Cdc42 and *sec10* Are Required for Normal Retinal Development in Zebrafish

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PURPOSE. To characterize the function and mechanisms of *cdc42* and *sec10* in eye development in zebrafish.

METHODS. Knockdown of zebrafish *cdc42* and *sec10* was carried out using antisense morpholino injection. The phenotype of morphants was characterized by histology, immunohistology, and transmission electron microscopy (TEM). To investigate a synergistic genetic interaction between *cdc42* and *sec10*, we titrated suboptimal doses of *cdc42* and *sec10* morpholinos, and coinjected both morpholinos. To study trafficking, a melanosome transport assay was performed using epinephrine.

RESULTS. *Cdc42* and *sec10* knockdown in zebrafish resulted in both abnormal eye development and increased retinal cell death. *Cdc42* morphants had a relatively normal retinal structure, aside from the absence of most connecting cilia and outer segments, whereas in *sec10* morphants, much of the outer nuclear layer, which is composed of the photoreceptor nuclei, was missing and RPE cell thickness was markedly irregular. Knockdown of *cdc42* and *sec10* also resulted in an intracellular transport defect affecting retrograde melanosome transport. Furthermore, there was a synergistic genetic interaction between zebrafish *cdc42* and *sec10*, suggesting that *cdc42* and *sec10* act in the same pathway in retinal development.

CONCLUSIONS. We propose a model whereby *sec10* and *cdc42* play a central role in development of the outer segment of the retinal photoreceptor cell by trafficking proteins necessary for ciliogenesis.

Keywords: zebrafish, connecting cilium, *cdc42*, *sec10*, retinal degeneration, protein trafficking

Cilia are rod-like, microtubule-based organelles found on most mammalian cell types. Cilia can be classified as motile or nonmotile (primary) cilia. Motile cilia function mainly as motor organelles, whereas primary cilia are sensory organelles.^{1–3} Dysfunction of primary cilia results in human disorders, such as Bardet-Biedl (BBS), Joubert, and Senior-Loken syndromes, that affect multiple organs, resulting in central nervous system malformation, cystic kidney disease, and retinal dystrophy.^{4–5} Zebrafish knockdown models for several ciliopathies, including *cep290*, *cc2d2a*, *inpp5e*, *ift57*, *ift88*, and *ift172*, have kidney and retinal phenotypes that suggest insight into the mechanisms underlying these defects.^{6–9}

The zebrafish eye is a well-laminated structure similar to the eyes of other vertebrates. Eye morphogenesis in the zebrafish starts at 11.5 hours post fertilization (hpf) and the eyecup is well-formed by 24 hpf. By 48 hpf, retinal lamination occurs in most of the retina.¹⁰ The vertebrate retina is organized into three laminae: the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). The photoreceptor cell in the ONL has a specialized morphology consisting of an inner segment (IS) and outer segment (OS) linked by connecting cilium, and is a highly polarized and light-sensitive

cell. The IS, where protein synthesis occurs, is connected by the cilium to the OS, which consists of a microtubule-based axoneme and membrane disc stacks containing opsin required for phototransduction.^{11–13} The connecting cilia and basal body in the IS are observed at 50 hpf, and the OS is visible by 54 hpf. The first visual responses are present at approximately 70 hpf, and photoreceptor cells reach adult size by 576 hpf (24 days).^{10,14}

The development and maintenance of photoreceptors requires intracellular trafficking in both photoreceptor and RPE cells. In photoreceptor cells, vesicles containing proteins destined for the OS traffic from the trans-Golgi network to the base of the connecting cilium via vesicular transport.^{15–17} In RPE cells, intracellular trafficking controls the phagocytosis of disk membranes shed from the tip of the OS.¹⁸ To replace lost membrane, the photoreceptor inner segments continuously provide material to the OS.⁹ The connecting cilium, therefore, plays an important role as the bridge between the IS and OS.

One of the proteins implicated in vesicular trafficking from the Golgi to the cilium is the small GTPase, Rab8.¹⁹ Disruption of Rab8 in *Xenopus* photoreceptor cells blocks rhodopsin-bearing post-Golgi vesicle trafficking, and results in the

abnormal accumulation of rhodopsin carrier vesicles at the base of connecting cilium.^{15,16} Rab proteins perform functions through downstream effectors, such as the exocyst, a highly conserved eight-protein trafficking complex.^{20,21} We previously demonstrated that the exocyst is required for ciliogenesis in MDCK cells, due to its role in targeting and docking vesicles carrying ciliary proteins.²² We also showed that Cdc42, another small GTPase, localizes with the exocyst at the primary cilium, and biochemically and genetically interacts with exocyst Sec10.²³ Although the roles of Cdc42 and Sec10 in epithelial cell biology are now better understood, their potential functions in eye development are still unknown. Interestingly, we recently found that knockdown of both *cdc42* and *sec10* in zebrafish resulted in small eyes, and knockdown of *cdc42* led to loss of photoreceptor cilia.^{24,25}

Here, we describe the role of *cdc42* and *sec10* in eye development using histological, functional, and embryonic manipulations in zebrafish. We find that *cdc42* and *sec10* knockdown results in increased retinal cell death, photoreceptor defects, and intracellular transport defects. We also demonstrate a synergistic genetic interaction between *cdc42* and *sec10*, suggesting that *cdc42* and *sec10* act in the same pathway in retinal development. These findings indicate that *sec10* and *cdc42* play a central role in trafficking ciliary proteins to the photoreceptor cell for ciliogenesis.

METHODS

Ethics Statement

Wild-type zebrafish embryos were provided by the University of Pennsylvania Zebrafish Core, and were raised at 28.5°C until the appropriate stages. All the zebrafish experiments were approved by the Institutional Animal Care and Use Committees at the University of Pennsylvania and the Philadelphia VAMC, and conform to the ARVO Animal Statement guidelines.

Microinjection for Knockdown and Rescue

Embryos were injected at the one- to two-cell stage, and morpholinos were diluted with phenol red tracer (P0290; Sigma-Aldrich Corp., St. Louis, MO, USA) at 0.05% and injected at 500 pL or 1 nL/embryo. The *cdc42* and *sec10* morpholinos, designed against zebrafish *cdc42* and *sec10*, were purchased from Gene Tools, LLC (Philomath, OR, USA): *cdc42*MO (5'-CAACGACGCACCTTGATCGTCTGCAT-3'), *sec10*MO (5'-AATATTCTGTAACTCACTTCTTAGG-3'). We described the *cdc42*MO and *sec10* morpholinos in previous publications.^{24,25} Morpholinos were injected either as single doses of 1, 2, or 3 ng *cdc42*MO (designated in the text as “*cdc42*MO”) or a combined dose of 1 or 2 ng *cdc42*MO + 7.5 ng *sec10*MO (designated in the text as “2 ng *cdc42*MO + 7.5 ng *sec10*MO”) per embryo. For the rescue experiments, capped human *SEC10* full-length mRNA was synthesized using the mMessage mMachine T7 kit per the instructions of the manufacturer (AM1344; Ambion, Grand Island, NY, USA); 25 to 150 pg of *SEC10* mRNA was coinjected with the morpholinos into two- to four-cell stage embryos.

Quantification of Eye/Body Size

To compare eye-to-body ratio between injection controls and morphant zebrafish embryos, the diameter of zebrafish eyes and the body length were measured with Fiji (ImageJ) software, version 1.47g (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) using images of whole embryos collected with a Leica M205 C microscope (Leica Microsystems, Wetzlar,

Germany) and a DFC450 digital camera (Leica Microsystems). The eye-to-body length ratios were determined by collecting one image at the area of widest diameter from each morphant or wild-type embryo.

Histological Analysis

Zebrafish embryos were fixed in 4% paraformaldehyde in 1× PBS at 4°C overnight. After gradual dehydration into ethanol, embryos were embedded in paraffin, and were sectioned at 4-μm thickness. For immunohistochemistry, the sections were deparaffinized and epitope retrieval was performed by heating the sections at 95°C in 10 mM sodium citrate buffer pH 6.0 for 10 minutes. After treating in 0.5% hydrogen peroxide for 5 minutes at room temperature, the sections were blocked by normal serum according to the instructions for the VECTA-STAIN Elite ABC kit (PK-6101 and PK-6102; Vector Laboratories, Burlingame, CA, USA). Rabbit anti-cleaved caspase-3 (9661; Cell Signaling, Danvers, MA, USA) was used to detect apoptosis, and hematoxylin was used for counterstaining.

Western Blot Analysis

Dechorionated zebrafish embryos at 120 hpf were homogenized in SDS sample buffer containing protease inhibitor cocktail (P2714; Sigma-Aldrich Corp.) and phosphatase inhibitor (78420; Thermo, Rockford, IL, USA) to perform Western blot analysis. The homogenized lysates were boiled for 5 minutes at 95°C followed by centrifugation at 17,136g for 20 minutes at 4°C, and the supernatants were collected and mixed with 3× Laemmli sample buffer for the protein electrophoresis. The protein samples were separated on NuPage 4% to 12% Bis-Tris gels (NP0336; Novex, Carlsbad, CA, USA) and then transferred to a nitrocellulose membrane (LC2000; Novex). The antibodies used in this study were mouse monoclonal anti-Cdc42 (610929; BD Transduction Laboratories, San Jose, CA, USA), mouse monoclonal anti-γ tubulin (ab11316; Abcam, Cambridge, MA, USA), mouse monoclonal anti-acetylated α-tubulin (T6793; Sigma-Aldrich Corp.), mouse anti-rhodopsin [1D4] (ab5417; Abcam), and mouse anti-Sec8 (ADI-VAM-SV016; Enzo, Farmingdale, NY, USA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and Thermo Fisher Scientific (Waltham, MA, USA).

Transmission Electron Microscopy (TEM)

Injection control and morphant larvae were fixed in a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, and postfixed with 2% osmium tetroxide. The fixed tissue was sectioned and rinsed with 100 mM cacodylate buffer, dehydrated through a graded ethanol series, and infiltrated with Epon resin. Samples were processed by the Electron Microscopy Resource Laboratory at the University of Pennsylvania.

Melanosome Transport Assay

The melanosome transport assay was performed as described.^{26,27} Briefly, 120 hpf embryos were exposed to epinephrine (50 mg/mL) at a final concentration of 2 mg/mL in a dark room, and melanosome retractions were observed under the bright field microscope. The aggregation endpoint was scored when all melanosomes in the head and trunk were perinuclear.

Imaging

All images were captured in TIF format and processed in Adobe Photoshop CS5.1 (Adobe Systems, Inc., San Jose, CA, USA). For immunofluorescence, zebrafish embryos were imaged on an

FIGURE 1. Small eyes are seen following knockdown of *cdc42* and *sec10*. (A) Live images of 72 hpf and 120 hpf injection control, *cdc42*, and *sec10* morphant embryos. Small eyes were seen in the *cdc42* and *sec10* morphants (arrows). (B) Percentages of control, *cdc42*, and *sec10* morphant embryos with small eyes at 120 hpf. (C) A boxplot of eye/body size ratios of control, *cdc42*, and *sec10* knockdown larvae at 120 hpf. (D) The *Cdc42* and *Sec10* proteins at 120 hpf were undetectable by Western blot in 3 ng *cdc42* and 15 ng *sec10* morphants, respectively. (E) The *sec10*MO morphants were rescued by coinjecting zebrafish *sec10* mRNA and human *SEC10* mRNA (hmRNA), which is resistant to the zebrafish *sec10*MO, due to a difference in primary base pair structure. * $P = 0.2964$; ** $P = 0.0087$; *** $P = 0.0012$. Scale bars: 1 mm.

Olympus BX41 (Tokyo, Japan) and a Zeiss Axio Observer D1m (Oberkochen, Germany). Histology samples were imaged using a Leica M205C light microscope.

Statistical Analysis

We compared the frequency of the small-eye phenotype across different exposures using logistic regression, clustered by trial. Synergistic effects and phenotype-rescue were explored using Fisher's exact test. These tests were performed using Stata software v12.1 (Stata Corp., College Station, TX, USA). For comparison of means, a Student's *t*-test was performed using Microsoft Office Excel (2007) (Microsoft Corp., Redmond, WA, USA). For all tests, *P* values less than 0.05 were considered statistically significant.

RESULTS

Knockdown of *cdc42* and *sec10* Reduces Eye Size in Zebrafish

We previously showed that the knockdown of *cdc42* and *sec10* in zebrafish is associated with ciliary defects, including tail curvature, glomerular expansion, and MAPK pathway activation.^{24,25} The phenotypic defects occur at varying levels of severity in *cdc42* and *sec10* morphants; however, the eye phenotype was very similar in the *cdc42* and *sec10* morphants at 72 hpf and 120 hpf (Fig. 1A). Most *cdc42* and *sec10* knockdown morphants had small eyes in comparison to their wild-type siblings; 0% of wild-type, 61.4% of 3 ng *cdc42*MO, and 71.4% of 15 ng *sec10*MO had small eyes ($n = 311$). To investigate how *cdc42* and *sec10* affect eye development, we measured eye size and body length at 28 hpf, 52 hpf, 72 hpf, and 120 hpf in *cdc42* and *sec10* morphants (Supplementary Fig. S1). Compared with

the embryos that were injected with dye only (controls), embryos injected with *cdc42* and *sec10* morpholinos displayed significantly smaller eyes from 28 hpf onward (Supplementary Fig. S1A). Interestingly, the body length of *cdc42* and *sec10* knockdown embryos was also somewhat shorter at the same time points (Supplementary Fig. S1B). Nevertheless, after normalizing the eye size with the body length of the embryos, the *cdc42* and *sec10* morphants still had a significantly smaller eye/body ratio compared with the controls after 72 hpf (data not shown). By 120 hpf, the eye/body ratio was further reduced in *cdc42* and *sec10* morphants (Fig. 1C).

Cdc42 and *Sec10* protein levels at 120 hpf were undetectable by Western blot in the *cdc42* and *sec10* morphant embryos (Fig. 1D). To rule out off-target effects of *sec10*MO, we added human *SEC10* mRNA, which is resistant to the zebrafish *sec10*MO due to a difference in primary base pair structure, to zebrafish embryos to see whether this could rescue the defect in *sec10* morphants. After injection of different amounts of human *SEC10* mRNA, eye size was partially rescued (Fig. 1E). The specificity of *cdc42*MO was confirmed in our previous study,²⁴ in which *cdc42*MO embryos were rescued by coinjecting with mouse *Cdc42* mRNA, which is resistant to the zebrafish *cdc42*MO.

Knockdown of *cdc42* and *sec10* Results in Retinal Degeneration

We next investigated the eye phenotype seen in Figure 1 in more detail by histological analysis. Examination of hematoxylin and eosin-stained histological sections at 120 hpf revealed a significant reduction in retinal size and cell number in *cdc42* and *sec10* morphants when compared with control embryos (Fig. 2). *Cdc42* morphants had relatively normal retinal structure except for the ONL thickness (Figs. 2A, 2B, arrow),

FIGURE 2. Knockdown of *cdc42* and *sec10* results in decreased retinal cell numbers in the developing zebrafish. (A–C) Hematoxylin-eosin (H&E) staining of transverse retinal paraffin sections of control, *cdc42*, and *sec10* morphants at 120 hpf. The *cdc42* morphants had relatively normal retinal structure except for the decreased ONL thickness (arrow). The OS is almost completely absent in *sec10* morphants. (D) Retinal cell number is reduced in *cdc42* and *sec10* morphants at 120 hpf. Scale bar: 10 μ m.

which is composed of the nuclei of photoreceptor cells. By contrast, we found that in *sec10* morphants, much of the ONL was missing and the RPE cell thickness was irregular (Fig. 2C). We next evaluated whether the reduced cell number was associated with cell death by performing immunohistochemistry on fixed retinal sections using antibody against activated caspase-3 (Fig. 3). High rates of apoptosis were seen in the retina of both *cdc42* and *sec10* morphants at 72 hpf (Figs. 3B, 3C). Whereas levels of apoptosis were low in control embryos (3.7%), 12.2% of cells stained positive for activated caspase-3 in the retinas of *cdc42* morphants, and 14.8% in the retinas of *sec10* morphants (Fig. 3D). In injection controls, the caspase-3-positive cells were mostly observed in the GCL (9.6%) and INL (1.6%), but were not found in the ONL (Fig. 3E). This is similar to previous studies, which showed that cell death in the GCL is highest at 72 hpf.²⁸ Most apoptotic cells in *cdc42* morphant retinas were located in the GCL (23.4%) and INL (12.0%), with only a few apoptotic cells found in ONL cells

(1.3%). In *sec10* morphant retinas, the apoptotic cells were found not only in GCL cells (23.5%) and INL (14.1%) cells, but also in ONL cells (6.2%). Interestingly, 72 hpf *sec10* morphants had relatively normal ONL structure (Fig. 3C) compared with 120 hpf morphants (Fig. 2C), although we found several apoptotic cells in *sec10* morphant ONL cells (Fig. 3C, yellow arrows). These results indicate that loss of *sec10* results in disruption of the ONL after 72 hpf. The cell number and apoptosis data, taken together, suggest that loss of *cdc42* and *sec10* results in lower retinal cell numbers due, at least in part, to increased apoptosis, leading to a smaller eye size.

To better evaluate the effects of the *cdc42* and *sec10* morpholinos on photoreceptor development, we performed immunofluorescence and TEM analysis on 120 hpf embryos. The connecting cilia of the OS of photoreceptor cells were clearly absent in *cdc42* and *sec10* morphant retinas when probed using antibody against acetylated alpha tubulin, which stains primary cilia (Fig. 4A). We next examined OSs with the

FIGURE 3. Increased apoptosis is seen in the retina of *cdc42* and *sec10* morphants at 72 hpf. (A–C) Caspase-3 staining reveals increased apoptotic cells within the retina of *cdc42* and *sec10* morphants. (D) The percentage of apoptotic retinal cells was significantly increased in *cdc42* and *sec10* morphants. (E) The frequency of apoptotic cells in the ONL, INL, and GCL of control, *cdc42*, and *sec10* morphants is shown. * $P < 0.001$. Scale bars: 10 μ m.

FIGURE 4. The *cdc42* and *sec10* morphant embryos display abnormal OS development. (A, B) Immunofluorescence analysis of control, *cdc42*, and *sec10* morphant zebrafish at 120 hpf. (A) Acetylated alpha tubulin (green), a marker for connecting cilia, localized to the OS region in control (arrow), but not *cdc42* and *sec10* morphants. (B) A marker for long double cones, 1D4 (red), localized to the OS region in control and *cdc42* mutants. Staining was rarely observed in shorter OSs of *cdc42* morphants (arrow), and no staining was observed in *sec10* morphants. (C) Transmission electron micrographs of transverse sections along the dorsal-ventral axis of control, *cdc42*, and *sec10* morphant zebrafish at 120 hpf. Low- and high-magnification images revealed longer OSs (arrow) in control embryos. Photoreceptor OSs were present in *cdc42* morphants, but were significantly shorter and smaller in number than in controls. The OS was not observed in *sec10* morphants. Scale bars: 10 μ m.

anti-rhodopsin antibody, 1D4. In controls, photoreceptor OSs were adjacent to the RPE (Fig. 4B, upper panel). In contrast, the OSs of *cdc42* morphant retinas were shorter and less numerous (Fig. 4B, middle and arrows). Transmission electron microscopy analysis of *cdc42* morphant embryos was significant for the absence of most ISs and OSs, along with a reduction of the remaining OS size (Fig. 4C, middle, arrows). In the *sec10* morphant embryos, OSs were completely absent due to the loss of ONL cells (Figs. 4B, 4C, bottom).

To determine the expression pattern of *cdc42* and *sec10* gene in zebrafish retina, immunofluorescence was carried out on embryos at 72 and 120 hpf. Unfortunately, the Cdc42 antibody and the Sec10 antibody, which we made, do not work well for immunofluorescence (despite working quite well for Western blot). We, therefore, performed immunofluorescence with exocyst Sec8 antibody, and demonstrated clear staining at the IS region, near the base of the cilium (Supplementary Fig. S2). The exocyst is thought to act as a holocomplex, so Sec8 staining should be a good surrogate for Sec10 staining, and indeed the entire exocyst complex.

cdc42 and *sec10* Genetically Interact During Eye Development

Our previous in vitro and in vivo studies supported a link between *sec10* and *cdc42*.^{22–24} The ciliary phenotypes shared

between *cdc42* and *sec10* morphant embryos also have been observed on knockdown of other ciliary proteins.^{29,30} We wanted to directly test for a specific genetic interaction in eye phenotype between these two genes. We titrated dosages of the *cdc42* and *sec10* morpholinos, and found suboptimal doses that did not result in gross phenotypes on their own (Figs. 5A, 5B, 5D). Importantly, when we coinjected both morpholinos at these reduced doses, we observed a synergistic effect on eye size reduction (Figs. 5C, 5D), and an increased number of apoptotic cells (data not shown). Disrupted photoreceptor OSs were readily observed in 2 ng *cdc42*MO + 7.5 ng *sec10*MO morphant retinas by TEM. At 120 hpf, only a small number of OSs were observed in 2 ng *cdc42*MO + 7.5 ng *sec10*MO morphant retinas (Figs. 5E, 5G, arrows), which was similar to the phenotype seen with 3 ng of *cdc42* morpholino (Fig. 4C). Moreover, low- (Fig. 5F) and high-magnification (Fig. 5H) images revealed regions of missing ONL, as seen in *sec10* morphant retinas. These results demonstrate a genetic interaction between *cdc42* and *sec10*, and suggest that *sec10* and *cdc42* act in the same pathway in retina development.

Knockdown of *cdc42* and *sec10* Delays Retrograde Intracellular Transport

Melanosome movement is an excellent method to examine microtubule-based ciliary transport. Melanosomes are lyso-

FIGURE 5. *cdc42* and *sec10* genetically interact. (A–C) Phenotype and H&E staining at 120 hpf shows a severe phenotype in the zebrafish injected with suboptimal doses of both *cdc42* and *sec10* morpholinos, whereas no effect is observed when the suboptimal doses were injected alone. Scale bar for left: 10 mm. Scale bar for right: 10 μ m. (D) The histograms quantified the effect of the MOs. (E–H) Transmission electron micrographs of transverse sections along the dorsal-ventral axis of control and 2 ng *cdc42*MO+7.5 ng *sec10*MO at 120 hpf.

some-related organelles whose bidirectional movements, toward the cell center (aggregation, retrograde transport) or toward the cell periphery (dispersion, anterograde transport), are carried out by different ciliary motor proteins.²⁶ Anterograde transport is accomplished by kinesin II motors, and retrograde transport is performed by dynein motors.^{31,32} Many ciliary proteins, such as BBS family members, are involved in retrograde melanosome transport in zebrafish.²⁶ Retrograde and anterograde trafficking of melanosomes is known to be stimulated by epinephrine and caffeine, respectively, due to their effects on cyclic AMP levels.³¹ In our analysis, embryos at 120 hpf displayed melanophore dispersion after overnight dark adaptation (Figs. 6A, 6B). When the injection control embryos were treated with epinephrine, the melanosomes rapidly aggregated and the area of pigmentation was reduced (Fig. 6C, arrow). At the maximum aggregation endpoint, all pigment granules showed perinuclear accumulation of melanosomes (Fig. 6D). The time to reach this pigment aggregation endpoint was measured as the readout. Completion of melanosome transport in wild-type embryos averaged 2.56 minutes ($n = 11$), whereas 3 ng *cdc42*MO ($n = 11$), 15 ng *sec10*MO ($n = 13$), and 2 ng *cdc42*MO+7.5 ng *sec10*MO ($n = 15$) morphant embryos showed a statistically significant delay with an average of 5.25 minutes, 7.50 minutes, and 4.94 minutes, respectively (Fig. 6E, $n = 50$). These data suggest that the *cdc42* and *sec10* are involved in retrograde transport.

DISCUSSION

In retinal degeneration, photoreceptor cell death is a common endpoint that is reached through a diverse range of cellular dysfunctions. Interestingly, almost one-quarter of known retinal degeneration genes in the Retinal Information Network (RetNet) are associated with ciliary function and trafficking.³³ Retinal ciliopathies seem to express their phenotypes through abnormalities in ciliary structure and trafficking, rather than defects in signaling or polarity. For example, proteins involved in Usher and Bardet-Biedl syndromes seem to affect ciliary transport through defects in the docking and loading of vesicles coming from the Golgi complex to the base of the connecting cilium.^{5,34,35} Primary ciliary dyskinesia, Joubert syndrome, and Senior-Loken syndrome/nephronophthisis involve proteins that are thought to function in microtubule associated protein transport in primary cilia and disk morphogenesis.^{36–39}

One factor implicated in vesicular transport to the connecting cilium is small GTPases that mediate the transport, docking, and fusion of carrier vesicles. These small GTPases are divided into several families, including, Rab, Rho, Rac, and Arf. The Rab family GTPases, Rab8 and Rab11, bind rhodopsin directly and traffic it to the cilium.⁴⁰ The Rho family GTPase, Rac1, has been implicated in light-induced photoreceptor degeneration as a proapoptotic factor, and also has been linked to the tethering and fusion of rhodopsin-bearing transport

FIGURE 6. Knockdown of *cdc42* and *sec10* delays retrograde intracellular transport. (A) Dorsal view of the head melanocytes of a 120-hpf dark-adapted embryo (pretreatment). The boxed region encompasses the area of high magnification seen in (B–D). (C) Embryos were treated with epinephrine, and the melanosomes rapidly decreased in size. (D) The endpoint occurred when the pigment granules showed perinuclear localization, and the size was maximally decreased. (E) Quantification of the response time to the endpoint for epinephrine treatment in control and *cdc42*, *sec10*, and *cdc42+sec10* morphants is shown. * $P = 0.0177$; ** $P = 0.0154$; *** $P = 0.0008$. This experiment was repeated three times with similar results.

carriers with moesin, actin, and Rab8.^{41,42} Another Rho family GTPase, Cdc42 is expressed in corneal epithelial cells and photoreceptor cells in mice,⁴³ and is also associated with photoreceptor cilia formation in the zebrafish retina.²⁴ Another eye-specific Cdc42 knockdown mouse model showed that lack of Cdc42 affected organization and cell survival in the developing eye, but that mature rod photoreceptors do not need intracellular Cdc42 for normal function.^{44,45} These studies suggest that Cdc42 is important during eye development, but its mechanism is still uncertain.

Another important component in vesicle trafficking is the exocyst, a highly conserved eight-protein tethering complex, that we showed localizes to cilia,^{22,46} and is regulated by multiple Rab and Rho family GTPases.^{47,48} In a recent study, a mutation in an exocyst component, *EXO84*, was found in a family with Joubert syndrome, a nephronophthisis-related disorder.⁴⁹ In addition, Sec10, a central component of the eight-protein exocyst complex,²² is necessary for ciliogenesis, and also is associated with eye development in zebrafish.²⁵ Interestingly, Sec10 interacts with Cdc42 in vitro and in vivo,^{23,24} and Sec10 and Cdc42 were identified in a photoreceptor sensory cilium proteomics study.⁵⁰ Together, these data strongly suggest that *sec10* and *cdc42* colocalize to the photoreceptor cell sensory cilium.

Here, we show that knockdown of *cdc42* in zebrafish resulted in a relatively normal retinal structure, but led to increased cell death and the absence of most connecting cilia and OSs. This suggests that the absence of cilia might be caused by defects in ciliary protein trafficking, which results in the loss of OSs. Lack of *sec10* resulted in severe and progressive degeneration of retinal cells, especially of photoreceptor cells. The 72 hpf *sec10* morphants still had ONL structure, but 120 hpf *sec10* morphants were completely

missing photoreceptor cells, due to apoptosis. Interestingly, we also found a genetic interaction between *cdc42* and *sec10* that is necessary for retinal development. Suboptimal doses of *cdc42* and *sec10* morpholinos, that alone had no effect, caused a severe retinal defect when given together. This synergistic result suggests that *cdc42* and *sec10* act in the same pathway. Retinal defects, similar to what we saw in *cdc42* and *sec10* morphants, have been similarly described in ciliopathy zebrafish models of intraflagellar transport. The *rpgr* and *inpp5e* mutants, which are associated with ciliogenesis and maintenance, have small eyes, cell death, connecting cilium defects, and short or absent OSs.^{6,27,51,52}

In this study, knockdown of *cdc42* and *sec10* resulted in disruption of the retrograde movement of skin melanosomes along microtubules. Importantly, some ciliary proteins capable of causing retinal degeneration, such as BBS1-8 and *Rpgr*, have been implicated in the regulation of retrograde melanosome transport by dynein motors, which also are associated with rhodopsin transport.^{26,27} Rhodopsin requires the dynein light chain, Tctex-1, which binds directly to the dynein intermediate chain and rhodopsin, targeting these proteins to the apical surface.⁵³ A recent study in *Xenopus* showed retrograde trafficking at the early stages of the rod OS, in which OSs are still cone-shaped.⁵⁴ This study suggests that opsin is trafficked in a retrograde fashion toward the basal portion of the OS in the early development of rod photoreceptors. In the zebrafish, OSs first appear at 60 hpf (cone-shaped) and the first indication of rod formation was evidenced at 192 hpf.¹⁴ This is very similar to OS development in *Xenopus*. Together, these data raise the possibility that *cdc42* and *sec10* are involved in retrograde transport, and OS formation, in the developing zebrafish.

FIGURE 7. Model for the role of *Cdc42* and exocyst *Sec10* in delivery of proteins. The connecting cilium originates from the basal body in the IS, and the axoneme extends into the OS. **(A)** An illustration of the photoreceptor and retinal pigmented cell structures. **(B)** The illustration focuses on the connecting cilium, a region of disk morphogenesis and protein transport. Vesicles containing rhodopsin/OS membrane proteins express Rab8 on the vesicle surface, and Rab8, in turn, is regulated by Rabin8 and Rab11. The exocyst complex is localized to the primary cilium by *Cdc42*, and then targets and docks vesicles carrying rhodopsin/OS membrane proteins. This occurs because exocyst *Sec15* interacts with Rab8 found on the vesicle surface. *Sec10* then binds to *Sec15*, and pulls the vesicle to the rest of the exocyst complex located at the cilium.

Together with our previous studies,^{23–25} our findings suggest a model in which *cdc42* and *sec10* cooperate in the transport and trafficking of proteins necessary for normal retina structure and function in photoreceptor cells (Fig. 7).

This study is the first to implicate ciliogenesis and photoreceptor development in *cdc42*- and *sec10*-related retinal degeneration. This study also confirms a role for *cdc42* and *sec10* in the development and function of the retina, and suggests trafficking mechanisms that may underlie retinal degeneration. Nevertheless, further studies regarding the association among retrograde trafficking, the exocyst, *cdc42*, and ciliogenesis are needed to clarify the precise role of *cdc42* and *sec10* in these processes.

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References

- Bhogaraju S, Engel BD, Lorentzen E. Intraflagellar transport complex structure and cargo interactions. *Cilia*. 2013;2:10.
- Badano JL, Mitsuma N, Beales PL, Katsanis N. The ciliopathies: an emerging class of human genetic disorders. *Annu Rev Genomics Hum Genet*. 2006;7:125–148.
- Cardenas-Rodriguez M, Badano JL. Ciliary biology: understanding the cellular and genetic basis of human ciliopathies. *Am J Med Genet C Semin Med Genet*. 2009;151:263–280.
- Baker K, Beales PL. Making sense of cilia in disease: the human ciliopathies. *Am J Med Genet C Semin Med Genet*. 2009;151:281–295.
- Blacque OE, Leroux MR. Bardet-Biedl syndrome: an emerging pathomechanism of intracellular transport. *Cell Mol Life Sci*. 2006;63:2145–2161.
- Luo N, Lu J, Sun Y. Evidence of a role of inositol polyphosphate 5-phosphatase INPP5E in cilia formation in zebrafish. *Vision Res*. 2012;75:98–107.
- Baye LM, Patrino X, Swaminathan S, et al. The N-terminal region of centrosomal protein 290 (CEP290) restores vision in

- a zebrafish model of human blindness. *Hum Mol Genet.* 2011;20:1467–1477.
8. Bachmann-Gagescu R, Phelps IG, Stearns G, et al. The ciliopathy gene *cc2d2a* controls zebrafish photoreceptor outer segment development through a role in Rab8-dependent vesicle trafficking. *Hum Mol Genet.* 2011;20:4041–4055.
 9. Sukumaran S, Perkins BD. Early defects in photoreceptor outer segment morphogenesis in zebrafish *ift57*, *ift88* and *ift172* Intraflagellar Transport mutants. *Vision Res.* 2009;49:479–489.
 10. Schmitt EA, Dowling JE. Early retinal development in the zebrafish, *Danio rerio*: light and electron microscopic analyses. *J Comp Neurol.* 1999;404:515–536.
 11. Besharse JC, Baker SA, Luby-Phelps K, Pazour GJ. Photoreceptor intersegmental transport and retinal degeneration: a conserved pathway common to motile and sensory cilia. *Adv Exp Med Biol.* 2003;533:157–164.
 12. Tsujikawa M, Malicki J. Genetics of photoreceptor development and function in zebrafish. *Int J Dev Biol.* 2004;48:925–934.
 13. Liu Q, Zhang Q, Pierce EA. Photoreceptor sensory cilia and inherited retinal degeneration. *Adv Exp Med Biol.* 2010;664:223–232.
 14. Branchek T, Bremiller R. The development of photoreceptors in the zebrafish, *Brachydanio rerio*. I. Structure. *J Comp Neurol.* 1984;224:107–115.
 15. Moritz OL, Tam BM, Hurd LL, Peranen J, Deretic D, Papermaster DS. Mutant *rab8* impairs docking and fusion of rhodopsin-bearing post-Golgi membranes and causes cell death of transgenic *Xenopus* rods. *Mol Biol Cell.* 2001;12:2341–2351.
 16. Deretic D, Huber LA, Ransom N, Mancini M, Simons K, Papermaster DS. *rab8* in retinal photoreceptors may participate in rhodopsin transport and in rod outer segment disk morphogenesis. *J Cell Sci.* 1995;108:215–224.
 17. Deretic D, Papermaster DS. Rab6 is associated with a compartment that transports rhodopsin from the trans-Golgi to the site of rod outer segment disk formation in frog retinal photoreceptors. *J Cell Sci.* 1993;106:803–813.
 18. Young RW. The renewal of photoreceptor cell outer segments. *J Cell Biol.* 1967;33:61–72.
 19. Nachury MV, Loktev AV, Zhang Q, et al. A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. *Cell.* 2007;129:1201–1213.
 20. Das A, Guo W. Rabs and the exocyst in ciliogenesis, tubulogenesis and beyond. *Trends Cell Biol.* 2011;21:383–386.
 21. Hutagalung AH, Novick PJ. Role of Rab GTPases in membrane traffic and cell physiology. *Physiol Rev.* 2011;91:119–149.
 22. Zuo X, Guo W, Lipschutz JH. The exocyst protein Sec10 is necessary for primary ciliogenesis and cystogenesis in vitro. *Mol Biol Cell.* 2009;20:2522–2529.
 23. Zuo X, Fogelgren B, Lipschutz JH. The small GTPase Cdc42 is necessary for primary ciliogenesis in renal tubular epithelial cells. *J Biol Chem.* 2011;286:22469–22477.
 24. Choi SY, Chacon-Heszele MF, Huang L, et al. Cdc42 deficiency causes ciliary abnormalities and cystic kidneys. *J Am Soc Nephrol.* 2013;24:1435–1450.
 25. Fogelgren B, Lin SY, Zuo X, et al. The exocyst protein Sec10 interacts with Polycystin-2 and knockdown causes PKD-phenotypes. *PLoS Genet.* 2011;7:e1001361.
 26. Yen HJ, Tayeh MK, Mullins RF, Stone EM, Sheffield VC, Slusarski DC. Bardet-Biedl syndrome genes are important in retrograde intracellular trafficking and Kupffer's vesicle cilia function. *Hum Mol Genet.* 2006;15:667–677.
 27. Shu X, Zeng Z, Gautier P, et al. Zebrafish *Rpgr* is required for normal retinal development and plays a role in dynein-based retrograde transport processes. *Hum Mol Genet.* 2010;19:657–670.
 28. Biehlermaier O, Neuhauss SC, Kohler K. Onset and time course of apoptosis in the developing zebrafish retina. *Cell Tissue Res.* 2001;306:199–207.
 29. Kramer-Zucker AG, Olale F, Haycraft CJ, Yoder BK, Schier AF, Drummond IA. Cilia-driven fluid flow in the zebrafish pronephros, brain and Kupffer's vesicle is required for normal organogenesis. *Development.* 2005;132:1907–1921.
 30. Serluca FC, Xu B, Okabe N, et al. Mutations in zebrafish leucine-rich repeat-containing six-like affect cilia motility and result in pronephric cysts, but have variable effects on left-right patterning. *Development.* 2009;136:1621–1631.
 31. Nascimento AA, Roland JT, Gelfand VI. Pigment cells: a model for the study of organelle transport. *Annu Rev Cell Dev Biol.* 2003;19:469–491.
 32. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn.* 1995;203:253–310.
 33. Wright AF, Chakarova CF, Abd El-Aziz MM, Bhattacharya SS. Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. *Nat Rev Genet.* 2010;11:273–284.
 34. Reiners J, Nagel-Wolfrum K, Jurgens K, Marker T, Wolfrum U. Molecular basis of human Usher syndrome: deciphering the meshes of the Usher protein network provides insights into the pathomechanisms of the Usher disease. *Exp Eye Res.* 2006;83:97–119.
 35. Williams DS. Usher syndrome: animal models, retinal function of Usher proteins, and prospects for gene therapy. *Vision Res.* 2008;48:433–441.
 36. Arts HH, Doherty D, van Beersum SE, et al. Mutations in the gene encoding the basal body protein RPGRIP1L, a nephrocystin-4 interactor, cause Joubert syndrome. *Nat Genet.* 2007;39:882–888.
 37. Adams NA, Awadein A, Toma HS. The retinal ciliopathies. *Ophthalmic Genet.* 2007;28:113–125.
 38. Moore A, Escudier E, Roger G, et al. RPGR is mutated in patients with a complex X linked phenotype combining primary ciliary dyskinesia and retinitis pigmentosa. *J Med Genet.* 2006;43:326–333.
 39. Roepman R, Letteboer SJ, Arts HH, et al. Interaction of nephrocystin-4 and RPGRIP1 is disrupted by nephronophthisis or Leber congenital amaurosis-associated mutations. *Proc Natl Acad Sci U S A.* 2005;102:18520–18525.
 40. Deretic D, Wang J. Molecular assemblies that control rhodopsin transport to the cilia. *Vision Res.* 2012;75:5–10.
 41. Haruta M, Bush RA, Kjellstrom S, et al. Depleting Rac1 in mouse rod photoreceptors protects them from photo-oxidative stress without affecting their structure or function. *Proc Natl Acad Sci U S A.* 2009;106:9397–9402.
 42. Deretic D, Traverso V, Parkins N, Jackson F, Rodriguez de Turco EB, Ransom N. Phosphoinositides ezrin/moesin, and rac1 regulate fusion of rhodopsin transport carriers in retinal photoreceptors. *Mol Biol Cell.* 2004;15:359–370.
 43. Mitchell DC, Bryan BA, Liu JP, et al. Developmental expression of three small GTPases in the mouse eye. *Mol Vis.* 2007;13:1144–1153.
 44. Heynen SR, Tanimoto N, Joly S, Seeliger MW, Samardzija M, Grimm C. Retinal degeneration modulates intracellular localization of CDC42 in photoreceptors. *Mol Vis.* 2011;17:2934–2946.
 45. Heynen SR, Meneau I, Caprara C, et al. CDC42 is required for tissue lamination and cell survival in the mouse retina. *PLoS One.* 2013;8:e53806.
 46. Rogers KK, Wilson PD, Snyder RW, et al. The exocyst localizes to the primary cilium in MDCK cells. *Biochem Biophys Res Commun.* 2004;319:138–143.

47. Knodler A, Feng S, Zhang J, et al. Coordination of Rab8 and Rab11 in primary ciliogenesis. *Proc Natl Acad Sci U S A*. 2010;107:6346–6351.
48. Lipschutz JH, Mostov KE. Exocytosis: the many masters of the exocyst. *Curr Biol*. 2002;12:R212–R214.
49. Dixon-Salazar TJ, Silhavy JL, Udpa N, et al. Exome sequencing can improve diagnosis and alter patient management. *Sci Transl Med*. 2012;4:138ra78.
50. Liu Q, Tan G, Levenkova N, et al. The proteome of the mouse photoreceptor sensory cilium complex. *Mol Cell Proteomics*. 2007;6:1299–1317.
51. Insinna C, Pathak N, Perkins B, Drummond I, Besharse JC. The homodimeric kinesin, Kif17, is essential for vertebrate photoreceptor sensory outer segment development. *Dev Biol*. 2008;316:160–170.
52. Tsujikawa M, Malicki J. Intraflagellar transport genes are essential for differentiation and survival of vertebrate sensory neurons. *Neuron*. 2004;42:703–716.
53. Yeh TY, Peretti D, Chuang JZ, Rodriguez-Boulan E, Sung CH. Regulatory dissociation of Tctex-1 light chain from dynein complex is essential for the apical delivery of rhodopsin. *Traffic*. 2006;7:1495–1502.
54. Tian G, Lodowski KH, Lee R, Imanishi Y. Retrograde intraciliary trafficking of opsin during the maintenance of cone-shaped photoreceptor outer segments of *Xenopus laevis*. *J Comp Neurol*. 2014;522:3577–3589.