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The nexin link and B-tubule glutamylation maintain the alignment of outer doublets in the ciliary axoneme

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Abstract

We developed quantitative assays to test the hypothesis that the N-DRC is required for integrity of the ciliary axoneme. We examined reactivated motility of demembranated drc cells, commonly termed “reactivated cell models.” ATP-induced reactivation of wild-type cells resulted in the forward swimming of ~90% of cell models. ATP-induced reactivation failed in a subset of drc cell models, despite forward motility in live drc cells. Dark-field light microscopic observations of drc cell models revealed various degrees of axonemal splaying. In contrast, >98% of axonemes from wild-type reactivated cell models remained intact. The sup-pf4 and drc3 mutants, unlike other drc mutants, retain most of the N-DRC linker that interconnects outer doublet microtubules. Reactivated sup-pf4 and drc3 cell models displayed nearly wild-type levels of forward motility. Thus, the N-DRC linker is required for axonemal integrity. We also examined reactivated motility and axonemal integrity in mutants defective in tubulin polyglutamylation. ATP-induced reactivation resulted in forward swimming of >75% of tpg cell models. Analysis of double mutants defective in tubulin polyglutamylation and different regions of the N-DRC indicate B-tubule polyglutamylation and the distal lobe of the linker region are both important for axonemal integrity and normal N-DRC function.

Keywords
cilia; axoneme; doublet microtubule; nexin; dynein; dynein regulatory complex; DRC; N-DRC

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**Introduction**

The nine outer doublet microtubules of motile cilia are composed of A- and B-microtubules connected by the dynein motors and the nexin links. Nexin was originally identified by electron microscopy as a thin filament extending between adjacent outer doublet microtubules to maintain the 9+2 ring structure of the axoneme (Gibbons 1963; Gibbons 1965; Stephens 1970; Witman et al. 1978). Electron microscopy also indicated the nexin links repeat at 96 nm intervals (Burgess et al. 1991; Goodenough and Heuser 1989; Warner 1983; Witman et al. 1978), and may be required to maintain the structural integrity of the axoneme and resist microtubule sliding driven by the dynein motors (Bozkurt and Woolley 1993; Brokaw 1980; Lindemann and Lesich 2010; Minoura et al. 1999; Olson and Linek 1977; Porter 2012; Warner 1976; Warner 1983). Protease treatment of the axonemes disrupted the structure of the nexin links (Summers and Gibbons 1971; Summers and Gibbons 1973), leading to the idea that nexin is an interdoublet linker that limits sliding between outer doublet microtubules to convert sliding into bending (Bower et al. 2013; Porter 2012; Satir et al. 2014).

Cryo electron tomography (cryo ET) revealed that the nexin (N) link coincides with the dynein regulatory complex (DRC; Gardner et al. 1994; Piperno et al. 1992) located near radial spoke 2 at the distal end of the 96 nm repeat (Heuser et al. 2009). The N-DRC is a conserved structure (Gardner et al. 1994; Mastronarde et al. 1992; Nicastro et al. 2006), and is composed of at least 11 different subunits organized into two major domains, a linker domain that contacts the B-subfiber of the neighboring doublet and a baseplate domain that associates with the A-microtubule (Fig. 1; Bower et al. 2013; Heuser et al. 2009; Lin et al. 2011; Wirschell et al. 2013). Analyses of drc mutants by biochemical and structural approaches have provided models for the localization of subunits within the substructures of the N-DRC (Fig. 1; Table S1; Awata et al. 2015; Bower et al. 2013; Heuser et al. 2009; Lin et al. 2011; Oda et al. 2015; Song et al. 2015). The N-DRC is considered one of the major regulatory hubs in the axoneme (Awata et al. 2015; Dymek et al. 2011; Heuser et al. 2012b; Heuser et al. 2009; Oda et al. 2013; Song et al. 2015), and may serve several functions, including physical alignment of outer doublet microtubules (Bower et al. 2013; Lin et al. 2015).

To test the hypothesis that the N-DRC is required for the structural integrity of the axoneme, we examined ATP-induced reactivation of demembranated *Chlamydomonas reinhardtii* cell models, and developed quantitative assays of reactivated motility and axonemal integrity using dark-field light microscopy. We predicted that ATP would induce detachment of the dynein cross-bridges (Sale and Gibbons 1979) and consequent splaying apart of the axoneme in drc cell models lacking the N-DRC linker domain (Fig. S1). We found that the linker domain is required for ATP-induced reactivated motility and maintenance of axonemal integrity. In addition, B-tubule polyglutamylation (Kubo et al. 2012; Kubo et al. 2014; Kubo et al. 2010) plays a role in axonemal integrity in concert with the distal lobe of the N-DRC linker.
Results and Discussion

Structural organization of the N-DRC linker and base plate domains

Cryo-ET has defined several structural domains within the N-DRC (Awata et al. 2015; Heuser et al. 2009; Oda et al. 2013; Oda et al. 2015; Song et al. 2015). In this study, we focus on the two major domains, the base plate that attaches to the underside of the A-tubule (Fig. 1, shown in blue) and the linker (Fig. 1, shown in green) that extends from the surface of the A-tubule to the B-tubule of the neighboring outer doublet. As the linker approaches the B-tubule, B_{n+1}, it bifurcates into two domains known as the proximal lobe (pL) and distal lobe (dL) (Fig. 1C). The majority of n-drc mutants studied thus far lack the linker domain (e.g. see Fig. 1, J-L, Table S1). However, two mutants, drc3 and sup-pf4, retain most of the linker domain but lack distinct regions of the distal lobe (Fig. 1, D–I). drc3 lacks the most distal portion of the distal lobe and a strut that connects that domain to the base of the linker (Fig. 1, D–F; (Awata et al. 2015; Song et al. 2015). sup-pf4 lacks the small region that connects the proximal and distal lobes of the linker (Fig. 1, G-I; (Heuser et al. 2009). Both of these defects reduce the area of contact with the neighboring B-tubule, but neither defect is as severe as that observed with the pf2 mutant, in which all contact points with the neighboring B-tubule are missing (Fig. 1, J–L(Heuser et al. 2009).

The nexin linker is required for ATP-induced reactivated motility in vitro

To test the role of the N-DRC in axonemal integrity and outer doublet alignment we reactivated wild-type and a variety of drc mutant cells in vitro with ATP and quantified forward swimming motility (Figs. S1, 2A). Demembranated cell models of wild-type (WT) cells could be reactivated 89.6 ± 8.3% of the time (Fig. 2B). In contrast, ATP-induced reactivation of pf3, ida6, sup-pf5, sup-pf3, and pf2 cell models failed (Fig. 2B), despite motility in live cells (Table S1; (Porter 2012). However, cell models of the sup-pf4 and drc3 mutants, which retain most of the N-DRC linker (Fig. 1; (Awata et al. 2015; Heuser et al. 2009; Song et al. 2015), could be reactivated like WT cells (91.5 ± 10.1% and 91.2 ± 4.8% of the time, respectively). The drc mutants pf3, ida6, sup-pf5, sup-pf3, and pf2 lack the linker domain (Fig. 1; (Austin-Tse et al. 2013; Heuser et al. 2009). Each mutant was rescued with the appropriate wild-type gene and then successfully reactivated in vitro (open bars, Fig. 2C). These results indicated that the N-DRC linker is required for in vitro reactivation of motility.

Consistent with this interpretation, addition of protease inhibitors did not change the ATP-induced reactivation results (data not shown). Previous sliding disintegration experiments relied on exogenous proteases to cleave the nexin link and cause ATP-induced microtubule sliding (Aoyama and Kamiya 2005; Brokaw 1980; Okagaki and Kamiya 1986; Summers and Gibbons 1971). Here, lack of in vitro reactivated motility in drc mutants is likely due to the defect in the N-DRC, rather than proteolysis. Curiously, live drc mutant cells lacking the nexin linker display robust motility (Gardner 1994; Piperno et al. 1992). Evidently in live cells, the ciliary membrane, including interactions between the distal axoneme and the membrane (Dentler 1980; Tammana et al. 2013), maintain the outer doublet alignment required for dynein-driven motility, even in the absence of the N-DRC linker.
The N-DRC linker is essential for axonemal integrity

We hypothesized that addition of ATP to drc mutants lacking the N-DRC linker (Fig. 1) would result in microtubule splaying (Fig. S1), consistent with previous observations of isolated axonemes (Bower et al., 2013). The idea was that in the absence of the N-DRC linker, the dynein arms maintain the 9+2 structure in isolated axonemes, and that addition of ATP would result in dynein detachment and microtubule splaying, even in the absence of added proteases. Cell models were fixed with glutaraldehyde immediately after ATP addition and observed by dark-field microscopy, thus it appears splaying occurs rapidly upon ATP addition (Fig. 3A). Consistently, direct dark-field observation of ATP-induced axonemal splaying also indicated that splaying is rapid, however, detailed analysis of splaying will require additional experimentation. Quantification was performed on dark-field images by counting the number of intact axonemes per 50 total. To better visualize axonemal splaying, reactivated cell models were also fixed with methanol and immunostained for acetylated α-tubulin (Fig. 3B). However, because of attachment to the cover slip and methanol fixation induced additional splaying as an artifact of the technique (Alford, unpublished results), axonemes of immunofluorescently labeled cell models were not used for quantification. Representative examples of intact WT axonemes and splayed sup-pf3 axonemes are shown in Figures 3A and 3B.

Consistent with WT reactivated motility, WT axonemes remained intact 95.2 ± 4.2% of the time (Fig. 3C). Similarly, axonemes from rescued strains remained intact like WT (open bars, Fig. 3B). However, axonemes of the drc mutants pf3, ida6, sup-pf5, sup-pf3, and pf2 underwent splaying (Fig. 3A and B), and remained intact less than 60% of the time after in vitro reactivation (Fig 3C). Splaying of the drc mutant axonemes was observed in the presence of protease inhibitors and only after ATP addition. The splaying of axonemes observed in these strains is consistent with their failure to reanimate above and earlier observations on isolated axonemes (Bower et al., 2013). Splaying of isolated drc- axonemes also occurred in the presence of 0.1 mM ATP and 5.0 μM vanadate, a vanadate concentration that blocked microtubule sliding (data not shown). This result indicated that dynein driven shear force is not required for axonemal splaying in drc- mutants missing the interdoublet linker.

In contrast to the other drc mutants, axonemes from sup-pf4 and drc3 remained intact after ATP-induced in vitro reactivation 98.1 ± 1.9% and 99.5 ± 1.4% of the time, respectively (Fig. 3C). Interestingly, sup-pf4 and drc3 lack only small portions of the N-DRC linker (Fig. 1). sup-pf4 lacks the region that connects the proximal and distal lobes of the linker, whereas drc3 lacks the distal lobe and the L1 projection that connects the distal lobe to central portion of the linker (Table S1, Fig. 1). The absence of splaying in drc3 and sup-pf4 axonemes is consistent with their ability to reanimate as described above. We have only observed splaying in isolated sup-pf4 axonemes by adding protease (Alford, unpublished), and thus the splaying of sup-pf4 axonemes observed previously (Bower et al., 2013) was likely the result of endogenous protease activity. Taken together, our data indicate that the nexin linker is required for ATP-induced reactivated motility (Fig. 2) and axonemal integrity (Fig. 3), but that small defects in the linker region, such as those observed with sup-pf4 and drc3, do not disrupt axoneme integrity or reactivation.
DRC5 and B-tubule polyglutamylation work together to maintain axonemal integrity

Recent studies revealed that ciliary motility is regulated by the B-microtubule modification polyglutamylation (Alper et al. 2014; Ikegami et al. 2010; Kubo et al. 2010; Suryavanshi et al. 2010) see also (Sirajuddin et al. 2014). Kubo et al., (2012) proposed that dynein e and/or the N-DRC might be regulated by B-tubule polyglutamylation. We reactivated the polyglutamylation mutants, tpg1 and tpg2, in vitro with ATP. Live tpg1 and tpg2 cells swim slower than WT, but faster than most drc mutants. Reactivated motility of the polyglutamylation mutants occurred significantly more frequently than drc mutants lacking the linker domain of the N-DRC (compare Fig. 2B and 4A).

Axonemes from tpg1 and tpg2 cell models remained intact (Fig. 4B, 85.4 ±11.8% and 85.5 ± 9.5% intact, respectively). Although tpg1 and tpg2 cell models occasionally showed some splaying at the very distal tip of the axoneme, this splaying was different from that observed with most drc mutants. Splaying in the tpg mutants was never longer than about 1 μm (Fig. 4C), whereas that for the drc mutants ranged from 3 – 9 μm (Fig. 3A). Similarly, the very few WT axonemes that splayed upon in vitro reactivation only exhibited this slight splaying at the distal tip and never complete splaying of the axoneme seen in drc mutants (compare Fig. 3A,B to Fig. 4C and Fig. 5D). Thus, the minor distal splaying at the distal tip of the axoneme does not dramatically affect reactivated movement.

To test the hypothesis that the N-DRC linker interacts with the glutamylated B-tubule to maintain axoneme integrity, we generated double mutants defective in tubulin polyglutamylation and the N-DRC (Table S1). The double mutant genotypes were confirmed by immunoblots and PCR (Fig. S2). Cell models were then tested for ATP-induced reactivation. The sup-pf3; tpg1 and ida6; tpg2 double mutants failed to reactivate motility (Fig. 5A, grey bars), similar to sup-pf3 and ida6 (Fig. 5A, open bars). The drc3; tpg1 and drc3; tpg2 double mutants reactivated motility in vitro, like the tpg and drc3 single mutants (Fig. 5A). However, the sup-pf4; tpg1 and sup-pf4; tpg2 double mutants failed to reactivate motility (Fig. 5A, grey bars), unlike the sup-pf4 (Fig. 5A, open bars; Fig. 2B), tpg1, and tpg2 (Fig. 5A, black bars; Fig. 4A) single mutants that reactivate like WT. Failure to reactivate in the sup-pf4; tpg double mutants is likely due to the lack of the portion of the distal lobe that contacts the B-tubule, including DRC5 and DRC6 (Fig. 1). Consistent with ATP-induced reactivated motility phenotypes (Fig. 5A), axonemes of the double mutants sup-pf3; tpg1, ida6; tpg2, sup-pf4; tpg1 and sup-pf4; tpg2 splayed after in vitro reactivation (Fig. 5B, grey bars, and Fig. 5C).

Comparison of the sup-pf4 and drc3 double mutants reveals that sup-pf4, which lacks the distal lobe of the N-DRC linker (Fig. 1I), is dependent upon tubulin polyglutamylation for reactivated motility and axonemal integrity. Notably, the splaying of axonemes in sup-pf4; tpg cell models is not only more frequent than drc3; tpg cell models (Fig. 5B, arrows and arrowheads, respectively), but also different and more extreme (compare Fig. 5C and D). Thus, the in vitro reactivation assays indicate that a connection between the proximal and distal lobes of the N-DRC linker (Fig. 1) and B-tubule glutamylation are, together, important for axonemal integrity and normal N-DRC function.

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Interestingly, one of the N-DRC subunits, DRC7, contains a conserved transglutaminase-like (TGL) peptidase domain that is predicted to bind tightly to glutamylated proteins (Bower et al. 2013; Zhang and Aravind 2012). This subunit is retained in both sup-pf4 and drc3, but missing in the other drc mutants (Awata et al. 2015; Bower et al. 2013). However, the biochemical stability of the complex, especially DRC7, appears to be compromised in sup-pf4 extracts (Bower et al. 2013). It remains to be demonstrated that DRC7 interacts directly with glutamylated tubulin, but it is an interesting possibility that would be consistent with our findings.

**Dynein e does not contribute to axonemal integrity**

*Chlamydomonas* mutants defective in subsets of inner dynein arms suggest that the function of inner dynein arm dynein e also depends on B-tubule polyglutamylation (Kubo et al. 2012). We tested the role of dynein e in axonemal integrity using our *in vitro* assays. Since no known mutant for dynein e exists, we compared ATP-induced reactivation of ida4 (missing inner arm dyneins a, c, d) and ida5 (missing inner arm dyneins a, c, d, and e) (Kamiya and Yagi 2014). Like WT, ida4 and ida5 cell models reactivated motility 83.7 ± 10.8% and 76.0 ± 11.9% of the time, respectively (Fig. S3A). Accordingly, axonemes of reactivated ida4 and ida5 cell models remain intact (Fig. S3B). Thus, the absence of dynein e does not have a significant effect on axonemal integrity. We also reactivated cell models of the ida5; tpg2 double mutants and quantified motility and axonemal integrity (Figs. S3C and S3D). Axonemal integrity is not dependent on dynein e in association with polyglutamylation on the adjacent B-tubule.

**Summary**

The results of these *in vitro* experiments are: 1) the N-DRC linker is required for integrity of the axoneme. In live cells, evidently the ciliary membrane (or other structures such as the microtubule caps at the distal tip of the axoneme; (Dentler 1980; Tammana et al. 2013) can maintain axonemal integrity, which is required for motility, in the absence of the N-DRC linker. 2) B-tubule polyglutamylation works in concert with the distal lobe of the N-DRC linker to maintain axonemal integrity between outer doublet microtubules. 3) Inner arm dynein e does not contribute to axonemal integrity. We postulate that the distal lobe of the N-DRC linker (Fig. 1C) interacts with the polyglutamylated B-tubule to maintain axonemal integrity and outer doublet alignment. Further work is required to understand the molecular interactions between the polypeptides located in the distal lobe of the N-DRC linker and the B-tubule.

**Materials and Methods**

**Strains and culture conditions**

Cells were obtained from the *Chlamydomonas* Resource Center (University of Minnesota, St. Paul, MN), as a gift from George Witman and Tomohiro Kubo, or generated for this study. The strains used in this study are listed in Table S1. Double mutants were recovered from either non-parental or tetratype tetrads as described previously (Dutcher 1995; Harris 2009). Strains were maintained on Tris-acetate phosphate (TAP) solid medium in constant illumination. For *in vitro* reactivation, swimming analysis, and axonemal isolation, cells
were transferred to liquid media with aeration on a 14:10 h light/dark cycle or under constant illumination.

**In vitro reactivation assay**

*In vitro* reactivation was performed as previously described in (Kamiya and Witman 1984) with modifications. Wild-type or mutant cells were grown to early log phase in either M or TAP media. 15 ml of cells were gently pelleted in a conical tube in a clinical centrifuge at medium speed for 1 minute. Pelleted cells were resuspended in HS buffer (10 mM HEPES pH 7.4, 4% sucrose) to a final concentration of 3 x 10^6 cells/ml. With a cut pipet tip, 10 μl of cells in HS were pipetted into 50 μl of demembranation buffer (30 mM HEPES pH 7.4, 5 mM MgSO_4_, 1 mM DTT, 1 mM EGTA, 50 mM potassium acetate, 1% PEG-8000, 0.1% NP-40) and let incubate at room temperature for 20 seconds. With the same cut pipet tip, 10 μl demembranated cells (termed cell models) were pipetted into reactivation buffer (30 mM HEPES pH 7.4, 5 mM MgSO_4_, 1 mM DTT, 2 mM EGTA, 50 mM potassium acetate, 1% PEG-8000, 0.1 mM ATP). Reactivated cell models were either: 1) pipetted immediately into a perfusion chamber as described in (Smith and Sale 1992) for swimming analysis by dark-field microscopy or 2) fixed by adding 15 μl 2% glutaraldehyde (final concentration 0.4%) for axonemal splaying analysis. In some experiments, protease inhibitors were added to the demembranation and reactivation buffers at the following concentrations: 1–10 mM phenylmethanesulfonyl fluoride (PMSF; Sigma, St. Louis, MO), 0.1–0.5 Trypsin Inhibitor Units (TIU)/mg aprotinin (from bovine lung; Sigma, St. Louis, MO), and 1–2 protease inhibitor cocktail tablets per 5ml buffer (cOmplete Mini, Roche, Indianapolis, IN). In additional control experiments, 1.0 – 5.0 μM vanadate was included with 0.1 mM ATP (Sale and Gibbons, 1979).

**Dark-field microscopy**

Images were recorded using a Zeiss Axiocvert 35 microscope equipped with dark-field optics, a Zeiss 40 or 10 Plan-Apo lens, and a silicon intensified camera (VE-1000; Dage-MTI, Michigan City, IN). The video images were converted to a digital format using Labview 7.1 software (National Instruments, Austin, TX). *Swimming velocity analysis of live cells*: Early log phase wild-type and mutant cells were imaged in perfusion chambers by time lapse for 30 seconds. Videos were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) for swimming speed in μm/s (Table S1). *Swimming analysis of reactivated cell models*: Reactivated cell models were imaged in perfusion chambers by time lapse in 30-second intervals at 10 objective magnification. Cell models were manually analyzed for forward progressive movement, swimming, over 30 – 120 s time lapses and scored. *Axonemal splaying analysis*: Fixed reactivated cell models were observed under 40 magnification for axonemal splaying. At least 50 axonemes in multiple fields were counted per experiment and scored as intact or splayed, without consideration of the degree of splaying.

**Statistical analysis**

GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) was used for graphing and statistical analysis. The Grubbs’ test was used to identify outliers within each data set and unpaired two-tailed t-tests (parametric assuming Gaussian distribution and equal standard
deviations) were used to compare reactivated motility and axonemal integrity between strains.

**Immunofluorescence**

To clearly demonstrate the splaying phenotypes observed in reactivated cell models, immunofluorescence was performed. Reactivated cell models were adhered to a 1% polyethylenimine-coated coverslip for 5 minutes and fixed by submersion into ice-cold (−20°C) methanol for 10 seconds. The coverslips were then air-dried, rehydrated with 0.1M HEPES, pH 7.4, blocked for 1 h at RT with blocking solution (1% fish skin gelatin, 2% BSA, 15% horse serum, 0.02% saponin, and 0.1% Triton X-100 in PBS, pH 7.0), and incubated with anti-acetylated tubulin antibody, clone 611B1, at 1:100 in block (Sigma-Aldrich, St. Louis, MO). Coverslips were subsequently washed 3X with blocking solution, incubated with a secondary antibody (Alexa Fluor 555–conjugated IgG, 1:1000; Invitrogen, Eugene, OR) for 1 h at RT, washed 3X with blocking solution, rinsed in buffer, then mounted with ProLong Antifade Gold (Invitrogen, Eugene, OR). Images were captured using a BX60 wide-field microscope (Olympus, Tokyo, Japan) with a 12-bit digital camera (Orca-ER, Hamamatsu, Bridgewater, NJ) and Slidebook software (Intelligent Imaging Innovations, Denver, CO). Image processing was performed in ImageJ (National Institutes of Health, Bethesda, MD). The frequency of splaying in reactivated cell models was increased compared to those observed by dark-field microscopy, likely an artifact of the methanol fixation. Thus, these reactivated cell models were not used for quantification.

**Cryo-ET**

Freshly isolated *C. reinhardtii* axonemes were plunge frozen and vitrified on holy grids (R2/2; 200 mesh; Quantifoil Micro Tools) as previously described (Heuser et al., 2009). In brief, the grids were glow discharged at −45 mA for 30 s and coated with 10-nm colloidal gold (Sigma-Aldrich). 3 −l of the axoneme sample and 1 −l of 10 times concentrated 10-nm colloidal gold solution were applied to the grid. Then, excess liquid was blotted away, and the grid was plunge frozen in liquid ethane that was cooled by liquid nitrogen. Grids were stored in liquid nitrogen until observed by EM.

Cryo-ET was performed on a transmission electron microscope (Tecnai F30; FEI) operated at 300 keV. Using the microscope control software SerialEM (Mastronarde, 2005), tomographic tilt series were collected from −65 to 65° with 1.5–2.5° increments using a −6- or −8–m defocus to increase the phase contrast. Tilt series were recorded at a nominal magnification of 13,500 using a 2,000 × 2,000–pixel charge-coupled device camera (resulting in a pixel size of 1 nm; Megascan 795; Gatan) and an energy filter (GIF; Gatan) operated in zero-loss mode with 20-eV slit width. The total electron dose used for a tilt series was limited to 100 e/Å².

Tomograms showing the 3D structure of *C. reinhardtii* axonemes were reconstructed using the IMOD software package (Kremer et al. 1996) with gold fiducial marker alignment and weighted back projection. Some of the tomograms were previously used for the analysis of other axonemal complexes (Heuser et al. 2012a; Heuser et al. 2009; Lin et al. 2012). Subtomogram averaging of the 96-nm axonemal repeats was performed using the Particle...
Axoneme isolation, SDS-PAGE and immunoblot

Deciliation of Chlamydomonas cells was induced by treating cells with dibucaine (Witman, 1986). Axonemes were isolated and prepared for immunoblot as previously described (Alford et al. 2013). SDS-PAGE was performed using 7.5% or 10% acrylamide gels. Gels were stained with Coomassie brilliant blue (CBB) or transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) for subsequent immuno-blotting. The membranes were blocked with 5% nonfat dry milk and incubated with various primary antibodies. Immunoreactive bands were detected using an HRP-conjugated secondary antibody (1:20,000; Bio-Rad Laboratories, Hercules, CA) and Pierce ECL western blotting chemiluminescent substrate (Thermo Scientific, Rockford, IL). Immunoblots were probed with the following antibodies (5–10 μg axonemes): anti-DRC5/FAP155 (1:500; (Bower et al. 2013)), anti-DRC3 (1:4000; (Awata et al. 2015)), and anti-tubulin clone B3 (1:1000; Abcam, Cambridge, MA).

Chlamydomonas colony PCR

A single colony of medium size was picked into 50 μl 10 mM EDTA pH 8.0. The tube was vortexed for 10 seconds, heated at 100°C for 10 min, vortexed again for 10 seconds, placed on ice for 1 min, and spun at 3000 rpm for 5 min; 1 μl of the supernatant was used as the template for the PCR reaction. The 50 μl PCR reaction mixture contained 1 pmol of each primer, 1X GoTaq reaction buffer (Promega Corp., Madison, WI), 1.25u GoTaq DNA Polymerase (Promega Corp., Madison, WI), 0.2 mM dNTPs (Promega Corp., Madison, WI), and 5% DMSO. Following an initial 3 min denaturation step at 95°C, the samples underwent 35 cycles of: 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min. PCR primers used were NPHP4-F1 and -R1 from (Awata et al. 2015).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structural comparison of WT and N-DRC mutants

Isosurface renderings of the averaged 96-nm axonemal repeat from wild-type (A–C), drc3 (D–F), sup-pf4 (G–I), and pf2 (J–L) Chlamydomonas flagella. The N-DRC linker (green) and base plate (blue) are shown in cross-section from distal (A, D, G, J), and in longitudinal views (B, E, H, K; proximal is on the left). The N-DRC linker connection to the neighboring (n+1) B-tubule (Bₙ) is highlighted in the enlarged longitudinal bottom views (C, F, I, L); to clearly show the N-DRC, the inner dynein arms (IDA, pink) and radial spoke 2 (RS2) have been cropped from these images. The three mutant axonemes show different degrees of structural defects: in drc3 the L1-linker arm and its connection to the distal lobe (dL) are missing (D, F), in sup-pf4 the distal lobe is reduced (I), and in pf2 a small projection of the base plate (J) and most of the linker, including the proximal and distal lobes (pL, dL) are missing, so that the N-DRC no longer connects to the neighboring B-tubule (J, L). Other labels: Aₜ, A-tubule; a-g, inner dyneins, including the two-headed (α,β) dynein f with its intermediate/light chain complex (ICLC); ODA, outer dynein arms.

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Figure 2. Dark-field microscopy of cell models revealed that the N-DRC linker is required for ATP-induced reactivated movement

(A) Time-lapse images of cell models after ATP-induced reactivation (cell bodies highlighted in yellow circles). WT cell models reactivated motility in the form of forward, progressive swimming. As predicted, the sup-pf3 cell models were unable to reactivate motility in the presence of ATP. ATP-induced reactivation also failed in pf3, ida6, sup-pf5, and pf2. In contrast, the sup-pf4 and drc3 cell models reactivated motility like WT. Scale bar = 50μm

(B) Forward progressive swimming as a result of ATP-induced in vitro reactivation was quantified for wild-type, the drc mutants, and control rescued strains. WT cell models exhibited reactivated motility 89.6 ± 8.3% of the time (n = 809; 12 independent experiments), while pf3, ida6, sup-pf5, sup-pf3, and pf2 reactivated less than 25% of the time (n >100; 2–9 independent experiments depending on cell type). In contrast to other drc mutants, sup-pf4 and drc3 cell models reactivated motility 91.5 ± 10.1% and 91.2 ± 4.8% of the time, respectively. (n >280; ≥6 independent experiments for each cell type; *p<0.0001 comparing pf3, ida6, sup-pf5, sup-pf3, and pf2 to WT, sup-pf4 and drc3.) Control, rescued strains (open bars) of drc mutant strains exhibited ATP-induced reactivated motility like WT. (n >40; ≥2 independent experiments for each cell type; *p<0.0001 comparing drc mutants to WT and corresponding rescue strains.)
Figure 3. Axonemal integrity requires the N-DRC linker and correlates with in vitro reactivated motility

(A) Representative dark-field microscopy images of cell models following ATP-induced in vitro reactivation and glutaraldehyde fixation. Axonemes remain intact in cell models from WT and drc mutants that retain the N-DRC linker (sup-pf4 shown as example). In contrast, axonemes from drc mutant cell models that fail to assemble the nexin linker (sup-pf3 shown as example) become splayed into individual or small groups of doublet microtubules after ATP-addition. Dashed lines define doublet microtubules out of the plane of focus to illustrate splaying of individual microtubules. Scale bar = 10 μm.

(B) Immunofluorescence of reactivated cell models after methanol fixation using an antibody to acetylated α-tubulin (611-B1). Splaying, typical of drc mutants lacking the N-DRC linker, occurs along the distal portion of the axoneme separating individual doublet microtubules. Scale bar = 5 μm.

(C) Quantification of axonemal integrity after in vitro reactivation of wild-type and drc mutant cell models viewed by dark-field microscopy. Wild-type and drc mutants that retain the N-DRC linker (sup-pf4, drc3) and control rescued axonemes were intact >95% of the time (n >200; >2 independent experiments for each cell type. In contrast, axonemes from drc mutants that lack the N-DRC linker domain (pf3, ida6, sup-pf5, sup-pf3, and pf2) splayed into doublets and were intact less than 70% of the time. (n >250; >2 independent experiments for each cell type; *p<0.0001, comparing pf3, ida6, sup-pf5, sup-pf3, and pf2 to WT, sup-pf4, drc3, and corresponding rescued strains.)
Figure 4. B-tubule polyglutamylation alone plays a minor role in axonemal integrity

(A) Quantification of ATP-induced reactivated motility of wild-type and polyglutamylation (tpg) mutants. Wild-type (CC-125) cell models exhibited reactivated motility 89.6 ± 8.3% of the time, while tpg1 exhibited reactivated motility 73.9 ± 17.0% and tpg2 71.3 ± 17.4% of the time (n >550; >9 independent experiments for each cell type; **p<0.001).

(B) Quantification of axonemal integrity after in vitro ATP-induced reactivation of wild-type and tpg mutant cell models. Dark-field microscopy images of reactivated cell models, depicted in Fig. 4C, were quantified for intact or splayed axonemal microtubules. Wild-type axonemes remained intact 95.2 ± 4.2% of the time. The polyglumatylation mutants remained intact 85.4 ± 11.8% (tpg1) and 85.5 ± 9.5% (tpg2) of the time (n >800; >6 independent experiments for each cell type; *p<0.01 **p<0.001).

(C) In contrast to splaying of the drc mutants lacking the N-DRC linker (Fig. 3A, B), splaying was restricted to the distal 1 μm in axonemes of tpg1 and tpg2 reactivated cell models. Scale bar = 10 μm.
Figure 5. The N-DRC linker and B-tubule polyglutamylation work together to maintain axoneme integrity

(A) Double mutants with drc and polyglutamylation defects (tpg) were analyzed for ATP-induced reactivated motility in vitro. The double mutant cell models of sup-pf3; tpg1 and ida6; tpg1 displayed minimal reactivated motility, like the single drc mutants (n = 100; ≥2 independent experiments for each double mutant cell type). Unlike the corresponding single mutants, both sup-pf4; tpg1 and sup-pf4; tpg2 cell models failed to reactivate motility in vitro (n >150; ≥3 independent experiments for each cell type). Notably, like tpg single mutants, double mutant drc3; tpg1 and drc3; tpg2 cell models reactivated motility in vitro (n >200; >3 independent experiments for each cell type). **p<0.0001 comparing double mutants to the corresponding single mutants.
(B) Axonemal integrity after ATP-induced \textit{in vitro} reactivation correlates with the motility phenotype. Specifically, axonemes from \textit{sup-pf3; tpg1, ida6; tpg1, sup-pf4; tpg1} and \textit{sup-pf4; tpg2} cell models remained intact <25\% of the time after ATP-induced \textit{in vitro} reactivation (n >190; ≥2 independent experiments for each cell type). In contrast, \textit{drc3; tpg1} and \textit{drc3; tpg2} cell models reactivated motility \textit{in vitro} (A) and axonemes remained intact >70\% of the time (n ≥500; ≥4 independent experiments for each cell type) compared to >85\% for the \textit{tpg} single mutants (B). The degree of axonemal splaying was distinct between \textit{sup-pf4; tpg} mutants (arrows, C) and \textit{drc3; tpg} mutants (arrowheads, D). *p<0.01 **p<0.0001 comparing double mutants to the corresponding single mutant with the most frequent splaying phenotype.  

(C) Splaying seen in \textit{drc} mutants lacking the N-DRC linker and \textit{sup-pf4; tpg} double mutants is represented. Immunofluorescence of acetylated α-tubulin illustrates splaying of individual or small groups of doublet microtubules. 

(D) Splaying of \textit{drc3; tpg} axonemes was similar to polyglutamylation mutants, limited to the distal 1 −m (compare Fig. 4C). Immunofluorescence of acetylated α-tubulin illustrated the minor splaying phenotype.