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Retrograde Intraflagellar Transport by Cytoplasmic Dynein-2 Is Required for Outer Segment Extension in Vertebrate Photoreceptors but Not Arrestin Translocation

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Abstract

Purpose—Anterograde intraflagellar transport (IFT) is essential for photoreceptor outer segment formation and maintenance, as well as for opsin trafficking. However, the role of retrograde IFT in vertebrate photoreceptors remains unclear. The purpose of this study was to evaluate zebrafish photoreceptors lacking the retrograde IFT motor, cytoplasmic dynein-2.

Methods—Morpholino oligonucleotides against the heavy chain (*dync2-h1*), light intermediate chain (*dync2-li1*), and intermediate chain (*dync2-i1*) subunits of cytoplasmic dynein-2 were injected into zebrafish embryos. Retinas and ciliated cells of these zebrafish morphants were analyzed by immunohisto-chemistry and transmission electron microscopy. Whole-field electroretinograms (ERGs) were performed on dynein morphants at 5 to 6 days after fertilization (dpf).

Results—Zebrafish lacking cytoplasmic dynein-2 function exhibited small eyes, kidney cysts, and short photoreceptor outer segments, some of which were disorganized with accumulated vesicles. Morphant photoreceptor connecting cilia were swollen, but neither opsin nor arrestin was mislocalized, although IFT88 accumulated in the distal region of the connecting cilium. Nasal cilia were shortened and displayed cytoplasmic swelling along the axoneme. Loss of cytoplasmic dynein-2 function resulted in a significant reduction in the amplitude of ERG a-, b-, and d-waves but no change in threshold response.

Conclusions—Retrograde IFT is essential for outer segment extension and IFT protein recycling in vertebrate photoreceptors. The results show, for the first time, that the *dync2-i1* subunit of cytoplasmic dynein-2 is necessary for retrograde IFT. In addition, arrestin translocation does not require retrograde IFT. Finally, the ERG results indicate that loss of cytoplasmic dynein-2 reduces the photoreceptor light response.

Vertebrate photoreceptor outer segments are modified sensory cilia,¹ and as is true of all other cilia, lack the cellular machinery for protein synthesis. The proteins required for phototransduction and that make up the outer segment, such as opsins, must pass through the connecting cilium. In addition to its function in phototransduction, opsin plays an important structural role in the outer segment² and the effective transport of proteins through the connecting cilium is critical to both the function and survival of photoreceptors. Failure of the

efficient transport of opsins to the outer segment leads to photoreceptor degeneration and death.^{3,4}

Intraflagellar transport (IFT) transports cargo through the connecting cilium to the outer segment.^{5,6} Studies of the zebrafish *ift57*, *ift88*, and *ift172* mutants have shown that the initial stages of outer segment formation require IFT and that IFT is also necessary for outer segment maintenance.⁷⁻⁹ Similarly, loss of IFT causes mislocalization of opsins and photoreceptor cell death. These studies, along with similar results in mouse *Ift88/polaris* mutants and kinesin-II conditional knockout mutants,^{5,6} reinforce the premise that the outer segment is, in fact, a modified cilium.⁶ As several diseases affecting cilia and ciliary trafficking result in blindness and retinal degeneration, understanding the role of IFT in vertebrate photoreceptors is of great importance.

In photoreceptor cell biology, anterograde IFT has received the most scrutiny, while the role of retrograde IFT remains largely unexplored.^{5,9,10} Evidence from several species indicates a conserved mechanism whereby kinesin-II¹¹ and *osm-3*¹² cooperate to drive anterograde IFT, whereas cytoplasmic dynein-2¹³ mediates retrograde IFT. The retrograde IFT motor is composed of an ATP-dependent homodimeric heavy chain, Dync2-h1 (previously termed DHC-1b)¹³⁻¹⁶, a cytoplasmic dynein-2 light intermediate chain, Dync2-li1,¹⁷⁻¹⁹ an intermediate chain, Dync2-i1,²⁰ and light chain known as LC8.^{20,21} Mutation of *Dync2-h1*, *Dync2-li1*, and LC8 all result in phenotypes consistent with defective retrograde IFT, with cells displaying shortened cilia that accumulate IFT particles. *Dync2-i1* was shown biochemically to associate with cytoplasmic dynein-2; however, the phenotypic consequence of *dync2-i1* dysfunction is unknown. Of importance, Dync2-h1 and Dync2-li1 localize to the connecting cilium of bovine photoreceptors,¹⁸ suggesting a role for retrograde IFT in photoreceptors.

Retrograde IFT recycles IFT proteins and other ciliary components by returning them to the basal body from the tip of the cilium.^{13,22} As photoreceptors shed approximately 10% of the outer segment material from the tips each day and most proteins move through the connecting cilium in a unidirectional manner (e.g., opsins), a role for retrograde IFT in photoreceptors has been sought. Retrograde IFT is necessary for other vertebrate cilia, as *Dync2-h1* and *Dync2-li1* mouse mutants have stumpy nodal cilia with swollen ciliary tips that contain disorganized microtubules, IFT proteins and cellular debris,^{23,24} reflecting the inability to return cargo to the ciliary base. Does retrograde IFT function in photoreceptors?

Although it is assumed that IFT components require retrograde IFT for recycling, arrestin and transducin also exhibit retrograde movement (reviewed in Calvert et al.²⁵). During light adaptation, arrestin moves from the inner segment to the outer segment through the connecting cilium, while transducin moves in a complementary fashion. During dark adaptation, arrestin translocates back to the inner segment, while transducin returns into the outer segment.²⁶⁻²⁸ Translocation of arrestin and transducin still occurs after ATP depletion of photoreceptors in retinal explants, suggesting that these proteins move via passive diffusion.²⁹ The kinetics of arrestin movement during light adaptation are 100 to 1000 times that of rhodopsin trafficking, suggesting that an active transport mechanism could not move arrestin to the outer segment so quickly.²⁵ However, the retrograde movement of arrestin during dark adaptation is much slower, raising the possibility that anterograde and retrograde arrestin movement are mediated by different processes. Furthermore, both the actin and microtubule cytoskeletons are required for the retrograde movements of arrestin and transducin, suggesting that motors could be involved.³⁰ As cytoplasmic dynein-2 is a microtubule minus-end motor located in the connecting cilium, IFT may mediate retrograde arrestin translocation through the photoreceptor connecting cilium.

We used morpholino oligonucleotides to eliminate the function of *dync2-h1*, *dync2-li1*, and *dync2-i1* in zebrafish embryos to understand the function of retrograde IFT in vertebrate photoreceptors. Retrograde IFT was necessary for outer segment extension, organization, and recycling of IFT proteins in photoreceptors. In addition, we describe robust light-dependent translocation of visual arrestin in larval zebrafish rods. Finally, we show that dynein morphant retinas exhibit reduced ERG a- and b-wave amplitudes, indicating that visual function requires cytoplasmic dynein-2.

Materials and Methods

Fish Maintenance and Breeding

Wild-type zebrafish of the AB strain were housed, bred, and staged according to standard procedures.³¹ The zebrafish were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cloning of Dynein Genes and Phylogenetic Analysis of *dync2-h1*

5' RACE (rapid amplification of cDNA ends) was performed on retinal cDNAs to clone the 5' end of *dync2-h1* using the primer *dync2-h1* RACE1R: 5' TGCAGCAGGACGGGGCTGTAGACCTGA 3' and the 5' end of *dync2-i1* using the nested primers *dync2-i1* RACE 1R: 5' TTTGGCCGCTCTGGTCTTGTGTTTG 3' and *dync2-i1*-nested-2R: 5'ATCGCCATCATCCACACGGCCAAAT3'. 5' RACE was performed (Gene Racer Kit; Invitrogen, Eugene, OR) according to the manufacturer's instructions. Initial *dync2-h1* sequence data were obtained from accession numbers: XR_029028 and XR_029028.2. The *dync2-h1* sequence was aligned against genomic contig NW_001514387 to annotate intron-exon boundaries for the 5' 1.3kb of the coding sequence for morpholino design. Sequence data for *dync2-i1* and *dync2-li1* was obtained from Ensembl, numbers ENDSARG00000057635 and ENSDAR00000039770, respectively.³² Phylogenetic analysis was performed with MEGA software (www.megasoftware.net/ Molecular Evolutionary Genetics Analysis, developed by S. Kumar, K. Tamura, and M. Nei and provided in the public domain by the Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ). GenBank accession numbers (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and Ensembl numbers are as follows: *Chlamydomonas reinhardtii dhc1b* (XP_001696428); *Caenorhabditis elegans DHC1* (NP_491363.1), CHE-3 (NP_492221.2); *Danio rerio dync1-h1* (NP_001036210.1); *Mus musculus Dync1-h1* (NP_084514.2), *Dync2-h1* (NP_084127.2); *Rattus norvegicus dync1-h1* (ENSNOG00000006178), *dync2-h1* (NP_075413.1); *Gallus gallus dync1-h1* (ENGALT00000037512), *dync2-h1* (ENSGALP00000027700); *Oryzias latipes dync1-h1* (ENSORLP00000015944), *dync2-h1* (ENSORLT00000013451); *Gasterosteus aculeatus dync1-h1* (ENSGALP00000014094), *dync2-h1* (ENSGALP00000010733); and *Takifugu rubripes dync1-h1* (ENSTRUP00000031697), *dync2-h1* (ENSTRUP00000010356).

Morpholino Microinjection and Design

One- to two-cell-stage zebrafish embryos were injected as described elsewhere.³³ Morpholinos were synthesized by Gene Tools, LLC (Philomath, OR). The sequences were: *dync2-h1*MO1: 5'CTGCTGTGAATGCATGTACCTGCTG3'; *dync2-h1*MO2: 5'GACAGGTGTGCAGTCTGACCTCCTC3'; *dync2-li1*MO1sp: 5'GGACTGTCACTTTTACCGTATTATG3'; *dync2-li1*MO2atg: 5'GCTTACTTTTCGGCATTATTTTACCG3'; *dync2-i1*MO1 5'ACAATAAACTGTTACCGGCCAAATC3'; and *dync2-i1*MO2 5'CCACACTAATCCCAGAAATACAACA3'.

In Situ Hybridization and Reverse Transcription-Polymerase Chain Reaction

Antisense riboprobes corresponding to *dync2-h1*, *dync2-li1*, and *dync2-il* were synthesized with SP6 RNA polymerase (Invitrogen) and in situ hybridization performed,³⁴ and RT-PCR was performed.⁸

Histology, Immunohistochemistry, and Immunocytochemistry

Embryos were processed for histology and electron microscopy as described elsewhere.³⁵ Wholemount immunostaining of zebrafish 30 hours after fertilization (hpf) was performed as previously described,³⁶ with an anti-acetylated tubulin antibody (1:50; Sigma-Aldrich, St. Louis, MO). Immunohistochemistry and imaging of frozen sections was performed as described⁸ with the following antibodies: anti-acetylated tubulin (1:500), anti-IFT88 (1:5000),⁸ 1d1 (monoclonal zebrafish rhodopsin antibody 1:100) (Fadool JM, et al. *IOVS* 1999;40:ARVO Abstract 1251), rabbit anti-zebrafish rhodopsin and rabbit anti-zebrafish blue cone opsin (both 1:200).³⁷ Staining with the mouse monoclonal anti-arrestin antibody, xArl-6, (1:25) staining was performed.³⁸

Antibody Generation

A rabbit polyclonal anti-*dync2-h1* antibody was generated against the synthetic peptide MAPGTDDPRK corresponding to the N-terminus of zebrafish *dync2-h1* and affinity-purified by Bethyl Laboratories (Montgomery, TX).

SDS-PAGE and Western Blot Analysis

Total protein from larval heads was generated,⁸ separated by electrophoresis on 5% to 15% gradient SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), and transferred to PVDF membranes. Rabbit anti-*dync2-h1* (1:5000) and anti- α -tubulin antibodies (Clone 12G10; 1:2000; Developmental Studies Hybridoma Bank, University of Iowa) were used and detected.⁸

Electroretinograms

Larval eyes were isolated with a tungsten wire loop³⁹ and ERGs performed.⁴⁰ Full-field light stimulation was prolonged for 500 ms under photopic conditions to ensure b- and d-wave separation. Light flashes were presented at intervals of 10 seconds to 3 minutes, depending on the recovery of the ERG from a test flash. Recordings from three sequential flashes were averaged to reduce noise and obtain the ERG trace. The ERG traces shown are representative of 5 to 10 separate trials of each morphant and wild-type fish. L-AP4 (L-(+)-2-amino-4-phosphonobutyric acid) and TBOA (DL-threo-b-benzyloxyaspartic acid) were purchased from ToCris Biosciences (Ellisville, MA). L-AP4 was dissolved in 0.1 M NaOH and TBOA dissolved in DMSO. Reagents were diluted in fish water to a final concentration of 0.2 mM TBOA and 0.4 mM L-AP4. Larval fish at 5 to 6 dpf were incubated in TBOA and L-AP4 for 30 minutes.

Results

Phylogenetic Analysis of *dync2-h1*

To identify the zebrafish *dync2-h1* orthologue, we performed a BLAST search of the zebrafish genome for sequences with homology to mouse *Dync2-h1*. Two partial mRNA sequences were recovered that represented 6.3 kb of the 5' end and 11.4 kb of the 3' end of *dync2-h1* (accession numbers XR_029028 and XR_029028.2, respectively). These two sequences combined yielded a predicted protein product of 4266 amino acids, which was 69% identical with mouse *Dync2-h1*. To verify this as a true zebrafish *dync2-h1* orthologue, we performed a phylogenetic analysis using the neighbor-joining method (Fig. 1) and found that the zebrafish sequence is

most closely related to other vertebrate *dync2-h1* genes. Sequence data for *dync2-li1* and *dync2-il1*, the zebrafish orthologue of *Chlamydomonas FAP133* were identified by using Ensembl.³²

Cytoplasmic dynein-2 Genes in the CNS and Pronephros

As cytoplasmic dynein-2 genes have not been described in zebrafish, we determined the expression pattern of *dync2-h1*, *dync2-li1*, and *dync2-il1* in 72 hpf embryos. Similar to the expression of other zebrafish IFT genes,⁴¹ expression of *dync2-h1*, *dync2-li1*, and *dync2-il1* was observed in the CNS and pronephros (Figs. 2A-C). Although *dync2-h1* and *dync2-li1* have been found in photoreceptors,¹⁸ it has been suggested that *dync2-il1* only functions in motile cilia.²⁰ We sectioned embryos after in situ hybridization and observed that *dync2-h1*, *dync2-li1*, and *dync2-il1* genes were expressed throughout the retina, including in photoreceptors (Figs. 2D-F).

IFT Phenotypes of Dynein Morphants

To disrupt retrograde IFT, we injected morpholino oligonucleotides against the *dync2-h1*, *dync2-li1*, and *dync2-il1* genes into one-cell zebrafish embryos. Zebrafish IFT mutants exhibit specific phenotypes due to ciliary dysfunction, including ventral curvature of the body axis, kidney cysts, and small eyes. Remarkably, *dync2-h1* morphants looked very similar to previously described IFT mutants, such as *ift88^{oval}* and *ift57^{hippi}*.^{9,42} The *dync2-h1* morphants had a distinctive ventral curvature of the body axis, small eyes, and 100% penetrance of kidney cysts at 4 days post fertilization (dpf) (Fig. 2G). The *dync2-li1* morphants similarly exhibited small eyes and a high penetrance of kidney cysts (Fig. 2H), although they did not exhibit ventral curvature of the body. Knockdown of *dync2-il1* (Fig. 2I) yielded morphants with small eyes, pronounced swelling in the hindbrain and a penetrance of kidney cysts similar to that of *dync2-li1* morphants (Table 1). Some *dync2-il1* morphants had a ventral curvature of the body while others did not, and both *dync2-li1* and *dync2-il1* morphants were shorter than age-matched control fish. Of importance, knockdown experiments with nonoverlapping morpholinos designed against each gene resulted in identical phenotypes, strongly suggesting that the dynein morphant phenotypes are specific (data not shown). These phenotypes are consistent with cytoplasmic dynein-2 function as the retrograde IFT motor.

Western blot analysis performed on heads of 4- and 5-dpf larvae, with a polyclonal antibody against zebrafish Dync2-h1, showed a high-molecular-weight band that was depleted in morphants at 4 and 5 dpf (Fig. 2J, black arrowhead). RT-PCR analysis of *dync2-h1* morphants showed similar results (Fig. 2J'). Because of the absence of suitable antibodies against *dync2-li1* and *dync2-il1*, we performed RT-PCR to assess the efficacy of morpholinos to interfere with splicing. Morpholinos against both *dync2-li1* and *dync2-il1* were effective at all time points assayed (Figs. 2K, 2L).

Features of Dynein Morphant Eyes

To determine how loss of cytoplasmic dynein-2 affects retinal organization, we analyzed histologic sections of 4-dpf morphants. Similar to that described in IFT mutants, retinal lamination was normal in all three dynein morphants but the eyes were smaller than control eyes. We did not observe pyknotic nuclei or other evidence of cell death within the photoreceptor layer of dynein morphants, unlike previously described IFT mutants^{8,9} (Fig. 3).

IFT plays a critical role in outer segment formation and maintenance, and we hypothesized that outer segment integrity would require retrograde transport. At 4 dpf, transmission electron microscopy (TEM) revealed that wild-type photoreceptors have robust outer segments that extend into the retinal pigment epithelium (Figs. 4A, 4B). In contrast, all the dynein morphants exhibited very short outer segments, with some photoreceptors completely lacking these structures (Figs. 4C, 4E, 4G). Higher magnification images revealed that connecting cilia in

all dynein morphants were swollen, perhaps because of the accumulation of IFT particles and cellular debris (Figs. 4D, 4F, 4H; arrows). Disc stacking was essentially normal in *dync2-h1* and *dync2-il* morphants. In contrast, many *dync2-li1* morphant photoreceptors had outer segments composed of disordered membranes, some of which also contained large vesicles (Figs. 4E, 4F). These structures were highly abnormal and were never observed in wild-type photoreceptors. We occasionally observed vertically stacked membranes within the outer segments of *dync2-li1* and *dync2-il* morphants (Figs. 4F, 4H). These data show that normal extension of the outer segments requires retrograde IFT and suggest that recycling of IFT proteins may be a limiting factor in outer segment growth.

Ciliogenesis in Other Tissues in Dynein Morphants

Retrograde IFT is essential for cilium formation and maintenance in *Chlamydomonas*, *C. elegans*, and mouse. Kidney cysts occurred with high penetrance in *dync2-h1*, *dync2-li1*, and *dync2-il* morphants, suggesting that pronephric cilia were impaired (Table 1). To directly evaluate the effect of cytoplasmic dynein-2 dysfunction on pronephric cilia, we performed wholemount immunolabeling on 30 hpf zebrafish embryos with an antibody against acetylated tubulin, a marker for cilia. The anterior zebrafish pronephros is highly ciliated, with numerous monocilia extending into the pronephric lumen (Fig. 5A). Most cilia were missing in *dync2-h1* morphants and those present were significantly shorter than wild-type cilia (Fig. 5B). In *dync2-li1* morphants, cilia were shorter and present at lower density than in the wild-type (Fig. 5C). Cilia density was also lower in *dync2-il* morphants, although cilia lengths were comparable to those in wild-type animals (Fig. 5D). These data correlate with the penetrance of kidney cysts observed at 4 dpf (Table 1).

The canonical ciliary phenotype of disrupted retrograde IFT is swollen ciliary axonemes in which accumulated cytoplasm, IFT proteins, and cellular debris are present.^{13,15,23} The zebrafish nasal epithelium is highly ciliated and provides an accessible tissue for analysis. TEM of the nasal epithelium of 4-dpf wild-type larvae revealed robust cilia (Fig. 5E). As expected, all three dynein morphants exhibited very short nasal cilia with swollen axonemes (Figs. 5F-H). Combined, these data show that loss of *dync2-h1*, *dync2-li1*, and *dync2-il* compromised retrograde IFT, which is essential for nasal cilium integrity.

Rod and Cone Opsins in Dynein Morphants

Rod and cone opsins mislocalize in kinesin-II, *ift88*, and *ift57* mutants, implying that opsins are cargo of IFT in photoreceptors.^{5,6,8,9} As opsins and many other known outer segment proteins move unidirectionally and do not return to the inner segment, it is unclear whether the loss of retrograde IFT would affect opsin trafficking. We performed immunohistochemistry on 4-dpf dynein morphants using antibodies directed against zebrafish rhodopsin and blue cone opsin. At 4 dpf, both rod and blue cone opsins localized nearly exclusively to the outer segment (Fig. 6A). Although outer segments were shorter, opsin trafficking was normal in *dync2-h1*, *dync2-li1*, and *dync2-il* morphants (Figs. 6B-D). We observed subtle mislocalization of opsins in some morphants, but this phenotype was observed only in the most severely affected photoreceptors and probably was a secondary consequence of severely shortened outer segments.

IFT88 Protein Distribution along Photoreceptor Connecting Cilia with Dynein Dysfunction

As disruption of retrograde IFT causes accumulation of IFT particles within cilia,^{13-15,21} we speculated that the debris we observed in photoreceptor cilia was IFT proteins. To investigate, we immunolabeled 4-dpf retinal sections with antibodies against IFT88 and acetylated tubulin. In wild-type photoreceptors, IFT88 colocalized with acetylated tubulin at the connecting cilium (Fig. 6E). Acetylated tubulin stained the connecting cilium and axoneme, and the staining often extended distally beyond the IFT88 staining (Fig. 6E). In the *dync2-h1* morphants, however,

IFT88 and acetylated tubulin staining often colocalized in a small puncta and failed to elongate in the proximal-distal axis (Fig. 6F, arrowhead). In *dync2-li1* and *dync2-il* morphants, IFT88 labeling was biased distally relative to acetylated tubulin, suggesting that IFT88 accumulates near the distal end of the connecting cilium (Figs. 6G, 6H, arrows). This mislocalization of IFT88 is consistent with the role cytoplasmic dynein-2 in IFT protein recycling and suggests that the axonemal swelling seen in the dynein morphants reflects accumulated IFT particles.

Effect of Disrupted Retrograde IFT on Arrestin Translocation

Although cytoplasmic dynein-2 does not participate in anterograde opsin trafficking, studies of mammalian photoreceptors have shown that transducin and arrestin move in a retrograde fashion through the connecting cilium. As an independent test of the previous results,²⁹ we sought to directly examine the role of cytoplasmic dynein-2 in retrograde movement of phototransduction components. Zebrafish cone transducin does not move to the inner segment during light adaptation,⁴³ but arrestin translocation has not been tested in zebrafish. To test light-dependent arrestin movement in zebrafish rods, we performed immunohistochemistry on 5-dpf zebrafish larvae with a monoclonal antibody against *Xenopus* arrestin.³⁸ After dark adaptation, arrestin was located predominantly in the rod inner segments (Fig. 7A). We observed robust light-dependent arrestin movement to the rod outer segment, with outer segment localization peaking after 15 minutes of light adaptation (Fig. 7B). With continued light exposure, arrestin progressively migrated back to the inner segment (Fig. 7C), with migration nearly complete after 60 minutes of light exposure (Fig. 7D). This is similar to the pattern of arrestin translocation in *Xenopus*.³⁸ To evaluate retrograde arrestin translocation, 5-dpf larvae were dark adapted for 3 hours, light adapted for 15 minutes, and then returned to the dark where arrestin translocation was analyzed by immunohistochemistry. Nearly all the arrestin translocated to the rod inner segments within 15 minutes of dark adaptation in 5-dpf wild-type larvae. Of note, arrestin translocation occurred normally in dynein morphant rods (Figs. 7E–L). These data indicate that cytoplasmic dynein-2 and, by extension, retrograde IFT do not mediate retrograde arrestin movement during dark adaptation.

Effect of Compromised Dynein Function on Retinal Electrophysiology

To examine visual function after loss of retrograde IFT, we recorded full-field electroretinograms on 5- and 6-dpf dynein morphants. Wild-type larvae have an ERG response characterized by a corneal negative a-wave, and a corneal positive b- and d-wave, which reflect the response of photoreceptors and on and off-bipolar cells respectively. The dynein morphant ERGs were variable, with some morphants exhibiting no response, whereas others had decreased b- and d-wave amplitudes (Fig. 8A). Inhibiting the photoreceptor transmission to ON-bipolar cells using the pharmacologic agents L-Ap4 and TBOA revealed that dynein morphants also displayed reduced photoreceptor potential (Fig. 8B). However, the threshold responses of dynein morphants were comparable to wild-type (Fig. 8A). These data show that visual function is impaired in dynein morphants.

Discussion

We investigated the function of retrograde IFT in vertebrate photoreceptors and nonphotoreceptor cilia through morpholino-mediated gene knockdown of three cytoplasmic dynein-2 subunits. Photoreceptors lacking cytoplasmic dynein-2 exhibit different phenotypes than those described in anterograde IFT mutants. First, *ift88* and *ift172* mutants fail to form outer segments,⁹ whereas *ift57* mutants produce short outer segments.^{7,8} Although the outer segments in dynein morphants appear shorter than those described for *ift57* mutants, they are not accompanied by significant opsin mislocalization or cell death, as was observed in *ift57* mutants. Second, the outer segments formed in *ift57* mutants had surprisingly normal

ultrastructure, whereas we often observed disorganized disc membranes with accumulated vesicles and debris in dynein morphant outer segments.

Our results represent the first direct evidence that retrograde IFT plays an important role in vertebrate photoreceptors. The outer segment sheds roughly 10% of its volume from the distal tips each day and it was assumed that most, if not all, active transport was unidirectional to the outer segment. The outer segment is nevertheless a modified sensory cilium⁴⁴ and several aspects of axoneme maintenance and dynamics require retrograde IFT. Recycling kinesin motors and IFT particles to the inner segment would reduce the metabolic demands of synthesizing large numbers of new IFT components. Within the outer segment the microtubule array of the axoneme diverges into singlets and doublets⁴⁴ and only extend approximately 80% of the length of toad outer segments.⁴⁵ The mechanisms underlying cilia assembly and disassembly at microtubule tips, particularly of microtubule singlets like those seen in photoreceptors, remains poorly understood, but retrograde IFT most likely returns axonemal turnover products to the base. Retrograde IFT plays an important role in signaling pathways that use cilia.^{46,47} Retrograde IFT could function in signaling pathways that control outer segment length and outer segment trafficking.

The mechanism underlying arrestin translocation has been the subject of several studies.^{26,29,30,38,48,49} Although other investigators concluded that arrestin translocation occurs via passive diffusion,²⁹ a direct test of retrograde IFT had not been performed. Although we observed several phenotypes consistent with disrupted retrograde IFT in dynein morphant photoreceptors, nasal cilia and pronephric cilia, translocation of rod arrestin occurred normally. Although consistent with previous reports,²⁹ our data directly showed that retrograde IFT did not participate in this process. To our knowledge, we are the first to describe light-dependent translocation of rod arrestin in zebrafish. The pattern of arrestin localization is similar to that described in *Xenopus*, where arrestin migrates to the outer segment during light adaptation and returns during prolonged light exposure.³⁸ This differs from murine systems where arrestin remains in the outer segment with continued light adaptation.^{26,28,48} This result suggests that retrograde arrestin translocation during constant light is common to teleosts and amphibians.

Testing the role of the accessory subunits of cytoplasmic dynein-2 is essential for a thorough understanding of the mechanism of retrograde IFT. Mutational studies in *Chlamydomonas*, *C. elegans*, and mouse have established a role for *dync2-h1*, *dync2-li1* and LC8 in retrograde IFT.^{13-15,21,23,46} Our phenotypic analysis of *dync2-il* morphants is the first, however, to show that the cytoplasmic dynein-2 intermediate chain is necessary for cytoplasmic dynein-2 function. *dync2-il* morphant photoreceptors and nasal cilia were shorter than wild-type and exhibited swollen axonemes, consistent with defects in retrograde IFT. Although the length of pronephric cilia did not appear shorter in *dync2-il* morphants, the density of cilia was reduced and the morphants developed kidney cysts.

Finally, we observed reduced amplitude in dynein morphant ERG a-, b- and d-waves, which is most likely explained by the presence of short photoreceptor outer segments and the overall reduction of eye size. The impairment of visual function after loss of dynein function also suggests that genes encoding components of the retrograde pathway may underlie certain forms of human blindness.

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Figure 1.

Phylogenetic analysis of zebrafish *dync2-h1*. A neighbor-joining tree was generated based on Clustal W protein sequence alignments of Cr, *Chlamydomonas reinhardtii*; Ce, *Caenorhabditis elegans*; Ga, *Gasterosteus aculeatus*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Ol, *Oryzias latipes*; Rn, *Rattus norvegicus*; and Tr, *Takifugu rubripes dync1-h1* and *dync2-h1* genes (Clustal W multiple sequence alignment program. European Bioinformatics Institute, European Molecular Biology Laboratory, Heidelberg, Germany; provided in the public domain at <http://www.ebi.ac.uk/clustalw/>). The bootstrap percentages (500 iterations) are shown at the branch points. Note how the *dync2-h1* gene identified in this study (arrow) lies closest to other vertebrate *dync2-h1* genes. Scale bar, 0.1 substitutions/amino acid.

Figure 2.

Dynein gene expression and morphant phenotypes. (A–C) Wholemount in situ hybridization performed on 72 hpf zebrafish revealed identical expression of all three dynein genes, with the strongest expression in the CNS and pronephros (arrowhead). (D–F) Transverse retinal sections from (A–C). Expression was observed in all retinal layers including photoreceptors (PL); dorsal is up. (G–I) Dynein morphant phenotypes showing kidney cysts (arrowheads). (J–L) Assessment of morpholino efficacy. (J) Western blot of cellular lysates from 4-dpf and 5-dpf wild-type and *dync2-h1* morphant heads, the anti-*dync2-h1* antibody recognizes a high-molecular-weight band (arrowhead), which is depleted in morphants at 4 and 5 dpf. (J') RT-PCR of *dync2-h1* morphants at 30 hpf and 4 dpf showing defective splicing. (K) RT-PCR analysis of *dync2-li1* morphants two shorter transcripts present (arrowhead), which reflect exclusion of 70-bp exon 4 and exclusion of both exon 4 and 35-bp exon 3. (L) RT-PCR analysis of *dync2-i1* morphants shows splice defect resulting in a longer transcript (arrowhead), resulting from inclusion of 71-bp exon 4. (M) Table of penetrance of kidney cysts observed in dynein morphants at 4 dpf. (*) in (D–F) the lens, and in (K, L) the 500-bp DNA standard. Scale bar: (A–C, G–I) 200 μ m; (D–F) 20 μ m.

Figure 3.

Transverse histologic sections through 4-dpf wild-type and dynein morphant retinas (**A–D**). The wild-type zebrafish retina (**A**) is highly organized by 4 dpf with all cellular layers readily observable, including the ganglion cell layer (GCL), inner nuclear layer (INL), photoreceptor layer (PL), and retinal pigmented epithelium (RPE). The organization of dynein morphant retinas (**B–D**) is unaffected, although their eyes are smaller than wild-type. IPL, inner plexiform layer; OPL, outer plexiform layer; ON, optic nerve. Scale bar, 100 μ m.

Figure 4.

Transmission electron microscopy of 4-dpf wild-type and dynein morphant retinas. (A, C, E, F) Lowmagnification electron micrographs of wild-type and dynein morphant retinas. (A) Wild-type photoreceptor outer segments (OS), inner segments (IS), nuclei (N) and connecting cilia (arrow) are readily observable. (B) Higher magnification highlights the basal body (arrowhead) and connecting cilium (arrow). (C) *dync2-h1* morphants had short outer segments with visible connecting cilia (long arrow) and cells lacking outer segments (short arrow). (D) *dync2-h1* morphant photoreceptors had swollen cilia (black arrow). (E) *dync2-li1* morphants had short outer segments. Cells with abnormal membranous structures and large electron-lucent vesicles were present (arrowhead). (E, inset) Boxed area magnified $\times 2.5$ showing a photoreceptor cilium with disorganized membrane at its distal end. The basal body (arrowhead) and axoneme (arrow) were evident. (F) *dync2-li1* morphant cilia were swollen (arrow), and disorganized outer segment membranes with accumulated vesicles (arrowheads) were evident. (G) *dync2-i1* morphants had short outer segments with connecting cilia present (long arrow) and abnormal outer segments with accumulated debris in them (arrowhead). Some *dync2-i1* morphant photoreceptors lacked outer segments (short arrow). (H) *dync2-i1* morphant connecting cilia were severely swollen (black arrows), and electron-lucent vesicles (black arrowheads) were visible at the distal tip of an outer segment. White arrows: photoreceptor membranes that were improperly oriented. Scale bar: (A, C, E, G) 1 μ m; (B, D, F, H) 500 nm.

Figure 5.

Analysis of nonphotoreceptor cilia. (A–D) The 30-hpf wild-type and dynein morphant embryos were immunolabeled with an antiacetylated tubulin antibody, a marker for cilia. Images were collected of the anterior pronephros. Anterior is to the left dorsal is up. (A) Wild-type embryos had numerous cilia that extended into the lumen of the pronephric duct. (B) *dync2-h1* morphant pronephric cilia were severely shortened, and (C) *dync2-li1* morphant cilia were shorter than wild-type. (D) *dync2-i1* cilia were less numerous than wild-type but were comparable in length. (E–H) Electron micrographs of 4-dpf wild-type and dynein morphant nasal cilia. (E) Wild-type nasal cilia (arrow) were homogeneous in diameter. (F) *dync2-h1*, *dync2-li1*, and *dync2-i1* morphant nasal cilia were short and displayed cytoplasmic swelling along the axoneme (arrow). The morphant cilia had a larger diameter than did the wild-type nasal cilia. (E–H arrowhead: basal body). Scale bar: (A–D) 20 μm; (E–H) 500 nm.

Figure 6.

Immunohistochemical analysis of wild-type and dynein morphant photoreceptors. (A–D) Immunohistochemical labeling of rhodopsin (red) and blue cone opsin (green) in 4-dpf wild-type and dynein morphant photoreceptors. (A) Wild-type photoreceptors have rhodopsin and blue cone opsin localized almost exclusively to the outer segment (arrow). Dynein morphants (B–D) have opsins highly concentrated in the outer segment (arrow), but have subtle mislocalization of opsins in some severely affected cells (arrowhead). (E–H) The 4-dpf retinal cryosections were stained with antibodies against IFT88 (red) and acetylated tubulin (green), which marks the connecting cilium. (E) Wild-type photoreceptors show marked colocalization of acetylated tubulin and IFT88 (yellow), although the acetylated tubulin staining often extends more distally than IFT88 (arrowhead). Staining of both acetylated tubulin and IFT88 were elongated along the proximal-distal axis of the cell. (F) *dync2-h1* morphants also showed colocalization of IFT88 and acetylated tubulin, but the staining was not elongated, and it appeared as a small sphere. IFT88 staining often appeared biased slightly apically relative to the acetylated tubulin stain (arrow). (G, H) *dync2-li1* and *dync2-i1* staining showed that IFT88 was mislocalized, accumulating at the apical end of the connecting cilium (arrows). (A–H) DAPI (blue)-labeled nuclei. Scale bar: (A–D) 20 μm; (E–H) 5 μm.

Figure 7.

Immunohistochemical analysis of arrestin translocation in 5-dpf wild-type zebrafish. (A—D) Arrestin moves to the outer segment during light adaptation. Animals were dark adapted for 3 hours, light adapted on a benchtop in a well-lit laboratory and fixed at regular intervals, and assayed for arrestin localization. A dark-adapted retina (LA0) had no arrestin (red) in the rod outer segment (white arrowhead), the apical part of the inner segment had a semicircular area of less intense staining, probably due to exclusion of cytoplasm by the mitochondria of the inner segment (green arrowhead). Staining along the photoreceptor cilium (white arrow) was apparent in some rods. At 15 minutes of light adaptation (B) arrestin was observed in the outer segment (white arrowhead). At 30 minutes of light adaptation (C), arrestin began to exit the outer segment, and by 60 minutes of light adaptation (D), nearly all the arrestin had left the outer segment. DAPI (blue) labeled the photoreceptor nuclei. (E—L) Assessment of arrestin migration during dark adaptation in wild-type and dynein morphant rods at 5 dpf. Larvae were dark adapted for 3 hours, light adapted for 15 minutes, then dark adapted, fixed at regular intervals, and immunolabeled for arrestin (red) and rhodopsin (green) to mark the outer segment. (E—H) With 15 minutes of light adaptation (L15/D0), arrestin was found in the outer segment (white arrowhead) in wild-type and dynein morphant rods. (E'—H') are merged images of arrestin and rhodopsin staining, note the colocalization of arrestin and rhodopsin in the outer segment (yellow). (I—L) With 15 minutes of dark adaptation, the arrestin observed in the outer segment at LA15/D0 had nearly all returned to the cell body. (I'—L') Merged image of arrestin and rhodopsin showing a lack of colocalization of arrestin and rhodopsin in the rod outer segments. (E—L, arrowheads) Outer segment. Scale bar, 10 μ m.

Figure 8.

Electrophysiological analysis of dynein morphants. ERGs of wild-type (wt), *dync2-h1*, *dync2-li1*, and *dync2-i1* morphant 5- to 6-dpf larvae ($n = 5-10$) under photopic conditions. **(A)** ERGs at increasing light intensities **(B)** ERGs after incubation in pharmacologic agents L-AP4 and TBOA to isolate the photoreceptor response and increasing light intensities. Bars: light on (white bar) and light off (black bar), with a 500-ms light stimulus.

Table 1

Frequency of Kidney Cysts in 4-dpf Dynein Morphants

Condition	Kidney Cysts
Wild-type	1/78 (1%)
dync2-h1MO1	53/53 (100%)
dync2-l1MO1	36/58 (62%)
dync2-i1MO1	32/51 (63%)

Data are the number affected/entire group (percentage affected).