Nano-scale resolution of native retinal rod disk membranes reveals differences in lipid composition

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Photoreceptors rely on distinct membrane compartments to support their specialized function. Unlike protein localization, identification of critical differences in membrane content has not yet been expanded to lipids, due to the difficulty of isolating domain-specific samples. We have overcome this by using SNA to coimmunopurify membrane proteins and their native lipids from two regions of photoreceptor ROS disks. Each sample’s copurified lipids were subjected to untargeted lipidomic and fatty acid analysis. Extensive differences between center (rhodopsin) and rim (ABCA4 and PRPH2/ROM1) samples included a lower PC to PE ratio and increased LC- and VLC-PUFAs in the center relative to the rim region, which was enriched in shorter, saturated FAs. The comparatively few differences between the two rim samples likely reflect specific protein–lipid interactions. High-resolution profiling of the ROS disk lipid composition gives new insights into how intricate membrane structure and protein activity are balanced within the ROS, and provides a model for future studies of other complex cellular structures.

Introduction

Rod photoreceptor cells of the retina are highly differentiated neurons that transduce visible light into a biochemical signaling cascade. These cells have an elongated cilium called the rod outer segment (ROS), which is composed of an internal stack of membranous disks surrounded by plasma membrane (PM). Two proteins are required to form the unique membrane structure: the light-receptive protein rhodopsin and a structural protein complex known as peripherin2–ROS membrane protein 1 (PRPH2/ROM1), which is essential for maintenance of the curved, bulbous rim of ROS disks (Goldberg and Molday, 1996a, b; Loewen and Molday, 2000; Kevany et al., 2013; Zulliger et al., 2018; Milstei et al., 2020). Roughly 40 million molecules of rhodopsin are packed into each ROS, and each light-activated rhodopsin is capable of binding and activating many molecules of the G protein transducin (Fung et al., 1981; Nathans, 1992; Polans et al., 1996; Heck and Hofmann, 2001). A third protein critical for photoreceptor function is the ATP-binding cassette (ABC) protein, family A, number 4 (ABCA4), which resides in the disk rim, like PRPH2/ROM1, and assists in retinaldehyde clearance from the ROS disk through its N-retinylidene-phosphatidylethanolamine flippase activity.

To accommodate this dynamic yet structured environment, ROS disks contain specialized lipids rich in long chain and very long chain polyunsaturated fatty acids (LC-PUFAs and VLC-PUFAs, respectively; Rotstein and Aveldaño, 1988). The location of these VLC-fatty acids (FAs) within the disk membrane interior or the disk rim membranes has become a point of debate in the field. VLC-PUFAs were hypothesized to provide flexibility in the disk membrane interior by folding back on themselves on one side of the bilayer (McMahon and Kedzierski, 2010; Molday and Zhang, 2010). This hypothesis was strengthened by findings that showed LC- and VLC-PUFAs remained with rhodopsin after hexane extraction of photoreceptor membranes (Aveldaño, 1988). Furthermore, rhodopsin exhibits maximal activity in a phospholipid environment with a high proportion of the VLC-PUFA, docosahexaenoic acid (DHA; 22:6; Mitchell et al., 1992). A subsequent theory suggested that because (1) elongation of VLC-FA protein 4 (ELOVL4) is responsible for VLC-PUFA production and (2) ELOVL4 and ABCA4 mutations both cause Stargardt...
disease (STGD3 and 1, respectively), VLC-PUFAs may reside in the ROS disk rims and affect ABCA4 activity, providing a potential link between two Stargardt mutations (Agbaga et al., 2010).

The disks are also known to have significantly higher levels of phosphatidylethanolamine (PE) than are typically found in PMs (Boesse-Battaglia and Albert, 1992; Daemen, 1973), which is compensated by a relative scarcity of phosphatidylcholine (PC) and phosphatidylserine (PS) in ROS membranes. This unusual phospholipid distribution is of functional importance as ABCA4 is optimally active when the membrane contains at least 40% PE (Sun and Nathans, 2001; Quazi and Molday, 2013; Quazi et al., 2012). Cholesterol has also been found to be necessary for rhodopsin activity, although high concentrations reduce its signaling efficiency (Mitchell et al., 1990, 1992; Palczewski, 2006). Indeed, many components of the membrane can have a profound impact on the function of the membrane proteins therein, making high-resolution study of membrane environments critical to the overall characterization of membrane proteins (Zimmerman and Keys, 1989; Bush et al., 1991; Gibson and Brown, 1993; Suh et al., 1994; Albert et al., 1998; Agbaga et al., 2008; Berdeaux et al., 2010; Giusto et al., 2010; Bennett et al., 2014; Goruszupudi et al., 2021; Hamano et al., 2021). Early work by Falk and Fatt (1969) on the ultra-structure of ROS membranes showed a remarkable ability of the outer rim region of ROS disks to resist disruption after OsO₄ fixation. Their work indicated that membranes in the rim region are distinct from the disk center, but concrete evidence in support of this idea is lacking.

The current paucity of knowledge regarding molecular differences between the center and rim regions of ROS disk membranes represents a significant bottleneck in the study of lipid synthesis, metabolism, and transport (Zhang et al., 2001; Edwards et al., 2001; Chen et al., 2005, 2007; Berdeaux et al., 2010; Sapieha et al., 2011; Chen et al., 2013, 2020). These processes modulate the impact of lipids on retinal degenerative diseases, such as STGD3, retinitis pigmentosa, diabetic retinopathy, and age-related macular degeneration (Simonelli et al., 1996; Bernstein et al., 2001; Seddon et al., 2003, 2006; SanGiovanni et al., 2007; Liu et al., 2010; Tikhonenko et al., 2010, 2013; Logan et al., 2013; Logan and Anderson, 2014; Hiieber et al., 2014). Mapping the possible lipid domains in which vision-related membrane proteins reside would be an invaluable contribution to the study of protein–lipid interactions.

The advent of styrene maleic acid (SMA) lipid particles (SMALPs) has made it possible to directly extract the membrane bilayer into discrete membrane disks containing the proteins therein (Knowles et al., 2009; Jamshad et al., 2011). It was initially unclear whether lipids “copurified” with native proteins represent the environment from which the protein was extracted. Accordingly, there have been reports on the lipid exchange dynamics of polymer-bound lipid nanodiscs (Cuevas Arenas et al., 2017; Schmidt and Sturgis, 2018; Danielczak and Keller, 2018). Initial studies showed that phospholipids extracted in SMALPs and diisobutylene maleic acid lipid particles can exchange more rapidly at ambient temperatures (i.e., 20–30°C) as compared with those prepared in large unilamellar vesicles or membrane scaffold protein nanodiscs. These findings suggested that native membrane proteins, once extracted by SMA, might reside in a lipid environment that reflects the average lipid environment of the extracted tissue. However, more recent data have provided evidence that SMALPs of various bacterial proteins formed under lower temperature conditions (≤4°C) have distinct lipid profiles (Teo et al., 2019), indicating the native local membrane environment composition is retained in samples prepared in this manner (local meaning a spatial resolution of 10–11 nm in diameter).

Here, we apply the SMA extraction method to enable detergent-free purification of functional rhodopsin, ABCA4, and PRPH2/ROM1 lipoprotein particles from bovine ROS disk membranes (Fig. 1, a and b). Untargeted lipidomic analysis of these samples reveals key differences in lipid composition between the central and rim regions of the disk that are likely of structural and functional importance.

**Results**

**SMA extraction of ROS membrane proteins and development of mAb against ABCA4**

We began by analyzing the ability of SMA to solubilize components of the ROS (Fig. 1). SMA showed a strong capacity for extracting ROS membrane proteins (Fig. S1 a). The high yield of total protein obtained from ROS extracted in SMA also showed near-complete extraction of the available ABCA4, as shown by immunoblot analysis (Fig. S1 b). Optimum extraction of ABCA4 in SMA occurred at 2.5% wt/vol and was essentially complete; by contrast, 2% laurel maltose neopentyl glycol (LMNG; roughly 2,000× the critical micelle concentration) resulted in roughly 50% solubilization.

The C-terminal region of ABCA4 contains a high-affinity binding epitope for the Rim3F4 antibody (YDLPILHPRT; Illing et al., 1997). The Rim3F4 antibody has high affinity for the C terminus of ABCA4, but immunopurification of ABCA4 proved difficult, given the low efficiency of elution from the column. We circumvented this limitation by generating a novel ABCA4 mAb (CL2), which was developed against a 26-amino acid peptide found at the C terminus of bovine ABCA4. Fig. S1 c shows the location and length of the resultant antibody binding site for CL2, in comparison to the locations of the antibody binding sites for Rim3F4 and TMR4 (Zhang et al., 2015), another antibody that targets the second extracytosolic domain. Dot blot analysis of CL2 confirmed that the paratope was different from that of Rim3F4 (Fig. S1 d). CL2 generated a reduced signal in the immunoblot of solubilized bovine ROS (Fig. S1 f), indicative of lower-affinity binding to the antigen.

The relatively weak binding of CL2 to ABCA4 was also apparent in murine samples (Fig. S1 e and g). The immunohistochemical analysis of murine retinas showed a gradual increase in ABCA4 signal intensity in samples stained with higher concentrations of CL2 (Fig. S1 e). CL2 showed a level of signal comparable to that of Rim3F4 for the same murine samples via immunoblots with comparable specificity (Fig. S1 g).

**Detergent-free purification of ABCA4 with CL2 antibody and EM imaging**

SMA-extracted bovine ROS was incubated with CL2-conjugated immunoaffinity resin (Fig. 1 c). Elution of ABCA4 with the
known epitope peptide produced a concentrated and pure sample of ABCA4 (Fig. 1 c, “Elu” and “EW” lanes), with large amounts of elution peptide and characteristic SMA smearing seen at the bottom of these lanes. The elution and subsequent wash from the immunoaffinity purification were then pooled and concentrated for size exclusion chromatography (SEC; Fig. 1 d). To characterize possible morphological changes to ABCA4 in the SMALP, the purified samples were prepared for negative stain transmission EM (nsTEM), which showed monodisperse, homogenous ABCA4 particles (Fig. S1 h, left). Clear 2D class averages were generated from particles selected by an unbiased autopicking feature of computational imaging system for

**Figure 1.** Detergent-free purification of native ABCA4 from bovine ROS by immunoaffinity chromatography. (a) Native lipids isolated by the SMALP coimmunopurification procedure. SMA extracts membrane proteins with their native lipids; the SMALPs may then be subjected to immunoaffinity chromatography for purification of native nanodiscs, enabling analysis of copurifying lipids. (b) The intricate membrane structure of ROS disks in rod photoreceptors. Three major membrane protein components are rhodopsin, ABCA4, and PRPH2/ROM1. (c) Detergent-free, immunoaffinity purification of ABCA4 using the CL2 mAb. L, soluble ROS (16 ml, 10 µl loaded); FT, flow-through (16 ml, 10 µl loaded); W1-4, washes 1-4 (each 15 ml, 10 µl loaded); Elu, elution (1 ml, 10 µl loaded); EW, wash of column after elution (1 ml, 10 µl loaded); Res, resin (1 ml, 10 µl loaded). Stained with Coomassie Blue R250. (d) Detergent-free SEC of combined elution fractions, 18–25 fraction numbers, 0.5 ml fractions from SEC, 10 µl loaded per lane. Stained with Coomassie Blue R250. (e) ABCA4 extracted and purified in SMALPs shows intrinsic Trp-quenching characteristic of ATP transporters in the presence of serially added ATP (dissociation constant $[K_a] = 133.5$ µM). Three separate experiments are shown with different symbols. Langmuir binding isotherm curve (black) fit to the average of three runs (maximum quench $[Q_{max}] = 11.85\%$, 17.91\%, and 10.00\% for black, red, and blue, respectively). Inset: One set of spectra for increasing concentrations of ATP, showing diminution of raw fluorescence.
transmission electron microscopy (cisTEM; Fig. S1 h, right; Grant et al., 2018). The resultant de novo 3D model, obtained using the cisTEM’s de novo reference map generator, showed significantly more density in the transmembrane domain (TMD) region than the prior nsTEM-generated structure of ABCA4 (Fig. S1 i). After refining, the roughly 4-nm-thick TMD showed a diameter of roughly 12 nm, which was larger than the previously published nsTEM model in the presence of detergent (EMDB-5497, orange; Fig. S1 j; Tsybovsky et al., 2019). The increased density did not confirm the presence of lipids in the TMD, and the possibility existed that more stain could have adhered to the TMD of the SMA-extracted protein. When considered in light of all of the results reported herein, however, we suspect the increased density was due to copurified lipids. The other proportions obtained agreed well with the published ABCA4 nsTEM model and the general size and shape of ABCA1 (EMDB-6724, purple ribbon; Fig. S1, i and j; Qian et al., 2017).

Assessing the activity of ABC transporters in SMA presents a challenge because the low millimolar concentrations of magnesium preferred for efficient coordination of ATP to the Walker A binding site of ABC transporters precipitates SMA (Olubwole et al., 2017). The correct folding and nucleotide binding of ABC transporters in SMALPs can be assessed via tryptophan fluorescence quenching with increasing concentrations of ATP in the absence of magnesium (Gulati et al., 2014). Using this assay, we confirmed that the SMA-purified ABCA4 is able to bind ATP (dissociation constant = 133.5 µM), albeit with lower affinity than reported in the presence of magnesium (Fig. 1 e; Ahn et al., 2000).

Detergent-free purification of PRPH2/ROM1 with novel nanobody Nb19

We developed a novel nanobody to pulldown PRPH2/ROM1 via an added His₆ tag on the nanobody (Fig. 2). All nanobodies share similar topology; they primarily vary in the hinge regions (H1, H2, etc.), which, upon folding, create complementarity-determining regions that constitute the paratope (Fig. 2, a–c). We selected, purified, and expressed 5 Nbs (Nb13, Nb19, Nb20, Nb28, and Nb32) representing different sequence families, each family grouped by complementarity-determining region sequences (Fig. 2, b and d; Fardon et al., 2014). All of the nanobodies bound tightly to prepurified PRPH2/ROM1 as monitored by SEC (Fig. 2 e). Nb19 proved to be the most efficient binder, as immunoprecipitation of PRPH2/ROM1 from extracted ROS (using the His₆ tag on the nanobody to bind Ni²⁺-nitriolotriacetic acid resin) gave the highest yield (Fig. 2 f). The resulting PRPH2/ROM1-Nb19 complex was of sufficient purity after elution from Ni²⁺-resin to analyze its copurifying lipids directly (Fig. 2, g and h).

SMALP-encapsulated rhodopsin retains ligand binding capacity

Rhodopsin is by far the most abundant membrane protein found in the central region of the ROS disk membrane, making it an excellent target protein to probe the lipid composition of the disk interior. Although rhodopsin is also found in the PM of the ROS, this represents a negligible fraction of total rhodopsin (i.e., <2%) that would not complicate its use to probe the lipid composition of the central disk (Kessler et al., 2014). We assessed the ability of rhodopsin to be purified in SMA with retention of bleaching and regeneration capacity (Fig. 3). Rhodopsin was purified using the ID4 antibody that had been developed previously and is well-established for protein purification in detergent-solubilized conditions (Fig. 1 g). SMA-solubilized rhodopsin purified by ID4 immunoaffinity chromatography (Molday and Molday, 2014) retained its chromophore when maintained in the dark, which suggested that the protein was structurally preserved. Moreover, SMA-purified rhodopsin could be photobleached, with and without hydroxylamine to scavenge the chromophore, showing that the protein either has sufficient free volume or the SMALP has enough flexibility to allow rhodopsin conformational changes required for these processes. The apo-opsin protein could also be regenerated efficiently with 9-cis-retinal, as shown by the reappearance of the characteristic absorbance peak of the opsin-chromophore complex at 487 nm (Hubbard and Wald, 1952). The regenerated samples were stable and soluble for days at room temperature. These results highlight the ability of SMALPs to efficiently extract this model G protein–coupled receptor (GPCR) in a stable form from its native, mammalian tissue, as has been done with other GPCRs (Bada Juarez et al., 1862; Jamshad et al., 2015; Gakhar et al., 2020; Ganapathy et al., 2020; Routledge et al., 2020; Ueta et al., 2020).

Untargeted lipidomic analysis of native SMALPs reveals different membrane environments for ABCA4, PRPH2/ROM1, and rhodopsin

With SMALP-extracted, immunopurified samples of these three representative membrane proteins in hand, we performed a high-resolution study of the lipid environments of each protein. Lipidomic analysis indicated that the SMALPs were able to extract many lipid classes from native ROS membranes, including phospholipid derivatives and other membranous lipid molecules in addition to phospholipids (Figs. 4, 5, S2, S3, S4, and S5). We detected many metabolites and other lipids, including acylcarnitines (AcCa), ceramides (Cer), cholesterol esters, monoaoylglycerols, diaoylglycerols, triaoylglycerols, free FAs (FFAs), cardiolipin, and several lyso-phospholipids (lyso-PLs: lyso-phosphatidylcholine [LPC], lyso-phosphatidylethanolamine [LPE], and lyso-phosphatic acid). There were many distinctions in the relative species composition within these lipid classes. In general, we observed the samples of SMA-extracted ROS (starting material) and rhodopsin had similar compositions (as would be expected given the large share of the ROS occupied by rhodopsin). Likewise, we found that the SMALPs of ABCA4 and PRPH2/ROM1, which both reside in the rim region, had similar species distributions within each lipid class. As a percentage of the total lipid class, the samples from the rim region lacked AcCa(16:0), which was balanced by a relative enrichment of AcCa(22:4) (Fig. 4 a). There was no gradual increase in the chain lengths up to AcCa(22:4) in the rim samples, suggesting that free carnitine becomes conjugated to the 22:4 FA directly, and that the resultant AcCa(22:4) is not metabolized as quickly as species of similar length. The
rim samples showed a relative abundance of Cer(d18:1_18:0) as compared with the rhodopsin samples, whereas the rhodopsin samples showed a relative abundance of Cer(d18:1_22:0), Cer(d18:1_24:1), and Cer(d18:2_24:0), suggesting a preference for longer chain lengths (Fig. 4b). The same relative preferences were seen with LPC and LPE analyses. The rim samples showed significant enrichment in LPC(18:0) and LPE(18:0), while LPC(22:5) and LPE(22:5), as well as LPC(22:6) and LPE(22:6), were several-fold

Figure 2. Biochemical characterization and purification of the SMALP-encapsulated PRPH2/ROM1/Nb19 complex. (a) The secondary structure of the Nb domain consists of nine β sheets separated by loop regions. H1, H2, and H3 are separated by four framework (FW) regions. (b) Each of the five delineated Nb families is defined by boxes around the clone names. Hypervariable region sequences H1, H2, and H3 are listed after each clone name and boxed in blue, green, and orange, respectively. (c) Robetta-homology modeled Nb19 is shown, highlighting extended complementarity-determining regions (CDRs) encoded by hypervariable regions defined in b. (d) 10 µg of purified Nb20, Nb19, Nb28, Nb32, and Nb13 were subjected to SDS-PAGE (stained with Coomassie Blue R250). (e) 10 µg of PRPH2/ROM1 was subjected to Superdex-200 gel filtration alone or after incubation with 20 µg of Nb. Nb19 caused the greatest shift in volume of elution. (f) Immunoprecipitation of PRPH2/ROM1 from solubilized ROS with Nbs. First lane, purified PRPH2/ROM1 (1.0 µg), was used as a positive control. Detection of PRPH2/ROM1 was performed by immunoblotting with the C6 (anti-PRPH2) and 2H5 (anti-ROM1) antibodies. Nb19-mediated immunoprecipitation produced the greatest quantity of PRPH2/ROM1. (g) Detergent-free, immunoaffinity purification of PRPH2/ROM1 (a) using the Nb19 nanobody (b). L, soluble ROS (10 ml, 10 µl loaded); FT, flow-through (10 ml, 10 µl loaded); W, wash (10 ml, 10 µl loaded); Elu, elution (2.5 ml, 2.5 µl loaded). Bottom: Anti-PRPH2 immunoblot of the above samples. (h) Detergent-free size exclusion chromatography of combined elution from Nb19-immunoaffinity purification. Left: PRPH2/ROM1 incubated with Nb19 (red) elutes earlier than PRPH2/ROM1 alone (black). Right: Peak PRPH2/ROM1/Nb19 fraction run on SDS-PAGE and stained with Coomassie Blue R250. Abs., antibodies; mAU, milliArbitrary Units.
higher in the rhodopsin samples (Fig. 4, c and d). Cholesterol levels were found to be higher in rhodopsin samples when compared with the PRPH2/ROM1 samples, while cholesterol ester (18:2) was relatively enriched in the PRPH2/ROM1 samples relative to the rhodopsin samples (Fig. 4 e).

The common phospholipids also displayed multiple significant differences between the rim region and the center (Table I), especially between PC and PE. There were many differences at the species level within each phospholipid class as well (Fig. 5). There were some instances of differences in PE species between the samples of the two rim proteins, where rhodopsin and PRPH2/ROM1 were relatively higher in PE(16:0_22:6) and PE(18:2_22:6) when compared with ABCA4 (Fig. 5 a). There were also significant differences among individual species in the phosphatidylinositol and PS classes (Fig. 5, b and c). Here, though, the rim samples had similar profiles and were both distinct from rhodopsin samples, further confirming the similarity between the rim sample membranes.

We further evaluated the aggregate relationship between each sample using the unbiased method of principal component analysis (PCA; Fig. 6). PCA produced linear combinations of the 199 separate species across all 14 lipid classes of the initial headgroup data (ROS: n = 2; rhodopsin: n = 3; ABCA4: n = 3; PRPH2/ROM1: n = 3). This global analysis confirmed the general similarity between the rim samples, whereas the center region samples localized to a distinct region of the PCA plot. PVC-1 explained a combined 77.7% of the variance in the system, with PVC accounting for >46%. When comparing PVC and PC2, there was obvious clustering of ABCA4 and PRPH2/ROM1 samples along PVC, far removed from rhodopsin on the PVC axis (Fig. 6 a). The same was true in a comparison of PVC-3 in an all-versus-all 3D plot (Fig. 6 b). The rhodopsin samples grouped tightly and associated more closely with the starting ROS samples with respect to PVC. Analysis of the PCA loadings suggested that PVC found strongest differences in species across classes containing palmitic and stearic acid (16:0 and 18:0, respectively; corresponding to the rim samples) and chain lengths of 20 or more containing 4-6 unsaturated bonds (rhodopsin samples; Fig. 6 d). We conclude that the lipid compositions of the rim and center regions of ROS disks are distinct at the lipid species level.

**Comparisons between the central and rim regions of ROS disks show differences in FA composition**

The PCA results suggested that FA chain length and/or unsaturation of the lipids residing in these two functionally distinct areas may be a key differentiator between their membranes. To address this fully, we performed lipid extractions from a new set of SMALP-protein samples (ROS: n = 3; rhodopsin: n = 5; ABCA4: n = 4; PRPH2/ROM1: n = 3), then hydrolyzed the head groups of all lipid species in each sample, followed by FA lipidomic analysis via liquid chromatography–mass spectrometry (LC/MS). The FA compositions of the lipids isolated from the two rim region proteins ABCA4 and PRPH2/ROM1 showed no statistically significant differences in relative molar percent for all chain lengths and saturations. There was considerable difference, however, in the FA composition of the rhodopsin-containing samples when compared with the rim proteins. The rim region proteins copurified with predominantly unsaturated and short chain length FAs, especially 16:0 and 18:0 (Fig. 7 a). Those two FA species accounted for >67% of the full FA content of the ABCA4 sample and >82% of the PRPH2/ROM1 sample. Conversely, the rhodopsin samples contained <30% of these two FAs.

DHA (22:6) is known to be essential to ROS disk health, facilitating rhodopsin activity (Bush et al., 1991; Organisciak et al., 1996; Litman et al., 2001). We found DHA was significantly higher in the central region than in the rim, with a DHA relative molar percentage of 13.5% for rhodopsin samples (Fig. 7 b). The rim region samples were enriched in LC-PUFAs more generally as well, whereas the rim samples contained only 1.6% or less molar percent LC-PUFAs.

Rhodopsin SMALPs also contained more VLC-PUFAs than those in the disk rim (Fig. 7 c). The most prominent VLC-PUFAs found in rhodopsin samples were dotriacontapentaenoic, dotriacontahexaenoic, tetratriacontapentaenoic, and tetratriacontahepaoenoic acids (32:5, 32:6, 34:5, and 34:6, respectively), with relative abundances between 0.6% and 1.3%. In contrast, the rim protein SMALPs were sparsely populated with VLC-PUFAs, accounting for 0.2% or less of their total FA content.

**Discussion**

The first question to be answered by this study is whether lipids that copurify in SMALPs containing purified membrane proteins faithfully represent the native membrane regions from which they are purified. There have been reports that SMALPs composed of pure phospholipids of different types (e.g., PC versus PE) rapidly exchange when incubated together at ambient temperatures, suggesting that native tissues, left solubilizing in SMA for 1–2 h at those temperatures, would result in an
equilibrated distribution of membrane components among the protein-containing SMALPs (Cuevas Arenas et al., 2017; Schmidt and Sturgis, 2018; Danielczak and Keller, 2018). Prior work on single-target proteins purified in SMALPs from membranes showed little difference between the mother membrane and the extracted, copurifying lipids (Dörre et al., 2014). A recent report on bacterial membrane proteins that associate with the membrane in three distinct ways showed that each purified protein copurified with distinct lipid profiles (Teo et al., 2019). Here, we document definitive differences between samples isolated from different regions of the same mammalian membrane tissue.

The most likely explanation for the preservation of differences between samples is that our purification is performed ≤4°C, which is far below the vast majority of lipid species’ main phase melting temperatures. To our knowledge, purification of proteins from native membranes using SMA at this temperature has only been done in one other study, and it reported differences in many species across phospholipid classes (Teo et al., 2019). With the addition of our findings, there remain no reports of lipid transfer in SMALP-protein nanodiscs. While we certainly affirm what was observed in purified bacterial membrane proteins, we provide further evidence that the lipid domains of continuous membranes are distinguishable with SMALPs by the purification and lipidomic analysis of two independent proteins from the same rim region, ABCA4 and PRPH2/ROM1, allowing comparison of their lipid profiles with that of rhodopsin.

Figure 4. Lipid compositions of SMALP-embedded ROS membrane proteins are particular to their native location in the membrane. (a–e) Percentages are shown of every detected species of AcCoA, Cer, LPC, LPE, and cholesterol (Chol)/cholesterol ester (ChE), respectively, extracted from SMALPs. Selected species are graphed (all species are shown in Figs. S2, S3, S4, and S5). Total ROS: black forward stripe; rhodopsin: red backward stripe; ABCA4: blue checker; PRPH2/ROM1: green diamond. ROS measured in duplicate as noted by individual data points (open circles). Percent composition was derived from each sample by dividing the area under the curve for each species in a class by the total area under the curve for the class reported via LC/MS after correction for variations in internal standard area, sample mass, and sample injection volume. Statistics were determined using two-way ANOVA with Tukey’s multiple comparisons post hoc test. Significance values are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
We hypothesized that if the local lipid environment is preserved in SMALPs, then samples of the rim region proteins should show similar lipid profiles to one another, distinct from that of rhodopsin. Our FA chain length/unsaturation analysis revealed no statistically significant differences between the two rim region samples, while clear differences were found between the FA arrays of the rhodopsin and rim protein samples. Furthermore, the cases of statistically significant differences between each rim sample and rhodopsin were nearly identical across all FA chain lengths and saturation levels. Therefore, this study demonstrates that SMA-extracted protein samples from native tissue retain the local environment from which they were isolated.

We approximated the amount of the ROS disk membrane accounted for by the SMALPs of our three chosen samples to assess the completeness of our analysis. We began with the fact that each SMALP will have a certain number of lipids copurifying with it. When ROS membranes are incubated with SMA, ∼12-nm-diameter disks of lipids encapsulate bilayer patches of the ROS, with each side of the bilayer representing roughly 113 nm² (equaling 226 nm² for the full surface area of each native nanodisc; Fig. 1 a). Because of the high density of proteins in the ROS disk membranes, we assumed protein-free SMALP-solubilized membranes would be negligible in amount. Then, with the ROS membrane separated into membrane protein-SMALPs, we estimate the number of lipids that can be accommodated within the SMALP in the presence of each of the known ROS membrane proteins, based the number of transmembrane helices that would occupy a portion of the total SMALP area ($T_n$). This is done using twice the average cross-sectional area of a transmembrane helix (1.4 nm²) to account for the helix displacing lipids in both bilayers (Eskandari et al., 1998; Swainsbury et al., 2014; Takamori et al., 2006). The transmembrane helix surface area is then subtracted from the total surface area of the nanodisc membrane to yield the available surface area for lipids within the SMALP. That surface area, divided by the average cross-sectional area of a single phospholipid (∼0.78 nm²), gives the number of lipids that should fit in the given ROS protein SMALP (Lee, 2003). The number of lipids that each membrane protein-SMALP can carry was then scaled by each protein’s relative abundance in the ROS membranes. The relative abundance, $A_n$, was calculated by cross-referencing the nine membrane proteins classified as ROS disk-specific with the ROS disk proteomics reported using absolute protein expression (APEX) measurements taken by tandem mass spectrometry (MS/MS; Skiba et al., 2013; Kwok et al., 2008). This is the estimation of what we call the weighted lipid contribution (WLC) of each protein (Eq. 1).

$$WLC_n = A_n \times \frac{226 \text{nm}^2 - \left( T_n \times 2.8 \text{nm}^2 \right)}{0.78 \text{nm}^2}$$

WLC$_{\text{rhodopsin}}$, WLC$_{\text{ABCA4}}$, and WLC$_{\text{PRPH2/ROM1}}$ were added together and divided by the sum of all WLCs, giving an approximate lipid contribution of 95% from the three samples studied here (Eq. 2 and Table 2).

$$WLC_{\text{total}} = \frac{WLC_{\text{rhodopsin}} + WLC_{\text{ABCA4}} + WLC_{\text{PRPH2/ROM1}}}{\sum WLC_n}$$

This estimation gives us confidence that we have studied the majority of the ROS disk membranes.

The stark contrast in the profiles of FA chain lengths between the rim and center of the disks is remarkable (Fig. 7).
of the disk is enriched with LC- and VLC-PUFAs relative to the disk rim. The relative abundance of DHA coincident with rhodopsin is consistent with the well-documented requirement of DHA for healthy rhodopsin activity (Mitchell et al., 1992). The relative abundance of eicosatetraenoyl acid (arachidonic acid, 20:4) in the disk center is consistent with its well-known role as a critical precursor for LC- and VLC-PUFAs (Grogan and Lam, 2004).

The presence of lyso-PLs has been reported at the tissue level (Henriksen et al., 2010; Rakshit et al., 2017). Our data show that injection of carnitine in the eye can be protective in a methylcellulose-induced ocular hypertension model, as measured by decreased levels of inducible nitrogen oxide synthase, malondialdehyde, and ubiquitin (Calandrella et al., 2010). Our results, which place AcCa in the immediate vicinity of rhodopsin in the membrane, suggest that carnitine may act as a check on normal oxidative stress in the outer segment (OS) disk membranes. Supplemental carnitine could increase the protective effect afforded the retina by endogenous levels of carnitine in the OS disks, but more evidence is needed to confirm this.

Table 1. Comparison of relative phospholipid compositions in native ROS membrane domains

| Phospholipid | Rhodopsin | ABCA4 | PRPH2/ROM1 | ROS disk | ROS PM |
|--------------|-----------|-------|------------|----------|--------|
| PC           | 39.6 ± 3.8| 60.9 ± 15.6 | 60.1 ± 11.8 | 45.3 ± 3.2 | 65.1 ± 3.8 |
| PE           | 54.0 ± 2.7| 34.7 ± 17.3 | 37.0 ± 11.4 | 41.6 ± 2.6 | 10.6 ± 2.8 |
| PG           | 0.2 ± 0.1 | 0.1 ± 0.1  | 0.1 ± 0.03  | -         | -      |
| PI           | 1.1 ± 0.6 | 0.6 ± 0.7  | 0.2 ± 0.1   | 2.5 ± 0.8 | <1.0   |
| PS           | 5.1 ± 3.4 | 3.7 ± 2.9  | 2.6 ± 0.6   | 13.7 ± 2.1| 24.1 ± 2.8|

PS, phosphatidylserine.

†Total values for all phospholipids detected in positive mode of LC/MS were used to estimate the relative phospholipid composition in each SMALP-extracted membrane region. Each value is presented as a mean percentage ± SD.

<sup>a</sup>Comparison values from prior ROS disk and PM isolations are taken from Boesze-Battaglia and Albert (1992). Values for PG not included in Boesze-Battaglia and Albert (1992) are noted with “<” and “< <1.0” refers to a value not reported for being <1%.
Figure 6. PCA groups rim region samples apart from central, rhodopsin samples. (a) PCA of 199 lipid species from 14 lipid classes shows clustering of rim samples away from rhodopsin samples, highlighting, in an unbiased manner, the similarity of the rim membrane lipids and the center region lipids. PC1 and PC2
various downstream, intracellular signaling pathways (Anliker and Chun, 2004; Xiang et al., 2013; Torkhovskaya et al., 2007; Li et al., 2016). Additional work has shown that light activation of bovine ROS leads to phospholipase A2 activation, which ties lyso-PL production to light-dependent pathways initiated by rhodopsin signaling (Jelsema, 1987). Rhodopsin is already known to be affected by the membrane composition when transitioning between the Meta I and Meta II states (Gibson and Brown, 1991a, 1991b, 1993; Botelho et al., 2002), but more research is needed to probe the possibility of alternative G protein interactions with rhodopsin for the propagation of lyso-PL signals.

The trend indicating enrichment of free cholesterol toward the center of the disks was surprising as prior data suggested that an exchange of disk cholesterol with the PM causes a gradient of cholesterol from high (nascent disks) to low (mature disks; Boesze-Battaglia et al., 1989). Therefore, we had expected to see a relative increase in free cholesterol in the rim of the disk. One way to explain our result is that the rim, with its highly curved structure, cannot maintain high levels of cholesterol. There may be a separate route for cholesterol movement between disks that allows for the diminution of free cholesterol in maturing disks, but this is only speculation. Regardless, all samples isolated from the disks showed lower relative levels of free cholesterol than the ROS starting material, which contained both disks and PM.

This study, to our knowledge, is the first to extract and purify mammalian membrane proteins along with their corresponding native membrane environment. We were able to document the precise, species-level differences between the two lipid domains of ROS disks (Fig. 8). Our results could provide more context for prior work done on detergent-resistant membranes (DRMs) of the ROS, where Triton X-100-resistant membranes low in rhodopsin and seemingly high in ABCA4 were isolated from the rest of the ROS disks (Martin et al., 2005). The DRMs were shown to have some of the same trends between DRM and fully solubilized regions as seen between the rim and center regions in this work.

Further work should be dedicated to studying physiological protein–lipid interactions of the retina, as many of the key proteins in the visual cycle and phototransduction are membrane proteins. To this end, the process of studying differential membrane composition based on native protein isolation in SMALPs should be expanded to other systems in the hope of uncovering detailed information on the preferred lipid environment of other membrane proteins. In particular, the use of high-resolution lipidomics may help explain pathologies involving critical protein–lipid interactions.

Materials and methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine, and were conducted in accordance with the Association for Research in Vision and Ophthalmology “Statement for the Use of Animals in Ophthalmical and Visual Research.” WT and Abca4−/−Rdh8−/− mice on a BALB/cj background were used in this study. All mice were housed in the University Laboratory Animal Resources facilities at the University of California, Irvine, and maintained in a 12-h/12-h light-dark cycle environment, and fed Teklad global soy protein-free extruded rodent diet (Envigo) chow and water ad libitum.

Production of mAb CL2 to optimize ABCA4 purification

The C-terminal region of ABCA4 is an accessible site that contains a high-affinity binding epitope for the Rim3F4 antibody. To develop a novel epitope here near this C terminus, a 26-amino acid peptide (NETYDLPLHPRTAGASRQAKEVDKGC) was synthesized and conjugated to keyhole limpet hemocyanin protein to induce an immunogenic response in mice (the procedure was performed by a Genescript antibody production service). CL2 showed lower signal in immunoblots of solubilized bovine ROS in comparison to Rim3F4 and TMR4. Dot blot analysis of the polypeptide used to generate CL2 was conducted to determine whether the epitope was different from that for Rim3F4. Various cleavage products of the polyepitope, purchased from Genescript and designed by sequentially omitting two amino acids from each end, were adsorbed onto the membrane to be probed for CL2 binding. Compared with the full-length peptide, none of the putative subepitopes bound CL2 with nearly the same affinity. When the first residues were removed (ΔF2-6), there was a complete loss of binding, suggesting that they are integral to CL2 recognition. The affinities of those peptides missing the last few residues (ΔL2-6) were much weaker than that of the full-length sequence, indicating that both ends of the epitope are important for robust binding of CL2, differentiating CL2 from Rim3F4.

Extraction of ROS proteins in SMA compared with LMNG solubilization

SMA (2:3:1 styrene:maleic acid ratio; XIRAN SL30010 P20; Polyscope Polymers B.V.) or detergent (LMNG; Anatrace) was incubated at varying concentrations (varied as shown in Fig. S1, a and b, where 2% LMNG was compared with 0.0, 0.5, 1.0, and 2.5% SMA) for 1 h with ROS (isolated as described previously) obtained from three or four bovine retinas in 1 ml of extraction buffer (20 mM bis-tris propane [BTP], pH 7.9, 10% glycerol, 300 mM NaCl, and 1 mM tris[2-carboxyethyl]phosphine) [TCEP]; Papermaster, 1982). The incubations with SMA were conducted at RT, and with detergent at 4°C. All samples were centrifuged at 100,000 g for 1 h, and the soluble fractions were separated. Each pellet was resuspended in 10% SDS-containing wash buffer. 10 μl were loaded for each sample.
onto a Mini-PROTEAN TGX precast gel, 4–20% gradient (Bio-Rad), and in the case of immunoblot analysis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking for 1 h in 5% (wt/vol) nonfat dry milk, anti-ABCA4 primary antibody TMR4 was added at 1:1,000 dilution from a 1 mg/ml stock, and incubated with the membrane overnight at 4°C. Membranes were washed with PBS containing 0.1% (vol/vol) Tween 20 (PBST), and then anti-mouse IgG (H&L) alkaline phosphatase-conjugated secondary antibody (Promega) was incubated with the membrane at a 1:5,000 dilution for 1 h at RT. After the membranes were again washed with PBST, the blots were developed with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega) for roughly 15 s, then quenched with ultrapure water.

**Immunoblotting of bovine ABCA4 to compare anti-ABCA4 antibodies**

ROS from 50 bovine retinas were isolated as described previously and suspended in extraction buffer (20 mM BTP, pH 7.9, 10% glycerol, 300 mM NaCl, and 1 mM TCEP) containing 2% LMNG (Anatrace; Papermaster, 1982). The soluble fraction was separated from insoluble material by centrifugation at 100,000 g for 1 h at 4°C. A 10-µl aliquot of the soluble fraction was loaded into each lane of a Mini-PROTEAN TGX precast gel, 4–20% gradient (Bio-Rad), and then proteins were transferred to PVDF membranes. After blocking for 1 h in 5% (wt/vol) nonfat dry milk, primary antibodies against ABCA4, namely CL2, Rim3F4, and TMR4 (Zhang et al., 2015), were added at dilutions of 1:10,000 from 1 mg/ml stocks, and incubated overnight at 4°C. Membranes were washed with PBST, and then anti-mouse IgG (H&L) alkaline phosphatase-conjugated secondary antibodies (Promega) were incubated with the blots at a dilution of 1:5,000 for 1 h at RT. After washing with PBST, blots were developed with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega) and imaged using an Odyssey Fc imager (LI-COR), using the 700-nm channel with a 2-min exposure time.

**Immunoblot of murine retinas to compare anti-ABCA4 antibodies**

Murine samples were obtained from the enucleated eyes of WT and Abca4−/−/Rdh8−/− mice according to a previously published protocol (Wei et al., 2016). Protein concentrations were determined with a BCA Assay kit (Bio-Rad), following the manufacturer’s instructions. Protein samples were mixed with NuPAGE LDS sample buffer and NuPAGE reducing agent, separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen), and transferred to PVDF membranes. Membranes were blocked with 5% (wt/vol) nonfat dry milk and incubated with the CL2 antibody overnight at 4°C. After washing with PBST, membranes were incubated with peroxidase-linked anti-mouse or anti-rabbit IgG (1:10,000; Jackson ImmunoResearch Laboratories) for 1 h at RT. Protein bands were visualized after exposure to SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific).

**Immunohistochemistry of retinal sections to compare anti-ABCA4 antibodies**

Mouse eye cups were fixed for 1 h in PBS containing 4% (wt/vol) paraformaldehyde (Sigma-Aldrich) at RT. After fixation, the eye cups were incubated sequentially in PBS containing 10, 20, or 30% (wt/vol) sucrose (Sigma-Aldrich) for 30 min at RT. Then, the eye cups were infiltrated with a 2:1 mixture of PBS containing 30% sucrose and optimal cutting temperature compound (VWR International) and frozen with dry ice. Retinal sections were cut at a thickness of 12 µm and stored at −80°C until use. The retinal sections were rehydrated with PBS and blocked with

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**Figure 7.** Comparison of FA chain lengths between the center and rim of ROS disks shows relative enrichment of shorter chain lengths in the rim and LC- and VLC-PUFAs in the center. (a) Relative molar percentages are shown for every detected class of FA molecule (C14-20) extracted from the SMALPs of each purified protein. (b) Relative molar percentages are shown for every detected class of FA molecule (C22-26) extracted from the SMALPs of each purified protein. (c) Relative molar percentages are shown for every detected class of FA molecule (C28-38) extracted from the SMALPs of each purified protein. Total ROS: black forward stripe; rhodopsin: red backward stripe; ABCA4: blue checker; PRPH2/ROM1: green diamond. The significance of differences between the means was determined using two-way ANOVA with Tukey’s multiple comparisons post hoc test. ROS: n = 3, rhodopsin: n = 5, ABCA4: n = 4, PRPH2/ROM1: n = 3. Significance values are indicated as follows: *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
PBS containing 5% (vol/vol) goat serum (Thermo Fisher Scientific) and 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich). After blocking, the sections were incubated with the appropriate primary antibodies diluted in PBS containing 5% goat serum overnight at 4°C. Primary antibodies used for immunohistochemistry were Rim3F4, TMR4, and CL2. The retinal sections were washed with PBS three times for 5 min each and then incubated with Alexa Fluor 488–conjugated goat anti-mouse IgG diluted in PBS containing 5% goat serum at 1:400. After incubation, the retinal sections were washed with PBS three times for 5 min each and then mounted with Vectashield Mounting Medium (Vector Laboratories). The images were acquired with a BZ-X810 Keyence microscope (Keyence) at 20× with numerical aperture of 0.75 at RT with no imaging medium and Alexa Fluor 488 used as the fluorochrome. The camera was built into the BZ-X810 Keyence microscope, and the BZ-X800 viewer from Keyence was the acquisition software. Adobe Photoshop was used to adjust the orientations and Adobe Illustrator to make the figure.

**Purification of native, bovine ABCA4 in SMA**

Every step of the following purifications was performed in a dark room under dim red light to prevent rhodopsin bleaching-induced aggregation. ROSs and sample-containing fractions were continuously on ice or at 4°C throughout the procedure. ROSs isolated from 50 bovine retinas (isolated as described previously) were extracted in 16 ml of ice-cold extraction buffer with 2.5% SMA (vol/vol; XIRAN SL30010 P20, product received at 1 g/ml; Polyscope Polymers B.V.) for 1 h at 4°C in the dark, followed by centrifugation at 100,000 g for 1 h at 4°C (Papermaster, 1982). 1 ml of ~8.0 mg/ml fresh immunoaffinity resin was prepared by conjugating purified, anti-ABCA4 antibody (CL2) to cyanogen bromide–activated Sepharose 4B beads (GE Healthcare Bio-Sciences) according to the manufacturer’s instructions. The extracted fraction of ROS in SMA was then mixed with the immunoaffinity resin, brought to 168 mM NaCl through dilution with SMA wash buffer (20 mM BTP, pH 7.9, 10% glycerol, 35 mM NaCl, and 1 mM TCEP) and incubated for 6 h. The flow-through was collected and used to purify rhodopsin or PRPH2/ROM1. After washing the column with 15 ml of SMA wash buffer, two successive 15-ml washes with high-salt SMA wash buffer (20 mM BTP, pH 7.9, 10% glycerol, 500 mM NaCl, and 1 mM TCEP) were passed over the column, followed by a 15-ml wash with SMA wash buffer. Elution buffer was made by adding 40 mg/ml of CL2 peptide (NETYDLHPRTAGASRQAKEVDKGC).

### Table 2. Comparison of WLC of ROS disk-specific membrane proteins

| Disk protein | APEXa | An | Tn | # lipids per protein | WLC | WLC (%) |
|-------------|-------|----|----|---------------------|-----|---------|
| Rhodopsin   | 0.1580841 | 0.663 | 7  | 264.6               | 175.3 | 68.3    |
| PRPH2/ROM1  | 0.0614938 | 0.258 | 16 | 232.3               | 59.9  | 23.3    |
| ABCA4       | 0.0073837 | 0.031 | 12 | 246.7               | 7.6   | 3.0     |
| GC-1        | 0.0073970 | 0.031 | 1  | 286.2               | 8.9   | 3.5     |
| R9AP        | 0.0016616 | 0.007 | 1  | 286.2               | 2.0   | 0.8     |
| ATP8A2      | 0.0013005 | 0.005 | 10 | 253.8               | 1.4   | 0.5     |
| GC-2        | 0.0012608 | 0.005 | 1  | 286.2               | 1.5   | 0.6     |

An is the APEX value of each protein, n, divided by the sum of all APEX values of disk-specific proteins. Tn is the number of transmembrane helices of each disk-specific protein. WLC is the calculated weighted lipid composition of the theoretical SMALP for each protein, as described by Eq. 1. GC-1 and -2 are guanylyl cyclase 1 and 2, respectively. R9AP is regulator of G protein signaling 9-binding protein. ATP8A2 is ATPase aminophospholipid transporter type 8A, member 2. Based on the estimation of WLC percent in the last column, rhodopsin, PRPH2/ROM1, and ABCA4 SMALPs account for 95% of the membrane lipids of ROS disks when extracted in SMA.

**Figure 8. ROS disks have regionally distinct micro-environments.** The center regions of ROS disks, rich in rhodopsin, have an abundance of long and unsaturated FAs. Rim regions of ROS disks have relatively high amounts of short and saturated FAs. There are many other distinctions in lipid species between the two regions, including relative amounts of PC and PE.
to 1 ml of wash buffer. After the elution step, the column was washed with 1 ml of SMA wash buffer, and then all proteins remaining on the resin were eluted with 1 column volume of 10% SDS. Each lane of the corresponding SDS-PAGE gel represents 10 μl of sample at the concentration of the sample, not adjusted to constant protein concentration across lanes.

Immunoaffinity elution and elution wash fractions of ABCA4 were pooled and concentrated to 0.5 ml and then centrifuged at 20,000 g for 10 min. The soluble fraction was then injected onto a Superdex 200 Increase 10/300 GL (GE Healthcare Bio-Sciences) SEC column to remove rhodopsin. SMA SEC buffer (20 mM BTP, pH 7.9, 10 mM NaCl, and 1 mM TCEP) was used as the mobile phase, and 0.5 ml fractions containing ABCA4 were pooled for use in other experiments.

Establishing nanobody (Nb) for PRPH2/ROM1 isolation

Washed ROS membranes from 50 frozen bovine retinas were thawed on ice and resuspended in a detergent-based solubilization buffer (20 mM BTP, pH 7.9, 300 mM NaCl, 2.5 mM DTT, and 25 mM n-dodecyl β-D-maltoside [DDM]) and incubated at 4 °C for 1 h with end-over-end mixing. To prevent reactions between free cysteine residues, the crude protein lysate was treated with 5.0 mM iodoacetamide for 30 min at RT. The solution was then quenched with an additional 5 mM DTT and immediately centrifuged at 150,000 g for 1 h at 4°C to clear insoluble material and aggregated proteins. The sample was incubated for 1 h at 4°C with end-over-end mixing with Nbs Nb20, Nb19, Nb28, Nb32, and Nb13 to a final ratio of PRPH2/ROM1:Nb of 1:2. 1.0 ml of preequilibrated Ni²⁺-resin (Sigma-Aldrich) was added to the solution and incubated for 1 h at 4°C with end-over-end mixing. The resultant suspension was transferred to a 5.0-ml gravity column. The resin was washed with 10 column volumes of 20 mM BTP, pH 7.9, 300 mM NaCl, 0.35 mM DDM, and 1.0 mM imidazole. Each PRPH2/ROM1/Nb complex was eluted with 4 column volumes elution buffer, comprised of the same wash buffer but with a final imidazole concentration of 300 mM. Aliquots of all samples along the stages of purification were saved for analysis. The resulting elution was then concentrated, and buffer exchanged into 20 mM BTP, pH 7.9, and 300 mM NaCl using a PD-10 column (GE Healthcare). The sample was concentrated to 1.0 mg/ml, frozen in liquid nitrogen, and stored at −80°C for future use.

Purification of native, bovine rhodopsin in SMA

Starting material for native PRPH2/ROM1 purification was either the flow-through fraction of the ABCA4 purification, or fresh ROS isolated from 50 bovine retinas were extracted in 16 ml of ice-cold extraction buffer with 2.5% SMA (vol/vol; XIRAN SL30010 P20; Polyscope Polymers B.V.) for 1 h at 4°C in the dark, followed by centrifugation at 100,000 g for 1 h at 4°C. 1 ml of ~8.0 mg/ml fresh immunoaffinity resin was prepared by conjugating purified anti-rhodopsin antibody (ID4) to cyanogen bromide-activated Sepharose 4B beads (GE Healthcare Bio-Sciences) according to the manufacturer’s instructions (Molday and Molday, 2014). The extracted fraction of ROS in SMA was then mixed with the immunoaffinity resin, brought to 168 mM NaCl through dilution with SMA wash buffer (20 mM BTP, pH 7.9, 10% glycerol, 35 mM NaCl, and 1 mM TCEP), and incubated for 6 h. The flow-through was collected and used to purify PRPH2/ROM1. After the column was washed with 15 ml of SMA wash buffer, two successive 15 ml washes with high-salt SMA wash buffer (20 mM BTP, pH 7.9, 10% glycerol, 500 mM NaCl, and 1 mM TCEP) were passed over the column, followed by a 15-ml wash with SMA wash buffer. Elution buffer was made by adding 40 mg/ml of ID4 peptide (TETSQVAPA) to 1 ml of wash buffer (Molday and Molday, 2014). After the elution step, the column was washed with 1 ml of SMA wash buffer, and then all proteins remaining on the resin were eluted with 1 column volume of 10% SDS. Each lane of the corresponding SDS-PAGE gel represents 10 μl of sample at the concentration of the sample, not adjusted to constant protein concentration across lanes.

Transmission EM

4 μl of the peak SEC fractions containing ABCA4 were adsorbed for 1 min on carbon-coated, glow-discharged grids (15 mAh 15 s; Electron Microscopy Sciences). The grids were washed with two 20-μl drops of ultrapure water and then stained with two 20-μl drops of 1% (wt/vol) uranyl acetate (Electron Microscopy Sciences), the first for 10 s and the second for 1 min. Data were collected with a JEOL JEM-2200fs microscope (JEOL), operated at 200 kV, and equipped with a Tietz TVIPS CCD Camera at 60,000× magnification. The pixel size was 2.131 Å.

Single particle reconstruction

De novo particle reconstruction of SMALP-imbedded ABCA4 was done using the program cisTEM following a published

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workflow ([Grant et al., 2018]). cisTEM auto-picked 71,088 particles that were then sorted by 2D classification into good classes containing 14,652 particles. The particles contained in these classes were then used for cisTEM’s ab initio 3D structure generation, which was then refined using cisTEM’s Auto Refine. Further structural analysis of ABCA4 was done in UCSF Chimera ([Pettersen et al., 2004]).

Trp fluorescence quenching assay
All measurements were performed on a PerkinElmer Life Sciences LS55 model fluorometer (PerkinElmer). Binding of ATP to purified ABCA4 in SMALPs was evaluated by monitoring the quenching of protein fluorescence at increasing concentrations of ATP (0–1.5 mM). With the excitation wavelength set at 290 nm, emission spectra were recorded at 330 nm over 1 min with 2-s intervals with bandwidths for excitation and emission fixed at 10 nm. Titrations were performed at 20°C in 20 mM BTP buffer, pH 7.9, containing 35 mM NaCl and 1 mM TCEP. ATP stock solution was diluted in ultrapure water. All binding data were corrected for background and self-absorption of excitation and emission light using a Varian Cary 50 Bio UV-Visible Spectrophotometer.

Rhodopsin absorption assay
All measurements were performed on a Varian Cary 50 Bio UV-Visible Spectrophotometer. Rhodopsin purified in the dark in SMALPs was measured by absorption from 250 to 600 nm. The sample was then incubated with hydroxylamine to a final concentration of 8 mM and allowed to bleach completely in light for 7 min, after which the absorption spectrum was taken. The sample was regenerated with 9-cis-retinal added to a final concentration of 70 µM and allowed to regenerate over 20 min, overnight, and for 2 d, with the spectrum taken at each time point.

Lipid extraction and untargeted lipidomics
Lipids were extracted using a modified version of the Bligh–Dyer method ([Bligh and Dyer, 1959]). Briefly, samples were shaken in a glass vial (VWR) with 1 ml PBS, 1 ml methanol, and 2 ml chloroform containing internal standards (13C16 palmitic acid and 2H7 cholesterol) for 30 s. The resulting mixture was vortexed for 15 s and centrifuged at 2,400 g for 6 min to achieve phase separation. The organic (bottom) layer was retrieved using a Pasteur pipette, dried under a gentle stream of nitrogen, and reconstituted in 2:1 chloroform:methanol for LC/MS analysis.

Lipid data analysis
Lipid identification was performed with LipidSearch (Thermo Fisher Scientific). Mass accuracy, chromatography, and peak integration of all LipidSearch-identified lipids and targeted lipids were verified with Skyline ([MacLean et al., 2010]). Peak areas were used in data reporting, and data were normalized using internal standards. Quantification of the FFAs was performed by measuring the area under the peak, and the species was included in the analysis if the area was greater than 0.1%. These relative molar percentages were used for all graphs and analyses. In cases of fewer than three samples for a particular species, the species was excluded from all ANOVA analysis. All lipid species found across all samples were used for PCA (199 total species, 14 classes). PCA scores, loadings, and variances were calculated using Graphpad Prism software.
(Graphpad). Principal components 1, 2, and 3 (PC1, PC2, and PC3, respectively) were included in our analysis because they passed Horn’s parallel analysis test (1,000 iterations; Horn, 1965). For two-way ANOVA measurements throughout, data distribution was assumed to be normal, but was not formally tested.

Online supplemental material
Fig. S1 shows SMA extraction of ROS, characterization of anti-ABCA4 mAb CL2 with murine and bovine samples, and nSPE analysis of ABCA4 purified with CL2 in SMA shows increased TMD density. Figs. S2, S3, S4, and S5 show the full list of lipid species detected, with each species amount graphed as the percent of the total for each particular class (PC, PE, etc.).

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Figure S1. Interrogation of ABCA4 extracted in SMA and the mAb generated for its immunoaffinity purification, CL2. (a) SDS-PAGE gel of the extraction of ROS proteins by various concentrations of SMA, or by the low critical micelle concentration detergent LMNG. Residual ROS pellets after initial detergent extraction were solubilized with 10% SDS. P, pellet; S, soluble. (b) Immunoblotting demonstrates a graded extraction of ABCA4 with increasing amounts of SMA. (c) Topographical map of ABCA4 highlighting the epitopes of three mAbs, TMR4, Rim3F4, and CL2. (d) Dot blots of polypeptides comprised of the amino acid chains shown to the right were used to confirm the novel epitope of CL2 on the C terminus of ABCA4. Truncations of the beginning of the sequence decreased the binding of CL2. The Rim3F4 epitope is depicted in blue. (e) Immunohistochemistry of retinal cryosections from 2-mo-old WT and Abca4−/−Rdh8−/− knockout (KO) mice, using CL2, Rim3F4, and TMR4 antibodies against ABCA4 (green) at three different dilutions. As expected, no fluorescence signal occurred with the KO mouse cryosections. With cryosections from WT mice, primary incubations with CL2 and Rim3F4 antibodies showed specific immunoreactivity with photoreceptor outer segments at all three dilutions, whereas TMR4 did not generate a fluorescence signal. Scale bar: 50 µm. (f) Relative amount of ABCA4 present in solubilized bovine ROS as assessed by immunoblotting. Stock concentrations of 1 mg/ml were used for all antibodies, and the dilution was 1:10,000 for each antibody tested. (g) Immunoblot of retinal and retinal pigment epithelium lysates obtained from 2-mo-old WT mouse using CL2, Rim3F4, and TMR4 antibodies. Probing with CL2 and Rim3F4 antibodies resulted in a specific band at 250 kD in the retinal samples, which corresponds to the size of ABCA4, whereas no positive signal was detected with TMR4. Retinal pigment epithelium-specific 65 kDa protein (RPE65) served as the control for tissue sample purity, and β-actin (42 kD) served as the loading control. (h) Negative stain micrograph of a representative SMA-CL2 preparation with 2D classes to the right; 60,000× magnification. SMALP-extracted ABCA4 shows an increase in TMD density, indicative of a native lipid belt. Scale bar: 86.2 nm. (i) 3D reconstruction of ABCA4 at ~18 Å resolution showing a putative bilayer thickness in the region of the SMALP. (j) SMALP-imbedded ABCA4 (gray) shows considerably more density within the predicted TMD region compared with (1) a prior ABCA4 negative-stained structure (EMDB-5497 [orange], solubilized in DDM and then switched into amphipol), and (2) the ABCA4 homologue, ABCA1 [EMDB-6724 [purple ribbon], solubilized in DDM and cholesterol hemisuccinate and then switched into digitonin). We interpret these differences to be explained by the SMALP nanodisc containing native lipids surrounding the TMD of ABCA4. IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer.

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Figure S2. Complete compositional analysis of detected species for AcCa, cardiolipin, Cer, and cholesterol (chol)/cholesterol ester (ChE). (a–d) Every detected species of lipid that copurified with each sample is shown as a percentage of each respective class (class noted on y axis). Cardiolipin chain lengths and unsaturation levels are summed together. Total ROS: black forward stripe; rhodopsin: red backward stripe; ABCA4: blue checker; PRPH2/ROM1: green diamond. The number of measurements for each sample of each species varies, as noted by the individual data points for each bar (open circles). Percent composition was calculated for each sample by dividing the area under the curve for each species in a class by the total area under the curve for that class, measured via LC/MS after correction for variations in internal standard area, sample mass, and sample injection volume. Statistics were determined using two-way ANOVA with Tukey’s multiple comparisons post hoc test between samples that had at least three detected replicates. Statistical significance values are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

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Figure S3. **Complete compositional analysis of detected species for mono- (MG), di- (DG), and triacylglycerides (TG).** (a–c) Every detected species of lipid that copurified with each sample is shown as a percentage of each respective class (class noted on y axis). Triacylglyceride chain lengths and unsaturation levels summed together. Total ROS: black forward stripe; rhodopsin: red backward stripe; ABCA4: blue checker; PRPH2/ROM1: green diamond. The number of measurements for each sample of each species varies, as noted by the individual data points for each bar. Percent composition was calculated for each sample by dividing the area under the curve for each species in a class by the total area under the curve for that class, measured via LC/MS after correction for variations in internal standard area, sample mass, and sample injection volume. Statistics are determined using two-way ANOVA with Tukey’s multiple comparisons post hoc test between samples that had at least three detected replicates. Statistical significance values are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure S4. Every detected species of FFA and Lyso-PL, relative to total species of each class. (a–f) Every detected species of FFA and lyso-PL that copurified with each sample is shown as a percentage of each respective class (class noted on the y axis). Total ROS: black forward stripe; rhodopsin: red backward stripe; ABCA4: blue checker; PRPH2/ROM1: green diamond. The number of measurements for each sample of each species varies, as noted by the individual data points for each bar (open circles). Percent composition was calculated for each sample by dividing the area under the curve for each species in a class by the total area under the curve for that class, measured via LC/MS after correction for variations in internal standard area, sample mass, and sample injection volume. Statistics were determined using two-way ANOVA with Tukey’s multiple comparisons post hoc test. Statistical significance values are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure S5. Every detected species of phospholipid, relative to total species of each class. (a–e) Every detected species of phospholipid that copurified with each sample is shown as a percentage of each respective class (class noted on y axis). Total ROS: black forward stripe; rhodopsin: red backward stripe; ABCA4: blue checker; PRPH2/ROM1: green diamond. The number of measurements for each sample at each species varies, as noted by the individual data points for each bar (open circles). Percent composition was calculated for each sample by dividing the area under the curve for each species in a class by the total area under the curve for that class, measured via LC/MS after correction for variations in internal standard area, sample mass, and sample injection volume. Statistics were determined using two-way ANOVA with Tukey’s multiple comparisons post hoc test. Statistical significance values are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. PI, phosphatidylinositol.