

PERIPHERIN-2 AND ROM1 SELF-ASSEMBLY SHAPES PHOTORECEPTOR
OUTER SEGMENT DISK RIM MEMBRANES

by

RUDRICK CASEY BOUCHER

A dissertation submitted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOLOGICAL AND BIOMEDICAL SCIENCES

2026

Oakland University
Rochester, Michigan

Doctoral Advisory Committee:

Andrew F. X. Goldberg, Ph.D., Chair

Adam Avery, Ph.D., Co-Chair

Fabia Battistuzzi, Ph.D.

Susan Brockerhoff, Ph.D.

Randal Westrick, Ph.D.

© Copyright by Rudrick Casey Boucher, 2026
All rights reserved

*To Evelyn and Hunter, my niece and nephew. May your generation inherit a world more
enlightened by endeavors such as this.*

ACKNOWLEDGEMENTS

I am deeply grateful to the many scientists whose centuries of foundational work have made this dissertation possible. The roughly 500 different authors who each contributed to the works cited here are necessarily only the most proximal of the giants upon whose shoulders I stand.

My greatest thanks go to my adviser, Andrew F. X. Goldberg, whose guidance, insight, and unwavering support shaped every stage of this project. I am equally indebted to my labmates—Michelle L. Milstein, Breyanna Cavanaugh, Laura Gunther, Michelle Tcherniak, and Alexander Seidel—for their intellectual contributions, technical expertise, and daily collaboration. Their efforts were essential to the conception, execution, and interpretation of the experiments presented here.

I also thank our collaborators, particularly Alex Sodt and Andrew Beaven, whose computational and biophysical insights provided a critical mechanistic framework for understanding the experimental findings presented here. I am likewise grateful to the faculty and staff of the Oakland University Eye Research Institute whose ongoing commitment to excellence has benefited every student here.

My parents, step-parents, grandparents, and my six brothers have provided love and encouragement throughout my education. I am also deeply grateful to my partner, Thomas, for his constant support, patience, and for keeping me out of trouble these past ten years.

I also thank the mentors who guided me through the early stages of my development as a scientist: Weibin Zhou, Friedhelm Hildebrandt, Sok Kean Khoo, and Yukiko Yamashita.

Finally, I thank the members of my doctoral committee for their time and support of this work.

PREFACE

This dissertation presents investigations into the self-assembly and organization of the tetraspanin peripherin-2 (Prph2) and its homolog Rom1 into the supramolecular transmembrane scaffold that shapes the characteristic extreme curvature of the rim domains of photoreceptor outer segment (OS) disk membranes, which is essential for vertebrate vision and human retinal health. This work was conducted in the laboratory of, and under the supervision of, Dr. Andrew F. X. Goldberg in the Eye Research Institute at Oakland University.

The primary results presented in Chapters 2-4 are the basis for the following manuscript recently published in the *Journal of Biological Chemistry*¹:

Boucher, R. C., Milstein, M. L., Hossein, A., Cavanaugh, B. L., Sodt, A. J., Beaven, A. H., & Goldberg, A. F. (2026). Photoreceptor outer segment disk rim curvature relies on a tetraspanin interaction web. *Journal of Biological Chemistry*, 302(6): 113136. PMID: 42119983.

Portions of this work were presented at ARVO 2024². I also presented this work in oral presentations at the Inaugural and Third Eye Research Retreat (Oakland University/OUWB, 2023 and 2025), as well as at the Clifford and Drusilla Harding lecture series (Oakland University, 2025).

ABSTRACT

PERIPHERIN-2 AND ROM1 SELF-ASSEMBLY SHAPES PHOTORECEPTOR OUTER SEGMENT DISK RIM MEMBRANES

By

Rudrick Casey Boucher

Adviser: Andrew Goldberg, Ph.D.

The outer segment (OS) is the highly specialized primary cilium that rod and cone photoreceptors use to sense light. Each OS comprises an evenly ordered stack of hundreds of membranous disks that scaffold the first steps of visual phototransduction. Disk structure, OS organization, and photoreceptor viability rely on a transmembrane scaffold of the tetraspanin peripherin-2 (Prph2) and its homologue Rom1 that self-assembles to shape the extreme membrane curvature that characterizes OS disk rims. Mutations in PRPH2 are among the leading causes of human inherited retinal degenerations (IRDs), yet the mechanisms underlying Prph2 and Rom1 self-assembly and membrane curvature scaffolding in both normal and disease states remain poorly understood.

Recent structural advances have revealed that the disk rim scaffold comprises three parallel belts of linear polymers of Prph2/Rom1 non-covalent dimers linked together by inter-dimer disulfide bonds. This dissertation describes complementary biochemical approaches for characterizing Prph2/Rom1 polymers in detergent extracts

from native rod outer segment (ROS) and transfected HEK293 cell membranes. A reoptimized velocity sedimentation protocol was developed to capture the complete size range and heterogeneity of the extracted Prph2/Rom1 polymers. We developed and validated a BN-PAGE method for resolving individual size species of Prph2/Rom1 polymers and then applied it to characterize Prph2 and Rom1 assembly in WT ROS, Rom1 knockout mice, and transfected HEK293 cells.

These approaches establish that native Prph2 assembles primarily as linear chains of disulfide-linked polymers of highly variable length and that Rom1 promotes higher-order Prph2 polymerization. Collectively, these findings provide a mechanistic framework for Prph2/Rom1-mediated disk rim morphogenesis and establish BN-PAGE as a robust, high-throughput platform for biochemical phenotyping of pathogenic PRPH2 variants of uncertain significance. This work advances fundamental understanding of photoreceptor membrane architecture and offers translational insights into the molecular basis of IRDs.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iv
PREFACE	v
ABSTRACT	vi
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xv
CHAPTER 1	
THE ESSENTIAL ROLE OF PRPH2 FOR VISION	1
Introduction	1
Function of Prph2 and Rom1	4
Biochemistry of Prph2 and Rom1	7
Cell biology of Prph2 and Rom1	13
Structural biology of Prph2, Rom1, and the disk rim	15
Knowledge gaps and aims of the work	16
CHAPTER 2	
A REOPTIMIZED SEDIMENTATION APPROACH ENHANCES REPRODUCIBILITY AND CAPTURES THE FULL RANGE OF PRPH2/ROM1 POLYMER SIZES	19
Introduction	19
Materials and Methods	21
Bovine ROS	21
Recombinant protein expression	22
Membrane protein solubilization	22
Velocity sedimentation	23
Semi-automated fractionation	24
SDS-PAGE and Western Blotting	26
Results	27
Reoptimized sedimentation of Triton X-100 solubilized bovine ROS	28
A semi-automated fractionation approach improves reproducibility	30
Detergent dependence of Prph2/Rom1 polymer sedimentation	31

TABLE OF CONTENTS—Continued

Reoptimized velocity sedimentation analysis of recombinant Prph2 missense variants overexpressed in HEK293	33
Discussion	35
CHAPTER 3	
A BLUE NATIVE PAGE APPROACH RESOLVES DETERGENT EXTRACTED PRPH2/ROM1 POLYMER SPECIES BY SIZE	38
Introduction	38
Methods	39
BN-PAGE electrophoresis	39
BN-PAGE blot development	41
Results	41
BN-PAGE method optimization	41
BN-PAGE captures the size heterogeneity of Prph2/Rom1 polymers seen on sedimentation	44
BN-PAGE resolves individual species of Prph2/Rom1 polymers in bovine ROS extracts	47
BN-PAGE resolves polymer species within sedimentation fractions	50
BN-PAGE reveals mutation induced self-assembly defects in recombinant Prph2 mutants	52
Discussion	55
CHAPTER 4	
ROM1 PROMOTES THE ASSEMBLY OF PRPH2 INTO CURVATURE-SHAPING SUPRAMOLECULAR FORMS	60
Introduction	60
Methods	61
Purification of mouse ROS	61
Immunopurification of Prph2 from bovine ROS extracts	62
High resolution clear native PAGE (hrCN-PAGE)	63
Reductant screen	64
Results	65

TABLE OF CONTENTS—Continued

Rom1 supports higher-order Prph2 polymerization	65
Free non-covalent dimers in bovine ROS are predominantly Prph2:Rom1 heterodimers reflecting a paucity of free homodimers	67
Thiol-specific reduction is consistent with S-glutathionylation of Rom1 in ROS membranes	71
Molecular modeling predicts non-covalent interactions supporting Prph2 dimer chain oligomerization	73
Molecular dynamics simulations predict spontaneous anisotropic curvature generation by Prph2 polymers	74
Molecular phenotyping of pathogenic Prph2 missense variants	76
Discussion	79
CHAPTER 5	
CONCLUSIONS	83
Summary of major conclusions	83
Prph2 is detergent extracted from its native membranes as linear disulfide-linked polymers of highly variable length	83
An optimized BN-PAGE method resolves Prph2/Rom1 polymers by size	84
Rom1 promotes higher-order Prph2 polymerization in OS membranes	84
Prph2 CTER-targeting monoclonal antibodies do not bind native non-covalent dimers	85
Heterodimers and heterotetramers predominate in WT ROS	85
Prph2 polymers generate spontaneous anisotropic membrane curvature	86
BN-PAGE offers a practical high-throughput platform for biochemically characterizing Prph2 VUSs	87
Key remaining knowledge gaps	88
How is polymer chain length regulated?	88
What is the native subunit organization of the disk rim scaffold?	89
What are the specific non-covalent interactions driving Prph2/Rom1 self-assembly?	90
Do Prph2 and Rom1 follow the same assembly rules in cones as in rods?	90

TABLE OF CONTENTS—Continued

What is the mechanism of membrane curvature generation by Prph2?	91
APPENDIX A: LIST OF PLASMIDS USED FOR TRANSFECTION	92
APPENDIX B: ANTIBODIES USED IN THESE STUDIES	93
APPENDIX C: KEY REAGENTS	94
APPENDIX D: APPROVAL FROM THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE	95
APPENDIX E: APPROVAL FROM THE INSTITUTIONAL BIOSAFETY COMMITTEE	96
REFERENCES	97

LIST OF TABLES

Table 1	Sucrose percentages and migration distances for sedimentation fractions with peak SS-dimer, monomer, or lane total immunoreactivities	32
Table 2	Reductants tested in Figure 22	73
Table 3	List of plasmids used for transfection	92
Table 4	List of primary antibodies	93
Table 5	List of secondary antibodies	93
Table 6	List of key reagents	94

LIST OF FIGURES

Figure 1	Photoreceptors and the outer segment	2
Figure 2	Transient overexpression of Prph2 drives the formation of highly curved intracellular membranes	5
Figure 3	Prph2 and Rom1 are required for normal OS structure	6
Figure 4	Cartoon of Prph2 topology	8
Figure 5	Model of Prph2/Rom1 assembly proposed by Loewen & Molday (2000)	10
Figure 6	Prph2 and Rom1 structure and polymerization	12
Figure 7	Cryo-ET of the native rod disk rim reveals three parallel belts of Prph2/Rom1 dimer chains	15
Figure 8	Current model of Prph2 and Rom1 scaffolding OS disk rims	17
Figure 9	Prph2 is detergent extracted as linear polymers of heterogeneous length	20
Figure 10	Reoptimized velocity sedimentation analysis of bovine ROS Triton X-100 extract	29
Figure 11	Semi-automated fractionation improves reproducibility	30
Figure 12	Comparison of reoptimized sedimentation with Triton X-100 or DDM bovine ROS extracts	31
Figure 13	Reoptimized sedimentation of recombinant Prph2 missense variants	34
Figure 14	Optimizing BN-PAGE for analysis of Prph2 polymers	43
Figure 15	BN-PAGE results recapitulate velocity sedimentation	46
Figure 16	BN-PAGE resolves heterogeneity and stoichiometry of Prph2/Rom1 polymers in bovine ROS detergent extracts	48

LIST OF FIGURES—Continued

Figure 17	Lane profiles from BN-PAGE analysis of Triton X-100 bovine ROS extracts	49
Figure 18	BN-PAGE resolves Prph2/Rom1 polymer size heterogeneity within velocity sedimentation fractions	51
Figure 19	BN-PAGE reveals self-assembly defects in recombinant Prph2 mutants	54
Figure 20	Rom1 is required for Prph2 polymerization into higher-order dimer chains in ROS	66
Figure 21	Prph2:Rom1 heterodimers and heterotetramers predominate in ROS membranes	70
Figure 22	Selective thiol reduction is consistent with S-glutathionylation of Rom1.	72
Figure 23	Molecular dynamics simulations predict anisotropic curvature generation by Prph2 polymers	75
Figure 24	Molecular phenotyping of pathogenic Prph2 missense variants overexpressed in HEK293	77

LIST OF ABBREVIATIONS

ϵ -Ahx	6-aminohexanoic acid
BN-PAGE	Blue native polyacrylamide gel electrophoresis
COS	Cone outer segment
Cryo-EM	Cryogenic electron microscopy
Cryo-ET	Cryogenic electron tomography
CTER	Prph2/Rom1 C-terminal domain
DDM	n-dodecylmaltoside
DTT	Dithiothreitol
EC2	Extracellular 2
hrCN-PAGE	High resolution clear-native polyacrylamide gel electrophoresis
IRD	Inherited retinal degeneration
LOESS	Locally estimated scatterplot smoothing
NEM	n-ethylmaleimide
OS	Outer segment
PBS	Phosphate buffered saline
POPC	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
<i>PRPH2</i>	Human peripherin-2, gene
PRPH2	Human peripherin-2, protein
<i>Prph2</i>	Peripherin-2, gene
Prph2	Peripherin-2, protein

LIST OF ABBREVIATIONS—Continued

Prph2/Rom1	Prph2 and/or Rom1 species as a general population
Prph2:Rom1	Heteromeric species of Prph2 and Rom1
<i>ROM1</i>	Human retinal outer segment membrane protein 1, gene
ROM1	Human retinal outer segment membrane protein 1, protein
<i>Rom1</i>	Retinal outer segment membrane protein 1, gene
Rom1	Retinal outer segment membrane protein 1, protein
Rom1 KO	Rom1 knockout mouse
ROS	Rod outer segment
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SS-dimer	Disulfide-linked dimeric
TEM	Transmission electron microscopy
TSPAN	Tetraspanin
TVN	Tubulovesicular network
WT	Wild type

CHAPTER 1

THE ESSENTIAL ROLE OF PRPH2 FOR VISION

Introduction

Vision begins when light passes through the transparent cornea, lens, and vitreous in the front of the eye and is focused onto a cup-shaped disk of brain tissue at the back of the eye: the retina. There, the focused light is detected by specialized sensory neurons: the rod and cone photoreceptors. Both rods and cones have a specialized organelle—the outer segment (OS)—that is the cell's light-sensing apparatus (Fig. 1A-C). When stimulated by light, the OS generates signals that are transmitted to, and processed by, the neural circuitry of the inner retina and ultimately conveyed to the rest of the brain for visual perception. Rods have a rod-shaped OS (rod outer segment, ROS) and mediate scotopic (low light or nighttime) vision. Cones have a cone-shaped OS (cone outer segment, COS) and mediate photopic (daytime) and color vision³. Each OS comprises a tightly ordered stack of hundreds of membranous disks^{4,5,6}. The flat surfaces (lamellae) of the disks scaffold the visual pigments^{7,8} (i.e. rhodopsin or the cone opsins); these lamellar membranes are the site of the initial steps of visual phototransduction⁹. Packaging these phototransductive membranes into stacked disks allows $\sim 6000 \mu\text{m}^2$ of light-sensitive surface area to fit into each OS—or a total phototransductive membrane surface area of $\sim 1.5 \text{ m}^2$ in each human eye¹⁰.

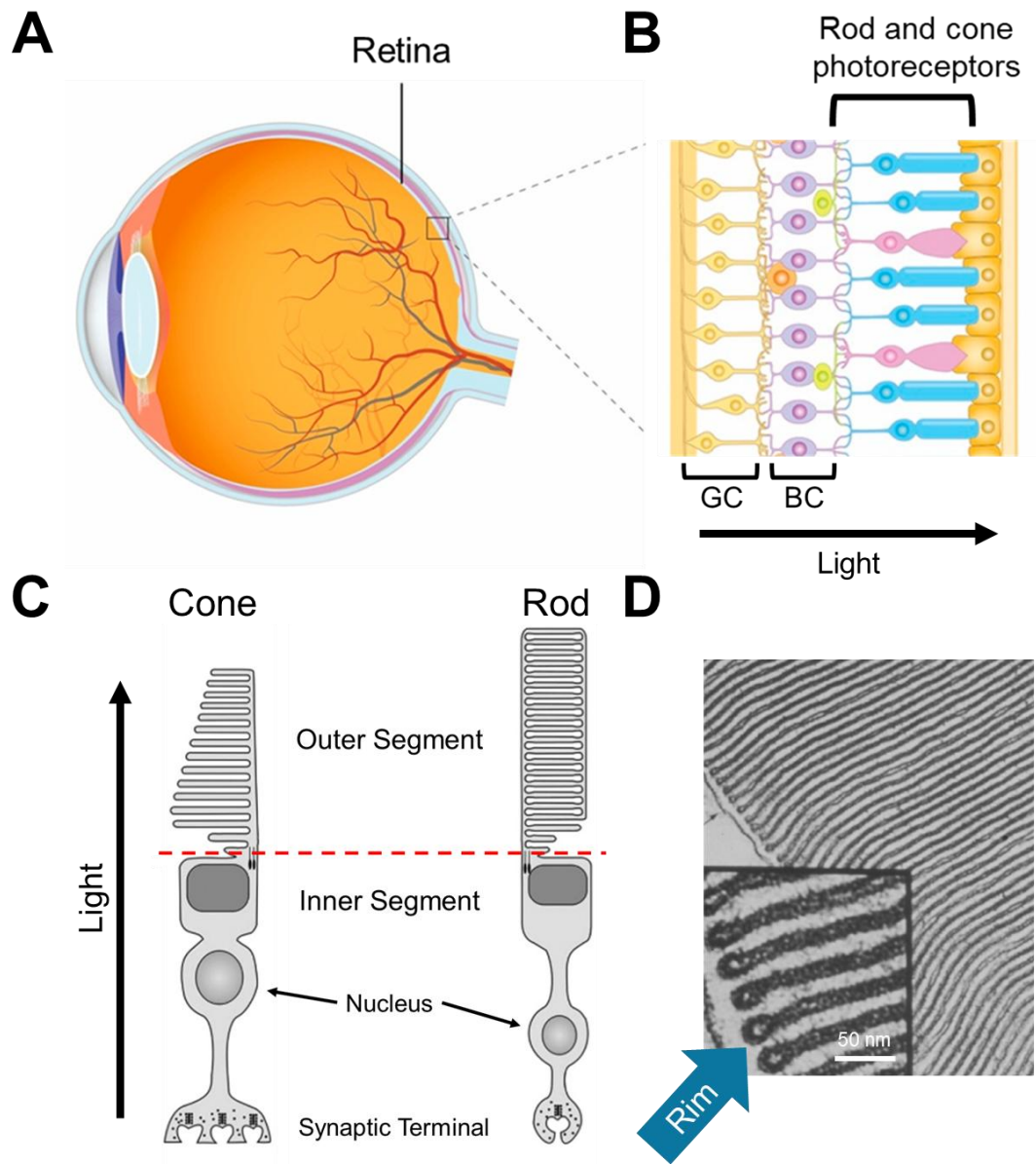


Figure 1: Photoreceptors and the outer segment. (A) Cartoon adapted from Sakai et al. (2022)¹⁴¹ showing the position of the retina in the back of the eye. (B) Cartoon illustrating the layers of neurons in the retina. Rod and cone photoreceptors are in the outer (back) retina. Nerve signals from photoreceptors are processed by the retinal neural circuitry, including bipolar cells (BCs) and retinal ganglion cells (GCs), and then transmitted via the optic nerve to the rest of the brain for visual perception. (C) Cartoon of rod and cone photoreceptor architecture. The light-detecting outer segments (OSs) are in the outermost (rear-most) layer of the retina. Rods have a rod-shaped outer segment and cones have a cone-shaped outer segment. Rod disks are fully enclosed and discontinuous with the OS plasma membrane. Cone disks are generally not fully enclosed, and their lumens are open to the interphotoreceptor matrix. Panel adapted from Lamb (2013)¹⁴² (D) Transmission electron micrograph of part of a rod OS showing the tight packing and even arrangement of the disks. The inset shows the disk rims (blue arrow) at a higher magnification.

Turnover of OS disk proteins is accomplished through a continuous mechanism of OS disk renewal¹¹. New disks form from evaginations of the connecting cilium plasma membrane at the base of the OS. As the disks mature, rod disks become fully enclosed within the ROS and discontinuous with the ROS plasma membrane. Cone disks remain open to the extracellular space¹², only becoming fully enclosed near the distal tip of the COS¹³. Mature disks are continuously displaced towards the tip of the OS as new disks are added at the base¹¹. Upon light onset every morning, enclosed packets of the oldest (~10 days old) disks are shed from the distal tip of the OS to be phagocytosed (or, more precisely, trogocytosed^{14,15}) by the underlying RPE^{11,16}. This renewal accounts for 10% of the OS length per day in mammalian photoreceptors.

To achieve the tight packing and even organization of the OS disks, the membrane around the periphery (rim) of each disk must curve sharply to a radius of 12 nm^{9,17} (Fig. 1D). This extreme level of curvature at OS disk rims is generated and maintained by a transmembrane protein scaffold composed of two photoreceptor-specific^{18,19} tetraspanins: Peripherin-2 (Prph2; aka P/rds and TSPAN22)²⁰ and Retinal outer segment membrane protein 1 (Rom1)²¹. In this role, both proteins are essential for OS morphogenesis and function. Photoreceptors lacking Prph2 do not form elaborated OS membranes (Fig. 3A), are negligibly responsive to light²², and degenerate within months of birth^{23,24}. Photoreceptors lacking Rom1 do form organized OSs and respond normally to light, but they exhibit dysregulated disk diameters (Fig. 3C) and impaired disk incisure formation, which disrupts OS organization and eventually leads to mild photoreceptor loss^{25,26}.

Mutations in the gene encoding human Prph2 (*PRPH2*) are one of the most common causes of inherited retinal degenerations (IRDs). Over 200 pathogenic *PRPH2* alleles have been reported²⁷, including over 93 missense mutations. These mutations are associated with a remarkable variety of human disease phenotypes²⁸, including retinitis pigmentosa^{29,30} (RP), central areolar choroidal dystrophy³¹ (CACD), vitelliform macular dystrophy³², and Leber congenital amaurosis³³ (LCA). Pathogenic mutations in Rom1 are much rarer and typically only cause mild vision loss late in life^{34,35}. However, retinitis pigmentosa resulting from heterozygosity for the Prph2 Leu185Pro (*PRPH2*^{L185P}) allele combined with heterozygosity for a Rom1 null allele is a widely referenced example of digenic inheritance^{36,37}. Numerous animal models corroborate the pathogenicity of *PRPH2* and *ROM1* mutations^{24,25,38,39,40,41,42,43}.

Function of Prph2 and Rom1

The molecular function of Prph2 is generating membrane curvature. Simply overexpressing recombinant Prph2 in transfected HEK293 cells is sufficient to drive the formation of an intracellular tubulovesicular network (TVN) of highly curved membranes of similar diameter to OS disk rims (Fig. 2A)⁴⁴. Heterologous overexpression of Rom1 does not drive TVN formation. Whereas Prph2 is necessary and sufficient to scaffold the robust membrane curvature at the disk rim, Rom1 is neither²⁵. The precise molecular function of Rom1 remains unclear; however, both proteins are required for normal OS and disk structure²⁶.

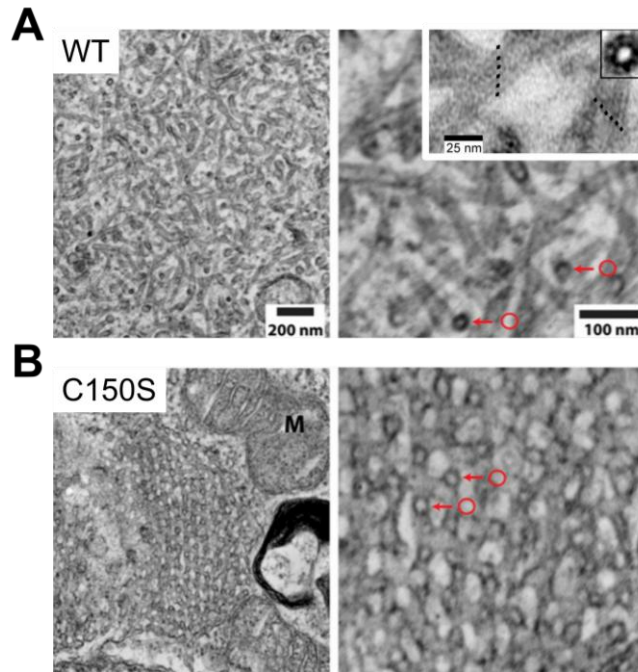


Figure 2: Transient overexpression of Prph2 in HEK293 drives the formation of highly curved intracellular membranes. Modified from Milstein et al. 2020⁴⁴. TEM micrographs showing the tubulovesicular network (TVN) of highly curved membranes in HEK293 cells transfected with (A) WT Prph2 or (B) Prph2^{C150S}, shown at lower (left) and higher (right) magnification. The inset in A shows the ~25 nm diameter of the tubules. Note that the TVN induced by Prph2^{C150S}, does not form elongated tubes like those seen with WT Prph2.

The first evidence for the fundamental significance of Prph2 to photoreceptor viability and function—and the first animal model of Prph2-related disease—came from the retinal degeneration slow (*rds*) mouse. The *rds* allele was identified as a spontaneously occurring mutation in the 020/A inbred mouse strain⁴⁵. *rds*

comprises a large retrotransposon insertion within the *Prph2* coding sequence and is a null allele^{46,47}.

As a null *Prph2* allele,

photoreceptors in homozygous

Prph2^{*rds/rds*} mice fail to form elaborated OS membranes (Fig. 3A)²³, are negligibly responsive to light²², and degenerate within months of birth. OS disk membranes in *Prph2*^{*rds/+*} mice form characteristic whorls that disrupt and disorganize the evenly layered structure of the outer segment (Fig. 3B), indicating a defect in disk morphogenesis²⁴. These defects lead to a decline in visual function^{42,48} and photoreceptor numbers²⁴ as the *rds/+* mice age; similar to the clinical course observed in human *PRPH2* associated autosomal dominant retinitis pigmentosa (adRP)²⁹. Transgenic overexpression of Prph2 in

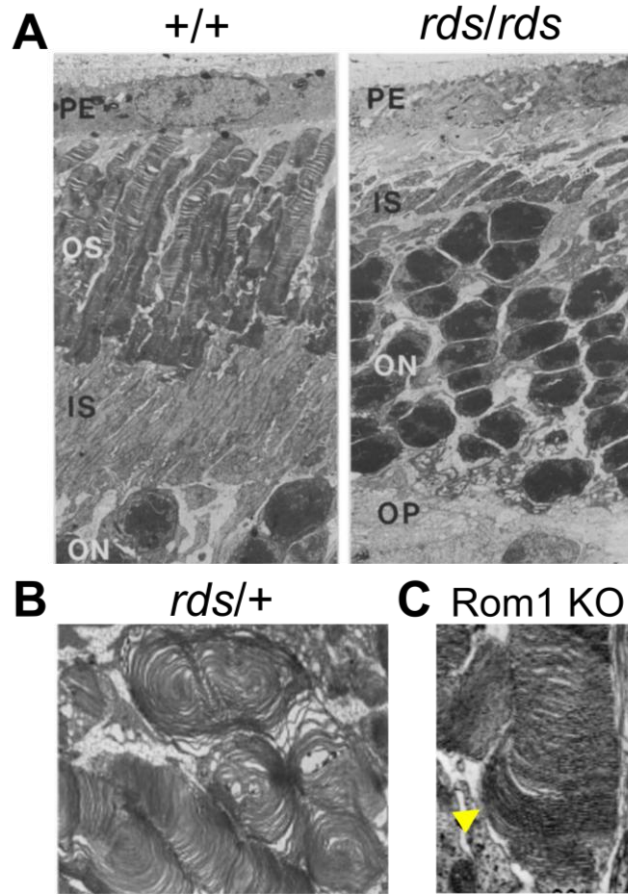


Figure 3: Prph2 and Rom1 are required for normal OS structure. TEM micrographs from mouse retinas. (A) Normal OS architecture in WT mice (left) and the absence of OSs in *rds/rds* mice²⁴. (B) Characteristic OS membrane whorls seen in heterozygous *rds* mice¹⁴³. (C) A packet of overelongated disks (yellow arrow) at the base of a Rom1 KO mouse rod OS²⁵.

rds mice rescues the *rds* phenotype⁴⁹, confirming the identification of the *Prph2* gene as the locus of the *rds* allele and also supporting the causal linkage between *PRPH2* mutations and adRP.

Rom1 was identified as an abundant novel transcript in a human retinal cDNA library screen and determined to be a Prph2-interacting, retina specific, OS disk rim localizing, Prph2 paralogue^{50,19}. Like Prph2, Rom1 was found to form disulfide-linked dimers in ROS.

Co-immunoprecipitation revealed the close structural interaction between the two proteins. The association of human retinitis pigmentosa with digenic double heterozygosity for mutations in *PRPH2* and *ROM1* firmly established the significance of the close functional relationship between both proteins to normal OS structure and healthy vision³⁶.

In contrast to the virtual absence of photoreceptor OSs seen in *Prph2^{rds/rds}* mice, photoreceptors in homozygous Rom1 knockout (*Rom1^{KO/KO}* or Rom1 KO) mice form organized and essentially normally functioning OSs²⁵; demonstrating that homomeric Prph2 is sufficient to support disk morphogenesis and OS architecture. However, OS disks in Rom1 KO mice do not form incisures²⁶ and the OSs in Rom1 KO mice contain discrete packets of disks with abnormally large diameters (Fig. 3C)²⁵. The resulting mild OS disorganization drives mild photoreceptor loss²⁵; consistent with the late age of onset reported in published cases of monogenic *ROM1* associated IRDs^{34,35}. Together, these findings suggest that whereas Prph2 self-assembly is the primary determinant of OS structure⁵¹, Rom1 serves a more auxiliary role in fine-tuning disk morphogenesis.

Biochemistry of Prph2 and Rom1

Prph2 and Rom1 are members of the tetraspanin (TSPAN) family of transmembrane proteins, which includes 33 members in humans⁵². Prph2 and Rom1 are very closely related paralogues that diverged from each other with the first whole genome duplication event at the base of the vertebrate lineage^{53,54}. They share 34.7% primary sequence identity in humans; within their large globular EC2 domains, they share 49% sequence identity and 71% residue similarity. Both Prph2 and Rom1 exhibit the canonical TSPAN topology (Fig. 4) comprising a cytosolic N-terminal domain (NTER), four transmembrane alpha helices (TM1-4), a small EC1 domain between TM1 and TM2 within the disk lumen, a small intracellular loop (SIL) between TM2 and TM3, a large globular EC2 domain within the disk lumen between TM3 and TM4, and an intrinsically

disordered cytosolic C-terminal domain (CTER). The Prph2 CTER contains an inducible amphipathic helix (AH)⁵⁵, the Rom1 CTER contains a putative amphipathic helix that has yet to be experimentally validated. Like all other TSPANs, the Prph2 and Rom1 EC2 domains have the characteristic TSPAN fold (helices A to E)^{56,57,58} and are stabilized by intramolecular disulfide bonds between an even number of EC2 cysteine residues⁵⁹. Unlike other TSPANs, both Prph2 and Rom1 have an additional EC2 cysteine (Prph2-C150 and Rom1-C153; Fig. 6 A & B) for forming intermolecular disulfide bonds between molecules of Prph2 and Rom1⁶⁰.

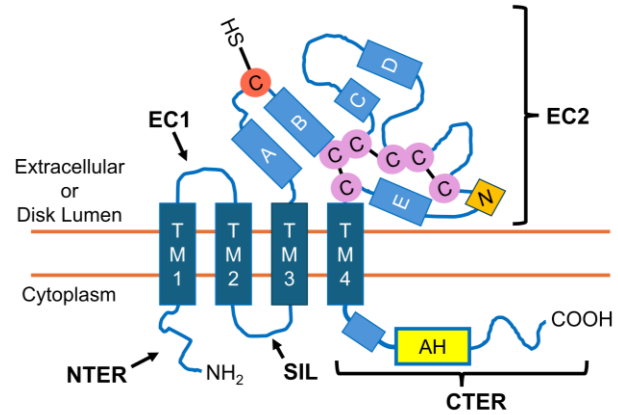


Figure 4: Cartoon of Prph2 topology. It shows the four transmembrane α -helices (TM1-4); the cytosolic N-terminus (NTER), short intracellular loop (SIL), and C-terminus (CTER) with an amphipathic helix (AH); and the extracellular/disk luminal EC1 and EC2 domains. The EC2 domain contains the five helices of the classical TSPAN fold (A-E), the magenta circles labeled 'c' represent the six intramolecular disulfide forming cysteines (C165, C166, C213, C215, C222, and C250) with short black lines representing their connectivities. C150, with its free sulfhydryl is shown in red. The orange diamond labeled "N" represents the Prph2-N229 glycosylation site. Rom1's topology is similar but it lacks the AH and an N-glycosylation site.

Prph2 and Rom1 function relies upon their self-assembly and oligomerization via protein-protein interactions between the EC2 domains of each molecule^{61,62}. Most of the reported pathogenic missense mutations in human PRPH2 occur within this vital oligomerization domain⁶³. Although Prph2 can self-assemble to generate membrane curvature in the absence of Rom1^{25,26}, Rom1 does not generate membrane curvature in the absence of Prph2. Prph2 self-assembly is critical to generating and maintaining the membrane curvature at the disk rim⁶⁴.

Early biochemical studies established the basic assembly pathway. Upon transient overexpression of bovine Prph2 or Rom1 in COS-1 cells, both proteins produce disulfide-linked dimers on non-reducing SDS-PAGE like they do ROS membranes⁶⁵. Velocity sedimentation showed that co-expression of the two proteins produced a stable non-covalently associated heteromeric complex. Immunofluorescence showed localization of both overexpressed proteins to COS-1 intracellular membranes⁶⁵. These experiments demonstrated that Prph2 and Rom1 can self-assemble into their non-covalent core subunit in the absence of other photoreceptor-specific factors. Site-directed cysteine mutagenesis studies in the COS-1 overexpression system established Prph2-C150 as the intermolecular disulfide forming residue; Prph2^{C150S}, which lacks the inter-dimer disulfide forming thiol, forms non-covalent core subunits but no intermolecular disulfide linkages⁶⁶. Significantly for the technical aims of this thesis, the reconstitution of native-like complexes by heterologous overexpression established an indispensable model system for subsequent investigations of Prph2/Rom1 assembly in normal and disease states.

Velocity sedimentation and gel exclusion chromatography of Prph2-containing complexes immunopurified from DTT-reduced ROS membranes using the C-terminus-directed monoclonal antibody mAb2B6 identified a single non-covalent species. In detergent micelles, this species exhibited an apparent molecular weight of 240 kDa, corresponding to an estimated protein mass of ~135 kDa⁶⁷. Given the 39 kDa and 37 kDa MWs of Prph2 and Rom1 respectively, these data suggested a tetrameric stoichiometry for the non-covalently associated smallest native form of Prph2/Rom1. More recent cryo-

EM studies have revised this model and established the dimeric stoichiometry of the native non-covalent Prph2/Rom1 core subunit⁵⁸.

The significance of Prph2 and Rom1 core subunit assembly was soon illustrated by characterization of the pathogenic Prph2^{L185P} variant. The adRP disease phenotype only co-segregates with the *PRPH2*^{L185P} allele when coinherited with a null *ROM1* allele, demonstrating a rare example of digenic inheritance⁶⁸. When overexpressed in COS-1 cells, Prph2^{L185P} failed to form native non-covalent core complexes like WT Prph2 when individually transfected but it retained its ability to form native non-covalent core complexes when co-transfected with Rom1, as verified by velocity sedimentation. This specific defect in homotypic non-covalent assembly provided a direct molecular rationale for the digenic inheritance pattern. Single heterozygotes form sufficiently homeostatic levels of functional Prph2/Rom1 complexes, double heterozygotes do not.

Loewen and Molday (2000) further refined this understanding by utilizing velocity sedimentation to compare Prph2/Rom1 oligomerization in DTT-reduced versus non-reduced ROS membranes⁶⁹. They found that the DTT reduced proteins exist as non-covalent homomeric Prph2 and heteromeric Prph2/Rom1

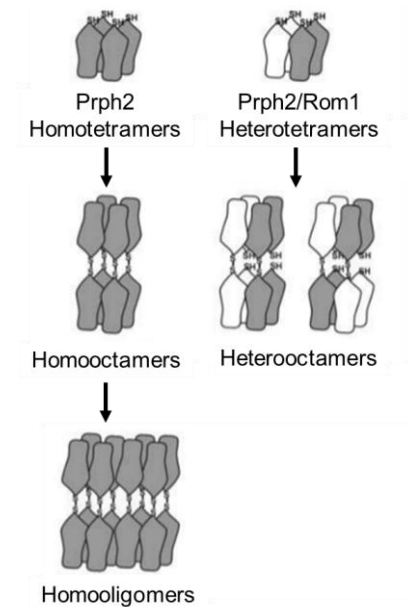


Figure 5: Model of Prph2/Rom1 assembly proposed by Loewen and Molday (2000). Under this model, non-covalent Prph2/Rom1 tetramers oligomerize via Prph2-C150/Rom1-C153 mediated intermolecular disulfide bonds. Both Prph2 and Rom1 are present in disulfide-linked intermediate oligomers, while only Prph2 is present in the higher-order forms.

core complexes. Under non-reducing conditions, these non-covalent core complexes further associate through Prph2-C150 (and Rom1-C153) mediated intermolecular disulfide bonds to form intermediate and higher-order polymers. Whereas Prph2 under non-reducing conditions is present as core complexes and polymers of every observed size, Rom1 is excluded from the higher-order oligomers (Fig. 5). The authors also noted (as has been confirmed by mass spectrometry⁷⁰) that Prph2 is twice as abundant in ROS membranes as Rom1.

More recent studies have shown that self-assembly proceeds in a strictly ordered, multistep process. The fundamental building block—the core subunit—of Prph2/Rom1 is the non-covalent dimer⁵⁸. Specifically, non-covalent Prph2 homodimers and Prph2:Rom1 heterodimers which exist in a 1:2 ratio in the rims of OS disks (Fig. 6C & D), mirroring the 2:1 absolute abundance ratio Prph2 to Rom1 in OS membranes^{69,70}. These non-covalent dimers assemble into linear polymeric dimer chains held together by inter-dimer disulfide bonds between the free cysteine residue (i.e. Prph2-C150 and Rom1-C153) within the EC2 domain of each monomer (Fig. 6E). Rom1 is restricted to the smallest oligomers, while Prph2 forms heterogeneously sized homomeric disulfide linked chains that can exceed ten dimers in length (Chapters 3 and 4). Heterogeneity of the polymer size is a native feature of the disk rim scaffold⁴⁴ but its physiological significance is not known. The long axis of the polymer chains is oriented along the circumference of the disk, perpendicular to the axis of the extreme membrane curvature at the rim. The chains associate end on end to form belts around the perimeter of the disk. Three belts interact laterally to form the disk rim scaffold (Figs. 7 & 8)⁷¹.

The Prph2 and Rom1 EC2 domains are essential for proper protein folding and non-covalent assembly⁶¹—a highly conserved feature of TSPANs^{72,56,57}. The presence of disk rims in homozygous *Prph2*^{C150S/C150S} mice and ectopic rims in transgenic *Xenopus*

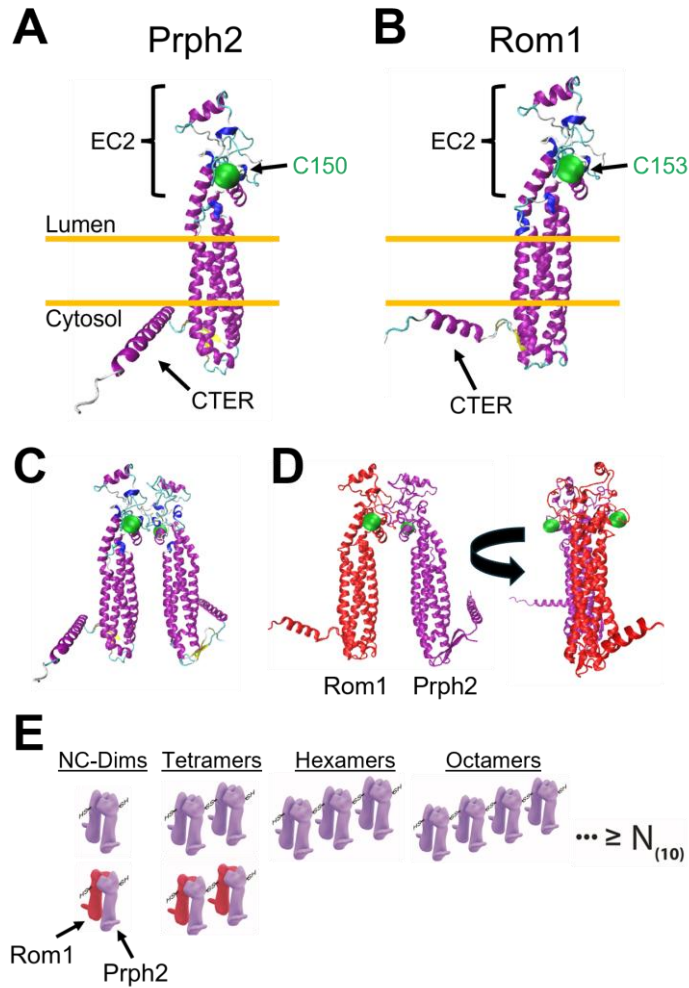


Figure 6: Prph2 and Rom1 structure and polymerization. (A) Model of the Prph2 monomer colored by secondary structure. C150 is highlighted in light green. (B) Cartoon of the Rom1 monomer, as in A. C153 is highlighted in light green. (C) Prph2 non-covalent homodimer, colored as in A. (D) Prph2:Rom1 non-covalent heterodimer, Prph2 is purple, Rom1 is red, free EC2 cysteines Prph2-C150 and Rom1-C153 are highlighted in light green. The rotated view on the right shows the accessibility of the free cysteines for forming inter-dimer disulfide bonds. (E) Model of disulfide-mediated Prph2/Rom1 polymerization. Colored as in D. Non-covalent homodimers of Prph2 form chains over 10 dimers in length. Rom1 is restricted to non-covalent dimers and tetramers.

overexpressing Prph2^{C150} indicate that non-covalent Prph2 interactions are sufficient to scaffold the extreme negative membrane curvature of the OS disk rim^{44,73,74}. These non-covalent Prph2 and Rom1 EC2 interactions remain largely uncharacterized beyond that mediating non-covalent dimerization. Identifying and better characterizing such interactions may provide valuable mechanistic insights into the pathogenesis of PRPH2-related disease.

Cell biology of Prph2 and Rom1

Non-covalent dimerization of Prph2 and Rom1 occurs in the ER and, as a prerequisite for ER exit, is a key checkpoint in disk rim biogenesis⁷⁵. The oligomeric disposition of Prph2 and Rom1 during trafficking is unknown; however, disulfide mediated Prph2 assembly into higher-order polymers occurs at the base of the OS during disk morphogenesis⁷⁶. Prph2 reaches the OS via two trafficking routes, determined by its dimerization partner. Homomeric Prph2, the pool destined to form higher-order oligomers, bypasses the Golgi and traffics unconventionally through the late endosomes⁷⁷. In contrast, heteromeric Prph2:Rom1 complexes traffic conventionally through the Golgi^{78,79}. In Rom1 KO mice, all Prph2 traffics unconventionally via the late endosomes⁷⁸. Prph2 mutations that shift the balance between homomeric and heteromeric forms compromise both trafficking efficiency and disk rim scaffold assembly^{40,68,78,80}. However, the potential significance of the separate trafficking pathways to Prph2/Rom1 self-assembly and organization in OS membranes remains an open question.

The precise mechanism(s) through which Prph2 and Rom1 scaffold this membrane curvature is unknown. Partitioning of an amphipathic helix into one leaflet of the membrane is a common curvature generating mechanism used by other proteins⁸¹. However, deletion of the Prph2 CTER amphipathic helix *increases* the propensity of the protein to form highly curved intracellular membranes upon overexpression in transfected HEK293⁵⁵. Another possibility is that Prph2 dimers intrinsically and spontaneously generate membrane curvature through hydrophobic mismatch. EC2 contacts between the belts of the disk rim scaffold may also contribute to membrane curvature.

The membrane curvature at the OS disk rims is highly anisotropic. Whereas the membrane at the rim is tightly negatively curved to a 12 nm radius towards the disk center, the membrane curvature along the disk circumference is negligible at the scale of individual dimers. Membrane curvature anisotropy can be driven by linear oligomerization of spontaneously curvature generating membrane proteins⁸². Transient overexpression of Prph2^{C150S} in HEK293 drives the appearance of highly curved intracellular membranes but these curved membranes are punctate and do not form elongated tubes like those seen in HEK293 cells overexpressing WT Prph2 (Fig. 2B)⁴⁴; suggesting a role for Prph2 polymerization in establishing the curvature anisotropy at the OS disk rim.

Prph2 self-assembly is mechanistically linked to multiple steps of OS disk morphogenesis. Disk morphogenesis begins with F-actin mediated evagination of the connecting cilium plasma membrane^{83,84,85}. Initiation of disk rim formation is mediated by the Prph2 CTER⁷⁸. In the absence of Prph2, OS disk material, most notably rhodopsin but also Rom1, is shed in ectosomes from the connecting cilium⁸⁶. Disk rim advance and disk enclosure are driven by disulfide-mediated Prph2 polymerization^{64,73,78}. Blocking disulfide mediated Prph2 polymerization drives the appearance of membranous whorls instead of evenly ordered disks in the OS⁷³, and perturbations in the efficiency of disulfide mediated Prph2 polymerization delay ROS disk enclosure²⁶.

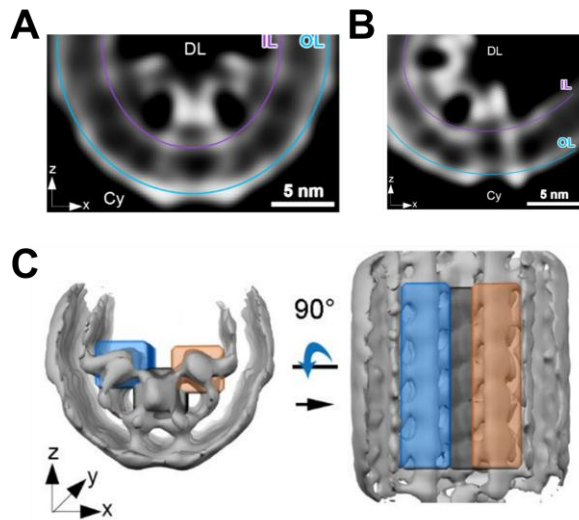


Figure 7: Cryo-ET of the native rod disk rim reveals three parallel belts of Prph2/Rom1 dimer chains. (A) Cross-section through the inner belt. (B) Cross-section through an outer belt. (C) 3D projection of the tridentate scaffold at the disk rim. All panels reproduced from Pöge et al. 2021⁷¹.

Structural biology of Prph2, Rom1, and the disk rim

High resolution single-particle cryo-EM has solved the structure of the Prph2:Rom1 heterodimer and established the dimeric stoichiometry of the non-covalent core subunit of disk rim scaffold assembly⁵⁸. The structure reveals the organization of the EC2 domains, including the positioning and accessibility of Prph2-C150 and Rom1-C153, and it

shows a closed arrangement of the TM domain bundles. The structure rationalizes decades of biochemical data on Prph2 and Rom1 assembly and it explains the assembly defect associated with missense mutations near the Prph2 dimerization interface (e.g. *PRPH2*^{L185P}). Significantly, the TM bundles of the monomers within the solved heterodimer cross the membrane almost in parallel, challenging previous conjectures of a V-shaped core complex⁴⁴.

The structure of the in-situ disk rim scaffold has been directly visualized by cryo-electron tomography (cryo-ET) of the in-situ scaffold in the rims of murine rod disks (Fig. 7)⁷¹. The disk rim scaffold is a ‘tridentate’ structure made of three parallel belts of polymerized Prph2/Rom1 dimers. The TM domains of the dimers in adjacent belts cross the membrane at a ~60° angle to each other. There appear to be EC2-mediated inter-belt

contacts within the resolved structure; possibly corresponding to contacts between the fin-like projections formed by EC2 helix D at the top of both proteins. This region of Prph2 is a hotspot for pathogenic missense mutations, such as *Prph2*^{R195L}. Mutations in this region may disrupt an unidentified inter-belt non-covalent interaction and may not significantly impact dimer chain polymerization efficiency.

Knowledge gaps and aims of the work

Although high resolution cryo-EM and cryo-ET have clarified the structure and Prph2/Rom1 subunit stoichiometry of the OS disk rim scaffold, key mechanistic knowledge gaps remain. The molecular function of Prph2 in generating membrane curvature is unambiguous, yet the mechanism(s) through which it does so remains elusive. Despite evidence suggesting that dimer chain oligomerization and scaffold maturation are supported by uncharacterized non-covalent Prph2/Rom1 interactions, these interactions remain hypothetical. In the absence of a biochemical method for resolving individual oligomeric species of Prph2 and Rom1, subunit stoichiometry in OS membranes is poorly defined.

Several pathogenic missense mutations in *PRPH2* are known to disrupt discrete stages of Prph2 and Rom1 self-assembly^{68,87}. However, the impact of most reportedly pathogenic *PRPH2* mutations on protein oligomerization and OS disk rim scaffold assembly has not been examined. The precise cellular, tissue homeostatic, and biomolecular mechanisms driving the pathogenesis of the many distinct IRDs associated with mutations in *PRPH2* (and *ROM1*) remain unknown. Clear correlations between

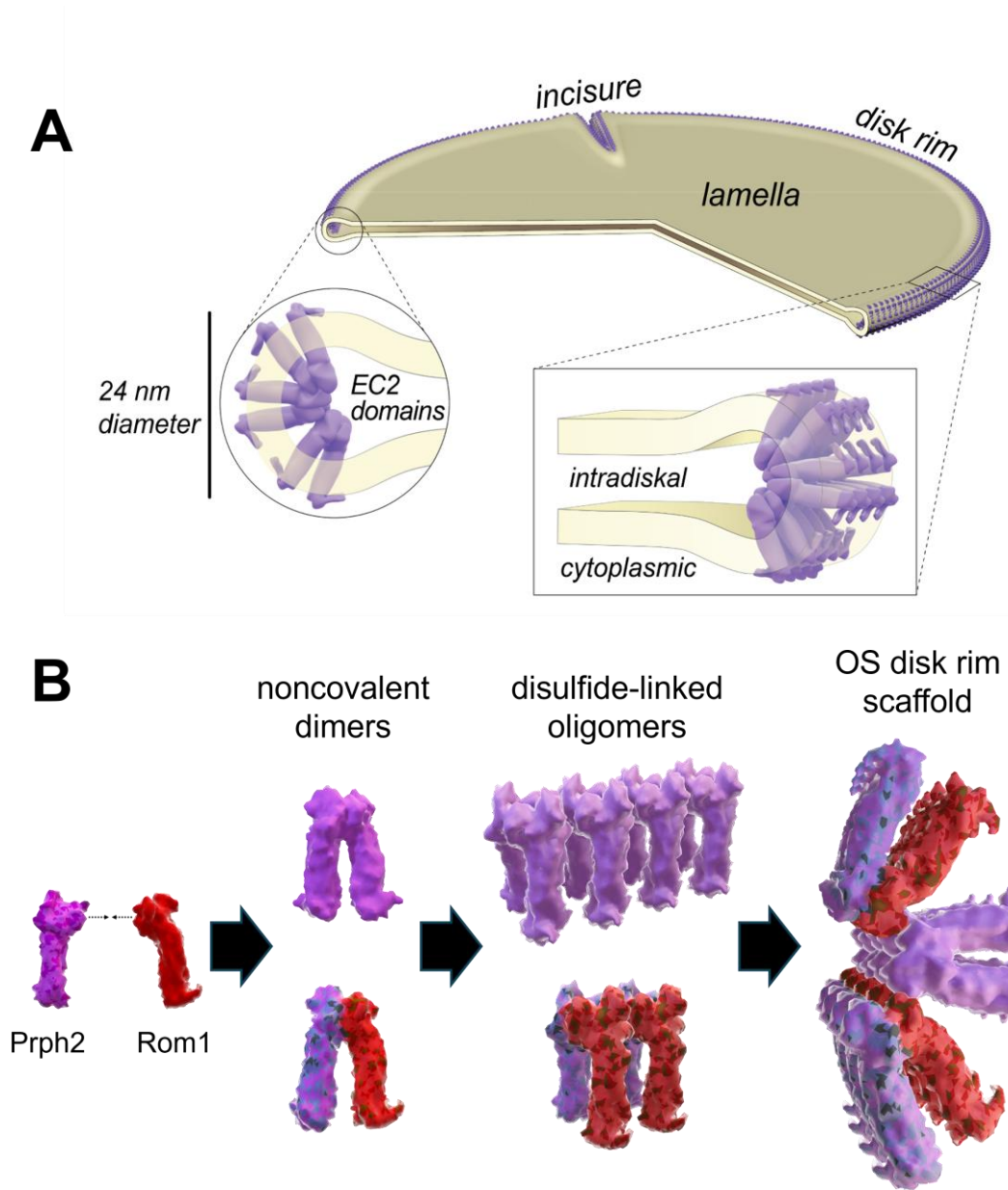


Figure 8: Current model of Prph2 and Rom1 scaffolding OS disk rims. (A) The extreme membrane curvature at the rims and incisures of OS disks is shaped by a transmembrane protein scaffold comprising three parallel belts of Prph2/Rom1 dimers. (B) Disk rim scaffold assembly is a multistep process. Prph2 and Rom1 associate into non-covalent (NC) heterodimers and Prph2 homodimers. The NC dimers polymerize via Prph2-C150/Rom1-C153 mediated inter-dimer disulfide bonding, giving rise to heterotetramers and Prph2 homopolymers of varying length. Three parallel belts of Prph2 and Rom1 polymers form the disk rim scaffold. Rom1 is shown in the scaffold model in B to illustrate relative abundances in OS membranes; the limited resolution of the current map precludes definitive assignment of individual subunits within the structure.

genotype and clinical phenotype remain elusive. Characterization of the growing number of *PRPH2* variants of unknown significance (VUSs) is severely bottlenecked by the low throughput of existing methods.

The studies presented in this thesis address these gaps by investigating Prph2/Rom1 self-assembly and the functional consequences of *PRPH2* mutations through biochemical, animal model, and computational approaches. The central hypothesis is that the OS disk rim scaffold emerges from a web of non-covalent interactions and inter-dimer disulfide bonding that drives multistep Prph2 and Rom1 self-assembly. Their assembly begins with non-covalent dimerization of Prph2 and Rom1 monomers into Prph2 homodimers and Prph2:Rom1 heterodimers that are present in a ~2:1 ratio in ROS membranes. These core non-covalent dimers subsequently polymerize into linear chains linked by intermolecular disulfide bonds mediated by the free cysteine (Prph2-C150 or Rom1-C153) within each EC2 domain. Longer polymers are exclusively homomeric Prph2, whereas Rom1 is restricted to the smallest oligomers (non-covalent dimers and heterotetramers) but is nevertheless required to support higher-order Prph2 homooligomerization¹⁸. These homo- and heterooligomers then associate non-covalently to form three belts that circumscribe the disk periphery and incisures (Fig. 8B). This supramolecular scaffold shapes the OS disk rim membranes.

The primary aims of this work are therefore (1) to refine methods for resolving Prph2/Rom1 polymer species and stoichiometry, (2) to dissect the mechanistic contributions of inter-dimer interactions to self-assembly and membrane curvature, and (3) to develop a high-throughput strategy for molecular phenotyping of *PRPH2* VUSs.

CHAPTER 2

A REOPTIMIZED SEDIMENTATION APPROACH ENHANCES REPRODUCIBILITY AND CAPTURES THE FULL RANGE OF PRPH2/ROM1 POLYMER SIZES

Introduction

Previous investigations into Prph2 and Rom1 structure determined that monomers of each self-assemble in at least two steps to form a large, disulfide-stabilized, linear, transmembrane complex at the outer segment disk rims^{44,69,67,66}. These self-assembly studies have relied on an established velocity sedimentation method to characterize detergent solubilized complexes^{44,69,65}. This approach has revealed that Prph2 and Rom1 are detergent extracted from their native membranes as polymers of varying size. The smallest form contains no intermolecular disulfide bonds⁶⁷. The ratio of intermolecular disulfide bonding increases with complex size in the intermediate forms. And the largest forms, which do not contain Rom1, comprise homomeric Prph2 that is completely disulfide bonded⁶⁹. Negative staining TEM published by our lab in 2020 demonstrated that these detergent-extracted polymers are linear chains of a non-covalently associated core subunit (Fig. 9C & D)⁴⁴. At that time, the core subunit was understood to be tetrameric based on a substantial body of historical work^{67,69,66,61}; however, more recent cryo-EM studies have established the dimeric stoichiometry of the non-covalent core subunit^{58,71}.

Recombinant Prph2 overexpressed in COS-1 or HEK293 cells forms disulfide-linked polymers that sediment like those seen in ROS^{44,65,55}. Insights from velocity

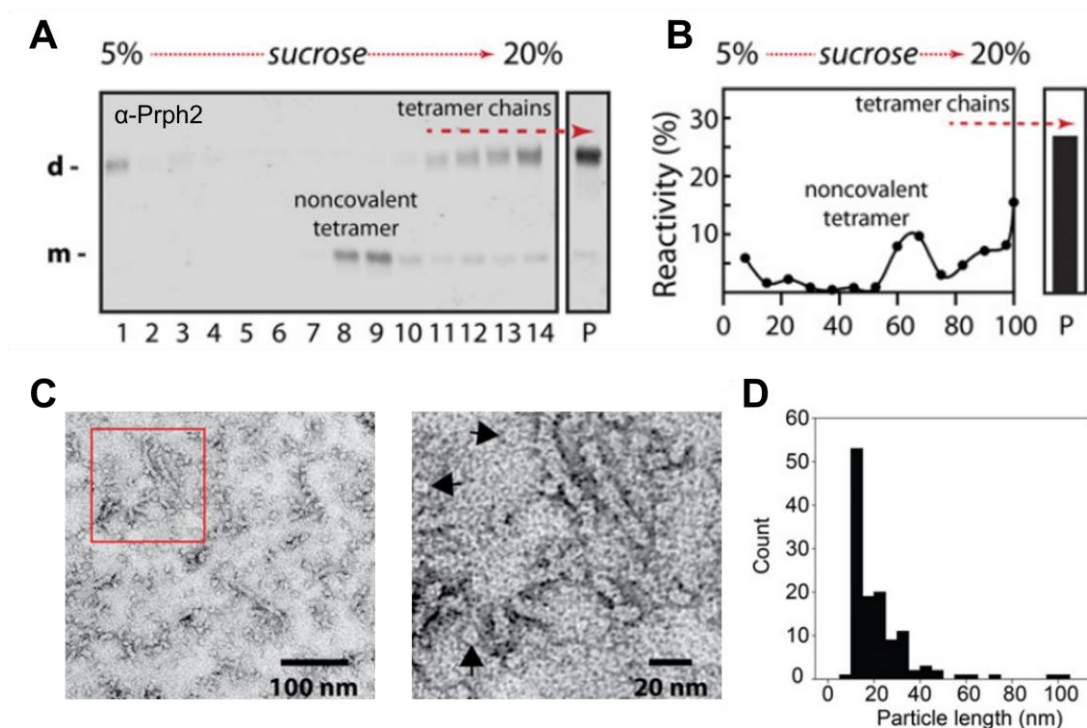


Figure 9: Prph2 is detergent extracted as linear polymers of heterogeneous length, the largest of which pellet under standard sedimentation conditions. All panels are modified from Milstein et al. (2020)⁴⁴ (A) Non-reducing SDS-PAGE Western blot of Triton X-100 solubilized wild-type recombinant Prph2 from HEK293 overexpression after velocity sedimentation under standard conditions. Under non-reducing conditions, Prph2 migrates as monomer ('m') and disulfide-linked dimer ('d') bands; a substantial proportion of the input Prph2 appears in the pellet fraction ('P'). (B) Sedimentation profile plotting α -Prph2 reactivity by fraction position within the gradient. The bar chart shows reactivity of the pellet. (C) Negative staining TEM of CHAPS solubilized Prph2 immunopurified from bovine ROS, revealing linear polymers of varying lengths. (D) Histogram of Prph2 polymer lengths measured from TEM images. Note that the vast majority of Prph2 polymers are under 50 nm. Panels were generated and interpreted under the historical tetrameric core subunit model prevailing at the time of publication.

sedimentation of heterologously expressed Prph2 variants are fundamental to the current understanding of Prph2 self-assembly. For example, Prph2^{L185P}, a missense variant causing retinitis pigmentosa in humans, was found to disrupt non-covalent subunit assembly, generating abnormally disulfide bound dimers⁶⁸. Similarly, velocity sedimentation analysis also demonstrated that Prph2^{C150S}, which lacks the EC2 cysteine responsible for the intermolecular disulfide bonds linking the polymers, and which

severely disrupts OS structure in transgenic mice⁷⁴, does not self-assemble beyond the non-covalently associated tetramer⁶⁶. These and other advances established velocity sedimentation as the gold standard approach for molecular phenotyping to determine the effects of Prph2 mutations on self-assembly.

However, despite these advances, the original sedimentation method has notable limitations that have limited the scope of the research questions to which it can be applied. Manual fractionation of small volume (~2 ml) sucrose gradients is technically challenging; reproducibility of sedimentation is limited by the dexterity of the experimenter. Additionally, a substantial proportion of larger Prph2 polymers pellet under standard sedimentation conditions (Fig. 9A & B)^{44,55}; greatly restricting the dynamic range of the assay. To address these limitations, we have reoptimized the velocity sedimentation method by (1) implementing a semi-automated mechanical approach to improve the reproducibility of gradient fractionation; and (2) reducing the centrifugation speed to prevent larger Prph2 polymers from pelleting, thus expanding the dynamic range of the assay.

Materials and Methods

Bovine ROS

Purified dark adapted bovine rod outer segments were obtained from InVision Bioresources (Seattle, Washington). Under dim red light, the frozen bulk ROS pellets were thawed and resuspended in chilled ROS aliquoting buffer (100 mM KPi, 1mM MgCl₂, 0.1 mM EDTA, 10% Sucrose, pH 7.0) to a final total protein concentration of ~3

mg/ml and aliquoted into 1.5 ml tubes. The ROS aliquots were frozen in either LN₂ or a dry ice ethanol slurry and stored at -80° until use.

Recombinant protein expression

HEK293 cell culture and recombinant protein expression were performed as previously described^{55,44}. Briefly, HEK293 cells were cultured in DMEM media containing 10% FBS and 5% penicillin/streptomycin, under standard conditions at 37° with 5% CO₂. All cells used for transient transfections had been passaged fewer than 20 times. Transient transfections were done using X-tremeGENE 9 reagent (Roche), according to the manufacturer's instructions. Constructs used for transfection are summarized in Appendix A.

Membrane protein solubilization

Purified ROS membranes were thawed and then photobleached for 20 minutes on ice under a desk lamp. The photobleached ROS was pelleted by centrifuging for 10 minutes at 10k rcf at 4°. The supernatants were discarded, and the pelleted ROS was resuspended to 3 mg/ml total protein in chilled resuspension buffer consisting of 1x phosphate buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 5.3 mM Na₂HPO₄, 0.7 mM KH₂PO₄, pH 7.4) supplemented with 50 mM NEM and 1x cOmplete ULTRA EDTA-free protease inhibitor cocktail. The resuspended ROS was incubated for 20 minutes at RT to allow the NEM to alkylate the free sulfhydryls, and then the ROS was returned to ice. An equal volume of chilled 2x solubilization buffer, comprising 1xPBS with 1x protease

inhibitor cocktail and either 2% w/v Triton X-100 or 2% w/v DDM-CHS, was incrementally added to the resuspensions with gentle vortexing; for final concentrations of 1% detergent and 1.5 mg/ml total protein. After solubilizing for 1 hour on ice with occasional gentle vortexing, the ROS was centrifuged for 30 minutes in a TLA-45 rotor in a Beckman Optima TLX ultracentrifuge at 45k rpm (90k rcf) at 4°. The supernatants, containing the detergent-extracted protein, were collected and either used immediately or stored overnight at 4° for use the following day. The pellets were resuspended by vortexing in Laemmli buffer and stored at 4° for SDS-PAGE analysis to confirm detergent extraction of Prph2 and Rom1.

Transfected HEK293 cell pellets were solubilized following essentially the same protocol, except they were initially resuspended to 20 mg/ml total protein and solubilized to 10 mg/ml final total protein.

Velocity sedimentation

Sucrose solutions (5%, 10%, 15%, and 20% w/w) were prepared in gradient buffer (1x PBS, 0.1% w/v detergent, 10 mM NEM, pH 7.4) and confirmed by refractometry before use. To pour the gradients, 500 µl of each sucrose solution was layered in order of decreasing sucrose percentage, in Ultraclear TLS-55 tubes. The gradients were then equilibrated for 1 hour at room temperature, rendering them continuous, and then placed in ice for at least 30 minutes before use.

100 µl of ROS or HEK293 cell detergent extract was layered on top of each gradient, bringing the final total volume of the loaded gradients to 2100 µl. The gradients

were then sedimented in a TLS-55 rotor in a Beckman Optima TLX ultracentrifuge for 16 hours at 25k rpm (41.5k rcf) at 4°. The bottoms of the gradient tubes were punctured and then the gradients were fractionated using a semi-automated method (described in the next section) and ~140 µl was collected in each fraction. The sucrose concentration in each fraction was measured by refractometry. Gradient fractions were stored at 4° until use.

To collect any pelleted material, the bottom of each gradient tube was sealed with parafilm and 55 µl 3xLSB was added. Next, the tubes were vortexed 3x 1 minute at maximum speed before 105 µl ddH₂O was added and the tubes were vortexed again. The resuspended insoluble fractions were then transferred to fresh 1.5 ml tubes and stored at 4° until use. Fractionated gradients were analyzed by SDS-PAGE as described below, or BN-PAGE (described in Chapter 3), and western blotting. Cumulative sedimentation profiles were modeled using locally estimated scatterplot smoothing (LOESS) regression in RStudio. Optimal LOESS spans for each experimental group were selected by minimizing the Akaike Information Criterion (AIC)⁸⁸.

Semi-automated fractionation

Gradient tubes (2.2 ml Ultraclear TLS-55) were marked along their seams prior to gradient preparation to ensure reproducible puncture placement. Following the 16 hour centrifugation at 25k rpm (41.5k rcf) at 4°, the buckets containing the gradients were transferred to sit upright in ice in their storage rack. Gradients remained in their buckets

in ice until immediately prior to fractionation and were handled exclusively with forceps to minimize warming.

Semi-automated fractionation utilized: (1) a 3D printed tube punch with a hole at its base for a 27 gauge needle. (2) A rubber sealing cap to connect the top of the tube to pneumatic tubing. (3) A screw drive precision Hamilton syringe pump (Harvard Apparatus, Pump 11 Plus). And (4) a three-way valve forming an airtight connection between the syringe and the tubing attached to the sealing cap.

To prepare the device, a clean 27G1/2 needle, with its hub plugged with parafilm, was inserted into the hole in the base of punch. For fractionation, the gradient tube was removed from its bucket and placed in the punch. The top of the tube was lightly lubricated with vacuum grease. The valve was set to vent the cap tubing to room air and then the sealing cap was applied to the aligned gradient tube, driving the needle through the bottom of the gradient tube. The valve was then closed to the cap tubing, then the capped and punctured gradient was transferred upright to a ring stand. The valve was switched to connect the syringe to the sealing cap, then the pump was activated at a constant and maintained flow rate of 1 ml/min, and ~140 μ l fractions were collected.

When the gradient was fully eluted, collected fractions (~15 per gradient) were placed in ice, and the pelleted material was collected as described in the preceding 'velocity sedimentation' section. Fraction volumes were measured by pipette, then stored at 4° until use.

SDS-PAGE and Western Blotting

Proteins in either detergent extracts or fractionated sucrose gradients were resolved by SDS-PAGE using 10% acrylamide TGX minigels (BioRad) under either reducing or non-reducing conditions. Briefly, samples were combined 2:1 with 3x Laemmli Sample Buffer (180 mM Tris-HCl pH 6.8, 24% v/v glycerol, 6% w/v SDS, 0.03% w/v bromophenol blue, $\pm$ 5% β -mercaptoethanol), then boiled for 10 minutes. The tubes were briefly cooled on ice and then spun for 2 min at 16.5k rcf prior to loading. The loaded gels were run in 1x SDS Tris-Glycine tank buffer (25 mM Tris, 192.3 mM glycine, 1% SDS, pH 8.3) at 80 V for 20 min, then 100 V for ~60 min. Following electrophoresis, the SDS-PAGE gels were removed from their cassettes and then equilibrated 3x5 min at RT in Tris-Glycine Towbin transfer buffer (25 mM Tris, 192.3 mM glycine, pH 8.3) with gentle rocking.

Proteins in the equilibrated gels were transferred to Immobilon-FL (Millipore) PVDF membranes in a Mini-Trans Blot Cell (BioRad) filled with chilled 1x Towbin transfer buffer, for one hour at 100 V on ice. After transfer, the membranes were rinsed 2x 5 min with ddH₂O at RT with gentle rocking and then placed on a clean piece of Whatman filter paper to dry overnight and stored until ready to develop.

Dried membranes were reactivated by rinsing for 30 sec in 100% meOH, followed by 30 sec in ddH₂O, then equilibrated 2x5 min in 1xPBS. Membranes were blocked in 1:2 PBS Odyssey Blocking Buffer (OBB; LiCor):1xPBS for one hour at RT with gentle rocking. The blocked membranes were then incubated in primary antibody mix comprising primary antibodies against Prph2 and/or Rom1 (Appendix B) diluted in

1:2 OBB:1xPBS and 0.1% Tween-20, for 1 hr at RT with gentle rocking. Primary antibodies were washed out by rinsing the blots 4x5 min in 1xPBST (1xPBS + 0.1% Tween-20). Next, the blots were incubated in secondary antibody mix, comprising host-specific IR800 and IR690 fluorescent secondary antibodies (Appendix B) diluted in 1:2 OBB:1xPBS, for 1 hr at RT with gentle rocking. Finally, the blots were washed 4x5 min in 1xPBST and then 2x5 min in 1x PBS at RT with gentle rocking. The developed blots were scanned on an AzureSapphire Biomolecular Imager. Densitometry measurements were done with either AzureSpot software or ImageJ. Densitometry data analysis was done in RStudio.

Results

Prph2 and Rom1 are detergent-extracted from their native membranes (either purified ROS or transfected HEK293 cells) as disulfide-linked linear polymers of varying lengths^{44,58}. Previous studies have characterized these polymers using an established velocity sedimentation method^{65,69,44}. However, the reproducibility of sedimentation has been limited by the difficulty of manually fractionating small volume (~2 ml) gradients. Additionally, a substantial proportion of the larger Prph2 polymers pellet at the standard centrifugation speed (Fig. 9A & C), which restricts the dynamic range of the assay^{55,44}. To overcome these limitations, we reoptimized the velocity sedimentation method by implementing a semi-automated fractionation approach to improve reproducibility. And we expanded the dynamic range of the assay by reducing the sedimentation centrifugation speed to retain the larger forms of Prph2 in the soluble fractions.

Reoptimized sedimentation of Triton X-100 solubilized bovine ROS

We validated the reoptimized sedimentation method using Triton X-100 extracts of bovine ROS (Fig. 10). Gradients were mechanically fractionated and the fractions were analyzed by non-reducing SDS-PAGE and western blotting with α -Prph2 (Fig. 10A) and α -Rom1 (Fig. 10C). Using this approach, both Prph2 and Rom1 were entirely retained within the soluble fractions and there was no pelleting of either protein (Fig. 10A & C; lane “P”). In contrast, ~30% of the Prph2 in WT Triton ROS extracts pellets under the original velocity sedimentation conditions⁴⁴. Consistent with prior studies, both Prph2⁸⁹ and Rom1⁵⁰ run as two bands on non-reducing SDS-PAGE, corresponding to monomeric and disulfide-linked dimeric (SS-dimer) forms (Fig. 10A). Neither the monomer nor the SS-dimer are native forms of WT Prph2 or Rom1; their appearance on SDS-PAGE is a product of non-covalent dimer and disulfide-linked oligomer denaturation with SDS. Prph2 and Rom1 in the lowest sucrose percentage fractions, which contain the smallest forms of both Prph2 and Rom1, are predominantly monomeric. The ratio of disulfide bound dimers SS- dimers to monomers increased with sucrose fraction sucrose percentage for both proteins. Rom1 is only present in lower sucrose fractions. The Prph2 in the highest sucrose fractions is entirely disulfide bonded.

Sedimentation profiles, plotting protein abundance in gradient fractions as a function of sucrose percentage, show that Prph2 sediments in two peaks: a taller, narrower peak of lower mobility and a shorter, much broader peak of higher mobility (Fig. 10B). Rom1 sediments in a single low mobility peak that overlaps with the lower mobility Prph2 peak (Fig. 10D & F). These findings are consistent with previous results using the original sedimentation method⁶⁹. Significantly, no Prph2 or Rom1 was detected

in the pellet fractions, highlighting the assay's expanded dynamic range that captures the broad and continuous distribution of disulfide-linked Prph2 polymer lengths.

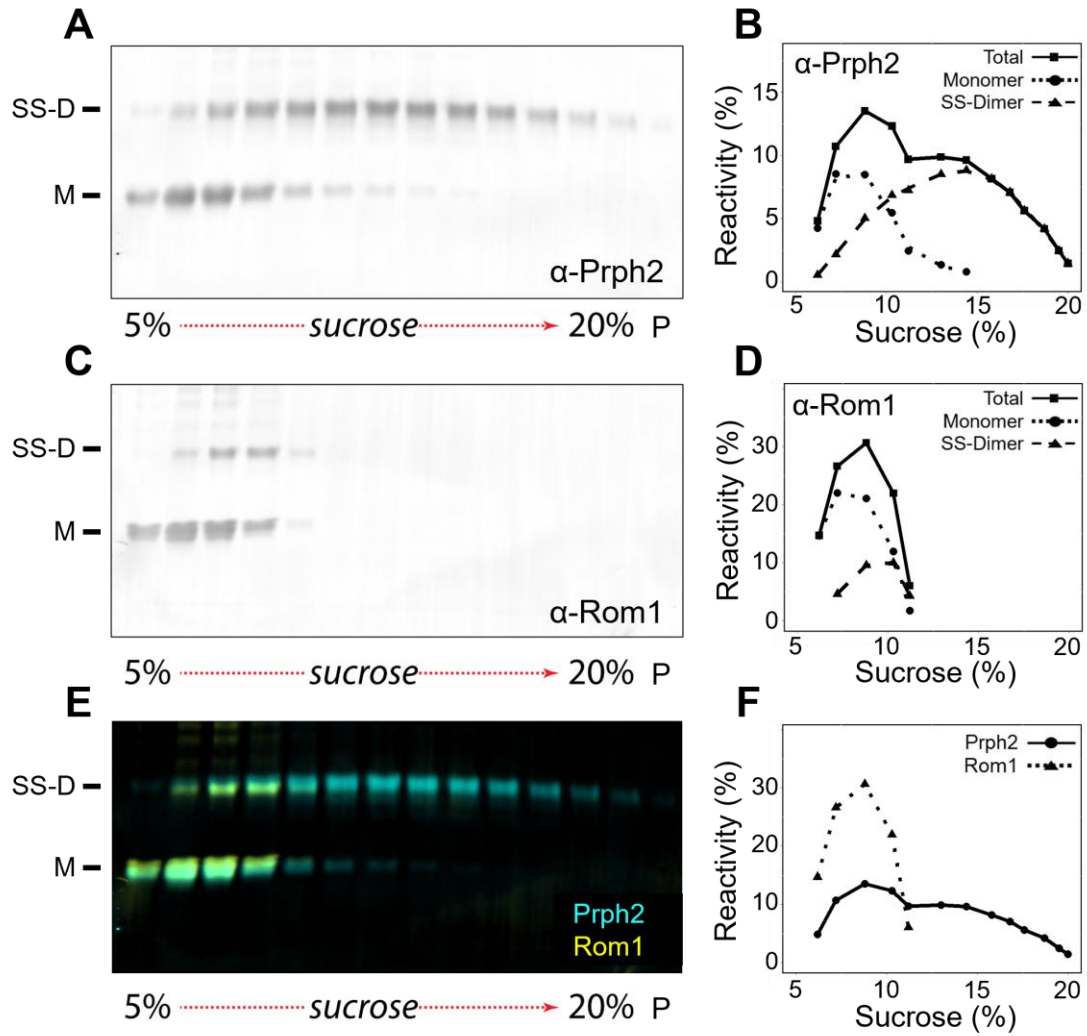


Figure 10: Reoptimized velocity sedimentation analysis of bovine ROS Triton X-100 extract. (A, C, E) Western blot from non-reducing SDS-PAGE of gradient fractions. The pellet fraction is in the last lane ('P') on the right. (A) The Prph2 channel. (B) Sedimentation profile of the data in panel A. Linear spline fits of SS-dimer (dashed), monomer (dotted), and lane total reactivity (solid). (C) The Rom1 channel. (D) Sedimentation profile the data in panel C. Plotted as in B. (E) Overlay of A and C. Prph2 is cyan and Rom1 is yellow. (F) Profile comparing lane total reactivities for Prph2 (solid) and Rom1 (dotted).

A semi-automated fractionation approach improves reproducibility

The semi-automated fractionation method decreases variability in the volumes of the collected fractions (Fig. 11A), enabling more robust comparisons between gradients sedimented in separate experiments. LOESS regressions of α -Prph2 reactivity (Fig. 11B) and α -Rom1 reactivity (Fig. 11C) from non-reducing SDS-PAGE analysis of n= 11 fractionated gradients of Triton extracted bovine ROS across five independent experiments show tight 95% confidence intervals; highlighting the high level of reproducibility that mechanical fractionation allows.

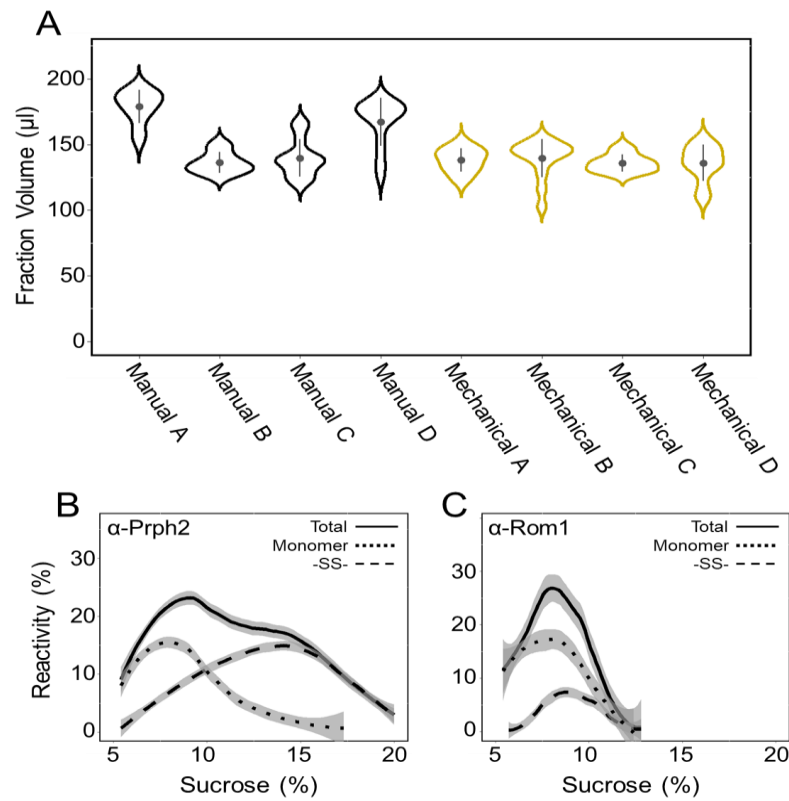


Figure 11: Semi-automated fractionation improves reproducibility. (A) Violin plot comparing gradient fraction volumes from manual (black) vs. semi-automated (gold) fractionation. (B) LOESS regressions of α -Prph2 reactivity from non-reducing SDS-PAGE analysis of 11 fractionated gradients of Triton bovine ROS extracts. Shading indicates 95% confidence intervals. The solid line represents lane total reactivity, the dotted line represents monomer reactivity, and the dashed line represents disulfide-linked dimer reactivity. (C) LOESS regressions of α -Rom1 reactivity from the gradients shown in B, plotted as in B.

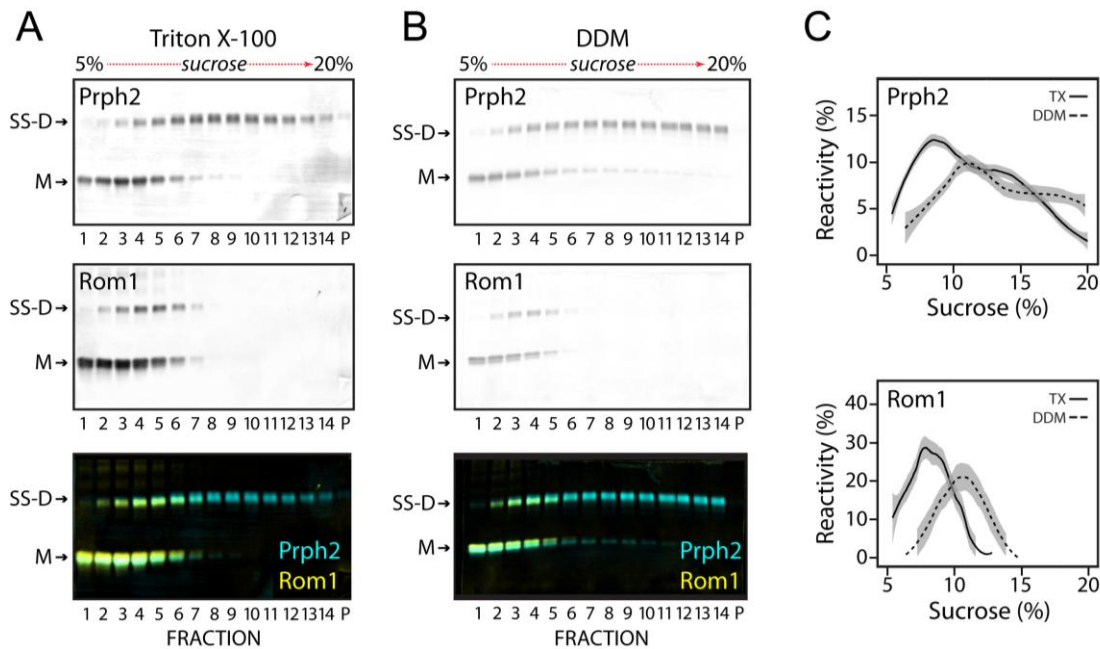


Figure 12: Comparison of reoptimized sedimentation with Triton X-100 or DDM bovine ROS extracts. (A) Western blot from non-reducing SDS-PAGE analysis of a fractionated gradient of Triton extracted bovine ROS. The Prph2 channel is on the top, the Rom1 channel is in the middle, and the overlay is on the bottom. (B) Western blot from non-reducing SDS-PAGE analysis of DDM extracted bovine ROS. (C) LOESS regressions of lane total α -Prph2 (top) and α -Rom1 (bottom) reactivity, comparing n=11 gradients of Triton extracted bovine ROS (“TX”, solid line) and n=3 gradients of DDM extracted bovine ROS (“DDM”, dashed line). Shading indicates 95% confidence intervals.

Detergent dependence of Prph2/Rom1 polymer sedimentation

Previous velocity sedimentation studies of Prph2 and Rom1 have used Triton X-100 for protein extraction^{55,44,69,78}, while recent cryo-EM structural studies have used n-dodecyl- β -D-maltoside (DDM)^{58,90} as the extraction detergent. To assess potential detergent effects on polymer appearance, we compared Triton and DDM bovine extracts using the reoptimized sedimentation method (Fig. 12).

As in Triton ROS extracts, DDM solubilized Prph2 and Rom1 resolved as monomeric and SS- dimeric bands on non-reducing SDS-PAGE (Fig. 12). In both detergents, the Prph2 and Rom1 in lowest sucrose percentage fractions were

predominantly monomeric, with the ratio of SS- dimers to monomers increasing with sucrose percentage. Rom1 was confined to the lower sucrose fractions (Fig. 12B).

Sedimentation peaks for DDM-extracted Prph2 and Rom1 are qualitatively similar to those in Triton but are slightly shifted towards a higher sucrose percentage. Whereas Prph2 monomers were undetectable in the highest sucrose fractions with Triton ROS extracts, Prph2 monomers were detectable in every soluble fraction with the DDM extracts (n = 4 for DDM, n = 11 for Triton).

Table 1 summarizes mean sucrose percentages, sedimentation distances, and gradient distance percentages for the sedimentation peaks of SS- dimer, monomer, and lane total reactivity peaks with α -Prph2 or α -Rom1 in each detergent. Fraction total α -Prph2 reactivity peaked at 9.0% sucrose (39.2% gradient distance) with Triton ROS extracts and at 11.5% sucrose (52.4% gradient distance) with DDM ROS extracts. Fraction total α -Rom1 reactivity peaked at 8.4% sucrose (35.6% gradient distance) in Triton and at 10.4% sucrose (47.3% gradient distance) in DDM.

Channel	α -Prph2	α -Prph2	α -Prph2	α -Prph2	α -Prph2	α -Prph2
Detergent	Triton	Triton	Triton	DDM	DDM	DDM
Band	SS-Dimer	Monomer	Lane Total	SS-Dimer	Monomer	Lane Total
% Sucrose	13.8	7.4	9	18.4	9.9	11.5
Distance (cm)	1.96	0.89	1.21	2.73	1.37	1.62
% Distance	63.4	28.7	39.1	88	44.3	52.4

Channel	α -Rom1	α -Rom1	α -Rom1	α -Rom1	α -Rom1	α -Rom1
Detergent	Triton	Triton	Triton	DDM	DDM	DDM
Band	SS-Dimer	Monomer	Lane Total	SS-Dimer	Monomer	Lane Total
% Sucrose	9.2	7.6	8.4	11.8	9.5	10.4
Distance (cm)	1.25	0.94	1.11	1.68	1.32	1.47
% Distance	40.4	30.4	35.8	54.1	42.4	47.3

Table 1: Average sucrose % and migration distances for sedimentation fractions with peak SS-dimer, monomer, or lane total immunoreactivity from gradients of bovine ROS extracts.

Reoptimized velocity sedimentation analysis of recombinant Prph2 missense variants overexpressed in HEK293

We leveraged the expanded dynamic range of the reoptimized sedimentation assay to revisit previous findings that recombinant Prph2 overexpressed in transfected HEK293 cells forms a similar complement of polymers chains as it does endogenously in ROS^{44,65}. To that end, we characterized Prph2 polymers in Triton extracts from transiently transfected HEK293 cells using the reoptimized sedimentation method (Fig. 13).

The western blot in Figure 13A shows a fractionated gradient of Triton-solubilized WT bovine Prph2 from HEK293 overexpression. The profile in Figure 13B shows the band intensities from that blot and a replicate gradient. The results are essentially comparable to those seen for Prph2 in Triton bovine ROS extracts (Fig. 10A). The recombinant WT Prph2 resolved as monomer and SS- dimer bands on non-reducing SDS-PAGE, and it sedimented in a single peak at ~9% sucrose, with a rightward skew indicating decreasing abundance with increasing polymer size.

Prph2^{C150S}, in which the intermolecular disulfide forming EC2 cysteine has been mutated to a serine, runs almost entirely as a monomer on non-reducing SDS-PAGE of fractionated gradients (Fig. 13C) and it sedimented within a single narrow peak in the lower sucrose fractions (Fig. 13D). This agrees with previously reported characterization of this variant, which concluded that Prph2^{C150S} is unable to polymerize into disulfide linked chains⁶⁶.

Prph2^{L185P} is a well characterized Prph2 mutant with defective non-covalent

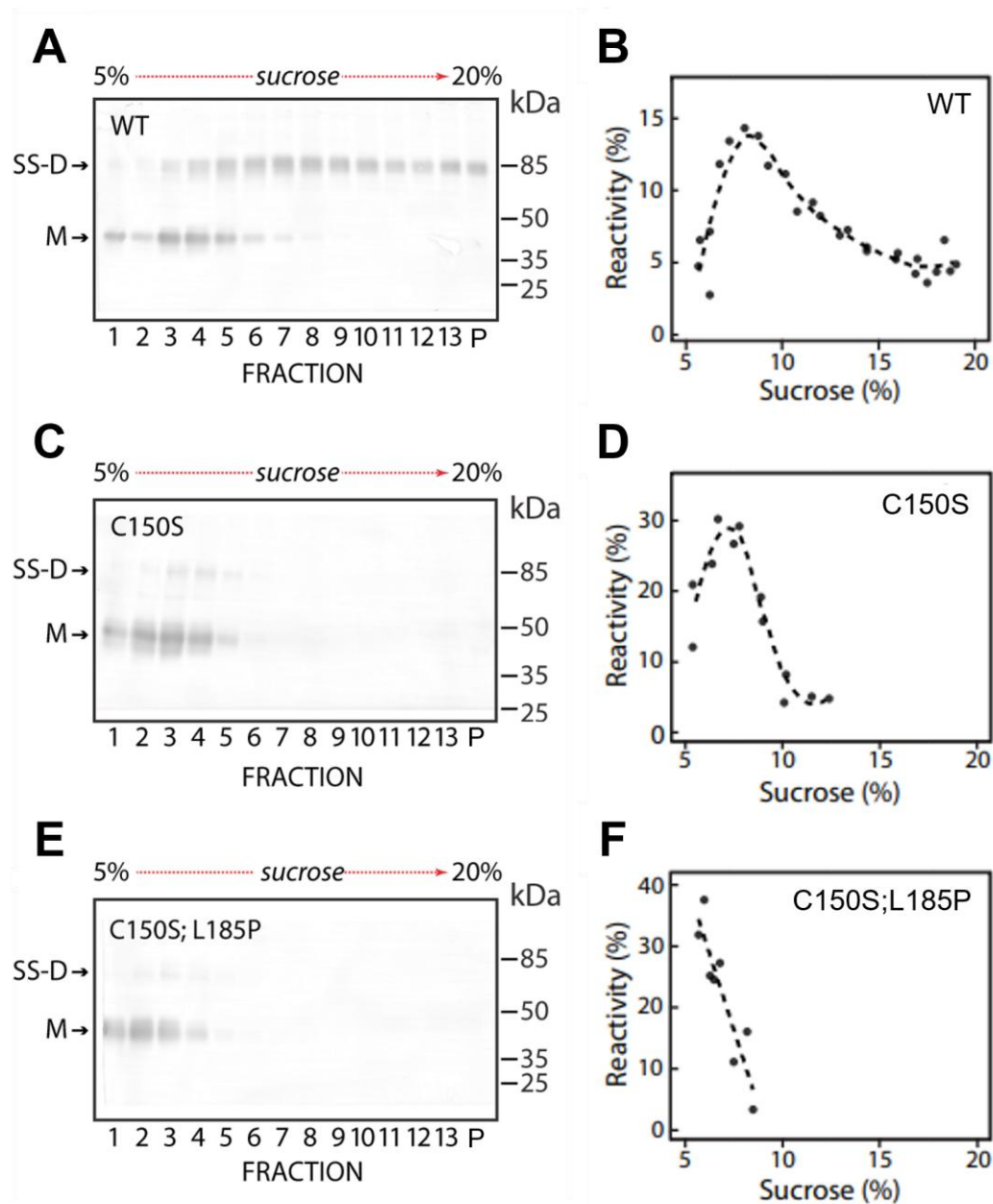


Figure. 13: Reoptimized sedimentation of recombinant Prph2 missense variants. (A) Western blot from non-reducing SDS-PAGE analysis of a fractionated gradient of recombinant WT Prph2 in transfected HEK293 Triton extract. (B) Scatterplot sedimentation profile of α -Prph2 reactivity by fraction sucrose percentage showing data from the blot in A and a replicate gradient. The dashed line is a LOESS regression of the plotted data. (C) Western blot showing a fractionated gradient of Triton extracted recombinant C150S-Prph2. (D) Scatterplot sedimentation profile showing the blot in C and a replicate gradient, as in B. (E) Western blot showing a fractionated gradient of Triton-extracted C150S-L185P-Prph2. (F) Scatterplot sedimentation profile of the data shown in E and a replicate gradient. No valid LOESS curve could be fit to the data in F, the trend is linear.

subunit assembly⁶⁸. We hypothesized that Prph2^{L185P} may disrupt non-covalent dimerization and, as such, double-mutant Prph2^{C150S;L185P} may be natively monomeric, unable to form either durable non-covalent associations or intermolecular disulfide bonds. Figure 13E shows a western blot of non-reducing SDS-PAGE analysis of a fractionated gradient loaded with Prph2^{C150S;L185P} HEK293 Triton extract. Like Prph2^{C150S}, Prph2^{C150S;L185P} resolved as a single monomeric band on non-reducing SDS-PAGE and sedimented entirely within the lowest sucrose fractions. The sedimentation profile in (Fig. 13F) revealed a steep linear decline in protein abundance within the lowest sucrose fractions across two replicate gradients. This indicates Prph2^{C150S;L185P} is likely present in a smaller form than Prph2^{C150} in the transfected cell extracts. However, an alternate method, such as native PAGE, capable of resolving individual Prph2 size species is required to determine if the double mutant is truly monomeric.

Discussion

This chapter describes the development of a reoptimized velocity sedimentation method that retains the complete size range of detergent-extracted Prph2/Rom1 polymers from ROS membranes or transfected HEK293 cells within the soluble fractions. These data also demonstrate that the new semi-automated approach to sucrose gradient fractionation improves assay reproducibility by reducing variability in collected fraction volume. The results are consistent with recent negative staining TEM results from Milstein et al. (2020), which indicate that Prph2 and Rom1 are detergent extracted from

their native membranes as a heterogeneous population of linear disulfide-linked polymer chains⁴⁴.

Sedimentation profiles of Triton X-100 and DDM ROS extracts were similar, demonstrating that solubilized Prph2 is extracted from its native membranes as disulfide-linked polymer chains in both detergents. However, DDM extracted polymers sedimented slightly faster than those solubilized in Triton (Fig. 12C). Because DDM:CHS micelles and Triton X-100 micelles have approximately equal mass, this difference is likely attributable to the smaller hydrodynamic radius of DDM:CHS under the experimental conditions^{91,92,93,94}. This micelle size difference may also explain the detection of Prph2 monomers in the highest sucrose fractions of DDM ROS extracts but not in Triton ROS extracts. Whether DDM additionally exerts a more direct effect on polymer structure cannot be distinguished from the present data; higher-resolution methods that permit direct comparison of polymer length distributions in the two detergents would be required to evaluate this possibility.

Despite the expanded dynamic range and enhanced reproducibility of the reoptimized velocity sedimentation method, the utility of velocity sedimentation remains limited as a tool for studies of Prph2/Rom1 assembly and organization and for molecular phenotyping of *Prph2* variants. The method is labor intensive and low throughput, severely restricting its scalability. More fundamentally, individual polymer species are not resolved within the broad peaks of sedimentation profiles.

Prph2 in ROS detergent extracts sediments in two broad peaks under the reoptimized sedimentation protocol (Figs. 11B & 12C). Given that negative staining

TEM reveals a wide distribution of polymer lengths (Fig. 9 C & D)⁴⁴, with well over a dozen distinct dimer chain lengths expected based on the 4.1 nm repeat length for each non-covalent dimer in a polymer chain^{58,71}, the near absence of Prph2 in the sedimentation pellets indicates that the two sedimentation peaks encompass substantial polymer heterogeneity. Additionally, as illustrated by the qualitative similarity between the sedimentation analyses of Prph2^{C150S} and Prph2^{C150S;L185P} (Fig. 13), the inability of sedimentation to identify Prph2/Rom1 species present within the sedimented extracts requires complementary methods for characterizing the structural consequences of Prph2 variants. To overcome these unresolved limitations, we developed a blue native polyacrylamide gel electrophoresis (BN-PAGE) method for characterizing detergent-solubilized Prph2/Rom1 polymers, which is described in the next chapter.

CHAPTER 3

A BLUE NATIVE PAGE APPROACH RESOLVES DETERGENT EXTRACTED PRPH2/ROM1 POLYMER SPECIES BY SIZE

Introduction

The rims of OS disks in both rods and cones are bent into a tight hairpin curve that is stabilized by a scaffold made of three parallel belts of heterogeneously-sized disulfide-linked linear polymeric chains of Prph2/Rom1 dimers^{44,58,71}. Prph2 and Rom1 are detergent extracted from their native membranes as these variously sized polymers. Even after the reoptimization in the previous chapter, velocity sedimentation yields only broad peaks within which the substantial size heterogeneity of Prph2/Rom1 polymers is not resolved. Although Cryo-EM has provided high resolution structures of the non-covalent dimer and the linear polymer architecture, its technical and resource demands make it impractical for routine analysis and for screening the effects of Prph2 variants. These limitations create a need for a complementary biochemical method capable of resolving and quantifying discrete Prph2/Rom1 species in detergent extracts from ROS membrane or transfected cells in a reproducible, scalable, and cost-effective manner.

Blue native PAGE (BN-PAGE) is a widely used method for separating protein complexes while preserving the non-covalent interactions underlying their assembly (i.e. the non-covalent interaction supporting Prph2/Rom1 dimerization)^{95,96,97,98}. Unlike SDS-PAGE, which uses SDS to denature proteins and impart them with a negative charge, BN-PAGE uses Coomassie Blue G-250 dye to impart a negative charge without denaturing

proteins, thereby preserving non-covalent protein-protein interactions underlying complex assembly. Earlier applications of BN-PAGE to study Prph2^{99,90} suggested that the method could, in principle, resolve individual polymer species if resolution and quantitative reproducibility could be improved.

The aims of the work described in this chapter were therefore to: (1) Develop and optimize a BN-PAGE protocol for analyzing Prph2/Rom1 polymers in detergent extracts of native ROS membranes and transfected HEK293 cells. (2) Validate the method through direct comparison with velocity sedimentation and biochemical confirmation of the dimeric core subunit. (3) Quantify polymer stoichiometry and relative abundances in ROS and transfected HEK293 cells. (4) Evaluate the feasibility of applying the BN-PAGE method for rapid, high-throughput molecular phenotyping of reportedly pathogenic and experimentally engineered Prph2 variants. Collectively, the work in this chapter complements the structural insights from cryo-EM with the power of ensemble measurements needed for functional and translational studies of Prph2/Rom1 oligomerization.

Methods

BN-PAGE electrophoresis

A blue native PAGE (BN-PAGE) method^{95,97} was optimized for resolving detergent-extracted Prph2/Rom1 polymers. All BN-PAGE was run on 4-20% acrylamide TGX minigels (BioRad), using Tris-Glycine running buffers (25 mM Tris + 192 mM glycine, pH 8.3; identical to Tris-Glycine Towbin transfer buffer), unless otherwise

indicated. To prevent band widening, the gel box was set up in ice and remained in ice until the end of the electrophoresis run. The cathode chamber was filled with chilled dark blue cathode buffer B (Tris-Glycine running buffer + 0.02% (w/v) G-250 and 2 mM ϵ -Ahx) and the anode chamber was filled with clear Tris-Glycine running buffer. Prior to loading, the samples, containing detergent solubilized Prph2/Rom1, were combined 2:1 with 3x BN-PAGE sample buffer (100 mM Tris, 60% (v/v) glycerol, 0.15% (w/v) bromophenol blue, 0.25% (w/v) Coomassie Blue G-250, 25 mM ϵ -Ahx, pH 7.2). Electrophoresis was started at 100 V for 30 minutes and then run at 150 V until the dark blue dye front had migrated $\sim 1/3$ of the way into the gel. Electrophoresis was then paused and the dark blue cathode buffer B was suctioned out and replaced with light blue cathode buffer B/10 (Tris-Glycine running buffer + 0.002% (w/v) G-250 and 0.2 mM ϵ -AHX). Electrophoresis was resumed at 100 V until the dark blue dye front had mostly migrated out of the gel.

After electrophoresis, the gel was removed from its cassette and placed in SDS pre-soak buffer (Towbin transfer buffer + 2% SDS) for 30 minutes at RT with gentle rocking. The pre-soaked gel was then equilibrated 3x 5 minutes in Towbin transfer buffer at RT with gentle rocking. Proteins were transferred to Immobilon-FL (Millipore) membranes as described within the SDS-PAGE method in chapter 2. After transfer, the membranes were rinsed in ultrapure water and air dried for storage until ready to develop.

BN-PAGE blot development

The procedure used for developing BN-PAGE western blots was essentially the same as described for SDS-PAGE western blots in chapter 2, except for the following changes. Dry blots were rinsed 2x30 seconds in 100% MeOH to remove excess G-250 dye from the membranes. To preserve the coomassie labeling of the NativeMark Unstained Protein standard (Invitrogen) the membranes were acidified for 15 minutes in destain solution (40% meOH, 7% AcOH). Destained membranes were rinsed for 30 seconds in ddH₂O, then equilibrated 3x 5 minutes in 1xPBS at RT with gentle rocking prior to blocking, immunodetection, image acquisition, and densitometry as described in chapter 3. Apparent molecular weights of species resolved by BN-PAGE were estimated by reference to logarithmic regression of the NativeMark Unstained Protein Standard (Invitrogen).

Results

BN-PAGE method optimization

Previously published BN-PAGE analyses of purified Prph2 from bovine ROS detergent extracts resolved only the two fastest-migrating bands, with most material appearing as high-molecular-weight smears⁹⁹. Moreover, the mobility of the resolved Prph2 bands relative to BSA standards was inconsistent between experiments⁹⁰. We noted that those experiments were performed under sub-optimal conditions using Bis-Tris gels and running buffers at pH 7.2, below the theoretical pI of Prph2 (~7.8*; Roml's

* 7.93 in *Homo sapiens*, 7.74 in *Mus musculus*, 7.75 in *Bos taurus*, 7.82 in *Xenopus laevis*.¹¹⁷

theoretical pI values are more variable but consistently < 6 in mammals). Anodic migration of basic proteins on BN-PAGE requires a net negative charge imparted by the binding of Coomassie Blue G-250 dye to the hydrophobic surfaces of the protein^{95,96}. G-250 binding is reversible. When BN-PAGE is run at a pH that is near or below a protein's pI, G-250 depletion during electrophoresis can cause migration reversal, streaking, loss of resolution, and reduced quantitative reproducibility⁹⁷. To prevent this, native PAGE experiments generally require a running buffer/gel pH ≥ 0.5 units above the protein pI^{100,101}. To improve performance, we adapted the BN-PAGE method to use 4-20% polyacrylamide gradient TGX gels and Tris-glycine running buffer at pH 8.3.

The initial BN-PAGE experiment analyzing Triton X-100-solubilized bovine ROS under these conditions, resolved seven discrete bands in the lane loaded with 2.7 μ g of ROS extract (Fig. 14A). The mobility of the fastest band relative to the BSA standard was comparable to that seen in previous BN-PAGE analysis of the size-purified Prph2/Rom1 core subunit from bovine ROS⁹⁰. Paradoxically, whereas AzureRed total protein stain intensity increased with the mass of ROS extract loaded to the lanes, Prph2 band intensity decreased at higher loads, likely due to in-gel Prph2 aggregation—a common issue in native PAGE with integral membrane proteins⁹⁷ that can be mitigated by adding the lysine analogue 6-aminohexanoic acid (ϵ -Ahx) to the samples and cathode buffers⁹⁷. Inclusion of ϵ -Ahx (8.3 mM final in loaded samples; 2 mM in dark blue cathode buffer B, and 0.2 mM in light blue cathode buffer B/10) dramatically improved both the resolution of higher-order Prph2 forms and the reproducibility of relative band intensities, while reducing the appearance of protein aggregates (Fig. 14B). Older, oxidized (yellowed), ϵ -

Ahx stocks impair the resolution/reproducibility of Prph2 bands on BN-PAGE and do not adequately mitigate in-gel aggregation (Fig. 14C).

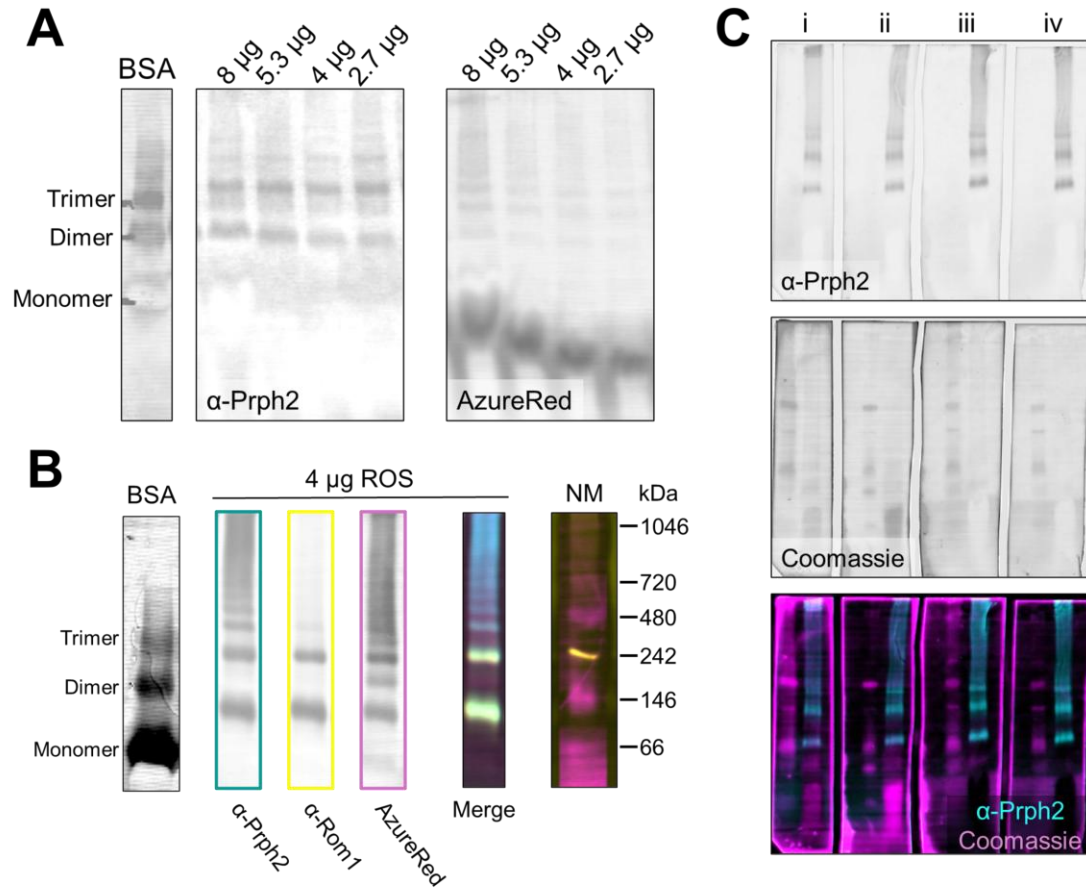


Figure 14: Optimizing BN-PAGE for analysis of Prph2 polymers. (A) BN-PAGE Western blot of Triton X-100 extracts of bovine ROS resolved in a pH 8.3 Tris-glycine gel and running buffer system. Lanes were loaded with the indicated total protein masses (μ g). Left panel: Coomassie-stained BSA standard lane. Center panel: α -Prph2 immunoblot of ROS lanes demonstrating the characteristic ladder pattern of Prph2 polymers. Right panel: AzureRed total protein stain of the corresponding ROS lanes. (B) Effect of supplementing the sample buffer and cathode buffer with ϵ -aminocaproic acid (ϵ -Ahx) on band resolution. Addition of fresh ϵ -Ahx markedly improves separation and detection of higher-order Prph2 polymers in Triton X-100 ROS extracts (ROS lane shown is the 1% Triton X-100 condition cropped from the blot presented in Fig. 2F). BSA standard is shown on the left; NativeMark molecular weight markers are shown on the right. (C) Optimization of post-transfer destaining conditions. Test strips were cut from a single non-destained PVDF membrane, each containing one lane of NativeMark standards and one lane loaded with 4 μ g Triton X-100 ROS extract. Strips were subjected to mild (i, ii), harsh (iv), or moderate (iii; approximating the final optimized protocol) destaining conditions prior to blocking and immunodetection with α -Prph2.

Post-transfer destaining of the membranes to remove the excess G-250 dye was optimized to balance dye removal with antibody epitope accessibility and marker label retention^{97,101,102,103,104}. Harsher destaining methods (e.g. prolonged 100% methanol washes⁹⁷, as in Fig. 14C blot iv, maximized the mouse α -Prph2 (mabC6) signal but consistently removed NativeMark labeling. NativeMark bands could not be reliably restored by total protein staining of the membrane after destaining and prior to blocking. Milder destaining conditions (e.g. extended rinses in 62.5 mM Tris-HCl, 2% SDS, pH 6.8¹⁰²) more consistently preserve marker labeling but with increased background (Fig. 4C blots i and ii). A moderate destaining method (Fig. 14C blot iii, approximating the final method) optimized performance across all primary antibodies used for immunodetection on the BN-PAGE western blots in this work while retaining marker labeling. These refinements enabled reproducible resolution and quantification of Prph2/Rom1 polymer ladders, as described below.

BN-PAGE captures the size heterogeneity of Prph2/Rom1 polymers seen on sedimentation

As an initial validation of the BN-PAGE method, we compared BN-PAGE results with those from the velocity sedimentation assay (Fig. 15). Because larger integral membrane protein complexes are generally less efficiently solubilized by detergents than smaller complexes¹⁰⁵, solubilizing bovine ROS extracts with lower concentrations of Triton X-100 than the standard 1% (w/v) is a convenient approach for generating samples with a reduced complement of higher-order forms of Prph2. Bovine ROS was solubilized

in 0.25%, 0.5%, 1%, and 2% Triton and analyzed by the reoptimized sedimentation method and BN-PAGE.

Solubilization efficiency of Prph2 and Rom1 at all four Triton concentrations was assessed by SDS-PAGE and densitometry comparing the relative Prph2 and Rom1 band intensities soluble clarified ROS extracts to the resuspended pellets from the centrifugation at the end of solubilization (Fig. 15D). Approximately 95% of the Prph2 was extracted from the ROS solubilized in 0.5%, 1%, and 2% Triton, and ~97% of the Rom1 was extracted under those three conditions. In contrast, only 80% of the Prph2 and 88% of the Rom1 was extracted in 0.25% Triton.

Non-reducing SDS-PAGE analysis of the fractionated gradients shows an absence of the largest disulfide-linked Prph2 polymers in 0.25% Triton (Fig. 15B) when compared to ROS solubilized under standard, 1% Triton, conditions (Fig. 15A). The same analysis for the 0.5% and 2% Triton extract gradients was comparable to that in 1% Triton and is not shown. Whereas Prph2 in the 0.5%, 1%, and 2% Triton extracts sedimented in the typical two broad peaks, the Prph2 in the 0.25% Triton sedimented in a single peak within the lighter fractions (Fig. 15C). Rom1 sedimentation profiles are essentially comparable in ROS solubilized at all four Triton concentrations and show a single peak in the lighter fractions. The ratio of Prph2 SS-dimer to monomers extracted in the 0.25% Triton ROS was ~1:2, compared to the ~3:2 Prph2 SS-dimer to monomer ratio seen under the other three conditions (Fig. 15E). The ratio of Rom1 -SS- dimers to monomers was ~1:3 under all four conditions. These data suggest that large disulfide-stabilized

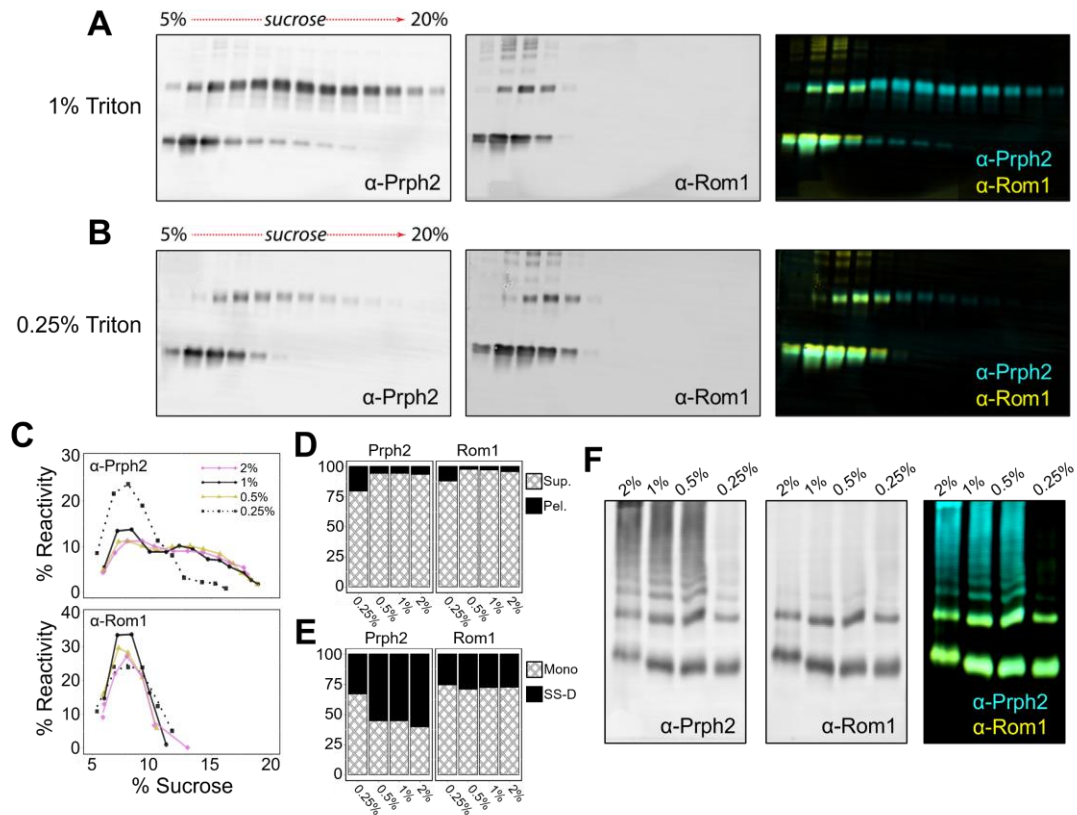


Figure 15: BN-PAGE results recapitulate velocity sedimentation. (A) Western blot from non-reducing SDS-PAGE analysis of a fractionated sedimentation gradient of bovine ROS solubilized in 1% (w/v) Triton X-100, the blot was probed with α -Prph2 (left) and α -Rom1 (center); the overlaid channels are on the right. (B) Western blot from non-reducing SDS-PAGE analysis of a fractionated sedimentation gradient of bovine ROS solubilized in 0.25% (w/v) Triton X-100, the blot was probed with α -Prph2 (left) and α -Rom1 (center); the overlaid channels are on the right. (C) Sedimentation profiles of Prph2 (top) and Rom1 (bottom) in bovine ROS solubilized in 0.25% (dotted black line), 0.5% (solid gold line), 1% (solid black line), and 2% (solid magenta line) Triton X-100. (D) Bar chart of solubilization efficiencies; comparing the percentages of Prph2 (left) and Rom1 (right) in the supernatant detergent extract (hatched) versus in the solubilization pellets for bovine ROS solubilized with the indicated concentration of Triton X-100. (E) Bar chart of Prph2 (left) and Rom1 (right) -SS-dimer (SS-D, solid) and monomer (hatched) percentages for bovine ROS solubilized with the indicated concentration of Triton X-100. (F) Western blot showing BN-PAGE of bovine ROS solubilized at the indicated concentrations of Triton X-100, the blot was probed with α -Prph2 (left) and α -Rom1 (center); the overlaid channels are on the right.

homomeric Prph2 polymers were extracted less efficiently in 0.25% Triton than at the higher Triton concentrations.

4 μ g total protein from each ROS extract was analyzed by BN-PAGE and western blotting for Prph2 and Rom1 (Fig. 15F). Rom1 migrated as two bands in all four extracts,

recapitulating the Rom1 sedimentation results (Fig. 15C). Prph2 in the 0.5%, 1%, and 2% Triton extracts migrated with the same ladderized banding pattern previously observed with 1% Triton ROS extracts. In contrast, higher-order Prph2 ladderizing was absent in the 0.25% Triton extract; which is consistent with the sedimentation finding of decreased higher-order Prph2 forms in 0.25% Triton when compared to the other three extracts. Together, these results demonstrate a direct correlation between the peaks observed in velocity sedimentation profiles and the discrete Prph2 and Rom1 bands resolved by BN-PAGE.

BN-PAGE resolves individual species of Prph2/Rom1 polymers in bovine ROS extracts

With the BN-PAGE method optimized and initially validated, we proceeded to analyze purified bovine ROS membranes solubilized in either 1% Triton X-100 or 1% DDM by BN-PAGE and western blotting. 2.2 μ g of ROS extract were loaded to each lane. In both detergents, Prph2 exhibited a characteristic ladderized pattern of bands corresponding to a wide distribution of polymer chain sizes. The fastest migrating bands were identified as non-covalent dimers by reference to the core subunit resolved by single particle cryo-EM⁵⁸ and apparent molecular weight relative to NativeMark standards. Prph2 was present in polymers ranging from individual non-covalent dimers up to eicosamers or larger (Fig. 16A–C). In contrast, Rom1 was largely confined to the lowest MW species, predominantly dimers and tetramers. For both proteins, the smallest forms (dimers and tetramers) were the most abundant. DTT reduction of the ROS extracts prior

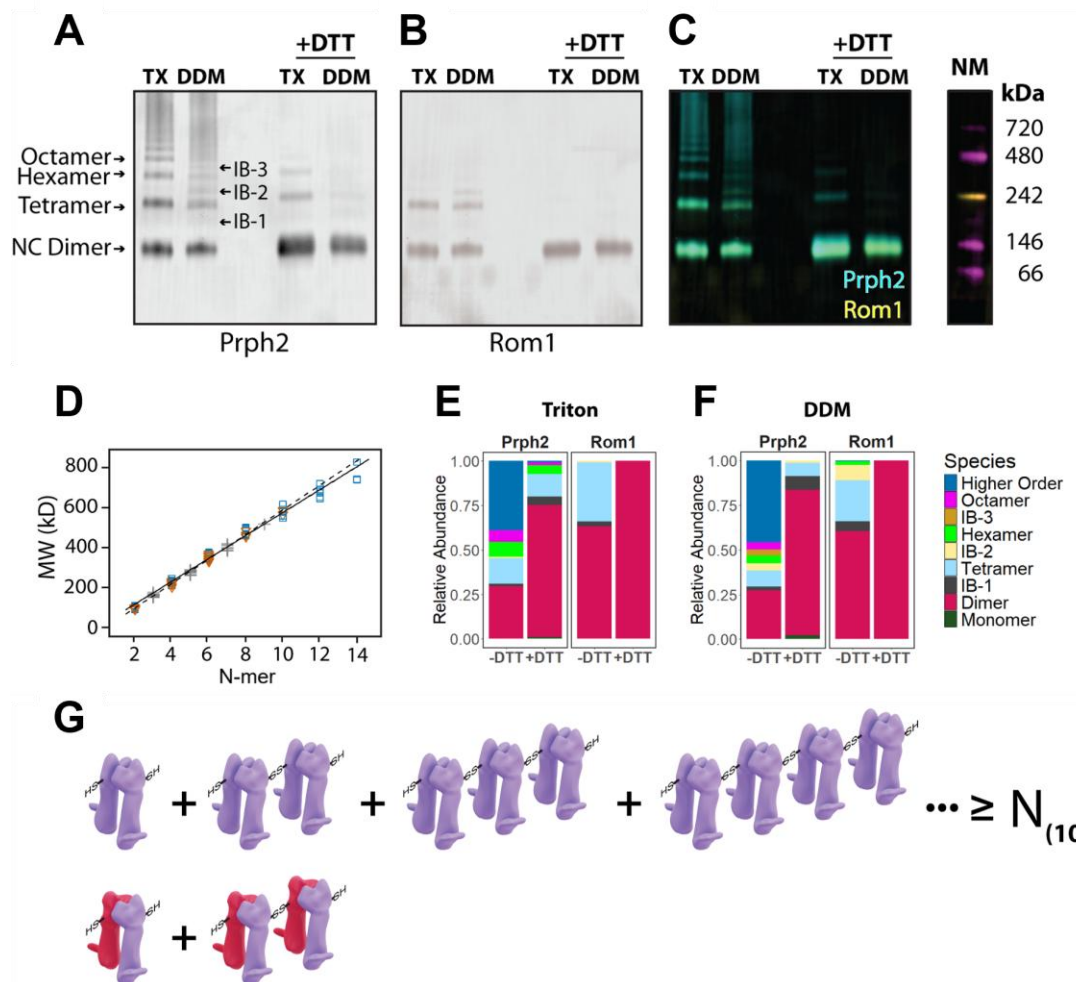


Figure 16: BN-PAGE resolves heterogeneity and stoichiometry of Prph2/Rom1 polymers in bovine ROS detergent extracts. (A-C) Purified bovine ROS membranes were solubilized in either 1% Triton X-100 or 1% DDM. The extracts were then analyzed by BN-PAGE and western blotting for Prph2 and Rom1. (A) Prph2 shows a regularly ladderred banding pattern in both detergents, reflecting polymers from 1 to ≥ 10 non-covalent dimers. DDM solubilization produced prominent intermediate bands (IBs, arrows) between the dimer chain species. DTT reduction of the samples prior to BN-PAGE collapsed the ladders to mostly dimers. (B) Rom1 is restricted to mainly dimers and tetramers in both detergents. DTT reduction of the samples prior to BN-PAGE almost entirely collapses Rom1 into dimers. (C) Overlay of panels A & B, Prph2 is cyan and Rom1 is yellow. (D) A scatter plot of apparent MW as a function of N-mer size for Prph2 dimer chains in Triton X-100 (blue squares) and DDM (orange triangles). Data includes replicate lanes from 3 separate BN-PAGE experiments (total $n = 11$ lanes for Triton, $n = 12$ lanes for DDM). Statistical regression shows a linear correlation in both Triton X-100 (solid line, $R^2 = 0.985$, $P < 0.001$) and DDM (dashed line, $R^2 = 0.997$, $P < 0.001$). IBs were excluded from the regressions but are plotted as odd-numbered N-mers with grey '+' symbols for reference. (E & F) Barcharts of relative species abundances on BN-PAGE of either Triton (E) or DDM (F) ROS extracts, data includes 4 replicate lanes for each reduced or nonreduced extract. (G) Model illustrating the polymers extracted from ROS membranes; including homo and heterodimers and variously sized disulfide-linked dimer chains.

to BN-PAGE collapsed the larger Prph2 polymers into the smallest forms (dimers to hexamers) (Fig. 16A) and collapsed the Rom1 bands to the dimer (Fig. 16B) in both detergents, supporting the identification of the ladder bands as disulfide stabilized dimer chains.

The non-covalent dimer migrates at an apparent MW of ~100 kDa on BN-PAGE, which is ~25 kDa larger than the true molecular weight of the non-covalent dimer; reflecting the contribution of the detergent micelle^{96,106}. The migration of the higher bands showed a highly reproducible linear, stepwise increase in apparent MW of ~120 kDa (~75 kDa protein + ~45 kDa detergent micelle) between the major bands in both detergents (Fig. 16D). The tetramer migrated at ~225 kDa, the hexamer at ~350 kDa, the octamer at ~470 kDa, the decamer at 590 kDa, and the dodecamer at ~700 kDa. These results are consistent with linear polymers of a non-covalent dimeric core subunit.

Relative polymer species abundance was determined by densitometry (Fig. 17). In non-reduced Triton X-100 ROS extracts, ~30% of the Prph2 is in the non-covalent dimers, ~14% is in the tetramers, ~8% is in the hexamers,

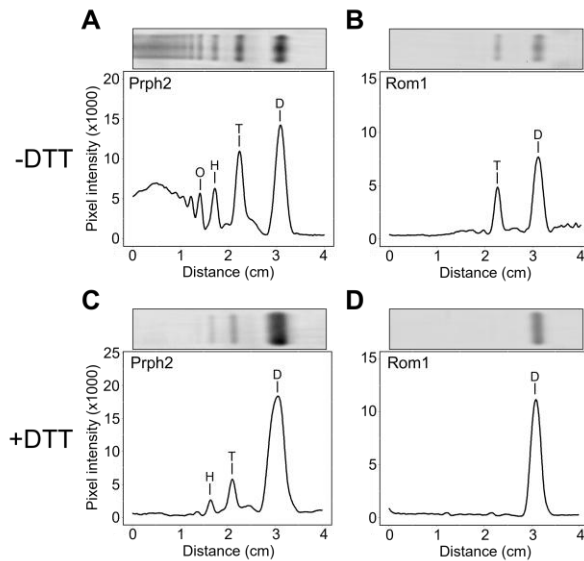


Figure 17: Lane profiles from BN-PAGE analysis of nonreduced (top panels, A & B) and reduced (bottom panels, C & D) Triton X-100 bovine ROS extracts. The α -Prph2 channel is shown in the left panels (A & C), the α -Rom1 channel is shown in the right panels (B & D). The positions of the peaks corresponding to the NC dimer (D), tetramer (T), hexamer (H), and octamer (O) are marked in the non-reduced ROS extract (A & B).

~6.5% is in the octamers, and ~40% is in polymers larger than octamer. In those same extracts, ~65% of the Rom1 is in the non-covalent dimers and ~30% is in the tetramers (Fig. 16E). Relative abundances in nonreduced DDM extracts are comparable to those in Triton; ~27% of the Prph2 is in the non-covalent dimers, ~10% of the Prph2 is in the tetramers, ~4.5% of the Prph2 is in the hexamer, ~3.5% of the Prph2 is in the octamers, and ~45% of the Prph2 is in polymers larger than the octamer. In those extracts, ~60% of the Rom1 is in the non-covalent dimers, ~25% of the Rom1 is in the tetramers, and ~2% of the Rom1 is in the hexamers (Fig. 16F).

Triton X-100 extraction produced the sharpest and most clearly resolved ladders. Additional intermediate bands (IBs), accounting for ~10% of total α -Prph2 reactivity and ~15% of total α -Rom1 reactivity, are present between the dimer chain bands in DDM. Within fresh ROS extracts, the relative intensity of the IBs is highly reproducible across multiple experiments. As shown in Fig. 16B, the IBs migrate at approximately the expected apparent MW of hypothetical odd-numbered polymers; however, their identity is unclear and they were not included in the regressions shown on that plot.

BN-PAGE resolves polymer species within sedimentation fractions

Velocity sedimentation has been the gold standard approach for assessing Prph2/Rom1 complex assembly. However, its inability to resolve highly heterogeneous complexes (Fig. 15) and its low throughput limit its utility for determining the molecular consequences of Prph2 mutations. Despite this, velocity sedimentation is better suited than BN-PAGE for certain applications (see discussion), and it will continue to be used

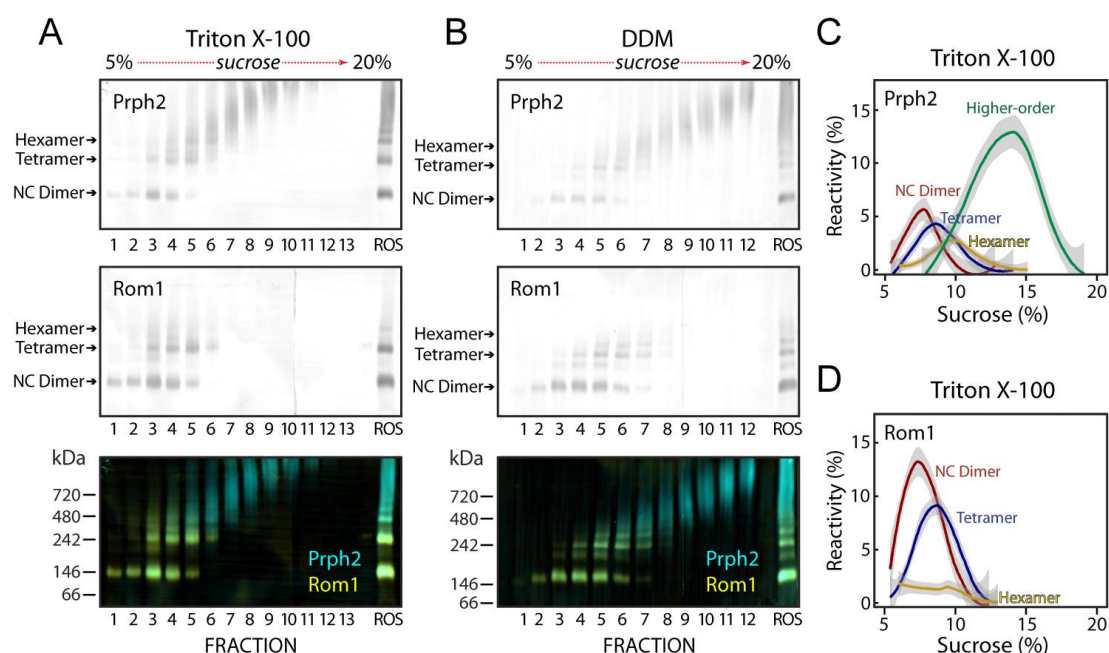


Figure 18: BN-PAGE resolves Prph2/Rom1 polymer size heterogeneity within velocity sedimentation fractions. Velocity sedimentation fractions from bovine ROS membranes solubilized in either 1% Triton X-100 (A) or 1% DDM (B) were analyzed by BN-PAGE and western blotting for Prph2 (top) and Rom1 (center), merged images are at the bottom. In both detergents, Prph2 is present in polymers ranging from 1 to ≥ 10 non-covalent dimers, while Rom1 is mostly restricted to non-covalent dimers and tetramers. (C & D) Plots showing the relative abundance of Prph2 (C) and Rom1 (D) polymer species within fractionated gradients of 1% Triton ROS extracts. Lines represent LOESS regressions of relative abundances for each size species indicated (Red: NC dimer; blue: tetramer; yellow: hexamer; green: higher-order). Data represent 8 independent sedimentation trials using Triton X-100 solubilized material. Grey shading indicates 95% confidence intervals.

for assaying Prph2/Rom1 self-assembly. To better guide those efforts, and to enable rational reinterpretation of the large body of velocity sedimentation data in the literature evidence in light of recent advances, we combined the reoptimized sedimentation method with BN-PAGE to assess the distribution of Prph2 and Rom1 polymer species in sedimentation fractions (Fig. 18).

Purified bovine ROS membranes were solubilized in either 1% Triton X-100 or 1% DDM and sedimented by the reoptimized method, and then gradient fractions were analyzed by BN-PAGE and western blotting (Fig. 18A & B). In both detergents, the blots

show a stair-like banding pattern of increasing polymer size with increasing gradient density. Prph2 is present in a broad range of polymer sizes. The heaviest Prph2 polymers in the densest soluble fractions, although detectable by denaturing SDS-PAGE analysis (Fig. 12), are too large (> 1.3 MDa) to resolve on the 4-20% polyacrylamide gradient gels used for the BN-PAGE and are lost when the gel combs are cut prior to transferring the proteins to the PVDF membranes. Rom1 was restricted to the smaller polymers (1 or 2 dimers) present in the lower density sedimentation fractions.

The results are qualitatively comparable between the two detergents. Complementing the non-reducing SDS-PAGE finding that sedimentation peaks in DDM extracts are slightly shifted toward the denser fractions relative to Triton (Fig. 12), BN-PAGE shows that the appearance of individual polymer species is slightly shifted towards the denser fractions in DDM extracts compared to Triton extracts (Fig. 18A & B). This observation likely reflects the smaller hydrodynamic radius of DDM-CHS micelles than Triton micelles^{91,92,93,94}.

Sedimentation profiles of individual polymer species (Fig. 18C & D) show the large degree of overlap between the peaks of different species. These data illustrate that velocity sedimentation does not resolve individual polymer species.

BN-PAGE reveals mutation induced self-assembly defects in recombinant Prph2 mutants

Recombinant overexpression in HEK293 has been a reliable tool for understanding the biochemical impact of pathogenic Prph2 mutations¹⁰⁷. To evaluate the feasibility of using BN-PAGE for molecular phenotyping of pathogenic Prph2 variants,

we analyzed self-assembly of WT and mutant recombinant Prph2 in detergent extracts of transiently transfected HEK293 cells by BN-PAGE and western blotting (Fig 19A).

In both Triton X-100 and DDM, the recombinant WT Prph2 produced ladderred bands ranging from one to > 10 dimers, like those seen in extracts from ROS membranes (Fig. 16). This is consistent with velocity sedimentation of recombinant WT Prph2, which shows self-assembly recapitulating Prph2 in native ROS membranes^{44,65} (Fig. 13). The Prph2^{C150S} mutant, in which the inter-dimer disulfide forming cysteine is mutated to a serine, exhibits no laddering on BN-PAGE. Instead C150S migrates as a single major band corresponding to the non-covalent dimer, with a minor tetramer band and a very faint, but reproducible, hexamer band (Fig. 19A & B). The lack of higher-order polymer laddering reflects the known significance of Prph2-C150 mediated intermolecular disulfide bonding for core subunit polymerization⁶⁶ and suggests a potential non-covalent inter-dimer interaction (Fig. 19A). Prph2^{L185P} is a well-characterized Prph2 mutation associated with retinitis pigmentosa and defective subunit assembly⁶⁸. Similar to Prph2^{C150S}, Prph2^{L185P} produced a single band on BN-PAGE that migrated at slightly lower mobility than the non-covalent dimer (Fig. 19A), consistent with the evidence that the L185P mutation restricts the protein to an abnormally oxidized form⁴⁴.

We hypothesized that the L185P mutation disrupts the non-covalent dimerization interaction interface within the Prph2 EC2 domain. To test this, we analyzed the double mutant Prph2^{C150S;L185P} by BN-PAGE; predicting that, because the protein would neither be able to form intermolecular disulfide bonds nor non-covalently dimerize, the double

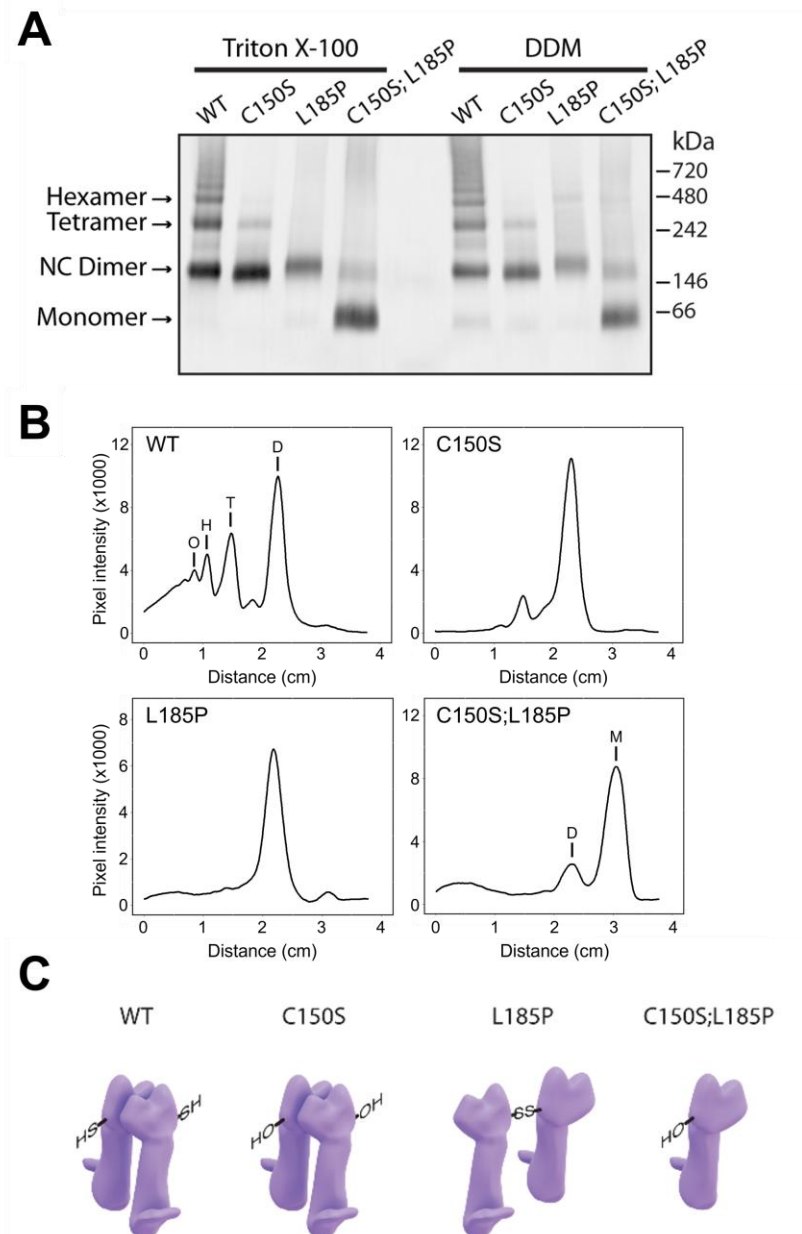


Figure 19: BN-PAGE reveals self-assembly defects in recombinant Prph2 mutants. (A) Prph2 variants were overexpressed in HEK293 cells and solubilized in either 1% Triton X-100 (left lanes) or 1% DDM (right lanes), and then analyzed by BN-PAGE and western blotting. The results are comparable between the two detergents. WT Prph2 generated a ladderred banding pattern, like in ROS detergent extracts. C150S, which lacks the free EC2 cysteine required for intermolecular disulfide bonding, primarily forms non-covalent dimers, with some tetramers. L185P, which disrupts non-covalent dimerization, forms abnormally oxidized covalent dimers. Double mutant C150S;L185P is natively predominantly monomeric. (B) Lane profiles of the Triton X-100 extracts for each variant. (C) Cartoon illustrating a physical model of the Prph2 variants. WT showing the non-covalent dimer only. C150S showing a non-covalent dimer lacking the intermolecular disulfide forming thiols. L185P showing an abnormally oxidized covalent dimer. And showing C150S;L185P as a native monomer.

mutant would be natively monomeric. BN-PAGE analysis shows that Prph2^{C150S;L185P} migrates as a single major band: a ~50 kDa monomer.

The BN-PAGE results for WT, C150S, and C150S;L185P expand upon and agree with the sedimentation shown in Fig. 13, and the results for L185P agree with previously published velocity sedimentation analysis of that variant⁴⁴. Together, these results demonstrate that BN-PAGE analysis of recombinant Prph2 variants offers a robust and efficient approach for screening and phenotyping the impact of Prph2 mutations on non-covalent dimerization and inter-dimer disulfide mediated polymerization.

Discussion

The optimized BN-PAGE method resolves individual size species of Prph2/Rom1 polymers that are detergent extracted from ROS membranes and transfected HEK293 cells. Laddered bands were reproducibly observed for WT Prph2, resolving disulfide linked chains ranging from 1 to ≥ 10 non-covalent dimers. Rom1 was largely restricted to dimers and tetramers (Fig. 16). The BN-PAGE findings corroborate and expand upon velocity sedimentation data (Fig. 12), which showed broad peaks suggesting a heterogeneous pool of polymers with a large size range. BN-PAGE analysis of sedimentation gradient fractions reveals the substantial overlap in the sedimentation peaks of individual polymer species (Fig. 18); both underscoring the limitations of the sedimentation method for precise molecular phenotyping and highlighting the utility of BN-PAGE for quantifying absolute polymer chain length and relative abundance.

Significantly, these data validate cryo-EM findings that suggest a dimeric, instead of a tetrameric, non-covalent core subunit of the disk rim scaffold⁵⁸. This resolution of core subunit stoichiometry fundamentally refines the molecular model of the photoreceptor OS disk rim scaffold, reconciling discrepancies between earlier hydrodynamic studies that inferred a tetrameric core subunit^{67,69} and high-resolution structural data demonstrating non-covalent dimers as the fundamental building blocks of disulfide linked Prph2 polymer chains⁵⁸. By enabling measurement of individual polymer species abundance, these findings provide a basis for interpreting Prph2 oligomerization defects in IRDs and provide a feasible approach for efficient molecular phenotyping of Prph2 variants of unknown significance.

The BN-PAGE method optimization steps described—pH 8.3 gels and running buffers to ensure the continuous anodic migration of Prph2 (pI ~7.8), the inclusion of fresh and properly stored ϵ -Ahx to mitigate in-gel protein aggregation, and the identification of a post-transfer destaining method balancing marker label retention and epitope accessibility for immunodetection—were essential for achieving reproducible high resolution ladders of Prph2 polymers. The detergent used for solubilization influenced band sharpness and the prominence of bands not identifiable as dimer chains (i.e. the intermediate bands), but the results in either detergent support the same core conclusions (Fig. 16). The comparable BN-PAGE results in Triton and DDM extracts suggest the presence of weak non-covalent interactions that are easily disrupted by gentle detergents. This is an important consideration when studying tetraspanins, which generally require specific detergents to maintain solubility without denaturation¹⁰⁸. Future BN-PAGE studies of Prph2 and Rom1 could explore detergent-free extraction strategies,

such as using SMALPs^{109,110} or nanodiscs^{111,112,113}, both to identify potential detergent-labile Prph2 interactions underlying rim scaffold assembly and to better mimic native lipid environments—potentially revealing lipid-dependent oligomerization dynamics.

Although BN-PAGE resolves Prph2/Rom1 polymers by size, it does not resolve them by composition. BN-PAGE analysis of non-reduced bovine ROS extracts indicates that ~2/3 of the Rom1 in the form of non-covalent dimers (Fig. 16E). In ROS, the abundance ratio of total Prph2 to Rom1 is ~2:1⁷⁰. Less than 14% of the total Rom1 in bovine ROS is in homomeric forms⁶⁹, and that upper limit is likely an overestimate^{58,114,78}. As such, ~30% of the Prph2 is expected to be in non-covalent heterodimers with Rom1. Interestingly, our data indicate that non-covalent Prph2 homodimers and heterodimers combine to account for ~30% of the Prph2 in ROS extracts. This suggests that a majority of the free non-covalent Prph2 dimers are heteromeric. A disproportionate paucity of homomeric Prph2 in the dimers and tetramers of WT ROS would support a model wherein higher-order Prph2 homopolymers are the *structurally* dominant feature of the rim scaffold, while Rom1 has a role in supporting the assembly of those Prph2 forms²⁶. Alternative approaches, such as the hrCN-PAGE presented in the next chapter (Fig. 21) are necessary to formally address the possibility of compositional bias within the smaller polymers in ROS membranes.

BN-PAGE allows efficient molecular phenotyping of recombinant mutant Prph2 expressed in HEK293 cells (Fig. 19). Prph2^{C150S}, which lacks the free EC2 cysteine required for inter-dimer disulfide bonding, predominantly formed non-covalent dimers with some tetramers but no higher-order chains; consistent with prior velocity

sedimentation and structural findings^{90,66}. The retinitis pigmentosa associated Prph2^{L185P} mutation produced abnormally migrating dimers on BN-PAGE without laddering, reflecting defective non-covalent core subunit assembly and aberrant oxidation^{44,68}. The novel Prph2^{C150S;L185P} double mutant variant migrated as a native monomer, confirming that the L185P mutation disrupts non-covalent Prph2 dimerization and that both non-covalent dimerization and inter-subunit disulfide bonding are required for higher-order assembly. Significantly, this also confirms that the intermolecular disulfide bonds in the abnormally oxidized dimers formed by Prph2^{L185P} are mediated by Prph2-C150. These BN-PAGE results agree with, and expand upon, the velocity sedimentation findings with these variants (Fig. 13).

The absence of Prph2-C150 mediated inter-dimer disulfide bonds does not entirely preclude dimer oligomerization. The presence of tetramers on BN-PAGE analysis of Prph2^{C150S} expressed in transfected HEK293 cell extracts implies that non-covalent interactions along the same axis as the inter-dimer disulfide bond may support dimer-dimer association into chains. More speculatively, such interactions may also contribute to the end-on-end organization of polymers into the belts of the scaffold. The precise mechanism through which Prph2 generates the extreme curvature at OS disk rims remains unknown. Cryo-ET of the in-situ disk rim scaffold suggests a possible EC2 interaction between the dimers of the parallel belts⁷¹ that may contribute some of the energy needed to bend the membrane. Alternatively, the non-covalent dimers may intrinsically promote spontaneous negative membrane curvature. Computational modeling performed by our collaborators, presented in the following chapter, addresses these questions.

The optimized BN-PAGE method represents a significant technical advance for the study of Prph2/Rom1 biology. By providing rapid and reproducible quantification of polymer chain length and relative abundance in native ROS membranes and heterologous expression systems, BN-PAGE overcomes the resolution limitations and low throughput of velocity sedimentation. The method's compatibility with multiple solubilizing detergents, its quantitative robustness, and its high throughput enable efficient screening of Prph2 variants for self-assembly defects. Additionally, its ability to detect subtle non-covalent interactions that persist in the absence of inter-dimer disulfide bonds opens new avenues for characterizing these interactions. Collectively, these data refine the current model of disk rim scaffold assembly, demonstrating that precisely controlled Prph2 oligomerization shapes the highly curved rims of OS disks morphogenesis and supports photoreceptor function and viability.

CHAPTER 4

ROM1 PROMOTES THE ASSEMBLY OF PRPH2 INTO CURVATURE-SHAPING SUPRAMOLECULAR FORMS

Introduction

The extreme membrane curvature at the rims of photoreceptor OS disks is shaped by a supramolecular scaffold of Prph2 and Rom1. The scaffold is composed of three parallel belts of linear disulfide-linked polymeric chains of non-covalent Prph2/Rom1 dimers. As revealed by BN-PAGE, Prph2 in OS membranes exists within polymers ranging from one to over ten dimers in length, while Rom1 is not present in forms larger than the tetramer. Inter-dimer disulfide mediated polymerization of Prph2 is fundamental to its rim-shaping function⁷³. Mutations blocking key steps of Prph2 self-assembly, such as non-covalent dimerization (i.e. Prph2^{L185P}) and inter-dimer disulfide bonding (i.e. Prph2^{C150S}) prevent the protein from scaffolding the highly anisotropic membrane curvature characterizing OS disk rims⁴⁴. Despite advances in our understanding of Prph2 and Rom1 structure, self-assembly, and function, many critical mechanistic questions persist. (1) What is the significance of Rom1 to Prph2 self-assembly? (2) How is inter-dimer disulfide bonding regulated? (3) How does Prph2 generate membrane curvature? (4) How do pathogenic mutations in *PRPH2* affect protein structure and function?

To address these gaps, we have performed: (1) Velocity sedimentation and BN-PAGE analysis of Rom1 KO mouse ROS detergent extracts to investigate the role of Rom1 in Prph2 self-assembly. (2) Selective reduction of bovine ROS extracts to investigate the regulation of inter-dimer disulfide bonding. (3) Molecular dynamics

simulations of Prph2 oligomers in model membranes to investigate the mechanism of membrane curvature generation. And (4) an initial BN-PAGE screen of uncharacterized pathogenic missense Prph2 variants to investigate the feasibility of high throughput molecular phenotyping of Prph2 VUSs.

Methods

Purification of mouse ROS

10-week-old mice were humanely euthanized, and the eyes were enucleated. Retinas were dissected and then frozen in LN₂ and stored at -80° until use. Frozen retinas were thawed in 100 µl / retina of resuspension buffer (1xPBS, 50 mM NEM, 1x cOmplete ULTRA EDTA-free protease inhibitor cocktail, pH 7.4) and then 4 thawed retinas per mouse ROS preparation were combined in round-bottom 2 ml tubes. The outer segments were sheared into the buffer by vortexing 3x 1 minute at max speed. The tubes were centrifuged for 1 minute at 500 rcf at 4° and the supernatants, containing the extracted outer segments, were collected. The retinas were resuspended and re-extracted twice; the extracted outer segments were pooled with those from the first extraction. The pooled ROS was spun for 15 minutes at 14k rcf at 4°, the supernatants were discarded, and the pelleted ROS was either stored at -80° or resuspended in 65 µl resuspension buffer for immediate use.

Immunopurification of Prph2 from bovine ROS extracts

Purified mouse monoclonal anti-bovine Prph2 CTER (mab2B6) was coupled to CNBr-activated sepharose 4B (Sigma) to ~2 mg mab2B6 / ml beads; giving a final theoretical capacity of ~1 mg Prph2 / ml beads. Mab2B6 beads were stored at 4° in quench buffer (1xPBS, 50 mM glycine, 0.02% sodium azide). Before use, the beads were equilibrated in Buffer S (50 mM NaPi, 100 mM NaCl, 2 mM EGTA, 1.2 mM MgCl₂, pH 7.4).

Bovine ROS membranes were thawed and resuspended in 1xPBS + 50 mM NEM and incubated for 20 minutes at RT to cap free sulfhydryls. The ROS was then pelleted and resuspended in hypotonic lysis buffer (10 mM KPi, 2 mM EGTA, 10 mM NEM, pH 7.4) and incubated for 10 minutes on ice. The hypotonically lysed ROS was resuspended in Buffer S and an equal volume of Buffer S and then solubilized in 1% DDM as described in Chapter 3.

Purifications were done in batch. To bind Prph2 to the beads, the solubilized ROS was combined with the equilibrated beads and incubated for 2 hours at 4° with gentle shaking. The unbound fraction was collected, and the beads were washed 3x in ~30 bed volumes of wash buffer (50 mM NaPi, 150 mM NaCl, 2 mM EGTA, 10 mM NEM, 0.1% DDM, pH 7.4). Bound Prph2 was eluted by resuspending the washed beads in elution buffer containing excess recombinant bovine Prph2 CTER antigen (Buffer W + 0.2 mg/ml peptide BCT¹¹⁵) and incubating for 1 hour at RT with gentle rocking; the beads were eluted twice. The immunopurification fractions were analyzed by SDS-PAGE and BN-PAGE followed by western blotting.

High resolution clear native PAGE (hrCN-PAGE)

High resolution clear native PAGE (hrCN-PAGE)¹¹⁶ analysis of bovine ROS detergent extracts was run on 4-20% acrylamide TGX minigels (BioRad), using Tris-glycine running buffers (25 mM Tris + 192 mM glycine, pH 8.3). Prior to loading, the samples were combined 2:1 with 3x Native PAGE sample buffer (100 mM Tris, 60% (v/v) glycerol, 0.15% (w/v) bromophenol blue, pH 7.2). The cathode chamber was filled with hrCN-PAGE cathode buffer (Tris-glycine running buffer + 4 mM ϵ -Ahx + 0.05% (w/v) sodium deoxycholate, pH 8.3) and the anode chamber was filled with Tris-glycine running buffer. Electrophoresis was run in ice at 150 V for ~6 hours.

After electrophoresis, the gel was removed from its cassette and placed in SDS pre-soak buffer (Towbin transfer buffer + 2% SDS) for 30 minutes at RT with gentle rocking. The pre-soaked gel was then equilibrated 3x 5 minutes in Towbin transfer buffer at RT with gentle rocking. Proteins were transferred to Immobilon-FL (Millipore) membranes as described within the SDS-PAGE method in chapter 3. After transfer, the membranes were rinsed in ultrapure water and air dried for storage.

For visualization of the NativeMark marker bands, the dry blots rinsed for 30 sec in 100% meOH and then stained in R-250 stain solution (40% meOH, 7% AcOH, 0.025% Coomassie Blue R-250) for 5 minutes at RT with gentle rocking. The blots were then destained 3x5 minutes in destain solution (40% meOH, 7% AcOH) at RT with gentle rocking. Destained membranes were rinsed for 30 seconds in ddH₂O, then equilibrated 3x 5 minutes in 1xPBS at RT with gentle rocking prior to blocking, immunodetection, image acquisition, and analysis as described in the Chapter 3 western blotting method.

Reductant screen

Thiol-specific reduction under denaturing conditions (Fig. 3A).

Bovine rod outer segment (ROS) membranes were solubilized in Triton X-100 as described in chapter 2. supplemented with a protease inhibitor cocktail. Reactions were set up to a final total protein concentration of 1 mg/ml, in 1x PBS with 2% (w/v) SDS and incubated at 50° for 30 minutes in the presence of 50 mM DTT, 50 mM sodium ascorbate, 50 mM hydroxylamine, or buffer alone (sham reduction). Reactions were then moved to ice, mixed with Laemmli sample buffer lacking reductant and immediately analyzed by non-reducing SDS-PAGE followed by western blotting using antibodies directed against Prph2 and Rom1.

Thiol-specific reduction under native conditions (Fig. 3B).

Triton X-100 and DDM bovine ROS extracts (1.5 mg/ml protein in 1x PBS containing 25 mM NEM) were prepared as described. For reduction experiments, 30 µl of extract was mixed on ice with 15 µl of 2x solubilization buffer (to maintain a final detergent concentration of 1%) and 15 µl of 100 mM reductant stock (DTT, L-cysteine, or reduced glutathione in 1xPBS, pH-adjusted for a final reaction pH of 7.0). This yielded final concentrations of 0.75 mg/ml total protein, 25 mM reductant, and 12.5 mM NEM in the 60 µl reaction volume (effective reductant concentration, [reductant] – [NEM], of 12.5 mM). Reactions were incubated in the dark for 60 min at room temperature. The reactions were then quenched by addition of 60 µl 100 mM N-ethylmaleimide and then analyzed by non-reducing SDS-PAGE and western blotting for Prph2 and Rom1.

Results

Rom1 supports higher-order Prph2 polymerization

Prph2 and Rom1 interact to form the OS disk rim scaffold, and both are required for normal OS structure²⁵. However, Prph2 is essential for OS morphogenesis, while Rom1 is auxiliary. The precise molecular function of Rom1 is unknown but it has been hypothesized to have a modulatory role in disk rim scaffold assembly^{69,44,21}.

To determine the significance of Rom1 to Prph2 structure, ROS membranes were purified from P72 WT and *Rom1*^{KO/KO} (Rom1 KO) mice, and then solubilized in 1% Triton X-100. Western blots from non-reducing SDS-PAGE analysis of the ROS extracts show Prph2 migrating as two bands, corresponding to monomers and SS-dimers (Fig. 20A), in extracts from mice of both genotypes. Rom1 monomers and SS-dimers are present in the WT extracts, but no Rom1 was detected in the Rom1 KO extracts, confirming the genotypes of the mice used for these experiments. The ratio of Prph2 monomers to SS-dimers was consistently ~1:2 between individual animals of both genotypes. Whereas the Prph2 monomer in WT mouse ROS extracts migrated as a single band on non-reducing SDS-PAGE, the Prph2 monomer in Rom1 KO extracts migrates as a doublet, with ~40% of the monomeric Prph2 in the upper band.

BN-PAGE analysis comparing WT and Rom1 KO extracts (Fig. 20B) reveals a striking absence of Prph2 polymers larger than the octamer in Rom1 KO. Whereas the polymerized forms in extracts of both genotypes migrated with the same mobility on BN-PAGE, the mobility of the non-covalent dimer was slightly reduced in Rom1 KO

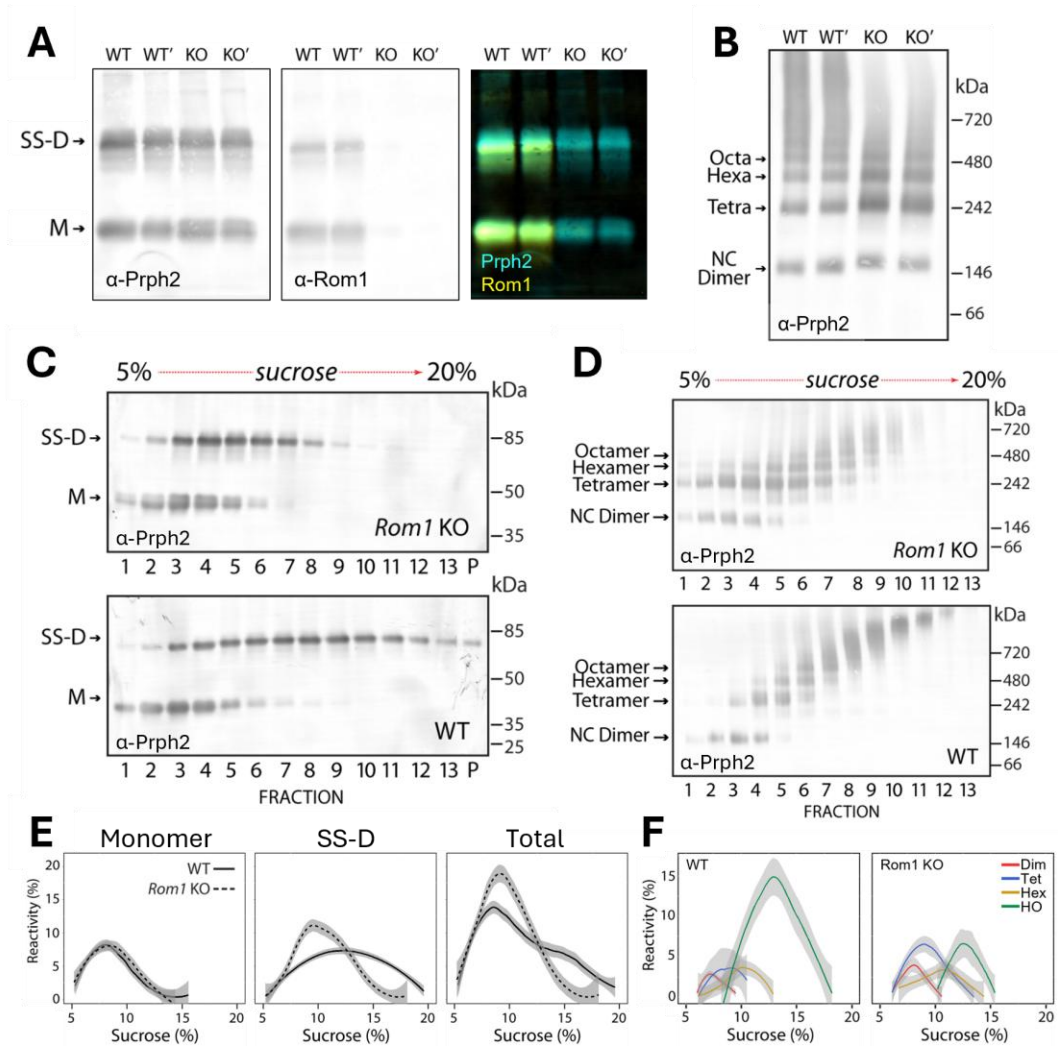


Figure 20: Rom1 is required for Prph2 polymerization into higher-order dimer chains in ROS. ROS membranes from wild-type (WT) and Rom1-knockout (Rom1 KO) mice were solubilized in 1% Triton X-100 and analyzed. (A) Western blot showing non-reducing SDS-PAGE of total ROS extracts probed with α -Prph2 (left), α -Rom1 (middle), and merged (right). Rom1 is absent in Rom1 KO extracts, confirming genotypes. Prph2 monomers resolve as a single band in WT but a doublet in Rom1 KO, mirroring the WT Rom1 monomer doublet. (B) Western blot from BN-PAGE analysis of total extracts showing Prph2 dimer chains up to the octamer in Rom1 KO ROS. The higher-order Prph2 bands seen in WT are absent in Rom1 KO. The non-covalent dimer migrates at a slightly higher apparent MW in KO. (C) Western blots from non-reducing SDS-PAGE analysis of fractionated velocity sedimentation gradients from WT (lower) and KO (upper) extracts. Prph2 is absent from the heavier sedimentation fractions in Rom1 KO. (D) Western blots from BN-PAGE analysis of fractionated gradients showing similar sedimentation of individual species between genotypes. (E) Sedimentation profiles showing LOESS regressions of monomer (left), disulfide-linked dimer (middle), and total α -Prph2 reactivity in WT (solid line) and Rom1 KO (dotted line). Shading indicates 95% confidence intervals. Data represents $n = 11$ WT and $n = 8$ Rom1 KO biological replicates. Monomer peaks are comparable, but the disulfide-linked dimer peak shifts to lower sucrose in KO. (F) LOESS regressions showing Prph2 species profiles from BN-PAGE analysis of $n = 2$ fractionated gradients for each genotype. Shading represents 95% confidence intervals.

compared to WT. The loss of Rom1 did not affect the relative abundance of Prph2 in non-covalent dimers; instead, the apparent loss of higher-order α -Prph2 reactivity in the Rom1-knockout lanes corresponded with an approximate doubling of the relative intensities of the tetramer, hexamer, and octamer bands in those lanes.

The BN-PAGE findings were validated by reoptimized velocity sedimentation experiments comparing WT and Rom1 KO ROS extracts. Non-reducing SDS-PAGE analysis of the fractionated gradients confirms the absence of higher-order Prph2 forms in the Rom1 KO, compared to WT (Fig. 20C). Sedimentation profiles show that while the Prph2 monomer peaks were comparable in intensity and position between genotypes, SS-dimer peak was of higher intensity and shifted to a lower sucrose percentage in Rom1 KO ROS extracts compared to WT (Fig. 20E). BN-PAGE analysis of fractionated sedimentation gradients (Fig. 20D & F) show that individual dimer-chain species sedimented similarly between genotypes. This result indicates that the differences in sedimentation profiles arise primarily from shorter Prph2 polymer lengths in Rom1 KO extracts, rather than from differences in polymer hydrodynamics. These findings agree with velocity sedimentation analysis of Rom1 KO total retina lysates published by another lab while this work was ongoing²⁶.

Free non-covalent dimers in bovine ROS are predominantly Prph2:Rom1 heterodimers reflecting a paucity of free homodimers

BN-PAGE analysis established that free non-covalent dimers are the most abundant form of both Prph2 and Rom1 in WT ROS membranes (Figs. 16 & 17). To

define their subunit composition, Prph2 was immunoprecipitated (IP'd) from bovine ROS DDM extracts using mAb2B6 (α -Prph2 CTER) at loads corresponding to 0.5x, 1x, and 2x the theoretical Prph2 binding capacity of the beads, followed by native elution with excess peptide BCT. Non-reducing SDS-PAGE and western blotting of the IP fractions demonstrated saturable, Prph2-dependent recovery: at 0.5x bead capacity, ~60% of the input Prph2 and ~20% of the input Rom1 were recovered. These values respectively declined to ~40% and ~5% at 1x bead capacity, and to 30% and ~2% at 2x bead capacity (Fig. 21A). Prph2 monomers on non-reducing SDS-PAGE were significantly overrepresented in the unbound fractions relative to the input ROS at all loads ($P < 0.05$, χ^2). BN-PAGE analysis of the IP fractions (Fig. 21B) revealed only negligible binding of non-covalent dimers at any load. Similarly, only 60% of the input Prph2 tetramers and 50% of the input Rom1 tetramers bound and eluted at 0.5x bead capacity, with tetramer binding efficiency decreasing at higher loads (Fig. 21B).

The ~80% of input Rom1 that was unbound at 0.5x bead capacity predicts that ~40% of input Prph2 should reside in unbound heteromeric complexes^{58,78}. The close match between this prediction and the observed ~40% of input Prph2 that remained unbound at that load therefore suggested a paucity of free Prph2 non-covalent homodimers in ROS membranes. To test this interpretation independently, Triton X-100 and DDM bovine ROS extracts were analyzed by high resolution clear native PAGE¹¹⁶ (hrCN-PAGE), which separates complexes by both size and intrinsic charge, followed by western blotting (Fig. 21C & D). Because Rom1 carries a significantly higher net negative charge than Prph2 at the running pH of 8.3¹¹⁷, heteropolymers migrate faster on hrCN-PAGE than equivalently sized Prph2 homopolymers.



Figure 21 : Prph2:Rom1 heterodimers and heterotetramers predominate in ROS membranes.

Prph2 was immunopurified from DDM extracts of bovine ROS using mAb2B6. (A) Western blot showing non-reducing SDS-PAGE of input (ROS), unbound (UB), and eluate fractions (Prph2 left, Rom1 middle, merged right). (B) Western blot from BN-PAGE of the same fractions showing negligible binding of noncovalent dimers. (C) Western blot from hrCN-PAGE of Triton X-100 (left) and DDM (right) ROS extracts. (D) Lane profiles of the Prph2 (solid) and Rom1 (dotted) channels. Peaks are labeled D (dimer), T (tetramer), H (hexamer), O (octamer), and IB1–IB3 (intermediate bands; unhyphenated for legibility). Both noncovalent dimer bands contained Prph2 and Rom1. The tetramer resolved as two bands, with only the faster-migrating band containing Rom1. Lighter intermediate bands (IB-1 and IB-2) showed similar splitting; IB-3 was homomeric and migrated as a single peak.

In both Triton and DDM ROS extracts, Prph2 formed ladder-like banding patterns on hrCN-PAGE (Fig. 21C), similar to those seen on BN-PAGE, while Rom1 was largely restricted to dimers and tetramers. Critically, the Prph2 tetramer migrated as two resolved bands, with only the faster-migrating band containing Rom1 (Fig. 21C & D). Larger Prph2 polymers (hexamers and above) migrated as single bands, consistent with their homomeric composition. A similar pattern was observed for the intermediate bands (IBs) in the DDM extracts; whereas IB-1 and IB-2 showed band splitting, IB-3 did not (Fig. 21D). Interestingly, although non-covalent dimers migrated as two bands in both detergents, both bands contained Prph2 and Rom1; possibly reflecting composition-independent charge heterogeneity in the non-covalent dimers (e.g. from an unidentified post-translational modification). Together, these data demonstrate the paucity of free non-covalent homodimers in bovine ROS membranes and indicate that non-disulfide bound Prph2 in ROS exists predominantly in non-covalent heterodimers with Rom1.

Thiol-specific reduction is consistent with S-glutathionylation of Rom1 in ROS membranes

Reversible thiol modifications at disulfide forming cysteines are a well-established mechanism for regulating intermolecular disulfide bonding of other proteins¹¹⁸. Rom1 monomers in detergent extracts of WT ROS migrate as a characteristic doublet under non-reducing SDS-PAGE conditions but resolve as a single band when the SDS-PAGE is run under reducing conditions. This doublet, and the similar Prph2 monomer doublet seen on non-reducing SDS-PAGE of Rom1 KO extracts (Fig. 20A & C), have been proposed to represent monomers with alternate EC2 intramolecular disulfide configurations²⁶. However, mutations in any of the six Prph2 EC2 intramolecular disulfide forming cysteines prevent proper folding, subunit assembly, and association with Rom1 in transfected COS-1 cells⁶⁶. Additionally, such mutations also prevent the mutant protein from trafficking to the OS in both mouse models^{119,120,121} and transgenic *Xenopus*⁷⁵. Such severe structural and biosynthetic defects make it unlikely that monomers of either protein with intramolecular EC2 disulfide configurations would be present in native polymers within ROS membranes. We therefore hypothesized that the Rom1 monomer doublet may instead reflect a reversible thiol modification.

To explore this possibility, we performed thiol modification-specific reduction of bovine ROS extracts. Under denaturing conditions (2% SDS at 50° for 30 minutes), 50 mM DTT collapsed Prph2 in Triton bovine ROS extract to a single monomer band and collapsed Rom1 to a single monomer band with faint high MW aggregates (Fig. 22A). The fully reduced Rom1 monomer migrated at a slightly higher apparent molecular weight than the lower band of the doublet observed under milder conditions, consistent

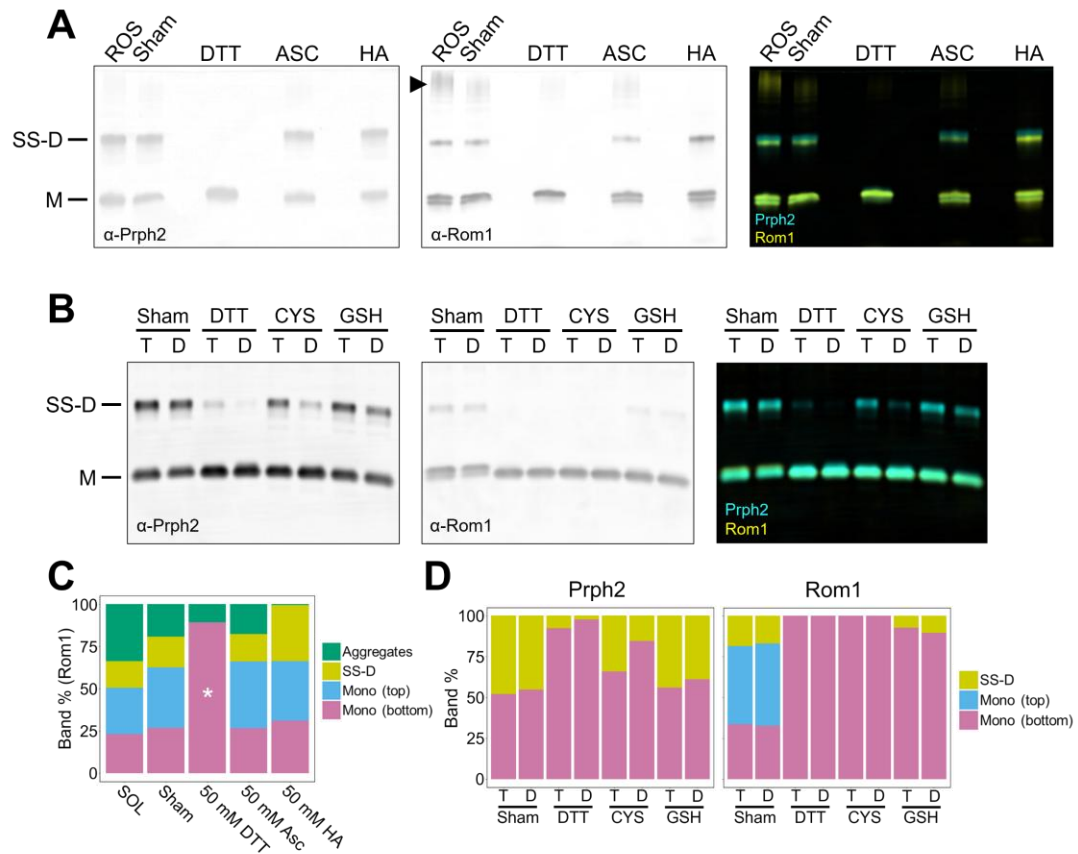


Figure 22. Selective thiol reduction is consistent with S-glutathionylation of Rom1. (A) Triton X-100 bovine ROS extract was reduced in 2% SDS at 50° for 30 minutes with 50 mM DTT (DTT), 50 mM sodium ascorbate (ASC), 50 mM hydroxylamine (HA), or sham-treated, then analyzed by non-reducing SDS-PAGE and western blotting for Prph2 (left) and Rom1 (middle); merged channels are shown at right. High MW aggregates of Rom1 in the untreated (ROS) and sham reduced ROS extracts (black arrowhead). (B) Triton X-100 ('T') and DDM ('D') bovine ROS extracts were reduced under native conditions for 60 min at room temperature with 12.5 mM DTT, 12.5 mM cysteine, 12.5 mM reduced glutathione (GSH), or sham-treated, then analyzed as in A. (C) Relative intensities of Rom1 aggregates, SS-dimers, and monomers from the Rom1 channel of the blot in A. Note that the Rom1 monomer in 50 mM DTT (white asterisk) is plotted as the bottom monomer band because it is a fully reduced form. (D) Relative intensities of disulfide-linked dimers and monomers from the Prph2 (left) and Rom1 (right) channels of the blot in B.

with complete reduction of intramolecular disulfides and full protein linearization.

Sodium ascorbate had no effect on the banding pattern generated on non-reducing SDS-PAGE by either protein. Hydroxylamine did not affect Prph2 or the Rom1 monomers but eliminated the high-molecular-weight Rom1 aggregates (Fig. 22A, black arrowhead); this

loss corresponded to an increase in the intensity of the disulfide-linked dimer band without a change in monomer intensity (Fig. 22C). This suggests S-acylation of disulfide-bound Rom1¹²² at one or more of its free cysteines outside of EC2.

Reductant	Specificity	Doublet Reactivity
DTT	Mixed disulfides, S-nitrosylation, S-sulfenylation, S-acylation, S-persulfidation	Yes
NEM	S-persulfidation	No
Hydroxylamine	S-acylation	No
Ascorbate	S-nitrosylation	No
GSH	S-glutathionylation	Yes
Cysteine	Mixed disulfides / S-cysteinylation	Yes

Table 2: Specificities of reductants tested in Figure 22 and their reactivity with the Rom1 monomer doublet.

Reducing Triton and DDM

bovine ROS extracts with 12.5 mM DTT, 12.5 mM reduced L-cysteine, and 12.5 mM reduced glutathione (GSH) under milder, native conditions eliminated the upper band of the Rom1 monomer doublet in both detergents (Fig. 22B). Nativeness of the reduced proteins was confirmed by BN-PAGE (not shown). Pretreatment of the ROS extracts with NEM precludes the possibility of S-persulfidation¹²³. Together, these data are most consistent with S-glutathionylation of non-disulfide-bound Rom1 in bovine ROS.

Molecular modeling predicts non-covalent interactions supporting Prph2 dimer chain oligomerization

Prph2 is detergent extracted from its native membranes as chains of noncovalent dimers linked together by C150 (or Rom1-C153) mediated inter-dimer disulfide bonds. Based on our observation that non-intermolecular disulfide forming Prph2^{C150S} can weakly associate into tetramers and hexamers, we hypothesized that WT noncovalent Prph2 dimers may also interact noncovalently in parallel with the inter-dimer disulfide

bonds. To address this, our collaborator Dr. Andrew Beaven (NICHD) used AlphaFold2-Multimer to identify potential interactions between Prph2 homodimers.

The top ranked model suggests a tetramer with the diagonally opposed Prph2-C150 residues in close proximity; mirroring the configuration of the linear dimer chains resolved by EM (Fig. 23A). The predicted dimer-dimer interaction is mediated by the Prph2 EC2 domains and appears to include a close contact between the D145/R183 salt bridges in the diagonally opposed monomers (Fig. 23B), in parallel with the arrangement of C150-C150 disulfide bonding. This result supports the hypothesis that dimers may interact noncovalently at that interface.

Molecular dynamics simulations predict spontaneous anisotropic curvature generation by Prph2 polymers

The extreme curvature of photoreceptor outer-segment (OS) disk rims is energetically unfavorable for a pure lipid bilayer. This curvature is highly anisotropic: the membrane undergoes a sharp radial bend in the direction of the Prph2/Rom1 EC2 domains towards the center of the disk (outer diameter ~24 nm), while circumferential curvature is negligible on the scale of individual Prph2 dimers given the >1 μm disk diameter. The essentially normal disk morphology observed in Rom1-knockout mice²⁵ indicates that homotypic Prph2 interactions are sufficient to supply the required bending energy. The interactions stabilizing dimer-chain oligomerization (i.e. Prph2-C150 and the predicted noncovalent EC2 interaction at that interface) are oriented perpendicular to the primary direction of curvature. Consequently, most of the bending energy must originate

from the noncovalent dimerization interface (via lipid packing defects induced by constraining the relative orientation of the TM bundles), from lateral interactions between the parallel constituent dimer chains of the belts, or from a combination of the two.

To address this, we reached out to our

collaborators in Dr. Alex Sodt's laboratory (NICHD)

who performed molecular dynamics simulations

evaluating spontaneous

curvature generation using a

Martini model¹²⁴ of Prph2

homodimers, tetramers, and

hexamers embedded in POPC bilayers at low protein coverage. Because coarse-grained

Martini protein models are prone to tertiary structure instability and unfolding, positional

restraints are routinely applied to maintain the proteins near their target conformation¹²⁵.

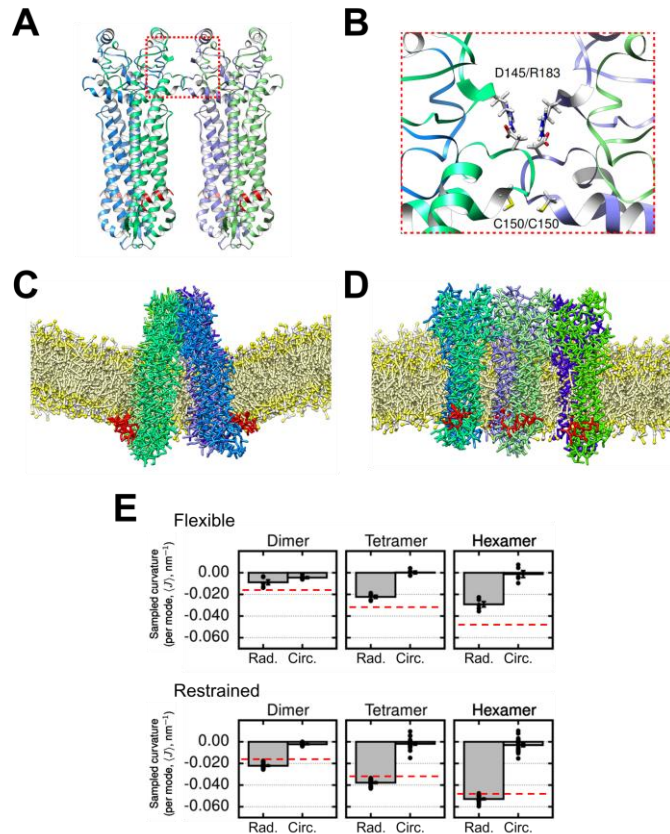


Figure 23: Molecular dynamics simulations predict anisotropic curvature generation by Prph2 polymers. (A) Perpendicular view of two Prph2 dimers showing the noncovalent interaction predicted by AlphaFold 2 Multimer. (B) Inset from A showing the predicted close contact between the D145/R183 intramolecular salt bridges of diagonally opposed monomers. (C) Individual dimers and dimer chains generate robust radial curvature. (D) Dimer chains do not generate circumferential curvature, mirroring the extreme anisotropy at the disk rim. (E) Barcharts showing the curvature sampled per mode in the molecular dynamics simulations. The dashed red lines indicate sampled curvature equivalent to a 12.5 nm extrapolated radius of curvature, approximating the energetic requirement for scaffolding the membrane curvature at the disk rim.

Simulations were run using two common restraint strategies: a loosely restrained model that maintains secondary structure elements but permits tertiary flexibility, and a tightly restrained model in which both tertiary structure and overall molecular shape are constrained to remain close to the initial AlphaFold-derived conformation (Fig. 23C-E).

In both cases, Prph2 oligomers induced robust spontaneous negative curvature in the membrane, similar to that seen at the rims of OS disks. The sampled curvature increases approximately linearly from the dimer to the tetramer to the hexamer (Fig. 23E). Tetramers and hexamers produced strong anisotropic curvature—pronounced negative curvature perpendicular to the polymer axis (i.e. disk radial, Fig. 23C) and only weak curvature parallel to the polymer axis (i.e. disk circumferential, Fig. 23D). These findings indicate that higher-order Prph2 polymerization is both necessary and sufficient to produce the extreme anisotropic curvature required for disk flattening; the loosely restrained model yielded roughly half the curvature, highlighting the importance of tertiary rigidity for spontaneous membrane curvature induction.

Molecular phenotyping of pathogenic Prph2 missense variants

The vast majority of reportedly pathogenic PRPH2 missense variants remain uncharacterized at the protein level. As a first step towards a larger scale screen aimed at addressing this critical knowledge gap, seven variants with strong evidence of pathogenicity (Y141C^{126,87,41}, R142W, R172W⁴³, L185P⁶⁸, R195L^{31,127}, G208D^{128,129}, and P216L³⁸) were overexpressed in transiently transfected HEK293 cells and the cell pellets were solubilized in 1% Triton X-100. The extracts were analyzed by SDS-PAGE to

determine relative protein expression levels and by BN-PAGE to assay Prph2 polymerization (Fig. 24).

Two codon optimized expression constructs were generated for each variant. One

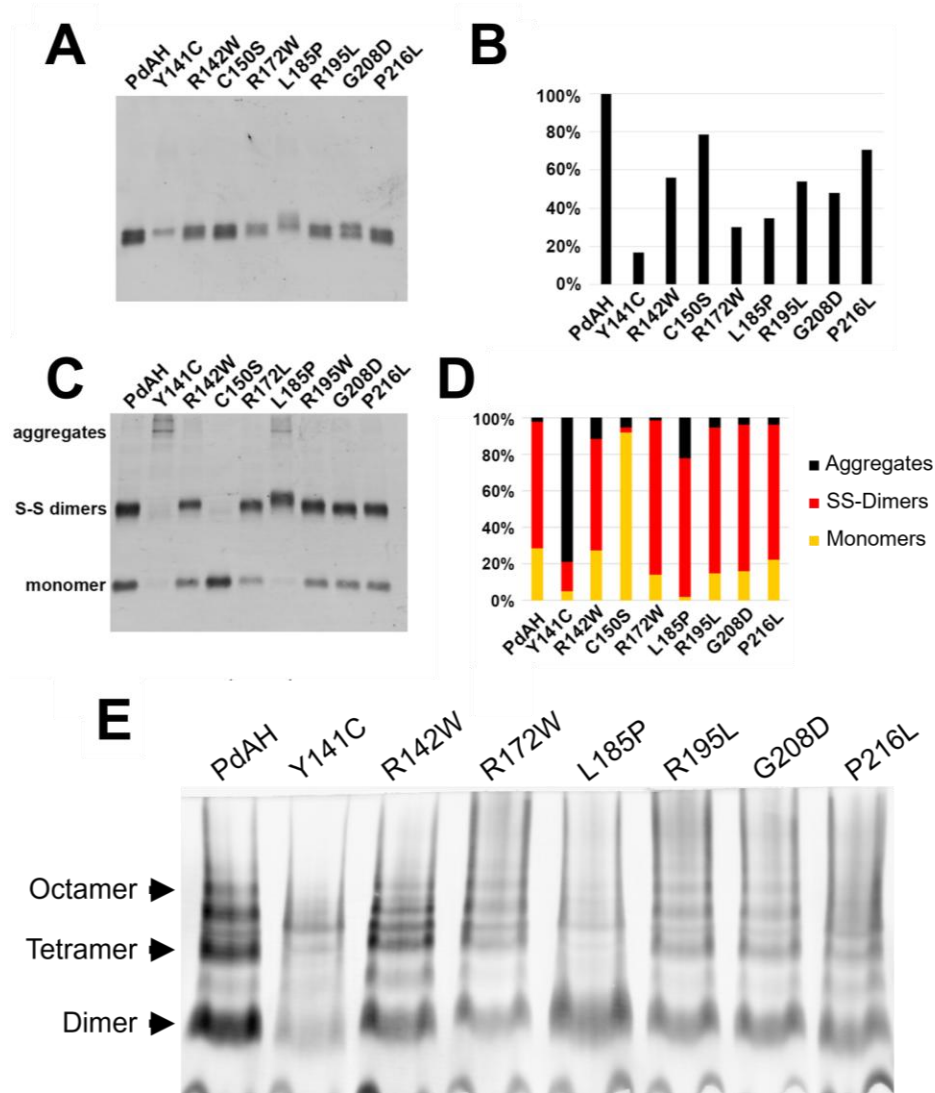


Figure 24: Molecular phenotyping of pathogenic Prph2 missense variants overexpressed in HEK293. (A) Western blot showing reducing SDS-PAGE analysis of PdAH missense variant expression level in HEK293. (B) Bar chart of mean relative band intensities (n = 3) from the analysis shown in A. (C) Western blot showing non-reducing SDS-PAGE analysis of the same extracts as shown in A. (D) Bar chart comparing mean relative intensities (n = 3) of monomers (yellow), SS-dimers (red), and high MW aggregates (black) from the analysis in C. (E) Western blot from BN-PAGE analysis of the PdAH variants in transfected HEK293 cell pellet extracts. Note that the prominent intermediate bands are a sample degradation product in Triton extracts of HEK293 cell pellets.

construct encodes the variant within the full length bovine Prph2 protein, and the other (PdAH) lacking the CTER amphipathic helix which activates curvature generation as assessed by TVN induction⁵⁵. PdAH forms generally have higher protein expression levels in HEK293 and produce more sharply resolved bands on BN-PAGE than their full-length counterparts (not shown). As such, to simplify data presentation, the dAH constructs were used for this initial proof-of-concept screen.

Reducing SDS-PAGE and western blotting of the HEK293 cell extracts shows that every missense variant exhibited decreased steady-state protein abundance levels when compared to the WT PdAH (Fig. 24A & B). Previous mouse studies suggest that alleles reducing Prph2 expression level below ~70% of wild type are expected to be pathogenic. All screened variants except P216L and the C150S control fell below that threshold. Non-reducing SDS-PAGE shows that intermolecular disulfide bonding efficiency was comparable to PdAH for all examined dAH variants except Y141C, C150S, and L185P (Fig. 24C & D). BN-PAGE analysis of the extracts showed that the R142W, R172W, R195L, G208D, and P216L variants formed polymers like those formed by WT PdAH (Fig. 24E). Together, these results suggest that many missense PRPH2 variants may exert their pathogenicity by reducing protein expression level, as opposed to disrupting Prph2 self-assembly.

Discussion

Rom1 is required for the appearance of higher-order Prph2 polymers in OS membranes. In Rom1 KO mouse ROS, Prph2 polymerization is limited to species no larger than the octamer. Rom1 is not required for the formation of disulfide-linked Prph2 dimer chains or for achieving a wild-type proportion of disulfide-bound Prph2; rather, it is required for the extension of smaller polymers into contiguous, higher-order homopolymers. The significance of and mechanistic basis for this polymer length limit in photoreceptors without Rom1 are unclear. Higher-order Prph2 polymerization occurs at the base of the OS during disk morphogenesis^{78,76,64}. Rom1:Prph2 heteromers traffic to the OS separately from homomeric Prph2^{78,77}. As a Rom1-naïve population of Prph2, the length-limited Prph2 polymers in Rom1 KO ROS membranes may represent trafficking-competent forms of Prph2.

The necessity of Rom1 for higher-order Prph2 polymerization in ROS appears paradoxical because Rom1 is only found in the smallest forms and also because WT Prph2 forms higher-order polymers in transfected HEK293 cells in the absence of Rom1. However, the Prph2 SS-dimer to monomer ratio is consistently higher in the transfected cells (~3:1, Fig. 24D) than in WT or Rom1 KO ROS (~3:2). Immunopurification with mAb2B6 and hrCN-PAGE demonstrate the virtual absence of free non-covalent Prph2 homodimers in WT ROS, indicating that ~ $\frac{3}{4}$ of the non-disulfide bound Prph2 in WT ROS is associated with Rom1. Consequently, the proportion of homomeric Prph2 that is disulfide linked in ROS is similarly elevated relative to the overall proportion of disulfide-bound Prph2. This implies that Prph2 inter-dimer disulfide bonding efficiency

in photoreceptors is mechanistically uncoupled from higher-order Prph2 polymerization and that Rom1 is significant to the latter, but not the former, process.

In ROS membranes, free noncovalent dimers are almost entirely Prph2:Rom1 heterodimers, tetramers are predominantly heteromeric, and all larger species (hexamers and above) are exclusively homomeric Prph2. As such, almost half of the tetraspanin molecules at the disk rim exist as free non-covalent heterodimers, whereas higher-order Prph2 polymers above the octamer account for only about one-quarter of the total tetraspanin content. Although the higher-order Prph2 homopolymers are functionally critical for generating the extreme anisotropic curvature of the disk rim membrane (Fig. 23), they are a minority component of the disk rim scaffold.

The early hydrodynamic studies that concluded a tetrameric stoichiometry for the core subunit of Prph2/Rom1 assembly were done with polymers that had been immunopurified from DTT-reduced bovine ROS Triton X-100 extracts using mAb2B6⁶⁷. Because mAb2B6 does not bind native non-covalent dimers, combined with the persistence of tetramers in DTT-reduced ROS extracts (Fig. 16), and because the apparent MW of the purified species matches that of the Triton-solubilized tetramer on BN-PAGE (~240 kDa), the species characterized in those studies was almost certainly a bona fide tetramer. A second Prph2 CTER targeting monoclonal antibody, mAbC6, similarly fails to bind native dimers (data not shown). The cryo-EM structures of El Mazouni et al. (2022) show the C-termini of polymer chains partitioned into a secondary micelle, promoting steric accessibility of both target epitopes, whereas the C-termini of the free dimers are partitioned into the primary micelle with the TM domains⁵⁸—a less sterically

accessible configuration. Development of a monoclonal antibody or nanobody that reliably recognizes a consistently natively accessible epitope of Prph2 (perhaps targeting EC2 helix D, which is both accessible in the polymers and divergent from Rom1) would provide an invaluable tool for future studies requiring purification of the protein.

Thiol modification specific reduction experiments demonstrate that the characteristic Rom1 monomer doublet seen on non-reducing SDS-PAGE of ROS detergent extracts is due to a reversible thiol modification. The reactivity profile of the monomer doublet, combined with the associated mobility shift^{130,131}, is most consistent with S-glutathionylation. Because this modification is present only on non-disulfide-bound Rom1 and is sterically accessible to glutathione on native dimers and tetramers, it is likely at the inter-dimer cysteine (C153); however, reduction with GSH lacks the specificity for definitive detection of S-glutathionylation¹³². Reducing the extracts with the S-glutathionylation-specific enzyme Grx1 followed by resin-assisted capture (SSG-RAC) would be the gold standard approach to validate this finding^{133,134}. Comparing the susceptibility of recombinant Rom1-C153S, Prph2-C150S, and the WT proteins to GSNO-induced glutathionylation in HEK293 cells¹³⁵ and GSSG-mediated glutathionylation in vitro¹³⁶, would determine whether the modification occurs at Rom1-C153/Prph2-C150. Investigations along these lines, aimed at identifying physiologically significant modifications occurring at those inter-dimer disulfide-forming thiols, will likely reveal the mechanisms underlying both the exclusion of Rom1 from higher-order polymers and the profound heterogeneity of Prph2 homopolymer lengths.

Molecular dynamics simulations establish that Prph2 polymerization is both necessary and sufficient to generate the anisotropic membrane curvature that defines photoreceptor disk rims. Although individual dimers generate robust curvature; only extended polymers reproduce the anisotropy characteristic of OS disk rim membranes. This provides the first direct mechanistic link between Prph2 polymer chain length and the physical architecture of the outer segment. Future extensions of these simulations will examine curvature induction by heteropolymers, the impact of deleting the Prph2 amphipathic helix (and the putative AH in Rom1), and the functional consequences of Prph2 VUSs.

Finally, high-throughput screening of previously uncharacterized PRPH2 variants demonstrates the feasibility of using optimized BN-PAGE for rapid functional assessment of PRPH2 VUSs. All screened alleles exhibited markedly reduced steady-state expression levels in HEK293 cells, potentially implicating pseudohaploinsufficiency as a major disease mechanism. Although most variants appeared to retain the ability to form higher-order polymers on BN-PAGE, the technical quality of these data was limited by material availability and polymer instability in Triton extracts. Repeating the BN-PAGE analysis with fresh extracts of both full-length and activated (dAH) constructs is necessary before definitive conclusions can be drawn regarding polymerization competence of the variants screened in this experiment.

CHAPTER 5

CONCLUSIONS

Summary of major conclusions

Prph2 is detergent extracted from its native membranes as linear disulfide-linked polymers of highly variable length

Previous investigations into Prph2 and Rom1 self-assembly and structure have relied on an established sucrose gradient velocity sedimentation assay to resolve Prph2 and Rom1 polymers in detergent extracts from their native membranes. The largest polymers, which are homomeric and comprise ~1/3 of the total Prph2 in WT extracts, pellet under the standard velocity sedimentation conditions, limiting the dynamic range of the assay. Additionally, the reproducibility of velocity sedimentation results has been limited by the high degree of manual dexterity and precision required to manually fractionate small-volume (2 ml) gradients. To expand the dynamic range of the assay, we reoptimized the velocity sedimentation conditions by reducing the sedimentation g-force to retain the entire size range of Prph2 polymers within the soluble fractions. To enhance reproducibility, we implemented a novel device for mechanical gradient fractionation that dramatically improves the precision of fraction collection (Fig. 11).

Applying the reoptimized velocity sedimentation method to analyze bovine ROS detergent extracts (Figs. 10 & 12) reproduced key previous findings from sedimentation experiments done under the standard conditions that (1) the smallest native form of Prph2 and Rom1 is non-covalently associated, (2) Rom1 is only present in smaller polymers, and (3) intermediate and higher-order polymers are stabilized by intermolecular disulfide

bonds⁶⁹. These experiments also reveal the striking range and diversity of Prph2 polymer sizes, biochemically validating negative staining TEM of purified Prph2 polymers previously published by our lab⁴⁴. This broad polydispersity of Prph2 polymers is seen regardless of whether Triton X-100 or DDM is used as the extraction detergent (Fig. 12). When applied to transfected HEK293 cell pellet detergent extracts, the reoptimized sedimentation results further demonstrate that recombinant WT Prph2 overexpressed in HEK293 forms polymers of similar polydispersity to those in ROS extracts.

An optimized BN-PAGE method resolves Prph2/Rom1 polymers by size

The velocity sedimentation approach is limited by its inability to resolve individual Prph2/Rom1 polymer species. This limitation hindered mechanistic studies of Prph2/Rom1 self-assembly as prevented rigorous biochemical characterization of pathogenic variants. To overcome this, we developed a BN-PAGE method optimized for the high-resolution separation and relative quantification of individual disulfide-linked Prph2/Rom1 polymer species. Systematic optimization of electrophoresis and immunoblotting conditions (Fig. 14) yielded highly reproducible results in both native bovine ROS extracts (Fig. 16) and recombinant overexpression systems (Fig. 19).

Rom1 promotes higher-order Prph2 polymerization in OS membranes

Velocity sedimentation and BN-PAGE analysis comparing WT and Rom1 KO mouse ROS extracts reveal that Rom1 is necessary for Prph2 polymerization into forms larger than the octamer (Fig. 20B-F). Non-reducing SDS-PAGE analysis of those extracts

shows that absence of higher-order Prph2 polymers in Rom1 KO ROS is not due to reduced efficiency of Prph2-C150 mediated inter-dimer disulfide bonding (Fig. 20A); implying that the absence of higher-order Prph2 polymers in Rom1 KO OS membranes arises from a defect inter-dimer disulfide contiguity instead of a defect in disulfide bond formation. Together, these results demonstrate that Rom1 is an important photoreceptor-specific Prph2 assembly factor that promotes higher-order Prph2 polymerization in OS membranes.

Prph2 CTER-targeting monoclonal antibodies do not bind native non-covalent dimers

The monoclonal α -Prph2 CTER antibody mAb2B6 does not efficiently bind native non-covalent Prph2 dimers (Fig. 21B). Because mAb2B6 preferentially recognizes higher-order, disulfide-linked polymers but fails to recognize the fundamental non-covalent dimer, earlier immunoaffinity purification strategies using this antibody inadvertently enriched for larger oligomeric species and thereby overestimated the size of the minimal core complex. This finding provides a clear explanation for the discrepancy between early hydrodynamic studies, which indicated a tetrameric core subunit stoichiometry based on experiments performed with mAb2B6-purified Prph2⁶⁷, and the dimeric core subunit stoichiometry established by recent cryo-EM studies⁵⁸.

Heterodimers and heterotetramers predominate in ROS membranes

Results from mAb2B6 immunopurification and hrCN-PAGE indicate a paucity of homomeric Prph2 tetramers and free non-covalent dimers in ROS (Fig. 21). Instead, the

vast majority of small Prph2/Rom1 complexes OS membranes exist as heterodimers and heterotetramers. When considered together with the finding that the overall proportion of disulfide-bound Prph2 in ROS does not change in the Rom1 KO mouse despite the absence of higher-order polymers (Fig. 20), these data indicate that Rom1 has the *apparent* effect of sequestering Prph2 monomers within the smallest, heteromeric forms. However, the mechanisms through which this apparent effect is achieved remain unknown.

Prph2 polymers generate spontaneous anisotropic membrane curvature

Molecular dynamics simulations suggest that while Prph2 homodimers can induce a high level of spontaneous membrane curvature, polymerization is necessary to achieve the curvature anisotropy characterizing OS disk rims (Fig. 23). These simulations suggest that a single belt of disulfide-linked Prph2 homodimers can provide most, if not all, of the energy required to scaffold the hairpin geometry of the disk rim membrane.

It is noteworthy that homomeric Prph2 accounts for approximately one-third of the total tetraspanin content at the disk rim and that the rim itself is organized into three parallel belts of polymers. These stoichiometric and structural observations raise the intriguing possibility—although rigorous experimental evidence is currently lacking—that Prph2 homopolymers may preferentially occupy the central belt. Regardless of the precise subunit organization within the disk rim scaffold, the simulations clearly indicate that the primary membrane-deforming activity of the scaffold is an intrinsic property of polymerized Prph2. Rom1, rather than being strictly required for curvature generation per

se, appears to function primarily by promoting the efficient formation of the longer Prph2 polymers needed to sustain anisotropic curvature and precise disk rim membrane architecture. Additional simulations evaluating the membrane curvature-inducing activity of heterodimers and heterotetramers will be required to test this hypothesis and further refine our understanding of the respective contributions of Prph2 and Rom1 to normal disk rim structure.

BN-PAGE offers a practical high-throughput platform for biochemically characterizing Prph2 VUSs

Applying the BN-PAGE method developed in Chapter 3 enables rapid and quantitative assessment of WT and variant Prph2 polymerization in transfected HEK293 cell pellet detergent extracts. By resolving discrete polymer species and enabling parallel analysis of multiple constructs with relatively modest amounts of material, this platform overcomes the low throughput and limited resolution of earlier methods. When applied to both pathogenic and experimental missense Prph2 variants, BN-PAGE readily distinguishes defects in non-covalent dimerization, inter-dimer disulfide bonding, and higher-order polymerization. These capabilities directly fulfill the third aim of this thesis and establish BN-PAGE as a powerful new tool for molecular phenotyping of *PRPH2* VUSs. Such biochemical classification will help rationalize the remarkable phenotypic heterogeneity observed in *PRPH2*-associated inherited retinal degenerations and may ultimately support improved clinical variant interpretation and guide clinical decision-making.

Key remaining knowledge gaps

How is polymer chain length regulated?

Although these studies have clarified several steps of Prph2/Rom1 polymerization, the determinants of polymer chain length remain unknown. In a highly oxidizing environment, like the OS or the interphotoreceptor matrix, unprotected juxtaposed cysteine thiols will disulfide bond. Prph2-C150 thiols between non-covalently interacting Prph2 dimers are in close proximity (Fig. 23 A & B), yet the inter-dimer disulfide bonding within the belts of the disk rim scaffold is highly discontinuous. This suggests that at least one of the terminal Prph2-C150/Rom1-C153 thiols on either end of each polymer is capped by a post-translational thiol modification.

Moreover, higher-order Prph2 polymerization occurs at the base of the OS⁷⁶. Prph2 homodimers assemble with their EC2 domains in the oxidizing environment of the ER lumen. These dimers (and / or small polymers, the precise trafficking forms are currently unknown) then traffic unconventionally to the OS^{137,77} via the oxidizing late endosomes¹³⁸. Upon reaching the base of the OS, where the EC2 domains are exposed to the highly oxidizing interphotoreceptor matrix¹³⁹, Prph2 homodimers only disulfide bonds with other Prph2 homodimers. The Prph2-C150 thiols must be prevented from premature interdimer disulfide bond formation during trafficking and from disulfide bonding with the wrong type of dimer when higher-order polymerization occurs during disk morphogenesis.

In WT ROS, ~1/3 of Rom1 molecules carry a reversible thiol modification, putatively identified as S-glutathionylation (Fig. 22). If this modification occurs at Rom1-

C153, then it would be sufficient to account for the absence of higher-order heteropolymers at the disk rim. The Prph2 monomer runs as a similar doublet on non-reducing SDS-PAGE analysis of both Rom1 KO ROS and WT total retina lysate, which may represent the trafficking end cap. However, this modification is not seen on Prph2 in WT ROS. Therefore, it remains to be determined if another thiol modification caps one or both ends of Prph2 homopolymers in ROS.

What is the native subunit organization of the disk rim scaffold?

The precise subunit organization of Prph2 and Rom1 within the three parallel belts of the disk rim scaffold is unclear. It is unknown whether Rom1 is evenly distributed throughout the belts, or if it preferentially occurs in the inner or outer belts. It is also unclear whether Rom1 has any tendency to alternate sides between dimers in the belts in which it occurs. The resolution of the current map does not allow definitive identification of subunits within the solved structure of the rim scaffold. However, the cryo-ET structure may provide a potential hint.

In both the solved cryo-EM structures and in AlphaFold models, Rom1-P126 induces a distinctive bend near the N-terminus of helix A at the base of the EC2 domain. Prph2 has a conserved arginine in that position instead and Prph2 helix A is not bent. The monomers on the inside of the outer belts in the cryo-ET of the in-situ rim scaffold appear to show a bend near the N-terminus of helix A; helix A does not appear to be bent in the other monomers within the structure (Fig. 7).

If Rom1 does have a sidedness preference in the belts where it occurs, then Rom1-C153 must also preferentially form heterotypic inter-dimer disulfide bonds with Prph2-C150. Because Prph2 is N-glycosylated and Rom1 is not, this prediction is extremely easy to test. PNGaseF digest of ROS detergent extract followed by non-reducing SDS-PAGE and western blotting would reveal whether the mobility of the Rom1 SS-dimer band shifts with the Prph2 SS-dimer band. Clarifying Rom1's position within the belts and rim scaffold will be important for understanding how Prph2 and Rom1 interact to achieve normal disk rim structure.

What are the specific non-covalent interactions driving Prph2/Rom1 self-assembly?

While inter-dimer disulfide bonds are clearly required for higher-order polymer formation, BN-PAGE analysis of the Prph2-C150S mutant and AlphaFold multimer predictions both point to the existence of additional non-covalent interactions that occur in parallel with these disulfides. These interactions require more rigorous empirical validation and characterization. Similarly, the precise interfaces that mediate interactions between the three parallel dimer-chain belts remain undefined.

Do Prph2 and Rom1 follow the same assembly rules in cones as in rods?

The biochemical analyses in this thesis were primarily performed on purified rod outer segments. Cone OS structure is more resilient to defective Prph2 polymerization than rod OS structure^{74,73}. Rod disk enclosure is tuned by Prph2 polymerization⁶⁴. Cone disks become fully enclosed at the distal tip of the cone OS prior to shedding¹³. This

raises the question of whether Prph2/Rom1 self-assembly is differently regulated and/or more (or less) dynamic in cones than in rods? Because *PRPH2* mutations are implicated in both rod-dominant and cone-dominant IRDs, answering this question will help rationalize the broad phenotypic diversity of *PRPH2*-related disease.

What is the mechanism of membrane curvature generation by Prph2?

Although our molecular dynamics simulations have begun to illuminate how Prph2 polymers scaffold the extreme anisotropic curvature at OS disk rims, and these studies are ongoing, this work has barely scratched the surface. The relative contributions of Rom1, the Prph2 CTER amphipathic helix, rim lipid composition, Prph2-N229 glycosylation (which may affect the rigidity of the EC2 domain), ions, and both the known and unknown heterotypic interacting proteins (e.g. GARP2) to membrane curvature generation at the disk rim remain almost completely unexplored.

APPENDIX A: LIST OF PLASMIDS USED FOR TRANSFECTION

Table 3: List of plasmids used for transfection.

Plasmid	Insert	Backbone	Resistance	Source
bP2	bovine Prph2	pD2610v4	kanamycin	ATUM
PdAH	bovine Prph2 dAH (deletion of the CTER amphipathic helix)	pD2610v4	kanamycin	ATUM
C150S-bP2	bovine Prph2-C150S	pD2610v4	kanamycin	ATUM
C150S-PdAH	bovine Prph2-C150S dAH	pD2610v4	kanamycin	ATUM
L185P-bP2	bovine Prph2-L185P	pD2610v4	kanamycin	ATUM
L185P-PdAH	bovine Prph2-L185P dAH	pD2610v4	kanamycin	ATUM
C150S-L185P-bP2	bovine Prph2-C150S;L185P	pD2610v4	kanamycin	ATUM
Y141C-bP2	bovine Prph2-Y141C	pD2610v4	kanamycin	ATUM
Y141C-PdAH	bovine Prph2-Y141C dAH	pD2610v4	kanamycin	ATUM
R142W-bP2	bovine Prph2-R142W	pD2610v4	kanamycin	ATUM
R142W-PdAH	bovine Prph2-R142W dAH	pD2610v4	kanamycin	ATUM
R172W-bP2	bovine Prph2-R172W	pD2610v4	kanamycin	ATUM
R172W-PdAH	bovine Prph2-R172W dAH	pD2610v4	kanamycin	ATUM
R195L-bP2	bovine Prph2-R195L	pD2610v4	kanamycin	ATUM
R195L-PdAH	bovine Prph2-R195L dAH	pD2610v4	kanamycin	ATUM
G208D-bP2	bovine Prph2-G208D	pD2610v4	kanamycin	ATUM
G208D-PdAH	bovine Prph2-G208D dAH	pD2610v4	kanamycin	ATUM
P216L-bP2	bovine Prph2-P216L	pD2610v4	kanamycin	ATUM
P216L-PdAH	bovine Prph2 P216L dAH	pD2610v4	kanamycin	ATUM
BovRom1	bovine Rom1	pD2610v4	kanamycin	ATUM
Rom1dCH	bovine Rom1 dCH (deletion of the putative CTER amphipathic helix)	pD2610v4	kanamycin	ATUM

APPENDIX B: ANTIBODIES USED IN THESE STUDIES

Table 4: List of primary antibodies

Antibody	Target	Host	Type	Source	Catalog #	Application
pAbMPCT	mouse Prph2	Rabbit	Polyclonal	ref. 136	N/A	WB
pAbMUTT	bovine Rom1	Rabbit	Polyclonal	ref. 57	N/A	WB
mAbC6	bovine Prph2	Mouse	Monoclonal	ref. 57	N/A	WB
mAb2B6	bovine Prph2	Mouse	Monoclonal	ref. 42	N/A	IP
mAb2H5	Rom1	Mouse	Monoclonal	Millipore-Sigma	MABN1757	WB
pAbBCT	bovine Prph2	Rabbit	Polyclonal	ref. 139	N/A	WB

Table 5: List of secondary antibodies

Antibody	Fluorophore	Vendor	Catalog #	Use
Goat α -rabbit 550	AzureSpectra 550	Azure Biosystems	AC2158	BN-PAGE WB
Goat α -rabbit 490	AzureSpectra 490	Azure Biosystems	AC2206	BN-PAGE WB
Goat α -rabbit 680	IRDye 680	LiCor	926-68071	SDS-PAGE WB
Goat α -mouse 800	IRDye 800CW	LiCor	926-32210	WB
Goat α -mouse 680	IRDye 680	LiCor	926-68070	SDS-PAGE WB

APPENDIX C: KEY REAGENTS

Table 6: List of key reagents.

Reagent	Vendor	Catalog #	Use
4–20% Mini-PROTEAN® TGX™ Gels	BioRad	4561094	BN-PAGE
6-Aminohexanoic acid	Millipore-Sigma	8001450005	BN-PAGE
B-phycoerythrin	Fisher Scientific	NC1048747	BN-PAGE
Coomassie Brilliant Blue G-250	BioRad	1610406	BN-PAGE
NativeMark™ Unstained Protein Standard	Fisher Scientific	LC0725	BN-PAGE
Cyanogen bromide-activated-Sephacrose™ 4B	Millipore-Sigma	C9142-1G	Immunopurification
10:1 Dodecyl Maltoside to Cholesteryl Hemisuccinate	Fisher Scientific	D310-CH210	Protein solubilization
cOmplete™ ULTRA Tablets, Mini, EDTA-free, <i>EASYpack</i> Protease Inhibitor Cocktail	Millipore-Sigma	5892791001	Protein solubilization
Triton™ X-100 Surfact-Amps™ Detergent Solution	Fisher Scientific	28314	Protein solubilization
X-tremeGENE™ 9 DNA Transfection Reagent	Millipore-Sigma	6365787001	Recombinant protein expression
10% Mini-PROTEAN® TGX™ Gels	BioRad	4561034	SDS-PAGE
Li-Cor Intercept (PBS) Blocking Buffer	Fisher Scientific	NC1660556	WB
Peptide BCT: VEAEGEDAGQAPAAG	Sigma-Genosys	N/A	Immunopurification

APPENDIX D: APPROVAL FROM THE INSTITUTIONAL ANIMAL CARE AND
USE COMMITTEE



Reference: IACUC Protocol # 2022-1176

Protocol Title: Role of rom1 for photoreceptor structure, function, and viability

Principal Investigator: Goldberg, Andrew

Approval Period: 07/19/2022 - 07/19/2025

Dear Professor Goldberg, Andrew:

The Institutional Animal Care and Use Committee (IACUC) has reviewed the original proposal, noted the required modifications provided by you upon committee's request (if applicable), and approved the project referenced above.

The proposed activities are approved for a period of three years, starting 07/19/2022 and ending 07/19/2025, and assigned the approval # IACUC-2022-1176. This is your formal approval notice; please save it for your records. Be aware that protocols expire at 12:01 AM on the expiration date. Ninety (90) days before the protocol approval expires, Cayuse will send you an alert email prompting you to submit a De Novo application to renew the project, if desired.

Any unanticipated problems, changes in, or halting of the activities proposed in the project, as described, must be promptly reported to the IACUC Administrator.

In addition, all faculty, staff, and students involved in the project must be trained and qualified to conduct the project in a responsible manner. Please note, in this regard, that you, the Principal Investigator, are fully responsible at all times.

Please let me know if you have any questions.

Sincerely,

Janet Schofding
Animal Research Facility Manager and IACUC Administrator

APPENDIX E: APPROVAL FROM THE INSTITUTIONAL BIOSAFETY COMMITTEE



Reference: IBC Protocol # IBC-00001116

Protocol Title: Y285x and rds mouse models for investigating inherited retinal degeneration

Principal Investigator: Goldberg, Andrew

Approval Period: 06/07/2023 - 06/07/2026

Dear Professor Goldberg, Andrew:

The Institutional Biosafety Committee (IBC), responsible for the review of research involving materials of human origin, recombinant DNA, and biohazardous, infectious or toxic materials, has reviewed the original proposal, noted the revisions provided by you upon committee's request (if applicable), and approved the project referenced above. This is your formal approval notice; please save it for your records.

The proposed activities are approved for a period of three years, starting 06/07/2023 and ending 06/07/2026, and assigned the approval # IBC-00001116. Please be aware that protocols expire at 12:01 AM on the expiration date. The highest BSL level approved for the protocol can be found on the Overview page of the protocol form.

Any unanticipated problems, changes in, or halting of the activities proposed in the project, as described, must be promptly reported to the IBC or the biosafety officer.

In addition, all faculty, staff, and students involved in the project must be trained and qualified to conduct the project in a responsible manner. Please note, in this regard, that you, the Principal Investigator, are fully responsible at all times for limiting or restricting access to laboratories and for assessing, in each circumstance, who may enter or work in a laboratory when biohazardous materials are in use.

Prior to initiating any work on this approved project, please contact me by email (luongo@oakland.edu) to schedule a startup Biosafety Audit. This audit will verify that all biosafety controls described in your application have been implemented.

Please let me know if you have any questions. Thank you.

Sincerely,

Domenic Luongo, CHMM
Director - Laboratory Safety and Compliance

REFERENCES

1. Boucher, R. C. *et al.* Photoreceptor outer segment disk rim curvature relies on a tetraspanin interaction web. *Journal of Biological Chemistry* **302**, 113136 (2026).
2. Boucher, R. C., Milstein, M., Cavanaugh, B. & Goldberg, A. F. X. New assays for peripherin-2/rom1 interactions shaping photoreceptor outer segment disk rims. *Invest. Ophthalmol. Vis. Sci.* **65**, 4736–4736 (2024).
3. Schultze, M. Ueber Stäbchen und Zapfen der Retina. *Archiv für Mikroskopische Anatomie* **3**, 215–247 (1867).
4. Schultze, M. Ueber die Nervenendigung in der Netzhaut des Auges bei Menschen und bei Thieren. *Archiv für Mikroskopische Anatomie* **5**, 379–403 (1869).
5. Sjöstrand, F. S. An electron microscope study of the retinal rods of the guinea pig eye. *J. Cell. Comp. Physiol.* **33**, 383–403 (1949).
6. Cohen, A. I. The fine structure of the extrafoveal receptors of the rhesus monkey. *Exp. Eye Res.* **1**, 128–136 (1961).
7. Kühne, W. Zur Photochemie der Netzhaut. *Untersuchungen aus dem Physiologischen Institute der Universität Heidelberg* **1**, 1–14 (1877).
8. Kühne, W. On the Stable Colours of the Retina. *J. Physiol.* **1**, 109–212 (1878).
9. Sjöstrand, F. S. The ultrastructure of the outer segments of rods and cones of the eye as revealed by the electron microscope. *J. Cell. Comp. Physiol.* **42**, 15–44 (1953).
10. Pattni, K., Wood, A., Cassels, N. & Margrain, T. Visual pigment concentration and photoreceptor outer segment length in the human retina. *Ophthalmic and Physiological Optics* **44**, 917–924 (2024).
11. Young, R. W. The renewal of photoreceptor cell outer segments. *J. Cell Biol.* **33**, 61–72 (1967).
12. Cohen, A. I. New evidence supporting the linkage to extracellular space of outer segment saccules of frog cones but not rods. *Journal of Cell Biology* **37**, 424–444 (1968).
13. Anderson, D. H., Fisher, S. K. & Steinberg, R. H. Mammalian cones: disc shedding, phagocytosis, and renewal. *Invest. Ophthalmol. Vis. Sci.* **17**, 117–133 (1978).

14. Umapathy, A. *et al.* Spatiotemporal live-cell analysis of photoreceptor outer segment membrane ingestion by the retinal pigment epithelium reveals actin-regulated scission. *Journal of Neuroscience* **43**, 2653–2664 (2023).
15. Paniagua, A. E., Chang, A. & Williams, D. S. Mechanism of photoreceptor outer segment tip ingestion: evidence of trogocytosis. *Adv. Exp. Med. Biol.* **1468**, 309–311 (2025).
16. Young, R. W. & Bok, D. Participation of the retinal pigment epithelium in the rod outer segment renewal process. *J. Cell Biol.* **42**, 392–403 (1969).
17. De Robertis, E. Electron microscope observations on the submicroscopic organization of the retinal rods. *J. Biophys. Biochem. Cytol.* **2**, 319–330 (1956).
18. Arikawa, K., Molday, L. L., Molday, R. S. & Williams, D. S. Localization of peripherin/rds in the disk membranes of cone and rod photoreceptors: relationship to disk membrane morphogenesis and retinal degeneration. *Journal of Cell Biology* **116**, 659–667 (1992).
19. Bascom, R. A. *et al.* Localization of the photoreceptor gene ROM1 to human chromosome 11 and mouse chromosome 19: sublocalization to human 11q13 between PGA and PYGM. *Am. J. Hum. Genet.* **51**, 1028 (1992).
20. Travis, G. H. Name dropping. *Nature*, **349**, 24 (1991).
21. Goldberg, A. F. X., Moritz, O. L. & Williams, D. S. Molecular basis for photoreceptor outer segment architecture. *Prog. Retin. Eye Res.* **55**, 52–81 (2016).
22. Reuter, J. H. & Sanyal, S. Development and degeneration of retina in rds mutant mice: The electroretinogram. *Neurosci. Lett.* **48**, 231–237 (1984).
23. Sanyal, S. & Jansen, H. G. Absence of receptor outer segments in the retina of rds mutant mice. *Neurosci. Lett.* **21**, 23–26 (1981).
24. Hawkins, R. K., Jansen, H. G. & Sanyal, S. Development and degeneration of retina in rds mutant mice: photoreceptor abnormalities in the heterozygotes. *Exp. Eye Res* vol. 41 (1985).
25. Clarke, G. *et al.* Rom-1 is required for rod photoreceptor viability and the regulation of disk morphogenesis. *Nat. Genet.* **25**, 67–73 (2000).
26. Lewis, T. *et al.* ROM1 is redundant to PRPH2 as a molecular building block of photoreceptor disc rims. *Elife* **12**, RP89444 (2023).

27. Alashwal, S. M., Yassin, S. H., Kalaw, F. G. P. & Borooah, S. PRPH2-associated retinal diseases: a systematic review of phenotypic findings. *Am. J. Ophthalmol.* **271**, 7–30 (2025).
28. Apfelstedt-Sylla, E. *et al.* Extensive intrafamilial and interfamilial phenotypic variation among patients with autosomal dominant retinal dystrophy and mutations in the human RDS/Peripherin Gene. *Journal of Ophthalmology* vol. 79 (1995).
29. Kajiwar, K. *et al.* Mutations in the human retinal degeneration slow gene in autosomal dominant retinitis pigmentosa. *Nature* **354**, 480–483 (1991).
30. Farrar, G. J. *et al.* A three-base-pair deletion in the peripherin-RDS gene in one form of retinitis pigmentosa. *Nature* **354**, 478–480 (1991).
31. Yanagihashi, S. *et al.* Autosomal dominant central areolar choroidal dystrophy and a novel Arg195Leu mutation in the peripherin/rds gene. *Archives of Ophthalmology* **121**, 1458–1461 (2023).
32. Wells, J. *et al.* Mutations in the human retinal degeneration slow (RDS) gene can cause either retinitis pigmentosa or macular dystrophy. *Nat. Genet.* **3**, 213–218 (1993).
33. Khan, A. O., Al Rashaed, S., Neuhaus, C., Bergmann, C. & Bolz, H. J. Peripherin mutations cause a distinct form of recessive Leber congenital amaurosis and dominant phenotypes in asymptomatic parents heterozygous for the mutation. *British Journal of Ophthalmology* **100**, 209–215 (2016).
34. Ma, C. J. *et al.* Late-onset pattern macular dystrophy mimicking ABCA4 and PRPH2 disease is caused by a homozygous frameshift mutation in ROM1. *Cold Spring Harb. Mol. Case Stud.* **5**, 1–8 (2019).
35. Sakuma, H. *et al.* A heterozygous putative null mutation in ROM1 without a mutation in peripherin/RDS in a family with retinitis pigmentosa. *Genomics* **27**, 384–386 (1995).
36. Kajiwar, K., Berson, E. L. & Dryja, T. P. Digenic retinitis pigmentosa due to mutations at the unlinked peripherin/RDS and ROM1 loci. *Science* (1979). **264**, 1604–1608 (1994).
37. Dryja, T. P., Hahn, L. B., Kajiwar, K. & Berson, E. L. *Dominant and Digenic Mutations in the Peripherin/RDS and ROM1 Genes in Retinitis Pigmentosa.*
38. Kedzierski, W., Lloyd, M., Birch, D. G., Bok, D. & Travis, G. H. Generation and analysis of transgenic mice expressing P216L-substituted Rds/Peripherin in rod photoreceptors. *Invest. Ophthalmol. Vis. Sci.* **38**, 498–509 (1997).

39. Chakraborty, D., Conley, S. M., Stuck, M. W. & Naash, M. I. Differences in RDS trafficking, assembly and function in cones versus rods: Insights from studies of C150S-RDS. *Hum. Mol. Genet.* **19**, 4799–4812 (2010).
40. Stuck, M. W., Conley, S. M. & Naash, M. I. Retinal Degeneration Slow (RDS) glycosylation plays a role in cone function and in the regulation of RDS·ROM-1 protein complex formation. *Journal of Biological Chemistry* **290**, 27901–27913 (2015).
41. Conley, S. M., Stuck, M. W., Watson, J. N. & Naash, M. I. Rom1 converts Y141C-Prph2-associated pattern dystrophy to retinitis pigmentosa. *Hum. Mol. Genet.* **26**, 509–518 (2017).
42. Cavanaugh, B. L. *et al.* A new mouse model for PRPH2 pattern dystrophy exhibits functional compensation prior and subsequent to retinal degeneration. *Hum. Mol. Genet.*
43. Ding, X. Q. *et al.* The R172W mutation in peripherin/rds causes a cone-rod dystrophy in transgenic mice. *Hum. Mol. Genet.* **13**, 2075–2087 (2004).
44. Milstein, M. L. *et al.* Multistep peripherin-2/rds self-assembly drives membrane curvature for outer segment disk architecture and photoreceptor viability. *Proceedings of the National Academy of Sciences* **117**, 201912513 (2020).
45. van Nie, R., Iványi, D. & Démant, P. A New H-2-Linked Mutation, rds, Causing Retinal Degeneration in the Mouse. *Tissue Antigens* **12**, 106–108 (1978).
46. Démant, P., Iványi, D. & van Nie, R. The Map Position of the rds Gene on the 17th Chromosome of the Mouse. *Tissue Antigens* **13**, 53–55 (1979).
47. Connell, G. *et al.* Photoreceptor peripherin is the normal product of the gene responsible for retinal degeneration in the rds mouse. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 723–726 (1991).
48. Cheng, T. *et al.* The effect of peripherin/rds haploinsufficiency on rod and cone photoreceptors. *Journal of Neuroscience* **17**, 8118–8128 (1997).
49. Travis, G. H., Groshan, K. R., Lloyd, M. & Bok, D. Complete rescue of photoreceptor dysplasia and degeneration in transgenic retinal degeneration slow (rds) mice. *Neuron* **9**, 113–119 (1992).
50. Bascom, R. A. *et al.* Cloning of the CDNA for a novel photoreceptor membrane protein (rom-1) identifies a disk rim protein family implicated in human retinopathies. *Neuron* **8**, 1171–1184 (1992).

51. Chakraborty, D., Conley, S. M., Al-Ubaidi, M. R. & Naash, M. I. Initiation of rod outer segment disc formation requires rds. *PLoS One* **9**, e98939 (2014).
52. Susa, K. J., Kruse, A. C. & Blacklow, S. C. Tetraspanins: structure, dynamics, and principles of partner-protein recognition. *Trends Cell Biol.* **34**, 509 (2023).
53. Garcia-España, A. *et al.* Appearance of new tetraspanin genes during vertebrate evolution. *Genomics* **91**, 326–334 (2008).
54. Lamb, T. D. Evolution of the genes mediating phototransduction in rod and cone photoreceptors. *Prog. Retin. Eye Res.* 100823 (2019).
55. Milstein, M. L., Kimler, V. A., Ghatak, C., Ladokhin, A. S. & Goldberg, A. F. X. An inducible amphipathic helix within the intrinsically disordered C terminus can participate in membrane curvature generation by peripherin-2/rds. *Journal of Biological Chemistry* **292**, 7850–7865 (2017).
56. Seigneuret, M., Delaguillaumie, A., Lagaudrière-Gesbert, C. & Conjeaud, H. Structure of the tetraspanin main extracellular domain: A partially conserved fold with a structurally variable domain insertion. *Journal of Biological Chemistry* **276**, 40055–40064 (2001).
57. Seigneuret, M., Conjeaud, H., Zhang, H. T. & Kong, X. P. Structural bases for tetraspanin functions. in *Tetraspanins* 1–29 (Springer Netherlands, 2013).
58. El Mazouni, D. & Gros, P. Cryo-EM structures of peripherin-2 and ROM1 suggest multiple roles in photoreceptor membrane morphogenesis. *Sci. Adv.* **8**, 3677 (2022).
59. DeSalle, R., Sun, T. T., Bergmann, T. & Garcia-España, A. The evolution of tetraspanins through a phylogenetic lens. in *Tetraspanins* 31–45 (Springer Netherlands, 2013).
60. Goldberg, A. F. X. Essential tetraspanin functions in the vertebrate retina. in *Tetraspanins* 321–343 (Springer, Dordrecht, 2013).
61. Goldberg, A. F. X., Fales, L. M., Hurley, J. B. & Khattree, N. Folding and subunit assembly of photoreceptor Peripherin/rds is mediated by determinants within the extracellular/intradiskal EC2 Domain: implications for heterogeneous molecular pathologies. *Journal of Biological Chemistry* **276**, 42700–42706 (2001).
62. Ding, X. Q., Stricker, H. M. & Naash, M. I. Role of the second intradiscal loop of peripherin/rds in homo and hetero associations. *Biochemistry* **44**, 4897–4904 (2005).

63. Reeves, M. J. *et al.* Genotype–phenotype associations in a large PRPH2-related retinopathy cohort. *Hum. Mutat.* **41**, 1528–1539 (2020).
64. Lewis, T. R. *et al.* Photoreceptor disc enclosure is tightly controlled by peripherin-2 oligomerization. *Journal of Neuroscience* **41**, 3588–3596 (2021).
65. Goldberg, A. F. X., Moritz, O. L. & Molday, R. S. Heterologous expression of photoreceptor Peripherin/rds and Rom-1 in COS-1 Cells: assembly, interactions, and localization of multisubunit complexes. *Biochemistry* **34**, 14213–14219 (1995).
66. Goldberg, A. F. X., Loewen, C. J. R. & Molday, R. S. Cysteine residues of photoreceptor peripherin/rds: Role in subunit assembly and autosomal dominant retinitis pigmentosa. *Biochemistry* **37**, 680–685 (1998).
67. Goldberg, A. F. X. & Molday, R. S. 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 (1996).
68. Goldberg, A. F. X. & Molday, R. S. Defective subunit assembly underlies a digenic form of retinitis pigmentosa linked to mutations in Peripherin/Rds and Rom-1. *Biochemistry* **93**, 13726–13730 (1996).
69. Loewen, C. J. R. & Molday, R. S. Disulfide-mediated oligomerization of peripherin/Rds and Rom-1 in photoreceptor disk membranes: Implications for photoreceptor outer segment morphogenesis and degeneration. *Journal of Biological Chemistry* **275**, 5370–5378 (2000).
70. Skiba, N. P. *et al.* Absolute Quantification of Photoreceptor Outer Segment Proteins. *J. Proteome Res.* **22**, 2703–2713 (2023).
71. Pöge, M. *et al.* Determinants shaping the nanoscale architecture of the mouse rod outer segment. *Elife* **10**, (2021).
72. Susa, K. J., Kruse, A. C. & Blacklow, S. C. Tetraspanins: structure, dynamics, and principles of partner-protein recognition. *Trends Cell Biol.* **34**, 509–522 (2024)
73. Zulliger, R., Conley, S. M., Mwoyosvi, M. L., Al-Ubaidi, M. R. & Naash, M. I. Oligomerization of Prph2 and Rom1 is essential for photoreceptor outer segment formation. *Hum. Mol. Genet.* **27**, 3507–3518 (2018).
74. Chakraborty, D., Ding, X. Q., Conley, S. M., Fliesler, S. J. & Naash, M. I. Differential requirements for retinal degeneration slow intermolecular disulfide-linked oligomerization in rods versus cones. *Hum. Mol. Genet.* **18**, 797–808 (2009).

75. Loewen, C. J. R., Moritz, O. L., Tam, B. M., Papermaster, D. S. & Molday, R. S. The role of subunit assembly in peripherin-2 targeting to rod photoreceptor disk membranes and retinitis pigmentosa. *Mol. Biol. Cell* **14**, 3400–3413 (2003).
76. Chakraborty, D., Ding, X. Q., Fliesler, S. J. & Naash, M. I. Outer segment oligomerization of Rds: Evidence from mouse models and subcellular fractionation. *Biochemistry* **47**, 1144–1156 (2008).
77. Tian, G. *et al.* An unconventional secretory pathway mediates the cilia targeting of peripherin/rds. *Journal of Neuroscience* **34**, 992–1006 (2014).
78. Conley, S. M. *et al.* Prph2 initiates outer segment morphogenesis but maturation requires Prph2/Rom1 oligomerization. *Hum. Mol. Genet.* **28**, 459–475 (2019).
79. Zulliger, R. *et al.* SNAREs interact with retinal degeneration slow and rod outer segment membrane protein-1 during conventional and unconventional outer segment targeting. *PLoS One* **10**, 1–24 (2015).
80. Böhm, S. *et al.* Peripherin-2 and Rom-1 have opposing effects on rod outer segment targeting of retinitis pigmentosa-linked peripherin-2 mutants. *Sci. Rep.* **7**, 1–13 (2017).
81. Drin, G. & Antonny, B. Amphipathic helices and membrane curvature. *FEBS Lett.* **584**, 1840–1847 (2010).
82. Xiang, Y., Lyu, R. & Hu, J. Oligomeric scaffolding for curvature generation by ER tubule-forming proteins. *Nat. Commun.* **14**, (2023).
83. Corral-Serrano, J. C. *et al.* PCARE and WASF3 regulate ciliary F-actin assembly that is required for the initiation of photoreceptor outer segment disk formation. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 9922–9931 (2020).
84. Bailly, M. *et al.* The F-actin side binding activity of the Arp2/3 complex is essential for actin nucleation and lamellipod extension. *Current Biology* **11**, 620–625 (2001).
85. Spencer, W. J. *et al.* The WAVE complex drives the morphogenesis of the photoreceptor outer segment cilium. *Proceedings of the National Academy of Sciences* **120**, (2023).
86. Salinas, R. Y. *et al.* Photoreceptor discs form through peripherin dependent suppression of ciliary ectosome release. *Journal of Cell Biology* **216**, 1489–1499 (2017).

87. Stuck, M. W., Conley, S. M. & Naash, M. I. The Y141C knockin mutation in RDS leads to complex phenotypes in the mouse. *Hum. Mol. Genet.* **23**, 6260–6274 (2014).
88. Cleveland, W. S. & Devlin, S. J. Locally weighted regression: An approach to regression analysis by local fitting. *J. Am. Stat. Assoc.* **83**, 596–610 (1988).
89. Molday, R. S., Hicks, D. & Molday, L. Peripherin. A rim-specific membrane protein of rod outer segment discs. *Invest. Ophthalmol. Vis. Sci.* **28**, 50–61 (1987).
90. Kevany, B. M. *et al.* Structural and functional analysis of the native peripherin-ROM1 complex isolated from photoreceptor cells. *Journal of Biological Chemistry* **288**, 36272–36284 (2013).
91. Stubičar, N., Matejaš, J., Zipper, P. & Wilfing, R. Size, Shape and internal structure of Triton X-100 micelles determined by light and small-angle X-Ray scattering techniques. *Surfactants in Solution* 181–195 (1989).
92. Thomas, M. J. *et al.* Lipid exchange between mixed micelles of phospholipid and triton X-100. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1417**, 144–156 (1999).
93. Thompson, A. A. *et al.* GPCR stabilization using the bicelle-like architecture of mixed sterol-detergent micelles. *Methods* **55**, 310–317 (2011).
94. Zhang, X. & Miller, K. W. Dodecyl Maltopyranoside Enabled Purification of Active Human GABA Type A Receptors for Deep and Direct Proteomic Sequencing. *Molecular & Cellular Proteomics* **14**, 724–738 (2015).
95. Schägger, H. & Von Jagow, G. Blue Native Electrophoresis for Isolation of Membrane Protein Complexes in Enzymatically Active Form. *Anal. Biochem.* **199**, 223–231 (1991).
96. Schägger, H., Cramer, W. A. & von Jagow, G. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **217**, 220–230 (1994).
97. Wittig, I., Braun, H. P. & Schägger, H. Blue native PAGE. *Nat. Protoc.* **1**, 418–428 (2006).
98. Wittig, I. & Schägger, H. Native electrophoretic techniques to identify protein-protein interactions. *Proteomics* **9**, 5214–5223 (2009).

99. Poetsch, A., Molday, L. L. & Molday, R. S. The cGMP-gated channel and related glutamic acid-rich proteins interact with peripherin-2 at the rim region of rod photoreceptor disc membranes. *Journal of Biological Chemistry* **276**, 48009–48016 (2001).
100. Garfin, D. E. One-dimensional gel electrophoresis. *Methods Enzymol.* **182**, 425–441 (1990).
101. Fiala, G. J., Schamel, W. W. A. & Blumenthal, B. Blue native polyacrylamide gel electrophoresis (BN-PAGE) for analysis of multiprotein complexes from cellular lysates. *J. Vis. Exp.* **48**, (2011).
102. Timón-Gómez, A. *et al.* Protocol for the analysis of yeast and human mitochondrial respiratory chain complexes and supercomplexes by blue native electrophoresis. *STAR Protoc.* **1**, 100089 (2020).
103. Pryor, J. L., Xu, W. & Hamilton, D. W. Immunodetection after complete destaining of Coomassie blue-stained proteins on immobilon-PVDF. *Anal. Biochem.* **202**, 100–104 (1992).
104. Na Ayutthaya, P. P., Lundberg, D., Weigel, D. & Li, L. Blue native polyacrylamide gel electrophoresis (BN-PAGE) for the analysis of protein oligomers in plants. *Curr. Protoc. Plant Biol.* **5**, e20107 (2020).
105. Pullara, F. *et al.* A general path for large-scale solubilization of cellular proteins: From membrane receptors to multiprotein complexes. *Protein Expr. Purif.* **87**, 111–119 (2013).
106. Wittig, I., Beckhaus, T., Wumaier, Z., Karas, M. & Schägger, H. Mass estimation of native proteins by blue native electrophoresis: Principles and practical hints. *Molecular and Cellular Proteomics* **9**, 2149–2161 (2010).
107. Goldberg, A. F. X. & Molday, R. S. Expression and characterization of peripherin/rds-rom-1 complexes and mutants implicated in retinal degenerative diseases. *Methods Enzymol.* **316**, 671–687 (2000).
108. Hemler, M. E. Tetraspanin functions and associated microdomains. *Nat. Rev. Mol. Cell Biol.* **6**, 801–811 (2005).
109. Postis, V. *et al.* The use of SMALPs as a novel membrane protein scaffold for structure study by negative stain electron microscopy. *Biochim. Biophys. Acta Biomembr.* **1848**, 496–501 (2015).

110. Pollock, N. L. *et al.* SMA-PAGE: A new method to examine complexes of membrane proteins using SMALP nano-encapsulation and native gel electrophoresis. *Biochim. Biophys. Acta Biomembr.* **1861**, 1437–1445 (2019).
111. Carlson, M. L. *et al.* The peptidisc, a simple method for stabilizing membrane proteins in detergent-free solution. *Elife* **7**, (2018).
112. Angiulli, G. *et al.* New approach for membrane protein reconstitution into peptidiscs and basis for their adaptability to different proteins. *Elife* **9**, (2020).
113. Ezsias, B. *et al.* Clear native gel electrophoresis for the purification of fluorescently labeled membrane proteins in native nanodiscs. *Anal. Chem.* **97**, 16796–16804 (2025).
114. Salinas, R. Y., Baker, S. A., Gospe, S. M. & Arshavsky, V. Y. A Single valine residue plays an essential role in Peripherin/rds targeting to photoreceptor outer segments. *PLoS One* **8**, e54292 (2013).
115. Molday, R. S. Peripherin/rds and rom-1: Molecular properties and role in photoreceptor cell degeneration. *Prog. Retin. Eye Res.* **13**, 271–299 (1994).
116. Wittig, I., Karas, M. & Schägger, H. High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. *Molecular and Cellular Proteomics* **6**, 1215–1225 (2007).
117. Halligan, B. D. *et al.* ProMoST (Protein Modification Screening Tool): a web-based tool for mapping protein modifications on two-dimensional gels. *Nucleic Acids Res.* **32**, W638–W644 (2004).
118. Cremers, C. M. & Jakob, U. Oxidant sensing by reversible disulfide bond formation. *Journal of Biological Chemistry* **288**, 26489–26496 (2013).
119. Chakraborty, D. *et al.* Novel molecular mechanisms for Prph2-associated pattern dystrophy. *The FASEB Journal* **34**, 1211–1230 (2020).
120. Stricker, H. M., Ding, X. Q., Quiambao, A., Fliesler, S. J. & Naash, M. I. The Cys214→Ser mutation in peripherin/rds causes a loss-of-function phenotype in transgenic mice. *Biochemical Journal* **388**, 605–613 (2005).
121. Ikelle, L. *et al.* Comparative study of PRPH2 D2 loop mutants reveals divergent disease mechanism in rods and cones. *Cellular and Molecular Life Sciences* **80**, 214- (2023).

122. Feig, C., Tchikov, V., Schütze, S. & Peter, M. E. Palmitoylation of CD95 facilitates formation of SDS-stable receptor aggregates that initiate apoptosis signaling. *EMBO J.* **26**, 221 (2006).
123. Schilling, D. *et al.* Commonly Used Alkylating Agents Limit Persulfide Detection by Converting Protein Persulfides into Thioethers. *Angew. Chem. Int. Ed Engl.* **61**, (2022).
124. Marrink, S. J., Risselada, H. J., Yefimov, S., Tieleman, D. P. & De Vries, A. H. The MARTINI Force Field: Coarse Grained Model for Biomolecular Simulations. *Journal of Physical Chemistry B* **111**, 7812–7824 (2007).
125. Kroon, P. C. *et al.* Martinize2 and Vermouth provide a unified framework for molecular topology generation. *Elife* **12**, (2025).
126. Vaclavik, V., Tran, H. V., Gaillard, M. C., Schorderet, D. F. & Munier, F. L. Pattern dystrophy with high intrafamilial variability associated with Y141C mutation in the peripherin/rds gene and successful treatment of subfoveal cnv related to multifocal pattern type with anti-VEGF (Ranibizumab) intravitreal injections. *Retina* **32**, 1942–1949 (2012).
127. Ruiz-Pastor, M. J. *et al.* Prph2 knock-in mice recapitulate human central areolar choroidal dystrophy retinal degeneration and exhibit aberrant synaptic remodeling and microglial activation. *Cell Death Dis.* **14**, (2023).
128. AlAshwal, S. M. *et al.* Early findings from a natural history study of patients with the pathogenic p.Gly208Asp PRPH2 variant associated with retinal dystrophy. *Retina* **46**, 521–532 (2025).
129. Woodard, D. *et al.* Evaluation of retinal phenotype in mice carrying the heterozygous p.Gly208Asp mutation in Prph2. *Invest. Ophthalmol. Vis. Sci.* **66**, 3818–3818 (2025).
130. Kim, Y. J. *et al.* S-Glutathionylation of Cysteine 99 in the APE1 Protein Impairs Abasic Endonuclease Activity. *J. Mol. Biol.* **414**, 313 (2011).
131. Mollica, J. P. *et al.* S-Glutathionylation of troponin I (fast) increases contractile apparatus Ca²⁺ sensitivity in fast-twitch muscle fibres of rats and humans. *Journal of Physiology* **590**, 1443–1463 (2012).
132. Ghezzi, P. Protein glutathionylation in health and disease. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1830**, 3165–3172 (2013).

133. Su, D. *et al.* Proteomic identification and quantification of S-glutathionylation in mouse macrophages using resin-assisted enrichment and isobaric labeling. *Free Radic. Biol. Med.* **67**, 460–470 (2014).
134. Guo, J. *et al.* Resin-Assisted enrichment of thiols as a general strategy for proteomic profiling of cysteine-based reversible modifications. *Nat. Protoc.* **9**, 64–75 (2014).
135. Samarasinghe, K. T. G. *et al.* A clickable glutathione approach for identification of protein glutathionylation in response to glucose metabolism. *Molecular BioSystems(MBS)* **12**, 2471–2480 (2016).
136. Gallogly, M. M. & Mieyal, J. J. Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr. Opin. Pharmacol.* **7**, 381–391 (2007).
137. Otsu, W., Hsu, Y. C., Chuang, J. Z. & Sung, C. H. The late endosomal pathway regulates the ciliary targeting of tetraspanin protein peripherin 2. *Journal of Neuroscience* **39**, 3376–3393 (2019).
138. Austin, C. D. *et al.* Oxidizing potential of endosomes and lysosomes limits intracellular cleavage of disulfide-based antibody–drug conjugates. *Proceedings of the National Academy of Sciences* **102**, 17987–17992 (2005).
139. Pan, W. W., Wubben, T. J. & Besirli, C. G. Photoreceptor metabolic reprogramming: current understanding and therapeutic implications. *Communications Biology* 2021 4:1 **4**, 245- (2021).
140. Sharma, Y. V. *et al.* Protective Gene Expression Changes Elicited by an Inherited Defect in Photoreceptor Structure. *PLoS One* **7**, e31371 (2012).
141. Sakai, D., Tomita, H. & Maeda, A. Optogenetic Therapy for Visual Restoration. *Int. J. Mol. Sci.* **23**, (2022).
142. Lamb, T. D. Evolution of phototransduction, vertebrate photoreceptors and retina. *Prog. Retin. Eye Res.* **36**, 52–119 (2013).
143. Goldberg, A. F. X. *et al.* An intramembrane glutamic acid governs peripherin/rds function for photoreceptor disk morphogenesis. *Invest. Ophthalmol. Vis. Sci.* **48**, 2975–2986 (2007).