GENERAL ARTICLE

Oligomerization of Prph2 and Rom1 is essential for photoreceptor outer segment formation

Rahel Zulliger^{1,†,‡}, Shannon M. Conley^{2,†}, Maggie L. Mwoyosvi¹, Muayyad R. Al-Ubaidi¹ and Muna I. Naash^{1,*}

¹Department of Biomedical Engineering, University of Houston, Houston, TX 77204, USA and ²Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

*To whom correspondence should be addressed at: 3517 Cullen Blvd, SERC Room 2005, Houston, TX 77204, USA. Tel: +1 7137431651; Fax: +1 7137430226; Email: mnaash@central.uh.edu

Abstract

Mutations in peripherin 2 (PRPH2, also known as Rds), a tetraspanin protein found in photoreceptor outer segments (OSs), cause retinal degeneration ranging from rod-dominant retinitis pigmentosa (RP) to cone-dominant macular dystrophy (MD). Understanding why some Prph2 mutants affect rods while others affect cones remains a critical unanswered question. Prph2 is essential for OS structure and function and exhibits a very specific pattern of oligomerization with its homolog Rom1. Non-covalent Prph2/Rom1 homo- and hetero-tetramers assemble into higher-order covalently linked complexes held together by an intermolecular disulfide bond at Prph2-C150/Rom1-C153. Here we disrupt this crucial bond using a C150S-Prph2 knockin mouse line to study the role of Prph2 higher-order complex formation. We find that C150S-Prph2 traffics to the OS, interacts with Rom1 and forms non-covalent tetramers, but alone cannot support normal OS structure and function. However, C150S-Prph2 supports the initiation or elaboration of OS disc structures, and improves rod OS ultrastructure in the presence of wild-type (WT) Prph2 (i.e. *Prph2*^{C150S/+} versus *Prph2*^{+/-}). *Prph2*^{C150S/+} animals exhibit haploinsufficiency in rods, but a dominant-negative phenotype in cones, suggesting cones have a different requirement for large Prph2 complexes than rods. Importantly, cone but not rod function can be improved by the addition of one *Prph2*^{Y141C} allele, a mutation responsible for pattern dystrophy owing to the extra cysteine. Combined these findings show that covalently linked Prph2 complexes are essential for OS formation, but not for Prph2 targeting to the OS, and that cones are especially sensitive to having a broad distribution of Prph2 complex types (i.e. tetramers and large complexes).

Introduction

Over 150 different pathogenic mutations in the photoreceptor-specific gene PRPH2 (also known as RDS or retinal degeneration slow) have been described in patients, leading to a variety of phenotypes spanning from MD to RP (1). PRPH2 and its homolog ROM1 are critical for the formation of the disc rim region of rod and cone photoreceptor outer segments (OSs) (2–4). Prph2 and Rom1 create and maintain the rim region of rod discs and cone

lamellae and help to regulate disc size and alignment. Data thus far show that both of these processes are dependent on the formation of specific types of Prph2 and Rom1 complexes (5–7). The two proteins initially form homomeric and heteromeric tetramers mediated by non-covalent interactions in the second extracellular/intradiscal (D2) loop (8–10). All tetraspanin proteins, including Prph2 and Rom1 have intramolecular disulfide bonds to stabilize the structure of the D2 loop (11,12); however, Prph2

[†]These authors contributed equally to this work.

[‡]Present address: Pharma Research and Early Development, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd, 4070 Basel, Switzerland.

Received: April 19, 2018. Revised: May 24, 2018. Accepted: June 4, 2018

© The Author(s) 2018. Published by Oxford University Press. All rights reserved.

For permissions, please email: journals.permissions@oup.com

and Rom1 each also have an additional D2 loop cysteine not involved in intramolecular disulfide bonding. This cysteine (C150 in Prph2 and C153 in Rom1) is involved in intermolecular disulfide bond formation and mediates assembly of Prph2/Rom1 tetramers into covalently linked higher-order complexes (5,10,12).

To understand the importance of C150-mediated complex assembly for OS structure and function, we previously used transgenic mouse models expressing oligomerization-incompetent (C150S) Prph2 in either rods (under the control of the mouse opsin promoter-MOP-T) or in cones (under the control of the cone opsin promoter-COP-T). Although C150S-Prph2 formed tetramers, it did not form higher-order oligomers and did not support OS formation. Importantly, C150S Prph2 led to a dominant-negative defect specifically on cone function and protein targeting (5,13). This finding suggested that Prph2/Rom1 complex formation may be differentially involved in the two cell types. Working out cone versus rod differences in Prph2 complex formation and behavior is central to understanding why some Prph2 mutants lead to rod-dominant disease while others lead to cone dominant disease. The C150S mutation is an essential structure-function model for this purpose, but our results were severely limited in their applicability because transgenic mouse models have uncontrolled levels of expression. This is critical both because Prph2 haploinsufficiency results in severe defects (14,15) independent of any mutation and because it is not possible to ensure that the MOP-T and COP-T promoters drive expression equally or in the same manner as the endogenous gene.

To overcome limitations of transgenic models, here we generated and evaluated a C150S knockin model. We confirm that C150S Prph2 leads to dominant-negative defects in cone but not in rod function, in spite of being trafficked properly to the OS. Interestingly, we find structural but not functional improvement in C150S heterozygous rods, suggesting that the Prph2 higher-order oligomers have roles beyond being simple structural components. We also utilize another Prph2 model (Y141C) to specifically evaluate the effects of the addition of a Prph2 allele with an extra cysteine and found that again rods and cones behave differently. These findings significantly refine and advance our understanding of the molecular defects underlying Prph2-associated pathologies and provide insight into the structural regulation of rod and cone photoreceptors.

Results

C150S does not support OS structure and leads to dominant-negative functional defects in cones

To eliminate the effects of variable levels of transgene expression, we generated a knockin mouse model carrying the C150S mutation in the *Prph2* locus (Supplementary Material, Fig. S1). To determine what effect this mutation had on retinal structure and function, we began by conducting light microscopy and electron microscopy (EM) analyses at postnatal day (P) 30 on retinas from C150S homozygous (*Prph2*^{C/C}) and heterozygous knockin mice (*Prph2*^{C/+}). Overall retinal lamination was not altered in mice carrying the C150S allele (Fig. 1A), and outer nuclear layer (ONL) thinning (owing to photoreceptor degeneration) was not evident; in contrast *Prph2*^{-/-} retinas at this age already showed reduction in ONL thickness (Fig. 1A, ~9–10 rows of photoreceptor nuclei in *Prph2*^{+/+}, *Prph2*^{+/-}, *Prph2*^{C/+} and *Prph2*^{C/C} and ~6 rows in *Prph2*^{-/-}). Examination by EM, however, revealed gross OS malformation in the *Prph2*^{C/C} retina (Fig. 1B and C; Supplementary Material, Fig. S2A). In contrast to the nicely stacked discs and elongated OSs of the WT,

the *Prph2*^{C/C} exhibited highly disorganized, very short OSs with elongated discs curving around into whorl-like structures that were nonetheless an improvement over the complete lack of OSs seen in the *Prph2*^{-/-}. *Prph2*^{C/C} OSs were similar in structure but smaller than those seen in the haploinsufficient *Prph2*^{+/-}. To understand whether these small structures were cones or rods (or both), we identified cone OSs by immunogold (IG) labeling at the EM level with anti-S-opsin antibodies (Fig. 1D), and found that cone OSs in the *Prph2*^{C/C} were structurally similar to rod OSs; namely they were quite small and exhibited whorl-like disc structures. C150S heterozygous animals also showed abnormal OSs, characterized by elongated, disorganized discs and vesicle-like structures (arrow in Fig. 1C). Importantly, although some OSs in the *Prph2*^{C/+} were still abnormal, most OSs were improved in ultrastructure when compared with the *Prph2*^{+/-} (Fig. 1C; Supplementary Material, Fig. S2B). These improvements included better disc stacking, orientation, and size, as well as reduction in the whorl-like phenotype seen in the *Prph2*^{C/C} and the *Prph2*^{+/-}.

To evaluate the effects of the C150S allele on retinal function, full-field scotopic and photopic electroretinograms (ERGs) were performed at P30 and P60 (Fig. 2A). Mean scotopic a- and b-wave ERG amplitudes in *Prph2*^{C/+} were significantly decreased by 50% (a-wave) or 30% (b-wave), respectively, when compared with WT, and were similar to those found in *Prph2*^{+/-} (Fig. 2B), suggesting that C150S acts as a loss-of-function allele in rods. To evaluate cone function, we measured photopic ERG responses (Fig. 2C). C150S heterozygous animals exhibited mean photopic responses ~25% lower than those in *Prph2*^{+/-} (which is normal at P30), suggesting that the C150S allele has dominant-negative or gain-of-function effects in cones, consistent with our previous findings (5). Rod and cone function in the *Prph2*^{C/+} did not change from P30 to P60, suggesting these retinas did not undergo rapid degeneration.

Scotopic function in the *Prph2*^{C/C} was slightly better than in the *Prph2*^{-/-} retina at P30, reaching ~25% of the WT levels, indicating that the small OSs we observed in these retinas were capable of phototransduction. However, rod function in the *Prph2*^{C/C} decreased at P60 suggesting these abnormal OSs may progressively degenerate (Fig. 2B). Cone function was not as affected as rod function in the *Prph2*^{C/C}, reaching 40% of WT at P30 (Fig. 2C). Combined these data indicate that C150S alone is not sufficient to support proper OS structure and function in rods or cones. In addition, we find that cones are adversely affected by C150S, even when WT Prph2 is also present.

The C150S mutation alters Prph2 protein levels and has a profound influence on Prph2 oligomerization

We next evaluated the molecular mechanism underlying the structural and functional defects associated with the C150S mutation, and our first step was to compare expression levels from the knockin allele to that of the WT allele. We evaluated transcript levels by qRT-PCR at P 10, 20 and 30 (time points that reflect OS development) and found that *Prph2* and *Rom1* transcript levels in *Prph2*^{C/C} retinas were not different from WT at any time points (Fig. 3A). In addition, co-localization studies showed that C150S protein was in the OSs of both rods and cones in the *Prph2*^{C/C} (Supplementary Material, Fig. S3). Combined these findings suggest that the mutation did not affect gene regulation and that expression from the knockin allele was properly regulated. In contrast, we found Prph2 and Rom1 protein levels were significantly decreased in the *Prph2*^{C/C} retinas. Because cones

Figure 1. C150S Prph2 does not support proper OS formation. Light microscopy (A) and EM (B, C) analysis on sections collected from eyes of the indicated genotypes at P30. (A) Images of toluidine blue-stained, plastic embedded and captured at $\times 40$. Images are aligned at the upper edge of the ONL for better comparison of outer nuclear layer thickness. (B, C) EM images were captured at $\times 7500$ (B) or $\times 15\,000$ (C) at the interface between the IS and OS. (D) IG staining with S-opsin antibodies coupled with EM imaging was performed on ultrathin sections from the WT and $Prph2^{C/C}$ retina to evaluate cone OS ultrastructure. Images were captured at $\times 50\,000$. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; CC, connecting cilium. Scale bars: 25 μm (A), 2 μm (B), 1 μm (C), and 500 nm (D).

make up 3–5% of photoreceptors in the WT retina, we assessed the effect of mutant Prph2 on total levels of Prph2 and Rom1 in retinas on WT (rod-dominant) and $Nrl^{-/-}$ (cone-dominant) (16) backgrounds to help understand differences in the two cell types. Prph2 protein levels in $Prph2^{C/C}$ retinas were only 7% of WT ($Prph2^{+/+}$, Fig. 3B and C), while Prph2 levels in the $Prph2^{C/C}Nrl^{-/-}$ were reduced even further, to 1% of those in the $Nrl^{-/-}$ (Fig. 3D and E). Decreases in Prph2 protein levels are frequently associated with a corresponding decrease in Rom1 levels (5,8,17,18), a finding recapitulated here; Rom1 levels in $Prph2^{C/C}$ were $\sim 10\%$ of WT (Fig. 3B and C). Interestingly, decreases in Rom1 associated with C150S were much less pronounced in the cone-dominant background: Rom1 levels in the $Prph2^{C/C}Nrl^{-/-}$ were 70% of those in the $Nrl^{-/-}$ (Fig. 3D and E). This relative preservation of Rom1 in cones does not appear to be owing to enhanced stability of Rom1 in cones in the absence of Prph2 per se, as Rom1 levels in

the $Prph2^{-/-}Nrl^{-/-}$ retina were very low, similar to levels in the $Prph2^{-/-}$ retina.

Prph2-associated macular disease is autosomal dominant, and patients are heterozygous for disease-causing mutations (1). Thus, understanding to what extent mutations affecting complex formation (such as C150S) can exert effects in the presence of the WT protein is critical. Since the only difference between the WT and mutant Prph2 is the conversion of cysteine at position 150 to serine, we are not able to distinguish between these 2 proteins in the heterozygous C150S retinas. We, therefore, evaluated total Prph2 levels in these retinas. C150S heterozygous animals on the WT (rod-dominant) background had reduced total Prph2 protein levels compared with WT (Fig. 3B and C), yet much higher levels than in the $Prph2^{+/-}$ retina. This is recapitulated in cones, as mean levels of Prph2 in the $Prph2^{C/+}Nrl^{-/-}$ retina were not statistically significantly different

Figure 2. C150S leads to dominant defects in cone but not rod function. Full-field ERGs were performed under scotopic and photopic conditions on animals of the indicated genotypes at either P30 or P60. (A) Representative ERG waveforms at P30 for scotopic and photopic conditions are shown. Plotted are maximum amplitudes (mean $\pm$ SEM) for scotopic (B) and photopic (C) recordings. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for one-way ANOVA with Tukey's post hoc test ($n = 5$ –15 animals).

from *Nrl*^{-/-} and were higher than those in the *Prph2*^{+/-}*Nrl*^{-/-} retina (Fig. 3D and E). Rom1 levels were not significantly affected in heterozygous animals in the WT or *Nrl*^{-/-} background. To independently evaluate C150S-Prph2 from the WT-Prph2 in the *Prph2*^{C/+} retina, we crossed C150S knockin mice with N229S Prph2 knockin mice. We previously showed that the N229S mutation does not affect OS structure, retinal function, or Prph2/Rom1 complex assembly at early timepoints (18), and since it abolishes Prph2 glycosylation, N229S-Prph2 can be distinguished from WT or C150S Prph2 by virtue of its smaller size. In *Prph2*^{C/N} retinas, overall Prph2 levels were ~75% of WT (similar to the *Prph2*^{C/+} as expected) with the C150S Prph2 accounting for ~25% (Fig. 3F).

In the retina, WT Prph2 assembles into various homomeric and heteromeric (with Rom1) complexes held together by both disulfide-linkages as well as non-covalent bonds, and was thus present as both a monomer and disulfide-linked dimer under non-reducing conditions (Fig. 3B and D). In contrast, Prph2 in the *Prph2*^{C/C} and *Prph2*^{C/C}*Nrl*^{-/-} was present as a monomer, consistent with the ablation of the cysteine involved in intermolecular disulfide bonding (Fig. 3B and D). Some Rom1 dimer remained in the *Prph2*^{C/C}, confirming the ability of Rom1 to form covalently linked homomeric complexes (17), however, no Rom1 dimer was found in the *Prph2*^{C/C}*Nrl*^{-/-} suggesting Rom1 may be involved in different types of complexes in rods versus cones.

To evaluate interactions between C150S and WT Prph2 in the *Prph2*^{C/C} retina, we performed reciprocal co-immunoprecipitation (IP). In work using the COP-T transgenic line on the *Prph2*^{-/-} background we previously observed that C150S protein in cones (but not rods) did not pull down Rom1 (5,13). However, here we do not find this cone versus rod difference; we see that C150S Prph2 pulled down Rom1 from *Prph2*^{C/C} and *Prph2*^{C/C}*Nrl*^{-/-} retinal extracts (Fig. 4A), a difference possibly attributable to low levels

of transgene expression in the COP-T retinas. To further understand what type of Prph2/Rom1 complexes are formed by C150S Prph2, we conducted non-reducing velocity sedimentation using 5–20% sucrose gradients and subsequently separated gradient fractions on reducing SDS-PAGE/western blots (WBs). The percent of total Prph2/Rom1 in each fraction was then plotted. In control retinas (WT and *Nrl*^{-/-}) Prph2 was present in large oligomers (gradient fractions 1–3), intermediate oligomers (gradient fractions 4–5), and tetramers (gradient fractions 6–9), while Rom1 was found in intermediate and tetrameric fractions (Fig. 4B and C). As expected, in the absence of WT Prph2 in both rods and cones, C150S Prph2 formed only tetramers (Fig. 4D and E). In addition to this shift in Prph2 complexes in the *Prph2*^{C/C} and *Prph2*^{C/C}*Nrl*^{-/-} we also saw a shift in the size of complexes containing Rom1. In *Prph2*^{C/C} retinas, only Rom1 tetramers were assembled, suggesting that although Rom1 normally participates in disulfide-linked intermediate sized heteromeric complexes with Prph2, on its own, it does not form intermediate-sized homomeric complexes.

We next asked whether C150S Prph2 could participate in larger, covalently linked complexes in the presence of WT Prph2 by utilizing the *Prph2*^{C/N}. In the *Prph2*^{C/N} very little N229S Prph2 was detected in fractions associated with higher order complexes (1–3), and no C150S Prph2 was detected in fractions 1–3 (Fig. 4F). This finding suggests that C150S Prph2 does not participate in covalently linked complexes with WT Prph2 (consistent with the lack of cysteine responsible for intermolecular disulfide bonding) and also that C150S Prph2 impairs the ability of WT Prph2 to assemble into larger oligomers. To confirm that this was a gain-of-function/dominant negative effect rather than simply an outcome of potential C150S loss-of-function, we evaluated Prph2/Rom1 complex assembly in the *Prph2*^{+/-} and found that large oligomers (Fractions 1–3) do assemble normally in this model (Fig. 4G). The *Prph2*^{C/N} retina also exhibited a

Figure 3. C150S transcript is expressed normally, but C150S protein levels are reduced. (A) cDNA prepared from total retinal RNA harvested at P10–P30 was evaluated for expression of *Prph2* and *Rom1*. Expression levels were normalized to *Hprt*, a housekeeping gene. (B, D) Proteins were extracted at P21 from the indicated genotypes and equal amounts of total proteins were loaded on non-reducing SDS-PAGE gels and blots were probed for *Prph2* (RDS-CT) and *Rom1* (2H5) to show the monomeric and dimeric (disulfide-linked) forms of *Prph2* and *Rom1*. (C, E) Protein levels in retinal extracts were calculated densitometrically and normalized to corresponding actin values. *Prph2* and *Rom1* dimers and monomers were quantified separately. (F) Retinal extracts from *Prph2*^{C/N} animals and controls were separated under non-reducing (left) and reducing (right) conditions. Levels of *Prph2* were calculated densitometrically from reducing blots where the size shift owing to the lack of glycosylation on the N229S protein can be easily visualized and normalized to actin. Graphs plot mean total *Prph2* and *Rom1* levels (i.e. dimer+monomer) ± SEM, *n* = 5 retinas/group. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 for one-way ANOVA with Tukey's post hoc test.

subtle right shift in the distribution of *Rom1*-containing complexes corresponding to fewer intermediate sized complexes and more tetrameric complexes (Fig. 4F). The shift in complex size between the *Prph2*^{C/C} and the *Prph2*^{C/N} (i.e. Fig. 4D versus Fig. 4F) suggests that the mutant protein is able to incorporate into non-covalently linked complexes with WT *Prph2*, but that overall, C150S suppresses the formation of larger disulfide linked *Prph2* complexes.

Given the defects in assembly of larger sized complexes in *Prph2*^{C/C} retinas, we asked whether the mutant protein was capable of localizing properly to the OS using IG labeling for *Prph2* (Fig. 5A) followed by EM. In the WT retina, *Prph2* and *Rom1* (Fig. 5A and B) properly localized to the rim region of the OS discs (arrows), while rhodopsin was found throughout the disc (Fig. 5C). This localization was preserved in the *Prph2*^{C/C} even though the disc structure was highly disorganized, with C150S *Prph2* and *Rom1* found at the rims of the discs (Fig. 5A, right three panels and Fig. 5B, right panel) and rhodopsin distributed

throughout (Fig. 4C, right panel). These findings indicate that even in the absence of higher-order *Prph2* complexes, the innate organization of the disc with its distinct protein distribution is preserved in the *Prph2*^{C/C} retina and that C150S *Prph2* retains the ability to target to the OS.

Heterogeneous complexes in a *Prph2*^{C/Y} double knockin mouse lead to rescue of cone but not rod function

One benefit of the C150S knockin allele is that we can use it to further our understanding of disease mechanisms in other *Prph2*-disease causing mutants such as that of the Y141C. The Y141C mutation has been of recent interest to us because it is associated with widely varying phenotypes in patients, some of whom present with milder forms of pattern dystrophy while others exhibit more severe RP. The Y141C knockin mouse line (*Prph2*^{Y/Y}) exhibits dominant gain-of-function defects in cones

Figure 4. C150S Prph2 interacts with Rom1 but exhibits abnormal complex formation. (A) IP was performed on retinal extracts collected at P21 of the indicated genotypes using antibodies for Prph2 (top) or Rom1 (bottom) and resultant blots were probed [immunoblot (IB)] for Prph2 or Rom1. FT, flow through/unbound. (B–G) Retinas from mice of the indicated genotypes were subjected to non-reducing velocity sedimentation for in-depth complex analysis. Resulting gradient fractions separated on reducing SDS-PAGE/WB. The amount of Prph2 (dashed line, B–E, G), or Rom1 (solid line, B–E, G) in each gradient fraction is plotted as a % of total Prph2 or Rom1, respectively. In (F) Rom1 is shown by a solid line, N229S Prph2 with a dotted line, and C150S Prph2 with a dashed line. Graphs show mean $\pm$ SEM, $n = 3$ –5 replicates/group.

Figure 5. C150S properly localizes to photoreceptor OSs. IG labeling with antibodies against Prph2 (A), Rom1 (B) and Rhodopsin (C) was performed coupled with EM imaging. Arrows indicate Prph2/Rom1 IG labeling at disc rims in both *Prph2*^{+/+} and *Prph2*^{C/C}. Images were captured of the OS layer at $\times 50\,000$, and the scale bar is 500 nm.

but a loss-of-function phenotype in rods (17,19), similar to what we see in the C150S knockin model. However, the effects of the Y141C mutation on complex formation are opposite to those of the C150S. Y141C retinas exhibit abnormally large disulfide-linked complexes which are not detected under reducing conditions, suggesting they are held together by excess disulfide bonds (17). This is consistent with the presence of an extra free cysteine in the D2 loop of the Y141C protein. Thus from a biochemical standpoint, the Y141C molecular phenotype in which Prph2 complexes are abnormally large and have too many intermolecular disulfide bonds is opposite to the C150S molecular phenotype in which Prph2 complexes are too small and lack intermolecular disulfide bonds. We thus asked whether molecular, cellular and functional phenotypes in the *Prph2*^{Y/Y} could be improved by crossing with the *Prph2*^{C/C}. We first confirmed that Y141C-Prph2 could interact with C150S Prph2 by performing reciprocal co-IP on lysates from HEK293 cells transfected with Myc- or Flag-tagged Prph2 constructs (Supplementary Material, Fig. S4) then performed non-reducing SDS-PAGE/WBs on retinal extracts from the *Prph2*^{C/Y} animals. We found that *Prph2*^{C/Y} retinas exhibited complexes characteristic of both the *Prph2*^{C/C} and the *Prph2*^{Y/Y} retinas. *Prph2*^{C/Y} retinas exhibited abnormally large molecular weight Prph2/Rom1 complexes like *Prph2*^{Y/+} and *Prph2*^{Y/Y} retinas (Fig. 6A, arrows), however, it completely lack dimers, in common with the *Prph2*^{C/C} (Fig. 6A, arrowheads). Co-expression of C150S and Y141C protein (*Prph2*^{C/Y}) did not substantially alter total Prph2 levels compared with *Prph2*^{C/C} or *Prph2*^{Y/Y}; all three genotypes exhibited Prph2 levels ~ 10 – 15% of WT. To further evaluate complex formation in these animals, we conducted non-reducing velocity sedimentation and separated gradient fractions on non-reducing gels. Prph2 was found in two broad pools in the *Prph2*^{C/Y}, a pool of disulfide linked abnormally large complexes in Fractions 1–3 and a pool of non-covalently linked tetramers in Fractions 6–9 (Fig. 6B). No Prph2 complex containing a single disulfide bond (i.e. dimers) was detected nor were any intermediate-sized (Fractions 4–5) complexes were detected. Again, this pattern is a combination of that seen in the *Prph2*^{Y/Y} (Fig. 6B) (17) and that seen in the *Prph2*^{C/C} (Fig. 6B).

To determine whether this distribution of Prph2 complexes had the ability to support retinal function, we performed ERG on *Prph2*^{C/Y} animals and controls (Fig. 6C, controls are replotted from Fig. 2 for comparison). Based on the abnormal complex assembly in the *Prph2*^{C/Y}, we predicted that rod and cone function

would be similar to that found in the *Prph2*^{C/C} or *Prph2*^{Y/Y}, i.e. quite poor. Indeed this was the case for rod function in the *Prph2*^{C/Y} which was significantly reduced compared with WT, *Prph2*^{C/+} and *Prph2*^{Y/+} and not significantly improved compared with the *Prph2*^{C/C} or *Prph2*^{Y/Y} (Fig. 6C, top). In stark contrast, cone function in the *Prph2*^{C/Y} was significantly improved compared with *Prph2*^{C/C} and *Prph2*^{Y/Y}, and was not significantly different from the *Prph2*^{Y/+} or *Prph2*^{C/+} at one month of age (Fig. 6C, bottom). The worsened rod phenotype in *Prph2*^{C/Y} versus *Prph2*^{C/+} or *Prph2*^{Y/+} is likely caused by a decrease in total Prph2 protein levels compared with *Prph2*^{Y/+} and *Prph2*^{C/+} as rods are very sensitive to Prph2 haploinsufficiency. However, the finding that cone function in the *Prph2*^{C/Y} is similar to *Prph2*^{C/+} or *Prph2*^{Y/+} suggests that cones benefit from having a mix of Prph2 complexes (i.e. some small and some very large) even if those complexes are still abnormal, rather than having all small or all abnormally large complexes.

Interestingly, OS ultrastructure in the *Prph2*^{C/Y} (Fig. 6D) was variable but largely worse than that seen in the *Prph2*^{C/+} or *Prph2*^{Y/+}, consistent with the severe defects we observed in rod function. Some *Prph2*^{C/Y} OSs were quite small (Fig. 6E, right image), with rounded discs similar to *Prph2*^{C/C} (Fig. 6F) and *Prph2*^{Y/Y} (Fig. 6F) while others were larger, similar to *Prph2*^{C/+} and *Prph2*^{Y/+} (Fig. 6F).

Discussion

As previously reported, here we find that C150S-Prph2 alone cannot support full OS formation, yet can form small nascent OS structures. *Prph2*^{C/C} retinas do not exhibit the typical ONL thinning that characterizes the *Prph2*^{-/-} at P30, suggesting that formation of even tiny OS structures can help delay photoreceptor cell death. From a functional standpoint, *Prph2*^{C/+} retinas exhibit a haploinsufficiency phenotype in rods and a dominant-negative phenotype in cones. Importantly, the C150S-Prph2 does traffic properly to the OSs of rods and cones and retains the ability to interact with Rom1 and form non-covalently linked tetramers. The primary molecular defect of C150S Prph2 is an inability to form covalently linked complexes (5,12), and the severe structural and functional defects in the *Prph2*^{C/+} and *Prph2*^{C/C} retinas clearly demonstrate the essential role for these large covalently-linked Prph2 complexes in proper OS morphogenesis. Importantly, although C150S is not a mutation found in patients with Prph2-associated disease, many patient

Figure 6. Co-expression of C150S and Y141C Prph2 improves cone but not rod function. Prph2^{C150S} animals were cross-bred with those carrying the Y141C mutation to generate Prph2^{C150S} animals. (A) Retinal lysates from P21 animals of the indicated genotypes, including Prph2^{+/+} and Prph2^{Y141C} as controls, were separated on non-reducing SDS-PAGE and resulting WBs were probed for Prph2 (left) or Rom1 (right). Arrows indicate abnormal high molecular weight complexes in retinas carrying the Y141C allele; arrowheads indicate absent or reduced dimers. (B) Retinas of the indicated genotypes underwent non-reducing sucrose gradient velocity sedimentation followed by separation of gradient fractions on non-reducing SDS-PAGE. Blots were probed for Prph2. (C) Full-field scotopic and photopic ERG was performed on animals of the indicated genotypes at P30 and P60. Control values are re-plotted from Figure 2 for comparison sake. Shown is comparison of retinal function between heterozygous Prph2^{C150S} at P30 and P60. Plotted are maximum amplitudes (mean $\pm$ SEM) for scotopic (left) and photopic (right) recordings. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for one-way ANOVA with Tukey's post hoc test ($n = 5-15$ animals). P30 retinal structure of the Prph2^{C150S} is shown at the light microscopy level (D, $\times 40$ magnification) and EM level (E, $\times 7500$ left image and $\times 15\,000$, for the middle and right) illustrating different types of OS structures seen in this model. (F) Representative images ($\times 15\,000$) from the indicated genotypes are shown for comparison. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer. Scale bars: D: 25 μ m, E: 2 μ m (left), 1 μ m (middle/right), F: 1 μ m.

mutations, including K153del, cause phenotypes that are quite similar to C150S (20).

Structurally, we observe that *Prph2*^{C/C} retinas exhibit very small whorl-like OSs that are nonetheless capable of low amounts of phototransduction. These small OSs were reminiscent of those seen in previous *Prph2* disease models such as the Y141C and the K153del in which the mutation alters complex formation (17,20). These data suggest that even though *Prph2* and *Rom1* levels are only at ~10% of WT levels, the small amount of tetrameric *Prph2* present in the *Prph2*^{C/C} is able to initiate OS formation. Likewise these findings suggest that properly formed large *Prph2* oligomers are not needed for initiation of disc formation, but are required for proper disc sizing, alignment, rim pinching and OS lengthening. This is consistent with recent work showing that *Prph2* plays a role in the initiation of disc formation by suppressing ectosome release at the connecting cilium, and may be involved in mediating membrane curvature. These functions reside in the C-terminus of *Prph2* (21–23), and thus would be intact in the various D2 loop mutants (C150S, K153del and Y141C) that exhibit altered *Prph2* complex formation but still initiate discs formation.

One of the critical advantages of using a knockin model (versus a transgenic) is that we confirmed that gene expression of the mutant allele on the mRNA level is similar to that of WT. In spite of this, *Prph2*^{C/C} animals have protein levels only ~10% of WT levels, suggesting that the C150S protein is rapidly degraded. It is possible that the C150S protein is inherently more unstable than WT *Prph2*, but it is also possible that in the absence of fully elaborated and elongated OSs, there is simply no place for the normal complement of *Prph2* to reside. This latter explanation also may account for the comparable reduction we observed in *Rom1* levels in *Prph2*^{C/C} retinas and is consistent with our previous observation that *Rom1* and rhodopsin levels are often reduced in *Prph2* mutants that have abnormal OSs (17, 24–26). On the other hand, we find cones do not follow this pattern precisely. C150S-*Prph2* levels in cones (i.e. *Prph2*^{C/C}*Nrl*^{-/-}) are comparable or even lower than those in rods (i.e. *Prph2*^{C/C}), yet *Rom1* levels are well preserved. This rod-cone divergence in the way *Rom1* responds to variations in *Prph2* suggests that rods and cones process and utilize *Prph2* and *Rom1* in different ways. This rod-cone difference is something we have observed previously, for example, rods lacking *Prph2* have no ERG function or OS structures, while cones lacking *Prph2* retain some function and open OS structures that lack lamellae (27). It is not clear what the different role of *Rom1* is in rods versus cones to account for these different protein levels in the presence of complex-inhibiting *Prph2* mutations like C150S since *Rom1* retains the ability to interact with C150S-*Prph2* in both rods and cones. However, we have previously observed rod-cone differences in the behavior of *Rom1* in the presence of *Prph2* mutations. In the case of the D2-loop mutant R172W (which causes cone-dominant MD in patients and mouse models) (25,28,29), we find that *Rom1*, in cones, is incorporated into abnormal, very high molecular weight *Prph2* complexes (25).

Our data suggest that rods and cones can be differentially affected by *Prph2* mutations that affect complex formation, and that deviations in the way that rods versus cones respond to altered complex formation may contribute to cell-specific differences in disease phenotypes. Studies on models of PRPH2-associated disease are consistent with this hypothesis. Studies on transgenic mouse lines carrying RP-causing mutations such as C214S or P216L (30,31) have suggested that rod-targeting mutations in *Prph2* act by a haploinsufficiency-based mechanism, either owing to loss-of-function or dominant negative

effects. In contrast, mutations that cause cone-dominant or macular defects appear to cause disease by more complex mechanisms. Like C150S, several cone-dominant disease mutations induce various alterations in *Prph2*/*Rom1* complex formation including R172W, K153del, and Y141C (17,19,20,25,28,32). While the C150S mutation causes a complete loss of higher-order complexes owing to the lack of the stabilizing disulfide bond, other mutations cause more subtle alterations in complex formation. For example, the R172W *Prph2* mutation induces the formation of abnormal intermediately sized complexes containing *Rom1*, while the Y141C mutation leads to the formation of abnormal large-sized disulfide linked *Prph2*/*Rom1* complexes. On the other hand, the K153del mutation affects complex formation and leads to other phenotypes similar to C150S, namely cone-dominant ERG defects and a lack of higher-order, disulfide-linked complexes (20). Together, these studies show that proper assembly of *Prph2*/*Rom1* oligomers is critical for the formation of rod and cone OSs, and cones especially are more sensitive to subtle changes in these complexes.

One of the key findings here is that ‘supplementation’ of the C150S model with large molecular weight complexes, albeit abnormal ones (e.g. from the Y141C in the *Prph2*^{C/Y}), specifically improves cone function compared with either the *Prph2*^{C/C} or *Prph2*^{Y/Y}. The Y141C mutation causes the formation of abnormal large disulfide-linked complexes, while the C150S mutation ablates them, and when expressed alone, C150S (*Prph2*^{C/C}) and Y141C (*Prph2*^{Y/Y}) can support only minor rod and cone function. Yet when expressed together in the *Prph2*^{C/Y}, cone function is significantly improved to levels seen in the *Prph2*^{Y/+} or *Prph2*^{C/+}; that is, in cones C150S and Y141C together can support function similar to a WT *Prph2* allele. This benefit is clearly cone-specific. We observe no significant improvement in rod function in *Prph2*^{C/Y} retinas when compared with *Prph2*^{C/C} or *Prph2*^{Y/Y}, highlighting the role of haploinsufficiency as a key determinant of rod function.

Here we observe a haploinsufficiency phenotype in rods expressing the C150S mutation, that is, similar rod function in *Prph2*^{+/-} and *Prph2*^{C/+}, yet *Prph2* protein levels in the *Prph2*^{C/+} are much higher than in the *Prph2*^{+/-}. Based on our earlier studies using a transgene that over-expresses WT *Prph2* wherein we showed levels above 60% of WT have the ability to form fully functional OSs (14), we conclude that the *Prph2*^{C/+} retina contains enough *Prph2* protein (~80%) to support normal rod function. Nevertheless, rod function in the *Prph2*^{C/+} is severely compromised, suggesting that the C150S protein is non-functional in rods. Yet it may be partially functional since scotopic ERG recordings are not different between *Prph2*^{+/-} and *Prph2*^{C/+} animals and OS ultrastructure is improved in the *Prph2*^{C/+} versus *Prph2*^{+/-}. Disc stacking, orientation and size are all improved in the *Prph2*^{C/+} compared with *Prph2*^{+/-}. We have previously observed this improvement in rod structure but not function in another one of our *Prph2* mutants with abnormalities in complex formation, the Y141C (17). The absence of functional improvement in the face of structural improvement suggests that these OSs have less efficient phototransduction signaling than WT OSs. Abnormally large (as in the Y141C model) and abnormally small (as in the C150S model) *Prph2* complexes are likely to lead to subtle alterations in rim strength and rigidity that may impair transduction of the light induced signal between the disc and the plasma membrane. Photoreceptor OSs rely on precise spatiotemporal organization for optimal phototransduction. For example, recent data indicate that cGMP-gated channels are concentrated in regions of the plasma membrane near areas of cGMP production (33).

PDE6, the protein responsible for hydrolyzing cGMP during phototransduction has been shown to translocate laterally from the rim region of the disc towards the center of the disc, a process thought to be important for changing rod sensitivity (34). In addition, a variety of key phototransduction components including PDE6, RGS-9, R9AP and the transducin alpha subunit are known to translocate to specialized detergent-resistant membrane (DRM) domains which do not contain Prph2/Rom1 (35) in response to light (36,37). These results highlight the extremely precise organization of transmembrane and membrane-associated proteins in the OS. Thus it is possible that Prph2 mutants that affect the structure, stability or rigidity of the rim membrane have the potential to alter or interfere with correct light-dependent movement of OS proteins, and thus with signal transduction.

In conclusion, these studies have significantly advanced our understanding of the role of large molecular weight Prph2 complexes, their divergent requirements in rods versus cones, and the consequences of eliminating or reducing them. Cones in particular can be improved by supplementation with large molecular weight Prph2 complexes, even abnormal ones, in spite of persistent Prph2 haploinsufficiency. In addition, we find that rod disc sizing and stacking can proceed even in the presence of reduced higher-order Prph2 oligomers, provided sufficient Prph2 is present, but that full OS function likely depends on the formation of a full complement of covalently linked Prph2 oligomers. Future studies will likely continue to focus on rod versus cone differences in the utilization of Prph2 and Rom1, particularly given the relative lack of attention paid to Rom1 thus far. The widely varying and untreatable rod and cone-dominant patient diseases associated with Prph2 mutations highlight the need for further investigations to understand pathogenic mechanisms associated with photoreceptor degeneration.

Materials and Methods

Animals

The Prph2 C150S knock-in mouse model (Prph2^{C/C}) was generated by inGenious Targeting Laboratory, Inc. (Ronkokoma, New York, NY, USA). A 10.14 kb region of genomic DNA was used from a bacterial artificial chromosome (BAC) clone identified earlier for another Prph2 knock-in mouse line to construct the targeting vector (17,19). The LoxP/FRT-neomycin selection cassette was inserted in intron 1383 bp downstream of Prph2 Exon 1 (which houses the C150S point mutation). The long-homology arm extended 6.9 kb 5' from the site of the mutation in Exon 1 while the short-homology arm extended 2.7 kb 3' of the end of the Neo cassette (for further information please see [Supplementary Material, Fig. S1](#)). The C150S point mutation (TGC to AGC) was introduced into the vector via PCR and sequencing confirmed that no other mutations were added. The BAC was sub-cloned into a 2.45 kb pSP72 (Promega, Madison, WI, USA) backbone vector (final target construct 14.3 kb), linearized with Not1 and electroporated into embryonic stem (ES) cells. The ES cells were subsequently screened for the presence of mutated Prph2 allele and positive clones were injected into C57BL/6 blastocysts. These blastocysts were implanted and subsequent chimeric founders were bred to identify mice with germline transmission. To remove the Neo cassette, the mice were bred to FLP^{er} expressing mice (Stock#003946, Jackson Labs, Bar Harbor, ME, USA). The Prph2 N229S (Prph2^{N/N}) and the Prph2 Y141C (Prph2^{Y/Y}) knock-in mouse lines were described in

detail in earlier publications (17,18). PCR genotyping confirmed that these mice do not carry the rd8 mutation. For this study, male and female animals were used and the animals were kept under a 12 h dark/light cycle at ~30 lux. All experimental and animal maintenance procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Oklahoma Health Sciences Center and the University of Houston and followed the guidelines introduced by the Association for Research in Vision and Ophthalmology.

Antibodies

For the experiments described in this paper, the following antibodies were used: RDS-CT (rabbit polyclonal, made in house, 1:1000) (14) for WB, IP and IG; Rom1-2H5 (mouse monoclonal, generated in house, 1:5) (25) for WB, IP and IG; S-Opsin (goat polyclonal, N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500) for immunofluorescence (IF); S-Opsin (a generous gift from Dr Cheryl Craft, Keck School of Medicine, University of Southern California) for IG; Flag-tag DYKDDDDK (cat# 2368, rabbit polyclonal, Cell Signaling Technology, Danvers, MA, USA, 1:1000) for IP and WB; Myc-tag 9B11 (cat# 2276, mouse monoclonal, Cell Signaling Technology, 1:1000) for IP and WB; and beta-actin (HRP-conjugated mAb, Sigma-Aldrich, Saint-Louis, MO, USA, 1:50 000) for WB. Secondary antibodies used were the following: HRP-conjugated mouse and rabbit IgG (goat polyclonal, 1:25 000, KPL, Gaithersburg, MD, USA) and Alexa 488/555-conjugated mouse, rabbit and goat IgG (donkey polyclonal, 1:1000, Life Technologies, Carlsbad, CA, USA).

Ultrathin sections for EM and IG labeling

Whole eyes were fixed in EM fixative (155 mM sodium cacodylate, 2 mM CaCl₂, 2% paraformaldehyde and 2% glutaraldehyde) or in IG fixative (1× PBS, pH 7.4, 2% paraformaldehyde and 0.1% glutaraldehyde) for 2 h as described previously (20,38). The cornea and lens were removed and the eye cups were embedded in Spurr's resin. Ultrathin sections (600–800 Å) were obtained with a glass or diamond knife on a Reichert-Jung Ultracut E microtome and collected on copper (EM) or nickel (IG) 75/300 mesh grids. Sections for EM analysis were stained with 2% (w/v) uranyl acetate and Reynold's lead citrate. Sections for IG were incubated in primary antibodies at the indicated above dilutions and in secondary antibodies (AuroProbe® 10 nm gold-conjugated goat anti-rabbit or anti-mouse IgG) at 1:50.

Electroretinography

Full-field ERG recordings were performed as described previously (19,25). Briefly, mice were dark-adapted overnight and then anesthetized with 85 mg/kg body weight ketamine and 14 mg/kg body weight xylazine (Butler Schein Animal Health, Dublin, OH, USA). Eyes were dilated with a 1% cyclogyl solution (Pharmaceutical Systems, Inc., Tulsa, OK, USA) and platinum wire loop electrode was placed on the cornea on a thin layer of methylcellulose (Pharmaceutical Systems, Inc.). ERG was recorded with a UTAS system (LKC, Gaithersburg, MD, USA) with a single flash stimulus of 157 cd s/m² for the scotopic response before the animals were light-adapted for 5 min and then flashed with a 29.03 cd/m² to bleach the rod response. Photopic (cone) response was measured with 25 flashes of white light at 157 cd s/m². Cone responses were measured against a white background light of 30 cd/m² (25).

RNA preparation and analysis

RNA was extracted from flash frozen retinas as described previously using TRIzol (Life Technologies) (24,27). Reverse transcription was performed using oligo dT primers and superscript III reverse transcriptase (Life Technologies) and the cDNA was used for qRT-PCR with SYBR® Green on a C1000 Thermal Cycler (Biorad Laboratories, Hercules, CA, USA). All the values were normalized to the housekeeping gene HPRT. Primer sequences are listed in [Supplementary Material, Table S1](#).

Protein extraction, IP and velocity sedimentation

Retinas were collected, flash frozen and stored at -80°C. For *in vitro* expression, HEK293 cells grown in DMEM (Life Technologies, Grand Island, NY, USA) with antibiotic/antimycotic solution (Corning Cellgro, Corning, NY, USA) and 5% fetal bovine serum were transfected with equimolar amounts of the following vectors: pKH3-Prph2-myc, pKH3-Prph2-Flag, pKH3-Prph2-C150S-myc and pKH3-Prph2-Y141C-flag. The DNA was added to 500 µl of a 250 mM CaCl₂ solution and an equal amount of 2× BBS (50 mM BES, 280 mM NaCl, 1.4 mM Na₂HPO₄, pH 6.96) was added while vortexing. The transfection mixture was incubated at room temperature for 20 min and then added to the cells. Cells were harvested after 48 h. Retinal proteins were extracted as described previously (17,19). Briefly, Cell lysates or retinas were collected in extraction buffer [PBS, pH 7.0, containing 1% (v/v) Triton X-100, 5 mM EDTA, 5 mg/ml N-ethyl maleimide and protease inhibitors], briefly sonicated, and insoluble material was removed by centrifugation. For quantification of protein levels, 10% SDS-PAGE gels were run with equal amounts of retinal protein (30 µg) and blotted on PVDF membrane using standard protocols. Protein detection was performed with the antibodies mentioned above and membranes were imaged with a ChemiDoc™ MP imaging system (Biorad). Densitometric analysis of the bands was performed on non-saturated bands with Image Lab software v4.1 (Biorad) and band intensities were normalized to beta-actin in the same lane.

For IP, 100 µg aliquot of total retinal proteins was used for WT retinas, and owing to the low amount of Prph2 and Rom1 in *Prph2^{C/C}* retinas, 600 µg aliquot of total retinal proteins was used as input for the mutant genotypes. For expressed protein from HEK293 cells, 200 µg protein aliquot was used as input. Briefly, the protein extract was incubated with anti-RDS-CT, Myc or Flag antibodies and protein A sepharose beads (GE Healthcare, Houston, TX, USA) or anti-Rom1-2H5 antibody conjugated to protein B sepharose beads (GE Healthcare). The bound protein was eluted with 2% SDS. A total of 7.5 µg of input (45 µg in case of *Prph2^{C/C}*), an equivalent amount of flow through, and half of the eluent from the beads were analyzed by SDS-PAGE/western blotting.

Velocity sedimentation was performed as described previously on 5–20% sucrose gradients (28). For WT retinas, 200 µg of retinal extract (obtained as described above) was used for the *Prph2^{C/C}* genotype extracts from two whole retinas were used per sedimentation. Sedimentations were repeated at least five times for each genotype.

Statistical analysis

For statistical analysis and graphical depiction of the data, GraphPad Prism 5 (GraphPad software, Inc., La Jolla, CA, USA) was used. All datasets were analyzed with a one-way ANOVA (after verifying that samples conformed to a Gaussian

distribution) to identify significant differences between groups ($P < 0.05$) with correction for multiple testing. For the identification of specific differences between two groups, Tukey's post-test was used for pairwise comparison. Graphs were drawn in GraphPad Prism and plotted as means plus or minus standard error of the mean (SEM).

Supplementary Material

[Supplementary Material](#) is available at HMG online.

Acknowledgements

The authors would like to thank Ms Jamie Watson and Ms Barb Nagel for technical support. We thank Dr Cheryl Craft for the provision of animal lines and reagents as indicated in the text.

Conflict of Interest statement. None declared.

Funding

National Eye Institute (R01EY010609 to M.I.N.); the Oklahoma Center for the Advancement of Science and Technology (HR14-150 to S.M.C.); and the Presbyterian Health Foundation to S.M.C.

References

- Boon, C.J., den Hollander, A.I., Hoyng, C.B., Cremers, F.P., Klevering, B.J. and Keunen, J.E. (2008) The spectrum of retinal dystrophies caused by mutations in the peripherin/RDS gene. *Prog. Retin. Eye Res.*, **27**, 213–235.
- Stuck, M.W., Conley, S.M. and Naash, M.I. (2016) PRPH2/RDS and ROM-1: historical context, current views and future considerations. *Prog. Retin. Eye Res.*, **52**, 47–63.
- Sanyal, S. and Jansen, H.G. (1981) Absence of receptor outer segments in the retina of rds mutant mice. *Neurosci. Lett.*, **21**, 23–26.
- Jansen, H.G. and Sanyal, S. (1984) Development and degeneration of retina in rds mutant mice: electron microscopy. *J. Comp. Neurol.*, **224**, 71–84.
- Chakraborty, D., Ding, X.Q., Conley, S.M., Fliesler, S.J. and Naash, M.I. (2009) Differential requirements for retinal degeneration slow intermolecular disulfide-linked oligomerization in rods versus cones. *Hum. Mol. Genet.*, **18**, 797–808.
- Clarke, G., Goldberg, A.F., Vidgen, D., Collins, L., Ploder, L., Schwarz, L., Molday, L.L., Rossant, J., Szel, A., Molday, R.S. et al. (2000) Rom-1 is required for rod photoreceptor viability and the regulation of disk morphogenesis. *Nat. Genet.*, **25**, 67–73.
- Wrigley, J.D., Ahmed, T., Nevett, C.L. and Findlay, J.B. (2000) Peripherin/rds influences membrane vesicle morphology. Implications for retinopathies. *J. Biol. Chem.*, **275**, 13191–13194.
- Chakraborty, D., Ding, X.Q., Fliesler, S.J. and Naash, M.I. (2008) Outer segment oligomerization of Rds: evidence from mouse models and subcellular fractionation. *Biochemistry*, **47**, 1144–1156.
- Ding, X.Q., Stricker, H.M. and Naash, M.I. (2005) Role of the second intradiscal loop of peripherin/rds in homo and hetero associations. *Biochemistry*, **44**, 4897–4904.
- Goldberg, A.F. and Molday, R.S. (1996) Subunit composition of the peripherin/rds-rom-1 disk rim complex from rod photoreceptors: hydrodynamic evidence for a tetrameric quaternary structure. *Biochemistry*, **35**, 6144–6149.

11. Hemler, M.E. (2003) Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annu. Rev. Cell Dev. Biol.*, **19**, 397–422.
12. Goldberg, A.F., Loewen, C.J. and Molday, R.S. (1998) Cysteine residues of photoreceptor peripherin/rds: role in subunit assembly and autosomal dominant retinitis pigmentosa. *Biochemistry*, **37**, 680–685.
13. Chakraborty, D., Conley, S.M., Stuck, M.W. and Naash, M.I. (2010) Differences in RDS trafficking, assembly and function in cones versus rods: insights from studies of C150S-RDS. *Hum. Mol. Genet.*, **19**, 4799–4812.
14. Nour, M., Ding, X.Q., Stricker, H., Fliesler, S.J. and Naash, M.I. (2004) Modulating expression of peripherin/rds in transgenic mice: critical levels and the effect of overexpression. *Invest. Ophthalmol. Vis. Sci.*, **45**, 2514–2521.
15. Cheng, T., Peachey, N.S., Li, S., Goto, Y., Cao, Y. and Naash, M.I. (1997) The effect of peripherin/rds haploin sufficiency on rod and cone photoreceptors. *J. Neurosci.*, **17**, 8118–8128.
16. Mears, A.J., Kondo, M., Swain, P.K., Takada, Y., Bush, R.A., Saunders, T.L., Sieving, P.A. and Swaroop, A. (2001) Nrl is required for rod photoreceptor development. *Nat. Genet.*, **29**, 447–452.
17. Stuck, M.W., Conley, S.M. and Naash, M.I. (2014) The Y141C knockin mutation in RDS leads to complex phenotypes in the mouse. *Hum. Mol. Genet.*, **23**, 6260–6274.
18. Stuck, M.W., Conley, S.M. and Naash, M.I. (2015) Retinal degeneration slow (RDS) glycosylation plays a role in cone function and in the regulation of RDS-ROM-1 protein complex formation. *J. Biol. Chem.*, **290**, 27901–27913.
19. Conley, S.M., Stuck, M.W., Watson, J.N. and Naash, M.I. (2017) Rom1 converts Y141C-Prph2-associated pattern dystrophy to retinitis pigmentosa. *Hum. Mol. Genet.*, **26**, 509–518.
20. Chakraborty, D., Conley, S.M., Zulliger, R. and Naash, M.I. (2016) The K153Del PRPH2 mutation differentially impacts photoreceptor structure and function. *Hum. Mol. Genet.*, **25**, 3500–3514.
21. Khattree, N., Ritter, L.M. and Goldberg, A.F. (2013) Membrane curvature generation by a C-terminal amphipathic helix in peripherin-2/rds, a tetraspanin required for photoreceptor sensory cilium morphogenesis. *J. Cell Sci.*, **126**, 4659–4670.
22. Salinas, R.Y., Pearing, J.N., Ding, J.D., Spencer, W.J., Hao, Y. and Arshavsky, V.Y. (2017) Photoreceptor discs form through peripherin-dependent suppression of ciliary ectosome release. *J. Cell Biol.*, **216**, 1489–1499.
23. Milstein, M.L., Kimler, V.A., Ghatak, C., Ladokhin, A.S. and Goldberg, A.F.X. (2017) An inducible amphipathic helix within the intrinsically disordered C terminus can participate in membrane curvature generation by peripherin-2/rds. *J. Biol. Chem.*, **292**, 7850–7865.
24. Cai, X., Nash, Z., Conley, S.M., Fliesler, S.J., Cooper, M.J. and Naash, M.I. (2009) A partial structural and functional rescue of a retinitis pigmentosa model with compacted DNA nanoparticles. *PLoS One*, **4**, e5290.
25. Conley, S.M., Stuck, M.W., Burnett, J.L., Chakraborty, D., Azadi, S., Fliesler, S.J. and Naash, M.I. (2014) Insights into the mechanisms of macular degeneration associated with the R172W mutation in RDS. *Hum. Mol. Genet.*, **23**, 3102–3114.
26. Chakraborty, D., Conley, S.M., DeRamus, M.L., Pittler, S.J. and Naash, M.I. (2015) Varying the GARP2-to-RDS ratio leads to defects in rim formation and rod and cone function. *Invest. Ophthalmol. Vis. Sci.*, **56**, 8187–8198.
27. Farjo, R., Skaggs, J.S., Nagel, B.A., Quiambao, A.B., Nash, Z.A., Fliesler, S.J. and Naash, M.I. (2006) Retention of function without normal disc morphogenesis occurs in cone but not rod photoreceptors. *J. Cell Biol.*, **173**, 59–68.
28. Ding, X.Q., Nour, M., Ritter, L.M., Goldberg, A.F., Fliesler, S.J. and Naash, M.I. (2004) The R172W mutation in peripherin/rds causes a cone-rod dystrophy in transgenic mice. *Hum. Mol. Genet.*, **13**, 2075–2087.
29. Michaelides, M., Holder, G.E., Bradshaw, K., Hunt, D.M. and Moore, A.T. (2005) Cone-rod dystrophy, intrafamilial variability, and incomplete penetrance associated with the R172W mutation in the peripherin/RDS gene. *Ophthalmology*, **112**, 1592–1598.
30. Stricker, H.M., Ding, X.Q., Quiambao, A., Fliesler, S.J. and Naash, M.I. (2005) The Cys214→Ser mutation in peripherin/rds causes a loss-of-function phenotype in transgenic mice. *Biochem. J.*, **388**, 605–613.
31. Kedzierski, W., Lloyd, M., Birch, D.G., Bok, D. and Travis, G.H. (1997) Generation and analysis of transgenic mice expressing P216L-substituted rds/peripherin in rod photoreceptors. *Invest. Ophthalmol. Vis. Sci.*, **38**, 498–509.
32. Conley, S., Nour, M., Fliesler, S.J. and Naash, M.I. (2007) Late-onset cone photoreceptor degeneration induced by R172W mutation in Rds and partial rescue by gene supplementation. *Invest. Ophthalmol. Vis. Sci.*, **48**, 5397–5407.
33. Nemet, I., Tian, G. and Imanishi, Y. (2014) Organization of cGMP sensing structures on the rod photoreceptor outer segment plasma membrane. *Channels (Austin)*, **8**, 528–535.
34. Chen, J., Yoshida, T. and Bitensky, M.W. (2008) Light-induced translocation of cyclic-GMP phosphodiesterase on rod disc membranes in rat retina. *Mol. Vis.*, **14**, 2509–2517.
35. Elliott, M.H., Nash, Z.A., Takemori, N., Fliesler, S.J., McClellan, M.E. and Naash, M.I. (2007) Differential distribution of proteins and lipids in detergent-resistant and detergent-soluble domains in rod outer segment plasma membranes and disks. *J. Neurochem.*, **104**, 336–352.
36. Nair, K.S., Balasubramanian, N. and Slepak, V.Z. (2002) Signal-dependent translocation of transducin, RGS9-1-Gbeta5L complex, and arrestin to detergent-resistant membrane rafts in photoreceptors. *Curr. Biol.*, **12**, 421–425.
37. Liu, H., Seno, K. and Hayashi, F. (2003) Active transducin alpha subunit carries PDE6 to detergent-resistant membranes in rod photoreceptor outer segments. *Biochem. Biophys. Res. Commun.*, **303**, 19–23.
38. Chakraborty, D., Conley, S.M., Fliesler, S.J. and Naash, M.I. (2010) The function of oligomerization-incompetent RDS in rods. *Adv. Exp. Med. Biol.*, **664**, 39–46.