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Progressive Rod-Cone Degeneration (PRCD) Protein Requires N-Terminal S-Acylation and Rhodopsin Binding for Photoreceptor Outer Segment Localization and Maintaining Intracellular Stability

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Abstract

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.

Graphical Abstract

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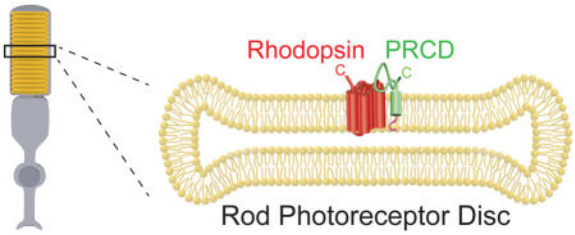

Progressive Rod-Cone Degeneration (PRCD) is a genetic disease initially identified in miniature poodles over 30 years ago and subsequently mapped to a single C2Y point mutation in a gene encoding a short protein of the same name.[1–3] The same mutation was identified in 35 different dog breeds and is among the most frequent causes of inherited dog blindness.[4–6] More recently, PRCD mutations, including C2Y, have been identified in human patients suffering from retinitis pigmentosa.[3, 7–11] 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.[3, 8–11]

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.[3] 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.[3, 12] 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.[3] 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.[12]

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.

EXPERIMENTAL PROCEDURES

Antibodies

We used the following antibodies for Western blotting: pAb anti-PRCD (described in [12]); mAb anti-Rhodopsin: epitopes 1D4 and 4D2 (abcam ab5417 and ab98887); pAb anti-FLAG (Sigma F7425); pAb anti-$G\alpha_t$ (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 [13]). 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-$G\alpha_t$ (described in [14]), and pAb anti-peripherin (described in [12]).

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,[15] 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).

Protease treatment of intact photoreceptor discs

Osmotically intact photoreceptor discs were purified from bovine retinas as described previously.[12] 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 $MgCl_2$, 1.2 mM $CaCl_2$, 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 × 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).

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 $MgCl_2$, and protease inhibitors (eComplete, Roche). The lysate was centrifuged at 100,000 × 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).

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 × 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.

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 [16 and 13]. 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.

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 × 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.

PRCD peptide pull down and mass spectrometry

Full length PRCD peptide with a biotin covalently attached to the C terminal lysine (used in [12]) 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 [12]), 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 [12, 17].

Identification of eluted proteins by LC-MS/MS analysis was performed as described previously[12] 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 ($HDMS^E$). 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 $HDMS^E$ 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.[18] 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.

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.[19] The elution rate was 400 μl/min, and fractions were collected every 1 minute for analysis by Western blotting.

Protein immunoprecipitation

Rhodopsin was precipitated using mouse monoclonal antibody 1D4 (Abcam) as described in [15], 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 ($G\alpha_t$) and peripherin was performed following the same protocol, using sheep anti-peripherin antibody described in [12] and sheep anti-$G\alpha_t$ antibody described in [14]. To avoid antibody cross-reactivity on Western blots, proteins were visualized with rabbit anti-$G\alpha_t$ (Sigma), and rabbit anti-peripherin antibody (gift from GH Travis, UCLA).

Immunofluorescence

Eyecups were dissected and fixed for agarose sectioning using a vibratome as described in [15]. Staining with Hoechst 33342 (Invitrogen), primary antibodies, and conjugation with appropriate Alexa Fluor secondary antibodies (Invitrogen 488 and 568) was performed as

described previously.[15] Images were taken with a Nikon Eclipse 90i microscope and C1 confocal scanner.

RESULTS

PRCD is bound to the cytosolic surface of discs

We previously identified that PRCD is tightly associated with outer segment disc membranes and not found in other subcellular compartments of the photoreceptor cell.[12] PRCD has a highly conserved N-terminus consisting of mostly hydrophobic amino acids that are predicted to form an α-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.[20] After proteinase treatment, we observed a complete disappearance of rhodopsin immunostaining when Western blots were probed with an antibody against its exposed, cytosolic epitope (Fig. 1A, 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 (Fig. 1A, 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 (Fig. 1A, bottom).

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 (Fig. 1B, 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 (Fig. 1B, 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 (Fig. 1B, lane 3). These bands consolidated into two more intense bands after phosphatase treatment (Fig. 1B, 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 (Fig. 1B, 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 (Fig. 1B, lane 6). Considering the complete lack of the lowest band without reducing agent in (Fig. 1B, 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.[21]

PRCD's N terminal cysteine is the site of S-acylation

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.[16] 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 (Fig. 2A top), while the C2Y PRCD construct did not shift (Fig. 2A 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.

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.[21] 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 (Fig. 2B).

PRCD C2Y mutation completely mislocalizes the protein from outer segments

The C2Y mutation in PRCD results in retinal degeneration of human and dog patients.[3] 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 (Fig. 3 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.[12] In contrast, the C2Y PRCD mutant was completely mislocalized from outer segments to other compartments of the rod cell (Fig. 3 bottom). Interestingly, mislocalized C2Y PRCD construct was detected in a smaller fraction of mCherry-positive cells than the wild type construct (Fig. 3 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.[22]

PRCD binds rhodopsin in photoreceptor discs

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 ($G\alpha_t$), peripherin and ROM1 (the latter two existing in a constitutive oligomeric complex in discs) (Table 1).

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 (Fig. 4A). It also significantly overlapped with the elution profile of $G\alpha_t$, but not with that of the peripherin-ROM1 complex.

Reciprocal immunoprecipitation of proteins from solubilized bovine discs using anti-rhodopsin antibody showed a significant PRCD fraction bound to rhodopsin (Fig. 4B). 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 (Fig. 4B, lane 5). Notably, a fraction of PRCD was not co-precipitated with rhodopsin (Fig. 4B, lane 2), despite a complete coincidence of elution peaks between the two proteins on gel filtration chromatography (Fig. 4A). 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[23], we tested the specificity of rhodopsin binding to immobilized PRCD peptide by repeating the experiment in serial dilutions of solubilized disc lysate (Fig. 4E). 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 $G\alpha_t$ or peripherin (Fig. 4C and 4D), 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 $G\alpha_t$, it is also possible that $G\alpha_t$ was actually associating with rhodopsin retained on these beads by PRCD, as these proteins are known to interact.

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.[15] 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.[24]

Immunostaining with anti-PRCD antibody revealed that PRCD is essentially absent from rods of rhodopsin knockout mice (Fig. 5A), and Western blotting confirmed that PRCD is reduced in rhodopsin knockout retinas by over 12-fold (Fig. 5B). 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; Fig. 1B), 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 (Fig. 5), consistent with previous reports.[15, 25] 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.[24] 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[15] and indicate that PRCD also requires rhodopsin for maintaining intracellular stability and, ultimately, outer segment delivery.

DISCUSSION

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.[21] 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;[12] 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.[8]

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.[26–28] 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.[26] 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.[29, 30] 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.[31]

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.[8] 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.[12] 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.

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.[23, 32] 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.[15]

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,[33] and continued long before their retinal degeneration phenotype was explained by the C2Y mutation in PRCD.[3] Mutant photoreceptors initially develop normally and then die, one after another, as the animal ages.[1, 34, 35] Photoreceptors of young C2Y dogs have normal ultrastructure[35] and unaffected visual function, as evident from electroretinographic analysis of their light responses.[1] 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.[7–11]

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,[36] mRNA expression, intracellular localization[37] and regeneration after bleaching.[38] On the other hand, rods of C2Y dogs were shown to have a somewhat reduced outer segment turnover rate, [1, 35, 39] 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[1] 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.[40–42] Another potential pathway downstream from rhodopsin involves light-dependent activation of Rac1, a signaling protein thought to regulate photoreceptor susceptibility to photo-oxidative stress.[43, 44]

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.[1, 35] 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.

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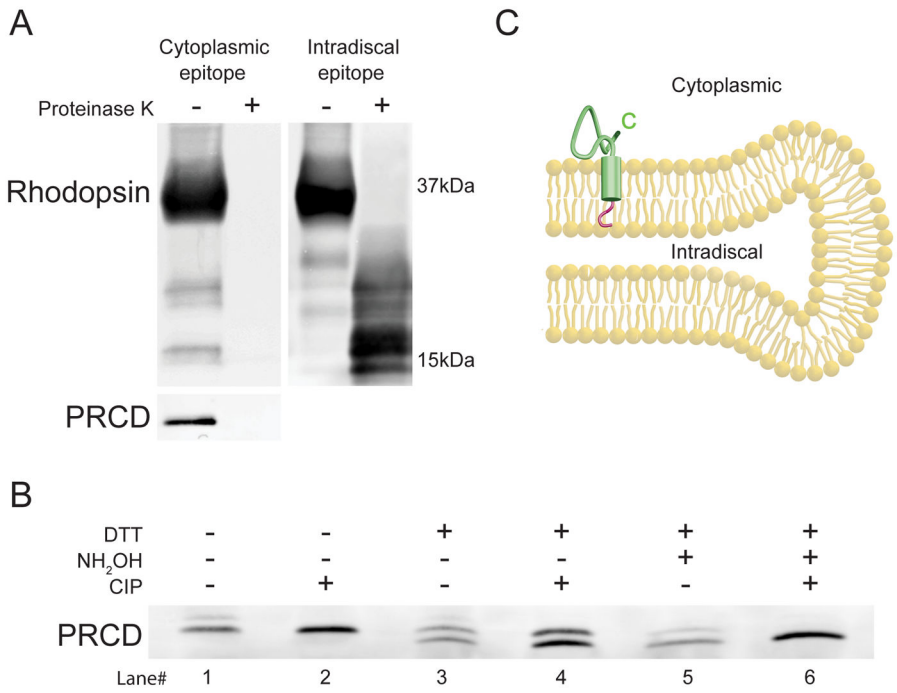

Figure 1.

Membrane topology and post-translational modifications of PRCD. *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 (NH_2OH, 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).

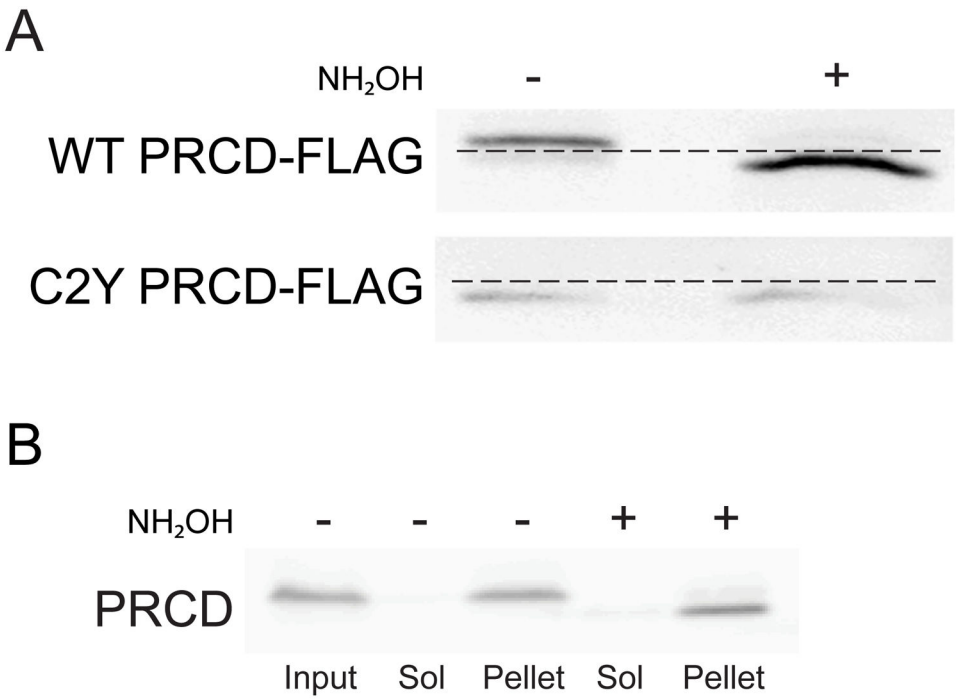

Figure 2.

PRCD S-acylation is not required for membrane association. *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_2OH, 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

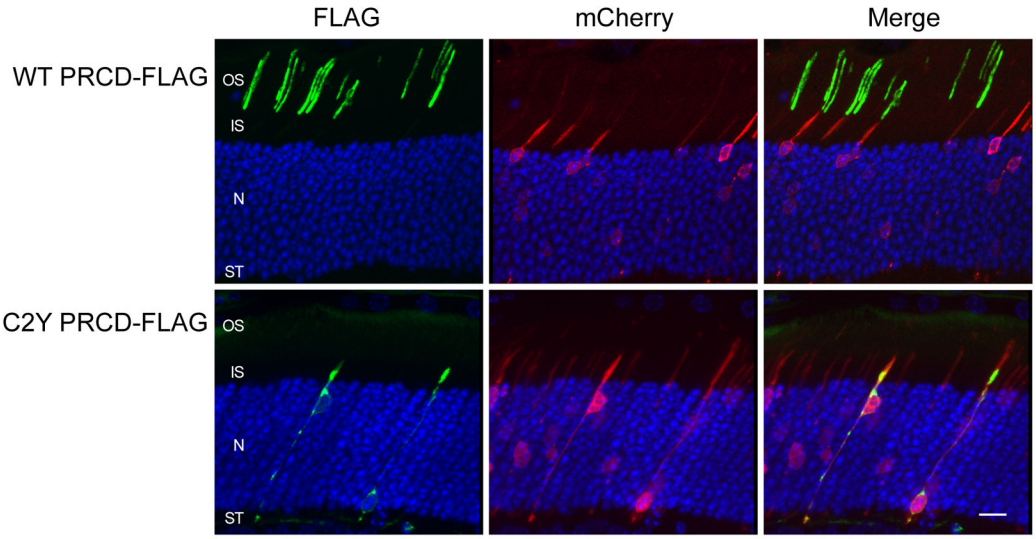

Figure 3.

Disease-causing PRCD mutant mislocalizes from rod outer segments. 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.

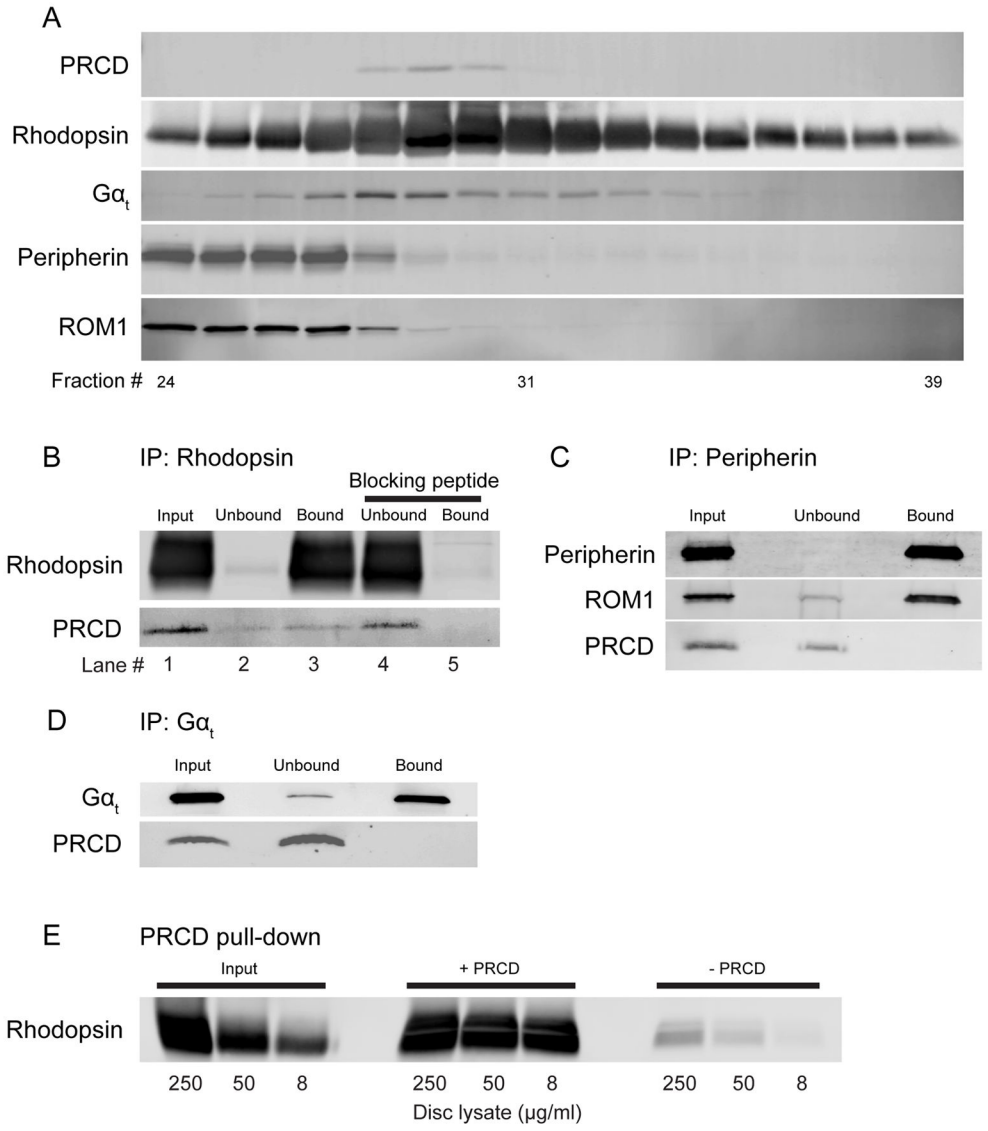

Figure 4.

Co-chromatography and co-precipitation of PRCD with its binding protein candidates. *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 antibodies against PRCD, rhodopsin, $G\alpha_t$, peripherin and ROM1. *B,* Proteins from bovine disc membranes were solubilized in 0.1% DDM (input, lane #1) and incubated with anti-rhodopsin antibody 1D4 bound to protein A/G magnetic beads. After incubation, the unbound lysate was collected (lane #2), the beads were washed, and bound proteins eluted (lane #3). In a control experiment, antibody was pre-incubated with its peptide antigen to block the antibody from precipitating rhodopsin, and unbound (lane #4) and bound (lane #5) lysate was collected. All loaded samples were normalized by volume. *C, D* Bovine discs solubilized in 0.1% DDM (input) were incubated with anti-$G\alpha_t$ (*C*) or anti-peripherin (*D*) antibodies attached to protein A/G magnetic beads. The unbound material was collected (unbound) before washing the beads and eluting bound proteins

(bound). Normalized volumes of input, unbound and bound fractions were loaded for Western blotting for PRCD, peripherin, ROM1, and $G\alpha_t$ using specific antibodies to each of these proteins. *E*, Streptavidin magnetic beads bound to PRCD peptide were incubated with three concentrations of disc lysate (250, 50 and 8 μg total protein/ml) solubilized in 0.7% CHAPS. Control experiment was performed with empty beads. Bound proteins were eluted and rhodopsin was detected by Western blotting with anti-rhodopsin antibody. Samples containing eluted proteins were normalized by volume; input samples were diluted by 70%. Each figure panel represents an image from at least 3 independent experiments.

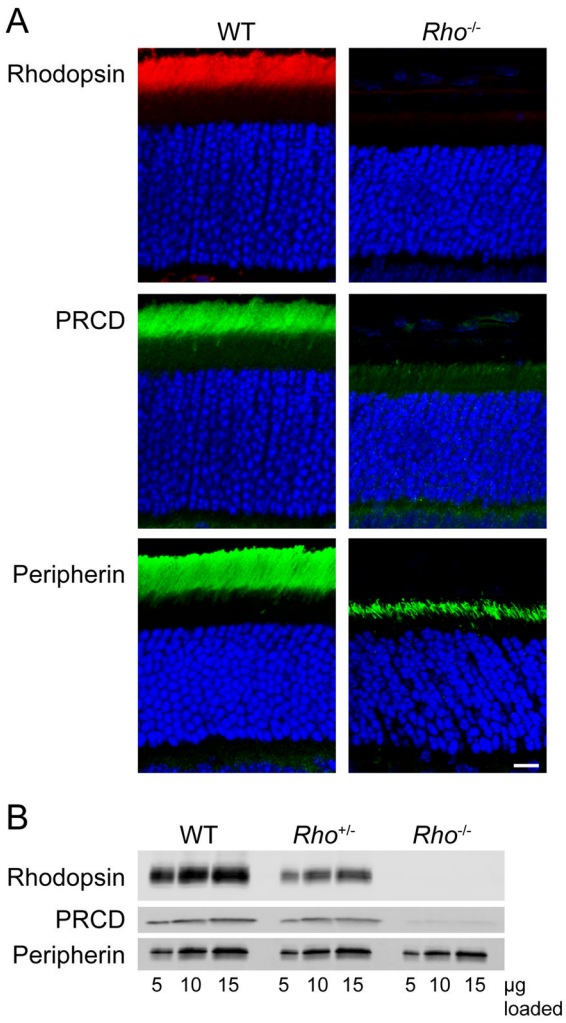

Figure 5.

PRCD is virtually absent from rhodopsin knockout rods. *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.

Table 1

PRCD peptide precipitates rhodopsin from solubilized disc membranes. 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.

	Experiment 1				Experiment 2			
	Total Ion Intensity		Fold Change	ANOVA (p)	Total Ion Intensity		Fold Change	ANOVA (p)
	+PRCD	−PRCD			+PRCD	−PRCD		
Rhodopsin	794839	15552	51	2E-07	322947	4946	65	1E-04
Peripherin	10363	102	101	2E-07	28126	34	817	0.008
ROM1	7522	153	49	8E-06	19558	16	1250	0.01
$G\alpha_t$	6702	234	29	1E-06	43176	249	173	0.003