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PRPH2/RDS and ROM-1: historical context, current views and future considerations

Michael W. Stuck[a], **Shannon M. Conley**[a], and **Muna I. Naash**[b,*]

[a]Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

[b]Department of Biomedical Engineering, University of Houston, Houston TX, 77204-5060, USA

Abstract

Peripherin2 (PRPH2), also known as RDS (retinal degeneration slow) is a photoreceptor specific glycoprotein which is essential for normal photoreceptor health and vision. PRPH2/RDS is necessary for the proper formation of both rod and cone photoreceptor outer segments, the organelle specialized for visual transduction. When PRPH2/RDS is defective or absent, outer segments become disorganized or fail to form entirely and the photoreceptors subsequently degenerate. Multiple *PRPH2/RDS* disease-causing mutations have been found in humans, and they are associated with various blinding diseases of the retina such as macular degeneration and retinitis pigmentosa, the vast majority of which are inherited dominantly, though recessive LCA and digenic RP have also been associated with RDS mutations. Since its initial discovery, the scientific community has dedicated a considerable amount of effort to understanding the molecular function and disease mechanisms of PRPH2/RDS. This work has led to an understanding of how the PRPH2/RDS molecule assembles into complexes and functions as a necessary part of the machinery that forms new outer segment discs, as well as leading to fundamental discoveries about the mechanisms that underlie OS biogenesis. Here we discuss PRPH2/RDS-associated research and how experimental results have driven the understanding of the PRPH2/RDS protein and its role in human disease.

Keywords

RDS; retinal degeneration; ROM-1; tetraspanin; photoreceptors PRPH2

1. Introduction

Peripherin 2 (PRPH2, also known as retinal degeneration slow or RDS) is a photoreceptor-specific transmembrane glycoprotein that is necessary for the proper formation of both rod

*To whom correspondence should be addressed: Muna Naash, Ph.D., John S. Dunn Professor of Biomedical Engineering, Department of Biomedical Engineering, University of Houston, 3517 Cullen Blvd. Room 2011, Houston, TX 77204-5060, BME Number: 832-842-8813, Cell Number: 405-821-6167, mnaash@central.uh.edu.

and cone photoreceptor outer segments (OS) (Cheng et al., 1997b; Connell et al., 1991; Demant et al., 1979; Hawkins et al., 1985; Molday et al., 1987; Sanyal et al., 1980; Sanyal and Jansen, 1981). Beyond its structural role in the formation of the OS, the human *PRPH2/RDS* gene has been associated with 151 individual disease-causing mutations (according to the Human Gene Mutation Database http://www.hgmd.cf.ac.uk/ac/gene.php?gene=PRPH2) with highly variable patient diagnoses ranging from retinitis pigmentosa to macular degeneration with additional disparity in severity and time of onset (Boon et al., 2008). These phenotypes are associated with degeneration of the neural retina as well as, in some cases, atrophy of the retinal pigment epithelium (RPE) and defects in the choroid. Currently no known treatment has been developed for *PRPH2/RDS*-associated disease. Additionally, while much work has been dedicated to understanding the role of PRPH2/RDS in OS formation; the details of this process remain largely a mystery, although exciting new work is leading to proposed mechanisms for PRPH2/RDS function. Given the central role of photoreceptor OSs in vision, the study of OS biogenesis in general and PRPH2/RDS specifically remains an active and controversial field of research with broad implications for the treatment of human blinding diseases. The current review will focus on a comprehensive analysis of PRPH2/RDS research to aid those interested in furthering their understanding of this critical photoreceptor protein.

In order to appreciate PRPH2/RDS and its importance, we must first establish the context within which PRPH2/RDS plays a significant role. The nomenclature in this field has been confusing, and is further discussed below in its historical context. Though the official gene name is *PRPH2,* much of the early work and work done using mouse models, has used the designation *Rds*. We will use the two forms of nomenclature judiciously, in an attempt to both minimize confusion for the reader and preserve the connection to the literature in the field. The first observation related to the *PRPH2/RDS* gene was that it was important for the survival of rod and cone photoreceptor cells of the neural retina in mice (van Nie et al., 1978). This critical importance of *PRPH2/RDS* would eventually be expanded to include the photoreceptors of all vertebrates (Conley et al., 2012b). Photoreceptors form the outermost neuronal layer in the retina and are the light sensitive cells which initiate phototransduction. The high metabolic, nutritional and environmental support that these cells require is maintained through a combination of the Müller glia and the adjacent RPE (Hoon et al., 2014; Strauss, 2005). Photoreceptors extend from the outer plexiform layer to the RPE and are polarized neuronal cells (Hoon et al., 2014). Their synapses are in the outer plexiform layer, their nuclei are in the outer nuclear layer, their cell bodies with most of the mitochondria, endosomes, and protein synthesis machinery are in the inner segment layer, and finally their sensory OSs extend toward the RPE from the inner segments (Hoon et al., 2014; LaVail, 1983; Mustafi et al., 2009; Steinberg et al., 1980; Sung and Chuang, 2010). The photoreceptor OSs are modified primary cilia which have evolved to detect incoming light and initiate a signal transduction cascade which is the first domino in the long chain of signaling and cellular communications necessary for vision (Arshavsky and Wensel, 2013; Gilliam et al., 2012; Hoon et al., 2014; Hubbell et al., 2003; Koch and Dell'Orco, 2015; Li et al., 2004; Palczewski et al., 2000; Papermaster and Dreyer, 1974; Sung and Chuang, 2010; Zhou et al., 2012).

Rod OSs are long and cylindrical consisting of approximately a thousand disc shaped membrane structures oriented perpendicular to the incoming light and covered with an outer sheath of plasma membrane (Carter-Dawson and LaVail, 1979a; Cohen, 1960; Nickell et al., 2007; Sung and Chuang, 2010). Cone OSs are generally shorter and more conical in shape than rod OS and their discs are referred to as lamellae because they are at least partially fused with the plasma membrane sheath and thus the intradiscal space is continuous with the extracellular environment (Carter-Dawson and LaVail, 1979a, b; Eckmiller, 1987; Mustafi et al., 2009). Because OSs go through a dynamic process of turnover in which the top tenth of the OS is phagocytosed by the RPE cells each day, biogenesis of new discs at the base of the OS is a constant process which requires an incredible level of coordination between various cellular components (Anderson et al., 1978; Insinna and Besharse, 2008; LaVail, 1976; Steinberg et al., 1980; Young, 1967).

The OS discs are packed with the photo-sensitive opsins (rhodopsin in rods and S-, M-, or L-wavelength opsins in cones) and other proteins that participate in the initial steps of phototransduction (Hubbell et al., 2003; Mustafi et al., 2009; Sakmar et al., 2002; Sung and Chuang, 2010), while the adjacent plasma membrane contains, among other things, cyclic nucleotide gated channels which convert the chemical phototransduction signal into an electrical one. However, a third membrane domain exists between these two regions. In both rods and cones the flattened surface of the disc is circumscribed by a rim region of membrane that makes a hairpin-like structure (Carter-Dawson and LaVail, 1979a; Nickell et al., 2007; Steinberg et al., 1980; Sung and Chuang, 2010), and this rim is the region where PRPH2/RDS plays its part. Though not a phototransduction protein, PRPH2/RDS is central to the entire process of vision because without it OSs completely fail to form (**Fig. 1**). Extensive research (further discussed below) has suggested that PRPH2/RDS functions differently in rods vs. cones, but that in both cell types its function depends on the formation of a wide variety of precisely tuned types of homo- and hetero-oligomers. Abnormalities in these oligomers and the difference in function in PRPH2/RDS between the two cell types is thought to underlie the vast phenotypic variation in patients with PRPH2/RDS-associated disease, and an understanding of the biochemical and cell biological function of PRPH2/RDS is critical for future development of effective therapies for patients. Here we will trace how the current understanding of PRPH2/RDS has developed over time and point the way towards exciting future developments in the field.

2. The discovery of *PRPH2/RDS*

2.1 *Rds*: phenotype to gene

Mouse inbred strains have provided some of the foundational understanding in almost every field of biomedical research and vision research is no exception (Chang et al., 2002). In 1977 it was discovered that the 020/A inbred mouse strain slowly lost the outer layers of the retina, with thinning becoming visually apparent around 5 weeks of age and only a single row of cone photoreceptors and no rod cells remaining by 7-10 months of age (van Nie et al., 1978). At this time, the new mutation represented only the second pure retinal phenotype (thus it is sometimes referred to as the *rd2* model) and only the fourth disorder which resulted in a retinal degeneration ever discovered (Chang et al., 2002). Linkage analysis

confirmed that the new disorder was the result of a hitherto unknown gene on mouse chromosome 17 and it was dubbed *rds* for retinal degeneration slow, (Demant et al., 1979; van Nie et al., 1978). The name was chosen because the observed degeneration in the 020/A mouse was significantly slower than the already known retinal degeneration (*rd1)*, in which rapid degeneration is caused by a mutation in the β subunit of cGMP-phosphodiesterase gene (Chang et al., 2002; Keeler, 1966). Despite the fact that it would be another 12 years before the gene (*Rds*) would be cloned and the protein identified (also termed RDS), characterization of the *rds* models in the intervening years provided a wealth of information. The mouse *rds* allele being studied in this early work would not be conclusively shown to be a null allele until the gene was cloned (discussed later), but for the sake of continuity, we will refer to the *rds/rds* line as the $rds^{-/-}$ and the associated heterozygous line as $rds^{+/-}$.

In addition to photoreceptor degeneration, the most striking structural phenotype in the $rds^{-/-}$ is that it does not form OSs (**Fig. 1**). However, it retains the connecting cilia, and because the retinas were able to convert cGMP to GMP in response to light (a critical step in phototransduction) it is thought that the photoreceptors retain some minimal signal transduction in response to light (Cohen, 1983; Sanyal and Jansen, 1981). In line with these results, when the $rds^{-/-}$ animals were tested using scotopic full-field ERG, the a-waves (generated by photoreceptors) were below the threshold of detection while the b-wave (generated by inner retinal responses) was greatly reduced and barely detectable (Reuter and Sanyal, 1984). In lieu of OSs, the subretinal space between the photoreceptors and the RPE was filled with abnormal extracellular vesicles which first appeared at the same time that OSs normally appear in the wild-type (WT) mouse (Jansen and Sanyal, 1984). Later analysis revealed that these vesicles stained positive for both arrestin and rhodopsin suggesting that they represented failed attempts to develop OS and/or fragmented and degenerated OSs (Agarwal et al., 1990; Jansen et al., 1990; Jansen et al., 1987). Consistent with this, over time the vesicles disappeared, mirroring both a decrease in rhodopsin expression and overall retinal degeneration (Nir et al., 1990). Detailed histological analysis of the $rds^{-/-}$ retina revealed that the photoreceptors loss began in the peripheral retina and proceeded along a peripheral to central gradient, resulting in complete loss of all photoreceptors in the periphery by ~9 months and in the central retina by ~12 months (Sanyal et al., 1980). As would be expected, in the $rds^{-/-}$ mouse the small detectable ERG b-wave response was slowly lost over the course of the degeneration and was completely lost between 9 and 12 months of age (Reuter and Sanyal, 1984). Interestingly it was also apparent that rod photoreceptors were lost more rapidly than cones resulting in a significant change in the rod/cone ratio over time (Sanyal et al., 1980). In the albino background, exposure to light exacerbated the loss of cells in the $rds^{-/-}$ and $rds^{+/-}$ mouse lines in the central retina but in contrast to some other retinal degenerative phenotypes, dark rearing was not protective (Sanyal and Hawkins, 1986).

Initially *Rds* was incorrectly thought to having a recessive inheritance pattern because the degeneration in the $rds^{+/-}$ (which is even slower than in the $rds^{-/-}$) was simply missed. However, it was shown that $rds^{+/-}$ mice displayed abnormally organized and shortened, whorl-like OSs (**Fig. 1**) coupled with very slow degeneration resulting in approximately 50% of the photoreceptors lost at 18 months of age (Hawkins et al., 1985). As in the $rds^{-/-}$,

the *rds*$^{+/-}$ mice were characterized by a slow loss of rods followed by a loss of cones (Hawkins et al., 1985). Careful examination of the rate of cone loss in the *rds*$^{+/-}$ suggested that the red/green cones degenerate at a greater rate than blue cones when RDS levels are low, however the mechanism for this remains a mystery (Cheng et al., 1997b). The phenotypes observed in the *rds*$^{+/-}$ when compared to the *rds*$^{-/-}$ mice were consistent with a simple dose-dependent, haploinsufficiency paradigm.

Early researchers were unsure whether *rds* was a photoreceptor-specific gene, and as a result work was done to characterize the phenotype in the RPE and inner retina as well. Much of this work was negative, for example no changes were observed in differentiation of the retinal cells or in the synaptic connections of the retina (Hawkins et al., 1985; Jansen and Sanyal, 1984; Sanyal et al., 1980). In contrast, though the RPE does not express RDS, significant dysregulation of the timing and processing of the RPE phagosomes which break down OS components were observed in the *rds*$^{+/-}$ mouse (Hawkins et al., 1985). In a normal retina, OSs are renewed from the base of the OS while older OS components at the tip are shed and consumed by RPE cells in a regulated processes. This is characterized by the uptake of OS tips of similar size in a burst immediately following light onset and a sharp decline in uptake throughout the rest of the day (LaVail, 1976; Young, 1967). In the *rds*$^{+/-}$ retina, phagosomes within the RPE are much larger and the total number of phagosomes is lower at light onset, with a peak near the end of the day (Hawkins et al., 1985). The size and shape of these phagosomes appeared to be very similar to the abnormal whorled OS structures observed in the *rds*$^{+/-}$ OS layer (Hawkins et al., 1985). It was proposed that OS structural defects resulted in the RPE consuming larger parts of the OS than normal which took longer to degrade and that this, combined with uptake throughout the day, led to abnormal build-up of phagosomes (Hawkins et al., 1985; Sanyal and Hawkins, 1988). It has never been clearly determined how much of this phenotype was due to structural changes in *rds*$^{+/-}$ OSs as opposed to defective regulation of the signaling supporting phagocytosis, however work that investigated changes in phagosome numbers in response to light suggested that both factors likely play a role (Sanyal and Hawkins, 1988, 1989). Recent work has suggested that some *PRPH2/RDS* mutations lead to RPE stress and has raised the possibility that abnormal phagocytosis of *PRPH2/RDS* mutations could play an important role in human disease (Conley et al., 2014b).

The early work on RDS, which focused on the *rds*$^{-/-}$ mouse, is conveniently wrapped up by discussing the cloning of the murine *Rds* gene (Travis et al., 1989). Based on the hypothesis that the *Rds* gene was a photoreceptor-specific transcript, work was done to identify specific mRNAs which were present in the WT retina but absent from the highly degenerated *rd/rd* retina (now known as *rd1*) which lacks all photoreceptor cells (Travis et al., 1989). From this pool several photoreceptor-specific clones were identified, and subsequently, one was identified whose transcript size was significantly altered on northern blots from *rds*$^{-/-}$ retinas. In the WT retina, this clone had two transcripts (~1.6 and 2.7 kb) due to alternative utilization of poly-A signal sequences, but a single much larger (~12 kb) transcript was detected in the *rds*$^{-/-}$ retina, suggesting that the *rds* mutation created a null allele (Cheng et al., 1997a; Travis et al., 1989). The insertion would later be identified as a t haplotype specific element and be confirmed to result in an altered gene which produced no protein product (Ma et al., 1995). The *Rds* gene has a conserved organization which in mouse

consists of three exons of 581, 247, and 213 bp, respectively, and two introns of approximately 8.6 and 3.7 kb (Cheng et al., 1997a).

Analysis of the transcript coding sequence allowed for examination of the protein for the first time. The *Rds* gene encoded a 346 amino acid (AA) long protein with possible transmembrane domains and multiple cysteine residues that could potentially be involved in inter or intra molecular disulfide bonds (Travis et al., 1989). Further work would map the human *PRPH2/RDS* gene to chromosome 6 and showed that it encoded a four pass transmembrane glycoprotein which formed dimers on non-reducing western blots (Travis et al., 1991a; Travis et al., 1991b). Formal proof that the gene underlying the *rds* phenotype had been identified came when the phenotype of the $rds^{-/-}$ and $rds^{+/-}$ mutant mouse was rescued by transgenically expressing WT *Rds* cDNA (Nour et al., 2004; Travis et al., 1992).

2.2 Peripherin 2: protein to gene

Independent of the research done on the $rds^{-/-}$ mouse, other work was being done to identify important constituents of the mammalian rod OS through biochemical purification of OS proteins and the generation of monoclonal antibodies. By injecting mice with purified bovine rod OSs, monoclonal antibodies were generated against individual components of the OS in a non-biased manner and subsequent screening of these antibodies allowed for the characterization of the proteins in question (Molday et al., 1987). Two of these antibodies (3B6 and 2B6) recognized a transmembrane protein that ran on SDS-PAGE at an apparent molecular weight of 35 kD under reducing conditions and as a predicted dimer at 67-69 kD under non-reducing conditions (Molday et al., 1987). Separation of the discs from the plasma membrane combined with immunogold electron microscopy (EM) showed that this protein localized to the rims and incisures of the flattened OS discs (Molday et al., 1987). Due to its localization, the new protein was dubbed peripherin 2 (PRPH2, since there was already an entirely unrelated protein called peripherin).

To clone the *PRPH2* cDNA, monoclonal antibodies 2B6 and 3B6 were mixed and used to screen a λgt11 bovine cDNA expression library for the presence of the protein of interest (Connell and Molday, 1990). Once the cDNA was identified, the hydrophobicity profile of the primary amino acid sequence was used to generate a hypothetical molecular model for the protein which included both N and C termini in the cytoplasm, four transmembrane domains, and two asymmetric extra cytosolic domains dubbed intradiscal (D) loop 1 and 2 (Connell and Molday, 1990) (**Fig. 2B**). Furthermore, PRPH2 was glycosylated and this glycosylation was sensitive to the glycosidase endoH, suggesting a high mannose sugar modification (Connell and Molday, 1990). Interestingly, the protein encoded by the cDNA was predicted to be 39kD in size, but would run at 33kD when rhodopsin was present and would run at 35 when rhodopsin was digested or immune-depleted (Connell and Molday, 1990).

With the bovine *PRPH2* cDNA sequence and primary amino acid sequence in hand it was realized that PRPH2 was the bovine homologue of the murine Rds protein (Connell et al., 1991). Importantly, this meant that the initial characterization of the gene and protein structure of PRPH2/RDS was independently verified (Travis et al., 1991a; Travis et al., 1991b). The concurrent evolution of this field has led to the sometimes confusing

nomenclature associated with the PRPH2/RDS. As mentioned above, the official gene name is now *PRPH2*, but due to decades of pre-existing work using the *rds* mouse model, many researchers continue to use this designation, particularly when referring to murine work.

3. Cellular and molecular characteristics of PRPH2/RDS

3.1 PRPH2/RDS trafficking and subcellular localization

Following this initial phase of basic characterization, researchers began to focus on the role of PRPH2/RDS in OS biogenesis. Very early work on the formation of rod OSs had shown that at the base of the OS new discs grow through an evagination process of growth outward from the connecting cilia axoneme, and that during this process the side of the disc abutting the connecting cilia was “closed” by the rim domain while the growing side of the disc was “open” as the newly formed disc extended toward its full size (Steinberg et al., 1980). Careful EM/immunogold work showed that in fully formed rod OS discs, PRPH2/RDS localizes throughout the rim (**Fig. 3A**), circumscribing the entire disc. However, within the nascent incompletely formed discs, PRPH2/RDS was enriched on the side of the newly forming discs that abutted the axoneme, i.e. the “closed” side and was completely absent from the growing edges of the “open” disc, (Arikawa et al., 1992) (**Fig. 3A**). While no direct evidence exists, this localization led to the hypothesis that PRPH2/RDS could regulate interactions between the “closed” disc rim and the axoneme during OS disc biogenesis (Arikawa et al., 1992). Very recent work has expanded upon and confirmed these results (Ding et al., 2015). While confirming the primary localization of PRPH2/RDS to the axonemal side of the growing discs, the new work used careful quantification of immunogold particles to show that the concentration of PRPH2/RDS in the axonemal side of the newly growing discs was 1.7 times higher than that observed in the mature disc rims (Ding et al., 2015). This suggests that during biogenesis of discs, PRPH2/RDS is initially concentrated at the axoneme and subsequently spreads throughout the rim (Ding et al., 2015).

In cones, even mature discs remain at least partially “open” and contiguous with the plasma membrane (**Fig. 3B**), thus to a certain extent they resemble newly forming rod discs. In the large cones of the *Xenopus laevis* retina, PRPH2/RDS localization was restricted to the region of the OS adjacent to the axoneme and surrounded by plamsa membrane (i.e. “closed”) and in opposition to another rim protein called prominin-1 which localized to the remainder of the “open” edge of the rim (Han et al., 2012), (**Fig. 3B**). This is consistent with immunogold labeling of transverse sections of ground squirrel cone OSs, showing that PRPH2/RDS is not necessarily evenly distributed throughout the rim (Arikawa et al., 1992). The idea that in cones PRPH2/RDS circumscribes only that portion of the lamellae which is “closed” is supported by the observation that the direction of membrane curvature of the open rim edges is opposite that of the closed edges and would thus not accommodate PRPH2/RDS (**Fig. 3C**). However, verification of this idea (that PRPH2/RDS in cones is restricted to the closed side of the rim) in mammals has been difficult due to the small number of cones in the WT mouse and the fact that both the portion of each disc that is “open” vs. “closed” and the number of discs that are at least partially contiguous with the plasma membrane vs. completely enclosed varies widely from species to species. In frogs

and other amphibians, the majority of the cone rim is open (Eckmiller, 1987), while in mice and other mammals most of the disc is enclosed by plasma membrane and many discs are completely enclosed (as in rods) (Anderson et al., 1978; Arikawa et al., 1992; Young, 1971).

Work using degenerative retinas showed mislocalization of rhodopsin and PRPH2/RDS occurred independently and suggested that these two major OS constituents were transported to the OS through partially different mechanisms (Fariss et al., 1997). This observation led to attempts to identify the sequence motifs in PRPH2/RDS responsible for its specific OS targeting. Chimeric GFP fusion proteins which contained various segments of the *Xenopus laevis* PRPH2/RDS homologue, xrds38, were expressed transgenically in frogs, and subcellular localization was tracked using fluorescent microscopy (Tam et al., 2004). PRPH2/RDS C-terminal amino acid residues 317-336 were shown to localize GFP to the disc while residues 307-346 localized GFP specifically to the rim and incisures regions of the discs (Tam et al., 2004) (**Fig. 2B**). Very recent work utilizing similar experiments showed that a single valine residue at position 332 within this domain is necessary for the proper targeting of PRPH2/RDS to the OS in both frogs and mice (Salinas et al., 2013) (**Fig. 2A**). Interestingly, expression of the PRPH2/RDS C-Terminal/GFP fusion proteins in the frog retina led to the abnormalities in the alignment of disc incisures suggesting that these chimeric proteins competitively disrupted an interaction important to disc alignment (Tam et al., 2004). The PRPH2/RDS C-terminal sequence which directed PRPH2/RDS to the rims and OSs was not related to other known OS targeting motifs such as the VxPx motif known to target rhodopsin (Wang and Deretic, 2014).

Further support for the idea that PRPH2/RDS traffics to the OS using a specialized transportation mechanism different from other OS components such as rhodopsin came from recent work showing that a large portion of PRPH2/RDS traffics to the OS through a non-conventional secretory pathway which bypasses the trans-Golgi (Tian et al., 2014). Hints that this might be the case had been observed many years earlier when it was discovered that the PRPH2/RDS N-glycan was sensitive to digestion by EndoH, a phenomenon usually associated with failure of a protein to be exposed to the enzyme Golgi mannosidase II in the medial to trans-Golgi (Connell and Molday, 1990; Velasco et al., 1993). Using a variety of PRPH2/RDS mutants, pharmacological agents which block protein transport at specific well characterized steps, and high quality fluorescence microscopy in a tissue culture system it was found that PRPH2/RDS could transport to the cilia when trans-Golgi exit was blocked, but does require COPII-mediated exit from the ER. Consistent with a key role for the PRPH2/RDS C-terminal in trafficking, PRPH2/RDS was retained in the cis-Golgi when the C-terminal targeting sequence was eliminated (Tian et al., 2014). Various unconventional secretory pathways have been described (Nickel, 2010; Nickel and Rabouille, 2009), and it is not yet clear which of these is utilized by PRPH2/RDS, however it has been shown that unconventional trafficking of PRPH2/RDS does not require GRASP55 (a known non-conventional secretory protein) (Gee et al., 2011; Tian et al., 2014), and our recent work has suggested that Syntaxin 3 may be involved (Zulliger et al., 2015). We also find that the distribution of conventionally vs. unconventionally secreted PRPH2/RDS varies with the type of PRPH2/RDS oligomerization (which will be further discussed below), suggesting there may be some tie between complex assembly and trafficking (Zulliger et al., 2015).

This observation is supported by other work showing that mutations which interrupt complex formation can cause cone-specific mislocalization of PRPH2/RDS to the inner segment and cell body suggesting differences in how PRPH2/RDS is trafficked between rods and cones (Chakraborty et al., 2009). Taken together these data suggest that PRPH2/RDS likely traffics from the ER to the cis-Golgi but then bypasses other components of the traditional secretory pathway (such as the trans-Golgi) to transport to the OS by incompletely understood mechanisms.

3.2 PRPH2/RDS oligomers and ROM-1

One of the most important discoveries in relation to PRPH2/RDS was the identification of ROM-1 (Bascom et al., 1992; Moritz and Molday, 1996). ROM-1 is a ~37 kD photoreceptor specific integral membrane protein with ~35% sequence identity to PRPH2/RDS. Like PRPH2/RDS, ROM-1 is localized to the rim region of both rod and cone OSs (despite the name) (Bascom et al., 1992; Moritz and Molday, 1996). PRPH2/RDS and ROM-1 have a highly conserved secondary and tertiary structure that includes four transmembrane domains and two non-symmetrical intradiscal loops (Bascom et al., 1992). In the D2 loop, the two proteins share a 15-16 residue highly conserved cysteine and proline rich region which is thought to promote a conserved 3D structure in the D2 loop, although ROM-1 lacks the N-glycosylation site and an important glutamic acid in the fourth transmembrane domain found in PRPH2/RDS (Bascom et al., 1992; Goldberg et al., 2007; Moritz and Molday, 1996). Both PRPH2/RDS and ROM-1 are members of the tetraspanin super family of proteins which are defined by this conserved tertiary architecture, although PRPH2/RDS and ROM-1 are both functionally distinct from other tetraspanin proteins (Conley et al., 2012b).

Importantly, PRPH2/RDS and ROM-1 form strong interactions *in vivo* (Bascom et al., 1992). Using reciprocal co-immunoprecipitation (IP) from bovine retinal extracts it was shown that when either protein was pulled down the other would come with it (Bascom et al., 1992). Similar to PRPH2/RDS, when ROM-1 was run on a non-reducing SDS-PAGE/western blot it ran as both a monomer and a disulfide linked dimer (Bascom et al., 1992). Early work showing that IP of either PRPH2/RDS or ROM-1 under denaturing conditions failed to pull down the other suggested that the interactions between the two proteins were non-covalent and that the disulfide-linked dimers present under denaturing but non-reducing conditions were RDS/RDS and ROM-1/ROM-1 homodimers rather than RDS/ROM-1 covalent heterodimers (Goldberg and Molday, 1996b). The non-covalent RDS/ROM-1 interactions were disrupted by strong denaturants or chaotropic salts but not high concentrations of NaCl, suggesting that the interaction is stabilized primarily by masking of hydrophobic domains. Combined these observations indicate that PRPH2/RDS complex assembly relied on both covalent (i.e. disulfide-linked) and non-covalent interactions.

After identifying these RDS/RDS and RDS/ROM-1 interactions it became important to identify the domain of PRPH2/RDS that regulates complex assembly. Likewise, though interactions between RDS/ROM-1 were non-covalent, the presence of disulfide-linked dimers under denaturing but non-reducing conditions suggested cysteines would be critical for complex formation. Using site directed mutagenesis combined with transient transfection of COS-1 cells, each of the 13 cysteine residues in bovine PRPH2/RDS was mutated to

determine its functional role in PRPH2/RDS complex formation (Goldberg et al., 1998). When the six non-conserved cysteine residues were mutated to serine no measurable effect was observed in terms of the PRPH2/RDS' behavior by western blot (Goldberg et al., 1998). However, mutation of any of the six highly conserved cysteine residues, all localized to the D2 loop of PRPH2/RDS, (C165, C166, C213, C214, C222, C250) (**Fig. 2B**), led to the formation of aggregates suggesting that these cysteine were important for the proper folding of the D2 loop (Goldberg et al., 1998). In contrast, mutation of the last conserved cysteine, also localized to the D2 loop (C150), did not cause aggregation of the protein but abolished its ability to form dimers (Goldberg et al., 1998) suggesting it was essential for intermolecular bonding between PRPH2/RDS molecules and suggesting that the D2 loop was a potential region of interaction for other types of bonds.

The D2 loop of PRPH2/RDS was a good candidate for mediating non-covalent RDS/RDS and RDS/ROM-1 complex formation. The first direct evidence to support this idea came from analysis of a transgenic mouse expressing an RDS/ROM-1 chimeric protein in which the D2 loop of PRPH2/RDS replaced the D2 loop of ROM-1 (Kedzierski et al., 1999b). The interactions between native PRPH2/RDS and the ROM-1/RDSD2 chimera were much more stable than those between native PRPH2/RDS and native ROM-1 (Kedzierski et al., 1999b; Zulliger et al., 2015). Furthermore, antibodies which recognized an epitope on the PRPH2/RDS D2 loop could only bind PRPH2/RDS once PRPH2/RDS and ROM-1 were disassociated (Kedzierski et al., 1999b). Later our group confirmed that RDS/RDS interactions were more stable than RDS/ROM-1 interactions and used GST fusion proteins and peptide competition assays to map the minimal domain of PRPH2/RDS needed for homomeric interactions to D2 loop residues 165-182, with maximal RDS/ROM-1 interactions requiring a slightly larger domain (residues 140-182) (Ding et al., 2005) (**Fig. 2B**).

Heterologous expression of PRPH2/RDS and ROM-1 would yield further insights into the nature of the PRPH2/RDS complexes. When co-expressed in the same cell systems, PRPH2/RDS and ROM-1 recapitulated the interactions which had been observed *in vivo* (Goldberg et al., 1995). Interestingly this interaction did not occur when PRPH2/RDS and ROM-1 were expressed separately and then mixed post isolation (Goldberg et al., 1995). The size of the PRPH2/RDS and ROM-1 homomeric complexes as well as the RDS/ROM-1 heteromeric complexes when run on a sucrose gradient under reducing conditions suggested that each of these complexes existed as a tetramer even in the absence of disulfide bonds (Goldberg et al., 1995). Later, a combination of gel exclusion chromatography and velocity sedimentation was used to show that RDS/ROM-1 complexes isolated from OSs under reducing conditions did indeed exist as tetramers and made up ~ 4% of the bovine rod OS protein constituents (Goldberg and Molday, 1996b). The key finding of this early work on PRPH2/RDS oligomerization was that PRPH2/RDS and ROM-1 formed stable homo or hetero tetramers which did not require inter-molecular disulfide bonds (Goldberg and Molday, 1996a, b; Goldberg et al., 1995).

Shared antibody epitopes between the WT PRPH2/RDS and the ROM-1/RDSD2 chimera, and between WT ROM-1 and the ROM-1/RDSD2 chimera also allowed for an estimate of the relative ratio of PRPH2/RDS to ROM-1, a question of relevance to the functional

assembly of the two proteins. Experiments using the ROM-1/RDSD2 protein as a normalizing factor suggested that PRPH2/RDS was 2.5 times more plentiful than ROM-1 in mice (Kedzierski et al., 1999b). This established that the OS must contain a significant number of PRPH2/RDS homo-tetramers which do not contain ROM-1. However, IP experiments targeting ROM-1 were able to deplete both PRPH2/RDS and ROM-1 despite the more than two fold greater number of PRPH2/RDS molecules and presence of PRPH2/RDS homo-tetramers (Kedzierski et al., 1999b). That fact that an IP targeting ROM-1 could pull down PRPH2/RDS in PRPH2/RDS homotetramers suggests that PRPH2/RDS homo-tetramers interact *in vivo* with RDS/ROM-1 heterotetramers to form higher-order complexes (Kedzierski et al., 1999b).

Work going forward directly tested the possibility that PRPH2/RDS and ROM-1 form larger oligomers by using a two-dimensional approach (Loewen and Molday, 2000). In the first dimension, proteins isolated from OS are sorted by density using a 5-20% sucrose gradient. In the second dimension individual gradient fractions are resolved on either reducing or non-reducing western blots to test for the presence or absence of PRPH2/RDS and ROM-1 monomers and dimers (Loewen and Molday, 2000). This enables separation of complexes, with larger complexes in heavier gradient fractions, and by type of bonds mediating the interactions, with non-covalently linked complexes running as monomers by SDS-PAGE and covalently linked complexes running as dimers. Using this method, three main types of PRPH2/RDS complexes were identified. The smallest complexes were tetramers which were largely free of intermolecular disulfide linkages. Intermediate complexes consisted of a mix of disulfide- and non-covalently linked PRPH2/RDS are predicted to be built from at least two tetramers. Finally, higher-order complexes were found in the heaviest gradient fractions and ran almost exclusively as dimers on gels (Loewen and Molday, 2000) (**Fig. 4**). Interestingly, ROM-1 did not exhibit this same pattern of complexes; it was found in tetrameric and intermediate gradient fractions but not the heaviest fractions where PRPH2/RDS higher-order oligomers were found. Tetramers have been shown by several methods to be the core PRPH2/RDS complex (Goldberg and Molday, 1996b; Kevany et al., 2013), and to further evaluate their assembly into larger complexes, OSs were incubated with a crosslinking agent either before or after treatment with the reducing agent DTT and RDS/ROM-1 complexes were then assessed on western blots (Loewen and Molday, 2000). Higher-order and intermediate complexes were only observed if the crosslinking occurred before treatment with reducing agent. This established disulfide bonds rather than non-covalent bonds link up neighboring tetramers to form the higher-order oligomers (Loewen and Molday, 2000) (**Fig. 4**). Support for the idea that the disulfide linked complexes are assembled by covalent linkages between tetramers comes from multiple studies showing elimination of C150 (by making a C150S mutation) results in the formation of only tetramers, no larger sized complexes (Chakraborty et al., 2009; Loewen and Molday, 2000). Together these data have led to the development of a model wherein PRPH2/RDS forms higher-order homo-oligomers, while PRPH2/RDS and ROM-1 form intermediate and tetramer-sized heteromers held together by a mixture of non-covalent and covalent interactions (**Fig. 4**).

However, this model raises several questions. First, sucrose gradient fractionation alone cannot differentiate between PRPH2/RDS or ROM-1 homotetramers and RDS/ROM-1 heterotetramers. *In vitro* both PRPH2/RDS alone and ROM-1 alone can form non-covalently linked tetramers (Loewen and Molday, 2000), and in the *rom1*$^{-/-}$ PRPH2/RDS continues to form all its normal type of complexes (Chakraborty et al., 2008). Other work shows that a population of ROM-1 not bound to PRPH2/RDS is present in the OS (Boesze-Battaglia et al., 2002). Combined, these data suggest that RDS/RDS and ROM-1/ROM-1 homotetramers likely occur *in vivo* in addition to the easily purified RDS/ROM-1 heterotetramers. The second question that arises from the model described above is whether covalent linkages occur only between two molecules of PRPH2/RDS and two molecules of ROM-1 or whether intermolecular linkages form between molecules of PRPH2/RDS and ROM-1. Early work suggested heteromeric disulfide linkages did not occur between PRPH2/RDS and ROM-1 (Goldberg and Molday, 1996b). However, later work by the same group used a more direct approach and presented data from IP experiments performed under denaturing but non-reducing conditions that suggested that PRPH2/RDS and ROM-1 do form disulfide bonds with each other (Loewen and Molday, 2000). We have recently used an alternate approach which supports the idea that PRPH2/RDS and ROM-1 form disulfide bonds with each other. To study the role of PRPH2/RDS glycosylation (further discussed below), we have generated a non-glycosylated (N229S) PRPH2/RDS knockin model. Non-glycosylated PRPH2/RDS retains the ability to bind ROM-1, but the lack of glycosylation leads to a readily detectable downshift in apparent molecular weight for both PRPH2/RDS monomer and covalently-linked dimer on non-reducing SDS-PAGE gels. We find that under non-reducing conditions in the N229S retina, ROM-1 dimer also significantly downshifts suggesting it is covalently linked to the smaller N229S PRPH2/RDS (Stuck et al., 2015). As expected, ROM-1 monomer does not shift in size, since non-covalent interactions between N229S PRPH2/RDS and ROM-1 would be interrupted by the denaturing conditions of the gels. Thus the burden of evidence clearly indicates that PRPH2/RDS and ROM-1 can form intermolecular disulfide bonds with each other.

Given the fact that different mutations in PRPH2/RDS can cause rod or cone dominant disease, it has been of interest to understand whether RDS/ROM-1 complex composition or complex assembly is different in the two cell types. This has been a somewhat difficult question to address directly since the murine and bovine retinas are rod-dominant, however the generation of the *nrl*$^{-/-}$ knockout line in which developing rod cells divert to a developmental fate of cone-like cells (Mears et al., 2001) has facilitated exploration of this issue. Using this model, we have found that cones form the same types of overall RDS/ROM-1 complexes as rods, however, cones have an increase in the relative amount of PRPH2/RDS found as higher-order oligomers compared to rods (Chakraborty et al., 2010; Conley et al., 2014b). In addition, studies from mice carrying rod- or cone-specific C150S PRPH2/RDS transgenes suggest that the assembly of complexes may be slightly different in both rods and cones (Chakraborty et al., 2010; Chakraborty et al., 2009). As mentioned above, when C150 is eliminated, no PRPH2/RDS intermolecular disulfide linkages are formed, but tetramers still assemble properly. This tetramer assembly occurs in both rods and cones carrying only C150S PRPH2/RDS (i.e. no WT PRPH2/RDS). However, in cones C150S PRPH2/RDS cannot bind to ROM-1 while C150S in rods retains the ability to bind

ROM-1 (Chakraborty et al., 2010; Chakraborty et al., 2009). These cone-specific defects in C150S complex assembly were also associated with substantial cone-specific mislocalization of PRPH2/RDS, cone opsins and cone transducin (Chakraborty et al., 2010; Chakraborty et al., 2009; Conley et al., 2012a). This suggested that intermolecular disulfide bond formation is a necessary early step for the formation of ROM-1 and PRPH2/RDS tetramers in cones, but not rods and that correct initial complex assembly is a prerequisite for OS targeting (Chakraborty et al., 2010).

The ability of PRPH2/RDS and ROM-1 to form oligomers is central to their ability to function, and when higher-order complex assembly is prevented, for example in the C150S model, no OSs are formed even though PRPH2/RDS tetramers are present (Chakraborty et al., 2009). It is important to understand, however that the relative importance, mechanisms and functions of the various PRPH2/RDS oligomers are not understood, and given the differences in distribution of complex type between rods and cones, the function of individual types of PRPH2/RDS complexes remains of great interest. Different organisms have different ratios of PRPH2/RDS to ROM-1 and many vertebrates lack ROM-1 entirely, although all the examined species which lack ROM-1 have more than one PRPH2/RDS gene (Kedzierski et al., 1996; Weng et al., 1998). It is likely that a complicated dynamic exists that establishes the relative ratios of each complex type based upon the ratios of the two PRPH2/RDS or ROM-1 proteins being expressed in a given organism and the structural requirements of the OS in question (i.e. disc size, incisure number, rod vs. cone OS), however this model has been difficult to test. Furthermore it isn't even clear what these changes in complex formation from organism to organism mean for the specific OS architecture. To understand PRPH2/RDS and ROM-1 oligomer formation much work remains to be done.

3.3 The role of PRPH2/RDS N-glycosylation

One of the striking differences between PRPH2/RDS and ROM-1 is that PRPH2/RDS has a highly conserved N-linked glycosylation within its D2 loop while ROM-1 does not (Bascom et al., 1992). In order to test the function of PRPH2/RDS glycosylation in rod photoreceptors, an unglycosylated form of the PRPH2/RDS protein was expressed using a transgenic approach with a promotor that was specific for rod photoreceptor cells (Kedzierski et al., 1999a). Surprisingly, when unglycosylated PRPH2/RDS was expressed in photoreceptors it formed normal covalent linkages, interacted robustly with ROM-1, and even rescued the structural and functional defects found in the $rds^{-/-}$ background (Kedzierski et al., 1999a). The lack of any gain-of-function phenotype combined with an ability to rescue the null mutant led to the conclusion that the N-glycan of PRPH2/RDS was not critical to PRPH2/RDS function in the OS (Kedzierski et al., 1999a).

However, given the high degree of conservation of the N-linked glycan and the differences in apparent PRPH2/RDS function and complex assembly in rods vs. cones, we hypothesized that PRPH2/RDS glycosylation might be necessary for PRPH2/RDS function in cones. We generated a knockin mouse model carrying the N229S mutation in the endogenous *Rds* locus (Stuck et al., 2015). Consistent with previous results, we found no adverse effect of non-glycosylated PRPH2/RDS in rods; $rds^{N229S/N229S}$ animals exhibited normal

PRPH2/RDS and ROM-1 levels and no defects in retinal structure or function. However, by six months of age, cone electroretinogram responses were decreased by ~40%. To study the biochemical defect in cones, the N229S animals were crossed onto the *nrl*$^{-/-}$ background, and we found that in cones expressing non-glycosylated PRPH2/RDS, PRPH2/RDS and ROM-1 levels were each reduced by ~60% (Stuck et al., 2015). These results suggest that PRPH2/RDS glycosylation is important for cone photoreceptors. Additional exploration is warranted to understand the mechanisms underlying this difference, but the different microenvironments for the PRPH2/RDS D2 loop in rods (intradiscal) vs. cones (extracellular) may play a key role.

4. Role of PRPH2/RDS in the photoreceptor

4.1 PRPH2/RDS as a regulator of OS membranes

The absence of OSs in the *rds*$^{-/-}$ mouse clearly establishes the importance of PRPH2/RDS to OS formation, however it doesn't reveal what PRPH2/RDS does to facilitate OS biogenesis (Sanyal and Jansen, 1981). One promising model for PRPH2/RDS function comes from work that examined the ability of rod OS membranes to undergo fusion events, a key process in the formation of new OS discs. When rod OS discs were isolated and suspended in the presence of calcium, about 5-10% were competent to initiate fusion events with similarly isolated plasma membranes (Boesze-Battaglia, 1997). The localization of PRPH2/RDS in the disc rim made it a good candidate to mediate the observed fusion events, and so it was purified from OSs and its ability to fuse membranes was tested *in vitro*. In these experiments, membrane fusion results in quantifiable changes in fluorescence, and two parameters are assessed: 1) a change in the lag time after mixing but before fusion occurs, and 2) the rate of membrane fusion. Not only did inclusion of purified PRPH2/RDS decrease the lag time, but it also increased the rate of fusion (Boesze-Battaglia et al., 1997). Interestingly, phosphorylated PRPH2/RDS seemed to have no effect on the lag time but did increase the fusion rate (Boesze-Battaglia et al., 1997). The orientation of PRPH2/RDS within the membrane made the PRPH2/RDS C-terminal region the most likely candidate to mediate these fusion events, and this was quickly confirmed (Boesze-Battaglia et al., 1998). Specifically, it was found that a 15 residue long amphipathic alpha helix corresponding to amino acids 311-325 within the C-terminal region of PRPH2/RDS, enhanced and regulated by the surrounding residues, promoted membrane fusion in the presence of calcium (Boesze-Battaglia et al., 1998; Damek-Poprawa et al., 2005; Muller-Weeks et al., 2002; Stefano et al., 2002).

It also became clear that PRPH2/RDS complex formation played an important role in promoting the ability of the PRPH2/RDS C-terminus to mediate membrane fusion events. First, a synthetic peptide corresponding to the PRPH2/RDS amphipathic alpha helix had the greatest effect on membrane fusion when self-associated as a tetramer, and adopted a more stable helical structure in the presence of lipids (Boesze-Battaglia et al., 2003; Boesze-Battaglia et al., 2000), suggesting that the presence of core tetramers of full-length PRPH2/RDS would facilitate this tetrameric arrangement of C-termini and facilitate membrane fusion. Support for the hypothesis that PRPH2/RDS complex formation is important for membrane fusion came from experiments showing that various disruptions in

the D2 loop and blocking of PRPH2/RDS disulfide mediated oligomerization disrupted the fusogenic activity of PRPH2/RDS (Boesze-Battagliaa and Stefano, 2002). In subsequent experiments using purified PRPH2/RDS and ROM-1 expressed in COS-1 cells, it was determined that ROM-1 could not promote membrane fusion alone but did enhance PRPH2/RDS fusogenic activity when in complex with PRPH2/RDS (Boesze-Battaglia et al., 2007b). These data led to a hypothesized model in which the PRPH2/RDS tetramers, intermediate and higher-order oligomers have variable abilities to initiate membrane fusion and together are involved directly in the complicated membrane dynamics at the base of the OS (Boesze-Battaglia et al., 1997).

In support of this idea, two binding partners were found for PRPH2/RDS which appeared to regulate the membrane fusion ability of the protein. The last five residues of the PRPH2/RDS C-terminus (which flank the amphipathic helix) bind melanoregulin (MREG), and MREG co-localizes with PRPH2/RDS at the base of the OS where disc biogenesis occurs (Boesze-Battaglia et al., 2007a). When MREG was added to PRPH2/RDS membrane fusion assays, the rate of membrane fusion was significantly reduced, suggesting that MREG acts as an inhibitor of PRPH2/RDS' fusogenic activity (Boesze-Battaglia et al., 2007a). The C-terminus can also associate with the calcium binding protein calmodulin in a Ca^{2+} dependent manner (Edrington et al., 2007b). Similarly to MREG, this binding seems to inhibit the fusion capacity of the PRPH2/RDS protein and binds to a similar region of the protein (Edrington et al., 2007b). The mechanisms of PRPH2/RDS fusogenic activity and its role in OS biogenesis is not fully elucidated, it is clear that PRPH2/RDS fusogenic activity involves the PRPH2/RDS C-terminus and can be regulated by various binding partners.

Recently, an alternative model to explain the role of PRPH2/RDS in OS biogenesis has developed. Early experiments using isolated PRPH2/RDS in lipid microsomes showed that WT-PRPH2/RDS induced the flattening of these microsomes *in vitro* (Wrigley et al., 2000). In this system, when PRPH2/RDS was mutated to eliminate disulfide bond formation (C150S) or to introduce pathogenic mutations (P216L and C165Y) this ability to flatten membranes was abolished (Wrigley et al., 2000). Recent work has extended this data to argue that the primary purpose of the PRPH2/RDS C-terminal amphipathic helix is to induce the membrane curvature necessary for OS disc biogenesis (Khattree et al., 2013). This model proposes that PRPH2/RDS complex formation acts to organize the PRPH2/RDS C-termini amphipathic helices within the membrane, allowing them to generate organized membrane curvature through hydrophobic insertion/wedge in the cytosolic leaflet of the lipid bilayer of the nascent disc (Khattree et al., 2013). In line with this model, recent work has shown that purified PRPH2/RDS from bovine OSs can induce membrane curvature (Kevany et al., 2013; Khattree et al., 2013). Experimentally it is clear that PRPH2/RDS has the ability to curve membranes, however it is not known whether this functionality is tied to PRPH2/RDS' ability to mediate membrane fusion and to what extent these activities are involved in OS biogenesis *in vivo*. Experiments showing that the PRPH2/RDS C-terminus is intrinsically disordered in isolation, but adopts stable 3D architecture in response to its environment suggest the possibility both factors could be in play (Edrington et al., 2007a; Ritter et al., 2005). While exact mechanisms are debated, clearly PRPH2/RDS is heavily involved in the processes required for disc formation and OS biogenesis. Other tetraspanins are also hypothesized to have a role in regulating membranes. Many tetraspanins are found in

exosomes and are hypothesized to have a role in their formation, as well as in the budding process that allows some viruses to be shed from cells (Andreu and Yanez-Mo, 2014).

4.2 PRPH2/RDS as a player in OS biogenesis and stabilization

One of the oldest proposed functions for PRPH2/RDS is that it interacts with other protein binding partners to help orient and stabilize the growing and mature discs in the OS (Arikawa et al., 1992). The localization of PRPH2/RDS on the “closed” rim facing the axoneme suggested a possible role in organizing the growing discs by attaching them to the axoneme (Arikawa et al., 1992). Additionally, freeze fracture experiments have revealed filamentous connections between neighboring disc rims and between the rims and the plasma membrane that are localized, and in the correct numbers, to potentially be organized by PRPH2/RDS (Goldberg and Molday, 1996b; Roof and Heuser, 1982). However no direct evidence has shown that PRPH2/RDS is directly involved in the formation of these filamentous structures and connections.

Other support for this stabilizing hypothesis comes from studies on the beta-subunit of the rod cyclic nucleotide gated channel (CNG). It has been shown that PRPH2/RDS can bind to the beta subunit of rod CNG, as well as two other non-membrane isoforms also coded for by the *Cngb1* gene called glutamic acid rich proteins (GARP1/2) (Poetsch et al., 2001; Ritter et al., 2011). The interactions between GARP and PRPH2/RDS were found to be strongest when PRPH2/RDS was in disulfide linked oligomers suggesting the larger PRPH2/RDS complexes might mediate this interaction (Poetsch et al., 2001). Given the localization of CNGB1 on the plasma membrane, PRPH2/RDS on the disc rim, and GARP1/2 as free cytosolic forms in between, it has been hypothesized that interactions between PRPH2/RDS and GARP could serve as a mechanism by which PRPH2/RDS could regulate the sizing and orientation of the rod discs. In support of this, elimination of GARP in rods causes disorganization in disc sizing and orientation (Zhang et al., 2009).

Work assessing the localization of PRPH2/RDS complexes in the photoreceptor cells found that the higher-order PRPH2/RDS oligomers likely don’t form until after trafficking to the OS (Chakraborty et al., 2008; Poetsch et al., 2001). Since covalently linked PRPH2/RDS has a higher affinity for GARP than non-covalently linked PRPH2/RDS, the assembly of large PRPH2/RDS oligomers in the OS could potentially play a role in spatially or temporally regulating the RDS/GARP interaction, and thus disc size and orientation. This idea is supported by experiments that utilized GFP chimeric proteins to map the subcellular compartments where RDS/CNGB1/GARP interactions took place and showed that while PRPH2/RDS and CNGB1 interact during trafficking, PRPH2/RDS and GARP only interacted in the OS (Ritter et al., 2011).

Interactions with GARP are another example of differing roles/function of PRPH2/RDS in rods vs. cones. The cone CNGB subunit does not have a GARP region or free GARP isoforms and PRPH2/RDS does not interact cone CNGB channel (Conley et al., 2010a). The precise role of PRPH2/RDS interactions with other proteins, such as GARP, to OS organization is not clearly established, however the model that these interactions play an important role in OS organization is well-supported. In addition, tetraspanins are frequently organizers of membrane domains that can mediate interactions with signaling molecules and

cellular structural components in other tissues, so a role for PRPH2/RDS in similar functions is consistent with its larger superfamily (Andreu and Yanez-Mo, 2014).

Though the mechanisms remain poorly understood, PRPH2/RDS also appears to play a differential role in OS biogenesis in rods vs. cones. It is well-established that rods without PRPH2/RDS fail to form any OSs at all (Sanyal and Zeilmaker, 1984), but the relatively low number of cones in the WT murine retina made cone OS biogenesis in the absence of PRPH2/RDS more difficult to study. However, after crossing the *rds*$^{-/-}$ onto the cone-dominant *nrl*$^{-/-}$ background (Farjo et al., 2006), it was found that cones lacking PRPH2/RDS behave quite differently from rods. First, cones in the *rds*$^{-/-}$/*nrl*$^{-/-}$ retained significant visual function (~50% of *nrl*$^{-/-}$). Second, they exhibited OSs, albeit highly dysmorphic. Specifically, OSs in the *rds*$^{-/-}$/*nrl*$^{-/-}$ were open balloon-like structures which completely failed to form any lamellae or rims (Farjo et al., 2006). Subsequent work demonstrated that in spite of the lack of lamellae/rim structures, OS proteins within this large balloon like structure retained the ability to segregate into separate “plasma membrane” and “disc” domains, with plasma membrane proteins such as the cone CNG channel residing in a separate region of the dysmorphic OS from disc proteins such as S-opsin and its associated g-protein cone transducin (Conley et al., 2014a). These data suggested that in cones, OS/lamellae morphogenesis is initiated by the addition of opsin-containing membrane, with rim/lamellae formation (mediated by PRPH2/RDS) occurring second. In contrast, in rods, OS biogenesis appears to require formation of an PRPH2/RDS-containing rim first with growth of discs via the addition of rhodopsin containing membrane occurring second. For example, in the absence of rhodopsin, though no fully formed discs are elaborated, an extensive network of small, flattened vesicles form in the OS space (Chakraborty et al., 2014). These attempts at disc formation contain PRPH2/RDS and are often aligned properly along the axoneme. In contrast, in the absence of PRPH2/RDS, rhodopsin is not capable of even initiating disc formation, and mislocalizes throughout the photoreceptor (Chakraborty et al., 2014). Thus experimental evidence suggests that there are fundamental differences in the mechanisms of rod vs. cone OS biogenesis, and that PRPH2/RDS plays a key role in these processes.

4.3 The importance of ROM-1

In spite of their structural similarities and overlapping localization, there is little functional redundancy between PRPH2/RDS and ROM-1, as evidenced by the dramatic structural and function defects that accompany PRPH2/RDS deficiency. Yet that leads to the question, what is the role of ROM-1? In the absence of ROM-1 (*rom1*$^{-/-}$), photoreceptors degenerated slowly, and exhibited mild (compared to the *rds*$^{+/-}$ and *rds*$^{-/-}$) defects in OS structure and function even though normal PRPH2/RDS was still present, suggesting that ROM-1 is needed for normal retinal structure (Clarke et al., 2000). Interestingly, the *rom1*$^{-/-}$ retina displayed OS disorganization, particularly abnormalities in disc sizing at the base of the OS, at P30, which worsened at P60, but by P120 this phenotype was largely corrected (Clarke et al., 2000). One interpretation of this finding is that OS biogenesis is fine-tuned by ROM-1 to ensure OSs optimally fill the subretinal space. Thus as photoreceptors die in the *rom1*$^{-/-}$ retina, the resulting extra space in the OS layer may reduce steric or packing pressure that earlier caused deformations in OSs whose size had not been properly regulated due to the

absence of ROM-1 (Clarke et al., 2000). However, the specifics of this model remain vague. When ROM-1 was over-expressed in the cone-dominant *nrl*$^{-/-}$ retina via transgenesis, the amount of PRPH2/RDS higher-order complexes was reduced and OS abnormalities were seen, suggesting that excess ROM-1 may be toxic to cells (Chakraborty et al., 2012). Taken together, these data suggest that though ROM-1 is not essential for OS formation, it does play an important role in fine tuning OS formation possibly through an intrinsic property of ROM-1 or by fine tuning the distribution of PRPH2/RDS complex types, and that the ratio of PRPH2/RDS to ROM-1 must be maintained (Clarke et al., 2000).

This fine-tuning effect of ROM-1 can also be seen by evaluating the PRPH2/RDS mutation that is associated with digenic RP, L185P (Kajiwara et al., 1994). In order to present the disease, a patient heterozygous for L185P has to also be homozygous for a null mutation in the ROM-1 gene (Kajiwara et al., 1994). To determine why this form of PRPH2/RDS was digenic, the ability of the L185P mutant PRPH2/RDS to form tetramers was assessed either in the presence or absence of ROM-1 in COS-1 cells (Goldberg and Molday, 1996a). When expressed alone, the L185P PRPH2/RDS could not form tetramers and appeared to be degraded, consistent with a misfolded protein product (Goldberg and Molday, 1996a; Loewen et al., 2001). When ROM-1 was expressed along with L185P PRPH2/RDS, stable PRPH2/RDS tetramers were observed leading to the model that ROM-1 interaction was able to aid in the folding and oligomerization of the mutant protein (Goldberg and Molday, 1996a; Loewen et al., 2001). Experiments expressing mutant forms of PRPH2/RDS in *Xenopus* correlated the ability to form tetramers with the ability to properly traffic to the OS and suggested that a tetramer checkpoint might exist during trafficking in photoreceptors (Loewen et al., 2003). Together these experiments helped to establish both that PRPH2/RDS haploinsufficiency leads to RP and that the formation of PRPH2/RDS tetramers is a critical first step in providing functional PRPH2/RDS to the OS.

5. Mechanisms of PRPH2/RDS associated disease

5.1 *PRPH2/RDS* and human disease

Gene mapping results suggested that *PRPH2/RDS* mutations could be associated with retinitis pigmentosa (RP) early in 1991 and this was shortly followed by the first direct link between *PRPH2/RDS* and autosomal dominant retinitis pigmentosa (RP), which identified the P216L, P219del and L185P pathogenic mutations (Farrar et al., 1991; Kajiwara et al., 1991) (**Fig. 2A**). This discovery was the first in a long line of medical research that linked over 80 individual pathogenic mutations in the *PRPH2/RDS* gene in human patients to a variety of retinal diseases (summarized at http://www.retina-international.org/files/sci-news//rdsmut.htm). Since the wide spectrum of retinal dystrophies caused by *PRPH2/RDS* has been reviewed outstandingly elsewhere (Boon et al., 2008), here we will focus on those mutations that have been important for developing our understanding of the function of *PRPH2/RDS*.

RP and other rod-cone dystrophies represent the first diseases to be associated with mutations in *PRPH2/RDS* (Kajiwara et al., 1991). RP itself is a disease of the retina caused by the death of rod photoreceptors and is usually associated with a secondary delayed loss of cones (Boon et al., 2008; Ferrari et al., 2011). The majority of *PRPH2/RDS*-associated RP

presents in the 3rd to 5th decades of life, although some cases manifest as early as the first decade (Boon et al., 2008). For example, N244K, D173V, and P216L *PRPH2/RDS* mutations (**Fig. 2A**) result in vision loss in the first decade of life, and are associated with some of the most severe visual phenotypes of all *PRPH2/RDS* mutations (Boon et al., 2008; Wells et al., 1993). However, critically, the timing and severity of vision loss can vary between patients with different *PRPH2/RDS* mutations as well as between patients with the same *PRPH2/RDS* mutation (Boon et al., 2008; Hartong et al., 2006). RP initially presents as loss of low light vision (night blindness) usually starting in the peripheral to mid-peripheral visual field accompanied by decreases in rod function as measured by full-field electroretinogram (ERG). This slowly progresses to loss of central vision and defects in cone ERGs at later stages of the disease when cones begin to die (Boon et al., 2008; Ferrari et al., 2011). While not necessarily true for all RP, animal studies have shown that *PRPH2/RDS* mutations associated with RP can also cause a primary functional deficit in cone photoreceptors, however this defect does not result in significant cone loss until late in the disease process after a significant number of rod cells have died (Farjo et al., 2007; Farjo et al., 2006). Most RP-causing mutations in *PRPH2/RDS* are autosomal dominant, although critically, digenic forms of the disease exist wherein mutations in *PRPH2/RDS* (**Fig. 2A**) co-exist with mutations in the ROM-1 (Kajiwara et al., 1994).

While RP mutations in *PRPH2/RDS* were identified first, it did not take long before it became evident that other mutations in *PRPH2/RDS,* such as R172W, could lead to cone and central-vision defects such as macular dystrophy (MD) (Wells et al., 1993). As is true with RP, *PRPH2/RDS* mutations associated with MD are autosomal dominant and present as subtle defects in central vision usually by the 5th decade of life and advance to very significant visual loss later in life (Boon et al., 2008). Given the high degree of variability in the clinical presentation of disorders classified broadly as MD, it is perhaps not surprising that inter and intra-familial phenotypic variability, both in terms of the different individual mutations and within populations with the same mutation, is extremely high (Duncan et al., 2011; Francis et al., 2005; Stuck et al., 2014). Patients with MD usually exhibit various hypo- or hyper-pigmented regions that appear as focal or multi-focal yellow, orange or grey spots on the fundus (Boon et al., 2008). The size and shape of these lesions can fluctuate or grow with time and the size and localization of these lesions is associated with the severity of vision loss (Boon et al., 2008). In some cases, for example the R172W mutation in *PRPH2/RDS*, pigmentation defects in the RPE can eventually advance to RPE atrophy associated with choriocapillaris atrophy/choroidal neovascularization and severe central vision loss (Michaelides et al., 2005; Wells et al., 1993; Wroblewski et al., 1994).

Patients with MD are usually classified into the various types of MD (pattern dystrophy, adult-onset foveomacular vitelliform dystrophy, etc.) using the timing of onset, the localization and nature of the observed pigmentation defects, and whether the disease is associated with atrophy of the RPE and choriocapillaris as well as choroidal neovascularization. The complexity of the MD disease process lends itself to being highly influenced by secondary genetic traits in other photoreceptor genes well as environmental factors, although the relative importance of these factors can vary dramatically from one disease-causing-mutation to the next (Leroy et al., 2007; Poloschek et al., 2010). The widely

varying disease phenotypes (from rod- to cone-dominant and from phenotypes primarily associated with photoreceptors to those involving the neighboring RPE and choroid) associated with different mutations in the same gene have prompted much investigation into the overall role of PRPH2/RDS and its specific role in the two photoreceptor subtypes.

5.2 PRPH2/RDS and RP

In general, research into the molecular mechanisms that link individual mutations in *PRPH2/RDS* with diseases in humans has focused on understanding the mechanisms associated with RP. Only recently has significant work begun to make headway into the mechanisms associated with MD. It was realized very early that the $rds^{+/-}$ mouse was a possible model for human RP based on the observed retinal phenotype (Hawkins et al., 1985). Just as in humans, $rds^{+/-}$ mice display a rod dominant degeneration followed by a later loss of cones and otherwise mimic RP pathology quite well (Cheng et al., 1997b; Hawkins et al., 1985). The mutant allele found in the $rds^{+/-}$ mouse is a simple null allele (Ma et al., 1995) and these results led to the hypothesis that RP pathology in patients was caused by PRPH2/RDS haploinsufficiency.

Work in transgenic mouse models that expressed human disease causing mutations in *PRPH2/RDS* would do a great deal to expand understanding about how these mutations contributed to PRPH2/RDS haploinsufficiency (Ding et al., 2004; Kedzierski et al., 2001; Nour et al., 2004; Stricker et al., 2005). Virtually all of the disease models that have been studied thus far have involved D2 loop mutations since these are extremely common, however, some have involved evaluation of mutations in other regions such as the C-terminal deletion/frameshift at amino acid 307 (McNally et al., 2002). In the simplest case, for example in the case of the C214S RP mutation (Stricker et al., 2005), the pathogenic mutation results a mutant protein that is unstable and degraded, resulting in an effective loss-of-function null allele. Consistent with this, genetic supplementation of WT PRPH2/RDS in C214S/$rds^{+/-}$ transgenic retinas can rescue disease phenotypes (Nour et al., 2008). However, in other cases, for example the P216L RP mutation, haploinsufficiency can be caused by apparent gain-of-function alleles. In the P216L PRPH2/RDS transgenic model, the P216L *PRPH2/RDS* transcript expressed at about 60% of the normal WT transcript levels per allele. When crossed onto the $rds^{+/-}$ mouse (Kedzierski et al., 2001), if the P216L mutant protein was stable one would expect to find roughly normal levels of total PRPH2/RDS protein (~50% mutant and ~50% WT). Likewise if the P216L was simply degraded like C214S PRPH2/RDS, one would expect ~50% of WT protein levels coming from the remaining WT allele. Experimentally however, the actual measured amount of PRPH2/RDS protein was only 8% of WT in P216L/$rds^{+/-}$ retinas (Kedzierski et al., 2001) suggesting that the mutant may exacerbate haploinsufficiency by promoting degradation of not only mutant proteins but the WT as well. This dominant mutant effect helps explain why some human pathological mutations in *PRPH2/RDS* lead to a more significant defect and early onset. Some mutations can also bind to and contribute to the degradation of WT PRPH2/RDS resulting in different steady state levels of PRPH2/RDS protein from mutation to mutation and thus different levels of RP severity. Current estimates suggest that about 60-80% of the normal WT PRPH2/RDS must be present for normal OS biogenesis, and the more significant the

decrease in PRPH2/RDS levels, the earlier the onset and more severe the RP pathology (Kedzierski et al., 2001; Nour et al., 2004).

5.3 PRPH2/RDS and MD

While the mechanisms that link individual *PRPH2/RDS* mutations with RP are clear and have been intensively investigated for years, our understanding of *PRPH2/RDS*-associated MD mutations has lagged behind. *In vitro* analysis of mutations in the asparagine at position 244 (N244) helped to illustrate how difficult it can be to determine the exact molecular mechanisms of *PRPH2/RDS* associated mutations. The N244H *PRPH2/RDS* mutation which is associated with MD in human patients, could not form normal higher-order oligomers yet could traffic out of the ER and associate with ROM-1 (Conley et al., 2010b). In contrast, the RP associated N244K *PRPH2/RDS* mutation loses all ROM-1 binding and becomes localized to the ER when expressed in COS-1 cells (Conley et al., 2010b). The fact that mutations in the same amino acid in the D2 loop can lead to MD or RP makes analysis and identification of MD associated pathology difficult.

The use of transgenic and knockin models has enabled us to begin elucidating disease mechanisms for some MD mutations. In some cases, MD mutations cause cell-type specific functional defects. For example, expression of the R172W *PRPH2/RDS* MD mutation in mice leads to a stable mutant protein product which is properly trafficked to the OS but results in a dominant negative phenotype on cone structure and function even when all WT PRPH2/RDS is present and there is no haploinsufficiency. Yet this mutant has no effect on rods and can even improve the structural and functional phenotypes associated with haploinsufficiency. For example at early timepoints rod ERG function is improved in the R172W/$rds^{+/-}$ compared to the $rds^{+/-}$ while cone function in the R172W/$rds^{+/-}$ and R172W/WT is significantly worse than in either the $rds^{+/-}$ or the non-transgenic WT (Conley et al., 2007; Ding et al., 2004). Patients carrying the R172W mutation exhibit reasonably good phenotypic consistency from patient to patient (at least compared to other *PRPH2/RDS* mutations), and patient phenotypes often include RPE atrophy and defects in the choroid and vasculature (Downes et al., 1999; Wickham et al., 2009; Wroblewski et al., 1994). In spite of the fact that the mouse does not have a macula, R172W transgenic animals also exhibit some of these changes (Conley et al., 2014b), making them a useful choice for studying the relationship between defects in photoreceptors and the neighboring tissues. Interestingly, as in patients, presentation of secondary phenotypes in R172W transgenic mice exhibit varying penetrance and severity (Conley et al., 2014b). **Fig. 5A** shows that the fundus in R172W transgenic mice is relatively normal on the $rds^{-/-}$ background and similar to the WT and $rds^{+/-}$. However, the retinal vasculature is highly abnormal: note the curly, attenuated, retinal vessels shown in the fundus fluorescein angiogram (**Fig. 5B**), in contrast with the normal vasculature in the WT and $rds^{+/-}$ RP model. While many animals exhibit a phenotype similar to this, others exhibit a much more severe vascular defect, while others exhibit normal retinal vasculature (Conley et al., 2014b).

In other cases, mutations that cause disease classified mainly as MD, such as the Y141C mutation, exhibit much more widely varying patient phenotypes and do not cause cell-type specific defects in rods vs. cones in animal models. For example knockin mice carrying one

Y141C mutant allele and one WT allele exhibit defects in rod and cone function as well as retinal degeneration in spite of having normal levels of PRPH2/RDS protein (i.e. the mutant protein is stable in the presence of WT) and normal targeting of WT and mutant PRPH2/RDS to the OS (Stuck et al., 2014). They also exhibit fundus features consistent with pattern dystrophy such as speckling (**Fig. 5A**, arrows and (Stuck et al., 2014)), but do not have detectable abnormalities in retinal vasculature (**Fig. 5B**), underlying the wide variation in phenotype from mutation to mutation.

Yet there do appear to be consistent features for MD-associated mutations in *PRPH2/RDS*. Thus far all those studied lead to the production of normal PRPH2/RDS protein levels (eliminating haploinsufficiency as a disease mechanism) and proper transport of mutant and WT PRPH2/RDS to the OS, yet somehow OS formation is disrupted (Conley et al., 2010b; Ding et al., 2004; Stuck et al., 2014). In addition, though PRPH2/RDS protein levels are normal in these cases, they do exhibit abnormalities in complex formation. For example, R172W PRPH2/RDS is slightly more sensitive to tryptic digestion than WT PRPH2/RDS (Ding et al., 2004) suggesting it may not fold properly, and the presence of R172W PRPH2/RDS results in the formation of intermediate RDS/ROM-1 complexes which contain abnormal disulfide linkages specifically in cones (Conley et al., 2014b). Y141C PRPH2/RDS also exhibits defects in RDS/ROM-1 oligomerization (likely due to the addition of an extra D2 loop cysteine), and forms extremely large RDS/ROM-1 oligomers that contain excess disulfide linkages and are found in the heaviest gradient fractions (Stuck et al., 2014). Combined these data suggest that MD mutations may exert their toxic effects by altering RDS/ROM-1 complex formation, and that differences in the effects of any given mutation on complex formation may contribute to variations in clinical phenotype.

The molecular causes that lead to cell death in animal models or patients with *PRPH2/RDS* mutations is not clearly established. It was discovered early that the photoreceptor cells die through regulated apoptosis (Chang et al., 1993; Portera-Cailliau et al., 1994), both caspase dependent and caspase-independent (Lohr et al., 2006), however there is very little understanding about what links defects in OS formation to apoptosis. Evaluation of chimeric mice has shed some light on this question although the results are not entirely clear. Chimeric mice generated through the fusion of $rds^{-/-}$ and WT morulae have retinas containing a mixed population of photoreceptors that either have the normal *PRPH2/RDS* gene or the null allele (Sanyal and Zeilmaker, 1984). In these mice, patches of $rds^{-/-}$ photoreceptors were observable within the context of neighboring WT photoreceptor cells. The $rds^{-/-}$ photoreceptors in this chimeric model degenerated similarly to those in the full $rds^{-/-}$ (Sanyal and Zeilmaker, 1984). Interestingly WT photoreceptors interspersed among $rds^{-/-}$ mutant photoreceptors cells had malformed OSs, suggesting that even with a normal load of PRPH2/RDS protein normal OS structure requires the physical structural support of neighboring OSs (Sanyal and Zeilmaker, 1984). In another case, a transgenic mouse in which a WT PRPH2/RDS transgene was incorporated into the X chromosome and resulted in a chimeric expression pattern due to random X-chromosome silencing was crossed onto the $rds^{-/-}$ background (Kedzierski et al., 1998). In this case photoreceptor cells expressing the transgene (i.e. with WT PRPH2/RDS) had apparently normal OSs but degenerated similarly to the surrounding photoreceptors which lacked PRPH2/RDS suggesting some

global cell-survival defect independent of individual mutant cells carrying defects in OS formation (Kedzierski et al., 1998). Generally this is thought to be due to the release of a toxic factor from the dying cells, the loss of a trophic factor from the cells that have died, or metabolic disruptions due to the loss of a large number of very metabolically active cells however it is important to remember that these mechanisms are not mutually exclusive and could be all occurring at once (Kedzierski et al., 1998; Vlachantoni et al., 2011). More recent work has focused on gaining a more in-depth understanding of the mechanisms that lead to cell death in the *rds*$^{-/-}$. Various pathways have been implicated in this process include nitric oxide-dependent pathways (Yang et al., 2007), complement-mediated lysis, oxidative stress, and autophagy (Lohr et al., 2006). Certainly further work to understand or retard the cell death process will be a key step in the development of effective therapies for *PRPH2/RDS*-associated disease.

6. Conclusions and future directions

The PRPH2/RDS protein is a critical component for normal vision through its role as a structural protein important for the proper formation of both rod and cone photoreceptor cells. While this is clearly established there is still considerable debate about how exactly PRPH2/RDS is able to fulfill its critical functions. A central remaining question is what the relative functions of the various PRPH2/RDS complexes are. When viewed as a whole, the body of work on the PRPH2/RDS protein strongly suggests that the protein is able to play a complex role in OS formation due to variations in binding affinity for other OS proteins and the relative concentration of the various PRPH2/RDS complexes, which each may possess intrinsic membrane curvature/fusion properties. For example, it is hypothetically possible that large PRPH2/RDS homomeric complexes are core structural components responsible for helping mediate and maintain the curvature of the rim region while smaller and intermediate RDS/ROM-1 complexes are responsible for mediating membrane fusion or organizing protein domains with interacting partners such as GARP to stabilize and properly structure the OS. While ample evidence discussed above would suggest that the PRPH2/RDS complexes have some level of distinct functional importance, no current evidence can adequately address what the functional distinctions and overlaps for the PRPH2/RDS complexes are. Further work will be needed to understand the functional discrepancies between the different PRPH2/RDS complexes and how changes to these complexes alter OS biogenesis.

In order to understand the role of PRPH2/RDS in OS biogenesis it is critical to think of PRPH2/RDS as part of a complex system involving multiple proteins and different lipid components that might not necessarily associated through direct interactions, yet must be coordinated and tuned to each other to promote proper OS biogenesis. The coordination of localization and function of PRPH2/RDS complexes with other important OS proteins involved in disc biogenesis remains a topic ripe for exploration using a systems-level approach. Further work is needed to clarify the importance of the membrane curvature/fusion properties of PRPH2/RDS and how protein-lipid interactions contribute to the proper formation of discs. It is also still unclear exactly what protein-protein interactions help to organize and size OS disc during biogenesis. The central role of PRPH2/RDS and the multiple functions of the protein conveyed by its complexes make it an attractive target for

understanding the entirety of OS biogenesis and how this process is modulated from species to species to result in the distinct intricate OSs observed in species from fish to humans.

In the case of human disease associated with PRPH2/RDS, it is unclear which approach should be taken to correct the problems in patients given the complicated disease processes that have been identified. While gene supplementation could be effective for PRPH2/RDS-associated haploinsufficiency in cases of loss-of-function mutations (Ali et al., 2000; Cai et al., 2010), the strict requirement for precise levels of PRPH2/RDS to avert haploinsufficiency make complete correction using this approach difficult. In addition, for gain-of-function mutations that create dominant-negative mutant proteins and defects in surrounding tissues as well as in photoreceptors, gene supplementation is not likely to be effective alone. If effective, long lasting treatments for PRPH2/RDS-associated disease are to be found, it is necessary to understand the basic science behind the role of PRPH2/RDS in OS biogenesis and the cell death which follows failures in OS biogenesis. Given the complexity of the system being disrupted it is unlikely that a non-targeted pharmacological agent or non-tailored gene therapy will be effective until the actual molecular defects are understood and can be addressed in a rational way. New potential therapeutic avenues may become apparent as we more clearly establish how disruptions of PRPH2/RDS function and OS disc biogenesis lead to the apoptosis of photoreceptor cells. Characterization of the signaling pathways activated in the various mouse models of PRPH2/RDS disease could identify potential targets for therapeutics and might be one of the most direct paths to human treatments. The field of PRPH2/RDS research is ripe for future discovery and while many questions remain, it is clear that dedicated work in this arena continues to answer the fundamental questions about photoreceptor cell biology and hopefully will lead to the development of treatments for associated blinding retinal disease.

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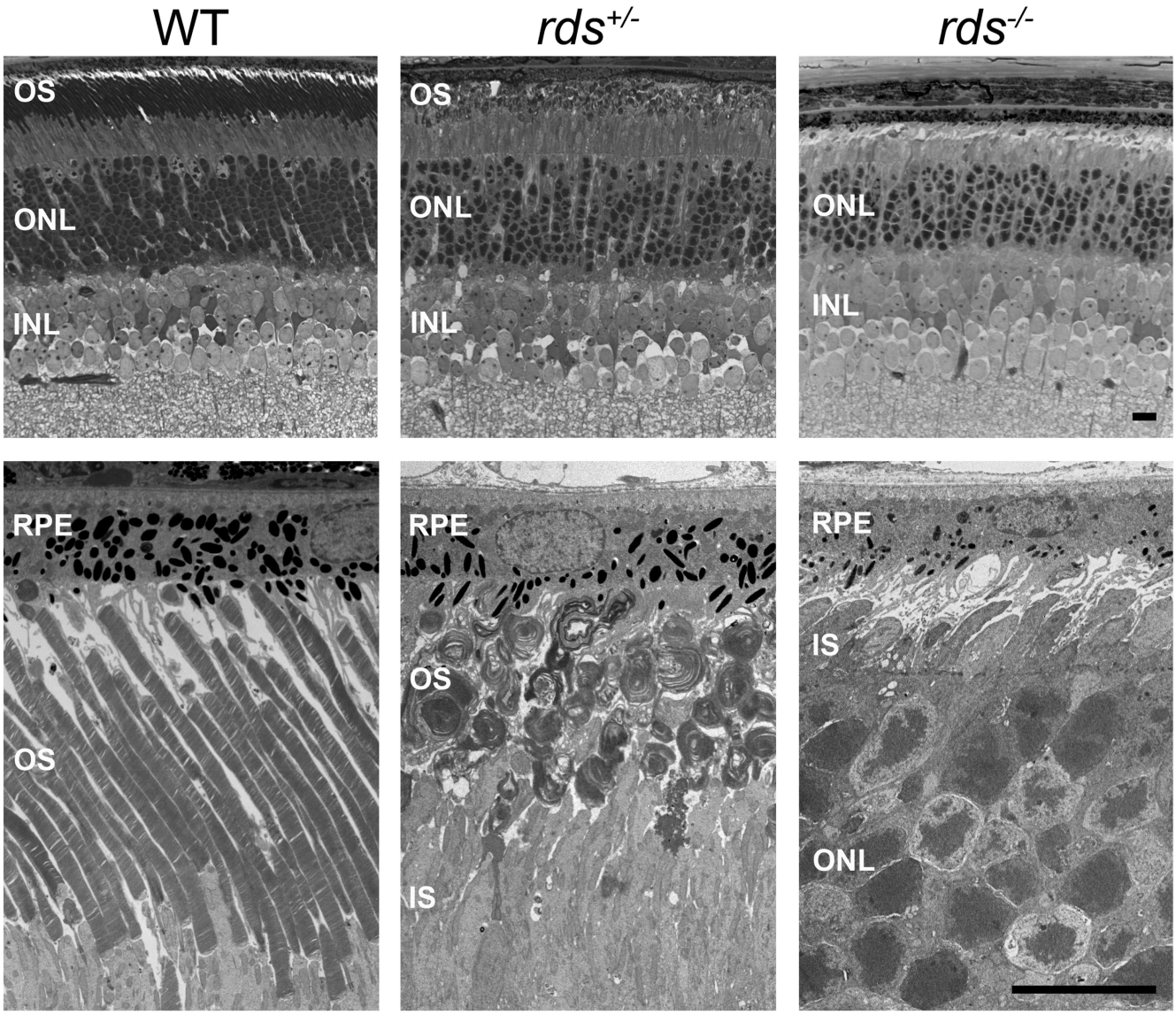

Figure 1. PRPH2/RDS deficiency leads to abnormalities in retinal structure

Retinal structure was evaluated at postnatal day 30 in murine retinas of the indicated genotypes. Top row shows light micrographs collected at 40×, bottom row shows transmission electron micrographs collected at 3,000×. Scale bars: 10μm. RPE: retinal pigment epithelium, OS: outer segment, IS: inner segment, ONL: outer nuclear layer, INL: inner nuclear layer.

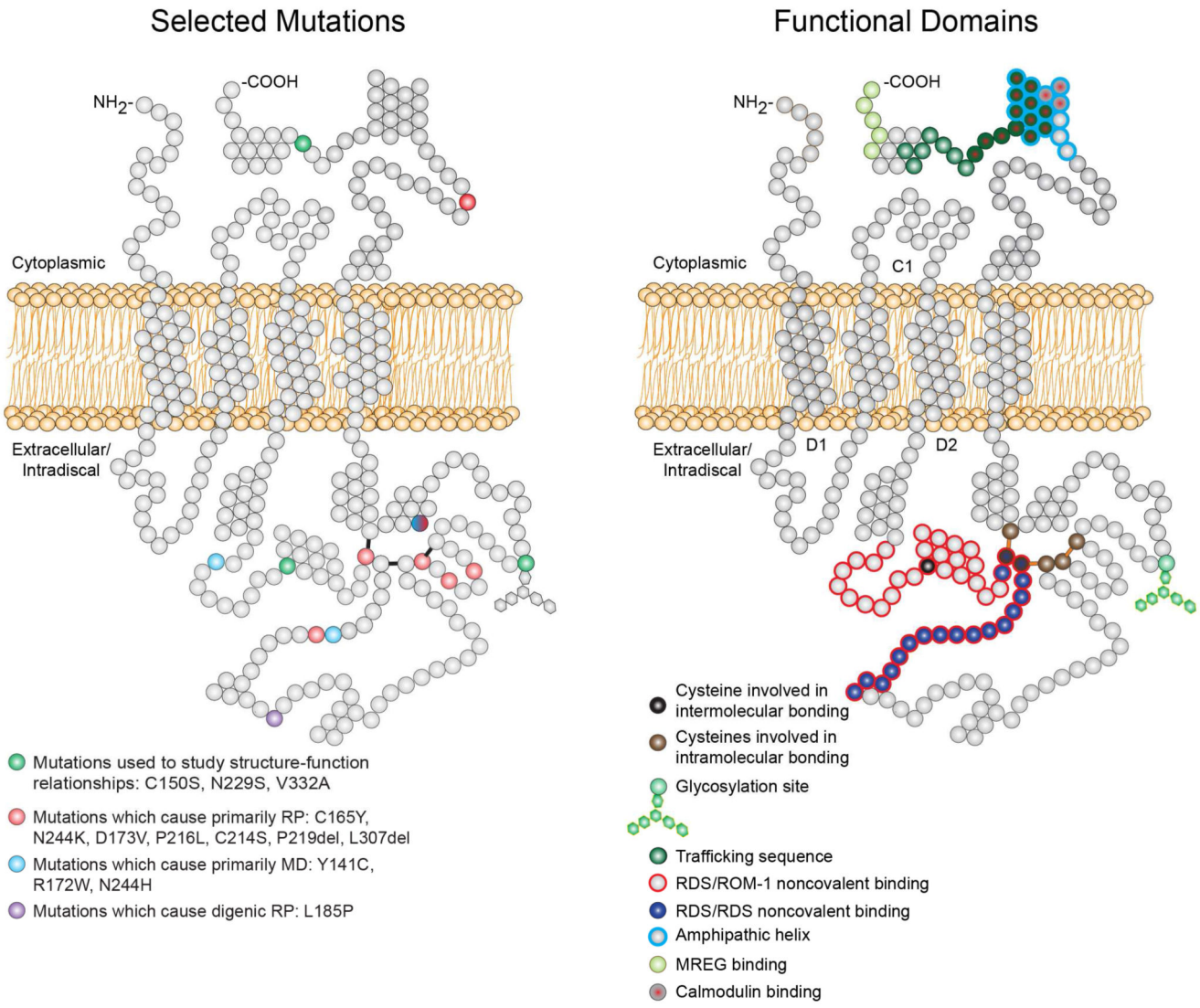

Figure 2. Mutations and functional domains within the PRPH2/RDS molecule

The PRPH2/RDS molecule is illustrated with selected mutations labeled on the left and functional domains labeled on the right. Mutations and functional diagrams on the illustration are those to which we refer directly here, additional pathogenic mutations in *PRPH2/RDS* exist and can be explored by visiting http://www.retina-international.org/files/sci-news/rdsmut.htm.

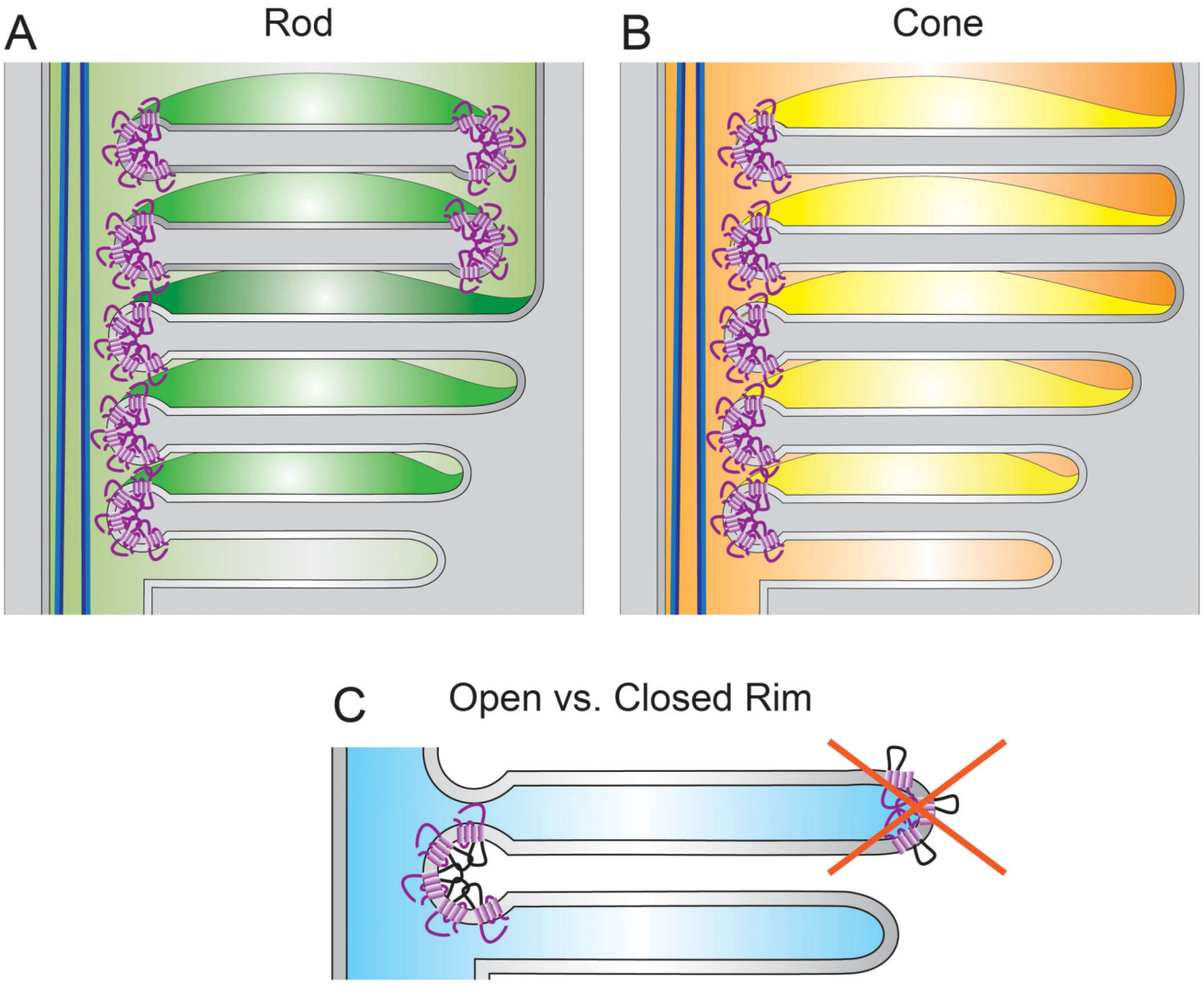

Figure 3. PRPH2/RDS localization in the rim. A-B

Shown are illustrations of the base of a rod (**A**) and cone (**B**) photoreceptor adjacent to the connecting cilium. Blue lines illustrate the axoneme, purple molecules indicate PRPH2/RDS. In rods, PRPH2/RDS is found on the “closed” edge of nascent discs and all the way around the circumference of fully formed discs. In cones, PRPH2/RDS is likewise found along “closed” rims only. **C**. Illustration showing that the curvature of the open rims is opposite that of the closed rims and would therefore not accommodate PRPH2/RDS in the orientation it is known to adopt in which the D2 loop (shown in black in **C**) mediates interactions between adjacent PRPH2/RDS molecules.

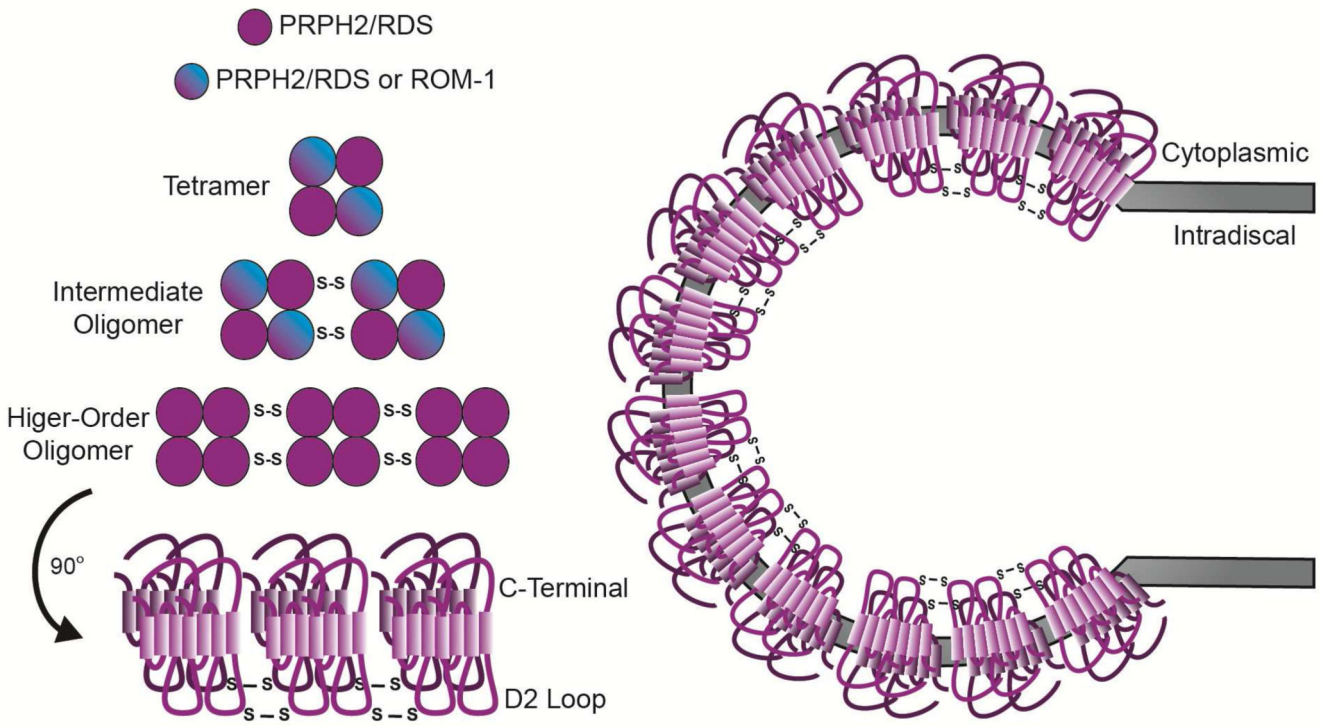

Figure 4. PRPH2/RDS and ROM-1 assemble into complexes

The core unit of RDS/ROM-1 complexes is the homo/heterotetramer. These tetramers assemble into disulfide linked RDS/ROM-1 hetero-oligomers of intermediate size and large higher-order homo-oligmers. These complexes are found in the disc rim with the disulfide-linked D2 loop protruding into the intradiscal space. Rim packing estimates are from (Kevany et al., 2013).

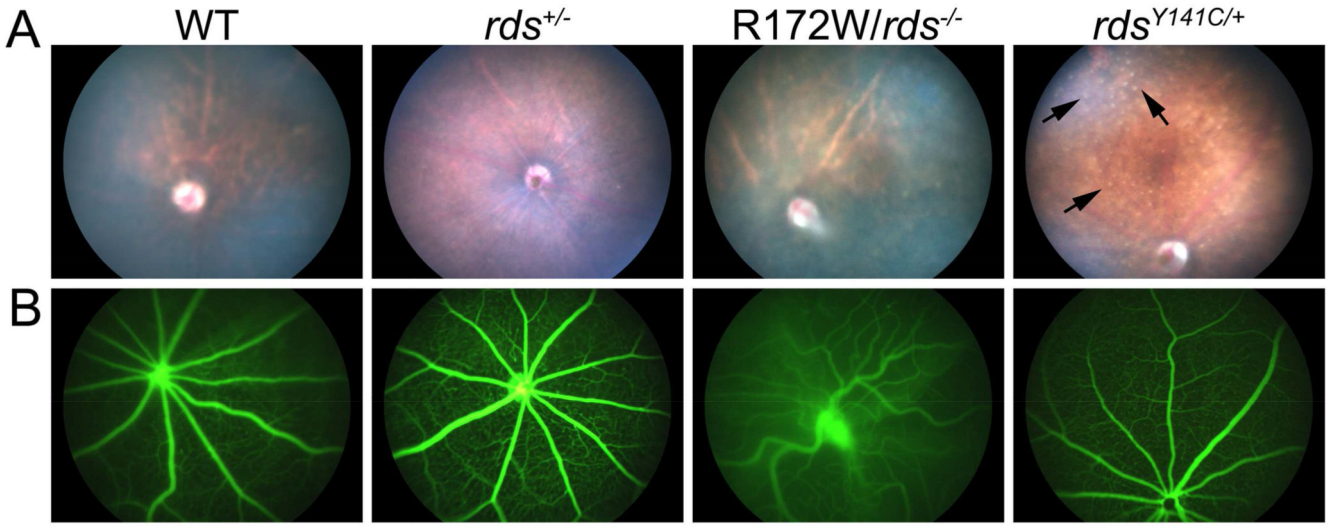

Figure 5. Mutations in *PRPH2/RDS* lead to phenotypic changes in the retina

Shown are brightfield fundus images (**A**) and fluorescein angiograms (**B**) from animals aged 5-6 months of the indicated genotypes. Though the *rds*$^{+/-}$ (a model of ADRP) exhibits no dramatic changes in the fundus/angiogram at this age, different MD mutations lead to striking phenotypes, including abnormalities in retinal vasculature in the case of R172W transgenic animals, and fundus speckling (arrows) in Y141C heterozygous knockin animals.