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Retrograde intraciliary trafficking of opsin during the maintenance of cone shaped photoreceptor outer segments of *Xenopus laevis*

Guilian Tian[#], Kerrie H. Lodowski[#], Richard Lee, and Yoshikazu Imanishi

Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4965, USA

[#] These authors contributed equally to this work.

Abstract

Photoreceptor outer segments (OSs) are essential for our visual perception, and take either rod or cone forms. The cell biological basis for the formation of rods is well established, however, the mechanism of cone formation is ill characterized. While *Xenopus* rods are called rods, they exhibit cone shaped OSs during the early process of development. To visualize the dynamic reorganization of disk membranes, opsin and peripherin/rds were fused to a fluorescent protein Dendra2 and expressed in early developing rod photoreceptors, in which OSs are still cone shaped. Dendra2 is a fluorescent protein which can be converted from green to red irreversibly, and thus allows spatiotemporal labeling of proteins. Using a photoconversion technique, we found that disk membranes are assembled at the base of cone shaped OSs. After incorporation into disks, however, Opsin-Dendra2 was also trafficked from old to new disk membranes, consistent with the hypothesis that retrograde trafficking of membrane components contributes to the larger disk membrane observed toward the base of the cone shaped OS. Such retrograde trafficking is cargo specific and was not observed for peripherin/rds-Dendra2. The trafficking is unlikely mediated by diffusion, since the disk membranes have a closed configuration, as evidenced by CNGA1 labeling of the plasma membrane. Consistent with retrograde trafficking, the axoneme, which potentially mediates retrograde intraflagellar trafficking, runs through the entire axis of OSs. This study provides an insight into the role of membrane reorganization in developing photoreceptor OSs, and proves that retrograde trafficking of membrane cargoes can occur there.

Correspondence to Yoshikazu Imanishi: yxi19@case.edu, Phone: 216-368-5226, Fax: 216-368-1300.

Role of authors.

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. G.T., K.H.L. and Y.I. designed research. G.T. and K.H.L. performed research. K.H.L. and Y.I. developed the technique used throughout the manuscript. G.T. and R.L. analyzed data. G.T. and Y.I. wrote the paper. Y.I. obtained funding for this project.

Conflict of interest statement

Authors declare that there is no conflict of interest associated with this study.

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Keywords

Dendra2; fluorescence; photoconversion; peripherin/rds; rod photoreceptor; disk membrane

Introduction

Rod and cone photoreceptors are two major photoreceptor cell types in vertebrates. As their names indicate, rod and cone photoreceptors have been classified based on the shape of their respective outer segments (OSs). In addition to their shapes, differences in physiological properties were observed between rods and cones. These differences were hypothesized to be derived from factors such as rod- and cone- specific proteins involved in phototransduction cascades (Kawamura and Tachibanaki, 2008). The topology of cone disk membranes appears to vary among species. In general, cone OSs of low vertebrate species show open disk membrane structures that are continuous with the plasma membrane, in contrast to closed rod disk membranes that are not continuous with the plasma membrane. In mammalian species, however, cone disks are suggested to take a mixture of open and closed disk structures (Anderson et al., 1978). Throughout the vertebrate species, rod OSs maintain the same width throughout their length, whereas cone OSs are tapered toward the distal end. While the processes of membrane renewal was characterized for rod shaped OSs more than 4 decades ago (Young, 1967), it is still unclear how cone shaped OS can be formed or renewed (Anderson et al., 1978).

Within the photoreceptor OSs, the intraflagellar transport (IFT) system plays important roles in trafficking of materials (Pazour et al., 2002) to disk membranes. Such a transport system operates in an anterograde fashion, and runs on the major cytoskeleton of cilia, the axoneme. Such anterograde transport is mediated by a kinesin II motor and is essential for carrying cargoes, such as opsin and other phototransduction components, to the disk membranes (Marszalek et al., 2000; Avasthi et al., 2009; Lopes et al., 2010; Trivedi et al., 2012). While anterograde trafficking is essential for carrying the constituents of the disk membranes, retrograde trafficking is also required for the maintenance of the cilia. For example, kinesin II motor and associated IFT proteins need to be retrogradely trafficked back to the cilia base, a trafficking process mediated by dynein motors (Rosenbaum and Witman, 2002; Krock et al., 2009). While this recycling process is well known, it is unclear if retrograde trafficking plays any additional role in the maintenance of photoreceptor OSs. It has been difficult to identify cargoes for retrograde trafficking due to the lack of methods to visualize retrograde trafficking of specific proteins in the OS.

The process of OS morphogenesis has been well documented for the *Xenopus laevis* rod photoreceptor which is large and amenable for microscopy observations. During development, the *Xenopus* rod OSs transform morphologically from cone to rod shape (Kinney and Fisher, 1978). Autoradiographic studies were instructive to understand the maintenance process of the OSs (Kinney and Fisher, 1978). Based on these studies, new proteins were preferably added to the base of both the rod and cone shaped OSs, and the rates of disk membrane addition, expressed by volume/time, appeared constant throughout the developmental process (Kinney and Fisher, 1978). Because of limited resolution and

background noise, however, the autoradiography did not provide an accurate view on how the cone shape was formed or maintained. In early developing rod OSs, radioactive signals, while condensed in the form of a band, were also observed throughout the OSs (Kinney and Fisher, 1978). Because radiolabeling occurs randomly for all the proteins being synthesized, autoradiography might have suffered from the lack of specific labeling of membrane proteins, and labeling of soluble proteins and existence of free radioactive amino acids partly contributed to the noise observed throughout the photoreceptor cells. Such noises are especially problematic in monitoring retrograde trafficking, which may also result in a diffuse signal through the height of the photoreceptor OSs. Further, autoradiography is designed to monitor newly synthesized proteins, but not suitable to visualize old and pre-existing proteins, which are potentially the cargoes for the retrograde trafficking mechanism.

We recently developed a method to monitor membrane protein trafficking by a photoconversion technique using a fluorescent protein Dendra2 (Lodowski et al., 2013). Unlike the autoradiography method, Dendra2 allows monitoring of both old and newly synthesized proteins in the photoreceptor OSs. In this manuscript, we used *Xenopus* rod photoreceptors early in their development as a model to address questions pertinent to the morphogenesis of cone shaped OSs. We asked how disk membrane proteins are renewed during the process of early OS development, and after maturation into the rod shaped OSs. A photoconvertible fluorescent protein, Dendra2 (Gurskaya et al., 2006; Chudakov et al., 2007), was used to track the renewal of disk membranes in developing cone shaped OSs. Then by using a photoconversion technique, we asked if retrograde trafficking can occur for disk membrane proteins to establish cone shaped OSs. We asked if those disks in primitive rods have open or closed disk configurations, by testing the localization of an OS plasma membrane protein, cGMP-gated channel. These studies provide insight into how membrane proteins are renewed and recycled to maintain cone shaped OSs in early developing rods.

Materials and Methods

Constructs

Human rod opsin and *Xenopus laevis* peripherin/rds (P/rds) fused with Dendra2 (Dend2) (Gurskaya et al., 2006) were generated as described previously (Lodowski et al., 2013; Tian et al., 2014). Briefly, the full-length human opsin cDNA was fused to Dendra2 (Clontech Laboratories, Inc., Mountain View, CA) followed by the last 8 aa of human opsin. This entire segment was inserted into a TOPO vector with the *Xenopus* rod opsin gene promoter to generate XOP-Opsin-Dend2. Following the coding region, an SV40 polyadenylation signal was added. Full-length peripherin/rds cDNA (GeneBank ID: AY062004, Rds38) was cloned from *Xenopus laevis* retina. Full-length bovine cyclic nucleotide gated channel alpha-1 (CNGA1) cDNA was a generous gift from Dr. William N. Zagotta (Department of Physiology & Biophysics, University of Washington, Seattle, WA). To generate pXOP-P/rds-Dend2 and pXOP-CNGA1-Dend2, the coding region of peripherin/rds and CNGA1 replaced the coding region of opsin in XOP-Opsin-Dend2. A stop codon TAA was added after the Dend2 coding region to prevent the 1D4 tag from being translated.

Xenopus laevis

All the experiments related to frogs and tadpoles were conducted following the protocol approved by the IACUC at Case Western Reserve University, and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult frogs were bought from Nasco (Fort Atkinson, WI) and housed at 16°C under a 12 h/12 h light/dark cycle. The tadpoles used for experiments were kept at 16°C in continuous darkness.

Generation of transgenic *Xenopus laevis*

Transgenic *Xenopus laevis* were generated as described previously (Lodowski et al., 2013). In short, eggs were injected with sperm nuclei mixed with transgenes. Several hours later, healthy embryos were selected and housed at 16°C in the dark. 6 to 7 days following fertilization, tadpoles expressing transgenes were identified by the presence of green fluorescence in their eyes. Tadpoles were staged according to Nieuwkoop and Faber's normal table (Nieuwkoop and Faber, 1967). The majority of tadpoles were stage 41 when they were 6-7 days post-fertilization (dpf), stage 45-46 when they were 9 dpf, and stage 47 when they were 21-22 dpf in our culture condition.

Photoconversion of Dend2 in tadpole eyes

The photoconversion procedure was described previously (Lodowski et al., 2013; Tian et al., 2014). Briefly, tadpoles were anesthetized by 0.026% tricaine methanesulfonate (Sigma) and placed into 6% methyl cellulose. A 405 nm laser pointer equipped with adjustable focus was secured to a ring stand and orientated downwards toward the head of the animal. Both eyes were exposed to 405 nm laser light for fifteen 1 minute intervals, each followed by a 20 second break.

Retina culture

As described previously (Lodowski et al., 2013; Tian et al., 2014), tadpoles were decapitated after anesthetizing in 0.026% tricaine methanesulfonate. Retinas were dissected and incubated in a modified version of Wolf amphibian culture medium inside a sealed chamber (DMIRB/E ONICS-D35, Tokai Hit CO., Ltd., Shizuoka-ken, Japan) during the imaging procedures. The Wolf medium was composed of 55% MEM (Invitrogen, Grand Island, NY), 31% Earle's sodium-free BBS, 10% FBS, 30mM NaHCO₃, and 700 mg/L D-glucose. The sealed chamber was used to maintain the proper temperature, humidity and gas concentrations (95% O₂ and 5% CO₂)

Immunofluorescence

The tadpoles expressing Opsin-Dend2 were decapitated after anesthetizing in 0.026% tricaine methanesulfonate. Heads were then fixed in 4% paraformaldehyde for 6 h at room temperature. The fixed heads were placed in 5%, 10% and 15% sucrose sequentially, and frozen in 10% sucrose with 50% OCT. All the following procedures were performed at room temperature and the washing buffer was PBS with 0.1% Triton X-100. Eye sections were blocked with 1.5% normal goat serum in washing buffer for 1 h, and then incubated with wheat germ agglutinin conjugated to Alexa Fluor 633 (Invitrogen, cat# W21404) in

combination with either mouse anti-*Xenopus laevis* peripherin/rds antibody (clone 2D4, raised against the c-terminus of *Xenopus* peripherin/rds, a generous gift from Dr. Robert Molday, RRID: AB_2315773) or mouse anti-acetylated tubulin (Sigma-Aldrich, cat# T6793, RRID: AB_477585) in washing buffer overnight. After washing, the sections were incubated with Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch, Cat #115-165-166) for 1h, and washed. The resulting sections were imaged by confocal microscopy.

Antibody Characterization

Xenopus laevis peripherin/rds antibody was characterized previously by western blots (Tian et al., 2014). The antibody detected bands with molecular weights 40 kilodaltons for glycosylated-form P/rds and 36 kilodaltons for deglycosylated-form peripherin/rds in *Xenopus* retinal lysate. The antibody detected a band with Endo H sensitivity, a unique characteristics of P/rds (Tian et al., 2014). The antibody stained the rim region of photoreceptor OS (see the result section) as reported previously for *Xenopus* peripherin/rds (Kedzierski et al., 1996; Han et al., 2012).

Image analysis and quantification

Single x-y panel images were processed by 2D blind deconvolution using AutoDeblur and AutoVisualize 9.3 (MediaCybernetics), and then the levels of red and green Opsin-Dend2 were quantified using ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2004, RRID: nif-0000-30467). Rod opsin promoter resulted in variable expression levels as reported previously (Moritz et al., 2001). Occasional overexpression of Opsin-Dend2 or P/rds-Dend2 caused distortion of the cell shape. Therefore, the cells that overexpressed Opsin-Dend2 or P/rds-Dend2 were not included in the analysis. A line corresponding to a width of 0.92 μm was drawn along the longitudinal axis of the OS and the intensity of both red and green fluorescence was plotted along the line. The average of 3 lowest intensities, along the line, was assigned as the background intensity for each color of fluorescence for each measurement. The backgrounds were then subtracted from all measurements. The fluorescence at each pixel was normalized to the highest intensity within the line. The crosspoint of red and green fluorescence was defined as the point with the closest percentage of both fluorescences. For the images which were taken right after photoconversion or without photoconversion, the crosspoint was defined as the point with fluorescence intensity closest to 50% of the highest intensity around the OS base. To calculate the distance that old Opsin-Dend2 retrogradely traveled (D_R), red fluorescence was normalized to the intensity of the crosspoint (100%) and the distance was calculated based on the number of pixels with intensities higher than 2% from the crosspoint toward the basal OS. D_G is the distance from the crosspoint to the first appreciable peak of green fluorescence. W_G represents the width of the newly synthesized disks measured by the distance from the crosspoint to the point where the level of new green Opsin-Dend2 became lower than 2%. To calculate the slope of green fluorescence around crosspoint, 5 intensities within a $\pm 0.73 \mu\text{m}$ region of the crosspoint were taken and fitted into the linear polynomial by Sigma plot 12.3.

Results

Visualization of *Xenopus* rod OSs by a fluorescent protein Dendra2

To visualize the morphology of rod OSs in the early stages of OS morphogenesis, we expressed opsin and peripherin/rds fused to Dend2 (Chudakov et al., 2007) in *Xenopus* retina using the rod opsin promoter (Batni et al., 1996; Knox et al., 1998; Lodowski et al., 2013). The labeling pattern of opsin was consistent with its localization in the disk lamellar region, whereas the labeling pattern of peripherin/rds was consistent with its localization in the disk rim and incisure regions (Fig 1A). At the early stage of development, 6-9 dpf, rod OSs are cone shaped as reported previously (Fig 1A, Opsin-Dend2, 7 dpf and 9 dpf; P/rds-Dend2, 6 dpf and 9 dpf). The diameters of those unfixed photoreceptor OSs ($5.49 \pm 1.35 \mu\text{m}$, mean $\pm$ SD; 30 rod photoreceptors from $n=3$ preparations) are close to the previously reported diameters ($3.85 \pm 0.49 \mu\text{m}$) (Kinney and Fisher, 1978) of fixed rod OSs at the same age. At an older age such as 21-22 dpf, both Opsin-Dend2 and P/rds-Dend2 labeled rod shaped OSs that are consistent with mature rod photoreceptors (Fig 1A, Opsin-Dend2 and P/rds-Dend2, 21-22 dpf). We confirmed that the rod opsin promoter drives the expression of proteins in rods as early as 6 dpf, as demonstrated by co-localization of Opsin-Dend2 with the immunofluorescence signal derived from an anti-frog P/rds antibody (2D4) (Fig 1B). P/rds localizes in the rim regions around the disks in rods (Fig 1B, arrowheads), to which Opsin-Dend2 also localized. As reported previously (Han et al., 2012), P/rds also localizes in the rim regions adjacent to the axoneme of cones, which was negative to Opsin-Dend2 and demonstrated much smaller diameter (Fig 1B, arrow). This labeling pattern of Opsin-Dend2 (Fig 1B) indicates that the rod opsin promoter drives the specific expression in rods. Thus those two markers, expressed by the rod opsin promoter, can accurately label rod photoreceptors throughout the process of morphological transformation from a cone to a rod shape.

Retrograde intraciliary trafficking hypothesis

Unlike rod shaped OSs, cone shaped OSs require more membranous materials, including opsin, toward the lower portion of the segments than the upper portion. To interrogate how more opsin can localize toward the basal portions, we designed two models (Fig. 2, models A and B). In model A (Fig. 2A), membrane components are retrogradely trafficked so that disks in the basal portions can become bigger. In model B (Fig. 2B), disk membranes are synthesized at gradually bigger sizes, causing a gradual increment in the disk diameter toward the bottom of the OS. The major difference between models A and B is the time dependent redistribution of old and new proteins (red and green, respectively), and the resulting axial gradient which would occur only in model A. The lack of the retrograde trafficking in model B would lead to maintenance of a sharp contrast between the old and new disk membranes.

Visualization of disk membrane morphogenesis in early developing OSs

At 6 dpf, *Xenopus* rod photoreceptors are cone shaped. In both the models (Fig. 2), newly synthesized disks are added to the base of the OSs. To study the dynamics of disk morphogenesis and the distribution of newly synthesized proteins at this stage, we photoconverted Opsin-Dend2 and P/rds-Dend2. Immediately after photoconversion, old

Opsin-Dend2 was converted to red (Fig. 3A). After photoconversion, trafficking of new protein was followed for up to 2 days during which the OS preserved the cone shape. Four and eight hours after photoconversion, the new green fluorescent Opsin-Dend2 accumulated at the base of the OSs (Fig. 3B and C), with the old red proteins enriched towards the tip. One day after photoconversion, the green band at the base of the OSs thickened (Fig. 3D), while two days (48 hours) after photoconversion, it further expanded towards the tip of the OS (Fig. 3E). The very base of the OS was devoid of old proteins (Fig. 3E), thus suggesting that new disk membranes are assembled and incorporate newly synthesized opsin. Newly synthesized peripherin/rds was observed at the base of the OSs (Fig. 3F). These observations suggest that new disk membranes are assembled and incorporate newly synthesized proteins to the rim, incisure, and also the core (lamellar) region of the disk membranes. We also monitored the disk morphogenesis at 21-22 dpf, in which the OS is mature and rod shaped. Opsin-Dend2 was photoconverted when *Xenopus laevis* was at 21-22 dpf, and we followed the OS renewal events for 2 - 6 days. Two days after photoconversion, basal green disk membranes displaced the old red disk membranes (Fig. 3G). Six days after photoconversion, the green band at the basal OS thickened further (Fig. 3H). Thus regardless of the age and the shape of the OSs, disk membranes are assembled at the base of the OSs. These observations are consistent with the addition of new opsin to the base of the OS, as predicted for both model A and B.

Retrograde Opsin trafficking in the OS of early developing rod photoreceptors

To experimentally discriminate the two models, we followed the distribution of red fluorescent Opsin-Dend2 at several time points after photoconversion. If retrograde trafficking is playing a role in the redistribution of OS proteins, an axial gradient of photoconverted proteins will develop and become shallower over time. Immediately after photoconversion at 6 dpf, red fluorescent Opsin-Dend2 demonstrated a sharp contrast at the bottom of the OS (Fig. 3A). However, 4 - 8 hours after photoconversion, the red fluorescent Opsin-Dend2 started to form a gradient at the red-green interface (Fig. 3B and C). One to two days after photoconversion, the gradient formed by red Opsin-Dend2 grew shallower on the longitudinal axis of the OS (Fig. 3D and E). These time dependent shifts of the distribution indicate that old Opsin-Dend2 in old disk membranes moved to newer disks. The retrograde trafficking was not apparent for P/rds-Dend2 even two days after photoconversion, since the gradient formed by red P/rds-Dend2 (Fig. 3F) was sharp and similar to that formed by red Opsin-Dend2 0 h after photoconversion (Fig. 3A). Thus retrograde trafficking appears to be cargo specific and was prominent for Opsin-Dend2 at 6 dpf and subsequent days, while rod OSs were cone shaped (Fig. 3A-E).

To quantitatively compare these two models, a few variables were defined in red and green fluorescence intensity profiles (Fig. 3 and Fig. 4A). One of the variables, D_R , is the length of the tail observed for red fluorescence intensity toward the base of the OS, and was used as an indicator of the retrograde trafficking. The longer the D_R , the further the old protein traveled toward the basal portion of the OS. To understand the time dependent shift in D_R , we compared D_R against the width of newly synthesized disk membranes (W_G) which increased over time. We found that D_R increased significantly over the time period of two days (6dpf-4h vs. 8h, 1d, and 2d, $P < 0.001$ by the Mann Whitney Rank Sum Test), during

which W_G also increased (Fig. 4B). This time dependent increase in D_R indicates that there was a retrograde trafficking of old opsin that existed at the time of photoconversion. The median of D_R was $2.29\ \mu\text{m}$ (interquartile range, 2.01 to $2.56\ \mu\text{m}$) at 8 hours post-photoconversion, however increased to $3.48\ \mu\text{m}$ (interquartile range, 2.79 to $3.80\ \mu\text{m}$) at 1 day post-photoconversion (Fig 4B). We obtained clear evidence that, 1 day after photoconversion, red Opsin-Dend2 moved to the newly synthesized disks which did not exist at the time of photoconversion. Accordingly, the median D_R value at 1 day post-photoconversion ($3.48\ \mu\text{m}$) was significantly higher ($P < 0.001$ by the Mann Whitney Rank Sum Test) than the median W_G value of $2.56\ \mu\text{m}$ (interquartile range, 2.38 to $2.88\ \mu\text{m}$) at 8 hours post-photoconversion (Fig 4B). This existence of red Opsin-Dend2 in these new disk membranes is not due to slow and gradual incorporation of red Opsin-Dend2 from the inner segments. Eight hours after photoconversion, the inner segment did not contain a detectable level of red Opsin-Dend2. These results quantitatively support model A (Fig. 2A) in which retrograde trafficking and membrane reorganization contribute to the cone shape. When the red Dendra2 fluorescence intensity was normalized to that of the crosspoint (100%) and plotted as a function of the distance from the crosspoint, the data points were well fitted to a single exponential function (decay) curve (Fig. 4C, left), with the constant gradually increasing over time from 0 hour to 2 days after photoconversion (Fig. 4C, right). Considering that retrograde trafficking resulted in a gradient of the Opsin-Dend2 concentration, this single exponential function relationship suggests that a similar fraction of Opsin-Dend2 moved from disks to disks at different locations on the axis of the OS. Accordingly the concentration of red Opsin-Dend2 was approximately 47.1% lower at the position $1\ \mu\text{m}$ below ($100\% - 52.9\% = 47.1\%$) the crosspoint 2 days post-photoconversion (Fig. 4C, right). Another 47.1% reduction was observed at a point $2\ \mu\text{m}$ below the crosspoint ($52.9\%/um \times 52.9\%/um = 28.0\%/2um$) (Fig. 4C, right). Based on the single exponential decay relationships obtained at different time points, an $8.5\%/um$ increment of red Opsin-Dend2 fluorescence intensity was observed from 8 hours to 1 day post-photoconversion, and a $10.4\%/um$ increment from 1 to 2 days post-photoconversion (Fig. 4C, right). Thus approximately 10% of Opsin-Dend2 moved a distance of $1\ \mu\text{m}$ in 1 day. Therefore, a relatively large degree of retrograde trafficking suggests that membrane reorganization is quite active and would be sufficient to change the overall shape of the developing rod OS.

The above analyses were conducted for red Opsin-Dend2, whose time dependent redistribution indicates the existence of retrograde trafficking of Opsin-Dend2. Model A also suggests that retrograde trafficking of Opsin-Dend2 leads to a time dependent redistribution of green Opsin-Dend2, which can be visualized at the red-green interface (Fig. 2A). To analyze the retrograde trafficking of green Opsin-Dend2, another variable, D_G , was defined (Fig. 4A). D_G is an axial distance measured from the crosspoint to the initial appreciable peak. If the retrograde trafficking of green Opsin-Dend2 is occurring (Fig. 2A), D_G shall increase over time. Consistent with model A, D_G increased from 4 hours to 2 days after photoconversion (Fig. 4D, 6dpf-4h vs. 1d and 2d, $P < 0.001$ by the Mann Whitney Rank Sum Test). We also measured the slope of the green fluorescence curve around the crosspoint (Fig. 4E), because expansion of the gradient (Fig. 2A) will lead to a shallower curve and slope. As expected from model A, the slope was significantly shallower and lower 2 days after photoconversion than 4 hours after photoconversion (Fig. 4E, $P < 0.001$ by the

Mann Whitney Rank Sum Test). Collectively, time dependent shifts in the axial concentrations of green and red Opsin-Dend2 are consistent with model A, in which Opsin-Dend2 is retrogradely trafficked.

Organization of membrane and cytoskeletal structures in developing OS of rod photoreceptors

Previously, electron microscopy analysis revealed closed disk membranes in *Xenopus laevis* of early developing rod photoreceptors (Kinney and Fisher, 1978). We further confirmed the topology of the OS membrane by labeling the α -subunit of a rod cGMP-gated channel (CNGA1), an OS plasma membrane marker, with Dend2 fluorescent protein (CNGA1-Dend2). As expected, the plasma membrane covers the entire length of the OS (Fig. 5A), suggesting that rod disks are in the closed configuration despite being cone shaped. Because of the closed configuration, disk membranes can exchange materials only by an active transport mechanism. Thus this observation is consistent with the retrograde trafficking being mediated by an active transport mechanism. We also obtained important structural insight into the retrograde trafficking. In the past, it was assumed that retrograde trafficking is mediated by axonemal microtubules. If retrograde trafficking is in operation throughout the length of the OS, the entire OS would be underlaid by the axoneme. Indeed, we found that the axonemal microtubule spans the entire length of the rod OS, as demonstrated by its intense labeling with anti-acetylated tubulin antibody (Fig. 5B). Thus, this structural configuration would be capable of supporting the retrograde trafficking which is likely required through the entire length of the cone shaped OS.

Lack of retrograde opsin trafficking in the mature rod OS

When tadpoles are 21-22 dpf, the OS of rod photoreceptors are maintained as a rod shape. The mixture of new green protein and old red parts of OS was minimal (Fig. 3G and H), and likely was contributed by an initial mixing of new and old protein at the growing evaginations. There is no retrograde trafficking at this age, because the slopes of old protein decay at the interface of green and red protein are similar at 2 days and 6 days post-photoconversion (Fig. 6A, 21-22 dpf-2d and 21-22 dpf-6d). Those slopes are similar to that observed for new proteins in newly synthesized disk membranes of mature rods (Fig. 6A, 21-22 dpf-0d), suggesting that the distribution of Opsin-Dend2 barely changed over time as the new disks moved toward the distal portion of the OS. The slope observed at 2 days and 6 days after conversion of 21-22 dpf animals (Fig. 6A) was also similar to the slope observed 0 hour after photoconversion of 6 dpf animals (Fig. 4C, 6dpf-0h), however, was much steeper than the slope observed 2 days after conversion of 6 dpf animals (Fig. 4C, 6dpf-2d). These comparisons indicate that there was no significant time-dependent redistribution of Opsin-Dend2 for several days, and no retrograde trafficking occurred at the stages later than 21-22 dpf. Retrograde trafficking occurred only when OSs maintained their cone shape during development, and did not occur after the OS acquired a rod shape in matured animals.

It was previously suggested that fluorescently tagged opsin is expressed in a time-dependent fashion, which leads to variation of protein expression levels on a daily or hourly basis (Moritz et al., 2001). More recently, light dependent changes in the disk morphogenesis rate

was suggested to cause such variation as well (Haeri et al., 2013). Regardless of the mechanisms involved, such variation led to a dramatic increase and decrease of Opsin-Dend2 concentrations along the axis in 21-22 dpf animals (Fig. 6B). However, such variation of Opsin-Dend2 concentrations was less pronounced in younger animals at the age of 7 dpf (Fig. 6B). To quantitatively compare these variations, we calculated the standard deviation of normalized Opsin-Dend2 fluorescence concentrations within a narrow region (1.28 μm) at the center of the OS (Fig. 6B, light green). The standard deviation of 21-22 dpf tadpoles is more than 1.5 fold higher than that of 7 dpf tadpoles (Fig. 6C, $n = 20$ cells both for 21-22 dpf and 7 dpf, $P < 0.001$ by the Mann Whitney Rank Sum Test), supporting the larger variation of Opsin-Dend2 concentration along the axis in older animals. Since retrograde trafficking would lead to averaging of concentration along the axis of the OS, observed high variation in the old animals, and less pronounced variation in the young animals are consistent with the absence and presence of retrograde trafficking at the respective ages of the animals.

Discussion

During the early stages of rod OS morphogenesis, opsin can be retrogradely trafficked from the older to the newer disk membranes. Retrograde trafficking was observed only at the early stages (6-9 dpf) of rod OS morphogenesis, when rod OSs were still cone shaped. Similar to mammalian cones (Anderson et al., 1978), those cone shaped OSs are taking closed disk membrane configurations as evidenced by coverage of the structure by CNGA1 positive plasma membrane and previous electron microscopy studies (Kinney and Fisher, 1978). During the early stages of development, retrograde trafficking would support the higher requirement of disk membrane contents toward the basal region of the OSs. With no such requirement in more mature rods at later stages (after 9 dpf), retrograde trafficking was no longer observed, and the OSs became rod shaped.

The retrograde trafficking mechanism appears to be cargo selective because another disk membrane protein, peripherin/rds, was not retrogradely trafficked. This selectivity is consistent with opsin and peripherin/rds having distinct trafficking signals and pathways (Tam et al., 2000; Tam et al., 2004; Tian et al., 2014). Such specific trafficking signals would be potentially required for recognition by retrograde trafficking machinery. While our study proves the existence of opsin trafficking from disk to disk, the mechanism of such trafficking is unclear. Since disk membranes are taking closed configurations, opsin cannot be trafficked by diffusion. Thus, vesicle budding and trafficking is a potential mechanism of carrying opsin. Intraciliary trafficking machineries are dependent on two major classes of motors, kinesin and dynein. We found that the axonemal microtubule spans the entire length of the OS, and thus may coordinate the microtubule motor mediated trafficking. However, this observation does not exclude the possible involvement of other OS microtubules which are located proximal to disk incisures and span the entire length of the OS (Eckmiller, 2000). In general, kinesin mediates the anterograde trafficking, whereas, dynein mediates the retrograde trafficking. Opsin can bind to the dynein motor through TcTex-1. Therefore dynein is one of the candidates for the retrograde trafficking of opsin (Tai et al., 1999). The importance of dynein in the maintenance and development of the OS was previously demonstrated (Krock et al., 2009; Insinna et al., 2010). Especially, dynein 2 is essential for

retrograde trafficking and IFT protein recycling. Morpholino knockdown of Dynein2, during the developmental processes, leads to morphological changes of the OS structures (Krock et al., 2009). Those previous studies are consistent with our observation that retrograde trafficking mediates the shift of disk membrane constituents toward the base of the OSs, thus contributing to the morphological reorganization of the OS.

The evidence for retrograde trafficking was obtained from experiments using a photoconversion technique. This photoconversion technique is suitable for monitoring the distribution of newly synthesized and old photoconverted proteins for several days. Old Opsin-Dend2, which existed in old disk membranes at the time of photoconversion, was retrogradely trafficked to the new disk membranes that did not exist at the time of photoconversion. In addition to the retrograde trafficking hypothesis, we sought an alternate interpretation of our data about the distribution of Opsin-Dend2, since a recent study by Haeri et al. suggested that the rate of disk assembly is regulated by light (Haeri et al., 2013). Such light regulation led to axial variation in the OS fluorescence intensity. In our study, however, *Xenopus* tadpoles were reared in darkness except when they received light for photoconversion of Opsin-Dend2. Despite this exposure to photoconversion light, we suspect that such light regulation would not affect the distribution of old and photoconverted Opsin-Dend2, since those disk membranes containing photoconverted Opsin-Dend2 were already synthesized at the time of completing photoconversion. It is also important to note that we observed a time-dependent shift in the distribution of old photoconverted opsin-Dend2. Gradually, old photoconverted Opsin-Dend2 shifted toward the basal portion of the OS. Such a time-dependent change in old protein is not explainable by changes in the rate of new disk membrane synthesis, which may cause variability in the distribution of newly synthesized opsin-Dend2, but not old preexisting Opsin-Dend2. We also observed the shift in the distribution of new protein that was synthesized around the time of photoconversion. Such a time-dependent shift is also not consistent with changes in disk membrane morphogenesis rates. Collectively, our observations based on the photoconversion technique are consistent with Opsin-Dend2 retrogradely trafficked from older to newer disk membranes.

In summary, we provided evidence that opsin is retrogradely trafficked toward the basal portion of the OS in early developing rod photoreceptors. At the stage we observed the retrograde trafficking, it appears that rod OSs are yet to be phagocytized. Previous study suggests that phagocytosis starts at stage 46 (Kinney and Fisher, 1978; Hollyfield and Rayborn, 1979), which is equivalent to 9-10 dpf in our study. Therefore, our observation made for 6-8 dpf would be relevant to the developmental process of the OS structure. After completing development, the tip of the OS becomes phagocytized. Because retrograde trafficking is relatively slow, older proteins are still distributed toward the tip of the OS and amenable for disposal by disk shedding. Thus the renewal of opsin molecules at the base of the OS is still possible during the critical period of development. It would be intriguing to test if similar retrograde trafficking occurs in cone photoreceptor OSs. In cone photoreceptor OSs, continuous remodeling of OS structure would be necessary to form a cone shape from a trapezoidal structure immediately after phagocytosis (Eckmiller, 1987). While disks in lower vertebrate cones take open configurations, the majority of disks in mammalian cones

are considered to take closed configurations (Anderson et al., 1978) similar to disks of developing *Xenopus* rods. Since cones and rods share an evolutionary origin, it would not be surprising if cones and rods utilize the same retrograde trafficking mechanism to either develop or maintain cone shaped OSs. Based on the molecular evolution of opsins, cones are predicted to be ancestral to rods (Imamoto and Shichida, 2014). The loss of retrograde opsin trafficking is likely a major contributing factor for transformation from cone to rod shape, as demonstrated for developing *Xenopus* rod photoreceptors in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Rod photoreceptor cells are cone shaped at the early stages of development. (A) Retinas of tadpoles expressing Opsin-Dend2 or P/rds-Dend2 in rod photoreceptors were imaged for Dend2 fluorescence at 6/7, 9 and 21-22 dpf. Rod photoreceptors are cone shaped at the early stages of development and become rod shaped as tadpoles age. (B) Eyes of tadpoles expressing Opsin-Dend2 (green) were fixed at 6 dpf and stained with Wheat Germ Agglutinin (WGA, blue) and an antibody against *Xenopus* peripherin/rds (P/rds, red). Peripherin/rds localizes to rod disk rim (arrowhead) and cone disk rim (arrow) regions that are structurally distinct. Rod and cone photoreceptor cells were indicated in the figure. Opsin-Dend2 is specifically expressed in rod cells. Scale bar, 10 μ m.

Figure 2.

Two models for formation of cone shaped OSs. Dend2 protein is shifted from green to red fluorescence by photoconversion. After photoconversion, newly synthesized Dend2 protein is green and is added to the base of the OS in both the models. (A) In model A, the cone shape is formed by the retrograde trafficking of protein from older disks to newer disks. Shortly after photoconversion, newly synthesized green Opsin-Dend2 is added to the evaginations formed before the photoconversion to form new disks, therefore, a few disks contain both green (new) and red (older) proteins generating yellow fluorescence (red + green = yellow). Due to the retrograde trafficking of opsin-Dend2, the number of disks with both old and new protein increases over time after photoconversion. (B) The cone shape is formed by the addition of gradually larger disks at the base of the OS. There is no trafficking between disks as indicated by a clear separation of proteins before and after photoconversion.

Figure 3.

The renewal of rod photoreceptor OSs. Tadpoles expressing Opsin-Dend2 were photoconverted at 6 dpf. Retinas were dissected and imaged for green and red fluorescence 0 h (immediately after photoconversion) (A), 4 h (B), 8h (C), 1 d (D), and 2 d (E) after photoconversion. (F) Tadpoles expressing P/rds-Dend2 were photoconverted at 6 dpf and the retinas were dissected and imaged for green and red fluorescence 2 d later. (G) and (H) Tadpoles expressing Opsin-Dend2 were photoconverted at 21-22 dpf and the retinas were dissected and imaged for green and red fluorescence 2 d (G) and 6 d (H) later, respectively. The images in the mid row are for red-Dend2 fluorescence, and represented by multiple colors. The intensity-color relationship is as shown in the middle panel of (A). In the bottom row of each image panel, the intensity of green and red fluorescence was measured in ImageJ and the percentage of Dend2 fusion protein along the longitudinal axis of the OS was calculated by normalizing to the highest concentration (100%) on the axis. Scale bar, 10 μm.

Figure 4.

The retrograde trafficking of opsin in rod photoreceptors during early development (6 – 8 dpf). (A) A diagram to define the terms used for the analysis. The sample diagram is about the normalized intensities of old and new Opsin-Dend2 (2 d after photoconversion at 6 dpf) along the longitudinal axis of the OS. Crosspoint represents the point where disks with intensity profiles of old and new Opsin-Dend2 intersect. D_R represents the distance between the crosspoint and the point where the level of old red Opsin-Dend2 became lower than 2%, in the direction towards where the new disks are being added. W_G represents the width of the newly synthesized disks measured by the distance from the crosspoint to the point where the level of new green Opsin-Dend2 became lower than 2%. D_G represents the distance between the crosspoint and the first appreciable peak of green. (B) Shift of D_R and W_G over time after photoconversion. The distance of old Opsin-Dend2 trafficking back into new disks increased with the growth of new disks. Note: While images were captured starting from 0 h, neither D_R nor W_G are definable at 0 h and hence not included in this graph. (C)

Red Opsin-Dend2 concentrations in the disks from the crosspoint to the base of the OS (formed after photoconversion) were normalized to the concentration at the crosspoint. Left; the intensity-distance relationships were fitted to single exponential function, and plotted on a liner graph for each time point after photoconversion. Right; the intensity-distance relationships were fitted to single exponential function and plotted on a semilog graph. The concentration of red Opsin-Dend2 in new disks at the same distance (e.g. 1 μm) from the crosspoint increased with the increase in time after photoconversion. The data points from 0 – 2 μm were used for these analyses. The data points are represented by mean $\pm$ SE. (D) D_G was averaged at different times after photoconversion. D_G increased over time, suggestive of retrograde trafficking of green Opsin-Dend2 at the red-green interface. (E) The slope of green Opsin-Dend2, around the crosspoint, was calculated at different time points after photoconversion. The slope decreased and the gradient of green Opsin-Dend2 became shallower over time. These time dependent changes indicate the retrograde trafficking of Opsin-Dend2 at the red-green interface. In B, D and E, box plots are shown with each interquartile range enclosed by colored boxes and median values indicated with white lines. The ends of whickers represent the 5th and 95th percentiles. Outliers were omitted for clarity. *** indicates $p < 0.001$ by the Mann Whitney Rank Sum Test. ns = not significant. . For 0 h, 4 h, 8 h and 1 d after photoconversion, n=20 rods from 3 tadpoles were analyzed. For 2 d after photoconversion, n=30 rods from 5 tadpoles were analyzed.

Figure 5.

Organization of membrane and cytoskeletal structures in OSs of developing rod photoreceptors. (A) Unfixed rod photoreceptor expressing CNGA1-Dend2, a plasma membrane marker, was imaged for Dend2 fluorescence at 6 dpf. The OS is outlined by plasma membrane, indicative of disks taking closed configurations. (B) Fixed rod photoreceptors of non-transgenic tadpoles were probed with Wheat Germ Agglutinin (WGA, red) and an antibody against acetylated tubulin (green, axoneme marker). The axoneme extended the whole length of the OS when tadpoles were 6 dpf. Images are from an x-y single focal plane. Multiple photoreceptors were analyzed and the representative images are shown in (A) and (B). Scale bars, 5 μ m.

Figure 6.

Lack of retrograde opsin trafficking in mature rod OSs. (A) Retinas were dissected and imaged for green and red fluorescence 2 d or 6 d after photoconversion at 21-22 dpf, or without photoconversion at 21-22 dpf. The intensity of green and red fluorescence was measured in ImageJ and the percentage of Opsin-Dend2 protein along the longitudinal axis of the OS was calculated by normalizing to the intensities at the crosspoint (100%). For OSs 2 d and 6 d after photoconversion, old Opsin-Dend2 protein concentrations in the disks from the crosspoint toward the base of the OS were normalized to their concentrations at the crosspoint. Likewise for non-photoconverted OSs (0 d), the concentration distribution of Opsin-Dend2 around the bottom of the OS was normalized to the intensity closest to 50% of the maximum concentration along the OS axis. There were no significant differences among 0 d, 2 d and 6 d, in terms of the distribution of Opsin-Dend2. Thus, there was no retrograde trafficking in 21-22 dpf rod photoreceptors. For 0 and 2 d after photoconversion, $n=20$ rod photoreceptors from 3 tadpoles were used for analysis. For 6 d after photoconversion, $n=10$ rod photoreceptors from 3 tadpoles were used for analysis. The data points are represented by mean $\pm$ SE. (B) The fluorescence intensities of Opsin-Dend2 in the rod OSs from the tadpoles at 7 (blue) and 21-22 (magenta) dpf without photoconversion were normalized to the highest intensities along the axes, and plotted along the longitudinal axis of the OS. (C) The central regions (1.3 μm width) of OSs, as shown in B (light green), were used to calculate the standard deviations of normalized fluorescence intensities. Concentrations of Opsin-Dend2 along the longitudinal axis of rod photoreceptors were less variable in 7 dpf than in 21-22 dpf tadpoles. For both 7 and 21-22 dpf, $n=20$ rod photoreceptors from 3 tadpoles were used for the calculations. ***, $p < 0.001$ by the Mann Whitney Rank Sum Test. Error bars represent standard deviation in C.