FAP20 is an inner junction protein of doublet microtubules essential for both the planar asymmetrical waveform and stability of flagella in *Chlamydomonas*

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**ABSTRACT** The axoneme—the conserved core of eukaryotic cilia and flagella—contains highly specialized doublet microtubules (DMTs). A long-standing question is what protein(s) compose the junctions between two tubules in DMT. Here we identify a highly conserved flagellar-associated protein (FAP), FAP20, as an inner junction (IJ) component. The flagella of *Chlamydomonas* FAP20 mutants have normal length but beat with an abnormal symmetrical three-dimensional pattern. In addition, the mutant axonemes are liable to disintegrate during beating, implying that interdoublet connections may be weakened. Conventional electron microscopy shows that the mutant axonemes lack the IJ, and cryo–electron tomography combined with a structural labeling method reveals that the labeled FAP20 localizes at the IJ. The mutant axonemes also lack doublet-specific beak structures, which are localized in the proximal portion of the axoneme and may be involved in planar asymmetric flagellar bending. FAP20 itself, however, may not be a beak component, because uniform localization of FAP20 along the entire length of all nine DMTs is inconsistent with the beak’s localization. FAP20 is the first confirmed component of the IJ. Our data also suggest that the IJ is important for both stabilizing the axoneme and scaffolding intra–B-tubular substructures required for a planar asymmetrical waveform.

**INTRODUCTION**

Cilia and flagella are conserved organelles projecting from the surface of nearly all eukaryotic cells and have been adapted for multiple uses, such as bulk fluid movement, cellular motility, and sensing of extracellular signals (Ishikawa and Marshall, 2011). These organelles are important for human health since ciliary defects have been implicated in a broad spectrum of human diseases, such as primary ciliary dyskinesia, nephronophthisis, retinal degeneration, situs inversus, hydrocephalus, polydactyly, and obesity (Hildebrandt et al., 2011).

Highly specialized doublet microtubules (DMTs), templated by the basal bodies, are cylindrically arranged to form the essential and conserved framework of cilia and flagella. Many structures, including outer and inner arm dyneins, radial spokes, and the dynein regulatory complex (DRC), are assembled onto DMTs in a highly specific manner to produce the integrated axoneme (Dutcher, 1995). A unique, asymmetrical morphology of DMTs should be important for both the site-specific attachment of these structures and mechanical properties of the axoneme. The A-tubules of DMTs are composed of 13 tubulin protofilaments (PFs), similar to cytoplasmic microtubules (MTs), whereas the B-tubules are incomplete, C-shaped tubules.
composed of 10 PFs and one smaller subunit (Nicastro et al., 2011). A long-standing question concerns how the junctions between the two tubules in the DMT are formed (Linck and Stephens, 2007). The two junctions between the A- and B-tubules are structurally different: the outer junction is formed by close, unusual interactions among three tubulin PFs, whereas the inner junction (IJ) is formed by the smaller subunit, composed of nontubulin protein(s) that bridge tubulin PFs of the A- and B-tubules (Nicastro et al., 2011). Formation of B-tubule-like “hooks” onto preexisting axonemal and mitotic spindle MTs can be induced by adding purified brain tubulins (Euteneuer and McIntosh, 1980). The hooks initiate by forming outer-like junctions but barely close by forming inner-like junctions, supporting the notion that nontubulin protein is indispensable for the IJ.

Biochemical and proteomic studies of the axonemes have identified potential candidates for the nontubulin protein(s) in the DMT. The PF ribbon, which is resistant to Sarkosyl extraction, contains tektins (Linck et al., 1982; Yanagisawa and Kamiya, 2004), Rib72/EFhc1 (Ikeda et al., 2003), and Rib43a (Norrander et al., 2000). Parkin co-regulated gene product (PACRG) was identified as an interacting protein of Rib72 (Ikeda et al., 2007). Because the PF ribbon is believed to derive from the A-tubule PFs near the IJ (Witman et al., 1972; Linck et al., 1982), it is possible that these proteins are involved in formation of the IJ. However, localization and functions of these proteins in the DMT are unclear due to lack of detailed structural analysis. In addition to these known proteins, we previously found several unidentified proteins tightly associated with the A-tubule after detergent extractions (Yanagisawa and Kamiya, 2004), which solubilize the B-tubule, leaving junctional PFs (Witman et al., 1972). The Chlamydomonas flagella proteome contains a large number of uncharacterized proteins in the salt-extracted axonemal fraction, which contained DMTs. Therefore these proteins were potential candidates for the junctional proteins of the DMT.

In this study, we focus on a highly conserved flagellar-associated protein (FAP), FAP20, found in the Chlamydomonas flagellar proteome and analyze its function using Chlamydomonas mutants that completely lack FAP20. The mutants have motility defects with an abnormal, symmetrical waveform and lack doublet-specific structures. In addition, the axonemes of the mutants exhibit reduced stability in the connection between DMTs. To explain these functional phenotypes, we structurally determined that FAP20 is a component of the IJ using both conventional electron microscopy and cryo-electron tomography. We discuss the function of the IJ in relation to the phenotypes of FAP20 mutants.

**RESULTS**

**FAP20 is a candidate for a junctional component of DMT**

To identify novel candidate proteins for the junctional proteins of the DMT, we used the Chlamydomonas flagellar proteome database, in which biochemically fractionated flagella were analyzed (Pazour et al., 2005). The candidates were selected according to three criteria: they are 1) mainly contained in the KCI-insoluble fraction; 2) as abundant as components of other known axonemal structures, for example, dyneins or radial spokes, but are not components of these structures; and 3) highly conserved among organisms with cilia and flagella. One of the candidates, FAP20, is predominantly detected in the KCl-insoluble fraction, and the number of detected peptides is comparable to those of radial spoke or dynein components. In addition, FAP20 has two prominent features: it is very basic, with a Pi of 9.6, and is extremely conserved across species; Chlamydomonas and human homologues share 89% identical and 94% similar amino acid sequences. FAP20 is also found in the Chlamydomonas basal body proteome (Keller et al., 2005) and annotated as BUG22p (basal-body proteins with upregulated genes 22). Studies in other organisms show that FAP20 homologues localize to the cilia or flagella (Laligne et al., 2010; Hodges et al., 2011; Ishikawa et al., 2012), and FAP20-depleted paramecia have motility defects with an altered waveform (Laligne et al., 2010). Therefore we focus on FAP20 here.

**FAP20 is mutated in motility mutants, dmj1**

We assumed that the FAP20 mutant cells can produce flagella but cannot swim forward because of altered rigidity of their flagella. We therefore first isolated the mutants that sink to the bottom of liquid cultures and then used a low-magnification microscope to identify the ones just trembling or rotating instead of swimming fast like wild-type cells. Four alleles of FAP20 mutants were isolated from different libraries of motility mutants. These mutants were named doublet microtubule junction 1 or dmj1 because FAP20 is a component of the inner junction of DMTs (data shown later). The first allele, dmj1-1, was created by insertional mutagenesis, but the plasmid insertion was not linked to the motility phenotype. Thus we mapped the mutation by amplified fragment length polymorphism (AFLP) analysis and sequencing of FAP genes (Pazour et al., 2005) contained in the mapped region. We found the dmj1-1 has a mutation in the gene encoding FAP20 (Figure 1A and Supplemental Figure S1, A and B). In addition to dmj1-1, two alleles, dmj1-2 and dmj1-3, were identified from similarity of the motility phenotype (Figure 1A).
and Supplemental Figure S1A). We also found that the strain RL-11 (Reverse Locomotion 11; Nakamura, 1981), which was reported as a backward-swimming mutant, is allelic to the dmj1 mutation (Figure 1A and Supplemental Figure S1A).

To characterize the localization and functions of the FAP20 in *Chlamydomonas*, we raised two rabbit polyclonal antibodies using the full-length recombinant FAP20 protein as antigens. Antibody #1 recognized a single 21-kDa band corresponding to FAP20 both in the whole-cell and axonemal samples, whereas antibody #2 recognized an additional 37-kDa nonspecific band only in the whole-cell sample (Supplemental Figure S2A). We thus mainly use antibody #1 in the following analyses. Because the hygromycin B resistance gene is inserted to the near 3’ end of the FAP20-coding sequence, the strain dmj1-3 is expected to produce a truncated FAP20 protein lacking the C-terminal 20 amino acids (Supplemental Figure S2B). To exclude the possibility that the weak signal for dmj1-3 on Western blots is due to the failure of antibody reacting to the C-terminal–truncated mutant protein, we used antibody #2 for analyses including the dmj1-3 strain.

We first biochemically examined the localization of FAP20. Western blot analysis clearly showed that most of FAP20 is present in the flagella, with a trace amount in cell bodies (Figure 1B). Another Western blot analysis of fractionated flagella showed that the FAP20 is associated with the axoneme (Figure 1C), consistent with the result of the *Chlamydomonas* flagellar proteome (Pazour et al., 2005). The axonomes of the three alleles, dmj1-1, dmj1-2, and RL-11, completely lacked the FAP20 protein, whereas dmj1-3 contained a reduced amount of the truncated FAP20 (Figure 1D and Supplemental Figure S2C).

We transformed dmj1-1 cells with FAP20 genes tagged with green fluorescent protein (GFP), biotin carboxyl carrier protein (BCCP), and triple hemagglutinin (3xHA) to produce the three rescued strains dmj1-1::FAP20-GFP, dmj1-1::FAP20-BCCP, and dmj1-1::FAP20-3xHA, respectively. The transgene products were incorporated into the axonomes at comparable amounts to that of wild type (Figure 1D). In the following analyses, we label dmj1-1 as fap20null, dmj1-3 as fap20ΔC, dmj1-1::FAP20-GFP as FAP20-GFP, dmj1-1::FAP20-BCCP as FAP20-BCCP, and dmj1-1::FAP20-3xHA as FAP20-3xHA (Table 1).

### FAP20 is not essential for flagellar assembly

Although FAP20 has been reported to be associated with both the flagella and the basal bodies, the FAP20 mutants did not exhibit any basal-body-related phenotypes, such as abnormality in number and positioning of flagella (unpublished data). The FAP20 mutants produced nearly wild-type length of flagella on average (Figure 2A). The RL-11 strain produced slightly longer flagella than wild type, which is consistent with the original report (Nakamura, 1981). The FAP20 mutants also regenerated their flagella normally (unpublished data). Flagella of the FAP20 mutant alleles had a wider distribution of lengths (p < 0.005 for fap20null and RL-11, p < 0.05 for fap20ΔC by F test of equality of variances) than did those of wild type. These results suggest that FAP20 is not essential for flagellar assembly in *Chlamydomonas*.

| Strain     | Abbreviation   | Phenotype       | FAP20 mutation                        |
|------------|----------------|-----------------|---------------------------------------|
| dmj1-1     | fap20null      | Trembling       | Null (TOC1 insertion)                 |
| dmj1-2     | –              | Trembling       | Null (UV, frame shift)                |
| dmj1-3     | fap20ΔC        | Mixed           | ΔC 20 amino acids (Hyg<sup>+</sup> insertion), reduced protein level |
| RL-11      | –              | Trembling       | Null (UV, splice site mutation)       |
| dmj1-1::FAP20-GFP | FAP20-GFP | Wild type       | Rescued                               |
| dmj1-1::FAP20-BCCP | FAP20-BCCP | Wild type       | Rescued                               |
| dmj1-1::FAP20-3xHA | FAP20-3xHA | Wild type       | Rescued                               |
| sup-pf1-1 dmj1-1::FAP20-GFP | sup-pf1 FAP20-GFP | Slow swimming | Rescued                               |

**Table 1:** *dmj1* alleles and rescued strains.
connections between DMTs are weakened. The base of the axonemes tended to remain connected after disintegration (Figure 2D), consistent with a previous report (Bower et al., 2013).

The axoneme of the fap20ΔC strain was unstable and liable to split or fray during beating. Demembranated axonemes of wild type and fap20null were reactivated with 0.1 mM ATP in presence of protease inhibitors. Bar, 10 μm. (E) Quantitative analysis of the disintegration of axonemes. Values in parentheses represent number of axonemes used for analyses.

FIGURE 2: FAP20 is essential for both asymmetrical waveform and stability of flagella. (A) FAP20 mutants produce wild-type length of the flagella (n = 33). (B) Introduction of the tagged FAP20 constructs rescued the motility defect of the FAP20 mutant (n = 30). Error bars represent SD. (C) Waveforms from representative cells of wild type and each mutant allele as traced from recorded videos. Time between frames 1 and 10 is 0.02, 0.02, 0.033, 0.02, and 0.01 s, respectively, for WT (asymmetrical), fap20ΔC (intermediate type), fap20null, mbo2, and WT (symmetrical). (D) The axoneme of fap20null strain was unstable and liable to split or fray during beating. Demembranated axonemes of wild type and fap20null were reactivated with 0.1 mM ATP in presence of protease inhibitors. Bar, 10 μm. (E) Quantitative analysis of the disintegration of axonemes. Values in parentheses represent number of axonemes used for analyses.
structures (Table 2), consistent with its mixed motility phenotype (Supplemental Video S3). The structures were restored in the axonemes of the rescued strains, FAP20-GFP and FAP20-BCCP (Figure 3 and Table 2), demonstrating the necessity of FAP20 for their formation. Further analysis of thin-section samples is given later.

**FAP20 localizes along the entire length of all DMTs**

Given that the FAP20 mutant axoneme lacked the beak structures, we examined the localization of FAP20 by immunofluorescence (Figure 4A) to compare it with proximal, DMT-specific localization of beak structures (Hoops and Witman, 1983; Nicastro et al., 2011; Pigino et al., 2012). FAP20 antibody clearly labeled the entire length of wild-type flagella in nucleoflagellar apparatuses. Such labeling was absent in the fap20null flagella. This result was verified by the rescued strains, FAP20-GFP (Figure 4B), FAP20-BCCP (Supplemental Figure S4B), and FAP20-3xHA (unpublished data). To investigate whether all nine DMTs contain FAP20 protein, we frayed the sup-pf1 FAP20-GFP axonemes by protease and ATP treatment. To ensure that the axonemes frayed well, we used a double mutant with a mutant of outer arm dynein, sup-pf1 (Huang et al., 1982; Porter et al., 1994; Mitchell et al., 2004), since its axonemes exhibit enhanced sliding efficiency (our unpublished observation). FAP20-GFP clearly localized to all nine DMTs of the frayed axoneme (Figure 4C). These observations suggest that FAP20 is not a component of the beak structure itself.

**FAP20 is tightly associated with DMTs**

To examine the relationship between FAP20 and known axonemal structures, we analyzed the axonemes of several representative axonemal mutants with FAP20 antibody (Figure 5A and Supplemental Table S1). FAP20 is present at wild-type levels in mutants affecting the assembly of the radial spokes (pf14), the central pairs (pf18), the beak structures (mbo1, mbo2, mbo3), the outer dynein (oda1), the inner dyneins (ida2, ida5), and the DRC (ida6, pf2, pf3, sup-pf3, sup-pf4, sup-pf5). Therefore FAP20 must assemble in the axoneme independently of these known structures.

We next checked the protein levels of known axonemal structures in the FAP20 mutants. The proteins of the radial spokes (RSP1), the outer dynein (IC2), the inner dyneins (IC140, p28 and centrin), the nexin–DRC (N-DRC; DRC2, DRC4, DRC7), and the PF ribbon (Rib72) appeared at the wild-type amounts in the FAP20 mutants (Figure 5B). The MBO2p, which is required for assembly of beaks in DMTs 5 and 6 (Tam and Lefebvre, 2002), was also unaltered. In contrast, two DMT proteins, tektin (Norlander et al., 1996; Yanagisawa and Kamiya, 2004) and PACRG (Dawe et al., 2005; Ikeda et al., 2007), were reduced in the FAP20 mutants, implying FAP20-dependent assembly on the DMT.

To further investigate FAP20's association with DMTs, we fractioned the axonemes by several treatments. Treatment with 0.6 M KCl, which removes most dynein arms but leaves DMTs intact, did not solubilize FAP20 (Figure 5C), indicating that FAP20 is tightly associated with DMTs. We then treated axonemes with increasing concentrations of Sarkosyl as previously described (Witman et al., 1972; Figure 5C). At concentrations of 0.1–0.2%, which solubilize the central pair and portions of B-tubules, FAP20 remained in the pellet, confirming the protein as a structural component of DMTs. At a concentration of 0.3%, which yields intact A-tubules with remnants of B-tubules attached at the junctions, about one-third of the FAP20 remained in the pellet. At high concentrations (0.5–0.7%), which solubilize the A-tubule, leaving the PF ribbon, FAP20 was completely solubilized. Tektin and PACRG exhibited extraction patterns quite similar to that of FAP20, supporting the notion of proximity of these three proteins on the DMT.

**FAP20 is a component of the IJ**

To further investigate FAP20 localization, we performed detailed structural analyses of DMTs. We first averaged cross-sectional images of embedded axonemes to identify missing structure(s) in the mutant DMTs (Figure 6, A–D). Because FAP20 localized along the length of all nine DMTs (Figure 4C), we included all DMTs except those with beak structures for averaging. The B-tubule of the wild-type DMT is composed of 10 tubulin PFs, and one smaller subunit

| Strain (n)      | 1+, 5+, 6− | 1+, 5−, 6+ | 1+, 5−, 6− | 1+, 5+, 6− | 1−, 5−, 6− |
|-----------------|------------|------------|------------|------------|------------|
| Wild type (12)  | 1.00       | 0.00       | 0.00       | 0.00       | 0.00       |
| mbo1 (10)*      | 0.00       | 0.00       | 0.10       | 0.90       | 0.00       |
| fap20null (47)  | 0.00       | 0.00       | 0.00       | 0.00       | 1.00       |
| fap20AC (60)    | 0.07       | 0.02       | 0.03       | 0.05       | 0.83       |
| FAP20-GFP (22)  | 0.95       | 0.05       | 0.00       | 0.00       | 0.00       |
| FAP20-BCCP (13) | 1.00       | 0.00       | 0.00       | 0.00       | 0.00       |

*From Segal et al. (1984).

**TABLE 2:** Quantitative analysis of patterns of beak structures.
Accessibility of the tag inside the axoneme was tested by labeling intact axonemes with a fluorescent streptavidin (Supplemental Figure S4B). For better visualization of the BCCP tag in cryo–electron tomography, we enlarged the tag by three-step treatments with streptavidin, biotinylated cytochrome C, and streptavidin (Oda et al., 2014). The enlarged tag was clearly localized to the IJ on comparing averaged subtomograms of the FAP20-BCCP and wild-type DMTs (Figure 6, E and F). These observations unambiguously demonstrated that FAP20 is a bona fide IJ protein.

To verify whether the amount of FAP20 on DMTs is sufficient to form the IJ, we estimated it by comparing the fluorescence intensity of FAP20-GFP in the axoneme with that of GFP-tagged RSP3, a subunit of the radial spokes. Introduction of a GFP-tagged RSP3 gene restored the radial spokes to the axoneme (Supplemental Figure S5A) and rescued the paralyzed phenotype of an RSP3 mutant, pf14 (Supplemental Figure S5B). Fluorescence intensity of FAP20-GFP in flagella was about three times higher than that of RSP3-GFP (Supplemental Figure S5, C and D). Previous biochemical analysis revealed that there are two radial spokes within a 96-nm longitudinal repeat of DMT, and each spoke has two or four RSP3 molecules (Yang et al., 2006; Diener et al., 2011; Oda et al., 2014). There should be four or eight RSP3-GFP proteins per 96 nm of DMT. Using RSP3-GFP intensity as the reference, there should be roughly 12 or 24 FAP20-GFP proteins per 96 nm of DMT, which is equivalent to one FAP20-GFP protein per 8 or 4 nm of DMT. The abundance of FAP20 seemed sufficient for forming the IJ continuously along the length of the DMT.

Assembly and turnover of FAP20 in dikaryon flagella
Flagellar assembly and maintenance require continuous transport of components to the tips by intraflagellar transport (IFT; Qin et al., 2004; Hou et al., 2007; Ahmed et al., 2008; Wren et al., 2013). Dikaryon studies show that the inner arm dyneins, the radial spokes, and the DRC are delivered to the tips, where they are assembled into the axoneme (Johnson and Rosenbaum, 1992; Pipperno et al., 1996; Bower et al., 2013). To examine whether FAP20 is transported by IFT to the flagellar tip for assembly, we mated FAP20-GFP gametes with both fap20null and wild-type gametes and observed dikaryons for the emergence of FAP20-GFP in the unlabeled flagella. In the dikaryons formed by FAP20-GFP and fap20null gametes, FAP20-GFP first appeared in the base of the fap20null flagella and gradually spread to the tip (Figure 7A). In the dikaryons formed by FAP20-GFP and wild-type gametes, no FAP20-GFP signal was observed in the wild-type flagella, indicating that in the presence of endogenous FAP20, no additional protein can be incorporated into the axoneme, and the turnover rate of FAP20 is very slow (Figure 7B). Taken together, these results hint that FAP20 is not transported into flagella by IFT but instead enters the flagella through diffusion and binds to the axoneme proximally to distally.

Localization of FAP20 on the basal bodies
FAP20 was also identified as a basal body protein through proteomic analysis (Keller et al., 2005). In live, deflagellated FAP20-GFP cells, GFP signals were clearly visible as two discrete dots at the basal bodies (Figure 4D). The GFP signals at the basal bodies were continuous with the flagellar axonemes in the flagellated cells (Figure 4B). To confirm the localization, we also analyzed the cell bodies of IFT and basal body mutants by Western blot (Supplemental Figure S3). FAP20 is present at wild-type levels in bld1 cells, which have normal basal bodies but cannot produce flagella because of the absence of IFT (Brazelton et al., 2001; Deane et al.,

FIGURE 4: FAP20 localizes along the entire length of flagellum and is present in all nine DMTs. (A) Immunofluorescence microscopy of nucleoflagellar apparatus (NFA) shows that FAP20 is evenly distributed along the entire length of wild-type flagella, but there is no signal in fap20null flagella. Acetylated α-tubulin was used to show the position of flagella. (B) Fluorescence image of the rescued strain FAP20-GFP. The FAP20-GFP signal is evenly distributed along the entire length of flagella. (C) The axonemes of sup-pf1 FAP20-GFP strain, frayed by treatment with ATP and a protease, show that FAP20-GFP localizes to all nine DMTs. The base of the axoneme is resistant to the protease and remains connected (asterisk). (D) Confocal images of a deflagellated FAP20-GFP cell and a wild-type cell with flagella. The FAP20-GFP signal remains on the basal bodies as two discrete dots after deflagellation. There is no GFP signal on the wild-type flagella and the basal bodies. Horseshoe-shaped fluorescence in the cell bodies (B and D) is autofluorescence of the chloroplast. Bars, 10 μm.
Morpholino knockdown of Gtl3/FAP20 caused a cilary phenotype in zebrafish

Vertebrate homologues of FAP20, GTL3/C16orf80, are highly expressed in the testis and other ciliated tissues (Rijkers and Ruther, 1996) and found in cilia proteomes (Ostrowski et al., 2002). To test whether GTL3/C16orf80 has a conserved function in vertebrate cilia, we performed morpholino-mediated knockdown experiments in zebrafish embryos. The gt3-knockdown fish showed a curved body axis (Supplemental Figure S6A), short somite length (Supplemental Figure S6, B and C), and defective heart-looping orientation (Supplemental Figure S6D). These phenotypes are consistent with ciliary dysfunction in zebrafish (Malicki et al., 2001). In contrast, FAP20 protein was dramatically reduced in bld10 cells, which completely lack the basal bodies (Matsuura et al., 2004). FAP20 protein was also greatly reduced in bld2 cells, which have short, singlet MTs instead of triplets in its basal bodies (Goodenough and St. Clair, 1975; Dutcher et al., 2002). These results suggest that association of FAP20 to the basal bodies requires integrity of the MT structures.

The integrity of the IJ is important for stability of the axoneme

Interdoublet connections have been reported to be mediated by the N-DRC (Heuser et al., 2009). A structural study showed that there is a hole along the IJ at 96-nm intervals in the wild-type axoneme, and the hole is filled in the axoneme of N-DRC mutants pif3 and ida6 (Nicastro et al., 2011). The mutated proteins, DRC1 and DRC2, compose the base plate that facilitates the attachment of the N-DRC to the A-tubule and extends to the IJ (Heuser et al., 2009). A structural study showed that the reduced amount of these proteins may partially maintain the IJ or rigidity of the outer junction, and the B-tubule wall may be enough to maintain the shape of the B-tubule.

FAP20 is an IJ protein of axonemal DMTs

High-resolution cryo-electron tomography analyses showed that the IJ of DMTs comprises smaller, nontubulin subunits (Nicastro et al., 2011; Pigino et al., 2012). We showed that the FAP20 mutant lacks the nontubulin subunit of the IJ (Figure 6B) and the tagged FAP20 localized to the IJ (Figure 6, E and F). These two observations clearly demonstrate that FAP20 is a component of the IJ. The stoichiometry of FAP20 to RSP3 supports the notion that FAP20 binds to the DMT with 8- or 4-nm periodicity (Supplemental Figure S5C). This also means that FAP20 is one of the most abundant proteins in the DMT besides tubulins. Two DMT proteins, tektin and PACRG, were reduced in the FAP20 mutant (Figure S5B) and cofractionated with FAP20 in the biochemical analyses (Figure 5C), suggesting that these proteins may interact with FAP20. Based on our calculation, one or two FAP20 proteins contribute 21 or 42 kDa/8 nm, which is too small to be the sole component of the IJ, since the size of the IJ is comparable to that of tubulin PFs (~100 kDa/8 nm). Thus tektin (58 kDa) and PACRG (33 kDa) may colocalize with FAP20 to form the IJ. We rarely observed any open B-tubule in the mutant axoneme, suggesting that the reduced amount of these proteins may partially maintain the IJ or rigidity of the outer junction, and the B-tubule wall may be enough to maintain the shape of the B-tubule.
FIGURE 6: FAP20 is an IJ protein. (A–D) The fap20null axoneme is defective in the IJ between A- and B-tubules. Images of DMTs in cross sections of Epon-embedded axonemes were averaged. DMT1, 5, and 6 were excluded from the analyses. The number of DMT images used for the averaging is 22, 22, 65, and 34, respectively, for wild type, fap20null, FAP20-GFP, and FAP20-BCCP. IDAs, inner dynein arms; IJ, inner junction (arrows); ODA, outer dynein arm; RS, radial spoke. (A) The wild-type B-tubule is composed of 10 tubulin PFs and one smaller, nontubulin subunit at the IJ. (B) The DMT of the fap20null clearly lacks the nontubulin subunit. (C, D) The nontubulin subunits are restored in the rescued strains. Bar, 20 nm. (E, F) FAP20 localized to the inner junction (IJ) of the DMT. (E, F) Wild-type and FAP20-BCCP axonemes were labeled by an enhanced streptavidin-label method. The position of the streptavidin–cytochrome C label (orange) in the DMT was visualized by comparing averaged subtomograms of the two strains. (E) Cross-sectional view from the base of the axoneme. (F) Lateral view from a direction indicated by an arrow in E. Bars, 25 nm. (G) Model of FAP20 localization and functions. FAP20 localizes to the IJ between A- and B-tubules and stabilizes the DMT. N-DRC components, DRC1/2, or its associated proteins may extend to the IJ and be anchored by FAP20. In DMT1, 5, and 6, FAP20 may work as a scaffold for intratubular proteins to produce the beak structures.

The IJ may function as a scaffold for assembly of beak structures
The FAP20 mutant completely lacks the doublet-specific beak structures (Figure 3), which are localized in the proximal portion of the axoneme and related to the direction of the bending. However, FAP20 is neither a beak component nor a determinant to specify DMTs with beaks; FAP20 itself localized uniformly along the length of all nine DMTs (Figure 4C), and the amount of FAP20 was unaffected in the axonemes of mbo mutants, which partially lack the beak structures (Figure 4A). Instead, FAP20 may function just as a scaffold for assembly of the beaks in DMTs specified by unknown factors (Figure 6G).

The underlying mechanism for generating the widely used planar asymmetrical beating of cilia or flagella is unknown. The beak structures required for asymmetrical waveform are found only in Chlamydomonas. It is possible that functionally equivalent structures are present in other organisms, given the following evidence: 1) MBO2p/CCDC146, the mutated protein of Chlamydomonas mbo2 mutant, is conserved among organisms with flagella or cilia (Tam and Lefebvre, 2002); and 2) knockdown of FAP20/BUG22 in Paramecium causes symmetrical ciliary beating (Lalig\-ne et al., 2010) similar to that of the Chlamydomonas mutant. Further functional studies of these proteins in other organisms are necessary to confirm whether a conserved mechanism is used to generate planar asymmetrical beating of cilia or flagella.

FIGURE 7: FAP20 is assembled into the flagella from base to tip. FAP20-GFP gametes were mated with both fap20null (A) and wild-type (B) gametes and dikaryons observed for the emergence of FAP20-GFP in the unlabeled flagella. Bright-field (BF) and GFP fluorescence images of dikaryons were recorded at 15 and 60 min after mixing. (A) FAP20-GFP fluorescence first appeared at the base of the fap20null flagella and gradually extended to the tip. (B) FAP20-GFP was not incorporated into wild-type flagella. Arrows indicate positions of flagellar tips.

FAP20 has a novel mode of assembly
In the dikaryon analyses (Figure 7), we found that FAP20 is assembled into the mutant axoneme from base to tip. This is opposite to the tip-to-base assembly of other axonemal components (Johnson and Rosenbaum, 1992; Piperno et al., 1996; Bower et al., 2013). The unique axonemal assembly pattern of FAP20 may reflect that it is a component of the DMT itself and involved in the assembly of intratubular beak structures. It will be interesting to examine whether IFT is involved in the flagellar entry of FAP20.

This unusual mode of assembly provides an explanation for several inconsistent results reported for tagged FAP20 localization in different organisms. In Trypanosoma (Hodges et al., 2011), FAP20 localized along the entire length of the axoneme, consistent with our observation (Figure 4, A and B, and Supplemental Figure S4B). In contrast, the tagged FAP20 only localized to the proximal portion of the cilia in Paramecium (Lalig\-ne et al., 2010), cultured mammalian cells (Ishikawa et al., 2012), and Chlamydomonas (Meng et al., 2013). In the these three studies, tagged transgenes were expressed in the presence of endogenous FAP20/GTL3 or the expression levels were lower than that of wild type. As we show in this study, FAP20 is incorporated into the axoneme from base
to tip and with very low turnover rate (Figure 7). Thus it is reasonable to observe that the tagged proteins localized only to the proximal portion of the axonemes in these studies because the expression level was low or not up-regulated upon growth of the axonemes.

**Basal body localization of FAP20 family proteins**

FAP20 was identified as a basal body component in a previous proteomic study (Keller et al., 2005). The basal bodies are arranged in a V shape in which the proximal ends are closely associated (Ringo, 1967). In contrast to the fused-single-dot staining observed with antibodies against proximal-end proteins (Matsuura et al., 2004; Nakazawa et al., 2007), FAP20 localized to the basal bodies as two discrete dots in deflagellated cells. These two dots were on the mature basal bodies but not the pro–basal bodies because the dots were continuous with the FAP20 staining on the axonemes in flagellated cells. The distance between the two dots was ∼0.8 μm, corresponding to the distance between the distal ends. FAP20 in the basal body is not essential for templating the axoneme since the FAP20 mutants exhibit no abnormality in flagellar assembly (Figure 2A). These observations suggest that FAP20 in the basal bodies may be localized to the distal ends, which are formed by DMTs instead of triplet MTs and are not essential for templating the axoneme.

FAP20 homologues are annotated as members of the domain of unknown function (DUF667) family in protein family databases (e.g., http://pfam.sanger.ac.uk/). Of interest, several proteins contain DUF667. One of the proteins, WDR90, is highly conserved among organisms with cilia and flagella. WDR90 is not in ciliary or flagellar proteomes but is instead found in the basal body proteome (Keller et al., 2005) and annotated as proteome of centriole 16 (POC16). WDR90/POC16 contains one DUF667 domain in its N-terminus, followed by a large C-terminal domain containing multiple WD40 repeats. A structural study of the basal bodies revealed that the IJ between the triplet A- and B-tubules scaffolds a large structure called the Y-shaped linker (Li et al., 2012). WDR90/POC16 may form both the IJ and part of the linker in the triplet MT.

**FAP20 may play a conserved role in modifying the functionality of MTs**

In this article, we showed that FAP20 forms the IJ of DMTs. The IJ seems to function as an essential hub for protein interactions in the DMT; the IJ likely anchors the N-DRC and scaffolds the intra–B-tubule bar structure. We propose that the flagellar stability and motility defects of FAP20 mutants are caused by the dysfunction/loss of these structures. Recently, a *Drosophila* FAP20 mutant was reported to be defective in tubulin polyglycylation in sperm MTs (Mendes et al., 2014). The IJ may work as a guide for tubulin modification enzymes. Moreover, FAP20 is also present in nonmotile primary cilia, which lack motility-specific proteins, including DRC subunits (Ishikawa et al., 2012). FAP20 likely forms the IJ of DMTs and might mainly strengthen the rigidity of axonemes in the primary cilia. Further studies are required to evaluate the role of the IJ in mechanical properties of DMTs.

**MATERIALS AND METHODS**

**Strains and culture conditions**

*Chlamydomonas reinhardtii* strains used for this study are listed in Supplemental Table S1. Cells were cultured on Tris-acetate-phosphate medium (TAP) plates with 1.5% agar or in liquid TAP with constant aeration at 22 or 25°C with a 12:12 h light:dark cycle. Gametes for dikaryon analyses were prepared by shaking cells in M-N (Sager and Granick minimum medium without nitrogen source) liquid medium for 4 h under illumination.

**Isolation and mapping of FAP20 mutants**

The first allele, *dmj1-1*, was isolated from a library of motility mutants generated by insertional mutagenesis using a marker plasmid, pSI103 (Sizova et al., 2001). The mutation was not linked to the marker plasmid and thus was mapped to the FAP20 gene by AFLP analysis (Kathir et al., 2003). The second allele, *dmj1-2*, was isolated from a library of motility mutants generated by ultraviolet (UV) mutagenesis. The third allele, *RL-11*, was previously described (Nakamura, 1981). Both *dmj1-2* and *RL-11* were identified as FAP20 mutants by sequencing of the FAP20 gene. The fourth allele, *dmj1-3*, was screened from a phototaxis-defective mutant library created by insertional mutagenesis of a hygromycin B–resistant gene (Berthold et al., 2002). The mutation was mapped to the FAP20 gene by restriction enzyme site-directed amplification PCR (Gonzalez-Ballester et al., 2005). Oligonucleotide primers used for amplification and sequencing of the FAP20 gene are listed in Supplemental Table S2. Each mutant was backcrossed at least twice for the experiments reported in this study.

**Antibodies**

The full-length FAP20 cDNA was amplified by reverse transcription-PCR with primers FAP20-BglNd-F and FAP20-Xho-R. The truncated FAP20 cDNA was amplified with primers FAP20-BglNd-F and FAP20-TR-Xho-R. The full-length product was digested with Ndel and Xhol and cloned into pET24a (Merck, Darmstadt, Germany) to produce FAP20full-His. Both the full-length and the truncated products were digested with Bgll and Xhol and cloned between BamHI and XhoI sites of pGEX6P2 (GE Healthcare, Little Chalfont, United Kingdom) to produce glutathione S-transferase (GST)–FAP20full and GST-FAP20ΔC20aa, respectively. Each of the three plasmids was transformed into BL21 (DE3). The expression of the recombinant proteins was induced by adding 1 mM isopropyl-β-D-thiogalactoside into a logarithmically growing culture. Almost all of the expressed proteins were contained within inclusion bodies. The inclusion bodies were purified as previously described (Yanagisawa and Kamiya, 2004). FAP20full-His was used to immunize two rabbits. The antibodies were affinity purified using GST-FAP20full or GST-FAP20C20aa blotted onto polyvinylidene difluoro membranes. Sequences of the primers used for the plasmid constructions are listed in Supplemental Table S2. Other antibodies used in this study are listed in Supplemental Table S3.

**Plasmid constructions**

To make FAP20 constructs with tags, an ~4.5-kb fragment containing the FAP20 gene was amplified from *Chlamydomonas* genomic DNA using primers FAP20-gDNA-F and FAP20-gDNA-R. The PCR product was cloned into the EcoRV site of pBluescript II to create the construct pBS-FAP20. A unique EcoRV site was introduced before the stop codon by PCR amplification of the entire plasmid with primers FAP20-gDNA-C-ERV-F and FAP20-gDNA-C-ERV-R. The
PCR product with 16-base cohesive ends was transformed into DH5α to make the construct pBS-FAP20-C-ERV. A GFP tag was cut out with BamHI from pCRGFP (Fuhrmann et al., 1999) and blunted with Klenow. A 3xHA tag was cut out with NruI and Scal from p3xHA (Silflow et al., 2001). Each of the two tag fragments was ligated into the EcoRV site of pBS-FAP20-C-ERV. A BCCP tag was amplified from pGenD-LC2-BCCP (Furuta et al., 2009) using primers FAP20-BCCP-F and FAP20-BCCP-R and cloned into the EcoRV site of pBS-FAP20-C-ERV using In-Fusion HD Cloning Kit (Clontech, Mountain View, CA). Each of the three FAP20 constructs was linearized with EcoRI and cotransformed with psI103 into the dmj1-1 cells by electroporation to produce the rescued strains dmj1-1::FAP20-GFP, dmj1-1::FAP20-3xHA, and dmj1-1::FAP20-BCCP. Sequences of the primers used for the plasmid constructions are listed in Supplemental Table S2.

Flagella length measurement

Cells were fixed with 2x Lugol’s iodine and placed in 4°C until the end of the experiment. The cells were observed by an Axioplan phase contrast microscope (Carl Zeiss, Oberkochen, Germany). Still images were taken with a Phantom Miro-eX2 (Vision Research, Wayne, NJ), and the length of a single flagellum from each cell was measured using the ImageJ (Schneider et al., 2012) segmented line tool.

Assessment of flagellar motility

For waveform analyses, cells were observed by an Axioplan phase contrast microscope or a BX51 dark-field microscope (Olympus, Tokyo, Japan). To prevent photoshock, a red filter was used. The filter was removed to induce waveform change in wild-type cells. Movies for waveform analysis were taken with a Phantom Miro-eX2 or an EoSens MC1362 (Mikrotron, Unterschleisheim, Germany) at either 600 or 1000 fps. To trace the waveforms, the movies were imported into ImageJ and trimmed to 10 frames with equal time elapsed between frames of a given set. Shapes of the flagella were traced over a lowered opacity using Illustrator (Adobe, San Jose, CA). For measurement of swimming velocity, cells were observed by a dark-field microscope and movies were recorded as described at 30 fps. Swimming velocity of the cells was measured using the MTrack2 plug-in (Klöffenstein and Vale, 2004) of ImageJ.

Immunofluorescence microscopy

Nucleoflagellar apparatus prepared as previously described (Taillon and Jarvik, 1995) was fixed with 2% formaldehyde for 10 min at room temperature, followed by treatment with cold acetone and 60% methanol (−20°C). Fixed samples were immunostained as previously described (Sanders and Salisbury, 1999). Images were acquired and processed as previously described (Oda and Kikkawa, 2013) with little modification. The details of the method was substituted by 50 mM Na-phosphate, pH 7.0. The samples were observed by a JEM-3100FEF transmission electron microscope (JEOL, Tokyo, Japan) equipped with TemCam-F416 (TVIPS, Gauting, Germany). Images were recorded at 300 keV, with ∼3-μm defocus, at a magnification of ×82,100 and a pixel size of 1.9 Å. Cross-sectional images in which the PFs were clearly visible were selected and used for averaging of DMTs. Alignment and averaging of DMTs were conducted using custom Ruby-Helix scripts (Metlagel et al., 2007) and the align2d program of EMAN (Ludtke et al., 1999).

Assessment of the BCCP-tagged strain

Biotinylation of the FAP20-BCCP in the dmj1-1::FAP20-BCCP axonemes was confirmed by Western blotting with streptavidin–horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA). Accessibility of the BCCP tag in the intact axoneme was assessed using fluorescence-labeled streptavidin. Demembranated axonemes were loaded to a flow cell and blocked with 1% bovine serum albumin (BSA) in HMDEK buffer. Then the flow cell was perfused with streptavidin–Alexa Fluor 546 (Life Technologies, Carlsbad, CA) diluted 1:1000 in the same buffer and washed with the buffer alone. Samples were observed by an IX70 fluorescence microscope as described.

Enhanced streptavidin labeling of the axonemes

Demembranated axonemes were incubated with 0.05 mg/ml streptavidin (Wako Pure Chemical Industries) for 15 min at 4°C in HMDEK buffer. Axonemes were washed five times with HMDEK buffer and incubated with 0.05 mg/ml biotinylated cytochrome C for 15 min at 4°C in the presence of 1 mg/ml BSA and 0.1 mg/ml unlabelled cytochrome C. Axonemes were washed five times with HMDEK again and incubated with 0.05 mg/ml streptavidin for 15 min at 4°C. Labeled axonemes were separated from unbound streptavidin by centrifugation and were resuspended in HMDEK buffer at a concentration of 0.02 mg/ml and mixed with equal amount of 15-nm colloidal gold suspension conjugated by BSA (Aurion, Wageningen, Netherlands). Home-made holey carbon grids were glow discharged and coated with 20-nm colloidal gold (BBInternational, Cardiff, United Kingdom). Suspended axonemes plus colloidal gold (5 μl) was loaded onto the grids and plunge frozen in liquid ethane at −180°C with an automated plunge-freezing device, EM GP (Leica Microsystems, Wetzlar, Germany). Images were acquired and processed as previously described (Oda and Kikkawa, 2013) with little modification. The details are described in the Supplemental Methods.

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