Understanding the role of Pten and the molecular mechanisms underlying Pten/PI3K/Akt/mTOR pathway during zebrafish retina regeneration.

A thesis submitted by

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Declaration

The work presented in this thesis has been carried out by me under the supervision of Dr. Rajesh Ramachandran at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, diploma, or a fellowship to any other university or institute.

Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in the bibliography.

Date-22.02.2021 **Place-**Mohali **Shivangi Gupta**

In my capacity as the supervisor of the candidate's thesis work, I certify that the above statements made by the candidate are true to the best of my knowledge.

Dr. Rajesh Ramachandran

(Supervisor)

Dedicated to my Beloved Mother and Father

“The woods are lovely, dark and deep.
But I have promises to keep,
And miles to go before I sleep,
And miles to go before I sleep.”

-Robert Frost, Stopping by Woods on a Snowy Evening

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If I were to describe the life of a Ph.D student and a researcher, I would say that it is actually based on the phrases from the poem "The Road not Taken", beautifully framed by the poet Robert Frost, which says that "Two roads diverged in the woods, and I, took the one less travelled by, And that has made all the difference." The path of research is a less chosen one, usually a less travelled road. A person, who here is a Ph.D. student chooses to tread on that path and invests their heart, soul, blood, body and time to reach till the other end. This chosen path is bleak and the traveller has no idea of coming back. But the end makes all the difference. The traveller on the way sows seeds of immence hardwork and at the end reaps great benefits and rewards. This end is the phase of the end of Ph.D. research which makes all the difference for the traveller Ph.D. student and enriches the garden of knowledge with his contributions. But this journey on this bleak path is like a roller-coaster ride, full of golden, happy, dark and gloomy moments, both during work and also on the personal front. There are times of utter confusion and times of extreme good luck. Even after being in Science, which works on the principles of facts and experiments, research life makes one to believe in luck and destiny also. The life during Ph.D. changes a student both physically and from within. On the way, apart from family many people come in their lives, who either prove to be helpful and supportive or become the demotivating factors or hurdles during the work. Thus, completing the Ph.D. thesis and the journey of Ph.D. research will be incomplete without acknowledging these people either good or bad, as we learn from all of them. In this section, I would like to thank all such people who became a part of my Ph.D journey.

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I hope that I stand up to all the expectations that others have from me and I myself have from me. I desire to contribute something good and valuable to the scientific community.

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Publications

1. **Shivangi Gupta**, Poonam Sharma[#], Mansi Chaudhary[#], Simran Kaur, Vijithkumar V., and Rajesh Ramachandran. (2021) Downregulation of Pten and its interplay with Mmp9/Notch signaling is essential for Müller glia reprograming and retina regeneration. (Manuscript in revision)

Thesis Abstract

Visual organs have been accorded as the most crucial organs of the living beings and vision impairment becomes a major loss to the body. Mammals have lost the ability to regenerate their Central Nervous System (CNS), and retina, being a part of it, upon injury or any insult cannot regenerate in mammals. This renders them permanently disabled. But the robust regenerative potential of a teleost, zebrafish (*Danio rerio*), becomes a ray of hope as studying the mechanism underlying regeneration in zebrafish can enable us to develop therapeutic interventions in mammals. Zebrafish has been a great tool to study genetics and for a long time researchers have been engaged in probing into its regenerative mechanisms, as almost all its organs can regenerate. Retina regeneration is a cascade of reprogramming events involving specialized cells named Müller Glia (MG) which get activated, lose their fate and also adopt a retinal stem cell-like state upon any injury or stress. These multipotent stem cell-like MG derived progenitor cells (MGPCs) can proliferate and also form various retinal cell types, thus facilitating visual function restoration. But, as the MGPCs proliferate, they are under stringent control by multiple factors, which keep a check on the proliferative pace of these cells and prevent the regeneration process from going erratic.

In this context, during retina regeneration, we have tried to explore the role of a tumor suppressor Pten which is a dual-specificity phosphatase. Till now Pten/PI3K/Akt/mTOR pathway has been well elucidated in various cancers, but it remains underexplored in retina regeneration. The pathway includes factors that promote proliferation like Akt and mTOR, and also factors that are anti-proliferative like Pten, all working in an orchestrated manner. Here we show that the downregulation of Pten from the MGPCs evokes a strong proliferative response by activating and increasing the levels of Akt. We observed that activated Akt leads to mTORC1 activation, which increases MGPCs proliferation. We propose the existence of a negative feedback control by mTORC1 on Akt. We delved deeper into the mechanism through which Pten contributes during retina regeneration and found the involvement of Mmp9/Notch signalling, β-Catenin, and some other pathways also to regulate MGPCs proliferation. We also found that Pten itself is regulated by the Mycb-Hdac1 repressive complex and this regulation is further fine-tuned by the Tgf-β signalling pathway. Thus, we conclude by highlighting the crucial role of Pten/PI3K/Akt/mTOR pathway in poising a successful regenerative response in the zebrafish retina.

Thesis Synopsis

Introduction and Literature Review

In this dynamic world where biological life forms thrive by depending on their sensory cues and body organisation to use the available resources on the earth, vision in the animals proves to be of immense importance to them, as it allows them to see and detect their surroundings, objects, food and predators. Thus, visual impairment becomes a major loss to the animals. The conditions which can be dealt with, involve invasive interventions but the ones which can't be, leave the animal blind. The evolutionarily primitive and simple organisms possess a highly sophisticated ability to regenerate their damaged tissue instead of rendering it unusable. Regeneration is a process which allows regaining the morphology and functionality of damaged tissue or organ without the need for any external factors. But unfortunately, along the evolutionary timeline animals have lost this ability or in some, it has got restricted to a few tissues that too under some specific conditions due to multiple molecular and epigenetic cues. The study of regeneration has become a major area of interest for research groups. It will be a boon for evolutionarily advanced animals if the mysteries and the science behind this phenomenon get unravelled. This would allow therapeutic interventions to circumvent various medical conditions.

Since years regeneration biologists had been actively engaged in exploring various model organisms, their abilities and molecular mechanisms underlying regeneration. Being inspired from Hydra in mythology, watching lizard's tail in their day-to-day lives and getting their own skin repaired and regenerated, scientists got intrigued by the beauty of regeneration. This led them to take their studies forward to the most complex biological systems of Central Nervous System (CNS), which in humans is incapable of regeneration. But in primitive vertebrates, it is possible to regenerate the injured or damaged nervous system and its parts. Zebrafish (*Danio rerio*) emerged to be an excellent model organism to study regeneration, since almost all its organs possess a remarkable ability to regenerate. Retina in the eye, which conveys the visual signals to the brain, forms the most accessible part of the CNS and can get regenerated in zebrafish preventing it from blindness, contrary to that seen in humans. Upon injury or any stress, the specialised cells named Müller Glia (MG) in the Inner Nuclear Layer (INL) of zebrafish retina get activated by dedifferentiation, assume a stem cell-like state and become progenitors (MGPCs). They start proliferating asymmetrically to repair the injury by forming

more MGPCs which could later also differentiate into various retinal cell-types to allow the retinal function restoration.

Till now scientists have investigated the roles of many transcription factors, pluripotency-inducing factors, growth factors, pro-proliferative pathways, chromatin and epigenetic modifiers and many other signalling pathways during zebrafish retina regeneration. But, the roles of phosphatases in this aspect remained underexplored, despite multiple crucial roles played by them to maintain homeostasis in the organisms. In our study, we have mainly focused on deciphering the roles played by Pten, a dual-specificity phosphatase and the second most mutated tumor suppressor, and its downstream Pten/PI3K/Akt/mTORC2 signalling pathway during zebrafish retina regeneration. This had been a well-established pathway in cancers, development, cell metabolism, cell migration, but had been overlooked in the context of retina regeneration. In mice, Pten plays anti-proliferative roles during axonal and spinal cord regeneration, similar to that found in zebrafish also in case of spinal cord injuries. There had been reports of Pten contributing during zebrafish embryogenesis, organism development and tissue homeostasis. Pten expression has been seen in zebrafish eyes and its deletion has been reported to cause ocular tumors. At the same time, we came across a report stating, Calcineurin, another phosphatase plays a role during fin regeneration in zebrafish. It has been established that there exists a fine line between cancerous cells dividing uncontrollably and regenerating cells dividing in a regulated manner. What exactly governs these borderlines has been an unsolved question. This question and all the previous pieces of evidence intrigued us about the role of Pten and this pathway during zebrafish retina regeneration.

Results

Pten induction after retinal injury and then its downregulation is necessary during zebrafish retina regeneration.

Zebrafish possesses 2 genes for Pten: *ptena* and *ptenb*, which code for proteins with 80% amino acid sequence similarity. Our work shows, through the temporal expression profiles of *ptena* and *ptenb* genes, that upon retinal injury by needle poke method both the genes got highly induced initially soon after the injury and showed a second shallow peak during the proliferative phase. We also observed spatial seclusion of *ptena* and *ptenb* expression from the MGPCs marked by PCNA. Similar trends were observed at the protein levels, with $PCNA^{+}$ MGPCs showing highly reduced Pten protein expression at 4dpi, unlike in uninjured retina

where Pten protein was uniformly expressed. This trend was validated in *1016tuba1a*:GFP transgenic line of zebrafish, where GFP expressing proliferating cells had very low expression levels of Pten. However, to maintain the retinal homeostasis, we found that Pten levels did not vary or get regulated in the entire retinal tissue. The drastic reduction in the Pten levels seen in the MGPCs intrigued us to modulate Pten levels further. For this, we blocked the Pten in zebrafish retina by its gene knockdown using *ptena*/*ptenb* MO as well as its functional blockade by pharmacological inhibitor SF1670. Interestingly, Pten blockade by both the ways led to a remarkable increase in the number as well as the span of the MGPCs marked by BrdU, in the retina at 4dpi. This effect was more profound upon combined knockdown of *ptena* and *ptenb* genes, as seen at 4dpi. Fortunately, we could trace these proliferating cells and found that these increased number of MGPCs formed upon Pten blockade continued to proliferate beyond 4dpi, till 30dpi, and even adopted various retinal cell fates. Thus, restoring the visual functions of the injured zebrafish retina after 30dpi.

Mechanism of involvement of Akt, PI3K, mTOR and β-Catenin during zebrafish retina regeneration.

Pten, despite being a dual-specificity phosphatase, conjures its major functions when it is membrane-anchored, by dephosphorylating the lipid molecule Phosphatidylinositol (3,4,5)-triphosphate (PIP3). Its actions are further mediated by the other members of this pathway which include a series of actions of kinases PI3K, Akt and mTOR complex, all of which are pro-proliferative in nature, opposite to Pten. We got interested in investigating the regulation and the roles of these proteins during regeneration of retina or upon Pten blockade during retina regeneration. Since our results reported downregulation of Pten in the MGPCs, we expected an increase in the levels of total Akt and activated Akt in these MGPCs. Akt is known to be activated upon phosphorylation at its 2 residues: Thr308 (Thr302 in zebrafish) by PDK1 (activated by PI3K) and Ser473 (Ser467 in zebrafish) by mTORC2. We found that the proliferating $PCNA^+$ MGPCs showed high expression of Akt and phosphorylated Akt at 4dpi, unlike the uninjured retina where the expression was negligible. Upon forced blockade of Pten by SF1670 and by combined *ptena*/*ptenb* knockdown, we observed a dose-dependent increase in the levels of phosphorylated Akt and total Akt in the retina at 4dpi. This regulation in Akt levels prompted us to elucidate its roles during the zebrafish retina regeneration. We proceeded to knockdown *akt1* using its translational blocker MO. We found a concentration dependent decrease in the number of MGPCs in the retina at 4dpi, which got reversed upon overexpression

of wild-type *akt1* and its constitutively active phosphomimetic mutant form as compared to the controls. These results were indicative of the necessity of Akt for supporting MGPCs proliferation and of the existence of Pten/Akt axis during retina regeneration.

Since Akt requires to be phosphorylated for its complete activation, the kinases: PI3K and mTORC2, contributing to this, seem to be indispensable too. Thus, we got inquisitive towards the roles of these kinases during retina regeneration and Akt activation. Our experiments further involved LY294002 drug-mediated blockade of PI3K, and Torin1 drug-mediated mTORC2 blockade in the retina. We observed that both these kinases were pro-proliferative, as their inhibition led to a drug dose-dependent decrease in the numbers of MGPCs, in the retina at 4dpi. We speculated that this decline in the MGPCs number may be a result of inactivation of Akt, occurring due to the reduction in its phosphorylation levels at both the annotated sites at 16hpi, 2dpi and 4dpi. These results hold a key to confirm the fact that activation of Akt by its phosphorylation becomes a prerequisite to a successful regenerative response. We wondered if PI3K/Akt/mTORC2 is the only arm of regulation through which Pten works during retina regeneration. To break further into this, we performed 2 sets of experiments, blocking Pten with mTORC2 and in another blocking Pten with PI3K, in the retina at 4dpi. Upon combined blockade of Pten with mTORC2, we found the number of MGPCs almost similar to the control, more than that in mTORC2 blockade and lesser than that in the Pten blockade alone. This suggests that Pten blockade leading to Akt activation is required for increasing the number of MGPCs. Other reason may be the inability of PI3K also to phosphorylate Akt at T302, since S467 was absent. pAktS467 is known to be a better substrate for PI3K for phosphorylating at T302, which couldn't happen in the current case. Surprisingly, in the other double blocker experiment, where Pten and PI3K were blocked in combination, we observed the number of MGPCs to be similar to that seen in the Pten blockade alone, which was significantly higher than that seen in the PI3K blockade alone and the control. This seemed to contradict our previous notion that the Pten blockade led to an increased level of phosphorylation of Akt, which proved to be conducive for the increased MGPCs proliferation during retina regeneration. These results suggested that Pten inhibition could influence MGPCs proliferation even in the absence of Akt activation by PI3K. This made us consider the chances of involvement of some other pro-proliferative mechanisms following Pten blockade in retina regeneration.

We came across the literature which gave us the evidence of the existence of Pten/PI3K/Akt/β-Catenin signalling axis during various cancerous and immunopathological conditions. This

made us delve deeper into Pten/PI3K/Akt pathway and its downstream effects during zebrafish retina regeneration. β-Catenin, a hallmark of proliferation-associated Wnt signalling pathway, is known to be itself highly associated with the MGPCs in the retina. In the same lines of thought, we checked the expression levels of β-Catenin and found that its levels got enhanced with the increased number of MGPCs in the Pten-blocked retina at 4dpi. These results were suggestive of the existence of Pten/PI3K/Akt/β-Catenin signalling axis in the regenerating retina of zebrafish also, supporting this increase in the MGPCs number upon Pten blockade.

Pten regulates MGPCs proliferation through Mmp9/Notch signalling and other parallel pathways.

As we already know from the previous works that Notch signalling is an anti-proliferative pathway during zebrafish retina regeneration, the same as that of Pten which restricts the number and the span of MGPCs proliferation. This made us explore if Pten plays its anti-proliferative actions through Notch signalling. We first analysed the expression levels of *her4.1*, an effector of the Notch signalling pathway, in Pten blocked conditions and found an anticipated decline in its levels in the retina at 4dpi. While the *her4.1* levels reduced upon Pten blockade, there was a significant increase in the levels of one of its direct targets, *mmp9.* The double blocker experiments done previously for Pten-mTORC2 and Pten-PI3K revealed a significant increase in *her4.1* levels in Pten-mTORC2 blockade. While in case of Pten-PI3K blocked retina *her4.1* level stayed similar to that in the control at 4dpi. The *mmp9* levels stayed high in both the blockades. These results directed us to the view that Pten blockade embarks its effects during retina regeneration through Notch signalling which in turn regulates *mmp9.*

There are abundant reports which state that Mmp9 can regulate Notch signalling through the activation of a series of effectors like TNFα/NF-κB, Adams, NICD. Thus, we checked if increased *mmp9* upon Pten blockade also regulated *her4.1*. We found a decline in the levels of *her4.1*, along with the *adam10a, adam17a* and *rbpja* in the SB3CT-mediated Mmp9-blocked retina at 16hpi and 2dpi. Further, the forced decrease in *her4.1* levels through Notch signalling blockade by DAPT and by MO-mediated *her4.1* knockdown led to a downregulation of *ptena* and *ptenb* transcripts as well as Pten protein at 2 and 4dpi. We also found that Pten levels declined appreciably upon Mmp9 blockade in the retina at 16hpi, a time associated with MG reprogramming, also when Mmp9 expression is at its peak. It is known that in cancerous conditions, Mmp9 upregulates NF-κB, which is a known positive regulator of Pten, which we also observed in our results. In the Mmp9 blockade alone and in combination with Pten

blockade, we observed that the Akt could not get phosphorylated, while it increased upon Pten blockade alone. This became supportive of the fact that Mmp9 was needed in activating Akt and thus also in facilitating Pten function through Notch signalling. We aimed at further dissecting out this network to conclude that Pten shows its effects to regulate MGPCs proliferation through Mmp9/Notch signalling.

We performed double blocker experiments again, blocking Pten using SF1670 along with Mmp9 using drug SB3CT. We got an expected increase in the proliferation upon Pten blockade, while a decline upon Mmp9 blockade. In the combined blockade, the number of MGPCs got reduced similar to that seen in the injured control retina at 4dpi. We should have found a drastic decrease in the number of MGPCs if Pten was regulating its function through Mmp9 only, which got prevented due to the involvement of some other parallel cellular pathway also, regulating MGPCs proliferation downstream of Pten. A similar trend was seen upon SF1670-mediated Pten blockade along with *mmp9* knockdown at 4dpi. On the similar lines, next, we overexpressed *pten* along with the blockade of Notch signalling, where the MGPCs number was seen to be similar to that of the control 4dpi. Notably, Pten overexpression alone retarded the MGPCs proliferation, while Notch signalling blockade by DAPT remarkably increased the MGPCs number. The combined blockade should have resulted in a consistent increase in the number of MGPCs which was not the case. Thus, confirming that Notch signalling was important for Pten to show its anti-proliferative effect, but the Pten carries this function through some other mediator also. Both these set of results were indicative of the functioning of parallel pathways also, other than the Mmp9/Notch signalling, through which Pten acts to regulate the MGPCs proliferation.

Fine-tuning of *pten* expression during retina regeneration.

In order to maintain the tissue homeostasis, *pten* gets upregulated post-injury, it decreases in the MGPCs and then increases back to the normalcy to allow the cells to exit the cell cycle and stop them from dividing persistently. To accomplish this, out of multiple regulatory cues known, some fine-tuned regulatory mechanism on *pten* gene is required. We probed onto the promoter sequences of *ptena* and *ptenb* genes and found multiple Mycb-binding sites. Mycb, as per its collaborative partners, is said to have transcriptional activation and repression roles. Mycb is known to collaborate with Hdac1, both of which get induced soon after the injury during retina regeneration, similar to the *pten* genes. This collaboration is reported to cause transcriptional repression. Thus, we analysed the effects of this interaction on *pten* genes, by

blocking the association of Myc-Max and function of Hdac1, by their pharmacological inhibitors 10058-F4 and TSA, respectively. We found significant upregulation in the levels of *pten* genes and protein upon Myc-Max and Hdac1 blockades in the retina at 2 and 4dpi, which directed us towards the repressive control of Mycb-Hdac1 complex on *pten* genes. We validated the binding of Mycb-Hdac1 complex on *pten* genes' promoters by doing Chromatin Immunoprecipitation (ChIP) assay and found the occupancy of Mycb-Hdac1 complex on *pten* genes' promoters. Thus, confirming the regulation of Pten by Mycb-Hdac1 complex.

Further exploring the refinement in the regulation on Pten, we selected the Tgf-β signalling pathway which mounts a pro-proliferative response during zebrafish retina regeneration and thus, should have a repressive effect on Pten function. Upon SB431542-mediated blockade of Tgf-β signalling, we observed a dose-dependent decline in the levels of *ptena* and *ptenb* genes. Upon exploring the *ptena* and *ptenb* gene promoters, we found 5GC sites on which pSmad3 binds to activate the gene expression and confirmed this by performing ChIP assay at 2dpi. These results were indicative of the fact that Tgf-β signalling could positively regulate the Pten expression. But this seemed like an enigma to us, since Tgf-β signalling is pro-proliferative, while Pten is anti-proliferative. This intrigued us to look closely into the *pten* gene promoter sequence. We found that *ptenb* promoter had Tgf-β inhibitory element (TIE) sequence on it and ChIP assay revealed that pSmad3 was bound to it. The literature states that pSmad3 binds to TIE sequence when Fos, a *cfos* gene product, is also recruited in the binding complex. The *cfos* gene also was seen to be induced just after the retinal injury in the MGPCs, where *pten* expression is highly reduced. We propose that binding of pSmad3 on TIE of *ptenb* promoter occurred through Fos in the MGPCs, downregulating the *ptenb* and thus allowing proliferation permissive environment, while Tgf-β signalling could positively regulate the Pten expression in the non-MGPCs. Further, upon Pten blockade we found SF1670 concentration-dependent decline in pSmad3 levels, which may be mediated through reduced *her4.1* levels. *her4.1* is a reporter of Notch signalling mediated by NICD, which is known to enhance the Tgf-β signalling through pSmad. Thus, we propose that Pten is able to positively regulate itself through this mechanism during retina regeneration.

Discussion

Our study identified the role of Pten and its downstream Pten/PI3K/Akt/mTORC2 pathway during zebrafish retina regeneration. Pten acts antagonistically to the other pro-proliferative molecules of this pathway during MGPCs proliferation. It gets induced upon injury to the retina

during the dedifferentiation phase, but stays secluded from the MGPCs. Its downregulation evokes the regenerative response in the retina, which is maintained by increased Akt activation through its phosphorylation. Our work further elucidates the roles of Akt, and PI3K and mTORC2 in Akt activation and eliciting MGPCs proliferation. We report that Pten initiates its functions mediated not only by PI3K/Akt/mTORC2, but a mechanistic link of Pten also exists through Mmp9/Notch signalling and some other parallel pathways to conjure the effects during retina regeneration. There lies a tight regulatory mechanism on the Pten also, to decrease and then upregulate the levels of *pten* during retina regeneration. We report that the Mycb-Hdac1 complex represses the expression of *pten*, while Pten positively regulates its own levels through Tgf-β signalling pathway. Thus, the findings from our study add deeper insights and clarity to the concept of regeneration by unravelling the roles of Pten and the associated gene-regulatory mechanisms.

Abbreviations

Ascl1a	Achaete-Scute Complex-Like 1a
ATF3	Activating transcription factor 3
β-*act*	beta-actin
BF	Bright-field
BHLH	Basic Helix Loop Helix
BMP	Bone Morphogenetic Proteins
BrdU	5-Bromo-2'-Deoxyuridine
BSA	Bovine Serum Albumin
ChIP	Chromatin Immuno-Preciptation
Chx10	Ceh-10 homeodomain-containing homolog, homeobox protein
CMZ	Ciliary Marginal Zone
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
Dig	Digoxigenin
Dkk1b	Dikkopf 1b
DNMTs	DNA Methyltransferases
dpf	Days Post Fertilization
dpi	Days Post Injury
ECM	Extracellular Matrix
EdU	5-Ethynyl-2'-deoxyuridine
EGF	Epidermal Growth Factor
ESC	Embryonic Stem Cell
FGF	Fibroblast Growth Factors
GAP	GTPase-activating proteins
GCL	Ganglion Cell Layer
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescence Protein
GS	Glutamine Synthetase
GSK-3β	Glycogen synthase kinase 3β

HB-EGF	Heparin-Binding EGF-like Growth Factor
Hdac1	Histone deacetylase 1
Her4.1	Hairy related 4, tandem duplicate 1
HES	Hairy enhancer-of-split
hpi	hours post-injury
HRP	Horseradish Peroxidase
IF	Immuno-Fluorescence
IGF	Insulin Grwoth Factor
Il6	Interleukin 6
INL	Inner Nuclear Layer
Insm1a	Insulinoma-Associated 1a
IPL	Inner Plexiform Layer
iPSC	Induced Pluripotent Stem Cell
Klf4	Kruppel-Like-Factor 4
LEF1	Lymphoid Enhancer-binding Factor-1
Lepa	Leptin a
Lepr	Leptin receptor
MAPK	Mitogen-Activated Protein Kinase
Max	Myc associated X
MEK	MAPK/ERK (extracellular signal-regulated kinases) Kinase
MG	Müller Glia
MGPC	Müller Glia derived Progenitor Cell
MMP	Matrix metalloproteinase
MO	Morpholino
mpi	minutes post injury
mTORC1/C2	Mammalian Target of Rapamycin Complex 1/2
NICD	Notch Intra-Cellular Domain
NF-κB	Nuclear Factor kappa light chain enhancer of activated B cells
NMDA	N-methyl-D-aspartate receptor
n.s.	not significant
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
Pax6	Paired Box 6
PBS	Phosphate Buffer Saline

PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDK1	3-Phosphoinositide-dependent kinase 1
PIF	Pluripotency inducing Factor
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphoinositide-4,5-Biphosphate
PIP3	Phosphoinositide-3,4,5-Triphosphate
PNS	Peripheral Nervous System
PPP	Phosphoprotein phosphatases
PPM	Metallo-dependent protein phosphatases
Pten	Phosphatase and Tensin Homologue
PTP	Protein-tyrosine phosphatases
qPCR	quantitative Polymerase Chain Reacion
RAGs	Regeneration-Associated genes
RGC	Retinal Ganglion Cell
RNFL	Retinal Nerve Fibre Layer
ROS	Reactive Oxidative Species
RPC	Rod Progenitor Cell
RPE	Retinal Pigment Epithelium
RT-PCR	Reverse Transcription Polymerase Chain Reacion
SD	Standard Deviation
Shh	Sonic hedgehog
SPRR1A	Small Proline Rich Protein 1A
Sox2	Sex Determining Region Y (SRY)-Box 2
Sox11	SRY-Box Transcription Factor 11
TCF	T-cell-specific factor
TEA	Triethanolamine hydrochloride
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor Alpha
TSA	Trichostatin A
TSC1/2	Tuberous Sclerosis Factor 1/2
Tuba1a	Tubulin, Alpha 1a
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UTR	Untranslated Region

VEGF	Vascular Endothelial Growth Factor
WNT	Wingless-related integration site
Zic2b	Zinc finger of cerebellum 2b
4E-BP1	4E-Binding Protein 1

Section 1.

Introduction and Literature Review

1.1 Tale of Regeneration

All the living organisms possess the ability to show response to a particular stimulus, which in mammals is facilitated by the presence of a highly developed nervous system. The response is surmounted by the presence of the five essential sense organs in the mammalian body, namely eyes, ears, nose, skin and tongue, all guided by a Master Regulator-the brain. Loss of any of the mentioned sense organs due to age, trauma, any disease or some genetic deformity leads to a tremendous loss to the organism (Mao & Mooney, 2015). There have been many scientific interventions and advancements over the years to combat these issues to restore these senses. These range from pharmacological aids to occupational therapy, from gene therapy to organ transplants and the stem cell sciences. A new dimension has been covered in the recent years with the emergence of the field of tissue engineering where knowledge and tools from various scientific arenas have been brought together to create substitutes and prototypes outside the living body (called organogenesis) to replace or help regenerate the damaged tissues (Berthiaume et al., 2011). However, none of these methods have proven to be completely successful or are extremely cost-intensive, or in some, the progression to the clinical product has been a slow process or sometimes the body's immune response rejects the transplant.

Now, exploring the possibility of using ones' innate ability to repair the injuries or loss of tissues and organs paves the way to two such self-healing processes: Regeneration and Wound healing. A better understanding of both the mechanisms will enable us to improve the repair and regain of function after injury in humans (Vibert et al., 2018), since these concepts are poorly elucidated, making the therapies limited for the same. We have mainly focused on regeneration paradigm in our study. It is reported that mammals especially humans regenerate or restore few of the body parts like skin, liver and endometrium, and haematopoietic system, which motivates us to reach another horizon of using the approach of the regenerative medicine (Eming et al., 2014). Using this approach, stem cells are used as a source to generate entire functional tissue or organ *in vivo* (Berthiaume et al., 2011). This enlightens the fact that the adult humans possess a minimal regeneration capacity as compared to the lower vertebrates, so it will be worth leveraging this innate healing response to promote regeneration of the other organs (Elder, 1979; Mao & Mooney, 2015). There lies a vast gap between the process of regeneration in the lower vertebrates and the regenerative biology of the advanced vertebrates and mammals (Kami & Gojo, 2014). It will be highly promising to work out the essential cues and the molecular and epigenetic mechanisms underlying the highly evolved regenerative

phenomenon in the lower vertebrates, which is limited in the higher vertebrates. This would enable us to extrapolate the information extracted out from the lower vertebrates to the mammalian regeneration phenomenon, to provide a therapeutic solution to improve the repair and regeneration capacity of the damaged or degenerated mammalian tissues/organs. This will primarily obviate the need for replacement of the organs (Brien & Barry, 2009).

1.1.1. Regeneration: An overview

We have been discussing briefly the novel strategies to overcome the problem of loss of functionally active organs or of healing of injury, with the regeneration of the organ being the most effective one. Now delving deeper into the terminology of regeneration will make us well versed in this field to explore it more. As has been beautifully quoted in 1969 by Professor Richard J. Goss, one of the pioneers in the fields of Experimental Zoology and Developmental Biology, in his book *Principles of Regeneration*, regarded as the primer for a person entering the field of regeneration biology (Dinsmore & Aronson, 1997),

> "If there were no regeneration there could be no life.
> If everything regenerated there would be no death."

It leaves us to ponder more on the sublimity and depth of this concept of Regeneration.

Regeneration is the process by which a damaged tissue or organ is able to restore its structure (morphology) and function (physiology) without the provision of any external factors or aids. During regeneration, the mother cells taking part in the process undergo asymmetric division, where one population of daughter cells can self-renew or divide and another population can form various cell types in order to restore the function (Morrison & Spradling, 2008). The maintenance of the balance between these two states of cells marks them for their primary, efficient and regulated role during tissue regeneration (Carlson, B. M. (2007). *Principles of Regenerative Biology*. London: Elsevier Inc.; Birnbaum & Sánchez Alvarado, 2008; Facchin et al., 2018). This peculiar property in the organism is achieved by its special cells called Stem cells which are found residing in the clusters in the specific niches in the body. Niches are those specific and instructive dynamic microenvironments in the body, progressive changes in which lead to stem cell activation (Xia et al., 2018). Stem cells have the potential to respond to a wide range of transcription factors, growth factors and other signalling molecules and then to trigger downstream signalling cascades. It has been hypothesised by the researchers that the space

within the niche is limited, and the signals from this microenvironment may specify stem cell self-renewal attribute. Due to this limited space, the stem cells upon division may place one daughter cell in the niche, but other daughter cells will be placed outside this niche. Thus, keeping those cells devoid of the factors for the self-renewal, where they may trigger differentiation. If the space in the niche itself or in the neighbouring niche is sufficient enough or is empty, both the daughter cells may retain their capacity to self-renew (Morrison & Spradling, 2008; Voog & Jones, 2010).

The regenerative capacity and the rate also depend upon the damage, insult, stress or trauma given to the tissue or the organ, apart from the age or complexity of the organisms. Damage to the tissue or the organ may be due to mechanical cues (stabbing, poking, cutting), chemical agents (NMDA, ouabain, acid, alkali burns and others), severe light or Ultraviolet rays. It may be just dermal, sub-dermal, may lead to disruption of the blood vessels; all requiring innervation and vascularisation for the function restoration. The extent or harshness of the injury also changes the efficiency of regeneration. If the tissue is damaged or injured to the extent that none of the cells are left to contribute to the regeneration or if the tissue is destroyed then this process is hampered. Citing an example, if the organs like limbs are cut or amputated more towards the proximal end, a stage is eventually attained when regeneration ceases. This happens since the entire territory of the tissue, or the cells which could help during the regeneration are removed (Goss, R. J. (1969). *Principles of Regeneration.* London: Academic Press, Inc. Ltd.).

Not only across the different phyla the regenerative potential varies, but within the same species also, the regenerative potential is different in different organs, like the mammalian skin regenerates fastest than the liver, which is faster than the heart (Fausto et al., 2006; Porrello et al., 2011). Regeneration has been unravelled to be maximum in the lower phyla (invertebrates and phylogenetically primitive vertebrates), which reduces as we move towards the evolutionarily complex organisms (like mammals) (Elder, 1979; Zhao et al., 2016; Xia et al., 2018). It has also been reported that regeneration capability also reduces with the ontogenic development or ageing of the organisms, like gradual loss of limb regeneration as we proceed from larval stages of frogs to adults (Mescher & Neff, 2005; Zhao et al., 2016).

Biologists during all these times have been trying to resolve the mystery behind this disparity. They have rendered us with some cues with the important ones being differences in the

numbers (Mahla, 2016) and the properties of stem cells, varying potentials of cell dedifferentiation and transdifferentiation, differences in the levels and chronology of the expression of different regeneration-associated genes (RAGs), epigenetic regulations on these genes and on their key controllers and the immune response elicited after the insult or trauma (Zhao et al., 2016; Xia et al., 2018).

The potential to regenerate relies on the selective expression or silencing of the RAGs, which might be expressed in the regenerative species and absent or silent in the non-regenerative ones (Zhao et al., 2016). This expression might be modulated by the epigenetic regulators in various organisms and during various aspects of regeneration (Gornikiewicz et al., 2013), example, in zebrafish the expression of *sonic hedgehog* (*shh*) gene makes the regenerative response quite strong as compared to the adult Xenopus, where its expression is lost due to hypermethylation of the enhancer region of Shh (Yakushiji et al., 2007; Kaur et al., 2018). As has been mentioned in the previous phrases, it has also been studied that with the development of a robust immune response and with the advent of the adaptive immunity in the organisms during evolution, the regenerative ability reduced (Mescher & Neff, 2005; Aurora & Olson, 2014; Julier et al., 2017), example, in zebrafish the CNS regeneration is higher and more potent than in the mammals, due to a weaker and shorter immune response triggered upon the CNS injury as compared to that in the mammals who possess a complex milieu of different immunological cell types and responses (Kyritsis et al., 2014) (Fig 1.1). Analysing and tweaking these perspectives will provide us with new connections and answers to promote regeneration in mammals.

In order to make the understanding of the regeneration phenomenon simple, the process has been divided into the following intermediate phases:

(i) acquisition of regeneration competence: where the cells near the site of injury are recruited in that region and become responsive to injury and form regeneration competent mass of cells. These cells either originate from the stem cells or they are formed from dedifferentiated cells. These cells are capable of dividing and forming various cell types,

(ii) the formation of progenitors: where upon perceiving the regulatory cues the cells of the competent tissue trigger the cell proliferation followed by re-specification of the stem cell niche leading to the progenitor formation by dedifferentiation, and

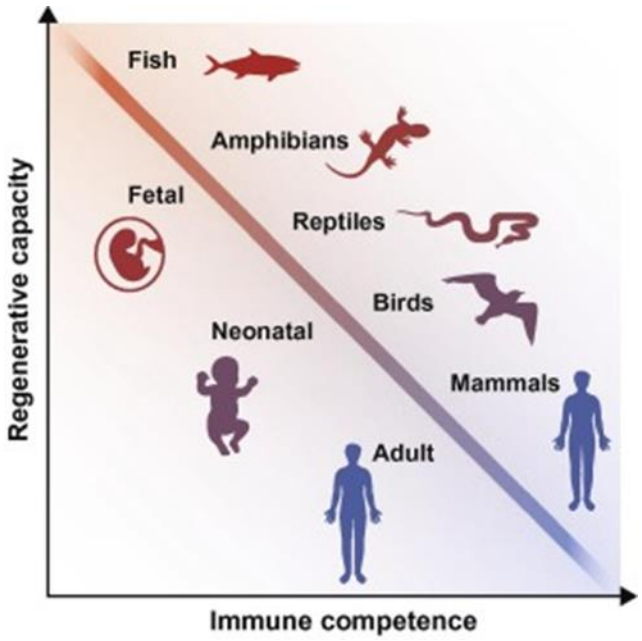

Fig 1.1 Inverse relationship between regenerative ability and immune capacities during evolution and development. Lower vertebrates such as fishes and amphibians are able to completely regenerate many of their tissues. In mammals, regenerative capacities depend on the developmental stage. Immune competence increases during evolution and also with age in mammals and thus, leads to a compromised regenerative ability. Taken from Julier et al., 2017.

(iii) cell fate specification and patterning, leading to organ formation: where re-specification of cell fate and re-patterning of an entire organ takes place. Here, tissue polarities are governed, cells migrate and acquire correct positional identities.
(Landge et al., 2018)

1.1.2. Regeneration: Types

To make the studies of regeneration easy, T.H. Morgan in 1898 classified the process into two types, based on the mechanistic ways in which it can occur. This division was as follows:

(i) **Morphollaxis:** In this process (a term coined in 1898 by T.H. Morgan and improved by him in 1901), regeneration occurs in the absence of an active cell proliferation, where missing or severed body parts are restored solely by the remodelling of pre-existing cells. This mechanism is best shown by Hydra where no blastema is formed on the wounded site, but the whole organismal body is remodelled drastically from the remaining body. The blastema (which is not formed during Morphollaxis) is the white coloured region growing on the wounded area that has a cluster of undifferentiated cells, which can get re-differentiated after responding to the regulatory cues.

(ii) **Epimorphosis:** In this process, active cell proliferation is necessary for the regeneration to occur. It can be further subdivided into two processes, as follows, out of which one may occur in an organism, (I) blastemal and (II) non-blastemal based regeneration.

Non-blastemal regeneration occurs by either of these:

- Transdifferentiation (conversion of one cell-type to another) of the remaining tissue after the injury back to the full missing structure.
- Dedifferentiation, which is followed by proliferation of the surviving cells in the tissue or organ after injury, a process by which a terminally-differentiated cell loses its own identity and becomes less differentiated, thereby, making it ready to enter into proliferation (Jopling et al., 2011).
- Proliferation and redifferentiation of the stem cells already present in the injured or damaged tissue.

Example, Liver and Bone regeneration in humans.

Blastemal regeneration involves the emergence of a specialised structure called Blastema, containing such undifferentiated cells that have the potential to re-differentiate and form various cell types. Blastema comprises of a fine outer layer of epithelial origin, and an underlying cell mass which is mesenchymal in origin, which helps in the restoration of the missing parts upon differentiation.

Example, Limb and tail regeneration in vertebrates, Planaria body regeneration.

(Agata et al., 2007; Sánchez Alvarado, 2000; Gilbert, S.F. (2000). *Developmental Biology*. Sunderland (MA): Sinauer Associates).

1.1.3. Wound Healing and Scarring: A 3-stage process

We have discussed the heterogeneity during the tissue repair mechanisms. This brings us to some organisms which are highly efficient in adopting self-healing by regeneration which elegantly either restores entire body part (as in Hydra and Planaria bodies) or heals the injury by cell proliferation (Limb regeneration in Salamanders, Liver regeneration in Humans). Surprisingly, in a vast range of evolutionarily advanced organisms and advanced vertebrates, this ability is minimal and has been widely taken over by wound healing or fibrosis, leading to scarring. Wound healing has been elaborately studied in Salamanders, Axolotls, Teleost fishes and Lizards. It follows through several overlapping phases mediated through (i) an inflammatory response and (ii) an associated cell migration, proliferation, matrix deposition, and (iii) tissue remodelling (Eming et al., 2014; Vibert et al., 2018), and has been found to have similarities with tumorigenesis and cancer (Kaplani et al., 2018). The inflammatory response is triggered immediately after the injury, by the circulating leukocytes leaked out of the damaged blood vessels near the injury spot into the wound. These leukocytes largely Neutrophils (Kim et al., 2008) help in cleansing and killing the invading microorganisms by generating Reactive oxidative species (ROS), which thereby induce many downstream genes leading to the formation of the fibrin clot and extracellular matrix (ECM), the process of angiogenesis and re-epithelialisation (Theilgard et al., 2004). Following this, the Macrophages come on the site to clear up the cell debris and the used neutrophils (Eming et al., 2007). The proliferation and migration of cells within the wound helps in achieving permanent closure of the wound gap and tissue replenishment (Shaw & Martin, 2009). Finally, the wound resolution or the tissue remodelling occurs, which restores the full function and the regular appearance of

the injured tissue. During this phase, blood vessels also refine and mature in the form of a functional network, haphazardly arranged ECM gets remodelled, giving a regular design to the dermis. Also, the Neutrophils get cleared off from the site by apoptosis and phagocytosis by macrophages (Haslett, 1992; Adams & Alitalo, 2007). Imperfect regulation of wound healing can lead to hyperproliferation, persistent inflammatory reaction leading to fibrosis and hypertrophic scar formation, all contributing to various pathological conditions (Shaw & Martin, 2009; Atala et al., 2011; Eming et al., 2014).

1.1.4. Regeneration: A boon over fibrotic wound healing

Regeneration is a type of scar-free wound healing, while the process of wound closure can be categorised as a non-regenerative type of tissue repair. A complete regenerative wound healing or regeneration process has many advantages over fibrosis or wound healing due to which some species have adopted it as their tissue repair mechanism. The first and the foremost benefit of regeneration is the short and fast initial response in the form of re-epithelialisation, which gets completed within 24hrs as in amphibians. At the same time, it is slow and delayed between 48-72 hrs in the mammals. This majorly happens because of the differential migration rates of keratinocytes, being fast in regeneration. Further beneath this epithelium, mesenchymal cells accumulate, which produce Hyaluronic acid, Tenascin, Fibronectin-rich ECM, all of which facilitate scar-less healing (Murawala et al., 2012). Wound repair involves an excessive production of collagen, which leads to only partial regeneration and the production of new non-functioning tissue (Wynn, 2007; Gurtner et al., 2008) with 80% similarity to the original tissue. On the other hand, the regenerative repair completely restores the morphological and functional originality (Emanuele, A. S., Giada, M., Alessio, F. & Ciprandi, G. (2019). From Tissue Repair to Tissue Regeneration. In K.H. Dogan (Ed.), *Wound Healing: Current Perspectives.* IntechOpen, DOI: 10.5772/intechopen.81291) (Fig 1.2). The regenerative response being a rapid process than scarring confers a survival advantage to the organism since it prevents the infectious microorganisms from attacking the wounded site and thus, inhibits the mechanical deformation of the wounded tissue (Gurtner et al., 2008).

In mammals, injury in heart, lungs, brain, retina and few other organs does not lead to their regenerative repair, only scar forms and makes that part of the tissue or entire organ non-functional (Jaźwińska & Sallin, 2016). Similarly, the mammalian Central Nervous System

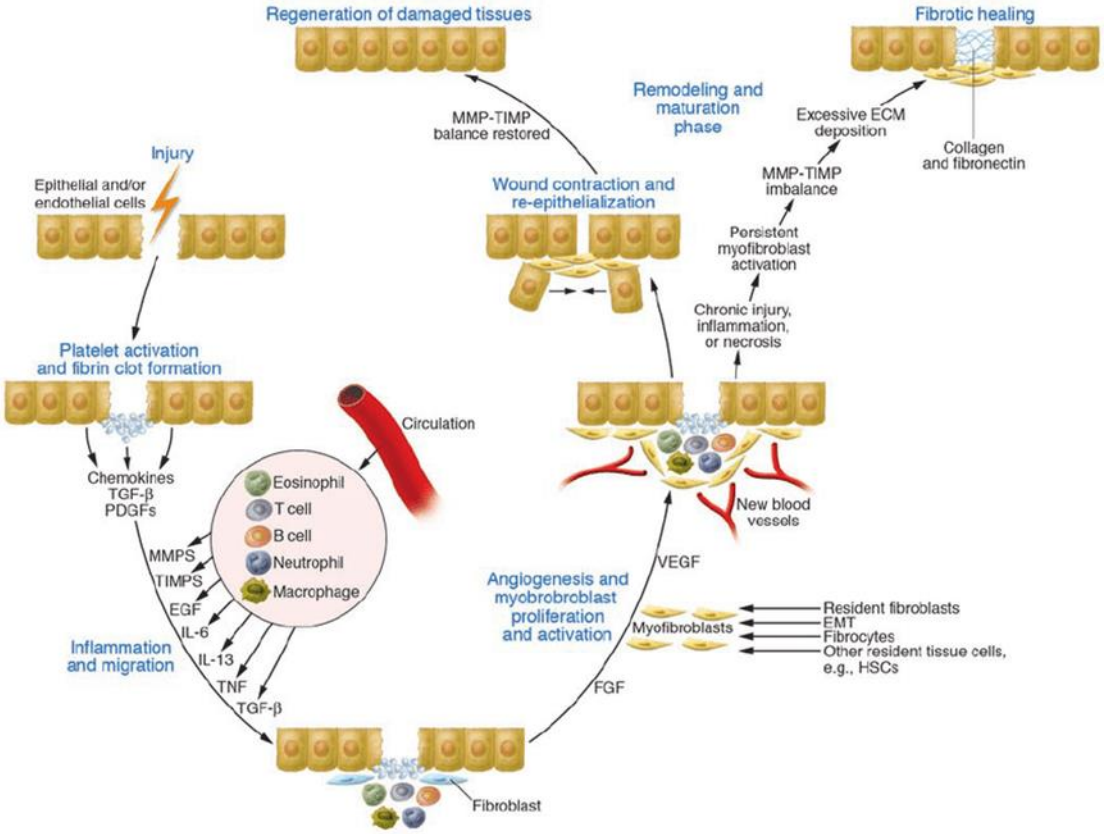

Fig 1.2 Regeneration & Fibrotic wound healing. Healing of injury by a process of either regeneration or by fibrotic wound healing (scarring) which is non-regenerative tissue repair. Taken from Wynn, 2007.

(CNS) also does not regenerate due to fibrosis, leading to organ failure or paralysis, while the Peripheral Nervous System (PNS), bones, skeletal muscles, urinary bladder, blood vessels possess ability to regenerate (Carlson, 2005). Thus, the regenerative response being a boon over scarring confers the structural and physiological integrity to the tissues or the organs.

1.1.5. Regeneration in the Peripheral Nervous system (PNS) and the Central Nervous system (CNS): An insight

The Nervous system is a highly complex part of the animal body specialised to coordinate all the actions of the body and to transfer information in the form of signals from one part of the body to the other. The existence of first nervous system dates back to the Pre-Cambrian ages (around 542 million years ago), in Ediacaran worm-like animals *Trichoplax*, which got extinct but left us some clues to investigate the origin of the nervous system (Jorgensen, 2014; Budd, 2015). The nervous system comprises of a well-connected framework of nervous tissue consisting of two main parts Central Nervous system (CNS) made up of Brain and Spinal Cord, and Peripheral Nervous system (PNS) made up of branched and interconnected cranial and spinal peripheral nerves, connecting all the body parts with the CNS. The nervous tissue is formed by the fundamental units called neurons. A Neuron is a cell having Dendrites, Cyton (cell body), Nucleus, Axon, Synaptic Terminal. The information or messages are transmitted from or to the various organs by these neurons in the form of electrochemical waves. These waves may either travel through the direct electrical connections between neurons or may migrate across the axons which cause the release of chemicals called neurotransmitters near the synaptic junctions. Nerves that carry the signals from the brain to the body are called efferent or motor nerves, while the one carrying information from the body to the CNS are termed as afferent or sensory nerves. There are some non-neuronal cells in the PNS with the supportive function called neuroglia or glial cells, which are of three types astrocytes, oligodendrocytes and microglia (Purves, D. et al. (2004). *Neuroscience.* Sunderland (MA): Sinauer Associates).

The intrinsic growth capacity of the CNS neurons is limited as compared to the PNS neurons due to some unidentified stop signals (Zhou & Snider, 2006). Following the brain injury or damage also, the neurons of the CNS fail to regenerate, while that of the PNS take charge. The microglia are the cells which respond by increasing their number and play the role like that of

macrophages in the immune system (Purves, D. et al. (2004). *Neuroscience.* Sunderland (MA): Sinauer Associates). In advanced mammals and humans, the traumatic brain injuries, spinal cord injuries, brain stroke and other CNS related conditions are devastating as these lead to loss of function due to axon connection disruption, which is surpassed in case of peripheral nerve injury by the long-distance axon regeneration, thus helping in conferring the neuronal functions **(**Huebner & Strittmatter, 2009). The studies by Ramón y Cajal, way back since 1928, also suggested that immature mammalian CNS might be able to regenerate, while adult CNS fails to do so. Cajal proposed the root causes behind this mystery to be the differences in the proliferative ability and the irreversibility of the intraprotoplasmic differentiation of the CNS neurons, which indicates that mature CNS neurons fail to attain the stem cell fate and to form the functional neuronal entities again (Nicholls et al., 1999; Cook et al., 2006). There are pieces of evidence which tell that such differences arise due to the expression of specific neurite-growth inhibitors in or near the injury area named as NI35/250 based on their molecular weight (Schnell & Shwab, 1993), myelin-associated glycoproteins, myelin-related inhibitors Nogo-A, semaphorins, chondroitin sulphate (Horner & Gage, 2000; Liu et al., 2006). All the mentioned factors lead to the formation of glial scar in the CNS neurons, across which axons cannot grow. The cell-autonomous factors also create a permissive environment for PNS neurons to grow or regenerate. The PNS neurons express and upregulate the growth or RAGs (c-Jun, ATF3, SPRR1A, sox11, GAP-43), which is not done to the same extent by CNS neurons. So, the ability to regenerate is lower in CNS neurons, even if the inhibitors are absent (Huebner & Strittmatter, 2009). Strategically, if it is intended to increase the expression of RAGs in CNS neurons or if the effect of inhibitors is diminished by subjecting them to the neutralising antibodies made against them, the regeneration potential of CNS neurons can be increased, and some degree of recovery from brain and spinal cord injury may be achieved. But these are not the only complications faced by the regeneration biologists, in the case of CNS neuron regeneration. Even if the neurons get regenerated, the formation of correct circuitry is indispensable to resume the normal physiology and to prevent various pathologies. Once connections are made, later targeted activation of the newly connected neurons will be needed by a rehabilitative training to ensure normal behavioural recovery (Cook et al., 2006). Thus, regeneration in the CNS neurons still poses a challenge to humanity, but scientific ventures are being made with full zeal to develop some therapeutic strategies to tackle with such CNS-related pathological conditions.

1.2. Retina Regeneration: An Overview

Being well-versed with the fact that there are many deficits in the regeneration in the CNS, we still know that there exist a few fields which do show either complete or partial regeneration like the neurons of the brain after lesion, Spinal Cord axon regeneration after transection or lesion and Retina upon photoreceptor or mechanical injury. Visual loss in organisms is one of the major losses since vision or eyes help to see the objects, the world and to maintain the balance of the body while moving. In humans, vision loss is marked due to the loss of retinal neurons owing to some traumatic injury, age-related weakness and retinal degenerative diseases like glaucoma, retinitis pigmentosa, diabetic retinopathy, macular degeneration, affecting millions of people worldwide. The clinicians and researchers across the world are trying to combat such physiological conditions by gene therapy, delivery of some anti-apoptotic or neurotrophic factors, invasive methods like retinal implants, transplantation of photoreceptors and retinal pigmented epithelium. However, these are not yet translated into wide-spectrum therapeutic intervention methods to cure blindness (Martin & Poché, 2019). The hopes remain on the alternative field of tissue regeneration, which is well established in the primitive vertebrates and then extrapolating the information to create similar permissive environments in the advanced vertebrates and humans. The aim is to awaken the dormant regenerative potential in the mammalian and human retina to invoke its self-repair paradigm. The retina, which is known to have specific neurogenic progenitor cells, can undergo self-healing by regeneration in primitive vertebrates, and thus the studies are being directed to use regenerative medicine in treating the retinal diseases.

1.2.1. Projector Screen of the Eye: Retina

The retina is the inner-most thin layer of light-sensitive tissue at the back of the eye. This part of the eye is the most crucial part on which image of the objects gets formed. This is done by the retina by employing the optics of the eye to capture signals, creating an image on itself and generating the electrical impulses to send the signal through the optic nerve to the brain.

(i) Retinal Anatomy: The retina has a sophisticated structure with a complex neural circuitry, comprising of multiple types of neurons all interconnected and arranged in a highly coordinated manner in various layers. The basic plan of the retina is conserved

across the vertebrate species. There are five types of neurons in the retina, having their cell bodies in the inner nuclear layer (INL), outer nuclear layer (ONL) and ganglion cell layer (GCL) while their processes and synaptic connections extended in inner plexiform layer (IPL) and outer plexiform layer (OPL). All these layers are stacked alternating each other. The five neurons are as follows:

- Photoreceptor cells: They comprise of two light-sensitive cells, Rod cells and Cone cells, where rods perceive light and dark signals and help in dim light vision and cones are responsible for the coloured vision. Rods can detect even a single photon, while cones are 100 times less sensitive than rods, but show faster signal transduction. These cells have an outer segment which lies covered with Retinal Pigmented Epithelium and an inner segment having contact with the Bipolar cells and Horizontal cells.
- Bipolar cells: These are the cells which transmit the signals from the photoreceptor cells to the retinal ganglion cells. Their cell bodies reside in INL, while the processes extend from Outer to Inner Plexiform Layers.
- Retinal ganglion cells: These are the inner-most cells in the retina, which perceive the signals from amacrine cells and bipolar cells and transmit them to the brain through the optic nerve. The long axons of the ganglion cells form the optic nerve, cell bodies are found in the GCL, and the cellular processes present opposite to the long axons are present in the IPL.
- Horizontal cells: These cells allow the lateral interactions between photoreceptors and bipolar cells, with the cell bodies in the INL and processes extending in the OPL. They maintain the sensitivity of the visual system to the luminance contrast over a broad range of light intensities.
- Amacrine cells: These cells form the connections and transmit the signals from the bipolar cells to the retinal ganglion cells. They have their cell bodies lying in the INL and processes extending in the IPL.

Retinal Pigmented Epithelium (RPE) is the outermost layer of the retina composed of the epithelial cells lying beneath the photoreceptor cells, creating a barrier to the blood vessels which are present in the choroid (a protective layer on the eye). RPE has the biochemical machinery to regenerate the pigment of the retina after each exposure to the light. Since the retina is a metabolically active tissue, its nourishment is taken care of by the blood supply to it managed by the capillaries present in the choroid underlying the RPE.

Additional to these layers, Retinal Nerve Fibre layer (RNFL) is present towards the inner surface of GCL, which collects and carries the axons of the Retinal ganglion cells, forming the optic nerve.

Apart from these neuronal cells, an endogenous population of stem cells is present in the retina comprising of Müller Glia (MG) cells and Ciliary Marginal Zone (CMZ) cells. MG cells reside in the INL, with their projections lying in an Inner Limiting layer. But these cells have been found in the fish and the amphibians by injuring the retina. These cells have the potential to lose their destined fate and attain stem-ness upon being triggered by some stress or insult to the tissue, followed by proliferation and re-differentiation. Thus, these cells play an essential role during retina regeneration (Fig 1.3).

(ii) Retinal Function: The retina is engaged in the role of receiving the information from the visual cues like light, dark and colours, and then transferring the signals to the brain by the optic nerve, which creates an image of the object on the retina, which acts as a projector screen. The light rays fall on the eye and get focused by the eye lens and reach the photoreceptors in the retina, where the signals get transduced in which a biochemical cascade is triggered that regulates the opening and closing of the ion channels in the membrane of the photoreceptors which release the neurotransmitters, received by the bipolar cells which then get depolarised. These action potentials mediated by Amacrine cells get transmitted to the Retinal Ganglion cells, whose axons constituting the optic nerve carry the signals to the visual centres of the brain. In the visual cortex of the brain, the signals are processed efficiently that the emphasis is laid on those aspects of the object which convey the maximum and the most explicit information.

(Purves, D. et al. (2004). *Neuroscience.* Sunderland (MA): Sinauer Associates; Tzekov et al., 2011; Hoon, Okawa, Santina & Wong, 2014; Jeon & Oh, 2015; Holmes, 2018)

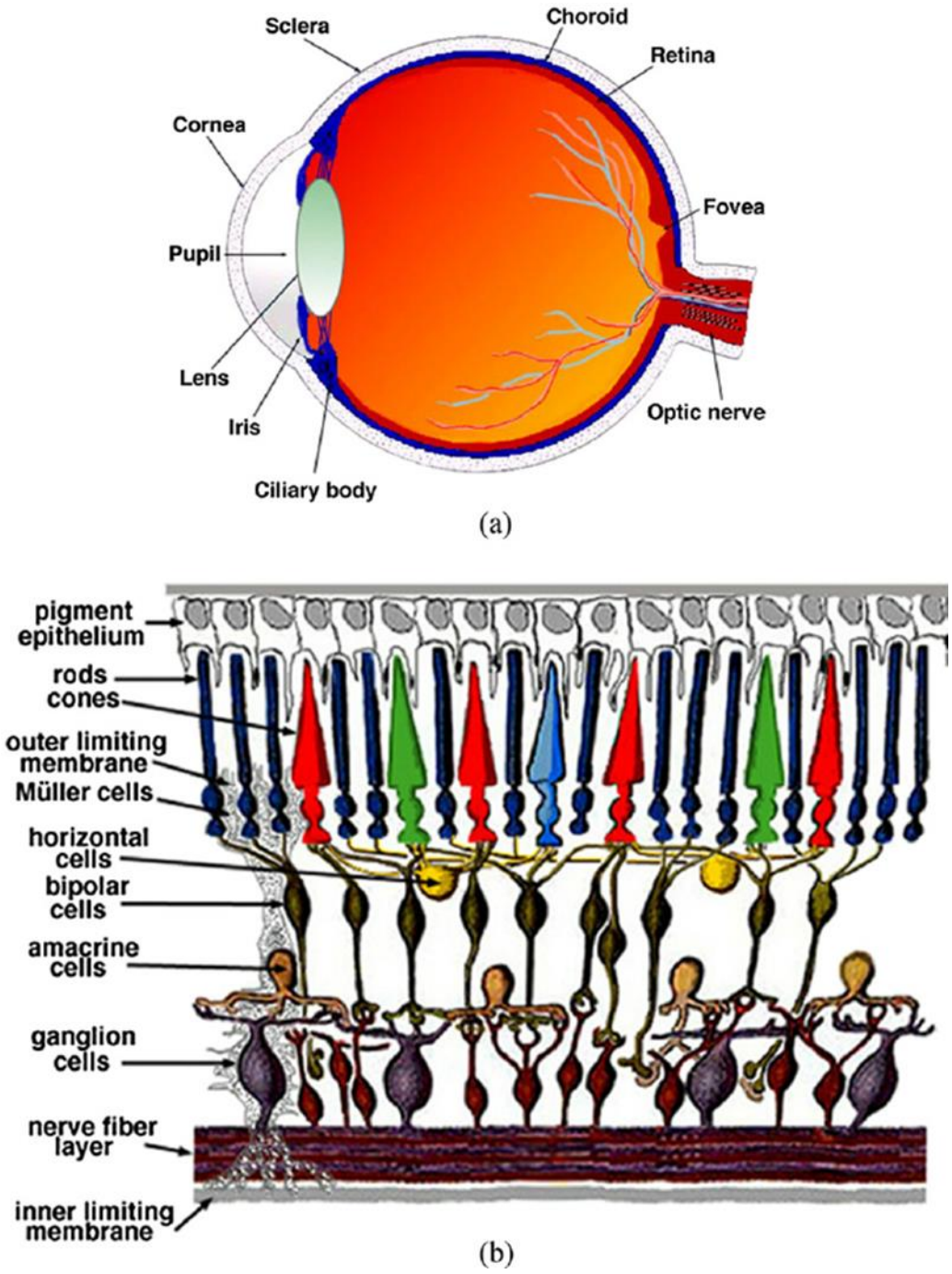

Fig 1.3 Human eye with retinal anatomy. (a) Human eye structure. (b) Human retinal anatomy. Taken from Tzekov et al., 2011.

1.2.2. Retina Regeneration

Retinal architecture, apart from various neuronal cells comprises of a population of stem cell-like cells: Müller Glia (MG) cells and Ciliary Marginal Zone (CMZ) cells. In our work we are more inclined towards the MG cells and their contribution to the maintenance of retinal homeostasis post-trauma.

Müller Glia cells have been named after their discoverer, the German anatomist Heinrich Müller. These cells are the only glial cells which have been derived from the neural retinal progenitors and play diverse roles during the developing and mature retina in normal as well as the traumatic state. MG cells have a radial morphology with their cell bodies in the INL and their projections lying in the inner limiting membrane. These cells are the last ones to be formed during retinogenesis and constitute 4-5% of the retinal cells. They help in the supporting and protecting functions in the retina by making the scaffold for the organisation of the retinal layers; by creation and maintenance of the blood-retinal barrier; by serving as the source of nourishment and energy in the form of glucose and lactate respectively for the active metabolism occurring in the retina. They also help in the maintenance of the homeostasis in the retina and remove the toxins from it (Adams, T. L. B., Chernoff, E. C., Wilson, J. M. & Dharmarajan, S. (2013). Reactive Muller Glia as Potential Retinal Progenitors. In L. Bonfanti (Ed.), *Neural Stem Cells- New Perspectives*. IntechOpen, DOI: 10.5772/55150).

These cells upon activation due to retinal injury, have the potential of getting dedifferentiated and forming the cells with neural progenitor or stem cell-like properties called Müller Glia Progenitor Cells (MGPCs). Culturing MG cells on the enriched medium as well as transplanting these into injured retina has shown the formation of multipotent microspheres and the cells with neuronal properties respectively, shedding light onto their stem cell-like features (Das et al., 2006). MG cells act fast and follow a particular regime upon activation. They show reactive gliosis followed by dedifferentiation, proliferation (asymmetric division), migration apically or basally based on the cell-type they have to form in the various retinal layers and finally re-differentiate and give rise to amacrine cells, retinal ganglions, photoreceptors, and MG cells also. These cells are a source of cytokines and other growth factors that help in regulating their regenerative processes in an autocrine/paracrine fashion (Wan et al., 2014). These properties allow them to be the leaders during the retina regeneration, curbing the retinal glial scar and hypertrophy. (Adams, T. L. B., Chernoff, E. C., Wilson, J. M.

& Dharmarajan, S. (2013). Reactive Muller Glia as Potential Retinal Progenitors. In L. Bonfanti (Ed.), *Neural Stem Cells- New Perspectives*. IntechOpen, DOI: 10.5772/55150; Hamon, Roger, Yang & Perron, 2016).

1.2.3. Retina Regeneration across the Vertebrates

As we move across the chordates, from primitive vertebrates to the advanced ones, there is a regression in the regenerative ability of all the organs, including the retina. There has been ongoing research to find out the reasons behind this and to tweak it to be used in the field of regenerative medicine, many of which we have discussed previously. This has been done by using few organisms from different classes like fishes, amphibians, birds, mammals of this sub-phylum, which have an immense potential to regenerate and are easy to maintain and propagate in the research laboratory. These tools are termed as model organisms. The teleost fish like goldfish, zebrafish are excellent models to study regeneration, amongst amphibians salamander, axolotl, frog having fascinating ability to regenerate are being used. In birds, since a long time developmental aspects of chicks have been elucidated, now regeneration is also being studied in them. Mammals such as mice systems and also human Müller cells in culture (Lawrence et al., 2007) are chosen as model organisms but have very limited regenerative potential owing to their complexity and epigenetic factors discussed previously.

We will discuss these classes and the respective model organisms in detail:

(i) **Fishes:** Teleost fishes have been known to possess a remarkable regenerative capacity. First such evidence was found in adult Goldfish by surgical ablation of a region of the retina, which regenerated the lost neurons. Further studies in goldfish and zebrafish showed the presence of stem cell-like cells in the germinal zones in the margins and in the inner nuclear layer as CMZ cells and MG cells, with MG cells having a neuronal origin. In normal physiological conditions, MG cells provide rod cells to the growing retina, while during regeneration these multipotent MG cells get reprogrammed and form all the retinal cells which are lost due to any cytotoxic, mechanical or light-induced injury in the retina (Lenkowski & Raymond, 2014). These MG cells change their epigenetic modifications and their gene expression patterns upon injury, making the environment conducive for MG reprogramming to convert them to MGPCs (Goldman,

2014). So far, roles of the genes like *ascl1a* (Fausett et al., 2008), *shha/b, zic2b* (Kaur et al., 2018), *myca/b* (Mitra et al., 2019), Tgf-β signalling factors (Lenkowski et al., 2013; Sharma et al., 2020), *stat3, lin28a/b* (Ramachandran et al., 2010), Notch signalling factors (Wan et al., 2012) and many more have been elucidated. Many cytokines like interleukin-6 family cytokines and growth factors like FGF (Wan et al., 2014), HB-EGF (Wan et al., 2012) also have been the key molecules initiating the regenerative response. A strong regulation of all these factors is conducted by epigenetic modifiers like Hdacs (Mitra et al., 2018), Apobecs, Dnmts (Powell et al., 2013). Even though much is known about fish retina regeneration, still many factors and aspects remain unexplored. Hence, it serves as a beautiful model organism to delve deeper into the studies of retina regeneration.

(ii) **Amphibians:** Urodelic amphibians like newts or salamanders and axolotls have fascinating regenerative ability for all their organs, including the retina. In contrast, anurans were considered to possess regenerative potential limited to their larval stages, which is lost post-metamorphosis. But recent studies prove that Xenopus retina can regenerate in post-larval stages, even after retinectomy. In the amphibian retina, the regeneration has been reported to be conducted by the cells in Retinal Pigmented Epithelium and also the ones in the ciliary marginal zones. However, their MG cells were thought to be quiescent under all the conditions of development as well as any injury repair. Eventually, there were reports that made the presence of proliferating cells evident not only RPE and ciliary margin but also in MG cells. Thus, it is stated that these MG cells also can get activated and enter into cell-cycle for injury repair, but the efficiency depends upon the extent of the damage to the retina and the age of the amphibian organism. In Xenopus tadpoles, mechanical injury to the retina and nitroreductase-mediated photoreceptor cell ablation have shown that MG cells in Xenopus retina can re-enter cell-cycle (Langhe et al., 2017). But the fact that this is ensured in adult Xenopus or in the urodeles, remains elusive. These models serve as a great tool to elucidate the mysteries of the molecular basis of retina regeneration in the amphibians as well as the vertebrates (Hamon et al., 2016).

(iii) **Birds:** In birds, the regenerative potential is compromised as compared to the zebrafish. The post-hatched chicks demonstrate the ability of MG cells to reprogram and proliferate upon neurotoxic injury and enter into hypertrophic condition. During this course, few

cells are able to get dedifferentiated, attain progenitor-like state and can express genes associated with retinal progenitors. These cells form MG cells and new retinal neurons like amacrine cells, bipolar cells, ganglion cells depending upon the type of injury done and the types of neurons destroyed in the retina. While there are no reports on the restoration of the photoreceptors, several vital molecules have been found to play significant roles during chick retina regeneration. Hedgehog signalling (Todd & Fischer, 2015), FGF/IGF/MAPK pathways (Fischer et al., 2009b; Fischer et al., 2002), Notch signalling and related factors Hes1 and Hes5 (Hayes et al., 2007), and others genes like Ascl1a, Pax6, Chx10, Six3 (Gallina et al., 2014) have been established to contribute during avian retina regeneration. The inflammatory signals and the complement system also influence the MG reactivation (Hamon et al., 2016; Wilken & Reh, 2016). This makes the post-natal chick a good model to study retina regeneration in birds.

(iv) **Mammals:** Mammalian retina regeneration is highly compromised due to many underlying molecular and epigenetic constraints and evolutionary advancements. The highly strengthened immune system also keeps the regenerative repair mechanism under check. Thus, any retinal injury or diseases in mammals and humans lead to vision loss. The mice are the mammalian model organism best suited to explore these aspects. The recent *in vitro* studies exhibit that some of the mammalian retinal cells do proliferate and may have neurogenic potential like the cells in the CMZ, RPE and the MG cells also. However, the proliferative efficiency of MG cells is too low, due to their quiescent state owing to the limited pro-mitogenic factors or other inhibitory cues. Upon giving the NMDA-based neurotoxic retinal injury coupled with specific growth factors, mammalian retina gave rise to a small number of bipolar cells, amacrine cells and photoreceptors also (Ooto et al., 2004; Karl et al., 2008). Since the *in vivo* mammalian retinal studies are challenging to conduct, due to the inefficient regenerative program in these systems due to the non-permissive environment in the retina (Fig 1.4), *ex vivo* models have been used in the form of retinal explants. The reports suggest clearly that in the retinal explants, the quiescent MG cells undergo reprogramming like in fish and chick where MG cells dedifferentiate, proliferate and form various retinal cell-types. But this was facilitated by the modulation of certain growth factors and upregulation of RAGs, like *Ascl1* which is not influenced in NMDA-induced injured mouse retina, but upregulates in explants (Löffler et al., 2015) and WNT, EGF, Notch, Hedgehog, BMP (Bone Morphogenetic Proteins), MEK, AKT signalling pathways (Hamon et al., 2016).

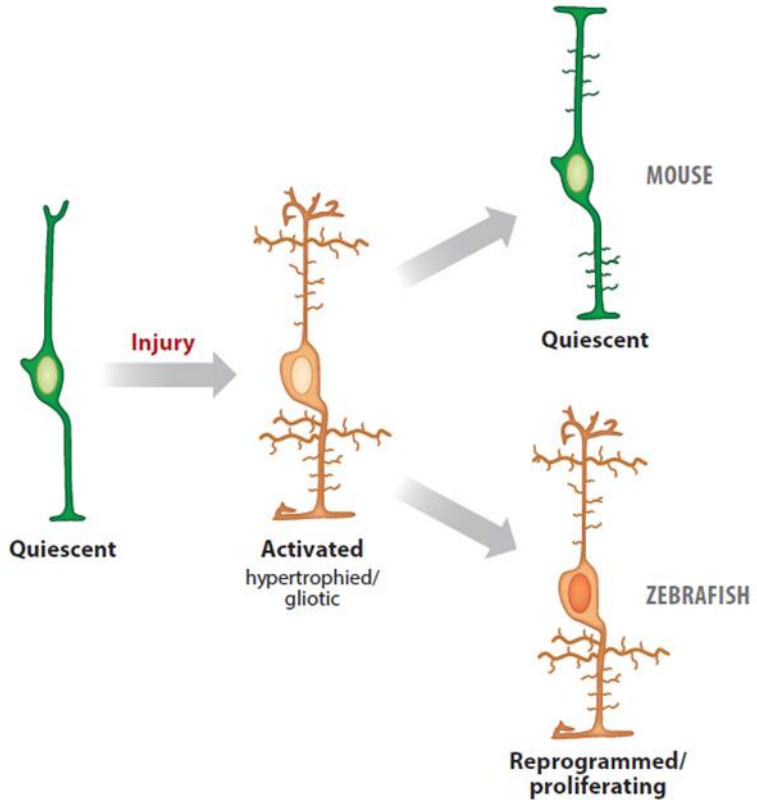

Fig 1.4 Comparison between the regenerative response in zebrafish and mice. Schematic of a quiescent Müller glia that gets activated upon retinal injury. In mice, the activated or gliotic Müller glia returns toward quiescence based on gene expression and morphology, in contrast in zebrafish, the activated Müller glia reprograms and proliferates. Taken from Lahne et al., 2020.

However, the intrinsic and extrinsic cues for regeneration paradigm in mammals still remain highly underexplored and difficult to be unravelled.

1.3. Zebrafish Retina Regeneration

As we go across the evolutionary timeline, we witness the increasing organismal complexity besides the reduction in the ability to regenerate the lost or injured organs. Considering this fact, humans possess a restricted regenerative potential, with skin, endometrium and liver regenerating the maximum. There have been many therapeutic interventions in this field over the years, and researchers seem to be intrigued by the phenomenon of regeneration. Stem cell-mediated therapies have been a boon but are not that extensively used. Another approach is based on exploring and studying various regeneration-based model organisms, and thus tweaking and extrapolating the information obtained from them to the humans. Zebrafish, over mice and Xenopus, has been an established model organism to study regeneration, because of its immense potential to regenerate almost all its organs post-injury or amputation, like heart, brain, fin, liver, pancreas, kidney, spinal cord, hair cells (lateral line).

Zebrafish, a tropical teleost fish from the family Cyprinidae, was introduced as a lab model system by George Streisinger (Meyers, 2018). It offers numerous benefits over other model organisms namely, its huge progeny size, fast *ex utero* growth and development of transparent embryos which allow easier screening, genetic manipulations and imaging, shorter generation time, a relatively smaller genome and its robust regenerative ability (Shi et al., 2015; Gemberling et al., 2013). Around 80% of mammalian disease-causing genes have their counterparts in zebrafish. The unique ability to regenerate all its lost body parts makes zebrafish an excellent model to study the ever-enigmatic phenomenon of regeneration. The ongoing research in the field of CNS regeneration in zebrafish brings us to investigate the paradigm of retinal injuries, retina regeneration and the molecular basis behind the same. The zebrafish eye size increases along with its body size, making it larger relative to the overall body. This gives the researchers more tissue size and feasible area to be genetically manipulated or handled. Its retina has many architectural similarities to that of the human retina, including its multi-layer manifestation (Chhetri et al., 2014) (Fig 1.5). Zebrafish has colour vision due to cone density closer to that of humans. Various genetic tools and development of sophisticated strategies like

a short oligonucleotide Morpholino-based gene knockdown, transcription activator-like effector nucleases (TALENs) or the clustered regularly interspaced short palindromic repeats (CRISPR) has led to the successful significant advances towards mutagenesis thereby making zebrafish a successful retinal disease model (Richardson et al., 2017).

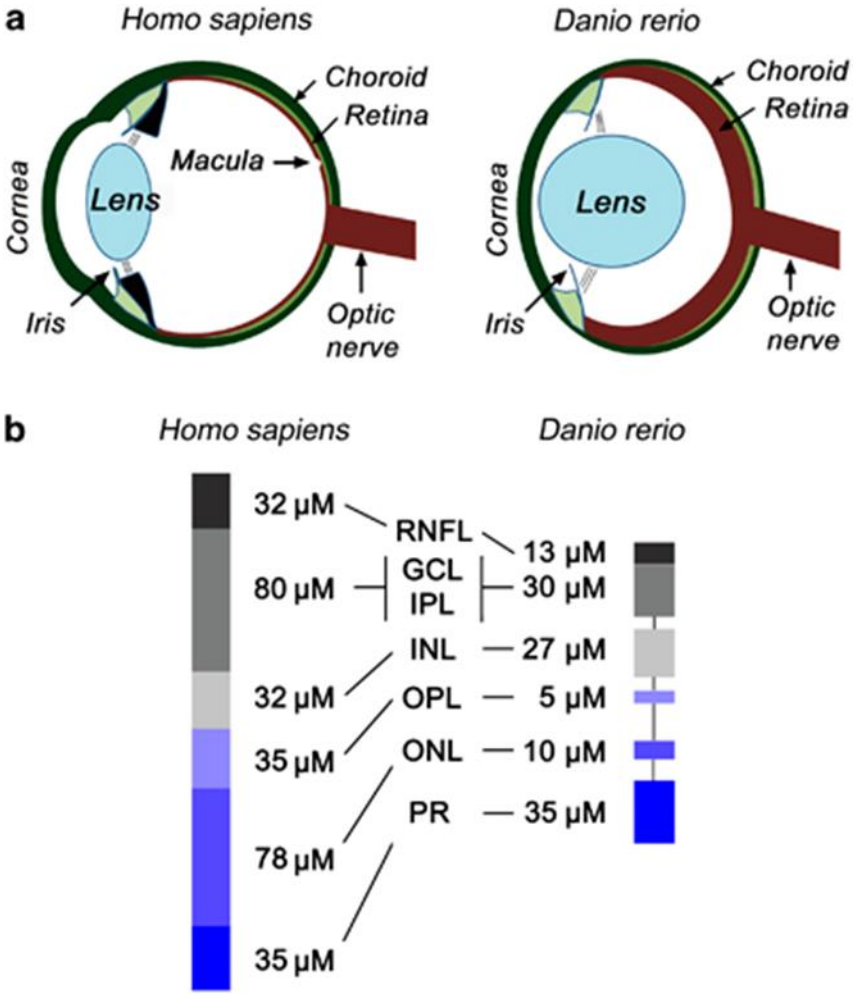

Fig 1.5 Comparison between Human eye and zebrafish eye. (a) Human and zebrafish eye structures. (b) Human and zebrafish retinal anatomy with the respective thickness of layers. Taken from Chhetri et al., 2014.

1.3.1. Müller Glia Reprogramming during Retina Regeneration

Upon perception of any chemical or light-mediated retinal stress or of injury by needle stab, the dying retinal cells signal the information to MG cells which respond first to it and initiate a gliotic response including hypertrophy and increased expression of cytoskeletal proteins like Glial Fibrillary Acidic Protein (Gfap). This is followed by a transient phase in which MG cells get reprogrammed and lose their determined state, thereby attaining a retinal stem cell-like fate. These dedifferentiated MG cells act as multipotent neuronal progenitors (MGPCs) and get activated to enter into the cell cycle. There occurs an interkinetic nuclear migration of MGPCs from INL to ONL for asymmetric division and their migration back to INL. In ONL, MGPCs are able to proliferate to maintain their own numbers as well produce a small population of cells which can further get differentiated into various functional retinal cell-types which finally exit the cell cycle. This leads to facilitated wound closure as well as visual function restoration. The reason for this migration is speculated to be a differential environment created in ONL by various cell proliferation and differentiation factors (Goldman, 2014; Wan & Goldman, 2016; Lahne et al., 2020) (Fig 1.6). The fact that MG cells are the source of the MGPCs which further form various retinal cell-types has been well elucidated by adopting the transgenic zebrafish line approach and BrdU-cell lineage tracing experiments (Fausett & Goldman, 2006).

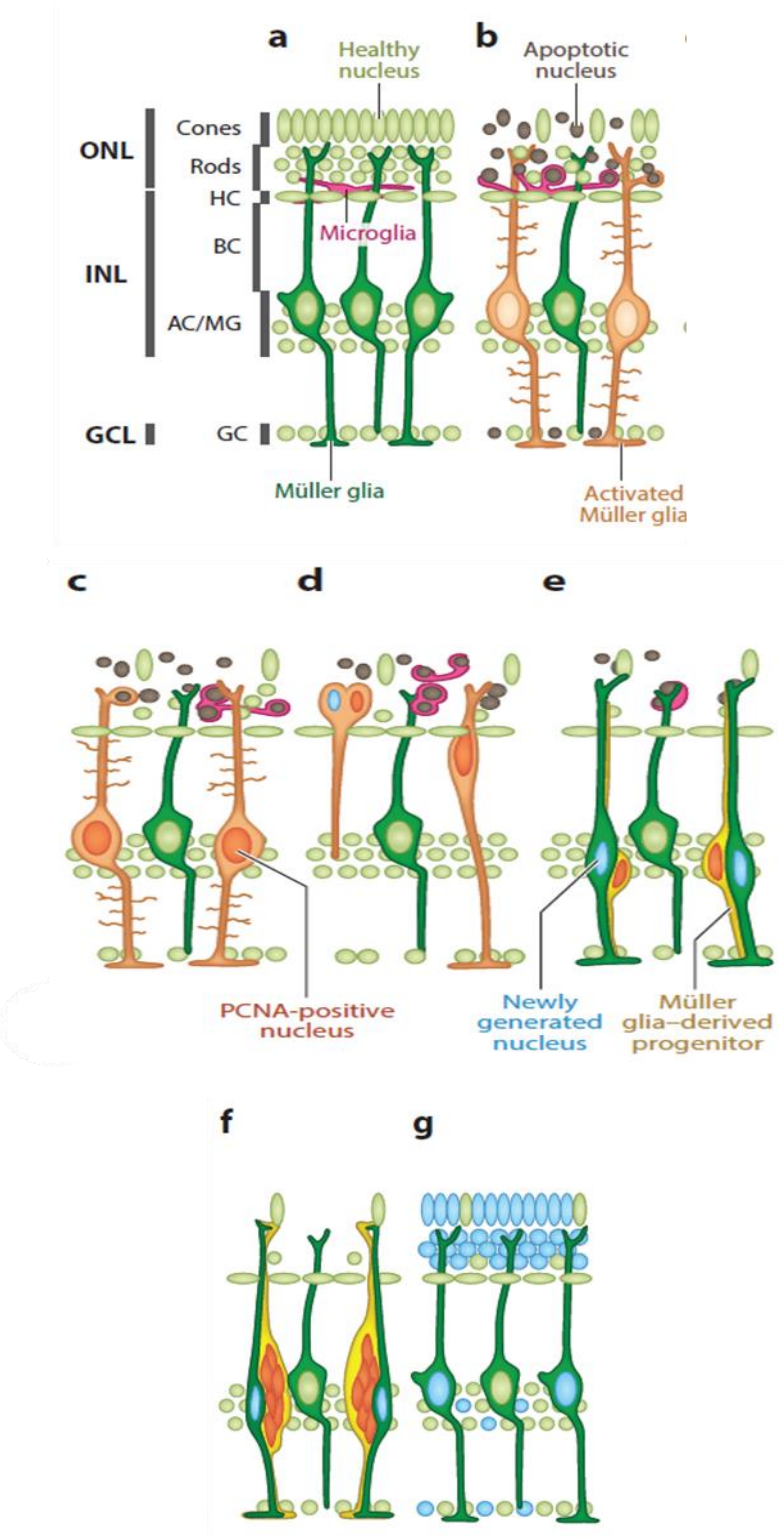

Fig 1.6 Events during Zebrafish Retina regeneration. (a) Uninjured retina. (b) Müller Glia (MG) reprogramming upon retinal injury. (c) Cell cycle re-entry of MGs, along with the PCNA expression. (d) Interkinetic nuclear migration of activated MGs to ONL. (e) MGPCs proliferation and migration back to INL. (f) MGPCs proliferation. (g) Re-differentiation and structural and functional restoration. Taken from Lahne et al., 2020.

1.3.2. Molecular cues behind MG Reprogramming and Retina Regeneration

The MG cells near the site of injury perceive the signals and respond first to the increased levels of cytokines (IL-6), cell-adhesion molecules like Cdh2 and matrix metalloproteinases like *mmp9*, *mmp13* and others, all of which help in the remodelling of retinal architecture and migration of the neuronal progenitors formed during reprogramming. This is followed by the upsurge of growth factors, transcription factors and other cytokines like Hbegf, Tnfα, Pax6b, Ascl1a, Myca/b, Leptin, IL-11 and many cell signalling pathways like Mapk-Erk, Egfr, Jak-Stat3, Wnt-Gsk3β-β-Catenin, Insulin signalling. All these processes acting in an autocrine or paracrine manner inside the MG lead to their reprogramming and consecutive proliferation. There is a cascade of these molecules being activated and then activating the other downstream molecules. Tnfα, upon getting activated leads to the induction of transcription factors Ascl1a and Stat3, both of which majorly contribute to the MGPCs production, which peaks during 4dpi phase. Further, Ascl1a is known to stimulate Lin28 expression, which is an RNA-binding protein and is highly associated with stem cell self-renewal and reprogramming of IPSCs. Lin28 suppresses *let-7*, a micro-RNA known to repress the expression of RAGs and associated with MG differentiation and their quiescence from the cell cycle. Surprisingly, it is reported that along with the induction or upregulation of the above-mentioned reprogramming and cell proliferation factors, there is an induction of anti-proliferative Notch signalling pathway and its component genes like *her4.1*, *delta A*, *delta B*, *delta C*, *notch1*, *notch3* which keeps a check on the rate and the span of MGPCs proliferation (Campbell et al., 2020). Thus, a balance is maintained between the numbers of MGPCs which divide and which exit the cell cycle. Once sufficient regenerative response for proliferation is mounted, MGPCs finally quit dividing and start differentiating. During this phase, the set of RAGs and other growth factors shut and switch to differentiation-inducing factors like *insm1a*, which downregulates the expression of cell-cycle associated genes. MicroRNAs like *let-7* which inhibits the expression of *lin28*, *ascl1a*, *oct4*, *c-myc*, *pax6b* and, *miR-203* which suppresses *pax6b* expression contribute to MGPCs differentiation (Ramachandran et al., 2010; Goldman, 2014; Zhao et al., 2014; Wan & Goldman, 2016).

Apart from the transcription factors and various other signalling molecules, epigenetic modifiers like Apobec2a/2b, Dnmts, Hdacs and others also regulate the regenerative response at DNA and chromatin level in order to control the different degrees of gene expression and to

regulate switching ON and OFF of the crucial RAGs. The cyclic changes occur in the methylation levels of various RAGs. During reprogramming, the methylation levels of these genes go down, which regains when the MGPCs re-differentiate or exit the cell cycle. DNA demethylation of these genes facilitates MG reprogramming and regenerative response while their methylation favours migration and differentiation. These epigenetic modifiers not only leave their marks on RAGs but also help in arresting the negative players of regeneration. If we explain this in simple terms, these marks also help in suppressing the suppressors of regeneration (Powell et al., 2013; Goldman, 2014; Mitra et al., 2018).

1.4. PTEN/PI3K/AKT/mTOR Signalling Pathway and its Role during Tissue Regeneration

Since the advent of research in the field of tissue regeneration, scientists have been focused mainly on elucidating the roles of various cell-adhesion molecules, growth factors, transcription factors, pluripotency-inducing factors, chromatin/epigenetic modifiers and many cell proliferation-associated signalling pathways also, and have been successful in deciphering their roles. But the roles of phosphatases have been undermined in this aspect, leaving them still underexplored. Phosphatases, unlike the kinases, have a broad range of substrates, making one a bit less inquisitive towards these molecules. Still, the life forms have been largely dependent on these enzymes, for various crucial steps. These enzymes maintain the homeostasis in various biological functions by reversing the molecular actions played by the kinases.

During the post-translational modifications, protein phosphorylation occurs to activate/deactivate them, which is mediated by protein kinases. This phosphorylation involves the attachment or transfer of phosphate (PO_4^{3-}) group to the R-group of Serine, Threonine or Tyrosine amino acids in the proteins (Ardito et al., 2017). Similar to them, lipid kinases also exist, which phosphorylate the lipids present either on the plasma membrane or on the organellar membranes and lead to their activation or deactivation or change in their localisation. These have two sub-types, namely Phosphatidylinositol Kinases, including PI3K (Sun et al., 2013), and Sphingosine Kinases which take part in Sphingosine metabolism (Cao et al., 2018). The reversible phosphorylation reaction performed by kinases is reverted back by

phosphatases, where the phosphate group is removed by phosphatases or the dephosphorylating enzymes. Like kinases, phosphatases also have proteins as well as lipids as their substrates. Unlike phosphorylation, the dephosphorylation kinetics is very rapid; thus, it becomes indispensable to use phosphatase inhibitors while working on any systems where phosphorylation is to be studied (Ardito et al., 2017). Protein phosphatases are grouped into three families, namely phosphoprotein phosphatases (PPP), metallo-dependent protein phosphatases (PPM) and protein-tyrosine phosphatases (PTP) (Ardito et al., 2017). Phosphatases using lipids as their substrates are classified into different groups based on the site on inositol ring from which the phosphate group is removed like Phosphoinositide 3-phosphatases which removed phosphate from D3 of the inositol ring, and many others (Sasaki et al., 2009).

The mechanisms of phosphorylation and dephosphorylation play vital roles in most cellular processes such as cell proliferation, cell migration, differentiation, cell growth, transcription, translation, signal transduction, cellular and vesicular trafficking, phagocytosis, immunological reactions, organismal development, ageing, DNA repair, cancer, apoptosis due to activation and deactivation of a cascade of receptors and other enzymes mediated by specific kinases and phosphatases (Sasaki et al., 2009; Sun et al., 2013; Ardito et al., 2017). This diversity of biological functions indicates the significance of these enzymes, which has long been overlooked in the context of tissue regeneration until a recent report shedding light on the role of a calcium and calmodulin-dependent serine/threonine protein phosphatase Calcineurin in zebrafish was published. Calcineurin or PPP3CA belonging to PP2B family regulates a coordinated growth and regeneration of zebrafish fin. Its pharmacological inhibition upregulates Retinoic acid signalling pathway and enhances the length of fin regenerate (Kujawski et al., 2014). This gave us clues about the functions of phosphatases during tissue regeneration and that too in zebrafish and made us question the potency of other phosphatases in the field of zebrafish tissue regeneration. It brought us to another interesting dual-specificity phosphatase Pten, the properties of which have been well-elucidated during tumorigenesis and cancer.

1.4.1. PTEN/PI3K/AKT/mTOR Signalling Pathway: An Overview

Phosphatase and Tensin Homolog deleted on Chromosome 10 (PTEN) is a dual-specificity phosphatase which can dephosphorylate protein but predominantly lipid substrates. It belongs to the superfamily of Protein-tyrosine phosphatases. Pten is well-established as the second most frequently mutated tumor suppressor gene after p53. It has been reported that it is majorly the loss of its lipid phosphatase activity that leads to tumorigenesis (Myers et al., 1998). PTEN holds the position of the negative regulator of the entire PTEN/PI3K/AKT/mTOR Pathway, working antagonistically to Phosphatidylinositol 3-Kinase (PI3K). Conclusively, it can be said that PTEN and PI3K work in equilibrium to maintain homeostasis of the cell. PTEN hydrolyses the second messenger Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) produced by PI3K to its inactive form Phosphatidylinositol (4,5)-bisphosphate (PIP2). Thus, inhibiting PIP3-dependent downstream signalling where 3-Phosphoinositide-dependent kinase 1 (PDK1) gets activated and further AKT gets recruited to the plasma membrane and activated (Song et al., 2012; Hopkins et al., 2014). All these events trigger cell growth, proliferation, cellular metabolism and protein synthesis upon their activation, which is prevented by the action of PTEN. The Ser-Thr kinase AKT (Protein Kinase B) is itself fully activated in a multi-step process involving the phosphorylation of its two sites at Thr308 by PDK1 (activated by PI3K) and at Ser473 by PDK2 (also known as Mammalian Target of Rapamycin Complex 2 or mTORC2), which has also been confirmed by its mutational analyses at these sites. The Thr308 phosphorylation changes the conformation of AKT, rendering it more susceptible to the phosphorylation at Ser473 by mTORC2, making AKT maximally activated (Liao & Hung, 2010; Hart & Vogt, 2011). Interestingly, some reports also suggest that phosphorylation of AKT at Thr308 by PDK1 is facilitated if it is plasma membrane-anchored and already phosphorylated at Ser473 by mTORC2 (Scheid et al., 2002; Sarbassov et al., 2005). The mTORC2 comprises of mTOR, DEPTOR, rapamycin-insensitive companion of mTOR (RICTOR), mLST8, and mammalian stress activated protein kinase-interacting protein (mSIN1) variants. Both RICTOR and mSIN1 are required for mTORC2 to phosphorylate AKT (Liao & Hung, 2010; Yoon & Choi, 2016). Upon its complete activation, AKT conjures a multitude of effects majorly, all of which are anti-apoptotic and pro-cell proliferative in nature. It controls cell survival or cell death by regulating Bad/Bcl2, death protease Caspase-9, FasL, IKK-NFκB, FoxO and p53. It monitors cell cycle progression also by regulating p27kip, cyclin D1, GSK-3β. Its activation also balances the protein synthesis and cell growth by regulating activities of Tuberous Sclerosis Complexes 1 and 2 (TSC1/2), mTOR complex, elongation-

initiation factor 4E binding protein-1 (4E-BP1), and S6K (Liao & Hung, 2010). The AKT phosphorylates and inhibits TSC leading to further activation of Mammalian Target of Rapamycin Complex 1 or mTORC1 mediated by Rheb-GAP activity of TSC (Manning & Toker, 2017). The active AKT, along with mTORC1, is accorded as the positive regulator of this entire pathway. The mTORC1 is a Ser/Thr kinase having mTOR, DEPTOR, mLST8, 40 kDa Pro-rich AKT1 substrate 1 (PRAS40; also known as AKT1S1) and regulatory associated protein of mTOR (RAPTOR) subunits. AKT can directly activate mTORC1 by inducing the inhibitory phosphorylation of PRAS40, which negatively regulates mTORC1 (Song et al., 2012; Manning & Toker, 2017). The active mTORC1 phosphorylates p70 ribosomal protein S6 kinase (S6K) and inhibitory 4E-binding protein 1 (4EBP1; also known as eIF4EBP1) to activate protein translation. Thus, activation of mTORC1, due to PTEN inactivation upstream leads to the enhanced translation of specific mRNAs critical for cell growth, proliferation and cell survival (Song et al., 2012) (Fig 1.7).

1.4.2. Role of PTEN as a Tumor Suppressor: Its Pivotal Function

The PTEN protein is a well-established tumor suppressor protein regulating the cellular microenvironment to control tumor growth and progression. PTEN is also known as MMAC1 (mutated in multiple advanced cancers-1) since it is the second most-mutated, deleted or silenced tumor suppressor gene after p53 in human cancerous conditions. These germline mutations in Pten occur on chromosomal locus 10q23 in the human genome and result in hereditary disorders such as Cowden disease, Bannayan-Riley-Ruvalcaba syndrome, Lhermitte-Ducros disease (LDD) and Proteus syndrome. All these syndromes are characterised by the development of multiple hamartomas and an increased risk of tumorigenesis, so these diseases have been collectively classified as types of “PTEN hamartoma tumor syndrome” (PHTS) (Song et al., 2012; Sasaki et al., 2009). Although protein dephosphorylation functions of PTEN are known to be of importance in signal transduction (Planchon et al., 2007), it is the loss of its lipid phosphatase activity that leads to tumorigenesis (Simpson & Parsons, 2001). In addition to that, there is no evidence proving the exclusive loss of its protein dephosphorylation

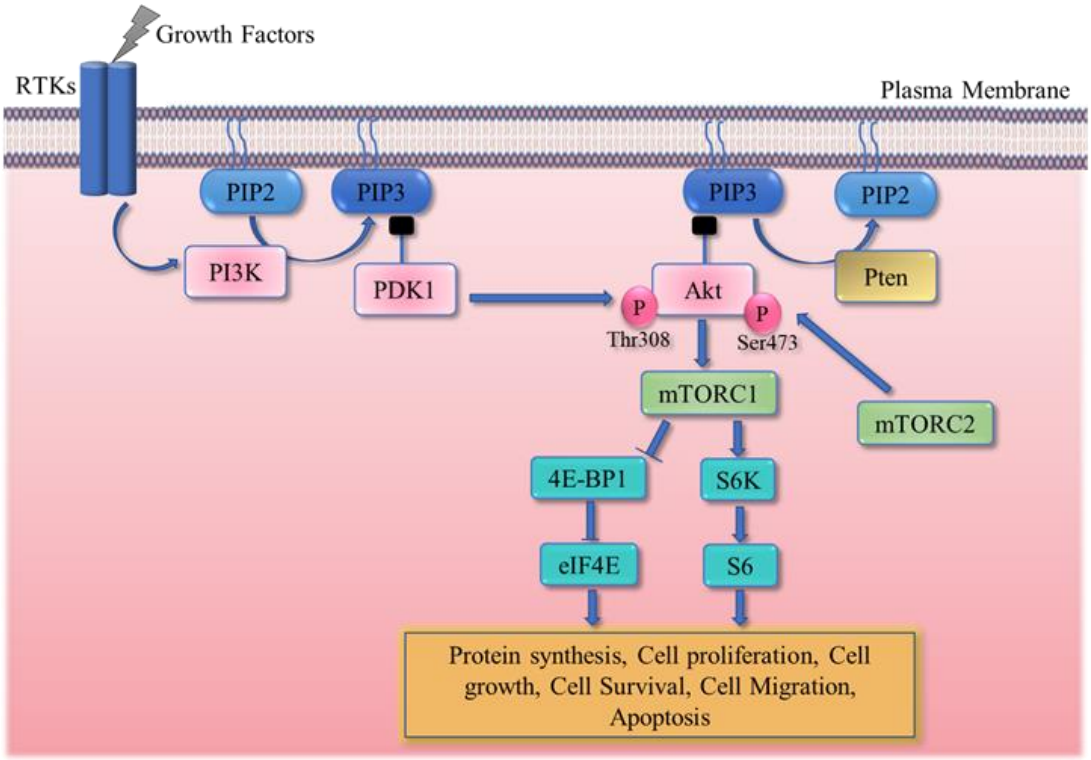

Fig 1.7 Pten/PI3K/Akt/mTOR Pathway. In this pathway, pro-proliferative factors like PI3K, Akt, mTORC1/C2 work in coordination with anti-proliferative Pten, in a cell.

activity during cancer (Myers et al., 1998). These reports clearly indicate that the loss of PTEN protein function leads to augmented cell proliferation, survival, migration, cell size, stem cell renewal, centromere instability, and DNA double-stranded breaks (Sasaki et al., 2009). These defects make the environment more permissive to transformation by making the affected cells more susceptible to carcinogens, making them prone to secondary genetic or epigenetic changes that lead to the cancer progression. Moreover, Pten haploinsufficiency makes the condition even severe, since, despite the retention of one wild-type Pten allele, mutation or partial loss of another allele predisposes the cell towards cancer (Sasaki et al., 2009; Choorapoikayil et al., 2012).

1.4.3. Other Cellular and Biological Roles of PTEN

The fact that PTEN widely acts as a tumor suppressor, overshadows its other significant roles. Nonetheless, PTEN has versatile cellular and biological implications in an organism. Many reports mention that PTEN maintains the normal physiology of the cells and the tissues. PTEN deficiency leads to many autoimmune diseases, insulin hypersensitivity, heart failure, macrocephaly (Sasaki et al., 2009). A complete loss of PTEN can lead to total DNA damage leading to cellular senescence (Song et al., 2012). PTEN regulates the cell size by regulating the actin-remodelling processes (Backman et al., 2002). It plays a role in maintaining a balance between self-renewal and differentiation of embryonic stem cells and hematopoietic stem cells (Shi et al., 2012). PTEN is highly involved in development also, since the homozygous Pten inactivation is embryonically lethal in multiple organisms, including mice, zebrafish, humans (Knobbe et al., 2008). PTEN also regulates apoptosis (Suzuki et al., 2008) and global gene expression by tuning chromatin dynamics (Chen et al., 2014). PTEN is known to dephosphorylate Dishevelled, a ciliogenesis regulator protein, to balance the multicilia formation and cilia disassembly (Shnitsar et al., 2015). There are reports which clearly indicate the role of PTEN in coordinating CNS development, retinal neurogenesis and in establishing a functional retinal architecture in mice and zebrafish also (Cantrup et al., 2012; Sakagami et al., 2012; Jo et al., 2012).

1.4.4. Role of PTEN/PI3K/AKT/mTOR Signalling Pathway during Tissue Regeneration

It is very well perceived, by delving into most of the available literature that PTEN acts as a pivot between cell proliferation and cell cycle exit. It maintains homeostasis in many processes pertaining to stemness, self-renewal and acquiring the cell fate. All these biological steps form a part of the phases of tissue regeneration also. Not only PTEN but the other proteins of this pathway also contribute to the cell proliferation and stemness. This gives us clues about the importance of this pathway during regeneration. Many groups have actively worked and elucidated the roles of various components of this pathway in regeneration. PTEN deletion, inhibition or its blockade by antagonistic peptide provoke robust axonal and spinal cord regeneration and growth after CNS injury (by injuring either optic nerve or spinal cord) in mice (Park et al., 2008; Ohtake et al., 2014; Ohtake et al., 2015; Singh et al., 2014; Borges et al., 2020; Mak et al., 2020). Although not much work is done to check the significance of PTEN and the related pathway in heart regeneration, still a few reports concur with this fact that blockade or silencing of PTEN facilitates cardiac stem-cell proliferation in rats and restricts the myocardial infarct size besides improving the heart function post-infarction in mice (Keyes et al., 2010; Shi et al., 2017). Pten maintains the adult muscle stem cell (Satellite Cells) pool. Deletion of Pten in quiescent satellite cells leads to their activation and premature differentiation surpassing proliferation, which results in a diminution of the stem cell pool and regenerative failure (Yue et al., 2017). Thus, PTEN and its downstream effectors maintain the homeostasis of the regenerating and differentiated cells.

Silencing and blockade of Akt alone also lead to a compromised regenerative response. A study unravels the role of Akt in tissue regeneration in nervous and excretory systems in planaria (Peiris et al., 2016). In Drosophila, Akt1 promotes imaginal disc regeneration mediated by Ask1 phosphorylation (Santabárbara et al., 2019). The mTOR, downstream of PTEN, also gets activated and promotes axonal regrowth and regeneration following CNS and PNS injury (Park et al., 2008; Hu, 2015; Diekmann et al., 2015). Conditional knockout of *mtor* in mice shows impaired muscle satellite cells activation, proliferation affecting skeletal muscle regeneration (Zhang et al., 2015). mTOR signalling has been reported to promote transdifferentiation of biliary epithelial cells and liver regeneration post-partial hepatectomy in zebrafish (Panasyuk et al., 2013; He et al., 2019). mTORC1 also facilitates blastema formation by enhancing cell proliferation, survival and differentiation in the fin regenerate of zebrafish (Hirose et al., 2014).

Hence, this pathway becomes notable for cell proliferation and differentiation during tissue regeneration.

1.4.5. Functions of Pten in Zebrafish

We have seen that PTEN/PI3K/AKT/mTOR Signalling Pathway balances out versatile functions from development to metabolism, from cell proliferation in regeneration to quiescence in various organisms. In zebrafish, Pten is reported to have two genes: *ptena* and *ptenb,* which had formed as a result of genome-wide duplication in teleost fishes (Croushore et al., 2005). Pten has been primarily known to contribute during embryogenesis and organismal development in zebrafish. The *ptenb* regulates actin polymerisation responsible for proper cell motility and migration, which controls cellular movements during gastrulation in zebrafish (Yeh et al., 2011). Pten contributes during angiogenesis and hematopoiesis, as also seen in mammalian systems (Stumpf et al., 2015). Pten/PI3K/Akt-axis along with Vegf signalling (Vascular Endothelial Growth Factor) and Bmp signalling fine-tune the sprouting of new blood vessels (vasculogenesis and angiogenesis) (Stumpf & Hertog, 2016). It also balances the proliferation and differentiation of the hematopoietic stem cells (Choorapoikayil et al., 2014). Although *ptena* and *ptenb* have overlapping and nonredundant functions and localisation not only during development but also in the adult (Faucherre et al., 2008), cumulatively Pten is reported to maintain the overall morphology of the zebrafish body. It is found to be expressed in somites, eyes, ears of zebrafish embryos and is also known to help in CNS and PNS development. Till date, it is not unravelled if PTEN genes are expressed in these tissues during mammalian embryogenesis (Croushore et al., 2005). Taking into consideration the mentioned functions of Pten in zebrafish, regeneration biologists have tried to decipher its role during tissue regeneration. A report mentions that Ptena, but not Ptenb, inhibits spinal cord regeneration in zebrafish after injury (Liu et al., 2014). On the contrary, the supportive function of Pten during caudal fin-fold regeneration of zebrafish embryos has also been elucidated (Hale et al., 2017). The fact that *pten* genes are haploinsufficient has been discussed previously, which stands conserved in the zebrafish also. It has been demonstrated that zebrafish *pten* mutants on three alleles $ptena^{+/-}ptenb^{-/-}$ or $ptena^{-/-}ptenb^{+/-}$ showed disturbed body homeostasis and development of hemangiosarcomas (Choorapoikayil et al., 2012). Earlier studies showed

the development of ocular tumors later in life in adult zebrafish with *ptenb*$^{-/-}$ mutations despite expressing *ptena* in the eye (Faucherre et al., 2008).

During regeneration, if chosen as a response to the tissue injury, cells proliferate, but there are definitive signalling programs and molecules which keep a check on the uncontrolled or imbalanced cell division. These create a borderline between a successful regenerative response and tumorigenesis. Such check-points are governed by cell cycle check-points, cell-cycle inhibitors, pro-apoptotic signals and tumor suppressor genes. Pten is one such tumor suppressor, which might be playing a role during regeneration. These facts direct us to investigate more about Pten in maintaining zebrafish eye homeostasis and also in the regeneration paradigm, as the role of this pathway in the field of retina regeneration is not yet probed.

Section 2.

Materials and Methods

2.1. Animal maintenance and breeding.

Zebrafish, used as a model organism in the study, were maintained and in-bred in the Zebrafish facility, IISER Mohali. The fish were reared in an automated water system, at a temperature of 25°C-28°C, under 14h:10h light and dark cycle conditions. The fish were fed with the live feed-Artemia which is an aquatic crustacean and also with Prawn feed pellets twice a day.

The adult fish of the age of 6-12months or above were used for all the experiments. Although for few experiments, Zebrafish embryos, obtained by crossing wild-type Zebrafish and maintained at 28°C, were also used. The *1016tuba1a*:GFP transgenic line, as developed and maintained in the lab at the same conditions, was also used for a few experiments.

2.2. Retinal injury and drugs delivery.

Out of the various ways to damage retina, we adopted the mechanical injury-based method using sterile 30-gauge surgical needle. The Zebrafish were anaesthetized using a light dosage solution of Tricaine Methanesulphonate (Acros Organics), by dipping, till the time the fish movement slows down. Following this, the fish eye is pressed slightly from one corner using forceps in order to tilt it at an elevated angle and stabbed using 30-gauge needle in 4-6 diagonally opposite directions from the back of the eye, which results in the damage in all the retinal layers. This stab wound is either treated as an injury, or from the same pokes 2ul of the desired pharmacological inhibitor drugs are injected into the vitreous humor of the eye, using Hamilton syringe, till the time eye is seen bulging, or small amount of blood oozes out. The experimental fish is put back in the water for its revival. The drugs used in the study are as follows: SF1670 (Sigma Aldrich; SML0684), LY294002 (ApexBio; A8250), Rapamycin (ApexBio; A8167), Torin1 (ApexBio; 8312), SB3CT (Sigma Aldrich; S1326), DAPT (Sigma Aldrich; D5942) which were all dissolved in DMSO.

2.3. Morpholino (MO) electroporation, mRNA transfection.

Lissamine-tagged Morpholinos (MOs) (Genetools) were used for gene-knockdown experiments in our study. For all the knockdown experiments, 0.5µl of 0.25-1.0mM of MOs were injected using Hamilton syringe at the time of injury. In order to facilitate the MO entry inside the nucleus, the electroporation was done, where positive electrode was placed on the fish eye in which MO was injected. During the electroporation, 5 pulses of 70V for 50ms each was given and fish was later released back to the water.
The sequences of the MOs used in the study are listed below:
Control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'
akt1 MO: 5'-GATCACCACATCTGTCGCCAT-3'
ptena MO: 5'-GTTTAGCAGTCATTGCCATGACAGC-3'
ptenb MO: 5'-ATTCCTTTATGATCGCAGCCATGGC-3'
her4.1 MO: 5'-TTGATCCAGTGATTGTAGGAGTCAT-3'

For overexpression and gene knockdown rescue experiments, mRNA transfection in retina was done; where a Transfection mix comprising of 2 solutions was made: (a) Equal volumes of 4-5µg mRNA mixed with 2X HBSS. (b) Equal volumes of Lipofectamine Messenger Max Reagent (Invitrogen, Catalogue Number LMRNA001) mixed with 2X HBSS. Both the solutions (a) and (b) were allowed to stand for 10min at room temperature (22°C-25°C), following this (a) and (b) were mixed together dropwise, and incubated for 30min at room temperature. This transfection mix was then mixed with that of the *gfp* mRNA mix (prepared in similar way as mentioned above, to confirm the efficiency of transfection). 0.5µl of the mix was injected in zebrafish retina using Hamilton syringe along with the injury, followed by the electroporation as mentioned earlier, to facilitate the access of mRNA transfection mix into the nucleus. In the control transfection experiments, *gfp* mRNA was transfected into the retina without the gene mRNA.

2.4. BrdU pulsing, EdU injection and eye or retina dissection.

In order to label the proliferating retinal cells (MGPCs in our study), BrdU (5-Bromo-2'-deoxyuridine) (Sigma; B5002) was used. Injured control and other experimental fish were

given a pulse of BrdU for 4 to 5hrs on suitable time points as per the experimental requirement. Similarly, EdU (5-ethynyl-2'-deoxyuridine) (Click-iT™ Plus EdU Cell Proliferation Kit for Imaging; ThermoFisher Scientific; C10638), a novel alternative molecule for BrdU was used for pulse labelling of the proliferating MGPCs. A 10mM EdU solution was injected from the front of the eye, beneath the lens, 3hrs prior to harvesting or as per the experimental timeline.

While harvesting the eye, the zebrafish was anaesthetized under a light dosage of Tricaine, the eye was pulled out of the socket using forceps, through the ventral side by exerting a slight pressure on the belly. The eye was then suspended in 1X PBS kept at 4°C in a Petri dish, kept on ice. The eye was held from the optic nerve or from the backside and stabilised firmly on the base of the Petri plate. Using a 30-gauge needle, the corneal layer was poked and was torn off using needle and forceps. The lens was plucked out and the surrounding tissue and the retinal pigmented epithelium was removed neatly, so as to reveal a cup shaped intact retina. This dissected retina was then suspended and stored in either Tri Reagent (for RNA isolation) or Laemmli buffer (for Western Blotting assays) in a 1.5ml microcentrifuge tube (MCT). Single retina was used as a sample set for RNA isolation and 2 or 3 retinae were used as a single sample set for the Western Blotting assays. This tissue was then stored in -80°C for future use, or used for further sample preparation and analysis.

2.5. Total RNA isolation.

For total RNA isolation from zebrafish retinal tissues, following reagents were used:

1) 10X Phosphate Buffer Saline (PBS):
   NaCl (Sigma)- 75.97g
   Na_2HPO_4 (Sigma)- 9.9372g
   NaH_2PO_4 (Sigma)- 3.59g
   The above solutes were dissolved in 800ml autoclaved MQ and its pH was set to 7.4. The final volume was made up to 1L by autoclaved MQ.
2) Tri Reagent (Sigma)
3) Chloroform (Merck)
4) Isopropanol (Merck)
5) Absolute Ethanol (Fisher brand)

6) DEPC water

Using the above reagents, the following protocol was adopted:

1) The dissected retinal tissue as mentioned in **4.** was suspended in 200µl Tri reagent under ice cold conditions and further homogenised by gentle pipetting.
2) The above suspension was allowed to stand at room temperature for 5min for better lysis.
3) Following lysis, 40µl Chloroform was added and the suspension was mixed vigorously 6-7 times, followed by phase separation by centrifugation at 10,000rcf for 10min at 4°C.
4) Around 30µl of the upper phase was collected in a fresh MCT using a cut tip. To this, an equal volume of Isopropanol was added and mixed vigorously, so as to precipitate the RNA. This mixture was either precipitated for 10min on ice or stored overnight in -80°C.
5) After precipitation, the solution was centrifuged at 10,000rcf for 20min at 4°C and the supernatant was then discarded.
6) The resultant faint thread-like pellet was then washed twice with 200µl of 80% Ethanol in DEPC water at 7600rcf for 10min each at 4°C.
7) The pellet was then allowed to get air-dried by keeping the MCT opened in a clean area.
8) Finally, the dried pellet was dissolved in 12µl DEPC MQ, by pipetting and incubating on ice and later stored in -80°C conditions.

For total RNA isolation from 24hours post fertilisation (hpf) zebrafish embryos, the embryos were collected post in-breeding and homogenised well in 200µl Tri Reagent at 24hpf using pestle. Following the same protocol, the final pellet obtained was dissolved in 30µl DEPC MQ and stored in -80°C.

2.6. cDNA synthesis from mRNA.

Using the Thermo Scientific RevertAid RT First-strand cDNA synthesis kit (K1612) and from the isolated mRNA from retina or 24hpf embryos, the complimentary DNA (cDNA) was synthesised.

The following steps were performed:

1) The dissolved mRNA was thawed by keeping on ice.
2) The components were mixed in PCR tubes, as follows:

	Components	**Amount**
a)	Template mRNA	up to 5µg of RNA
b)	Oligo (dT) Primers	1µl
c)	Random Hexamers	1µl
d)	Nuclease-free water	up to 12µl

3) The PCR tubes were incubated for 5min at 65°C, to open up the secondary structures of mRNA, and transferred immediately to ice after this incubation.
4) This solution was incubated on ice for 1-2minutes, and to it the following component mixture was further added:

	Components	**Amount**
a)	5X Reaction Buffer	4µl
b)	RiboLock RNase Inhibitor	1µl
c)	10mM dNTPs	2µl
d)	RevertAid M-MulV RT Enzyme	1µl

Making the final reaction volume to 20µl. This reaction was scaled down to the lower volumes of 5µl or 10µl (in different experiments) depending upon the cDNA requirements.

5) The above components were mixed properly and spun down, and subjected to the following PCR Thermocycler conditions:

Temperature	**Duration**
25°C	5min
42°C	60min
70°C	5min
4°C	∞

6) The cDNA was diluted in Nuclease-free water in a ratio of 1:4 or 1:8 and then used for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Quantitative PCR (qPCR) analyses and later stored in -20°C.
7) The rest of the neat cDNA was stored in -80°C safely.

2.7. Primers for PCR and cloning.

The Forward and Reverse primers used in this study for performing all the RT-PCR and qPCR reactions and for cloning the genes' coding sequences (CDS) and promoters are listed in Appendix.

2.8. Genomic DNA (gDNA) isolation.

1) The zebrafish fin was cut and the tissue was suspended in 200µl TEN buffer take in a 1.5ml microcentrifuge tube.
   TEN buffer Composition:

Components	**Amount**
1M Tris Cl (pH 8.0) (Sigma)	40mM
0.5M EDTA (pH8.0) (Sigma)	1mM
5M NaCl (Sigma)	150mM

2) The tissue was homogenised for around 1minute, after adding 1% Sodium dodecyl sulphate (SDS) (Sigma) (v/v) to it.
3) Proteinase K solution (10mg/ml) (Roche) was added to the homogenised tissue to a final concentration of 100-200µg/ml (in order to digest the protein) and tapped gently.
4) The above suspension was incubated at 37°C either overnight or for 3hrs in water bath.
5) An equal volume of Phenol:Chloroform:Isoamyl alcohol (PCI) was added and mixed by inverting the tube.
6) The tube was then spun at 6000rpm at room temperature for 5min.
7) The upper aqueous phase was collected using a cut tip.
8) To this aqueous phase, $1/10^{th}$ volume of 3N Sodium acetate (Sigma) buffer was added and mixed gently by inverting the tube.
9) Finally, 2volumes of Isopropanol or absolute ethanol was added alongside the wall, not directly to the solution and was invert mixed gently.
10) A noodle-like mesh formed was collected using a tip, a process named as Spooling.
11) This noodle-like mesh collected in previous step was instantly put into 70% Ethanol.
12) The tube was then spun at 6000rpm at room temperature for 5min.

13) The ethanol was decanted by opening and inverting the tube and the pellet of genomic DNA collected was dried at room temperature.
14) Following the drying step, the gDNA obtained was dissolved in 50µl Nuclease-Free Water, without harsh tapping.
15) It was left overnight at 4°C for proper dissolution.

2.9. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

In order to amplify cDNA template for a particular gene, the following reaction mixture was made:

Components	**Amount**
20X PCR Buffer	0.5µl
2.5mM dNTPs	1.0µl
10pM Forward Primer	0.1µl
10pM Reverse Primer	0.1µl
Template	X µl
Taq Polymerase	0.1µl
MilliQ	Y µl
Total volume	up to 10µl

The above reaction mixture was subjected to the following program in PCR thermocycler:

Steps	**Temperature**	**Time**	
Initial Denaturation	95°C	2min	
Denaturation	95°C	20s	
Annealing	58°C	30s	25-36cycles
Extension	68°C	1kb/min	
Final Extension	72°C	7min	
Hold	4°C	∞	

The PCR product obtained was then loaded in wells in 1% Agarose gel, run under 61V condition in 1X Tris Acetate EDTA (TAE) buffer and sample movement was tracked by the migration of Bromophenol Blue and Xylene Cyanol present in the 2X gel loading dye. The final gel image was captured in UVP UV GelDoc system.

2.10. Quantitative PCR (qPCR).

In order to quantify the fold-change in the gene levels, qPCR was performed. The cDNA obtained was amplified in Applied Biosystems QuantStudio3 qPCR machine.

The following reaction mixture was made and loaded in the wells in qPCR plate:

Components	**Amount**
2X PowerUP SYBR Mix (ThermoFisher Scientific; A25742)	2.5µl
10pM Forward Primer	0.1µl
10pM Reverse Primer	0.1µl
Template	Xµl
MilliQ	Yµl
Total volume	up to 5µl

A protocol was run on Applied Biosystems QuantStudio3 qPCR machine and the data was analysed in MS excel by ΔΔ Ct method.

2.11. Cloning of gene coding sequence (CDS) and promoters.

The gene CDSs for *akt1, ptena, ptenb, ascl1a, her4.1, mmp9* were amplified using respective gene primers as listed in Appendix and 24hpf zebrafish embryonic cDNA as the template. The different gene promoters for *akt1, ptena, ptenb* were amplified using respective promoter primers and gDNA as the template. These inserts as amplified were purified from the agarose gel after agarose gel electrophoresis by either manual gel elution method or by Nucleo-pore SureExtract PCR Clean-up/Gel Extraction Kit (Cat.#NP-36107). The purified inserts were then digested by the respective restriction enzymes as per the restriction sites added in the primers, to create the sticky ends. These restriction sites being compatible to the restriction sites in the Multiple Cloning region (MCS) in the vector, further allow the ligation of the amplified insert with the digested vector at the sticky end.

The cDNAs were cloned in the pCS2+ vector under the *cmv* promoter by using conventional T4-DNA Ligase (NEB #M0202S) based method or in pCRII-TOPO vector by using TOPO-TA cloning kit (Invitrogen, catalogue number 45-0640), to be used for mRNA *in vitro* transcription reactions to make probes to be used in mRNA *in situ* hybridisation or for making mRNA for *in vivo* overexpression studies.

The different gene promoters for *akt1*, *ptena*, *ptenb* were cloned in the pEL luciferase expression vector to create *gfp:luciferase* reporter constructs driven by respective promoters, using T4-DNA Ligase.

2.12. Site-Directed Mutagenesis (SDM) based on PCR.

In order to mutate one or few bases in a gene CDS, Site-directed Mutagenesis was done using PCR method.

Following reaction was set:

Components	**Amount**
Plasmid DNA	50-100ng
Forward Primer	20pM
Reverse Primer	20pM
10mM dNTPs	2µl
10X Reaction Buffer	2.5µl
Taq Polymerase (For extender system)	0.2µl [Not added in the Control Reaction]
Nuclease Free Water	Make up the volume
Total volume	25µl

Alternately, the following reaction was set up:

Components	**Amount**
Plasmid DNA	1µl
Forward Primer+ Reverse Primer	2.5µl (10pM each)
GoTaq Long PCR Master Mix (2X) (Promega, M4021)	12.5µl [Not added in the Control Reaction]

Nuclease Free Water	Make up the volume
Total volume	25µl

Experimental reaction was set up in duplicates 25µl each, which were clubbed together after the PCR cycle.

The reaction was subjected to the following PCR cycle:

Steps	**Temperature**	**Time**	
Initial Denaturation	93°C	2min	
Denaturation	93°C	15s	18 cycles
Annealing	55°C	1min 30s	
Extension	72°C	1kb/min	
Final Extension	72°C	20min	
Hold	4°C	∞	

Following PCR cycle, the PCR products (Control and Experimental) were purified using Nucleo-pore SureExtract PCR Clean-up/Gel Extraction Kit (Cat.#NP-36107). The cleaned PCR products were then treated with DpnI enzyme (1µl) for 3hrs at 37°C, to cleave the unmutated strands. The digested products (15µl) were then transformed in DH5α Ultracompetent cells. The colonies obtained from experimental plates were inoculated in LB media and grown overnight in 37°C, at 200rpm shaking. The plasmids isolated from the above cultures were sent for sequencing to detect the mutagenized constructs.

2.13. Plasmid Isolation.

1) 1.5ml culture was taken in MCT and centrifuged at 13,400rpm for 2min at room temperature.
2) The supernatant was discarded and pellet was dissolved completely in 100µl chilled autoclaved MilliQ by vortexing.
3) To this bacterial suspension, 100µl Lysis Buffer was added and invert mixed gently, to lyse bacterial cell wall. The sample was not vortexed or tapped to prevent genomic DNA shearing.

   Lysis Buffer Composition:

Components	Amount
20% SDS (Sigma)	50µl
0.5mM EDTA (Sigma)	20µl
10N NaOH (Sigma)	20µl
MilliQ	910µl

4) The above solution was boiled for 2-3min at 100°C on thermo-block, until it became clear.

5) After lysis, 50µl 0.5M $MgCl_2$ (Sigma) was added to the above clear solution, gently invert mixed and incubated for 2min on ice, to precipitate the genomic DNA.

6) The solution was centrifuged at 13,400rpm for 2min at room temperature.

7) To the supernatant, 50µl 3N Potassium Acetate was added and gently invert mixed to renature the plasmid DNA.

3N Potassium Acetate Buffer Composition:

Components	Amount
5M Potassium Acetate (Sigma)	60ml
Glacial Acetic acid (Merck)	11.5ml
MilliQ	28.5ml
Total Volume	100ml

Stored in 4°C.

8) The above solution was centrifuged at 13,400rpm for 2min at room temperature and the supernatant was transferred to a fresh MCT having 600µl Isopropanol chilled on ice, to precipitate the plasmid.

9) It was mixed by vigorous shaking, incubated for 5min on ice and centrifuged at 13,400rpm for 3-4min at room temperature.

10) The pellet obtained was washed using 200µl 70% Ethanol by tapping, followed by centrifugation at 13,400rpm for 10min at room temperature.

11) The pellet was air dried at room temperature and finally dissolved in 50µl autoclaved MilliQ.

2.14. Ultra-competent cells preparation.

1) 5ml primary culture of DH5α strain of *E.coli* was incubated at 37°C overnight with continuous shaking at 200rpm.
2) 1% of primary culture was inoculated in 250ml L.B. media and incubated as secondary culture at 18°C, 200rpm shaking till OD_{600} reached the value of 0.6-0.8.
3) The secondary culture was then incubated on ice for 10-15min.
4) It was then centrifuged at 2500rcf for 10min at 4°C.
5) The pellet obtained was very gently resuspended in 80ml ice-cold TB Buffer, by swirling the falcon on ice and using a cut tip.

   TB Buffer Composition:

Components	**Amount**
PIPES (Sigma)	10mM
$CaCl_2.2H_2O$ (Sigma)	15mM
KCl (Sigma)	250mM
$MnCl_2.4H_2O$ (Sigma)	55mM

   pH set to 6.8 using 1N KOH.
6) It was kept on ice for 10min and then centrifuged at 2500rcf for 10min at 4°C.
7) The pellet was again resuspended in 20ml ice-cold TB Buffer, using a cut tip and gentle swirling on ice.
8) The cells obtained from this final resuspension were cryoprotected by diluting in DMSO (Himedia) to a final concentration of 7% (1.4mL DMSO+18.6mL of cells in TB buffer).
9) 100µl aliquots of these ultracompetent cells were made and stored at -80°C immediately.

2.15. Bacterial Transformation.

1) The Ultracompetent cells were thawed on ice.
2) DNA to be transformed was added to the comp cells, tapped vigorously and incubated on ice for 30min.
3) The Heat shock was given to the cells for 75s in 42°C static waterbath.

4) The cells were immediately plunged into ice for 5min.
5) After 5min, 1ml L.B. media was added to the cells and incubated at 37°C for 1hr at constant shaking of 200rpm.
6) The cells were pelleted down after 1hr, by centrifugation at 4000rpm for 4min.
7) Pellet was resuspended in 100µl media and plated on LB Agar plates.
8) The LB Agar plates were incubated in 37°C overnight to allow bacterial colonies to grow.

2.16. Restriction Digestion.

The reaction was set up in an MCT as follows:

Components	**Amount**
Plasmid DNA	1µg
10X Cutsmart or Fast Digest Buffer	1µl
Restriction Enzyme	0.2µl
MilliQ	To make up the volume
Total Volume	10µl

The MCT was tapped well, given a short spin and incubated for 3hrs to overnight (depending on the reaction volume) in 37°C waterbath. The digested products were checked on 1% Agarose gel, loaded with the ladder and undigested sample for the comparison or later isolated from the gel using Manual gel extraction method.

2.17. Manual Gel Extraction.

1) The digested plasmid or the PCR product was run on 0.8-1% Agarose gel for a long duration to allow maximum resolution, at 70-90V.
2) The desired DNA band was cut and collected in an MCT.
3) A fresh MCT was taken and poked gently at its base using a needle to create fine pore.

4) This MCT was filled 1/4th with finely cut pieces of sterile aluminium foil, to be used as a Gel Extraction Column, which was mounted on top of another MCT to be used as a Collection Tube.
5) The cut gel piece was kept in the Gel Extraction Column and centrifuged for 3min at 13,400rpm at room temperature.
6) The flow-through was collected in the lower Collection tube.
7) The flow-through was mixed with equal volume PCI and tapped vigorously. Centrifuged for 10min at 13,400rpm at room temperature.
8) The upper aqueous layer was carefully collected in a fresh MCT, using a cut tip.
9) It was again mixed with equal volume of Chloroform by tapping vigorously, centrifuged for 10min at 13,400rpm at room temperature.
10) The upper aqueous layer was again carefully collected in another MCT, using a cut tip.
11) This aqueous layer was precipitated by adding 3N Ammonium acetate (final concentration), 70% Ethanol (final concentration) and 1µl 10mg/ml Glycogen.
12) The solution was tapped and mixed vigorously and precipitated in -80°C overnight.
13) Then it was centrifuged for 30min at 13,400rpm at 4°C.
14) Later the pellet obtained was washed twice with 500µl of 70% Ethanol by tapping, followed by centrifugation at 13,400rpm for 10min at 4°C.
15) The pellet was dried at room temperature and dissolved in 20µl Nuclease Free Water or DEPC water as per the need.

2.18. The *in vitro* mRNA synthesis reaction for overexpression studies.

The linearised plasmid from the desired CDS was used as the template to transcribe sense mRNA *in vitro*, using mMESSAGE mMACHINE® Kit (Invitrogen AM1340).

1) The reaction was set as follows:

Components	**Amount**
Linearised template DNA	0.1-1µg
10X Reaction Buffer	2µl
2X NTP/CAP	10µl
Enzyme Mix	2µl
Nuclease free water	Make up the volume to 20µl

2) The above reaction mix was tapped and incubated at 37°C for 2hrs in waterbath.
3) To the reaction, 1µl TURBO DNase was added, mixed well and incubated in 37°C for 15min.
4) The product was then checked on 1% Agarose gel and O.D. was measured.
5) The mRNA was precipitated by adding 30µl Nuclease Free Water and 30µl LiCl Precipitation Solution.
6) To enhance the precipitation efficiency 70% Ethanol (final concentration) was added, tapped well and stored overnight in -20°C.
7) It was then centrifuged for 30min at 13,400rpm at 4°C.
8) The pellet obtained was washed twice with 500µl of 80% Ethanol by tapping, followed by centrifugation at 13,400rpm for 10min at 4°C and pellet obtained was air dried.
9) The mRNA pellet was then dissolved in 10µl Nuclease Free Water and stored in -80°C.

2.19. The *in vitro* mRNA transcription reaction for probe synthesis.

1) The following reaction was set in an MCT:

Components	**Amount**
Linearised template DNA	1µg
10X Transcription Reaction Buffer	4µl
Digoxigenin or Fluorescein RNA labelling mix	2µl
RiboLock RNase Inhibitor	0.5µl
SP6/T7/T3 RNA Polymerase	2µl
DEPC Water	To make up the volume
Total volume	40µl

2) The reaction was tapped, mixed and incubated for 4hrs at 37°C in waterbath.
3) After 4hrs, 1µl probe was checked on 1% Agarose gel and O.D. was measured.
4) The reaction was stopped and the probe was precipitated by adding the following components, by mixing in between:

Components	**Amount**
Tris-EDTA Buffer (pH8.0)	4µl
LiCl	4µl
Glycogen (10mg/ml)	2µl

100% Ethanol	70µl

5) The above mix was tapped well and incubated overnight in -80°C.
6) It was then centrifuged for 30min at 13,400rpm at 4°C.
7) The pellet obtained was twice washed with 500µl of 80% Ethanol, followed by centrifugation at 13,400rpm for 10min at 4°C and pellet obtained was air dried.
8) The mRNA probe pellet was then dissolved in 20-30µl DEPC Water and stored in -80°C.

2.20. Tissue cryofixation, sucrose washing and cryo-sectioning of the eye tissue.

Reagents used:

1) 1X Phosphate Buffer (PB) (pH 7.4)

   Made from 5X Phosphate Buffer (PB) (pH 7.4)

Components	**Amount**
$NaH_2PO_4.2H_2O$ (Sigma)	1.3g
Na_2HPO_4 (Sigma)	5.575g

   Dissolved in 70ml Autoclaved MilliQ. Its pH was set to 7.4 and volume was made up to 100ml with MilliQ.

2) 4% PFA

   4g Paraformaldehyde (PFA) (Sigma) was dissolved in 100ml 1X PB by heating in 65°C waterbath and shaking vigorously. Made fresh before use. Stored as small volume aliquots in -20°C.

3) 5% Sucrose and 20% Sucrose (Sigma) solutions

   Made using 1X PB as solvent. Stored in -20°C.

4) OCT

The uninjured or experimental eye was pulled out of the eye socket as mentioned previously and suspended in 4% Paraformaldehyde (PFA) in ice cold conditions. In a similar manner as mentioned above, the lens was carefully pulled off, without disturbing the other layers of the eye or pulling out the retina. This eye tissue was then suspended in 1ml 4% PFA in an MCT and kept overnight under static condition or under rotation in 4°C.

After PFA fixation, the eye tissue was preserved by a gradient of sucrose solution washes. During each wash, the sample was rotated on a rotator at room temperature for a duration of 45min. The series solutions used was as follows:

5% Sucrose (1000µl): 20% Sucrose (0µl)

5% Sucrose (800µl): 20% Sucrose (400µl)

5% Sucrose (500µl): 20% Sucrose (500µl)

5% Sucrose (400µl): 20% Sucrose (800µl)

5% Sucrose (0µl): 20% Sucrose (1000µl)

To this final 20% Sucrose, OCT was added in a ratio of 2:1 and eyes were washed by rotation at room temperature for 30min. Using aluminium foil, cubical moulds/ blocks were made and filled 3/4th with the OCT and labelled with paper tags on one edge. The eye tissue was embedded in OCT in the blocks and frozen and stored immediately in -80°C.

The above made tissue blocks were thawed for a few minutes in the Leica cryostat machine CM3050 S, then fixed on the stamp and placed firmly on the block holder arm of the cryostat. Using a fine cryo-microtomy blade, under the cryostat chamber temperature (CT) conditions of -23°C, the 10-12µm thick eye tissue sections were obtained on Fisherbrand Superfrost Plus Microscope Slides. The slides were dried overnight at room temperature in dark, and then stored at -20°C condition or used for immunostaining or mRNA *in situ* hybridisation.

2.21. mRNA *in situ* Hybridisation for Cryosections.

Day 1:

Reagents made:

1) 100% Ethanol
2) 95% Ethanol
3) 70% Ethanol
4) 50% Ethanol
5) 2X SSC (Made from 20X SSC solution)

   The 20X SSC solution was made by dissolving 8.76g NaCl in 35ml DEPC water, followed by dissolving 4.412g Sodium citrate and making the volume up to 50ml with DEPC water.

6) Proteinase K Buffer

Components	Amount
1M TrisCl (pH 8.0)	5ml
0.5M EDTA (pH 8.0)	5ml

Made the volume up to 50ml using DEPC water

7) Proteinase K Enzyme (10mg/ml)

160µl Proteinase K enzyme was added to 50ml Proteinase K Buffer.

8) TEA Solution

0.93g Triethanolamine Hydrochloride was dissolved in 45ml DEPC water. To it, 173µl 10N NaOH was added to set the pH to 8.0. Final volume was made up to 50ml with DEPC water.

9) TEN Solution

Components	Amount
1M TrisCl (pH 7.5)	5ml
0.5M EDTA (pH 8.0)	1ml
5M NaCl	30ml
Total volume	36ml

10) 5X Maleate Buffer

2.9g Maleic Acid was dissolved in 42.5ml DEPC water, by setting the pH to 7.5 with 10N NaOH. Once Maleic Acid was dissolved by setting the pH, 2.19g NaCl was added to it, dissolved and the volume was made up to 50ml with DEPC water.

11) 10% RMB Blocker

10g RMB Blocker powder (Blocking Reagent; Roche 1096176) was dissolved in 35ml 1X Maleate buffer and was mixed by intermittent shaking or heating in microwave and setting the pH to 7.4 by 10N NaOH. This was stored at -20°C.

12) Hybridisation Solution

Components	Amount
TEN Solution	3.6ml
100% Formamide	25ml
50% Dextran Sulphate	10ml
10% RMB Blocker	5ml
DEPC water	6.4ml

Store at -20°C.

13) 50% Formamide/5X SSC Solution

Components	Amount
100% Formamide	25ml
20X SSC Solution	12.5ml

Made the volume up to 50ml with DEPC water.

Protocol followed:

1) The slides were hydrated step-wise in a series of Ethanol of various dilutions, followed by 2X SSC for 1min each:
   i. 100% Ethanol
   ii. 100% Ethanol
   iii. 95% Ethanol
   iv. 70% Ethanol
   v. 50% Ethanol
   vi. 2X SSC
2) Proteinase K Buffer was pre-warmed in 37°C. Just before using 160µl 10mg/ml Proteinase K enzyme was added to 50ml Proteinase K Buffer. The slides were incubated in this buffer for 5-6min (for 10-12µm cryosections) at 37°C.
3) The effect of Proteinase K was washed off by rinsing the slides for 1min in DEPC water.
4) Further the slides were rinsed in 0.1M TEA pH8.0 for 3min.
5) The slides were then treated for 10min with 50ml TEA solution having 130µl Acetic Anhydride.
6) Then the slides were dehydrated step-wise in 2X SSC and a series of Ethanol of various dilutions, for 1min each:
   i. 2X SSC
   ii. 50% Ethanol
   iii. 70% Ethanol
   iv. 95% Ethanol
   v. 100% Ethanol
   vi. 100% Ethanol
7) The slides were air-dried at room temperature for 1hr in a clean isolated place.
8) Meanwhile, the Hybridisation solution was prewarmed at 56°C.
9) 100-500ng mRNA probe was boiled in 300µl pre-warmed Hybridisation buffer for each slide for 10min at 100°C, immediately kept on ice and then added dropwise to the slides.

10) The slides were coverslipped using HybriSlips and incubated overnight in 56°C, in a humidified chamber made by pouring 50% Formamide/5X SSC Solution in it.

Day 2:

Reagents made:

1) 2X SSC
2) 50% Formamide/2X SSC Solution

Components	**Amount**
100% Formamide	25ml
20X SSC Solution	5ml

Made the volume up to 50ml with DEPC water.

3) RNase Buffer

Components	**Amount**
5M NaCl	5ml
1M Tris-Cl (pH 7.5)	500µl
0.5M EDTA (pH 8.0)	100µl

Volume made up to 50ml.

4) RNase A Enzyme (10mg/ml)

100µl RNase A enzyme was added to 50ml RNase Buffer.

5) 1X Maleate/0.05% Triton X-100/1% RMB Blocker Solution

Components	**Amount**
5X Maleate Buffer	2ml
Triton X-100	5µl
10% RMB Blocker	1ml

3ml aliquots of Blocking solution were made and stored in -20°C. Before using 3ml aliquots were diluted by adding 7ml MilliQ.

6) 1X Maleate (Made from 5X Maleate).

Protocol followed:

1) The coverslipped slides were washed in 2X SSC at Room Temperature on Shaker Plate for 30min. After around 15min, the coverslips were gently teased off from the slides using forceps, if they did not come out.
2) Then the slides were rinsed in 50% Formamide/2X SSC Solution (preheated at 65°C), for 30min at 65°C, with gentle agitation for first 5min.

3) The slides were rinsed twice in 2XSSC (prewarmed at 37°C) for 10min at 37°C.
4) RNase Buffer was prewarmed at 37°C. Just before the use, 100µl RNase A enzyme (10mg/ml) was added to 50ml RNase Buffer. The slides were then incubated in this buffer for 30min at 37°C.
5) Sequentially the slides were washed in RNase Buffer in 65°C for 30min.
7) The slides were blocked for 2-3hrs with diluted 1X Maleate/0.05% Triton X-100/1% RMB Blocker Solution.
8) Following this blocking, the slides were washed twice with 1X Maleate for 10min each.
9) Further, the slides were incubated overnight at room temperature, with 500µl Anti-Dig/ Anti-Fl AP antibody dissolved in 1:2500 dilution in diluted 1X Maleate/0.05% Triton X-100/1% RMB Blocker Solution. These slides were kept in a moist chamber.

Day 3:

Reagents made:

1) 1X Maleate (Made from 5X Maleate).
2) Genius Buffer

Components	**Amount**
1M Tris-Cl (pH 9.5)	5ml
5M NaCl	1ml
0.5M $MgCl_2$	5ml

Volume made up to 50ml with MilliQ.

Protocol followed:

1) The antibody was removed and slides were washed twice with 1X Maleate for 10min each.
2) Followed by 2 washes of Genius Buffer for 10min.
3) Then the slides were incubated in dark with NBT/BCIP substrate (1:50 diluted in Genius Buffer), till the time colour reaction takes place.
4) After signal detection under the microscope, either the slides were stored or washed with 1XPBS, dried and mounted with 80µl DABCO.

2.22. Immunohistochemistry.

Day 1:

Reagents made:

1) 1X Phosphate Buffered Saline (1X PBS)
   Made from 10X PBS (pH 7.4), as mentioned previously.
2) 4% Paraformaldehyde (4% PFA)
   Made in 1X PB as mentioned previously.
3) 2N HCl
   Made from 12N HCl.
4) 10mM Sodium Citrate (Sigma) (pH 6.5)
5) 0.1M Sodium Borate solution (pH 8.5)
   19.0685g Sodium Borate salt was dissolved in 450ml Autoclaved MilliQ. pH was set to 8.5 and volume was made up to 500ml with MilliQ.
6) 3% BSA-1X PBST (1X PBS with 0.1% Triton X-100)
7) 1% BSA-1X PBST (1X PBS with 0.1% Triton X-100)
8) Primary antibody: The primary antibodies used in the study are, Rabbit monoclonal antibody against Pten (138G6) (Cell Signalling Technologies, 9559); Rabbit monoclonal antibody against Phospho-Akt (Ser473) (D9E) XP® (Cell Signalling Technologies, 4060); Rabbit monoclonal antibody against Phospho-Akt (Thr308) (D25E6) XP® (Cell Signalling Technologies, 13038); Rabbit polyclonal antibody against Akt (Cell Signalling Technologies, 9272); Mouse monoclonal antibody against Akt (pan) (40D4) (Cell Signalling Technologies, 2920); Rabbit polyclonal antibody against beta Catenin (Abcam, ab6302); Rabbit polyclonal antibody against pSmad3 (Abcam, ab52903).

Steps followed:

1) The slides with the cryosections were dried overnight in dark at room temperature or dried in 37°C incubator.
2) The dried slides were washed thrice with 1X PBS, for 10min each.
3) The slides were incubated with 4% PFA for 20min, if Morpholino or any previous staining had to be fixed.
4) The slides were washed once with 1XPBS for 10min to wash off the PFA.

5) Epitope Retrieval: For nuclear antigens, epitopes were exposed or retrieved by various methods like-

a) 2N HCl was prewarmed in a Coplin Jar at 37°C for 20-30min. Slides were incubated in it for 20min in 37°C incubator, or

b) 10mM Sodium Citrate (pH6.5) was boiled at 100°C in a glass beaker on hot-plate. Slides were constantly boiled in this buffer for 20-30min.

6) Immediately after epitope retrieval, slides were washed well twice with 0.1M Sodium Borate buffer to neutralise the acidic pH, for 10min each.

7) The slides were again washed once with 1X PBS for 5min.

8) The slides were then blocked for 1hr with 3% BSA-1X PBST.

9) Primary antibody against the respective antigen was dissolved as per the recommended or standardised dilution in 1% BSA-1X PBST.

10) After the blocking, antibody was overlaid on the slides and incubated overnight in 4°C.

Day 2:

Reagents made:

1) 1X PBST (1X PBS with 0.1% Triton X-100)
2) 1% BSA-1X PBST (1X PBS with 0.1% Triton X-100)
3) Secondary antibody
4) MilliQ
5) DABCO

Steps followed:

1) After the overnight primary antibody incubation, the slides were washed thrice with 1X PBST for 10min each.
2) Following the washes, the slides were incubated in dark conditions with the respective Secondary antibody diluted in 1:1000 ratio in 1% BSA-1X PBST, for 2-3hrs at room temperature or overnight at 4°C.
3) After this incubation, the slides were washed thrice in dark with 1X PBST for 10min each and the fluorescence signals were checked under a fluorescence microscope.
4) For a few experiments, DAPI or Hoechst were used as the reference nuclear stains and washed off using 3 washes, 10min each of 1X PBS.
5) Finally, the slides were washed once with MilliQ for 10min and dried in dark.

6) The slides were mounted with 80µl DABCO under glass coverslips and left overnight for drying, followed by either storage in -20°C or imaging with Nikon A1 confocal imaging system.

2.23. TUNEL Assay.

The detection and quantification of the cell death in retina post-injury was done by *in situ* cell death detection kit (Roche 11684795910). It was used to perform Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.

1) The slides were washed thrice for 10min each with 1X PBS.
2) In case of Morpholino-bearing sections, the slides were fixed for 20min with 4% PFA, followed by washing it off with 1X PBS for 10min.
3) Then the retinal sections were permeabilised with 1ml pre-warmed Trypsin in 37°C for 15min.
4) After permeabilization, the slides were overlaid with a mixture of 45µl Label Solution (Fluorescein-dUTP) and 5µl Enzyme Solution (TdT), coverslipped and incubated in 37°C for 1hr, in a humidified chamber in dark.
5) Finally, the slides were washed twice with 1X PBS for 10min each and the fluorescence signals for TUNEL +ve cells were checked under a fluorescence microscope.
6) The slides were dried and mounted with 80µl DABCO and either stored in -20°C or imaged with Nikon A1 confocal imaging system.

2.24. Western Blotting Assay.

Sample Preparation:

Reagents required:

1) 2X Laemmli Buffer

Components	**Amount**
10% SDS	4ml
Glycerol	2ml
1M TrisCl (pH 6.8)	1.2ml

Bromophenol Blue	0.002g
β-Mercaptoethanol	1ml

Volume was made up to 10ml with MilliQ. The buffer had a pH of 6.8.

Steps followed:

1) The retinae were harvested by the previously mentioned protocol in 1X PBS and suspended in 150-200µl 2X Laemmli Buffer in an MCT.
2) Using a sterile piston, the tissue was homogenised well so as to make the solution clear.
3) After homogenisation, the sample was vortexed for 30s with intermittent incubation on ice for 1min for total 20min.
4) This was followed by boiling the sample at 100°C for 10min on thermo-block.
5) The prepared samples were either stored in -20°C or used directly for Western Blotting experiment.

Western Blotting experiment:

Reagents and apparatus required:

1) Western Blotting Gel Apparatus
2) 30% Acrylamide:Bis-acrylamide Solution
   29.2g Acrylamide powder (Sigma) was mixed with 0.8g Bis-Acrylamide powder (Sigma). Shaken well and kept overnight in 37°C incubator for uniform and complete mixing.
3) 4X Resolving Buffer (1.5M TrisCl, pH 8.8)
   18.17g Tris-base was dissolved in 70ml MilliQ. Its pH was set to 8.8 with HCl and the volume was made up to 100ml with MilliQ.
4) 1X Stacking Buffer (1M TrisCl, pH 6.8)
   12.14g Tris base was dissolved in 70ml MilliQ. Its pH was set to 6.8 with HCl and the volume was made up to 100ml with MilliQ.
5) 10% SDS
6) 10% Ammonium Persulphate (APS)
7) TEMED
8) 10% Resolving Gel composition

Components	**Amount**
Resolving Buffer	2.5ml
30% Acrylamide	3.33ml

Milli Q water	3.97ml
10% SDS	100µl
10% APS	100µl
TEMED	6µl
Total volume	10ml

9) Stacking Gel composition

Components	**Amount**
Stacking Buffer	625µl
30% Acrylamide	667µl
Milli Q water	3603µl
10% SDS	50µl
10% APS	50µl
TEMED	5µl
Total volume	5ml

10) 1X SDS Running Buffer

Made from 10X SDS Running Buffer

Components	**Amount**
Tris Base	30.30g
Glycine	144.10g
SDS	10g

Mixed using magnetic stirrer bead and made volume up to 1L using MilliQ.

11) Transfer Buffer

Components	**Amount**
Methanol	400ml
Tris Base	6g
Glycine	28.8g

Mixed well and made the volume up to 2L using MilliQ and stored in 4°C.

12) PVDF Membrane (BioRad)

13) Methanol (For charging the PVDF membrane)

14) 1X PBS with 0.05% Tween-20

15) 1X PBS with 0.3% Tween-20

16) 5% or 10% Skimmed Milk

Made using 1X PBS with 0.05% Tween-20. Stored in 4°C, while longer storage done in -20°C.

17) 5% BSA in 1X PBS with 0.1% Tween-20

Steps followed:

1) The Gel casting glass plates were set and the Resolving Gel was casted between the glass plates.
2) The Resolving Gel was allowed to solidify, on it Stacking Gel was poured and gel combs were placed for forming the wells.
3) Once the Stacking gel got solidified, the gel comb was removed and the wells formed were washed with MilliQ.
4) Entire set-up was placed in the Electrophoretic tank half-filled with 1X SDS Running Buffer. 1X SDS Running Buffer was also filled between the glass plates.
5) To level the wells, 2X Laemmli Buffer was loaded in the wells.
6) The desired volumes of the tissue lysate samples were loaded in the wells and samples were subjected to electrophoresis under a constant voltage of 70-90V.
7) Once the dye-front reached the base of the glass plates, the run was switched off.
8) The gel was taken or cut out of the opened set-up of the Gel casting glass plates and washed thrice with water to remove SDS.
9) The gel was then rinsed and kept in the Transfer buffer.
10) In parallel, the required sized PVDF membrane was cut and activated by washing in Methanol for 5min, followed by 2 washes with Milli Q.
11) The proteins from the SDS gel were transferred to the PVDF membrane, mediated by the transfer buffer in ice cold conditions, for 2hrs at 70V.
12) After the successful transfer, blocking of the membrane was done either in the 5% or 10% Skimmed Milk, with constant slow shaking at room temperature for 1hr.
13) The blocking was followed by washing off the milk by 3 washes of 1X PBS with 0.05% Tween-20, for 10min each at room temperature with constant fast shaking.
14) Primary antibody against a particular antigen was diluted either in 5% or 10% Skimmed Milk or in 5% BSA in 1X PBS with 0.1% Tween-20 as per the recommended or the standardised dilutions.
15) The membrane/blots were incubated in this primary antibody either for 1-2hrs at room temperature or overnight in 4°C, with constant slow shaking.
16) After primary staining, the unbound antibody was washed off with 3 washes each of 10min with 1X PBS with 0.05% Tween-20, with constant fast shaking.
17) Secondary antibody conjugated with Horse Radish Peroxidase (HRP) was diluted in 1:10000 ratio in 1X PBS with 0.05% Tween-20.

18) The blots were incubated in Secondary antibody for 2hrs with constant slow shaking at room temperature.
19) After secondary staining, the unbound antibody was washed off with 3 washes each of 10min with 1X PBS with 0.3% Tween-20, with constant fast shaking.
20) Blots were developed using Chemiluminescence method of ImageQuant LAS4000.

2.25. Microscopy, cell counting, and statistical analysis and softwares.

The Nikon N*i*-E fluorescence microscope installed with fluorescence optics and Nikon A1 confocal imaging system was used for the retinal imaging experiments. The *in situ* signal bearing cells were visualised and imaged using the Bright field microscopy in the same microscope. The $PCNA^+$, $BrdU^+$, Cell-type specific $antigen^+$, other $protein^+$ and $TUNEL^+$ cells were visualised and counted directly by looking at the fluorescence from the stained retinal sections. Each section of the retinal tissue was considered for counting for the fluorescence and ISH signals. Three or more retinae were used for each experiment.

The densitometry plots made for the western blot data and the fluorescence intensity plots made for a few immunohistochemistry images were done using ImageJ image processing & analysis software.

The statistical analysis of the data for all the experiments was done using a two-tailed unpaired students' *t*-test. Comparison based studies were done using Analysis of Variance test (ANOVA), and further a Bonferroni/Dunn *post hoc t*-test was performed using Stat View Software. Error bars in all the histograms represent the standard deviation in between the different datasets.

2.26. Chromatin Immuno-Precipitation (ChIP) Assay.

1) The experimental retina was harvested, suspended in 200µl 1X PBS/1mM PMSF/1X PI cocktail solution and was homogenised by pipetting.

2) The cells were crosslinked immediately by adding Formaldehyde to 1% v/v final concentration, gently tapped and incubated for 10min at room temperature with slow rotation.
3) Glycine was added to make its final concentration to 0.125M, to quench the formaldehyde. The tube was tapped gently and incubated on ice for 5min. From this step onwards, all the steps were performed on ice or in 4°C condition.
4) The tube was centrifuged at 1730rpm for 10min at 4°C, to sediment the debris and the cells from the chorion. The supernatant was carefully removed and discarded with a 1ml pipette with a cut tip.
5) 500µl PBS/PMSF/PI cocktail solution was added to the pellet and cells were resuspended by gentle tapping. It was again centrifuged at 1730rpm for 10min at 4°C and supernatant was discarded.
6) Another 500µl PBS/PMSF/PI cocktail solution was added to the pellet, cells were again resuspended by gentle tapping. It was again centrifuged at 1730rpm for 10min at 4°C and supernatant was discarded.
7) The cells were stored as a dry pellet at -80°C or proceeded further for the next steps.
8) 600µl of Nuclear Lysis Buffer was added to the pellet and it was resuspended by rotation/ rubbing by hands while avoiding too much frothing.

   Nuclear Lysis Buffer Composition: For 10ml

Components	**Amount**	**Final Concentration**
1M TrisCl pH 7.5	500µl	50mM TrisCl
0.5M EDTA pH 8.0	200µl	10mM EDTA
20% SDS	500µl	1% SDS

9) The tissue was sonicated for 8X 30s with 30s pauses on ice between each rounds of sonication. This was performed with a power of 4.3.
10) The sonicated sample was centrifuged at 13000rpm for 10min at 4°C and supernatant was removed and placed in a clean tube. From this, 100µl sample was collected and stored as Input Control in -80°C.
11) From the remaining sonicated sample, 2µl was checked on 0.8% Agarose gel run along a ladder and its concentration was also measured, using Nuclear Lysis Buffer as the blank.
12) The sample was diluted with 250µl or more IP Dilution Buffer/PMSF/PI cocktail solution (to make up the sample volume up to 1ml). This was done to increase the rotation and binding efficiency after antibody mixing.

IP Dilution Buffer Composition: For 10ml

Components	Amount	Final Concentration
1M TrisCl pH 7.5	167µl	16.7mM TrisCl
0.5M EDTA pH 8.0	24µl	1.2mM EDTA
5M NaCl	334µl	167mM NaCl
20% SDS	5µl	0.01% SDS
100% Triton X-100	110µl	1.1% Triton X-100

13) To this above solution, 2µg of the desired antibody was added and allowed to rotate and mix overnight at 4°C.
14) 20µl of Protein Agarose A beads (BioRad) were added to the above solution and allowed to interact by rotation for 2hrs at 4°C.
15) After 2hrs, the tube was centrifuged for 1s and the immune complexes were captured by placing the tube in the chilled magnetic rack.
16) The supernatant was discarded and the pellet was resuspended in 500µl of ice-cold IP Dilution Buffer/PMSF/PI cocktail solution by gentle manual agitation. Then the tube was rotated on a rotator at 40rpm for 4min at 4°C.
17) Step 15 was repeated.
18) The supernatant was discarded and pellet was washed in Wash Buffer/PMSF/PI cocktail solution. The complex was resuspended by gentle manual agitation. Then the tube was rotated on a rotator at 40rpm for 4min at 4°C.
19) Step 17 was repeated.

Wash Buffer Composition: For 10ml

Components	Amount	Final Concentration
1M TrisCl pH 8.0	1ml	100mM TrisCl
0.5M EDTA pH 8.0	40µl	2mM EDTA
5M LiCl	1ml	500mM LiCl
100% NP-40	100µl	1% NP-40

20) The supernatant was removed and 500µl TE Buffer was added to the beads and these were incubated on rotator for 4min at 4°C.

TE buffer Composition:

Components	Amount
1M Tris Cl (pH 8.0)	100mM
0.5M EDTA (pH8.0)	1mM

21) The supernatant was removed.

22) To the ChIP reaction beads as well as to the Input Control, 150µl ChIP Elution Buffer was added, tapped gently and incubated on thermomixer at 1300rpm for 2hrs at 37°C.

ChIP Elution Buffer Composition: For 5ml

Components	**Amount**	**Final Concentration**
1M TrisCl pH 7.5	100µl	20mM TrisCl
0.5M EDTA pH 8.0	50µl	5mM EDTA
5M NaCl	50µl	50mM NaCl
20% SDS	7.5µl (For 150µl)	1% SDS
10mg/ml Proteinase K	0.75µl (For 150µl)	50µg/ml

SDS and Proteinase K enzyme were added just before the use.

23) The tube was spin down for 1s, beads were captured in the magnetic rack at room temperature and the eluate from the tube was transferred to a clean tube.
24) 150µl ChIP Elution Buffer was again added to the beads, and incubated for 15min on thermomixer as in step 21.
25) The tube was again spin down for 1s, beads were captured in the magnetic rack and the eluate from the tube was removed and pooled with the first eluate from the step 22.
26) To the pooled eluate (300µl), 200µl ChIP Elution Buffer was added.
27) Equal volume (as that of the sample) of PCI was added to the Input chromatin as well as to the Pull-down chromatin samples. Vortexed and centrifuged at 13000 for 5min at room temperature.
28) The aqueous phase was transferred to a fresh tube and step 26 was repeated with Chloroform.
29) Finally, to the aqueous phase collected now, 1µl Glycerol, 1/10th volume of 3N Sodium Acetate, twice the volume of Ethanol was added to precipitate. Samples were tapped and precipitated overnight in -80°C.
30) Then the samples were centrifuged for 30-40min at 13,400rpm at 4°C.
31) Later the pellet obtained was washed twice with 500µl of 70% Ethanol by tapping, followed by centrifugation at 13,400rpm for 10min at 4°C.
32) The pellet was dried at room temperature and dissolved in 5-6µl Nuclease Free Water.
33) Using this DNA as the template, under normal PCR conditions, PCR was done and the binding sites were analysed as per the amplicon obtained in pull down samples along with the Input control and the negative control.

Section 3.

Results

Chapter 1

Pten induction after retinal injury and then its downregulation is necessary during zebrafish retina regeneration.

3.1.1. Pten is induced in the retina post retinal injury.

In zebrafish, Pten gets translated by two genes: *ptena* and *ptenb,* which resulted from genome-wide evolutionary duplication in teleosts (Croushore et al., 2005). The proteins Ptena and Ptenb share 80% amino acid sequence similarity as analysed by CLUSTALW sequence alignment software (Fig 3.1.1.1 A), and are known for their roles during zebrafish embryogenesis, tumorigenesis and maintaining homeostasis (Croushore et al., 2005; Faucherre et al., 2008; Choorapoikayil et al., 2012).

In order to unravel the role of Pten during zebrafish retina regeneration, we first checked whether the *ptena* and *ptenb* transcripts got regulated upon retinal injury, both spatially and temporally. For this, the fish retina was injured by needle stab method, and mRNA was isolated from retina harvested at different hours post injury (hpi), days post injury (dpi) and from the retina without any injury (uninjured control or uc). Using these RNA samples, first strand cDNA was synthesised and these cDNAs were subjected to quantitative Polymerase Chain Reaction (qPCR) using gene-specific primers, to analyse the relative change in transcript levels of *ptena* and *ptenb* post retinal injury. *β-actin* was used as the normalisation control. We observed dual peaks of induction of *ptena* (Fig 3.1.1.1 B) and *ptenb* (Fig 3.1.1.1 C), with a higher peak of induction in the early phase of reprogramming soon after the injury (dedifferentiation phase) and a shorter one during the proliferative phase at 4dpi as compared to the uninjured control, while the second peak was not that significant for *ptenb*. This suggests that Pten, which is necessary for maintaining cellular homeostasis, gets regulated after retinal injury. This happens probably to restore the normalcy of the tissue by allowing the Müller Glia (MG) cells to proliferate at a reasonable rate during the proliferative phase.

A

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Ptena           MTAKLKEIVSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLDSK
Ptenb           MAAIIKEFVSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLDSK
                *:* :**:**************************************************

Ptena           HKNHYKIYNLCAERHYDASKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSEDENHVA
Ptenb           HKDHYKIYNLCAERHYDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDKWLSENDNHVA
                **:**************::*******************************:****::****

Ptena           AIHCKAGKGRTGVMICAYLLHRKKFAEAQEALDFYGEVRTRDKKGVTIPSQRRYVYYYSY
Ptenb           AIHCKAGKGRTGVMICAYLLHRGKFKKAQEALDFYDEVRTRDKKGVTIPSQRRYVYYYSY
                ********************** ** :********.***********************

Ptena           LLKNKLDYKPVALLFHKMVFQTLPMFSGGTCRDSTVKNRSEVNPQGFKKGSWHWDPQFVV
Ptenb           LLRNKLEYKPVALLFHKMVFETVPMFSGGTCRDSAVKSKSEQIPHGFRKTNWHWDPQFVV
                **:***:*************:*:***********:**.:**  *:**:* .*********

Ptena           YQLKVKIHTSNPAHTRREEKHMIFEFPQPLPVCGDIKVEFFHKQSKMMKKDKMFHFWINT
Ptenb           YQLKVKIHTSNPAHTRREEKYMFFEFPQPLPVCGDIKVEFFHKQNKMMKKEKMFHFWVNT
                ********************:*:**********************.*****:******:**

Ptena           FFIPGPEEAGDKVENGSVVNDVDSLSCQSQSSPAERDREKERAVAAAALDRERGEQRADG
Ptenb           FFIPGSEESTEKVENGGLVKDLDGIQTAE--------------------------RGEN
                *****.**: :*****.:*:*:*.:.  .                           *.:.

Ptena           EKDYLILTLTKNDLDKANKDKANRYFSPNFKVKLYFTKTVEEPSNSEASTSTSVTPDVSD
Ptenb           DKDYLILTLSKTDLDKANKDKANRYFSPNFKVKMFFTKTVEEPSNSEASTSTSVTPDVSD
                :********:*.*********************::************************

Ptena           NEPDHYRYSDTTDSDPENEPFDEEQHAQITKV
Ptenb           NEPDHYRYSDTTDSDPENEQYDEEQITKV---
                ****************** :**** :::
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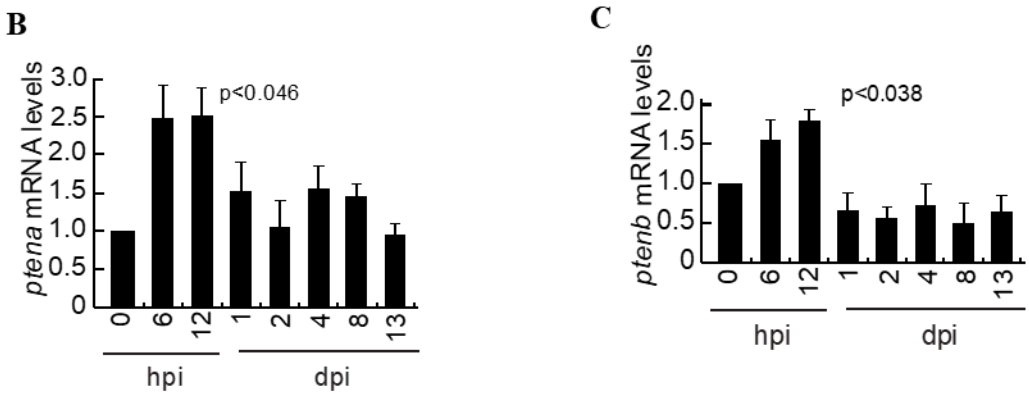


Fig 3.1.1.1 Pten is induced in the zebrafish retina post retinal injury. (**A**) CLUSTALW analysis shows the amino acid sequence alignment between zebrafish Ptena and Ptenb proteins. (**B** and **C**) The qPCR analyses of *ptena* (**B**) and *ptenb* (**C**) genes in the retina at various time points post-retinal injury; p<0.04, n=5 biological replicates. hpi, hours post injury; dpi, days post injury.

3.1.2. Pten is induced in retina post-injury but remains secluded from the MGPCs.

As we saw that both the *pten* transcripts showed temporal regulation after retinal injury, we went ahead to examine the spatial localisation of *ptena* and *ptenb* in the injured retinal tissues. For this, the cryosections of retinae prepared from eyes fixed at various times post injury were used, and mRNA *in situ* hybridisation was performed on them. Intriguingly, we found that *ptena* and *ptenb* genes showed an exclusion from the proliferating population of Müller Glia Progenitor Cells (MGPCs) marked by Proliferating Cell Nuclear Antigen (PCNA) in the retina at 2, 4 and 6dpi. The quantitative analysis also revealed a significantly reduced co-labelling of *ptena* (Fig 3.1.2.1 A, B) and *ptenb* (Fig 3.1.2.1 C, D) expression with $PCNA^+$ MGPCs. Interestingly, the cells flanking MGPCs seemed to express more *pten* mRNAs. We also observed that the MGPCs entering the M-phase labelled by phospho Histone 3 (pH3) also had negligibly low levels of *ptena* (Fig 3.1.2.2 A, B) and *ptenb* (Fig 3.1.2.2 C, D) genes, also validated quantitatively.

We checked if this exclusion shown at the gene level was also validated at the protein level. The immunohistochemistry for the Pten protein was done on the cryosections prepared from retinae collected at 4dpi and uninjured control, using the antibody against the Pten protein. We observed that while Pten was uniformly expressed in the uninjured retina, its expression was significantly secluded from the $PCNA^+$ MGPCs in 4dpi retina (Fig 3.1.2.3 A, B). These results of the expression status of Pten obtained from the wild-type (WT) fish were further validated in *1016tuba1a*:GFP, a transgenic line of zebrafish, already stabilised in our zebrafish facility. The α-tubulin family of proteins, belonging to tubulin protein superfamily, polymerises with other members of the superfamily to form microtubules crucial for mitosis. The α-tubulin family has various isoforms, out of which one is *tuba1a* gene, which expresses mainly during the neuronal development. A fragment of 1016bp region of *tuba1a* gene promoter fused with GFP which expresses specifically in the CNS of the developing zebrafish embryo has been reported to be also expressed in the proliferating MGPCs of zebrafish retina (Ramachandran et al., 2010b). Thus, this 1016bp fragment of tuba1a promoter also acts as a reporter for the proliferating MGPCs (Kaur et al., 2018; Mitra et al., 2019; Sharma et al., 2020). In concurrence with the trend of Pten expression in the WT zebrafish retina, we found the expression of Pten to be significantly diminished in the GFP^+ cells in the retina of transgenic fish at 4dpi, by immunohistochemistry (Fig 3.1.2.2 C, D). Although reduced levels of Pten protein was seen in

the proliferating MGPCs, western blotting assay showed no such regulation or change in its levels in the entire retinal tissue on different time points post retinal injury (Fig 3.1.2.3 E). This might be due to the meagre level of representation of MGPCs formed in the retina by needle poke method of injury and due to the role of Pten in maintaining tissue homeostasis.

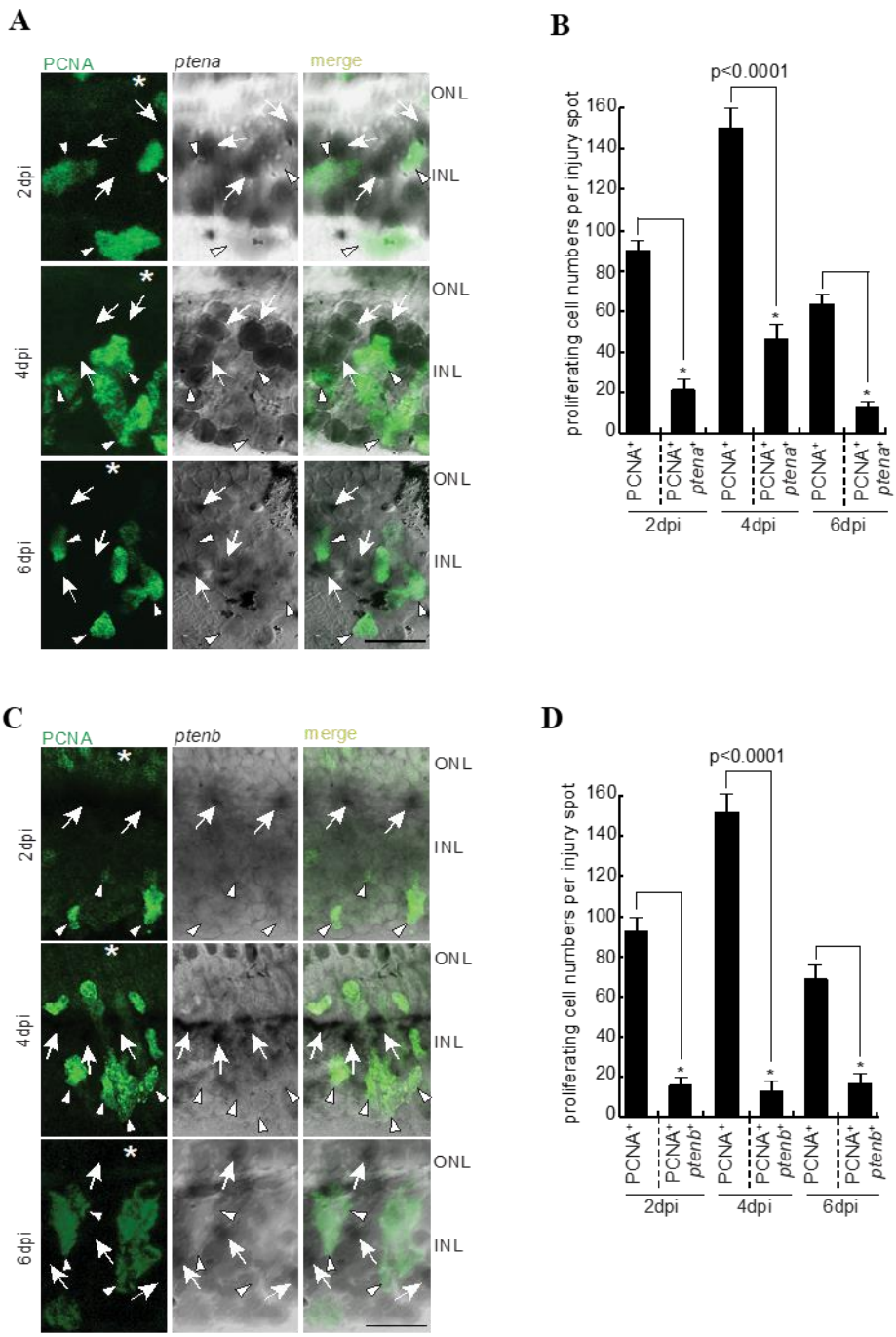


Fig 3.1.2.1 *ptena* and *ptenb* are expressed in the whole retina and secluded from the proliferating MGPCs. (**A** and **B**) Brightfield (BF) and Confocal microscopy images (high-magnification) of retinal cross-sections show the mRNA *in-situ* hybridisation (ISH) which reveals the expression of *ptena* mRNA in the neighbouring cells of PCNA$^+$ MGPCs at 2dpi, 4dpi and 6dpi (**A**), which is quantified (**B**); *p < 0.0001; n=6 biological replicates. (**C** and **D**) BF and Confocal microscopy images (high-magnification) of retinal cross-sections show the mRNA ISH which reveals the expression of *ptenb* mRNA in the neighbouring cells of PCNA$^+$ MGPCs at 2dpi, 4dpi and 6dpi (**C**), which is quantified (**D**); *p < 0.0001; n=6 biological replicates. Scale bars represent 10µm in (**A**, **C**); the asterisk marks the injury site and INL, inner nuclear layer; ONL, outer nuclear layer (**A**, **C**); white arrowheads mark PCNA$^+$/*ptena*$^-$ (**A**), PCNA$^+$/*ptenb*$^-$ (**C**) cells; white arrows mark *ptena*$^+$/PCNA$^-$ (**A**), *ptenb*$^+$/PCNA$^-$ (**C**) cells; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**, **C**).

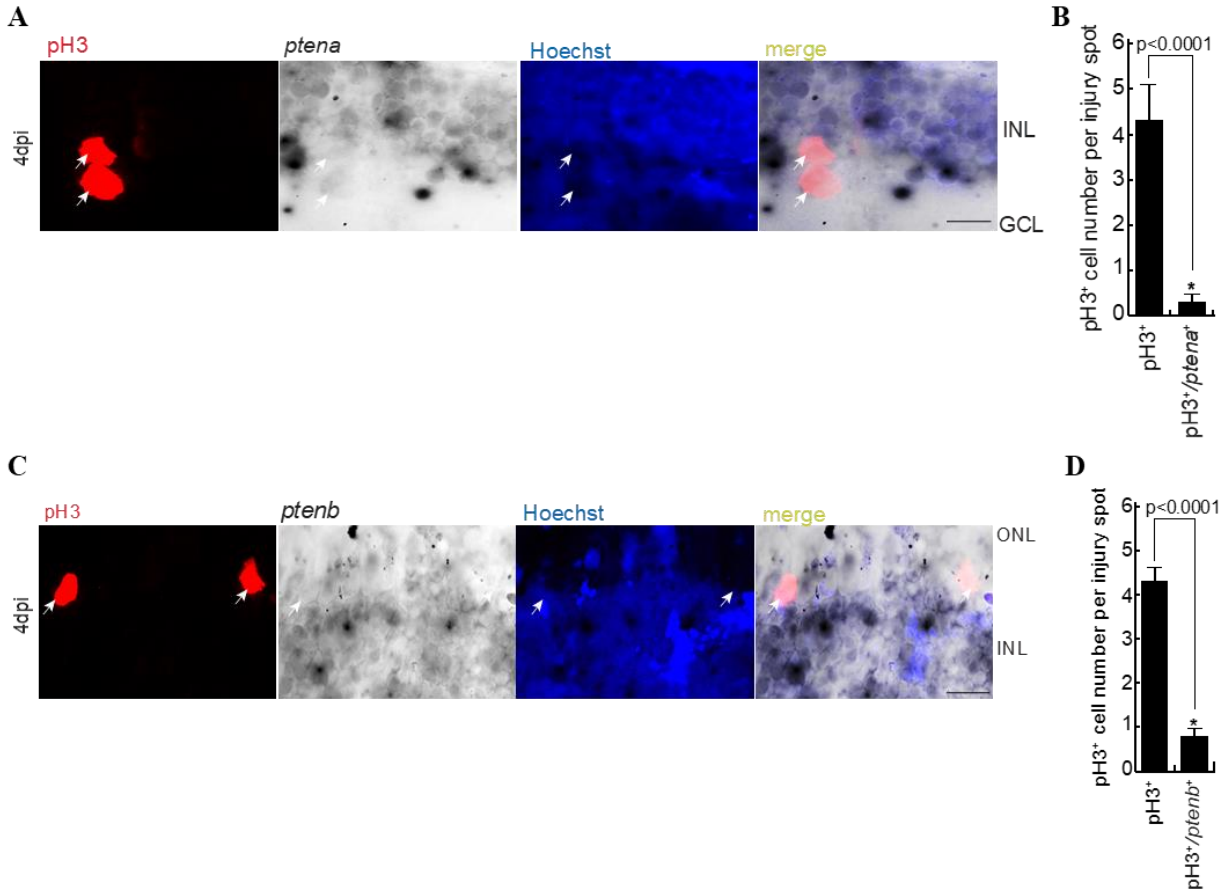


Fig 3.1.2.2 *ptena* and *ptenb* expression is also majorly secluded from the pH3$^+$ MGPCs. (**A** and **B**) Brightfield (BF) and Confocal microscopy images (high-magnification) of retinal cross-sections show the mRNA *in-situ* hybridisation (ISH) which reveals the expression of *ptena* mRNA in the neighbouring cells of pH3$^+$ MGPCs at 4dpi (**A**), which is quantified (**B**); *p < 0.0001; n=6 biological replicates. (**C** and **D)** Brightfield (BF) and Confocal microscopy images (high-magnification) of retinal cross-sections show the mRNA *in-situ* hybridisation (ISH) which reveals the expression of *ptenb* mRNA in the neighbouring cells of pH3$^+$ MGPCs at 4dpi (**C**), which is quantified (**D**); *p < 0.0001; n=6 biological replicates. Scale bars represent 10µm in (**A**, **C**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**, **C**); white arrows mark pH3$^+$/*ptena*$^-$ (**A**), pH3$^+$/*ptenb*$^-$ (**C**) cells. Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**, **C**). Hoechst staining is the reference nuclear staining.

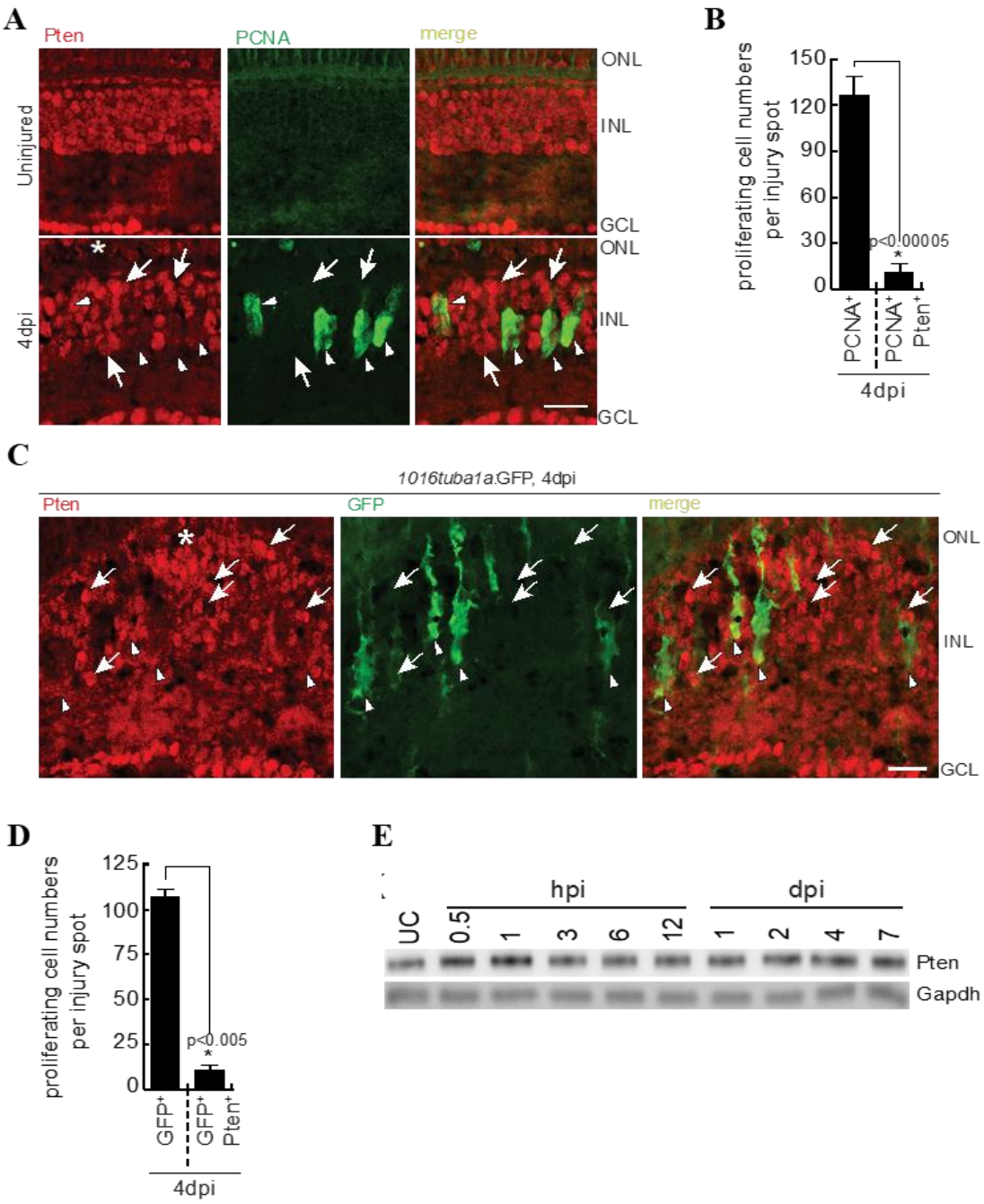


Fig 3.1.2.3 Pten protein is expressed in the whole retina and secluded from the MGPCs. (**A** and **B**) Confocal microscopy images of retinal cross-sections show the pan-retinal expression of Pten in the uninjured retina, while being highly reduced in the PCNA^{+} MGPCs in 4dpi retina (**A**), which is quantified (**B**); *p < 0.00005; n=6 biological replicates. (**C** and **D**) Confocal microscopy images of a retinal cross-section show reduced expression of Pten in the GFP^{+} MGPCs of *1016tuba1a*:GFP at 4dpi (**C**), which is quantified (**D**); *p < 0.005; n=3 biological replicates. (**E**) Western Blot analysis of Pten from retinal lysates prepared at different time points post-injury. Gapdh is the loading control. Scale bars represent 10µm in (**A**, **C**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**, **C**); hpi, hours post injury; dpi, days post injury; white arrowheads mark PCNA^{+}/Pten^{-} (**A**), GFP^{+}/Pten^{-} (**C**) cells; white arrows mark Pten^{+}/PCNA^{-} (**A**) and Pten^{+}/GFP^{-} (**C**) cells; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**, **C**).

3.1.3. Downregulation of Pten facilitates increase in the MGPCs proliferation.

Our previous results gave us a clue that Pten might have anti-proliferative properties during zebrafish retina regeneration also, since Pten was found to be majorly secluded from the MGPCs. Thus, we intended to tweak the levels of functional Pten in the zebrafish retina and then study its effects on the MGPCs proliferation. For this, we adopted two approaches to reduce the levels and efficiency of Pten protein. In the first one, we used the Morpholino (MO)-based gene knockdown approach. Morpholinos are antisense oligonucleotide stretches which work on the principle of RNA interference. In our study, we used a translational blocker MO which is a strand of oligonucleotides which binds to the complimentary target mRNA sequence. This sterically hinders the translation initiation machinery from translating the mRNA to a functional protein. The MO is structurally different from the nucleic acids since it replaces ribose or deoxyribose sugars with methylenemorpholine ring. MO is also tagged with a fluorescently labelled molecule Lissamine, which makes it easy to track the entry of MO into the cell. Being positively charged Lissamine allows the entry of negatively charged MO oligo into a negatively charged nucleus mediated by microinjection and electroporation (Moulton & Yan, 2008). In our second approach, we used a small molecule pharmacological inhibitor of Pten protein function SF1670 (Spinelli et al., 2015), to ensure the blockade of all the functional Pten protein in the cell.

We first checked the effects of knocking down *ptena* and *ptenb* genes on the MGPCs proliferation. For this, we injured the retina and injected and electroporated the various concentrations of the MOs against *ptena* and *ptenb* genes in it at the time of injury (Fig 3.1.3.1 A). We observed a moderate increase in the number of proliferating MGPCs labelled with BrdU, upon treatment of zebrafish retina with 0.25mM and 0.5mM *ptena* and *ptenb* MOs, while a drastic enhancement in the number of $BrdU^+$ MGPCs was seen at 4dpi, upon *ptena* and *ptenb* knockdowns using maximum concentration (1mM) of *ptena* and *ptenb* MOs separately in the retina (Fig 3.1.3.1 B, C; Fig 3.1.3.2 A, B). BrdU, denoted for Bromodeoxyuridine, is a synthetic thymidine analogue used to detect the proliferating cells in the living tissues. It gets incorporated in the newly synthesised DNA of the replicating cells during S-phase of the cell cycle, substituting for thymidine during DNA replication and can be detected by BrdU assay using the antibody against BrdU. Further, the double knockdown of *ptena* and *ptenb* genes had a more pronounced effect on the MGPCs proliferation in the retina at 4dpi (Fig 3.1.3.3 A, B).

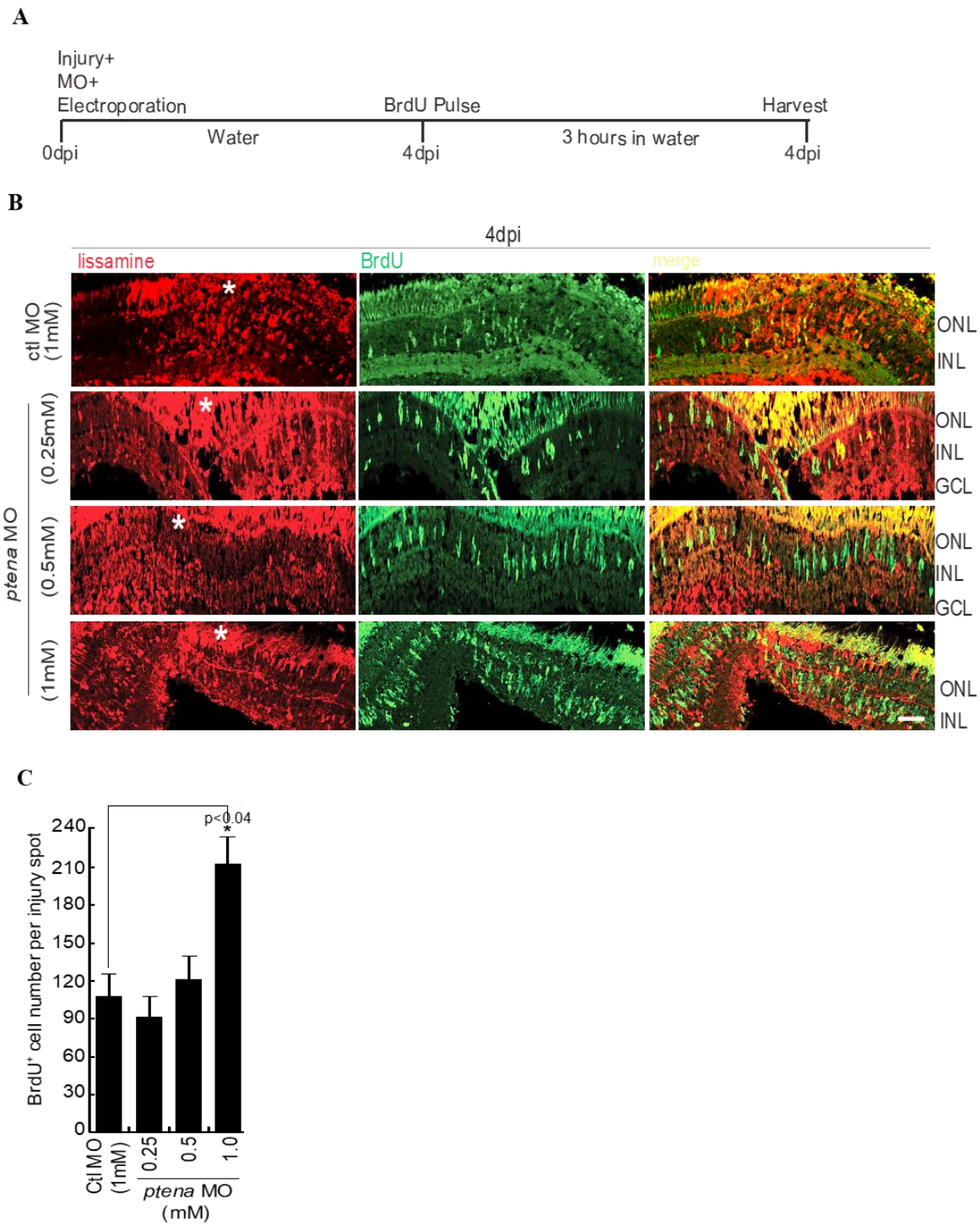


Fig 3.1.3.1 Knockdown of *ptena* enhances MGPCs proliferation. (**A**) An experimental timeline that describes injury, Morpholino (MO) delivery, electroporation at 0dpi, BrdU pulse for 4hrs at 4dpi, followed by harvesting after 3hrs dipping in water. (**B** and **C**) Confocal microscopy images of retinal cross-sections show an increase in the number of $BrdU^+$ MGPCs with the increasing concentrations of *ptena* MO (Lissamine tag) at 4dpi (**B**), which is quantified (**C**); *$p < 0.04$; n=6 biological replicates. Scale bars represent 10µm in (**B**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**B**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**B**).

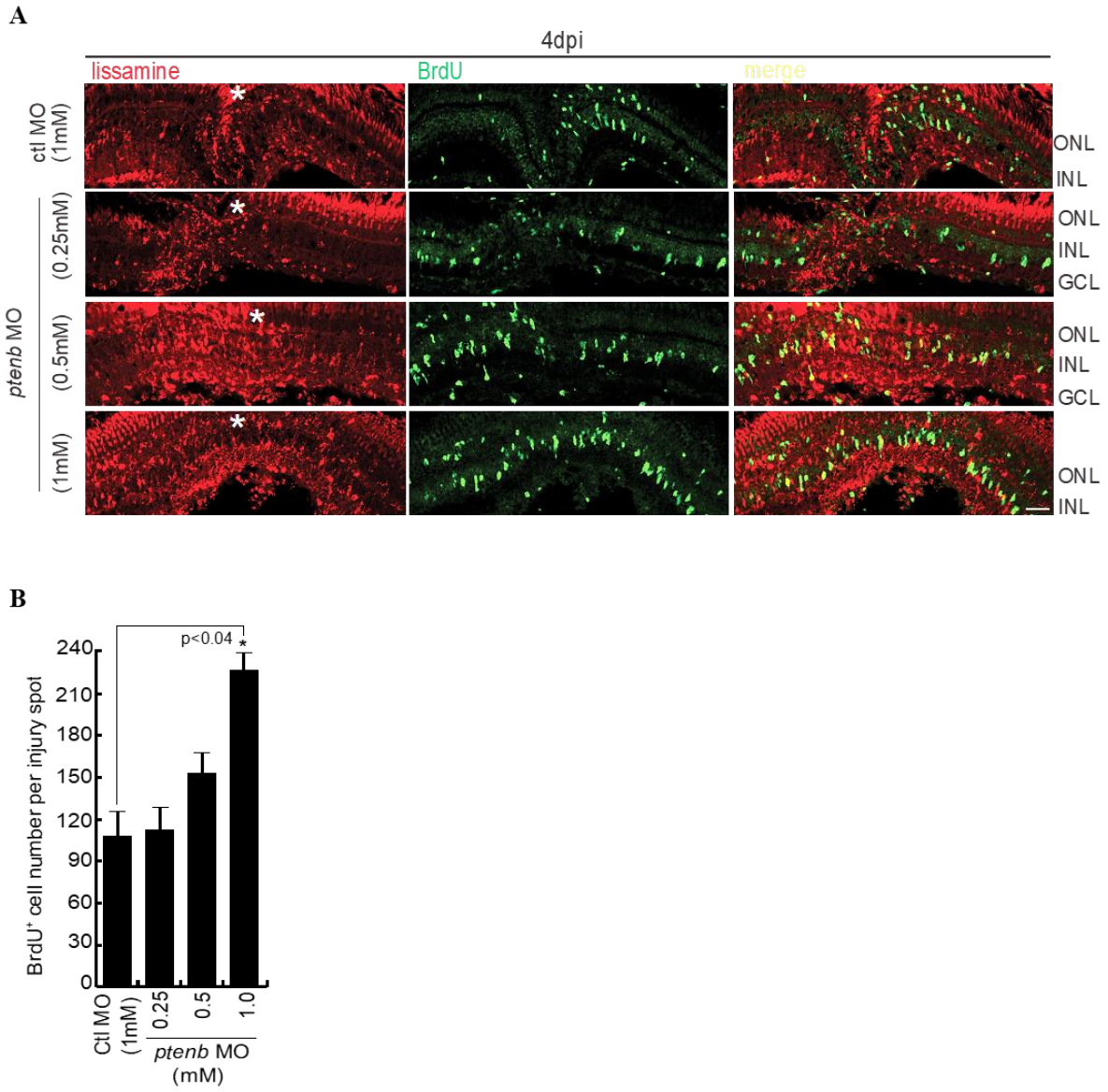


Fig 3.1.3.2 Knockdown of *ptenb* enhances MGPCs proliferation. (**A** and **B**) Confocal microscopy images of retinal cross-sections show an increase in the number of $BrdU^+$ MGPCs with the increasing concentrations of *ptenb* MO (Lissamine tag) at 4dpi (**A**), which is quantified (**B**); *p < 0.04; n=6 biological replicates. Scale bars represent 10µm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**).

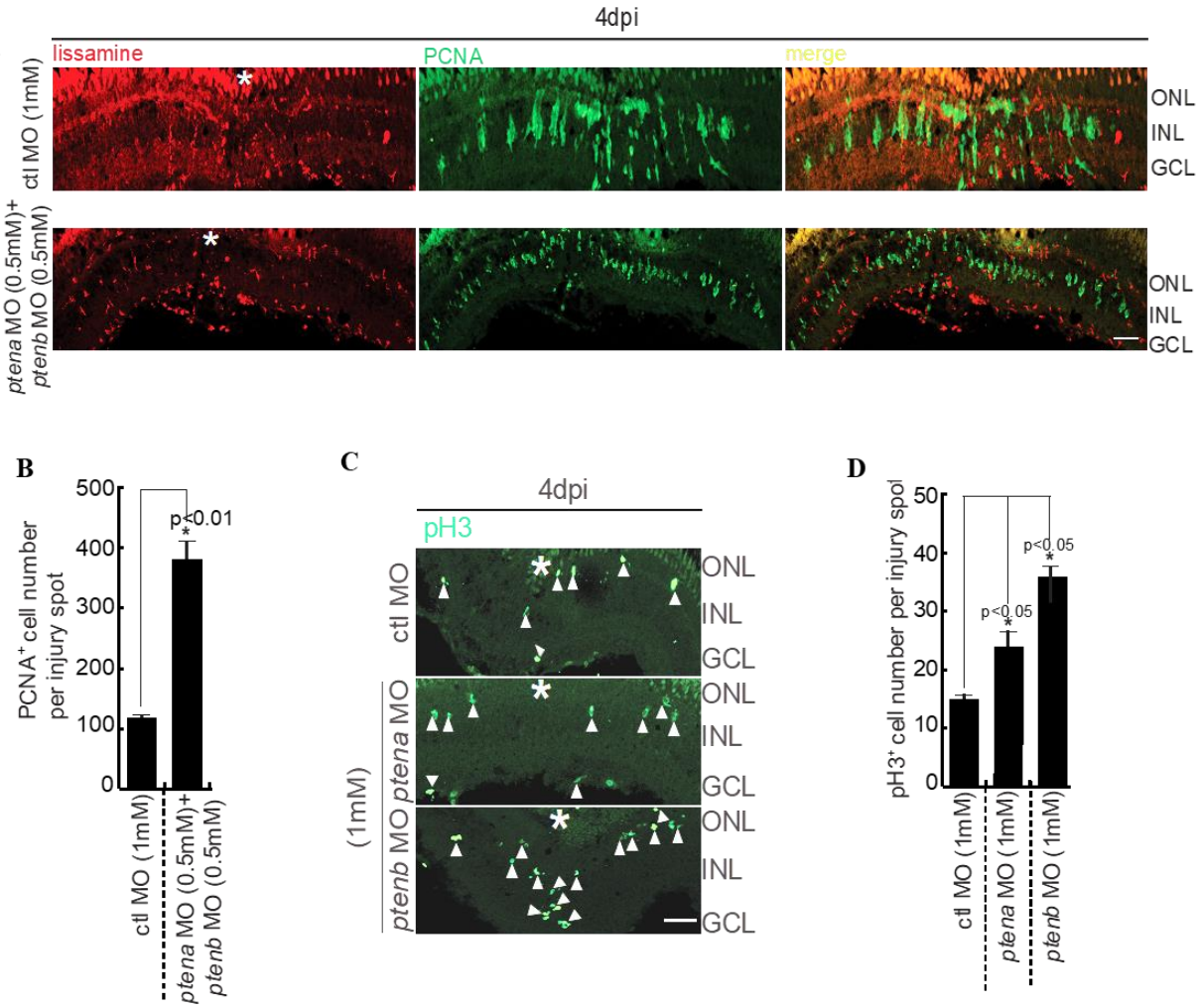


Fig 3.1.3.3 Knockdown of *pten* genes enhances the number of MGPCs entering S-phase and also in M-phase. (**A** and **B**) Confocal microscopy images of retinal cross-sections show an increase in the number of PCNA$^+$ MGPCs in the combined knockdown of *ptena* and *ptenb* in retina at 4dpi (**A**), which is quantified (**B**); *p < 0.01, n=3 biological replicates. (**C** and **D**) Confocal microscopy images of retinal cross-sections show an increase in the number of mitotically active pH3$^+$ cells in *ptena* and *ptenb* knockdown in retina at 4dpi (**C**), which is quantified (**D**); *p < 0.05, n=3 biological replicates. Scale bars represent 10µm in (**A, C**); arrowheads mark the pH3$^+$ cells in (**C**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A, C**); dpi, days post injury. Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A, C**).

Besides studying the effects of *pten* genes on the MGPCs in S-phase, we were also fascinated to check their impact on the mitotically active MGPCs. For this, we knocked down *ptena* and *ptenb* in the retina at the time of injury and checked the effect on $pH3^+$ cells. We observed an increase in the $pH3^+$ cells reflective of enhanced active mitosis upon *pten* knockdown (Fig 3.1.3.3 C, D).

We wondered if overexpression of these *pten* genes could reverse the impact as shown by knocking them down. For this, we overexpressed both *ptena* and *ptenb* genes together by *in vivo* mRNA transfection into the injured retina along with a *gfp* mRNA reporter at 4dpi. This drastically reduced the number of proliferating MGPCs in the retina at 4dpi (Fig 3.1.3.4 A, B). These results suggest that *pten* genes keep a check on the rate and span of MGPCs proliferation.

We then tried to rescue the effect of *ptenb* knockdown, by overexpressing the *ptenb* mRNA in the retina with the knocked down *ptenb* gene at 4dpi. For this, we mutated the *ptenb* MO-binding site (BS) by Site-directed Mutagenesis (SDM) on the plasmid DNA having *ptenb* CDS cloned in pCS2+ vector. This could be later *in vitro* transcribed to a functional *ptenb* mRNA and transfected into the retina along with the *ptenb* MO electroporation and *gfp* mRNA reporter at 4dpi. Unlike an increase seen in the number of MGPCs upon *ptenb* knockdown, we observed the number of MGPCs equivalent to that of the control in the rescue experiment (Fig 3.1.3.5 A, B). The rescue experiment consolidated the trends observed using *ptenb* MO, also ruling out its off-target effects in the regenerating retina. Similarly, we could successfully rescue the effects of *ptena* MO by overexpressing *ptena* mRNA in the retina with the knocked down *ptena* gene at 4dpi (Fig 3.1.3.6 A, B).

Further, as per our second approach to dissect out the effect of Pten on the regenerating retina, we blocked its function using the pharmacological inhibitor SF1670. For this, we injected the different concentrations of the drug in the retina at the time of injury. We obtained a similar set of results as with the *pten* gene knockdown, where a drug dose-dependent enhancement in the number and the span of the proliferating MGPCs marked by PCNA was seen in the retina at 4dpi (Fig 3.1.3.7 A, B). These results were validated in the *1016tuba1a*:GFP transgenic zebrafish retina also. The transgenic retina showed an increase in the GFP expression upon Pten blockade with SF1670 at 4dpi, supporting the increased number of MGPCs as seen in the WT zebrafish retina (Fig 3.1.3.7 C).

Interestingly, Pten blockade by SF1670 did not mount any proliferative response in the uninjured retina (Fig 3.1.3.8 A). This is suggestive of the fact that injury which is the initial

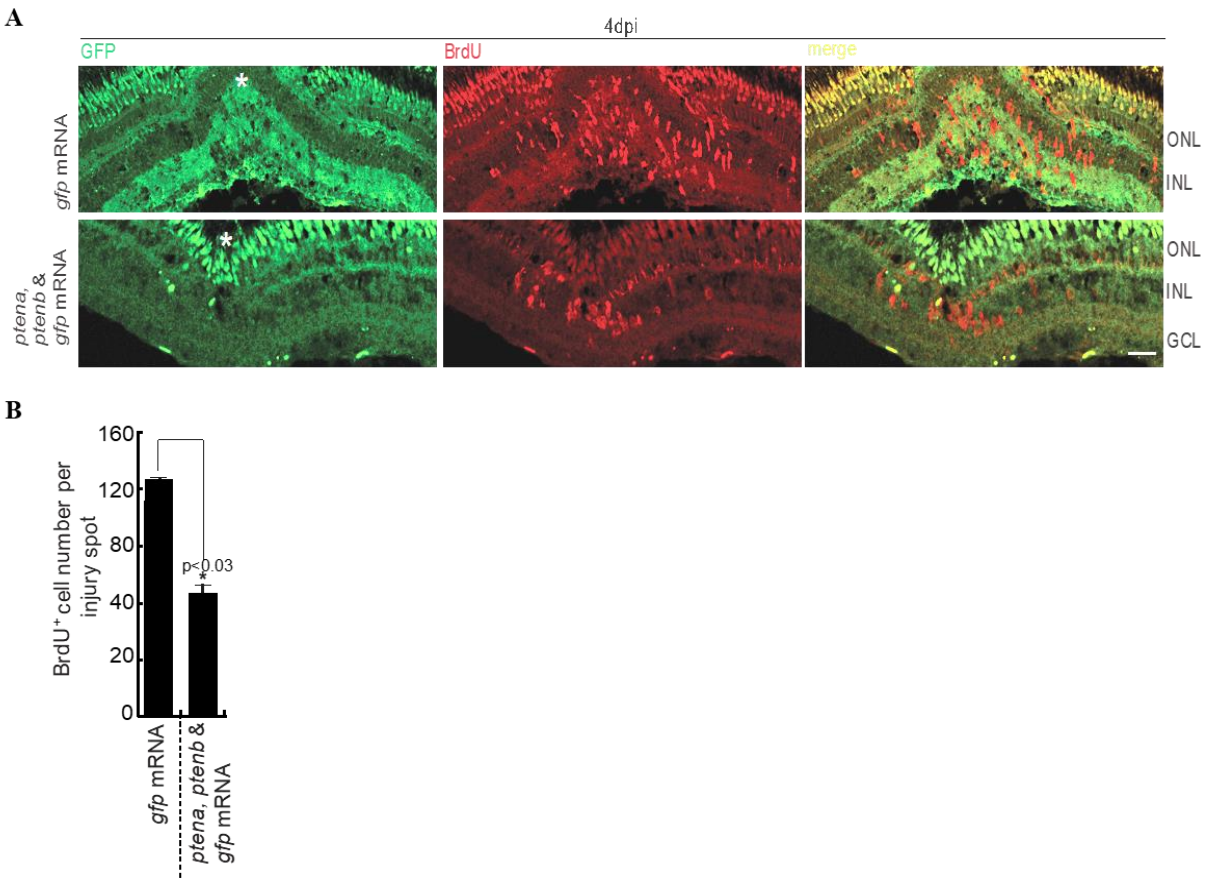


Fig 3.1.3.4 *pten* overexpression keeps a check on MGPCs proliferation. (**A** and **B**) Confocal microscopy images of retinal cross-sections show the reduction in the number of $BrdU^+$ MGPCs upon *pten* gene overexpression in retina at 4dpi (**A**), which is quantified (**B**); *p < 0.03, n=3 biological replicates. Scale bars represent 10µm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**).

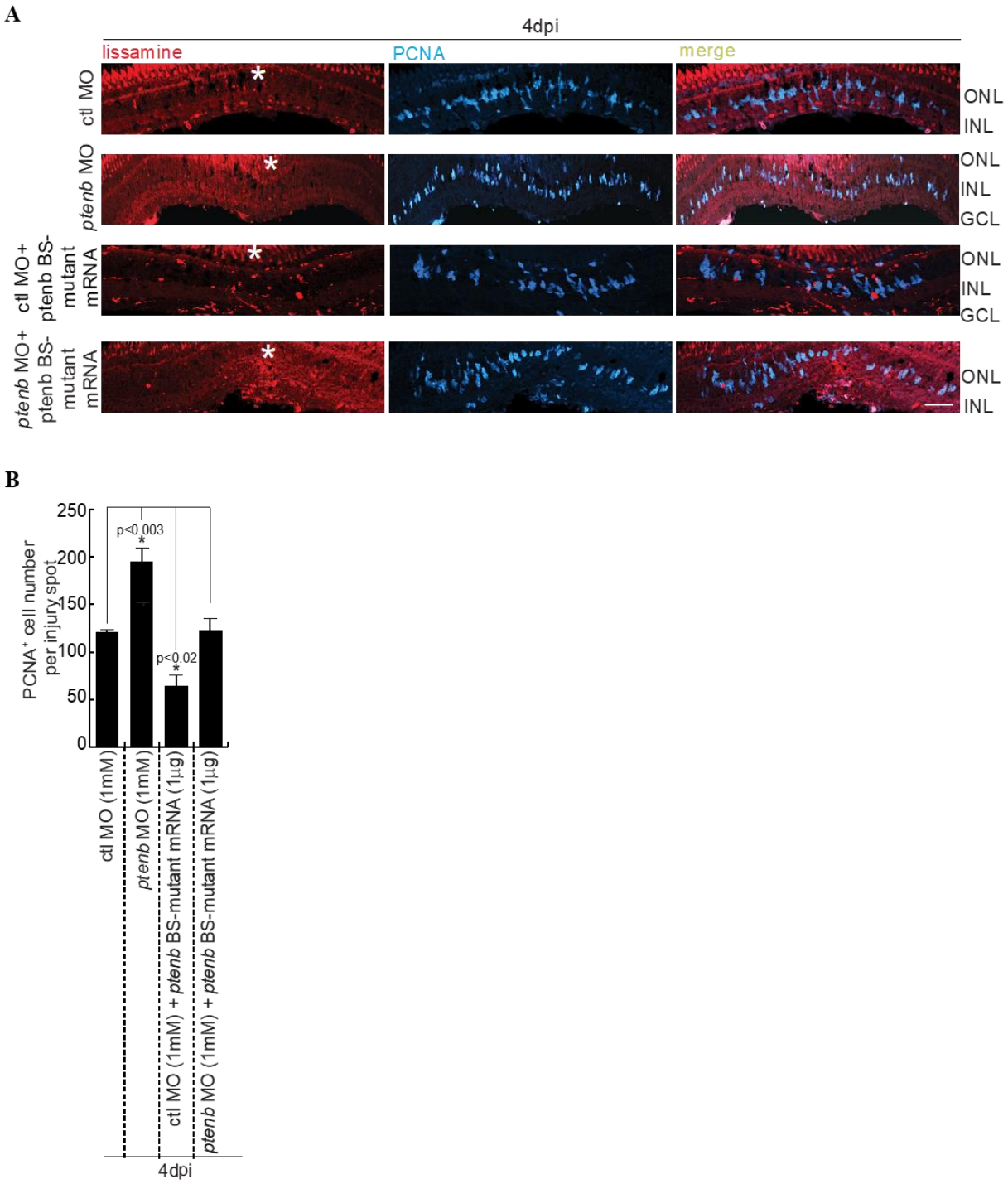


Fig 3.1.3.5 Effect of *ptenb* MO in regenerating retina can be rescued. (**A** and **B**) Confocal microscopy images of retinal cross-sections show the rescue of *ptenb* MO effect by the *ptenb* MO-binding site mutated mRNA transfected in retina at 4dpi (**A**), which is quantified (**B**); *p < 0.015, n=3 biological replicates. Scale bars represent 10µm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**).

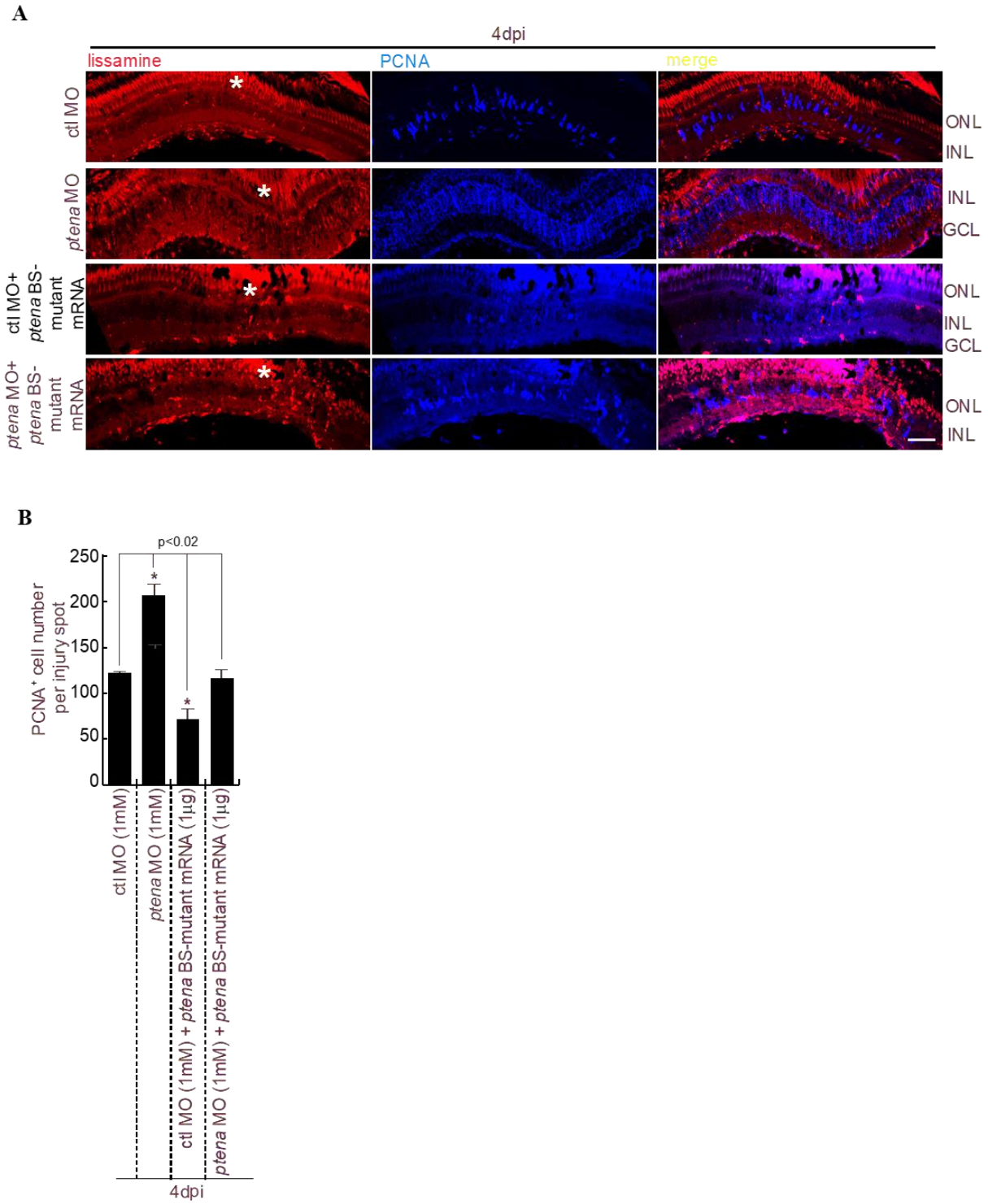


Fig 3.1.3.6 Effect of *ptena* MO in regenerating retina can be rescued. (**A** and **B**) Confocal microscopy images of retinal cross-sections show the rescue of *ptena* MO effect by the *ptena* MO-binding site mutated mRNA transfected in retina at 4dpi (**A**), which is quantified (**B**); *p < 0.02, n=3 biological replicates. Scale bars represent 10µm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**).

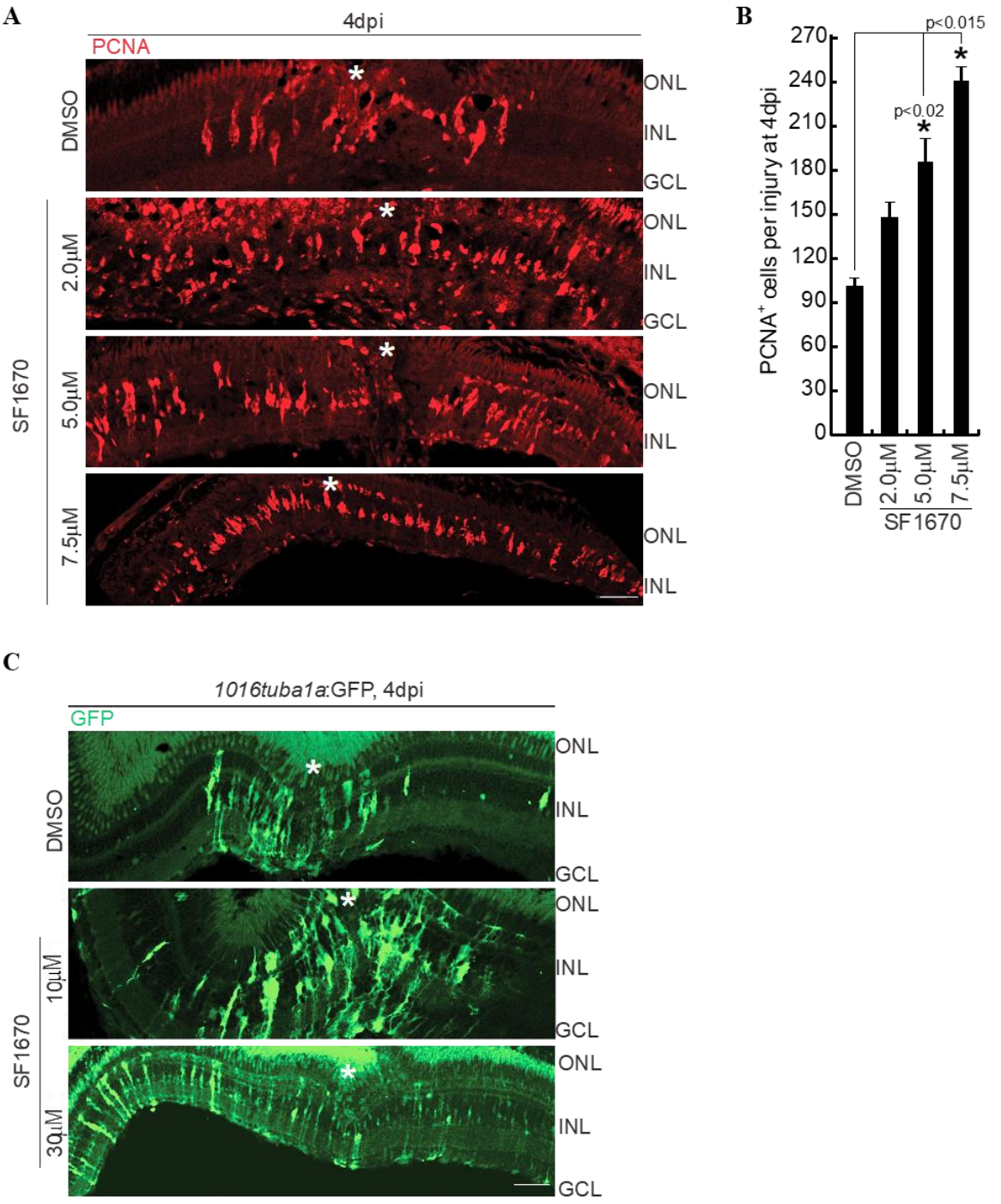


Fig 3.1.3.7 Inhibition of Pten activity enhances MGPCs proliferation. (**A** and **B**) Confocal microscopy images of retinal cross-sections show an increase in the number of PCNA^{+} MGPCs with the increasing concentrations of SF1670 at 4dpi (**A**), which is quantified (**B**); *p < 0.03; n=6 biological replicates. (**C**) Confocal microscopy images of retinal cross-sections of the transgenic zebrafish line *1016tuba1a*:GFP show a concentration dependent increase in the expression of GFP upon treatment with SF1670 at 4dpi; n=3 biological replicates. Scale bars represent 10µm in (**A**, **C**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**, **C**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**, **C**).

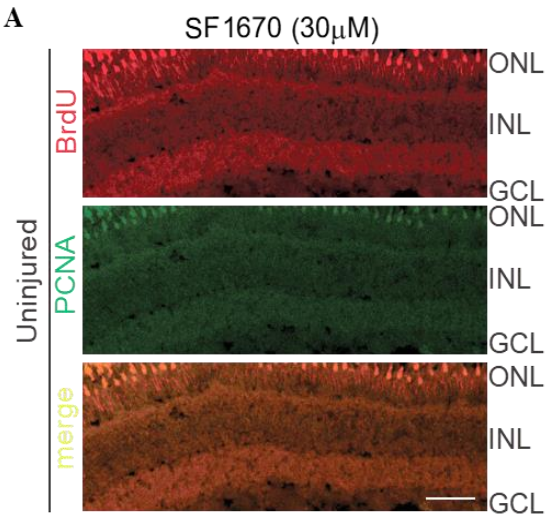


Fig 3.1.3.8 Pten blockade does not affect uninjured retina. (**A**) Confocal microscopy images of a retinal cross-section show that the proliferative response marked by $PCNA^{+}$ MGPCs is absent in the uninjured retina treated with SF1670; n=3 biological replicates. Scale bars represent 10μm in (**A**); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**). A single 0.5-μm-thick optical section was taken in (**A**).

trigger or an activating signal for the MG cells is required for the initiation of PI3K/Pten/Akt/mTOR pathway to embark its effect on the regenerating retina. Since it is reported that changes in the levels of growth factors and cytokine upon any stress or injury are perceived by Receptor Tyrosine Kinases (RTKs) on the plasma membrane, which further leads to an activation of the PI3K/Pten/Akt/mTOR pathway.

We proceeded to verify that MGPCs which are proliferating after *pten* gene knockdown and upon drug-mediated Pten blockade are as a result of the regenerative response and not as a defence mechanism against apoptosis. To address this, Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay on the cryosections from *ptena*/*ptenb* MO-electroporated and SF1670-treated retina was performed. TUNEL assay allows detection of the cells undergoing apoptosis, by labelling the fragmented DNA at their 3'-ends (nicks) by a fluorescently-tagged dUTP, mediated by Terminal deoxynucleotidyl transferase enzyme. We did not find many $TUNEL^{+}$ cells upon *pten* knockdown (Fig 3.1.3.9 A) and upon Pten blockade at 2dpi (Fig 3.1.3.9 B), ruling out the possibility of apoptosis leading to MGPCs proliferation.

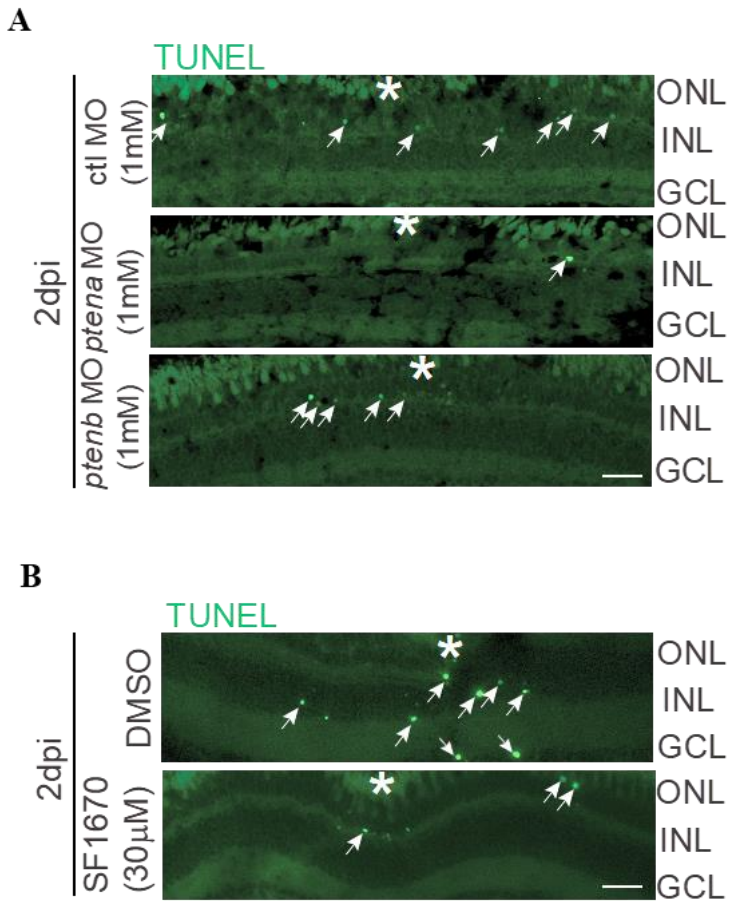


Fig 3.1.3.9 *pten* knockdown and Pten blockade does not lead to apoptosis in the retina. (**A** and **B**) Confocal microscopy images of retinal cross-sections show an insignificant number of TUNEL^{+} cells in *ptena* and *ptenb* knockdowns (**A**) and in SF1670 treatment (**B**) as compared to the control retina at 2dpi; n=3 biological replicates. Scale bars represent 10µm in (**A**, **B**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**, **B**); dpi, days post injury. A single 0.5-µm-thick optical section was taken in (**A**, **B**).

3.1.4. Lineage tracing of the cells formed after Pten blockade.

If we again ponder over the definition of the term “regeneration”, it mentions that damaged tissue regains its original attributes in terms of structure and the functions. In the context of retina regeneration, a regenerating retina not only should allow the progenitor cells to proliferate but also to re-differentiate into the functional retinal cell types to restore the visual function. These multipotent progenitors having the stem cell-like properties should divide asymmetrically, to propagate their numbers and also to differentiate into different retinal cell-types. From our initial experiments, we found an increase in the number of proliferating MGPCs upon Pten blockade. We wanted to explore if these increased number of MGPCs formed at 4dpi could continue to proliferate and can stay viable till 30dpi. For this, we traced the lineage of these proliferating MGPCs at 30dpi. We electroporated *ptenb* MO, and also injected SF1670 in separate experiments, at the time of injury, and labelled the proliferating MGPCs with BrdU for 3, 4 and 5dpi and finally harvested the eye on 30dpi (Fig 3.1.4.1 A). We saw an increase in the number of BrdU-labelled MGPCs, which remained viable, till 30dpi upon *ptenb* knockdown done during the initial time, as compared to the control 30dpi (Fig 3.1.4.1 B, C). The slides with SF1670 treatment, following the above mentioned experimental regime, were also subjected to immunostaining with the antibodies against Glutamine synthetase (GS), a marker for MG; Protein Kinase C (PKC), a marker for bipolar cells; and HuD, a marker for amacrine cells, along with BrdU. We observed that the MGPCs (analysed by their BrdU label), as seen in SF1670 treatment in retina, could form various retinal cell-types on 30dpi (Fig 3.1.4.2 A, B). These results indicate that Pten blockade not only leads to enhanced proliferation of MGPCs but also leads to the functional restoration of the tissue. The multipotent retinal progenitor cells that were formed as a part of regenerative response in the Pten blocked retina were viable and could get differentiated into various retinal cell-types also.

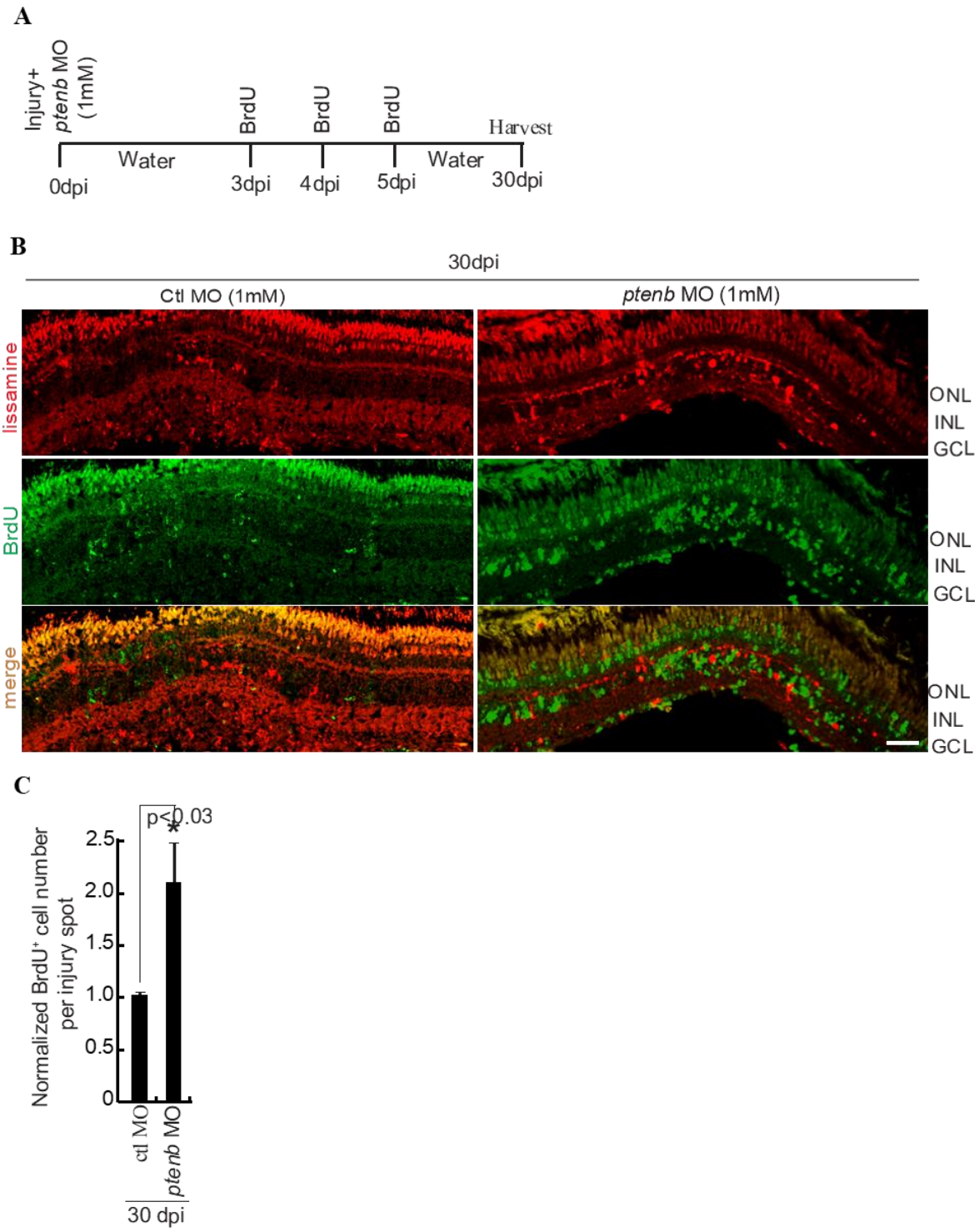


Fig 3.1.4.1 MGPCs formed in *ptenb* knockdown retina are viable till later stages of regeneration. (**A**) An experimental timeline that describes injury and *ptenb* MO injection at the time of injury, BrdU exposure for 4hrs at 3, 4 and 5dpi, followed by harvesting at 30dpi. (**B** and **C**) Confocal microscopy images of retinal cross-sections show an increased number of $BrdU^+$ cells at 30dpi in *ptenb* knockdown done at the time of retinal injury (**B**), as compared to the control, which is quantified (**C**); *p < 0.03; n=5 biological replicates. Scale bars represent 10µm in (**B**); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**B**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**B**).

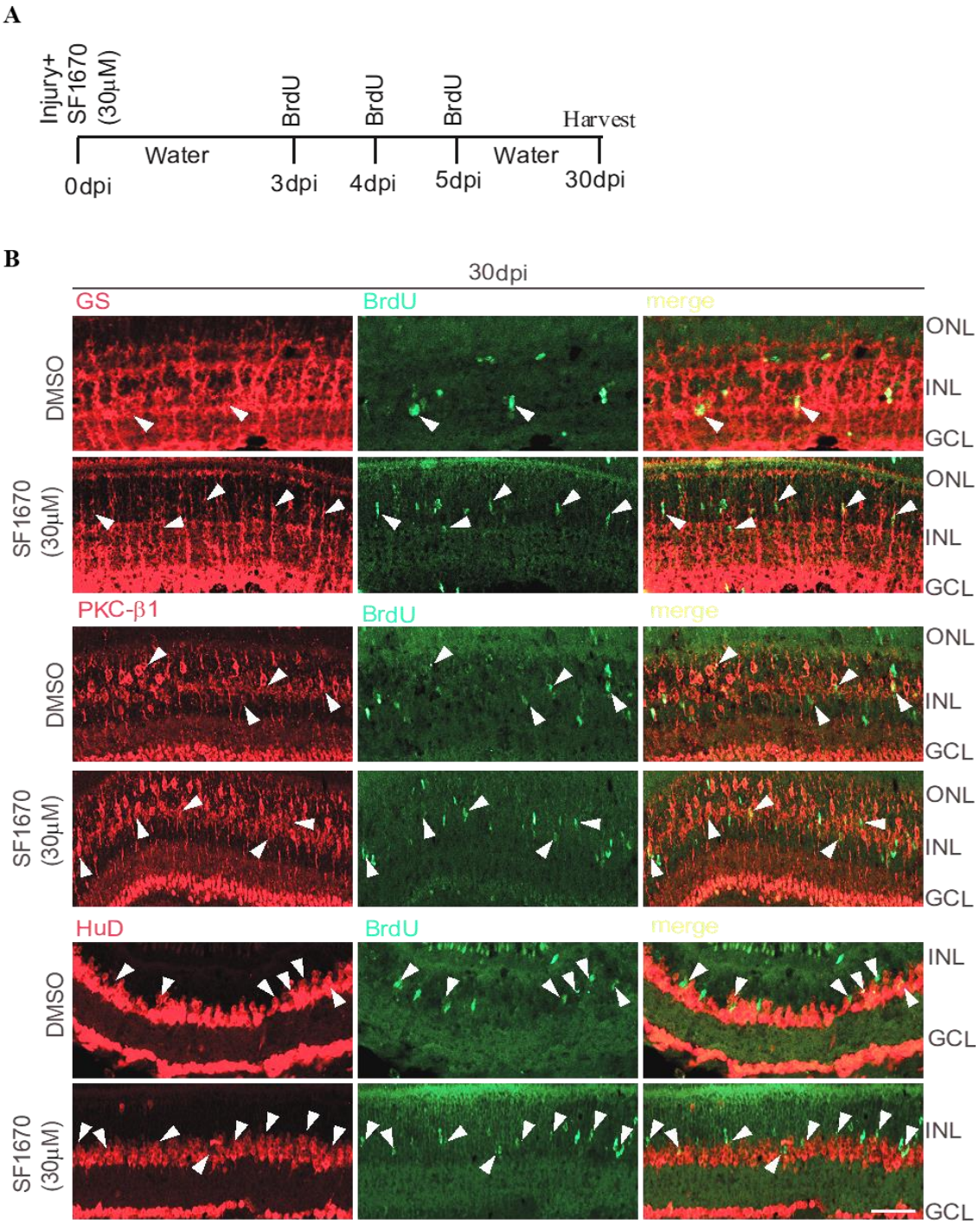


Fig 3.1.4.2 MGPCs formed in Pten inhibited retina are able to form various retinal cell-types. (**A**) An experimental timeline that describes drug delivery at the time of injury, BrdU exposure for 4hrs at 3, 4 and 5dpi, followed by harvesting at 30dpi. (**B**) Confocal microscopy images of retinal cross-sections show that $BrdU^{+}$ cells in the SF1670-treated retina make various retinal cell types at 30dpi, where Müller Glia are marked by Glutamine Synthetase (GS), Bipolar cells are marked by PKC-β1, Amacrine cells are marked by HuD, as compared to the control. Scale bars represent 10μm in (**B**); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**B**); dpi, days post injury; Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**B**).

Discussion

Pten is a tumor suppressor gene well known for its anti-proliferative signalling functions (Hill & Wu, 2009; Stumpf et al., 2015). Our results where *ptena* and *ptenb* transcripts as well as Pten protein are excluded from the proliferating MGPCs, also support this fact. These could be the cells which are either entering the cell cycle or are exiting it and are lacking Pten which could have otherwise prevented them from proliferating. While being mainly secluded from the proliferating MGPCs, marked by PCNA or BrdU, Pten remains uniform in the uninjured retina, in the entire retinal tissue away from the injury site and also in the neighbouring cells of MGPCs. This directs us to the necessity of Pten in maintaining tissue homeostasis and integrity. It prevents the cells from becoming tumorigenic. Our results also convincingly prove the fact that Pten keeps a check on MGPCs proliferation and thus, its downregulation and functional blockade lead to an increase in the rate and the number of proliferating MGPCs in the regenerating retina. At the same time, its overexpression helps in ensuring the anti-proliferative environment in the retina. We also found that the proliferating MGPCs in Pten blocked retina are not only to restore the damaged tissue structure, rather they are the viable cells which take up various retinal cell lineages later and form functional entities which aid in the restoration of the visual function of the zebrafish retina also. These results suggest that Pten expression gets regulated during zebrafish retina regeneration and its forced downregulation or inhibition leads to a remarkable increase in the number of MGPCs to facilitate the process of retina regeneration (Fig 3A and B).

Chapter 2

Mechanism of involvement of Akt, PI3K, mTOR and β-Catenin during zebrafish retina regeneration.

3.2.1. Pten downregulation leads to an increase in the levels of active and total Akt.

From the previous set of experiments, we derived a conclusion that Pten downregulation leads to an increase in the number of MGPCs which contribute to the successful restoration of structure and function of the damaged retina in the zebrafish. The vast literature available establishes a fact of the increased activity of Akt and total Akt levels upon Pten inhibition in various systems (Song et al., 2012; Hopkins et al., 2014). So, we intended to ponder more on the regulation of Akt downstream to Pten. We also came across a report which mentions that in zebrafish the two important phosphorylation sites leading to Akt activation are located at Thr302 (Thr308 in mammals) and Ser467 (Ser473 in mammals) (Cheng et al., 2013). Henceforth, in our work, we will be annotating these sites accordingly. First, we checked the spatial localisation of Akt in the retinal tissue and by performing immunohistochemistry using antibodies against pAkt302, pAkt467 and Akt we found that activated Akt and total Akt did not express in uninjured retinal tissue. In contrast, we could see the expression of these proteins to be colocalised with the $PCNA^{+}$ MGPCs in the retina at 4dpi (Fig 3.2.1.1 A, B, C). We also found an exclusion of the Akt expression from the Pten expressing cells. Since Pten is expressed in the cells flanking the MGPCs, while Akt, as seen from the above experiments, stays localised in the proliferating MGPCs (Fig 3.2.1.2 A). This colocalised expression of Akt in proliferating MGPCs made us to speculate that the increased MGPCs proliferation upon Pten inhibition might be linked to the increased levels of Akt. For this, we performed Western Blotting assay to check the levels of phosphorylated and total Akt upon *pten* knockdown and Pten blockade in the retina at 4dpi. Although, we could not observe any significant changes in the expression of phosphorylated and total Akt upon *ptena* knockdown (with 0.5mM *ptena* MO), *ptenb* knockdown (with 0.5mM *ptenb* MO) led to an increase in their levels (Fig 3.2.1.3 A, B). Parallelly, we also observed an increase in the levels of phosphorylated and total Akt upon SF1670-mediated Pten blockade (Fig 3.2.1.4 A, B). However, here we also observed an unanticipated dip in the levels of phosphorylated and total Akt upon combined *ptena* and *ptenb* knockdown and also upon a very strong drug-mediated inhibition of Pten (Fig 3.2.1.3 A, B; Fig 3.2.1.4 A, B), which we suspect might be because of some negative feedback regulation on the Akt levels. By performing immunohistochemistry in the retinal tissue also, we observed an increase in the active Akt and total Akt levels with the SF1670 mediated-Pten blockade at 4dpi as compared to the DMSO control (Fig 3.2.1.5 A, B, C, D).

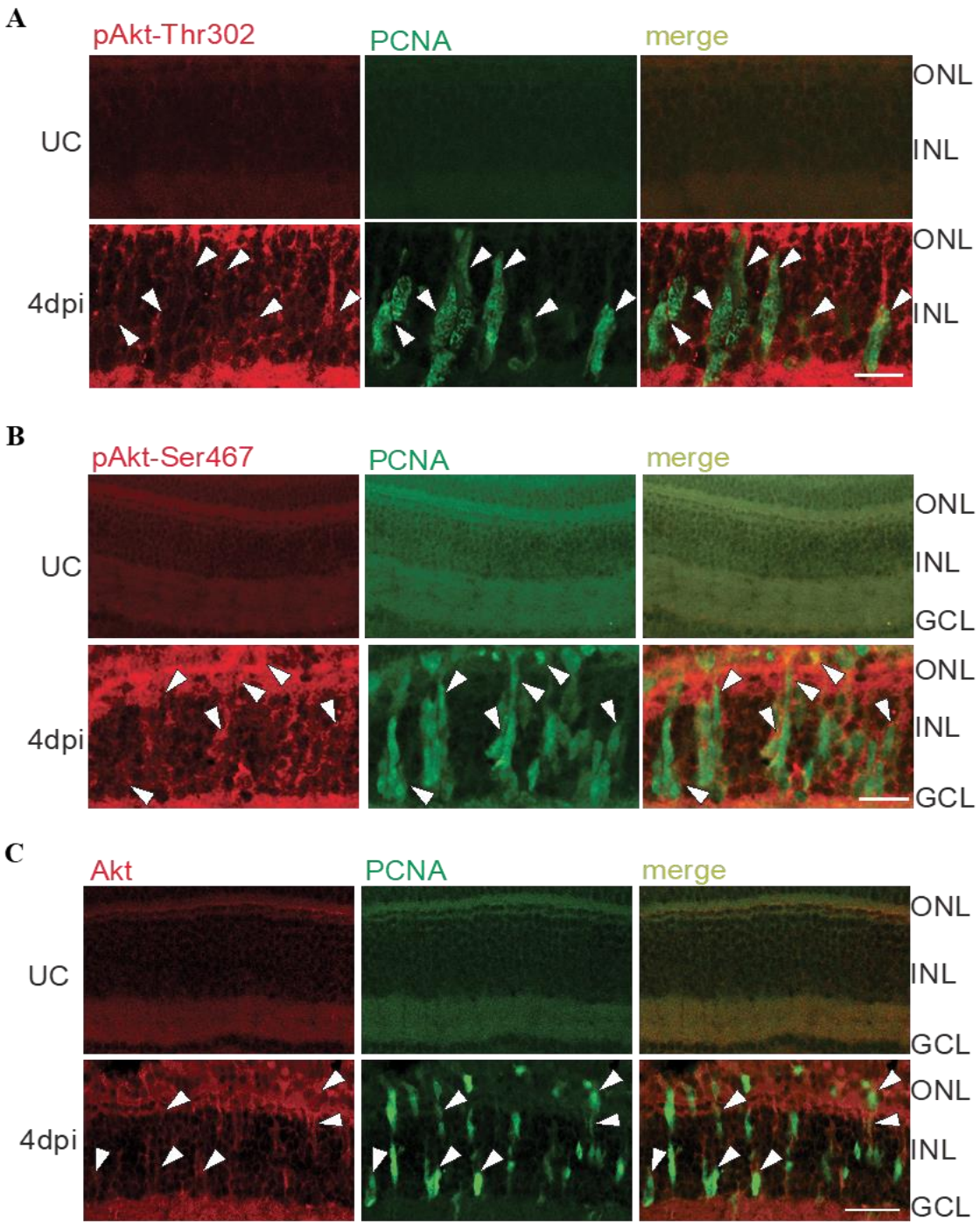


Fig 3.2.1.1 Colocalised expression of pAkt and Akt in proliferating MGPCs. (**A-C**) Confocal microscopy images of retinal cross-sections show the expression of pAkt-Thr302 (**A**), pAkt-Ser467 (**B**) and Akt (**C**) in the PCNA^{+} MGPCs in 4dpi retina, while being absent in uninjured retina. Scale bars represent 10µm in (**A, B, C**); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A, B, C**); dpi, days post injury; white arrowheads mark pAkt-Thr302^{+}/PCNA^{+} (**A**), pAkt-Ser467^{+}/PCNA^{+} (**B**) and Akt^{+}/PCNA^{+} (**C**) cells. A single 0.5-µm-thick optical section was taken in (**A**, **B**, **C**).

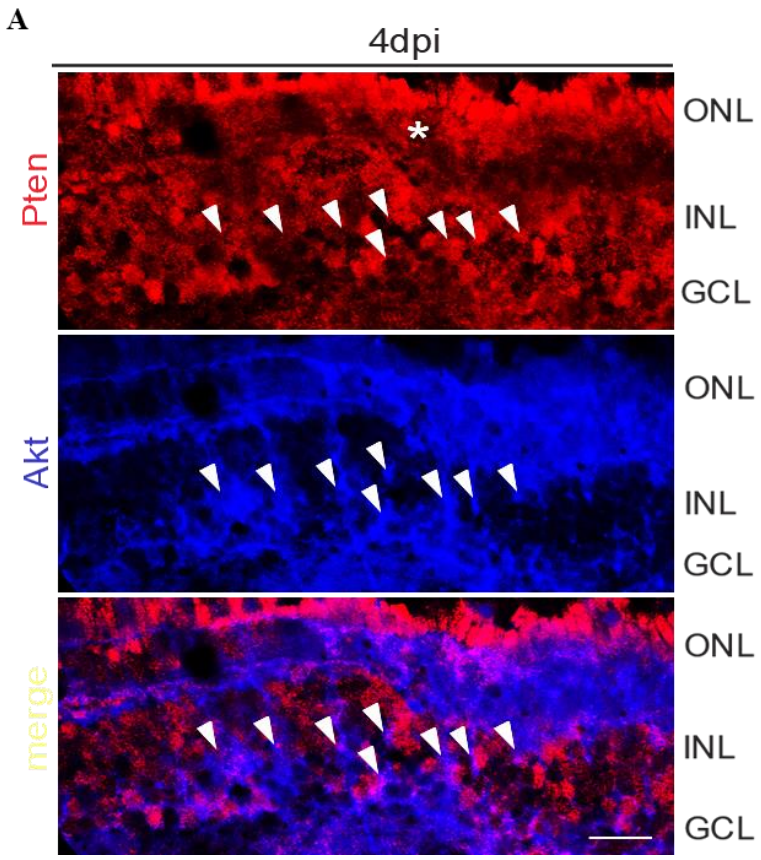


Fig 3.2.1.2 Akt expression is excluded from Pten expressing cells. (**A**) Confocal microscopy images of a retinal cross-section show the exclusion of expression of Akt from the Pten expressing cells. Scale bars represent 10µm in (**A**); the asterisk marks the injury site in (**A**); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**); dpi, days post injury; white arrowheads mark $Akt^{+}/Pten^{-}$ (**A**) cells. A single 0.5-µm-thick optical section was taken in (**A**).

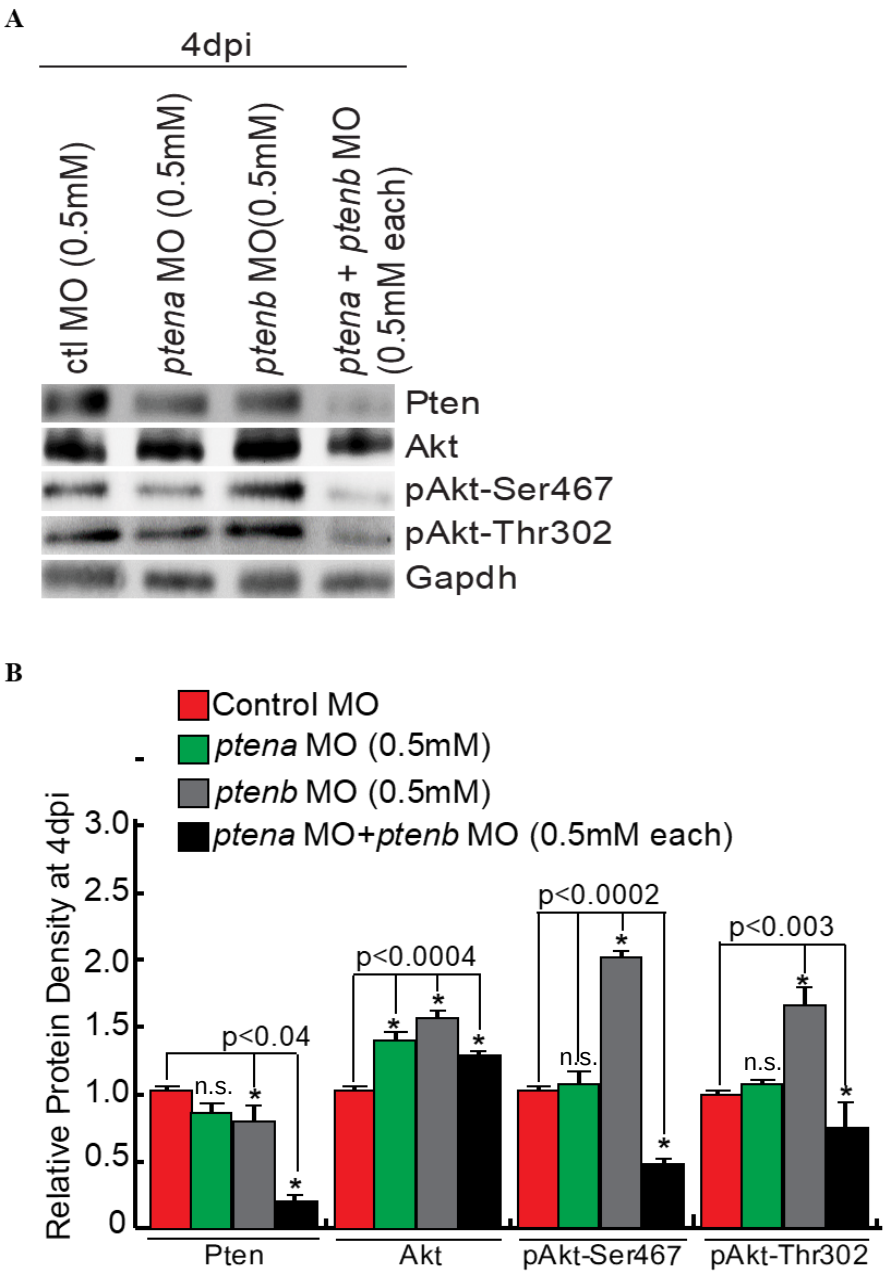


Fig 3.2.1.3 Downregulation of *ptenb* activates Akt. (**A**) Western Blot analyses of Pten, Akt, pAkt-Ser467, pAkt-Thr302 from retinal extracts collected after *ptena*/ *ptenb* knockdown in retinae at 4dpi. (**B**) Densitometry plots showing the relative expression of various proteins upon *ptena* and *ptenb* knockdowns, normalised to the control MO-electroporated retina at 4dpi; *p<0.04 (and as shown in the figure); n=3. Gapdh is the loading control. dpi, days post injury; n.s., not significant. Error bars represent SD.

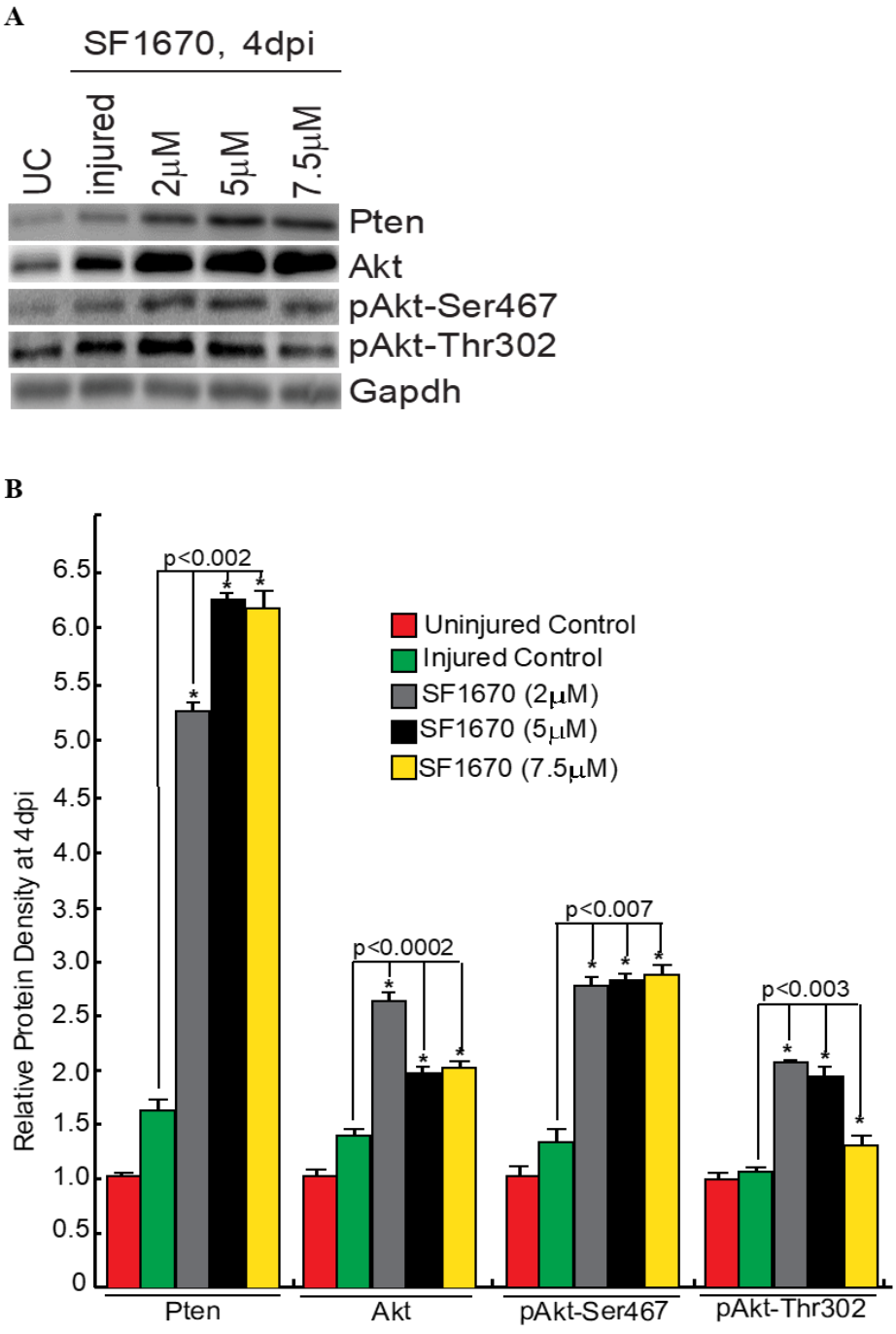


Fig 3.2.1.4 Pten inhibition leads to activation of Akt. (**A**) Western Blot analyses of Pten, Akt, pAkt-Ser467, pAkt-Thr302 from retinal extracts prepared from retinae injected with different concentrations of SF1670 at 4dpi. Gapdh is the loading control. (**B**) Densitometry plots showing the relative expression of various proteins upon Pten blockade, normalised to the uninjured control retina; *p<0.007 (and as shown in the figure); n=3. dpi, days post injury; UC, Uninjured Control; Error bars represent SD.

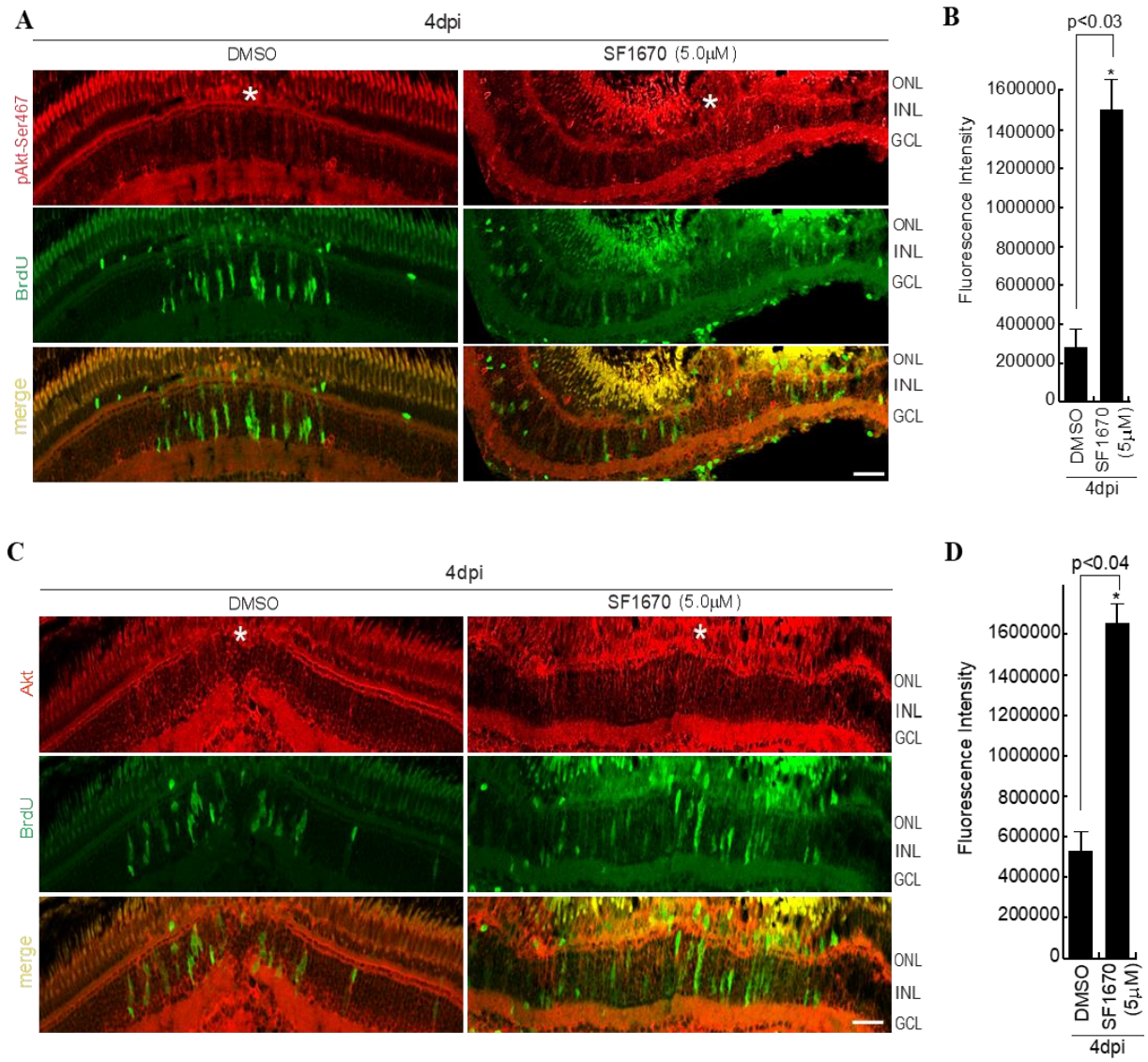


Fig 3.2.1.5 Pten inhibition increases pAktS467 and total Akt levels with an increase in the number of MGPCs. (**A** and **B**) Confocal microscopy images of retinal cross-sections show the increase in the expression of pAkt-Ser467 (**A**) and its fluorescence intensity plot (**B**) with the enhanced number of MGPCs upon Pten blockade as compared to the DMSO control in retina at 4dpi; *p < 0.03; n=3 biological replicates. (**C** and **D**) Confocal microscopy images of retinal cross-sections show the increase in the expression of Akt (**C**) and its fluorescence intensity plot (**D**) with the enhanced number of MGPCs upon Pten blockade as compared to the DMSO control in retina at 4dpi; *p < 0.04; n=3 biological replicates. Scale bars represent 10μm in (**A**, **C**); the asterisk marks the injury site in (**A**, **C**); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**, **C**); dpi, days post injury. A single 0.5-μm-thick optical section was taken in (**A**, **C**); Error bars represent SD.

3.2.2. Akt and its activation play a role in mounting a successful regenerative response in the retina.

From the findings mentioned above, it was evident that Akt expression was in association with the MGPCs. However, we wanted to find out further the role played by Akt during retina regeneration. For this, we knocked down *akt1* using *akt1* MO (Fig 3.2.2.1 A) and observed *akt1* MO concentration-dependent decline in the number of $PCNA^{+}$ MGPCs in the retina at 4dpi (Fig 3.2.2.1 B, C), suggestive of the pro-proliferative role of Akt during retina regeneration. The literature mentions about the abundance of *akt1* out of the three isoforms of *akt* namely, *akt1*, *akt2* and *akt3*, and about the significant involvement of *akt1*/Akt1 in the majority of cancers in mammalian systems and during cell growth and survival (Gonzalez & McGraw, 2009; Cheng et al., 2013). We could successfully rescue the effects of *akt* MO by overexpressing *akt* mRNA in the retina with the knocked down *akt* gene at 4dpi (Fig 3.2.2.2 A, B).

We were also intrigued by the role played by the two phosphorylation sites of Akt1 in activating it and facilitating it to evoke a regenerative response in the retina. For this, we systematically mutated the Thr302 and Ser467 on Akt1 to Alanine (A), which is unable to get phosphorylated, and this is known as a neutral mutation. We also mutated these sites to Aspartic acid (D), which also cannot get phosphorylated, but it structurally mimics the pThr and pSer, and this is known as a phosphomimetic mutation (Hart & Vogt, 2011). This seems to interact with PDK1, promoting activation and thereby bypassing the need for the actual phosphorylation of the hydrophobic motif of the Aspartic acid residue (Fig 3.2.2.3 A). We proceeded with the *in vitro* mRNA transcription from these constructs using SP6 polymerase. Next, using the mRNA as mentioned above, we performed *in vivo* mRNA transfection in the zebrafish retina. We observed an increase in the number of MGPCs with the WT *akt1* overexpression, as compared to the *gfp* mRNA-transfected control at 4dpi (Fig 3.2.2.3 B, C). Parallelly, upon transfecting the *akt1* mRNA with neutral mutation into the retina, we found the number of MGPCs similar to that seen in the *gfp* mRNA control at 4dpi (Fig 3.2.2.3 B, C). At the same time, we observed a remarkable increase in the proliferation of MGPCs with the *akt1* mRNA with phosphomimetic mutation compared to the *gfp* mRNA control as well as WT *akt1* mRNA at 4dpi (Fig 3.2.2.3 B, C). These results are indicative of the significance of Akt1 phosphorylation at both its sites Thr302 and Ser467 for its activation and thus facilitating Akt1 in mounting a robust regenerative response.

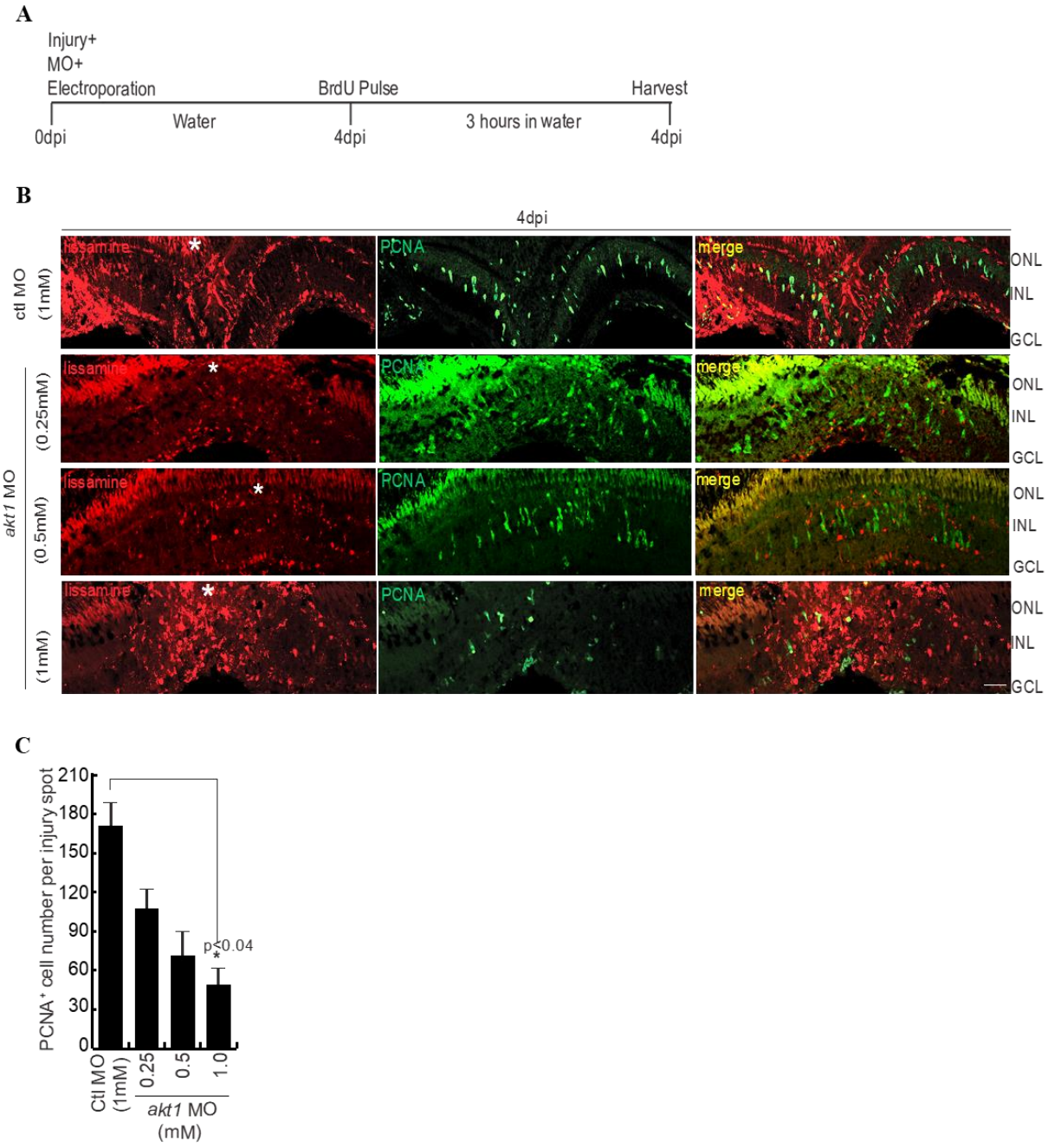


Fig 3.2.2.1 Knockdown of *akt1* leads to a reduction in the number of proliferating MGPCs during retina regeneration. (**A**) An experimental timeline that describes injury, Morpholino (MO) delivery, electroporation at 0dpi, BrdU pulse for 4hrs at 4dpi, followed by harvesting after 3hrs dipping in water. (**B** and **C**) Confocal microscopy images of retinal cross-sections show a decline in the number of $PCNA^+$ MGPCs with the increasing concentrations of *akt1* MO at 4dpi (**B**), which is quantified (**C**); *p < 0.04; n=3 biological replicates. Scale bars represent 10µm in (**B**); the asterisk marks the injury site in (**B**); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**B**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**B**).

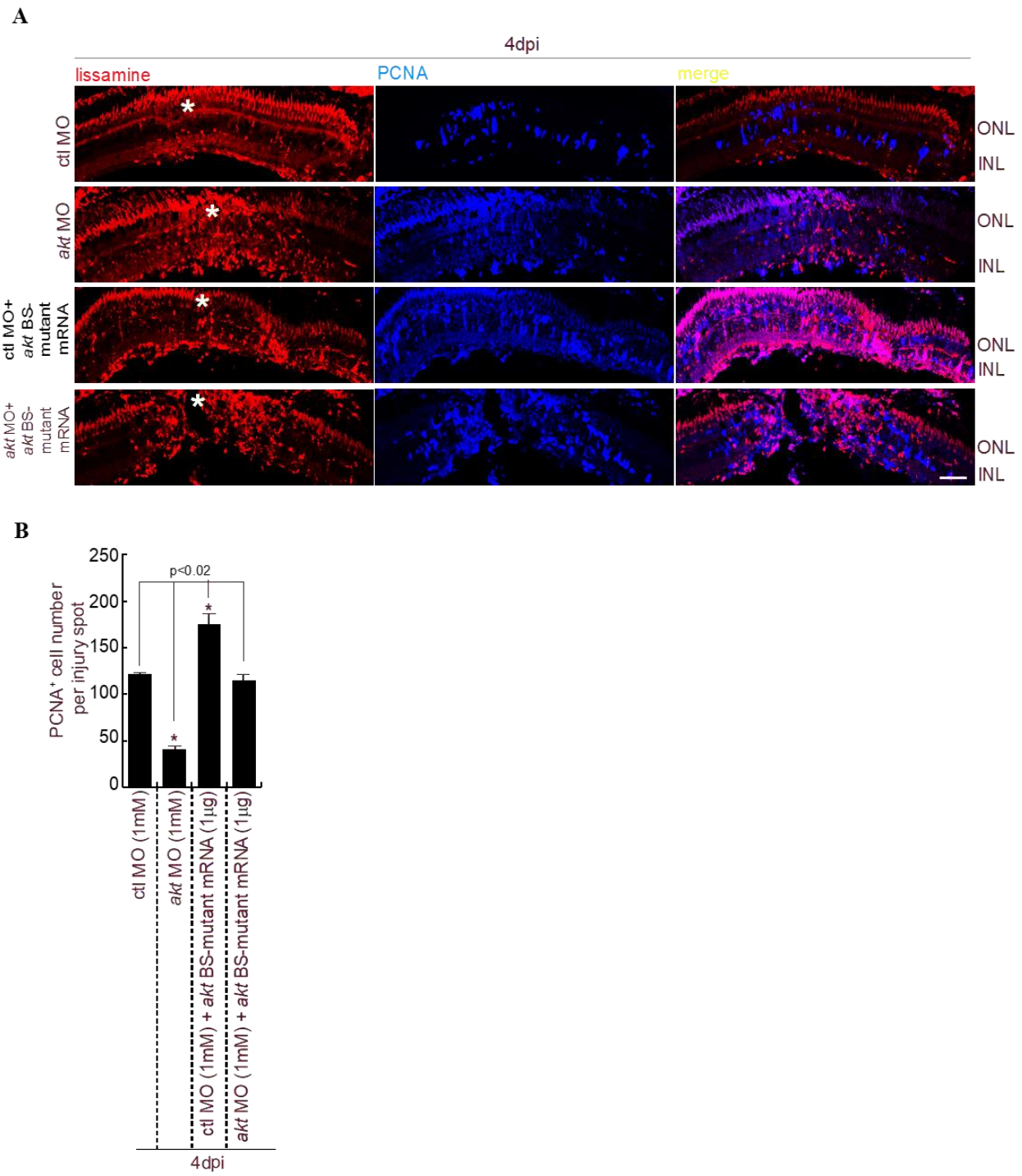


Fig 3.2.2.2 Effect of *akt* MO in regenerating retina can be rescued. (**A** and **B**) Confocal microscopy images of retinal cross-sections show the rescue of *akt* MO effect by the *akt* MO-binding site mutated mRNA transfected in retina at 4dpi (**A**), which is quantified (**B**); *p < 0.02, n=3 biological replicates. Scale bars represent 10μm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**A**); dpi, days post injury; Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**A**).

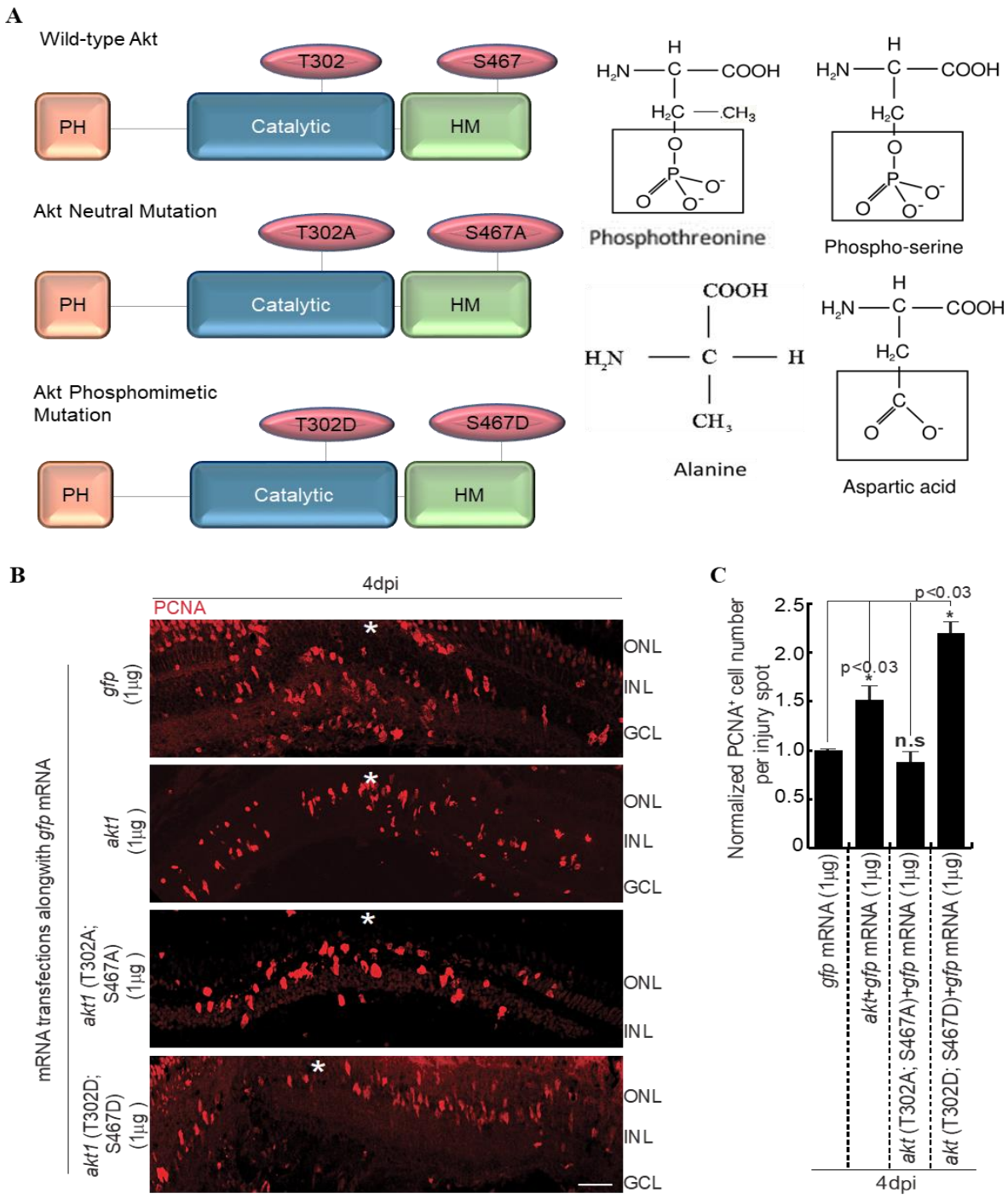


Fig 3.2.2.3 Active Akt plays a pro-proliferative role during retina regeneration. (**A**) Schematics representing Wild-type Akt, Akt with Neutral mutation and with Phosphomimetic mutations and the respective amino acids structures. (**B** and **C**) Confocal microscopy images of retinal cross-sections show the number of PCNA$^+$ MGPCs upon transfection of retina with *akt1* wild-type mRNA or with phosphomimetic form of *akt1* mRNA which increases, while the cell number remains unchanged upon retinal transfection with neutral mutation-bearing *akt1* mRNA at 4dpi (**B**), as compared to *gfp*-transfected control, which is quantified (**C**); *p < 0.03; n=6 biological replicates. Scale bars represent 10µm in (**B**); the asterisk marks the injury site in (**B**); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (**B**); dpi, days post injury; Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**B**).

3.2.3. PI3K and mTORC2 are involved in promoting MGPCs proliferation during retina regeneration.

But we further wanted to delve deeper into the mechanism of activation of Akt1 by its phosphorylation at Thr302 and Ser467 by kinases PI3K and mTOR-complex 2 (mTORC2) respectively. We adopted the drug mediated-protein function blockade strategy to study the roles of these kinases. First, we intended to block the function of PI3K by its selective pharmacological inhibitor LY294002 (Chen et al., 2017). LY294002 competes with ATP for binding the PI3K active site (Wang et al., 2017). We injected different concentrations of this drug into the zebrafish retina at the time of injury. We found LY294002 concentration-dependent decline in the number of $PCNA^{+}$ MGPCs in the retina at 4dpi (Fig 3.2.3.1 A, B), with a corresponding decrease in the levels of pAktThr302 in the retina at 16hpi (Fig 3.2.3.2 A, B) and 4dpi (Fig 3.2.3.2 C, D).

The blockade of mTORC2 function using its selective inhibitor drug Torin1, which is an ATP-competitive inhibitor of mTORC2 (Thoreen et al., 2009; Zhang et al., 2020), administered to the fish by dipping it in the different dosages of this drug from the time of injury till the harvest at 4dpi, led to a Torin1 concentration-dependent decrease in the number of MGPCs in the retina at 4dpi (Fig 3.2.3.3 A, B). Along with this, we found a reduction in the levels of pAktSer467 in the mTORC2-blocked retina at 16hpi (Fig 3.2.3.4 A, B) and 4dpi (Fig 3.2.3.4 C, D). We also observed a decrease in the levels of Akt also. This regulation of mTORC2 on total Akt levels might be through some unknown factor or pathway. There might be existing a factor/s which is a target of mTORC2 and which might be regulating the total Akt levels also. These results suggested that the mTORC2 is important for the Akt phosphorylation at Ser467 through known mechanism and parallelly also effects the total Akt levels through some unknown factor.

Thus, we could confirm that PI3K and mTORC2 play a supportive function during zebrafish retina regeneration by activating Akt1, through its phosphorylation.

But still, we wondered if Pten inhibition during retina regeneration conducts its pro-proliferative functions only through the Akt1 activation by PI3K and mTORC2, or if this is the primary purpose of Pten inhibition. We explored this possibility by performing a series of double-blocker experiments. In the first set of experiments, we blocked Pten along with mTORC2, using a combination of SF1670 (5µM) and Torin1 (0.5µM) in the retina (Fig 3.2.3.5 A). We observed that as the number of MGPCs was remarkably high upon SF1670-mediated

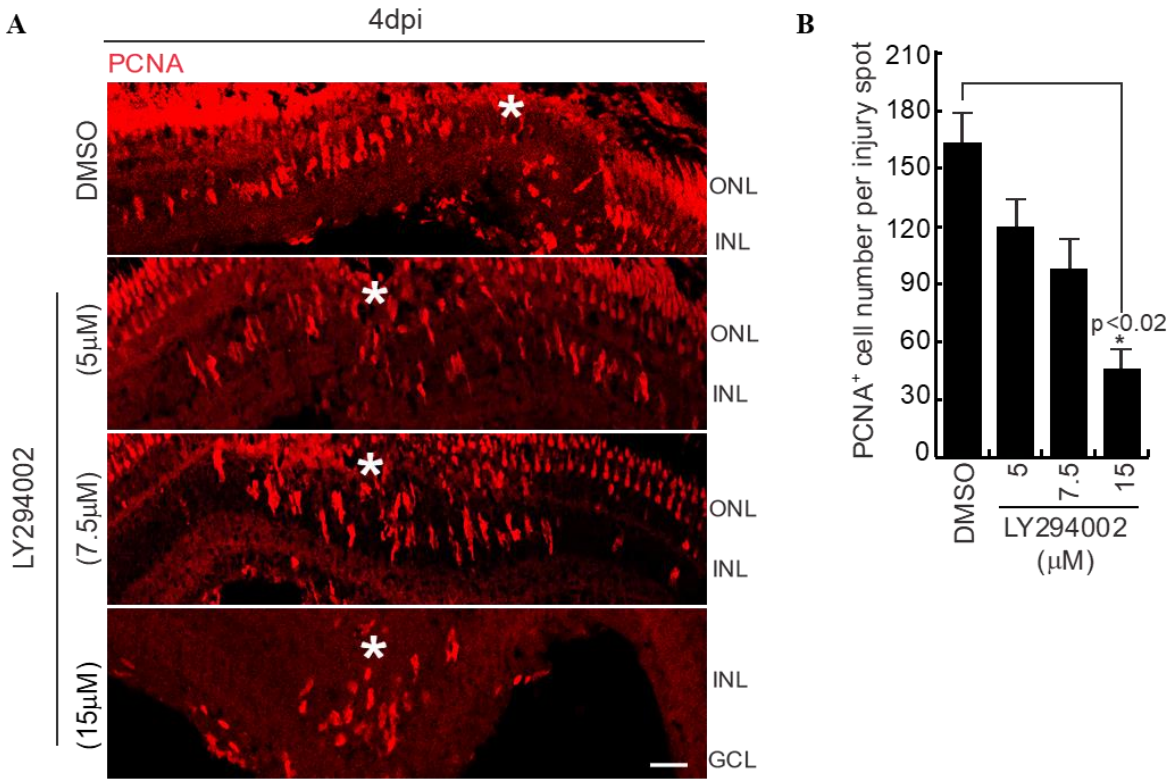


Fig 3.2.3.1 PI3K blockade leads to a decline in the number of proliferating MGPCs. (**A** and **B**) Confocal microscopy images of retinal cross-sections show a decline in the number of PCNA$^+$ MGPCs with the increasing concentrations of LY294002 at 4dpi (**A**), which is quantified (**B**); *p < 0.02, n=3 biological replicates. Scale bars represent 10μm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**A**).

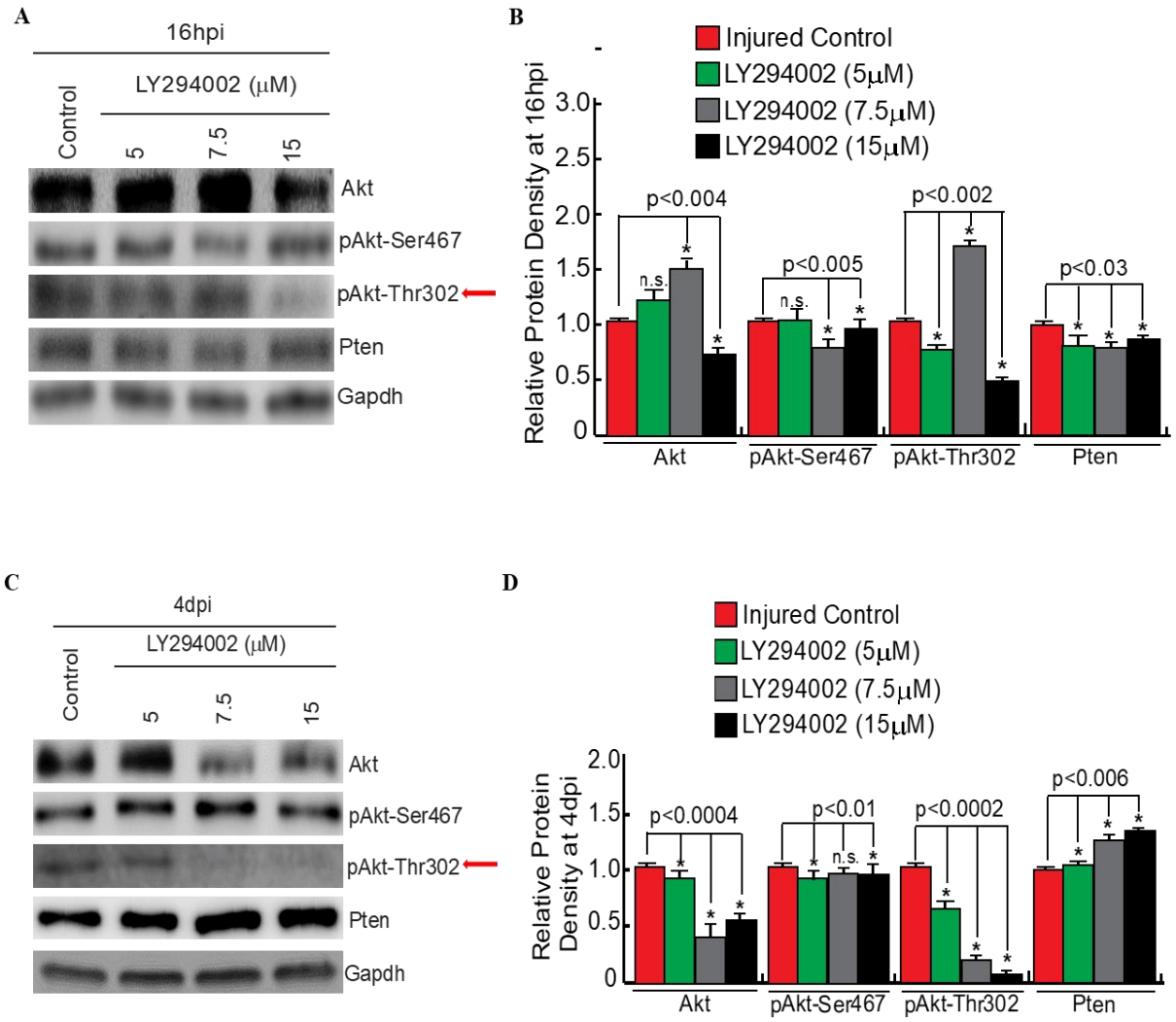


Fig 3.2.3.2 PI3K blockade affects Akt levels and Akt phosphorylation at Thr302. (**A** and **C**) Western Blot analyses of Akt, pAkt-Ser467, pAkt-Thr302, Pten from retinal extracts prepared from retinae treated with different concentrations of LY294002 at 16hpi (**A**) and 4dpi (**C**). (**B** and **D**) Densitometry plots showing the relative expression of various proteins upon PI3K blockade, normalised to the injured control retina at 16hpi and 4dpi; *p<0.03 and *p<0.01 (and as shown in the figure); n=2. Gapdh is the loading control. hpi, hours post injury; dpi, days post injury; n.s., not significant. Error bars represent SD.

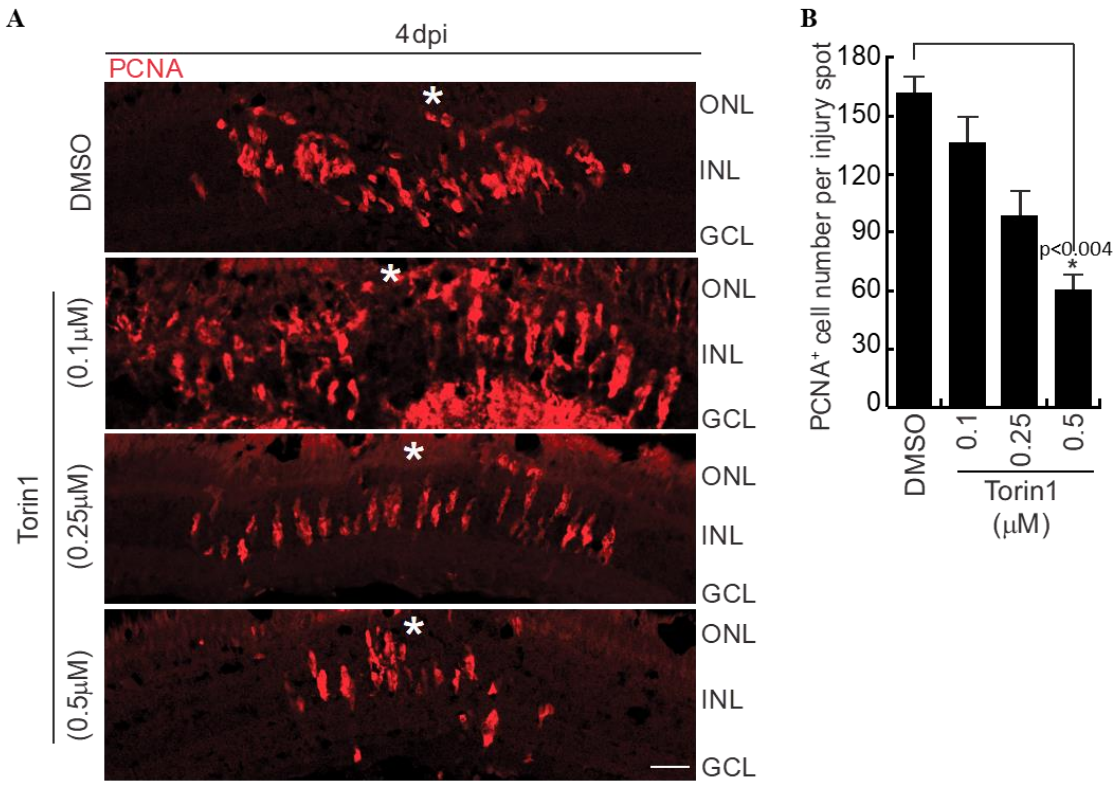


Fig 3.2.3.3 mTORC2 blockade suppresses MGPCs proliferation. (**A** and **B**) Confocal microscopy images of retinal cross-sections show a decline in the number of PCNA$^+$ MGPCs with the increasing concentrations of Torin1 at 4dpi (**A**), which is quantified (**B**); *p < 0.004, n=3 biological replicates. Scale bars represent 10μm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**A**).

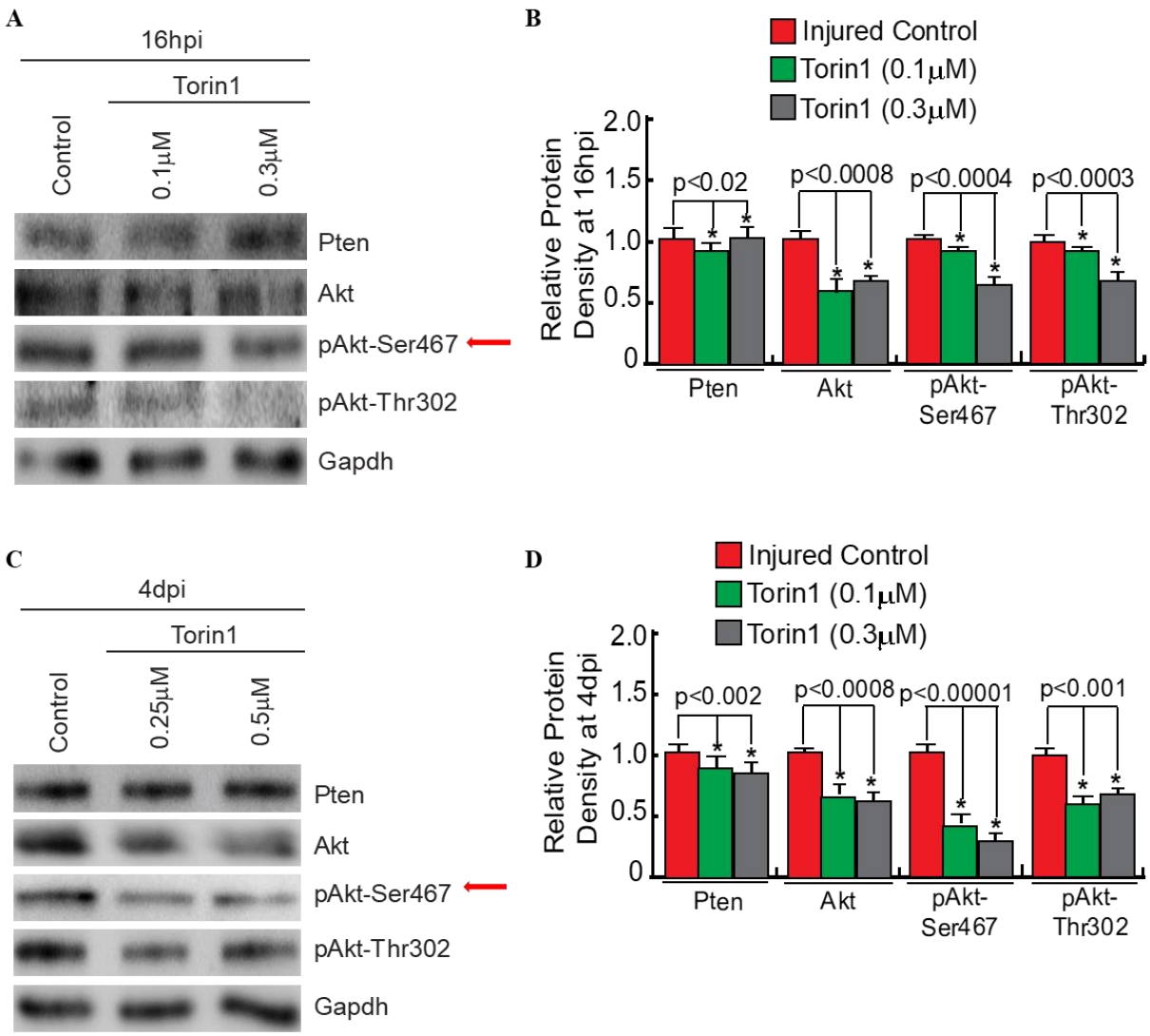


Fig 3.2.3.4 mTORC2 blockade also influences Akt levels and Akt phosphorylation at Ser467. (**A** and **C**) Western Blot analyses of Pten, Akt, pAkt-Ser467, pAkt-Thr302 from retinal extracts prepared from retinae treated with different concentrations of Torin1 at 16hpi (**A**) and 4dpi (**C**). (**B** and **D**) Densitometry plots showing the relative expression of various proteins upon mTORC2 blockade, normalised to the injured control retina at 16hpi and 4dpi; *p<0.008 and *p<0.002 (and as shown in the figure); n=2. Gapdh is the loading control. hpi, hours post injury; dpi, days post injury. Error bars represent SD.

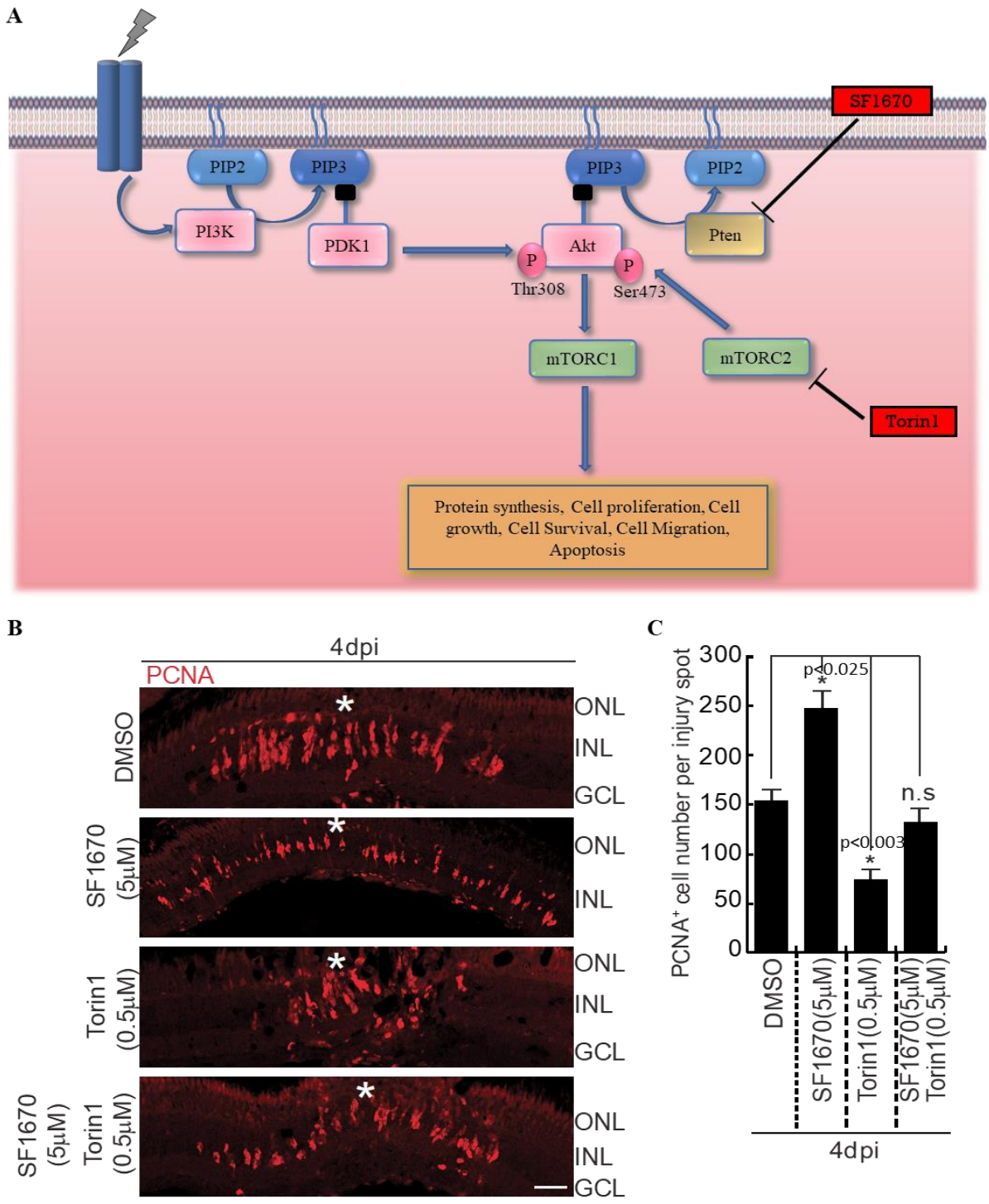


Fig 3.2.3.5 Pten blockade mediates its pro-proliferative roles through mechanisms other than mTORC2-mediated Akt activation. (**A**) A model representing double blockade of Pten with mTORC2. (**B** and **C**) Confocal microscopy images of retinal cross-sections show increased PCNA$^+$ MGPCs with SF1670 treatment, which reduce drastically with the treatment of Torin1, while again increase slightly in the combination of SF1670 and Torin1 as compared to the DMSO control at 4dpi (**B**), which is quantified (**C**); *p < 0.03, n=3 biological replicates. Scale bars represent 10μm in (**B**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**B**); dpi, days post injury, n.s., not significant. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**B**).

Pten blockade and it got nullified upon mTORC2 blockade, upon the double blockade of these two proteins the MGPCs number was elevated back to that of the control retina at 4dpi (Fig 3.2.3.5 B, C). Pten inhibition was still conjuring its pro-proliferative functions even if mTORC2-mediated Akt activation was not taking place. If Pten inhibition were dependent solely on Akt1 activation, we would have obtained a reduction in the number of MGPCs like that seen in mTORC2 blockade alone. This directs us to a fact that in the absence of Pten, there might be some mechanisms apart from the active Akt1 which are supporting the MGPCs proliferation.

In the next set of double blocker experiment, we blocked Pten along with PI3K, using a combination of SF1670 (5µM) and LY294002 (15µM) in the retina (Fig 3.2.3.6 A). In this experiment, we observed that the number of MGPCs upon double blockade of both the proteins was similar to that observed upon Pten blockade alone in the retina at 4dpi (Fig 3.2.3.6 B, C). This gave us a clue of the involvement of some other factor which in the absence of Pten increases the MGPCs number, without the aid of Akt1 activation by PI3K. If Pten inhibition had worked only in an active Akt1-dependent manner, we would have observed a decline in the number of MGPCs upon Pten-PI3K double blockade. Thus, Pten inhibition mediates its pro-proliferative effects during retina regeneration through some other factors also.

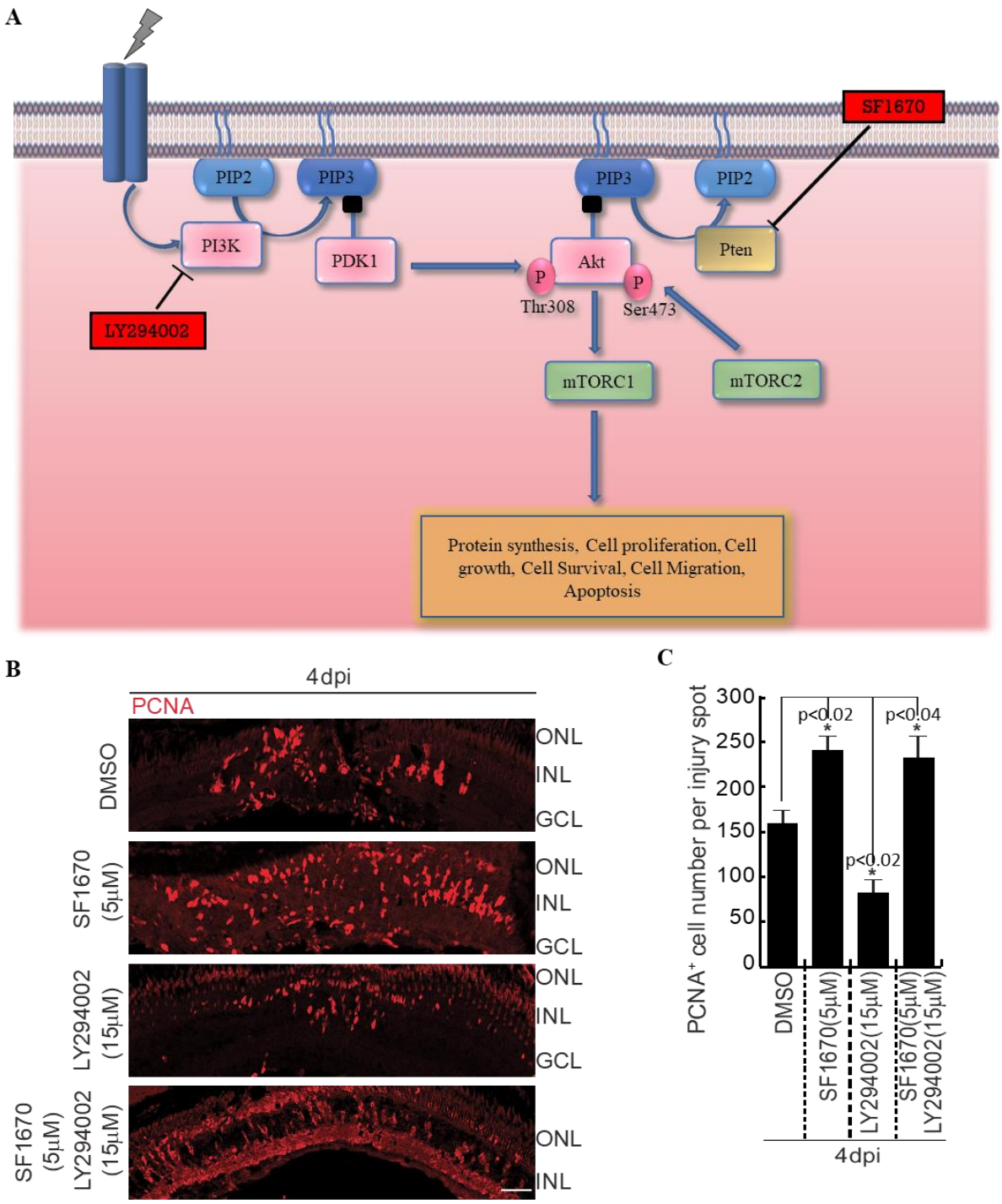


Fig 3.2.3.6 Pten blockade mediates its pro-proliferative roles through mechanisms other than PI3K-mediated Akt activation. (**A**) A model representing double blockade of Pten with PI3K. (**B** and **C**) Confocal microscopy images of retinal cross-sections show the reduction in the number of $PCNA^{+}$ MGPCs with the treatment of LY294002, while an increase with SF1670 treatment, which is maintained upon the treatment with the combination of SF1670 and LY294002, as compared to the DMSO control at 4dpi (**B**), which is quantified (**C**); *$p < 0.04$, n=3 biological replicates. Scale bars represent 10μm in (**B**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**B**); dpi, days post injury. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**B**).

3.2.4. mTORC1 activation leads to an enhancement in the number of MGPCs.

From the previous experiments, we found the involvement of Akt1 and its activation upon Pten inhibition during zebrafish retina regeneration, which made us curious to check the role of its downstream effector mTOR Complex 1 (mTORC1). mTORC1 is known to be activated as TSC1/2 get inhibited by the active Akt1, where TSC1/2 keep a check on the activation of mTORC1. Thus, we intended to study the role of mTORC1 during retina regeneration. For this, we blocked mTORC1 using its selective inhibitor Rapamycin. We administered the drug to the fish by dipping them in different dosages of this drug from the time of injury until the harvest at 4dpi. We found that Rapamycin-mediated mTORC1 blockade led to a concentration-dependent decline in the number of $PCNA^{+}$ MGPCs in the retina at 4dpi (Fig 3.2.4.1 A, B). Thus, we could infer that mTORC1 acts as a pro-proliferative kinase during retina regeneration also similar to its role in cancer systems.

Parallelly, we checked the levels of active and total Akt upon Rapamycin mediated-mTORC1 blockade by Western Blotting assays. For this, we treated the retinae with different concentrations of Rapamycin and performed Western Blotting assay from the retinal lysates prepared at 4dpi. We found Rapamycin concentration-dependent increase in the levels of pAkt302, pAkt467 and Akt (Fig 3.2.4.1 C, D). This could be due to the existence of a negative feedback regulation of mTORC1 or its downstream effectors on PI3K and mTORC2 which phosphorylate Akt1 at its two sites. Upon blockade of mTORC1, this negative feedback regulation might have got lifted from phosphorylation of Akt1, which would have led to the increase in the levels of phosphorylated Akt1. Such a negative feedback regulation mechanism of mTORC1 or its downstream effectors on PI3K and mTORC2 is also known to exist during mice axonal regeneration (Miao et al., 2016) and in many cancerous conditions (Efeyan & Sabatini, 2010). This feedback regulation may exist to maintain a homeostatic state in the cell, to prevent hyperproliferation of the cells due to mTORC1 activity, which otherwise will get continuous ON signals itself from activated Akt1, in case of absence of this negative feedback mechanism.

We also speculate that this negative feedback regulation emanating from mTORC1 on Akt1 phosphorylation might be the reason for the unanticipated decrease in the levels of phosphorylated Akt1 that we found upon strong Pten blockade or combined *pten* knockdown

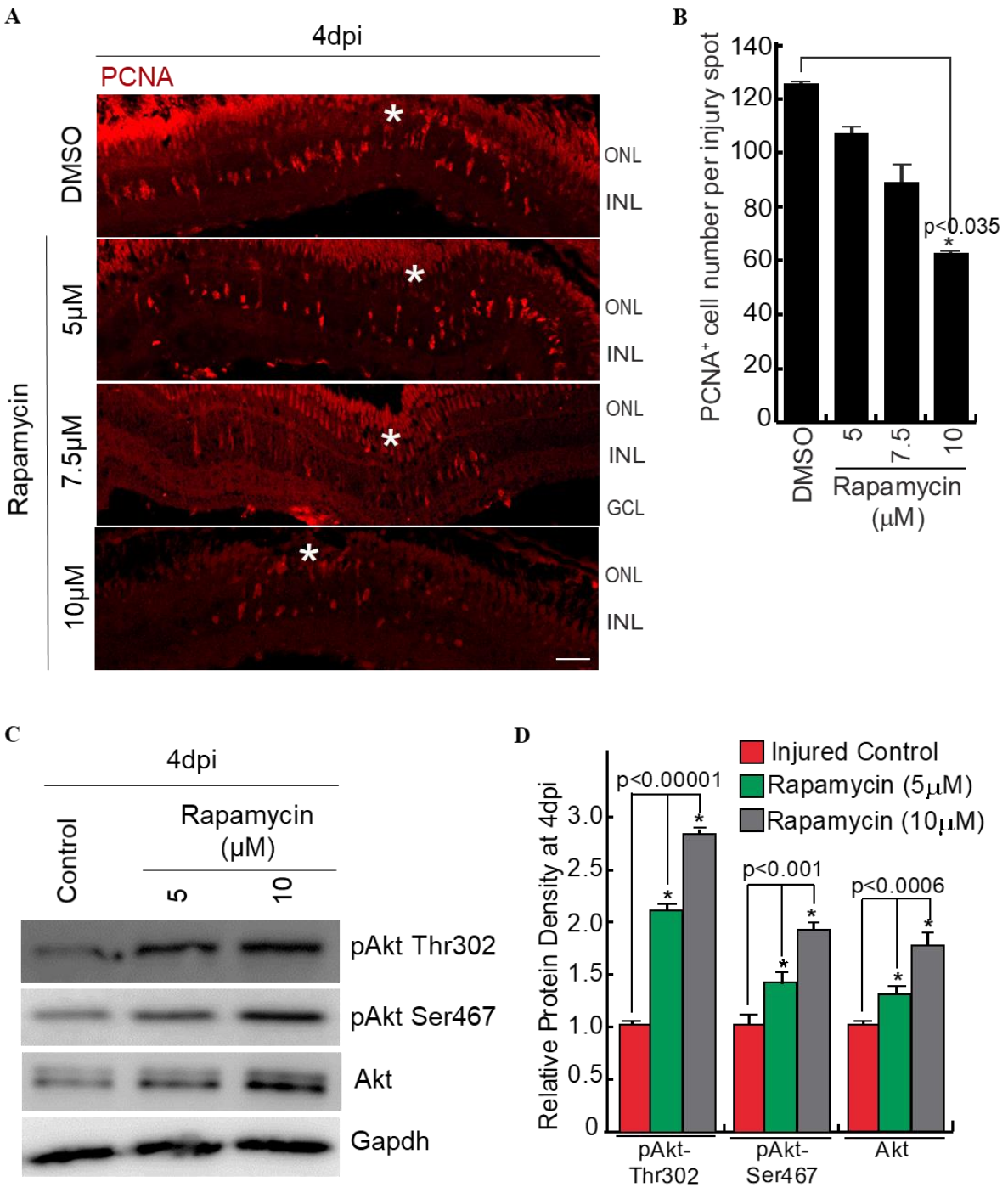


Fig 3.2.4.1 mTORC1 blockade leads to a reduction in the MGPCs proliferation. (**A** and **B**) Confocal microscopy images of retinal cross-sections show a decline in the number of PCNA$^+$ MGPCs with the increasing concentrations of Rapamycin at 4dpi (**A**), which is quantified (**B**); *p < 0.035, n=3 biological replicates. (**C**) Western Blot analyses of pAkt-Thr302, pAkt-Ser467, Akt from retinal extracts prepared from retinae treated with different concentrations of Rapamycin at 4dpi. (**D**) Densitometry plots showing the relative expression of various proteins upon mTORC1 blockade, normalised to the injured control retina at 4dpi; *p<0.001 (and as shown in the figure); n=3. Gapdh is the loading control. Scale bars represent 10µm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury. Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**).

as seen in Fig 3.2.1.2. This protective mechanism must have played an essential role in defining a fine line between cell replication during cancers and during retina regeneration upon Pten blockade, where this regulation might have got haywire in cancerous states.

Next, we aimed to confirm that Pten inhibition mediates its effects through mTORC1 activity downstream. We again blocked Pten along with mTORC1 using a combination of SF1670 (30µM) and Rapamycin (10µM) in the retina (Fig 3.2.4.2 A). We observed that the increase in the number of MGPCs seen upon Pten inhibition was alleviated upon a combinational mTORC1 blockade, as was also seen upon mTORC1 blockade alone in the retina at 4dpi (Fig 3.2.4.2 B, C). This result is suggestive of the presence and activation of mTORC1 downstream of Pten and acting antagonistically to it. It also states that Pten inhibition is dependent on the mTORC1 activity to embark its pro-proliferative effects during retina regeneration.

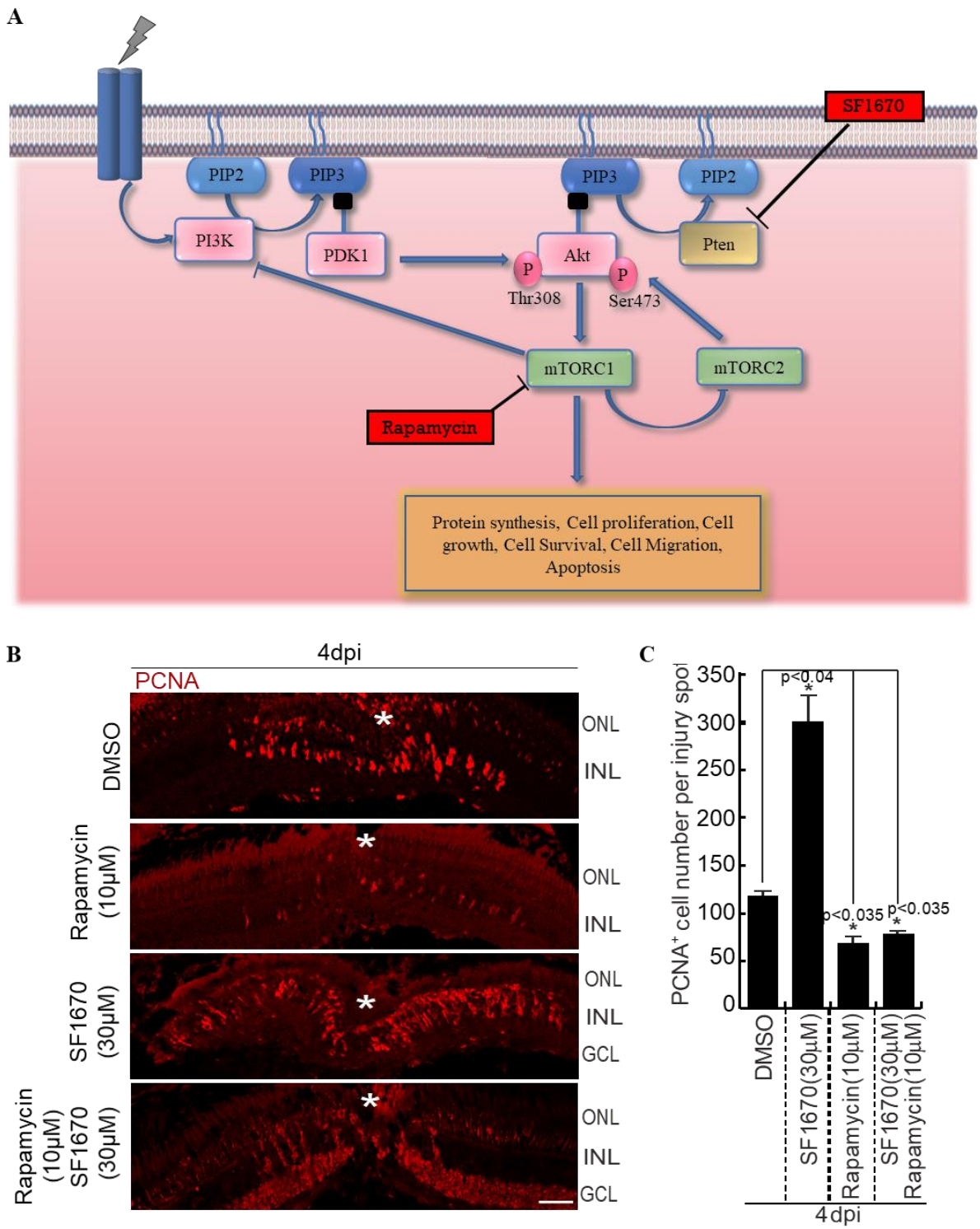


Fig 3.2.4.2 Pten inhibition mediates its pro-proliferative effects on MGPCs through mTORC1. (**A**) A model representing double blockade of Pten with mTORC1. (**B** and **C**) Confocal microscopy images of retinal cross-sections show an increase in the number of PCNA^{+} MGPCs with SF1670 treatment, which reduces significantly with the Rapamycin treatment, which is consistently low in the combination of SF1670 and Rapamycin as compared to the DMSO control at 4dpi (**B**), which is quantified (**C**); *$p < 0.04$, n=3 biological replicates. Scale bars represent 10μm in (**B**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**B**); dpi, days post injury. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**B**).

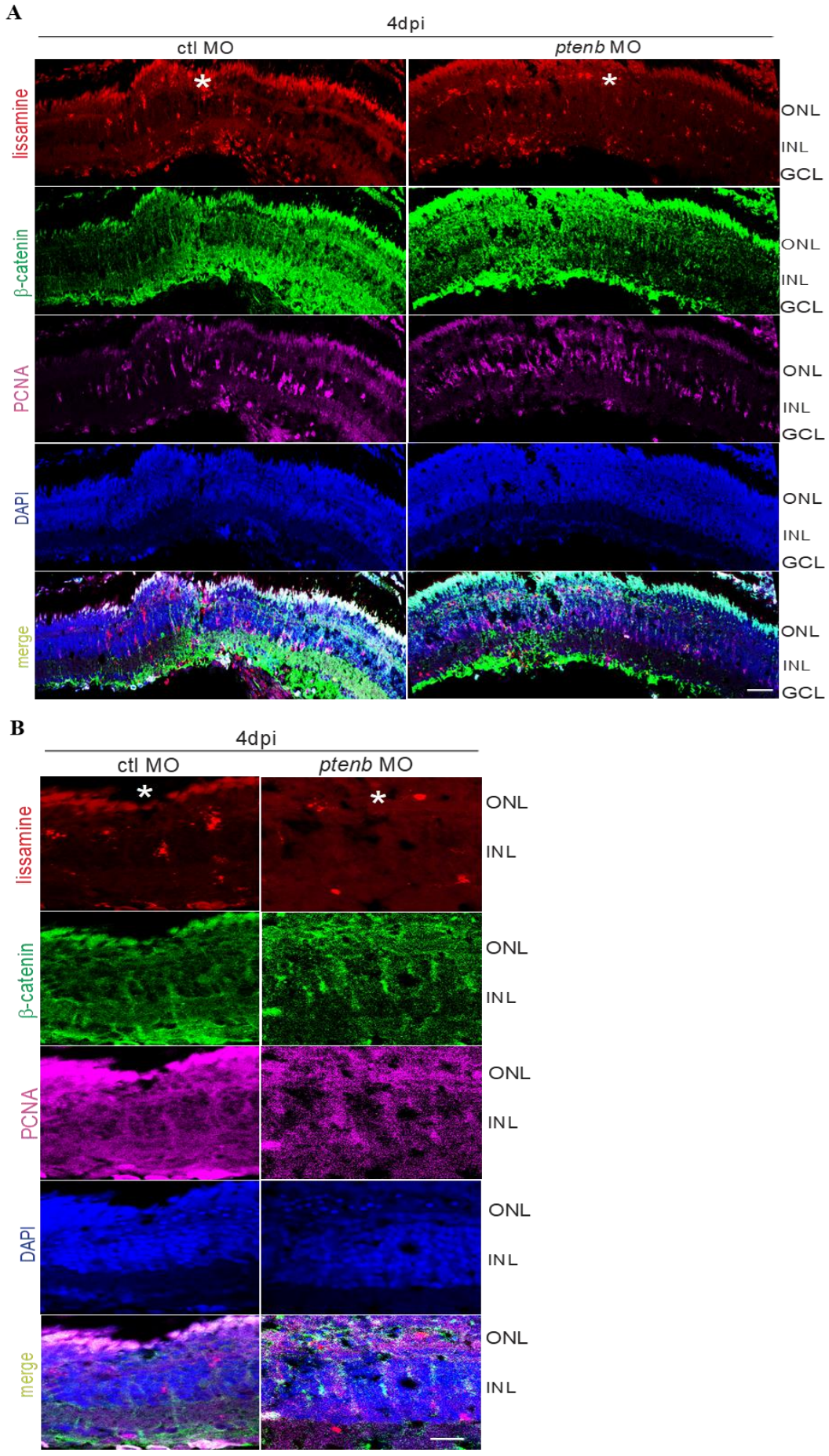
3.2.5. Pten/PI3K/Akt/β-Catenin axis is supportive of the increased number of MGPCs upon Pten inhibition.

The literature suggests that during the onset of cancerous conditions, a cascade of events takes place which majorly involves the blockade of the functions of tumor suppressors or cell cycle-inhibitors along with the highly induced expression of oncogenes or pro-proliferative pathways, since all the signalling pathways are interlinked. Wnt signalling is one such pathway which gets dysregulated during cancer. β-Catenin, a hallmark protein of canonical Wnt signalling acts as an oncoprotein and pro-proliferative factor which gets upregulated during cancer and promotes malignant transformation. PTEN/PI3K/Akt pathway is known to regulate the subcellular localisation and thus the activity of β-Catenin by regulating the stability of Glycogen synthase kinase 3β (GSK3β) (Huang et al., 2007; Persad et al., 2016). It is also reported that the nuclear stabilisation of β-Catenin is positively associated with dedifferentiation and proliferation of MGPCs in regenerating zebrafish retina (Ramachandran et al., 2011). Our results showed that Pten blockade led to an increase in the number of proliferating MGPCs in the regenerating retina. Therefore, we proceeded to check the levels of β-Catenin upon Pten blockade. For this, we performed immunohistochemistry and western blotting assay on tissues treated with *ptena*/*ptenb* MO and SF1670, using the antibody against β-Catenin. We observed that with the enhancement in the number and the span of proliferating MGPCs marked by PCNA in the retina upon *ptenb* knockdown (Fig 3.2.5.1 A, B) and Pten inhibition (Fig 3.2.5.3 A, B), there was a striking increase in the expression of β-Catenin also. This suggested that increased expression of β-Catenin, supported the MGPCs proliferation due to Pten inhibition.

We also tried to investigate the aspect of the nuclear stabilisation of β-Catenin, during Pten inhibition in the retina. For this, we performed immunohistochemistry on the tissues, where we retrieved the epitope for β-Catenin protein by boiling the cryosections in 10mM Sodium Citrate (pH 6.0) for 45min. We observed the β-Catenin nuclear stabilisation in the respective controls at 4dpi as well as upon *ptena*/*ptenb* knockdown (Fig 3.2.5.2 A) and Pten function blockade (Fig 3.2.5.4 A). But we observed that even after adopting strong method of epitope retrieval, complete nuclear β-Catenin could not be retrieved due to some technical limitations in tissue based systems and some cytoplasmic β-Catenin was still seen. The cytoplasmic β-Catenin is a regulatory intercellular adhesion protein alongwith α-Catenin and Integrin, and important for cell-cell communication. Its nuclear roles involve its activity as a nuclear transcriptional co-

activator for the lymphoid enhancer-binding factor-1 (LEF1)/T-cell-specific factor (TCF) family of DNA-binding proteins, contributing in gene transcription. Although, in response to WNT signalling, excess β-catenin which is not used in cell–cell junctions can get accumulated in nucleus and assumes its second function to facilitate cell proliferation (Kobielak & Fuchs, 2004). However, at a particular time, both cytoplasmic and nuclear β-Catenin can co-express in the cells, since both play different essential cellular and intercellular roles (Ramachandran et al., 2011). In our system also, during regeneration we observed the expression of cytoplasmic as well as nuclear stabilised β-Catenin in the proliferating MGPCs, where nuclear β-Catenin is one of the factors which supports the MGPCs proliferation upon Pten inhibition. However, we could not observe any increase in the nuclear stabilisation of β-Catenin in the MGPCs, in Pten blocked condition, when MGPCs number enhances as compared to the control at 4dpi.

Along with this, we observed a *ptenb* MO concentration-dependent (Fig 3.2.5.5 A, B) and SF1670 drug dose-dependent (Fig 3.2.5.5 C, D) increase in the levels of β-Catenin even in the western blotting assay. On the contrary, surprisingly, *ptena* knockdown in the retina at 4dpi did not seem to have much impact on the levels of β-Catenin, as seen by western blotting assay (Fig 3.2.5.5 A, B). These results not only become a validation for the increase in the number of MGPCs that we found with Pten blockade but rather they also suggest the functional existence of Pten/PI3K/Akt/β-Catenin axis during zebrafish retina regeneration. Such a link was till now known to play a role in various inflammatory responses in diseases (Tokuhira et al., 2015) and cancerous conditions (Cubrey et al., 2016).


A
4dpi
ctl MO
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lissamine
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PCNA
DAPI
merge
ONL
INL
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ptenb MO
lissamine
β-catenin
PCNA
DAPI
merge
ONL
INL

Fig 3.2.5.1 β-Catenin expression increases upon *ptenb* knockdown. (**A** and **B**) Confocal microscopy images taken at 20X (**A**) and 60X (**B**) of retinal cross-sections show an increase in the expression of β-Catenin upon *ptenb* knockdown in the retina**,** at 4dpi; n=3 biological replicates. Scale bars represent 10µm in (**A**, **B**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**, **B**); dpi, days post injury. A single 0.5-µm-thick optical section was taken in (**A**, **B**). DAPI is the reference nuclear staining.

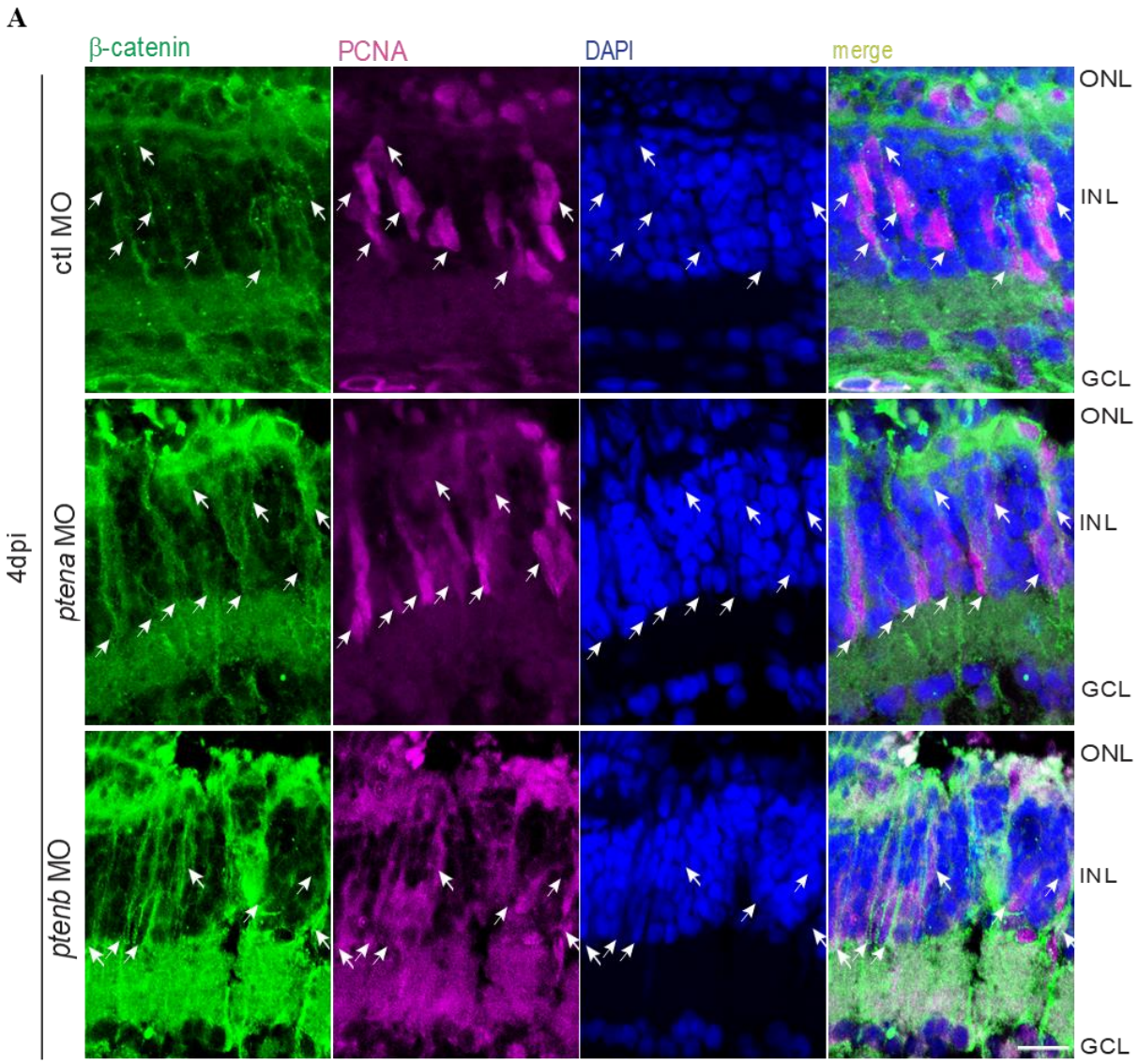

Fig 3.2.5.2 β-Catenin expression by strong epitope retrieval method upon *ptena* and *ptenb* knockdown. (**A**) Confocal microscopy images of retinal cross-sections taken at 60X showing nuclear localisation of β-Catenin in the *ptena* and *ptenb* knocked-down retina**,** at 4dpi, where epitope is retrieved by boiling in Sodium citrate. Scale bars represent 10µm in (**A**); arrows mark MGPCs with nuclear localised β-Catenin, with PCNA; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury. A single 0.5-µm-thick optical section was taken in (**A**). DAPI is the reference nuclear staining.

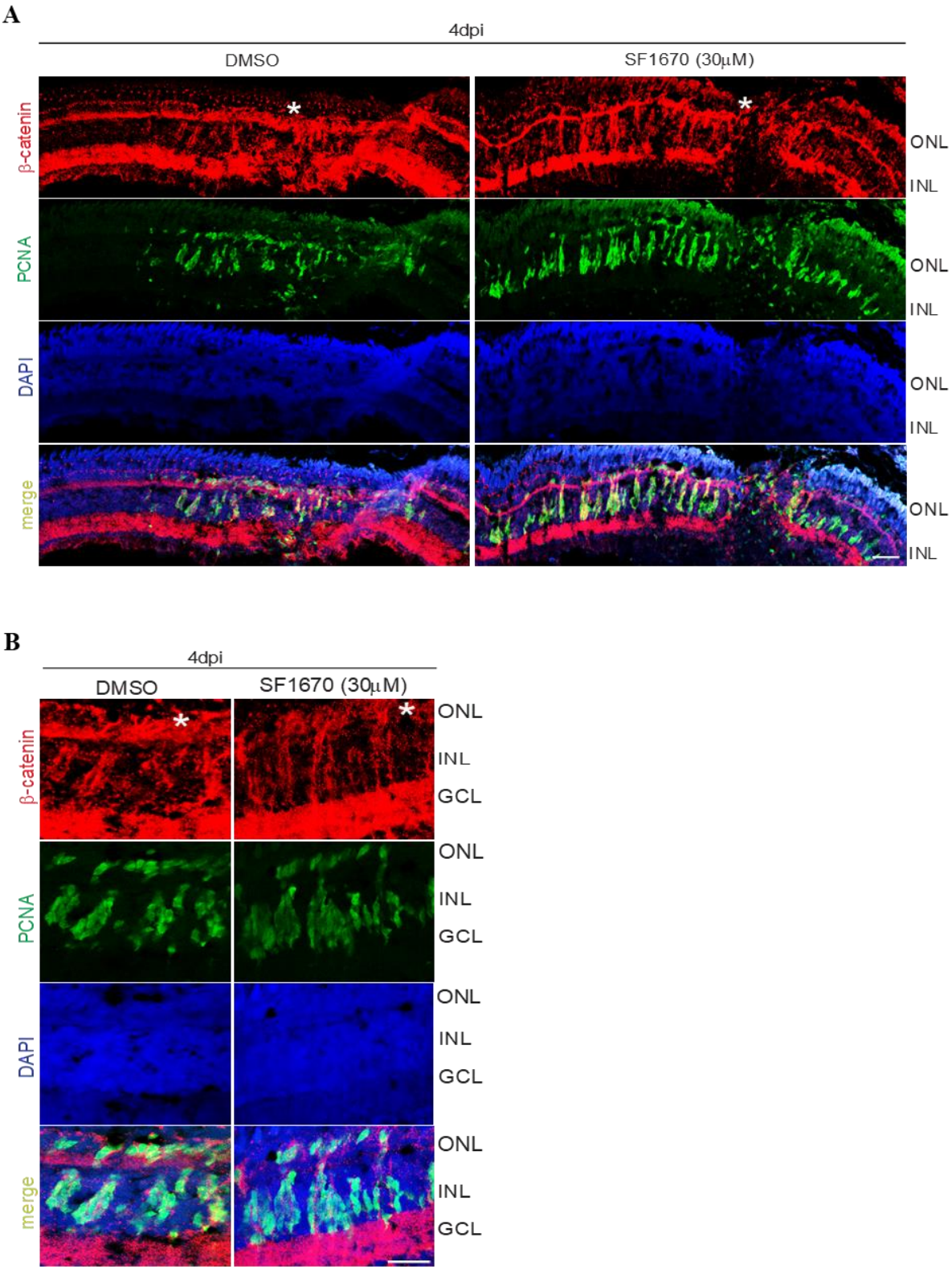

Fig 3.2.5.3 β-Catenin expression increases upon Pten function inhibition. (**A** and **B**) Confocal microscopy images taken at 20X (**A**) and 60X (**B**) of retinal cross-sections show an increase in the expression of β-Catenin in the SF1670 treated retina**,** at 4dpi; n=3 biological replicates. Scale bars represent 10µm in (**A**, **B**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**, **B**); dpi, days post injury. A single 0.5-µm-thick optical section was taken in (**A**, **B**). DAPI is the reference nuclear staining.

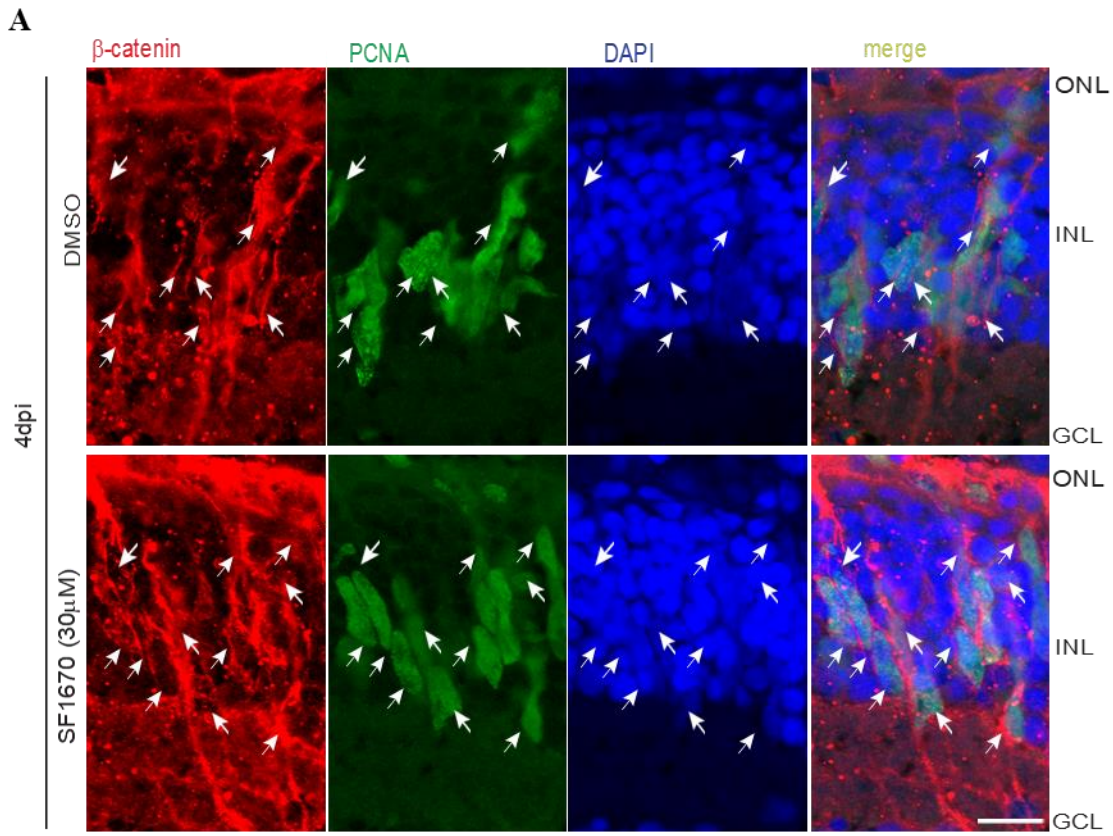

Fig 3.2.5.4 β-Catenin expression by strong epitope retrieval method upon Pten function inhibition. (**A**) Confocal microscopy images of retinal cross-sections taken at 60X showing nuclear localisation of β-Catenin in the SF1670 treated retina**,** at 4dpi, where epitope is retrieved by boiling in Sodium citrate. Scale bars represent 10μm in (**A**); arrows mark MGPCs with nuclear localised β-Catenin, with PCNA; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury. A single 0.5-μm-thick optical section was taken in (**A**). DAPI is the reference nuclear staining.

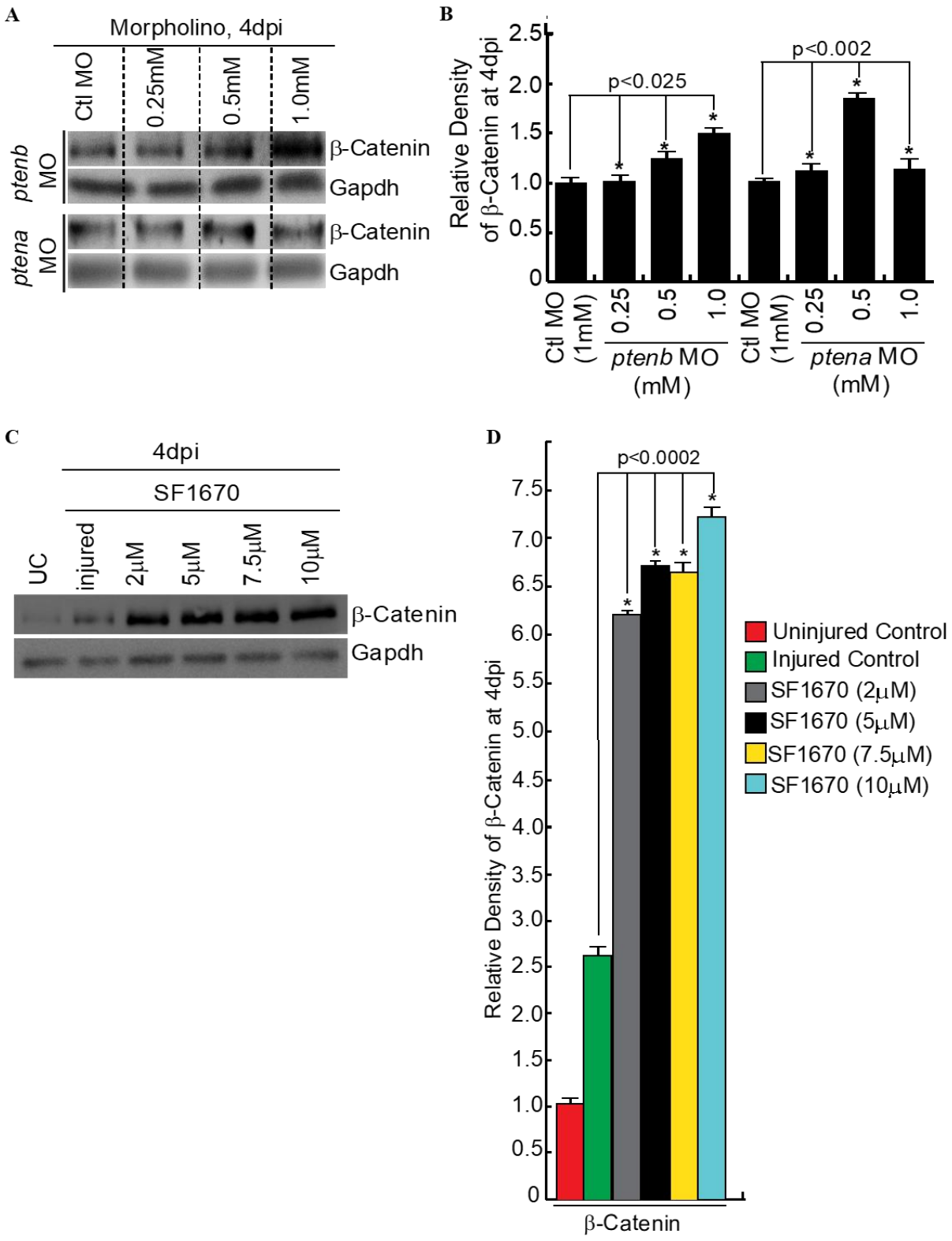

Fig 3.2.5.5 Western blot analyses showing increased β-Catenin expression upon *pten* knockdown and Pten function inhibition. (**A** and **C**) Western Blot analyses of β-Catenin in retinal extracts collected at 4dpi after *ptena*/*ptenb* knockdown and from retinae treated with different concentrations of SF1670. (**B** and **D**) Densitometry plots showing the relative expression of β-Catenin protein in the retina at 4dpi, upon *ptena*/*ptenb* knockdown and SF1670 treatment, normalised to the uninjured retina; *p<0.025 (and as shown in the figure); n=3, n=4. Gapdh is the loading control. dpi, days post injury; UC, uninjured control; Error bars represent SD.

Discussion

The above set of experiments are suggestive of the functioning of Pten/PI3K/Akt/mTOR pathway during zebrafish retina regeneration. We observed that as the number of MGPCs increases upon Pten blockade, the active and total Akt levels associated with the MGPCs also increase, which lead to the activation of mTORC1, thereby, initiating a cell proliferative response post retinal injury. However, excessive Pten blockade triggers a negative feedback regulation mechanism by mTORC1 and its downstream factors on Akt1 activation, which leads to a decrease in the phosphorylation status of Akt1 at Thr302 and Ser467 by PDK1 (activated by PI3K) and mTORC2, respectively. We deduce that this negative feedback loop which has been reported during mice axonal regeneration also creates a fine line of balancing between the rate of cell proliferation in cancerous state and successful retina regeneration in zebrafish. Interestingly, we also found that PI3K and mTORC2 are also the pro-proliferative kinases as their blockade results in a reduction in the number of MGPCs in the regenerating retina, along with a respective decline in the levels of pAktThr302 and pAktSer467. Thus, the increase in MGPCs proliferation upon Pten blockade is mediated through the activated Akt1 and mTORC1 downstream. In this pathway, PI3K, Akt1 and mTOR act as pro-proliferative factors while Pten is an anti-proliferative factor during retina regeneration in zebrafish, all beautifully orchestrated in the same pathway to regulate cell proliferation, cell growth and survival responses. But, surprisingly, upon delving deeper into the Akt1 activation mechanism by PI3K and mTORC2 upon inhibition of Pten, we found that apart from both these molecules there are some other factors which play their roles upon Pten inhibition to mediate the pro-proliferative effects on MGPCs in the regenerating retina of zebrafish. Even if the PI3K and mTORC2 activity seemed lost, MGPCs could still proliferate and enhance their numbers and span upon Pten blockade, as some other pro-proliferative molecules contribute towards MGPCs proliferation in co-ordination with the inhibited Pten. Lastly, we also report that the increase in the number of proliferating MGPCs upon downregulation of *ptenb* and Pten function blockade might be also supported by the involvement of Pten/PI3K/Akt/β-Catenin axis which was very well documented in the other systems till now. These reports mention that Pten/PI3K pathway mediated through Akt interfere with the GSK3β activation and stability, which further alters the nuclear localisation of β-Catenin, thereby affecting the dividing cell population. In our system also, we see similar trends of increase in the expression levels of β-Catenin upon Pten blockade, promoting the number of proliferating MGPCs (Fig 3A and B).

Chapter 3

Pten regulates MGPCs proliferation through Mmp9/Notch signalling and other parallel pathways.

3.3.1. Notch signalling and Mmp9 get perturbed by Pten during retina regeneration.

At the end of the previous Chapter, we concluded about the existence and activity of some other factors which could support the pro-proliferative action in the absence of Pten, even if Akt was not active. We explored the literature and decided to probe into the regulation of Notch signalling, upon Pten blockade. Notch signalling has been well documented and a highly conserved cell signalling system present in most animals. It has been accounted for playing a vital role in cell proliferation and differentiation during embryonic development and in lateral inhibition during cancers. This juxtacrine cell signalling, important for the cell-cell communication, is dependent on the interaction of Notch receptors present on the signal-receiving cells with the Delta ligand present on the signal sending cells. A recent report states that Notch3 receptor and Delta B ligand play a role in maintaining MG quiescence, and these are the negative regulators of regeneration during the light-mediated regenerative response in the zebrafish retina. Their knockdown has shown to increase MGPCs proliferation (Campbell et al., 2020). Along with this, the existing literature reports of the anti-proliferative roles of Notch signalling and its effector *her4.1* during zebrafish retina regeneration, upon mechanical injury to the retina, just similar to the role of Pten. Notch signalling blockade in *1016tuba1a*:GFP transgenic zebrafish retina, by a γ-secretase inhibitor DAPT, showed an increase in the number of GFP^+ MGPCs at 4dpi (Wan et al., 2012). We also found this increase in the number and the span of MGPCs upon *her4.1* knockdown in the retina at 4dpi (Fig 3.3.1.1 A, B). Thus, we speculated that Pten might be mediating its anti-proliferative functions through the Notch signalling and upon Pten blockade the Notch signalling must be getting perturbed during zebrafish retina regeneration.

Parallelly, we also tried to address the effects of Pten tumor suppressor on the regulation of a metastatic cancer marker, Matrix Metalloproteinase 9 (Mmp9). Mmp9 is an extra-cellular matrix (ECM) protein, well known for its proteolytic activities on several inactive signalling molecules like Transforming Growth Factor-β (TGF-β), Tumor Necrosis Factor-α (TNF-α) and others, to activate them and to facilitate the downstream pro-proliferative pathways (Sharma et al., 2020; Yabluchanskiy et al., 2013). Mmp9 activity preludes the MGPCs proliferation and is also known to be regulated by the Notch signalling during zebrafish retina regeneration (Kaur

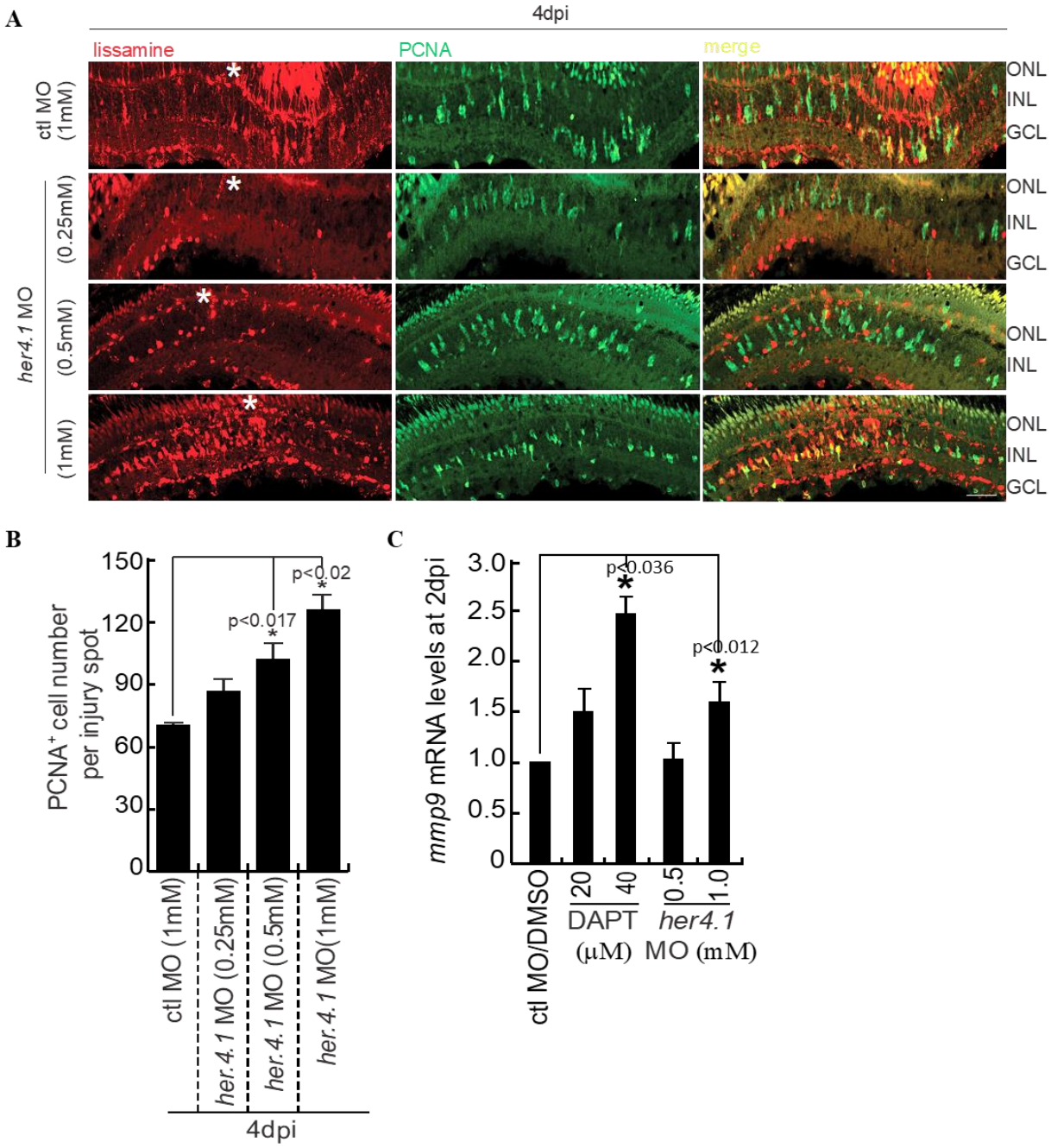

Fig 3.3.1.1 Knockdown of *her4.1* leads to an enhancement in the number of proliferating MGPCs and *mmp9* upregulation. (**A** and **B**) Confocal microscopy images of retinal cross-sections show a concentration-dependent increase in the number of PCNA$^+$ MGPCs in *her4.1* knockdown retina at 4dpi (**A**), which is quantified (**B**); *p < 0.02, n=3 biological replicates. (**C**) The qPCR analyses of *mmp9* mRNA levels in DAPT-treated and *her4.1* knockdown retina at 2dpi; *p < 0.04, n=5 biological replicates. Scale bars represent 10μm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**A**).

et al., 2018). This regulation was validated by us also by qPCR in the retina at 2dpi (Fig 3.3.1.1 C).

In these lines, first, we checked the effects of the SF1670-mediated Pten blockade on the expression of *her4.1* and found SF1670 concentration-dependent decline in the levels of *her4.1*, while an upregulation in the levels of *mmp9* in the retina by mRNA *in situ* hybridisation at 4dpi (Fig 3.3.1.2 A) as well as qPCR at 2dpi (Fig 3.3.1.2 B, C). This suggests that the increase in the number of proliferating MGPCs which were seen upon Pten blockade as in 3.1.3 might be due to an increase in the levels of *mmp9* mediated through the decreased levels of *her4.1*.

Further, we also analysed the levels of *her4.1* and *mmp9*, upon double blockade of Pten-PI3K and Pten-mTORC2 as done previously in 3.2.3 for assessing their effects on the MGPCs proliferation. We found a decrease in the levels of *her4.1* upon SF1670-mediated Pten blockade, with an upregulation seen upon Torin1-mediated mTORC2 blockade alone and in combination with SF1670, and also in LY294002-mediated PI3K blockade alone. While there was no change in the *her4.1* expression level upon LY294002 treatment with SF1670, as seen by qPCR done in the retina at 2dpi (Fig 3.3.1.3 A). Contrary to this, we observed an increase in the expression of *mmp9* in all the experimental set-ups at 2dpi (Fig 3.3.1.3 B). Such a regulation might be existing owing to the selective shifts or flux change in the interaction of PDK1 either with Akt (also known as PKB) or with PKC (another kinase responsible for pro-survival signals) and subject to the Akt phosphorylation at its Ser467 by mTORC2. Upon Torin1 treatment, Akt is not phosphorylated, leading to the flux changing of PDK1 from Akt to PKC, thus allowing Notch activation through Adams (Steinbuck & Winandy, 2018), leading to upregulation of *her4.1*, thereby keeping a check on the number of MGPCs as was seen upon Torin1 treatment in 3.2.3. There might be a feedback signal due to this decrease in the MGPCs number, to elevate the levels of *mmp9*, which is a pro-proliferative factor, to maintain a balance in the rate of MGPCs proliferation during retina regeneration. While upon combined blockade of Torin1 along with Pten, PDK1 tried to keep the *her4.1* expression high through Adam mediated Notch activation, but *her4.1* expression faced a slight suppression in its levels based on Pten blockade. In this condition, we observed an increase in the *mmp9* expression levels due to the blockade of Pten, which further tried to bring *her4.1* levels up. In the case of LY294002 treatment, Pten drove the *her4.1* expression, even if PDK1-based Notch signalling activation was absent, thus, reducing the MGPCs proliferation also. Still, in this condition also *mmp9* levels were unexpectedly staying high might be as a result of a feedback mechanism. Interestingly, upon combined blockade of Pten with PI3K, there are no significant differences

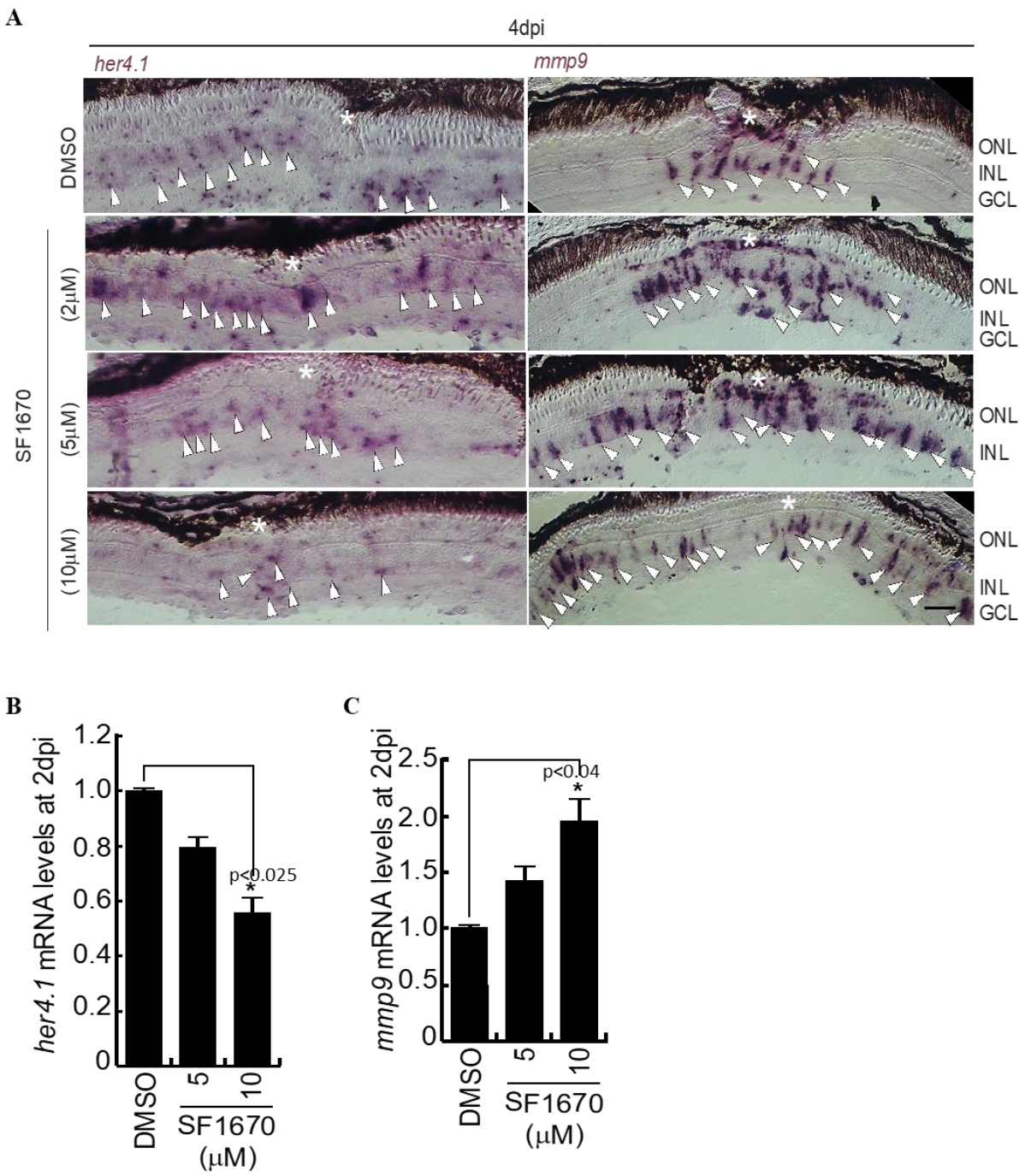

Fig 3.3.1.2 Pten blockade regulates *mmp9* and *her4.1* levels during retina regeneration. (**A**) BF microscopy images of retinal cross-sections show the mRNA ISH of the *her4.1* and *mmp9* mRNAs in the retina treated with SF1670 at 4dpi. (**B** and **C**) The qPCR analyses of *her4.1* (**B**) and *mmp9* (**C**) mRNA levels in SF1670-treated retina at 2dpi; *p<0.04, n=4 biological replicates. Scale bars represent 10μm in (**A**); arrowheads mark the *her4.1* and *mmp9* mRNA *in situ* hybridisation signal; the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**A**).

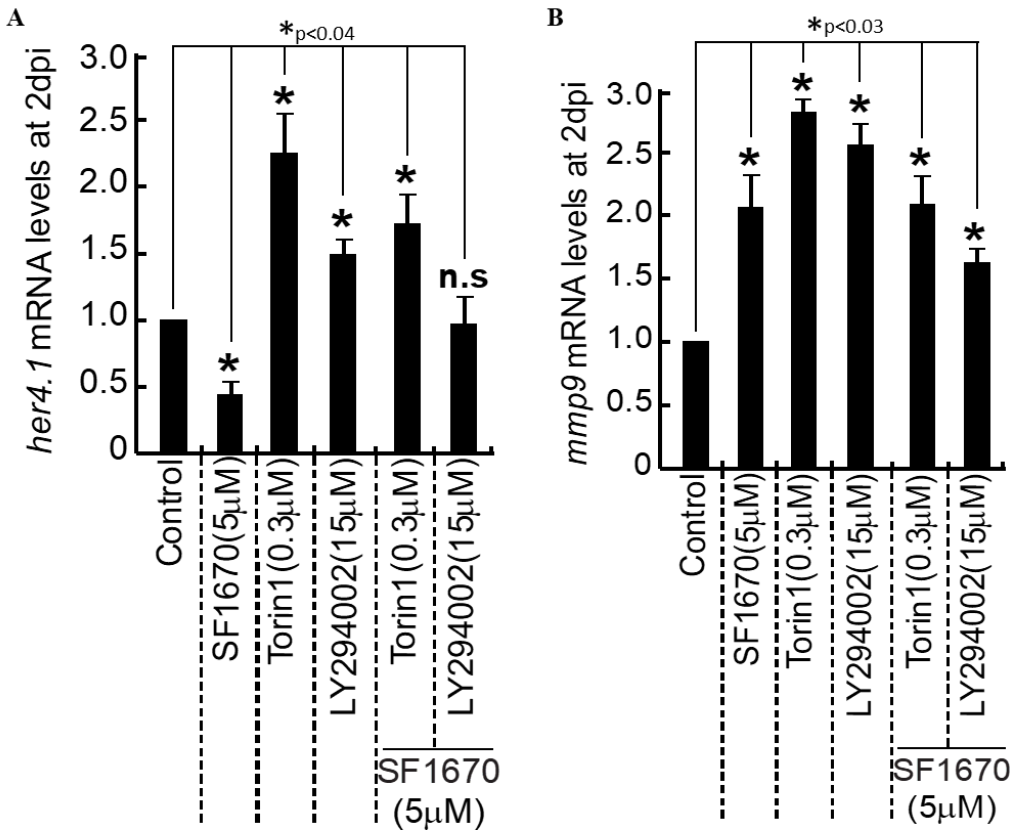

Fig 3.3.1.3 Pten blockade along with PI3K-mTORC2 blockade regulate *her4.1* and *mmp9* levels during retina regeneration. (**A** and **B**) The qPCR analyses of *her4.1* (**A**) and *mmp9* (**B**) mRNA levels in retina treated with Torin1, LY294002 alone and in combination with SF1670 at 2dpi; *p < 0.03, n=3 biological replicates. n.s., not significant; dpi, days post injury. Error bars represent SD.

in the levels of PIP3 and PIP2, and there is no activation of PDK1 also, thus, we observed the level of *her4.1* to be reduced similar to that of the control, as a mechanism of tissue homeostasis. While in this condition, *mmp9* expression was high owing to the Pten blockade and reduction in the levels of its negative regulator *her4.1*, which might be a reason for the increase in the number of MGPCs in this case similar to that seen upon Pten blockade alone in 3.2.3.

3.3.2. Mmp9 positively regulates Notch signalling and its effector gene *her4.1*.

There is ample literature available which mentions that the Mmp9 regulates Notch signalling by a cascade of processes and multiple effectors involved. Mmp9 proteolytically cleaves the inactive TNF-α to activate and release it (Yabluchanskiy et al., 2013), which further induces a transcription factor NF-κB (Nuclear Factor kappa light chain enhancer of activated B cells) (Fujisawa et al., 1996; Pozniak et al., 2014). Further, NF-κB upregulates the transcription of *mmp9* (Rhee et al., 2007) and also of *adam10* (Zhu et al., 2014) and *adam17* (Wawro et al., 2019), which are a disintegrin and metalloproteinase family genes, which code for cell surface proteins responsible for the ectodomain shedding of a variety of substrates. The Adam10 and Adam17 proteins are known for their S2 proteolytic cleavage on the Notch receptors on the cells rendering this processed Notch receptor susceptible to the S3 cleavage by γ-Secretase, which leads to the internalisation of Notch Intracellular Domain (NICD) into the nucleus (Gibb et al., 2011; Groot & Vooijs, 2012; Steinbuck & Winandy, 2018). Inside the nucleus, NICD binds to the DNA along with the transcription factor Recombining binding protein suppressor of hairless (RBPJ) and a protein Mastermind homologue (MAML), which activate the transcription of the Notch signalling effector genes one of which is *hairy-related 4.1* (*her4.1*) (Groot & Vooijs, 2012).

In consistence with the existing literature, we aimed to check the regulation of Notch signalling and its effector *her4.1* by Mmp9 during zebrafish retina regeneration. For this, we blocked the Mmp9 function by injection of its pharmacological inhibitor SB3CT in the zebrafish retina. In the Mmp9-blocked condition, we assayed the levels of *adam10a*, *adam17a*, *rbpja* and *her4.1*. The qPCR was done at 16hpi (Fig 3.3.2.1 A) and 2dpi (Fig 3.3.2.1 B) and revealed a downregulation in the levels of all these genes. While *mmp9*-overexpression in the retina showed an opposite trend of an increase in the levels of these genes at 2dpi (Fig 3.3.2.1 C). These results are suggestive of the conserved mechanism of activation of Notch signalling by Mmp9 and Adams during zebrafish retina regeneration, as has been well known in cancer models and also in mice. This also made us conclude that Mmp9 tries to regulate its own levels by activating Notch signalling which tries to suppress Mmp9, thereby creating an anti-proliferative environment.

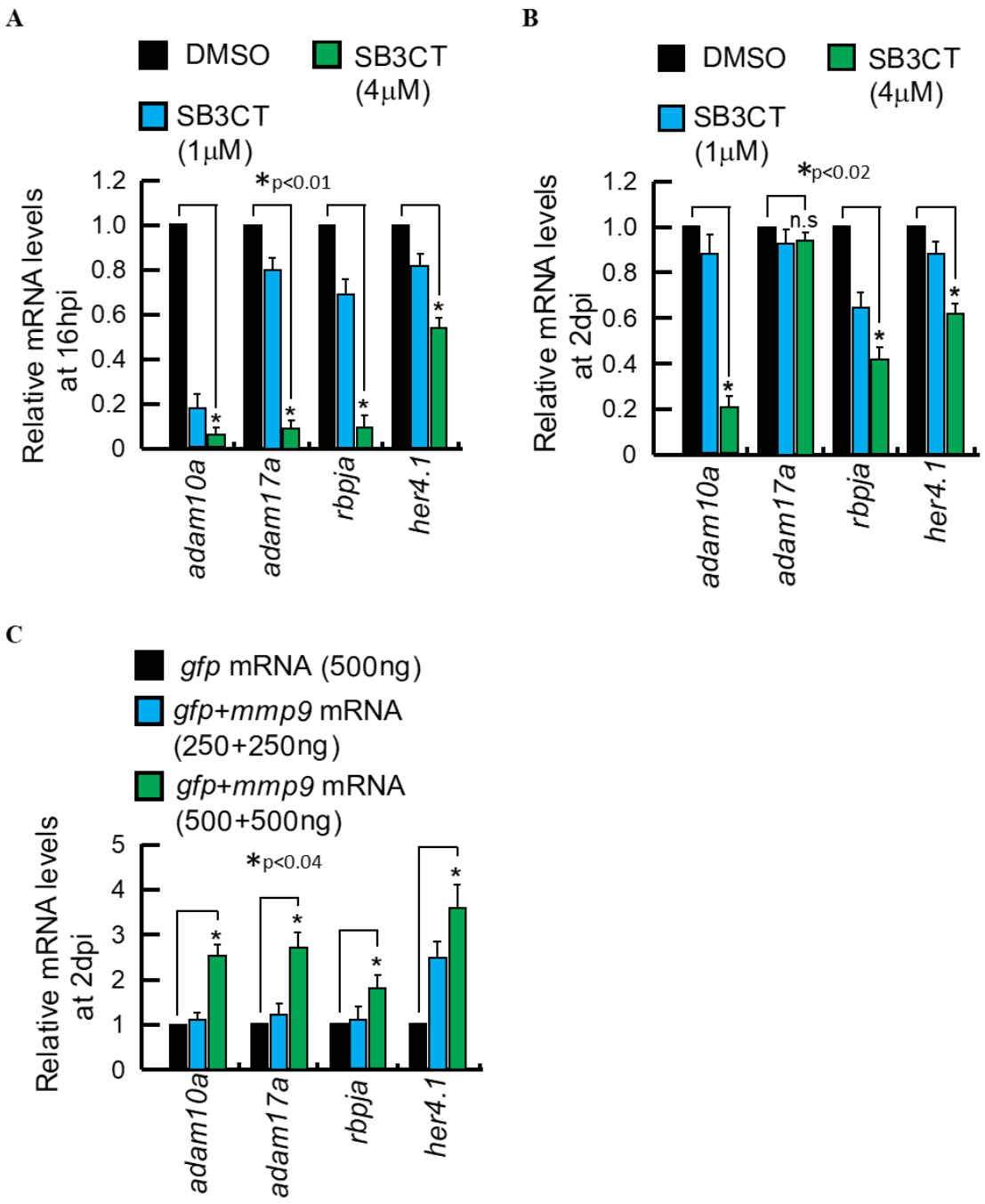

Fig 3.3.2.1 Mmp9 regulates *adam*s and Notch signalling effector genes during retina regeneration. (**A**) The qPCR analyses of *adam10a*, *adam17a*, *rbpja* and *her4.1* in SB3CT-treated retina at 16hpi; *p < 0.01, n=6 biological replicates. (**B**) The qPCR analyses of *adam10a*, *adam17a*, *rbpja*, *her4.1* in SB3CT-treated retina at 2dpi; *p < 0.02, n=3 biological replicates. (**C**) The qPCR analyses of *adam10a*, *adam17a*, *rbpja* and *her4.1* in *mmp9*-overexpressed retina at 2dpi; *p < 0.04, n=5 biological replicates; n.s., not significant. Error bars represent SD.

3.3.3. Notch signalling and Mmp9 positively regulate Pten.

We had previously found that the Pten blockade, which enhanced the MGPCs proliferation led to a decrease in the levels of *her4.1*. Next, we tried to investigate the effects of Notch signalling blockade on Pten expression. Notch signalling blockade also downregulates *her4.1* and shows a similar impact on MGPCs proliferation as shown by Pten blockade. Upon DAPT-mediated Notch signalling blockade, we observed a decrease in the levels of *ptena* and *ptenb* by performing qPCR (Fig 3.3.3.1 A) and of Pten protein by western blotting assay (Fig 3.3.3.1 B) at 2 and 4dpi. Similar downregulation of *ptenb* was seen in qPCR done at 2dpi (Fig 3.3.3.1 C), with a parallel decrease in its protein as seen in western blotting assay (Fig 3.3.3.1 D) at 2 and 4dpi upon MO-mediated *her4.1* knockdown in the retina. This suggests that Notch signalling and its effector gene *her4.1* might be regulating Pten positively to keep the number of MGPCs under a check during retina regeneration. However, *her4.1* knockdown done at 2dpi, with lower MO concentrations (0.25mM and 0.5mM), could not show this declining effect on the Pten protein levels. We theorize that there might be an underlying condition at 2dpi where Pten slightly increases its levels trying to keep the MGPCs proliferation under check, upon lower extent of *her4.1* knockdown, so as to ensure the tissue homeostasis (since *her4.1* knockdown allows the increase in rate and the span of the MGPCs proliferation). These increased levels of Pten protein might be trying to functionally compensate the effects of knockdown of *her4.1*. But this compensatory mechanism ceases upon strong *her4.1* knockdown, leading to a decrease in the expression levels of Pten.

We also explored the effects of Notch signalling blockade on the activation of Akt. By performing western blotting assay from DAPT-treated retinal lysates, we observed an increase in the levels of pAktThr302, pAktSer467 and total Akt also at 4dpi, as compared to the respective levels of these proteins in the DMSO control at 4dpi (Fig 3.3.3.2 A). This is directive of the fact that Notch signalling keeps a check on the MGPCs proliferation by preventing the Akt from getting phosphorylated. Thus, Notch signalling inhibition becomes an important event to initiate Akt phosphorylation and to increase total Akt levels during retina regeneration in zebrafish. Interestingly, upon DAPT treatment in the NMDA-mediated injured retina of mice, we found only a moderate increase in the MGPCs proliferation (Fig 3.3.3.3 A, B). This was accompanied by a slight decrease in the PTEN protein levels, while moderate changes in the levels of AKT were seen, despite an increase in the cMYC levels in the mice retina at 2.5dpi (Fig 3.3.3.3 C, D). This might be due to the involvement of some factors other than the Notch

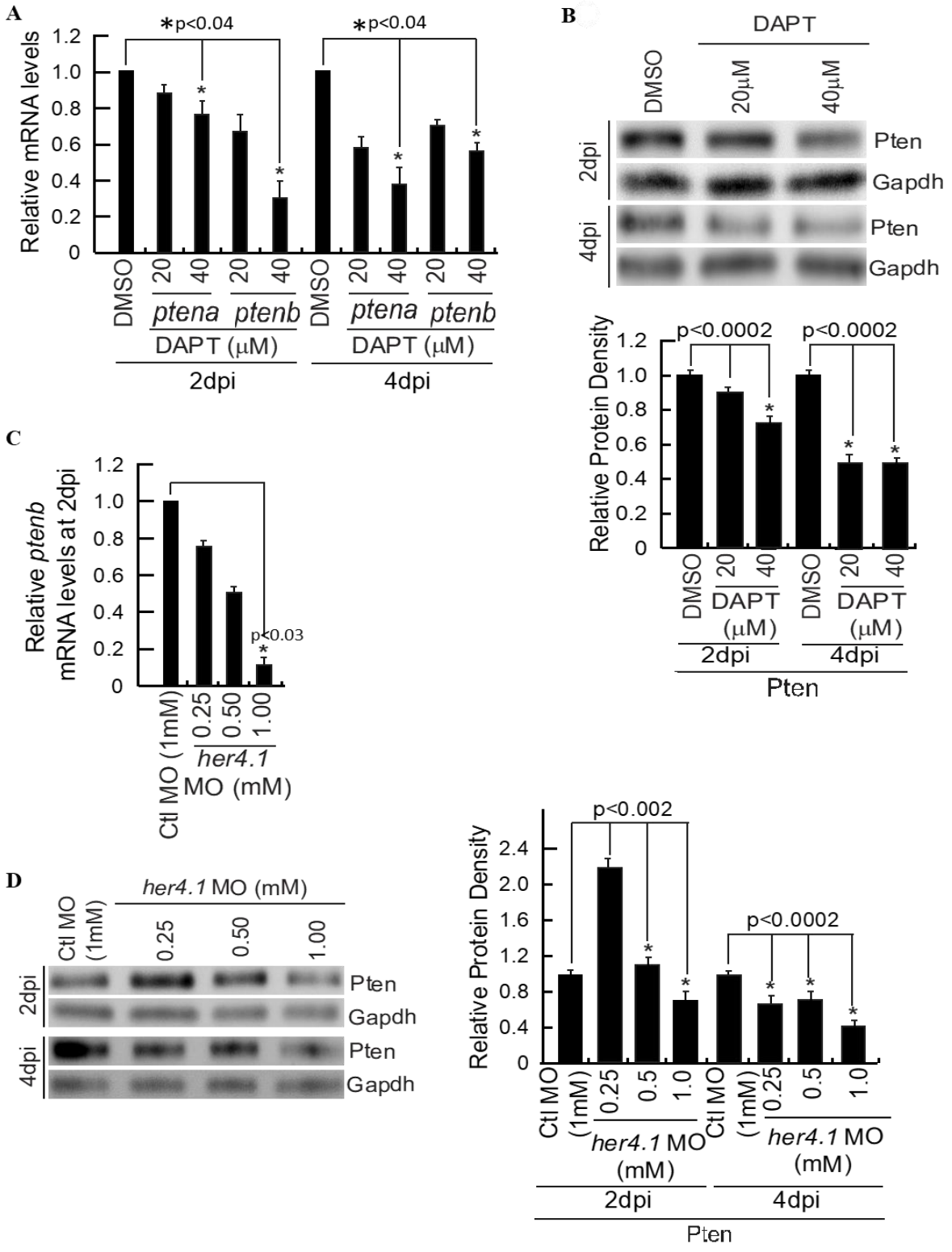

Fig 3.3.3.1 Notch signalling regulates Pten expression. (**A**) The qPCR analyses of *ptena* and *ptenb* mRNA levels in DAPT-treated retina at 2dpi and 4dpi; *p < 0.04, n=5 biological replicates. (**B**) Western blot analyses (upper) and densitometry plots (lower) of Pten protein in DAPT-treated retina at 2dpi and 4dpi; *p < 0.0002 (and as shown in the figure); n=2. (**C**) The qPCR analysis of *ptenb* mRNA levels at 2dpi in *her4.1* knockdown retina; *p < 0.03, n=6 biological replicates. (**D**) Western blot analyses (left) and densitometry plots (right) of Pten protein at 2dpi and 4dpi in *her4.1* knockdown retina; *p < 0.002 (and as shown in the figure); n=4. Gapdh is the loading control. dpi, days post injury. Error bars represent SD.

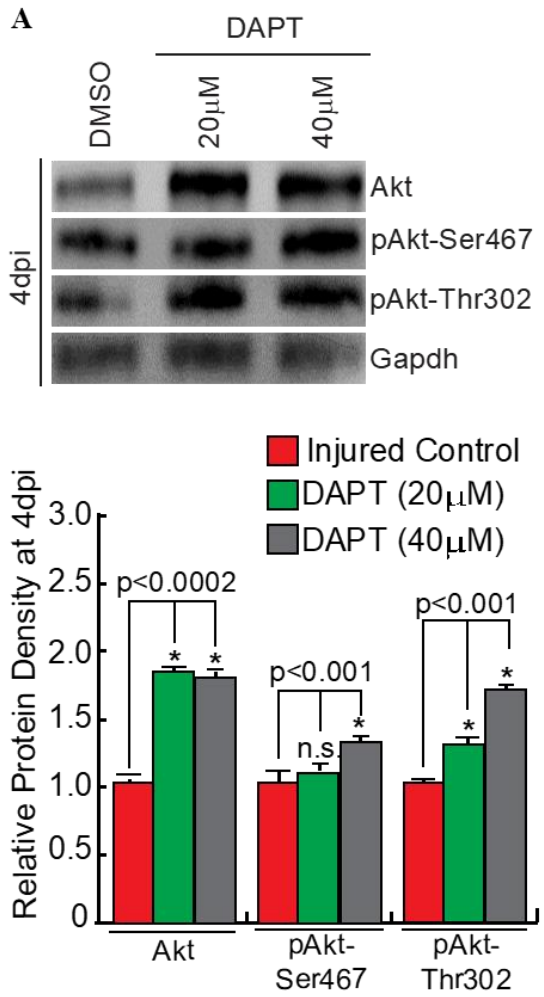

Fig 3.3.3.2 Notch signalling blockade leads to an increase in Akt expression and its phosphorylation. (**A**) Western Blot analyses (upper) and densitometry plots (lower) of Akt, pAkt-Ser467, pAkt-Thr302 from retinal extracts prepared from retinae injected with different concentrations of DAPT at 4dpi; *p < 0.001 (and as shown in the figure); n=2. Gapdh is the loading control. dpi, days post injury; n.s., not significant. Error bars represent SD.

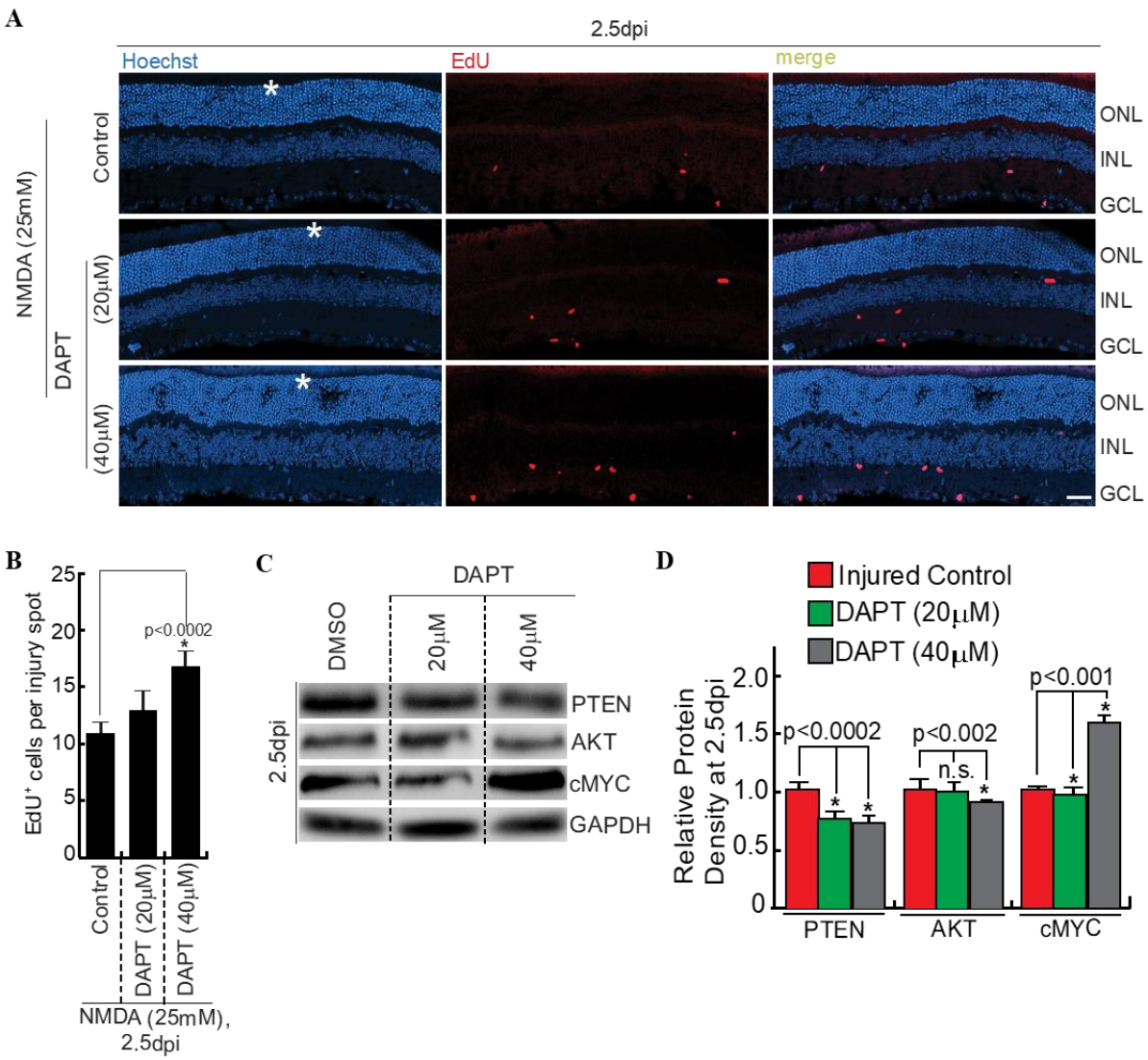

Fig 3.3.3.3 Effect of Notch signalling blockade on MGPCs proliferation and PTEN/AKT expression in mice. (**A** and **B**) Confocal microscopy images of retinal cross-sections show a concentration-dependent increase in the number of EdU^+ MGPCs with DAPT treatment in mice retina compared to the control retina at 2.5dpi (**A**), which is quantified (**B**); *p < 0.0002; n=3 biological replicates. (**C** and **D**) The western blot analyses (**C**) and the densitometry plots (**D**) of PTEN, AKT, cMYC from the lysates collected from mice retina treated with different concentrations of DAPT at 2.5dpi; *p < 0.002 (and as shown in the figure); n=2. GAPDH is the loading control. Scale bars represent 10µm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury; n.s., not significant. Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**A**). Hoechst is the reference nuclear staining.

signalling influencing the regulation of the PTEN/AKT levels during mice retina regeneration.

From the previous set of results in 3.3.2, we had already observed a positive regulation of Notch signalling and *her4.1* by Mmp9, along with this we also found Notch signalling promoting the levels and the activity of Pten. So, we speculated that the Mmp9 could be positively regulating the Pten levels also. We analysed the expression levels of *pten* genes in SB3CT-mediated Mmp9 blockade in the retina and found a decline in the levels of *ptena* and *ptenb* by qPCR done at 16hpi (Fig 3.3.3.4 A, B). This decline was consistent at the translational levels also, as confirmed by western blotting assay and its densitometry plots for Pten protein at 16hpi and 2dpi from the SB3CT-treated retinal lysates (Fig 3.3.3.4 C). Here, Mmp9 was blocked at 16hpi, which is the time of the peak of *mmp9* expression, as well as of MG reprogramming. On the contrary, we found an increase in the expression of *ptena* (not much significant) and *ptenb* upon *mmp9* overexpression in the retina at 2dpi (Fig 3.3.3.4 D). This suggests that as Pten tries to lower down the *mmp9* level, Mmp9, on the other hand, keeps the level of its suppressor Pten high, which keeps the level of Mmp9 and thus, the associated MGPCs proliferation under a check. This defines a fair act of balancing between the rate of proliferation and cell migration during cancerous conditions and the regenerative response in the retina.

Next, we explored the effects of Mmp9, which is a pro-proliferative factor, on the levels of Akt. For this, we checked the levels of active and total Akt by western blotting assay in the SB3CT-mediated Mmp9 blocked retina and observed an abolishment in the levels of phosphorylation of Akt at 2 of its sites at 4dpi. This loss of phosphorylation on Akt was persistent even upon Pten blockade along with Mmp9 blockade at 4dpi, keeping a note of the fact that Pten blockade increases the Akt activation (Fig 3.3.3.3 E). This made us conclude that the Mmp9 activity along with Pten downregulation is essential for the Akt phosphorylation at its Thr302 and Ser467, which elicits a successful regenerative response during zebrafish retina regeneration.

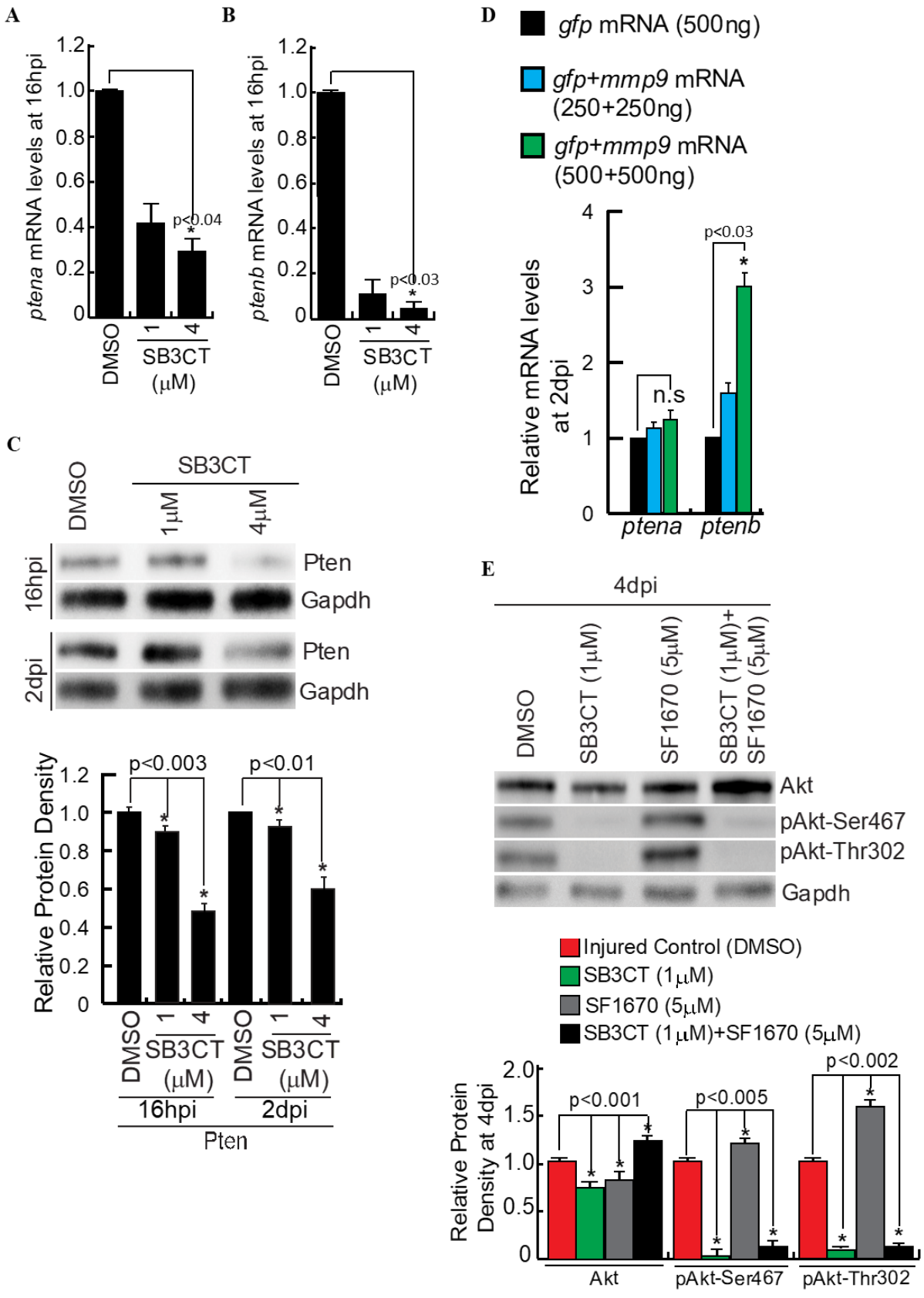
A
ptena mRNA levels at 16hpi
p<0.04
DMSO
1
4
SB3CT (μM)
B
ptenb mRNA levels at 16hpi
p<0.03
C
DMSO
SB3CT
1μM
4μM
16hpi
2dpi
Pten
Gapdh
Relative Protein Density
p<0.003
p<0.01
16hpi
2dpi
D
gfp mRNA (500ng)
gfp+mmp9 mRNA (250+250ng)
gfp+mmp9 mRNA (500+500ng)
Relative mRNA levels at 2dpi
n.s
p<0.03
ptena
ptenb
E
4dpi
DMSO
SB3CT (1μM)
SF1670 (5μM)
SB3CT (1μM)+ SF1670 (5μM)
Akt
pAkt-Ser467
pAkt-Thr302
Gapdh
Injured Control (DMSO)
SB3CT (1μM)
SF1670 (5μM)
SB3CT (1μM)+SF1670 (5μM)
Relative Protein Density at 4dpi
p<0.001
p<0.005
p<0.002

Fig 3.3.3.4 Mmp9 regulates Pten and Akt expression and Akt activation during retina regeneration. (**A** and **B**) The qPCR analyses of *ptena* (**A**) and *ptenb* (**B**) mRNA levels at 16hpi in SB3CT-treated retina; *p < 0.04, n=5 biological replicates. (**C**) Western blot analyses (upper) and densitometry plots (lower) of Pten protein in SB3CT-treated retina, at 16hpi and 2dpi; *p < 0.01; n=3. (**D**) The qPCR analyses of *ptena* and *ptenb* levels in *mmp9*-overexpressed retina at 2dpi; *p < 0.03, n=5 biological replicates. (**E**) Western Blot analyses (upper) and densitometry plots (lower) of Akt, pAkt-Ser467, pAkt-Thr302 from retinal extracts collected from retinae treated with SB3CT alone and in combination with SF1670 at 4dpi; *p < 0.005; n=3. Gapdh is the loading control. hpi, hours post injury; dpi, days post injury; n.s., not significant. Error bars represent SD.

3.3.4. Pten also mediates its functions through pathways other than Notch signalling and Mmp9.

The above-mentioned results direct us to a view that Pten inhibition conjures its pro-proliferative effect on MGPCs through a controlled Notch signalling leading to upregulation and activation of Mmp9. But we wanted to confirm this effect on the number of MGPCs in this condition. Thus, we blocked the Mmp9 using SB3CT (1µM) along with Pten using its selective blocker SF1670 (30µM). Pten blockade led to an increase in the number of MGPCs as already observed, which was nullified and drastically reduced upon Mmp9 blockade in the retina at 4dpi. The combined blockade of both the proteins did not exert any significant effect on the number of MGPCs as it was found to be similar to that in the DMSO control retina at 4dpi (Fig 3.3.4.1 A, B). This suggests the possible existence of some other parallel pathways apart from Mmp9 through which the blocked Pten enhances the number of MGPCs. These parallel pathways or some factor apart from Mmp9 works in the absence of Pten and also in the absence of active Akt and tries to rescue the number of proliferating MGPCs. If Mmp9 was the only factor involved through which the blocked Pten was mediating its pro-proliferative functions. In that case, there should have been a negative effect on the proliferation as Mmp9 was also blocked. Similar results were obtained upon combined *mmp9* knockdown along with Pten blockade in the retina, with no significant difference in the MGPCs proliferation as compared to the control MO-injected retina at 4dpi (Fig 3.3.4.1 C, D), owing to the pro-proliferative role played by a factor other than Mmp9 or a parallel pathway, in the absence of Pten activity.

Similar to this experiment, in the next strategy, we investigated the effect of *pten* overexpression along with DAPT-mediated Notch signalling blockade. We observed a low number of proliferating MGPCs upon *in vivo ptena*/*ptenb* mRNA transfection, which enhanced drastically upon DAPT treatment (40µM) in the retina at 4dpi. But upon *pten* overexpression along with the DAPT treatment, the number of MGPCs got reduced back to that seen in the *gfp* mRNA-transfected control retina at 4dpi (Fig 3.3.4.2 A, B). This must be due to the involvement of some other pathway apart from Notch signalling, working upon its inactivation and creating an anti-proliferative effect along with overexpressed *pten* genes. If Pten was mediating its anti-proliferative functions only through Notch signalling, we should have observed an enhanced number of proliferating MGPCs as was seen with the DAPT-treatment alone.

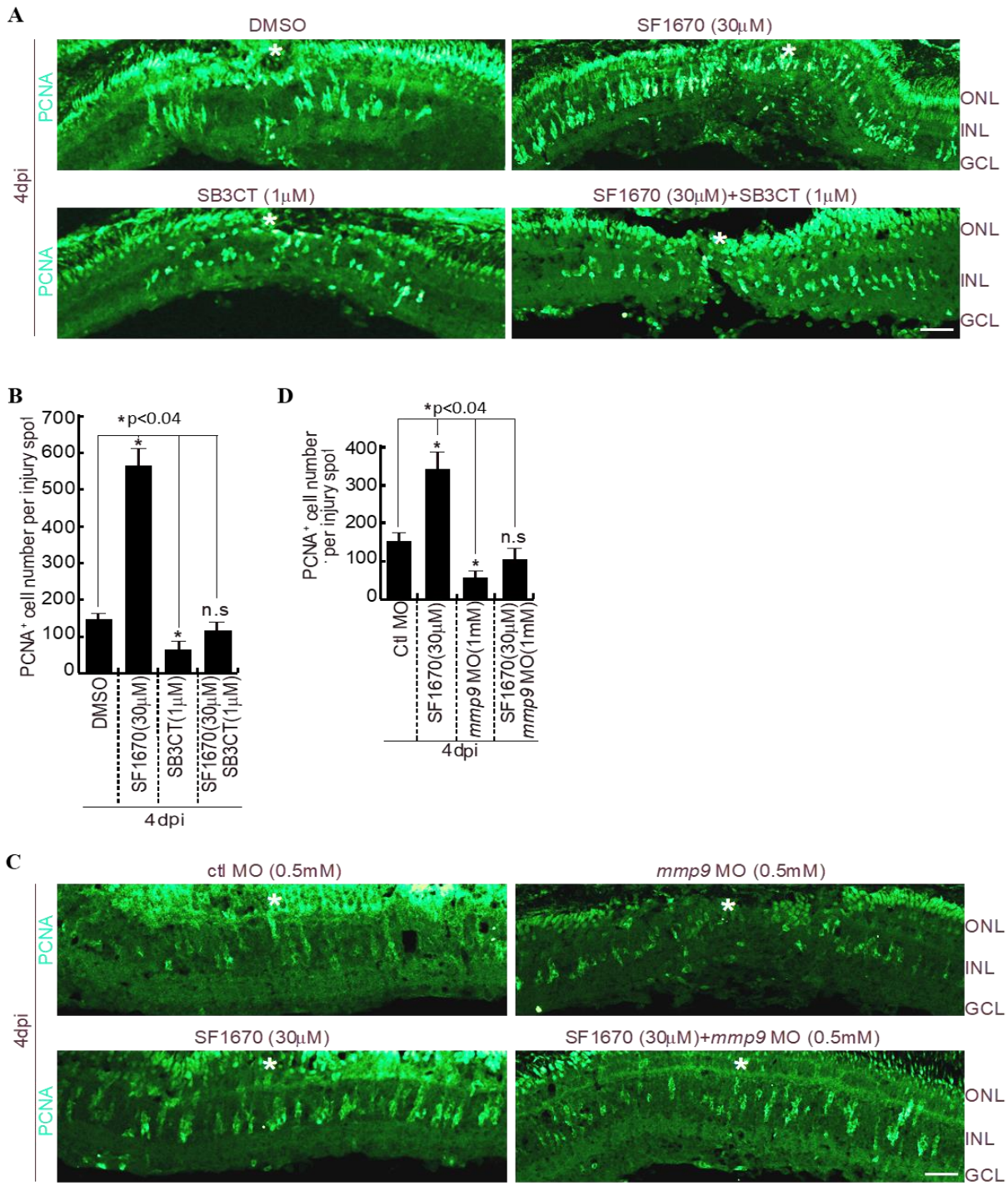

Fig 3.3.4.1 Pten mediates its functions through factors other than Mmp9 during retina regeneration. (**A** and **B**) Confocal microscopy images of retinal cross-sections show an increase in the number of PCNA$^+$ MGPCs with SF1670 treatment, which reduces drastically with the treatment of SB3CT, while the number is elevated close to the DMSO control in the combination of SF1670 and SB3CT at 4dpi (**A**), which is quantified (**B**); *p < 0.04, n=3 biological replicates. (**C** and **D**) Confocal microscopy images of retinal cross-sections show an increase in the PCNA$^+$ MGPCs with SF1670 treatment, which decreases with the *mmp9* knockdown, while this number is elevated close to the DMSO control in the combination of SF1670 and *mmp9* MO in 4dpi retina (**C**), which is quantified (**D**); *p < 0.04, n=3 biological replicates. Scale bars represent 10μm in (**A, C**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A, C**); dpi, days post injury; n.s., not significant. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**A**, **C**).

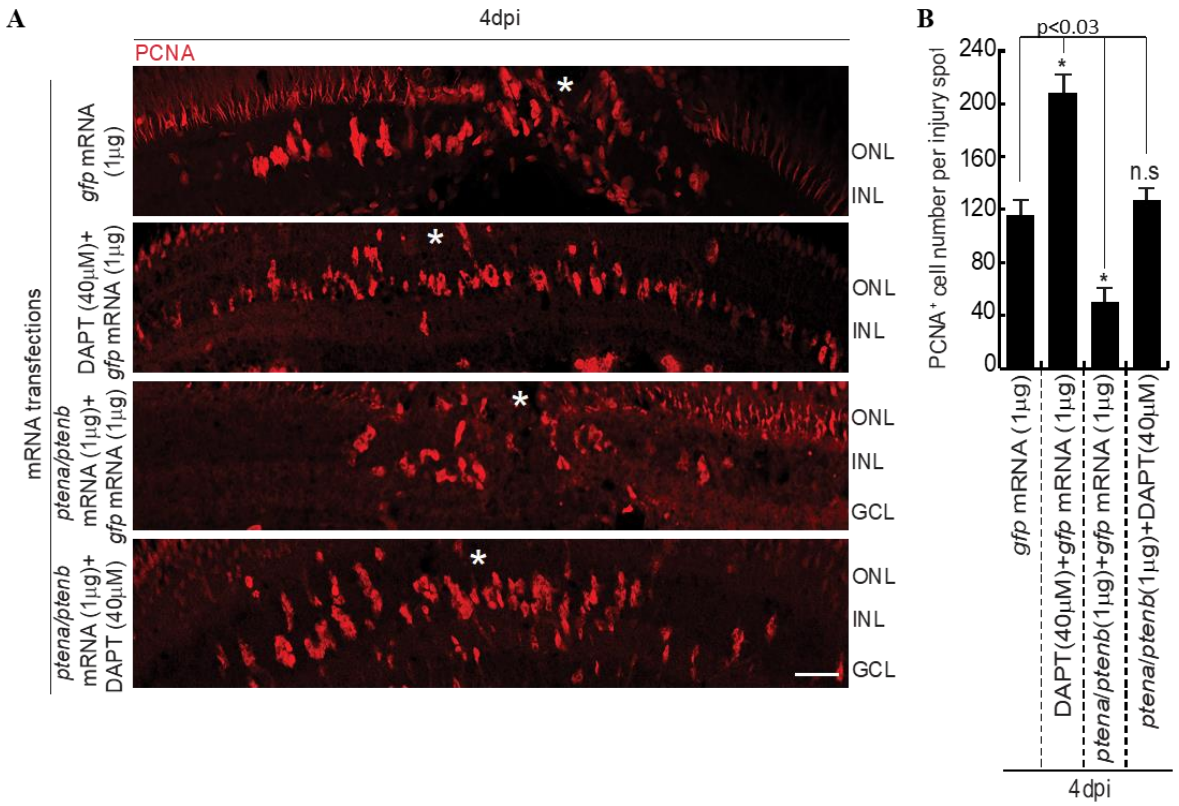

Fig 3.3.4.2 Pten mediates its functions through pathways other than Notch signalling during retina regeneration. (**A** and **B**) Confocal microscopy images of retinal cross-sections show a significant increase in the number of PCNA$^+$ MGPCs with DAPT treatment, which decreases with the *ptena*/*ptenb* overexpression and gets close to the DMSO control in the combination of DAPT and *ptena*+*ptenb* mRNAs-treated retina at 4dpi (**A**), which is quantified (**B**); *p < 0.03, n=3 biological replicates. Scale bars represent 10μm in (**A**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**A**); dpi, days post injury; n.s., not significant. Error bars represent SD. A single 0.5-μm-thick optical section was taken in (**A**).

Discussion

It has been observed in a multitude of cancers, where mutations in Pten lead to tumorigenesis; this is accompanied by several changes in the gene regulatory networks of the system. The normal cellular program gets switched to a more chaotic one. The anti-proliferative genes get silenced or downregulated, along with the activation of the pro-proliferative machinery, which thus leads to uncontrolled growth. But during tissue regeneration, all these pathways are under stringent control, holding true for retina regeneration also. We found that the proliferation permissive environment for MGPCs is created upon Pten downregulation not only through Akt activation by PI3K and mTORC2 but by the coordinated activity of other factors also. We intended to explore in these lines and found that the increase in the MGPCs proliferation due to inhibition of Pten is further strengthened by perturbation of Notch signalling and downregulation of its effector *her4.1*, with an upregulation in the levels of a pro-proliferative factor Mmp9 during retina regeneration. During malignant cancers also, the upregulation of Mmp9 leads to degradation of the ECM, facilitating the invasion of the distant tissue by the tumorigenic cells. Further, *her4.1* and *mmp9* are found to be altered by the combined blockade of Pten-PI3K and Pten-mTORC2 also. As Mmp9 is upregulated, it further activates the other metalloproteinases Adam10 and Adam17, leading to the enhanced expression of transcription factor RBPJ and of Notch signalling effector *her4.1*. This increase in the *her4.1* expression contributes to keep a check on the expression of *mmp9* and thus the MGPCs proliferation also. Along with this, there lies a mutual positive regulation between *her4.1* and Pten, which keeps MGPCs proliferation under control. On the other hand, the increase in the number of MGPCs seen upon DAPT-mediated Notch signalling blockade owes to the increase in the activation of Akt and a decrease in the Pten levels. We also observed that the Pten downregulation leading to increased Mmp9 activity is crucial for an efficient Akt phosphorylation at its Thr302 and Ser467, which manifests a pro-proliferative condition during zebrafish retina regeneration. Besides this we speculate that Pten does not mediate its effects through Notch signalling/Mmp9 axis only, there possibly exist parallel pathways and factors which support the MGPCs proliferation upon Pten blockade even if Mmp9 is inactive, during retina regeneration. There must be parallel pathways supporting the anti-proliferative function of Pten even if Notch signalling is blocked. Taken together, we can infer that Pten could impact the MGPCs proliferation during zebrafish retina regeneration through means and pathways other than Notch signalling and Mmp9 also (Fig 3A and B).

Chapter 4

Fine-tuning of *pten* expression during retina regeneration.

3.4.1. Regulation of *pten* expression by Myc-Hdac1 complex during retina regeneration.

In the previous sections, we had already found that the absence of Pten from the MGPCs is an important event for their increased proliferation during retina regeneration. Still, its expression needs to be regulated stringently at the transcriptional and at the translational level also. Keeping this in view, lastly, we aimed at investigating the factors which control the levels of Pten during zebrafish retina regeneration. To address this, we analysed the *ptena*/*ptenb* promoter sequences and interestingly, found several putative Myc-binding sites (BSs) (CACGTG) on them (Fig 3.4.1.1 A). The literature suggests that Myc is a proto-oncogene involved in cellular transformation during cancers (Miller et al., 2012). Myc is a transcription factor which can act as a transcriptional activator or a repressor for the gene regulation (Mitra et al., 2019). This made us check the effect of Myc on the expression of Pten tumor suppressor during retina regeneration. For this, we blocked the Myc-Max interaction using pharmacological inhibitor 10058-F4 (Huang et al., 2006; Lin et al., 2007; Mitra et al., 2019). We observed that 10058-F4-mediated Myc blockade led to an upregulation in the expression level of *ptena* and *ptenb*, as seen by RT-PCR and qPCR done in the retina at 2dpi (Fig 3.4.1.1 B, C). This increase was consistent at the translational level also as analysed by Western blotting assay for the Pten protein, in the 10058-F4-treated retinal lysates at 4dpi (Fig 3.4.1.1 D, E). Thus, we could conclude that Myc tries to repress the *pten* expression.

The literature also suggests that Myc while binding to the gene promoters, co-recruits bona fide transcription repressors like Histone deacetylases (Hdacs) to suppress its target genes (Kurland & Tansey, 2008; Mitra et al., 2019). We speculated that a similar mechanism of action might be mediating Myc-based repression of *pten* genes. To check this, we performed Chromatin Immunoprecipitation (ChIP) assay using anti-Myca/b and anti-Hdac1 antibodies and primers for Myc-BS on *ptena*/*ptenb* promoters. Interestingly, we found co-occupancy of both Myc and Hdac1 on all the Myc-BS on *ptena*/*ptenb* promoters at 2dpi (Fig 3.4.1.1 F). This is suggestive of the fact that Myc-Hdac1 collaborate in a binding complex and get recruited on the *ptena*/*ptenb* promoters to repress the *pten* gene expression in the MGPCs during retina regeneration.

Parallelly, we observed increase in the levels of *ptena* and *ptenb* genes by RT-PCR and qPCR performed at 2dpi (Fig 3.4.1.2 A, B), as well as an increase in the Pten protein as analysed by

western blotting assay done at 4dpi (Fig 3.4.1.2 C, D), upon Trichostatin A (TSA)-mediated Hdac1 blockade in the zebrafish retina. Thus, confirming the repressive regulation of Hdac1 on *pten* expression, which in this case is ensured when Hdac1 co-occupies the *pten* promoter along with Myc.

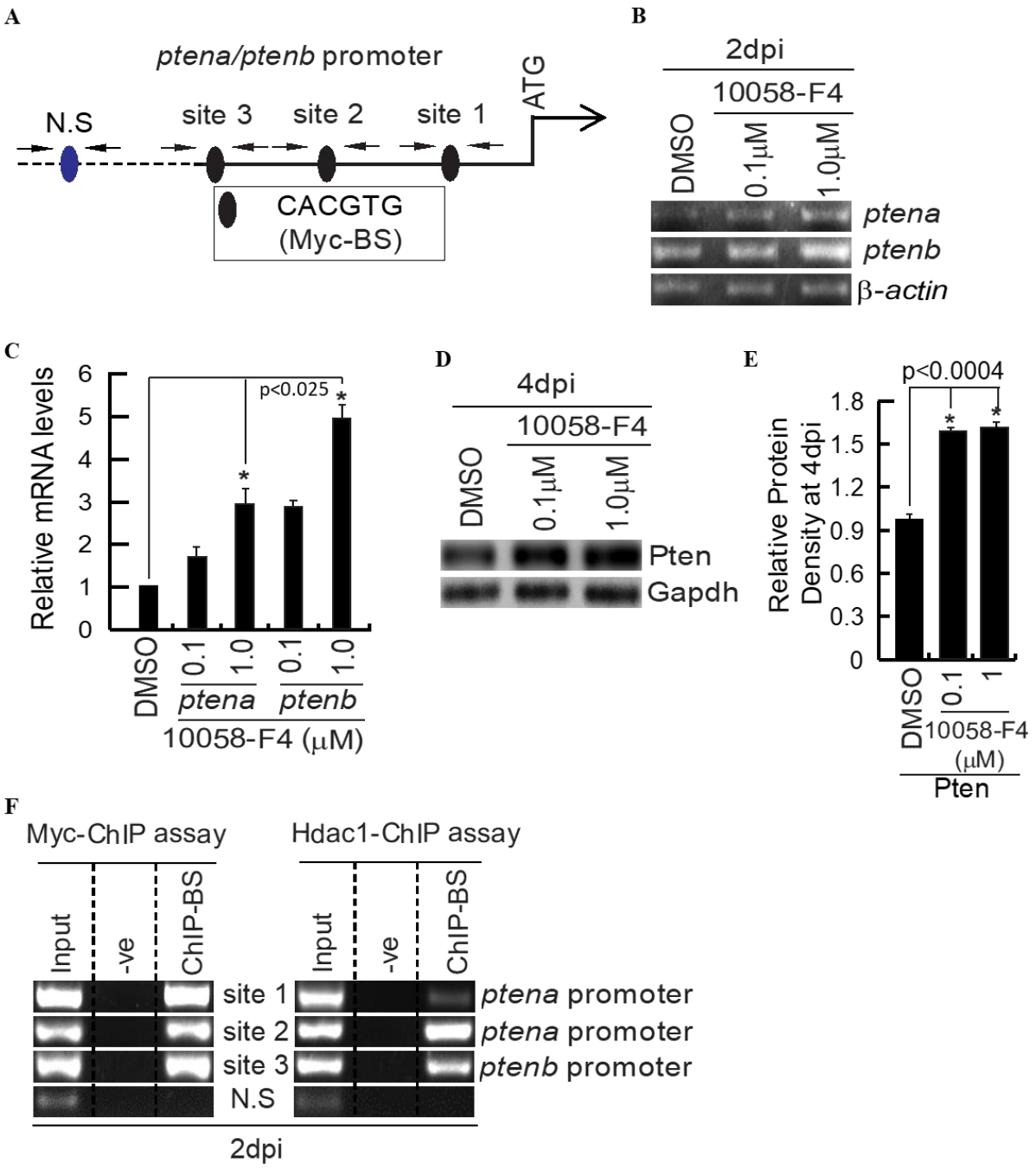

Fig 3.4.1.1 Repressive regulation of Myc-Hdac1 complex on Pten during retina regeneration. (**A**) The *ptena/ptenb* promoter schematic reveals the Myc-binding sites (BS) on the promoters. (**B** and **C**) The RT-PCR (**B**) and qPCR (**C**) analyses of *ptena* and *ptenb* mRNA levels in 10058-F4-treated retina, at 2dpi; *p < 0.025, n=3 biological replicates. (**D** and **E**) Western blot analysis (**D**) and protein densitometry plot (**E**) of Pten protein in 10058-F4-treated retina, at 4dpi; *p < 0.004; n=3. (**F**) The retinal ChIP assays confirm the physical binding of Mycb along with Hdac1 to the Myc BS on *ptena/ptenb* promoter in 2dpi retina. N.S marks the negative control, and capital letters mark putative Mycb-BS. Gapdh is the loading control; dpi, days post injury; Arrows mark ChIP primers in (**A**); Error bars represent SD.

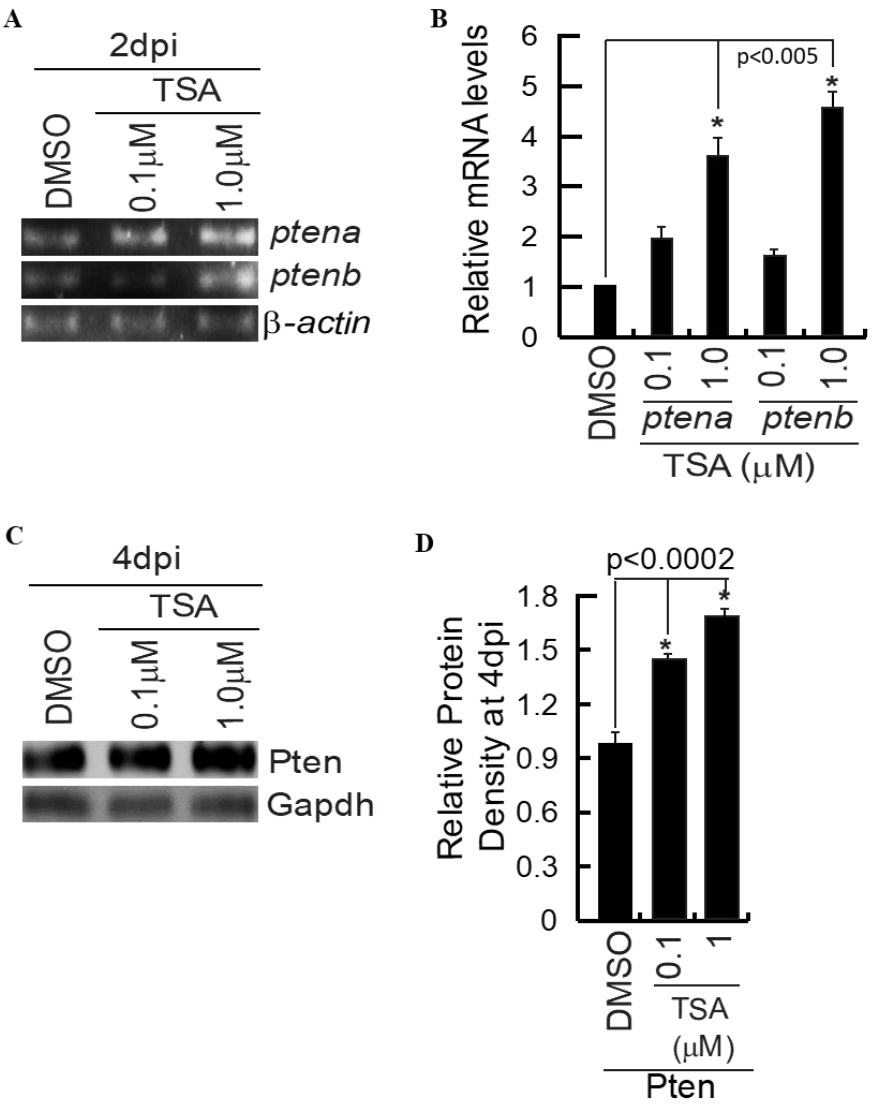

Fig 3.4.1.2 Effect of Hdac1 inhibition on Pten expression during retina regeneration. (**A** and **B**) The RT-PCR (**A**) and qPCR (**B**) analyses of *ptena* and *ptenb* mRNA levels in TSA-treated retina, at 2dpi; *p < 0.005, n=3 biological replicates. (**C** and **D**) The western blot analysis (**C**) and protein densitometry plot (**D**) of Pten protein in TSA-treated retina, at 4dpi; *p < 0.0002; n=3. Gapdh is the loading control. dpi, days post injury; Error bars represent SD.

3.4.2. Regulation of *pten* expression by Tgf-β signalling during retina regeneration.

The Pten lost in MGPCs eventually should come back to its expressive state also and must be under a regulated control of various factors, to allow MGPCs cell cycle exit and quiescence and thus, balance the rate of proliferation and cell cycle exit. Next, we chose to study the effects of Tgf-β signalling pathway on *pten* expression. During zebrafish retina regeneration, Tgf-β signalling has been attributed to be a pro-proliferative pathway, as its blockade using a pharmacological inhibitor SB431542 shows a remarkable reduction in the number of proliferating MGPCs (Sharma et al., 2020). We speculated that since Tgf-β signalling pathway and its effectors are pro-proliferative in nature, they must be negatively regulating the *pten* expression levels. We observed that upon SB431542-mediated Tgf-β signalling blockade in the retina, there was a decline in the levels of *ptena* and *ptenb* as seen by qPCR at 2dpi (Fig 3.4.2.1 A), which was also consistently seen in the Pten protein level by Western blotting assay at 2dpi (Fig 3.4.2.1 B). This directed us to a view that there is a positive regulation of *pten* by Tgf-β signalling pathway. Added to this, the analysis of *ptena*/*ptenb* promoters revealed the presence of 5GC element sequences (Fig 3.4.2.1 C). 5GC elements are the putative sites on a gene promoter to which pSmad3, which is a messenger molecule of active Tgf-β signalling, binds to regulate it positively. Further, we performed ChIP assay using pSmad3 antibody, and found the occupancy of pSmad3 on those 5GC element sites (GGCGC) on *ptena*/*ptenb* promoter in the retina at 2dpi (Fig 3.4.2.1 D), leading to *pten* gene upregulation by Tgf-β signalling. But this became an enigmatic situation since a pro-proliferative factor was positively regulating the expression of an anti-proliferative factor.

To solve this confusion, we analysed the *ptena*/*ptenb* promoter sequences closely and found the presence of Tgf-β Inhibitory Element (TIE) sequences on *ptenb* promoter (Fig 3.4.2.2 A). These are the unique sequences on gene promoters to which once pSmad3 is bound, it leads to the negative regulation of that gene. We performed ChIP assay using the antibody against pSmad3 and found the occupancy of pSmad3 on the TIE sequences on *ptenb* promoter in the retina at 2dpi (Fig 3.4.2.2 B). But we came across the literature which mentions that binding of pSmad3 to the TIE sequences on a gene promoter is mediated through Fos, which is a product of *cfos* proto-oncogene (Kerr et al., 1990). We analysed the spatiotemporal expression pattern of *cfos* gene and by RT-PCR found its early induction in the retina post-injury (Fig 3.4.2.2 C).

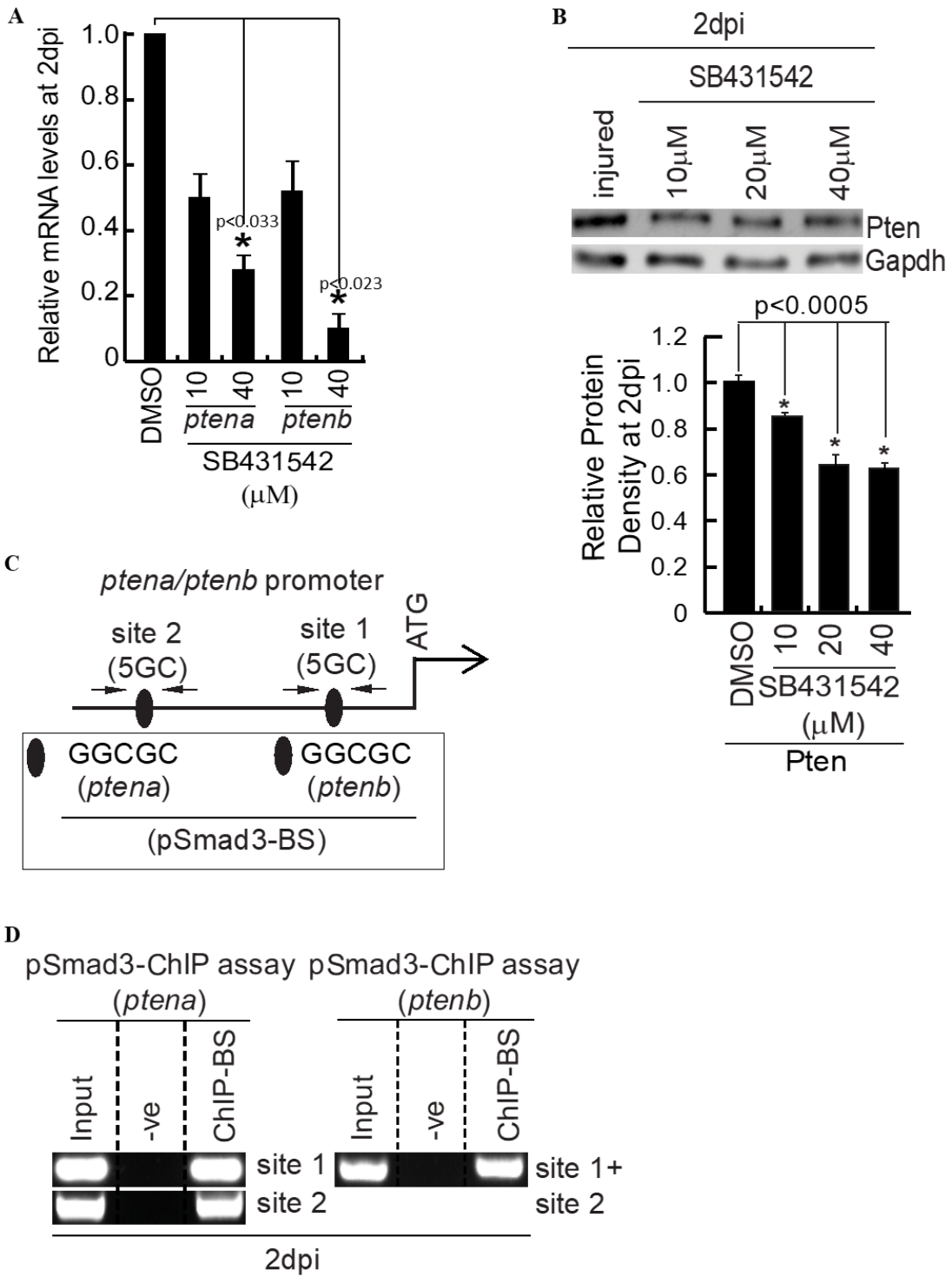

Fig 3.4.2.1 Fine-tuned regulation of Pten by Tgf-β signalling during retina regeneration. (**A**) The qPCR analyses of *ptena* and *ptenb* levels in SB431542-treated retina at 2dpi; *p < 0.03, n=3 biological replicates. (**B**) The western blot analysis (upper) and the densitometry plot (lower) of Pten in SB431542-treated retina at 2dpi; *p < 0.0005; n=3. (**C** and **D**) The *ptena/ptenb* promoter schematic reveals the typical 5GC sites (**C**) and the retinal ChIP assays confirm the physical binding of pSmad3 to the 5GC sites in 2dpi retina (**D**). Capital letters mark 5GC sequence. Gapdh is the loading control. dpi, days post injury; Arrows mark ChIP primers in (**C**); Error bars represent SD.

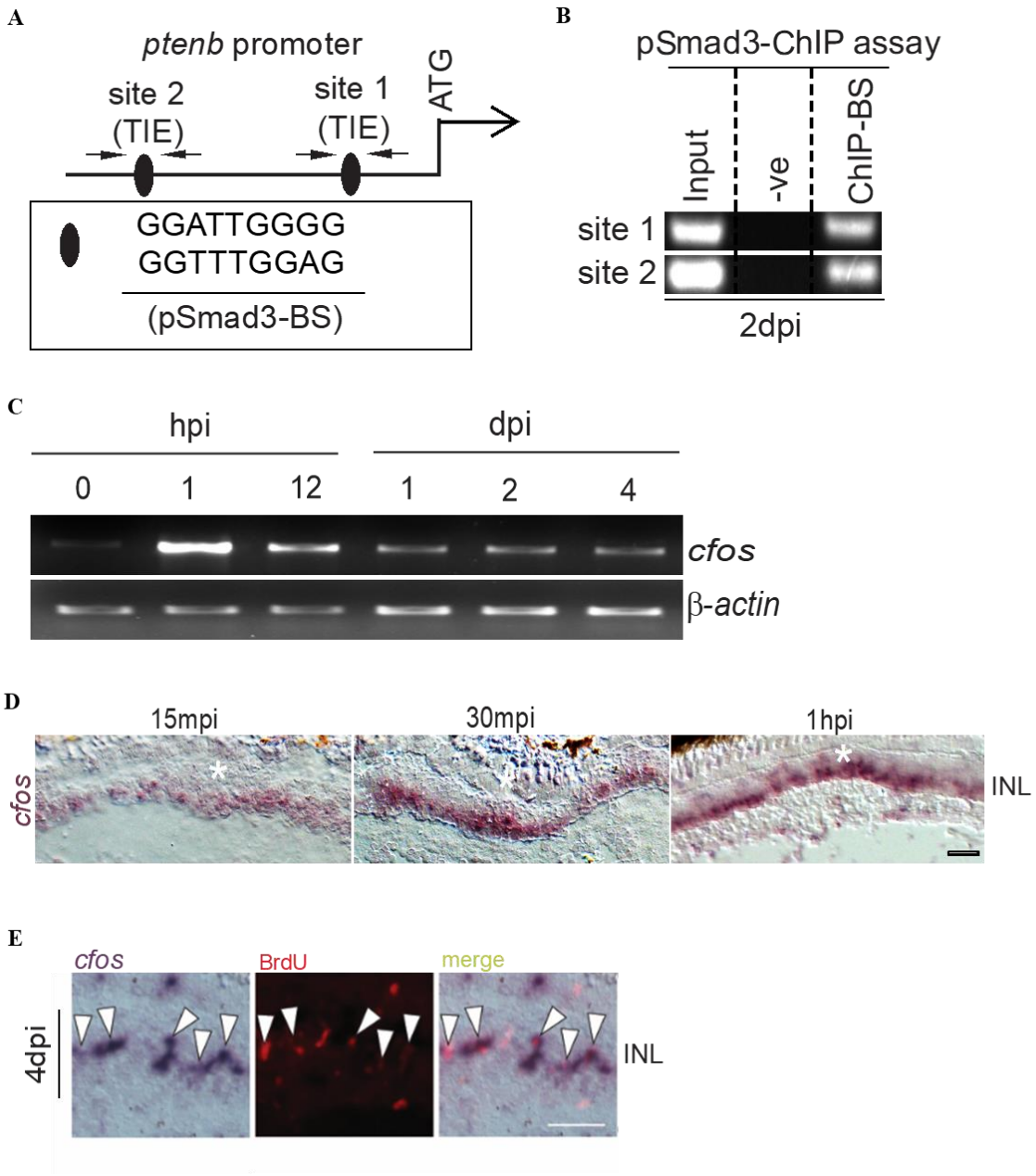

Fig 3.4.2.2 pSmad3 binding on TIE sequences of *ptenb* promoter in MGPCs in the presence of *cfos* during retina regeneration. (**A** and **B**) The *ptenb* promoter schematic reveals the typical TIE sequence (**A**) and the retinal ChIP assay confirms the physical binding of pSmad3 at the TIE sites in 2dpi retina (**B**). Capital letters mark the TIE sequences. (**C**) Semi-quantitative PCR analysis of *cfos* gene expression at various time points post-retinal injury. (**D**) Brightfield (BF) microscopy images of retinal cross-sections show the mRNA *in-situ* hybridisation (ISH) of the *cfos* mRNA in the retina at 0.25, 0.5 and 1hpi. White arrowheads mark *cfos* mRNA signal. (**E**) BF and immunofluorescence (IF) microscopy images of a retinal cross-section show the mRNA ISH of the *cfos* mRNA in the $BrdU^{+}$ cells in the retina at 4dpi. White arrowheads mark co-labelled *cfos* mRNA and BrdU signal. Scale bars represent 10µm in (**D**, **E**); the asterisk marks the injury site and GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer in (**D**, **E**); mpi, minutes post injury; hpi, hours post injury; dpi, days post injury. Arrows mark ChIP primers in (**A**); Error bars represent SD. A single 0.5-µm-thick optical section was taken in (**D**, **E**).

This expression was found throughout the INL of the retina, as seen by *cfos* mRNA *in situ* hybridisation in retinal sections done at 15 minutes post-injury (mpi), 30mpi, 1hpi (Fig 3.4.2.2 D), just like *pten.* Surprisingly, this *cfos* expression got restricted to the $BrdU^+$ MGPCs by 4dpi, as revealed by *cfos* mRNA *in situ* hybridisation (Fig 3.4.2.2 E). This suggests that pSmad3 binds to the TIE sequences on the *ptenb* promoter, along with Fos as a binding partner in proliferating MGPCs. While in order to bring back the levels of *pten* gene in neighbouring cells of MGPCs, Tgf-β signalling positively regulates it through pSmad3 binding to 5GC elements on *pten* genes.

We also got intrigued to find out the mechanism of action of Pten on Tgf-β signalling. We blocked Pten using its selective inhibitor SF1670 and checked its effect on the expression of Tgf-β effector genes *smad3a*, *smad3b* and *tgfbi*. We found that there was a downregulation of *smad3a* and *smad3b*, with an insignificant effect on *tgfbi* as compared to the control retina at 2dpi, as analysed by qPCR (Fig 3.4.2.3 A). In SF1670-treatment in the retina, we saw a decrease in the levels of pSmad3 protein by western blotting assay done at 2dpi (Fig 3.4.2.3 B). This is indicative of the fact that Pten tries to control its own expression in the neighbouring cells of MGPCs by positively regulating Tgf-β signalling, where pSmad3 binds to 5GC element sequences on *pten* gene promoters.

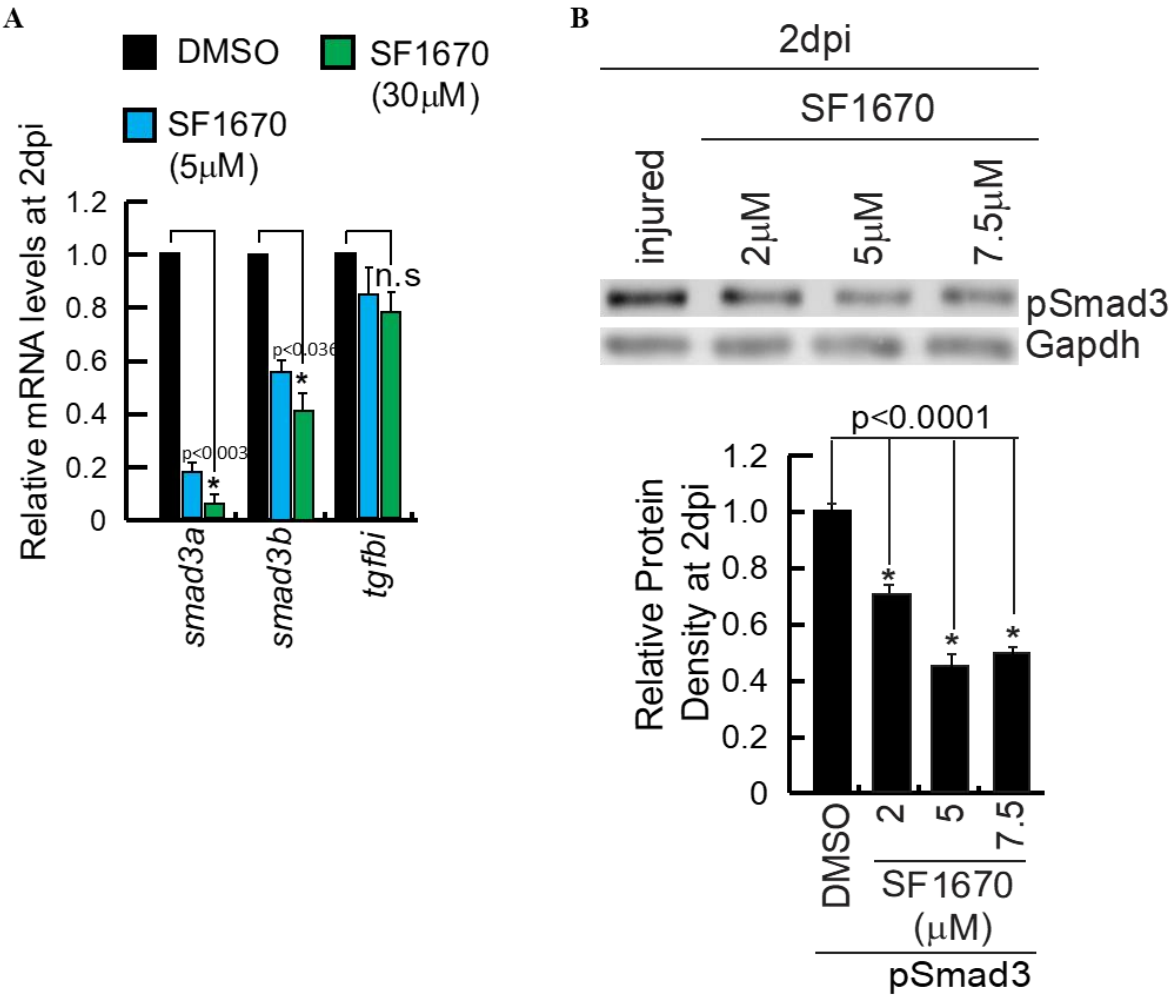

Fig 3.4.2.3 Pten blockade reduces the expressions of Tgf-β signalling effectors during retina regeneration. (**A** and **B**) The qPCR analyses of Tgf-β signalling reporter genes *smad3a*, *smad3b* and *tgfbi* mRNA levels; *p < 0.04, n=4 biological replicates (**A**), and western blot analysis (upper) and densitometry plot (lower) of pSmad3 (**B**) in SF1670-treated retina at 2dpi; *p < 0.0001; n=3. Gapdh is the loading control. dpi, days post injury; n.s., not significant. Error bars represent SD.

Discussion

As the *pten* expression was found to be missing from the MGPCs, we suspected robust repressive machinery recruited to negatively regulate its expression. But we speculated that the *pten* expression must be fine-tuned by some factors, which allow *pten* to express in quiescent MG or post-proliferative MGPCs which are cells in the neighbourhood of proliferating MGPCs. Such a regulation is needed to prevent a situation of hyperproliferation of MGPCs during the retina regeneration. Upon *pten* gene promoter analysis, we found the binding sites for several factors, like Myc-Hdac1 and pSmad3 (which is a messenger of Tgf-β signalling pathway). Myc and Tgf-β signalling pathway effectors are oncogenic, transforming and pro-proliferative factors which play a role opposite to that of Pten, which is an anti-proliferative factor. Thus, these should have a negative effect on the expression of *pten*. In our work, we proved that Myc-Hdac1 repressive complex after getting recruited to the *ptena/ptenb* promoters suppresses their expression during retina regeneration. This repression thus keeps a check on the levels of Pten, allowing the activation of Akt in the MGPCs and efficient proliferation of MGPCs. While we found that Tgf-β signalling pathway regulates *pten* in 2 contrasting ways. Tgf-β signalling pathway messenger molecule pSmad3 binds to 5GC elements on *pten* genes' promoters, thereby positively regulating them. While Pten also tries to upregulate Tgf-β signalling effectors, to maintain its own levels. Such a regulation to fine-tune *pten* levels exists in the neighbouring cells of MGPCs. While Tgf-β signalling negatively regulates *pten* expression in the MGPCs by binding of pSmad3 along with Fos to the TIE sequences on to the *ptenb* promoter during zebrafish retina regeneration. Such a stringent regulation occurs to maintain the *pten* expression in the proliferating MGPCs and in the cells flanking these MGPCs during retina regeneration to maintain the rate of proliferation and to initiate a successful regenerative response, without allowing the MGPCs to enter into a mode of hyperproliferation (Fig 3A and B).

Conclusion

From the available literature in the field as well as from the results obtained from our study, we could deduce a working model (Fig 3A) and a simplified schematic representation (Fig 3B). We could bring forward a spatially segregated mechanistic pathway in the MGPCs and their neighbouring cells which plays role during the zebrafish retina regeneration. We observe that the *pten* genes as well as Pten protein are spatially secluded from the proliferating MGPCs, and rather expressed in the non-proliferating neighbouring cells of MGPCs. After skimming through the literature, we found that Myc acts as a transcriptional repressor in collaboration with Hdac1 in the MGPCs and they co-occupy a target gene promoter to downregulate it (Mitra et al., 2019). We found that in the MGPCs, Myc-Hdac1 complex keeps the levels of *pten* genes and Pten protein under a repression, by binding on to *pten* genes' promoter regions on various Myc binding sites. Along with this check, a pro-proliferative Tgf-β signalling also plays role to regulate Pten levels in the MGPCs. We observed that the messenger molecule of Tgf-β signalling pathway, pSmad3, binds to the Tgf-β Inhibitory Element (TIE) sites present on *ptenb* promoter region, in the presence of Fos, a *c-fos* gene family product. This might be keeping a check on the levels of Pten in the proliferating MGPCs. The highly reduced expression or absence of Pten from the MGPCs, allows the activation of the lipid substrate PIP2 to its phosphorylated and activated form PIP3 by PI3K as reported in multiple cancer systems and mice axonal regeneration (Song et al., 2012; Ohtake et al., 2015). As PIP3 is active, it leads to membrane anchoring and activation of Akt by step-wise phosphorylation events occurring at its Ser467 by mTORC2, followed by phosphorylation at Thr302 by PDK1. Akt activation in the MGPCs leads to an increase in the proliferation, mediated by downstream mechanisms in the regenerating retina. The phosphorylated Akt further activates mTORC1 and also β-Catenin expression, both of which are seen to be associated with the MGPCs proliferation. The nuclear localised β-Catenin along with the transcription activation machinery of Lef1/TCF might also be upregulating the pro-proliferative genes. As mTORC1 activation also, creates a pro-proliferative environment, we found that simultaneously it might be imposing a negative feedback regulation on the PI3K and mTORC2, for preventing the phosphorylation of Akt at 2 of its sites, as seen during mice axon regeneration also (Miao et al., 2016). This maintains tissue homeostasis and avoids the situation of hyper-proliferation, thus, preventing an oncogenic transformation in the retinal tissue in the absence of Pten.

The literature suggests that *mmp9* is a pro-proliferative gene, which is majorly expressed in the MGPCs and its substantial levels are found in the neighbouring cells of MGPCs along with *her4.1*, an effector of Notch signalling (Kaur et al., 2018). Upon activation, the Mmp9 protein acts as an Extracellular Matrix (ECM) protein, which is known to be responsible for getting upregulated in multiple cancers during metastasis in order to cleave the ECM (Mehner et al., 2014). During zebrafish retina regeneration, anti-proliferative Notch signalling pathway is known to downregulate *mmp9* (Kaur et al., 2018), thereby ensuring a check on the MGPCs proliferation. We also found that Notch signalling and its effectors playing role in the non-proliferating MGPCs and neighbouring cells of MGPCs, try to positively regulate the levels of Pten in these cells. Pten also maintains the levels of Notch signalling effector *her4.1*, in these neighbouring cells in order to maintain homeostasis. While both Notch signalling and Pten, downregulate the *mmp9* levels in the neighbouring cells of MGPCs. The Mmp9 protein expressed in the ECM might be acting indirectly to keep the levels of Pten high in the neighbouring cells of MGPCs, in order to bring down its own levels and to prevent ECM cleavage. We also speculate that the Pten levels might be positively regulated in the neighbouring cells of MGPCs, by the pSmad3 binding to the GC elements on *ptena*/*ptenb* promoter regions. The active Pten in these cells does not allow Akt to express and also to get activated in non-proliferating MGPCs and neighbouring cells of MGPCs (Fig 3A and 3B). A similar trend is seen throughout the uninjured retina also. Thus, Pten/PI3K/Akt/mTOR pathway acts in co-ordination with various other pro-proliferative and anti-proliferative factors and regulate zebrafish retina regeneration.

A

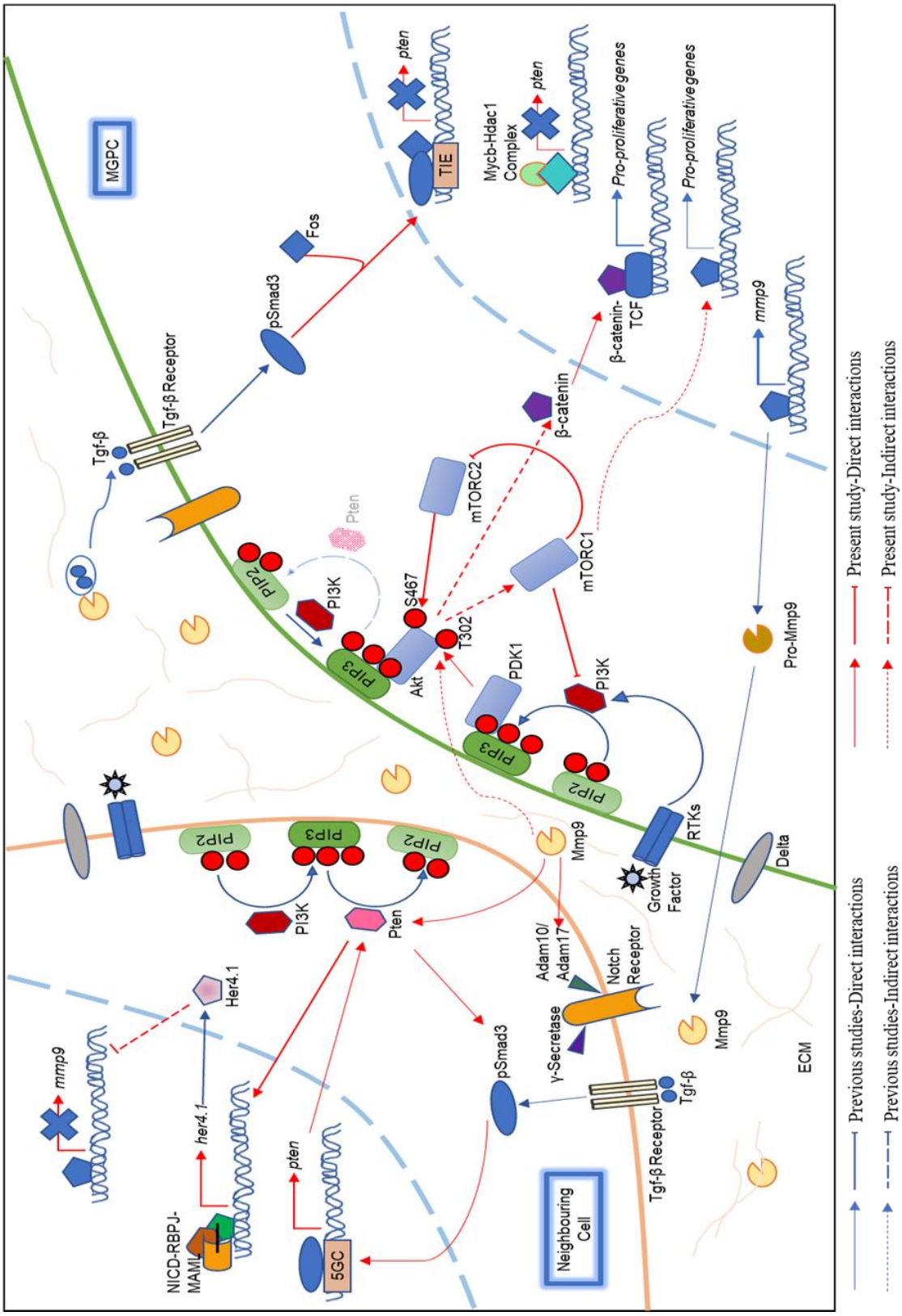

Fig 3A A model depicting the mechanism of action of Pten during retina regeneration.

B

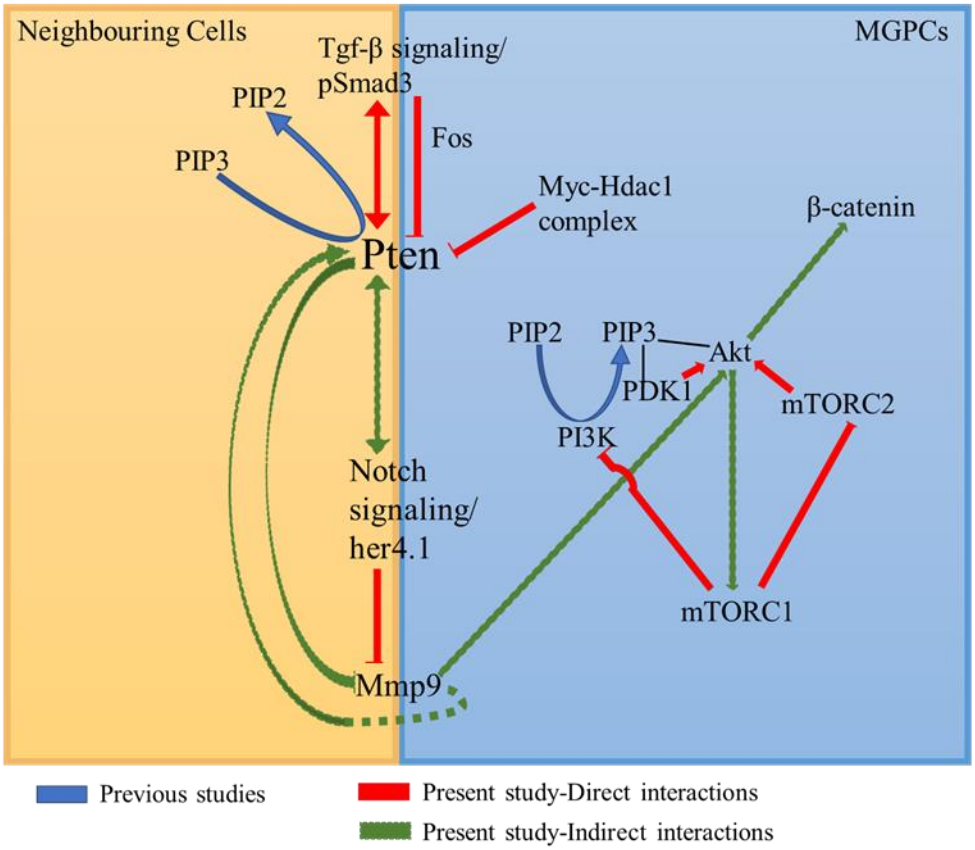

Fig 3B A simplified schematic representation (**B**) depicting the mechanism of action of Pten during retina regeneration.

Section 4.

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Section 5.

Appendix

Appendix 1:

Primer Table

qPCR and RT primers	Ensembl ID	Sequence (5'-3')
RT_akt1_Short_Fwd	ENSDART00000165641.2	AGAGTATCTGGCGCCCGAGGTGC
RT_akt1_Short_Rev		GCTTGAACGGAGGAACCAACTTC
RT_ptena_Fwd_new	ENSDART00000122209.4	ATGTTCAGTGGGGGCACCTGCAGGGAC
RT_ptena_Rev_new		TCAGGCCCCGGGATGAAGAACGTG
RT_ptenb_Fwd_new	ENSDART00000079144.5	GTTCAGTGGAGGGACCTGCAGGGATTC
RT_ptenb_Rev_New		TGTCCGAATAACGATAATGGTCCGGTTC
RT_her4.1_Fwd	ENSDARG00000056732	GCTGATATCCTGGAGATGACG
RT_her4.1_Rev		GACTGTGGGCTGGAGTGTGTT
RT_mmp9_Fwd	ENSDART00000062845.5	GGAGAAAACTTCTGGAGACTTG
RT_mmp9_Rev		CACTGAAGAGAAACGGTTTCC
RT_ascl1a_Fwd	ENSDARG00000038386	ATCTCCCAAAACTACTCTAATGACATGAACTCTAT
RT_ascl1a_Rev		CAAGCGAGTGCTGATATTTTTAAGTTTCCTTTTAC
RT_b-actin_Fwd	ENSDARG00000037746	GCAGAAGGAGATCACATCCCTGGC
RT_b-actin_Rev		CATTGCCGTCACCTTCACCGTTC
RT_tgfbi_Fwd	ENSDART00000105933.4	CGCTGACCTCAACAAACTCATGAGAG
RT_tgfbi_Rev		TGGTCACTCACAATTTTAGGAGGCAG
RT_smad3a_Fwd	ENSDART00000186800.1	TCCTGCCCACAACAATTTAGATCTC
RT_smad3a_Rev		TACAAGCGCACACCCCGGCCAATGTGTC
RT_smad3b_Fwd	ENSDART00000043455.5	TCTCCGGCAAACAGCAACCTAGATCTG

RT_smad3b_Rev		TACAAACGCACGCCTCGGCCGATGTG
RT_adam10a_Fwd	ENSDART00000173954.2	AAGCACCTGCAGCAGCACTAGCTC
RT_adam10a_Rev		TCTTCTTCTCTTTAGCGTGCCTGG
RT_adam10b_Fwd	ENSDART00000170892.2	ACACAACTTCGGCTCTCCGCAC
RT_adam10b_Rev		TGCACAGACCCTGATGAGCACACTC
RT_adam17a_Fwd	ENSDART00000063437.4	AGGAAACAGGCTCAGTGCAGTGAC
RT_adam17a_Rev		ACTTGTTTCATGCATTTGCCAGCC
RT_adam17b_Fwd	ENSDART00000153339.2	AGTCCTGTGCTTGCAATGAGAC
RT_adam17b_Rev		TGCTCAGCAACTCAGCATTGCTG
RT_rbpja_Fwd	ENSDART00000005067.10	ATGGCTCTACCCCGACTGATC
RT_rbpja_Rev		AACATCTCCTCCTCCATTCAGCTG
RT_rbpjb_Fwd	ENSDART00000092580.7	ACTGGCATGGCTCTTCCTAGACTGATC
RT_rbpjb_Rev		TACATCTCCACCACCATTCAACTG
RT_cfos_Fwd	ENSDART00000043298.8	CAAGAGAAGCACAGATCTGAACAGCTTTC
RT_cfos_Rev		ATCTTCTAACTGGTCGGTTTCATTTTG
Cloning primers		
Akt1_BamHI_FL_Fwd	ENSDART00000165641.2	ATGCTAGCGGATCCACCATGGCGACAGATGTGGTGATCGTG
Akt1_XbaI_FL_Rev		ATGCTAGCTCTAGATCATGCTGTTCCGCTGGCCGAGTATGAGAA
Ptena_FL_BamHI_Fwd_pcs2	ENSDART00000122209.4	ATGCTAGCGGATCCACCATGGCAATGACTGCTAAACTAAAAG

Ptena_FL_XhoI_Rev_pcs2		ATGCTAGCCTCGAGTCAGACTTTTGTAATCTGTGCGTG
Ptenb_FL_EcoRI_Fwd_pcs2	ENSDART00000079144.5	ATGCTAGCGAATTCACCATGGCTGCGATCATAAAGGAATTTGTC
Ptenb_FL_XhoI_Rev_pcs2		ATGCTAGCCTCGAGAACTTTAGTAATCTGTTCTTCATCGTACTG
ptena fwd fl primer	ENSDART00000122209.4	GCTGTCATGGCAATGAC
ptena rev fl primer		TCAGACTTTTGTAATCTGTGCG
ptenb fwd fl primer	ENSDART00000079144.5	GACTCCTGTCACAGCCATGGCTGCG
ptenb rev fl primer		CTTCCCATAAAAATATTTCAAC
ascl1a FL Fwd	ENSDARG00000038386	ATGGACATCACCGCCAAGATGGAAATAAGCG
ascl1a FL Rev		TCAAAACCAGTTGGTGAAGTCCAGGAGCTC
her4.1 FL Fwd	ENSDARG00000056732	GAAACTCTACTGACAAACAAGCTG
her4.1 FL Rev		GATGTTGTCCATCTTCGTTTAGTGC
mmp9 FL Fwd	ENSDART00000062845.5	ATGAGACTTGGAGTCCTGGCGTTTCTGGTTCTG
mmp9 FL Rev		CTATAGAGAATGAATGTCACTGCATTTCAG
Mmp9_FL_BamHI_Fwd	ENSDART00000062845.5	GGATCCAATGAGACTTGGAGTCCTGG
Mmp9_FL_XhoI_Rev		CTCGAGGGTCTATAGAGAATGAATGTCACTGC
cfos_FL_BamHI_Fwd	ENSDART00000043298.8	ATGCGTGAGGATCCACCATGATGTTTACCAGCCTTAACGC
cfos_FL_XhoI_Rev		ATGCTGGACTCGAGAAGAGTGAGGAGGGTTGGGGAATTCAAG
Mutation Primers		
Akt1_T302D_Fwd	ENSDART00000165641.2	agccactatgaagGActtctgcgggactcc
Akt1_T302D_Rev		ggagtcccgcagaagTCcttcatagtggct
Akt1_T302A_Fwd	ENSDART00000165641.2	agccactatgaagGccttctgcgggactcc

Akt1_T302A_Rev		ggagtcccgcagaaggCcttcatagtggct
Akt1_S467D_Fwd	ENSDART00000165641.2	atttcccacaattcGACtactcggccagc
Akt1_S467D_Rev		gctggccgagtaGTCgaattgtgggaaat
Akt1_S467A_Fwd	ENSDART00000165641.2	atttcccacaattcGcatactcggccagc
Akt1_S467A_Rev		gctggccgagtatgCgaattgtgggaaat
ptenb MO BS_2 Mut_Fwd_1	ENSDART00000079144.5	ATGGCcGCaATCATAAAGGAATTTG
ptenb MO BS_2 Mut_Rev_1		CAAATTCCTTTATGATtGCgGCCAT
ptenb MO BS_4 Mut_Fwd	ENSDART00000079144.5	ATGGCcGCaATCATtAAGGAgTTTGTCAGTAG
ptenb MO BS_4 Mut_Rev		CTACTGACAAAcTCCTTaATGATtGCgGCCAT
akt1MOBS3Mut_newFwd	ENSDART00000165641.2	atggcgacTgaCgtCgtgatcgtgaag
akt1MOBS3Mut_newRev		cttcacgatcacGacGtcAgtcgccat
ptenaMOBS3Mut_NewFwd	ENSDART00000122209.4	gGATCCACCATGGCAATcACaGCgAAACTAAAAG
ptenaMOBS3Mut_NewRev		CTTTTAGTTTcGCtGTgATTGCCATGGTGGATCc
Promoter Cloning Primers		
Ptenb promoter_XhoI_Fwd	ENSDART00000079144.5	ATGCTAGCCTCGAGAAGCTTTTCACTCATCATAGCACACGTGCTGC
Ptenb promoter_BamHI_Rev		ATGCTAGCGGATCCGGCTGTGACAGGAGTCTTTAGGGTTTTTAGTG
Akt1 promoter_XhoI_Fwd	ENSDART00000165641.2	ATGCTAGCCTCGAGCACATTATCAGTCAAACTCTGCAC
Akt1 promoter_BamHI_Rev		ATGCTAGCGGATCCGCTGGCCGTCTATCCAGATCTG
ChIP Primers		
MycBS_PtenbProS1_F	ENSDART00000079144.5	AAGCTTTTCACTCATCATAGCACACGTGC
MycBS_PtenbProS1_R		TCCATTAAAATCCACACCTCCCCC
MycBS_PtenbProS2_F	ENSDART00000079144.5	AAGGAAACGACAATGTTGGAGC

MycBS_PtenbProS2_R		GCATTCTTGTTGAGCTGACATGGC
MycBS_PtenaProS1_F	ENSDART00000122209.4	ATTGGTGATGTGGTGGCACAGTAGG
MycBS_PtenaProS1_R		TGATTCACACACATAACCTACAGACAG
MycBS_PtenaProS2_F	ENSDART00000122209.4	ACCCTCGAGGCTTAGCTGTAGAAATC
MycBS_PtenaProS2_R		TCGCGTTGAAGGATATGAGCGGAAGG
5GC on PtenaproS1_Fwd	ENSDART00000122209.4	ACGCTGCCTGCTTTAAAAGTTGC
5GC on PtenaProS1_Rev		ATGGCTTCCCACAGAGGAAAAAAGAAC
5GC on PtenbProS1+2_Fwd	ENSDART00000079144.5	ATGTTTGGTTTTAGGCCCTTCTTAC
5GC on PtenbProS1+2_Rev		AGGAAATTACTGAAATGCTTACGCC
TIE on PtenbProS1_Fwd	ENSDART00000079144.5	AGTTCCAGACGTGGGAAGTTGC
TIE on PtenbProS1_Rev		ATTGTTGCTTTCGGACGGTCGGTC
TIE on PtenbProS2_Fwd	ENSDART00000079144.5	AGGCAAGATGTCCTTGCTGCAGTC
TIE on PtenbProS2_Rev		AGGTGCTTCCTATCCCTGGAC