

# A microtubule depolymerizing kinesin functions during both flagellar disassembly and flagellar assembly in *Chlamydomonas*

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**Cilia and flagella are dynamic organelles that are assembled and disassembled during cell differentiation, during stress, and during the cell cycle. Although intraflagellar transport (IFT) is well documented to be responsible for transport of ciliary/flagellar precursors from the cell body to the flagella, little is known about the molecular mechanisms for mobilizing the cell body-localized precursors to make them available for transport during organelle assembly or for disassembling the microtubule-based axoneme during shortening. Here, we show that *Chlamydomonas* kinesin-13 (CrKinesin-13), a member of the kinesin-13 family of microtubule depolymerizing kinesins best known for their roles in the cell cycle, functions in flagellar disassembly and flagellar assembly. Activation of a cell to generate new flagella induces rapid phosphorylation of CrKinesin-13, and activation of flagellar shortening induces the immediate transport of CrKinesin-13 via intraflagellar transport from the cell body into the flagella. Cells depleted of CrKinesin-13 by RNAi assemble flagella after cell division but are incapable of the rapid assembly of flagella that normally occurs after flagellar detachment. Furthermore, they are inhibited in flagellar shortening. Thus, CrKinesin-13 is dynamically regulated during flagellar assembly and disassembly in *Chlamydomonas* and functions in each.**

cilia | flagella | intraflagellar transport | Kinesin-13

Cilia and flagella play pivotal roles in cell motility and cell signaling during development and homeostasis (1–3). Even though we know much about the functions of these ubiquitous organelles, we are only just beginning to understand the molecular mechanisms that regulate their assembly and disassembly. After cell division is completed, the primary cilium is formed by assembly of ciliary components onto the distal end of the basal body, a modified centriole. Ciliary precursors in the cell body are delivered to the tip of the growing organelle by intraflagellar transport (IFT), a bidirectional transport system composed of protein complexes (called IFT particles) and their cargo (flagellar components such as tubulin and dynein arms). Anterograde movement from the cell body to the flagellar tip is driven by kinesin-2, whereas retrograde movement from the tip to the cell body depends on cytoplasmic dynein (4–6).

Regulation of the formation and removal of cilia and flagella is highly complex. During entry into the cell cycle, cilia, and flagella (which are functionally and structurally similar organelles whose names are used interchangeably) are resorbed (7–9). Many cells also modify their cilia in response to environmental or developmental cues (10, 11). *Chlamydomonas*, a unicellular biflagellated green alga, shortens its flagella in response to changes in the ionic properties of its medium and during zygote development. Recently, an aurora-like protein kinase (CALK) was shown to be required for flagellar resorption and rapidly phosphorylated during shortening induced by changes in the medium and after zygote formation (12). The resorption of primary cilia in mammalian cells that occurs during reentry into the cell cycle also requires an aurora kinase (9).

Cells also are capable of rapidly regenerating cilia after shortening or detachment. Studies on primary cilia of fibroblasts have shown that within 1 hour after growth factor-triggered loss of cilia, the fibroblasts regenerate the organelles (13). Early, classic studies on *Chlamydomonas* uncovered a cellular pool of flagellar precursors used for formation of new flagella. *Chlamydomonas* cells experimentally induced to detach their flagella by pH shock initiate flagellar regeneration immediately and grow two new flagella within 1–2 hours. Even in the absence of new protein synthesis, the cells can form two half-length flagella solely from a cell body pool of flagellar precursors (14). The mechanisms that regulate flagellar regeneration, including the accompanying up-regulation of transcription of flagellar genes, are likely to be complex (15–17). It is not known, for example, whether the tubulin dimers in the precursor pool are soluble or in the form of polymerized cytoplasmic microtubules. Wilson and Lefebvre reported that glycogen synthase kinase 3 (GSK3) was regulated during flagellar detachment and regeneration, and suggested that GSK3 might influence cargo loading in the IFT system (18).

In studies of the cellular and molecular events that underlie flagellar formation and removal, we observed that CrKinesin-13, the *Chlamydomonas* member of the kinesin-13 family of microtubule depolymerizing kinesins, is dynamically regulated during flagellar regeneration and during flagellar shortening. Within seconds after cells are triggered to generate new flagella, CrKinesin-13 in the cell body is posttranslationally modified, and modification is reversed as flagella reach nearly full-length. Cells depleted by RNAi of CrKinesin-13 fail to assemble flagella immediately after deflagellation and exhibit a  $\approx 2$  hours lag in initiation of assembly. During flagellar disassembly, CrKinesin-13, which lacks the neck domain implicated in diffusional translocation, is transported by IFT from the cell body into the flagella. Depletion of CrKinesin-13 inhibits flagellar shortening. Thus, in *Chlamydomonas*, Kinesin-13 functions both during flagellar assembly and during flagellar disassembly.

## Results

### Posttranslational Modification of Kinesin-13 in *Chlamydomonas*.

Based on the known roles of “mitotic kinases” in cilia and flagella (9, 12, 19, 20), we searched for additional mitosis-linked proteins that appeared after pH shock-induced flagellar detachment/regeneration using the MPM2 antibody, which recognizes

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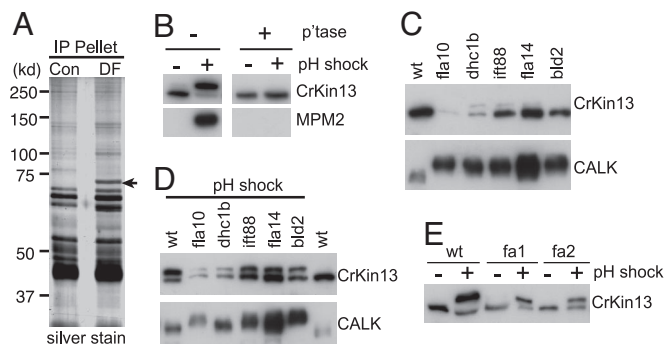
The authors declare no conflict of interest.

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**Fig. 1.** Posttranslational modification of CrKinesin-13 (A) MPM2 antibody immunoprecipitates a 70-kDa protein from pH-shocked cells. Cell lysates from control (con) cells and pH-shocked cells (DF) were incubated with MPM2 antibody, and the immunoprecipitates were analyzed by immunoblotting (Fig. S1) and silver staining. (B) CrKinesin-13 is phosphorylated during pH shock, and the phosphorylated CrKinesin-13 is the antigen recognized by the MPM2 antibody. Cell lysates from control and deflagellated cells were treated with or without phosphatase followed by immunoblot analysis with anti-CrKinesin-13 and mAb-MPM2. (C) CrKinesin-13 properties in mutants defective in flagellar assembly. The indicated mutant and wild-type (21gr) cells were analyzed by immunoblotting using the CrKinesin-13 antibody. (D) CrKinesin-13 is modified during the pathways activated by pH shock. The flagellar mutants were subjected to pH shock along with wild-type cells, frozen within 1 minute, and subsequently analyzed by SDS/PAGE and immunoblotting. (E) The *fa1* and *fa2* mutants, which are defective in flagellar detachment, undergo pH shock-induced modification of CrKinesin-13. Immunoblot analysis of 21gr, *fa1*, and *fa2* mutant cells was carried out before and after pH shock.

a phospho-epitope (pS/T -P) present on a set of proteins, including microtubule-associated proteins MAP-1, MAP-2, and NIMA kinase, that regulate microtubule dynamics during the cell cycle (21). Immunoblotting and immunoprecipitation with the MPM2 antibody (Fig. 1A), MALDI-TOF mass spectrometry, and BLAST analysis identified a 70 kDa, MPM2-binding protein that appeared after pH shock as a 647 aa-containing kinesin-like protein with a highly conserved 343 aa kinesin motor domain in the middle of the protein (Fig. S1). Analysis by BLAST and alignment with other kinesin family members showed that the protein, which we have named CrKinesin-13, is a member of the microtubule depolymerizing kinesin-13 family. Kinesin-13 family members in other organisms are well established as key regulators of mitotic microtubules and enforce the proper length of the spindle and regulate chromosome segregation by catalyzing a 100-fold increase in the rate of depolymerization of microtubules (22–25).

CrKinesin-13 contains all of the conserved domains of the kinesin-13 family members except the neck domain, (SI Text and Fig. S2). Kinesin-13 family members recently reported to be present in the flagella of the unicellular pathogens *Leishmania* and *Giardia* also are missing the neck domain (26, 27). Because the neck domain is implicated in movement of kinesin-13s along microtubules in mitosis (23), cells that possess neckless kinesin-13 family members must use other mechanisms for transporting kinesin-13.

As shown in Fig. 1B (upper-left panel), immunoblotting with an anti-CrKinesin-13 antibody that we generated (Fig. S3) showed that the lower isoform of CrKinesin-13 was converted to the upper form when cells were exposed to a pH shock. Incubation of lysates with a protein phosphatase converted the upper isoform to the lower isoform (Fig. 1B, upper-right panel), indicating that the upper band was a phosphorylated form of the protein. Immunoblotting with the MPM2 antibody confirmed the anti-CrKinesin-13 results (Fig. 1B, lower panels).

CrKinesin-13 modification upon pH shock is unrelated to flagellar detachment per se. Previous work from several labo-

ratories has established that exposure of *Chlamydomonas* cells to treatments (such as pH shock) that activate signals for deflagellation also activate pathways for flagellar regeneration (15, 17). To determine whether the pH shock-induced posttranslational modification of CrKinesin-13 was related to deflagellation per se or to the flagellar regeneration that occurs immediately after deflagellation, we studied CrKinesin-13 in several mutants defective in genes essential for flagellar assembly or flagellar detachment. The mutants were a *fla10* null mutant (flagella-less; defective in anterograde IFT motor kinesin2) (28); *dhc1b* (flagella-less; defective in retrograde IFT motor cytoplasmic dynein 1b) (29); *fla14* (short, stumpy flagella; defective in the retrograde motor cytoplasmic dynein light chain) (30, 31); *ift88* (flagella-less; defective in IFT particle protein IFT88) (32); *bld2* (flagella-less; defective in epsilon tubulin essential for basal body assembly) (33); and *fa1* and *fa2* (flagella of normal length; defective in the FA1 and FA2 proteins required for pH shock-induced flagellar detachment) (19).

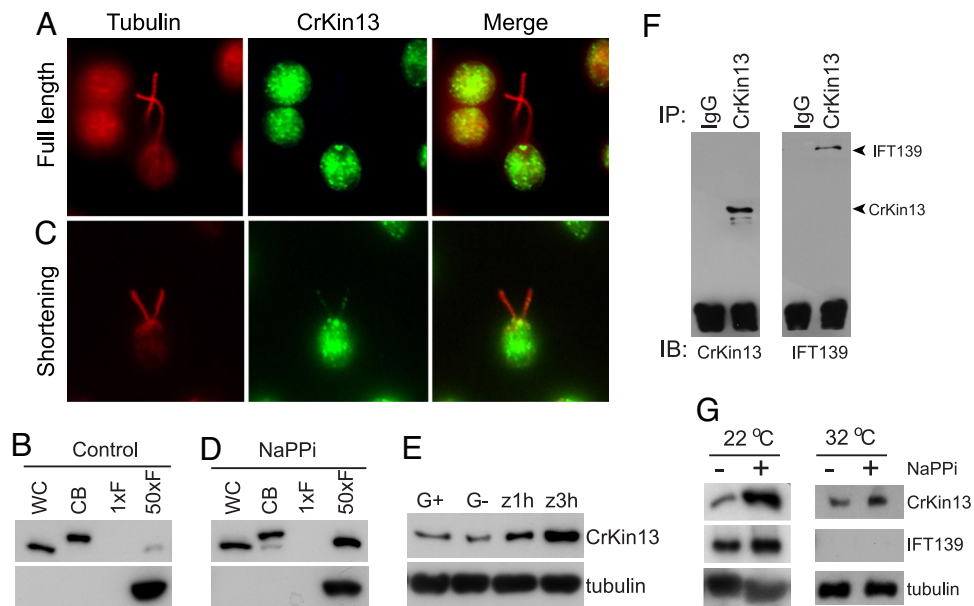
Similar to wild-type cells, the phosphorylated form of CrKinesin-13 was essentially undetectable in all of the mutants under standard culture conditions (Fig. 1C, upper panel). (We should note that, unlike CrKinesin-13, the aurora-like protein kinase CALK was phosphorylated in all of the flagella-less mutants before pH shock (Fig. 1C, lower panel) (12), indicating that CALK phosphorylation and CrKinesin-13 phosphorylation were decoupled in these cells.) When we subjected the mutants to pH shock, CrKinesin-13 was phosphorylated in each (Figs. 1D and 1E). Thus, these results indicated that the pH shock-induced posttranslational modification of the kinesin was unrelated to flagellar detachment per se.

**CrKinesin-13 and Flagellar Regeneration.** Analysis of CrKinesin-13 in cells regenerating their flagella implicated the protein in flagellar growth. We examined flagellar regeneration in cells induced to detach their flagella by pH shock (Fig. 2A); in agar-grown cells, which are aflagellate but can be activated to regenerate flagella by transfer to liquid medium (Fig. 2B); and in cells previously treated (see below) to bring their flagella to half length and then returned to standard culture conditions to allow re-growth of the organelles to full length (Fig. 2C). In cells regenerating flagella in all three conditions, only the lower isoform of CrKinesin-13 was present before regeneration was induced. Importantly, the upper form appeared immediately after regeneration was triggered. In addition, when the flagella began to approach full length, the CrKinesin-13 in all 3 sets of regenerating cells returned to the lower form. These experiments strongly linked cellular regulation of CrKinesin-13 to flagellar regeneration.

**CrKinesin-13 Is Dispensable for Flagellar Detachment but Functions During Flagellar Assembly.** To examine the function of kinesin-13 in flagellar regeneration, we used RNAi methods to deplete CrKinesin-13, which is encoded by a single copy gene (34, 35) (see SI Text). Several transformants (92, 93, 99, 115, and 116) showed reduced levels of CrKinesin-13 protein by immunoblotting (Fig. 3A). Examination by phase contrast microscopy (not shown) indicated that the RNAi transformants also possessed shorter flagella, whereas clones with normal expression levels of CrKinesin-13 did not. Four of the transformants (92, 93, 99, and 115) that were examined in more detail possessed flagella that ranged in average length from 5.6 to 7.5  $\mu\text{m}$ , whereas the average length of wild-type cells is  $\approx 12 \mu\text{m}$  (see SI Text). Recovering cells with a short flagella phenotype is relatively uncommon in *Chlamydomonas* mutant screens (36). Cultures of 99 and 115 sometimes contained many undivided or non-flagellated cells. All four transformants behaved similarly in the experiments to be described below, but transformants 92 and 93 exhibited







**Fig. 4.** Transport of CrKinesin-13 into flagella is triggered when the flagellar shortening pathway is activated and transport requires IFT. (A) Immunofluorescence of control cells under standard culture conditions. Staining with anti-CrKinesin-13 was detected in the basal body region, and little if any was detected in the flagella. (B) Immunoblot of whole cells (WC), cell bodies (CB), and flagella (F) with anti-CrKinesin-13. CrKinesin-13 was predominantly localized to the cell body (note that the pH shock used for deflagellation caused CrKinesin-13 phosphorylation). (1x: One cell equivalent of flagella; 50x: 50 cell equivalents of flagella.) Staining with alpha tubulin antibody documented equal loading. (C) Immunofluorescence of cells undergoing flagellar disassembly. CrKinesin-13 staining was detected along the length of the flagella and at the tip. (D) CrKinesin-13 in the flagella increased within 5 minutes after cells were placed in NaPPI. (E) Immunoblots of flagella from gametes and the zygotes that formed at 1 hour and 3 hours after gametes were mixed. CrKinesin-13 in the flagella increased during zygotic flagellar disassembly. G+, mt+ gametes; G-, mt- gametes; Z, zygotes. Alpha tubulin was used as loading control. (F) Co-immunoprecipitation of CrKinesin-13 and IFT particles. The flagellar membrane/matrix fractions of flagella isolated 5 minutes after transfer of cells to NaPPI were used for immunoprecipitation with anti-CrKinesin-13 or preimmune IgG. The immunoprecipitates were analyzed with anti-CrKinesin-13 and anti-IFT139 antibodies. (G) Failure of CrKinesin-13 to be transported into flagella in the *fla10-1* mutant at the non-permissive temperature after induction of flagellar disassembly by NaPPI. *fla10-1* Cells were incubated at 22 °C or 32 °C for 1 hour followed by incubation in 20 mM NaPPI for 5 minutes at the same temperatures, and the flagella were isolated and analyzed by immunoblotting. Alpha tubulin was used as a loading control and an anti-IFT139 antibody was used to detect IFT particles.

staining of CrKinesin-13, which is similar to the distribution of IFT particles in flagella (38), was consistent with this idea. To test directly for a relationship between CrKinesin-13 and IFT, we immunoprecipitated CrKinesin-13 from shortening flagella and immunoblotted the immunoprecipitate with an antibody against IFT particle protein IFT139. As shown in Fig. 4F, the IFT particle protein was present in the immunoprecipitates prepared using anti-CrKinesin-13 serum and was absent in those prepared using preimmune serum. These results indicated that CrKinesin-13 interacted with IFT particles.

We also took advantage of the conditional kinesin-2 mutant, *fla10-1* to determine whether IFT was required for CrKinesin-13 transport (39). At the permissive temperature (22 °C), IFT is functional, but when transferred to the non-permissive temperature (32 °C), IFT gradually diminishes; and after  $\approx 1$  hours, IFT becomes undetectable microscopically and the amounts of IFT particle proteins are substantially reduced in flagella. The flagella also gradually begin to shorten, albeit at rates (1–1.5  $\mu\text{m}/\text{h}$ ) that are 3–4-fold lower than in wild-type cells undergoing NaPPI-induced shortening (4–5  $\mu\text{m}/\text{h}$ ) (12, 31, 40–42).

To test for a role of IFT in transport of CrKinesin-13, we preincubated *fla10-1* cells at the permissive and non-permissive temperatures for 1 hour, added NaPPI for 5 minutes to stimulate flagellar disassembly, and then determined the amounts of CrKinesin-13 in isolated flagella by immunoblotting. As expected, activation of flagellar disassembly at 22 °C resulted in a several-fold increase of CrKinesin-13 in flagella compared with non-NaPPI treated cells (Fig. 4G). Also as expected, the IFT particles were reduced substantially in flagella isolated from the *fla10-1* cells treated with NaPPI at the non-permissive temperature (Fig. 4G). Importantly, however, although the amount of

CrKinesin-13 in the flagella increased slightly upon NaPPI treatment at the non-permissive temperature [which was consistent with a small NaPPI-induced increase in shortening rate at 32 °C (37)], the increase was substantially less than that at the permissive temperature (Fig. 4G). Thus, our results indicated that the transport of CrKinesin-13 into flagella during flagellar shortening depended on IFT.

**CrKinesin-13 Functions During Flagellar Shortening.** Finally, we investigated the function of CrKinesin-13 in flagellar shortening using the RNAi cells described above. As shown in Fig. 3C, wt cells shortened their flagella rapidly (4.2  $\mu\text{m}/\text{h}$ ) when incubated in NaPPI. On the other hand, although the RNAi cells underwent flagellar shortening, the rate of shortening (2  $\mu\text{m}/\text{h}$ ) was less than 50% of that of wild-type cells and was closer to the basal rate of flagellar disassembly (41). Thus, in addition to functioning during rapid re-growth of flagella, CrKinesin-13 also functions during the disassembly of the axoneme that occurs during shortening.

## Discussion

Our major findings are the following: (i) CrKinesin-13 was phosphorylated when flagellar growth was initiated and dephosphorylated as the flagella reached full length. (ii) The protein was transported by IFT into flagella during flagellar shortening. (iii) CrKinesin-13-depleted cells exhibited a nearly 2-h lag in initiation of flagellar regeneration. (iv) Flagellar shortening was inhibited in CrKinesin-13-depleted cells.

**CrKinesin-13 Posttranslational Modifications Track Flagellar Length During Regeneration.** Our results that three independent methods for inducing flagellar growth in cells all led to CrKinesin-13

phosphorylation, which was followed by dephosphorylation as the flagella reached nearly full length, strongly linked the protein to flagellar assembly. CrKinesin-13 thus joins a short list of proteins whose posttranslational modifications parallel flagellar length during regeneration. Previous work showed that after pH shock-induced flagellar detachment, the ratio of tyrosine-phosphorylated glycogen synthase kinase 3 (GSK3) to non-phosphorylated GSK3 increased  $\approx 1.4$ -fold within 5 minutes after deflagellation and then decreased to predeflagellation levels at 30 minutes (18). Given the implication of GSK3 in flagellar length control, it will be interesting to determine if the protein kinase is functionally linked to CrKinesin-13.

Moreover, the phosphorylation and activation of *Chlamydomonas* aurora-like kinase CALK during deflagellation and flagellar regeneration (Fig. 1) (12), suggest that CrKinesin-13 could be a substrate for CALK. The action of aurora protein kinases on mammalian kinesin-13 regulates its localization and microtubule depolymerization activity (43), which are essential for mitotic spindle assembly and chromosome segregation (23, 24). We found, however, that although CALK was phosphorylated in cells defective in flagellar assembly, CrKinesin-13 was not modified until the mutants were subjected to pH shock (Fig. 1). This evidence does not rule out a possible role for CALK in phosphorylating CrKinesin-13, but it does indicate that the relationship, if any, between CALK and CrKinesin-13 is complex.

#### CrKinesin-13 Is Translocated by IFT Into Flagella During Shortening.

Although our results showed that CrKinesin-13 is a cell body protein, when flagellar shortening was triggered by environmental or developmental cues, it was rapidly transported into flagella by IFT (Fig. 4). Given that many kinesin-13 family members require the neck domain for diffusional translocation along microtubules, it might have been expected that this “neckless” CrKinesin-13 would fail to translocate into flagella on its own. Even though it possesses a neck domain, Kif2a, another member of the kinesin-13 family, is transported to the spindle pole by a cytoplasmic dynein motor complex (44). Thus, both kinesin and dynein motors have now been shown to mediate the translocation of kinesin-13 family members.

#### CrKinesin-13 Functions During Flagellar Shortening and During Flagellar Growth.

Our studies of CrKinesin-13-depleted cells demonstrated that the protein functions during both flagellar assembly and flagellar disassembly (Fig. 3). Even though the CrKinesin-13-depleted cells failed to initiate flagellar growth for nearly 2 hours, they retained the ability to regenerate flagella and by 4 hours had grown flagella of predetachment length. These results suggested that the signaling pathway for regeneration was intact, and that mobilization of the pool of flagellar precursors required CrKinesin-13.

Moreover, the rate of shortening in the CrKinesin-13-depleted cells was reduced more than 2-fold compared with that in wild-type cells (Fig. 3C). The reduced rate of shortening in the knockdown cells was similar to the basal rate of shortening observed in *fla10-1* cells at the restrictive temperature (37, 40).

This result, coupled with the short-flagella rather than a long-flagella phenotype in the knockdown cells, suggests that the basal rate of microtubule depolymerization in *Chlamydomonas* flagella of steady-state length might be independent of CrKinesin-13.

Our results that CrKinesin-13 is posttranslationally modified in the cell body during regeneration and functions during regeneration raise the possibility that microtubule dynamics in the cell body might be just as important for flagellar growth and length control as microtubule dynamics in the flagellum.

## Materials and Methods

**Strains, Culture Conditions, and Special Chemicals.** *Chlamydomonas reinhardtii* strains and growth conditions, methods for inducing flagellar disassembly, and special chemicals were essentially as described previously (12).

**Cell Culture, Cell Fractionation, and Flagellar Length Measurements.** For pH shock treatment, the cultures were treated with 0.2 M HAc to reduce the pH to 4.5 rapidly and after 30 seconds the cultures were brought to pH 7.2 with 0.2 M KOH. To induce flagellar shortening, cultures were incubated in medium containing 20 mM NaPPI. Flagella and cell bodies were isolated as previously described (37). Cell samples for flagellar length measurements were fixed in a final 1% glutaraldehyde solution and imaged by differential interference contrast with a 40 $\times$  lens on a Zeiss Axio Observer Z1 microscope. Flagellar length was measured by using ImageJ software (National Institutes of Health) and the length was calibrated with a micrometer. Flagellar length results were graphed using GraphPad Prism version 5.0a for Mac OS S.

#### Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblotting were performed essentially as described previously (12). Antibodies used were mouse anti- $\alpha$ -tubulin (1:5000, Sigma), mouse MPM2 (1:2000, DakoCytomation), mouse anti-IFT139 (1:5000), and rabbit anti-CALK (1:2000). IFT antibodies were gifts from Dennis Deiner and Joel Rosenbaum (Yale University) and Douglas Cole (University of Idaho). Phosphatase treatment of the cell lysate was essentially as described previously (12).

#### Immunofluorescence Microscopy.

We followed a protocol provided by Susan Dutcher (33) for cell extraction and labeling with the antibodies. A Zeiss Axiovert 200M inverted Imaging Microscope equipped with a CCD camera (Zeiss AxioCam) and an AxioVision image analysis system were used to acquire images. The images were processed in AxioVision and/or Adobe Photoshop. Primary antibodies used were anti-CrKinesin-13 (1:50), anti-acetylated  $\alpha$ -tubulin (1:200, Sigma), anti-IFT139 (1:50). Secondary antibodies used were Alexa fluor 488 goat anti-rabbit IgG, and Texas Red goat anti-mouse IgG (all 1:400; Molecular Probes).

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