Chlamydomonas ODA10 is a conserved axonemal protein that plays a unique role in outer dynein arm assembly

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ABSTRACT **Assembly of outer dynein arms (ODAs) requires multiple steps and involves multiple proteins in addition to dynein subunits. The *Chlamydomonas ODA10*, *ODA5*, and *ODA8* loci genetically interact and are hypothesized to function as an axonemal accessory complex, but only ODA5p was previously characterized. We positionally cloned *ODA10* and identified the gene by rescuing an *oda10* mutant with a hemagglutinin-tagged cDNA. *ODA10* sequence predicts a conserved coiled-coil protein homologous to mouse ccdc151. ODA10p is present in cytoplasm and flagella, remains axonemal after detergent treatment, and is extracted with 0.6 M NaCl. Both outer arm dynein and ODA10p rebound to the axonemes when desalted extracts are mixed with *oda10*-mutant axonemes. Sucrose gradient separation of these extracts shows that ODA10p sediments near the top of the gradient, not with 23S outer dynein arm proteins. Unexpectedly, dynein and ODA10p fractions are able to bind individually to *oda10* axonemes. ODA10p is present on *oda8*-mutant flagella at wild-type levels. However, ODA10p does not assemble into *oda5* flagella and is absent from *oda5* cytoplasm, suggesting a necessity of ODA5p for stability of ODA10p in vivo. The results suggest that ODA10p does not function as a part of a traditionally defined docking complex.**

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INTRODUCTION

The cilium is a highly conserved organelle in eukaryotic cells. Mammals have cilia in almost all cells for sensory and/or motility purposes, and disruptions of ciliary function are the foundation of a group of pleiotropic diseases collectively called ciliopathies that affect developmental, sensory, reproductive, and respiratory functions (Hildebrandt *et al.*, 2011). To treat and accommodate patients who are affected by ciliopathies, it is crucial to understand the mechanism of ciliary assembly.

Dyneins are highly conserved minus end–directed, microtubule-associated motor complexes that have AAA ATPase domains. Axonemal dyneins are essential for proper functioning of motile cilia. Defects in dynein assembly disrupt motility and are one cause of human primary ciliary dyskinesia (PCD), which is characterized by infertility, respiratory tract infections, left–right asymmetry malformation, and hydrocephalus. Axonemal dyneins can be divided into subfamilies based on their structural and functional differences (Gokhale *et al.*, 2012; Kamiya, 2012). Multiple unique inner dynein arms are distributed, each with 96-nm periodicity, and mutations in components of inner dynein arms typically affect flagellar waveform. In contrast, one (*Chlamydomonas*) or two (human) types of outer dynein arm are found along each doublet microtubule with 24-nm periodicity, and mutations in components of outer dynein arms reduce beat frequency. By far the most common genetic defects associated with PCD in humans are mutations that disrupt the assembly of outer dynein arms (Hildebrandt *et al.*, 2011).

Assembly of the outer dynein arm complex has been extensively studied in *Chlamydomonas reinhardtii*. In *Chlamydomonas*, outer dynein arms consist of three heavy, two intermediate, and multiple light chains. In addition to their core components, outer dynein arms require a docking complex (DC) that facilitates their attachment onto microtubules (Takada and Kamiya, 1994; Takada *et al.*, 2002; Koutoulis *et al.*, 1997; Casey *et al.*, 2003), cytoplasmic chaperones that assist preassembly of dynein complexes (Freshour *et al.*,

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Abbreviations used: AC, accessory complex; DC, docking complex; ODA, outer dynein arm.

2007; Omran *et al.*, 2008; Duquesnoy *et al.*, 2009; Mitchison *et al.*, 2012), and an intraflagellar transport (IFT) adaptor protein specific for transportation of outer arm complexes (Ahmed and Mitchell, 2005; Ahmed *et al.*, 2008).

Other gene products required for assembly of outer dynein arms (ODAs) in *Chlamydomonas* may form an accessory complex (AC) composed of three subunits: ODA5p, ODA8p, and ODA10p (Kamiya, 1988; Fowkes and Mitchell, 1998; Wirschell *et al.*, 2004). Previous work on the *oda5*, *oda8*, and *oda10* mutants and biochemical characterization of ODA5p supported the existence of a complex containing all three gene products that is localized on doublet microtubules. Genetic evidence for this complex comes from cytoplasmic complementation analysis in temporary dikaryons, quadraflagellate diploid cells that form when two haploid gametes fuse during mating. When two mutants that do not affect the same complex or pathway form a temporary dikaryon, a wild-type version of each protein is provided by its mating partner in the fused cytoplasm. Three groups of *oda* mutants were defined by the lack of cytoplasmic complementation seen when temporary dikaryons were formed between any two mutants in a group (Kamiya, 1988). One group was later discovered to include subunits of the ODA complex itself (Fowkes and Mitchell, 1998) and cytoplasmic preassembly chaperones required for complex formation (Omran *et al.*, 2008; Mitchison *et al.*, 2012). A second group included *oda1* and *oda3*, which were later shown to encode subunits of the doublet-associated DC (Takada *et al.*, 2002). A third group (*oda5*, *oda8*, and *oda10*) defines the proposed AC (Kamiya, 1988; Fowkes and Mitchell, 1998; Wirschell *et al.*, 2004).

ODA5p is the only gene defining this AC that has been characterized. Consistent with its proposed function as the subunit of a doublet-associated AC, ODA5p is an axonemal coiled-coil protein, distantly homologous to two of the three DC subunits. Assembly of ODA5p in flagella is dependent on ODA10p but independent of ODA8p, the DC, or axonemal dynein complexes (Wirschell *et al.*, 2004). In *oda5*, *oda8*, and *oda10* mutant cells, dynein subunits are preassembled in the cytoplasm but do not assemble in flagella (Fowkes and Mitchell, 1998). However, outer dynein arms that are extracted from wild-type flagella not only can rebind to *oda5*, *oda8*, and *oda10* axonemes, but they can also rescue beat frequency up to 52 Hz in vitro (Sakakibara and Kamiya, 1989). Further, purified 12S and 18S dynein fractions are also able to rebind to *oda5* axonemes and rescue beat frequency (Takada and Kamiya, 1994). These rebinding studies suggest that dynein may rebind in the absence of the AC and appear to be in conflict with previous models that proposed a role for the AC as a second docking complex for binding of outer dynein arms in flagella (Fowkes and Mitchell, 1998; Wirschell *et al.*, 2004). Furthermore, although outer dynein arm complexes form in the *oda5* cytoplasm (Fowkes and Mitchell, 1998) and these complexes can rescue the motility of flagella in vivo in dikaryons with subunit-defective strains (Kamiya, 1988), their cytoplasmic abundance is reduced compared with wild-type or DC-mutant strains (Fowkes and Mitchell, 1998). This result suggests a unique role for ODA5p in the cytoplasm instead of, or in addition to, a role as part of an accessory docking complex in the flagellum.

Here we characterize the ODA10 protein and described its role in assembly of outer dynein arms. Our data support a new model in which ODA5, ODA8, and ODA10 proteins modify outer dynein arms into a form that binds with high affinity to axonemal binding sites.

RESULTS

Positional cloning of *ODA10* locus

Oda10 was previously mapped near *pf3* on chromosome 8 (parental ditype to nonparental ditype to tetratype = 132:0:5; Harris *et al.*, 1987), and the *pf3* locus was physically mapped to scaffold 29 of the *Chlamydomonas* genomic sequence version 3, but this genomic region remains incompletely assembled in current versions of the *Chlamydomonas* genome (Wirschell *et al.*, 2013). We further mapped 67 progeny from a cross between *oda10-1* and strain *S1D2* (*CC-2290*). Linkage data were verified with previously characterized molecular markers: PSBQ, MCA1, and LI818 (Rymarquis *et al.*, 2005). The results identified a general candidate region for *ODA10* between markers LI818 and MCA1 on chromosome 8. The linkage between *pf3* and *ODA10* was genetically verified to be ~1 cM, which corresponds to a physical distance of ~100 kb (Rymarquis *et al.*, 2005). Based on these linkage data, two candidate regions were identified within ~100 kb of the predicted location of *pf3* locus. Using an additional marker within one candidate region, V3S294, we mapped *ODA10* between V3S294 and MCA1 (see Supplemental Table S1 for marker details). A candidate gene in this interval, C_290012 in JGI *Chlamydomonas* Genome Version 4, was selected for further analysis based on its homology to genes found only in organisms and tissues with motile cilia. A marker, ODA10-6, near the candidate gene was tested and showed no recombination with the *ODA10* locus in all 67 test progeny. These results strongly support that C_290012 was the *ODA10* locus.

A BLAST search with C_290012 identified two cDNA clones in the Kazusa database (Asamizu *et al.*, 1999). One of these clones (AV640669) was confirmed to be a full-length cDNA of candidate gene C_290012. To express this cDNA in *C. reinhardtii*, we cloned the predicted coding region into a *C. reinhardtii* expression vector that adds three hemagglutinin (HA) epitopes at the C terminus. When transformed into *oda10-2* cells this construct rescued the beat frequency of *oda10-2(V87.2)* mutant (~24 Hz) to that of wild type (~60 Hz; Figure 1A) and restored assembly of outer dynein arms to wild-type levels as detected by Western blotting (Figure 1B). Comparison of multiple independent transformants showed a correlation between the amount of ODA10HAp and the amount of outer arm dynein in flagella. We conclude that gene C_290012 on chromosome 8 is the *ODA10* locus, and the level of outer dynein arm assembly is dependent on the amount of ODA10p present in the flagella.

ODA10p is a conserved coiled-coil protein

ODA10p is predicted to be a 56-kDa protein with pI of 7.93 and a high probability to form multiple α-helical coiled coils (Figure 1C). Direct BLASTp comparison of ODA10p to predicted *Chlamydomonas* proteins did not reveal strong similarity to any other proteins; however the overall predicted structural similarity suggested that ODA10p might share some evolutionary relationship to other predicted coiled-coil proteins important for dynein assembly. These include another protein genetically associated with the AC (ODA5p) and two proteins in the DC (ODA1p = DC2 and ODA3p = DC1). The distribution of coiled-coil domains in these proteins is shown in Figure 1C. All homologues of these four *Chlamydomonas* proteins (selected by BLASTp with a cutoff of 1E-10) in an alga (*Micromonas*), a basal chordate (*Ciona*), a fish (*Danio*), and a mammal (*Mus*) were aligned, and an unrooted tree was generated to display relative sequence similarities (Figure 1D). A single close homologue of ODA10p appeared in each genome. The other *Chlamydomonas* AC protein, ODA5p, appears to be distantly related to *Chlamydomonas* DC protein ODA1p (*E* value of 3E-22), and two ODA1p homologues were also apparent in the *Micromonas*, *Ciona*, and *Mus* genomes. Branch order suggests that the metazoan homologues arose before the divergence of chordates but after the divergence of metazoa from their common ancestor with algae and that one ODA1p homologue may have been lost in the *Danio* lineage.

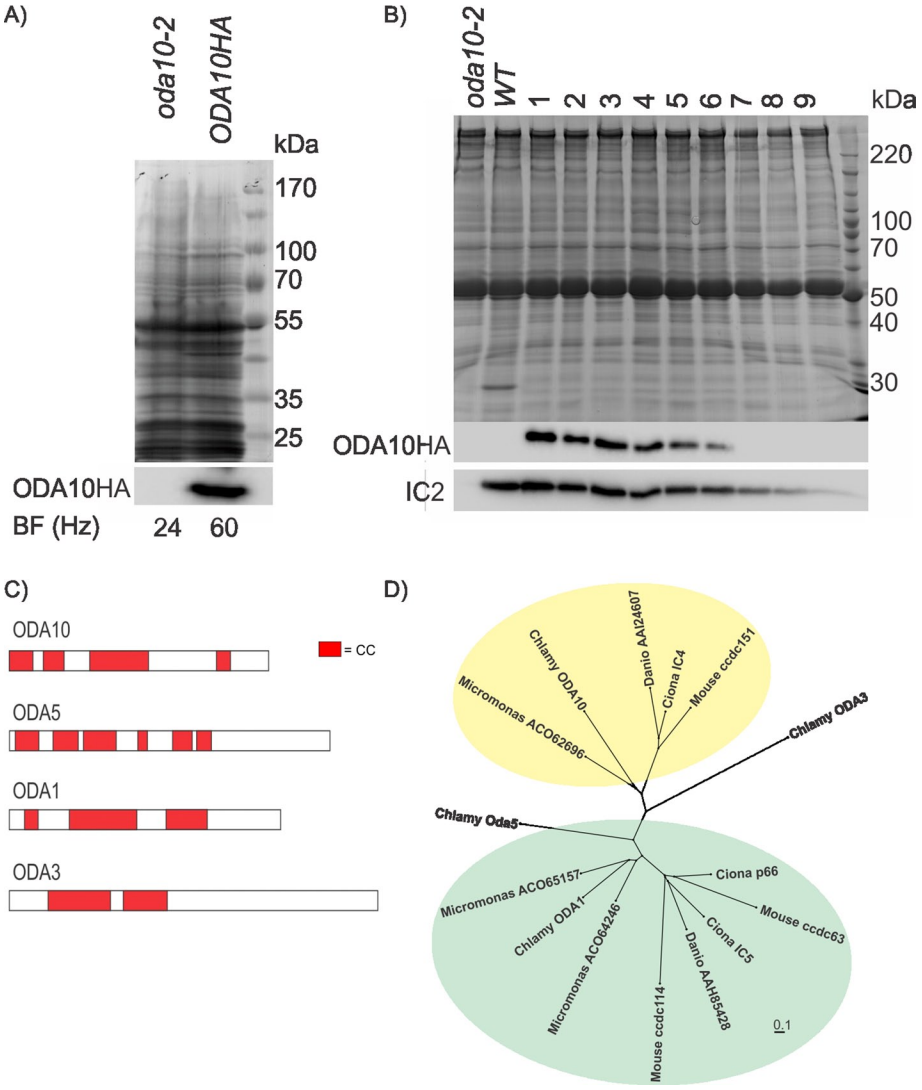

FIGURE 1: ODA10p is a conserved coiled-coil protein. (A) A Coomassie-stained gel (top) and Western blot (bottom), probed with an antibody against HA epitope, of equally loaded (by cell number) whole-cell samples of *oda10-2,ODA10HA* and *oda10-2* cells show expression of ODA10HAp in a strain with rescued beat frequency. (B) A Coomassie-stained gel (top) and western blot (bottom), probed with antibodies against HA epitope and IC2, of flagellar samples of mutant (*oda10-2*), wild type (*137c*), and rescued transformants (*oda10-2,ODA10HA* 1-9) show restored assembly of outer dynein arms to wild-type levels in transformant cells expressing the highest levels of ODA10HAp. (C) The distribution of predicted coiled-coil domains of ODA1p, ODA3p, ODA5p, and ODA10p. (D) All homologues of ODA10p and of three *Chlamydomonas* ODA10p paralogues (ODA5p, ODA1p [= DC2], ODA3p [= DC1]) in an alga (*Micromonas*), a basal chordate (*Ciona*), a fish (*Danio*), and a mammal (*Mus*) were aligned, and an unrooted tree was generated to display relative sequence similarities. Green background highlights homologues of ODA1p; yellow background highlights homologues of ODA10p. Protein sequences used in the alignments that are not given in the figure were Q8CDV6 (ccdc63), AAI60348 (ccdc114), AAH57069 (ccdc151), NP_001027646 (*Ciona* p66), NP_001072015 (*Ciona* IC4), NP_001071888 (*Ciona* IC5), AAC49732 (ODA3), AAK72125 (ODA1), AAS10183 (ODA5), and AGW18228 (ODA10).

In contrast, no close homologues of ODA3p appear in any of these genomes, suggesting that it might be a recent invention in the *Chlamydomonas* lineage.

Biochemical fractionation of ODA10p

To determine the localization of ODA10p, cytoplasmic extract, flagella, axoneme, and membrane matrix fractions of *oda10-2,ODA10HA* cells were prepared at 1:1 stoichiometric ratios, and the abundance of ODA10p was detected with Western blots using an antibody against the HA epitope. ODA10p was present in cytoplasm and flagella at ~1:1 ratio (Figure 2A) and stayed in the axonemal microtubule-associated fraction after treatment of flagella with detergent to remove membranes and soluble matrix (Figure 2B). When *oda10-2,ODA10HA* axonemes were extracted with NaCl, ODA10p was extracted quantitatively at concentrations >0.4 M NaCl (Figure 2C), similar to the concentration of NaCl required to extract outer dynein arms from axonemes.

Similar to *Chlamydomonas* docking complex, the *Ciona* ODA10p orthologue (IC4) copurifies with outer row dyneins on sucrose gradients (Hozumi *et al.*, 2006). Because ODA10p not only showed some evolutionary relationship with docking complex proteins (Figure 1D), but was also hypothesized to have a similar function (Wirschell *et al.*, 2004), we tested whether *Chlamydomonas* ODA10p cofractionates with the 23S dynein fraction, which has intact outer row dyneins together with docking complexes (Takada *et al.*, 2002). Fractionation of dialyzed high-salt extract of *ODA10HA* axonemes by sucrose gradient centrifugation shows that ODA10HAp cosediments with tubulin at the top of the gradient (~6 S), whereas outer row dynein sediments at ~23 S, represented by its IC (IC2; Figure 2D).

Effects of *oda5* and *oda8* mutations on ODA10p

To test the effect of other outer dynein arm mutants on assembly of ODA10p, we probed flagellar samples of *pf28*, *oda5*, *oda8*, and *oda10-1* for the presence of ODA10p using a polyclonal antibody against endogenous ODA10p. The results show that flagellar assembly of ODA10p was significantly reduced in *oda5* but not in oda8 or dynein heavy-chain mutant *pf28* (Figure 3A). To confirm the effect of *oda5* and *oda8* on assembly of ODA10p, we probed samples of *oda5,oda10* and *oda8,oda10* double-mutant cells expressing an HA-tagged ODA10p (designated here as *oda5,ODA10HA* and *oda8,ODA10HA*) for the presence of ODA10p with the more sensitive antibody against the HA epitope. A Western blot with equally loaded flagellar samples of *oda10,ODA10HA*, *oda5,ODA10HA*, and *oda8,ODA10HA* cells showed that ODA10p did not assemble in *oda5*-mutant flagella, whereas ODA10p did assemble to wild-type levels in flagella of *oda8*-mutant cells (Figure 3B). To elucidate the reason for lack of assembly of ODA10p in *oda5* mutants, we tested the level of ODA10p in the cytoplasm. The results showed an

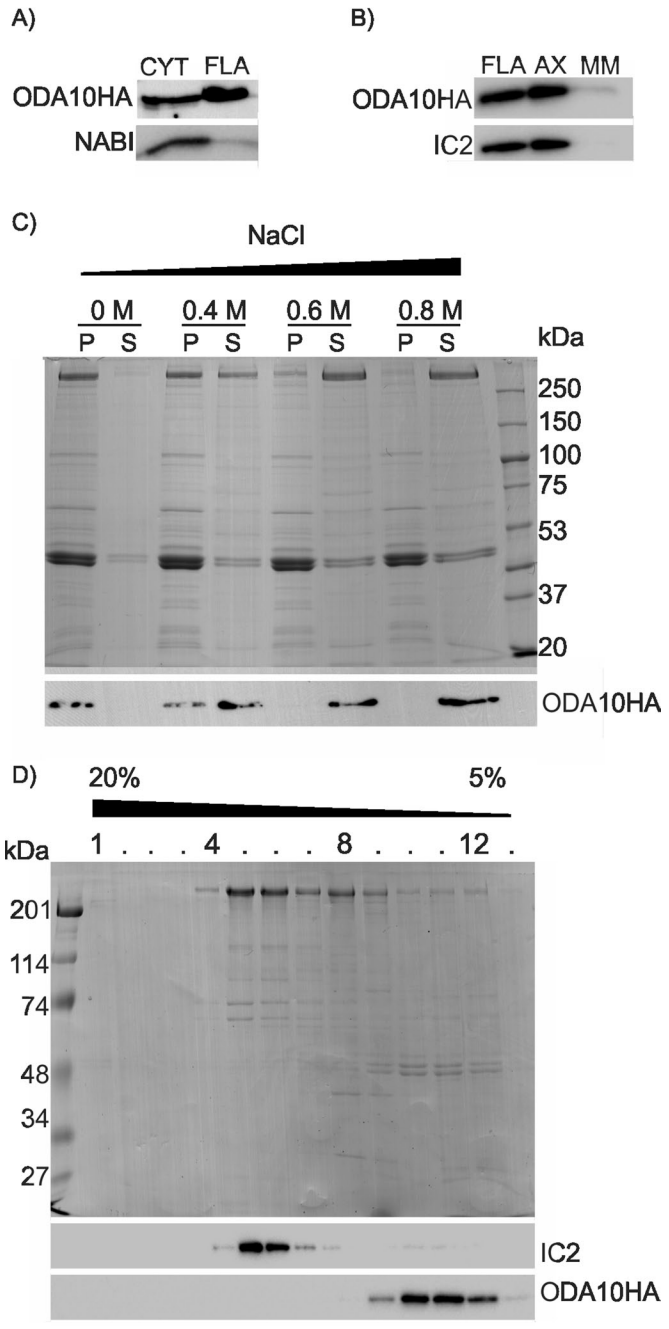

FIGURE 2: ODA10p is a salt-extractable axonemal protein that does not cofractionate with dynein. (A) Western blots of cytoplasmic extract and flagellar samples from equal numbers of cells, probed with antibodies against HA (ODA10p) and NAB1 (a cytoplasmic protein used as a control for cell body contamination in the flagellar fraction), show ODA10p distribution in cytoplasm and flagella. (B) Western blots of flagellar, detergent-resistant axonemal, and detergent-soluble membrane and matrix fractions, probed with antibodies against HA and IC2, show that ODA10p remains microtubule associated, similar to IC2. (C) A Coomassie-stained gel (top) and Western blot of soluble and insoluble fractions of wild-type axonemes after exposure to increasing salt concentrations, probed with an antibody against HA, show extraction of ODA10p >0.4 M NaCl. (D) A Coomassie-stained gel (top) and Western blot (bottom) of sucrose gradient fractions of a 0.6 M NaCl extract of ODA10HA axonemes. Outer row dynein (IC2) does not cosediment with ODA10p (ODA10HA).

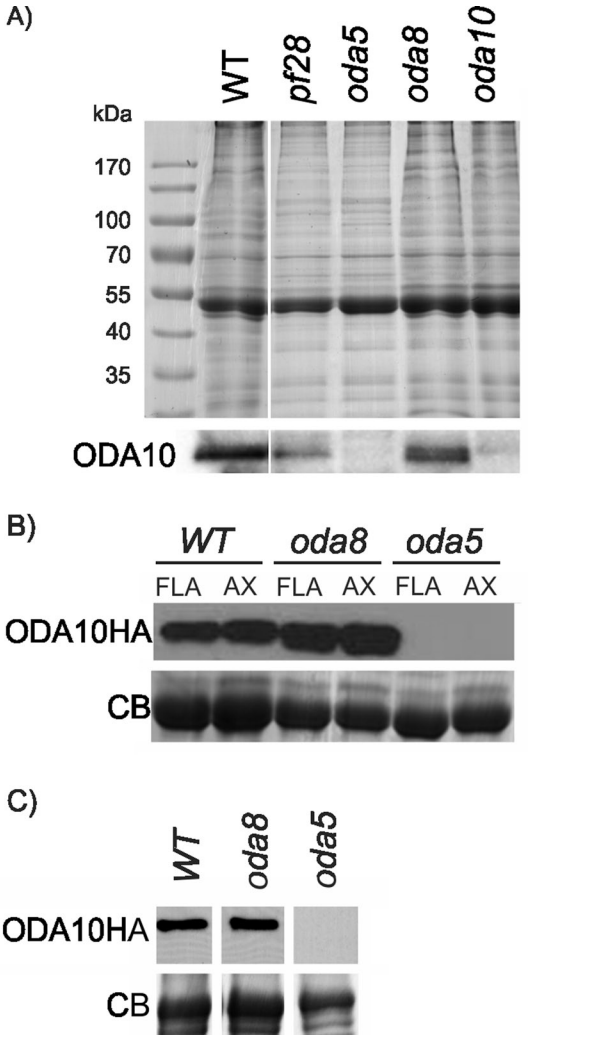

FIGURE 3: ODA10p is unstable in *oda5* mutant. (A) A Coomassie-stained gel (top) and Western blot (bottom) of flagellar samples from the indicated strains, probed with a polyclonal antibody against ODA10p, show that ODA10p is missing from *oda5* as well as *oda10* flagella. (B) Flagellar and axonemal samples of *oda8,ODA10HA oda5,ODA10HA* and *oda10,ODA10HA* were probed with antibody against the HA epitope. The tubulin region of a Coomassie-stained gel (bottom) serves as a loading control. (C) A Western blot (top) of deflagellated cell body samples of *oda10,ODA10HA oda8,ODA10HA* and *oda5,ODA10HA* probed for ODA10HAp. The amount of ODA10p in cytoplasm of *oda5* was greatly reduced but was near wild type in *oda8*. A portion of the Coomassie-stained gel serves as a loading control.

almost undetectable amount of ODA10p in the cytoplasm of *oda5*, whereas cytoplasmic abundance of ODA10p in *oda8* was maintained at wild-type levels (Figure 3C). Thus ODA10p is unstable in the absence of ODA5p, whereas its stability in cytoplasm and assembly in flagella were not affected by lack of ODA8p. Because ODA complexes fail to assemble in either *pf28* or *oda8* flagella, the absence of outer dynein arms did not alter assembly of ODA10p.

ODA10p and 23S dynein stably bind to *oda10* axonemes independently in vitro

Sakakibara and Kamiya (1989) showed that a dialyzed high-salt extract of wild-type axonemes was sufficient to restore wild-type motility to axonemes of *oda* cell models, including *oda10*. We

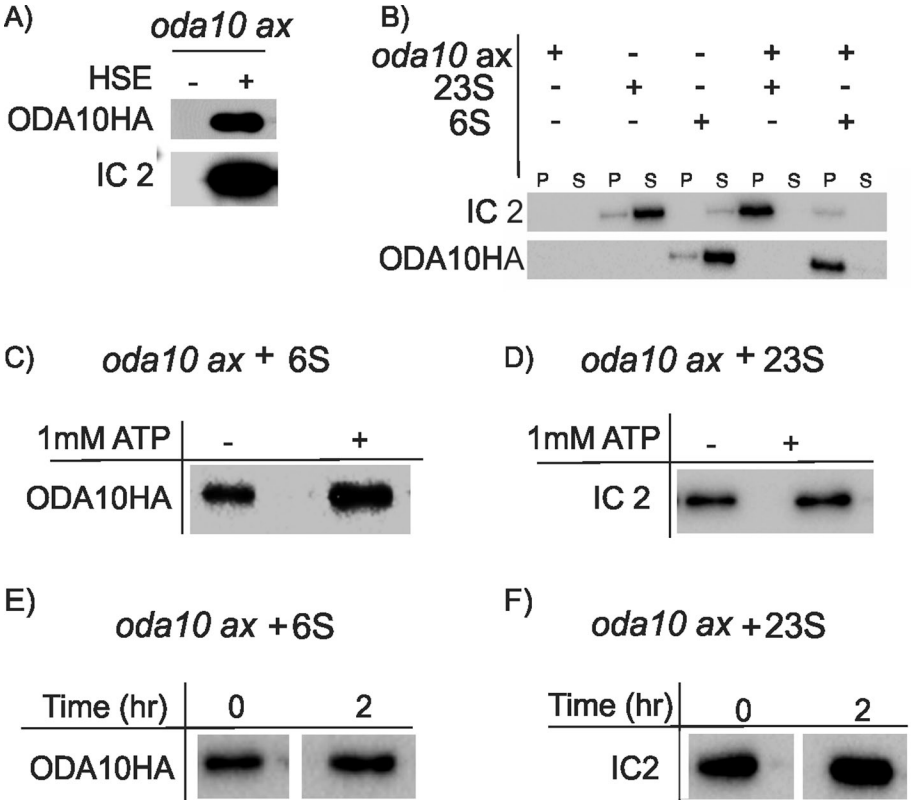

FIGURE 4: ODA10p and outer arm dynein stably assemble onto *oda10* axonemes independently in vitro. Western blots probed with anti-IC2 and anti-HA detect proteins bound in vitro to *oda10* axonemes. (A) *oda10* axonemes were incubated with buffer alone (–) or a dialyzed HSE of wild-type axonemes from ODA10HA cells (+) for 1 h, and pellet fractions were examined. Both IC2 and ODA10HAp are present in the pellet. (B) *oda10* axonemes were incubated on ice for 1 h with separate sucrose gradient fractions of outer row dyneins (~23 S) or ODA10HAp (~6 S) or buffer alone. Both pellet (P) and supernatant (S) were analyzed. (C, D) The binding experiment in B was repeated in presence of 1 mM ATP, and pellets were probed for ODA10HA or IC2. ODA10HA and outer dynein arms pellet with axonemes, regardless of presence of ATP. (E, F) *oda10* axonemes previously incubated with either 6S (E) or 23S (F) fractions were pelleted, and half of each sample was resuspended in fresh buffer, incubated on ice for 2 h, and pelleted again. Equal amounts of ODA10HAp (E) and IC2 (F) are associated with *oda10* axonemes before (0) and after (2) incubation.

tested the ability of ODA10p to rebind to *oda10* axonemes under these conditions. If ODA10p cannot rebind to the axonemes, then we could conclude that ODA10p is unnecessary for functional dynein binding in vitro. Mixing a dialyzed salt extract containing axonemal dynein with *oda10* axonemes shows that dynein and ODA10HAp both rebound to *oda10* axonemes in vitro (Figure 4A). To see whether ODA10p is necessary for dynein binding, we fractionated extracts by sucrose gradient centrifugation under conditions that preserve a 23S dynein fraction, which has been shown to functionally rescue *oda1* axonemes in vitro (Takada *et al.*, 1992). Binding experiments with independently pooled 23S and 6S fractions show that ODA10p and outer dynein arms each binds independently onto *oda10* axonemes in vitro (Figure 4B). Therefore ODA10p is not required for the initial binding of outer dynein arms onto *oda* axonemes in vitro. Addition of 1 mM ATP did not affect binding of either ODA10p or outer dynein arms to *oda10*-mutant axonemes (Figure 4, C and D), ruling out dynein binding only by ATP-sensitive motor domain interaction with doublet microtubules (Haimo *et al.*, 1979; Haimo and Fenton, 1988). To further test the stability of outer dynein arm and ODA10p axoneme association, we resuspended axonemes with bound proteins in buffer alone, incubated them on ice, and repelleted them. Neither ODA10p nor outer dynein arms dissociated from *oda10* axonemes even after 2 h in buffer alone (Figure 4, E and F). Thus outer dynein arms extracted from wild-type axonemes can rebind to *oda10* axonemes at high affinity in the absence of ODA10p.

DISCUSSION

Assembly of ODA motor complexes appears to be a complicated, multistep process that involves chaperoning steps in the cytoplasm (Mitchison *et al.*, 2012), IFT-based transport (Ahmed *et al.*, 2008), and specific docking sites on doublet microtubules (Takada and Kamiya, 1994; Takada *et al.*, 2002; Wakabayashi *et al.*, 2001). An additional step involving a hypothesized AC composed minimally of ODA5p, ODA8p, and ODA10p is required for assembly of outer dynein arms, but the specific role of the AC in assembly is yet to be defined.

ODA10p is a coiled-coil protein with weak similarity to ODA5p and DC proteins ODA1p and ODA3p. A single ODA10p orthologue occurs in the genomes of most organisms with motile cilia, including a diatom (*Thalassiosira*) that lacks inner dynein arms, whereas no orthologues are found in a moss (*Physcomitrella*) that lacks outer dynein arms, supporting a conserved role for ODA10p in outer dynein arm assembly or function. In contrast, clear orthologues of ODA5p were not detected in organisms more distantly related than *Volvox*, suggesting that the *ODA5* gene may have been created relatively recently from a duplication of *ODA1* within the Chlorophyte algae lineage. Independent duplications of *ODA1* homologues have occurred in the Prasinophyte algae (*Micromonas*) and the Metazoa (*Ciona*, *Mus*), as seen in Figure 1. It will be interesting to determine whether any of these gene duplications also created a gene product functionally equivalent to ODA5p and thus interacting genetically with ODA10p. Alternatively, these gene duplication events might have created proteins that interact with each other to create a docking complex equivalent to the *Chlamydomonas* DC, which contains a heterodimer between coiled-coil proteins ODA1p and ODA3p (Wakabayashi *et al.*, 2001), or proteins with tissue-specific roles, as suggested by the cilia-specific expression of ccdc114 and testis-specific expression of ccdc63 in humans (Onoufriadis *et al.*, 2013). Evidence from purification of *Ciona* sperm outer dynein arms (Hozumi *et al.*, 2006) shows that the *Ciona* ODA10p orthologue (IC4) copurifies with two *Ciona* ODA1p orthologues (IC5 and p66), but only one of these, IC5, is present at 1:1 stoichiometry with IC4. In *Ciona* IC4/IC5 may therefore be AC subunits equivalent to *Chlamydomonas* ODA10/ODA5.

Purification of outer dynein arm complexes from a wide range of organisms has not presented a consistent pattern of copurifying intermediate chains. Sea urchin ODA complexes typically have three ICs. Two of these are WD-repeat proteins homologous to *Chlamydomonas* IC1 and IC2, which are the only universal components

of all outer dynein arms, and the third is a hybrid thioredoxin/nucleoside diphosphate kinase (TNDK) that copurifies with ODAs from the sea urchin *Anthocidaris*, the trout *Oncorhynchus*, the ascidian *Halocynthia*, and the mussel *Mytilus* (Ogawa *et al.*, 1996), as well as the ascidian *Ciona* (Hozumi *et al.*, 2006), but is not present in ODA fractions of *Chlamydomonas* (Pfister and Witman, 1984) or of ciliates such as *Paramecium* (Walczak *et al.*, 1993). Database searches indicate that this TNDK-type IC is present in the genomes of a choanozoan (*Monosiga*) and a fungus (*Batrachochytrium*) in addition to the metazoa listed earlier but not in any other branches of eukaryotes and thus appears to be an Opisthokont invention. No other bands typically copurify with ODA complexes from ciliates, *Chlamydomonas*, sea urchin sperm, or mussel gill cilia. However, one or two additional unidentified bands are seen in fish sperm dynein fractions (Ogawa *et al.*, 1996; Ogawa and Inaba, 2006), the three coiled-coil proteins already discussed cofractionate with *Ciona* sperm dynein (Hozumi *et al.*, 2006), and under select buffer conditions and fractionation methods the coiled-coil DC1 and DC2 proteins copurify with *Chlamydomonas* outer arms (Takada *et al.*, 2002). On the basis of the copurification of an ODA10p homologue with ODA complexes from *Ciona* sperm, we speculate that *Chlamydomonas* ODA10p also interacts directly with outer dynein arms but that this interaction is relatively weak and not maintained under typical extraction and purification conditions.

ODA10p has only some of the properties expected of a subunit of an accessory docking complex. In *Chlamydomonas*, ODA10p remains associated with flagella after detergent treatment and is extractable in half-molar salt solutions, but unlike the DC, ODA10p fails to copurify with outer dynein arms (Figure 2). In addition, the flagellar assembly of ODA10p is independent of the assembly of outer dynein arms, the docking complex, or the presence of the *ODA8* gene product, consistent with the model of the AC as an independent axonemal complex (Wirschell *et al.*, 2004). The instability of ODA10p in the *oda5* mutant suggests the existence of a mutually dependent coiled-coil dimer between ODA10p and ODA5p, which may be similar to the dimer between mutually dependent coiled-coil proteins DC1 and DC2 in the docking complex (Wakabayashi *et al.*, 2001).

Surprisingly, our functional tests do not support the model of ODA10p as the subunit of a complex involved in anchoring dynein onto axonemal microtubules. Our observation that dynein extracted from wild-type axonemes can rebind to *oda10* axonemes at high affinity and in an ATP-insensitive manner, even though ODA10p is not present (Figure 4), combined with previous experiments showing that fractions containing only 12S and 18S dynein are sufficient to reconstitute functional outer dynein arms onto *oda5* axonemes (Takada and Kamiya, 1994), shows that dynein that has already assembled once onto doublets in vivo retains its high-affinity binding state for doublet docking sites after salt extraction and no longer requires ODA5p and ODA10p to rebind. In contrast, experiments with DC mutants such as *oda1* and *oda3* show that the DC is required for outer dynein arm binding in vitro (Takada and Kamiya, 1994; Takada *et al.*, 2002). We therefore hypothesize that the AC is an essential complex for the initial in vivo assembly of outer dynein arms but is not needed for dynein function postassembly. Because assembled outer dynein arm complexes can be immunoprecipitated from cytoplasmic extracts of *oda5*, *oda8*, and *oda10* cells (Fowkes and Mitchell, 1998), the AC is also not essential for the initial cytoplasmic assembly steps, but further tests are needed to see whether the dynein that assembles in an *oda10* cytoplasm is fully competent to bind to axonemes in vitro. The essential function of the AC thus could occur in the cytoplasm, during IFT transport, or at a late step of final association of dynein complexes with axonemal binding sites.

MATERIALS AND METHODS

Strains and culture conditions

The following strains of *Chlamydomonas reinhardtii* were obtained from the *Chlamydomonas* Genetics Center (University of Minnesota, Minneapolis, MN): wild-type *137c,mt- (CC-124)*, *137c,mt+ (CC-125)*, *S1D2,mt- (CC-2290)*, mutant strains *pf28,mt- (CC-1877)* (an allele of *oda2*; Mitchell and Rosenbaum, 1985), *oda1,mt+ (CC-2228)*, *oda5,mt+(CC-2236)*, *oda8,mt+(CC-2242)*, *oda10-1,mt+ (CC-2246)*, and *oda10-1,mt-(CC-2247)* (Kamiya, 1988); strains *oda5,ODA5HA (CC-4030)* and insertional mutants *oda10-2,mt-* and *oda10-2,mt-,arg7* (Wirschell *et al.*, 2004) were obtained from George Witman (University of Massachusetts Medical School, Worcester, MA). *oda5,oda10*, *oda5,oda8*, *oda8,oda10*, *oda5,oda10,ODA10HA*, and *oda8,oda10,ODA10HA* were constructed in our laboratory. Strains with *oda* phenotype, *oda5,oda10*, *oda8,oda10*, and *oda5,oda8*, were selected from nonparental ditype tetrads of the crosses between *oda5* and *oda10*, *oda8* and *oda10*, and *oda5* and *oda8*, respectively. The double mutant strains were crossed with *oda10,ODA10HA* to create *oda5,oda10,ODA10HA* and *oda8,oda10,ODA10HA* from nonparental ditype tetrads. *oda* mutant strains that expressed *ODA10HA* were selected using whole-cell Western blots probed with anti-HA antibody (see *Antibodies and immunodetection* for details). *oda10-2,ODA10HA* was created by cotransformation of HA-tagged *ODA10* cDNA with ARG2 gene plasmid pJD67 into an arginine-dependent *oda10-2,arg7* strain. Mating and tetrad analyses were performed according to standard protocols (Harris, 1989; Dutcher, 1995).

Antibodies and immunodetection

Antibodies used include rat monoclonal anti-HA epitope 3F10 (Roche, Indianapolis, IN), NAB1 (Agrisera AB, Vannas, Sweden), anti-ODA-IC2 (C11.4; Mitchell and Rosenbaum, 1986), and rabbit polyclonal anti-ODA10. Rabbit polyclonal anti-ODA10 was created by subcloning a cDNA region encoding an N-terminal fragment of ODA10p into the pMAL-p2 vector (New England BioLabs, Ipswich, MA) to make bacterially expressed SDS–PAGE-purified maltose-binding fusion proteins of ODA10p (amino acids 41–133), which was used to immunize rabbits for antibody production (Covance, Berkeley, CA). Antibodies were affinity purified against glutathione *S*-transferase fusion protein of the full-length ODA10 protein (Supplemental Figure S1). For Western blots, horseradish peroxidase–labeled secondary antibodies were detected using Super Signal West Dura Extend Duration (Pierce Chemical, Rockford, IL). The chemiluminescent signals were detected using either radiography or Chemidoc MP Imager (Bio-Rad, Hercules, CA). The blots were quantified using ImageJ (National Institutes of Health, Bethesda, MD) or Chemidoc software (Bio-Rad).

Positional cloning

ODA10 locus was positionally cloned using previously described amplification polymorphisms (Rymarquis *et al.*, 2005). To do linkage analysis between markers and the *oda10* locus, we isolated DNA from 67 fast-swimming recombinant progeny from a cross between *oda10-1* and polymorphic strain *S1D2*. We initially used known markers on chromosome 8 and then designed markers to map additional loci on chromosome 8 contigs predicted by the *Chlamydomonas* genome assemblies 3.0 and 4.0. Markers PBSQ, LI818, and MCA1 from a molecular mapping kit (Rymarquis *et al.*, 2005) were used to locate candidate regions for *ODA10* between

LI818 and MCA1. An additional marker, V3S294 on scaffold 29 of version 3 of the *C. reinhardtii* map (www.jgi.doe.gov/; Merchant *et al.*, 2007), was designed to amplify between markers LI818 and MCA1 and used to map *ODA10* between V3S294 and MCA1. Amplification probe ODA10-6 was selected to identify a locus near candidate gene C_290012. V3S294 and ODA10-6 were designed by the Primer-BLAST Primer designing tool, using standard procedures (Rymarquis *et al.*, 2005). Information regarding the markers is shown in Supplemental Table S1. Amplification was carried at 94°C for 1 min, the chosen temperature range or annealing temperature for 1 min, 72°C for 1 min (40 cycles), and 72°C for 10 min. NEB Taq polymerase with either NEB buffer or Advantage buffer SA, or Advantage Buffer with Advantage Polymerase (Clontech, Mountain View, CA), was used as noted.

cDNA cloning

Candidate gene model C_290012 on scaffold 29 on version 3 of the *C. reinhardtii* map was used to identify two expressed sequence tag (EST) sequences (AV640669 and AV640714) deposited by the Kazusa DNA Research Institute (Asamizu *et al.*, 1999). The full insert of EST AV640669 clone HCL020c08_r was sequenced, and sequences were deposited at the National Center for Biotechnology Information (Accession No. KF442967). When this sequence was compared with the predicted mRNA encoded by C_290012 (XM_00169594), it extended one exon and identified five more exons that were not predicted by Joint Genome Institute (JGI) version 3 or 4 of the *Chlamydomonas* genome. The coding region in this cDNA has two plausible start codons within the same reading frame. The second start codon was selected for generating expression constructs, based on alignment of predicted translation products from homologues in closely related algae (*Volvox*, *Micromonas*). Restriction sites *Nde*I and *Nru*I were inserted, respectively, at the 5′ and 3′ ends of this predicted coding region, using a QuikChange II XL Site-directed Mutagenesis kit (Invitrogen, Carlsbad, CA). The coding region was cloned between *Nde*I and *Nru*I sites in expression vector pGenDlinkerHA (Duquesnoy *et al.*, 2009).

Transformations

DNA was introduced into *Chlamydomonas* using the glass bead method as described previously (Kindle et al., 1989). Tagged ODA10 expression plasmids were cotransformed with pJD67 plasmid into *oda10-2,arg7,mt-(51b)* mutant. Individual transformant colonies were screened for rescued phenotype in liquid under a light microscope. Cotransformation rates were 4.7–6.8%. Expression levels of tagged ODA10p in rescued strains were determined using anti-HA Western blots on whole-cell samples.

Protein extraction and fractionation

Flagella and flagellar fractions were prepared as described previously (Ahmed and Mitchell, 2005). High-salt extract of axonemes of *oda10,ODA10HA* prepared with 0.6 M NaCl were dialyzed against 500× volume HMDEK (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 M $MgSO_4$, 1 mM dithiothreitol [DTT], 0.5 mM K acetate, pH 7.4) overnight at 4°C with one buffer change after the first hour. The high-salt extract was frozen in liquid nitrogen and kept at –75°C until use. The dialyzed dynein extracts were separated in sucrose gradient as described before (Takada *et al.*, 1994). Cytoplasmic extracts were made as described previously (Ahmed and Mitchell, 2005). Broken cells were pelleted in a microcentrifuge for 15 min, and supernatants were spun again for 30 min. The cytoplasmic extracts were either used immediately or frozen in liquid nitrogen and stored at –75°C until use.

Binding and dissociation assay

Axonemes were used within 24 h of preparation and were incubated with either crude dynein extract or pooled sucrose gradient fractions at ~1:1 stoichiometric ratio (by cell count) on ice for 1 h, with or without 1 mM ATP. These mixtures were spun in a microcentrifuge at top speed for 5 min. The pellet was resuspended in fresh HMDEK. For dissociation assay, the resuspended pellets were incubated on the ice for the noted length of time and microcentrifuged at top speed for 5 min. The pellets were resuspended in HMDEK and mixed with equal amount of 2× SDS sample buffer. Supernatants were concentrated by acetone precipitation and resuspended in 1XSDS-SB, so that the final volumes of pellets and corresponding supernatants were equal.

Motility analysis

Swimming speed was measure using videomicroscopy (30 frames/s) of at least 50 cells under a Zeiss Axioskop light microscope (Carl Zeiss, Jena, Germany) with dark-field illumination through a 635-nm cutoff filter. Motility paths were tracked and average swimming speeds were measured using CellTrak 1.5 software (Motion Analysis, Santa Rosa, CA). Beat frequency was measured with stroboscopic illumination.

Computational analysis

The BLAST server at www.ncbi.nlm.nih.gov/BLAST (Altschul *et al.*, 1997) and www.genome.jgi.doe.gov (Grigoriev *et al.*, 2012) were used to search for homologous sequences. PairCoil (Berger *et al.*, 1995) was used to determine whether a protein has a high probability of forming coiled coils, and COILS (Lupas *et al.*, 1991) was used to determine regions of high coiled-coil probability. Antigenicity of the peptide was determined using web.expasy.org/protparam/ (Gasteiger *et al.*, 2005). Multiple sequence alignments of protein sequences selected with BLASTp (scores <1E-10) in the genomes of an alga (*Micromonas*), a basal chordate (*Ciona*), a fish (*Danio*), and a mammal (*Mus*) using *Chlamydomonas* ODA1, ODA3, ODA5, or ODA10 as bait were generated by MAFFT using MEGA5 (Tamura *et al.*, 2011). An unrooted tree was generated from the alignment by the PHYLIP neighbor-joining method with a Jones–Taylor–Thornton distance matrix using SeaView (Gouy *et al.*, 2010).

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REFERENCES

Ahmed NT, Gao C, Lucker BF, Cole DG, Mitchell DR (2008). ODA16 aids axonemal outer row dynein assembly through an interaction with the intraflagellar transport machinery. J Cell Biol 183, 313–322.

Ahmed NT, Mitchell DR (2005). ODA16p, a *Chlamydomonas* flagellar protein needed for dynein assembly. Mol Biol Cell 16, 5004–5012.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25, 3389–3402.

Asamizu E, Nakamura Y, Sato S, Fukuzawa H, Tabata S (1999). A large scale structural analysis of cDNAs in a unicellular green alga, *Chlamydomonas reinhardtii*. I. Generation of 3433 non-redundant expressed sequence tags. DNA Res 6, 369–373.

Berger B, Wilson DB, Wolf E, Tonchev T, Milla M, Kim PS (1995). Predicting coiled coils by use of pairwise residue correlations. Proc Natl Acad Sci USA 92, 8259–8263.

Casey DM, Inaba K, Pazour GJ, Takada S, Wakabayashi K, Wilkerson CG, Kamiya R, Witman GB (2003). DC3, the 21-kDa subunit of the outer dynein arm-docking complex (ODA-DC), is a novel EF-hand protein important for assembly of both the outer arm and the ODA-DC. Mol Biol Cell 14, 3650–3663.

Duquesnoy P *et al.* (2009). Loss-of-function mutations in the human ortholog of *Chlamydomonas reinhardtii* ODA7 disrupt dynein arm assembly and cause primary ciliary dyskinesia. Am J Hum Genet 85, 890–896.

Dutcher SK (1995). Mating and tetrad analysis in *Chlamydomonas reinhardtii*. Methods Cell Biol 47, 531–540.

Fowkes ME, Mitchell DR (1998). The role of preassembled cytoplasmic complexes in assembly of flagellar dynein subunits. Mol Biol Cell 9, 2337–2347.

Freshour J, Yokoyama R, Mitchell DR (2007). *Chlamydomonas* flagellar outer row dynein assembly protein ODA7 interacts with both outer row and I1 inner row dyneins. J Biol Chem 282, 5404–5412.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005). Protein identification and analysis tools on the ExPASy server. In: The Proteomics Protocols Handbook, ed. JM Walker, Totowa, NJ: Humana Press, 571–607.

Gokhale A, Wirschell M, Sale W, Mitchell DR (2012). Dynein motility in cilia and flagella. In: Handbook of Dynein, ed. K Hirose and LA Amos, Singapore: Pan Stanford, 203–244.

Gouy M, Guindon S, Gascuel O (2010). SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27, 221–224.

Grigoriev IV *et al.* (2012). The genome portal of the Department of Energy Joint Genome Institute. Nucleic Acids Res 40, D26–D32.

Haimo LT, Fenton RD (1988). Interaction of *Chlamydomonas* dynein with tubulin. Cell Motil Cytoskeleton 9, 129–139.

Haimo LT, Telzer BR, Rosenbaum JL (1979). Dynein binds to and crossbridges cytoplasmic microtubules. Proc Natl Acad Sci USA 76, 5759–5763.

Harris EH (1989). The *Chlamydomonas* Source Book: A Comprehensive Guide to Biology and Laboratory Use, San Diego, CA: Academic Press.

Harris EH, Boynton JE, Gillham NW (1987). *Chlamydomonas* nuclear gene loci. In: Genetic Maps, ed. SJ O'Brien, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 258–265.

Hildebrandt F, Benzing T, Katsanis N (2011). Ciliopathies. N Engl J Med 364, 1533–1543.

Hozumi A, Satouh Y, Makino Y, Toda T, Ide H, Ogawa K, King SM, Inaba K (2006). Molecular characterization of *Ciona* sperm outer arm dynein reveals multiple components related to outer arm docking complex protein 2. Cell Motil Cytoskeleton 63, 591–603.

Kamiya R (1988). Mutations at twelve independent loci result in absence of outer dynein arms in *Chlamydomonas reinhardtii*. J Cell Biol 107, 2253–2258.

Kamiya R (2012). Functional diversity of axonemal dyneins. In: Handbook of Dynein, ed. K Hirose and LA Amos, Singapore: Pan Stanford, 267–284.

Kindle KL, Schnell RA, Fernandez E, Lefebvre PA (1989). Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. J Cell Biol 109, 2589–2601.

Koutoulis A, Pazour GJ, Wilkerson CG, Inaba K, Sheng H, Takada S, Witman GB (1997). The *Chlamydomonas reinhardtii* ODA3 gene encodes a protein of the outer dynein arm docking complex. J Cell Biol 137, 1069–1080.

Lupas A, Van DM, Stock J (1991). Predicting coiled coils from protein sequences. Science 252, 1162–1164.

Merchant SS *et al.* (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318, 245–250.

Mitchell DR, Rosenbaum JL (1985). A motile *Chlamydomonas* flagellar mutant that lacks outer dynein arms. J Cell Biol 100, 1228–1234.

Mitchell DR, Rosenbaum JL (1986). Protein-protein interactions in the 18S ATPase of *Chlamydomonas* outer dynein arms. Cell Motil Cytoskeleton 6, 510–520.

Mitchison HM *et al.* (2012). Mutations in axonemal dynein assembly factor DNAAF3 cause primary ciliary dyskinesia. Nat Genet 44, 381–382.

Ogawa K, Inaba K (2006). Ap58: A novel in situ outer dynein arm-binding protein. Biochem Biophys Res Commun 343, 385–390.

Ogawa K, Takai H, Ogiwara A, Yokota E, Shimizu T, Inaba K, Mohri H (1996). Is outer arm dynein intermediate chain 1 multifunctional? Mol Biol Cell 7, 1895–1907.

Omran H *et al.* (2008). Ktu/PF13 is required for cytoplasmic pre-assembly of axonemal dyneins. Nature 456, 611–616.

Onoufriadis A *et al.* (2013). Splice-site mutations in the axonemal outer dynein arm docking complex gene *CCDC114* cause primary ciliary dyskinesia. Am J Hum Genet 92, 88–98.

Pfister KK, Witman GB (1984). Subfractionation of *Chlamydomonas* 18 S dynein into two unique subunits containing ATPase activity. J Biol Chem 259, 12072–12080.

Rymarquis LA, Handley JM, Thomas M, Stern DB (2005). Beyond complementation. Map-based cloning in *Chlamydomonas reinhardtii*. Plant Physiol 137, 557–566.

Sakakibara H, Kamiya R (1989). Functional recombination of outer dynein arms with outer arm-missing flagellar axonemes of a *Chlamydomonas* mutant. J Cell Sci 92, 77–83.

Takada S, Kamiya R (1994). Functional reconstitution of *Chlamydomonas* outer dynein arms from alpha-beta and gamma subunits: requirement of a third factor. J Cell Biol 126, 737–745.

Takada S, Sakakibara H, Kamiya R (1992). Three-headed outer arm dynein from *Chlamydomonas* that can functionally combine with outer-arm-missing axonemes. J Biochem 111, 758–762.

Takada S, Wilkerson CG, Wakabayashi K, Kamiya R, Witman GB (2002). The outer dynein arm-docking complex: composition and characterization of a subunit (oda1) necessary for outer arm assembly. Mol Biol Cell 13, 1015–1029.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28, 2731–2739.

Wakabayashi K, Takada S, Witman GB, Kamiya R (2001). Transport and arrangement of the outer-dynein-arm docking complex in the flagella of *Chlamydomonas* mutants that lack outer dynein arms. Cell Motil Cytoskeleton 48, 277–286.

Walczak CE, Marchese-Ragona SP, Nelson DL (1993). Immunological comparison of 22S, 19S, and 12S dyneins from *Paramecium* cilia. Cell Motil Cytoskeleton 24, 17–28.

Wirschell M *et al.* (2013). The nexin-dynein regulatory complex subunit DRC1 is essential for motile cilia function in algae and humans. Nat Genet 45, 262–268.

Wirschell M, Pazour G, Yoda A, Hirono M, Kamiya R, Witman GB (2004). Oda5p, a novel axonemal protein required for assembly of the outer dynein arm and an associated adenylate kinase. Mol Biol Cell 15, 2729–2741.