Establishing Zebrafish as an Animal Model to Study Drusen

by

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Abstract

Drusen are extracellular deposits that accumulate in the retina and are a hallmark of age-related macular degeneration (AMD), a leading cause of blindness in the elderly. Sometimes drusen have also been detected in otherwise healthy eyes of younger and elderly patients. Despite their clinical significance, the underlying mechanisms that lead to drusen formation and their impact on retinal function remain poorly understood. Therefore, specific diagnostic tests and treatment are also unavailable.

Animal models can be an essential tool for studying drusen. Zebrafish (*Danio rerio*), from its first published used in 1981, have emerged as a valuable model organism due to their genetic similarity to humans, ease of genetic manipulation, and fast development. They also share similarity with human eyes and visual system. Rodents are the most common, animal model for vision research. However, they are not well-suited for drusen research because rodents are nocturnal, and their retina is structurally different than the part of human retina where drusen appear. This presents some challenge with using rodents for drusen studies because drusen appears in and degenerates within cone-rich macula of human eye. Zebrafish retina, is cone-rich and more adapted to diurnal light variations, thus overcoming the challenge we face with rodents.

This project determines the earliest age when drusen-like deposits become detectable in *rp1l1*$^{-/-}$ zebrafish using histological staining techniques. It is the zebrafish line where a mutation has been introduced into the *Retinitis pigmentosa 1-like 1* (*rp1l1*) gene to demonstrate the ocular disease, retinitis pigmentosa. Oil Red O-stained deposits were first detected sporadically in *rp1l1*$^{-/-}$ fish at 55 days post-fertilization (dpf) and progressed

to appear bilaterally in the following months. These progressively increased in size with age, increasing about 3-fold in area in fish up to 1-year post fertilization age. Coincident with the accumulating drusen, the data revealed progressive decreases in the size of the zebrafish Outer Nuclear Layer (ONL).

Unexpectedly, drusen were also detected infrequently in older (1 year old) otherwise normal wildtype zebrafish retinas. This suggests that zebrafish might also be used to model the drusen that appear in healthy human non-AMD retinas, as well as providing the chance to explore structural components of drusen in AMD. Finally, the presence of drusen in 4-year post fertilization WT and *rp1l1*$^{-/-}$ fish supports the age-related drusen events successfully replicating into zebrafish.

New and advanced animal models are always essential to understand human conditions – both developmental and pathological. Zebrafish is a well-accepted animal model, and this project has laid the groundwork that using the novel *rp1l1*$^{-/-}$ zebrafish can provide us with the opportunity to understand a complex polygenic condition like drusen and to explore AMD in a comprehensive manner.

Preface

This thesis is an original work by Tasnuva Humaira. No part of this thesis has been previously published. All figures and tables are prepared by the author of this thesis. One figure is collected with Creative Commons BY SA.

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

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In the name of Allah, Most Gracious, Most Merciful. I am sincerely grateful to my Lord for granting me the opportunity to study in the University of Alberta.

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List of Abbreviations

AMD – Age-related Macular Degeneration

Anti-VEGF – Anti-Vascular Endothelial Growth Factor

BBB – Blood Brain Barrier

BM – Bruch's Membrane

bp – base pair

BRB – Blood Retinal Barrier

CaMPARI – Calcium Modulated Photoactivatable Ratiometric Integrator

Cas9 – CRISPR Associated Protein 9

CMZ – Ciliary Marginal Zone

CNS – Central Nervous System

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

dpf – days post fertilization

FD-OCT – Fourier Domain/Frequency Domain - Optical Coherence Tomography

gDNA – genomic Deoxyribonucleic acid

GFP – Green Fluorescence Protein

hpf – hour post fertilization

INL – Inner Nuclear Layer

IS – Inner Segment

LED – Light-emitting diode

mpf – month post fertilization

OCT – Optical Coherence Tomography

ONL – Outer Nuclear Layer

OS – Outer Segment

PBS – Phosphate buffered saline

PCR – Polymerase Chain Reaction

PDT – Photodynamic Therapy

PFA – Paraformaldehyde

ROS – Reactive Oxygen Species

RP1L1 – Retinitis Pigmentosa 1-Like 1

RPE – Retinal Pigment Epithelium

SASS – Science Animal Support Services

TBI – Traumatic Brain Injury

UV – Ultraviolet

WT – Wild Type

ypf – year post fertilization

Chapter 1: Introduction

1.1. Drusen in Age-related Macular Degeneration (AMD)

1.1.1. Age-related Macular Degeneration (AMD)

Drusen is a key hallmark of AMD, which is one of the major causes of visual impairment all around the world (Wong et al., 2014). This vascular-metabolic disease manifests with advancing age, and is likely to present symptoms in people age 50 years and older (*Age-Related Macular Degeneration (AMD) | National Eye Institute*, n.d.). AMD is a progressive eye disease, and is commonly diagnosed when visual impairment reaches an advanced stage (*Age-Related Macular Degeneration (AMD) | National Eye Institute*, n.d.; "Diagnosing Age-Related Macular Degeneration - AMDF," n.d.). Patients usually complain of blurred vision, with or without lines and black woolly appearances, in the visual field. Symptoms also include loss of central vision in advanced stages (Virgili et al., 2015) due to the irreversible damage to the macula, which is a cone-rich region of the eye. Since it has abundance of cone-cells, the macula it is the region that is most active in daytime conditions and in color vision. (Bowes Rickman et al., 2013; *Photoreceptors by Helga Kolb – Webvision*, n.d.; *The Architecture of the Human Fovea By Helga Kolb, Ralph Nelson, Peter Ahnelt, Isabel Ortuño-Lizarán and Nicolas Cuenca – Webvision*, n.d.). Symptoms appear slowly in affected individuals and it is often ignored until it reaches an advanced stage, thus making it a silent disease with the severe consequence of visual loss, without effective diagnostic interventions ("Diagnosis," 2018) at its early stage. AMD is the primary cause of blindness in developed countries (Pennington & DeAngelis, 2016; W. L. Wong et al., 2014), the third leading cause of blindness in developing and underdeveloped countries (Pascolini & Mariotti, 2012), within the population group older than 60 years of age. It is estimated that an astounding 288 million

people will live with AMD by 2040 (W. L. Wong et al., 2014). Despite bearing such heavy disease load, the cause and progression of AMD are unknown; therefore, understanding its pathogenesis and therefore identifying specific therapeutic have been extremely challenging. Understanding drusen is likely to provide insights into AMD development, and molecular basis of drusen can be the tool to unlock the pathogenic processes eyes go through in the diseased condition.

1.1.2. Types of AMD

AMD can be categorized into two main types: dry AMD and wet AMD ("Diagnosis," 2018). Dry AMD, also known as non-neovascular AMD, is the most common form and is characterized by the presence of drusen, yellow deposits under the retina. It progresses slowly and may cause central vision loss over time. Wet AMD, also called neovascular AMD, is a more severe form where abnormal blood vessels grow beneath the retina (*Age-Related Macular Degeneration (AMD) | National Eye Institute*, n.d.; "Diagnosing Age-Related Macular Degeneration - AMDF," n.d.), causing fluid leakage and scarring, leading to rapid vision loss. Timely detection and management of AMD are essential to preserve vision and quality of life.

1.1.3. What are Drusen?

Drusen are a biomarker for AMD. These appear as extracellular deposits and the largest part of its contents are neutral lipids with esterified and non-esterified cholesterol (Rudolf et al., 2008). These lipids accumulate along with some amount of carbohydrates, proteins, and cellular apoptotic waste that form deposits underneath the Retinal Pigment

Epithelium (RPE) and Bruch's membrane in retina and form drusen (Cukras et al., 2010). The presence of larger, abundant confluent drusen in macula is often considered as one of the major risk factors of AMD (Crabb et al., 2002). Histological studies of drusen by Bressler et al (Bressler et al., 1994), showed for the first time how different components, debris and entities can be mispresented by drusen, using common staining and imaging techniques. The confusion persists. Fundoscopic examination commonly detects yellow-white appearance of drusen in patients from 50-65 years of age (Klein et al., 2020; Rudolf et al., 2008). Morphologically drusen can be categorized into two groups – hard drusen and soft drusen (Age-Related Eye Disease Study Research Group, 2005a; Klein et al., 1991, 2020). Hard drusen are non-neovascular, smaller, and can be seen with defined well-demarked area. They are a common phenomenon of the eye changes is advanced age. Soft drusen, on the other hand, are associated with neovascularization, rapidly growing, larger, more spread out and without a defined margin. They almost always lead to severe AMD and vision impairment (Spaide et al., 2018; Spaide & Curcio, 2010; VanDenLangenberg & Carson, 2022).

However, appearance of drusen is not always the confirmatory sign of AMD, as drusen can also be a normal physiological change of aging. This makes it even more important to understand the pathophysiology of drusen development, so it is possible to differentiate between AMD-pathogenesis related drusen with the other forms.

1.1.4. Types of Drusen

Drusen deposits can vary in size, composition, and distribution, leading to different types of drusen that can be observed during clinical examinations.

The first type is hard drusen, which are small, discrete deposits that typically measure less than 63 micrometers in size. Hard drusen are commonly found in the macula of aging eyes and are often considered a normal part of the aging process. They are characterized by their distinct borders and homogeneous appearance when viewed through imaging techniques such as fundus photography or optical coherence tomography (OCT) (Spaide & Curcio, 2010; Van Den Langenberg & Carson, 2022; Rudolf et al., 2008).

Soft drusen, on the other hand, are larger and more confluent deposits. They tend to be greater than 125 micrometers in size and can merge, forming larger areas of deposition. Soft drusen are commonly associated with the development and progression of AMD (Mrejen et al., 2013; Curcio, 2018; Landreville et al., 2008). Unlike hard drusen, soft drusen have a more irregular appearance and can exhibit a range of characteristics, including fuzzy borders and a heterogeneous composition (Rudolf et al., 2008). They are considered a hallmark feature of AMD and are often associated with the deterioration of the macula and the loss of central vision.

Intermediate drusen fall between the size and characteristics of hard and soft drusen. They are typically larger than hard drusen but smaller than soft drusen. Intermediate drusen can have varying appearances, with some exhibiting features of hard drusen and

others showing characteristics closer to soft drusen. These drusen can be an indication of an increased risk of developing AMD or progressing to more advanced stages.

1.1.5. Development of Drusen

Drusen have been a part of ocular studies for a long time (Bressler et al., 1994; VanDenLangenberg & Carson, 2022), however the exact cause of drusen remains unknown. It is assumed that age-related metabolic changes in retina is the key factor in development of drusen (Curcio, 2018; Landreville et al., 2008; Spaide et al., 2018; Spaide & Curcio, 2010). It is often hypothesized that the apoptotic cell wastes, degraded parts of retinal components or remnants from a disbalanced lipid homeostasis may result in accumulation and coalescence of drusen under the laminar basement membrane of RPE (Doucette et al., 2021; Malicki et al., 1996; Rudolf et al., 2008; W. L. Wong et al., 2014). There are different environmental elements that can contribute to development of drusen including hereditary qualities, smoking, and hypertension (Crabb, 2014; Crabb et al., 2002; Zarbin, 2004). Complement activation with the changes associated with aging may also be involved with drusen formation (Luibl et al., 2006). While the presence of drusen does not mean that the patient will get AMD, bigger and more various drusen can indicate an increased risk for a more severe form of the disease. An advanced AMD is almost always accompanied by the presence of drusen in macula within the retinal layers (Jiang et al., 2012; Wang et al., 2010).

1.1.6. Symptoms, Ocular Examinations, and Treatment of Drusen

1.1.6.1. Symptoms Associated with Appearance of Drusen in AMD Patients

Elderly patients with AMD do not usually have a drastic change in their visual field at early stage, hence they do not seek urgent medical attention. Sometimes affected individuals complain of blurring of vision and appearance of cotton-woolly appearance in their visual field. It is frequently accompanied by the presence of dark and wavy lines in front of them. Blurring of vision appears and progresses very slowly and is often ignored. At the advance stage, patients may complain of complete loss of central vision which is the complete inability to see the faces of someone they are talking with or inability to read when they look at any written text. This is usually when patients reach out and seek medical help. Unfortunately, once vision loss happens, there is nothing that can be done to replace it.

1.1.6.2. Clinical Examination of Eye

Most of the time drusen deposits are detected during a regular ophthalmic examination and fundal imaging, making it a silent cause of visual loss without specific or noticeable symptoms. **Figure 1.1** is an example of how drusen appear in fundoscopy images, **Figure 1.1A** shows a normal fundus and **Figure 1.1B-C** are examples of hard and soft drusen in fundal images. When elderly patients seek medical help to address visual discomforts, their fundal examination usually show appearance of drusen. Often fundoscopic examination detects drusen which are well-demarked, relatively smaller and similar to what **Figure 1.1B** presents. They are categorized as hard drusen. Sparse, spread and without a well-defined margin like **Figure 1.1C** are categorized to be soft drusen which are the more severe form.

Alongside fundal imaging, Optic Coherence Tomography (OCT) has recently become a very useful investigative tool to detect drusen and the changes they bring to the retina (Mrejen et al., 2013). It is a non-invasive procedure that measures how much infrared light is reflected off different structures in the retina; and measures of the thickness of retinal layers and optic nerve can be estimated from the light reflected. Drusen and other similar inclusions come up as a change in regular retinal structures. Fluorescence angiography can also be done where contrast medium is injected into the ocular vasculature and a detailed scan of retina and macula are collected. However, none of these tests are specific for drusen, and as a result there is still no effective way to know whether a person possesses drusen in their eyes or what is the nature of those.

1.1.6.3. Treatment of Drusen-associated AMD

Despite bearing such a huge disease burden, specific treatments for AMD are still unavailable. The supportive treatment regimen commonly includes close observation of the eyes and dealing with any hidden medical issue that might add to their turn of events. Vitamin and mineral supplements are often advised. Anti-VEGF (vascular endothelial growth factor) which is hostile anti-angiogenic medication and laser-assisted subthreshold photocoagulation of abnormal blood vessels, and photodynamic therapy (PDT) or any combinations of these have been utilized to slow the progression of AMD in people with huge soft drusen (Age-Related Eye Disease Study Research Group, 2005a, 2005b). Nevertheless, none of them show significant change in the disease course, all possess hazardous and severe side effects, and is not often accepted by every patient

because of the uncertain outcomes of AMD (Alibhai et al., 2018; Novais et al., 2015; Virgili et al., 2015).

1.1.7. Gaps in Understanding Drusen

Drusen are a pathological condition and a substantial risk factor for dire outcomes, yet the development and pathogenesis of drusen are still enigmatic. Not knowing about the disease etiology thwarts implementation of specific diagnostic and prognostic measures at early stages of drusen-related AMD, and even hinders efficacious treatment once they are incidentally diagnosed. This requires more *in vivo* studies where it is possible to project the drusen morphogenesis into experimental animal models, so the molecular mechanism is well-understood. And the changes and effects of drusen may have on other retinal structures can be studied.

It is very hard to identify a single cause for the development of drusen. It is also difficult to demonstrate how drusen contribute to AMD. Several genetic factors are likely involved in the pathogenesis, and all of which may not be an exacerbation of normal aging process. The enigmatic molecular process for the development of drusen, when associated with appropriate environmental conditions (Okubo et al., 1999), ultimately result in full-blown AMD in affected individuals.

1.2. Eye Changes Accompanying Drusen

1.2.1. Retina: Layers and Light Pathway in Normal Condition

Retina is the posterior part of the eye. A healthy retina consists of ten distinct layers with interconnected neurons, and the cells are categorized into three groups – photoreceptors, neuronal and glial cells (Mahabadi & Al Khalili, 2023). **Figure 1.2B** is a schematic representation of the functional retinal layers, and how presence of drusen can interrupt the light pathway in retinal layers is demonstrated in **Figure 1.2C**. When light enters the eye through the lens, and the waves fall onto the photoreceptors adjacent to the Retinal Pigment Epithelium (RPE). Pigment molecules present in the outer segment (OS) captures it and phototransduction takes place. It is the cascade of molecular changes for the conversion of light energy into an electrochemical and neural format comprehensible by brain (Palczewski, 2012). Then these chemical impulses travel through bipolar cells, ganglion cells and through the optic nerve driven by a multitude of molecular events to reach the visual cortex of the brain and the image becomes coherent. Any interruption, structural and chemical, of the pathway inside the retina may impair normal vision; and how drusen interrupts retinal normal structures is shown in **Figure 1.2C**. In any retinal pathology, and in normal aging conditions several changes take place within the retinal layers.

1.2.2. Age-related Changes in RPE and in Bruch's membrane (BM)

Aging increases cumulative oxidative stress in body tissue, and the retina is not excluded from this effect (Harman, 1956; Verzar, 1957; Wallace, 1999). Changes in the cellular environment, alter their phenotypes (Bartfai et al., 2019; Robert, 1998). RPE cells

accumulate lipofuscin, a group of auto-fluorescent lipid-proteins during life (Feeney-Burns et al., 1984), and it occupies about 19% of the cytoplasmic volume of the RPE cells by 80 years of age (Zarbin, 2004) which is much higher than that of earlier ages. Reduction of functional volume of RPE cells is likely to compromise normal cellular functions like phagocytosis (Sundelin et al., 1998) and apoptosis by which waste products are removed. That may also be associated with more photoreceptor death than usual (Bujakowska et al., 2017; *Introduction to Xenopus - Xenbase*, n.d.; Kennedy et al., 1995; Kha et al., 2018; Lee et al., 2012; Molday & Moritz, 2015; Zarbin, 2004), especially degradation of the outer segment. Increased lipofuscin may exert more oxidative stress on RPE and surrounding tissues (Luibl et al., 2006). There may also be inhibition of lysosomal activities in RPE cells. Recent studies imply that the presence of nonfibrillar amyloid oligomers, forming amyloid plaques, may lead to drusen formation (Crabb, 2014; Crabb et al., 2002).

Bruch's membrane (BM) histologically consists of five layers between the RPE and the choroid (Mrej, 1971) – basement membrane, inner collagen layer, elastin layer, outer collagen layer and basement membrane of choriocapillaris. All the layers go through significant changes in aging process (Booij et al., 2010). Being an acellular layer, BM is likely to depend on RPE cells for their nutrition and for the suitable environmental constituents for it to function (Alabduljalil et al., 2019). Almost all cellular waste products from RPE and choroid, and biomolecules go through BM. As age advances, waste products can become abundant, transport mechanisms may be hindered, BM morphology is likely to alter affecting the diffusion properties, or any combinations of these event may take place; thereby increasing the chance of accumulation of these at any place in the pathway (Booij et al., 2010; Okubo et al., 1999; *Studies on the Human Macula: IV. Aging*

Changes in Bruch's Membrane | JAMA Ophthalmology | JAMA Network, n.d.). Retinal structures show an increase in the cross-linkage between collagen fibers, thus reducing the elasticity (Klein et al., 2020) with age.

1.2.3. Drusen-associated Changes in Aging Retina

Age is a normal phenomenon, and the changes associated with aging are inevitable. Considering the physiological changes of RPE and BM, it can be guessed why drusen deposit appear in AMD. However, drusen have been identified in younger eyes exhibiting all the features of AMD (Doucette et al., 2021; Noel et al., 2022), and presence of drusen has not always been a confirmatory diagnosis of AMD in elderly patients. The unknown etiology of drusen development also raises questions on the mechanisms responsible for turning the presence of drusen into a grave illness resulting in visual impairment. Therefore, appearance of drusen has remained a mystery to both the scientist and clinical communities. It is also imperative to explore whether the age-related changes in retina are the contributing factors behind drusen formation or drusen is one of the causes of normal aging changes taking a dramatic turn to full-blown AMD.

1.3. Animal Models for AMD and Drusen

1.3.1. Using Animal Models in Research

The enigmatic origin, associated pathology and progression of drusen beg exploring into the molecular etiology. AMD disease burden becomes very heavy to not only the affected individuals but also on people around them. Healthcare system and communities are economically affected as they provide support and rehabilitation of AMD patients. Without a proper knowledge base, it is difficult to diagnose drusen in the early stage, as well as to provide specific treatments for this condition. Preventive measures for drusen have solely been raising awareness and lifestyle modification to improve general health conditions such as quitting smoking. Any link between the inheritance pattern or genetic predisposition goes undetected owing to the absence of credible knowledge on the drusen pathophysiology. Therefore *in vivo* studies on drusen are crucial for our understanding and to prepare a strategic plan for diagnostic, treatment and preventive purposes.

1.3.2. Current Animal Models for Ocular Research

AMD is a heterogeneous disease (Pennesi et al., 2012). Like all *in vivo* studies with animals as disease models, an ideal model would mimic the histological and functional changes of the disease of interest (M. K. Davidson et al., 1987). They should also possess anatomical analogy, developmental homology and functional similarity with the tissue affected by the disease (Mizui & Tsokos, 2014). An ideal animal model must also be inexpensive to breed and maintain, as it is imperative to have the experimental samples evolve in a rapid course of time for resourceful studies (Festing & Altman, 2002). Handling

the animal models must fulfil the ethical requirements and should be done in the most humane way possible (Ferreira et al., 2019).

Considering the complex nature of AMD and drusen, selecting a suitable animal model has been very challenging. Multiple genetic and environmental factors are associated with the disease progression, such as genetic defects and polymorphism, and oxidative stress, inflammation and imbalance in metabolic homeostasis (Crabb, 2014; Pennington & DeAngelis, 2016; Wang et al., 2010). It is also crucial to consider the anatomical and morphological differences between human retina and that of the animal models for *in vivo* studies on drusen-driven AMD.

Rodents, rabbits, pigs, African clawed frogs and non-human primates (NHP) have been developed to model drusen and AMD throughout a long course of time (Carr & Moritz, 2020; Edwards & Malek, 2007; Elizabeth Rakoczy et al., 2006; Grossniklaus et al., 2010; Marmorstein & Marmorstein, 2007; Ramkumar et al., 2010; Zeiss, 2010); all of which have their own merits and disadvantages. However, while studying a human disease in an animal model, it should always be considered that the animals may not represent the true etiology of the disease for the reason that they have been developed in a laboratory through a vast array of genetic manipulation (Pennesi et al., 2012). **Table 1** is a summary of the benefits and drawback of using current animal models that are being used to study drusen.

1.4. Zebrafish in Ocular Studies

Zebrafish (*Danio rerio*) has been an effective animal model to study human developmental biology and disease pathology since its first introduction in 1981 (Streisinger et al., 1981). They possess several advantages compared to other vertebrate models. With the advancement of protocols such as mutagenesis techniques, next-generation sequencing (NGS), and phenotypic screening, zebrafish has become one of the most effective and utilized animal models to explore the causal connection between the genotype and phenotype of human conditions (Choi et al., 2021).

Zebrafish also possess several advantages as an animal model. They have at least 70% orthologues of human genes, and there is at least one zebrafish counterpart for 84% of human pathogenic genes (Bradford et al., 2017). Zebrafish have human internal organ counterparts, and they go through similar developmental events (Kimmel et al., 1995). Many embryos can be generated from each breeding between a male and a female adult zebrafish. Zebrafish embryos are transparent and they fertilize externally, which is extremely beneficial for administering microinjections at earlier stages during development. This characteristic also makes imaging very convenient (Martin et al., 2019). Zebrafish genome is well-known and there are established genetic manipulation techniques, which helps to plan, develop and implement new methods of experiments (*ZFIN Lab: Sanger Centre Zebrafish Project*, n.d.). Last but not least, breeding, maintaining and handling zebrafish are relatively simple and inexpensive compared to those of other vertebrate animal models (M. K. Davidson et al., 1987). Zebrafish can also be utilized and euthanized in a very humane way, and the ethical protocols related to

using zebrafish as an animal model are easy to follow and obey. These benefits make zebrafish an ideal candidate to explore pathophysiology of human disease and disorders.

1.4.1. Zebrafish Eye

1.4.1.1. Retina and Photoreceptors

Retina derives from external neural ectoderm at approximately 20hpf (hours post fertilization) of embryonic development as a part of posterior segment development (Cassar et al., 2020). Since AMD features and its biomarker, drusen, accumulate in macular region of retina, its structural, biophysiological changes and morphology are of our particular interest. This is also the region for sensory detection; therefore scientists have always been fascinated with the similarity and dissimilarity of zebrafish retinal structures to that of human's (Rosa et al., 2023). **Figure 1.3** is a comparison between the human retinal layers and zebrafish retinal layers, using one of the images from this project. Understandably there must be some difference between human eye and aquatic zebrafish eye, however a striking similarity is also observed in anatomical arrangement of the layers (Gestri et al., 2012; Richardson et al., 2017).

Ganglion cells differentiate first at 28hpf, followed by amacrine, horizontal and Müller glial cells by 2dpf (days post fertilization) age. Rod and cone cells become evident around the same time and synaptic structures show functional maturation by approximately 60hpf age (Cassar et al., 2020). By 5dpf, structurally mature photoreceptors become functionally competent with active signal transmission (E. A. Schmitt & Dowling, 1999).

Photoreceptor cells, rods and cones, have light-sensitive outer segment (OS), mitochondria-rich inner segment (IS) with cellular organelles for metabolic functions, and a connecting cilium between them (Noel et al., 2022; Noel & MacDonald, 2020). These cells lie adjacent to the RPE, and depend on it for nutrition, waste removal, and other metabolic supports (Jaroszynska et al., 2021). RPE constituent melanin provides a protective support to the photoreceptors from light-induced damage, and any change in RPE exerts an effect on the photoreceptors (Jaroszynska et al., 2021; Noel et al., 2022; Rosa et al., 2023; E. A. Schmitt & Dowling, 1999).

1.4.1.2. Zebrafish Eye: Anterior Segment

First step of zebrafish eye development takes place with anterior neural plate specification under the influence of several transcription factors. Components of anterior segment of the eye such as cornea, lens, ciliary body, hyaloid vasculature follow the similar developmental events that take place in human eye because of the involvement of neuroectoderm, superficial ectoderm and mesenchyme. There have been a number of successful studies done using zebrafish visual system as they share structural and functional similarity with human visual components (Gestri et al., 2012). Zebrafish cornea is a layered structure and is avascular like that of the other vertebrates. Its histological and morphological characteristics have been thoroughly studied in relation to anterior segment development (Akhtar et al., 2008; Soules & Link, 2005; Swamynathan et al., 2003; Zhao et al., 2006). However, a significant difference between zebrafish cornea and cornea of other vertebrates is the presence of fingerprint-like hexagonal cells in the external capsule replacing microvilli structures that are present in other vertebrates (Collin

& Collin, 2000). Lens in adult zebrafish eye present similar important morphological hallmarks to other vertebrates (Greiling & Clark, 2008; Vihtelic, 2008), despite some important differences such as lens shape being more spherical with a covering of elongated fiber cells – which leads to abrupt disassembly of organelles (Dahm et al., 2007). Regardless of the dissimilarities, comprehensive studies on the corneal and anterior segment development of zebrafish eye strongly support conservation of multiple molecular mechanisms with other vertebrates because of shared embryonic germ-layer origin (Langenberg et al., 2008; Soules & Link, 2005).

1.4.1.3. Similarity and Dissimilarity between Human and Zebrafish Eye

The human eye is strongly rod dominant, but the vital part of the eye is the macula and it is abundant in cone cells. When light enters the eyeball and reaches the fovea, at the center of the macula, it triggers visual response and initiates the complex cascade to capture light intensities. Fovea is the part of retina where the only neurons are the cone-cells (*The Architecture of the Human Fovea By Helga Kolb, Ralph Nelson, Peter Ahnelt, Isabel Ortuño-Lizarán and Nicolas Cuenca – Webvision*, n.d.). The macula is incidentally the region where drusen appears. Therefore, replicating drusen characteristics into an ideal animal model required a cone-rich environment, which is lacking in most of the ocular animal models, including rodents – the most common model in vision research.

The retina of zebrafish is cone-rich, similar to that of the human macula; as such which zebrafish is a better model for examination of drusen than the commonly used ocular models like rodents, which are nocturnal and without macula in their eyes (Volland et al., 2015). On the other hand, zebrafish have double cones, which are absent in human and

in other mammalian retina (Mahabadi & Al Khalili, 2023). Zebrafish photoreceptor mosaic pattern is quite different than human photoreceptor mosaic pattern, presenting a challenge when translating human macular degeneration to zebrafish (Noel et al., 2022). Zebrafish do not have a distinct retinal region like human fovea, which is located in the center of macula, and consists of closely-packed cone cells (*Webvision: Simple Anatomy of the Retina*, n.d.). However, zebrafish larvae do have a functionally similar region to human fovea in their retina which takes part in high-acuity vision acquisition (Yoshimatsu et al., 2020).

Another significant dissimilarity between human and zebrafish retina is the capability of robust regeneration in the latter (Chrystal et al., 2021; Fraser et al., 2013; Hitchcock & Raymond, 2004; Noel et al., 2022; Oel et al., 2020). Any neuron loss is irreversible in human retina, and it may lead to loss of vision.

Despite these dissimilarities, the anatomical resemblance of the retinal layers shown in **Figure 1.3**, cone-rich environment like the human retina and common benefits over others, diurnal zebrafish are one the most accepted animal models for vision research purpose.

1.5. Homozygous *rp1l1* Mutant Zebrafish, *rp1l1*$^{-/-}$

Pathogenesis of drusen and AMD remains enigmatic, however it is acknowledged that multiple factors, both molecular and environmental, contribute to the condition. The first zebrafish line that exhibit detectable drusen is the mutant line developed by Dr. Nicole Noel (Noel et al., 2020) which may give us an excellent opportunity to explore the pathogenesis and molecular mechanism of drusen formation.

1.5.1. Human *Retinitis Pigmentosa 1-like 1, RP1L1*

The human *RP1L1* gene encodes a retina-specific protein which is expressed in the connecting cilium of the photoreceptors (PubChem, accessed on April 22, 2023, from https:// www.ncbi.nlm.nih.gov/gene/94137). The gene expands to 50kb and is in chromosome 8. The mRNA is over 7kb, however the exact length is variable because of several length polymorphs (Bowne et al., 2003). RP1L1 protein consists of approximately 2400 amino acids with an estimated molecular weight of 252 kDa. **Figure 1.4** represents the location of RP1L1 in photoreceptor cilium. This protein have synergistic role on photosensitivity and outer segment morphogenesis in rod cells (Datasets, accessed on April 22, 2023, from https:// www.ncbi.nlm.nih.gov/datasets/tables/genes/?table_type= transcripts&key= aeb5b78e98754aebd0cf3de359731360n.d.). *RP1L1* mutations have an autosomal dominant (Akahori et al., 2010), sometimes recessive (Noel & MacDonald, 2020) inheritance pattern, and its mutation causes occult macular dystrophy (Miyake & Tsunoda, 2015), and its zebrafish variant *rp1l1* has been linked with retinitis pigmentosa (Noel et al., 2020).

1.5.2. Mutation in Zebrafish *rp1l1*

Zebrafish Rp1l1 is a 2394 amino acid with a well-conserved doublecortin and RP1 domain; and is predicted to be active in axoneme (ZFIN Gene: Rp1l1a, accessed on April 22, 2023, from https://zfin.org/ZDB-GENE-120711-1). Noel et al reported a 16 base-pair (bp) deletion in the first doublecortin domain of *rp1l1* (Noel et al., 2020), targeting its homologous region of it, using CRISPR/Cas9 genome editing. This novel mutant zebrafish line showed progressive photoreceptor dysfunction, retinal structural abnormalities, disorganization of photoreceptor OS; and it linked retinitis pigmentosa with the *rp1l1*$^{-/-}$. A serendipitous discovery was made in retinal cross-sections of mutant fish when Oil-red O (ORO)-stained lipid deposits appear at 14mpf age. These relatively small deposits were detected between the photoreceptor OS and the RPE and appeared to have a circular and well-marked margin. This provides an extremely valuable opportunity to study these lipid droplets as they bear many characteristics of drusen, or at least of subretinal drusenoid deposits, neither of which have ever been observed in zebrafish before. These droplets are established to be lipid-rich because Oil Red O is a type of Sudan lysochrome that stains neutral lipid and triglyceride contents in fixed and frozen tissue sections (Curcio, 2018; Z. Zheng et al., 2011).

The pathogenesis of a complex multifactorial disease like AMD, and its biomarker drusen, remains a mystery. It is crucial to understand its pathophysiology, which has been discussed in detail in **Section 1.1.5**. Zebrafish has been an effective animal model to study human developmental attributes and they provide valuable insights into numerous human diseases and disorders. Success of zebrafish as an animal model, to understand

human biology, its benefits as an ocular disease-model and the similarity to human retinal structures (discussed under **Sections of 1.4**) inspire us to explore whether the *rp1l1*$^{-/-}$ zebrafish can be a candidate to conduct drusen-study with them.

1.6. Purpose of the Project: Hypotheses and Aims

The purpose of the project is to establish and characterize the novel homozygous *rp1l1*$^{-/-}$ mutant zebrafish as an animal model to study drusen.

1.6.1. Hypothesis 1

The first hypothesis tested is that there are detectable drusen appearing within the retinal layers of the *rp1l1*$^{-/-}$ zebrafish. ORO staining is one of the most accepted methods to detect lipid-rich components in tissue, and almost 90% of drusen components are esterified and non-esterified cholesterol. Therefore ORO-stained retinal cross sections collected from *rp1l1*$^{-/-}$ should show detectable drusen-like deposits if they are present.

1.6.2. Hypothesis 2

The second hypothesis is to test whether the detectable drusen exhibit age-related manifestation. Drusen are a hallmark of AMD, however drusen have also been detected in otherwise normal retina in younger and aged individuals. Detecting drusen in *rp1l1*$^{-/-}$ zebrafish at an earlier age than they are expected to appear would support that *rp1l1*$^{-}$ is inducing earlier presentation of detectable drusen, i.e. the lack of rp1l1 may be creating a developmental phenotype that would not be a strong match for the typical AMD patient drusen history.

1.6.3. Hypothesis 3

The Third hypothesis is that by recording the continuous progression, and increasing accumulation or incidence, of *rp1l1*$^{-/-}$ drusen will be observable over a considerable period over the mutant fish lifespan.

1.6.4. Hypothesis 4

Rp1l1 is expressed in the photoreceptors. Mutation in *rp1l1* can exert a number of effects on the photoreceptor morphogenesis, and the adjacent retinal structures should reflect the changes as well as the photoreceptors. Therefore, the fourth hypothesis is to determine the changes in photoreceptor-adjacent structures with the presence of detectable *rp1l1*$^{-}$-induced drusen.

The findings from the experiments are likely be very useful to establishing homozygous *rp1l1*$^{-/-}$ zebrafish as an applicable, instrumental and befitting animal model to study drusen.

Figures for Chapter 1

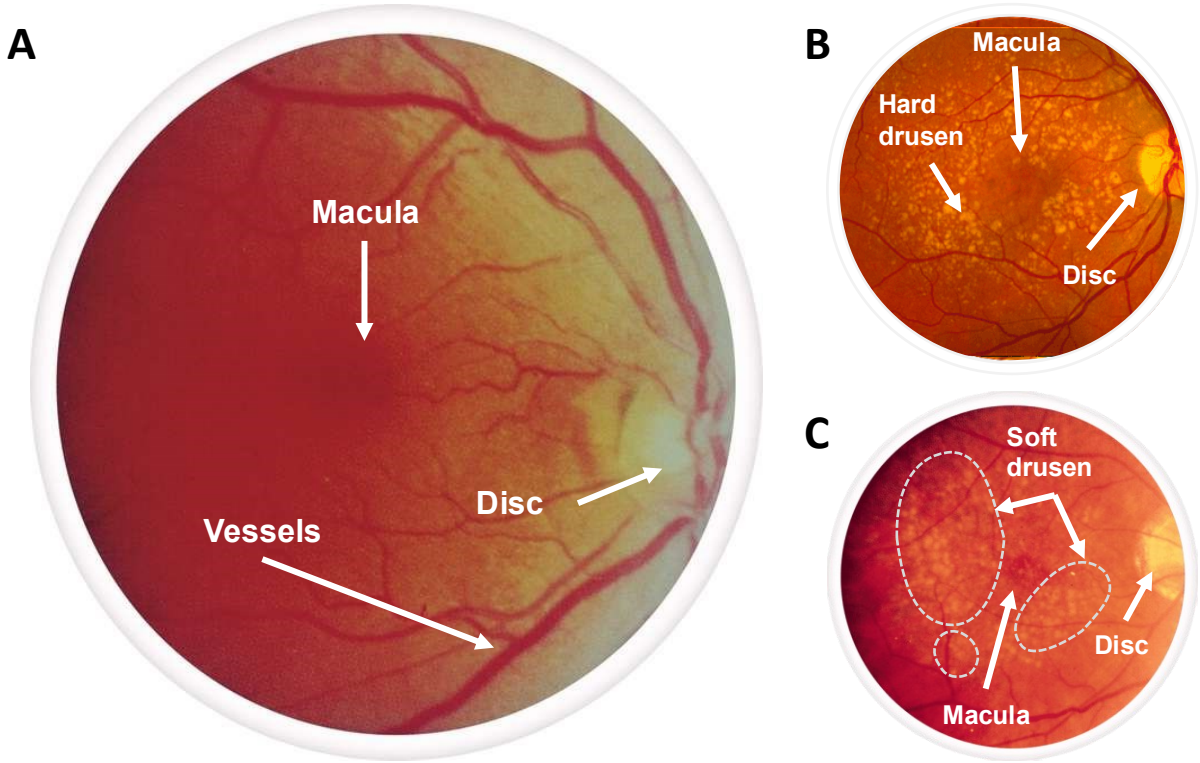

Figure 1.1. Fundal images of human eye. The fundal images are reused from under the Creative Commons by 4.0, collected from https://commons.wikimedia.org/wiki/File: Macularhard_drusen.png and https://en.wikipedia.org/wiki/Macular_degeneration **A)** shows a fundal image of a normal human right eye. The dark red-orange color of the image is retina, the white area pointed as disc is the continuation of optic nerve from the eyeball. Optic vessels are clear. Macula is the darker region, and fovea is the center of it. **(B)** shows a fundal image of human right eye with pale-yellow hard drusen appearing in the macular region. Drusen droplets are punctate in appearance and seem to have well-defined margin. **(C)** is a fundal image of human right eye with soft drusen, highlighted within the dotted margins.

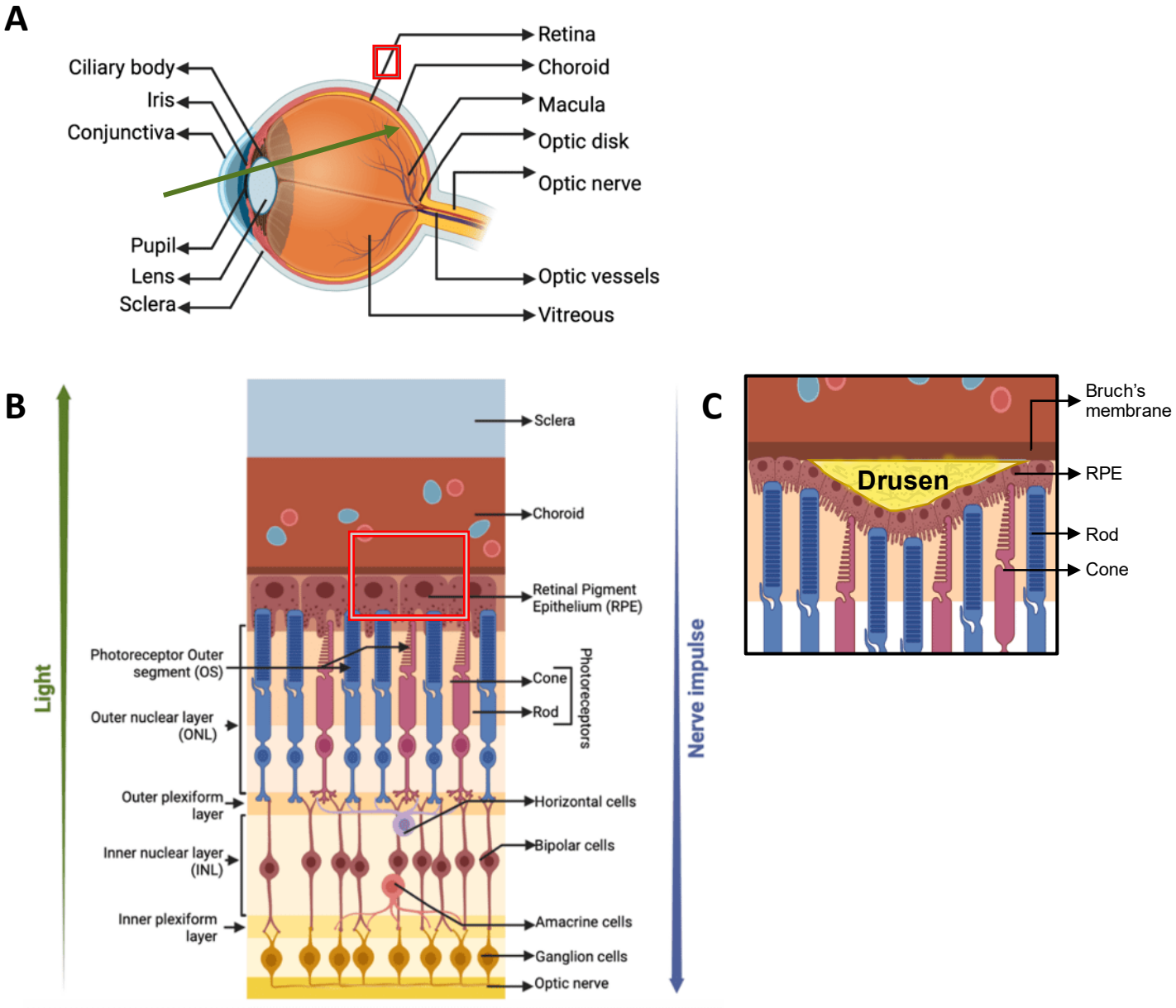

Figure 1.2. Normal eye, retinal structures and area affected by drusen. Figures are prepared on Biorender.com. **(A)** is a schematic representation of healthy eyeball. Retina is in its posterior part. Light, represented by the arrow enters the eyeball through the opening of pupil and falls on to the retina passing the lens. **(B)** is the depiction of small

red squared area in panel **(A)** where the retinal layers are presented. Light rays are captured by the outer segment of the photoreceptor, phototransduction converts the light energy into nerve impulse in chemical form. It travels along bipolar cells and ganglion cells reaching the optic nerve which carries it to the visual cortex of brain and the image is perceived. **(C)** is an example of accumulation of lipid-rich drusen between the Retinal Pigment Epithelium (RPE) and Bruch's membrane of choroid vasculature. Drusen in this location has the potential to interrupt the light pathway through retina by simply disrupting normal layered arrangement and organization.

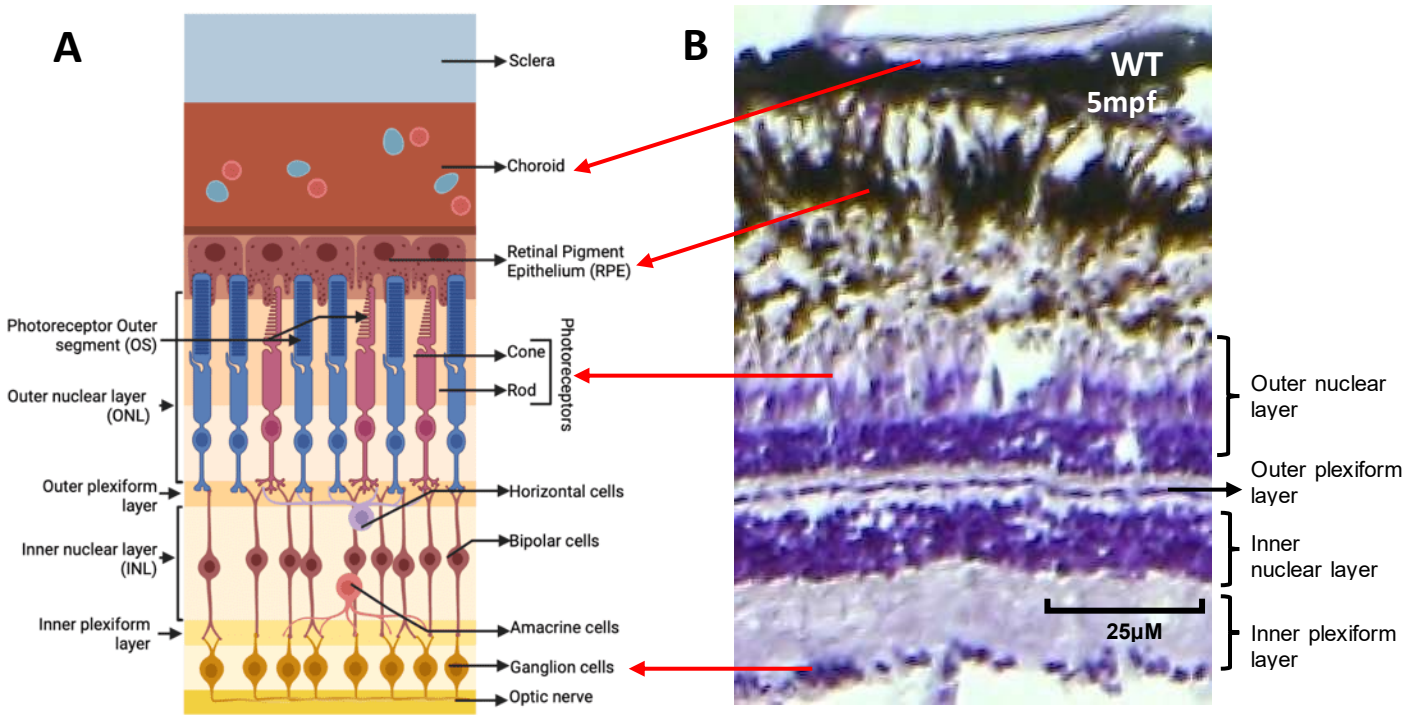

Figure 1.3. Retinal layers in zebrafish. (A) shows schematic presentation of human retinal layers; prepared in Biorender.com. **(B)** shows an Oil-Red O (ORO) and hematoxylin-stained retinal layers from a Wild Type (WT) zebrafish at 5-month post fertilization (mpf) age. Layers of zebrafish retina are distinguished by the arrangements of cell nuclei stained by hematoxylin. Similar retinal structures – a part of the choroid vasculature, Retinal Pigment Epithelium (RPE), photoreceptor cells, ganglion cells, outer and inner nuclear layers, and outer and inner plexiform layers are compared between the prepared retinal image and the zebrafish retinal cross section.

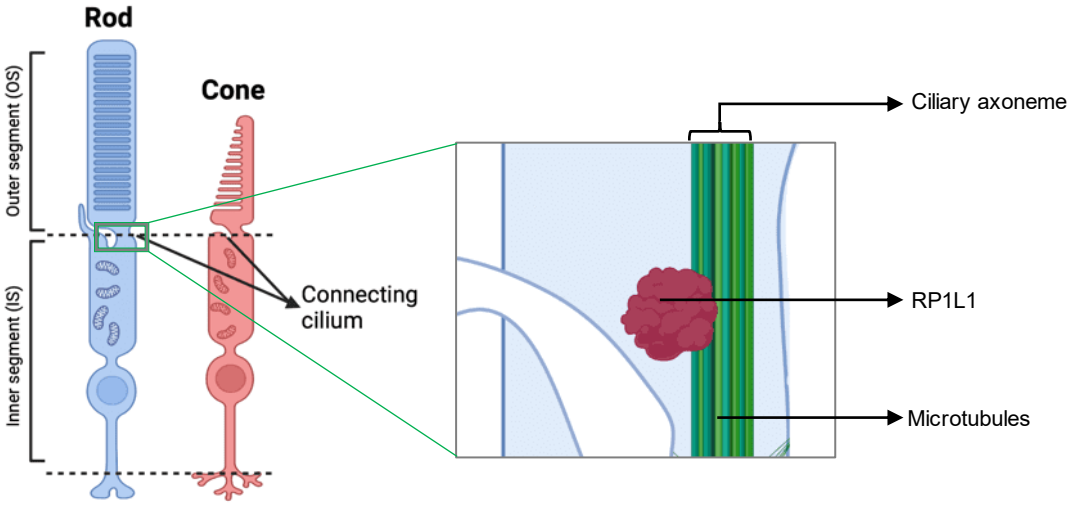

Figure 1.4. Location of *RP1L1* expression. It shows the outer segment (OS) and inner segment (IS) of rod and cone photoreceptors. OS and IS are connected through a cilium which is represented by the enlarged boxed area. Ciliary axoneme consists of microtubules where the RP1L1 protein, coded from *RP1L1* is expressed. It has important synergistic role in microtubule polymerization and OS morphogenesis of photoreceptors.

Table 1. Comparison among the current animal models of drusen, with their benefits and challenges

Model Animal	Demonstrated AMD-related findings & benefits	Challenges
Rodents	• First mouse model demonstrating the heritable retinal degeneration was developed almost 100 years ago (Keeler, 1924), and they are proven to be a quite effective animal model to study drusen. It still is the most common animal to use for drusen studies. • Rodent model allows to see the effects of complement factors (Ross et al., 2008) and inflammation cascade (Coffey et al., 2007) in the mechanism of drusen formation. • Chemokines and cytokines involved in the process of leukocyte migration are hypothesized to induce the Retinal Pigment Epithelium (RPE) changes in AMD (Age-related Macular Degeneration) (Raoul et al., 2010; Yang et al., 2010). Mice models are	• Mouse retina does not have a macula (Volland et al., 2015). Human retina has a distinct cone-rich dense area (Curcio et al., 1990), which can be a very important factor for drusen development. Photoreceptor degradation may even be the primary cause of drusen accumulation and mice not having a macula is a great challenge while using them for drusen studies. • Rodents are mostly nocturnal animal. It is very difficult to compare the effects of drusen on cone-rich human retina to rodent retina which is not adapted to well-lit conditions during a big part of their circadian cycle (Banks & Nolan, 2011; Jud et al., 2005). • Drusen and other AMD manifestations appear in

	available and studied to observe the effects of chemokines and cytokines (Despriet et al., 2008; Luhmann et al., 2009). • Mice model demonstrates drusen forming from the degradation of retinal endothelial, Müller and ganglion cells (Combadière et al., 2007; Yang et al., 2010). • Thinning of Retinal Pigment Epithelium (RPE), Bruch's membrane (BM), photoreceptor atrophy are demonstrated in association of drusen in mice model (Ross et al., 2008). • Oxidative stress effects are demonstratable in mice model (Dunaief, 2006; Imamura et al., 2006; Wenzel et al., 2005). Dietary modifications (Dithmar et al., 2000; Klein et al., 2020; Weikel et al., 2012), dysregulation of lipid/glucose metabolism (Markovets et al., 2011), exposure to smoking environment (Pons & Marin-Castaño, 2011; Thornton et al., 2005), dietary supplements (Luhmann et al., 2009; Markovets et al., 2011) have	relatively older age (Pennesi et al., 2012; Tuo et al., 2007). • Different genetic background in mice can react to mutations or rearing conditions differently. • Sometimes homozygous mutant mice do not demonstrate phenotype differences from that of the single knockout mice, which suggests selective breeding of animals are capable to produce altered outcomes of the mutation (Raoul et al., 2010).

	been manipulated in mice models to ascertain their effects on drusen . • Polygenic rodent models are available (Pennesi et al., 2012). AMD is a complex disease with the involvement of multiple genes and different environmental conditions, which can be demonstrated in polygenic models at relative ease. • Rodents are relatively inexpensive and easy to maintain and breed in a laboratory setting, which make them a very effective animal model for not only retinal studies, but for other *in vivo* studies as well (M. K. Davidson et al., 1987).	
Rabbits	• Rabbits have larger eyeballs, which is very advantageous for drusen-studies (Muraoka et al., 2012), as administering subretinal injections is easier and less hazardous. • They are also relatively easy to handle and breed; and less expensive than breeding non-human primates (NHP).	• Rabbit lacks macula, which is one of the most important locations for translating human drusen and other AMD effects into animal models. Instead of a macula, rabbits possess a dense streak of rods and cones (Muraoka et al., 2012; Qiu et al., 2006). • Rabbit model demonstrating wet AMD phenotype has not

	• Although conventional methods of introducing Copy Number Variations (CNV) have not been a great success in rabbits, a novel method of injecting matrix-mixture into retina seemed effective (Qiu et al., 2006), suggesting rabbit disease model can bring about newer techniques and novel ideas for genetic manipulation.	been as successful as rodent and NHP models, using conventional methods (Qiu et al., 2006).
Nonhuman Primates (NHP)	• Retinal structures closely resembling human retina, having most similar anatomy and physiology to those of the humans are some of the great advantages of NHP models. • NHP eyes have cone-dense macula, hence they overcome one of the major limitations of any other animal model for drusen studies. • NHPs share similarity in the organization of their visual pathway to humans (Pennesi et al., 2012). The only source of nutrition of macula is choroid plexus (Moshiri et al., 2019; Picaud et al., 2019), it is also the mechanisms for waste removal; which have similar characteristics of human retina,	• Genetic manipulation is extremely challenging. • Expensive to breed and maintain, and extremely difficult to handle to conduct regular consecutive experiments (Moshiri et al., 2019; Muraoka et al., 2012). • Relatively longer disease course, which is not an ideal criteria for animal models (M. K. Davidson et al., 1987). • More ethical constraints are associated with using NHPs as an animal model to study a disease process (Colman, 2018).

	and hypothesized to play very important role on drusen formation (Doucette et al., 2021). • NHPs share genetic risk factors and diet with those of human, which provide a very effective environment to translate the pathophysiology and therapeutics of a disease between the animal models and human (Age-Related Eye Disease Study 2 Research Group, 2013; Pennesi et al., 2012). • Rhesus monkey has shown spontaneous development of drusen in their retina (Yiu et al., 2017), sharing similar locations and compositions (Fletcher et al., 2014). • Chronic neovascularization has been observed in NHPs following intravitreal injections, that may be an excellent model to see short and long-term effects of locally administrable therapeutics (Patel et al., 2020).	
Amphibians	• Easily bred and maintained (Kha et al., 2018). • Longevity can prolong up to 30 years in laboratory captivity,	• Very diverse genus, that includes Anurans (frogs and toads), Urodeles (salamanders) and

	providing an opportunity to observe long term effects of disease process and therapeutics (Blum & Ott, 2018; Burggren & Warburton, 2007). • Breeding can be induced by gonadotropin injections administered to the female *Xenopus laevis* (Blum & Ott, 2018; Burggren & Warburton, 2007). • Eggs and embryos are moderately large and robust; therefore, microinjections into embryos or a particular cell are relatively easier. And they can tolerate extreme manipulation (*Introduction to Xenopus - Xenbase*, Accessed on April 22, 2023. from https://www.xenbase.org/entry/anatomy/intro.do.). • External fertilization of embryos provides much advantage towards imaging studies, surgical manipulation and protein and other chemical treatments. • Retinal cells are large and a good platform to study the morphogenic changes (*Introduction to Xenopus -*	Gymnophiona (caecilians or apodans). It provides excellent variety of species (A. F. Bennett, 2003), however translating the findings is difficult to and from human. • They breed seasonally, making it hard to obtain enough suitable eggs, embryos, and larvae for experiments throughout the year (*Developmental Biology: Xenopus as a Model System*, Accessed on April 22, 2023, from http://people.ucalgary.ca/~browder/frogsrus.html); with the exception of *Xenopus laevis* (Blum & Ott, 2018). • Developing transgenic lines has been very challenging (*Developmental Biology: Xenopus as a Model System*, Accessed on April 22, 2023, from http://people.ucalgary.ca/~browder/frogsrus.html) • Amphibians do not have eyelids. • They are fully aquatic animal. • Amphibians have diploid and allotetraploid cells, which

	Xenbase, Accessed on April 22, 2023. from https://www.xenbase.org/entry/anatomy/intro.do..; Lee et al., 2012). • *In vivo* retinal degeneration has been observed in *Xenopus* using Fourier domain optical coherence tomography (FD-OCT) (Lee et al., 2010), making it an efficient model to see regeneration effects over a period. • *Xenopus* has a ciliary marginal zone (CMZ) in peripheral areas of the retina which contain stem cells and progenitor cells that contribute to developing larvae and adult eye (Ail & Perron, 2017). • Excellent model to study regeneration biology and environmental toxicology (*Introduction to Xenopus - Xenbase*, accessed on April 22, 2023. from https://www.xenbase.org/entry/anatomy/intro.do.). *Xenopus* embryos regrow full-sized eye within five-days of tissue removal (Kha et al., 2018).	makes translation of human pathophysiology complex and complicated. • Amphibians lack anatomical macula like human do. They also do not possess specialized intraretinal structures like fovea. In some frog species, a streak of high cell density exists instead of the cone-rich macula in human retina (Dicke & Roth, 2009). • Amphibians have four types of photoreceptors (*ARCHIVE - Amphibians - Comparative Physiology of Vision*, Accessed on 22 April, 2023, from https:// archives.evergreen.edu/webpages/curricular/2011-2012/m2o1112/web/amphibians.html; Dowling & Werblin, 1969); hence the color and light perception are different in them than those of human. This can be an important thing to consider while translating disease pathophysiology between amphibians and human. • They are nocturnal. It has been reported that *Xenopus*

		show light-induced retinal degeneration due to autophagy (Bogéa et al., 2015). Human retina is adaptive to different light conditions, and translating a human retinal event into an amphibian retina where the effects of light are so drastic possesses a big challenge. • Difficult to confirm whether an induced effect is an effect from regeneration point of view or a developmental point of view.

Chapter 2: Materials and Methods

2.1. Ethical Approval

BioSciences Animal Care and Use Committee at the University of Alberta (Animal Use Protocol number: AUP00000077) approved the work in this study, which adheres to the Canadian Council for the Animal Care Guidelines. Zebrafish were bred and maintained in the Science Animal Support Services (SASS) facility of the Biological Sciences.

2.2. Zebrafish Collection and Care

This study required both larval and adult zebrafish (*Danio rerio*). Embryos were harvested by both pair-mating of healthy adult male and female zebrafish, and in mixed-parentage technique to in-cross the same fish line producing large number of embryos from a maximum of five adult fish in a larger breeding tank. Fertilized eggs were collected, kept in petri-dishes and grown in E3 media (ZFIN Publication: Westerfield, 2000, n.d.). The plates were kept in 28ºC in an incubator. Media were changed every 24 hours until 6dpf age, afterwards larvae were moved to the nursery in the SASS facility and fed standardized food set for zebrafish husbandry.

2.3. Zebrafish Line

Zebrafish mutant line with homozygous *rp1l1*$^{-/-}$ has been used for the study, with allele designation ua5017 and ZFin ID ZDB-ALT-210506-4. The fish line was developed by Dr. Nicole Noel (Noel et al., 2020) by introducing a mutation in zebrafish homologue of 2394bp long *rp1l1* by using CRISPR/Cas9, generating 16bp deletion after nucleotide 139. This frameshift mutation is expected to lead to an extremely truncated Rp1l1 protein that

lacks all recognizable functional domains (Noel et al., 2020). Wild type (WT) zebrafish (AB strain) were bred and nurtured following the same protocols to maintain fish line and used for the experiments as control samples.

2.4. Cryopreservation and Cryosection

Fish were euthanized at different timepoints, within 11am-12pm on those days when cryopreservation process started. The time was kept constant for the purpose of photo adaptation, since environmental light may affect the changes in the Retinal Pigment Epithelium (RPE). Adult zebrafish eyeballs after enucleation and larvae up to 1mpf age were fixed in 4% Paraformaldehyde (PFA) with 3% sucrose in 0.1M phosphate buffer solution overnight at 4ºC. A series of washes followed with 12.5% and 20% sucrose in 0.1M phosphate buffer at room temperature and finally in 30% sucrose in 0.1M phosphate buffer overnight at 4ºC. On the following day, samples were placed for three hours in a solution of equal part 30% sucrose in 0.1M phosphate buffer and OCT (embedding compound; VWR® Premium Frozen Section Compound, Cat. No. 95057-838; referred as OCT hereafter) at 4ºC. After fixing a half of 1mL centrifuge tubes onto a glass slide with nail polish, they were filled with OCT solution and samples were flash frozen in it using dry ice. All the samples in respective OCT molds were stored in -80ºC temperature for at least 24-hour before they were sectioned and imaged. Each OCT mold with embedded samples were sectioned at 10µm thickness using the cryostat (Leica CM 1900 UV cryostat). The chamber temperature was maintained at -20ºC during the sectioning process. Ribbons of OCT molds were placed on Superfrost Plus microscope slides from

ThermoFisher Scientific, Cat. No. 12-550-15; and stored in -80ºC for at least 24 hours until used.

2.5. Oil-Red O and Hematoxylin Staining

Frozen sections on Superfrost Plus glass slides were thawed for at least an hour in room temperature, followed by a cycle of three eight minute-washes with PBS to remove the OCT compounds. Tissues were treated with 4% PFA for 10 minutes, washed with distilled water and 60% isopropyl alcohol until they were prepared for Oil-Red O (ORO) staining. ORO stock solution was prepared by dissolving 0.5g ORO powder into 100mL of isopropanol; and a 0.3% working solution was prepared by diluting the stock solution in 60% isopropanol. After filtering, the freshly prepared working solution was added to a Coplin jar and incubated with slides for one hour; and later rinsed with 60% isopropanol. Nuclei were stained with hematoxylin for one minute, and samples were rinsed with distilled water. Finally, samples were mounted in 80% glycerol, cover-slipped and sealed with nail polish. Images were obtained using a ZEISS AXIO A1 Compound Light Microscope (Zeiss, Toronto, ON, Canada) with a SeBaCam 5.1MP Camera (Laxco, Bothell, WA, USA).

2.6. Image Processing, Data Collection, and Statistical Analysis

Obtained images were processed as TIF files, and data were collected using ImageJ software (version 1.53t, NIH and LOCI, University of Wisconsin, US). To obtain the drusen size, ORO^{+} stained areas were selected individually, and their pixel area were converted to square micrometers. The width of RPE, ONL and INL were obtained by drawing a

vertical line at respective ORO^{+} location of the retinal cross sections, pixel length of the drawn lines was converted to square micrometer. All analyses were done using the converted μm^2 and μm measures from the retinal cross section images.

Analysis was done using GraphPad Prism (v9.5.1-528, San Diego, CA, CA, USA). One-way ANOVA parametric test, Kruskal-Wallis non-parametric test, and simple t-test were performed for the collected data, and to present the findings in a graphical format.

ORO and hematoxylin-stained retinal cross-sections from the *rp1l1*$^{-/-}$ zebrafish were examined. Once ORO+ drusen was detected in a section, consecutive eight sections were collected, imaged and measurements from the drusen deposits were obtained for analysis. Similar workflow was followed for WT-ORO+ drusen analysis.

For the analysis of Outer and Inner Nuclear Layer differences, five fish from *rp1l1*$^{-/-}$ and WT were randomly selected at each timepoint. Among all slides containing retinal cross sections from the selected five fish, 10 sections were randomly selected and measurements were collected for analysis.

2.7. Genotyping Sample Fish

To confirm that the experimental fish have homogenous mutant genotype, genomic DNA (gDNA) was collected from all the adult *rp1l1*$^{-/-}$ fish bred in the nursery and sequenced. Randomly collected 10 larvae were selected among the newly bred groups and their genotype was also analyzed to confirm the inheritance. Adult fin-clips and tail-clips, and juvenile tail-clips were collected after 1mpf of age to extract gDNA. Samples were put into 20-50μl of 50mM NaOH, boiled at 95ºC for 20 minutes, then incubated on ice. For

neutralizing, 1M Tris-HCl was added at the 1/10th of the NaOH volume after correcting its pH to 8.0. After subsequent centrifuging, supernatants were collected as template for PCR thermocycle. gDNA were saved at -20ºC until the thermocycle step for PCR.

Extracted gDNA was used as template to amplify a 479bp fragment of *rp1l1* using 5'-GGCTTTTTCGACGCTGATCC-3' as forward primer, and 5'-AATCCTTTTGGGGTGCCGAT-3' as reverse primer (Invitrogen, Waltham, MA, USA) as per previous genotyping (Noel et al., 2020). PCR Purification Kit (Qiagen, Hilden, Germany) was used to purify the amplified DNA for Sanger sequencing. Collected gDNA was amplified using the following PCR protocol – 40 cycles of 94ºC for 3 minutes, 94ºC for 1 minute, 58ºC for 30 seconds, and 72ºC for 1 minute; 72ºC for 10 minutes; and finally cooling down to 4ºC as the final step. To confirm the targeted construct in each sample, a gel electrophoresis was run for the PCR products. 1% agarose gel was prepared with EtBr, and 20µL 50bp DNA ladder were used, and electric current were run at 90volts for 45minutes. Afterwards DNA bands were visualized and imaged in the ultraviolet chamber of transilluminator.

2.8. Sequence Analysis

Sequencing results were collected via email from Molecular Biology Facility (MBSU), University of Alberta. Each sample was assigned with an identification. Sequencing data was visualized and analyzed using SnapGene Viewer software (SnapGene 6.2, 2023. Dotmatics, MA, USA). Sequence results from the samples were compared with the original sequence of *rp1l1*$^{-/-}$ introduced into zebrafish developed by Dr. Nicole Noel (Noel et al., 2020).

Chapter 3: Results

3.1. Location of Detectable Drusen in *rp1l1*$^{-/-}$ Retina

RP1L1 codes for a protein that is expressed in the axonemes of retinal photoreceptor (Yamashita et al., 2009). Dr. Nicole Noel from the Allison lab introduced a mutation in its zebrafish homolog and developed the homozygous *rp1l1*$^{-/-}$ fish line (Noel et al., 2020). Zebrafish from this mutant line manifest progressive photoreceptor dysfunction and disorganization of the outer segment of photoreceptors (Noel et al., 2020). Unexpectedly, in the retinal cross sections at 14mpf age, drusen deposits were serendipitously discovered. Since drusen development and progress have remained a mystery despite it being closely linked with Age-related Macular Degeneration (AMD), the *rp1l1*$^{-/-}$ fish line can be an excellent opportunity to explore into the molecular basis of the disease pathology.

Drusen or drusen-like deposits are identified by their characteristic red color of Oil-Red O (ORO); and the cell nuclei in the retinal layers are counter-stained by hematoxylin, thus making different layers distinguishable. Thus, making ORO^{+} drusen clearly visible. **Figure 3.1** shows sample retinal cross sections where drusen were detected in *rp1l1*$^{-/-}$ zebrafish.

Drusen in *rp1l1*$^{-/-}$ are located around the Retinal Pigment Epithelial (RPE) layer. The ORO+ droplets appear to have a defined area and distinguished margin, suggesting these share the morphology with hard drusen.

RPE, ONL and INL, which are the structures surrounding the ORO^{+} droplets were also observed. Experimental samples were chosen from different ages, and the changes in those structures are also monitored with those of the WT. These characteristics remain

constant from 55dpf when drusen are first detected, and throughout the entire timeline for the experiment. Therefore, supporting *rp1l1*$^{-/-}$ zebrafish exhibit changes in their retina that are homologous to the diseases that have already been linked with *RP1L1*, and detecting drusen in them can be explored into in relation with other retinal conditions.

10 WT and *rp1l1*$^{-/-}$ zebrafish were observed and analyzed from 10dpf age to 1ypf age. **Figures 3.2 – 3.3** are some examples of how the images were collected, and the different layers of retina were measured for the analyses discussed afterwards.

3.2. Detection of Drusen in *rp1l1*$^{-/-}$ Zebrafish

One of the purposes of this project was to ascertain the earliest age when drusen can be detected in the *rp1l1*$^{-/-}$ zebrafish retina. Zebrafish larvae bred from in-crossing adult *rp1l1*$^{-/-}$, were observed from 10dpf, and along the progression of age up to 1ypf - at 90dpf, 5mpf, 6mpf, 9mpf and 1ypf age. *RP1L1* has been linked with a number of inherited disorders such as rod and cone dystrophy which is likely to occur in childhood; maculopathies which are mostly age-related; and retinitis pigmentosa that usually appears within 10-35 years of age (Akahori et al., 2010; A. E. Davidson et al., 2013; Oishi et al., 2014). Since *RP1L1*-associated diseases have been known to manifest at different age, it is important to analyze the earliest age when *RP1L1*-associated drusen are detectable, and whether age is a factor for the development of drusen associated with it. Therefore, the first set of experiments was to determine the earliest age when drusen are detectable in *rp1l1*$^{-/-}$ zebrafish. Larval, juvenile and adult fish (staged as per (*ZFIN Zebrafish Developmental Stages*, Accessed on 22 April, 2023, https://zfin.org/

zf_info/zfbook/stages) bred from the homozygous *rp1l1*$^{-/-}$ were observed - 10 fish were selected from the mutants at each timepoint, while using 10 WT fish as controls.

In *rp1l1*$^{-/-}$ mutants drusen are detected as red lipid-rich droplets stained with ORO around the RPE (**Figures 3.1B, 3.2 and 3.3**). The earliest age when drusen are detected is 55dpf **(Figures 3.2D, 3.4A)**. Among the 10 *rp1l1*$^{-/-}$ fish observed, three show detectable drusen in one of the eyes of a single fish **(Figure 3.4C).** Mutant fish at every timepoint afterwards show detectable drusen **(Figures 3.2A and 3.2C)**. From 60dpf age, drusen are detectable bilaterally (in both eyes), and all *rp1l1*$^{-/-}$ fish by 6mpf age show detectable drusen **(Figure 3.4C).**

3.3. Detection of Drusen in WT Zebrafish

One of the most important findings from the experiment is detecting drusen in WT fish. Drusen is an age-related accumulation of lipid droplet. In the experiment, WT fish show detectable drusen at 9month-post fertilization (mpf) age, which was unilateral and in a single fish. At 1ypf age, four WT fish are detected with drusen and only one of them show it bilaterally **(Figures 3.3D and 3.2B)**. Selected WT fish did not show any significant phenotype changes or characteristics. ORO+ drusen were also observed in 4ypf WT (**Figure 3.8**).

Detectable drusen in older WT correlates with the age-dependent characteristics of drusen morphogenesis. At the same time *rp1l1*$^{-/-}$ fish show detectable drusen at noteworthy earlier age than when they appear in WT, suggesting that introducing *rp1l1*$^{-}$ into zebrafish genome resulted in early onset of drusen.

3.4. Progression of Drusen Size with Age in *rp1l1*$^{-/-}$ Zebrafish

Once the earliest timepoint has been established at 55dpf age in *rp1l1*$^{-/-}$ zebrafish to show detectable drusen-like deposits, progression of the changes associated with it were recorded. At 55dpf, *rp1l1*$^{-/-}$ drusen sizes are relatively small, around 10μm^2, and they increase with the progression of age **(Figure 3.5A).** Throughout the experimental timeline, drusen size in *rp1l1*$^{-/-}$ zebrafish remained <40μm^2. When compared to the WT drusen size separately at 9mpf and 1ypf ages, *rp1l1*$^{-/-}$ drusen appear to be much larger (**Figure 3.5A)**. WT-ORO+ drusen appear to be within 10-20μm^2 in both 9mpf and 1ypf zebrafish. Progression of the WT drusen also appears to be stable.

WT exhibit detectable drusen at only two ages during the experimental duration. More data is required to analyze the progression of WT-drusen size over long period.

These findings provide support that the *rp1l1*$^{-}$ is likely to be involved with altering the size of drusen as well as its course, and therefore, suggesting the *rp1l1*$^{-/-}$ zebrafish is a good candidate for *in vivo* study of drusen-associated AMD.

3.5. RPE-changes with Detectable Drusen

One of the characteristic changes that takes place in patient's retinal layers following the accumulation of lipid-rich drusen is detachment of the RPE away from Bruch's membrane (Cukras et al., 2010; Mrejen et al., 2013). It is likely due to the expansion with or without the merging of soft drusen (Balaratnasingam et al., 2016). Although drusen detected in *rp1l1*$^{-/-}$ zebrafish appear to have defined margin and distinct area **(Figures 3.1B, 3.2 and**

3.3), which are the characteristics of hard drusen. But the location of where they were detected may also suggest similarities with soft drusen location.

Although it is yet to be seen if the RPE changes are associated with *rp1l1*$^{-}$-drusen or due to the changes in the photoreceptors. Doucette et al, proposed that pathways related to lipid homeostasis and protein functions in scavenging apoptotic cells designated to remove the extracellular waste, are involved in the process of drusen formation (Doucette et al., 2021). That may be the link to connect the cause behind the RPE thinning in *rp1l1*$^{-/-}$. Imaging-histology correlation studies with clinical samples showed a varied range of RPE changes, both at structural and functional competency (K. C. Chen et al., 2016; Vogt et al., 2011). Therefore, RPE changes in *rp1l1*$^{-/-}$ necessitate exploring into when retinal layers show detectable drusen.

RPE width of the *rp1l1*$^{-/-}$ and WT zebrafish were individually analyzed **(Figure 3.6B)**. At the earlier timepoints, from 10dpf-50dpf ages, the difference between the width of RPE in WT and *rp1l1*$^{-/-}$ do not appear to be very significant. The ORO+ drusen were first detected in the *rp1l1*$^{-/-}$ zebrafish at 55dpf age, and from 55dpf-1ypf age, *rp1l1*$^{-/-}$ zebrafish show marked thinning of the RPE width compared to that of the WT using one-way ANOVA with 95% CI, post-hoc Kruskal-Wallis analysis was performed to determine the significant difference between the WT and *rp1l1*$^{-/-}$ zebrafish RPE width at each age in the experimental timeline.

The inference from the RPE width changes in WT fish can be that the drusen in *rp1l1*$^{-/-}$ is likely to have higher detrimental effects on the RPE structure and organization, however it requires more studies to connect if this is related to the drusen development or not.

The retinal, specifically RPE effects observed in homozygous *rp1l1*$^{-/-}$ strongly support the efficacy of zebrafish as a drusen-model since notable significant changes are evident, measurable, and comparable with those of WT samples.

Drusen area were compared with the width of the RPE layer in respective retinal cross sections from *rp1l1*$^{-/-}$, using WT as controls, in 10 samples at each timepoint **(Figures 3.6)**. At 55dpf age, when drusen were first detected in *rp1l1*$^{-/-}$ fish, the ratio between drusen area and the RPE width is very significant, in relation to the drop in the ratio afterwards. The drop of drusen:RPE is recorded up to 85dpf age, and then it increases progressively which appears to in somewhat stable manner **(Figure 3.6)**. WT drusen appearing at 6mpf and 1ypf age also show a similar pattern with a high drusen:RPE ratio initially followed by a drop. However, this change does not appear to be as drastic as the *rp1l1*$^{-/-}$ fish, and it requires further experiment to check the continuity of the trend.

3.6. Changes in the Outer Nuclear Layer (ONL) in *rp1l1*$^{-/-}$

Photoreceptors are specialized neurons where the initial step of vision takes place. They lie adjacent to the RPE and extend to the Outer Nuclear Layer (ONL) of retina (Mj, 1971). There are three parts of a photoreceptor **(Figure 1.4)** – outer segment (OS) to capture and convert light to electrical signals by phototransduction; inner segment (IS) containing cellular and metabolic machinery including nucleus; and the cilium connecting OS and IS (Molday & Moritz, 2015) where RP1L1 is expressed (Conte et al., 2003).

Following detection of drusen in *rp1l1*$^{-/-}$ zebrafish at an earlier age and comparing *rp1l1*$^{-/-}$ drusen development and morphology with those of WT, ONL width and the relative width between the ONL and INL were analyzed. The progression of WT ONL width appears to be progressive, likely as a developmental event. Comparing to that, the *rp1l1*$^{-/-}$ ONL width initially increases from 10dpf-60dpf age **(Figure 3.7A)**, and then decreases until 1ypf age. Although the INL does not overtly contain any part of photoreceptors, *rp1l1* is a specific gene in which, *rp1l1*$^{-/-}$ zebrafish are also observed to show thinning of the INL.

ONL:INL in the WT fish show a progressive and stable increase with age. In contrast, *rp1l1*$^{-/-}$ fish show a gradual increase between 10dpf-60dpf age, followed by progressive decrease at 90dpf which continued to 9mpf and 1ypf. **Figures 3.7C** shows the striking difference between the WT and *rp1l1*$^{-/-}$ ONL:INL progression with age.

This is a very important finding because previous studies have shown a strong relation between the appearance of drusen and RPE changes; reasons behind it are likely to be accumulation and coalescence of soft drusen (Balaratnasingam et al., 2016; K. C. Chen

et al., 2016; A. E. Davidson et al., 2013). *rp1l1*$^{-/-}$ zebrafish drusen also show a change in the normal RPE structures. At the same time, ONL where photoreceptor OS extends to, is significantly altered.

The width of ONL and INL, and their comparative progression in *rp1l1*$^{-/-}$ zebrafish with age **(Figures 3.7)** can also be interpreted that *rp1l1*$^{-}$ may have the aptitude to bring changes in the overall retinal morphology. Although it is difficult to conclude whether these changes are related to drusen development without further studies. And *rp1l1*$^{-/-}$ zebrafish gives the opportunity to explore even more drusen-associated retinal changes in AMD.

3.7. Detection of Drusen in 4ypf Zebrafish

Three *rp1l1*$^{-/-}$ and three WT eyeballs from four-year-old zebrafish were sectioned and observed following ORO and hematoxylin staining **(Figure 3.8)**. There were numerous, visibly larger and more dispersed drusen detected in all *rp1l1*$^{-/-}$ samples. All WT fish had ORO+ drusen in them as well, suggesting that WT drusen also persist for a long time. These finding strongly suggests the relatability of age-associated drusen in zebrafish, making this a suitable animal model for drusen research.

Data from those fish were not included for analysis, because the gap from the oldest fish in the experimental timeline (1ypf) and 4ypf is rather long. It is necessary to continue monitoring zebrafish drusen, both in WT and in *rp1l1*$^{-/-}$, and drusen-associated changes consecutively in order to relate them with human drusen pathophysiology.

Figures for Chapter 3

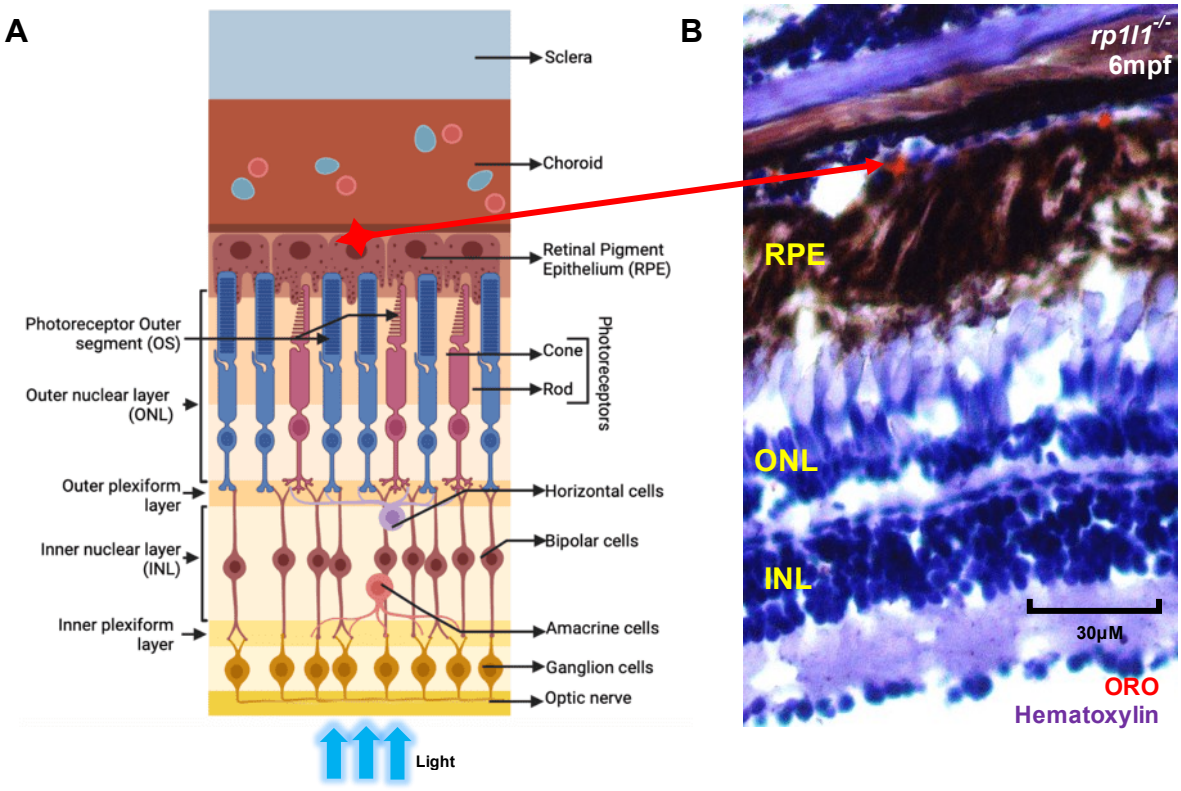

Figure 3.1. Detectable drusen in zebrafish eye. A shows the schematic presentation of the location of drusen using a red star on the diagram of retinal layers prepared in Biorender.com and **B** shows a retinal cryosection of 6-month post fertilization (mpf) *rp1l1*$^{-/-}$ zebrafish eye, stained using Oil-Red O (ORO) and hematoxylin. ORO was used to stain the lipid-rich drusen deposits, and hematoxylin was used to differentiate the retinal layers. The dark brown areas are the Retinal Pigment Epithelium (RPE). Arrow extending to **B** is directing to a detected drusen in *rp1l1*$^{-/-}$ sample. Retinal layers – RPE, Outer Nuclear Layer (ONL) and Inner Nuclear Layer (INL) measured for analyses are also labelled in **B**.

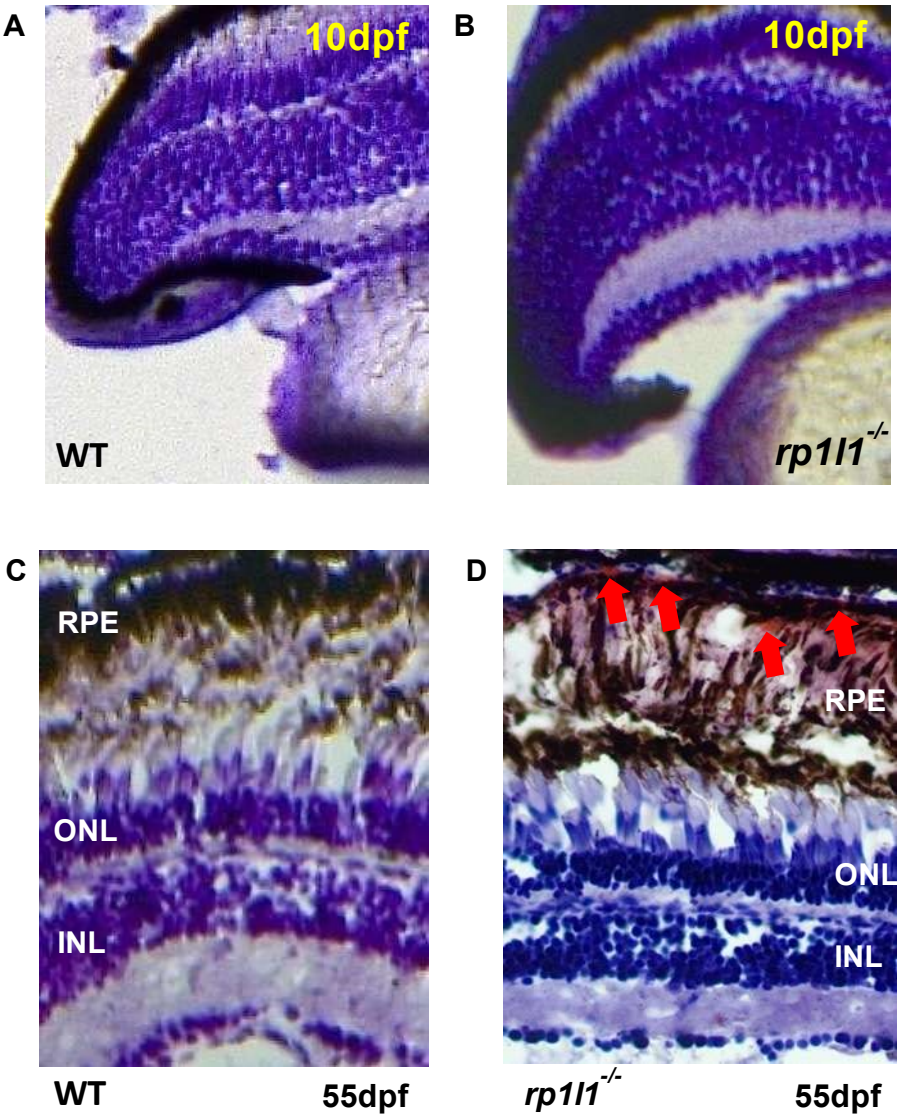

Figure 3.2. Hematoxylin and ORO-stained retinal cross sections of 10-days post fertilization (dpf) and 55dpf zebrafish. A and **B** show Wild Type (WT) and *rp1l1*$^{-/-}$ retinal layers respectively, at 10dpf; without much visible difference between them. **C** and **D** are WT and *rp1l1*$^{-/-}$ retinal cross sections, at 55dpf –the earliest age when drusen is detected in the mutant. Retinal cross section presented in **D** is from one of the three *rp1l1*$^{-/-}$ fish exhibiting ORO+ drusen in unilateral eye. Red arrows in **D** are pointing toward the ORO+ drusen.

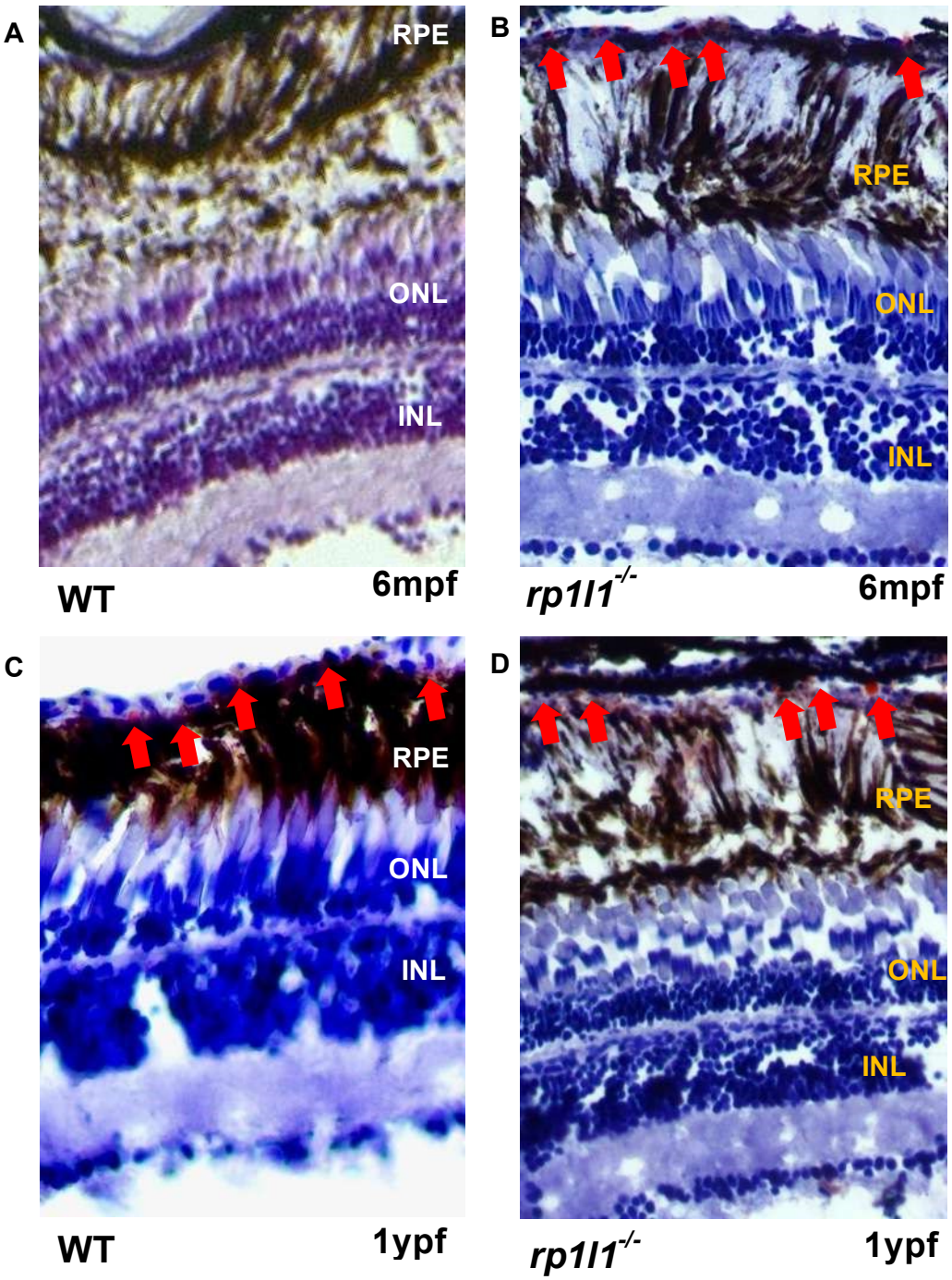

Figure 3.3. Hematoxylin and ORO-stained retinal cross sections of 6mpf and 1-year post fertilization/ypf zebrafish. A and **B** show Wild Type (WT) and *rp1l1*$^{-/-}$ retinal layers respectively, at 6mpf. *rp1l1*$^{-/-}$ sample shows ORO+ drusen, pointed by the red arrows. **C**

and **D** are WT and *rp1l1*$^{-/-}$ retinal cross sections, at 1ypf. Retinal cross section presented in **C** is from one of the three WT fish exhibiting ORO+ drusen. Red arrows in **C** and **D** are pointing toward the ORO+ drusen.

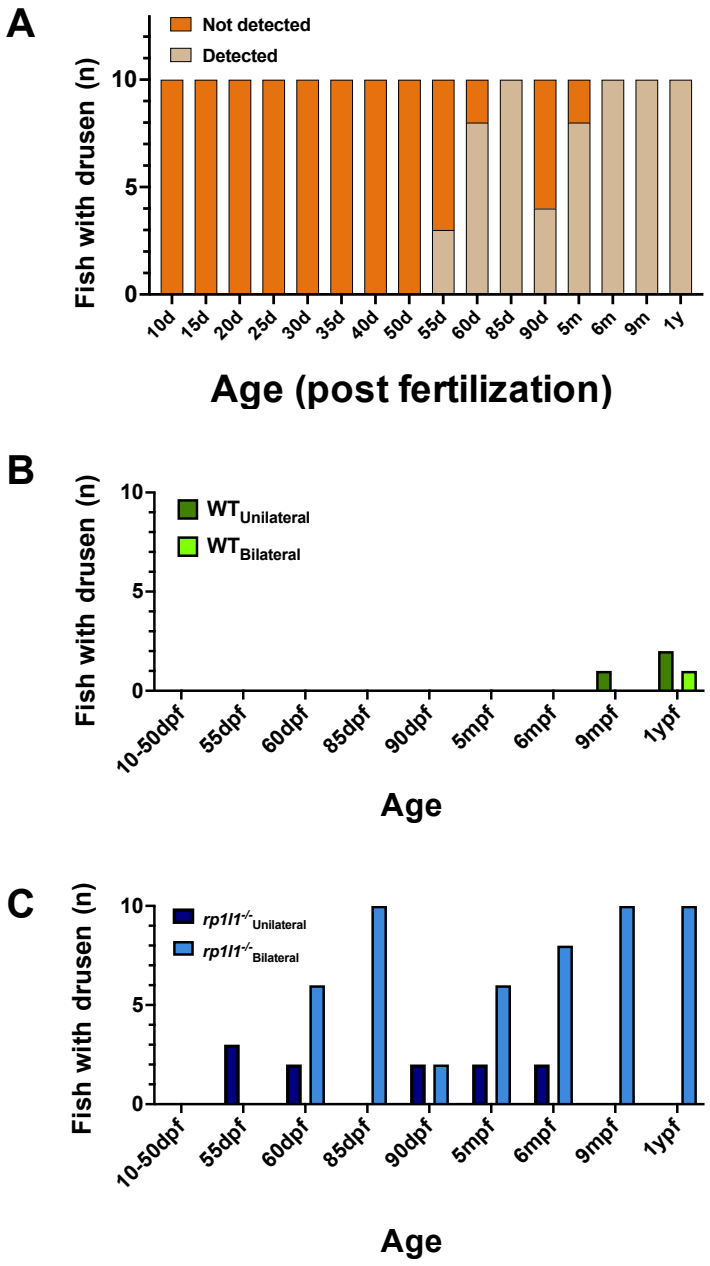

Figure 3.4. Progressive accumulation of drusen with age in zebrafish. **A** shows the number of *rp1l1*$^{-/-}$ fish among the samples that had detectable drusen over the experimental timeline. Earliest age when drusen were detected is at 55dpf. With the progression of age, more samples have detectable drusen in their retinas. **B** shows the

number of WT fish at each time-point that exhibited detectable drusen. From 10dpf-6-month post fertilization (mpf), WT fish didn't have detectable drusen. At 9mpf age, one WT fish showed detectable drusen in unilateral eye. And at 1ypf, three WT fish had drusen, among them only one had them in both eyes. **C** shows the presentation of detectable drusen in *rp1l1*$^{-/-}$ fish (same data as panel **A**). At 55dpf, unilateral eye was affected among the samples. Afterwards, bilaterally affected eyes were observed, and at 9mpf-1ypf age groups all the sample had bilaterally affected eyes.

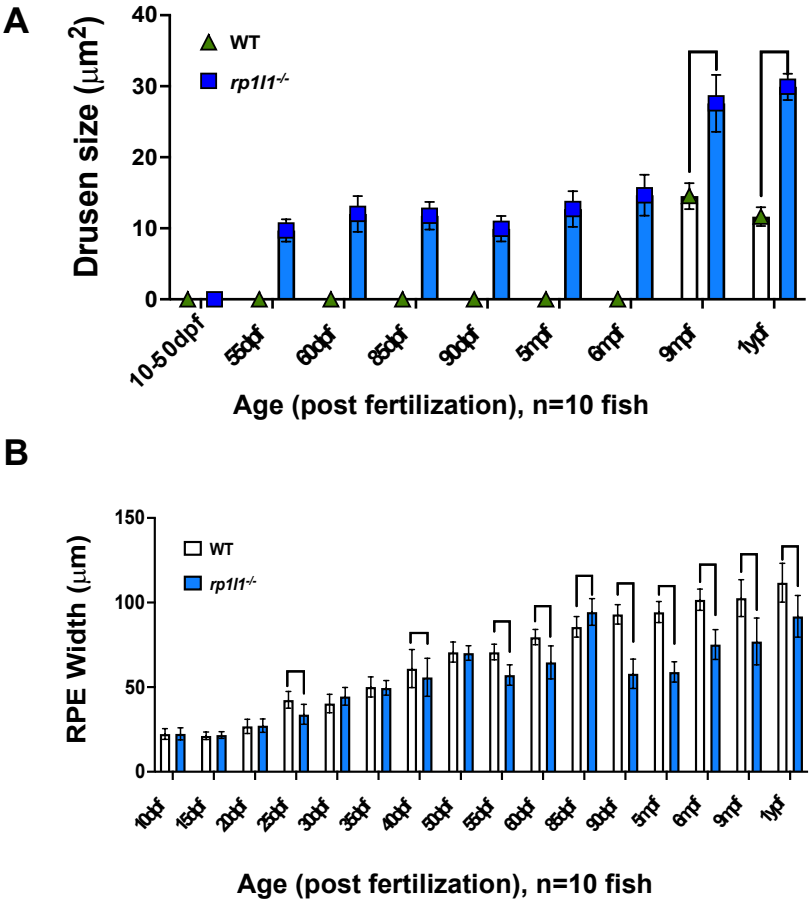

Figure 3.5. Progression and comparison of detectable drusen size in Wild Type (WT) and *rp1l1*$^{-/-}$ zebrafish with their respective Retinal Pigment Epithelium (RPE) width. A shows the progression of drusen size in *rp1l1*$^{-/-}$ and wildtype (WT) zebrafish with age. After the earliest detection of drusen at 55dpf, *rp1l1*$^{-/-}$ ORO+ drusen area gradually increases. WT drusen are detected at 9mpf age, but they appear to be smaller in 1ypf samples. After the earliest detection of drusen at 55dpf, ORO+ drusen area measuring approximately 10μm^2, drusen size shows progressive enlargement. Still during the experimental timeline ORO+ area remains <40μm^2. When compared to the WT detectable drusen-size at 9mpf-1ypf, *rp1l1*$^{-/-}$ drusen sizes are much larger, and their progression is also drastic. WT-ORO+ drusen appear to be within 10-20μm^2 in both 9mpf

and 1ypf zebrafish. Comparison of drusen size within *rp1l1*$^{-/-}$ show significance difference for earlier ages with 9mpf and 1ypf fish (using two-way ANOVA, 95% CI calculated assuming each drusen is a statistically independent entity). Comparing WT drusen size with *rp1l1*$^{-/-}$ drusen size at 9mpf (*, P-value <0.03, 95% CI) and 1ypf (**, P-value <0.002, 95% CI) also show significance differences between them. **B** shows RPE width was examined to compare the changes that appear with the progression of age in *rp1l1*$^{-/-}$ zebrafish and to explore the impact of ORO+ drusen on RPE. The RPE width in both fish groups appear to be similar in the earlier timepoints. *rp1l1*$^{-/-}$ zebrafish were detected with ORO+ drusen from 55dpf age. And the difference between the RPE width of the mutant and WT fish are significantly different in all ages after 55dpf (using one-way ANOVA with 95% CI, post-hoc Kruskal-Wallis analysis was performed to determine the significant difference between the WT and *rp1l1*$^{-/-}$ zebrafish RPE width at each age in the experimental timeline).

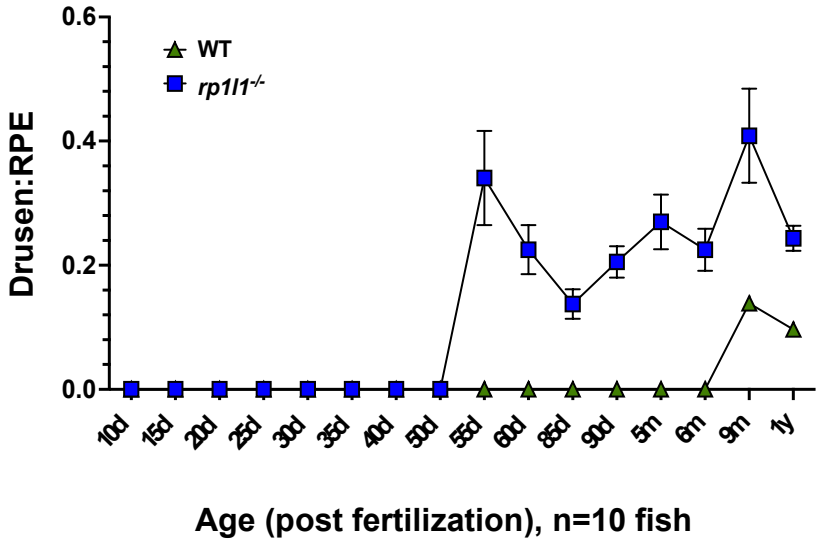

Figure 3.6. Comparison of the ORO+ drusen size and the Retinal Pigment Epithelium (RPE) width in Wild Type (WT) and *rp1l1*$^{-/-}$ zebrafish. The drusen sizes were normalized against Retinal Pigment Epithelium (RPE) size to reduce any potential artefacts of sectioning angle.

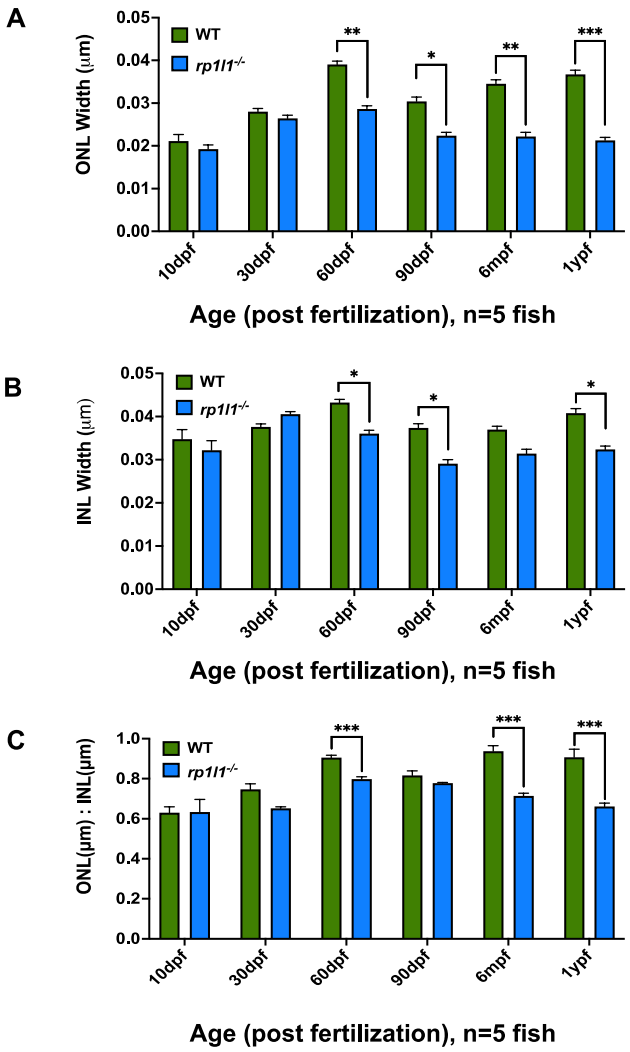

Figure 3.7. Comparison among the width of Outer Nuclear Layer (ONL) and Inner Nuclear Layer (INL) in WT and rp1l1-/- zebrafish. A shows the progression of the width of Outer Nuclear Layer (ONL) in WT and *rp1l1*$^{-/-}$ zebrafish. ONL is the layers where the photoreceptor Outer Segments (OS) extend to. Significant decrease in the ONL length in the mutant zebrafish can be explained as a result of the mutation in the gene that is essential for photoreceptor structural integrity (using one-way ANOVA with 95% CI, post-hoc Kruskal-Wallis analysis was performed to determine the significant difference

between the WT and *rp1l1*$^{-/-}$ zebrafish ONL width at each age in the experimental timeline). **B** shows the comparison among the width of the Inner Nuclear Layer (INL) in WT and *rp1l1*$^{-/-}$ zebrafish. Decreased width of INL in *rp1l1*$^{-/-}$ compared to that of WT is visible, however, it is not as marked as the ONL width decrease between these groups of fish. **C** shows the ratio between the Outer Nuclear Layer (ONL) and Inner Nuclear Layer (INL) is significantly altered in *rp1l1*$^{-/-}$ fish comparing to those of the WT. This suggests that there is some loss in morphological integrity of functional retinal structures in the *rp1l1*$^{-/-}$, which is otherwise intact in WT. Statistical analysis shows the difference is significant between the *rp1l1*$^{-/-}$ and the WT zebrafish (using one-way ANOVA with 95% CI, post-hoc Kruskal-Wallis analysis was performed to determine the significant difference between the WT and *rp1l1*$^{-/-}$ zebrafish INL width at each age in the experimental timeline). **C** shows the progression of the ONL:INL (both measured in μm) within the retinal layers. Progression in WT with age is gradual and stable, whereas *rp1l1*$^{-/-}$ characteristic suggests a radical decrease as age progresses.

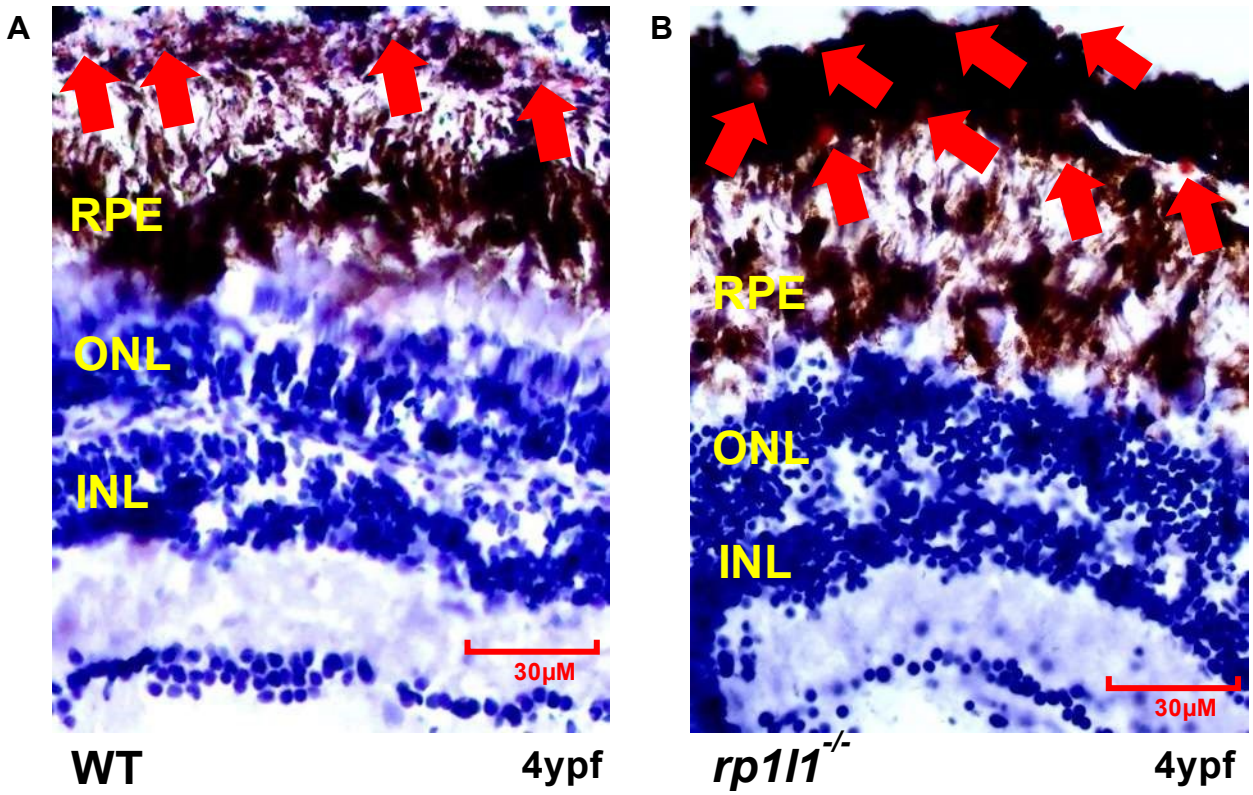

Figure 3.8. Hematoxylin and ORO-stained retinal cross sections from 4ypf zebrafish. **A** shows ORO and hematoxylin-stained retinal cross section of a WT zebrafish. Three WT fish were observed at 4ypf age, to show the comparison with three *rp1l1*$^{-/-}$ fish of same age. All WT fish had detectable ORO+ drusen, eyes were affected bilaterally**. B** shows a 4ypf *rp1l1*$^{-/-}$ retinal cross section with significant number of ORO+ drusen. Data from these observations were not included for analysis since the last age of the experimental timeline is 1ypf, and there is a significant gap in the age between 1ypf fish and these 4ypf fish. Consecutive analysis is required to connect and correlate ORO+ drusen and associated findings. However, detecting ORO+ drusen in both WT and *rp1l1*$^{-/-}$ fish suggest that the novel mutant line shows age-related characteristic of drusen, which are present in human – appearance of drusen in otherwise normal but elderly patient samples.

Chapter 4: Discussion

4.1. Necessities of Novel Animal Models for Drusen Study

Age-related Macular Degeneration (AMD) is a complex disease, with polygenic influences and contributions of several environmental factors (*Age-Related Macular Degeneration (AMD)*, 2021). It is true that influences of age and genetics are almost impossible to alter and modify. Detection of drusen in younger (<49 years) patient retinal samples (Silvestri et al., 2005) indicates that there may be other mechanism such as genetic mutations and other molecular pathogenesis which are involved in the process of drusen formation.

Drusen has always been considered an enigma for both biologists and clinicians. It is a hallmark of AMD, and various groups of scientists have been relentlessly exploring into the pathophysiology of drusen. There are several ideas shared by drusen-researchers, and experiments are also being conducted to validate, update and modify them every day. Patient sample analysis and *in vitro* studies are hardly sufficient to understand a complex human disease like AMD that has severe consequences like visual impairment. Therefore, several animal models are being used for *in vivo* research.

Translating a human disease into an animal model is always hard. There is strong genetic similarity between human genome with the genomes of currently established model animals. Rodents, rabbits, murine, non-human primates (NHP), amphibians and zebrafish are a few of the animal models that have their own benefits and successes in the scientist community. Nonetheless, human biology always has structural, functional, and physiological differences with the biology of other animals. We must always consider this while studying a disease animal model. Studying a disease using multiple animal models support the findings from each other. Results from more than one animal models

are advantageous and valuable to decide best possible pathophysiological explanation reflecting the events taking place in human cells and to decide the best therapeutics which should be adapted to human.

4.2. Zebrafish as a Novel Candidate for Drusen Studies

Zebrafish has been an established animal model to study human pathology since 1981 (Streisinger et al., 1981). There is a lot of similarity between zebrafish and human genome. Zebrafish genome project was undertaken in 2001, and comparative analysis between human and zebrafish genome was published in 2013 (Howe et al., 2013). Zebrafish embryos are transparent, and they fertilize externally, which makes applying genetic manipulation techniques such as microinjections, continuous imaging and manipulating specific cell lineage much easier than several other vertebrate models like rodents, rabbits and murine. Short generation time is another excellent advantage of zebrafish that has made experiments with numerous samples possible within a very short time. The expense to breed and maintain zebrafish in a laboratory setting is relatively cheaper than mammalian animal models.

Zebrafish visual system also shares numerous similarities with human visual systems, which have been discussed in detail in section 1.4.1. Although there are many zebrafish lines that are being used for several human pathophysiology, AMD studies have not been successful using zebrafish yet. Using zebrafish can give the scientist community a considerable advantage because most of the established drusen-models are either nocturnal, or they lack macula – the cone-rich area of human retina where drusen appear commonly.

4.3. Zebrafish Line with Homozygous *rp1l1*$^{-/-}$ Mutation

A new zebrafish line with a mutation in *rp1l1* has been developed by Dr. Nicole Noel in Allison lab (Noel et al., 2020). This novel mutant line has efficiently exhibited retinitis pigmentosa pathophysiology. Additionally, the presence of ORO+ drusen in 14mpf fish has opened the scope to consider *rp1l1*$^{-/-}$ mutant fish as a candidate to study drusen. This is the first zebrafish line that exhibits drusen, a hallmark of AMD. The objective has been to analyze the *rp1l1*$^{-/-}$ drusen characteristics and learn whether this novel mutant zebrafish line can be studied to understand drusen.

4.4. Detectable Drusen in *rp1l1*$^{-/-}$ Zebrafish

Drusen is the accumulation of debris beneath RPE. It consists of mostly lipid. Drusen detected in *rp1l1*$^{-/-}$ zebrafish are stained by Oil Red O (ORO) shown in **Figures 3.1B, 3.2, 3.3, and 3.8.** ORO is a specific lysochrome that selectivity stains lipid. Detecting red droplets in *rp1l1*$^{-/-}$ fish strongly supports that their constituent is lipid. Comparing the histological images with other reports where drusen has been detected by ORO staining (Curcio, 2018; Haimovici et al., 2001; Huang et al., 2017; Noel et al., 2020) is also supporting the hypothesis that they are likely to be drusen.

Drusen were detected in 55dpf old *rp1l1*$^{-/-}$ fish and were detectable throughout the whole duration of the experimental timeline afterwards. At this age, drusen were detected unilaterally in three mutant fish. From 60dpf, both eyes of *rp1l1*$^{-/-}$ fish started exhibiting detectable drusen; and each group of *rp1l1*$^{-/-}$ afterwards had bilaterally affected eyes. To

summarize, *rp1l1*$^{-/-}$ zebrafish eyes have shown gradually increasing impact of drusen with age, supported by the observation of older fish exhibiting drusen bilaterally.

4.5. Detectable Drusen in WT Zebrafish

WT zebrafish were used as control samples at every experimental step with *rp1l1*$^{-/-}$ fish. Initially our expectation was to compare the retinal layers of the WT fish with that of the *rp1l1*$^{-/-}$ fish. But the results show a very curious finding when drusen were detected in one WT fish of 9mpf age. The fish exhibited ORO+ drusen in unilateral eye. Drusen is considered a biomarker for AMD because they usually appear in older age. Detecting drusen in a relatively older WT fish support that zebrafish exhibit drusen and introducing *rp1l1*$^{-}$ into zebrafish genome resulted in early onset of it. This is a strong support for our effort to establish *rp1l1*$^{-/-}$ zebrafish line as a suitable candidate to study drusen. WT fish also exhibited drusen at 1ypf age, suggesting that the presence of detectable drusen in 9mpf WT fish is unlikely a technical error, cryosection or staining procedure artefact. In 1ypf WT fish drusen were detected in both eyes, supporting that progression of age can increase drusen pathology in WT fish as well.

4.6. Drusen Progression with Age in *rp1l1*$^{-/-}$ Zebrafish

Clinical prevalence studies have shown that drusen size increases with age (Jonasson et al., 2011; Silvestri et al., 2005). ORO+ drusen in *rp1l1*$^{-/-}$ zebrafish were analyzed based on their size at each timepoint in the experiment. The size of drusen detected in *rp1l1*$^{-/-}$ zebrafish gradually increased with age (**Figure 3.5A**). Rp1l1 is expressed in the connecting cilium of the photoreceptors and actively takes part in the morphogenesis of

Outer Segment (OS). Although zebrafish retina has an effective regeneration system (Hitchcock & Raymond, 2004), it appears that the *rp1l1*$^{-/-}$ photoreceptors do not share the benefit. This may also indicate that a large part of the drusen component originate from the degraded photoreceptor parts; and the imbalance between lipid homeostatic and insufficient apoptotic cell waste removal, which have been hypothesized to be one of the mechanisms behind drusen accumulation by (Doucette et al., 2021) are the causes behind gradual increases of drusen size with age. However, more studies are required to link these two events with drusen formation, and with each other.

4.7. RPE Changes in *rp1l1*$^{-/-}$ Zebrafish

AMD is commonly associated with thinning of the RPE layer of retina (Somasundaran et al., 2020; Zekavat et al., 2022). RPE is the supporting layer providing nutrition to the photoreceptors. Degradation of dysregulation of RPE is another important hallmark of AMD beside drusen. Although some RPE changes are observed as a part of aging process in retina, AMD changes are more severe and significant (Gao & Hollyfield, 1992). Other AMD-related RPE changes include alteration of cellular shape, reduction of melanosome, and accumulation of lipofuscin (Eldred & Katz, 1988). It was proposed that the changes take place due the loss of photoreceptor cells and thickening of Bruch's membrane (Ugarte et al., 2006).

Experimental samples show significant thinning of RPE in the mutant group **(Figure 3.5B)**. Drusen area in *rp1l1*$^{-/-}$ zebrafish and their corresponding RPE width were compared over the course of time in the experiment. Earlier timepoint, 55dpf age *rp1l1*$^{-/-}$ drusen size appears to be larger in relation to the RPE width (**Figure 3.6**). As age

progressed drusen:RPE creates the impression that the difference between drusen area and RPE is decreasing.

This result can be interpreted as an associated event of photoreceptor degradation because of the mutation in *rp1l1*. As age progresses, degradation products from photoreceptor gradually accumulate and the cellular waste excretion mechanism may overwhelm. Therefore, RPE is provoked with the effects of a vicious cycle of more apoptotic waste depositing alongside normal physiological effects of aging. This proposition is also supported by the decrease of the drusen:RPE in WT fish from 9mpf to 1ypf age (**Figure 3.6**). Nonetheless, more experiments, directing to RPE changes must be conducted to connect the changes with drusen formation. It is also an interesting foundation to investigate whether RPE changes are because of drusen, they are actually contributing to formation of drusen, and their interdependency.

4.8. Outer Nuclear Layer (ONL) and Inner Nuclear Layer (INL) Changes in *rp1l1*$^{-/-}$ Zebrafish

Photoreceptor cells lie on RPE and their OS contribute to the ONL of retina (Curcio et al., 1990; Mahabadi & Al Khalili, 2023; *Webvision: Simple Anatomy of the Retina*, Accessed on 22 April, 2023, from https:// web.archive.org/ web/20110315083230/ http:// webvision .med.utah.edu/sretina.html). Since Rp1l1 is expressed in the connecting cilium between the OS and IS of photoreceptors, mutation in *rp1l1* disrupts the structural integrity of rod and cone cells (Noel et al., 2020). To test the fourth hypothesis, the width of *rp1l1*$^{-/-}$ ONL was measured and analyzed in comparison with the INL width over time.

As age progressed, WT zebrafish ONL width showed a progressive and stable increase in width (**Figure 3.7A**), which is likely to be a normal growth and development with age. On the other hand, *rp1l1*$^{-/-}$ ONL show an increase in the earlier ages (up to 60dpf) in experimental timeline, and then a gradual drop in ONL persists until 1ypf. This suggests that ONL, containing the photoreceptor OS, is affected in *rp1l1*$^{-/-}$ zebrafish. Width of ONL was also compared with INL width of respective fish (**Figure 3.7C**); as well as ONL and INL width were compared between the WT and rp1l1-/- group individually **(Figures 3.7A and B)**. Decrease in the ONL:INL ratio at 90dpf and afterwards suggest that the reduction of the ONL width is also associated with some level of shortening of INL. This illustrates that although *rp1l1*$^{-}$ affects photoreceptor, and in turn alter ONL morphology, it may have the potential to influence other retinal layers as well. Although the INL does not overtly contain any part of photoreceptors, *rp1l1* is a specific gene in which, *rp1l1*$^{-/-}$ zebrafish are also observed to show thinning of the INL; which can be an interesting avenue to explore as an influence of drusen formation.

RPE thinning and changes have already been linked with AMD, although the pathophysiology behind it is not determined exclusively. Novel *rp1l1*$^{-/-}$ zebrafish exhibits appearance of detectable drusen, alongside change in RPE and change in ONL, suggesting the mutant fish line have the potential to be an excellent candidate to analyze AMD and drusen's effect on multiple retinal structures and to determine the connection amongst them.

4.9. Drusen in Old Zebrafish

Although the data were not included for analysis, three *rp1l1*$^{-/-}$ and three WT fish were observed at 4ypf age. This is nearing the age of senescence for zebrafish. All showed ORO+ drusen **(Figure 3.8),** and eyes were affected bilaterally. Number of drusen appeared to be more on visible inspection in *rp1l1*$^{-/-}$ samples, compared to WT. However, these findings are merely a qualitative observation. Oldest zebrafish that were used in the experimental timeline were 1ypf age. It is a significantly large gap between the age of experimental samples and these older fish of 4ypf. To correlate and compare experimental findings, consecutive observation is essential.

Presence of ORO+ drusen in both WT and *rp1l1*$^{-/-}$ zebrafish of 4ypf age is very significant, because this shows that WT fish also develop drusen with age and they persist for a long time. Drusen is an important biomarker of AMD, however, its presence does not conclusively diagnose AMD. Samples collected from elderly, but of otherwise normal eyes, also show presence of drusen. Zebrafish exhibiting similar pathophysiology (presence of ORO+ drusen in older WT fish) strongly supports the competency of zebrafish as an animal model to study drusen etiology.

4.10. Future Direction

The experiments in this project were planned centered around ORO+ drusen in *rp1l1*$^{-/-}$. This is just an initial step to establish the novel *rp1l1*$^{-/-}$ zebrafish as an animal model to study drusen. More research is required to justify and demonstrate effective drusen-related changes in this fish line.

One of the future experiments must be measuring the RPE width using Optical Coherence Topography (OCT), thus determining specific and significant changes in RPE driven by the *rp1l1*$^{-}$. This will also indicate specific changes in RPE in the exact location where drusen are detected.

If *rp1l1*$^{-}$ is indeed a causal factor behind drusen formation, then it is expected that introducing the mutation into other ocular model of zebrafish will show an early onset of detectable drusen in their eyes. If similar events are observed in other zebrafish line, then it will be a strong support to establish connection between photoreceptor degradation and drusen development.

Rp1l1 is a retina specific protein, and it is expressed in light-sensitive photoreceptors. Keeping *rp1l1*$^{-/-}$ zebrafish in constant dark and lit conditions will be another dynamic approach to determine the relationship between photoreceptors and drusen, which is yet to be explored into.

If rescuing the *rp1l1*$^{-}$ reduces detectable drusen, in number or in size, it will also provide stronger support to understand the pathogenesis of drusen formation by linking a photoreceptor gene with AMD pathogenesis.

It will also be interesting to explore whether drusen-sensitive *rp1l1*$^{-/-}$ zebrafish exhibit any change in its drusen pathophysiology when they are kept in medication-enriched environment. Few of the medications that can be used are anti-cholesterol medicines and drusen contains large concentration of lipid, and common medicines used for ciliopathies.

Chapter 5: Conclusion

The project aimed at determining the support for establishing novel homozygous *rp1l1*$^{-/-}$ zebrafish as an animal model to study drusen. A fortuitous discovery of ORO+ drusen in this novel zebrafish line provided the scope to study drusen and changes associated with it using zebrafish. Zebrafish are being used for numerous research into human developmental biology and disease pathophysiology. There are zebrafish models to study ocular conditions, however it has not yet been used for drusen and AMD studies.

This thesis discussed the benefits of using zebrafish to understand AMD and drusen morphology, over other current animal models of rodents, rabbits, non-human primates and amphibians. The intention is not to invalidate the contributions of other animal models, but to show the advantages of using zebrafish parallelly to understand drusen, which is still a mystery to the scientific community.

Establishing a novel mutant model to study a disease requires a number of trials and careful comparison of its findings with the disease pathophysiology and outcomes. It also requires comparison of its findings with other animal models. Homozygous *rp1l1*$^{-/-}$ zebrafish necessitates more research to understand the effects of its mutation, and only then we can confidently compare its efficacy for drusen studies. Nevertheless, the results reported here provide a strong foundation to establish a baseline of its usefulness, practicality, and applicability for drusen research.

The results have shown lipid-rich detectable drusen in *rp1l1*$^{-/-}$ zebrafish, compared its ORO+ drusen characteristics with those of the WT ORO+ drusen, recorded the progression of ORO+ drusen size over time, analyzed two vital retinal layers – RPE and ONL changes. It has also shown the appearance of detectable drusen WT zebrafish when

the reach adulthood and at 4ypf age. Based on these results, the next course of action is laid out so more support can be gathered to validate *rp1l1*$^{-/-}$ zebrafish to be explored into for understanding drusen in a more comprehensive and effective way.

Age-related Macular Degeneration (AMD) can significantly impact overall health and well-being of individuals, communities, and nations. There is potential to gain a deeper understanding of AMD and prevent it in the future by using novel *rp1l1*$^{-/-}$ zebrafish. This project has established the groundwork for the continuation of drusen-research to achieving this goal in near future.

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Chapter 6: Appendix

Post-Traumatic Brain Injury and Neural Hyperactivity Measured using Calcium-modulated Photoactivatable Ratiometric Integrator (CaMPARI)

Abstract

Traumatic brain injury (TBI) is a major public health concern affecting people worldwide. One of the most significant complications of TBI is post-traumatic epilepsy (PTE), which is characterized by recurrent seizures that can occur months or even years after the initial injury. PTE can have a significant impact on the individual's quality of life and can be difficult to treat. Researchers are constantly trying to look for new ways to better understand PTE and develop effective treatments. One of the reasons why we still know not much about PTE is because it is hard to determine the mechanisms of how brain reacts in and after a TBI event.

CaMPARI is a relatively new imaging technique that allows researchers to visualize and track changes in neural activity in response to specific stimuli. It works by using a fluorescent protein that changes color in response to changes in calcium levels within the neuron. This allows researchers to track the activity of individual neurons over time and identify patterns of activity that may be associated with the development of PTE.

One of the major advantages of the CaMPARI technique is that it can be used to study neural activities in unrestrained animals, which, in turn, can provide valuable insights into the underlying mechanisms of PTE by explaining the changes that occur immediately after TBI, and how much of that persists. Researchers can use this information to develop new treatments such as deep brain stimulation or targeted drug therapies.

The project used zebrafish stable line expressing the CaMPARI protein and attempted to measure neural hyperactivity that followed delivery of TBI to these fish in a controlled environment. The CaMPARI measurement under this project provides a preliminary

report of an increase in post-TBI neural activity in a single group of zebrafish. It also shows the need for proper optimization of a highly sensitive technique like CaMPARI.

A new technique requires numerous testing and optimization. Although in a relatively small group, CaMPARI detection of post-TBI neural hyperactivity provides a promising dynamic scope to understand the mechanisms behind PTE, and hopefully to determine an effective approach to prevent them.

Chapter 6.1: Introduction

6.1.1. Traumatic Brain Injury (TBI)

Traumatic brain injury or TBI is an umbrella term used to refer to a varying range of physical insults to the brain starting from a simple blow to the head to a penetrating injury (Georges & M Das, 2023). TBI is one of the conditions that has been causing a heavy burden in the global population leading to hospitalization and death. It has also been identified as the “silent epidemic”, contributing to death and disability, more than any other traumatic insults (Dewan et al., 2018). TBI not only has an acute, often severe outcomes, but also leads to continuous chronic irreversible consequences resulting in physical and cognitive disabilities, long-term morbidities and reduced life expectancy (Bergeson et al., 2004; Guenthner et al., 2013; Levin et al., 1987; Masel & DeWitt, 2010). Changes followed by a traumatic event continue to persist for a long time even after cessation of the direct force applied to brain (Bramlett & Dietrich, 2015), and these insults cause various secondary conditions including seizures, sleep disturbances, neurodegenerative disorders and even psychiatric problems (Annegers et al., 1998; Masel et al., 2001; McLean et al., 1984).

6.1.2. Post-TBI Seizure

TBI possesses not only an immediate danger but also exerts long-term effects that impact life of any individual. There are several conditions that have been linked with TBI, the effects of which are difficult to manage. Cognitive impairment like normal thinking and memory; diminished vision, speech and balance; emotional changes like anxiety, irritability and depression; and sleep disturbance may significantly recede the quality of life (CDC, 2019). However, a major concern is the post-TBI epilepsy. Brain undergoes a

cascade of changes as soon as it experiences an injury, which are recorded by electroencephalography (EEG) (S. Schmitt & Dichter, 2015), and seizure prophylaxis are administered immediately (Gabriel & Rowe, 2014). This has shown some benefit in preventing single incidence of seizure attack, but little to no effect on seizure events occurring after a week following the injury (Lucke-Wold et al., 2015). Not all individuals with TBI develop post traumatic seizure (PTS), and one of the reasons for this can be explained by genetic variation (Ding et al., 2016). There are also the possibilities of focal brain insults like contusions or intracranial hematoma affecting the suitable environment for brain inside the skull (Lowenstein, 1996), inflammation of surrounding tissue structures (Yablon, 1993), alteration of blood-brain barrier (Anwer et al., n.d.; Payan et al., 1970), neuroplasticity driven by glutamate transporter gene mutations (Ritter et al., 2016). Vast majority of the PTS are resistant to medications, extremely complex to understand, and hardly treatable – which make this a vital topic that urges more research into its pathophysiology in order to prevent and manage it properly (Anwer et al., n.d.).

6.1.3. Molecular Changes in Brain Following TBI

Advancement of molecular biology techniques has expanded our understanding of the changes in brain following an event of TBI. However, there is still a gap existing between our existing knowledge about numerous changes occurring at neural level and translating those to direct clinical interventions (Veenith et al., 2009).

Following an event of TBI, a complex cascade of response systems are activated involving growth factors, catecholamines, neurokinins, and cytokines (Ghirnikar et al., 1998). Thus making it challenging to predict and manage the aftermaths based on the

clinical presentations only (E. R. Bennett et al., 2016). The acute injury responses can disrupt the physical and histological structures of brain tissue, mostly by disintegration of the blood brain barrier (BBB) (Schmidt, 2019; Winter et al., 2004). The chronic or secondary phase of the injury is the systemic response at molecular level involving complex network pathways that have been linked with severe and several genetic influences (McAllister, 2010). Identifying and understanding these genetic networks and the changes they cause at molecular level can be a vital tool to plan and design preventive measures to reduce the chronic consequences of TBI. A vast knowledge gap persists in our understanding of numerous ways post-TBI changes keep occurring and one of the methods to reduce this gap is optimizing a suitable animal model, studying which can give us more insight into these molecular changes.

6.1.4. Calcium-modulated Photoactivatable Ratiometric Integrator (CaMPARI)

CaMPARI, short for calcium-modulated photoactivatable ratiometric integrator, is a genetically encoded fluorescent protein that enables researchers to track the activity of neurons in real-time (Fosque et al., 2015). Developed by a team of researchers at the Howard Hughes Medical Institute in 2015, CaMPARI works by changing fluorescent color emission in response to changes in Ca2+ levels within cells (Fosque et al., 2015; Moeyaert et al., 2018).

Brain functions are a combination of responses to stimuli, learning - storing, and recalling memory through coordinated neuronal activities. Identifying the method by which the neurons are activated is imperative to expand the knowledge of our understanding of

brain functions. Genetically encoded Calcium indicators (GECIs) are used to analyze neural activity (J. L. Chen et al., 2013; Tian et al., 2012), but continuous monitoring is required to record Ca2+ influx in neurons which makes the GECIs transient. Thus, making the process highly specific and sensitive.

Fosque et al, by introducing CaMPARI protein, developed a new class of genetically encoded Ca2+ indicators optimized for monitoring in vivo neural activity, with an improved dynamic range and advanced temporal resolution.

CaMPARI is a fluorescent protein that changes color in response to the changes in intracellular Ca2+ concentration, allowing it to serve as a sensor. When exposed to blue light of 405nm wavelength, CaMPARI undergoes a photoconversion from green to red fluorescence and the ratio of red to green fluorescence provides a readout of Ca2+ transportation across cellular membrane over time, reported by the changes in intracellular Ca2+ through a circulatory permuted green fluorescent protein (cpGFP). One of the key advantages of CaMPARI is its ability to store information about neural activity over time. Through photoconverting the protein in response to a specific stimulus of 405nm wavelength UV-light, active neurons can be marked and labelled, and then can be tracked over time to see how their activity patterns change. This allows a more detailed and dynamic understanding of neural circuit function than it is possible with other methods (Fosque et al., 2015).

6.1.5. TBI and CaMPARI

Dr. Hadeel Alyenbaawi from the Allison lab has contributed significantly studying seizure as one of the chronic consequences of TBI. Seizures are abnormally abundant neuronal activation (Stafstrom & Carmant, 2015). CaMPARI reporter imaging following a TBI event provided a deep insight into the sudden burst of excessive neural activity, recorded by permanent and stable conversion of cpGFP to red fluorescent expressed throughout the Central Nervous System (CNS), while studying TBI-associated effects of seizure and tauopathy. The technique allows robust quantification of neural activity by an increase of the red:green ratio for fluorescence emission, especially at hindbrain region (Alyenbaawi et al., 2021a).

Thus, suggesting the technique to be exceptionally useful while studying other brain conditions with altered neural activity, which can open the possibility to develop and modify different preventive and treatment modalities to battle the chronic consequences of TBI.

CaMPARI protein was developed through structure guided mutagenesis, where the conversion takes place at the exposure of 405nm UV-light. The conversion of the green to red is dependent upon the presence of Ca2+, and the conversion of Ca-bound and Ca-free states are different (Fosque et al., 2015). The conversion from green to red is also permanent and stable.

My goal is to optimize a zebrafish line for CaMPARI technique following TBI and lay a baseline of the neural activity in post-TBI conditions. Our understanding of the neural

activities, extent of their variations and their characteristics defined by a highly technical, methodical, dynamic and specific procedure of CaMPARI may provide us with scopes to plan and design treatment and preventive measures of post-traumatic seizures.

Chapter 6.2: Materials and Methods

6.2.1. Ethical Approval

BioSciences Animal Care and Use Committee at the University of Alberta (Animal Use Protocol number: AUP00000077) approved the work in this study, which adheres to the Canadian Council for the Animal Care Guidelines. Zebrafish were bred and maintained in the Science Animal Support Services (SASS) facility of the Biological Sciences.

6.2.2. Zebrafish Breeding and Husbandry

Zebrafish larvae at 4dpf age were used for the experiments. Adult fish from CaMPARI line of zebrafish were in-bred, and out-bred with Casper line following breeding protocols. Upon collection of the fertilized eggs, they were placed in petri-dishes with E3 medium ("E3 Medium (for Zebrafish Embryos)," 2011). Dead larvae, unfertilized eggs and amnionic sac remnants were expelled out and medium was changed every 24-hour. The dishes were kept in incubator at 28ºC until the experiment. After the experiments, and following the TBI, larvae were euthanized following the humane protocol set by SASS.

6.2.3. Zebrafish Lines

Alison laboratory re-developed the Tg[elavl3:CaMPARI (W291F+V398L]ua3144 (Hanwell et al., 2016) using Tol2 transgenic system, where the vector pDestTol2-elavl3:CaMPARI (W391F+V398L) was gifted from Eric Schreiter's laboratory (Fosque et al., 2015). Larvae obtained from this transgenic zebrafish line expressed CaMPARI, which is a modified green fluorescence protein (GFP) in the central nervous system, which was used to screen the larvae at an early age. Dr. Richard Kanyo of Allison lab developed a stable

zebrafish line using transgenic Tg[elavl3:CaMPARI (W391F+V398L)] ua3144 (Alyenbaawi et al., 2021a). Larvae bred from in-crossing adult zebrafish with this unique genetic background were used as experimental samples for the experiment. To use as control samples, wild type Casper zebrafish were bred and maintained following the same protocol.

6.2.4. Inducing Traumatic Brain Injury

At 4dpf age of larvae with GFP expression, TBI was induced using methods slightly modified from Alyenbaawi (2021). A 10cc syringe was used and 10-15 larvae were aspirated into it with E3 media they were kept in. The volume of the E3 media was kept constant at 1cc. After 10 minutes to stabilize the larvae in a new environment, the syringe was placed in a foam block with the tip directing downwards and the plunger directing upwards. The barrel of the syringe was closed using a valve so the contents cannot expel out. A cardboard tube was mounted vertically above the foam block where the syringe was placed so the openings of the tube lies directly above the plunger of the syringe (Fig 1A). The length of the tube was 48cm, which was kept constant in all the experiments. A 300 g weight was released into the upper opening of the tube, so it drops directly onto the plunger of the syringe which was placed at the lower opening of the tube. This was repeated thrice for each experimental setup. After each drop, the plunger of the syringe was pulled with the tip still closed by the valve, to return the larvae stuck at the tip of the syringe to the barrel. This was done to reduce the fatality from constricting the larvae in a relatively smaller place where the pressure wave from the wright-drop could crush them. **Figure 6.1** presents a scheme of TBI-induction to zebrafish larvae.

6.2.5. Photoconversion

Following administering TBI to the GFP+ CaMPARI larvae at 4dpf age, they were placed into the wells of a 96-well plate with 150µl of E3 medium without the methylene blue to avoid its effect on the images obtained. Control groups, CaMPARI+ larvae that were not exposed to TBI, and CaMPARI- larvae without TBI were added in consecutive wells to the larvae administered with TBI. Multiple larvae were placed into each well in some instances. The 96-well plate was kept in room temperature for at least two hours for the acclimatization of the larvae following TBI. Afterwards a water bath was prepared at room temperature, and the 96-well plate was placed on the surface of water carefully, so it rests evenly and remained immobile. Foam blocks were placed directly above the plate, so the distance between the water surface and the UV light-source remains constant. To alter the intensity of the light, three distances were used for photoconversion – 10, 5 and 2.5 cm. Larvae were exposed to the LED UV-rays (Loctite) for 30, 60, and 100 seconds in consecutive attempts. Following the exposure, the 96-well plate was removed from the water bath and the third control group, CaMPARI+ without TBI and not-photoconverted, were added into another column of the consecutive wells to the larvae with TBI+photoconverted groups. All larvae were anaesthetized with 0.24mg/mL MS-222 (tricaine; ethyl 3-aminobenzoate methanesulfonate; ©Sigma-Aldrich, catalog no. E10521) added with the 150 µL E3 medium without methylene blue. Finally, the plate was placed on the rotator for two minutes for the contents to mix well, until the samples were ready for imaging.

6.2.6. Confocal Imaging

The 96-well plates were loaded onto the platform for imaging in the LSM 710 confocal microscope (Zeiss). After optimizing the measures for the borders and width of the wells on the plate, images were obtained with DAPI-FITC and Cy3 filters. The FITC filter acquired the cpGFP recording the Ca2+ changes when CaMPARI protein was activated, and Cy3 acquired the green to red fluorescence after the conversion. Images were collected for the whole 96-well plate.

6.2.7. Data Collection

From the obtained images maximum intensity projections were acquired from Z-stacks (7µm width) using a 20x/0.8 objective and a laser-point scanning confocal microscope (Zeiss LSM 710). After isolating the brain regions with the GFP+ expression that went under photoconversion, a 3D area was created with MetaXpress PowerCore v1.5.0.18 (®Molecular Devices, San Jose, CA, USA) and the mean of the green and red channels intensities were calculated. The red/green ratios for each individual fish were collected and interpreted as relative neural activity, and were defined as the acitivity of CaMPARI (Fosque et al., 2015), which were analyzed and presented as results.

6.2.8. Data Analysis

GraphPad Prism (v9.5.1-528, San Diego, CA, CA, USA) was used for statistical analysis. Simple unpaired t-test, and two-way ANOVA were performed among the experimental and control groups.

Chapter 6.3: Results

Based on CaMPARI principles, the neural activity following the TBI was measured using the Standard Error of the Mean (SEM) for the red:green ratios **(Figures 6.2.E-L)** in the brain of zebrafish larvae shown in **Figure 6.1**.

While comparing the photoconversion rates within the TBI group and the no-TBI groups, an increased activity was recorded for the TBI group when the distance was 5cm and the larvae were exposed to UV-light for 60 seconds, which was statistically significant with a P-value 0.0203, at 95% confidence interval (**Figure 2.L**). The other groups were observed with no measurable increase in the neural activity between the TBI and no-TBI groups (**Figure 2.E-K**). It suggests that TBI caused quantifiable neuronal hyperactivity only within this group. However, it supports the hypothesis that induction of TBI increases brain activity, and it is measurable using CaMPARI.

Figures for Chapter 6.

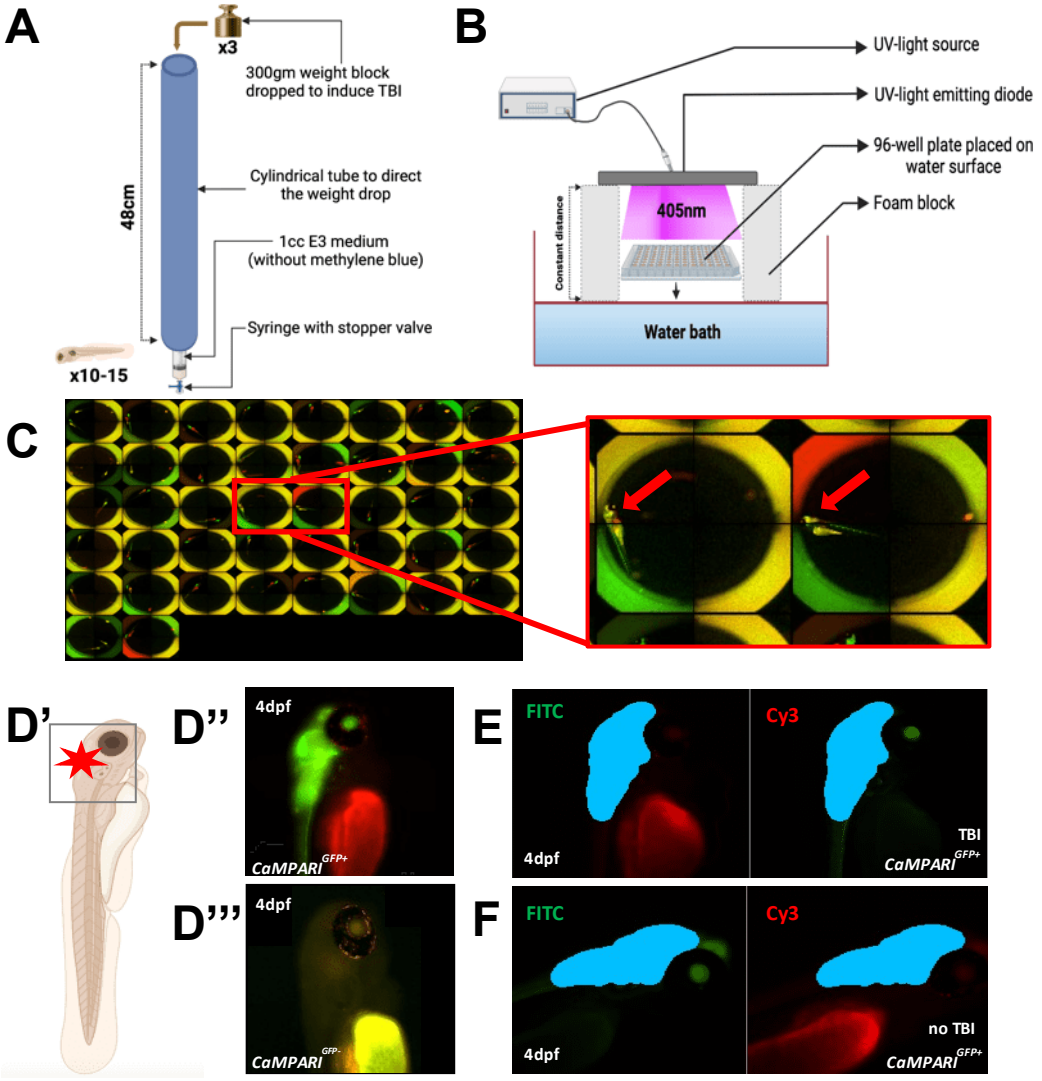

Figure 6.1. CaMPARI activation following TBI in zebrafish larvae. (A) shows the scheme for inducing TBI to zebrafish larvae; 10-15 larvae are aspirated into a closed syringe with 1cc E_3 medium (without methylene blue), 300gm weight was dropped directly on the syringe thrice from 48cm height. **(B)** shows the schematic representation of photoconversion; 96-well plates with larvae is placed on the surface of the water bath and

exposed under 405nm UV-light from above. **(A)** and **(B)** were prepared in Biorender.com. **(C)** shows the acquired composite image of individual larvae in individual wells in a 96-well plate. **(D')** is a cartoon zebrafish larva with a boxed area depicting the region of interest, and the star representing the hindbrain which is mostly affected in TBI. It was prepared in eDrawSoft (© 2023 Edrawsoft, AZ, USA). **(D'')** is a CaMPARI^{+} larva; **(D''')** is a CaMPARI^{-} larva. **(E)** shows region of interest in blue that is optimized to obtain data for red to green (R:G) conversion following TBI. This is crucial because unless the parameters are selected correctly, autofluorescence from yolk sac and surrounding areas may interfere with quantification. **(F)** is a CaMPARI^{+} larva but without TBI; it shows how comparing the R/G conversion can be a challenge for a TBI sample, since no virtual difference can be detected within the brain region with naked eyes. All larvae in this figure are at 4dpf age.

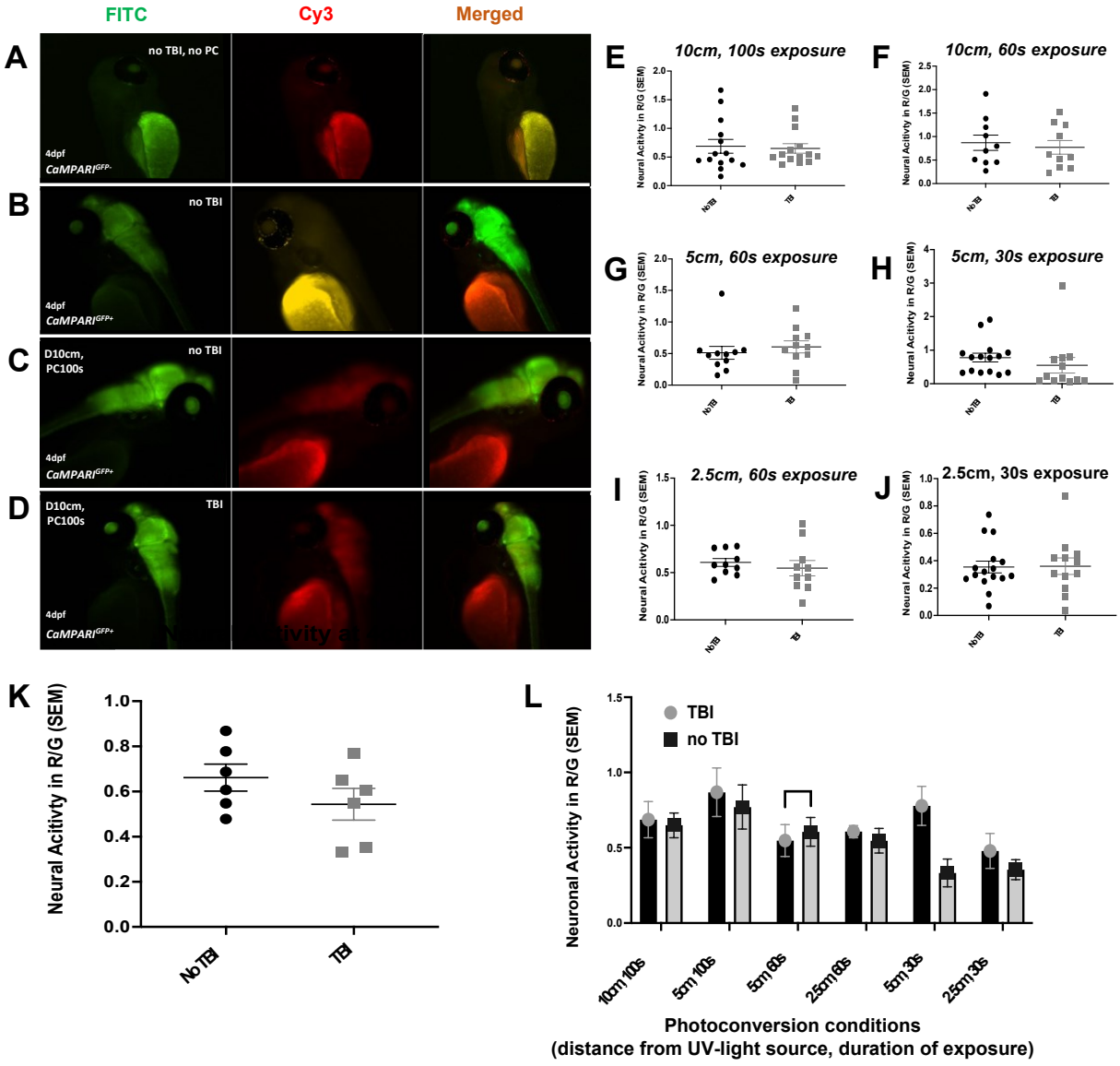

Figure 6.2. CaMPARI activity in red:green ratio. A-D show examples for the analysis of neural activity in zebrafish CaMPARI larvae at 4dpf age. A shows offspring that is GFP^- and is not exposed to UV-light. Rest are GFP^+ and have gone through photoconversion. **B** and **C** are not introduced to TBI. **D** is one of the samples from TBI group. **C-D** are references of how different larvae groups with TBI and no-TBI are photoconverted for different durations of the exposure positioning the light source at different distances. FITC filter is used to calculate the green expression and Cy3 was used to measure permanent

and stable red conversion. **E-J** are comparison of neural activity at different conditions. Unpaired t-test was used for analyzing the SEM of red:green between the TBI and no-TBI groups at 95% confidence interval, which showed no significant difference. **K** is summarized results compiling all data in different conditions. **L** is the result of two-way ANOVA analysis for the CaMPARI activity within all the groups, where a significant difference is noticed (95% CI) between the TBI/no-TBI groups when they are exposed to UV-light from 5cm distance and for 60 seconds, suggests quantifiable neuronal hyperactivity only within this group, providing preliminary support for the hypothesis that induction of TBI increases brain activity.

Chapter 6.4: Discussion

TBI causes a cascade of molecular changes in the brain, and in our larval zebrafish model most of the activities are located at hindbrain region (Alyenbaawi et al., 2021a). The changes can be due to oxidative stress, inflammation, excitotoxicty and altered gene expressions (Veenith et al., 2009). Neural hyperactivity following a TBI event can explain the immediate changes occurring in brain and the extent of the changes that persist resulting in the long-term effects it bears. Post-traumatic seizures are likely to be the outcome of this neural hyperactivity that initiates a vicious cycle of continuing injury to brain tissue.

Oxidative stress is following an injury creates an imbalance between production and neutralization of Reactive Oxygen Species (ROS) (Ismail et al., 2020), which can be due to TBI-induced increase of ROS production. These attacks cell membrane and alters protein synthesis and DNA damage (Rodríguez-Rodríguez et al., 2014). After a TBI event, immune cells are activated, which leads to the release of pro-inflammatory cytokines and cellular mediators (Postolache et al., 2020; Shi et al., 2019). Thus resulting in progressive tissue damage and further injury to the already weakened brain (Schimmel et al., 2017; R. Zheng et al., 2022). Excitotoxicity is a grave condition of the brain, when there is excessive injury from dendrite degeneration and neuronal death due to the overactivation of glutamate receptors, resulting in sudden influx of Ca^{2+} and Na^{+} across the cell membranes (Mattson, 2019; Narayanan et al., 2022, p. 7). These processes have the potential to increase the neural activity following TBI and may be the cause behind several serious post-traumatic consequences like seizures and dementia.

All these events, by themselves or acting together cause chronic and often permanent alteration of gene expressions (Hellmich et al., 2013; Kukacka et al., 2006; Samal et al., 2015; Vonder Haar et al., 2014; Y.-H. Wong et al., 2016, 2016).

TBI results in neural hyperactivation, and all evidence suggests that the neural activity is increased following an event of traumatic injury. Molecular changes in TBI are very complex and multifaceted. They often contribute to further injury even after the cessation of traumatic forces on the brain; and often result in significant physical and cognitive deficit in an affected individual. Which can also be interpreted as a continuous ongoing process where several molecular cascades remain activated.

CaMPARI measures brain activity by detecting changes in the concentration of Ca2+ in neurons, which is a key step in neural activity. When a neuron fires, it experiences a brief influx of Ca2+, which triggers a cascade of biochemical events that result in the release of neurotransmitters and the transmission of signals to other neurons (Alyenbaawi et al., 2021b; Fosque et al., 2015). CaMPARI protein Exhibits 60-40 green to red fluorescence brightness of mEos2, which photoconverts to the red form 21 times as fast in the presence of Ca2+ (Fosque et al., 2015) by a short pulse of ultraviolet light. Green fluorescence represents a low active state associated with low levels of Ca2+, while the red fluorescence indicates high Ca2+ levels.

Data and molecular changes at neural level following a TBI suggest that there should be a sudden increase of the Ca2+ influx in cells, which is stable and permanent. However, the preliminary data from the experiment here appear to contradict that expectation, however they must be interpreted with great caution until further optimization of the

technique is complete. Previous work by Dr. Hadeel Alyenbaawi also suggests that there is likely to be an increase of the brain activity, measurable by the CaMPARI activation through an increase of the red:green ratio.

Chapter 6.5: Conclusion

A new technique such as CaMPARI has provided the scientist community the scope to record and monitor the neural hyperactivity in many adverse conditions. It allows the researcher to gather information, and to set up a baseline to proceed further to analyze and translate the findings as an explanation of the changes occurring at molecular level. TBI bears significant danger in acute conditions, but its long-term effects can be even more debilitating than the trauma itself. Post-traumatic seizures can keep injuring the brain tissue after the fact, and it does not have much effective preventive and treatment options.

The first step following introduction of a new technique for any scientific experiment is to check whether it is recording the changes of our initial hypothesis. In the instances when it does not, it needs to be checked whether the technique is applicable for the experiment. Results presented here support that CaMPARI recognized the neural hyperactivity following TBI in zebrafish. However, reviewing previous studies and hypothesized events in post-TBI brain suggest that brain remains in a constant agitated and active state, the initiation of which is immediately following TBI, and should be recognizable by overactivation of CaMPARI protein. Our experiment discussed in this section does not yet recapitulate that effect in zebrafish larvae brain.

Although the data from the experiment shows further optimization of CaMPARI is required before it can provide information about associations between TBI and brain activity, CaMPARI photoconversion experiment provides a promising and applicable technique to measure and analyze active state of brain following any neural event.

The findings could not correlate with previous work, it could be due to imaging complexity, sensitivity and specificity of zebrafish larvae, requirement of more photoconversion conditions and more detailed ways to consistently chose what brain regions are working.

CaMPARI photoconversion is an extremely sensitive, specific and skillful technique. The distance of the UV-light source from the samples, duration of the exposure, wavelength specificity of the LED light and consistent experimental conditions are crucial for this technique. Prior to photoconversion, larvae were anaesthetized. Since this technique measures the brain activity, anesthetic usage and technical errors while anesthetizing the larvae may have altered the Ca2+ levels. Activation of the CaMPARI protein is measured by precise and detailed imaging. Zebrafish larvae were kept in E3 medium prior to TBI-induction, which contains methylene blue to prevent fungal infection. Although very important for larvae maintenance, presence of even a small amount of methylene blue can interfere with image acquisition (Figure 2.D-F).

Last but not the least, autofluorescence from the zebrafish larvae, plate materials and the medium, although is excluded of methylene blue, can alter the optimization of the image processing for data collection.

As a future direction to this part of the project, a larger sample size for the TBI-affected larvae, varying environmental settings for photoconversion and multiple attempts with more controlled samples can provide the opportunity to improve skills for this technique. This is a highly advanced avant-garde technique to observe and analyze brain activity at molecular level, and practicing this technique is an illustrious and striking method to gain insight on several brain conditions, including but not limited to traumatic brain injury.

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