Spring 5-2017

Dynamic Protein Movement Involved in Photoreceptors

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A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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and Renée Crown University Honors
Spring 2017

Honors Capstone Project in Biotechnology

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Abstract

Ciliopathies are a class of genetic diseases which affect the function of primary cilium within multiple organs throughout the body. Although ciliopathies are known to present high morbidity and mortality rates, there are no current cures and only a limited amount of treatments. A universal function of cilia is to regulate cellular activities through the transduction of signals. Disruption of the localization of proteins following the reception of a stimuli can be responsible for the phenotypic effects of dysfunctioning cilia in ciliopathies. In order to address the pathogenesis, we must first understand how proteins move within the cilia, so that we can begin to postulate treatments for ciliopathies.

In vision, the light-sensing photoreceptors have a connecting cilium which is disrupted in retinal degeneration. As retinal degeneration is a phenotypic effect of ciliopathies, the inspection of how cilia function to transduce signals, would provide insight on the treatment of this class of diseases.

By utilizing transgenic rod photoreceptors from the model organism of Xenopus laevis, we were able to investigate properties of proteins potentially responsible for their translocation via the connecting cilium. Through the manipulation of characteristics generally responsible for cell localization such as charge, hydrophobicity, and size, we were able to determine patterns in the resulting localization of such protein probes. Analysis of these experiments in conjunction with current literature regarding the characteristics of the proteins involved in the phototransduction cascade allowed for a deeper understanding of the applicability to ciliopathy patients.

Specifically, my research involved relating the translocation of transducin, phosphodiesterase 6, recoverin, and rhodopsin kinase to the experimental evidence of translocation of genetically engineered protein probes within the rod photoreceptors of Xenopus laevis. It was apparent the presence of the hypothesized characteristics of lipidation, poly-charged regions, and size of the proteins had an effect on their localization via the connecting cilium, however, there appeared to be overwhelming evidence which indicated conformational changes in response to protein-protein interactions within the cascade altered such characteristics, and consequentially their localization.

This analysis and literature review provides evidence to suggest for the development of further experimental protein models, which in combination with past results can elucidate the specific influence these characteristics have. Given future experimental models which are able to account for specific conformational changes and protein-protein interactions, the relative roles of protein characteristics could quantitatively describe the role of connecting cilia. By understanding the mechanisms of the phototransduction cascade in regards to the ciliated rods, we may eventually be able to generate probes capable of specific localization for treatment of ciliopathies.

Acknowledgements

I have been immensely lucky to work within the Calvert Lab at Upstate Medical University. I would specifically like to thank Dr. Peter Calvert for his ongoing support throughout my two years as a research assistant. Initially my research advisor, and subsequently my capstone advisor, Dr. Calvert has enabled me to progress with my research further than I could have imagined. I am thankful I've had the opportunity to be involved in such substantial research, and am excited to see the future work that comes from the Calvert Lab. Dr. Calvert's encouragement and support gave me the confidence to conduct this literature review, and the independence to feel capable of accomplishing future projects.

Additionally, I would like to thank the members of the Calvert Lab: Nycole Maza, Dr. Rajib Pramanik, Cassandra Barnes, and Sungsu Lee. Throughout my time as a research assistant, each member of the Calvert Lab has at one point offered support or guidance. Not limited to this specific project, the cohesive environment within the Calvert Lab provided me with a wonderful support system, to which I could turn to with essentially anything. I could not be more proud to have been a part of a community as warm and inspiring as the Calvert Lab.

I would also like to thank Dr. Corey White for agreeing to fill the role as my capstone reader. Although I was only recently acquainted with professor White in my Neuroscience capstone class, he has provided an unwavering support throughout the my last two semesters and allowed me to maintain peace of mind and a relaxed approach to my future aspirations.

Producing a piece as daunting as this capstone was no easy task. By challenging myself to formulate a research piece on a topic as complicated as the phototransduction cascade took dedication, support, and a lot of trial and error. I am fortunate to have had to compose such a body of work because the process taught me lessons which I could not have learned in any other way.

Executive Summary

Ciliopathies are defined as disruptions to ciliary structure or function, which lead to multi-organ diseases. Ciliopathies can be categorized within a class of genetic diseases which specifically present malfunctions of non-motile, or primary cilium, and result in dysfunction in functioning, and thus cilia-mediated signaling. These diseases are understood to frequently impact vision, the renal system, and cortical functioning (Tobin, 2009). Research on the mechanisms of cilia function has enabled scientists to uncover potential applications from model organisms to human diseases.

When assessing the relevance of ciliopathies to retinal degeneration, the anatomy of photoreceptors, the light-sensing neurons of the retina, explain cilia's impact. Photoreceptor cells are sub-divided into inner segments and outer segments, linked by a connecting cilium. Known to develop from a primary cilium, photoreceptor outer segments are generally considered to be modified cilia; The outer segments therefore are responsible for the link between ciliopathies and retinal defects such as retinitis pigmentosa (Tobin, 2009).

Retinitis pigmentosa is a sensory deficit that is a common feature of many ciliopathies. This disease is progressive, and can result in complete blindness. Degeneration of the connecting cilium in rod cells results in the inability to transfer rhodopsin molecules from the inner segments. This translocation of rhodopsin occurs due to the distribution of organelles. The inner segments of rod cells contain all of the cell machinery capable of the production and modification of proteins, such as the ER, nucleus, and Golgi. In contrast, the outer segments consist of disc membranes imbedded with proteins necessary for phototransduction. In order for the pho-

toreceptors to distribute proteins to the outer segment, there must be translocation via the connecting cilium. Thus, deficits in the primary cilia of rod photoreceptors disable such cells from integrating photon stimuli to synaptic transmission to the visual neurons (Tobin, 2009).

Cilia and flagella have long been known to be of importance for cellular motility and organization, however immotile primary cilia, previously thought to be primarily useful as a cellular extension have been shown to be necessary in sensory processes and transducin information.

There are many characteristics of cilia that must be considered before analyzing how the presence of ciliated cells, characteristics of rod outer segment disk membranes, and qualities of phototransduction proteins work together to enable vision in specific model organisms. The basic research regarding size, lipidation, and charge of peripheral membrane proteins is pertinent not only to understanding how specific diseases may be disrupt vision, but also to providing insight on how proteins can localize through primary cilia, thus revealing implications for the future treatment of ciliopathies.

Project Body

Introduction

This article aims to present a comprehensive perspective on the mechanisms responsible for the functional translocation of rod phototransduction cascade proteins. This piece addresses the topic of interest through an analytical literature review with respect to the research I have assisted in the Calvert Lab at Upstate Medical University. Through determining connections between the observed protein localization in Xenopus Laevis in the Calvert Lab, and current research regarding cohesive properties of phototransduction proteins, anatomy and physiology of rod photoreceptors, and apparent adaptive and non-adaptive phenomena of protein localization through the connecting cilium, the relevance of protein characteristics can be further conceptualized.

Within this review, I address the experimentally significant qualitative and quantitative impact of protein charge and lipid characteristics on the movement of proteins involved in the phototransduction cascade in rod photoreceptors. To provide context for the relevance of such localization, I provide an overview of the phototransduction cascade, anatomy of rod photoreceptors, and pertinent characteristics of proteins governing their respective localizations.

Following a general overview of the properties of the rod phototransduction cascade, I present evidence from the recent studies which I assisted in within the Calvert Lab. The experimental data will provide background for the current literature regarding protein translocation within photoreceptors. The juxtaposition of experimental evidence from our models and external research provides a platform for a thorough analysis of the translocation of specific rod photoreceptor proteins.

I subsequently address the functions and structures of four of the photoreceptor proteins: *Transducin, Phosphodiesterase 6, Recoverin, and Rhodopsin Kinase* with implications for how they could affect their translocation. I, then, integrate this background information by presenting evidence of differential translocation between phototransduction proteins. By connecting the literature review to experimental evidence, I can begin to hypothesize how respective protein characteristics might dictate their distribution.

The translocation of proteins via primary cilia is currently not-well understood. This collaborative literature review and analysis of in-vivo protein translocation enables the proposition of future experiments to further elucidate phototransduction translocation. Once fully understood, these trends will be able to address phenotypic effects of ciliopathies and retinal degeneration, and generate pertinent information their treatment.

An Overview of Photoreceptors, the Phototransduction Cascade, and the methodology for the generation of structure of the photoreceptors

Overview of photoreceptors:

When analyzing the mechanisms of phototransduction and vision it is important to understand how components of the neural retina interact, conduct sensory stimuli, and readjust following a reaction to a stimulus. The photoreceptors, classified as rods and cones, lie within the retina. The photoreceptors are stimulated by photons and relay information to the ganglion cells via interneurons. Ganglion cells are then able to carry the signal to the brain as their axons create the optic nerve (Kolb, 1981).

Rods and cones can be broken down into two parts: the outer segment and the inner segment. The outer segment of a photoreceptor can be categorized as a modified cilium with a cilium-like arrangement of microtubules which connect the outer and inner segment (Pearring, 2013). The outer segment is filled with discs of photoreceptive membranes that contain high concentrations of rhodopsin photosensitive complexes composed of opsin protein and retinal visual pigment. Retinal, an aldehyde of vitamin A, is the light-absorbing chromophore of the photopigment (Purves, 2012).

Overview of the Phototransduction Cascade:

In rods, rhodopsin is an embedded seven-pass transmembrane molecule which is a member of the G-coupled protein receptor family. When rhodopsin is activated by a photon of light, it's covalently attached 11-cis retinal chromophore isomerize to all- trans. This isomerization alters the shape of the retinal, conformationally changing the shape of the opsin. Such change alters the G-protein transducin (Gt) and transducin's α subunit dissociates. The α subunit goes on to activate the cyclic GMP phosphodiesterase which in turn hydrolyzes cyclic GMP, causing the cGMP levels to fall. This drop in cGMP levels causes the cGMP gated Na+/K+/Ca+ion channels to close. In a dark state, these channels are kept open due to the bound cyclic GMP, however, in light, the drop in cGMP causes a hyper-polarization of the outer outer segment, which inhibits synaptic signaling.

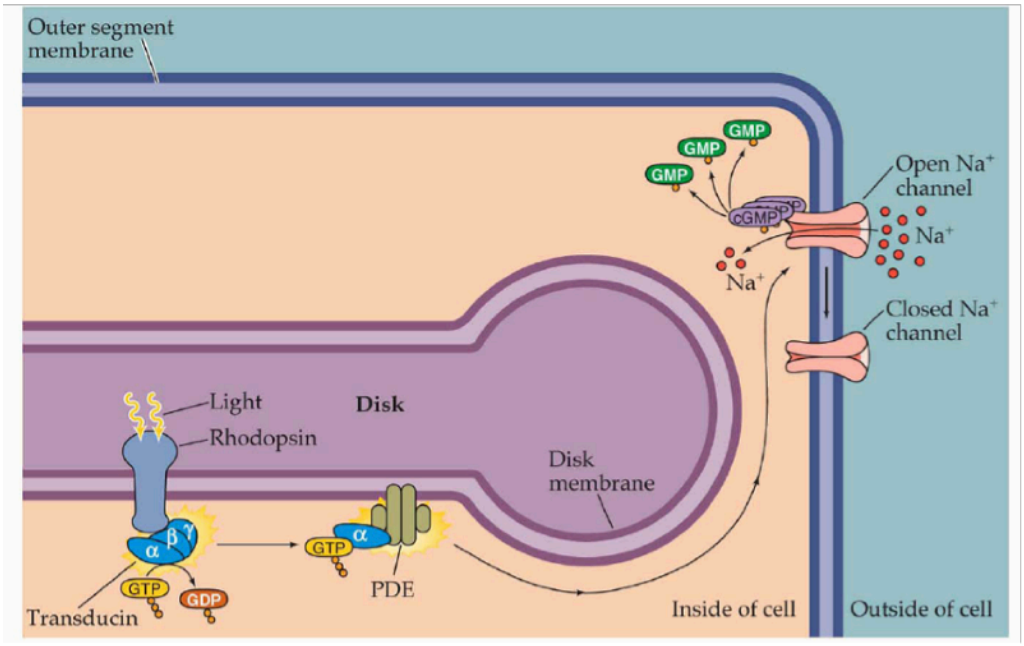


Figure 1: Rod phototransduction cascade (Purves, 2012)

Regeneration of the resting-state of rod photoreceptors:

In order to regenerate the depolarized, dark-adapted state of rod outer segments, several proteins function to quickly revert the cell. One protein is a rhodopsin-specific kinase which phosphorylates the cytosolic tail of rhodopsin on serine residues, thereby inhibiting the ability of rhodopsin to activate transducin. Once phosphorylated, *arrestin* can bind to the phosphorylated rhodopsin which further inhibits rhodopsin's activity. Additionally, RGS proteins- *regulator of G protein signaling*- act to shut off the G-protein-mediated responses of eukaryotes. They work by stimulating Transducin to hydrolyze it's bound GTP to GDP and return Transducin to its inactive state. Additionally, when the cell is in the hyperpolarized state with closed Na+ channels, the normal influx of Ca2+ is also inhibited. This decrease of intracellular Ca2+ causes *Recoverin* – which is inactive when Ca2+ is bound and active when it is Ca+ free, to inhibit rhodopsin kinase.

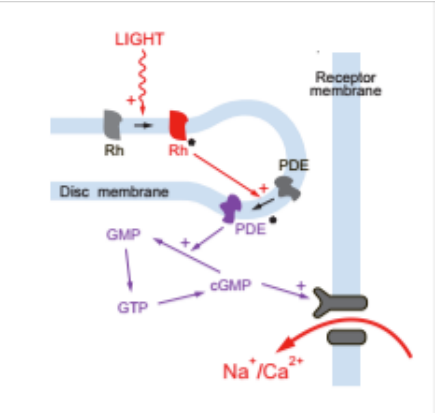


Figure 2: PDE6 involvement in phototransduction cascade (Purves, 2012)

The inner segment contains a high concentration of mitochondria and the nucleus, as well as forms the synaptic junction with horizontal cells and bipolar cells. These interneurons then relay the message to the ganglion cells that are able to conduct the message further to convey the signal to the brain. Photoreceptors are cells that do not have a strong mitotic ability, but the proteins of the phototransduction cascade, such as rhodopsin, are continuously degraded and replaced (Purves, 2012).

Mechanism of transport

It is well known that membrane-associated proteins mediate their movement based on their interactions by intraflagellar transport (Pedersen, 2008), binding to membrane structures (Francis, 2011), and barriers to diffusion (Dishinger, 2010). However, specific physical, chemical, charge, and hydrophobic properties of each of the rod photoreceptor proteins involved in the initiation and degradation of the signal is less well known.

In this review, a comprehensive overview of the effects of lipidation and charge accumulation, as well as movement of the main proteins in the rod phototransduction cascade will be

analyzed, in order to shed light on what properties may be responsible for the localization of the various proteins.

Photoreceptor Formation

In order to gain insight for how the outer and inner segments of rods originate and associate, it is important to address their creation. Rods are known to be thin rod-shaped cells consisting of –as stated previously- an outer segment composed of stacks of membranes containing the visual pigment molecules of rhodopsin, an inner segment containing ribosomes and membranes where opsin molecules are assembled and transferred to other outer segment disks, the cell body, containing the nucleus, and the point of neurotransmission to other neurons involved in phototransduction: the synaptic terminal.

The rod outer segment is generated as the result of evaginations and invaginations originating from the base of the cilium which connects the outer and inner segment of the photoreceptor. This process can be called outpouching (Kolb, 2005). Throughout the process of creating such structure, the plates become detached disks inside the outer segment- causing them to appear to be folded double membranes (Kolb, 2005).

An important phenomenon to note is that new disks are added to the outer segment in a recycling manner, with new disks being added to the base of the outer segment at the cilium, and old disks phagocytized within the pigment epithelium (Francke, 2001).

The disks are the residential areas of the phototransduction proteins. This arrangement of the outer segment of rod photoreceptors brings the question of translocation of the proteins and pigments present in the outer segment during the phototransduction cascade.

Considering that the inner segment is the main portion of the photoreceptors that contains the nucleus: responsible for the transcription of mRNA, ribosomes – responsible for the translation of proteins- and the Golgi: the responsible unit for the post translational modifications, including lipidation and glycosylation of proteins, it is important to look into *how* exactly it is that these proteins get to be present in the outer segment.

The Anatomy of the Rod Photoreceptor: Generation of Structure

Many molecular pathways have been shown to be disrupted following ciliary pathology. In the retina, photoreceptors, ciliogenesis and transport have revealed disrupted signaling. In photoreceptors, this dysfunctions could result from the various phenotypes that have occurred with ciliopathies such as a failure of connecting cilium formation or improper disk assembly (Madhivanan, 2014) which teams of researchers have analyzed through the visualization of transport. The figure below shows the compartments in the primary cilia. Figure taken from Rachel (A) shows how the primary cilium parallels the stacks of the outer segment disks. New discs are assembled at the base of the OS by incorporating proteins and lipids from the synthesizing components of the rod inner segment. As the discs mature, they migrate to the end of the photoreceptor; discs are eventually shed and degraded by the pigment epithelial cells. It has been reported that a "vesicular targeting model" is responsible for the regeneration of disc membranes and integration of rhodopsin at the base of the outer segment. Additionally, Chuange et. Al., 2011, found such vesicular structures to be concentrated near the junctions between the distal cilium and OS base (Chuang, 2011). This anatomy of the connection and dynamic regeneration

between the inner and outer segments of photoreceptors seems to be unique from other neuronal systems.

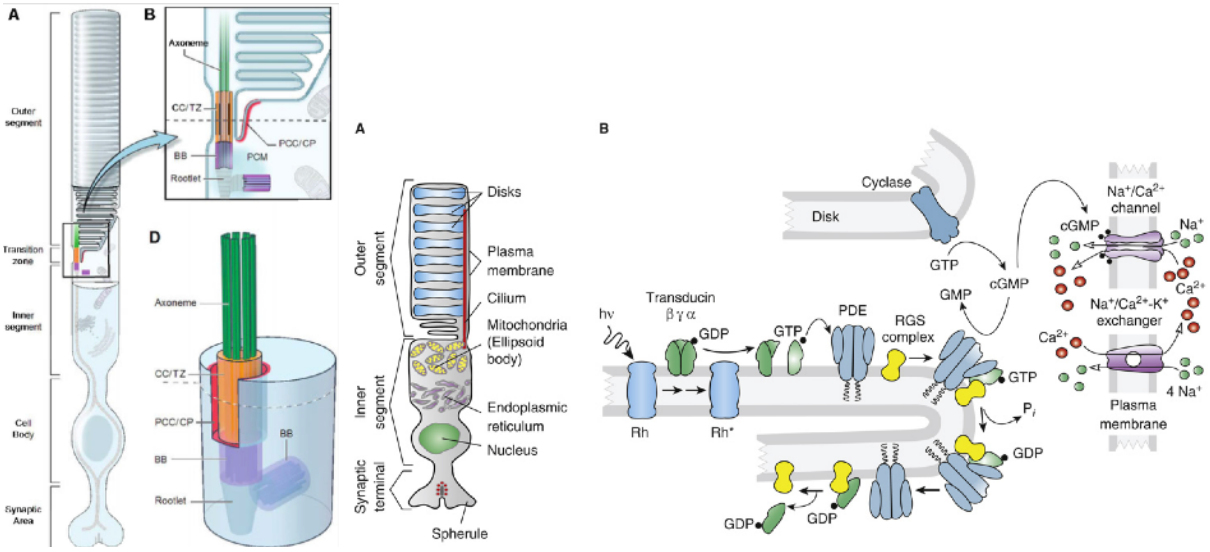


Figure 3:(left) (**A**) Photoreceptor anatomy, highlighting compartmentalization by the separation of cellular domains (1) distal cilium or axoneme (Axo; green); (2) connecting cilium(CC; orange); (3) basal body (BB; purple); and (4) ciliary pocket (CP; red) (**B**) The Connecting Cilium (Rachel, 2010)
Figure 4: (right) **(A**) Anatomy of representative vertebrate rod. (**B**) Major proteins and mechanisms in vertebrate rod transduction (Fain, 2012)

You can visualize the major sections of the vertebrate rod photoreceptor and begin to see the necessity of the connecting cilium. As the internal composition of the inner segment of such photoreceptors contains organelles such as ER, Golgi, mitochondria, and a nucleus, the outer segment does not. The implications of this compartmentalization of the segments of the photoreceptor highlight the importance of translocation. We must address how, given the presence of proteins involved in phototransduction cascade within the outer segment, it is that these proteins specifically propagate there. The outer segment contains no cell machinery capable of protein

transcription or translation, thus the phototransduction proteins must gain access to the outer segment by traveling through the connecting cilium.

The two segments not only differ in visualization, but additionally differ in diffusion properties, electrostatic properties, and hydrophobic properties: all of these differences in composition between the inner segment, outer segment, and connecting cilium, will prove to be relevant in the analysis of characteristics that might determine localization within the different sub cellular regions.

On the right, another visualization of the phototransduction cascade is shown, highlighting the interaction between transducin, PDE, and the cGMP gated channel. Other proteins will also be important in our aim to understand the outcomes of various lipid and charge characteristics in innate proteins compared to experimental probes.

How protein properties affect translocation and membrane association

In order to understand the physical properties of proteins involved in the phototransduction cascade, an important circumstance to acknowledge is that, of the proteins we will be discussing in this review, most excluding *Arrestin* are associated with membranes (Alberts, 2002).

In a communication of interplay between the chemical and physical properties of membranes and proteins, the compositions of both are important in terms of the association of proteins with membranes. In order to eventually begin to understand how proteins involved in specifically phototransduction of rods translocate, we must first address how membrane proteins generally associate with membranes in terms of a general overview, and then specifically (a) lipidation and (b) charge characteristics.

Membrane proteins and the lipid bilayer

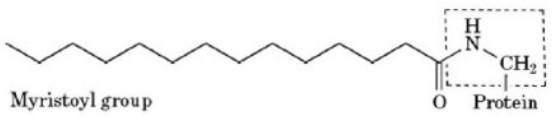
There are several ways in which proteins can be associated with the plasma membrane. While most proteins that are defined as transmembrane proteins tend to be thought of as proteins with a single α helix, or multiple α helices extending across the membrane. Additionally, membrane proteins can have multiple β sheets rolled into a barrel formation which extend across the membrane's lipid bilayer. Membrane associated proteins, however, do not need to fulfill these requirements and can simply be associated with the membrane.

Typically, these proteins are only associated with one side of the membrane with an extension of their hydrophobic portion of the protein, or attached to the bilayer by means of a covalently linked lipid chain, an oligosaccharide, or simply by non-lipidated covalent interactions with other membrane proteins.

Lipid-linked proteins

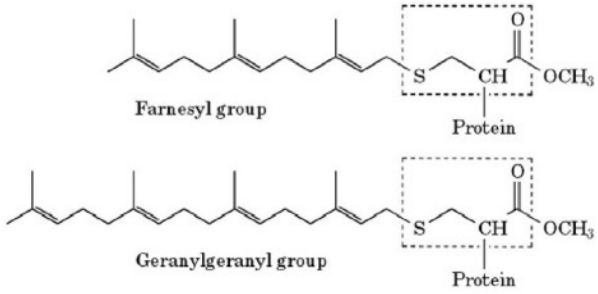
When a precursor to a lipid-linked protein is made, it is initially made as soluble proteins in the cytosol, and later directed to the membrane by attachment of a fatty acid or prenyl group.

Examples of fatty acid covalent attachments are titled myristic acid and are attached to the N- terminal glycine of a protein via an amide linkage. We will later bring up how the attachments of these fatty acids play in to the specific localization of *Recoverin* and how an addition of charge may affect its localization of GFP in vivo.



Myristoyl group: The-Crankshaft Publishing's

Proteins anchored to the membrane via prenyl groups are further typically split up into the addition of a farnesyl group or geranylgeranyl attachment. Both of these groups can be attached by a thiol-ester linkage to a cysteine residue, four residues from the C-terminus of the protein. These three residues are then cleaved off following prenylation, and the group is methylated, so that the be easily inserted into the membrane.



Prenyl groups: The-Crankshaft Publishing's

Although there are more methods of attaching lipid linkers to proteins, these are the more common two seen in several of the membrane associated proteins that we will be looking at, so we only will go into the explanation of these.

Experimental Assessment of "What directs localization"

In order to address how proteins imperative to the phototransduction cascade are sub- cellularly localized, our lab sought to construct protein probes that would shed light on what factors influence the localization of proteins. Primarily, we identified the properties that are known to affect association of proteins with membrane localization. Next, the lab members created probes

that would incorporate such modifications into a neutral protein construct EGFP. In order to assess how specific sequences of phototransduction proteins affected their localization, genetic constructs were created. The methods used to generate probes of XOP_EGFP constructed green flourescent proteins, linked to various linkers are largely standardized and explained below. The results from previous work analyzing the effects of charged and lipidated EGFP probes are relevant to our discussion of the properties of phototransduction proteins and must now be referenced and analyzed, so that we can then begin to understand how the properties of phototransduction proteins might affect their localization.

Methods:

In previous experiments within the Calvert lab, the addition of electrostatic charges and lipid moieties to EGFP protein were assessed in Xenopus Laevis rod photoreceptors with confocal microscopy in-order to image and statistically represent how specific characteristics affect the localization of such modified proteins. We produced transgenic African Claw frogs (Xenopus laevis) using a fragment of the Xenopus rod opsin gene to regulate the production of EGFP specifically in rod photoreceptor cells (Knox, 1998). Transgenic Xenopus species are used frequently in photoreceptor research (Jin, 2003) due to the rapid development of photoreceptors in pre-metamorphic tadpoles. EGFP is a soluble, 32 kDa, flourescent protein with a relatively even surface charge; EGFP functions to localize within the rod photoreceptor without localization, while providing fluorescence for quantification.

Due to the varying compositions of the IS and OS of rod photoreceptors, protein affinity could depend on the interaction and compatibility between the protein and the segment of the

photoreceptor. Specifically, the initial modifications the Calvert lab made to address the interactions of probe proteins and various sub cellular regions of rods relied on the alterations of size, charge, and hydrophobicity.

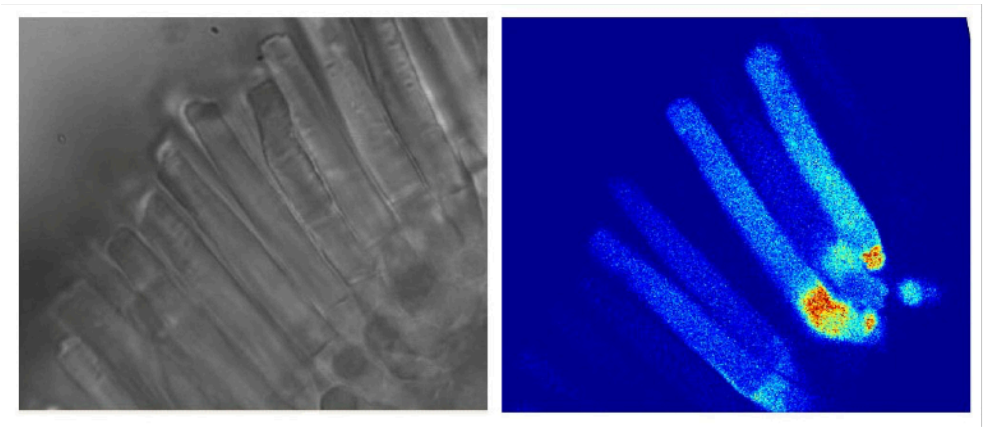


Figure 3: Images of live Xenopus Laevis retinal slices and the distribution of soluble EGFP protein images are infared (left) and confocal (right) (Calvert, 2011)

Electrostatic probes were constructed by ligating sequences conferring for specific charges localized to either the N or C-terminus of EGFP probes. Lipidated probes were generated by constructing sequences that enabled the addition of either farnesyl, gernanylgeranyl groups, or myristoyl groups. The lipidated probes were mutated so that the linkers were neutral- this mutation was incorporated in order to isolate the effects of lipidation separate from charge characteristic.

In order to understand how proteins with electrostatically charged sequences localize in the photoreceptor, probes designed to express either poly-basic, neutral, or negative charges were produced. Arginines were incorporated for positive charges and glutamic acid for negative charges. Neutral charges were established using glutamine. The charge linkers were expressed in

EGFPs in Xenopus rod photoreceptors via degradation and ligation using restriction enzyme mediated transgenics. Under the control of the Xenopus opsin promotor for rod photoreceptor expression, the probes were integrated into the respective plasmids. Tadpoles were screened for fluorescence in the eye, and those expressing our probes were grown into frogs and than imaged with confocal and fluorescence microscopy.

The sequence for each probe was inserted behind the XOP promoter, and introduced to Xenopus sperm nuclei in vitro using restriction enzyme mediated hydrolization and integration. The microinjection of the plasmid DNA under control of the XOP enabled the integration of the transgene for the production of transgenic Xenopus Laevis offspring.

Once the retinas from the mature tadpoles were harvested, the rods seen in these images were created using confocal microscopy and multi-photon fluorescence relaxation after photoactivation (mpFRAPa).

Once the images were created, MATLAB was utilized to statistically analyze the confocal images of the rods. The fluorescence intensity can be visualize by viewing the red coloring as the highest intensity of fluorescence and the dark blue as the lowest intensity of fluoresces. The assessments of charge distribution were then calculated and normalized to the fluorescence of the outer segment.

Results:

Size modification:

Previously shown by (Najafi, Maza, Calvert, 2012). Size of the protein can affect it's localization. When adding additional components of soluble GFP protein, and subsequently

increasing the protein size from 27kDa to 54 kDa and finally to 81kDa, a distribution shift was revealed. The larger the soluble protein showed a preferential distribution to the inner segment. This contrasted to the distribution of a 27kDa, XOP_EGFP protein which displayed a more even distribution throughout the entire photo receptor. This could be explained by the differences in free space within each compartment: within the inner segment, the free space is much higher, which would accommodate larger proteins, while the outer segment is densely packed with disc membranes.

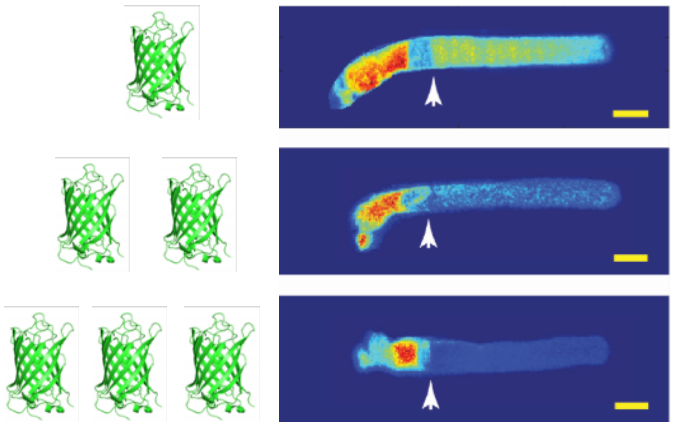


Figure 4: Crystal structure of GFP, green florescent protein (27kDa) (Ormö, 1996)

Figure 5: Images of live Xenopus Laevis retinal slices and the distribution of soluble (top to bottom) GFP, GFP_GFP, and GFP_GFP_GPF protein. The highest concentration of GFP is red, whereas the lowest is darker blue. It can be visualized with the confocal images that the relationship between outer segment localization of protein and size of protein is inverse. EGFP diffuses throughout the whole cell, but GFPX3 displays inner segment localization. (Calvert 2011)

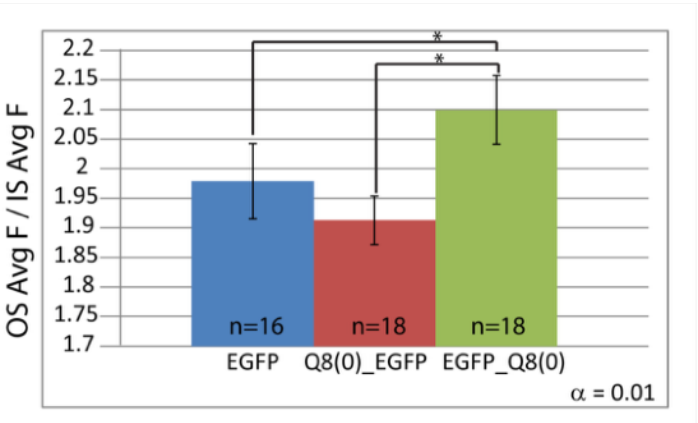
Electrostatic Charge Modification:

Electrostatic charges conditionally have revealed impacts in protein localization. Generally, it has been seen that positive charges have affinity for negatively charged membranes, but the presence of positive poly-basic sequences can also result in nuclear localization. Electrostatic affects were determined by comparing the localizations of neutral EGFP proteins linked to elec-

trostatic probes. The initial comparison was made between (1) EGFP protein (2) EGFP protein with a neutral linker on the C-term (3) EGFP and a neutral linker on the N-term.

When comparing the effects of adding neutral linkers to soluble EGFP proteins, the addition of the neutral linker to the C-terminus had a significant affect on the localization of EGFP to the rod outer segment.

Figure 6: Linkers added to create electrostatic probes. Added amino acids were arginines (+), glutamic acid (-), and glutamine (0). (Maza, 2015)



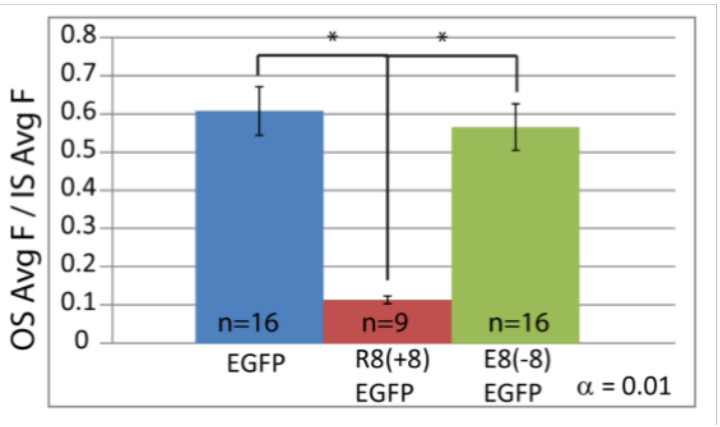


Figure 7: Distribution of EGFP and neutral EGFP soluble proteins, EGFP ligated to a neutral linker on the C-terminal displayed significantly higher localization to the outer segment than EGFP or N-terminal neutral linkers to EGFP (Maza, 2015)

Figure 8: Distribution of EGFP compared to positively and negatively charged protein probes. Positively charged proteins displayed localization in the inner segment. Negatively charged probes displayed localization statistically equivalent to soluble EGFP protein (Maza, 2015)

The addition of negative and positive charges to EGFP probes was assessed. Between the three varieties of probes, the addition of the negative charged probe resulted in a distribution similar to the soluble EGFP. Whereas, the addition of the positively linked probe resulted in inner segment localization, which could be attributed to the positive poly-basic characteristics of nuclear localization sequences.

Lipidation Modification: Myristoyl

Because photoreceptor transduction cascade proteins are often associated with membranes due to their post-translational lipidation, assessment of the addition of lipid moieties of different kinds is necessary to elucidate their impacts. Najafi et. Al reveal that the addition of a myristoyl group to the *N-terminus* of the 3xEGFP probe restored its affinity for the OS.

When assessing the impact of lipidation, we must recall the varying forms of lipidation. These varieties include, but are not limited to: Myristoylation, farnesylation, and geranylgeranylation. The hydrophobicity of each lipid moiety can be defined according to the number of carbons the moiety has. Myristoyl (14 C), farnesyl (15 C), and geranylgeranyl (20 C). These lipids are attached either during translation, or post-translationally by the ER or cytosol, meaning within the inner segment, due to its translational machinery.

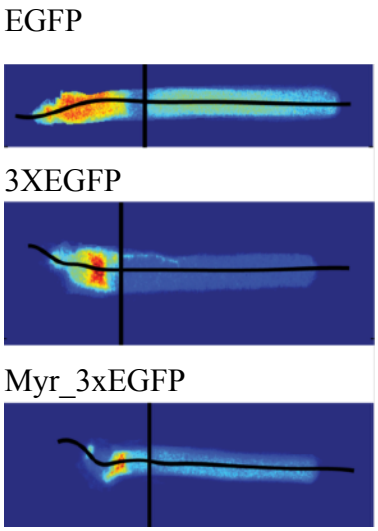


Figure 9: EGFP (top), 3xEGFP (second), Myr_3xEGFP (bottom) displayed with confocal microscopy to compare the effects of lipidation on a larger EGFP protein. The lipidated 3xEGFP showed an increased outer segment localization than non-lipidated 3xEGFP (Maza, 2015)

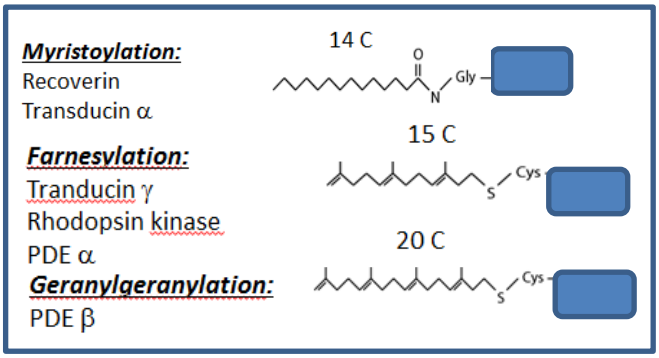


Figure 10: Protein lipidation moieties and phototransduction proteins associated with each (Maza, 2015)

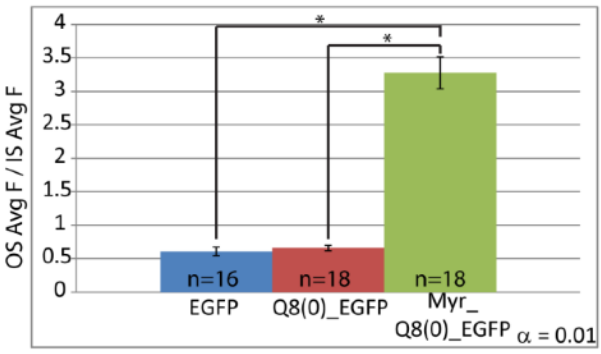


Figure 11: Myristoylation concentration in the outer segment, significance of 0.01 (Maza, 2015)

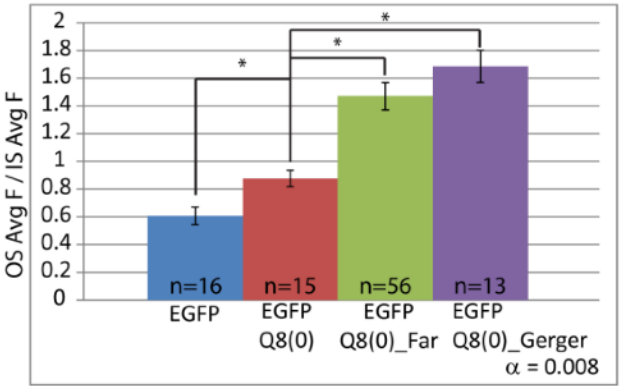


Figure 12: Geranylgeranyl lipidation increases outer segment localization significance of 0.08. (Maza, 2015)

(1)The Calvert Lab assessed the impact of attaching a myristoyl group to an EGFP protein with a neutrally charged linker and apparent outer segment localization occurred.

(2) Additionally, when the lab tested how prenylation (farnesylation and geranylgeranylation) affect the distribution of neutral EGFP, similar results of outer segment localization occurred.

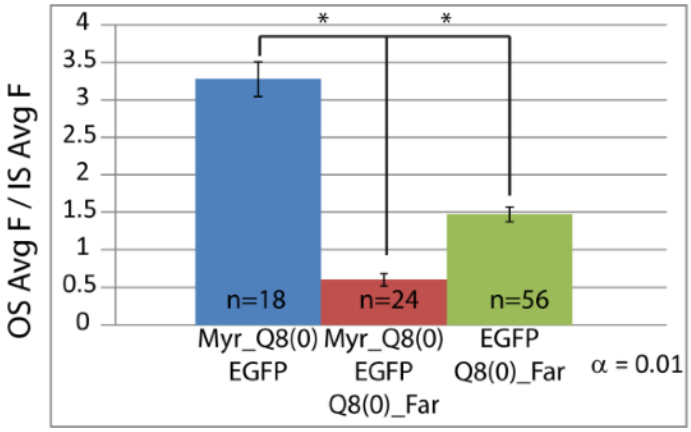


Figure 13: Dually lipidated (myristoylated and farnesylated) probes displayed in the inner segment localization with a significance of 0.01 (Maza, 2015)

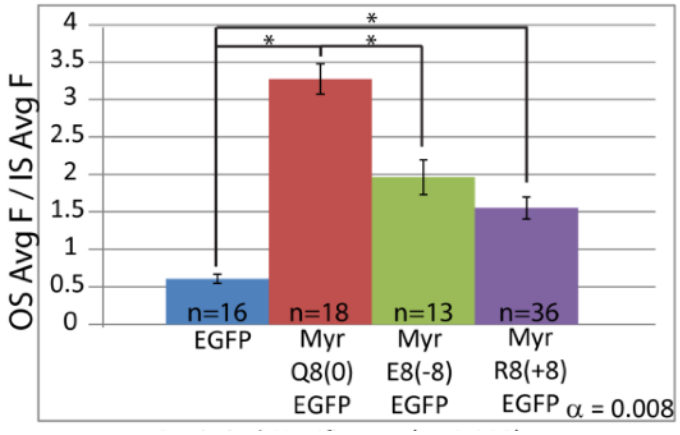


Figure 14: Myristoylated proteins localize depending on charge with discrepancies between positively and negatively charged probes. significance (0.08) (Maza, 2015)

(3) An important test to note was the assessment of the effects of dual lipidation. When the soluble EGFP was linked to both a Myristoyl group on it's N-terminus and a Farnesyl group on it's C-terminus, the model revealed inner segment localization.

(4) Myristoylated proteins were then shown to differentially localize depending on the addition of charge. With the addition of positive charge also enhancing association with the synapse.

This differential pattern, which our lab proved to be statistically significant, shows that the addition and possibly placement of the lipid group has different outcomes on the distribution of the protein. Following in-depth analysis using a 3-D model to visualize the distribution of positively charged, farnesylated-EGFP, imaging reveals that the distribution has a high concentration in the connecting cilium.

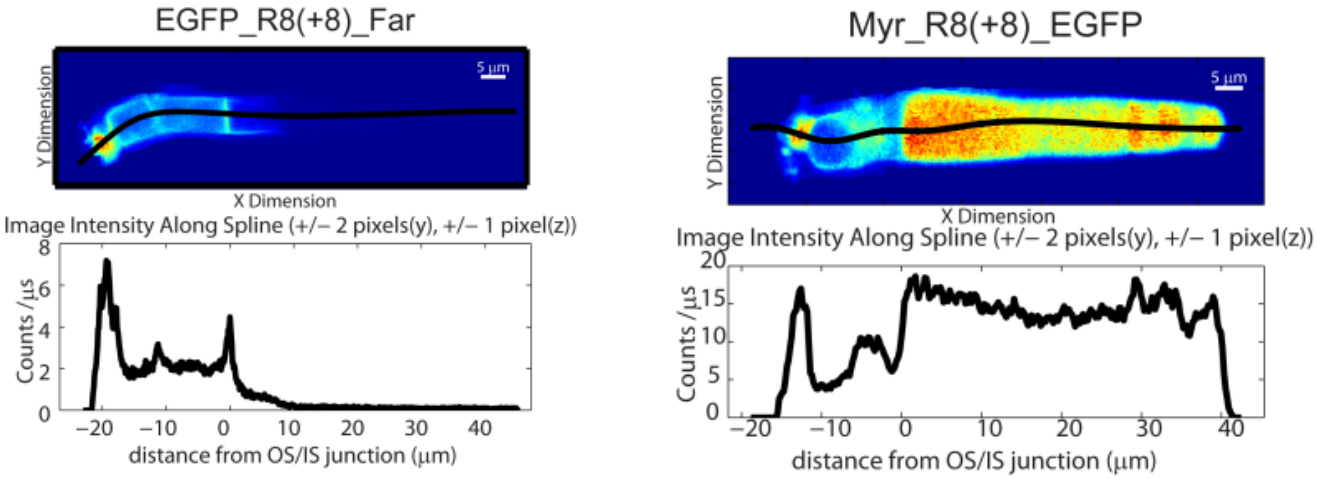


Figure 14: Confocal images showed nuclear localization of probes with both positive charge linkers and farnesylation. Myristoylated positive probes did not show the same inner segment or nuclear localization distribution (Maza, 2015)

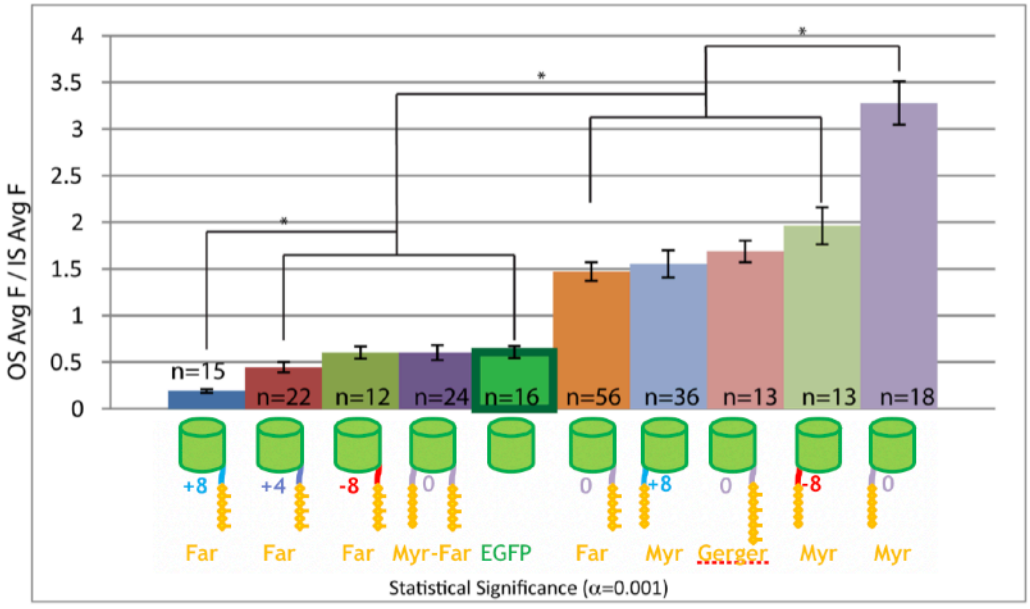


Figure 15: Comparing all of the probes within the Calvert lab with data thus far, Farnesylated probes with charge and dually lipidated probes with myristoyl and farnesyl groups displayed inner segment localization, whereas neutral farnesylated, myristoylated (charged and neutral), and geranylgeranylated proteins localized to the outer segment with varying concentrations. (Maza, 2015)

When comparing all of the tests from the Calvert lab, (Maza, 2015) created all the comparisons of the varying distributions of proteins. We can see that both charge and lipidation affect the outer segment to inner segment distribution ratio. These variations should be kept in mind and utilized when comparing the different rod photoreceptor proteins involved in the visual transduction cascade.

The important notes to take away from these background experiments are that Myristoylation results in outer segment enrichment regardless of charge and Farnesylation with a positive charge is synapse and IS localized. Finally, diffusion experiments enabled the group to ascertain that the charge distributions were not fixed and peripheral membrane protein distribution was not entirely based on membrane affinity.

Phototransduction Membrane Associated Protein Properties

Review and relevance of Transducin, PDE6, Recoverin, and GRK1

Transducin

Following the activation of rhodopsin by a photon, the G-protein, Transducin, is subsequently activated. In it's inactive form, Transducin is peripherally attached to the membranes of the rod outer segment, however upon activation by light, Transducin is known to translocate from the outer segment to the inner segment of rod photoreceptors. In order to understand this translocation in respect to the findings from the Calvert lab, we must understand the electrostatic and lipidation properties of Transducin (Gt) in both the heterotrimeric and dissociated subunit forms.

As a heterotrimer, transducin is peripherally - but reversibly - attached to the lipid bilayer by lipid modifications of the Myristoylated N terminus of the α subunit and the farnesylated C terminus of the γ subunit (Bigay, 1994, Seitz, 1999). Additionally, electrostatic interactions between either the heterotrimer or subunits of transducin have also been shown to interact with the negatively charged membrane (Lambright, 1996).

Electrostatic Properties of Transducin Heterotrimer:

Through analysis of electrostatically favorable orientations of Transducin to the membrane, it has been determined that there is a small positively charged path around the attachment points of the two lipid anchors, with the portion of Gt with negative potential oriented away from the membrane, generating a weak electrostatic attraction when bound. This distribution of charge not only plays a role in the association with the membrane, but also Transducin's orientation to the membrane, by revealing that the electrostatics of the heterotimer are insignificant (Kosloff, 2008). These results indicate that the heterotrimer is likely to be membrane associated due to the lipid anchors of the heterotrimer when oriented in its electrostatically favorable ways.

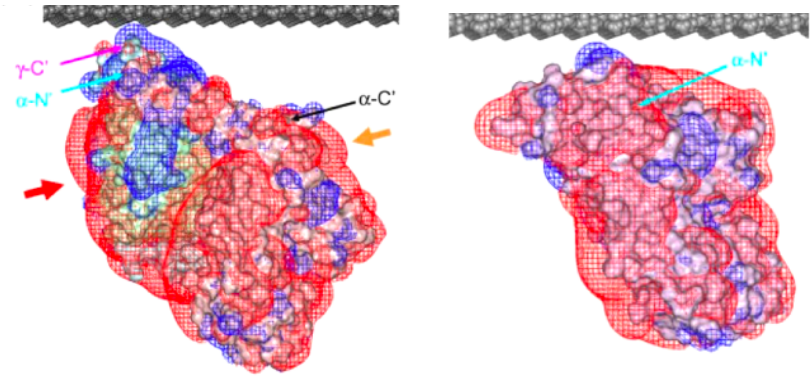


Figure 16: Interactions of the G_t heterotrimer with the membrane, electrostatic potential map of the G_t heterotrimer in the orientation of minimum free energy (left), electrostatic interactions between the alpha subunit and the membrane (right). (Kosloff, 2008)

Dissociated:

It has been shown that upon dissociation from Gα, G$\beta\gamma$ is attracted to the membrane, which allows many more electrostatically favorable interactions between the membrane and the subunit. Experimentally, it has been shown that the contributions of lipid anchor and electrostatics to the free energy of G$\beta\gamma$ are both independent and additive.

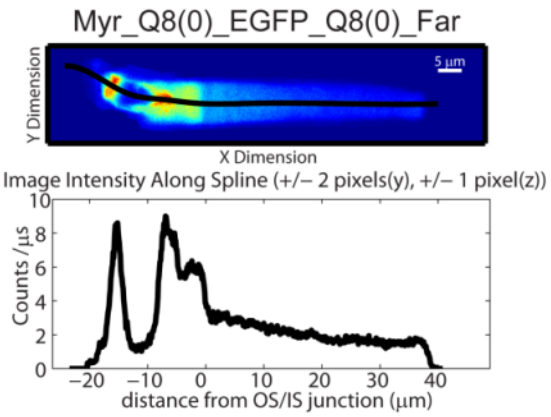


Figure 17: Confocal images show inner segment localization of probes with both myristoylation on the N-terminus and farnesylation on the C-terminus. (Maza, 2015)

Gα, when within the heterotimer, shown with the teal arrow, is conformed so that it is stable with an extended N-terminus, allowing for the interaction between the Myristoyl group and the membrane. This stabilization is due to the interaction between the subunits and is refolded into a compact conformation when Gα dissociates from G$\beta\gamma$ (Kosloff, 2008). When modeling the electrostatic effects of Gα, revealed inevitable repulsion due to the entire surface being dominated by negative charges. These repulsions due to the negative Gα charge, and conformational change cause Gα to behave similarly to a soluble protein.

Implications for Translocation:

The light-dependent translocation of Transducin has been well established, indicating the apparent necessary dissociation of the subunits of G_t and is possible via diffusion (Calvert, 2006). Such diffusion has notably been seen to occur at rates similar to soluble GFP (Nair, 2005) despite possible lipid and charge interactions. This diffusion coefficient can be reasoned due to the established conformation change of Gα, shielding the myristoyl group from interacting with the plasma membrane, and from G$\beta\gamma$'s phosducin dependent translocation (Solokov, 2004), which allows for the stabilization of the farnesyl moiety and attraction to the membrane.

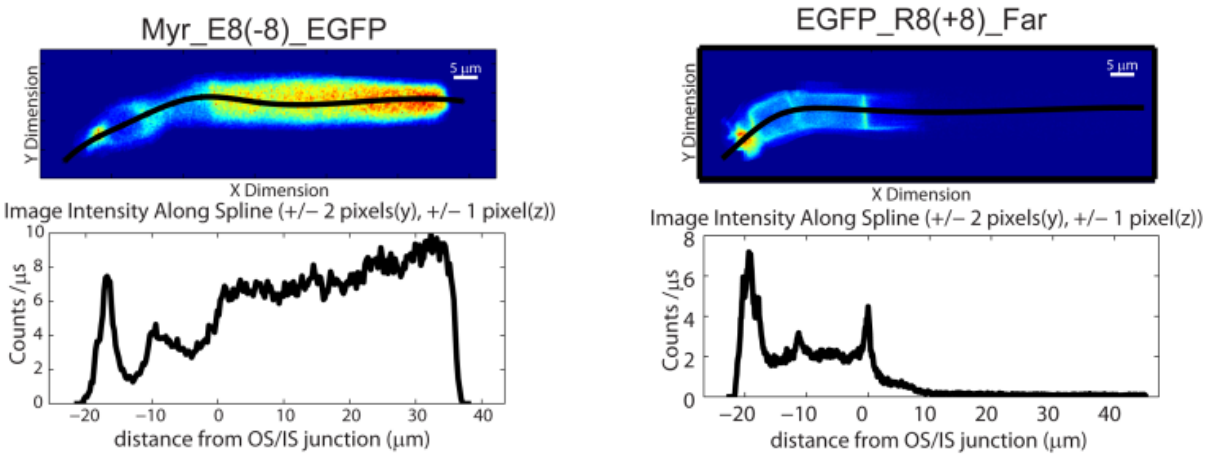


Figure 18: Confocal images showed a outer segment distribution with EGFP probes with negative myristoylated linkers (left) and nuclear localization of probes with both positive charge linkers and farnesylation (right) (Maza, 2015)

Relevance to Experimental Evidence:

As a heterotrimer, Transducin contains the components of a N-terminal myristoyl group, a C-terminal farnesyl group, and an overall electrostatic interaction that is insignificant. When taking in the results from the Calvert lab, we should expect a distribution from Transducin that is

similar to the dually lipidated EGFP probe. However, such probe revealed inner segment localization, this comparison of the distribution between the neutral probe favoring the IS, and the native Transducin favoring the OS, enhances previously shown research that electrostatics, or the addition of poly-basic charges enhances membrane affinity (Peitzsch, 1993), and that the positive region of the heterotrimer of Transducin must play a role in OS localization rather than simply being dependent on the presence of the two lipids, contrary to previous literature.

I constructed models of the Transducin heterotrimer using Pymol software. The red residues confer to negative residues and blue residues, positive. The distribution of charge shows a relatively even and neutral configuration with a positive charge localized to the lipidation site of the heterotrimer. This area, highlighted by yellow, represents the C-terminal of Gγ and the N-terminal of Gα, both with lipidation, and a slight positive charge.

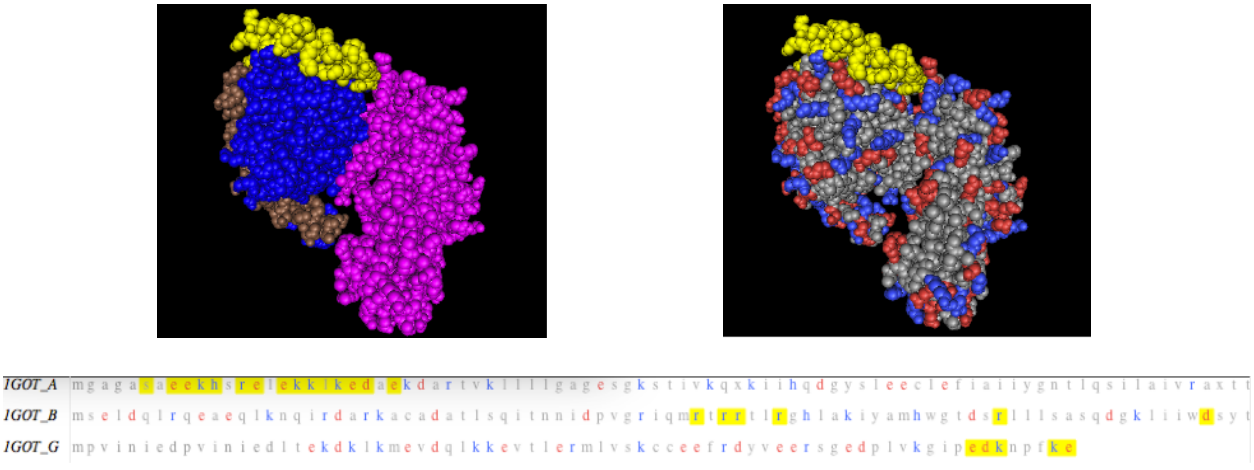


Figure 19: Pymol created crystal structures using Bos Tarus sequences for transducin (1GOT) with the highlighted residues correlating to the membrane interacting region. Heterotrimer (right) is color coordinated with the subunits expressing different colors, beta (blue), gamma (brown), alpha (pink). The heterotrimer (right) shows charge distribution with negative residues as red and positive residues as blue.

When deciphering how the charge and lipidation of the subunits might impact transducin light dependent translocation, we must again compare the properties of the subunits to the probes. The Gα subunit of transducin, anatomically, has a myristoyl group extended when included in the heterotrimer but remains apparently soluble upon dissociation from G$\beta\gamma$, translocating to the inner segment. Even more, the Gα subunit is larger in size than its binding partner G$\beta\gamma$; this property, in combination with the myristoylation and positive charge would not reflect the rapid translocation of Gα to the inner segment in response to light. The conformational change following heterotrimer dissociation could explain the discrepancies between the distribution of myristoylated probes and the phototransduction proteins, in this case the Gα subunit of transducin.

With G$\beta\gamma$, the subunit was explained to reveal both a farnesyl group and moderate attraction to the membrane. These co-characteristics of a positive charge and farnesyl group, if similar to models in the Calvert lab, should show nuclear localization. However, the movement of G$\beta\gamma$ appears to be both slower and less repelled as the Gα subunit. This discontinuity with the modeled results points to the association of the G$\beta\gamma$ subunit with an additional protein. In literature, G$\beta\gamma$, phosducin interaction has been referenced, this differential translocation could support such data.

Given that the Transducin heterotrimer confers a protein that has a size that reduces following activation of the phototransduction cascade, but subsequently results in inner segment

localization of the subunits, the two events do not appear to occur in consequence of one another. Therefore, the properties of the distribution of Transducin provide ample evidence that there are more interactions between proteins in the rod phototransduction cascade that are responsible for localization of proteins than just hydrophobicity and charge. In order to fully understand how consistent the trend of the differences between the experimental constructs and the localization of innate phototransduction cascade proteins, we must both increase the literature research on protein in vivo translocation and the modifications of our probes to see how proteins may act under various conditions.

Phosphodiesterase 6: PDE6

As the primary regulator of intracellular cGMP and therefore calcium levels, phosphodiesterase 6, is the main effector of Transducin. The photoexcitation of rhodopsin from Rh to Rh* and subsequent activation of G protein Transducin has been previously discussed. We will now discuss the following processes involved in the hyperpolarization of rod photo receptors.

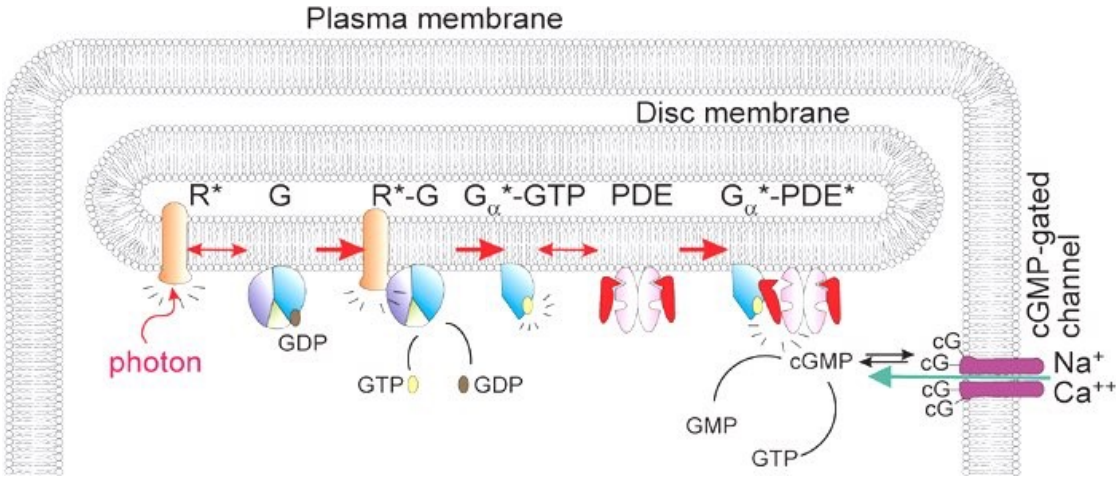


Figure 20: Phosphodiesterase involvement in the rod phototransduction cascade (Cote, 2004)

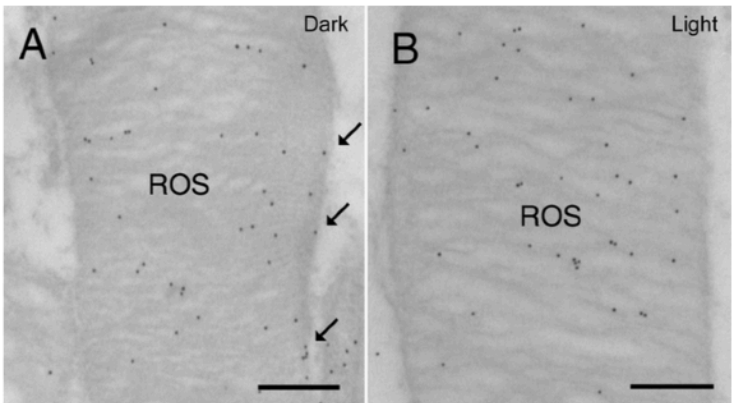
When the Gα subunit of Transducin binds to GTP, it is then able to bind to it's effector: PDE6. PDE6 is a holoenzyme in rod photoreceptors which consists of two catalytic subunits α and β, bound to two identical inhibitory subunits, γ (αβγ2) (Deterre, 1988). The membrane attachment of PDE6 is mediated by farnesylation of the PDE6α C-terminus and geranylgeranylation of the PDE6β C-terminus (Anant, 1992). PDE6 has been reported to be weakly associated to the disk membrane (Wasmuth, 2017). Although dually lipidated, the geranylgeranylated C terminus of PDE6β is more hydrophobic and thus critical to the membrane attachment of PDE6 (Wasmuth, 2017). PDE6 is released from the membrane when the PDE6B C terminus is removed by limited proteolysis (Cote, 2012). PDE is the first enzyme shown to be modified by both types of prenyl groups.

PDE6 is activated by the GTP-bound transducin α-subunit (Gαt-GTP) which interacts with PDE6 so that the γ subunit is displaced from the active site of the enzyme. This conformational modification due to the interaction between Gαt-GTP and PDE6, relieves the inhibition of the enzyme (Burns, 2005). PDE6, once relieved of inhibition, functions to degrade cytolytic cGMP to 5'GMP (Cote, 2004). A drop in cGMP levels decreases the amount of cGMP that are associated with the cGMP gated ion channels responsible for the maintenance of high intracellular calcium. As channels close, Ca2+ levels drop and the rod outer segments become hyperpolarized and continue on to generate a receptor potential at the synapse.

Implications for Translocation:

In previous research, PDE6 was hypothesized to be randomly distributed throughout the disc membranes of rod photoreceptors, but it has more recently been elucidated using Long-

Evans rat rod photoreceptors and immunogold electron microscopy that PDE6 does undergo light dependent translocation (Chen, 2008). The experiment revealed that PDE concentration in rod outer segments was significantly higher in dark-adapted retina than in the light-adapted counter parts (Chen, 2008). Additionally, it was revealed to have a translocation speed faster than that of Transducin. The translocation of PDE6 seemed to be due not only the presence of light, but to an extent to the presence of hydrolyzable GTP.



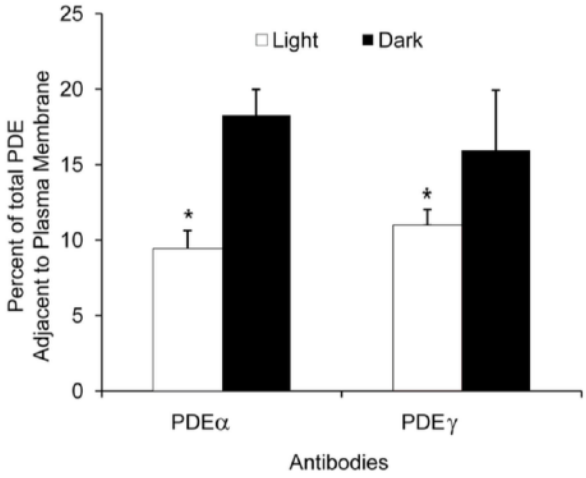


Figure 21: (left) Labeling of light- and dark- adapted rat retina, utilizing immunogold labeling with PDEα antibody to to identify PDE in (**A**) Dark- adapted rod outer segments and (**B**) light-adapted rod outer segments. There is an increase of phosphodiesterase 6 in dark-adapted conditions. (Chen, 2008)

Figure 22: Shows the percentage of PDE adjacent to plasma membranes of rod outer segments, labeling for either PDEα and PDEγ to show light- and dark- adapted localization of the respective subunits (Chen, 2008)

The figure to the left was composed following the study of PDE6 translocation reveals that PDE6 is more concentrated in the outer segment of rods in dark adapted cells, and upon illumination, it translocates to the inner segment. The group assessed if the alteration in distribu-

tion was due to the translocation of the entire PDE6 protein or simply the translocation of one portion of the protein: PDE6α being the catalytic subunit and PDE6 γ being the inhibitory subunit.

Electrostatic and Lipidation

In order to make correlations to the translocation within the presented study, Genomic rat DNA sequences for PDE6α and β were assessed for charge characteristics at the site of lipid modification, or the C-term. I assessed the C-terminal residues of PDE6β as a geranylgeranyl-lipid linked protein for charge characteristics. By analyzing the last 35 AA residues, the overall charge comes out to be -1. However, in analysis, there does not seem to be an overwhelming pattern of charge cluster next to the link to the lipid moiety (Wasmuth, 2017). Regarding electrostatic analysis of the final 40 amino acids of PDE6α, the charge clustering is also not significant, resulting in a +1 charge (Wasmuth, 2017).

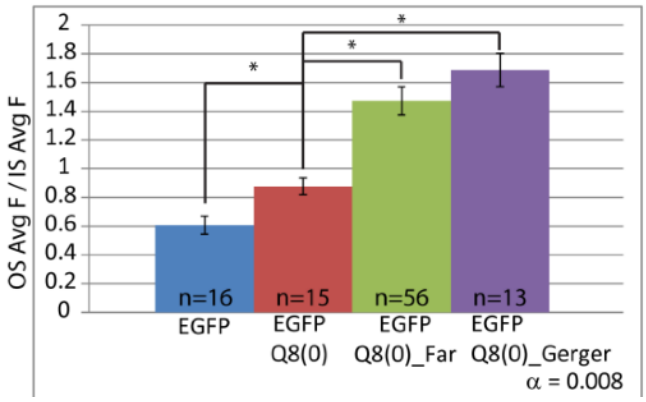


Figure 23: EGFP linked to neutral linkers and either geranylgeranyl lipid moieties or farnesylated moieties shows increasing outer segment association with carbon number, geranylgeranyl moieties have 20 carbons, whereas farnesyl groups have 15 carbon. (Maza, 2015)

Relevance to Experimental Evidence:

When relating the in vivo translocation of PDE6 following illumination of rod photoreceptors, we can relate such pattern to the movement of transducin in light-adapted rods. Similar to how transducin is a dually lipidated protein, PDE6 consists of a holoenzyme lipidated by a geranylgeranyl moiety on PDE6β and a farnesyl moiety on PDE6α.

When relating these moieties to the experimental probes within the work of the Calvert lab, we can see how the attachment of a geranylgeranyl and a farnesyl group both independently cause the outer segment localization of proteins. It was also shown using a dually lipidated protein model probe with myristoyl and farnesyl, that dual lipidation resulted in inner segment localization of the probe.

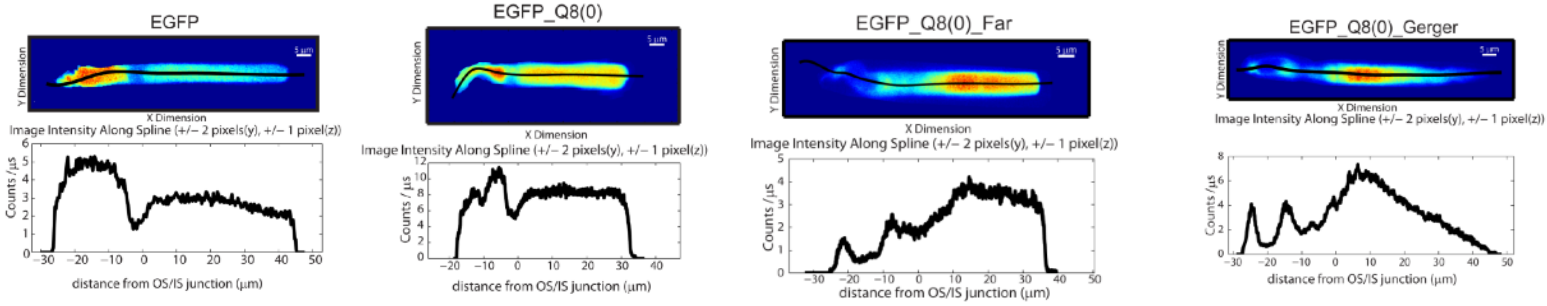


Figure 24: Confocal images of rod photoreceptors with (left to right) EGFP, EGFP (neutrally linked), EGFP with a. neutral linker and farnesyl group, and EGFP with a neutral linker and geranylgeranyl group. (Maza, 2015)

Although PDE6 is not lipidated on either side because both modifications to the protein subunits reside on the C-terminus, whereas this probe maintains lipid moieties on both the C-terminus and the N-terminus, this pattern of inner segment localization with the dual lipidation of proteins could potentially be related to the inner segment localization of PDE6 following illumination.

Similar to transducin, PDE6 reveals light dependent translocation to the inner segment and maintains two lipid moieties that somewhat resemble the experimental pattern of the probe. For future work it would be interesting to see more specific reasons why dually lipidated proteins might translocate to the inner segment only following light stimulation. It leads me to suspect that there are likely protein-protein interactions during dark-adapted cells which combat the factors that electrostatic and lipidation contribute.

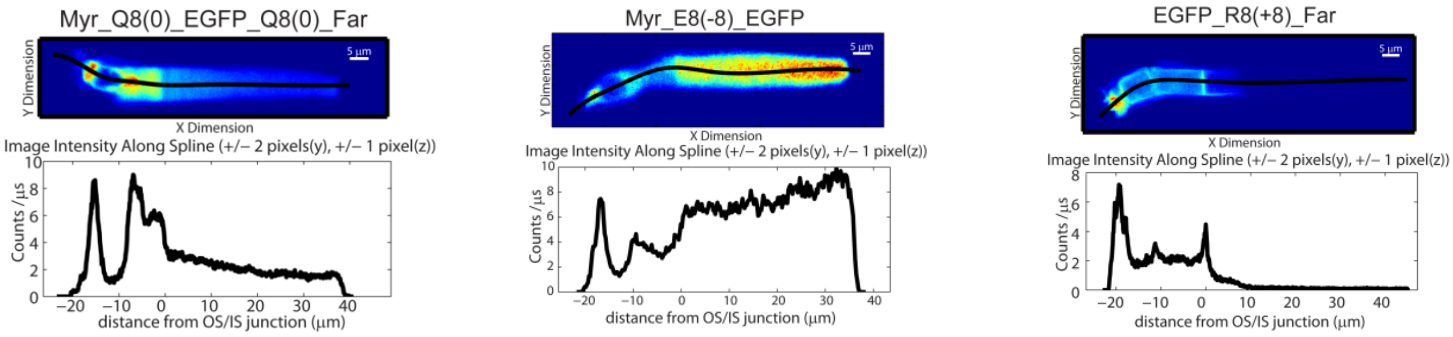


Figure 25: Confocal images of rod photoreceptors with (left to right) dually lipidated EGFP (myristoyl and farnesyl), negatively linked myristoylated EGFP, and positively linked farnesylated EGFP (Maza, 2015)

Recoverin and Rhodopsin Kinase: Association and Implications

Following activation, in order to shut off the activated phototransduction cascade and return to normal rod signaling, activated rhodopsin (Rh*) is targeted by several proteins as to stop downstream effects. Termination of the phototransduction cascade is initiated by the phosphorylation of Rh* by Rhodopsin Kinase 1 (GRK1). Rh* recognizes the N-terminus of GRK1, which sequentially leads to a conformational change in Rh*, such that the C-terminus of Rh* is able to interact with the catalytic groove of GRK1 and be phosphorylated (Komolov, 2010).

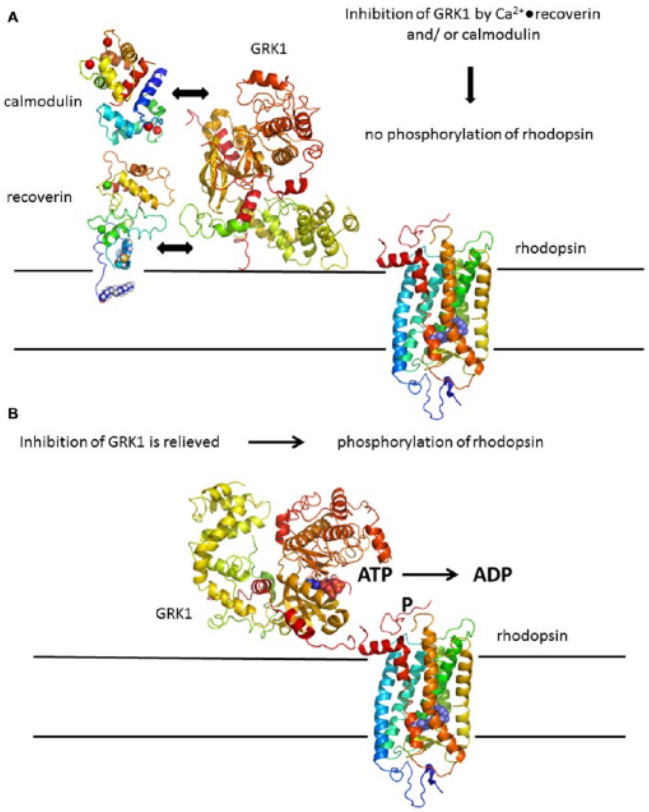


Figure 26: Rhodopsin phosphorylation by GRK1. (A) Deactivation of rhodopsin due to Ca2+ binding to recoverin and inhibition of GRK1 (B) Phosphorylation of rhodopsin by GRK1 following dissociation of recoverin or calmodulin. (Koch, 2015)

This phosphorylation then promotes the binding of another protein - arrestin - to rhodopsin which functions primarily to decrease the coupling of Transducin activation. The events of phosphorylation and arrestin binding serve to deactivate photo-activated rhodopsin faster than decay of the photoproduct and are vital for terminating the cascade. GRK1 selectively phosphorylates light-stimulated rhodopsin at the C-terminus in a calcium dependent manner. Such calcium dependence is due to the interaction between GRK1 and the calcium sensing protein, Recoverin. Recoverin is a myristoylated protein that inhibits GRK1 activity when in the presence of Ca2+ (Koch, 2015). Ca2+ acts as a ligand which enables the extrusion of the myristoyl group and subsequent membrane and GRK1 association. By binding to the N-terminus of GRK1, Ca2+-recoverin inhibits GRK1 exposure of the N-terminus (responsible for the interaction between Rh* and the active site of GRK1) (Zernii, 2011); therefore, Ca2+-recoverin blocks phosphorylation of Rh*.

Recoverin Electrostatic and Lipophilic Properties:

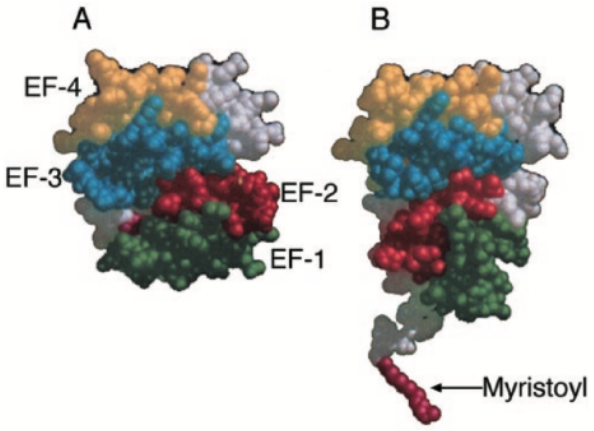


Figure 27: (A) Calcium-free Recoverin (B) Ca^2 -bound state of myristoylated recoverin (Desmeules, 2002)

Dark-adapted rod photoreceptors are depolarized. When the phototransduction cascade is not activated, cGMP cation channels are responsible for maintaining a higher intracellular concentration of Ca2+. This high level of Ca2+ allows for the binding of 2 Ca2+ by the calcium sensing Recoverin. The binding of Ca2+ to Recoverin induces the extrusion of the N-terminal myristoyl group from Recoverin's hydrophobic core (Zernii, 2011), allowing for a conformational change in Recoverin. Ca2+-recoverin preferentially associates with plasma membrane disc and GRK1 and seises the phosphorylation of Rh* by GRK1.

Following the hydrolysis of cGMP by phosphodiesterase, cGMP gated cation channels close, and the concentration of cations such as Ca2+ drops significantly. Ca2+-recoverin interacts with GRK1 in the mechanism shown below - recoverin (yellow) and GRK1 (magenta), where GRK1 occupies a hydrophobic cleft, which in the calcium-free state is responsible for binding the myristoyl moiety (Ames, 1994; Flaherty, 1993, Tanaka, 1995, Ames, 2006). The inward folding of the myristoyl group in calcium-free recoverin dissociates the protein from the membrane, and allows for the phosphorylation of Rh*, which is schematically represented by the figure below.

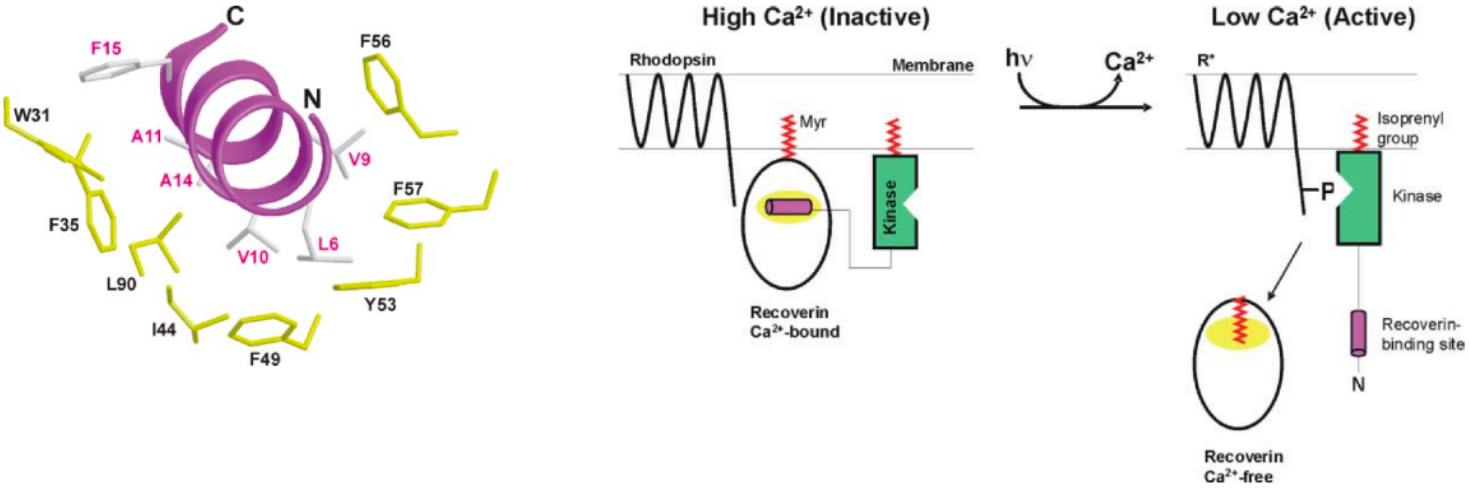


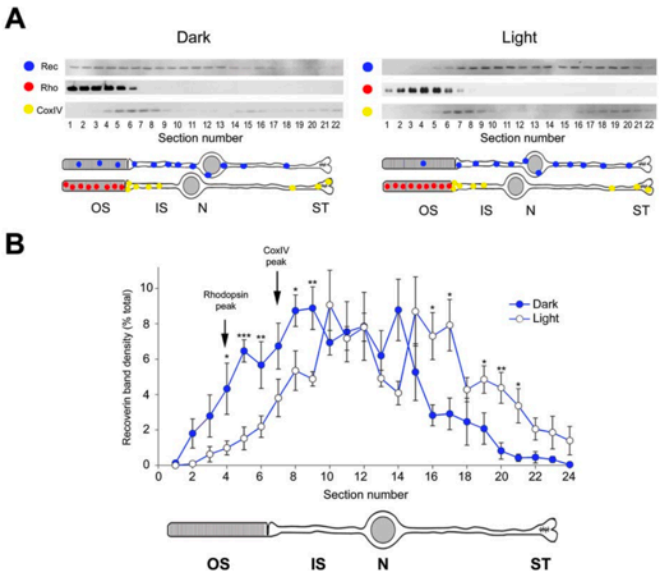
Figure 28: (Left) Interactions between recoverin and hydrophobic region Rhodopsin Kinase with residues RK25. Recoverin (*yellow*) and Rhodopsin Kinase (*magenta*) (Ames, 2006)

Figure 29: (Right) Ca^2 -induced inhibition of rhodopsin kinase represented in model. Ca^2 -bound recoverin binds to both the membrane surface via myristoylation (*red*) and the N-terminus of rhodopsin kinase (*magenta*), blocking the active site of rhodopsin kinase from phosphorylating rhodopsin. When the dark current is stopped by the phototransduction cascade, cytosolic Ca^2 drops. Recoverin undergoes a conformational change into its calcium free form. Rhodopsin kinase is then capable of phosphorylating and deactivating rhodopsin. Ca^2 -free recoverin, following the sequestration of the myristoyl group, dissociates from the membrane surface. (Ames, 2006)

Light Dependent Translocation of Recoverin and Rhodopsin Kinase (GRK1)

It has been shown that Recoverin undergoes a light-dependent transformation which is independent of the translocation of GRK1. In an experiment using retinas of dark- and light-adapted mice, it was observed that Recoverin undergoes light dependent translocation (shown in the figure below).

When compared to light-adapted rods, dark-adapted rods revealed significantly more recoverin in the outer segment (left). The data also showed that although recoverin revealed light-dependent translocation, it's binding partner, GRK1, remained confined to the outer segment regardless of illumination conditions (right).



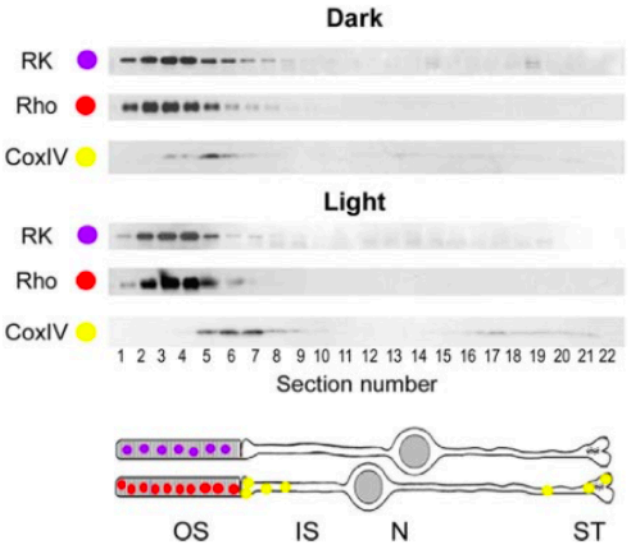


Figure 30: (Left) Recoverin undergoes light-dependent translocation in rods from the outer segment to the inner segment (A) displayed such results by conducting experiments using dark- and light- adapted retinas from mice. Using Western blot to determine relative concentrations of recoverin to rhodopsin cellular markers (indicative of the outer segment), and cytochorome oxidase indicating mitochondria (inner segment). The drawings contrast the distribution of recoverin (blue), rhodopsin (red), and cytochrome oxidase (yellow) where OS *(*outer segment); IS *(*inner segment); N (nucleus); ST (synaptic terminal). (B) the band density distribution of recoverin was averaged as a percentage of the total protein present in each section. (Strissel, 2005)

Figure 31: (Right) Rhodopsin kinase localizes in the outer segment of both dark- and light-adapted mice. Rhodospin kinase (purple*)* (Strissel, 2005)

Electrostatic and Lipophilic Properties of Recoverin: Ca2+-recoverin and calcium-free

In dark-adapted rods, the binding of Ca2+, followed by the extrusion of the myristoyl group and interaction with GRK1 cause Ca2+-recoverin to maintain a membrane associated conformation that maintains recoverin in the outer segment of the rod in greater concentration than in light-adapted conditions, where recoverin is more likely to be calcium-free.

Recoverin is myristoylated by way of acylation of the N-terminal glycine residue. Electrostatically, it is important to note that across species, it can be seen that there is a cluster of positively charged amino acids at the N-terminus. These residues next to the myristoyl group are postulated to interact with the negatively charged disc membranes. When analyzing the cross-species conservation of such residues, complete homology can be seen between K5, K11, K37, R43 of the N-terminus of 5 species (Bos taurus, Human, Mouse, Xenopus Laevis, and Tropicalis) with all protein sequences gathered from Uniprot and shown in supplemental data (Ames, 1995). The four figures below show Cn3D models of Recoverin in Bos taurus, with highlighted domains revealing the sequestration of positive charges in the calcium-free recoverin in the first figure, and the N-terminal Glycine in the middle two. The red and blue residues reveal the negative and positive charges respectively. In the fourth figure, I attempted to determine a general over-view of the apparent surface charge in calcium-free recoverin. The highlighted residues consist of all of the charged residues and the apparent surface charge was -8.

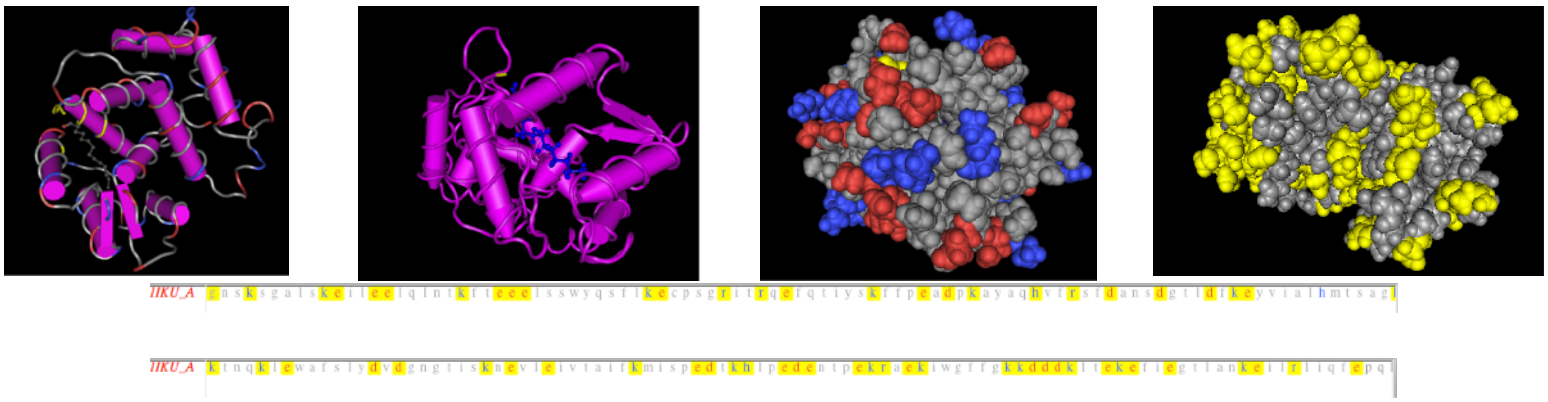


Figure 32: Left to right recoverin Bos Taurus (1IKU), showing the structure and charge distribution of calcium-free recoverin, resulting in sequestration of the myristoyl group. The highlighted residues are the charged external residues

Electrostatic and Lipophilic Properties of Recoverin bound to Rhodopsin Kinase: Ca2+-recoverin

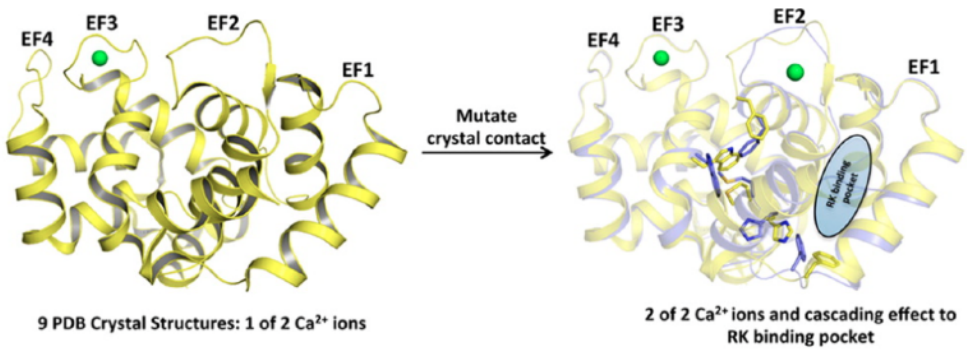


Figure 33: X-ray crystal structure showing calcium ions binding to specific residues within the conformational EF hands of recoverin which mutate the crystal structure and enable the interaction with rhodopsin kinase at the binding pocket (Kumar, 2015)

When recoverin is bound to calcium, it's structural conformation results in a differential charge distribution. The association between the N-terminus of GRK1 and the hydrophobic cleft of recoverin (which shields the myristoyl group in calcium free-recoverin shown by the first figure), results in a significant modification in charge distribution due to the exposure of hydrophobic residues. Recoverin is known to be a member of the EF-hand superfamily, where Ca2+ binds to preferentially to calcium pockets of the Ef-3 and Ef-2 domains. Upon binding to the EF-2 loop, the second coordinated Ca2+ is responsible for the conformational change of recoverin (Kumar, 2015).

Below are four crystal structures which show the differential charge distribution, when analyzing the combined structure of recoverin and GRK1. The three models on the left highlight the N-terminal of recoverin and C-terminal domain of GRK1.

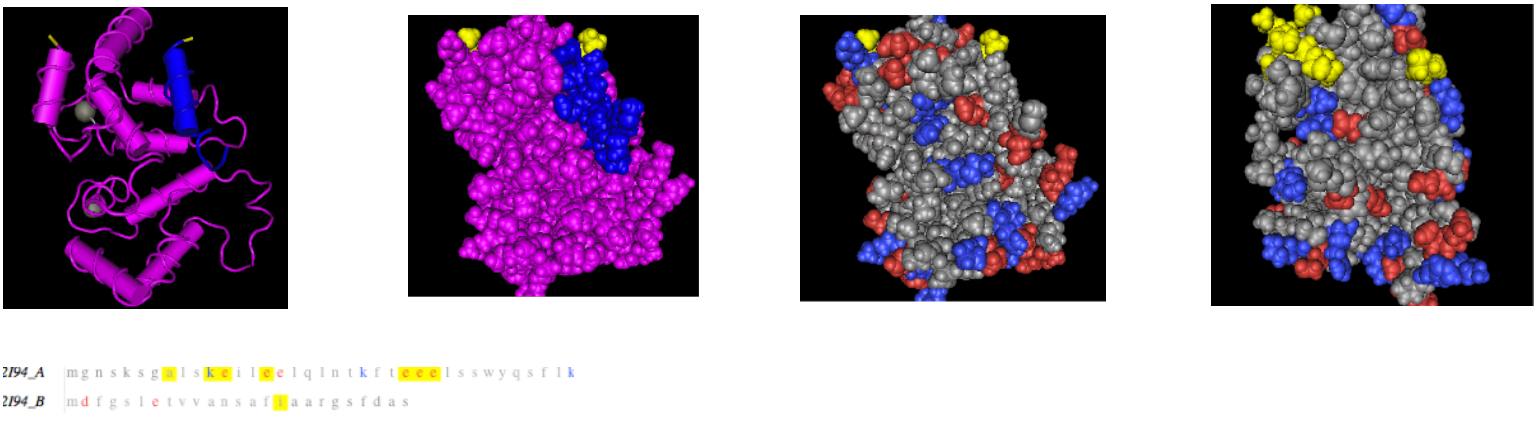


Figure 34: (left to right) Crystal structure of recoverin bound to GRK1 in Bos Taurus (2I94) Recoverin (pink) is bound to two calcium ions, and therefore in it's extended form, bound to GRK1 (blue). Highlighted in yellow are the residues which would flank the myristoyl group, and display a negative charge of approximately -5.

The fourth figure on the right highlights the surface charge of the combined proteins and how the conformational change potentially could impact the electrostatics- based on my analysis of the model, the charge near the myristoyl group decreased upon association with GRK1.

Next, I observed the electrostatic characteristics surrounding the C-terminal region of Rhodopsin Kinase, or the farnesylated area. Using Bovine GRK1 protein sequences to model the kinase, the highlighted region shows the region to which would flank the farnesyl group, if included in the model. When observing the residues of GRK1, the C-terminal region maintains an approximate +3 charge at the point of membrane association.

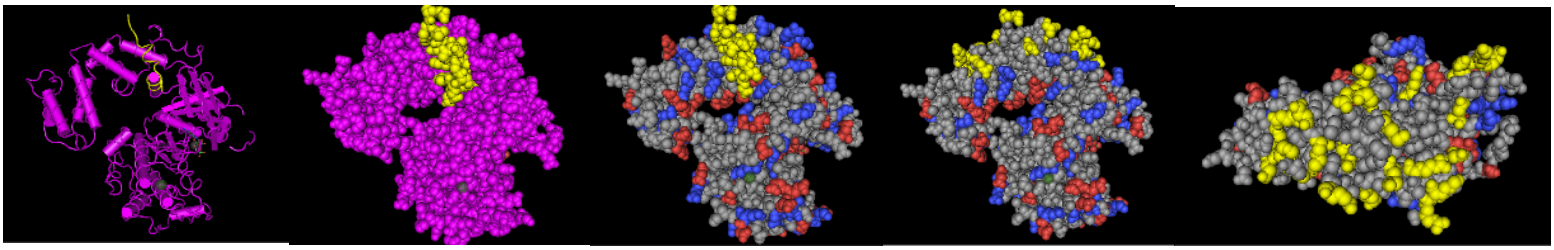


Figure 35: (left to right) Crystal structure of bovine rhodopsin kinase (GKR1) with highlighted C-terminal region where farnesyl group would be.

Relevance to Experimental Evidence:

Understanding properties about the two membrane associated proteins will allow us to further analyze how such electrostatic and lipidiuations might contribute to experimental evidence of the differential translocation of recoverin and Rhodopsin Kinase.

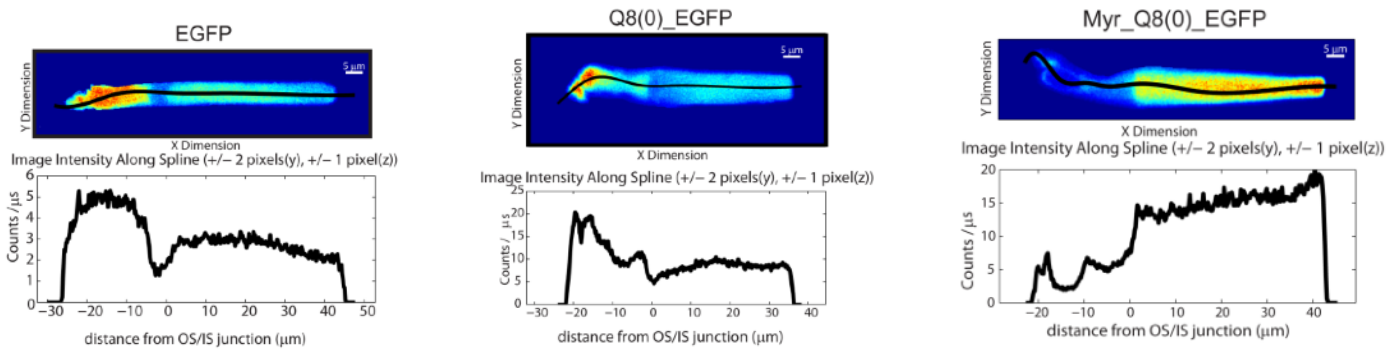


Figure 36: Confocal images of rod photoreceptors with (left to right) soluble EGFP, neutrally linked EGFP, and neutrally myristoylated EGFP. The myristoylated EGFP protein was seen to reveal outer segment localization. (Maza, 2015)

Recoverin, in it's calcium bound form, serves as an interesting protein for comparison to our experimental probes. Ca2+-recoverin maintains both an extended myristoyl moiety with a neighboring positive charge on its N-terminus. Recalling the experimental evidence from the Calvert lab, properties of recoverin can be connected to the probe models to compare if they re-semble translocation seen in vivo.

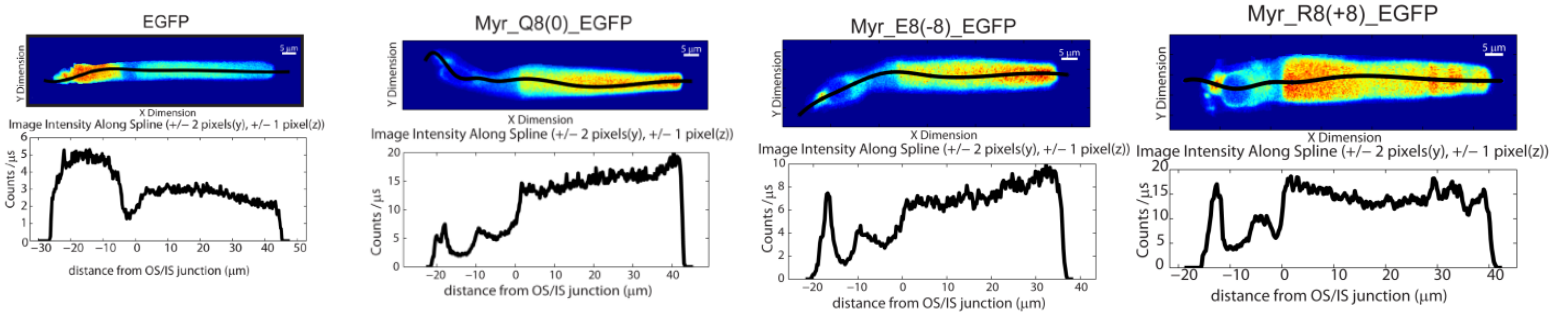


Figure 37: Confocal images of rod photoreceptors with (left to right) soluble EGFP, neutrally, negatively charged, and positively charged myristoylated EGFP. All myristoylated EGFP protein were seen to maintain outer segment localization. (Maza, 2015)

These con-focal images, taken of Xenopus Laevis transgenic rod photoreceptors display the effects of N-terminal myristoylation of a neutral protein on its translocation. In these experiments, it can be observed that the addition of a myristoyl group increases the membrane associative properties of an otherwise soluble protein. Due to the densely membranous outer segment, it is apparent, with an $\alpha=0.01$, that the myristoylation significantly increased outer segment localization.

Recoverin, in addition to being a myristoylated protein, also revealed an apparent positive charge near to the myristoyl moiety. The Calvert lab tested such modifications on lipidated probes, by adding charge to a myristoylated EGFP via incorporation of arginines and glutamic acid for positive and negative charges respectively. In this case, in addition to movement to the outer segment, positively charged Myr_R8(+8)_EGFP revealed synaptic enrichment when compared to the other probes. Although this phenomenon has was not notably explained by the referenced experiments on the translocation of recoverin, there remains potential that the simultaneous modifications are relevant when contrasting to the translocation of proteins.

The visualization of the effects of lipid and charge mediated protein translocation reflect the properties of the light dependent translocation of recoverin. Recoverin within the dark-adapted rods of mice revealed outer segment localization when compared to the light-adapted rods. This can be explained by the sequestration of the lipid moiety following light-adaption and the decrease in intracellular calcium. During light-adaption, recoverin should dissociate from calcium due to it’s intracellular drop in concentration. The myristoyl group of calcium-free recoverin

then “switches” into the hydrophobic cleft of recoverin and dissociates from GRK1 and the membrane. This switch should seemingly temporarily make recoverin translocate in a similar manner to a soluble lipid-free protein. This myristoyl switch of recoverin can explain how the slightly charged lipid-linked protein behaves similar to Myr_R8(+8)_EGFP in dark-adaption, and more like EGFP in light-adaption.

Rhodopsin Kinase on the other hand possess a farnesyl group, and based on my calculations, an apparent positive charge on the flanking the C-terminal region. Experimentation with the lipid-linked probes revealed that the distribution of farnesylated proteins depends heavily on charge. While neutral farnesylated EGFP probes displayed outer segment localization, the addition of a positive (or negative) charge caused the probe to be essentially excluded from the outer segment. This distribution has been postulated to be due to the characteristics of nuclear signaling proteins, which includes the combination of farnesylation and positive charge. However, this charge dependent pattern does not reflect the translocation of GRK1 under light- and dark-adapted rods.

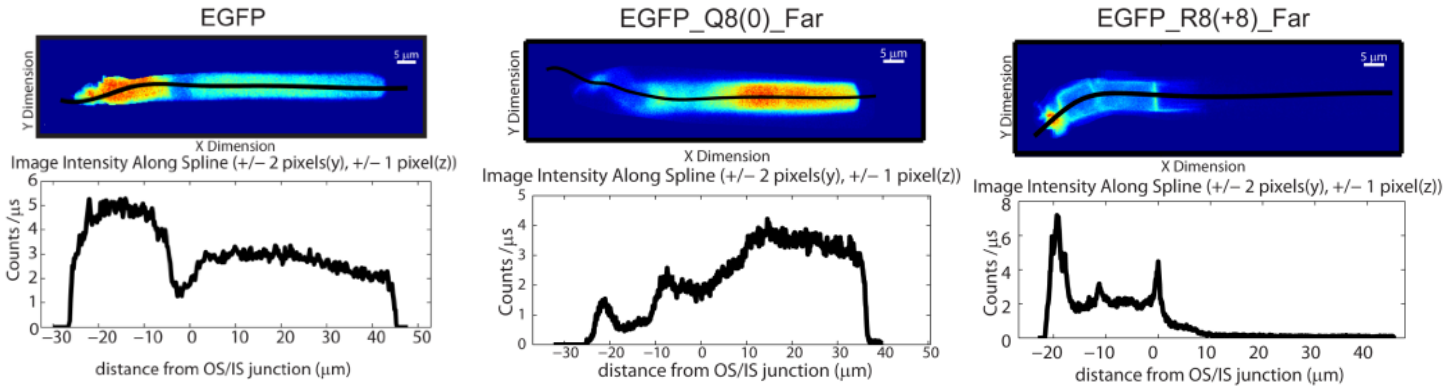


Figure 38: Confocal images of rod photoreceptors with (left to right) soluble EGFP, neutrally linked farnesylated EGFP, and positively charged farnesylated EGFP. The positively charged farnesylated EGFP protein was seen to reveal inner segment or nuclear localization sequences. (Maza, 2015)

GRK1 was shown to be localized to the outer segment independent of the manipulation of illumination. GRK1 outer segment localization could be attributed to its association with other proteins. Despite the apparent charge I found to be localized on the C-terminus of GRK1, there are many contributing factors that might diminish how they might mimic the characteristics of a truly charged farnesylated protein. First, hydrophobic regions of GRK1 are either associated with recoverin or rhodopsin and therefore might not display characteristics that can entirely be explained by its isolated electrostatic and lipid characteristics. Additionally, I used an independent Bovine GRK1 model to assess charge; although the use of models can be effective for generalizations, in vivo experimentation can more realistically display how the impact of potential interactions between the protein might affect its movement. Finally, although my charge quantification does not align with the results of the Calvert lab, assuming either my calculated positive charge is either diminished by interaction, or negligible, the translocation of EGFP_Q8(0)_Far resembles the experimental translocation of GRK1 in vivo.

Conclusions

In conclusion, investigation of the true characteristics of rod photoreceptor proteins following light and dark experimentation in vivo is necessary to assess the validity and reproducibility of protein models. While the models within the Calvert lab use charge, lipid, and size modifications to better understand the translocation of photoreceptor proteins within the biochemical atmosphere of the outer segment, connecting cilium, and inner segment, it is important

to maintain a sense of awareness of alternative characteristics of the proteins involved in phototransduction that differ from the protein models.

From my analysis of current literature displaying the various light dependent and independent translocations of transducin, phosphodiesterase 6, recoverin, and rhodopsin kinase, I can see both similarities and discrepancies between the movements of the model proteins and the proteins observed in their natural state. While gathering more information regarding the interactions between various proteins in the cascade seems to have an effect on protein translocation, one of the primary differences I found seemed to be due to the conformational changes that alter the localization of charge.

Transducin contains an N-terminal myristoyl group, a C-terminal farnesyl group, and an overall neutral charge. We should expect a distribution from Transducin that is similar to the dually lipidated EGFP probe. However, such probe revealed inner segment localization. This comparison of the distribution enhances previously shown research that electrostatics, or the addition of poly-basic charges enhances membrane affinity (Ramasamy, 2015), and that the positive region of the heterotrimer of Transducin must play a role in OS localization rather than simply being dependent on the presence of the two lipids. Gα, a larger subunit, has a myristoyl group extended when included in the heterotrimer, however, following the conformational change it undergoes following the activation and dissociation of the subunits. This conformational change could explain the difference between the distribution of myristoylated model proteins, which should yield concentration in the inner segment. With G$\beta\gamma$, the subunit revealed both a farnesyl group and moderate attraction to the membrane. These co-characteristics of a positive charge and

farnesyl group, should show nuclear localization. However, the movement of G$\beta\gamma$ appears to be both slower and less repelled as the Gα subunit.

When comparing the light dependent translocation of PDE 6, we can see that the dually lipidated PDE 6 localizes to the outer segment in the dark, but localizes to the inner segment during light exposure. This differential distribution suggests that there may be protein-protein interactions that maintain PDE 6 within the outer segment during dark adaption, but release the dually lipidated portion for translocation to the inner segment following light exposure. In our experimental evidence, this pattern of dual lipidation, tested with opposing N terminal and C terminal myristoyl and farnesyl moieties results in inner segment localization. Since PDE 6 following light exposure resembles this, future experiments within the Calvert lab would benefit to assess the dual lipidation that mirrors PDE6. When assessing the difference and localization of these attached lipid moieties, we will be able to see whether or not the light adapted PDE 6 mirrors model probes and determine further potential targets that may be affecting the localization of PDE 6.

Recoverin is a myristoylated protein that inhibits GRK1 activity when in the presence of Ca2+ (Koch, 2015). Ca2+ acts as a ligand which enables the extrusion of the myristoyl group and subsequent membrane and GRK1 association. By binding to the N-terminus of GRK1 Ca2+-recoverin inhibits GRK1 exposure of the N-terminus (responsible for the interaction between Rh* and the active site of GRK1)(In dark-adapted rods, the binding of Ca2+, followed by the extrusion of the myristoyl group and interaction with GRK1 cause Ca2+-recoverin to maintain a membrane associated conformation that maintains recoverin in the outer segment of the rod in

greater concentration than in light-adapted conditions, where recoverin is more likely to be calcium-free.

When permitted to observe major discrepancies in protein localization between the experimental probes and the proteins in the phototransduction cascade themselves, the two major themes of difference seem to depend on the presence of either protein protein interactions or conformational changes. While the protein-protein interactions may be difficult to control for in future experiments without modifying charge or hydrophobicity characteristics, consideration of deletion of specific residues responsible for the interactions to other proteins could be valuable. Additionally, the impact of protein conformational change is important in terms of the difference between protein localization in light adapted conditions compared to dark adapted conditions. Further utilization of experimental probes will enable us as researchers too draw comparisons from genetically engineered experimental models and the translocation of proteins in non-transgenic organisms.

The experiments aimed to elucidate the localization of rod phototransduction proteins are vital to or understanding of the treatment of diseases of cilia. If we are aware of the specific mechanisms to which proteins which enable the initiation and cessation of visual cues, we might be able to one day restore vision due to the understanding of the exact interactions and translocations of proteins. Given this information, treatment options lie in the future, if we are able to elucidate mechanisms that may be responsible for the specific pattern of protein association.

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