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

Tomohiro Kubo[a,b], Haru-aki Yanagisawa[c], Zhongmei Liu[a], Rie Shibuya[a], Masafumi Hirono[a], and Ritsu Kamiya[a,d]

[a]Department of Biological Sciences, Graduate School of Science, and [c]Department of Cell Biology and Anatomy, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan; [b]Department of Cell and Developmental Biology, University of Massachusetts Medical School, Worcester, MA 01655; [d]Department of Life Science, Faculty of Science, Gakushuin University, Toshima-ku, Tokyo 171-8588, Japan

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.**

Monitoring Editor
Keith G. Kozminski
University of Virginia

Received: Jul 31, 2013
Revised: Oct 21, 2013
Accepted: Oct 29, 2013

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). These enzymes catalyze either the addition of a glutamic acid to the γ-carboxyl group of glutamate in the C-terminal region of tubulin or the elongation of branched glutamate chains by successive addition of glutamates to the α-carboxyl group. The former reaction is carried out by "initiase" TTLLs, which include TTLL4, TTLL5, and TTLL7, whereas the latter reaction is carried out by "elongase" TTLLs, which include TTLL6, TTLL9, and TTLL11 (van Dijk *et al.*, 2007). Some TTLLs, such as TTLL1, catalyze both reactions (Janke *et al.*, 2008).

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-07-0424) on November 6, 2013.

Address correspondence to: Ritsu Kamiya (ritsu.kamiya@gmail.com).

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

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; Wloga *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* (*tpg1*), which lacks TTLL9 (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, *tpg2*, which has a phenotype similar to that of TTLL9-lacking *tpg1* (Kubo *et al*., 2010). Genetic and biochemical analyses show that *tpg2* possesses a mutation in FAP234, a conserved flagella-associated protein of unknown function, and that FAP234 forms a complex with TTLL9 in the axoneme and cytoplasm. Furthermore, by using a temperature-sensitive kinesin II–deficient mutant, we find that the TTLL9-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 polyglutamylase in the axoneme.

RESULTS

A novel *Chlamydomonas* mutant, *tpg2*, exhibits a tubulin polyglutamylation defect

Two mutants that were phenotypically similar to *tpg1* were isolated using ultraviolet (UV) mutagenesis of wild-type cells and screening for slow-swimming phenotypes. Because these two mutants were genetically distinct from *tpg1* and carried mutations in a gene involved in axonemal tubulin polyglutamylation (see later discussion), we designated the mutants as *tpg2-1* and *tpg2-2*. In most experiments, we used the mutants interchangeably. Thus we here refer to the mutants as *tpg2*, unless it is necessary to distinguish between them. The motility of *tpg2* was indistinguishable from that of *tpg1*; 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 *tpg1*, *tpg2* was completely no-motile in the background of a mutation causing the loss of outer-arm dynein (Supplemental Figure S1, A and B). Also similar to *tpg1*, *tpg2* showed a normal composition of axonemal dyneins (Supplemental Figure S2).

Polyglutamylated tubulin was significantly reduced in the *tpg2* 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 *tpg1* and *tpg2* 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 *tpg1* and *tpg2* axonemes than in wild-type axonemes. Immunofluorescence microscopy of *tpg2* 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 *tpg1* axoneme (Figure 1D; Kubo *et al*., 2010).

tpg2 has a mutation in FAP234, a conserved flagella-associated protein

Both *tpg2-1* and *tpg2-2* 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 *tpg2-1* and *tpg2-2* possess mutations in the gene encoding FAP234, a 177-kDa flagella-associated protein of unknown function. The *tpg2-1* mutant contained a deletion between exons 26 and 36, whereas *tpg2-2* 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 TTLL9, 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 *tpg2-1*; thus the antibody should detect a truncated version of the protein if one were produced in *tpg2*. 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 *tpg2* 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 *tpg2* must have been degraded in the cytoplasm. Despite repeated trials, we were unable to detect FAP234 signals in wild-type cells or axonemes by

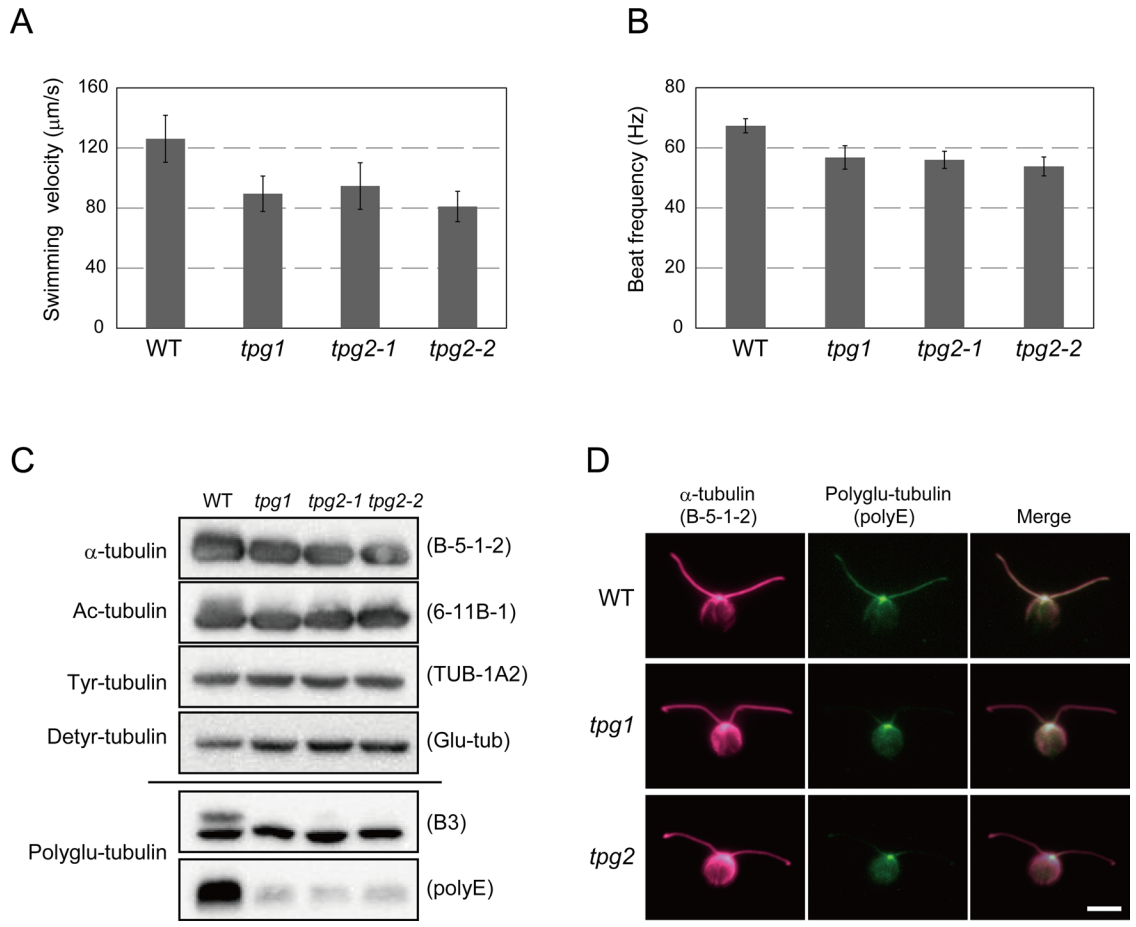

FIGURE 1: The novel *Chlamydomonas* mutant *tpg2* is phenotypically similar to *tpg1*, a mutant deficient in the tubulin polyglutamylase TTLL9. (A) Swimming velocities of the wild type (WT), *tpg1*, *tpg2-1*, and *tpg2-2*. The averages and SDs (error bars) measured in >20 cells for each sample. (B) Beat frequencies estimated from the vibration of cell bodies. (C) Western blotting analysis of the axonemes. One microgram each of WT, *tpg1*, *tpg2-1*, and *tpg2-2* was run on SDS–PAGE and analyzed using antibodies specific to anti–α-tubulin (B-5-1-2), anti–acetylated tubulin (6-11B-1), anti–tyrosinated tubulin (TUB-1A2), anti–detyrosinated tubulin (Glu-tub), and anti–polyglutamylated tubulin (B3 and polyE). Marked differences were detected by the B3 and polyE antibodies between WT and *tpg* mutants. (D) Immunofluorescence microscopy using the polyE antibody. The cells of WT, *tpg1*, and *tpg2-1* (*tpg2*) were fixed and double stained with anti–α-tubulin antibody (B-5-1-2) and polyE antibody. The signal of tubulin possessing a long polyglutamylated chain was detected by the polyE antibody along the entire length of the wild-type axoneme. In contrast, only very weak signals were observed in the axonemes of *tpg1* and *tpg2* mutants. Similar intensities of polyE signals were observed in the basal bodies of WT, *tpg1*, and *tpg2* cells, indicating that polyglutamylase(s) other than TTLL9 are present to polyglutamylate the basal body tubulin. Scale bar, 10 μm.

immunofluorescence microscopy using these antibodies (unpublished data).

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.

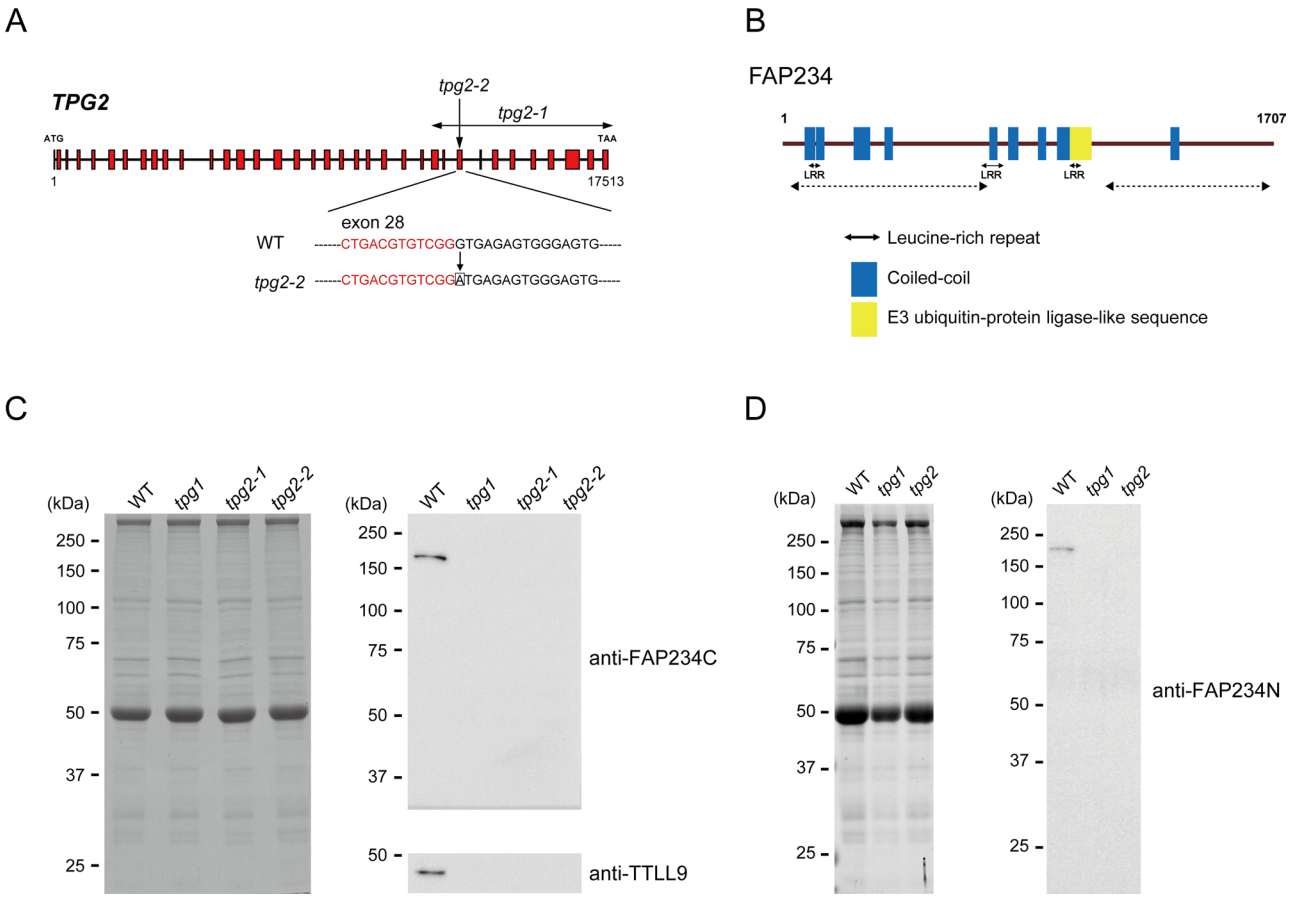

FIGURE 2: The mutant *tpg2* carries a mutation in the gene encoding FAP234, a flagella-associated protein of unknown function. (A) Schematic illustration of the genomic DNA sequence of FAP234. The red regions indicate exons. RT-PCR analyses showed that *tpg2-1* cDNA contains a deletion between exons 26 exon 36, and *tpg2-2* cDNA has a single-base substitution near the 5′ end of exon 28, resulting in deletion of the entire exon 28. (B) Known motifs in FAP234. Secondary structure prediction using $SOSUI_{coil}$ suggested that FAP234 has nine coiled-coil domains (shown in blue), a leucine-rich repeat (double arrows in solid line), and an E3 ubiquitin protein ligase–like sequence (yellow). The two double arrows in dotted line indicate sequences used to produce antibodies against N- and C-terminal portions (termed FAP234N and FAP234C antibodies). (C, D) CBB staining (left) and Western blotting analysis (right) of the WT, *tpg1*, *tpg2-1*, and *tpg2-2* axonemes using (C) the anti-FAP234C antibody and anti-TTLL9 antibody and (D) the anti-FAP234N antibody (only the result of the *tpg2-1* is shown). Five micrograms of samples was used for each lane. Bands corresponding to FAP234 (177 kDa) and TTLL9 (50 kDa) were detected in wild-type axonemes, whereas both signals were absent in *tpg1*, *tpg2-1*, and *tpg2-2* axonemes.

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

Organism	Name	Locus	Maximum score	BLAST *E* value
Homo sapiens	Unnamed protein product	BAC87242.1	450	2e-138
Mus musculus	Leucine-rich repeat–containing protein 9 isoform 1	NP_001136200.1	588	0.0
Danio rerio	Leucine-rich repeat–containing protein 9	NP_001107274.2	616	0.0
Tetrahymena thermophila	Leucine-rich repeat family protein	XM_001022238.1	474	1e-145
Trypanosoma cruzi	Hypothetical protein	XP_815521.1	388	4e-115
Caenorhabditis elegans	Protein K 10D2.8	NM_001267995.1	104	5e-28

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).

TABLE 1: Putative homologues of FAP234.

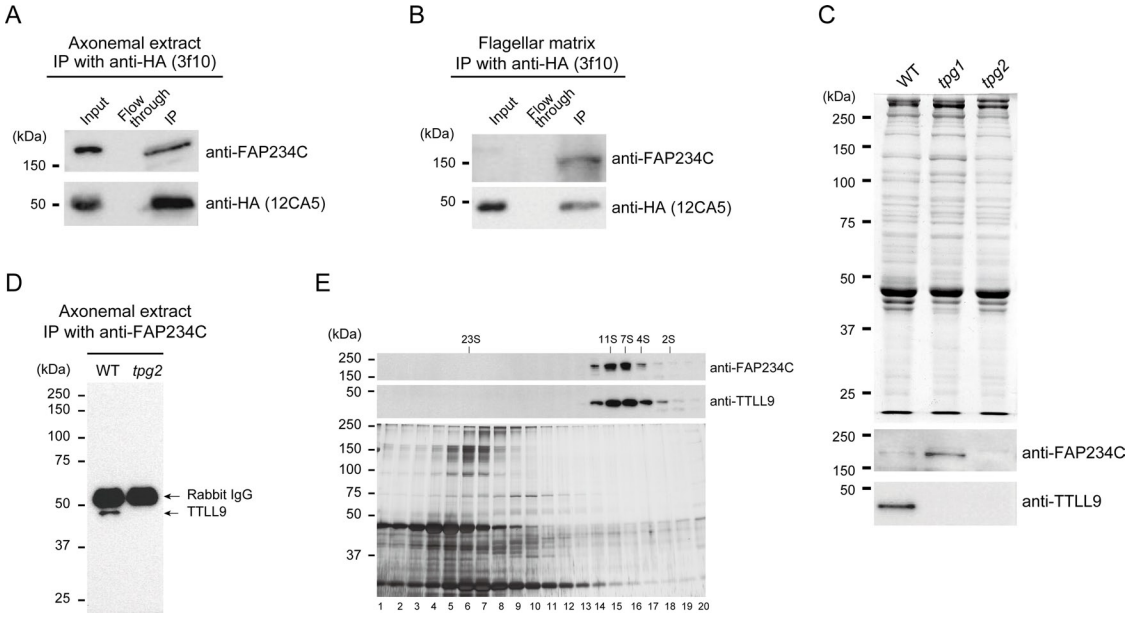

FIGURE 3: TTLL9 and FAP234 interact in the axoneme and flagellar matrix. (A, B) Coimmunoprecipitation of FAP234 with TTLL9 from the high-salt axonemal extract (A) and flagellar matrix fraction (B). A high-salt axonemal extract and a flagellar matrix fraction were prepared from *oda2tpg1::TTLL9HA* and 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 (Fan *et al.*, 2010) 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 (M_r = 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.

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.

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 the precipitates formed by addition of anti-HA antibody (3f10)–conjugated beads (Figure 4B), indicating that TTLL9 and FAP234 are associated in the cytoplasm. Furthermore, when the 1H cell lysate was centrifuged on a sucrose density gradient, TTLL9HA and FAP234 peaked in the same fraction (7–11S; Figure 4C), similar to the observation with the axonemal extract (Figure 3E). Thus TTLL9 and FAP234 most likely form a complex in the cytoplasm.

Band intensities of TTLL9 in the axonemal and cytoplasmic extracts, corrected for the total volume of the samples, suggested that the amount of TTLL9 in the cytoplasm was ~10 times greater than the amount in the axoneme (Figure 4D).

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

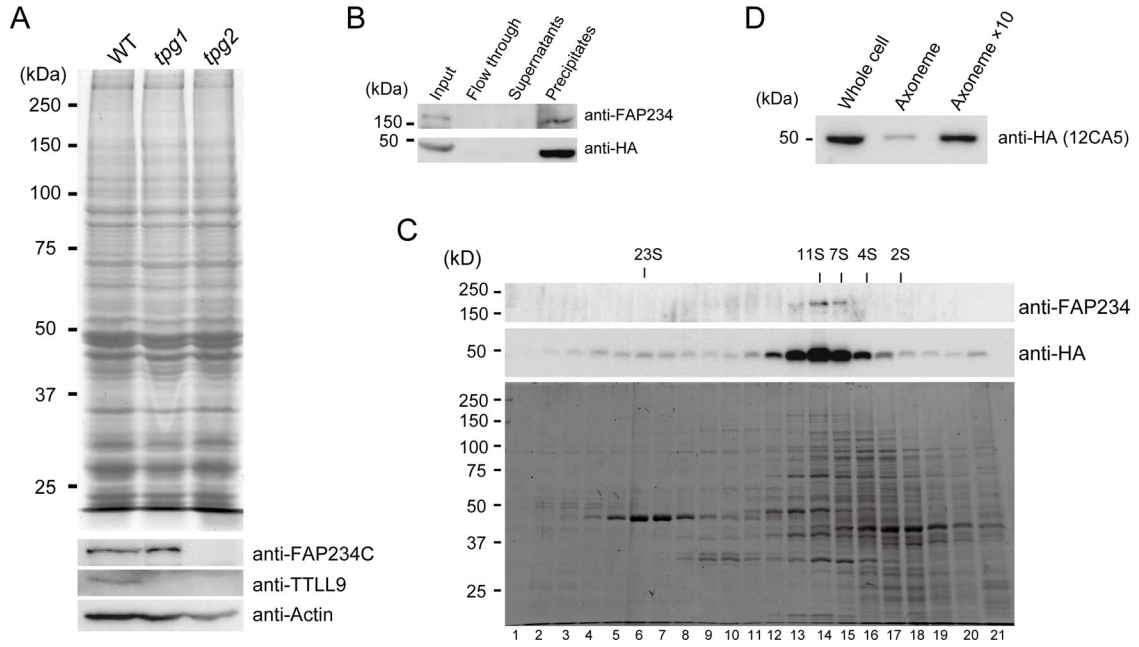

FIGURE 4: TTLL9 and FAP234 associate in the cytoplasm. (A) Western blotting analysis of the cytoplasmic proteins from the WT, *tpg1*, and *tpg2* with anti-TTLL9 and anti-FAP234C antibodies. Bands corresponding to TTLL9 and FAP234 were detected in the WT cytoplasm, whereas only FAP234 was detected in the *tpg1* cytoplasm, and neither protein was detected in the *tpg2* cytoplasm. These patterns suggest that FAP234 is stable in the cytoplasm without associating with TTLL9 but that TTLL9 is unstable in the absence of FAP234. (B) Western blotting analysis of the 1H (*oda2tpg1::TTLL9HA*) cell lysate immunoprecipitated with an anti–HA tag antibody (3f10). Input, flowthrough, supernatant, and immunoprecipitated fractions were blotted with the anti-FAP234 antibody (top) or the anti–HA tag antibody (12CA5) (bottom). Gel was stained with CBB (bottom). FAP234 was detected in the immunoprecipitate. (C) Sedimentation of 1H cell lysate on a 5–20% sucrose density gradient. Aliquots were blotted with the anti–HA tag antibody (12CA5) and the anti-FAP234C antibody. As in the experiment with the axonemal extract (Figure 3), TTLL9HA and FAP234 signals showed peaks at 7–11S. (D) Estimated ratio of the amount of TTLL9 in the cell body and that in the axoneme. Loaded on each lane are 10 µg of whole-cell body proteins (left), axonemal proteins equivalent to those isolated from 10 µg of cell bodies (middle), and a 10-fold amount of the axoneme (right).

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

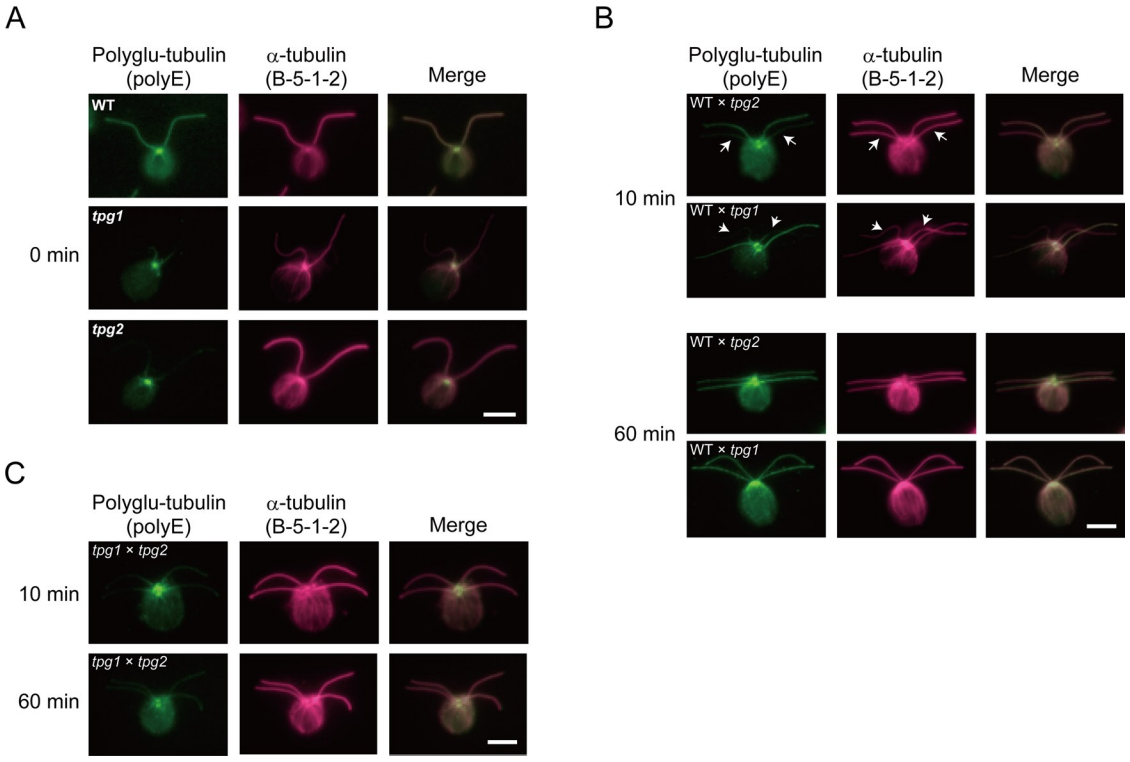

FIGURE 5: Tubulin polyglutamylation occurs on assembled axonemal microtubules. (A) Indirect fluorescence observation of the WT, *tpg1*, and *tpg2* gametes before fertilization. Methanol-fixed cells were treated with anti–polyglutamylated tubulin antibody (polyE) and anti–α-tubulin antibody (B-5-1-2). 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.

polyglutamylated tubulin did not change quickly after *fla10* cells were transferred to nonpermissive temperatures; polyE antibody-staining intensity did not noticeably change 4 h after the onset of exposure to 33°C, when no TTLL9-FAP234 complex was detectable in the axoneme or the flagellar matrix (Figure 6C). This observation suggests that polyglutamylation turnover in the axoneme, if any, proceeds rather slowly.

The experiment on *fla10* raised another question: how are the TTLL9-FAP234 complexes removed from the flagella when kinesin II is blocked, or in other words, how is the complex being turned over under normal conditions? One possibility is that the removal is caused by retrograde IFT. To examine this, we used *d1blic*, a mutant lacking the light IC (LIC) of cytoplasmic dynein 1b (Hou *et al*., 2004), and *dhc1b-2*, a mutant possessing a hypomorphic allele of the dynein 1b heavy chain gene (Lechtreck *et al*., 2009, 2013; Witman, 2012). These mutants are defective in retrograde IFT due to the dysfunction of cytoplasmic dynein 1b and display abnormal accumulation of IFT particle proteins in the flagella (Hou *et al*., 2004; Lechtreck *et al*., 2013). We reasoned that TTLL9 and FAP234, as well as IFT particles, would accumulate in these mutants' flagella if retrograde IFT were responsible for the export of the TTLL9-FAP234 complex from the flagella. However, contrary to our expectation, the amounts of TTLL9 and FAP234 in both *d1blic* and *dhc1b-2* flagella appeared to be slightly decreased, rather than increased, 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_r ≈ 50 kDa) and

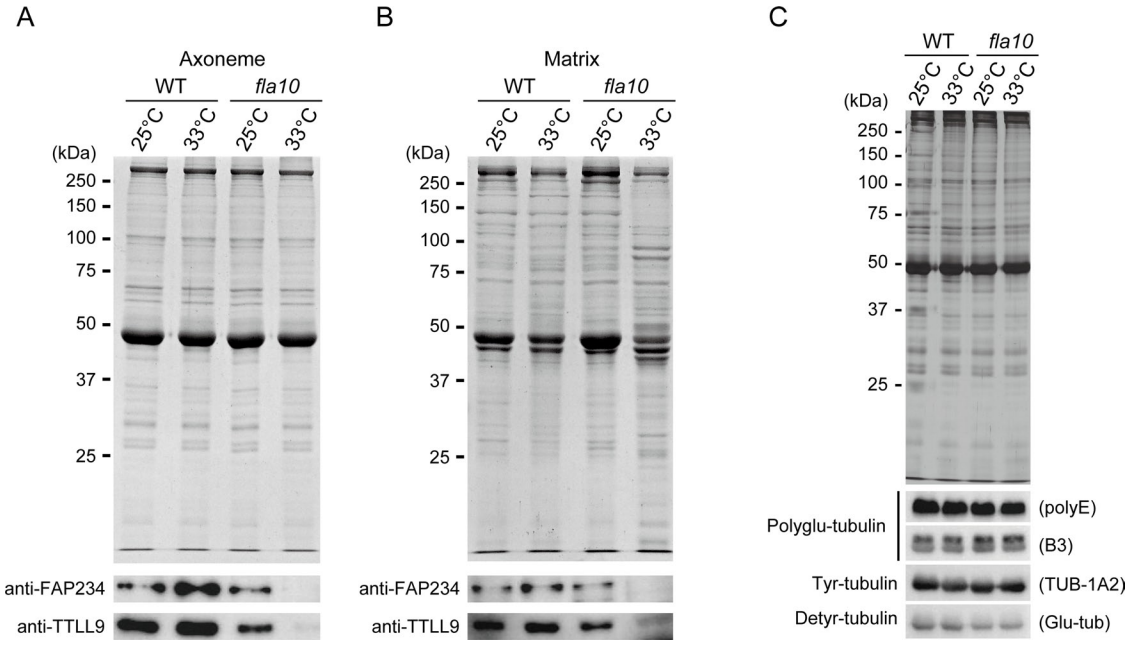

FIGURE 6: Transport of TTLL9-FAP234 to the flagellum depends on IFT. (A) Change in the quantity of axonemal TTLL9 and FAP234 after cell incubation at two different temperatures. SDS–PAGE pattern stained with CBB (top) and Western blotting patterns (bottom) of axonemes of WT and *fla10* cells that had been cultured at a permissive temperature (25°C) for 3 d and kept at the same temperature or exposed to a nonpermissive temperature (33°C) for additional 4 h. Immunoblotting was performed using anti-FAP234C and anti-TTLL9 antibodies. After incubation at a permissive temperature, FAP234 and TTLL9 signals were detected in both WT and *fla10* axonemes. After incubation at a nonpermissive temperature, the signals of both proteins were observed in the axoneme of WT but not in the axoneme of *fla10*, a mutant with a temperature-sensitive mutation in a subunit of the motor protein kinesin II. (B) Flagellar matrix fraction after cell incubation at two different temperatures. CBB staining pattern (top) and Western blotting analysis (bottom) of flagellar matrix of WT and *fla10* by using anti-FAP234C and anti-TTLL9 antibodies. (C) Tubulin polyglutamylation in axonemes after cell incubation at two different temperatures. Silver staining (top) and Western blotting of axonemes (bottom) using anti–polyglutamylated tubulin (B3 and polyE), anti–tyrosinated tubulin (TUB-1A2), and anti–detyrosinated tubulin (Glu-tub). No obvious differences were observed between WT and *fla10* axonemes, suggesting that loss of the TTLL9-FAP234 complex from the axoneme does not immediately affect the level of tubulin polyglutamylation.

FAP234 (~177 kDa) sedimented in the same fraction between 7S and 11S (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.

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

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

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. *T. pseudonana* (which lacks inner arm dynein) lacks these proteins, whereas *P. patens* (which lacks outer arm dynein) retains them. This is consistent with TTLL9/FAP234 functioning in the regulation of inner arm dynein. - : No similarity found or Max score = 0 ~ 149 in Protein Blast search, ±: 150 ~ 299, +: 300 ~, M: motile cilia/flagella, I: immotile cilia.

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

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 the polyglutamylation level 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.

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.

We previously reported that long-chain polyglutamylation by TTLL9 affects the activity of a specific kind of inner-arm dynein known as dynein e, an inner-arm dynein associated with the dynein regulatory complex (DRC), but does not significantly affect the function of other kinds of dyneins (Kubo *et al.*, 2012). TTLL9-FAP234 does not seem to modulate IFT because the kinetics of flagellar regeneration in *tpg1* was nearly identical to that in the wild type (Kubo *et al.*, 2010). Regarding the possible function of 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-polyglutamylating system that regulates the

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 TTLL9 (FAP267), *oda2tpg1::TTLL9HA*, was constructed by introducing the genomic TTLL9 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 flagellar proteome 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 *Bam*HI and *Hin*dIII 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 analyzed by SDS–PAGE. The band corresponding to the N-terminal sequence (684 amino acids) or the C-terminal sequence (572 amino acids) of FAP234 (Figure 2B) was removed from the gel and used to immunize two rabbits. The resulting antisera were blot purified using the antigen protein blotted onto a polyvinylidene fluoride membrane (Olmsted, 1981).

Preparation of flagellar proteins and cytoplasmic proteins

Axonemes were isolated using the method described by Witman *et al.* (1978). A flagellar matrix fraction was isolated based on the freeze-thaw method described by Fan *et al.* (2010). The resulting fractions were centrifuged to remove flagella and, if necessary, concentrated by chloroform and methanol treatment (Wessel and Flügge, 1984). Cytoplasmic proteins were prepared according to Fowkes and Mitchell (1998). Briefly, cells were treated with methanol and chloroform to remove DNA and RNA. Cytoplasmic proteins were solubilized in a solution containing 5 M urea, 2 M thiourea, and 0.05% Triton X-100.

Immunoprecipitation

Immunoprecipitation from axonemal and cytoplasmic extracts was performed using an anti-FAP234 antibody or an anti–HA tag antibody (12CA5 and 3f10; Roche, Basel, Switzerland). Because the two anti–HA tag antibodies differed in titer and specificity, different antibodies were used for different types of experiments. Axonemal extract was obtained by treating axonemes with 0.6 M KCl in HMDEK solution (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM ethylene glycol tetraacetic acid, and 50 mM K acetate). Protein A agarose beads (Roche) were mixed with desalted axonemal extract and anti-FAP234 antibody, and the mixture was incubated for 1 h. The beads were washed three times with HMDEK before Western blotting was performed. The cytoplasmic extract was obtained by sonication of *oda2tpg1::TTLL9HA* 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.

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). Sedimentation coefficients were estimated using ribonuclease A (~2S), bovine serum albumin (~4S), aldolase (~7S), catalase (~11S), and three-headed outer-arm dynein in the axonemal extract (~23S; Takada *et al.*, 1992).

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 polyE 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

We thank Naomi Miyamoto for excellent technical assistance and George B. Witman (University of Massachusetts Medical School, Worcester, MA) for critically reading the manuscript and providing antibodies for various IFT proteins. This study was supported by grants-in-aid from the Japan Society for Promotion of Science (23657046 and 24370079 to M.H. and 23570189 to R.K.), a Japan Society for the Promotion of Science Postdoctoral Fellowship, and a Uehara Memorial Foundation Research Fellowship for Research Abroad (to T.K.).

REFERENCES

Dentler W (2013). A role for the membrane in regulating *Chlamydomonas* flagellar length. PLoS One 8, E53366.

Fan ZC, Behal RH, Geimer S, Wang Z, Williamson SM, Zhang H, Cole DG, Qin H (2010). *Chlamydomonas* IFT70/CrDYF-1 is a core component of IFT particle complex B and is required for flagellar assembly. Mol Biol Cell 21, 2696–2706.

Fowkes ME, Mitchell DR (1998). The role of preassembled cytoplasmic complexes in assembly of flagellar dynein subunits. Mol Biol Cell 9, 2337–2347.

Harris E (2009). The *Chlamydomonas* Sourcebook, Vol.1, 2nd ed., Amsterdam: Academic Press.

Hou Y, Pazour GJ, Witman GB (2004). A dynein light intermediate chain, D1bLIC is required for retrograde intraflagellar transport. Mol Biol Cell 15, 4382–4394.

Ikegami K *et al.* (2007). Loss of alpha-tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function. Proc Natl Acad Sci USA 104, 3213–3218.

Ikegami K, Sato S, Nakamura K, Ostrowski LE, Setou M (2010). Tubulin polyglutamylation is essential for airway ciliary function through the regulation of beating asymmetry. Proc Natl Acad Sci USA 107, 10490–10495.

Janke C, Bulinski JC (2011). Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. Nat Rev Mol Cell Biol 12, 773–786.

Janke C, Rogowski K, van Dijk J (2008). Polyglutamylation: a fine-regulator of protein function? "Protein modifications: beyond the usual suspects" review series. EMBO Rep 9, 636–641.

Janke C *et al.* (2005). Tubulin polyglutamylase enzymes are members of the TTL domain protein family. Science 308, 1758–1762.

Kamiya R (2000). Analysis of cell vibration for assessing axonemal motility in *Chlamydomonas*. Methods 22, 383–387.

Kathir P, LaVoie M, Brazelton WJ, Haas NA, Lefebvre PA, Silflow CD (2003). Molecular map of the *Chlamydomonas reinhardtii* nuclear genome. Eukaryot Cell 2, 362–379.

Kimura Y, Kurabe N, Ikegami K, Tsutsumi K, Konishi Y, Kaplan OI, Kunitomo H, Iino Y, Blacque OE, Setou M (2010). Identification of tubulin deglutamylase among *Caenorhabditis elegans* and mammalian cytosolic carboxypeptidases (CCPs). J Biol Chem. 285, 22936–22941.

Konno A, Setou M, Ikegami K (2012). Ciliary and flagellar structure and function—their regulations by posttranslational modifications of axonemal tubulin. Int Rev Cell Mol Biol 294, 133–170.

Kozminski KG, Beech PL, Rosenbaum JL (1995). The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. J Cell Biol 131, 1517–1527.

Kubo T, Yagi T, Kamiya R (2012). Tubulin polyglutamylation regulates flagellar motility by controlling a specific inner-arm dynein that interacts with the dynein regulatory complex. Cytoskeleton 69, 1059–1068.

Kubo T, Yanagisawa HA, Yagi T, Hirono M, Kamiya R (2010). Tubulin polyglutamylation regulates axonemal motility by modulating activities of inner-arm dyneins. Curr Biol 20, 441–445.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lechtreck KF, Geimer S (2000). Distribution of polyglutamylated tubulin in the flagellar apparatus of green flagellates. Cell Motil Cytoskeleton 47, 219–235.

Lechtreck KF, Johnson EC, Sakai T, Cochran D, Ballif BA, Rush J, Pazour GJ, Ikebe M, Witman GB (2009). The *Chlamydomonas reinhardtii* BBSome is an IFT cargo required for export of specific signaling proteins from flagella. J Cell Biol 187, 1117–1132.

Lechtreck KF, Brown JM, Sampaio JL, Craft JM, Shevchenko A, Evans JE, Witman GB (2013). Cycling of the signaling protein phospholipase D through cilia requires the BBSome only for the export phase. J Cell Biol 201, 249–261.

Lee JE *et al.* (2012). CEP41 is mutated in Joubert syndrome and is required for tubulin glutamylation at the cilium. Nat Gene 44, 193–199.

Merchant SS *et al.* (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318, 245–250.

O'Hagan R, Piasecki BP, Silva M, Phirke P, Nguyen KC, Hall DH, Swoboda P, Barr MM (2011). The tubulin deglutamylase CCPP-1 regulates the function and stability of sensory cilia in *C. elegans*. Curr Biol 21, 1685–1694.

Olmsted JB (1981). Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. J Biol Chem 256, 11955–11957.

Pathak N, Austin CA, Drummond IA (2011). Tubulin tyrosine ligase-like genes ttll3 and ttll6 maintain zebrafish cilia structure and motility. J Biol Chem 286, 11685–11695.

Pathak N, Obara T, Mangos S, Liu Y, Drummond IA (2007). The zebrafish fleer gene encodes an essential regulator of cilia tubulin polyglutamylation. Mol Cell Biol 18, 4345–4364.

Pazour GJ, Agrin N, Leszyk J, Witman GB (2005). Proteomic analysis of a eukaryotic cilium. J Cell Biol 170, 103–113.

Rogowski K *et al.* (2010). A family of protein-deglutamylating enzymes associated with neurodegeneration. Cell 143, 564–578.

Rosenbaum JL, Witman GB (2002). Intraflagellar transport. Nat Rev Mol Cell Biol 3, 813–825.

Sanders MA, Salisbury JL (1995). Immunofluorescence microscopy of cilia and flagella. Methods Cell Biol 47, 163–169.

Suryavanshi S *et al.* (2010). Tubulin glutamylation regulates ciliary motility by altering inner dynein arm activity. Curr Biol 20, 435–440.

Takada S, Sakakibara H, Kamiya R (1992). Three-headed outer arm dynein from *Chlamydomonas* that can functionally combine with outer-arm-missing axonemes. J Biochem 111, 758–762.

Towbin H, Staehelin T, Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76, 4350–4354.

van Dijk J, Rogowski K, Miro J, Lacroix B, Eddé B, Janke C (2007). A targeted multienzyme mechanism for selective microtubule polyglutamylation. Mol Cell 26, 437–448.

Walther Z, Vashishtha M, Hall JL (1994). The *Chlamydomonas* FLA10 gene encodes a novel kinesin-homologous protein. J Cell Biol 126, 175–188.

Wessel D, Flügge UI (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138, 141–143.

Witman GB (2012). Dynein and intraflagellar transport. In: Dyneins: Structure, Biology and Disease, ed. SM King, New York: Elsevier, 395–421.

Witman GB, Plummer J, Sander G (1978). *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules. Structure, composition, and function of specific axonemal components. J Cell Biol 76, 729–747.

Wloga D, Dave D, Meagley J, Rogowski K, Jerka-Dziadosz M, Gaertig J (2010). Hyperglutamylation of tubulin can either stabilize or destabilize microtubules in the same cell. Eukaryot Cell 9, 184–193.