A molecular ruler determines the repeat length in eukaryotic cilia and flagella

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Existence of cellular structures with specific size raises a fundamental question in biology: How do cells measure length? One conceptual answer to this question is by a molecular ruler, but examples of such rulers in eukaryotes are lacking. In this work, we identified a molecular ruler in eukaryotic cilia and flagella. Using cryo-electron tomography, we found that FAP59 and FAP172 form a 96-nanometer (nm)–long complex in *Chlamydomonas* flagella and that the absence of the complex disrupted 96-nm repeats of axonemes. Furthermore, lengthening of the FAP59/172 complex by domain duplication resulted in extension of the repeats up to 128 nm, as well as duplication of specific axonemal components. Thus, the FAP59/172 complex is the molecular ruler that determines the 96-nm repeat length and arrangements of components in cilia and flagella.

Cilia and flagella are motile organelles that play crucial roles in the generation of fluid flow by beating motion and in the development of mammals (1, 2). Their doublet structure has 96-nm repeats, which are very accurately specified. One explanation for this precise length control is that a molecular ruler exists (3). In bacteria and bacteriophages, ruler molecules have been shown to determine the length of the injectisome needles and the tail structures (4, 5). In eukaryotes, however, titins and nebulins have been proposed to be the rulers for muscle (6, 7), but this has been controversial (5–10).

To investigate the mechanism of ciliary and flagellar assembly, we focused on two *Chlamydomonas* proteins, FAP59 and FAP172. A lack of these proteins results in axonemal disorganization in various mammals and zebrafish (11–14). We first isolated a FAP172-missing mutant (strain 5-8) by screening *Chlamydomonas* motility mutants that lack FAP59 and FAP172 (table S1 and fig. S1, A and B; also see supplementary materials and methods). The mutant produced short and immotile flagella. Another four mutants that exhibit similar phenotypes to strain 5-8 were identified, including FAP172-deficient (pf8) or FAP59-deficient (pf8, hA24, and gam2) mutants (Fig. 1A, table S1, and fig. S1, B and C). Hereafter, we labeled pf8 as fap59 and pf7 as fap172. Transformation of hemagglutinin (HA)–tagged genes to fap59 and fap172 mutants recovered wild-type (WT) motility and flagella length, confirming the direct relation between mutations in the two genes and the phenotypes (Fig. 1B, table S1, and fig. S1D). In fap59 and fap172 flagella, inner dynein arm (IDA) and nexin-dynein regulatory complex (N-DRC) components were absent or severely reduced (Fig. 1C). On the other hand, FAP59 and FAP172 were retained by mutant strains deficient in major axonemal components (Fig. 1D and table S2). Biochemical characterization revealed that FAP59 and FAP172 are mainly localized in flagella and released by treatment with 0.2 to ~0.4 M NaI and that FAP59 protein is phosphorylated in flagella (fig. S1, E to G) (14, 15).

Next we examined whether FAP59 and FAP172 form a complex because the flagellar localization of each protein depends on the other (Fig. 1C) (16).
We labeled the two proteins with biotin carboxyl carrier protein (BCCP) tags and performed pull-down assays using streptavidin beads (Fig. 1E). BCCP-tagged FAP59 pulled down FAP172 and vice versa, showing that FAP59 and FAP172 form a complex in flagella.

To examine the structural defects caused by a loss of the FAP59/172 complex, we used cryo-electron tomography (cryo-ET) to observe the mutant axonemes. In WT axonemes, the doublet microtubule (DMT) components are arranged with 96-nm periodicity, as represented by radial spokes (RSs) (Fig. 2, A and B) (16, 17). In fap59 and fap172, however, RSs were attached to DMTs with an irregular periodicity of ~32 nm instead of 96 nm (Fig. 2, C and D, and fig. S2, A and B). In agreement with our immunoblot analyses (Fig. 1C) and previous reports (12, 19), IDAs and the N-DRC were missing in the mutant axonemes (fig. S2, C and D, and fig. S2, A and B). Thus, the FAP59/172 complex is essential for the establishment of 96-nm repeats in DMTs. The 24-nm repeats of outer dynein arms (ODAs) were unaltered in these mutants (fig. S3, C to E), suggesting that the arrangement of ODAs does not depend on the FAP59/172 complex. Therefore, we hypothesized that the FAP59/172 complex serves as a molecular ruler for the 96-nm repeats of DMTs.

To test an alternative hypothesis that tektin is a 96-nm molecular ruler (18, 19), we examined a N-DRC mutant, sup-pf-5 (20, 21). Tektin is missing in sup-pf-5 flagella (Fig. 2F), but its axonemes retain the 96-nm periodicity of RSs (Fig. 2E). Thus, tektin does not define the 96-nm repeats of DMTs.

To examine the possible role of the FAP59/172 complex as a molecular ruler, we inserted BCCP tags into the two proteins at different sites and determined their three-dimensional (3D) positions on DMTs using cryo-ET and an enhanced streptavidin-labeling method (Fig. 3 and fig. S4) (22, 23). The labels on FAP59 and FAP172 were similarly positioned along the protofilament(s), and each label appeared once per 96-nm repeat (Fig. 3, B and C). The labels in the middle segment were located approximately halfway between the N and C termini. Thus, both FAP59 and FAP172 form 96-nm-long structures and lie along the long axes of DMTs, consistent with the idea that the FAP59/172 complex is a 96-nm molecular ruler.

To test whether the FAP59/172 complex defines the 96-nm DMT repeats, we expressed longer versions of the complex in fap59 fap172 double mutants and used cryo-ET to observe their axonomeral ultrastructures (Fig. 4). The FAP59/172 complex was lengthened by duplicating the coiled-coil domains at the N terminal, at the middle, or near the C-terminal part. The short flagella phenotype of the double mutant was rescued by expressing the elongated proteins (fig. S5, A to E). Cryo-ET of the axonemes of the rescued strains—named FAP59/172-EL1, -EL2, and -EL3—revealed that the repeat length was extended to ~128 nm (FAP59/172-EL1 and -EL3) or ~120 nm (FAP59/172-EL2) (Fig. 4, C to H). Extension of the repeats by 32 nm in FAP59/172-EL1 and -EL3, and by 24 nm in FAP59/172-EL2, is approximately proportional to the number of amino acids duplicated in each mutant (~1.3 Å per residue).

Elongation of the repeats was accompanied by duplication of specific structures in the flagella. In FAP59/172-EL1, the IC2 (Fig. 4D, deep blue), IDA a (blue), the intermediate chain–light chain (IC-LC) complex (light blue), and RS1 (RS1’2) were duplicated. In FAP59/172-EL2, the gap between IDAs f and d was widened (Fig. 4F, square brackets), and weak densities of additional IDA-like structures (purple) were observed. In FAP59/172-EL3, the single-headed IDAs c and e (Fig. 4H, green), N-DRC (orange), and RS2 (RS2’) were duplicated. These results strongly support the idea that each domain of the FAP59/FAP172 complex recruits specific axonomeral components to the IDTs.

Taken together, our results indicate that the FAP59/FAP172 complex uses very intricate folding mechanisms for the correct alignment of
Fig. 2. The 96-nm periodicity of radial spokes was lost in *fap59* and *fap172* axonemes. (A) The 96-nm repeating units of DMTs in a *Chlamydomonas* WT axoneme. Each 96-nm repeat has two RSs (RS1 and RS2), seven IDAs (a to e, fa, fb, g, and an IC-LC complex of f), four ODAs, and one N-DRC. (B to E) Slices of tomograms showing positions of the RSs (white arrowheads). (B) The WT axoneme shows the regular 96-nm periodicity of the RSs. Distal ends are to the left. CP, central pair microtubules. (C and D) Both *fap59* and *fap172* axonemes show irregular alignment of the RSs with ~32-nm gaps. (E and F) *sup-pf-5* axonemes show a normal 96-nm repeat, in spite of tektin protein deficiency. (F) Immunoblot analysis of N-DRC mutant axonemes. An absence of tektin proteins in the *pf3* and *ida6* mutants were observed. WB, Western blot.

Fig. 3. Both FAP59 and FAP172 take on a 96-nm-long extended conformation. (A) Coiled-coil structure prediction of FAP59 and FAP172 using the CoilScan program (24). The predicted coiled-coil domains were roughly divided into three parts. (B and C) FAP59 and FAP172 were structurally tagged, and their positions were visualized by comparing the averaged subtomograms of the WT DMT (gray) with those of the labeled DMTs. The positions of tags (indicated by stars) are shown above each tomogram. Views on the right were rotated 90° around the vertical axis. (B) BCCP tags were added to the N terminus (red, after Met22) and the C terminus (yellow, before the stop codon) or inserted into the middle (orange, after Asp420) of FAP59. (C) BCCP tags were added to the N terminus (green, before Met1) and the C terminus (blue, before the stop codon) or inserted into the middle (light blue, after Leu381) of FAP172. Distal ends are to the left. The orientation of the proteins relative to the DMTs was determined in combination with the next experiment (Fig. 4).
RSs, IDAs, and N-DRCs (fig. S6). In the absence of the FAP59/172 complex, an excessive number of RSs bind along the specific protofilament(s) of the A-tubule, indicating that binding of the RSs to the A-tubule does not depend on the FAP59/172 complex. Therefore, the FAP59/172 complex can be seen as a kind of negative regulator for RSs, as the complex masks most of the RS-binding regions. At the same time, the complex leaves “holes” that allow for binding of RSs in appropriate locations. In contrast, the absence of the FAP59/172 complex causes loss of IDAs and the N-DRC, suggesting that the complex works as a positive regulator and provides anchoring sites for IDAs and N-DRCs.

REFERENCES AND NOTES


ACKNOWLEDGMENTS

We thank M. Hirota (University of Tokyo) and T. Yagi (Prefectural University of Hiroshima) for technical assistance and valuable advice. This work was supported by CREST, Japan Science and Technology Agency (to M.K.), the Takeda Science Foundation (to M.K. and T.O.), the Kazato Research Foundation (to T.O.), and Japan Society for the Promotion of Science KAKENHI grant 20770129 (to H.Y.). We declare no competing financial interests. The electron microscopy maps of ODAs and RSs are available in the EM Data Bank (www.emdbank.org) under accession numbers EMD-6328 to EMD-6371.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Supplementary Text

Figs. S1 to S6

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References (25–59)

20 August 2014; accepted 14 October 2014

10.1126/science.1260214
A molecular ruler determines the repeat length in eukaryotic cilia and flagella
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Science 346, 857 (2014);
DOI: 10.1126/science.1260214

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