

INVESTIGATION OF THE CELLULAR PATHOLOGY UNDERLYING THE OPTIC
NEUROPATHY IN A MOUSE MODEL OF FAMILIAL DYSAUTONOMIA

by

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DEDICATION

I would like to dedicate this work to the Familial Dysautonomia Foundation and to all the patients, families, and friends affected by this devastating disease. Your strength and perseverance are inspiring.

Additionally, I would like to dedicate this work to my husband, Andrew Baranak, for his unwavering support, understanding, and inspiration. I couldn't have done it without you.

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ABSTRACT

Familial dysautonomia (FD) is a rare, recessive, progressive autosomal disorder that affects the nervous system. This neurological disorder is caused by a splice mutation in the Elongator complex I (*ELP1*) gene.¹ The mutation results in a tissue-specific reduction of ELP1 protein due to unstable mRNA targeted for nonsense-mediated decay. ELP1 is a highly conserved scaffolding protein and core subunit of the six-subunit Elongator complex required for normal translation, neuronal development, and survival.^{2,3} Insufficient ELP1 leads to the developmental death of neurons in the peripheral and autonomic nervous systems in addition to central and peripheral nervous system neurodegeneration.^{1,4,5} Patients suffer from congenital and progressive neuropathies, such as cardiovascular dysfunction, reduced peripheral sensory function, poor growth, and digestive and respiratory problems. Outside of the risk of death in early adulthood, one of the most debilitating conditions affecting patients' quality of life is progressive blindness marked by continual loss of retinal ganglion cells (RGCs).^{1,6} Within the FD community, there is a concerted effort to develop treatments to prevent the loss of RGCs, thereby improving patients' quality of life. This study aims (1) to elucidate mechanisms underlying the death of RGCs in the absence of Elp1 and (2) to obtain pre-clinical intervention data that can eventually be translated into therapeutics for rescuing RGCs in FD. Using histology and confocal microscopy in conjunction with biochemistry, this study provides evidence for disrupted cellular homeostasis and inflammation preceding RGC death, and as the disease progresses, the retinal cells fail to mount a correct stress response to restore neuronal homeostasis. Furthermore, this study provides first-of-its-kind pre-clinical data using targeted gene therapies to rescue RGCs. Understanding the biological crosstalk and signaling mechanisms underlying the death of RGCs in the absence of Elp1 will allow for more targeted and effective therapeutics that will benefit not only the FD community but also individuals affected by other retinal diseases and neurological diseases that result from a faulty Elongator complex. This study provides a novel characterization of the FD retina and establishes baseline methods to further investigate rescuing RGCs.

CHAPTER ONE

INTRODUCTION

Familial Dysautonomia

Familial dysautonomia (FD), also known as “Riley-Day syndrome” or Hereditary Sensory and Autonomic Neuropathy (HSAN) type III, is an autosomal recessive, congenital sensory and autonomic neuropathy that primarily affects individuals of Eastern European or Ashkenazi Jewish decent,^{7,8} with an estimated 1:3600 live births.⁹ FD is both a neurodevelopmental and a progressive disorder with incomplete development and continual loss of sensory and autonomic neurons throughout life.¹⁰ FD is also characterized by the gradual loss of retinal ganglion cells (RGCs) in the neural retina, which is part of the central nervous system (CNS).

All FD patients are homozygous for a mutation in Elongator Acetyltransferase Complex Subunit I (*ELP1*), formerly known as IKBAP with over 99% being homozygous for the original founder mutation which is a T to C transition in the donor splice-site of intron 20 in the *ELP1* gene (IVS20+6C > C), resulting in mRNA in which exon 20 is excluded, which leads to a frameshift and premature stop codon. There is evidence that mutant mRNA is targeted for nonsense-mediated decay,^{4,11} which results in reduced levels of ELP1 protein. There is variable tissue-specific skipping of exon 20, leading to reduced full-length ELP1 in all tissue types however, the nervous system is most affected, displaying a severe reduction in exon 20 inclusion.^{8,12,13} In short, the deficiency in ELP1 affects the development of afferent processes in the peripheral and central nervous systems and presents varied pathological manifestations

among individuals. However, a clinical hallmark that affects 100% of patients is visual impairment marked by a reduction in the macular retinal ganglion cell (RGC) layer and their axons in the retinal nerve fiber layer (RNFL). So, why are neurons unable to correctly splice without ELP1 while other cells seemingly adapt and function normally?

Elp1 and the Elongator Complex

The Elongator complex consists of six subunits (ELP1-ELP6) and is organized into two three-subunit complexes; the core sub-complex ELP1-ELP2-ELP3 and the accessory complex ELP4-ELP5-ELP6.² Elongator subunits are highly evolutionarily conserved from yeast to humans in sequence and their interaction with the other subunits. ELP1 is the largest subunit, forming a scaffold for the other Elongator proteins. ELP2 is the second largest subunit, stabilizing the core sub-complex Elp123 and integrates signals that regulate Elongator activity. ELP3 is the enzymatic core of Elongator and is essential for Elongator function. The functional integrity of Elongator is compromised and associated with multiple neurological disorders when any genes encoding the subunits are experimentally deleted or absent, indicating the tight functional association between protein subunits.¹⁴ As previously noted, mutations in *ELP1* result in FD. Mutations in *ELP2* are associated with intellectual disability (ID), epilepsy, and autism.^{2,15-17} Mutations in *ELP3* have been associated with Amyotrophic lateral sclerosis (ALS)¹⁸, and non-coding mutations in *ELP4* are associated with Rolandic epilepsy.^{19,20}

Several models for the function of ELP1 and Elongator have been proposed. Over two decades ago, Elongator was first identified from yeast chromatin as a component of RNA polymerase II (RNAPII) holo-enzyme involved in transcription elongation.²¹ Initial studies associated Elongator with hyperphosphorylated RNAPII and mutations in the complex resulted

in delayed activation of several genes, and ELP3 has histone acetyltransferase activity.^{22–25} So, one school of thought is that Elongator is involved in histone acetylation and transcription.

However, subsequent findings showed that the Elongator is primarily cytoplasmic, could not coimmunoprecipitate chromatin²⁶, and plays a direct role in transfer RNA (tRNA) modification via synthesis of the 5-carboxymethyluridine (cm⁵U) on uridines at the wobble position.²⁷ Multiple studies have shown that Elongator is required to translate codon-biased genes and is essential for the chemical modification of wobble uridine (U₃₄) in the tRNA to enhance the translation efficiency of AA-ending codons.^{25,27,28} The loss of Elp1 results in dysregulated translation with an increase in small proteins whose mRNA exhibit small AA:AG ratios and a reduction in the percentage of large proteins that are encoded by transcripts with AA:AG ratios in the DRG of the *Wnt1-Cre; Elp1^{loxp/loxp}* conditional knock-out mice.²⁹ Furthermore, it was observed that genes involved in DNA damage repair are codon biased, and a loss of Elongator function results in elevated levels of DNA damage.²⁹ Additionally, there is evidence from fission yeast that (1) the lack of Elongator results in stress phenotypes due to its role in tRNA modification and subsequent translation inefficiency^{27,30} and (2) there is reciprocal regulation of TOR (target of rapamycin) signaling and Elongator, indicating a function for Elongator in cell growth and differentiation.^{14,31,32} Another hypothesis suggests that Elp1 might be involved in kinase signaling, specifically c-Jun N-terminal kinase (JNK) mediated stress signaling.³³ However, this is not well documented, and further evidence is needed to support this claim.

The Retina and Vision Loss in Familial Dysautonomia

The retina is the unique component of the central nervous system (CNS) that converts visual stimuli (photons) into chemical and electrical signals, which are transmitted along neuronal pathways to the brain via the optic nerve. The retinal structure is highly conserved, from fish to humans³⁴, and is divided into three distinct cell body layers; the outer nuclear layer (ONL) contains the cell bodies of photoreceptors: rods and cones; the inner nuclear layer (INL) contains the cell bodies of the bipolar, horizontal, and amacrine cells, and the ganglion cell layer (GCL) contains the cell bodies of ganglion cells and displaced amacrine cells. Two synaptic neuropil layers separate the nuclear layers; the outer plexiform layer (OPL) contains synapses between photoreceptors, horizontal, and bipolar cells; the inner plexiform layer (IPL) contains synaptic contacts between bipolar, amacrine, and ganglion cells.³⁵ Each retinal layer achieves one step of vertical synaptic transmission, and each neuronal subtype fulfills a specific functional role in signal processing. The functional aspects of retinal neurons impose a tremendous metabolic requirement in the retina, which must be met without interfering with signal transduction.³⁶ Therefore, retinal neurons rely heavily on effective vascular structures and glial cells for trophic support and waste removal.³⁷

In humans, the macula is the region of the retina that receives input from our central field of vision. The fovea is the center of the macula and contains the highest density of cone receptors, which are coupled in a one-to-one relationship with bipolar and ganglion cells, allowing this cone system pathway to mediate high visual acuity.³⁸ The retinal ganglion cell axons leave the retina, form the optic nerve and are the sole cell directly transmitting visual information to the brain.

The visual impairment seen in FD is universal, meaning every patient experiences a progressive visual decline. Optical coherence tomography (OCT) measurements and histopathological findings from patients confirm that visual impairment is correlated with RGC death, and our current understanding is that the RGC death manifests as low visual acuity of 20/70, central visual field deficits, and a red-green color deficiency.^{6,39} Mendoza-Santiesteben et al. 2014 showed significant reductions in the thickness of the RNFL in the temporal regions of FD patients (average age 27) compared to carriers (heterozygous for the mutation) and healthy age-matched controls. Importantly, this study confirmed that visual loss is associated with optic nerve dysfunction. This significant reduction of axonal fibers in the temporal region indicates a loss of RGCs from the macula, the more metabolically active cells involved with high acuity vision and our central field of view. Interestingly, data from a young FD patient (9yo) with normal visual acuity showed reduced temporal RNFL thickness. Suggesting that this maculopapillary RNFL loss begins early in life. Determining when this initial loss occurs could be critical for the most optimal and efficient therapeutics. The FD optic neuropathy seen in patients presents similarly to the mitochondrial optic neuropathies, Leber hereditary optic neuropathy (LHON), and autosomal dominant optic atrophy (DOA), in which mitochondrial structure and function are affected.^{40,41} We can use these diseases and models to glean information on therapeutics and potential triggers of RGC death in FD.

The Pax6-Cre; Elp1^{loxp/loxp} Mouse Model

A mouse retinal model of FD was developed using a retina-specific *Pax6-Cre* to address the pathophysiologic mechanisms underlying visual impairment in FD patients.⁴² Pax6 is a transcription factor expressed throughout retinal development, including an early expression in

retinal progenitors, which includes all retinal neurons and Müller glial cells.⁴³ Therefore, in the Pax6-Cre; Elp1^{loxp/loxp} model, Elp1 is conditionally knocked out only in the peripheral retina (the Cre is only active early in development and restricted to the periphery). These mice show ~70% reduction in retinal ELP1 expression throughout the peripheral retina⁴² and recapitulate the phenotype of progressive RGC loss seen in patients. This is an attractive model because of (1) the robust effect of the *Pax6-Cre* in knocking out Elp1 in the retina, (2) mice that are null for Elp1 die by embryonic day 10 (E10), and (3) these mice do not present with any additional FD phenotype, while other models with more a systemic knock out of Elp1 result in extreme FD phenotypes and reduced lifespan which makes studying the optic neuropathy challenging. Previous studies using the *Pax6-Cre; Elp1^{loxp/loxp}* have corroborated patient data showing a progressive decline in RGC number, mitochondrial dysfunction, and reduced ATP.⁴² While different cell types have been characterized in the *Pax6-Cre; Elp1^{loxp/loxp}* model, characterization of signaling pathways and molecular mechanisms underlying RGC death is still needed.

Aims and Objectives

While the FD population is small and patients' life expectancy has increased from an average age of 5 to 25 thanks to informed and effective medical interventions and elucidation of the underlying mechanisms of the diseases, it is still a fatal disease with no existing cure. FD shares similarities with other optic neuropathies and retinal diseases, e.g., mitochondrial dysfunction and the preferential loss of RGCs.

Current research on FD has led to better characterization of neuronal function impacted by the loss of ELP1. However, the role of ELP1 protein in the retina has not been fully determined. Elucidating the molecular mechanisms that lead to RGC death will shed light on our

understanding of ELP1 function in the retina, our understanding of the pathological state in the FD retina, and our understanding of neurodegenerative and neurodevelopmental disorders in general. Therefore, using the Pax6-Cre; Elp1^{loxp/loxp} mouse model, the project's first objective was to characterize and identify cellular events and upstream signals that trigger RGC death. The second objective was to determine whether RGC degeneration can be prevented *in vivo* by (1) overexpressing healthy copies of *Elp1* in the retina and (2) manipulating defective cellular energetics and aberrant signaling. In short, this project aims to identify biomarkers of RGC death and obtain pre-clinical data that can be translated into therapeutics to rescue RGCs in FD.

CHAPTER TWO

REDUCTION OF RETINAL GANGLION CELL DEATH IN MOUSE MODELS OF
FAMILIAL DYSAUTONOMIA USING AAV-2 MEDIATED GENE THERAPY AND
SPLICING MODULATORSContribution of Authors and Co-Authors

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REDUCTION OF RETINAL GANGLION CELL DEATH IN MOUSE MODELS OF FAMILIAL DYSAUTONOMIA USING AAV-MEDIATED GENE THERAPY AND SPLICING MODULATORS

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Abstract

Familial dysautonomia (FD) is a rare neurodevelopmental and neurodegenerative disease caused by a splicing mutation in the Elongator Acetyltransferase Complex Subunit 1 (*ELP1*) gene. The reduction in ELP1 mRNA and protein leads to the death of retinal ganglion cells (RGCs) and visual impairment in all FD patients. Currently, patient symptoms are managed, but there is no treatment for the disease. We sought to test the hypothesis that restoring levels of Elp1 would thwart the death of RGCs in FD. To this end, we tested the effectiveness of two therapeutic strategies for rescuing RGCs. Here we provide proof-of-concept data that gene replacement therapy and small molecule splicing modifiers effectively reduce the death of RGCs in mouse models for FD and provide pre-clinical data foundation for translation to FD patients.

Introduction

Familial dysautonomia (FD) is a rare recessive autonomic and sensory neuropathy. Over 99% of FD patients are homozygous for the “founder” mutation in intron 20 of the *ELP1* gene (c.2204 + 6T > C; formerly called “*IKBKAP*”), causing the “skipping” of exon 20 from the mature mRNA coding sequence [1, 2]. This mutant mRNA is then targeted for non-sense mediated decay [3]. The mis-splicing occurs in a tissue-specific manner, with peripheral neurons being most impacted, producing almost no functional protein [4, 5]. The hallmarks of FD include reduced pain and temperature sensation, gait ataxia, cardiovascular instability, swallowing impairment, gastrointestinal dysfunction, and eventual blindness [6]. As patients age, visual impairment becomes one of the most debilitating symptoms, given that it is accompanied by loss of balance and gait ataxia. The optic neuropathy in FD results from the progressive death of

retinal ganglion cells (RGCs) and the loss of their axons from the nerve fiber layer (NFL) in the retina's macular region [7-9].

It is necessary to develop therapeutics to reduce the loss of RGCs that occurs in all FD patients. Being a monogenic disease, FD is a strong candidate for genetic therapies. Gene replacement therapies for retinal diseases show tremendous potential due to the accessibility and immune-privileged status of the eye [10]. Preclinical and clinical studies have demonstrated that adeno-associated viruses (AAVs) are efficient viral vectors for delivering functional genes in hereditary retinal diseases [10-12]. In addition to gene therapy, a potent strategy for treating genetic diseases is using splicing modulator compounds (SMCs) that correct mutations at the messenger RNA level. Such a modifier was recently approved to restore correct splicing in the neurological disorder spinal muscle atrophy [13, 14]. SMCs effectively reduce disease phenotypes in FD mouse models [15-17]. However, to date, no gene therapy approaches have been used to introduce wild-type copies of *ELP1* to correct disease phenotypes. Here we present pre-clinical data demonstrating the effectiveness of gene therapy and a novel splicing compound in preventing RGC death in FD mouse models.

Results

Using two complementary FD mouse models, mice were treated either with (1) an intravitreal injection of an AAV2 (highly tropic for RGCs) engineered to express a wild type copy of *ELP1* [18-20] (Figure 1A) or (2) a novel small molecule splicing modulator to restore correct *ELP1* splicing [16] (Figure 1B).

To test whether a gene replacement strategy could prevent the death of RGCs in our previously established *Pax6-cre;Elp1^{loxp/loxp}* mouse model [21], we generated two AAV2 vectors,

one expressing the murine *Elp1* gene (*mElp1*) and the other expressing eGFP, both driven by the U1a promoter (see Methods). We first determined that the AAV2-U1a-*mElp1* could drive the expression of *Elp1* in the mouse retina and increase Elp1 protein expression *in vitro* (Supplemental Fig. 1).

Next, cohorts of *Pax6cre;Elp1^{loxp/loxp}* mice received intravitreal injections of AAV2-U1a-*mElp1* or control (eGFP) vector at postnatal day 21 (P21) before any significant loss of RGCs is observed [21]. Three months following injection, RGCs were quantified by flat-mount analysis (Figure 2A). This time point was selected since prior work established that, by this age, 40-60% of RGCs have died [21]. We observed a significant response with a 137% increase in RGC number (Mean: 5696 ± 252 , $p < 0.0001$) with the higher AAV2-U1a-*mElp1* vector dose ($\times 10^9$ vg/eye) and a 131% increase in RGC number (Mean: 5563 ± 172 , $p < 0.0001$) with the lower AAV2-U1a-*mElp1* vector dose ($\times 10^8$ vg/eye) in *Pax6cre; Elp1^{loxp/loxp}* retinæ compared to untreated *Pax6cre; Elp1^{loxp/loxp}* retinæ (Mean: 2404 ± 405) (Figure 2B). Interestingly, eyes injected with the control AAV2-U1a-eGFP showed an 82% increase in RGCs (Mean: 4386 ± 534) compared to the untreated *Pax6cre; Elp1^{loxp/loxp}* (Figure 2B). However, the *mElp1-virus* significantly increased RGC numbers by 30% ($p = 0.04$) above the eGFP-injected eyes. This suggests that (1) injection of a virus in a dose-dependent manner alone induces a protective response in the retina, most likely by stimulating a wound response [22-24], but (2) that the *Elp1* virus increased RGCs significantly above the virus-alone-induced protective response. These data provide promising evidence that gene replacement therapy, with further optimization, could be used to ameliorate RGC death in FD.

Based on these data, we generated an AAV2 vector driving the expression of the human *ELP1* gene (*hELP1*). Again, we confirmed that the AAV2-U1a-*hELP1* could increase ELP1 protein *in vitro* (Supplemental Fig. 2A,B) and drive *ELP1* mRNA expression in wild-type mouse retinas *in vivo* (Supplemental Fig. 2C). Subsequently, *Pax6cre;Elp1^{loxp/loxp}* and littermate controls were intravitreally injected with three different viral titers of the human *ELP1* virus. All mice received bilateral injections at P21, and RGCs were quantified three months later (Figure 3A). There was a significant increase in RGC survival in the *Pax6cre;Elp1^{loxp/loxp}* retinae that received the highest vector dose (5.4×10^8 vg/eye), showing a 39% increase in RGC number (Mean: 4925 ± 439 , $p = 0.02$) (Figure 3B). There was no significant difference between the uninjected retina and those injected with the AAV2-U1a-*eGFP* construct, which was used at a lower dose than in the mouse *Elp1* virus experiments. To visualize the AAV2-U1a-*hELP1* infected RGCs, we double immunolabeled retinal sections with antibodies to RGCs and human ELP1. In the retinal sections infected with AAV2-U1a-*hELP1*, approximately 26% of RGCs expressed the human ELP1 protein (Figure 3C), visually confirming that driving *ELP1* expression can efficiently produce human ELP1 protein.

Another validated approach for treating genetic disease is using orally administered small molecules that modify splicing [25, 26]. To test the efficacy of a novel small splicing compound PTC680, an analog to the highly specific *ELP1* splicing modulator PTC258 [16], we generated a new hybrid mouse model in which we introduced the human *FD ELP1* original founder splice mutation (*TgFD9*) transgene into the *Pax6cre;Elp1^{loxp/loxp}* conditional knockout mouse model. This new model recapitulates the FD retinal phenotype while recreating the tissue-specific mis-splicing observed in FD patients. To assess the therapeutic effect of PTC680, we administered an

oral treatment formulated to dose each *TgFD9;Pax6cre;Elp1^{loxp/loxp}* mouse 1.2 mg/kg/day. Treatment started at three different time points, (a) Embryonic day 18.5 (E18.5), (b) P21, and (c) P90, to test the therapeutic efficacy throughout the lifespan and disease progression (Figure 1B). In all cases, mice consumed either vehicle or PTC680 special chow for 3 months. RGCs were quantified at the end of each 3-month treatment regimen using retinal flat-mount analysis (Figure 4A). PTC680 treatment increased RGC number by up to 57% depending on the age at which treatment began (Figure 4B). The most significant increase in RGCs occurred when treatment began at the youngest age (E18.5). Notably, a significant rescue of RGCs occurred even when treatment started at 3 months old. Thus, daily consumption of PTC680 prevents significant RGC death in both mature and immature retinas.

To assess whether RGC rescue was associated with increased splicing efficiency of the mutant FD *ELP1* gene, we analyzed *ELP1* splicing in retinal tissue from vehicle- and PTC680-treated FD mice. As demonstrated with PTC258 [16], PTC680 significantly increases *ELP1* exon 20 inclusion in the FD retina (Figure 4C), providing evidence that this novel splicing modulator mitigates a primary FD phenotype by increasing *ELP1* in the retina.

Discussion

We demonstrate that both an intravitreally-delivered RGC-specific gene transfer and the systemic delivery of a novel small molecule splicing modulator can prevent the progressive death of RGCs in models of FD. This is the first gene therapy study in an FD model and lays the foundation for a translational path for mitigating RGC loss in FD patients. Additionally, we show that in a new mouse model, the oral administration of the novel PTC680 compound significantly

improved RGC survival throughout multiple stages of disease progression. These data are encouraging as most FD patients do not report vision loss until they are teenagers.

Our data show that approximately 30% of RGCs were infected with the human *ELP1* virus and expressed the human ELP1 protein, which is in the same range as the percentage of RGCs rescued (39%). Moreover, we provide the first evidence that morphology is not disrupted in RGCs expressing virally transduced *ELP1*, suggesting that overexpression of human *ELP1* is not harmful to retinal neurons. Future studies will be required to test the extent of RGC rescue, i.e., physiological function. One concern is whether the reintroduction of Elp1 protein in RGCs is sufficient to support circuit formation with RGC targets in the brain; a second concern, given the broad expression of Elp1 in the brain, is whether visual processing and perception will be improved if RGC inputs are restored.

These data justify the further optimization of an *ELP1*-vector for translation to a clinical setting. Potential paths forward include optimizing the AAV2 capsid properties via chemical modifications or assembling mosaic capsids consisting of different serotypes could improve targeting specificity and tropism to achieve higher transduction efficiency [27-29]. Alternatively, concatamerization of dual AAV vectors [30-34] can be considered as the *ELP1* transgene is large (4 kb), making packaging challenging. This would provide more flexibility in promoter choice to maximize transduction efficiency in RGCs.

FD is a complex developmental and degenerative disease, yet because it is caused by a single splicing-associated point mutation, it is an excellent candidate disease for both gene therapy and small molecule splicing modulators. In summary, the data presented here provide a

rationale for translating gene replacement therapy and splicing modulator compounds into the clinic to prevent the progressive optic neuropathy that plagues all FD patients.

Materials and Methods

Animals

All mice were housed in the AALAC-accredited Animal Resource Center at Montana State University. All animal use protocols were approved by the Montana State University Institutional Animal Care and Use Committee (IACUC) (protocol No. 2020-15-IA; Bozeman, MT). The study fulfilled the ARRIVE guidelines, and all experiments complied with relevant guidelines and regulations. The generation of the *Pax6cre⁺;Elp1^{lox/lox}* CKO is described in Ueki et al., 2018. Briefly, retina-specific *Elp1* CKO mice were generated by crossing *Elp1* floxed (International Knockout Mouse Consortium, Wellcome Sanger Institute, UK) and α Pax6 promoter-driven Cre (*Pax6-Cre*) mice [35], which were a gift from the original founders (Drs Peter Gruss and Ruth Ashery-Padan). This is a useful mouse model to study the FD optic neuropathy due to the restricted deletion of *Elp1* only in retinal neurons with a time course of RGC loss consistent with patients while maintaining a phenotypically healthy mouse. A new hybrid mouse model, the *TgFD9;Pax6cre;Elp1^{loxp/loxp}* was generated to analyze the splicing compound in a mouse model that expressed the FD founder mutation on a mouse background that was null for mouse *Elp1*. To this end, we crossed our original *Pax6-cre;Elp1^{loxp/loxp}* mice, with *FD9/FD9; Elp1^{loxp/loxp}* transgenic line that expresses nine copies of the human FD *ELP1* mutation [36]. To ensure that crossing in the *FD9* transgene did not, by itself, rescue the loss of RGCs in the *Pax6cre;Elp1^{loxp/loxp}* model, we quantified RGCs in both models at

3 months and found a ~55% reduction, indicating that the low levels of *ELPI* transcribed from the human gene was not sufficient to prevent the death of RGCs.

The sample size for all experiments was based on an A priori power analysis of 90% with an effect size of 2 and α of 0.05 to detect a statistical difference between the experimental and control groups [21; Ueki and Lefcort, unpublished]. Both male and female mice were used at the ages indicated (E18.5 up to 6 months), and all controls were littermate *Pax6cre⁻;Elp1^{loxp/loxp}*. Mice were randomly assigned to a treatment group based on sex and genotype. All mice were housed in a 12-hour light-dark cycle and were fed chow and water ad libitum.

AAV Vector Design and Production

All AAV viruses were made at the Vector Core in the Gene Therapy Center at the University of Massachusetts Medical School. AAV2 was selected based on its reported tropism for mouse retinal ganglion cells and its use and tolerance in the human eye [37-39] and preliminary experiments by Yumi Ueki et al. (unpublished) revealing its higher expression efficiency in retinal ganglion cells in our mouse line compared to AAV9. The AAV2 was used at vector doses ranging from 8×10^8 to 1.7×10^9 vector genomes per eye (vg/eye) per mouse for the mouse *Elp1*, while the human *ELPI* was used at vector doses ranging from 2.7×10^7 to 5.4×10^8 vg/eye. Vector doses were based on preliminary experiments showing potential viral toxicity at higher vector doses and evidence for cell rescue at lower vector doses (data not shown). Each virus expressed the full-length sequence for either mouse *Elp1* (Origene MC202501, NM 026079) or human *ELPI* (Origene RC2076868), *ELP1* (NM_003640), and both were driven by a murine small nuclear RNA promoter (U1a). The U1a promoter was selected based on its small size (~250 bp) and ability to effectively transduce cells in the central nervous system [40].

Cell Culture

Human embryonic kidney cells (HEK293) were gifted from Carolyn Machamer at Johns Hopkins, and the Chinese hamster ovary cell (CHO) cells were gifted from Ira Mellman at Yale University. HEK293 were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco #12430-054) supplemented with 10% fetal bovine serum (FBS, Gibco #16000-036), 100 U/ml penicillin/100 µg/ml streptomycin (Corning, #30-002-CI), and 2 mM L-Glutamine (Gibco, #25030-081). HEK293 cells submitted to American Type Culture Collection (ATCC) for STR profiling were an 88% match for the HEK293 cell line, CRL-1573.

In Vitro AAV transduction

HEK293 cells were seeded onto a 12-well plate at a density of 2.5×10^4 cells/well for 24hr. Two viral solutions were diluted (1) AAV2-U1a-h*ELPI* stock to 2.7×10^9 VG/ml in DMEM and (2) AAV2-U1a-eGFP stock to 8.8×10^9 VG/ml in DMEM. The *ELPI* solution was serially diluted to three different multiplicities of infection (m.o.i.) at 20K, 100K, and 400K. M.O.I. was calculated by dividing the initial seeding density by the total viral particles. At 24 hours, the complete medium was removed and supplemented with the viral medium. Cells proliferated for 6 hours in the viral medium. At 30 hours, the viral medium was removed and replaced by the complete medium. Cells were harvested 4 days post-transduction (PTD).

Western Blot

The western blot analysis was performed as previously described (*Ueki et al., 2018*). Briefly, cells were extracted on ice with lysis buffer (25 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 5% glycerol) containing 1X HALT protease inhibitor cocktail, 1 mM PMSF, and 10 µM Leupeptine. A Bradford 1X protein-dye reagent (Bio-Rad #5000205) was

used to determine the protein concentration. 25 µg of protein was separated by electrophoresis in an SDS-polyacrylamide gel, and the proteins were transferred onto a PVDF membrane. The membrane was blocked for 1 hour in 5% non-fat dry milk (NFDM) in 1X Tris-buffered saline containing 0.1% Tween-20. The membrane was incubated with primary antibodies overnight at 4°C. The primary antibodies to rabbit anti-Elp1 (1:1500) (Anaspec, #AS_54494)) and rabbit anti-GFP (1:2000) (Invitrogen, RRID: AB_221569) were diluted in 1X TBS containing 5% BSA and 0.1% Tween-20. Following primary incubation, membranes were probed with a secondary antibody (Horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Laboratories, #111-035-144), 1:10,000 dilution, in 1X TBS containing 5% NFDM and 0.1% Tween-20. Quantitative analysis was performed using Image J (NIH, USA) software, and bands were normalized to total protein and the housekeeping gene *Gapdh* (1:10,000, Millipore, NP_002037).).

RT-qPCR

Real-time PCR (RT-qPCR) was used to quantify mRNA collected from snap-frozen retinal tissue. Total RNA was isolated and purified using the Direct-zol RNA mini-prep kit (Zymo Research, #R2050). The RNA concentration and purity was measured using BioTek Epoch 2 microplate spectrophotometer. 25 ng of purified RNA was reverse transcribed using SuperScript IV VILO mastermix (Invitrogen, # 11756050). qPCR was performed with cDNA equivalent of 25ng of transcribed RNA using SYBR Green master mix (Bio-Rad, #336501) using the 7500 Fast Real-Time PCR system (Fisher Scientific, Applied Biosystems, #4351106). The cycling condition was performed per the manufacturer's protocol (Bio-Rad). All primers (Supplemental file 1) were designed using NIH Primer-Blast tool. The relative expression

analysis was performed using the delta delta CT method ($\Delta\Delta CT$) and expressed as fold change normalized to the average of the two housekeeping genes.

RT-PCR Analysis of *ELP1* Transcripts

Retinae were snap-frozen in a dry-ice bath with 95% EtOH upon collection. Tissues were homogenized in ice-cold TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), using a TissueLyser (Qiagen). Total RNA was extracted using the TRI reagent procedure provided by the manufacturer. RNA quality and yield was determined using a Nanodrop ND-1000 spectrophotometer. Reverse transcription was performed with 500ng of total RNA, random primers, and SuperscriptTMIII reverse transcriptase (Invitrogen). PCR was performed for the splicing analysis with the 3 μ l of cDNA in a total volume of 20 μ l with GoTaq Polymerase 2X (Promega) and 32 amplification cycles. We used human-specific ELP1 primers – forward, 5' - CCTGAGCAG CAATCATGTG- 3'; reverse, 5' -TACATGG TCTTCGTGACATC- 3' to amplify human ELP1 expressed from the transgene. The PCR products were separated on 1.5% agarose gels for 2.5 hours at 90V. The relative amounts of WT and mutant ($\Delta 20$) ELP1 spliced isoforms in a single PCR were determined using ImageJ and the integrated density value for each band as previously described [41, 42]. The relative proportion of the WT isoform detected in a sample was calculated as a percentage.

Intravitreal Injections

Three-week-old mice were anesthetized by isoflurane inhalation. Prior to anesthesia the pupil was dilated with one drop of phenylephrine (Phenylephrine Hydrochloride Ophthalmic Solution, USP 10%; National Drug Code, [NDC] 42702-103-05, Paragon, BioTeck, Inc) and one drop of tropicamide (Tropicamide Ophthalmic Solution, USP 1%, [NDC] 17478-102-12, Akorn

Pharmaceuticals). Two different intravitreal injection techniques (*Manual Hamilton Syringe* and *WPI-UMP3 with Mirco2T Injector*) were used to determine the optimal system for viral delivery. Each eye was treated as an independent experimental endpoint. All control-treated eyes were injected with an AAV2 preparation expressing eGFP at 1.6×10^9 or 8.0×10^8 viral genomes. Experimental-treated eyes were tested in a dose-dependent manner to optimize transduction efficiency and RGC rescue. Experimental-treated eyes receiving the AAV2-U1a-*Elp1* were injected with 1.6×10^9 , 1.77×10^9 , 8.3×10^8 , or 8.6×10^8 viral genomes of murine *Elp1*. Experimental-treated eyes receiving the AAV2-U1a-*ELP1* were injected with either 5.4×10^8 , 2.7×10^8 , or 2.7×10^7 vector genomes of human *ELP1*.

Manual Hamilton Syringe: Using forceps, the eyelids were pushed back to expose the cornea. With a 32-G, a small hole was made at the margin of the cornea and sclera. This was necessary because the A 34-G needle (#W1690678) had a blunt tip. The 5 μ l calibrated Hamilton #65 needle was inserted at the margin of the sclera and cornea and into the vitreous cavity and 1 μ l of the viral solution was injected slowly into the cavity. The needle was left in position for another 15s and removed slowly.

WPI-UMP3 with Mirco2T Injector (#0916C): Using forceps, the eyelids were pushed back to expose the cornea. A 36-G beveled needle (NF36BV) attached to a 10 μ l Hamilton NANOFIL syringe (WPI, lot# 08C) was inserted at the margin of the sclera and cornea and into the vitreous cavity. Each eye was injected with 600 nL of AAV preparation bilaterally with a 200 nL/sec delivery rate. The needle was left in position for another 10s to allow the pressure in the injection system to equilibrate with the pressure in the eye and removed slowly. After all surgeries, Puralube® Vet Ointment (Dechra Veterinary Products) was placed on the eye per

IACUC guidelines. Mice were monitored and kept on a 37°C heating pad until awake and recovered and then transferred to their home cage and monitored for 1 to 2 hours. Mice remained in their home cage with cage mates until analysis, 3 months after treatment.

Oral Administration of Splicing Compound using a Formulated Diet

The newly hybrid transgenic mice carrying the human *ELP1* transgene with FD mutation *TgFD9;Pax6cre;Elp1^{flox/flox}* were fed a special diet containing 0.0008% of PTC-409680 (Purina Prolab 3000 with PTC-409680, #21032505A2i) corresponding to a daily consumption of 1.2 mg/kg/day.

Retinal Histology following Ocular Gene Therapy with AAV2-U1a-*mElp1*

Retina flat mount dissection and histology were performed as previously described [43, 44]. In brief, Mice were euthanized with CO₂ and the temporal surface of the eye was marked with a green tattoo dye (Ketchum Manufacturing) prior to enucleation. After the eyes were enucleated, a hole was made at the sclera-cornea margin and fixed overnight at 4°C in 4% PFA. Before antibody staining, the neural retinal layer was detached from the retinal pigment epithelium (RPE)/choroid layer. Rabbit anti-Brn3a (1:300; Synaptic Systems, Cat#411 003) was used as the primary antibody. Cy3 conjugated anti-rabbit (1:500, donkey) secondary antibody was purchased from Jackson Immuno Research. All antibodies were diluted in 1X PBS with 0.3% Triton X-100 and 5% bovine serum albumin (BSA, Cell Signaling Technology).

Retinal Histology following Ocular Gene Therapy with AAV2-Ula-hELP1

Mice were euthanized as stated above. Eyes were fixed at room temperature (RT) for 1 hour in 4% PFA. Retinae were then removed, with the temporal region marked by a small cut, and incubated in 4% PFA for an additional 15 minutes at RT. Tissue was permeabilized in 1X PBS containing 0.5% Triton X-100 for 30 minutes at RT. Non-specific binding was blocked by incubation with 5% BSA containing 0.5% Triton X-100 for 3 hours at RT. All retinae were incubated for 2 nights at 4°C in a primary antibody solution containing 2% BSA, 0.2% Triton X-100, an anti-Brn3 antibody (1:200, Santa Cruz, #sc-6026), and an anti-ELP1(IKAP) antibody (2µm/ml, Invitrogen, #PA5-111296, RRID AB_2856706). After overnight incubation at 4°C with secondary antibodies (Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 and Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 from Thermo Fisher Scientific, catalog # A-21447, RRID AB_2535864 and catalog # A10042, RRID AB_2534017), four identical cuts were made to flatten the tissue into four lobes, and the tissue was mounted with Prolong Gold (Invitrogen).

RGC Quantification

Flat mounts were imaged and tiled using a Leica DM6 Thunder microscope at a 10X magnification. For the AAV2-hELP1 injected retinae, flat mount images were obtained with a Leica DMI8 THUNDER imaging system using the navigator spiral scan at a 10X magnification. IMARIS software (Oxford Instruments) was used to quantify the number of Brn3a⁺ cells. To identify Brn3a⁺ cells, the spots module was used. The detected diameter was set at 8 µm in the peripheral region determined by a 1 mm² box placed at the edge of each quadrant in regions with the most cell death. If a specific region was damaged, the RGCs directly adjacent were counted.

If any complication with the intravitreal injection, retinal harvest, or histology resulted in overall damaged retinal tissue, the sample was excluded and omitted from the analysis. The experimenters were blind to genotype and treatment group for all RGC quantification. The total peripheral cell count is the sum of the cells counted in the four peripheral regions. We analyzed and counted all the Brn3a⁺ RGCs across the entire retina but only report data from the peripheral retina because the *Pax6-Cre* expression is not expressed in the central retina. Therefore, the loss of *Elp1* is restricted to the periphery [21].

Statistics

All data are expressed as mean $\pm$ SEM. Column data are plotted as a scattered dot-bar plot to show variability, and n shows the sample size for each data set. GraphPad Prism (version 9) was used to make all graphs. $P < 0.05$ was considered to represent a significant difference. Following confirmation of normal distribution, data were analyzed using an unpaired two-tailed student's t-test and/or one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

For AAV2-h*ELPI* injection data, we visually checked normality, found a non-normal distribution, and confirmed with a Shapiro test ($p = 7.89e^{-8}$). Due to non-normal distribution, we ran a Kruskal Wallis non-parametric test ($p=0.0005$). Followed by a post hoc Dunn test with Bonferroni correction, we found significance only between controls and *Pax6cre;Elp1^{loxp/loxp}* mice, regardless of treatment. Since we had a small sample size with unequal variance and were most interested in the relationship between the uninjected *Pax6cre;Elp1^{loxp/loxp}* and the *Pax6cre;Elp1^{loxp/loxp}* receiving the higher dose of 5.4×10^8 vg/eye, we tested for normality and found a non-normal distribution. Therefore, using a Mann-Whitney U test, we reject the null

hypothesis that the distributions are equal and conclude there is a difference between uninjected *Pax6cre;Elp1^{loxp/loxp}* and the *Pax6cre;Elp1^{loxp/loxp}* that received the highest vector dose ($p = 0.02$). All statistics were performed using GraphPad Prism (version 9) and R version 4.2.1 (R Core Team, 2022).

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Materials availability

The newly designed AAV2 vectors are publicly available from Vector Core in the University of Massachusetts Medical School Gene Therapy Center.

Data availability

All data needed to assess the conclusions from the study are included in the paper and/or supplemental files. When applicable, individual data points are shown on each graph. Please address any requests for raw data counts to the corresponding author.

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Additional Information

The authors declare competing financial interests.

Xin Zhao, Jana Narasimhan, and Marla Weetall are employees of PTC Therapeutics, Inc., a biotechnology company. In connection with such employment, the authors receive salary,

benefits, and stock-based compensation, including stock options, restricted stock, other stock-related grants, and the right to purchase discounted stock through PTC's employee stock purchase plan.

Susan A. Slaughaupt is a paid consultant to PTC Therapeutics and is an inventor on several U.S. and foreign patents and patent applications assigned to the Massachusetts General Hospital, including U.S Patents 8,729,025 and 9,265,766, both entitled “Methods for altering mRNA splicing and treating familial dysautonomia by administering benzyladenine,” filed on August 31, 2012 and May 19, 2014 and related to use of kinetin; and U.S. Patent 10,675,475 entitled, “Compounds for improving mRNA splicing” filed on July 14, 2017 and related to use of BPN-15477.

Elisabetta Morini and Susan A. Slaughaupt are inventors on an International Patent Application Number PCT/US2021/012103, assigned to Massachusetts General Hospital and PTC Therapeutics entitled "RNA Splicing Modulation" related to use of BPN-15477 in modulating splicing.

All other authors declare no competing interests.

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Figures

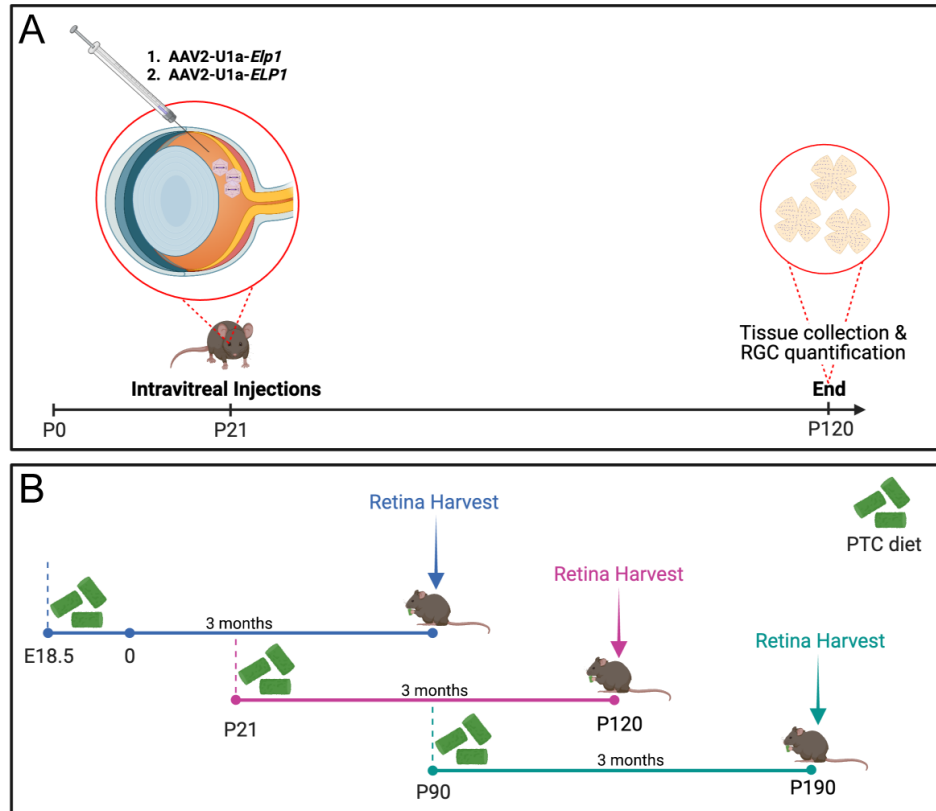


Figure 1: Experimental workflow.

(A) Mice were intravitreally injected with an AAV2-vector in both eyes at P21, and the retinal ganglion cell (RGC) number was quantified 3 months later. An experimental or control virus was injected into the vitreous humor of the mouse eye, where it can diffuse into the retina and target RGCs. (B) FD mutant mice were divided randomly into two groups: control and experimental. The control group was fed a standard chow diet, and the experimental group with a specially formulated chow diet containing a novel splicing modulator compound, PTC680. Groups were started on treatment at three different ages: E18.5, P21 or P90. After 3 months on either the control or experimental diet, retinas were analyzed and quantified for exon 20 inclusion and RGC numbers, respectively. Created with BioRender.com

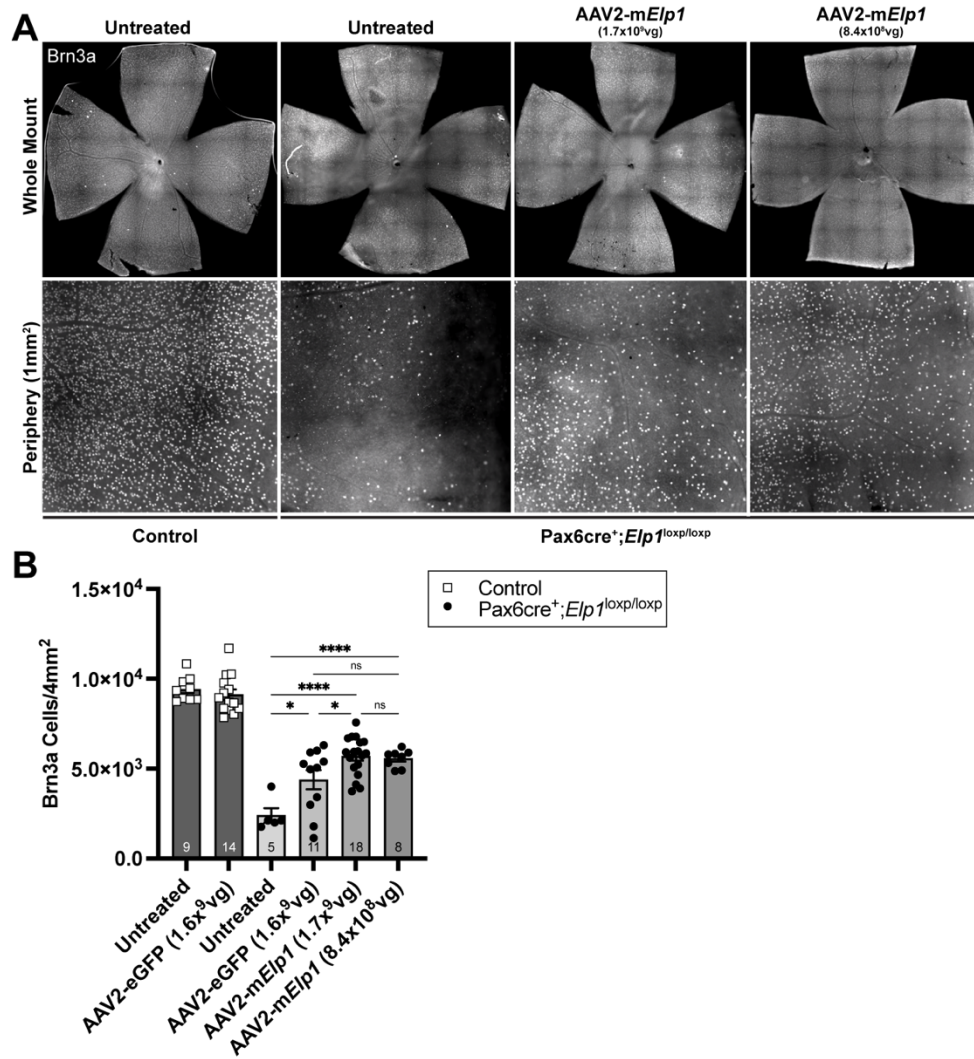


Figure 2: Viral transduction of murine Elp1 rescues RGCs in a mouse model of FD. (A) Representative retinal whole mount (top) and peripheral retina (bottom) 3 months after intravitreal injection with AAV2-U1a-mElp1. RGCs were immunolabeled using an antibody against Brn3a. Scale bar, 500 μ m. (B) Quantification of Brn3a + RGCs from control mice (dark gray) compared to FD mice (lighter gray gradient) receiving injections at P21 with either the eGFP (1.6x10⁹ vg/eye) or experimental mElp1 virus (1.6x10⁹ vg/eye, 1.7x10⁹ vg/eye, 8.4x10⁸ vg/eye). Brn3a + cells were counted in a 1mm² area in each quadrant of the peripheral retina. The adjusted p-values are displayed. ns p = 0.2, 0.99, *p = 0.02, 0.04, **** p < 0.0001, one-way ANOVA with Tukey's multiple comparisons follow-up test. Data are shown as average $\pm$ SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar.

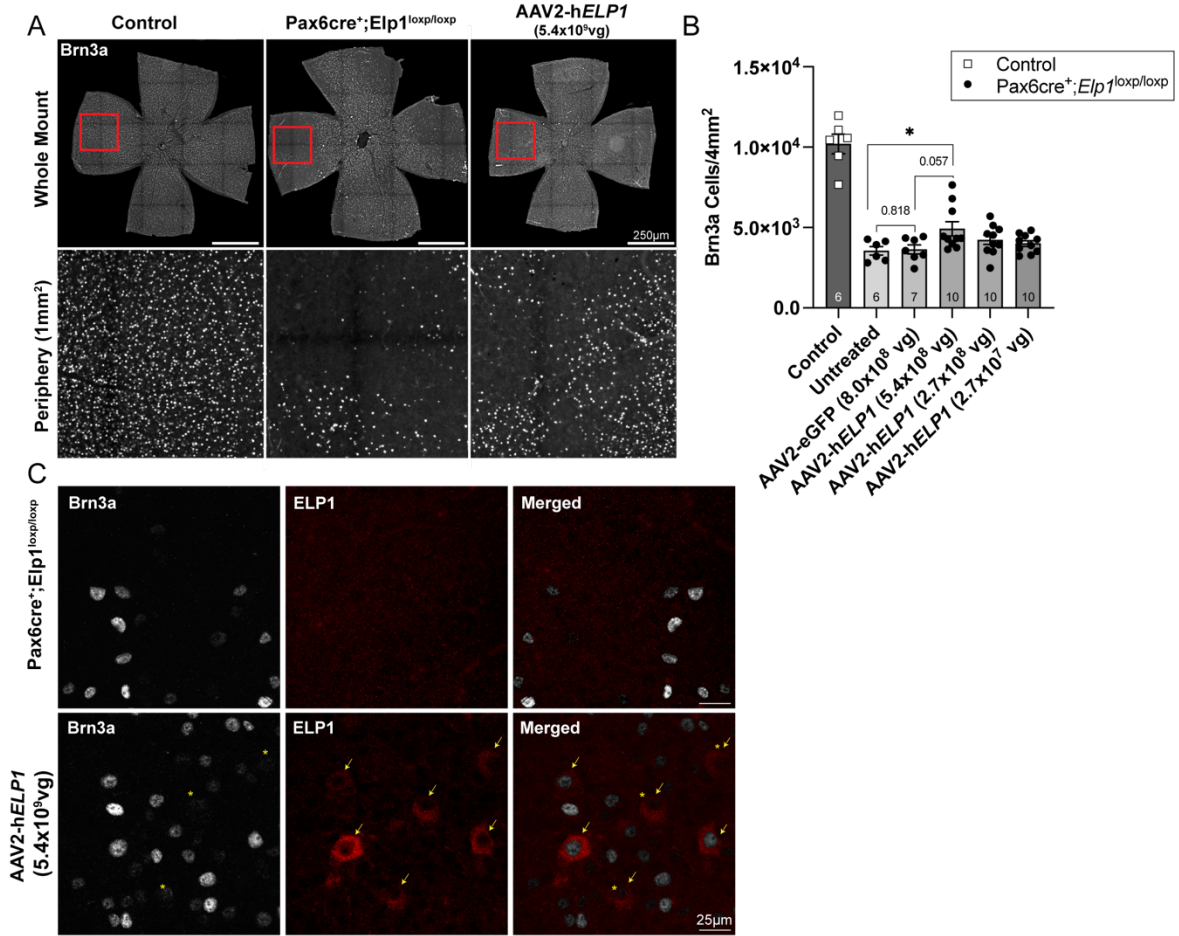


Figure 3: In vivo expression of human ELP1 prevents loss of RGCs in a mouse model of FD. (A) Representative retinal whole mount (top) and peripheral retina (bottom) 3 months after intravitreal injections with *hELP1*. RGCs were immunolabeled using an antibody against Brn3a. Scale bar, 250μm. (B) Quantification of Brn3a + RGCs from control littermate mice (dark gray) compared to FD mice (lighter gray gradient) receiving injections at P21 with either the eGFP (8.0×10^8 vg/eye) or experimental *ELP1* (5.4×10^8 vg/eye, 2.7×10^8 vg/eye, 2.7×10^7 vg/eye) virus. Brn3a + cells were counted in a 1mm² area in each quadrant of the peripheral retina. (C) Representative images of Brn3a+ RGCs (gray, yellow asterisks represent lower Brn3a intensities) and the human ELP1 protein expression (red, yellow arrows) 3 months after intravitreal injections into mutant mice. Scale bar, 25μm. The adjusted p-value is displayed. *p = 0.02, ns = 0.818, ns = 0.057; unpaired, two-sided Mann-Whitney U test. The only statistically significant difference is between the untreated mice and mice receiving the higher dose of 5.4×10^8 vg/eye. Data are shown as average ± SEM, and each data point represents an individual retina. The sample size for individual groups is represented by *n* within each bar.

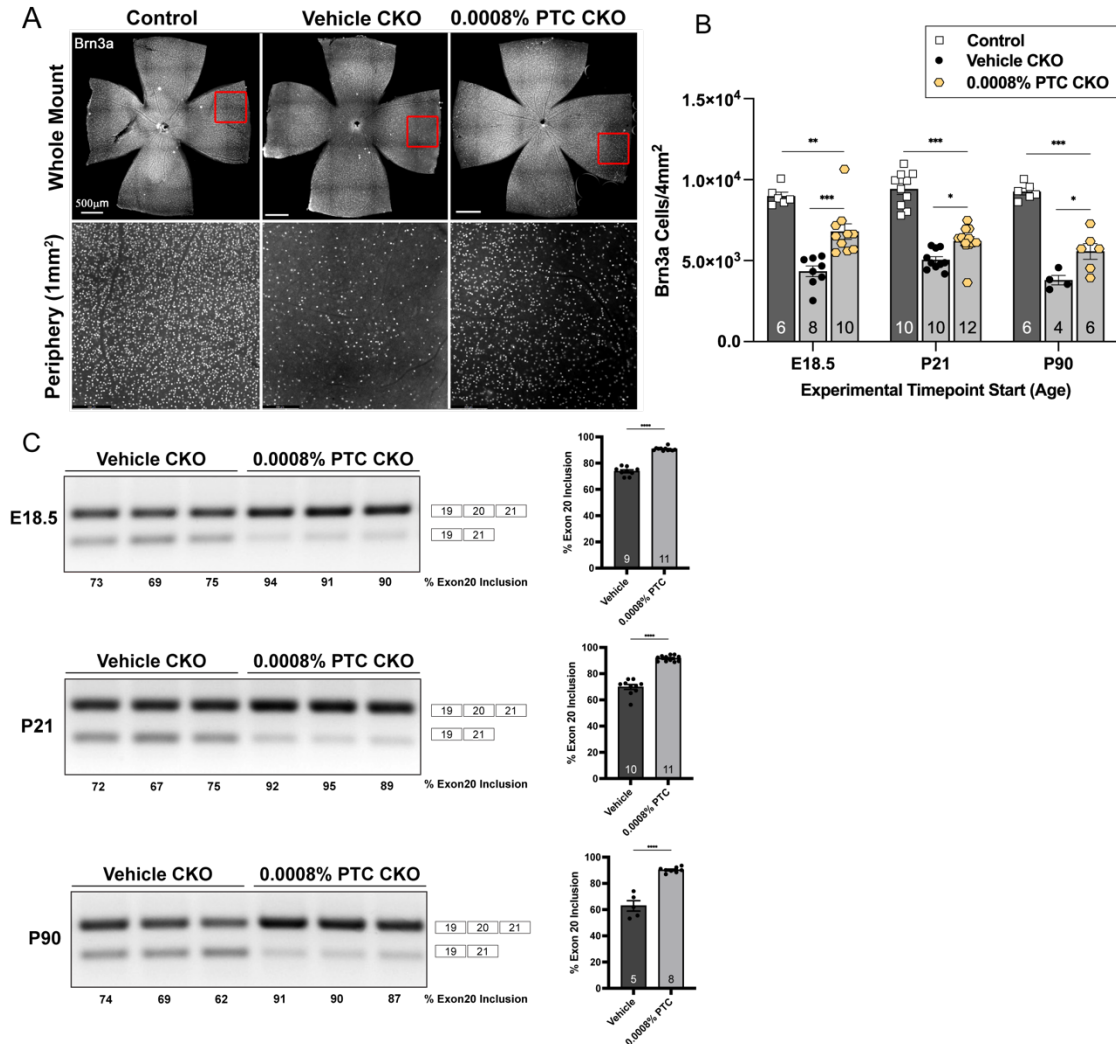
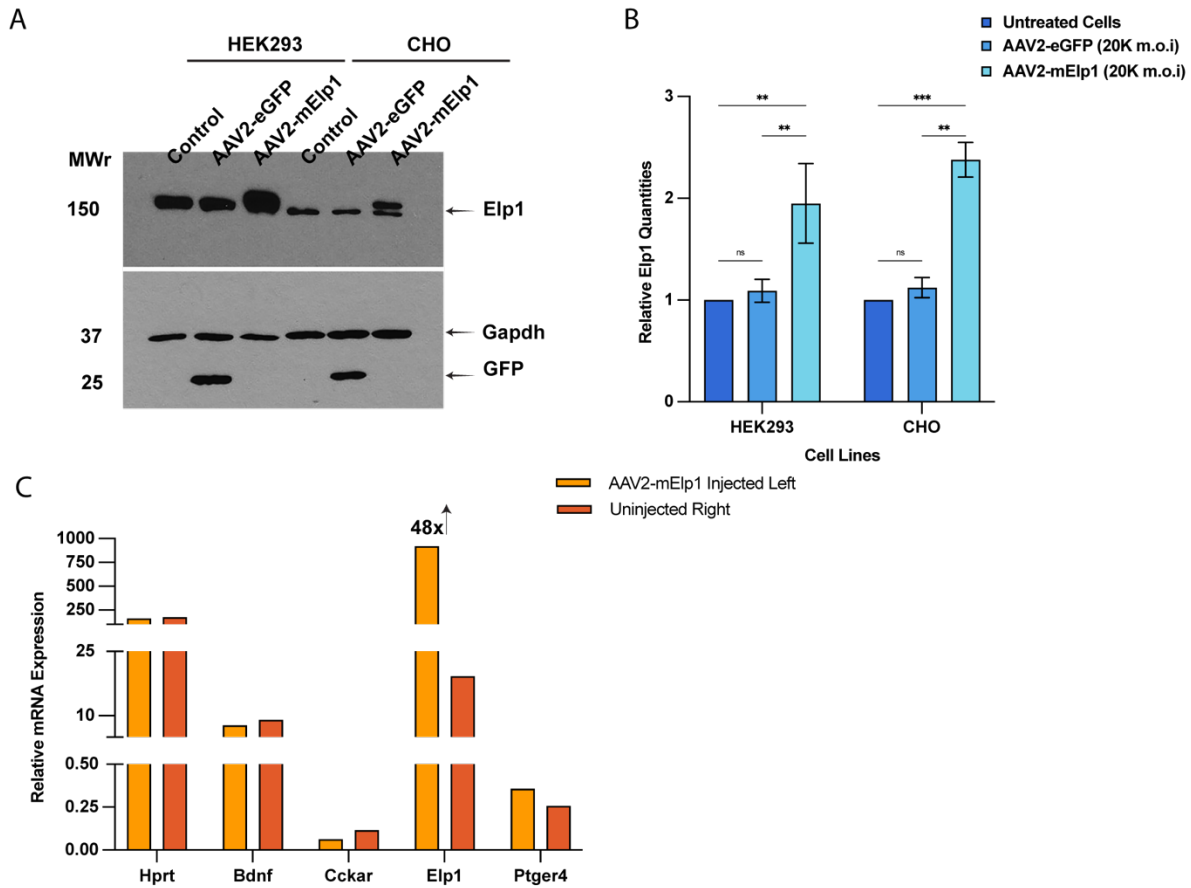


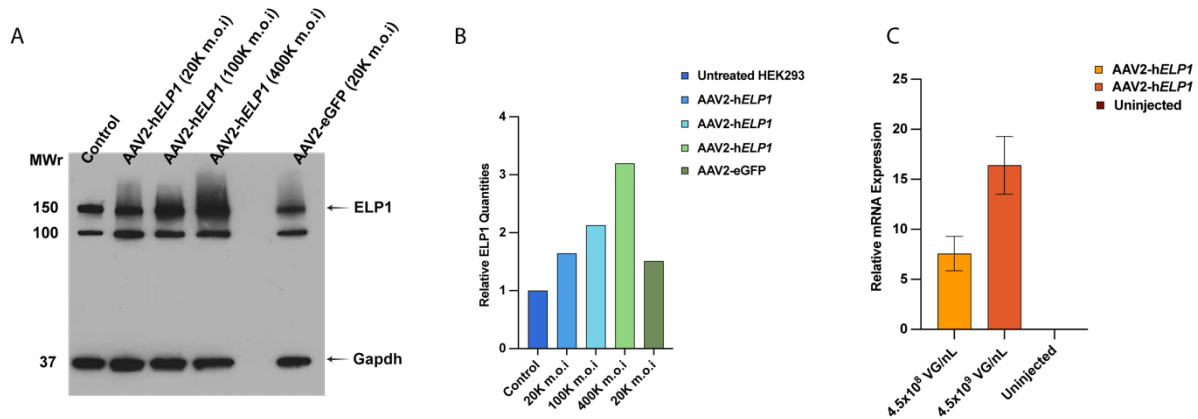
Figure 4: Daily PTC680 consumption protects retinal ganglion cells from cell death and increases full-length ELP1 transcript in a new retinal FD mouse model.

(A) Representative retinal whole mount (top) and peripheral retina (bottom) after daily consumption of PTC680 for 3 months. Scale bar, 500µm. (B) Quantification of Brn3a+ cells from control littermate mice (dark gray) and FD mice (light gray) on the vehicle or experimental chow. All mice consumed chow for 3 months, but each experiment started when the mice were at a different age: E18.5 (**p = 0.004, ***p < 0.001), P21 (*p = 0.01, ***p = 0.0000) or P90 (*p = 0.02, ***p = 0.0000), to test the efficacy of starting treatment at different time points within disease progression. The adjusted p-values are displayed, one-way ANOVA with Tukey's multiple comparisons follow-up test (C) Representative splicing analysis of human *ELP1* transcripts and percent of exon 20 inclusion in retinas from vehicle-treated (dark gray) and PTC 0.0008%-treated (light gray) FD mice at the different experimental start times. The adjusted p-values are displayed. ****p < 0.0001, two-tailed unpaired Student's *t*-test. Data are shown as average ± SEM, and each data point represents an individual retina. The sample size for individual groups is represented by *n* within each bar. This experiment was conducted three times. Conditional knockout (CKO) = Pax6cre⁺;Elp1^{lox/lox}



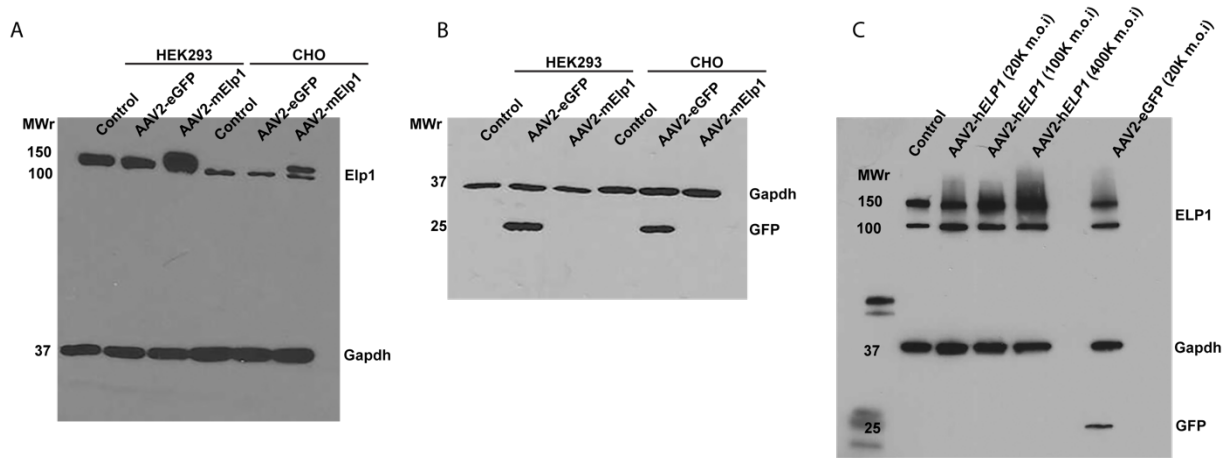
Supplemental Figure 1:

(A) Representative immunoblot showing the expression of murine Elp1 in two different cell lines 4 days post-transduction with AAV2-U1a-m*Elp1* and AAV2-U1a-eGFP. This is a compiled image from two separate gels run simultaneously (see supplemental figure 3 for a full gel image). There is an increase in Elp1 expression in the transduced cells. 20µg of protein was loaded in each well, and GAPDH was used as the loading control. (B) Densitometric analysis of Elp1 protein in HEK293 and CHO cells transduced with AAV2-U1a-m*Elp1* compared to untreated cells (HEK293: **p = 0.004, 0.005; CHO: **p = 0.001, ***p = 0.0008, two-way ANOVA with Tukey's multiple comparisons follow-up test. The experiment was done in triplicate. (C) RT-qPCR analysis of *Elp1* expression in retinal homogenates 1 month after intravitreal injections of AAV2-U1a-m*Elp1*. Only Elp1 is strongly increased.



Supplemental Figure 2:

(A) Representative immunoblot showing the upregulation of human ELP1 in HEK293 cells 4 days post-transduction with AAV2-U1a-h*ELP1*. Note the gradual increase in ELP1 expression as the multiplicity of infection (m.o.i.) increases. Equal amounts of protein were loaded in each well, and GAPDH was used as the loading control. This is a cropped gel image (See supplemental figure 3 for a full gel image). (B) Densitometric analysis of ELP1 protein in HEK293 cells transduced with AAV2-U1a-h*ELP1* compared to untreated cells. The experiment was done five times. (C) RT-qPCR analysis of *ELP1* expression in retinal homogenates (n=2) 1 month after intravitreal injections of AAV2-U1a-h*ELP1*. As expected, we saw no expression of the human ELP1 gene in the uninjected control homogenates, whereas the control mice receiving AAV2-U1a-h*ELP1* showed increasing levels of human ELP1 protein or mRNA.



Supplemental Figure 3: Representative immunoblot showing full gel images.

(A) The full gel image from supplementary figure 1 uses a 7.5% gel showing the expression of murine Elp1 in HEK293 and CHO cells. The PVDF membrane was cut into 2 pieces before antibody hybridization. (B) The full gel image from supplementary figure 1 uses a 12% gel; immunoblot shows the expression of GFP in HEK293 and CHO cells. Only the bottom half of the PVDF membrane containing the smaller proteins was used and was cut into 2 pieces before antibody hybridization. (C) The full gel image from supplementary figure 2 uses a 4-15% gradient gel; immunoblot shows GFP expression in HEK293 cells. The PVDF membrane was cut into 3 pieces before antibody hybridization. For all western blot procedures, the individual membrane pieces were developed simultaneously on a single piece of X-ray film.

Supplemental Table 1: List of oligonucleotide primers

Sequence Name		Seq 5' to 3'	Accession No.
ELP1	F	CTCTGCAGTCTCAGCACACA	NM_003640.5
ELP1	R	CTGCTCCAGGATTGGCTCAA	
Elp1	F	GGTGACAGTCTTTCGGCAGA	NM_026079.3
Elp1	R	GATCAGCAGCCGGTAGGTAC	
Cckar	F	TGAACAAACGCTTTCGCCTG	NM_009827
Cckar	R	TGGCTGTAGGAATACCGGGA	
Ptger4	F	CACCACCTCGCTGAGAACTT	NM_008965
Ptger4	R	TCCTTTAGAGGCAGGCTCCT	
Hprt	F	TCAGTCAACGGGGGACATAAA	NM_013556
Hprt	R	GGGGCTGTACTGCTTAACCAG	
Actb	F	AACCCTAAGGCCAACCGTGAA	NM_007393
Actb	R	TCACGCACGATTTCCCTCTCA	
Bdnf	F	ACTGCAGTGGACATGTCTGG	NM_007540
Bdnf	R	AGTTGGCCTTTGGATACCGG	

Supplemental Table 1: Forward (F) and reverse (R) primer sequences used for RT-qPCR experiments on RNA extracted from retinal tissue.

CHAPTER THREE

ALTERATIONS IN THE RETINAL NEUROVASCULAR UNIT IN FAMILIAL
DYSAUTONOMIA

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Manuscript Information Page

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Introduction

Familial dysautonomia (FD) is a progressive autosomal recessive disorder that devastates nervous system function. It is caused by a point mutation in a splice site of the *ELP1* gene¹, leading to the skipping of exon 20 and a tissue-specific reduction of ELP1 protein due to nonsense-mediated decay of this mutant mRNA.^{5,12,13} This tissue-specific defect primarily affects the nervous system. ELP1 is a highly conserved scaffolding protein and is a core subunit of the six-subunit Elongator complex that regulates translation by chemically modifying transfer RNAs (tRNAs), specifically wobble uridines.^{27,28,44} Consequently, insufficient ELP1 protein and loss of Elongator function leads to the developmental death of neurons in the sensory and autonomic nervous systems and progressive neurodegeneration in the peripheral and central nervous systems.^{1,7,8}

FD patients have a complex neurological phenotype with gait ataxia, diminished pain and temperature perception, impaired sense of taste, decreased corneal sensitivities, and dry eyes.^{6,7} These phenotypes are variable from patient to patient. However, a clinical hallmark of FD is progressive visual impairment marked by reduced visual acuity, central visual field deficits, and temporal optic nerve pallor.^{39,45} Clinical findings show a significant reduction in the retinal nerve fiber layer (RNFL) of the maculopapillary region, suggesting that the visual impairment is primarily due to progressive loss in retinal ganglion cells (RGCs). The preferential loss of RGCs and optic nerve damage found in FD patients resembles other mitochondrial optic neuropathies, such as Leber hereditary optic neuropathy (LHON) and autosomal-dominant optic atrophy (DOA). Both of these latter disorders display pronounced mitochondrial dysfunction with phenotypes similar to FD marked by an early loss of retinal nerve fiber layer (RNFL) thickness,

particularly in the papillomacular bundle, along with retinal vascular tortuosity in ~25% of patients.^{6,39–41}

Previous studies have quantified some mechanisms underlying the loss of RGCs, primarily in mouse models of FD showing the progressive loss of RGCs and confirming impairment in mitochondrial function and Complex I electron transport chain that recapitulate these deficits seen in patients.^{42,46} FD patients present with a hypermetabolic state, lower body mass index (BMI), increased resting energy expenditure levels, gastrointestinal dysfunction, and dysfunction in the gut-brain-liver metabolism axis.^{47–49} Suggesting an overall alteration in the patient's metabolism.

Energy generation in the retina is modulated dynamically in response to various stimuli: (1) long-term processes like development and aging and (2) short-term processes, which can be psychological or pathological, like light adaptation, hypoxia, or ischemia.⁵⁰ Three distinct retinal cell populations work in unison to respond to changes in metabolic and energy demands: (1) neurons, (2) glia, and (3) blood vessels⁵¹, often referred to as the neurovascular unit. Neuronal activity can induce changes in and directly regulate this coupling between the glial cells and blood vessels.³⁷ While neurons can modulate this system, the glial cells synapse with both neurons and vasculature, thus becoming an interface to regulate crosstalk between these cells.³⁷ Furthermore, studies have shown that glial cell dysfunction in retinal pathologies results directly or indirectly in neuronal and vascular damage.^{52–55} Additionally, there is evidence that mitochondrial activity is critical for angiogenesis, and dysfunction can lead to retinal vascular malformations however, these mechanisms are not well understood.⁵⁶

Therefore, given the mitochondrial dysfunction, metabolic alterations, and a preferential loss of RGCs via RNFL thinning in the papillomacular bundle in FD, a detailed investigation into potential indirect cellular mechanisms by which RGCs die in the FD retina is needed. In response to the dire need for developing therapeutics to reduce the inevitable loss of RGCs, this study aims to elucidate non-neuronal cellular perturbations involved with RGC death. We show here that alterations in the neurovascular unit develop within days of birth and precede the death of RGCs, which then undergo changes in cellular signaling (Schultz and Lefcort, in preparation).

Results

Alterations in Glial Cell Populations Precede Significant RGC Death and Continue throughout Disease Progression in a Mouse Model of FD.

The retina is a highly complex and tightly regulated tissue requiring large amounts of energy for function. It is comprised of ten layers from the outside to inside: retinal pigment epithelium (RPE), outer limited membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), nerve fiber layer (NFL), and inner limited membrane (ILM), forming an organized and very laminar structure. As one of the most metabolically active tissues, a multi-heterocellular structure of neurons (photoreceptors, horizontal and bipolar cells, amacrine cells, and ganglion cells), glial cells (Müller cells, astrocytes, and microglia), and endothelial cells form the metabolic unit known as the neurovascular unit (NVU) to this energetic requirement. The NVU is recognized as providing this functionality in the central nervous system (CNS) by regulating metabolism, providing trophic support, and removing waste from neurons.³⁷ However, understanding the metabolic exchanges between individual retinal cell types is poorly understood.⁵⁷

To identify potential triggers of RGC death, i.e., gliosis and inflammation, we analyzed the morphology and density of glial cells throughout disease progression. Work in our previously established Pax6-cre;Elp1^{loxp/loxp} mouse model, herein referred to as the FD retina, shows that there is significant RGC death by postnatal day 30 (P30).⁴² Therefore, we focused on three-time points (1) postnatal day 7 (P7), an age prior to the death of RGCs, (2) P30, an age that marks the period of rapid RGC death. And (3) P90 when cell death begins to plateau with an observed 50-60% reduction in RGCs.

Using immunofluorescent chemistry and confocal imaging, we assessed the expression pattern of three glial-specific markers, glial fibrillary acidic protein (GFAP), glutamine synthetase (GS), and ionized calcium-binding adaptor molecule 1 (Iba1) to identify the main glial subtypes: astrocytes, Müller cells, and microglia, respectively. Due to their close juxtaposition to RGC axons and blood vessels and their critical role in retinal development⁵⁰ we first examined astrocytes in the FD and control littermate retina. To our surprise, P7 – a time when neurogenesis and astrocyte migration is complete, and the reduction in the number of RGCs in the FD retina is not significant - we observed chaotic organization of astrocytes along with an upregulation in GFAP-positive signal in the FD retina compared to the control (Fig. 1A, B). Interestingly, the density of GFAP-positive astrocytes was not significantly different (Fig. 1C; $p=0.11$, Mann-Whitney), most likely due to the variability between samples. However, the density of GFAP-positive astrocytes trends higher in the FD retina than in the control littermate retinae. Whereas the GFAP signal intensity was significantly increased in the FD retinae compared to the controls (Fig. 1D; $p = 0.032$, Mann-Whitney). When activated, glia can produce

neurotrophic and growth factors.^{58,59} However, chronic gliosis can accelerate neurodegeneration and is a hallmark of disease onset.⁶⁰⁻⁶²

To determine whether this early perturbation of increased GFAP-positive fibers and labeling intensity leads to chronic astrogliosis, we immunolabeled retinal flat mounts at P30 (Fig. 2A) and at P90 (Fig. 2B). In both normal and diseased retinas, astrocytes showed the characteristic stellate morphology. However, at P30, we saw a significant increase in GFAP immunoreactivity in the nerve fiber layer of the FD retina compared to the control (Fig. 2C; * $p = 0.020$, unpaired multiple t-tests). Moreover, we continued to see increased GFAP immunoreactivity at P90 (Fig. 2C; ** $p = 0.0057$, unpaired multiple t-tests). At this older time point, areas with the most RGC degeneration also had increased GFAP-positive fibers and increased labeling intensity, characteristic of reactive gliosis higher densities of astrocytes (Fig. 2B)

The upregulation of intermediate filament proteins in glial cells is a hallmark of gliosis.^{63,64} When Müller glia are activated in a pathological state, they express high levels of GFAP.⁵³ Importantly, we do not see any GFAP-positive Müller glial end feet in the FD retinal whole mounts (Fig. 3A). Therefore, we hypothesize that there is a potential failure in Müller glia recognition of, and/or response to, a stressed environment. To investigate the involvement of the Müller glia in the CKO, we immunolabeled retinal cross-sections at P30, the age when a significant change in RGC number is first detected. Double labeling with GFAP and glutamine synthetase (GS), enriched in Müller cells, allowed visualization of the morphology of the entire Müller glia. Surprisingly, GFAP was not upregulated in the FD Müller glia (Fig. 3B) and the morphology of the Müller cells, revealed by staining with GS, remained relatively unchanged in

the CKO. No significant difference in GS expression between the FD and control retinæ was found (Fig. 3C). However, we did find a trend that GS expression is reduced in the FD retina compared to the control (data not shown). Furthermore, it is known that the low-affinity neurotrophin receptor, p75, is increased in Müller glia in response to chronic stress or alterations in their environment.^{65,66} Therefore, we explored if there were differences in p75 immunoreactivity in the FD retina. Again, we found no difference in p75 expression between our FD and control retinas (data not shown).

Both astrocytes and Müller cells are involved in the activation of microglia cells⁶⁷, and we have previously shown that the microglial marker, Iba1, is upregulated in the optic nerve of a 9-month-old of a different mouse model of FD⁶⁸. Therefore, we sought to investigate the distribution and morphology of microglia in the FD retina. Immunolabeling with Iba1 at three different time points shows that the overall number of microglia changes as the disease progresses and neurons die. At P7, quantification of Iba1-positive cells in retinal cross-sections (Fig. 4A) showed no significant difference between the FD and control retina (Fig. 4B; $p = 0.3906$, unpaired t-test). However, a significantly higher proportion of microglia (Iba1-positive cells) were found in the GCL of the FD retina (Fig. 4C; Supplemental Fig. 1A; $p = 0.0002$, unpaired t-test) compared to control, with 82% of total Iba1 cells located in the GCL in the FD retina compared to the 50% located in the GCL of the control.

Next, the morphology of the Iba1-positive cells was analyzed. The percentage of Iba1-positive cells with an amoeboid or activated shape was significantly elevated in the FD retina (Fig. 4D, E; $p < 0.0001$, unpaired t-test), with 78% of microglia being “activated” in the FD retina compared to 30% of microglia “activated” in the control retina.

Interestingly, as the disease progresses and mice age, a more uniform distribution of Iba1-positive cells was detected throughout the FD retina (Supplemental Fig. 1B), more in line with the distribution seen in the controls at both ages (P7 and P30). However, there were still significantly more microglial cells in the GCL (Supplemental Fig. 1B; $p = 0.000049$) and fewer Iba1-positive cells in the INL (Supplemental Fig. 1B; $p = 0.0012$). While the overall number of Iba1-positive cells is not significantly different between the control and FD retina at P30 (Fig. 4F, G; $p = 0.1901$, unpaired t-test), there was a decrease in Iba1-positive cells in the FD retina compared to the control. Again, 53% of all microglia were found in the GCL in the FD retina compared to 25% of all microglia being found in the GCL of the control (Fig. 4H). Moreover, more of these microglia were “activated” in the mutant than in the control retina (Fig. 4I, J; $p = 0.0571$, Mann-Whitney).

By P90, when the rate of RGC death plateaus, there was a change in the morphology pattern of Iba1+ cells and a significant increase in the number of microglia in the GCL of the FD retina compared to the control (Fig. 5A, B; $p = 0.0098$, unpaired t-test). There was, however, no difference in the number of morphologically “activated” or amoeboid cells in the FD retina (Fig. 5C; $p = 0.4672$, unpaired t-test).

Taken together, these data suggest that retinal homeostasis in the FD model is altered, in part, by perturbations in the macro and micro-glia cell populations within the retina in a few ways—first, the initial and constant upregulation of GFAP expression within astrocytes. Second, the Müller cells are (1) not activated nor exhibit a typical gliosis response to chronic neuronal stress and are (2) in a potential state of dysfunction, thereby not responding nor maintaining retinal neurons and the microenvironment, possibly leading to the FD pathological state. And

finally, the microglia populations appear to be heavily asymmetrically localized to the GCL with an amoeboid morphology, suggesting either an increased production in cytokines, chemokines, or neurotrophic factors and/or eliminating dysregulated synapses or phagocytosing dead cells and cellular debris. These findings raise the question of whether this difference in spatial localization is due to a cellular response to the microenvironment, or a delayed migration since, as mice age, the distribution of microglia becomes more uniform between the FD and control retinas. Furthermore, the alterations in the microenvironment potentially impact the neurons' ability to receive the trophic support necessary to respond to the chronic stress caused by a loss of Elp1.

Glial Alterations Coincide with Vascular Remodeling in the Retina.

Given the disruption and changes to the glia in the FD retina and their tight coupling with the retina vasculature, the retinal vasculature was analyzed over the same time course, P7-P90. Primarily focusing on the superficial plexus due to its direct interaction with RGCs, the density, morphology, and distribution of retinal vessels were analyzed in whole mount retinæ from control and FD mice. Retinal whole mounts were immunolabeled using the endothelial cell marker CD31 (Pecam-1), and noticeable vascular abnormalities were found at four different ages throughout disease progression (Fig. 6).

Starting at P7, the superficial plexus showed increased vessel outgrowth and hyper-sprouting in the FD retina compared to the control (Fig.6A I-II). Subsequently, at P19 – a time when all retinal development is complete, and the mice have opened their eyes - the superficial plexus continued to show dramatic abnormalities in the main veins and the arterial beds (Fig. 6B I-II). These alterations seen at P19 corroborate the results seen early in development at P7.

These morphological abnormalities continued through P30 (Fig. 6C I-II) and up until P90 (Fig. 6D I-II), with increased vessel densities and capillary branching from the veins in the FD retina compared to the control. Furthermore, the arterial beds were more pronounced with a greater distance from the main artery to sprouts in the FD retina at all ages (Fig. 6 A-D).

Quantitative analysis of capillary density using REAVER⁶⁹ showed a significant increase in the relative capillary surface area at P7 (Supplemental Fig. 2A; ** $p=0.0045$, unpaired t-test). As the mice aged and the disease progressed, vessel density was significantly increased across the FD retina compared to controls (Supplemental Fig. 2B-D; ** $p=0.0012$, *** $p=0.0006$, unpaired t-test). Based on the variation in the vascular distribution in the superficial plexus, a Sholl analysis was conducted using the ImageJ neuroanatomy plugin.⁷⁰ Strikingly, quantification via Sholl analysis at P7 revealed no difference in the number of intersections (Fig. 7A-B) despite the significant increase in vessel density from the branch count quantification (Supplemental Fig. 2E) and differences in the pattern of vascular development (Fig. 6A). However, all older ages (P19, P30, P90) showed significantly increased vascular branching and intersections in the FD retina compared to the control (Fig. 7C-H).

Consequently, we sought to understand the relationship between the vascular network and the progressive RGC death. Using CD31 and the retinal ganglion cell marker RNA binding protein with multiple splicing (RBPMS), the association between endothelial cells and RGCs at two older ages, P30 and P90 (Fig. 8A) was analyzed. Not only was increased vascular branching seen at P30 and P90 in the FD retina, but this vascular remodeling also occurred in such a pattern that vascular regression was observed in areas of severe RGC degeneration, alongside an increase in vessels in areas with surviving RGCs (Fig. 8B).

Discussion

Our results lay a foundation for an initial understanding of the mechanisms involved with RGC loss in FD concerning mitochondrial dysfunction and metabolic impairments. The complexity of the retina makes this tissue extremely vulnerable to any cellular alterations, resulting in cell signals that lead to morphological and functional changes.⁵⁵ In this case, we show that due to a loss of Elp1, there are morphological changes in glial cells along with vascular remodeling to potentially meet the RGCs' changing metabolic needs. Furthermore, an initial insult with an early upregulation of GFAP continues as the disease progresses, suggesting chronic gliosis. We also reveal information about microglia distribution in the retina. Microglia are consistently distributed within the GCL, IPL, and OPL in the control retina. In comparison, there is an asymmetrical distribution of microglia in the CKO and an increased percentage in amoeboid Iba1-positive cells, specifically in the GCL, indicative of the pathological state in the GCL, suggesting that the reactive morphology represents recruitment to clean up dying retinal ganglion cells, before when we see significant cell death—giving insight into an initial assault to the ganglion cells. This is the first time that glial distribution and morphology changes have been described in the FD retina. Imbalanced microglial activation is implicated not only in eye diseases e.g., glaucoma and diabetic retinopathy, but also in neurological disorders e.g., Alzheimer's, Parkinson's, and multiple sclerosis.⁷¹ An alternative explanation proposes a potential migration problem. Microglia primarily reside in the IPL and OPL^{71,72} and, during development enter the retina through the vitreal surface prior to vascularization and will invade the inner retinal layers before migrating to the outer layer.⁷³ Interestingly, Müller cells share a lineage with retinal neurons and, therefore, are directly affected by our Elp1 conditional

knockout in the *Pax6-cre;Elp1^{loxp/loxp}* mouse model. Therefore, Müller glia are directly affected by a loss of Elp1, while the astrocytes and microglia respond secondarily to the loss of Elp1 based on environmental cues.

Retinal astrocytes are required to pattern the developing vasculature, and the astrocyte pattern, in turn, depends on migration cues from RGCs. In *Math5* mutants (RGC knockouts), astrocytes fail to colonize the peripheral retina and subsequent angiogenesis⁷⁴, meaning astrocytes depend on RGC axons' cues as a template for migration and angiogenesis. Therefore, aberrant cues from the RGCs without Elp1 could influence the differences in astrocytic pattern and increased vessel density we observe in the CKO. Further studies are needed to confirm this.

The vision loss in FD presents similarly to other optic neuropathies, such as Leber's hereditary optic neuropathy (LHON) and dominant optic atrophy (DOA), with inflammation and changes to vasculature structure mediating the phenotype and providing disease biomarkers.^{75–77} As the disease progresses, the arterial branches will dilate and form torturous arteriovenous shunts, and as the ganglion cells atrophy and energy demand falls, the pathogenic vessels will withdraw.⁵⁰

These data shed light on neuron-glia interactions in the FD retina and provide evidence for downstream bidirectional signaling between cells, which we hypothesize exacerbates the disease state. Future work is needed to fully understand the subsequent effects of neuron/vascular communication, but these implications are broader than FD. Data collected here could provide insight into potential interventions for the FD optic neuropathy and other retinal diseases with vascular abnormalities, like glaucoma and diabetic retinopathy.

Materials and Methods

Animals

The generation of the *Pax6cre⁺;Elp1^{fllox/fllox}* CKO is described in Ueki et al., 2018. Briefly, retina-specific *Elp1* CKO mice were generated by crossing *Elp1* floxed (International Knockout Mouse Consortium, Wellcome Sanger Institute, UK) and α Pax6 promoter-driven Cre (*Pax6-Cre*) mice⁴³, which were a gift from Drs Peter Gruss and Ruth Ashery-Padan). All animal use protocols were approved by the Montana State University Institutional Animal Care and Use Committee (IACUC) (protocol No. 2020-15-IA; Bozeman, MT). Roughly equal numbers of males and females were used for this study (males = 28, females = 22). All mice were housed in the AALAC-accredited Animal Resource Center at Montana State University, kept on a 12-hour light-dark cycle and fed chow and water *ad libitum*.

Tissue Processing and Preparation for Cryosectioning

Eyes were enucleated, and the cornea and lens were removed, and the remaining eyecup was fixed for one hour in a 4% paraformaldehyde (PFA) in 1X phosphate-buffered saline (PBS) at room temperature (RT) with rotation. Eyecups were washed in 1X PBS for 15 minutes and cryopreserved incrementally, first in 10%, then 20%, and followed by a 30% sucrose (VWR, #0335-1KG) until the tissue sank at 4C. Following the sucrose gradient, the eyecups were flash-frozen in Tissue-Tek O.C.T Compound (Sakura, #4583) and stored at -80C. Using a Leica CM 1950 cryostat, 14µm coronal sections were cut at -22C and collected on microscope slides.

Retinal Immunocytochemistry for Cross-Section Preparations

As described above, eyes were harvested and fixed in 4% PFA for 1 hour. Slides with 14µm coronal sections stored at -20C were air dried at RT for 1 hour and rehydrated in 1X PBS for 20 minutes, followed by a 15-minute permeabilization in 1X PBS containing 0.5% Triton X-100 and blocked in 5% BSA containing 0.5% Triton X-100 for 1 hour at RT. Retinal sections were then incubated overnight at 4C in primary antibodies (Table 1) diluted in 1% BSA, 0.1% Triton X-100 in 1X PBS. The tissue was then washed in 1X PBS and incubated for 2 hours at RT in secondary antibodies (Table 2).

Confocal Microscopy and Image Analysis in Retinal Cross-Sections

Glutamine Synthetase (GS) (Müller Glia): Retinal sections following immunohistochemical staining with anti-GS (Table 1) were imaged using a Leica SP8 confocal microscope. Two regions of interest (ROIs) in three tissue sections per microscope slide (representative of an entire retina) for a total of 6 images per retina were collected using a 20X oil-immersion objective with a zoom of 1.5, resolution of 1024 x 1024, line average of 6. Z-stacks spanned 10µm in-depth and comprised 5 images per series. The ROI was determined by the structure and integrity of the DAPI+ tissue to not bias ROIs based on anti-GS signal. The pixel intensity of GS immunoreactivity was quantified between control and CKO tissue using Fiji (ImageJ, NIH). All images were imported in Fiji, and a scale of 2.6 pixels/µm was applied to all images to ensure similar measurements throughout the samples. All images were rotated so that the GCL was positioned horizontally at the top retinal layer. Using a threshold and particle analysis in ImageJ, we quantified how much of the stain was in the entire ROI (393.85µm X 393.85µm) by thresholding the pixel intensity. A threshold was set based on an average of the

most intense and dimmest stains. The same threshold was applied to all images. The first round of quantifications accounted for all pixel intensities in the NFL, GCL, IPL, INL, OPL, and ONL, as the Müller cells span the entire length of retinal tissue. The second quantification focused on the GCL due to our primary interest in cellular interactions with the ganglion cells. Again, a threshold was set based on an average of the most intense and dimmest stains in the GCL. The ROI was created by delineating the nuclei in the GCL using DAPI+ nuclei.

Iba1 (Microglia): Retinal sections following immunohistochemical staining with anti-Iba1 (Table 1) were imaged using a Leica SP8 confocal microscope. Two regions of interest (ROIs) in three tissue sections per microscope slide (representative of an entire retina) for a total of 6 images per retina were collected using a 20X oil-immersion objective with a resolution of 1024 x 1024, line average of 8. Z-stacks spanned 10µm in-depth and comprised 5 images per series. Cells were manually counted in Fiji (ImageJ). All images were imported into Fiji, and a 1.75 pixels/µm scale was applied to all images. All Iba1-positive cells that could clearly be identified as amoeboid, intermediate, or ramified were counted in each retinal layer. Each cell morphology was assigned “Type 1”, “Type 2”, or “Type 3”. Type 1 corresponded to an amoeboid shape defined by a large, bodied blob, no or few short thick extensions, no long fine processes, and relatively large round to variable shaped cell body. Type 2 corresponds to an intermediate morphology distinguished by larger soma, bushier, or stout extending processes, a hypertrophied or elongated cell body. Type 3 corresponds to a ramified or “resting” morphology distinguished by small oval or round somas, fine branches, and/or long waving processes (Rawlinson et al., 2020; Guo et al., 2022) If the cell morphology was not clear or discernable based on those criteria, they were excluded from the total count and analysis. Per retina, cells were counted in a

total area of 2mm². At post-natal day 7, 393 control Iba1+ cells and 371 CKO Iba1+ cells were counted. At P30, 157 control Iba1+ cells and 137 CKO Iba1+ cells were counted. Each cell type was binned according to each layer it was localized in. Using that information, we determined the asymmetrical distribution of amoeboid Iba1-positive cells in the GCL of the CKO compared to the control.

Retinal Immunocytochemistry for Flat Mount Preparations

Mice were euthanized with CO₂, and the temporal surface of the eye was marked with a green tattoo dye (Ketchum Manufacturing) prior to enucleation. After the eyes were enucleated, a hole was made at the sclera-cornea margin and fixed in 4% PFA at room temperature (RT) for 1 hour. The neural retinal layer was removed, and the temporal region was marked by a small 15-degree cut and incubated in 4% PFA for an additional 15 minutes at RT. Tissue was permeabilized in 1X PBS containing 0.5% Triton-X 100 for 30 minutes at RT with gentle shaking. Non-specific binding was blocked with 1X PBS containing 0.5% Triton-X 100 and 5% BSA at RT for 3 hr with gentle shaking. All retinæ were incubated for 2 nights at 4°C in primary antibodies (Table 1) diluted in 2% BSA, 0.2% Triton X-100 in 1X. Followed by overnight incubation at 4°C with secondary antibodies (Table 2). Four identical cuts were made to flatten the tissue into four lobes, and the tissue was mounted with ganglion cell layer-up on microscope slides with Prolong Gold (Invitrogen).

Confocal Microscopy and Image Analysis of Astrocytes in Retinal Flat Mounts.

Retinal flat mounts were imaged using a Leica TCS SP8 confocal microscope with an oil immersion objective at a resolution of 1024 x 1024 and a line average of 8. Z-stacks spanned

6 μ m in-depth with an image acquired every 1.5 μ m for a total of 5 images per Z-series and were kept constant between all retinas and timepoints. All settings were kept constant for a given antibody across retinas at each age (P7, P30, P90). However, the staining intensity cannot be directly compared between age groups because different signal intensities were used. For quantifying GFAP positive fiber signal intensity in the flat mounts, images were taken 1mm from the optic nerve in the periphery of the temporal, nasal, superior, and inferior regions. Fiji (ImageJ) was used to quantify the amount of stain in each image, i.e., the ROI percentage containing anti-GFAP (Table 1) positive pixels. The data is reported as density or intensity. The density is the area of the image containing a positive signal based on the designated threshold. The intensity is the intensity of the staining signal of each pixel above the threshold.

Microscopy and Image Analysis of Vasculature in Retinal Flat Mounts.

Morphological Remodeling in Superficial Plexus: Retinal flat mount images were obtained with a Leica DMI8 THUNDER imaging system using the navigator spiral scan at 20X/0.80 dry magnification. All images were acquired with 16-bit acquisition and 2x2 binning with a consistent illumination across samples. Z-stacks spanned the thickness of tissue (RNFL & GCL). Therefore, the Z-stack was set according to each retina, and we primarily focused on the superficial plexus based on its direct proximity to the ganglion cells. However, a step size of 1.5 μ m was kept consistent between all samples. Due to a difference in tissue thickness across samples, no analyses were included that require a consistent Z-stack, and all images were normalized to the ganglion cell layer. The entire superficial plexus, but no other vascular layer, was analyzed in these retinas.

Sholl Analysis: Traditionally, Sholl analysis is used by neuroscientists to measure complex dendritic arbors of neurons to better understand neural circuits and information processing. However, it can also be used to measure other structures with a defined focus point, e.g., retinal vasculature⁷⁰. All images were pulled into Fiji (ImageJ), the scale was set for each image as each retina is a slightly different size, images were converted from RGB to 8-bit, and a threshold was applied. The threshold was kept consistent. Using the Neuroanatomy ImageJ plugin⁷⁰, and the Legacy program, the center of the retina at the optic nerve was the focus point and center radius, and the outer radius was determined by the longest distance to the periphery of one lobe. For each retina, the radius step size was 0.15mm and was kept consistent for every image.

Statistics

Unless otherwise indicated, all data are expressed as mean $\pm$ SEM. Column data are plotted as scatter dot-bar plots to show variability, and *n* shows the sample size for each data set. GraphPad Prism (version 9) was used to make all graphs. $P < 0.05$ was considered to represent a significant difference. Following confirmation of normal distribution, data were analyzed using an unpaired two-tailed student's t-test and/or one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. All data follow a normal distribution unless otherwise indicated.

Figures

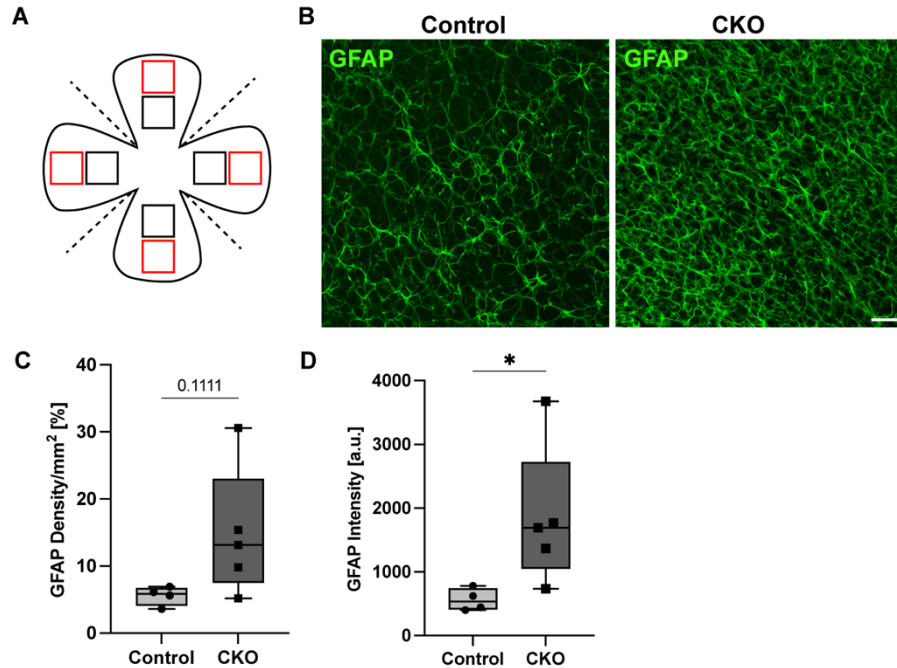


Figure 1: Morphological differences in retinal astrocytes in early postnatal life (P7).

(A) A retinal whole mount schematic representing the regions of interest (ROI) selected in each retina, primarily focusing on the ROIs in red. (B) Representative confocal images of control and FD retinas labeled with anti-GFAP (astrocytes) demonstrating the structural changes in the FD retina at P7. Scale bar, 50µm. (C) Boxplot for quantification of cell density of astrocytes (GFAP expression) in whole-mounted retinas showing the percentage of area occupied by the stain. $P = 0.1111$ (D) Boxplot showing quantification of GFAP signal intensity in whole-mounted retinas. $*p = 0.032$. P values are from the Mann-Whitney U test between control and corresponding FD groups due to non-normal data distribution. Whiskers: min to max. $n=4$ control; $n=5$ CKO.

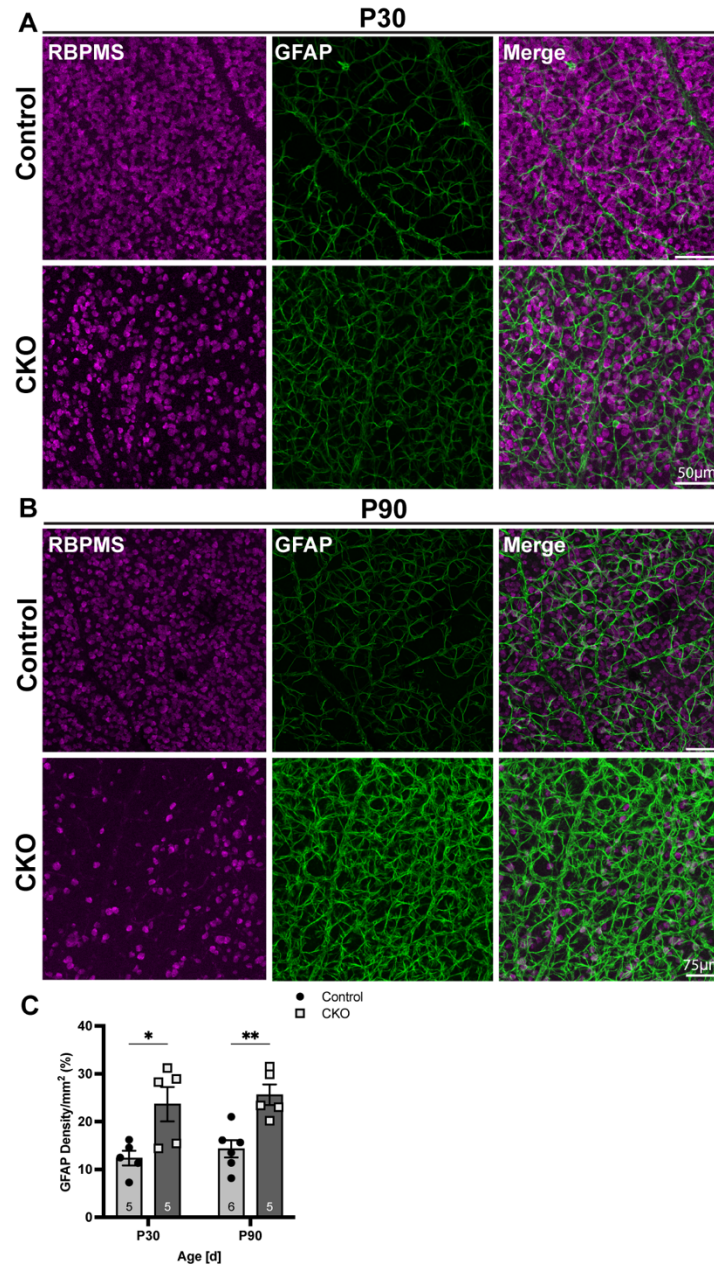


Figure 2: GFAP expression in FD retina throughout disease progression. (A) Representative confocal images of control (top panels) and CKO (bottom panels labeled with anti-GFAP (astrocytes, green) and anti-RBPMS (retinal ganglion cells, magenta) at P30 showing the localization and change in astrocyte morphology and the initial loss of retinal ganglion cells. Scale bar, 50 μ m. (B) Representative confocal images of control (top panels) and CKO (bottom panels) labeled with anti-GFAP (astrocytes, green) and anti-RBPMS (retinal ganglion cells, magenta) at P90 showing a more progressive loss of ganglion cells and an increase in GFAP-positive fibers in CKO. Scale bar, 75 μ m. (C) Quantification of pixel density showing how much area GFAP-positive signal occupies. The adjusted p-value is displayed. *p = 0.020 and **p

= 0.0057, multiple unpaired t-tests. Data are shown as mean $\pm$ SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar.

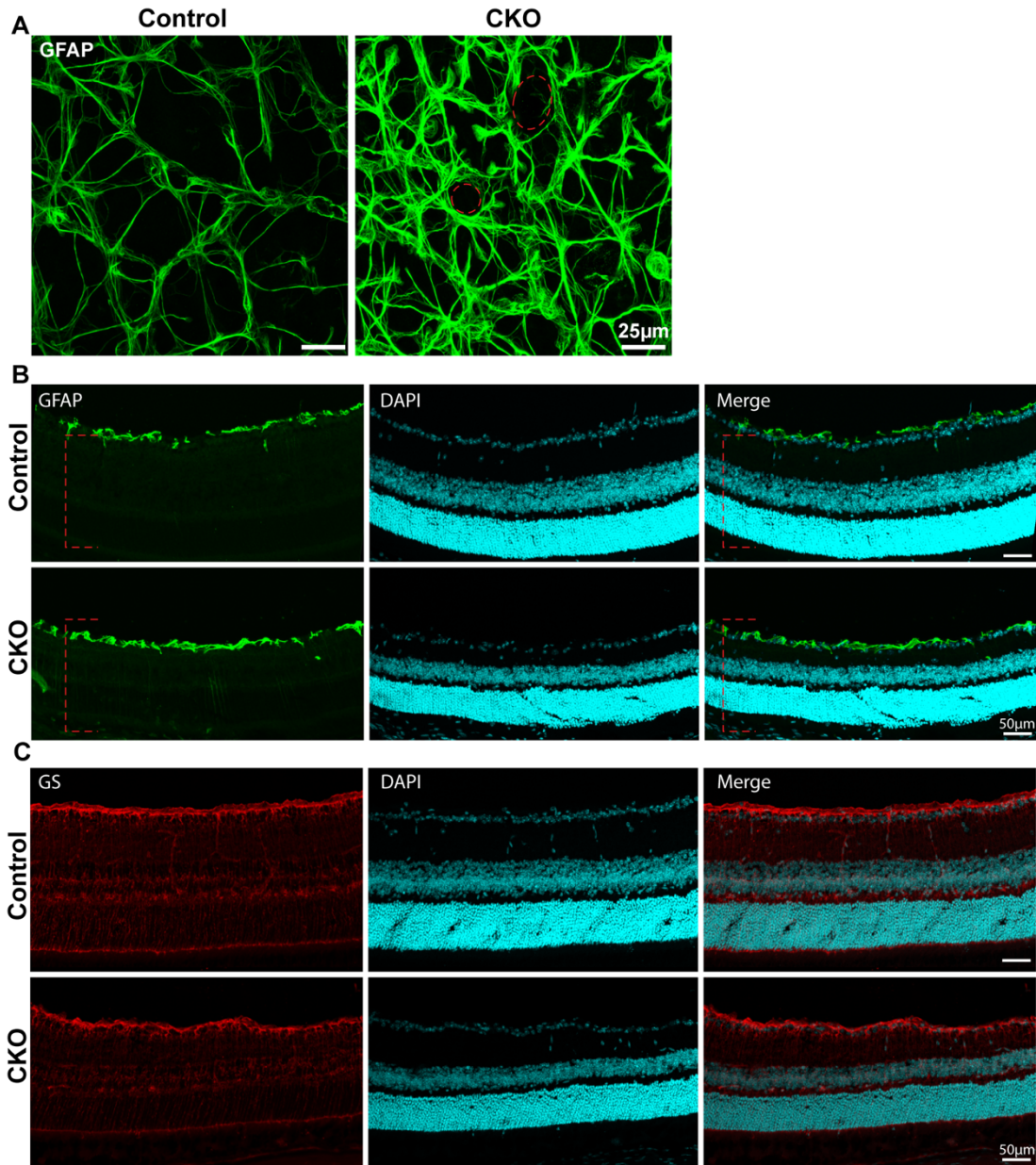


Figure 3: FD Müller glia are not changed at P30.

(A) Whole mounted retinae from a P30 control and FD mouse showed an increase in retinal astrocyte density (green) in the nerve fiber layer of the CKO compared to the control. Note the absence of GFAP positive Müller cell end-feet in both retinae (dashed red circles). Astrocytes are labeled with antibodies against GFAP. Scale bar, 25µm. (B-C) Immunostaining with GFAP (green) and Glutamine Synthetase (GS; red) shows no reactive change in the Müller cells in the FD retina compared to the control (dashed red lines) and no morphological changes or reduction in GS expression associated with the FD retinal pathology. Nuclei are stained with a nuclear marker 4'6-diamidino-2-phenylindole (DAPI; Cyan). Scale bar, 50µm.

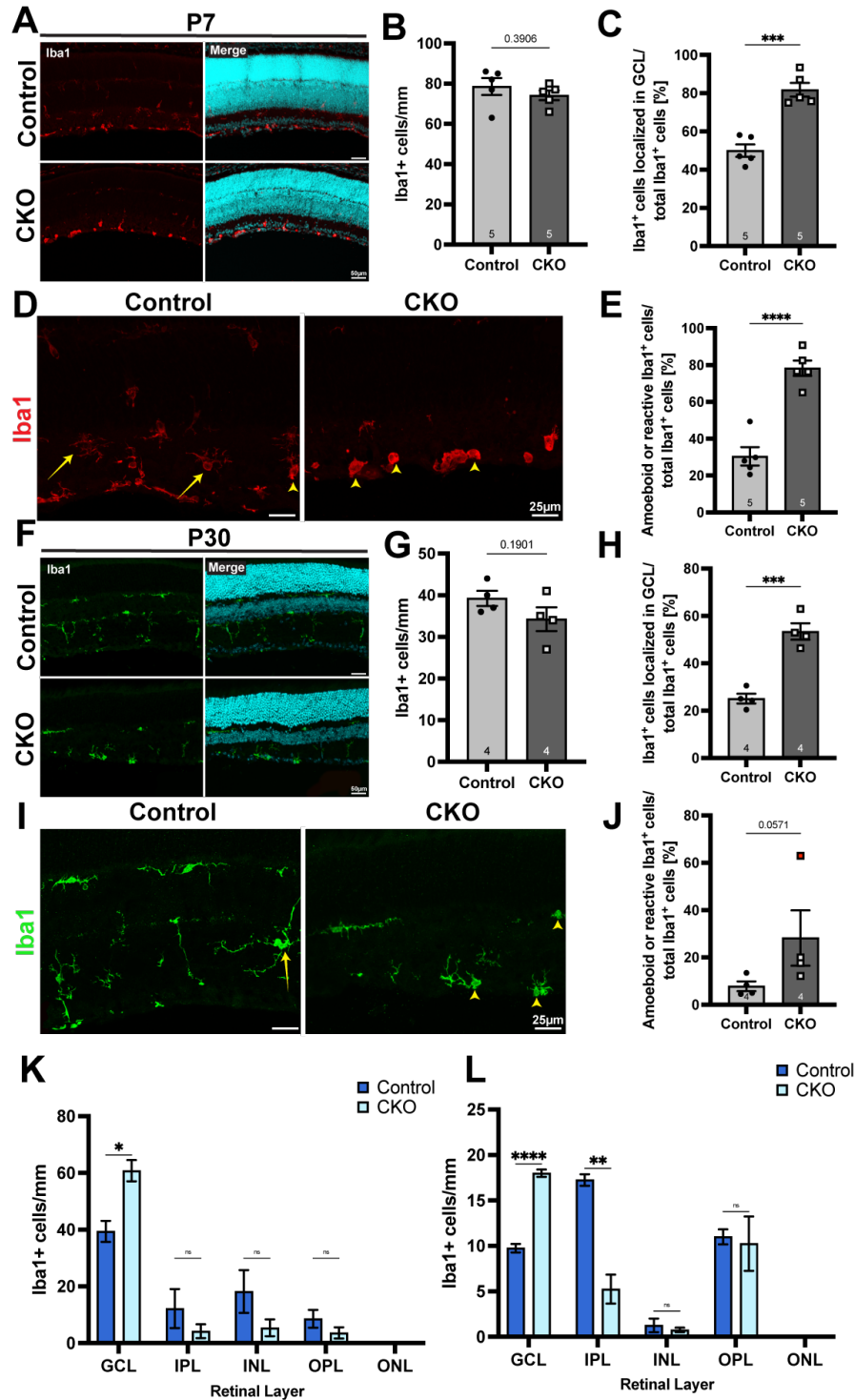


Figure 4: Microglia become activated and localize to the GCL as the disease progresses. (A) Representative images from a P7 healthy control retina and a P7 FD retina (CKO) immunolabeled with anti-Iba1 (red) and 4'6-diamidino-2-phenylindole (DAPI; Cyan). Scale bar,

50 μ m. (B) Quantification of positively stained microglial cells in retinal cross sections between control and FD retinae at P7. ns, $p = 0.3906$. (C) The average number of positively stained microglia cells localized in the GCL of the retinae analyzed in B. *** $p = 0.0002$. (D) High magnification images of Iba1 immunolabeling (red) in retinal sections show that prior to significant RGC death in the FD retina, microglia exhibit amoeboid/activated morphology (arrowhead) primarily localized to the GCL compared to more ramified/resting morphology (arrows) localized throughout the retinal layers in the control retinae. Scale bar, 25 μ m. (E) Quantification of amoeboid morphology microglial cells compared to the total number of microglia quantified in B. **** $p = <0.0001$ (F) Representative images from a P30 healthy control retina and a P30 FD retina immunolabeled with anti-Iba1 (green) and 4'6-diamidino-2-phenylindole (DAPI; Cyan). Scale bar, 50 μ m. (G) Quantifying positively stained microglial cells in retinal cross sections between control and FD retinae at P30, $p = 0.1901$. (H) The average number of positively stained microglia cells localized in the GCL of the retinae analyzed in G. *** $p = 0.0004$. (I) High magnification images of Iba1 immunolabeling (green) in retinal sections show that once there is significant RGC death in the FD retina, microglia continue to exhibit amoeboid/activated morphology (arrowhead) primarily localized to the GCL compared to more ramified/resting morphology (arrows) localized throughout the retinal layers in the control retinae. Scale bar, 25 μ m. (J) Quantification of amoeboid morphology microglial cells compared to the total number of microglia quantified in G. Red data point indicates a statistical outlier. $p = 0.0571$, Mann-Whitney U. (K) Microglia distribution by retinal layer at P7. (L) Microglia distribution by retinal layer at P30. P values are from unpaired t-tests unless otherwise noted. Data are shown as mean $\pm$ SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

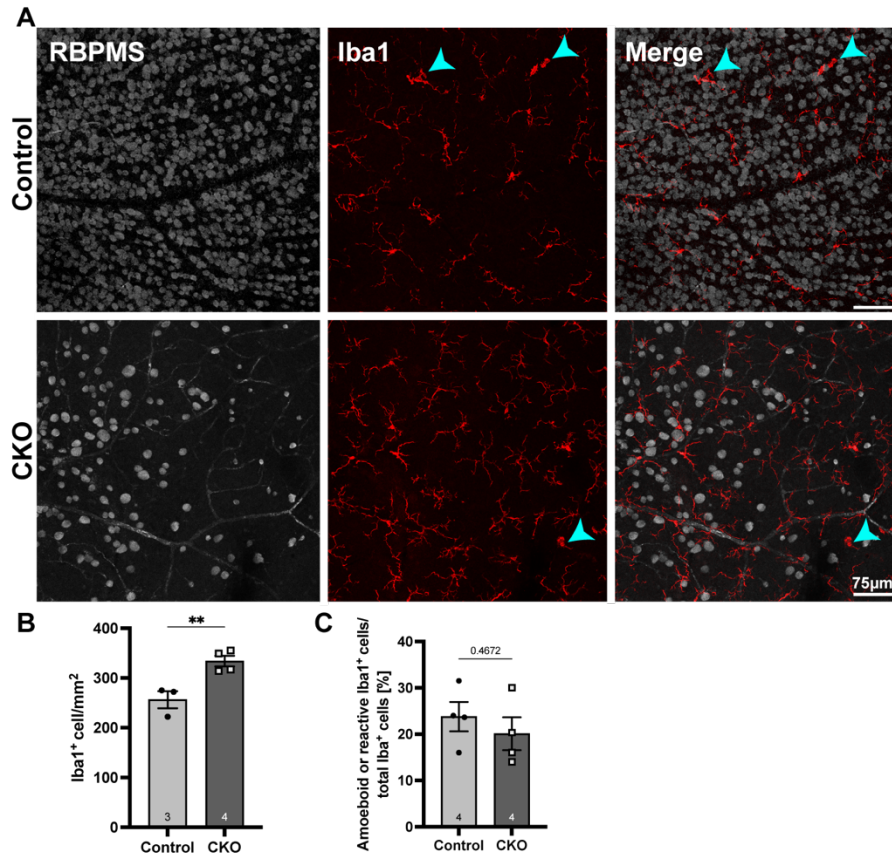


Figure 5: Microglia in the GCL increase in number and become more ramified towards the end of rapid RGC death.

(A) Representative confocal images of control and FD retina (CKO) labeled with anti-RBPMS (retinal ganglion cells, gray) anti-Iba1 (microglia, red) at P90 showing an increase in cell number and a reduction in the presence of the amoeboid morphology (blue arrowhead). Scale bar, 75 μ m. (B) Quantification of positively stained microglia cells (Iba1) in retinal whole mounts between control and FD retinas in the GCL, ** $p = 0.0098$. (C) Quantification of amoeboid morphology microglial cells compared to the total number of microglia quantified in B. ns, $p = 0.4672$. GCL, ganglion cell layer. P values are from unpaired t-tests. Data are shown as mean $\pm$ SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar.

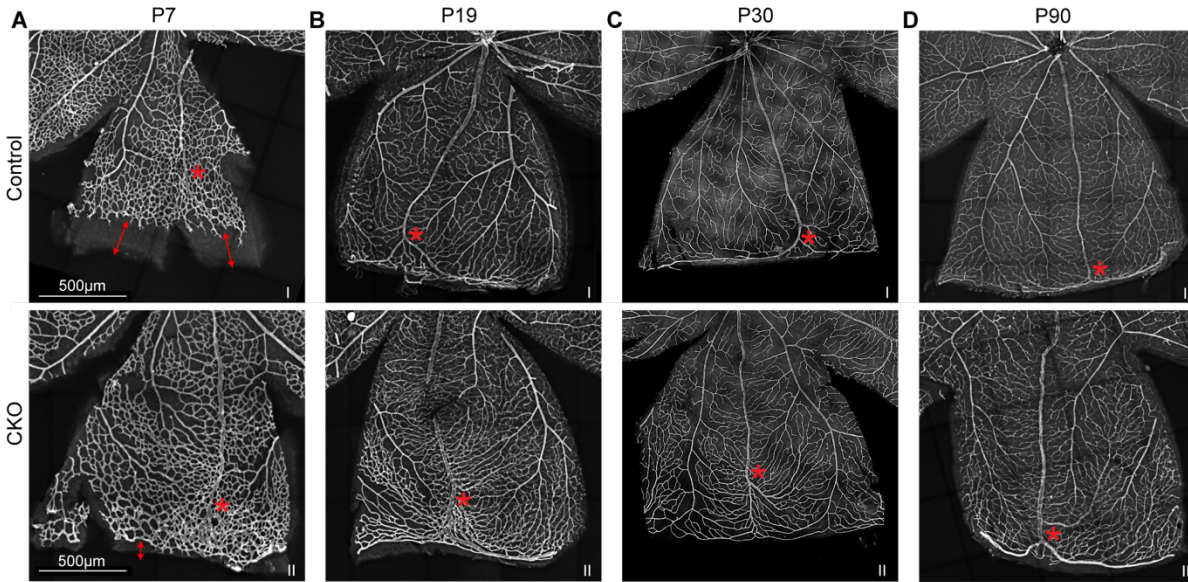


Figure 6: Morphological differences in the retinal vasculature in the FD retina.

(A) representative CD31 (Pecam-1) stained retinal whole mount preparations of control and FD retinæ (CKO) at P7 showing increased vessel outgrowth of superficial plexus (arrowed lines). (A-D) representative retinal whole mount preparations of control and FD retinæ throughout disease progression, revealing consistent vascular alterations in the vein morphology with increased branching (asterisks), vessel tortuosity, and vein thickness. In parallel with an observed increase in the distance from the main artery. Scale bar, 500µm.

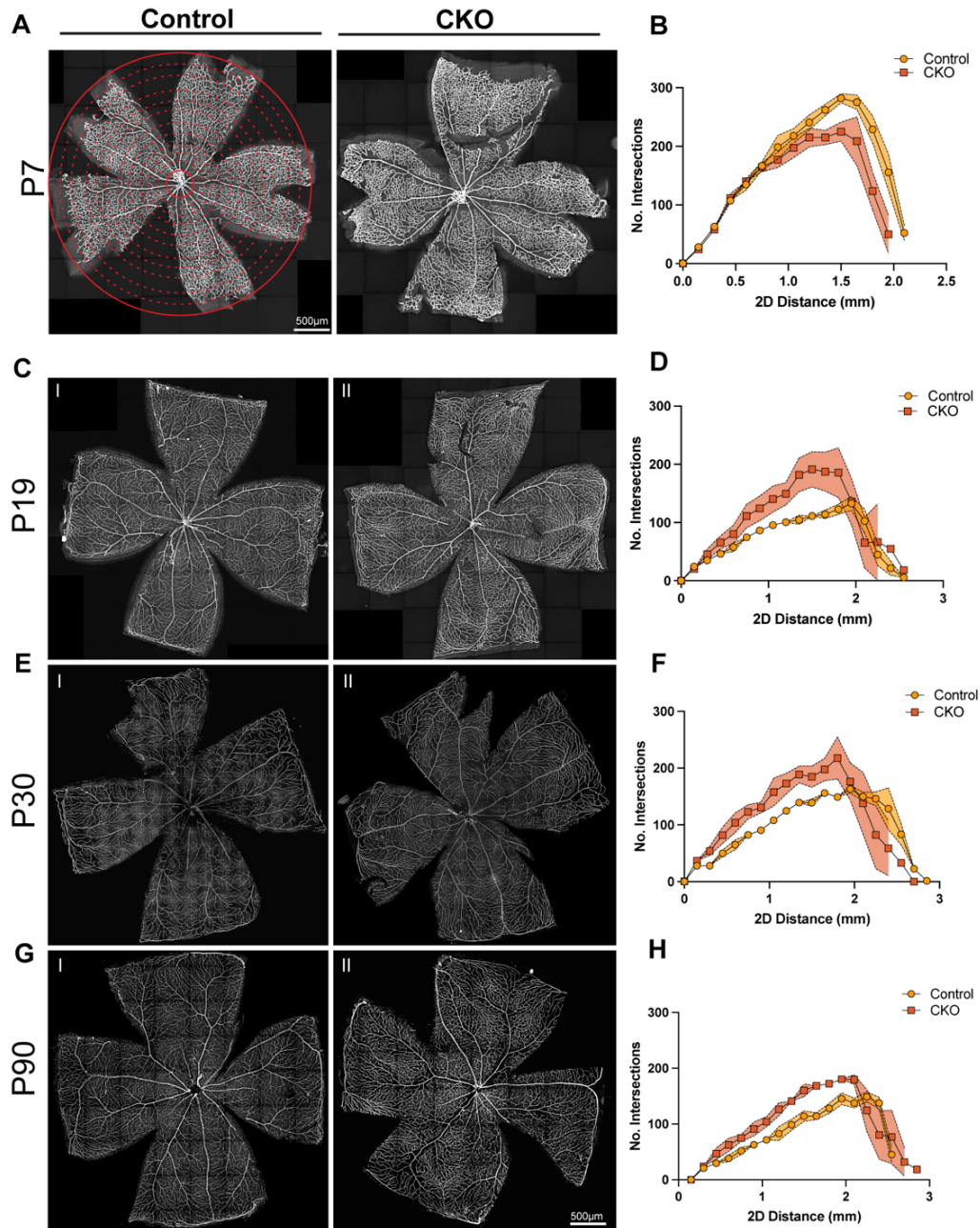


Figure 7: Sholl analysis reveals changes in retinal vasculature throughout disease progression. (A) Representative images of control and FD retinas at P7 stained for CD31 (white) and example of concentric circles used for Sholl analysis in red. (B) Linear Sholl plot at P7 between the control and CKO retinas. (C,E,G) Representative images of control and CKO retinas at 3 different time points stained for CD31 (D,F,H) Linear Sholl plots for representative time points,

showing the mean (symbol connected with solid line) and $\pm$ SEM (fill area). The Sholl analysis counts the number of vessels (intersections) as a function of distance from the optic nerve (distance expressed in mm) in 2D. Scale bar, 500 μ m. P7 control n = 4, CKO = 3. P19 control n=4, CKO n=4. P30 control n=4, CKO n=4. P90 control n=3, CKO n = 3.

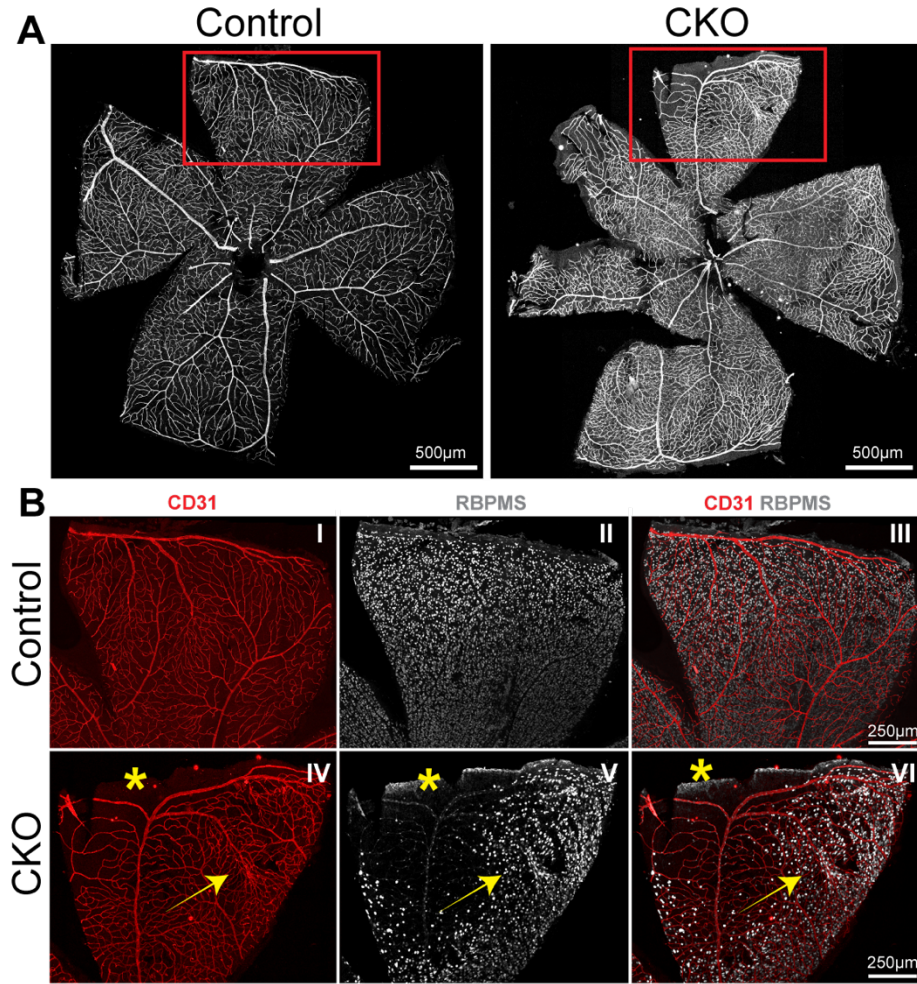
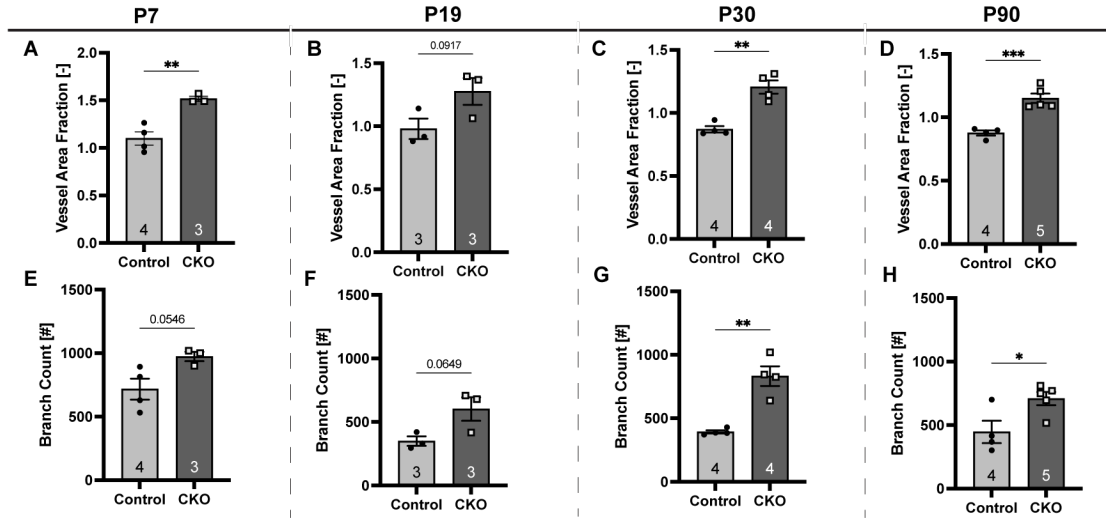


Figure 8: Vessel atrophy is associated with neuronal degeneration in the FD retina. (A) Representative whole mounts at P90 are shown. Vasculature was immunolabeled with CD31. Scale bar, 500µm. Area boxed in red is shown in higher magnification in in B. (B) Vasculature and retinal ganglion cells were immunolabeled with CD31 (red) and RBPMS (white), respectively, for control (I-III) and CKO (IV-VI) derived retinæ. Yellow asterisks indicate regions of vessel atrophy and corresponding retinal ganglion cell loss in the FD retina. Yellow arrows indicate areas with remaining retinal ganglion cells and increased microvessel branching. Scale bar, 250µm.

Supplemental Figure



Supplemental Figure 1: REAVER analysis.

Columns indicate different timepoints. Top panel shows vessel area fraction analysis between control and CKO. Bottom panel shows branch count analysis between control and CKO.

CHAPTER FOUR

IMPLICATIONS FOR A NONCANONICAL STRESS RESPONSE IN THE PATHOGENESIS OF THE OPTIC NEUROPATHY IN A MOUSE MODEL OF FAMILIAL DYSAUTONOMIA

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

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Contributions: Study design, methodology, conducted experiment, data collection & analysis, writing – original draft, writing – review and editing

Co-Author: Marta Chaverra

Contributions: data collection and analysis, writing: review & editing

Co-Author: Jamie Morgan

Contributions: data collection and analysis, writing: review & editing

Co-Author: Frances Lefcort

Contributions: supervised and oversaw the experimental approach, conceptualization, study design, writing – original draft, reviewing & editing

Manuscript Information Page

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Status of Manuscript:

- ☒ In preparation for submission to a peer-reviewed journal
- ☐ Officially submitted to a peer-reviewed journal
- ☐ Accepted by a peer-reviewed journal
- ☐ Published in a peer-reviewed journal

Introduction

Familial dysautonomia (FD) is a rare, recessive, progressive autosomal disorder that devastates nervous system function. FD is caused by a point mutation in a splice site of the Elongator Acetyltransferase Complex Subunit 1 (*ELP1*) gene.^{1,5} The mutation results in a tissue-specific reduction of Elp1 protein due to unstable mRNA targeted for nonsense-mediated decay.^{2,5} Elp1 is a highly conserved scaffolding protein and is a core subunit of the six-subunit Elongator complex. Elp1-Elp2-Elp3 forms the two-lobe core scaffold and asymmetrically binds Elp4-Elp5-Elp6. Across organisms, transfer RNAs (tRNAs) contain modified nucleosides, and when a uridine is present in the wobble position (U₃₄), the residue is almost universally modified.^{25,78} This six-subunit Elongator complex is required to modify these wobble uridines (U₃₄). These modifications facilitate specific codon-anti-codon interactions, providing precise translation rates for AA or AG-ending codons, with codon-biased genes exhibiting dependence on Elongator for normal translation rates. Importantly, all six subunits are required for a functional complex, and in the absence of any subunit, the entire complex is inactive, resulting in a diverse range of phenotypes.¹⁴ Consequently, loss of Elongator function in multiple cell types, including central and peripheral neurons, impairs translation efficiency and the integrity of the proteome and cellular homeostasis.

In FD, insufficient levels of ELP1 lead to the developmental death of neurons and progenitors in the sensory and autonomic nervous system (ANS) in addition to progressive central (CNS) and peripheral nervous system (PNS) neurodegeneration.^{1,7,8} One of the clinical hallmarks of FD is the progressive visual impairment marked by a steady loss of retinal ganglion cells (RGCs).^{6,45} The selective loss of RGCs seen in FD is similar to other progressive optic

neuropathies such as Leber hereditary optic neuropathy (LHON) and autosomal-dominant optic atrophy (DOA), both mitochondrial disorders with pathological similarities marked by an early loss of retinal nerve fiber layer (RNFL) thickness particularly the papillomacular bundle.^{6,79} Although ELP1 is expressed in all retinal neurons, the energetically demanding RGCs are the most devastated in FD. We have previously shown that Elp1 is not required for the normal development of retinal neurons.⁴² Instead, the RGCs begin to die soon after birth and continue to do so as mice age, which recapitulates the disease progression seen in patients.³⁹ So, why are post-natal RGCs dependent on Elp1? And what is responsible for triggering the neuronal degeneration seen in our mouse models and patients?

Previous studies have begun identifying the mechanisms underlying the loss of RGCs. Mouse models of FD that recapitulate the progressive loss of RGCs confirm the impairment of mitochondrial function and Complex I electron transport chain deficits seen in patients (unpublished correspondence). Moreover, in the FD mouse retina, ATP levels are reduced, and mitochondria are depolarized.⁴² Given that the mutation for FD causes hypomodification of tRNAs, it is unclear how the loss of Elongator function leads to mitochondrial stress and subsequent cell death; it could be directly caused by the hypomodification of tRNAs and consequential alteration in levels of particular proteins required for mitochondrial function and/or indirectly due to an altered proteome and transcriptome^{29,80}. We have shown that DNA repair genes are codon biased and reduced in the FD DRG, resulting in an increase in DNA damage in addition to mitochondrial and ER stress.^{29,81} Elongator function is highly evolutionarily conserved and required for normal response to cellular stress.^{82–86} While precise and tightly regulated stress responses exist, dysregulated signaling leading to cell death has been

associated with numerous human disorders.⁸⁷ Due to mounting interest in developing treatments, a detailed investigation of signaling pathways and underlying mechanisms by which RGCs die in the FD retina is needed. This study aimed to delineate dysregulated signaling pathways in the FD retina associated with the death of RGCs.

Results

Evidence that Metabolic Alterations Lead to an Increase in mTOR Signaling.

Based on patient data and previous studies in the *Pax6-cre;Elp1^{loxp/loxp}* mouse model⁴² showing a (1) loss of axons and thinning in the RNFL, reduced ATP, reduced complex I function, and (2) the essential function of Elp1 modification of wobble uridines on tRNAs facilitating translation, we hypothesized energetic signaling in the retina is impaired.

Goffena et al., 2018 showed the ramifications of a comprised ability to translate codon-biased mRNA in neurons lacking Elp1 and the relationship between tRNA modifications and the ratio of AA-ending to AG-ending codons that regulate translational efficiency in specific FD neurons. Focusing on proteins differentially expressed in the dorsal root ganglion (DRG) involved in metabolism, we found 17 proteins with a significant fold change (fold change <2 and > -2) in the CKO DRG. Interestingly, 13 out of the 17 proteins are involved in the mammalian (or mechanistic) target of rapamycin (mTOR) signaling cascade. mTOR lies at the center of a key signaling hub that controls cellular growth, metabolism, and survival in response to environmental cues, which, when dysregulated, contributes to neurodegenerative diseases.⁸⁸ mTOR forms the core component of two functionally and structurally different signaling complexes, mTORC1 and mTORC2.^{31,89} mTORC1 is the metabolic master regulator, and

mTORC2 promotes cell survival via phosphorylation of the AGC protein kinase families⁹⁰ in retinal development and axonal survival after injury. Importantly, mTORC1 and mTORC2 proteins are highly expressed in normal rodent and human RGCs⁹¹, and in yeast, the Elongator complex has been shown to be a critical regulator of the balance between the two mTOR complexes based on nutrient availability.³¹

Our previous study⁴² determined the time course of RGC degeneration in the *Pax6-cre;Elp1^{loxp/loxp}* mouse model. There is no significant difference in RGC number at post-natal day 14 (P14) but by P30 there is ~25% reduction in the number of RGCs followed by a rapid period of cell death over the following 2 months and by P90 there is ~50% reduction in RGCs. The degeneration then plateaus and we see ~60% reduction by P270 (9 months). Thus, here we focused on P30 during the onset of RGC death to assess molecular changes that may be responsible for triggering peak RGC death in the FD retina.

To assess whether mTOR signaling is elevated in the CKO retina, we immunolabeled whole mount retinæ at P30 with an antibody to phosphorylated ribosomal protein S6 (pS6) (Figure 1A), an indirect but robust assay as the downstream effector of mTORC1 activity. Using a generalized linear model to predict the relationship between pS6 intensity and soma size, we found a significantly positive relationship between pS6 signal intensity and soma size (Figure 1B; $R^2 = 0.65$; GLM). The pS6 RBPMS-positive cells in the FD retina were 38% larger than the cells in control, supporting that pS6 signal intensity corresponds to hypertrophic RGCs in the CKO retina (Figure 1C; $**p=0.0015$, unpaired t-test). To further investigate the energetic state of RGCs in the FD retina and gather insight into the potential causality for cell death, we immunolabeled retinæ at P7 (Supplemental Figure 1A) and P90 (Supplemental Figure 1C) and

found elevated pS6 intensity and soma size before RGC death is significant (Supplemental Figure 1B), and the trend continued at P90 (Supplemental Figure 1A) with the remaining 40-50% of RGCs showing more intense pS6 signal along with larger somas.

A major target of the mTOR signaling cascade is Akt kinase. Akt has two phosphorylation sites, S473 and T308. PDK1 phosphorylates at the T308, and mTORC2 phosphorylates the S473 site. Following full activation, Akt activates mTOR by at least two different pathways. One is the TSC1/2-Rheb pathway, and the other is the PRAS40 pathway.^{92,93} Based on the proteomic analysis in the DRG, PDK2, and PDK3 isoenzymes are increased 247 and 10-fold, respectively, the TSC complex is decreased 13 and 36 fold (Tsc1 and Tsc2), and Rheb is increased 2 fold. This and the increase in pS6 signal intensity suggest the potential for unchecked mTORC1 activation in the retina. Therefore, western blot and densitometric analyses for Akt (Figure 2A) were conducted, and we found that while there was no difference in phosphorylated Akt S473 in the mutants (Figure 2B; ns = 0.1442, unpaired t-test), there was a significant decrease in total Akt expression (Figure 2C; *p=0.024, unpaired t-test) and the relative expression of pAkt(S473) to total Akt protein is increased in retinal lysates at P30 in the FD retina (Figure 2D; **p=0.0035).

Localization of phosphorylated Akt T308 in the GCL using confocal microscopy (Figure 2E) showed no difference in the number of double-labeled Brn3a+ pAkt (T308) positive cells at P30 (Figure 2F). However, at P30, there is a significant reduction in the number of Brn3a+ cells in the FD retina (Figure 10G; *p=0.014). Consequently, we analyzed the ratio of double-labeled cells to the total number of Brn3a cells and found a significant increase in the number of RGCs expressing phosphorylated Akt T308 in the FD retina compared to that in littermate controls

(Figure 2H; * $p=0.040$, unpaired t-test). Collectively, these data support increased mTORC1 activity in the FD retina and/or dysregulation in protein regulation.

Canonical Stress Responses are Altered in the Retina of a Mouse Model of FD.

ER stress contributes to cell death in multiple retinal diseases, such as glaucoma and diabetic retinopathy. Using Gene Ontology (GO) along with the DRG the transcriptomic and proteomic studies²⁹, we focused on genes and proteins that are involved with MAPK signaling and endoplasmic reticulum (ER) stress and found Map2k5, PERK, Mapk10, Mapk13, Map3k7, Ddit3, Eif2 α were dysregulated. Both Jnk signaling and ER stress have been implicated in RGC death in mouse models of glaucoma and diabetic retinopathy.⁹⁴⁻⁹⁸ *Jnk (Mapk10)* is downregulated in the DRG transcriptome. Jnk activation can initiate contradictory signals in the neuronal stress response. Additionally, Elp1 has been shown to associate and regulate Jnk activation via a binding domain at amino acids 1216 - 1279 in the C terminus.³³ In the absence of Elp1, is Jnk activation misregulated? To assess any changes in Jnk expression or protein localization in the FD retina, we immunolabeled P30 cross-sections and found that Jnk expression is reduced in the FD retina, with the majority of signal in the inner plexiform layer (IPL), and some faint signal observed in the GCL (Figure 3A).

Jnk intensity was quantified in the neurites of both control and FD retinae using IPLaminator, an ImageJ plugin for quantifying and binning retinal lamination.⁹⁹ When plotted to “conventional” IPL bands, the neurite bands map at layers 4 and 8 in the control retina compared to layers 5 and 7 in the FD retina, indicating an organizational difference in the FD retina (Figure 3B). The image-based quantification shows a clear difference when plotting Jnk stain intensity - the neurite stripes emerge as paired and disparate histogram peaks between the control and FD

retinae (Figure 3B, C). The two peak intensities for Jnk in the control retinae displayed 25% in layer 4 and 22% in layer 8 of Jnk intensity to total IPL intensity. In contrast, the FD retinae exhibited Jnk peak intensities of 11% in layer 5 and 11% in layer 7 to total IPL intensity (Figure 3C; *** $p=0.0005$, ** $p=0.041$; one-way ANOVA). Using a generalized additive model, the relationship between Jnk intensity and IPL depth was predicted based on genotype and a significantly different relationship between Jnk intensity in the neurites and the IPL thickness was found (Figure 3D; $R^2 = 0.62$; GAM). Western blot and densitometric analysis corroborated these results (Figure 3D) and showed reduced Jnk and pJnk expression in the entire FD retina (Figure 3E; ** $p=0.0039$; unpaired t-test), (Figure 3F; * $p=0.012$; unpaired t-test) compared to controls. These data suggest that reduced Jnk activation in the FD retina may be due to the loss of Elp1 binding to Jnk, which might prevent FD RGCs from mounting a normal stress response.

If Elp1-depleted neurons cannot respond to stress, can they activate or initiate disparate signaling pathways in a noncanonical fashion? In response to diverse stress stimuli, eukaryotic cells activate a common adaptive pathway, the integrated stress response (ISR), to restore cellular homeostasis.¹⁰⁰ The core event in the ISR is the phosphorylation of the regulatory hub, eukaryotic translation initiation factor 2 alpha (eIF2 α)^{100,101} and interestingly, from our GO analysis with the embryonic DRG proteome, we found that Gadd34/Ppp1r15a is decreased 6-fold while both Eif2a and Ddit3 are increased 9-fold in the mutant. Furthermore, Gadd34 is known to directly bind to and dephosphorylate eIF2 α following the relief of stress.^{102,103} To determine if the retina exhibits similar expression changes to that in the DRG, we immunolabeled retinal cross-sections with an antibody to p-eIF2 α . We found p-eIF2 α primarily localized to the GCL with some signal in the INL (Figure 4A). Similar to the pAkt(T308) staining pattern, we found

no difference in the number of p-eIF2 α , Brn3a positive cells between the control and FD retina (Figure 4B) but did find that the ratio of double-labeled RGCs to total RGCs is significantly increased in the FD retina (Figure 4C-D; ***p=0.0004, *p=0.021; unpaired t-test). If protein homeostasis is not restored, prolonged activation of ISR or ER stress may initiate the up-regulation of pro-apoptotic C/EBP homologous protein (CHOP), also known as DNA damage-inducible transcript 3 (DDIT3). Since we see increased p-eIF2 α and Ddit3 is known to be highly induced in response to ER stress and is an important molecule for ER stress-mediated apoptosis, we immunoblotted whole retinal lysates at P30 to assess Ddit3 levels. The relative expression level of Ddit3 in the FD retina was increased by 99% compared to the relative expression found in the control lysates (Figure 5A, B; *p=0.0247; unpaired t-test). Indicating the involvement of Ddit3 in potential downstream signaling events leading to RGC death in the FD retina. However, to our surprise, we did not find an increase in caspase-3-dependent apoptosis in the FD retina (data not shown), suggesting that RGC death could be caspase-independent.

Poly(ADP-ribose) (PAR) polymerase-1 (PARP-1) is a nuclear enzyme best known to facilitate DNA base excision repair and plays critical roles in the regulation of cell death in caspase-independent cell death.^{104,105} Interestingly, PARP-1 is also involved in the regulatory process of sensing and responding to cellular stress across the transcriptional, post-transcriptional, and post-translational levels.¹⁰⁵ Western blotting showed that PARP-1 protein levels were increased by 59% in the CKO lysates compared to controls (Figure 6A, B). PARP-1 activation was examined by levels of poly(ADP-ribose)lated protein in retina lysates (Figure 6C, D). The increase in PARP-1 levels was accompanied by a significant increase in levels of

poly(ADP-ribose)lated proteins at a higher protein concentration (20µg) in CKO lysates (Figure 6E, F).

Discussion

Familial dysautonomia is an autosomal recessive neuropathy caused by an intronic mutation in the *ELP1* gene, resulting in a tissue-specific reduction of the Elp1 protein.⁸ FD patients face many adversities, but one of the most debilitating symptoms is progressive blindness. Patients present with a significant reduction in RNFL thickness in the temporal quadrant as early as 6 years old. There is a preferential loss of small axons that originate from the parvocellular RGCs in the macula early in the disease, and this progresses until severe retinal damage is observed in the third decade of life.^{6,39,46,106} Why neurons die in the absence of Elp1 and whether this death results from direct or indirect impairment of tRNA modification is not resolved. Here we provide the first molecular evidence for dysregulated stress signaling in the post-natal neurons of the FD retina. While the data reveal a dysregulation in major signaling pathways, they do not culminate in canonical responses to chronic stress. We posit that given the upstream position of Elp1 in regulating the cell proteome, and indirectly, the transcriptome, that potentially multiple downstream pathways are misregulated that ultimately cause the death of RGCs according to non-canonical signaling mechanisms.

While we do not completely understand ELP1 functions within the cell, it is known that ELP1 is highly evolutionarily conserved and broadly expressed in various tissues and cell types, indicating it as a critical cellular protein. ELP1 is predominantly located in the cytoplasm which is consistent with its critical role as the scaffolding subunit of the Elongator complex which binds and modifies tRNA^{25,28}, although it has also been found to associate with RNA Pol II and

to be involved in transcript elongation.^{21,107} Evidence in yeast reveals that ELP1 interacts with mTOR signaling and that mTORC1 signaling activity is relatively high in rodent and human RGCs, and glial cells.^{31,32,91,108} Here we provide for the first time, that in the absence of Elp1, retinal ganglion cells upregulate pS6, a robust downstream effector of mTORC1 activity along with full Akt activation, indicating elevated mTORC1 activity. Further studies are required to understand the underlying implication of mTOR activity in relation to RGC death in FD.

Interestingly, mTORC1 activity is required for the high rate of protein synthesis that occurs in RGCs, particularly 5' CAP-dependent mRNA translation¹⁰⁸ yet this function might be mitigated by the seemingly contradictory increase in p-Eif2 α we found in the FD retina. When Eif2 α is phosphorylated, general translation is shut off, and the translation of specific transcription factors is upregulated, in an attempt to restore homeostasis.^{100,102} Therefore, in the extremely susceptible RGCs, we provide evidence for potentially dysregulated communication via signaling molecules; ER stress leads to the phosphorylation of Eif2 α , which then reduces general translation and, in a chronically stressful state, leads to the upregulation of pro-apoptotic Ddit3. Yet, despite repeated attempts, we did not see any caspase-3-dependent apoptosis in the RGCs (data not shown). The presence of activated mTORC1 target signaling (elevated pS6) during a period of chronic ER stress could disrupt the normal communication between these two cellular pathways and result in hypertrophic RGCs that are unable to restore homeostasis and ultimately die.

The Jnk signaling pathway can mediate metabolism, growth, cell migration, repair, proliferation, and death.¹⁰⁹ Studies have shown Jnk's involvement in synapse dysfunction, which can be an initiating event in neurodegenerative diseases.^{110,111} It has been proposed that ELP1

may be an important physiological regulator of stress-associated-Jnk signaling due to Jnk's ability to bind ELP1.³³ Phospho-Jnk was also found to colocalize with Elp1 at axon terminals in DRG neurons growing *in vitro*¹¹² and given Jnk's ability to stabilize microtubules, reduced Jnk activation might lead to enhanced microtubule turnover, destabilization, and shorter neurites in FD neurons.¹¹³ Moreover, these authors show that when Elp1 levels were reduced, the spatial localization of pJnk was altered and it aggregated. Here we show *in vivo* that Jnk expression is reduced primarily in the neurites in the FD retina and that this reduction in protein is correlated with a reduced IPL thickness (figure 3B). The IPL is divided into ON and OFF sublamellae (Figure 3B) and reflects the functional segregation of the ON and OFF pathways. The neurites in the OFF layer appear to show an increased reduction in Jnk protein compared to the neurites in the ON layer. It will be of interest to assess the synaptic physiological function of RGCs in FD retinæ in the future.

We also show here that PARP-1, a nuclease that functions to sense DNA damage, is also increased in the FD retina. Overactivation of PARP-1 can lead to parthanatos, PARP-1-dependent cell death. Canonical parthanatos is characterized by an accumulation of PAR and a nuclear translocation of AIF from mitochondria and has been linked with retinal degeneration.^{96,114,115} Furthermore, PARP-1 is known to interact directly with Jnk, and Jnk is required for PARP-1-induced cell death by AIF translocation to the nucleus.¹¹⁶ We show a reduction in Jnk and an increase in PARP-1 in retinal lysates and no difference in the localization of AIF into the nucleus (data not shown). There is evidence for a PARP-1-mediated necrotic pathway distinct from the canonical parthanatos that is involved in the pathogenesis of dry age-related macular degeneration.¹¹⁷ Collectively, in conjunction with the dysregulated stress

response, these data suggest a model in which an initial death signal mediated by DNA damage and PARP-1 activation, may lead to mitochondrial dysfunction⁴², but in the absence of sufficient Jnk levels, AIF is unable to translocate to the nucleus which prevents canonical parthanatos from causing the death of RGCs in the FD retina. Meanwhile, ER stress is triggered perhaps due to problems with protein homeostasis due to altered translation^{2,15,17,32}, which cannot be resolved, thereby activating Ddit3, but for reasons unclear, does not lead to activation of Caspase3. Another interesting pathological process that starts in the post-natal mouse FD retina is gliosis and vascular remodeling (Schultz and Lefcort, in preparation), which, given the tight units that these cells form with neurons, are also leading to inflammatory pathways that could promote RGC death. In summary, these data indicate that more than one deleterious pathway is activated in RGCs once the mouse eyes open, and visual processing taxes the energetics of RGCs. Future studies are needed to clarify the details of these pathways, with the goal of providing insights into translational intervention strategies.

Materials and Methods

Animals

All mice were housed in the AALAC-accredited Animal Resource Center at Montana State University. All animal use protocols were approved by the Montana State University Institutional Animal Care and Use Committee (IACUC) (protocol No. 2020-15-IA; Bozeman, MT). The generation of the *Pax6cre*⁺;*Elp1*^{flox/flox} CKO is described in Ueki et al., 2018. Briefly, retina-specific *Elp1* CKO mice were generated by crossing *Elp1* floxed (International Knockout Mouse Consortium, Wellcome Sanger Institute, UK) and α Pax6 promoter-driven Cre (*Pax6-Cre*)

mice, which were a gift from Drs Peter Gruss and Ruth Ashery-Padan). All mice were housed in a 12-hour light-dark cycle and fed chow and water *ad libitum*.

Tissue Processing and Preparation for Cryosectioning

Eyes were enucleated and the cornea and lens were removed, and the remaining eyecup was fixed for one hour in a 4% paraformaldehyde (PFA) in 1X phosphate-buffered saline (PBS) at room temperature (RT) with rotation. Eyecups were washed in 1X PBS for 15 minutes and cryopreserved incrementally, first in 10%, then 20%, and followed by a 30% sucrose (VWR, #0335-1KG) until the tissue sank at 4°C. Following the sucrose gradient, the eyecups were flash-frozen in Tissue-Tek O.C.T Compound (Sakura, #4583) and stored at -80°C. Using a Leica CM 1950 cryostat, 14µm coronal sections were cut at -22°C and collected on microscope slides.

Retinal Immunocytochemistry for Flat Mount Preparations

Mice were euthanized with CO₂, and the temporal surface of the eye was marked with a green tattoo dye (Ketchum Manufacturing) prior to enucleation. After the eyes were enucleated, a hole was made at the sclera-cornea margin and fixed in 4% PFA at room temperature (RT) for 1 hour. The neural retinal layer was removed, and the temporal region was marked by a small 15-degree cut and incubated in 4% PFA for an additional 15 minutes at RT. Tissue was permeabilized in 1X TBS containing 0.1% Saponin (MP Biomedicals, Cat#102855) for 30 minutes at RT. At RT, non-specific binding was blocked by incubation with 1X TBS containing 5% BSA and 0.1% Saponin for 3 hours. All retinæ were incubated for 3 nights at 4°C in a primary antibody solution containing block solution, an anti-RBPMS antibody (1:500, PhosphoSolutions, 1832-RBPMS, #NB1121a), and an anti-Phospho-S6 ribosomal protein (Ser235/236) antibody (1:100, Cell Signaling Technology, #2211). After overnight incubation at

4°C with secondary antibodies (Donkey anti-Guinea Pig IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 and Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 from Thermo Fisher Scientific, catalog #A-21447, RRID AB_2535864 and catalog # A10042, RRID AB_2534017), four identical cuts were made to flatten the tissue into four lobes, and the tissue was mounted with ganglion cell layer-up on microscope slides with Prolong Gold (Invitrogen).

Retinal Immunocytochemistry for Cross-Section Preparations

As described above, eyes were harvested and fixed in 4% PFA for 1 hour. Slides with 14µm coronal sections stored at -20°C were air dried at RT for 1 hour and rehydrated in 1X PBS for 20 minutes, followed by a 15-minute permeabilization in 1X PBS containing 0.5% Triton X-100 and blocked in 5% BSA containing 0.5% Triton X-100 for 1 hour at RT. Retinal sections were then incubated overnight at 4°C in primary antibodies (Table 1) diluted in 1% BSA, 0.1% Triton X-100 in 1X PBS. The tissue was then washed in 1X PBS and incubated for 2 hours at RT in secondary antibodies (Table 1).

Western Blot

Immunoblotting was used to quantify protein expression from snap-frozen retinal tissue. Retinae were extracted on ice with lysis buffer (25 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 5% glycerol) containing 1X HALT protease inhibitor cocktail, 1 mM PMSF, and 10 µM Leupeptine. Protein concentration was quantified with either a Bradford 1X protein-dye reagent (Bio-Rad #5000205), or a BCA™ protein assay kit (Pierce, #23225 and #23227). Protein was separated by electrophoresis using a Mini-Protean TGX 4-15% gel (BioRad, #4561086), and the proteins were transferred onto a PVDF membrane. The membrane

was blocked for 1 hour in 5% non-fat dry milk (NFDM) in 1X Tris-buffered saline containing 0.1% Tween-20. The primary antibodies (Table 1) were diluted in 1X TBS containing 5% BSA and 0.1% Tween-20, and the membrane was incubated in primary antibody overnight at 4°C with gentle shaking. Following primary incubation, membranes were probed with a secondary antibody (Table 1) in 1X TBS containing 5% NFDM and 0.1% Tween-20. Quantitative analysis was performed using Image J (NIH, USA) software. All protein levels were quantitated relative to total protein based on Ponceau S dye (5% acetic acid, 0.1% Ponceau S). The expression of target proteins in both control and CKO groups was normalized to the total protein area, and the normalized density is reported as a 'relative expression' unless otherwise noted. All gel images show the developed X-ray film in grayscale from portions of the PVDF membrane that were cut prior to antibody hybridization. No post-processing of the images occurred.

Confocal Microscopy and Image Analysis of Ganglion Cells and Phospho-S6 Ribosomal Protein in Retinal Flat Mounts.

Retinal flat mounts were imaged using a Leica TCS SP8 confocal microscope with an oil immersion objective at a resolution of 1024 x 1024. Z-stacks spanned 6µm in-depth with an image acquired every 2µm for a total of 4 images per Z-series. All settings were kept constant for a given antibody across sections. For quantifying pS6 in RBPMS positive cells in the flat mounts, images were taken 1 FOV away from the edge of the tissue in the periphery of the temporal, nasal, superior, and inferior regions. ImageJ was used to manually count the number of pS6 RBPMS positive cells, signal intensity, and soma size analysis in each image.

Using the freehand selection tool, the signal intensity of individual cells was measured for all the double-labeled cells in a total of 1.35mm² by tracing each cell using the freehand selection tool at a magnification of 200%. The area of each cell was recorded based on the

outline, and the intensity of the individual cells was measured using the raw integrated density output. The corrected total cell fluorescence (CTCF) was used to compare the fluorescent intensity between cells, regions, and genotypes for the relationship between signal intensity and cell size. Using a generalized linear regression to show the relationship between pS6 signal intensity and RGC soma size, we found a significantly positive relationship between cell size and signal intensity.

Confocal Microscopy and Image Analysis in Retinal Cross-Sections

Akt Signaling: Retinal sections following immunohistochemical staining with anti-pAkt(T308) (Table 1) were imaged using a Leica SP8 confocal microscope. Four regions of interest (ROIs) in four sections per retina for a total of 16 images per retina were collected using a 63X oil-immersion objective with a resolution of 1024 x 1024. Z-stacks spanned 8µm in-depth and comprised 5 images per series. The ROI was determined by the structure and integrity of the DAPI+ tissue to not bias ROIs based on the protein of interest signal. Final cell counts were conducted on the LasX program and divided into three steps. Each image was treated identically, and the experimenter was blind to the genotype. First, all pAkt(T308)-positive RGCs were counted; requirements included a distinct cytoplasmic stain that was confirmed with a DAPI+ nucleus. Next, all Brn3a positive cells were counted, and different intensities were all considered positive signals; requirements included a distinct nuclear stain that coincided with a DAPI+ nucleus. Lastly, all double-labeled cells were recorded.

Jnk Signaling: Retinal sections following immunohistochemical staining with anti-Jnk (Table 1) were imaged using a Leica SP8 confocal microscope. 12 single focal plane images per retina were collected using a 20X oil-immersion objective with a resolution of 1024 x 1024. As

previously stated, the ROI was determined by DAPI staining in two peripheral regions and two more central regions of the eyecup.

All images were imported into Fiji, and the scale of 1.75 pixels/ μm was applied to all images to ensure similar measurements throughout the samples. All images were rotated so that the neurite stripes were vertical and positioned to the right of the ganglion cell layer (Figure 3B). Using the IPLaminator plugin (Li et al., 2016), a rectangular ROI was selected to include an area that spanned the width of the IPL. The same ROI was used for every image. The settings were selected to reduce background noise; for each image, a dark area was selected as background noise. The output resulted in a histogram/plot profile for each image created with the ImageJ Profile Plot class and represents the binning of peak signal intensity of the neurites (Jnk signal) and the lowest intensity location between these two peaks.

The values from the normalized intensity output were used for statistical analysis between genotypes. Each binning layer was analyzed using a one-way ANOVA with multiple comparisons to show signal intensity differences between the littermate control and CKO. Furthermore, to show the relationship between Jnk signal intensity and the IPL depth i.e., the neurite stratification and expression of Jnk in each layer, we conducted a generalized additive model due to the nonlinear data distribution. The model's explanatory power is substantial, with an $R^2 = 0.62$, and shows the effect of CKO is statistically significant ($\beta=6.04$, 95% CI [4.57, 7.50], $p < 0.001$) and different than the control ($\beta=10.35$, 95% CI [9.04, 11.66], $p < 0.001$). This indicates that the IPL and distance between neurites in the CKO is thinner than the controls, along with reduced Jnk signal intensity in the CKO compared to the control.

p-Eif2 α Signaling: Retinal sections following immunohistochemical staining with anti-pEif2 α (Ser51) (Table 1) were imaged using a Leica SP8 confocal microscope. Four ROIs in three sections per retina for a total of 12 images per retina were collected using a 63X oil-immersion objective with a resolution of 1024 x 1024. Z-stacks spanned 9 μ m in-depth and comprised 4 images per series. As previously stated, the ROI was determined by DAPI staining in two peripheral regions and two more central regions of the eyecup. Final cell counts were conducted on the LasX program and divided into three steps. Each image was treated identically, and the experimenter was blind to the genotype. First, all pEif2 α (Ser51) were counted, and different intensities were all considered positive signals; requirements included a distinct cytoplasmic stain and was confirmed with DAPI+ nucleus. Second, all Brn3a positive cells were counted, and different intensities were all considered positive signals; requirements included a distinct nuclear stain that coincided with a DAPI+ nucleus. Again, all double-labeled cells were recorded.

Statistics

Unless otherwise indicated, all data are expressed as mean $\pm$ SEM. Column data are plotted as scatter dot-bar plots to show variability, and *n* shows the sample size for each data set. GraphPad Prism (version 9) was used to make all graphs. $P < 0.05$ was considered to represent a significant difference. Following confirmation of normal distribution, data were analyzed using an unpaired two-tailed student's t-test and/or one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. All data follow a normal distribution unless otherwise indicated.

Linear Regression

To isolate the effect and test the relationship between soma size and signal intensity between control and CKO cells. We used a linear regression model with status (control or CKO) as the predicting factor. We visually checked normality and confirmed results with a Shapiro-Wilk test for modeling data. If any non-normal distributions were observed in the data sets, we took the log of all values to account for the non-normality of the data and followed up with visual confirmation of the residuals. The residuals are the difference between the predicted value within the model and the actual values of the data and show a normalization. We report the intercept, 95% CI, and R^2 value for each control and CKO group.

Generalized Additive Model

To help understand the relationship between tissue thickness and signal intensity of the inner plexiform (IPL) based on genotype. We performed a generalized additive model using the R package **mgcv** in R version 4.2.1 (R Core Team, 2022). The model selection was based on minimizing the Akaike Information Criteria (AIC), which measures model quality and informs how well our model fits the data set without over-fitting it and the predicted R^2 value. Significance was determined on 95% CI and the quality of the predicted R^2 value.

Figures

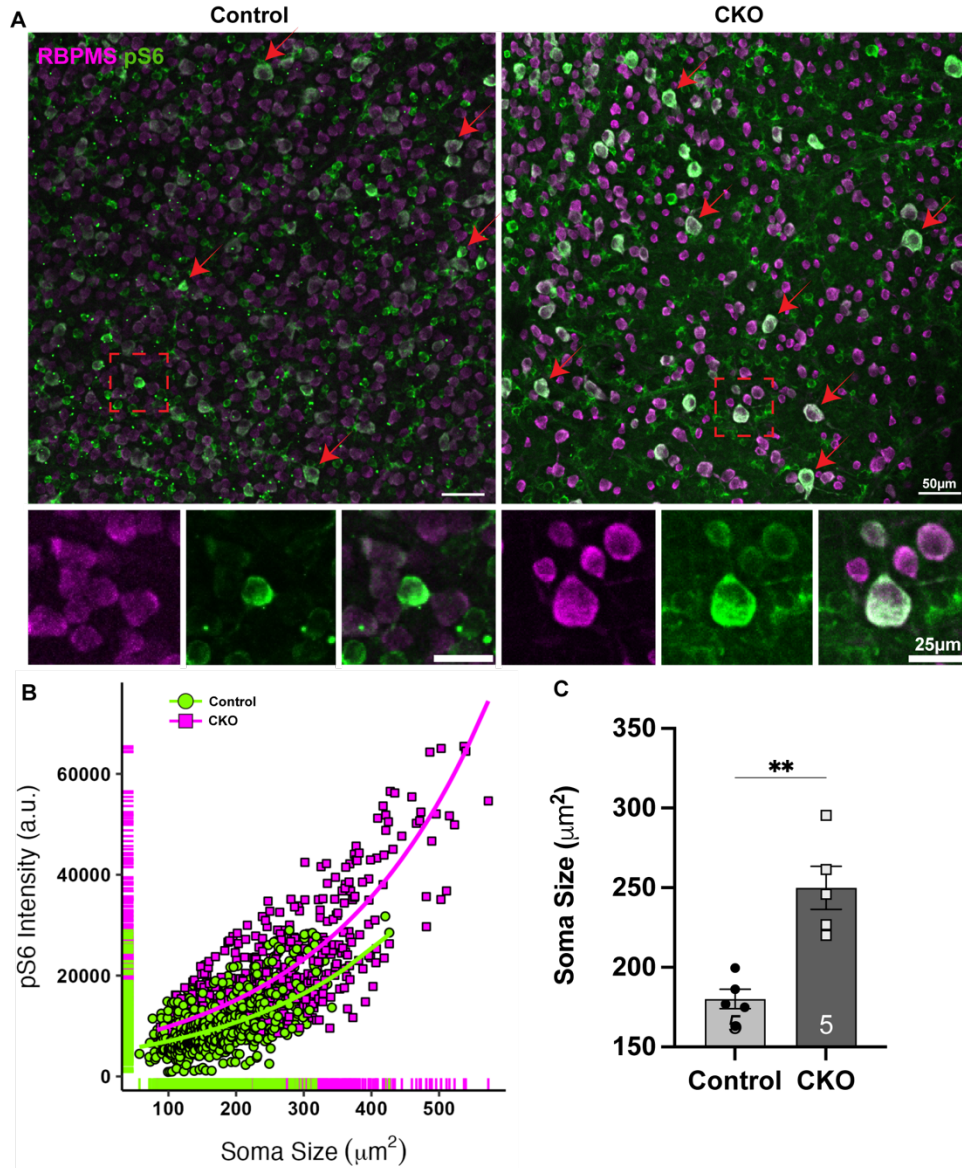


Figure 1: Increased pS6 signal at P30.

(A) Representative confocal images of retinal whole mounts from control and CKO mice stained for phosphorylated ribosomal protein S6 (pS6; green) and RBPMS (magenta) to visualize RGCs. The top row (lower magnification) depicts signal intensity and soma sizes in the control and CKO retinae (red arrows). Scale bar, 50μm. The bottom row (higher magnification) depicts the region boxed in red (dashed), showing the larger soma sizes of pS6-positive RGCs in the CKO retina. Scale bar, 25μm. (B) The intensity of cellular pS6 expression and the area of RBPMS expression were quantified for each cell ($n = 1537$ control RGCs, $n = 789$ CKO RGCs). A positive correlation was observed between pS6 staining intensity and soma size ($R^2 = 0.65$, linear

regression; CKO: $\beta=8.77$, 95% CI [8.70, 8.85], Control: $\beta= -0.34$, 95% CI [-0.39, -0.30]). Magenta line = CKO predicted values, green line = control predicted values. **(C)** Quantification of soma size between the control and CKO retinae shows the hypertrophy of pS6-positive ganglion cells. Data for **(C)** are shown as mean $\pm$ SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar. ($p=0.0015$; unpaired t-test).

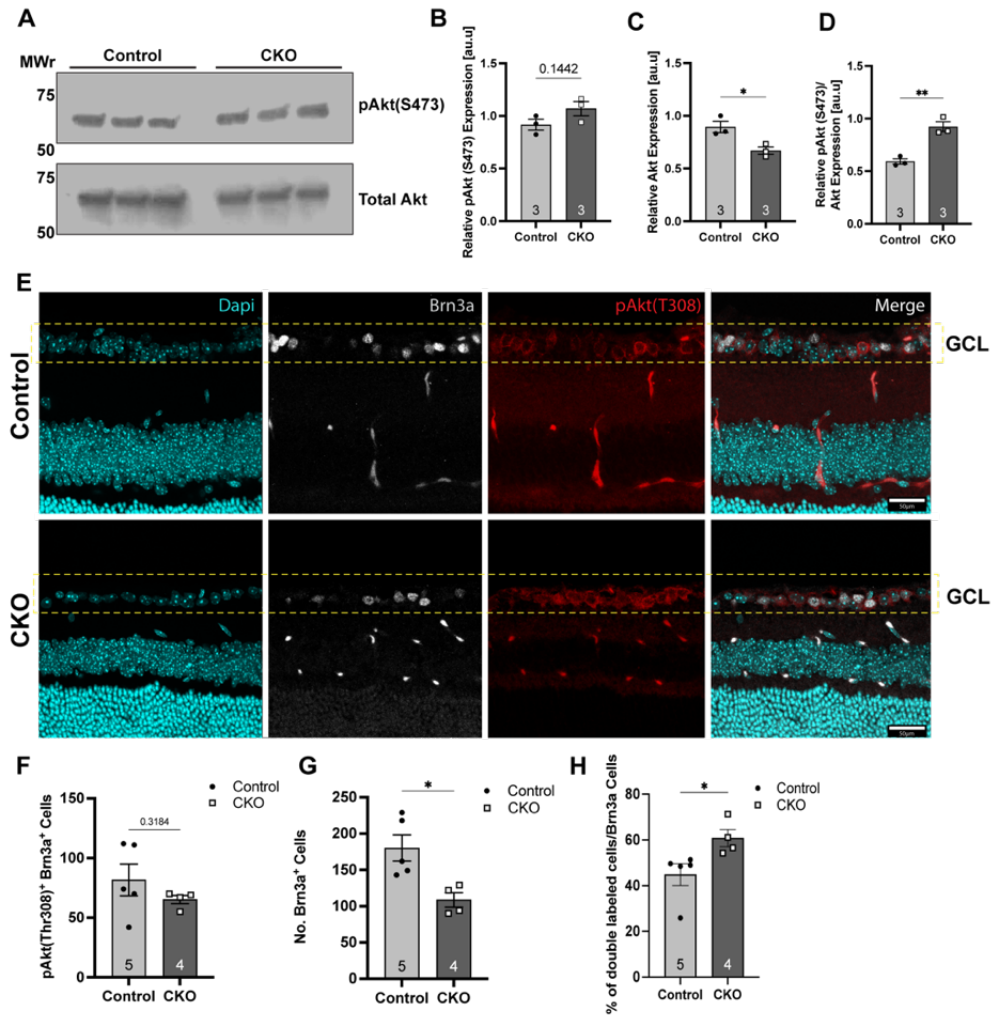


Figure 2: Alterations in Akt signaling at P30.

(A) Immunoblot showing the phosphorylation of Akt S473 and a slight reduction in total Akt protein in CKO retinal lysates. Equal amounts of protein (15µg) were loaded in each well. (B, C) Densitometric analysis of p-Akt(S473) (B) and Akt (C) in control and CKO retinas showed no significant difference in the amount of pAkt (S473), while a significant reduction in total Akt expression is observed in the CKO. (D) Densitometric analysis of the ratio of p-Akt(S473) to total Akt shows that the amount of total phosphorylated Akt S473 increases in the CKO lysates. Total protein amounts were used for normalization and as a loading control (Supplemental Figure 1A). * $p=0.024$, ** $p=0.0035$, unpaired t-test. (E) Representative confocal images of RGCs (Brn3a) and p-Akt(T308) expression in retinal sections of control and CKO retinas with a focus on the GCL (yellow rectangle). Scale bar, 50µm. (F) Quantification of double-labeled cells in the GCL. All cells were counted in eight fields of view (FOV). The number of double-labeled cells did not differ between the control and CKO retina (G) Quantification of Brn3a-positive cells per 1.5mm. There is a significant reduction in the Brn3a-positive cells. * $p=0.014$. (H) The ratio of double-labeled cells to total Brn3a-positive cells. Proportionally to the number of RGCs, there

are significantly more double-labeled cells in the CKO compared to the control. * $p=0.040$. Data are shown as mean $\pm$ SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar. GCL = ganglion cell layer. 1 FOV = $184.52\mu\text{m}^2$

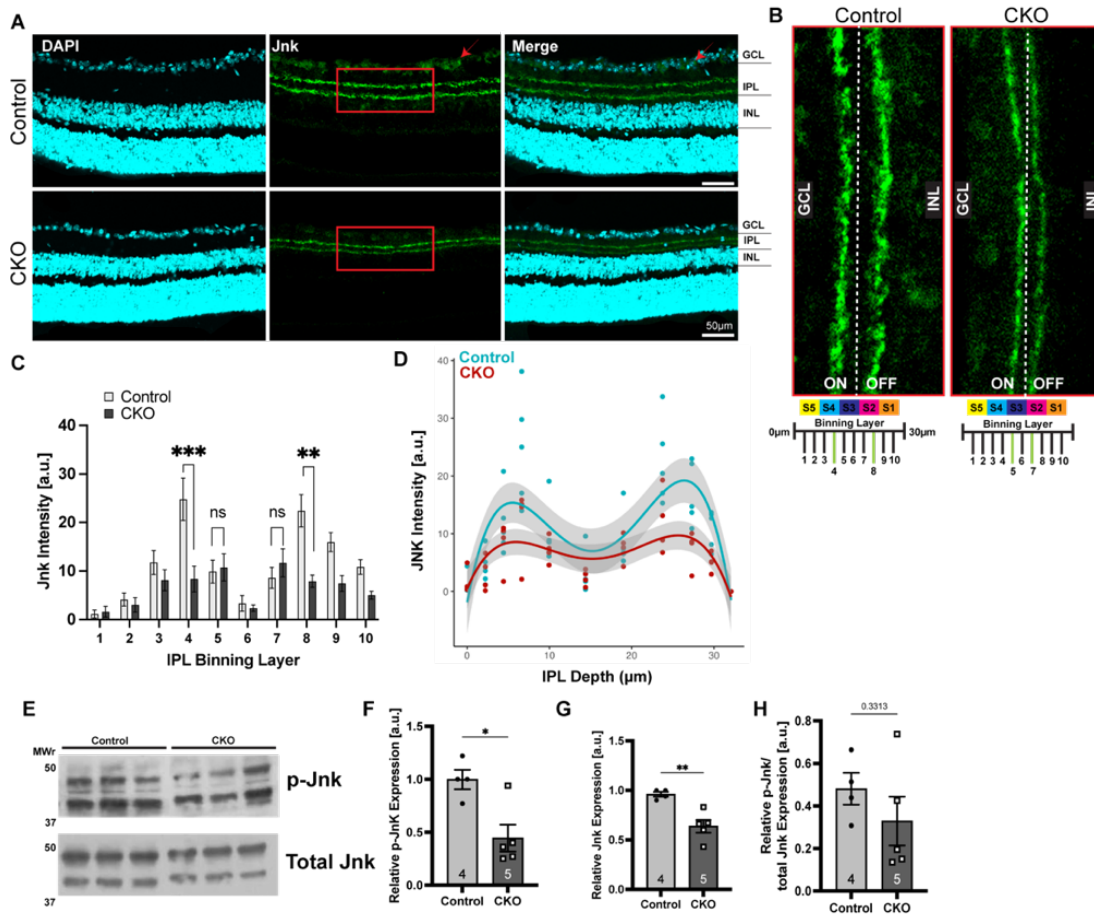


Figure 3: Alterations in Jnk expression at P30.

(A) Representative images of control and CKO retinal cross-sections stained with an antibody to Jnk (green) and the nuclear stain DAPI (cyan). Jnk signal was primarily located in the IPL and certain GCL somas (red arrows). Red boxes highlight the IPL region of interest (ROI) used for quantification and is shown in higher magnification in B. Scale bar, 50μm. (B) High magnification representation of the IPL with a schematic showing the synaptic layers divided into five conventional strata (S1-S5), the binning layers used for quantification, and the IPL depth. (C) Quantification of the Jnk signal intensity in the neurites of the IPL using IPLaminator (ImageJ). Each conventional strata (S1-S5) corresponds to two binning layers. Data for (C) are shown as mean ± SEM; the sample size for individual groups is $n=5$ control, $n=4$ CKO. (D) Using a generalized additive model to visualize the relationship between signal intensity and IPL depth, there is a correlation between a thinner IPL and reduced Jnk staining in the CKO. $p < 0.001$, $R^2 = 0.62$, CKO: $\beta=6.04$, 95% CI [4.57, 7.50], Control: $\beta= 10.35$, 95% CI [9.04, 11.66]. (E) Representative immunoblot showing reduced p-Jnk and total Jnk in CKO retinal lysates. Equal amounts of protein (15μg) were loaded in each well. (F,G) Densitometric analysis of p-Jnk (F) and Jnk (G) in retinal lysates shows reduced phosphorylated and total protein levels. However, the relative quantities of pJnk to total Jnk (H) is not significantly different. Total

protein amounts were used for normalization and as a loading control (Supplemental Figure 1B). * $p=0.012$, ** $p=0.0039$, unpaired t-test. Data for **(F, G)** are shown as mean $\pm$ SEM; each data point represents an individual retina. The sample size for individual groups is represented by n within each bar. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer.

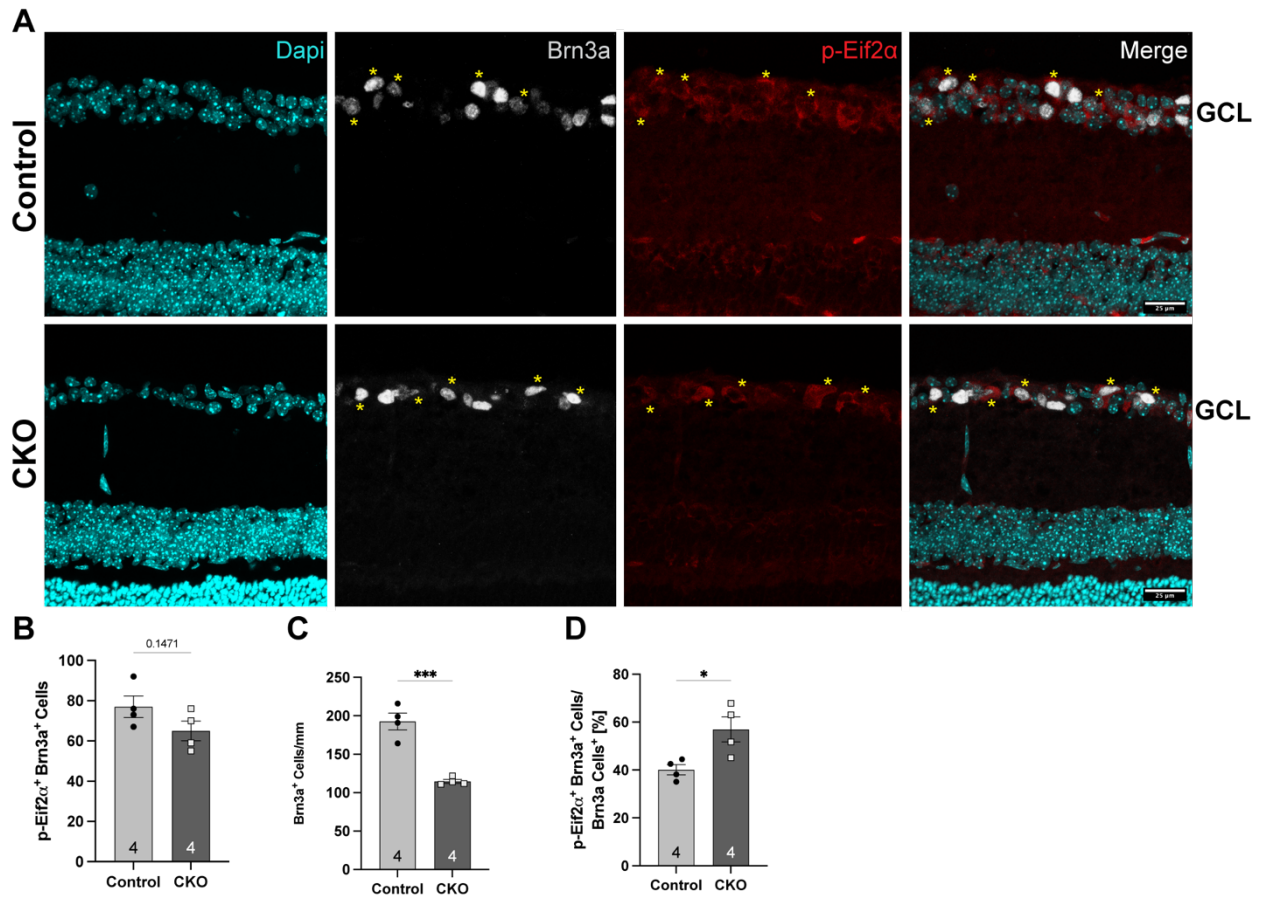


Figure 4: Evidence for an integrated stress response at P30 in the FD retina.

(A) Representative confocal images of RGCs (Brn3a) and p-Eif2α (red) expression in retinal sections of control and CKO retinas with a focus on the GCL and RGCs of all different intensities (yellow asterisks). Scale bar, 25μm. (B) Quantification of double-labeled cells in the GCL. All cells were counted in eight fields of view (FOV). The number of double-labeled cells did not differ between the control and CKO retina (C) Quantification of Brn3a-positive cells per 1.5mm. There is a significant reduction in the Brn3a-positive cells in the CKO. ***p=0.0004. (D) The ratio of double-labeled cells to total Brn3a-positive cells is significantly increased in the CKO compared to the control. *p=0.0021. Data are shown as mean ± SEM, and each data point represents an individual retina. The sample size for individual groups is represented by *n* within each bar. GCL = ganglion cell layer. 1 FOV = 184.52μm².

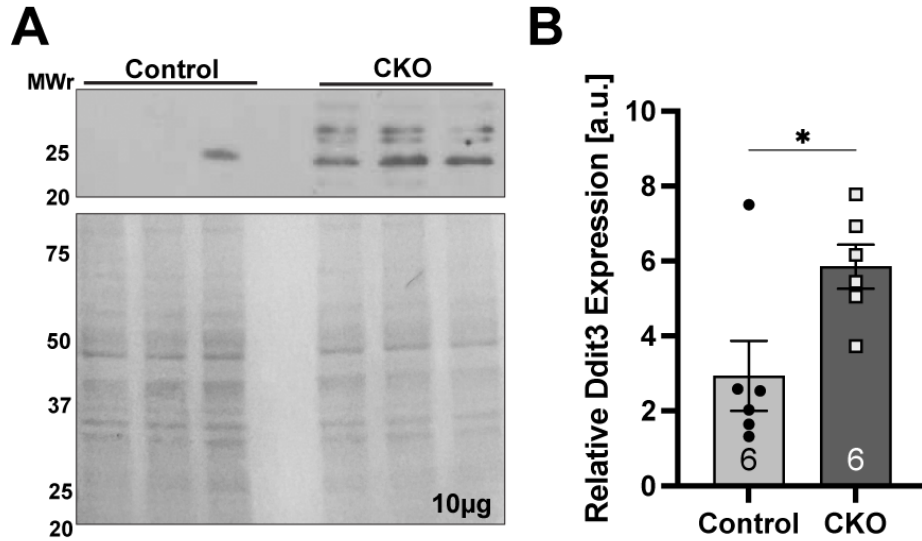


Figure 5: Increase in retinal Ddit3 expression at P30 in the FD mutant.

(A) Representative Western blots of Ddit3 (top) and total protein (10µg) for each retinal sample (bottom). **(B)** Densitometric quantitative results of increased Ddit3 protein in the FD retinal lysates (* $p=0.0247$, unpaired t-test). The Ddit3 protein band was normalized to total protein (Supplemental Figure 1C). Data are shown as mean $\pm$ SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar.

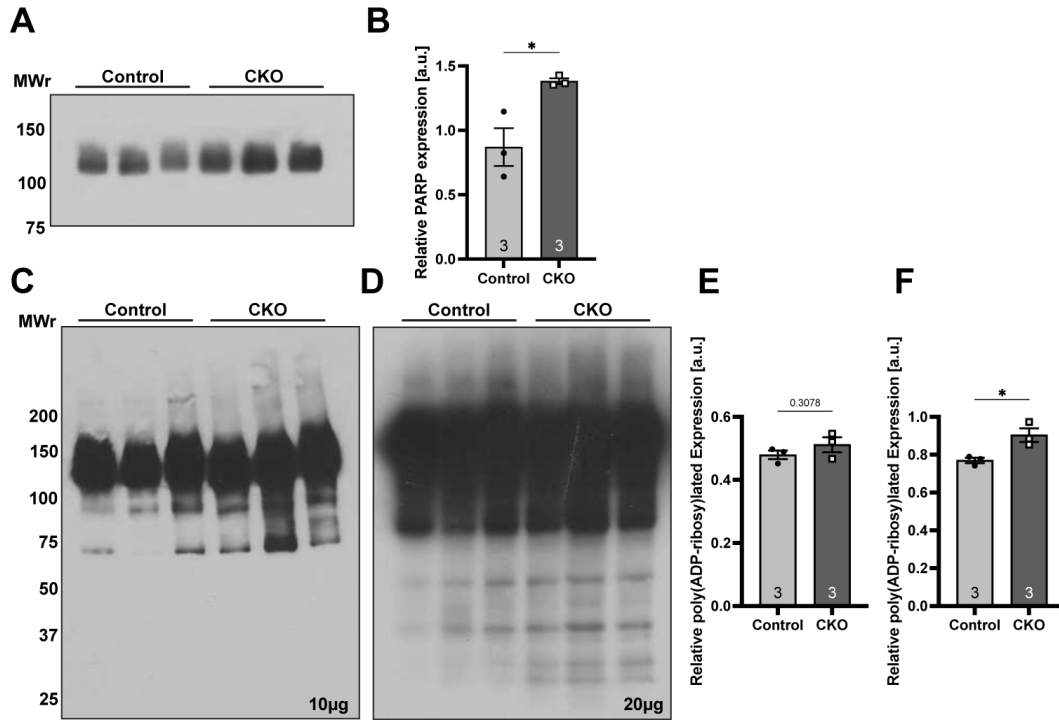
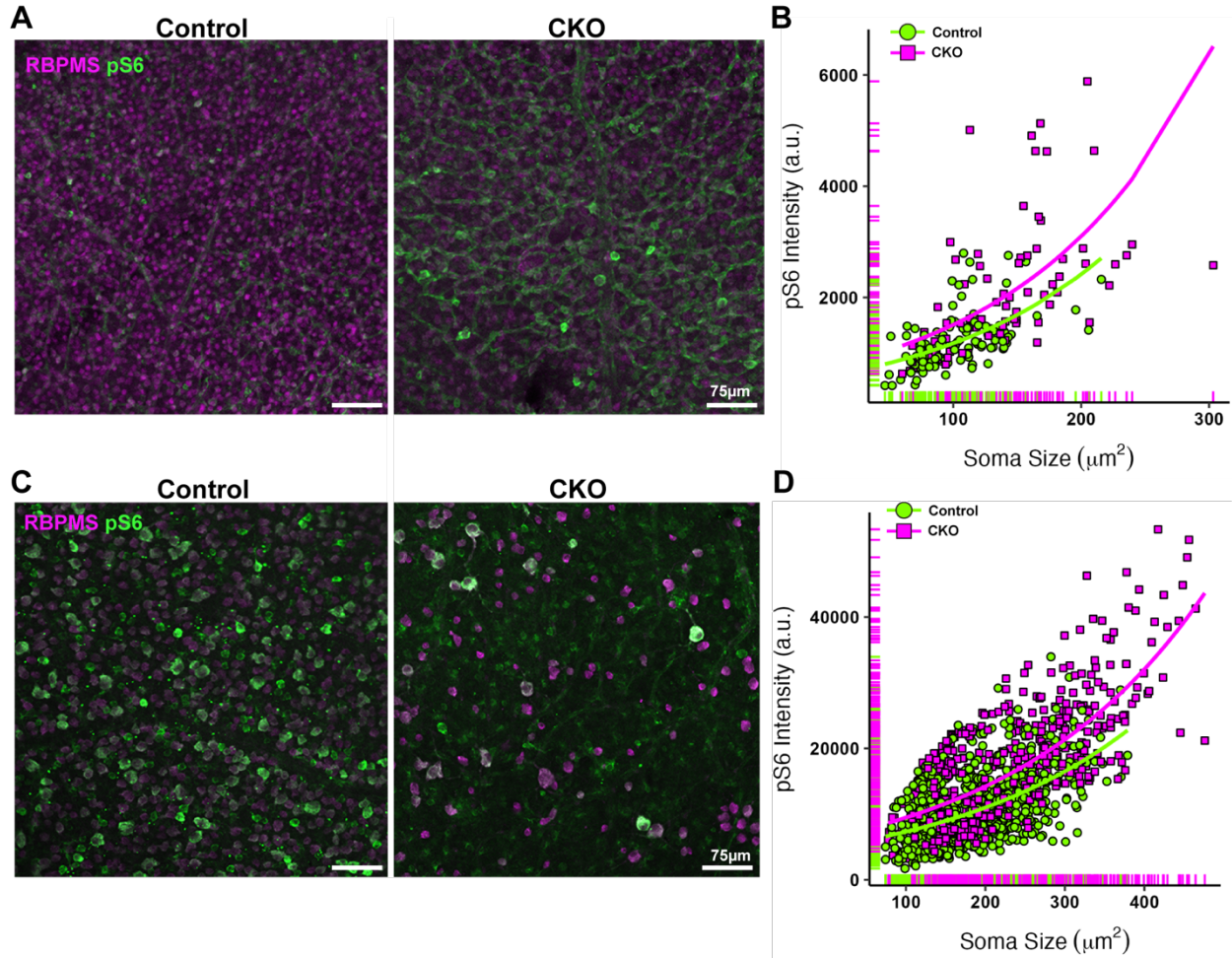


Figure 6: Increase in retinal PARP expression and ADP-Ribosylation at P30.

(A) Immunoblot of PARP in retinal lysates. **(B)** Densitometric quantitative results showed increased full-length PARP (116 kD) in the CKO lysates (* $p = 0.0265$, unpaired t-test). **(C)** Western blots of poly(ADP-ribose)ylated proteins (10µg of total protein per lane). **(D)** Western blots of poly(ADP-ribose)ylated proteins with 20µg of total protein. **(E)** Densitometric quantitative results showed no difference in ribosylated protein in the CKO lysates with 10µg of total protein (ns = 0.3078, unpaired t-test). **(F)** Densitometric quantitative results showed a significant increase in ribosylated protein with 20µg of total protein (* $p = 0.0260$, unpaired t-test). PARP and ADP-ribose expression was normalized to total protein (Supplemental Figure 1D-F). Data are shown as mean $\pm$ SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar.

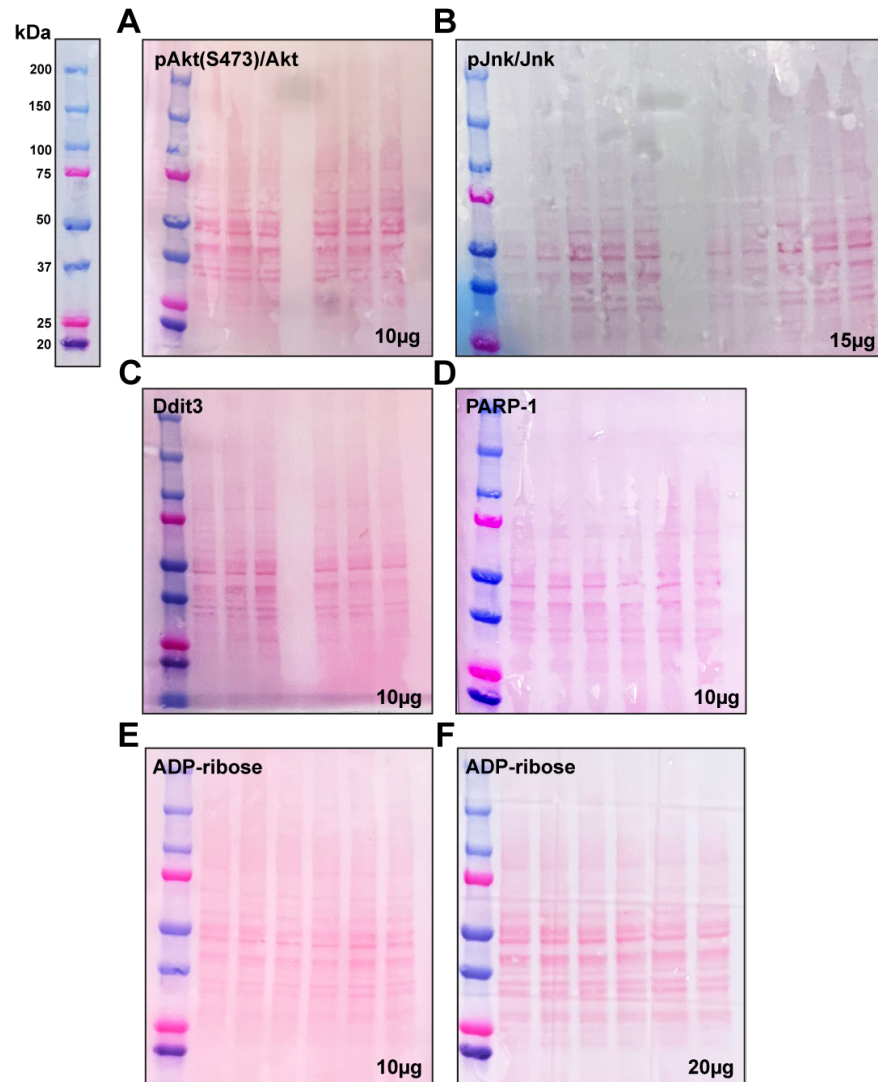
Supplemental Figures



Supplemental Figure 1: pS6 signal is elevated early at P7, continues throughout disease progression at P90, and is associated with larger soma sizes.

(A, C) Representative confocal images of retinal whole mounts from control and CKO mice at P7 and P90 stained for phosphorylated ribosomal protein S6 (pS6; green and RBPMs (magenta) to visualize RGCs. Notice the increased pS6 signal associated with the vasculature structure at P7. Scale bar, 75 μ m. (B) Quantification of pS6-positive RGCs by measuring the intensity of the pS6 stain and the area of RBPMs for each cell ($n= 103$ control RGCs, $n= 78$ CKO RGCs) and using a generalized linear model to visualize the relationship between signal intensity and cell size and while we see a positive relationship between soma size and pS6 intensity the relationship between the control and CKO is significantly different. $p < 0.001$, Nagelkerke's $R^2 = 0.54$, CKO: $\beta=6.60$, 95% CI [6.35, 6.85], Control: $\beta= -0.25$, 95% CI [-0.38, -0.12]. (D) Quantification of pS6-positive RGCs by measuring the intensity of the pS6 stain and the area of RBPMs for each cell ($n= 599$ control RGCs, $n= 556$ CKO RGCs) and using a generalized linear model to visualize the relationship between signal intensity and cell size and while we see a

positive relationship between soma size and pS6 intensity the relationship between the control and CKO is significantly different. $p < 0.001$, Nagelkerke's $R^2 = 0.50$, CKO: $\beta = 8.75$, 95% CI [8.67, 8.83], Control: $\beta = -0.26$, 95% CI [-0.30, -0.21]. Notice the increase in smaller pS6 cells compared to the younger ages; interestingly, by P90, ~50-60% of RGCs have died. This result suggests that as the disease progresses and RGCs die, the smaller remaining cells continue upregulating pS6.



Supplemental Figure 2: Ponceau S staining.

(A-F) PVDF membranes with total protein were used for the quantitative densitometry analysis for all western blots in this study.

Tables

Primary Antibody	Catalog No.	RRID	Concentration	Company	Technique
Rabbit anti - SAPK/JNK	9252	AB_2250373	1:200	Cell Signaling Technology	IFC & WB
Rabbit anti - Phospho SAPK/JNK (Thr183/Tyr185)	9251	AB_331659	1:1000	Cell Signaling Technology	WB
Rabbit anti - Phospho-S6 Ribosomal Protein (Ser235/236)	2211	AB_331679	1:100	Cell Signaling Technology	IFC
Guinea pig anti - RBPMS	1832-RBPMS	AB_249226	1:500	PhosphoSolutions	IFC
Goat anti - Brn3	SC-6026	AB_673441	1:200	Santa Cruz	IFC
Rabbit anti - PARP1 (46D11)	9532	AB_659884	1:1000	Cell Signaling Technology	WB
Rabbit anti - Poly/mono ADP-ribose (E6F6A)	83732	AB_2749858	1:1000	Cell Signaling Technology	WB
Rabbit anti - Phospho Efi2a (S51) (119A11)	3597	AB_390740	1:200	Cell Signaling Technology	IFC
Rabbit anti - Phospho Akt (Thr308) (D25E6) XP	13038	AB_2629447	1:400	Cell Signaling Technology	IFC
Rabbit anti - Phospho Akt (S473)	9271	AB_329825	1:1000	Cell Signaling Technology	WB
Rabbit anti - Akt	9272	AB_329827	1:1000	Cell Signaling Technology	WB
Mouse anti - Ddit3 [9C8]	ab11419	AB_298023	5µg/mL	Abcam	WB

Table 1: Primary antibody list with concentration and catalog information.

Secondary Antibody	Catalog No.	RRID	Concentration	Company
Donkey anti - rabbit 568	A10042	AB_2534017	1:2000	Thermo Fisher
Donkey anti - rabbit 647	A-31573	AB_2536183	1:2000	Thermo Fisher
Donkey anti - rabbit 488	A-21206	AB_2535792	1:2000	Thermo Fisher
Donkey anti - goat 647	706-605-147	AB_2340437	1:1000	Jackson Laboratories
Donkey anti - guinea pig 647	A-21450	AB_2735091	1:2000	Thermo Fisher

Table 2: Secondary antibody list with concentration and catalog information.

CHAPTER FIVE

ADDITIONAL FINDINGS

Introduction

Familial dysautonomia (FD) is a progressive neurodevelopmental and neurodegenerative disorder caused by a point mutation in the ELP1 gene. Patients present with reduced peripheral sensory function, congenital and progressive neuropathies, such as reduced peripheral sensory function, poor growth, and digestive and respiratory problems. However, progressive visual impairment is one of the most debilitating conditions affecting patients' quality of life. This visual impairment is marked by a continual loss of retinal ganglion cells (RGCs), resulting in severely decreased visual acuity, a central field of view decline, and color vision defects. Clinical findings show reduced papillomacular retinal nerve fiber layer (RNFL) thickness and mouse models have corroborated these findings by showing mitochondrial dysfunction and reduced ATP^{6,39,46,118}, all indicative of optic neuropathy.

In neurodegenerative retinal diseases such as glaucoma and diabetic retinopathy, RGCs are the primary cell type affected. However, studies have shown observed cell death in other retinal layers, i.e. the amacrine cells of the inner nuclear layer (INL).^{119,120} Amacrine cells are interneurons that are synaptically active in the inner plexiform layer (IPL), integrating and modulating information in a time-dependent manner to the RGCs.^{121,122} Amacrine cells have myriad subtypes, and each plays an integral role in signal transmission in the retina. Dopamine (DA) neurotransmission in the retina is primarily regulated by a single subpopulation of amacrine cells¹²², the dopaminergic amacrine cells (DACs). DA plays a crucial role in the

modulation of light adaptation and color vision, along with synaptic formation and transmission.¹²³

In Parkinson's disease (PD), a neurodegenerative disease characterized by the death of dopaminergic neurons in the Substantia nigra, many patients present with visual impairment like FD, including decreased visual acuity, color blind defects, and difficulties performing more complex visual tasks.¹²⁴ Moreover, PD patients also show a reduction in the RNFL indicative of altered mitochondrial function^{124–126} which is similar to the clinical OCT findings of axonal loss in the papillo-macular bundle found in Leber hereditary optic neuropathy (LHON), dominant optic atrophy (DOA), and FD.

Of particular interest is understanding the overall dysregulation seen in neurons. Furthermore, we have shown reduced TH-positive cells in the nodose-petrosal ganglia of three FD mouse models.¹²⁷ Therefore, immunostaining whole-mount retinas with tyrosine hydroxylase (TH), the rate-limiting step in DA synthesis. We found alternations in the TH-positive dopaminergic amacrine cell populations.

Results

To determine whether there were any other cell types affected in the FD retina, control, and CKO retinæ at three different time points were immunolabeled with an antibody to TH (rabbit anti-Tyrosine Hydroxylase, Millipore, #AB152). Interestingly, at postnatal day 7 (P7), before mice have opened their eyes and all amacrine cells have been born, we do not see a difference in the number of TH-positive cells between the control and CKO (Figure 1A, B). However, by P30, when we first start to see significant RGC death, we found significantly less TH-positive amacrine cells in the CKO (Figure 1A, B; **** p < 0.0001). This trend continued

until P90, with a similar number of TH-positive cells in the CKO, and this was significantly reduced in the CKO compared to the control (Figure 1A, B; **** $p < 0.0001$).

Discussion

This small study provides the first evidence that specific subtypes of retinal neurons may also be affected by the loss of Elp1. Furthermore, this data might help explain the decline in visual acuity and color-vision deficits in patients.^{6,45} Interestingly, it does not appear that the DA amacrine cells are dying because the number of TH-positive cells in the CKO is consistent throughout disease progression, whereas in the control, we see an increase in roughly 400 cells from P7 to P30.

Amacrine cells are the most diverse cell type in the retina, and despite their morphological differences, most amacrine cells are inhibitory interneurons and can be divided into two groups based on whether they use GABA or Glycine as their main neurotransmitter. Furthermore, the GABAergic cells and glycinergic cells can also express additional neurotransmitters.^{128–130} Dopaminergic amacrine cells are also GABAergic, and GABAergic amacrine cells are “born” at distinct times. The majority of the TH-positive amacrine cells are born by E16.¹²⁸ Therefore, by P7 all amacrine cells fated to be GABA⁺ TH⁺ should have undergone terminal mitosis and begun differentiating. Taken together, this indicates a potential problem with differentiation in a small subtype of neurons in the absence of Elp1. Additionally, the alterations in cell number could lead to modifications in receptive properties of RGCs, which may result in additional visual processing dysfunction seen in FD patients. This preliminary experiment with exciting results warrants further investigation to understand the impact of DA in

the FD retina, confirm potential alterations in neuronal differentiation in the absence of Elp1, and elucidate yet another biological function of Elp1 and the Elongator complex.

Materials and Methods

Imaging

Retina flat mount images were obtained with a Leica DMI8 THUNDER imaging system using the navigator spiral scan function with a 20X objective under brightfield illumination with false color. Confocal images of flat-mount retinas were obtained using Leica TSC SP8 with a 20X oil immersion objective with a resolution of 1024 X 1024—a single image 1mm from the peripheral edge of the tissue in each retinal quadrant.

Cell Counting

Retinal images were processed using ImageJ plugin: Fiji. The Total TH⁺ cells in each retina were counted manually. All images were randomized, ensuring the experimenter was blind to the genotype. The data is reported as the total number of TH⁺ amacrine cells per retina.

Statistics

For pairwise comparisons, unpaired two-tailed t-tests were used. Significance was concluded when $p < 0.05$. All data are expressed as mean $\pm$ SEM. Column data are plotted as scatter dot-bar plots to show variability, and n shows the sample size for each data set. GraphPad Prism (version 9) was used to make the graph.

Figures

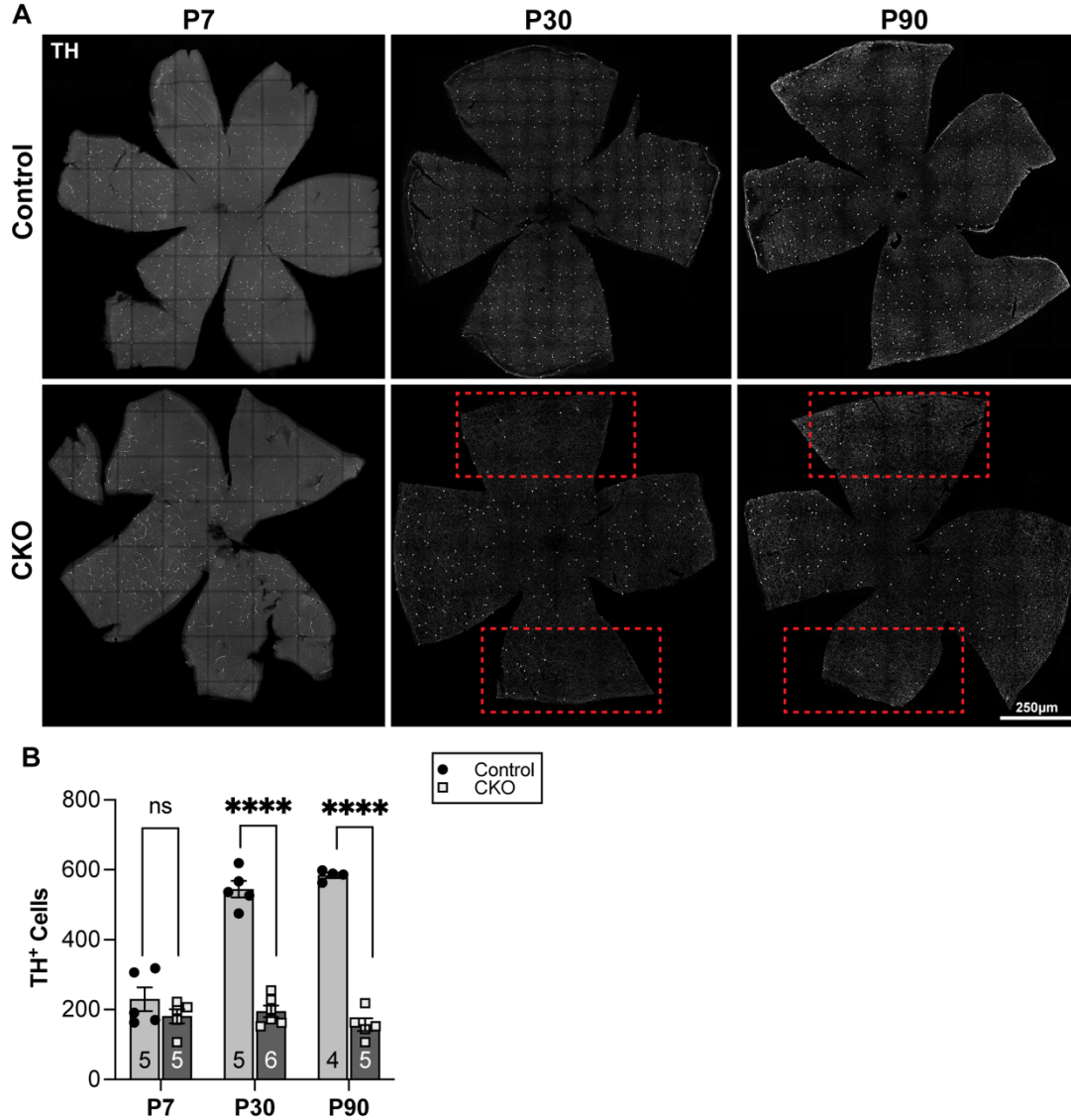


Figure 1: Changes in TH⁺ cell number throughout disease progression.

(A) Representative retinal flat mount stained with anti-TH at three different ages (columns) showing changes in cell number as the disease progresses between control (top panel) and CKO (bottom panel). Red boxes indicate regions with reduced TH cells in the CKO. Scale bar, 250µm. (B) Quantification of TH⁺ amacrine cells from control mice compared to CKO mice. TH⁺ cells were counted in the entire retina. ns = 0.2545, **** p < 0.0001, unpaired t-test. Data are shown as average ± SEM, and each data point represents an individual retina. The sample size for individual groups is represented by n within each bar.

CHAPTER SIX

CONCLUSION

Summary

Our data reveal that in the FD retina, there are alterations in glial cell populations and vascular remodeling and provides direct evidence for ER stress and non-canonical stress signaling in retinal neurons, and presents an additional cell type that is affected by the loss of Elp1. Collectively, our data show a multifaceted contribution to the preferential death of RGCs and propose that the implications of reduced Elp1 in the retina are more far-reaching than the death of RGCs. Furthermore, we present promising preliminary data that RGCs can be rescued *in vivo* in our FD retinal mouse model.

Specifically, we demonstrate that all retinal glial cells are subsequently affected by a loss of Elp1. We see an initial gliosis event characterized by upregulation of GFAP in astrocytes, which is maintained throughout disease progression. Concurrently, there is an increase in amoeboid or “activated” microglia that are asymmetrically localized in the FD retina to the ganglion cell layer early in post-natal life, and the proliferation of microglia increases as the loss of RGCs progresses. Most interestingly, we do not see Müller glia activation, and there is a trend of reduced glutamine synthetase, indicating a potential reduction in overall Müller cell function.

Additionally, we provide evidence for the indirect effect of loss of Elp1 in the retina based on the vascular remodeling observed throughout the disease progression. The blood vessels are born outside of the retina and are not directly affected by the conditional knockout in this model, yet we observe significant changes in the superficial vascular plexus, suggesting the

subsequent effect of neurons on vascular health and that bidirectional signaling could be exacerbating the disease state. In this project, we report for the first time in the retina that signaling molecules related to stress responses are altered and that the death of RGCs may result from noncanonical signaling due to Elongator's function in translation efficiency. However, additional studies are required to confirm this. Mitochondrial dysfunction^{6,42}, mTOR signaling, ER stress, and caspase-independent cell death have all been shown to be involved in neurodegenerative diseases. Here, we present evidence for increased mTORC1 activation that could be leading to increased protein synthesis, activation of the integrated stress response with an increase in p-Eif2a, and potential for ER stress due to the presence of Ddit3 in retinal lysates, and increased PARP-1 activity. These pathways can offer specific cellular and molecular targets for therapeutic development in FD. While we could not directly address this here, it provides a template for future studies.

Lastly, we show the efficacy of RGC-specific gene transfer and systemic delivery of a small molecule splicing modulator in preventing RGC death in models of FD and provide the rationale for translating gene therapy and splicing compounds, post optimization, into clinic trials to prevent the optic neuropathy in FD and provide potential treatments for other common ocular diseases like glaucoma, diabetic retinopathy.

Future Studies

Our results provide a two-fold template for future investigations into the cellular mechanisms responsible for neuronal death, understanding the retinal pathophysiology in FD, and the potential for targeted therapies.

First, addressing the cellular pathology, (1) to what extent does the bidirectional signaling of the vasculature, glial cells, and *all* retinal neurons exacerbate the disease state and contribute to RGC death, and (2) are the signaling pathways uncovered here executing RGC death or do we see these alterations in response to cell death, and (3) to what degree do these findings contribute to the metabolic and mitochondrial dysfunction found in FD or is mitochondrial dysfunction the initial activator of metabolic dysfunction and RGC death? and (4) a more precise timeline to understand the causality of retinal degeneration could inform about the most effective therapies.

Second, we demonstrate that an AAV2 viral vector driven under a U1a promoter and the oral administration of a small splicing compound can prevent RGC death in mouse models of FD. Thus, how do these therapeutics affect the underlying cellular pathologies identified in this study? Are we able to correct gliosis and prevent vascular remodeling? Are aberrant signaling pathways restored to promote cellular homeostasis instead of cellular stress and death? Does early administration of the splicing compound increase the differentiation of dopaminergic amacrine cells? Even more importantly, are we restoring function to cells, and do we have functional target innervation by RGCs even though we see increased RGC cell bodies?

Of important note are future investigations focusing on the target innervation and axonal transport in retinal ganglion cells. Therapeutics targeting RGCs will be more effective and long-term if we have information about the function of Elp1 in axon development, transport, and target innervation. While we know retinal ganglion cell bodies develop in normal numbers in the FD mouse model^{42,68} and patients are born with normal visual acuity, there is evidence in patients that axonal loss precedes visual decline.⁴⁶ It is unknown whether these neurons (1) innervate their target appropriately and proceed to retract processes due to a lack of trophic

support or additional environmental cues without Elp1 or (2) if initially, a percentage of axons are unable to reach and innervate the brain and therefore retract their processes. Interestingly, FD patients experience ophthalmic symptoms that are not directly related to visual impairment caused by RGC loss. Patients have decreased lacrimation (a reduction in the secretion of tears/fluid from the eyes) and reduced corneal sensitivities.^{45,46} These functions rely on input from the ophthalmic division of the trigeminal ganglion, and there is evidence that Elp1 is required for axonal outgrowth and proper innervation in the trigeminal ganglion.¹³¹

Final Remarks

The retina is a tightly regulated system, and cells vary in their cellular proteome. While there is redundancy in the genetic code to protect against damage and pathological states, we still see progressive death of RGCs and indirect cellular effects of reduced Elp1. The retina is an optimal system for therapy due to its accessibility, the variety of cell types, and the blood-retinal barrier, allowing the retina to be an immune-privileged tissue. Here, we provide a novel characterization of the FD retina and establish a baseline for future studies and therapeutic interventions.

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