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The MIA complex is a conserved and novel dynein regulator essential for normal ciliary motility

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xonomel dyneins must be precisely regulated and coordinated to produce ordered ciliary/flagellar motility, but how this is achieved is not understood. We analyzed two Chlamydomonas reinhardtii mutants, mia1 and mia2, which display slow swimming and low flagellar beat frequency. We found that the MIA1 and MIA2 genes encode conserved coiled-coil proteins, FAP100 and FAP73, respectively, which form the modifier of inner arms (MIA) complex in flagella. Cryo-electron tomography of mia mutant axonemes revealed that the MIA complex was located immediately distal to the intermediate/light chain complex of I1 dynein and structurally appeared to connect with the nexin–dynein regulatory complex. In axonemes from mutants that lack both the outer dynein arms and the MIA complex, I1 dynein failed to assemble, suggesting physical interactions between these three axonemal complexes and a role for the MIA complex in the stable assembly of I1 dynein. The MIA complex appears to regulate I1 dynein and possibly outer arm dyneins, which are both essential for normal motility.

Introduction

Motile cilia and flagella are complex microtubule-based organelles critical for embryonic development and organ functions (Satir and Christensen, 2007; Roy, 2009; Lee, 2011; Smith and Rohatgi, 2011). Ciliary motility is generated by the coordinated activities of both the axonemal outer dynein arms (ODAs) and inner dynein arms (IDAs), which are regulated in a precise spatial and temporal manner (Brokaw, 1994; Kamiya, 2002; King and Kamiya, 2009; Lindemann, 2011). Although the ODAs generate the main force for control of ciliary beat frequency, the IDAs mainly contribute to control of the size and shape of the ciliary bend, parameters referred to as waveform (Brokaw and Kamiya, 1987; King and Kamiya, 2009). Failure in proper coordination and regulation of the dyneins results in abnormal ciliary motility, which is implicated in a wide range of human diseases known as ciliopathies (Marshall, 2008; Hildebrandt et al., 2011; Drummond, 2012; Hirokawa et al., 2012; Oh and Katsanis, 2012). However, we have little understanding of the mechanisms that coordinate the activity among the different ciliary dynein arms or that regulate the activity of each dynein subform (Kamiya, 2002; Brokaw, 2009; Lindemann and Lesich, 2010; Mitchison and Mitchison, 2010).

Theoretical and experimental analyses have indicated the oscillatory movement of cilia is, in part, an inherent property of the dynein motors (Yagi et al., 1994; Shingyoji et al., 2004; Shingyoji, 2008; Marshall, 2008; Hildebrandt et al., 2011; Drummond, 2012; Hirokawa et al., 2012; Oh and Katsanis, 2012). However, we have little understanding of the mechanisms that coordinate the activity among the different ciliary dynein arms or that regulate the activity of each dynein subform (Kamiya, 2002; Brokaw, 2009; Lindemann and Lesich, 2010; Mitchison and Mitchison, 2010).

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axonemal complexes have been identified that regulate normal ciliary motility. They include the central pair (CP) apparatus, radial spokes (RSs), the nexin–dynein regulatory complex (N-DRC; Heuser et al., 2012a), the calmodulin- and spoke-associated complex (CSC; Dymek et al., 2011; Heuser et al., 2012a), and the two-headed IDA called I1 dynein, also known as dynein f (Piperno, 1995; Porter and Sale, 2000). The regulatory mechanisms are thought to involve structural and chemical signals that begin in the CP and are then transmitted to the outer doublet microtubules (DMTs) by the RSs (Fig. 1, A and B; Smith and Yang, 2004). Although the composition and structure of the RSs have recently been revealed (Pigino et al., 2011; Lin et al., 2012a), how the RSs transmit signals to the outer DMTs is not understood.

The CSC, which is required for regulation of dynein and for calcium regulation of motility (Dymek et al., 2011; Heuser et al., 2012a), is associated with RS2 and the N-DRC in the distal region of the axonemal 96-nm repeat structure (Gardner et al., 2012a; Heuser et al., 2009), the mechanisms for regulation and signal transduction remain a mystery.

In the proximal part of the axonemal 96-nm repeat, the I1 dynein is the corresponding key regulator of dynein-driven microtubule sliding, relaying signals that are required for normal ciliary waveform (Wirschell et al., 2007; VanderWaal et al., 2011). I1 dynein is the only two-headed IDA, and the functional mechanism involves changes in phosphorylation of its regulatory intermediate chain (IC) IC138 by axonemal kinases and phosphatases (Gokhale et al., 2009; Elam et al., 2011) and is also regulated by the CP and RS (Smith and Yang, 2004).

Based on additional genetic analysis (Porter et al., 1992) and measurement of microtubule sliding (Smith and Sale, 1992; Smith, 2002; Bower et al., 2009; Toba et al., 2011), the CP/RS structures also appear to regulate the ODAs, possibly mediated by I1 dynein (Kikushima, 2009). Consistent with this idea, recent cryo–electron tomography (cryo-ET) studies have revealed intriguing structural links between I1 dynein and the ODAs (Nicastro et al., 2006; Bui et al., 2008, 2012; Heuser et al., 2012b), possibly to coordinate activities between these dyneins. However, neither the proteins nor the functional roles of these linkers have been elucidated, nor have regulatory mechanisms that coordinate various axonemal dyneins been identified. One idea is that I1 dynein predominantly plays a regulatory role, like the N-DRC, controlling the activity of other dyneins (Kotani et al., 2007; Bower et al., 2009; Toba et al., 2011; Heuser et al., 2012b). However, the mechanism of transmission of signals from the RSs to I1 dynein and regulation of I1 dynein is not understood.

To further investigate the mechanism for how I1 dynein is regulated, we analyzed two Chlamydomonas reinhardtii motility mutants, mia1 and mia2. The mia mutants were identified in genetic screens for cells with defects in axonemal dynein regulation (King and Dutcher, 1997). The mutants fail to perform normal phototaxis and display abnormal phosphorylation of IC138, indicative of a defect in regulation of I1 dynein and control of ciliary waveform (Bower et al., 2009; VanderWaal et al., 2011). Here, we report that the gene products of mia1 and mia2 are the conserved coiled-coil proteins, FAP100 and FAP73, respectively, that form a tight complex in the axoneme, which we named the modifier of inner arms (MIA) complex. Cryo-ET indicates that the MIA complex is localized to every 96-nm axonemal repeat just distal to the I1 dynein IC/light chain (LC) complex and proximal to the N-DRC. Biochemical data demonstrate that the MIA complex interacts with I1 dynein components, and structural data suggest that I1 dynein is structurally more flexible in the mia mutants. The mia mutants also display reduced ciliary beat frequencies indicative of defects in regulation of the ODAs. These results are consistent with the idea that the MIA complex functions in a regulatory pathway that controls the activities of I1 dynein and possibly of the ODAs.

Figure 1. Schematic model of motility regulation in C. reinhardtii axonemes. (A and B) Models depicting mechanical/chemical signaling pathways (dotted red arrows) in C. reinhardtii axonemes. Transverse (A) and longitudinal (B) sections are shown. Based on genetic and pharmacological experiments, signals are transmitted from the CP, through the RS1 to I1 dynein, and through RS2 to the CSC and N-DRC. Signals are also thought to be transmitted from I1 dynein and N-DRC to ODAs through OIDLs (blue lines in B). The question addressed here is how signals are transmitted to I1 dynein (question marks in A and B).
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The MIA1 gene encodes the coiled-coil protein FAP100

While searching for C. reinhardtii IDA-deficient mutants from our UV-mutagenized library (Kamiya et al., 1991), we isolated three motility mutants, 317, 623, and 834, which showed slightly jerky, slow-swimming phenotypes, reduced flagellar beat frequencies, and defective phototaxis. The mutations mapped to linkage group II/chromosome 2 near the previously mapped location for the mia1 mutant (King and Dutcher, 1997). Of the candidate genes in this region, FAP100 was of particular interest because it is highly conserved in organisms with motile cilia, and its predicted mass (65.2 kD with an isoelectric point of 5.37) is consistent with that previously reported for a protein missing in mia1 (King and Dutcher, 1997). Sequence analysis revealed mutations in the FAP100 gene for all three strains as well as the original mia1: strain 317 has a T to A substitution, which creates a premature stop at codon 127; strain 623 has a one-base deletion in codon 534 (GTG to GG), resulting in a frame shift mutation; and strain 834 has a two-base (GG to AA) substitution, which alters the 5' splice site of exon 2 (Fig. 2, A and B). The original mia1 mutant (King and Dutcher, 1997) has the ~560-bp “Bill” transposon (Kim et al., 2006) inserted in the first exon of FAP100, resulting in failure of expression of this protein (Fig. 3 A). Because 317, 623, and 834 are alleles of mia1, we renamed the original mia1 to mia1-1, 317 to mia1-2, 623 to mia1-3, and 834 to mia1-4. We made an antibody against the FAP100 protein (Fig. S1), and immunoblotting revealed that FAP100 is missing from axonemes from all three of these strains as well as the original mia1-1 (Fig. 3 A). Using the wild-type FAP100 genomic fragment with a triple HA (3×HA) tag at the C terminus of the coding region, we rescued the mia1 phenotype. The rescued strain, mia1R, had nearly wild-type motility and phototaxis (unpublished data). Furthermore, immunoblot analysis revealed that FAP100-HA is assembled in the axonemes from mia1R (Fig. 3 B). The data definitively reveal that MIA1 encodes FAP100.

FAP100/Mia1p interacts with the coiled-coil protein FAP73/Mia2p to form the MIA complex

To identify FAP100-interacting proteins, we immunoprecipitated FAP100-HA from high salt extracts derived from mia1R axonemes using anti-HA antibodies (Fig. 3 C). Comparison between precipitates from mia1R and wild type, which does not express HA-tagged FAP100, clearly identified an ~76-kD protein and an ~35-kD protein that were exclusively pulled down in precipitates from mia1R extracts. Mass spectrometry (MS) analysis revealed the 76-kD band to be FAP100-HA and the 35-kD band to be FAP73. Both are coiled-coil proteins (Fig. 3 D) conserved in organisms with motile cilia and flagella.
(Fig. S1) confirmed that FAP73 is missing from mia2 axonemes (Figs. 3 A and 4 A), demonstrating that mia2 is a null allele. We rescued the phototaxis and motility defects (not depicted) in mia2 with a HA-tagged FAP73 protein (mia2R; Fig. 3 B). The data definitively reveal that MIA2 encodes FAP73.

Although FAP100 and FAP73 are both absent or greatly reduced from mia1 axonemes, FAP100 is present in mia2 axonemes (Figs. 3 A and 4 A). Based on the coimmunoprecipitation and other biochemical data, FAP100 and FAP73 form an
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The MIA complex localizes to the outer DMTs
To determine where the MIA complex localizes, we performed immunoblotting experiments using mutants lacking specific axonemal structures (Fig. 4 A). The MIA complex was present in axonemes defective in the CP (pf18), RS (pf14 and pf17), ODA (oda1), I1 dynein (ida1), single-headed inner arms (ida5), the N-DRC (pf3), beak structures (mbo1 and mbo2), and the protein phosphatase PP2A (pf4). Double mutants oda1 × ida1, oda1 × ida2, and ida1 × ida5 also had normal amounts of FAP100 and FAP73. Thus, the MIA complex is located on the outer DMTs.

Given the slow-swimming phenotype of mia strains, we analyzed mia mutant axonemes for the presence of other notable axonemal structures. Biochemical analyses indicate that mia mutant axonemes have normal amounts of outer arms (IC69), single-headed inner arms (p28 and actin), RS (RSP1; Fig. 4 B), and a normal dynein heavy chain (HC) profile (Fig. 4 C). In addition, assembly of I1 dynein (HCs, IC140, and IC97) in mia mutants appeared relatively normal (Fig. 4, B and C), although there are slight differences from culture to culture and allele to allele. Furthermore, the mia mutants manifest a highly phosphorylated IC138 (Fig. 5 A) consistent with a previous study (King and Dutcher, 1997), suggesting a defect in I1 dynein regulation. However, immunoblots indicate normal levels of signaling components (CK1, PP1, and PP2A; Fig. 5 B) important in the CP–RS pathway that regulates phosphorylation of IC138 (e.g., Gokhale et al., 2009; Elam et al., 2011). Thus, failure in assembly of the MIA complex does not result in failure of assembly of other axonemal proteins, including the kinases and phosphatases that regulate I1 dynein.

The MIA complex interacts with I1 dynein in the axoneme
To identify proteins that interact with the MIA complex in the axoneme, we used chemical cross-linking with EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) followed
The MIA complex functions in the I1 dynein phosphoregulatory pathway and interacts with multiple axonemal proteins. [A] Untreated flagella (ftl), untreated axonemes (axo), and axonemes treated with the kinase inhibitor DRB were probed with the anti-IC138 antibody. In the mia untreated axonemes, IC138 migrates in multiple forms in a manner similar to ptl. In contrast, wild-type axonemes show a compact IC138 profile. DRB treatment results in a shift in migration of IC138, indicating that these multiple forms of IC138 are caused by phosphorylation. (B) Immunoblot analyses indicate that CK1, PP2A (B-sub), and PP1 are assembled normally in the mia mutants. (C) Isolated wild-type axonemes treated with or without 5 mM EDC were probed with the FAP100 and FAP73 antibodies. Upon EDC exposure, both Mia proteins form some cross-linked products (red arrowheads), indicating that the MIA complex is in direct contact with multiple axonemal proteins. We could not detect the cross-linked products between I1 dynein subunits and the Mia proteins as solid bands on these blots. Black arrows indicate the un–cross-linked FAP100 and FAP73. Ab, antibody; wt, wild type.

Cryo-ET reveals that the MIA complex is located distal to the IC/LC complex of I1 dynein, possibly extending across the middle of the 96-nm repeat to the N-DRC. Immunofluorescence of nucleoflagellar apparatuses from mia1R clearly showed that FAP100 is present along the entire length of both cilia, which is the same localization pattern obtained for I1 dynein (Fig. 6A; Bui et al., 2012). To definitively determine structural changes and to localize the MIA complex, we used cryo-ET and subtomogram averaging of the 96-nm axonomal repeat to analyze the 3D structures of mia axonemes and compare them with pseudo–wild type (pWT) and pf9-3/ida1 (Videos 1 and 2). Consistent with our EDC cross-linking results (Fig. 5C and Table S1), cryotomographic averages of both mia1 and mia2 axonemes revealed structural defects in the distal portion of the I1 dynein IC/LC complex and in the structure located between I1 dynein and the N-DRC (Figs. 6, B and C; and S2, A–C; and Videos 3, 4, and 5). These structural defects are more severe in mia1 than in mia2, supporting our biochemical results that mia1 axonemes lack both FAP100 and FAP73, whereas mia2 lacks only FAP73. Consistent with our biochemical analysis (Fig. 4, B and C), other structures including the RSs, N-DRC, and the dynein structures appear fully assembled as in the pWT control (Figs. 6, B and C; and S2, A–C; and Videos 1, 3, and 4).

Consistent with previous data (Nicastro et al., 2006; Bower et al., 2009; Heuser et al., 2012b), axonemes of the pf9-3/ida1 mutant lack the entire I1 dynein, including the distal portion of the IC/LC complex that is reduced in the mia mutants. In contrast, the structure that bridges between the I1 dynein and N-DRC, which is missing or greatly reduced in the mia mutants, is present in pf9-3/ida1 and pWT axonemes (Figs. 6 C and S2, A–C; and Videos 1, 2, 3, 4, and 5). Therefore, failure in I1 dynein assembly does not alter assembly of the MIA complex.

The MIA complex is important for the stable attachment of I1 dynein to the axoneme. The majority of the I1 dynein density is present in mia mutants, but we observed by cryo-ET that the density of the I1 dynein appeared weaker and blurred compared with pWT (Figs. 6 B and S2, A–C). This blurring was only observed for the I1 dynein and not for other axonemal structures. The blurred density of I1 dynein in the mia averages indicates heterogeneity between the averaged axonomal repeats, either because of assembly or positional differences, leading to bad alignment of just the I1 dynein in relation to the other axonemal structures. Classification of all averaged subtomogram volumes concerning presence or absence of the I1 dynein detected only occasional missing of the I1 dynein, mostly in random 96-nm repeats of both mia mutants (unpublished data). To test whether the position of I1 dynein is more flexible in mia axonemes than in wild type, we calculated local averages of the I1 dynein by masking...
Figure 6. **The MIA complex is localized to a unique position in each 96-nm repeat.** (A) Nucleoflagellar apparatuses from *oda1* and *mia1R × oda1* were stained with the anti-HA antibody. The HA antibody detects FAP100-HA in both flagella in the *mia1R × oda1* strain (fluorescence image on the bottom). Very weak or no signal was detected in *oda1* flagella that do not express the FAP100-HA protein. Both basal bodies and nuclei have some nonspecific staining. (top) Differential interference contrast images show that all cells possess flagella. Arrowheads indicate cis- and trans-flagella, showing that both flagella are staining. 

(B) Longitudinal tomographic slices of the averaged 96-nm axonemal repeats from DMTs 1–9 of pWT, *mia2*, *mia1*, and *pf9-3/ida1* reveal structural defects in the mutant axonemes. The density of the I1 dynein (red outlines) is completely missing in *pf9-3/ida1* (white outlines) and appears reduced in the *mia* mutants. These defects are more prominent in *mia1* than in *mia2*. The densities of other major axonemal structures, such as the ODAs, single-headed inner dynein arms (IDAs 2–6 and X), and the N-DRC are not significantly changed in the *mia* mutants. (C) 3D isosurface renderings of the averaged 96-nm axonemal repeats from pWT, *mia2*, *mia1*, and *pf9-3/ida1* reveal the structural defects in more detail. Regions that are reduced in the *mia* averages are colored green; the most obvious defects are the distal density of the IC/IC of I1 dynein (orange) and the density between the I1 dynein and the N-DRC (yellow), which is reduced in *mia2*, missing in *mia1*, but fully assembled in pWT and *pf9-3/ida1*. The pWT and *pf9-3/ida1* data were refined from data originally reported by Heuser et al. (2012b). Proximal is on the left in B and C. Bars: (A) 10 µm; (B) 25 nm.
Figure 7. The MIA complex affects I1 dynein stability in the axoneme. [A] I1 dynein is missing along the entire length of DMT1 in the mia mutants. Tomographic slices of axonemal average of individual outer DMTs from pWT (top row), mia2 (middle row), and mia1 (bottom row). The first two columns show the axonemal repeat of DMT1 in the proximal (left) and distal (middle) regions; the right column depicts the combined average of the axonemal

B

C

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Figure 7. The MIA complex affects I1 dynein stability in the axoneme. [A] I1 dynein is missing along the entire length of DMT1 in the mia mutants. Tomographic slices of axonemal average of individual outer DMTs from pWT (top row), mia2 (middle row), and mia1 (bottom row). The first two columns show the axonemal repeat of DMT1 in the proximal (left) and distal (middle) regions; the right column depicts the combined average of the axonemal
all other structures during the alignment process. In contrast to the global averages in which the alignment is dominated by the density of the DMTs and other structures in the 96-nm repeat, in the local average of both mial1 and mial2, the density of the I1 dynein appears very similar to the density in pWT, whereas the surrounding axonemal structures are greatly blurred because of alignment errors (unpublished data). This suggests that the I1 dynein density in the mia averages is mostly reduced because the position of the I1 dynein is more variable in the mia mutants than in pWT, which is consistent with a structural interaction between I1 dynein and the MIA complex.

In addition, doublet-specific averaging revealed an intriguing feature of mial mutant axonemes (Figs. 7 A and S3). DMTs 2–9 assemble all the IDA and ODA structures, and the defects noticeable are the missing densities distal of I1 dynein as described in the previous paragraph. Doublet-specific averages of DMTs 2–9 did not reveal obvious differences between these doublets in regards to the structural defects found in the mial mutants (Fig. S3). However, DMT1 of mial1 and mial2 axonemes shows additional defects compared with pWT. In wild-type C. reinhardtii flagella, DMT1 is known to exhibit unique features compared with DMTs 2–9: (a) the ODAs and the outer–inner dynein linkers (OIDLs) are missing along the entire length of DMT1, (b) in the proximal quarter of the flagellum, the ODAs are substituted by the 1–2 bridge that links DMTs 1 and 2 (Fig. 7 A; Hoops and Witman, 1983; Bui et al., 2009, 2012; Lin et al., 2012b), and (c) in the proximal region where the 1–2 bridge is present, the I1 dynein is missing (Bui et al., 2012; Lin et al., 2012b). In the mial mutants, however, I1 dynein is missing along the entire length of DMT1.

The results suggested that the MIA complex is located on all outer doublets (Fig. S3). However, one interpretation of the doublet-specific mial mutant defect is that I1 dynein assembly and stability are dependent on assembly of both the ODAs and the MIA complex, with the ODAs missing from DMT1. To test this, we used protein fractionation and immunoblots to compare the amount of I1 dynein assembled in axonemes from a mial1 × oda6 double mutant (lacking the MIA complex and ODAs) to oda1 or oda6 lacking only the ODAs. Chromatographic separation of flagellar dyneins revealed that the amount of I1 dynein is indeed reduced in axonemes from mial1 × oda6 compared with that of oda1 (Fig. 7 B). In addition, immunoblots using antibodies to the I1 dynein HC and IC97 revealed a similar reduction of I1 dynein in other mial × oda double mutants (Fig. 7 C). These results indicate that I1 dynein assembly fails when both the ODA and MIA complex are missing and is dependent on both structures. Among these two stabilizing complexes, the MIA complex seems to be more crucial for stable I1 dynein assembly because, in contrast to the MIA complex, the absence of ODAs alone does not cause increased flexibility of the I1 dynein (Fig. 7 A, distal portion of DMT1 in pWT).

The MIA complex controls ciliary/flagellar motility through regulation of dynein activity

The mial mutants were first isolated as slow-swimming, non-phototactic cells (King and Dutcher, 1997). These are motility phenotypes common to defective I1 dynein assembly or regulation (Okita et al., 2005; Elam et al., 2011; VanderWaal et al., 2011) and that display excessive phosphorylated forms of IC138 (King and Dutcher, 1997). These observations are consistent with a role for the MIA complex in regulation of I1 dynein. In addition, from our analyses, the mial mutants have both swimming speed (∼60 µm/s) and beat frequency (40–50 Hz) defects (Fig. S4 and Table 1) that are more severe than those observed for I1 dynein mutants (swimming velocity: ∼100 µm/s; beat frequency: ∼60 Hz; Table 1; Hendrickson et al., 2004; VanderWaal et al., 2011), indicating that the MIA complex may regulate multiple dyneins, including direct or indirect regulation of the ODAs.

To test whether the MIA complex regulates the function of more than one species of axonemal dynein, we analyzed motility in a series of C. reinhardtii double mutants defective in both the MIA complex and selected dynein arms. As expected, doubles between mial mutants (mial1 × mial2) and I1 dynein–deficient mutants (mial1 × ida1, mial1 × ida2, mial1 × bop5, and mial2 × bop5) showed quite similar motility phenotypes to those of mial alone (Fig. S4 and Table 1). These results strongly suggest a functional interaction between the MIA complex and I1 dynein and indicate that the I1 dynein is inactive in the mial mutants (see Discussion). The double mutants between the mial mutants and ida4 (lacking dynein a, c, and d), ida6 (lacking dynein c and having a defect in N-DRC; Porter, 2011), and ida9 (lacking dynein c) all showed more severe motility phenotypes (nearly nonmotile) than either parent (Table 1). Similarly, doubles between the mial mutants and oda mutants show very low beat frequencies (10–20 Hz), indicating a more severe phenotype than either parent (Fig. S4 and Table 1). These results indicate that the MIA complex operates in the same pathway as I1 dynein but that the MIA

repeats from DMTs 2–9 that do not show obvious structural differences between the proximal and distal regions (for individual DMT averages, see Fig. S3). In wild type, I1 dynein is missing from the proximal region of DMT1 [white outlines] but is present in the distal region [red outlines]. In contrast, in both mial mutants, I1 dynein is missing along the entire length of DMT1 [white outlines]. The pWT data were refined from data originally reported by Heuser et al. (2009) and Lin et al. (2012b). Other structures labeled are single-headed inner dynein arms (IDAs 2–6 and X), the N-DRC, and the inner SUB5-6 bridge. Bars, 25 nm. (B) Representative ion-exchange chromatography [Mono Q; GE Healthcare] elution profiles of axonemal dyneins show that I1 dynein levels ([black arrows] appear relatively normal in mial2 extracts compared with that of wild-type extracts [left graph], consistent with the immunoblots results using I1 dynein subunit antibodies [Fig. 4 B]. In contrast, I1 dynein is drastically reduced in the mial2 × oda6 double mutant [red arrow] compared with the oda1 single mutant [right graph], suggesting that I1 dynein becomes unstable without both ODAs and the MIA complex. Typical Mono Q elution patterns are shown [A280 protein estimate in arbitrary units is indicated to the right of each scan [y axis]; elution time in minutes is shown on the x axis], and at least two experiments were performed for each strain. Elution of dynein complexes began at ∼12 min. (C) Immunoblot analysis of isolated axonemes using the antiHC1 (I1 dynein HC-c) and IC97 antibodies confirms the reduction of I1 dynein in mial × oda double mutants. I1 dynein appears normal to slightly reduced in the single mial mutants relative to wild type [wt].

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complex does not solely regulate the single-headed IDAs or the ODAs.

To determine how the MIA complex contributes to regulation of dynein motor function, we used a sliding disintegration assay to measure microtubule sliding velocities in isolated axonemes (Okagaki and Kamiya, 1986). The microtubule sliding velocities of *mia1* mutants were similar to wild type, although there was some slight variation in the different alleles. In contrast, microtubule sliding velocities of *mia2* were significantly reduced (Fig. 8). *II* dynein plays a role in control of microtubule sliding by a regulatory pathway that involves the CP, RS, and axonemal kinases and phosphatases (Fig. 1, A and B; Wirschell et al., 2007). Dynein-driven microtubule sliding is globally inhibited in isolated, paralyzed axonemes from *RS* mutants, whereas these treatments successfully rescued the microtubule sliding of *pf17*. In addition, kinase inhibitor treatment resulted in a decrease in microtubule sliding velocities in *mia1* (Fig. 8). As discussed in the next section, these results, although revealing the unexpected features of the MIA complex, are consistent with a role for the MIA complex in regulation of dyneins by a pathway that includes the CP/RS/II dynein phosphoregulatory mechanism.

### Discussion

In this study, we addressed the hypothesis that the *MIA1* and *MIA2* genes encode proteins required for regulation of *II* dynein. Consistent with this, these genes encode conserved coiled-coil proteins FAP100 and FAP73, respectively (Fig. 2, A and B), which interact to form the MIA complex (Fig. 3 C), a novel regulator of dynein-driven ciliary/flagellar motility. The MIA complex localizes along the entire length of both flagella (Fig. 6 A), and Table S1), and is localized near the IC/LC complex–IC138 coil proteins FAP100 and FAP73, respectively (Fig. 2, A and B), which interact to form the MIA complex (Fig. 3 C), a novel regulator of dynein-driven ciliary/flagellar motility. The MIA complex localizes along the entire length of both flagella (Fig. 6 A), although revealing the unexpected features of the MIA complex, are consistent with a role for the MIA complex in regulation of dyneins by a pathway that includes the CP/RS/II dynein phosphoregulatory mechanism.

### Table 1. Phenotypes of the *mia* mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Defect</th>
<th>Phenotype</th>
<th>Typical beat frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>None</td>
<td>Slightly jerky or smooth, fast swimming</td>
<td>60–70 Hz</td>
</tr>
<tr>
<td><em>mia1</em></td>
<td>MIA complex</td>
<td>Slightly jerky, slow swimming</td>
<td>40–50 Hz</td>
</tr>
<tr>
<td><em>mia1-2</em></td>
<td>MIA complex</td>
<td>Slightly jerky or jerky (some cells), slow swimming</td>
<td>~40 Hz</td>
</tr>
<tr>
<td><em>mia1-3</em></td>
<td>MIA complex</td>
<td>Slightly jerky or jerky (some cells), slow swimming</td>
<td>40–50 Hz</td>
</tr>
<tr>
<td><em>mia1-4</em></td>
<td>MIA complex</td>
<td>Slightly jerky or jerky (some cells), slow swimming</td>
<td>40–50 Hz</td>
</tr>
<tr>
<td><em>mia2</em></td>
<td>MIA complex</td>
<td>Nearly nonmotile (more severe than <em>mia1</em></td>
<td>&lt;10 Hz</td>
</tr>
<tr>
<td><em>mia1-1 × mia2</em></td>
<td>MIA complex</td>
<td>Nearly nonmotile (more severe than <em>mia1</em></td>
<td>&lt;10 Hz</td>
</tr>
<tr>
<td><em>mia1-1 × bop5-4</em></td>
<td>MIA complex, IC138 complex</td>
<td>Beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-3 × ida1</em></td>
<td>MIA complex, II dynein</td>
<td>Beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-2 × ida2</em></td>
<td>MIA complex, II dynein</td>
<td>Beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-2 × ida4</em></td>
<td>MIA complex, dynein α, c, and d</td>
<td>Nearly nonmotile (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-4 × ida4</em></td>
<td>MIA complex, dynein α, c, and d</td>
<td>Nearly nonmotile (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-1 × ida9</em></td>
<td>MIA complex, dynein c</td>
<td>Nonmotile or beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-2 × oda1</em></td>
<td>MIA complex, ODA</td>
<td>Beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-3 × oda2</em></td>
<td>MIA complex, ODA</td>
<td>Beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-1 × oda6</em></td>
<td>MIA complex, ODA</td>
<td>Beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia1-1 × pf4</em></td>
<td>MIA complex, PP2A phosphatase</td>
<td>Beat at low beat frequency (more severe than <em>mia1</em>)</td>
<td>~30 Hz</td>
</tr>
<tr>
<td><em>mia1-1 × pf17</em></td>
<td>MIA complex, RS head</td>
<td>Nonmotile (more severe than <em>mia1</em>)</td>
<td>N/A</td>
</tr>
<tr>
<td><em>mia2 × bop5-3</em></td>
<td>MIA complex, IC138 complex</td>
<td>Slightly jerky or smooth (some cells), slow swimming</td>
<td>40–50 Hz</td>
</tr>
<tr>
<td><em>mia2 × ida6</em></td>
<td>MIA complex, N-DRC, dynein e</td>
<td>Nonmotile or sporadic twitching (more severe than <em>mia1</em>)</td>
<td>N/A</td>
</tr>
<tr>
<td><em>mia2 × ida9</em></td>
<td>MIA complex, dynein c</td>
<td>Nonmotile or beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>&lt;10 Hz</td>
</tr>
<tr>
<td><em>mia2 × oda6</em></td>
<td>MIA complex, ODA</td>
<td>Beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia2 × oda7</em></td>
<td>MIA complex, ODA</td>
<td>Beat at very low beat frequency (more severe than <em>mia1</em>)</td>
<td>10–20 Hz</td>
</tr>
<tr>
<td><em>mia2 × pf4</em></td>
<td>MIA complex, PP2A phosphatase</td>
<td>Very short flagella (more severe than <em>mia1</em>)</td>
<td>N/A</td>
</tr>
<tr>
<td><em>mia2 × pf17</em></td>
<td>MIA complex, RS head</td>
<td>Nonmotile (more severe than <em>mia1</em>)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Typical beat frequencies of cells were measured at room temperature (~25°C). When cells had no flagella, cells were kept rotating in distilled water for several hours to grow flagella. N/A, not analyzable.*
requires assembly of both the MIA complex and ODA (Fig. 7, A–C), consistent with the presence of a structural link between I1 dynein and ODA (Nicastro et al., 2006; Bui et al., 2012; Heuser et al., 2012b). Together, the data indicate a structural and functional interaction between I1 dynein and the MIA complex, which is required for normal ciliary motility. As illustrated in Fig. 9 (A and B), we postulate that the MIA complex physically positions the I1 dynein/IC138 (Bower et al., 2009) relative to the predicted position of the axonemal kinases and phosphatases for regulation of IC138 phosphorylation and control of ciliary waveform (King and Dutcher, 1997; Bayly et al., 2010; VanderWaal et al., 2011).

The MIA complex affects the structure and stability of I1 dynein

Among the primary results of this study was the discovery of the MIA complex. The MIA complex assembles independent of other major axonemal structures: the mia mutants do not have defects in assembly of other major axonemal structures (Figs. 4, B and C; and 6 C), and, in turn, the MIA complex assembly is unaffected in mutants of other major axonemal complexes (Fig. 4 A). Moreover, the kinases (CK1) and phosphatases (PP2A and PP1) involved in the CP–RS–I1 regulatory pathway (Yang et al., 2000; Gokhale et al., 2009; Elam et al., 2011) are assembled at wild-type levels (Fig. 5 B). Consistent with our biochemical analyses, King and Dutcher (1997) found that only an ~65-kD protein (FAP100), an ~34-kD protein (FAP73), and an unknown ~35-kD protein (also missing in ida1) are missing or reduced in mia mutant axonemes. Although mia2 only lacks FAP73, mia1 has defects in both FAP100 and FAP73 in the axoneme, indicating FAP100 is required for ciliary localization of FAP73 but not vice versa (Figs. 3 A and 4 A).

Cryo-ET of mia axonemes identified the putative location of the MIA complex on the outer DMTs. The mia mutant axonemes have structural defects in the region between the IC/LC complex of I1 dynein and the N-DRC (Fig. 6 C, green; and Video 5). The simplest interpretation is that the MIA complex is located adjacent to the IC/LC domain of I1 dynein, an ideal location for regulation of I1 dynein. The reduced densities further distal toward the N-DRC could be downstream structural assembly defects when the MIA complex is missing. Moreover, cryo-ET analyses clearly demonstrate that, in axonemes from the mia mutants, I1 dynein is specifically missing along the entire length of DMT1. ODAs are also missing from DMT1 (Hoops and Witman, 1983; Bui et al., 2009, 2012; Lin et al., 2012b). Thus, one possible explanation is that assembly or stability of I1 dynein is affected by structural interactions with both the ODA and the MIA complex. This hypothesis is supported by analyses of mia × oda double mutants, which show a reduced amount of I1 dynein relative to oda and mia single mutants (Fig. 7, B and C).

Our results indicate that the MIA complex structurally supports I1 dynein (Fig. 9, A and B). Consistent with this interpretation, I1 dynein structure is also altered in mia axonemes. This is illustrated by the reduced and blurred density of the I1 dynein in mia mutants (Figs. 6 B, 7 A, and S3). Thus, despite the assembly of I1 dynein on DMTs 2–9 in axonemes from mia mutants (Figs. 4, B and C; and 7, B and C), the I1 structure appears more flexible compared with that in wild type (Fig. 9 B). These observations are also consistent with our recent cryo-ET study revealing that the IC/LC complex appears to be suspended from the A tubule of outer DMTs with only a single, narrow connection (Video 1; Heuser et al., 2012b). Our observations suggest that the distal connection to the MIA complex is crucial for stable attachment of the I1 dynein IC/LC complex to the axoneme.

The MIA complex may coordinate multiple axonemal pathways for regulation of dyneins

To test whether the MIA complex functions in the signaling pathways that regulate dynein activity in vivo (e.g., in cooperation with the N-DRC for control of ODA and beat frequency [Porter, 2011] or with the CP–RS–I1 phosphorylation pathway...

Figure 8. Regulation of dynein-driven microtubule sliding is disrupted in the mia mutants. ATP- and protease-induced microtubule sliding measurements show the characteristic sliding velocities for wild-type (wt) and pf17 mutant axonemes. The reduced sliding velocity of pf17 is rescued to wild-type levels by the addition of a kinase inhibitor (Ki). Microtubule sliding velocities of mia1 (mia1-1 and mia1-3) are similar or only slightly reduced relative to that of wild type but greatly reduced when coupled with the pf17 mutation (mia1-1 × pf17). Microtubule sliding velocities of mia2 axonemes are significantly reduced and are not altered by kinase inhibitor treatment (mia2 + kinase inhibitor). Error bars show standard deviations (n = 4–36).
lacks the MIA complex, several N-DRC components, and IDA e, we isolated and characterized double mutants between the mutants for control of I1 dynein and waveform (Wirschell et al., 2007), we isolated and characterized double mutants between the mia mutants and the I1 dynein mutants ida1, ida2, and bop5 showed a similar motility phenotype to mia alone (Fig. S4 and Table 1). In addition, the mia × bop5 double mutants fail to perform phototaxis similar to the single mia mutants (VanderWaal et al., 2011). The bop5 mutant is particularly informative because it only affects the assembly of the IC138 complex, whereas the remaining I1 dynein subunits assemble as in wild-type axonemes (Bower et al., 2009). Moreover, the bop5 mutants revealed that the IC138 complex is required for control of flagellar waveform but is not required for phototaxis (VanderWaal et al., 2011). Thus, the MIA complex must have an essential role in phototactic behavior that is independent of the IC138 complex.

We observed reduced beat frequencies (~40 Hz) and slightly jerky or jerky phenotypes of the mia mutants (Fig. S4 and Table 1; King and Dutcher, 1997). The simplest explanation for this observation is that the MIA complex either directly or indirectly regulates ODAs because the ODAs control ciliary beat frequency (Brokaw and Kamiya, 1987). We postulate that the MIA complex affects beat frequency and ODA activity via its effects on I1 dynein. Consistent with this hypothesis, defective assembly of I1 dynein results in greatly reduced microtubule sliding velocity in isolated axonemes, suggesting a functional interaction between I1 dynein and ODAs (e.g., Kikushima, 2009; Toba et al., 2011). The functional interaction between I1 dynein and the ODAs could be mediated by the OIDLs observed by cryo-ET (Nicastro et al., 2006; Bui et al., 2012).

**Possible regulatory mechanism of the MIA complex**

A primary functional role of the MIA complex appears to be the regulation of I1 dynein. One model, illustrated in Fig. 9 A, indicates a direct interaction of the I1 IC138 complex (Bower et al., 2009) with the MIA complex and stable positioning of I1 dynein relative to RS1 and presumably relative to the kinase and phosphatase for IC138 (Gokhale et al., 2009; Elam et al., 2011). In agreement with this model, structural analysis of the mia axonemes reveals defective structure just distal to the location of the IC/LC complex (Figs. 6 C and S2, A–C) and altered structure of I1 dynein on DMTs 2–9 (Figs. 6 B, 7 A, and S3). In addition, cross-linking analysis reveals interaction between the Mia proteins and IC138 (Table S1).

Diverse evidence has revealed a correlation between the velocity of microtubule sliding and the degree of IC138 phosphorylation: increased IC138 phosphorylation correlates with inhibition of microtubule sliding (Habermacher and Sale, 1997; Wirschell et al., 2007). Thus, the microtubule sliding results of the mia mutants were surprising: despite increased phosphorylation of IC138 (Fig. 5 A) and deficits in structure and assembly of both FAP100 and FAP73 (Figs. 3 A, 4 A, and 6 C), microtubule sliding velocity was rapid in axonemes from mia1 alleles.
potential structural defects only with the loss of FAP73 (Figs. 3 A, 4 A, and 6 C) and increased phosphorylation of IC138, microtubule sliding in mia2 axonemes was greatly reduced (Fig. 8). Because the in vivo motility phenotypes of mia1 and mia2 are similar, it was surprising to observe the large difference in microtubule sliding between mia1 and mia2 mutations. In addition, we and others (King and Dutcher, 1997) observed that despite more limited axonemal structural defects, the phosphorylation state of IC138 was more excessive in mia2 than in mia1 (Fig. 5 A). Furthermore, addition of kinase inhibitors to mia1 axonemes inhibited microtubule sliding, whereas neither DRB nor PKI altered or rescued microtubule sliding in mia2 axonemes regardless of reduced IC138 phosphorylation (Fig. 8). These results reveal that the key axonemal phosphorytase for IC138 is at least present in mia axonemes and can dephosphorylate IC138 in vitro (Fig. 5 A).

One explanation for these discrepancies is that in axonemes from the mia mutants, phosphorylation of IC138 is uncoupled from changes in microtubule sliding. In addition, the mia mutants may reveal an important in vivo regulatory mechanism that cannot be studied by the in vitro microtubule sliding assay. For example, changes in IC138 phosphorylation may be very rapid in vivo (e.g., in response to signals from the external environment required for control of waveform) and that assembly of the MIA complex is required to ensure close and efficient interaction between IC138 and axonemal kinases and phosphatases. In contrast, in in vitro assays, changes in phosphorylation may be slow. In any case, further understanding of the apparent discrepancy between live-cell motility and in vitro microtubule sliding in the mia mutants will require future analysis of the key, motility-related phosphoresidues in IC138 (Bower et al., 2009; VanderWaal et al., 2011).

**Potential functions of the MIA complex in higher organisms**

Mia proteins are well conserved from *C. reinhardtii* to mammals, suggesting the MIA complex regulates ciliary motility and/or assembly also in higher organisms. The Mia proteins each have two potential homologues in humans; CCDC37 and CCDC38 for FAP100 and CCDC42A and CCDC42B for FAP73 (Fig. S5, A and B). The CCDC42A gene is expressed in mouse testes, and mutations in CCDC42A result in a failure in flagellar assembly (Yoder, B., personal communication). In addition, CCDC37 has been recently reported to be one of five hypermethylated and downregulated genes in human lung squamous cell carcinoma (Kwon et al., 2012). Also, CCDC38 has been recently reported as one of 16 loci that are tightly related to normal pulmonary function (Soler Artigas et al., 2012). These studies suggest that potential Mia protein homologues have important functions in mammalian tissues/cells that bear motile cilia.

**Materials and methods**

**Strains, DNA, and culture conditions**

The strains used in this study are listed in Table S2. The mia1-2, mia1-3, and mia1-4 mutants were all produced by standard UV mutagenesis and strain selection as described previously (Kamiya et al., 1991) and used for the first time in this study. The double mutants were constructed by standard methods (Dutcher, 1995). Cells were cultured in liquid L or Tris-acetate-phosphate medium and grown with aeration on a 14h/10h light/dark cycle or under constant illumination. The cDNA sequences of *C. reinhardtii* FAP100 and FAP73 have been deposited in the DNA Data Bank of Japan upon accession number AB692780 and AB692781, respectively.

**Preparation of flagella and axonemes**

Deflagellation of *C. reinhardtii* cells was induced by treating cells with dimethyl sulfoxide (DMSO). Cells were pelleted by centrifugation and resuspended in medium containing 10% DMSO. Cells were then shifted to 20°C for 2 h and pelleted by centrifugation. The supernatant was removed, and the pelleted cells were resuspended in 20% DMSO in the presence of 0.1 mM EDTA and then treated with 0.1% Nonidet P-40 in HMDEK (30 mM Hepes, 5 mM MgSO4, 1 mM DTT, 1 mM EGTA, and 50 mM potassium acetate, pH 7.4) and centrifuged to remove the membrane and matrix fraction. Flagella used for cryo-ET were isolated by the pH shock method, and axonemes were isolated after flagellar demembranation (Vesel et al., 1972). Purified axonemes were resuspended in 30 mM Hepes, pH 7.4, 25 mM KCl, 5 mM MgSO4, 1 mM EGTA, and 0.1 mM EDTA and processed within 24 h.

**SDS-PAGE and immunoblotting**

SDS-PAGE and immunoblotting were performed using standard procedures. SDS-PAGE was performed using normal acrylamide gels or 3–5% gradient gels with a 3–8 M urea gradient (Laemmli, 1970; Jurvick and Rosenbaum, 1980). Gels were stained with Coomassie brilliant blue (CBB) or silver. For immunoblotting, protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). 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 (Bio-Rad Laboratories) and a 3,3′,5,5′-tetramethybenzidine peroxidase substrate kit (Vector Laboratories). Antibodies used were anti-DHC1 (11 dynein HC-a; rabbit; Myster et al., 1997), anti-IC140 (rabbit; Yang and Sole, 1998), anti-IC138 (rabbit; Hendrickson et al., 2004), anti-IC97 (rabbit; Wirschell et al., 2009), anti-IC69 (mouse; King et al., 1985), antianticain (rabbit; Kato-Minoura et al., 1997), anti-p28 (rabbit; LeDizet and Piperno, 1995a), anti-RPS1 (rabbit; Kohno et al., 2011), anti-FAP100 (rabbit; this study), anti-FAP73 (rabbit; this study), anti-P22A B subunit (rabbit; Elam et al., 2011), anti-P22A C subunit (mouse; Yu et al., 2001), anti-P1 (rabbit; Yang et al., 2000), anti-C1 (rabbit; Gekkale et al., 2009), and anti-HA clone 3F10 (rat; Roche).

**Polyclonal antibody production**

To produce FAP100 and FAP73 polyclonal antibodies, the coding region of FAP100 or FAP73 cDNA from wild-type cells was ligated into the BamHI and EcoRI sites of the pCold bacterial expression vector (Takara Bio Inc.). Protein expression was induced by IPTG. The primer pairs used to amplify the coding regions of FAP100 and FAP73 cDNA were FAP100-1F, 5′-CTGGATCCTCAATCTCCGCGCGACGCCAGCGACGTC-3′; FAP73-1F, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′; FAP100-1R, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′; FAP73-1R, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′; FAP100-2R, 5′-CTGGATCCTCGAGCGCGCGACGCCAGCGACGTC-3′; FAP73-2R, 5′-CTGGATCCTCGAGCGCGCGACGCCAGCGACGTC-3′; FAP100-3R, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′; FAP73-3R, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′; FAP100-4R, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′; FAP73-4R, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′; and FAP73-5R, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′.

**Phenotypic rescue of mia mutants**

For phenotypic rescue, mia1-2 was transformed with an ~5.4-kb HindIII-KpnI genomic fragment, which was excised from BAC 16N2 (Clemson University Genomics Institute) and cloned into the pKF19k-2 vector (Takara Bio Inc.). This fragment contained the whole FAP100 gene. A 3xHA tag was inserted into the Dral site at the C terminus of the FAP100 gene. The genomic fragment with the HA tag was incorporated in mia1-2 cells by electroporation as previously described (Shimogawara et al., 1998). For phenotypic rescue of mia2, a modified pGenD plasmid vector was used (Fischer and Rochaix, 2001; Yamamoto et al., 2010). The coding region of FAP73 was amplified by PCR with the primer pair FAP73-NF1, 5′-CTGGATCCTCGAGCGCGCGACGCCAGCGACGTC-3′; FAP73-R1, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′; and FAP73-R2, 5′-GAGAATTCATGTAAGTCCGCGCGACGCCAGCGACGTC-3′, which

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contained the recognition sites for BamHI, NdeI, and EcoRI (underlined). The Ndel-EcoRI cDNA fragment of FAP73 was ligated into the pGenD vector and used for transformation of mia2 cells by electroporation.

Immunoprecipitation
Immunoprecipitation under non–cross-linked conditions was performed as described previously (Yamamoto et al., 2008). In brief, the anti-FAP100, anti-FAP73, or anti-HA antibody (Roche) was attached to protein A or G beads and incubated with three times diluted 0.6 M KCl axonemal extracts at 4°C for several hours. The precipitates were washed several times, and the samples were boiled and processed for SDS-PAGE. Immunoprecipitation under cross-linked conditions was performed by the modified method of Sakato (2009). Axonemes from mia1R and mia2R were cross-linked with 5 mM EDC for 1 h at room temperature, boiled in SDS sample buffer containing 1% SDS, diluted 10 times with 1% NP-40 in TBS, and mixed with protein G beads (Santa Cruz Biotechnology, Inc.) with the anti-HA antibody. The mixture was kept rotating overnight at 4°C, washed several times, and subjected to SDS-PAGE. Precipitates were run to the length of ~10 nm on the gels, stained briefly with CBB, and cut out for the ESI/liquid chromatography/MS/MS analysis. Non–cross-linked mia1R and mia2R axonemes were processed in the same way as controls. The proteins identified only in EDC cross-linked conditions, but not in non–cross-linked controls, are summarized as the potential Mia complex proteins in Table S1.

Immunofluorescence microscopy
Immunofluorescence microscopy and nucleoflagellar apparatus preparations were performed as previously described (Sanders and Salisbury, 1995; Taillon and Jarvik, 1995). Nucleoflagellar apparatuses were fixed with 2% formaldehyde at room temperature followed by treatment with cold acetone and methanol (–20°C). Fixed samples were stained with an anti-HA antibody (12CA5, Roche) in blocking buffer (10 mM sodium phosphate, pH 7.2, 5% normal goat serum, 5% glycerol, 1% cold fish gelatin, and 0.004% sodium azide) and then with the tetratetramethyl rhodamine isothiocyanate–labeled anti–mouse IgG antibody (Kirkegaard & Perry Laboratories). Images were acquired on a fluorescence microscope (Axioplan; Carl Zeiss) with a 63×/1.4 NA Plan Apochromat objective lens at room temperature (Carl Zeiss) and recorded with a charge-coupled device camera (CoolSNAP; Roper Scientific).

Microtubule sliding assay
Microtubule sliding assays were performed as described previously (Okaegaki and Kamiya, 1986; Gokhale et al., 2009). Sliding was triggered by the addition of HMDEP [10 mM Hepes, 1 mM MgSO4, 1 mM DTT, 0.5 mM EDTA, 50 mM potassium acetate, and 1% of 20,000 polyethylene glycol] buffer containing 1 mM ATP and 5 µg/ml subtilisin A type VIII protease (Sigma-Aldrich) to axonemes in perfusion chambers. For inhibition experiments, we used 50 µM DRB (Enzo Life Sciences) and 100 mM PKI (Enzo Life Sciences) as kinase inhibitors. Images were recorded with a microscope (Axioplan) equipped with dark-field optics, a 40× Plan Apochromat lens, and a cooled charge-coupled device camera (FX-1000; Dage-MTI). Video images were converted to a digital format using LabVIEW 7.1 software (National Instruments), and sliding velocity was determined manually by measuring microtubule displacement on tracings calibrated with a micrometer.

Beat frequency measurement
Flagellar beat frequency of live C. reinhardtii cells was determined using the method of Kamiya (2000) by measuring the vibration frequency of cell bodies with a fast Fourier transform analyzer.

Cryo-ET
Freshly isolated C. reinhardtii axonemes were plunged frozen and vitriﬁed onto 200 mesh Quantifoil MicrO TwIsTos by vapor deposition of 72%/28% ethane gas (R2/2; 200 mesh; Quantifoil Micro Tools) as previously described (Heuser et al., 2009). In brief, the grids were glow discharged at ~40 mA for 30 s and coated with 10-nm colloidal gold (Sigma-Aldrich). 3 µl of the axoneme sample and 1 µl of 10 times concentrated 10-nm colloidal gold solution were applied to the grid. Then, excess liquid was blotted away, and the grid was plunged frozen in liquid ethane that was cooled by liquid nitrogen. Grids were stored in liquid nitrogen until observed by EM.

Cryo-ET was performed on a transmission electron microscope (Tecnai F30; FEI) operated at 300 keV. Using the microscope control software SerialEM (Mastronarde, 2005), tomographic tilt series were collected from −65 to 65° with 1.5–2.5° increments using a ~6–8–9 µm focal area to increase the phase contrast. Tilt series were recorded at a nominal magnification of 13,500 using a 2,000 × 2,000-pixel charge-coupled device camera (resolution in a pixel size of 1 nm; Megascan 795; Gatan) and an energy filter (GIF; Gatan) operated in zero-loss mode with 20-eV slit width. The total electron dose used for a tilt series was limited to ~100 e−/Å2. Tomograms showing the 3D structure of C. reinhardtii axonemes were reconstructed using the IMOD software package (Kremer et al., 1996) with gold fiducial marker alignment and weighted back projection. Some of the tomograms (pW7 and pF9/3/ida1) were previously used for the analysis of other axonemal complexes (Heuser et al., 2009; 2012b; Lin et al., 2012b). Subtomogram averaging of the 96-nm axonemal repeat was performed using the Particle Estimation for Electron Tomography software [Nicastro et al., 2006] available from the Boulder Laboratory for 3D Electron Microscopy of Cell (Colorado). The University of California, San Francisco, Chimera package (Petterson et al., 2004) was used for 3D visualization by isosurface rendering and data analysis. Resolutions of 3D structures obtained by cryo-ET were summarized in Table S3.

Computational methods
Protein domains, motifs, and families were searched using SMART (simple modular architecture research tool; European Molecular Biology Labora-