The Oligomeric Outer Dynein Arm Assembly Factor CCDC103 Is Tightly Integrated within the Ciliary Axoneme and Exhibits Periodic Binding to Microtubules*

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Background: CCDC103 is necessary for the assembly of outer dynein arms on ciliary microtubules.

Results: CCDC103 is arrayed along the axoneme, self-assembles, and binds microtubules with a 12-nm periodicity.

Conclusion: CCDC103 is an integral component of the axoneme that underlies dynein assembly.

Significance: Defining how dynein arms are incorporated is necessary to understand the complex patterning of motile cilia.

CCDC103 is an ~29-kDa protein consisting of a central RPAP3_C domain flanked by N- and C-terminal coiled coils. Defects in CCDC103 lead to primary ciliary dyskinesia caused by the loss of outer dynein arms. This protein is present along the entire length of the ciliary axoneme and does not require other dynein or docking complex components for its integration. Unlike other known dynein assembly factors within the axoneme, CCDC103 is not solubilized by 0.6 M NaCl and requires more chaotropic conditions, such as 0.5 M KI. Alternatively, it can be extracted using 0.3% sarkosyl. CCDC103 forms stable dimers and other oligomers in solution through interactions involving the central domain. The smallest particle observed by dynamic light scattering has a hydrodynamic diameter of ~25 nm. Furthermore, CCDC103 binds microtubules directly, forming ~9-nm diameter particles that exhibit a 12-nm spacing on the microtubule lattice, suggesting that there may be two CCDC103 units per outer arm dynein repeat. Although the outer dynein arm docking complex is necessary to form arrays of dyneins along microtubules, it is not sufficient to set up a single array in a precise location on each axonemal doublet. We propose that CCDC103 helps generate a high-affinity site on the doublets for outer arm assembly, either through direct interactions or indirectly, perhaps by modifying the underlying microtubule lattice.

In vertebrates, defects in the assembly of outer and/or inner dynein arms within the axonemes of cilia and flagella lead to primary ciliary dyskinesia with phenotypes that include infertility, hydrocephalus, situs inversus, and chronic bronchial problems (1, 2). In addition to components of the dynein arms themselves, a variety of additional factors and complexes have been identified as being required for this process, either in vertebrates directly or in model organisms such as Chlamydomonas reinhardtii. Although the precise functional role of many of these assembly factors remains to be fully elucidated, they appear to fall into several general categories, including cytoplasmic components needed for preassembly of dynein complexes (e.g. DNAAF2 and DYX1C1 (3, 4)), adaptors that allow dyneins to be transported into the cilium by intraflagellar transport (e.g. ODA16 (5)), a protein in the ciliary matrix of completely unknown activity (C21orf59/FBB18 (6)), and structural components of the axoneme that enable the dynein arms to be docked at appropriate sites within the superstructure (e.g. the trimeric outer arm docking complex (7)). However, a convincing molecular model for how dyneins and the docking complex can be incorporated only at very specific locations on each axonomal doublet microtubule rather than at ectopic sites on the microtubule lattice has remained elusive.

In C. reinhardtii, the outer dynein arm is an ~2-MDa complex that contains three different heavy chain motor units, two WD-repeat intermediate chains (ICs), and a series of light chain (LC) components that are involved in assembly and/or regulation (8). However, although this complex can bind microtubules in an ATP-sensitive manner, it is not sufficient for the structural or ATP-independent association that is required for assembly within the axonomal superstructure even though two components, IC1 and LC1, interact with tubulin directly in a nucleotide-independent manner (9, 10). Genetic and biochemical studies have demonstrated that a trimeric docking complex containing two coiled coil proteins and a calmodulin homologue is needed for dynein assembly (7, 11–13) and acts as an adaptor to attach the motor to the microtubule wall (14). In addition, the C. reinhardtii ODA5/ODA10 proteins, which form part of an additional structure now known as the accessory complex (15), are necessary for outer arms to bind to axonomal doublet microtubules in vivo (15–17). Intriguingly, although the C. reinhardtii accessory complex is located within the axoneme, purified outer dynein arms can rebind in vitro to preformed axonemes that completely lack this structure (15). This suggests that it does not act as a canonical docking factor but, rather, plays a more subtle role in the assembly process.

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perhaps by converting dyneins transported into the growing organelle to an assembly-competent form (15). Furthermore, because isolated outer arm complexes from crude extracts that include the docking complex can bind at multiple sites around the circumferential surface of cytoplasmic microtubules (18), these docking factors, although essential, cannot be sufficient to define the precise axonemal sites of outer arm assembly on doublet microtubules. Together, these observations suggest that at least one, and probably more, additional step(s) and/or component(s) in the in vivo assembly reaction remain(s) to be identified.

Recently, defects in a small, well conserved, ~29-kDa predicted coiled coil protein (CCDC103) were identified in zebrafish and humans as leading to primary ciliary dyskinesia because of the loss of outer dynein arms (19). Initial biochemical studies of the C. reinhardtii orthologue (19) revealed that this protein is located within the axoneme and is present in wild-type amounts even in mutant strains lacking docking complex, accessory complex, or dynein proteins, suggesting that it is not dependent on any of these other systems for its axonemal localization. Furthermore, C. reinhardtii CCDC103 exhibited rather extraordinary biophysical properties, including a $T_m$ of ~80 °C, refolding to its native conformation following heating at 100 °C in the presence of SDS, and migrating as a dimer or higher-order oligomer following SDS-PAGE. Together, these findings suggest that CCDC103 is an essential component of the complex mechanism required to dock outer dynein arms at precise axonemal locations and likely plays a previously unanticipated role in this process.

In an attempt to further define the functional activity of CCDC103 in the outer arm dynein assembly reaction, we analyzed the biochemical and self-assembly properties of this protein in vitro. We demonstrate here that CCDC103 is indeed very tightly associated with the axoneme and is arrayed along the entire axonemal length. In solution, CCDC103 forms dimers and higher-order oligomers that bind microtubules in a concerted manner with a periodicity of 12 nm, suggesting there may be two CCDC103 units per outer arm dynein. This is consistent with our dynamic light scattering data that identified particles with a hydrodynamic diameter of ~25 nm. Furthermore, we found that oligomerization is a property of the CCDC103 central region and does not require either the N- or C-terminal coiled coil regions. On the basis of these data, we propose a model in which CCDC103 acts as a self-assembling template that helps define the sites of outer dynein arm incorporation within the growing axoneme.

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of Recombinant Proteins*—The coding regions for both C. reinhardtii and Homo sapiens CCDC103 were synthesized using the Escherichia coli codon bias and subcloned into the pET16b vector across the XmnI/ XbaI sites. Following transformation into E. coli strain BL21 (DE3), protein expression was induced by addition of 2 mM isopropyl 1-thio-β-D-galactopyranoside for 2 h or longer. Cell pellets were resuspended in 20 mM Tris (pH 8.0) 150 mM NaCl, frozen overnight, and, following defrosting, disrupted by sonication. The His$_{10}$-tagged proteins in the pellet were dissolved in 8 M urea, slowly diluted into 1 liter of 20 mM Tris (pH 8.0) 150 mM NaCl, and then purified by Ni$^{2+}$ affinity chromatography using 20 mM Tris pH 8.0, 500 mM NaCl, 250 mM imidazole for elution. Samples were subsequently concentrated in Amicon Ultra-4 ultrafiltration units. The N- and C-terminal regions of CCDC103 were fused to LC1 and maltose-binding protein using a LC1-pET16b construct made previously (20) and the standard pMal-c2 vector. Gel filtration chromatography of the recombinant proteins was achieved using Superose 6 10/300 and Superdex 200 10/300 columns attached to an Äkta Purifier 10 chromatography work station. Dynamic light scattering analysis of purified C. reinhardtii CCDC103 was performed using a Zetasizer ZSP (Malvern Instruments).

*Preparation of C. reinhardtii Flagellar Extracts*—C. reinhardtii wild-type strains cc124/125 were grown in R medium, harvested, and deflagellated with dibucaine using standard methods (21). Following demembranation with 1% Igepal-CA630 in 30 mM Hepes (pH 7.5), 5 mM MgSO$_4$, 1 mM EDTA, 25 mM KCl (HMEK), flagellar axonemes were extracted with either 0.6 M NaCl, 0.5 M KI or 1.0 M KI in HMEK. The 0.5 M KI extract was further fractionated by gel filtration chromatography using a Superose 6 10/300 column. For the pulldown assays, extracts were diluted 10-fold, and recombinant CCDC103 was added. Samples were then incubated with Ni$^{2+}$-charged beads, and, following several washes, bound proteins were eluted by heating in the presence of gel sample buffer.

Alternatively, axonemes were treated with sarkosyl (sodium N-lauroyl sarcosine, 0.3–1.0%) (22) in 30 mM Tris-Cl (pH 8.0), 0.5 mM EDTA, 150 mM NaCl, 5 mM Tris (2-carboxyethyl) phosphine for 2 h on ice. Extracted proteins and the axonemal remnants were separated by centrifugation in a microfuge.

*Electrophoresis and Immunoblotting*—For most experiments, protein samples were separated in either 10 or 12.5% polyacrylamide gels containing SDS. To examine dynein heavy chains extracted using sarkosyl, samples were separated in 4% acrylamide, 4 M urea gels without SDS in either the stacking or separating gels. Gels were subsequently either stained with Coomassie Blue or transferred to nitrocellulose using our standard methods. Blots were blocked with 5% dry milk 0.1% Tween 20 in Tris-buffered saline and then probed with blot-purified rabbit antibody (CT285) that was made against C. reinhardtii CCDC103. Following incubation with a peroxidase-conjugated goat anti-rabbit secondary antibody, antibody reactivity was detected by chemiluminescence using a LAS4000 digital imaging system (GE Healthcare). Quantitation of band intensities was performed using ImageQuant TL.

*Immunofluorescence*—Immunofluorescence of flagella from wild-type (cc125) and moda1 C. reinhardtii was performed essentially as described previously (23). In brief, isolated flagella were attached to poly-lysine-covered coverslips, fixed in methanol at ~20 °C, and air-dried. Samples were then blocked with 3% goat serum, 1% bovine serum albumin, 1% cold-water fish gelatin, 0.1% Igepal-CA630, and 0.05% Tween 20 in PBS. Samples were incubated with blot-purified CT285 rabbit antibody against CCDC103 and the 1869A mouse monoclonal antibody that specifically reacts with IC2 from the outer arm (24). Following addition of Alexa Fluor 488- and Alexa Fluor 594-conjugated second antibodies and multiple washes with PBS, sam-
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Domain Organization of CCDC103—CCDC103 is a mostly α-helical protein (19) consisting of three distinct structural domains (Fig. 1a). Predicted coiled coil regions are located at both the N and C termini of the H. sapiens protein (residues 6–46 and 224–240, respectively) (Fig. 1b), whereas the central segment of the molecule contains a RPAP3_C domain (residues 96–189, E value = 8.15e−28, pfam 13877). This latter domain has a very limited distribution and was originally described in RNA polymerase II-associated protein 3 (RPAP3) (28), which has since been shown to interact with reptin (29), a protein interacting with Hsp90 (PIH) domain-containing protein (PIHD1) (30) and a WD-repeat protein termed Monad or WDR92 (28), which we have found recently to be involved in ciliary assembly in planaria.3 Intriguingly, the RPAP3_C domain is also present in sperm-associated antigen 1 (SPAG1), which is required for axonemal dynein assembly in mammals (31). In both RPAP3 and SPAG1, the RPAP3_C domain is located C-terminal of several tetratricopeptide repeats. Secondary structure predictions for the C. reinhardtii and H. sapiens CCDC103 orthologues made using PREDICTPROTEIN suggest that there are ten discrete helices. Although these proteins share only 32% identity, the predictions for both molecules were highly similar except for an enlarged loop located between helices α6 and α7 in the C. reinhardtii orthologue (Fig. 1c). Hydrophobic cluster analysis (Fig. 1d) supports this general secondary structure assignment and the presence of an extended apparently unstructured region encompassing residues 60–100 between helices α2 and α3.

CCDC103 Is Tightly Associated with the Axoneme—We observed previously that CCDC103 was associated with the C. reinhardtii flagellar axoneme and that it was present in mutants lacking the outer arm, the outer arm docking complex, and the ODA5 protein, which is part of the accessory complex and also required for outer arm dynein assembly (19). Therefore, we concluded that CCDC103 does not require any of these other outer arm dynein components or assembly factors for its integration within the flagellar axoneme. To further test this idea, we first treated isolated axonemes with 0.6 M NaCl—conditions which remove the outer dynein arm, docking complex, and ODA5—and again found that essentially all CCDC103 remained associated with the salt-extracted axonemes. Subsequently, axonemes were treated with either 0.5 or 1.0 M KI, which is significantly more chaotropic than NaCl because it is further along the Hofmeister series. Under these conditions, most of the axonemal proteins, including tubulin, were solubilized, as was essentially all CCDC103 (Fig. 2a). When the 0.5 M KI extract was fractionated by gel filtration, we found that CCDC103 migrated as a large complex in the 400–500 kDa range (Fig. 2, b and c). Solubilized tubulin formed a single broad peak centered on a mass of ~400 kDa, indicating that it remains mainly oligomeric (Fig. 2, b and c). Therefore, in contrast to other known outer arm dynein assembly components, CCDC103 is very tightly integrated within the axonemal superstructure, requiring highly chaotropic high-salt conditions for solubilization.

To further address the location of CCDC103, we extracted purified wild-type axonemes with 0–1% sarkosyl, which sequentially solubilizes axonemal doublets and associated structures (Fig. 3). Under our buffer conditions, essentially all of the outer dynein arms were solubilized by 0.3% sarkosyl, as was CCDC103. In contrast, the protofilament ribbon protein Rib72 was almost completely resistant to extraction and remained in the pellet even after treatment with 1.0% sarkosyl.

Immunofluorescence analysis of a cell wall-less strain of C. reinhardtii using antibody CT285 (raised against C. reinhardtii CCDC103 fused to maltose-binding protein) revealed

3 R. S. Patel-King and S. M. King, unpublished results.
that CCDC103 was indeed present within both the flagella and cell body (Fig. 4a). Further analysis of purified wild-type flagella revealed that CCDC103 is located along the entire axonemal length but with an increased signal at both ends that likely derives from enhanced antigen accessibility at the exposed proximal and distal tips of the doublet microtubules (Fig. 4b). In contrast, although outer arm dynein IC2 was also arrayed along the axonemal shaft, it did not stain either terminal region. When we examined flagella from the oda1 strain that lacks the outer arms and docking complex, we found that the CCDC103 signal was essentially constant along the entire length, and we also occasionally observed enhanced staining at the tips (Fig. 4c), confirming that the outer arm docking complex is not required for CCDC103 localization.

In the cell body, the CT285 antibody against CCDC103 gave a diffuse staining pattern with multiple bright puncta throughout the cytoplasm but without a major localization to the basal body region. This is generally similar to the patterns observed
for outer arm dynein components such as IC2 (Fig. 4a) but unlike the ODA16 transport adaptor (5) or Lis1 (32), which, in cytoplasm, are both localized mainly to the flagellar base. In addition, CCDC103 was clearly present in a linear structure that spanned the region between the bases of the two flagella (Fig. 4d, arrow and insets). This structure was not stained by antibodies against either IC2 or acetylated tubulin and may represent the distal striated fiber (33, 34).

**Recombinant CCDC103 Forms Oligomers**—We found previously that both *C. reinhardtii* and zebrafish CCDC103 formed dimers that were stable under the conditions used for SDS-PAGE (19). To further examine this self-association, full-length *H. sapiens* CCDC103 containing an N-terminal His10 tag was expressed from the pET16b vector, and soluble protein was purified by Ni²⁺ affinity chromatography. Subsequently, the purified protein was fractionated by gel filtration chromatography using a Superose 6 column (Fig. 5a). Three distinct peaks of CCDC103 were obtained with calculated molecular weights consistent with dimer (~60 kDa), a potential octamer (~250 kDa), and very large complexes that eluted in the void volume with masses of >~2 MDa. The ~250-kDa peak was broad and smooth, suggesting that it may consist of a complex mixture of oligomeric species. We also prepared *C. reinhardtii* CCDC103 using the same methods and obtained a very comparable gel filtration profile containing the same three peaks (Fig. 5b), suggesting that both proteins behave similarly in solution. In addition, the *C. reinhardtii* profile exhibited a fourth major peak with a mass of ~120 kDa, which is consistent with a tetrameric species. Dynamic light scattering of the putative tetramer peak revealed that soluble *C. reinhardtii* CCDC103 could form par-
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FIGURE 3. Sarkosyl-mediated fractionation of axonemes. Purified wild-type axonemes were treated on ice for 2 h with 0–1% sarkosyl, and solubilized proteins and the axonemal remnants were separated by centrifugation. The top and center panels show the resulting supernatant (S) and pellets (P) separated in either a 4% acrylamide 4M urea gel with no SDS or a 10% acrylamide SDS gel. The location of the outer arm dynein heavy chains (DHCs), Rib72, and tubulin (Tub) are indicated. The bottom panel shows an immunoblot of similar samples probed with CT285 to detect CCDC103. Essential all of the outer arm dynein heavy chains were extracted by 0.3% sarkosyl, as was CCDC103. In contrast, the protofilament ribbon protein Rib72 remains in the pellet even after treatment with 1.0% sarkosyl.

To determine which region of CCDC103 is responsible for self-association, we prepared a series of truncated forms of the protein lacking either or both terminal coiled coil domains. When the soluble proteins were fractionated by gel filtration in a Superdex 200 column, all eluted as large oligomers, suggesting that the central region (residues 35–215) is both necessary and sufficient for self-association and that the coiled coil domains are not involved. To further test this, we prepared two additional proteins consisting of the CCDC103 C-terminal domain fused to the C terminus of maltose-binding protein and the N-terminal coiled coil attached to the N terminus of C. reinhardtii outer arm dynein LC1. Both fusions were soluble and eluted from the Superdex 200 gel filtration column as monomers when fractionated either individually or as a 1:1 mixture, indicating that, under these solution conditions, the CCDC103 coiled coil regions do not form either homo- or heterodimers (Fig. 6).

CCDC103 Associates Directly with Microtubules—Because CCDC103 is tightly bound to the axoneme, we then examined whether it might bind microtubules directly using a simple sedimentation assay with preformed Taxol-stabilized bovine brain microtubules (Fig. 7a). In the absence of microtubules, H. sapiens CCDC103 remained in the supernatant. However, when CCDC103 was incubated with microtubules, most protein bound and was present in the pellet. Furthermore, when CCDC103 was added to tubulin that was actively polymerizing following addition of 2 mM GTP and warming to 37 °C, nearly all of the CCDC103 protein was found in the pellet, although only a small fraction of tubulin had actually assembled during the assay (Fig. 7b). This strongly suggests that CCDC103 specifically binds polymerized microtubules and has little or no affinity for unpolymerized tubulin dimers. Neither of the CCDC103 coiled coil domains exhibited microtubule-binding activity, confirming that this activity is also a property of the central region containing the RPAP3-C domain.

Because axonemal doublet microtubules are highly resistant to depolymerization, we next examined whether CCDC103 might act as a microtubule-stabilizing factor. Brain microtubules were assembled at 37 °C by addition of 2 mM GTP in the presence or absence of CCDC103. The microtubules were pelleted, resuspended in fresh prewarmed buffer, and then placed at 4 °C for 20 min, followed by sedimentation in the airfuge (Fig. 7c). Under these conditions, in the absence of CCDC103, most microtubules depolymerized, and the resulting soluble tubulin was present in the supernatant. However, in the presence of CCDC103, ~37% more microtubules (on the basis of quantitation of Coomassie Blue-stained samples) were resistant to depolymerization, and essentially all CCDC103 was found associated with the microtubule pellet. Therefore, microtubules with CCDC103 bound are indeed resistant to cold depolymerization, as are axonemal microtubule doublets. Furthermore, because only a relatively small fraction of microtubules was stabilized but all CCDC103 was found in the pellet, CCDC103 binding may occur in a highly cooperative manner so that only a subset of microtubules has CCDC103 associated (see below).

To test whether CCDC103 could also interact with axonemal microtubules, we prepared 0.6 M NaCl and 0.5 M KI extracts of C. reinhardtii axonemes, reduced the salt concentration 10-fold by dilution, incubated them with His-tagged C. reinhardtii CCDC103, and isolated interacting proteins using Ni2+-charged beads (Fig. 7d). No major components of the NaCl extract were greatly enriched in the pulldown. However, a very significant increase (~2.5-fold) in the amount of tubulin obtained from the 0.5 M KI extract, which contains large amounts of tubulin oligomers from the solubilized axonemes (Fig. 2c), was observed in the presence of CCDC103.

Within the axoneme, outer dynein arms are assembled in linear rows with a precise spacing of 24 nm. Therefore, if CCDC103 were also to bind in a periodic manner, this might provide insight into its role in the assembly process. Consequently, we examined H. sapiens CCDC103-microtubule complexes by negative stain electron microscopy (Fig. 8) to determine whether CCDC103 could bind in a regular manner without any additional dynein components or assembly factors. We observed that, in this minimal system consisting only of polymerized tubulin and CCDC103, CCDC103 oligomers appeared as particles ~9 nm in diameter and formed linear arrays that decorated the microtubule wall. The oligomeric units bound with a spacing of 12 nm, suggesting that there are eight per 96-nm axonemal repeat (i.e. two per outer dynein arm) (Fig. 8, c and e, inset). We occasionally observed arrays of...
CCDC103 oligomers that appeared to be attached only at one end to the microtubule wall, with the remaining units extending away from the surface in a chain (Fig. 8e, arrow). In the absence of CCDC103, many short microtubules were present, likely as a result of shearing during sample preparation (Fig. 8a). However, in the presence of CCDC103, very few short microtubules were observed. Indeed, even when CCDC103-microtubule complexes had clearly broken during sample preparation, as evidenced by an abrupt acute change in polymer orientation on the carbon film (Fig. 8b, arrows), the resulting polymer ends remained in close proximity, presumably because of the tethering action of CCDC103 oligomers.

**DISCUSSION**

In this report, we analyzed the domain organization, *in situ* localization, and *in vitro* interaction properties of the outer dynein arm assembly factor CCDC103, which is essential for incorporating this motor into the ciliary axonemal superstructure (19). We find that CCDC103 is arrayed along the entire axoneme and forms dimers and higher-order oligomers. CCDC103 self-association is a property of the central region that includes an RPAP3_C domain and does not require either the N- or C-terminal coiled coils. Furthermore, CCDC103 binds directly and specifically to microtubules, forming long arrays that serve to stabilize the polymeric structure. In con-
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Contrast, it shows little or no affinity for free tubulin dimers. The observed CCDC103 spacing on the microtubule lattice is 12 nm, suggesting that two CCDC103 units assemble per outer arm dynein repeat of 24 nm. This is also consistent with the hydrodynamic diameter of the smallest particle observed in solution by dynamic light scattering. As discussed below, these data support a general model in which CCDC103 acts as a self-assembling system that is involved in defining the axonemal sites of outer dynein arm incorporation.

CCDC103 Oligomerization and Association with Microtubules—CCDC103 exhibits an apparently unique domain architecture with a central RPAP3_C domain flanked by N- and C-terminal coiled coil regions. The RPAP3_C domain has a very limited distribution, and, intriguingly, all proteins in which it has been found (CCDC103, RNA polymerase II-associated protein 3, and SPAG1) are either directly involved in ciliary assembly (CCDC103 (19) and SPAG1 (31)) or, in the case of RPAP3, interact with proteins (reptin (35) and WDR923) required for this process. Using various truncated recombinant forms and fusion proteins containing the coiled coil segments, we determined that intermonomer associations and microtubule binding involve the central region containing the RPAP3_C domain and that the coiled coils do not appear to form either homo- or heterodimers. In zebrafish, the schmalhans mutation results in expression of a truncated version of CCDC103 encompassing only part of the N-terminal coiled coil. Intriguingly, when schmalhans mutant mRNA was microinjected into the progeny of a schmalhans heterozygote cross (smhi/+ × smhi/+), it resulted in a more penetrant primary ciliary dyskinesia phenotype (19). Therefore, the future identi-
fication of the axonemal targets of these terminal segments will likely provide essential insights into the overall function of CCDC103.

When soluble *C. reinhardtii* CCDC103 was examined by dynamic light scattering, we observed three peaks corresponding to distinct oligomeric configurations. The smallest had an average hydrodynamic radius of 12.9 \( \pm \) 2.6 nm, which gives a particle diameter (25.8 \( \pm \) 5.2 nm) indistinguishable, within experimental error, from the outer arm dynein repeat distance within the axoneme of 24 nm. Upon examining CCDC103

![Graph showing oligomerization](image)

**FIGURE 6. Oligomerization requires the CCDC103 central domain.** a, soluble *H. sapiens* CCDC103 encoding residues 1–215, 25–242, and 35–215, which lack either the N- or C-terminal coiled coil domains or both, were fractionated in a Superdex 200 10/300 gel filtration column. All proteins migrated as complexes with masses of >200 kDa, suggesting that the central segment containing the RPAP3_C domain is required for oligomerization. Note that only a relatively small amount of the 35–215 protein was obtained. b, to test whether the CCDC103 coiled coils might be involved in dimer formation, the CCDC103 N-terminal coiled coil region (residues 1–40) was fused to the N-terminal residue of outer arm dynein LC1, and the CCDC103 C-terminal coiled coil was attached to the C terminus of maltose-binding protein (MBP). When applied to a Superdex 200 gel filtration column, both proteins eluted as monomers with no significant formation of homodimers. Furthermore, when mixed in a 1:1 ratio prior to chromatography, these proteins eluted as two overlapping peaks with no evidence of heterodimer formation. Electrophoretic analysis of the overlapping peaks is shown in the inset (Coomassie Blue stain).

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bound to microtubules by negative stain electron microscopy, we observed small particles (9 nm in diameter) arrayed in a linear manner along the microtubule long axis with a 12-nm spacing. Together, these data suggest a model in which CCDC103 oligomers form a bilobed structure 24 nm long.

The larger complexes observed by gel filtration and light scattering might then simply derive from further addition of these units to essentially form oligomeric chains. When combined with our previous observation (19) that CCDC103 reforms dimers and other oligomers following heat denaturation in SDS gel sample buffer, this suggests that CCDC103 is capable of self-assembly into polymeric arrays.

**CCDC103 as an Integral Component of the Axoneme**—We observed previously that CCDC103 was present in the *C. reinhardtii* axoneme and that mutants lacking the outer arm, the outer arm docking complex, or the accessory complex all retain wild-type levels of this protein (19). Therefore, CCDC103 can become incorporated into the axoneme in the complete absence of any other known dynein assembly factor. Our immunofluorescence data indicate that CCDC103 is actually present along the entire axonemal length. However, we did observe a very distinct enhanced signal at the ends of isolated flagella that could reflect an increased absolute amount of protein in these locations or, perhaps more likely because a greater signal was not observed at the flagellar base of intact cells, enhanced antibody accessibility for CCDC103 antigens at the ends of the doublet microtubules. This latter possibility might be expected if CCDC103 is located within the microtubule lumen, integrated within the doublet wall, or, more generally, rendered inaccessible by other overlying axonemal components. This latter interpretation would also be consistent with our observation that removing CCDC103 from the axoneme required highly chaotropic salt conditions that essentially solubilized the entire superstructure, which is in sharp contrast to the other known outer arm docking factors that can be almost completely removed by relatively mild treatment with 0.6 M NaCl. We also treated axonemes with sarkosyl, which was used to sequentially solubilize first the B tubule and then the A tubule, ultimately leaving a protofilament ribbon remnant (22). We found that CCDC103 and all dynein arms were removed by 0.3% sarkosyl, whereas most tubulin remained in the pellet. Therefore, CCDC103 is sufficiently accessible that it can be removed without solubilizing the entire outer doublet.

**FIGURE 7.** CCDC103 Associates directly with microtubules. *a*, *H. sapiens* CCDC103 in the presence or absence of Taxol-stabilized microtubules (MTs) was spun for 5 min in an airfuge. Nearly all CCDC103 was found to be associated with the microtubule pellet (P), whereas it remained in the supernatant (S) in the absence of microtubules. *b*, bovine brain tubulin with or without *H. sapiens* CCDC103 was placed at 37 °C in the presence of 2 mM GTP. Following sedimentation in the airfuge, essentially all CCDC103 was found to be associated with the small amount of tubulin that polymerized. *c*, microtubules were polymerized by addition of 2 mM GTP at 37 °C and sedimented in an airfuge. The pellets were resuspended in fresh microtubule polymerization buffer and placed on ice for 20 min. Nearly all microtubules depolymerized under these conditions. However, essentially all *H. sapiens* CCDC103 was found to be associated with the small amount of tubulin that did not depolymerize, suggesting that it acts to stabilize microtubules against cold depolymerization. *d*, recombinant His-tagged *C. reinhardtii* CCDC103 was incubated with either 0.6 M NaCl or 0.5 M KI extracts that had been diluted 10-fold to reduce the salt concentration. Following pulldown of CCDC103 using Ni²⁺-charged beads, a significant increase in the amount of tubulin present was observed from the 0.5 M KI extract. This suggests that *C. reinhardtii* CCDC103 also binds tubulin oligomers and that this is, therefore, a conserved property of this protein. All samples were electrophoresed in 12.5% (*a–c*) or 10% (*d*) SDS-polyacrylamide gels and stained with Coomassie Blue.
FIGURE 8. CCDC103 forms linear arrays along microtubules. Shown are transmission electron micrographs of Taxol-stabilized microtubules incubated with buffer alone (a and c) or with *H. sapiens* CCDC103 (b, d and e). CCDC103 forms arrays of circular or C-shaped particles (~9 nm in diameter) that bind along the microtubules. The average spacing between particles is 12 nm. In some cases, what appear to be linear arrays of CCDC103 particles are attached to the microtubule at one end and extend away from the surface (e, arrow). The arrows in the inset in e indicate the CCDC103 particles arrayed in a periodic manner, and the bars mark the tubulin protofilaments. In the absence of CCDC103, many short microtubules are evident (a), whereas those with CCDC103 bound appear longer, and even when clearly broken, presumably during sample preparation (b, arrows), the fractured ends remain associated. Scale bars = 2 µm (a and b), 100 nm (c and d), 50 nm (e), and 25 nm (e, inset).
Compared with brain cytoplasmic microtubules, axonemal outer doublets exhibit extraordinary resistance to cold depolymerization in vitro, suggesting that one or more non-tubulin components act to stabilize the structure. We found that brain cytoplasmic microtubules with bound CCDC103 were almost completely resistant to cold depolymerization, which raises the intriguing possibility that the presence of CCDC103 contributes directly to axonemal stability.

**A Model for CCDC103 Function in Outer Arm Dynein Assembly**—There are two distinct but related modes by which axonemal dyneins can become incorporated into the axoneme. The first occurs as the flagellum is actively growing and dynein arms are added, likely sequentially, as components are trafficked into the organelle, presumably by intraflagellar transport, and the structure elongates. In this situation, there is continual addition of dynein components as the structure grows, and we predict that there will be little axonemal doublet length near the growing distal tip on which dyneins are not fully assembled. The second mode, although it also occurs in vivo, might be considered somewhat artificial in the sense that it only apparently occurs when cells of a strain lacking, for example, dynein arms and/or docking complex proteins fuse to a wild-type or different mutant strain, generating a quadriflagellate cell with a shared cytoplasm. In this situation, dynein components become trafficked into a preformed, full-length organelle with empty binding sites exposed and available along the entire structure. Under these conditions, docking complex proteins have been observed to add sequentially from base to tip in the flagella that originally lacked them, whereas the dynein arms themselves appeared to add randomly along the flagellar length, provided the docking complex had already been incorporated. These observations led to the clear and well supported hypothesis that sequential cooperative docking complex assembly underlies dynein arm incorporation (14). However, what is much less apparent is how the initial sites of docking complex attachment are patterned and why docking complexes do not assemble at inappropriate or ectopic locations on the microtubule doublets.

Although the docking complex is capable of binding cytoplasmic microtubules directly, the $K_d$ for this interaction ($1.6 \times 10^{-7}$ M$^{-1}$) is one order of magnitude greater than that for the association of the docking complex with axonemes from the $oda1$ mutant ($0.78 \times 10^{-8}$ M$^{-1}$), which lack this structure (14). Therefore, one potential role for CCDC103 is to modify the doublet microtubule in a manner to provide a very high affinity binding site for the docking complex. In this scenario, we predict that the linear self-assembly of CCDC103 during axonemal growth would provide a single very high affinity track for docking complex incorporation on each doublet. This might be achieved either through direct interactions between CCDC103 and the docking complex or indirectly, perhaps by altering the conformation of the microtubule wall itself or some other com-
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ponent to promote docking complex association. A model for the components and interactions needed for outer dynein arm incorporation in the axoneme is shown in Fig. 9. However, this model still leaves open the key question of how the initial site of CCDC103 incorporation could be set up. Because this protein is very tightly integrated with the microtubule, one intriguing possibility is that incorporation is templated by the basal body from which the axonemal doublet microtubules grow directly. Alternatively, CCDC103 incorporation might be patterned from the distal region of the transition zone beyond which dynein arm addition occurs. In either scenario, when incorporation is initiated, CCDC103 self-assembly would then generate (directly or indirectly) the high-affinity sites needed for dynein attachment.

In conclusion, we found that CCDC103 forms linear arrays along microtubules with a regular spacing consistent with the axonemal outer dynein arm repeat distance. Both inter-CCDC103 and CCDC103-microtubule interactions involve the central region, which includes a RPAP3_C domain, whereas the targets of the N- and C-terminal coiled coil sections remain unknown. We propose a model in which the self-assembly of CCDC103 in the growing axoneme either acts directly as an association site or modifies the doublet microtubule to provide a single array of high-affinity binding sites for the outer arm docking complex. Finally, although CCDC103 has been found to play an essential, and perhaps foundational, role in outer dynein arm assembly, it is clear that there is much that remains to be learned about the function of this enigmatic protein in dynein and ciliary biology.

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REFERENCES

1. Fliegauf, M., Benzing, T., and Omran, H. (2007) When cilia go bad: cilia defects and ciliopathies. Nat. Rev. Mol. Cell Biol. 8, 880–893
2. Hildebrandt, F., Benzing, T., and Katsanis, N. (2011) Ciliopathies. New Engl. J. Med. 364, 1533–1543
3. Omran, H., Kobayashi, D., Olbrich, H., Tsukahara, T., Loges, N. T., Hagiwa, H., Zhang, Q., Leblond, G., O’Toole, E., Hara, C., Mizuno, H., Kawano, H., Fliegauf, M., Yagi, T., Koshida, S., Miyawaki, A., Zentgraf, H., Seithe, H., Reinhardt, R., Watanabe, Y., Kamiya, R., Mitchell, D. R., and Takeda, H. (2008) Ktu/PF13 is required for cytoplasmic pre-assembly of axonemal protein important for assembly of both the outer arm and the ODA-DC. J. Biol. Chem. 283, 283–295
4. Nakamura, H., Takada, S., Wilkerson, C. G., Wakabayashi, K., Kamiya, R., and Wittman, G. B. (2002) The outer arm docking complex: composition and characterization of a subunit (oda1) necessary for outer arm assembly. Mol. Biol. Cell 13, 1015–1029
5. Koutoulis, A., Pazour, G. J., Wilkerson, C. G., Inaba, K., Sheng, H., Takada, S., and Wittman, G. B. (1997) The Chlamydomonas reinhardtii ODA3 gene encodes a protein of the outer dynein arm docking complex. J. Cell Biol. 137, 1069–1080
6. Casey, D. M., Inaba, K., Pazour, G. J., Takada, S., Wakabayashi, K., Wilkerson, C. G., Kamiya, R., and Wittman, G. B. (2003) DC3, the 21-kD subunit of the outer 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
7. Owa, M., Furuta, A., Usukura, J., Arisaka, F., King, S. M., Wittman, G. B., Kamiya, R., and Wakabayashi, K. (2014) Cooperative binding of the outer arm-docking complex underlies the regular arrangement of outer arm dynein in the axoneme. Proc. Natl. Acad. Sci. U.S.A. 111, 9461–9466
8. Dean, A. B., and Mitchell, D. R. (2013) Chlamydomonas ODA10 is a conserved axonemal protein that plays a unique role in outer dynein arm assembly. Mol. Biol. Cell 24, 3689–3696
9. Kamiya, R. (1988) Mutations at twelve independent loci result in absence of outer dynein arms in Chlamydomonas reinhardtii. J. Cell Biol. 107, 2253–2258
10. Wirtschell, M., Pazour, G., Yoda, A., Hirono, M., Kamiya, R., and Wittman, G. (2004) Oda5p, a novel axonemal protein required for assembly of the outer dynein arm and an associated adenylyl kinase. Mol. Biol. Cell 15, 2729–2741
11. Haimo, L. T., and Fenton, R. D. (1988) Interaction of Chlamydomonas dynein with tubulin. Cell Motil. Cytoskeleton 9, 129–139
12. Panizzi, J. R., Becker-Heck, A., Castleman, V. H., Al-Mutairi, D. A., Liu, Y., Loges, N. T., Pathak, N., Austin-Tse, C., Elder, K., Schwarting, M., Lohmer, S., Brown, P., Frey, C., and Witman, G. B. (1997) Solution structure of a dynein motor domain of the outer dynein arm-docking complex. J. Biol. Chem. 272, 1069–1080
13. King, S. M., Wilkerson, C. G., and Witman, G. B. (1991) The Mr 78,000 intermediate chain of Chlamydomonas outer arm dynein interacts with α-tubulin in situ. J. Biol. Chem. 266, 8401–8407
14. Patel-King, R. S., and King, S. M. (2009) An outer arm dynein light chain acts in a conformational switch for flagellar motility. J. Cell Biol. 186, 737–745
15. Collins, C. M., Johnson, A. R., Pfeffer, S. M., King, S. M., and Witman, G. B. (1993) Defects in axonemal dyneins. Cell Motil. Cytoskeleton 24, 47–60
16. Kamiya, R. (1988) Mutations at twelve independent loci result in absence of outer dynein arms in Chlamydomonas reinhardtii. J. Cell Biol. 107, 2253–2258
17. Wirtschell, M., Pazour, G., Yoda, A., Hirono, M., Kamiya, R., and Wittman, G. (2004) Oda5p, a novel axonemal protein required for assembly of the outer dynein arm and an associated adenylyl kinase. Mol. Biol. Cell 15, 2729–2741
18. Haimo, L. T., and Fenton, R. D. (1988) Interaction of Chlamydomonas dynein with tubulin. Cell Motil. Cytoskeleton 9, 129–139
19. Panizzi, J. R., Becker-Heck, A., Castleman, V. H., Al-Mutairi, D. A., Liu, Y., Loges, N. T., Pathak, N., Austin-Tse, C., Elder, K., Schwarting, M., Lohmer, S., Brown, P., Frey, C., and Witman, G. B. (1997) Solution structure of a dynein motor domain of the outer dynein arm-docking complex. J. Biol. Chem. 272, 1069–1080
20. Patel-King, R. S., and King, S. M. (2009) An outer arm dynein light chain acts in a conformational switch for flagellar motility. J. Cell Biol. 186, 737–745
21. King, S. M. (1995) Large-scale isolation of Chlamydomonas flagella. Methods Cell Biol. 47, 9–12
22. Witman, G. B., Carlson, K., Berliner, J., and Rosenbaum, J. L. (1972) Chla-mydomonas flagella: I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigemesones. J. Cell Biol. 54, 507–539
23. Rompolas, P., Pedersen, L. B., Patel-King, R. S., and King, S. M. (2007) Chlamydomonas FAP133 is a dynein intermediate chain associated with...
the retrograde intraflagellar transport motor. *J. Cell Sci.* **120**, 3653–3665
24. King, S. M., Otter, T., and Witman, G. B. (1986) Purification and characterization of *Chlamydomonas* flagellar dyneins. *Methods Enzymol.* **134**, 291–306
25. Rost, B., and Sander, C. (1993) Prediction of protein structure at better than 70% accuracy. *J. Mol. Biol.* **232**, 584–599
26. Lupas, A., Van Dyke, M., and Stock, J. (1991) Predicting coiled coils from protein sequences. *Science* **252**, 1162–1164
27. Gaboriaud, C., Bissery, V., Benchetrit, T., and Mornon, J. (1987) Hydrophobic cluster analysis: an efficient new way to compare and analyze protein sequences. *FEBS Lett.* **224**, 149–155
28. Itsuki, Y., Saeki, M., Nakahara, H., Egusa, H., Irie, Y., Terao, Y., Kawabata, S., Yatani, H., and Kamisaki, Y. (2008) Molecular cloning of novel Monad binding protein containing tetratricopeptide repeat domains. *FEBS Lett.* **582**, 2365–2370
29. Ni, L., Saeki, M., Xu, L., Nakahara, H., Suijo, M., Tanaka, K., and Kamisaki, Y. (2009) RPAP3 interacts with Reptin to regulate UV-induced phosphorylation of H2AX and DNA damage. *J. Cell. Biochem.* **106**, 920–928
30. Inoue, M., Otter, T., and Witman, G. B. (1983) Outer doublet heterogeneity reveals structural polarity related to beat direction in *Chlamydomonas* flagella. *J. Mol. Biol.* **232**, 584–599
31. Taillon, B. E., Adler, S. A., Suhan, J. P., and Jarvik, J. W. (1992) Mutational analysis of centrin: an EF-hand protein associated with three distinct contractile fibers in the basal body apparatus of *Chlamydomonas*. *J. Cell Biol.* **119**, 1613–1624
32. Zhao, L., Yuan, S., Cao, Y., Kallakuri, S., Li, Y., Kishimoto, N., DiBella, L., and Sun, Z. (2013) Reptin/RuvB12 is a Lrrc6/Seahorse interactor essential for cilia motility. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 12697–12702

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