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The *Chlamydomonas* mutant *pf27* reveals novel features of ciliary radial spoke assembly

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Abstract

To address the mechanisms of ciliary radial spoke assembly, we took advantage of the *Chlamydomonas pf27* mutant. The radial spokes that assemble in *pf27* are localized to the proximal quarter of the axoneme, but otherwise are fully assembled into 20S radial spoke complexes competent to bind spokeless axonemes *in vitro*. Thus, *pf27* is not defective in radial spoke assembly or docking to the axoneme. Rather, our results suggest that *pf27* is defective in the transport of spoke complexes. During ciliary regeneration in *pf27*, radial spoke assembly occurs asynchronously from other axonemal components. In contrast, during ciliary regeneration in wild-type *Chlamydomonas*, radial spokes and other axonemal components assemble concurrently as the axoneme grows. Complementation in temporary dikaryons between wild-type and *pf27* reveals rescue of radial spoke assembly that begins at the distal tip, allowing further assembly to proceed from tip to base of the axoneme. Notably, rescued assembly of radial spokes occurred independently of the established proximal radial spokes in *pf27* axonemes in dikaryons. These results reveal that 20S radial spokes can assemble proximally in the *pf27* cilium but as the cilium lengthens, spoke assembly requires transport. We postulate that *PF27* encodes an adaptor or modifier protein required for radial spoke – IFT interaction.

Keywords

cilia; flagella; axonemes; microtubules; radial spokes

Introduction

Despite the growing body of information about the molecules involved in intraflagellar transport (IFT) in the assembly of cilia (Ishikawa and Marshall 2011; Pazour and Rosenbaum 2002), there is little known about the molecular details of assembly. Our goal is to understand these mechanisms by focusing on the assembly of the axonemal radial spoke, an essential structure required for normal ciliary motility (Smith and Yang 2004). Motile cilia play an indispensable role in human development, such as left-right patterning, and in adult health (Drummond 2012; Oh and Katsanis 2012; Roy 2009). Defects in ciliary movement due to improper or incomplete assembly of axonemal components lead to a variety of human diseases including primary cilia dyskinesia (PCD), infertility, and patterning defects (Fliege et al. 2007; Hildebrandt et al. 2011; Lee and Gleeson 2011;

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Marshall 2008). However, we are just beginning to understand the precise assembly of axonemal structures required for ciliary movement.

The radial spoke is an important regulator of ciliary motility that transduces signals between the central pair apparatus and the outer doublet microtubules for control of dynein-driven axonemal motility and regulation of bending waveform (Dymek et al. 2011; Dymek and Smith 2007; Smith and Yang 2004; Wirschell et al. 2011). The radial spokes are targeted and anchored on the outer doublet microtubules in precise positions in the axonemal 96 nm repeat (Barber et al. 2012; Pigino et al. 2011). Each radial spoke is made up of more than 23 polypeptides, organized in a T-shaped complex composed of a stalk and head domains, that can be isolated as a 20S complex (Diener et al. 2011; Lin et al. 2012; Yang et al. 2001; Yang et al. 2006). Many of the individual radial spoke proteins assemble into complexes in the cytoplasm before transit to the ciliary compartment, where the final steps of radial spoke assembly occur (Diener et al. 2011; Gupta et al. 2012; Qin et al. 2004; Yang et al. 2005). However, the exact mechanisms behind these steps of assembly and docking on the doublet microtubules are not well understood.

In the cell body a subset of radial spoke proteins is assembled into a precursor complex that sediments at 12S (Diener et al. 2011; Qin et al. 2004). By unknown mechanisms, the 12S radial spoke precursor complex, as well as additional radial spoke proteins, are transported to the base of the cilium and then enter the ciliary compartment, passing a predicted barrier at the transition zone (Kee et al. 2012; Lin et al. 2013). Following passage into the ciliary compartment, evidence suggests that the 12S radial spoke precursor complex is transported by IFT to the axonemal tip for assembly (Qin et al. 2004). For example, taking advantage of temporary dikaryons between wild-type and paralyzed *Chlamydomonas* mutant cells lacking the radial spokes, Johnson and Rosenbaum (1992) visualized rescue of radial spoke assembly starting at the tip of the cilium and then progressing toward the base. The simplest interpretation is that radial spoke precursors are loaded onto and transported by IFT to the distal tip of the cilium for further assembly steps (Gupta et al. 2012). Consistent with this model, radial spoke proteins have been shown to interact with IFT complexes (Qin et al. 2004). Furthermore, interruption of IFT at non-permissive temperature in the *fla10* *Chlamydomonas* mutant, which is defective in anterograde IFT, results in a decrease in the presence of 12S radial spoke precursors in the cilium (Qin et al. 2004). However, the mechanism for radial spoke interaction with the IFT protein subunits is not known.

In addition to transport by IFT, radial spoke precursors undergo modification in the ciliary compartment to form a fully assembled 20S radial spoke complex. One hypothesis is that a phosphorylation event allows the 12S complexes dimerize (Diener et al. 2011), leading to formation of the 20S radial spoke (Gupta et al. 2012). Consistent with the idea that the radial spoke is assembled as a dimer of 12S precursors, recent cryo-electron tomography (cryo-ET) studies have revealed a two-fold symmetry of the 20S axonemal radial spoke (Barber et al. 2012; Pigino et al. 2011). The details of dimer formation are not well understood but are believed to occur at the tip of the cilium (Dentler and Rosenbaum 1977; Pedersen et al. 2003; Sloboda 2005). Recent evidence indicates that radial spoke protein subunits LC8 and RSP3 (Gupta et al. 2012) and radial spoke protein 16, a HSP40 family member (Yang et al. 2005), play key roles in assembly of the mature 20S radial spoke. Notably, *in vitro* reconstitution studies revealed the 20S radial spoke complex is competent to dock to the axoneme, thus indicating the 20S radial spoke complex comprises the fully assembled spoke (Yang et al. 2001). In contrast, the 12S radial spoke precursor complex will not rebind to axonemal microtubules (L. Alford and W. Sale, unpublished data).

Following formation of the 20S radial spoke complex, the stalk domain docks the radial spoke in very precise positions within the 96 nm repeat on the outer doublet microtubules

(Barber et al. 2012; Pigino et al. 2011). The targeting and docking of the radial spokes are not well understood. However, docking requires radial spoke protein 3 (RSP3) and interaction with unidentified spoke docking proteins on the axonemal outer doublet microtubules. For example, the paralyzed *Chlamydomonas* mutant *pf14* is defective in RSP3 and fails to assemble the radial spokes (Diener et al. 1993; Luck et al. 1977; Wirschell et al. 2008; Witman et al. 1978). Furthermore, Diener and colleagues (1993) not only identified that the N-terminus of RSP3 binds to axonemes, but also found binding to be dependent on axonemal outer doublet components. Cryo-ET of the base of the radial spoke stalk, which is responsible for docking on the doublet microtubule, recently revealed structures that likely contain candidate proteins for radial spoke targeting and docking (Barber et al. 2012; Pigino et al. 2011). For example, one such candidate complex is the CSC (calmodulin and spoke associated complex) required for assembly of radial spoke 2 (Dymek et al. 2011; Heuser et al. 2012). Although these are important advances, additional work is required to define how the radial spokes are targeted and docked on the doublet microtubule.

Taken together, radial spoke assembly involves a sequence of distinct, complex steps that begin in the cytoplasm and are then completed in the ciliary compartment (Diener et al. 2011; Gupta et al. 2012). To further investigate the steps of assembly, we analyzed the radial spoke mutant *pf27*. The *pf27* mutant was isolated over thirty years ago and characterized by a reduction of radial spoke proteins and decreased phosphorylation of five axonemal radial spoke polypeptides (Huang et al. 1981). Despite extensive whole-genome sequencing (Dutcher et al. 2012) and genetic, biochemical and proteomic analysis, we have not identified the *PF27* gene (Supp. Fig. S1). However, phenotypic analysis of *pf27* has been productive and has revealed new features of radial spoke assembly. In particular, the few spokes that assemble in *pf27* axonemes are only localized to the proximal cilium. This observation indicates that axonemal radial spoke assembly is uncoupled from IFT in *pf27*. Our model suggests that diffusion is sufficient for transit, processing, and assembly of the radial spokes in short cilia, but as the cilium lengthens active transport is required. We predict *PF27* encodes a protein required for the interaction between radial spoke precursor complexes and IFT.

Results

The *pf27* mutant has reduced number of axonemal radial spokes

To characterize the radial spoke assembly defect in *pf27*, we first compared axonemes from wild-type and *pf27* by immunoblot analysis of radial spoke proteins (RSPs) and other axonemal components. Antibodies available to radial spoke head proteins 1 and 10 and spoke stalk proteins 2, 3, 8, 11 and 16 reveal a reduction in each of these RSPs (Fig. 1A). Densitometry of the immunoblot bands confirmed a 76.1% reduction in *pf27* axonemal RSPs 1, 2, 3, 8, 10, 11, and 16 ($n = 22$; Table I). This 3-fold reduction in *pf27* axonemal RSPs varies among preparations with a standard deviation of 12.5% (Table I).

Consistently, ultrastructural analysis revealed reduced numbers of axonemal radial spokes in *pf27* (Fig. 1B). Cross-sections from *pf27* axonemes examined by electron microscopy ($n = 160$) were grouped into three phenotypic categories: 1) all radial spoke structures missing (55.1%; Fig 1B, top panel), 2) all 9 intact radial spokes present (14.4%; Fig 1B, bottom panel), or 3) a fraction of the radial spokes missing (30.5%; Fig 1B, middle panel). Notably, partially assembled spokes (e.g. radial spoke stalks) were rarely observed by EM. Thus, the radial spoke deficiency in *pf27* appears to be due to failure in assembly of whole spokes onto the axoneme. To define the location of *pf27* cross-sections containing radial spokes along the axoneme, we used the “beak” structure, characteristic of the proximal end of the axoneme (Hoops and Witman 1983; Segal et al. 1984; Tam and Lefebvre 2002). The beak structure is present in the lumen of the B-tubule on microtubule outer doublets numbers 1, 5,

and 6 in the proximal *Chlamydomonas* axoneme. In cross-sections containing all 9 radial spokes an identifiable beak structure was present in doublets 1, 5, and 6 (a flattened, electron dense structure, red arrows, Fig. 1B, bottom panel). In contrast, beak structures were not seen in cross-sections lacking radial spokes (Fig. 1B, top panel). The simplest interpretation is that the radial spokes are assembled primarily at the proximal end of the *pf27* axoneme (Fig. 1C and shown below).

To examine the integrity of the remainder of the *pf27* axoneme, other axonemal components were analyzed by immunoblot and electron microscopy (Fig. 1B and D). The outer dynein arm (IC69), I1 dynein (IC138), the dynein regulatory complex (DRC1), and single-headed inner dynein arms (actin and p28) were all assembled at wild-type levels in the *pf27* axoneme. In addition, ultrastructural analysis of *pf27* axonemes did not reveal a visible defect in any of these structures, and the central pair complex was present in each image (Fig. 1B). Thus, the *pf27* mutant is only deficient in radial spoke assembly onto the axoneme.

The axonemal radial spokes in *pf27* are located at the proximal end of the axoneme

Phenotypic analysis revealed that beak structures are coincident with radial spokes in *pf27* axonemal cross-sections viewed by EM (Fig. 1B and C). We therefore hypothesized that radial spokes are localized to the base of the *pf27* ciliary axoneme. To test this idea, we performed immunofluorescence analysis of *pf27* and wild-type cells to localize ciliary radial spokes (Fig. 2A). Using a double label approach, the cilia were visualized with a monoclonal antibody to the outer dynein arm subunit IC69 (Fig. 2A, left column) and with a polyclonal antibody to RSP3 (Fig. 2A, middle column). Like the outer dynein arms (Ahmed et al. 2008), radial spokes (RSP3) appear to assemble along the entire length of the wild-type cilia (Fig. 2A and B). As expected, the outer dynein arms also appear to assemble along the entire length of *pf27* cilia. However, RSP3 staining was only present at the proximal end of the *pf27* cilium (Fig. 2A). To quantify the extent of radial spokes assembled along the axoneme in *pf27* cilia, we generated fluorescence intensity profiles of RSP3 staining along the length of the cilium (see Methods). The average length of RSP3 staining in *pf27* cilia was the proximal 2.26 μm ($n = 82$ from 6 independent experiments; Fig. 2C), whereas wild-type radial spoke staining was seen along the entire length of the cilium (Fig. 2B). The *pf27* cilia are slightly shorter than wild-type cilia (Supp. Fig. S2). Examples of the corresponding fluorescence intensity profiles of the cells imaged in Figure 2A are shown in Figures 2B and 2C.

There was variation between *pf27* cells in the length of radial spoke assembly along the proximal axoneme (standard deviation = 1.03 μm). This variation in proximal radial spoke staining is also reflected in our densitometry analysis of reduced RSPs in *pf27* axonemes (Table I). In addition, 30.5% of *pf27* cross-sections examined by electron microscopy contained a variable number of spokes (Fig. 1B). These data suggest that the length of radial spoke assembly at the proximal end of the *pf27* cilium is variable among cells, possibly consistent with diffusion of radial spoke precursors in short cilia (see Table I and Discussion).

Rescue of radial spoke assembly in *pf27* dikaryons occurs at the distal axoneme, independent of proximal radial spokes

We performed dikaryon rescue experiments to examine the location of radial spoke assembly in *pf27* axonemes during cytoplasmic complementation (see Johnson and Rosenbaum, 1992). Wild-type x *pf27* temporary dikaryons were collected at specific time points and fixed for immunofluorescence analysis of radial spoke assembly as described above. In *pf27* x wild-type dikaryons, RSP3 ciliary staining distinguished wild-type and *pf27*

cilia (Fig. 3A, green arrows). Rescue of radial spoke assembly consistently occurred in a tip down fashion (Fig. 3A, red arrowheads) until full recovery was seen (Fig. 3A, bottom row). This result is consistent with previous data demonstrating that radial spokes are transported by an IFT-mediated mechanism for assembly at the distal tip of the axoneme (Gupta et al. 2012; Johnson and Rosenbaum 1992; Qin et al. 2004). In addition, dikaryons between *pf27* and *pf14*, a mutant defective in RSP3 and completely lacking radial spokes, showed the same tip to base pattern of radial spoke assembly upon rescue (data not shown).

We also investigated whether new radial spoke assembly in *pf27* × wild-type dikaryons simultaneously occurs at the proximal axoneme end. We measured fluorescence intensity of RSP3 staining along the length of the *pf27* cilia in dikaryons (Fig. 3B). Similar to unmated cells (Fig. 2), the average length of proximal RSP3 staining in *pf27* cilia is $2.81 \pm 1.03 \mu\text{m}$ 45 minutes after mixing gametes ($n = 21$) and $2.87 \pm 0.91 \mu\text{m}$ 85 minutes after mixing gametes ($n = 62$). A vertical scatter plot of the range and similarity between lengths of proximal RSP3 staining in unmated versus dikaryon cells is shown in Figure 3C. Relative to the total ciliary length ($\sim 9 \mu\text{m}$), a minor ($\sim 0.6 \mu\text{m}$) difference in proximal staining was seen in *pf27* unmated cells versus dikaryons. Notably, the variability in proximal radial spoke staining is similar in unmated and dikaryon *pf27* cells, indicating rescue of radial spoke assembly in dikaryons occurs primarily at the distal tip of the axoneme with relatively insignificant additional assembly at the proximal axoneme.

The axonemal radial spokes in *pf27* are fully assembled 20S complexes

One question is whether the radial spokes at the proximal end of the *pf27* cilia are fully assembled 20S radial spoke complexes (Diener et al. 2011; Yang et al. 2001). Our ultrastructural analysis of *pf27* cross-sections reveal characteristic T-shaped radial spokes in the axoneme when present, suggesting that the few radial spokes that dock are fully assembled 20S spoke structures (Fig. 1B). In addition, longitudinal sections show fully assembled radial spokes often arranged in pairs, repeating at 96 nm along the length of the axoneme (Fig. 4A). As expected, longitudinal sections of whole cells near the cell body displayed fully assembled radial spokes just beyond the transition zone (Fig. 4B). This result is consistent with the immunofluorescence data illustrating proximal radial spoke assembly (Fig. 2A).

We also used a complimentary biochemical approach to test whether *pf27* axonemal radial spokes are fully assembled into a 20S complex characteristic of radial spokes (Diener et al. 2011; Yang et al. 2001). Radial spokes were isolated from wild-type and *pf27* axonemes by KI extraction and analyzed by velocity sedimentation on sucrose gradients (Fig. 4C). Axonemal radial spoke fractions from wild-type and *pf27* were probed for RSP1, RSP3, and RSP16 by immunoblot. Supporting our EM results, isolated radial spokes from both wild-type and *pf27* axonemes cosedimented in the sucrose gradients at 20S indicating that *pf27* spokes are also fully assembled (Fig. 4C, arrow). In addition to analyzing the assembly of the 20S RS complex in *pf27*, we found no difference in the assembly of the 12S RS precursor in wild-type and *pf27* cytoplasm by velocity sedimentation (Fig. 4D).

To test if *pf27* is defective in the docking of radial spokes to the axoneme, we used an *in vitro* reconstitution approach (Diener et al. 2011; Yang et al. 2001). Radial spokes derived from the wild-type axoneme bind the *pf14* spokeless axoneme to saturation, suggesting precise docking of radial spokes to the axoneme (Yang et al. 2001). Similarly, isolated wild-type axonemal radial spokes bind the *pf27* axoneme to saturation (Fig. 5). As expected, saturation of radial spoke binding to the *pf27* axoneme was reached more quickly than onto *pf14* axonemes, due to assembled radial spokes occupying proximal docking sites on the *pf27* axoneme. We also reconstituted isolated *pf27* axonemal radial spokes onto *pf14* axonemes. Radial spokes derived from the *pf27* axoneme bound the *pf14* axoneme to

saturation (Fig. 5, lower panel). Although we cannot exclude an issue in the experimental design, it appears isolated *pf27* radial spokes saturate the *pf14* axoneme at a lower ratio.

Together, these data indicate that the radial spokes at the proximal end of the *pf27* axoneme are fully assembled into the 20S mature complex competent to bind the axoneme. Furthermore, these results indicate that the defect in *pf27* is not in the axonemal radial spoke docking mechanism. Consistent with this, our preliminary data reveal that CaM-IP2 and CaM-IP3 of the CSC complex are assembled in *pf27* axonemes (Dymek et al. 2011; Dymek and Smith 2007; Heuser et al. 2012).

Radial spoke assembly occurs asynchronously from other axonemal components during ciliary regeneration in *pf27*

Although fewer in number, *pf27* axonemal radial spokes appear intact. Given these data, we then asked how the proximally localized radial spokes come to be assembled in the growing *pf27* axoneme. To examine this, we deflagellated wild-type and *pf27* cells and collected axonemes at specific time points during ciliary regeneration. The axonemes were analyzed by immunoblot to measure the rate of radial spoke assembly relative to other axonemal complexes including the outer and inner dynein arms and the N-DRC (nexin – dynein regulatory complex).

For wild-type cells we predicted that all axonemal complexes assemble at the same continual rate as the cilium grows. This is illustrated in Figure 6A. As predicted for wild-type, the radial spokes (RSP1, 3, and 16), N-DRC (DRC1), and outer (IC69) and inner (IC138, p28) dyneins were restored at a constant rate as the cilium grew (Fig. 6B). Densitometry confirmed the relative band intensity of each time point, compared to the pre-deflagellation band intensity, was very close to 1, indicating no significant difference in the assembly rate for axonemal components analyzed (Fig. 6C). The simplest interpretation of these results is a coordinated, continual transport and assembly of the complexes, including the radial spokes as the wild-type cilium grows (Fig. 6A).

We predicted two models for radial spoke assembly during ciliary regeneration in *pf27*. Prediction 1 posits that *pf27* axonemal radial spokes (black) assemble like wild-type, concurrent with other axonemal components (red), but assembly halts once the cilium reaches ~2-3 microns. In prediction 2 we hypothesize that *pf27* radial spokes (black) assemble progressively at the proximal axoneme, but more slowly, and asynchronously from other axonemal components (red) as the cilium grows (Fig. 6C, Prediction 2).

In contrast to the coordinated, continual assembly of all axonemal complexes in wild-type cilia, immunoblot analysis of axonemes during *pf27* regeneration illustrate a relatively slow but progressive increase in RSPs over time, whereas other axonemal components assemble at a constant rate (Fig. 6D, F). As with wild-type, band intensity for axonemal assembly was quantified by densitometry and non-radial spoke components consistently had a relative density near 1 (Fig. 6F). Thus, radial spoke incorporation into the *pf27* axoneme is gradual and asynchronous from other axonemal components, yet occurs progressively (Fig. 6F). Regeneration of wild-type and *pf27* cilia occurs at a similar rate (Supp. Fig. S2). The data support prediction 2 (Fig. 6C) revealing an independent mechanism of radial spoke assembly from other axonemal components in *pf27* and an apparent failure of assembly of radial spokes components at the distal end as the cilium grows.

Discussion

Summary of the Results: Efficient Radial Spoke Assembly Requires Transport

The *pf27* mutant is deficient in axonemal radial spoke assembly and contains approximately 25% the amount of wild-type axonemal radial spokes. One of our most important observations is that the few radial spokes assembled in *pf27* axonemes are fully formed and properly docked (Figs. 4 and 5), but only assemble at the proximal end of the axoneme. In addition, ciliary regeneration experiments revealed that the proximal assembly of radial spokes in *pf27* axonemes occurs asynchronously from assembly of other axonemal components (Fig. 6). Since other axonemal complexes, such as the outer dynein arms (Hou et al. 2007; Omran et al. 2008) are assembled in *pf27* axonemes (Fig. 1), IFT appears to be operating normally for these structures as in wild-type cells. Moreover, dikaryon rescue experiments revealed rescue of spoke assembly at the distal tip, presumably mediated by IFT (Johnson and Rosenbaum 1992; Qin et al. 2004), independent of the proximal assembly of radial spokes in *pf27* (Fig. 3). These observations appear contrary to the established idea that radial spokes are transported by IFT to the distal tip of the ciliary axoneme where final assembly takes place (Diener et al. 2011; Gupta et al. 2012; Qin et al. 2004). A simple explanation for assembly of the proximally localized radial spokes in *pf27* is that in short cilia diffusion of radial spoke precursors is sufficient for assembly (Fig. 7A, C), whereas radial spoke assembly within longer cilia requires active transport of radial spoke precursors by IFT (Fig. 7B and see Diener et al. 2011; Qin et al. 2004). Thus, *pf27* has revealed a potential novel mechanism of radial spoke assembly in short and growing cilia that involves diffusion and does not require active transport (Fig. 7).

Our data indicate that radial spoke assembly is uncoupled from IFT in *pf27* preventing further radial spoke assembly as the cilium grows. We postulate that *PF27* encodes a protein required for facilitating interaction between radial spoke precursors and the ciliary IFT transport machinery. However, further direct tests of this idea require identification of the *PF27* gene. Despite careful mapping of *pf27* to chromosome 12 near the centromere (Supp. Table SI; Dutcher et al. 1991; Huang et al. 1981), repeated whole-genome sequencing analysis of *pf27* (Supp. Fig. S1) and transformation with BACs mapped to chromosome 12, we have failed to identify the gene. Identification of *PF27* is likely to be difficult due to *PF27*'s location near the centromere and gaps or misassembly of the assembly of the genome of chromosome 12 (Kwan et al. 2009).

Model for Radial Spoke Assembly

Radial spoke assembly includes a complex series of steps that begins in the cytoplasm with the assembly of a subset of spoke proteins into a 12S complex (Diener et al. 2011; Qin et al. 2004) followed by subsequent processing to form the mature 20S radial spoke (Fig. 7; Diener et al. 2011; Gupta et al. 2012; Yang et al. 2005). We found no difference in assembly of the 12S radial spoke precursor complex in the cytoplasm of wild-type and *pf27* (Fig. 4D). The mature 20S radial spoke then docks on the outer doublet microtubules of the axoneme (Diener et al. 1993). This series of steps, illustrated in Figure 7B, includes entry to the ciliary compartment (step 2); IFT-mediated transit to the distal tip of the cilium (steps 3 and 4), unloading, processing, and final assembly of the mature 20S radial spoke (step 5), followed by docking on the axonemal microtubules (step 6).

Assembly of the ciliary barrier and radial spoke precursor entry

Although diverse evidence supports this general sequence of events for radial spoke assembly, many questions remain. The regulation or facilitation of protein and protein complex passage is not understood (Nachury et al. 2010; Obado and Rout 2012; Reiter et al. 2012), and could involve IFT and/or IFT-independent transporters (Belzile et al. 2013;

Dishinger et al. 2010; Ludington et al. 2013; Reiter et al. 2012; Ye et al. 2013). One model, known as the avalanche model, posits that accumulation of IFT components at the ciliary base correlates with the rate of entry into the cilium (Ludington et al. 2013). Additional new evidence indicates the presence of a size barrier at the proximal end of the cilium at the transition zone (Kee et al. 2012; Lin et al. 2013).

Since radial spokes assemble in the *pf27* axoneme, radial spoke precursors enter the ciliary compartment, but this entry is likely less efficient. One idea is that the ciliary barrier may assemble more slowly than the axoneme during ciliary growth, which permits temporary free access for radial spoke precursors to the ciliary compartment. Following assembly and maturation of the postulated barrier, radial spoke precursor entry to the cilium may then require a protein encoded by *PF27*. However, our regeneration data may be inconsistent with this model since we predict an abrupt halt in axonemal radial spoke assembly when the putative barrier matures and becomes selective (Fig. 6D, Prediction 1).

Rather, we observed gradual assembly of axonemal radial spokes throughout regeneration in *pf27* as illustrated in Prediction 2 (Fig. 6). In this case, radial spoke assembly is progressive but asynchronous with assembly of the remainder of the axoneme, and is curtailed when spokes assemble to about 25% of the final length of the axoneme. Radial spoke assembly onto the *pf27* axoneme throughout regeneration may be due to inefficient docking of 20S radial spoke complexes, contained in the ciliary compartment, to the axoneme. However, our data do not address the timing and mechanism of radial spoke entry in *pf27*. Further direct tests of radial spoke entry and assembly will require live cell imaging and a suitable radial spoke transgene.

Diffusion and transport of radial spoke precursors and possible functions for PF27p

Diffusion within the ciliary compartment may account for the variability in radial spoke assembly from cell to cell in *pf27* cilia. Specifically, the distance of proximal RSP immunostaining in *pf27* cilia varied by approximately 0.6 microns in single unmated cells and dikaryons (Fig. 3C). If radial spoke precursors are free to diffuse in the cilium, the question remains: what inhibits radial spoke assembly along the remainder of the axoneme in *pf27*? One possibility is that as other axonemal complexes assemble, including the dynein arms, nexin-dynein regulatory complex and central pair apparatus, further diffusion of radial spoke precursors is physically impeded. Alternatively, radial spokes of *pf27* may reach the center of the axoneme base through the basal body or through the sides of the ring of outer doublets.

As described above, one model is that *PF27* encodes a protein adaptor required for IFT-mediated radial spoke precursor transport. The idea for an IFT – radial spoke adaptor is derived from studies of outer dynein arm transport by IFT (Ahmed et al. 2008; Hou et al. 2007). The outer dynein requires an adaptor protein, ODA16p, for efficient dynein transport and assembly (Ahmed et al. 2008; Ahmed and Mitchell 2005; Gao et al. 2010). Similar to the radial spoke abundance in *pf27* cilia, low abundance of the outer dynein arm is found in the ciliary compartment of *oda16* mutant cells. Moreover, IFT46p is required for specific assembly of the outer dynein arms (Hou et al. 2007), and ODA16p associates with IFT46p thereby suggesting an interaction between IFT machinery and the ODA16p adaptor to transport outer dynein arms (Ahmed et al. 2008). Thus, it is possible that ciliated cells express classes of adaptor proteins, each responsible for linking a specialized subset of axonemal proteins / complexes to IFT. In the case of the radial spokes, we speculate that *PF27p* could serve this purpose.

Alternatively, *PF27* may encode a protein modifier that facilitates IFT – radial spoke interaction. For example, radial spoke assembly appears to involve changes in radial spoke

protein phosphorylation, particularly RSP3, that seems to occur at the ciliary tip (Gupta et al. 2012). In addition, posttranslational modification may play a role in IFT-mediated transport of radial spoke components (Diener et al. 2011). Thus, it remains possible that *pf27* is defective in a protein kinase required for normal transport of spoke proteins. Consistent with this idea, radial spoke proteins exhibit hypo-phosphorylation in *pf27* axonemes (Huang et al. 1981). Specifically, radial spoke proteins 2, 3, 5, 13, and 17 were absent or present in trace quantities by ^{32}P radioactive labeling in the *pf27* axoneme compared to wild-type. Using SDS-PAGE and immunoblots, our preliminary data also show altered migration of RSP3 in *pf27* axonemes (Fig. 1A) consistent with hypophosphorylation (Gupta et al. 2012). Thus, an alternative idea is that *PF27* encodes a protein kinase required for normal transport of the radial spoke precursors within the cilium. Either as adaptor or a modifier, PF27p would be extrinsic to the assembled radial spoke and axoneme as predicted by Huang et al., (1981).

Materials and Methods

Strains and culture conditions

Chlamydomonas reinhardtii strains used in this study include wild-type (CC-125, CC-620, CC-621), the polymorphic strain S1C5 (CC-1952; (Gross et al. 1988)), *pf27* (CC-1387, CC-3321), and *pf14* (CC-613, CC-1032). All strains were obtained from the *Chlamydomonas* Resource Center (University of Minnesota, St. Paul, MN). Cells were cultured in tris-acetate-phosphate (TAP) medium with aeration on a 14:10 h light/dark cycle or under constant illumination, unless otherwise noted. Dikaryons between wild-type and *pf27* were formed by mixing gametes of each cell type that were first differentiated 4-16 h in M-N medium (Harris 2009).

Preparations of axonemes

Deflagellation of *C. reinhardtii* cells was induced by treating cells with dibucaine (Witman 1986). Flagella/cilia were collected by subsequent centrifugation. To prepare axonemes, flagella were demembranated with 0.1% Nonidet P-40 in HMDEgS (10 mM Hepes, 5 mM MgSO_4 , 1 mM DTT, 0.5 mM EGTA, and 4% sucrose, pH 7.4) and centrifuged to remove the membrane and matrix fraction. Axoneme pellets were processed immediately for electron microscopy, immunoblotting, or radial spoke isolation. For immunoblotting, purified axonemes were resuspended in Na-low (10 mM Hepes, 5 mM MgSO_4 , 1 mM DTT, 0.5 mM EDTA, and 30mM NaCl, pH 7.4) and fixed with Laemmli sample buffer at a concentration of 1 mg/ml.

Electron microscopy

Axonemes: For thin section electron microscopy, pelleted axonemes were fixed and embedded as described previously (Mitchell and Sale 1999). Blocks were thin sectioned, stained with uranyl acetate and lead citrate, and viewed using a Hitachi H7500 transmission electron microscope. Images were acquired using a Gatan 792 BioScan CCD camera with Gatan Digital Micrograph software (Gatan, Pleasanton, CA). **Whole cells:** Pelleted intact cells were fixed and prepared for ultrastructural analysis as previously described (Craig et al. 2010). Briefly, cells in media were fixed with 1% glutaraldehyde (final concentration). The cells were then thoroughly washed with 100 mM sodium cacodylate, pH 7.2. After the final wash, a cell/agarose suspension was made and solidified on ice. The cell/agarose block was then cut into $\sim 1 \text{ mm}^3$ pieces, postfixed for 1 h on ice with 1% OsO_4 in 50 mM sodium cacodylate, washed three times with ice-cold water, and stained overnight at 4°C in freshly prepared 1% uranyl acetate in water.

SDS-PAGE and immunoblot analysis

SDS-PAGE and immunoblotting were performed using standard procedures. SDS-PAGE was performed using 7.5% or 10% acrylamide gels. Gels were stained with Coomassie brilliant blue (CBB) or transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) for subsequent immunoblotting. The membranes were blocked with 5% nonfat dry milk and incubated with various primary antibodies. Immunoreactive bands were detected using an HRP-conjugated secondary antibody (1:20,000; Bio-Rad Laboratories, Hercules, CA) and a 3,3',5,5'-tetramethylbenzidine peroxidase substrate kit (Vector Laboratories, Burlingame, CA) or Pierce ECL western blotting chemiluminescent substrate (Thermo Scientific, Rockford, IL). Immunoblots were probed with the following antibodies (5–10 μ g axonemes): anti-RSP1 (1:10,000; Kohno et al. 2011), anti-RSP2 (1:5000; Yang et al. 2004), anti-RSP3 (1:4000; Wirschell et al. 2008), anti-RSP8 (1:300), anti-RSP10 (1:1000), anti-RSP11 (1:1000; Yang et al. 2006), anti-RSP16 (1:3000; Yang et al. 2005), anti-DRC1 (1:500; Wirschell et al. 2013), anti-IC138 (1:10,000; Hendrickson et al. 2004), anti-IC69 (1:100,000; monoclonal anti-dynein 1869A hybridoma, Sigma, St. Louis, MO), anti-actin (1:300; Kato-Minoura et al. 1997), anti-p28 (1:500; LeDizet and Piperno 1995) and anti-CSC antibodies CaM-IP2 (1:500) and CaM-IP3 (1:1000; Dymek et al. 2011; Dymek and Smith 2007). Densitometry of immunoblot analysis was performed on 8-bit images taken with an AlphaImager HP using FluorChem SP software (ProteinSimple, Santa Clara, CA).

Immunofluorescence microscopy and quantification

For immunofluorescence of individual cells and dikaryons, cells were allowed to adhere for 5 min to polyethylenimine-coated coverslips, excess cells were rinsed off by submersion in 0.1M HEPES, pH 7.4, and the coverslips were submerged in -20°C methanol for 5 min. The coverslips were then air-dried, rehydrated with 0.1M HEPES, pH 7.4, blocked for 1 h at RT with blocking solution (1% fish skin gelatin, 2% BSA, 15% horse serum, 0.02% saponin, and 0.1% Triton X-100 in PBS, pH 7.0), and incubated with primary antibodies (either 30–60 min at RT or 4°C overnight). Antibodies were diluted in blocking buffer and used at the following concentrations: affinity purified RSP3 at 1:100 (Wirschell et al. 2008) and IC69 at 1:1000 (monoclonal anti-dynein 1869A hybridoma, Sigma, St. Louis, MO). Coverslips were subsequently washed 3X with blocking solution, incubated with secondary antibodies (Alexa Fluor–conjugated IgG, 1:1000; Invitrogen, Eugene, OR) for 1 h at RT, washed 3X with blocking solution, rinsed in buffer, then mounted with ProLong Antifade Gold (Invitrogen, Eugene, OR). Images were captured using a BX60 widefield microscope (Olympus, Tokyo, Japan) with a 12-bit digital camera (Orca-ER, Hamamatsu, Bridgewater, NJ) and Slidebook software (Intelligent Imaging Innovations, Denver, CO). Image processing and fluorescent linescans of flagella were performed in ImageJ (National Institutes of Health, Bethesda, MD). Analysis of flagellar linescans was conducted in Matlab (Math Works, Natick, MA). A rolling average (window=3 pixels) was applied to the linescans and they were normalized to the brightest pixel. The length of RSP3 staining was defined as the first point at which the intensity dropped and stayed below 20% for at least 3 consecutive pixels, because of the punctate pattern of fluorescence. Probability was calculated using multiple statistical tests, including the Student's t-test.

Cytoplasmic extract fractionation

Cells cultured for 3 days were lysed by the glass bead method previously described (Ahmed et al. 2008). Broken cells were spun at 10,000 rpm for 10 minutes and the supernatant was clarified at 22.5K rpm for 2h (Type-40 fixed angle rotor, Beckman Coulter, Fullerton, CA). The supernatant, cytoplasmic extract, was loaded on an 11.5 ml 5–20% continuous sucrose gradient, sedimented at 32.5K rpm (SW41Ti rotor; Beckman Coulter, Fullerton, CA) for 16

h, and fractionated into 22 0.5 ml fractions. For SDS-PAGE, fractions were fixed with 5X Laemmli sample buffer.

Isolation of axonemal radial spokes, fractionation, and in vitro reconstitution

Radial spoke isolation, fractionation, and in vitro reconstitution were slightly modified from published protocols (Kelekar et al. 2009). Axonemal pellets (wild-type or *pf27*, preparation described above) were resuspended to 5mg/ml in Na-hi (10 mM Hepes, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EDTA, and 0.6M NaCl) and incubated for 15 min on ice to remove dynein arms. The extraction was repeated to remove residual dynein components. The extracted axonemes were resuspended in 0.5M potassium iodide (KI) to 8-10 mg/ml, incubated for 30 min on ice, and pelleted for 10 min at max speed in a microcentrifuge. The supernatant, isolated radial spokes, was dialyzed in Na-med (10 mM Hepes, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EDTA, and 0.2M NaCl) for 2 h at 4°C and then clarified by centrifugation. For fractionation, 0.6 ml supernatant (dialyzed KI extract) was loaded on an 11.5 ml 5–25% continuous sucrose gradient in Na-med, sedimented at 32.5K rpm (SW41Ti rotor; Beckman Coulter, Fullerton, CA) for 16 h, and fractionated into 22 0.5 ml fractions. For SDS-PAGE, fractions were fixed with 5X Laemmli sample buffer. For in vitro reconstitution (Yang et al. 2001), increasing amounts of the dialyzed KI extract were added to a fixed quantity (50µl, 1 mg/ml) of *pf14* or *pf27* axonemes first extracted with 0.6M NaCl to remove dynein arms. The final volume was adjusted to 90µl, and after 30 min incubation on ice, the mixture was centrifuged at 12,000 g for 10 min. Pellets and supernatant were processed as described above for immunoblot analysis.

Ciliary regeneration

Cells were cultured in L-media for ease of deflagellation, collected by centrifugation, and resuspended in deflagellation buffer (10 mM Tris, 5% sucrose, 1 mM CaCl₂, pH 7.5). Cells were deflagellated by pH shock (Lefebvre 1995), washed once with L-media, and resuspended in L-media to allow for regeneration of flagella on constant light (Lefebvre 1995). At specific timepoints, cells were deflagellated by the dibucaine method (Witman 1986) and axonemes were prepared for SDS-PAGE as described above.

Whole-genome sequencing and read alignment (Supplemental Material)

Chlamydomonas genomic DNA was prepared as previously described (Dutcher et al. 2012). Approximately 10⁸ cells were used in DNA preparation. About three µg of genomic DNA from each strain was submitted to Genome Technology Access Core (Department of Genetics, Washington University in St. Louis) for Illumina sequencing (Illumina Inc., San Diego, CA). The 101 bp paired-end sequencing was performed with the Illumina HiSeq platform. The indexed sequencing reads were de-multiplexed before being subjected to read alignment. For read alignment, the genome sequence of *Chlamydomonas* v5.3.1 (*C. reinhardtii_236.fa.gz*) was downloaded from <http://www.phytozome.net/chlamy.php> (Merchant et al. 2007). The building of an indexed database and SNP calling is described elsewhere (Lin et al., in press).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The *pf27* mutant has reduced axonemal radial spokes compared to wild-type
(A) Immunoblots of wild-type and *pf27* axonemes show a $76.1 \pm 12.5\%$ reduction in *pf27* radial spoke proteins using antibodies to the radial spokehead proteins 1 and 10 and spoke stalk proteins 2, 3, 8, 11, and 16 ($n = 22$). CBB = Coomassie Brilliant Blue load control. **(B)** Electron microscopy of *pf27* axonemes: non-serial cross-sections are shown. In 55.1% of cross-sections no radial spokes were present (top panel, $n = 160$). 30.5% of cross-sections showed a reduced number of radial spokes; between 1 and 8 spokes were missing (middle panel). 14.4% of cross-sections contained all 9 radial spokes (bottom panel). When present, radial spokes typically appeared as intact T-shaped structures. Red arrows indicate beak structures in the B-tubule of outer doublets 1, 5, and 6, a unique structure to the proximal

end of the axoneme. Beak structures were present in cross-sections that contained all 9 radial spokes and occasionally in cross-sections with some spokes present. Scale bar = 50nm. (C) Schematic of predicted location of non-serial *pf27* cross-sections along the length of the axoneme. The spokes appear as T-shaped 20S intact complexes and are represented as such in the model. The hatched box represents the basal body. Since beak structures correlate with the presence of all 9 radial spokes in the *pf27* axoneme, those 14.4% of cross-sections are presumed to be at the proximal end of the axoneme. In contrast, when spokes are missing, beaks structures are not present, so those cross-sections are likely at the distal end. In 30.5% of cross-sections with one or more spokes are missing and beak structures were intermittently seen. (D) Other axonemal components were examined for integrity of the *pf27* axoneme including the outer dynein arm (IC69), I1 dynein (IC138), the dynein regulatory complex (DRC1), and single-headed inner dynein arms (actin and p28). Each of these components is present at near equal levels in wild-type and *pf27* axonemes, indicating the *pf27* defect is in radial spoke assembly only.

Figure 2. Radial spokes of *pf27* are localized to the proximal end of the cilium

(A) Immunofluorescence of RSP3 (green) in wild-type (WT) and *pf27* cilium. The outer dynein arm (IC69; red) was used as a marker for cilia. RSP3 and IC69 colocalize along the length of the cilium in wild-type cells (top panels). In contrast, RSP3 only localizes to the proximal end of *pf27* cilium (green arrows, bottom panels). Full-length cilia are present on *pf27* cells indicated by IC69 staining along the length, although *pf27* cilia are slightly shorter than wild-type (Supp. Fig. 2). Scale bar = 2 μm . (B) Quantification of RSP3 immunofluorescence along the length of the cilium. Distance from the cell body in microns is along the x-axis. The relative normalized intensity (AU) is plotted against distance along the cilium for one cilium of the wild-type cell shown in part A. Since RSP3 localizes along the entire length of the cilium in a punctate pattern, the relative intensity remains above the 0.2 threshold (dashed line). (C) Graph of one cilium from the *pf27* cell shown in part A. The graph illustrates the proximal pattern of RSP3 staining in *pf27* cilium as well as the average distance (2.26 μm , dotted line) at which spoke staining drops below the 0.2 threshold ($n = 82$). The shaded box shows the standard deviation (1.03 μm) of the distance of proximal staining in *pf27* and the high variability.

Figure 3. Dikaryon rescue of *pf27* radial spoke assembly occurs from the distal end
(A) Immunofluorescence of RSP3 in *pf27* x wild-type (WT) dikaryons. A dikaryon fixed 45 minutes after mixing *pf27* and wild-type gametes is shown in the top panels. The *pf27* cilia with proximal staining (green arrows) are distinct from wild-type cilia. Recovery of RSP3 can be seen at the distal end of the *pf27* cilia (red arrowheads) 85 minutes post-mixing in addition to the characteristic proximal staining (green arrows). Radial spoke assembly fully recovers approximately 100 minutes post-mixing (bottom panels). Scale bar = 2 μ m. **(B)** Quantification of RSP3 immunofluorescence along the length of *pf27* cilium in dikaryons as described in Fig. 3. The plots of relative fluorescence intensity in *pf27* cilium versus distance from the cell body correspond to the dikaryons in part A. The average length of *pf27* proximal RSP3 staining at 45 minutes post-mixing is 2.81 μ m (dotted line; SD = 1.03 μ m, shaded box) and that at 85 minutes post-mixing is 2.98 μ m (dotted line; SD = 0.88 μ m, shaded box). **(C)** Comparison of proximal RSP3 staining in *pf27* cilia from gametes versus dikaryons. Each dot of the vertical scatter plot represents the distance of RSP3 staining from one cilium. Relative to the total ciliary length, a minor (~ 0.6 μ m) change in proximal staining was seen in *pf27* gametes versus dikaryons at 45 minutes ($p = 0.0348$) and *pf27* gametes versus dikaryons at 85 minutes ($p = 0.0001$). Thus, rescue of radial spoke assembly occurs first at the distal axoneme.

Figure 4. Axonemal radial spokes from *pf27* are fully assembled and intact

(A) Longitudinal section from electron microscopy of *pf27* axonemes reveals pairs (red asterisks) of radial spokes fully assembled into the 20S T-shaped structure. Scale bar = 100 μ m. (B) Electron microscopy section of whole *pf27* cells at the transition zone. Axonemal radial spokes of *pf27* are assembled in pairs near the cell body (red asterisks). Scale bar = 50 μ m. (C) Immunoblots of fractions from velocity sedimentation of isolated axonemal radial spokes were analyzed. Axonemal radial spokes from *pf27* cosediment with those from wild-type at fraction 8 (arrow) when analyzed for a spokehead protein (RSP1) and the spoke stalk proteins RSP3 and RSP16. (D) Fractions from velocity sedimentation of cytoplasmic extracts were analyzed by immunoblot. *pf27* 12S precursor subunits RSP1 and RSP3

cosediment with WT RSP1 and RSP3 at fractions 13-14 (arrow). Thus, the 12S precursor complex is assembled like wild-type in the *pf27* cytoplasm.

Figure 5. Radial spoke docking in *pf27* is intact

In vitro reconstitution of isolated axonemal radial spokes onto extracted axonemes. Varying ratios of radial spoke extract to extracted axonemes (RS:Axoneme) were combined, incubated on ice for 30 minutes, and probed for RSP3 by immunoblot. Wild-type radial spokes reconstituted onto spokeless *pf14* axonemes result in spoke binding (P = pellet) to saturation (S = supernatant/unbound RS). Wild-type radial spokes reconstituted onto *pf27* axonemes also bind (P) to saturation and the excess unbound fraction (S) is the result of less spoke binding sites on the *pf27* axoneme due to assembled spokes occupying proximal docking sites. Isolated axonemal spokes from *pf27* bind the *pf14* axoneme to saturation.

Figure 6. The proximal radial spokes in the *pf27* axoneme assemble asynchronously from the rest of the axoneme during ciliary regeneration

(A) Schematic model of axonemal assembly in regenerating wild-type cilia. After deflagellation, wild-type axonemal components are predicted to assemble concurrently during regeneration. Specifically, the radial spokes (black) assemble at the same rate as other axonemal components (red). (B) Immunoblot analysis of regenerating wild-type cilia. Axonemes isolated before and three time points after deflagellation were probed for radial spoke proteins 1, 3, and 16. Other axonemal components including the dynein regulatory complex (DRC1), outer dynein arm (IC69), II dynein (IC138), and single-headed inner dynein arms (p28) were also examined. Band intensity for each of these axonemal

components remains unchanged during regeneration. Consistent with the model, axonemal components assembled in the regenerating cilium at the same rate as quantified by densitometry (graph in C). Ponceau staining was used as a load control for densitometry. (C) Quantification graph of band density over time illustrates concurrent assembly of axonemal components in wild-type. (D) Schematic model of axonemal assembly in regenerating *pf27* cilia. Since *pf27* axonemes have less radial spokes than wild-type, we present two predictions for *pf27* axonemal radial spoke assembly relative to other axonemal components. Prediction 1 illustrates radial spoke (black) assembly concurrent with other axonemal components (red) in the early stages of regeneration. At about 2 μ m, radial spoke assembly ceases while other axonemal components continue to assemble. Prediction 2 illustrates a progressive and independent assembly of *pf27* axonemal radial spokes (black) compared to other axonemal components (red). (E) Immunoblot analysis of regenerating *pf27* cilia support prediction 2. Band intensity for radial spoke proteins 1, 3, and 16 increased progressively during regeneration while band intensity for other axonemal components (DRC1, IC69, IC138, and p28) remained unchanged. Densitometry confirmed the independent assembly of *pf27* axonemal radial spokes from other axonemal components as in prediction 2 (graph in F). (F) Quantification of band density illustrates progressive and independent assembly of *pf27* axonemal radial spokes compared to other axonemal components.

Figure 7. Model for axonemal radial spoke assembly as revealed by the *pf27* mutant

(A) Short cilia: Axonemal radial spokes first assemble as a precursor 12S complex in the cell body (1). Passage of RS precursors into the ciliary compartment (basal body = hatched box) is followed by diffusion (2). In short cilia, diffusion is sufficient for 12S precursor complexes to reach the tip of the axoneme where unknown factors are present for modification and final assembly into a fully assembled 20S spoke (3). Therefore, the 20S spoke is competent to dock onto the axoneme (4). (B) Wild-type cilia: When the growing cilium reaches approximately 2 μm in length, the 12S radial spoke precursors cannot reach the tip by diffusion. Therefore, concurrent with diffusion (2), RS precursors are loaded onto IFT (3) for transport to the tip (4). Modification and final assembly of the radial spoke

occurs at the growing tip at all lengths of ciliary assembly and the fully assembled 20S spoke is competent to dock onto the axoneme (6). (C) *pf27* cilia: Without IFT-mediated transport, radial spoke precursors in the *pf27* ciliary compartment cannot reach the distal tip of the assembling axoneme beyond 2 μ m for processing and final assembly. Since 12S precursor complexes are not competent to bind the axoneme, diffusion to the tip during ciliary construction allows radial spokes of the *pf27* axoneme to localize proximally.

Table I
Reduction of *pf27* axonemal radial spokes

Radial spokes are consistently reduced in axonemes from *pf27* compared to wild-type when analyzed by immunoblot and by microscopy.

Experiment	% Assembled Compared to Wild-type	St. Dev.
Immunoblot	23.9	12.5
EM	14 - 45	NA ^a
IF: Single unmated cells	19	10.3
IF: Dikaryons	28.1	10.3

^aBased on the type of analysis (categorization) of EM cross-sections, it was not possible to calculate a standard deviation.