

Association of Lis1 with outer arm dynein is modulated in response to alterations in flagellar motility

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ABSTRACT The cytoplasmic dynein regulatory factor Lis1, which induces a persistent tight binding to microtubules and allows for transport of cargoes under high-load conditions, is also present in motile cilia/flagella. We observed that Lis1 levels in flagella of *Chlamydomonas* strains that exhibit defective motility due to mutation of various axonemal substructures were greatly enhanced compared with wild type; this increase was absolutely dependent on the presence within the flagellum of the outer arm dynein α heavy chain/light chain 5 thioredoxin unit. To assess whether cells might interpret defective motility as a “high-load environment,” we reduced the flagellar beat frequency of wild-type cells through enhanced viscous load and by reductive stress; both treatments resulted in increased levels of flagellar Lis1, which altered the intrinsic beat frequency of the *trans* flagellum. Differential extraction of Lis1 from wild-type and mutant axonemes suggests that the affinity of outer arm dynein for Lis1 is directly modulated. In cytoplasm, Lis1 localized to two punctate structures, one of which was located near the base of the flagella. These data reveal that the cell actively monitors motility and dynamically modulates flagellar levels of the dynein regulatory factor Lis1 in response to imposed alterations in beat parameters.

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INTRODUCTION

Multiple lines of evidence indicate that the lissencephaly protein Lis1 is a key factor in the regulation of cytoplasmic dynein. This protein was identified as NUDF in a screen for nuclear distribution mutants in *Aspergillus* (Xiang *et al.*, 1995) and found to interact genetically with both NUDC and NUDE. In addition, mutations in the cytoplasmic dynein heavy chain gene (NUDA) were able to suppress the phenotype caused by defects in Lis1 (NUDF; Willins *et al.*, 1997). Subsequently, mammalian Lis1 together with NudE or its close homologue NudEL1 was demonstrated to associate with the cytoplas-

mic dynein motor domain (Sasaki *et al.*, 2000; Tai *et al.*, 2002) and induce a persistent high-force-generating state that is required for the slow transport of large cargoes such as nuclei within cells and/or for the generation of cortical tension (McKenney *et al.*, 2010); in essence, Lis1 association increases the microtubule-binding affinity of cytoplasmic dynein. Lis1 is essential in mammals, and homozygous Lis1-knockout mice die in utero (Hirotsune *et al.*, 1998). Microinjection experiments in dividing mammalian cells also support a role for Lis1 during cell division (Faulkner *et al.*, 2000). In humans, Lis1 haploinsufficiency causes aberrant nuclear migration in neurons and results in a smooth-brain phenotype with devastating neurological consequences (Wynshaw-Boris and Gambello, 2001).

In addition to its interactions with cytoplasmic dynein, Lis1 has also been identified in motile mammalian cilia but found to be specifically absent from primary or sensory cilia that lack axonemal dyneins, radial spokes, and the central pair microtubule complex and consequently do not beat (Pedersen *et al.*, 2007). Furthermore, a Lis1 orthologue (encoded at *DOI1*; Hom *et al.*, 2011) was found in *Chlamydomonas reinhardtii* (Pedersen *et al.*, 2007) that does not express most components of the canonical cytoplasmic dynein/dynactin complex, including the Lis1-interacting proteins Nde1 and

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Abbreviations used: HC, heavy chain; IC, intermediate chain; IFT, intraflagellar transport; LC, light chain; MBP, maltose-binding protein.

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Ndel1 (Merchant *et al.*, 2007; King and Kamiya, 2009). Lis1 was identified within the flagellum of wild-type *Chlamydomonas* but was missing from the flagella of mutants that do not assemble outer dynein arms or the α heavy chain (HC)/light chain 5 (LC5) thioredoxin subcomplex; in contrast, Lis1 was observed in flagella of mutants that lack other axonemal substructures (Pedersen *et al.*, 2007). In addition, *Chlamydomonas* Lis1 was demonstrated to interact directly with mammalian NudC, which is highly expressed in ciliated epithelial cells (Gocke *et al.*, 2000) and is part of the cytoplasmic dynein regulatory pathway identified in *Aspergillus* (Morris *et al.*, 1998).

Axonemal dyneins are subject to multiple levels of regulation that ultimately control the beating of cilia and flagella (for recent reviews see Alford *et al.*, 2012; Porter, 2012; Wakabayashi, 2012). Currently recognized mechanisms involve signals from the radial spoke/central pair microtubule complex (Smith and Yang, 2004) and responses to the phosphorylation of individual dynein components (Porter and Sale, 2000), changes in Ca^{2+} levels (Hyams and Borisov, 1978), alterations in cellular redox poise (Wakabayashi and King, 2006), and direct action of the dynein regulatory complex (Piperno *et al.*, 1992). Furthermore, inner and outer arm dyneins also respond to mechanical cues that enable these motors to propagate a wave of activity along the axoneme (Hayashibe *et al.*, 1997; Patel-King and King, 2009), and their activity may be modulated by changes in ATP/ADP ratios (Kinoshita *et al.*, 1995). Given that Lis1 has been implicated in controlling the ability of cytoplasmic dynein to generate force by stabilizing the dynein–microtubule interaction, we reasoned that Lis1 in the flagellum might act in a similar manner to control the force-generating properties of outer arm dynein. This hypothesis would be consistent with biochemical studies that demonstrated that the α HC exerts a dominant-negative control over the microtubule-binding properties of other motor units within the outer arm (Sakato and King, 2003).

Here we demonstrate that imposed alterations in flagellar beat frequency due to mutation of several different axonemal subsystems, increased viscous load, or enhanced reductive stress all result in a dynamic increase in Lis1 levels within the flagellum. This implies that the cell monitors beat frequency and responds to an enforced reduction in this parameter by increasing intraflagellar and axonemal dynein-associated levels of Lis1. Because Lis1 binding to cytoplasmic dynein induces a long-term force-generating state, in the flagellum this would lead to persistent cross-bridges between outer arm dynein and the microtubule doublets that may modulate beat frequency and/or flagellar stiffness and thus help maintain the waveform and force output even in a high-load environment.

RESULTS

Previously we demonstrated that Lis1 (also termed CrLis1) is present in the wild-type *Chlamydomonas* flagellum but absent from the flagella of mutants lacking the entire outer dynein arm (*oda1*, *oda2*) or the α HC/LC5 subcomplex (*oda11*; Pedersen *et al.*, 2007). This prior analysis used an antibody raised against a hexahistidine-tagged version of Lis1 that also reacted with a second axonemal protein missing in the central pair-defective mutant *pf18*. To further analyze Lis1 function, we therefore raised a new antibody (CT273) against a maltose-binding protein (MBP)–Lis1 fusion. After blot purification, this antibody was used to probe flagella preparations from small-scale (200 ml or 2 l) cultures of wild-type *Chlamydomonas* and a mutant (*pf14*) lacking the radial spokes. The purified CT273 antibody was highly specific and recognized a single band in whole-flagella samples from *pf14* (Figure 1). However, we also consistently observed that Lis1 levels in wild-type flagella were greatly reduced

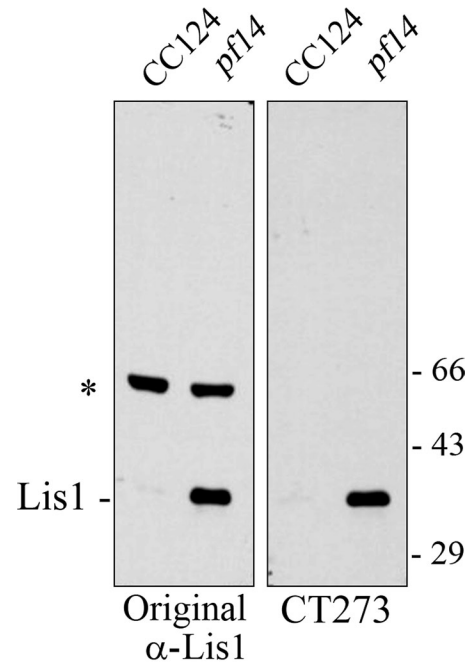


FIGURE 1: Specificity of antibodies against Lis1. Nitrocellulose blots of flagella samples from *Chlamydomonas* wild-type strain cc124 and the *pf14* mutant lacking radial spokes were probed with the anti-Lis1 antibody used previously (Pedersen *et al.*, 2007) or with CT273 raised against MBP–Lis1 fusion protein. Both antibodies yielded a very prominent band in *pf14* flagella of the appropriate molecular weight, but Lis1 was not detected in the wild-type samples at this exposure. The original α -Lis1 antibody also reacts with a second band (indicated by the asterisk), which acts as a loading control, whereas CT273 does not. As described previously (Pedersen *et al.*, 2007), this second band is absent from *pf18* flagella and thus presumably represents a component of the central pair apparatus.

and, in some samples (such as those shown in Figure 1), barely detectable at normal exposures compared with the mutant. In our previous experiments (Pedersen *et al.*, 2007), we detected a strong Lis1 signal in wild-type flagella samples that were obtained from a large-scale (~30 l) culture. These cells had been concentrated by tangential flow filtration and consequently subject to a high shear stress before centrifugation. Thus the small-scale preparations used here and the large-scale preparations used previously differ significantly in how cells were concentrated and also in the length of time they remained as either highly concentrated samples or as centrifuged pellets before deflagellation.

To further address this surprising observation, we prepared flagella samples from wild-type (cc124) cells and from mutants lacking part or all of the outer arm (*oda2-t*, *oda4-s7*, *oda6*, *oda9*, *oda11*), inner arm *I1/f* (*ida1*), monomeric inner arms containing the p28 LC (*ida4*), radial spokes (*pf14*), and the central pair microtubule complex (*pf18*); of importance, all strains were grown under identical culture conditions and harvested by methods that allowed the cells to be rapidly deflagellated. Lis1 was detectable in the wild-type samples but completely missing in *oda6*, *oda9*, and *oda11*, as described previously (Pedersen *et al.*, 2007), suggesting that it requires the α HC for flagellar localization (Figure 2a). The *oda2-t* mutant lacking the γ HC motor unit that is believed to act as an ATP-dependent brake in situ had very low amounts of Lis1 (Figure 2b). In contrast, Lis1 levels were very clearly enhanced over wild type in *oda4-s7*, which lacks the β HC motor domain but assembles the

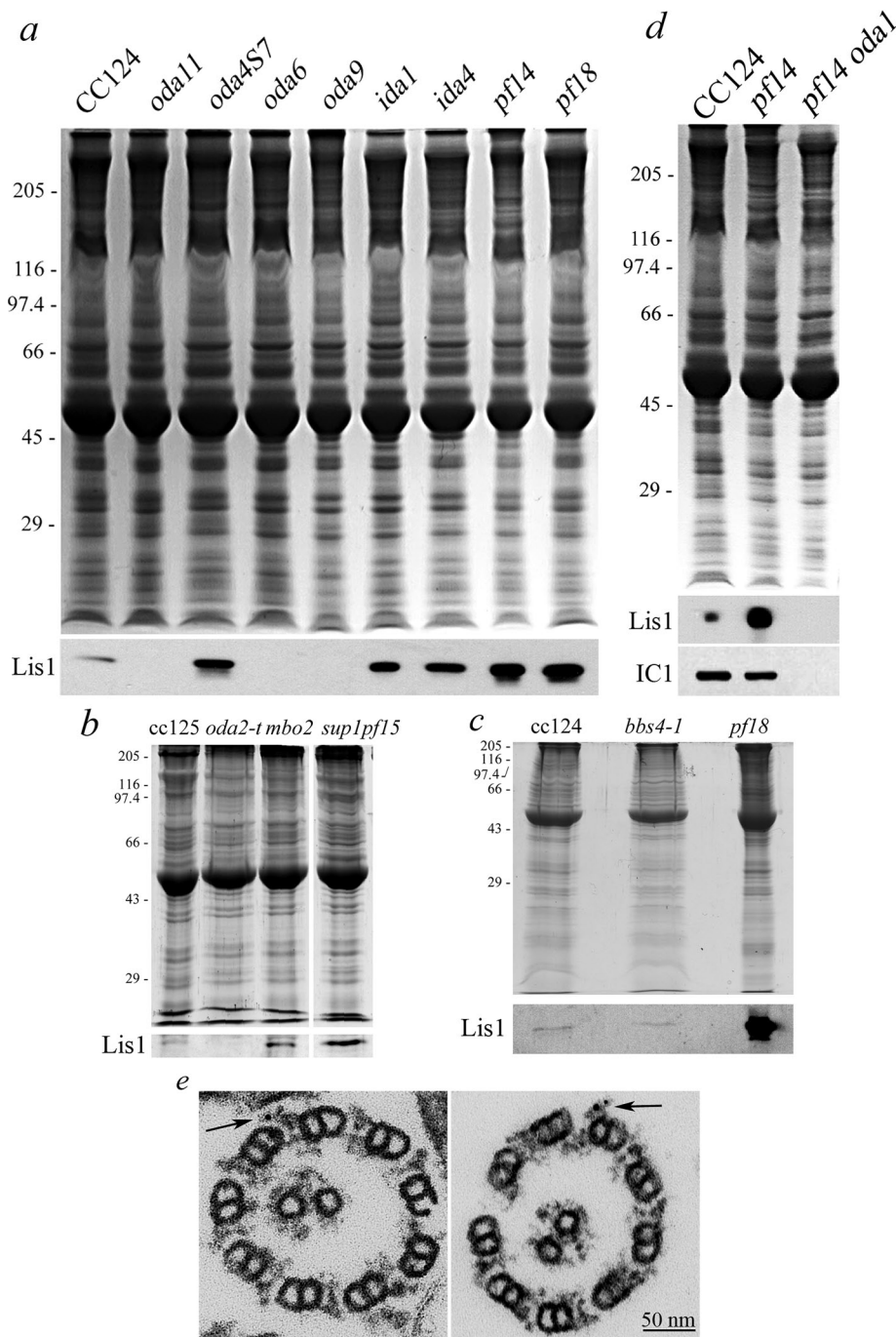


FIGURE 2: Central pair-, radial spoke-, and inner arm-defective flagella contain enhanced levels of Lis1. (a) Flagella were purified from wild-type (cc124) cells and from mutants lacking the entire outer arm (*oda6* and *oda9*), the outer arm α HC and LC5 (*oda11*), the outer arm β HC motor domain (*oda4-s7*), subsets of inner arms (*ida1* and *ida4*), the radial spokes (*pf14*), and the central pair microtubule complex (*pf18*). After electrophoresis, samples were stained with Coomassie blue (top) or blotted and probed with CT273 (bottom). The levels of Lis1 were greatly enhanced in mutants lacking the central pair or radial spokes, but this protein was completely absent from flagella of mutants that do not assemble outer arms. Lis1 levels were also elevated in *oda4-s7*, which lacks the β HC motor domain and exhibits defective motility, and the inner arm-defective mutants *ida1* and *ida4*. (b) Flagella samples from *oda2-t*, *mbo2*, and *sup1pf15* were separated in a 10% SDS gel and stained with Coomassie blue or probed for Lis1. The *oda2-t* mutant had very low levels of Lis1, whereas enhanced levels occurred in cells that cannot undergo forward swimming (*mbo2*). Furthermore, Lis1 was not reduced to wild-type levels in a paralyzed strain that had some degree of motility restored (in *sup1pf15*). (c) Analysis of flagella from the *bbs4-1* mutant (defective in the BBSome IFT adaptor) separated in a 12.5% SDS gel revealed a similar level of Lis1 to the cc124 wild type. Because *bbs4-1* is in a genetic

remainder of the outer arm and exhibits low beat frequency (Sakakibara *et al.*, 1993), and the *ida1* and *ida4* inner arm mutants, which have a reduced beat amplitude (Kamiya *et al.*, 1991). Even stronger Lis1 signals were observed in flagella of radial spoke- and central pair-deficient strains (*pf14* and *pf18*, respectively), which are immotile (Figure 2a). Lis1 was also enhanced in a *sup1pf15* double mutant (which has restored motility) and in *mbo2*, which swims only with a symmetric waveform (Figure 2b). A mutant defective in the BBSome intraflagellar transport (IFT) adaptor (*bbs4-1*) had wild-type Lis1 levels (Figure 2c). Quantitative analysis of the chemiluminescence signals derived from *pf18* flagella and from known amounts of MBP-Lis1 and recombinant outer arm dynein component LC1 revealed an approximate Lis1:LC1 molar ratio of 0.3:1. Thus, as there are two copies of LC1 per outer arm (King and Witman, 1989), Lis1 is present in close-to-stoichiometric amounts with respect to outer arm dynein particles within mutant flagella.

Because Lis1 was undetectable in outer arm-deficient mutants but greatly enhanced in reduced-motility or paralyzed strains lacking other substructures, we next tested which axonemal subsystem exhibited a dominant effect on Lis1 levels in the flagellum. To this end, we prepared flagella from wild-type, *pf14*, and *pf14 oda1* cells grown under identical conditions and examined the amounts of Lis1 and outer arm dynein

background derived from the 21gr wild type, a low level of Lis1 is not a specific property of the cc124 wild-type strain. (d) To determine whether lack of outer arms or radial spokes exerts dominant control over Lis1 levels within the flagellum, flagella were obtained from wild-type, *pf14*, and *pf14 oda1* (lacks radial spokes, outer arms, and the outer arm docking complex) mutant flagella. Flagella from the *pf14 oda1* double mutant contained no detectable Lis1 or outer arm IC1. Similar results were obtained with a *pf18 oda1* double mutant (unpublished data). These observations suggest that outer arms are required for both wild-type and mutant-enhanced levels of Lis1 localization within the flagellum. (e) Cross-section electron micrographs of *pf14* axonemes probed with CT273 and labeled with 5-nm gold particles are shown. Gold particles associate with the external face of the outer dynein arm, consistent with the predicted binding to the outermost (α) HC within the outer arm. Note that the central pair microtubules are mislocalized due to the absence of radial spokes in this mutant.

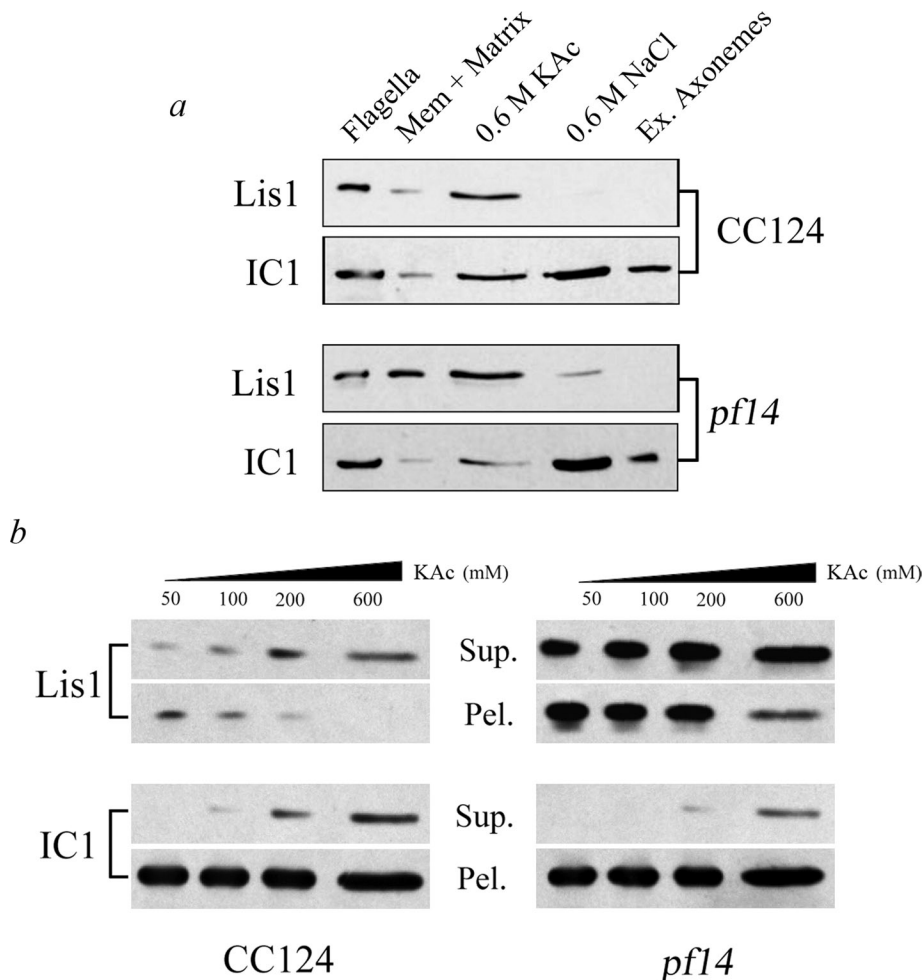


FIGURE 3: Association of Lis1 with wild-type and *pf14* mutant axonemes. (a) Wild-type and *pf14* flagella were demembrated with IGEPAL CA-630 and the resulting axonemes sequentially extracted with 0.6 M K acetate and 0.6 M NaCl. Equivalent amounts of the various samples were electrophoresed and probed with CT273 and 1878A to detect Lis1 and IC1, respectively. Blot exposures for the two antibodies were adjusted to achieve approximately equal band intensities in whole-flagella samples. (b) To further assess the association of Lis1 with the outer arm, wild-type and *pf14* mutant axonemes were extracted with various concentrations of K acetate and the resulting supernatants and pellets probed to detect IC1 and Lis1. The results indicate that Lis1 is somewhat more tightly associated with *pf14* axonemes than with wild type, suggesting that the interaction has been modulated.

intermediate chain 1 (IC1) present in the samples (Figure 2d). IC1 levels were essentially identical in wild-type and *pf14* flagella, and this protein was completely absent from *pf14 oda1* flagella, as expected. In contrast, Lis1 levels were greatly elevated in *pf14* flagella compared with wild type. However, Lis1 was also not detected in the double mutant that lacks both outer arms and radial spokes (Figure 2d). A similar result was obtained with *pf18 oda1* mutant flagella. This indicates that the enhanced levels of Lis1 observed in motility-deficient mutants require the presence of outer arm dynein for flagellar localization and/or retention. Localization of Lis1 within *pf14* axonemes by immuno-gold electron microscopy using the CT273 antibody revealed gold particles associated with the external face of the outer dynein arm, consistent with the predicted binding to the α HC (Figure 2e).

Flagellar Lis1 was present in two pools: a small amount was found in the detergent-soluble membrane/matrix fraction, but the majority was associated with the detergent-insoluble microtubular axoneme. We found previously that the Lis1–outer arm dynein inter-

action is completely disrupted by 0.6 M NaCl (Pedersen *et al.*, 2007). Given that motility-deficient flagella have altered amounts of Lis1, we examined whether Lis1 levels in these pools were changed by sequential extraction of flagella with detergent, 0.6 M K acetate, and 0.6 M NaCl; IC1 was used as a marker for integral outer arm dynein proteins (Figure 3a). An increase in detergent-soluble Lis1 was evident in the *pf14* samples. Furthermore, a small fraction of Lis1 in the *pf14* samples was resistant to extraction with K acetate but could be solubilized with 0.6 M NaCl, whereas no detectable Lis1 resisted K acetate extraction from wild-type axonemes. To further evaluate this apparent difference in solubility, we treated axonemes with increasing concentrations of K acetate to extract Lis1 (Figure 3b). In wild-type samples, most Lis1 was extracted with 0.1 M K acetate, and none could be detected in axoneme samples treated with 0.6 M K acetate. In contrast, *pf14* axonemes treated with 0.2 M K acetate contained significant quantities of Lis1, and indeed this protein was still readily detectable in axonemes after treatment with 0.6 M K acetate.

Immunoblot analysis of cell body extracts revealed that both Lis1 and IC1 levels were approximately equivalent in wild-type and *pf14* cells (Figure 4a), suggesting that the enhanced levels of Lis1 found in *pf14* flagella were not simply the result of increased synthesis. We next used the CT273 antibody to locate Lis1 within wild-type, *pf14*, and *oda6* cells (Figure 4b); these cells were adhered to the glass by their flagella for several minutes before fixation and thus experienced very high load conditions. In all three strains we obtained a strong signal within the cell body located at the base of the flagella and often observed a second stained punctate structure at the opposite end of the cell and possibly located within the chloroplast (Figure 4, b and c). At exposures of 100 ms, no signal was observed in the flagella of any strain. However, following a 1-s exposure, weak signals were seen in flagella from both wild type and *pf14* but not in those from *oda6*. The discrete localization at the flagellar base is very distinct from the diffuse cytoplasmic localization of integral outer arm dynein components such as LC1 (Figure 4c; and see Piperno *et al.*, 1996; Ahmed *et al.*, 2008), suggesting that Lis1 may not be directly associated with integral outer arm components within cytoplasm.

Therefore, to further examine the organization of Lis1 in *Chlamydomonas*, we fractionated extracts of whole-cell lysates from deflagellated wild-type cells and both *oda6* and *oda11* mutants using a Superose 6 gel filtration column that can separate multimegadalton complexes (Figure 5). Quantification of Lis1 levels using a LAS4000 digital imaging system to measure the chemiluminescence signal revealed three major peaks of Lis1 in wild-type extracts. These have estimated masses of ~1.1 MDa, 158 kDa, and 40 kDa, respectively. The last peak presumably represents monomeric Lis1, whereas the

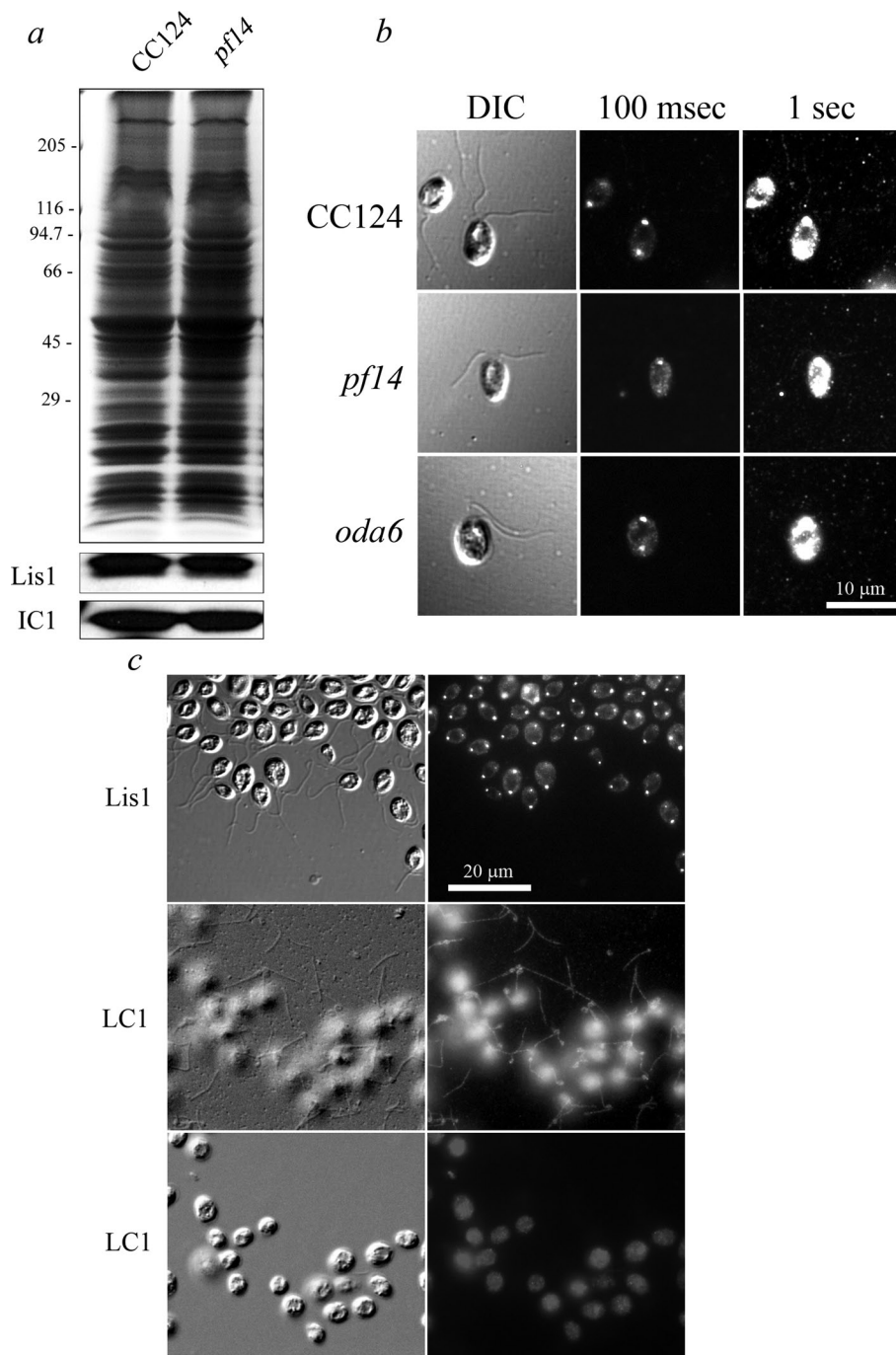


FIGURE 4: Localization of Lis1 in wild-type and mutant *Chlamydomonas*. (a) Cell-body extracts were prepared from *cc124* and *pf14* strains and probed for outer arm IC1 and Lis1. The relative levels of both proteins were similar in *cc124* and *pf14*, suggesting that the enhanced levels of Lis1 observed in *pf14* flagella are not the result of increased cytoplasmic synthesis. (b) Differential interference contrast (DIC) and immunofluorescence micrographs of *cc124* wild-type cells and radial spoke-defective (*pf14*) and outer arm-less (*oda6*) mutants stained with CT273. Micrographs of the three strains were all taken with the same settings: for the fluorescence images, two different exposures are shown. The Lis1 signal is concentrated near the base of the flagella and does not vary significantly between wild type and mutants. In addition, a second stained spot was often observed at the opposite end of the cell. On prolonged exposure, a weak signal was detected in wild-type and *pf14* flagella but not in *oda6*. (c) Survey micrographs (DIC and fluorescence) of CT273-stained wild-type cells illustrating that almost every cell in the population contains two Lis1-positive punctate structures (top). Wild-type cells stained for outer arm dynein LC1 using the R5932 antibody and focusing on the flagella (middle) or cell bodies (bottom) are also shown. LC1 is found along the entire axonemal length but does not show a clear localization to the basal body region.

other peaks reflect higher-order complexes. All of the outer arm IC2 component migrated at ~150 kDa and was not observed as part of an intact outer arm dynein complex, which elutes much nearer the void volume (Rompolas *et al.*, 2007); IC2 did not precisely coelute with the second Lis1 peak, suggesting they are not present in the same complex. Similarly, a peak of outer arm LC2 was also observed at ~150 kDa coeluting with IC2, indicating that this complex indeed represents the IC/LC outer arm subassembly.

We next examined Lis1 levels in *oda6* extracts and found that the majority of Lis1 was present in the 40-kDa peak, and the two larger peaks seen in wild-type were almost completely absent. Furthermore, even though equivalent loadings and exposure times were used, the total Lis1 signal was less than one-third that observed in wild-type extracts. With *oda11*, which lacks the α HC with which Lis1 is believed to associate within the flagellum, we observed a single peak of Lis1 that coincided with a minor peak in the wild-type samples migrating between the major 158- and 40-kDa peaks. The low signal present in the *oda6* and *oda11* extracts suggests that Lis1 expression, solubility, or stability is reduced in the absence of assembly-competent outer arm dynein α HC.

Because Lis1 levels were enhanced in the flagella of a variety of motility-deficient mutants except those lacking outer arms and this did not correlate with increased cytoplasmic synthesis, we next tested whether altering the ability of wild-type flagella to beat normally changed the amount of Lis1 present. Therefore we placed cells in either 0 or 16% Ficoll to increase the viscosity of the medium (from 1 to 12 cP) and subsequently purified and fractionated the flagella (Figure 6a). Under these conditions, wild-type cells have a beat frequency of ~50 Hz at 1 cP (0% Ficoll) or ~10 Hz at 12 cP (16% Ficoll; Patel-King and King, 2009; Figure 6b). We found that flagella from cells subject to increased viscosity had enhanced levels of Lis1 and that this increase occurred in both the detergent-soluble and axoneme-associated pools; in contrast, the amount of outer arm IC1 present did not change in response to the increased viscous load.

To assess whether enhanced Lis1 altered flagella beating, we treated cells with either 0 or 16% Ficoll for 90 min. Ficoll was then removed and demembrated cell models prepared. When reactivated, we observed that models of cells previously treated with 0% Ficoll, and thus containing low levels of Lis1, gave two peaks in the beat frequency power spectrum of 38 ± 1.4 and 47 ± 1.9 Hz

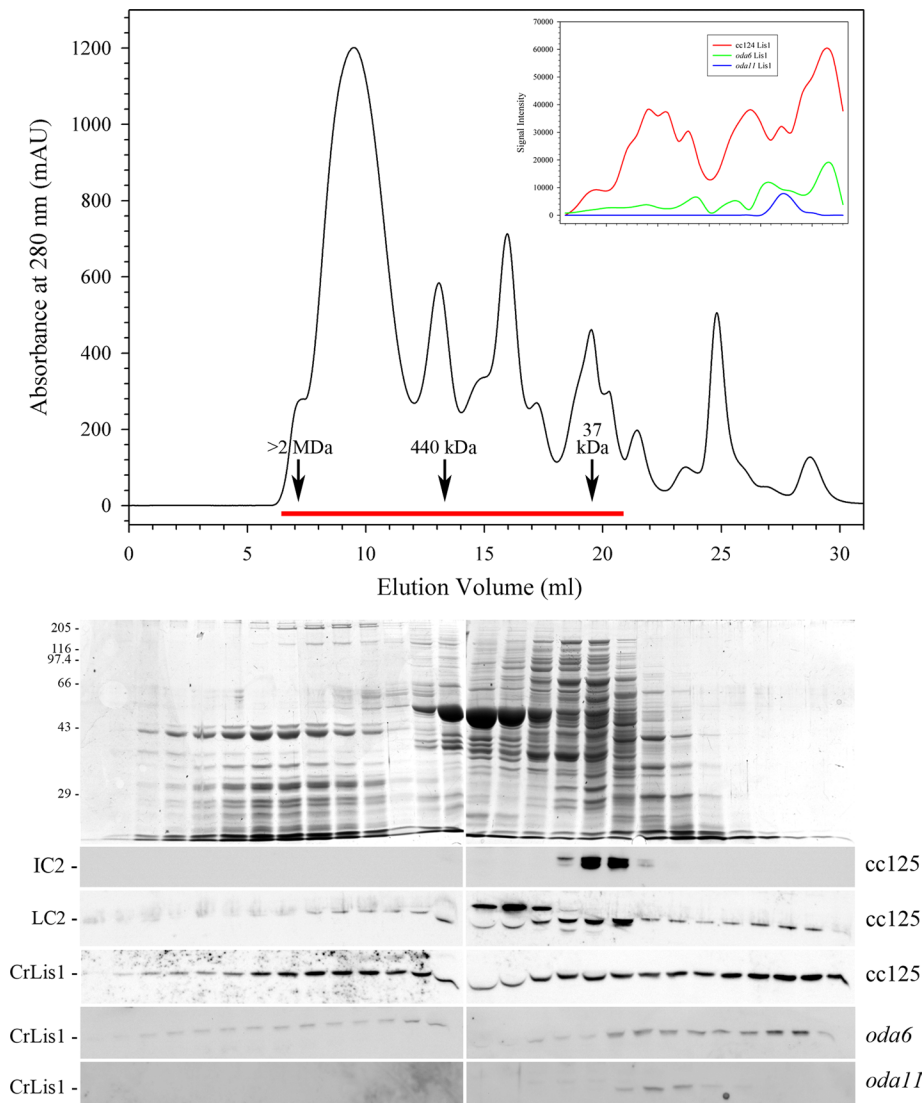


FIGURE 5: Lis1 is present in multiple cytoplasmic complexes. Top, the 280-nm absorbance trace for a wild-type (cc125) cell body extract fractionated in a Superose 6 column; the positions at which blue dextran (>2 MDa), ferritin (440 kDa), and monomeric Lis1 (37 kDa) migrated are indicated. Five major peaks and multiple minor peaks are evident; the large initial peak absorbs strongly, as it contains photosynthetic pigment(s). Fractionation of *oda6* and *oda11* extracts gave similar profiles (unpublished data). Samples from the region indicated by the red bar for each fractionated extract (0.5-ml fractions) were electrophoresed in 10% SDS–polyacrylamide gels and either stained with Coomassie blue or blotted to nitrocellulose, stained with reactive brown 10, and then probed with CT273, 1869A, and R5391 to detect Lis1, IC2, and LC2, respectively. Quantitative analysis of the Lis1 chemiluminescence signal in each fraction of the extracts is plotted as spline curves in the inset: red, wild type; green, *oda6*; blue, *oda11*.

(mean \pm SD; $n = 8$); these represent the intrinsic frequencies of the *cis* and *trans* flagella (Kamiya and Hasegawa, 1987) under our conditions. Models prepared from cells pretreated with 16% Ficoll and containing enhanced levels of Lis1 gave peaks of 39 ± 1.6 and 54 ± 3.6 Hz ($n = 8$; Figure 6c). Thus *trans* flagella exposed to high viscous load exhibit a statistically significant (Student's *t* test; $p = 0.0029$) increase in their intrinsic beat frequency (power output per unit time), whereas the *cis* flagella do not ($p = 0.15$).

Lis1 is missing in *oda11* flagella and thus is predicted to interact with the α HC, which also associates with a tightly bound, redox-active thioredoxin (Pfister and Witman, 1984; Patel-King et al., 1996). Therefore we next subjected cc125 wild-type cells

to oxidative and reductive stress, which is known to modulate flagellar beat frequency (Wakabayashi and King, 2006) and control the sign of the phototactic response (Wakabayashi et al., 2011). Treatment with 250 μ M H₂O₂ reduced beat frequency by 5–10 Hz and resulted in a moderate, approximately twofold increase in flagellar Lis1 levels (Figure 7a). In contrast, when cells were placed under high reductive stress (75 mM dimethylthiourea [DMTU]), beat frequency was reduced by \sim 20 Hz within a few minutes, and nearly all cells were essentially immotile within 2 h; the few remaining motile cells had beat frequencies of \sim 20 Hz or less (Figure 7b). Quantitative analysis revealed that flagella from DMTU-treated cells had Lis1 levels \sim 4.8-fold above that of the untreated controls (Figure 7a). The enhancement in response to reductive stress was relatively slow, and Lis1 levels approximately doubled after 60 min of treatment with DMTU. The *agg1* mutation, which confers negative phototaxis under low light, is carried by the cc124 wild-type strain but not by cc125 (Harris, 2009). Consequently, this experiment also revealed that the cell's ability to enhance flagellar levels of Lis1 is independent of *AGG1/agg1* status.

DISCUSSION

Lis1 is a key regulator of cytoplasmic dynein that alters the microtubule-binding affinity of this motor such that it can function under high load and thus move large cargoes (Mesngon et al., 2006; McKenney et al., 2010; Torisawa et al., 2011). The identification of Lis1 in motile mammalian cilia and demonstration that the *Chlamydomonas* orthologue is absent from flagella of strains lacking outer dynein arms suggested that this component also plays a key role in ciliary/flagellar motility (Pedersen et al., 2007). Here we demonstrated that Lis1 levels in the flagellum are dependent on the motile behavior of this organelle and that the cell dynamically modulates the quaternary structure of the outer arm. Thus, under normal conditions, flagellar levels of Lis1

are very low. However, when cells were subjected to conditions that disrupt the normal flagellar beat cycle (induced experimentally by mutation of other axonemal subsystems or by enhancing viscous load or reductive stress), intraflagellar Lis1 levels increased dramatically to levels almost stoichiometric with its target HC within the outer arm (Figure 8a). For example, paralyzed mutants lacking either the radial spokes or the central pair complex had large amounts of Lis1 compared with wild type. Furthermore, the outer arm was absolutely required for enhanced Lis1 localization within the flagellum, and double mutants lacking both radial spokes/central pair and outer arm dynein were completely devoid of this protein.

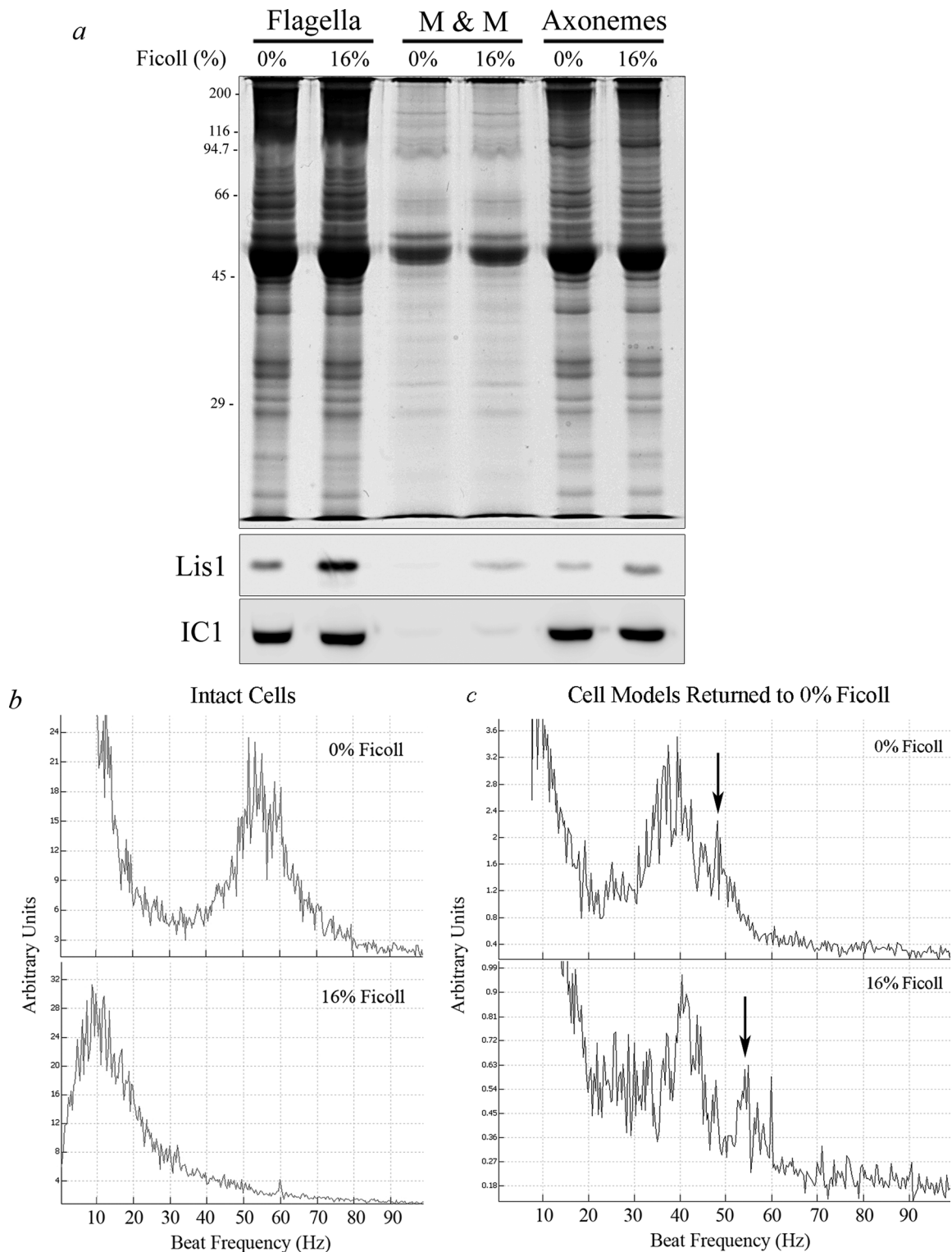


FIGURE 6: Flagellar levels of Lis1 increase in response to high viscous load. (a) Wild-type (*cc124*) cells were placed in buffer containing 0 or 16% Ficoll (1 or 12 cP, respectively) for 60 min and then deflagellated with dibucaine. The resulting flagella were purified and detergent extracted using standard methods. Equivalent amounts of each sample were loaded onto 10% polyacrylamide SDS gels and either stained with Coomassie blue or transferred to nitrocellulose and probed with CT273 and 1878A to detect Lis1 and IC1, respectively. Although incubation in viscous media had no effect on the levels of IC1, both detergent-soluble and axoneme-bound pools of Lis1 were significantly enhanced under high-load conditions. (b) Power spectra illustrating the beat frequency of *cc124* wild-type cells in the presence of 0 or 16% Ficoll. (c) Power spectra illustrating the beat frequency of wild-type cell models that had been pretreated by incubation in either 0 or 16% Ficoll for 90 min before washing and demembration. Two peaks representing the intrinsic beat frequencies of the *cis* and *trans* flagella are evident in both spectra. An increase in *trans* flagella frequency of 5–7 Hz was consistently observed, whereas increases of at most 1–2 Hz were seen for the *cis* flagellum.

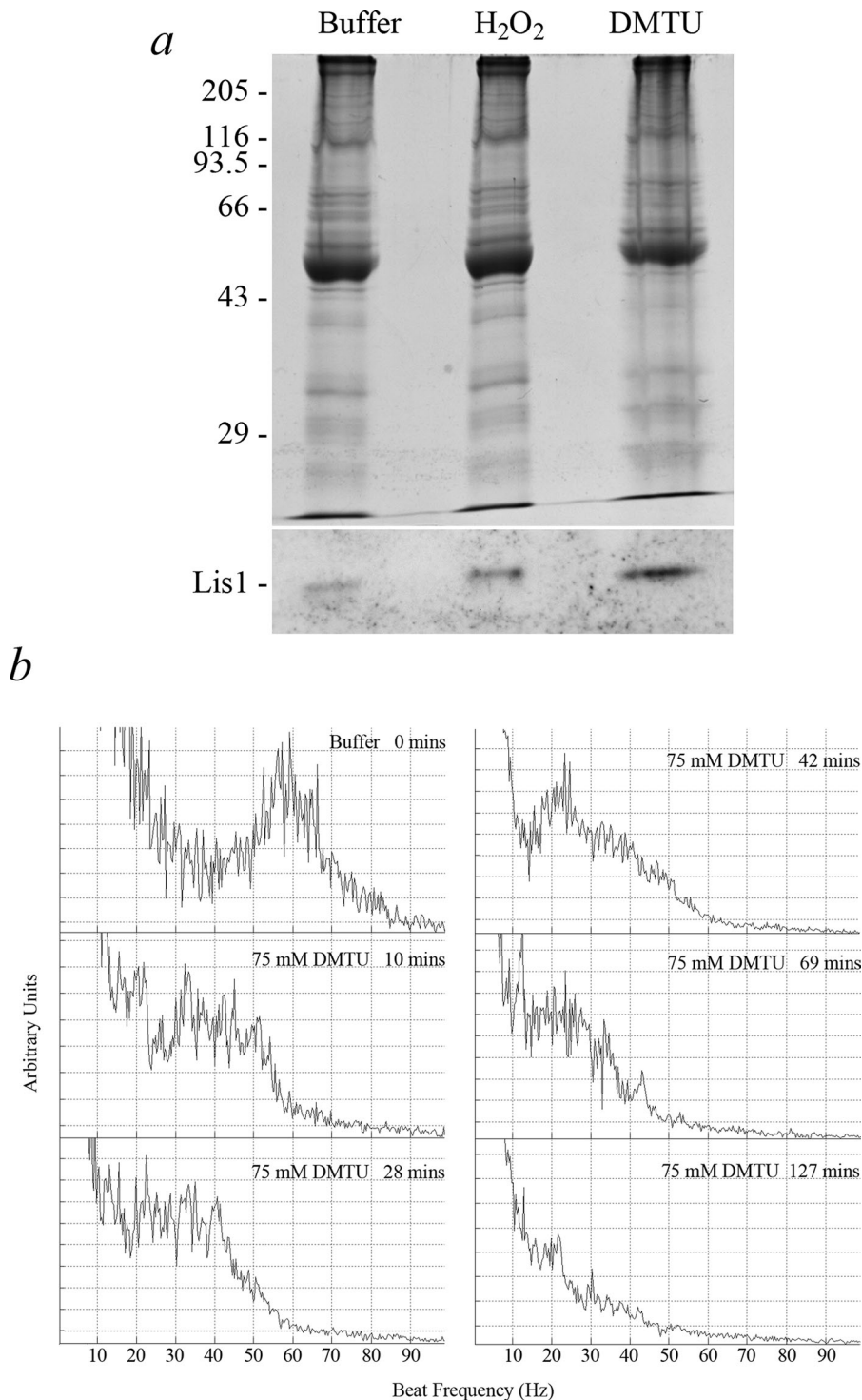


FIGURE 7: Oxidative and reductive stress enhance flagellar levels of Lis1. (a) Flagella from cells treated with buffer alone, 250 μ M H₂O₂, or 75 mM DMTU were electrophoresed in a 10% polyacrylamide gel and stained with Coomassie blue (top) or blotted and probed with CT273 to detect Lis1 (bottom). Lis1 levels were only slightly raised under oxidizing conditions but increased almost fivefold under high reductive stress. (b) Beat frequency power spectra for populations of wild-type cells in buffer or under high reductive stress after addition of 75 mM DMTU for the indicated times. DMTU treatment resulted in a severe time-dependent decrease in flagellar beat frequency.

These observations suggest several intriguing cellular mechanisms. First, the data imply that the cell directly monitors motility and can modify the amount of Lis1 import into, or perhaps its reten-

tion within, the flagellum in response to imposed changes. Because the increases in flagellar Lis1 were not related to alterations in the total cytoplasmic Lis1 pool, this suggests that levels are modulated by a dynamic process rather than representing a passive change in response to an increased concentration in the cytoplasm. Second, based on its role in cytoplasmic dynein mechanochemistry, it is likely that enhanced levels of axoneme-bound Lis1 alter the motor properties of the outer dynein arm and most likely of the α HC with which Lis1 directly associates. The outer dynein arm is a low-duty-ratio motor, spending only ~8% of the mechanochemical cycle time tightly attached to microtubules (Furuta *et al.*, 2009). Because Lis1 binding to cytoplasmic dynein increases the percentage of cycle time spent in this high-affinity, microtubule-bound state (McKenney *et al.*, 2010), a similar alteration in the flagellum would lead to an increase in the number of persistent cross-bridges between adjacent microtubule doublets mediated by the α HC, while the β and γ HCs remained competent to undergo normal cycles (Figure 8b). Thus, enhanced flagellar Lis1 might be essential for maintaining the optimal beat frequency and/or waveform for high power output in a high-load environment. Consistent with this hypothesis, we observed that demembrated models prepared from cells pretreated with high-viscosity media and thus containing enhanced levels of Lis1 exhibited a higher beat frequency of the *trans* flagellum than models of control cells subject to normal viscosity conditions. Thus Lis1 appears to modulate the intrinsic beat frequency difference between the *cis* and *trans* flagella; this difference is lost in the *oda11* mutant that lacks the α HC, LC5 thioredoxin, and Lis1 (Sakakibara *et al.*, 1991).

The three HCs within the *Chlamydomonas* outer dynein arm play very distinct roles in the generation of motile force. This is evidenced by the major differences in the swimming velocities of cells lacking various outer arm motor units (Sakakibara *et al.*, 1991, 1993; Liu *et al.*, 2008) and also in the rates at which these HC-deficient dyneins hydrolyze ATP and translocate microtubules *in vitro* (Furuta *et al.*, 2009). Lack of the β HC (in the *oda4-s7* mutant; Sakakibara *et al.*, 1993) reduces both microtubule gliding and swimming velocity significantly, and the purified enzyme has an ATPase activity only ~20% that of wild type. In contrast, the γ HC appears to act as a negative regulator of outer arm function, as its absence (in the *oda2-t* mutant; Liu *et al.*, 2008) increases both ATPase activity and the rate of microtubule translocation by the remaining motors. Intriguingly, lack of the α HC (in the *oda11* mutant; Sakakibara *et al.*, 1991) *in vitro* results in the most severe decrease in microtubule translocation rate

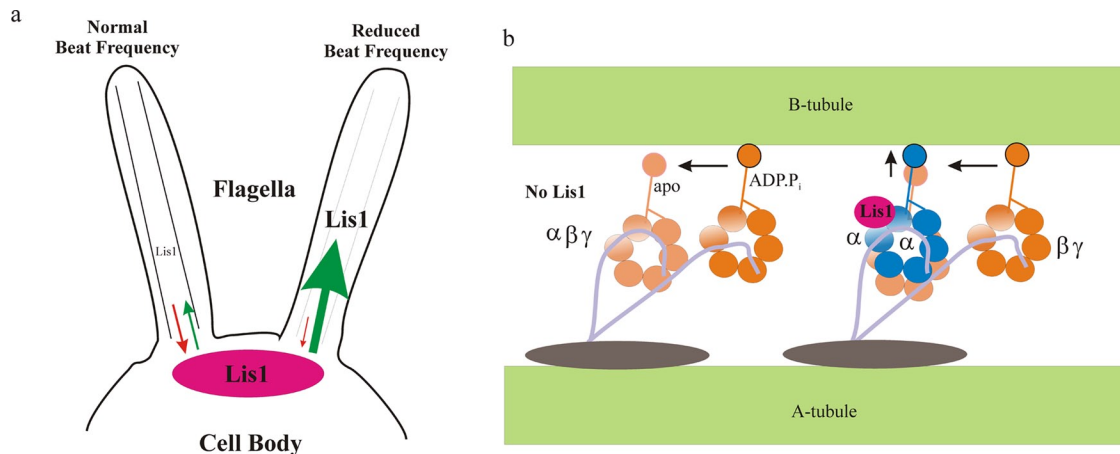


FIGURE 8: Models for the dynamics and role of Lis1 in the flagellum. (a) A pool of Lis1 is present in cytoplasm near the base of the flagella. Under normal motility conditions, intraflagellar levels of Lis1 are low. However, when motility is reduced there is a large increase in the amount of this protein that enters and is retained within the flagellum. (b) In the absence of Lis1, all three HCs within the outer arm are able to undergo a standard mechanochemical cycle to generate force through transient ATP-dependent interactions with the B-tubule (only one HC is shown for clarity; the others would stack on top). However, based on studies of cytoplasmic dynein, enhanced levels of Lis1 would lead to decoupling of the ATPase cycle from microtubule-binding activity for the α HC but not for the β or γ HCs. This in turn would result in the formation of persistent cross-bridges that might limit the degree of sliding generated by other motors and/or increase flagellar stiffness.

but with only relatively minor effects on ATPase and in vivo swimming velocity. In addition, the α HC has been demonstrated to exhibit a dominant-negative control over the ATP-dependent microtubule-binding activity of the purified outer arm (Sakato and King, 2003) and also to down-regulate the ATPase activity of the β HC (Pfister and Witman, 1984). The modulated interaction of Lis1 with the α HC provides further support for the hypothesis that this HC exerts a key influence over outer arm function in vivo.

Some outer arm dyneins (e.g., those from mammals and sea urchins) contain only two HCs rather than the three found in *Chlamydomonas* and ciliates such as *Tetrahymena*. The innermost HC of these dyneins is orthologous to the *Chlamydomonas* γ HC, whereas recent phylogenetic studies suggest that the outermost HC is orthologous to both the *Chlamydomonas* α and β HCs; that is, *Chlamydomonas* α and β HC motor domain sequences are so closely related that phylogenetic analysis cannot place them into distinct monophyletic groups (Wickstead and Gull, 2012). Thus we predict that the Lis1 present in motile mammalian cilia is most likely associated with the outermost HC and potentially might aid the motility of these organelles when beating under high-viscosity conditions such as those found in the respiratory tract.

Increased viscosity led to the recruitment of Lis1 onto outer arm dynein, although mutants that lack the outer arm show essentially no reduction in force output as viscosity is increased (Minoura and Kamiya, 1995; Patel-King and King, 2009). However, inner arms alone are capable of generating only ~25% the power output of wild-type flagella and, furthermore, one monomeric inner arm species (dynein c) appears to be specialized for functioning under high-load conditions (Yagi et al., 2005). Thus the outer and inner arm systems employ distinct mechanisms to allow them to function under high load.

Placing cells under high reductive stress also led to a reduction in beat frequency and an increase in flagellar Lis1, suggesting an interconnection between outer arm redox signaling and Lis1 function. Previously we found that demembrated cell models from *oda11* (which completely lack Lis1) show an attenuated response to altera-

tions in redox poise, that is, beat frequency is lower than wild type under reducing conditions but better than wild type in a more oxidizing environment (Wakabayashi and King, 2006). It is likely that these wild-type cell models also contained little Lis1, as they were prepared from small-scale cultures using similar methods to those used here. In addition, *oda11* flagella also lack the LC5 thioredoxin, which is believed to act as one of the outer arm redox sensors (Patel-King et al., 1996), and thus this in vitro result may reflect impaired redox signaling to the remaining outer arm HCs rather than the lack of a Lis1-modified α HC activity.

Intriguingly, although Lis1 can directly bind the mammalian cytoplasmic dynein HC, this association is relatively weak in the absence of the additional factor NudE (McKenney et al., 2010). However, *Chlamydomonas* lacks cytoplasmic dynein 1/dynactin, and its genome does not encode a NudE orthologue (Merchant et al., 2007). Thus we propose that NudE is required only by the cytoplasmic enzyme and that the dynamic interaction of Lis1 with outer arm dynein is controlled in a different manner. Although dimerization of mammalian Lis1 through the LisH domain is required for its regulatory effects on cytoplasmic dynein (Torisawa et al., 2011), *Chlamydomonas* Lis1 lacks the LisH domain and is apparently monomeric. This likely reflects both the lack of cytoplasmic dynein and the observation that Lis1 binds to only one HC within the outer arm, which is a heterotrimer of HCs, rather than to both HCs of the cytoplasmic dynein HC homodimer. We found that Lis1 is more tightly bound to *pf14* dynein than to wild type, and one possibility is that modification of outer arm dynein (or possibly Lis1 itself) controls the affinity. In this regard, the α HC, which is required for Lis1 localization, is known to be phosphorylated at six or more sites that are located near/within the motor domain (King and Witman, 1994). Alternatively, the interaction may be modulated solely by altering the intraflagellar concentration of Lis1. In either case, this represents a key difference between the cytoplasmic and axonemal enzymes.

Because *Chlamydomonas* does not encode most components of the canonical cytoplasmic dynein/dynactin system (Merchant

et al., 2007), this organism provides an excellent model in which to analyze Lis1-containing cytoplasmic complexes specifically involved in the functioning of cilia/flagella. We found that wild-type cells contain three major and two minor distinct cytoplasmic pools of Lis1. The most prominent peak, at ~40 kDa, likely represents monomeric Lis1; however, two others (of 1.1 MDa and 158 kDa) clearly represent multimeric complexes. Both of these peaks were almost completely missing from both *oda6* and *oda11* cytoplasm. When we examined these samples for the integral outer arm dynein component IC2, we found that all detectable IC2 protein migrated in a complex of ~150 kDa, which also contained LC2 and thus likely represents the outer arm IC/LC complex. LC2 was also present in both larger complex(es) (see also Panizzi et al., 2012) and as an apparent monomer. These observations suggest that outer arm dynein exists within cytoplasm as a series of partly assembled sub-complexes and is consistent with our previous finding that the cytoplasmic pool of the γ HC-associated protein LC1 migrated in the upper portion of sucrose density gradients (Tanner et al., 2008) and as a monomer upon gel filtration chromatography (Panizzi et al., 2012). Similarly, IC1 was also found in the mid-upper region of the sucrose gradients rather than at 18–20 S (Tanner et al., 2008). However, other studies indicated that the HC and IC components of outer arm dynein can be immunoprecipitated together from cytoplasmic extracts by anti-IC and anti-HC antibodies, implying that at least some fraction of outer arm dynein is preassembled within the cytoplasm (Fowkes and Mitchell, 1998; Mitchison et al., 2012). Given that outer arm dynein derived from flagella can be purified by gel filtration chromatography (e.g., Rompolas et al., 2007), it is possible that the complex in cytoplasm is less stable than the flagellar form and/or that the assembly pathway is more complex than currently envisaged.

Lis1 is distinct from integral outer arm dynein components in that it is concentrated at the basal body region, as are intraflagellar transport proteins (Deane et al., 2001), whereas other outer arm dynein proteins show a more diffuse cytoplasmic staining (Piperno et al., 1996; Ahmed et al., 2008). This discrete localization at the flagellar base may be related to the dynamic behavior of Lis1, allowing the cell to rapidly increase intraflagellar Lis1 levels in response to imposed alterations in motility. In contrast there is little evidence that the outer arm is undergoing a similarly large-scale redistribution in fully assembled flagella.

Our observations also have implications for analysis of the phenotypes of various *Chlamydomonas* motility mutants and potentially those in other model organisms as well. In general, phenotypes have been interpreted based on the implicit assumption that only the mutated structure in the flagellum has been altered. However, as Lis1 levels were significantly increased over those of wild type in all the motility mutants we tested (with the exception of those lacking outer dynein arms), we must now consider whether this might also contribute to the phenotypes observed. For example, might the observation that *ida1*-mutant cells missing the I1/f dynein exhibit altered waveform but approximately wild-type beat frequency be due, at least in part, to a change in outer arm motor function as a consequence of the increase in Lis1 in addition to the lack of the I1/f dynein?

In conclusion, we describe here the dynamic association of the cytoplasmic dynein regulatory protein Lis1 with the outer dynein arm of cilia/flagella after treatments that alter the motile properties of these organelles. Thus Lis1 represents a novel regulatory factor for ciliary/flagellar motility whose effects must be integrated with other known responses to Ca^{2+} , redox, phosphorylation, and mechanical signals to modulate dynein motor output.

MATERIALS AND METHODS

Strains and culture conditions

The following *Chlamydomonas reinhardtii* strains were used in this study: cc124/125 (wild-type of – and + mating types, respectively; cc124 is *agg⁻*, whereas cc125 is *AGG⁺*), *bbs4-1*, *ida1*, *ida4*, *mbo2*, *oda1*, *oda2*, *oda2-t*, *oda3*, *oda4-s7*, *oda6*, *oda11*, *pf14*, *pf14 oda1*, *pf18*, *pf18 oda1*, and *sup1pf15*. The *pf14 oda1* and *pf18 oda1* double mutants were the generous gift of Ritsu Kamiya (University of Tokyo, Tokyo, Japan). All other strains can be obtained from the *Chlamydomonas* Resource Center (<http://chlamycollection.org>). Two-liter or 200-ml *C. reinhardtii* cultures were grown in R medium with continuous bubbling with 5% CO_2 /95% air under a 15/9-h light/dark cycle (Witman, 1986), and the cells harvested by low-speed centrifugation (2000 rpm, 5 min, using a F14 6x250y Fiberlite rotor). Beat frequency power spectra of live cells and demembrated cell models were obtained using the fast Fourier transform method (Kamiya, 2000).

Flagellar isolation and fractionation

Flagella from wild-type and mutant *C. reinhardtii* strains were isolated as described previously (Witman, 1986; King, 1995) and demembrated with 1% IGEPAL-630 (Sigma-Aldrich, St. Louis, MO) in HMEK buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 5 mM MgSO_4 , 25 mM KCl, 0.5 mM EDTA, pH 7.4). After centrifugation, the supernatant was removed (M&M fraction), and the pellet was further extracted with 0.6 M K acetate. This second supernatant was removed (K acetate fraction), and the pellet was resuspended in 0.6 M NaCl to solubilize the dynein arms from the remaining axonemal pellet (King et al., 1986). For titration of K acetate extraction of axonemal components, whole flagella were isolated and demembrated and the resulting axonemes split into four equal parts. Individual samples were then extracted with 0.05, 0.1, 0.2, or 0.6 M K acetate for 10 min on ice.

To examine the levels of Lis1 in the flagellum under high viscous loads, wild-type cells were grown in 1-l liquid R medium cultures to a density of $\sim 1.0 \times 10^6$ cells/ml, briefly washed with 10 mM HEPES, pH 7.5, and then split into two equal parts and resuspended in 50 ml of either 0 or 16% Ficoll in 10 mM HEPES, pH 7.5. Cell suspensions were gently rocked for 1 h at room temperature before harvesting; flagella were then purified as described. Wild-type cells were also placed under oxidative or reductive stress by addition of H_2O_2 or dimethylthiourea, respectively, as described by Wakabayashi et al. (2011), before deflagellation.

Reactivation of cell models

To test whether the enhanced levels of Lis1 altered flagella function, we placed intact wild-type cells (cc125) in 0 or 16% Ficoll for 90 min. Cell models were then prepared as described by Kamiya (2009). Cells were rapidly harvested by centrifugation, placed on ice, and washed with ice-cold 10 mM HEPES, pH 7.4, 1 mM ethylene glycol tetraacetic acid (EGTA), and 4% sucrose to remove Ca^{2+} . Care was taken to keep cells as centrifuged pellets for as short a time as possible. Cells were demembrated with a small volume of 0.1% IGEPAL CA-630 in 30 mM HEPES, pH 7.4, 5 mM MgSO_4 , 1 mM dithiothreitol, 1 mM EGTA, and 50 mM K acetate (HMDEK). Cell models were diluted in HMDEK buffer containing 1% (wt/vol) polyethylene glycol (molecular weight, 8000) and reactivated with 1 mM ATP (final concentration). Beat frequency was then measured as detailed above.

Whole-cell extracts and gel filtration chromatography

To compare levels of Lis1 in wild-type, *oda11*, and *pf14* cells, we prepared whole-cell extracts essentially as described by Fowkes and

Mitchell (1998), with minor modifications. Briefly, *Chlamydomonas* cells were grown to a density of $\sim 1.0 \times 10^6$ cells/ml in 500 ml of R medium, washed with 10 mM HEPES, pH 7.5, buffer, and harvested. The cell suspension was homogenized with an equal volume of acid-washed glass beads (diameter, ~ 1 mm) by vortexing for 1 min at maximum speed. The homogenate was clarified in a TLA100.2 rotor (Beckman, Fullerton, CA) at 33,000 rpm for 2 h at 4°C.

For fractionation experiments, washed cells were deflagellated and then resuspended in a small volume of 10 mM HEPES, pH 7.5, and 150 mM NaCl. Subsequently, cells were filtered through cheese cloth and passed three times at 40 psi through an Emulsiflex C3 high-pressure disrupter or through a French press. The samples were centrifuged at 14,000 rpm in a Fiberlite F21S 8x50y rotor and extracts concentrated using Amicon Ultra 4 ultrafiltration units (10,000-Da cutoff) at 4000 rpm in the same rotor. Concentrated samples (500 μ l) were separated by size-exclusion chromatography using a Superose 6 10/300 GL column equilibrated with 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 5 mM MgSO₄, and 150 mM NaCl. Fractions of 0.5 ml were collected. The column was calibrated using blue dextran ($>2 \times 10^6$ Da), ferritin (440,000 Da), and monomeric Lis1 (37,211 Da).

Antibodies

Rabbit antiserum raised against hexahistidine-tagged Lis1, the CT248 antibody that reacts with D1bIC(FAP133), the R5932 and R5391 antibodies against outer arm LC1 and LC2, and the 1878A and 1869A mouse monoclonals against outer arm IC1 and IC2 have been described (King *et al.*, 1985; Patel-King *et al.*, 1997; Benashski *et al.*, 1999; Pedersen *et al.*, 2007; Rompolas *et al.*, 2007). Because the original α -Lis1 antibody also detected a second protein in whole-flagella samples, the entire Lis1 coding region was subcloned onto the pMAL-c2 vector (New England Biolabs, Ipswich, MA) across the *XmnI/XbaI* restriction sites. This resulted in the fusion of Lis1 to the C-terminus of maltose-binding protein (MBP) via a hydrophilic linker that incorporated a factor Xa proteolytic cleavage site. The full-length MBP fusion protein was used as the immunogen for production of rabbit antibody CT273 (Covance Immunology Services, Denver, PA), and specific antibody was subsequently obtained from serum by blot purification using recombinant Lis1.

Gel electrophoresis and immunoblotting

Samples were separated in 5–15% gradient or 10/12.5% SDS polyacrylamide gels. Gels were stained with Coomassie blue or transferred to nitrocellulose and stained with 0.1% reactive brown 10 before immunoblotting. Blots were blocked and washed with 5% dry milk and 0.1% Tween 20 in Tris-buffered saline (TBS). After a final wash with 0.5% Triton X-100 in TBS, immunoreactive bands were identified by chemiluminescence. In some cases, blots were imaged using Fuji RX film and the resulting films scanned. Alternatively, the chemiluminescent signal was detected directly using an ImageQuant LAS4000 cooled, charge-coupled device (CCD) digital imaging system (GE Healthcare, Piscataway, NJ). Quantification of band intensities was performed using ImageQuant TL software.

Immunofluorescence microscopy and immuno-gold labeling

Immunofluorescence analysis of *C. reinhardtii* cells was performed as described previously (Rompolas *et al.*, 2007). Briefly, cells were adhered for 5 min to poly-L-lysine-treated slides, fixed with methanol at -20°C for 10 min, and then air dried at room temperature for an additional 10 min. Samples were subsequently incubated for 1 h

at room temperature with 3% normal goat serum, 1% bovine serum albumin (BSA), 1% cold water fish gelatin, 0.1% IGEPAL CA-630, and 0.05% Tween-20 in PBS. All antibodies used for immunofluorescence microscopy were diluted in PBS buffer containing 1% BSA, 0.1% cold water fish gelatin, and 0.05% Tween-20. Cells were incubated with primary antibody against Lis1 or LC1 for 1–2 h at 22°C or for 16 h at 4°C. After washes with PBS, samples were incubated with Alexa Fluor 488- or 568-conjugated second antibody for 1 h and examined with an Olympus BX51 epifluorescence microscope (Olympus America, Center Valley, PA), using PlanApo 60x/1.40 and 100x/1.35 oil immersion lenses. Images were captured with a MagnaFire cooled CCD (Optronics, Goleta, CA) or a ProgRes CFscan (Jenoptik, Jena, Germany) digital camera. Brightness and contrast adjustments were performed using Photoshop CS4 (Adobe, San Jose, CA).

Immuno-gold labeling of *pf14* axonemes with CT273, fixation, and preparation for transmission electron microscopy were performed as described by Patel-King and King (2009).

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