Cryoelectron tomography reveals doublet-specific structures and unique interactions in the I1 dynein

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Cilia and flagella are highly conserved motile and sensory organelles in eukaryotes, and defects in ciliary assembly and motility cause many ciliopathies. The two-headed I1 inner arm dynein is a critical regulator of ciliary and flagellar beating. To understand I1 architecture and function better, we analyzed the 3D structure and composition of the I1 dynein in *Chlamydomonas* axonemes by cryoelectron tomography and subtomogram averaging. Our data revealed several connections from the I1 dynein to neighboring structures that are likely to be important for assembly and/or regulation, including a tether linking one I1 motor domain to the doublet microtubule and doublet-specific differences potentially contributing to the asymmetrical distribution of dynein activity required for ciliary beating. We also imaged three I1 mutants and analyzed their polypeptide composition using 2D gel-based proteomics. Structural and biochemical comparisons revealed the likely location of the regulatory IC138 phosphoprotein and its associated subcomplex. Overall, our studies demonstrate that I1 dynein is connected to multiple structures within the axoneme, and therefore ideally positioned to integrate signals that regulate ciliary motility.

dynein *f* | flagella

Cilia and flagella are highly conserved organelles with roles in cellular movement and signal transduction. In humans, defects in cilia can lead to a number of diseases, such as polycystic kidney disease and primary ciliary dyskinesia (1, 2). The axoneme core of most motile cilia and flagella consists of nine doublet microtubules (DMTs) surrounding two single microtubules (MTs) known as the central pair complex (CPC) (Fig. 1). DMTs are highly periodic with a 96-nm-long unit that repeats along the MT length. They are decorated with two rows of dynein motors, the inner dynein arms (IDAs) and the outer dynein arms (ODAs), which drive MT sliding and axoneme bending (3). Generating the diverse ciliary and flagellar waveforms requires the precise coordination of the activity of thousands of dynein motors within a single organelle (4).

The primary signaling pathway known to regulate dynein function in axonemes involves signals traveling from the CPC through radial spokes (RSs) to the IDAs and ODAs (reviewed in 5, 6). Many CPC and RS mutants are paralyzed (7). However, MT sliding can be restored to WT levels in isolated CPC/RS mutant axonemes using protein kinase inhibitors (8, 9). These observations, together with biochemical evidence of phosphorylation of dynein subunits, have implicated the dyneins as a major signaling target of the CPC/RS signaling pathway (10, 11). However, the regulatory mechanisms and physical interactions that participate in signal transduction to the dynein targets are not well understood.

Isolated axonemes require only ATP for reactivation, suggesting that direct interactions between the dyneins and their regulators are physically built into the axoneme (4). Therefore, studies that visualize axonemal structures and their connections at high resolution can provide new insights into interactions that

might be important for assembly and/or regulation. For example, a recent cryoelectron tomography (cryo-ET) study of *Chlamydomonas* axonemes has shed new light on the function of the nexin-dynein regulatory complex (N-DRC) and further defined its role as a highly connected regulatory hub in the distal part of the axonemal repeat (12).

The I1 dynein (dynein *f*) is the only two-headed IDA in axonemes. Defects in the I1 dynein reduce the swimming velocity of *Chlamydomonas* and paralyze the flagellum of *Trypanosoma brucei* (13, 14). The I1 dynein shares some unusual features with the N-DRC. Like N-DRC mutants, I1 mutants were also isolated as suppressors of paralyzed flagella in CPC/RS mutants (15, 16), suggesting that the I1 dynein is not just a motor but has a regulatory function. Biochemical studies and MT sliding assays have demonstrated that both casein kinase 1 (CK1) and type 2 protein phosphatase regulate dynein activity and, either directly or indirectly, phosphorylate I1 intermediate chain IC138, a subunit of the I1 intermediate chain and light chain complex (ICLC) (9, 17, 18). MT sliding assays showed that the phosphorylation status of IC138 correlates with dynein activity, whereby the phosphorylated IC138 appears to be the inactive form (19, 20). Despite continuing studies, the locations of the proteins involved in I1-dependent dynein regulation, and which components and direct interactions participate in signal transduction, remain unknown.

Classic 2D EM of chemically fixed specimens (11, 13, 21–26) and averaged 3D cryo-ET data (27) have demonstrated that the I1 dynein is located at the proximal end of the axonemal repeat between RS1 and the ODA row. The I1 dynein has a “trilobed” structure composed of the two dynein heavy chains (HCs), 1 α HC and 1 β HC, and the ICLC (Fig. 1). A number of I1 components have been identified biochemically, including three intermediate chains (IC140, IC138, and IC97), several light chains (LC8, LC7a, LC7b, Tctex1, and Tctex2b), and the accessory protein FAP120 (11, 13, 16, 23–25, 28–34). A recent study of an IC138 null mutant (*bop5-2*) revealed that IC138 is required for assembly of an “IC138 subcomplex” consisting of IC138, IC97, LC7b, and FAP120 (21). However, the precise location of this subcomplex within the I1 ICLC is unknown.

To obtain more detailed information about the specific positions and interactions of the I1 subunits, we analyzed the 3D

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Fig. 1. Organization of the *Chlamydomonas* flagellum. (A) Differential interference contrast light microscopy (LM) image shows a *Chlamydomonas* cell with its two flagella. (B–D) The ultrastructure of the axoneme is visible in EM images; slices from a cryotomogram show longitudinal (B), 3D (C), and cross-sectional (D) views. Key features are highlighted, such as the cylindrical arrangement of nine DMTs (red box in D) and the organization of 96-nm repeats (red box in B) along the length of the axoneme. (E and F) Schematic representations of a 96-nm repeat modified from Nicastro et al. (27) are shown in longitudinal front (E) and cross-sectional (F) views observed from proximal (flagellar base) toward distal (flagellar tip). These orientations are maintained in all figures. The IDAs are numbered in E, and the A- and B-tubule (A_t and B_t) protofilament numbers are indicated in F. [E and F from ref. 27. Adapted with permission from AAAS.] (Scale bar: 50 nm, valid for B and D.)

structure and protein abundance in *Chlamydomonas* axonemes from WT and three I1 mutants (*bop5-1*, *bop5-2*, and *pf9-3*) using state-of-the-art cryo-ET complemented by gel-based proteomics. Consistent with previous studies that implicated I1's importance for proper motility of cilia and flagella, our averages reveal the I1 dynein as a highly connected regulatory node on the proximal end of the axonemal repeat. We also discovered unique and previously undescribed features of the I1 complex in WT axonemes, such as doublet-specific differences and a large complex called the tether and tether head, which links the 1α HC motor domain directly to the A-tubule. Through correlation of biochemical and 3D structural data, we localized both IC138 and the IC138 subcomplex within the ICLC. These results provide a high-resolution view of the I1 dynein as well as unique insights into the distribution of CPC/RS signals to axonemal dyneins and the mechanism of I1-dependent regulation of axoneme motility.

Results

Using cryo-ET combined with subtomogram averaging, we analyzed the 3D structure of the I1 complex in five *Chlamydomonas* strains with different degrees of I1 defects. For each strain, well-preserved frozen-hydrated axonemes were reconstructed (Fig. S1) and hundreds of repeat units were averaged to increase the signal-to-noise ratio, and thus the image resolution (more details are provided in Table S1). The pseudo-WT (pWT) strain was generated by transforming the N-DRC mutant *pf2* with a GFP-tagged WT PF2 construct; the presence of the PF2-GFP transgene rescues the motility, as well as biochemical and structural defects in the *pf2* mutant, and yields averages of the axonemal repeat that are basically identical to WT (side-by-side comparison in Fig. S2) (12). Because of higher resolution in the pWT average (Table S1), some structural details are clearer in the pWT than in the WT averages; therefore, both averages were used to determine the boundaries, 3D structure, and connections of the I1 complex. To determine the location of I1 subunits, we also correlated our structural data with the relative abundance of I1 subunits in the same five stains, as established by gel-based proteomics.

Cryo-ET Reveals the 3D Ultrastructure of the I1 Dynein and a Tether of a Dynein Motor Domain to the DMT. The boundaries of the I1 complex (i.e., which densities form the I1 dynein) were determined by comparing the structures present in the WT/pWT

axonemal repeat relative to those missing in the 96-nm repeat from the I1 mutant strain *pf9-3* (Fig. 2, Table 1, and Movies S1 and S2). This I1 mutant is missing all the known I1 components (13, 23, 35); therefore, we defined all structures missing in *pf9-3* as part of the I1 complex. The overall “trilobed” shape of the I1 dynein (22) is confirmed by our results. However, the increased resolution of our 3D averages provides previously undescribed details and identifies unique features of this two-headed dynein complex (Figs. 2–4 and Figs. S2–S6). The ICLC is associated with the tail domains of the I1 dynein HCs and forms the ATP-insensitive binding site to the A-tubule of the DMTs (36). Given I1's large mass of ~ 2.5 MDa, we expected that the ICLC would be robustly attached to the A-tubule. Surprisingly, the I1 ICLC is a sheet-like structure that appears to be suspended at a distance of ~ 5 nm from the A-tubule, with only a single narrow connection (connection 1 in Fig. 4 and Fig. S5) spanning the distance between the ICLC and the A-tubule (Fig. 4, Figs. S3 and S5, and Movie S1). Interestingly, the same protofilament that the ICLC connects to also links to the density of a MT inner protein, MIP1 (27, 37) (Fig. 2 and Figs. S2 and S3). The protein composition and biological function of MIP1 are unknown. The ICLC has three protrusions toward the neighboring DMT, termed the proximal, central, and distal protrusions (Fig. 24), as well as a small hole near the distal protrusion (red arrow in Fig. S5H).

Consistent with previous studies, the 1α and 1β HC motor domains are located at the proximal end of I1 (13, 24, 27, 38) (Fig. 24 and Fig. S24). The two I1 dynein heads are not only connected to the ICLC but to each other (Fig. 24 and Fig. S24 A and B) by interactions similar to those seen between the stacked heads of the ODAs (27, 38). A previously undescribed density, named the tether head in this study (colored orange in Fig. 2), is attached to the proximal side of the 1α HC motor domain. The tether head is connected to the A-tubule through another large, previously undescribed density we call the tether (Fig. 2A and C and Fig. S3). The tether is not considered part of the I1 complex because it is present in *pf9-3* (Fig. 2 B and D).

Doublet-Specific Differences of the I1 ICLC Structure in WT Axonemes.

Previous studies have documented some structures that are present only on subsets of doublets in *Chlamydomonas* axonemes, such as the B-tubule projections (beaks or beak-MIPs) in DMTs 1/5/6, the ODAs missing from DMT 1, and the inner arm (IA) dy-

Fig. 2. Defining the WT structure of I1 dynein. Isosurface renderings show averaged axonemal repeats in longitudinal (A and B) and cross-sectional (C and D) views. The pWT strain (A and C) is indistinguishable from WT (Fig. S2). The *pf9-3* (B and D) mutant lacks the entire I1 dynein. A comparison of both strains is used to define the boundaries of the I1 complex. Both dynein HCs (α and β), the ICLC, and the tether head (TH) are part of the I1 complex because they are present in pWT but missing in *pf9-3*. The tether (T) is present in both strains, and is thus not considered part of the I1 complex. The three protrusions of the ICLC were named proximal (black arrowhead), central (white arrow), and distal (black arrow) protrusions in A. Note the location of MIP1 (white arrowheads) (C and D). Dynein IAX (X) is only present in some doublets and thus appears weaker and variable in density (A and B). The A- and B-tubules (A_t and B_t) are also labeled. (Scale bar: D, 20 nm.)

nein IAX missing from some DMTs (37, 39–43). To investigate if the I1 complex has doublet-specific structures, we generated 3D averages of each individual DMT from two different strains with wild-type phenotypes, pWT and pWT2 (Table S1). The doublet-specific averages of both strains confirmed the same overall 3D structure of the I1 complex on all nine DMTs (Fig. S4). The ICLC central protrusions of DMTs 1, 2, and 5–9 are short; have a mass of ~150 kDa; and end ~5 nm from the neighboring B-tubule (Fig. 3 C and E and Fig. S4). The central protrusions of DMTs 3 and 4, however, have an additional density of ~100 kDa that projects from the central protrusion toward the distal protrusion, forming a ring-shaped structure that extends to within ~3 nm of the neighboring doublet (Fig. 3 D and F and Fig. S4).

Dynein Heads and ICLC of the I1 Complex Are Highly Connected to Neighboring Axonemal Structures. As described above, we used a comparison between WT/pWT and *pf9-3* to establish the

boundaries of the I1 complex. We identified several connections based on continuous electron density between the I1 complex and neighboring axonemal structures. At least seven connections to neighboring structures can be identified, which is a unique feature of the I1 dynein in comparison to all the other axonemal dyneins. The first four connections originate from the ICLC, and the remaining three originate from the 1α HC motor domain. Connection 1 (described above) is the attachment of the ICLC to the A-tubule (Fig. 4 and Fig. S5 B, C, and G). Connection 2 is the proximal outer-inner dynein (OID) linker that connects the ICLC to the row of ODAs (27) (Fig. 4 and Fig. S5 A, D, and H). Connection 3 is the linkage of the distal end of the ICLC to a neighboring, unidentified density that ultimately links to the N-DRC (12) (Fig. 4, and Figs. S3N and S5 A and H). This unidentified density also connects to the doublet-specific dynein IAX (Figs. 2A and 4 A and B). The last ICLC connection 4 is large and links between the ICLC and the dynein head of IA2 (dynein *a/d*) (Fig. 4 and Fig. S5 A and D).

Connections 5–7 are unusual because they originate from the 1α HC motor domain. Connection 5 links the dynein head domains of I1 α and IA2 (Fig. 4 and Fig. S5 A and I), similar to interactions seen between the stacked heads of the ODAs (27, 38). This connection, however, is smaller than connection 4 between IA2 and the ICLC. Connections 6 and 7 both link the 1α dynein head to the newly described tether density, and ultimately to the A-tubule. The tether consists of two filamentous parts: One links to the 1α motor domain at connection 6 (Fig. 4 and Fig. S5 B and E), whereas the second links to the tether head at connection 7 (Fig. 4 and Fig. S5 B and F). Both parts of the tether are interconnected and attach to multiple protofilaments of the A-tubule (Fig. 4 and Fig. S5 B, E, and F).

Table 1. Structural phenotypes of I1 mutants determined by cryo-ET

Component	Strain			
	WT/pWT	<i>bop5-1</i>	<i>bop5-2</i>	<i>pf9-3</i>
α HC	+ (500 kDa)	+	+	–
β HC	+ (500 kDa)	+	+	–
ICLC	+ (1.3 MDa)	± (750 kDa)	± (650 kDa)	–
Tether head	+ (100–150 kDa)	+	+	–
Tether	+ (700 kDa)	+	+	+

Mass estimates were calculated from the electron densities according to Quillin and Matthews (68). +, present; –, absent; ±, reduced.

Having characterized the WT I1 protein components, we turned to analysis of the biochemical defects in the *bop5* mutants. In addition to the previously reported *bop5-1* deficiencies, our 2DE gels and Western blots of *bop5-1* suggest that IC138 and IC97 are significantly reduced compared with WT (Fig. 6). These observations suggest that the C-terminal domain of IC138, which is missing in *bop5-1*, influences the assembly of IC138 and IC97 into the axoneme. In *bop5-2*, the levels of I1 ICs detected by 2DE and Western blot (Fig. 6) largely confirmed previous reports (21): IC97 and IC138 are missing, and IC140 appears slightly reduced. However, trace amounts of FAP120 (previously believed to be absent in the *bop5* mutants) were detected in both *bop5* mutants (Fig. 64). Table 2 and Fig. 7

stereo images

Fig. 4. Connections between the I1 complex and neighboring structures. Isosurface renderings of averaged axonemal repeats from pWT show seven connections (magenta-colored densities and numbers) between the I1 complex and adjacent structures in longitudinal (A–B') and cross-sectional (C–D') views. Note that B and B', as well as D and D', are pairs of stereo images. A summary of connections is indicated in magenta: 1, ICLC attachment to the A-tubule (A_t); 2, proximal outer-inner dynein (OID) linker between the ICLC and ODAs; 3, bridge (magenta arrowhead in A) between the distal part of the I1 complex and the N-DRC; 4, ICLC to the IA2 dynein; 5, I1 α motor domain to IA2; 6, I1 α motor domain to tether; 7, tether head (TH) to tether. Other axonemal structures are also labeled: β, I1 β motor domain; B_t, B-tubule. See also Fig. S5.

summarize the subunit composition of the I1 complex in different strains. These proteomics results agree well with our structural data, increasing confidence in our data interpretation.

Discussion

Dynein Regulatory Network and Major Regulatory Hubs in the Axoneme: I1 Dynein and N-DRC. Cryo-ET allowed us to reconstruct the 3D structure of the I1 dynein in WT and mutant axonemes to the highest resolution currently available. The tomographic averages revealed unique details about the I1 dynein, including its large overall size and shape, its high connectivity to other axonemal structures, and also a surprisingly small attachment of the ICLC to the A-tubule. The large number of connections of the I1 dynein with neighboring structures, as well as its demonstrated role in regulating flagellar motility (reviewed in 45), suggests that the I1 complex is a major regulatory hub in the axoneme and is the proximal counterpart of the distal regulatory node, the N-DRC (12). Both structures form links to the ODAs and other IDAs, and they are also connected to one another through an unidentified density (12) (Fig. 4A and Fig. S3N), which may allow coordination/integration of regulatory signals. The major differences are that the I1 complex is itself a dynein and the I1 complex does not connect directly to a RS.

CPC/RS Signaling and Phosphorylation of IC138. Both I1 and N-DRC mutants have been identified as suppressors of paralyzed CPC/RS mutants, indicating that the I1 complex and the N-DRC regulate dynein activity in response to CPC/RS signaling (15, 16, 46). Although the N-DRC makes a small, direct connection to

RS2 (connection 8 in figures 3 F and G of ref. 12), we found no evidence of a direct connection between RS1 and the I1 complex. The only obvious connections between the I1 ICLC and RSs are indirect, either through the ICLC-to-IA2 connection (connection 4), because IA2's tail is attached to the base of RS1 (47) (Fig. S3J), or through ICLC's distal connection (connection 3) to the N-DRC that links to RS2. Therefore, these connections may be critical for phosphorylation of IC138 and regulation of I1 dynein activity.

Many protein kinases have been implicated in the regulation of I1; however, their locations and targets remain unclear (4, 9, 17, 18, 48, 49). Of these kinases, CK1 is thought to be the most likely to phosphorylate IC138 directly (18, 19). Here, we show that IC138 is located in the center of the ICLC. This is surprising, because previous studies have shown that CK1 is present in all known I1 mutants (18), suggesting it is not part of the ICLC and thus not in a position to directly interact with IC138. Similar to previous RNA interference studies (50–52), future CK1 knock-downs may provide insights into the axonemal location of this kinase.

1α HC Tether. Only a few cases have been reported where proteins are thought to interact directly with the motor domain of a dynein (53–55). Based on biochemical evidence, Patel-King and King (55) have reported that the 22-kDa LC1 likely forms a bridge between the ODA γ HC motor domain and the DMT in axonemes, possibly providing limiting doublet sliding in the axonemal bend. Our study identified a unique structure tethering the I1α HC motor domain to the A-tubule. The identities of the

Fig. 5. Comparisons of I1 structure between WT and IC138 mutants. Three-dimensional structure of the averaged axonemal repeat from WT (A and D) is compared with the averages from *bop5-1* (B and E) and *bop5-2* (C and F). The IC138 subcomplex, which is part of the ICLC, is reduced in *bop5-1* and missing in *bop5-2*. Isosurface renderings show close-ups of the I1 complex in longitudinal (A–C) and cross-sectional (D–F) views. Note that in comparison to WT, the size of the ICLC is smaller in *bop5-1* and even more reduced in *bop5-2*. Other I1 components, such as the α and β HCs and the tether head (TH), and other axonemal structures, including the tether (T), IDA (including IA2), ODA, RS, and A-tubule (A_t), look similar in all three strains. (Scale bars: 10 nm.) See also [Fig. S6](#).

proteins that form the tether and tether head are unknown. Because the tether is present in all strains we have analyzed by cryo-ET thus far, it is not identifiable by comparative proteomics of I1 mutants. The tether head, with a predicted size of 100–150 kDa, appears to be missing in *pf9-3* averages (Fig. 2), but comparison of WT and *pf9-3* 2DE gels did not reveal a polypeptide that could correspond to the tether head. This could be attributable to inherent limitations of the gel-based approach (e.g., protein size). Alternatively, but less likely, the tether head (or parts of it) may remain in *pf9-3* but is not visible in the averages because of increased flexibility in the absence of the I1 dynein.

Although the function of the I1 α tether in the axoneme is unknown, it would be in an ideal position to work as a tension sensor detecting relative movement between the I1 dynein and the A-tubule during dynein stepping or axoneme bending. Thus, tethering may serve as part of a mechanical feedback mechanism for dynein coordination. This is consistent with previous studies of I1 HC mutants reporting distinct roles for the I1 α and I1 β HCs: The I1 β HC seems to be mostly responsible for generating motor force, whereas the I1 α HC is suggested to have a regulatory and/or restraining function (13, 24, 25, 35). Alternatively, the substantial connection between the I1 motor domain and the A-tubule may serve to stabilize the I1 complex.

Table 2. Abundance of I1 complex proteins in WT and mutant axonemes determined by 2DE analysis

	WT	<i>bop5-1</i>	<i>bop5-2</i>	<i>pf9-3</i>
α HC*	+	+	+	—
β HC*	+	+	+	—
IC138	+	$\pm$	—	—
IC97	+	$\pm$	—	—
IC140 [†]	+	+	$\pm$	—
FAP120	+	$\pm$	$\pm$	—
No. of gels	17	8	4	8

+, present; −, absent; ±, reduced.

*Based on results from previous studies (13, 24) (not visualized in our 2DE gels).

[†]Abundance of IC140 was determined by the 2DE analysis shown in this study and from previously published work (21, 25, 31).

Asymmetry Provided by Doublet-Specific Differences of the I1 ICLC Could Be Important for Generating the Effective Stroke. Based on sliding disintegration assays, the activity of dyneins on doublets 2–4 is believed to be crucial for generating interdoublet sliding during the effective stroke in *Chlamydomonas* flagella, whereas the activity of dyneins on doublets 6–8 drives the recovery stroke (56). It has been suggested that the generation and direction of the effective and recovery stroke are regulated by structural components or signals that provide inherent asymmetry to the axonemes (40, 56–59). Our doublet-specific averages identified just such a unique structure of unknown protein composition associated with the ICLC on DMTs 3 and 4. Here, the central ICLC protrusion is part of a larger ring structure that extends closer to the adjacent B-tubule compared with the ICLC seen on all other DMTs (Fig. 3 and Fig. S4). Although we did not observe a connection from the ICLC protrusions on DMTs 3 and 4 to the neighboring B-tubule,

Fig. 6. Assembly of I1 dynein subunits in WT and mutant axonemes. (A) Close-up images of 2DE gels show the changes in the abundance of I1 dynein subunits in different strains (examples of full-sized 2DE gels are shown in Fig. S7). Arrowheads highlight the position(s) of the protein of interest. (B, Upper) One-dimensional Western blot analysis shows that IC138 is present in WT, reduced in *bop5-1*, and completely missing in *bop5-2* and *pf9-3*. (B, Lower) Coomassie-stained gel of the same samples shows the tubulin bands demonstrating equal protein loading for all samples.

we cannot exclude a small linkage beyond our resolution limit. This doublet-specific structure could play a role in generating the asymmetrical distribution of dynein activity required for effective motility, which is supported by recent studies of *bop5* mutants. These studies revealed defects in both MT sliding and the flagellar waveform of *bop5* mutants that are consistent with a role for the IC138 subcomplex in the selective activation or inactivation of dyneins during flagellar bending (21, 60).

Internal Architecture of the I1 Complex and Localization of I1 Subunits. By combining our 3D structural data with both previously published biochemical results and those described in this study (summarized in Fig. 7 A–H) to compare the WT and mutant I1 dyneins, we can propose a likely location of both IC138 and the IC138 subcomplex within the I1 ICLC. Fig. 7 I–K and Movie S5 depict our summary model by showing color-coded regions for proposed subunit locations projected onto the WT structure.

Bop5-2 axonemes lack the entire IC138 subcomplex: IC138, IC97, LC7b, and FAP120 (21) (Fig. 6). *Bop5-1* was previously thought to be missing only the C-terminal 8% of IC138, LC7b, and FAP120 (2–3 copies per I1), that is, roughly a difference of 110–150 kDa compared with WT (31, 32). It was therefore surprising that our *bop5-1* averages suggested a loss of ~500 kDa compared with WT (Fig. 5 A and B). Such a large discrepancy made interpretation of the structures difficult. However, our biochemical studies here revealed that IC138 and IC97 are also significantly reduced in *bop5-1* axonemes (Fig. 6 and Table 2), which is consistent with the structural defect seen in the ICLC of *bop5-1*. Additional possibilities that could explain part of the missing mass include the following: (i) some of the structure is present but blurred out as a result of increased flexibility, and thus appears smaller in our averages; (ii) some of the ICLC subunits are present in multiple copies, resulting in an underestimation of the total missing mass; or (iii) there are additional subunits in the IC138 subcomplex that have not yet been identified.

Most of the structural difference between *bop5-1* and *bop5-2*, which consists of a central region of the ICLC, is likely caused by different amounts of IC138 and IC97 in the two mutants. IC97 is known to bind tubulin (34), and is therefore probably located near connection 1. If IC97 is indeed part of connection 1, this could explain why connection 1 appeared weaker in *bop5-2* axonemes. In previous analyses, IC97 and IC140 were both thought to be potential ICLC-to-MT anchors because IC97 interacts directly with tubulin (34) and purified IC140 cross-links to I1-depleted axonemes (36). Both IC140 and IC97 could form extensions into connection 1; however, IC140 was reported to be present at near-WT levels in all the *bop5* mutants (21, 31, 32, 34,

60). This suggests that IC140 is located in the upper ICLC density present in both *bop5* mutants and that IC97 is likely located more centrally, at or below the ICLC-to-MT connection (connection 1).

In both *bop5* mutants, the ICLC-to-IA2 connection 4 is clearly missing. Meanwhile, the largest protein mass missing in both *bop5* mutants should be FAP120 (estimated mass of ~40 kDa), which is believed to be present in two to three copies per I1 complex (32). Genetic evidence supports an association between FAP120 and the ICLC because it is reduced in I1 mutants (32). However, instead of copurifying with the I1 dynein (dynein f) during FPLC ion exchange chromatography, FAP120 coelutes with dyneins d and e (32). Dynein e is located at the IA4 position next to the N-DRC (12, 61), making an association of FAP120 with dynein e unlikely. The precise position of dynein d is not known, but IA2 and IA6 are possible locations (38, 61). One interesting interpretation of these data is that FAP120 associates with dynein d at the ICLC-to-IA2 connection 4, but additional work is needed to test this hypothesis. FAP120 is not reduced in *ida5*, which is missing dyneins a, c, d, and e (32). However, this is not necessarily inconsistent with the proposed localization for FAP120, because the I1 ICLC could be sufficient for proper FAP120 localization even when FAP120 cannot connect to IA2. The ICLC-to-IA2 connection 4 is one possible pathway for RS signals to reach the I1 dynein, but further study of FAP120 isoforms in different mutant backgrounds will be required to understand FAP120's role in the pathway(s) that regulate I1 activity.

The suspension of the I1 complex above the A-tubule through connections on all sides, like a spider web, does not seem ideal for providing resistance to force production by the two I1 dynein heads. However, its high connectivity and flexibility in combination with the tethering of the 1 α motor domain to the MT suggest important roles in signal transduction, and possibly tension sensing, in a regulatory system that can generate and modify complex waveforms during motility.

Our results provide a unique, high-resolution view into the 3D structure and subunit organization of the I1 dynein, revealing probable pathways for regulatory signals. Overall, we demonstrate that I1 dynein is a highly connected structure within the axoneme, ideally positioned to integrate signals that regulate axonemal dynein activity. A deeper insight into the structure and protein composition of the I1 dynein is an important step toward understanding how axonemal movement is regulated and how it fails in disease.

Materials and Methods

Axonemal Sample Preparation. *Chlamydomonas reinhardtii* strains used in this study are listed in Table S1. Axonemes were isolated and purified from WT and mutant *C. reinhardtii* cells as previously described (12, 21) with minimal

Sigma-Aldrich). Cell bodies were collected at $1,800 \times g$ at 4°C for 10 min, and flagella were purified by two additional centrifugation steps over 20% (wt/wt) sucrose cushions. Purified flagella were demembranated with 0.1% Nonidet-P-40 or 1% (vol/vol) IGEPAL CA-630 (Sigma-Aldrich) for 30 min at 4°C , and axonemes were collected by centrifugation at $35,000 \times g$ for 1 h. The isolation procedure was performed in the absence of ATP. Axonemes for cryo-ET were prepared by resuspension in 10 mM Hepes (pH 7.4), 25 mM NaCl, 4 mM MgSO_4 , 1 mM EGTA, and 0.1 mM EDTA and processed within 24 h.

For 1D and 2D gel analysis, proteins were dissolved directly from axoneme pellets with 2DE lysis buffer [7 M urea, 2 M thiourea, 4% (wt/wt) CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate], 65 mM DTT, and 2% (vol/vol) IPG (immobilized pH gradient) buffer (pH 3–10NL; GE Healthcare)] with vigorous stirring for 30 min. Cell debris and insoluble substances were removed by centrifugation at $45,000 \times g$ for 1 h. Protein concentrations were determined using a 2-D Quant Kit (GE Healthcare). The supernatant was aliquoted and stored at -70°C until further use.

Proteomic Analysis. Protein samples (70 μg for analytical gels or 300 μg for semipreparative gels) were separated with 13-cm, immobilized, pH 3–10 nonlinear gradient dry strips (GE Healthcare) for the first dimension and 10% SDS/PAGE gels for the second dimension. All samples (2–7 axoneme preparations for each strain) were run at least in duplicate to confirm reproducibility. Analytical gels were stained with silver nitrate, and semipreparative gels were stained with colloidal Coomassie blue. The 2DE images were analyzed using ImageMaster 2D Platinum software (version 5.0; GE Healthcare). Two-sample *t* tests were used to analyze differences in protein abundance between WT and mutant samples. *P* values of less than 0.05 were considered statistically significant. Spots that showed significant changes in abundance between WT and I1 mutants (greater than ± 1.5 -fold) were identified as affected I1 dynein components.

Spots of interest were excised from the semipreparative gels, trypsin-digested as described by Lin et al. (63), and analyzed using a Bruker Daltonics Microflex mass spectrometer. Peptide mass fingerprinting was interpreted by the Mascot sequence database search engine configured with a mass tolerance of 50 ppm and the variable modifications “carbamidomethylation of cysteine and oxidation of methionine” against the National Center for Biotechnology Information database. Probability-based Mascot scores were used to evaluate MS results: peptide mixtures that yielded statistically significant search scores ($>95\%$ confidence interval, which is equivalent to Mascot expected values <0.05) and accounted for the majority of ions present in the mass spectra were defined as positive identifications.

For 1D immunoblotting of IC138, a total of 15 μg of axonemal protein for each cell line was separated on 10% SDS/PAGE minigels under standard denaturing conditions. A polyclonal antibody against IC138 was used in a 1:10,000 dilution (31). Signals were visualized using the ECL detection system (Pierce).

Preparation of Cryosamples and EM. Cryosamples were prepared, examined by EM, and processed as previously described (12, 42). Briefly, Quantifoil holey carbon grids (Quantifoil Micro Tools GmbH) were glow-discharged and coated with 10 nm of colloidal gold (Sigma-Aldrich). Grids were loaded in a homemade plunge freezer; 3 μL of axoneme sample and 1 μL of 10-fold concentrated 10-nm colloidal gold were added to the grid, blotted with a filter paper from the front side, and immediately plunge-frozen in liquid ethane. Grids were stored in liquid nitrogen until examined by EM.

Cryosamples were transferred into a transmission electron microscope (Tecnai F30; FEI) with a cryoholder (Gatan, Inc.). Tilt series were recorded by rotating the specimen from -65° to $+65^\circ$ with 1.5° to 2.5° angular increments. The accumulative electron dose was limited to $\sim 100 \text{ e}/\text{\AA}^2$. Using the microscope control software SerialEM (64), samples were imaged at 300 keV, with $-6 \mu\text{m}$ or $-8 \mu\text{m}$ defocus, under low-dose conditions and in zero loss mode of the energy filter (Gatan, Inc.) (slit width of 20 eV). All images were digitally recorded with a $2,000 \times 2,000$ pixel CCD camera (Gatan, Inc.) at a magnification of 13,500 $\times$, resulting in a pixel size of $\sim 1 \text{ nm}$.

Image Processing. Cryoelectron tomograms were reconstructed using fiducial alignment and the IMOD software package (65). Only tomograms of intact and noncompressed axonemes were used for further data analysis (Fig. S1). The software PEET (Particle Estimation for Electron Tomography) (27) was used for subtomogram averaging of the 96-nm repeats. The extracted subvolumes were aligned and averaged with missing wedge compensation to increase the signal-to-noise ratio, and thus the resolution (27).

Two different alignment strategies were applied and compared during subtomogram averaging: The “global alignment” approach used the entire 96-nm repeat unit (volume size: $100 \text{ nm} \times 100 \text{ nm} \times 80 \text{ nm}$) for 3D alignment, whereas the “local alignment” approach used only the region surrounding the I1 complex for alignment (volume size: $60 \text{ nm} \times 36 \text{ nm} \times 40 \text{ nm}$). Both strategies resulted in comparable but slightly different averages. The strongest differences between alignment strategies were seen in *bop5-1* (Fig. S6E and Movie S3) and *bop5-2* (Fig. S6F and Movie S4). The local alignment yielded the best I1 averages for both *bop5* strains and pWT, whereas the global alignment was slightly better for WT. Because of the absence of the entire I1 complex in the *pf9-3* mutant, only global alignment was used for this strain. The I1 structures described in this study refer to the best averages obtained for each strain (i.e., global alignment for *pf9-3* and WT, local alignment for all other strains).

The number of tomograms and repeat units included in the final averages, as well as the estimated resolution, is summarized in Table S1. Resolution was measured at the base of RS1 in close proximity to the I1 complex. This density, which is present in all strains, permits a direct comparison among all averages. The resolution was estimated using the Fourier shell correlation method with a criterion of 0.5 (66). For 3D visualization by isosurface rendering of axonemal averages, analysis, animation, and volume measurements, we used the University of California, San Francisco Chimera software package (67). For mass estimations, an average protein density of $1.43 \text{ g}/\text{cm}^3$ was assumed (68). The 3D structure of the WT I1 dynein from this publication has been submitted to the Electron Microscopy Data Bank (EMDB; <http://www.emdatabank.org>) and assigned the EMD accession code EMD-5330.

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