A conserved flagella-associated protein in Chlamydomonas, FAP234, is essential for axonemal localization of tubulin polyglutamylase TTLL9

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A conserved flagella-associated protein in Chlamydomonas, FAP234, is essential for axonemal localization of tubulin polyglutamylase TTLL9

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A conserved flagella-associated protein in *Chlamydomonas*, FAP234, is essential for axonemal localization of tubulin polyglutamylase TTLL9

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ABSTRACT Tubulin undergoes various posttranslational modifications, including polyglutamylation, which is catalyzed by enzymes belonging to the tubulin tyrosine ligase–like protein (TTLL) family. A previously isolated *Chlamydomonas reinhardtii* mutant, tpg1, carries a mutation in a gene encoding a homologue of mammalian TTLL9 and displays lowered motility because of decreased polyglutamylation of axonemal tubulin. Here we identify a novel tpg1-like mutant, tpg2, which carries a mutation in the gene encoding FAP234, a flagella-associated protein of unknown function. Immunoprecipitation and sucrose density gradient centrifugation experiments show that FAP234 and TTLL9 form a complex. The mutant tpg1 retains FAP234 in the cell body and flagellar matrix but lacks it in the axoneme. In contrast, tpg2 lacks both TTLL9 and FAP234 in all fractions. In fla10, a temperature-sensitive mutant deficient in intraflagellar transport (IFT), both TTLL9 and FAP234 are lost from the flagellum at nonpermissive temperatures. These and other results suggest that FAP234 functions in stabilization and IFT-dependent transport of TTLL9. Both TTLL9 and FAP234 are conserved in most ciliated organisms. We propose that they constitute a polyglutamylation complex specialized for regulation of ciliary motility.

INTRODUCTION Tubulin undergoes various types of posttranslational modification, which fine tune the properties of microtubules involved in diverse functions in various cells and organelles (Janke and Bulinski, 2011). One such modification, polyglutamylation, generates polyglutamate chains that branch from the C-terminal region of α- and/or β-tubulin. Microtubules contained in the mitotic spindle, neurons, centrioles, and cilia/flagella axonemes are abundantly polyglutamylated. Several studies demonstrated the importance of polyglutamylation in the interaction between microtubules and associated proteins such as the motor proteins kinesin (Ikegami et al., 2007; Konno et al., 2012) and dynein (Kubo et al., 2010; Suryavanshi et al., 2010). The enzymes that carry out the polyglutamylation belong to the tubulin tyrosine ligase–like protein (TTLL) family, which shares a conserved TTL domain containing an ATP-binding site (Janke et al., 2005; van Dijk et al., 2007). Some TTLLs, such as TTLL1, catalyze both reactions (Janke et al., 2008).

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Abbreviations used: DRC, dynein regulatory complex; FAP, flagella-associated protein; HA, hemagglutinin; IFT, intraflagellar transport; TTLL, tubulin tyrosine ligase–like.

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Tubulin polyglutamylation is important for the assembly and function of cilia/flagella. Organisms in which a specific TTLL protein is knocked out or overexpressed show various defects in the axoneme, including absence of the central pair and abnormal arrangements of the outer doublet microtubules (Pathak et al., 2007, 2011; Wloka et al., 2010). Motility deficiencies have been observed in TTLL6-knockout Tetrahymena (Suryavanshi et al., 2010), TTLL1-knockout mice (Ikegami et al., 2010), and the Chlamydomonas mutant *tubulin polyglutamylation-deficient 1* (*tpg*1), which lacks TTTL9 (Kubo et al., 2010, 2012). Despite the clear importance of TTLL enzymes for the assembly and function of axonemes, however, the mechanisms that underlie the recruitment of the TTLL proteins to the cilia/flagella and the regulation of polyglutamylation remain to be elucidated. In addition, how the sites of polyglutamylation are determined is also not known. Of interest, long polyglutamate side chains have been detected exclusively on the B-tubule of outer doublets (Lechtreck and Geimer, 2000; Kubo et al., 2010).

An effective approach for answering these questions is to identify proteins that interact with TTLL proteins. Previous studies showed that mouse TTLL1 interacts with four proteins (Janke et al., 2005) and zebrafish TTLL6 interacts with another protein, CEP41, which regulates the entry of TTLL6 into the cilia from the basal body (Lee et al., 2012). In the present study, we isolate and analyze a novel *Chlamydomonas* mutant, *tpg*2, which has a phenotype similar to that of TTTL9-lacking *tpg*1 (Kubo et al., 2010). Genetic and biochemical analyses show that *tpg*2 possesses a mutation in FAP234, a conserved flagella-associated protein of unknown function, and that FAP234 forms a complex with TTTL9 in the axoneme and cytoplasm. Furthermore, by using a temperature-sensitive kinesin II-deficient mutant, we find that the TTTL9-FAP234 complex is recruited to the axoneme by intraflagellar transport (IFT). Thus we show that FAP234 is a novel protein required for proper localization of polyglutamylation in the axoneme.

### RESULTS

**A novel *Chlamydomonas* mutant, *tpg*2, exhibits a tubulin polyglutamylation defect**

Two mutants that were phenotypically similar to *tpg*1 were isolated using ultraviolet (UV) mutagenesis of wild-type cells and screening for slow-swimming phenotypes. Because these two mutants were genetically distinct from *tpg*1 and carried mutations in a gene involved in axonemal tubulin polyglutamylation (see later discussion), we designated the mutants as *tpg*2-1 and *tpg*2-2. In most experiments, we used the mutants interchangeably. Thus we here refer to the mutants as *tpg*2, unless it is necessary to distinguish between them. The motility of *tpg*2 was indistinguishable from that of *tpg*1; swimming velocity and flagellar beat frequency of both mutants were ~70% of those of wild-type cells (Figure 1, A and B). Furthermore, similar to *tpg*1, *tpg*2 was completely motile in the background of a mutation causing the loss of outer-arm dynein (Supplemental Figure S1, A and B). Also similar to *tpg*1, *tpg*2 showed a normal composition of axonemal dyneins (Supplemental Figure S2).

Polyglutamylation of tubulin was significantly reduced in the *tpg*2 axoneme, as detected by Western blot analysis (Figure 1C). Immunoblotting with the B3 antibody, which recognizes α-tubulin that has side chains with two or more glutamates (van Dijk et al., 2007), detected two bands in the wild-type axoneme but only one band in both *tpg*1 and *tpg*2 axonemes. The upper band observed in the wild-type axoneme corresponded to α-tubulin with a long polyglutamate chain (Kubo et al., 2010). The lower band, which was detected in both wild-type and *tpg* mutants, most likely corresponded to α-tubulin with a short polyglutamate chain. Immunoblotting with the polyE antibody, which recognizes long side chains with three or more glutamates (van Dijk et al., 2007), showed that the band intensities were weaker in *tpg*1 and *tpg*2 axonemes than in wild-type axonemes. Immunofluorescence microscopy of *tpg*2 cells using the polyE antibody also showed significantly reduced tubulin glutamylation in the flagella (Figure 1D). In contrast, the staining intensity in the basal body was similar to that observed for the wild type. These staining features are similar to those observed in the *tpg*1 axoneme (Figure 1D; Kubo et al., 2010).

*tpg*2 has a mutation in FAP234, a conserved flagella-associated protein

Both *tpg*2-1 and *tpg*2-2 mutations were mapped to a region in linkage group I by using Amplified-fragment-length polymorphism (AFLP) analysis after a genetic cross with the S1-D2 strain. This region contained two proteins listed in the *Chlamydomonas* flagellar proteome database (http://labs.umassmed.edu/chlamyfp/index.php; Pazour et al., 2005). Sequence analysis of the cDNA and genomic DNA of these proteins revealed that both *tpg*2-1 and *tpg*2-2 possess mutations in the gene encoding FAP234, a 177-kDa flagella-associated protein of unknown function. The *tpg*2-1 mutant contained a deletion between exons 26 and 36, whereas *tpg*2-2 showed a single-base substitution in the intron immediately after exon 28, which causes a splicing defect that completely eliminates exon 28 (Figure 2A). A BLAST search of the protein databases of the National Center for Biotechnology Information (NCBI) and the Joint Genome Institute indicated that FAP234 is a protein highly conserved among organisms possessing cilia and flagella. Caenorhabditis elegans, which has only nonmotile cilia, also possesses a FAP234 homologue, although it is more diverged than the homologues in other ciliated organisms (Supplemental Figure S3 and Table 1; also see Table 2 later in the paper). Secondary structure prediction using the NCBI BLAST service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and a coiled-coil prediction system, SOSUI coil (http://bp.nuap.nagoya-u.ac.jp/sosui/coil/submit.html), suggested that FAP234 contains three leucine-rich repeats, one E3 ubiquitin protein ligase–like sequence, and nine coiled-coil regions (Figure 2B). The *Chlamydomonas reinhardtii* flagellar proteome database indicated that FAP234 is an axoneme-associated protein. Similar to TTTL9, FAP234 in the axoneme increased in amount after deflagellation (Supplemental Figure S4).

FAP234 is localized to the flagella

To facilitate localization of FAP234 and detection of its interactions with other proteins, we raised two kinds of rabbit polyclonal antibodies, anti-FAP234N and anti-FAP234C, which recognize the 684 N-terminal and 572 C-terminal amino acids, respectively (Figure 2B). The C-terminal amino acid sequence used as the antigen for the anti-FAP234C antibody is longer than the deleted portion in *tpg*2-1; thus the antibody should detect a truncated version of the protein if one were produced in *tpg*2. Western blotting of wild-type axoneme by using either antibody detected a single band corresponding to the size of predicted full-length FAP234 (Figure 2, C and D). Furthermore, the FAP234C antibody also detected the FAP234 signal in the cytoplasmic extract from wild-type cells (see later discussion), although the anti-FAP234N antibody, which had a lower titer, did not detect the signal. In *tpg*2 mutants, these antibodies did not detect signals corresponding to FAP234 or its truncated variants in the axoneme (Figure 2, C and D). Any mutated FAP234 protein(s) potentially produced in *tpg*2 must have been degraded in the cytoplasm. Despite repeated trials, we were unable to detect FAP234 signals in wild-type cells or axonemes by
FAP234 forms a complex with TTLL9

Western blot analysis revealed that tpg2 axonemes lacked not only FAP234, as expected, but also TTLL9, which was not expected (Figure 2, C and D). Similarly, TTLL9-deficient tpg1 axonemes also lacked FAP234 (Figure 2C). These results suggest that TTLL9 and FAP234 localize to the axoneme interdependently, perhaps through an association between the two.

The potential FAP234-TTLL9 interaction was examined using an oda2 transformant (a mutant lacking outer-arm dynein) expressing TTLL9--hemagglutinin (HA), oda2tpg1::TTLL9HA, which enabled sensitive detection of TTLL9. This strain will be referred to as transformant 1H. In contrast to the nonmotile double mutant oda2tpg1, transformant 1H displayed nearly the same level of motility as oda2. A high-salt extract from the transformant's axonemes was immunoprecipitated using antibodies against the HA tag or FAP234. A FAP234 signal was detected in the high-salt extracts of 1H axonemes precipitated using the anti-HA antibody (Figure 3A). Conversely, a TTLL9 signal was detected in wild-type axonemal extracts precipitated using the anti-FAP234C antibody (Figure 3D). These results indicate that TTLL9 and FAP234 directly or indirectly associate with each other in the axonemal extracts.

The association between TTLL9 and FAP234 was further analyzed by sedimentation velocity measurements. A high-salt extract from wild-type axonemes was centrifuged on a sucrose density gradient and analyzed by Western blotting. Both TTLL9 and FAP234 signals peaked at the same fraction between 7S and 11S (Figure 3E), confirming that the two proteins are components of a single complex.
Because use of the transformant 1H expressing TTLL9-HA provided higher sensitivity in immunodetection by using the anti–HA tag antibody, we reexamined TTLL9 localization in flagellar fractions separated using a freeze-thaw method (Fan et al., 2010). Western blot with the anti–HA tag indicated that TTLL9-HA TTLL9-FAP234 complex is present in the flagellar matrix and membrane fractions.

In our previous study, Western blot analysis of flagella showed that TTLL9 is present predominantly in the axoneme fraction and not significantly in the detergent-soluble membrane/matrix fraction (Kubo et al., 2010). Because use of the transformant 1H expressing TTLL9-HA provided higher sensitivity in immunodetection by using the anti–HA tag antibody, we reexamined TTLL9 localization in flagellar fractions separated using a freeze-thaw method (Fan et al., 2010). Western blot with the anti–HA tag indicated that TTLL9-HA TTLL9-FAP234 complex is present in the flagellar matrix and membrane fractions.

### TABLE 1: Putative homologues of FAP234.

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Putative homologues of FAP234 among ciliated organisms. Maximum scores and BLAST E values were obtained from the NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
was present in both the membrane and the matrix (soluble) fractions, as well as in the axoneme, although the amounts in the former two fractions were much lower than in the axoneme (unpublished data). The anti-HA antibody (3f10) was able to immunoprecipitate both FAP234 and TTLL9 from the matrix fraction of 1H flagella (Figure 3B). These results suggest that TTLL9 and FAP234 also form a complex in the matrix fraction.

The amounts of TTLL9 and FAP234 in the flagellar matrix fraction were compared in the wild-type, tpg1, and tpg2 by Western blotting (see Materials and Methods for details). Both TTLL9 and FAP234 were detected in the wild type, whereas neither protein was detected in tpg2 lacking FAP234 (Figure 3C). Unexpectedly, the matrix fraction of tpg1 lacked a TTLL9 signal but showed a FAP234 signal, which was significantly stronger than in the wild type (Figure 3C). Because tpg1 lacks FAP234 in the axoneme (Figure 2, C and D), this result suggests that FAP234 can be transported into the flagella without TTLL9 but cannot bind the axoneme alone. The increased FAP234 signal in tpg1 possibly reflects FAP234 molecules that would be incorporated into the axoneme if TTLL9 were available.

**FIGURE 3:** TTLL9 and FAP234 interact in the axoneme and flagellar matrix. (A, B) Coimmunoprecipitation of FAP234 with TTLL9 from the high-salt axonemal extract (A) and flagellar matrix fraction (B). A high-salt axonemal extract and a flagellar matrix fraction were prepared from oda2tpg1::TTLL9HA and subjected to immunoprecipitation with the anti-HA tag antibody 3f10. Precipitates were analyzed with the FAP234C antibody and another anti-HA tag antibody, 12CA5. An FAP234 signal, as well as the HA-tag signal, was detected in the precipitate in both the axonemal extract (A) and the flagellar matrix fraction (B). (C) Western blotting analysis of flagellar matrix fractions from WT and polyglutamylation-deficient mutants. The flagellar matrix fractions were obtained by the freeze-and-thaw method and sedimented and concentrated using chloroform and methanol (Wessel and Flügge, 1984). (D) Immunodetection of TTLL9 in the axonemal extract precipitated with anti-FAP234C antibody. A band corresponding to TTLL9 (Mr = 49.6 kDa) was detected in WT, whereas no band was detected in tpg2 other than the rabbit IgG band. (E) Western blotting analysis of the WT axonemal extract fractionated by centrifugation on a 5–20% sucrose density gradient. Fractionated aliquots were analyzed using SDS–PAGE (bottom; stained with silver) and immunoblotted with the anti-TTLL9 and anti-FAP234 antibodies. Signals of both proteins peaked at 7–11 S, suggesting that TTLL9 and FAP234 are in the same complex.

**FAP234 binds and stabilizes TTLL9 in the cytoplasm**

We next examined whether TTLL9 and FAP234 are associated in the cellular cytoplasm. Immunoblotting analysis of these proteins in the cytoplasm gave results that were qualitatively similar to those obtained from the flagellar matrix fraction. A 177-kDa band corresponding to FAP234 and a 50-kDa band corresponding to TTLL9 were detected in the wild type, whereas only FAP234 was detected in tpg1, and neither protein was detected in tpg2 (Figure 4A). The absence of both proteins in the cytoplasm of tpg2 cells suggests that TTLL9 is stabilized in the cytoplasm by forming a complex containing FAP234 and that it is quickly degraded when FAP234 is absent.

To confirm the association between TTLL9 and FAP234 in the cytoplasm, we subjected cell body lysate from the transformant 1H to an immunoprecipitation assay. A FAP234 signal was detected in tpg1, and neither protein was detected in tpg2 (Figure 4A). The absence of both proteins in the cytoplasm of tpg2 cells suggests that TTLL9 is stabilized in the cytoplasm by forming a complex containing FAP234 and that it is quickly degraded when FAP234 is absent.

**Tubulin polyglutamylation occurs in assembled axonemes**

We examined whether tubulin polyglutamylation can take place in fully grown axonemes by investigating polyglutamylation in the axonemes of temporary dikaryons formed between the wild type...
and tpg1 or tpg2. Temporary dikaryons are fused gametes with four flagella, which are transiently formed upon mating of two gametes of opposite mating types. When gametes of a flagellar component–deficient mutant are mated with wild-type gametes, the mutant’s flagella often recover normal function as the lacking component(s) are supplied from the wild-type cytoplasm. This phenomenon is known as temporary dikaryon rescue. In this experiment, the gametes of tpg1 or tpg2 and those of the wild type were mated, incubated, and observed by indirect fluorescence microscopy.

In dikaryons formed between the wild-type and tpg1 gametes or between the wild-type and tpg2 gametes, the mutant flagella showed only a very low level of polyglutamylation signal when observed within 10 min of mating (Figure 5, A and B). However, in dikaryons observed 60 min after mating, the tpg1 and tpg2 flagella showed staining as strong as in the wild type flagella (Figure 5B). Staining was fairly uniform all along the length of the axoneme; no distinct directionality was observed in the increased polyglutamylation signal any time after the mating. This result indicates that polyglutamylation can take place in assembled axonemes and that the polyglutamylation proceeds almost uniformly along the flagellar length. The TTLL9 and FAP234 proteins, derived from the wild-type cytoplasm, may be quickly transported from the cytoplasm across the entire length of the mutant flagella. In contrast to dikaryons formed between the wild type and tpg1 or tpg2, dikaryons formed between tpg1 and tpg2 did not recover polyglutamylation in any flagella for at least 60 min (Figure 5C). Consistent with this observation, motility was not recovered in temporary dikaryons. Rescue may have failed in this case because the cytoplasmic concentration of TTLL9 was extremely low in the cytoplasm of tpg2 and the fused gametes.

### Flagellar import but not export of the FAP234-TTLL9 complex depends on IFT

The dikaryon rescue experiment described earlier suggests the presence of a mechanism underlying the prompt recovery of polyglutamylation in flagella. One possibility is that the IFT system, which is responsible for generating and maintaining cilia and flagella (Rosenbaum and Witman, 2002), functions to transport the TTLL9-FAP234 complex.

To examine this possibility, we used fla10, a temperature-sensitive mutant whose anterograde IFT motor protein, kinesin II, loses its function at nonpermissive temperatures (≥33°C) but not at permissive temperatures (≤25°C; Walther et al., 1994; Kozminski et al., 1995). When this mutant was incubated at 25°C, TTLL9 and FAP234 signals were clearly observed in both the axoneme and the flagellar matrix fraction; however, the signals disappeared after the cells had been incubated at 33°C for 4 h, although the mutant cells remained flagellated (Figure 6, A and B). In contrast with fla10, wild-type cells showed TTLL9 and FAP234 signals in both axoneme and matrix fractions after incubation at 33°C for the same period. Thus the TTLL9-FAP234 complex is most likely recruited to the flagella by the IFT system. However, the level of
compared with wild type (Supplemental Figure S5, A and B). This result does not support the idea that TTLL9-FAP234 complexes are removed by retrograde IFT.

As another mechanism for the TTLL9-FAP234 removal from the flagella, we also examined the possibility that flagellar membrane vesicles containing these proteins are released from the flagellar tip. We collected flagellar vesicles from the culture medium of wild-type cells by the method of Dentler (2013). However, no TTLL9 or FAP234 signals were detected by Western blot analysis in the vesicles, although a flagellar membrane marker, FMG-1B, and small amounts of IFT proteins were detected (Supplemental Figure S5C). Therefore, it seems unlikely that TTLL9-FAP234 complexes are removed by excretion of membrane vesicles containing these proteins.

**DISCUSSION**

**Possible function of FAP234**

Isolation of a novel motility mutant, tpg2, led to the identification of FAP234 as a protein essential for the axonemal localization of the tubulin polyglutamylating enzyme TTLL9. Our results indicated that FAP234 forms a complex with TTLL9 in the axoneme, flagellar matrix, and cellular cytoplasm, suggesting its involvement in the stabilization and transport of TTLL9. However, the exact function of FAP234 and the structure of the FAP234-TTLL9 complex remain to be studied. Regarding the structure, TTLL9 (M, ~ 50 kDa) and

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**FIGURE 5:** Tubulin polyglutamylation occurs on assembled axonemal microtubules. (A) Indirect fluorescence observation of the WT, tpg1, and tpg2 gametes before fertilization. Methanol-fixed cells were treated with anti–polyglutamylated tubulin antibody (polyE) and anti–α-tubulin antibody (B-5-1-2). As shown in Figure 1D, the signal intensity of polyglutamylated tubulin was much lower in tpg1 and tpg2 flagella than in WT flagella. (B) Temporary dikaryons formed between WT and tpg2, and between WT and tpg1. Dikaryons were fixed with methanol at 10 or 60 min after onset of mating. Note that the flagella derived from tpg2 and tpg1 (arrows) showed increased polyglutamylation signals at ~60 min after mating. (C) Temporary dikaryons formed between tpg1 and tpg2. In contrast with dikaryons formed between WT and tpg mutants, polyglutamylation was only minimally recovered by ~60 min in either axoneme based on signal intensity compared with control intensity. Scale bars, 10 μm.
speculate that the axoneme contains a structure that binds the TTLL9-FAP234 complex but not FAP234 alone. Another possible function of FAP234 is to enhance the catalytic activity of TTLL9. In a study evaluating the catalytic activities of various TTLL proteins overexpressed in HeLa cells, van Dijk et al. (2007) showed that TTLL4, -5, -6, and -7 display high activities in tubulin polyglutamylation reactions, whereas TTLL1, -2, and -9 show only low activities. Of interest, TTLLs with high catalytic activities are significantly larger than those with low activities, leading these authors to suggest that additional factor(s) are necessary for the activity of the latter group (van Dijk et al. (2007)). In fact, TTLL1, an enzyme that catalyzes tubulin polyglutamylation in neurons, is believed to associate with four other subunits for its enzymatic activity (Janke et al., 2005). Although none of these proteins is structurally related to FAP234, TTLL9 may be catalytically active only when associated with other proteins, including FAP234.

Localization and dynamics of TTLL9-FAP234

The TTLL9-FAP234 complex appears to undergo dynamic IFT-dependent turnover in the flagellum. Dynamic localization of TTLL9-FAP234 is supported by the observations that it is present in the flagellar matrix fraction as well as in the axoneme, tubulin polyglutamylation occurs in the flagella of tpG1 or tpG2 gametes 60 min after fertilization. FAP234 (~177 kDa) sedimented in the same fraction between 75 and 115 (Figure 3E). These sedimentation coefficients were calibrated with aldolase (7 S, 156 kDa) and catalase (11 S, 240 kDa), both of which are globular tetrameric proteins. According to the molecular weights of subunits, the TTLL9-FAP234 complex may consist of a single molecule of each protein, assuming that its structure is fairly globular. However, if the structure is significantly elongated, it is possible that the complex contains two TTLL9 molecules and a single FAP234 or contains a third protein.

The observation that the cytoplasm of tpG2 lacks both FAP234 and TTLL9 (Figure 4A) suggests a function of FAP234 as a stabilizer of TTLL9 in the cytoplasm. Because this function can explain the absence of TTLL9 from the flagellar axoneme and matrix of tpG2 (Figure 3C), as well as the lack of dikaryon rescue capability between tpG1 and tpG2 gametes, the sole function of FAP234 could be to stabilize TTLL9 in the cytoplasm. However, the presence of FAP234 in the matrix fraction of the TTLL9-lacking tpG1 flagella indicates that FAP234 can be transported into flagella by itself and suggests its involvement in the intraflagellar transport of TTLL9; it may well function as a carrier or adapter for IFT. How it functions in the transport is an important subject of future studies. Our observation indicates that FAP234 is unable to bind to axonemes in the absence of TTLL9, although it is associated with axonemes in the wild type. We speculate that the axoneme contains a structure that binds the TTLL9-FAP234 complex but not FAP234 alone.

Another possible function of FAP234 is to enhance the catalytic activity of TTLL9. In a study evaluating the catalytic activities of various TTLL proteins overexpressed in HeLa cells, van Dijk et al. (2007) showed that TTLL4, -5, -6, and -7 display high activities in tubulin polyglutamylation reactions, whereas TTLL1, -2, and -9 show only low activities. Of interest, TTLLs with high catalytic activities are significantly larger than those with low activities, leading these authors to suggest that additional factor(s) are necessary for the activity of the latter group (van Dijk et al. (2007)). In fact, TTLL1, an enzyme that catalyzes tubulin polyglutamylation in neurons, is believed to associate with four other subunits for its enzymatic activity (Janke et al., 2005). Although none of these proteins is structurally related to FAP234, TTLL9 may be catalytically active only when associated with other proteins, including FAP234.
after mating with wild-type gametes, and TTLL9 and FAP234 rapidly disappear from the flagella of fla10 at nonpermissive temperatures. The last observation indicates that the flagellar entrance of TTLL9-FAP234 requires anterograde IFT. In contrast, our analyses indicated that the removal of these proteins from the flagella does not depend on retrograde IFT or vesicle excretion from flagella (Supplemental Figure S5). These observations favor the view that the TTLL9-FAP234 complex imported by anterograde IFT is subsequently lost from the flagella through proteolytic degradation. The ubiquitin ligase-like sequence in FAP234 may play a role in this putative degradation. However, whether these proteins actually undergo degradation in the flagella remains to be determined.

In contrast to dynamic localization of TTLL9 and FAP234 in the axoneme, long polyglutamyl side chains on the B-tubule apparently did not undergo rapid turnover because they persisted for a significantly longer period after TTLL9 and FAP234 disappeared from the flagellum of fla10 after transfer to nonpermissive temperatures. Several recent studies identified deglutamylases, which are enzymes that remove polyglutamate side chains from modified tubulin, and their results imply that tubulin polyglutamylation is a reversible process (Rogowski et al., 2010; Kimura et al., 2010). In C. elegans, a homologue of human deglutamylase CCP1 functions to regulate tubulin polyglutamylation in the axoneme to maintain optimal sensory activity (O’Hagan et al., 2011). Our results indicate that deglutamylation in the flagellum, if it occurs, is a slow process.

**TABLE 2: Conservation of TTLL9 and FAP234 among various organisms.**

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Phylum</th>
<th>Organism</th>
<th>Cilia/flagella</th>
<th>TTLL9</th>
<th>FAP234</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals</td>
<td>Chordata</td>
<td>Homo sapiens</td>
<td>M + +</td>
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<td></td>
<td></td>
<td>Mus musculus</td>
<td>M + +</td>
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<td></td>
<td></td>
<td>Monodelphis domestica</td>
<td>M + +</td>
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<tr>
<td></td>
<td></td>
<td>Bos taurus</td>
<td>M + +</td>
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<td></td>
<td></td>
<td>Danio rerio</td>
<td>M + +</td>
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<tr>
<td></td>
<td></td>
<td>Gallus gallus</td>
<td>M + +</td>
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<td></td>
<td></td>
<td>Ciona intestinalis</td>
<td>M + +</td>
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<td>Arthropoda</td>
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<td>Drosophila melanogaster</td>
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<tr>
<td></td>
<td></td>
<td>Caenorhabditis elegans</td>
<td>I + –</td>
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<tr>
<td>Cnidaria</td>
<td>Hydra magnipapillata</td>
<td>M – +</td>
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<tr>
<td>Protists</td>
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<tr>
<td></td>
<td>Tetrahymena thermophila</td>
<td>M + +</td>
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<td></td>
<td>Trypanosoma brucei</td>
<td>M + +</td>
<td></td>
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<tr>
<td>Plants</td>
<td>Oryza sativa</td>
<td>– – –</td>
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<td></td>
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<tr>
<td></td>
<td>Arabidopsis thaliana</td>
<td>– – –</td>
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<td></td>
<td></td>
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<tr>
<td>Diatom</td>
<td>Thalassiosira pseudonana</td>
<td>M ± –</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Green alga</td>
<td>Chlamydomonas reinhardtii</td>
<td>M + +</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Moss</td>
<td>Physcomitrella patens</td>
<td>M + +</td>
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<tr>
<td>Fungi</td>
<td>Aspergillus clavatus</td>
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<tr>
<td></td>
<td>Saccharomyces cerevisiae</td>
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<td></td>
<td>Schizosaccharomyces pombe</td>
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</table>

Amino acid sequence of TTLL9 and FAP234 were subjected to Blast search to find homologues in various organisms. Both TTLL9 and FAP234 are highly conserved among organisms possessing cilia and flagella. These data suggest that FAP234 functions in combination with TTLL9 in most ciliated/flagellated organisms. TTLL9 in the regulation of inner-arm dynein, it is particularly interesting that no homologues are present in the diatom Thalassiosira pseudonana, which has motile flagella and genes of outer-arm dynein subunits but no genes of inner-arm dynein subunits (Merchant et al., 2007). Although TTLL9 and FAP234 may have some motility-independent function in organisms with nonmotile cilia, these data prompt us to propose that TTLL9-FAP234 complex functions as a unique tubulin-polyglutamylation system that regulates the

**TTLL9-FAP234 is conserved as a cilia-specific tubulin polyglutamylation system.**

A database search indicated that homologues of TTLL9 and FAP234 are present in most organisms that have cilia but absent in organisms that do not have cilia (Table 2). It is interesting to note that the pair of TTLL9 and FAP234 is best conserved in organisms that have motile cilia, although not all organisms having motile cilia retain the pair, and C. elegans has an orthologue of TTLL9 despite possessing only nonmotile cilia.
activity of inner-arm dyneins, particularly those that influence the function of DRC.

**MATERIALS AND METHODS**

**Strains and culture**

The strains used in this study included *C. reinhardtii* wild-type CC-124 and the mutants listed in Supplemental Table S1. Two alleles of the mutant tpg2 were produced in our laboratory by UV mutagenesis of wild-type *C. reinhardtii* (137c), followed by screening for slow-swimming phenotypes. A double mutant of tpg2 with oda6 was produced using standard procedures (Harris, 2009). A transformant (designated “1H”) expressing HA-tagged TTL9 (FAP267), oda2tpg1::TTL9HA, was constructed by introducing the genomic TTL9 sequence fused with a triple HA tag sequence into nonmotile *oda2tpg1* and selecting clones that showed recovery of flagellar motility. Cells were grown in Tris–acetate–phosphate medium with aeration on a 12 h/12 h light/dark cycle.

**Identification of the tpg2 mutation**

AFLP analysis of progeny from the S1-D2 strain crosses (Kathir et al., 2003) mapped both tpg2-1 and tpg2-2 to a 531-kb genomic region on linkage group I. This region contained two genes registered in the *C. reinhardtii* database (Pazour et al., 2005), and FAP234 was mutated in both alleles. The FAP234 gene was confirmed to cause the tpg2 mutation by the observation of motility recovery in nonmotile *oda6tpg2* cells transformed with FAP234 cDNA.

**Cloning of FAP234 cDNA and production of antibodies**

The coding region of FAP234 cDNA was amplified by reverse transcription PCR (RT-PCR) using mRNA from wild-type cells. Primers used to sequence FAP234 cDNA are listed in Supplemental Table S2. The FAP234 cDNA sequence has been deposited in the DNA Data Bank of Japan under accession number AB781330. PCR products were digested with BamHI and HindIII at restriction sites within the primer sequences and ligated into the bacterial expression vector pCold I (Takara, Shiga, Japan). Expression of the recombinant proteins was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside to the bacterial culture, followed by cold shock according to the manufacturer’s instructions. Nearly all expressed proteins were present in inclusion bodies, which were pelleted by centrifugation and fractionated into 19–21 aliquots (0.25 ml). Sedimentation coefficients were estimated using ribonuclease A (−25S), bovine serum albumin (−4S), aldolase (−7S), catalase (−11S), and three-headed outer-arm dynein in the axonemal extract (−23S; Takada et al., 1992).

**Sucrose density gradient centrifugation**

Soluble fractions from the axoneme or the cell lysates were fractionated through 5 ml of 5–20% sucrose density gradients in HMDEK. The gradients were centrifuged at 240,000 × g for 5 h, 30 min and fractionated into 19–21 aliquots (0.25 ml). Samples were washed three times with HMDEK before Western blotting was performed. The cytoplasmic extract was obtained by sonication of *oda2tpg1::TTL9HA* cells followed by centrifugation to remove the insoluble materials. The extract was mixed with Protein G agarose (Roche) and anti–HA tag antibody (3f10; Roche) and processed as described.

**Gel electrophoresis and Western blotting**

Axonemal and cytoplasmic proteins were resolved using SDS–PAGE on 7.5 or 9% gels (Laemmli, 1970). Gels were stained with Coomassie brilliant blue (CBB) or silver. Western blotting was performed as described by Towbin et al. (1979). Primary antibodies used are listed in Supplemental Table S3.

**Immunofluorescence microscopy**

Immunofluorescence microscopy was carried out according to the method described by Sanders and Salisbury (1995). In most experiments, samples were double stained with rabbit polyclonal polyclonal antibody (kind gift from M. A. Gorovsky, University of Rochester, Rochester, NY) and mouse monoclonal anti–α-tubulin (B-5-1-2; Sigma-Aldrich, St. Louis, MO) or mouse monoclonal anti-acetylated α-tubulin antibody (6-11B-1; Abcam, Cambridge, MA). The secondary antibodies used were anti-rabbit immunoglobulin G (IgG) antibody conjugated with Alexa 488 (Invitrogen, Carlsbad, CA) and anti-mouse IgG antibody conjugated with rhodamine (Santa Cruz Biotechnology, Santa Cruz, CA).

**Assessment of flagellar motility**

Swimming velocities were measured by tracking images of swimming cells acquired using a dark-field microscope with a 40× objective and a charge-coupled device camera. Flagellar beat frequencies were estimated from the frequencies of cell body vibration (Kamiya, 2000).

**ACKNOWLEDGMENTS**

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