplasm (supplemental Fig. S1). We found that the filamentous distribution of FLAG-MARCH10a was abolished by treatment with the microtubule-depolymerizing agent nocodazole (Fig. 6A). Moreover, when overexpressed in COS7 cells, an EGFP fusion protein of MARCH10a (EGFP-MARCH10a) was colocalized with the microtubules (Fig. 6B). Thus, the MARCH10a-associated structures are likely to be microtubules. The E3 activity of several of the MARCH proteins has been shown to contribute to their subcellular localization (17, 24, 27). To test if the E3 activity of MARCH10a has such an effect, a mutant FLAG-MARCH10a containing serine substitutions at the conserved catalytic cysteine residues (C641S and C644S; FLAG-RINGmut) was transfected into COS7 cells, and the localization was determined by immunofluorescence microscopy. As shown in Fig. 6C, FLAG-RINGmut exhibited a filamentous pattern similar to that of FLAG-MARCH10a suggesting that the E3 activity of MARCH10a is not essential for its subcellular localization. Next, to investigate the region responsible for the microtubule localization, we performed deletion analysis of MARCH10a. The localization of a series of MARCH10a fragments fused to EGFP was determined in COS7 cells by immunofluorescence microscopy (Fig. 7A). As shown in Fig. 7B, none of the deletion constructs tested exhibited microtubule localization; the N-terminal deletion mutants were preferably localized in the nucleus (top 4 panels) and the...
C-terminal deletion resulted in a cytosolic distribution pattern (bottom). These results suggest that the entire region of MARCH10a is required for its microtubule localization. To confirm the microtubule association of MARCH10a, we performed an in vitro microtubule cosedimentation assay. Purified tubulin and in vitro translated MARCH10a or BSA (used as a nonbinding control protein) were incubated at 37 °C in the presence or absence of GTP and Taxol, a microtubule-stabilizing reagent. The polymerized microtubules were then allowed to sediment through a sucrose cushion, after which the resulting supernatants and pellets were analyzed by Coomassie Brilliant Blue staining and Western blotting with anti-MAR10N antiserum. As shown in Fig. 8, MARCH10a was detected in the pellets in the presence of polymerized microtubules, whereas BSA was not found to be cosedimented. Taken together, these results suggest that MARCH10a directly associates with microtubules.

Microtubule-dependent E3 Ub Ligase Activity of MARCH10a—To assess the E3 Ub ligase activity of the RING finger domain of MARCH10a, we performed an in vitro ubiquitination assay with E1, E2, and Ub in the presence or absence of a recombinant GST fusion protein of the RING finger (GST-RING). We used recombinant UBE2B as an E2 enzyme, because UBE2B is known to be expressed in spermatids (36, 37). Western blotting of the reaction samples revealed that the GST-RING catalyzed dense, polyubiquitinated products (Fig. 9A, lane 2). This polyubiquitin formation was abolished by the C641S and C644S mutations (GST-RINGmut; Fig. 9A, lane 3). Various MARCH members have been shown to mediate self-ubiquitination in transfected cultured cells (25, 27, 38). To obtain evidence on the in vivo E3 activity of MARCH10a, FLAG-MARCH10a was transfected into COS7 cells along with HA-tagged Ub. After cells were treated with the proteasome inhibitor MG132 for 3 h, the whole cell lysates were immunoprecipitated with anti-FLAG antibody followed by Western blotting with anti-HA antibody. As shown in Fig. 9B, a smear signal was detected in the anti-FLAG immunoprecipitates with the anti-HA antibody (lane 2), indicating that MARCH10a undergoes self-ubiquitination. Interestingly, when the cells were treated with nocodazole, this self-ubiquitination activity was strongly repressed (Fig. 9B, lane 3). These results suggest that the E3 activity of MARCH10a is dependent on intact microtubules. To determine whether the self-ubiquitination has the capacity to catalyze protein degradation, we compared the stability of FLAG-MARCH10a with FLAG-RINGmut using the protein synthesis inhibitor cycloheximide (CHX). COS7 cells transiently transfected with FLAG-MARCH10a or FLAG-RINGmut were treated with CHX, and their expression levels were analyzed by Western blotting with the anti-FLAG antibody. As shown in Fig. 9C, ~50% of the FLAG-MARCH10a proteins were degraded within 12 h (lanes 1–3), whereas FLAG-RINGmut...
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FIGURE 8. Microtubule association of MARCH10a in vitro. Porcine brain tubulin proteins were mixed with either in vitro translated MARCH10a or BSA in PEM buffer. The mixtures were incubated at 37 °C for 30 min in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of GTP and Taxol, after which polymerized microtubules were then sedimented through a 1 m sucrose cushion by ultracentrifugation. The resulting supernatants (100 μl) were recovered, and the pellets were dissolved with 100 μl of sample buffer. The supernatant (20 μl; lanes 1 and 3) and pellet samples (20 μl; P, lanes 2 and 4) were separated by SDS-PAGE followed by Western blotting with anti-MAR10N antiserum (top) and Coomassie Brilliant Blue staining to visualize BSA and tubulin (bottom).

was much more stable (lanes 7–9). The degradation of FLAG-MARCH10a was blocked by MG132 (Fig. 9C, lanes 4 – 6). The protein quantification data is shown in Fig. 9D. These results indicate that self-ubiquitination of MARCH10a accelerates its proteasomal degradation.

DISCUSSION

In this study, we identified two isoforms of the March10 gene products that are predominantly expressed in the testis. March10a encodes a long RING finger protein having E3 Ub ligase activity, and March10b encodes a short proline-rich protein lacking the RING finger domain. The following observations suggest that MARCH10a is a microtubule-associated protein. First, when overexpressed in COS7 cells, MARCH10a was localized along the microtubules, and this pattern of distribution was dispersed by microtubule depolymerization. Second, MARCH10a formed a complex with microtubules in vitro. Deletion analysis failed to identify the region responsible for the microtubule binding but suggested that the tertiary structure of MARCH10a would likely be important. In this regard, it seems reasonable that MARCH10b exhibited diffuse cytoplasmic distribution in COS7 cells. We demonstrated that the E3 Ub ligase activity of MARCH10a is not essential for its subcellular localization, but the microtubule association does appear to be important to the E3 activity. Therefore, it is plausible that MARCH10a is a microtubule-associated E3 Ub ligase that is active on microtubule structures. The cytoplasmic localization and lack of the E3 activity of MARCH10b suggest that it has a function that is distinct from MARCH10a. Proline-rich sequences, which are found in the C-terminal region of MARCH10b, have been shown to mediate protein-protein interactions with, for example, the Src homology 3, WW, and EVH1 domains (39). MARCH10b may thus act as an adaptor or scaffold protein.

Immunohistochemical studies demonstrated that MARCH10 proteins are abundantly expressed in elongating and elongated spermatids and disappear in epididymal spermatozoa, suggesting that MARCH10 is required for spermatid maturation. Immunostaining of the smear samples of rat testis revealed that the signals for MARCH10 were detected in the cytoplasmic lobes and the principal piece of the flagella. In this experiment, two polyclonal antisera, which stained the same regions of the spermatids with different degrees of immunoreactivity, were used as follows: anti-MAR10N antiserum stained the cytoplasm and the principal piece quite evenly, whereas anti-MAR10M antiserum stained the principal piece more heavily. Therefore, MARCH10a and MARCH10b are preferentially localized in the tail and the cytoplasmic lobe, respectively. MARCH10 expression was initially observed in the cytoplasm at step 10, followed by the tail region. The delayed localization to the flagella suggests that the MARCH10 proteins are synthesized in the cytoplasm and then transported to the developing flagella via the intraflagellar transport system (40). Microscopic studies provided detailed information on the morphological development of the spermatid flagella, which takes place during the early stages of spermiogenesis (i.e. steps 1–3) and continues...
until step 19 (1). The longitudinal columns of the FS are deposited along the axoneme from the distal end to the proximal end of the principal piece at steps 2–10, and the ribs are assembled at steps 11–17 (41, 42). The ODF first appear in the most proximal portion of the axoneme at step 9 and continue to extend to the end of the principal piece until step 19 (42, 43). MARCH10 proteins are unlikely to be essential structural components of the flagellum, because their expression is considerably reduced in the late stages of spermiogenesis. Rather, they may help regulate the organization of the ODF and FS. In this context, it is interesting that MARCH10a exerted the E3 activity with the E2 enzyme UBE2B (also known as HR6B). It has been reported that mouse Ube2b mRNA is expressed during meiosis and spermiogenesis, and its disruption causes structural abnormalities in both the sperm head and flagella, in which the longitudinal columns are assembled in an incorrect manner (10–12). Our data thus suggest that MARCH10a might be an as-yet-undefined E3 partner of the spermatid UBE2B in the course of FS formation.

Given the microtubule association of MARCH10a, its E3 substrate protein(s) may be present in proximity to the axoneme and/or the inner sides of the ODF and FS. In support of this notion, our three-dimensional SIM imaging suggests that MARCH10 seems to be present in the inner part of the longitudinal columns of the FS (Fig. 5D). E3 Ub ligases have been shown to bind to microtubules and to regulate the function and maintenance of microtubules in somatic cells. For example, MID1, which is associated with Opitz syndrome, controls the phosphorylation of microtubule-associated proteins by targeting the catalytic subunit of protein phosphatase 2A (PPP2CA) for ubiquitination and subsequent degradation (44). PARK2 (also known as Parkin), which is mutated in Parkinson disease, participates in the quality control of microtubules by promoting tubulin ubiquitination (45, 46). MARCH10a may participate in this quality control activity by targeting damaged and defective flagellar proteins for Ub-mediated degradation, thereby ensuring proper flagellar assembly. Recent work by Huang et al. (47) has reported a role for ubiquitination in the resorption of the flagella of Chlamydomonas reinhardtii. Before cell division starts, ubiquitination occurs on flagellar proteins, including α-tubulin, a subunit of the outer dynein arm, and also on signaling molecules, which likely gives the cue for disassembly of the flagella without any need of proteasomal degradation. This ubiquitination has been hypothesized to facilitate the retrograde transport of disassembled flagellar proteins to the cell body. It is important to determine whether this activity is conserved in other species, including mammals. Another role of flagellar ubiquitination is known to prevent the paternal inheritance of mitochondrial DNA; sperm mitochondria are highly ubiquitinated, which takes place during the late stages of spermiogenesis, and thus undergo degradation after fertilization (7–9). The ODF and FS are also destroyed in fertilized eggs (48), but their ubiquitination has not been observed in spermatids or spermatozoa. Nevertheless, we cannot exclude the possibility that MARCH10a and/or other E3 Ub ligases might tag ODF/FS proteins, even if to a much lesser extent than mitochondria, for degradation after fertilization. Self-ubiquitination of MARCH10a promotes its turnover by proteasomal degradation. Thus, the elimination of MARCH10 expression before sperm maturation may be regulated by the Ub-proteasome system. If so, it is possible that the MARCH10a activity is suppressed during spermiogenesis in a manner dependent on microtubule binding.

Three-dimensional SIM imaging revealed the localization of MARCH10 in the annulus, an electron-dense structure surrounding the axoneme between the midpiece and the principal piece. The annulus is assembled from septins, which are polymerizing GTP-binding proteins, and serves as a physical barrier to prevent the diffusion of the components of the midpiece and principal piece (33, 49–53). This structure is essential for sperm flagellar morphology and motility (33, 49). Although less is known about the involvement of the Ub-proteasome system, MARCH10 may contribute to the assembly and integrity of the annulus.

These results provide supporting evidence for the importance of the Ub-proteasomal system in spermiogenesis. To clarify the exact mechanisms underlying the formation of the flagella, it will be necessary to determine the detailed subcellular localization and substrate protein(s) of MARCH10.

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