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The Ciliary Inner Dynein Arm, I1 dynein, is assembled in the Cytoplasm and Transported by IFT before Axonemal Docking

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Abstract

To determine mechanisms of assembly of ciliary dyneins, we focused on the *Chlamydomonas* inner dynein arm, I1 dynein, also known as dynein f. I1 dynein assembles in the cytoplasm as a 20S complex similar to the 20S I1 dynein complex isolated from the axoneme. The intermediate chain subunit, IC140 (*IDA7*), and heavy chains (*IDA1*, *IDA2*) are required for 20S I1 dynein preassembly in the cytoplasm. Unlike I1 dynein derived from the axoneme, the cytoplasmic 20S I1 complex will not rebind I1-deficient axonemes *in vitro*. To test the hypothesis that I1 dynein is transported to the distal tip of the cilia for assembly in the axoneme, we performed cytoplasmic complementation in dikaryons formed between wild-type and I1 dynein mutant cells. Rescue of I1 dynein assembly in mutant cilia occurred first at the distal tip and then proceeded toward the proximal axoneme. Notably, in contrast to other combinations, I1 dynein assembly was significantly delayed in dikaryons formed between *ida7* and *ida3*. Furthermore, rescue of I1 dynein assembly required new protein synthesis in the *ida7 x ida3* dikaryons. Based on additional observations, we postulate that *IDA3* is required for 20S I1 dynein transport. Cytoplasmic complementation in dikaryons using the conditional kinesin-2 mutant, *fla10-1* revealed that transport of I1 dynein is dependent on kinesin-2 activity. Thus, I1 dynein complex assembly depends upon IFT for transport to the ciliary distal tip prior to docking in the axoneme.

Keywords

Cilia; flagella; axonemes; *Chlamydomonas* dikaryon zygotes; I1 dynein

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Introduction

Motile cilia/flagella play critical roles in vertebrate development and normal function of organs in the adult (Drummond 2012; Koefoed et al. 2014; Satir and Christensen 2007; Wallingford 2012). Ciliary motility is driven by several different axonemal dynein motors, which are organized into the outer dynein arms (ODAs) and inner dynein arms (IDAs) (Gokhale et al. 2009; King and Kamiya 2009; Porter and Sale 2000) in most organisms. The axonemal dyneins are large multi-subunit complexes, and mutations in genes that encode dynein subunits, or components required for the assembly, transport or docking of the axonemal dyneins, result in a wide range of developmental defects and diseases in the adult (Horani et al. 2014; Zariwala et al. 2007). For example, defective assembly of the dyneins can result in primary cilia dyskinesia (PCD) due to a failure in normal ciliary motility (Knowles et al. 2013b; Zariwala et al. 2011). However, we are just beginning to understand the molecular mechanisms by which dyneins, and other large axonemal complexes, are assembled and targeted in the cilium.

The best-studied axonemal dynein is the ODA, a large complex of at least 16 subunits in *Chlamydomonas* axonemes including three heavy chains (Hom et al. 2011; King and Kamiya 2009). The ODA complex assembles in a step-wise manner beginning with preassembly in the cytoplasm ("preassembly" is defined as cytoplasmic assembly of complexes destined for the axoneme) (Ahmed and Mitchell 2005; Fowkes and Mitchell 1998) followed by transport to and within the ciliary compartment by the intraflagellar transport (IFT) machinery (Ahmed et al. 2008; Hou et al. 2007). The preassembly of the ODA also requires a number of additional, conserved proteins, which when defective result in a failure in ODA assembly in the axoneme and in many cases PCD (Austin-Tse et al. 2013; Horani et al. 2013; Horani et al. 2012; Hornef et al. 2006; Knowles et al. 2013a; Knowles et al. 2013b; Kobayashi and Takeda 2012; Loges et al. 2009; Loges et al. 2008; Mitchison et al. 2012; Moore et al. 2013; Omran et al. 2008; Onoufriadis et al. 2014; Panizzi et al. 2012; Zariwala et al. 2006). For transport, the *ODA16* gene is thought to encode an adapter required for interaction between the preassembled ODA and IFT, also mediated by IFT46 (Ahmed et al. 2008; Hou et al. 2007). Moreover, assembly of the ODA in the axoneme requires additional proteins for docking on the outer doublet microtubule (Casey et al. 2003; Dean and Mitchell 2013; Ide et al. 2013; Onoufriadis et al. 2013; Owa et al. 2014; Panizzi et al. 2012; Wirschell et al. 2004). However, despite these advances, little is known about the role that non-dynein cytoplasmic factors play in the preassembly of the ODA and we do not understand the mechanism of loading and unloading of the ODA from IFT.

Compared to the ODAs, which are relatively homogeneous in structure and composition, repeating every 24 nm, the IDAs are heterogeneous, organized into seven dynein structures (dyneins a, b, c, d, e, f/I1 and g), each distinct in composition and each targeted to a unique position in the axonemal 96 nm repeat (Bui et al. 2012; Goodenough and Heuser 1985; King and Kamiya 2009). Based on phenotypic analysis of *Chlamydomonas* mutants, the IDAs appear to each contribute to different parameters and regulation of axonemal motility (Brokaw and Kamiya 1987; Kamiya 1991; Kamiya et al. 1991; Kubo et al. 2012; Porter et al. 1992; VanderWaal et al. 2011; Yagi et al. 2005). The mutants also reveal that failure in assembly of one IDA type results in a gap in inner arm structure that repeats every 96 nm

(Bui et al. 2012; Heuser et al. 2012; Piperno et al. 1990; Porter et al. 1992). This result indicated that each IDA is targeted to a unique position on the outer doublet microtubule and assembles independent of the other IDAs. Consistent with this interpretation, *in vitro* reconstitution of IDAs with axonemes lacking a specific subset of inner arm structures also demonstrated that each IDA type is targeted to a unique position in the axonemal 96 nm repeat (Smith and Sale 1992; Yamamoto et al. 2006). Recent evidence also indicates that preassembly of IDAs requires additional non-dynein cytoplasmic proteins (Tarkar et al. 2013; Yamamoto et al. 2010) and the IFT complex B protein TTC26/DYF13 (Ishikawa et al. 2014). However, in contrast to the ODA, little is known about the assembly, transport and targeted docking of the IDAs.

To address questions of assembly of the IDAs, we focused on the I1 dynein, also known as dynein f (Kagami and Kamiya 1992; Piperno et al. 1990; Wirschell et al. 2007). I1 dynein is a complex located at the proximal most position in the 96 nm repeat, and unlike the other IDAs, which are single headed-dyneins each containing a single heavy chain, I1 dynein is a two headed dynein containing two distinct heavy chains, 1 α and 1 β (Myster et al. 1997; Myster et al. 1999; Perrone et al. 2000) that are distinct in function (Kotani et al. 2007; Toba et al. 2011). In addition to the 1 α and 1 β heavy chains, I1 dynein is composed of three intermediate chains IC140, IC138 and IC97 (Bower et al. 2009; Hendrickson et al. 2004; Perrone et al. 1998; Porter et al. 1992; VanderWaal et al. 2011; Wirschell et al. 2009; Yang and Sale 1998), five different light chains, TcTex1, TcTex 2b, LC7a, LC7b and LC8 (Bowman et al. 1999; DiBella et al. 2001; DiBella et al. 2004a; DiBella et al. 2004b; Harrison et al. 1998; Wirschell et al. 2009) and FAP120 (Ikeda et al. 2009). When isolated from the axoneme, I1 dynein sediments as a 20S complex (Piperno et al. 1990). While the molecular composition of I1 dynein is known, several questions remain unanswered. Where is the 20S I1 complex assembled? Is I1 dynein transported by IFT for assembly in the axoneme? How is I1 dynein localized and docked on the outer doublet microtubule?

In this study, we take advantage of *Chlamydomonas* wild-type and select mutant cells defective in I1 dynein assembly and test the hypotheses that the I1 complex preassembles in the cytoplasm and is then transported by IFT for docking in the axoneme (Fig. 1). We show that I1 dynein preassembles as a 20S complex in the cytoplasm, that preassembly of the 20S complex requires the heavy chains and IC140 and that the 20S complex is transported to the distal tip of the cilium for docking in the axoneme. We further show that transport requires IFT and predict that the I1 dynein – IFT interaction also requires the gene product encoded by *IDA3*.

Results

I1 dynein preassembles as a 20S complex in the cytoplasm

Axonemal I1 dynein is a multisubunit complex that, when fully assembled, sediments at 20S (Piperno et al. 1990; Porter et al. 1992; Smith and Sale 1991). To test the hypothesis that I1 dynein preassembles as a 20S complex in the cytoplasm prior to transport to the cilia and axoneme, we fractionated cytoplasmic extracts from wild-type cells by velocity sedimentation on sucrose gradients (Fig. 2A). Similar to the axonemal I1 dynein, cytoplasmic I1 dynein sediments at 20S, suggesting the complex is fully assembled in the

cell body (Fig. 2). Axonemal and cytoplasmic fractions were probed with antibodies to various I1 subunits to confirm assembly. The 20S complex from the axoneme and cytoplasm contains both heavy chains (HC1 α and HC1 β) (data not shown) and three intermediate chains (IC138, IC140 and IC97) (Fig. 2A). Immunoblots of the cytoplasmic 20S I1 dynein also revealed the presence of the light chains TcTex1, TcTex 2b, LC7a, LC7b and LC8 in the complex (data not shown).

Given that cytoplasmic extracts contain a 12S radial spoke precursor complex and an intact 20S radial spoke that has been shown to be recycled from the ciliary axoneme (Diener et al. 2011; Qin et al. 2004), it is possible that the 20S I1 complex observed in the cytoplasm is also a recycled axonemal product. To test this idea, cytoplasmic extracts from the aflagellate mutant *bld1* (Brazelton et al. 2001), which lacks cilia and recycled axonemal components, were fractionated and examined for the presence of the 20S I1 complex. The sedimentation profile from *bld1* extracts shows that the 20S I1 complex forms as in wild-type extracts (Fig. 2B) indicating that the cytoplasmic 20S I1 complex is formed in the cytoplasm before transport to the cilium.

The assembly of I1 dynein in the axoneme is dependent on the heavy chains (Myster et al. 1997; Myster et al. 1999; Perrone et al. 2000) and the intermediate chain IC140 (Perrone et al. 1998). We hypothesized that HC1 α , HC1 β and IC140 are also required for the cytoplasmic preassembly of the 20S I1 dynein complex. To test this, we examined I1 dynein assembly in cytoplasmic extract fractions from *pf9-3/ida1* (HC1 α -null), *ida2-6* (HC1 β -null) and *ida7* (IC140-null) (Myster et al. 1997; Myster et al. 1999; Perrone et al. 2000; Perrone et al. 1998). As predicted, the 20S I1 dynein complex failed to assemble in cytoplasmic extracts from *ida1*, *ida2* (data not shown) and *ida7* (lower panel, Fig. 2B). Therefore, both the heavy chains and IC140 are required for 20S complex preassembly (Fig. 2B).

The cytoplasmic 20S I1 dynein complex preassembles in *ida3* but is defective in entry to the ciliary compartment

In the *ida3* mutant, I1 dynein fails to assemble in the axoneme (Kamiya et al. 1991). The *IDA3* gene is not yet identified, however the *ida3* mutant has proven to be informative. We reasoned that failure in assembly in the axoneme is a consequence of defective 20S I1 complex assembly in the cytoplasm. To test this, we compared wild-type and *ida3* cytoplasmic fractions for the presence of the 20S I1 complex. Immunoblot analysis indicated that I1 dynein from *ida3* cytoplasmic extracts forms a 20S complex that co-sediments with the I1 complex from wild-type extracts (Fig. 2B). Moreover, the 20S I1 complex in the *ida3* cytoplasm has the same heavy and intermediate chain composition as in wild type (data not shown). Thus, the *ida3* mutant is not defective in preassembly of the 20S I1 complex.

We next tested the hypothesis that *ida3* is defective in I1 docking in the axoneme. We applied the *in vitro* reconstitution assay, which takes advantage of I1 dynein's ability to rebind to I1 depleted axonemes at precise locations along the axoneme *in vitro* (Smith and Sale 1992). Varying amounts of I1 dynein isolated from *oda2* axonemes (Fig. 3, HSE) or cytoplasm (Fig. 3, cyt) were combined with a fixed amount of *ida3* axonemes and *ida7* control axonemes in the presence of ATP (Toba et al. 2011; Yamamoto et al. 2006). Immunoblot analysis with the I1 intermediate chain IC140 indicated that I1 dynein from

oda2 axonemal extracts (Fig. 3, HSE) is capable of rebinding equally to *ida3* and *ida7* mutant axonemes (Fig. 3, P). In both cases, I1 bound the axonemes to saturation, indicating that I1 is binding to specific sites along the axoneme. This result demonstrated that *ida3* axonemes are able to support I1 docking in the axoneme, and IDA3 is not likely an axonemal docking component.

We then used *in vitro* reconstitution to test the hypothesis that 20S cytoplasmic I1 dynein assembles in a form competent to rebind axoneme. Cytoplasmic extracts from *oda2* (containing wild-type 20S I1 dynein) were combined with a fixed amount of *ida3* or *ida7* axonemes in the presence of ATP. In contrast to isolated axonemal I1 dynein (Fig. 3A), cytoplasmic I1 dynein, from either *oda2* or *ida3* cytoplasm, failed to reconstitute with either *ida3* or *ida7* axonemes (Fig. 3B). The same results were obtained using the antibody to IC138 to assess *in vitro* reconstitution. Thus, the preassembled 20S I1 dynein, despite assembling as a 20S complex, is not competent to bind the axoneme.

Since *ida3* is neither deficient in the preassembly of 20S I1 dynein (Fig. 2B) nor in *in vitro* docking of axonemal I1 dynein (Fig. 3A), we tested whether I1 dynein is present in the ciliary compartment in *ida3*. We first confirmed that the preassembled 20S I1 complexes were present at a similar concentration in *ida3* and wild-type extracts (data not shown). We examined the membrane plus matrix (M+M) fractions, the ciliary fraction in which IFT complexes and their cargoes are typically present (Cole et al. 1998; Piperno and Mead 1997; Qin et al. 2004). Detergent (NP-40) generated wild-type and *ida3* M+M fractions were loaded at five times the relative volume of the cilia and axoneme fractions, to detect I1 dynein in the M+M. The fractions were then analyzed by immunoblots for the presence of I1 dynein (Fig. 4). As expected, I1 dynein was significantly reduced in *ida3* cilia and axonemes compared to wild type. In addition, I1 dynein is present in the wild-type M+M fraction (Fig. 4, top left panels). In contrast, at the same protein concentration, I1 dynein is missing in M+M fraction from *ida3* (Fig. 4, top right panels). The same results were obtained using a freeze-thaw method to generate the M+M fraction (data not shown) (Behal and Cole 2013). Thus, compared to wild-type, the 20S I1 complex does not appear to enter the ciliary compartment in *ida3* mutant cells.

I1 dynein is transported to the distal tip of the cilium prior to docking onto the axoneme

Since IFT typically transports cargo to the distal tip of the cilium prior to docking onto the axoneme (Wren et al. 2013), we performed dikaryon rescue experiments to determine the pattern of I1 dynein assembly (Bower et al. 2013; Johnson and Rosenbaum 1992; Piperno et al. 1996); reviewed in (Dutcher 2014). Wild-type and I1-deficient (*ida1*, *ida3* and *ida7*) cells were mated to allow for cytoplasmic complementation in temporary dikaryons. Resulting dikaryons were fixed at 30, 60, and 90 minutes for immunofluorescence with an antibody to IC140 as a marker for I1 dynein (Fig. 5A). By 30 minutes, I1 dynein assembly began at the distal tip of the *ida7* axoneme and proceeded toward the proximal end (Fig. 5A, yellow arrows). I1 dynein assembled along the entire length of the axoneme in less than 90 minutes (Fig. 5A, bottom panels). We estimated the rate of I1 dynein assembly along the axoneme from tip to base by generating fluorescence intensity profiles of individual cilia undergoing I1 dynein rescued-assembly at each time point (Fig. 5B). The length of I1 dynein assembly

along the axoneme was measured from the distal tip to a point defined when the normalized fluorescence intensity dropped below 0.2 AU (Fig. 5B, dashed lines; see (Alford et al. 2013). Quantification of I1 staining from the tip revealed that assembly occurred progressively starting at the distal tip and proceeding to proximal end of the axoneme (Fig. 5C, blue line). Therefore, I1 dynein is transported to the tip of the cilium before incorporation into the axoneme.

We also tested whether cytoplasmic complementation could rescue the assembly of I1 dynein in *ida3* axonemes. Dikaryons between wild-type and *ida3* illustrated rescue in the same tip to base assembly pattern compared to wild-type x *ida7* dikaryons (Fig. 5C, compare black circle and triangle line). Similar to wild-type x *ida7* and wild-type x *ida3* dikaryons, rescue of axonemal I1 dynein assembly was seen in *ida3* x *ida1* dikaryons (Fig. 5C, black diamond line). In addition, in *ida3* x *ida1* and *ida3* x *ida7* dikaryons, the rescue of I1 dynein assembly was synchronous in all four cilia. However, in contrast to other combinations, the rate of I1 axonemal assembly is delayed in *ida3* x *ida7* dikaryons relative to other dikaryon combinations examined (Fig. 5C, red line).

We hypothesized that the delay in rescued assembly of I1 dynein in the *ida7* x *ida3* dikaryons was due to the requirement of new protein synthesis. To test this, we repeated dikaryon rescue in the presence of the protein synthesis inhibitor cycloheximide (Fig. 6). In wild-type x *ida3*, wild-type x *ida7*, and *ida3* x *ida1* dikaryons, rescued axonemal I1 dynein assembly occurred at the same rate and in the same tip to base assembly pattern irrespective of the presence (Fig. 6A, lower panel and Fig. 6B) or absence (Fig. 6A, upper panel, Fig. 6B) of cycloheximide. In contrast to other dikaryon combinations tested in the presence of cycloheximide, rescue of I1 dynein assembly was blocked in *ida3* x *ida7* dikaryons (Fig. 6A, yellow circles; 6B, red line and boxes). Thus, the delay in rescue of I1 dynein assembly is due to a requirement for new protein synthesis in the *ida3* x *ida7* dikaryon. One interpretation of these results is that IC140 and IDA3 form a complex that requires the presence of one or both proteins for complex formation and stability (see Discussion).

Transport of I1 dynein requires the IFT anterograde motor, kinesin-2

Based on our observations that I1 dynein is transported to the distal tip before docking to the axoneme in dikaryons, and recent evidence showing axonemal components such as the DRC are transported by IFT (Wren et al. 2013), we proposed that I1 transport to the distal tip is mediated by IFT. To test this idea, we took advantage of the conditional mutant, *fla10-1*, a temperature-sensitive allele of the KHP1 locus encoding the heterotrimeric kinesin-2 motor required for anterograde IFT (Kozminski et al. 1995; Walther et al. 1994). An *ida3*; *fla10-1* double mutant was generated to perform cytoplasmic complementation in dikaryons. The design of the experiment and potential outcomes are illustrated in Fig. 7A (Here we use a semicolon to separate each unlinked mutation in the double mutants - [www.cell.com/trends/genetics/pdf/S0168-9525\(98\)80010-3.pdf](http://www.cell.com/trends/genetics/pdf/S0168-9525(98)80010-3.pdf)).

We analyzed *fla10-1* x *ida3*; *fla10-1* dikaryons at permissive (21°C) and restrictive (32°C) temperatures for rescue of axonemal I1 dynein assembly from the ciliary tip. Dikaryons were fixed at two time-points post-mixing for immunofluorescence with the IC140 antibody. As described before (Pan and Snell 2002; Piperno et al. 1996), the number of zygotes

formed at restrictive temperature (32°C) was reduced as compared to permissive temperature (21°C). However, sufficient numbers of *fla10-1* x *ida3*; *fla10-1* dikaryons were formed for analysis. At permissive temperature (21°C), I1 assembly was observed at the distal tip of *ida3*; *fla10-1* mutant cilia in the dikaryon (Fig. 7B, arrows) and progressed towards the proximal base end of the axoneme (Fig. 7C, blue line). Complete rescue of I1 assembly along the length of *ida3*; *fla10-1* cilia was seen after 90 minutes (data not shown). In contrast, at restrictive temperature (32°C) rescue of I1 assembly did not occur in the *ida3*; *fla10-1* mutant cilia (Fig. 7B, dashed circles; Fig. 7B, red line). This result provides strong evidence that I1 dynein transport to the distal tip of the cilium requires functional kinesin-2 and IFT.

To control for secondary effects of temperature variation on dikaryon rescue, *fla10-1* x *ida3* and wild-type x *ida3* dikaryons were also analyzed by immunofluorescence for rescued I1 assembly at permissive and restrictive temperatures. As expected, we observed rescue of I1 dynein in the dikaryons formed between *fla10-1* x *ida3* and WT x *ida3*, at both temperatures. Rescue of I1 assembly occurred at comparable rates from tip to base, indicating that increase in temperature had no effect on the function of the wild type proteins, FLA10 and IDA3 (Fig. 7C). As an additional essential control, *ida3* x *ida3*; *fla10-1* dikaryons (lacking IDA3) were analyzed since rescue is not expected at either temperature. Consistent with this prediction, *ida3* x *ida3*; *fla10-1* dikaryons do not rescue I1 assembly in any of the four ciliary axonemes at either temperature (Fig. 7C, black dashed lines). Together, these results indicate that that kinesin-2/IFT is required for I1 transport to the distal tip of the axonemes.

Discussion

Our goal was to define the mechanisms of assembly of ciliary I1 dynein. The primary conclusions from the data presented here include: the 20S I1 complex is preassembled in the cytoplasm; assembly of the cytoplasmic 20S I1 complex requires the heavy chains and intermediate chain IC140 and is not a recycled product of the axoneme; the 20S I1 is transported by IFT to the distal tip of the cilium before docking in the axoneme. In addition, as indicated in the model (Fig. 1), IFT and IDA3 are required for transport of I1 dynein. Other evidence also indicates transport of I1 dynein is facilitated by the TTC26/DYF13/IFT56 subunit of the IFT B complex (Ishikawa et al. 2014), which may be functionally analogous to the IFT46 subunit required for ODA transport (Hou et al. 2007).

One of the surprising observations in this study includes the failure of the 20S I1 from the cytoplasm to reconstitute I1-deficient axonemes in the presence of ATP (Fig. 3B). This result is in contrast to reconstitution using the 20S I1 dynein derived from the axoneme: I1 dynein derived from the axoneme specifically restores I1 dynein to I1 dynein-depleted axonemes (Fig. 3A and (Smith and Sale 1992; Yamamoto et al. 2006). One possibility is that the cytoplasmic 20S I1 complex lacks one of the light chains required for docking in the axoneme. While the light chains have been detected in the cytoplasmic 20S complex, we cannot definitively assign these light chains to the I1 dynein complex: the same light chains are shared with the outer dynein arms, radial spokes and the cytoplasmic dynein (King and Kamiya 2009). Thus, refined purification of the cytoplasmic I1 dynein (e.g. (Toba et al.

2011) is required to test this idea and to definitively determine the light chain composition of the cytoplasmic complex.

It is equally possible that the I1 complex must be modified before docking onto the axoneme. For example, before docking on the axoneme, the radial spokes undergo additional assembly and modification at the ciliary distal tip involving protein assembly and altered phosphorylation (Diener et al. 2011; Gupta et al. 2012; Yang et al. 2005). Similarly, I1 dynein may require further modification at the distal tip prior to docking to the axoneme. Future studies will be directed towards understanding how these large preassembled complexes acquire microtubule binding competency in the axoneme.

The *ida3* mutant is defective in entry and/or transport of the I1 dynein complex

The *ida3* mutant was isolated in a screen to identify mutations in assembly or function of the inner dynein arms (Kamiya et al. 1991). We confirmed that the *ida3* mutant is defective in I1 dynein assembly in the axoneme (Fig. 4) and that the *ida3* mutation maps to chromosome 3 (our unpublished data). While we have not yet identified the *IDA3* gene, the *ida3* mutant has proven to be very informative with regard to I1 dynein assembly. A key observation is that the 20S I1 complex preassembles in the *ida3* cytoplasm as in wild-type (Fig. 2B). The simplest interpretation of this result is that *IDA3* is not a factor required for I1 dynein preassembly. Another possibility is that *IDA3* encodes an axonemal protein required for docking I1 dynein, analogous to ODA-DC proteins (Casey et al. 2003; Dean and Mitchell 2013; Ide et al. 2013; Owa et al. 2014; Takada et al. 2002; Wirschell et al. 2004). However, our *in vitro* reconstitution analysis does not support this model and indicates that the *ida3* axoneme is not defective in I1 dynein docking.

Based on our observation that I1 dynein preassembles in the *ida3* mutant cytoplasm but not in the axoneme, the simplest model is that *IDA3* is required for transport of I1 dynein into the ciliary compartment (Fig. 1). Consistent with this model, the I1 dynein complex is preassembled in the cytoplasmic compartment (Fig. 2B), but I1 dynein does not appear in the M+M fraction of *ida3* cilia (Fig. 4). As illustrated in Figure 1, *IDA3* may be an adapter that selectively links I1 dynein to IFT for entry and transport within the ciliary compartment. The adapter model is founded on studies of ODA16 as an adapter that mediates interaction between the ODA and IFT46 (Ahmed et al. 2008; Hou et al. 2007). Additionally, new evidence indicates IFT, and associated axonemal cargoes (such as the radial spokes), are carried with membrane vesicles destined to the cilium (Wood and Rosenbaum 2014). Furthermore, based on studies of N-DRC (nexin-dynein regulatory complex) transport (Wren et al. 2013), IFT is required for both entry into and transport within the ciliary compartment. An alternative to the adapter model is that the *IDA3* protein is a modifier, such as a protein kinase, that is required for selective control of interaction between the I1 complex and IFT and entry into the cilium. Definitive tests of these ideas require the identification of *IDA3* and characterization of the *IDA3* protein.

I1 dynein is transported to the distal tip by IFT

Cilia assemble by addition of tubulin and protein complexes at the distal end of the growing axoneme (Johnson and Rosenbaum 1992; Witman 1975). Although not necessarily identical

to de novo ciliary assembly, analysis of rescued assembly of I1 dynein in the cilia of *Chlamydomonas* quadraflagellate dikaryons has been a particularly powerful approach demonstrating many proteins are transported to the distal tip of the cilium for incorporation in the axoneme (Bower et al. 2013; Johnson and Rosenbaum 1992; Piperno et al. 1996; Wren et al. 2013); reviewed in (Dutcher 2014). Consistently, analysis of dikaryons, generated using at least one I1 dynein assembly mutant such as *ida1*, *ida3* or *ida7*, revealed that the I1 dynein complex is transported to the ciliary distal tip before incorporation in the axoneme (Fig. 5). Furthermore, quantification of assembly as a function of time demonstrated that assembly begins at the distal axoneme and then proceeds progressively toward the proximal axoneme until rescue is complete (Fig. 5C). With the exception of one dikaryon combination, *ida3* x *ida7*, which displayed a delayed rescue of I1 dynein assembly, the relative rate of tip to base rescue was uniform across several different dikaryon combinations (Fig. 5C, wild-type x *ida3*; wild-type x *ida7*; *ida1* x *ida3*). Furthermore, in our assay the rate of rescue of I1 dynein was synchronous for both cilia in dikaryons made from complementary I1 dynein assembly mutants (e.g. *ida1* x *ida3*; *ida1* x *ida7*).

Our assay also resolved a delay in rescue in I1 dynein assembly in the *ida3* x *ida7* dikaryons (Fig. 5C, red boxes). We determined that the delay in I1 dynein assembly in the axonemes from the *ida3* x *ida7* dikaryon combination is due to a requirement of new protein synthesis (Fig. 6). In contrast, new protein synthesis was not required for I1 dynein assembly in axonemes from the *ida3* x *ida1* dikaryon (Fig. 6B). One interpretation of these results is that IC140, the gene product of *IDA7*, and the IDA3 protein form a complex, and that this interaction cannot be complemented in the *ida3* x *ida7* dikaryon without new protein synthesis. This hypothesis may be consistent with a model in which IDA3 interacts with IC140 as an adapter required for I1 dynein transport into the ciliary compartment (Fig. 1).

Upon entry into the cilium, the 20S I1 dynein is transported to the ciliary tip by IFT. This conclusion is based on the failure of rescue of I1 dynein assembly upon inactivation of the IFT anterograde motor kinesin-2 at restrictive temperature (see Methods and (Kozminski et al. 1995; Piperno et al. 1996). Additionally, failure of I1 dynein rescue in the *ida3* and *ida3*; *fla10-1* dikaryons (Fig. 7C) revealed that a functional copy of IDA3 is essential for I1 assembly in the axoneme. Once I1 is unloaded from the transport complex, I1 dynein may diffuse towards the base (dashed arrow, Fig. 1), similar to DRC4, followed by incorporation into the axoneme at unoccupied I1 dynein docking sites (Wren et al. 2013). This result also indicates that the majority of the I1 dynein complex remains associated with IFT until reaching the distal tip where unloading of cargo presumably occurs. This interpretation is consistent with the live cell imaging study of Wren et al., (Wren et al. 2013) in which the majority of the N-DRC is transported as a cargo to the distal tip of the axoneme prior to release and diffusion. However, diffusion of I1 dynein, prior to docking in the axoneme, cannot be assessed by the assays we employed in this paper. Live cell imaging will be required for further tests of how I1 dynein enters the ciliary compartment and to determine the sites of loading, rate of assembly and diffusion after unloading from IFT.

Materials and Methods

Cell Strains and Culture

Chlamydomonas strains used in this study were wild-type (CC-125, CC-124, CC-620 and CC-621), *ida3* (CC-2668, CC-2669), *ida7* (CC-3921 5b10; *ida7-1*), *fla10* (CC-1919, CC-4617), *ida3; fla10-1* (this study), *ida1* (CC-2664) and *ida2* (CC-2666). All strains except for *ida3; fla10-1* were obtained from the *Chlamydomonas* Resource Center (University of Minnesota, St. Paul, MN). Cells were grown in tris-acetate-phosphate (TAP) medium with aeration on a 14:10h light/dark cycle. Dikaryons between wild type and I1-deficient cells were generated by mixing gametes produced by differentiating each cell type for 4 hours in M-N medium (Harris 1989). The *ida3; fla10-1* double mutant was generated by crossing *fla10-1* (–) and *ida3* (+) followed by screening progeny based on the *ida3* motility and ciliary resorption at 32°C. PCR and Sanger sequencing confirmed the selected candidates for the *fla10-1* mutation (Vashishtha et al. 1996).

Preparation of Axonemes and Membrane + Matrix

Cells were grown to mid-log phase and deciliated by treating the cells with 25mM dibucaine (Witman 1986) followed by centrifugation to separate the cilia and cell bodies. To prepare axonemes, the isolated cilia were demembranated by 1% Nonidet P-40 (Darmstadt, Germany) in HMDE + 25mM NaCl (10mM Hepes, 5mM MgSO₄, 1mM DTT, 0.5mM EGTA, pH 7.4) and the axonemal pellet was resuspended/dissolved in HMDE + NaCl (10mM Hepes, 5mM MgSO₄, 1mM DTT, 0.5mM EDTA, 25mM NaCl and protease inhibitors, pH 7.4) and fixed with Laemmli sample buffer at a concentration of 1mg/mL. The membrane+matrix (M+M) fraction was obtained by demembranating the pelleted cilia with 1% Nonidet-P40 in HMDE + 25mM NaCl buffer and fixed with Laemmli sample buffer for immunoblotting. For detection of axonemal subunits (IC69, IC140, IC138), the M +M was loaded at five times the relative volume of cilia and axonemes.

Preparation and Fractionation of Axonemal and Cytoplasmic Extracts

Axonemal Extracts: Wild type and I1-deficient axonemes were isolated as described above. Axonemes were then treated with HMDE + 0.6M NaCl for 30 minutes to solubilize the dyneins. The extracted dyneins in the high-salt extract (HSE) were dialyzed against HMDE + 25mM NaCl for 2 × 30mins. Cytoplasmic Extracts from wild type and I1-deficient whole cells were prepared at using the method described in (Alford et al. 2013). Cells were lysed by mechanical agitation by glass beads at room temperature. The axonemal HSE and cytoplasmic extracts were fractionated by velocity sedimentation on 5–20% sucrose gradients as described in Alford et al. 2013 or utilized for reconstitution experiments as described below.

In vitro Reconstitution

Varying amounts of extracts isolated as described above were mixed with a fixed amount of extracted I1-dynein deficient axonemes in the presence of 1mM ATP. The final volumes of all the reconstitutions were equalized with buffer. The reconstitution mixtures were separated by centrifugation into supernatant (S, unbound I1) and pellet (P, bound I1)

fractions. The axonemal pellets were resuspended to the original volume and fixed for SDS-PAGE.

Antibodies and Immunoblots

SDS-PAGE and immunoblotting were performed using standard procedures. Primary antibodies used in this study include the following: mouse monoclonal against IC69 (clone 1869A hybridoma, Sigma, St. Louis, MO); rabbit polyclonal antibodies against IC140 (Yang and Sale 1998), IC138 (Hendrickson et al. 2004), IC97 (Wirschell et al. 2009) and IFT46 clone 17600 (Hou et al. 2007). The secondary antibodies (goat anti-mouse or rabbit) used in immunoblots were purchased from BioRad (Hercules, CA). The secondary anti rabbit or goat conjugated with Alex Fluor 488 or 555 were purchased from Invitrogen (Eugene, OR).

Immunofluorescence microscopy and quantification

For immunofluorescence of whole cells, cells were allowed to adhere for 5 min to poly-L-lysine coated cover slips, excess cells were wicked off, and the cover slips were submerged in -20°C methanol for 5 min. The cover slips were then air-dried, rehydrated with 1X PBS, blocked for 30 min at room temperature with blocking solution (6% fish skin gelatin, 1% BSA, and 0.05% Tween-20 in PBS, pH 7.0), incubated with primary antibodies (4°C overnight), washed 3X with blocking solution, washed 3X, incubated with secondary antibodies (Alexa Fluor-conjugated IgG; 1:1000; Invitrogen, Eugene, OR) for 30 min at RT, washed 3x with PBS, then mounted with ProLong Antifade Gold (Invitrogen, Eugene, OR). Images were captured using a BX60 wide field microscope (Olympus, Tokyo, Japan) under a 60x objective, and with a digital camera (Orca-ER, Hamamatsu, Bridgewater, NJ) and Slidebook software (Intelligent Imaging Innovations, Denver, CO). Quantifying I1 dynein assembly from the tip of mutant cilia in dikaryons was performed as described in Alford et al., (Alford et al. 2013). All the image analysis was performed using Image J (FIJI, National Institutes of Health, Bethesda, MD). For each combination of dikaryons (Ex. WT x *ida3*, *ida3* x *ida7*), the fluorescence signal (Arbitrary units, AU) starting from the tip of the rescuing cilia was measured as function of distance (μm). The fluorescence intensity values corresponding to every pixel along the length were normalized to the brightest intensity value in the data set and plotted against the length of the cilium (μm) as illustrated in the line scan (Figure 5B). A threshold value of 0.2 was established, and distance at which the normalized fluorescence intensity value dropped and stayed below 0.2 for at least three consecutive data points was recorded as the absolute distance at which the IC140 staining from the tip ended (μm). The progressive rescue of I1 dynein assembly from the tip as a function of time after mixing the gametes was plotted using Kaliedograph (Reading, PA). The standard error and p-values were calculated in MS Excel (Redmond, WA).

Dikaryon rescue using the *fla10-1* mutants was performed as described (Piperno et al. 1996). Briefly both gametes, *ida3*; *fla10-1* and *fla10-1* were preincubated at the restrictive temperature, 32°C , for at least 38 minutes prior to mixing in order to inactivate the kinesin. Secondly, the dikaryons involving *fla10-1* and *ida3*; *fla10-1* double mutant gametes could be analyzed for I1 assembly for a maximum time of 60 minutes after mixing, after which dikaryons began to lose their cilia due to resorption.

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Figure 1. Schematic model depicting steps leading to I1 dynein assembly in the axoneme
 We tested the hypotheses that I1 dynein preassembles in the cytoplasm and that the preassembled I1 complex is transported to the distal tip of the cilium by IFT. Based on the characterization of the *ida3* mutant in this paper, we hypothesize that IDA3, a cytoplasmic protein is required for the entry or transport of I1 dynein. IDA3 could be a modifier required for I1 dynein transport or an adapter linking I1 dynein to IFT (both possibilities indicated by a “?”). The postulated barrier between the cytoplasm and the ciliary compartment is shown as black boxes.

Figure 2. I1 dynein assemblies in the cytoplasm as a 20S complex

(A) Immunoblots of fractions from velocity sedimentation of axonemal high salt extracts (HSE) and cytoplasmic extracts (CE) were analyzed. The I1 dynein subunits IC140, IC138 and IC97 co-sediment at fraction 5 (arrows) in both axonemal high salt extracts and cytoplasmic extracts from wild-type cells. (B) Cytoplasmic extracts derived from *bld1*, *ida1*, *ida3* and *ida7* were fractionated by velocity sedimentation and analyzed by immunoblots using IC138 or IC140 as a marker of I1 dynein. Cytoplasmic I1 dynein from *bld1* and *ida3* cosediment with wild-type 20S I1 complex. The 20S I1 complex is missing in the I1 dynein heavy chain mutant *ida1* and *ida2* (data not shown) and in the IC140-null mutant *ida7*.

Figure 3. The *ida3* mutant is not defective in docking of I1 dynein to the axonemes *in vitro*
(A) High salt extracts (HSE) containing I1 dynein from *oda2* axonemes were reconstituted onto salt-extracted I1-deficient *ida3* and *ida7* axonemes in the presence of 1mM ATP. Varying ratios of axonemal I1 (HSE) to extracted axonemes (I1: Axonemes) were combined in an ATP-containing buffer, incubated on ice for 30 min and probed with IC140 as a marker for I1 dynein in immunoblots analysis. Each mixture was centrifuged to separate the supernatant (S = unbound I1 dynein) and pellet (P = bound I1 dynein) fractions. Axonemal I1 (HSE) reconstituted onto both *ida3* and *ida7* axonemes results in I1 dynein binding to the axonemes to saturation (P = pellet/bound I1). (B) I1 dynein-containing cytoplasmic extracts (cyt.) from *oda2* were reconstituted onto *ida3* and *ida7* axonemes in the presence of ATP. Cytoplasmic I1 dynein (cyt.) does not bind to the axoneme as indicated by I1 presence in the supernatant (S) only.

Figure 4. The 20S I1 dynein assembled in the *ida3* mutant cytoplasm is defective in entry to the ciliary compartment

(A) Immunoblots comparing I1 intermediate chains, IC138 and IC140, in wild-type and *ida3* cytoplasmic extracts. NAB1 was used as a marker and loading control (Mussnug et al. 2005)

(B) Immunoblots of ciliary (Cil), axonemal (Axo) and membrane + matrix (M+M) fractions were analyzed using antibodies to the IC138 and IC140 subunits of I1 dynein. For detection of axonemal subunits (IC69, IC140, IC138), the M+M was loaded at five times the relative amount of cilia and axonemes. Analysis showed a significant reduction in ciliary and axonemal I1 dynein in *ida3* compared to wild-type. In addition, M+M fractions show that I1 dynein is absent in *ida3* compared to wild-type indicating inefficient entry into the ciliary compartment. The M+M samples from WT and *ida3* were also examined by immunoblots at

twice the protein load (data not shown), and I1 dynein subunits were never present in *ida3*. The outer arm component, IC69, is present in equivalent amounts in WT and *ida3* M+M and serves as a control for IFT cargo. The IC69 immunoblot also validates that the defect in *ida3* is specific to I1 dynein. IFT46 serves as a positive control for M+M fractionation. CBB = Coomassie Brilliant Blue loading control.

Figure 5. Dikaryon rescue of I1 dynein assembly occurs from the distal tip

(A) Immunofluorescence of IC140 in *ida7* x wild-type (WT) dikaryons. Cytoplasmic complementation in WT and I1-deficient dikaryons results in the rescue of I1 dynein assembly (arrows) from the distal tip to base in the *ida7* mutant cilia. Scale bar = 2 μ m. (B) Quantification of I1 dynein staining along the cilia of I1 mutants at various time points post mating. Normalized fluorescence intensity is plotted against the distance of rescue from the tip for one rescuing mutant cilium of the WT x *ida7* dikaryon shown in part A. The length of I1 assembly at the ciliary tip (vertical dotted line) increased progressively over time: 2.5 μ m at 30m, 5.25 μ m at 60m, and 8.75 μ m at 90m. (C) Comparison of dikaryon rescue rates of I1 assembly in various mutant combinations. The length of IC140 staining at the distal end of the cilium is plotted versus time. WT x *ida3* (blue circles, n = 83) and WT x *ida7* (black

circles, n = 29) dikaryons demonstrate progressive rescue of axonemal I1 dynein assembly with complete recovery at 90m. Dikaryons between *ida3* and *ida1* show this same pattern of rescue along all four cilia (black triangles, n = 59). Rescue of axonemal I1 dynein assembly is delayed in *ida3* x *ida7* dikaryons (red squares, n = 30), but occurs from the distal tip like other dikaryon combinations.

Figure 6. Dikaryon rescue of axonemal I1 assembly requires protein synthesis of IC140 and/or IDA3

(A) Immunofluorescence of IC140 in *ida3* x *ida7* dikaryons 60 minutes after mixing gametes. Rescue of axonemal I1 dynein assembly is seen at the distal end as in Figure 5A (arrows, top panel). In contrast, in the presence of the protein synthesis inhibitor, cyclohexamide (CHX), the recovery of I1 dynein is not seen in the cilia (dotted circle, bottom panel). Scale bar = 2μm. (B) Quantification of IC140 staining from the distal tip of I1-deficient cilia as in Figure 5B in the presence of CHX. Axonemal I1 dynein assembly occurs progressively from the distal tip to base in WT x *ida3* (blue circles, n = 25), wild-type

x *ida7* (black circles, n = 25) and *ida3* x *ida1* (black triangles, n = 22) dikaryons. In contrast I1 dynein is not assembled on the axoneme in *ida3* x *ida7* dikaryons (red squares, n = 29).

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Figure 7. Transport of I1 to the tip of the cilium requires kinesin-2

(A) Diagram of dikaryon rescue experimental design using the temperature sensitive mutant, *fla10-1*. (B) Immunofluorescence of IC140 in *fla10-1 x ida3; fla10-1* dikaryons 60 minutes after mixing gametes. At the permissive temperature (21°C), recovery of axonemal I1 dynein assembly occurs from the distal end of *ida3* cilia (arrows). At the restrictive temperature (32°C), I1 dynein assembly on the axoneme is not seen in *ida3; fla10-1* cilia (dotted circles). Scale bar = 2µm. (C) Quantification of IC140 staining at the distal end of I1-deficient cilia at permissive (21°C) and restrictive (32°C) temperatures. Axonemal I1 dynein assembly occurs progressively from the distal tip to base in wild-type x *ida3* (n = 19)

and *fla10-1* x *ida3* (black diamonds and black circles, n = 39) dikaryons at both permissive and restrictive temperatures. While I1 assembly at the distal tip is seen at the permissive temperature in *fla10-1* x *ida3*; *fla10-1* dikaryons (blue circles, n = 52), I1 assembly at the tip does not occur at restrictive temperature (red circles, n = 19). As a control, *ida3*; *fla10-1* x *ida3* dikaryons were tested for a lack of I1 axonemal assembly at both temperatures (black dotted triangles and squares, n = 46).