Role of "CAAX" protein processing in the retina

Jeffrey Rowan Christiansen
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Role of “CAAX” protein processing in the retina

Jeffrey Rowan Christiansen

Dissertation submitted to the
School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
In
Neuroscience

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Neuroscience Graduate Program
Morgantown, West Virginia
2011

Keywords: prenylation, retinal degeneration, PDE6, RCE1, posttranslational modification
ABSTRACT

Role of “CAAX” protein processing in the retina

Jeffrey Rowan Christiansen

Prenylation is a posttranslational modification which adds a lipid, either a farnesyl or geranylgeranyl group, to the C-terminal cysteine of proteins ending in a “CAAX” motif. Two additional steps referred to as “CAAX” protein processing follow prenylation. First, the terminal three amino acids are cleaved by the endoprotease RAS converting enzyme1 (RCE1), then isoprenylcysteine methyltransferase (ICMT) adds a methyl group to the cysteine. While it is clear that the addition of the prenyl group is required for the association of proteins with membranes, the function of proteolysis and methylation is not obvious. Two lines of evidence suggest a critical role for “CAAX” protein processing, particularly in neurons. First, the brain has the highest methyltransferase activity of all tissues tested and second, mice lacking RCE1 or ICMT are embryonic lethal. The objective of this study is to investigate the importance of “CAAX” protein processing in a neuronal system. The two enzymes responsible for the processing steps were genetically eliminated from the developing retina to evaluate the role of proteolysis and methylation in the stability, assembly, and localization of retinal proteins.

Retinas lacking Rce1 were morphologically similar to littermate controls at early ages. However, by the time the mice open their eyes there is a reduction in the number of photoreceptor cell nuclei which continues until the entire photoreceptor cell layer has degenerated by adulthood. Degeneration is exclusive to photoreceptor cells as other downstream neurons in the retina do not exhibit any signs of cell death in the absence of RCE1-mediated endoproteolysis. The rod and cone photoreceptor neurons remaining at post-natal day (P) 14 do not function as measured by electroretinography (ERG). Our studies also reveal that phosphodiesterase 6 (PDE6), a prenylated heteromeric protein cannot be transported to the outer segment (OS) in the absence of Rce1. This is in contrast to other prenylated proteins, such as the G-protein transducin or rhodopsin kinase, which are transported normally. Our studies conclusively demonstrate that RCE1-mediated endoproteolysis is critical for proper functioning and stability of photoreceptor neurons and more specifically it is needed for transport of PDE6.

In contrast, retinas lacking Icmt showed much more subtle defects than those observed upon elimination of Rce1. Elimination of Icmt message was confirmed by qPCR. To our surprise, no developmental defects were observed even though Six3-Cre eliminates Icmt at early embryonic ages. The earliest noted defect was a reduction in the
rod photoreceptor response as measured by ERG. Loss of rod function is likely a consequence of reduction of prenylated phototransduction proteins, PDE6, GRK1 and most dramatically, transducin. Transducin γ-subunit levels were reduced by 90% in animals lacking Icmt. Interestingly, PDE6 reduction was not associated with a trafficking defect as observed in the conditional knockout of Rce1 in the retina.

These are the first direct investigations of “CAAX” protein processing in an in vivo neuronal system. This is significant because neurons are known to contain a large population of unknown methylated proteins and express “CAAX” processing enzymes at high levels. Our studies are the first to show that RCE1-mediated proteolysis is required for transport of PDE6. In contrast, studies using the conditional knockout of Icmt demonstrate that PDE6 does not require methyl esterification for transport. Our findings suggest the presence of a unique PDE6 transport pathway that is distinct from previously characterized phototransduction protein transport pathways. Another contribution of this research has been to demonstrate that the PDE6 holoenzyme is assembled in the inner segment of photoreceptor cells. Altogether, the results add to our basic understanding of the contributions of posttranslational modifications of proteins to cellular function.
ACKNOWLEDGEMENTS

I would like to express my gratitude to a whole host of people who have been instrumental in supporting me throughout my graduate training. First, I need to thank Vishy for being an excellent mentor and sharing his undying enthusiasm for science. You have taught me many valuable skills that will stay with me throughout life from public speaking to designing well controlled experiments.

I feel lucky to have had the guidance of many other faculty members over the years. From Janet who patiently introduced me to scientific research to the members of my committee who have been very helpful in many aspects from experimental to career advice. Friday morning research meetings in the Eye Institute were like mini-committee meetings. They were a great forum to ask questions, share ideas, and hear what other folks were up to. Spending many an hour in the lab has been made more enjoyable and fruitful by my labmates, Cristy, Ratnesh and Saro. I would also like to thank Lori, who has been extremely helpful in proofreading documents for me at the last minute as well as being a great friend. Martin has been instrumental in sharing mice and revising manuscripts.

Last but not least, Thank You Family. Heather your patience, understanding and undying support have made this adventure possible. I couldn’t have done it without you. Rowan and Shea, thank you for making me laugh and helping me keep everything in perspective. Finally, thanks Mom and Dad. Your creativity, independence and perseverance have inspired me throughout life and have been instrumental in my scientific life as well.
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## Glossary

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CC</td>
<td>Connecting cilia</td>
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<tr>
<td>CKO</td>
<td>Conditional knockout</td>
</tr>
<tr>
<td>CNGA</td>
<td>Cyclic nucleotide gated channel α</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
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<tr>
<td>GCL</td>
<td>Ganglion Cell Layer</td>
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<tr>
<td>GDI</td>
<td>GDP dissociation inhibitor</td>
</tr>
<tr>
<td>GGTaseII</td>
<td>Geranylgeranyl transferase II</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GRK1</td>
<td>Rhodopsin kinase</td>
</tr>
<tr>
<td>ICMT</td>
<td>Isoprenylcysteine methyltransferase</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>IS</td>
<td>Inner segment</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LCA</td>
<td>Lebers congenital amoursis</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>Nm</td>
<td>Nanometers</td>
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<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
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<tr>
<td>OS</td>
<td>Outer segment</td>
</tr>
<tr>
<td>P</td>
<td>Post-natal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDE6</td>
<td>Phosphodiesterase 6</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut agglutinin</td>
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<tr>
<td>PrBP/δ</td>
<td>Prenyl binding protein/δ</td>
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<tr>
<td>PTM</td>
<td>Posttranslational modification</td>
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<tr>
<td>RCE1</td>
<td>RAS-converting enzyme 1</td>
</tr>
<tr>
<td>ROS</td>
<td>Rod outer segment</td>
</tr>
<tr>
<td>RP</td>
<td>Retinitis Pigmentosa</td>
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<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ZMPSTE24</td>
<td>Zinc metalloproteinase sterile 24</td>
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</table>
Chapter 1. Literature Review

I. Prenylation; a lipid posttranslational modification

The cellular milieu in which proteins must interact consists of two diverse environments; the aqueous and the membranous. Hydrophilic proteins utilize various strategies to interact with membranous environments. One such strategy is lipid posttranslational modification (PTM). In general, a lipid group is covalently attached to a protein at a consensus sequence to confer affinity for membrane domains or protein interacting partners. Examples of lipid PTMs are acylation (including palmitoylation and myristoylation), glycosylphosphatidylinositol (GPI) anchoring, and the focus of this review, prenylation.

Prenylation is a posttranslational lipid modification that adds a C\textsubscript{15} farnesyl or C\textsubscript{20} geranygeranyl to proteins terminating in a “CAAX” motif (Glomset, Gelb et al. 1990). A farnesyl group is added to the cysteine if X is serine, methionine, glutamine or alanine. Alternatively, a geranylgeranyl group is added to the C-terminus if the amino acid in the X position is leucine or phenylalanine (Fig. 1, step 1) (Svensson, Casey et al. 2006). Addition of a prenyl group increases the hydrophobicity of proteins, enabling their interaction with membranes (Winter-Vann and Casey 2005). Traditionally, prenyl groups have been thought to directly intercalate into the phospholipid bilayer, however the double bonded nature of prenyl groups makes this interaction less likely (Magee and Seabra 2003). Another role of prenylation is to aid in protein/protein interactions (Marshall 1993). One example of protein/protein interactions being facilitated by prenylation is the interaction between prenylated proteins and proteins with prenyl
binding pockets such as PrBP/δ (Kloog and Cox 2004; Norton, Hosier et al. 2005).

PrBP/δ acts as a chaperone to escort the hydrophobic prenyl moiety to specific membrane compartments.

**Geranylgeranyl transferase II**

In addition to singly geranylgeranylated proteins, there are over 60 RAB proteins ending in “CXC”, “XCC”, “CCXX”, “CCXXX”, or “CCX”, that are doubly geranylgeranylated by RAB Geranylgeranyl transferase II (GGTaseII). GGTaseII is composed of a unique α and β-subunit which binds two geranylgeranyl groups. RAB escort protein (REP) accompanies RAB proteins to the GGTaseII enzyme to catalyze the thioether linkage of the geranylgeranyl groups to both C-terminal cysteines (Figure 2) (Leung, Baron et al. 2006). REP then acts as a chaperone to protect the highly hydrophobic geranylgeranyl residues in transit to their target membrane compartment.

**Prenylated proteins require additional processing for maturation.**

Unlike other lipid modifications, prenylated proteins undergo additional processing steps during the course of maturation. After addition of the prenyl group, the last three amino acids are cleaved by the protease RAS-converting enzyme 1 (RCE1). Subsequently, isoprenylcysteine methyltransferase (ICMT) catalyzes the addition of a methyl group to the newly exposed isoprenylcysteine. “CAAX” processing further increases the hydrophobicity of prenylated proteins and aids in their interaction with other proteins (Wright and Philips 2006). For instance, *in vitro* liposome binding studies
have shown farnesylated proteins exhibit a 20 fold increase in binding affinity when they are methyl esterified (Silvius and l'Heureux 1994).

**RCE1-mediated endoproteolysis**

After prenylation, proteins proceed to the endoplasmic reticulum (ER) for cleavage of the last three amino acids of the “CAAX” motif (Fig. 1, Step 2). RCE1 is the enzyme responsible for endoproteolytic cleavage of “CAAX” proteins. RCE1 is a multipass integral ER membrane zinc metalloproteinase that was identified in yeast in a sterile yeast mutant screen (Zhang and Casey 1996). Mutants were unable to reproduce due to defective postprenylation processing of a-factor mating pheromone. A homolog of RCE1 has also been characterized, AFC1P in yeast or ZMPSTE24 in mammals (Trueblood, Boyartchuk et al. 2000). Substrate specificity is distinctive, but overlapping for these two homologues. For example, in yeast both RCE1 and AFC1P require the aliphatic amino acid V, L, I, C, or M in the a₁ or a₂ position for cleavage, but each protease was also able to process additional CAAX mutants that the other was unable to process (Trueblood, Boyartchuk et al. 2000). In vertebrates the only known substrate for ZMPSTE24 is nuclear lamin A (Barrowman and Michaelis 2009).

The importance of RCE1 in mammals was established through genetic knockout of \( Rce1 \) which resulted in embryonic lethality (Kim, Ambroziak et al. 1999). Embryos survived until embryonic day (E15.5) without any gross morphological defects. Subsequent conditional knockout (CKO) studies revealed the importance of RCE1 in the heart, but liver and bone marrow CKO were unaffected (Bergo, Lieu et al. 2004; Wahlstrom, Cutts et al. 2007; Christiansen, Kolandaivelu et al. 2011). The cause of lethality in the germline and heart knockout of \( Rce1 \) has been suggested to be due to
defects in Ras membrane association (Bergo, Lieu et al. 2004). Indeed, studies investigating the localization of K-Ras in MEFs from germline KO mice have shown that K-Ras is not localized at the plasma membrane (Michaelson, Ali et al. 2005).

**Methyl Esterification**

The final event of “CAAX” protein processing is methyl esterification of the newly exposed isoprenyl cysteine by ICMT (Fig. 1, Step 3) (Hrycyna, Sapperstein et al. 1991). ICMT catalyzes the addition of a methyl group utilizing S-adenosyl methionine (SAM) as the methyl donor. ICMT is a multipass integral ER membrane protein that is present as a dimer or higher order oligomer (Griggs, Hahne et al. 2010). It is a highly conserved protein throughout evolution as the yeast homolog STE14 can be functionally replaced by the human protein (Dai, Choy et al. 1998).

Initially, a milder phenotype was expected from an Icmt KO than the Rce1 KO, because multiple methyltransferase enzymes were predicted. However, elimination of Icmt resulted in embryonic lethality of pups five days earlier than Rce1 KO (Bergo, Leung et al. 2001). In addition to catalyzing the methyl esterification of FTase and GGTase substrates, ICMT also catalyzes the methyl esterification of GGTaseII substrates (Fig. 2). An implication of this result is that ICMT processes more proteins than RCE1 (Bergo, Leung et al. 2001). Another possible cause of increased lethality is that lack of methylation leaves an exposed carboxylate anion adjacent to the prenylated cysteine residue. A negatively charged carboxylate anion in this position has been proposed to inhibit the ability of the prenyl group to interact with membranes leading to a toxicity referred to as the “Carboxylate anion positioning hypothesis” (Bergo, Leung et al. 2001).
Another consequence of Icmt elimination is an increased rate of protein turnover (Bergo, Gavino et al. 2004).

**Methylation, a regulator for signaling pathways?**

Increasingly, it is becoming apparent that a subset of lipid modifications can be regulated in an activity-dependent manner (Rando 1996; Salaun, Greaves et al. 2010). For example, glutamate stimulation of hippocampal cultures increases depalmitoylation of PSD95, leading to endocytosis of AMPA receptors and downregulation of signaling (Fukata and Fukata 2010). Prenylation is another lipid PTM that can potentially be dynamically regulated. Unlike the covalent attachment of the prenyl group, methyl esterification is physiologically reversible (Clarke, Vogel et al. 1988). Recently, a methylesterase responsible for removal of the methyl group from prenylated proteins in *Arabidopsis thaliana* was identified (Deem, Bultema et al. 2006). Reversible methyl esterification in plants is thought to play a role in drought resistance by inactivating negative regulators of abscisic acid (ABA) signaling pathway through demethylation (Huizinga, Omosegbon et al. 2008). Reversibility of carboxyl methylation presents an intriguing possibility for regulation of signaling pathways via methylation.

**Photoreceptor neurons are enriched in prenylated proteins**

Photoreceptor cells are highly specialized neurons capable of detecting a single photon of light and converting it into an electrical response. The unique structure that enables this remarkable feat is a modified cilia with tightly packed membrane stacks referred to as the outer segment (OS) (Fig. 3). The membrane stacks of the OS are
critical for phototransduction because key signaling proteins are integrally and peripherally associated with the membrane. Interestingly, the translational and transcriptional apparatus necessary to synthesize phototransduction proteins is present in a different cellular compartment, the inner segment (IS) (Fig. 3). Transport of proteins and membranes from IS to OS occurs along the connecting cilia (CC) and the membrane sheath surrounding the CC. Maintenance of the OS is energetically demanding due to continual signaling and the replacement of the entire OS and its contents every 10 days (Young 1967; Linton, Holzhausen et al. 2010). Replacement is necessary due to phagocytosis of approximately 100 OS discs including the associated lipids and proteins every day by the retinal pigmented epithelium (RPE) (LaVail 1976; Besharse and Hollyfield 1979).

Two types of photoreceptor cells are present in the retina, rods to detect light in low light conditions and cones for detection of color and light under bright light conditions. Both cell types utilize similar signal transduction pathways, but the specific proteins and their posttranslational modifications differ between rod and cone cells. Phototransduction pathway is initiated when a photon of light alters the conformation of an opsin molecule through conversion of chromophore, 11-cis retinal to all-trans retinal (Yoshizawa and Wald 1963; Palczewski, Kumasaka et al. 2000). Activated opsin exchanges GDP for GTP on heterotrimeric G-protein transducin α-subunit (G$_\text{at1}$) (Fig. 4) (Kwok-Keung Fung and Stryer 1980; Fung, Hurley et al. 1981). GTP-bound G$_\text{at1}$ dissociates from G$_{\beta\gamma}$ and relieves the inhibitory constraint imposed by phosphodiesterase6 (PDE6) γ–subunit on the catalytic PDE6α β–subunits (Hurley and Stryer 1982; Deterre, Bigay et al. 1988). Hydrolysis of cGMP by activated PDE6 leads
to lower levels of cGMP in photoreceptor OS. Reduction of cGMP closes cyclic nucleotide gated channel (CNGA), causing the photoreceptor cell to become hyperpolarized and initiate downstream signaling (Fesenko, Kolesnikov et al. 1985; Burns and Arshavsky 2005). Rhodopsin kinase (GRK1) attenuates rhodopsin signaling by phosphorylating rhodopsin (Kuhn and Wilden 1982). Further attenuation of rhodopsin signaling is achieved by binding of arrestin (Ar) to phosphorylated rhodopsin (Wilden, Hall et al. 1986). Three of the above proteins, Gγ1, PDE6αβ, and GRK1, are prenylated and undergo “CAAX” protein processing (Swanson and Applebury 1983; Anant, Ong et al. 1992; Inglese, Koch et al. 1992).

**Transducin**

Amplification of signaling requires proper assembly and localization of transducin in relationship to its signaling partners. This is likely achieved through a series of PTMs which includes myristoylation of the catalytic Gαt1-subunit, and farnesylation of the Gγ1-subunit of the obligate βγ heterodimer (Fukada, Takao et al. 1990). Peripheral membrane proteins generally require at least two lipophilic signals for membrane association (Hancock, Cadwallader et al. 1991). Several lines of evidence suggest lipid PTMs of transducin are essential for its membrane association. First, the absence of the myristoyl group on Gαt1 resulted in a gross mislocalization as well as a marked reduction in phototransduction (Kerov, Rubin et al. 2007). Furthermore, genetic elimination of Gγ1 resulted in increased solubility of Gαt1 and decreased amplification of the signal transduction cascade (Kolesnikov, Rikimaru et al. 2011). The prenyl group alone is insufficient for assembly and localization of heterotrimeric G-proteins as *in vitro* studies utilizing ICMT-/- fibroblasts demonstrated farnesylated Gγ1 requires methyl
esterification for endomembrane association, while the more hydrophobic geranylgeranylated \( G_{\gamma2} \) does not (Michaelson, Ahearn et al. 2002). Collectively, these data suggest that rod transducin requires myristoylation of \( G_{\alpha1} \) and complete “CAAX” processing of \( G_{\gamma1} \) for membrane localization as none of the modifications alone are sufficient.

**Phosphodiesterase6 (PDE6):**

PDE6 the effector enzyme downstream of transducin is a heterotetramer composed of a farnesylated \( \alpha \)−subunit, a geranylgeranylated \( \beta \)−subunit as well as two inhibitory \( \gamma \)−subunits (Baehr, Devlin et al. 1979; Anant, Ong et al. 1992). PDE6 is the only known protein to be differentially prenylated, however the contributions of differential prenylation to PDE6 function have not been conclusively determined (Ong, Ota et al. 1989).

The final two steps of “CAAX” protein processing also appear to be critical to PDE6 transport and maintenance in the OS. Purification of PDE6 from bovine retinal extracts identified a putative fourth subunit, later referred to as prenyl binding protein/\( \delta \) (PrBP/\( \delta \)) (Gillespie, Prusti et al. 1989). PrBP/\( \delta \) has been shown to be able to extract PDE6 from rod outer segment membrane preparations and is thought to be important for PDE6 transport to the OS in cones (Zhang, Hosier et al. 2005; Zhang, Li et al. 2007). To identify the site of interaction between PDE6 and PrBP/\( \delta \), peptides corresponding to the last six amino acids of PDE6 catalytic subunits were tested for binding affinity with PrBP/\( \delta \). Prenylated and methylated peptides were able to interact with PrBP/\( \delta \); however lack of either prenylation or methyl esterification was sufficient to
abolish this interaction in vitro (Cook, Ghomashchi et al. 2000). Interestingly, absence of ICMT-mediated methyl esterification did not affect the transport of PDE6 to OS, but did affect PDE6 protein stability in the OS (Chapter 3). Altogether, these results implicate the importance of prenylation and postprenylation processing in the transport, maintenance and function of PDE6.

In contrast to rod PDE6, cone photoreceptor cells express a catalytic core of PDE6 α’ as a homodimer. In addition, cone PDE6 catalytic subunits are thought to be geranylgeranylated. Recent studies indicate that rod and cone PDE6 catalytic subunits are interchangeable in cone photoreceptor cells (Kolandaivelu, Chang et al. 2011).

**Rhodopsin kinase (GRK1):**

Rhodopsin signaling is inactivated by phosphorylation of rhodopsin by GRK1. Phosphorylation allows for the binding of arrestin which blocks rhodopsin’s interaction with transducin (Fig. 4). GRK1 is a farnesylated and methyl esterified monomeric kinase whose ability to phosphorylates rhodopsin is enhanced by its PTMs (Inglese, Glickman et al. 1992) (Inglese, Koch et al. 1992). Mutations in GRK1 lead to Oguchi disease in humans, a form of congenital stationary night blindness (Chen, Burns et al. 1999). Interestingly, elimination of PrBP/δ in mouse retina leads to severe reduction in GRK1 levels. (Zhang, Li et al. 2007). PrBP/δ binds to multiple prenylated proteins, but the specific loss of GRK1 suggests a requirement for prenylation and/or postprenylation processing in the maintenance and transport of GRK1.
Defects in prenylation lead to retinal degenerative diseases:

The importance of prenylation in photoreceptor neurons is highlighted by the fact that defects in this process cause choroideremia, a disease primarily affecting rod photoreceptor cells (Pereira-Leal, Hume et al. 2001). In addition, mutations in aryl-hydrocarbon interacting protein-like 1 (AIPL1), a protein thought to enhance farnesylation of proteins causes a severe childhood blinding disease called Leber congenital amaurosis (LCA) (Ramamurthy, Niemi et al. 2004). Further evidence for the importance of prenylation in retinal function comes from a study showing a link between changes in rod PDEβ subunit and rod-cone dysplasia (Petersen-Jones, Entz et al. 1999). In this canine model for retinitis pigmentosa (RP), a non-sense mutation that removed the last 49 amino acid residues including the “CAAX” box resulted in non-functional PDE and severe degeneration of rod photoreceptors (Suber, Pittler et al. 1993). Clinical evidence suggests PDE6 requires differential prenylation because a patient with night blindness had a mutation in the final amino acid of the PDEβ gene (Veske, Orth et al. 1995). The patient was diagnosed with RP at age 12, had difficulty seeing at night, but no loss of visual acuity or color vision. The mutation would be predicted to alter the prenyl group from a geranylgeranyl group to a farnesyl group (Trueblood, Boyartchuk et al. 2000). Animal models that replicate a similar mutation in PDE6 β subunit show progressive rod cell degeneration and dysfunction (Singh, R and Ramamurthy, V, unpublished studies). These studies show the importance of differential prenylation and/or geranylgeranylation of PDE6 in proper functioning of rod cells.
Objective

Daily shedding of OS discs necessitates rapid and efficient vectorial transport of proteins synthesized in the IS along the narrow connecting cilia to repopulate the OS. Protein-lipid modifications likely play a crucial role in assembly of multimeric protein complexes and their transport and maintenance in the proper location and orientation in the OS disc membranes. Among various lipid modifications, the following two studies focus on deciphering the importance of the “CAAX” protein processing steps that follow prenylation in maintenance and function of photoreceptor cells. Study 1 described in Chapter 2 is an analysis of a retina conditional knockout of the “CAAX”-protease, RCE1. Retinas from animals lacking Rce1 were assessed for their developmental profile, function as measured by ERG, and protein expression pattern. Study 2 described in Chapter 3 compares the elimination of the “CAAX”-methyltransferase, ICMT, from the retina to the phenotype observed in the elimination of Rce1. Chapter 4 concludes with a summary of the findings from both studies and the implications of those results.
Fig. 1. Schematic representation of prenylation and postprenylation processing. Phosphodiesterase6 (PDE6α) is farnesylated. (1) Cytosolic farnesyl transferase (FTase) adds a farnesyl (FAR) to the cysteine of the CAAX motif. (2) RCE1-mediated endoproteolysis cleaves the last three AAX amino acids. (3) ICMT catalyzes the methyl esterification of the prenylcysteine residue. The methyl donor S-adenosyl-methionine (SAM) is converted into S-adenosyl-homocysteine (SAH). Adapted from (Wright and Philips 2006).
Fig. 2. RAB Geranylgeranyl transferase pathway. RAB proteins are escorted through the cytosol by RAB escort protein (REP1) to geranylgeranyl transferase II enzyme (GGTaseII). GGTaseII adds a geranylgeranyl (GG) group to each of the cysteines before RAB proteins are transported to the endoplasmic reticulum (ER). Unlike GGTase I substrates, RABs are directly methylated, skipping the proteolysis step. REP1 is then displaced by RAB GDP Dissociation Inhibitor (GDI) to deliver RABs to various membrane compartments. Adapted from (Konstantinopoulos, Karamouzis et al. 2007).
Rod photoreceptor neurons are polarized cells. Photons are absorbed by rhodopsin in the disc membranes of the outer segment (OS). Phototransduction results in membrane hyperpolarization that is transmitted to bipolar cells at the synaptic terminal. Proteins are synthesized and processed in the inner segment (IS). Transport of proteins occurs along the connecting cilia (CC) at the transition zone. Retinal pigmented epithelium (RPE) cells phagocytose OS disc membranes. Nucleus is present in the cell body. (Adapted from (Liu, Tan et al. 2007)).
Fig. 4. Rod phototransduction cascade. Light-activated rhodopsin interacts with heterotrimeric transducin resulting in the exchange of GDP for GTP on Tα. Nucleotide exchange results in dissociation of Tα and the obligate βγ heterodimer. GTP-bound cytosolic Tα activates PDE6, resulting in hydrolysis of cGMP produced by guanylyl cyclase. Intracellular concentrations of cGMP are lowered leading to closure of CNGA channels and hyperpolarization of the plasma membrane. (Adapted from (Farber 1995))
Chapter 2. Study 1: RCE1-mediated endoproteolysis is required for trafficking of rod PDE6 to photoreceptor outer segments.

Abstract

Prenylation is the posttranslational modification of a carboxyl-terminal cysteine residue of proteins that terminate with a “CAAX” motif. Following prenylation, the last three amino acids are cleaved off by the endoprotease, RCE1, and the prenylcysteine residue is methylated. Although it is clear that prenylation increases membrane affinity of CAAX proteins, less is known about the importance of the postprenylation processing steps. RCE1 function has been studied in a variety of tissues but not in neuronal cells. To approach this issue, we generated mice lacking Rce1 in the retina. Retinal development proceeded normally in the absence of Rce1 but photoreceptor cells failed to respond to light and subsequently degenerated in a rapid fashion. In contrast, the inner nuclear and ganglion cell layers were unaffected. We found that the multimeric rod phosphodiesterase PDE6, a prenylated protein and RCE1 substrate, was unable to be transported to the outer segments in Rce1-deficient photoreceptor cells. PDE6 present in the inner segment of Rce1-deficient photoreceptor cells was assembled and functional. Synthesis and transport of transducin, and GRK1, also prenylated substrates of RCE1,
was unaffected by *Rce1* deficiency. We conclude that RCE1 is essential for the intracellular trafficking of PDE6 and survival of photoreceptor cells.
Introduction

Posttranslational modifications increase a protein’s functional repertoire, regulating protein–protein interactions and targeting to cellular components. One such posttranslational modification is prenylation, a three-step process which first adds a farnesyl or geranylgeranyl lipid to the C-terminal cysteine of proteins ending in a “CAAX” motif (C-Cysteine, A-Aliphatic amino acid, X- any amino acid residue) (Glomset, Gelb et al. 1990; Zhang and Casey 1996; Lane and Beese 2006). The next step is proteolysis of the last three amino acids (-AAX) by the protease RAS-converting enzyme 1 (RCE1) or zinc metalloproteinase sterile-24 homologue (ZMPSTE24) (Wright and Philips 2006; Barrowman and Michaelis 2009). The final step is the methyl esterification of the newly exposed isoprenylcysteine residue catalyzed by isoprenylcysteine carboxyl methyltransferase (ICMT) (Wright and Philips 2006).

Prenylation enables peripheral membrane proteins to interact with membranes and facilitates their interaction with protein partners (Zhang and Casey 1996). The significance of the proteolysis and methylation steps is not entirely clear (Wright and Philips 2006). In general, farnesylated proteins require postprenylation processing for proper localization, while the more hydrophobic geranylgeranylated proteins do not (Michaelson, Ali et al. 2005). Germline knockout of Rce1 or Icmnt results in embryonic lethality in mice demonstrating the importance of these processing steps during development (Kim, Ambroziak et al. 1999; Bergo, Leung et al. 2001). Subsequent conditional knockout studies revealed that proteolysis is crucial in the heart but is dispensable in liver and bone marrow (Bergo, Lieu et al. 2004; Wahlstrom, Cutts et al.)
Although Rce1 is expressed in the brain and eye, the role of RCE1-mediated endoproteolysis in neuronal tissue in vivo is not known (Kim, Ambroziak et al. 1999).

Several phototransduction signaling proteins in the retina are prenylated RCE1 substrates. These proteins include transducin (Tγ), rhodopsin kinase (GRK1), and phosphodiesterase 6 (PDE6) catalytic subunits (αβ) (Swanson and Applebury 1983; Ong, Ota et al. 1989; Fukada, Takao et al. 1990; Anant, Ong et al. 1992; Inglese, Koch et al. 1992; Qin and Baehr 1994). Transducin, a heterotrimeric G-protein in the photoreceptor cells, relays the light response from the activated G-protein coupled receptor (GPCR) rhodopsin to the effector enzyme PDE6 (Burns and Arshavsky 2005). The transducin heterotrimer consists of a myristoylated Gαt1-subunit (Tα), a Gβ1-subunit (Tβ), and a farnesylated Gγ1-subunit (Fukada, Takao et al. 1990). In vitro studies show that methyl esterification of Gγ1 is required for efficient assembly of the heterotrimer as well as targeting to the plasma membrane (Fukada, Matsuda et al. 1994; Michaelson, Ahearn et al. 2002).

PDE6 is the phototransduction effector enzyme that hydrolyzes cGMP to GMP in response to light-activated transducin (Burns and Arshavsky 2005). Rod PDE6 heterotetramer is composed of catalytic α and β-subunits and two inhibitory γ-subunits (Wensel 2008). A unique feature of PDE6 is the differential prenylation of catalytic subunits: the α-subunit is farnesylated and the β-subunit geranylgeranylated (Anant, Ong et al. 1992). In contrast, the catalytic subunit of cone PDE6, a homodimer is thought to be geranylgeranylated (Wensel 2008). The importance of PDE6 prenylation in retinal function is implied by studies on a canine model for retinitis pigmentosa (Suber, Pittler et al. 1993). The disease is caused by a nonsense mutation in PDE6 that truncates the C-
terminal 49 amino acid residues including the “CAAX” box. This results in an unstable protein and leads to degeneration of photoreceptor cells (Suber, Pittler et al. 1993). Lack of a functional heterologous expression system and suitable animal models has hampered the in-depth analysis needed to understand the role of postprenylation processing in PDE6 function (Muradov, Boyd et al. 2010).

The role of prenylation and methyl esterification of proteins in the maintenance of retinal neurons has been investigated by injecting Lovastatin, an inhibitor of HMG-CoA reductase and N-acetyl-S-farnesyl-L-cysteine (AFC), an inhibitor of ICMT, into the eye (Pittler, Fliesler et al. 1995). After four days, the retinas were severely disorganized and formed rosette-like structures (Pittler, Fliesler et al. 1995). However, the results of the study are confounded by the fact that lovastatin and AFC may also affect endothelial cells in blood vessels, leading to apoptosis of photoreceptor cells (Kramer, Harrington et al. 2003). Consistent with this idea, blood vessel shunting was observed in injected eyes (Pittler, Fliesler et al. 1995).

To define the importance of postprenylation processing of CAAX proteins in the eye, we used a genetic approach to eliminate RCE1-mediated proteolysis and subsequent methyl esterification specifically in retinal neurons. To accomplish this, we bred conditional Rce1 knockout mice on a background of a retina-specific Cre transgene and analyzed the impact of Rce1 deficiency in retinal neurons.
Materials and Methods

Animal Models. Rce1^{fl/fl} mice were described previously (Bergo, Ambroziak et al. 2002; Bergo, Lieu et al. 2004). To enhance the efficiency of recombination by Six3-Cre, we generated heterozygous Rce1^{wt/null} mice by crossing Rce1^{fl/fl} mice with transgenic mice expressing Cre under a ubiquitous β-actin promoter (Meyers, Lewandoski et al. 1998). Rce1^{wt/null} mice developed normally and did not exhibit any visual deficits. The Rce1^{wt/null} mice were crossed with Six3-Cre mice to obtain Rce1^{wt/null}Six3-Cre mice. These mice were then crossed with Rce1^{fl/fl} mice for experimental and control samples. Aipl1^{null/null} mice were previously described (Ramamurthy, Niemi et al. 2004).

Message Analysis. Retinas, dissected from freshly enucleated eyes, were flash frozen in dry ice in the presence of Trizol (Invitrogen). RNA was extracted from frozen retinas and used to generate cDNA using SuperScript III (Invitrogen). 300 μg of cDNA from heterozygous and knockout littermates in triplicate was used as a template for Quantitative PCR using MyiQ PCR cycler (Bio-Rad) and MyiQ SYBR Green Supermix (Bio-Rad). Rce1 was amplified using primers 5’-AGTGTGGGAAGTATCTTCGTGTCT-3’ and 5’-CTGTTCTTTCCAAAAGCATACAAA-3’ to generate a 289-base pair (bp) product. Zinc metalloproteinase sterile 24 (Zmpste24), the homologue of Rce1, was amplified using primers 5’-TGCCTGGCTGTTCACATTAG-3’ and 5’-TCACTGTCCCCTTCACCTTC-3’ to generate a 336 bp product. Threshold values were normalized to hypoxanthine phosphoribosyltransferase (Hprt) gene expression levels with the following primers 5’-CAAACCTTGCTTTCCCTGTGTG-3’ and 5’-CAAGGGCATATCCCAACAACA-3’ (250-bp product).
Histology. Mice were euthanized by CO$_2$ inhalation and eyes were enucleated. A 2-mm hole was made at the corneal limbus and eyes were fixed (1.6% paraformaldehyde, 2.5% glutaraldehyde, 0.05% MgCl$_2$, 0.04 M sucrose in 0.08 M PIPES buffer pH 7.0) for 5 minutes before dissecting the anterior chamber and removing the lens. Eyecups for semi-thin plastic sections were prepared according to JB-4 (Electron Microscopy Sciences) embedding protocol. In brief, eyecups were fixed for 12 hours at 4 °C. Following a 30 minute wash in phosphate-buffered saline (PBS), eyecups were dehydrated in a graded alcohol series. Two changes of infiltration solution were performed before an overnight incubation in fresh infiltration solution. Eyecups were embedded in JB-4 in a BEEM capsule (Electron Microscopy Sciences) on ice. 3 μm sections were cut on a Leica ultramicrotome and mounted on Superfrost plus slides (Fisher Scientific). Hematoxylin and eosin (H&E) staining was carried out by the WVU pathology department and images were captured on an Olympus AX70 transmitted light microscope.

Immunohistochemistry. Eyecups for cryosections were dissected as previously stated, then fixed for 2 hours in 4% paraformaldehyde in PBS before cryoprotection in 30% sucrose overnight at 4 °C. Eyecups were embedded in Tissue-Tek O.C.T. Compound (Sakura) and fast frozen in dry ice ethanol bath. Blocks were sectioned with a Leica CM1850 Cryostat and 15-20 μm sections were mounted on Superfrost plus slides. Cryosections were washed in PBS, and then incubated in blocking buffer (2 % goat serum (Invitrogen), 0.1% Triton X-100, and 0.05% sodium azide in PBS) for 1 hour. Primary antibodies were incubated for 2 hours at room temperature or overnight at 4 °C. Excess antibody was removed by three 10 minute washes in PBST (PBS, 0.1% Triton X-100) before incubation with secondary antibody for 1 hour at room temperature. Slides
were washed twice for 10 minutes with PBST and for 10 minutes in PBS. ProLong Gold antifade reagent (Invitrogen) was applied to each section then coverslips were mounted. Images were collected on a Zeiss LSM 510 Meta confocal microscope using 488, 543 and 633 nm laser lines. The following antibodies were used: anti-CNGA1/3 (UC Davis/NIH NeuroMab Facility); anti-PDE β (Affinity Bioreagents); anti-Tγ (Santa Cruz); anti-GRK1 (Thermo Fisher); anti-GC-E (David Garbers). TO-PRO-3 nuclear stain (Invitrogen) was added to dilutions of Alexa Fluor secondary antibodies (Invitrogen) in antibody dilution buffer (0.05% goat serum, 0.1% Triton X-100, and 0.05% sodium azide in 1X PBS).

**Electroretinography (ERG).** Littermates were dark adapted overnight, then eyes were dilated (1:1 Phenylephrine: Tropicamide) for 10 minutes. Isoflurane anesthesia (1.5% in 2.5% oxygen) was administered via nose cone on a 37 °C platform. A reference electrode was placed subcutaneously in the scalp and silver wire electrodes were positioned above the cornea, with contact being made by methylcellulose solution. Light flashes were presented by placing the mouse in a Ganzfield apparatus. Corneal evoked potentials were collected using UTAS-E4000 Visual Electrodiagnostic Test System and EMWIN 8.1.1 software (LKC Technologies). Background light (30 cd · m⁻²) was presented for 10 minutes before recording flicker responses in the presence of the background light. Grabdata (Dr. Machelle Pardue, Emory University) was used to import raw data into Excel (Microsoft) for final analysis of response curves. Representative waveforms are shown.

**Immunoblot.** Flash frozen retinal samples were solubilized in 6 M Urea buffer (6 M urea, 4% SDS, 0.5 M Tris pH 6.8, 10 mg/ml DTT, and 2% Bromophenol Blue) using
sonication for 6 pulses of 20 milliseconds at power setting 6 (Misonix XL-2000). The protein concentration was estimated using a NanoDrop (Thermo Scientific) spectrophotometer. 150 μg total protein samples were resolved on 4-20% Criterion (Bio-Rad) polyacrylamide gels. Proteins were then transferred to Immobilon-FL membrane (Millipore) and probed with primary antibodies against desired target proteins. The following antibodies were used; anti-PDE6αβγ (MOE, Cytosignal), anti-Tγ (Santa Cruz), anti-GRK1 (Thermo Fisher), anti-Rab8 (BD Bioscience), anti-PDE6α’ (Kirschman, Kolandaivelu et al. 2010), anti-Rhodopsin (Robert Molday), anti-Tα (Santa Cruz), anti-Tβ (Affinity Bioreagents), anti-Cone Tα (Santa Cruz), anti-GC-E (James Hurley), anti-arrestin (Affinity Bioreagents), anti-peripherin (Gabriel Travis), anti-AIPL1 (Ramamurthy, Roberts et al. 2003), anti-GOα (Santa Cruz), and anti-PKCα (Affinity Bioreagents). Odyssey goat anti-rabbit Alexa 680 and Odyssey goat anti-mouse Alexa 680 secondary antibodies (LI-COR Biosciences) were used at 1:50,000 dilutions to label primary antibodies. Membranes were scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences). Images are representative from at least three independent experiments.

**Immunoprecipitation.** PDE6 assembly was assessed by immunoprecipitation with ROS-1 monoclonal antibody as previously described (Kolandaivelu, Huang et al. 2009). Briefly, retinas were homogenized in PBS containing Complete Mini EDTA-Free protease inhibitor tablet (Roche Biochemicals) using a pellet pestle (VWR) in an Eppendorf tube on ice. To solubilize the proteins, 1% Triton X-100 was added and samples were nutated for 30 minutes at 4 °C. Samples were clarified by centrifugation for 5 minutes at 10,000 x g. Supernatants from the previous centrifugation step were
incubated with ROS-1 coupled Protein A/G beads for 3 hours. Unbound proteins were removed and beads were washed in Triton buffer (1% Triton X-100, 50 mM Tris pH 7.5, 300 mM NaCl, 5 mM EDTA, 0.02% NaN₃). PDE6 subunits bound to ROS-1 were eluted from the beads by adding SDS-PAGE sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue, 5% 2-mercaptoethanol) and boiling for 5 minutes. Eluates were resolved on polyacrylamide gels and immunblotted as above with MOE antibody (1:4000) which recognizes rod PDE6 α, β, and γ-subunits.

**PDE Activity Assay.** Cyclic GMP hydrolysis by trypsin-activated PDE6 was performed as previously described (Hurley and Stryer 1982; Ramamurthy, Niemi et al. 2004; MacKenzie 2010). Briefly, two retinas were homogenized with a pellet-pestle (VWR) in 100 μl ROS buffer (20 mM Hepes pH 7.2, 2 mM MgCl₂, 60 mM KCl, 30 mM NaCl, 1 mM DTT, 0.1 mM ATP). 5 μg of retinal homogenate was treated with trypsin (Sigma), final concentration 40 μg/ml, at room temperature for 2 minutes. Reactions were stopped by the addition of five-fold excess of soybean trypsin inhibitor (Sigma). [³H]-cGMP (Perkin Elmer) in Tris-buffered cGMP (100 mM Tris pH 8.0, 4.8 mM MgCl₂, 60 mM cGMP) was added to each sample and incubated at 30 °C for 4 minutes before boiling for 3 minutes. Snake venom (Sigma) was then added to each reaction for 15 minutes at 30 °C to cleave the phosphate group from 5’GMP. Samples were mixed with Dowex (Sigma) ion exchange column and incubated for 30 minutes (MacKenzie 2010). After centrifugation the supernatant was added to scintillation fluid and counted on a Wallacs 1410 liquid scintillation counter.

**cGMP Assay.** Two retinas per sample were homogenized in 0.1 N HCl and protein concentration was estimated using NanoDrop spectrophotometer. Retinal homogenates
were boiled for 5 minutes and clarified by centrifugation at 6,000 x g for 5 minutes at 4
°C. Supernatant was neutralized in 0.5 M Tris pH 8.0. Equal amounts of samples (1 mg
of total protein) were used to measure cyclic GMP levels using the Direct Cyclic GMP
Enzyme Immunoassay Kit (Assay Designs) as described by the manufacturer.
Results

Generation of Mice Lacking \textit{Rce1} in the Retina. To eliminate \textit{Rce1} expression in the retina we used mice carrying the conditional \textit{Rce1}^{fl} allele and the \textit{Six3-Cre} transgene mice which express Cre recombinase in cells of the nascent neural retina beginning at embryonic day 10 (Furuta, Lagutin et al. 2000; Bergo, Ambroziak et al. 2002). The efficiency of Cre recombination was assessed by breeding the \textit{Six3-Cre} mice with a tdTomato reporter line (Fig. 1) (Madisen, Zwingman et al. 2009). Uniform expression of tdTomato was observed throughout all cell layers in retinas of adult mice, demonstrating efficient Cre-mediated recombination. We bred \textit{Rce1}^{wt/null}\textit{Six3-Cre} males with \textit{Rce1}^{fl/fl} females to generate \textit{Rce1}^{fl/null}\textit{Six3-Cre} mice (designated \textit{Rce1}^{−/−}). Littermate \textit{Rce1}^{fl/wt}\textit{Six3-Cre}, \textit{Rce1}^{fl/wt}, and \textit{Rce1}^{fl/null} mice (collectively designated \textit{Rce1}^{+/−}) were indistinguishable from wild-type mice and were used as controls. \textit{Rce1}^{+/−} and \textit{Rce1}^{−/−} littermates did not differ in size, mortality or gross eye morphology.

\textit{Rce1} expression in retinas of \textit{Rce1}^{−/−} mice was reduced by 90\% as judged by Quantitative Polymerase Chain Reaction (qPCR) (Fig. 2A). The expression of \textit{Zmpste24}, a related endoprotease, which served as a control, was similar in \textit{Rce1}^{+/−} and \textit{Rce1}^{−/−} retinas (Fig. 2A).

\textbf{RCE1-mediated Endoproteolysis is Required for Viability of Photoreceptor Cells.}
To determine if the development of the laminated cell layers of the retina was affected by \textit{Rce1} deficiency, we examined retinal morphology using semi-thin plastic sections stained with H&E from post-natal day (P) 08 and P16 \textit{Rce1}^{+/−} and \textit{Rce1}^{−/−} mice. At P08 we observed similar numbers of nuclei in all three cell layers (outer, inner and ganglion) in \textit{Rce1}^{+/−} and \textit{Rce1}^{−/−} retinas and no signs of cell death (Fig. 2B and Fig. 3). Both rod
and cone photoreceptor cells were present (Fig. 4). At P16, however, the number of photoreceptor cell nuclei was dramatically reduced (Fig. 2B). This reduction was limited to the outer nuclear layer, as the thickness of the inner and ganglion cell layers was unaltered. Indeed, no cell death or reduction in cell numbers was observed in the inner and ganglion layer up to one year of age (Fig. 3). The development and survival of horizontal, amacrine, and ganglion cells was unaffected by \textit{Rce1} deficiency as judged by immunostaining of P30 retinal cryosections (Fig. 5). We conclude that \textit{Rce1} is dispensable for the development of retinal neurons but required for the subsequent viability of photoreceptor cells.

\textbf{Rce1 Deficiency Reduces Light-Evoked Responses.} We performed electroretinography (ERG) to evaluate light responses of \textit{Rce1}-deficient photoreceptor cells. The \textit{a}-wave of an ERG is generated by the collective response of photoreceptor cells in response to a flash of light. This is followed by a \textit{b}-wave, which is primarily shaped by response from bipolar cells (Nusinowitz 2002). Scotopic (rod-mediated) conditions produced robust \textit{a}- and \textit{b}-waves in P14 \textit{Rce1\textsuperscript{+/–}} mice at various light intensities (Fig. 6A). Photopic (cone-mediated) conditions also produced a characteristic \textit{b}-wave in \textit{Rce1\textsuperscript{+/–}} mice (Fig. 6B). In contrast, recordings from \textit{Rce1\textsuperscript{–/–}} mice failed to produce measurable \textit{a}-waves under any illumination condition and only minimal \textit{b}-wave responses (Fig. 6A and B). In agreement with the morphological analysis, which showed complete degeneration of photoreceptor cells, light-evoked responses were also abolished at P35 (Fig. 7). Collectively, these results demonstrate that \textit{Rce1} is required for the proper function of photoreceptor cells.
The Absence of RCE1-mediated Proteolytic Cleavage Does Not Affect the Stability of Prenylated Photoreceptor Cell Proteins. The ERG results suggest that the absence of Rce1 affects proteins involved in the light response. Therefore, we examined the levels of prenylated proteins involved in phototransduction (Fig. 8A). We reasoned that a major destabilization and degradation of either Tγ or PDE6 could lead to the loss of function we observed, similar to the knockout of either of these genes (Farber 1995; Lobanova, Finkelstein et al. 2008). However, the levels of Tγ, PDE6αβ, cone PDE6α’ and GRK1 in P08 retinal homogenates, before any detectable cell death, was similar in Rce1+/– and Rce1–/– retinas (Fig. 8A). The transducin γ-subunit from Rce1–/– retinal homogenates exhibited a reduced electrophoretic mobility (Fig. 8A), a characteristic of unprocessed forms of RCE1 substrates (Kim, Ambroziak et al. 1999; Bergo, Ambroziak et al. 2002). Although the steady-state levels of rhodopsin were reduced by 30% in Rce1-deficient retinas, the levels of the outer segment (OS) phototransduction proteins transducin, guanylate cyclase (GC-E), and arrestin, the OS structural protein peripherin, the inner segment (IS) protein AIPL1, and the bipolar cell proteins GOα and PKCα were unaffected by Rce1 deficiency (Fig. 8B). Thus, the lack of light response in Rce1-deficient photoreceptor cells does not appear to be due to changes in steady-state levels of prenylated RCE1 substrates.

Rce1 is Required for Intracellular Transport of PDE6. We next tested if the absence of Rce1 affected the transport of phototransduction proteins from the inner to the outer segment. We compared the localization of PDE6, Tγ, and GRK1 in Rce1+/– and Rce1–/– retinas by immunofluorescence. Cyclic nucleotide-gated channel alpha 1 (CNGA1) was used as a marker for the OS (Molday, Molday et al. 1991). In Rce1+/– retinas, the PDE6
β subunit colocalized with CNGA1 in the OS (Fig. 9A). In contrast, the PDE6 β-subunit in \textit{Rce1}⁻/⁻ retinas did not colocalize with CNGA1 and could only be detected in the IS and outer nuclear layer (Fig. 9B). No significant accumulation of PDE6 was observed in synaptic layer of photoreceptor cells (Fig. 10). The α and γ subunits of PDE6 were also confined to the IS and outer nuclear layer in \textit{Rce1}⁻/⁻ retinas (Fig. 10). Our attempts to localize cone PDE6α’ was inconclusive due to rapid degeneration of photoreceptor cells (Data not shown). The localizations of GRK1 and Tγ were unaffected by \textit{Rce1} deficiency (Fig. 9 C-F). Moreover, GC-E, an OS integral membrane protein thought to be transported in association with PDE6, co-localized with CNGA1 in \textit{Rce1}⁻/⁻ retinas (Zhang, Li et al. 2007) (Fig. 9 G and H). These results suggest that unprocessed rod PDE6 subunits accumulate in the IS in \textit{Rce1}⁻/⁻ retinas and that their transport to the OS is blocked (Fig. 9A).

\textbf{Normal Assembly and Activity of PDE6 in \textit{Rce1}-deficient Retina.} Posttranslational modifications play a role in the assembly of multimeric proteins (Winter-Vann and Casey 2005; Marrari, Crouthamel et al. 2007). To determine if the absence of \textit{Rce1} affected PDE6 assembly we immunoprecipitated PDE6 heterotetramers with ROS1, monoclonal antibody that only recognizes assembled PDE6. Immunoprecipitated proteins were separated on a SDS-PAGE gel followed by western blotting using a polyclonal antibody that recognizes all three PDE6 subunits (Hurwitz, Bunt-Milam et al. 1984; Kolandaivelu, Huang et al. 2009). We found that \textit{Rce1}⁺/⁻ and \textit{Rce1}⁻/⁻ immunoprecipitation samples had similar levels of PDE6, indicating that the assembly of PDE6 multimers does not require \textit{Rce1} (Fig. 11A).
To assess the ability of the assembled PDE6 enzyme to hydrolyze cGMP, we measured the enzymatic activity of PDE6 and the concentration of cGMP in the retina. These highly sensitive assays demonstrated that there were no significant differences in the ability of PDE6 in $Rce1^{+/−}$ and $Rce1^{−/−}$ retinas to hydrolyze cGMP. Consistent with our enzymatic assays, the level of cGMP was not significantly different in the presence or absence of RCE1 (Fig. 11 B and C).
Discussion

In this study, we show that the retina displays a unique requirement for RCE1-mediated endoproteolysis. *Rce1* is not needed for the development of retinal neurons but is specifically required for the maintenance of photoreceptor cells. *Rce1*−/− retinas exhibited minimal light responses, as measured by ERG, demonstrating the importance of postprenylation processing in phototransduction. The lack of light responses is likely due to the mislocalization of PDE6, a prenylated protein complex critical to phototransduction. The data suggest that although the PDE6 heterotetramer is assembled and activated in the IS of *Rce1*−/− retinas, the complex fails to be transported to the photoreceptor cell OS.

Interestingly, despite the absence of *Rce1* from early stages of retinal development, we observed no deficits in the formation of retinal cell layers at P08. At all tested ages, survival of the neurons in the inner retina was unaffected by the lack of *Rce1*. However, the photoreceptor cells in *Rce1*−/− retina do not survive beyond P30.

The rapid photoreceptor cell degeneration observed in *Rce1*−/− retinas is reminiscent of the photoreceptor cell loss seen in mice with mutations in PDE6 subunits, such as *rd/rd* mice, or in mice lacking *Aipl1* (Farber 1995; Ramamurthy, Niemi et al. 2004). However, in these mice, photoreceptor cell degeneration was linked to the destabilization of PDE6 and subsequent accumulation of cGMP (Ramamurthy, Niemi et al. 2004). In *Rce1*−/− retinas, by contrast, the stability of PDE6 was unaffected and cGMP levels were unchanged (Fig. 8A and Fig 11C). In addition, loss of photoreceptor cells was not accompanied by reduced stability of any of the major phototransduction proteins except rhodopsin, which was reduced by 30%. It is unlikely that reduced rhodopsin levels in
*Rce1*−/− retinas was the cause of the rapid photoreceptor cell degeneration as a 50% reduction in rhodopsin levels leads to only a slow degeneration (Lem, Krasnoperova et al. 1999).

PDE6 does not seem to require RCE1 processing for catalytic activity as we observed only minor reductions in trypsin-activated cGMP hydrolytic activity in *Rce1*−/− retinas. Furthermore, cGMP level was not altered, consistent with the presence of functional PDE6 (Fig. 11C). At this point, the likeliest explanation for the photoreceptor cell dysfunction is that PDE6 accumulates in the IS and fails to reach its site of action in the OS. However, we can’t rule out the possibility that dysfunction of other prenylated photoreceptor cell signaling proteins are at the root of the phenotypes of *Rce1*-deficient retinas. This possibility seems unlikely, however, because the localization and stability of other prenylated substrates, including Tγ and GRK1, were unaffected by the absence of *Rce1*.

The OS is the default location for lipid-modified proteins and the highly selective loss of PDE6 transport in *Rce1*-deficient retinas is intriguing (Baker, Haeri et al. 2008). One potential explanation is that there is an active mechanism preventing the transport of unprocessed PDE6. Another is that RCE1-mediated endoproteolysis of an as-yet unidentified CAAX protein is required for the transport of PDE6 following assembly in the IS. A third, potential explanation is that the PDE6 transport mechanism requires that the carboxyl terminus of PDE6 is cleaved by RCE1 and methylated by ICMT. For example, the lack of RCE1-mediated proteolysis of PDE6 might disrupt its interaction with a protein binding partner or membrane domain in the IS that normally promotes the transport of PDE6 to the OS. PrBP/δ and AIPL1 are well-known binding partners of
PDE6 (Gillespie, Prusti et al. 1989; Kolandaivelu, Huang et al. 2009). Previous studies have shown that the prenylcysteine methyl group of PDE6 peptides is important for the interaction with PrBP/δ (Cook, Ghomashchi et al. 2000). However, PrBP/δ knockout mice exhibit only a minor defect in rod PDE6 transport (Zhang, Li et al. 2007). AIPL1, on the other hand, is critical in early folding and assembly of PDE6 subunits (Ramamurthy, Niemi et al. 2004; Kolandaivelu, Huang et al. 2009). PDE6 in Rce1–/– retinal homogenates could assemble into functional heterotetramers in the IS (Fig. 11A), suggesting that the interaction with AIPL1 is not affected. Thus, the mechanisms behind the selective loss of PDE6 transport remains to be determined.

The sequence of events from PDE6 synthesis to its insertion into the OS membrane is beginning to emerge. Our previous studies show that AIPL1 is involved in initial folding and/or assembly of PDE6 subunits (Kolandaivelu, Huang et al. 2009). The present study clearly demonstrates that RCE1 is required for the transport of the fully assembled enzyme from the IS to the OS. Our model suggests that PDE6 subunits are assembled into a functional heterotetramer in the IS of the photoreceptor cell before being exported to the OS.
ACKNOWLEDGEMENTS We thank Dr. Peter Mathers for the experimental advice and generous sharing of mice and Dr. Karen Martin for advice and the use of the WVU Microscopic Imaging Facility. For critical reading of the manuscript we thank Drs. Lori Kang and James Hurley. We would also like to thank the members of the Ramamurthy laboratory. We thank Drs. Ariel Agmon, Joseph Beavo, David Garbers, James Hurley, Robert Molday, Gabriel Travis, and Theodore Wensel for their generous donation of reagents. This work was supported by National Institutes of Health grants RO1EY017035 (VR), P30RR031155 (George Spirou), West Virginia Lions, and an Unrestricted Research to Prevent Blindness (RPB) challenge grant (WVU). The authors have no conflict of interest.
Figures:

Fig. 1

Fig. 1. Uniform expression of Cre recombinase in entire retina from P19 mice. Ai9 tdTomato reporter mice were crossed with Six3-Cre mice (Furuta, Lagutin et al. 2000; Madisen, Zwingman et al. 2009). Cryosections of retinal tissue from mice heterozygous for tdTomato and Six3-cre transgene were stained with DAPI, a nuclear marker (blue). TdTomato expression is shown in red. Inset shows a higher magnification view of the photoreceptor cells. Sections were imaged on a Zeiss LSM 510 Meta confocal microscope using the 405 and 543 nm laser lines.
Fig. 2. Elimination of Rce1 in the retina leads to rapid photoreceptor cell degeneration.

(A) RT-PCR from P08 retinal cDNA with primers for Rce1 and Zmpste24 normalized to Hprt. (B) Semi-thin plastic sections of P08 and P16 Rce1+/− and Rce1−/− littermates were stained with H&E and imaged on an Olympus AX70 epifluorescent/transmitted light microscope. RPE: retinal pigment epithelium, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer.
Fig. 3

$Rce1^{+/ -}$  $Rce1^{-/-}$

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Fig. 3. Progressive and rapid degeneration of photoreceptor cells in the absence of Rce1. Retinal cryosections stained with propidium iodide (red), to delineate the nuclei, at various time points. Thickness of ONL is equal between Rce1+/− and Rce1−/− retina at P8 but reduced by P12 until complete loss of this layer at P30. Note the presence of inner and ganglion cell layers up to one year of age. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer.
**Fig. 4.** Rod and cone photoreceptor cells are present in retina lacking *Rce1*. P10 cryosections of *Rce1*+/− and *Rce1*−/− retinal tissue were stained with DAPI (blue) to identify the nuclear layer. (A) Rod photoreceptor cells (green) were labeled with anti-rhodopsin mouse monoclonal antibody (4D2, from Dr.Robert Molday). (B) Cone photoreceptor cells (red) were marked by staining with peanut agglutinin (PNA, Vector).
Fig. 5. RCE1-mediated endoproteolysis is dispensable for formation and survival of inner retinal neurons. Immunohistochemistry using P30 retinal cryosections. Staining with propidium iodide (red) is used to identify the cell nuclei. (A and B) Anti-Calretinin (Millipore) labels amacrine cells and their processes (green). (C and D) Sections stained with antibody against neurofilament 200 (NF200, Sigma). NF200 staining (green) identifies horizontal and ganglion cells. (E and F) Anti-Protein kinase C α–subunit (PKCα, Affinity Bioreagents) labels bipolar cells (green).
Fig. 6

Fig. 6. Functional vision loss in mice lacking Rce1 in the retina. (A) Scotopic ERG responses to increasing light intensities were recorded from P14 Rce1+/− and Rce1−/− littermates (n=3). Average responses to log intensity (cd s⁻¹/m²) flashes are shown. (B) Photopic ERG responses from Rce1+/− and Rce1−/− pups at log intensity 2.4 cd s⁻¹/m² (n=3).
Fig. 7. Photoreceptor cells do not respond to light at P35 in Rce1<sup>−/−</sup> mice. (A) Scotopic rod ERG responses from P35 Rce1<sup>+/−</sup> and Rce1<sup>−/−</sup> littermates were recorded as above (n=3). (B) Photopic cone ERG responses in presence of background light to suppress rod responses measured in log intensity (cd s<sup>−1</sup>/m<sup>2</sup>).
Fig. 8

Stability of prenylated proteins is maintained in the absence of RCE1-mediated endoproteolysis. Immunoblots of P08 Rce1+/– and Rce1−/− retinal lysates from littermates. Equal amounts of protein (150 μg) were loaded for each sample. (A) Western blots were probed with the indicated antibodies against prenylated proteins. Tγ displays a reduced electrophoretic mobility in Rce1−/− retinal lysates. (B) Levels of non-prenylated proteins of the retina were assessed in littermate pairs of P08 retinal lysates. PDE6αβ: cGMP phosphodiesterase 6, Tγ: Transducin γ-subunit, GRK1: Rhodopsin kinase, PDE6α′: cone cGMP phosphodiesterase, Rho: Rhodopsin, Tα: Transducin α-subunit, Tβ: Transducin β-subunit, Cone Tα: cone transducin α-subunit, GC-E: Guanylate cyclase, AIPL1: Aryl hydrocarbon receptor interacting protein-like 1, GOα: Guanine nucleotide-binding protein alpha activating activity polypeptide O, PKCα: Protein kinase C α-subunit.
Fig. 9. Intracellular transport of PDE6 requires proteolysis by RCE1 for proper localization. Immunohistochemistry using indicated antibodies on cryosections from P12 retina. Nuclei are labeled by TO-PRO-3 and appear in blue. OS are labeled with cyclic-nucleotide gated channel alpha 1 (CNGA1) and appear in green. (A) Co-localization of PDE6 β-subunit and CNGA1 in the OS of photoreceptor cells from Rce1+/– retinal sections (red). (B) Mis-localization of PDE6 β-subunit in the IS of rod photoreceptor cells of Rce1–/– retinal sections (red). (C and D) Colocalization of Tγ and CNGA1 in the OS of photoreceptor cells in both Rce1+/– and Rce1–/– retinal sections (yellow). (E and F) Rhodopsin kinase (GRK1) is localized in the OS and IS equally between Rce1+/– and Rce1–/– retinas. (G and H) Guanylate cyclase (GC-E) colocalizes with CNGA in the
photoreceptor cell OS in both $Rce1^{+/-}$ and $Rce1^{-/-}$ retinas. Scale bar is 10 μm. OS: outer segment, IS: inner segment, ONL: outer nuclear layer.
Fig. 10. PDE6 subunits accumulate in IS, but not the synapse of photoreceptor cells lacking Rce1. P12 Rce1<sup>−/−</sup> retinal cryosections labeled with anti-cyclic nucleotide-gated channel alpha 1 (CNGA1) in green and TO-PRO-3 nuclear stain in blue. (A) 40x image of PDE6 β-subunit (red) in the IS and nuclear layer, but not in the synaptic layer. (B) PDE6 α-subunit (red) (Affinity Bioreagents) is predominantly present in IS. (C) PDE6 γ-subunit (red) (Theodore Wensel) immunofluorescence reveals a punctate labeling pattern in the IS of photoreceptor cells of Rce1<sup>−/−</sup> retinas, similar to labeling of both α and β-subunits of rod PDE6.
Fig. 11. PDE6 is assembled and functional in retina lacking Rce1. (A)

Immunoprecipitation (IP) from P08 retinal extracts with ROS-1 monoclonal antibody.
ROS-1 precipitates assembled PDE6 heteromer. Western blots of eluted IP extracts probed with MOE antibody that recognizes individual PDE6 subunits (αβγ). Total samples represent the input to each IP reaction. (B) Trypsin-activated PDE activity from P9 Rce1+/− and Rce1−/− retinal homogenates measured in triplicate. The difference in enzymatic activity between Rce1+/− and Rce1−/− retinas was not statistically significant (P=0.35). (C) Competitive immunoassay for cGMP content (Assay Design) in P10
retinal extracts from $Rce1^{+/−}$, $Rce1^{−/−}$ and $Aipl1^{−/−}$ mice. No significant change in cGMP level was found between $Rce1^{+/−}$ and $Rce1^{−/−}$ retinal extracts ($P=0.18$). However, cGMP level in $Aipl1^{−/−}$ retina was significantly higher ($P=0.03$).
Chapter 3. Study 2: Methyl esterification of retinal proteins is essential for rod-mediated vision.

Abstract

Proteins ending in a “CAAX” box are first prenylated at their c-terminal cysteine and then RAS-converting enzyme 1 (RCE1) cleaves the final three amino acids before isoprenylcysteine methyltransferase (ICMT) catalyzes the methyl esterification of the newly prenylated cysteine residue. Rce1-mediated proteolysis of prenylated proteins plays a crucial role in phototransduction. In the absence of Rce1, photoreceptors do not function and rapidly degenerate. To determine if the requirement for RCE1-mediated proteolysis in photoreceptor cell function was related to the lack of proteolysis, lack of methyl esterification, or both we analyzed the retinas of mice hypomorphic for Icmt. Photoreceptor cells survived despite reduction in Icmt and we did not observe any evidence of photoreceptor degeneration. However, at low light levels, visual response mediated by rod cells was reduced corresponding to reductions in Icmt levels. In agreement with the loss of visual response, phototransduction protein turnover was increased. Interestingly, the stability of transducin γ−subunit was dramatically reduced by 90%. Unlike retina lacking Rce1, PDE6 was transported normally to photoreceptor outer segments. This results suggests that the presence of –AAX at the C-termini of PDE6 catalytic subunits is detrimental for PDE6 transport. Based of our results, we conclude that methyl esterification of prenylated proteins is required for efficient light signaling in photoreceptor neurons.
Introduction

Almost 30 years ago a subset of proteins in photoreceptor rod outer segments was identified that were able to incorporate a radioactive methyl group (Swanson and Applebury 1983). The identity of the proteins and the site of methylation has been extensively studied (Ong, Ota et al. 1989; Ohguro, Fukada et al. 1991; Inglese, Koch et al. 1992). Rod phosphodiesterase 6 (PDE6), the effector enzyme of the visual signal transduction cascade, was the first methylated protein to be identified in retinal lysates (Swanson and Applebury 1983). The C-termini of PDE6 catalytic subunits contain “CAAX” motifs that specify prenylation, a lipid posttranslational modification. Prenylation is the addition of a prenyl group to the cysteine residue of the “CAAX” motif, followed by two additional processing steps. Prenylated proteins undergo proteolytic cleavage of the final three amino acids by the protease RCE1 and methyl esterification of the cysteine residue by isoprenylcysteine methyl transferase (ICMT) (Lane and Beese 2006). PDE6 catalytic subunits are differentially prenylated with PDE6α being farnesylated and PDE6β geranylgeranylated (Anant, Ong et al. 1992). Interestingly, rod PDE6 α- subunit incorporated a methyl group using in-vitro radioactive S-Adenosyl Methionine (SAM) incorporation assay whereas the β-subunit was not methylated (Anant, Ong et al. 1992). In addition to PDE6, the γ–subunit of heterotrimeric G-protein, transducin, (Tγ) and rhodopsin kinase (GRK1) are farnesylated and undergo “CAAX” processing (Fukada, Takao et al. 1990; Inglese, Koch et al. 1992).

In vitro studies indicate that methyl esterification of the prenylated cysteine residue further increases the hydrophobicity of a protein (Parish and Rando 1996). “CAAX” processing facilitates the protein’s interaction with membranes and enhances
protein/protein interactions, such as the interaction between PDE6 and prenyl binding protein/δ (PrBP/δ) (Cook, Ghomashchi et al. 2000). Transducin isolated from bovine rod outer segment preparations also shows an increased ability to interact with rhodopsin if it is carboxyl methylated (Ohguro, Fukada et al. 1991). GRK1 also had stronger interactions with rhodopsin when it was methylated (Inglese, Freedman et al. 1993).

In our previous study (chapter 2), we demonstrated the essential nature of “CAAX” processing in functioning and maintenance of retinal neurons by eliminating the “CAAX” endoprotease, RCE1 in the neural retina. We also observed the transport of assembled and functional PDE6 was aborted in the absence the “CAAX” protein processing. Since proteolysis and methylation of prenylated proteins is linked, we were unable to conclude whether the need for “CAAX” processing in retinal neurons is due to lack of RCE1-mediated proteolysis, lack of methyl esterification, or a combination of the two.

The role of prenylation and “CAAX” processing in the retina has been previously studied utilizing injections of pharmacologic inhibitors of both processes, Lovastatin and AFC, respectively. Both inhibitors caused rapid degeneration and disorganization of retinal laminations (Pittler, Fliesler et al. 1995). Injection of pharmacologic inhibitors into the retina is complicated by the coincident effects upon the vasculature as blood vessel shunting was also observed (Pittler, Fliesler et al. 1995). Therefore, direct in vivo evidence for the function of methyl esterification of “CAAX” proteins in the retina is lacking.
To specifically investigate the role of methyl esterification on retinal proteins we genetically eliminated the “CAAX” methyl transferase, ICMT, in the retina. Our model was generated by breeding $Icm^{\theta}$ mice with mice expressing cre recombinase under the retina- and forebrain- specific promoter, $Six3$ (Furuta, Lagutin et al. 2000; Bergo, Gavino et al. 2004). Histology, electroretinography, and biochemistry experiments were carried out to analyze the specific contribution of methyl esterification to maintenance and function of the retina.
Materials and Methods

Animal Models. *Icm*/*Icm* males were crossed with *Six3-Cre* males to obtain *Icm*/*Six3-Cre* animals (Furuta, Lagutin et al. 2000; Bergo, Gavino et al. 2004). Crossing *Icm*/*Six3-Cre* males with *Icm*/* females produced *Icm*, *Icm*, *Icm*/*Six3-Cre*, and *Icm*/*Six3-Cre* for experimental littermate comparisons.

Message Analysis. Retinas, dissected from freshly enucleated eyes, were flash frozen in dry ice in the presence of Trizol (Invitrogen). RNA was extracted from frozen retinas and used to generate cDNA using Qscript (Quanta Bioscience). 300 μg of cDNA from heterozygous and knockout littermates in triplicate was used as a template for Quantitative PCR using MyiQ PCR cycler (Bio-Rad) and MyiQ SYBR Green Supermix (Bio-Rad). *Icm* was amplified using primers 5’-CGCCTCAGCCTCGCTACATT-3’ and 5’- TTGGAGCCAGCCGTAAACAT -3’ to generate a 509-base pair (bp) product. Threshold values were normalized to hypoxanthine phosphoribosyltransferase (*Hprt*) gene expression levels with the following primers 5’-CAAACTTTGCTTTCCCTGGT-3’ and 5’-CAAGGGCATATCCAACAACA-3’ (250-bp product).

Immunohistochemistry. Mice were euthanized by CO₂ inhalation and eyes were enucleated. A 2-mm hole was made at the corneal limbus and eyes were fixed (4% paraformaldehyde in PBS) for 5 minutes before dissecting the anterior chamber and removing the lens. Fixation continued for 2 hours before cryoprotection in 30% sucrose overnight at 4 °C. Eyecups were embedded in Tissue-Tek O.C.T. Compound (Sakura) and fast frozen in dry ice ethanol bath. Blocks were sectioned with a Leica CM1850 Cryostat and 15-20 μm sections were mounted on Superfrost plus slides. Cryosections were washed in PBS, and then incubated in blocking buffer (2 % goat serum (Invitrogen),
0.1% Triton X-100, and 0.05% sodium azide in PBS) for 1 hour. Primary antibodies were incubated for 2 hours at room temperature or overnight at 4 °C. Excess antibody was removed by three 10 minute washes in PBST (PBS, 0.1% Triton X-100) before incubation with secondary antibody for 1 hour at room temperature. Slides were washed twice for 10 minutes with PBST and for 10 minutes in PBS. ProLong Gold antifade reagent (Invitrogen) was applied to each section then coverslips were mounted. Images were collected on a Zeiss LSM 510 Meta confocal microscope using 488, 543 and 633 nm laser lines. The following antibodies were used: anti-CNGA1/3 (UC Davis/NIH NeuroMab Facility); anti-PDE β (Affinity Bioreagents); anti-γ (Santa Cruz); anti-Calretinin (Millipore); anti-Nestin (Neuromics); anti-PKCα (Affinity Bioreagents); and the lectin PNA (Vector Labs). TO-PRO-3 nuclear stain (Invitrogen) was added to dilutions of Alexa Fluor secondary antibodies (Invitrogen) in antibody dilution buffer (0.05% goat serum, 0.1% Triton X-100, and 0.05% sodium azide in 1X PBS).

Electroretinography (ERG). Littermates were dark-adapted overnight, then eyes were dilated (1:1 Phenylephrine: Tropicamide) for 10 minutes. Isoflurane anesthesia (1.5% in 2.5% oxygen) was administered via nose cone on a 37 °C platform. A reference electrode was placed subcutaneously in the scalp and silver wire electrodes were positioned above the cornea, with contact being made by methylcellulose solution. Light flashes were presented by placing the mouse in a Ganzfield apparatus. Corneal evoked potentials were collected using UTAS-E4000 Visual Electrodiagnostic Test System and EMWIN 8.1.1 software (LKC Technologies). Background light (30 cd · m⁻²) was presented for 10 minutes before recording flicker responses in the presence of the background light. Representative waveforms are shown.
**Immunoblot.** Flash frozen retinal samples were solubilized in 6 M Urea buffer (6 M urea, 4% SDS, 0.5 M Tris pH 6.8, 10 mg/ml DTT, and 2% Bromophenol Blue) using sonication for 6 pulses of 20 milliseconds at power setting 6 (Misonix XL-2000). The protein concentration was estimated using a NanoDrop (Thermo Scientific) spectrophotometer. 7.5 μg total protein samples were resolved on 4-20% Criterion (Bio-Rad) polyacrylamide gels. Proteins were then transferred to Immobilon-FL membrane (Millipore) and probed with primary antibodies against desired target proteins. The following antibodies were used; anti-PDE6αβγ (MOE, Cytosignal), anti-Tγ (Santa Cruz), anti-GRK1 (Thermo Fisher), anti-Tα (Santa Cruz), anti-Tβ (Affinity Bioreagents), anti-arrestin (Affinity Bioreagents). Odyssey goat anti-rabbit Alexa 680 and Odyssey goat anti-mouse Alexa 680 secondary antibodies (LI-COR Biosciences) were used at 1:50,000 dilutions to label primary antibodies. Membranes were scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences). Images are representative from at least three independent experiments.
Results

Retina Conditional Knockout of Icmt. To study the role of methyl esterification of proteins in the retina, we generated a retina CKO of Icmt by breeding animals with floxed alleles of Icmt to those carrying the Six3-cre transgene. In our animal model, Six3-cre expression eliminates Icmt in the retina and the forebrain at embryonic day 10 (Furuta, Lagutin et al. 2000; Bergo, Gavino et al. 2004). After the initial cross, animals were genotyped and Icmt^{fl/wt}Six3-Cre males were crossed with Icmt^{fl/fl} females to obtain experimental Icmt^{fl/fl}Six3-Cre animals, as well as littermate controls, Icmt^{wt/fl}Six3-Cre, Icmt^{fl/fl}, Icmt^{wt/fl} (Fig. 1). This cross produced the highest percentage of experimental animals and Icmt^{wt/fl} animals, although expressing less Icmt, were phenotypically similar to wild-type animals.

Quantitative Polymerase Chain Reaction (qPCR) was performed to confirm the elimination of Icmt in the retina. In agreement with previous reports, expression of Icmt from cDNA of control animals was hypomorphic (Svensson, Casey et al. 2006). We observed stepwise reductions in Icmt expression from Icmt^{wt/fl}, Icmt^{wt/fl}Six3-Cre, and Icmt^{fl/fl}, from highest to lowest, with negligible expression in Icmt^{fl/fl}Six3-Cre retinas (Fig. 2A).

Photoreceptor Cells Do Not Require ICMT-Mediated Methyl Esterification for Survival. Elimination of Rce1 from the retina led to rapid degeneration of photoreceptors cells while cells of the inner nuclear layer (INL) were unaffected. We were unable to conclude whether our results were due to loss of RCE1-mediated proteolysis or the inability of proteins to be methyl esterified. To specifically test the role...
of methyl esterification examined the presence of rod and cone specific markers by
immunofluorescence on adult retinas lacking Icmt (Fig. 2B). Rod and cone photoreceptor
cells were present in normal distribution in Icmt CKO retinas as shown by the presence of
cyclic-nucleotide gated channel alpha 1 (CNGA1), a marker for rod cells, and peanut
agglutinin (PNA), a marker for cone cells (Fig. 2B). Outer nuclear layer (ONL), INL,
and ganglion cell layer (GCL) thickness was unaltered by the absence of Icmt (Fig. 3).
Furthermore, the three characteristic laminations of the inner plexiform layer (IPL) were
labeled by calretinin in both Icmtwt/flSix3-Cre and Icmtfl/flSix3-Cre (arrowheads Fig. 3A).
We also observed the presence of higher than usual numbers of mis-localized nuclei in
the IPL (Arrows Fig. 3C). Interestingly, an occasional calretinin-positive neurite
extended from the ganglion cell layer through both the INL and ONL onto cells in the
RPE (arrows Fig. 3A).

**Light-Evoked Response Is Reduced Proportional to the Level of Icmt in the Retina.**
Electroretinography (ERG) was used to measure the response of photoreceptor and
bipolar cells to increasing flashes of light from Icmtwt/fl and Icmtfl/flSix3-Cre animals.
The hyperpolarized a-wave of scotopic ERGs is generated by rod photoreceptor cells in
response to light. Subsequent signaling to downstream bipolar cells is indicated by the
depolarizing b-wave of ERGs. At all light levels the maximal rod photoreceptor response
from Icmtfl/flSix3-Cre was reduced in comparison to Icmtwt/fl response. For example, a-
wave amplitudes were reduced by 48% at 0 dB (Fig. 4). B-wave amplitudes were also
reduced by approximately 50% throughout the flash series and the latency to b-wave was
prolonged in the absence of Icmt (Fig. 4). Collectively, these results suggest the rod
signal transduction pathway is disrupted when proteins have not undergone complete “CAAX” processing.

**Stability of Prenylated Photoreceptor Cell Proteins Is Reduced in the Absence of Methyl Esterification.** We reasoned that decreased visual response may result from a reduction in the levels of prenylated proteins involved in phototransduction. Immunoblots of retinal lysates from animals expressing decreasing levels of *Icmt* were probed with antibodies against prenylated and non-prenylated phototransduction proteins. The reduction of *Icmt* message in each genotype corresponded to the changes in protein levels of PDE6, GRK1 and Tα (Fig. 5A, B). For instance, PDE6 levels in *Icmt*fl/flSix3-Cre animals were 34% of *Icmt*wt/fl level, GRK1 levels were 63% and Tα was 45% of levels observed in *Icmt*wt/fl animals. In contrast, arrestin, a soluble photoreceptor protein that is not prenylated was not appreciably different between various genotypes (Fig. 5A, B). Tα is not prenylated but forms a heterotrimeric complex with farnesylated Tγ, so we measured the levels of Tγ at P17 and P35. We observed a 73% reduction in the levels of Tγ in *Icmt*fl/flSix3-Cre animals at P17 in comparison to *Icmt*wt/fl P17 and a 90% reduction at P35 in comparsion to *Icmt*wt/flSix3-Cre animals (Fig. 5C, D). Lack of a corresponding reduction in Tβ was not surprising as it is also present in INL and GCL (Fig. 5C, D). Our results suggest that lack of methyl esterification by ICMT causes “CAAX” proteins of photoreceptor cells to undergo increased protein turnover with Tγ showing the most dramatic reduction.

**Trafficking of PDE6 Does Not Require Icmt.** Phototransduction protein levels remained stable in retinas lacking RCE1-mediated proteolysis prior to retinal
degeneration. However, a striking accumulation of PDE6 was observed in the IS of retinas lacking Rce1 (Christiansen, Kolandaivelu et al. 2011). This prompted us to investigate if the reduction in levels of PDE6 in Icmtfl/flSix3-Cre was due to a similar trafficking defect. Immunolocalization of PDE6β was compared to that of CNGA, a marker of OS (Fig. 6A, B). Merged images demonstrated that PDE6 was transported to the OS and colocalized with CNGA in retinas lacking Icmt.

**Transducin γ-subunit is Transported to the OS in the Absence of ICMT-Mediated Methyl Esterification.** To examine if the loss of Tγ was caused by an inability to be transported to the OS, immunolabeling of Tγ on Icmtfl/flSix3-Cre retinas was compared to the labeling pattern of CNGA (Fig. 6 C, D). While labeling was faint, as would be expected from the level of reduction observed by immunoblot, residual Tγ colocalized with CNGA in the OS and was not present in the nuclear layer or IS. Taken together these results suggest methyl esterification is not required for trafficking of prenylated phototransduction proteins.
Discussion

Our findings highlight the complexity of the effects posttranslational modifications impose upon individual proteins and cell types. This is the first reported comparison between *in vivo* elimination of *Icmt* and *Rce1* in the same tissue. Deletion of *Icmt* thus far displays a more severe phenotype than deletion of *Rce1* (Kim, Ambroziak et al. 1999; Bergo, Leung et al. 2001; Bergo, Gavino et al. 2004; Michaelson, Ali et al. 2005). Unexpectedly, the retinal CKO of *Icmt* did not phenocopy the retinal CKO of *Rce1* and actually displayed a less severe phenotype. Elimination of *Icmt* in the retina did not result in rapid degeneration, complete loss of vision, or defects in trafficking of PDE6 as we observed in *Rce1* CKO animals. The most significant defects in the *Icmt* retina CKO animals were reduced ERG sensitivity and a significant reduction in *Tγ* protein level. The milder phenotype was particularly surprising given that chimeric mice generated with *Icmt*−/− stem cells did not incorporate any *Icmt*−/− stem cells in the brain, implicating an essential function for ICMT in neurons (Bergo, Leung et al. 2001).

ERG recordings displayed two notable features, a reduction in the amplitude of *a*- and *b*-waves and an increased latency to the peak of the *a*-wave in animals lacking *Icmt*, indicating a reduction in photoreceptor response sensitivity. The reduced *b*-wave amplitude may be a consequence of reduced *a*-wave amplitude or may indicate a disruption in the bipolar cell signal transduction pathway. A candidate protein would be a farnesylated *Gγ* critical to bipolar cell signaling that is destabilized similar to *Tγ*.

Reduced rod sensitivity is likely a consequence of reduction in phototransduction protein levels. An increased rate of turnover for PDE6, GRK1 and transducin in the absence of *Icmt* is expected as methyl esterification is proposed to contribute to protein
stability (Young SG 2000). For instance, RhoA undergoes higher rates of turnover without methyl esterification, while K-Ras is more stable in the absence of Icmt (Bergo, Gavino et al. 2004).

Icmt retina CKO animals share many phenotypic features with a recently published Tγ knockout animal (Kolesnikov, Rikimaru et al. 2011). The notable phenotype of these animals was slow retinal degeneration starting at six months, reduced ERG sensitivity, and stable protein expression profile. Complete loss of Tγ did not result in a major destabilization of photoreceptor proteins, similar to our observations of Icmt retina CKO animals. Reduced levels of transducin may be related to a remarkable feature of rod photoreceptor cells that allows the redistribution of rod transducin from the OS to IS under intense light conditions (Sokolov, Lyubarsky et al. 2002). Methylated and unmethylated Tγ have been detected in OS preparations, prompting speculation that the two different forms may play a regulatory role in phototransduction (Perez-Sala, Tan et al. 1991). As the only step of “CAAX” protein processing that is potentially reversible, it is intriguing to speculate that transducin translocation is dynamically regulated by removal of the methyl group by an unidentified methyl esterase to aid in light adaptation.

Overall, CKO of Icmt in the retina is proving to be an informative approach to studying the importance of “CAAX” protein processing in the retina because the extent and rate of degeneration are not as severe or rapid as that of the retina CKO of Rce1.
Fig. 1. Breeding of Icmt retina CKO animals. Experimental and littermate controls were generated by breeding an $Icmt^{fl/fl}$ female with an $Icmt^{wt/fl}$ Six3Cre male. $Icmt^{wt/fl}$ animals were phenotypically similar to wild type animals and thus served as controls.
Fig. 2. Retina is maintained in the absence of Icmt. (A) RT-PCR from P60 retinal cDNA with primers for Icmt normalized to Hprt. Icmt primers were designed with the forward primer in exon 1 and reverse primer in exon 4 (N=3). (B) Cryosections of P90 Icmt<sup>wt/−</sup>Six3-Cre and Icmt<sup>fl/fl</sup>Six3-Cre littermates labeled with cyclic- nucleotide gated channel alpha 1 (CNGA1), Peanut agglutinin (PNA) and TO-PRO-3. Images were captured on a Zeiss LSM 510 confocal.
Fig. 3. Inner neural retina morphology in the absence of Icmt. Immunofluorescence of 
$Icmt^{wt/}\text{Six3-Cre}$ and $Icmt^{fl/fl}\text{Six3-Cre}$ littermates at P90 with cell nuclei labeled by 
propidium iodide in red. (A) Amacrine cells are labeled by calretinin (Green). 
Laminations in the IPL are marked by arrowheads. Neurite (arrows) extending from 
amacrine cell in ganglion cell layer through INL, ONL and into RPE (n=1). Compressed 
z-stack. (B) Nestin (Green) is a marker for Mueller glia. (C) Labeling of bipolar cell 
processes by protein kinase C subunit-\(\alpha\)(Green). Arrows mark cell nuclei in the IPL. 
Images were collected on a Zeiss LSM 510 confocal microscope.
Fig. 4. Visual deficit in mice lacking Icmt in the retina. Scotopic ERG responses to increasing light intensities were recorded from P60 Icmt$^{wt/fl}$ and Icmt$^{fl/fl}$-Six3-Cre littermates (n=2). Representative waveforms from (A) -40 dB intensity flash. (B) -32 dB intensity flash. (C) -20 dB intensity flash. (D) 0 dB intensity flash.
Fig. 5. Levels of prenylated proteins are reduced in retina lacking Icmt. Immunoblots of equal amounts (7.5 μg) of P60 retinal lysates from Icmt littermates. (A) Western blots were probed with antibodies against the indicated proteins. (B) Y axis of the bar graph is the % of integrated intensity value normalized to Icmt<sup>wt/fl</sup> as measured using Odyssey imaging software (n=1). (C) P17 and P35 Icmt retinal lysates were compared to P08 Rce1<sup>+/−</sup> and Rce1<sup>−/−</sup> retinal lysates (150μg). Note altered electrophoretic mobility of Tγ in Rce1<sup>−/−</sup> lysates but not Icmt<sup>−/−</sup> lysates. (D) Integrated intensity values normalized to littermate controls for each representative immunoblot band (n=1). PDE6αβ: cGMP
phosphodiesterase 6, Ty: Transducin γ-subunit, GRK1: Rhodopsin kinase, Tα: Transducin α-subunit, Tβ: Transducin β-subunit.
Fig. 6

PDE6 and Tγ do not require ICMT-mediated methyl esterification for transport to the OS. Immunofluorescent labeling of P90 Icmt<sup>wt/fl</sup> Six3-Cre and Icmt<sup>fl/fl</sup> Six3-Cre littermates. (A,B) CNGA1 in green labels the OS, PDE6 β-subunit in red and nuclei are stained with TO-PRO-3 in blue. PDE6 β-subunit is co-localized with CNGA1 in the OS of photoreceptor cells of both retinas as observed by the yellow color in the merge panel. (C, D) In agreement with Western blot results, Tγ in red is significantly reduced compared to Icmt<sup>wt/fl</sup> Six3-Cre. Tγ is present in the OS in both Icmt<sup>wt/fl</sup> Six3-Cre and Icmt<sup>fl/fl</sup> Six3-Cre animals.
Chapter 4. General Discussion, Conclusions and Future Work:

Study 1: Lack of RCE1-mediated proteolysis leads to retinal dysfunction

“CAAX” protein processing is critical for the assembly and targeting of phototransduction proteins. This was the initial hypothesis that formed the basis of the previous two studies. To test our hypothesis, we began by investigating the role of RCE1-mediated proteolysis in the function and maintenance of prenylated proteins in the retina. Our model system was a CKO that removed Rce1 from the retina and forebrain at embryonic day 10 (Christiansen, Kolandaivelu et al. 2011). To our surprise, we did not observe any changes in retinal morphology until P 09. Rods and cones formed normally but started to degenerate as the ciliated OS elaborated. Timing of degeneration corresponded to the requirement for efficient protein trafficking from IS to OS. We also found that PDE6 requires RCE1-mediated proteolysis for transport to the OS while the other farnesylated proteins Tγ and GRK1 are transported normally (Christiansen, Kolandaivelu et al. 2011). Mislocalized PDE6 appeared as discrete vesicular structures at the distal end of the inner segment below the connecting cilia. Our preliminary ultrastructural studies confirm the presence of these vesicular structures in retina lacking Rce1. Several animal models that exhibit defective protein trafficking to the OS accumulate excess protein at the synaptic termini. For example, rod PDE6 transport is partially defective in the prenyl binding protein/δ (PrBP/δ) knockout and PDE6 is immunolocalized in the IS and synaptic termini (Zhang, Li et al. 2007). In contrast, PDE6 was exclusively present in IS in retina lacking Rce1.
Does PDE6 transport require a chaperone?

Altogether, these findings suggest a model where vesicles containing assembled and functional PDE6 likely interacts with a protein binding partner (akin to Rho GDI) and is not able to release and dock with ciliary transport machinery for further transport to OS. Alternatively, a prenyl binding protein that recognizes the methyl esterified isoprenyl cysteine group of PDE6 is needed for its transport to OS. We do not believe this prenyl binding protein to be PrBP/δ, because defects in rod PDE6 localization were partial in the absence of PrBP/δ (Zhang, Li et al. 2007). Investigating the composition and identity of proteins present in the vesicular structures seen by light and electron microscopy is crucial in defining the PDE6 transport pathway.

Previous studies suggest that PDE6 is transported to OS along with guanylyl cyclase (GC) containing vesicles (Karan, Zhang et al. 2008). However, transport of GC was unaffected in the absence of Rce1. This finding coupled with presence of discrete vesicular structures in our animal model suggests that there is a unique pathway for PDE6 trafficking in rod cells. We are in the early stages of deciphering how multimeric proteins such as PDE6 are assembled, targeted to transport vesicles, trafficked and integrated into photoreceptor discs in the OS. Resolving the mechanism behind the requirement for proteolysis and/or methylation of PDE6 is the first step in this process and will lead to a greater understanding of the role of postprenylation processing in photoreceptor cells and protein transport in general. The immediate question to be answered is whether this is strictly a PDE6 defect or a secondary defect such as, defective interaction with a small GTPase responsible for PDE6 vesicle docking. To test this question, PDE6 mutants that cannot undergo proteolysis by RCE1 will need to be
expressed in the retina. Immunolabeling experiments on retina expressing PDE6 ending in CAMQ will answer if the PDE6 defect is a direct or indirect result or elimination of Rce1.

**Study 2: Icmt disruption destabilizes prenylated phototransduction proteins**

To address the question of whether the PDE6 defect in Rce1 CKO animals is caused by lack of proteolysis or lack of methylation we generated a retina CKO of Icmt. These animals specifically lack the ability to methylate prenylated proteins in the retina from an early embryonic age. Methyl esterification of retinal proteins is not critical for photoreceptor cell development or baseline functioning. ERGs on six month and one year old animals will need to be performed to know if photoreceptor maintenance is affected by lack of ICMT. Photoreceptor response measured by ERG was reduced in the absence of Icmt. Reduction of Tγ likely accounts for the observed reduction of a-wave amplitude in Icmt0/+/Six3-Cre animals as KO of rod Tγ showed a reduced sensitivity to light by ERG (Kolesnikov, Rikimaru et al. 2011). Defective signal transduction could be due to insufficient Tγ levels or a reduced ability of the transducin heterotrimer to reassemble and return to its proper orientation with rhodopsin after GTP-induced dissociation. Immunofluorescence images suggest Tγ and PDE6 are not defective in their ability to traffic to the OS.

Regardless of the fact that RCE1 and ICMT are both obligatory sequential steps following prenylation, it is becoming apparent that “CAAX” processed proteins have unique requirements for each step. For instance, PDE6 is transported to OS without
ICMT-mediated methyl esterification, but is not transported in the absence of RCE1-mediated endoproteolysis.

**Summary:**

The role of the individual steps of “CAAX” protein processing has been difficult to study *in vivo*, particularly in neuronal systems. Studies utilizing pharmacologic inhibitors have nonspecific affects on other cell types as well as the inability to target specific steps of “CAAX” processing. Conditional elimination of *Rce1* and *Icmt* has provided unique opportunities to probe their function particularly in a system such as photoreceptors which are not amenable to culturing *ex vivo*.

Although not the original goal of the project, a striking outcome was a greater understanding of the requirements for PDE6 transport to the OS. Our results demonstrated that RCE1-mediated proteolysis was required for transport to the OS, whereas methyl esterification was not required. Combined, the results suggest that a transport partner/adapter protein interacts with prenylated PDE6 regardless of the methylation status; however, if the last three amino acids are uncleaved, the interaction is inhibited and transport is arrested. Identification of this unknown transport partner will certainly be an interesting finding for photoreceptor biology as well as the field of protein trafficking. It also leaves open the possibility that methylation status plays a role in the regulation of PDE6 function.
Fig. 1. Model for biosynthesis and transport of PDE6 in rods. A) Polarized rod cell with different compartments. ROS=rod outer segment, RIS=rod inner segment, RER=rough endoplasmic reticulum, V=Vesicle and RPE=retinal pigment epithelium. B) Model depicting biosynthesis and trafficking of rod PDE6. Prenylation and downstream processing is a three-step process. (I) Farnesyltransferase (FTase) adds a C$_{15}$ farnesyl to the C-terminal cysteine of PDE6$\alpha$. (II) The last three amino acids are cleaved by the protease RAS-converting enzyme 1 (RCE1). (III) Isoprenylcysteine methyltransferase (ICMT) catalyzes the addition of a methyl group to the newly exposed prenylcysteine.
Adenosyl methionine serves as the methyl donor for the reaction. PDE6β subunit undergoes similar modification except it gets modified by a geranylgeranyl group. Assembly of the holoenzyme takes place in the inner segment before transport to the OS by an unknown mechanism. AIPL1, a chaperone binds to PDE6α subunit after farnesylation in the cytosol to promote its folding and/or assembly with other PDE6 subunits. Absence of RCE1, preventing the last two steps of CAAX processing, leads to accumulation of PDE6 containing vesicles in IS.
References:


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WVU Center for Neuroscience Retreat - Greasing the protein trafficking machinery of neurons. Roanoke, WV June 7, 2011.

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