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Role Of Developmental Protein, Nudc, In The Retina

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ABSTRACT

Rod photoreceptors are the most prominent light sensitive cell in the retina of most mammals. The outer segments of photoreceptors contain the cellular machinery necessary for phototransduction to occur. The process of generating the phototransduction components occurs in the inner segment before being trafficked to the outer segment. These processes are highly regulated and require many different trafficking proteins and chaperones. The developmental protein nuclear distribution gene C (nudC) was found to be present in the photoreceptor, but its function is yet to be determined. Here we confirm that nudC is present in the retina and necessary in the photoreceptors. We also explore its possible functions in the post-mitotic photoreceptor cells using iCre75-nudC knockout mice.

Keywords: Photoreceptor, outer segments, disk formation, nudC, outer segment trafficking

DEDICATION

This thesis is dedicated to my parents, David and Beth Boehm, whose passion for education and life have given me the desire and will to pursue this Master's Degree.

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TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iii

ACKNOWLEDGEMENTS iv

LIST OF FIGURES vi

INTRODUCTION 1

Overview of the eye 1

Photoreceptors 2

NudC 6

MATERIALS AND METHODS 9

RESULTS 13

DISCUSSION AND CONCLUSION 18

LIST OF REFERENCES 23

APPENDIX: IACUC APPROVAL FORMS 29

LIST OF FIGURES

Figure Page

1. Western blot for nudC in bovine, murine, and anuran retinal samples 13

2. ApopTag on nudC heterozygous and homozygous knockout retinal sections 15

3. hTERT RPE1 cells transfected with nudC-L280P and nudC-shRNA 16

4. Rhodopsin mislocalization in nudC homozygous and heterozygous knockout 17

INTRODUCTION

OVERVIEW OF THE EYE

Eyes are the source of our brains preeminent source of sensory information. Almost all animals have developed a light sensing mechanism (1). The human eye has a highly complex structure which allows for incoming light to be converted to a chemical signal that can be transmitted to the visual cortex in the brain which allows for the visual perception of objects in our field of view. In the front of the eye, the cornea and the lens focus incoming light onto the back of the eye where the retina, which is the light sensitive tissue layer in the eye, is able to transduce the light energy into a physiological response which is transmitted to the brain via the optic nerve (2).

The retina is composed of seven highly specialized layers: the nerve fiber layer (NFL), the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL), and the retinal pigmented epithelium (RPE). The NFL is made of the axons that originate in the ganglion cell bodies which make up the GCL. These axons will all culminate to form the optic nerve and leave the eye at the optic disc. The dendrites of the ganglion cells receive nerve impulses from the axons of bipolar cells in the IPL. The bipolar cell bodies occupy the INL. Their dendrites exist in the OPL where they synapse with the axons of the light sensing photoreceptor cells. The ONL is composed of the nuclei of photoreceptors. The light sensing portion of the photoreceptors extends towards the RPE which is a

monolayer of cells that function to support the photoreceptors (2). The area with the highest concentration of photoreceptors is the macula, with an even more highly concentrated region in the center of the macula called the fovea.

PHOTORECEPTORS

Photoreceptors are the retinal cell type that initiate the transduction of light into nerve impulses and begin the process of vision. There are two main photoreceptor types: rod cells and cone cells. Rod cells are highly sensitive to light and function in low levels of light, while cone cells function in bright and ambient light and are responsible for differentiating between colors. Although the retina is rod photoreceptor dominated, cones are more highly concentrated in the macula, and the fovea is entirely made up of cone photoreceptors (3). Photoreceptors are made up of 5 sections: the outer segment (OS), connecting cilium (CC), inner segment (IS), nuclear body, and the synaptic region. The OS are modified primary cilia which hold the components necessary for the conversion of light into electrical impulses which is known as phototransduction. The CC connects the outer segment to the IS and allows for proper protein trafficking between the two. The IS contains the mitochondria, necessary to meet the metabolic demands of the photoreceptors, and the components necessary for protein synthesis, such as the Golgi and endoplasmic reticulum. The nuclear body of course houses the nucleus, and the synaptic region synapses with the dendrites of the bipolar cells using synaptic ribbons which release glutamate (3, 4).

Phototransduction

Phototransduction is the conversion of light energy to an electrical signal which will propagate further physiological responses. The phototransduction cascade begins when light causes the isomerization of 11-cis retinal to all-trans retinal (3). The conformational change caused by this isomerization causes the activation of the G-coupled protein receptor, rhodopsin. Activated rhodopsin catalyzes an exchange of the guanosine diphosphate (GDP) bound to the α-subunit of G-protein transducin to guanosine triphosphate (GTP). This causes the α-subunit of transducin α-subunit to dissociate from its βγ subunits. This GTP-transducin α activates phosphodiesterase 6 (PDE6), which hydrolyzes cyclic guanosine monophosphate (cGMP). The reduction of cytosolic cGMP causes the closure of the cyclic nucleotide gated (CNG) channels located in the plasma membrane which remain open in high concentrations of cGMP. The closure of the CNG channels causes a decrease in intracellular Na^+ and Ca^{2+}, which causes hyperpolarization of the photoreceptor. This causes a release of glutamate at the ribbon synapse onto bipolar cells or secondary neurons such as amacrine cells and propagation of the light activation (5).

To return the photoreceptor to the dark state, a recovery process takes place. Rhodopsin kinase phosphorylates rhodopsin, which allows arrestin to bind to rhodopsin which inhibits it from further activating transducin (3). The GTP bound to transducin is hydrolyzed by GTPase and RGS-9, allowing the βγ transducin subunits to bind with the α-subunit (6). Once transducin is no longer in its active state, PDE6 becomes inactive. cGMP levels are returned to the dark level by guanylate cyclase (GC) which gets activated by the calcium level-sensitive guanylate cyclase activating protein (GCAP) (7).

When cGMP levels rise, the CGN channels reopen and the dark state is reassumed (3). All-trans retinal must then be converted back to 11-cis retinal which is accomplished by the visual cycle which involves multiple reactions in both the photoreceptors and the RPE (3).

Protein trafficking in the photoreceptor

Many proteins must be trafficked to the OS of photoreceptors to maintain its function in vision, including the light sensitive protein rhodopsin which is the most abundant protein in rod outer segments. This is accomplished through vesicular transport with the help of trafficking proteins that help to shepherd necessary components of phototransduction to the outer segment (8, 9). To transport rhodopsin to the OS, it must be synthesized in the IS, and then a rhodopsin-containing vesicle must fuse with the IS plasma membrane at the base of the CC in an area called the pericilliary ridge complex (10, 11). Small G-proteins have been found to be involved in the trafficking process (12, 13). Our research group has identified rab11a as a protein that binds to the C-terminal of rhodopsin and is involved in the canonical trafficking of rhodopsin to the OS (12). The regulation of the transfer of proteins from the IS through the CC to the OS occurs in an area of the CC called the transition zone which acts as a gate keeper for the proteins that can be trafficked to the OS (14). The trafficking of the phototransduction components is highly regulated and mutations in many of the regulatory proteins in the CC or those involved in transport to the CC can result in blindness (15).

Outer segment disk formation

The OS of vertebrate rod photoreceptors are modified primary cilia which contain membranous disks that contain the necessary components for phototransduction. The distal tips of the outer segments of photoreceptors are phagocytized by the retinal pigmented epithelium (RPE) which allows for the removal of portions of the OS that contain photo-damaged molecules and photo-oxidative radicals (16). However, this means that the OS must constantly be renewed (17, 18). In order to achieve this, the components to form the new OS disks must be trafficked from the IS of the photoreceptors where protein synthesis occurs, to the CC at the proximal base of the OS where they will be incorporated into the plasma membrane (8, 9, 19). The disk membranes are formed by the evagination of the plasma membrane, followed by a fusion with the plasma membrane to form an enclosed disk (20). The entire renewal of OS disk membranes takes 10 days in mice, rats, and *Xenopus laevis* (21).

Retinal degeneration as a result of mutations in photoreceptor trafficking

As a result of mistrafficking, retinal degeneration can occur, beginning with retinal remodeling. Retinal degeneration generally occurs in three phases (22, 23). The first phase initiates as a molecular response from the photoreceptors, RPE, or Mueller cells to an insult such as buildup of mislocalized protein. This causes a cascade of events that result in cell stress and early cell death. The second phase exhibits the phagocytic ablation of the photoreceptor layer as well as death of Mueller cells. In the third phase, large scale dysregulation of the retinal layers can be seen, as bipolar cells and amacrine cells may be observed migrating into the ganglion cell layer, and likewise ganglion cells

can be observed in the inner nuclear layer. As phase three progresses, neurons may migrate through breaks in Bruch's membrane and leave the neural retina (24).

Mislocalization of proteins is a major cause of cellular stress which could result in retinal remodeling and degeneration. Mutations causing the loss of the ciliary targeting domain on the C-terminal of rhodopsin (a VXPX motif), as exhibited by the Q344Ter mutation, results in mislocalization of rhodopsin to the IS, ONL, and even to the synaptic region (25-27). This occurs as a result of a loss of association between rhodopsin and the trafficking proteins (28). Another example of this is the Ter349Glu mutation, which causes the VXPX motif to be occluded (29, 30). Retinitis pigmentosa GTPase regulator (GPGR) is a protein in the transition zone that plays a role in trafficking by interacting with trafficking proteins (31). Mutations in GPGR have been shown to be associated with early onset retinitis pigmentosa as well as Leber's congenital amaurosis (32, 33). Centrosomal protein 90 (CEP290) is involved in microtubule-based protein trafficking, and mutations in CEP290 have been associated with a variety of blinding diseases.

NUCLEAR DISTRIBUTION PROTEIN C

Nuclear distribution protein C (nudC) was first identified in the fungus *Aspergillus nidulans* as a regulator of nuclear migration involved in the dynein/dynactin complex. In healthy *A. nidulans*, nuclei replicate within a single cell and then translocate along the growing hyphae and establish new cells. When temperature sensitive nudC mutation was expressed at permissive temperatures, the replicated nuclei were clustered centrally and unable to translocate resulting in a single cell (34, 35). The larger family of Nud genes in *A. nidulans* are recognized as coding for components of dynein, including

the light, intermediate and heavy chains, as well as dynactin complexes The structure of nudC is highly conserved in all eukaryotes, both structurally and functionally, though the N-terminal region is expanded in. In *A. nidulans*, nudC is 198 amino acids in length, while in humans it is 331 amino acids in length (36). NudC has been shown to be a co-chaperone of hsp90 for lis1, which is involved in classical lissencephaly (37). The presence of a CS (CHORD-containing proteins and SGT1) domain in nudC protein structure suggests similarities in function to small heat shock proteins like p23 and hsp20 which act as co-chaperones with hsp90. NudC also showed independent chaperone activity such as suppressing the aggregation of luciferase and citrate synthase in vitro in a chaperone activity assay (36). NudC has been associated with microtubule organization with research groups finding it to be involved in mitosis and cytokinesis (38, 39). Zhang and colleagues also report that nudC is involved in ciliogenesis via regulation of actin dynamincs by operating as a chaperone for cofilin1 (40). Overall, the common feature in these various functions appears to be the stabilization of microtubule/dynein/dynactin complexes.

NudC interaction with rab11a in the retina

NudC has been discovered in the photoreceptors by proteomic analysis, but its function has not been elucidated (41, 42). Our research group was studying the function of the protein rab11a in the rod photoreceptors and performed an immunoprecipitation to see if rhodopsin bound to rab11a. When that was performed, nudC was also found to be pulled down by rab11a (Boitet et al, publication under review). To confirm this interaction, an immunoprecipitation of the reverse order was performed, using a nudC antibody as bait. This experiment revealed that nudC was also capable of pulling down

rab11a and rhodopsin. This suggests that nudC interacts in a complex with rab11a/rhodopsin. To further examine this, transgenic *Xenopus laevis* tadpoles expressing a mutant version of nudC, nudC^{L280P}, and an shRNA targeting nudC were created. Examination of retinal sections from these transgenic tadpoles showed rhodopsin mislocalization and malformed OS disks. These results support the idea that nudC functions as a chaperone for the rab11a/rhodopsin complex. However, given the microtubule associated roles ascribed to nudC by previous research groups and the presence of the f-actin rich area at the base of photoreceptor OS (43), the question must be asked whether there are multiple functions of nudC in the photoreceptor. It is possible that it is a chaperone or co-chaperone for an array of proteins with diverse functions within the retina. In this thesis, the possible roles of nudC in the retina will be explored and discussed.

MATERIALS AND METHODS

Research animals

All animal studies were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Embryonic stem cells used to generate nudC conditional knockout mice were acquired in collaboration with Wolfgang Baehr, University of Utah, from the International Mouse Phenotyping Consortium (IMPC). Once the conditional nudC knockout mouse line was established, mice with a floxed nudC gene were crossed with the iCre75 transgenic mouse line (20), in which iCre expression is driven by the rhodopsin promoter, to generate a mouse that no longer expresses nudC in rod cells after the expression of rhodopsin begins at postnatal day 7. Breeding pairs were setup to ensure only one copy of the iCre75 gene was present in mice tested in order to avoid Cre toxicity.

Cryosectioning of mouse eyes

Mice were anesthetized by isoflourane before being sacrificed by cervical dislocation. Eyes were enucleated and fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS, pH 7.4) overnight at 4°C. Samples were cryoprotected by submersion in 30% sucrose for overnight at 4°C. Eyes were placed in optimal cutting temperature (OCT) medium and flash frozen in isopentane on dry ice. Samples frozen in

OCT medium were allowed to equilibrate to -18°C before being sectioned at 12 μm intervals using a cryomicrotome. Slides were rinsed to remove OCT residue, dried overnight, and stored at -20°C.

Immunohistochemistry

Sectioned eyes were treated with ApopTag Red In Situ Apoptosis Detection kit (cat# S7165, Burlington, MA, USA) as per the manufacturer's instructions. Following treatment with the kit, samples were also stained with wheat germ agglutinin conjugated to Alex Fluor 555 (WGA, 1:650 dilution) for detection of outer segments and 4′, 6-diamidino-2-phenylindole (DAPI) for detection of nuclei. For experiments regarding nudC's effect on rhodopsin mislocalization, retinal sections were stained with anti-rhodopsin antibody (B630N) along with an antibody against nudC and DAPI. Images of stained retinal sections were acquired on an Olympus IX81spinning disk confocal microscope with 60X oil-immersion objective. ImageJ software was used to compile maximum projection z- stacks and process acquired images.

Cell culture and transfection

hTERT RPE1 cells from American Type Culture Collection (ATCC) (Manassas, VA, USA) were maintained in complete DMEM F12 (10% fetal bovine serum (FBS), 1% penicillin/streptomycin) at 37°C at 5% CO_2. Once cells reached 70% confluency cells were electroporated with 40 μg of plasmid DNA in electroporation buffer (135 mM KCl, 2 mM MgCl 2 , 20 mM HEPES pH 7.4). Transfected plasmids used in these experiments contained mCherry-nudC (for overexpression of nudC), mCherry-shRNA targeting nudC, or mCherry-nudC L280P. Electroporation was carried out using an exponential decay

protocol with a 4mm cuvette; 150V, 3000μF capacitance, resistance was set at infinite. Cells were then plated in 6-well plates with a glass cover slip at the bottom in complete DMEM F12 and incubated for 24 hours. In order to ciliate the cells, serum-free DMEM F12 was added to the cells for at least 24 hours.

Immunocytochemistry

Media was aspirated and cells were washed with PBS before being fixed with 4% PFA in PBS for 15 minutes. Cells were incubated in blocking buffer of 10% BSA with 0.2% Triton X-100 for 30 minutes at room temperature. Cells were stained with polyclonal rabbit antibody against rab11a from Life Technologies (Carlsbad, CA, USA) (1:500) and mouse monoclonal anti-arl13b from Abcam (Cambridge, MA, USA) (1:750) followed by goat anti-rabbit 488 (1:500) and goat anti-mouse 568 (1:500). Images of stained cells were acquired on an Olympus IX81 spinning disk confocal microscope. Image J software was used to compile maximum projection z-stacks and measure cilia length.

Western Blotting

All bovine, mouse, and frog tissue samples were prepared by homogenization in Eppendorf tube with plastic homogenizer in PBS with a protease inhibitor cocktail. Samples were vortexed then centrifuged at max speed in table top centrifuge for 5 minutes. A sodium dodecyl sulfate (SDS) loading buffer with β-mercaptoethanol was added to samples before they were boiled and spun down. Samples were loaded into a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Samples were stacked at 80V for 18 min then resolved at 145V for 1 hour and 45 minutes in a gel electrophoresis chamber. Gel was transferred to a nitrocellulose membrane at 270 mA for 75 minutes at

4°C. Membrane was blocked in 3% milk for 1 hour at room temperature. Membrane washes were performed in TBST. The blot was then probed with a monoclonal antibody against nudC produced in rabbit from Abcam (Cambridge, MA, USA) in 3% milk (1:1000). The secondary antibody used was goat anti-mouse HRP (1:10,000) from Life Technologies (Carlsbad, CA, USA). Blot was developed exposure to ECLs for 1 minute followed by exposure to x-ray film for 2 minutes and 30 seconds. An anti-GAPDH antibody from Abcam (Cambridge, MA, USA) was used as a loading control.

Xenopus laevis transgenesis

Transgenic *X. laevis* tadpoles were generated as described in Sparrow et al., 2006. The Xenopus rod opsin expression vector, or pXOPO.8, a gift from Orson Mortiz (University of British Columbia, BC, Canada), was modified to insert I-SceI recognition sites near the PciI and AflII sites of the vector to generate pXOPO.8/I2. The vectors pCDNA3.1 mC-nudC and pCDNA3.1 mC-nudCL280P were created by amplifying nudC from mouse retinal RNA and introducing the L280P mutation by site-directed mutagenesis. The mC-nudC and mC-nudCL280P fusions were then digested with NheI/NotIand ligated into the NheI/NotI sites of pN1/I2 or the unmethylated XbaI/NotI sites of pXOP0.8/I2.For transgenesis, DNA was mixed with *X. laevis* sperm and injected together into eggs.

RESULTS

Detection of nudC in bovine, murine, and anuran retinal samples

Our research group found nudC to be associated with the rab11a:rhodopsin trafficking complex, but its function in the photoreceptors is unclear as of yet (Boitet et al, publication under review).To further confirm nudC's presence in the retina, a Western blot probed with an anti-nudC antibody was performed with retinal samples from three different animal types: bovine, murine, and anuran. All three retinal samples showed bands present at ~39 kDa, which is the molecular weight of nudC (Fig. 1). This result, in context with other reports (17, 18) further confirms that there is nudC present in the retina of all three animals. Although this result does not confirm the specific layer of the retina in which nudC is present, our research group has identified it in the photoreceptors by immunohistochemical analysis (Boitet et al, publication under review).

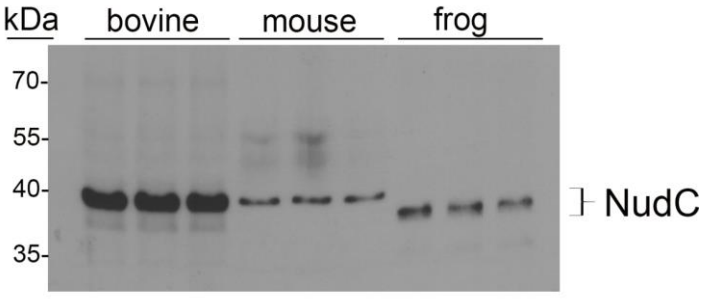

Figure 1. Western blot for nudC in bovine, murine, and *X. laevis* retinal samples. Triplicates of each sample were loaded into a 12% polyacrylamide gel. After samples were run and the blot was stained with an anti-nudC antibody, a band was detected in all three samples at ~39 kDa, the molecular weight of nudC.

Detection of cell death via apoptosis in mice with retinal nudC knockout

To uncover whether or not nudC was necessary for the survival of photoreceptors, an ApopTag Red In Situ Apoptosis Detection kit was used to detect apoptosis occurring in retinal sections of heterozygous and homozygous iCre75-nudC knockout mice as well as the iCre75 mouse as a negative control. In the iCre75-nudC knockout mouse line, nudC is not expressed in in cells expressing rhodopsin, which in mouse rod cells begins at postnatal day 7 (20). This allowed for canonical embryonic development of the retina, in which nudC may have a vital role, but also allowed for the role of nudC in the developed retina to be evaluated. The ApopTag apoptosis detection kit shows positive staining in cells that are undergoing apoptosis and have free 3'OH DNA ends in a fashion similar to Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining (45). The iCre75 mouse retina showed no positive ApopTag staining (Fig. 2A), while the heterozygous nudC knockout showed positive ApopTag staining (Fig. 2B). The homozygous nudC knockout showed even further positive ApopTag staining, which indicated the increase in the presence of apoptotic cells (Fig. 2C). Staining is present in high amounts in the ONL as well as the INL. This indicates a high amount of cell death in both retinal layers.

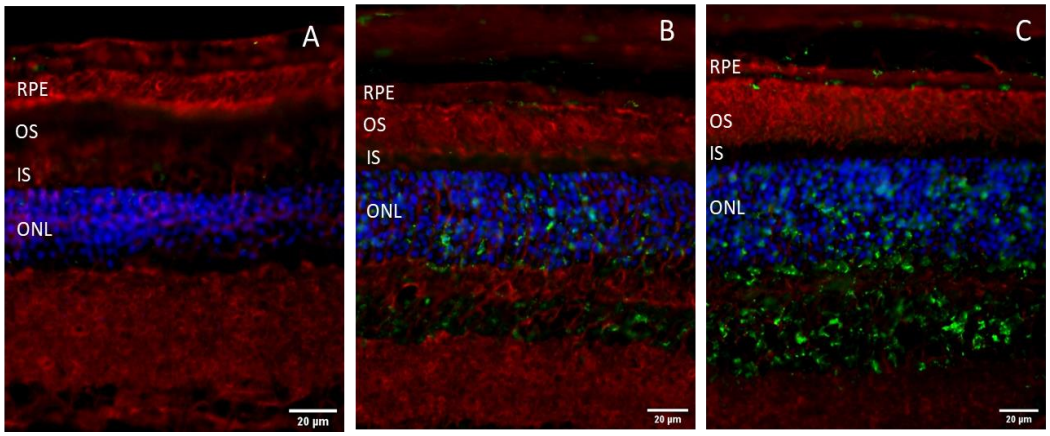

Figure 2. ApopTag on nudC heterozygous and homozygous knockout retinal sections. Retinal sections from iCre75-nudC mice were stained using the ApopTag Red In Situ Apoptosis Detection kit. Positive ApopTag staining (shown in green) is absent from the iCre75 mouse but can be seen in the ONL of both the heterozygous (B) and homozygous (C) nudC knockouts. Positive ApopTag staining: green, WGA (outersegments): red, DAPI: blue. Images are all 40x.

Evaluation of nudC's effect on cilia growth in hTERT RPE1 cell line

After confirming nudC's presence in the retina and its necessity for retinal health, we sought to evaluate the role of a protein thought to be involved in cell division in retinal tissue which is mainly post-mitotic. The Zhou research group has reported that nudC may have a role in regulating ciliogenesis due to its association with cofilin1, a protein which regulates actin dynamics involved in the growth of cilia (14). To evaluate its role in ciliogenesis, we transfected the hTERT-RPE1 cells with plasmids containing a nudC-shRNA, a mutant form of nudC, nudC-L280P, as well overexpressing nudC and measured the cilia growth in response to serum starving (Fig. 3). However, we did not find a significant increase in the cilia length of cells transfected with nudC-shRNA when compared with the cilia of untransfected cells, with the average cilia length of nudC-

shRNA transfected cells being 3.122 µm and the untransfected cilia being 3.023 µm (n of untransfected cells=20, n of transfected cells=4, p= 0.822). An increase in cilia length was not observed in the cells transfected with the mutant nudC-L280P plasmid compared to untransfected cells, nor did we see a decrease in cilia length when nudC was overexpressed (data not shown).

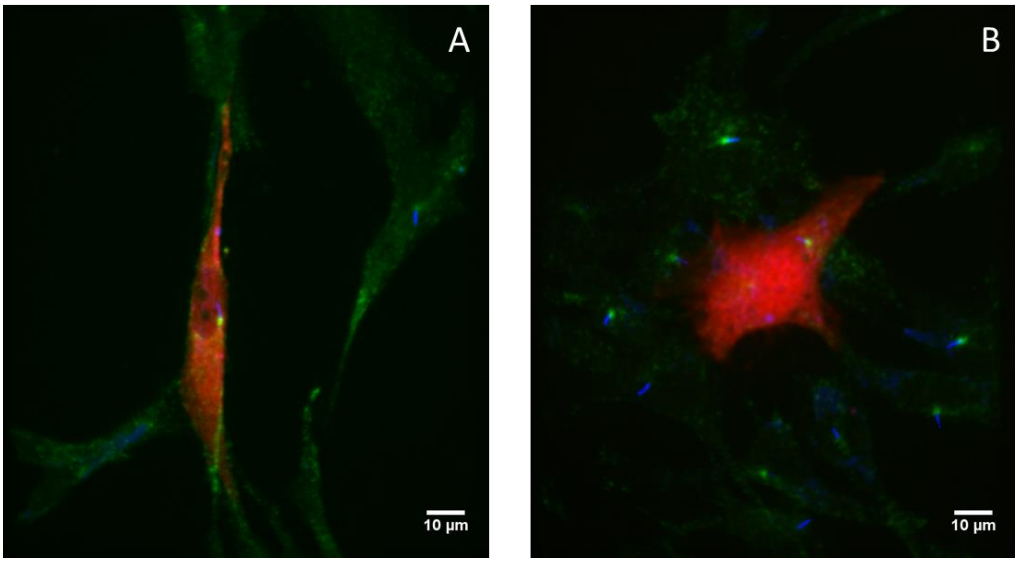

Figure 3. hTERT RPE1 cells transfected with nudC-L280P and nudC-shRNA. No significant change in cilia length was observed between untransfected cells and cells transfected with plasmids containing genes for nudC-L280P (left) or nudC-shRNA (right). Plasmids were tagged with mCherry. Transfected cells: red, arl13b (cilia): blue, rab11a: green. Images are 60x.

Rhodopsin mislocalization in nudC-shRNA knockdown

In an attempt to understand the role nudC has in maintaining photoreceptor homeostasis, rhodopsin was stained for in the retinal sections of *X. laevis* tadpoles expressing an shRNA targeting nudC on the rhodopsin promoter and compared with wild type. The

retinal sections of the wild type control tadpole shows typical staining for rhodopsin in the outer segments of photoreceptors which can be visualized in Figure 4A. However, in the retinal sections of tadpoles expressing the nudC-shRNA, an abnormal band of positive staining can consistently be seen at the base of the OS as well as being highly present in inner segment (Fig. 4B). This abnormality suggests a possible role for nudC in the localization of rhodopsin.

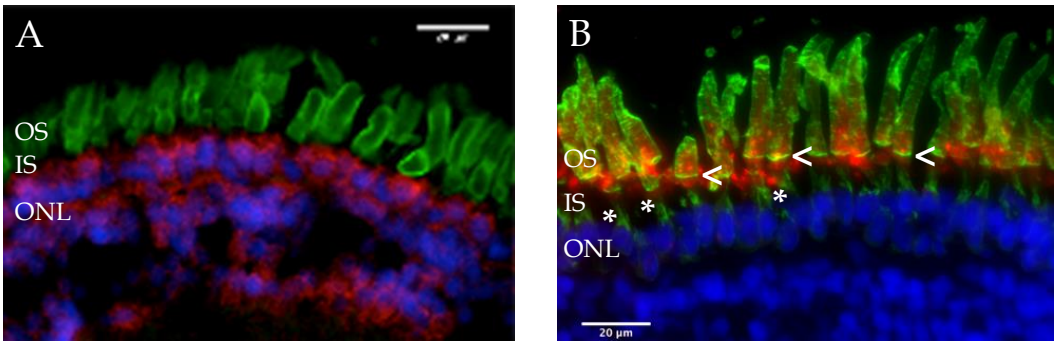

Figure 4. Rhodopsin mislocalization in nudC homozygous and heterozygous knockout. The retinal sections of the 2 week post-fertilization wild type *X. laevis* tadpoles show positive IHC staining for rhodopsin (green) throughout the OS and is also stained for nudC (red) (A). In the retinal sections of the *X. laevis* tadpoles expressing the nudC-shRNA (shRNA construct expression shown in red), the bottom of the OS shows a band of atypical rhodopsin staining (arrows) as well as rhodopsin present in the IS (asterisks) (B). In 4A NudC staining: red, in 4B nudC-shRNA construct expressing m-Cherry: red, rhodopsin: green, DAPI: blue. Scale bars = 20 µm.

DISCUSSION AND CONCLUSION

NudC was first identified in the retina in an attempt to identify the entire proteome of the photoreceptor cilium complex (17). Afterwards, by our research group, it was found as part of a complex with rab11a and rhodopsin in an immunoprecipitation assay (publication in review). The retina is a highly metabolic tissue with many signaling events and constant protein trafficking. It is also comprised mainly of post-mitotic cells. While nudC has many diverse functions, it was long thought to mainly be involved in cellular development and division. Therefore its presence in the developed retina could suggest an alternative area of function for the protein.

First, to further confirm nudC's presence in the retina, a Western blot was performed for nudC on samples from three different animal types, bovine, murine, and amphibian. As expected, all three species showed positive binding of the anti-nudC antibody at approximately 39 kDa, which is the molecular weight of nudC. Thus confirming that nudC is present in the retina as well as being conserved among multiple species from different evolutionary lineages. Because the Western blot was performed on retinal samples and not isolated photoreceptor or outer segment samples, the specific location of nudC in the retina is not confirmed by this result, however, our research group has shown via immunohistochemistry that nudC is present in photoreceptors (Boitet et al, publication under review).

We next tested whether nudC was necessary for the retina to maintain viability using an ApopTag Red In Situ Apoptosis Detection kit to detect apoptosis in retinal sections of iCre75-nudC knockout mice that were both heterozygous and homozygous for the nudC knockout. The kit detects nicked DNA, similarly to Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining. After performing the test, it was revealed that the retina was highly apoptotic in both the heterozygous and homozygous nudC knockout mice compared to the iCre75 control mouse. This suggests that the presence of nudC is necessary for the mouse retina to maintain homeostasis and viability. Indeed, the homozygous mouse did show further increased apoptosis than the heterozygote which indicates that one copy of the nudC gene is insufficient for the retina to retain viability.

The Zhou research group found that nudC was involved in the regulation of cilia growth via stabilization of cofilin1 which regulates actin dynamics (14). We considered the possibility that nudC's function in the retina was to regulate the growth of outer segments which are modified primary cilia. Before studying this *in vivo*, we first wanted to test whether we could reproduce the results of the paper by knocking down nudC, expressing a mutant version of nudC, and overexpressing nudC to evaluate the effect of changing nudC's expression levels on the ciliogenesis of hTERT-RPE1 cells. Surprisingly, we did not see a significant change in the cilia length of transfected cells. This could potentially be because of a low number of transfected cells because there was a very slight difference in cilia length between the cells expressing a shRNA targeting nudC and untransfected cells, so possibly with a higher number of sample cells, that difference may have become significant.

Continuing to probe for the possible function of nudC in the retina, we next stained for rhodopsin in the retinas of 2 week post-fertilization *X. laevis* tadpoles expressing an shRNA targeting nudC and compared the staining to that of wild type tadpoles. We found that in the tadpoles expressing the nudC-shRNA, there were abnormal bands of positive rhodopsin highly concentrated at the base of the OS which was not present in the wild type tadpole OS. A possible cause of a rhodopsin mislocalization is that nudC is somehow involved in the regulation or maintenance of the rab11a:rhodopsin complex which is necessary for the correct localization of rhodopsin to the OS (12). Another possible reason, however is that we are picking up the rhodopsin present in the over grown OS discs that we have seen in transmission electron microscopy images of tadpoles that have nudC knocked down (Boitet et al, publication under review).

The malformed OS disks in tadpoles with an shRNA nudC knockdown are thought to be the result of the lack of nudC to stabilize cofilin-1 which regulates the actin dynamics of new OS disk growth (40, Boitet et al, manuscript being written). We believe that cofilin -1 is involved in helping to maintain a branched actin structure in the OS disks, and without cofilin-1 to depolymerize actin in the newly forming disks, long fibrous actin forms causing the overgrown disks rather than the correctly sized disks.

In addition to the malformed OS disks in the tadpoles with a nudC-shRNA knockdown, we have also found that the iCre75-nudC knockout mice have almost no electroretinogram (ERG) response at six weeks of age and have a severely degenerated ONL (Boitet et al, manuscript being written). These results support both the findings that the ONL is undergoing massive apoptosis and that there may be a problem with protein

trafficking as a result of the retinal knockout of nudC. Possibly, the role of nudC is to stabilize and prevent the ubiquitination of proteins with various functions in the retina, such as rab11a which complexes with and transports rhodopsin and cofilin-1 which we believe helps maintain proper disk formation.

Conclusions

Our data suggests that nudC is not only present in the retina, but that it also has functions which are crucial for maintaining cellular homeostasis. We have shown for the first time that the knockout of nudC results in rampant apoptosis, which may be due to a number of possible disruptions to normal cellular functions. This work expands the knowledge in the field of photoreceptor trafficking and photoreceptor homeostasis, as nudC plays a previously undiscovered role in both functions. The specific role that nudC plays in these areas requires further study, but our research has shown that there is a necessity for nudC in the developed retina.

NudC's specific role in the photoreceptor could be further tested by first examining other protein interactions that nudC may have by immunoprecipitation assays and/or proximity ligation assays on an array of proteins such as cofilin-1. Another area that needs to be examined is a temporal delineation of the degeneration of the retina in the iCre75-nudC knockout mice. It would be useful to see what morphological changes occur early that lead to the apoptosis of the retinal layers and eventual entire retinal degeneration in order to fully understand the function that nudC may have.

The importance of understanding the molecular mechanisms of canonical photoreceptor trafficking and maintenance cannot be overstated. Knowing the importance

of specific proteins and how they function is critical to developing new therapies for degenerative diseases. Not knowing the molecular events involved in maintaining homeostasis in our photoreceptors could have serious consequences if molecular therapies are developed that could potentially interfere with these molecular mechanisms.

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APPENDIX

IACUC APPROVAL

Protocol PI: Alecia K. Gross Gutierrez

Title: Rhodopsin Trafficking in Transgenic Tadpoles

Sponsor: UAB DEPARTMENT

Animal Project Number (APN): IACUC-21624

On 1/30/19, the UAB Institutional Animal Care and Use Committee (IACUC) approved the proposed modification: Personnel- Skyler Boehm . The sponsor for this project may require notification of modification(s) approved by the IACUC, but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary.