INVESTIGATING DISEASE PRESENTATION AND MECHANISM IN *RP1L1*-ASSOCIATED PHOTORECEPTOR DEGENERATION

by

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ABSTRACT

Photoreceptor disease results in irreparable vision loss and blindness, which has a dramatic impact on quality of life. Retinitis pigmentosa 1-like 1 (RP1L1) is a component of the photoreceptor axoneme, the backbone structure of the photoreceptor's light-sensing outer segment. Pathogenic variants in *RP1L1* lead to a cone disease called occult macular dystrophy (OMD) and a rod disease known as retinitis pigmentosa (RP). This indicates that RP1L1 has important, but distinct, roles in rod and cone biology, though its exact function is unknown. In this thesis, I summarize reported *RP1L1*-associated photoreceptor conditions and report novel associations between *RP1L1* and additional photoreceptor conditions outside of OMD and RP. I found that OMD can progress to an *RP1L1* maculopathy with visible fundus findings in patients with the most common OMD-causing *RP1L1* mutation. Further, I report *RP1L1* maculopathies with novel *RP1L1* variants which may have initially started as OMD and progressed to more severe macular degenerations. In addition, *RP1L1* was associated with a rod-cone dystrophy. This is the first report of simultaneous rod and cone disease due to *RP1L1* mutations.

Zebrafish are an instrumental system for the generation of photoreceptor degeneration models, which can be utilized to determine underlying causes of photoreceptor dysfunction and death. To investigate the potential function of RP1L1 in photoreceptors and model *RP1L1*-associated disease, I generated an *rp1l1* zebrafish mutant using CRISPR/Cas9 genome editing. The introduced *rp1l1* mutation is predicted to severely truncate the Rp1l1 protein and abolish all functional domains. Homozygous *rp1l1* mutant zebrafish had progressive photoreceptor functional defects that began with the rod photoreceptors, as determined by electroretinographic assessment. Live imaging via optical coherence tomography revealed that *rp1l1* homozygotes had gaps in the photoreceptor layer, disrupted photoreceptor mosaics, and thin retinas, indicative of outer retinal degeneration. Mutant photoreceptor outer segments were disorganized and lacked uniformity, with wavy discs, and in some cases appeared extremely abnormal with outer segment

vesicles or atypical morphology. Intriguingly, the *rp1l1* mutants also had lipid-rich subretinal deposits between the photoreceptors and retinal pigment epithelium. The *rp1l1* mutant is a novel model of *RP1L1*-associated photoreceptor disease and the first zebrafish model of photoreceptor degeneration with reported subretinal drusenoid-like deposits, a feature of age-related macular degeneration. This model could be further utilized to determine the mechanism underlying photoreceptor degeneration in *RP1L1*-associated RP. Additionally, as a unique zebrafish model of photoreceptor disease with subretinal lipid deposits, these *rp1l1* mutants could be further utilized to inform etiology of ocular deposits observed in other retinal degenerative conditions.

PREFACE

This thesis is an original work by Nicole C. L. Noel.

Approval for this study was obtained from the Animal Care and Use Committee: BioSciences, under protocol AUP00000077.

A portion of Chapter 1 is modified from the publication: Nicole C. L. Noel, Ian M. MacDonald, and W. Ted Allison. (2021) *Zebrafish models of photoreceptor dysfunction and degeneration*. Biomolecules, 11(1): 78.

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Chapter 3 is modified from the published manuscript: Nicole C. L. Noel, Nathan J. Nadolski, Jennifer C. Hocking, Ian M. MacDonald, and W. Ted Allison. (2020) *Progressive photoreceptor dysfunction and age-related macular degeneration-like features in* rp1l1 *mutant zebrafish*. Cells, 9(10): 2214.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS AND INITIALISMS

aa	amino acid
AMD	age-related macular degeneration
AOS	accessory outer segment
bp	base pair
Cas	CRISPR-associated protein
CC	connecting cilium
cDNA	complementary DNA
CMZ	ciliary marginal zone
CNS	central nervous system
CORD	cone-rod dystrophy
CRISPR	clusters of regularly interspaced short palindromic repeats
dpf	days post fertilization
EdU	5-Ethynyl-2´-deoxyuridine
EM	electron microscopy
ERG	electroretinogram
ESC	embryonic stem cell
EZ	ellipsoid zone
gDNA	genomic DNA
GFP	green fluorescent protein
gRNA	guide RNA
H&E	haematoxylin and eosin
HDACs	histone deacetylases
hpf	hours post fertilization
IFT	intraflagellar transport

INL	inner nuclear layer
IPL	inner plexiform layer
iPSC	induced pluripotent stem cell
IS	inner segment
IZ	interdigitation zone
LCA	Leber congenital amaurosis
lws	long wavelength sensitive
mCFP	membrane-targeted cyan fluorescent protein
MNU	*N*-methyl-*N*-nitrosourea
MTZ	metronidazole
OCT	optical coherence tomography
OKR	optokinetic response
OMD	occult macular dystrophy
OMR	optomotor response
ONL	outer nuclear layer
OPL	outer plexiform layer
OS	outer segment
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RETGC1	retinal guanylate cyclase-1
RFLP	restriction fragment length polymorphism
rh2	rhodopsin 2
rho	rhodopsin
RP	retinitis pigmentosa
RP1	retinitis pigmentosa 1

RP1L1	retinitis pigmentosa 1-like 1
RPE	retinal pigment epithelium
SNP	single nucleotide polymorphisms
sws	short wavelength sensitive
TALEN	transcription activator-like effector nuclease
Tg	transgene/transgenic
UV	ultraviolet
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VMR	visual motor response
WT	wild-type
XOPS	*Xenopus* rhodopsin
ZFN	zinc finger nuclease

1 INTRODUCTION TO PHOTORECEPTORS AND ZEBRAFISH MODELS OF PHOTORECEPTOR DISEASE

This chapter contains content modified from the published manuscript: Nicole C. L. Noel, Ian M. MacDonald, and W. Ted Allison. (2021) *Zebrafish models of photoreceptor dysfunction and degeneration*. Biomolecules, 11(1): 78.

1.1 PHOTORECEPTORS AND THEIR DISEASES

Vision is possible due to specialized light-detecting cells in the eye called photoreceptors. The degeneration of photoreceptors or the retinal pigment epithelium (RPE), essential retinal support cells, results in permanent vision loss and blindness. Photoreceptor diseases are extremely phenotypically and genetically diverse, which creates challenges for development of treatments to prevent or reverse vision loss [1]. Photoreceptors are sensory neurons responsible for converting light into signals that are transmitted to the brain for interpretation, allowing the perception of visual information. There are two types of retinal photoreceptors, named after their distinct morphologies: cones, which are maximally sensitive to particular wavelengths of light; and rods, which are extremely sensitive to low levels of light and can detect single photons. Cones allow for high-acuity daytime and colour vision, while rods facilitate vision under dim-light and night conditions. Rods and cones have an outer segment (OS), the light-sensitive part of the photoreceptor; an inner segment (IS), a specialized compartment packed with mitochondria that is connected to the OS by a structure called the connecting cilium (CC); a cell body; and a synaptic terminal, where downstream photoreceptors innervate downstream cells (Figure 1A). The OS is a modified cilium that is responsible for the photoreceptor names – cones have a cone-shaped OS,

while rods have a rod-shaped OS. The OS contains many membranous discs that are packed with opsin, the light-sensitive protein that triggers a signalling cascade, which results in signal transmission to downstream neurons. In cones, OS discs are contiguous with the plasma membrane, whereas rod OS discs are internalized within the plasma membrane.

The human retina has three cone photoreceptor subtypes: blue light-sensitive (*OPN1SW1* opsin gene), green light-sensitive (*OPN1MW1* opsin gene), and red light-sensitive (*OPN1LW1* opsin gene) cones [2,3]. The photoreceptors are organized such that the peripheral retina is rod-dense with cones interspersed throughout, and the central retina is comparatively cone-dense [4,5] (Figure 1B). This central region is called the macula, in the middle of which is a pit that contains exclusively cone photoreceptors, known as the fovea [6]. The fovea is important for high-acuity central vision. Fovea-like structures are rarely seen outside of primate species.

Photoreceptors are supported by the RPE. The RPE plays many essential roles in the retina, including maintenance of the blood-retina barrier, absorption of stray photons, transport of materials to and from the underlying choroid vessels, phagocytosis of shed photoreceptor OS fragments, and recycling of chromophores necessary for the visual cycle [7]. The RPE also secretes factors and signalling molecules important for photoreceptor function and neuroprotection, including pigment epithelium-derived factor (PEDF), which aids in photoreceptor survival [8–10].

Photoreceptor dysfunction and degeneration can be caused by mutations in many aspects of the photoreceptor cells, including structural factors, cilium components, phototransduction machinery, and ion channels [1]. In addition, RPE dysfunction leads to photoreceptor disease as the RPE has numerous vital roles in photoreceptor maintenance and function. The complexities of photoreceptor development, function, and maintenance can result in extreme genetic and phenotypic heterogeneity for photoreceptor conditions. Moreover, photoreceptor disease can manifest as part of multi-system conditions, such as ciliopathies [11].

In the following sections, photoreceptor diseases with a genetic component are described. Inherited photoreceptor diseases cumulatively affect 1/2000 to 1/3000 individuals, and age-related macular degeneration, a multifactorial condition with a genetic component, is the leading cause of vision loss in the ageing population [12–14]. Vision loss has an immense impact on quality of life, and unfortunately these conditions have few effective therapies. This necessitates animal models of photoreceptor disease, as targeted therapeutics cannot be developed without an understanding of disease pathology and underlying mechanisms.

1.1.1 RETINITIS PIGMENTOSA

Retinitis pigmentosa (RP) is a group of rod degenerative diseases, characterized by night blindness and peripheral vision loss. RP is the most common inherited photoreceptor condition, affecting 1/3500 to 1/5000 individuals. During RP progression, the RPE becomes degenerate and pigment granules can translocate into the retina, resulting in a feature called bone spicule. Cones are spared initially in RP, but eventually degenerate, leading to total blindness. Over 70 genes have been associated with RP, and RP can be inherited in an autosomal recessive, dominant, X-linked, multigenic, or mitochondrial manner [1]. Additionally, RP can be caused by mutations that directly affect the rod photoreceptors or the RPE. RP can manifest alone or in combination with extraocular phenotypes in syndromes.

1.1.2 LEBER CONGENITAL AMAUROSIS

Leber congenital amaurosis (LCA) is a severe, early-onset disease of the RPE and photoreceptors. LCA is caused by mutations in essential RPE and photoreceptor genes. The genes most commonly mutated in LCA patients are *RPE65*, *GUCY2D*, and *CEP290* [15–18]. The *RPE65* gene produces an RPE-specific retinoid isomerohydrolase, which is essential for recycling of components required for the phototransduction cascade [19]. *GUCY2D* produces the protein retinal guanylate cyclase-1 (RETGC1), which is also needed for phototransduction [20,21].

CEP290 encodes a centrosomal protein important for development of the centrosome and cilium [22].

1.1.3 CHOROIDEREMIA

Choroideremia is an X-linked condition characterized by degeneration of the photoreceptors, RPE, and underlying choroid vasculature. Choroideremia is caused predominantly by mutations in the gene *CHM*, which encodes Rab escort protein 1 (REP1) [23]. REP1 posttranslationally modifies Rab proteins, which are involved in membrane trafficking; protein modifications are essential for Rab function. Defects in Rab protein function would impact processes in many retinal cell types, but there is evidence from animal models that suggest it is specifically the REP1-related RPE defects that lead to photoreceptor degeneration in choroideremia [24,25].

1.1.4 CONE-ROD DYSTROPHY

Cone-rod dystrophies (CORD) are conditions where both the cones and rods are affected, with cone disease manifesting prior to rod disease. CORD patients can present with visual acuity loss, light sensitivity, colour vision defects, central vision loss, and partial peripheral vision loss.

1.1.5 ENHANCED S-CONE SYNDROME

Enhanced S-cone syndrome is a unique condition, as it presents with increased photoreceptor function. Enhanced S-cone syndrome is caused by mutations in the transcription factors *NR2E3* (*nuclear receptor subfamily 2 group E member 3*) or *NRL* (*neural retina-specific leucine zipper*), which are required for rod development [26–29]. Patients with enhanced S-cone syndrome therefore have cone-only retinas with enhanced blue cone sensitivity and experience night blindness and photoreceptor degeneration.

1.1.6 CONE DYSTROPHY

Cone dystrophies are conditions that affect the cone photoreceptors, leading to their dysfunction and degeneration. Cone dystrophies typically present with visual acuity defects, colour vision abnormalities, and reduced cone responses, measured by electroretinography (ERG). Although extremely rare, there are reported cases of peripheral cone dystrophies, whereby the cones in the peripheral retina are more affected than those in the central macula [30].

Macular degenerations involve loss of cones in the macula and leads to visual acuity decline, colour vision disturbances, and central vision loss. Cone loss can result in macular atrophy, which is visible on routine fundoscopy imaging. There are many different types of macular degeneration, the most common of which is age-related macular degeneration (AMD).

AMD is the leading cause of vision loss in the ageing population [12]. AMD is a multifactorial condition, and a combination of genetic and environmental factors influence a person's likelihood of developing AMD. There are two forms of AMD: dry and wet. Dry AMD is the most common, characterized by geographic atrophy that results from the degeneration of the RPE, photoreceptors, and choroid vessels. Dry AMD can progress to wet AMD, which is characterized by choroidal neovascularization. The aberrant vessels are fragile and prone to leaking or bursting, which can lead to fluid buildup and retinal scarring. Deposits, called drusen and subretinal drusenoid deposits, are hallmark features of AMD. Drusen develop between the RPE and underlying choroid vasculature, while subretinal drusenoid deposits occur between the photoreceptor OSs and RPE. Although characteristic features of AMD, the impact that drusen and subretinal drusenoid deposits have on the retina and photoreceptor biology is unclear.

1.1.7 ACHROMATOPSIA

Achromatopsia is a condition characterized by lack of colour vision. Achromatopsia can either be complete or incomplete; individuals with complete achromatopisa have no functioning cone photoreceptors, while those with incomplete achromatopsia have partial cone function.

Achromatopsia is generally caused by mutations in cone-specific phototransduction machinery or ion channels that prevent the cones from relaying messages to downstream neurons [31,32].

1.2 PHOTORECEPTOR DISEASE TREATMENTS + CHALLENGES

Due to the diversity of photoreceptor and RPE diseases, developing treatments to prevent or reverse vision loss has been challenging. However, there are some promising therapeutic avenues.

1.2.1 GENE THERAPY

Gene therapy is a potential therapeutic option for inherited blinding conditions caused by mutations in a single identified gene, particularly when disease is caused by nonsense mutations or an inadequate quantity of gene product. Gene therapy involves introduction of a functional gene copy; this must be accomplished using a delivery vector, typically viral vectors. The specific viral vector can be selected based on its genomic capacity, immunogenicity, ability to transduce the target cell type, and whether genetic integration is a desired outcome or not. Although visual recovery post gene therapy is often minimal, gene therapy does have some potential to reverse vision loss when the photoreceptors are thriving but dysfunctional.

Although an encouraging therapeutic avenue, gene therapy has its limitations. It is ideal for single gene conditions with nonsense mutations, but not for disease caused by gain-of-function mutations or conditions with multigenic inheritance. Viral vectors also pose unique challenges, as vectors that were considered non-immunogenic and commonly utilized for gene therapy approaches have been reported to trigger inflammatory responses [33–35]. In addition, the genetic heterogeneity of photoreceptor disease means that many conditions will not have a gene therapy option in the foreseeable future, as gene therapy development and testing is expensive and time consuming.

1.2.2 CELL TRANSPLANTATION

1.2.2.1 PHOTORECEPTOR PRECURSOR TRANSPLANTATION

Transplantation of photoreceptor precursor cells is a promising therapy to replace degenerated photoreceptors and restore vision. Precursor cells are limited in the number of divisions they can undergo and cell types they can produce, which makes it unlikely for unwanted cell types to be generated. Photoreceptor precursor transplantation is applicable to many photoreceptor diseases regardless of genetic etiology and is one of the few approaches that has the potential to restore sight after significant cellular loss. Development of human induced pluripotent stem cells (iPSCs) and retinal organoids is encouraging for the production of retinal stem cells and photoreceptor progenitor cells that could be utilized for transplantation into damaged retinas, however patient-derived cells – which have the lowest risk of rejection – would require genome editing or gene therapy approaches to overcome pathogenic disease-causing mutations. Nevertheless, although it is a therapeutic ideal, generation of stem cells from individual patients would require much time and space and be extremely expensive and is likely not feasible.

Donor photoreceptor precursor cells have been reported to survive, appropriately localize, mature, and integrate into host retina [36–45]. However, it is difficult to assess whether introduced cells that appear to localize correctly form appropriate neural connections *in vivo*; part of this is due to the complexities of the retinal synaptic layers, retinal remodelling that can occur during disease, and challenges assessing functional output of specific introduced cells. A unique confound to research on cellular integration in diseased retina is material transfer. In mice, transplanted photoreceptor precursors and host retinal cells can transfer material, resulting in host cells containing material only expressed in donor cells, or the opposite [46–48]. Whether material transfer occurs in the human retina is not known, but could pose a novel therapeutic strategy. Introduced cells could deliver essential material to the host photoreceptors, thereby

providing two simultaneous avenues of treatment – precursors that generate new photoreceptor cells as well as delivery of necessary components to the remaining photoreceptors.

1.2.2.2 RPE TRANSPLANTATION

Transplantation of RPE cells into the retina to replace areas of RPE atrophy and support the persisting photoreceptors is a potential treatment avenue to preserve photoreceptor function and survival, particularly for conditions with focal RPE dysfunction such as macular degenerations. Transplantation of RPE typically involves either subretinal injection of suspended embryonic stem cell (ESC)-derived RPE cells [49,50] or surgical insertion of an ESC-RPE monolayer via an implant [51,52]. Initial clinical trials for human ESC-derived RPE transplantation into macular degeneration patients have reported moderate success; the transplanted cells were successfully grafted, there was limited evidence of rejection, and improved visual acuity was reported in a subset of patients [49–52]. However, some individuals had adverse events, including retinal thinning [49] and vitreal inflammation [50]. A challenge of using RPE derived from ESCs is rejection; recipients of these treatments must be monitored closely for inflammatory response. Whether RPE transplantation will be effective at maintaining vision long-term is unknown and requires further follow-up.

1.2.3 CRISPR/CAS GENOME EDITING

CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated protein) genome editing has immense potential for treating a wealth of inherited diseases, including diverse ocular diseases. The CRISPR/Cas genome editing strategy utilizes a synthetic guide RNA (gRNA) and Cas protein. The gRNA contains a sequence-specific region that allows for gene targeting and specificity while the Cas protein cleaves the DNA. Cut DNA can subsequently be repaired by several mechanisms, most notably non-homologous end joining,

which typically leads to small deletions or insertions, or homology-directed repair, which uses a repair template to introduce specific genetic changes.

The CRISPR/Cas system has potential for treating genetic blinding diseases, although needs to be combined with other therapeutic avenues. Combining iPSC technology with CRISPR/Cas gene editing, transgenesis technology, or gene therapy approaches would facilitate use of patient cells to create photoreceptor progenitor cells without disease-causing mutations. In the case of CRISPR/Cas, cultured cells allow for sampling of iPSC subpopulations to assess off-target cutting. While using CRISPR/Cas on cultured patient iPSCs allows for thorough assessment of off-target activity, it is difficult to effectively determine whether non-target sequences have been manipulated without whole genome sequencing, which is time consuming, expensive, and not widely available.

Alternatively, CRISPR/Cas could be introduced into patient retina via a gene therapy delivery method, which has been accomplished in mice [53,54]. However, using CRISPR/Cas in endogenous cells comes with risks. CRISPR/Cas off-target activity is difficult to predict and could lead to disruption of important genes or oncogene activation. Additionally, delivery of CRISPR/Cas components to intact retinal tissue using viral vector could lead to integration of the *Cas* gene into the genome or persistence of Cas protein longer than is ideal. This may result in subsequent modification of non-target genes, which is an important consideration as whether some endogenous RNA molecules could be co-opted by Cas as gRNAs is unclear. Recently, a study reported that use of CRISPR/Cas on human embryos to edit the *EYS* gene – mutations in which lead to photoreceptor degeneration – can result in unrepaired DNA and loss of large chromosomal regions [55]. This is a major concern, particularly when CRISPR/Cas is used to generate stem cells or progenitor cells that could divide post ocular transplantation. Such extreme genomic changes post CRISPR/Cas editing have not been reported in non-dividing cells, but may occur. Nevertheless, genome editing of human cells has a lot of potential and therapeutic strategies involving CRISPR/Cas are under development.

1.2.4 DRUGS

The heterogeneity of photoreceptor diseases makes development and testing for pharmaceuticals challenging. Currently, the only condition with a proven drug treatment is wet AMD. Neovascularization in wet AMD can be prevented with drugs that prevent blood vessel growth, specifically anti-vascular endothelial growth factor (VEGF) therapies [56].

1.3 ZEBRAFISH AS A MODEL FOR PHOTORECEPTOR DISEASE

Zebrafish are a powerful model for photoreceptor development and disease. The zebrafish retina is remarkably conserved to the mammalian retina in terms of development, structure, and function. Researchers can study cone photoreceptor development and disease in zebrafish, which is challenging in nocturnal rodent models that have few cones. There are many tools for assessing zebrafish visual responses, photoreceptor function, and morphology in live animals. Additionally, there are well-established technologies for generating transgenic and mutant animals. These features are outlined below. For reviews that compare retinal features of different animal species used to model disease, see [57,58].

1.3.1 THE ZEBRAFISH VISUAL SYSTEM

Zebrafish have a visual system that develops rapidly and larval zebrafish can be assessed for visual function at early ages. Zebrafish embryos develop externally and embryos are transparent, meaning that visual system development can be easily observed. Eye patterning begins at 28 hours post fertilization (hpf), and the developing retina expresses opsin genes as early as 51 hpf [59,60]. At 5 days post fertilization (dpf), zebrafish can carry out complex visually-mediated behaviours such as prey capture [61], allowing for larvae to be assessed for visual defects very early in life.

The zebrafish retina is cone-rich, similar to the human macula. This is a huge benefit since other commonly used models, such as mice and rats, are nocturnal with few cones and no macula-

like region. The abundance of cone photoreceptors allows for modeling of cone diseases in zebrafish. However, there are nevertheless notable differences between zebrafish and humans. Zebrafish have double cones, which are not observed in the mammalian retina. Additionally, the zebrafish photoreceptor mosaic is organized differently than the human mosaic, and while it can model general cone dystrophies, cannot accurately recapitulate macular degeneration. Although zebrafish have uneven distributions of cones at some stages, their retina does not have a macular region with increased cones and a central cone-only foveal pit [62]. It is important to note that although zebrafish do not have a retinal region that is structurally similar to the fovea, larvae do have a region that is important for high-acuity vision and is therefore functionally similar to the human fovea [63].

The zebrafish photoreceptor mosaic is differentially organized between larval and adult stages. Zebrafish have four cone subtypes: ultraviolet (*sws1* opsin gene, homolog of mammalian *OPN1SW*), blue (*sws2*), green (*rh2*), and red (*lws*, homolog of mammalian *OPN1MW* and *OPN1LW*). The green and red cones exist as physically fused double cones. Larval zebrafish have a cone-dominant retina with few rods [62,64]. As the larval zebrafish age, they develop more rods, and in adulthood there are roughly equal proportions of cones and rods [62]. Adult zebrafish photoreceptors are organized into a highly structured row mosaic, with UV and blue cones alternating in their rows, and red and green cones alternating in neighbouring rows [62,65] (Figure 2A,B). Some opsins have multiple copies that are differentially expressed according to retinal location and age. For example, zebrafish have four green opsin genes (*rh2-1, rh2-2, rh2-3,* and *rh2-4*), two red opsin genes (*lws1* and *lws2*), and two rhodopsin genes (*rho* and *rhol*), which have different spatial localizations depending on developmental stage [66–68]. There are many tools available for visualizing zebrafish photoreceptors, including transgenic lines expressing fluorescent proteins in specific cell types and antibodies for immunolabelling (Figure 2B,C). A subset of commonly used transgenic lines (Table 1) and antibodies (Table 2) are highlighted here; available zebrafish tools are catalogued on the Zebrafish Information Network (ZFIN) [69]. The

well-characterized promoters in these transgenes can also be utilized to drive expression of other genes, such as disease or apoptotic genes.

1.3.2 VISUALLY-MEDIATED RESPONSES AS DIAGNOSTICS IN ZEBRAFISH

Visual function can be investigated in zebrafish larvae by several behavioural tests. Visually-mediated responses in zebrafish larvae include the optokinetic response (OKR), optomotor response (OMR), and visual motor response (VMR). OKR tracks the eye movements of the larvae in response to visual stimuli. OKR involves immobilizing a larvae and placing the animal inside of a drum with moving black and white stripes [70–73]. As the drum moves, the zebrafish's eyes will track the movement then saccade back to the origin. OKR can be detected as early as 3 dpf [72,73]. OMR uses a visual stimulus to elicit a swim response. Larvae are placed inside narrow tracks on top of a flat screen, a stimulus of moving lines plays on the screen, and the fish swim in the direction of the moving stimulus [73–75]. OMR is detectable as early as 4 dpf, but is more robust at 6–7 dpf [74]. VMR is a visually-mediated startle response triggered by rapid changes in light. Individual larvae are placed into wells of a 96-well plate and then the plate is inserted into a recording chamber with lights that can be turned on and off [76]. Larvae will become active when the lights come on, followed by returned to baseline; similarly, when the lights are turned off, there is an increase in activity followed by a return to baseline. VMR can be elicited from larvae as early as 4 dpf.

1.3.3 RETINAL IMAGING AND FUNCTIONAL ASSESSMENT FOR ZEBRAFISH

Zebrafish have many tools available for the assessment of photoreceptor health in live animals. These techniques include optical coherence tomography (OCT), ERG, calcium imaging, and fundoscopy.

OCT is a live imaging technique that uses the differential reflectivity of the various retinal cell types to capture images of the retinal layers and photoreceptor mosaic. Photoreceptor ISs are

highly reflective due to the abundance of densely packed mitochondria, thereby facilitating visualization of the photoreceptor layer [77]. Refractive properties of the zebrafish lens cause zebrafish OCT to be very high resolution, and individual photoreceptor ISs can be observed [78,79]. *En face* projections provide a view of the photoreceptor mosaic and the distinct photoreceptor rows.

Electroretinography is an electrophysiological method for assessing retinal function. ERG involves placing an electrode on the subject's eye and using light stimuli to elicit a response. The testing conditions and light stimuli can be modified to assess specific photoreceptor types. ERG has been utilized to assess both larval and adult zebrafish [80–84]. Calcium imaging techniques can also be used to assess photoreceptor activity in transgenic zebrafish larvae expressing a Ca^{2+} sensor fused to a fluorescent protein [85,86]. When Ca^{2+} is present in the cell, such as during neuronal activity, the protein undergoes a conformational change that increases fluorescence. This is a useful tool to assess whether photoreceptor cells are responding to stimuli, although it cannot easily be used in live adult zebrafish due to eye size and significant pigmentation.

Fundoscopy involves shining a light into the eye to observe the back of the eye, termed the fundus. Zebrafish fundoscopy enables visualization of adult zebrafish retinal cells in transgenic animals expressing fluorescent proteins [87]. This can be used to observe progressive photoreceptor degeneration and regeneration.

1.3.4 GENERATION OF TRANSGENICS AND MUTANTS

External fertilization allows for zebrafish embryos to be easily accessed during early development. This makes generation of transgenic or mutant animals straightforward, as constructs can be injected directly into the single-cell embryo [88]. The generation of transgenic zebrafish can be accomplished using transposase and Tol2 transposable element sites or I-SceI meganuclease [89–91]. However, some transgene sequences are susceptible to silencing in zebrafish. The Gal4/UAS transcriptional activation system is a gene/enhancer trap system; the

zebrafish-adapted version is KalTA4/UAS [92]. This involves the tissue-specific activation of the KalTA4 transcription factor, which then recognizes the UAS promoter and drives expression of the downstream transgene – for example, a fluorescent protein. Unfortunately, the UAS promoter becomes silenced throughout subsequent generations, and silencing can even be observed in individual fish; larvae may have high levels of expression, but expression can decrease drastically in adulthood due to silencing [93,94]. However, silencing seems to be a relatively uncommon occurrence when transgenes are generated using endogenous zebrafish promoter sequences. Promoter silencing has also created challenges for generation of conditional knockout lines in zebrafish. Cre-lox recombination utilizes Cre recombinase and lox target sites to create conditional loss-of-function alleles. Cre can be expressed ubiquitously or under tissue-specific promoters. Cre-lox has been successfully utilized in zebrafish and there are increasing numbers of tools and zebrafish Cre-driver lines [95–100]. However, it has been difficult to find tissue-specific promoters that are not silenced over time, as well as issues activating inducible Cre in zebrafish. In addition, it is challenging to insert lox sites around zebrafish target genes. As a result, conditional knockout zebrafish are difficult to generate and rare.

There are many means by which mutations have been introduced to zebrafish, including in forward genetic screens using mutagens such as ethylnitrosourea, or targeted gene mutations with zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas [101–104]. However, mutants isolated from forward genetic screens require extensive mapping in order to locate the affected gene(s), and some lines never have a mutated gene identified. ZFN and TALEN editing strategies require fusing a nuclease to a sequence-specific DNA binding domain to target genomic regions; this process can therefore be strenuous, but resulting mutants are unlikely to have off-target mutations introduced. CRISPR/Cas systems are easily adaptable to different target genes, but off-target cutting is difficult to predict, though there are methodologies that help limit off-target mutagenesis [105–108]. While there are many means by which to introduce mutations in target genes, there are nevertheless challenges

associated with the zebrafish genome. The teleost lineage underwent a genome duplication event, meaning that many genes have two copies. This creates complexities when making mutants, and functional redundancy between the paralogs can necessitate generation of double mutants. Conversely, there can be sub-specialization of the paralogs. It is not uncommon for one gene to be specific to cones while the other is either specific to rods, have pan-photoreceptor expression, or be expressed elsewhere in the retina or nervous system entirely [109–112].

1.3.5 REGENERATIVE RESPONSE

The mature human retina has very limited capacity to replace lost neurons, meaning that loss of any retinal neurons is irreversible and can lead to vision impairment. In contrast, zebrafish retinas have a robust regenerative capacity [113]. This is both a benefit and challenge, as it permits investigation into mechanisms of neuronal regeneration, but also means that the zebrafish retina responds differently to acute retinal lesions than the human system. Assessment of disease progression can be complicated by regeneration of lost photoreceptors; there may be cases where degeneration is underestimated due to regenerated photoreceptors restoring visual function. It is important for researchers to be aware of how regenerative responses may affect their assessment of retinal damage longitudinally. However, factors necessary for regenerative responses can be pharmacologically blocked or knocked down to allow for disease progression more comparable to humans [114–116], although blocking regeneration may cause additional issues with retinal biology. Potential regenerative responses are important to be aware of when investigating models for potential degeneration. Mechanisms underlying the zebrafish retinal regenerative response will be discussed further in Section 1.5.3.

1.4 ZEBRAFISH MODELS OF PHOTORECEPTOR DISEASE

There are many zebrafish models of photoreceptor disease, including models where degeneration is induced by exposure to light or toxins, transgenic lines expressing deleterious components, and mutants, which are reviewed here.

1.4.1 LIGHT ABLATION

Light ablation allows for photoreceptor ablation while the remaining neural retina stays relatively intact [113]. There are also differences in susceptibility of photoreceptor types depending on retina location, potentially due to the amount of light that reaches specific areas [117–122]. Light ablation typically causes generalized destruction of the photoreceptors, though targeted laser ablation can be performed to target specific photoreceptor types [123]. A robust regenerative response occurs post light ablation [123].

1.4.2 TOXIC LESIONS

1.4.2.1 OUABAIN

Retinal lesion can be achieved with injection of ouabain, which inhibits Na^+/K^+-ATPase and thereby reduces available ATP for cellular processes [124]. Dosage determines the retinal layers that are affected. High doses can penetrate far into the retina, damaging many retinal cell types including photoreceptors, while lower doses only reach inner retinal layers [124–126]. This method cannot easily be limited to only one cell type, although ouabain could be injected subretinally to cause more targeted photoreceptor and RPE damage. In zebrafish, ouabain has been used successfully to ablate retinal neurons, including photoreceptors, and the resultant damage stimulates a regenerative response [125].

1.4.2.2 N-METHYL-N-NITROSOUREA

N-methyl-*N*-nitrosourea (MNU) is an alkylating agent that transfers its methyl group to nucleic acids, resulting in DNA mutations and damage. It is a carcinogen, typically used to induce tumours in cancer models [127,128]. MNU treatment in animals induces photoreceptor cell death, presumably through DNA damage [129–131]. MNU treatment primarily affects the rod photoreceptors and therefore produces rod degeneration akin to RP [129]; the acute loss of rods results in a regenerative response in zebrafish. Why MNU treatment is more damaging to rods than cones is unknown.

1.4.3 HYPOXIA-INDUCED NEOVASCULARIZATION

Exposing adult zebrafish to a hypoxic environment results in retinal neovascularization [132]. Angiogenic sprouts begin to grow around day 2 and continue to grow throughout hypoxia treatment. Similar to wet AMD neovascularization, the aberrant vessel growth can be minimalized with anti-VEGF treatment. How the neovascularization impacts retina function in adult zebrafish is poorly investigated.

1.4.4 GENE KNOCKDOWN (MORPHOLINOS)

Morpholinos are antisense oligonucleotide analogs, usually 20-25 bases in length, which complement and bind mRNA to prevent the production of protein products. Morpholinos can either block mRNA splicing or translation [133,134]. They can be easily introduced to zebrafish embryos by microinjection at the single-cell stage. However, morpholinos can have off-target effects and morpholino experiments therefore need to be carefully controlled. It has been reported that morpholino phenotypes are often not recapitulated in genetic mutants [135]; the reason for this is unclear, but may be due to lack of genetic compensation. When there are genomic mutations, there is genetic compensation in response to the genetic lesions – however, this compensation does not occur in a gene knockdown scenario [136]. Despite their limitations,

morpholinos provide the opportunity for preliminary assessment of potential gene function when properly controlled, which is extremely valuable for expeditious assessment of putative disease-causing genes.

1.4.5 TRANSGENIC MODELS

1.4.5.1 XOPS:MCFP

The Tg(*XOPS:mCFP*) transgenic zebrafish line expresses membrane-targeted cyan fluorescent protein (mCFP) under the *Xenopus* rhodopsin promoter (*XOPS*) [137] (Table 3). The mCFP is overexpressed, resulting in toxicity and rod degeneration. Interestingly, there is no sign of cone death, despite rod loss. This differs from what is observed in humans with RP, where cone degeneration follows rod death. The reason for cone persistence despite rod degeneration in the Tg(*XOPS:mCFP*) zebrafish is unclear, though could be due to the cone-rich environment of the zebrafish retina, compared to the rod-rich human retina. The phenomenon of cone survival in zebrafish provides opportunities to investigate how cone photoreceptors respond to rod loss, and may provide insight into factors contributing to cone death in human RP.

1.4.5.2 NITROREDUCTASE ABLATION

Nitroreductase (NTR, *nfsB* gene) is a bacterial enzyme that can reduce otherwise inert prodrugs, such as metronidazole (MTZ), into DNA cross-linking agents, allowing for the targeted ablation of cells expressing NTR [138]. Importantly, there appears to be no toxic bystander effect as the result of NTR ablation, and neighbouring (non-NTR expressing) cells are not damaged by the treatment [75,139–141]. The NTR mechanism of ablation is thus one of the few methods that allows for ablation of targeted cell subtypes. Additionally, as NTR-fluorescent protein fusions have been made, the degeneration of target cells can be followed, and, where possible, regeneration of these cells can similarly be observed. It is also possible to delay, or prevent, the

regeneration of the target cell type through continued treatment with MTZ, since regenerating cells will undergo apoptosis once they begin to express NTR.

The NTR method has been used to ablate UV, blue, and red cones as well as rods in zebrafish [75,140–143]. Intriguingly, ablation of specific photoreceptor subtypes has revealed thresholds for triggering regenerative responses. In larval zebrafish, NTR-mediated blue cone ablation did not stimulate appreciable amounts of regeneration [143]. However, simultaneously ablating both blue and red cones using the NTR mechanism successfully induced robust regeneration. Conversely, ablating solely UV or red cones successfully stimulated a regenerative response [140,143,144]. Regenerated cones can be of multiple subtypes, and are not restricted to the ablated cone subtype [144]. How NTR ablation of specific cone subtypes triggers regenerative responses in the adult zebrafish retina has not been investigated. The ablation of rods in the adult zebrafish retina demonstrated that there is similarly a threshold of damage that needs to be reached to trigger regenerative responses. Ablating the vast majority of rod photoreceptors stimulated a regenerative response, but incomplete ablation of rods did not [141].

NTR ablation of RPE resulted in RPE degeneration as well as subsequent photoreceptor degeneration [145]; this is similarly observed in diseases of the RPE, where post RPE degeneration the photoreceptors also deteriorate. RPE loss by the NTR method triggered a regenerative response, and animals were able to re-establish an RPE monolayer. While regenerative capacity for retinal neurons in zebrafish is well established, this was the first study to show RPE regeneration post degeneration.

1.4.5.3 DISEASE GENES

Transgenesis technology allows for insertion of a disease-causing gene from humans or other species into the zebrafish genome. This can be used to generate models of photoreceptor disease, as well as investigate how the mutated protein behaves within and impacts photoreceptor cells.

There are several transgenic zebrafish models of RP-like photoreceptor disease (Table 3). Zebrafish with transgenically introduced mouse rhodopsin with a common RP mutation [146–149], Tg(*rho:msRho-P23H-flag*), have notable rod loss by early adulthood [150]. Similarly, a line expressing human rhodopsin with the RP-causing Q344X mutation in rod photoreceptors, Tg(*rho:hsaRHO-Q344X*), has rapid rod degeneration [151,152]. Adcy (adenylyl cyclase) is an enzyme normally found in the IS that is related to mechanisms of photoreceptor cell death; antagonists of Adcy increase photoreceptor survival in degeneration models [152]. An RP-like transgenic line that expresses Adcy2b with a C-terminal rhodopsin tail, targeting the protein to the OS, undergoes rod degeneration [152]. *PRPF31* (*pre-mRNA processing factor 31*) is involved in splicing of pre-mRNA and has been associated with RP [153]. Tg(*rho:prpf3-AD5-mCherry*) zebrafish express mutant *prpf31* in rods and have abnormal splicing for a subset of important photoreceptor genes and increased cell death in the photoreceptor layer [154]. A single nucleotide polymorphism in the *HTRA1* (*HtrA serine peptidase 1*) promoter region increases an individual's likelihood of developing AMD [155,156]. Overexpression of HTRA1 in mouse RPE has also been reported to induce choroidal neovascularization, similar to what is observed in AMD [157]. Zebrafish larvae transgenically expressing human *HTRA1* in rod photoreceptors have rod cell death [158]. Of interest, the zebrafish model of RP expressing human mutant rhodopsin have increased *htra1a* expression [158].

There is a transgenic CORD model with mutant human *GUCY2D*, which produces the protein retinal guanylate cyclase-1 (RETGC1) [159] (Table 3). Cones first degenerate in larval zebrafish, followed by rod degeneration. In early adulthood, rods look dysmorphic, but whether rods continue to degenerate in aged adult retina has not been reported.

1.4.6 GENETIC MUTANTS

Genetic screens and genome editing strategies have fostered the discovery and generation of many zebrafish mutants with photoreceptor phenotypes. Of note, there are instances where the

disease phenotype in zebrafish differs from what is observed in human patients with mutations in the same gene. This phenomenon may be due to disparities in the type of genetic lesions present; loss-of-function mutations are most commonly engineered in animal models, but disease-causing mutations observed in humans may instead be missense, splice site mutations, or compound. Alternatively, these divergences could be related to gene duplication and subspecialization/neospecialization in zebrafish, or differences between the human and zebrafish photoreceptor mosaic.

Mutations in genes affecting cilia or other proteins expressed in many tissue and cell types may have extraretinal phenotypes and early lethality. Only the photoreceptor phenotypes will be focused on here. For previous reviews on zebrafish models of ocular disease, please see [160–164].

Please note that the following zebrafish mutants are categorized based on the condition that their phenotype is most similar to, and this may not necessarily align with the phenotypes observed in human patients with mutations in the same gene. In order to be included here, the zebrafish mutant lines had to have an identified mutated gene and present with photoreceptor degeneration, photoreceptor dysfunction, or otherwise fit into a photoreceptor condition category based on retinal findings.

1.4.6.1 RETINITIS PIGMENTOSA

Mutations in the rod-specific opsin gene, rhodopsin (*RHO*), are a frequent cause of RP [146,148,149,165]. Recently, several *rho* mutant zebrafish lines have been generated which model dominant or recessive RP [166,167] (Table 4 for list of zebrafish models of RP). These animals have rod degeneration beginning soon after rod development and continuing into adulthood [166,167]. Cones are unaffected in *rho* mutants.

RP2 is a cilium-associated protein and its mutation causes X-linked RP [168–171]. Knockout of *rp2* in zebrafish results in photoreceptor functional defects in larval animals, as well as progressive rod OS degeneration, followed by cone OS degeneration [172]. Mutations in *ceramide*

kinase-like (*CERKL*), a gene involved in metabolism of components important for neuron survival, can cause RP [173]. Zebrafish *cerkl* knockout larvae have photoreceptor defects detected by electrophysiological assessment [174]. Young adults have OS defects, primarily affecting the rods, which progresses to rod degeneration and eventual cone OS defects. *KIF3B* encodes a kinesin motor involved in transport through the cilium and mutations in *KIF3B* have recently been associated with a ciliopathy that presents with RP [175]. *kif3b* mutant zebrafish have rapid rod degeneration and delayed OS genesis, but cones appear normal [176,177]. The retinitis pigmentosa GTPase regulator-interacting protein 1 (RPGRIP1) localizes to the CC and OS and mutations in *RPGRIP1* cause several different photoreceptor diseases, including LCA, RP, and CORD [178–181]. Zebrafish *rpgrip1* mutants do not have proper rod OS development, and no rods are observed at 5 dpf [182]. Rod cells were almost entirely absent at 3 months of age. Cones appear normal at 7 dpf but have functional defects, and cone degeneration is clear by 6 months. By 23 months, almost all photoreceptors are lost. Interestingly, double cones degenerate before single cones. *Retinitis pigmentosa 1-like 1* (*RP1L1*) is a photoreceptor cilium-associated protein, mutations in which lead to RP and maculopathies [183–185]. Mutant *rp1l1* zebrafish have rod functional defects at 6 months, which worsen over time [186]. By 12 months, animals have disorganized rod OSs, photoreceptor loss, and subretinal lipid deposits. (This model was developed and characterized as part of this thesis; see Chapter 3 for additional information.)

MYO7A encodes a myosin protein expressed in human photoreceptors and RPE [187]. *MYO7A* mutations lead to a subtype of Usher syndrome, a condition characterized by hearing and vision loss [188,189]. *myo7aa* mutant larvae have decreased photoreceptor function and rod photoreceptor loss [190]. Mutations in *USH2A* also cause a subtype of Usher syndrome [191]. *USH2A* encodes the transmembrane protein usherin, the exact function of which is unknown [191,192]. *ush2a* mutant zebrafish have photoreceptor functional defects in larvae, and progressive photoreceptor degeneration in adulthood [193,194]. Rods begin to degenerate prior to cone degeneration [193].

Hairy-related 9 (*her9*) is a transcriptional regulator that has increased expression in Tg(*XOPS:mCFP*) retinas undergoing rod degeneration [195]. *her9* mutant larvae have a lower abundance of rods and there is evidence that red cones do not develop appropriately or rapidly degenerate [196]. After rods begin to decrease in number, green cones are reduced in number and appear to have small OSs, while blue and UV cones appear unaffected. Animals do not survive past 13 dpf. *her9* orthologs have not been associated with human photoreceptor disease.

1.4.6.2 LEBER CONGENITAL AMAUROSIS

Mutations in genes involved in ciliogenesis initiation, cilia elongation, transport of cilium components, or photoreceptor physiological processes can result in LCA or an LCA-like phenotype in animal models. Intraflagellar transport (IFT) proteins play crucial roles in movement of cargo in the cilium, which is facilitated by kinesin and dynein motors. *ift88* and *ift172* zebrafish mutants have no photoreceptor OS development and the photoreceptors degenerate [197–200] (Table 5 lists LCA zebrafish models). Sensory cilia in general do not develop in these animals [199]. Similarly, *cluap1* (also known as *ift28*) mutant zebrafish have ciliogenesis defects that result in no OS development and rapid photoreceptor degeneration [201]. Fish with mutations in *ift57* develop photoreceptor OSs, although they are shorter than normal, and photoreceptors degenerate soon after developing [200,202]. *ift122* mutant zebrafish have normal photoreceptor development, but similarly undergo degeneration [203]. Zebrafish *kif3a* (kinesin family 3a) mutant larvae fail to develop photoreceptor OSs, the photoreceptors rapidly degenerate, and the animals have an extinguished ERG [177,204,205]. *NSF attachment protein beta* (*napbb*) encodes a protein important for synaptic vesicle fusion [206] and *napbb* mutant photoreceptors undergo cell death almost immediately after specification [207,208]. No photoreceptor layer is detectable at early larval stages, with only a few photoreceptors observed at the ciliary margin [207,208]. *IFT88*, *CLUAP1*, *IFT57*, *IFT122*, *KIF3A*, and *NAPB*, mutations

have not been associated with human photoreceptor disease, likely because of essential roles in development. *IFT172* mutations have been associated with RP [209].

KIAA0586 is a basal body component required for ciliogenesis and *KIAA0586* mutations underlie a ciliopathy called Joubert syndrome, which presents with brain abnormalities and photoreceptor degeneration [210]. Developing photoreceptors in *kiaa0586* mutant zebrafish have abnormal OS development and degenerate rapidly after development [211]. Mutant larvae have significantly reduced ERG responses. Transmembrane protein 216 (TMEM216) is required for cilia assembly and *TMEM216* mutations also cause Joubert syndrome [212,213]. Knockout of *tmem216* results in decreased cone OS development, short OSs, disorganized OS discs, and photoreceptor degeneration [214]. *Growth differentiation factor 6* (*GDF6*) encodes a morphogen and its mutation causes LCA and juvenile RP in humans [215]. *gdf6a* mutant zebrafish larvae are functionally blind at 7 dpf as determined by OMR, and have short, dysmorphic cone OSs and ISs, as well as overgrown, disorganized rod OSs and short ISs [215,216]. Photoreceptors do not undergo degeneration in these animals [216].

1.4.6.3 CHOROIDEREMIA

Zebrafish *rep1* knockout larvae have RPE that lacks uniformity with hypertrophic and de-pigmented regions [24] (Table 6). Additionally, the RPE has large vacuoles and accumulation of undigested OS fragments. The photoreceptor layer is disorganized with dysmorphic, functionally defective photoreceptors. The photoreceptor abnormalities appear to result from RPE dysfunction in these animals, as wild-type photoreceptors transplanted into mutant retina still become dysmorphic and degenerate, while *rep1* mutant photoreceptors transplanted into wild-type retinas are morphologically normal. *rep1* mutant zebrafish do not survive to adulthood.

1.4.6.4 CONE-ROD DYSTROPHY

Coiled-coil and C2 domain containing 2A (*CC2D2A*), *Abelson helper integration site 1* (*AHI1*), and *ADP-ribosylation factor-like GTPase 13B* (*ARL13B*) encode basal body or cilia-related proteins and their mutation causes forms of Joubert syndrome [217–219]. *cc2d2a* knockout zebrafish larvae have disorganized photoreceptor OSs and functional defects [220] (see Table 6 for list of zebrafish CORD mutants). Of interest, cilia assembly occurs normally in mutants, but trafficking of cilia components is impaired. Zebrafish larvae with *ahi1* mutations have abnormal cone OSs but normal visual function, as assessed by OKR [221]. Cone OS morphology recovers by early adulthood, but cone morphology defects and degeneration are seen in 5-month-old animals. At this age, rod photoreceptors have mislocalization of rhodopsin. Zebrafish a*rl13b* mutant larval photoreceptors have short OSs, however the *arl13b* mutants do not survive past 9 dpf – to assess the impact of *arl13b* mutation on photoreceptors in aged animals, transplantation experiments were performed [222]. Transplanted *arl13b* mutant photoreceptors had noticeable degeneration by 30 dpf. LCA5 is also a ciliary protein and mutations in the *LCA5* gene cause LCA [223]. However, knockout of zebrafish *lca5* results in a CORD phenotype rather than LCA [224]. *lca5* mutant larvae have decreased photoreceptor responses, with cone OS abnormalities obvious at 1 month of age and observed rod OS defects at 7 months. Mutations in *PCARE*, a regulator of photoreceptor cilium actin, cause RP [225]. Zebrafish larvae with mutations in *pcare1* have abnormal OS morphology, photoreceptor functional defects, and decreased OKR [226]. Adult *pcare1* mutants have dysmorphic photoreceptor OSs and thin photoreceptor layers, indicative of degeneration. *Eyes shut* (*EYS*) mutations typically cause RP in human patients, although *EYS* mutations have also been associated with a CORD [227–229]. The function of EYS is unknown, but a recent study found evidence that EYS localizes to the photoreceptor cilium and may be involved in cilium stability [230]. Zebrafish *eys* mutants have CORD, with cone degeneration in 6-month-old animals and

rod degeneration at 8-14 months [231–233]. *Barbet-Biedl syndrome 2* (*BBS2*) is a basal body-associated protein necessary for proper ciliogenesis, and mutations in *BBS2* lead to RP and a ciliopathy called Bardet-Biedl syndrome [234,235]. Larval *bbs2* mutants have photoreceptor deficits observed by OKR and short, disorganized photoreceptor OSs [236]. In adulthood, photoreceptor degeneration is observed, along with reported regeneration of rods but no appreciable regeneration of cones.

Potassium inwardly-rectifying channel subfamily J member 13 (*KCNJ13*) encodes a channel protein found in the RPE and mutations in *KCNJ13* lead to LCA [237]. Adult zebrafish with mutated *kcnj13* have photoreceptor loss and RPE abnormalities by 6-12 months [238]. Specifically, the RPE has increased phagosomes, enlarged mitochondria, and changes in melanosome localization, while cone photoreceptors have abnormal mitochondria and degenerate in aged animals. Protein O-linked-mannose beta-1,2-N-acetylglucosaminyl-transferase 1 (POMGNT1) is an enzyme that post-translationally modifies other proteins, and *POMGNT1* mutations cause RP [239]. Zebrafish with *pomgnt1* have cone and rod degeneration at 6 months of age [240].

1.4.6.5 ENHANCED S-CONE SYNDROME

A similar phenotype to human patients with enhanced S-cone syndrome is observed in *nr2e3* mutant zebrafish. Mutant animals lack rod photoreceptors and undergo progressive cone degeneration starting at 1 month [241] (Table 6). Interestingly, green and red cones degenerate while UV and blue cones persist. *nrl* mutant larvae also do not develop rods, but intriguingly have increased UV cone photoreceptors [242]. Adult *nrl* mutants unexpectedly have rods, but the rods have abnormal synapses. Whether these rods are functional is unknown.

1.4.6.6 CONE DYSTROPHY

Prominin 1 (PROM1) plays a critical role in OS disc morphogenesis and *PROM1* mutations cause a variety of photoreceptor diseases, such as RP, CORD, and macular degeneration [243–245]. Zebrafish with *prom1b* mutations have a cone degeneration, starting with red and green cones and affecting blue and UV cones soon after [246] (Table 7; see for list of zebrafish mutant models of cone dystrophy). While rods do not appear to degenerate in *prom1b* mutants, they have overgrown OSs. *CEP290* encodes a centrosomal protein and *CEP290* mutations are the most frequent cause of LCA [17]. Interestingly, *cep290* mutant zebrafish have cone OS disorganization and degeneration in adulthood that is severe by 12 months, but normal rod OS morphology [247]. Why *cep290* results in an adult-onset, cone-specific phenotype in zebrafish is unknown, but may be due to alternative photoreceptor centrosomal proteins in zebrafish.

Phosphodiesterase 6C (*PDE6C*) is an essential component of the cone phototransduction pathway and results in cone dystrophy when mutated [248]. *pde6c*-deficient zebrafish have rapid cone degeneration as well as dysmorphic, degenerate rods in larval stages [208,249]. In adulthood, mutants have severe cone degeneration but normal rod photoreceptors. Aryl hydrocarbon receptor interacting protein like 1 (AIPL1) is required for maintenance of photoreceptor phototransduction machinery and *AIPL1* mutations can lead to LCA, RP, and CORD [180,250,251]. Zebrafish have two copies of this gene: *aipl1a*, which is expressed in rods and potentially UV cones; and *aipl1b*, which is expressed in all cones [252]. Zebrafish with mutations in *aipl1b* have progressive cone degeneration starting in larvae, and by early adulthood have only a few single cones remaining. Rod photoreceptors are unaffected. ATPase H^+ transporting V0 subunit E1 (ATP6V0E1) is a subunit of a proton pump and *atp6v0e1* mutant zebrafish larvae have RPE pigment loss, outer retinal holes, visual defects determined by OKR, photoreceptor functional deficiencies assessed by ERG, and short OSs [253]. Rods were not assessed in these animals.

WRB (tryptophan rich basic protein) is involved in recruitment of proteins to the endoplasmic reticulum, although its exact role in photoreceptors is unclear [254]. *wrb* mutant zebrafish larvae have holes in their photoreceptor layer, decreased or absent OMR, photoreceptor function deficits, and short cone ribbon synapses in early life [255,256]. Rods have not been investigated in these animals. Mutations in *WRB* have not been associated with human disease.

1.4.6.7 ACHROMATOPSIA

G protein subunit alpha transducin 2 (GNAT2) is an essential component of the cone phototransduction pathway and pathogenic variants in *GNAT2* lead to achromatopsia [32]. *gnat2* mutant zebrafish have cone dysfunction but the photoreceptors do not have overt morphological defects [257] (see Table 7 for zebrafish achromatopsia models). Electrophysiological assessment indicates that *gnat2* mutant cones are ~1000 times less sensitive than wild-type cones. Rods are unaffected in these animals.

Pathogenic mutations in the retinal voltage-gated calcium channel subunit gene *CACNA2D4* (*calcium channel, voltage-dependent, alpha 2/delta subunit 4*) lead to cone dystrophy [258]. Zebrafish have two paralogs of *CACNA2D4*: *cacna2d4a* and *cacna2d4b* [110]. Knocking out each paralog individually does not impact photoreceptor function in early development, but double knockout larvae have reduced cone function and more untethered ribbon synapses at 5 dpf; photoreceptors do not degenerate in these animals. Mutations in *calcium channel, voltage-dependent, alpha 1F subunit* (*CACNA1F*), another voltage-gated calcium channel gene, cause CORD and congenital stationary night blindness, a non-progressive condition characterized by rod dysfunction [259–261]. CACNA1F protein is required at the photoreceptor synapse [111,262]. Zebrafish *cacna1fa* is cone-specific and 7 dpf larval mutant retinas have thin photoreceptor synaptic layers and decreased photoreceptor responses [111]. *cacna1fa* mutant cones do not develop synaptic ribbons. Similarly, zebrafish *synaptojanin 1* (*synj1)* mutants have a thin photoreceptor synaptic layer and abnormal cone pedicles with

unanchored synaptic ribbons [263–265]. The cone IS and OS appear normal. Mutant zebrafish do not survive long enough to develop appreciable numbers of functional rods, however a transplantation study demonstrated that *synj1* is required for cone synapse formation but not rod synapses [263]. A related phenotype is seen in *period circadian regulator 2* (*per2*) knockout zebrafish; *per2* mutant larvae have an abnormal VMR, untethered ribbon synapses, and decreases in cone opsin expression which may indicate cone degeneration [266]. *Per2* mutant animals have not been aged for further investigation of cone and rod viability.

1.4.6.8 AGE-RELATED MACULAR DEGENERATION

AMD models are challenging to generate as AMD is a multifactorial condition. However, there are zebrafish models with features of AMD. Recently, *rp1l1* mutant zebrafish were reported to have progressive photoreceptor dysfunction and subretinal lipid deposits [186] (Table 4; see Chapter 3 for additional information on the mutant line). This is the first report of subretinal drusenoid-like deposits in zebrafish, and may provide insight into how subretinal lipid deposits develop and impact photoreceptor function. In addition, models of retinal neovascularization are relevant to wet AMD. Zebrafish larvae with mutations in *vhl* (*Von Hippel–Lindau tumor suppressor*) present with retinal neovascularization, leaky vessels, and develop oedema and retinal detachment [267] (Table 7).

1.4.7 RELEVANCE TO HUMAN DISEASE

In order to understand and prevent photoreceptor disease, a keen knowledge of photoreceptor development, function, and maintenance is required. Zebrafish have provided additional information about factors involved in these aspects of photoreceptor biology, as lines with mutations in genes not associated with human photoreceptor disease – potentially due to lethality, ascertainment bias, or lack of inclusion on genetic panels – have impaired or degenerate photoreceptors. Additionally, the cellular abnormalities and cause of photoreceptor death can be

investigated in these models. For example, genetically mosaic zebrafish generated by transplantation experiments with zebrafish *rep1* mutant cells determined that *rep1* loss in photoreceptors does not lead to photoreceptor degeneration, but rather that *rep1* is required for normal RPE function, and photoreceptors die due to RPE dysfunction in *rep1* mutant retinas [24]. The RPE should therefore be the primary target of therapies aiming to prevent photoreceptor degeneration in choroideremia patients with *CHM* mutations.

Models of cone disease are desperately needed, as preservation of the high-acuity central vision is a priority for patients and physicians. Nocturnal rodent models are not an ideal system to recapitulate these conditions, as they have a low density of cones and their cones have some properties that are distinct from human cones, such as expression of multiple opsins in a single cell. Indeed, some mouse models of cone diseases do not have the expected cone degeneration phenotype [268]. The zebrafish retina allows for the investigation of cone diseases, and the numerous genetic models available provide the opportunity to determine disease mechanisms based on the mutated gene.

In addition, the cone-rich zebrafish retina enables the assessment of how rod loss impacts cone photoreceptors. Cone persistence post rod death in adult zebrafish RP models permits determination of how cones respond to rod loss and the factors that influence their health when neighbouring photoreceptors degenerate. Additionally, while cones appear viable post rod loss in RP zebrafish models, *pde6c* mutant larvae have rod degeneration post cone loss despite the cone-specific genetic lesion [208,249]. This suggests that the minority photoreceptor type is inherently susceptible to death, as the cone-dominant larval zebrafish retina undergoes rod degeneration post cone loss while the rod-dominant human retina experiences cone degeneration post rod loss. The *pde6c* mutants can therefore be utilized to determine why the minority photoreceptor type is sensitive to degeneration and provide avenues to preserve the cells, which is relevant to efforts aimed at protecting cones in late-stage RP.

Interestingly, several zebrafish models of photoreceptor disease have red and green cone degeneration prior to UV and blue cone degeneration [182,196,241,246,252]. Whether this is due to structural distinctions between double and single cones or if there are specific features of longer wavelength-sensitive cones that make them more susceptible to cell death than short wavelength cones is unknown. Further assessment is required and may inform macular degeneration pathologies. The central macula is comprised of primarily red and green cones, and understanding what makes these cones vulnerable to disease is important for preventing maculopathies including AMD.

Drusen and subretinal drusenoid deposits are characteristic features of AMD but how they develop and affect photoreceptor health is unknown. Animal models of drusen and drusenoid deposits are difficult to develop and there are few photoreceptor disease models that acquire them. Subretinal drusenoid deposits in particular are poorly investigated, as they are relatively newly characterized. A zebrafish model of photoreceptor disease with accompanying subretinal deposits provides the opportunity to investigate how ocular lipid accumulations develop *in vivo* [186].

1.5 ZEBRAFISH CONTRIBUTIONS TO RETINAL THERAPEUTICS

Zebrafish provide a model to test therapeutic strategies and pharmaceuticals. Numerous disease models combined with the small size of zebrafish larvae and visual assessment tools – such as VMR, OMR, and OKR – allow for screening of compounds. Below, the contributions from zebrafish models are outlined.

1.5.1 PHARMACEUTICALS

1.5.1.1 ANTI-ANGIOGENIC COMPOUNDS

Zebrafish models of retinal neovascularization, such as the hypoxia-induced model and *vhl* mutants, provide the opportunity to screen for anti-angiogenic compounds that could be employed in patients with wet AMD. Indeed, Van Rooijen and colleagues found that treating *vhl* mutants with vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors typically used in cancer treatment successfully blocked pathogenic angiogenesis [267], which could be further tested in mammalian models of retinal neovascularization.

1.5.1.2 HISTONE DEACETYLASE INHIBITION

Cellular stress markers can activate cell death pathways, resulting in apoptosis, or trigger elimination by immune cells. This can result in loss of cells that are stressed, but otherwise functional, and has been observed in the retina [269]. Overactivation of histone deacetylases (HDACs) has been detected in models of photoreceptor degeneration [270]. Indeed, HDAC inhibitors have been effective for treating neuronal pathologies *in vitro* and in animal models of CNS disease, including photoreceptor degeneration models [271–273]. However, it is important to note that there are also HDACs that inhibit photoreceptor death after light injury [274]; it is therefore important to target specific HDACs.

HDAC6 inhibition has been reported to prevent neurodegeneration in peripheral neuropathy models [275]. In the *atp6v0e* mutant zebrafish model of cone photoreceptor disease, HDAC6 inhibitors successfully restored OKR, decreased the number of apoptotic cells in the photoreceptor layer, and increased OS persistence [253,276,277]. Similar results are observed in a mouse model of photoreceptor disease [276,277], further supporting HDAC6 as a potential therapeutic target for treating photoreceptor degenerations.

1.5.1.3 NOVEL COMPOUND SCREENING

Zebrafish models of photoreceptor disease provide a platform for discovery of novel neuroprotective drugs. Zebrafish have been employed for numerous large drug screens, such as screens to identify compounds that impact neuronal activity [278,279]. Zebrafish can therefore be utilized to identify novel compounds in large-scale screens. Through behavioural screens and histological approaches, several compounds have been identified as protective against photoreceptor dysfunction and degeneration in zebrafish.

Ganzen and colleagues utilized Tg(*rho:hsaRHO-Q344X*) and transgenics with NTR-ablatable rods to screen a library of potential drugs for potential RP treatment [280] (bioRxiv preprint). VMR assessment identified carvedilol as beneficial, and carvedilol-treated Tg(*rho:hsaRHO-Q344X*) larvae had increased rod survival compared to control animals. As RP is the most common inherited photoreceptor disease, a pharmacological treatment may have applicability to many patients experiencing progressive vision loss.

Cone degeneration in *pde6c* mutant zebrafish larvae occurs through necroptosis (inflammatory cell death, a programmed form of necrosis), while the subsequent rod degeneration is apoptosis-mediated [281]. Blocking necroptosis factors by either using morpholinos to knockdown relevant genes or pharmacologically with necrostatin-1s or necrosulfonamide at 10 hpf until 7 dpf results in a reduction in cone death. However, whether vision is preserved due to necrostatin-1s or necrosulfonamide treatment is unknown. Further investigation is required, but pharmacological repression of necroptosis has applicability to many different photoreceptor diseases that involve necroptosis pathways.

Studies using zebrafish models of photoreceptor disease have also identified schisandrin B and gypenoside, plant-derived compounds with antioxidant and anti-inflammatory properties, as potential neuroprotective compounds [281–287]. These examples highlight the benefits of

utilizing zebrafish models of photoreceptor disease to screen novel therapeutics and specific drugs.

1.5.1.4 OCULOTOXICITY

Many drugs have retinal toxicity, meaning that prolonged exposure can result in retinal damage and vision loss. Zebrafish have provided a relatively high-throughput tool to assess retinal toxicity of pharmaceuticals. Deeti and colleagues used zebrafish larvae to develop a methodology for assessing the impact of pharmaceuticals on the zebrafish visual system [288]. 3 dpf zebrafish larvae were treated with compounds for 2 days in 48-well plates, and then assessed for visual impairment using VMR and OKR. 5/6 known oculotoxic drugs reduced the visual responses of zebrafish. However, all of the oculotoxic drugs resulted in a reduction in zebrafish touch response, suggesting defects in other sensory neurons. Therefore, zebrafish provide a viable tool for screening drugs for retinal toxicity.

1.5.2 ANTISENSE OLIGONUCLEOTIDE THERAPY

Antisense oligonucleotide therapy can block aberrant splicing in genes with mutations in splice donor or acceptor sites. A frequent Usher syndrome-causing *USH2A* mutation results in incorporation of a pseudoexon and truncation of the USH2A protein. Morpholino treatment successfully prevented aberrant splicing in mosaic zebrafish with *ush2a* containing a CRISPR/Cas-introduced region of human mutant *USH2A* [289]. In addition, this group recently investigated morpholino-induced skipping of a mutated exon in *ush2a* mutant zebrafish [290] (BioRxiv preprint); specifically, they skipped mutant exon 13, which is commonly mutated in Usher syndrome [194,291]. Exon skipping was successful and partially restored protein expression [290] (BioRxiv preprint). This therapeutic approach of using antisense oligonucleotides to partially restore *USH2A* function by skipping the problematic exon 13 is currently in clinical trial (ID: NCT03780257).

1.5.3 PHOTORECEPTOR REGENERATIVE PATHWAYS

Zebrafish can regenerate lost retinal neurons, including photoreceptors [292]. Investigating the zebrafish regenerative response provides information on activation of endogenous retinal stem cells as well as factors that promote photoreceptor differentiation and integration of newly generated cells, which is invaluable for effective development and deployment of stem cell therapies.

Zebrafish have two distinct stem cell pools: the ciliary marginal zone (CMZ) and Müller glia.

1.5.3.1 CILIARY MARGINAL ZONE

The CMZ is a pool of retinal stem cells located at the peripheral edges of the retina. In many species, this pool becomes inactive once the retina has matured. However, in some vertebrates, such as zebrafish and other species that undergo indeterminate growth, the CMZ self-renews and constantly adds new retinal neurons at the edges of the retina. The CMZ contains both retinal stem cells, which can divide indeterminately, and retinal progenitor cells, which can only divide a certain number of times and lose contact with the CMZ as differentiation occurs [118,292–294]. Retinal stem cells sit in the edges of the CMZ, and typically undergo asymmetrical division to produce a retinal stem cell and a retinal progenitor cell.

Mammals have a CMZ-like region that gives rise to some retinal neurons during development [295], although whether it has the capacity to produce photoreceptors is unclear. In the mature retina, these retinal margin cells seem to lose neurogenesis capacity or become quiescent. However, there are cells with stem cell-like properties in the mammalian/human peripheral retinal margin next to the pars plana; specifically, Müller glia and ciliary epithelium cells that express neural progenitor markers [296–298]. Whether these cell populations could be stimulated to enter a stem cell-like fate in the damaged retina requires further investigation.

1.5.3.2 MÜLLER GLIA

Müller glia are support cells that play many essential roles in the retina, including ion regulation, neurotransmitter uptake and degradation, cellular debris removal, and neuronal insulation. In zebrafish and other teleost fishes, Müller glia also act as endogenous stem cells by undergoing de-differentiation, proliferation, and specification to produce many different retinal neuron types, including photoreceptors. Zebrafish Müller glia division is asymmetrical, allowing for replenishment of lost retinal neurons while maintaining the stem cell pool [113,118,122,294,299–303]. As mentioned above, there is a threshold of damage that must be met to trigger the Müller glial regenerative response, which has been observed in NTR ablation studies [140–144]. Interestingly, some mutant zebrafish have cone degeneration but Müller glia do not undergo proliferation to appreciably regenerate the lost photoreceptors [236,247]. Further investigation into one of these lines, the *bbs2* mutants, found that the endogenous Müller glia do have the capacity to regenerate retinal neurons in *bbs2* mutant retina, as evident by light ablation experiments [236]. Why photoreceptor degeneration triggers a regenerative response in some mutant models but not others is unclear, but may be related to the speed of degeneration or an inflammatory requirement for Müller glia activation.

Zebrafish Müller glial behaviour must be carefully controlled in order to prevent aberrant proliferation and production of unneeded neurons. In the uninjured retina, the reprograming pathways of Müller glia are inhibited [304]. After retinal injury, Müller glia respond to factors released by damaged cells – including TNFα [305] – and undergo reprogramming, followed by proliferation and neurogenesis. Studying the regenerative pathways in zebrafish has provided insight into stem cell and fate-determining factors that can be utilized to generate photoreceptor precursor cells for transplantation.

As Müller glia are interspersed within the human retina, how the stem cell behaviour of Müller glia is activated and whether mature mammalian Müller glia can be reprogrammed to a

stem cell fate to treat retinal degenerations is an area of active interest. Human Müller glia detect and respond to damage and have some characteristics of stem cells, but do not undergo neurogenesis *in vivo* [296,306]. Müller glia proliferation rarely occurs in the mammalian retina, and is typically associated with glial scar formation [307]. When cultured, human Müller glia treated with growth and differentiation factors can adopt stem cell properties and differentiate into rod photoreceptor cells [296,306,308]. Human Müller glia-derived rod photoreceptors have been transplanted successfully into a rodent RP model and the rods migrate into the photoreceptor layer then restore some visual function [309]. Further investigations into Müller glial response to photoreceptor loss, photoreceptor cell differentiation, and integration will be essential for application of stem cell therapies in human disease, and zebrafish provide this unique avenue. For in-depth reviews of photoreceptor regeneration in zebrafish and how these mechanisms relate to mammalian systems, please see [161,303,310–313].

1.6 OBJECTIVES + PURPOSE OF THESIS

Photoreceptor diseases are extremely genetically and phenotypically heterogeneous. The objective of this project was to characterize inherited photoreceptor degenerations caused by *RP1L1*. Previously, *RP1L1* had only been associated with occult macular dystrophy – a maculopathy with normal fundus – and retinitis pigmentosa. To determine whether *RP1L1* might be causative in additional photoreceptor diseases, I utilized a database with patient clinical and genetic information to ascertain a patient with a severe maculopathy and compound heterozygous *RP1L1* mutations. In addition, two patients seen by Dr. MacDonald were found to have a dominantly inherited maculopathy associated with a missense *RP1L1* mutation. Finally, I report a rod-cone dystrophy patient with a homozygous *RP1L1* mutation.

Although many patients with *RP1L1* mutations have been identified and RP1L1 appears to cause a diversity of photoreceptor disease, the mechanism underlying *RP1L1*-associated disease is unknown and the function of RP1L1 in the photoreceptor axoneme has not been determined.

To address this, I aimed to generate mutant *rp1l1* zebrafish to model photoreceptor disease. Zebrafish with mutations in *rp1l1* were generated using CRISPR/Cas9 genome editing. These animals had progressive photoreceptor dysfunction, retinal degeneration, and abnormal, disorganized photoreceptor OSs. Interestingly, subretinal lipid deposits were observed in these animals. Why a photoreceptor-specific protein could lead to these lipid accumulations is unclear, but may have relevance to retinal diseases characterized by ocular lipid deposit accumulation, such as age-related macular degeneration.

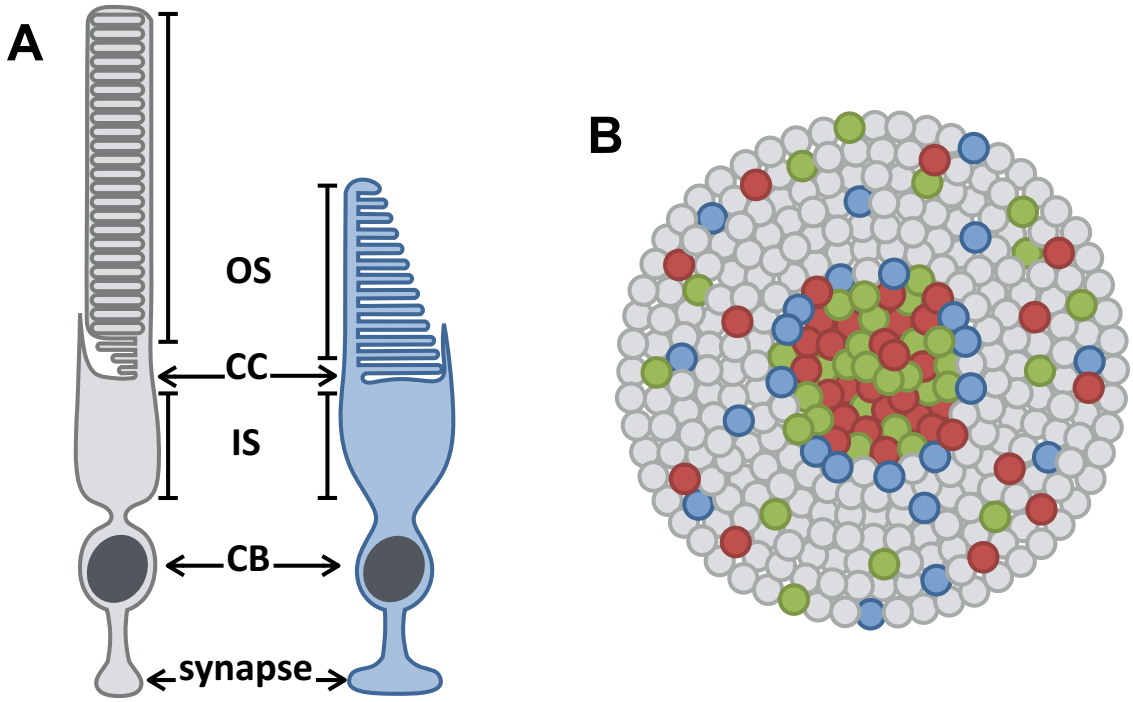

Figure 1. Anatomy of rod and cone photoreceptors and their organization in the human retina.

(A) Cartoons of a rod (grey) and cone (blue) photoreceptor. Photoreceptors have an outer segment (OS) that is packed with light-sensitive opsin proteins, a connecting cilium (CC) that connects the OS with the mitochondria-rich inner segment (IS), a cell body (CB), and a synapse. **(B)** Cartoon of the human photoreceptor mosaic. Humans have three types of cones: red, green, and blue, depicted in those respective colours. The peripheral retina is rod-dense with cones interspersed throughout, while the central retina is comparatively cone-dense.

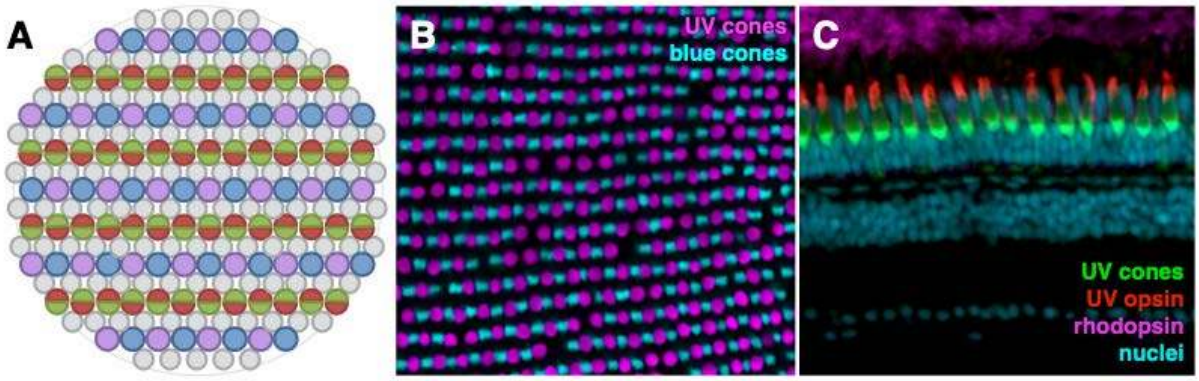

Figure 2. Zebrafish photoreceptor mosaic organization.

(A) Cartoon depiction of the zebrafish photoreceptor mosaic. UV and blue cones, depicted in purple and blue respectively, alternate in their rows while red and green double cones alternate in their rows. Rods are studded throughout. **(B)** Fluorescent image of a flat-mounted adult transgenic zebrafish retina, with GFP expressed in UV cones (magenta) and mCherry expressed in blue cones (cyan). The alternation of UV and blue cones in their rows is clear. **(C)** Immunofluorescent image of a cryosectioned adult zebrafish retina with GFP in UV cones (green). UV opsin (red) and rod outer segments (magenta) are labelled, as well as nuclei (cyan).

Table 1. Examples of common zebrafish transgenic lines with labelled photoreceptors.

Transgene	**Description**	**Reference**
Tg(*3.2gnat2:eGFP*)	Enhanced GFP expressed in all cone photoreceptors	[314]
Tg(*rho:eGFP*)	Enhanced GFP expressed in rod photoreceptors	[315]
Tg(*sws1:GFP*)	GFP expressed in UV cones	[316]
Tg(*sws2:mCherry*)	mCherry expressed in blue cones	[87]

Table 2. Examples of commonly used antibodies that label zebrafish photoreceptors.

Antibody	**Antigen**	**Labels**	**References**
4c12	N/A	Rods	[249]
10c9.1	UV opsin	UV cone OSs	[317]
1D4	Red opsin	Red cone OSs	[318]
zpr1	Arrestin3a	Red/green double cones	[319,320]
zpr3	N/A	Rod and double cone OSs	[321]

Table 3. Transgenic zebrafish models of photoreceptor disease.

Photoreceptor Disease	**Transgene**	**Photoreceptor Features**	**Reference**
Cone-rod dystrophy	Tg(*3.2gnat2:hsa.GUCY2D-E837D R838S*)	Dysmorphic cones at 5 dpf. 3-month-old animals have a thin photoreceptor layer in the central retina, dysmorphic cones, and less cone and rod staining	[159]
Retinitis pigmentosa	Tg(*rho:hsaRHO-Q344X*)	Rod degeneration detectable at 5 dpf, rapid progression.	[152]
Retinitis pigmentosa	Tg(*rho:msRho-P23H-flag*)	Rod degeneration at 3 months.	[150]
Retinitis pigmentosa	Tg(*rho:prpf3-AD5-mCherry*)	Increased photoreceptor cell death at 5 months.	[154]
Retinitis pigmentosa	Tg(*rho:adcy2b-rho-tail*)	Rod degeneration noted at 14 dpf.	[152]
Retinitis pigmentosa	Tg(*rho:hsaHTRA1*)	Rod degeneration at 5 dpf.	[158]
Retinitis pigmentosa	Tg(*XOPS:mCFP*)	Rapid rod degeneration by 5 dpf. Adults do not have a rod response on ERG.	[137]

Table 4. Zebrafish mutant models of retinitis pigmentosa-like photoreceptor disease.

Gene	Photoreceptor Features	Reference
cerkl	Photoreceptor functional defects at 7 dpf. Rod OS defects at 3 months, cone OS defects at 7 months. Notable thinning of the photoreceptor layer and cell death by 12 months.	[174]
her9	Decrease in rod photoreceptors at 5 dpf. Few double cones with short OSs at 12 dpf.	[196]
kif3b	Delayed OS development. Rapid rod degeneration by 5 dpf.	[176,177]
myo7aa	Decreased photoreceptor function at 5dpf. Reduced rods at 8 dpf.	[190]
rho	Rod loss observed at 6 dpf. Degeneration continues into adulthood.	[166,167]
rp1l1	Rod dysfunction at 6 months. Subretinal drusenoid deposits at 11 months. Photoreceptor loss observed at 12 months.	[186]
rp2	Photoreceptor functional defects at 7 dpf. Short rod OSs at 2 months; cone OS defects at 4 months; significant rod OS loss and decreased cone OSs by 7 months.	[172]
rpgrip1	No rod OSs at 5 dpf. Cone dysfunction at 7 dpf. Severe rod degeneration by 3 months, followed by cone degeneration. By 23 months, majority of photoreceptors have degenerated.	[182]
ush2a	Decreased photoreceptor function at 5-7 dpf and increased photoreceptor apoptosis at 8 dpf. Notable rod OS degeneration at 12 months, cone OS degeneration observed at 20 months.	[193,194]

Table 5. Zebrafish mutant models of Leber congenital amaurosis-like photoreceptor disease.

Gene	Photoreceptor Features	Reference
cluap1	No OS development, rapid photoreceptor degeneration.	[201]
gdf6a	Short, dysmorphic cones and expanded, disorganized rods at 7 dpf.	[215,216]
ift57	Short OSs with normal disc stacking at 4 dpf. Central retina photoreceptor degeneration at 5 dpf.	[200,202]
ift88	No OS development, rapid photoreceptor degeneration.	[197–200]
ift122	Normal photoreceptor OS development. Degeneration starting at 7 dpf, severe degeneration by 10 dpf.	[203]
ift172	No OS development, rapid photoreceptor degeneration.	[197,198,200]
kiaa0586	Fewer OSs observed at 3 dpf, photoreceptor degeneration observed at 4 dpf. Decreased photoreceptor function detected at 6 dpf.	[211]
kif3a	No OS development and rapid rod photoreceptor degeneration by 5 dpf, subsequent cone degeneration. Extinguished photoreceptor response at 7 dpf.	[177,204,205]
napbb	Immediate, severe photoreceptor degeneration; cell death observed at 3 dpf, no distinct photoreceptor layer at 6 dpf, few photoreceptors in ciliary margin.	[207,208]
tmem216	Short cilia and disorganized OS discs by 7 dpf, rapid degeneration. Few photoreceptors remaining by 14 dpf.	[214]

Table 6. Zebrafish mutant models of choroideremia, cone-rod dystrophy, and enhanced S-cone syndrome.

Photoreceptor Disease	Gene	Photoreceptor + RPE Features	Reference
Choroideremia	*rep1*	RPE irregularity and pigment loss. Disorganized photoreceptor layers, dysmorphic photoreceptors, and significantly reduced photoreceptor responses at 5 dpf.	[24]
Cone-rod dystrophy	*ahi1*	Disorganized, short OSs, but normal visual function at 5 dpf. Thin photoreceptor nuclear layer, cone degeneration, and dysmorphic rods at 5 months.	[221]
Cone-rod dystrophy	*arl13b*	Short OSs at 4dpf. Cone degeneration observed at 30 dpf in mosaic animals.	[222]
Cone-rod dystrophy	*bbs2*	Short, disorganized photoreceptor OSs and visual deficits at 5 dpf. Photoreceptor degeneration observed in adulthood.	[236]
Cone-rod dystrophy	*cc2d2a*	Dysmorphic photoreceptor OSs and functional defects at 5 dpf.	[220]
Cone-rod dystrophy	*eys*	Progressive photoreceptor loss; cone degeneration observed at 6 months, rod degeneration observed at 14 months	[231–233]
Cone-rod dystrophy	*kcnj13*	Increased phagosomes in the RPE at 3 months, enlarged RPE mitochondria at 6 months, and abnormal melanosome localization under dark adaptation at 12 months. Cone mitochondria abnormalities at 6 months and photoreceptor loss at 12 months.	[238]
Cone-rod dystrophy	*lca5*	Photoreceptor functional defects at 7 dpf. Cone OS defects at 1 month, rod OS defects at 7 months, and progressive photoreceptor loss.	[224]
Cone-rod dystrophy	*pcare1*	Dysmorphic OSs and dysfunctional photoreceptors at 5 dpf. Abnormal OS morphology and thinner photoreceptor layer at 6 months.	[226]
Cone-rod dystrophy	*pomgnt1*	Reduction in cones and rods at 6 months.	[240]
Enhanced S-cone syndrome	*nr2e3*	No rods. Green and red cone degeneration started at 1 month.	[241]
Enhanced S-cone syndrome	*nrl*	Rods fail to develop and UV cones are over-abundant in larvae. Adult zebrafish surprisingly have rods, but with synaptic defects.	[242]

Table 7. Zebrafish mutant models of cone dystrophy, achromatopsia, and retinal neovascularization.

Photoreceptor Disease	**Gene**	**Photoreceptor, RPE, or Vascular Features**	**Reference**
Achromatopsia	*cacna1fa*	Thin photoreceptor synaptic layer. Photoreceptor functional defects at 5-6 dpf. Cone synapses do not develop synaptic ribbons.	[111]
Achromatopsia	*cacna2d4a* + *cacna2d4b*	Mild cone dysfunction at 5 dpf. Increased untethered synaptic ribbons.	[110]
Achromatopsia	*gnat2*	Cone functional defects under dim to moderate light intensities.	[257]
Achromatopsia	*per2*	Visually-mediated behaviour deficiencies at 5-6 dpf. Unanchored cone ribbon synapses and decreased cone opsin expression.	[266]
Achromatopsia	*synj1*	Thin photoreceptor synaptic layer and abnormal, unanchored cone ribbon synapses at 6 dpf.	[263–265]
Cone dystrophy	*aipl1*	Thin photoreceptor layer and OS defects at 7 dpf. Severe cone degeneration by 3 months.	[252]
Cone dystrophy	*atp6v0e1*	Hypopigmented RPE, reduced or absent visual response, decreased photoreceptor function, short OSs, and outer retinal holes at 5 dpf.	[253]
Cone dystrophy	*cep290*	Cone OS abnormalities and degeneration at 3 months, severe degeneration at 12 months. Normal rods.	[247]
Cone dystrophy	*pde6c*	Cone degeneration at 4 dpf and rod subsequently become dysmorphic and degenerate in the larval retina. At 3 months, cones are degenerate and largely missing, rods are normal.	[208,249]
Cone dystrophy	*prom1b*	Progressive cone degeneration. Reduced red and green cones at 7 dpf; reduced UV and blue cones at 1 month. Overgrown rod OSs.	[246]
Cone dystrophy	*wrb*	Holes in the photoreceptor layer at 4dpf. Vision defects at 5 dpf, photoreceptor function defects at 4-5 dpf. Small cone ribbon synapses	[255,256]
Retinal neovascularization	*vhl*	Retinal neovascularization, leaky blood vessels, retinal oedema, and retinal detachment at 7-8 dpf.	[267]

2 RP1L1 AND INHERITED PHOTORECEPTOR DISEASE

This chapter is modified from the published manuscript: Nicole C. L. Noel and Ian M. MacDonald. (2020) RP1L1 *and inherited photoreceptor disease: A review*. Survey of Ophthalmology, 60(6): 725-739.

2.1 INTRODUCTION

Retinitis Pigmentosa 1-Like 1 (*RP1L1*) encodes a component of the photoreceptor axoneme [322,323], the core structure within the photoreceptor cilium comprised of microtubules and associated proteins. The photoreceptor axoneme is an essential structure for proper OS development and maintenance; it allows transport of materials between the IS and OS, which is necessary for construction of OS discs and biochemical homeostasis of the photoreceptor. Pathogenic variants within, or deletion of, cilia components are well-known causes of photoreceptor dysfunction and degeneration, either in combination with systemic cilia abnormalities (ciliopathies) or photoreceptor-specific disease [324,325]. *RP1L1* is no exception to this case, as pathogenic variants in *RP1L1* result in progressive photoreceptor degenerative diseases.

RP1L1 expression has only been detected in retinal photoreceptors, suggesting that it is a photoreceptor-unique cilia component. Despite *RP1L1* expression in both rods and cones, *RP1L1* variants typically result in either rod or cone disease; combined rod and cone disease due to *RP1L1* variants have not been previously reported in the literature. This observation suggests that how photoreceptor disease manifests in patients with *RP1L1* variants may be influenced by other genetic or environmental factors, or that RP1L1 has similar, but distinct, roles in the different photoreceptor types.

The pathological mechanisms underlying *RP1L1*-associated photoreceptor disease are not known, largely due to poor understanding of RP1L1 protein function. RP1L1 protein contains two doublecortin (microtubule-binding) domains, an RP1 domain, and two repetitive regions containing 16 amino acid repeats [322] (Figure 1). One repeat region is variable in length (1-6 repeats have been reported); RP1L1 protein therefore has a minimum length of 2400 amino acids and a maximal length of 2480 amino acids. Whether or not specific numbers of repeats are associated with increased susceptibility to photoreceptor disease is not known. As its name indicates, *RP1L1* is highly similar to the Retinitis Pigmentosa 1 (*RP1*) gene in terms of gene structure, size, domains, and localization (Figure 3). Pathogenic variants in *RP1* are well-known causes of autosomal dominant RP, which manifests due to abnormalities in OS development and maintenance [326,327]. RP1 is an axoneme-associated protein, and interacts with the photoreceptor cilium microtubules through doublecortin domains [326,328]. As RP1L1 and RP1 share such extensive similarities, they could play comparable roles within the photoreceptor cell. Research conducted using mutant mouse lines suggests synergistic or partially redundant roles of RP1 and RP1L1 [323]. In mice, knockout of *RP1L1* resulted in short photoreceptor OSs, disorganized OS discs, and progressive photoreceptor degeneration. Inheritance of both *RP1* and *RP1L1* heterozygous loss-of-function mutations resulted in an exacerbated photoreceptor degeneration phenotype in the mice. *RP1L1* thus appears to be involved in maintaining OS organization and structure, and its loss or mutation can lead to OS dysregulation and structural impairment.

Variants in *RP1L1* have been associated with occult macular dystrophy (OMD) and RP [184,185]. This chapter summarizes *RP1L1*-associated photoreceptor disease and reports novel associations between *RP1L1* and photoreceptor degenerations.

2.2 OCCULT MACULAR DYSTROPHY

2.2.1 CLINICAL FEATURES

OMD was first characterized as a hereditary macular dystrophy without apparent fundus abnormalities [185,329–331] (Figure 4); patients present with normal appearing fundi despite experiencing loss of central vision, visual acuity decline, photophobia, and colour vision disturbances [184,331–345]. Typically, testing reveals a normal full-field ERG, reduced multi-focal (macular) ERG, normal fluorescein angiography, macular photoreceptor layer thinning observed by OCT – indicating cone photoreceptor cell loss – and decreased foveal sensitivity detected by microperimetry [329–331,337–339,345–351]. Central vision loss and visual acuity decline worsens over time. As the condition progresses, OCT assessment shows that macular thinning becomes more pronounced, along with further disruption of the ellipsoid zone (EZ; photoreceptor inner segment region) and loss of the interdigitation zone (IZ; location of contact between OSs and RPE), signifying progressive cone photoreceptor loss [336–338,340,352,353]. OMD is typically dominantly inherited, with incomplete penetrance and a wide range of onset [185]. However, asymptomatic family members with OMD genetic variants can have abnormal ellipsoid and interdigitation zones in the parafovea, which indicates that the photoreceptors may possess structural abnormalities or have undergone a sub-clinical amount of degeneration [350,354]. While OMD is characterized by a normal appearing fundus, I found that OMD patients with pathogenic *RP1L1* variants can slowly develop clinically evident macular disruptions over time (Figure 5). By definition, patients who progress to have fundus findings no longer have an "occult" phenotype, and instead have what I refer to as an *RP1L1* maculopathy – a maculopathy with clinical fundus findings caused by variants in *RP1L1*.

2.2.2 OMD-ASSOCIATED *RP1L1* VARIANTS

About 50% of patients with OMD have a discernable genetic cause, all of which have been reported to be pathogenic variants in *RP1L1* [184,185,333,335,340,342,345,346,355–357] (Table 8). The most common OMD-causing variant is p.R45W [185]. This is the most N-terminal variant reported in *RP1L1*, and it is found inside the first microtubule-binding domain of the RP1L1 protein. The p.R45W variant may prevent effective axoneme microtubule association. A second region of interest that is prone to OMD-associated variants falls between amino acid 1172-1258. Many variants have been reported in this region, including several missense variants that lead to different amino acid changes of the same residue [184,335,345,346,356] (Table 8). This observation is intriguing, as this region of *RP1L1* has no discernable protein domains. This area is potentially an important binding site for other (currently unidentified) proteins and its disruption may result in deleterious alterations in binding affinity. Alternatively, variants within this region may lead to RP1L1 protein misfolding. No phenotypic differences have been noted between patients with *RP1L1* mutations in different regions [345].

2.3 RETINITIS PIGMENTOSA

2.3.1 CLINICAL FEATURES

RP is a genetically heterogeneous photoreceptor degenerative disease that primarily affects rod photoreceptors. Inheritance of RP can be dominant, recessive, X-linked, mitochondrial, or multigenic, and RP can be associated with systemic disease, often due to ciliopathies or metabolic disorders [1,358]. RP patients experience progressive night blindness and loss of peripheral vision due to rod cell degeneration. Central vision and visual acuity can be preserved or unaffected due to sparing of the macula. However, patients can also lose central vision in late-stage disease, as cone photoreceptors subsequently degenerate post rod loss through complex, poorly understood mechanisms [359]. Characteristics of RP include a reduced full-field ERG, loss of visual field, and

abnormal pigment deposition (called bone spicules) due to RPE cell death and pigment migration (Figure 6).

2.3.2 RECESSIVE RP-ASSOCIATED *RP1L1* VARIANTS

RP1L1 variants are associated with autosomal recessive RP, and are often predicted loss-of-function mutations such as frameshift or nonsense mutations, though exceptions have been reported [184,355,357,360–363] (Table 9). In the Japanese population, up to 8% of autosomal recessive RP is predicted to be caused by variants in *RP1L1* [360,361]; *RP1L1* variants are thus a significant contributor to inherited photoreceptor disease in certain populations [357,361]. Heterozygous *RP1L1* loss-of-function mutations do not appear to cause disease on their own, as they can be relatively frequent. Despite *RP1L1* being reported as a significant contributor to autosomal recessive RP in some populations, many commercially available panels used in clinical genetic testing for RP do not include *RP1L1*; this could have resulted in under-reporting of RP-associated *RP1L1* variants in the literature.

2.4 OTHER CONDITIONS (ORIGINAL DATA)

2.4.1 *RP1L1* MACULOPATHY

By searching the repository of the National Ophthalmic Disease Genotyping and Phenotyping Network (eyeGENE®), I found a patient clinically diagnosed with Stargardt disease (Figure 7) who carried compound heterozygous variants in *RP1L1* (c.1370C>G (p.S457C) and c.4396G>T (p.E1466*)) (Tables 10-11). The missense p.S457C variant is predicted to be damaging by PolyPhen-2, SIFT, and PROVEAN, while MutationTaster, MutPred2, and PANTHER predict it to be benign, similar to the outputs from the p.R45W variant (Table 11). The variant satisfies moderate and supporting pathogenicity criteria from the American College of Medical Genetics and Genomics (ACMG) guidelines for classifying variants [364] (Table 11), categorizing it as a

variant of uncertain significance; however, as RP1L1 has extremely little structural and functional information available and protein modeling has either not been attempted or been unsuccessful, many of the pathogenicity criteria cannot be assessed. No reported coding or intronic variants were reported in *ABCA4*, the most common Stargardt disease-causing gene, in this patient. Severe geographic atrophy is seen in the macula of both eyes (Figure 7A). Spectral domain OCT reveals loss of the ellipsoid zone, retinal thinning, and loss of RPE (Figure 7B). In one eye, there is a loss of uniform retinal lamination and gaps within the photoreceptor layer. Areas of increased autofluoresence that are characteristic of Stargardt disease, commonly seen as reflective flecks on fundi, are absent. While this patient was classified as having Stargardt disease in the database, the phenotype is more similar to geographic atrophy or a severe (non-Stargardt) macular degeneration. I suggest that these *RP1L1* variants, in combination, have the potential to be major contributors to the patient phenotype. However, there may be additional genetic interactions between maculopathy and retinopathy-associated genes underlying the phenotypic presentation in this patient, as the observed degeneration is severe.

I also report two patients, a mother and daughter, who presented with what appeared to be either adult pseudovitelliform macular dystrophy or Best disease (a progressive macular dystrophy caused by dominant mutations in *BEST1*). The patients had bilateral sub-foveal disruption as seen by OCT, indicative of cone cell loss; foveal mottling was also observed in the mother's fundus (Figure 8). The bilateral presentation and similar phenotype in both mother and daughter suggested a dominantly inherited condition. Next generation sequencing of the mother's DNA using a maculopathy panel revealed no mutations in *BEST1* or other maculopathy-associated genes, and a heterozygous variant of uncertain significance in *RP1L1* (c.1994C>G; p.P665R) (Tables 10-11). DNA from the affected daughter was subsequently sequenced and she was found to also carry the same c.1994C>G; p.P665R *RP1L1* variant. The *RP1L1* variant thus segregates with the disease, and is the most likely cause of the phenotype. Pathogenicity predictor programs reported results for the p.P665R variant similar to the most common OMD variant

p.R45W, with PolyPhen-2, SIFT, and PROVEAN predicting the variant to be damaging and ACMG criteria classifying it as likely pathogenic (Table 11). These patients could potentially have had *RP1L1*-associated OMD that progressed to a visible retinal phenotype – an *RP1L1* maculopathy – similar to what has been observed in other OMD patients (Figure 5).

2.4.2 ROD-CONE DYSTROPHY

A patient with a rod-cone dystrophy presented with adult-onset nyctalopia and bilateral macular involvement (Figure 9). The patient's visual acuity in both eyes was 20/30. Fundus examination of the patient showed thinning of the peripheral retina and vasculature attenuation, suggesting degeneration of the peripheral retina, as well as a small hypopigmented area under the fovea resembling an area of retinal atrophy in the left eye. OCT imaging revealed loss of foveal anatomy. Full-field ERG testing revealed severe reduction in both the rod and cone response. Sequencing uncovered a homozygous variant in *RP1L1* (c.1215T>G, p.Y405*) (Table 10). This is the first reported patient with simultaneous rod and cone involvement associated with variants in *RP1L1*.

2.5 DISCUSSION

2.5.1 OCCULT MACULAR DYSTROPHY PROGRESSION TO AN *RP1L1* MACULOPATHY

The term "occult macular dystrophy" was coined due to the absence of fundus findings despite central vision loss [329,330]. However, the OMD patient phenotype can progress to have clear retinal phenotypes visible by fundoscopy examination (Figure 5). This is not entirely surprising, since vision loss in OMD patients is progressive; if cones in the macula continue to degenerate throughout the life of the patient, one would expect their fundus to evolve accordingly, not remain static. Several patients with the canonical OMD variant in *RP1L1* (c.133C>T, p.R45W) that presented with OMD but whose phenotype slowly transformed into a maculopathy with

retinal findings visible by fundoscopy are reported here. I suggest that in these cases, the patients' conditions be referred to as an *RP1L1* maculopathy, as they cease to fit the "occult" descriptor of OMD.

Additionally, the reported case of novel *RP1L1* variants and pseudovitelliform macular degeneration (Figure 8) is potentially an example of *RP1L1*-associated OMD progressing to a more severe maculopathy with fundus findings. Factors that influence the penetrance, severity, and onset of *RP1L1*-associated photoreceptor degeneration remain poorly understood; other genetic factors likely explain the considerable variation in presentation. I suggest that maculopathies with fundus abnormalities seen in patients who carry *RP1L1* variants could be referred to as *RP1L1* maculopathies.

2.5.2 *RP1L1* VARIANTS ARE ASSOCIATED WITH ADDITIONAL PHOTORECEPTOR DISEASES

RP1L1 variants have historically been associated with OMD and RP. However, there is increasing evidence that *RP1L1* variants may play a role in additional photoreceptor diseases and/or that OMD may be an early stage of more severe macular degenerations. As examples, I report a patient with severe macular degeneration, a mother and daughter with adult pseudovitelliform macular dystrophy, and a patient with a rod-cone dystrophy, all with variants in *RP1L1*.

It is not surprising that *RP1L1* variants are associated with a rod-cone dystrophy, as *RP1L1* is expressed in all photoreceptor types. Rather, it is intriguing that a gene expressed in all photoreceptors is typically associated with either cone or rod disease; this observation suggests that *RP1L1* may play divergent roles in different photoreceptor subtypes, or that additional variants – perhaps in rod- or cone-specific genes – influence where, how, and when disease manifests.

2.5.3 *RP1L1* MUTATIONS MAY COMPOUND WITH VARIANTS IN OTHER PHOTORECEPTOR-RELATED GENES

Variants in *RP1L1* have the potential to compound with variants in other photoreceptor or cilia genes. Mutational load has been described as a key factor underlying the diversity of ciliopathy phenotypes [325]. Mutational load refers to the phenomenon whereby all the variants (including single nucleotide polymorphisms, deletions, and copy number variants) in cilium-related genes contribute to disease presentation. This effect has been seen in Bardet-Biedl Syndrome, as mutation types, locations, and copy number variants modify phenotypic presentation [365]. Incomplete penetrance of OMD, variable disease onset age, and differing rates of disease progression may plausibly result from combinations of discrete, otherwise non-pathogenic single nucleotide polymorphisms, deletions, and copy number variants in additional photoreceptor-specific or cilium component genes. The unique combination of cilium- or photoreceptor-specific variants carried by an individual with disease-associated *RP1L1* variants could then play a determining role as to whether an individual develops photoreceptor pathology, and may contribute to when disease presents, how disease progresses, and specific pathological features. Additional photoreceptor cilium gene variants may also impact how disease manifests in terms of the geographic presentation and photoreceptor types affected. This concept could address why variants in *RP1L1* are associated with a diversity of photoreceptor degenerative diseases and the incomplete penetrance of OMD.

The idea of mutational load or combinations of variants impacting phenotypic outcome with *RP1L1* is supported by findings in mouse models and human patients. In mice, heterozygous *Rp1l1* (*Rp1l1*$^{-/+}$) or *Rp1* (*Rp1*$^{-/+}$) loss-of-function variants are relatively benign, and do not result in photoreceptor morphology changes [323]. However, mice with heterozygous variants in both *Rp1l1* and *Rp1* (*Rp1l1*$^{-/+}$;*Rp1*$^{-/+}$) undergo photoreceptor degeneration. This is a complex example, as specific *RP1* variants can cause dominantly inherited photoreceptor disease in human patients;

nevertheless, the mouse example demonstrates that *RP1L1* interacts with other axonemal components and that phenotypic presentation may be modified based on otherwise benign variants in additional photoreceptor cilium genes. There have been additional reports of patients presenting with photoreceptor degeneration carrying variants in *RP1L1* and other photoreceptor genes. Liu and colleagues reported a patient with an inherited retinal dystrophy associated with variants in *RP1L1* and *C2ORF71*, a regulator of photoreceptor cilium actin [362]. Individually, the mutations do not seem to cause disease (the parents were unaffected), however in combination they appear to be deleterious. There is also a reported case of OMD with a pathogenic variant in *RP1L1* – p.R45W specifically, the most frequently reported OMD-causing variant – and compound heterozygous mutations in *KCNV2* [366]. *KCNV2* is a component of the voltage-gated potassium channel in the photoreceptors; variants in *KCNV2* are associated with autosomal recessive cone dystrophy [367]. Therefore, this combination of variants within different essential photoreceptor components may contribute to disease penetrance and its relatively early onset (27 years). Similarly, a pedigree with *RP1L1* (p.R45W) and *ABCA4* (p.R2040Q) variants has also been reported [368]. *ABCA4* variants are well-known causes of Stargardt macular degeneration and ABCA4 protein is found at the edges of photoreceptor OS discs, where it is involved in retinoid recycling [369]. The phenomenon of genetic variants in many genes impacting disease onset, progression, and pathology is certainly a more common occurrence than is currently appreciated, particularly for diseases with phenotypic variability and genes with many interactors.

2.5.4 OMD PRESENTING WITHOUT GENETIC FINDINGS

About 50% of OMD patients do not have genetic findings. OMD presenting without *RP1L1* variants could be the result of variants in *RP1L1* non-coding elements. Pathogenic variants in the regulatory or intronic regions of *RP1L1* have not yet been reported in the literature. Reliance on exome sequencing rather than whole genome sequencing can cause pathogenic non-coding variants to be missed. *RP1L1* is a four-exon gene that produces a large protein. Splicing errors

would result in the production of abnormal length RP1L1 protein, and potentially lead to mislocalization, dysregulation, atypical binding, and/or accumulation within the photoreceptor cells. Any of these scenarios could lead to structural defects in the photoreceptor axoneme or OS and subsequent photoreceptor loss.

Complex, changing pathologies could also underlie an OMD-like phenotype without *RP1L1* variants. OMD may not be a singular disease, but may represent early stages of numerous macular diseases; in other words, many retinal diseases may present with an OMD-like phenotype during first onset. Individually observed ocular phenotypes could be in evolution, and patients may have starkly different clinical presentations as disease progresses. Clinical re-assessment of their phenotype at later stages and the examination of other family members could result in a more accurate clinical diagnosis, and allow for a specific genetic diagnosis. Early stage Stargardt disease has been reported to present with an OMD-like phenotype [370]. Indeed, this has also occurred in our experience; a patient presented with what appeared to be *RP1L1*-negative OMD, but upon re-assessment at a later date was clinically diagnosed with Stargardt macular degeneration and subsequently confirmed to carry compound heterozygous variants in *ABCA4*.

OMD can also be a feature of more complex neurological disease. Patients with spinocerebellar ataxia, a progressive inherited neurodegenerative disorder, can have *RP1L1*-negative OMD as a retinal manifestation [371]. This could potentially be true for additional neurological conditions, whereby a retinal presentation mimics OMD.

2.6 CONCLUSION

RP1L1 variants can lead to a variety of photoreceptor diseases (Figure 10). *RP1L1* has been associated primarily with dominant OMD and recessive RP. While isolated cases of recessive OMD have been reported (Table 8), *RP1L1* has not been linked to dominantly inherited RP. However, it is important to note that many commercially available RP gene panels currently do

not include *RP1L1*, and this may have resulted in under-reporting of RP-associated *RP1L1* variants.

I observed that *RP1L1* variants are associated with non-OMD maculopathies and a rod-cone dystrophy, which indicates that *RP1L1* variants do not exclusively cause OMD and RP. Additionally, the phenotypic presentation of OMD patients is not static, and some patients progress to an *RP1L1* maculopathy with visible fundus findings. This is an important observation, and *RP1L1* should be considered as a gene candidate in patients with visible fundus findings.

The photoreceptor axoneme is essential for photoreceptor cell function. Cilium components have complex, poorly understood relationships to each other and photoreceptor transduction machinery. As an axonemal protein, *RP1L1* variants may modify the onset or progression of other photoreceptor diseases, and variants in additional photoreceptor or cilia genes may impact the presentation of *RP1L1*-associated disease. Further investigation into RP1L1 functionality is required for its influence on photoreceptor health and disease to be fully appreciated.

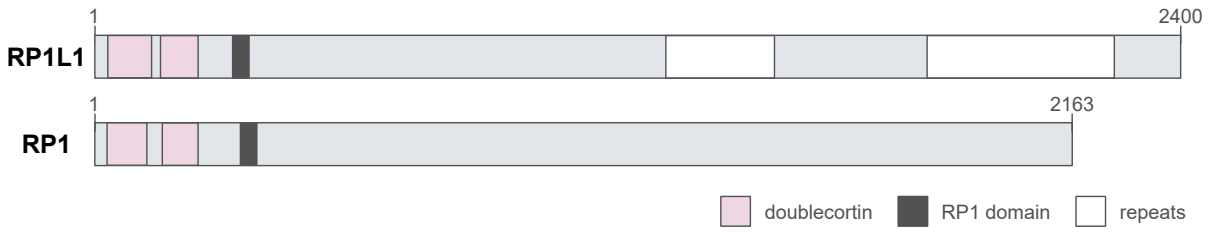

Figure 3. Comparison of RP1L1 and RP1 proteins.

RP1L1 and RP1 proteins are similar in size and domains. RP1L1 protein has a minimum length of 2400 amino acids, depicted here, and a maximum of 2480 amino acids. RP1 protein is 2163 amino acids in length. RP1L1 and RP1 both contain two N-terminal doublecortin domains (pink) and a unique RP1 domain (dark grey). RP1L1 also has two repeat domains, shown in white, which RP1 lacks.

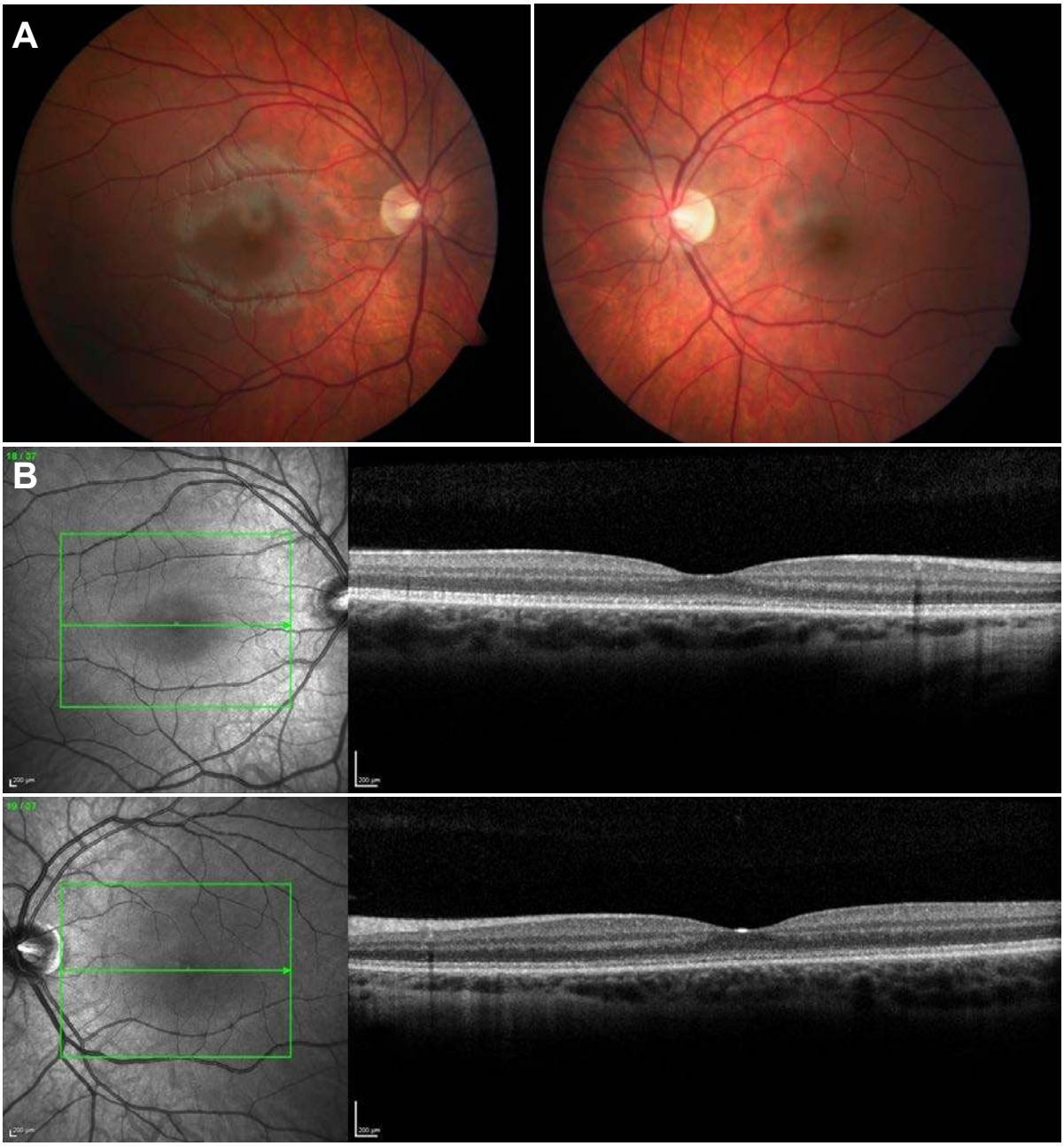

Figure 4. Occult macular dystrophy retinal phenotypes.

(A) A 15-year-old patient with the p.R45W variant in *RP1L1*. Fundus appears normal, as is typical for OMD patients. Characteristic of young patients, the images show sheen from the inner limiting membrane and foveal light reflex, suggesting normal foveal contour. **(B)** OCT shows loss of the ellipsoid zone in the sub-foveal region, consistent with cone loss.

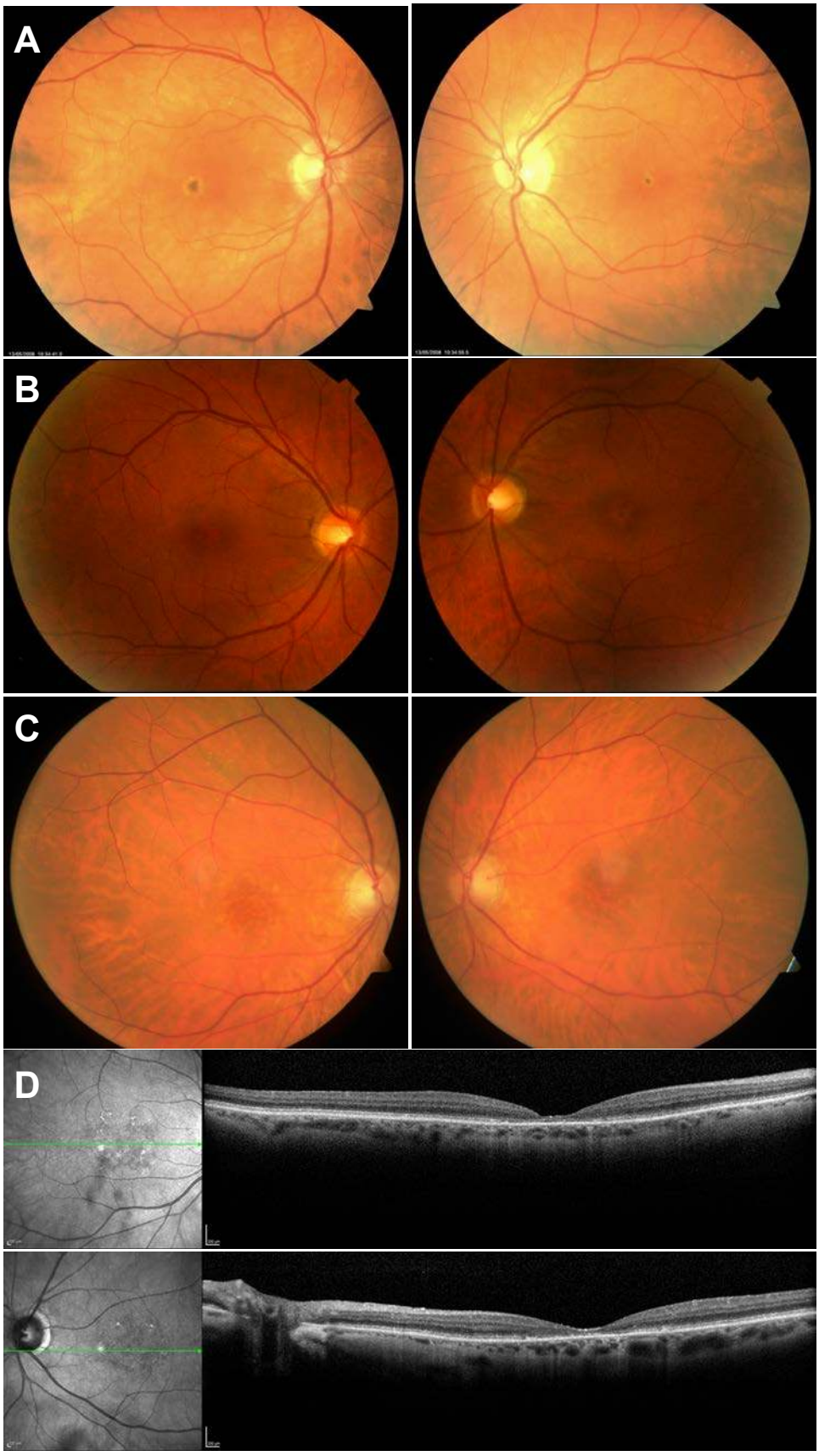
A
B
C
D

Figure 5. Patients with the most common OMD *RP1L1* variant (p.R45W) that progressed to an *RP1L1* maculopathy with retinal findings visible by fundoscopy.
(A) Fundus images from an 85-year-old patient with central macular findings. **(B)** 61-year old patient, son of patient in (A) with changes in the fovea. **(C)** Retina of a 64-year-old patient with macular mottling corresponding with photoreceptor loss. **(D)** OCT of the patient in (C), showing loss of the ellipsoid zone in the macular region, indicating photoreceptor loss.

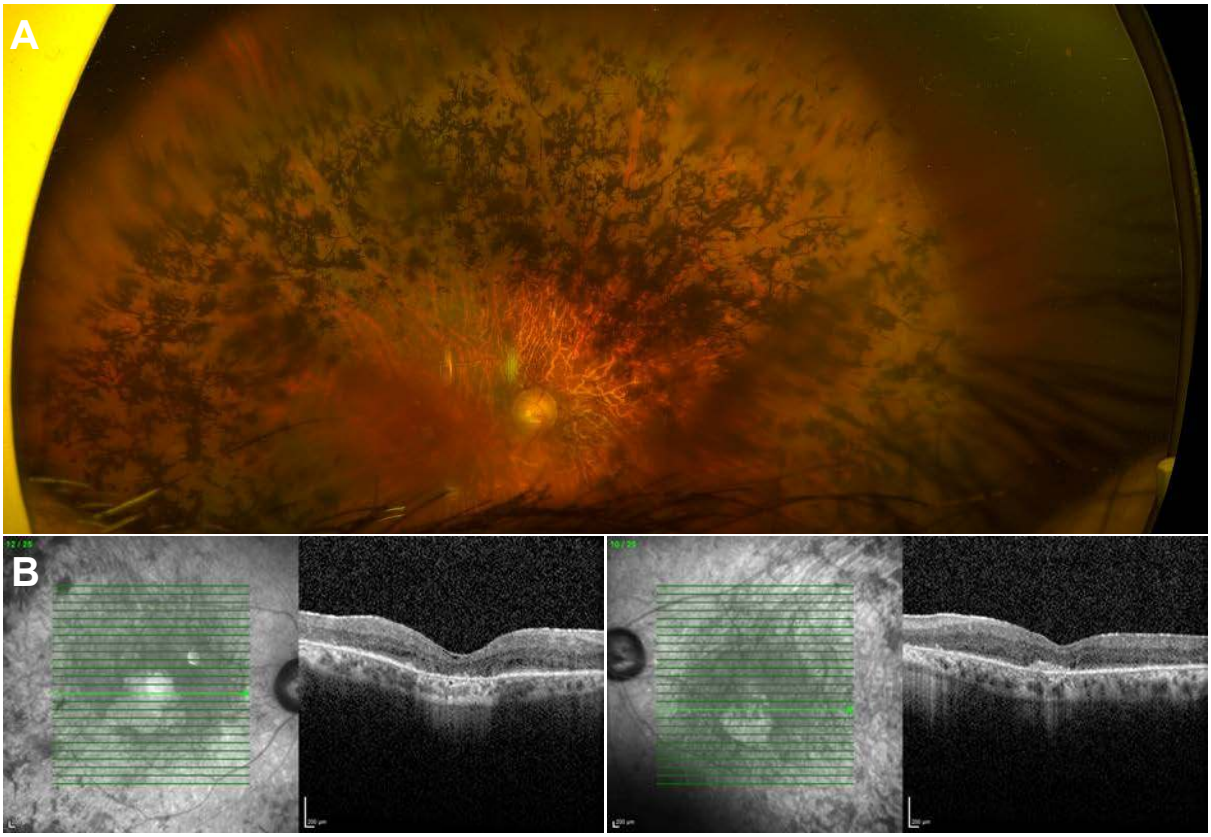

Figure 6. Fundus of a patient with retinitis pigmentosa associated with homozygous Lys203Argfs*28 variants *RP1L1* variants.

(A) Optos wide-field fundus images of the 79-year-old patient's retina show characteristic features of RP such as retinal thinning, attenuated blood vessels, and bone spicules. **(B)** SD-OCT images of both eyes show delimitation of the retinal layers, absence of the ellipsoid layer, attenuated RPE, and intra-retinal cystic changes. In the left eye, there is also a region of subretinal hyper-reflective material.

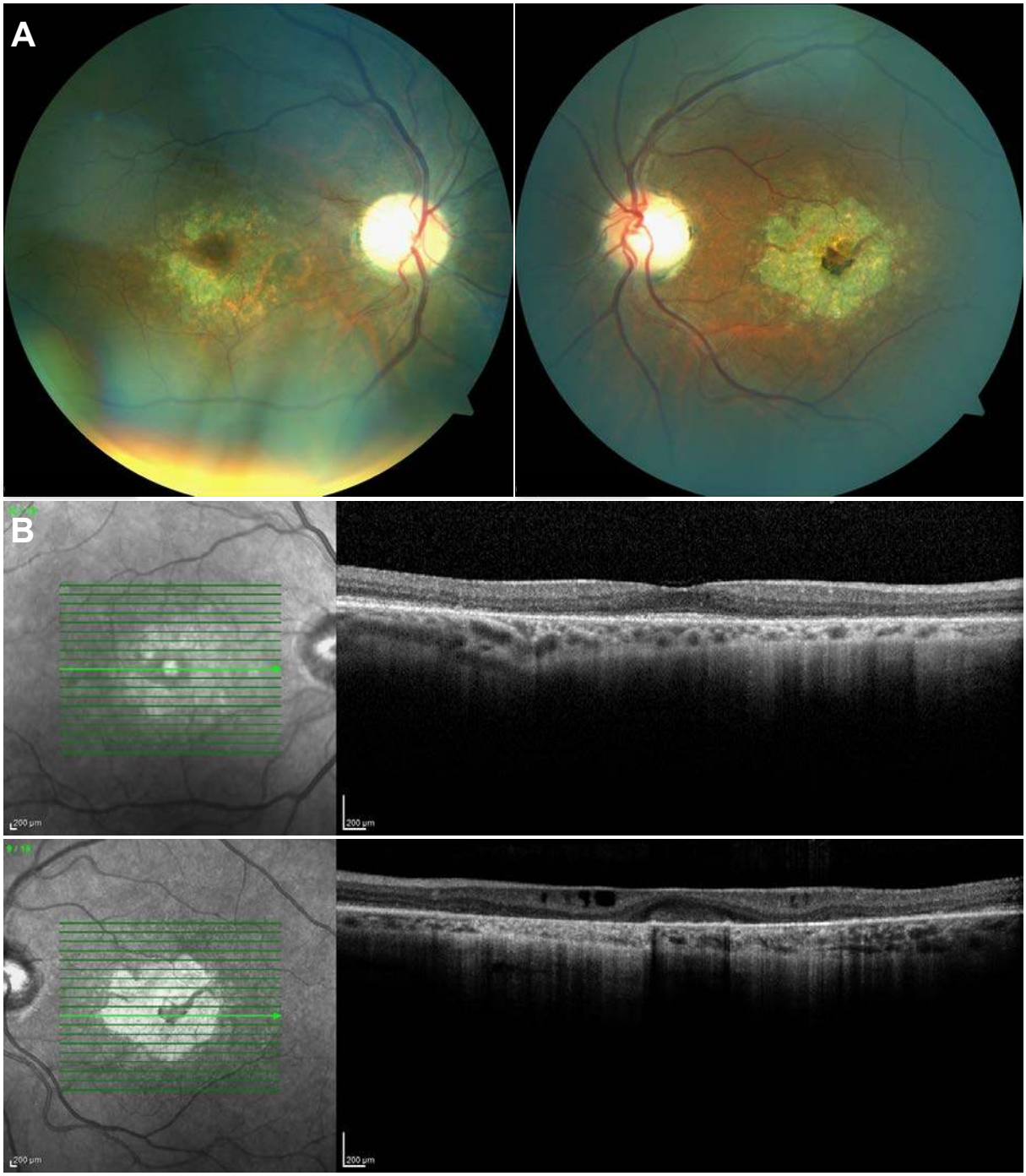
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Figure 7. Fundus and OCT of the patient ascertained from the eyeGENE database was clinically diagnosed with Stargardt disease and carried two mutations in *RP1L1*. **(A)** Patient was 58 years of age when examined. Geographic atrophy can be seen in both eyes around the fovea. **(B)** OCT shows that both eyes have loss of photoreceptors in the macula, accompanied by retinal thinning, RPE loss, and increased reflectivity of the choroid in the area adjacent to the fovea where the RPE is absent. The left eye has anomalous retinal lamination, areas of lucency in the inner plexiform layer, and severe loss of foveal anatomy.

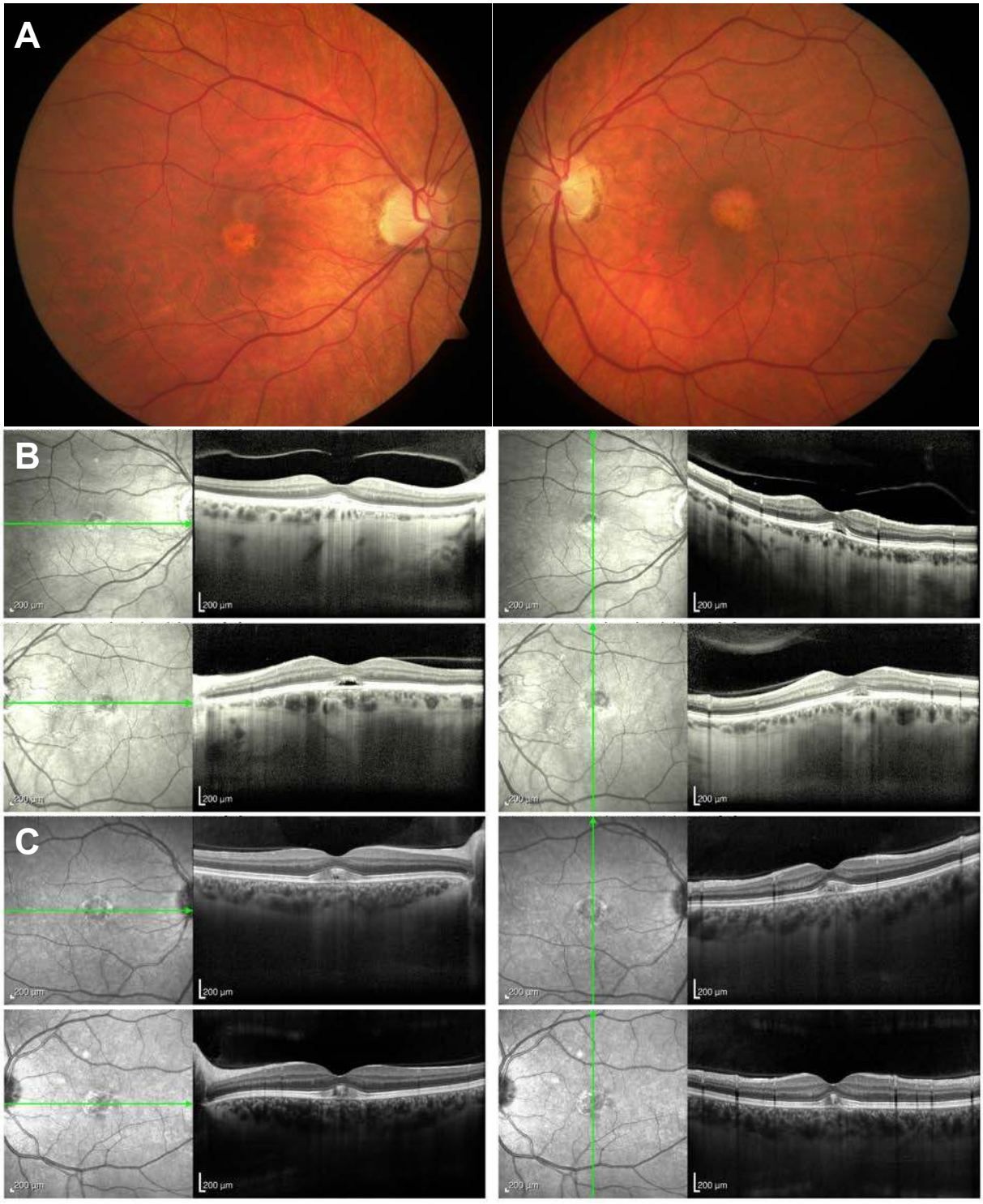
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Figure 8. Retinas of patients presenting with adult pseudovitelliform dystrophy with heterozygous *RP1L1* mutations.

(A) Fundus of the mother shows a bilateral, circular area of outer retinal mottling in the fovea, indicative of cone loss. Patient was 60 years old at examination. **(B)** In both eyes, the OCT shows sub-foveal outer retinal disruption with hyper-reflective debris, representing loss of the cone photoreceptors. **(C)** OCT of the affect daughter's eyes. Similar to the mother, this patient presented with sub-foveal outer retinal disruption with hyper-reflective debris, representing loss of the cone photoreceptors. Patient was examined when she was 38 years old.

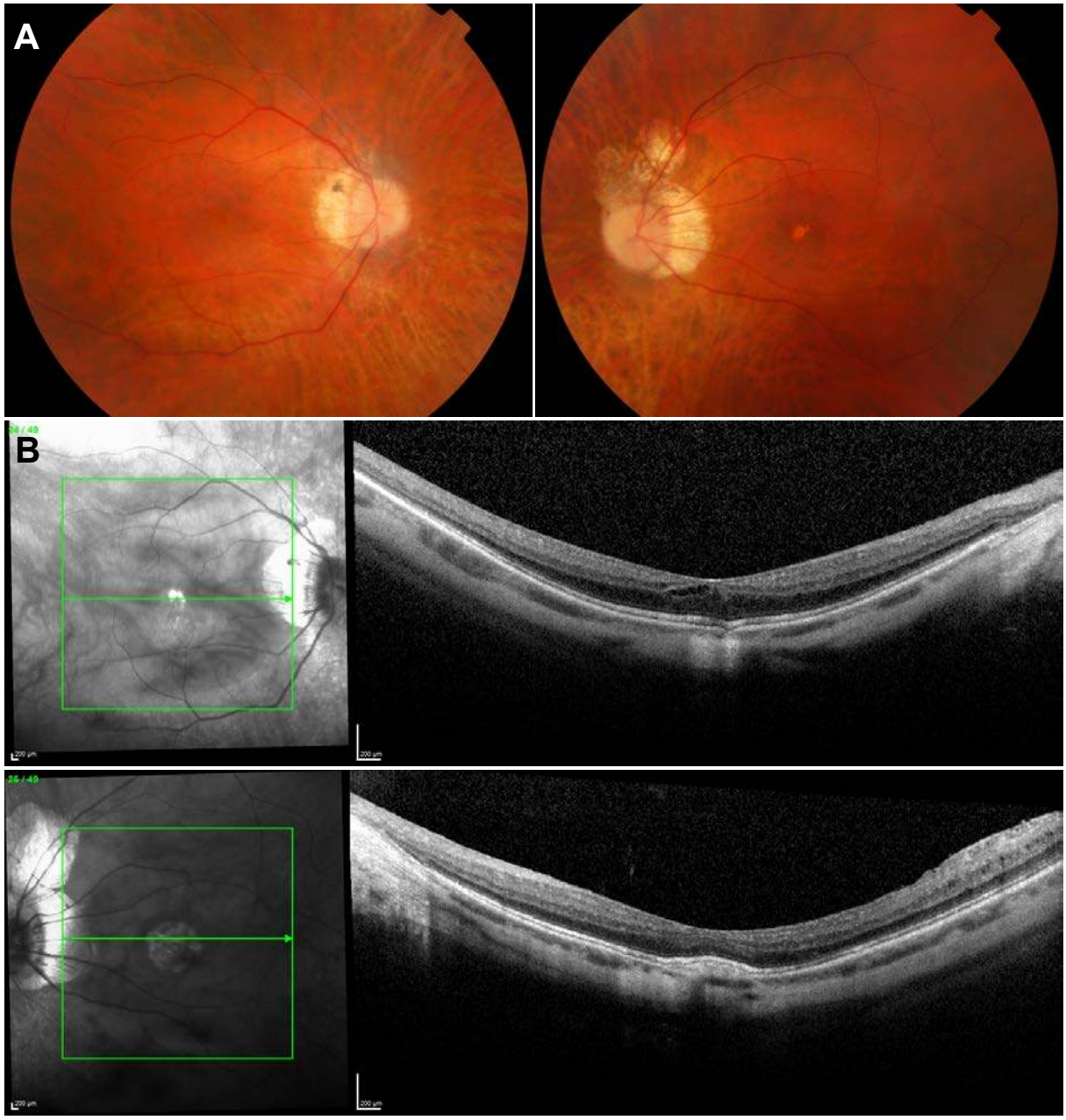

Figure 9. Rod-cone dystrophy associated with recessive *RP1L1* variants.

(A) Fundus of 55-year-old patient shows pale optic nerves, peripapillary atrophy, vascular attenuation, and peripheral retinal thinning. The left eye has a small region of mottling of the central macula. **(B)** SD-OCT through the macular region shows loss of foveal anatomy in both eyes. In the right eye, there is loss of ONL volume, and cystic structures in the central OPL. In the left eye, there are sub-RPE deposits with blocked fluorescence in the macular region.

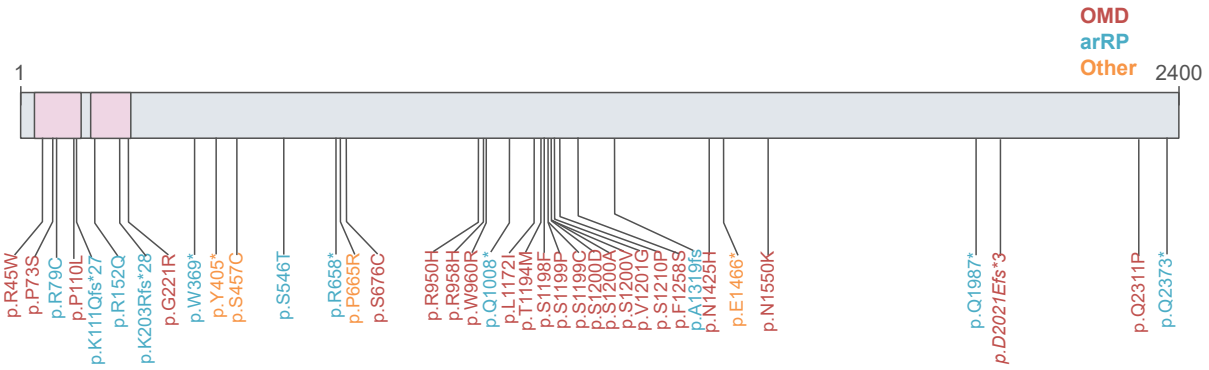

Figure 10. Map of RP1L1 variants onto the RP1L1 protein.

Variants in RP1L1 can be associated with OMD (red), RP (blue), or other retinal conditions (orange). Protein domains other than the doublecortin domains (pink) were omitted for simplicity.

Table 8. Disease-causing variants associated with occult macular dystrophy reported in the literature.

*Indicates that the variants were all found within a single patient.

Base Change	Amino Acid	Mutation Type	Allelic State	Literature Source (First Report)	Allele Frequency
133C>T	R45W	Missense	Heterozygous; single reported homozygous case [357]	[185]	2.03e-05
217C>T	P73S	Missense	Heterozygous	[340]	1.65e-03
329C>T	P110L	Missense	Heterozygous	[184]	1.05e-04
661G>A	G221R	Missense	Heterozygous	[346]	2.41e-05
2026A>T	S676C	Missense	Heterozygous	[333]	1.2e-05
2849G>A	R950H	Missense	Heterozygous	[184]	2.72e-04
2873G>A	R958H	Missense	Heterozygous	[184]	2.43e-05
3107T>C	W960R	Missense	Heterozygous	[185]	1.21e-05
3514C>A	L1172I	Missense	Heterozygous	[346]	2.79e-03
3581C>T	T1194M	Missense	*Heterozygous; in *cis* with T1196I	[346]	5.61e-05
3587C>T	T1196I	Missense	*Heterozygous; in *cis* with T1194M	[346]	4.01e-06
3593C>T	S1198F	Missense	Heterozygous	[345]	N/A
3595T>C	S1199P	Missense	Heterozygous	[342]	N/A
3596C>G	S1199C	Missense	Heterozygous	[356]	N/A
3599G>A	G1200D	Missense	Heterozygous	[346]	N/A
3599G>C	G1200A	Missense	Heterozygous	[184]	N/A
3599G>T	G1200V	Missense	Heterozygous	[346]	N/A
3602T>G	V1201G	Missense	Heterozygous	[346]	N/A
3628T>C	S1210P	Missense	Homozygous	[335]	N/A
3773T>C	F1258S	Missense	Heterozygous	[184]	3.01e-04
4273G>C	N1425H	Missense	Heterozygous	[333]	3.74e-04
4650T>G	N1550K	Missense	Heterozygous	[346]	8.16e-06
6063delC	D2021Efs*3	Frameshift	*Homozygous	[346]	1.24e-04
6932A>C	Q2311P	Missense	Heterozygous	[333]	2.0e-05

Table 9. RP1L1 variants associated with autosomal recessive retinitis pigmentosa.

Base Change	Amino Acid	Mutation Type	Allelic State	Literature Source (First Report)	Allele Frequency
235C>T	R79C	Missense	Homozygous	[361]	1.32e-04
326_327insT	K111Qfs*27	Frameshift	Heterozygous; in *cis* with Q2373*, putative digenic inheritance with *C2orf71*	[362]	4.08e-05
455G>A	R152Q	Missense	Compound heterozygous with c.5959C>T; p.Q1987*	[357]	3.57e-04
601delG	K203Rfs*28	Frameshift	Homozygous	[184]	2.41e-05
1107G>A	W369*	Nonsense	Homozygous	[357]	3.22e-05
1637G>C	S546T	Missense	Homozygous	[184]	4.7e-05
1972C>T	R658*	Nonsense	Homozygous	[361]	2.41e-05
3022C>T	Q1008*	Nonsense	Homozygous	[357]	N/A
3955_3956insGGACTAAAGTAATAGAAGGGCTGCAAGAAGAGAGGGTGCAGTTAGAGG	A1319fs	Frameshift	Homozygous	[363]	N/A
5959C>T	Q1987*	Nonsense	Compound heterozygous with c.455G>A; p.R152Q	[357]	1.55e-03
7117C>T	Q2373*	Nonsense	Heterozygous; in *cis* with Lys111Glnfs*27, putative digenic inheritance with *C2orf71*	[362]	4.01e-06

Table 10. RP1L1 mutations associated with other (non-OMD, non-RP) retinal conditions.

Disease	**Base Change**	**Amino Acid**	**Mutation Type**	**Allelic State**	**Allele Frequency**
Best-like	1994C>G	P665R	Missense	Heterozygous	2.39e-05
Severe Macular Degeneration/ Geographic Atrophy	1370C>G	S457C	Missense	Compound heterozygous with c.4396G>T; p.E1466*	4.15e-06
Severe Macular Degeneration/ Geographic Atrophy	4396G>T	E1466*	Nonsense	Compound heterozygous with c.1370C>G; S457C	5.27e-05
Rod-Cone Dystrophy	1215T>G	Y405*	Nonsense	Homozygous	1.61e-05

Table 11. Outcomes of pathogenicity predictors for missense variants in RP1L1.

Lines in italics are novel patient variants reported here.

Protein Change	PolyPhen-2	Mutation Taster	MutPred2	PANTHER	SIFT	PROVEAN	ACMG
R45W	damaging	polymorphism	likely benign	likely benign	damaging	deleterious	—
P110L	damaging	disease causing	benign	likely benign	tolerated	neutral	—
W960R	damaging	disease causing	likely pathogenic	likely damaging	damaging	deleterious	—
S1199C	damaging	disease causing	likely benign	likely benign	damaging	neutral	—
S457C	*damaging*	*polymorphism*	*likely benign*	*likely benign*	*damaging*	*deleterious*	variant of uncertain significance*
P665W	*damaging*	*polymorphism*	*likely benign*	*likely benign*	*damaging*	*deleterious*	likely pathogenic**

3 PROGRESSIVE PHOTORECEPTOR DYSFUNCTION AND AGE-RELATED MACULAR DEGENERATION-LIKE FEATURES IN *RP1L1* MUTANT ZEBRAFISH

This chapter is modified from the published manuscript: Nicole C. L. Noel, Nathan J. Nadolski, Jennifer C. Hocking, Ian M. MacDonald, and W. Ted Allison. (2020) *Progressive photoreceptor dysfunction and age-related macular degeneration-like features in* rp1l1 *mutant zebrafish*. Cells, 9(10): 2214.

3.1 INTRODUCTION

Photoreceptors are sensory neurons that detect light via an elaborated cilium, the OS. The photoreceptor OS contains membranous discs densely packed with opsins, the photosensitive proteins. As the OS is a modified cilium, pathogenic variants in ciliary components are common causes of photoreceptor disease [324,372]. The morphology of the OS distinguishes the two types of photoreceptors, cones and rods. Cones allow for high-acuity daytime and colour vision, while rods are responsible for low light vision. The human retina is organized such that the peripheral retina is rod dense and the central retina, termed the macula, is cone dense. Diseases that lead to degeneration or dysfunction of the cone photoreceptors in the macula are called maculopathies.

The most common maculopathy is AMD. AMD is the leading cause of vision loss for individuals over 50 in North America, affecting ~2% of people [12]. Patients with AMD experience visual acuity decline, colour vision deficits, and central vision loss. There are two types of AMD: dry and wet AMD. Dry AMD is characterized by accumulation of drusen and subretinal drusenoid

deposits, which are deposits that occur in specific retinal locations. Drusen are lipid-rich deposits that collect between the RPE and underlying choroid vasculature, while subretinal drusenoid deposits build up between the photoreceptor OSs and the RPE (Figure 11). In advanced cases, dry AMD can progress to wet AMD, characterized by retinal neovascularization. Neovascularization can lead to leakage of blood into the retina and further tissue damage. Despite AMD being a common cause of vision loss, there are no effective treatments for dry AMD and only angiogenesis inhibitors for blocking aberrant vessel growth in wet AMD. The lack of therapeutics results in part from challenges surrounding generation of animal models to investigate disease mechanisms underlying AMD progression. AMD is a multi-factorial condition, with many genetic and environmental contributors, making it difficult to establish a genetic model. Additionally, commonly used animal models for studying human disease, such as mouse and rat, have minimal cones and no macula and rarely exhibit many of the hallmark features of AMD during retinal degeneration progression, like drusen or subretinal drusenoid deposits.

OMD is a rare inherited maculopathy. Similar to AMD, OMD is characterized by progressive central vision loss and colour vision disturbances [329,330,373]. OMD was initially termed "occult" because OMD patients had normal appearing maculas, as observed by clinical fundus imaging, despite central vision loss [329,330,373]. Modern OCT imaging revealed that OMD patients do in fact exhibit loss of macular photoreceptors and retinal thinning [374]. Recently, it was reported that OMD patients can progress to a maculopathy with fundus findings [183].

OMD is caused by mutations in the gene *RP1L1* [185]. RP1L1 is a component of the photoreceptor axoneme [322,323,375]. It is a microtubule-binding protein unique to photoreceptors [323,328]. The function of RP1L1 in the axoneme is unknown, although it is believed to bind the axoneme microtubules and associate with other (currently unidentified) proteins [183,323,375]. Evidence from mouse models suggests that RP1L1 may play a role in maintaining OS disc structure or tether the discs to the photoreceptor axoneme, as *Rp1l1* loss resulted in disorganized OSs [323]. Therefore, pathogenic variants in *RP1L1* may lead to axonemal

instability, loss of OS structural integrity, and subsequent OS degeneration. *RP1L1* mutations have been reported in cone and rod disease, including OMD, *RP1L1* maculopathies, rod-cone dystrophy, and the rod degenerative disease RP [183–185].

RP is the most common inherited photoreceptor degenerative disease, characterized by peripheral vision loss and night blindness due to rod photoreceptor dysfunction and death [1,358]. RP is extremely genetically heterogeneous, with pathogenic mutations in many genes leading to disease. During RP progression, rod defects and degeneration lead to RPE atrophy, which causes intra-retinal pigment translocation called bone spicules. Cones degenerate during late stage RP via poorly understood processes, even when the disease is caused by rod-specific genetic lesions [359].

Zebrafish are an advantageous model of photoreceptor disease, as they are diurnal vertebrates with an abundance of cone photoreceptors and retinas conserved with the mammalian retina in terms of structure and function. Mice, while a commonly utilized mammalian model for human disease, are nocturnal, and due to adaptation for night activity have rod dominant retinas with few cones and limited cone-cone interactions. Zebrafish have roughly equal proportions of cones and rods, making their retinas more similar to the human macula than other retinal regions. This allows zebrafish to be utilized for dissecting etiology of cone and rod disease, as well as assessment of how rod loss impacts cone photoreceptors.

I developed a zebrafish model of *rp1l1*-associated photoreceptor disease using CRISPR/Cas9 genome editing. Mutant animals presented with progressive photoreceptor dysfunction and rod OS disorganization. During late-stage disease, *rp1l1* mutant retinas had subretinal lipid deposits, a feature similar to AMD pathologies.

3.2 METHODS

3.2.1 ZEBRAFISH CARE

Zebrafish were maintained in the University of Alberta aquatics facility using protocols approved by the Animal Care and Use Committee: Biosciences, under guidelines from the Canadian Council of Animal Care (protocol AUP00000077). The fish were maintained at 28°C, under a 14/10 light/dark cycle. All control fish were of the AB line and the mutant line was generated on the AB background. Stock solution of tricaine anesthetic was mixed using 400 mg of tricaine powder (Sigma Aldrich, St. Louis, MO, USA) in 97.9 mL of distilled water. 2.1 mL 1 M Tris, pH 9, was added and pH adjusted to 7.3.

3.2.2 GDNA EXTRACTION

Extraction of gDNA was performed as previously described [376]. 50 mM NaOH was added to the tissue, boiled at 95°C for 20 minutes, then cooled on ice. 1M Tris-HCl, pH 8, was added to neutralize the pH, at a 1/10 volume of NaOH. Samples were subsequently centrifuged and the supernatant isolated for DNA. For larval finclips, 20 µL of NaOH was used, 50 µL for adult finclips, and 100 µL for pooled embryos.

3.2.3 CRISPR/CAS9 GENOME EDITING

CRISPR sites were identified in the first coding exon of *rp1l1* (Gene ID: 101882236) using Geneious R9 (Biomatters Inc., San Diego, CA, USA). Previously, there were two *rp1l1* homologues annotated in zebrafish: *rp1l1a* and *rp1l1b*. However, *rp1l1b* did not resemble *rp1l1* genes in other organisms (including human, mouse, cow, pig, dog, chicken, and *Xenopus*) as it was missing defining features that identify *rp1l1* and was almost twice the size. In addition, in more recent versions of the zebrafish genome (z10), *rp1l1b* is no longer annotated. Therefore, it appears that zebrafish have one *rp1l1* homolog, formerly annotated as *rp1l1a*, which I targeted for my

experiments. The guide RNA (gRNA; DNA-targetting sequence: 5'-CATCTTGACGCCTTTGAACT-3') was synthesized as previously described [377], using the mMessage mMachine SP6 Transcription Kit (Invitrogen, Waltham, MA, USA). Embryos were injected at the single-cell stage using a glass needle mounted on a micromanipulator. The injection mix consisted of: 1µL (>1500 ng/µL) gRNA, 2 µL Cas9 nuclease, *S. pyogenes* (New England Biolabs, Ipswich, MA, USA), 0.5 µL Cas9 buffer (New England Biolabs, Ipswich, MA, USA), and 1.5 µL 1.5M KCl.

3.2.4 FINCLIPS AND GENOTYPING

To select for CRISPR/Cas9-injected fish that could produce mutant offspring, mosaic P0 CRISPR-injected animals were grown to adulthood and crossed to uninjected AB fish. The 5 dpf larval zebrafish were pooled in groups of 25 for gDNA extraction. The extracted DNA was used as template to PCR amplify a 479 bp fragment of *rp1l1* (forward primer: 5'-GGCTTTTTCGACGCTGATCC-3'; reverse primer: 5'-AATCCTTTTGGGGTGCCGAT-3') which was then TOPO cloned (Invitrogen, Waltham, MA, USA), and transformed into One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen, Waltham, MA, USA). Colony PCR was performed on 10-20 colonies per larval gDNA pool, and the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) for Sanger sequencing. Once individuals with germline mutations were identified, they were bred to AB fish and the progeny screened for mutations. To genotype the progeny, 3-4 dpf larvae were individually placed in a droplet of embryo media in a plastic petri dish [378]. A sharp scalpel blade was used to remove a small piece of the tail. Single larvae were transferred to a 24-well dish with fresh embryo media, and the tail fragment was transferred to a 1.5 mL microfuge tube using a 200 µL pipette that had PBS + 0.1% Tween aspirated into it to prevent the tissue from sticking. To isolate mutant alleles, a region of *rp1l1* was PCR amplified using the aforementioned primers, TOPO cloned (Invitrogen, Waltham, MA, USA), transformed into One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen, Waltham, MA, USA), and sequenced. Once animals with mutant alleles were isolated, they were

grown, crossed to AB fish, and their progeny in-crossed. Homozygosity for the 16 basepair (bp) deletion allele (ua5017) used for this work was confirmed in adult animals through finclip, PCR amplification using the same primers, and RFLP using BtsIMutI (New England Biolabs, Ipswich, MA, USA).

3.2.5 QPCR

5 dpf embryos were pooled in groups of 50 and flash frozen in liquid nitrogen. RNA was extracted using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was generated using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The qPCR was set up according to the Luminaris HiGreen High ROX qPCR kit (Thermo Fisher Scientific, Waltham, MA, USA) and performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Results were analyzed using the ΔΔCT method.

3.2.6 ELECTRORETINOGRAPHY

Electroretinographic measurements were obtained as previously described [81]. Briefly, zebrafish were dark adapted for 20 minutes in a dim, red-illuminated room, and anaesthetized in a working solution of 4% stock tricaine/aquatics facility water for 2-3 minutes. An anaesthetized fish was then transferred to a moistened PVA sponge on the testing platform and positioned on its side. The reference electrode (PN: EP08, World Precision Instruments Inc., Sarasota, FL, USA) was positioned underneath the sponge while the Ag/AgCl recording electrode was carefully placed on the centre of the cornea using a micromanipulator (Narishige International USA Inc., Amityville, NY, USA). The testing platform was then inserted into the Ganzfeld light stimulator connected to an E3 Electrophysiology System (Diagnosys LLC, Lowell, MA, USA). The testing protocol was an increasing scotopic series of 0.2, 1.0, 3.0, and 10 cd·s/m^2 stimuli, each step consisting of 5 single flashes of white light separated by 5000 ms. A band-pass filter of 0.3-300

Hz was applied to reduce system noise. Following the testing protocol, the fish was removed from the platform and placed into a recovery tank. Zebrafish survival was maximized by gently pumping water past the gills using a 1 mL transfer pipette until normal gilling and swimming behaviour was regained. Waveforms were generated by averaging 5 flashes for each stimulus intensity. The b-wave amplitude was measured as the difference in amplitude between the trough of the initial negative a-wave and the peak of the b-wave. If the a-wave was not present, the b-wave was measured from the baseline.

3.2.7 OPTICAL COHERENCE TOMOGRAPHY

Zebrafish were anaesthetized using 4% tricaine stock solution/aquatics facility water and placed on a damp, triangular sponge in a plastic chamber, with the right eye facing upwards for imaging. The subject was secured with a strip of damp gauze with small weights on both sides and covered with tricaine/fish water solution for the procedure. The handheld Envisu R-Series OCT (Bioptigen Inc., Durham, NC, USA) was mounted perpendicularly to the zebrafish, thereby directed at the eye. InVivoVue 2.4 OCT Management Software (Bioptigen Inc., Durham, NC, USA) was used for image acquisition and analysis. After the procedure, animals were transferred to a recovery tank and aquatics facility water was gently passed over the gills with a 1 mL transfer pipette to resuscitate them. Fish were observed until normal swimming behaviour was maintained.

3.2.8 ELECTRON MICROSCOPY

Zebrafish were euthanized with 8.4% tricaine stock solution in aquatics facility water, underwent surgical cervical dislocation, and enucleation. Lenses were removed with forceps and the eyes were placed in fixative (2.5% glutaraldehyde/2% paraformaldehyde) for a minimum 24 hours before processing. Post fixation but prior to dehydration and processing, the eyes were cut into halves to allow for appropriate penetration of solutions. Tissue was washed with 0.1M

phosphate buffer three times for 10-15 minutes each, stained with 1% osmium tetroxide for 1 hour, and dehydrated through an ethanol series (50%, 70%, 90%, 100%, 100%, 100%) allowing 15-25 minutes for each step. The tissue was treated with a 1:1 ratio of ethanol: Spurr resin mixture for 1 hour, then the mixture was removed and replaced with pure Spurr resin for overnight. The Spurr resin was replaced with fresh resin two more times the next day, and the tissue subsequently embedded in resin in flat moulds and cured overnight at 70°C. Blocks were sectioned into 70-90nm sections using a Reichert-Jung Ultracut E Ultramicrotome and placed on grids. Sections were stained with uranyl acetate for 20 minutes, rinsed with water, then stained with lead citrate stain for 7 minutes, rinsed with water, dried, and imaged using a Morgagni 268 transmission electron microscope (Philips/FEI, Hillsboro, OR, USA) with a Gatan CCD camera (Gatan Inc., Pleasanton, CA, USA).

3.2.9 PARAFFIN PROCESSING AND HEMATOXYLIN AND EOSIN STAINING

Whole zebrafish heads were fixed in 4% paraformaldehyde for 48 hours prior to paraffin processing. Samples were transferred into 50% ethanol for 1-3 hours, then placed into a cassette and loaded into a Leica Tissue Processor 1020. The processing program steps were: 1 hour in 70% ethanol, 1 hour in 90% ethanol, 1.5 hours in 100% ethanol (twice), 1.25 hours in equal parts ethanol: toluene, 0.5 hours in toluene (twice), and 2 hours in wax. Tissue was embedded in paraffin blocks, sectioned with a microtome, placed on slides, and dried in a 37°C oven.

For staining, slides were treated with toluene for 10 minutes to remove paraffin wax, then rehydrated using an ethanol gradient (100%, 90%, 70%, 50% ethanol for 2 minutes each) and washed with distilled water. Slides were stained with Haematoxylin Gill III for 2 minutes, rinsed with distilled water, washed with cold tap water for 15 minutes, and washed with 70% ethanol for 2 minutes. Slides were treated with eosin for 30 seconds and washed twice in 100% ethanol for 2 minutes each, followed by two toluene washes for 2 minutes each. Slides were covered with Dibutyl Pthalate Xylene and a coverslip, then kept at 37°C overnight for media solidification.

Haematoxylin and Eosin (H&E) stained sections were imaged using a ZEISS AXIO A1 Compound Light Microscope (Zeiss, Toronto, ON, Canada) with a SeBaCam 5.1MP Camera (Laxco Inc., Bothell, WA, USA).

3.2.10 OIL RED O STAINING ON CRYOPRESERVED TISSUE

Zebrafish eyes were fixed in 4% paraformaldehyde overnight then dehydrated in 30% sucrose in phosphate buffer for 24 hours. The sucrose was removed and replaced with Optimum Cutting Temperature compound (Fisher Scientific, Toronto, ON, Canada). Eyes were embedded, flash frozen on dry ice, and stored at -80°C until sectioning. Tissue was then cryosectioned at 10 µm, placed on SuperFrost Plus microscope slides (Fisher Scientific, Toronto, ON, Canada), and stored at -80°C until use.

In preparation for staining, sections were allowed to thaw at room temperature for 1 hour. Tissue was treated with 4% paraformaldehyde for 20 minutes, washed with water, and rinsed with 60% isopropanol. A working solution of Oil Red O (0.3%) was prepared in 60% isopropanol and slides were stained for 15 minutes in a Coplin jar. Slides were rinsed with 60% isopropanol, nuclei stained with haematoxylin for 1 minute, and rinsed with distilled water. Mount Quick aqueous mountant (Electron Microscopy Sciences, Hatfield, PA, USA) was added to the slides, coverslipped, and sealed with nail polish. Images of sections were captured using a ZEISS AXIO A1 Compound Light Microscope (Zeiss, Toronto, ON, Canada) with a SeBaCam 5.1MP Camera (Laxco, Bothell, WA, USA).

3.2.11 STATISTICAL ANALYSIS

Statistical analysis was performed using Prism version 8.0 (GraphPad Software Inc., San Diego, CA, USA). For OCT measurements, Mann-Whitney tests were performed; for ERG results, unpaired T-tests were used to compare the genotypes and repeated measures ANOVAs with Tukey post-hoc comparison to investigate differences within the groups. The qPCR data was analysed

using the ΔΔCT method and statistical analysis was performed on the ΔΔCT values using Mann-Whitney tests. Propagation of error was calculated to determine upper and lower limits of error.

3.3 RESULTS

3.3.1 GENERATION OF *RP1L1* MUTANT ZEBRAFISH

Human RP1L1 is a 2400 amino acid protein with two doublecortin (microtubule-binding) domains and an RP1 domain towards the N-terminus. Zebrafish Rp1l1 is similar in size at 2394 amino acids in length (Figure 12), with the most well conserved sequences in the doublecortin and RP1 domains (Figure 13). As the most frequent OMD-causing mutation occurs in the first doublecortin domain of human RP1L1 and this region is well conserved between the species, I targetted the homologous region in zebrafish *rp1l1* using CRISPR/Cas9 genome editing. I generated a 16 bp deletion (ua5017 allele number) in the first doublecortin domain of *rp1l1* (Figure 12C). This deletion is predicted to severely truncate the Rp1l1 protein and abolish all functional domains (Figure 12B, Figure 13). Homozygous mutant fish were viable, fertile, and had no overt ocular defects at any developmental stage. Homozygous *rp1l1* mutants were used for all experiments (hereafter referred to as *rp1l1* mutants or mutants).

To assess whether the mutant *rp1l1* transcript undergoes nonsense-mediated decay, I utilized qPCR. The relative abundance of *rp1l1* transcript was not decreased in mutant zebrafish (average of 2.36 fold increase, lower limit = 1.6, upper limit = 3.4; n=4). Mutant transcript was not obviously lost via nonsense-mediated decay, but the frameshift mutation in the transcript is predicted to encode a nonfunctional Rp1l1 protein lacking all recognizable domains (Figure 12).

3.3.2 *RP1L1* MUTANTS HAVE PROGRESSIVE PHOTORECEPTOR DYSFUNCTION

Functional assessment of the photoreceptors was obtained with ERG under scotopic (dark-adapted) conditions. An amplitude reduction in ERG waveforms suggests an inability of the

photoreceptor to appropriately respond to the light stimulus and transmit information to downstream neurons. Light stimuli intensities used for ERG testing are 0.2 cd·s/m^2, which elicits a rod-dominated response, 1 cd·s/m^2, 3 cd·s/m^2, and 10 cd·s/m^2, which elicit mixed rod-cone responses.

1-month-old (juvenile) and 3-month-old (young adult) mutant animals had scotopic b-wave amplitudes comparable to wild-type (WT) under all intensities tested (Figure 14), suggesting photoreceptor function comparable to WT. However, the b-wave onset was significantly delayed in 1-month-old mutants for the 0.2, 1, and 3 cd·s/m^2 intensities (Figure 14C); this recovered by 3 months of age (Figure 14F). Taken together, the ERG assessment suggests that young *rp1l1* mutants have functional photoreceptors.

At 6 months of age, mutant animals had significantly decreased b-wave amplitudes when exposed to the 0.2 cd·s/m^2 intensity, with the average b-wave amplitude decreased by 57.9%. Responses were comparable to WT under all other intensities (Figure 15). The reduced dim stimulus amplitude indicates that 6-month-old *rp1l1* mutants have defects in rod photoreceptors. Intriguingly, the b-wave implicit time was increased for the 1 cd·s/m^2 and 3 cd·s/m^2 intensities in the mutants, suggesting that the photoreceptors were not able to transmit information at a typical rate for those intensities (Figure 15C).

12-month-old mutant zebrafish had significantly decreased responses under all light intensities tested (Figure 16). The average b-wave amplitudes for mutants were decreased by 25.8% for the 0.2 cd·s/m^2 intensity stimulus; 35.7% for 1 cd·s/m^2 intensity; 28.7% for 3 cd·s/m^2 intensity; and 31.5% for 10 cd·s/m^2 intensity. The decrease in b-wave amplitude at higher intensities was not observed at 6 months, and suggests progressive photoreceptor deficits in the *rp1l1* mutants. Mutants had significantly faster b-wave onset than WT for the 0.2 cd·s/m^2 intensity stimulus, but not the other intensities (Figure 16C). Figure 17 shows repeated measures analysis for WT and *rp1l1* mutants for all ERG intensities.

Taken together, these functional assessments suggest that *rp1l1* mutant zebrafish have progressive photoreceptor dysfunction. In light of this, I focused further investigations on aged *rp1l1* mutant zebrafish.

3.3.3 LIVE IMAGING REVEALS ABNORMALITIES IN THE OUTER RETINAS OF *RP1L1* MUTANT ZEBRAFISH

I assessed retinal structure in live animals to investigate how the functional deficits related to retinal structure in aged zebrafish using OCT. Photoreceptor ISs are highly reflective due to the abundance of densely packed mitochondria and appear as bright punctae on zebrafish OCT. The hyper-reflective punctae form banding patterns due to the different heights of the photoreceptor subtypes, and this region is equivalent to the EZ region in humans. In WT animals, the hyper-reflective punctae in the EZ region are numerous and form the expected banding pattern (Figure 18A). However, 12-month-old mutant zebrafish had visibly fewer punctae on sectional OCT scans (Figure 18A). This was similarly observed in *en face* projections, which provide a view of the photoreceptor mosaic. The WT *en face* projections show a full mosaic with clearly distinguishable rows of photoreceptors (Figure 18B). Conversely, mutant *en face* images have fewer punctae and the rows are difficult to distinguish (Figure 18B). Of note, some of the mutants also had large hyper-reflective blebs in their OCT images, characteristic of activated immune cells that have translocated to the outer retina [33,379–381]. Immune cell translocation is commonly seen in response to photoreceptor disease or outer retinal injury [33,269,379–382].

Next, I measured retinal layer thickness in the OCT images (Figure 19). Mutant animals had a significantly thinner EZ, which contributed to thinner retinas overall (Figure 19). Measurements were also taken for the outer nuclear layer (ONL) where the photoreceptor cell bodies are found, the outer plexiform layer (OPL) where the photoreceptors synapse with downstream interneurons, and inner nuclear layer (INL) where the interneuron cell bodies reside. These retinal regions were not statistically different in measurement between the WT and mutant

zebrafish, although the ONL and INL were trending towards a decrease in the mutants. The lack of statistically significant differences between genotypes for ONL and INL thicknesses may be attributable to modest sample size. These findings suggests that the photoreceptor EZ region is disrupted in the mutant animals, but that the cell bodies may remain intact. Taken together, the OCT results show that the mutant animals had abnormal photoreceptors at 12 months of age.

3.3.4 *RP1L1* IS REQUIRED FOR NORMAL ORGANIZATION OF PHOTORECEPTOR OUTER SEGMENTS

As Rp1l1 is a structural component of the photoreceptor OS, I assessed how OS morphology was impacted in the *rp1l1* mutant zebrafish. WT OS discs observed by electron microscopy (EM) were densely packed with few visible spaces between them, and discs uniformly spanned the OS width (Figure 20). EM of 11-month-old mutant zebrafish retinas showed disorganized photoreceptor OSs with many gaps and uneven, swirling discs (Figure 20). The vast majority of photoreceptor OSs were disorganized in mutant retinas, and few OSs had uniformly organized discs throughout the whole OS. In some OSs, I observed regions of normal appearing OS disc organization next to disorganized areas. The regions of normally organized OS discs were often at the most apical tip of the OS in distally positioned photoreceptors, which are most likely rods. While normal rod OSs were not typically observed, cone OSs sometimes appeared mildly affected, with largely normal OS disc morphology and small defects in uniformity and stacking (Figure 21). A subset of OSs were extremely abnormal; some contained vesicles instead of stacked discs, while others had multilayered membranes surrounding the OSs and an abnormal OS shape (Figure 22).

3.3.5 DISEASED ZEBRAFISH RETINAS HAVE SUBRETINAL LIPID DEPOSITS

I observed deposits between the photoreceptor OS and RPE in the EM images (Figure 20). The deposits were globular and typically 3-10 μm in diameter. EM thick sections stained with azure blue also showed the deposits by light microscopy (Figure 23A,B). H&E staining similarly

showed accumulations between the OSs and RPE, as well as short OSs and visibly thinner ONLs, in 12-month-old animals (Figure 23C,D). On H&E, but not the EM thick or ultrathin sections, the deposits appeared to be empty holes, suggesting that paraffin processing stripped the contents of the accumulations out of the tissue. Paraffin processing is known to have such an effect on lipid-rich materials. To assess whether these subretinal deposits in the mutant animal retinas were indeed lipid-rich, I next used Oil Red O staining. I found that in 14-month-old animals, the subretinal deposits stained with Oil Red O, indicating that they were lipid-rich (Figure 24). The location and positive staining for lipids suggests that these deposits may be comparable to drusen or subretinal drusenoid deposits observed in human patients. No such deposits were observed in age-matched WT zebrafish retinas using any of the described methods.

3.4 DISCUSSION

Animal models that can provide insight into photoreceptor disease and drusen formation are urgently needed to allow for the development and characterization of therapeutic strategies to prevent vision loss. I report a novel model of *rp1l1*-associated photoreceptor disease that has progressive photoreceptor dysfunction, seemingly caused by structural abnormalities at the level of the photoreceptor OS. In a previous study, knockdown of *rp1l1* was performed in larval zebrafish, but the authors did not characterize photoreceptor function or retinal features [362]. This study provides novel insight into the function of *rp1l1* in the aging zebrafish retina. To my knowledge, this is the first report of a zebrafish model of photoreceptor disease exhibiting subretinal drusenoid deposits. Photoreceptor defects similar to what is observed in RP and AMD-like lipid deposits in this model provide the opportunity to investigate means by which photoreceptors can be preserved and how lipid accumulations develop and impact the diseased retina. Additionally, the cone-rich zebrafish retina allows for assessment of how drusenoid deposits influence cones and how cones respond to rod dysfunction.

Electrophysiological assessment revealed progressive photoreceptor dysfunction in the *rp1l1* mutant zebrafish. The b-wave amplitude was comparable to WT in 1- and 3-month-old animals, abnormal under the dimmest intensity at 6 months, and abnormal under all tested light intensities at 12 months of age. However, a delay in b-wave implicit time was observed at 1 month that recovered by 3 months. The cause and functional impact of the delayed b-wave in 1-month-old *rp1l1* mutants is difficult to interpret. Recovery of the b-wave implicit time by 3 months may be due to changes in photoreceptor proportions, organization, and retinal circuitry. At 1 month, zebrafish photoreceptors are primarily organized in a larval mosaic, which has many more cones than rods at a ratio of ~4:1 [62,64,383]. At 3 months of age, the photoreceptors are organized in a row mosaic, with roughly equal proportions of cones to rods [62]. These differences between the ages may result in the changes observed in the b-wave implicit time in the mutants. Interestingly, at 12 months, after photoreceptor dysfunction progressed to impact photoreceptors under all tested light intensities, b-wave onset was increased in speed for the dimmest intensity. Why this was observed and what impact a shorter implicit time would have on the photoreceptors is unknown and requires further study.

The function of RP1L1 protein is unknown. However, evidence from mutant mice suggests that it is involved, either directly or indirectly, in maintaining OS morphology; *Rp1l1* mutant mice had progressive photoreceptor degeneration, thinner outer retinas, and subsets of disorganized OSs with wavy discs [323]. As RP1L1 is a microtubule-associated protein that localizes to the photoreceptor axoneme, it may be tethering the OS discs to the cilium core through its interaction with protein binding partners [183,323,375]. I observed disorganized photoreceptor OSs in the *rp1l1* mutant zebrafish. This observation coincides with data found in *Rp1l1*-deficient mice [323]; however, only a subset of photoreceptor OSs in *Rp1l1* mutant mice were disorganized, whereas the majority of OSs in *rp1l1* mutant zebrafish were affected. Interestingly, some photoreceptors in the *rp1l1* mutant zebrafish had disorganized discs through the length of the OS except for a small stretch of discs at the apical tip that appeared normally organized. The axoneme does not

extend through the entire rod photoreceptor OS, and the discs in the most apical OS region may be organized by RP1L1-independent means.

RP1L1 has a synergistic role with RP1, another photoreceptor-specific component of the axoneme that shares many features with RP1L1 [323,326,384,385]. RP1L1 and RP1 proteins are comparable in terms of size, domains, and localization, and both result in OS disorganization when mutated in animal models [323,384,385]. *RP1L1* mutations have been reported to cause a diversity of human photoreceptor disease that can affect cones and rods [183–185], while *RP1* mutations have primarily been reported in retinitis pigmentosa cases, though there have been some cone diseases associated with *RP1* [1,326,363,386]. RP1L1 likely has unique roles or binding partners that it does not share with RP1, and may have functions specific to cones or rods. Specialized subfunctions for RP1L1 in different photoreceptor types could explain why *RP1L1* mutations can cause both cone and rod disease in patients, in a mutation-specific manner.

I report accumulation of subretinal lipid deposits between the photoreceptor OS and RPE in the mutant animals. Subretinal drusenoid deposits and drusen are common in aging humans and are defining features of AMD, but how they impact retinal disease is not known [387]. Subretinal drusenoid deposits (also called reticular drusen, reticular pseudo-drusen, or reticular lesions) are a newly characterized feature of aging and AMD and are therefore not as well investigated as drusen [387–390]; however, presence of subretinal drusenoid deposits has been reported to more than double an individual's likelihood of developing AMD [387]. AMD patients can have both subretinal drusenoid deposits and drusen, but may alternatively have one and not the other. Drusen most frequently develop in the cone-rich central macula in humans, while subretinal drusenoid deposits typically occur in the comparatively rod-rich outer macula, though this is not always the case. These trends suggest that the photoreceptor types influence the development of drusen and subretinal drusenoid deposits. Thus, drusen and subretinal drusenoid deposits may be infrequently reported in small animal models due to the photoreceptor types present and their organization.

Small animal models of drusen and drusen-like deposits have been sought after for decades. There are some mouse models that develop drusen similar to what is observed in humans in terms of composition and location [391–394]. Recently, a mouse model with photoreceptor dysfunction and subretinal drusenoid deposits due to impaired cholesterol metabolism was reported [395]. However, some presumed models of drusen unfortunately had deposits that differed greatly from drusen observed in the human retina. In some cases, murine drusen-like accumulations were expanded macrophages rather than lipids [396]. Additionally, white lesions observed on funduscopy appeared drusen-like but were actually indicators of retinal atrophy or intra-retinal lesions, not lipid accumulation [397–399]. Of note, mouse models that develop drusen or subretinal drusenoid deposits are often complex double mutants or have mutations in genes not associated with AMD [391–395]. This zebrafish model of photoreceptor disease in a cone-rich retina has mutations in a gene known to cause photoreceptor degeneration in humans. Prior to this study, whether diseased zebrafish retinas could develop drusen or subretinal drusenoid-like deposits was unknown.

The origin of drusen and drusenoid deposits is not well understood. Drusen and drusenoid deposits may both result from RPE cell metabolic dysfunction in a diseased retina. As drusen develop between the choroidal vasculature and the RPE, it has been postulated that they may be derived from choroid-deposited materials. Indeed, the protein components of drusen are typically found in the blood and RPE [400]. However, the blood-retina barrier protects the retina from leakage of substances from the blood into the eye, and this hypothesis alone does not explain the components of drusen that seem RPE-derived. It is possible that in the healthy retina drusen-forming materials are normally deposited by the choroid and then taken up and broken down by the RPE, but that with age and disease the RPE loses cells or becomes dysfunctional, resulting in ineffective RPE phagocytosis and accumulation of deposited materials. In contrast, drusenoid deposits are found between the OS and RPE, making a vascular origin much less likely. There is evidence that regression of subretinal drusenoid deposits can coincide with severe outer retinal

thinning and loss of photoreceptor OSs in AMD patients [401]; this could suggest that photoreceptor OSs, or OS-RPE interactions, are key for development of subretinal drusenoid deposits. Drusenoid deposits contain some of the same components as drusen, including complement factor H, vitronectin, and ApoE [389]. The drusenoid-like deposits observed in this model provide the opportunity to investigate the cellular origins and protein composition of drusenoid deposits. This is an important finding, as animal models rarely develop drusen or drusen-like deposits. This model may be relevant to AMD by providing the unique opportunity for study of subretinal lipid deposits in photoreceptor disease progression, and how this impacts photoreceptor and RPE health and function over time.

Photoreceptor cell death is an irreversible cause of vision loss and blindness. Investigating disease mechanisms and genetic factors that impact disease onset and progression is essential for combating vision loss and generating effective therapies for patients. *RP1L1* mutations underlie a spectrum of photoreceptor disease and treatments for *RP1L1*-associated conditions cannot be developed without a keen understanding of the function of RP1L1 in photoreceptors. This zebrafish model of *RP1L1*-associated photoreceptor disease provides the opportunity to investigate the role of Rp1l1 in photoreceptor disease and the generation and impact of subretinal lipid deposits in diseased retina.

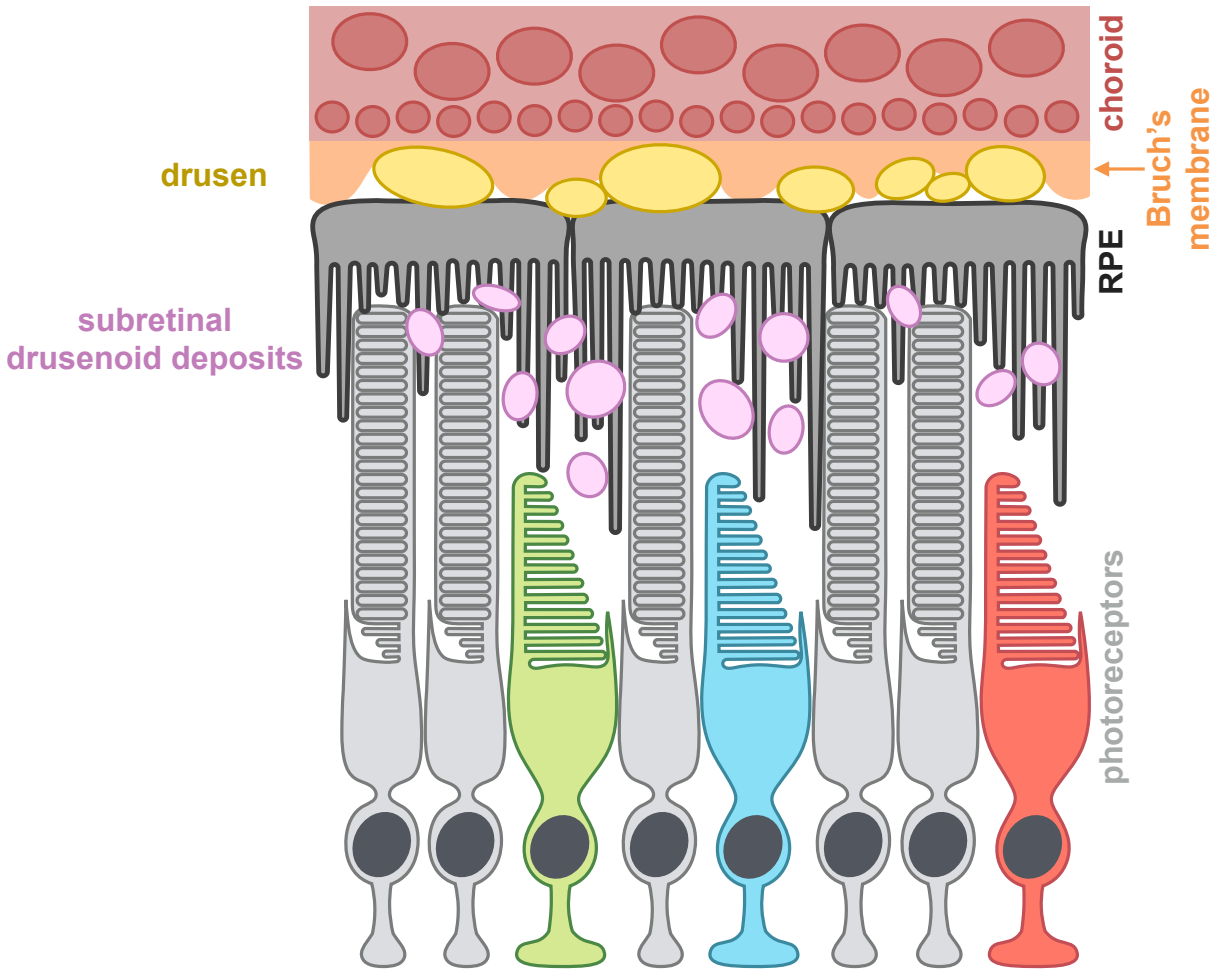

Figure 11. Drusen and subretinal drusenoid deposits in the retina.

Drusen (yellow) are lipid-rich deposits that occur between the choroid vasculature and the retinal pigment epithelium. Subretinal drusenoid deposits (pink) develop between the retinal pigment epithelium and photoreceptors, and contain membranous inclusions and lipids [402].

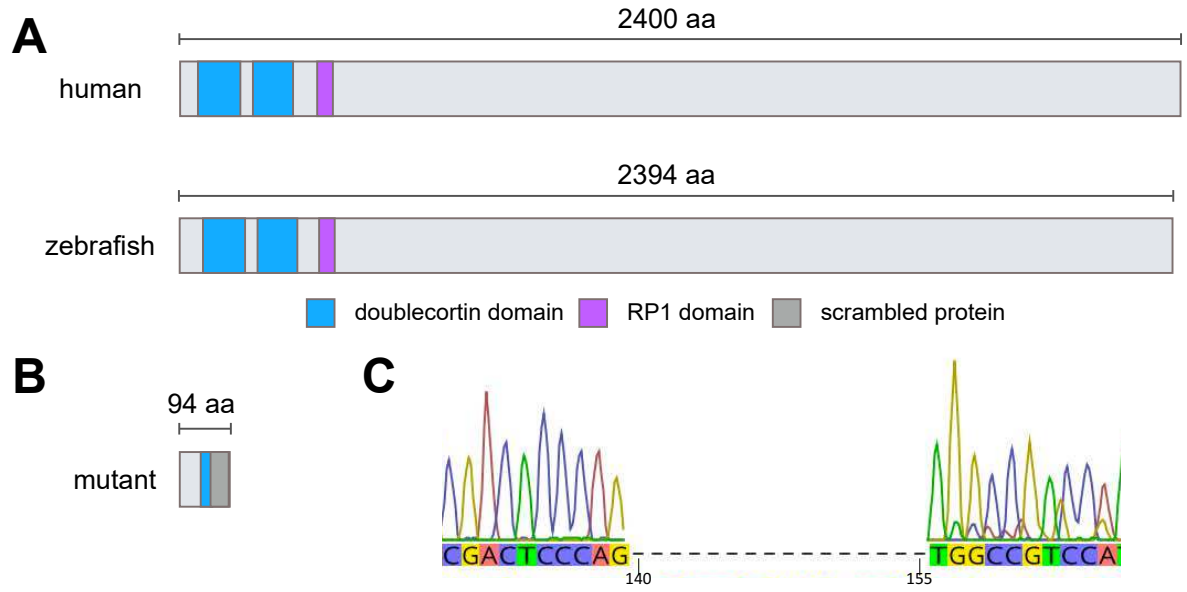

Figure 12. Human and zebrafish RP1L1 proteins have conserved domains, which are predicted to be eliminated in *rp1l1* mutant zebrafish.

(A) Cartoon of human and wild-type zebrafish protein. Human and zebrafish RP1L1 have conserved domains, and are similar sizes, with human RP1L1 protein being 2400 amino acids (aa) and zebrafish Rp1l1 protein being 2394 amino acids in length. **(B)** The mutation in zebrafish *rp1l1* is predicted to result in a severely truncated, nonfunctional Rp1l1 protein. The mutation occurs in the first doublecortin domain of Rp1l1 after amino acid 52, resulting in significant loss of more than half of the doublecortin domain, which is followed by an abnormal protein extension and termination at 94 amino acids in length. **(C)** The CRISPR/Cas9-generated 16 bp deletion in zebrafish *rp1l1* occurs after nucleotide 139.

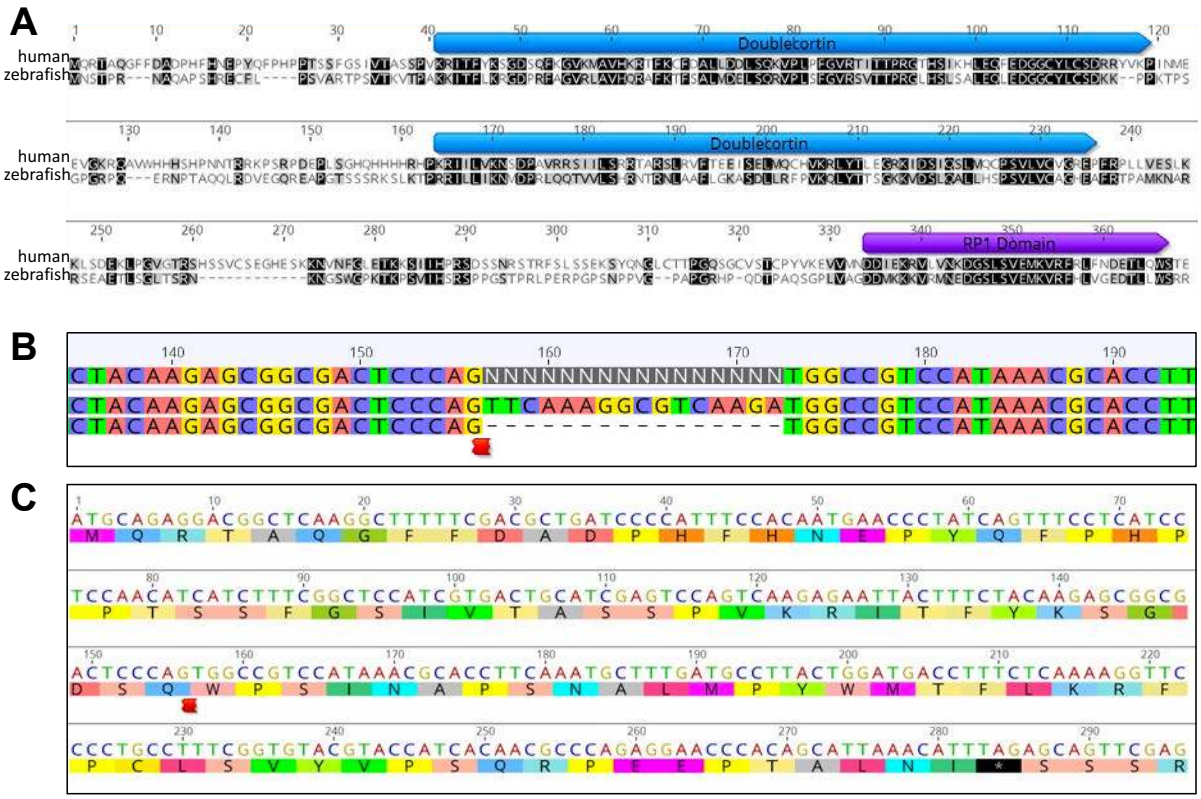

Figure 13. CRISPR/Cas9-induced mutation in zebrafish *rp1l1*.

(A) Alignment of human RP1L1 (top) and zebrafish Rp1l1 (bottom) proteins, showing conversation in the doublecortin and RP1 domains. **(B)** The CRISPR/Cas9-induced 16 bp deletion in the first coding exon of *rp1l1* in the mutant zebrafish. **(C)** Location of the deletion in the Rp1l1 protein and its predicted consequence. The mutation results in a scrambled protein sequence after amino acid 52 and a premature stop codon after amino acid 94.

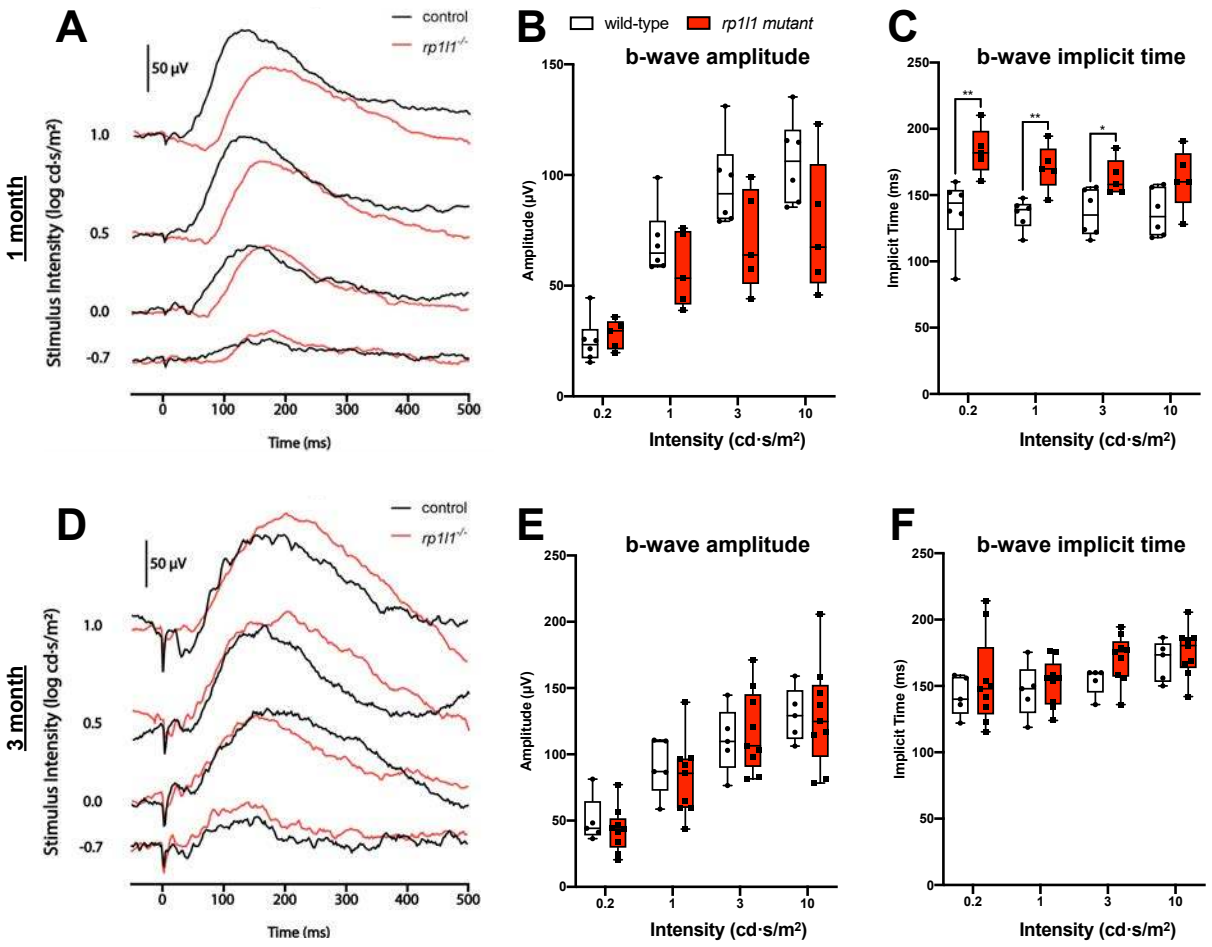

Figure 14. *rp1l1* mutants have photoreceptor responses comparable to wild-type zebrafish at 1 and 3 months.

(A) Sample traces from one mutant and one wild-type animal at 1 month of age. **(B)** b-wave amplitude is not different between wild-type and mutant fish. **(C)** 1-month-old mutant zebrafish have increased b-wave implicit time under 0.2, 1, and 3 intensities, indicating a delay in response. n=6 for wild-type, n=5 for mutant. **(D)** Sample traces from a 3-month-old wild-type and mutant animal. **(E)** b-wave amplitude and **(F)** implicit time are not significantly different between the groups at 3 months of age. The box and whiskers plots depict median (centre line), upper and lower quartile (box), and distribution (bars). Each dot is an individual animal. n=5 for wild-type, n=9 for mutants. *p<0.05, **p<0.01

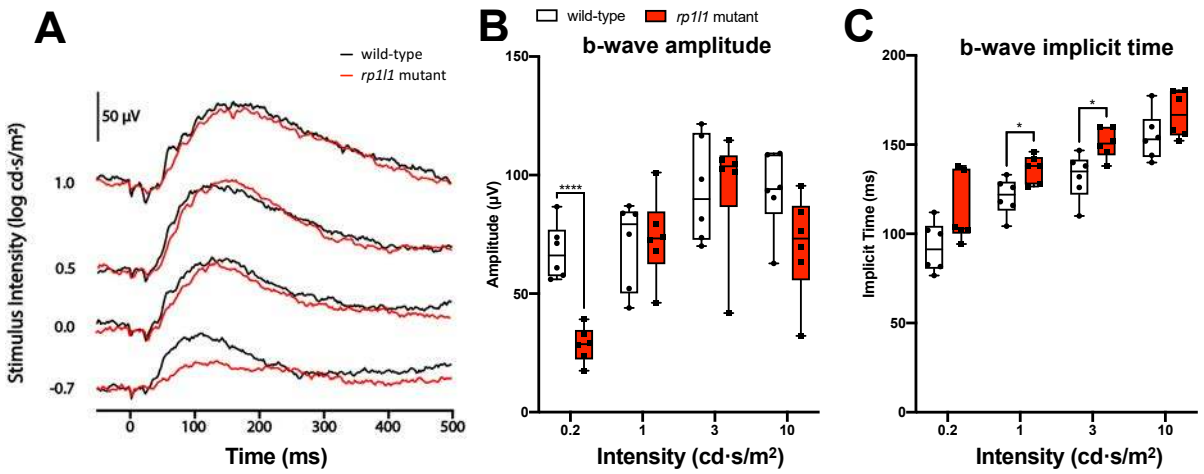

Figure 15. *rp1l1* mutants have reduced dim light responses at 6 months.

(A) Sample traces from a wild-type and an *rp1l1* mutant zebrafish at 6 months old. **(B)** Amplitudes for the b-waves for wild-type and mutant groups. Mutant animals have a reduced b-wave when given the dim light stimulus (0.2 cd·s/m² intensity), but normal waveforms under brighter intensities. **(C)** Implicit time for the b-waves at the tested intensities. The mutants had delayed b-waves for the mixed rod-cone (1 and 3 cd·s/m²) intensities. The box and whiskers plots depict median and distribution, with all animals plotted. n=6 for both groups. *p<0.05, ****p<0.0001

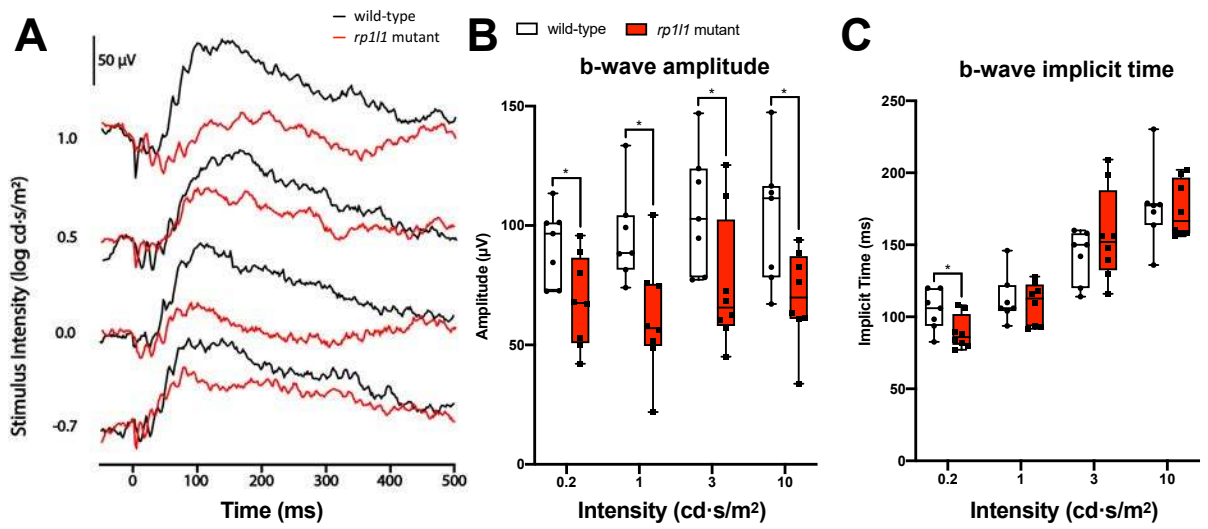

Figure 16. *rp1l1* is required for normal photoreceptor physiological response in aged animals.

(A) Sample traces from a 12-month-old wild-type and *rp1l1* mutant fish. **(B)** b-wave amplitude for wild-type and *rp1l1* mutants. The *rp1l1* mutant zebrafish have a significantly decreased b-wave under all light intensities. **(C)** b-wave implicit time for the wild-type and mutant zebrafish. The mutants have a significantly faster b-wave under the 0.2 cd·s/m² intensity. The box and whiskers plots depict median and distribution, with all animals plotted. n=7 for wild-type, n=8 for mutants. *p<0.05

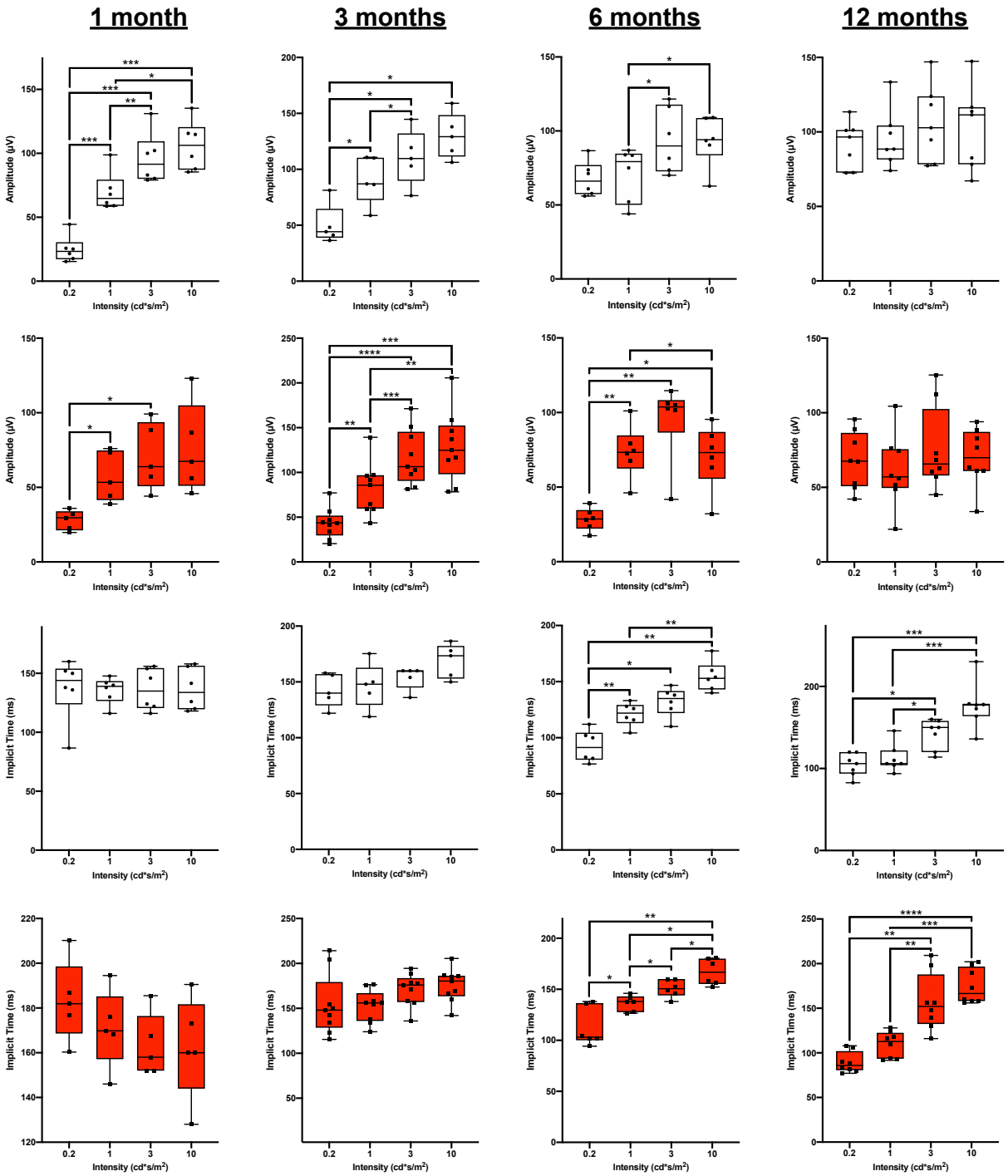

Figure 17. Differences in b-wave amplitude and implicit time between stimuli intensities for each group, using repeated measures ANOVA.

Wild-type in white, *rp1l1* mutants in red. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

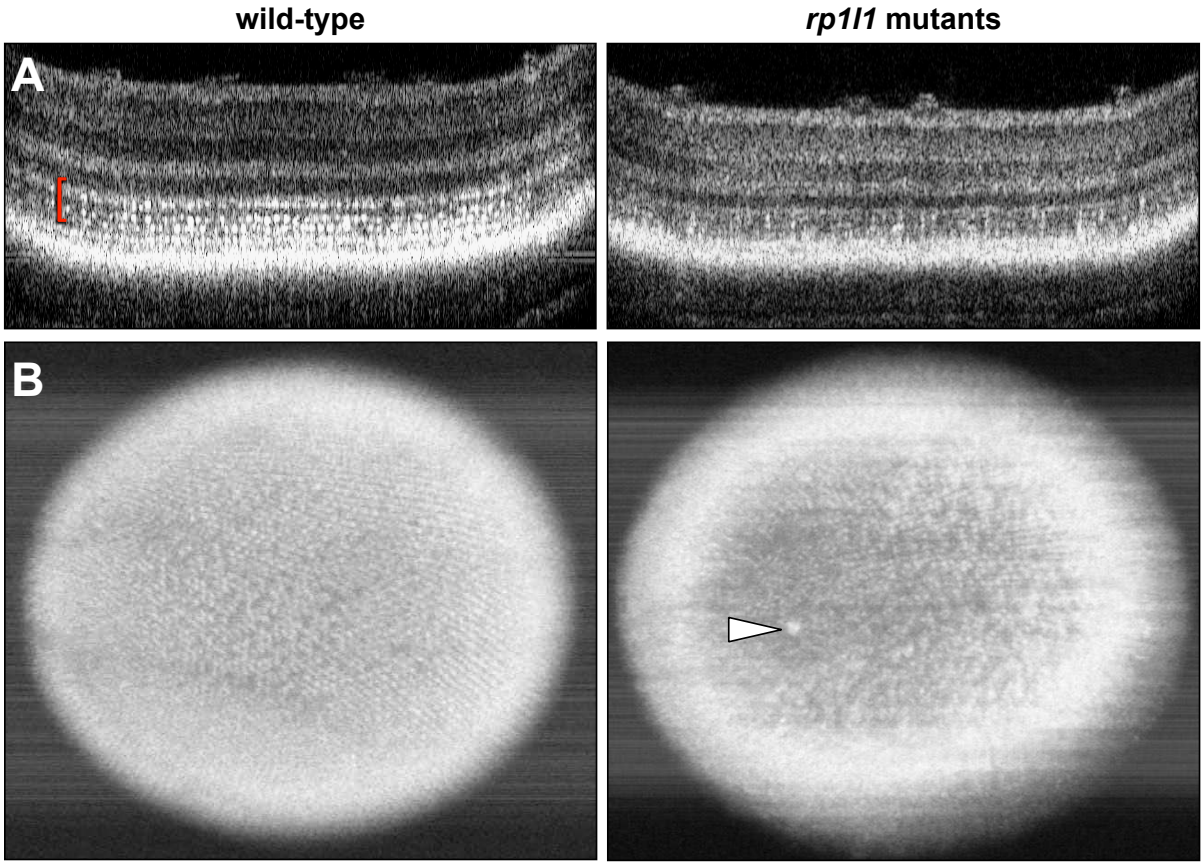

Figure 18. *rp1l1* mutant zebrafish have fewer hyper-reflective inner segment punctae and gaps in the photoreceptor mosaic.

OCT of wild-type and *rp1l1* mutant zebrafish retinas at 12 months. **(A)** OCT B-scans through zebrafish retinas. In the wild-type animals, the photoreceptors inner segments (seen as hyper-reflective dots, red bracket) are abundant and uniformly distributed. In the mutant animals, this part of the retina is very patchy and appears to have fewer punctae. **(B)** *En face* projections through the retina. In the mutant animals, the mosaic appears patchy and disrupted. There is also a large hyper-reflective speck in the retina of the mutant animal (arrowhead), which typically indicates an immune cell response.

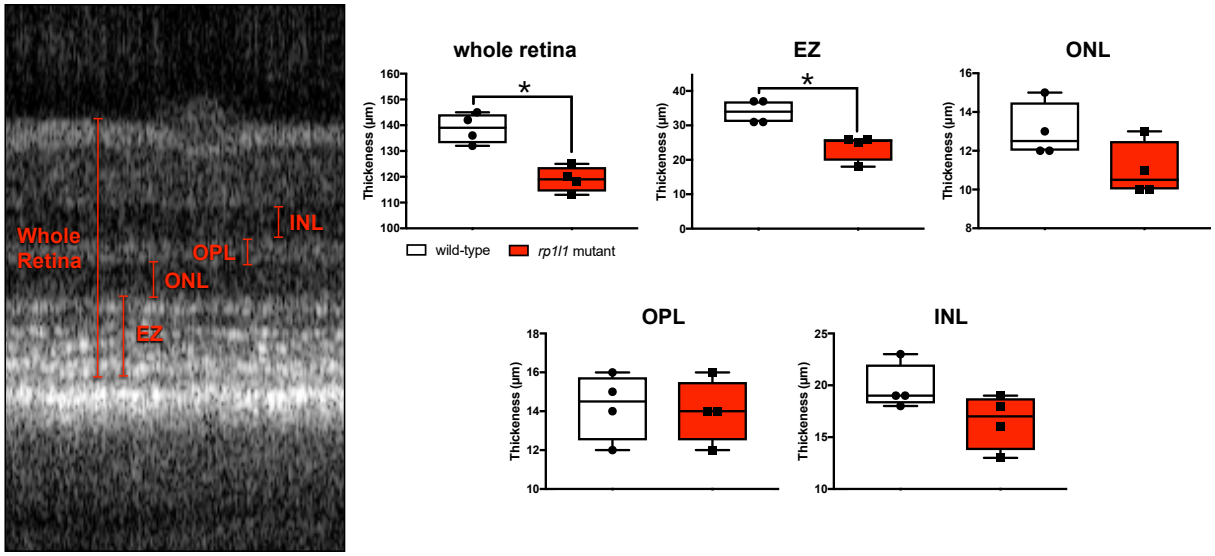

Figure 19. *rp1l1* mutant zebrafish have thin retinas.

Measurements of retinal thickness from OCT scans of 12-month-old wild-type and *rp1l1* mutant zebrafish. Mutants have statistically significantly thinner retinas and EZ areas. Other regions were not statistically different. The box and whiskers plots depict median and distribution, with all animals plotted. n=4 individuals/group; *p<0.05; EZ = ellipsoid zone; ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer

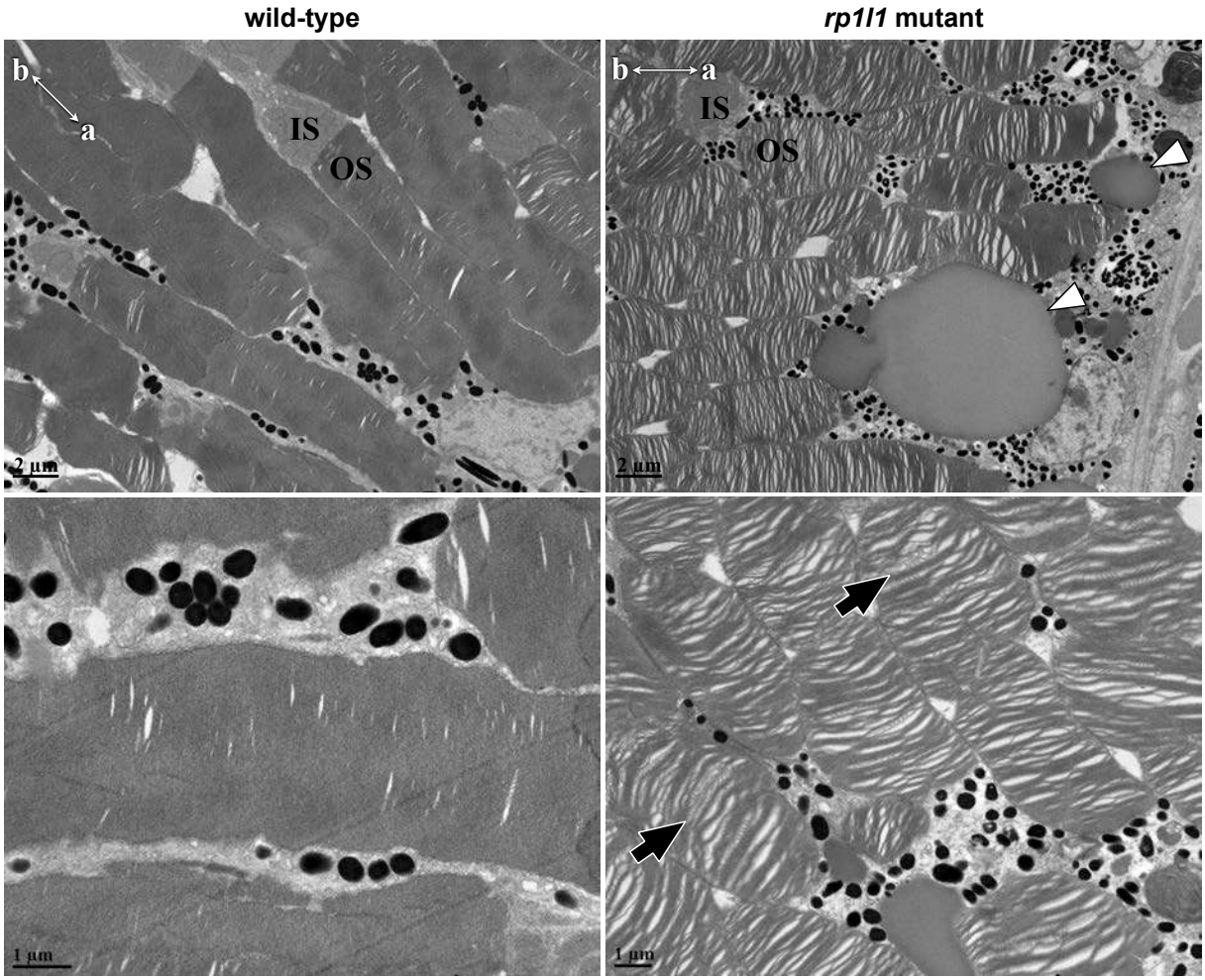

Figure 20. *rp1l1* mutant zebrafish have disorganized photoreceptor outer segments and deposits between the photoreceptors and RPE.

Electron microscopy of 11-month-old mutant and wild-type photoreceptors. Mutant photoreceptors appear disorganized, with gaps between the discs, and discs waving or swirling in some outer segments (black arrows). Additionally, there are deposits between the photoreceptor outer segments and RPE (white arrowheads). IS = inner segment; OS = outer segment. Compass in top left corners shows apical (a) and basal (b) photoreceptor orientation for the images.

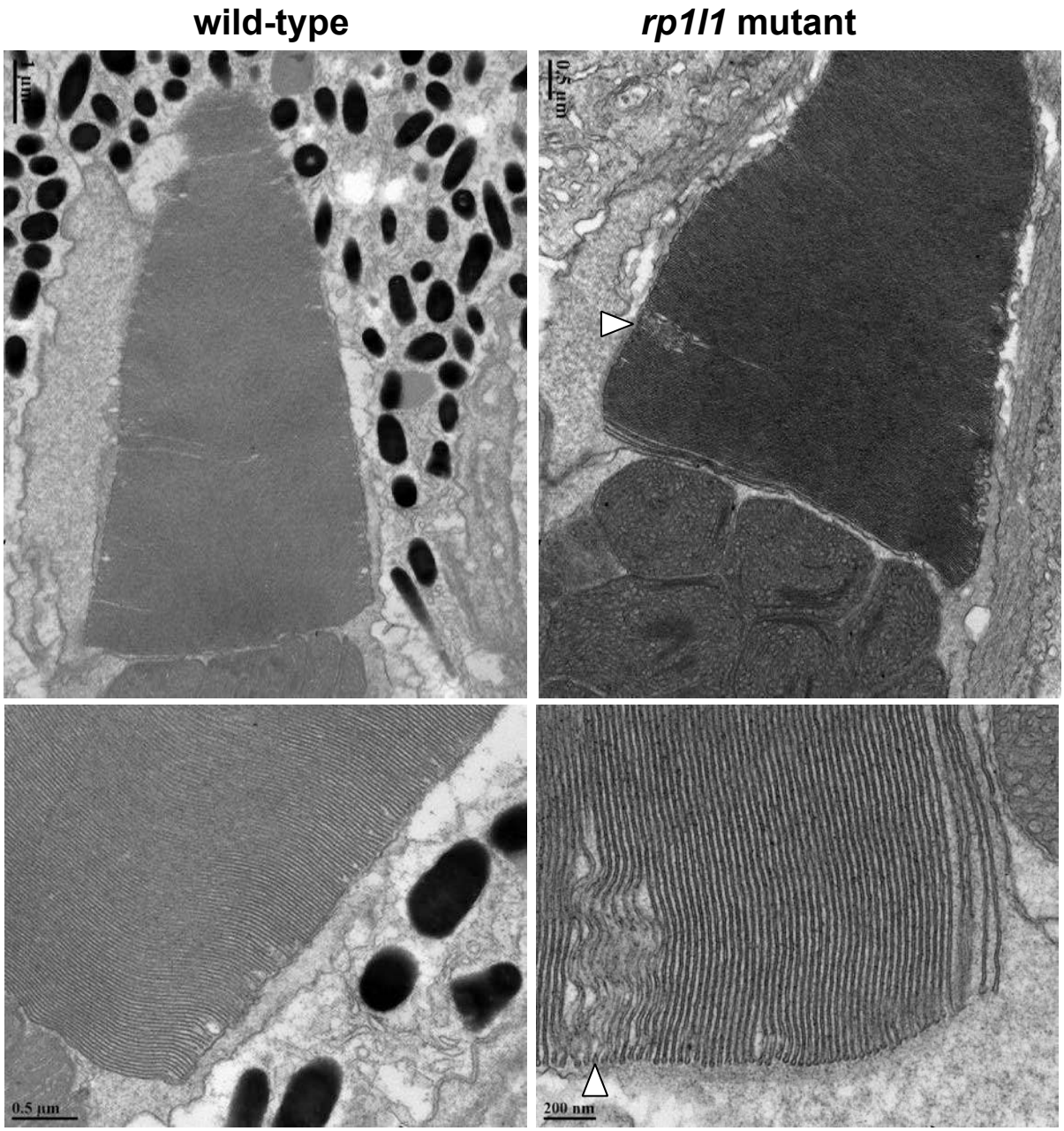

Figure 21. Cone photoreceptor outer segments can be well organized in *rp1l1* mutants.

Wild-type (left) and *rp1l1* mutant (right) cone photoreceptors observed via electron microscopy in 11-month-old animals. Mutant cone outer segments can appear quite normal, with small defects in disc uniformity or stacking (arrowhead).

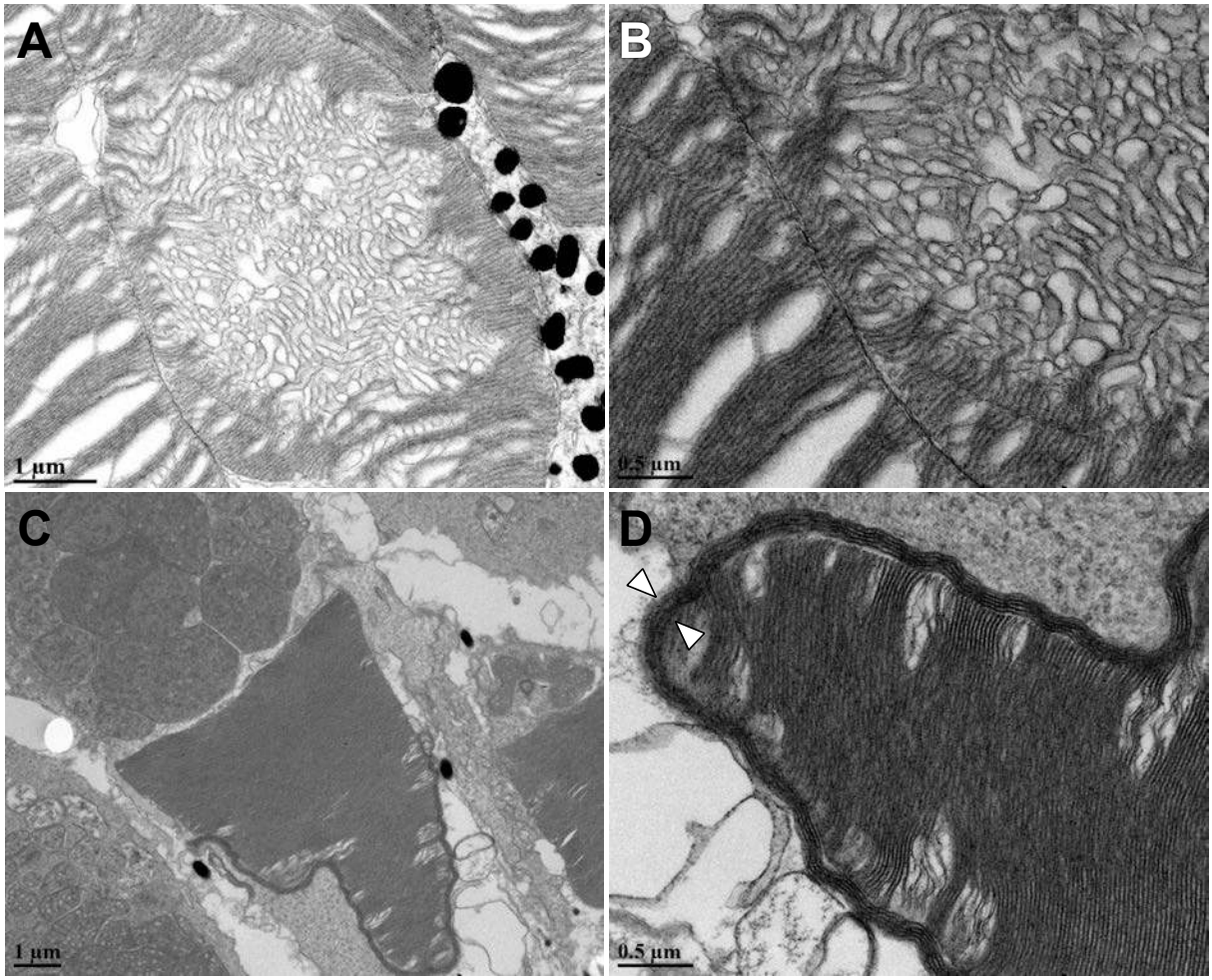

Figure 22. Abnormal outer segment membrane organization in *rp1l1* mutant photoreceptors.

A subset of 11-month-old *rp1l1* mutant photoreceptors have extremely abnormal outer segment membrane organization. **(A,B)** Outer segments in *rp1l1* mutants containing vesicles rather than uniform discs. The discs appear relatively normal at the outer segment periphery. **(C,D)** Cone photoreceptor with an abnormal, multi-layered membrane surrounding the outer segment (arrowheads). Note the lack of uniformity in the shape of the OS.

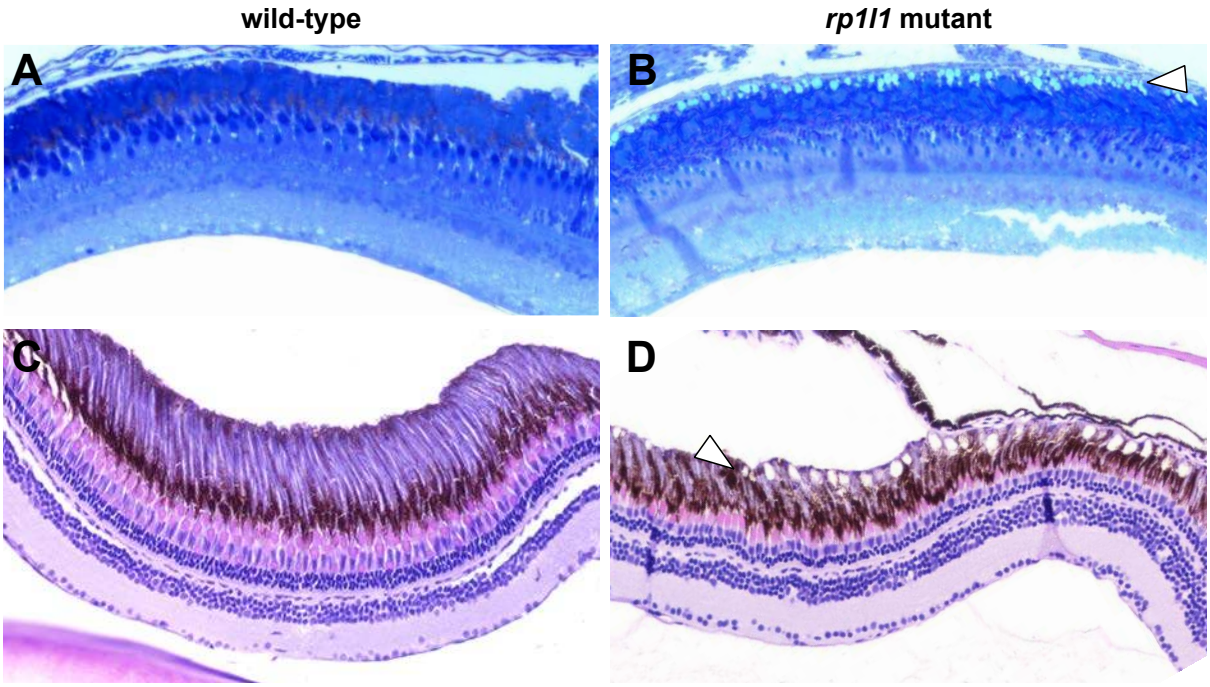

Figure 23. Mutant zebrafish retinas have subretinal deposits and short outer segments.

Wild-type and mutant retina sections, stained with azure blue **(A,B)** or hematoxylin and eosin (H&E) **(C,D)**. Deposits can be observed in the 11-month-old mutant azure blue-stained (B) sections between the photoreceptor outer segments and retinal pigment epithelium (arrowhead). H&E-stained sections of 12-month-old mutant retinas (D) show similar accumulations (arrowhead); however, the material that had filled the deposits was stripped from the tissue during processing. *rp1l1* mutant retinas appear to have shorter outer segments and thinner outer nuclear layers (D). No deposits were observed in the wild-type retinas (A,C).

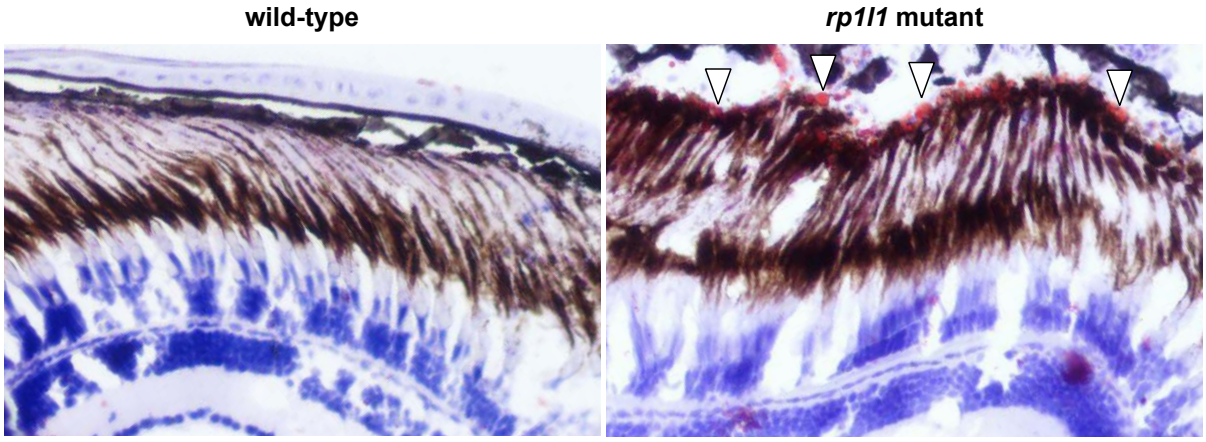

Figure 24. *rp1l1* mutants have lipid-rich subretinal drusenoid-like deposits.

Cryosections of 14-month-old wild-type and mutant zebrafish retinas, treated with Oil Red O to stain lipids and haematoxylin to stain nuclei (blue). The deposits in the mutant retina stained red (arrowheads), demonstrating that they are lipid-rich.

4 DISCUSSION + FUTURE DIRECTIONS

4.1 SPECULATION ABOUT DIFFERENCES BETWEEN THE ROD AND CONE CILIUM

Extensive research has gone into investigating the photoreceptor cilium due to the specialized morphology of the OS and importance for visual function. More recently, numerous genetic disorders, including photoreceptor diseases, have been associated with genes encoding cilium components. Despite extensive research into photoreceptor OS biology, cone photoreceptors are generally understudied compared to rods, especially in terms of OS development and composition. This is partially due to the historical use of rod-dominant models, such as mouse and rat, for photoreceptor characterization. What is observed in rods is often presumed to relate to cones, though whether this is truly the case is infrequently confirmed. There are many instances where both rods and cones express the same protein, but its localization and function are not assessed between the two photoreceptor types. Indeed, there is increasing evidence from disease manifestation that there are different features of the rod and cone cilium.

The distinct morphology of rods and cones necessitates unique mechanisms for growth and maintenance of their OS architecture. There are two hypothesised mechanisms for how the rod OS adds discs and maintains its shape. The first postulates that vesicles migrate to the basal OS and fuse to form new discs that are enclosed within the plasma membrane [403]. However, there is concern that evidence for this method may have been the result of artifacts of tissue preservation, rather than a biological process. The second, more well-supported mechanism suggests that the membrane that will become new discs evaginates from the cilium at OS base [404–406]. These membrane outgrowths expand to the width of the OS then are eventually enclosed within the plasma membrane and lose their connection to the cilium, becoming free-floating discs. A similar mechanism is supported in cone OSs, although the discs remain contiguous with the plasma membrane and do not become enclosed.

How cone OSs achieve and maintain their tapered morphology is unclear, but may be the result of several mechanisms. In terms of early photoreceptor development, the OSs between immature rods and cones can be similarly shaped; therefore, the cone OS taper is likely established after initial OS growth by maintenance mechanisms. The OS shape must be constantly modified and re-established as new lamellae are added via membrane evagination at the OS base [407] and apical lamellae are shed and phagocytosed by the RPE. Thus, lamellae must become reduced in diameter as they move up the OS. The molecular means by which this occurs has not been elucidated. The distal lamellae may acquire their taper by continually losing membrane as they move apically through the OS [408,409]. In some species, such as *Xenopus*, this may be aided by the development of distal invaginations – cone discs can sometimes invaginate at the disc edge [408], although whether this creates two fully formed discs or the disc remains partially split is uncertain. To conclude, the tapered cone OS morphology may be maintained through a combination of new, larger discs being added at the basal OS, distal invagination of OS discs, and loss of membrane content as the lamellae travel towards the tip of the OS.

An important consideration for cone OS growth and turnover is that the lamellae are contiguous with the plasma membrane, not free-floating discs that are enclosed in a membrane. Thus, to facilitate movement of the discs apically to be phagocytosed, there must be axonemal mechanisms that facilitate the movement. Either the axoneme must move along with the associated lamellae as they move towards the RPE, there is a mechanism that allows for the lamellae to slide up the axoneme, or the membrane-axoneme connections must be regularly broken and re-established to accommodate disc movement. A similar methodology could also exist in rods where the OS discs sit next to the axoneme to facilitate apical movement, although it would not require as much coordination between the plasma membrane and axoneme. The unique rod and cone OS disc morphologies may be why the cone axoneme extends to the OS tip while the rod axoneme only extends partway into the OS. The cone axoneme likely plays a different role in OS turnover than the rod axoneme.

4.1.1 DISEASE PHENOTYPES SUGGEST DIVERGENT CILIUM COMPOSITION BETWEEN RODS AND CONES

Mutation of cilium components found in both rods and cones can cause pan-photoreceptor disorders, but often results in either rod or cone-specific disease, sometimes in a mutation-dependent manner. In certain cases, the types of mutations (nonsense versus missense) and inheritance pattern can differ between rod and cone-related disease caused by mutations in the same gene. However, in other instances there is no clear link between the type of genetic lesion and resultant photoreceptor disease that could indicate why rods or cones are affected distinctly. Further, there is often a predominant disease manifestation – such as RP – due to mutations in a specific gene, despite it being in both photoreceptor types. Together, this suggests that these photoreceptor cilium components may have unique isoforms, subcellular localization, functions, and/or binding partners between rods and cones that may not have been identified as of yet.

Alternative splicing and tissue-specific isoforms have been noted for numerous genes important for photoreceptor development, function, and structure [410–416]. While some genes have retina or photoreceptor-specific isoforms, few have been investigated for rod versus cone specificity. Several cilium proteins have multiple isoforms, some of which are retina-specific, such as *BBS5* [413]. *BBS5* appears to have different roles in rods and cones, as *Bbs5* mutant mice have severely reduced cone function and only a mild decrease in rod function [417]. This may indicate that Bbs5 is more integral for cone OS structure and function, that it is a larger contributor to cone than rod BBSome stability, or that Bbs5 has unique roles in transport of phototransduction machinery in mouse cones. A similar phenomenon has been observed in zebrafish with mutations in components of the BBSome; loss of *bbs2* affects primarily cone photoreceptors [236]. This finding could suggest that other BBSome components may have partially redundant roles with Bbs2 in zebrafish rods. Additionally, differential localization of specific photoreceptor cilium components can occur between rods and cones. In *Xenopus*, RPGR protein localizes to the rod

axoneme, but in cones it localizes to both the axoneme and IS [418]. Similarly, in zebrafish the Eys protein localizes to the axoneme of both rods and cones, but also is present in the cone ciliary pocket [232]. This indicates that RPGR and EYS may have functions unique to cones in *Xenopus* and zebrafish, respectively, which would influence how disease manifests in mutant models. A similar phenomenon may be occurring in human photoreceptors as well.

IFT is required for photoreceptor function and maintenance and is another potential source of divergent cilium component function between rods and cones. Kinesin motors are needed for anterograde IFT and *KIF3A* encodes a subunit of the kinesin-II motor protein. Mice with conditional knockout of *Kif3a* in photoreceptors undergo rapid photoreceptor degeneration with severely reduced cone responses and moderately reduced rod responses [419], which indicates a differential impact of Kif3a in the photoreceptor types. Furthermore, Kif3a-deficient cone photoreceptors had defective trafficking of OS components while no OS protein mislocalization was observed in rods. Kif3a is therefore important for anterograde transport in mouse cones but not rods, highlighting that IFT components in the cilium can have differential roles between the photoreceptor subtypes.

It is important to acknowledge that there are species-specific differences in photoreceptor morphology, OS component genes, RNA isoforms, and protein localization. *Xenopus* have increased IS expression of BBS5 compared to mice [420]. Mice lack the *EYS* gene and have a unique isoform of *RPGRIP1* not detected in humans [421]. In addition, while RP1L1 is found in all retinal photoreceptors in humans and non-human primates, in mice it has only been detected in rods [184,185,323]. Interestingly, mice do not have photoreceptor calyceal processes – microvilli-like structures that surround the OS base – which means that there are undoubtedly dissimilarities in expression and localization of photoreceptor structural factors between mice and other organisms, including humans. These unique features of mouse photoreceptors may be adaptations for nocturnal life. Photoreceptors in zebrafish and other teleost fishes can have

accessory outer segments (AOSs), which are cilium-like structures that originate from the IS [422]. The function of the photoreceptor AOS is not known.

It is essential to acknowledge the unique photoreceptor features across different species when assessing photoreceptor development and disease manifestation. In some situations, human photoreceptors may be more comparable to diurnal non-mammalian photoreceptors than nocturnal mammalian photoreceptors.

4.2 RP1L1 IN THE PHOTORECEPTOR CILIUM

The function of RP1L1 in photoreceptors has yet to be elucidated. It may be tethering OS discs to the axoneme by binding the axoneme microtubules and forming a complex with proteins that maintain OS disc morphology, such as peripherin. Further investigations are required to determine the exact role of RP1L1 in photoreceptors.

Disease presentations suggest that there are divergent functions or protein associates of RP1L1 in rods versus cones. Dominant missense mutations generally lead to cone disease, while recessive nonsense mutations typically result in rod disease. Therefore, it seems that cones are sensitive to potential changes in RP1L1 protein folding or binding, while rods are less so; conversely, cones seem relatively resistant to loss of RP1L1, while rods are sensitized. *RP1L1* is a four exon gene, and three of these exons are coding; no functional alternatively spliced isoforms of *RP1L1* have been reported, and any changes in splicing would remove functional domains or severely truncate the RP1L1 protein, making *RP1L1* isoforms with photoreceptor type specificity unlikely.

The proteins that RP1L1 associated with in rods versus cones could be the source of divergent responses to *RP1L1* mutations between the photoreceptor types. The most common OMD-causing variant is p.R45W, which is located in the first microtubule-binding domain of RP1L1 [184,185]; this mutation may therefore prevent effective binding to the cone axoneme (Figure 25). The mislocalized mutant RP1L1 protein may subsequently aggregate within the

photoreceptor cell and cause further cellular dysfunction, as is commonly observed in neurodegenerative diseases [423,424]. A second mutational hotspot in the RP1L1 protein is the region between amino acid 1172-1258 (Table 8, Figure 10). As this region has no discernable protein domains, it is likely a site for protein binding. Mutations in this region may result in loss of binding with normal binding partners and aberrant binding with additional proteins (Figure 25). This could interfere with many necessary cilium processes, depending on the identity of the proteins that RP1L1 binds. However, rods seem largely unaffected by missense mutations in either of these regions. In the rod axoneme, RP1L1 may be closely associated with other microtubule-binding proteins that it does not interact with in the cone axoneme (Figure 26). This could allow for RP1L1 with the p.R45W mutation to maintain its axoneme association, and prevent rod disease. Mutations in the second RP1L1 mutational hotspot, amino acid 1172-1258, may have less of an impact on rod photoreceptors due to rod-specific binding partners within this region. Alternatively, RP1L1 may not have binding partners in this region in rod photoreceptors. Conversely, when RP1L1 is absent – such as in the case of recessive RP caused by nonsense *RP1L1* variants – there may be proteins with similar functions to RP1L1, for example RP1, that can maintain cone OS structure (Figure 25), while rods are unable to compensate for RP1L1 loss (Figure 26).

To further assess potential functions of RP1L1, co-immunoprecipitation (co-IP) could be used to determine its binding partners. The identity of the proteins that RP1L1 interacts with would indicate its particular function in the cilium. For example, if RP1L1 binds peripherin, then it would suggest that RP1L1 is involved in maintaining the structure of the OS discs and tethering them to the axoneme. There are challenges with the co-IP approach, however. In order to perform a co-IP, specific, efficacious antibodies are required. Currently, there are commercially available anti-human RP1L1 antibodies that work for immunohistochemistry, but no antibodies that have been proven to work on denatured RP1L1 for Western blot. This means that pull-downs cannot be easily confirmed and makes this approach challenging. Unfortunately, there is limited protein

conservation between species outside of the doublecortin domains of RP1L1, which poses challenges for utilizing antibodies that may be successful in one species for use in another. Further, as there are suspected differences between RP1L1 function and/or binding partners between rods and cones, it would be ideal to separate rods from cones and perform co-IPs on both pools separately. This would determine whether RP1L1 does indeed have different binding partners between rods and cones and the identities of the associated proteins.

4.3 REGENERATION IN *RP1L1* MUTANT ZEBRAFISH

Zebrafish have a robust regenerative capacity, although it has been reported that chronic photoreceptor degeneration in mutant zebrafish does not always trigger a regenerative response [236]. While the exact reason for this is unclear, it may be due to the damage threshold not being reached to trigger Müller glial proliferation. Regenerative capacity could be assessed in the *rp1l1* mutant zebrafish to determine whether lost photoreceptors are being replaced and causing the degeneration to appear slower than it is due to cell repopulation. Photoreceptor regeneration in *rp1l1* mutant zebrafish could be assessed using EdU treatment. When cells divide, EdU is incorporated into their DNA. Thus, EdU treatment can show Müller cell proliferation and newly regenerated photoreceptors. Alternatively, there are antibodies that label dividing cells that could be utilized for this purpose, such as antibodies against proliferating cell nuclear antigen (PCNA), which is an essential component of DNA replication.

The requirements for regenerative response activation in the zebrafish retina are poorly understood, and whether or not the chronic photoreceptor degeneration in *rp1l1* mutant zebrafish reaches the threshold to trigger regeneration may provide insight into what is necessary for this process. Assessment of regenerative capacity in *rp1l1* mutants would also inform disease pathology. Humans are not able to regenerate lost neurons, and a lack of retinal regeneration in the *rp1l1* mutants would increase the applicability of this model to human disease. Alternatively, if there is photoreceptor regeneration occurring at a level that is inadequate to prevent retinal

defects but nevertheless could be delaying visual dysfunction, the response could be further investigated to elucidate molecular mechanisms involved in Müller glial proliferation in the chronically ill teleost retina.

4.4 IMPACT OF DRUSEN AND SUBRETINAL DRUSENOID DEPOSITS ON RETINAL HEALTH

How drusen and subretinal drusenoid deposits develop is unclear. However, both drusen and subretinal drusenoid deposits are dynamic; they can grow, shrink, and resolve [388,402]. The biology and influence of these deposits on the retina is challenging to assess in human patients, and necessitates the use of animal models – however, few animals develop drusen and subretinal drusenoid deposits. Why small animal models of photoreceptor disease infrequently develop drusen and subretinal drusenoid deposits is unknown, but could be due to many factors. Small animals of course have a much smaller eye size and typically a short lifespan compared to humans. Lifespan and ageing biology may be important factors for developing drusen and subretinal drusenoid deposits; the comparatively short lifespans of zebrafish (2-5 years) and mice (1.5-3 years) may prevent opportunity for age-related RPE dysfunction and lipid deposit accumulation. Large eyes and long lifespan provide more space and time for deposits to develop. Photoreceptor mosaic organization also differs between humans and small animal models. Zebrafish have an organized mosaic comprised of 50-60% cones that are organized in predictable rows, while mice have a rod-dominant retina with cones interspersed throughout [62,425]. In humans, drusen develop primarily in the cone-rich regions of the retina, while subretinal drusenoid deposits often develop in more rod-dense regions.

Immune response may also pay a role in drusen and subretinal drusenoid deposit formation. The innate immune system is implicated in AMD pathology; there are pro-inflammatory components found within drusen and subretinal drusenoid deposits, and single

nucleotide polymorphisms (SNPs) within complement factors increase an individual's likelihood of developing AMD [426–432]. Mouse eyes have very different immunological responses than human eyes; some mouse models of retinal dystrophy with AMD-like pathologies have less retinal complement protein than controls, and retinal detachment and exposure to viral vectors does not typically trigger appreciable levels of inflammation [391,433,434].

The effect of drusen and subretinal drusenoid deposits on retinal function is unknown. In early stages, when the deposits are small, they may simply reflect low levels of retinal dysfunction – that the photoreceptors and/or RPE have some difficulty carrying out functions or are experiencing metabolic deregulation to a certain degree, leading to lipid accumulations. Indeed, drusen and subretinal drusenoid deposits can be observed in individuals with normal vision, and though important risk factors for developing AMD, some individuals with such deposits do not develop clinically detectable photoreceptor loss. At later stages, when the deposits become numerous or large, they may also cause functional defects. Multiple large drusen between the RPE and choroid may prevent transport of components between the blood stream and retina. Similarly, the location of subretinal drusenoid deposits between the photoreceptor OSs and RPE may create barriers that prevent optimal clearance of shed OS discs and transport of nutrients to the photoreceptor cells. This would be detrimental to RPE and photoreceptor function, and the OSs fragments that are not phagocytosed by the RPE may further feed into the development of additional subretinal drusenoid deposits.

4.5 SUBRETINAL LIPID DEPOSITS IN *RP1L1* MUTANT ZEBRAFISH

The *rp1l1* mutant zebrafish have subretinal lipid deposits. These deposits differ from subretinal drusenoid deposits observed in humans, in that they are lipid-rich; human subretinal drusenoid deposits contain lipids but are not so lipid-dense that they stain clearly with Oil Red O the way drusen do [402]. In this regard, the deposits in *rp1l1* mutant zebrafish have an interesting

combination of features – their location is akin to subretinal drusenoid deposits, but their composition is more similar to drusen, which are lipid-rich.

It is unknown when the subretinal lipid deposits appear in relation to retinal dysfunction in the *rp1l1* mutant zebrafish. The deposits may appear prior to detectable retinal dysfunction, similar to how drusen or subretinal drusenoid deposits can be precursors to photoreceptor disease in humans. Mutant zebrafish could be assessed for subretinal drusenoid-like deposits at 3 months old, when retinal function was normal, and 6 months old, when photoreceptor dysfunction was first observed. This would provide an initial idea of whether the lipid deposits are present in young, normally functioning mutant retina, or only manifest in early disease stages. Naturally, if deposits were observed at 6 months but not 3 months, further functional and histological assessments would be required at intermediate age-points to determine when the deposits manifest in relation to retinal dysfunction.

Further assessment of the lipid deposits could be performed to determine the lipid composition, such as by lipid mass spectrometry. This would inform potential sources of the deposits, as specific lipids have limited sources within the retina, and could indicate whether the accumulations are RPE-derived, photoreceptor-derived, or derived from a combination of sources.

There are several environmental risk factors for developing AMD, which include diet and smoking/oxidative stress. Ocular lipid deposit formation may be impacted by diet, and high fat diets contribute to sub-RPE deposit development in mice [435–437]. Whether this is similar in zebrafish is unknown, but could easily be tested by feeding zebrafish an altered diet with increased fat content, such as readily available tropical fish food pellets. A high fat diet may increase the production of subretinal lipid deposits, and potentially lead to the production of drusen-like deposits on the basal side of the RPE. Additionally, how oxidative stress impacts deposit accumulation could also be investigated. In humans, smoking is a major risk factor for AMD due to oxidative stress [438,439]. Oxidative stress can be induced in fish by exposure to certain

compounds, such as fluoride, sulfamethoxazole, and cypermethrin [440,441]. Oxidative stress on *rp1l1* mutants may result in earlier disease onset and/or increased lipid deposits within the retina due to damaged RPE.

Retinal neovascularization can be induced by hypoxia in both larval and adult zebrafish [132,442], although how neovascularization may cause or exacerbate visual defects in zebrafish has not been assessed. However, it is likely that aberrant vessels will result in retinal damage due to leakage or growth into new regions that inhibit retinal cell function. Leaky vessels may further exacerbate development of deposits in the eye if they have small or transient leaks. Inducing neovascularization in the zebrafish *rp1l1* photoreceptor disease model could be a potential avenue to relate a zebrafish disease model to wet AMD.

4.6 CONCLUSION

I have investigated *RP1L1* disease presentation in humans and modelled *RP1L1*-associated retinal disease in zebrafish. In doing so, I found that *RP1L1* can cause diverse photoreceptor diseases, including occult macular dystrophy, retinitis pigmentosa, *RP1L1* maculopathies, and rod-cone dystrophy. Loss of *rp1l1* in zebrafish resulted in progressive photoreceptor functional defects, retinal degeneration, disorganized photoreceptor outer segments, and subretinal lipid accumulations. This novel model appears to recapitulate retinitis pigmentosa as it presents with rod functional defects in adulthood, but has features relevant to age-related macular degeneration, specifically subretinal deposits.

Differential disease presentation suggests that the rod and cone cilium have distinct components or functions. This has not been well investigated, but RP1L1 could be utilized to uncover potential rod and cone-specific cilium interactors. Generally, dominantly inherited *RP1L1* missense mutations lead to cone degeneration while recessively inherited nonsense *RP1L1* mutations lead to rod degeneration, indicating unique RP1L1 binding partners or functions

between the photoreceptor types. Isolating RP1L1 protein in rods versus cones and identifying protein interactors could help determine differences between the rod and cone cilium.

Why loss of *rp1l1* – a photoreceptor-specific cilium component – leads to subretinal deposits in zebrafish is unclear, but may be due to RPE stress caused by abnormal photoreceptor outer segments. In the future, the lipid deposits could be investigated in several ways: when the deposits occur in relation to photoreceptor functional defects could be assessed; the lipid components of the deposits defined to determine potential sources; and methods undertaken to attempt to enhance the deposit accumulation and/or retinal dysfunction.

In summary, this work has expanded the clinical presentations associated with *RP1L1* and modelled related photoreceptor disease in zebrafish. This is important for genetic assessment and diagnosis of patients; *RP1L1* should be included on genetic panels for maculopathies, retinitis pigmentosa, and rod-cone dystrophy. Additionally, the zebrafish *rp1l1* mutant model can be further utilized to uncover disease mechanisms, elucidate Rp1l1 function, investigate AMD-related pathologies, and provide a platform for therapeutic testing.

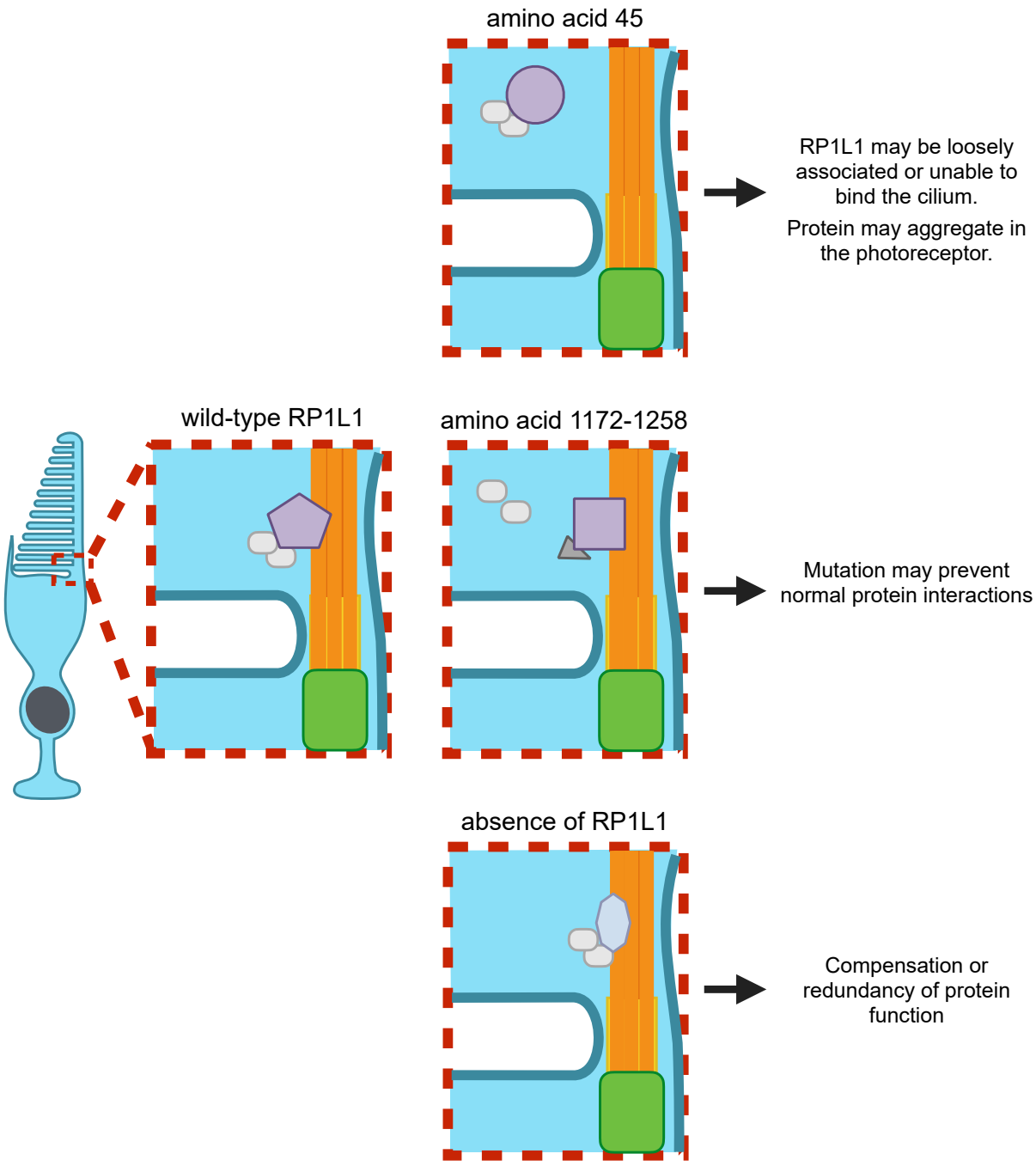
amino acid 45
RP1L1 may be loosely associated or unable to bind the cilium.
Protein may aggregate in the photoreceptor.
wild-type RP1L1
amino acid 1172-1258
Mutation may prevent normal protein interactions
absence of RP1L1
Compensation or redundancy of protein function

Figure 25. Potential consequence of *RP1L1* variants on cones.

RP1L1 (purple) localizes to the axoneme (orange) and presumably binds other protein partners (grey). The most common disease-associated RP1L1 variant, p.R45W, is located within the first microtubule-binding domain of RP1L1. This variant therefore has the potential to render mutant RP1L1 unable to bind the axoneme microtubules, and may result in mutant RP1L1 protein aggregation within the photoreceptor. However, the second mutational hotspot in RP1L1 – between amino acids 1172-1258 – has no noted domains and is likely a binding site for other proteins. Variants in this region could impact the ability of RP1L1 to bind its regular protein partners and increase aberrant binding (dark grey). Conversely, when RP1L1 is lost entirely within cones, there may be compensation by other axonemal proteins that prevent cone disease (blue).

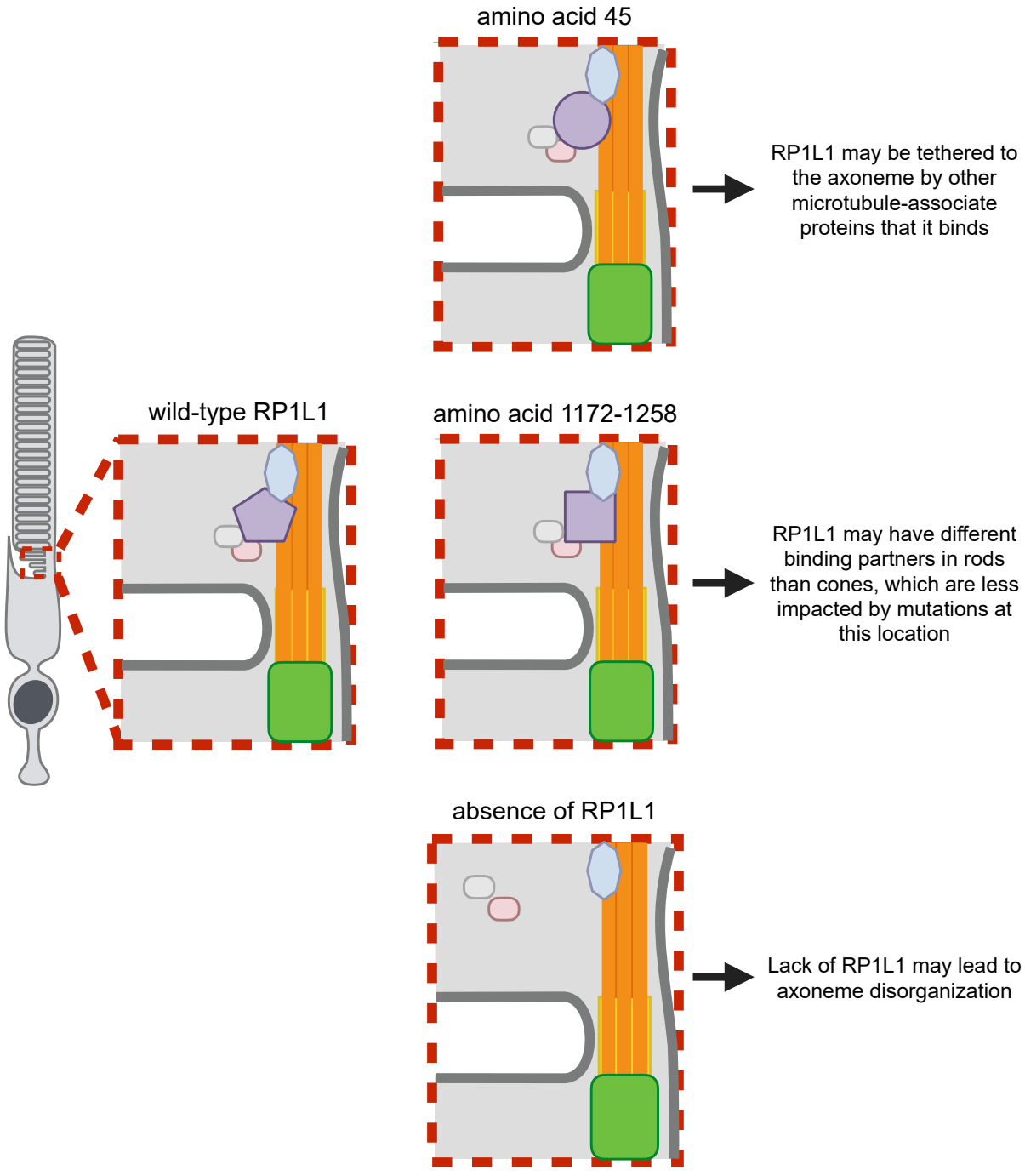
amino acid 45
RP1L1 may be tethered to the axoneme by other microtubule-associate proteins that it binds
wild-type RP1L1
amino acid 1172-1258
RP1L1 may have different binding partners in rods than cones, which are less impacted by mutations at this location
absence of RP1L1
Lack of RP1L1 may lead to axoneme disorganization

Figure 26. Potential consequence of *RP1L1* variants on rods.

RP1L1 (purple) may have unique protein associates in rods that it does not have in cones (blue, pink). RP1L1 could have axoneme-binding protein associates that are unique to rods and help tether RP1L1 with the p.R45W mutation to the cilium, thereby preventing rod dysfunction and degeneration. RP1L1 may have other rod-specific binding partners that are less affected by mutations in the second hotspot between amino acids 1172-1258. However, when RP1L1 is absent, the proteins it typically binds to may be unable to associate with the axoneme and disrupt axonemal organization.

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