Statins and Protein Prenylation in the Retina

by

Jennifer Yun Mu Ling

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

Master of Science

Department of Pharmacology

University of Alberta

Abstract

Protein prenylation is the post-translational addition of isoprenoid lipid moieties to proteins, which regulates their subcellular localization and function. Farnesyl or geranylgeranyl isoprenoids are covalently linked to cysteines residues in a C-terminal prenylation recognition sequence in selected proteins. A host of inherited retinal diseases, such as choroideremia (CHM), results from prenylation defects suggesting that prenylation is of heightened importance in the eye. Yet, in many cases, it is unclear what drives retinal degeneration.

Small GTPases represent the most important group of prenylated proteins and among them Rabs GTPases (Rabs) fulfill crucial cellular functions. Rabs regulate many steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion. These processes are essential for endocytosis, autophagy and phagocytosis, among others cellular activities. Phagocytosis of the photoreceptor outer segments (OS) is one of the main functions of the retinal pigment epithelium (RPE). This process requires the activity of several Rabs, at different stages, *e.g.* Rab5 and Rab7 participate in phagosome maturation and Rab7 is required for the fusion of phagosomes and lysosomes to allow degradation of the phagosome cargo.

Previous work in our laboratory has demonstrated that inhibition of Rab prenylation impairs the endocytic-autophagic pathway. Here, we hypothesize that inhibiting protein prenylation in the RPE will lead to decreased degradation of OS following phagocytosis.

The isoprenoids attached to proteins during prenylation are synthesized by the mevalonate pathway that also generates cholesterol. The mevalonate pathway can be inhibited by statins, which are competitive inhibitors of the rate limiting enzyme Hydroxy-methyl glutaryl CoA Reductase. Statins may inhibit synthesis of both cholesterol and isoprenoids or just cholesterol. Therefore, some statins inhibit protein prenylation. We use two type of statins to regulate protein prenylation in retina-derived cells and in fibroblasts from patients with CHM.

First, we demonstrate that simvastatin, but not pravastatin, inhibits protein prenylation in ARPE- 19 cells, a model of the RPE. This inhibititon can be prevented by geranylgeranyl pyrophosphate (GGPP), suggesting that prenylation inhibition is due to a shortage of isoprenoids. Second, we examined the effect of statins on OS degradation. Simvastatin, but not pravastatin, inhibited the degradation of OS in ARPE- 19, though this effect was not fully prevented by the addition of GGPP. Third, we extend our work to CHM, a retinal degeneration that results from the absence of Rab Escort Protein-1 (REP-1). REP-1 is an essential chaperone for prenylation of newly synthesized Rabs. We examined the effects of statins on Rab prenylation in dermal fibroblasts from age-matched choroideremia patients and healthy participants. Protein prenylation in fibroblasts from CHM patients was significantly more sensitive to inhibition by simvastatin than fibroblasts from healthy individuals.

Our studies suggest that inhibition of protein prenylation in the RPE may lead to decreased OS degradation, and that CHM patients might be more sensitive treatment with statins.

Acknowledgements

I am grateful for the opportunity to perform graduate studies at the University of Alberta in the Department of Pharmacology. There are many people to whom I am greatly and forever indebted, and I must thank them for their contribution.

First, I need to thank my supervisors, Dr Elena Posse de Chaves and Dr Ian MacDonald. Dr Posse de Chaves accepted me into her laboratory and provided outstanding mentorship and encouragement for me to complete my studies. I have grown in my ability to think critically, experiment carefully and communicate scientific knowledge. I am confident the lifelong knowledge and skills I have gained will continue to be useful. Dr Ian MacDonald has been a pleasure to work with. He has been a great help by providing expert knowledge with respect to my research, and is a living exemplar of how to be a scholar and a teacher. It has been a tremendous pleasure being their student, and I will not forget the lessons anytime soon.

Great thanks must be given to my supervisory committee: Dr Basil Hubbard and Dr Yves Sauvé, for their insightful comments during our meetings and continual support during my studies. Each of you provided great scientific expertise that enabled me to complete my research and enriched my learning experience. I am thankful toward Dr Luc Berthiaume for being the external examiner at my defence.

There are many current and previous lab members whom I need to thank for their diverse contributions. From the Posse de Chaves lab, I would like to thank Dr Amany Mohamed, Sarah Samuelson, Federico Sacchi, Karl Narvacan and Dr Valeria Sgambati, for their help on my project. From them, I learned many research techniques

and I thank them for their collegiality. From the MacDonald lab, I would like to thank Alina Radziwon and Chris St Laurent, for their generosity, expertise in genetics research, and for providing me with valuable materials for my thesis.

Thank you to Kevin Thorburn, Luis Carlos, Danny Galleguillos, Diego Ordonez Pasaje, Melanie Alpaugh, Kevan Smith and Anissa Viveiros for the technical assistance, scientific discussion and bountiful friendship.

I am grateful to Alberta Innovates Health Solutions for funding this project under the Collaborative Research and Innovation Opportunities grants.

Finally, I am so thankful for my wonderful family and friends. They have supported me every step of the way and their encouragement has made it all possible. It is a fortune and privilege to have you all and I am forever obliged.

Table of Contents

List of Figures

List of Abbreviations

AAV	Adeno-Associated Virus
AIPL1	Aryl-hydrocarbon interacting protein 1
AMD	Age-related Macular Degeneration
Anx	Annexin
CBR	C-terminal Binding Protein
CC	Connecting Cillia
CCZ1	Vacuolar Fusion Protein CCZ1
CDC42	Cell Division Control Protein 42
CENP	Centromere Protein
cGMP	Cyclic GMP
CHM	Choroideremia
CLN3	Neuronal Ceroid Lipofusinosis 3
CRALBP	Cellular Retinaldehyde Binding Protein
CRBP	Cellular Retinol Binding Protein
DMPP	Dimethylallyl Pyrophosphate
ECM	Extracellular Matrix
ER	Endoplasmic Reticulum
FAF	Fundus Autofluorescence
FPP	Farnesyl Pyrophosphate
FPPS	Farnesyl Pyrophosphate Synthase
FTase	Farnesyl Transferase
FTI	Farnesyl Transferase Inhibitor
FYCO	Fab1-YotB-Vac1p-EEA1 and Coiled-Coil Domain Containing Protein
GAP	GTPase-Activating Protein
GDI	Guanosine Nucleotide Dissociation Inhibitor
GEF	Guanosine Nucleotide Exchange Factor
GGPP	Geranylgeranyl Pyrophosphate
GGPPS	Geranylgeranyl Pyrophosphate Synthase
GGTase	Geranylgeranyl Pyrophosphate Transferase
GPP	Geranyl Pyrophosphate
GRK1	Rhodopsin Kinase
hfRPE	Human Fetal RPE
HIDS	Hyper-immunoglobulin D and Periodic Fever Syndrome
HMG CoA	3-Hydroxyl-3-Methylglutaryl CoA
HMGR	HMG CoA Reductase

ICMT	Isoprenyl Cysteine Carboxylmethyltransferase
IL-8	Interleukin 8
IPP	Isopentenyl Pyrophosphate
iPSC	Induced Pluripotent Stem Cell
IRBC	Interstitial Retinal Binding Protein
IS	Inner Segment
LAMP1	Lysosomal-Associated Membrane Protein 1
LC/MS	Liquid Chromatography/Mass Spectrometry
LC3	Microtubule-Associated Protein 1A/1B-Light Chain 3
LCA	Leber's Congenital Amaurosis
LRAT	Lecitin:Retinol Transferase
Mertk	Tyrosine-protein Kinase Mer
MFG-E8	Milk Fat Globulin E8
MK	Mevalonate Kinase
Mon1	Vacuolar Fusion Protein 1
OAT	Organic Anion Transporter
OCT	Optical Coherence Tomography
ONL	Outer Nulcear Layer
OS	Outer Segment
PDE6	Rod cGMP-specific-3',5',-cyclic Phosphodiesterase
PEDF	Pigment Epithelium-Derived Growth Factor
RBP	Rab Binding Platform
RCE1	Ras-Converting Endopeptidase 1
RDH5	11-cis Retinol Dehydrogenase
REP	Rab Escort Protein
RILP	Rab-Interacting Lysosome Protein
RP	Retinitis Pigmentosa
RPE	Retinal Pigment Epithelium
RPE65	RPE 65 kDa
RPGR	Retinitis Pigmentosa GTPase Regulator
TNFα	Tumor Necrosis Factor Alpha
VA	Visual Acuity
VEGF	Vascular Endothelial Growth Factor
VF	Visual Field

CHAPTER 1

INTRODUCTION

1.1 Introduction

1.1.1 *The Retina*

The retina is a thin layer of tissue in the eye that detects light signals and relays this information to the brain via the optic nerve. There are many neuronal and non-neuronal cell layers in the retina that work together to transmit visual information (**Figure 1**). The conversion of light to an electrical signal occurs in the OS of the photoreceptor, which is the neuronal cell type closest to the back of the eye (Kolb 1995). Photoreceptors consist of two subtypes: rods and cones. Rods enable dim light vision while cones mediate colour vision in bright light (Masland 2001). Cones are largely concentrated in the fovea of the retina, whereas rods are spread throughout (Henrickson 2005). Anterior to the photoreceptors, closer to the lens, are the bipolar cells, horizontal cells and amacrine cells. Bipolar cells receive synaptic input from the photoreceptors and many synapse directly onto retinal ganglion cells (Hubel 1995). Some bipolar cells synapse onto amacrine cells, which then synapse onto retinal ganglion cells (Hubel 1995). Horizontal cells receive synaptic input from multiple photoreceptors to integrate and relay their signals onto retinal ganglion cells. The axons of the retinal ganglion cells make up the optic nerve (Masland 2001).

Behind the photoreceptors is the RPE. The RPE contains melanin and rests on the Bruch's membrane and the choroid (Strauss 1995). The Bruch's membrane is a multilayered matrix in between the basal membrane of the RPE, and the fenestrated capillaries of the choroid (Guymer, Luthert et al. 1999). Together, the RPE, the Bruch's membrane and the choroid connect the neural retina with the systemic circulation,

receiving nutrients and eliminating waste. The photoreceptors and the RPE are highly dependent upon each other; the dysfunction of either layer can lead to loss of the other.

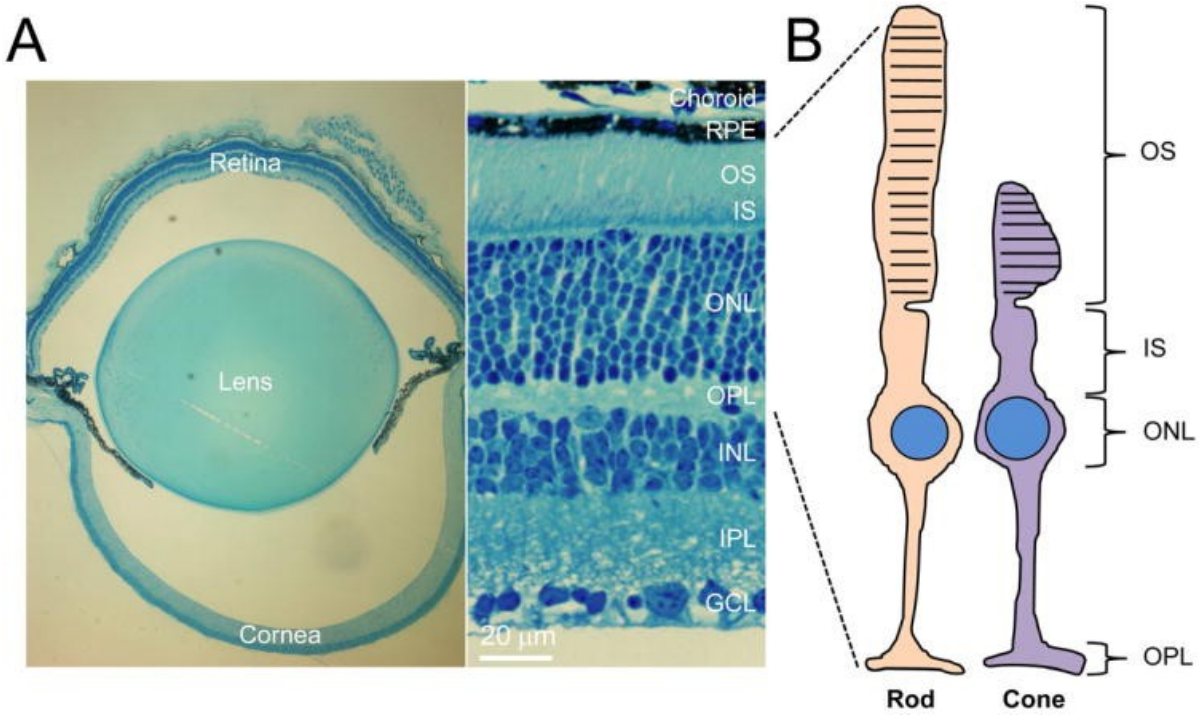

Figure 1: **Mouse Retina and Photoreceptor Cell Morphology. (A)** The retina is located in the posterior portion of the eyecup (left) and is organized into multiple distinct layers (right). **(B)** Schematic of rod and cone photoreceptor cells. RPE: retinal pigment epithelium; OS: outer segment; IS: inner segment; ONL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; OPL: outer plexiform layer. *From Tang, P. H., Kono, M., Koutalos, Y., Ablonczy, Z. & Crouch, R. K. New insights into retinoid metabolism and cycling within the retina. Prog Retin Eye Res 32, 48-63, doi:10.1016/j.preteyeres.2012.09.002 (2013).*

1.1.2 Photoreceptors

Retinal photoreceptor cells are essential to visual transduction by detecting light and translating it into an electrical response (Kolb 1995). Photoreceptors are polarized cells: the apical OS are adjacent to the RPE and detect light, while the cell body and

synaptic endings are proximal to ganglion cells. The OS contains key visual transduction proteins, such as rhodopsin. These proteins are produced in the inner segments (IS), which contain mitochondria and ribosomes, and then transported along the connecting cilia (CC) between the OS and IS.

Each day, at the onset of light and dark, the photoreceptors shed the photoreceptor OS, a membranous particle containing stacks of individualized disks of rhodopsin-embedded membrane (Reme and Sulser 1977). A molecule of retinal is bound to each rhodopsin molecule, which is converted from 11-*cis*-retinal to all-*trans*-retinal when light hits, changing the rhodopsin conformation so that it can activate its associated G-protein, transducin (Okada, Ernst et al. 2001, Mustafi and Palczewski 2009). Rhodopsin is phosphorylated to terminate its action. The photoreceptors are able to isomerize the all-*trans* retinal back to the 11-cis conformation, but still need to synthesize new OS to replace shed particles. Spent all-*trans*-retinal, within rhodopsin, is moved further from the photoreceptor soma (Young 1967, Young and Bok 1969). Outer segments are phagocytosed by the RPE, which degrade the contents of the OS and isomerize the retinal back to the 11-*cis* conformation (Travis, Golczak et al. 2007). When phagocytosis is impaired or segment shedding is excessive, shortening of photoreceptor segments is seen as photoreceptors attempt to remove photo-oxidative products and generate new segments. This is seen in retinal degenerative diseases such as Oguchi disease and retinitis pigmentosa (Kajiwara, Hahn et al. 1991, Kedzierski, Lloyd et al. 1997, Yamamoto, Sippel et al. 1997).

1.1.3 The Retinal Pigment Epithelium

The RPE is a single layer of pigmented hexagonal cells, connected by tight junctions. Its apical membrane faces the photoreceptors and its basolateral membrane rests on the Bruch's membrane (Strauss 1995) (**Figure 2**). It is full of melanosomes which move from the cytosol to the apical processes in the bright conditions (Back, Donner et al. 1965, Burnside 2001). Photo-oxidation occurs in the photoreceptor OS (Wright, Chakarova et al. 2010), which need to be renewed, so the RPE phagocytose the OS and neutralize the oxidation products and free radicals to maintain retinal health. In addition, photothermal damage may occur in the retina as pigments such as melanin in the absorb photons and generate heat (Youssef, Sheibani et al. 2011). The choroid is highly perfused, allowing the cooling of the RPE. The RPE also has significant roles in transport of essential components such as retinol and glucose from the choroidal blood supply, as well as secretion of metabolic end products such as ions and lactic acid (Strauss 1995).

The RPE plays an important role in the renewal of all-*trans*-retinol for the visual cycle (Tang, Kono et al. 2013). All-*trans*-retinol, bound to the carrier interphotoreceptor retinal binding protein (IRBP), enters the RPE (Pepperberg, Okajima et al. 1991). All-*trans*-retinol is then bound to cellular retinol binding protein (CRBP), which carries the retinol to a protein complex (Kawaguchi, Yu et al. 2007). This protein complex is composed of lecithin:retinol transferase (LRAT), RPE protein 65 kDa (RPE65) and 11-cis retinol dehydrogenase (RDH5), to be converted back to 11-*cis* retinal (Moiseyev, Chen et al. 2005, Kiser, Golczak et al. 2014). Also within this complex is cellular retinaldehyde binding protein (CRALBP) which accelerates the process. The 11-*cis*-retinol re-binds to IRBP and is transported back to the photoreceptor.

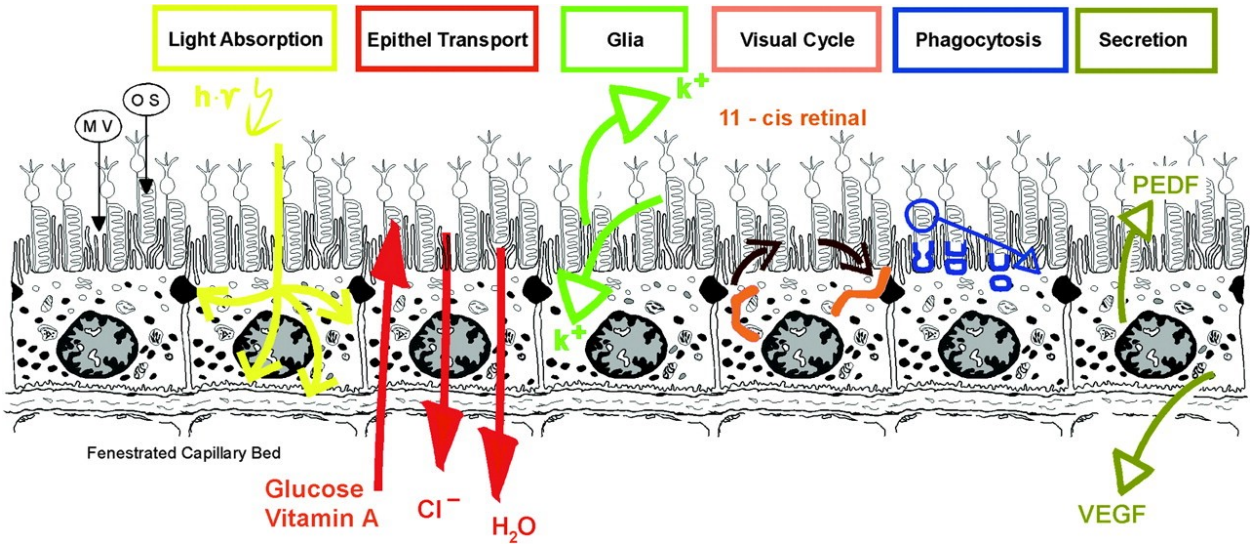

Figure 2: **Summary of Retinal Pigment Epithelium (RPE) Functions.** PEDF, pigment epithelium-derived growth factor; VEGF, vascular epithelium growth factor; Epithel, epithelium. *From Strauss, O. The retinal pigment epithelium in visual function. Physiological reviews 85, 845-881, doi:10.1152/physrev.00021.2004 (2004).*

The RPE is a component of the blood-retina barrier (Strauss 1995). It is a physical barrier between the choroid blood supply and the retina due to its tight junctions, and it also ensures the inner eye is immune privileged by interacting with the immune cells of the retina or the periphery (Wenkel and Streilein 2000, Streilein, Ma et al. 2002). The second component of the blood-retinal barrier is formed by the tight junctions between the retinal capillary endothelial cells (Cunha-Vaz, Bernardes et al. 2011). The barrier tightly controls the passage of fluids and solutes. This system is very similar to the blood-brain barrier in many aspects, such as pharmacological entrance and efflux, and structure, despite different developmental origins (Steuer, Jaworski et al. 2005, Cunha-Vaz, Bernardes et al. 2011).

1.1.4 Phagocytosis of Photoreceptor Outer Segments

Phagocytosis of photoreceptor OS requires a sequence of steps: recognition, engulfment, maturation and degradation. Each step is defined by characteristic protein players (**Figure 3**).

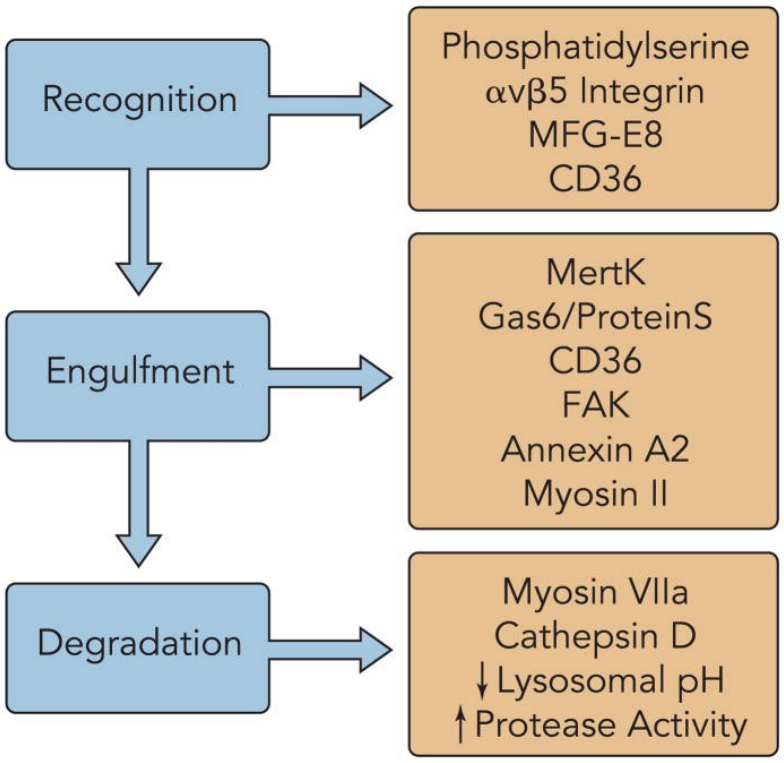

Figure 3: Components of OS Phagocytosis. Components are categorized by their involvement in photoreceptor recognition, engulfment, and degradation, the stages of the renewal process. *Taken from (Kevany and Palczewski 2010).*

Two ligand pairs on the RPE recognize OS: receptor tyrosine kinase Mer precursor (MerTK) and its secreted ligands Gas6 and protein S, and the integrin receptor αvβ5, and its secreted ligands milk fat globulin-E8 (MFG-E8). These ligands recognize phosphatidylserine on the OS, in a mechanism similar to that involved in apoptotic phagocytosis (Fadeel, Quinn et al. 2007). There may be additional roles for CDC36 (Kevany and Palczewski 2010). Without these receptors, RPE either do not engulf OS

at all or change their diurnal pattern of phagocytosis greatly, depending on which ligand-receptor pair is defective (Mullen and LaVail 1976, Feng, Yasumura et al. 2002, Nandrot, Kim et al. 2004, Nandrot, Anand et al. 2007, Burstyn-Cohen, Lew et al. 2012). **(Figure 4).**

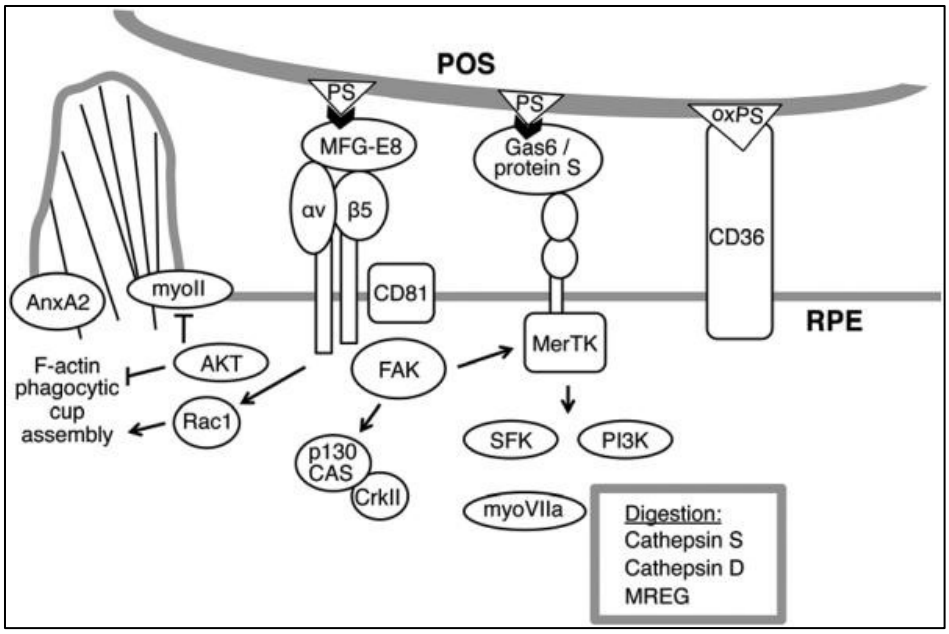

Figure 4: OS Phagocytosis by RPE Cells Requires Coordinated Activities of Numerous Cell Surface and Cytosolic Proteins. The scheme illustrates the current knowledge on proteins involved in OS uptake gained from studies of both RPE phagocytosis *in situ* and in cell culture. Abbreviations: AnxA2, annexin A2; myoII, myosin II; myoVIIa, myosin VIIa; PI3K, PI3 kinases; SFK, Src family kinases. *Taken from (Mazzoni, Safa et al. 2014)*

On phagosomes, MerTK colocalizes with Rab5 and/or GDP dissociation factor alpha 1 (GDI1) (Shelby, Feathers et al. 2015). Engulfment requires significant cytoskeletal rearrangement. Annexins (Anx), particularly Anx A2 and A4, are seen to be enriched in RPE cells capable of phagocytosis (Law, Ling et al. 2009). Anx A2 is a regulator of actin dynamics and is detected in new phagosomes in the RPE. Furthermore, siRNA-knockdown of Anx A2 abolishes the ability to phagocytose in RPE cells (Law, Ling et al. 2009).

Once internalized by RPE, phagosomes with OS must undergo maturation and degradation steps to breakdown their protein and lipid components. Generally, phagosomes will go through the endosome-lysosome pathway, a series of fusion events that include fusing with early endosomes, late endosomes and lysosomes, finally forming a phagolysosome (Ng, Gan et al. 2012). Each step is characterized by the subset of proteins on the membrane and lumen of the vesicle, as well as the acidification of the lumen. RPE cells are polarized, so phagocytosis always occurs on their apical side. The phagosomes move from the apical side to the basal side, where lysosomes are, to be degraded (Herman and Steinberg 1982, Gibbs, Kitamoto et al. 2003). A defect in this process is seen in Usher syndrome 1B, which is caused by mutations in myosin VIIA. Mutant myosin VIIA cannot traffic phagosomes from the apical side and retinal degeneration ensues (Gibbs, Kitamoto et al. 2003). Lysosomes contain cathepsin D, an acidic protease that will cleave opsin following phagosome-lysosome fusion (Wavre-Shapton, Meschede et al. 2014). Balifomycin A1, which inhibits vacuolar-type H^+-ATPase on lysosomes and prevents their acidification, inhibit such degradation. Rather, phagolysosomes have increased pH and decreased OS degradation when cells are treated with bafilomycin A1 (Deguchi, Yamamoto et al. 1994). The phagocytosis of OS by RPE is crucial to phototransduction in the long term.

1.2 Protein Prenylation

1.2.1 Protein Prenylation

Protein prenylation is the attachment of isoprenoid lipid moieties to a cysteine residue within the C-terminal motif of certain proteins, which is essential for their

association with hydrophobic lipid membranes (Zhang and Casey 1996, Amaya, Baranova et al. 2011). Post-translational prenylation is essential for the proper subcellular distribution and membrane localization of proteins. Prenylation has come to significant attention due to its crucial role in protein function, as well as its implication in a myriad of diseases such as progeria, cancer and bacterial infection (De Sandre-Giovannoli, Bernard et al. 2003, Amaya, Baranova et al. 2011, Berndt, Hamilton et al. 2011, Wang and Casey 2016).

Prenylation is divided into two subcategories: farnesylation and geranylgeranylation. Farnesylation involves the attachment of the 15-carbon farnesyl lipid. It is catalyzed by farnesyltransferase (FTase), which condenses a farnesyl pyrophosphate (FPP) molecule and a cysteine residue to form a thioether linkage (Dunten, Kammlott et al. 1998). Geranylgeranylation is defined as the attachment of the 20-carbon geranylgeranyl lipid to a cysteine residue by either geranylgeranyltransferase I (GGTase I) or geranylgeranyltransferase II (GGTase II) (Guo, Wu et al. 2008).

The three types of prenyltransferases differ in their peptide recognition motif and the isoprenoid species they attach (Casey and Seabra 1996, McTaggart 2006). Farnesyl transferase (FTase) and geranylgeranyl transferase type I (GGTase I) prenylate proteins with C-terminal CAAX motifs, where C is cysteine, A is generally an aliphatic amino acid and X determines the isoprenoid attached. Proteins in which the X is a methionine, as seen in Ras proteins are farnesylated. Proteins in which X is leucine, as seen in Rho proteins, or phenylalanine, are geranylgeranylated by GGTase I (Seabra, Goldstein et al. 1992, Lane and Beese 2006). The Rab proteins are double-geranylgeranylated by geranylgeranyl transferase type II (GGTase II) on CC or CXC

motifs (Seabra, Goldstein et al. 1992, McTaggart 2006). A notable exception is Rab28, which contains a –CAAX motif and is farnesylated (Roosing, Rohrschneider et al. 2013). There are over 300 human proteins that may be prenylated based on their C-terminal motifs (Sebti 2005), but small GTPases represent the largest group of prenylated proteins.

1.2.2 Prenyltransferases and Accessory Proteins

Protein prenylation is catalyzed by prenyltransferase proteins (Casey and Seabra 1996) (**Figure 5**). All prenyltransferase proteins are heterodimers composed of an α-subunit and a β-subunit. FTase and GGTase I share the same α-subunit but have different β-subunits. The β-subunit contains the specific lipid-binding site and a peptide binding site that requires a Zn^{2+} ion (Reiss, Brown et al. 1992). It is more energetically favourable for these prenyltransferases to first bind the isoprenoid moiety than to bind the peptide (Casey and Seabra 1996). Differences in the conformation of both the lipid- and peptide-binding sites of each protein allow each prenyltransferase to be specific for its own substrates, although overexpression of either FTase or GGTase I show there is some functional overlap between the two for the same substrates. Yeast overexpressing GGTase I but lacking FTase show increased membrane localization of Ras, which is typically an FTase substrate (Trueblood, Ohya et al. 1993). Experiments involving the removal of the GGTase I gene have shown that FTase is able to prenylate the substrates of GGTase I (Sjogren, Andersson et al. 2007). Interestingly, some proteins, such as K-Ras and N-Ras, can be farnesylated or geranylgeranylated if either mechanism is inhibited (Lerner, Zhang et al. 1997, Rowell, Kowalczyk et al. 1997,

Whyte, Kirschmeier et al. 1997). Some proteins may physiologically be either farnesylated or geranylgeranylated, as in the case of RhoB (Armstrong, Hannah et al. 1995, Konstantinopoulos, Karamouzis et al. 2007).

GGTase II is also known as Rab Geranylgeranyltransferase (RabGGTase) as it exclusively prenylates small Rab GTPases. It is a heterodimer like the other two prenyltransferases, but it shares limited homology. The α- subunit sharing 27% amino acid identity with FTase and GGTase I, and the β-subunit sharing 29% amino acid identity with the β-subunit of FTase (Zhang and Casey 1996). The enzyme only binds GGPP, and not FPP nor geranyl pyrophosphate (GPP) (Schafer and Rine 1992). It will transfer two molecules of GGPP by attaching the geranylgeranyl onto each cysteine in the –CC or –CXC prenylation motif and releasing the pyrophosphate molecule. Rab GGTase does this sequentially, as the mono-geranylgeranylated intermediate is quite stable and is unlikely to dissociate. There is not a clear preference which cysteine is prenylated first (Shen and Seabra 1996).

Rab GGTase does not stably bind its substrate Rab proteins by itself (Shen and Seabra 1996), but rather binds a complex consisting of a Rab protein and a Rab Escort Protein (REP) (Seabra, Brown et al. 1992, Andres, Seabra et al. 1993). REPs are accessory proteins that bind a newly synthesized Rab protein and deliver it to Rab GGTase. Following prenylation by Rab GGTase, REP protects the newly hydrophobic tail and delivers the Rab protein to its appropriate target membrane (Alexandrov, Horiuchi et al. 1994). The REP is then freed of the prenylated Rab and able to bind another unprenylated Rab.

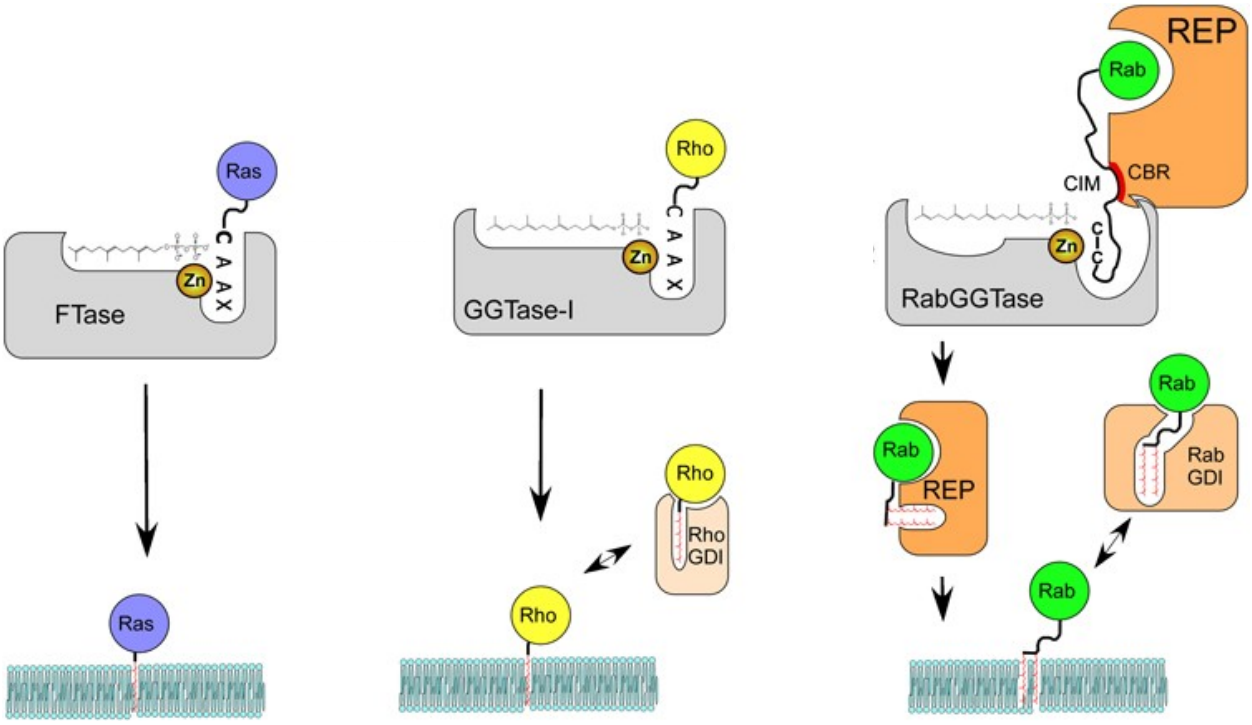

Figure 5: The Types of Protein Prenylation. Farnesyltransferase adds farnesyl pyrophosphate (FPP) to Ras GTPases. Geranylgeranyltransferase I adds a single geranylngeranyl pyrophosphate (GGPP) to the Rho/Rab GTPases. Geranylgeranyltransferase II adds two GGPPs to the Rab GTPases that are brought by Rab Escort Proteins (REP). *Derived from Guo, Z. et al. Structures of RabGGTase–substrate/product complexes provide insights into the evolution of protein prenylation. EMBO J. 27, 2444-2456. DOI 10.1038/emboj.2008.164 (2008).*

Prenylation alone is not sufficient to cause stable membrane association; post-prenylation processing is often required for prenylated proteins to function (Gelb, Brunsveld et al. 2006). Two main modifications occur following prenylation (Schafer and Rine 1992, Leung, Baron et al. 2007). First, Ras-Converting CAAX Endopeptidase (RCE1) cleaves the –AAX amino acids off the prenylation motif. This is followed by isoprenylcysteine carboxylmethyltransferase (ICMT) which methylates the carboxyl group on the carboxy-terminal isoprenoid modified Cys residue (Ashby 1998, Winter-Vann and Casey 2005). Both RCE1 and ICMT reside on the endoplasmic reticulum (ER) (Wang and Casey 2016), suggesting that newly prenylated proteins are proximal to the

cytosolic face of the ER, unless there is an intermediate sequestering step. Without RCE1, fetal mice die in late gestation or within the first week of life (Kim, Ambroziak et al. 1999). Deletion of RCE1 in the retina resulted in the rapid degeneration of the photoreceptors, which was attributed to mislocalization of the geranylgeranylated rod cGMP-specific phosphodiesterase 6 (PDE6) as it was not transported to the OS, while farnesylated proteins such as transducin and rhodopsin kinase were localized properly (Christiansen, Kolandaivelu et al. 2011). Similarly, deletion of ICMT resulted in even earlier deaths of the affected mice, as its deletion impacts Rab proteins, in addition to –CAAX prenylated proteins. ICMT deletion in the retina leads to photoreceptor degeneration (Christiansen, Pendse et al. 2016). Moreover, some proteins need further modifications, such as palmitoylation and myristolyation, for stable membrane attachment. For example, N-Ras and H-Ras are palmitoylated in the Golgi (Hancock, Magee et al. 1989).

1.2.3 Biological Roles of Prenylated Proteins

Many proteins with diverse functions are prenylated. The largest family of prenylated proteins is the intracellular GTP-binding proteins. This family includes heterotrimeric G protein γ subunits, such as the transducin γ subunit, and the monomeric small GTPases superfamily (Konstantinopoulos, Karamouzis et al. 2007). Farnesylated proteins include the Ras GTPases, the nuclear lamins, and the kinetochore centromere proteins (CENP). Geranylgeranylated proteins include other small GTPase proteins, such as the Rho proteins, Rac proteins, cell division control protein 42 homolog (CDC42), R-Ras and the Rab proteins (Sebti 2005).

There are several heterotrimeric G proteins involved in visual transduction. Rhodopsin, a receptor that translates visual light into a biochemical signal, is a G-protein couple receptor and its associated G-protein, transucin, contains a farnesylated γ subunit (Lai, Perez-Sala et al. 1990, Kisselev, Ermolaeva et al. 1995). The action of transducin leads to the activation of PDE6; PDE6 is farnesylated on its α subunit and geranylgeranylated on its β subunit (Anant, Ong et al. 1992). X-linked retinitis pigmentosa GTPase regulator (RPGR) is a geranylgeranylated interactor of PDE6D subunit (Lee and Seo 2015). Rhodopsin kinase (GRK1) is another farnesylated protein that serves to phosphorylate rhodopsin in dim light to slow rhodopsin deactivation (Anant and Fung 1992).

Within small G proteins, the Ras superfamily is divided into subfamilies Ras, Rho/Rac, Rab, Sar1/ARF, and Ran (Segev 2011). All of these are involved in diverse cellular functions: Ras members regulate gene expression and cell proliferation, the Rho/Rac members are responsible for cytoskeletal arrangements, the Rab family regulates intracellular vesicle trafficking, and the Ran family regulates nucleocytoplasmic transport (Takai, Sasaki et al. 2001, Segev 2011). Rab proteins are of particular importance in retinal degeneration because of their role in OS phagosome trafficking in the RPE. Rab proteins are important in signaling pathways, and in vesicle trafficking processes including vesicle formation, transport and fusion to the acceptor membrane (Seabra, Mules et al. 2002). The localization of each Rab species to distinct membrane compartments is determined by its hypervariable C-terminal domain (Chavrier, Gorvel et al. 1991).

Small GTPase proteins are molecular switches that give spatial and temporal specificity to cellular events such as signal transduction and vesicular trafficking. Small GTPases function by hydrolyzing GTP. When bound to GTP, Rab proteins are active, and are inactive when bound to GDP (Stenmark 2009). The nucleotide hydrolysis and exchange cycle allows temporal and spatial regulation of membrane transport. When GTP is hydrolyzed, the GDP-bound Rab may undergo nucleotide exchange by a guanidine nucleotide exchange factor (GEF) or be removed from its attached membrane by a Rab GDP-dissociation factor (GDI) (Muller and Goody 2017). The hydrolysis of GTP is catalyzed by GTPase-activating proteins (GAP) to return the GTPase to the inactive GDP-bound form (Fukuda 2011). These regulators of small GTPases are essential for the temporal specificity of their actions. Activating mutations of Ras proteins are seen in ~20% of human cancers. Mutated Ras proteins may be constitutively GTP-bound and are continuously active, driving uncontrolled cell division (Konstantinopoulos, Karamouzis et al. 2007). Four GDI proteins have been identified in humans: three Rho GDIs and one Rab GDI. These proteins bind prenylated small GTPases and hold them in the cytosol. This prevents GEFs replacing the GDP bound to GTPases (Sasaki, Kikuchi et al. 1990). It is also possible for GDIs to bind GTP-bound GTPases to prevent the actions of GTPase effectors. GEFs catalyze the exchange of GDP for GTP on small GTPases; this exchange is the rate-limiting step the cycle of GTPases. GEFs regulate the temporal and spatial specificity of small GTPase signaling (Cherfils and Zeghouf 2013).

1.2.4 Rab GTPases in Phagocytosis

Various small GTPases orchestrate steps in the vital process of phagocytosis (Flannagan, Jaumouille et al. 2012, Gutierrez 2013). Engulfment involving the extension of pseudopods requires CDC42, Rac1 and Rac2 to reorganize the cytoskeleton (Hoppe and Swanson 2004). Following engulfment, a series of Rab GTPases are recruited to the phagosome, which is essential for the degradation and recycling of its components (**Figure 6**). In the early phagosome, Rab5 is recruited to mediate the fusion of the phagosome with the early endosome (Duclos, Diez et al. 2000). When Rab5 is associated to this early phagosome, it recruits vacuolar fusion protein 1 (Mon1). Mon1, in complex with vacuolar fusion protein CCZ1 and Rab5, recruit Rab7 by acting as a GEF and removing Rab7 from its inactive GDI-bound state (Poteryaev, Datta et al. 2010). Rab7 and its associated effectors mature the early phagosome into a late phagosome, allowing it to recruit late endosome/lysosome markers such as lysosome-associated membrane protein 1 (LAMP1) and move towards the microtubule-organizing centre (Huynh, Eskelinen et al. 2007, Johansson, Rocha et al. 2007). Rab7 is also present on late endosomes and lysosomes on the microtubule-organizing centre, facilitating the fusion of late phagosomes to these vesicles. The newly fused phagolysosome degrades the luminal contents. The lumen is acidic (pH <5) and contains degradative enzymes, such as proteases from the cathepsin family, nucleases and lipases (Botelho and Grinstein 2011). The degradation products may be recycled and this recycling may be essential, as seen in other phagocytic processes such as the recycling of iron from erythrocytes (Kong, Lei et al. 2013), but this area requires further investigation in the retina.

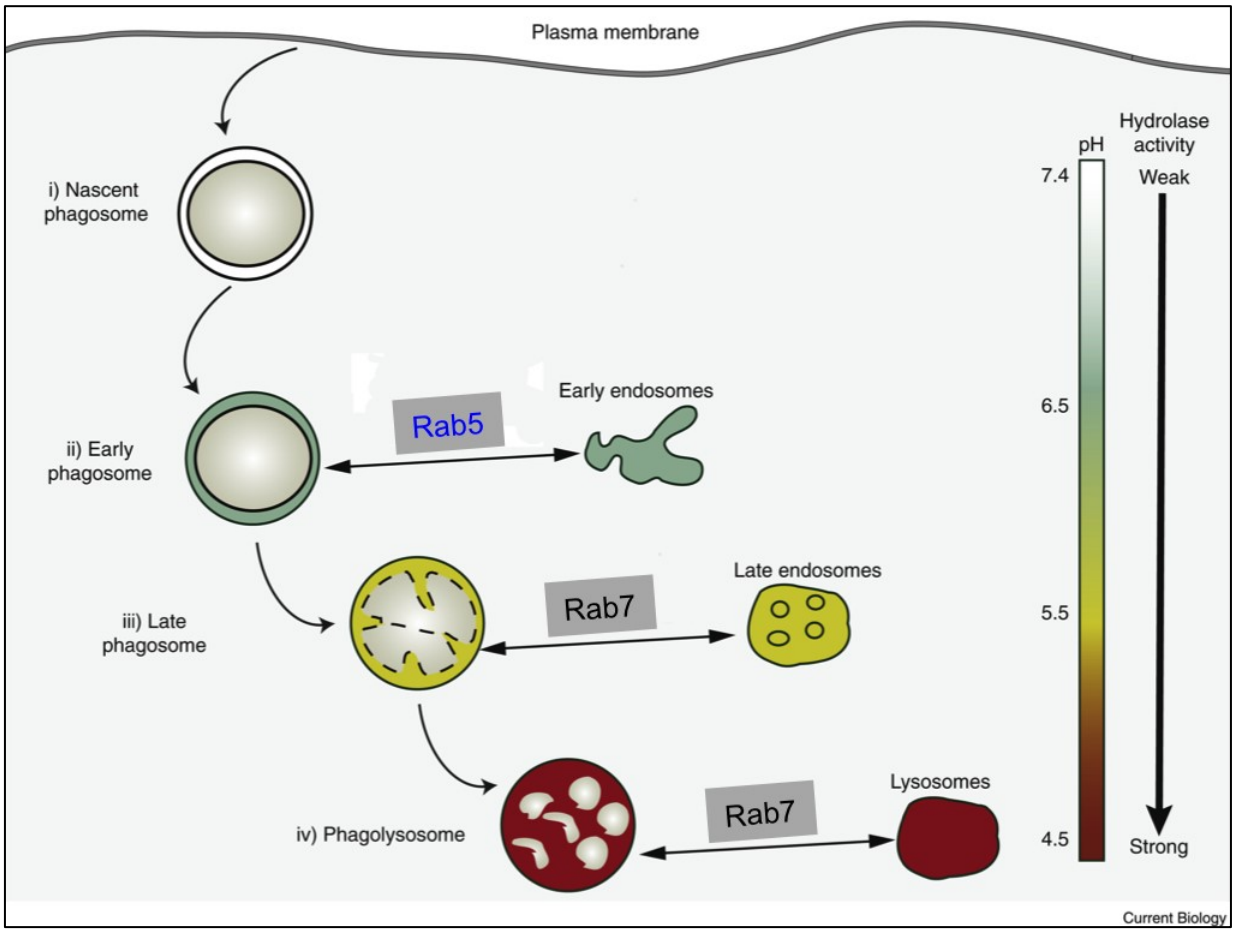

Figure 6: Rabs in Phagosome Maturation. i) The membrane of the nascent phagosome has a few notable differences compared with the plasma membrane. **ii)** Early phagosomes acquire many of the molecular markers of early endosomes including Rab5. **iii)** Subsequently, phagosomes exchange early endosomal markers and transition into a late endosome-like organelle, with a more acidic lumen. Rab7 is recruited at this stage. **iv)** Lastly, phagosomes mature into phagolysosomes; these are highly acidic and hydrolase-rich organelles that degrade the internalized particle. Rab7 is also required at this late stage. *Adapted from (Botelho and Grinstein 2011).*

1.3 The Mevalonate Pathway

The isoprenoids attached to proteins during prenylation are produced by the mevalonate pathway that also synthesizes cholesterol. The steps of the mevalonate pathway were first discovered by Konrad Bloch and Feodor Lynen, for which they were

awarded the Nobel Prize in Physiology or Medicine in 1964 (Zetterstrom 2009). Dubbed the mevalonate pathway, the sequential actions of over twenty enzymes in forming cholesterol and isoprenoids from acetyl-CoA was further elucidated by Joseph Goldstein and Michael Brown (Goldstein and Brown 1990), culminating in a Nobel Prize in the same category in 1985. Akira Endo discovered inhibitors for the mevalonate pathway (Endo and Kuroda 1976) that lead to the development of many statin species. Soon after, John Glomset showed that mevalonate pathway inhibitors arrest cells in the S phase of cell division, and that exogenously supplied mevalonate is converted to isoprenoid, which are then attached to membrane-anchored Ras and Rho proteins (Raiteri, Arnaboldi et al. 1997).

The early pre-squalene portion of the mevalonate pathway produces a 15-carbon isoprenoid lipid named farnesyl pyrophosphate (FPP), before diverging into two separate pathways: (1) the post-squalene pathway which produces cholesterol and (2) the non-sterol isoprenoid pathway (**Figure 7**).

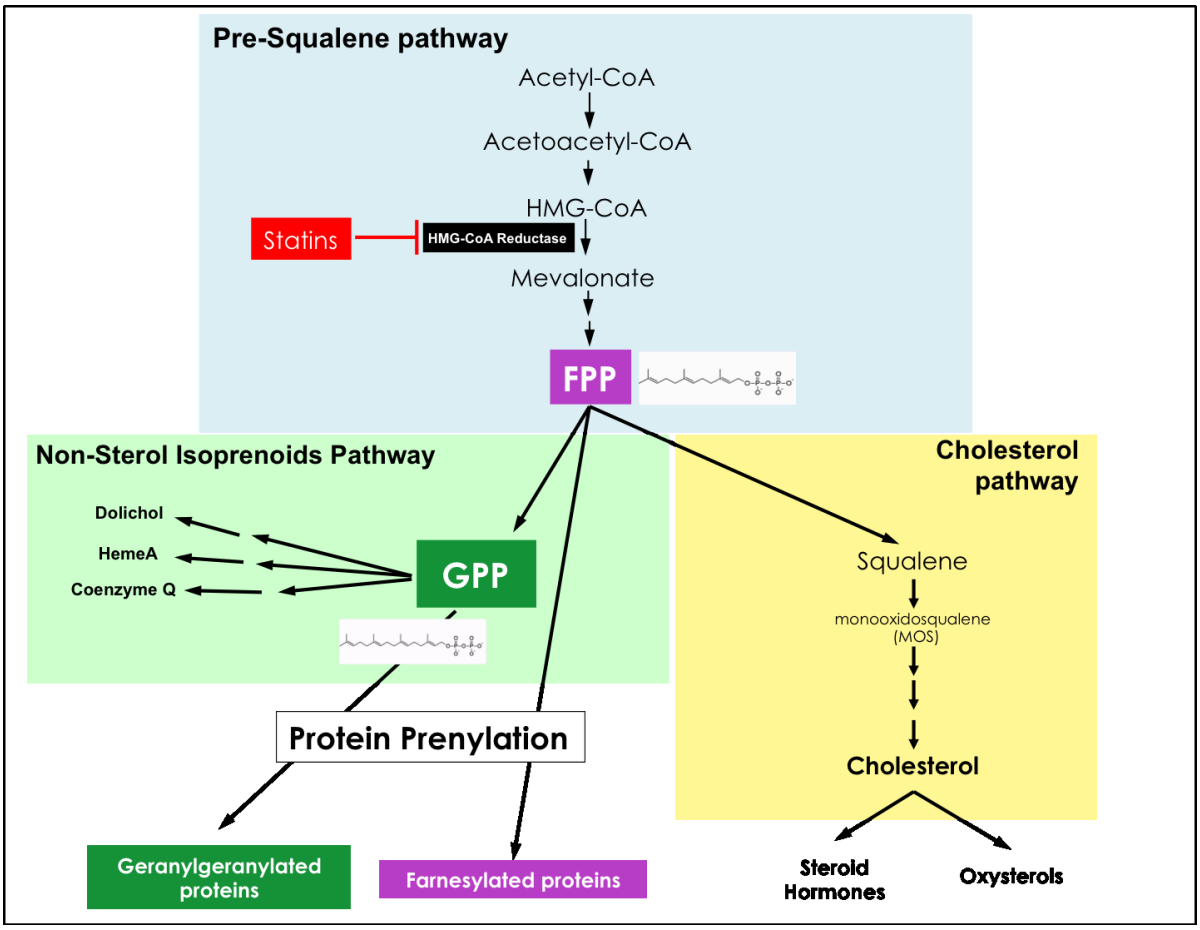

Figure 7: The Mevalonate Pathway. *Adapted from Amany Mohamed, Kevan Smith and Elena Posse de Chaves (2015). The Mevalonate Pathway in Alzheimer Disease — Cholesterol and Non-Sterol Isoprenoids, Alzheimer Disease - Challenges for the Future, Prof. Inga Zerr (Ed.), InTech, DOI: 10.5772/59904. Available from: https://www.intechopen.com/books/alzheimer-s-disease-challenges-for-the-future/the-mevalonate-pathway-in-alzheimer-s-disease-cholesterol-and-non-sterol-isoprenoids*

1.3.1 The Pre-Squalene Pathway

The pre-squalene pathway begins with the reaction between two acetyl CoA molecules to form acetoacetyl CoA by acetoacetyl CoA thiolase. Another acetyl CoA is added to the acetoacetyl CoA to form 3-hydroxy-3 methylglutaryl CoA (HMG CoA). HMG CoA reductase (HMGR) converts HMG CoA to mevalonic acid (Ferguson, Durr et al. 1959); this is the rate limiting step of the mevalonate pathway and is one of the most

regulated enzymes in nature (Rodwell, Nordstrom et al. 1976, Goldstein and Brown 1990, Mohamed A 2015). Mevalonic acid, also known as mevalonate, is then phosphorylated to form 5-phosphomevalonate by mevalonate kinase (MK), which in turn is phosphorylated to 5-pyrophosphomevalonate by phosphomevalonate kinase (Tchen 1958). This is then turned into isopentenylpyrophosphate (IPP) by mevalonate diphosphate decarboxylase. IPP is isomerized to dimethylallyl pyrophosphate (DMPP) in the presence of IPP isomerase (Agranoff, Eggerer et al. 1960). One molecule of IPP and one molecule of DMPP form geranyl pyrophosphate (GPP). Finally, farnesyl pyrophosphate (FPP) is formed from a molecule of GPP and IPP. Both GPP and FPP are formed by farnesyl pyrophosphate synthase (FPPS) (Wang and Ohnuma 2000, Holstein and Hohl 2004).

Farnesyl pyrophosphate synthase (FPPS) is regulated by product-feedback competitive inhibition as its product, FPP, will compete with its substrate, GPP, for the active site (Laskovics, Krafcik et al. 1979). GGPPS is allosterically inhibited by GGPP (Lutz, McLain et al. 1992).

1.3.2 The Cholesterol Pathway

The post-squalene pathway ultimately produces cholesterol. It begins with the first committed step to cholesterol synthesis: conversion of FPP to squalene by squalene synthase (Do, Kiss et al. 2009). Squalene is converted to mono-oxidosqualene by squalene monooxygenase, which is then converted to lanosterol by squalene epoxidase. Lanosterol is the first sterol species in cholesterol biosynthesis, and forms the fundamental carbon backbone for steroids in animals. Lanosterol is

metabolized to cholesterol in 19 enzymatic steps (Mohamed, Smith et al. 2015). In the retina, cholesterol is formed by both the Kandutsch-Russel pathway from 7-dehydrocholesterol and lathosterol, or by the Bloch pathway from desmosterol, as enzymes from both pathways are seen to be expressed (Zheng, Reem et al. 2012). The post-squalene pathway is not the focus of this thesis, but cholesterol homeostasis is vital for retinal function. Decreased cholesterol removal from the retina results in the deposition of ApoB-rich lipid deposits dubbed drusen in the Bruch's membrane between the RPE and the choroid, a hallmark of many retinal degeneration diseases such as aged-related macular degeneration (AMD) (Malek, Li et al. 2003).

1.3.3 Non-Sterol Isoprenoid Pathway

Isoprenoids are a diverse class of important molecules that are present in all lifeforms. Some are small, such as the 5-carbon isopentenyl diphosphate (IPP), which is condensed into larger isoprenoids that are crucial to life, such as heme and ubiquinone (Edwards and Ericsson 1999). Many diseases are associated with the dysfunction of the non-sterol isoprenoid pathway: Alzheimer Disease, Parkinson Disease, and cancer (Rajanikant, Zemke et al. 2007, Wiemer, Hohl et al. 2009, Hooff, Peters et al. 2010).

Following the reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid by HMGR, mevalonic acid is then converted to farnesyl pyrophosphate (FPP, 15 carbons) and geranylgeranyl pyrophosphate (GGPP, 20 carbons) in a series of steps. Prenyltransferases catalyze the consecutive condensations of IPPs with allylic substrates to form the linear backbone of FPP and its derivative isoprenoids (Wang and Ohnuma 2000). FPP is converted to GGPP by GGPP synthase (GGPPS) (Kandutsch,

Paulus et al. 1964). FPP and GGPP are important for post-translational prenylation (i.e farnesylation and geranylgeranylation) of proteins.

1.3.4 Coordination of the Regulation of Post-Squalene and Non-Sterol Isoprenoid Branches of the Mevalonate Pathway

Intracellular cholesterol may be synthesized from the mevalonate pathway or may be taken up systemically from the extracellular milieu. On the other hand, non-sterol isoprenoids can only be generated by intracellular synthesis. The mevalonate pathway must ensure the adequate production of non-sterol isoprenoids for cellular functions without the accumulation of potentially toxic cholesterol. The mevalonate pathway must maintain a constant isoprenoid pool, with or without the production of cholesterol. Analysis of the affinity of the enzymes of the mevalonate pathway demonstrates how such balance is achieved. The affinity of GGPPS for its substrate, FPP (K_M = 0.6 μM) (Ericsson, Runquist et al. 1993), is more than an order of magnitude higher than the affinity of squalene synthase for FPP ($K_M \cong 15$ μM) (Biller, Forster et al. 1988). In addition, saturation of coenzyme Q and dolichol synthesis occurs at lower concentrations of isoprene intermediates than the concentration required to saturate cholesterol synthesis (Gold and Olson 1966, James and Kandutsch 1980). Under limited mevalonate and FPP, the non-sterol isoprenoid pathway is favoured. Inhibition of mevalonate synthesis first decreases the availability of FPP for cholesterol synthesis while the non-sterol pathway may or may not be affected.

1.3.5 The Mevalonate Pathway in the Retina

Studying cholesterol in the retina has been challenging due to its compartmentalization and high level of complexity, so research has been largely limited to systemic manipulations of cholesterol in animal models. However, recent progress has provided evidence that the retina obtains cholesterol by local synthesis and from the circulation and expresses all the genes required for cholesterol homeostasis (Fliesler 2015, Zheng, Mast et al. 2015). Staining histological sections of the retina reveals that enzymes of the mevalonate pathway are largely expressed in the inner retina, in neuronal cell types and accompanying glia (ganglion cells, Müller glia), and less so in the outer retina (photoreceptors, RPE) (Fliesler and Bretillon 2010, Zheng, Reem et al. 2012). The inner retina has a separate blood supply stemming from the central retinal artery that runs along the optic nerve. It contains no fenestrations and feeds all layers starting at and including the Ganglion cell layer (Anand-Apte 2011). Studies manipulating the mevalonate pathway through high fat diet and mevalonate pathway inhibitors show that the retina maintains its own cholesterol homeostasis mainly through local synthesis and elimination (Zheng, Mast et al. 2015, Lin, Mast et al. 2016). This is speculated to be due to the inner retina having tight junctions in the vasculature, like the blood-brain barrier, that do not allow the passage of large lipoproteins and must generate cholesterol by local synthesis (Fliesler 2015). The outer retina is fed by the choroicapillaris, which is fenestrated, exchanges lipoproteins with the adjacent Bruch's membrane and RPE (Tserentsoodol, Gordiyenko et al. 2006).

1.4 Statins

1.4.1 General Considerations

Statins are cholesterol synthesis inhibitors that target HMGR, the rate-limiting enzyme of the mevalonate pathway (Rodwell, Nordstrom et al. 1976, Goldstein and Brown 1990). Statins are competitive inhibitors of HMGR at the HMG CoA binding site, reducing the conversion of HMG CoA to mevalonate (Istvan and Deisenhofer 2001). Inhibitors of HMG CoA-reductase were first derived from fungal cultures in 1968 (Endo and Kuroda 1976). All statins have the same mechanism of action but differ greatly in their structures, pharmacokinetic properties and efficacy (Sirtori 2014). Lovastatin, pravastatin and simvastatin were the first statins discovered, while fully synthetic statins, such as atorvastatin and rosuvastatin, came later.

Statins are widely used to treat hypercholesterolemia, as high blood cholesterol is a key contributor to atherosclerosis and heart attacks (Endo 2010). However, some patients receiving statins experience muscle pain (Law and Rudnicka 2006), visual impairment (Mizranita and Pratisto 2015), and a multitude of other detrimental side effects. Despite this, the use of statins is widespread as they are very effective drugs to treat hyperlipidemia and have fewer side effects than other drugs used to reduce lipids (Hajar 2011, Force, Bibbins-Domingo et al. 2016). It is important to understand the mechanism of its detrimental effects in order to design targeted therapeutics or circumvent such phenomena.

1.4.2 Pharmacology of Statins

The structure of statins consists of three main features: (1) analog of HMG CoA, the enzyme substrate, (2) a hydrophobic ring that binds the analog to the enzyme and (3) side groups that affect the pharmacokinetic profile of the drug (Istvan and Deisenhofer

2001). Type I statins have a decalin-ring like structure, in contrast to type II statins that have a larger ring structures for their hydrophobic ring (**Figure 8**) (McTaggart, Buckett et al. 2001). The affinity of statins for HMGR is approximately three orders of magnitude greater than that of the natural substrate, HMG CoA. Atorvastatin and simvastatin are lipophilic, while pravastatin and rosuvastatin are more hydrophilic due to their polar side groups (McTavish and Sorkin 1991). Hydrophilicity affects the mechanism of cellular uptake: lipophilic statins enter hepatocytes by passive diffusion while hydrophilic statins enter by carrier-mediated uptake (Hamelin and Turgeon 1998, Nezasa, Higaki et al. 2003).

The pharmacokinetics of statin vary greatly (Pasha, Muzeeb et al. 2006). All are absorbed rapidly, reaching peak plasma concentration within 4 hours (Pan, DeVault et al. 1990, Tse, Jaffe et al. 1992, Cilla, Gibson et al. 1996). Some statins that have half-lives of 3 hours or less need to be taken in the evening to increase their lipid-lowering effects as cholesterol synthesis is higher at night, while newer statins have longer half-lives (Schachter 2005). The systemic bioavailability is generally low for statins as they are extensively taken up in the liver, due to the first-pass effect. Notable exceptions include cerivastatin (60% bioavailability) and pivastatin (80% bioavailability) (Muck, Ritter et al. 1997, Kajinami, Mabuchi et al. 2000). Statins in circulation are extensively bound to plasma proteins, and systemic exposure to statins is relatively low (Corsini, Bellosta et al. 1999). Unbound lipophilic statins will readily pass through cellular membranes outside of the liver, while hydrophilic statins do not and appear to impact peripheral tissues to a lesser extent (Rosenson and Tangney 1998).

In the liver, the cytochrome P_{450} (CYP450) enzymes metabolize statins so that they may be predominantly excreted via the bile. Many have active metabolites; for example, simvastatin is metabolized by CYP3A4 to its β-hydroxy acid form which retains 40% of simvastatin's lipid lowering activity (Garcia, Reinoso et al. 2003).

1.4.3 Statins and the Retina

The blood-brain barrier is similar to the blood-retina barrier, so some parallels may be drawn. First, both lipophilic and hydrophilic statins are able to cross the blood-brain barrier but the lipophilic statins cross more readily whereas high concentrations of hydrophilic statins in the blood are required to be detected in the brain, even by very sensitive methods such as liquid-chromatography/mass spectrometry (LC/MS) (Saheki, Terasaki et al. 1994, Wood, Eckert et al. 2010). Hydrophilic statins are mainly transported by organic anion transporters (OATs) into the brain (Hasegawa, Kusuhara et al. 2003), though many other transporters have roles (Tomi and Hosoya 2010).

The lipophilicity of statins changes their biological effects. Mohamed *et al.* demonstrated that pravastatin inhibits cholesterol synthesis in primary neurons without affecting protein prenylation (Mohamed, Saavedra et al. 2012). Prenylation may be reduced by other statins that more potently inhibit HMGR due to their increased lipophilicity and ability to cross the plasma membrane, especially with respect to the blood-retina barrier (Fujii, Setoguchi et al. 2015). Protein farnesylation is usually preserved under statin treatment (Winter-Vann and Casey 2005, Rauthan and Pilon 2011), due to the high affinity of protein farnesyl transferase for FPP ($K_M < 0.1$

μM)(Sinensky, Beck et al. 1990) and thus deficient geranylgeranylation is a more significant concern.

It is controversial whether statins are beneficial or detrimental in the brain. There is evidence that statins are protective in a variety of conditions, such as excitotoxicity (Bosel, Gandor et al. 2005), amyloid-beta insults (Johnson-Anuna, Eckert et al. 2007), and ischemic injuries (Bosel, Gandor et al. 2005). On the other hand, statins have been shown to cause toxicity and cell death in the brain, especially at high concentrations (Marz, Otten et al. 2007). It is unclear whether these effects are due to the effects of statins on the cholesterol pathway or the non-sterol isoprenoid pathway. The anti-inflammatory effects of effects of statins are attributed to the decreased prenylation of Ras and Rho GTPases (Weitz-Schmidt 2002).

Decreasing the pool of isoprenoids or blocking the function of prenylated proteins has serious deleterious consequences for retina. Rapid retinal degeneration occurs when the eye is injected intravitreally with inhibitors of HMGR (statins) or prenylprotein-specific carboxylmethyltransferases, but not with inhibitors of the sterol branch (Pittler, Fliesler et al. 1992, Fliesler and Keller 1997). There is conflicting evidence for the use of statins in diseased states. In many retinal diseases, from age-related macular degeneration (AMD) to diabetic retinopathy, it is unclear whether statins should be recommended (Gehlbach, Li et al. 2016, Modjtahedi, Bose et al. 2016, Vavvas, Daniels et al. 2016, Ioannidou, Tseriotis et al. 2017).

Figure 8: Legend on next page.

Figure 8: **Structure of Statins**. **(A)** Structural formulas of statins and the enzyme substrate HMG-CoA. Compactin and simvastatin are examples of type 1 statins; not shown are the other type 1 statins, lovastatin and pravastatin. Fluvastatin, cerivastatin, atorvastatin, and rosuvastatin are type 2 statins. The HMG-like moiety that is conserved in all statins is colored in red. The IC50 (median inhibitory concentration) values of the statins and the K_M value of HMG-CoA are indicated.
(B) Chemical structure of rosuvastatin, indicating the relative lipophilicity of different statins, as expressed by log D at pH 7.4. Both rosuvastatin and pravastatin have lower log D compared to other statins and thus exhibit significant hydrophilicity. *Adapted from Istvan & Deisenhofer, 2001 and Sirtori, 2014.*

1.5 Prenylation-Associated Retinal Disease

1.5.1 Prenylation-Associated Retinal Diseases

Many inherited retinal degenerative diseases arise from defects in prenylated proteins (Roosing, Collin et al. 2014) (**Figure 9**). This shared aspect highlights the importance of prenylation in the retina. When lovastatin was injected intravitreally into the eye, photoreceptor degeneration preceded rapid degeneration of the retina, while the injection of sterol synthesis inhibitors had no effect, suggesting that protein prenylation is crucial to homeostasis in the retina (Pittler, Fliesler et al. 1992, Pittler, Fliesler et al. 1995).

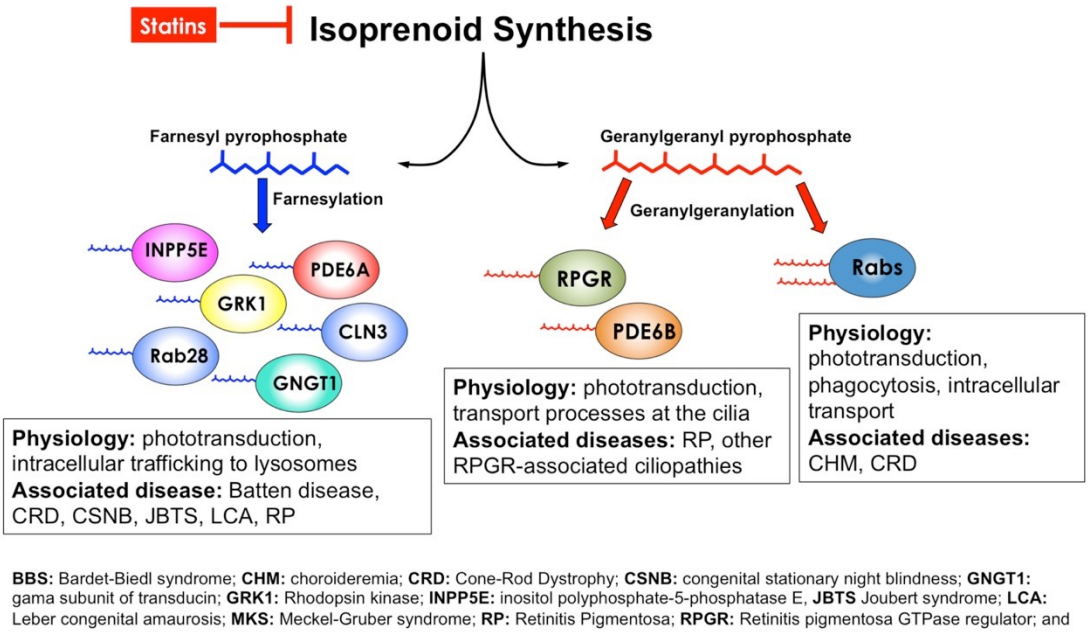

Figure 9: Prenylation-associated Retinal Diseases. The two types of protein prenylation are presented, as well as the proteins that are prenylated.

Many visual transduction proteins are prenylated. Transducin, the heterotrimeric G-protein coupled to rhodopsin activation, is farnesylated on its γ subnit for membrane

association (Fukada, Takao et al. 1990). When transducin is activated, it will translocate from the photoreceptor outer segments to the inner segments to dampen light sensitivity (Kassai, Aiba et al. 2005). Under inhibition of the mevalonate pathway, the farnesyl-producing pre-squalene pathway is largely preserved while downstream products are more affected. The Rab subclass of small GTPases all undergo digeranylgeranylation at their C-terminal sequence motifs –CXC or –CC, which are recognized by the Rab GGTase II. Rab6 and Rab8 are suggested to facilitate the delivery of rhodopsin from the *trans*-Golgi network to the disk membrane assembly at the start of the OS (Deretic and Papermaster 1993).

Mutations in proteins involved in farnesylation can cause inherited retinal degeneration. Aryl-hydrocarbon interacting protein 1 (AIPL1) is not prenylated itself but it is a chaperone for farnesylated proteins (Sohocki, Perrault et al. 2000); it is mutated in autosomal-recessive LCA or autosomal-dominant rod-cone dystrophy (Ramamurthy, Niemi et al. 2004). It chaperones important visual transduction proteins such as gamma-transducin and the PDE6 alpha and beta subunits. Prenyl binding protein δ (PDE6D) facilitates intracellular trafficking and solubilizes multiple proteins such as RPGR and PDEα and β; it is implicated in Joubert syndrome (Thomas, Wright et al. 2014). Overall, PDE proteins have significant roles in the retina (Baehr 2014).

Mutations in mevalonate kinase are implicated in late-onset non-syndromic retinitis pigmentosa (RP), or could result in either mevalonic aciduria or hyper-immunoglobulin D and periodic fever syndrome (HIDS), both of which have retinal dystrophy as a consequence (Horvat, McWhir et al. 2011, van der Burgh, Ter Haar et al. 2013). These mutations result in the accumulation of mevalonate to toxic levels and

lead to a decrease of mevalonate pathway products such as isoprenoids and cholesterol, though it is presently unclear how the disease phenotypes are generated and why there is such great variability. Under this condition, attempts to abrogate the accumulation of mevalonate by using statins have yielded conflicting results: in mevalonic aciduria, lovastatin treatment led to high fever and diarrhea amongst other medical crises (Hoffmann, Charpentier et al. 1993, Simon, Drewe et al. 2004), while in HIDS, a decrease in febrile days was observed (Simon, Drewe et al. 2004). There do not seem to be attempts to give statins as a therapy for this form of RP.

The γ subunit of transducin is farnesylated (Lai, Perez-Sala et al. 1990). When mutated, photoreceptors degenerate as seen in Nougaret-type congenital stationary night blindness (Dryja, Hahn et al. 1996). Transducin is a heterotrimer GTPase, consisting of α-, β-, and γ-subunits. Farnesylation of the γ-subunit is important for translocation of transducin to the OS during dark adaptation (Whelan and McGinnis 1988).

The phosphodiesterase 6 α-subunit is farnesylated and β- subunit is geranylgeranylated (Anant, Ong et al. 1992). The other subunits of PDE6, particularly the two γ-subunits, are involved in reducing cyclic GMP (cGMP) levels and hyperpolarization in the photoreceptor. Mutations in the α- and β- subunits are implicated in autosomal-recessive RP (Zhang, Hanke-Gogokhia et al. 2015).

Rab28 is a farnesylated small GTPase containing an –CAAX motif (Roosing, Rohrschneider et al. 2013), unlike other Rab GTPases that are typically geranylgeranylated. Mutations in Rab28 cause cone-rod dystrophy. Rab3A and Rab6 transport rhodopsin to the outer segment (Deretic 1997). Mutations in Rab3A and its

associated Rab3-interacting protein 1 (RIM1) are associated with dominant cone-rod dystrophy (Johnson, Halford et al. 2003). These degenerative phenotypes are limited to the eye.

Retinitis pigmentosa GTPase regulator (RPGR) is geranylgeranylated and localized to the Golgi (Yan, Swain et al. 1998, Dutta and Seo 2016). RPGR can act as a GEF for Rab8, a GTPase involved in rhodopsin trafficking, or is alternatively spliced to the ORF15 isoform. Mutations within the ORF15 isoform are associated with X-linked retinal dystrophy (Ebenezer, Michaelides et al. 2005), but this form is not prenylated. Other mutations do not affect the GEF action of RPGR, suggesting that RPGR may have additional roles in the retina that are currently unknown.

Rhodopsin kinase (GRK1) is farnesylated and bound to rhodopsin. When the conformation of rhodopsin changes, GRK1 phosphorylates it to release its G protein. GRK1 mutations are a cause of Oguchi disease, a form of night blindness (Yamamoto, Sippel et al. 1997).

Ceroid lipofuscinosis neuronal 3 (CLN3) is a farnesylated lysosomal transmembrane protein (Pullarkat and Morris 1997). In Batten disease, vision loss is often the first symptom, leading to neurodegeneration. Mutations in CLN3 are associated with Batten disease and non-syndromic LCA.

There are currently a number of gene therapy clinical trials that seek to introduce a functional copy of the affected gene into the retina, such as trials for Leber's congential amaurosis (LCA) or CHM, so it is additionally important to maintain adequate protein prenylation and ensure the success of these efforts. Recent interest in genetic disorders of the eye has increased as the eye is an ideal target organ for gene therapy. The eye is

easily accessible, compartmentalized and immune-privileged due to the blood-retina barrier (Zhou and Caspi 2010). The current clinical trial by groups in the UK, USA and Canada seek to deliver functional copies of the CHM cDNA in an Adeno-Associated Virus (AAV) by subretinal injection into RPE and photoreceptor cells to permanently reverse genetic defects, stopping the progression of disease.

1.5.2 Choroideremia is a Prenylation-Associated Retinal Disease

CHM is an inherited eye disease that causes retinal degeneration leading to blindness. CHM affects approximately 1 in 50,000 men (MacDonald, Sereda et al. 2004). The RPE, photoreceptors and choroid layers in the eye are affected. It is an X-linked recessive disease that is 100% penetrant in hemizygous males (MacDonald, Hume et al. 1993). CHM was first described by Dr Ludwig Mauthner in 1872, who observed the almost total lack of choroid vessels above the white sclera at late stages of the disease in males. Initial studies in 1985 using linkage analysis suggested the gene causing CHM was located on the X-chromosome, spanning Xq13-21 (Nussbaum, Lewis et al. 1985). It was discovered that the *CHM* gene exists at Xq21.2 (Merry, Lesko et al. 1989), and it was first successfully cloned in 1990 (Cremers, van de Pol et al. 1990).

CHM is a disorder of defective intracellular trafficking; deficient Rab geranylgeranylation has been ascribed to this disease (Seabra, Ho et al. 1995). Typically, affected males develop night-blindness during adolescence, then experience progressive loss of peripheral vision until complete blindness by their 70's (MacDonald, Russell et al. 2009). CHM is caused by loss-of-function mutations in the *CHM* gene,

which encodes for REP-1 (Seabra, Brown et al. 1992). REP-1 is ubiquitously expressed in all tissues, but in CHM, the only significant phenotype is retinal degeneration.

Despite advances towards a cure for CHM, its pathogenesis is incompletely understood. Clinical observations have hinted at how disease progresses within the eye but the difficulty in obtaining patient samples of retinal tissues presents an ongoing challenge. An understanding of the pathogenesis of CHM is vital to identify factors that may alter the progression of disease and ensure the success of future treatments.

Earlier stages of the disease indicated that the eye develops normally, and that CHM is a progressive degenerative disease (Sorsby, Franceschetti et al. 1952). Affected males have characteristic fundus changes: patchy mottling of the RPE progresses to loss of large areas of RPE and retina in the periphery. This is followed by atrophy of the choroicapillaris. The macula is relatively preserved until late stages of the disease, when it at last degenerates. FAF will show remaining islands of RPE tissue as they accumulate intralysosomal lipofuscin fluorophores consisting of cross-linked protein residues, diverse lipid entities and iron (Brunk and Terman 2002). Optical Coherence Tomography (OCT) shows the loss of the outer nuclear layer (ONL) containing photoreceptor cell bodies, photoreceptor IS and OS, RPE and choroid. Photoreceptor OS loss precedes ONL and RPE loss (Lazow, Hood et al. 2011). The retina thickens in affected young males, which is thought to be caused by inflammation resulting from Müller glial activation or early photoreceptor defects. The inner layers of the retina are preserved until late stages of the disease, consisting of neuronal cell types, including the retinal nerve fibre layer, ganglion cell layer and inner nuclear layer (Genead, Pasadhika et al. 2009).

Heterozygous female carriers usually do not experience significant vision impairment, but imaging of the RPE reveals patches of depigmentation and degeneration which has been attributed to regions of random X-chromosome inactivation. Female carriers have a mild phenotype and do not have clinically significant changes in visual acuity (VA) or visual field (VF) (Jacobson, Cideciyan et al. 2006, Huang, Kim et al. 2012). There may be patchy pigmentation of the peripheral fundus which is more apparent with FAF imaging; this is sometimes the only sign that a female is a carrier (Preising, Wegscheider et al. 2009). However, this rarely progresses to RPE or photoreceptor degeneration (Renner, Fiebig et al. 2009). The relative preservation of the retina in female carriers is attributed to lyonization of the X-chromosome. However, there has been some evidence that lyonization is unbalanced and preferential inactivation of the X-chromosome with the functional *CHM* gene occurs (Lyon 1961, Majid, Horsborough et al. 1998, Rudolph, Preising et al. 2003). Thus, affected female carriers may experience a range of symptoms, from mild nyctalopia to severe phenotypes akin to affected males.

The general order of cell atrophy in CHM is unknown, whether atrophy of all three layers occurs concurrently and primarily, or if one or more layers atrophy secondarily. There have been many attempts to recapitulate CHM in animal models, but none fully mirror all of its ramifications. However, the current models for CHM offer significant insight into the progression of the disease. In zebrafish containing a *rep1* gene mutation, photoreceptor outer segment material was not degraded fully and RPE vacuolization was observed, while opsin trafficking in photoreceptors appeared normal (Krock, Bilotta et al. 2007). In a conditional knock-out model in mice, the *CHM* gene was disrupted in

either photoreceptors or RPE. These layers degenerated independently without greatly affecting the other, but RPE degeneration accelerated photoreceptor degeneration (Tolmachova, Anders et al. 2006, Tolmachova, Wavre-Shapton et al. 2010). Substantial evidence suggests the RPE is the first and more important site of degeneration, and the photoreceptors and choroid degenerate secondarily.

A functional parallel emerged between the *CHM* gene product and Rab GGTase (Seabra, Brown et al. 1992). By 1993, the *CHM* gene product was dubbed Rab Escort Protein based on its speculated function (Andres, Seabra et al. 1993). CHM is caused by a functional loss of the gene product. Mutations include deletions, translocations and subtler changes such as splice site mutations. Deletion size spans a few kilobases removing a single exon, to the entire CHM gene and beyond, manifesting in contiguous gene syndromes that involve the deletion or duplication of multiple genes that result in disorders in addition to CHM (van den Hurk, Schwartz et al. 1997). Various splice site mutations have been documented, such as the insertion of a retrotransposon insertion into exon 6. While maintaining the reading frame, this mutation resulted in direct splicing of exon 5 to exon 7, resulting in a truncated protein (van den Hurk, van de Pol et al. 2003). Most of the mutations discovered thus far result in a premature stop codon. This results in a prematurely truncated mRNA, which is either degraded via nonsense-mediated decay (Kervestin and Jacobson 2012) or translated into a truncated REP-1 protein, which is degraded via the unfolded protein response (UPR) (Cao and Kaufman 2012).

Very few missense mutations have been found. Two mutations have resulted in very low levels of REP-1 expression: c.1520A>G (p.His507Arg) and c.1649T>C

(p.Leu550Pro). The REP-1$^{p.His507Arg}$ could not bind to Rab GGTase II and thus considered non-functional (Esposito, De Falco et al. 2011). The REP-1$^{p.Leu550Pro}$ expression was virtually undetectable in fibroblasts and undetectable in lymphoblasts (Sergeev, Smaoui et al. 2009). Three other missense mutations in *CHM* have been reported but lack various aspects of characterization to determine the expression or activity of REP-1 (NGRL, Manchester LOVD, available at: https://secure.ngrl.org.uk/LOVDv.2.0/home.php?select_db=CHM). In the few known cases of missense mutations in the *CHM* gene, there are no cases with normal or partial REP-1 expression or function.

1.5.3 Rab Escort Proteins

REP-1 is an obligatory chaperone protein that binds nascent Rab proteins. It facilitates prenylation of Rab proteins by binding unprenylated Rabs with its Rab Binding Platform (RBP) and C-terminal binding region (CBR), and, when in complex with Rab Geranylgeranyltransferase II (Rab GGTase II), it captures unprenylated Rabs. Rab GGTase II then catalyzes the transfer of two geranylgeranyl pyrophosphate (GGPP) to the CXC or CC sequence, where 'C' is a cysteine, in the carboxy-terminus of Rab proteins. The binding of a new GGPP to the Rab GGTase II induces the dissociation of the Rab:REP-1 complex. The newly prenylated Rab stays bound to REP-1, its hydrophobic tail shielded from the aqueous milieu, and is then delivered to its target membrane. It is possible for Rab proteins to directly bind Rab GGTase II and become prenylated (Seabra, Goldstein et al. 1992), but it is not known whether this happens physiologically; and the protein complex formed is very unstable. Some studies have

shown that it is unlikely that REP-1 directly captures newly synthesized Rab proteins to bring them to the Rab GGTase II, as this process is energetically unfavourable (Baron and Seabra 2008). Further examination of this reaction is needed.

REP-1 is ubiquitously expressed throughout the body, yet the deficiency of REP-1 only produces a clinically significant phenotype in the eye. Interestingly, prenylated Rab proteins are still seen in tissues from CHM patients despite the lack of REP-1. This led to the identification of a second REP protein. REP-2. REP-2 is encoded in the *CHML* gene on chromosome 1, that is functionally similar to REP-1 (van Bokhoven, van Genderen et al. 1994). Early studies demonstrated the equivalent ability of REP-2 to facilitate the prenylation of some Rabs such as Rab 1A, Rab5A and Rab6, but decreased ability towards other Rabs such as Rab3A (Cremers, Armstrong et al. 1994). As CHM is a retinal disease, this suggests that REP-2 is not able to compensate for the loss of REP-1 in the retina. It is currently unclear to what extent REP-2 is expressed in the retina. This is likely due to the difference in affinities of each REP protein for specific Rabs, as Rab27 is prenylated less efficiently by REP-2 than REP-1 (a fourfold difference) (Rak, Pylypenko et al. 2004). In the presence of both REP proteins, a hierarchy of Rab prenylation exists as Rabs compete for binding to the prenylation complex (Kohnke, Delon et al. 2013). Rab Escort Protein 2 (REP-2) compensates for the loss of REP-1 in most tissues except the retina (MacDonald, Sereda et al. 2004). Consequently, various Rab GTPases are underprenylated in the retina in CHM (Seabra, Ho et al. 1995).

1.5.4 Rab GTPase Prenylation in Choroideremia

Of particular interest to CHM is Rab27. It came to attention when Dr Miguel Seabra demonstrated that it is preferentially prenylated with the assistance of REP-1 rather than REP-2 in lymphoblasts from choroideremia patients (Seabra, Ho et al. 1995). While it is possible for Rab27 to be prenylated by REP-2, it is likely outcompeted by other Rabs with stronger affinities. In addition, it was found that Rab27 is predominantly membrane bound in normal lymphoblasts but largely cytosolic in CHM lymphoblasts (Kohnke, Delon et al. 2013). It is indeed highly expressed in the RPE and choroid, two important sites of degeneration in CHM. Within the RPE, Rab27a controls the distribution of melanosomes. It, along with its effector myosin VIIa, facilitate the trafficking of melanosomes to the apical processes of the RPE that surround the photoreceptor OS following light exposure. In the absence of Rab27a or myosin VIIa, melanosomes remain in the perinuclear region (Futter, Ramalho et al. 2004). However, some evidence suggests Rab27 hypoprenylation does not cause retinal degeneration. Griscelli syndrome is caused by mutations in the *RAB27* gene (Corbeel and Freson 2008). It is a disease characterized by albinism and uncontrolled T-lymphocyte and macrophage activation (Griscelli, Durandy et al. 1978). In Griscelli syndrome patients, no ocular phenotype is observed.

It is likely that retinal degeneration in CHM is a result of underprenylation of multiple Rab proteins, resulting in a defect in phagocytosis and degradation of OS by the RPE. While retinal tissue from CHM patients is rarely donated and vary in the extent of degeneration, fibroblasts and blood monocytes offer some insight into the disease. Compared to control participants, monocytes from CHM patients have significantly increased lysosomal pH and slower proteolytic degradation. Their dermal fibroblasts

also displayed changes in cytokine and growth factor secretion, with decreases in tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8) (Strunnikova, Barb et al. 2009). There is some evidence that the extent of these effects correlates with their clinical severity of disease (Strunnikova, Zein et al. 2012), but broader studies would further such an understanding of disease progression.

To study the CHM retina, cell and mouse models have been particularly helpful. The phagocytic and degradation pathways in the RPE are likely impaired given the accumulation of photoreceptor OS and debris at the level of the RPE (Gordiyenko, Fariss et al. 2010). There is little literature on CHM models derived from RPE cell lines, but the reprogramming of CHM human dermal fibroblasts into induced pluripotent stem cells (iPSCs), then iPSCs into RPE, was successful (Cereso, Pequignot et al. 2014). While REP-1 was absent, some REP-2 was present which may or may not reflect the levels of REP-2 in the RPE *in vivo*. As expected, Rab27A prenylation decreased and less was membrane-bound. In addition, it was seen that the overall amount of Rabs was increased. In a CHM siRNA knock-down model in human fetal RPE (hfRPE), OS degradation was altered (Gordiyenko, Fariss et al. 2010). Internalization of OS seemed normal but the extent of degradation was decreased in CHM hfRPE. This was attributed to reduced acidification of phagolysosomes, possibly due to impaired fusion with late endosomes and lysosomes because of decreased Rab7A and lysosomal-associated membrane protein 1 (LAMP-1).

Similar phenomena are found in mouse models of CHM. In an RPE-only knockout model of REP-1 in mice, a decrease in melanosomes was seen in the apical processes of RPE which was attributed to decreased Rab27A prenylation (Wavre-

Shapton, Tolmachova et al. 2013). There was, as well, a delay in phagosome processing. It is not known whether this delay was due to phagosome processing maturation, delivery to the lysosome, or lysosomal acidification. It was attributed to the premature signs of aging seen in this mouse model, as lipofuscin and lipid accumulated in vacuoles in the RPE. As well, there was accumulation of extracellular basal deposits, whether by increased secretion of extracellular matrix (ECM) by the RPE or reduced elimination by the choroid. While most studies of CHM pathogenesis have focused on decreased prenylation of Rab27A, it is evident that OS phagocytosis involves other suspect Rab GTPases, such as Rab 7 and Rab5.

1.6 Hypothesis and Objectives

Rationale: Several proteins that participate in retinal function are prenylated and therefore susceptible to be affected by statins. There is initial evidence suggesting that statins used to treat hypercholesterolemia may accelerate the retinal degeneration in CHM (Zhou, Weis et al. 2013). The exact role of statins in the progression of CHM is currently unknown, but they could further impair prenylation in CHM patients who have a pre-existing defect in the geranylgeranylation of Rab proteins. Prenylated Rabs are essential for phagocytosis, a cellular process significantly affected in the RPE of CHM patients.

Thus, we hypothesize that statins decrease the degradation of photoreceptor OS by the RPE due to their inhibition of protein prenylation.

Here, we present evidence to demonstrate that:

1. Statins inhibit protein prenylation by decreasing isoprenoid lipids in retina-derived cells;
2. Inhibiting protein prenylation decreases photoreceptor outer segment degradation;
3. In the context of CHM, the defective prenylation machinery leads to an increased sensitivity to inhibition of protein prenylation by statins.

CHAPTER 2

METHODOLOGY

2.1 Reagents and Antibodies

Simvastatin was purchased from Calbiochem (567011). GGPP was purchased from Sigma-Aldrich (G6025). The same lot number was used throughout these studies. Immobilon polyvinylidene difluoride (PVDF) was from Bio-Rad. Chemiluminescence reagents were from Bio- Rad (170-5060). GGPP was purchased from Sigma-Aldrich (G6025). DMEM (11995-065), DMEM/F12 (11330-057), Opti-MEM (31958-070), Non-Essential Amino Acids (NEAA, 11140-050), penicillin/streptomycin (penstrep, 15140-122), fetal bovine serum (FBS, 12483-020), and trypsin-EDTA (25200-072) were all purchased from Gibco. Primary antibodies used were β-Actin (1:2000, Cell Signaling Technology, 4967S), β-tubulin (1:1000, Sigma-Aldrich, T4026), COX IV (1:2000, Abcam, ab62164), farnesyl (1:2000, Millipore Sigma, AB4073), GAPDH (1:5000, Abcam, mAbcam 9484), LC3 (1:2000, Novus Biologicals, NB100-2220), Rab7 (1:2000, Sigma, R8779), and rhodopsin (1:2000, Novus Biologicals, NBP2-25159).

2.2 Cell Culture

2.2.1 Culture of Immortalized Retinal Pigmented Epithelium Cells ARPE-19

Spontaneously-immortalized human retinal pigmented epithelium cells were maintained in culture at 37C and 5% $CO_{2(g)}$. The growth medium contained DMEM with Ham's F12 supplement, with the addition of 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were plated in 6 well plates at 0.2 x 10^6 cells/well and cultured for 18h before starting indicated treatments. To generate polarized cells, cells were plated at confluence, at a density of 4 x 10^4 cells/well on 96 well plates, according

to (Mao and Finnemann 2013). Growth medium was changed every 3 days for 14 days before each experiment.

2.2.2 Culture of Primary Human Dermal Fibroblasts from CHM Patients and Age-Matched Control Participants

The fibroblasts were a gift from the lab of Dr Ian MacDonald. Briefly, fibroblasts were isolated from skin biopsies obtained with written informed consent from patient and control participants. The procedure was approved by the Health Research Ethics Board of the University of Alberta and observed the guidelines of the Declaration of Helsinki and ARVO guidelines about research involving human subjects. The growth medium consisted of DMEM with 10% FBS, 1% penicillin/streptomycin and 1% non-essential amino acids. Fibroblasts were cultured at 37C, 5% CO_2. Fresh medium was given to cultures every 3 days.

2.3 Protein Quantification

The protein concentration of harvested cells was determined by the bicinchoninic acid (BCA) assay method according to the manufacturer instructions (Pierce, 23228).

2.4 Immunoblot Analysis

Proteins were separated by molecular weight using 16% poly-acrylamide, 0.1% SDS-PAGE gels. Proteins were transferred from the gel to PVDF membranes at 4°C in 25 mM Tris, 192 mM glycine, and 16% methanol buffer. Experiments blotting for COX IV were transferred at 4°C in 500 mM CAPS and 10% methanol, pH 11 buffer. Following

the transfer, membranes were blocked in 5% non-fat milk tris-buffered saline with 0.1% Tween 20 (T-TBS). Membranes were rinsed with two 5 minute washes of TBS, two 5 minute washes of T-TBS, then two 5 minute washes of TBS. Membranes were incubated with secondary antibodies (1:2000) for 1 hr at room temperature, then rinsed again. The membranes were imaged by incubating with Clarity Western ECL Substrate (Bio-Rad, 170-5060) and scanned using the Licor C-Digit Blot Scanner. Semiquantification of band density was done using Licor C-Digit Image Studio software.

2.5 Determination of Protein Prenylation

Two methods were used to assess the extent of protein prenylation: (1) capture with Rab GDI and (2) extraction with Triton X-114 detergent.

2.5.1 GDI-Capture of Rabs

Rab GDI capture was performed as published in (Narita, Choudhury et al. 2005, Mohamed, Saavedra et al. 2012). Briefly, cells were harvested in GDI-capture buffer containing 30 mM HEPES, 75 mM potassium acetate, 5 mM $MgCl_2$ and cOmplete™ EDTA-free protease-inhibitor cocktail (Roche, 04693159001). Cells were sonicated on ice, and centrifuged at 5000 x g for 10 seconds to pellet cellular debris. 'Input' samples prepared using 10 ug of lysate protein, and 'extract' samples were prepared using 30 ug of lysate protein. The extracts were diluted fivefold with reaction mix (75 mM potassium acetate, 30 mM HEPES, 5 mM MgCl2, 100 µM ATP, 500 µM GDP) containing 100 µM GDI-GST, and then incubated at 30°C for 20 min. Prenylated Rab proteins bound to GDI-GST were pulled down with Glutathione-sepharose 4B beads (GE Healthcare, 17-

0756). The beads were eluted by boiling with SDS-sample buffer for 5 minutes, followed by analysis with 16% SDS-PAGE and immunoblot as described previously.

2.5.2 Triton X-114 Extraction

Triton X-114 partition was performed as previously published in (Coxon, Ebetino et al. 2005, Mohamed, Saavedra et al. 2012). Triton X-114 is a non-ionic detergent that forms large micelles above its cloud point temperature (~20°C), becoming turbid and separating into two phases in aqueous solutions. Lipophilic, prenylated Rab proteins are in the detergent-rich phase, while the unprenylated Rab proteins remain in the aqueous phase. Cells were harvested after the corresponding treatments in Tris buffer containing Triton X-114 (20 mM Tris-HCl, pH 7.5, 150 mM Triton X-114, 1% Triton X-114 and protease inhibitor cocktail). After incubating on ice for 15 minutes, lysates were cleared by centrifugation at 13 000 x g for 15 minutes at 4°C. Protein content was determined in the clear lysates. Equal amounts of protein were adjusted to 40 uL with the Tris buffer containing Triton X-114, then loaded on top of a cushion solution (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 6% sucrose, 0.06% Triton X-114). Samples were then centrifuged at 16 000 x g for 5 min, and the top aqueous clear layer was separated from the translucent, detergent-rich droplet. Both layers were boiled with SDS-sample buffer, and proteins were separated by SDS-PAGE. Prenylated Rab proteins run faster than unprenylated Rabs under SDS-PAGE conditions; the detergent phase has a lower band than that in the aqueous phase.

2.6 Preparation of Photoreceptor Outer Segments

2.6.1 Isolation of OS

OS were isolated from porcine and/or bovine eyes as published by (Mao and Finnemann 2013). The retinas were dissected from the eyes under dim red light into homogenization solution (20% w/v sucrose, 20 mM Tris-Ac pH 7.2, 2 mM $MgCl_2$, 10 mM glucose, 5mM taurine). The retina suspension was vigorously shaken for 2 minutes, and filtered 3 times through 1 layer gauze to remove large tissue fragments. The filtered solution was ultracentrifuged on a 25-60% sucrose gradient, with 20 mM Tris-Ac pH 7.2, 10 mM glucose, 5 mM taurine, at 114 000 x g for 48 minutes at 4°C. The single pink band in the upper third of the gradient contains the purified OS. This band was isolated and diluted 5 volumes of 20 mM Tris-Ac pH 7.2, 5 mM taurine, then centrifuged at 300 x g for 10 minutes at 4°C. The OS were then washed twice with 10% sucrose, 20 mM Tris-Acetate pH 7.2, 5 mM taurine. Lastly, the OS were washed twice with 10% sucrose, 20 mM phosphate buffer pH 7.2, 5 mM taurine. OS were counted on a hemocytometer, and store at 10 x 10^6 OS/mL at -80°C in DMEM with 2.5% sucrose until usage.

2.7 Photoreceptor Phagocytosis Assay

2.7.1 Detecting Internalized Outer Segments

This assay is derived from Mao & Finneman (2013). OS aliquots from -80°C were thawed and centrifuged at 2 400 x g for 5 minutes at room temperature. OS were resuspended in serum free DMEM with or without pharmacological agents. To detect only internalized OS, cells were washed with PBS containing 2 mM EDTA for 10 minutes to remove bound OS, and rinsed 3 times with PBS with 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$. Following the OS challenge, cells received fresh DMEM/F12 with 10% BGS

and 1% penicillin/streptomycin, with or without pharmacological agents. Cells were harvested in HNTG buffer with protease inhibitor cocktail (50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1% Triton X-100) and lysed. Protein content was quantified and samples with equal amounts of protein were prepared. SDS-sample buffer was added without boiling to prevent rhodopsin aggregation, and samples were run on SDS-PAGE and immunoblotting was performed.

CHAPTER 3

RESULTS

3.1 Statins inhibit Rab Prenylation in ARPE-19 Cells

Previous work from our laboratory has demonstrated that some, but not all statins inhibit protein prenylation in cortical neurons and neuron-like N2a cells (Mohamed, Saavedra et al. 2012). Our goal was to investigate the effect of statins on retinal cells, in particular RPE. Over the past 20 years, numerous RPE cells lines have been generated and used successfully as models of RPE (Mazzoni, Safa et al. 2014). Here we use ARPE-19 cells derived spontaneously from RPE of a 19-year-old human donor with no known eye disease (Dunn, Aotaki-Keen et al. 1996). ARPE-19 cells readily proliferate to confluence and they exhibit contact inhibition (Dunn, Aotaki-Keen et al. 1996). They may develop morphological and some functional polarity after at least 2 weeks in culture (Mao and Finnemann 2013). ARPE-19 cells retain many of the characteristics of RPE, including cell morphology (Dunn, Aotaki-Keen et al. 1996), functional tight junctions (Dunn, Aotaki-Keen et al. 1996) and the ability to phagocytose isolated OS prepared from cow or pig eyes (Finnemann, Bonilha et al. 1997). There is evidence that ARPE-19 cells use the MFG-E8-α v β 5-integrin-FAK mechanism to recognize and bind OS (Finnemann, Bonilha et al. 1997, Chowers, Kim et al. 2004, Qin and Rodrigues 2012, Olchawa, Herrnreiter et al. 2013). They engulf bound OS but whether ARPE-19 cells express and use the engulfment receptor MerTK is unclear (Carr, Vugler et al. 2009, Mazzoni, Safa et al. 2014).

The RPE *in vivo* is a polarized monolayer of pigmented cells with an apical side that is closely associated with the photoreceptor and contains receptors for OS phagocytosis. To mimic this phenotype *in vitro*, post-confluent ARPE-19 cells were cultured for two weeks to establish polarity (**Figure 10A**). Exposure of proliferative as

well as polarized ARPE-19 to different concentrations of simvastatin resulted in significant inhibition of Rab7 prenylation in a dose-dependent manner (**Figure 10, B-D**).

We hypothesized that statins reduce protein prenylation by decreasing the pool of isoprenoid moieties due to their inhibitory effect on the mevalonate pathway. Thus, if the pool of GGPP is maintained by the addition of exogenous GGPP, protein prenylation should be protected. The treatment of polarized ARPE19 cells with simvastatin and GGPP concurrently prevented the inhibition of Rab7 by simvastatin (**Figure 11**).

As stated before, the efficiency of statins to regulate the mevalonate pathway is very diverse and depends on, among several factors, their hydrophobicity. Thus, we assessed the effects of pravastatin, a much more hydrophilic statin as compared to simvastatin (Jacobson 2004), on Rab prenylation. At all of the concentrations tested, pravastatin failed to inhibit Rab7 prenylation in cultured ARPE-19 cells (**Figure 12**). Further experimentation is needed to determine whether this is because pravastatin has not entered these cells or if pravastatin has entered cells but is not able to inhibit protein prenylation as the affinity for the non-sterol isoprenoid branch of the mevalonate pathway for FPP is much higher than that of the cholesterol pathway.

All together these experiments indicate that hydrophobic statins such as simvastatin but not the more hydrophilic pravastatin inhibit Rab7 prenylation in ARPE-19 cells by a mechanism that depends on GGPP shortage.

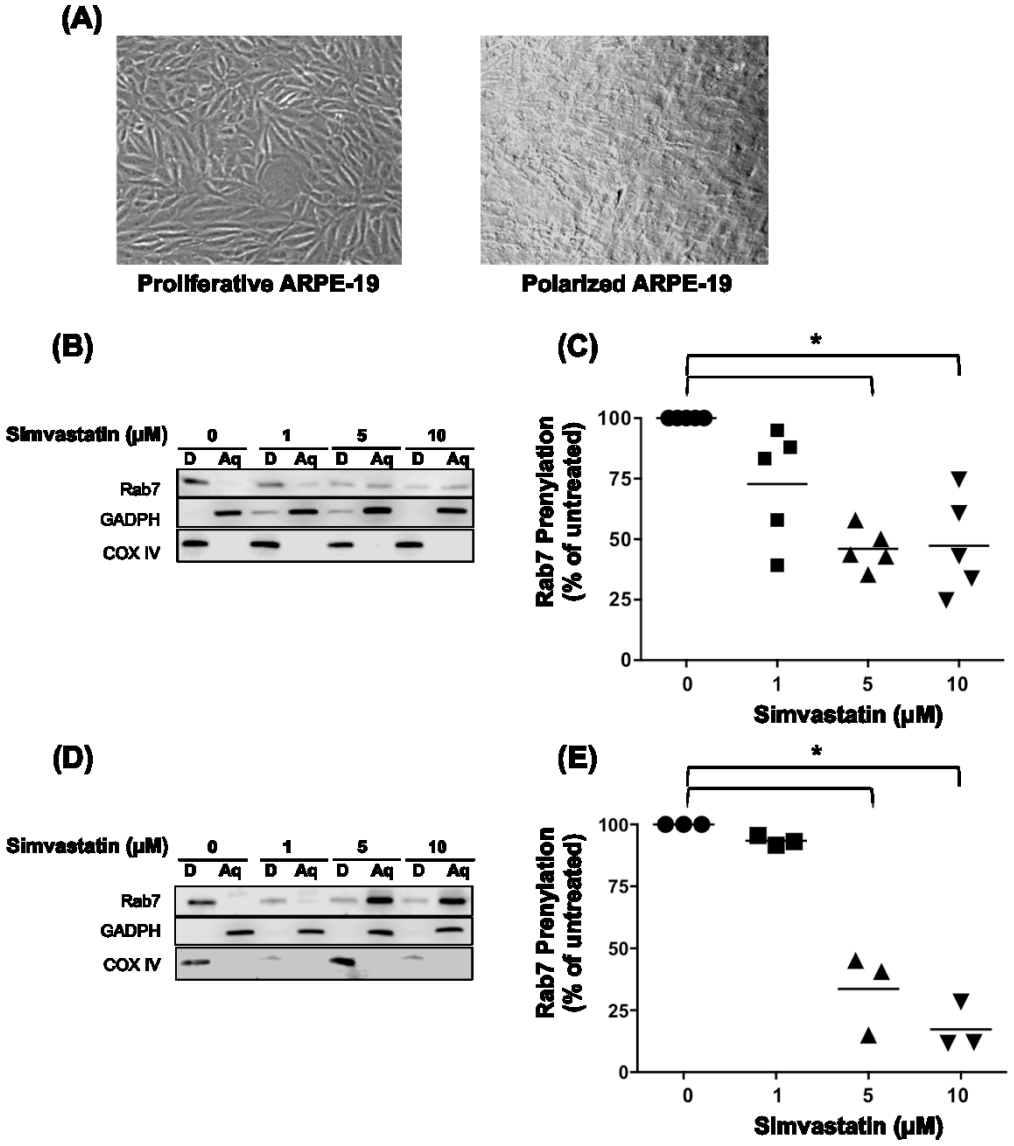

Figure 10: Simvastatin Inhibits Rab7 Prenylation in ARPE-19 Cells. (A) Phase contrast images of ARPE-19 cells cultured for 18h (proliferative ARPE- 19 cells) or 2 weeks (polarized ARPE-19 cells). Proliferative ARPE-19 cells **(B, C)** or polarized ARPE19 cells **(D, E)** were treated with various concentrations of simvastatin for 24h. **(B, D)** Analysis of Rab prenylation was performed by extraction with Triton X-114: ARPE-19 lysates (equal amount of protein) were extracted with Triton X-114. Proteins in detergent (D, prenylated proteins) and aqueous (Aq, unprenylated proteins) phases were analyzed by SDS-PAGE and immunoblotting. Proper phase separation was confirmed by immunoblotting of marker proteins GAPDH for the aqueous phase and COX IV for the detergent phase. **(C, E)** Data quantification was performed by calculating ratios between total pixels of Rab7 in detergent/total pixels of Rab 7 in detergent plus aqueous, and referred to the ratio in untreated ARPE-19 cells. Data from individual experiments and the mean value (line) are depicted. * $p < 0.05$, one-way ANOVA.

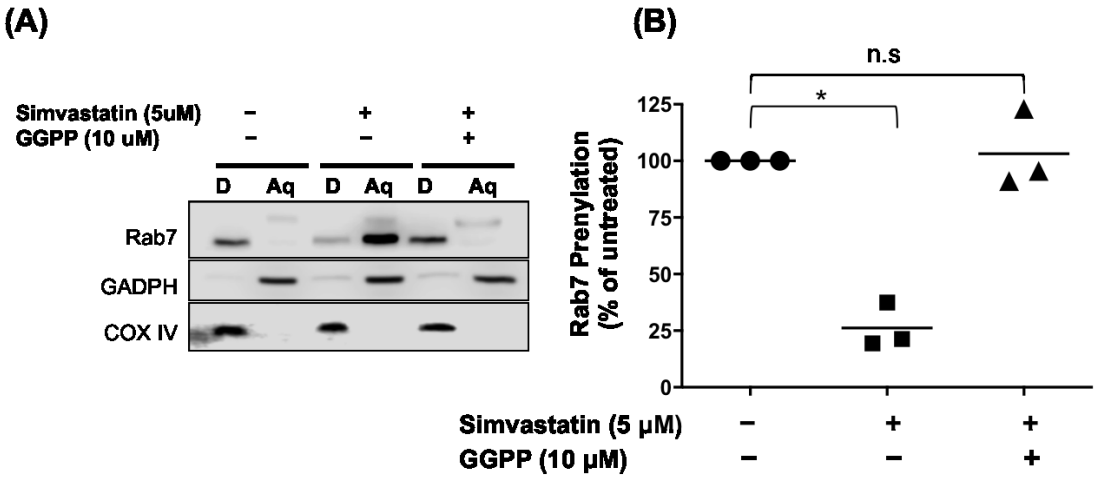

Figure 11: GGPP Prevents Inhibition of Rab7 Prenylation by Simvastatin. Proliferative ARPE-19 cells were treated with simvastatin with or without GGPP for 24h. **(A)** Analysis of Rab prenylation was performed by extraction with Triton X-114: ARPE-19 lysates (equal amount of protein) were extracted with Triton X-114. Proteins in detergent (D, prenylated proteins) and aqueous (Aq, unprenylated proteins) phases were analyzed by SDS-PAGE and immunoblotting. Proper phase separation was confirmed by immunoblotting of marker proteins GAPDH for the aqueous phase and COX IV for the detergent phase. **(B)** Data quantification was performed by calculating ratios between total pixels of Rab7 in detergent/total pixels of Rab 7 in detergent plus aqueous, and referred as percentage of prenylated Rab7 in untreated ARPE-19 cells. Data from individual experiments and the mean value (line) are depicted. * $p < 0.05$, one-way ANOVA.

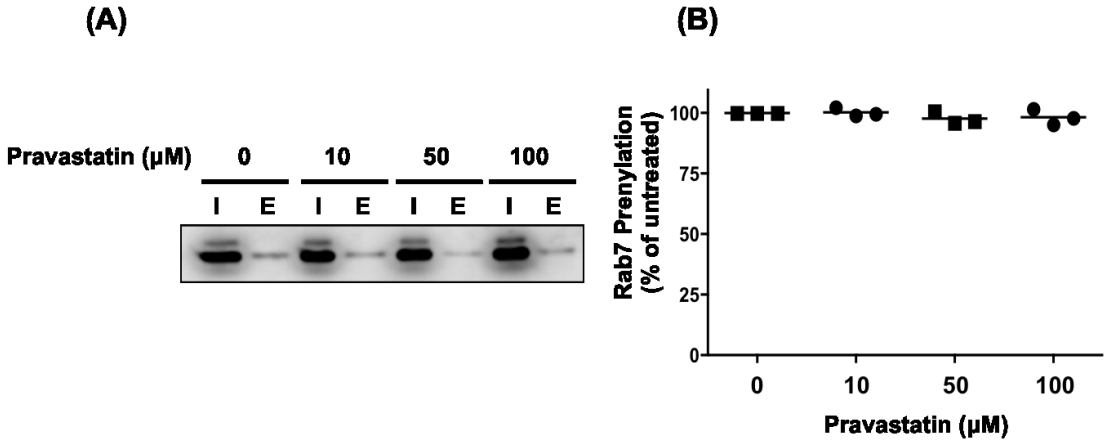

Figure 12: Pravastatin Does Not Affect Rab7 Prenylation. Proliferative ARPE-19 cells were treated with different concentrations of pravastatin for 24h. **(A)** Analysis of Rab prenylation was performed by GDI- capture analysis: Aliquots with equal amount of proteins corresponding to approximately 1/10 of the total protein content were used to assess total protein input (I). The rest of the cell lysates were used to extract prenylated proteins with low concentration of recombinant GST–GDI (E). Proteins were analyzed by immunoblot analysis. **(B)** Ratios between total pixels of Rab7 in GDI-bound (E)/total pixels of Rab7 in lysate before extraction (I) were calculated. Densitometric analysis of I included both bands. Data are presented as percentage control (untreated cells).

3.2 Impaired Protein Prenylation Inhibits Phagocytosis of Photoreceptor Outer Segments

Inhibiting prenylation in the retina has significant consequences. Several retinal diseases are linked to prenylation defects in proteins that participate in diverse retinal processes that take place at different layers of the retina (Roosing, Collin et al. 2014).

As mentioned above, one of the main functions of RPE is OS phagocytosis (Strauss 2005). There is evidence that different Rabs are recruited to maturing phagosomes and participate at multiple steps of the phagocytic pathway by regulating interactions with multiple components of the endocytic and other intracellular compartments (Vieira, Bucci et al. 2003, Gutierrez 2013, Yeo, Wall et al. 2016). Yet, the function of the majority of phagosomal Rabs, the specific organelle interactions that they regulate and the functional significance of those interactions remain poorly characterised (Wavre-Shapton, Meschede et al. 2014). Moreover, to our knowledge the importance of protein prenylation for proper function of Rabs in phagocytosis and the effect of statins has not been directly studied. Yet, previous indirect evidence suggested its potential relevance. For example, in cultured cells and in mice, silencing of the CHM gene, which results in lack of REP-1 protein and would reduce Rab prenylation, causes a significant reduction of OS degradation (Gordiyenko, Fariss et al. 2010, Wavre-Shapton, Tolmachova et al. 2013).

Here we investigate if simvastatin-induced inhibition of Rab prenylation affects OS phagocytosis by ARPE-19 cells. As a measure of OS phagocytosis, we examine the levels of rhodopsin protein. Rhodopsin has been identified as an ideal phagosome marker because it is by far the most abundant protein in rod outer segments; there are a

number of well characterized monoclonal antibodies specific to rhodopsin that are commercially available; and RPE cells and ARPE-19 cells do not express rhodopsin (Mazzoni, Safa et al. 2014).

We first determined the experimental conditions to treat polarized ARPE-19 cells with OS. We tested two procedures to eliminate the OS that remain attached to the cell surface so that we only evaluate internalized OS. EDTA has been used extensively to eliminate the OS attached to the surface of RPE or ARPE cells (Mao and Finnemann 2013). In other studies, a mild treatment with trypsin successfully eliminated material externally attached to cells (Baumann and Doyle 1979, Rothe, Liguori et al. 2010). We tested EDTA and trypsin in ARPE-19 cells pulsed with OS for a very short time to avoid significant internalization. EDTA but not trypsin was able to eliminate the surface OS (**Figure 13 A**). We next determined the minimum time of OS pulse required to achieve sufficient OS internalization, before intracellular OS degradation is significant. Based on the results shown in **Figure 13 B**, we selected 30 min for further experiments of POS degradation.

Next, we investigated the effect of simvastatin and pravastatin on POS degradation. Simvastatin, but not pravastatin caused a delay in POS degradation (**Figure 14**). This result suggested that inhibition of prenylation may account for the different behaviour of the statins. However, GGPP was unable to prevent the effect of simvastatin on OS degradation.

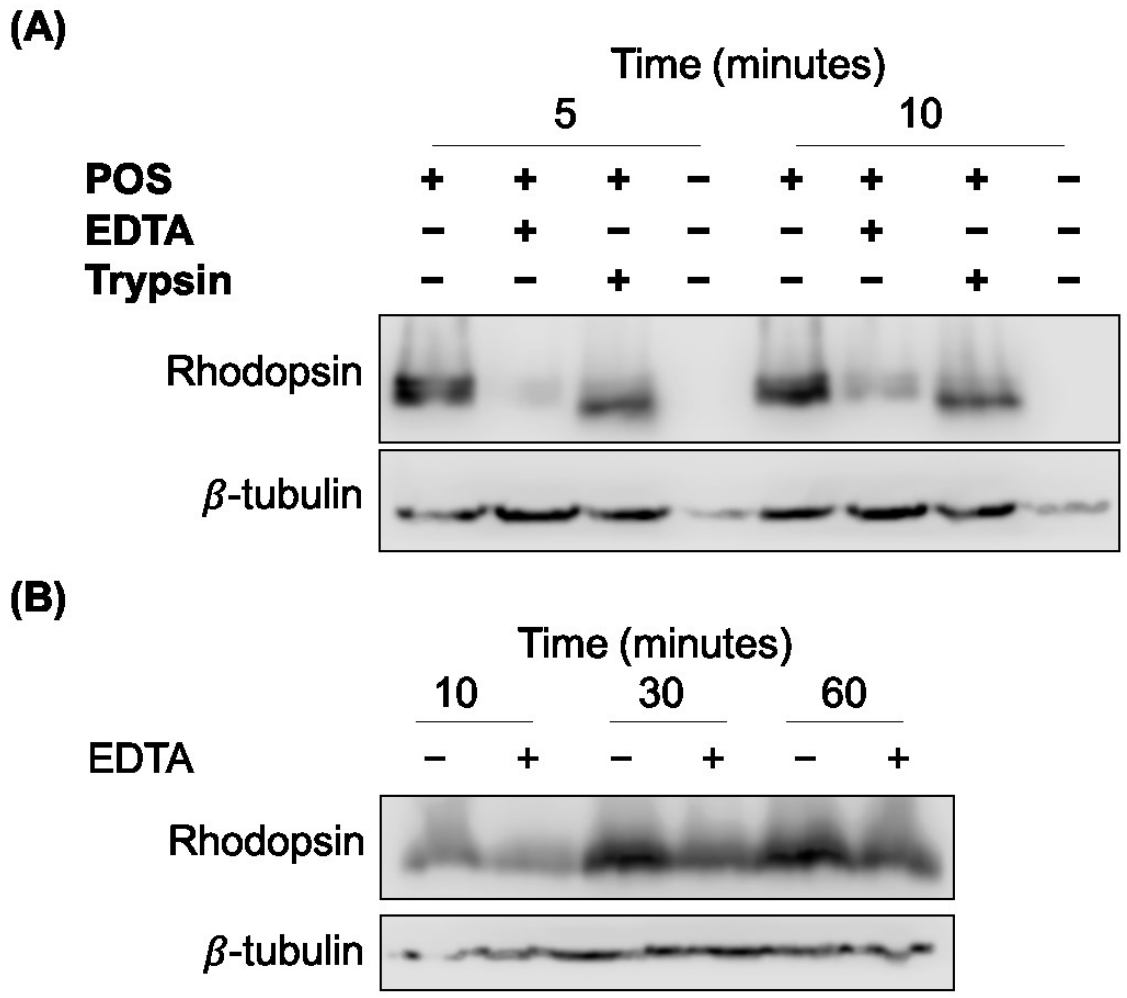

Figure 13: Determining Experimental Conditions for OS Degradation. (A) Polarized ARPE-19 cells were treated with OS (10 segments/cell) for 5 or 10 min followed by treatment with EDTA or trypsin as indicated under Materials and Methods. Rhodopsin was examined by immunoblotting. **(B)** Polarized ARPE-19 cells were treated with OS (10 segments/cell) for different times followed by treatment with EDTA. Rhodopsin was examined by immunoblotting.

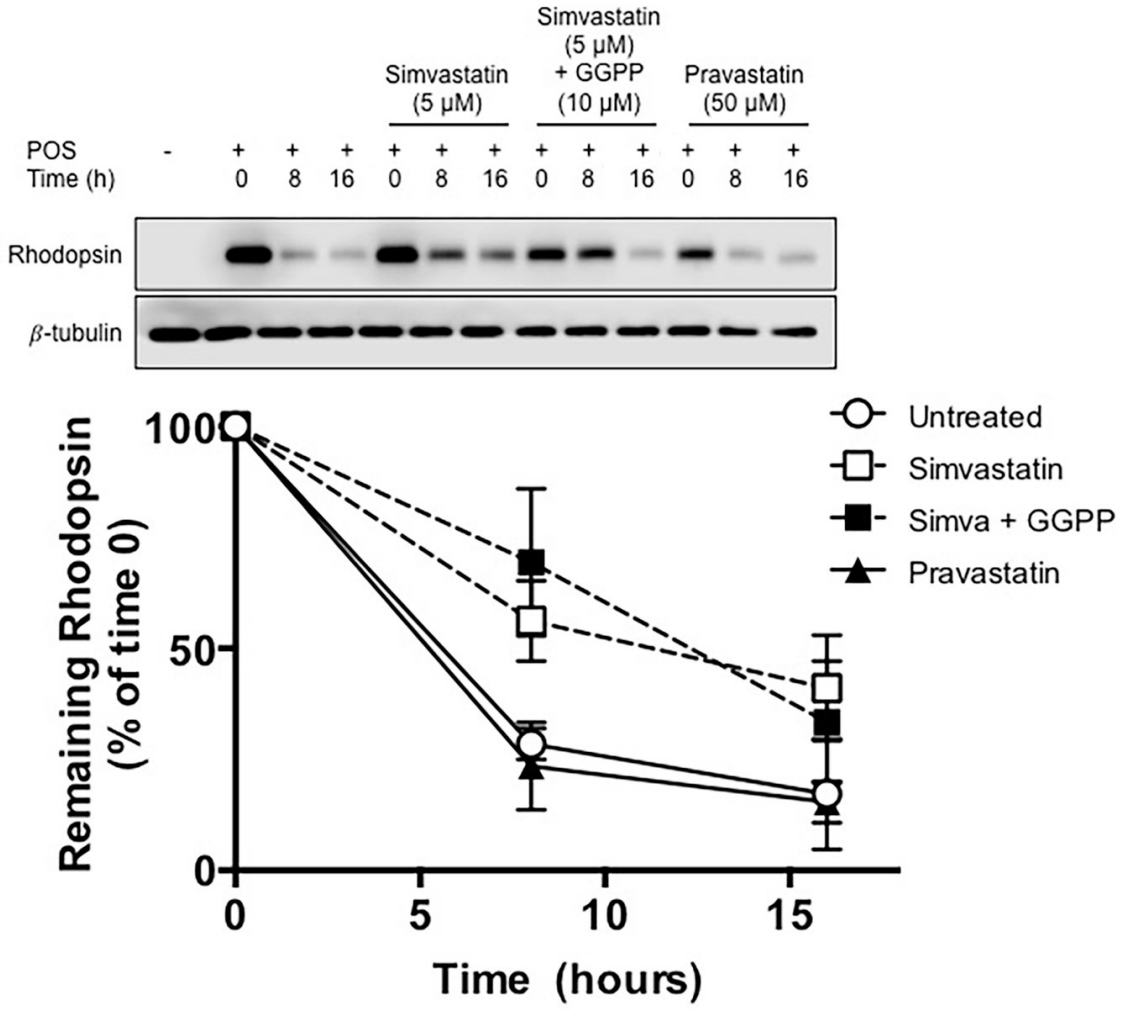

Figure 14: Simvastatin But Not Pravastatin Reduces OS Degradation. Polarized ARPE-19 cells were treated with simvastatin or pravastatin with or without GGPP for 12 h, followed by a 30min pulse with OS (10 segments/cell). Degradation of rhodopsin was examined at intervals up to 18h. Pharmacological treatments were present during degradation. **(A)** Immunoblot analysis of rhodopsin. **(B)** Densitometric rhodopsin levels have been referred to β-tubulin and then referred to the ratio rhodopsin/β- tubulin at time 0 for the corresponding treatment. Data have been analyzed by ordinary one way ANOVA. * $p<0.05$

Recent studies have demonstrated a role for non-canonical autophagy in OS degradation (Kim, Zhao et al. 2013, Ferguson and Green 2014). The evidence indicates that the morning burst of RPE phagocytosis coincides with the conversion of autophagy protein LC3 (LC3I) to its lipidated form (LC3II) (Kim, Zhao et al. 2013). LC3II is recruited to single-membrane phagosomes containing engulfed OS. This type of phagocytosis has been designated LC3- associated phagocytosis or LAP (Kim, Zhao et al. 2013,

Ferguson and Green 2014, Munz 2015). Our laboratory has shown that in neurons, inhibition of protein prenylation by simvastatin or by inhibitors of GGTase II results in an increase of LC3II causing deregulation of canonical autophagy (Smith and Posse de Chaves, unpublished). Thus, we examined if simvastatin alters LC3 in ARPE-19 cells. We found that the levels of LC3II are increased in simvastatin-treated ARPE-19 cells and that this effect is prevented by GGPP, therefore it is mediated likely by inhibition of protein prenylation (**Figure 15 A**). We next investigated the behaviour of LC3 in ARPE-19 cells challenged with OS in the absence or presence of statins (**Figure 15 B, C**). The addition of OS caused an initial significant increase in LC3II, which return to normal values with time. This suggests that LAP was activated. Simvastatin did not affect the elevation of LC3II caused by OS but prevented the decrease of LC3II over time, suggesting that phagocytosis flux is impaired. GGPP counteracted the effect of simvastatin, which indicates that the effect might be mediated by reduction of prenylation.

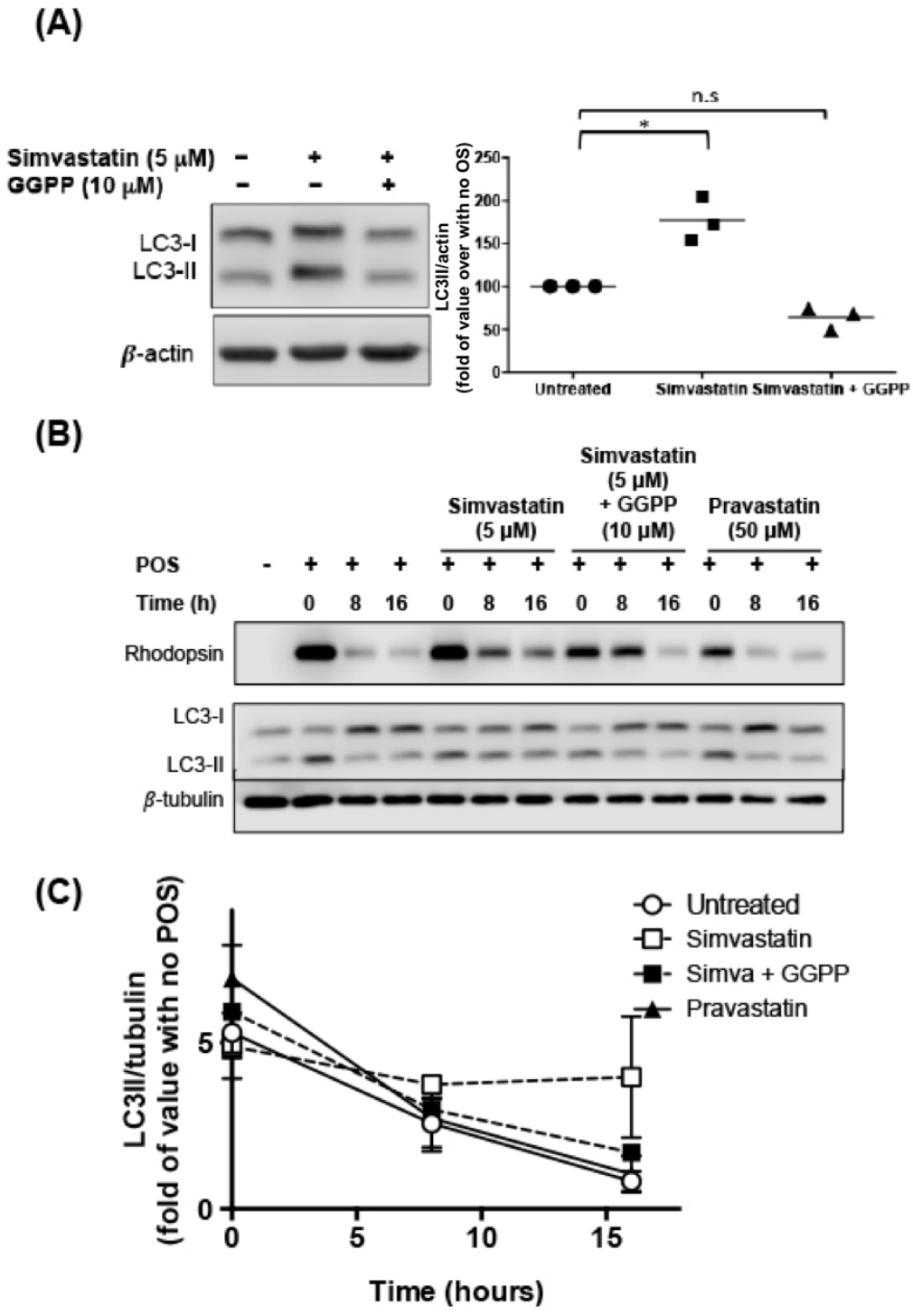

Figure 15: Simvastatin Affects LC3-mediated Mechanisms. Polarized ARPE-19 cells were treated with simvastatin and pravastatin with or without GGPP for 12 h, followed by a 30min pulse with OS (10 segments/cell). Degradation of rhodopsin was examined at intervals up to 18h. Pharmacological treatments were present during degradation. **(A)** Immunoblot analysis of rhodopsin. **(B)** Densitometric rhodopsin levels have been referred to β-tubulin and then referred to the ratio rhodopsin/β- tubulin at time 0 for the corresponding treatment. Data have been analyzed by ordinary one way ANOVA. * $p<0.05$.

3.3 Protein prenylation in fibroblasts from choroideremia patients is more sensitive to statins

Recent work suggests CHM patients experience accelerated vision deterioration when taking statins (Zhou, Weis et al. 2013). We hypothesize that CHM patients are more sensitive to statins that limit the generation of isoprenoid moieties.

To test this hypothesis, we use dermal fibroblasts from CHM patients. These fibroblasts present several advantages since although they have the same genetic mutation of CHM RPE, they are readily available in comparison with retinal tissue, and they divide when cultured. Human dermal fibroblasts from CHM patients do not have REP-1, but do express REP-2; fibroblasts from healthy participants, which are used as controls express both REP-1 and REP-2 (Strunnikova, Barb et al. 2009). Importantly, the severity of trafficking defects in dermal fibroblasts correlate with the extent of retinopathy in CHM, validating their use as a model (Strunnikova, Zein et al. 2012). Without REP-1, all prenylation reactions with Rab GGTase must be catalyzed by REP-2.

We examined the effect of simvastatin on Rab7 prenylation in two pairs of fibroblasts derived from age-matched CHM patients and healthy individuals. At each dose of simvastatin, protein prenylation was significantly more inhibited in CHM fibroblasts than in control fibroblasts **(Figure 16).** This was seen in both pairs of age-matched fibroblast pairs, suggesting this phenomenon may extend to all CHM patients. Our results suggest that REP-2 cannot adequately compensate for the loss of REP-1 in CHM when protein prenylation is inhibited. Prenylation inhibition may be particularly detrimental in CHM, but the examination of the role of other Rab GTPases is needed.

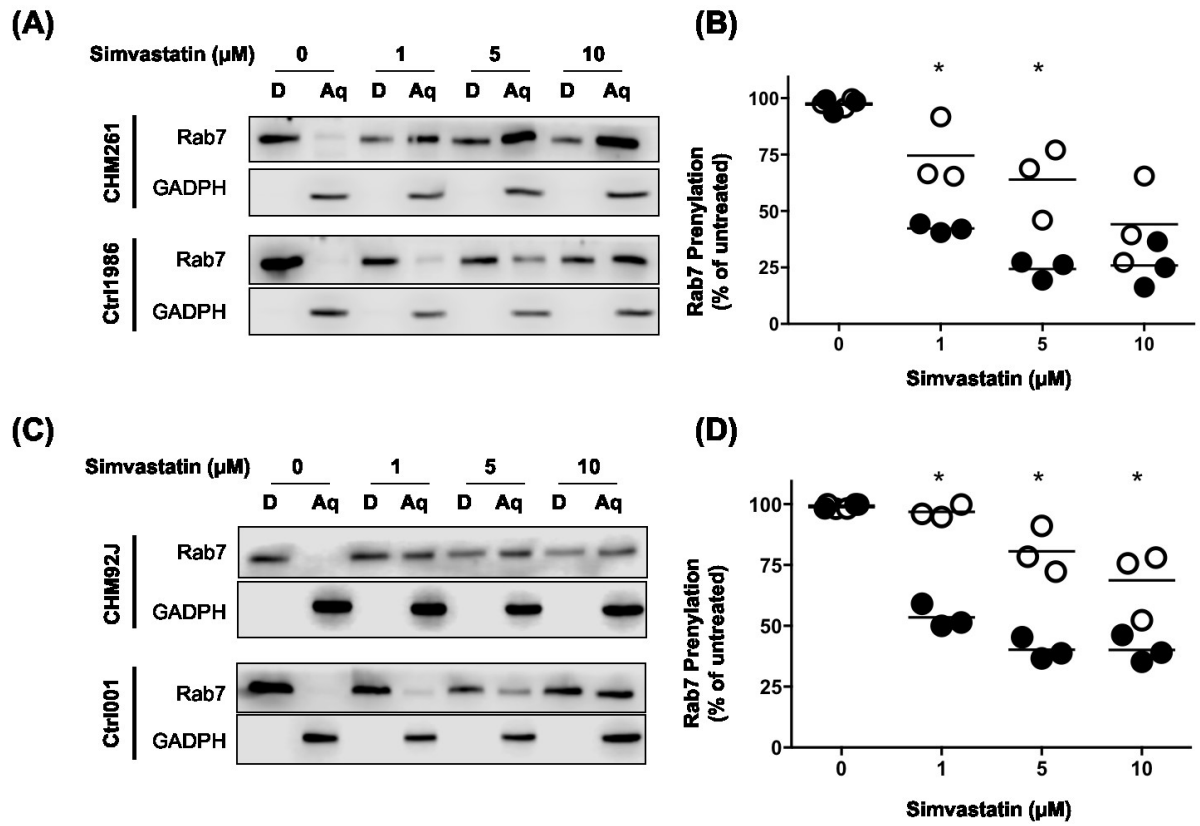

Figure 16: Fibroblasts from CHM Patients are More Sensitive to Statins. Two distinct pairs of age-matched human dermal fibroblasts were treated with various concentrations of simvastatin for 24h. **(A, C)** Triton X-114 extraction was performed to examine Rab prenylation; fibroblast lysates (equal amounts of protein) were extracted with Triton X-114. Proteins were separated into detergent (D) and aqueous (Aq) phases, and analyzed by SDS-PAGE and immunoblotting. Proper phase separation was confirmed by immunoblotting of GAPDH for the aqueous phase. **(B, D)** Data quantification was performed by calculating ratios between the total pixels of Rab7 in the detergent phase/total Rab7 (detergent plus aqueous), and referred to the ratio of the untreated condition. Data from individual experiments are depicted and the mean value (line) is shown. Closed circles represent CHM fibroblasts and open circles represent control fibroblasts. *p<0.05, one-way ANOVA.

CHAPTER 4

DISCUSSION

Numerous retinal diseases arise from defects in prenylated proteins, highlighting the potential importance of prenylation in the retina. Relevant prenylated proteins in the retina include the visual transduction proteins, such as transducin and PDE proteins, and the small monomeric GTPases, such as Rho and Rab GTPases. The direct implication of protein prenylation in the retina is understudied although some information can be obtained from work using statins. All statins inhibit the mevalonate pathway through the same mechanism, but they differ greatly in properties such as hydrophilicity, half-lives and cell mechanisms of uptake (Schachter 2005). Many beneficial effects for statins have been shown in retinal diseases from anti-inflammatory effects to induction of heat-shock proteins to prevent cell death (Honjo, Tanihara et al. 2002, Kretz, Schmeer et al. 2006). Conversely, many detrimental effects have been described as well. Intravitreal injection of lovastatin directly to the eye results in massive retinal degeneration beginning with the photoreceptors, while injections of sterol synthesis inhibitors did not have an effect. The retinal degeneration caused by lovastatin was attributed to the inhibition of protein prenylation (Pittler, Fliesler et al. 1995). Understanding the homeostasis of protein prenylation is important in preventing retinal degeneration.

4.1 Statins and Protein Prenylation in the Retinal Pigment Epithelium

Many inherited retinal diseases are due to a mutation in the genes of prenylated proteins. As a significant portion of the population will develop hyperlipidemia and needs to be treated with lipid-lowering statins (Force, Bibbins-Domingo et al. 2016), it is important to understand whether statins will accelerate the progression of these

inherited retinal diseases. Previous work in our laboratory has demonstrated that statins inhibit protein prenylation in primary cortical neurons; the studies presented here extend this hypothesis to the retinal cells.

For our experiments, we utilized the ARPE-19 cell line. This cell line arose spontaneously from human RPE and is widely used in vision research (Dunn, Aotaki-Keen et al. 1996). ARPE-19 cells are phenotypically similar to the RPE when they are maintained as confluent monolayers in culture, as they become polarized and acquire many RPE characteristics (Dunn, Marmorstein et al. 1998). Other RPE models have been used to study OS phagocytosis, including (1) primary cultures of animal RPE, (2) other RPE cells lines, such as the rat RPE cell line RPE-J, and (3) stem cell-derived RPE cells. These models differ in their time for OS phagocytosis and degradation, and vary in other aspects such as cell junction formation. Primary cultures of the RPE often do not divide extensively and dedifferentiate after isolation from the eye, but RPE cell lines take longer to phagocytose and degrade OS. Stem cell-derived RPE have potential as therapeutics for RPE atrophy, but they internalize OS at a very slow rate compared to immortalized cell lines and are heterogeneous as demonstrated by the finding that only half of the stem cell-derived RPE express phagocytic receptors for OS (Mazzoni, Safa et al. 2014).

We found that simvastatin, but not pravastatin inhibits protein prenylation in ARPE-19 cells. Simvastatin inhibits the mevalonate pathway by acting as a competitive inhibitor of HMGR, the rate-limiting enzyme, to decrease the prenylation substrate GGPP. Pravastatin has been shown to inhibit cholesterol synthesis but not protein prenylation in neurons (Mohamed, Saavedra et al. 2012). Similarly, pravastatin failed to

inhibit protein prenylation in ARPE- 19 cells even at concentrations an order of magnitude higher than simvastatin. However, in order to conclude that pravastatin is inefficient in regulating protein prenylation more experiments are required. First, we need to confirm the lack of effect of pravastatin on protein prenylation by including a positive control in the GDI-capture technique, such as cells treated with simvastatin. In parallel the results should be confirmed using other techniques to detect protein prenylation, such as Triton X-114 extraction.

In an *in vivo* context, not only is statins hydrophilicity a factor, but also the ability of each statin species to cross the blood-retinal barrier is under question. Experience from the blood-brain barrier shows that lipophilic statins, such as simvastatin, readily cross while hydrophilic statins require significantly higher concentrations to cross the blood-brain barrier and to reduce cholesterol synthesis to the same extent (Saheki, Terasaki et al. 1994, Sirtori 2014).

To affirm the finding that simvastatin inhibits Rab prenylationby causing a shortage of isoprenoids, the addition of GGPP was able to prevent prenylation inhibition by simvastatin. There is substantial evidence that GGPP is able to enter cultured cells and prevent some effects of mevalonate pathway inhibitors. GGPP is taken up from the medium and incorporated into cellular proteins, and is able to recover protein prenylation in animal models of mevalonate kinase deficiency (Kukar, Murphy et al. 2005, Marcuzzi, Pontillo et al. 2008, Marcuzzi, Crovella et al. 2011, Mohamed, Saavedra et al. 2012).

We have chosen to use Rab7 as a reporter of protein prenylation in our studies because of its role in phagosome maturation (Gutierrez, Munafo et al. 2004, Gutierrez

2013). Without prenylated Rab7, phagosomes should accumulate in the cytosol, unable to proceed with degradation. Studies in cells that expressed mutant dominant-negative GDP-bound inactive Rab7T22N, showed intracellular accumulation of intermediates of the endosome-lysosome pathway such as autophagosomes (Press, Feng et al. 1998). This is likely due to Rab7 mutant improper localization and inability to mediate fusion events. Knockdown of Rab7 with siRNA yielded similar results (Zhou, Tan et al. 2013).

Inhibition of Rab7 prenylation has detrimental downstream effects, since Rab 7 fulfills several important functions. First, prenylated Rab7 in the late phagosome is important for the recruitment of many characteristic proteins and lipids, such as LAMP1 and lysobisphosphatidic acid (Kobayashi, Startchev et al. 2001). Vacuolar-ATPases are inserted into the vesicle membrane and are important to the acidification of late phagosomes due to their proton pump action. To move the vesicle towards the lysosome-containing microtubule-organizing centre, Rab7 recruits RILP (Rab-interacting lysosomal protein) and FYCO (Fab1-YotB-Vac1p-EEA1 and coiled-coil domain containing protein) (Cantalupo, Alifano et al. 2001, Wang and Hong 2006, Johansson, Rocha et al. 2007, Zhang, Fishel Ben Kenan et al. 2013). These proteins recruit kinesin and dynein/dynactin motor complexes, respectively. The microtubule-organizing centre has lysosomes, and Rab7 mediates the fusion of the late phagosome and lysosome.

4.2 Impaired Protein Prenylation inhibits Outer Segment Degradation

We hypothesize the underprenylation of Rab proteins inhibits the degradation of photoreceptor OS. The role of Rab GTPases has been studied in phagocytosis of other materials, from bacteria to apoptotic bodies (Botelho and Grinstein 2011), but has not

been well characterized in OS phagocytosis. Some prenylation-associated retinal diseases, such as CHM, show defects in OS phagocytosis and degradation. The monocytes from CHM patients have an increased lysosomal pH due to a failure to acidify the lysosome and have decreased ability to degrade OS material (Strunnikova, Barb et al. 2009). Thus, it is important whether simvastatin compounds such inhibition of phagocytosis and degradation.

In our experiments we sought to mimic the short pulse of OS following by a long degradation period that occurs in the eye. Previous protocols and guidelines on the use of OS for studying phagocytosis have been published (Mao and Finnemann 2013, Mazzoni, Safa et al. 2014). We established PBS-EDTA as a good wash to remove externally bound OS and that 30 minutes is enough time to allow OS binding to ARPE-19 cells without greatly increasing internalization. Then, we turned our focus to OS degradation. Simvastatin, but not pravastatin inhibited OS degradation. GGPP was not able to fully prevent the inhibition of degradation. The lack of effect of GGPP on OS degradation suggests that the recovery of geranylgeranylation is not sufficient to normalize OS degradation. Alternatively, GGPP may not fully recover prenylation of all Rabs under the conditions tested. The concentration of GGPP may need to be adjusted to prevent inhibition of prenylation in a broader set of Rabs, based on the previously discovered hierarchy of Rab prenylation (Kohnke, Delon et al. 2013). This is similar to what was demonstrated in mice, in which REP-1 ablation in mice delayed phagosome processing and decreased the lysosomal degradative capacity of RPE by a mechanism that seems to be independent of reduced prenylation of Rab27a (Wavre-Shapton,

Tolmachova et al. 2013). Our experiments do not preclude that inhibition of prenylation of other proteins that are perhaps farnesylated may play a role.

Contrary to our discoveries, a recent study demonstrated that atorvastatin protects ARPE-19 from impairment of phagocytosis induced by cholesterol crystals and ox-LDL (Tian, Al-Moujahed et al. 2017). This represents a different disease model in which lipophilic statins seem to promote phagocytosis and decrease the pro-inflammatory response in the RPE. This study, however, has some experimental shortcomings. Specifically, the authors examined the uptake of polystyrene beads in proliferating ARPE-19 cells. The use of polystyrene beads to study phagocytosis has been previously criticized as polystyrene beads are internalized via a pathway distinct from OS phagocytosis. The lack of digestibility of these beads is also a concern as their accumulation in the phagosome/lysosome system may stress the cells and produce secondary effects unrelated to the phagocytic process (Edwards and Szamier 1977, Mazzoni, Safa et al. 2014). For our work, we chose to use polarized ARPE-19 cells challenged with isolated photoreceptors OS, a strategy that is recommended as it is more similar to phagocytosis in the retina (Mazzoni, Safa et al. 2014).

Recently, it has been shown that some OS undergo the LC3-associated phagocytosis, a noncanonical form of autophagy (Kim, Zhao et al. 2013, Frost, Lopes et al. 2015). LAP is characterized by the recruitment of LC3-II on the phagosomes. In other phagocytic cells, such as macrophages, this process efficiently clears phagocytosed materials (Martinez, Almendinger et al. 2011). We demonstrated that prenylation inhibition by simvastatin leads to the increase of LC3-II in our RPE model. When challenged with OS, there is an initial increase of LC3-II, followed by a decrease

over time, suggesting that LAP is activated and LAP flux is normal, allowing protein degradation. Simvastatin did not affect the initial increase in LC3-II, but did prevent its return to normal levels, suggesting prenylation inhibits LAP flux. The observation that GGPP was able to recover normal LC3-II degradation suggests that the effect of simvastatin could be due to protein prenylation inhibition.

The RPE serves many important functions in the retina, from clearing shed photoreceptor OS and recycling its contents, such as amino acids and all-*trans*-retinal, to mediating the transport of nutrients between the choroid and the retina. Impairment of degradative processes in the RPE have detrimental effects and may lead to retinal degeneration. Much work has been focused on the lipofuscin component A2E. A2E is suspected to be generated from incomplete digestion by the lysosome (Finnemann, Leung et al. 2002); it has been shown to inhibit the V-ATPase on lysosome and increase lysosomal pH (Liu, Lu et al. 2008), resulting in the inhibition of pH-dependent degradative enzymes in the lumen (Bergmann, Schutt et al. 2004). This work is corroborated by observations in chloroquine-induced retinal degeneration. Chloroquine accumulates in lysosomes, especially in its nonprotonated form, increasing lysosomal pH, and leads to the accumulation of undegraded materials (Mahon, Anderson et al. 2004).

4.3 Increased Sensitivity to Statins in Fibroblasts from CHM Patients

Among the retinal degenerative diseases characterized by defects in prenylated proteins, CHM is of particular interest because it is due to a defect in the prenylation machinery. The defective gene product, REP-1 is essential for the prenylation of Rab

GTPases. REP-1 has a homolog, REP-2, that has similar but evidently not identical functions in Rab prenylation, and cannot fully compensate for the loss of REP-1 (MacDonald, Hume et al. 1993). The leading hypothesis of the field is that some Rabs are more efficiently prenylated by REP-1 than REP-2. This has been shown by the decreased Rab27 prenylation in lymphoblasts from CHM patients (Seabra, Ho et al. 1995), though other Rabs have not been widely studied to our knowledge. However, peripheral tissues from CHM patients and healthy individuals do not fully recapitulate the disease model as peripheral tissues do not degenerate in CHM. Peripheral tissue does have some advantages, as retina tissue from CHM patient is difficult to come by and maintain *ex vivo.* Here, we used human dermal fibroblasts from CHM patients and age-matched controls.

Simvastatin inhibited protein prenylation in fibroblasts from CHM patients and healthy individuals. However, as compared to fibroblasts from healthy individuals, fibroblasts from CHM patients displayed significantly more inhibition of protein prenylation at the same dose of simvastatin. Since GGPP was able to prevent the inhibition of protein prenylation in ARPE-19, we speculate that this effect will also occur in the fibroblasts but experimentation is needed. A decrease in the available isoprenoid lipids secondary to statin treatment, compounded by the absence of REP-1, would lead to a more severe loss of prenylated Rab7. This suggests that not only are some Rabs hypoprenylated in the absence of REP-1, but also when prenylation is inhibited, REP-2 is not able to compensate to the full extent. Future work in the lab may investigate whether this phenomenon holds true for all Rab GTPases and to what extent, as the order of prenylation for Rab GTPases exist in a hierarchy (Kohnke, Delon et al. 2013).

Ongoing work is looking at the effect of simvastatin on retinal function and protein prenylation in a mouse model of CHM. Knockdown of REP-1 with siRNA in human fetal RPE resulted in decreased localization of Rab5A and Rab7A to the early/late phagosomes (Gordiyenko, Fariss et al. 2010), which is consistent with our findings. It is evident that geranylgeranylation is inhibited by statins, but it is unclear to what extent farnesylation is impacted by statins. Studies have suggested that both farnesylation and geranylgeranylation are impacted by the administration of mevalonate pathway inhibitors (Pittler, Fliesler et al. 1995).

4.4 Conclusion and Future Directions

Protein prenylation is a post-translational modification that is vital for the association of many proteins to cell membranes. It is evident that protein prenylation is important in the retina, but it is unclear in which cells and functions it is most vital. The implication of many prenylated-proteins in inherited retinal degenerations has highlighted the importance of prenylation in the retina. Recent work using statins as treatments for various diseases such as AMD and diabetic retinopathy has drawn inconclusive results (Gehlbach, Li et al. 2016), suggesting a further understanding of the effects of statins in the retina is needed.

There are many unanswered questions regarding the effects of statins in the retina, but the role of protein prenylation in the retina is receiving increasing attention due to the ongoing clinical trials that seek to remedy inherited retinal degenerations such as CHM. Further work is needed to understand the effect of statins on other prenylation modifications, such as farnesylation, especially in the retina. Treatment of primary RPE

cells with lovastatin decreased cell proliferation, changed cell phenotype and induced apoptosis, while treatment with farnesyl inhibitors only affected cell proliferation (Capeans, Pineiro et al. 2001). It is likely that farnesylation of more importance in cell division, while the morphological changes were attributed to actin depolymerisation; actin polymerization is controlled by prenylation Rac, Rho and CDC42 (Schmidt, Glomset et al. 1982, Ridley and Hall 1992). It is also unclear how LC3 plays a role in OS phagocytosis and degradation, why only some phagosomes recruit LC3 and not others (Kim, Zhao et al. 2013, Frost, Lopes et al. 2015). Further investigation is needed. This thesis demonstrates that statins inhibit Rab7 prenylation, which in turn inhibits the degradation of OS. However, it is unknown whether statins affect OS uptake. These results were extended to a model of CHM, to probe for novel mechanisms of disease. Thus, we have elucidated a role of protein prenylation in OS phagocytosis, and highlighted the importance of maintaining homeostasis of protein prenylation in the retina.

5. Chapter 5: References

Agranoff, B. W., H. Eggerer, U. Henning and F. Lynen (1960). "Biosynthesis of terpenes. VII. Isopentenyl pyrophosphate isomerase." J Biol Chem **235**: 326-332.

Alexandrov, K., H. Horiuchi, O. Steele-Mortimer, M. C. Seabra and M. Zerial (1994). "Rab escort protein-1 is a multifunctional protein that accompanies newly prenylated rab proteins to their target membranes." EMBO J **13**(22): 5262-5273.

Amaya, M., A. Baranova and M. L. van Hoek (2011). "Protein prenylation: a new mode of host-pathogen interaction." Biochem Biophys Res Commun **416**(1-2): 1-6.

Anand-Apte, B., Hollyfield, J.G. (2011). Developmental anatomy of the retinal and choroidal vasculature. Immunology, Inflammation and Diseases of the Eye. D. A. D. R. D. P. D. A. J. Niederkorn, Elsevier**:** 186-192.

Anant, J. S. and B. K. Fung (1992). "In vivo farnesylation of rat rhodopsin kinase." Biochem Biophys Res Commun **183**(2): 468-473.

Anant, J. S., O. C. Ong, H. Y. Xie, S. Clarke, P. J. O'Brien and B. K. Fung (1992). "In vivo differential prenylation of retinal cyclic GMP phosphodiesterase catalytic subunits." J Biol Chem **267**(2): 687-690.

Andres, D. A., M. C. Seabra, M. S. Brown, S. A. Armstrong, T. E. Smeland, F. P. Cremers and J. L. Goldstein (1993). "cDNA cloning of component A of Rab geranylgeranyl transferase and demonstration of its role as a Rab escort protein." Cell **73**(6): 1091-1099.

Armstrong, S. A., V. C. Hannah, J. L. Goldstein and M. S. Brown (1995). "CAAX geranylgeranyl transferase transfers farnesyl as efficiently as geranylgeranyl to RhoB." J Biol Chem **270**(14): 7864-7868.

Ashby, M. N. (1998). "CaaX converting enzymes." Curr Opin Lipidol **9**(2): 99-102.

Back, I., K. O. Donner and T. Reuter (1965). "The screening effect of the pigment epithelium on the retinal rods in the frog." Vision Res **5**(3): 101-111.

Baehr, W. (2014). "Membrane protein transport in photoreceptors: the function of PDEdelta: the Proctor lecture." Invest Ophthalmol Vis Sci **55**(12): 8653-8666.

Baron, R. A. and M. C. Seabra (2008). "Rab geranylgeranylation occurs preferentially via the pre-formed REP-RGGT complex and is regulated by geranylgeranyl pyrophosphate." Biochem J **415**(1): 67-75.

Baumann, H. and D. Doyle (1979). "Effect of trypsin on the cell surface proteins of hepatoma tissue culture cells. Characterization of a carbohydrate-rich glycopeptide released from a calcium binding membrane glycoprotein." J Biol Chem **254**(10): 3935-3946.

Bergmann, M., F. Schutt, F. G. Holz and J. Kopitz (2004). "Inhibition of the ATP-driven proton pump in RPE lysosomes by the major lipofuscin fluorophore A2-E may contribute to the pathogenesis of age-related macular degeneration." FASEB J **18**(3): 562-564.

Berndt, N., A. D. Hamilton and S. M. Sebti (2011). "Targeting protein prenylation for cancer therapy." Nat Rev Cancer **11**(11): 775-791.

Biller, S. A., C. Forster, E. M. Gordon, T. Harrity, W. A. Scott and C. P. Ciosek, Jr. (1988). "Isoprenoid (phosphinylmethyl)phosphonates as inhibitors of squalene synthetase." J Med Chem **31**(10): 1869-1871.

Bonilha, V. L., K. M. Trzupek, Y. Li, P. J. Francis, J. G. Hollyfield, M. E. Rayborn, N. Smaoui and R. G. Weleber (2008). "Choroideremia: analysis of the retina from a female symptomatic carrier." Ophthalmic Genet **29**(3): 99-110.
Bosel, J., F. Gandor, C. Harms, M. Synowitz, U. Harms, P. C. Djoufack, D. Megow, U. Dirnagl, H. Hortnagl, K. B. Fink and M. Endres (2005). "Neuroprotective effects of atorvastatin against glutamate-induced excitotoxicity in primary cortical neurones." J Neurochem **92**(6): 1386-1398.
Botelho, R. J. and S. Grinstein (2011). "Phagocytosis." Curr Biol **21**(14): R533-538.
Brunk, U. T. and A. Terman (2002). "Lipofuscin: mechanisms of age-related accumulation and influence on cell function." Free Radic Biol Med **33**(5): 611-619.
Burnside, B. (2001). "Light and circadian regulation of retinomotor movement." Prog Brain Res **131**: 477-485.
Burstyn-Cohen, T., E. D. Lew, P. G. Traves, P. G. Burrola, J. C. Hash and G. Lemke (2012). "Genetic dissection of TAM receptor-ligand interaction in retinal pigment epithelial cell phagocytosis." Neuron **76**(6): 1123-1132.
Cantalupo, G., P. Alifano, V. Roberti, C. B. Bruni and C. Bucci (2001). "Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes." EMBO J **20**(4): 683-693.
Cao, S. S. and R. J. Kaufman (2012). "Unfolded protein response." Curr Biol **22**(16): R622-626.
Capeans, C., A. Pineiro, M. Pardo, C. Carneiro, M. J. Blanco, J. M. Vinuela, M. Sanchez Salorio and F. Dominguez (2001). "Role of inhibitors of isoprenylation in proliferation, phenotype and apoptosis of human retinal pigment epithelium." Graefes Arch Clin Exp Ophthalmol **239**(3): 188-198.
Carr, A. J., A. Vugler, J. Lawrence, L. L. Chen, A. Ahmado, F. K. Chen, M. Semo, C. Gias, L. da Cruz, H. D. Moore, J. Walsh and P. J. Coffey (2009). "Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay." Mol Vis **15**: 283-295.
Casey, P. J. and M. C. Seabra (1996). "Protein prenyltransferases." J Biol Chem **271**(10): 5289-5292.
Cereso, N., M. O. Pequignot, L. Robert, F. Becker, V. De Luca, N. Nabholz, V. Rigau, J. De Vos, C. P. Hamel and V. Kalatzis (2014). "Proof of concept for AAV2/5-mediated gene therapy in iPSC-derived retinal pigment epithelium of a choroideremia patient." Mol Ther Methods Clin Dev **1**: 14011.
Chavrier, P., J. P. Gorvel, E. Stelzer, K. Simons, J. Gruenberg and M. Zerial (1991). "Hypervariable C-terminal domain of rab proteins acts as a targeting signal." Nature **353**(6346): 769-772.
Cherfils, J. and M. Zeghouf (2013). "Regulation of small GTPases by GEFs, GAPs, and GDIs." Physiol Rev **93**(1): 269-309.
Chowers, I., Y. Kim, R. H. Farkas, T. L. Gunatilaka, A. S. Hackam, P. A. Campochiaro, S. C. Finnemann and D. J. Zack (2004). "Changes in retinal pigment epithelial gene expression induced by rod outer segment uptake." Invest Ophthalmol Vis Sci **45**(7): 2098-2106.
Christiansen, J. R., S. Kolandaivelu, M. O. Bergo and V. Ramamurthy (2011). "RAS-converting enzyme 1-mediated endoproteolysis is required for trafficking of rod

phosphodiesterase 6 to photoreceptor outer segments." Proc Natl Acad Sci U S A **108**(21): 8862-8866.
Christiansen, J. R., N. D. Pendse, S. Kolandaivelu, M. O. Bergo, S. G. Young and V. Ramamurthy (2016). "Deficiency of Isoprenylcysteine Carboxyl Methyltransferase (ICMT) Leads to Progressive Loss of Photoreceptor Function." J Neurosci **36**(18): 5107-5114.
Cilla, D. D., Jr., D. M. Gibson, L. R. Whitfield and A. J. Sedman (1996). "Pharmacodynamic effects and pharmacokinetics of atorvastatin after administration to normocholesterolemic subjects in the morning and evening." J Clin Pharmacol **36**(7): 604-609.
Corbeel, L. and K. Freson (2008). "Rab proteins and Rab-associated proteins: major actors in the mechanism of protein-trafficking disorders." Eur J Pediatr **167**(7): 723-729.
Corsini, A., S. Bellosta, R. Baetta, R. Fumagalli, R. Paoletti and F. Bernini (1999). "New insights into the pharmacodynamic and pharmacokinetic properties of statins." Pharmacol Ther **84**(3): 413-428.
Coxon, F. P., F. H. Ebetino, E. H. Mules, M. C. Seabra, C. E. McKenna and M. J. Rogers (2005). "Phosphonocarboxylate inhibitors of Rab geranylgeranyl transferase disrupt the prenylation and membrane localization of Rab proteins in osteoclasts in vitro and in vivo." Bone **37**(3): 349-358.
Cremers, F. P., S. A. Armstrong, M. C. Seabra, M. S. Brown and J. L. Goldstein (1994). "REP-2, a Rab escort protein encoded by the choroideremia-like gene." J Biol Chem **269**(3): 2111-2117.
Cremers, F. P., D. J. van de Pol, L. P. van Kerkhoff, B. Wieringa and H. H. Ropers (1990). "Cloning of a gene that is rearranged in patients with choroideraemia." Nature **347**(6294): 674-677.
Cunha-Vaz, J., R. Bernardes and C. Lobo (2011). "Blood-retinal barrier." Eur J Ophthalmol **21 Suppl 6**: S3-9.
De Sandre-Giovannoli, A., R. Bernard, P. Cau, C. Navarro, J. Amiel, I. Boccaccio, S. Lyonnet, C. L. Stewart, A. Munnich, M. Le Merrer and N. Levy (2003). "Lamin a truncation in Hutchinson-Gilford progeria." Science **300**(5628): 2055.
Deguchi, J., A. Yamamoto, T. Yoshimori, K. Sugasawa, Y. Moriyama, M. Futai, T. Suzuki, K. Kato, M. Uyama and Y. Tashiro (1994). "Acidification of phagosomes and degradation of rod outer segments in rat retinal pigment epithelium." Invest Ophthalmol Vis Sci **35**(2): 568-579.
Deretic, D. (1997). "Rab proteins and post-Golgi trafficking of rhodopsin in photoreceptor cells." Electrophoresis **18**(14): 2537-2541.
Deretic, D. and D. S. Papermaster (1993). "Rab6 is associated with a compartment that transports rhodopsin from the trans-Golgi to the site of rod outer segment disk formation in frog retinal photoreceptors." J Cell Sci **106 (Pt 3)**: 803-813.
Do, R., R. S. Kiss, D. Gaudet and J. C. Engert (2009). "Squalene synthase: a critical enzyme in the cholesterol biosynthesis pathway." Clin Genet **75**(1): 19-29.
Dryja, T. P., L. B. Hahn, T. Reboul and B. Arnaud (1996). "Missense mutation in the gene encoding the alpha subunit of rod transducin in the Nougaret form of congenital stationary night blindness." Nat Genet **13**(3): 358-360.
Duclos, S., R. Diez, J. Garin, B. Papadopoulou, A. Descoteaux, H. Stenmark and M. Desjardins (2000). "Rab5 regulates the kiss and run fusion between phagosomes and

endosomes and the acquisition of phagosome leishmanicidal properties in RAW 264.7 macrophages." J Cell Sci **113 Pt 19**: 3531-3541.
Dunn, K. C., A. E. Aotaki-Keen, F. R. Putkey and L. M. Hjelmeland (1996). "ARPE-19, a human retinal pigment epithelial cell line with differentiated properties." Exp Eye Res **62**(2): 155-169.
Dunn, K. C., A. D. Marmorstein, V. L. Bonilha, E. Rodriguez-Boulan, F. Giordano and L. M. Hjelmeland (1998). "Use of the ARPE-19 cell line as a model of RPE polarity: basolateral secretion of FGF5." Invest Ophthalmol Vis Sci **39**(13): 2744-2749.
Dunten, P., U. Kammlott, R. Crowther, D. Weber, R. Palermo and J. Birktoft (1998). "Protein farnesyltransferase: structure and implications for substrate binding." Biochemistry **37**(37): 13042.
Dutta, N. and S. Seo (2016). "RPGR, a prenylated retinal ciliopathy protein, is targeted to cilia in a prenylation- and PDE6D-dependent manner." Biol Open **5**(9): 1283-1289.
Ebenezer, N. D., M. Michaelides, S. A. Jenkins, I. Audo, A. R. Webster, M. E. Cheetham, A. Stockman, E. R. Maher, J. R. Ainsworth, J. R. Yates, K. Bradshaw, G. E. Holder, A. T. Moore and A. J. Hardcastle (2005). "Identification of novel RPGR ORF15 mutations in X-linked progressive cone-rod dystrophy (XLCORD) families." Invest Ophthalmol Vis Sci **46**(6): 1891-1898.
Edwards, P. A. and J. Ericsson (1999). "Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway." Annu Rev Biochem **68**: 157-185.
Edwards, R. B. and R. B. Szamier (1977). "Defective phagocytosis of isolated rod outer segments by RCS rat retinal pigment epithelium in culture." Science **197**(4307): 1001-1003.
Endo, A. (2010). "A historical perspective on the discovery of statins." Proc Jpn Acad Ser B Phys Biol Sci **86**(5): 484-493.
Endo, A. and M. Kuroda (1976). "Citrinin, an inhibitor of cholesterol synthesis." J Antibiot (Tokyo) **29**(8): 841-843.
Ericsson, J., M. Runquist, A. Thelin, M. Andersson, T. Chojnacki and G. Dallner (1993). "Distribution of prenyltransferases in rat tissues. Evidence for a cytosolic all-trans-geranylgeranyl diphosphate synthase." J Biol Chem **268**(2): 832-838.
Esposito, G., F. De Falco, N. Tinto, F. Testa, L. Vitagliano, I. C. Tandurella, L. Iannone, S. Rossi, E. Rinaldi, F. Simonelli, A. Zagari and F. Salvatore (2011). "Comprehensive mutation analysis (20 families) of the choroideremia gene reveals a missense variant that prevents the binding of REP1 with Rab geranylgeranyl transferase." Hum Mutat **32**(12): 1460-1469.
Fadeel, B., P. Quinn, D. Xue and V. Kagan (2007). "Fat(al) attraction: oxidized lipids act as "eat-me" signals." HFSP J **1**(4): 225-229.
Feng, W., D. Yasumura, M. T. Matthes, M. M. LaVail and D. Vollrath (2002). "Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells." J Biol Chem **277**(19): 17016-17022.
Ferguson, J. J., I. F. Durr and H. Rudney (1959). "The Biosynthesis of Mevalonic Acid." Proc Natl Acad Sci U S A **45**(4): 499-504.
Ferguson, T. A. and D. R. Green (2014). "Autophagy and phagocytosis converge for better vision." Autophagy **10**(1): 165-167.
Finnemann, S. C., V. L. Bonilha, A. D. Marmorstein and E. Rodriguez-Boulan (1997). "Phagocytosis of rod outer segments by retinal pigment epithelial cells requires

alpha(v)beta5 integrin for binding but not for internalization." Proc Natl Acad Sci U S A **94**(24): 12932-12937.
Finnemann, S. C., L. W. Leung and E. Rodriguez-Boulan (2002). "The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium." Proc Natl Acad Sci U S A **99**(6): 3842-3847.
Flannagan, R. S., V. Jaumouille and S. Grinstein (2012). "The cell biology of phagocytosis." Annu Rev Pathol **7**: 61-98.
Flannery, J. G., A. C. Bird, D. B. Farber, R. G. Weleber and D. Bok (1990). "A histopathologic study of a choroideremia carrier." Invest Ophthalmol Vis Sci **31**(2): 229-236.
Fliesler, S. J. (2015). "Cholesterol homeostasis in the retina: seeing is believing." J Lipid Res **56**(1): 1-4.
Fliesler, S. J. and L. Bretillon (2010). "The ins and outs of cholesterol in the vertebrate retina." J Lipid Res **51**(12): 3399-3413.
Fliesler, S. J. and R. K. Keller (1997). "Isoprenoid metabolism in the vertebrate retina." Int J Biochem Cell Biol **29**(6): 877-894.
Force, U. S. P. S. T., K. Bibbins-Domingo, D. C. Grossman, S. J. Curry, K. W. Davidson, J. W. Epling, Jr., F. A. Garcia, M. W. Gillman, A. R. Kemper, A. H. Krist, A. E. Kurth, C. S. Landefeld, M. L. LeFevre, C. M. Mangione, W. R. Phillips, D. K. Owens, M. G. Phipps and M. P. Pignone (2016). "Statin Use for the Primary Prevention of Cardiovascular Disease in Adults: US Preventive Services Task Force Recommendation Statement." JAMA **316**(19): 1997-2007.
Frost, L. S., V. S. Lopes, A. Bragin, J. Reyes-Reveles, J. Brancato, A. Cohen, C. H. Mitchell, D. S. Williams and K. Boesze-Battaglia (2015). "The Contribution of Melanoregulin to Microtubule-Associated Protein 1 Light Chain 3 (LC3) Associated Phagocytosis in Retinal Pigment Epithelium." Mol Neurobiol **52**(3): 1135-1151.
Fujii, S., C. Setoguchi, K. Kawazu and K. Hosoya (2015). "Functional Characterization of Carrier-Mediated Transport of Pravastatin across the Blood-Retinal Barrier in Rats." Drug Metab Dispos **43**(12): 1956-1959.
Fukada, Y., T. Takao, H. Ohguro, T. Yoshizawa, T. Akino and Y. Shimonishi (1990). "Farnesylated gamma-subunit of photoreceptor G protein indispensable for GTP-binding." Nature **346**(6285): 658-660.
Fukuda, M. (2011). "TBC proteins: GAPs for mammalian small GTPase Rab?" Biosci Rep **31**(3): 159-168.
Futter, C. E., J. S. Ramalho, G. B. Jaissle, M. W. Seeliger and M. C. Seabra (2004). "The role of Rab27a in the regulation of melanosome distribution within retinal pigment epithelial cells." Mol Biol Cell **15**(5): 2264-2275.
Garcia, M. J., R. F. Reinoso, A. Sanchez Navarro and J. R. Prous (2003). "Clinical pharmacokinetics of statins." Methods Find Exp Clin Pharmacol **25**(6): 457-481.
Gehlbach, P., T. Li and E. Hatef (2016). "Statins for age-related macular degeneration." Cochrane Database Syst Rev(8): CD006927.
Gelb, M. H., L. Brunsveld, C. A. Hrycyna, S. Michaelis, F. Tamanoi, W. C. Van Voorhis and H. Waldmann (2006). "Therapeutic intervention based on protein prenylation and associated modifications." Nat Chem Biol **2**(10): 518-528.

Genead, M. A., S. Pasadhika and G. A. Fishman (2009). "Retinal nerve fibre layer thickness analysis in X-linked retinoschisis using Fourier-domain OCT." Eye (Lond) **23**(5): 1019-1027.
Gibbs, D., J. Kitamoto and D. S. Williams (2003). "Abnormal phagocytosis by retinal pigmented epithelium that lacks myosin VIIa, the Usher syndrome 1B protein." Proc Natl Acad Sci U S A **100**(11): 6481-6486.
Gold, P. H. and R. E. Olson (1966). "Studies on coenzyme Q. The biosynthesis of coenzyme Q9 in rat tissue slices." J Biol Chem **241**(15): 3507-3516.
Goldstein, J. L. and M. S. Brown (1990). "Regulation of the mevalonate pathway." Nature **343**(6257): 425-430.
Gordiyenko, N. V., R. N. Fariss, C. Zhi and I. M. MacDonald (2010). "Silencing of the CHM gene alters phagocytic and secretory pathways in the retinal pigment epithelium." Invest Ophthalmol Vis Sci **51**(2): 1143-1150.
Griscelli, C., A. Durandy, D. Guy-Grand, F. Daguillard, C. Herzog and M. Prunieras (1978). "A syndrome associating partial albinism and immunodeficiency." Am J Med **65**(4): 691-702.
Guo, Z., Y. W. Wu, D. Das, C. Delon, J. Cramer, S. Yu, S. Thuns, N. Lupilova, H. Waldmann, L. Brunsveld, R. S. Goody, K. Alexandrov and W. Blankenfeldt (2008). "Structures of RabGGTase-substrate/product complexes provide insights into the evolution of protein prenylation." EMBO J **27**(18): 2444-2456.
Gutierrez, M. G. (2013). "Functional role(s) of phagosomal Rab GTPases." Small GTPases **4**(3): 148-158.
Gutierrez, M. G., D. B. Munafo, W. Beron and M. I. Colombo (2004). "Rab7 is required for the normal progression of the autophagic pathway in mammalian cells." J Cell Sci **117**(Pt 13): 2687-2697.
Guymer, R., P. Luthert and A. Bird (1999). "Changes in Bruch's membrane and related structures with age." Prog Retin Eye Res **18**(1): 59-90.
Hajar, R. (2011). "Statins: past and present." Heart Views **12**(3): 121-127.
Hamelin, B. A. and J. Turgeon (1998). "Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors." Trends Pharmacol Sci **19**(1): 26-37.
Hancock, J. F., A. I. Magee, J. E. Childs and C. J. Marshall (1989). "All Ras Proteins Are Polyisoprenylated but Only Some Are Palmitoylated." Cell **57**(7): 1167-1177.
Hasegawa, M., H. Kusuhara, H. Endou and Y. Sugiyama (2003). "Contribution of organic anion transporters to the renal uptake of anionic compounds and nucleoside derivatives in rat." J Pharmacol Exp Ther **305**(3): 1087-1097.
Henrickson, A. (2005). Organization of the Adult Primate Fovea. Macular Degeneration. P. L. Penfold, Provis, J.M. Berlin, Springer**:** 1-23.
Herman, K. G. and R. H. Steinberg (1982). "Phagosome degradation in the tapetal retinal pigment epithelium of the opossum." Invest Ophthalmol Vis Sci **23**(3): 291-304.
Hoffmann, G. F., C. Charpentier, E. Mayatepek, J. Mancini, M. Leichsenring, K. M. Gibson, P. Divry, M. Hrebicek, W. Lehnert, K. Sartor and et al. (1993). "Clinical and biochemical phenotype in 11 patients with mevalonic aciduria." Pediatrics **91**(5): 915-921.
Holstein, S. A. and R. J. Hohl (2004). "Isoprenoids: remarkable diversity of form and function." Lipids **39**(4): 293-309.

Honjo, M., H. Tanihara, K. Nishijima, J. Kiryu, Y. Honda, B. Y. Yue and T. Sawamura (2002). "Statin inhibits leukocyte-endothelial interaction and prevents neuronal death induced by ischemia-reperfusion injury in the rat retina." Arch Ophthalmol **120**(12): 1707-1713.
Hooff, G. P., I. Peters, W. G. Wood, W. E. Muller and G. P. Eckert (2010). "Modulation of cholesterol, farnesylpyrophosphate, and geranylgeranylpyrophosphate in neuroblastoma SH-SY5Y-APP695 cells: impact on amyloid beta-protein production." Mol Neurobiol **41**(2-3): 341-350.
Hoppe, A. D. and J. A. Swanson (2004). "Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis." Mol Biol Cell **15**(8): 3509-3519.
Horvat, S., J. McWhir and D. Rozman (2011). "Defects in cholesterol synthesis genes in mouse and in humans: lessons for drug development and safer treatments." Drug Metab Rev **43**(1): 69-90.
Huang, A. S., L. A. Kim and A. A. Fawzi (2012). "Clinical characteristics of a large choroideremia pedigree carrying a novel CHM mutation." Arch Ophthalmol **130**(9): 1184-1189.
Hubel, D. H. (1995). Amacrine Cells. Eye, Brain and Vision, Henry Holt and Company.
Hubel, D. H. (1995). Bipolar Cells and Horizontal Cells. Eye, Brain and Vision, Henry Holt and Company.
Huynh, K. K., E. L. Eskelinen, C. C. Scott, A. Malevanets, P. Saftig and S. Grinstein (2007). "LAMP proteins are required for fusion of lysosomes with phagosomes." EMBO J **26**(2): 313-324.
Ioannidou, E., V. S. Tseriotis and K. Tziomalos (2017). "Role of lipid-lowering agents in the management of diabetic retinopathy." World J Diabetes **8**(1): 1-6.
Istvan, E. S. and J. Deisenhofer (2001). "Structural mechanism for statin inhibition of HMG-CoA reductase." Science **292**(5519): 1160-1164.
Jacobson, S. G., A. V. Cideciyan, A. Sumaroka, T. S. Aleman, S. B. Schwartz, E. A. Windsor, A. J. Roman, E. M. Stone and I. M. MacDonald (2006). "Remodeling of the human retina in choroideremia: rab escort protein 1 (REP-1) mutations." Invest Ophthalmol Vis Sci **47**(9): 4113-4120.
Jacobson, T. A. (2004). "Comparative pharmacokinetic interaction profiles of pravastatin, simvastatin, and atorvastatin when coadministered with cytochrome P450 inhibitors." Am J Cardiol **94**(9): 1140-1146.
James, M. J. and A. A. Kandutsch (1980). "Regulation of hepatic dolichol synthesis by beta-hydroxy-beta-methylglutaryl coenzyme A reductase." J Biol Chem **255**(18): 8618-8622.
Johansson, M., N. Rocha, W. Zwart, I. Jordens, L. Janssen, C. Kuijl, V. M. Olkkonen and J. Neefjes (2007). "Activation of endosomal dynein motors by stepwise assembly of Rab7-RILP-p150Glued, ORP1L, and the receptor betalII spectrin." J Cell Biol **176**(4): 459-471.
Johnson, S., S. Halford, A. G. Morris, R. J. Patel, S. E. Wilkie, A. J. Hardcastle, A. T. Moore, K. Zhang and D. M. Hunt (2003). "Genomic organisation and alternative splicing of human RIM1, a gene implicated in autosomal dominant cone-rod dystrophy (CORD7)." Genomics **81**(3): 304-314.

Johnson-Anuna, L. N., G. P. Eckert, C. Franke, U. Igbavboa, W. E. Muller and W. G. Wood (2007). "Simvastatin protects neurons from cytotoxicity by up-regulating Bcl-2 mRNA and protein." J Neurochem **101**(1): 77-86.
Kajinami, K., H. Mabuchi and Y. Saito (2000). "NK-104: a novel synthetic HMG-CoA reductase inhibitor." Expert Opin Investig Drugs **9**(11): 2653-2661.
Kajiwara, K., L. B. Hahn, S. Mukai, G. H. Travis, E. L. Berson and T. P. Dryja (1991). "Mutations in the human retinal degeneration slow gene in autosomal dominant retinitis pigmentosa." Nature **354**(6353): 480-483.
Kandutsch, A. A., H. Paulus, E. Levin and K. Bloch (1964). "Purification of Geranylgeranyl Pyrophosphate Synthetase from Micrococcus Lysodeikticus." J Biol Chem **239**: 2507-2515.
Kassai, H., A. Aiba, K. Nakao, K. Nakamura, M. Katsuki, W. H. Xiong, K. W. Yau, H. Imai, Y. Shichida, Y. Satomi, T. Takao, T. Okano and Y. Fukada (2005). "Farnesylation of retinal transducin underlies its translocation during light adaptation." Neuron **47**(4): 529-539.
Kawaguchi, R., J. Yu, J. Honda, J. Hu, J. Whitelegge, P. Ping, P. Wiita, D. Bok and H. Sun (2007). "A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A." Science **315**(5813): 820-825.
Kedzierski, W., M. Lloyd, D. G. Birch, D. Bok and G. H. Travis (1997). "Generation and analysis of transgenic mice expressing P216L-substituted rds/peripherin in rod photoreceptors." Invest Ophthalmol Vis Sci **38**(2): 498-509.
Kervestin, S. and A. Jacobson (2012). "NMD: a multifaceted response to premature translational termination." Nat Rev Mol Cell Biol **13**(11): 700-712.
Kevany, B. M. and K. Palczewski (2010). "Phagocytosis of retinal rod and cone photoreceptors." Physiology (Bethesda) **25**(1): 8-15.
Kim, E., P. Ambroziak, J. C. Otto, B. Taylor, M. Ashby, K. Shannon, P. J. Casey and S. G. Young (1999). "Disruption of the mouse Rce1 gene results in defective Ras processing and mislocalization of Ras within cells." J Biol Chem **274**(13): 8383-8390.
Kim, J. Y., H. Zhao, J. Martinez, T. A. Doggett, A. V. Kolesnikov, P. H. Tang, Z. Ablonczy, C. C. Chan, Z. Zhou, D. R. Green and T. A. Ferguson (2013). "Noncanonical autophagy promotes the visual cycle." Cell **154**(2): 365-376.
Kiser, P. D., M. Golczak and K. Palczewski (2014). "Chemistry of the retinoid (visual) cycle." Chem Rev **114**(1): 194-232.
Kisselev, O., M. Ermolaeva and N. Gautam (1995). "Efficient interaction with a receptor requires a specific type of prenyl group on the G protein gamma subunit." J Biol Chem **270**(43): 25356-25358.
Kobayashi, T., K. Startchev, A. J. Whitney and J. Gruenber (2001). "Localization of lysobisphosphatidic acid-rich membrane domains in late endosomes." Biol Chem **382**(3): 483-485.
Kohnke, M., C. Delon, M. L. Hastie, U. T. Nguyen, Y. W. Wu, H. Waldmann, R. S. Goody, J. J. Gorman and K. Alexandrov (2013). "Rab GTPase prenylation hierarchy and its potential role in choroideremia disease." PLoS One **8**(12): e81758.
Kolb, H. (1995). Photoreceptors. Webvision: The Organization of the Retina and Visual System. H. Kolb, E. Fernandez and R. Nelson. Salt Lake City (UT).
Kong, W. N., Y. H. Lei and Y. Z. Chang (2013). "The regulation of iron metabolism in the mononuclear phagocyte system." Expert Rev Hematol **6**(4): 411-418.

Konstantinopoulos, P. A., M. V. Karamouzis and A. G. Papavassiliou (2007). "Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets." Nat Rev Drug Discov **6**(7): 541-555.
Kretz, A., C. Schmeer, S. Tausch and S. Isenmann (2006). "Simvastatin promotes heat shock protein 27 expression and Akt activation in the rat retina and protects axotomized retinal ganglion cells in vivo." Neurobiol Dis **21**(2): 421-430.
Krock, B. L., J. Bilotta and B. D. Perkins (2007). "Noncell-autonomous photoreceptor degeneration in a zebrafish model of choroideremia." Proc Natl Acad Sci U S A **104**(11): 4600-4605.
Kukar, T., M. P. Murphy, J. L. Eriksen, S. A. Sagi, S. Weggen, T. E. Smith, T. Ladd, M. A. Khan, R. Kache, J. Beard, M. Dodson, S. Merit, V. V. Ozols, P. Z. Anastasiadis, P. Das, A. Fauq, E. H. Koo and T. E. Golde (2005). "Diverse compounds mimic Alzheimer disease-causing mutations by augmenting Abeta42 production." Nat Med **11**(5): 545-550.
Lai, R. K., D. Perez-Sala, F. J. Canada and R. R. Rando (1990). "The gamma subunit of transducin is farnesylated." Proc Natl Acad Sci U S A **87**(19): 7673-7677.
Lane, K. T. and L. S. Beese (2006). "Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I." J Lipid Res **47**(4): 681-699.
Laskovics, F. M., J. M. Krafcik and C. D. Poulter (1979). "Prenyltransferase. Kinetic studies of the 1'-4 coupling reaction with avian liver enzyme." J Biol Chem **254**(19): 9458-9463.
Law, A. L., Q. Ling, K. A. Hajjar, C. E. Futter, J. Greenwood, P. Adamson, S. T. Wavre-Shapton, S. E. Moss and M. J. Hayes (2009). "Annexin A2 regulates phagocytosis of photoreceptor outer segments in the mouse retina." Mol Biol Cell **20**(17): 3896-3904.
Law, M. and A. R. Rudnicka (2006). "Statin safety: a systematic review." Am J Cardiol **97**(8A): 52C-60C.
Lazow, M. A., D. C. Hood, R. Ramachandran, T. R. Burke, Y. Z. Wang, V. C. Greenstein and D. G. Birch (2011). "Transition zones between healthy and diseased retina in choroideremia (CHM) and Stargardt disease (STGD) as compared to retinitis pigmentosa (RP)." Invest Ophthalmol Vis Sci **52**(13): 9581-9590.
Lee, J. J. and S. Seo (2015). "PDE6D binds to the C-terminus of RPGR in a prenylation-dependent manner." EMBO Rep **16**(12): 1581-1582.
Lerner, E. C., T. T. Zhang, D. B. Knowles, Y. Qian, A. D. Hamilton and S. M. Sebti (1997). "Inhibition of the prenylation of K-Ras, but not H- or N-Ras, is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase and a geranylgeranyltransferase I inhibitor in human tumor cell lines." Oncogene **15**(11): 1283-1288.
Leung, K. F., R. Baron, B. R. Ali, A. I. Magee and M. C. Seabra (2007). "Rab GTPases containing a CAAX motif are processed post-geranylgeranylation by proteolysis and methylation." J Biol Chem **282**(2): 1487-1497.
Lin, J. B., N. Mast, I. R. Bederman, Y. Li, H. Brunengraber, I. Bjorkhem and I. A. Pikuleva (2016). "Cholesterol in mouse retina originates primarily from in situ de novo biosynthesis." J Lipid Res **57**(2): 258-264.
Liu, J., W. Lu, D. Reigada, J. Nguyen, A. M. Laties and C. H. Mitchell (2008). "Restoration of lysosomal pH in RPE cells from cultured human and ABCA4(-/-) mice:

pharmacologic approaches and functional recovery." Invest Ophthalmol Vis Sci **49**(2): 772-780.
Lutz, R. J., T. M. McLain and M. Sinensky (1992). "Feedback inhibition of polyisoprenyl pyrophosphate synthesis from mevalonate in vitro. Implications for protein prenylation." J Biol Chem **267**(12): 7983-7986.
Lyon, M. F. (1961). "Gene action in the X-chromosome of the mouse (Mus musculus L.)." Nature **190**: 372-373.
MacDonald, I. M., S. Hume, S. Chan and M. C. Seabra (1993). Choroideremia. GeneReviews(R). R. A. Pagon, M. P. Adam, H. H. Ardinger et al. Seattle (WA).
MacDonald, I. M., L. Russell and C. C. Chan (2009). "Choroideremia: new findings from ocular pathology and review of recent literature." Surv Ophthalmol **54**(3): 401-407.
MacDonald, I. M., C. Sereda, K. McTaggart and D. Mah (2004). "Choroideremia gene testing." Expert Rev Mol Diagn **4**(4): 478-484.
Mahon, G. J., H. R. Anderson, T. A. Gardiner, S. McFarlane, D. B. Archer and A. W. Stitt (2004). "Chloroquine causes lysosomal dysfunction in neural retina and RPE: implications for retinopathy." Curr Eye Res **28**(4): 277-284.
Majid, M. A., B. Horsborough and R. H. Gray (1998). "Unusual macular findings in a known choroideremia carrier." Eye (Lond) **12 (Pt 4)**: 740-741.
Malek, G., C. M. Li, C. Guidry, N. E. Medeiros and C. A. Curcio (2003). "Apolipoprotein B in cholesterol-containing drusen and basal deposits of human eyes with age-related maculopathy." Am J Pathol **162**(2): 413-425.
Mao, Y. and S. C. Finnemann (2013). "Analysis of photoreceptor outer segment phagocytosis by RPE cells in culture." Methods Mol Biol **935**: 285-295.
Marcuzzi, A., S. Crovella and A. Pontillo (2011). "Geraniol rescues inflammation in cellular and animal models of mevalonate kinase deficiency." In Vivo **25**(1): 87-92.
Marcuzzi, A., A. Pontillo, L. De Leo, A. Tommasini, G. Decorti, T. Not and A. Ventura (2008). "Natural isoprenoids are able to reduce inflammation in a mouse model of mevalonate kinase deficiency." Pediatr Res **64**(2): 177-182.
Martinez, J., J. Almendinger, A. Oberst, R. Ness, C. P. Dillon, P. Fitzgerald, M. O. Hengartner and D. R. Green (2011). "Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells." Proc Natl Acad Sci U S A **108**(42): 17396-17401.
Marz, P., U. Otten and A. R. Miserez (2007). "Statins induce differentiation and cell death in neurons and astroglia." Glia **55**(1): 1-12.
Masland, R. H. (2001). "The fundamental plan of the retina." Nat Neurosci **4**(9): 877-886.
Mazzoni, F., H. Safa and S. C. Finnemann (2014). "Understanding photoreceptor outer segment phagocytosis: use and utility of RPE cells in culture." Exp Eye Res **126**: 51-60.
McTaggart, F., L. Buckett, R. Davidson, G. Holdgate, A. McCormick, D. Schneck, G. Smith and M. Warwick (2001). "Preclinical and clinical pharmacology of Rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor." Am J Cardiol **87**(5A): 28B-32B.
McTaggart, S. J. (2006). "Isoprenylated proteins." Cell Mol Life Sci **63**(3): 255-267.
McTavish, D. and E. M. Sorkin (1991). "Pravastatin. A review of its pharmacological properties and therapeutic potential in hypercholesterolaemia." Drugs **42**(1): 65-89.
Merry, D. E., J. G. Lesko, D. M. Sosnoski, R. A. Lewis, M. Lubinsky, B. Trask, G. van den Engh, F. S. Collins and R. L. Nussbaum (1989). "Choroideremia and deafness with

stapes fixation: a contiguous gene deletion syndrome in Xq21." Am J Hum Genet **45**(4): 530-540.
Mizranita, V. and E. H. Pratisto (2015). "Statin-associated ocular disorders: the FDA and ADRAC data." Int J Clin Pharm **37**(5): 844-850.
Modjtahedi, B. S., N. Bose, T. D. Papakostas, L. Morse, D. G. Vavvas and A. U. Kishan (2016). "Lipids and Diabetic Retinopathy." Semin Ophthalmol **31**(1-2): 10-18.
Mohamed, A., L. Saavedra, A. Di Pardo, S. Sipione and E. Posse de Chaves (2012). "beta-amyloid inhibits protein prenylation and induces cholesterol sequestration by impairing SREBP-2 cleavage." J Neurosci **32**(19): 6490-6500.
Mohamed A, S. K., Posse de Chaves, E (2015). The Mevalonate Pathway in Alzheimer's Disease — Cholesterol and Non-Sterol Isoprenoids. Alzheimer's Disease - Challenges for the Future. I. Zerr, INTECH Open Science.
Mohamed, A., K. Smith and E. Posse de Chaves (2015). The Mevalonate Pathway in Alzheimer's Disease — Cholesterol and Non-Sterol Isoprenoids. Alzheimer's Disease - Challenges for the Future. I. Zerr, InTechOpen.
Moiseyev, G., Y. Chen, Y. Takahashi, B. X. Wu and J. X. Ma (2005). "RPE65 is the isomerohydrolase in the retinoid visual cycle." Proc Natl Acad Sci U S A **102**(35): 12413-12418.
Muck, W., W. Ritter, K. Ochmann, S. Unger, G. Ahr, W. Wingender and J. Kuhlmann (1997). "Absolute and relative bioavailability of the HMG-CoA reductase inhibitor cerivastatin." Int J Clin Pharmacol Ther **35**(6): 255-260.
Mullen, R. J. and M. M. LaVail (1976). "Inherited retinal dystrophy: primary defect in pigment epithelium determined with experimental rat chimeras." Science **192**(4241): 799-801.
Muller, M. P. and R. S. Goody (2017). "Molecular control of Rab activity by GEFs, GAPs and GDI." Small GTPases: 1-17.
Munz, C. (2015). "Of LAP, CUPS, and DRibbles - Unconventional Use of Autophagy Proteins for MHC Restricted Antigen Presentation." Front Immunol **6**: 200.
Mustafi, D. and K. Palczewski (2009). "Topology of class A G protein-coupled receptors: insights gained from crystal structures of rhodopsins, adrenergic and adenosine receptors." Mol Pharmacol **75**(1): 1-12.
Nandrot, E. F., M. Anand, D. Almeida, K. Atabai, D. Sheppard and S. C. Finnemann (2007). "Essential role for MFG-E8 as ligand for alphavbeta5 integrin in diurnal retinal phagocytosis." Proc Natl Acad Sci U S A **104**(29): 12005-12010.
Nandrot, E. F., Y. Kim, S. E. Brodie, X. Huang, D. Sheppard and S. C. Finnemann (2004). "Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking alphavbeta5 integrin." J Exp Med **200**(12): 1539-1545.
Narita, K., A. Choudhury, K. Dobrenis, D. K. Sharma, E. L. Holicky, D. L. Marks, S. U. Walkley and R. E. Pagano (2005). "Protein transduction of Rab9 in Niemann-Pick C cells reduces cholesterol storage." Faseb J **19**(11): 1558-1560.
Nezasa, K., K. Higaki, M. Takeuchi, M. Nakano and M. Koike (2003). "Uptake of rosuvastatin by isolated rat hepatocytes: comparison with pravastatin." Xenobiotica **33**(4): 379-388.
Ng, E. L., B. Q. Gan, F. Ng and B. L. Tang (2012). "Rab GTPases regulating receptor trafficking at the late endosome-lysosome membranes." Cell Biochem Funct **30**(6): 515-523.

Nussbaum, R. L., R. A. Lewis, J. G. Lesko and R. Ferrell (1985). "Choroideremia is linked to the restriction fragment length polymorphism DXYS1 at XQ13-21." Am J Hum Genet **37**(3): 473-481.
Okada, T., O. P. Ernst, K. Palczewski and K. P. Hofmann (2001). "Activation of rhodopsin: new insights from structural and biochemical studies." Trends Biochem Sci **26**(5): 318-324.
Olchawa, M. M., A. M. Herrnreiter, C. M. Skumatz, M. Zareba, T. J. Sarna and J. M. Burke (2013). "Photosensitized oxidative stress to ARPE-19 cells decreases protein receptors that mediate photoreceptor outer segment phagocytosis." Invest Ophthalmol Vis Sci **54**(3): 2276-2287.
Pan, H. Y., A. R. DeVault, D. Wang-Iverson, E. Ivashkiv, B. N. Swanson and A. A. Sugerman (1990). "Comparative pharmacokinetics and pharmacodynamics of pravastatin and lovastatin." J Clin Pharmacol **30**(12): 1128-1135.
Pasha, M. K., S. Muzeeb, S. J. Basha, D. Shashikumar, R. Mullangi and N. R. Srinivas (2006). "Analysis of five HMG-CoA reductase inhibitors-- atorvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin: pharmacological, pharmacokinetic and analytical overview and development of a new method for use in pharmaceutical formulations analysis and in vitro metabolism studies." Biomed Chromatogr **20**(3): 282-293.
Pepperberg, D. R., T. L. Okajima, H. Ripps, G. J. Chader and B. Wiggert (1991). "Functional properties of interphotoreceptor retinoid-binding protein." Photochem Photobiol **54**(6): 1057-1060.
Pittler, S. J., S. J. Fliesler, P. L. Fisher, P. K. Keller and L. M. Rapp (1995). "In vivo requirement of protein prenylation for maintenance of retinal cytoarchitecture and photoreceptor structure." J Cell Biol **130**(2): 431-439.
Pittler, S. J., S. J. Fliesler and L. M. Rapp (1992). "Novel morphological changes in rat retina induced by intravitreal injection of lovastatin." Exp Eye Res **54**(1): 149-152.
Poteryaev, D., S. Datta, K. Ackema, M. Zerial and A. Spang (2010). "Identification of the switch in early-to-late endosome transition." Cell **141**(3): 497-508.
Preising, M. N., E. Wegscheider, C. Friedburg, C. M. Poloschek, B. K. Wabbels and B. Lorenz (2009). "Fundus autofluorescence in carriers of choroideremia and correlation with electrophysiologic and psychophysical data." Ophthalmology **116**(6): 1201-1209 e1201-1202.
Press, B., Y. Feng, B. Hoflack and A. Wandinger-Ness (1998). "Mutant Rab7 causes the accumulation of cathepsin D and cation-independent mannose 6-phosphate receptor in an early endocytic compartment." J Cell Biol **140**(5): 1075-1089.
Pullarkat, R. K. and G. N. Morris (1997). "Farnesylation of Batten disease CLN3 protein." Neuropediatrics **28**(1): 42-44.
Qin, S. and G. A. Rodrigues (2012). "Roles of alphavbeta5, FAK and MerTK in oxidative stress inhibition of RPE cell phagocytosis." Exp Eye Res **94**(1): 63-70.
Raiteri, M., L. Arnaboldi, P. McGeady, M. H. Gelb, D. Verri, C. Tagliabue, P. Quarato, P. Ferraboschi, E. Santaniello, R. Paoletti, R. Fumagalli and A. Corsini (1997). "Pharmacological control of the mevalonate pathway: effect on arterial smooth muscle cell proliferation." J Pharmacol Exp Ther **281**(3): 1144-1153.
Rajanikant, G. K., D. Zemke, M. Kassab and A. Majid (2007). "The therapeutic potential of statins in neurological disorders." Curr Med Chem **14**(1): 103-112.

Rak, A., O. Pylypenko, A. Niculae, K. Pyatkov, R. S. Goody and K. Alexandrov (2004). "Structure of the Rab7:REP-1 complex: insights into the mechanism of Rab prenylation and choroideremia disease." Cell **117**(6): 749-760.
Ramamurthy, V., G. A. Niemi, T. A. Reh and J. B. Hurley (2004). "Leber congenital amaurosis linked to AIPL1: a mouse model reveals destabilization of cGMP phosphodiesterase." Proc Natl Acad Sci U S A **101**(38): 13897-13902.
Rauthan, M. and M. Pilon (2011). "The mevalonate pathway in C. elegans." Lipids Health Dis **10**: 243.
Reiss, Y., M. S. Brown and J. L. Goldstein (1992). "Divalent cation and prenyl pyrophosphate specificities of the protein farnesyltransferase from rat brain, a zinc metalloenzyme." J Biol Chem **267**(9): 6403-6408.
Reme, C. E. and M. Sulser (1977). "Diurnal variation of autophagy in rod visual cells in the rat." Albrecht Von Graefes Arch Klin Exp Ophthalmol **203**(3-4): 261-270.
Renner, A. B., B. S. Fiebig, E. Cropp, B. H. Weber and U. Kellner (2009). "Progression of retinal pigment epithelial alterations during long-term follow-up in female carriers of choroideremia and report of a novel CHM mutation." Arch Ophthalmol **127**(7): 907-912.
Renner, A. B., U. Kellner, E. Cropp, M. N. Preising, I. M. MacDonald, J. A. van den Hurk, F. P. Cremers and M. H. Foerster (2006). "Choroideremia: variability of clinical and electrophysiological characteristics and first report of a negative electroretinogram." Ophthalmology **113**(11): 2066 e2061-2010.
Ridley, A. J. and A. Hall (1992). "The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors." Cell **70**(3): 389-399.
Rodwell, V. W., J. L. Nordstrom and J. J. Mitschelen (1976). "Regulation of HMG-CoA reductase." Adv Lipid Res **14**: 1-74.
Roosing, S., R. W. Collin, A. I. den Hollander, F. P. Cremers and A. M. Siemiatkowska (2014). "Prenylation defects in inherited retinal diseases." J Med Genet **51**(3): 143-151.
Roosing, S., K. Rohrschneider, A. Beryozkin, D. Sharon, N. Weisschuh, J. Staller, S. Kohl, L. Zelinger, T. A. Peters, K. Neveling, T. M. Strom, C. European Retinal Disease, L. I. van den Born, C. B. Hoyng, C. C. Klaver, R. Roepman, B. Wissinger, E. Banin, F. P. Cremers and A. I. den Hollander (2013). "Mutations in RAB28, encoding a farnesylated small GTPase, are associated with autosomal-recessive cone-rod dystrophy." Am J Hum Genet **93**(1): 110-117.
Rosenson, R. S. and C. C. Tangney (1998). "Antiatherothrombotic properties of statins: implications for cardiovascular event reduction." JAMA **279**(20): 1643-1650.
Rothe, R., L. Liguori, A. Villegas-Mendez, B. Marques, D. Grunwald, E. Drouet and J. L. Lenormand (2010). "Characterization of the cell-penetrating properties of the Epstein-Barr virus ZEBRA trans-activator." J Biol Chem **285**(26): 20224-20233.
Rowell, C. A., J. J. Kowalczyk, M. D. Lewis and A. M. Garcia (1997). "Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo." J Biol Chem **272**(22): 14093-14097.
Rudolph, G., M. Preising, P. Kalpadakis, C. Haritoglou, G. E. Lang and B. Lorenz (2003). "Phenotypic variability in three carriers from a family with choroideremia and a frameshift mutation 1388delCCinsG in the REP-1 gene." Ophthalmic Genet **24**(4): 203-214.

Saheki, A., T. Terasaki, I. Tamai and A. Tsuji (1994). "In vivo and in vitro blood-brain barrier transport of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors." Pharm Res **11**(2): 305-311.
Sasaki, T., A. Kikuchi, S. Araki, Y. Hata, M. Isomura, S. Kuroda and Y. Takai (1990). "Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to smg p25A, a ras p21-like GTP-binding protein." J Biol Chem **265**(4): 2333-2337.
Schachter, M. (2005). "Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update." Fundam Clin Pharmacol **19**(1): 117-125.
Schafer, W. R. and J. Rine (1992). "Protein prenylation: genes, enzymes, targets, and functions." Annu Rev Genet **26**: 209-237.
Schmidt, R. A., J. A. Glomset, T. N. Wight, A. J. Habenicht and R. Ross (1982). "A study of the Influence of mevalonic acid and its metabolites on the morphology of swiss 3T3 cells." J Cell Biol **95**(1): 144-153.
Seabra, M. C., M. S. Brown, C. A. Slaughter, T. C. Sudhof and J. L. Goldstein (1992). "Purification of component A of Rab geranylgeranyl transferase: possible identity with the choroideremia gene product." Cell **70**(6): 1049-1057.
Seabra, M. C., J. L. Goldstein, T. C. Sudhof and M. S. Brown (1992). "Rab geranylgeranyl transferase. A multisubunit enzyme that prenylates GTP-binding proteins terminating in Cys-X-Cys or Cys-Cys." J Biol Chem **267**(20): 14497-14503.
Seabra, M. C., Y. K. Ho and J. S. Anant (1995). "Deficient geranylgeranylation of Ram/Rab27 in choroideremia." J Biol Chem **270**(41): 24420-24427.
Seabra, M. C., E. H. Mules and A. N. Hume (2002). "Rab GTPases, intracellular traffic and disease." Trends Mol Med **8**(1): 23-30.
Sebti, S. M. (2005). "Protein farnesylation: implications for normal physiology, malignant transformation, and cancer therapy." Cancer Cell **7**(4): 297-300.
Segev, N. (2011). "GTPases in intracellular trafficking: an overview." Semin Cell Dev Biol **22**(1): 1-2.
Sergeev, Y. V., N. Smaoui, R. Sui, D. Stiles, N. Gordiyenko, N. Strunnikova and I. M. Macdonald (2009). "The functional effect of pathogenic mutations in Rab escort protein 1." Mutat Res **665**(1-2): 44-50.
Shelby, S. J., K. L. Feathers, A. M. Ganios, L. Jia, J. M. Miller and D. A. Thompson (2015). "MERTK signaling in the retinal pigment epithelium regulates the tyrosine phosphorylation of GDP dissociation inhibitor alpha from the GDI/CHM family of RAB GTPase effectors." Exp Eye Res **140**: 28-40.
Shen, F. and M. C. Seabra (1996). "Mechanism of digeranylgeranylation of Rab proteins. Formation of a complex between monogeranylgeranyl-Rab and Rab escort protein." J Biol Chem **271**(7): 3692-3698.
Simon, A., E. Drewe, J. W. van der Meer, R. J. Powell, R. I. Kelley, A. F. Stalenhoef and J. P. Drenth (2004). "Simvastatin treatment for inflammatory attacks of the hyperimmunoglobulinemia D and periodic fever syndrome." Clin Pharmacol Ther **75**(5): 476-483.
Sinensky, M., L. A. Beck, S. Leonard and R. Evans (1990). "Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis." J Biol Chem **265**(32): 19937-19941.
Sirtori, C. R. (2014). "The pharmacology of statins." Pharmacol Res **88**: 3-11.

Sjogren, A. K., K. M. Andersson, M. Liu, B. A. Cutts, C. Karlsson, A. M. Wahlstrom, M. Dalin, C. Weinbaum, P. J. Casey, A. Tarkowski, B. Swolin, S. G. Young and M. O. Bergo (2007). "GGTase-I deficiency reduces tumor formation and improves survival in mice with K-RAS-induced lung cancer." J Clin Invest **117**(5): 1294-1304.
Sohocki, M. M., I. Perrault, B. P. Leroy, A. M. Payne, S. Dharmaraj, S. S. Bhattacharya, J. Kaplan, I. H. Maumenee, R. Koenekoop, F. M. Meire, D. G. Birch, J. R. Heckenlively and S. P. Daiger (2000). "Prevalence of AIPL1 mutations in inherited retinal degenerative disease." Mol Genet Metab **70**(2): 142-150.
Sorsby, A., A. Franceschetti, R. Joseph and J. B. Davey (1952). "Choroideremia; clinical and genetic aspects." Br J Ophthalmol **36**(10): 547-581.
Stenmark, H. (2009). "Rab GTPases as coordinators of vesicle traffic." Nat Rev Mol Cell Biol **10**(8): 513-525.
Steuer, H., A. Jaworski, B. Elger, M. Kaussmann, J. Keldenich, H. Schneider, D. Stoll and B. Schlosshauer (2005). "Functional characterization and comparison of the outer blood-retina barrier and the blood-brain barrier." Invest Ophthalmol Vis Sci **46**(3): 1047-1053.
Strauss, O. (1995). The Retinal Pigment Epithelium. Webvision: The Organization of the Retina and Visual System. H. Kolb, E. Fernandez and R. Nelson. Salt Lake City (UT).
Strauss, O. (2005). "The retinal pigment epithelium in visual function." Physiol Rev **85**(3): 845-881.
Streilein, J. W., N. Ma, H. Wenkel, T. F. Ng and P. Zamiri (2002). "Immunobiology and privilege of neuronal retina and pigment epithelium transplants." Vision Res **42**(4): 487-495.
Strunnikova, N., W. M. Zein, C. Silvin and I. M. MacDonald (2012). "Serum biomarkers and trafficking defects in peripheral tissues reflect the severity of retinopathy in three brothers affected by choroideremia." Adv Exp Med Biol **723**: 381-387.
Strunnikova, N. V., J. Barb, Y. V. Sergeev, A. Thiagarajasubramanian, C. Silvin, P. J. Munson and I. M. Macdonald (2009). "Loss-of-function mutations in Rab escort protein 1 (REP-1) affect intracellular transport in fibroblasts and monocytes of choroideremia patients." PLoS One **4**(12): e8402.
Takai, Y., T. Sasaki and T. Matozaki (2001). "Small GTP-binding proteins." Physiol Rev **81**(1): 153-208.
Tang, P. H., M. Kono, Y. Koutalos, Z. Ablonczy and R. K. Crouch (2013). "New insights into retinoid metabolism and cycling within the retina." Prog Retin Eye Res **32**: 48-63.
Tchen, T. T. (1958). "Mevalonic kinase: purification and properties." J Biol Chem **233**(5): 1100-1103.
Thomas, S., K. J. Wright, S. Le Corre, A. Micalizzi, M. Romani, A. Abhyankar, J. Saada, I. Perrault, J. Amiel, J. Litzler, E. Filhol, N. Elkhartoufi, M. Kwong, J. L. Casanova, N. Boddaert, W. Baehr, S. Lyonnet, A. Munnich, L. Burglen, N. Chassaing, F. Encha-Ravazi, M. Vekemans, J. G. Gleeson, E. M. Valente, P. K. Jackson, I. A. Drummond, S. Saunier and T. Attie-Bitach (2014). "A homozygous PDE6D mutation in Joubert syndrome impairs targeting of farnesylated INPP5E protein to the primary cilium." Hum Mutat **35**(1): 137-146.
Tian, B., A. Al-Moujahed, P. Bouzika, Y. Hu, S. Notomi, P. Tsoka, J. W. Miller, H. Lin and D. G. Vavvas (2017). "Atorvastatin Promotes Phagocytosis and Attenuates Pro-Inflammatory Response in Human Retinal Pigment Epithelial Cells." Sci Rep **7**(1): 2329.

Tolmachova, T., R. Anders, M. Abrink, L. Bugeon, M. J. Dallman, C. E. Futter, J. S. Ramalho, F. Tonagel, N. Tanimoto, M. W. Seeliger, C. Huxley and M. C. Seabra (2006). "Independent degeneration of photoreceptors and retinal pigment epithelium in conditional knockout mouse models of choroideremia." J Clin Invest **116**(2): 386-394.
Tolmachova, T., S. T. Wavre-Shapton, A. R. Barnard, R. E. MacLaren, C. E. Futter and M. C. Seabra (2010). "Retinal pigment epithelium defects accelerate photoreceptor degeneration in cell type-specific knockout mouse models of choroideremia." Invest Ophthalmol Vis Sci **51**(10): 4913-4920.
Tomi, M. and K. Hosoya (2010). "The role of blood-ocular barrier transporters in retinal drug disposition: an overview." Expert Opin Drug Metab Toxicol **6**(9): 1111-1124.
Travis, G. H., M. Golczak, A. R. Moise and K. Palczewski (2007). "Diseases caused by defects in the visual cycle: retinoids as potential therapeutic agents." Annu Rev Pharmacol Toxicol **47**: 469-512.
Trueblood, C. E., Y. Ohya and J. Rine (1993). "Genetic evidence for in vivo cross-specificity of the CaaX-box protein prenyltransferases farnesyltransferase and geranylgeranyltransferase-I in Saccharomyces cerevisiae." Mol Cell Biol **13**(7): 4260-4275.
Tse, F. L., J. M. Jaffe and A. Troendle (1992). "Pharmacokinetics of fluvastatin after single and multiple doses in normal volunteers." J Clin Pharmacol **32**(7): 630-638.
Tserentsoodol, N., N. V. Gordiyenko, I. Pascual, J. W. Lee, S. J. Fliesler and I. R. Rodriguez (2006). "Intraretinal lipid transport is dependent on high density lipoprotein-like particles and class B scavenger receptors." Mol Vis **12**: 1319-1333.
van Bokhoven, H., C. van Genderen, C. M. Molloy, D. J. van de Pol, C. W. Cremers, A. von Aarem, M. Schwartz, T. Rosenberg, A. H. Geurts van Kessel, H. H. Ropers and et al. (1994). "Mapping of the choroideremia-like (CHML) gene at 1q42-qter and mutation analysis in patients with Usher syndrome type II." Genomics **19**(2): 385-387.
van den Hurk, J. A., M. Schwartz, H. van Bokhoven, T. J. van de Pol, L. Bogerd, A. J. Pinckers, E. M. Bleeker-Wagemakers, I. H. Pawlowitzki, K. Ruther, H. H. Ropers and F. P. Cremers (1997). "Molecular basis of choroideremia (CHM): mutations involving the Rab escort protein-1 (REP-1) gene." Hum Mutat **9**(2): 110-117.
van den Hurk, J. A., D. J. van de Pol, B. Wissinger, M. A. van Driel, L. H. Hoefsloot, I. J. de Wijs, L. I. van den Born, J. R. Heckenlively, H. G. Brunner, E. Zrenner, H. H. Ropers and F. P. Cremers (2003). "Novel types of mutation in the choroideremia (CHM) gene: a full-length L1 insertion and an intronic mutation activating a cryptic exon." Hum Genet **113**(3): 268-275.
van der Burgh, R., N. M. Ter Haar, M. L. Boes and J. Frenkel (2013). "Mevalonate kinase deficiency, a metabolic autoinflammatory disease." Clin Immunol **147**(3): 197-206.
Vavvas, D. G., A. B. Daniels, Z. G. Kapsala, J. W. Goldfarb, E. Ganotakis, J. I. Loewenstein, L. H. Young, E. S. Gragoudas, D. Eliott, I. K. Kim, M. K. Tsilimbaris and J. W. Miller (2016). "Regression of Some High-risk Features of Age-related Macular Degeneration (AMD) in Patients Receiving Intensive Statin Treatment." EBioMedicine **5**: 198-203.
Vieira, O. V., C. Bucci, R. E. Harrison, W. S. Trimble, L. Lanzetti, J. Gruenberg, A. D. Schreiber, P. D. Stahl and S. Grinstein (2003). "Modulation of Rab5 and Rab7

recruitment to phagosomes by phosphatidylinositol 3-kinase." Mol Cell Biol **23**(7): 2501-2514.
Wang, K. C. and S. Ohnuma (2000). "Isoprenyl diphosphate synthases." Biochim Biophys Acta **1529**(1-3): 33-48.
Wang, M. and P. J. Casey (2016). "Protein prenylation: unique fats make their mark on biology." Nat Rev Mol Cell Biol **17**(2): 110-122.
Wang, T. and W. Hong (2006). "RILP interacts with VPS22 and VPS36 of ESCRT-II and regulates their membrane recruitment." Biochem Biophys Res Commun **350**(2): 413-423.
Wavre-Shapton, S. T., I. P. Meschede, M. C. Seabra and C. E. Futter (2014). "Phagosome maturation during endosome interaction revealed by partial rhodopsin processing in retinal pigment epithelium." J Cell Sci **127**(Pt 17): 3852-3861.
Wavre-Shapton, S. T., T. Tolmachova, M. Lopes da Silva, C. E. Futter and M. C. Seabra (2013). "Conditional ablation of the choroideremia gene causes age-related changes in mouse retinal pigment epithelium." PLoS One **8**(2): e57769.
Weitz-Schmidt, G. (2002). "Statins as anti-inflammatory agents." Trends Pharmacol Sci **23**(10): 482-486.
Wenkel, H. and J. W. Streilein (2000). "Evidence that retinal pigment epithelium functions as an immune-privileged tissue." Invest Ophthalmol Vis Sci **41**(11): 3467-3473.
Whelan, J. P. and J. F. McGinnis (1988). "Light-dependent subcellular movement of photoreceptor proteins." J Neurosci Res **20**(2): 263-270.
Whyte, D. B., P. Kirschmeier, T. N. Hockenberry, I. Nunez-Oliva, L. James, J. J. Catino, W. R. Bishop and J. K. Pai (1997). "K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors." J Biol Chem **272**(22): 14459-14464.
Wiemer, A. J., R. J. Hohl and D. F. Wiemer (2009). "The intermediate enzymes of isoprenoid metabolism as anticancer targets." Anticancer Agents Med Chem **9**(5): 526-542.
Winter-Vann, A. M. and P. J. Casey (2005). "Post-prenylation-processing enzymes as new targets in oncogenesis." Nat Rev Cancer **5**(5): 405-412.
Wood, W. G., G. P. Eckert, U. Igbavboa and W. E. Muller (2010). "Statins and neuroprotection: a prescription to move the field forward." Ann N Y Acad Sci **1199**: 69-76.
Wright, A. F., C. F. Chakarova, M. M. Abd El-Aziz and S. S. Bhattacharya (2010). "Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait." Nat Rev Genet **11**(4): 273-284.
Wu, T., M. Fujihara, J. Tian, M. Jovanovic, C. Grayson, M. Cano, P. Gehlbach, P. Margaron and J. T. Handa (2010). "Apolipoprotein B100 secretion by cultured ARPE-19 cells is modulated by alteration of cholesterol levels." J Neurochem **114**(6): 1734-1744.
Yamamoto, S., K. C. Sippel, E. L. Berson and T. P. Dryja (1997). "Defects in the rhodopsin kinase gene in the Oguchi form of stationary night blindness." Nat Genet **15**(2): 175-178.
Yan, D., P. K. Swain, D. Breuer, R. M. Tucker, W. Wu, R. Fujita, A. Rehemtulla, D. Burke and A. Swaroop (1998). "Biochemical characterization and subcellular localization of the mouse retinitis pigmentosa GTPase regulator (mRpgr)." J Biol Chem **273**(31): 19656-19663.

Yeo, J. C., A. A. Wall, L. Luo and J. L. Stow (2016). "Sequential recruitment of Rab GTPases during early stages of phagocytosis." Cell Logist **6**(1): e1140615.
Young, R. W. (1967). "The renewal of photoreceptor cell outer segments." J Cell Biol **33**(1): 61-72.
Young, R. W. and D. Bok (1969). "Participation of the retinal pigment epithelium in the rod outer segment renewal process." J Cell Biol **42**(2): 392-403.
Youssef, P. N., N. Sheibani and D. M. Albert (2011). "Retinal light toxicity." Eye (Lond) **25**(1): 1-14.
Zetterstrom, R. (2009). "The 1964 Nobel Prize for the discovery of the biosynthesis of cholesterol." Acta Paediatr **98**(7): 1223-1227.
Zhang, F. L. and P. J. Casey (1996). "Protein prenylation: molecular mechanisms and functional consequences." Annu Rev Biochem **65**: 241-269.
Zhang, H., C. Hanke-Gogokhia, L. Jiang, X. Li, P. Wang, C. D. Gerstner, J. M. Frederick, Z. Yang and W. Baehr (2015). "Mistrafficking of prenylated proteins causes retinitis pigmentosa 2." FASEB J **29**(3): 932-942.
Zhang, K., R. Fishel Ben Kenan, Y. Osakada, W. Xu, R. S. Sinit, L. Chen, X. Zhao, J. Y. Chen, B. Cui and C. Wu (2013). "Defective axonal transport of Rab7 GTPase results in dysregulated trophic signaling." J Neurosci **33**(17): 7451-7462.
Zheng, W., N. Mast, A. Saadane and I. A. Pikuleva (2015). "Pathways of cholesterol homeostasis in mouse retina responsive to dietary and pharmacologic treatments." J Lipid Res **56**(1): 81-97.
Zheng, W., R. E. Reem, S. Omarova, S. Huang, P. L. DiPatre, C. D. Charvet, C. A. Curcio and I. A. Pikuleva (2012). "Spatial distribution of the pathways of cholesterol homeostasis in human retina." PLoS One **7**(5): e37926.
Zhou, J., S. H. Tan, V. Nicolas, C. Bauvy, N. D. Yang, J. Zhang, Y. Xue, P. Codogno and H. M. Shen (2013). "Activation of lysosomal function in the course of autophagy via mTORC1 suppression and autophagosome-lysosome fusion." Cell Res **23**(4): 508-523.
Zhou, Q., E. Weis, M. Ye, S. Benjaminy and I. M. MacDonald (2013). "An internet-based health survey on the co-morbidities of choroideremia patients." Ophthalmic Physiol Opt **33**(2): 157-163.
Zhou, R. and R. R. Caspi (2010). "Ocular immune privilege." F1000 Biol Rep **2**.