Current Biology 20, 441–445, March 9, 2010 ©2010 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.12.058

Report

Tubulin Polyglutamylation Regulates Axonemal Motility by Modulating Activities of Inner-Arm Dyneins

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Summary

Tubulin polyglutamylation is a modification that adds multiple glutamates to the γ-carboxyl group of a glutamate residue in the C-terminal tails of α- and β-tubulin [1, 2]. This modification has been implicated in the regulation of axonal transport and ciliary motility. However, its molecular function in cilia remains unknown. Here, using a novel *Chlamydomonas reinhardtii* mutant (*tpg1*) that lacks a homolog of human TTLL9, a glutamic acid ligase enzyme [3], we found that the lack of a long polyglutamate side chain in α-tubulin moderately weakens flagellar motility without noticeably impairing the axonemal structure. Furthermore, the double mutant of *tpg1* with *oda2*, a mutation that leads to loss of outer-arm dynein, completely lacks motility. More surprisingly, when treated with protease and ATP, the axoneme of this paralyzed double mutant displayed faster microtubule sliding than the motile *oda2* axoneme. These and other results suggest that polyglutamylation directly regulates microtubule-dynein interaction mainly by modulating the function of inner-arm dyneins.

Results and Discussion

Tubulin Polyglutamylation Is Decreased in a TTLL9-Deficient Mutant

We isolated a novel *Chlamydomonas* mutant, *tpg1*, by screening ultraviolet irradiation-mutagenized cells for low motility [4]. Amplified fragment length polymorphism analysis after genetic cross with the S1-D2 strain [5] mapped this mutation to a region on linkage group XIX. This region contained three genes coding for proteins FAP59, RPL24, and FAP267, registered in the flagellar proteome database [6]. Sequence analysis of genomic DNA revealed that the isolate has a 502 bp deletion that covers the entire exon 7 sequence of the gene encoding FAP267, a homolog of TTLL9 of mammals (Figures 1A and 1B; see also Figures S1A and S1B available online). TTLL9 is one of tubulin tyrosine ligase-like proteins (TTLLs) with tubulin glutamic acid ligase activity on α-tubulin [7, 8]. The *tpg1* mutation was found to have a deletion in the C-terminal third of this protein (CrTTLL9) where the ATP binding site is located (Figure 1A). Hence, the product of the mutated gene, if any, should lack the enzymatic activity.

Immunoblot with the polyE antibody, which recognizes tubulin with a side chain of three or more glutamates [8], detected a significantly weaker band in the *tpg1* axoneme than in the wild-type axoneme (Figure 1C). In wild-type axonemes, this antibody detected only a weak band of polyglutamylated β-tubulin compared with that of polyglutamylated α-tubulin (Figure S1C). In two-dimensional SDS polyacrylamide gel electrophoresis patterns, α-tubulin separated into at least seven discrete spots in the wild-type axoneme, whereas only about four spots were detected in *tpg1*. In addition, upon close examination, both α- and β-tubulin spots were associated with faint long smears on the acidic side in the wild-type, but not in the *tpg1*, axoneme sample (Figure 1D). However, the β-tubulin spot did not separate into discrete spots. These results indicate that the *tpg1* mutation causes loss of long-chain polyglutamylation mostly in α-tubulin. It is likely that CrTTLL9 catalyzes the elongation of polyglutamylate side chains as shown for the TTLL9 ortholog in *Tetrahymena thermophila* [8]. Initiation of polyglutamylation is probably carried out by some TTLL protein(s) other than CrTTLL9; in other organisms, different TTLL proteins have been assigned to the initiation and elongation of polyglutamylation [1]. Database search indicates that *Chlamydomonas* genome contains ~10 TTLL proteins.

Indirect fluorescent microscopy with the polyE antibody showed staining of the wild-type axoneme over the entire length (Figure 2A). In extensively frayed axonemes, staining occurred on single or bundled microtubules except for the central pair, which can be distinguished from the outer doublet by its strongly curved shape [9] (Figure S2). Because we encountered no outer doublet that lacked polyE staining, it is likely that all of the nine outer doublets are polyglutamylated. The staining intensity of doublet bundles in frayed axonemes did not show a gradient, unlike the staining pattern of the whole axoneme (Figure 2A). Thus, the origin of the graded staining pattern in the wild-type axoneme is not clear. In a striking contrast with the wild-type axoneme, the *tpg1* axoneme was stained only in the proximal 1–2 μm region of the 10–11 μm flagellum (Figures 2A and 2B). This observation is consistent with the immunoblot results showing that long polyglutamate side chains are greatly decreased but not completely eliminated in *tpg1*. The polyglutamylation at the proximal portion may well be carried out by other TTLL protein(s).

Immunoelectron microscopy with colloid gold indicated that long-chain polyglutamylation takes place predominantly on the B-tubule of outer doublet in the wild-type axoneme (Figure 2C). These observations are consistent with the results of a previous study [10]. In contrast, *tpg1* axonemes showed very low levels of staining (Figure 2C).

CrTTLL9 Is Associated with the Axoneme and Is Upregulated upon Deflagellation

We raised polyclonal antibodies against the total length of CrTTLL9 expressed in *E. coli*. Western blot of detergent-extracted wild-type flagella showed that this protein is present mostly in the axoneme fraction and is extractable with 0.6 M KCl (Figures 3A and 3B). In *tpg1*, this antibody did not detect bands corresponding to CrTTLL9 or truncated products in either axoneme or detergent-soluble fractions.

Because the expression level of the *CrTTLL9* gene (the FAP267-encoding gene) has been shown to increase upon

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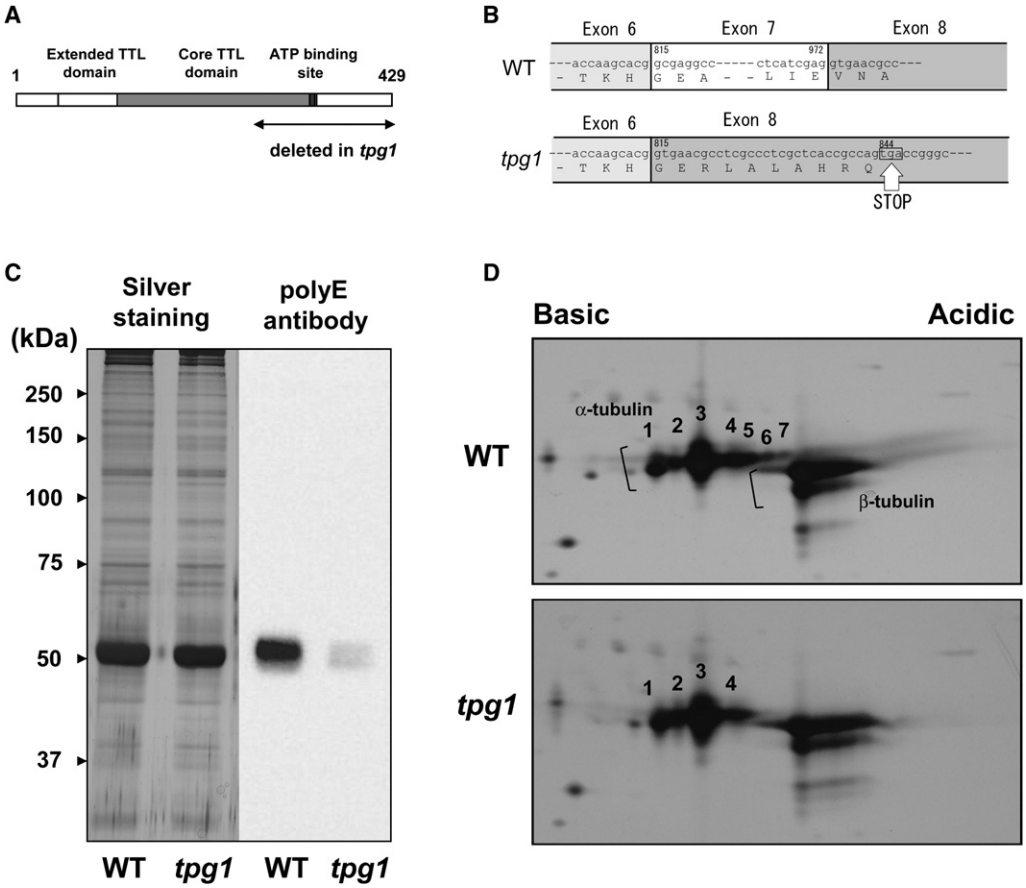

Figure 1. Mutation in *tpg1*

tpg1 is deficient in an enzyme (CrTTLL9) that elongates the polyglutamate side chain on α-tubulin.

(A) Schematic drawing of the CrTTLL9 sequence. The TTL domains and ATP binding site were identified as described [3]. The arrow indicates the region deleted in *tpg1*.

(B) The *tpg1* mutation causes the loss of exon 7, which results in premature stop codon (arrow) (Figure S1A).

(C) Western blot of wild-type (WT) and *tpg1* axonemes with polyE antibody, which recognizes α-tubulin with a polyglutamate side chain of three or more residues.

(D) Two-dimensional SDS polyacrylamide gel electrophoresis patterns of wild-type and *tpg1* axonemes. The α-tubulin spots are separated into seven or more discrete spots, of which ~3 are missing in *tpg1*. In addition, only the wild-type pattern shows long smears extending from the rightmost α- and β-tubulin spots in the upper right direction, suggesting that CrTTLL9 catalyzes formation of long glutamate side chains on β-tubulin as well as on α-tubulin, although modification of β-tubulin is much less extensive (Figure S1C).

deflagellation [6], we examined the change in the amount of the flagellar CrTTLL9 protein after deflagellation in the wild-type axoneme. As shown in Figures 3C and 3D, the amount of CrTTLL9 in a constant weight of axoneme increased after deflagellation by pH shock and decreased to the basic level following flagellar regeneration. The relative CrTTLL9 amount per flagellum can be estimated by multiplying the protein amount by the average flagellar length. This value also showed an increase immediately after deflagellation and a decrease following the flagellar growth, indicating that excess CrTTLL9 protein is removed as flagella grow (Figure 3D). With the same antibodies, we were unable to detect CrTTLL9 in the wild-type axonemes via immunofluorescence.

The *tpg1* Axoneme Has a Normal Structure

To examine whether the loss of CrTTLL9 causes any defects in axonemal structure, we examined isolated axonemes by electron microscopy (data not shown), analyzed dynein composition by ion-exchange chromatography (Figures S3A and S3B), and measured the average length (Figure S3C) and the time course of flagellar growth after deflagellation (Figure S3D). None of these assays detected any differences between wild-type and mutant axonemes. Therefore, we conclude that loss of long-chain tubulin polyglutamylation does not interfere with the formation of axoneme and assembly of dyneins.

The *tpg1* Mutation Severely Affects the Function of Inner-Arm Dyneins

The *tpg1* mutant swam at a velocity that was 70%–80% as fast as the wild-type velocity (Figure 4A). The flagellar beat frequency in *tpg1* was reduced to a similar extent, indicating that the *tpg1* and wild-type cells move for a similar distance per flagellar beat (Figure 4B). Thus, the flagellar beat pattern does not appear to greatly differ between the two strains (Movies S1 and S2), although direct waveform analysis must be performed to conclude it. Strikingly, however, the double mutant of *tpg1* and the mutant *oda2* lacking outer-arm dynein completely lacked motility, whereas *oda2* swam at about 30% of the wild-type velocity (Figure 4A; Movies S3 and S4). In contrast to the difference in motility, axonemal ATPase

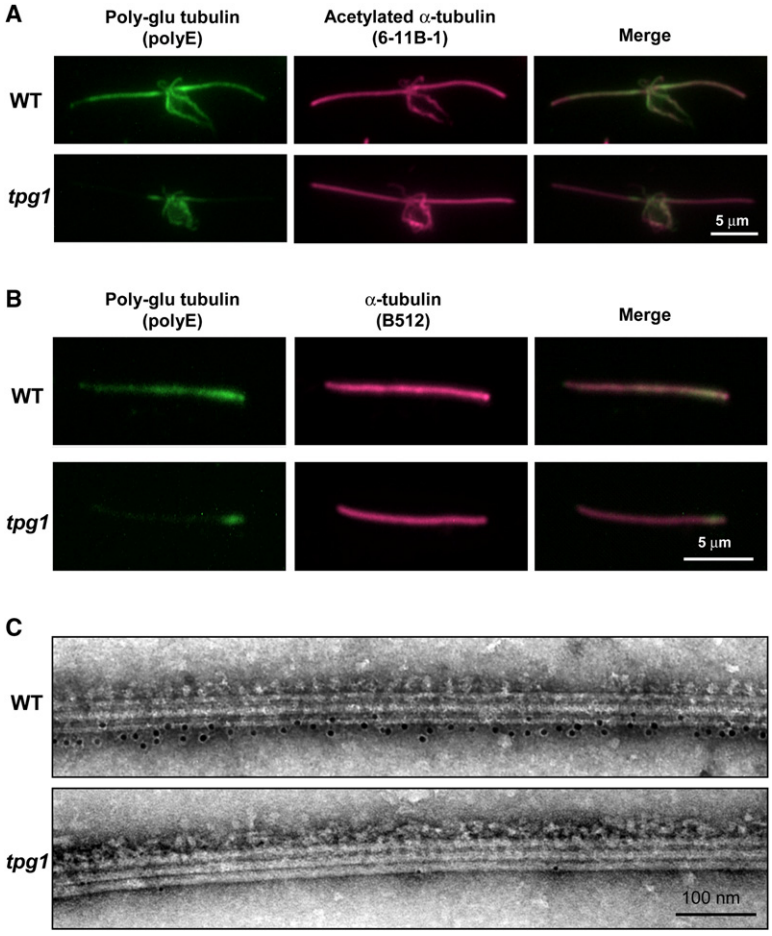

Figure 2. Localization of Polyglutamylated Tubulin in Wild-Type and *tpg1* Axonemes

(A) The nucleoflagellar apparatus (a complex of two flagella, the basal body, and the nucleus) was stained with the polyE antibody and an acetylated α-tubulin antibody (6-11B-1).

(B) Localization in isolated axonemes. In *tpg1*, tubulin with a long polyglutamate side chain is present only in the basal ~1–2 μm portion (on the right side of the image). In wild-type, the polyglutamylated tubulin signal decreases toward the flagellar tip. However, such a gradient is not seen in outer doublets in frayed axonemes (Figure S2).

(C) Immunogold electron microscopy with polyE antibody (1:100) and anti-rabbit IgG conjugated with 10 nm gold (1:100). Isolated doublet microtubules were negatively stained with 1% uranyl acetate. In wild-type, gold particles are predominantly present on the B-tubule, which can be distinguished from the A-tubule by the absence of attached dyneins. In *tpg1*, far fewer gold particles were attached.

activities were almost the same between wild-type and *tpg1* and between *oda2* and *oda2tpg1* (Table S1).

To explore the function of polyglutamylation on axonemal motility, we next examined the microtubule sliding in disintegrating axonemes induced by treatment with protease and ATP [11, 12] (Figure 4C). The *tpg1* axoneme underwent sliding at almost the same velocity as the wild-type axoneme. Unexpectedly, however, the axoneme of paralyzed *oda2tpg1* displayed much faster sliding than the axoneme of motile *oda2*. Thus, long-chain tubulin polyglutamylation in *oda2* axonemes apparently functions to suppress microtubule sliding produced by inner-arm dyneins under low-load conditions wherein no axonemal bending takes place. The presence or absence of long polyglutamate chains must be severely affecting the function of inner-arm dyneins, but not so much that of outer-arm dynein.

One-Headed Inner-Arm Dynein Species Is More Sensitively Affected Than Two-Headed Species

Chlamydomonas has seven major species of inner-arm dyneins called dyneins a–g [13, 14]. Of these, dynein f (also called I1) is the only two-headed species containing two heavy chains, and all others are one-headed species containing a single heavy chain. The subunit composition of dynein f/I1 is totally different from other inner-arm dyneins. To examine which type of inner-arm dynein species is most strongly affected by tubulin polyglutamylation, we next examined the effect of the *tpg1* mutation on the motility of two inner-arm-deficient mutants, *ida1* and *ida5*. The mutant *ida1* lacks dynein species f/I1 because of a mutation in one of its heavy chains [15], whereas *ida5* lacks species a, c, d, and e because of the loss of actin [16], a common subunit of one-headed inner-arm dyneins. We found that the double mutant *ida1tpg1* swam 5–6 times slower than *ida1* (Figure 4A), with flagella beating at about 70% frequency of *ida1* (Figure 4B). The reduction in swimming velocity is apparently caused by a significant decrease in flagellar bend angle, as is evident from the extremely small amplitude of the envelope of beating flagella (data not shown).

The other double mutant, *ida5tpg1*, displayed a distinct motility phenotype; like *ida5* cells, the *ida5tpg1* cells swam slowly, but unlike *ida5* mutants alone, double mutants tended to stick to the glass surface a few seconds after the onset of observation under the microscope. A fraction (~40%) of cells remained motile when observation was performed under red (>630 nm) light. In this case, the average velocity of swimming cells was ~50 μm/s, i.e., ~70% of the *ida5* swimming velocity (Figure 4A). Thus, in this mutant, flagellar beating did not appear to be so greatly impaired as in *ida1tpg1* as long as the cells did not stick to the surface in response to light. The reason for this light sensitivity is not understood. These observations suggest that polyglutamylation must be affecting the dynein species remaining in *ida1* (outer-arm dynein and one-headed inner-arm dynein species a–e and g) more severely than those remaining in *ida5* (outer-arm dynein, two-headed inner-arm dynein species f, and one-headed species b and g). From the comparison of the dynein species involved, we suppose that polyglutamylation affects one-headed dyneins more strongly than two-headed dynein. However, these observations do not rule out the possibility that polyglutamylation also affects two-headed inner-arm dyneins to some extent, because *ida5tpg1* displayed weaker motility than *ida5*.

Microtubule sliding velocity in disintegrating axonemes also showed a difference between the two inner-arm mutants; the sliding velocity in *ida1* slightly increased in the background of *tpg1* mutation, whereas no change occurred in *ida5* (Figure 4C). Thus, in this experiment also, the *tpg1* mutation affects the *ida1* axoneme more significantly than the *ida5* axoneme.

How the *tpg1* mutation lowers flagellar motility while increasing microtubule sliding velocity in some mutant

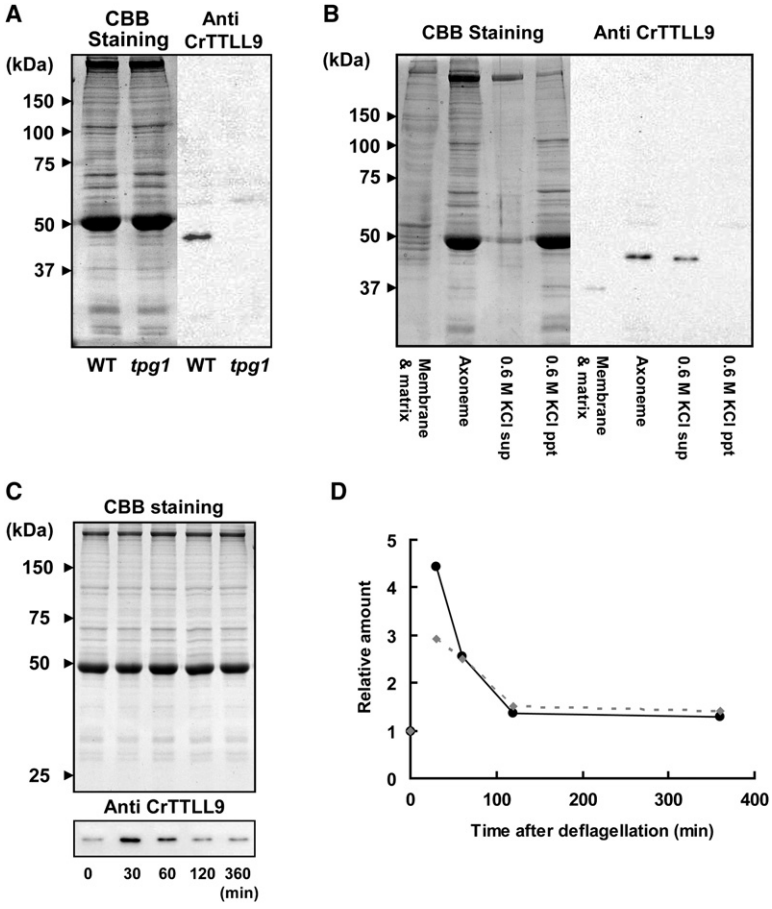

Figure 3. CrTTLL9 Is an Axonemal Protein That Undergoes Upregulation upon Deflagellation

(A) Immunoblot of axonemes with anti-CrTTLL9 antibodies raised against full-length recombinant protein. In the wild-type axoneme, a single major band is detected at the position corresponding to the size of CrTTLL9 (49.6 kDa). The *tpg1* axoneme entirely lacks a full-length or truncated CrTTLL9 protein.

(B) CrTTLL9 is present predominantly in the axoneme and can be solubilized with 0.6 M KCl.

(C and D) The CrTTLL9 in axonemes greatly increases upon deflagellation and decreases to the baseline within 2 hr. The graph shows the change in band intensity in a constant amount of flagella (solid line) after deflagellation and the change in the amount per flagellum (dotted line) relative to the control value.

axonemes remains to be studied. It is conceivable that lack of long, negatively charged polyglutamate side chains from the microtubule causes a significant effect on the strength of dynein-microtubule interaction. Electrostatic interaction between dynein and the C-terminal portion of tubulin has been suggested to be critical for the processive movements in inner-arm dynein species c [17], as well as in cytoplasmic dynein [18]. The function of other dyneins may well also critically depend on the surface charge of the microtubule. Interestingly, the microtubule binding site of dynein, the stalk tip, is positively charged in all kinds of inner-arm dyneins, and the stalk tip of a one-headed species, dynein e, has the highest pI among all dyneins (T.Y., unpublished data). In the background of the *tpg1* mutation, electrostatic dynein-microtubule interaction should decrease. We speculate that the decreased interaction may increase sliding velocity by preventing some slow-moving dynein from entering into a strong binding state or by decreasing interdoublet friction that acts as a drag against sliding. At the same time, a strong interaction of inner-arm dyneins with microtubules may be prerequisite for axonemal beating, which requires stronger force generation than simple microtubule sliding. However, the exact mechanism by which the *tpg1* mutation accelerates microtubule sliding and inhibits flagellar beating must await further studies.

In summary, the novel *Chlamydomonas* mutant *tpg1* lacking a tubulin polyglutamylating enzyme revealed that lack of long polyglutamate side chains in doublet microtubules specifically interferes with motility. In particular, the *tpg1* mutation causes complete loss of motility in *oda2* lacking outer-arm dynein and an extremely slow swimming velocity in *ida1* lacking two-headed inner-arm dyneins. Curiously, the mutation increased microtubule sliding velocity in the background of *oda2* or *ida1*. These findings support the idea that CrTTLL9 tubulin glutamic acid ligase most significantly affects the function of one-headed inner-arm dyneins. In accordance with this conclusion, a recent study in *Tetrahymena* concluded that tubulin polyglutamylation mediated by TTLL6, a chain elongase for β-tubulin, regulates ciliary motility by restraining the activity of inner-arm dynein (Suryavanshi et al. [19], this issue of *Current Biology*). Different sensitivities to polyglutamylation among different dyneins may be due to the difference in intrinsic properties of dyneins. Alternatively, it may be due to a biased localization of polyglutamylated tubulin in the B-tubule. How tubulin polyglutamylation affects dynein function, as well as how it takes place specifically on the B-tubule, must await further studies.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, one table, and four movies and can be found with this article online at doi:10.1016/j.cub.2009.12.058.

Acknowledgments

We thank M.A. Gorovsky (University of Rochester) for providing polyE antibody and J. Gaertig (University of Georgia) and W. Sale (Emory University) for discussion and critical reading of the manuscript. This study has been supported by a grant from the Japan Society for the Promotion of Science (JSPS).

Received: November 20, 2009
Revised: December 25, 2009
Accepted: December 30, 2009
Published online: February 25, 2010

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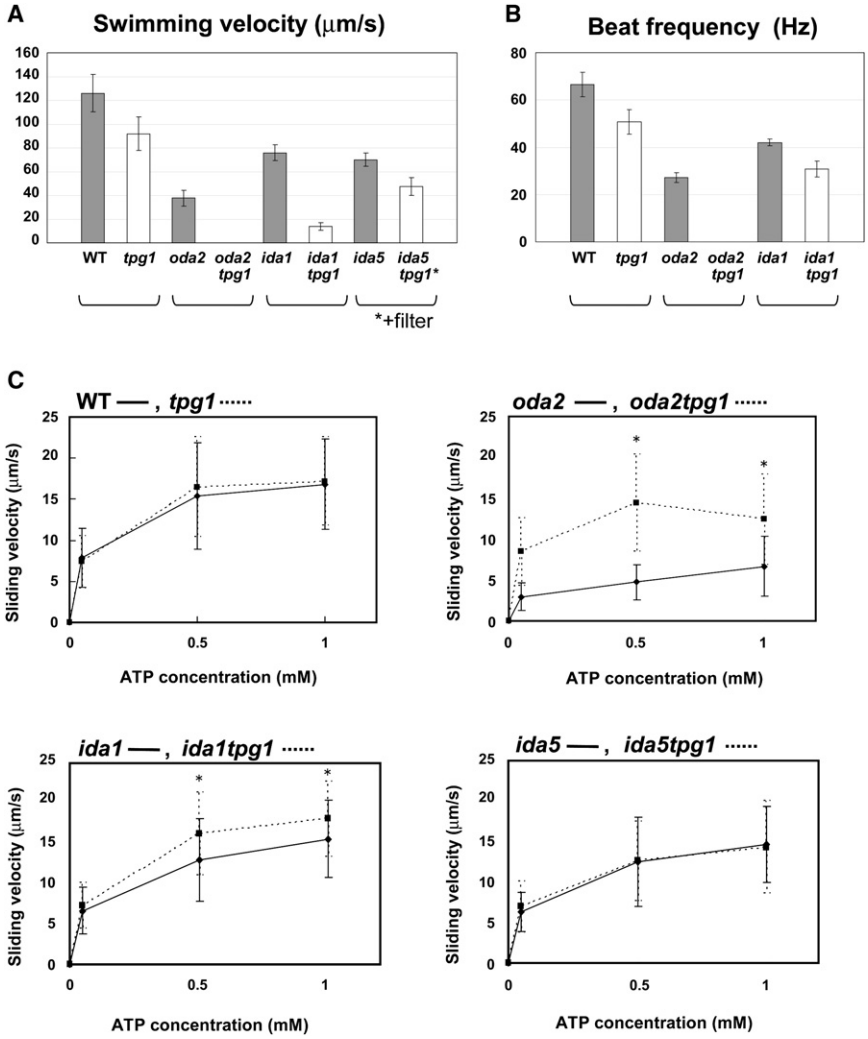

Figure 4. Effects of the *tpg1* Mutation on the Motility of Dynein-Deficient Mutants

(A) Swimming velocity of wild-type (WT), *oda2* (lacking outer-arm dynein), *ida1* (lacking dynein f/I1), and *ida5* (lacking dyneins a, c, d, and e) with and without the *tpg1* mutation. The averages and standard deviations (bars) measured in >20 cells are shown. *oda2* becomes completely nonmotile when combined with the *tpg1* mutation. The difference in each pair of data was found to be statistically significant ($p < 0.01$).

(B) Beat frequency measured by analyzing the vibration of cell body [20]. The mutant *ida5tpg1* displayed irregular beating, which did not allow frequency measurements.

(C) Microtubule sliding velocities in disintegrating axonemes. Wild-type and mutant axonemes with and without the *tpg1* mutation were fragmented by sonication. The samples were perfused with nagarse (type XXVII protease, Sigma) and various concentrations of ATP while images were being recorded with an SIT video camera [12, 21]. Each data point shows the average and the standard deviation (bar) measured in >40 axonemes. Asterisks indicate where the difference is statistically significant ($p < 0.01$).

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