mTOR activity is essential for retinal pigment epithelium regeneration in zebrafish

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ABSTRACT:

The retinal pigment epithelium (RPE) plays numerous critical roles in maintaining vision and this is underscored by the prevalence of degenerative blinding diseases like age-related macular degeneration (AMD), in which visual impairment is caused by progressive loss of RPE cells. In contrast to mammals, zebrafish possess the ability to intrinsically regenerate a functional RPE layer after severe injury. The molecular underpinnings of this regenerative process remain largely unknown yet hold tremendous potential for developing treatment strategies to stimulate endogenous regeneration in the human eye. In this study, we demonstrate that the mTOR pathway is activated in RPE cells post-genetic ablation. Pharmacological and genetic inhibition of mTOR activity impaired RPE regeneration, while mTOR activation enhanced RPE recovery post-injury, demonstrating that mTOR activity is necessary and sufficient for RPE regeneration in zebrafish. RNA-seq of RPE isolated from mTOR-inhibited larvae identified a number of genes and pathways dependent on mTOR activity at early and late stages of regeneration; amongst these were components of the immune system, which is emerging as a key regulator of regenerative responses across various tissue and model systems. Our results identify crosstalk between macrophages/microglia and the RPE, wherein mTOR activity in the RPE is required for recruitment of macrophages/microglia to the injury site. In turn, these macrophages/microglia reinforce mTOR activity in regenerating RPE cells. Interestingly, the function of macrophages/microglia in maintaining mTOR activity in the RPE appeared to be inflammation-independent. Taken together, these data identify mTOR activity as a key regulator of RPE regeneration and link the mTOR pathway to immune responses in facilitating RPE regeneration.

AUTHOR SUMMARY:

Age-related macular degeneration (AMD) is a leading cause of blindness world-wide, with incidences predicted to rise substantially over the next few decades. Cells of the retinal pigment epithelium (RPE) are affected in AMD and there are currently no effective therapies that slow RPE cell death or restore lost RPE cells in advanced-stage AMD. An exciting potential approach to treat many diseases of the eye, including AMD, is to stimulate endogenous regeneration to restore cells lost to disease. For this to become possible, we must first understand the molecular and cellular underpinnings of the regenerative response. In this study, we utilize zebrafish as a model system, which possess tremendous regenerative potential in multiple cell and tissue types, including the RPE. Our results identify the mTOR signaling pathway as a key regulator of RPE regeneration. We identify a link between mTOR signaling and immune responses, which are known to modulate regeneration of a variety of tissues and organs. Together, our results identify one of the first molecular mechanisms facilitating intrinsic RPE regeneration and these data could serve as a foundation for the development of new therapies aimed at stimulating the regeneration of RPE cells in the diseased eye.

INTRODUCTION:

The retinal pigment epithelium (RPE) is a monolayer of ocular cells located between the vascular choriocapillaris and neural retina. The RPE plays myriad critical roles during vision and this is highlighted by the prevalence of degenerative blinding diseases like age-related macular degeneration (AMD), in which the RPE is primarily affected. AMD is a leading cause of blindness in the developed world; indeed, advanced AMD with geographic atrophy (so called “dry” AMD) accounts for the majority of AMD cases worldwide (1). Functions of the RPE during vision include: phagocytosis of photoreceptor outer segments and recycling of key components of the visual cascade; transport of nutrients and ions between the choriocapillaris and retina; secretion of growth factors; and contributing to the blood-retina barrier, amongst others (2). Despite the importance and involvement of the RPE in human blinding diseases, there are currently no FDA-approved treatments available to treat RPE loss in diseases like AMD.

In recent years, RPE cell replacement therapies like transplantation of stem cell-derived RPE, autologous peripheral RPE, or RPE progenitor cells have been shown to restore some visual function in animal models of human disease, and some are in early-phase human trials (3). Unfortunately, these therapies have not yet been proven effective enough for use as an AMD treatment, and immune rejection and limited long-term survival and function of the grafts *in vivo* are challenges that remain to be overcome before these can be clinically available (4). A complementary reparative strategy to explore for use in human patients is to stimulate intrinsic RPE regeneration. However, in mammals, the RPE shows an extremely limited capacity to regenerate after injury (5–8). RPE cells terminally differentiate during the early stages of eye development and remain mitotically inactive in adults, under most conditions. RPE cells do have a modest capacity to proliferate in some injury contexts; however, this can turn pathological in conditions like proliferative vitreoretinopathy (9). Recently, it was shown that a subpopulation of adult human RPE cells, termed retinal pigment epithelial stem cells (RPESCs), were capable of self-renewal and differentiation into neural and mesenchymal progeny *in vitro* (10,11). This was an exciting result as it demonstrated that there could be an intrinsic, but latent, potential for human

RPE to be induced to regenerate.

In contrast to mammals, zebrafish possess remarkable regenerative capacity after injury in multiple tissues (8,12), including the RPE (13,14). Previous work from our laboratory established a transgenic zebrafish RPE ablation model (*rpe65a:nfsB-eGFP*) and demonstrated that RPE could intrinsically regenerate into a functional monolayer after widespread genetic ablation (13). Using this model, Wnt signaling and innate immune system activity have been shown to facilitate RPE regeneration (13,14). Despite these advances, the molecular mechanisms underlying intrinsic RPE regeneration in zebrafish are still unclear. Identifying these mechanisms could have a significant impact on developing strategies to stimulate endogenous regeneration in the human eye.

The mechanistic target of rapamycin (mTOR) pathway is activated by a number of intracellular and extracellular inputs that include growth factors, stress, energy, oxygen, and nutrient levels to regulate cell growth and metabolism during development and in a variety of physiological processes (15,16). mTOR activity is also known to contribute to RPE function. For example, mTOR is activated in the RPE physiologically by the phagocytosis and digestion of photoreceptor outer segments (17–19) and pathologically by stress, which leads to RPE cell dedifferentiation and hypertrophy (20). Moreover, *in vitro* studies using cultured RPE have demonstrated that mTOR activity is essential for the survival, migration and proliferation of RPE cells after damage (21–23). mTOR activity is also associated with regeneration in several organs and tissues across Metazoa (24). For instance, in zebrafish, mTOR activity regulates fin (25), liver (26), and heart regeneration (27). In mammals, mTOR plays important roles in optic nerve regeneration (28–30) and this function is conserved in human induced pluripotent stem cell (iPSC)-derived retinal ganglion cells (31). In zebrafish and chicken, mTOR activity has also been shown to facilitate retinal regeneration (32,33).

Functions of mTOR during RPE regeneration have not been reported but given the association with regenerative events in other contexts, and known functions in the RPE, we hypothesized that mTOR might facilitate RPE regeneration. Taking advantage of our zebrafish RPE ablation-regeneration

model, here we demonstrate that mTOR is rapidly activated in RPE cells post-ablation. Through pharmacological and genetic manipulations, we show that mTOR activity is necessary for RPE regeneration and that ectopic activation of the mTOR pathway is sufficient to expedite RPE regeneration post-injury. Mechanistically, mTOR activity after RPE injury triggers the recruitment of macrophages/microglia to the injured RPE, which then further reinforce mTOR activity in the regenerating RPE. Taken together, these data highlight a novel role for mTOR during RPE regeneration and reveal another avenue through which therapies could be developed to restore RPE lost to degenerative ocular disease.

RESULTS:

Dynamics of mTOR activity after RPE ablation

To stimulate RPE injury and subsequent regenerative responses, we utilized *rpe65a:nfsB-eGFP* transgenic zebrafish and metronidazole (MTZ)-mediated cell ablation (13). In this model, MTZ addition results in ablation of approximately the central two-thirds of the RPE, which stimulates a regenerative response over the following two weeks. After onset of RPE ablation at 5 days post-fertilization (dpf), the spatial and temporal activity of mTOR was quantified using a monoclonal antibody against phosphorylated 40S ribosomal protein S6 (p-S6), a downstream effector of the mTOR pathway that is routinely used as a readout of mTORC1 activation (26,34). We focused on post-injury time points ranging from 3 hours (hpi) to 4 days (dpi), which encompass the peak phases of the RPE regenerative response (13). In unablated (MTZ⁻) larvae, low basal levels of p-S6 were detected in the RPE layer from 3hpi to 12hpi, and p-S6 was almost undetectable in the RPE layer after 12hpi (Fig 1A-G). By contrast, p-S6 levels were significantly increased in the RPE layer of ablated (MTZ⁺) larvae from 6hpi to 3dpi, with peak activation detected during 6-12hpi (Fig 1H-L, O). From 6hpi to 1dpi, p-S6 colocalized with eGFP in RPE cells, confirming that the mTOR pathway was activated in the RPE after ablation (Fig 1I-K). However, at 2 and 3dpi, while p-S6 remained enriched in eGFP⁺ RPE, some p-S6⁺ cells also occupied the central region where the ablated RPE had been cleared (Fig 1L,M), suggesting that mTOR could also be active in regenerating RPE and/or cells that infiltrated the RPE layer during the response to injury. In addition to the RPE, p-S6 signal was also observed in the retinae of MTZ-treated larvae (e.g. Fig 1H-L; arrowheads). Given that RPE ablation leads to rapid degeneration of the photoreceptors (13), these p-S6⁺ cells are likely Müller glia that have initiated a regenerative response to repair the retinal damage (35), a process known to involve mTOR activation (32,33).

mTOR activity is required for RPE regeneration

To determine whether mTOR activity was required during RPE regeneration, we utilized complementary pharmacological and genetic perturbations to mTOR and assessed the regenerative ability of the RPE. To pharmacologically inhibit mTOR activity, rapamycin, an allosteric inhibitor of

mTORC1 (36,37), and INK128, an adenosine triphosphate competitive inhibitor targeting both mTORC1 and mTORC2 (21), were utilized. Larvae were immersed in system water containing 2 μ M rapamycin, 0.9 μ M INK128, or DMSO as a vehicle control, from 24 hours prior to RPE ablation until they were sacrificed for analyses (Fig 2A,G). p-S6 distribution in the RPE at 2dpi was completely abolished by rapamycin or INK128 treatment (Fig 2B-F), demonstrating the efficacy of these treatments in blocking mTOR activity in zebrafish. To evaluate the effects of mTOR inhibition on RPE regeneration, we first assessed RPE proliferation post-ablation, which has been used previously as a quantitative readout of the regenerative response (13,14). We performed 24-hour BrdU incorporation assays and assessed pigment recovery in rapamycin- or INK128-treated and DMSO-treated MTZ⁺ and MTZ⁻ larvae at 4dpi, the time at which proliferation within RPE layer peaks post-injury (13). Quantification of the number of BrdU⁺ cells within the RPE layer showed that rapamycin and INK128 treatments significantly decreased proliferation post-injury, when compared to 4dpi DMSO-treated controls (Fig 2H-L). There were no obvious differences between rapamycin- or INK128-treated or DMSO-treated MTZ⁻ larvae (S1A-D,I Fig). Next, we quantified RPE pigment recovery as a readout of the extent to which the RPE had regenerated (13,14). Consistent with BrdU incorporation results, RPE pigment recovery was also significantly diminished in rapamycin- and INK128-treated larvae when compared to DMSO-treated controls (Fig 2M-Q). Rapamycin and INK128 showed no effect on RPE pigmentation in MTZ⁻ controls (S1E-H Fig). Importantly, mTOR inhibitors did not affect MTZ-mediated RPE ablation, as TUNEL staining at 1dpi showed no significant differences between rapamycin- and INK128-treated larvae and DMSO controls (S2 Fig).

Pharmacological inhibition of mTOR resulted in impaired RPE regeneration, supporting a role for mTOR activity in the regenerative response. We next wanted to confirm these data genetically; here, we took advantage of a zebrafish *mtor* mutant allele (*mtor*^{sal16755}), which contains a nonsense mutation that is predicted to truncate the mTOR protein by 64% (38), and crossed the *rpe65a:nfsB-eGFP* ablation transgene into this mutant background. Previous studies showed that *Mtor* knockout in mouse and *Drosophila* (*dtor*) resulted in embryonic lethality (39,40), and in a different zebrafish *mtor* mutant allele (*mtor*^{xu015}), most homozygous *mtor* mutants died by 10dpf (41). In our assays, *rpe65a:nfsB-eGFP*;

mtor^{sal16755} larvae (hereafter referred to as *mtor*^{-/-}) survived to at least 8dpf, with some *mtor*^{-/-} larvae even inflating their swim bladders. However, at 9dpf, roughly half of the homozygous mutants had died, with the remainder appearing generally healthy. Therefore, our analyses of RPE regeneration were necessarily limited to these 9dpf/4dpi *mtor*^{-/-} surviving larvae, which could have lower expressivity of *mtor* loss-of-function phenotypes. Importantly, mTOR activity after MTZ-mediated ablation, as assessed by p-S6 immunostaining at 2dpi, was significantly reduced in *mtor*^{-/-} larvae, validating the utility of the *mtor*^{sal16755} allele (Fig 3A-C). As with the pharmacological perturbations, 24-hour BrdU incorporation from 3-4dpi and subsequent immunohistochemistry revealed significant decreases in proliferative cells within the RPE layer of *mtor*^{-/-} larvae, when compared to *mtor*^{+/+} controls (Fig 3D-F). There were no significant differences in BrdU incorporation between *mtor*^{+/+} and *mtor*^{-/-} MTZ⁻ controls (S1J-L Fig). Finally, RPE regeneration was also significantly impaired in *mtor*^{-/-} larvae, as measured by both eGFP recovery and ZPR2 expression (Fig 3G-I). These findings confirm the results from pharmacological inhibition of mTOR activity (Fig 2) and, collectively, provide three independent perturbations that support a requirement for mTOR activity in facilitating RPE regeneration.

Activation of mTOR signaling is sufficient to enhance RPE regeneration

To test the sufficiency for mTOR signaling to enhance RPE regeneration, we utilized an mTOR agonist, MHY1485, to activate mTOR signaling (42). Larvae were placed into either DMSO- or 2μM MHY1485-containing system water from 24 hours prior to RPE ablation until they were sacrificed for analyses (Fig 4A,E). To confirm the efficacy of MHY1485-mediated mTOR activation, p-S6 levels in the RPE layer were quantified at 2dpi and shown to be significantly increased when compared to MTZ⁺ DMSO-treated controls (Fig 4B-D). To evaluate the effect of MHY1485 on proliferation within the RPE layer post-injury, we quantified the number of BrdU⁺ cells within the RPE layer at 2, 3, and 4dpi. While there were no significant differences between MTZ⁺ MHY1485-treated and DMSO-treated groups at 2dpi or 4dpi (S3 Fig), at 3dpi, MTZ⁺ MHY1485-treated larvae possessed significantly more BrdU⁺ cells in the RPE layer when compared with DMSO-treated siblings (Fig 4H-J). Indeed, MHY1485 treatment pushed forward the peak of proliferation in the RPE post-ablation to 2-3dpi, one day earlier than in normal regenerating conditions (13). Notably, MHY1485 showed no impact on

proliferation in unablated larvae (Fig 4F,G,J). To determine the effect of MHY1485 treatment on RPE regeneration, we quantified central ZPR2 recovery at 3dpi and detected a significant increase in overall regeneration in the MHY1485-treated larvae when compared to DMSO controls (Fig 4K-O). Importantly, MHY1485 treatment did not impair MTZ-mediated ablation, as shown by TUNEL staining (S2C,F Fig). When combined with our loss-of-function studies, these data support a model in which mTOR activity is both necessary and sufficient for RPE regeneration in zebrafish.

Immune-related gene expression is downregulated when mTOR activity is impaired during RPE during regeneration

We next sought to identify the mechanisms by which mTOR regulates RPE regeneration. To achieve this, we performed RNA-seq analyses on fluorescence-activated cell sorting (FACS)-isolated eGFP⁺ RPE cells from MTZ⁻ and MTZ⁺ DMSO-treated and rapamycin-treated *rpe65a:nfsB-eGFP* larvae at 2dpi/7dpf and 4dpi/9dpf (Fig 5A; S1-4 Tables). These time points represent early (2dpi) and peak (4dpi) regenerative stages (13). Significantly differentially expressed genes (DEGs) were identified by comparing MTZ⁺ DMSO- and rapamycin-treated expression profiles, with MTZ⁺ DMSO replicates set as the control group. A total of 252 filtered DEGs were derived from the 2dpi dataset, among which 108 genes were upregulated and 144 were downregulated in rapamycin-treated RPE (Fig 5B; S1,2 Tables). In the 4dpi dataset, 948 filtered DEGs were identified, among which 195 genes were upregulated and 753 were downregulated in rapamycin-treated RPE (Fig 5B; S3,4 Tables). To examine the mTOR-dependent mechanisms that modulate RPE regeneration, we performed pathway enrichment analyses using STRING (43) and focused on downregulated DEGs in rapamycin-treated larvae. Interestingly, the most downregulated DEGs in rapamycin-treated larvae at 2dpi populated immune-related Reactome pathways (Fig 5C). DEGs within these immune-related pathways included proinflammatory cytokines (e.g. *il34*, *cxc118a.1*), which are important signals for recruitment and chemotaxis of leukocytes (44,45), matrix remodeling proteins (e.g. *mmp9*, *qsox1*), which are crucial for the migration of RPE cells as well as macrophages (46–48), neutrophil regulation genes (e.g. *serpinb1*, *mpx*), amongst other genes associated with regulating an immune response (e.g. *iqgap2*, *itgb5*, *krt8*) (Fig 5D; S5 Table) (49–51). Importantly, most of these immune-related DEGs were upregulated in

MTZ⁺ DMSO-treated controls, when compared to MTZ⁻ DMSO-treated controls (Fig 5D; S6 Table), consistent with our previous study (14). Taken together, these data support a model in which immune system activity is upregulated in RPE cells at an early stage of regeneration (2dpi) in an mTOR-dependent manner.

At 4dpi, immune-related pathways were no longer enriched in the downregulated 4dpi dataset when comparing rapamycin- to DMSO-treated controls (Fig 4C). Downregulated genes at this time point comprised pathways related to small molecule transport and cell-cell communication pathways, including GABA, neurotransmitter, and G-protein signaling (Fig 5C; S7 Table). Taken together, these data suggest a model in which mTOR acts early during regeneration as a regulator of the immune response, while later, mTOR-dependent gene regulation perhaps facilitates some of the restoration/reintegration of regenerated RPE cells into a functional tissue.

mTOR signaling regulates the recruitment of macrophages/microglia during RPE regeneration

Recently, we have shown that macrophages/microglia infiltrate the injury site post-RPE ablation and are important regulators of zebrafish RPE regeneration (14). That rapamycin impaired the expression of immune-related genes and pathways suggests that mTOR activity may be required for leukocyte recruitment and/or function during RPE regeneration. To test this hypothesis, we utilized *mpeg1:mCherry;rpe65:nfsB-eGFP* transgenic fish, in which macrophages and microglia are labeled by mCherry (52). MTZ⁺ and MTZ⁻ control *mpeg1:mCherry;rpe65:nfsB-eGFP* larvae were treated with rapamycin or DMSO from 4dpf until 3dpi (Fig 6A), the time at which macrophage/microglia infiltration peaks after RPE ablation (14). Immunostaining for mCherry revealed no visible accumulation of macrophages/microglia in the RPE layer of DMSO-treated MTZ⁻ larvae (Fig 6B), while, as expected, significant macrophage/microglia recruitment to the RPE layer in DMSO-treated MTZ⁺ larvae was observed (Fig 6D,G). By contrast, macrophage/microglia presence in the RPE layer was significantly reduced in both rapamycin-treated MTZ⁻ and MTZ⁺ larvae (Fig 6D-F). However, due to the significant recruitment of additional macrophages/microglia to the RPE layer of MTZ⁺ larvae over resident populations in MTZ⁻ larvae, the magnitude of decrease was more substantial in rapamycin-treated MTZ⁺

larvae when compared to DMSO-treated MTZ⁺ controls (i.e. a decrease from 4.072% to 1.069% of the RPE layer occupied by mCherry⁺ cells in rapamycin-treated MTZ⁺ larvae, p=0.0089; Fig 6F). These data demonstrate that mTOR activity is required for leukocyte recruitment to the injured/regenerating RPE.

Macrophage/microglia function is required to maintain mTOR activity in the RPE during regeneration in an inflammation-independent manner

mTOR activation within the RPE layer peaks between 6-12hpi (Fig 1) and mTOR activity is required for the recruitment of macrophages/microglia to the RPE post-injury (Fig 6). However, significant macrophage/microglia recruitment to the injured RPE does not occur until 2dpi, and peaks at 3dpi (14), supporting a model in which early mTOR activation in the RPE contributes to the recruitment of macrophages/microglia to the injury site. As mTOR activity remains stably elevated in the regenerating RPE until 3dpi, and cytokine signaling is a well-known extrinsic inducer of mTOR activity in a variety of cell types (53,54), we hypothesized that there may be crosstalk between recruited macrophages/microglia and the RPE, whereby macrophages/microglia could also play a role in maintaining mTOR activation in the RPE at later stages post-injury. Indeed, during retinal regeneration in zebrafish, macrophage/microglia-mediated inflammation promotes mTOR activation in Müller glia and mTOR activity is required for regeneration (32). To test this hypothesis and explore the relationship between macrophages/microglia and later mTOR activity in the RPE, we used the CSF1R inhibitor, pexidartinib (PLX3397), to deplete macrophages/microglia, an approach that has been shown to be effective in zebrafish (55–57), and assessed the effects on mTOR activation. Importantly, the *csflra* and *csflrb* genes were not significantly upregulated in the MTZ⁺ RPE at 2dpi and therefore unlikely to be directly involved in the regenerative response of RPE cells at this time (S8 Table). Experimentally, larvae were exposed to 1μM PLX3397 or DMSO from 72 hours prior to ablation until 2dpi (Fig 7A), the time when significant recruitment of macrophages/microglia to the RPE post-injury was first observed (14). As expected, MTZ⁺ larvae exposed to PLX3397 showed a significant reduction in *mpeg1*-driven mCherry signal in the RPE when compared to DMSO-treated controls (Fig 7B-E,H). Having confirmed the efficacy of PLX3397 in depleting macrophages/microglia, we next sought to

determine whether macrophage/microglia depletion affected mTOR activation in the RPE post-injury. p-S6 levels in the RPE were significantly suppressed by PLX3397 treatment at 2dpi, when compared to DMSO controls (Fig 7B,C,F,G,I). These data demonstrate that there is crosstalk between the injured/regenerating RPE and macrophages/microglia. RPE damage induces an mTOR-dependent recruitment of macrophages/microglia to the injury site and macrophages/microglia reinforce mTOR activity in the regenerating RPE. Interestingly, mTOR signaling also appeared to be activated in macrophages/microglia at 2dpi, as shown in MTZ⁺ DMSO-treated controls (Fig 7B',D',F'). As noted above, p-S6⁺ cells were detected at 2dpi and 3dpi in the region where the ablated RPE had been cleared (Fig 1L,M). This observation suggests that the injury-dependent recruitment of macrophages/microglia to the ablated RPE also activates mTOR in the macrophages/microglia, perhaps modulating their behaviors during the injury/regenerative response.

In many tissue injury paradigms, the recruitment and activation of leukocytes triggers inflammation (58–61). Inflammation is a complex process that, when acute and/or chronic, can be detrimental to tissue survival; if properly resolved, however, inflammation can stimulate pro-regenerative responses (62,63). Indeed, inflammation is necessary for RPE and retinal regeneration in zebrafish (14,32,64). With this in mind, we utilized Dexamethasone (Dex) to dampen inflammation systemically (14,64) to determine whether inflammation was responsible for mTOR activity in the RPE layer at later stages post-injury. As expected, DMSO-treated MTZ⁺ larvae showed significantly increased p-S6 levels in the RPE layer at 2dpi when compared to MTZ⁻ controls (Fig 7K,M,O). Interestingly, there were no significant differences in mTOR activity between MTZ⁺ Dex-treated and DMSO-treated larvae (Fig 7L,N,O). Dex-treatment does not affect macrophage/microglia recruitment post-RPE ablation (14), and therefore these data support a model in which it is an inflammation-independent function of macrophages/microglia that maintains mTOR activity in the RPE layer during regeneration.

DISCUSSION

AMD is a progressive blinding disease characterized by dysfunction of the RPE and choroid which causes a gradual loss of these tissues, of the photoreceptors, and eventually of central vision (65). Currently, there are few options for treating AMD (66); however, an innovative potential direction for therapeutic development is the stimulation of intrinsic regeneration of the RPE (8). Previously, our lab established a genetic RPE ablation paradigm in zebrafish and demonstrated that zebrafish are capable of regenerating a functional RPE monolayer, thus providing a model through which the molecular underpinnings of the RPE regenerative response can be identified (13).

In this study, we focused on activation of mTOR signaling, which has been associated with tissue and organ regeneration in a number of contexts and model systems (24,67). mTOR activity was rapidly induced in zebrafish RPE cells within 6 hours after the onset of RPE ablation, and the mTOR pathway remained active through 3dpi, the time of peak proliferation within the regenerating RPE layer (13) (Fig 8A,B). Through a combination of pharmacological and genetic loss-of-function and gain-of-function assays, our results demonstrate that mTOR activity is both necessary and sufficient for RPE regeneration in zebrafish. These results are consistent with findings in other systems, where the mTOR pathway has been shown to exert a pro-regenerative role. Indeed, in the zebrafish retina, mTOR is activated in Müller glia within 6 hours after injury and expression is maintained in proliferating Müller glia and Müller glia-derived progenitor cells (MGPCs) (32). In this system, mTOR activity is required both for the dedifferentiation of Müller glia to generate progenitors as well as the proliferation of the progenitor cells themselves. Similarly, in the chicken retina, mTOR is also required for the formation of MGPCs (33). These results parallel our observations in the injured and regenerating RPE, as well as those in cultured RPE cells where mTOR activity is required for proliferation and migration of RPE cells after a laser photocoagulation injury (22). Taken together, these data further support an evolutionarily conserved role for mTOR in modulating regenerative responses and extend this to include the RPE.

Interestingly, our results link mTOR activation in the RPE after injury to immune system

activity (Fig 8C,D). Involvement of the immune system in regeneration is well documented in several tissues and organs (68), including the zebrafish RPE (14). In RPE-ablated larvae where mTOR activity was inhibited, the recruitment of macrophages/microglia to the injured RPE was impaired. This suggests that mTOR-dependent regulation of gene expression in damaged RPE facilitates signaling events that recruit leukocytes to the injury site. Amongst the DEGs downregulated in the RPE of rapamycin-treated larvae, several genes involved in immune response activation were identified, and these include *il34* and *mmp9*. IL-34 is a proinflammatory cytokine that regulates macrophage differentiation, proliferation, migration, and polarization (44,69), and has been shown to be upregulated in the zebrafish RPE post-ablation (14). Matrix metalloproteinase-9 (Mmp-9) is a secreted gelatinase that acts on extracellular molecules, such as extracellular matrix, growth factors, and cytokines (70,71). Mmp-9 also plays a critical role in recruiting leukocytes to sites of injury after peripheral nerve crush in rats (72), glomerulonephritis in mice (73), and cryoinjury in zebrafish (48). *mmp-9* also regulates heart regeneration in zebrafish by mediating leukocyte recruitment, possibly via the processing of chemokines into active forms (48). Our data demonstrate that *il34* and *mmp9* are upregulated in an mTOR-dependent fashion, and these factors could stimulate macrophage/microglia recruitment to the injured RPE during the early stages of the injury response.

It is known that macrophages/microglia directly modulate the RPE regenerative response (14) and here, we demonstrate that these cells also reinforce mTOR activity in regenerating RPE cells during the later stages of regeneration. Interestingly, our results indicate that the macrophage/microglia-dependent maintenance of mTOR in the RPE is inflammation-independent. This is in contrast to mTOR activation during retinal regeneration, which is dependent on inflammation (32). Cytokines have been shown to activate the mTOR pathway (54), and physiologically, macrophages are the main cellular source of many cytokines (74), including in zebrafish post-RPE injury (14). Thus, it is possible that macrophages/microglia recruited to the RPE injury site release cytokines that function to maintain elevated mTOR activity in RPE cells as they regenerate. In this context, however, these cytokines would likely be of the anti-inflammatory subtype given that mTOR maintenance is inflammation-independent. Additionally, the mTOR pathway can be activated by a variety of intracellular and

extracellular signals, the latter of which includes components of the insulin/insulin-like growth factor (IGF) pathway (75), Wnt pathway (76), and macrophage-derived glutamine (77). Thus, it is also possible that one or more of these pathways could be stimulated by injury-induced macrophages/microglia and function to maintain mTOR activity in the regenerating RPE. Several zebrafish studies have identified injury-induced gene expression profiles in isolated leukocytes (14,78–80), and it will be of interest to screen candidates for potential mTOR maintenance roles during RPE regeneration. Finally, with respect to the immune system, we detected mTOR activity in RPE-localized macrophages/microglia at 2dpi (Fig 7B',D',F') and also observed that rapamycin treatment decreased the resident population of macrophages/microglia in unablated larvae (Fig 6F). Thus, we cannot rule out the possibility that genetic and pharmacological modulation of mTOR activity also affected the ability of macrophages/microglia to respond to RPE ablation. Indeed, mTOR activity in leukocytes is known to modulate their migratory abilities, supporting this notion (81).

Mechanistically, mTOR function during RPE regeneration may be bi-phasic, with an early stage that stimulates innate immune system activity and a later stage that directly regulates regeneration of a functional RPE monolayer. Indeed, RNA-seq data from 4dpi rapamycin-treated samples identified pathways that could function in RPE cells directly, at later stages of the regenerative response. For example, GABA signaling and neurotransmitter release pathways were significantly downregulated in the rapamycin-treated group. GABA has a well known role in synaptic transmission in the central nervous system, including in the retina (82). GABA receptors are also expressed in cultured human RPE cells (83) and in the chick RPE (84), where signaling regulates the intracellular calcium concentration. Calcium signaling plays a number of important roles during tissue regeneration (85) and wound healing (86), and therefore mTOR-dependent activation of GABA signaling could serve as a trigger to induce migration of regenerating RPE cells and/or reformation of the RPE monolayer. GABA signaling has also recently been shown to be involved in tissue regeneration, including in the zebrafish retina (87,88). mTOR is a critical mediator of cellular growth control via its ability to facilitate anabolism (protein synthesis) and inhibit catabolism (autophagy) in response to environmental inputs (16). There is significant proliferation in the RPE layer during regeneration (13), which undoubtedly

requires coordinated protein synthesis, cellular growth, and substantial energy requirements and it is likely that mTOR activity modulates these processes during RPE regeneration. In addition to its role in leukocyte recruitment, mmp-9 has also been shown to be involved in regulating cell migration by remodeling the extracellular matrix (46,89,90). Indeed, in cultured RPE cells, TNF α -induced MMP-9 expression promotes RPE cell migration, which is controlled by activation of Akt/mTORC1 signaling (46). During RPE regeneration, mTOR activity could stimulate mmp-9-mediated modulation of RPE cell migration to enable the reformation of a functional RPE monolayer. Future studies focusing on these and other candidates will shed light on the mTOR-dependent processes that facilitate RPE regeneration in zebrafish and whether any of these processes can be stimulated in the mammalian RPE to trigger a regenerative response.

FIGURES AND LEGENDS:

Fig 1. The mTOR pathway is activated in the RPE after ablation. (A-G) Immunofluorescent images of p-S6 staining on transverse cryosections from unablated (MTZ⁻) and (H-L) ablated (MTZ⁺) larvae from 3hpi to 4dpi. *White arrows* indicate the p-S6⁺ eGFP⁻ cells in the RPE layer. *White arrowheads* indicate p-S6⁺ cells in the retinae. (I'-I''') High-magnification images showing the colocalization of p-S6 and eGFP at 6hpi, 12hpi (J'-J''') and 1dpi (K'-K'''). Nuclei (white), eGFP (green), p-S6 (magenta). (O) Quantification of the p-S6⁺ area in the RPE layer revealed that expression was low in the RPE of unablated larvae but increased significantly in ablated larvae from 3hpi to 3dpi. Dorsal is up and distal is left. Scale bar = 50μm.

Fig 2. Pharmacological inhibition of mTOR activity impairs RPE regeneration.

(A,G) Schematic of the experimental paradigm showing the timeline for chemical treatments and ablation. (B-E) Immunofluorescent images of p-S6 staining on transverse cryosections from MTZ⁺ DMSO-, rapamycin- or INK128-treated larvae at 2dpi. Nuclei (white), p-S6 (magenta) (F) p-S6 levels in the RPE layer were significantly decreased in rapamycin-treated and INK128-treated larvae when compared with DMSO-treated controls. (H-K) BrdU immunostaining of cryosections from MTZ⁺ DMSO-, rapamycin- or INK128 -treated larvae at 4dpi. *White arrowheads* indicate BrdU⁺ cells in the RPE layer. Nuclei (white), BrdU (magenta). (L) Quantification of BrdU⁺ cells in the RPE layer showed significantly fewer in rapamycin- and INK128-treated larvae when compared to DMSO-treated controls. (M-P) Brightfield images of cryosections from MTZ⁺ DMSO-, rapamycin- or INK128-treated larvae at 4dpi. *White arrows* indicate the edges of pigment recovery. (Q) Quantification of percent RPE recovery showed a significant impairment in pigment recovery in rapamycin- or INK128-treated larvae. Dorsal is up and distal is left. Scale bar = 50μm.

Fig 3. Mutation in *mtor* impairs RPE regeneration.

(A,B) Immunofluorescent images of p-S6 staining on transverse cryosections from ablated *mtor*^{+/+} and *mtor*^{-/-} at 2dpi. Nuclei (white), p-S6 (magenta). (C) Quantification of the p-S6 signal in the RPE layer

revealed a significant decrease in MTZ⁺ *mtor*^{-/-} larvae, when compared to MTZ⁺ *mtor*^{+/+} siblings. (D,E) Immunofluorescent images of BrdU staining on transverse cryosections from MTZ⁺ *mtor*^{+/+} and *mtor*^{-/-} larvae at 4dpi. Nuclei (white), BrdU (magenta). (F) Quantification of BrdU⁺ cells in the RPE layer showed a significant decrease in MTZ⁺ *mtor*^{-/-} larvae when compared to MTZ⁺ *mtor*^{+/+} siblings. (G-H) Immunofluorescent images of ZPR2 staining on transverse cryosections from MTZ⁺ *mtor*^{+/+} and *mtor*^{-/-} larvae at 4dpi. Single channel (G' H') *rpe65a:nfsB*-eGFP images and (G" H") ZPR2 images. Nuclei (white), eGFP (green), ZPR2 (magenta). *White arrows* highlight the edges of eGFP or ZPR2 recovery in the RPE. (I) Quantification of the eGFP expression recovery showed a significant decrease in MTZ⁺ *mtor*^{-/-} larvae, compared to *mtor*^{+/+} siblings. Dorsal is up and distal is left. Scale bar = 50μm.

Fig 4: Pharmacological activation of mTOR signaling enhances RPE regeneration.

(A,E) Schematic of the experimental paradigm showing the timeline for chemical treatments and ablation. (B,C) Immunofluorescent images of p-S6 staining on transverse cryosections from MTZ⁺ DMSO- and MHY1485-treated larvae at 2dpi. Nuclei (white), p-S6 (magenta). (D) Quantification of p-S6 signal in the RPE layer showed a significant increase of p-S6 levels in MHY1485-treated larvae when compared to DMSO-treated controls. (F-I) Fluorescent images of BrdU immunostaining on cryosections from MTZ⁻ and MTZ⁺ DMSO- and MHY1485-treated larvae at 3dpi. *White arrowheads* highlight BrdU⁺ cells in the RPE layer. Nuclei (white), BrdU (magenta). (J) BrdU⁺ cells in the RPE layer are significantly increased in the RPE of MTZ⁺ MHY1485-treated larvae when compared to DMSO-treated controls. There was no significant effect of MHY1485 treatments on MTZ⁻ larvae. (K-N) Fluorescent images of ZPR2 immunostaining on cryosections from MTZ⁺ DMSO- and MHY1485-treated larvae at 3dpi. *White arrows* highlight the edges of ZPR2 signal recovery. Nuclei (white), eGFP (green), ZPR2 (magenta). (O) Quantification of percent ZPR2 recovery showed a significant increase in MHY1485-treated larvae over DMSO-treated controls. Dorsal is up and distal is left. Scale bar = 50μm.

Fig 5. Inhibition of mTOR signaling affects the expression of immune related genes in regenerating RPE cells.

(A) Representative FACS plots from MTZ⁻ and MTZ⁺ samples to show gating parameters used to isolate GFP⁺ PI⁻ RPE cells (B) Volcano plots showing differentially expressed genes between MTZ⁺ rapamycin-treated and DMSO-treated groups at 2dpi and 4dpi. Dashed lines indicate the threshold criteria. Each dot represents an individual gene; red dots represent significantly upregulated DEGs, blue dots represent significantly downregulated DEGs, and black dots represent non-significant genes. (C) Pathway enrichment analysis on the significantly downregulated 2dpi and 4dpi DEGs. Numbers in parentheses are the gene counts enriched in each pathway. (D) Hierarchical clustering heatmap of the 15 genes enriched in the 2dpi immune system Reactome pathway across all four experimental groups showing these immune related genes are downregulated by rapamycin treatment. Heatmap legend represents log (Counts per million mapped reads; CPM).

Fig 6. Inhibition of mTOR activity impaired macrophage/microglia recruitment to the RPE layer post-ablation

(A) Schematic of the experimental paradigm showing the timeline for chemical treatments and ablation on transgenic larvae (*mpeg1:mCherry;rpe65a:nfsB-eGFP*). (B-E) Immunofluorescent images of mCherry staining on transverse cryosections from MTZ⁻ and MTZ⁺ DMSO- and rapamycin-treated larvae at 3dpi. Nuclei (white), mCherry (magenta). (F) Quantification of the mCherry signal in the RPE layer showed significant increases in MTZ⁺ DMSO- and Rapamycin-treated larvae, when compared to matched MTZ⁻ controls. However, the percent of the RPE covered by mCherry⁺ cells was significantly reduced in rapamycin-treated larvae when compared to DMSO-treated controls. Dorsal is up and distal is left. Scale bar = 50µm.

Fig 7. Macrophage/microglia function is required for the maintenance of mTOR activity in an inflammation-independent manner.

(A,J) Schematic of the experimental paradigm showing the timeline for chemical treatments and

ablation on transgenic larvae (*mpeg1:mCherry; rpe65a:nfsB-eGFP*). (B-G) MTZ⁺ DMSO- and PLX3397-treated larvae at 2dpi. Single channel immunofluorescent images of mCherry (D,E) and p-S6 (F,G) are shown. (B',D',F') are high-magnification images showing the colocalization of p-S6 and mCherry. Nuclei (white), eGFP (green), p-S6 (magenta), mCherry (yellow). (H) Quantification of mCherry signal in the RPE layer showed a significant depletion of mCherry⁺ macrophages/microglia in the RPE after PLX3397 treatment in MTZ⁺ larvae, compared to DMSO-treated controls. (I) Quantification of p-S6 levels in the RPE layer showed a significant decrease in PLX3397-treated larvae, when compared to DMSO-treated controls. (K-N) Fluorescent images of p-S6 immunostaining on cryosections from MTZ⁻ and MTZ⁺ DMSO- and Dex-treated larvae at 7dpf/2dpi. Nuclei (white), eGFP (green), p-S6 (magenta). (O) Quantification of p-S6 level in the RPE layer showed significant increases in MTZ⁺ larvae when compared to MTZ⁻ controls, but no significant differences in Dex-treated MTZ⁻ or MTZ⁺ larvae. Dorsal is up and distal is left. Scale bar = 50µm.

Fig 8. Model of mTOR activity during RPE regeneration

(A) mTOR activity is rapidly induced in damaged/regenerating RPE cells between 6hpi and 1dpi. (B) Activation of mTOR signaling in RPE cells regulates the expression of immune-related genes (e.g. *mmp9*, *il34*, *cxcl18a.1*). (C) mTOR activity is required for the recruitment of macrophages/microglia to the injured/regenerating RPE. (D) mTOR is also activated in macrophages/microglia recruited to the RPE post-injury and these cells are required to maintain mTOR activity in RPE cells during the later stages of the regenerative response. This maintenance occurs in an inflammation-independent manner

MATERIALS AND METHODS:

Fish maintenance and husbandry

Adult zebrafish were maintained at 28.5°C under a 14-hour light/10-hour dark cycle. Embryos and larvae used for subsequent experiments were kept in an incubator at 28.5°C in the dark until being euthanized by tricaine (MS-222; Fisher Scientific). All experiments were performed in accordance with the relevant guidelines and regulations by the University of Pittsburgh School of Medicine Institutional Animal Care and Use Committee.

Genotyping

mtor^{sal16755} (38) was purchased from the Zebrafish International Resource Center (ZIRC;). *mtor*^{+/-} fish were crossed to: *rpe65a:nfsB-eGFP* fish (13) to generate *mtor*^{+/-};*rpe65a:nfsB-eGFP*, which were identified by eGFP screening and genotyping via PCR-restriction fragment length polymorphism-based analysis (PCR-RFLP). *mtor*^{+/-};*rpe65a:nfsB-eGFP* fishes were then incrossed to generate *mtor*^{-/-};*rpe65a:nfsB-eGFP* larvae, which were genotyped by PCR-RFLP after euthanasia. For genotyping, genomic DNA was extracted from adult fins or larval tails by incubating with 50ul of 50mM NaOH at 95°C for 10 minutes. After cooling to 4°C, 5ul of 1M Tris-HCl (pH8) was added for neutralization and this mixture was used as a template for PCR amplification. The following primers were used:

Forward primer (5'-GATAACGTAGAATGCAGTGGGACAG-3')

Reverse primer (5'-CGGGCCCAAACGTATTATGCATAC-3') .

For PCR-RFLP analysis, 10ul of PCR product was digested with 5 units of MseI (New England Biolabs, Ipswich, Massachusetts) for one hour. The digested PCR products were separated on 2% agarose gel. Uncut, 391bp fragments, indicated *mtor*^{+/+};*rpe65a:nfsB-eGFP* and cut, 223 and 168 bp fragments, indicated *mtor*^{-/-};*rpe65a:nfsB-eGFP*. Genotyping results from PCR-RFLP were confirmed by Sanger sequencing in a subset of larvae.

RPE ablation

RPE ablation followed the protocol in Hanovice et al., (2019)(13). Briefly, embryos derived from

rpe65a:nfsB-eGFP crosses were treated with 1-phenyl-2-thiourea (PTU; Sigma-Aldrich) from 6hpf to 5dpf to inhibit pigmentation. At 4dpf, larvae carrying the *nfsB-eGFP* transgene were selected by eGFP screening under a fluorescent microscope (Zeiss AxioZoom V.16). At 5dpf, larvae were incubated with 10mM metronidazole (MTZ; Sigma-Aldrich) in system water for 24 hours. Then, larvae were removed from MTZ and maintained in system water until euthanized for experiments.

Pharmacological treatments

Concentrations of rapamycin (2 μ M in DMSO; Sigma-Aldrich), INK128 (0.9 μ M in DMSO; Fisher Scientific) and MHY1485 (2 μ M; Sigma-Aldrich) were determined based on levels in system water that allowed larvae to develop normally from 4dpf-9dpf and whose efficacy was validated through p-S6 immunohistochemistry. Concentrations of dexamethasone (50 μ M; Sigma-Aldrich) and PLX3397 (1 μ M; Fisher Scientific) were based on previous studies (14). Matched volumes of DMSO were used as a vehicle control for each drug. For multiple day exposures, system water was changed daily and supplemented with fresh compounds.

BrdU incorporation and TUNEL assays

For bromodeoxyuridine (BrdU) incorporation, larvae were immersed in system water containing 10mM BrdU (Sigma-Aldrich) for 24 hours prior to euthanasia. For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, the In Situ Cell Death Detection Kit, TMR red (Sigma-Aldrich) assay was used according to manufacturer's instructions to detect apoptotic cells.

RPE cell isolation and sequencing

Dissected eye tissues were pooled from unablated (MTZ⁻) and ablated (MTZ⁺) DMSO-treated control and rapamycin-treated larvae in triplicate, with each biological replicate consisting of around 80 eyes (40 larvae). Cell dissociation was performed as previously described (14). Briefly, pooled eyes were dissociated mechanically in a 0.25% trypsin in 1X PBS solution with a 1mL syringe and 27 1/2-gauge needle, strained through a 70 μ M cell strainer, and spun at 450g for 5 min to pellet the cells. Then, cell pellets were resuspended and washed in 1X PBS three times and resuspended in a final 1X PBS 5%

fetal bovine serum solution. Before sorting, propidium iodide (PI) live/dead stain was added and incubated at room temperature for 5 minutes. Sorting gates were established using unstained (GFP⁻ and PI⁻) and single channel controls (GFP⁺/PI⁻; GFP⁻/PI⁺) on a FACS Aria IIu cell sorter (BD Biosciences). FACS was performed at the Flow Cytometry Core at the University of Pittsburgh School of Medicine Department of Pediatrics. Sorting gates were maintained for each biological replicate and a maximum of 1000 cells was collected per sample. cDNA was generated using the Smart-seq ultra-Low input RNA kit (Takara Bio USA, Inc.). cDNA quality control was assessed using a TapeStation 2200 system (Agilent Technologies, Inc.). A Nextera XT DNA Library Preparation Kit (Illumina, Inc.) was used to generate indexed 2×75bp paired-end cDNA libraries for subsequent sequencing on NextSeq 500 sequencing platform (Illumina, Inc.). Library preparation, quality control analysis, and next generation sequencing were performed by the Health Sciences Sequencing Core at Children's Hospital of Pittsburgh. 60-100 million reads were obtained per sample. Raw read and processed data files are available in GEO under accession number GSE174538.

Bioinformatics

Bioinformatics analyses were performed on the CLC Genomics Workbench (Qiagen Digital Insights) licensed through the Molecular Biology Information Service of the Health Sciences Library System at the University of Pittsburgh. Raw Illumina reads (FASTQ file format) were imported to CLC Genomics Workbench. Quality checks were performed on imported raw reads and trimmed reads; samples showed average Phred scores of >20. Trimmed paired-end reads were then aligned to the zebrafish reference genome sequence (GRCz11) and differentially expressed genes (DEGs) were determined using filters as follows: 1) log₂ fold change absolute value >1; 2) false discovery rate (FDR) p-value <0.05; 3) maximum group mean ≥1. Using the Ensembl gene identifiers of the filtered downregulated DEGs as input parameters, STRING v.11 (<https://string-db.org>) was used to perform functional pathway enrichment analysis. Reactome datasets were used and the significant enriched Reactome pathways were selected with the thresholds of FDR p-value < 0.05 and gene counts ≥5.

Immunohistochemistry

Larvae were euthanized with tricaine and fixed in 4% paraformaldehyde (PFA) at room temperature for 2-3 hours or overnight at 4°C. Fixed larvae were cryoprotected in a 25% then 35% sucrose in 1X PBS and subsequently embedded in optimal cutting temperature (OCT) compound (Uribe and Gross, 2007). Frozen cryosections (12 µm) were cut on a Leica CM1850 cryostat (Leica Biosystems) and mounted onto poly-L-lysine pre-coated glass slides (Superfrost plus; Thermo Fisher). For p-S6 immunostaining, sections were refixed in 4% PFA at room temperature for ten minutes, followed by three washes with 0.1% PBST (1XPBS with 0.1% and Triton X-100). For BrdU immunostaining, after rehydration in 1X PBS, slides were incubated in 4N HCl for 8 minutes at 37°C for antigen retrieval, and then washed three times with PBTD (1XPBS with 0.1% Tween-20 and 1% DMSO). For general immunostaining, slides were rehydrated in 1X PBS for 5 minutes and then washed three times with PBTD. After blocking with 5% normal goat serum (NGS) in PBTD for at least 2 hours, slides were incubated with either rabbit anti-pS6 (Ser235/236) (1:75; Cell Signaling #4857), rat anti-BrdU (1:250; Abcam; ab6326), mouse anti-ZPR2 (1:250, ZIRC), or mouse anti-mCherry (1:200; Fisher Scientific; NC9580775) antibodies diluted in 5% NGS in PBTD overnight at 4°C. After three washes with PBTD, slides were incubated with anti-rabbit Alexa Fluor 647 (1:500, Fisher Scientific; A21244), anti-mouse or anti-goat Cy3 (1:250; Jackson ImmunoResearch Laboratories; #115-165-166) secondary antibodies in 5% NGS in PBTD for 2-3 hours at room temperature. After incubation, slides were washed three times with PBTD. DAPI in 5% NGS in PBTD was added onto slides between the first and second washes and slides were incubated in DAPI for 10 minutes. After the third wash, slides were mounted with Vectashield (Vector Laboratories) and sealed with nail polish. All fluorescent images of cryosections were captured using an Olympus Fluoview FV1200 laser scanning microscope and a 40X (1.30 NA) objective (Olympus Corporation).

Image quantification

Raw z-stack confocal images were processed for quantification using FIJI (ImageJ) (91). As described previously (14), the number of BrdU⁺ cells in the RPE layer was counted manually by using the Cell Counter plugin in FIJI. The counting area (RPE layer) was determined using the DAPI channel to outline the outer edges of photoreceptor outer segments and using the bright field channel to outline the

basal RPE pigment edges. For quantification of TUNEL⁺ cells, backgrounds were subtracted (rolling ball radius, 60 pixels) from the TUNEL channel. Images were then converted to binary under the same thresholds (60/255) for watershed separation of merged particles. The number of TUNEL⁺ puncta between the outer plexiform layer and RPE were counted on the segmented images using Analyze Particles in FIJI with the following parameters: size:pixel²: 5-infinity; circularity: 0.00-1.00. The quantification of p-S6 and mCherry signals in RPE layer were analyzed on 8-bit images by selecting the Region Of Interest (ROI) and the percent area was measured on the p-S6 or mCherry channel under constant thresholds (40/255 and 50/255, respectively). Pigmentation, eGFP, and ZPR2 were quantified for RPE recovery based on angle measurement by using the angle tool in FIJI. The edges of RPE recovery were determined based on contiguous pigmentation or contiguous expression of eGFP or ZPR2 signals. The measurements of signal percent area and percentage of RPE recovery were made on the central-most section of each larva. The numbers of BrdU⁺ cells and TUNEL⁺ puncta were the averages from three consecutive central sections per larva.

Statistics

All datasets for statistical analysis came from at least three independent experiments. Statistical analyses were performed using Prism 8 (GraphPad Software). For each dataset, a D'Agostino-Pearson omnibus normality test was performed to determine whether data obeyed normal (Gaussian) distributions. For the datasets that obeyed normal distribution, an unpaired student's t-test with Welch's correction was used, otherwise, nonparametric Mann-Whitney tests were used to determine statistical significance. For all box plots, top and bottom whiskers represent the maximum and minimum value of each dataset. Median and mean of the dataset were displayed by the line and plus within the box, respectively. Inside triangles represent biological replicates or individual larvae. P-values of >0.05 were considered not significant, and significant p-values were represented as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, and **** ≤ 0.0001. Statistical tests, biological replicates (n) and p-values for each dataset/comparison can be found in S9 Table.

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REFERENCES:

1. Wong WL, Su X, Li X, Cheung CMG, Klein R, Cheng C-Y, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *The Lancet Global Health* [Internet]. 2014 Feb 1;2(2):e106–16. Available from: <https://www.sciencedirect.com/science/article/pii/S2214109X13701451>
2. Lakkaraju A, Umapathy A, Tan LX, Daniele L, Philp NJ, Boesze-Battaglia K, et al. The cell biology of the retinal pigment epithelium. *Prog Retin Eye Res* [Internet]. 2020;100846. Available from: <http://dx.doi.org/10.1016/j.preteyeres.2020.100846>
3. Chichagova V, Hallam D, Collin J, Zerti D, Dorgau B, Felemban M, et al. Cellular regeneration strategies for macular degeneration: past, present and future. *Eye* [Internet]. 2018 May 1;32(5):946–71. Available from: <https://doi.org/10.1038/s41433-018-0061-z>
4. Zarbin M, Sugino I, Townes-Anderson E. Concise Review: Update on Retinal Pigment Epithelium Transplantation for Age-Related Macular Degeneration. *Stem Cells Transl Med* [Internet]. 2019;8(5):466–77. Available from: <https://stemcellsjournals.onlinelibrary.wiley.com/doi/abs/10.1002/sctm.18-0282>
5. Kampik D, Basche M, Luhmann UFO, Nishiguchi KM, Williams JAE, Greenwood J, et al. In situ regeneration of retinal pigment epithelium by gene transfer of E2F2: a potential strategy for treatment of macular degenerations. *Gene Ther* [Internet]. 2017 Dec 1;24(12):810–8. Available from: <https://doi.org/10.1038/gt.2017.89>
6. Machalińska A, Lejkowska R, Duchnik M, Kawa M, Rogińska D, Wiszniewska B, et al. Dose-dependent retinal changes following sodium iodate administration: application of spectral-domain optical coherence tomography for monitoring of retinal injury and endogenous regeneration. *Curr Eye Res* [Internet]. 2014 Oct;39(10):1033–41. Available from: <http://dx.doi.org/10.3109/02713683.2014.892996>
7. Machalińska A, Lubiński W, Kłos P, Kawa M, Baumert B, Penkala K, et al. Sodium iodate selectively injures the posterior pole of the retina in a dose-dependent manner: morphological and electrophysiological study. *Neurochem Res* [Internet]. 2010 Nov;35(11):1819–27. Available from: <http://dx.doi.org/10.1007/s11064-010-0248-6>
8. George SM, Lu F, Rao M, Leach LL, Gross JM. The retinal pigment epithelium: Development, injury responses, and regenerative potential in mammalian and non-mammalian systems. *Prog Retin Eye Res* [Internet]. 2021;100969. Available from: <http://dx.doi.org/10.1016/j.preteyeres.2021.100969>
9. Tamiya S, Kaplan HJ. Role of epithelial-mesenchymal transition in proliferative vitreoretinopathy. *Exp Eye Res* [Internet]. 2016 Jan;142:26–31. Available from: <http://dx.doi.org/10.1016/j.exer.2015.02.008>
10. Saini JS, Temple S, Stern JH. Human Retinal Pigment Epithelium Stem Cell (RPESC). *Adv Exp Med Biol* [Internet]. 2016;854:557–62. Available from: http://dx.doi.org/10.1007/978-3-319-17121-0_74
11. Salero E, Blenkinsop TA, Corneo B, Harris A, Rabin D, Stern JH, et al. Adult human RPE can be activated into a multipotent stem cell that produces mesenchymal derivatives. *Cell Stem Cell* [Internet]. 2012;10(1):88–95. Available from: <http://dx.doi.org/10.1016/j.stem.2011.11.018>
12. Marques IJ, Lupi E, Mercader N. Model systems for regeneration: zebrafish. *Development* [Internet]. 2019;146(18):dev167692. Available from: <http://dev.biologists.org/content/146/18/dev167692.abstract>

13. Hanovice NJ, Leach LL, Slater K, Gabriel AE, Romanovicz D, Shao E, et al. Regeneration of the zebrafish retinal pigment epithelium after widespread genetic ablation. *PLoS Genet* [Internet]. 2019 Jan;15(1):e1007939. Available from: <http://dx.doi.org/10.1371/journal.pgen.1007939>
14. Leach LL, Hanovice NJ, George SM, Gabriel AE, Gross JM. The immune response is a critical regulator of zebrafish retinal pigment epithelium regeneration. *Proc Natl Acad Sci U S A* [Internet]. 2021 May 25;118(21). Available from: <https://www.pnas.org/content/118/21/e2017198118>
15. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* [Internet]. 2012;149(2):274–93. Available from: <http://dx.doi.org/10.1016/j.cell.2012.03.017>
16. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. *Cell* [Internet]. 2017;168(6):960–76. Available from: <https://pubmed.ncbi.nlm.nih.gov/28283069>
17. Chiang C-K, Tworak A, Kevany BM, Xu B, Mayne J, Ning Z, et al. Quantitative phosphoproteomics reveals involvement of multiple signaling pathways in early phagocytosis by the retinal pigmented epithelium. *J Biol Chem* [Internet]. 2017 Dec 1;292(48):19826–39. Available from: <https://www.sciencedirect.com/science/article/pii/S0021925820328775>
18. Yu B, Egbejimi A, Dharmat R, Xu P, Zhao Z, Long B, et al. Phagocytosed photoreceptor outer segments activate mTORC1 in the retinal pigment epithelium. *Sci Signal* [Internet]. 2018;11(532):eaag3315. Available from: <http://stke.sciencemag.org/content/11/532/eaag3315.abstract>
19. Campbell LJ, West MC, Jensen AM. A high content, small molecule screen identifies candidate molecular pathways that regulate rod photoreceptor outer segment renewal. *Sci Rep* [Internet]. 2018;8(1):14017–14017. Available from: <https://pubmed.ncbi.nlm.nih.gov/30228302>
20. Zhao C, Yasumura D, Li X, Matthes M, Lloyd M, Nielsen G, et al. mTOR-mediated dedifferentiation of the retinal pigment epithelium initiates photoreceptor degeneration in mice. *J Clin Invest* [Internet]. 2011;121(1):369–83. Available from: <https://pubmed.ncbi.nlm.nih.gov/21135502>
21. Calton MA, Vollrath D. The mTOR Kinase Inhibitor INK128 Blunts Migration of Cultured Retinal Pigment Epithelial Cells. LaVail MM, Anderson RE, Grimm C, Hollyfield J, Ash J, editors. Cham: Springer International Publishing; 2016. 709-715 p. (Retinal Degenerative Diseases).
22. Madрахimov SB, Yang JY, Park HY, Park TK. Essential Role of mTOR Signaling in Human Retinal Pigment Epithelial Cell Regeneration After Laser Photocoagulation. *Lasers Med Sci* [Internet]. 2019 Jul;34(5):1019–29. Available from: <http://dx.doi.org/10.1007/s10103-018-2692-5>
23. Faghiri Z, Bazan NG. PI3K/Akt and mTOR/p70S6K pathways mediate neuroprotectin D1-induced retinal pigment epithelial cell survival during oxidative stress-induced apoptosis. *Exp Eye Res* [Internet]. 2010 Jun 1;90(6):718–25. Available from: <https://www.sciencedirect.com/science/article/pii/S0014483510000771>
24. Lund-Ricard Y, Cormier P, Morales J, Boutet A. mTOR Signaling at the Crossroad between Metazoan Regeneration and Human Diseases. *Int J Mol Sci* [Internet]. 2020;21(8):2718. Available from: <https://pubmed.ncbi.nlm.nih.gov/32295297>
25. Hirose K, Shiomi T, Hozumi S, Kikuchi Y. Mechanistic target of rapamycin complex 1 signaling regulates cell proliferation, cell survival, and differentiation in regenerating zebrafish fins. *BMC Dev Biol* [Internet]. 2014;14:42–42. Available from: <https://pubmed.ncbi.nlm.nih.gov/25480380>

26. He J, Chen J, Wei X, Leng H, Mu H, Cai P, et al. Mammalian Target of Rapamycin Complex 1 Signaling Is Required for the Dedifferentiation From Biliary Cell to Bipotential Progenitor Cell in Zebrafish Liver Regeneration. *Hepatology* [Internet]. 2019;70(6):2092–106. Available from: <https://aasldpubs.onlinelibrary.wiley.com/doi/abs/10.1002/hep.30790>
27. Xu S, Xie F, Tian L, Manno SHC, Manno FAM, Cheng SH. Prolonged neutrophil retention in the wound impairs zebrafish heart regeneration after cryoinjury. *Fish Shellfish Immunol* [Internet]. 2019 Nov 1;94:447–54. Available from: <https://www.sciencedirect.com/science/article/pii/S1050464819309143>
28. Duan X, Qiao M, Bei F, Kim I-J, He Z, Sanes JR. Subtype-specific regeneration of retinal ganglion cells following axotomy: effects of osteopontin and mTOR signaling. *Neuron* [Internet]. 2015 Mar 18;85(6):1244–56. Available from: <http://dx.doi.org/10.1016/j.neuron.2015.02.017>
29. Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, et al. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science* [Internet]. 2008 Nov 7;322(5903):963–6. Available from: <http://dx.doi.org/10.1126/science.1161566>
30. Pita-Thomas W, Mahar M, Joshi A, Gan D, Cavalli V. HDAC5 promotes optic nerve regeneration by activating the mTOR pathway. *Exp Neurol* [Internet]. 2019 Jul;317:271–83. Available from: <http://dx.doi.org/10.1016/j.expneurol.2019.03.011>
31. Teotia P, Van Hook MJ, Fischer D, Ahmad I. Human retinal ganglion cell axon regeneration by recapitulating developmental mechanisms: effects of recruitment of the mTOR pathway. *Development* [Internet]. 2019;146(13). Available from: <https://doi.org/10.1242/dev.178012>
32. Zhang Z, Hou H, Yu S, Zhou C, Zhang X, Li N, et al. Inflammation-induced mammalian target of rapamycin signaling is essential for retina regeneration. *Glia* [Internet]. 2020 Jan;68(1):111–27. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1002/glia.23707>
33. Zelinka CP, Volkov L, Goodman ZA, Todd L, Palazzo I, Bishop WA, et al. mTor signaling is required for the formation of proliferating Müller glia-derived progenitor cells in the chick retina. *Development* [Internet]. 2016;143(11):1859–73. Available from: <https://pubmed.ncbi.nlm.nih.gov/27068108>
34. Diekmann H, Kalbhen P, Fischer D. Active mechanistic target of rapamycin plays an ancillary rather than essential role in zebrafish CNS axon regeneration. *Front Cell Neurosci* [Internet]. 2015;9(251). Available from: <https://www.frontiersin.org/article/10.3389/fncel.2015.00251>
35. Fausett BV, Goldman D. A Role for α 1 Tubulin-Expressing Müller Glia in Regeneration of the Injured Zebrafish Retina. *J Neurosci* [Internet]. 2006;26(23):6303. Available from: <http://www.jneurosci.org/content/26/23/6303.abstract>
36. Huang S, Houghton PJ. Inhibitors of mammalian target of rapamycin as novel antitumor agents: from bench to clinic. *Curr Opin Investig Drugs*. 2002 Feb;3(2):295–304.
37. Vézina C, Kudelski A, Sehgal SN. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiot* [Internet]. 1975 Oct;28(10):721–6. Available from: <http://dx.doi.org/10.7164/antibiotics.28.721>
38. Busch-Nentwich E, Kettleborough R, Dooley CM, Scahill C, Sealy I, White R, et al. Sanger institute zebrafish mutation project mutant data submission. ZFIN direct data submission. 2013;
39. Gangloff YG, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz JF, et al. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell

- development. *Mol Cell Biol* [Internet]. 2004 Nov;24(21):9508–16. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC522282/pdf/1475-04.pdf>
40. Oldham S, Montagne J, Radimerski T, Thomas G, Hafen E. Genetic and biochemical characterization of dTOR, the *Drosophila* homolog of the target of rapamycin. *Genes Dev* [Internet]. 2000;14(21):2689–94. Available from: <https://pubmed.ncbi.nlm.nih.gov/11069885>
41. Ding Y, Sun X, Huang W, Hoage T, Redfield M, Kushwaha S, et al. Haploinsufficiency of target of rapamycin attenuates cardiomyopathies in adult zebrafish. *Circ Res* [Internet]. 2011;109(6):658–69. Available from: <http://dx.doi.org/10.1161/circresaha.111.248260>
42. Choi YJ, Park YJ, Park JY, Jeong HO, Kim DH, Ha YM, et al. Inhibitory effect of mTOR activator MHY1485 on autophagy: suppression of lysosomal fusion. *PLoS One* [Internet]. 2012;7(8):e43418. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3425474/pdf/pone.0043418.pdf>
43. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res* [Internet]. 2018;47(D1):D607–13. Available from: <https://doi.org/10.1093/nar/gky1131>
44. Baghdadi M, Umeyama Y, Hama N, Kobayashi T, Han N, Wada H, et al. Interleukin-34, a comprehensive review. *J Leukoc Biol* [Internet]. 2018;104(5):931–51. Available from: <https://jlb.onlinelibrary.wiley.com/doi/abs/10.1002/JLB.MR1117-457R>
45. Fu Q, Zeng Q, Li Y, Yang Y, Li C, Liu S, et al. The chemokine superfamily in channel catfish: I. CXC subfamily and their involvement in disease defense and hypoxia responses. *Fish Shellfish Immunol* [Internet]. 2017 Jan;60:380–90. Available from: <http://dx.doi.org/10.1016/j.fsi.2016.12.004>
46. Wang C-H, Cao G-F, Jiang Q, Yao J. TNF- α promotes human retinal pigment epithelial (RPE) cell migration by inducing matrix metalloproteinase 9 (MMP-9) expression through activation of Akt/mTORC1 signaling. *Biochem Biophys Res Commun* [Internet]. 2012 Aug 17;425(1):33–8. Available from: <http://dx.doi.org/10.1016/j.bbrc.2012.07.044>
47. Valoskova K, Biebl J, Roblek M, Emtenani S, Gyoergy A, Misova M, et al. A conserved major facilitator superfamily member orchestrates a subset of O-glycosylation to aid macrophage tissue invasion. *Elife* [Internet]. 2019;8:e41801. Available from: <https://pubmed.ncbi.nlm.nih.gov/30910009>
48. Xu S, Webb SE, Lau TCK, Cheng SH. Matrix metalloproteinases (MMPs) mediate leukocyte recruitment during the inflammatory phase of zebrafish heart regeneration. *Sci Rep* [Internet]. 2018 May 8;8(1):7199. Available from: <https://doi.org/10.1038/s41598-018-25490-w>
49. Ghaleb AM, Bialkowska AB, Snider AJ, Gnatenko DV, Hannun YA, Yang VW, et al. IQ Motif-Containing GTPase-Activating Protein 2 (IQGAP2) Is a Novel Regulator of Colonic Inflammation in Mice. *PLoS One* [Internet]. 2015;10(6):e0129314–e0129314. Available from: <https://pubmed.ncbi.nlm.nih.gov/26047140>
50. Podok P, Wang H, Xu L, Xu D, Lu L. Characterization of myeloid-specific peroxidase, keratin 8, and dual specificity phosphatase 1 as innate immune genes involved in the resistance of crucian carp (*Carassius auratus gibelio*) to Cyprinid herpesvirus 2 infection. *Fish Shellfish Immunol* [Internet]. 2014 Dec 1;41(2):531–40. Available from: <https://www.sciencedirect.com/science/article/pii/S1050464814003751>
51. Zhang L-Y, Guo Q, Guan G-F, Cheng W, Cheng P, Wu A-H. Integrin Beta 5 Is a Prognostic

- 857 Biomarker and Potential Therapeutic Target in Glioblastoma. *Front Oncol* [Internet].
858 2019;9(904). Available from: <https://www.frontiersin.org/article/10.3389/fonc.2019.00904>
- 859 52. Ellett F, Pase L, Hayman JW, Andrianopoulos A, Lieschke GJ. mpeg1 promoter transgenes
860 direct macrophage-lineage expression in zebrafish. *Blood* [Internet]. 2011 Jan 27;117(4):e49–56.
861 Available from: <http://dx.doi.org/10.1182/blood-2010-10-314120>
- 862 53. Sedda S, Dinallo V, Marafini I, Franzè E, Paoluzi OA, Izzo R, et al. mTOR sustains
863 inflammatory response in celiac disease. *Sci Rep* [Internet]. 2020 Jul 1;10(1):10798. Available
864 from: <https://doi.org/10.1038/s41598-020-67889-4>
- 865 54. Weichhart T, Hengstschläger M, Linke M. Regulation of innate immune cell function by mTOR.
866 *Nat Rev Immunol* [Internet]. 2015;15(10):599–614. Available from:
867 <https://pubmed.ncbi.nlm.nih.gov/26403194>
- 868 55. Chen S, Lathrop KL, Kuwajima T, Gross JM. Retinal ganglion cell survival after severe optic
869 nerve injury is modulated by crosstalk between JAK/STAT signaling and innate immune
870 responses in the zebrafish retina [Internet]. *bioRxiv*. 2021 [cited 2021 May 18]. p.
871 2021.04.08.439090. Available from:
872 <https://www.biorxiv.org/content/10.1101/2021.04.08.439090v1>
- 873 56. Conedera FM, Pousa AMQ, Mercader N, Tschopp M, Enzmann V. Retinal microglia signaling
874 affects Müller cell behavior in the zebrafish following laser injury induction. *Glia* [Internet].
875 2019 Jun;67(6):1150–66. Available from: <http://dx.doi.org/10.1002/glia.23601>
- 876 57. Van Dyck A, Bollaerts I, Beckers A, Vanhunsel S, Glorian N, van Houcke J, et al. Müller glia-
877 myeloid cell crosstalk accelerates optic nerve regeneration in the adult zebrafish. *Glia* [Internet].
878 2021 Jun;69(6):1444–63. Available from: <http://dx.doi.org/10.1002/glia.23972>
- 879 58. Desgeorges T, Caratti G, Mounier R, Tuckermann J, Chazaud B. Glucocorticoids Shape
880 Macrophage Phenotype for Tissue Repair. *Front Immunol* [Internet]. 2019;10(1591). Available
881 from: <https://www.frontiersin.org/article/10.3389/fimmu.2019.01591>
- 882 59. Li L, Yan B, Shi Y-Q, Zhang W-Q, Wen Z-L. Live imaging reveals differing roles of
883 macrophages and neutrophils during zebrafish tail fin regeneration. *J Biol Chem* [Internet]. 2012
884 Jul 20;287(30):25353–60. Available from: <http://dx.doi.org/10.1074/jbc.M112.349126>
- 885 60. Rice RA, Pham J, Lee RJ, Najafi AR, West BL, Green KN. Microglial repopulation resolves
886 inflammation and promotes brain recovery after injury. *Glia* [Internet]. 2017;65(6):931–44.
887 Available from: <https://pubmed.ncbi.nlm.nih.gov/28251674>
- 888 61. Silva NJ, Nagashima M, Li J, Kakuk-Atkins L, Ashrafzadeh M, Hyde DR, et al. Inflammation
889 and matrix metalloproteinase 9 (Mmp-9) regulate photoreceptor regeneration in adult zebrafish.
890 *Glia* [Internet]. 2020;68(7):1445–65. Available from: <https://pubmed.ncbi.nlm.nih.gov/32034934>
- 891 62. Var SR, Byrd-Jacobs CA. Role of Macrophages and Microglia in Zebrafish Regeneration. *Int J*
892 *Mol Sci* [Internet]. 2020;21(13):4768. Available from:
893 <https://pubmed.ncbi.nlm.nih.gov/32635596>
- 894 63. Vannella KM, Wynn TA. Mechanisms of Organ Injury and Repair by Macrophages. *Annu Rev*
895 *Physiol* [Internet]. 2017;79:593–617. Available from: <http://dx.doi.org/10.1146/annurev-physiol-022516-034356>
- 896
897 64. White DT, Sengupta S, Saxena MT, Xu Q, Hanes J, Ding D, et al. Immunomodulation-
898 accelerated neuronal regeneration following selective rod photoreceptor cell ablation in the
899 zebrafish retina. *Proceedings of the National Academy of Sciences* [Internet].

- 2017;114(18):E3719. Available from: <http://www.pnas.org/content/114/18/E3719.abstract>
65. Ruan Y, Jiang S, Gericke A. Age-Related Macular Degeneration: Role of Oxidative Stress and Blood Vessels. *Int J Mol Sci* [Internet]. 2021;22(3):1296. Available from: <https://pubmed.ncbi.nlm.nih.gov/33525498>
66. Akyol E, Lotery A. Gene, Cell and Antibody-Based Therapies for the Treatment of Age-Related Macular Degeneration. *Biologics* [Internet]. 2020;14:83–94. Available from: <https://pubmed.ncbi.nlm.nih.gov/32982165>
67. Wei X, Luo L, Chen J. Roles of mTOR Signaling in Tissue Regeneration. *Cells* [Internet]. 2019;8(9). Available from: <http://dx.doi.org/10.3390/cells8091075>
68. Mescher AL, Neff AW, King MW. Inflammation and immunity in organ regeneration. *Dev Comp Immunol* [Internet]. 2017 Jan;66:98–110. Available from: <http://dx.doi.org/10.1016/j.dci.2016.02.015>
69. Lelios I, Cansever D, Utz SG, Mildenerberger W, Stifter SA, Greter M. Emerging roles of IL-34 in health and disease. *J Exp Med* [Internet]. 2020;217(3):e20190290. Available from: <https://pubmed.ncbi.nlm.nih.gov/31940023>
70. Deleon-Pennell KY, Altara R, Yabluchanskiy A, Modesti A, Lindsey ML. The circular relationship between matrix metalloproteinase-9 and inflammation following myocardial infarction. *IUBMB Life* [Internet]. 2015 Aug;67(8):611–8. Available from: <http://dx.doi.org/10.1002/iub.1408>
71. Van Lint P, Libert C. Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. *J Leukoc Biol* [Internet]. 2007 Dec;82(6):1375–81. Available from: <http://dx.doi.org/10.1189/jlb.0607338>
72. Shubayev VI, Angert M, Dolkas J, Campana WM, Palenscar K, Myers RR. TNFalpha-induced MMP-9 promotes macrophage recruitment into injured peripheral nerve. *Mol Cell Neurosci* [Internet]. 2006 Mar;31(3):407–15. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4431648/pdf/nihms688508.pdf>
73. Kui Tan T, Zheng G, Hsu T-T, Ra Lee S, Zhang J, Zhao Y, et al. Matrix metalloproteinase-9 of tubular and macrophage origin contributes to the pathogenesis of renal fibrosis via macrophage recruitment through osteopontin cleavage. *Lab Invest* [Internet]. 2013 Apr 1;93(4):434–49. Available from: <https://doi.org/10.1038/labinvest.2013.3>
74. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol* [Internet]. 2014;5:491–491. Available from: <https://pubmed.ncbi.nlm.nih.gov/25339958>
75. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* [Internet]. 2001 Nov;3(11):1009–13. Available from: <http://dx.doi.org/10.1038/ncb1101-1009>
76. Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, et al. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* [Internet]. 2006;126(5):955–68. Available from: <http://dx.doi.org/10.1016/j.cell.2006.06.055>
77. Shang M, Cappellesso F, Amorim R, Serneels J, Virga F, Eelen G, et al. Macrophage-derived glutamine boosts satellite cells and muscle regeneration. *Nature* [Internet]. 2020

- 943 Nov;587(7835):626–31. Available from: <http://dx.doi.org/10.1038/s41586-020-2857-9>
- 944 78. Issaka Salia O, Mitchell DM. Bioinformatic analysis and functional predictions of selected
945 regeneration-associated transcripts expressed by zebrafish microglia. BMC Genomics [Internet].
946 2020 Dec 7;21(1):870. Available from: <http://dx.doi.org/10.1186/s12864-020-07273-8>
- 947 79. Mitchell DM, Sun C, Hunter SS, New DD, Stenkamp DL. Regeneration associated
948 transcriptional signature of retinal microglia and macrophages. Sci Rep [Internet]. 2019 Mar
949 18;9(1):4768. Available from: <http://dx.doi.org/10.1038/s41598-019-41298-8>
- 950 80. Oosterhof N, Holtman IR, Kuil LE, van der Linde HC, Boddeke EWGM, Eggen BJL, et al.
951 Identification of a conserved and acute neurodegeneration-specific microglial transcriptome in
952 the zebrafish. Glia [Internet]. 2017 Jan;65(1):138–49. Available from:
953 <http://dx.doi.org/10.1002/glia.23083>
- 954 81. Jin J, Zhao Q. Emerging role of mTOR in tumor immune contexture: Impact on chemokine-
955 related immune cells migration. Theranostics [Internet]. 2020 May 15;10(14):6231–44.
956 Available from: <http://www.thno.org/v10p6231.htm>
- 957 82. Brandstätter JH, Hack I. Localization of glutamate receptors at a complex synapse. Cell Tissue
958 Res [Internet]. 2001 Jan 1;303(1):1–14. Available from: <https://doi.org/10.1007/s004410000304>
- 959 83. Cheng Z-Y, Wang X-P, Schmid KL, Han X-G, Song H, Tang X. GABAA α 1 and GABAA ρ 1
960 subunits are expressed in cultured human RPE cells and GABAA receptor agents modify the
961 intracellular calcium concentration. Mol Vis [Internet]. 2015;21:939–47. Available from:
962 <https://pubmed.ncbi.nlm.nih.gov/26321868>
- 963 84. Cheng ZY, Wang XP, Schmid KL, Liu L. Identification of GABA receptors in chick retinal
964 pigment epithelium. Neurosci Lett [Internet]. 2013;539:43–7. Available from:
965 <http://dx.doi.org/10.1016/j.neulet.2013.01.038>
- 966 85. Liu Y, Lou WP-K, Fei J-F. The engine initiating tissue regeneration: does a common mechanism
967 exist during evolution? Cell Regen [Internet]. 2021 Apr 5;10(1):12. Available from:
968 <http://dx.doi.org/10.1186/s13619-020-00073-1>
- 969 86. Ghilardi SJ, O'Reilly BM, Sgro AE. Intracellular signaling dynamics and their role in
970 coordinating tissue repair. Wiley Interdiscip Rev Syst Biol Med [Internet]. 2020
971 May;12(3):e1479. Available from: <http://dx.doi.org/10.1002/wsbm.1479>
- 972 87. Kent MR, Kara N, Patton JG. Inhibition of GABA(A)- ρ receptors induces retina regeneration in
973 zebrafish. Neural Regeneration Res [Internet]. 2021 Feb;16(2):367–74. Available from:
974 <http://dx.doi.org/10.4103/1673-5374.286972>
- 975 88. Romaus-Sanjurjo D, Ledo-García R, Fernández-López B, Hanslik K, Morgan JR, Barreiro-
976 Iglesias A, et al. GABA promotes survival and axonal regeneration in identifiable descending
977 neurons after spinal cord injury in larval lampreys. Cell Death Dis [Internet]. 2018 Jun
978 28;9(6):663. Available from: <https://doi.org/10.1038/s41419-018-0704-9>
- 979 89. Legrand C, Gilles C, Zahm JM, Polette M, Buisson AC, Kaplan H, et al. Airway epithelial cell
980 migration dynamics. MMP-9 role in cell-extracellular matrix remodeling. J Cell Biol [Internet].
981 1999 Jul 26;146(2):517–29. Available from: <http://dx.doi.org/10.1083/jcb.146.2.517>
- 982 90. Zhang J-F, Wang P, Yan Y-J, Li Y, Guan M-W, Yu J-J, et al. IL-33 enhances glioma cell
983 migration and invasion by upregulation of MMP2 and MMP9 via the ST2-NF- κ B pathway.
984 Oncol Rep [Internet]. 2017 Oct;38(4):2033–42. Available from:
985 <http://dx.doi.org/10.3892/or.2017.5926>

91. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods [Internet]. 2012 Jul 1;9(7):676–82. Available from: <https://doi.org/10.1038/nmeth.2019>

SUPPORTING INFORMATION

S1 Fig. Pharmacological and genetic inhibition of mTOR activity does not affect RPE proliferation in unablated larvae

(A-F) Immunofluorescent images of BrdU staining on transverse cryosections from MTZ⁻ DMSO-, rapamycin and INK128--treated larvae and (J-K) from MTZ⁻ *mtor*^{+/+} and *mtor*^{-/-} larvae at 9dpf. (E-F) Brightfield images of cryosections from MTZ⁻ DMSO-, rapamycin- or INK128-treated larvae at 4dpi. (I) Quantification of BrdU⁺ cells in the RPE layer showed no significant differences between inhibitor-treated and DMSO-treated larvae. (K) Quantification of BrdU⁺ cells in the RPE layer showed no significant differences between MTZ⁻ *mtor*^{+/+} and *mtor*^{-/-} larvae. Dorsal is up and distal is left. Scale bar = 50µm.

S2 Fig. Rapamycin, INK128 and MHY1485 do not affect MTZ-induced apoptosis.

(A-G) Fluorescent images of TUNEL staining on cryosections from MTZ⁺ 0.04%DMSO-, 2µM rapamycin-, 2µM MHY1485- and 0.018% DMSO and 0.9µM INK-treated larvae at 1dpi. (F) Quantification of TUNEL⁺ puncta between outer plexiform layer and RPE layer showed no significant differences between DMSO-treated and drug-treated groups. Dorsal is up and distal is left. Scale bar = 50µm.

S3 Fig: Pharmacological activation of mTOR signaling by MHY1485 does not affect RPE proliferation at 2dpi and 4dpi

(A-D) Fluorescent images of BrdU immunostaining on cryosections from unablated and ablated DMSO- and MHY1485-treated larvae at 7dpf/2dpi and (E-H) 9dpf/4dpi. *White arrowheads* highlight BrdU⁺ cells in the RPE layer. (I,Q) Quantification of BrdU⁺ cells in the RPE layer showed significant increases by treatment of MTZ in ablated larvae at 2dpi, when compared to age-matched MTZ-unablated controls. But it showed no significant differences between ablated MHY1485-treated larvae and DMSO-treated siblings at 2dpi and 4dpi. Dorsal is up and distal is left. Scale bar = 50µm.

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1022 **S1 Table: MTZ⁺ 2dpi rapamycin vs. DMSO downregulated genes (top 100)**

1023 **S2 Table: MTZ⁺ 2dpi rapamycin vs. DMSO upregulated genes (top 100)**

1024 **S3 Table: MTZ⁺ 4dpi rapamycin vs. DMSO downregulated genes (top 100)**

1025 **S4 Table: MTZ⁺ 4dpi rapamycin vs. DMSO upregulated genes (top 100)**

1026 **S5 Table: 2dpi rapamycin vs. DMSO enriched downregulated Reactome pathways**

1027 **S6 Table: MTZ⁺ 2dpi DMSO vs. 7dpf MTZ⁻ DMSO upregulated genes (top 100)**

1028 **S7 Table: 4dpi rapamycin vs. DMSO enriched downregulated Reactome pathways (top 10)**

1029 **S8 Table: Differential expression of *csf1ra*, *csf1rb* in MTZ⁺ DMSO vs. MTZ⁻ DMSO RPE cells**

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1031 **S9 Table: Statistics**

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Fig. 1

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 2