DRC2/CCDC65 is a central hub for assembly of the nexin–dynein regulatory complex and other regulators of ciliary and flagellar motility

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INTRODUCTION

Cilia and flagella are microtubule-based organelles that play essential roles in human health and development, serving both as sensory antennae and as motile structures. Defects in the assembly, signaling, or motility of these organelles have severe consequences for an organism, and lead to a diverse spectrum of diseases known as ciliopathies in humans (reviewed in Mitchison and Valente, 2017; Reiter and Leroux, 2017). Ciliary and flagellar motility are critical for the clearance of mucus and foreign particles from the respiratory tract, fertilization in the reproductive tract, circulation of fluid in the brain and spinal cord, and the determination of the left–right body axis during embryonic development. Genomic and proteomic approaches have identified more than 600 polypeptides as structural subunits of the ciliary axoneme, and several other proteins contribute to the assembly and transport of these components into the axoneme (Li et al., 2004; Pazour et al., 2005; Ishikawa et al., 2012; Blackburn et al., 2017). Advances in the development of high-resolution structural techniques, combined with the highly ordered and repetitive nature of axoneme structure, have yielded insights into the location and interactions of several polypeptides (reviewed in Mizuno et al., 2012), but thus far only a fraction of the ciliary proteins have been clearly correlated with a specific structure and/or function.

Most motile cilia and flagella contain nine outer doublet microtubules arranged in a ring that surrounds two singlet microtubules in the central pair (CP) to form the 9 + 2 axoneme. The outer and inner
dynein arms (ODA and IDA) are large, multisubunit motor proteins that drive interdoublet sliding (Gibbons, 1965), and they form two distinct rows along the length of the A-tubule of each outer doublet. Both the ODAs and IDAs walk along the surface of the B-tubule of the neighboring outer doublet in an ATP-sensitive manner, pushing the adjacent doublets toward the tip of the axoneme (Satir, 1968). In an intact cell, the outer doublet microtubules are attached to basal bodies at their minus ends, and the outer doublets are also cross-linked by nexin links every 96 nm along their length. These structures limit the extent of interdoublet sliding and convert microtubule sliding into axonemal bending. However, given that all dyneins are minus-end-directed motors, the geometry of the axoneme dictates that the site of dynein activity must switch between opposite sides of the axoneme for effective bending to occur (Sale and Satir, 1977; Satir, 1985; Fox and Sale, 1987). In addition, most organisms can modify the pattern of axonemal bending in response to internal or external stimuli.

The study of motility mutants in the unicellular alga Chlamydomonas has identified several axonemal complexes as important regulators of dynein activity and flagellar motility, such as the CP microtubules and the radial spokes (RS) that transmit signals from the CP projections to the dynein arms on the outer doublet microtubules (reviewed in Smith and Yang, 2004). Mutants that lack either the CP or the RS are often paralyzed, and the velocity of dynein-driven sliding in sliding–disintegration assays is also reduced (Witman et al., 1978; Smith and Sale, 1992; Smith 2002). The prevailing model is that the projections of the CP microtubules make contact with the RS in an asymmetric manner during flagellar bending. These signals are then transduced through the RS to regulatory complexes located at the base of the RS to coordinate the activity of the multiple dynein motors (Smith and Yang, 2004). The study of extragenic suppressors that restore limited motility to paralyzed CP/RS mutants has identified two of these regulatory complexes (Huang et al., 1982). The first is the intermediate chain/light chain (IC/LC) complex of the two-headed 11 dynein, which is located at the proximal end of the 96-nm repeat, at the junction between the base of RS1, several IDAs, the MIA complex, and the row of ODAs (Bower et al., 2009; Heuser et al., 2012a; Yamamoto et al., 2013). The second is the nexin–dynein regulatory complex (N-DRc), which is located at the distal end of the 96-nm repeat, at the junction between the bases of RS2 and the shorter RS3S, several other IDAs, the calmodulin- and spoke-associated complex (CSC), and the row of ODAs (Mastronarde et al., 1992; Piperno et al., 1992; Gardner et al., 1994; Heuser et al., 2009, 2012b).

The N-DRc is a large 1.5-MDa structure composed of at least 11 distinct subunits (Heuser et al., 2009; Lin et al., 2011; Bower et al., 2013). It contains a base plate that attaches to the surface of the outer doublet, beginning at the inner junction between the A- and B-tubule and wrapping around the A-tubule to protofilament A4, and a linker that extends across the interdoublet space to the adjacent B-tubule. Both the base plate and the linker make contacts with other substructures on the outer doublet (Heuser et al., 2009). The precise arrangement of the 11 subunits in the structure of the N-DRc is unknown, but recent work with epitope-tagged subunits has revealed the approximate locations of DRC1-5. The C-termini of DRC1, DRC2, and DRC4 are located in the base plate, near the A- and B-tubule inner junction, and the N-termini extend through the linker toward the neighboring B-tubule (Oda et al., 2015; Song et al., 2015). DRC3 and DRC5 are located in the linker region (Awata et al., 2015; Oda et al., 2015; Song et al., 2015). Mutations in individual DRC subunits have diverse phenotypes (Table 1). drc3 and drc5 (sup-pf4) mutations have limited impact on the assembly of other DRC subunits and subtle effects on motility (Huang et al., 1982; Awata et al., 2015), whereas drc1 (pf3) and drc4 (pf2, sup-pf3) mutations have more dramatic effects on motility and the assembly of other DRC subunits and associated IDAs (Gardner et al., 1994; Piperno et al., 1994; Heuser et al., 2009; Lin et al., 2011; Bower et al., 2013; Wirschell et al., 2013). Defects in the assembly of the ODAs, IDAs, CP projections, RS, and N-DRc have been linked to ciliary motility defects and primary ciliary dyskinesia (PCD) in human patients (reviewed in Mitchison and Valente, 2017). However, detailed characterization of biochemical and structural defects in human cilia is challenging due to the limited quantity of purified material available for analysis. In these cases, comparative studies of mutant phenotypes in model organisms have proven to be valuable in determining the likely impact of a given disease-causing mutation. For example, PCD mutations have been identified in DRC1/CCDC164 and DRC4/GAS5, and analysis of the motility, biochemical, and structural phenotypes observed with related drc mutations in Chlamydomonas has provided insights into the defects that lead to human disease (Wirschell et al., 2013; Olbrich et al., 2015; Jeanson et al., 2016; Lewis et al., 2016). PCD mutations have also been identified in CCDC65, the human orthologue of DRC2, but in Chlamydomonas, DRC2 is linked to an inner arm motility mutant known as ida6 (Austin-Tse et al., 2013; Horani et al., 2013). The ida6 mutant was first described as a slow-swimming strain with a defect in the assembly of single IDA known as dyneine e (Kato et al., 1993). Identification of ida6 as a drc2 mutation prompted a reevaluation of axoneme structure by thin section transmission electron microscopy (TEM). The two-dimensional (2D) averaging of longitudinal images revealed the loss of a crescent-shaped structure above RS2, similar to that seen in previous images of other drc mutants (Austin-Tse et al., 2013).

To obtain more complete information about the function of DRC2 and its role in the assembly of the N-DRc and IDAs, we have characterized the molecular, biochemical, and structural phenotypes of two drc2 mutations in detail. First, we generated and characterized several epitope-tagged strains for analysis of N-DRc assembly in vivo and in vitro. We then used iTRAQ labeling, quantitative mass spectrometry, and spectral counting to identify the polypeptide defects in mutant axonemes and more precisely define the IDAs most closely associated with the N-DRc. Finally, we employed high-resolution cryo-ET and classification analysis to more rigorously describe the structural defects in drc2/ida6 mutant axonemes and to compare drc2/ida6 with another closely related n-drc mutant, drc1/pf3. These studies reveal the importance of both DRC1 and DRC2 in stabilizing interactions between the N-DRc base plate, multiple IDAs, the RS, and the CSC. They also indicate how defects in DRC1 and DRC2 can lead to such profound changes in ciliary motility in other organisms and result in PCD.

RESULTS
sup-pf5 is a drc2 mutation
We previously identified DRC2 as the mutant gene product of the IDA6 locus (Austin-Tse et al., 2013). However, there is little information about the impact of a defective DRC2 subunit (also known as FAP250) on the assembly of other axonemal proteins in the ida6 strain. The ida6 mutation is caused by a single nucleotide substitution in the stop codon and is predicted to add 101 amino acids to the C-terminus of the DRC2 polypeptide, but the resulting protein is not assembled into the axoneme (Figure 1, A and B; see also Austin-Tse et al., 2013). The sup-pf5 mutant is a slow-swimming strain that was isolated as an extragenic suppressor of paralyzed RS/CP mutants (Piperno et al., 1992). The sup-pf5 mutation is a
single-base-pair change (TGG to TGA) in exon 7 that results in a premature stop codon that is predicted to truncate DRC2 at amino acid 291 in the middle of the protein, specifically in the third coiled-coil domain (Figure 1, B–D). Western blot analysis confirmed that DRC2 is truncated and significantly reduced in sup-pf5 axonemes (Figure 1E). However, both DRC2-GFP and DRC2-HA assembled into the axoneme in rescued strains and migrated at positions near WT levels (Figure 2, C and D).

**Rescue of ida6 and sup-pf5 with epitope-tagged DRC2**

We showed previously that DRC4 and DRC5 are distributed along the length of the flagella and near the basal body region in Chlamydomonas cells (Rupp and Porter, 2003; Bower et al., 2013). To determine the distribution of DRC2, we transformed ida6 and sup-pf5 with HA- and GFP-tagged constructs of the wild-type (WT) DRC2 gene (Figure 1B) and screened for transformants with WT motility. As expected, DRC2-GFP and DRC2-HA were found along the length of the flagella and in the basal body region (Figure 2, A and B). Measurements of flagellar motility showed that the epitope-tagged DRC2 constructs rescued both strains and increased swimming velocities to near WT levels (Figure 2, C and D).

To determine the impact of drc2 mutations on the assembly of other DRC subunits, we analyzed isolated axonemes from WT, mutant, and rescued strains on Western blots. As shown in Figure 2E, DRC2 migrated at ∼65 kD in WT and the DRC2-rescued strain but could not be detected in ida6, similar to previous results (Austin-Tse et al., 2013). However, both DRC2-GFP and DRC2-HA assembled into the axoneme in rescued strains and migrated at positions near WT levels (Figure 2, C and D).

**TABLE 1: Polypeptide defects observed in n–drc mutants.**

| Polypeptide          | Size (kDa) | Wild-type (drc3) | sup–pf3 (drc4) | ida6 (drc2) | sup–pf3 (drc4) | pf2 (drc4) | sup–pf4 (drc5) |
|----------------------|------------|------------------|---------------|-------------|---------------|------------|---------------|
| DRC1                 | 79         | +                | −             | −           | +             | +          | +             |
| DRC2                 | 65         | +                | −             | −           | +             | +          | +             |
| DRC3                 | 60         | +                | ±             | ±           | −             | ±          | −             |
| DRC4                 | 55         | +                | +             | +           | +             | ±          | −             |
| DRC5                 | 43         | +                | −             | −           | +             | −          | −             |
| DRC6                 | 28         | +                | −             | ND          | +             | −          | −             |
| DRC7                 | 177        | +                | ±             | ±           | +             | ±          | −             |
| DRC8                 | 19         | +                | ±             | −           | +             | ±          | ±             |
| DRC9                 | 46         | +                | ±             | ND          | +             | ±          | ±             |
| DRC10                | 41         | +                | ±             | ND          | +             | ND         | ND            |
| DRC11                | 95         | +                | −             | −           | +             | −          | −             |

Monomeric dyneins

- +: Present at WT levels; −: not detected; ±: reduced; ND = not determined.
- DRC subunit data compiled from Huang et al., 1982; Piperno et al., 1992, 1994; Lin et al., 2011; Bower et al., 2013; Wirschell et al., 2013; Awata et al., 2015; this study.
- Dynein subunit data compiled from Piperno et al., 1992, 1994; Kato et al., 1993; Piperno, 1995; Gardner et al., 1994; Bui et al., 2012; Bower et al., 2013; Wirschell et al., 2013; this study.
- CaM–IP3 data compiled from Bower et al., 2013; Wirschell et al., 2013; this study.
- Tektin data compiled from Yanagisawa and Kamiya, 2004; Austin–Tse et al., 2013; Wirschell et al., 2013; this study.
Assembly and turnover of DRC2 subunits in dikaryon flagella

The dikaryon rescue experiments are shown in Figure 2, G and H. Cells expressing DRC2-GFP were mated to unlabeled WT cells or ida6 cells to form quadriflagellate dikaryons, fixed at various time points after mating, and then analyzed by immunofluorescence for the appearance of GFP signal in the two flagella of the unlabeled cells. When DRC2-GFP cells were mated to WT cells, very little GFP signal could be detected in the two flagella from the WT parent, even 1 h after mating (Figure 2G). Turnover of endogenous DRC2 in WT flagella is therefore relatively slow compared with the time course of intraflagellar transport (IFT) of DRC2-GFP subunits observed by TIRF microscopy (Wren et al., 2013). In contrast, when DRC2-GFP cells were mated to ida6 cells, a strong GFP signal could be detected along the length of both flagella from the ida6 parent within 30 min (Figure 2H).

Consistent with the predicted sizes of the epitope-tagged polypeptides (Figure 2E), similar results were observed with DRC2-HA subunits in sup-pf5 rescued strains (Figure 2F). When the region of the blot containing the truncated DRC2 subunit (DRC2*) was overexposed, the mutant band was easily detected in sup-pf5, but reduced in the five DRC2-HA strains, demonstrating that DRC2-HA displaced the truncated DRC2* subunits in the axonemes of the rescued strains.

The absence or truncation of DRC2 in ida6 and sup-pf5 also correlated with a decrease in the assembly of at least two other axoneme polypeptides, DRC1 and tektin (Figures 1E and 2, E and F). Both DRC1 and tektin were restored to WT levels in the DRC2-rescued strains (Figure 2, E and F). However, we did not observe a major decrease in the amount of DRC4 in either mutant (Figures 1E and 2, E and F). These results suggested that drc2 mutations might alter the assembly of a different group of polypeptides than drc4 mutations (Bower et al., 2013). These observations also raised the possibility that the pathway of assembly for DRC2 might differ from that previously described for DRC4 (Bower et al., 2013; Wren et al., 2013). To test the first hypothesis, we compared the polypeptide composition of WT, mutant, and rescued axonemes by iTRAQ labeling and tandem mass spectrometry (see below). To determine if the pathway for DRC2 assembly might be different from that for DRC4 assembly, we analyzed the time course of DRC2-GFP assembly into the flagella using dikaryon rescue.

FIGURE 1: Both ida6 and sup-pf5 are drc2 mutations. (A) Diagram of genes contained within BAC clone 31N21. This clone was digested with DraI and SpeI to release a genomic fragment encoding DRC2 (FAP250). (B) Diagram of the subclone containing DRC2 showing the location of the sup-pf5 and ida6 mutations and the site of the HA or GFP epitope tags. (C) Diagram of the predicted secondary structure of the DRC2 polypeptide showing the four coiled-coil domains in the wild-type (WT) sequence, the addition of 101 amino acids to the C-terminus of the ida6 sequence, and the truncation of the third coiled-coil domain in the sup-pf5 sequence. (D) The aligned amino acid sequences of the third coiled-coil domains in the Chlamydomonas (Cr) and Homo sapiens (Hs) DRC2 polypeptides. The asterisks indicate the site of the Chlamydomonas sup-pf5 mutation (top) and the H. sapiens mutation (bottom) linked to PCD by Austin-Tse et al. (2013). (E) Western blot of WT and sup-pf5 axonemes probed with antibodies to different polypeptides reveals that truncation of DRC2 (indicated by an asterisk in the DRC2 panel) reduces the assembly of multiple N-DRC subunits, the CSC (FAP61), and tektin, but does not alter assembly of ODA subunits (IC2/IC69), RS subunits (RSP16), or the molecular ruler CCDC39 (FAP59).
Characterization of DRC2 associated proteins by iTRAQ analysis and quantitative mass spectrometry

To more completely characterize the polypeptide defects in the drc2 mutants and compare them with other drc mutants, we isolated axonemes from WT, mutant, and rescued strains and subjected them to iTRAQ labeling and tandem mass spectrometry (MS/MS) using an improved protocol (see Materials and Methods for details). This approach makes it possible to compare complex protein mixtures and identify a small subset of polypeptides that are quantitatively different between samples (Portman et al., 2009; Bower et al., 2013; Reck et al., 2016). In the first experiment, duplicate samples of WT, ida6, DRC2-HA, and p33 axonemes were digested with trypsin, labeled with eight different iTRAQ tags, and then mixed together. The p33 sample was included because previous work had shown that DRC2 is also reduced in this strain (Lin et al., 2011; Bower et al., 2013; see Table 1). The p33 mutation has been identified as a premature stop in the transcript encoding the DRC1 subunit of the N-DRC (Wirschell et al., 2013). The pooled iTRAQ mixture was first fractionated by liquid chromatography and then loaded into a mass spectrophotometer. A total of 481 proteins were identified with high confidence at a false discovery rate of 5% in the 8-plex experiment, and for most proteins, no significant differences were observed between the WT and ida6 samples. However, a small number of proteins were reproducibly reduced in ida6 and the closely related drc1 mutant p33 and restored to WT levels in the DRC2-HA rescued sample (Tables 1 and 2).

To increase the number of proteins detected at high confidence, we repeated the iTRAQ analysis using duplicate samples of ida6 and the DRC2-HA rescued strain and labeled these samples with four different iTRAQ labels. A total of 1089 proteins were identified in the second 4-plex experiment, but again only a small number were reproducibly reduced in ida6 compared with the rescued strain (Table 2). Based on the iTRAQ analysis, the proteins most significantly reduced in ida6 and p33 axonemes are those previously identified to be associated with the N-DRC complex, DHC8, and tektin (Table 2). In addition, four RS-associated proteins (RS8, FAP206, FAP251/CaM-IP4, FAP91/CaM-IP2) and three other polypeptides (FAP241, FAP273, and Cre09.g397993) were decreased. However, most RS proteins or other doublet microtubule-associated proteins were not consistently altered in a statistically significant manner (p < 0.05).

To confirm and extend these observations, we probed Western blots of axonemes isolated from WT, ida6, DRC2-HA, and p33 with antibodies to N-DRC subunits. DRC1, DRC2, DRC5, DRC8, and DRC11 were barely detected in ida6 and p33 axonemes, and DRC3, DRC4, and DRC7 were reduced to varying degrees (Figure 3A). Similar results were observed with sup-pf5 axonemes (Figure 1E). The signals observed on the blots were consistent with the protein ratios predicted by iTRAQ analysis (Table 2). Probing the blots with antibodies for subunits of the outer dynein arms (IC2/IC69), the I1 inner arm dynein (IC140), and the radial spokes (RSP16) confirmed that these proteins were unchanged in all samples, consistent with the iTRAQ results (Figures 1E and 3A and Table 2). Although the ida6 and sup-pf5 mutations affect different regions of the DRC2 polypeptide, the two mutations have similar consequences for the assembly of other DRC subunits. The defects seen in ida6 and sup-pf5 are also very similar to those observed in the drc1 mutant, p33 (Figure 3A; see also Lin et al., 2011; Wirschell et al., 2013), and far more severe than those observed in p2, sup-p33, drc3, and sup-pf4 (Table 1; Piperno et al., 1994; Lin et al., 2011; Bower et al., 2013; Awata et al., 2015).

To confirm that the defects observed in ida6 were the result of a failure to assemble N-DRC subunits onto the axoneme and not an indirect effect on the stability of N-DRC subunits in the cytoplasm, we also analyzed whole-cell lysates on Western blots (Figure 3B). Probing WT and ida6 lysates with the DRC2 antibody demonstrated that the ida6 mutation (which adds an additional 101 amino acids to the C-terminus of the DRC2 sequence) dramatically reduced the amount of DRC2 protein in the cytoplasm. Probing a duplicate blot with a DRC1 antibody revealed that the DRC1 protein was also reduced in ida6 lysates. Duplicate blots probed with antibodies to several other DRC subunits (DRC5, DRC7, and DRC11) showed that the cytoplasmic levels of these proteins were unaffected by the ida6 mutation. These results suggest that DRC1 and DRC2 form a subcomplex in the cytoplasm prior to assembly into the axoneme. However, the presence or absence of this subcomplex does not affect the stability of the other N-DRC subunits.

Because ida6 was originally identified as an inner arm motility mutant defective in assembly of dynein e (Kato et al., 1993), we tested several other motility mutants for possible defects in assembly of N-DRC subunits. The pf28/oda2 mutation in the γ DHC disrupts the assembly of the ODAs (Mitchell and Rosenbaum, 1985). In contrast, the assembly of different subsets of IDAs is disrupted in the inner arm mutants ida2, ida4, ida5, and IO (Huang et al., 1979; Kamiya et al., 1991; Kato et al., 1993, Yamamoto et al., 2017), including some of the DHcs affected in ida6 (see below). The pf14 mutation disrupts the assembly of the R5 (Huang et al., 1977), and mbo2 and pf12 disrupt the assembly of beak structures in outer doublets #5 and #6 (Segal et al., 1984; Tam and Lefebvre, 2002). No defects in the assembly of N-DRC subunits were detected in any of these strains (Figure 3B). Thus, the drc2 mutants ida6 and sup-pf5 are more similar to the drc1 mutant p33 than any other motility mutants.

Given the numerous connections that the N-DRC makes with other structures in the axoneme (Heuser et al., 2009, 2012b), we analyzed the iTRAQ data to identify other components that could potentially interact with the N-DRC subunits. As expected, DHC8 was significantly reduced in both ida6 and p33, but several DHCs were not detected or not consistently altered in either iTRAQ experiment. Because the DHCs are extremely large polypeptides that vary widely in abundance, their dynamic range can make it challenging to interpret the ratios obtained from different iTRAQ experiments against the background of other axonemal proteins. We therefore reanalyzed the DHC content of the axonemes more directly using SDS–PAGE and spectral counting (Zhu et al., 2010; Bower et al., 2013). WT, ida6, DRC2-HA, and DRC2-GFP axonemes were fractionated by SDS–PAGE, and the high-molecular-weight region was excised from gel to enrich for DHCs relative to other proteins in the axonemes (Bower et al., 2013; Wirschell et al., 2013). Each sample was extracted and digested with trypsin, and four replicates per sample were analyzed by tandem mass spectrometry to determine the total number of assigned spectra for each DHC. Because the two I1 DHCs were constant between samples, the total number of assigned spectra for each DHC was normalized relative to the total number of assigned spectra for the I1 DHCs. The results are shown in Figure 4A, and the relative abundance of each DHC in the four samples is expressed as a percentage of the I1 DHCs present. No significant differences in DHC3, DHC4, or DHC5 were observed between the four strains. As expected, DHC8 was missing in ida6 (Kato et al., 1993) but restored to WT levels in the rescued strains. DHC2, DHC6, DHC7, and DHC9 were reduced to varying degrees in ida6 and restored to WT levels in the rescued strains. In contrast, DHC11 was elevated in ida6 but decreased to WT levels in
FIGURE 2: Transformation with HA- or GFP-tagged DRC2 rescues ida6 and sup-pf5. (A) Live cell images of an ida6 strain rescued with DRC2-GFP transgene as viewed by DIC (left) or confocal (right) microscopy show the distribution of DRC2 in the basal body region and along the length of both flagella. (B) Images of a fixed sup-pf5 cell (bottom) or DRC2-HA rescued cell (top) stained with an antibody to the HA epitope tag as viewed by DIC (left) or fluorescence (right) microscopy demonstrate the presence of DRC2-HA in the flagella of the rescued strain. The basal body region is out of the plane of focus in this image. (C, D) Measurements of motility as viewed by phase contrast microscopy indicate that transformation of either ida6 (C) or sup-pf5 (D) with WT or epitope-tagged DRC2 transgenes increased forward swimming velocities to near WT levels. All strains indicated by asterisks were slower than WT, but the DRC2 rescued strains were significantly faster than ida6 or sup-pf5 ($p < 0.05$). (E, F) Western blots of axonemes from WT, mutant, and rescued strains were probed with antibodies against DRC subunits and tektin. Loss or truncation of DRC2 was
the rescued strains (Figure 4A). The spectral counting data indicate that the DHC defects in ida6 are much more extensive than previously reported (Kato et al., 1993), but similar to the DHC defects observed in pf3 (Bower et al., 2013; Wirschell et al., 2013).

Western blots of isolated axonemes probed with antibodies to several inner arm subunits confirmed these overall trends (Figures 3A and 4B). No obvious differences in the levels of outer arm (IC2) or IC1 dynein subunits (IC140 and IC138) were observed in ida6 axonemes. Similarly, DHC5 levels were not noticeably affected. However, the intermediate and light chains (p44, p38, p28) associated with IDAs a, c, and d (DHC6, DHC9, and DHC2) were slightly decreased in ida6, and DHC11 was slightly increased in ida6, consistent with the spectral counting data (Figures 3A and 4B).

Cryo–electron tomography (Cryo–ET) has demonstrated that the N-DRC is closely connected to the base of RS2 and the CSC that interconnects RS2 with the neighboring short RS3 (RS3S) (Dymek et al., 2011; Heuser et al., 2012b). We therefore analyzed the iTRAQ data for possible changes in RS and CSC subunits to identify other polypeptides that might potentially interact with the N-DRC. No statistically significant differences were observed for most RS subunits, with the exception of RS8, CaM-IP4/FAP251, and CaM-IP2/FAP91, which were reduced in both ida6 and pf3 (Table 2). Western blots of isolated axonemes confirmed that most RS subunits were unchanged in ida6, except for a consistent decrease in RSP8 (Figure 4C). Variable results were observed for CaM-IP3/FAP61 (aka as RSP19). CaM-IP3/FAP61 was significantly reduced in both ida6 and pf3 in only one of the two iTRAQ experiments (Table 2). However, multiple Western blots probed with antibodies against CaM-IP3 suggested that CaM-IP3 was reduced in sup-pf5, ida6, and pf3 axonemes, but increased to WT levels in rescued strains (Figures 1E, 3A, and 4C). Analysis of the iTRAQ data also identified four other polypeptides that were reduced in ida6 and pf3 (Table 2). Most are not well characterized, but one protein, FAP206, was previously described as a conserved polypeptide whose phosphorylation state was altered in several drc mutants (Lin et al., 2011). FAP206 is also missing in the RS mutant pf14 (Lin et al., 2011) and coimmunoprecipitates with the docking protein RSP3 (Gupta et al., 2012). Recent study of a gap206 mutant in Tetrahymena has shown that FAP206 is a docking protein for RS2 (Vasudevan et al., 2015). Taken together, these observations and the reductions in CaM-IP3, CaM-IP4, RSP8, and FAP206 seen in ida6 and pf3 strongly suggest that the connections between the base of the N-DRC, the base of RS2, and the CSC might be destabilized in these two drc mutants.

Fractionation of N-DRC subunits in drc2 and drc4 mutants

Previous work demonstrated that N-DRC subunits are tightly bound to the outer doublet microtubules (Piperno et al., 1994; Bower et al., 2013). Following extraction by 04–0.6 M NaI and dialysis to lower ionic strength, most N-DRC subunits cosediment as a large complex on sucrose density gradients, but some subunits can dissociate from the larger complex, depending on buffer conditions or mutant background (Bower et al., 2013; Wirschell et al., 2013; Awata et al., 2015). We therefore analyzed the solubility and sedimentation behavior of N-DRC subunits in ida6 and sup-pf5 extracts. As shown in Figure 5A, DRC4 could be extracted from ida6 and sup-pf5 axonemes with 0.6 M NaCl, but required 0.4–0.6 M NaI for extraction from WT or rescued strains. Thus, the absence of DRC2 (and DRC1) subunits in ida6 and sup-pf5 axonemes appeared to destabilize the association of DRC4 with the outer doublets. Similar results were observed with drc4 mutant pf2. Both DRC1 and DRC2 could be readily extracted from pf2 axonemes with 0.6M NaCl, but following rescue of pf2 with DRC4-GFP, both DRC1 and DRC2 became more resistant to extraction with 0.6M NaCl (Figure 5A). These results indicated that the absence of DRC2 weakens the binding of DRC4 to the outer doublet and vice versa, even though neither subunit requires the other for assembly.

To determine how loss or truncation of DRC2 influences the stability of the isolated N-DRC, we analyzed DRC-containing extracts on sucrose density gradients (Figure 5). When extracts were prepared from either WT or pf14 axonemes, most N-DRC subunits sedimented as a large complex near the bottom of the gradient, although a significant fraction of both DRC7 and DRC3 was sometimes observed closer to the top of the gradient (Figure 5, B and E). This pattern is similar to that seen previously with extracts from DRC4-HA and DRC5-HA rescued strains (Bower et al., 2013). However, when extracts were prepared from either ida6 or sup-pf5 axonemes lacking DRC1 and DRC2, all of the DRC3, DRC4, and DRC7 subunits sedimneted near the top of the gradient. In contrast, RSP16 still sedimented at ~20S, demonstrating that the RS were largely intact in these drc mutants (Figure 5, C and D). In extracts prepared from a DRC2-HA rescued strain, most N-DRC subunits reassocited and cosedimented as part of a larger complex, although a portion of DRC3 and DRC7 still sedimented at the top of the gradient (Figure 5F). These observations demonstrate that DRC1 and DRC2 are important for maintaining the integrity of the isolated N-DRC and further suggest that the N-DRC may be assembled in vivo from smaller sub-complexes (see the Discussion).

Cryo–electron tomography reveals defects in the assembly of multiple substructures

Analysis of WT and mutant axonemes by thin section TEM and 2D image averaging of longitudinal sections indicated that the crescent-shaped N-DRC structure was missing in ida6 (Austin-Tse et al., 2013). To obtain a clearer view of the impact of the ida6 mutation on all the substructures within the axoneme, we reanalyzed the WT, pf3, ida6, and DRC2-GFP rescued strains by cryo-ET. Subtomogram averaging of 300–800 axonemal repeats per strain resulted in three-dimensional (3D) structures with resolution 3.4 nm and revealed significant defects in the assembly of multiple substructures (Figure 6, A–T).

Closer examination of the N-DRC demonstrated that ida6 axonemes lack most of the region on the underside of the A-tubule known as the base plate (Figure 6, M–T). A defect in the base plate affects in the assembly of multiple substructures (Figure 6, A–T).
| Protein         | Peptides | WT/WT | ida6/WT | HA/WT | pf3/WT | Peptides | HA/HA | ida6/HA |
|-----------------|----------|-------|---------|-------|--------|----------|-------|--------|
| **DRC subunits**|          |       |         |       |        |          |       |        |
| DRC1            | 17 (22)  | 0.98  | 0.29    | 0.97  | 0.29   | 36 (92)  | 1.10  | 0.31   |
| DRC2 (FAP250)   | 11 (14)  | 1.01  | 0.29    | 1.03  | 0.31   | 25 (50)  | 1.12  | 0.31   |
| DRC3 (FAP134)   | 11 (8)   | 0.98  | 0.50    | 1.08  | 0.44   | 48 (79)  | 1.04  | 0.51   |
| DRC4 (PF2)      | 16 (35)  | 0.96  | 0.57    | 0.96  | 0.44   | 69 (112)| 1.00  | 0.57   |
| DRC5 (FAP155)   | 2 (8)    | 1.01  | 0.25    | 0.97  | 0.27   | 17 (24)  | 0.97  | 0.32   |
| DRC6 (FAP169)   | 5 (9)    | 1.01  | 0.35    | 1.06  | 0.41   | 21 (34)  | 1.09  | 0.21   |
| DRC7 (FAP50)    | 32 (59)  | 1.01  | 0.40    | 0.83  | 0.37   | 103 (205)| 1.03  | 0.67   |
| DRC8 (FAP200)   | 5 (6)    | 0.95  | 0.22    | 0.88  | 0.26   | 10 (29)  | 1.18  | 0.24   |
| DRC9 (FAP122)   | 12 (28)  | 1.03  | 0.50    | 1.02  | 0.43   | 33 (50)  | 1.03  | 0.61   |
| DRC10 (FAP84)   | 4 (16)   | 1.03  | 0.58    | 0.94  | 0.48   | 20 (40)  | 1.06  | 0.59   |
| DRC11 (FAP82)   | 19 (24)  | 0.97  | 0.35    | 0.87  | 0.33   | 63 (117)| 1.02  | 0.38   |
| **MT doublet proteins** | |       |         |       |        |          |       |        |
| Tektin*         | 29 (49)  | 1.00  | 0.42    | 0.91  | 0.56   | 46 (105)| 1.08  | 0.22   |
| CCDC39 (FAP59)  | 24 (46)  | 0.98  | 0.86    | 0.87  | 0.87   | 60 (107)| 1.08  | 1.21   |
| CCDC40 (FAP172) | 31 (31)  | 0.96  | 0.87    | 0.82  | 0.68   | 74 (127)| 1.09  | 1.24   |
| Rib72           | 100 (146)| 0.97  | 0.90    | 0.79  | 0.82   | 170 (259)| 0.97  | 1.07   |
| Rib43           | 34 (42)  | 1.00  | 0.90    | 0.93  | 0.85   | 41 (77) | 1.09  | 1.13   |
| PACRG           | 36 (72)  | 1.02  | 1.04    | 0.98  | 1.02   | 57 (161)| 1.01  | 1.13   |
| MBO2            | 20 (28)  | 1.04  | 0.88    | 0.90  | 0.78   | 73 (110)| 1.06  | 1.22   |
| FAP20           | 12 (15)  | 1.18  | 0.93    | 0.86  | 1.09   | 24 (44) | 0.98  | 1.11   |
| TUB             | 502 (892)| 1.04  | 1.06    | 1.07  | 0.95   | 534 (1344)| 0.95  | 0.98   |
| TUA             | 343 (465)| 1.02  | 0.94    | 0.93  | 1.07   | 517 (1390)| 1.06  | 1.03   |
| **Radial spokes/CSC**| |       |         |       |        |          |       |        |
| RSP1            | 69 (61)  | 0.99  | 0.88    | 0.99  | 0.94   | 88 (203)| 1.17  | 1.12   |
| RSP2            | 41 (35)  | 0.95  | 0.79    | 0.90  | 0.82   | 102 (158)| 1.17  | 1.12   |
| RSP3            | 41 (64)  | 0.97  | 0.87    | 1.00  | 0.84   | 65 (114)| 1.09  | 1.09   |
| RSP4            | 17 (15)  | 0.97  | 0.75    | 0.77  | 0.77   | 42 (75) | 1.10  | 1.05   |
| RSP5            | 19 (32)  | 0.86  | 0.65    | 0.76  | 0.65   | 53 (135)| 1.05  | 1.07   |
| RSP6            | 8 (18)   | 1.01  | 0.87    | 0.80  | 0.88   | 40 (40) | 0.94  | 0.88   |
| RSP7            | 29 (25)  | 0.97  | 0.84    | 0.95  | 0.87   | 53 (64) | 1.03  | 1.80   |
| **RSP8**        | 7 (17)   | 0.99  | 0.55    | 0.96  | 0.61   | 22 (49) | 1.04  | 0.51   |
| RSP9            | 21 (29)  | 1.06  | 0.83    | 0.84  | 0.90   | 55 (98) | 0.93  | 1.06   |
| RSP10           | 1 (3)    | 1.06  | 0.98    | 1.03  | 1.07   | 23 (30) | 1.10  | 1.18   |
| RSP11           | 4 (8)    | 0.99  | 0.87    | 0.96  | 0.83   | 26 (37) | 0.98  | 1.10   |
| RSP12           | 2 (4)    | 0.99  | 0.84    | 0.94  | 0.86   | 15 (15) | 1.06  | 1.28   |
| RSP14           | 6 (27)   | 1.00  | 1.05    | 1.01  | 1.08   | 18 (49) | 1.02  | 1.35   |
| RSP16           | 30 (58)  | 0.96  | 0.82    | 0.89  | 0.81   | 58 (112)| 1.13  | 1.21   |
| RSP17           | 26 (26)  | 0.99  | 0.70    | 0.84  | 0.67   | 61 (95) | 1.07  | 0.88   |
| RSP19/CaM-IP3/FAP61 | 11 (15)  | 1.00  | 0.55    | 0.87  | 0.53   | 27 (49) | 1.00  | 0.92   |
| RSP20/CaM       | 7 (5)    | 0.88  | 0.67    | 1.05  | 0.73   | 36 (47) | 1.00  | 0.89   |
| RSP22/LC8       | 15 (46)  | 0.98  | 0.78    | 0.82  | 0.91   | 34 (67) | 1.04  | 0.92   |
| RSP23/NDK       | 16 (24)  | 0.94  | 0.85    | 0.98  | 0.87   | 37 (65) | 1.09  | 1.21   |

**TABLE 2**: iTRAQ protein ratios in WT and *drc* mutant axonemes.
with the observations that DRC1 and DRC2 are uniquely missing in pf3, sup-pf5, and ida6 (Figure 3; see also Bower et al., 2013). However, significant defects were also observed in the assembly of the linker region in ida6 and pf3 (Figure 6, J, K, and Q–T). The latter is consistent with the decreases observed in most of the other DRC subunits (Figure 3A and Table 2). Only a small region at the base of the linker remains (Figure 6, J, K, R, and S), consistent with the presence of residual DRC4 in pf3 and ida6 (Figure 3A).

The loss of the base plate also correlated with the loss of the “hole” seen at the A/B-tubule inner junction in WT axonemes (Figure 6, M–P). The hole was “filled” in pf3 and ida6 axonemes, but reappeared in the DRC2-GFP rescued axonemes. Most of the proteins associated with this region of the outer doublet are unknown, but the hole is located in close proximity to the protofilament ribbon recently observed near the A/B-tubule partition (Linck et al., 2014) and the inner junction protein FAP20 (Yanagisawa et al., 2014). No defects were observed in the assembly of ribbon proteins or FAP20 in either pf3 or ida6 (Table 2). However, tektin is reduced in pf3, ida6, sup-pf5, and the fap20 (bug22) mutant (Figures 1–3; see also Yanagisawa and Kamiya, 2004; Yanagisawa et al., 2014).

We also observed defects in the assembly of multiple IDA structures in ida6 axonemes (Figure 6). As expected, inner arm IA4 corresponding to dynein e (DHC8) was missing in both pf3 and ida6 and restored in the DRC2-GFP rescued strain (Figure 6, A–L). This IDA is directly associated with the N-DRC (Gardner et al., 1994; Heuser et al., 2009). Two other inner arm structures closely associated with the N-DRC, IA5 (corresponding to dynein g or DHC7) and IA6 (corresponding to dynein d or DHC2), were also reduced in both pf3 and ida6 (Figure 6, B, C, F, G, J, and K). We also noted a decrease in intensity of the IA2 structure in pf3 (Figure 6, C and G). IA2 (corresponding to dynein a or DHC6) is located in close proximity to RS1, well separated from both RS2 and the N-DRC. In addition, the decrease in IA2 seen in the ida6 tomograms

### TABLE 2: iTRAQ protein ratios in WT and drc mutant axonemes. Continued

| Protein            | Peptides | WT/WT | ida6/WT | HA/WT | pf3/WT | Peptides  | HA/HA | ida6/HA |
|--------------------|----------|-------|---------|-------|--------|-----------|-------|---------|
| CaM-IP4/FAP251*    | 19 (18)  | 1.03  | 0.72    | 0.91  | 0.81   | 26 (56)   | 0.99  | 0.79    |
| Cam-IP2/FAP91*     | 18 (27)  | 0.96  | 0.53    | 0.94  | 0.50   | 38 (84)   | 1.04  | 0.73    |
| FAP206*            | 16 (31)  | 0.99  | 0.57    | 0.92  | 0.54   | 20 (56)   | 0.98  | 0.72    |
| Other              |          |       |         |       |        |           |       |         |
| FAP241*            | 14 (18)  | 0.93  | 0.55    | 0.88  | 0.61   | 32 (63)   | 1.09  | 0.59    |
| FAP273*            | 11 (10)  | 1.02  | 0.71    | 0.90  | 0.99   | 17 (25)   | 1.06  | 0.76    |
| Cre09.g397993*     | 11 (9)   | 0.99  | 0.54    | 0.77  | 0.54   | 19 (41)   | 1.04  | 0.55    |

The amount of protein in each sample was compared with that present in WT (or HA rescued sample) to obtain a protein ratio. The WT/WT and HA/HA ratios indicate the variability in iTRAQ labeling and protein loading between replicates of the same sample (typically less than 10%). For each protein, duplicate mutant/WT ratios were averaged, and those ratios that were statistically significant (p < 0.05) in both samples are indicated in bold. Peptides indicate the number used for protein ID (95% confidence) followed by the number used for iTRAQ quantification (in parentheses). Non-DRC subunits that are significantly reduced in 8-plex and the 4-plex iTRAQ experiments are indicated in bold type with an asterisk (*).
was consistent with the reduction in DHC6 observed by spectral counting (Figure 4A). The multiple DHC defects in conjunction with the reduction in tektin indicate that drc2 mutations can have both local effects (e.g., defects in assembly of the N-DRC) and broader impacts (e.g., defects in the assembly of tektin and multiple IDAs) beyond the immediate vicinity of the N-DRC.

**Heterogeneity in the assembly of the radial spokes and CSC**

The base plate of the N-DRC connects to the base of RS2, RS3S, and the CSC (Heuser et al., 2012b). Analysis of CSC knockdown strains has shown that the CSC interconnects the base of RS2 to the neighboring RS3S, and that defects in the assembly of the CSC subunits lead to misplacement of the RSs in the 96-nm repeat (Heuser et al., 2012b; Urbanska et al., 2015). In earlier studies of pf3, we also noted that RSs were misplaced in a subset of longitudinal sections (Gardner et al., 1994). Yet when we averaged all of the 96-nm repeat in pf3 or ida6, no obvious differences in the assembly of the RS or CSC were detected (Figure 6, I–P). These observations raised the possibility that the averaging procedure might be masking genuine variability in the assembly of RS and the CSC in pf3 and ida6 axonemes. We therefore reviewed the images of the RS in the individual tomograms for the WT, pf3, ida6, and rescued DRC2-GFP strain. As shown in Supplemental Figure S1, all of the 96-nm repeats in the WT and rescued strain were consistent with the reduction in DHC6 observed by spectral counting (Figure 4A). The multiple DHC defects in conjunction with the reduction in tektin indicate that drc2 mutations can have both local effects (e.g., defects in assembly of the N-DRC) and broader impacts (e.g., defects in the assembly of tektin and multiple IDAs) beyond the immediate vicinity of the N-DRC.

**DISCUSSION**

**The impact of mutations in the DRC2 polypeptide**

The N-DRC is a large, multisubunit complex critical for the regulation of ciliary motility, but little is known about the specific function of each subunit. Here we analyzed the phenotypes of two drc2 mutant alleles in detail. DRC2 is a coiled-coil protein that extends from one end of the N-DRC linker to the opposite end of the base plate (Oda et al., 2015). The ida6 mutation is a single-base pair change in the stop codon that results in the addition of 101 amino acids to the DRC2 sequence (Figure 1). The mutant DRC2 polypeptide cannot assemble into the axoneme (Austin-Tse et al., 2013), and the assembly of several other N-DRC subunits is reduced (Table 2; Figure 3). However, the addition of an HA (45 amino acids) or GFP (244 amino acids) tag to the C-terminus does not interfere with the ability of these constructs to rescue ida6 and restore all the missing polypeptides and structures (Figures 2, 4, and 6). Therefore, increasing the length of DRC2 does not by itself inhibit DRC2 assembly. However, the additional 101 amino acids in ida6 significantly increase the predicted isoelectric point of the DRC2 protein. Such a change could interfere with the preassembly of the N-DRC complex in the cytoplasm, its transport into the flagellar compartment, or its binding to the axoneme. The C-terminal half of the DRC2 polypeptide clearly plays a role in the attachment of the N-DRC to the outer doublet. Truncation of DRC2 as seen in sup-pf5 reduces the amount of DRC2 and several other N-DRC subunits in the axoneme (Figure 1E). Given that the sup-pf5 mutation is very close to the site of the drc2/ccdc65 mutation found in several families with PCD (Figure 1D; see also Austin-Tse et al., 2013; Horani et al., 2013), the sup-pf5 results suggest that the assembly of multiple N-DRC subunits is compromised in the respiratory cilia of the PCD patients.

**Coassembly of DRC1 and DRC2 in the base plate of the N-DRC**

Our experiments further demonstrate that DRC2 coassembles with DRC1 to form the N-DRC base plate on the A-tubule of the outer doublet. Loss or truncation of DRC2 inhibits the assembly of DRC1, and loss of DRC1 inhibits the assembly of DRC2 (Table 2; Figures 1–3). In addition, DRC2 and DRC1 cosediment as a subcomplex in extracts prepared from WT (Figure 5) and other drc mutant axonemes (e.g., pf2, sup-pf3, sup-pf4 as described in Bower et al., 2013). However, another component of the N-DRC base plate, DRC4, is still present in both drc2 and drc1 mutant axonemes (Figures 1 and 3 and Table 2; Song et al., 2015). Moreover, even though DRC4 extends from the bottom of the base plate to the tip

![Image](image-url)
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DRC2 connects several axoneme structures

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et al., 2009; Bui et al., 2012; Wirschell et al., 2013). Cryo-ET indicated that at least one other IDA structure (IA5) was reduced in pf3/drc1 (Heuser et al., 2009). Mass spectrometry and spectral counting further suggested that there might be defects in several other IDAs in pf3 (Wirschell et al., 2013). Cryo-ET and spectral counting of ida6 axonemes have now shown that not only the IDAs closest to the N-DRC are reduced (IA4/DHC8, IA5/DHC7, IA6/DHC2), but IA2/DHC6 is also decreased. In contrast, a minor dynein, DHC11, located in the proximal portion of the axoneme appears to be elevated in ida6. The position of DHC11 in the 96-nm axoneme repeat is unknown, but previous work has suggested that DHC11 can bind to sites normally occupied by the other inner arm DHCs of the linker region (Oda et al., 2015; Song et al., 2015), the loss of DRC4 in the pf2 mutant does not significantly affect the assembly of DRC1, DRC2, the base plate, or other nearby structures (Heuser et al., 2009; Lin et al., 2011; Bower et al., 2013; Awata et al., 2015; Song et al., 2015). Taken together, these observations suggest that DRC1 and DRC2 coassemble to form a central scaffold needed for assembly of the N-DRC and its attachment to the outer doublet.

DRC1 and DRC2 stabilize the assembly of multiple IDAs and the RS

Previous work showed that pf3/drc1 and ida6/drc2 axonemes lack dynein e/DHC8/IA4 (Kato et al., 1993; Gardner et al., 1994; Heuser et al., 2009; Bui et al., 2012; Wirschell et al., 2013). Cryo-ET indicated that at least one other IDA structure (IA5) was reduced in pf3/drc1 (Heuser et al., 2009). Mass spectrometry and spectral counting further suggested that there might be defects in several other IDAs in pf3 (Wirschell et al., 2013). Cryo-ET and spectral counting of ida6 axonemes have now shown that not only the IDAs closest to the N-DRC are reduced (IA4/DHC8, IA5/DHC7, IA6/DHC2), but IA2/DHC6 is also decreased. In contrast, a minor dynein, DHC11, located in the proximal portion of the axoneme appears to be elevated in ida6. The position of DHC11 in the 96-nm axoneme repeat is unknown, but previous work has suggested that DHC11 can bind to sites normally occupied by the other inner arm DHCs.

![FIGURE 5: DRC1 and DRC2 form a distinct subcomplex whose loss alters the extraction or sedimentation behavior of other subunits of the N-DRC. (A) Whole axonemes (WA) were isolated from WT, three drc mutants (ida6, sup-pf5, and pf2), and three rescued strains (ida6 rescued with DRC2-HA or DRC2-GFP and pf2 rescued with DRC4-GFP) and then subjected to sequential extraction with 0.6 M NaCl (HS), followed by 0.2, 0.4, and 0.6 M NaI. The resulting extracts and final pellets of outer doublets (OD) were analyzed on Western blots probed with antibodies to DRC subunits. Note that DRC4 is more readily extracted from isolated axonemes in the absence of DRC1 and DRC2 and vice versa. (B–F) Nal extracts (0.5 M) of axonemes from WT (B), ida6 (C), sup-pf5 (D), pf14 (E), and an ida6 strain rescued with DRC2-HA (F) were fractionated by sucrose density gradient centrifugation and analyzed on Western blots probed with antibodies to different DRC subunits. Note the changes in sedimentation behavior of other DRC subunits, especially DRC4, in the absence of DRC1 and DRC2 in ida6 and sup-pf5.](image-url)
The impact of drc1 and drc2 mutations on other components of the outer doublet microtubule

Tektins, Rib43, and Rib72 are the major components of the protofilament ribbon associated with the A-tubule of the outer doublet, and they are located in close proximity to protofilaments A11-A12-A13 or A12-A13-A1 (Linck et al., 2014). Chlamydomonas tektin is significantly reduced in drc1 and drc2 mutant axonemes (Yanagisawa and Kamiya, 2004; Austin-Tse et al., 2013; Wirschell et al., 2013) and restored to WT levels after rescue with the appropriate gene (Austin-Tse et al., 2013; this study, Figure 2 and Table 2). In addition, tektin-t mutations in mice have been linked to defects in the assembly of IDAs (Tanaka et al., 2004). Ikeda et al. (2003) proposed that the protofilament ribbon plays a role in templating the assembly of the N-DRC and other structures. More recent work has shown that the coiled-coil proteins CCDC39/FAP59 and CCDC40/FAP172 located on the surface of the A-tubule function as a molecular ruler to establish the spacing of the assembly of multiple IDAs, either directly by disrupting binding sites associated with the N-DRC or indirectly by altering the assembly of other proteins associated with the outer doublets (see below).

Recent studies of the CSC and FAP206 have shown that the three RS in each 96-nm repeat are structurally and biochemically distinct (Lin et al., 2012; Heuser et al., 2012b; Urbanska et al., 2015; Vasudevan et al., 2015). The CSC subunits link the base of RS2 with the base of RS3S (or RS3), and knockdown (by RNA interference [RNAi]) or knockout of CSC subunits and FAP206 result in defects in RS assembly (Heuser et al., 2012b; Urbanska et al., 2015; Vasudevan et al., 2015). We previously observed misplaced RS in a subset of pf3 axonemes by thin-section TEM (Gardner et al., 1994). Following the identification of defects in the assembly of FAP206, RS8, and several CSC subunits in both pf3 and ida6 axonemes by iTRAQ labeling and mass spectrometry (Table 2; Figure 4), we analyzed several drc mutants by cryo-ET to identify potential defects in RS assembly. Class averaging of pf2 (drc4) tomograms showed no evidence of RS defects in any of the axoneme repeats. However, class averaging of ida6 and pf3 tomograms revealed that ~25% of the repeats had defects in the assembly of RS2 and RS3S. We predict that DRC1 and DRC2 interact directly or indirectly with FAP206 and CSC subunits near the base of the RS to stabilize the attachment of RS2 and RS3S to the axoneme, and that the reduction in FAP206 and CSC subunits is the primary cause for the heterogeneity in RS assembly observed in pf3 and ida6.

FIGURE 6: Cryo-ET reveals defects in the assembly of multiple structures in ida6 and pf3 axonemes. (A–T) Tomographic slices (A–D) and isosurface renderings (E–T) show the 3D structures of averaged 96-nm repeats of axonemes isolated from WT, pf3 (drc1), ida6 (drc2), and the DRC2-GFP rescued strain. Longitudinal views (A–P) show an overview of the entire repeat (A–H) and close-ups of the N-DRC (I–P) viewed from the front (E–L) and the bottom (Y–T) show the axonemal repeat viewed from the distal end of the N-DRC. Most structures, such as the ODAs or the I1 dynein, including α- and β-motor domains as well as the ICLC complex, appear very similar in all strains. However, some IDAs are reduced in both drc mutants: IA4 is missing, and IA5 and IA6 are reduced in both pf3 and ida6 (see rose arrowheads in B, C, F, G, J, K). Additionally, ida6 shows a reduction in IA2 (C, G). Note that IAx is only present in a subset of doublets and is therefore more variable and weaker than the other IDAs (A–H). The N-DRC is severely reduced in both drc mutants: the linker region and most of the base plate are missing in pf3 and ida6, and only a small portion of the N-DRC remains (J, K, N, O, R, S). In WT, the B-tubule has a small hole (cyan arrow in M) at the inner junction between the A- and B-tubules (A, B), which is not present in pf3 and ida6 (dashed cyan circles in N, O). All of the structural defects (IDAs, N-DRC, B-tubule hole) were repaired in the DRC2-GFP rescued strain, which appears indistinguishable from WT. The CSC (red) is located between the base of RS2 and the shorter RS3S and shows no obvious differences in the averages of all repeats. Scale bar (D) is 20 nm. Similar views of the WT and pf3 data were previously published (Heuser et al., 2009).

(Sequeira et al., 2012). Variable changes in DHC11 levels have also been observed in a subset of long flagella (lf) mutants (Sequeira et al., 2016). The factors that determine the levels and locations of each inner arm DHC are not well defined, but all of the DHCs affected in ida6 are restored to WT levels in the DRC2 rescued strains (Figures 4 and 6). Thus, the absence of DRC1 and DRC2 impacts the assembly of multiple IDAs, either directly by disrupting binding sites associated with the N-DRC or indirectly by altering the assembly of other proteins associated with the outer doublets (see below).
The biochemical and structural phenotypes of pf3, ida6, and sup-pf5 have revealed that mutations in DRC1 and DRC2 destabilize the assembly of multiple components in the Chlamydomonas axoneme (Figures 1–7). The drc1 and drc2 mutations also have profound effects on flagellar motility. Both forward swimming velocities and the shear amplitudes of the flagellar waveform are significantly reduced (Figure 2; Brokaw and Kamiya, 1987; Kato et al., 1993). The localization of DRC1 and DRC2 by epitope tagging and cryo-ET has further demonstrated that they coassemble in situ with DRC4 to form a central portion of the N-DRC extending from the base plate on the A-tubule through the linker region toward the neighboring B-tubule (Oda et al., 2015; Song et al., 2015). Disrupting this structure would be expected to reduce the resistance to dynein-driven microtubule sliding. In vitro studies of isolated axonemes and cell models have shown that mutations in drc1, drc2, and drc4 inhibit reactivated motility and lead to the slippage of outer doublet microtubules (Bower et al., 2013; Alford et al., 2016). Measurements of flexural rigidity and shear stiffness in vanadate-treated axonemes have confirmed that elastic resistance is reduced in pf3 axonemes (Xu et al., 2016).

DRC1 and DRC2 are two of the most highly conserved and widely distributed N-DRC subunits in organisms with motile axonemes (Bower et al., 2013; Nevers et al., 2017). Given these observations and the central role of DRC1 and DRC2 in the assembly of the N-DRC described above, it is not surprising that drc1 and drc2 mutations have been linked to motility defects in other species. RNAi-mediated knockdown of DRC2 leads to motility defects in both trypanosome flagella and zebrafish cilia (Kabututu et al., 2010; Austin-Tse et al., 2013). Similarly, PCD causing mutations in CCDC164 (DRC1) and CCDC65 (DRC2) results in altered ciliary beat patterns in isolated respiratory cilia (Austin-Tse et al., 2013; Horani et al., 2013; Wirschell et al., 2013). However, subtle defects in ciliary ultrastructure are very difficult to detect in images of axoneme cross-sections obtained by standard transmission electron microscopy in the absence of any computational averaging procedures (Austin-Tse et al., 2013; Horani et al., 2013; Wirschell et al., 2013; Shapiro and Leigh, 2017). We predict that the PCD causing mutations in DRC1 and DRC2 likely disrupts the assembly of multiple axonemal substructures in human cilia similarly to that described here in Chlamydomonas. Additional structural studies of patient cilia by cryo-ET and computational averaging could be used to address this question.

**Functions of other N-DRC subunits**

Less is known about the specific functions of the other N-DRC subunits. Like DRC1 and DRC2, DRC3–DRC7 are structural proteins with coiled-coil or leucine-rich repeat domains predicted to be important for subunit interactions (Lin et al., 2011; Bower et al., 2013). DRC8–DRC11 also contain EF hand or IQ motifs predicted to bind calcium or calmodulin-like proteins (Bower et al., 2013). They may interact with CSC and RS subunits, and play a role in calcium-mediated changes in flagellar motility, but their exact location in the N-DRC is still unknown.

The phenotypes of the Chlamydomonas drc3 and drc5/sup-pf4 mutations are subtle (see Table 1): forward swimming velocities are only slightly reduced, and small domains near the distal end of the linker region are missing (Heuser et al., 2009; Bower et al., 2013; Awata et al., 2015). Mutations in the human orthologues for DRC3 (LRRC48) and DRC5 (TCTE1) have not yet been identified, but PCD-like symptoms have been observed in a mouse Lrrc48 mutant with hydrocephalus, left–right laterality defects, sinusitis, and male infertility (Ha et al., 2016), and tcte1 mutations in mice have recently been associated with defects in sperm motility (Castaneda et al., 2017). The Chlamydomonas drc4 mutations (pf2, sup-pf3) primarily disrupt the N-DRC linker (Heuser et al., 2009; Bower et al., 2013), and the pf2 motility defects are quite severe (Brokaw and Kamiya, 1987; Rupp and Porter, 2003; Bower et al., 2013). Knockdown of DRC4 orthologues in trypanosomes, zebrafish, and mice results in severe motility defects (Hutchings et al., 2002;
Colantonio et al., 2009; Lewis et al., 2016), and truncating mutations in the human gene (GAS8) have been linked to PCD (Olbrich et al., 2015; Jeanson et al., 2016; Lewis et al., 2016). The preliminary biochemical and structural characterization of gas8 cilia is consistent with defects in assembly of the N-DRC linker, as was observed previously for drc4 mutants in Chlamydomonas (Heuser et al., 2009; Bower et al., 2013).

No mutations in DRC6–DRC11 have yet been characterized in Chlamydomonas, but mutations in DRC7, DRC9, and DRC11 have been linked to motility defects in flies, mice, and trypanosomes, respectively (Yang et al., 2011; Nguyen et al., 2013; Harris et al., 2014; Li et al., 2014). Additional work is needed to localize each subunit within the substructure of the N-DRC and identify its specific function. Interestingly, comprehensive phylogenetic profiling has identified a subset of N-DRC subunits that are more widely distributed in organisms with motile axonemes than in others. For example, orthologues of DRC1, DRC2, DRC4, DRC7, and DRC11 are present in Thalassiosera (Nevers et al., 2017), a centric diatom with flagellated sperm and ODAs, but no IDAs, RS, or CP microtubules (Armbrust et al., 2004). One intriguing possibility is that these subunits represent a minimal functional core unit of the N-DRC required for regulation of dynein-driven microtubule sliding. Further study of the composition and structure of the N-DRC in organisms with more simplified axonemes may also provide new insights into the specific functions of N-DRC subunits.

MATERIALS AND METHODS

Culture conditions, genetic analyses, and strain construction

Strains used in this study are listed in Supplemental Table S1. Cells were maintained on Tris-acetate phosphate medium, but occasionally resuspended overnight in liquid minimal medium or 10 mM HEPES, pH 7.6, to facilitate flagellar assembly and mating. The site of the drc2 mutation in sup-p52 was identified by PCR of genomic DNA and reverse transcriptase PCR (RT-PCR) of cDNA as previously described (Austen-Tse et al., 2013; Bower et al., 2013; see Supplemental Table S2). A genomic clone containing the full-length DRC2/FAP250 gene was subcloned from BAC 31n21 (Figure 1A; Austin-Tse et al., 2013). For the purpose of epitope tagging, a BglII site was created in the stop codon of the DRC2 gene using the Quick Change II XL mutagenesis kit (Stratagene) and the primers listed in Supplemental Table S2. A triple-HA epitope tag was amplified from the plasmid p3HA (gift of Carolyn Silflow, University of Minnesota), and a GFP tag was amplified from the pHisCrGFP plasmid (Fuhrmann et al., 1999) using the primers listed in Supplemental Table S2. Both tags were inserted into the BglIII site and then sequenced to verify their orientation and sequence. The resulting changes in the DRC2 amino acid sequence are shown in Supplemental Table S2. All constructs were linearized with NotI prior to transformation.

Phase contrast and fluorescence microscopy, swimming velocities, and dikaryon rescues

Forward swimming velocities were assessed by phase contrast microscopy using a 40x objective on a Zeiss Axioskop as described by Bower et al. (2013). The distribution of DRC2-GFP in live cells immobilized in 1% low-melting-point agarose was analyzed using a Zeiss Axioskop with 100x, 1.3 N.A. Plan Neofluor objective, a CoolSNAP-ES monochrome CCD Camera (Photometrics), and the Metamorph software package, version 7.1.7.0 (Molecular Devices). Some images were collected on a Nikon Eclipse E800 photomicroscope using a 100x, 1.3 N.A. plan apo objective, a Photometrics Cascade 512B camera, and the ImagePro Plus software package available at the University Imaging Center (University of Minnesota). Image stacks were collected using z-steps of 0.339 μm. For the image shown in Figure 2A, some sections were deconvolved using ImagePro Plus 6.2 Sharp Stack and then used to make a maximum intensity projection.

Cells or dikaryons were fixed using ice-cold methanol (Sanders and Salisbury, 1995) and stained for immunofluorescence using a mouse monoclonal antibody (mAb) to GFP (Molecular Probes) or a rat mAb to HA (Roche Clone 3F10) and an Alexafluor-488 conjugated secondary antibody (Molecular Probes; Mueller et al., 2005; Bower et al., 2013). Images were collected on the Zeiss Axioskop as described above. Selected images were cropped, rotated, and labeled in Adobe Photoshop.

Isolation of axonemes, fractionation of the N-DRC, SDS–PAGE, and Western blot analyses

Chlamydomonas whole cell lysates, isolated flagella, and demembranated axonemes were prepared as previously described (Witman, 1986; Bower et al., 2013; Reck et al., 2016). Purified axonemes were resuspended in HMEME (10 mM HEPES, pH 7.4, 5 mM MgSO4, 1 mM EGTA, 0.1 mM EDTA, 30 mM NaCl plus 1 mM dithiothreitol (DTT)) and 0.1 μg/ml protease inhibitors (leupeptin, aprotonin, pepstatin), and extracted with HMEME containing 0.6 M NaCl or 0.2, 0.4, or 0.6 M NaCl. N-DRC-containing extracts were dialyzed against HMEME, clarified by centrifugation, and fractionated on 5–20% sucrose density gradients (Bower et al., 2013). Samples were separated on 5–15% polyacrylamide gradient gels, transcribed to Immobilon P, and probed with different antibodies (Bower et al., 2013). Antibody sources and dilutions used are listed in Supplemental Table S3.

Preparation of samples for iTRAQ labeling and mass spectrometry

Isolated axonemes were washed briefly in 10 mM HEPES, pH 7.4, to remove salt, DTT, and protease inhibitors, resuspended in 0.5 M triethylammonium bicarbonate, pH 8.5, and processed for trypsin digestion and iTRAQ labeling as described in detail in Bower et al. (2013) and Reck et al. (2016). Samples were combined and fractionated offline using high-pH C18 reversed-phase chromatography (Reck et al., 2016). Column fractions were vacuum-dried, resuspended in solvent (98:2:0.1, water:acetonitrile:formic acid), and loaded in aliquots of 1–1.5 μg for capillary LC using a C18 column at low pH. The C18 column was mounted in a nanospray source directly in line with a Velos Orbitrap mass spectrometer (Thermo Fisher Scientific). Online capillary LC, MS/MS, database searching, and protein identification were performed as previously described (Lin-Moshier et al., 2013; Reck et al., 2016) using ProteinPilot software version 4.5 (AB Sciex) and the most recent version (v5.5) of the Chlamydomonas database (https://phytozome.jgi.doe.gov/pz/portal.html). The bias factors for all samples were normalized to alpha and beta tubulin (Reck et al., 2016).

Because the DHCs vary widely in abundance in isolated axonemes, not all inner arm DHCs were detected in both iTRAQ experiments. To improve the signal-to-noise for the DHCs, isolated axonemes were fractionated by SDS–PAGE and stained briefly with Coomassie blue, and the DHC region was excised from the gel (Bower et al., 2013). Following extraction and trypsin digestion, three to five replicates per sample were analyzed by tandem MS/MS. Both the total number of peptides and total number of assigned spectra per DHC isoform were analyzed. The relative abundance of each DHC was estimated by spectral counting (Zhu et al., 2010) and expressed as a percentage of the total spectra identified for the 1-alpha and 1-beta DHCs of the I1 dynein (Bower et al., 2013; Wirschell et al., 2013).
Cryo–electron tomography and image processing
Sample preparation, cryo-ET, and image processing were done as previously described (Nicastro et al., 2006; Heuser et al., 2009; Nicastro, 2009). Briefly, Quantifoil copper grids (Quantifoil Micro Tools, Jena, Germany) with a holey carbon film (R2/2, 200 mesh) were glow-discharged for 30 s at –40 mA and loaded with 3 μl of axoneme sample and 1 μl of 10-fold-concentrated 10 nm colloidal gold (Sigma-Aldrich). After brief mixing, grids were blotted from the front with filter paper for 2 s and plunged-frozen in liquid ethane using a homemade plunger. Vitrified samples were stored in liquid nitrogen and transferred to a Tecnai F30 transmission electron microscope (FEI) using a cryo holder (Gatan) for imaging. Single-axis tilt series of noncompressed, intact axonemes were acquired using the software package SerialEM (Mastronarde, 2005). Typically, 60–100 images were recorded at 13,500-fold magnification (~1 nm pixel size) with ~6 to ~8 μm defocus while the specimen was tilted from about ~65° to +65° in 1.5–2.5° increments. The microscope was operated in low-dose mode at 300 keV and the accumulated electron dose of the sample was restricted to ~100 e/Å² to minimize radiation damage. Electron micrographs were recorded digitally with a 2 k x 2 k CCD camera (Gatan) after passing a post-column energy filter (Gatan) in zero-loss mode with a slit width of 20 μm.

The IMOD software package (Kremer et al., 1996) was utilized to reconstruct tomograms from the recorded tilt series. The highly repetitive 96-nm repeat units of the axoneme were used for subtomogram averaging with the program PEET (Particle Estimation for Electron Tomography) (Nicastro et al., 2006). At least 300–800 axonemal repeat units from two to six tomograms of each strain were aligned and averaged, resulting in 3D structures with compensated missing wedge effect, reduced noise, and thus increased resolution. The 3D averages for WT included four tomograms with 612 repeats, for pf3 three tomograms with 500 repeats, for ida6 six tomograms with 800 repeats, and for the ida6 rescued strain (DR22-GFP) two tomograms with 300 repeats. The 3D visualization of the averaged 96-nm repeat units was achieved by isosurface rendering with the UCSF Chimera package (Petterson et al., 2004) using the microtubule mass to normalize the isosurface-rendering threshold of all averages. Classification of RS2 used a principal component–based clustering analysis integrated into the PEET software package (Heumann et al., 2011) and was performed as previously described (Heuser et al., 2012b).

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DRC2 connects several axoneme structures.