The Role of PRCD in Building the Photoreceptor Outer Segment

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Human vision begins in the retina, where ~100 million photoreceptor neurons absorb light and respond to it, transferring the information to the brain where ultimately an image is created. Just like a camera’s sensor, an essential quality of photoreceptors for functional vision is their incredible sensitivity—our rod photoreceptors can detect the smallest unit of light possible, a single photon. To achieve this level of sensitivity, the photoreceptor evolved a primary cilium-derived light sensor organelle called the outer segment, which is a massive 30µm-long cylinder filled with a stack of ~1000 perfectly flattened disc membranes. The disc membrane houses the protein machinery necessary for generating light responses, including rhodopsin, the transmembrane photopigment protein responsible for absorbing light. By stacking 1000 discs, each with two rhodopsin containing membrane bilayers, the light absorbing membrane surface area of the retina is increased ~2000 fold, enabling the incredible sensitivity of the rod photoreceptor.

We completed a mass spectrometry project identifying the proteins which specifically reside in the photoreceptor disc. Except for one 6 kDa protein called PRCD, the proteins identified were previously known to reside in photoreceptor discs and their functions were well studied. PRCD is a recently discovered protein whose mutations are linked to retinal degeneration in canine and human patients, and had previously unknown localization in any cell type. Virtually nothing was known about this protein, so this dissertation sought to biochemically characterize the protein, understand how its
mutation leads to blindness in dogs and humans, and elucidate its function in photoreceptor discs.

To biochemically characterize the protein, we generated an antibody to its C terminus, and used it to confirm its localization specific to discs by immunohistochemistry. By analyzing the multiple bands PRCD protein produces on Western blot, we discovered that the protein is post translationally modified by lipid acylation and phosphorylation. Furthermore, mutagenesis experiments determined that the lipid is attached to the single cysteine residue of PRCD, which is mutated in blind canine and human patients. This disease-causing mutation results in complete mislocalization of PRCD from the outer segment, and its degradation—effectively resulting in a null \( PRCD \) mutant allele.

Pull down experiments revealed PRCD specifically binding to rhodopsin, which was confirmed by reciprocal immunoprecipitation and co-chromatography experiments. Bolstering this result, we found that PRCD was nearly absent from rhodopsin knockout mouse retinas and without outer segment localization. This result contrasted a large cohort of other outer segment proteins; all of them except guanylate cyclase 1 were trafficked to the outer segment and expressed in relative abundance. Through reciprocal co-immunoprecipitations, we discovered that guanylate cyclase 1 is also a rhodopsin binding protein, and that this interaction is dependent on gentle detergent conditions, likely hindering its identification in the past. These results reveal that the bulk of disc
specific proteins have their own, uncharacterized trafficking pathway(s), independent of rhodopsin.

To elucidate the function of PRCD in photoreceptor discs, we generated and characterized a PRCD knockout mouse, which develops a normally layered retina. The abundance and localization of disc proteins is normal in young animals, and so are their rod photoresponses. PRCD knockout mouse photoreceptors degenerate extremely slowly. By electron microscopy, outer segments from PRCD knockout mice are disorganized and display a phenotype similar to dogs containing C2Y mutation in PRCD. The studies presented in this dissertation are the first to lay the biochemical ground work for characterizing PRCD, and elucidate its function in photoreceptor disc membranes.
Dedication

I would like to dedicate this dissertation to my wife, Kaila. Her love and support is unwavering, and I cannot stress enough how special she is to me.
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1. Introduction

Human vision is an incredible product of evolution. Our vision is highly sensitive—rod photoreceptors can detect the smallest unit of light, a single photon (Baylor, Lamb, and Yau 1979). An equally impressive feat is the dynamic range of light intensity from which our vision can function properly, operating through 10 orders of magnitude of light intensity from dim conditions (e.g. moonlight) to bright (e.g. sunny day at the beach) (Arshavsky and Burns 2012). Human vision is also remarkably fast, accurately processing images displayed for a duration of only 13 milliseconds (Potter et al. 2014). Human vision encompasses a wide range of the electromagnetic spectrum from about 400nm (violet) to 750nm (red) of the electromagnetic spectrum, and combines inputs from three peaks of this spectrum to produce millions of colors. Finally, human vision has excellent spatial resolution constructed from each eye having over 120 million photoreceptors (Mustafi, Engel, and Palczewski 2009; Curcio et al. 1990), which for comparison, a typical digital camera in 2017 has ~12 million pixels.

Our vision is arguably our most important sense, making it the most debilitating to live without. Its importance is highlighted by the fact that roughly half of the human brain can be purposed to processing visual information, in addition to the direct and dedicated processing by the visual cortex (Kastner and Ungerleider 2000; Toth et al. 1996). Unfortunately, blindness is a relatively common and incapacitating occurrence.
The most common visual impairments, are refractive errors and cataracts, both of which are relatively easy to fully correct with eyeglasses, contacts, or routine surgery. In contrast, blindness caused by retinal degeneration such as retinitis pigmentosa is incurable, and occurs at a rate of 1 in 4000 (Hartong, Berson, and Dryja 2006). Retinitis pigmentosa (RP) is caused by mutations in genes essential for the functioning and survival of photoreceptors in the retina. Human patients typically lose night vision young in life, followed by the loss of peripheral vision and finally complete blindness. There are currently over 100 genes whose mutations are linked to the disease, but about half of RP patients presumably have uncharacterized mutations in these genes or other genes entirely (Hartong, Berson, and Dryja 2006). Through the course of retinitis pigmentosa, the loss of night vision and peripheral vision occurs first, because rod photoreceptors, which are responsible for these aspects of vision, die first, followed by cone photoreceptors. Future therapies will depend on furthering our basic understanding of how photoreceptors function at the molecular level, especially filling the void in our knowledge about how proteins are trafficked in these cells, how their elaborate light sensitive compartments are built, and what specific stressors result in their death.

The subject of this dissertation is a study of the rod photoreceptor, in the context of PRCD, a small protein whose mutations cause retinitis pigmentosa in humans, and an
analogous and common disease of canine patients called progressive rod-cone
degeneration (from which PRCD was discovered and named). PRCD had unknown
localization in any cell type until we discovered it as a unique photoreceptor disc
resident protein—the only one with completely unknown function. Given virtually
nothing was known about this protein, this study goes extensively through its
biochemical characterization, how its disease-causing mutation affects the protein, its
molecular interaction with other proteins, and a detailed analysis of photoreceptors
which lack this protein.

**1.1 The retina is a multilayered neural tissue**

Vision begins when light enters the eye, passing through the cornea and pupil
after which it is refracted by the lens. The lens is double convex, so an inverted and
focused light image is projected to the back of the eye where it is then absorbed by the
retina (Figure 1A,B). The retina is a ~250 µm thick tissue composed of distinct layers of
neurons and their synapses. The outer nuclear layer (ONL) is composed of the nuclei of
rod and cone photoreceptor neurons, which are responsible for sensing light and
beginning the transmission of a visual signal to the brain, ultimately initiating vision. To
transmit their light response, the photoreceptors form synaptic connections with the
bipolar cells at a distinct layer of the retina called the outer plexiform layer (OPL). The
bipolar cells relay the light signal from photoreceptors, transmitting it to the ganglion
cells while also integrating information about the light stimulus, such as contrast, temporal and chromatic information (Euler et al. 2014). Bipolar cell nuclei largely populate the inner nuclear layer (INL), but also amacr ine and horizontal cell nuclei. Horizontal cells are interneurons which widely project laterally along the OPL to photoreceptors, providing negative feedback to adjust the light response in dim or bright conditions (Masland 2012). Amacrine cells also modulate the light response in various ways (e.g. direction selectivity), and project along the inner plexiform layer (IPL), a layer of synapses between bipolar, amacr ine and ganglion cells (Masland 2012). Ganglion cells transmit the light response to the brain, through their axons which are bundled together forming the optic nerve (Figure 1B).
Figure 1: Anatomy of the retina. (A) A light microscope image of a full mouse retinal cross section. Note the purple and green box showing orientation consistent between panels A, B and C. (B) A cartoon depiction of human eye anatomy, with the same orientation as the retinal cross section in A. (C) A zoomed and partially rotated view of the image in A corresponding to the purple/green box. All layers of the retina, and RPE/choroid are shown. (D) A zoomed view of C indicated by the dashed lines, which
shows the photoreceptor layer next to a cartoon of a single rod photoreceptor cell as it is positioned in the retina.

Light must pass through all the aforementioned layers of neurons to reach its final site of absorption, the outer segments of photoreceptors. This counter intuitive layout ensures the juxtaposition of photoreceptor outer segments with the retinal pigment epithelium (RPE) (Figure 1D). This placement is ideal for at least two of the RPE’s functions. First, the RPE contains numerous light-absorbing melanin granules (Figure 1D) which allow it to prevent light scattering in the eye by fully absorbing any light which isn’t absorbed by the retina. Second, the RPE slowly phagocytoses the outer tips of photoreceptor outer segments—a process which coupled to the continuous growth of the outer segment at the base functions to replace the entire outer segment every ~10 days. This process is necessary to ensure proper outer segment function, despite continuous photooxidative damage.

There are two types of photoreceptors, rods and cones, which are named after the shapes of their light sensitive outer segments. Rod photoreceptors have larger, rod shaped outer segments which are suited for extremely high-sensitivity photon absorption making them ideal for nighttime, or scotopic vision. In contrast, cones have smaller outer segments, and contain a photopigment (cone opsin) which absorbs light at a different wavelength than the photopigment of rods (rhodopsin). Furthermore, many
animals have multiple types of cones (humans have three), each containing a cone opsin with unique absorption spectra peaks—the basis for color vision. Humans contain ~120 million rod photoreceptors, and ~6 million cone photoreceptors in each retina, which are distributed unevenly (Curcio et al. 1990). The center of the human retina, called the macula, is cone dominated and responsible for high acuity vision, while the periphery of the retina is rod dominated and responsible for peripheral/nighttime vision. The mouse retina does not have a macula, its cones are distributed evenly across the retina.

1.2 The photoreceptor is a sensory neuron

Rod and cone photoreceptors have four highly specialized segments: the outer segment, the inner segment, the nucleus, and the synaptic terminal (Figure 2).
Figure 2: The rod photoreceptor cell. On the left, the four compartments of the mouse rod photoreceptor are shown, roughly to scale in relative proportion. The dashed lines correspond to the zoomed cartoon in the middle, showing the inner and outer segment as a cartoon cross section, which is not to scale in relative proportion. The inner segment houses machinery needed to support the outer segment, including endoplasmic reticulum (ER), golgi apparatus, and mitochondria. The basal body has a pair of centrioles which are anchored to the plasma membrane, and extend microtubules forming the axoneme through the connecting cilium (shown as CC). From the cilia plasma membrane, discs begin as evaginations before they are enclosed inside the outer segment, trapping extracellular space inside—note the blue colored extracellular space, and the yellow colored cytosolic space. Also note the “hairpin” shape of the disc edges, which is only present at the axonemal side of evaginating discs. On the right of this figure, dashed lines connected to the middle cartoon correspond to a zoomed electron
micrograph of rod photoreceptor discs packed in the outer segment. There are ~1000 discs packed into a single outer segment.

1.2.1 The photoreceptor cell body

The synaptic terminal of a photoreceptor is responsible for transmitting the light responses to bipolar cells of the inner retina. To accomplish this under the conditions of a rapid and sustained transmission which is typical of a light response, photoreceptors have a specialized type called ribbon synapses. These synapses contain a structural protein called RIBEYE, which forms a scaffold that positions synaptic vesicles in close proximity to their release sites, enabling fast transmission (Magupalli et al. 2008; tom Dieck and Brandstatter 2006).

The nucleus of photoreceptors does what nuclei do—make mRNA transcripts of the proteins needed for the photoreceptor. Of special note, the nucleus of mammalian photoreceptors has a larger diameter than that of its outer and inner segment (Figure 2). If the photoreceptors were arranged in a single row, they would have large gaps between their outer and inner segments. To overcome this potential packing inefficiency, the photoreceptor nuclei are stacked above and below each other, creating an outer nuclear layer that is ~10 nuclei thick (Figure 1 C,D). In some vertebrates with especially large outer segments, such as frogs, the photoreceptor nuclei are in a single row because their nuclei have the same diameter as their outer segments.
The inner segment of the photoreceptor lies between the nucleus and outer segment. It houses all the biosynthetic machinery and mitochondria which are necessary for sustaining the extreme metabolic demands of the photoreceptor outer segment. This includes sustaining light responses, and continuously building and trafficking the protein and lipid components of the outer segment as it is renewed at the base of the outer segment. The large anabolic demand on the inner segment is due to the fact that the outer segment replaces 10% of itself every day, to replenish its photo-oxidized lipids and proteins (Kevany and Palczewski 2010). This high anabolic demand is comparable to that of a rapidly dividing cancer cell, and coincidently, photoreceptors predominately express pyruvate kinase M2, which is a specialized glycolytic enzyme commonly over expressed in tumor cells which redirects glucose to anabolic pathways rather than efficient ATP production by the TCA cycle, even in the presence of abundant oxygen (Lindsay et al. 2014; Rajala et al. 2016; Winkler 1981). One advantage of this aerobic glycolysis (called the Warburg effect in cancer cells) for photoreceptors, is the ability to produce more NADPH which is required for the lipid synthesis needed to build new discs (Punzo, Xiong, and Cepko 2012). Conversely, photoreceptors are neurons which require ATP to drive the proton pumps necessary to maintain their membrane potentials, which necessitates that ATP production is also high in these cells. For these reasons, photoreceptors have been touted as the highest energy consuming cells of the
1.2.2 The outer segment is the light sensitive organelle of the photoreceptor

The outer segment is a large, modified primary cilium responsible for absorbing light and generating the light response (Pearring et al. 2013). As a primary cilium, the outer segment protrudes from the apical plasma membrane by a projection of microtubules from the basal body. The basal body consists of a pair of centrioles anchored to the plasma membrane, where nine groups of triplet microtubules are bundled in a cylindrical structure. Nearly all primary cilia are non-motile, and do not have a pair of microtubules in the center of the basal body, thus they are in a “9+0” configuration rather than the “9+2” type observed in motile cilia. These microtubules serve as a nucleation site for building doublet microtubule extensions called the axoneme which protrude the plasma membrane. The axoneme of photoreceptors is exceptionally long, and extends approximately half the length of the outer segment (~10 \text{ \mu m} in mouse). The first segment of the axoneme and surrounding ciliary membrane forms the connecting cilium (Figure 2), which is the narrow passageway that all molecules must pass through to be delivered to the outer segment. Perhaps a function of this connecting cilium, there exists a diffusional barrier to the cilium, analogous to tight
junctions, which prevents membrane proteins from freely diffusing into the cilium.

Furthermore, a dense meshwork of Y-link structures are found in the connecting cilium of photoreceptors, and in the transition zones of primary cilia, which may be responsible for the observed limitation of cytosolic protein diffusion to primary cilia and photoreceptor outer segment. This cytosolic barrier limits cytosolic diffusion between compartments to soluble proteins smaller than ~81 kDa in photoreceptors (Najafi and Calvert 2012), and in ~67 kDa primary cilia (Kee and Verhey 2013). The exact molecular components of membrane and/or cytosolic diffusional barriers are unknown, but one important protein may be CEP290, which anchors transition zone microtubules to the ciliary membrane, localizes to the Y-link structures (Craige et al. 2010), and is a commonly mutated gene in inherited retinal degeneration patients (den Hollander et al. 2006).

Distal to the connecting cilium, is the site of disc morphogenesis. The discs begin as evaginations from the ciliary membrane, which then grow to an equal diameter of the outer segment before being fused with the ciliary membrane and enclosed inside (described in detail in the next section). Through this process the outer segment grows as a large cylinder, packed with enclosed lamellar disc shaped membranes. The function of this structure is to create the largest membrane surface area possible for housing photopigment (rhodopsin in rods) to maximize the probability that a photon will be
absorbed. In humans, there are ~100 million rod photoreceptors (Curcio et al. 1990), each of which contains an outer segment that is ~30 µm in length (Kocaoglu et al. 2016) filled with discs regularly spaced at an interval of 32nm (Gilliam et al. 2012) yielding ~1000 discs per outer segment. Since each disc contains two layers of photopigment-housing membrane, this configuration effectively increases the surface area for light capture in the retina ~2000 fold, and since the retina is ~12 cm² (Panda-Jonas et al. 1994) this equates to ~2.4 m² of light absorbing membrane surface. Plants convergently evolved a similar structure for efficient light capture called thylakoid discs which are lamellar membranes grouped into stacks called grana and maximize light absorption for photosynthesis (Pribil, Labs, and Leister 2014).

Photoreceptor discs, are round, but have one or more infolds/indentations at their edges called incisures, which are aligned between discs. While mice only have one incisure per disc, frogs, have multiple incisures in their discs and contain microtubules separate from the axoneme which run longitudinally through them, perhaps functioning to structurally support their large outer segments (Eckmiller 2000). Another function of incisures may be to form longitudinal channels to enhance the diffusion of soluble phototransduction components (Makino et al. 2012).
1.3 Photoreceptor discs are formed through the retention of ciliary ectosomes*1

Photoreceptor discs form by the evagination of the ciliary plasma membrane, a process which requires the photoreceptor disc specific protein, peripherin. Primary cilia have the capacity to release small vesicles as outward buds from their ciliary plasma membrane, called ciliary ectosomes. In photoreceptors, there is a tremendous capacity to release these ectosomes from the photoreceptor’s cilia, but the specific expression of peripherin enables these ectosomes to be retained, and morphed into photoreceptor discs. This process therefore begins as outward buds, or evaginations from the ciliary membrane which are open to the cytosolic space, before they are fully elongated and enclosed in the outer segment.

1.3.1 Photoreceptor discs form as evaginations of the ciliary plasma membrane

The morphogenesis of photoreceptor discs has been described as the invagination of the ciliary plasma membrane (Nilsson 1964), the evagination of the ciliary plasma membrane (Steinberg, Fisher, and Anderson 1980), and by vesicle fusion from within the outer segment (Chuang, Zhao, and Sung 2007). Recently, the vesicle

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1. This section is largely an adaptation from Salinas RY, Pearring JN, Ding JD, Spencer WJ, Hao Y, Arshavsky VY. Photoreceptor discs form through peripherin-dependent suppression of ciliary ectosome release. *The Journal of Cell Biology.* DOI: 10.1083/jcb.201608081 | Published April 5, 2017
fusion model, has been disproven by showing that newly forming discs are exposed to the cytosolic space (see blue-colored cytosolic space in Figure 2) by using a weakly membrane permeable contrasting agent during the fixation process for transmission electron microscopy which showed intense staining of the newly formed discs indicating that they were open, and accessible to the stain (Ding, Salinas, and Arshavsky 2015). Furthermore, the artefactual observation of small vesicles inside the outer segment was dependent on harsher fixation protocols, and completely absent in protocols known to preserve membrane structure the best (perfusion fixation gives best preservation). With high quality fixation, evaginating disc membrane was observed contiguously extending from the ciliary outer segment plasma membrane, below the enclosed outer segment. This observation was confirmed by two other publications in the same year (Burgoyne et al. 2015; Volland et al. 2015).

Several proteins are implicated in the process of forming photoreceptor discs, exemplified by their knockout mouse models which have improperly structured outer segments. Rhodopsin, the visual pigment in rod photoreceptors, is a seven-transmembrane g-protein coupled receptor (GPCR) which is not only responsible for initiating phototransduction, but also required for the proper structure of the outer segment. In rhodopsin knockout mice, the outer segment is a shortened ciliary stalk with an enclosed bulge full of disorganized membranes, which is ~10 fold smaller than a wild
type mouse outer segment. In this mouse, discs are not formed, but rather there is a
disorganized mass of membranes packed at a high density with most other disc
proteins. Given the extremely high expression of rhodopsin, accounting for 90% of the
protein material in the outer segment, this protein likely functions, at the very least, as a
material scaffold to construct the outer segment. Supporting this concept, in mice
expressing a single copy of the rhodopsin gene, the outer segment is roughly half the
diameter as wild type. Another proposed role of rhodopsin in disc structure, which has
not been confirmed experimentally, is that it helps hold discs together in a flat, lamellar
shape through homotypic interaction of its N-terminus in trans orientation. Since the N-
terminus of rhodopsin is exposed to the intradiscal space of mature discs, and the
cytosolic space in evaginating, new discs, this would effectively “glue” mature discs on
the inside, and “glue” evaginating discs to each other.

Two other proteins which are implicated in disc morphogenesis are prominin 1
and protocadherin 21, which are interacting proteins specifically localized to the round,
blunt tips of newly evaginating discs. In mice which lack these proteins, discs are
extremely long, somewhat disordered and are often sagittally oriented (parallel to the
axoneme). It is largely unknown what their function is in the disc morphogenesis
process, but in other cells promin is found to localize and stabilize the curved tips of
microvilli, while protocadherin 21 has been proposed to form connections between the
tips of evaginating discs and the enveloping inner segment. In contrast to this hypothesis, in the mechanosensitive microvilli of cells responsible for detecting sound waves in the ear, protocadherins are known to form “tip links”, which are protein-protein (e.g. protocadherin 23 binds protocadherin 15) interactions between the tip of one microvilli, and the juxtaposed membrane of the neighboring microvilli. These microvilli to microvilli connections are essential for maintaining microvilli structure, and mutations in these proteins result in deafness.

1.3.2 Peripherin enables the morphing of ectosomes into discs

Another protein required for proper disc formation is peripherin, a highly expressed protein of photoreceptor cells which specifically resides in discs (Skiba et al. 2013). It is a tetraspanin transmembrane protein which forms tetramers with another disc specific protein and close homolog, Rom-1 (Conley and Naash 2009), and specifically localizes to the edges of discs, where it is thought to form higher order oligomers which stabilize the rims/edges and incisures (Kevany et al. 2013). It is thought that this complex at the disc rims forms the “hairpin” shape of photoreceptor discs along their edges (Figure 2). This notion is consistent with the observation that peripherin is found along the axoneme side of the ciliary plasma membrane in newly forming discs, where a hairpin can be observed, but is absent from the leading edge of newly forming discs which are bluntly rounded (Figure 2) (Ding, Salinas, and Arshavsky 2015).
Figure 3: The photoreceptor has an innate capacity to produce tremendous numbers of ciliary ectosomes. (A) Retinal EM images from wild type and rds<sup>−/−</sup> mice, bar is 500nm. (B) Diameter of extracellular vesicles in rds<sup>−/−</sup> mice. (C) Budding of vesicle from rds<sup>−/−</sup> cilia, bar is 200 nm. (D) Rhodopsin immunogold labeling from wild type and rds<sup>−/−</sup> retinal sections, bar is 100nm. Figure from Salinas et al. 2017
In peripherin knockout mice (rds−/− mouse: a functional peripherin knockout created by point mutation) the photoreceptors degenerate and, an outer segment is not formed, but rather a primary cilium forms which is surrounded by vesciculated membrane material, but lacks any membrane enclosed inside (Figure 3A). The vesciculated material contains a high abundance of photoreceptor disc proteins (e.g. rhodopsin immunogold, Figure 3D), and was previously thought to be cell debris from dying photoreceptors. Upon closer examination of the vesicles using improved mass spectrometry techniques, their appearance and size is consistent with ciliary ectosomes (Figure 3B), and not cell debris, and could even be observed evaginating from the ciliary membrane (Figure 3C). Ciliary ectosomes are small vesicles (~200nm) which evaginate (push outwards from the ciliary cytoplasm) as buds, and then pinch-off (scission) from the membrane. These newly described vesicles have a range of bioactive properties, which may include releasing signaling molecules, exchanging protein and nucleic acid material, or selectively removing proteins from the cilia (Wood et al. 2013; Wang and Barr 2016; Nager et al. 2017). To confirm that the vesicles in peripherin knockout mice are indeed ectosomes, the membrane topology was tested and confirmed to expose the N-terminus of rhodopsin to the extracellular space, while the C-terminus faced inside, which is consistent with ectosome-like outward budding of the membrane. This membrane topology contrasts with photoreceptor discs, which sequester the N-terminus
of rhodopsin towards the disc lumen (inside of the disc). Furthermore, the vesicles were found to specifically contain disc proteins, but not those from the inner segment plasma membrane, meaning they were released specifically from the ciliary membrane.

To ascertain which part of peripherin is required for morphing ciliary ectosomes into discs, the core body of peripherin was expressed with its C terminus replaced with rhodopsin’s C terminus. This construct was incapable of retaining ciliary ectosomes, while a construct with peripherin’s C terminus fused to rhodopsin’s core did retain ectosomes and produced an enlarged cilia. This work proved that peripherin’s C terminus is responsible for retaining ectosomes, while the body of peripherin likely performs the membrane curvature and scaffolding functions of the protein.

In summary, the photoreceptor has a robust capability for producing ciliary ectosomes, a conserved machinery of primary cilia. Due to the specific expression of peripherin, the photoreceptor has evolved to repurpose the production of ectosomes to build photoreceptor discs and its light sensitive outer segment (Figure 4). The machinery involved in the production of ciliary ectosomes is largely unknown.
Figure 4: The photoreceptor morphs ectosomes into discs in a peripherin dependent manner. Figure from Salinas et al. 2017

1.4 Proteomic identification of unique photoreceptor disc components reveals the presence of PRCD, a protein linked to retinal degeneration*²

The protein composition of photoreceptor discs has been a subject of controversy. While biochemical studies indicated that discs are built with a handful of

*² This section is a direct adaptation from (Skiba et al. 2013)
highly specialized proteins, proteomic studies yielded databases consisting of hundreds of entries. We revisited the disc proteome by using protein correlation profiling, a methodology allowing us to identify unique components of organelles that can be fractionated but not purified to absolute homogeneity. Discs were subjected to sequential steps of fractionation and the relative amounts of proteins identified in each fraction were measured by label-free quantitative mass spectrometry. Despite several hundred proteins being identified in even the most pure disc fraction, only eleven proteins satisfied the hallmark criterion for being unique disc-resident components: the retention of a constant molar ratio among themselves across fractionation steps. Ten of them are known signaling, structural or lipid flippase proteins. The eleventh was PRCD, a protein whose mutations cause progressive photoreceptor degeneration in dogs and human patients. The remaining proteins reduced in abundance as disc purity increased, classifying them as impurities or proteins shared among multiple cellular organelles. These data demonstrate that the composition of unique disc-resident proteins is indeed very simple. The addition of PRCD to this group of highly specialized proteins opens doors toward understanding its functional role and the pathobiological significance of its mutations.
1.4.1 Proteomes of rod photoreceptor outer segments

Rod outer segments can be easily detached from the rest of the cell and obtained in preparative amounts sufficient for biochemical experiments (Smith, Stubbs, and Litman 1975; Papermaster 1982); discs can be further purified from these preparations, following simple membrane fractionation procedures. This ease of membrane purification facilitated progress in understanding visual signal transduction and placed photoreceptors among the most productive model systems for studying general principles of cell signaling (Arshavsky, Lamb, and Pugh 2002). Several decades of intensive studies have identified a number of outer segment-specific proteins, those involved in visual signaling and those responsible for maintaining the outer segment structure. However, systematic analysis of outer segment and disc proteomes has become possible only in recent years, following advances in mass spectrometry.

Proteomes of rod outer segments have been identified in three independent studies (Liu et al. 2007; Panfoli et al. 2008; Kwok et al. 2008), with the latter two addressing the proteomes of photoreceptor discs as well. These studies reported very large databases, including many hundreds of individual proteins. Notably, even the smaller proteomes of photoreceptor discs contained over 200 entries. These results do not appear intuitive: the outer segment is a highly-specialized organelle fulfilling a single physiological function. Furthermore, biochemical studies consistently stressed
that photoreceptor discs have a relatively simple protein composition. One plausible explanation for such a discrepancy is that membrane preparations analyzed in these proteomic studies were contaminated by other membranes present in retinal homogenates. This explanation is consistent with each published proteome containing multiple entries for proteins normally confined to mitochondria, nuclei, endoplasmic reticulum, or synaptic terminals, rather than outer segments. We should stress that these contaminants were identified not because outer segments or discs analyzed in these studies lacked traditionally acceptable biochemical purity, but due to the very high sensitivity of modern mass spectrometry techniques. Therefore, the actual number of proteins residing specifically in photoreceptor outer segments and discs is likely to be significantly smaller than reported, and non-traditional methodologies should be employed for their reliable identification.

In this study, we revisited the photoreceptor disc proteome by utilizing a mass spectrometry approach known as protein correlation profiling (Andersen et al. 2003). We subjected photoreceptor discs to three sequential purification steps, identified protein compositions of each preparation, and determined the relative amounts of identified proteins across disc preparations using label-free quantitative proteomics. We reasoned that the abundance of unique disc-resident proteins must remain constant across all disc preparations, whereas the abundance of contaminating or non-unique proteins should
decrease at each purification step. The total number of proteins identified in disc fraction ranged from 1000 to over 300 hundred. However, only eleven satisfied the hallmark criterion of being unique disc-resident components. Ten of these proteins have been well-characterized in previous studies and can be divided into three groups: phototransduction proteins, lipid flippases, and proteins supporting the structure of disc rims. The eleventh member of this group is PRCD (progressive rod-cone degeneration) – a protein linked to retinal degenerations in humans and dogs (Zangerl et al. 2006; Nevet et al. 2010), whose localization in the retina was completely unknown.

1.4.2 Protein Composition of Disc Membranes Subjected to Multiple Purification Steps

![Figure 5: Increasingly pure photoreceptor disc biochemical preparations.](image)

Figure 5: Increasingly pure photoreceptor disc biochemical preparations. Three steps of disc purification are shown as D1, D2 and D3, with the corresponding number of proteins identified in each by mass spectrometry. D3 is the most pure disc prep, and D1 the least pure disc prep. Figure from (Skiba et al. 2013)
The goal of this study was to identify the proteome of transmembrane or otherwise tightly membrane-associated proteins uniquely residing in photoreceptor discs. The initial disc preparation (D1; Figure 5) was obtained by osmotically shocking rod outer segments, followed by flotation of sealed disc membranes in 6% Ficoll – the methodology used for decades to purify discs in biochemical studies (Smith, Stubbs, and Litman 1975). These discs were subjected to two additional fractionation steps designed to remove or reduce their contamination by other organelles and membrane fragments. We then subjected D1 to centrifugation on a linear 1-7% Ficoll density gradient, which produced a distinct band of pink-colored discs just above another white-colored band containing membrane material of slightly higher density. Discs collected from this gradient (preparation D2) were then precipitated by antibodies against peripherin, a protein uniquely located at disc rims, to yield the final disc preparation (D3).

Proteins from each disc preparation were subjected to trypsin digestion, followed by peptide identification by LC/MS-MS using the data-dependent analysis (DDA) workflow described in Materials and Methods. Consistent with increasing disc purity, the number of confidently identified proteins (protein FDR<0.1%, minimum 2 peptides) was 1017, 526 and 335 in D1, D2 and D3. However, even D3, likely the purest photoreceptor disc membranes analyzed to date, contained a large number of proteins residing in other membrane organelles, such as mitochondria, synapses, or ER. This
convinced us that conventional membrane purification techniques are unlikely to ever allow removing disc contaminants below the detection level of modern mass spectrometers.

1.4.3 Protein correlation profiling across disc preparations of various purity

A powerful alternative approach to analyzing multi-protein complexes or organelles that can be fractionated but not purified to homogeneity is protein correlation profiling. This approach was introduced to identify resident components of centrosomes by analyzing the relative protein compositions of sucrose gradient fractions containing these organelles (Andersen et al. 2003). The method was subsequently expanded for protein assignment to other organelles (e.g. (Foster et al. 2006; Wiese et al. 2007; Borner et al. 2012)), and additional applications such as conducting subcellular proteomic profiling of a layered tissue (Reidel et al. 2011).
In the context of the disc proteome, we reasoned that unique disc-resident proteins should remain at a constant molar ratio among themselves across the D1-D3 preparations, whereas the relative content of contaminating proteins should decrease as disc purity increases. We illustrate this principle in Figure 6, which shows how the quantities of representative peptides from either resident or contaminating protein vary across the disc preparations. The upper panel of Figure 6 shows that the relative amount of a peptide from the disc-specific protein Rom1 remains nearly unchanged across disc preparations. In contrast, the abundance of another peptide representing the ADP/ATP
translocase, a contaminating protein residing in the inner mitochondrial membrane, decreases at each disc purification step (Figure 6; lower panel). The same panel illustrates that disc purification was accompanied by a disappearance of many small contaminants found around the peptide of interest.

Assuming that proteins absent from the purest D3 preparation cannot represent unique constitutive disc components, we devoted the subsequent quantitative analysis to 335 proteins identified in D3. The relative amount of each protein in each disc preparation was calculated as a sum of ion intensities from the LC/MS runs for all peptides confidently assigned to this protein (see Materials and Methods and (Reidel et al. 2011) for details) and normalized by the amount of disc-resident protein ABCA4 (which produced at least 36 easily quantifiable peptides). To maximize our chances to identify minor disc components, we did not restrict this analysis to any minimal number of quantifiable peptides since all proteins in this group have been already identified by DDA with high stringency.
Figure 7: Only eleven proteins retained abundance through the disc purification steps. Rankings from each protein identified in the D3 disc prep, of the ratio of the relative abundance in D3 to D1. The eleven listed proteins met the statistical criteria to be disc unique. A “group of five,” represents a cluster of enriched proteins but not unique to discs by the criteria. Figure from (Skiba et al. 2013)

Once the relative quantities of all 335 proteins were calculated, we ranked them based on their molar ratios between the most pure (D3) and the least pure (D1) disc preparations (Figure 7). The coefficient of variation for the entire dataset (an average of the standard deviation-to-mean ratios for all proteins) was 23.7%. Remarkably, only eleven proteins yielded the normalized D3/D1 ratio within this variation range from the value of 1 and, therefore, are disc-resident protein candidates. The difference between the D3/D1 ratio of these eleven proteins and any other of the remaining 324 proteins was highly statistically significant (p<0.0001; see Material and Methods for details of
statistical analysis). The identities of proteins forming the “top eleven” will be discussed in the next section.

1.4.4 Eleven unique disc membrane proteins

![Image of eleven unique disc proteins](Image)

Out of the eleven unique disc proteins identified (Figure 8), only one was previously unknown to reside in photoreceptor discs—PRCD.

1.4.4.1 Six phototransduction proteins

Six of them are critical components of the phototransduction pathway (see (Wensel 2008; Palczewski 2012; Arshavsky and Burns 2012) for reviews on structure and function of phototransduction proteins). They include: the visual pigment rhodopsin; retinal guanylate cyclase isoforms 1 and 2 (RetGC1&2) responsible for the synthesis of cGMP which is the second messenger in phototransduction; three proteins comprising the RGS9-Gβ5-R9AP GTPase activating complex for transducin. Interestingly, RGS9 and Gβ5 displayed the lowest values of the D3/D1 ratio in this group. Unlike all other
proteins in this group, RGS9 and Gβ5 do not contain transmembrane domains and instead are tethered on the disc surface by the membrane anchor R9AP. Thus, it is conceivable that a small RGS9-Gβ5 fraction is lost from the membranes upon disc purification.

1.4.4.2 Two disc rim proteins and two lipid flippases

The next two entries of the top eleven are the structural proteins peripherin (also known as peripherin-2 or rds protein) and rom-1. These proteins form large heterooligomeric complexes responsible for maintaining the curvature of disc edges (reviewed in (Farjo and Naash 2006; Goldberg 2006; Molday et al. 1999)). In addition, peripherin was shown to possess membrane fusogenic activity in model systems (Boesze-Battaglia et al. 2003), which suggest its potential involvement in disc morphogenesis.

The last two known proteins found in top eleven entries are lipid flippases ABCA4 and ATP8A2. ABCA4 is responsible for transporting all-trans-retinol and its phosphatidylethanolamine conjugates from the intradiscal to the cytoplasmic leaflet of the disc membrane (Weng et al. 1999; Sun, Molday, and Nathans 1999). This process is thought to play a critical role in detoxification of disc membranes from compounds that may poison retinal pigment epithelium, the cell type performing the phagocytosis of the photoreceptor outer segment tips (Sparrow, Hicks, and Hamel 2010; Kevany and Palczewski 2010). ATP8A2 is a relatively new member of the P-type ATPase family.
localized in disc membranes (Coleman, Kwok, and Molday 2009). It is thought to be involved in the ATP-dependent translocation of amino-phospholipids across disc membranes and in maintaining the phosphatidylserine asymmetry across the membrane, which could be important for normal phototransduction.

1.4.4.3 PRCD is a novel disc-specific protein

The eleventh protein satisfying the most stringent criteria for being an integral part of photoreceptor discs is PRCD (progressive rod-cone degeneration) – a protein linked to retinal degenerations in humans and dogs (Zangerl et al. 2006; Nevet et al. 2010) whose intracellular localization and cellular function had not been addressed. The Cys2Tyr mutation of the PRCD gene is commonly associated with numerous dog breeds and identified in a human patient (Zangerl et al. 2006; Dostal, Hrdlicova, and Horak 2011; Kohyama et al. 2015). Recently, other mutations in PRCD have been shown to cause retinitis pigmentosa in human patients (Beheshtian et al. 2015; Remez et al. 2014; Pach et al. 2013; Nevet et al. 2010). PRCD is the subject of this dissertation.
Figure 9: Immunolocalization of PRCD in mouse retina. Fixed retinal sections were stained with anti-PRCD antibody and costained with either WGA or PNA, lectins which bind rods or cones respectively. Merged images shown on the right, with arrows indicating PRCD staining in cone outer segments. Figure from (Skiba et al. 2013)

To confirm that PRCD indeed resides in photoreceptor discs, we generated polyclonal antibodies against the PRCD C-terminus and conducted immunohistochemical detection of this protein in the mouse retina (Figure 9). PRCD immunostaining nearly completely overlapped with that of the lectin, WGA, decorating primarily rod outer segments. We next interrogated whether PRCD is also expressed in the outer segments of cones, as may be predicted from the disease phenotype affecting
both photoreceptor types. Retina cross-sections were stained for PRCD and another lectin, PNA, which decorates primarily cone inner segments. The images in Figure 9 demonstrate strong PRCD staining in cone outer segments, which appears as an extension of the PNA-positive cone inner segment structures.

1.4.5 The detection limit of protein correlation profiling

A critical question regarding the completeness of the disc-specific proteome is the detection limit of protein correlation profiling methodology. Our study identified each previously known unique disc-resident protein, including the least abundant ATP8A2, guanylate cyclase 1 and guanylate cyclase 2 expressed at ~1:3,000, 1:1,400 and 1:5,800 molar ratios with rhodopsin, respectively (Peshenko et al. 2011; Coleman, Kwok, and Molday 2009). Provided that a mammalian rod photoreceptor disc contains fewer than 100,000 rhodopsin molecules (Lyubarsky, Daniele, and Pugh 2004; Nickell et al. 2007), these molar ratios suggest that we were able to confidently identify proteins expressed in the amount of at least ~15-50 copies/disc. While we cannot exclude that less abundant disc-specific proteins escaped our detection limits, there is no evidence in the literature that they may exist.
1.5 *PRCD is a 6 kDa protein whose mutations cause blindness in canine and human patients*

The disease progressive rod-cone degeneration causes late-onset blindness in dogs and was characterized in-depth by Dr. Gustavo Aguirre in dozens of publications dating back to the 1970s (Aquirre and Rubin 1972). The disease is well-known in the veterinary clinic, as it is the one of the most common causes of blindness in canine patients (Gentilini, Rovesti, and Turba 2009; Mellersh 2014). In 2006, the disease was linked to a single point mutation, in an unknown gene, which was subsequently named, PRCD, after the disease from which it was discovered (Zangerl et al. 2006). Also in this study, the exact point mutation which was linked to disease in dogs was also found in a blind human patient with a diagnosis of retinitis pigmentosa. In five subsequent publications, additional blind human patients have been identified containing other mutations in PRCD (Pach et al. 2013; Nevet et al. 2010; Remez et al. 2014; Beheshtian et al. 2015; Fu et al. 2013). The gene codes for a small, 6 kDa protein, which is the subject of this dissertation.

1.5.1 *Progressive rod-cone degeneration is a common canine retinal disease which causes late-onset blindness*

Progressive rod-cone degeneration disease dates to 1972 in a study of miniature poodles suffering from a blinding set of symptoms classified as progressive retinal atrophy (Aquirre and Rubin 1972), and later was more specifically termed progressive...
rod-cone degeneration (prcd) after an in-depth study of the pathogenesis (Aguirre et al. 1982). The canine disease presents as late-onset blindness, characterized by the slow, progressive reduction in visual responses measured by electroretinograms beginning at 28 weeks, and near complete loss at 18 months (Aguirre et al. 1982). In a subsequent study using full-field electroretinograms, it was calculated that rods lost 7.2% of their response amplitude per month, while cones lost 2% (Sandberg, Pawlyk, and Berson 1986). There is considerable variation for the age of onset between dog breeds, and between individuals of a given dog breed, but overall the disease relatively slow (Aguirre and Acland 1988; Dostal, Hrdlicova, and Horak 2011). Hearing in prcd affected dogs remains normal (Acland, Marsh, and Northington 1985).

By transmission electron microscopy of fixed dog retinas affected by the disease, morphological defects were observed in the structure of photoreceptor outer segments, including misoriented discs, invasion of phagocytic cells, and “vesicular profiles” accumulating in the interphotoreceptor matrix (small extracellular vesicles)(Aguirre et al. 1982; Aguirre and O’Brien 1986; Parkes et al. 1982a; Aguirre and Acland 1988). Interestingly, the outer segments developed normally, and early in the disease, most photoreceptors looked indistinguishable from those of wild type littermates in these studies. One phenotype of the disease consistently observed even in young, morphologically normal dog retinas, was a reduced ~40% rate of rod photoreceptor disc
renewal. Following an established technique (Young 1967), this rate was measured by injecting $^3$H-leucine (incorporates into nascent disc proteins) in the vitreous of control and prcd affected dogs, followed by autoradiograph several days later of a cross section of the retina to measure the scleral displacement of the radioactive band of disc protein. The result was confirmed in subsequent studies using $^3$H-fucose (Aguirre and O’Brien 1986) and Nomarski optics (Aguirre and Andrews 1987). At a young age, despite this reduction in the rate of rod disc renewal, the protein quantity, absorption spectra, and mRNA expression of rhodopsin in the affected dog retinas remains normal (Parkes et al. 1982a; Kemp and Jacobson 1992; Huang, Chesselet, and Aguirre 1994). Normal distribution of interphotoreceptor matrix lectins (Mieziewska, van Veen, and Aguirre 1993; Long and Aguirre 1991) and several other photoreceptor proteins was confirmed during early stage of prcd disease, specifically IRBP, arrestin, T$_{b}$, phosducin and PDE$^\gamma$ (Wiggert et al. 1991; Gropp, Huang, and Aguirre 1997).

Dogs affected by prcd have a ~25% reduced blood serum concentration of docosahexaenoic acid (DHA, a major lipid component of the brain and retina) concordant with a ~50% increase in the ratio of DPA (DHA precursor) to DHA (Anderson et al. 1991). This study sparked several further investigations which ultimately found: DHA synthesis in prcd affected dog retinas is normal (Alvarez et al. 1994; Chen et al. 1999) and oral DHA supplementation fails to rescue the disease.
A~20% reduction in DHA from outer segments purified from prcd affected dogs was reported, however this result was determined to be secondary and observed in multiple models of retinal degeneration (Anderson, Maude, and Bok 2001). Low DHA concentration in plasma or outer segments does not seem to have a causal role in the pathogenesis of prcd dogs, and is likely a secondary consequence (Anderson et al. 1999).

1.5.2 Discovery of the PRCD gene

The discovery of the PRCD gene is an example of the power of the canine model for genetic mapping studies. Centuries of strong phenotypic selection has created ~350 breeds of dog with large differences in coat color, size, bone structure, propensity for specific diseases, behavior and numerous other morphological traits, each with associated genotypes (Rimbault and Ostrander 2012). The advantage of a large variation in genetic background for these studies comes from the fact that two loci (e.g. genes or single-nucleotide polymorphisms(SNP)) which are close in proximity on the same chromosome, may be inherited together in a non-random fashion after chromosomal crossover, thereby putting them in linkage disequilibrium. By comparing the inheritance pattern of SNPs and their association with a specific phenotype (e.g. progressive rod-cone degeneration disease), a geneticist can narrow a region of the genome which co-segregates with the phenotype. This linkage disequilibrium analysis is much more
powerful when the genetic background is diverse between individuals, as is the case for
dogs. Furthermore, the linkage disequilibrium distance (the genomic distance between
loci after which there is 50% chance of co-segregation therefore placing them in
equilibrium) in dogs is much longer than that for humans (0.4-3.2 Mb for dogs, ~0.1 Mb
for humans) (Sutter et al. 2004). This larger linkage disequilibrium distance necessitates
a proportionally easier workload in genetic studies—less markers (e.g. SNPs,
microsatellites, RAPD) are needed for linking with a disease phenotype. A recent, large
 genetic study using over 4,000 dogs across 150 different breeds and following 180,000
SNPs identified loci associated with body size, fur length, and diseases such as hip
dysplasia and epilepsy (Hayward et al. 2016).

The progressive rod-cone degeneration disease in dogs follows an autosomal
recessive mode of inheritance, only homozygous dogs display a disease phenotype,
while no evidence of disease is observed in heterozygous animals (Aguirre et al. 1982;
Aguirre and O’Brien 1986). The disease was initially characterized in miniature poodles,
but subsequently identified English cocker spaniels and confirmed to be associated with
the prcd locus through cross-breeding experiments (Aguirre and Acland 1988). The
disease follows Mendelian expected ratios of inheritance when bred heterozygous sire to
heterozygous dam, and heterozygous sire to affected dam, but has a somewhat lower
than expected segregation ratio while breeding affected sire and heterozygous dam
(Acland et al. 1990). Although it’s possible there is a viability preference for non-prcd ova during fertilization/development, it’s likely that the disease phenotype was missed in some offspring, as it is late-onset, and varies between individuals making it much more difficult to ascertain without knowing the exact mutation (Aguirre and Acland 1988; Dostal, Hrdlicova, and Horak 2011).

The series of studies to identify the specific gene responsible for prcd disease initially involved elimination of gene candidates which are not responsible, including: RDS/peripherin (Ray, Acland, and Aguirre 1996), phosducin (Lin, Petersen-Jones, and Sargan 1998), transducin alpha (Ray et al. 1997) and apolipoprotein H (Gu et al. 1999). In the first two unbiased studies, DNA from three-generation pedigree of prcd affected dogs with large genetic background differences was obtained, which was used for linkage analysis between the prcd locus and 100 microsatellite markers (Acland et al. 1998) or random amplified polymorphic DNA (Gu et al. 1998). These studies narrowed the prcd locus to the centrometric end of canine chromosome 9. This region was further refined to 1.5 Mb using additional gene markers and microsatellites (Sidjanin et al. 2003), and subsequently by tracking SNPs and insertions/deletions between 14 different dog breeds all affected by prcd, the region was narrowed to just 106 kb (Goldstein et al. 2006). Finally, using expressed sequence tags from canine cDNA a novel gene was identified in the 106 kb region and cloned from human, mouse and dog, which
contained a single point mutation concordant with prcd disease in 18 different dog breeds (Zangerl et al. 2006).

The newly discovered retinal gene linked to prcd disease was named PRCD, and contains a ~600 bp transcript in the dog genome containing 4 exons, of which a 54 amino-acid protein is coded in exons 1-3. A single TGC to TAC nucleotide mutation which codes for a C2Y amino acid mutation results in the canine disease, and the same mutation was found in a blind human patient suffering from retinitis pigmentosa (Zangerl et al. 2006).

### 1.5.3 PRCD gene encodes a 6 kDa protein

![Alignment of PRCD protein amino acid sequences](#)

**Figure 10: Alignment of PRCD protein amino acid sequences.** Human disease causing mutations are shown with arrows. Jalview alignment with Clustal W, coloring scheme including: blue, hydrophobic residues, red, positively charged residues, purple, negatively charged residues. Jalview reference: (Waterhouse et al. 2009)

The PRCD gene encodes a 6 kDa protein, which is 54 amino acids long in dog, human and cow, and 53 amino acids in mouse (Figure 10). The point mutation which results in prcd disease in dogs, and also identified in a blind human patient, results in the replacement of the protein’s only cysteine (residue 2) for tyrosine (Zangerl et al. 2006).
Several other mutations have been identified in human patients, all diagnosed with retinitis pigmentosa, including a proline to threonine mutation at residue 25, and early stop codons at residues 1, 18 and 23 (Fu et al. 2013; Pach et al. 2013; Remez et al. 2014; Beheshtian et al. 2015). The N-terminus, which harbors the C2Y mutation, is highly conserved across vertebrate species, highly hydrophobic (residues 1-15), and was predicted to form a membrane anchor domain (Zangerl et al. 2006; Spencer et al. 2016). The putative membrane anchor cannot extend past the three positively charged arginines (residues 16-18). The C-terminus of PRCD contains numerous positively and negatively charged amino acids, and is highly conserved within the last ~15 residues. Mouse PRCD has a theoretical molecular weight of 5931 Da and pI of 9.3.
2. Materials and methods*3

2.1 Antibodies

We used the following antibodies for Western blotting: pAb anti-PRCD (described in (Skiba et al. 2013)); mAb anti-Rhodopsin: epitopes 1D4 and 4D2 (abcam ab5417 and ab98887); pAb anti-FLAG (Sigma F7425); pAb anti-Gαt (Sigma G5290); pAb anti-peripherin (residues 296-346 from Gabriel Travis, University of California Los Angeles); pAb anti-R9AP (residues 144-223 from Stefan Heller, Stanford University); pAb anti-ROM1 (described in (Gospe et al. 2011)). For immunohistochemistry, we used mAb anti-FLAG (Sigma F3165), pAb anti-PRCD, and mAb anti-rhodopsin (abcam ab5417). For immunoprecipitation we used mAb anti-rhodopsin (abcam ab5417), pAb anti-Gαt (described in (Lobanova et al. 2007)), and pAb anti-peripherin (described in (Skiba et al. 2013)).

The following antibodies were generously provided by: David Garbers, University of Texas Southwestern (pAb L670, anti-GC2); Alexander Dizhoor, Salus University (pAb KHD, anti-RetGC1); Wolfgang Baehr, University of Utah (mAb 1S4, anti-RetGC1); Robert Molday, University of British Columbia (mAb 1D1 PMC, anti-CNGα1); Steven Pittler, University of Alabama at Birmingham (pAb, anti-CNGβ1);

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*3 Methods include those from (Spencer et al. 2016), (Pearring et al. 2015) and (Skiba et al. 2013)
Gabriel Travis, University of California Los Angeles (pAb, anti-peripherin residues 296–346); Jeremy Nathans, Johns Hopkins University (pAb, anti-protocadherin 21 C-terminus); Stefan Heller, Stanford University (pAb, anti-R9AP residues 144–223).

The polyclonal antibody against Rom-1 was generated in our laboratory (Gospe et al. 2011). Commercial antibodies were: mAb 1D4, anti-rhodopsin (Abcam, Cambridge, MA); pAb, anti-rhodopsin N-terminus (Sigma, St. Louis, MO); pAb M-18, anti-ABCA4 (Santa Cruz, Dallas, TX); pAb, anti-ABCA4 C-terminus (Everest Biotech, Ramona, CA); mAb 13A4, anti-prominin (eBioscience, San Diego, CA); mAb M2, anti-FLAG (Sigma) and pAb, anti-FLAG (Pierce, Grand Island, NY); pAb, anti-GFP conjugated to Alex Fluor 488 (Molecular Probes, Grand Island, NY); pAb 71D10, anti-Myc-Tag (Cell Signaling, Danvers, MA); pAb, anti-DnaJ-B6 (Thermo Scientific, Grand Island, NY).

Polyclonal anti-PRCD antibodies were generated in rabbit against the peptide: CDGTVVGSDDTDLQSTGREKGPVK representing the mouse PRCD sequence. The antibody was affinity-purified using the corresponding peptides attached to SulfoLink beads (Thermo Scientific) via the N-terminal cysteine residues.

2.2 Western blotting

Western blotting was performed using polyacrylamide gels and PVDF from Bio-Rad. Comparison of PRCD and peripherin expression between C57BL/6J, Rho+/−, and Rho−/− mice was performed as described previously, (Pearring et al. 2015) with the addition of
phosphatase and hydroxylamine treatments to fully remove PRCD post-translational modifications. Western blots were imaged using the Odyssey infrared imaging system (LiCor Bioscience) using Alexa Fluor 680 or 800 secondary antibodies (Invitrogen) and the intensities of protein bands were quantified with Image Studio software (LiCor Bioscience). For total protein quantification we used the RC DC Protein Assay kit (Bio-Rad).

For western blots comparing protein quantities between wild type and rhodopsin knockout mouse retinas, retinas from C57BL/6J or Rho−/− mice were collected at P21 and sonicated in 250 µl of 2% sodium dodecyl sulfate and 1× cOmplete protease inhibitor mixture (Roche, Indianapolis, IN) in phosphate-buffered saline (PBS). Lysates were cleared at 5,000 g for 10 min at 22°C. Total protein concentration was measured using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA) and serial dilutions of each lysate were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (with samples not boiled). For most proteins, Western blotting was performed using secondary goat or donkey antibodies conjugated with Alexa Fluor 680 or 800 (Invitrogen) and bands were visualized and quantified using the Odyssey infrared imaging system (LiCor Bioscience). Bands of PCDH21 and CNGβ1 were visualized using goat or donkey secondary antibodies conjugated with horseradish
peroxidase (HRP) for enhanced chemiluminescence (ECL) detection (ECL Prime, GE Healthcare, Pittsburgh, PA).

2.3 Protease treatment of intact photoreceptor discs

Osmotically intact photoreceptor discs were purified from bovine retinas as described previously. (Skiba et al. 2013) Briefly, a crude disc preparation was obtained by floating osmotically shocked rod outer segment membranes in 6% Ficoll in water, and then discs were further purified on a continuous gradient of 1-7% Ficoll in water. Discs were suspended at a protein concentration of 1 mg/ml in buffer containing 130 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 10 mM HEPES (pH 7.2), and proteinase K (QIAGEN) at 8 µg/ml was included or excluded. The disc suspension was rotated at room temperature for 1 hour prior to dilution in the same buffer without proteinase and centrifugation at 100,000 x g for 15 minutes. The pellet was rinsed once to remove proteinase before adding the SDS-PAGE sample buffer with 100 mM DTT and protease inhibitors (eComplete, Roche).

2.4 Removal of PRCD phosphorylation and S-acylation modifications

Retinas from wild type C57BL/6J mice (Jackson Labs) were pulled and sonicated in 2% SDS, 20 mM Tris (pH 7.0), 100 mM NaCl, 5 mM MgCl₂, and protease inhibitors (eComplete, Roche). The lysate was centrifuged at 100,000 x g for 20 minutes before
adding either calf intestinal phosphatase at 10 units/ml (New England Biolabs) or phosphatase inhibitors (PhosSTOP, Roche) and incubated at 37 °C for 1 hour. Next, SDS-PAGE sample buffer was added to the lysates with or without DTT (Sigma) at 100 mM final concentration and hydroxylamine (Sigma) (pH adjusted to 7.0 with NaOH) at 500 mM final concentration. The lysates were incubated at 50°C for 1 hour before loading equal amounts (not exceeding 5 µg total retinal protein) from each condition on an 18 well 10-20% Tris-HCl polyacrylamide gel (Bio-Rad). Standard Western blotting techniques were used with the following modification: transfer buffer with 40% methanol, 25 mM Tris (pH 8.3), 192 mM glycine and 30 min transfer at 260 mA (Bio-Rad Criterion).

2.5 Removal of PRCD S-acylation prior to centrifugation

Bovine discs were resuspended in PBS, and treated with or without hydroxylamine (Sigma) at a final concentration of 500 mM (pH adjusted to 7.0) for 1 hour at 50°C. The suspension was centrifuged at 16,000 x g for 20 minutes. The supernatant was collected, and the pellet resuspended in equal volume of PBS as the supernatant. SDS-PAGE sample buffer was added, and Western blotting for PRCD was performed on normalized amounts of input, pellet and supernatant.
2.7 DNA constructs and in vivo electroporation

Mouse PRCD was cloned using primer overlap extension PCR, either wild type, or incorporating a C2Y mutation. A single FLAG tag was added directly to the C terminus of PRCD, and the DNA construct was incorporated between 5’ Age1 and 3’ Not1 sites of the pRho plasmid containing the bovine rhodopsin promoter (gift from CL Cepko, Harvard University; Addgene plasmid #11156). These FLAG-tagged PRCD constructs (4 mg/ml) were injected subretinally into neonatal wild type CD-1 mice (Charles River), as described previously in (Matsuda and Cepko 2004) and (Gospe et al. 2011). To identify electroporated patches by fluorescence, a construct expressing soluble mCherry (2 mg/ml) was included. At postnatal day 21, the mice were sacrificed for experiments. All animal research in this study has been reviewed and approved by The Institutional Animal Care and Use Committee, Duke University.

2.8 PRCD immunoprecipitation from electroporated mouse retinas

For the immunoprecipitation of FLAG-tagged PRCD constructs from electroporated retinas, we dissected eyecups at P21 in ice cold mouse Ringer’s solution and carefully cut mCherry-positive patches under a fluorescent dissecting microscope (Leica M165 FC). The electroporated patches of retinas from the entire electroporated litter (~10 mice) were combined and sonicated in 1 ml of 1% DDM (n-Dodecyl-β-D-
maltoside, ThermoFisher), PBS and protease inhibitors (Roche). The lysate was centrifuged at 16,000 x g for 15 minutes, and incubated overnight with 20 µl of anti-FLAG magnetic beads (Sigma), rotating at 4 °C. The beads were washed once with lysis buffer, before eluting proteins with 2% SDS. The eluted constructs were treated with phosphatase and reducing agents as described above to determine their S-acylation status by Western blot.

2.9 PRCD peptide pull down and mass spectrometry

Full length PRCD peptide with a biotin covalently attached to the C terminal lysine (used in (Skiba et al. 2013)) was bound to streptavidin magnetic beads (Pierce). The beads were washed to remove any unbound PRCD peptide before incubation with bovine photoreceptor discs (purified as described in (Skiba et al. 2013)), solubilized at a concentration of 250 µg/ml in PBS containing 0.7% CHAPS (ThermoFisher) and protease inhibitors (Roche), overnight, rotating at 4 °C. The beads were washed three times with the same buffer before eluting proteins with 2% SDS before conducting their tryptic hydrolysis for mass spectrometry as described in (Wisniewski et al. 2009; Skiba et al. 2013). Identification of eluted proteins by LC-MS/MS analysis was performed as described previously (Skiba et al. 2013) in two separate purifications with three technical repeats for each. Briefly, peptides were analyzed using a nanoAcquity UPLC system coupled to a Synapt G2 HDMS mass spectrometer (Waters, Inc.) employing the LC-
MS/MS experiment in a data independent acquisition mode complemented with ion mobility separation (HDMSE). In triplicate repeats, peptide digests were separated on a C18 BEH column (Waters Inc.) using a 90 min gradient of 8% to 35% of acetonitrile in 0.1% formic acid at a flow rate of 0.3 ml/min at 45 °C. Eluting peptides were sprayed into the ion source of the Synapt G2 using the 10 µm PicoTip emitter (Waters Inc.) at a voltage of 2.75 kV. For robust peak detection and alignment of individual peptides across all HDMSE runs, we used automatic alignment of ion chromatography peaks representing the same mass/retention time features. To perform peptide assignment to the features, PLGS 2.5.1 was used to generate searchable files that were submitted to the IdentityE search engine incorporated into Progenesis QI Proteomics. For peptide identification we searched against IPI database (2013 release). Protein abundances were calculated from the sum of all unique peptide ion intensities normalized to the total ion current of all peptides in the sample.(Reidel et al. 2011) Conflicting peptides were excluded from the calculations. Progenesis software was used to determine the significance level of fold changes for each protein identification as p-values calculated by repeated measures ANOVA.

2.10 Gel filtration chromatography

Photoreceptor discs were solubilized in PBS containing 0.1% DDM and subjected to gel filtration chromatography on a Superose-12 column (Amersham) connected to a
FPLC system (Pharmacia), as described previously. (Lobanova et al. 2013) The elution rate was 400 µl/min, and fractions were collected every 1 minute for analysis by Western blotting.

2.11 Protein immunoprecipitation

Rhodopsin was precipitated using mouse monoclonal antibody 1D4 (Abcam) as described in (Pearring et al. 2015), except for using purified bovine photoreceptor discs solubilized in PBS containing 0.1% DDM in place of mouse retina lysates. Solubilized disc membranes were incubated with anti-rhodopsin antibody overnight at 4 °C before addition of protein A/G magnetic beads (Pierce). After incubation for 1 hour at 22 °C, the beads were separated from the lysate using a magnet, and the unbound material was collected before washing the beads with the same buffer. The bound proteins were eluted from the beads using an equal volume of PBS containing 2% SDS for elution. By eluting bound material in an equal volume as input, all fractions (input, unbound, and bound) were normalized prior to Western blotting. Immunoprecipitation of the α-subunit of transducin (Gαt) and peripherin was performed following the same protocol, using sheep anti-peripherin antibody described in (Skiba et al. 2013) and sheep anti-Gαt antibody described in (Lobanova et al. 2007). To avoid antibody cross-reactivity on
Western blots, proteins were visualized with rabbit anti-\(\text{G} \alpha\) (Sigma), and rabbit anti-peripherin antibody (gift from GH Travis, UCLA).

For the co-immunoprecipitation of rhodopsin with guanylate cyclase 1, one C57BL/6J or \(Rho^+/-\) retina was homogenized in 200 µl of PBS with 1× phosphatase inhibitor cocktail (PhosSTOP, Roche), 1× cOmplete protease inhibitor mixture (Roche), and either n-dodecyl β-D-maltoside or Triton X-100 at desired concentration. Gentle homogenization was performed using a pestle on ice without vortexing or sonication. Lysates were cleared at 100,000 g for 20 min at 4°C and 20 µl aliquots were incubated with primary antibodies overnight at 4°C under continuous rotation (5 µg of anti-rhodopsin antibody 1D4, 0.2 µg of anti-GC-1 antibody 1S4, or mouse monoclonal IgG, Santa Cruz). For epitope blocking, rhodopsin 1D4 peptide (AnaSpec, Fremont, CA) was added to lysate with 1D4 antibody at a final concentration of 2 mM. Protein A/G magnetic beads (Pierce) were incubated with the lysate under rotation for 15 min at 22°C; 25 µl of beads were used to precipitate antibodies bound to rhodopsin, while 5 µl of beads were used to precipitate GC-1. Flow through was collected and beads were washed in 100 µl of the corresponding lysate buffer before being eluted with 20 µl of 2% sodium dodecyl sulfate (SDS) in PBS. Finally, 5 µl of 6× sample buffer with 100 mM dithiothreitol (DTT) were added to each sample (input, flow through, eluate) for SDS-PAGE. Samples were not boiled.
2.12 Immunofluorescence

Eyecups were dissected and fixed for agarose sectioning using a vibratome as described in (Pearring et al. 2015). Staining with Hoechst 33342 (Invitrogen), primary antibodies, and conjugation with appropriate Alexa Fluor secondary antibodies (Invitrogen 488 and 568) was performed as described previously. (Pearring et al. 2015) Images were taken with a Nikon Eclipse 90i microscope and C1 confocal scanner.

Immunofluorescence of disc proteins to compare between wild type and rhodopsin knockout mice were performed from C57BL/6J or Rho^-^- mice fixed for 1 hr with 4% paraformaldehyde in mouse Ringer’s solution, rinsed three times in Ringer’s, and embedded in 4% UltraPure agarose (Invitrogen, Grand Island, NY). Cross-sections of 100 µm were collected using a vibratome (Leica Biosystems, Buffalo Grove, IL), placed in 24-well plates, and blocked in 5% goat serum and 0.5% Triton X-100 in PBS for 1 hr at 22°C. Sections were incubated with primary antibodies in blocking solution overnight at 4°C, rinsed three times, and incubated with goat secondary antibodies conjugated with Alexa Fluor 488, 568, or 647 (Invitrogen) in blocking solution for 2 hr at 22°C. To stain nuclei, 5 µg/ml Hoechst - (33342, Invitrogen) was used. To stain mouse cones, 1 µg/ml lectin peptide nucleic acid (PNA) conjugated to Alexa Fluor 488 (Molecular Probes) was used. Sections were mounted with Immu-Mount (Thermo
Scientific) and cover-slipped. Images were acquired using a Nikon Eclipse 90i microscope and a C1 confocal scanner controlled by EZ-C1, version 3.10 software.

2.13 Isolation of osmotically intact rod outer segments from bovine retinas

All procedures were performed under dim red light illumination at 4°C, following the strategy described in (McDowell 1993) with modifications. 100 frozen retinas (T.A. & W.L. Lawson Co., Lincoln, NE) were thawed, re-suspended in 180 ml of the Ringer’s solution (10 mM HEPES, 130 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, and 0.02 mM EDTA pH 7.4) containing 20% OptiPrep (Axis-Shield PoC AC, Oslo). Outer segments were detached from the retinas by swirling in 1 l Erlenmeyer flask and separated from retinal debris by centrifugation at 3,700g for 6 min. The supernatant was diluted with an equal volume of Ringer’s, and rod outer segments were pelleted by centrifugation at 6,300g for 8 min. The pellet was re-suspended in Ringer’s, applied on the 12/20% step gradient of OptiPrep and centrifuged for 1 h in a SW-28 rotor at 27,000 rpm. Rod outer segments were collected from the 12/20% OptiPrep interphase and used to prepare disc membranes.

2.14 Photoreceptor disc purification

The initial disc preparation (D1) was obtained from bovine rod outer segments as described in (Smith, Stubbs, and Litman 1975) by subjecting rod outer segments to
osmotic shock in water and floating discs on the 6% Ficoll solution in water. Discs were next enriched on a 1-7% continuous gradient of Ficoll in water, using a SW-28 rotor at 27,000 rpm for 2 h. Discs migrated as a pink band located ~⅓ from the top of the gradient just above another diffused whitish band representing microsomal contaminations. A small grayish pellet of additional contaminating material was sedimented at the bottom of the tube. This pink band was collected in the dark, diluted with 40 ml buffer containing 10 mM HEPES, 120 mM NaCl, 8 mM MgCl₂ (pH 7.5) (this buffer was used at all subsequent disc purification steps) and spun down at 20,000 rpm for 20 min. To remove the residual Ficoll, the membrane pellet was washed in another 45 ml buffer. The pellet was re-suspended in 1-2 ml buffer and was defined as D2. The final disc preparation (D3) was obtained by immunoprecipitation of D2 with antibodies against the disc-specific protein, peripherin. D2 membranes containing 450 µg of total protein were incubated overnight with 150 µg anti-peripherin antibodies, followed by pull-down with Protein A/G magnetic beads (200 µl the 50/50 slurry; Thermo Scientific). The slurry was gently shaken for 2 h at 20°C and the beads were collected using a magnet, washed 3 times with the disc purification buffer and once with the same buffer containing 0.5 M NaCl. To maximize protein recovery from the beads, we solubilized discs attached to the beads with SDS-PAGE sample buffer (100 µl of 0.125 M Tris-HCl
(pH 6.8), 2% SDS and 10% glycerol). Total protein concentration in all disc fractions was determined using BCA assay (Thermo Scientific).

**2.15 Quantitative Western blotting**

PRCD quantification in the D2 disc preparation was determined essentially as described in (Lobanova et al. 2008). Disc aliquots containing known amounts of rhodopsin were analyzed alongside with synthetic full-length bovine PRCD peptide (purchased from Alpha Diagnostic Int.). The peptide concentration was determined spectrophotometrically using the molar extinction coefficient $\varepsilon_{280}=5,580$. PRCD bands were visualized with our anti-PRCD antibody and secondary goat anti-rabbit antibodies conjugated with Alexa Fluor 680 (Invitrogen). Protein bands quantification was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences).
2.16 Generation and confirmation of PRCD knockout mouse

The PRCD knockout mouse was generated in collaboration with the Duke Transgenic Mouse Facility. A PRCD genomic targeting construct was generated by BAC recombineering which contained 8 kb 5’ homology, and 2 kb 3’ homology arms flanking exons 1-3 of PRCD gene, which contain the entire coding region. The targeting construct contained a neomycin cassette between the homology arms flanked by LoxP sites. The construct was injected into G4 ES cells and selected as described previously (DeChiara et al. 2010), and positive clones were identified by two PCR reactions: (1) 5’ long arm primers, TGGTATCAGGGAGGAAGGGTGTAG and AAGGGTTATTGAATATGATCGGAATTGG, (2) 3’ short arm primers catgcctcttgacgagtcttc and GGCTAGACAGAGGCATCTCTGCTG. These PCR reactions extend from the flanking genomic locus through the entire length of the homology arms (8 kb and 2 kb) to the targeted locus, so only targeting vector incorporated at the PRCD locus would produce a PCR product.

Final verification of PRCD knockout was performed by Southern blot using previously described methods (Southern 2006). Briefly, 20 µg of DNA from wild type, PRCD heterozygous or PRCD mock knockout BAC construct was fully digested with Pst1 restriction enzyme, loaded on an agarose gel and transferred to a nylon membrane.
Next, a 1 kb radio-labeled probe was generated within the 3' homology arm of the targeting construct using random primer DNA labeling kit (Takara), and incubated with the nylon membrane before exposure to x-ray film. A ruler was used to align the film with the DNA ladder, to identify the presence of targeted or wild type PRCD genomic constructs. See Figure 22 for detailed description of targeting construct, primers, probe and Figure 23 for the results of Southern blot experiments.

PRCD knockout mice were backcrossed at least five generations with C57BL/6 mice purchased from Jackson Labs.

**2.17 Genotyping strategy for PRCD knockout mice**

PRCD knockout mice were genotyped using three primers in one PCR reaction (TAAGTCCTCAAGTGCTTG, CCAACCCACACCACCTAGCTC, and AAGGGTGATTGAATATGATCGGAATTGG) which produces a 600bp PCR product in wild type mice, and 300bp product in PRCD knockout mice. Heterozygous mice produce both 300bp and 600bp products. See Figure 22 for primer design cartoon, and Figure 23 for an example PCR genotyping result between wild type, PRCD knockout and PRCD heterozygous mice.

**2.18 Electron microscopy**

All electron microscopy experiments were performed using the techniques described and developed previously by our laboratory (Ding, Salinas, and Arshavsky...
2015). Briefly, mice were perfused with fixative containing 2% paraformaldehyde, 2% glutaraldehyde, and 0.05% CaCl2 in 50 mM MOPS buffer, pH 7.4, for about 10 minutes with ~15mL of fixative. The eyes were carefully dissected from the mouse being careful not to put any pressure on the eye, and post fixed in the same fixative buffer for 1 hour at room temperature. For tissue processing, tannic acid-uranyl acetate was incorporated as a contrasting agent.

Postembedment immunogold labeling with anti-rhodopsin antibodies was also performed exactly as described previously (Ding, Salinas, and Arshavsky 2015), using 1D4 and 4D2 mouse monoclonal anti-rhodopsin antibodies, at a concentration of 1:5000. For image acquisition, a JEM-1400 microscope was used.

2.19 Phase contrast microscopy and live-imaging microfluidics of isolated mouse rod outer segments

Osmotically intact rod outer segments were purified from wild type and PRCD knockout mice as described previously (Tsang et al. 1998), with modification. Retinas were pulled and placed in cold mouse ringers solution, in a 1.5 mL centrifuge tube, and vortexed for 45 seconds to break off outer segments. Large retinal debris was allowed to sediment by gravity, and rod outer segments were collected and kept on ice. Aliquots from the isolated outer segments were attached to microfluidic channel slides containing 50 µL channels (ibidi, Cat. No. 80166, µ-Slide I 0.2 Luer ibiTreat). Channel slides were
pretreated with poly-lysine solution to enhance the attachment of the outer segments.

The channel slides were connected to a peristaltic pump, and unbound outer segments were washed away by pumping isotonic mouse ringers through the channel slide. A 100x phase contrast microscope (well calibrated, Zeiss Axio Observer A1 with 100x/1.4 Ph3 440781-02: Plan-Apochromat, NA: 1.40, oil, Ph3) was used to live-image the rod outer segments as buffer was perfused over them by taking images every 0.1 seconds.

To hypotonically shock the outer segments, buffer containing 1mM Tris pH 7.4 was perfused over the rod outer segments, which lysed them, clearly swelling discs. The flow rate was increased to impart shear stress over the rod outer segments using the equation \( T \ (\text{dyn/cm}^2) = 0.0072 \ (\text{dyn*s/cm}^2) \times 512.9 \times \Phi \ (\text{mL/min}) \). A maximum shear stress of 4 dyn/cm\(^2\) was used, corresponding to approximately 1 mL/min flow rate through the channel slide at room temperature.
3. PRCD requires N-terminal S-acylation and rhodopsin binding for photoreceptor outer segment localization and maintaining intracellular stability

This section contains an adaptation from (Spencer et al. 2016).


Figure 11: PRCD interacts with rhodopsin, Cartoon from (Spencer et al. 2016)

3.1 Introduction

The light-sensing outer segments of photoreceptor cells harbor hundreds of flattened membranous discs containing the visual pigment, rhodopsin, and all the proteins necessary for visual signal transduction. PRCD (Progressive Rod-Cone Degeneration) is one of a few proteins residing specifically in photoreceptor discs, and
the only one with completely unknown function. The importance of PRCD is highlighted by its mutations causing photoreceptor degeneration and blindness in canine and human patients. Here we report that PRCD is S-acylated at its N-terminal cysteine and anchored to the cytosolic surface of disc membranes. We also showed that mutating the S-acylated cysteine to tyrosine, a common cause of blindness in dogs and found in affected human families, causes PRCD to be completely mislocalized from the photoreceptor outer segment. We next undertook a proteomic search for PRCD interacting partners in disc membranes and found that it binds rhodopsin. This interaction was confirmed by reciprocal precipitation and co-chromatography experiments. We further demonstrated this interaction to be critically important for supporting the intracellular stability of PRCD, as the knockout of rhodopsin caused a drastic reduction in the photoreceptor content of PRCD. These data reveal the cause of photoreceptor disease in PRCD mutant dogs, and implicate rhodopsin to be involved in PRCD’s unknown, yet essential function in photoreceptors.

Progressive Rod-Cone Degeneration (PRCD) is a genetic disease initially identified in miniature poodles over 30 years ago and subsequently mapped to a single C2Y point mutation in a gene encoding a short protein of the same name. (Aguirre et al. 1982; Goldstein et al. 2006; Zangerl et al. 2006) The same mutation was identified in 35 different dog breeds and is among the most frequent causes of inherited dog
blindness. (Kohyama et al. 2015; Gentilini, Rovesti, and Turba 2009; Dostal, Hrdlicova, and Horak 2011) More recently, PRCD mutations, including C2Y, have been identified in human patients suffering from retinitis pigmentosa. (Behesthian et al. 2015; Remez et al. 2014; Zangerl et al. 2006; Nevet et al. 2010; Fu et al. 2013; Pach et al. 2013) As evident from its name, the disease affects both rod and cone photoreceptors. It starts with impairment of rod function and eventually causes complete blindness when cones become affected. The time course of this disease varies across both dog and human patients, but overall, is relatively slow when compared to other inherited retinal degenerations. (Remez et al. 2014; Pach et al. 2013; Zangerl et al. 2006; Nevet et al. 2010; Fu et al. 2013)

In humans and dogs, PRCD is a 54 amino acid protein, while mouse PRCD encodes 53 amino acids. The 15 amino acids on the N-terminus of PRCD are largely hydrophobic and predicted to form an α-helical signal sequence domain. (Zangerl et al. 2006) Given that PRCD is a membrane-associated protein tightly bound to the photoreceptor disc, its N-terminus was proposed to serve as a membrane domain. (Zangerl et al. 2006; Skiba et al. 2013) However, these 15 amino acids are followed by a cluster of three positively charged arginines making this domain too short to completely span the membrane. Thus, the N-terminus is more likely to serve as a membrane anchor for PRCD rather than a typical transmembrane domain. The
remaining C-terminal part of PRCD is mostly hydrophilic without any clear secondary structure prediction. Although highly conserved across vertebrate species, PRCD has no clear homology with other proteins, which makes it hard to predict its functional role. PRCD transcript is highly expressed in the retina and has little, if any expression in other tissues. (Zangerl et al. 2006) Our recent study identified PRCD belonging to a handful of membrane proteins residing exclusively in photoreceptor discs and not in other cellular compartments of rods and cones. (Skiba et al. 2013)

In this study, we found that PRCD is adhered to the cytosolic surface of discs, is S-acylated at the N-terminal cysteine and phosphorylated. When we mutated the N-terminal cysteine to tyrosine, thereby introducing a disease-causing point mutation, the mutant PRCD was mislocalized from outer segments of mouse rods. To identify proteins which may interact with PRCD, we used biotin-tagged PRCD peptide as bait to pull down potential interacting partners from photoreceptor discs and identified them by mass spectrometry. We found that PRCD is bound to rhodopsin and confirmed this interaction by reciprocal co-immunoprecipitation and co-chromatography experiments. Finally, we showed that the PRCD-rhodopsin complex is essential for the intracellular stability of PRCD, given that PRCD is nearly absent from photoreceptors of rhodopsin knockout mice.
3.2 Results

3.2.1 Discs contain approximately 350 PRCD molecules

Figure 12: PRCD to rhodopsin ratio is ~1:300. Calibration curve for quantitative Western blotting with full length synthetic PRCD peptide standards and purified photoreceptor discs with known amounts of rhodopsin. Figure from (Skiba et al. 2013)

We determined the amount of PRCD in bovine photoreceptor discs (D2) by quantitative Western blotting, using a synthetic PRCD peptide standard to obtain a calibration curve (Figure 12). These measurements revealed that the molar ratio between PRCD and rhodopsin is 1:290±40 (SD), which is on the same order as the corresponding ratios for PDE6 (Pentia, Hosier, and Cote 2006) and the members of the GTPase
activating complex (Martemyanov et al. 2008). This ratio equates to approximately 350 PRCD molecules per bovine disc, given that there are about 100,000 rhodopsin molecules in a disc (Nickell et al. 2007).

3.2.1 PRCD is bound to the cytosolic surface of discs

Figure 13: Membrane topology and post-translational modifications of PRCD. Figure from (Spencer et al. 2016) A, Osmotically intact discs were treated with membrane impermeable proteinase K (at 8 µg/ml) followed by Western blotting with antibodies against PRCD and rhodopsin (recognizing its cytoplasmic or intradiscal epitopes). The experiment was performed with two technical repeats for each of two independently obtained biological disc preparations. B, Mouse retina lysates were treated with combinations of DTT (100 mM), hydroxylamine (NH₂OH, 500 mM), and calf intestinal phosphatase (CIP, 10 units/ml) followed by Western blotting with anti-PRCD antibody. The experiment was performed with three individual mouse retinas. C, A cartoon
depicting PRCD orientation in the disc membrane and the site of its S-acylation. The C terminus of PRCD is exposed on the cytoplasmic surface of discs, while the N terminus is anchored in the membrane and contains a lipidation attached by S-acylation (red).

We previously identified that PRCD is tightly associated with outer segment disc membranes and not found in other subcellular compartments of the photoreceptor cell. (Skiba et al. 2013) PRCD has a highly conserved N-terminus consisting of mostly hydrophobic amino acids that are predicted to form an alpha helix. This domain may serve as a membrane anchor but is too short to fully span the membrane. The rest of PRCD could reside on either the cytosolic surface, or within the intradiscal lumen of photoreceptor discs. To distinguish these scenarios, we treated osmotically intact discs with proteinase K, a membrane impermeable enzyme that cleaves peptide bonds adjacent to aliphatic and aromatic amino acids. (Ebeling et al. 1974) After proteinase treatment, we observed a complete disappearance of rhodopsin immunostaining when Western blots were probed with an antibody against its exposed, cytosolic epitope (Figure 13A, top left). In contrast, the intradiscal epitope of rhodopsin was protected from proteinase treatment, as shown by preservation of the corresponding rhodopsin peptides visualized with anti-rhodopsin antibody against its intradiscal N-terminus (Figure 13A, top right). These controls show that proteinase K treatment of osmotically intact discs proteolyzed parts of proteins exposed on the disc surface, while sparing the parts confined to the intradiscal space. In this experiment, the PRCD band on Western
blot completely disappeared after proteinase K treatment, demonstrating that the C-terminal domain of PRCD is exposed to the cytosolic space, and all PRCD molecules adhere to the cytosolic surface of photoreceptor discs (Figure 13A, bottom).

3.2.2 PRCD is S-acylated and phosphorylated

By carefully examining PRCD bands from mouse retina lysates by Western blot, we identified that PRCD is S-acylated and phosphorylated. When lysates were loaded without reducing agent treatment, PRCD migrated as a double band (Figure 13B, lane 1). When the same amount of lysate was treated with calf intestinal phosphatase, it resulted in a single, more intense PRCD band migrating alongside with the lower band prior to treatment (Figure 13B, lane 2). This indicates that the PRCD extracted from the retina represented a mixture of its phosphorylated and non-phosphorylated forms. The significant increase in band intensity could be due to both a consolidation of PRCD molecules and an increase in antibody affinity to unphosphorylated PRCD. The PRCD antibody was generated using an unphosphorylated peptide that encompasses 25 amino acids of PRCD’s C terminus, and contains most of PRCD’s serines and threonines which could be sites of phosphorylation.

When we treated the same amount of mouse retina lysate with the reducing agent, dithiothreitol (DTT), we observed multiple PRCD bands (Figure 13B, lane 3). These bands consolidated into two more intense bands after phosphatase treatment.
(Figure 13B, lane 4). Because PRCD has a single cysteine residue, DTT, by a reduction reaction could either break a disulfide bond between PRCD and another protein (such as a PRCD dimer), or remove a fatty acid attached by S-acylation to its cysteine. Given that the DTT-induced shift in electrophoretic mobility is very small, we reasoned that DTT treatment must be removing a fatty acid attached to its cysteine by S-acylation. To explain the persistence of two bands after DTT treatment (Figure 13B, lane 4), we considered that the reducing strength of DTT alone was insufficient to fully deacylate all PRCD molecules. Therefore, we treated retinal lysates with a stronger reducing agent, hydroxylamine, which ultimately shifted PRCD to a single lower band when phosphatase was also present (Figure 13B, lane 6). Considering the complete lack of the lowest band without reducing agent in (Figure 13B, lane 2), we conclude that all PRCD molecules contain a fatty acid acylation at their cysteine residue, and that a significant fraction of PRCD molecules are phosphorylated.

We attempted to determine the exact nature of the S-acylation by both electrospray ionization and MALDI mass spectrometry, but found that the modified full length protein and its N-terminal trypsinized peptide were completely insoluble under multiple experimental conditions. While it is reasonable to expect that PRCD is palmitoylated, as the majority of S-acylated proteins are, it remains conceivable that a
different fatty acid is attached to cysteine by a thioester bond. (Chamberlain and Shipston 2015)

### 3.3.3 PRCD’s N terminal cysteine is the site of S-acylation

A, PRCD-FLAG constructs, either wild type or C2Y mutant, were expressed in mouse retinas by in vivo electroporation and immunoprecipitated using an anti-FLAG antibody (a total of ten injected and expressing mouse retinas were used from ten different mice in one experiment). The constructs were treated by hydroxylamine (NH$_2$OH, 500 mM) to fully remove S-acylation. The post-treatment shift of the PRCD band (or the lack thereof) on Western blots was documented using anti-FLAG antibody. The dashed lines were drawn to assist in observing the band shift present in the wild type construct and absent in the C2Y mutant construct. B, Bovine discs were treated with hydroxylamine to fully remove PRCD S-acylation, followed by membrane sedimentation. Membranes were re-suspended in the same volume as the initial sample, and equal aliquots from the input material, soluble (Sol), and pellet fractions were analyzed by Western blotting using anti-PRCD antibody. The experiment was performed in two technical repeats for two independently purified disc preparations.

To further demonstrate that the N terminal cysteine of PRCD is a site of S-acylation in vivo, we expressed the entire mouse PRCD protein with a C terminal FLAG
tag, either wild type or bearing a C2Y mutation. The constructs were introduced by in vivo electroporation of neonatal mouse retinas. (Matsuda and Cepko 2004) The electroporated plasmid contained the rhodopsin promoter, resulting in specific expression in rod photoreceptors. Mice were harvested at postnatal day 21 after rods had fully developed. Using these mouse retinal lysates, we immunoprecipitated the constructs with anti-FLAG antibodies and subjected the lysates to reducing or non-reducing conditions after phosphatase treatment. The wild type PRCD construct showed a band shift after hydroxylamine treatment (Figure 14A top), while the C2Y PRCD construct did not shift (Figure 14A bottom). This shows that PRCD’s N-terminal cysteine is the only site of S-acylation, and is entirely responsible for the PRCD band shift after DTT and/or hydroxylamine treatment.

3.2.4 PRCD is not released from disc membranes upon its deacylation

In some instances, fatty acid S-acylation is a reversible mechanism for a protein to alternate between membrane-bound and soluble cytosolic states. (Chamberlain and Shipston 2015) To test if PRCD’s solubility depends on its S-acylation, we treated photoreceptor discs with hydroxylamine sufficient to remove all S-acylation and collected membrane and soluble fractions after centrifugation. All of PRCD was found in the insoluble fraction, regardless of its acylation status (Figure 14B).
3.2.5 PRCD C2Y mutation completely mislocalizes the protein from outer segments

Figure 15: Disease-causing PRCD mutant mislocalizes from rod outer segments. Figure from (Spencer et al. 2016) Recombinant constructs coding either wild type PRCD or its C2Y mutant behind the rhodopsin promoter were electroporated into the retinas of neonatal mice, and immunostained at P21 with an anti-FLAG antibody (green). Co-transfection with a construct coding soluble mCherry (red) allowed screening for electroporated areas of the retina prior to immunostaining for PRCD (note that the total number of cells expressing WT PRCD was higher than that expressing mCherry because the ratio between the corresponding DNA constructs upon electroporation was 2:1). A merged image is shown on the right. Nuclei are stained by Hoescht. At least three electroporated mice were analyzed for each construct and yielded similar immunolocalization patterns. Scale bar, 10 µm.

The C2Y mutation in PRCD results in retinal degeneration of human and dog patients. (Zangerl et al. 2006) In the next experiment, we investigated whether this mutation alters the cellular localization of PRCD in vivo. We expressed wild type and C2Y PRCD, each containing a C-terminal FLAG tag, by electroporation and
immunostained transfected retinas using anti-FLAG antibody. The wild type PRCD construct localized exclusively to the outer segments of rod photoreceptors (Figure 15 top; note that only a subset of rods become transfected by this technique), consistent with our previous result that PRCD is a unique photoreceptor disc protein. (Skiba et al. 2013) In contrast, the C2Y PRCD mutant was completely mislocalized from outer segments to other compartments of the rod cell (Figure 15 bottom). Interestingly, mislocalized C2Y PRCD construct was detected in a smaller fraction of mCherry-positive cells than the wild type construct (Figure 15 merged images). This likely reflects the mislocalized mutant PRCD being actively degraded, and only found in cells with especially high expression of the construct. Expression from electroporated DNA vectors inherently varies from cell to cell based on the number of incorporated DNA copies. (Itasaki, Bel-Vialar, and Krumlauf 1999)

### 3.2.6 PRCD binds rhodopsin in photoreceptor discs

<table>
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<th>Protein</th>
<th>Total Ion Intensity +PRCD</th>
<th>Fold Change</th>
<th>Anova (p)</th>
<th>Total Ion Intensity +PRCD</th>
<th>Fold Change</th>
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<td>249</td>
</tr>
</tbody>
</table>

**Figure 16:** PRCD peptide precipitates rhodopsin from solubilized disc membranes. **Figure from** (Spencer et al. 2016) Full-length PRCD peptide containing a covalently attached C-terminal biotin was used to precipitate proteins from bovine disc membranes solubilized in 0.7% CHAPS. The peptide was attached to streptavidin magnetic beads.
while the beads alone without peptide were used as a control. Precipitating proteins were analyzed by LC-MS/MS in three technical repeats, and precipitating proteins from both experiments are shown. The experiment was repeated twice with two independently purified disc membrane preparations.

Since PRCD resides exclusively in photoreceptor discs, we screened for potential PRCD interacting partners in purified disc membranes. We incubated disc lysates with synthetic, full length PRCD protein containing a biotin covalently attached to its C terminal lysine as bait in pull down experiments using streptavidin magnetic beads. As a control, the disc lysate was incubated with the streptavidin beads alone without biotinylated PRCD. The precipitating proteins were analyzed by mass spectrometry in two independent experiments, the total ion current for peptides representing all identified proteins was calculated, and the following criteria for selecting potential PRCD-binding protein candidates were applied: 1) a potential candidate is identified in both experiments on the basis of at least three peptides; and 2) at least a 2-fold increase in protein binding is observed when PRCD is present with a p-value not greater than 0.05. Based on these criteria, we identified four potential candidates: rhodopsin, the α-subunit of transducin (Gαt), peripherin and ROM1 (the latter two existing in a constitutive oligomeric complex in discs) (Figure 16).

To further evaluate whether any of the four candidate proteins identified in the pull-down assay are indeed PRCD partners, we subjected proteins solubilized from
purified bovine discs to gel filtration chromatography and compared their elution profiles to that of endogenous PRCD. The elution peak of PRCD coincided with that of rhodopsin, consistent with rhodopsin being the top candidate in the pull-down assay (Figure 17A). It also significantly overlapped with the elution profile of G\(\alpha\)<sub>t</sub>, but not with that of the peripherin-ROM1 complex.
Figure 17: Co-chromatography and co-precipitation of PRCD with its binding protein candidates. Figure from (Spencer et al. 2016) A, Purified bovine discs were solubilized in 0.1% DDM and subjected to gel filtration chromatography on a Superose 12 column. 400 µl fractions were collected and aliquots were used for Western blotting with
Reciprocal immunoprecipitation of proteins from solubilized bovine discs using anti-rhodopsin antibody showed a significant PRCD fraction bound to rhodopsin (Figure 17B). The density of PRCD band in precipitated fraction was 2.3±0.4-fold more intense than in unbound fraction (mean±SEM, n=3). This result was not dependent on the status of rhodopsin bleaching (data not shown). As a control, the antibody was blocked with its antigen peptide, and neither rhodopsin nor PRCD were bound non-specifically (Figure 17B, lane 5). Notably, a fraction of PRCD was not co-precipitated with rhodopsin (Figure 17B, lane 2), despite a complete coincidence of elution peaks between the two proteins on gel filtration chromatography (Figure 17A). Perhaps, this
could be explained by precipitation conditions being less favorable for preserving the PRCD-rhodopsin complex. Since rhodopsin is an extremely abundant component of photoreceptor discs (Pearring et al. 2013), we tested the specificity of rhodopsin binding to immobilized PRCD peptide by repeating the experiment in serial dilutions of solubilized disc lysate (Figure 17E). Rhodopsin binding to PRCD peptide was retained after dilution, while nonspecific rhodopsin binding to the empty streptavidin beads decreased proportional to the rhodopsin concentration in the lysate.

In contrast to rhodopsin, endogenous PRCD did not co-immunoprecipitate with either Gαt or peripherin (Figure 17C and 17D), despite the constitutive peripherin-ROM1 complex being well-preserved. This result suggests that association of these proteins with PRCD-containing beads was either non-specific or so weak that it was only possible with a large molar excess of PRCD (as in experiments with PRCD-loaded beads). In the case of Gαt, it is also possible that Gαt was actually associating with rhodopsin retained on these beads by PRCD, as these proteins are known to interact.

### 3.2.7 Rhodopsin is required for maintaining PRCD’s intracellular stability

Using the model of rhodopsin knockout mouse, we recently demonstrated that the majority of membrane proteins residing specifically in rod outer segments do not require the expression of rhodopsin for their intracellular stability and
localization. (Pearring et al. 2015) The only exception was guanylate cyclase 1 (GC-1), which is a rhodopsin-binding protein relying on this interaction for both intracellular stability and outer segment delivery. Since our data indicate that PRCD also binds rhodopsin, we examined PRCD expression and localization in photoreceptors of rhodopsin knockout mice, using parallel measurements with peripherin as a control. Rods of these mice do not form normal outer segments, but instead develop small ciliary extensions filled with disorganized membrane material. (Lem et al. 1999)

Immunostaining with anti-PRCD antibody revealed that PRCD is essentially absent from rods of rhodopsin knockout mice (Figure 18A), and Western blotting confirmed that PRCD is reduced in rhodopsin knockout retinas by over 12-fold (Figure 18B). To ensure that the observed reduction of the PRCD band on Western blot was not due to a change in PRCD’s phosphorylation and/or S-acylation status (both affecting the PRCD band intensity; Figure 13B), the lysates used in these experiments were treated with phosphatase and hydroxylamine to fully remove these post-translational modifications. This was different from peripherin, which was delivered to the photoreceptor outer segment ciliary extensions of these mice and retained a relatively high abundance of ~60% WT (Figure 18), consistent with previous reports. (Lee, Burnside, and Flannery 2006; Pearring et al. 2015) We also assessed the PRCD content in the retinas of mice expressing a single copy of the rhodopsin gene (Rho+/- mice) which
contain one half of normal rhodopsin content. (Lem et al. 1999) PRCD amount in these rods was reduced, but to a lesser extent than rhodopsin. This lack of strong correlation is likely explained by the presence of a large molar excess of rhodopsin in both mouse types. In summary, these results mirror our previous findings with GC-1 (Pearring et al. 2015) and indicate that PRCD also requires rhodopsin for maintaining intracellular stability and, ultimately, outer segment delivery.
Figure 18: PRCD is virtually absent from rhodopsin knockout rods. Figure from (Spencer et al. 2016) A, Immunofluorescence staining of PRCD in cross-sections of WT (left) and Rho-/- (right) mouse retinas collected at P21. Staining of rhodopsin (red), PRCD (green) and peripherin (green) was performed with anti-rhodopsin, anti-PRCD, and anti-peripherin antibodies, respectively. Nuclei are stained by Hoescht. Scale bar, 10 µm. B, Western blot of rhodopsin, PRCD and peripherin in serial dilutions of mouse retinal lysates from WT, Rho+/- and Rho-/- mice. The lysates were treated with phosphatase and hydroxylamine to fully remove PRCD post-translational modifications. Representative images are taken from three independent experiments.

3.2.8 The correct outer segment localization of other disc proteins is maintained in rhodopsin knockout mouse rods, with the exception of guanylate cyclase 1

Despite our deep understanding of visual signal transduction, little is known about how the outer segment is populated by proteins performing this function. Indeed, nearly all mechanistic studies of outer segment protein trafficking have been devoted to rhodopsin (Nemet, Ropelewski, and Imanishi 2015; Wang and Deretic 2014), which is a GPCR visual pigment comprising the majority of the outer segment membrane protein mass (Palczewski 2006). The mechanisms responsible for outer segment delivery of other transmembrane proteins remain essentially unknown. Some of them contain short outer segment targeting signals, which can be identified through site-specific mutagenesis (Deretic et al. 1998; Li et al. 1996; Pearring et al. 2014; Salinas et al. 2013; Sung et al. 1994; Tam et al. 2000; Tam, Moritz, and Papermaster 2004). A documented

4 This section is adapted and reprinted from Pearring, J. N., W. J. Spencer, E. C. Lieu, and V. Y. Arshavsky. 2015. ‘Guanylate cyclase 1 relies on rhodopsin for intracellular stability and ciliary trafficking’, Elife, 4. under Creative Commons Attribution License, Copyright 2015.
exception is retinal guanylate cyclase 1 (GC-1), whose exhaustive mutagenesis did not yield a distinct outer segment targeting motif (Karan et al. 2011).

GC-1 is a critical component of the phototransduction machinery responsible for synthesizing the second messenger, cGMP (Wen, Dizhoor, and Makino 2014). GC-1 is the only guanylate cyclase isoform expressed in the outer segments of cones and the predominant isoform in rods (Baehr et al. 2007; Yang et al. 1999). GC-1 knockout in mice is characterized by severe degeneration of cones and abnormal light-response recovery kinetics in rods (Yang et al. 1999). Furthermore, a very large number of GC-1 mutations found in human patients causes one of the most severe forms of early onset retinal dystrophy, called Leber’s congenital amaurosis (Boye 2014; Kitiratschky et al. 2008). Many of these mutations are located outside the catalytic site of GC-1, which raises great interest in understanding the mechanisms of its intracellular processing and trafficking.

In this study, we demonstrate that, rather than relying on its own targeting motif, GC-1 is transported to the outer segment in a complex with rhodopsin. We conducted a comprehensive screen of outer segment protein localization in rod photoreceptors of rhodopsin knockout (Rho-/-) mice and found that GC-1 was the only protein severely affected by this knockout. Next, we showed that this unique property of GC-1 is explained by its interaction with rhodopsin, which likely initiates in the biosynthetic membranes and supports both intracellular stability and outer segment delivery of this
enzyme. These findings explain how GC-1 reaches its specific intracellular destination and also expand the role of rhodopsin in supporting normal vision by showing that it guides trafficking of another key phototransduction protein.

3.2.8.1 Outer segment localization of disc proteins in rhodopsin knockout rods

This study was initiated by testing the hypothesis that rhodopsin, by far the most abundant outer segment protein whose proper transport is essential for formation of this organelle, may affect trafficking and abundance of outer segment membrane proteins. This was accomplished by a comprehensive examination of outer segment protein localization in rods of Rho⁻/⁻ mice. Normal outer segments are cylindrical structures filled with an ordered stack of several hundred membrane discs (Figure 19A). In contrast, Rho⁻/⁻ rods only develop small ciliary extensions filled with disorganized membrane material (Humphries et al. 1997; Lee, Burnside, and Flannery 2006; Lem et al. 1999) (Figure 19A). Despite this morphological defect, two outer segment-specific proteins, peripherin and R9AP, have been previously shown to reliably target to this ciliary extension (Lee, Burnside, and Flannery 2006; Pearring et al. 2014). We broadened this analysis to include the majority of transmembrane outer segment proteins.

We analyzed ten proteins, whose antibodies have been verified in the corresponding knockout controls. Five of these proteins are components of the phototransduction cascade (R9AP, GC-1, GC-2, CNGα1, and CNGβ1), two support disc
structure (peripherin and Rom1), one is a membrane lipid flippase (ATP-binding cassette transporter A4, ABCA4), and the last two are thought to participate in photoreceptor disc morphogenesis (protocadherin 21 and prominin). All experiments were performed with animals sacrificed on postnatal day 21 when the rudimentary outer segments of Rho-/ rods are fully formed, but photoreceptor degeneration that eventually occurs in these mice remains minimal.
Figure 19: Localization of outer segment membrane proteins in wild-type (WT) and Rho-/- retinas. (A) Electron micrographs showing the outer segment and connecting cilium in WT and Rho-/- rods (scale bar 500 nm). (B–K) Immunofluorescent localization
of individual outer segment proteins in WT and Rho-/- retinal cross-sections: (B) Rom-1; (C) ABCA4; (D) guanylate cyclase 2 (GC-2); (E) cyclic nucleotide gated (CNG) α1; (F) CNGβ1; (G) prominin; (H) protocadherin 21 (PCDH21); (I) peripherin; (J) R9AP; and (K) GC-1. (L) Double labeling of GC-1 (green) and the cone maker, PNA (magenta). Here and in the following figures, the identity of antibodies used in each panel is indicated in ‘Materials and methods’. Scale bars, 10 µm. Nuclei are stained by Hoechst (blue). Figure from (Pearring et al. 2015)

Remarkably, nine out of ten proteins were localized specifically to the ciliary extensions of the Rho-/- rods. They included Rom1, ABCA4, GC-2, CNGα1, CNGβ1, protocadherin 21, and prominin (Figure 19 B-H), as well as previously reported R9AP and peripherin (Figure 19 I,J). A striking exception was GC-1, which displayed a punctate pattern in the outer segment layer with no distinct signal in rod ciliary extensions (Figure 19K). Further analysis using a cone marker, peanut agglutinin, revealed that the GC-1-positive puncta corresponds to cone outer segments (Figure 19L; note that cone outer segments in Rho-/- mice are smaller than normal).

3.2.8.2 The abundance of disc proteins in rhodopsin knockout mouse retinas

We then used quantitative Western blotting to measure the amounts of outer segment proteins in the retinas of Rho-/- knockout mice. Availability of suitable antibodies allowed us to analyze eight of the initial ten proteins (Figure 20). Serial dilutions of retinal lysates from wild-type (WT) and Rho-/- mice were run on the same blot (such as examples in Figure 20A) and the relative protein amounts were calculated using WT data to generate calibration curves. We found that proteins retaining their
normal outer segment localization (Figure 19) were all expressed at 40–80% WT levels (Figure 20B). Considering how small the ciliary extensions of Rho−/− rods are, this amount of protein expression is quite remarkable and suggests a high density of protein packing.

Figure 20: Quantification of outer segment transmembrane proteins in Rho−/− retinas at P21. (A) Representative Western blots show serial dilutions of wild-type (WT) and Rho−/− retinal lysates for three proteins (guanylate cyclase 1 [GC-1], GC-2, and peripherin). The fluorescent signal produced by each band in the serial dilution was plotted and used to calculate the amount of each protein in Rho−/− lysate. In these examples, GC-1 was to 10% of its WT content, GC-2 to 38%, and peripherin to 57%. (B) Expression levels of outer segment transmembrane proteins in Rho−/− retinal lysates calculated as %WT. A minimum of four independent experiments was performed for each protein. Error bars represent SEM. Figure from (Pearring et al. 2015)

Once again the outlier was GC-1 whose content in Rho−/− retinas was only 10 ± 3% (SEM, n=5) of WT (Figure 20A,B). Considering that (1) a large fraction of this GC-1 is expressed in cones (Figure 19L); (2) cones comprise 3% of mouse photoreceptors; and (3) cones express more GC-1 than rods (Dizhoor et al. 1994), our most conservative estimate is that GC-1 in Rho−/− rods is reduced by at least 95%.
Taken together, the data reported in Figures 19 and 20 demonstrate that GC-1 is unique among outer segment transmembrane proteins in its reliance on rhodopsin for intracellular stability and outer segment localization. We next addressed the mechanistic basis for this phenomenon.

3.2.8.3 GC-1 is a rhodopsin-interacting protein

We investigated whether GC-1 and rhodopsin interact with one another by co-precipitating them from mouse retinal membranes solubilized in a mild detergent, dodecyl maltoside. The experiment in Figure 21A demonstrates that a large fraction of GC-1 can be co-precipitated with rhodopsin using the monoclonal anti-rhodopsin antibody 1D4. The specificity of this co-precipitation was established by replacing 1D4 with non-immune mouse IgG and by performing the experiment in the presence of the 1D4 epitope-blocking peptide (Hodges et al. 1988). Neither rhodopsin nor GC-1 was precipitated under these conditions. We also probed the 1D4 precipitate for the chaperone protein, DnaJB6 (Figure 21A), which was previously shown to link GC-1 to the intraflagellar transport (IFT) particle for ciliary transport (Bhowmick et al. 2009). Whereas a strong DnaJB6 staining was identified in the retinal lysate, it did not precipitate with the rhodopsin-GC-1 complex. This suggests that, unlike the interaction with rhodopsin, the GC-1 interaction with DnaJB6 is transient. Following a recent report that another rhodopsin-binding protein is peripherin (Becirovic et al. 2014), we also
probed the rhodopsin precipitate for peripherin. However, no appreciable fraction of peripherin was found in precipitate, both under our experimental conditions and in membranes dissolved in Triton X-100 as in their study. Given that these authors did not show what fraction of total peripherin was precipitating with rhodopsin in their assays, it is hard to fully reconcile these observations, although it is highly unlikely that rhodopsin’s interaction with peripherin could be as prominent as that with GC-1.
Figure 21: Guanylate cyclase 1 (GC-1) co-precipitation with rhodopsin from mouse retinal lysate. (A) GC-1 and rhodopsin co-precipitation by monoclonal anti-rhodopsin antibody 1D4. Wild-type (WT) mouse retinal lysate (Input) was incubated with 1D4 antibody and then bound to protein A/G beads. After the unbound material in flow through (FT) was removed, the beads were washed and bound proteins were eluted (Eluate) and analyzed by Western blotting for GC-1, rhodopsin, DnaJB6, and peripherin. Non-specific protein binding was probed using either non-immune mouse IgG or 1D4 antibody treated with its epitope blocking peptide. (B) Co-precipitation of GC-1 and rhodopsin by the 1D4 antibody from retinal membranes solubilized under different detergent conditions. (C) Rhodopsin and GC-1 co-precipitation by monoclonal anti-GC-1 antibody 1S4. Rho+/- mouse retinal lysate (Input) was incubated with 1S4 antibody bound to protein A/G beads. After the unbound material in flow through (FT) was removed, bound proteins were eluted from the beads (Eluate) and analyzed by Western
blotting for GC-1 and rhodopsin. Non-specific rhodopsin binding was probed using non-immune mouse IgG. Protein loading on each lane was normalized to input in all panels. Figure from (Pearing et al. 2015)

Importantly, the efficiency of GC-1 co-precipitation with rhodopsin was highly dependent on the type and concentration of detergent used for membrane solubilization. The co-precipitated fraction of GC-1 diminished when we increased the concentration of dodecyl maltoside, or used Triton X-100 instead (Figure 21B). Such instability of membrane protein complexes in detergent solutions is a common phenomenon (Prive 2007), and we believe that this likely hindered identification of GC-1’s interaction with rhodopsin in the past.

The reciprocal co-precipitation of rhodopsin by the monoclonal anti-GC-1 antibody 1S4 is shown in Figure 21C. The challenge of this experiment was that rhodopsin in mouse rods is expressed at a 1400-fold molar excess over GC-1 (Peshenko et al. 2011). Therefore, the theoretical limit of rhodopsin bound to GC-1 is only 0.07% of its total amount, and its detection requires special measures to reduce non-specific rhodopsin binding to the beads. We addressed this issue by employing two strategies. First, we improved the molar ratio between GC-1 and rhodopsin by using retinal lysates from Rho<sup>−/−</sup> mice, which express twice less rhodopsin without affecting the amounts of other outer segment proteins (Calvert et al. 2001; Liang et al. 2004). Second, we used a minimal amount of beads fully saturated with anti-GC-1 antibodies, just sufficient to
precipitate the majority of GC-1 in the lysate. Under these conditions, rhodopsin precipitation with anti-GC-1 antibody exceeded non-specific background with non-immune IgG by over six-fold (6.5 ± 1.7, n=3).

Taken together and combined with our finding that the intracellular stability of GC-1 is critically dependent on the presence of rhodopsin, these data demonstrate that rhodopsin and GC-1 form a complex in photoreceptor cells.

3.3 Discussion

3.3.1 PRCD lipidation is required for its correct targeting to the outer segment

The first major finding of this study is that PRCD in photoreceptors is S-acylated at the N terminal cysteine, and that this modification is required for maintaining PRCD’s intracellular stability and outer segment localization. In some proteins S-acylation is reversible, switching them between membrane-bound and soluble states which often modulates their function. (Chamberlain and Shipston 2015) We believe that this is not the case with PRCD for several reasons: (a) all PRCD molecules are S-acylated in photoreceptors; (b) endogenous PRCD remains insoluble after complete reduction of its S-acylation; (c) the non-S-acylated synthetic full-length PRCD peptide is completely insoluble; (Skiba et al. 2013) and (d) the C2Y PRCD mutant does not co-immunostain with soluble mCherry expressed in the same cell. It is, therefore, likely that S-acylation is
a constitutive property of PRCD required not just for its membrane attachment, but also for correct processing in biosynthetic membranes, and that without it PRCD is retained in these membranes and ultimately targeted for intracellular degradation. Consistent with this hypothesis, the C2Y PRCD mutant is found in much lower abundance than wild type PRCD when expressed in both electroporated rods and cell culture. (Remez et al. 2014)

One purpose of PRCD’s S-acylation may be to effectively lengthen its N-terminal membrane anchor domain, which is only 15 amino acids long (or 14 if N-terminal methionine is cleaved). Membrane domain lengths are known to modulate subcellular protein localization. (Baker et al. 2008; Watson and Pessin 2001; Osborn, Grigoriev, and Crew 1997) For example, GFP-tagged cytochrome b5 with a 14 amino acid membrane domain was shown to be completely retained in the ER of transgenic Xenopus rods, while lengthening of the membrane domain to 18 amino acids shifted the protein to outer segments. (Baker et al. 2008) It is less likely that S-acylation of PRCD is required for rhodopsin binding because robust binding to rhodopsin was observed with non-S-acylated PRCD peptide.

Unfortunately, our efforts to determine the exact nature of PRCD’s S-acylation moiety by mass spectrometry were hindered by complete insolubility of the acylated peptides. Other methods, such as acyl biotin exchange or acyl resin-assisted capture
(RAC) are well-suited for determining whether a given protein or peptide is S-acylated, but cannot identify the nature of their original modification. (Wan et al. 2007; Forrester et al. 2011) Experiments using radiolabeled palmitate are similarly used to determine whether a given protein can be in principle palmitoylated, but they do not address the patterns of endogenous lipidation and may in fact affect these patterns. For example, a recent study found that the endogenous pool of lipids attached by S-acylation to platelet proteins was significantly altered by incubation with exogenous palmitate. (Muszbek et al. 1999)

### 3.3.2 PRCD is neither secreted nor is its signal peptide cleaved in vivo

A recent study reported that cell culture expression of recombinant PRCD fused to six MYC tags resulted in secretion of PRCD into the media. The secreted PRCD had lower molecular weight, which was interpreted as evidence of N-terminal signal peptide cleavage. (Remez et al. 2014) These findings do not reflect the properties of PRCD in photoreceptors, since endogenous PRCD is always associated with photoreceptor disc membranes and migrates by electrophoresis as a single uncleaved polypeptide alongside synthetic full-length PRCD. (Skiba et al. 2013) Our results show that the presence of multiple PRCD bands on Western blot is explained by its phosphorylation and/or S-acylation, rather than a consequence of signal peptide cleavage.
3.3.3 PRCD interacts with rhodopsin in photoreceptors

The second major finding of this study is that PRCD binds rhodopsin and relies on rhodopsin for intracellular stability in photoreceptors. Rhodopsin is a major protein precipitated by the PRCD peptide from lysed photoreceptor discs and the only one whose binding was observed by reciprocal immunoprecipitation. Bolstering the conclusion that PRCD is a rhodopsin binding protein, we found that PRCD is completely absent from rods of rhodopsin knockout mice.

At the very least, the function of PRCD’s interaction with rhodopsin is to stabilize PRCD in photoreceptor cells. It is also plausible that the subsequent outer segment delivery of the rhodopsin-PRCD complex utilizes the very well-characterized rhodopsin trafficking pathway. (Pearring et al. 2013; Wang and Deretic 2014)

Highlighting the specificity of this function, rhodopsin is not required for stabilization and targeting of nine other proteins residing exclusively in the outer segment, with the exception of GC-1 which also binds to rhodopsin. (Pearring et al. 2015)

3.3.4 PRCD has an essential yet unknown function in photoreceptor outer segments

Attempts to understand the functional role of PRCD began over four decades ago in a study of mutant dogs, (Aquirre and Rubin 1972) and continued long before their retinal degeneration phenotype was explained by the C2Y mutation in PRCD. (Zangerl et
Mutant photoreceptors initially develop normally and then die, one after another, as the animal ages. Photoreceptors of young C2Y dogs have normal ultrastructure and unaffected visual function, as evident from electroretinographic analysis of their light responses. The autosomal recessive nature of this disease, with heterozygous dogs and human patients never developing pathology, argues that degeneration arises from the loss of PRCD function and not toxicity of its mutant. This is entirely consistent with our result that the C2Y mutation results in complete PRCD mislocalization from the outer segment and with findings that some of the human patients are homozygous for early PRCD truncations.

What does PRCD do in rods and cones and how does our discovery that PRCD is a rhodopsin-binding protein shape future attempts to answer this question? Unlike PRCD’s dependency on rhodopsin for maintaining intracellular stability, rhodopsin is unlikely to reciprocally require PRCD. Young C2Y dogs are characterized by normal rhodopsin content, mRNA expression, intracellular localization and regeneration after bleaching. On the other hand, rods of C2Y dogs were shown to have a somewhat reduced outer segment turnover rate.
which in principle could be connected with a reduced rate of rhodopsin delivery to this compartment. This suggests that a more subtle PRCD involvement in rhodopsin biosynthesis or trafficking cannot be completely dismissed.

Normal electroretinograms in young C2Y dogs (Aguirre et al. 1982) argue against a major involvement of PRCD in phototransduction, although the possibility remains that the phenotype is subtle and could only be revealed by single cell recordings which have yet to be performed. A potentially exciting alternative is that PRCD endows rhodopsin with an ability to stimulate signaling pathways other than phototransduction. One example of such an alternative rhodopsin-mediated signaling is stimulation of the insulin receptor pathway, which occurs independently of transducin activation; this phenomenon is thought to serve as a pro-survival mechanism in rods (Rajala et al. 2013; Rajala et al. 2008; Rajala et al. 2009). Another potential pathway downstream from rhodopsin involves light-dependent activation of Rac1, a signaling protein thought to regulate photoreceptor susceptibility to photo-oxidative stress (Haruta et al. 2009; Balasubramanian and Slepak 2003).

It is also conceivable that the complex of rhodopsin with PRCD contributes to maintaining the structural integrity of the outer segment, which is consistent with the prevalence of disorganized discs in degenerating photoreceptors of C2Y dogs (Aguirre et al. 1982).
et al. 1982; Aguirre and O'Brien 1986) Finally, PRCD phosphorylation, which we observed in mouse retinal preparations analyzed in this study, may prove to be an integral part of any putative mechanism discussed in this section.

In conclusion, this study reveals PRCD as an S-acylated rhodopsin binding partner, a discovery that will guide future efforts to understand the functional role of this protein in photoreceptors, which is clearly significant for maintaining the healthy status of these cells.
4. PRCD supports the lamellar structure of photoreceptor discs

By maintaining a flat, lamellar shape, photoreceptor discs are packed at the highest efficiency inside outer segments of photoreceptor cells, creating vast membrane surfaces for efficient light capture and sensitive photo responses. Previously we showed that only a handful of proteins specifically localize to discs, including PRCD, a 6 kDa rhodopsin-binding protein with unknown function whose mutations result in retinal degeneration of canine and human patients. Here we generate and characterize the PRCD knockout mouse, whose photoreceptors slowly die but develop normally, have normal localization and abundance of disc proteins, and have normal single-cell photoresponses. With high-quality electron microscopy, a morphological defect is evident in the flattening of newly evaginating photoreceptor discs in PRCD knockout mice. In this mouse most newly evaginating discs are elongated, flattened and enclosed, but some of them are prematurely ectocytosed and accumulate at the base of outer segments, causing a retraction of the enveloping inner segment to outer segment juncture.

4.1 Introduction

Light-sensitive photoreceptor outer segments house hundreds of flattened, lamellar disc membranes which specifically contain only a handful of proteins—six
phototransduction proteins (e.g. rhodopsin), two lipid flippases, two rim-specific proteins (peripherin, Rom-1), and PRCD (Skiba et al. 2013). With the exception of PRCD, the function of these disc specific proteins is relatively well described, including the recent discovery that the knockout mouse of peripherin results in a massive production of ectosomes, previously thought to be cell debris, from photoreceptor cilia. Ciliary ectosomes are a conserved mechanism of the primary cilia (Wood and Rosenbaum 2015) to release vesicles to the extracellular space for a variety of functions, and a process by which the photoreceptor cilium has adapted, in a peripherin-dependent manner, for the production of photoreceptor discs. Like ciliary ectosomes, discs form as evaginations from the ciliary plasma membrane (Ding, Salinas, and Arshavsky 2015), but instead of membrane scission/release, they are completely flattened, elongated to the diameter of the outer segment, and finally enclosed inside.

PRCD is a 6 kDa protein named after progressive rod-cone degeneration, which is one of the most common causes of blindness in dogs (Dostal, Hrdlicova, and Horak 2011; Zangerl et al. 2006). The disease results in the slow death of rod and cone photoreceptor cells, with a hallmark characteristic that disc renewal is up to ~40% slower than normal prior to widespread degeneration (Aguirre et al. 1982; Aguirre and O’Brien 1986; Aguirre and Andrews 1987). This reduction of disc renewal rate early in disease-stage occurs despite a normal mRNA and protein expression level of rhodopsin (Huang,
Chesselet, and Aguirre 1994; Kemp and Jacobson 1992). The disease was eventually linked to a single C2Y point mutation in the previously unknown PRCD protein. This exact point mutation, and several others, have been identified in human patients diagnosed with retinitis pigmentosa. We found this cysteine harbors a lipidation on all PRCD molecules and that its mutation results in a complete mislocalization of PRCD from the outer segment, and its degradation. Furthermore, we found that PRCD is a rhodopsin-binding protein, and that it requires this interaction for its delivery and stability in rod photoreceptors (Spencer et al. 2016).

To understand PRCD’s function, we generated a PRCD knockout mouse, which was viable and without any overt, extra-ocular phenotype. We found that the retina develops normally, disc proteins, including its binding partner, rhodopsin, are expressed and localized normally, and single-cell photoresponses are normal. Rod photoreceptors died very slowly, losing half of the ONL thickness by 18 months, while loss of cones was not evident during this time. By high-quality electron microscopy, a defect in the flattening of newly forming discs was evident, resulting in the release of discs as ectosomes which accumulated in the extracellular space and caused a retraction of the inner to outer segment juncture.
4.2 Results

4.2.1 Generation of the PRCD knockout mouse

Figure 22: Targeting vector design to create PRCD knockout mouse. Targeting vector design, corresponding PRCD genomic locus, and resulting targeted locus are shown. Primers \(a, b, \) and \(c\) shown with arrows were used for genotyping for the presence of targeted allele. Asterisks indicate the cut sites for PstI restriction enzyme used for digestion of genomic DNA during Southern blot confirmation. Neo shows the neomycin cassette which replaces PRCD coding region, shown in black, within exons 1, 2 and 3. Non-coding exon 5 is not close to the targeting locus, and therefore not shown.

To create a mouse lacking the PRCD gene, a targeting vector was designed encoding an 8 kb 5’-homology arm and 2 kb 3’-homology arm which complement the genomic region flanking the entire coding region (exons 1-3) of the mouse prcd gene (Figure 22). A neomycin cassette was inserted between the homology arms, and used for positive selection of ES cell clones expressing the targeting vector, while MC1-HSV-TK
was used for negative selection of ES cell clones with random insertions of the targeting vector. The properly targeted construct effectively replaces the entire PRCD coding region with a neomycin cassette. To confirm the correct insertion of the targeting construct, primers in the neomycin cassette and outside the homology arms were used in long-range PCR to obtain 8kb and 2kb products (data not shown). This PCR confirmation can only verify the correct insertion of the targeting vector at the prcd gene, and cannot confirm the lack of incorporated targeting vector at other genomic loci, which may disrupt other genes. Misincorporated knockout construct can be eliminated by breeding, assuming it is not linked to the targeted region.

**Figure 23: Confirmation of PRCD knockout mouse.** (A) PCR genotype of PRCD+/+, PRCD+/-, and PRCD-/- mice shown using three primers together in one reaction, indicated
in Figure 22. (B) Southern blot with radiolabeled DNA probe indicated in Figure 22, of fully PstI-digested DNA from PRCD+/+ mice, PRCD+/- mice, and PRCD-/- control. (C) Western blot of equal amounts of retinal lysate from PRCD+/+, PRCD+/-, and PRCD-/- mice with anti-PRCD and anti-GAPDH antibodies

To ensure single insertion of the knockout construct at the prcd locus, a Southern blot was performed using a probe which targets a restriction enzyme digested fragment of genomic DNA encompassing the prcd deleted region and targeting vector. The probe hybridized to both 5.3 kb WT and 3.8 kb KO fragments and did not bind any other fragments of the fully digested genomic DNA in the PRCD +/- mouse, indicating that single and correct insertion of the vector had occurred (Figure 23B). A three-primer genotyping strategy was developed to easily discern WT, heterozygous and homozygous PRCD knockout mice in a single PCR reaction (Figure 23A). Western blot of mouse retinal lysates using anti-PRCD antibody verified the complete lack of a PRCD band in PRCD knockout genotyped mice (Figure 23C). Mice which are heterozygous for PRCD were one-half reduced in PRCD protein band intensity, indicating that two PRCD alleles are required for full expression of the protein (Figure 23C). PRCD knockout mice overtly develop normal, and have Mendelian inheritance with expected ratios of WT, knockout and heterozygous genotypes when bred heterozygous male to heterozygous female.
4.2.2 Disc proteins have normal abundance and localization in young PRCD knockout mice

By light microscopy of fixed retinal sections, retinas from PRCD knockout mice develop normally, and have normal thicknesses of all retinal layers, including inner and outer nuclear layers, inner segments and outer segments. While outer segments have normal thickness, some appear less ordered than in wild type (Figure 24A).
Figure 24: PRCD knockout mouse retina develops normally, with normal abundance of disc proteins. (A) Light microscope image from fixed retinal sections of wild type and PRCD knockout retina, (B) Confocal images of immunostained fixed retinal sections from wild type and PRCD knockout mice with antibodies against rhodopsin, peripherin, and GC1. (C) Left, Westerns for disc proteins shown from equal amounts of retina lysate loaded from PRCD knockout and wild type mice, Right, coomassie stained protein gel loaded with equal amounts of material, normalized by rhodopsin difference spectroscopy, of purified ROS from PRCD knockout and wild type mice.
Since we identified that PRCD is a rhodopsin binding protein, and requires rhodopsin for its proper expression and localization to the outer segment, we tested rhodopsin’s localization and abundance in PRCD knockout mice. By immunohistochemistry with anti-rhodopsin antibodies against fixed retinal sections at P21 we observed no changes in rhodopsin localization between wild type and PRCD+/− mice (Figure 24B). There was no difference in rhodopsin content between young wild type and PRCD+/− mice as measured by Western blotting equal amounts of retinal lysate (Figure 24C). Furthermore, rhodopsin content was measured quantitively by rhodopsin absorption difference spectroscopy, which found no difference in rhodopsin quantity between 3 month old wild type (590±50 pmol), PRCD+/− (610±50 pmol), and PRCD+/− (630±70 pmol) mice, and no difference in the shapes of their absorption spectra.

Guanylate cyclase 1 is a unique disc protein which like PRCD, also requires rhodopsin interaction for delivery to the outer segment (Pearring et al. 2015). We tested the hypothesis that PRCD is a trafficking adapter between rhodopsin and GC1 in rod photoreceptors required for its delivery to the outer segment. By Western blotting equal amounts of retinal lysate and immunohistochemistry of retinal sections with anti-GC1 antibodies we found GC1 to maintain proper outer segment localization and abundance in PRCD+/− mice as compared to wild type, proving that guanylate cyclase 1 traffics to the outer segment independently of PRCD (Figure 24B,C). Given that the molar ratio of GC1
to rhodopsin is ~1:1400 (Peshenko et al. 2011) and PRCD to rhodopsin is ~1:300 (Skiba et al. 2013), this large abundance of rhodopsin is consistent with both outer segment proteins independently requiring rhodopsin interaction for delivery to the outer segment.

We tested the abundance of numerous other outer segment proteins between wild type and PRCD⁻/⁻ mice, finding no differences in any of those tested (Figure 24C). We also performed immunohistochemistry in wild type and PRCD⁻/⁻ mice for peripherin, a disc rim-specific protein required for morphing ciliary ectosomes into photoreceptor discs, and observed a normal localization pattern complete with increased staining along the outer segment edges where the normally protein resides (Figure 24B). To compare outer segment protein abundances in an unbiased manner, we purified osmotically intact rod outer segments by Optiprep density gradient fractionation from dark-adapted wild type and PRCD⁻/⁻ mice. Creating lysates of these preparations and normalizing by rhodopsin difference spectroscopy, we compared outer segment protein abundances by coomassie staining. Confirming the purity of the preparation, an equally intense coomassie stained band at 37 kDa corresponding to the mass of rhodopsin was observed in both preparations. We observed no differences in any protein’s band intensity between wild type and PRCD⁻/⁻ mice (Figure 24).
4.2.3 Rod photoreceptors have normal photoresponses in PRCD knockout mice

Dogs which are homozygous for a C2Y mutation in PRCD display normal electroretinograms (ERG), followed by their progressive loss of response (Sandberg, Pawlyk, and Berson 1986). Human patients with mutations in PRCD also begin life without apparent visual symptoms but later develop retinitis pigmentosa and have unresponsive ERGs (Zangerl et al. 2006; Nevet et al. 2010). Since we identified PRCD as a rhodopsin binding protein which exclusively resides with phototransduction machinery in photoreceptor discs, we tested if PRCD modulates phototransduction in any manner by quantitatively measuring photoresponses from single-rod photoreceptors of 8-week PRCD<sup>−/−</sup> mice using a suction electrode. The photoresponses between wild type littermate and PRCD<sup>−/−</sup> mice were similar, and not sufficiently different to implicate PRCD in any phototransduction step, nor implicate aberrant phototransduction as the cause of degeneration in PRCD<sup>−/−</sup> mice or PRCD mutant affected patients (Figure 25). Diminished ERGs in these mice and patients must be a secondary effect resulting from photoreceptor cell death caused by loss of PRCD’s function unrelated to phototransduction.
Figure 25: Photoresponses from single rod-photoreceptors of PRCD<sup>−/−</sup> mice. Single rod photoreceptors from young PRCD knockout and wild type littermate mice were isolated by suction electrode from dissected retinas, and subsequently subjected to calibrated light flashes while recording photoresponses with respect to current (pA) and time. This photoresponse data is shown in the graphs and table.

During the single-cell recording procedure, a suction electrode is placed over a single rod photoreceptor outer segment while it is still attached to the photoreceptor cell body and retina. It was noted during the procedure, that PRCD<sup>−/−</sup> mice, in comparison to their wild type littermates, have a much greater propensity to break at their connecting cilium from the photoreceptor cell body, perhaps reflecting a structural defect at this juncture.
4.2.4 Slow and progressive death of photoreceptors in PRCD knockout mice

Figure 26: Loss of photoreceptor cells in PRCD$^{-/-}$ mouse shown by light microscopy of fixed retinal sections. Left, Light microscope images of fixed retinal sections from wild type and PRCD knockout mice at different ages showing all layers of the retina and RPE. The retinal sections were aligned to the photoreceptor synapses to clearly show the decrease in photoreceptor layer thickness in PRCD knockout mice. Right, images showing the photoreceptor layers of wild type and PRCD knockout mice at 17 months of age.

After ageing PRCD knockout mice, we continually observed disordered morphology, characterized by the presence of matter between wavy-shaped outer segments, from p21 to 17 months of the outer segment layer by light microscopy of fixed retinal sections (Figures 24A, 26). Despite this early presence of mildly disordered outer
segments, degeneration of photoreceptor cells occurs extremely slow compared to other mouse models of retinal degeneration (Veleri et al. 2015). By 17 months, we observed shortening of the outer segments and significant thinning of the outer nuclear layer caused by death of photoreceptor cells and loss of their nuclei (Figure 26). The only abnormal morphology observed and degeneration is within the photoreceptor layer, while the inner retinal layers retain normal morphology and thicknesses, even after 17 months of age (Figure 26). The outer limiting membrane, inner segments, and RPE appear morphologically normal (Figure 26).
Figure 27: Cone photoreceptors in wild type PRCD knockout mouse retinal flat mounts. *Left*, 160µm x 160µm confocal images of PNA stained flat mounts taken randomly from the superior and inferior retinas of wild type and PRCD knockout mice. *Right*, whole retinal flat mount tile scans of PNA stained cones in wild type and PRCD knockout mice at age of 17 months. All four quadrants of the retina from each tile scan are labeled on the edges of the tile scans.
Given that rod photoreceptors constitute greater than 90% of the nuclei in the mouse retina’s outer nuclear layer, the substantial loss of nuclei in the outer nuclear layer can only be explained by the loss of rod photoreceptor cell nuclei. To determine if the loss of photoreceptor nuclei results in the death of cone photoreceptors in addition to rods, we labeled cones with fluorphore-conjugated lectin peanut agglutinin (PNA) in whole flat-mount retinas from wild type and PRCD knockout mice at p21, 3 months and 17 months of age. Tile-stitched images of the entire flat-mount retina from 17 month wild type and PRCD knockout mice, show no overt differences in cone density at any location, including superior, inferior, nasal and temporal quadrants (Figure 27, right). Furthermore, zoomed images of 160x160 µm taken from the inferior and superior of retinas of wild type and PRCD knockout mice show no differences in PNA labeled cone density (Figure 27, left). Given that dogs containing C2Y mutations in PRCD lose rod photoreceptors first shown by ERG (Sandberg, Pawlyk, and Berson 1986) and morphology (Aguirre and O’Brien 1986)), and human patients containing mutations in PRCD display a retinitis pigmentosa pathology (Zangerl et al. 2006; Pach et al. 2013), cone photoreceptors must die secondarily to rod photoreceptor cells as a result of the loss of PRCD’s function in photoreceptor cells.
4.2.5 Electron microscopy reveals defect in flattening and retention of discs during their morphogenesis; many are ectocytosed

To view the disordered outer segments observed by plastic sections in greater detail, we performed transmission electron microscopy on 2 month aged wild type and PRCD knockout mouse retinal sections. We fixed the retinas by mouse perfusion using our recently-developed protocol which best preserves outer segment structure, especially of newly forming disc membranes (Ding, Salinas, and Arshavsky 2015). This protocol also features the use of tannic acid contrasting agent, which is weakly membrane permeable and therefore highlights/darkens the membranes which are exposed to the extracellular space, such as newly evaginating disc membranes (Figure 28, top left).
Wild type photoreceptors have tightly packed disc membranes, and an inner segment that closely cups the newly evaginating discs which are darkly labeled by tannic acid. In contrast, rod photoreceptors lacking PRCD, darkly labeled and fully flattened evaginating disc membranes are present with ectosomes accumulated between them and the inner segment, causing a full retraction of the inner segment (Figure 28, top right), perhaps explaining the fragility observed at this junction during single-cell suction electrode recordings. The axoneme, outer segment membrane, and many mature and enclosed photoreceptor discs appear morphologically normal in PRCD knockout mouse rods, while others are clearly degenerating severely with sagitally oriented disc membranes and complete loss of outer segment structure (Figure 28, top right). Some of the mature, enclosed discs in PRCD knockout rods appear more swollen than in wild type, but this necessitates other fixation techniques, because enclosed discs can also be swollen in wild type rods using our perfusion fixation technique (Ding, Salinas, and Arshavsky 2015). The defect observed consistently, even in otherwise normal looking rods, is the vesicular accumulation at the base of the outer segment, which leads to a
tremendous accumulation of these vesicles in the interphotoreceptor space (Figure 28, bottom). Curiously, the vesicles appear to ectocytose from the first and most basal of the newly evaginating disc membranes, with the co-presence of full flattened, tannic acid labeled evaginating discs (Figure 28). A high magnification micrograph of newly forming disc membranes in a PRCD knockout mouse rod photoreceptor cell which has not yet retracted its inner segment clearly shows the delamination of newly forming discs, their ectocytosis and accumulation in this space (Figure 29).
Figure 29: Some of the newly evaginating discs in PRCD knockout mouse rod photoreceptors are incompletely flattened, and ectocytosed. An electron micrograph of a retinal section from a 2 month aged PRCD knockout mouse. The base of the outer segment, and site of newly evaginating discs is shown from a rod photoreceptor cell.
Some of the discs bud off as ectosomes, and accumulate in the extracellular space between the outer segment and inner segment. The transmission electron micrograph is shown at 100,000x.

To confirm that the vesicles observed at the base of the outer segment are indeed, evaginating discs which have been ectocytosed, we immunostained fixed retinal sections with anti-rhodopsin antibodies followed by gold-particle conjugated secondary antibody, as described previously (Ding, Salinas, and Arshavsky 2015). After this immunogold staining, we performed transmission electron microscopy on these sections and detected dense gold-particle labeling of the vesicles, corresponding to their abundant rhodopsin content (Figure 30). The staining intensity is comparable to that of mature, enclosed discs and contrasts the lack of staining present in the inner segment, indicating that these vesicles originate from the newly evaginating discs, and therefore are photoreceptor ciliary ectosomes. The specificity of the rhodopsin immunogold labeling is highlighted by the presence of a cone outer segment juxtaposed to a rod in (Figure 30, top left). The lack of severe morphological defects in the cone outer segment observed in this image coincides with the lack of cone degeneration observed in PRCD knockout mice (Figure 27).
Figure 30: Immunogold electron microscopy with anti-rhodopsin antibody of PRCD knockout outer segments. Fixed retinal sections were labeled with anti-rhodopsin antibodies (clones 1D4, or 4D2), secondary antibodies containing gold particles, and
imaged with transmission electron microscope at 40,000x top images, 60,000x bottom. *Top left,* A cone outer segment is not labeled with the anti-rhodopsin gold particles demonstrating the specificity of the staining.

### 4.2.6 Disc adhesion is maintained in PRCD knockout mice

The striking phenotype present in PRCD knockout rods is the delamination of newly forming discs, followed by their scission and release as ectosomes. Given that PRCD is entirely exposed to the cytoplasmic face of a photoreceptor disc (Spencer et al. 2016) corresponding to the inside of an evaginating disc, we decided to test the hypothesis that PRCD is the molecular component responsible for forming the disc-to-disc linking filaments which have been observed by freeze-fracture and cryoelectron electron microscopy for decades (Corless and Schneider 1987; Meller 1984; Nickell et al. 2007; Roof, Korenbrot, and Heuser 1982). Typically, these filaments, whose molecular component(s) are unknown, have been observed at the disc rims and incisures, but were observed throughout the lamellar region of mouse rod photoreceptors (Nickell et al. 2007). They are thought to act structurally, to hold discs to adjacent discs, but since newly evaginating discs have inverted topology, they may act to “glue” these evaginations from within to keep their flat, lamellar shape as they elongate.
Figure 31: Discs retain their adhesive nature in PRCD knockout mice. Left, mouse ROS purified osmotically intact from wild type and PRCD knockout mice, and adhered to a flow chamber live-microscopy slide. Right, images of the osmotically lysed ROS after flow of hypotonic buffer with sheer stress of 4 dyn/cm²

To test if PRCD is responsible for these linker filaments, we tested how well discs stay adhered together in wild type and PRCD knockout mouse rods after bursting their ROS and applying shear stress over the aggregated discs. Electron microscopy studies of osmotically lysed ROS indicate that the discs swell but remain aggregated (Heller,
Ostwald, and Bok 1971). To accomplish this, we purified osmotically intact rod outer segments from these mice as described previously (Tsang et al. 1998), and then adhered them to a poly-lysine coated, live-imaging flow chamber. With high-powered phase contrast microscopy, we could live-image unfixed ROS while perfusing buffers of desired osmolality with a controllable flow rate and consequential shear stress (shear force). In contrast to wild type mice, the ROS from PRCD knockout mice had wavy, indented edges (Figure 31), as similarly reported for dogs containing a C2Y mutation in PRCD (Aguirre and Andrews 1987). Upon perfusion of hypotonic buffer, PRCD knockout and wild type ROS bend and curl, eventually forming a round mass enclosed by the outer segment’s plasma membrane, which finally ruptures as photoreceptor discs swell and are visible by phase contrast microscopy (Figure 31, right). After rupture, swollen and uncontained discs from both wild type and PRCD knockout ROS remained adhered together, despite constant flow and shear stress (0.9 dyn/cm²) over them. Increasing the shear stress to 4 dyn/cm² still did not break disc aggregation (Figure 31, right), but did begin to break some of the ROS’s adhesion to the flow chamber. By starting and stopping the flow, and watching the entire ROS oscillate/pivot on its attachment point to the flow chamber, it was clear that the adhesive properties of the flow chamber were not responsible for the disc aggregation.
4.3 Discussion

In this study, we generated and characterized the PRCD knockout mouse, which displayed a similar overall phenotype to that of dogs containing a C2Y mutation in PRCD. Likely this is because the C2Y mutation effectively creates a null PRCD allele by completely mislocalizing the coded protein and resulting in its degradation (Spencer et al. 2016). With two null copies, autosomal recessive disease occurs in canine and human patients. It is possible that there is additional toxicity from the mislocalized C2Y PRCD protein in addition to complete loss of its function, although the lack of any phenotype associated with heterozygous C2Y PRCD dogs suggests otherwise. PRCD knockout mice appear to degenerate even slower than dogs containing C2Y mutations by species adjusted rate, although the disease in dogs varies considerably in age of onset in different breeds (Dostal, Hrdlicova, and Horak 2011).

The PRCD knockout mouse and PRCD C2Y mutant dogs develop normal layered retinas, and have normal expression of rhodopsin. Early ERGs in C2Y dogs are normal (Sandberg, Pawlyk, and Berson 1986), and the normal single-cell recordings from PRCD rod photoreceptors conclude that the protein is not involved in phototransduction. In the C2Y dogs rod photoreceptors die first (Aguirre and O’Brien 1986), followed by cones, while we didn’t detect death of cone photoreceptors even in 17 month aged PRCD knockout mice, likely reflecting the slower rate of overall
degeneration in the mouse. This result is consistent with the function of PRCD to be important for rods, but not cones, despite its expression and localization in cone outer segments (Skiba et al. 2013).

The accumulation of “vesicular profiles” was noted in numerous studies of C2Y PRCD affected dogs, after observing them in the interphotoreceptor space (Aguirre et al. 1982; Aguirre and O’Brien 1986; Aguirre and Acland 1988). Here we show that these vesicles are ciliary ectosomes, released from the base of the outer segment at the site of disc morphogenesis in PRCD knockout mice. The widespread prevalence these ectosomes and of rod outer segments with retracted inner segments is not consistent with their immediate death given the extremely slow rate of degeneration. Likely, some of the newly evaginating discs are shed as ectosomes, while others are fully flattened and enclosed in the outer segment. This may explain the hallmark phenotype described in C2Y PRCD dogs that rods have up to a 40% reduced rate of scleral disc displacement at a young age before widespread degeneration and despite the expression of rhodopsin mRNA and protein remaining normal (Aguirre et al. 1982; Aguirre and O’Brien 1986; Aguirre and Andrews 1987; Parkes et al. 1982b; Huang, Chesselet, and Aguirre 1994). By inadvertently allocating protein and membrane material to the co-production of ectosomes, the PRCD-lacking photoreceptor displaces discs slower.
The co-production of ectosomes from the base of the outer segment may present several problems resulting in the eventual degeneration and death of rod photoreceptors lacking PRCD. First, the ROS renewal rate is greatly slowed, which is needed to discard and renew toxic, oxidized proteins and lipids from the photoreceptor cell (Kevany and Palczewski 2010). Second, the accumulation of ectosomes in the interphotoreceptor space may not be cleared by the RPE, and result in inflammation. This is consistent with the recruitment of phagocytic cells into the outer segment layer, at an early age of 2 months, before the significant death of photoreceptors (Appendix, Figure 32). Third, the inner segment is fully retracted from the outer segment in many PRCD knockout photoreceptor cells, and it was noted during single-cell recordings that this juncture was easily broken compared to wild type. In any case, with retinal disease resulting from PRCD mutations being slow, and a result of its loss of function rather than toxicity, it may be effective to treat with gene replacement therapy. It is convenient that PRCD protein is extremely small, and would easily fit into an AAV viral vector.
5. Conclusions

5.1 Biochemical characterization of PRCD protein

This dissertation biochemically characterizes the PRCD protein with the following findings and key points:

(1) PRCD is a 6 kDa protein which exclusively resides in photoreceptor discs
(2) PRCD is expressed at 1:290 ratio with rhodopsin
(3) All PRCD molecules reside on the cytoplasmic surface (interdiscal) of discs
(4) All PRCD molecules are modified by lipid acylation of the protein’s only cysteine residue (position 2), which is mutated to tyrosine in retinal disease
(5) Mutation of PRCD’s lipidated cysteine results in complete mislocalization of the protein from outer segments, and its degradation—results in disease
(6) PRCD is not soluble upon release of its lipid
(7) PRCD’s predicted signal peptide is not cleaved, nor is the protein secreted
(8) PRCD contains multiple phosphorylated residues, but not all PRCD molecules are phosphorylated—S27, S38, S44, and T45

5.1.1 PRCD’s role in cones

We identified PRCD as a unique photoreceptor disc protein, and the only one of eleven which had unknown localization in this compartment. Furthermore, we showed that it is also expressed and localized to the outer segments of cone photoreceptors, but
we did not see degeneration of cones in PRCD knockout mice, suggesting that PRCD is important primarily for the survival of rod photoreceptors. This is consistent with the observation that rods die first in dogs which contain C2Y mutations in PRCD, and humans with mutations in PRCD develop retinitis pigmentosa, with the loss of rod function before cone (Aguirre and O’Brien 1986; Zangerl et al. 2006; Beheshtian et al. 2015). Future studies will be required to elucidate the functioning of PRCD in cones in contrast to rods, and why its loss in cones does not result in their degradation.

5.1.2 PRCD’s lipidation moiety

Reported in this document, all PRCD molecules contain a lipidation of its single cysteine reside at position 2, which is the same residue which when mutated to tyrosine results in complete mislocalization and degradation of the protein (Spencer et al. 2016). Furthermore, this exact residue and mutation results in blindness in canine and human patients (Zangerl et al. 2006). Our efforts to identify the exact nature of this lipid were large unsuccessful. We were unable to detect the extremely hydrophobic N-terminal tryptic peptide of PRCD by LC-MS/MS and found that the full-length peptide aggregated in the capillary tubes of our chromatography system. To circumvent this issue, we attempted MALDI-TOF mass spectrometry of the endogenous, full-length protein either with, or without the acyl moiety straight from a PVDF membrane (Chang et al. 2006). We were able to detect a mass corresponding to full length PRCD peptide,
but were unable to accurately detect the size of the endogenous protein with attached lipid, given the extremely low intensity of the peak.

Recently, a study was published claiming PRCD’s lipid is a palmitoyl (Murphy and Kolandaivelu 2016), shown by metabolic labeling with palmitoyl analogue, acyl-RAC to precipitate all acyl modified cysteines, and identification of zDHHC3 which enhances the expression of PRCD. It should be stressed, that these studies do not show that endogenous PRCD is palmitoylated, as acyl-RAC pull downs all S-acylated proteins regardless of their attached moiety. Incubation with radioactive palmitate (or palmitoyl analogue) followed by precipitation of the protein, proves that a given protein can be palmitoylated, but it does not reflect the endogenous lipid species, and has been shown to artificially shift the entire pool of lipid in a sample (Muszbek et al. 1999). Finally, the identification of a zDHHC3 enzyme which enhances expression of the protein is not an indicator that the protein is palmitoylated, as zDHHC enzymes have broad fatty acid selectivity (Chamberlain and Shipston 2015). In the specific case of zDHHC3, the strongest preference of fatty acid is for 14-carbon myristate (Greaves et al. 2017). It is a misnomer to call these enzymes “palmitoyl” transferases; instead they should be called S-acyltransferases.

Future studies are needed to confirm the endogenous nature of the PRCD lipidation by mass spectrometry—perhaps by immunoprecipitation of mouse PRCD
protein followed by hydroxylamine treatment to release the attached fatty acid followed by fatty acid mass spectrometry analysis without the PRCD protein attached. An essential control will be the immunoprecipitation from PRCD knockout mice in parallel, to ensure that the identified fatty acid is specific to PRCD protein, and not something non-specifically bound during the immunoprecipitation.

5.2 PRCD is a rhodopsin binding protein

We identified PRCD as a rhodopsin binding partner, and requires this interaction for its stability and trafficking in rod photoreceptor cells (Spencer et al. 2016). This result mirrors and complements our study that guanylate cyclase 1 also binds to rhodopsin, and requires this interaction for its delivery to the outer segment (Pearring et al. 2015). Both of these examples are in stark contrast to the all other tested outer segment proteins, which were abundantly expressed and robustly delivered to the outer segment structure in rhodopsin knockout mouse rod photoreceptors. At the very least, these interactions probably evolved to traffic these proteins to the outer segment where they are unique disc proteins. Given that PRCD and guanylate cyclase 1 are expressed at much lower levels than rhodopsin (1:290 for PRCD), they are likely delivered independently, in separate complexes. Guanylate cyclase 1 expression and localization is completely normal in PRCD knockout mouse photoreceptors.
PRCD likely has a more involved function with rhodopsin than just its delivery to the outer segment. There are three main functions ascribed to rhodopsin: (1) phototransduction, (2) alternative signaling pathways (e.g. Rac1, insulin receptor), (3) structural support of the outer segment. We ruled out the first possibility by performing single-cell photoresponses from rod photoreceptors of PRCD knockout mice, which were normal. Next, by breeding PRCD knockout mice in complete darkness, and observing persistence of retinal degeneration, we reasoned that PRCD likely is not involved in light protective photoreceptor survival signaling pathways. The data in this dissertation is consistent with PRCD have a structural role in photoreceptor discs, perhaps involving rhodopsin complex.

The structural function of rhodopsin can be highlighted by the fact that a proper outer segment structure is not formed in rhodopsin knockout rod photoreceptors, but rather a large ciliary stalk is formed which is filled with disorganized membranes packed with other outer segment proteins (Figure 19A). This highly disordered phenotype is difficult to directly link to the less pronounced phenotype of ectocytosed discs in PRCD knockout mouse rods, but one rhodopsin mutant, P347S has a remarkably similar phenotype to PRCD knockout mice (Li et al. 1996), characterized by the accumulation of vesicles at the base of outer segments in the interphotoreceptor space. The mutant rhodopsin is delivered to morphologically normal outer segments, before
shortening and degeneration of the outer segments. Furthermore, this rhodopsin mutant retains normal phototransduction activity, including normal ERG, transducin activation, arrestin binding, and phosphorylation by rho kinase (Weiss et al. 1995). The P347 residue, is at the end of the C terminus of rhodopsin, and exposed to the cytosolic surface of discs, in the same space that PRCD orients. Future studies should assay if this residue is a site of PRCD interaction with rhodopsin, and if it is involved in the high-fidelity retention of ectosomes as discs. A closer investigation of the P347S mouse model in parallel with PRCD knockout with high quality EMs and immunogold can verify their similarity.

5.3 PRCD is required for the lamellar structure of photoreceptor discs

Here we report that PRCD is required for the lamellar structure of photoreceptor discs, particularly as they evaginate from the ciliary plasma membrane during their morphogenesis. Some of the evaginating discs are not full flattened, followed by their scission and release into the extracellular space as ectosomes, which greatly accumulate in this space. This is the earliest detected defect in rod outer segments and slowly, this process follows with the complete degeneration and death of rod photoreceptors.

Our initial hypothesis was that PRCD forms the molecular component of the disc to disc linking filaments observed by cryo electron microscopy of mouse rod
photoreceptors throughout the lamellar surface of a disc (Nickell et al. 2007) and analogous to the filaments observed at disc rims by freeze fracture EMs (Roof, Korenbrot, and Heuser 1982). These linking filaments are thought to serve as structural support for the entire stack of discs and may help newly evaginating discs flatten, since the topology of the membrane surface in newly evaginating discs is inverse to that of mature discs—PRCD resides within a new disc, and between mature discs. By applying shear stress to discs of osmotically lysed wild type and PRCD knockout ROS, the disc adhesion and aggregation does not seem to be lost, and is remarkably strong. It is plausible that additional linkers, independent of PRCD, remain present in PRCD knockout and are sufficient to maintain disc aggregation during this experiment. One example is GARP domain mediated protein-protein connection between CNG channel in the plasma membrane, and peripherin in the disc rim, which presumably anchors discs to the plasma membrane (Poetsch, Molday, and Molday 2001). This may necessitate future studies disrupting this connection in addition to loss of PRCD to test for disc aggregation. However, the observation of mature, swollen discs in many PRCD knockout ROS by electron microscopy is another piece of evidence observed in this study which contradicts the linkers hypothesis (Figure 28, bottom left). This suggests that PRCD flattens disc membranes regardless of its orientation on the membrane surface. Future studies which best preserve mature disc structure will be valuable in
making this conclusion, such as freeze-fracture electron microscopy which circumvents the osmotic stress of hydration/dehydration steps used in traditional transmission electron microscopy (Ding, Salinas, and Arshavsky 2015).

### 5.4 Clues to mechanism of PRCD’s function

The mechanism for PRCD’s function to ensure the high-fidelity retention of ectosomes and flattening of disc membranes remains enigmatic, but one striking example of similar membrane architecture may provide clues—pulmonary surfactant (Perez-Gil and Weaver 2010). Pulmonary surfactant are lamellar membrane bilayers, with striking resemblance to photoreceptor discs, which are secreted en masse from granules of the alveolar cells of the lung. Alveoli are small sacs which exchange gasses from blood to air in the lung. This anatomy creates an air-liquid interface which would collapse because of the surface tension of water, but is stabilized by the lipid film created by the pulmonary surfactant which is constantly secreted from these alveolar cells. Disruption of this process leads to respiratory distress syndrome (RDS) and death. The mass associated with pulmonary surfactant is simple—phospholipids, ~10% cholesterol, and two hydroboic peptides called surfactant protein B/ C. Surfactant protein C is a hydrophobic 35 amino acid peptide, largely dispensable for surfactant structure in mice but necessary in humans (Glasser et al. 2001). Surfactant protein B, is a hydrophobic, 79 amino acid protein which is absolutely required for the proper lamellar structure of
pulmonary surfactant, and mice lacking the protein die shortly after birth of respiratory stress (Stahlman et al. 2000). The pulmonary surfactant in surfactant B knockout mice (or hemizygotes), loses its lamellar, flattened membranes and instead consists of small vesicles, reminiscent of the ectosomes observed in PRCD knockout mice.

The exact mechanism for surfactant protein B’s ability to flatten membranes is debated (Parra and Perez-Gil 2015). The protein is highly hydrophobic, has a positive charge, is amphiphilic and partially penetrates the membrane (Parra and Perez-Gil 2015). It’s mechanism of action in vitro corresponds to its tendency to aggregate (Cruz et al. 2004); in vitro, as the protein aggregates on the lipid surface, lipid vesicles are first permeabilized, then begin to flatten and aggregate (Hawgood, Derrick, and Poulain 1998). The membrane-active detergent like effects of the protein are ascribed to its amphipathic nature, and the protein is included in a class of other membrane active proteins called SAPLIP (saposin-like) (Olmeda, Garcia-Alvarez, and Perez-Gil 2013).

PRCD is an amphipathic (N terminus hydrophobic, C terminus hydrophilic), and highly hydrophobic peptide which specifically resides in photoreceptor discs. The data presented in this document are consistent with PRCD being a membrane active peptide, analogous to surfactant protein, which functions to flatten disc membranes, preventing their premature ectocytosis. Future studies should study the PRCD protein in vitro, to determine if its presence alone is sufficient to exert membrane active properties,
consistent with other membrane active peptides, including permeabilization of membranes, aggregation, and/or flattening. Note should be taken if these properties are exerted in a PRCD concentration dependent manner. Key to these experiments will be to obtain PRCD complete with its lipid modification, and phosphorylation sites—both translational modifications of this protein which are present in photoreceptor disc membranes. In any case, structural studies of PRCD protein should give insight to its mechanism of action.
Appendix

1. Phosphorylation sites of PRCD

Figure 32: Phosphorylation sites of PRCD—S27, S38, S44, and T45. The full mouse PRCD amino acid sequence is shown. Phosphorylated residues are in dark gray, and mass spec peptide coverage in light gray.

To ascertain which residues are responsible for the phosphorylation in PRCD shown by Western blot (Figure 13), we purified rod outer segments from wild type mouse retinas by sucrose gradient fractionation in the presence of phosphatase inhibitors, followed by separation of the proteins by gel electrophoresis. One lane of the gel was cut for Western blot of PRCD protein, which was then aligned with the protein gel to make an exact cut of the gel band containing all phospho-PRCD residues. This method was necessary because of the propensity of phosho-PRCD to oligomerize, and run at a higher mass than expected. The gel band was processed by in-gel tryptic digestion followed by phosho-peptide enrichment by titanium dioxide chromatography. This method provided the sensitivity needed for identification of phospho peptides of PRCD, and identified four phosphorylated peptides encompassing PRCD’s C terminus, each containing one phosphorylation. Since the N-terminus of PRCD is likely a
membrane anchor domain (Spencer et al. 2016), and could not be phosphorylated, the mass spectrometer peptide coverage (Figure 32, light gray residues) encompassed all phospho-predicted residues except one threonine at position 19.
2. Invasion of phagocytic cells into the outer segment layer of PRCD knockout mouse retinas

Figure 33: Infiltration of phagocytic cells in the outer segment layer of PRCD knockout mice. Left, light microscopy image of fixed retinal section from 17m PRCD knockout mouse. scale bar is 10 µm, Right and middle, transmission electron micrographs from fixed retinal sections of 2m PRCD knockout mice, scale bar is 1 µm

By light microscopy at 17 months of age, large and swollen round-shaped cells appear sparsely throughout the outer segment layer (Figure 33, left). Furthermore, these cells are observed by electron microscopy at 2 months of age in the outer segment layer, although they are much smaller in diameter at this age. Likely, these are phagocytic cells (microglia/macrophages), often observed in the outer segment layer of other mouse models of retinal degeneration (Chen, Yang, and Kijlstra 2002), which are infiltrating the outer segment layer to remove ectocytosed discs which have accumulated in the interphotoreceptor space.
3. **PRCD knockout mice raised in complete darkness have degenerating photoreceptors**

**Figure 34:** PRCD knockout mice raised in complete darkness (IR light only) have **degenerating outer segments**. EMs of retinal sections from 1 month aged mice either wild type, *left*, or PRCD knockout, *right*. All mice were born and raised under complete darkness, with only the use of infrared light and night vision goggles for handling. Scale bar is 1 µm.

Since we identified PRCD as a rhodopsin binding protein (Spencer et al. 2016), and subsequently found that it is not involved in phototransduction, we tested the...
hypothesis that PRCD is involved alternative rhodopsin-dependent, light activated neural protective signaling pathways (Rajala and Anderson 2010), and may not degenerate when raised under complete darkness. We found that 1 month aged PRCD knockout mice have degenerating outer segments after the mice were raised in complete darkness, suggesting that the function of PRCD is independent of the light activation status of rhodopsin.


membrane fusion and changes conformation upon membrane association', Experimental Eye Research, 77: 505-14.


Boye, S. E. 2014. 'Insights gained from gene therapy in animal models of retGC1 deficiency', Frontiers in Molecular Neuroscience, 7: 43.


Ding, J. D., R. Y. Salinas, and V. Y. Arshavsky. 2015. 'Discs of mammalian rod photoreceptors form through the membrane evagination mechanism', Journal of Cell Biology: in press.


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Haruta, M., R. A. Bush, S. Kjellstrom, C. Vijayasarathy, Y. Zeng, Y. Z. Le, and P. A. Sieving. 2009. 'Depleting Rac1 in mouse rod photoreceptors protects them from photo-oxidative stress without affecting their structure or function', *Proceedings of the National Academy of Sciences of the United States of America*, 106: 9397-402.


Hodges, R. S., R. J. Heaton, J. M. Parker, L. Molday, and R. S. Molday. 1988. 'Antigen-antibody interaction. Synthetic peptides define linear antigenic determinants recognized by monoclonal antibodies directed to the cytoplasmic carboxyl terminus of rhodopsin', *Journal of Biological Chemistry*, 263: 11768-75.


Meller, K. 1984. 'The ultrastructure of the developing inner and outer segments of the photoreceptors of chick embryo retina as revealed by the rapid-freezing and deep-etching techniques', *Anatomy and Embryology, 169*: 141-50.
Mellersh, C. S. 2014. 'The genetics of eye disorders in the dog', *Canine Genet Epidemiol*, 1: 3.


Biography

William James Spencer was born in Augusta, GA on March 21st, 1987. In 2009, Will graduated with honors from the University of South Carolina with a Bachelor of Science double-major in Chemistry and Biology, and was inducted into the Phi Beta Kappa national honor society. Then he spent two years working as a research technician in the laboratory of Dr. Joseph Janicki at the University of South Carolina School of Medicine. In 2011, Will enrolled in the Cell and Molecular Biology PhD program at Duke University, and subsequently affiliated with the Pharmacology and Cancer Biology department and joined the laboratory of Dr. Vadim Arshavsky. In 2013, Will was awarded the Research to Prevent Blindness Duke Ophthalmology small grant award. In 2014, Will won Duke Eye Center’s Best Research Presentation Award. In 2015, Will was awarded the Ruth L. Kirschstein National Research Service Award (NRSA) from the National Institute of Health, and a Fitzgerald Academic Achievement Award from Duke University department of pharmacology. Will’s NRSA fellowship was awarded for three years of funding, continuing through 2017.

2. **Spencer WJ**, Pearring JN, Salinas RY, Loiselle DR, Skiba NP, Arshavsky VY. 
   


   
   Proteomic identification of unique photoreceptor disc components reveals the presence of PRCD, a protein linked to retinal degeneration. *J Proteome Res*. 2013

5. McLarty JL, Meléndez GC, **Spencer WJ**, Levick SP, Brower GL, Janicki JS.