Published in final edited form as:

Cytoskeleton (Hoboken). 2016 June ; 73(7): 331–340. doi:10.1002/cm.21301.

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

Lea M. Alford^{*}, Daniel Stoddard^{^,&}, Jennifer H. Li^{*}, Emily L. Hunter^{*}, Douglas Tritschler[@], Raquel Bower[@], Daniela Nicastro[&], Mary E. Porter[@], and Winfield S. Sale^{*,#}

^{*}Department of Cell Biology, Emory University, 465 Whitehead Biomedical Research Building, 615 Michael Street, Atlanta, GA 30322

[^]Biology Department, Brandeis University, Rosenstiel Basic Medical Science Research Center, 415 South Street, Waltham, MA 02454

[@]Department of Genetics, Cell Biology and Development, University of Minnesota Medical School, 6-160 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455

[&]Dept. of Cell Biology and Biophysics, University of Texas Southwestern Medical School, Dallas, TX 75390

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 axoneme 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

[#]Corresponding author: Phone 404-727-6265; wsale@emory.edu.

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 Linck 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 interdoubt 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 $89.6 \pm 8.3\%$ 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 ($91.5 \pm 10.1\%$ and $91.2 \pm 4.8\%$ 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 $95.2 \pm 4.2\%$ 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 reactivate 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 μ M 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 interdoublt linker.

In contrast to the other *drc* mutants, axonemes from *sup-pf4* and *drc3* remained intact after ATP-induced *in vitro* reactivation $98.1 \pm 1.9\%$ and $99.5 \pm 1.4\%$ 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 reactivate 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, $85.4 \pm 11.8\%$ and $85.5 \pm 9.5\%$ 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.

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* cells models reactivated motility $83.7 \pm 10.8\%$ and $76.0 \pm 11.9\%$ 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 3×10^6 cells/ml. With a cut pipet tip, 10 μ l of cells in HS were pipetted into 50 μ l of demembration 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 μ l demembrated 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 μ l 2% glutaraldehyde (final concentration 0.4%) for axonemal splaying analysis. In some experiments, protease inhibitors were added to the demembration 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 μ M vanadate was included with 0.1 mM ATP (Sale and Gibbons, 1979).

Dark-field microscopy

Images were recorded using a Zeiss Axiovert 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 $\mu\text{m/s}$ (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 holey 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 μl of the axoneme sample and 1 μl 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 (Mastrorade, 2005), tomographic tilt series were collected from -65 to 65° with 1.5 – 2.5° increments using a -6 – -8 μm defocus to increase the phase contrast. Tilt series were recorded at a nominal magnification of 13,500 using a $2,000 \times 2,000$ -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 $100\text{ e}/\text{\AA}^2$.

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

Estimation for Electron Tomography software (Nicastró et al., 2006) available from the Boulder Laboratory for 3D Electron Microscopy of Cell (Colorado). The University of California, San Francisco, Chimera package (Pettersen et al. 2004) was used for 3D visualization by isosurface rendering and data analysis.

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 –g 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 –l 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 –l of the supernatant was used as the template for the PCR reaction. The 50 –l 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

We are grateful to Drs. George Witman, Junya Awata, Tomohiro Kubo (University of Massachusetts Medical Center, Worcester) for sharing data before publication and mutant cells. L.M.A. was supported by a training grant from the NIH (Emory University FIRST Postdoctoral Career Development Award K12 GM000608). E.L.H. was supported by a predoctoral fellowship from American Heart Association. The work was also supported by Department of Pediatrics and the Pediatric Research Center, Children's Hospital of Atlanta (CHOA) Pilot Grant NIAAA P50 AA013757 10 pilot project 00006748, and grants from the NIH (W.S.S., NIH GM051173; M.E.P., GM-055667 and D.N. GM083122).

References

Alford LM, Matheyses AL, Hunter EL, Lin HW, Dutcher SK, Sale WS. The *Chlamydomonas* Mutant pf27 Reveals Novel Features of Ciliary Radial Spoke Assembly. *Cytoskeleton*. 2013; 70(12):804–818. [PubMed: 24124175]

Cytoskeleton (Hoboken). Author manuscript; available in PMC 2017 June 13.

- Alper JD, Decker F, Agana B, Howard J. The motility of axonemal dynein is regulated by the tubulin code. *Biophys J*. 2014; 107(12):2872–80. [PubMed: 25658008]
- Aoyama S, Kamiya R. Cyclical interactions between two outer doublet microtubules in split flagellar axonemes. *Biophys J*. 2005; 89(5):3261–8. [PubMed: 16113117]
- Austin-Tse C, Halbritter J, Zariwala MA, Gilberti RM, Gee HY, Hellman N, Pathak N, Liu Y, Panizzi JR, Patel-King RS, et al. Zebrafish Ciliopathy Screen Plus Human Mutational Analysis Identifies C21orf59 and CCDC65 Defects as Causing Primary Ciliary Dyskinesia. *Am J Hum Genet*. 2013; 93(4):672–86. [PubMed: 24094744]
- Awata J, Song K, Lin J, King SM, Sanderson MJ, Nicastro D, Witman GB. DRC3 connects the N-DRC to dynein g to regulate flagellar waveform. *Mol Biol Cell*. 2015; 26(15):2788–2800. [PubMed: 26063732]
- Bower R, Tritschler D, Vanderwaal K, Perrone CA, Mueller J, Fox L, Sale WS, Porter ME. The N-DRC forms a conserved biochemical complex that maintains outer doublet alignment and limits microtubule sliding in motile axonemes. *Mol Biol Cell*. 2013; 24(8):1134–52. [PubMed: 23427265]
- Bozkurt HH, Woolley DM. Morphology of Nexin Links in Relation to Interdoublet Sliding in the Sperm Flagellum. *Cell Motility and the Cytoskeleton*. 1993; 24(2):109–118. [PubMed: 8440024]
- Brokaw CJ. Elastase digestion of demembranated sperm flagella. *Science*. 1980; 207(4437):1365–7. [PubMed: 6898364]
- Burgess SA, Carter DA, Dover SD, Woolley DM. The inner dynein arm complex: compatible images from freeze-etch and thin section methods of microscopy. *J Cell Sci*. 1991; 100(Pt 2):319–28. [PubMed: 1836791]
- Dentler WL. Structures linking the tips of ciliary and flagellar microtubules to the membrane. *J Cell Sci*. 1980; 42:207–20. [PubMed: 6772653]
- Dutcher SK. Mating and tetrad analysis in *Chlamydomonas reinhardtii*. *Methods Cell Biol*. 1995; 47:531–40. [PubMed: 7476541]
- Dymek EE, Heuser T, Nicastro D, Smith EF. The CSC is required for complete radial spoke assembly and wild-type ciliary motility. *Mol Biol Cell*. 2011; 22(14):2520–31. [PubMed: 21613541]
- Gardner LC, O'Toole E, Perrone CA, Giddings T, Porter ME. Components of a “dynein regulatory complex” are located at the junction between the radial spokes and the dynein arms in *Chlamydomonas* flagella. *J Cell Biol*. 1994; 127(5):1311–25. [PubMed: 7962092]
- Gardner LC, O'Toole E, Perrone CA, Giddings T, Porter ME. Components of a “dynein regulatory complex” are located at the junction between the radial spokes and the dynein arms in *Chlamydomonas* flagella. *J Cell Biol*. 1994; 127:1311–1325. [PubMed: 7962092]
- Gibbons IR. Studies on the Protein Components of Cilia from *Tetrahymena Pyriformis*. *Proc Natl Acad Sci U S A*. 1963; 50:1002–10. [PubMed: 14082342]
- Gibbons IR. Chemical dissection of cilia. *Arch Biol (Liege)*. 1965; 76(2):317–52. [PubMed: 5323574]
- Goodenough, UW.; Heuser, JE. Structure of the Soluble and In Situ Ciliary Dyneins Visualized by Quick-Freeze Deep-Etch Microscopy. In: Warner, FD.; Satir, P.; Gibbons, IR., editors. *Cell Movement*. New York: Alan R. Liss; 1989. p. 121-140.
- Harris, EH. The *Chlamydomonas* Sourcebook: Introduction to *Chlamydomonas* and Its Laboratory Use. Harris, EH., editor. Oxford: Academic Press; 2009.
- Heuser T, Barber CF, Lin J, Krell J, Rebesco M, Porter ME, Nicastro D. Cryoelectron tomography reveals doublet-specific structures and unique interactions in the II dynein. *Proc Natl Acad Sci U S A*. 2012a; 109(30):E2067–76. [PubMed: 22733763]
- Heuser T, Dymek EE, Lin J, Smith EF, Nicastro D. The CSC connects three major axonemal complexes involved in dynein regulation. *Mol Biol Cell*. 2012b; 23(16):3143–55. [PubMed: 22740634]
- Heuser T, Raytchev M, Krell J, Porter ME, Nicastro D. The dynein regulatory complex is the nexin link and a major regulatory node in cilia and flagella. *J Cell Biol*. 2009; 187(6):921–33. [PubMed: 20008568]
- Ikegami K, Sato S, Nakamura K, Ostrowski LE, Setou M. Tubulin polyglutamylation is essential for airway ciliary function through the regulation of beating asymmetry. *Proc Natl Acad Sci U S A*. 2010; 107(23):10490–5. [PubMed: 20498047]

- Kamiya R, Witman GB. Submicromolar levels of calcium control the balance of beating between the two flagella in demembranated models of *Chlamydomonas*. *J Cell Biol.* 1984; 98(1):97–107. [PubMed: 6707098]
- Kamiya R, Yagi T. Functional Diversity of Axonemal Dyneins As Assessed by In Vitro and In Vivo Motility Assays of *Chlamydomonas* Mutants. *Zoological Science.* 2014; 31(10):633–644. [PubMed: 25284382]
- Kremer JR, Mastronarde DN, McIntosh JR. Computer visualization of three-dimensional image data using IMOD. *J Struct Biol.* 1996; 116(1):71–6. [PubMed: 8742726]
- Kubo T, Yagi T, Kamiya R. Tubulin polyglutamylation regulates flagellar motility by controlling a specific inner-arm dynein that interacts with the dynein regulatory complex. *Cytoskeleton (Hoboken).* 2012; 69(12):1059–68. [PubMed: 23047862]
- Kubo T, Yanagisawa HA, Liu Z, Shibuya R, Hirono M, Kamiya R. A conserved flagella-associated protein in *Chlamydomonas*, FAP234, is essential for axonemal localization of tubulin polyglutamylase TTL9. *Mol Biol Cell.* 2014; 25(1):107–17. [PubMed: 24196831]
- Kubo T, Yanagisawa HA, Yagi T, Hirono M, Kamiya R. Tubulin polyglutamylation regulates axonemal motility by modulating activities of inner-arm dyneins. *Curr Biol.* 2010; 20(5):441–5. [PubMed: 20188560]
- Lin H, Zhang Z, Guo S, Chen F, Kessler JM, Wang YM, Dutcher SK. A NIMA-Related Kinase Suppresses the Flagellar Instability Associated with the Loss of Multiple Axonemal Structures. *PLoS Genet.* 2015; 11(9):e1005508. [PubMed: 26348919]
- Lin J, Heuser T, Song K, Fu X, Nicastro D. One of the nine doublet microtubules of eukaryotic flagella exhibits unique and partially conserved structures. *PLoS One.* 2012; 7(10):e46494. [PubMed: 23071579]
- Lin J, Tritschler D, Song K, Barber CF, Cobb JS, Porter ME, Nicastro D. Building blocks of the nexin-dynein regulatory complex in *Chlamydomonas* flagella. *J Biol Chem.* 2011; 286(33):29175–91. [PubMed: 21700706]
- Lindemann CB, Lesich KA. Flagellar and ciliary beating: the proven and the possible. *J Cell Sci.* 2010; 123(Pt 4):519–28. [PubMed: 20145000]
- Mastronarde DN, O'Toole ET, McDonald KL, McIntosh JR, Porter ME. Arrangement of inner dynein arms in wild-type and mutant flagella of *Chlamydomonas*. *J Cell Biol.* 1992; 118(5):1145–62. [PubMed: 1387403]
- Minoura I, Yagi T, Kamiya R. Direct measurement of inter-doublet elasticity in flagellar axonemes. *Cell Struct Funct.* 1999; 24(1):27–33. [PubMed: 10355876]
- Nicastro D, Schwartz C, Pierson J, Gaudette R, Porter ME, McIntosh JR. The molecular architecture of axonemes revealed by cryoelectron tomography. *Science.* 2006; 313(5789):944–8. [PubMed: 16917055]
- Oda T, Yagi T, Yanagisawa H, Kikkawa M. Identification of the outer-inner Dynein linker as a hub controller for axonemal Dynein activities. *Curr Biol.* 2013; 23(8):656–64. [PubMed: 23583547]
- Oda T, Yanagisawa H, Kikkawa M. Detailed structural and biochemical characterization of the nexin-dynein regulatory complex. *Mol Biol Cell.* 2015; 26(2):294–304. [PubMed: 25411337]
- Okagaki T, Kamiya R. Microtubule sliding in mutant *Chlamydomonas* axonemes devoid of outer or inner dynein arms. *J Cell Biol.* 1986; 103(5):1895–902. [PubMed: 2946702]
- Olson GE, Linck RW. Observations of the structural components of flagellar axonemes and central pair microtubules from rat sperm. *J Ultrastruct Res.* 1977; 61(1):21–43. [PubMed: 915974]
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem.* 2004; 25(13):1605–12. [PubMed: 15264254]
- Piperno G, Mead K, Shestak W. The inner dynein arms I2 interact with a “dynein regulatory complex” in *Chlamydomonas* flagella. *J Cell Biol.* 1992; 118(6):1455–63. [PubMed: 1387875]
- Porter, ME. Flagellar Motility and the Dynein Regulatory Complex. In: King, SM., editor. *Dyneins: Structure, Biology and Disease.* Amsterdam: Elsevier; 2012. p. 337–365.
- Sale WS, Gibbons IR. Study of the mechanism of vanadate inhibition of the dynein cross-bridge cycle in sea urchin sperm flagella. *J Cell Biol.* 1979; 82(1):291–8. [PubMed: 158028]

- Satir P, Heuser T, Sale WS. A Structural Basis for How Motile Cilia Beat. *Bioscience*. 2014; 64(12): 1073–1083. [PubMed: 26955066]
- Sirajuddin M, Rice LM, Vale RD. Regulation of microtubule motors by tubulin isotypes and post-translational modifications. *Nat Cell Biol*. 2014; 16(4):335–44. [PubMed: 24633327]
- Smith EF, Sale WS. Structural and functional reconstitution of inner dynein arms in *Chlamydomonas* flagellar axonemes. *J Cell Biol*. 1992; 117(3):573–81. [PubMed: 1533396]
- Song K, Awata J, Tritschler D, Bower R, Witman GB, Porter ME, Nicastro D. In situ localization of N and C termini of subunits of the flagellar nexin-dynein regulatory complex (N-DRC) using SNAP tag and cryo-electron tomography. *J Biol Chem*. 2015; 290(9):5341–53. [PubMed: 25564608]
- Stephens RE. Isolation of Nexin - Linkage Protein Responsible for Maintenance of Nine-Fold Configuration of Flagellar Axonemes. *Biological Bulletin*. 1970; 139(2):438.
- Summers KE, Gibbons IR. Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. *Proc Natl Acad Sci U S A*. 1971; 68(12):3092–6. [PubMed: 5289252]
- Summers KE, Gibbons IR. Effects of trypsin digestion on flagellar structures and their relationship to motility. *J Cell Biol*. 1973; 58(3):618–29. [PubMed: 4747919]
- Suryavanshi S, Edde B, Fox LA, Guerrero S, Hard R, Hennessey T, Kabi A, Malison D, Pennock D, Sale WS, et al. Tubulin Glutamylation Regulates Ciliary Motility by Altering Inner Dynein Arm Activity. *Current Biology*. 2010; 20(5):435–440. [PubMed: 20189389]
- Tammana TV, Tammana D, Diener DR, Rosenbaum J. Centrosomal protein CEP104 (*Chlamydomonas* FAP256) moves to the ciliary tip during ciliary assembly. *Journal of Cell Science*. 2013; 126(21):5018–5029. [PubMed: 23970417]
- Warner FD. Ciliary inter-microtubule bridges. *J Cell Sci*. 1976; 20(1):101–14. [PubMed: 129478]
- Warner FD. Organization of Interdoublet Links in Tetrahymena Cilia. *Cell Motility and the Cytoskeleton*. 1983; 3(4):321–332.
- Wirschell M, Olbrich H, Werner C, Tritschler D, Bower R, Sale WS, Loges NT, Pennekamp P, Lindberg S, Stenram U, et al. The nexin-dynein regulatory complex subunit DRC1 is essential for motile cilia function in algae and humans. *Nat Genet*. 2013; 45(3):262–8. [PubMed: 23354437]
- Witman GB. Isolation of *Chlamydomonas* flagella and flagellar axonemes. *Methods in Enzymology*. 1986; 134:280–290. [PubMed: 3821567]
- Witman GB, Plummer J, Sander G. *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules. Structure, composition, and function of specific axonemal components. *J Cell Biol*. 1978; 76(3):729–47. [PubMed: 632325]
- Zhang D, Aravind L. Novel transglutaminase-like peptidase and C2 domains elucidate the structure, biogenesis and evolution of the ciliary compartment. *Cell Cycle*. 2012; 11(20):3861–75. [PubMed: 22983010]

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 ($B_t(n+1)$) 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: A_t , 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.

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 $89.6 \pm 8.3\%$ 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 $91.5 \pm 10.1\%$ and $91.2 \pm 4.8\%$ 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 μ m.

(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 μ m

(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 $89.6 \pm 8.3\%$ of the time, while *tpg1* exhibited reactivated motility $73.9 \pm 17.0\%$ and *tpg2* $71.3 \pm 17.4\%$ 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 $95.2 \pm 4.2\%$ of the time. The polyglutamylation mutants remained intact $85.4 \pm 11.8\%$ (*tpg1*) and $85.5 \pm 9.5\%$ (*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 $1 \mu\text{m}$ in axonemes of *tpg1* and *tpg2* reactivated cell models. Scale bar = $10 \mu\text{m}$.

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 *in vitro* reactivation correlates with the motility phenotype. Specifically, axonemes from *sup-pf3; tpg1, ida6; tpg1, sup-pf4; tpg1* and *sup-pf4; tpg2* cell models remained intact <25% of the time after ATP-induced *in vitro* reactivation (n >190; 2 independent experiments for each cell type). In contrast, *drc3; tpg1* and *drc3; tpg2* cell models reactivated motility *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 *tpg* single mutants (B). The degree of axonemal splaying was distinct between *sup-pf4; tpg* mutants (arrows, C) and *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 *drc* mutants lacking the N-DRC linker and *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 *drc3; tpg* axonemes was similar to polyglutamylation mutants, limited to the distal 1 μ m (compare Fig. 4C). Immunofluorescence of acetylated α -tubulin illustrated the minor splaying phenotype.