Understanding the molecular mechanisms of Mycs and Hdacs to control Her4.1/Lin28a/*let-7* regulatory axis during zebrafish retina regeneration

A thesis submitted by

SOUMITRA MITRA

for the partial fulfilment of the degree of

DOCTOR OF PHILOSOPHY

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

September 2018

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 **Place** **Soumitra Mitra**

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 Mother and Father

“IF THERE WERE NO REGENERATION THERE COULD BE NO LIFE.

IF EVERYTHING REGENERATED THERE WOULD BE NO DEATH”

- RICHARD J. GOSS, PRINCIPLES OF REGENERATION (1969).

Acknowledgements

I believe I could have written this thesis in a better way, had the anonymous reviewers not disturbed my peace of mind, somewhere in between Section 2 and stolen several thought processes which otherwise could have very well been used for the betterment of the thesis. Thanks to them, those thoughts are gone, and so are a few more extra unnecessary pages.

Although sometimes I am not entirely sure about whether to be thankful for the various events or things which instilled in my mind the idea of pursuing Ph.D., I am definitely deeply in debt to countless marvelous individuals who have helped me in this adventure of mine. In this section, it is my desire to thank all of them, genuinely from the core of my heart, for adding to this wonderful journey, a sense of purpose and a definite shape which also helped in building me, both as a person and a student of science.

First and foremost, much thanks go to Dr. Rajesh Ramachandran, my Ph.D. supervisor, who believed in me, my qualities, and gave me a unique opportunity to be his first student and set off on an adventure of a lifetime with a smile! Thinking about getting the opportunity to work with him, I cannot help but wonder at my extremely fantastic luck! At times of grave desperation, it was his constant motivation, contagious enthusiasm and relentless passion for science that dragged me out of the dark and placed right on track again. While he gave me complete independence regarding the execution of my research work which helped to build my scientific mind to a great extent, I never found his office door shut for a quick discussion or a long pending trouble-shooting. I sincerely thank him for all his efforts, in developing and shaping me as a student, a presenter, and a human being, above all.

I want to thank Dr. Kavita Babu and Dr. Kuljeet Singh Sandhu, the members of my Ph.D. committee, for their invaluable and timely suggestions and unconditional mentorships. Thank you for your support and advice that set the stage for my dissertation work.

I am grateful to Prof. N. Sathyamurthy, former Director, IISER Mohali, for providing me the opportunity to work in this esteemed institute. I would also like to extend my gratitude towards Prof. Debi Prasad Sarkar, Director IISER Mohali, for giving me an opportunity to use the excellent infrastructures available in this premier research institute for smooth execution of my experimental works.

My honest thanks to IISER Mohali, and Department of Biological Sciences, for giving me a chance to work in this wonderful environment and more so for providing financial support throughout the span of my graduate studies.

I am thankful to IISER Mohali for providing me with excellent facilities and faculties who helped in shaping my understanding of science to a great extent. I thank all of my teachers at IISER Mohali, who trained me in various fields of biology, and helped to build a scientific attitude and often taught me how to approach a scientific problem, which I am sure would remain indispensable in my future endeavors.

I would like to thank Dr. Arunika Mukhopadhyay and her lab members at IISER Mohali for allowing me to use the qPCR machine, even in extremely odd hours; and Dr. Daniel Goldman's lab in University of Michigan for providing us with some of the crucial clones used in this study, making my life a lot easier.

I consider myself extremely fortunate to be a part of the Regeneration Biology Lab at IISER Mohali. My heartfelt gratitude to all lab members for creating an amazing scientific and fun-filled environment in the lab which nurtures a strong sense of community and co-operativity, above all odds. I would also like to especially thank Dr. Poonam "Crazy-Lady" Sharma, who joined our lab as a PDF and not only filled the lab atmosphere with her positive and hard-working attitude but also helped me with numerous experiments. Her optimistic perspective towards life and scientific outlook towards tackling a problem are as inspiring as her ability to work hard, but with a brain! I thank Simran and Anwar, two of my "ancient" lab-mates and friends who always stood strong beside me in all ups and downs of lab-work. It was truly a wonderful experience setting up the lab and sharing this adventurous journey of Ph.D. with them. As far as learning is concerned, their contributions towards making me sound about the field, both theoretically and practically, are only second to that of Rajesh. I also thank both of them for helping me out with numerous experiments and troubleshooting and even performing a few, when it came to that. I would also like to extend my sincere thanks to Shivangi and Mansi (read Maataa and Mausi), another two of the graduate students in the lab, for their unconditional love, support, willingness to help, and for taking care of literally everything in the lab that has to be taken care of. Starting a day's work with the smile they used to greet me with, often lessened the burden of mysteriously failing experiments. I am also thankful to share my days in the lab with Bindia and Sharanya for they maintained a joyful and healthy aura in the lab and Ashish for his contributions of extremely rich humor in the lab atmosphere!

Although graduate students definitely form the core of any lab in this part of the world, no lab can deny its debt to the undergrads. I have been fortunate enough to work with a number of exceptionally bright and hard-working undergraduate students. This dissertation work would not have been feasible for me to carry out without the unconditional and unquestioning help I received from Pratibha, and Riya. I thank them for they were simply amazing in making my last two years of research work enjoyable beyond expectation, and it was an absolute privilege working with them. I am also grateful to Akshai for taking the pain of standardizing the FACS protocol and helping me out with experiments involving cell sorting. I also thank Swathi, Praveen, Roja, Vijith, Priyanka, Evelyn, Kranti, Sukanya, Sapna, and Mayank Kamboj for extending similar helping hands whenever asked for, and always with a smile.

It will be right-out lying if I say that my voyage of Ph.D. never faced a bottomless pit. This would have definitely felt deeper, had I not been fortunate enough to have met people like Zeeshan, Martik, Promit, Manaoj, Panda, Priyanka (the same one who worked in our lab), TJ, Ruchika, Dagar, Divanshu (not sure if I am missing out anybody) and many other residents of the four great hostels of IISER Mohali. I am sure they not only enlightened me about the various other important (or unimportant) things happening in the outer world other than science but also must have played a significant role in prolonging my sufferings through their constant motivation, encouragement and carefree celebration of the exuberance of life! Without this

amazing group of people, I can see neither me nor this dissertation reaching their current state of existences.

I also thank Gupta Ji, for believing in the system of "credit" and for all those extra cups of tea, which often proved to be an elixir!

For me, it was a matter of great happiness that I could start the journey of my Ph.D. along with my friend Krishna, who had the uncanny ability to ease out (or complicate, sometimes) any kind of distress or frustration just with his presence. Thank you for being my room-mate in the first three months of my Ph.D. life and for being my neighbor for the rest of the past six years and also for being a weirdo at times! I would also like to thank Rohan and Shashank, two of my batchmates who never hesitated to share my laughters or sorrows and stood beside me as the strongest supports that any person can ever ask for.

I Started this journey with a bunch of super-excited and dedicated graduate students. Today, after years of sharing many of our joys and sufferings, together, I am thankful to Poonam, Preeti, Prashant, Shivani, Pallavi, Prince, Manisha, Bhupinder, Rivi, Aastha, Madhu, Avneet, and Devashish (not in any particular order!).

My stay at IISER Mohali was wonderful, thanks to a lot of people who regularly, without fail, took care of our daily affairs. I thank Mr. Brijesh, caretaker of Hostel 5, for his friendly nature; all the mess workers for providing us with food (I could have been a bit more grateful to them had the food quality been better); and all the workers who took care of the hygiene of the residential as well as the academic buildings.

Now that I have successfully (at least I hope so!) completed thanking all the people from the lab and the institute, let me take this opportunity to express my gratitude towards my family. My sincere thanks to my father Subrata Mitra, for letting me nurture the desire to be a science student and for the very first time allowing a scientific nourishment of my mind as a child. I thank my mother Shyamali Mitra, well, just for being my mother, actually! I definitely cannot thank her enough for all the sacrifices she had to make throughout her life, to date, for making me what I am today.

I bow to the Mountains for keeping me alive!

At last (but whether it is least is up to the reader), I thank the elegant and elusive kingdom of Nature which has woven an intricate piece of art-work around it where its very existence is balanced upon its knowledge about itself and for allowing us to be bemused in its infinite and marvelous spectacles.

Contents

Publications

1. **Mitra S.**, Sharma P., Kaur S., Khursheed M. A., Gupta S., Ahuja R., Kurup A. J., Chaudhary M., Ramachandran R. (2019) **Dual regulation of lin28a by Myc is necessary during zebrafish retina regeneration.** *Jornal of Cell Biology*, 218(2): 489-507.

2. **Mitra S.**, Sharma P., Kaur S., Khursheed M. A., Gupta S., Ahuja R., Kurup A. J., Chaudhary M., Ramachandran R. (2018) **Histone deacetylase-mediated Muller glia reprogramming through Her4.1/Lin28a axis is essential for retina regeneration in zebrafish.** *iScience,* 7: 68-84.

3. Kaur S. Gupta S, Chaudhary M, Khursheed M. A., **Mitra S**., Kurup A. J., Ramachandran R. (2018) ***let-7*** **microRNA mediated regulation of Shh signalling and the gene regulatory network is essential for retina regeneration.** *Cell Rep*orts, 23: 1409-1423.

Thesis Abstract

Vision loss due to retinal damage, to date, proves to be a major health problem. In mammals, retina, being a part of the Central Nervous System (CNS), does not possess the ability to regenerate after an insult. Surprisingly, other vertebrates such as teleosts or urodeles possess remarkable regenerative potential in various tissues or organs. Zebrafish, being one of the most extensively studied teleosts, serves as a great model organism to study regeneration of retina. Besides, being one of the most easily accessible parts of the CNS, retina serves as an ideal model system for studying the detailed molecular mechanisms underlying a successful retinal regeneration. Following an injury, Muller Glia (MG) cells, the only type of glial cells, present in the retina, de-differentiate to form Muller glial derived progenitor cells (MGPCs) with stem cell-like properties which further proliferate and differentiate to all retinal cell types across every retinal layer, including MG itself, to compensate for the damage. Till now, a plethora of genetic factors including transcriptional activators (e.g. Ascl1a), transcriptional repressors (e.g. Insm1a, Her4.1), pluripotency-inducing factors (e.g. Lin28a), growth factors (e.g. Hb-egf), cytokines (e.g. interleukins), and epigenetic modifiers (e.g. Dnmts) have been identified to play significant roles regulating the cellular process of retina regeneration. In spite of accumulation of this vast knowledge about the molecular regulators of retina regeneration in zebrafish, therapeutic interventions towards successful mammalian retinal regeneration still remains an unsolved enigma in mammals, demanding further investigation. In this study, we report rapid and MGPCs-associated induction of zebrafish Myc genes, namely *myca* and *mycb* which are necessary for a successful retinal repair. We also show the stringent regulation of *mycb* by previously characterized Ascl1a/Insm1a regulatory axis. Further, our study places Mycb, which is a *de facto* transcriptional activator, as a dual regulator acting on regeneration associated Lin28a/*let-7* regulatory axis. We also show regeneration associated Delta/Notch

signalling controls the extent of the injury responsive zone by negatively regulating *mycb*. Further to elucidate the mechanism underlying the negative regulation of Lin28a by Mycb, we show physical collaboration of Histone de-acetylase1 (Hdac1) with Mycb to repress *lin28a* and control proliferation. Besides Hdac1, we also found that several other Hdacs to be regulated post retinal injury and inhibition of Hdacs resulted in an impaired but reversible blockade of MGPCs proliferation fine-tuned by Her4.1/cytokines axis. Taken together, our study not only places Myca/b and Hdacs as key regulators of the molecular mechanisms underlying zebrafish retina regeneration, but it also opens new possibilities for therapeutic interventions towards successful mammalian retina regeneration.

Thesis Synopsis

Introduction and Review of literature

Regeneration is the complete restoration of morphology and functionality of a tissue or organ after an injury. Animals across different phyla exhibit varying regenerative capacity which also differs in different organs or tissues of a single organism. While invertebrates and lower vertebrates are able to display a robust regenerative capacity, higher vertebrates, in this regard, has lost most of it in the course of evolution. As the organs evolved more and more complex, regeneration through restoring accurate spatiotemporal connections of cells became more difficult for higher organisms. For regenerating a complex system like the vertebrate central nervous system (CNS), the formation of any kind of wrong connection would cause havoc on the organism. And probably that is the reason why in higher vertebrates, complex tissues, or systems, or organs do not regenerate while simpler tissues like skin and liver still possess limited regenerative capacity.

Injury to the central nervous system can be fatal depending upon the severity of the injury. This makes studying regeneration of CNS, an important aspect of regeneration biology. Compared to higher vertebrates, lower vertebrates like teleost fishes possess immense regenerative capacity in their CNS. Keeping this in mind, previous studies have established Zebrafish (*Danio rerio*) as an excellent model system to study regeneration of CNS.

Retina, being an easily accessible part of the CNS, is one of the most convenient tissues to study CNS regeneration. Besides, injury to the retina does not cause lethality in zebrafish as compared to the brain or spinal cord injury. The advantage of studying retina regeneration in zebrafish is manifold as zebrafish retina bears significant structural similarities to its

mammalian counterpart. The retina can be broadly divided into three layers: Outer Nuclear Layer (ONL), Inner Nuclear layer (INL) and Ganglion Cell Layer (GCL). Axons from the GCL ultimately form the optic nerve. Unlike mammalian retina, fish retina continues to grow with age by producing new retinal cells from a group of stem cells known as Ciliary Marginal Zone (CMZ). Surprisingly, during regeneration in adult stage, these stem cells do not take part to heal the wound by producing new retinal cells. This job has been taken up by Muller Glial (MG); the only glial cell type present in the retina. They maintain retinal homeostasis through a variety of processes like ion balance, recycling neurotransmitters, controlling molecular transfer between other cells, phagocytising, etc. These cells, residing in INL and encompassing all retinal layers by wrapping around other cells in the vicinity, take up the job to de-differentiate into Muller Glia derived Progenitor Cells (MGPCs) with stem-cell-like characteristics. MGPCs then proliferate, migrate to all retinal cell layers and differentiate to generate all retinal cell types including MG itself.

To date, quite a few genes and signalling pathways have been attributed for reprogramming of Muller Glia. It has been reported that, following injury, phagocytosis of the dead tissue by Muller glial cells is essential for a successful regenerative response. This initiates a cascade of signalling events comprising up-regulation of various cytokines, growth factors, and their receptors. Through MAP-kinase pathway, all of these converge to upregulate Ascl1a, an E-box binding transcriptional activator and one of the key regulators of retina regeneration. Ascl1a, upon binding to E-box binding-proteins, induces the expression of a number of genes in different pathways to bring about the necessary changes in cellular level. In one of the pathways, it induces the expression of Lin28a, an RNA binding protein which binds to *let-7* microRNA. *The let-7* maintains the differentiated state of the cell. Lin28a inhibits mature *let-7* miRNA formation and also causes its degradation thus priming the cell towards de-differentiation. Ascl1a also was found to upregulate the expression of Insm1a, a transcriptional

repressor which in turn downregulates Dkk1b, a Wnt antagonist, thus activating Wnt signalling in Muller Glia derived progenitor cells (MGPC). Wnt signalling acting through stabilization of β-catenin is another crucial and essential signalling pathway active during retina regeneration in zebrafish. Notch signalling has also been reported to be upregulated in injury responsive zone for restricting the zone of proliferation by downregulating Ascl1a.

In spite of this vast volume of knowledge about the signalling mechanisms active during zebrafish retina regeneration, implementing these findings towards the successful repair of mammalian retina remains yet to be achieved. Clearly, these findings are only part of the massive reprogramming that is essential for a successful retina regeneration. The involvement of epigenetic regulators and their interaction with genetic factors remains greatly under-explored with this regard. Hdacs, being global regulators of transcription, are hypothesized to have critical roles during zebrafish retina regeneration. Although Hdacs are reported to have been required for *Xenopus* tail regeneration, its exact roles during zebrafish retina regeneration remain unknown. Also, like Lin28a, the involvement of Myc genes, another group of pluripotency-inducing factors, remain greatly under-explored. Also, the epigenetic properties of Myc make it more interesting to investigate its role in retina regeneration.

Results

Induction and regulation of *myca* and *mycb* are essential for Muller Glia de-differentiation and retina regeneration.

Since we know that Wnt signalling is upregulated in MGPCs, and c-Myc, a well-studied pluripotency inducing factor, is one of the downstream targets of Wnt signalling, we wanted to check the status of expression of Mycb during retina regeneration. At 12 hours post retinal injury (12 hpi), we performed an RNAseq analysis which showed upregulated transcript levels of both *myca* and *mycb*, the two isoforms of c-Myc present in zebrafish. In a time-course expression pattern of these two over a period of 21 days after injury, we found both to be upregulated very early during the de-differentiation phase. Knocking down Myca or Mycb or pharmacological inhibition of Myc/Max binding at the early or late stage during de-differentiation and proliferation, caused a significant reduction in the number of MGPCs.

To further investigate the mechanism by which the expression of Myca and Mycb is regulated post retinal injury, we identified the already reported Ascl1a/Insm1a regulatory axis also modulates the expression of Myc genes in proliferative MGPCs. Insm1a, a transcriptional repressor, binds to Mycb promoter to control its transcription. We also have found that Ascl1a physically binds to Mycb promoter to upregulate its transcription and Mycb in turn also regulate Ascl1a by directly binding to its promoter, thus forming a feedback signalling loop.

Lin28a has also been reported previously to be upregulated by pluripotency factors like Myc and Sox in various other systems. We wanted to check if similar regulation exists in retina regeneration. Surprisingly, in spite of Myc being a *de facto* transcriptional activator, we found induction of *lin28a* in Mycb inhibited retinae. Promoter activity assay also showed an increase

in promoter activity in the presence of Mycb mRNA as well as MO against it. This prompted us to decipher the mechanism of Myc acting as a transcriptional repressor. We found that Mycb physically binds to HDAC1 and recruits it to Lin28a promoter to downregulate its expression. These results made us hypothesize that in proliferating and non-proliferating cells; regulation of Lin28a by Mycb differs depending upon the availability of HDAC1. This was proven right when we found HDAC1 protein is depleted from proliferating cells at 4dpi. Further, we also identified Her4.1 as another target of Mycb and we show that Myc regulates her4.1 in a similar fashion by which it regulates Lin28a.

Similar to the regulation of *lin28a*, we also found Myc and Hdac1 collaborating to regulate another important regeneration-associated gene *her4.1*. In this way, in one hand, Mycb can downregulate an important gene *lin28a*, which facilitates de-differentiation and proliferation, but on the other hand, also downregulates *her4.1*, the absence of which results in increased number of proliferating cells.

Regulation of histone deacetylases is essential for Muller Glia de-differentiation and retina regeneration.

This physical collaboration between Mycb and HDAC1 prompted us to check for transcriptional regulation of all HDACs. Time course analysis of *hdac*s mRNA fold change following retinal injury revealed *hdac1* to be downregulated. mRNA *in situ* hybridization at different time points post injury showed a drastic depletion of *hdac1* from all layers of the retina, but at around 4dpi its expression was restricted to mostly non-proliferating cells in the injury responsive zone.

RT-PCR and mRNA *in situ* hybridization at various time points post retinal injury also revealed *hdac1, hdac4, hdac7, and hdac9* to be downregulated, but *hdac3, hdac5,* and *hdac6* to be

upregulated. *hdac3* showed higher expression in non-proliferating cells in injury responsive zone at 4dpi compared to its pan retinal expression pattern in uninjured retinae.

Knocking down *hdac1* caused a slight but significant increase in the number of proliferating cells at 4dpi which was supported by increased expression of regeneration-associated genes like *ascl1a*, *lin28a* and *mycb* as revealed by RT-PCR, q-PCR, and mRNA *in situ* hybridization at 2 and 4dpi.

Contrary to our previous finding, we found a significant reduction in the number of proliferating cells at 2dpi after knocking down *hdac1* following injury. Surprisingly it was also accompanied by increased expression of *ascl1a* and *mycb*, but a decrease in the expression of *lin28a*.

We have got decreased proliferation at 4dpi by treating retinae with HDACs inhibitors like valproic acid (VPA), sodium butyrate (NaB, class I and IIa HDACs inhibitor), or trichostatin-A (TSA, pan HDACs inhibitor). This was surprising when we found transcripts of regeneration-associated genes like *ascl1a*, *mycb*, *zic2b*, *mmp9*, *shha*, *sox2* to be upregulated. But more surprisingly we found the cytokines and *lin28a* to be down-regulated. Suspecting downregulation of *lin28a* to cause less translation from the upregulated mRNAs by accumulation of *let-7* microRNA, we checked protein levels of Ascl1a and Mycb and found them not to be upregulated as their mRNA counterpart. We also found a dose-depended increase in the levels of *let-7* microRNA.

As another potential reason for the reduction of proliferation in Hdacs inhibited retina, we found upregulated transcript levels of *her4.1* which is also a downstream target of Delta/Notch signalling. Her4.1, being a transcriptional repressor, regulates Lin28a to control proliferation.

Withdrawal of the inhibitors after 4 days of injury resulted in new cells entering cell cycles which were able to migrate to all retinal cell layers. When we checked the level of *lin28a* in

this background, we found it to be upregulated following the withdrawal of Hdacs inhibition. This supported the occurrence of new proliferating MGPCs. We also found the cytokines mRNAs to be upregulated in this background.

Blockade of HDACs later after injury, while de-differentiation stage, resulted in deregulated cell migration with a huge bias towards ONL and very little towards GCL. This suggests a possible role of HDACs during cell migration.

Delta/Notch signalling restricts de-differentiation and proliferation in the injury responsive zone by suppressing *mycb* and *lin28a* through a regulatory network which cross-talks to HDACs.

Previous studies have shown that DAPT mediated blockade of Delta/Notch signalling causes copious proliferation in the injured retina which was associated by an upregulation of *ascl1a*. We showed that this increased proliferation in Delta/Notch compromised retina is also associated with increased expression of *mycb* and *lin28a*. In fact, we identified Mycb as a necessary molecular player which mediate the effect of Notch signalling. We also found Lin28a along with Mycb to be another important regeneration-associated gene which gets downregulated by Delta/Notch signalling as a mechanism to control MGPCs proliferation. This was further supported by double mRNA ISH which showed mutual exclusion of *myca* and *lin28a* with *her4.1*, a Delta/Notch signalling target gene.

As a final support to our hypothesis, we found an active Her4.1 binding site on *lin28a* promoter which was found to be regulated by Her4.1.

Discussion

Our study identified c-Myc and HDAC1 as novel regulators of zebrafish retina regeneration which they achieve through physical interaction to regulate Her4.1/Lin28a/*let-7* regulatory axis. We also identified novel regulations of Myc genes in transcriptional levels by Ascl1a/Insm1a axis to regulate reprogramming of MGPCs by achieving fine-tuned expression of *myca* and *mycb*. We also showed transcriptional and translational regulations of different *hdac*s and their relevance with respect to proliferation. This current study, elucidating the roles played by c-Myc to regulate retina regeneration, provides crucial insights into its complex and fascinating molecular mechanisms. Further, depletion of HDAC1 from proliferating cells might explain why all Muller glial cells do not proliferate following injury. We also show a regulation of cytokines through HDACs/Her4.1 axis. As a whole, besides identifying new players in retina regeneration, our present study also links them to some of the key regulators and signalling pathways reported previously. Taken together, our study identifies novel signalling pathways regulating crucial genes for a successful regenerative response which might pave the way towards successful intervention of mammalian retina regeneration.

Abbreviations

Ascl1a	Achaete-Scute Complex-Like 1a
β-act	beta-actin
BHLH	Basic Helix Loop Helix
BrdU	5-Bromo-2'-Deoxyuridine
BSA	Bovine Serum Albumin
ChIP	Chromatin Immuno-Preciptation
CMZ	Ciliary Marginal Zone
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
CRLF	Cytokine receptor-like factor
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
Dig	Digoxigenin
Dkk1b	Dikkopf 1b
dpf	Days Post Fertilization
dpi	Days Post Injury
ESC	Embryonic Stem Cell
FACS	Fluorescence-Activated Cell Sorting
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
HDAC	Histone de-acetylase
Her4.1	Hairy related 4, tandem duplicate 1
HES	Hairy enhancer-of-split
HRP	Horseradish Peroxidase
IF	Immuno-Fluorescence
Il6	Interleukin 6
Il6r	Interleukin 6 receptor
INL	Inner Nuclear Layer
Insm1a	Insulinoma-Associated 1a
IPL	Inner Plexiform Layer
iPSC	Induced Pluripotent Stem Cell
Klf4	Kruppel-Like-Factor 4
Lepa	Leptin a
Lepr	Leptin receptor
Max	Myc associated X
MG	Müller Glia
MGPC	Müller Glia derived Progenitor Cell
MMP	Matrix metalloproteinase
MO	Morpholino
mpi	minutes post injury
NaB	Sodium butyrate
NICD	Notch Intra-Cellular Domain

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
PIF	Pluripotency inducing Factor
PNS	Peripheral Nervous System
qPCR	quantitative Polymerase Chain Reacion
RGC	Retinal Ganglion Cell
RNFL	Retinal Nerve Fibre Layer
RPC	Rod Progenitor Cell
RPE	Retinal Pigment Epithelium
RT-PCR	Reverse Transcription Polymerase Chain Reacion
Shh	Sonic hedgehog
Sox2	Sex Determining Region Y-Box 2
SV40	Simian Vacuolating Virus 40
TEA	Triethylamine hydrochloride
TNF-α	Tumor Necrosis Factor Alpha
TSA	Trichostatin A
Tuba1a	Tubulin, Alpha 1a
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UTR	Untranslated Region
VPA	Valproic acid
Zic2b	Zinc finger of cerebellum 2b

Section 1

Introduction and Review of Literature

1.1 Regeneration: the phenomenon and the field of study

Regeneration, in its comprehensive sense, is essentially renewal of a lost or damaged part of any system achieved by the system itself without any direct foreign aids. In biology, as long as we go by this definition, virtually all organisms, be it as primitive as a protozoon or as complex as a human, are capable of regeneration, if not for the complete organism, for specific parts at least, making it one of biology's most fascinating and yet most complex and poorly understood spectacle.

If the words coined by Richard J. Goss in 1969 are to be believed, all organisms exist in between two extremes, one being able to regenerate absolutely every part of the body thus getting immortality, and the other being not able to regenerate any of the body-parts at all, which naturally would result in death. So, in a way regeneration acts as a fulcrum, on which the existence of life should be finely balanced. But life, in general, shows a bias against regeneration as evident from the loss of regenerative capability through evolution, most probably because of the fundamental differences between the purpose of regeneration and life. Life cannot exist in its current form without reproduction and that will be redundant if regeneration is absolute. So, with the price of regeneration, forced by natural selection and reproduction, life has opted for less regeneration in the course of evolution.

Surprisingly, the field of studying regeneration with a scientific outlook is one of the earliest fields in biology, dating back to the 18^{th} century (Denismore, 1991; Vorontsova and liosner, 1960). Some of the earliest known records of regeneration studies, although might not be strictly scientific in today's definition of science, include that of Aristotle and Pliny. In fact, some scientists could account for enough reasons to claim that essentially the field of regeneration studies initiated, in a way, the whole field of developmental biology and embryology. Unfortunately, due to lack of appropriate tools to manipulate and investigate

regenerative organisms to uncover the underlying cellular and molecular mechanisms, the field lost its initial status and underwent a repressive period only to be revived by the arrival of tools for genetic manipulation (Carlson, 2007).

1.2 Types of regeneration

In a succinct and synoptic way, regeneration, in biology, is the "reproduction or reconstitution of a lost or injured part, or a form of asexual reproduction". As a matter of fact, dramatically different biological phenomena could be incorporated in this definition of regeneration. Naturally, for better understanding, delineating the different processes based on mechanistic similarities and classifying them accordingly becomes imperative. The whole plethora of different regenerative responses brought about by damage or loss of tissue has been simplistically categorized into four major classes, namely physiological regeneration, hypertrophy, morphallaxis and reparative regeneration (Fig 1.1) (Carlson, 2007).

Physiological regeneration is the natural renewal of deteriorated body parts such as replacement of blood cells, or replenishment of the endometrium after a menstrual period, or annual regeneration of deer antlers. These processes, although varies tremendously in genetic and cellular levels, share a common feature of adjusting themselves according to the physiological needs such as increased haematopoiesis in higher altitudes.

The term hypertrophy is generally attributed to the increase in the functional volume of an organ due to enlargement of the size of its component cells. One classic example of Hypertrophy was demonstrated by Addis and Lew in 1940 in which hypertrophy was

established by showing an almost two-third increase in the size of a kidney when the other kidney was removed surgically.

Morphallaxis, on the other hand, as can be seen in the Cnidarian hydra, is the fascinating transformation of severed body parts into a whole organism or part of it, often without any apparent aid from proliferation at the injured surface. This phenomenon is mostly restricted to invertebrate regenerating systems.

Clearly, retina regeneration following an acute injury cannot be considered in any of these three above-mentioned categories of regeneration. Retina regeneration is classified into one of the sub-categories under reparative regeneration, named tissue regeneration, the other two being epimorphic regeneration and cellular regeneration. In general, mostly all post-traumatic regenerative processes can be classified as reparative regeneration occurring at various levels from single cell to major body parts. Epimorphic regeneration necessitates the formation of a blastema, an accumulation of de-differentiated cells, to replace a lost part of an organ. Regeneration of limb or tail in amphibians or reptiles is a classic example of epimorphic regeneration. On the other hand, renewal of a single damaged cell is termed as cellular regeneration mostly seen in protozoans such as *Stentor sp.* or the green alga *Acetabularia sp.* Tissue regeneration, interestingly, does not necessitate the formation of blastema and often associates with inflammatory ques as initial regulators of cellular de-differentiation and proliferation which are further regulated by a plethora of developmentally important genetic and epigenetic pathways.

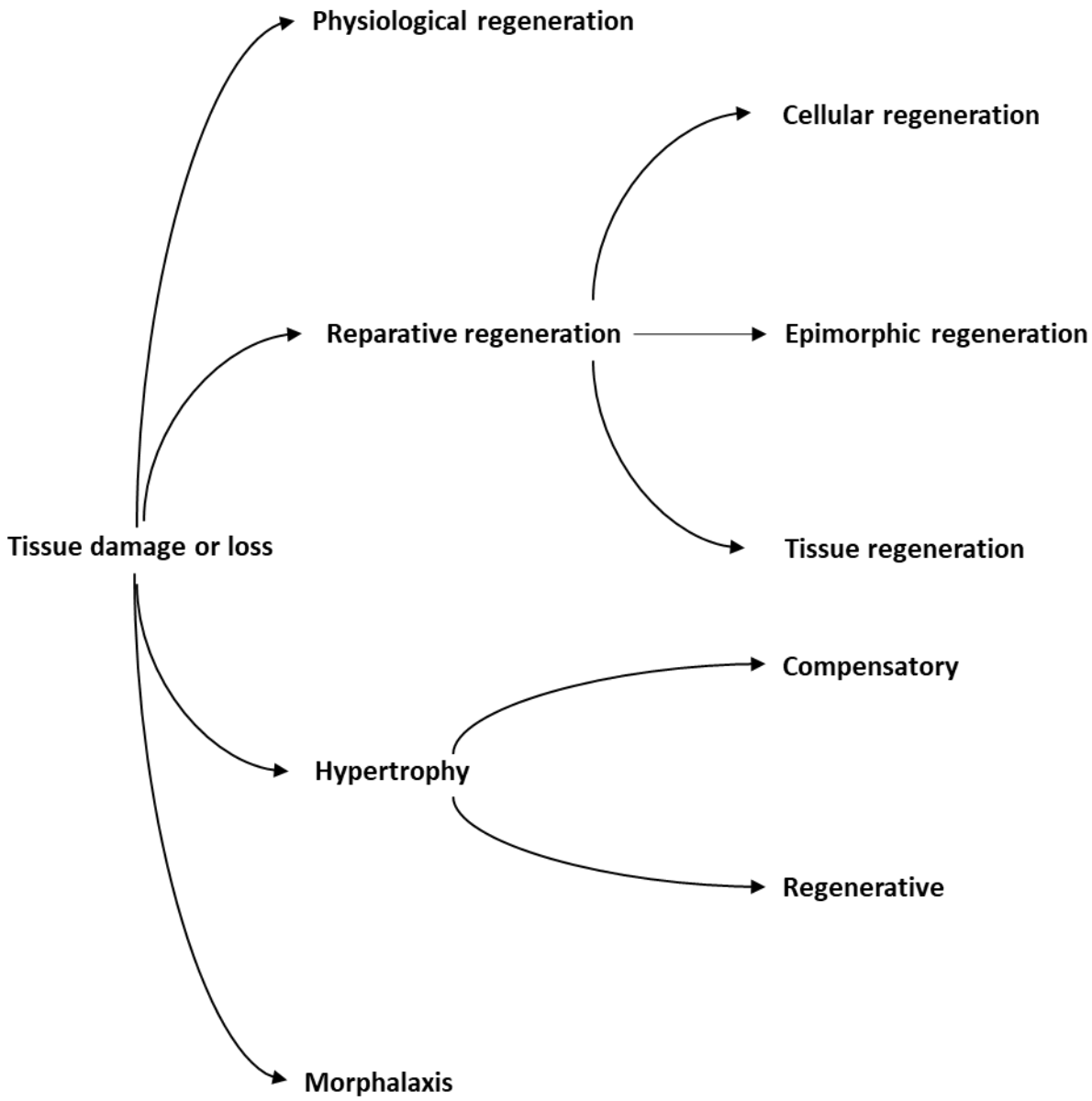


Fig 1.1: Classification of Regenerative responses. (Modified from Principles of Regenerative Biology, authored by Bruce M. Carlson, 2007, Academic Press, ISBN: 9780123694393).

1.3 Tissue regeneration and wound healing

A tissue is termed wounded when it loses the physical continuity of the functional tissue which is generally caused by any chemical, metabolic, physical, electrical or even any kind of optical destructive stimuli (White et al., 2010). A variety of inter-cellular and intra-cellular interactions coordinate the response of a tissue to injury which is one of the fundamental properties of any tissue. Regeneration happens when the lost part can be reverted back to the original functional tissue. But, instead of that, if the wounded part is replaced by a scar or less functional tissue, the tissue is said to be undergoing tissue repair and not regeneration. In case of wound healing or scar formation or fibrosis, the newly recovered part of the tissue consists mainly of fibroblasts and de-organized extracellular matrix, predominantly collagen. Surprisingly, while many of the eukaryotic organisms, like teleosts and urodeles, can undergo a very complex and poorly-understood process of cellular reprogramming to recapitulate the original tissue structure and functionality, humans, during adult life, lose this ability to regenerate and instead opt for fibrosis. Wound healing or fibrosis is achieved through three main stages of development, namely *inflammation*, *formation of new tissue* and *remodelling* (Gurtner et al., 2008). While inflammation is mainly guided by the components of the coagulation cascade, inflammatory pathways, and immune system, the formation of new tissue is guided by upregulation and secretion of various growth factors which also help in angiogenesis. The third and last step of wound healing or remodelling is achieved through systemically retracting all the responses that had already been in action for the previous two stages. Surprisingly, the set of molecular players involved in the process of wound healing have been identified to be of significant similarities with that of malignancy, suggesting the importance of studying both these phenomena in great details. The process of wound healing in humans also exerts a great deal of negative impact on the health of people in general and also consecutively can display an economic impact (White et al., 2010; Gurtner et al., 2008).

Interestingly, wound healing happens in mammals at the cost of compromised regeneration. Much like teleosts and urodeles, many of the molecular players or regeneration are still conserved in mammals and in fact following injury start to be regulated and in turn, regulate other pathways or molecules. Unfortunately, fibrosis facilitates scar formation and inhibits regeneration.

1.4 Zebrafish retina as a model system to study regeneration

Retina is the most easily accessible part of the Central Nervous System (CNS) and upon receiving injury does not cause lethality to the organism. These attributes make retina one of the best model systems suitable for studying regeneration of CNS. Besides, while mammals have lost regenerative capacity of several of their tissues or organs through the course of evolution and replaced tissue regeneration with tissue repair, other vertebrates like teleosts and urodeles have this regenerative capacity well conserved in most of their tissues and organs (Goldman, 2014; Ail and Perron, 2017; Gemberling et al., 2013; Kyritsis et al., 2012; Mokalled et al., 2016; Poss et al., 2002; Rabinowitz et al., 2017; Singh et al., 2012; Wan and Goldman, 2016). For this same reason, human CNS does not possess any regenerative capacity but that of fish can demonstrate remarkable regenerative response after receiving an acute mechanical, optical or chemical injury. The structural similarities between teleost and mammalian retina make the study of fish retinal regeneration even more significant (Fadool et al., 2008). Like mammalian retina, the zebrafish retina can also be broadly divided into three nuclear layers, namely Outer Nuclear Layer (ONL), Inner Nuclear Layer (INL) and Ganglion Cell Layer (GCL) (Fig 1.2). All major retinal cell types position themselves with their nuclei in any of

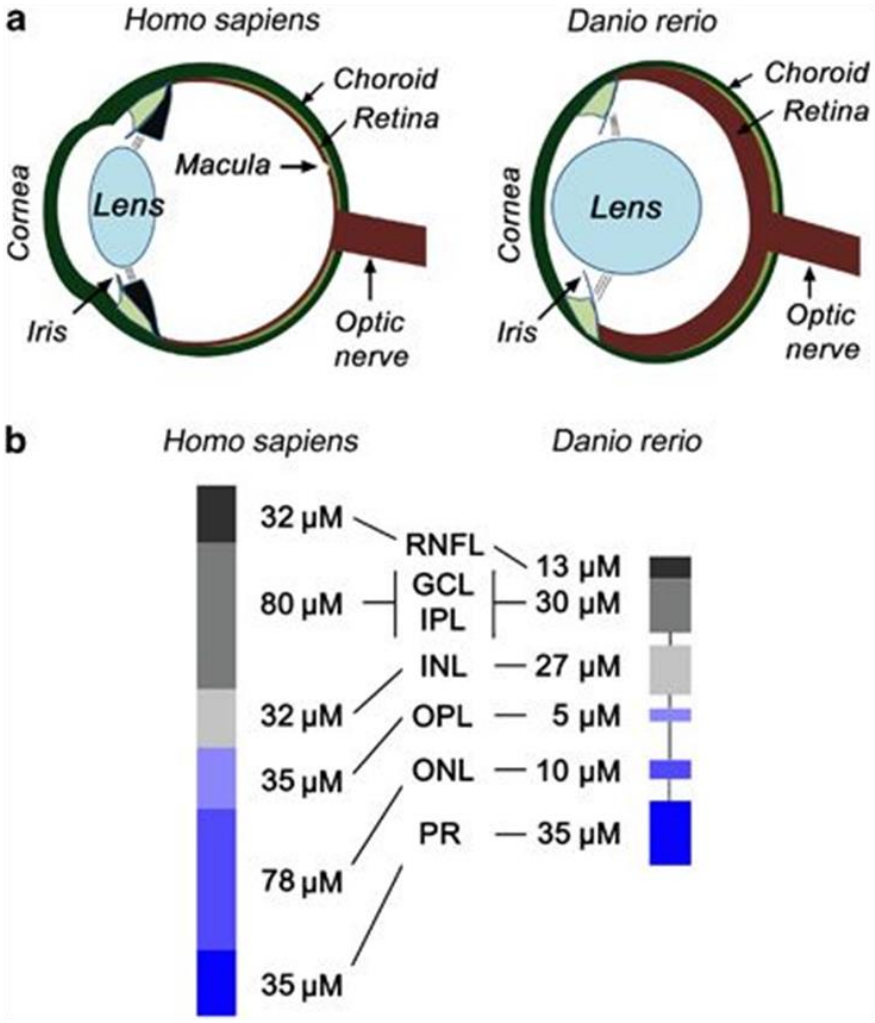


Fig 1.2: Structural similarities of Human and fish retina (a) overall structure of human and fish eye displaying sililar parts. (b) comparison between human and piscine retinal cell layers. Taken from Chetri et al., 2014.

these layers. Whereas ONL consists of rod and cone cells, INL is made up of bipolar cells, horizontal cells, amacrine cells and Muller glial cells, the only glial cells present in the retina. The axons of ganglion cells extend from the GCL to make the optic nerve (Gaia et al., 2012; Kolb et al., 2001). Taken together, the structural similarities of zebrafish and mammalian retina, being the most easily accessible part of CNS, and, above all the capacity to demonstrate a successful regenerative response which is lacking heavily in higher vertebrates, make zebrafish retina one of the best suitable model system to study retina regeneration aiming towards successful medical intervention of mammalian retinal repair.

1.5 Anatomy of the zebrafish neural retina

Developmentally, eye is one of the most prominent structures developed as soon as 3 days post fertilization (dpf) and the cellular organization of the retina also becomes quite apparent at that stage (Gaia et al., 2012). The neural retina resides beneath the pigment epithelium (RPE) which acts as a light absorber. The zebrafish neural retinae, in which six neuronal cell types and one single glial cell, all derived from the neural ectoderm, are arranged in three nuclear layers separated by two synaptic or plexiform layers, demonstrate a structure which is also canonical to the vertebrate retinae (Fadool et al., 2008). These cells are namely, rod cells, cone cells, bipolar cells, amacrine cells, horizontal cells, ganglion cells, and Muller glia cells. The neural retina can be broadly classified into three nuclear layers, namely outer nuclear layer (ONL) which is the farthest layer from the lens, inner nuclear layer (INL) and ganglion cell layer (GCL) which is closest to the retina. The somata of all retinal cell types reside within these three layers (Fig 1.3) (Fadool et al., 2008). Compared to the true complexity demonstrated by

the extensive neuronal circuitry of the retina, the categorization of the retinal layer in aforementioned order might seem a bit too simplistic (Kolb et al., 2001; Masland, 2001a/b).

Cell bodies of one type of rod and four types of cones reside in the ONL, whereas those of Muller glial cells, and the interneurons such as horizontal cells, bipolar cells and amacrine cells can be found in the INL. The GCL comprises cell bodies of the ganglion cells and a few displaced amacrine cells. Scientists, today, still believe that the complete structure and all the types or sub-types of the cells present in the INL are still not fully understood. The comparatively thinner outer plexiform layer which can be seen in between the ONL and INL is formed by the numerous synapses between the cells of these two nuclear layers. Similarly, outer plexiform layer, the significantly thicker and more complex one, is formed in between the INL and the GCL and contains all synapses formed in between the cells of these two nuclear layers. The axons of the ganglion cells form the optic nerve which connects to the arborization fields and the optic tectum of the midbrain, which is the piscine counter-part of mammalian superior colliculus. Apart from all these similarities, the zebrafish retina differs from its mammalian counterpart in respect of development, as it continues to grow bigger as the retina never stops proliferating. The circumferential germinal zone at the ciliary margin contains stem cells that continue to proliferate in the adult retina by generating all retinal cell types through-out the life-span of the fish (Gaia et al., 2012).

1.6 Function of the zebrafish neural retina

Developmentally, the retina is one of the fastest growing tissues in the larval structure. And this fast growth is also associated with the functionality of different neurons which also

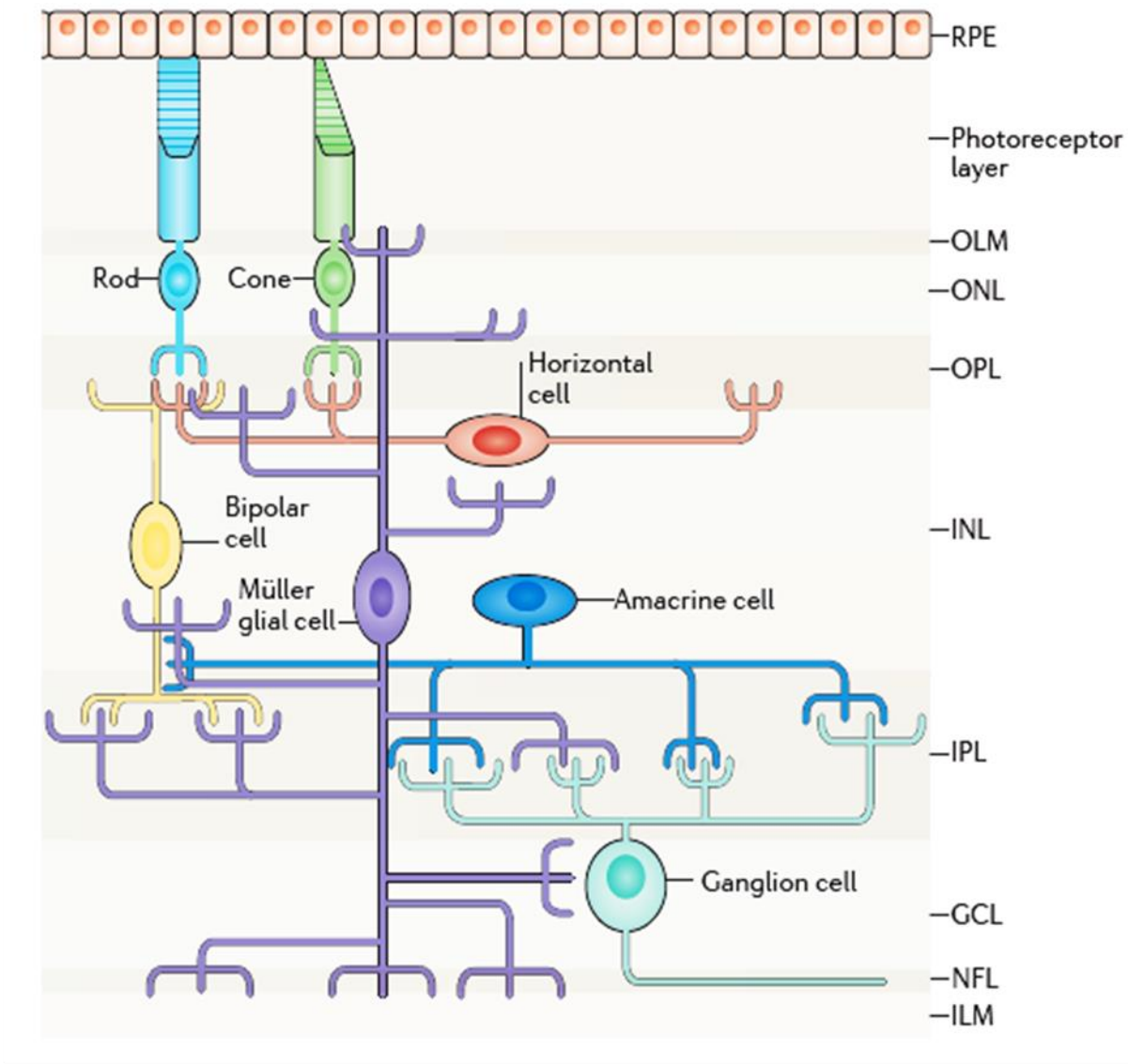


Fig 1.3: Retinal anatomy of zebrafish. Taken from Goldman, 2014.

becomes prominent very early during the development as many of the early behaviours of the larval zebrafish is driven by visually guided ques. The retinal pigment epithelium helps in absorbing light which has to travels through all retinal cell layers as the RPE is the outer-most layer which envelops the neural retina. The rod cells are capable of mediating vision in the low light level while the four types of cones, namely the red-green light sensitive double cones, blue light sensitive long cones, and UV light sensitive short cones are responsible in mediating vision in bright light (Raymond et al., 1993; Robinson et al., 1993). Larval vision is mainly guided through the cone cells while the rod cells start functioning at an age of 15 dpf (Gaia et al., 2012).

This complexity of five different cell types in the ONL is as fascinating as the formation and functionality of the cells residing in the INL, if not less. The extent of our understanding about the functionality of the INL cells is arguably still in its infancy. To date, there are a total of four sub-types of horizontal cells in the INL that can be distinguished by different cell-specific markers and specific type of these cells form synapses with specific types of cones or rod cells and induce lateral inhibition in the dendritic end (Song et al., 2007). Around a total of 70 different types of amacrine cells also induce lateral inhibition, but at the axonal end (Marc and Cameron, 2001; Yazulla and Studholme, 2001). As of now, a total of 17 types of bipolar cells, classified in 3 different categories, have been distinguished and are believed to mediate the nerve impulse between the photoreceptors and the ganglion cells (Connaughton and Nelson, 2000). The ganglion cell layer contains at least 11 types of morphologically distinguishable ganglion cells and the axons arising from these cells form the optic nerve and subsequently the optic tract (Mangrum et al., 2002).

In contrast to the vastly diverse population of neuronal cell types present in the retina, and the complexity of their functionality, Muller glial cells are the only type of glial cells present in the retina. But unlike their morphological similarities, they perform a plethora of diverse

functions (Goldman, 2014). The cellular extensions of these specialized radial glial cells span through all retinal layers and ensheath the somata and processes of all retinal cell types, thus, forming numerous sub-compartments within the retina which contains different retinal cells and allow their interaction to be limited within a fixed territory (Bringmann et al., 2006; Reichenbach et al., 2010). The Muller glia cells also regulate metabolism, provide nutrients to the retinal cells, help in the formation of the blood-retinal barrier, recycle neurotransmitters, release factors like glutamate and D-serine to controls excitability of neurons, maintain ion balance and water homeostasis, and also aids in regenerating injured retinae (Bringmann et al., 2006). Furthermore, these cells can produce proinflammatory cytokines in response to infection (Kumar et al., 2013; Escobar et al., 2017; Shamsuddin et al., 2011; Kumar et al., 2012) or injury (Thummel et al., 2008; Ramachandran et al., 2010), and also phagocytose dead cell fragments or foreign materials (Escobar et al., 2017). Unlike mammals, zebrafish Muller glia cells do not undergo gliosis, rather prepare to de-differentiate through a very complex process of genetic and epigenetic landscape alteration (Goldman, 2014; Wan and Goldman, 2016). The Muller glia respond to and help cure a variety of retinal diseases and insults such as diabetic retinopathy, retinal detachment, macular edema, proliferative retinopathy, ischemia-reperfusion, hepatic retinopathy, retinoschisis, and retinitis pigmentosa or even mechanical injury (Bringman et al., 2001). All these attributes make Muller glial cells one of the most studied cells in the retina as understanding and adapting the molecular mechanisms exhibited by these cells are of immense importance for therapeutic interventions of injured or diseased mammalian retina.

1.7 Response of MG cells to retinal injury: a comparative analysis between different vertebrate phyla

Vision loss due to retinal dysfunction is one of the major health-related issues with excessive impact in society and economy (Tailer et al., 2006). One way to overcome this is by developing technologies which will allow mammalian Muller glia to heal itself. Thus, studying the mechanisms underlying natural retinal regeneration in other species, such as fish, becomes imperative. Muller glia reprogramming-driven retina regeneration in zebrafish has given scientists new hope towards inducing a successful regenerative response in the injured human retina (Wu et al., 2001). Muller glial cells, named after their discoverer Heinrich Muller (Muller, 1857), a German anatomist, are believed to be specialized radial fibres or radial glia, which are capable of performing diverse functions to maintain the homeostasis and functionality of the neural retina (Reichenbach et al., 2013). These cells are also the last ones to be originating during the development of the neural retina and are indeed very similar to multipotent late retinal progenitors with respect to their proteome signature, explaining the existence of a mechanism by which these cells can reprogram themselves and aid in proliferation during retina regeneration to recreate all types of lost retinal cells in certain species (Jadhav et al., 2009; Blackshaw et al., 2004; Roesch et al., 2008).

1.7.1 Response of the Muller glial cells to retinal injury in Mammals

Although several cells from different regions of the adult mammalian retina possess varying potential to generate neurons (Gallina et al., 2013; Chohan et al., 2017), mostly, the mammalian retina is unable to self-repair and demonstrate permanent visual ailment following acute injury. Thus, studying the *in vivo* response of the mammalian MG cells is especially challenging

pertaining to their incapability to completely regenerate the retina. The Muller glia in adult mammalian retina, instead of reprogramming themselves to form Muller glia-derived progenitor cells (MGPCs), undergoes reactive gliosis which is characterized by changes in morphology, de-differentiation, and nuclear migration, but very rarely proliferation (Dyer and Cepko, 2000; Bringmann et al., 2009). Disproving the former common belief about the incapability of mammalian MG cells to re-enter the cell cycle, preliminary studies have shown that, following NMDA or MNU mediated retinal injury, the mammalian MG cells, although proliferate rarely, can initiate a cellular and molecular response accompanied by changes in morphology, de-differentiation, and nuclear migration; which closely resembles the initial response of teleost MG cells to injury (Ooto et al., 2004, Wan et al., 2008). *In vitro* and *ex* vivo studies have also shown that not only rodent and human MG cells can be persuaded to generate both glial cells and neurons, but mammalian MG cells can also migrate and integrate into the correct cell layer when transplanted into retina with photoreceptor, amacrine or ganglion cells depletion, and can aid in the functionality of photoreceptors, amacrine or ganglion cells (Singhal et al., 2012; Jayaram et al., 2014).

ASCL1 is one of the most studied molecules in regeneration in various model systems including mammals. But unlike fish, following injury, *Ascl1* was found to be induced not in adult mice but in young mice with NMDA administration (Karl et al., 2008, Loffler et al., 2015). This differential expression pattern could account for the lesser regenerative potential in adult mice compared to the younger ones (Ueki et al., 2015; Loffler et al., 2015). Interestingly, ASCL1 overexpression could preprogramme MG cells *in vitro* and initiate a neurogenic signalling cascade (Pollak et al., 2013). Besides genetic reprogramming, *Ascl1* overexpression was reported to induce a change in the epigenetic landscape and chromatin remodelling of its own targets (Pollak et al., 2013). Besides ASCL1, subtoxic level of glutamate has also been shown to be able to reprogramme mammalian MG cells for a neurogenic fate

(Takeda et al., 2008). Other studies have shown that just like fishes, proliferation of mammalian MG can also be influenced by various exogenous growth factors such as EGF, FGF or insulin (Ooto et al., 2004, Close et al., 2006, Karl et al., 2008, Fischer and Bongini, 2010), or even by some of the developmentally important signalling pathways like Wnt (Das et al., 2006; Osakada et al., 2007; del Debbio et al., 2010; Liu et al., 2013), Notch (Das et al., 2006; del Debbio et al., 2010), and Hedgehog (Wan et al., 2007).

1.7.2 Response of the Muller glial cells to retinal injury in Aves

For a long time, the scientific community believed the avian retina could not demonstrate any proliferative response following an acute injury. But this came to an end when regeneration of few neurons like ganglion cells, amacrine cells, or bipolar cells by post-natal chick MG cells was reported to be associated with proliferative response (Fischer and Reh, 2002). But the regeneration of photoreceptors has still not been well documented in avian retinal injury. Also, while neonatal chick MG possesses limited ability to regenerate the retina following activation by different growth factors, MG cells of adult chick have arguably lost this ability (Todd and Fischer, 2015).

Recent studies have revealed the activation of microglia and macrophages in chick MG cells de-differentiation and re-entry to the cell cycle which is achieved by induction of several of the pro-inflammatory cytokines and the components of the complementary systems (Fischer et al., 2014; Haynes et al., 2013). In support of this, glucocorticoid receptor with an anti-inflammatory response was reported to exert a negative regulation on avian MG cells reprogramming (Gallina et al., 2014b). Besides FGF2, MAP kinase pathway, and insulin were also reported to promote MG cells de-differentiation and cell cycle entry (Fischer et al., 2002, Fischer and Reh 2002, Fischer et al., 2009a, b, Fischer et al., 2010, Gallina et al., 2014a).

Induction of *Ascl1*, different *sox* and *pax* genes, mTOR, Notch and Hedgehog signalling have also been reported to be induced in avian retina following injury to promote MG cells de-differentiation (Hayes et al., 2007, Ghai et al., 2010, Gallina et al., 2014, Todd and Fischer, 2015). In spite of this accumulation of knowledge about chick retina regeneration, the intrinsic factors responsible for de-differentiation of MG cells have not yet been well characterized.

1.7.3 Response of the Muller glial cells to retinal injury in Amphibians

Unlike mammals or birds, the urodele retinae like that of the salamanders or newts possess remarkable and fascinating regenerative ability (Mitashov, 1996, Yoshii et al., 2007), which is mediated by RPE and CMZ regions of the retina. Although a direct involvement of the MG cells in mediating retinal regeneration following any kind of insult is yet to be observed in the amphibian retina, the existence of proliferation in the INL which is associated with MG cells has been observed (Grigorian and Poplinskaia, 1999; Novikova et al., 2008). In some specific injury models, amphibian MG cells have been observed to undergo hypertrophy and do not enter cell cycle (Choi et al., 2011).

1.7.4 Response of the Muller glial cells to retinal injury in Pisces

The ever-growing fish retina gets its constant supply of new neurons from the CMZ region of the fish retina. Besides CMZ, some previously unknown cells, later to be identified as Muller glia, residing in the INL, have also been observed giving rise to new rod photoreceptors (Johns and Fernald, 1981; Johns, 1982; Julian et al., 1998; Otteson et al., 2001; Otteson and Hitchcock, 2003, Lenkowski and Raymond, 2014). But this proliferation is extremely sporadic and slow when compared to the proliferative response show-cased by piscine MG cells following retinal

injury. Some of the earliest known examples of retina regeneration in teleosts include that of the goldfish retina, following surgical removal of one-fourth of it (Lombardo, 1968). Regeneration of all types of lost neurons and MG itself in any kind of injury paradigm has made zebrafish the most favourite model organism to study regeneration (Goldman, 2014). Muller glia cells were finally coaxed to retina regeneration and identified as the sole source of new neurons using lineage tracing in transgenic zebrafish (Raymond et al., 1988; Wu, 2001; Braisted, 1994; Otteson, 2001). Till date a plethora of different regeneration associated molecules and signalling pathways have been identified to play crucial roles in zebrafish retina regeneration making zebrafish retina as one of the most studied model systems in the field of retina regeneration.

1.8 Retina regeneration in zebrafish.

If we compare the initial cues which help zebrafish MG cells to embark on a reprogramming journey in a strictly scientific sense, with the not-so-scientific re-emerging of the mythological bird Phoenix from its own ashes, we certainly cannot help but be bewildered at the similarities between these two as the MG reprogramming is also initiated by dying cells informing the neighbouring MG cells about their demise through several secreted factors. The complete process of retina regeneration through Muller glia reprogramming can be broadly classified in three phases, namely *de-differentiation, proliferation* and *re-differentiation* (Fig 1.4).

The phagocytic properties of the zebrafish MG cells play crucial role in initiating the de-differentiation process (Bailey et al., 2010). Zebrafish MG cells phagocytose dead cells secreting TNF-α following an acute injury, and start secreting various growth factors and cytokines such as Hb-egf, Insulin, Igf-1, Leptin and Interleukins family of cytokines which are

also induced in the transcriptional level in the MGPCs itself (Wan et al., 2016; Wan et al., 2012; Zhao et al., 2014; Wan et al., 2014; Nelson et al., 2013; Conner et al., 2014). These autocrine and paracrine signalling pathways converge to induce Ascl1a, a transcriptional activator of immense importance in retina regeneration, through Jak/Stat3 signalling pathway. It is interesting to note that while in fish, the interleukins and Jak/Stat3 mediated signalling is important for initiating a regenerative response, the same in mammalian and avian retina demonstrate reactive gliosis which also has a protective function for the retina (Peterson et al., 2000; Rhee et al., 2013). Another of the earliest signalling pathways in retina regeneration involves MAP kinase which, along with PI3K, regulates retina regeneration through β-catenin and pStat3 expression (Zhao et al., 2014; Wan et al., 2014).

Once the MG cells start de-differentiating they start to display a wide range of genetic and epigenetic transformations that bring forth MGPCs proliferation. Among the genetic regulators, Ascl1a has been shown to be of pivotal importance and can regulate a plethora of downstream target molecules. Initially, *ascl1a* shows a pan-retinal expression pattern following an injury which is restricted to the injury responsive zone by 4dpi (Ramachandran et al., 2010). One of the recently characterized function of Ascl1a in retina regeneration is to regulate Lin28a, an RNA binding protein, and an embryonic stem cell marker during development. Lin28a has been shown to bind and degrade *let-7* microRNA which, if active, maintains the differentiated state of the cell through microRNA mediated blockade of translation of several important regeneration associated genes such as *myca*, *mycb*, *pax6*, *hspd1*, *lin28a*, *ascl1a*, *oct4*, *zic2b* and several genes of the Sonic hedgehog signalling pathways such as *shha*, *ptch1*, and *smo*. Once Lin28a brings down the level of *let-7* microRNA in an injury-responsive MG, newly started transcription of all these genes can now be coupled with subsequent translation, thus

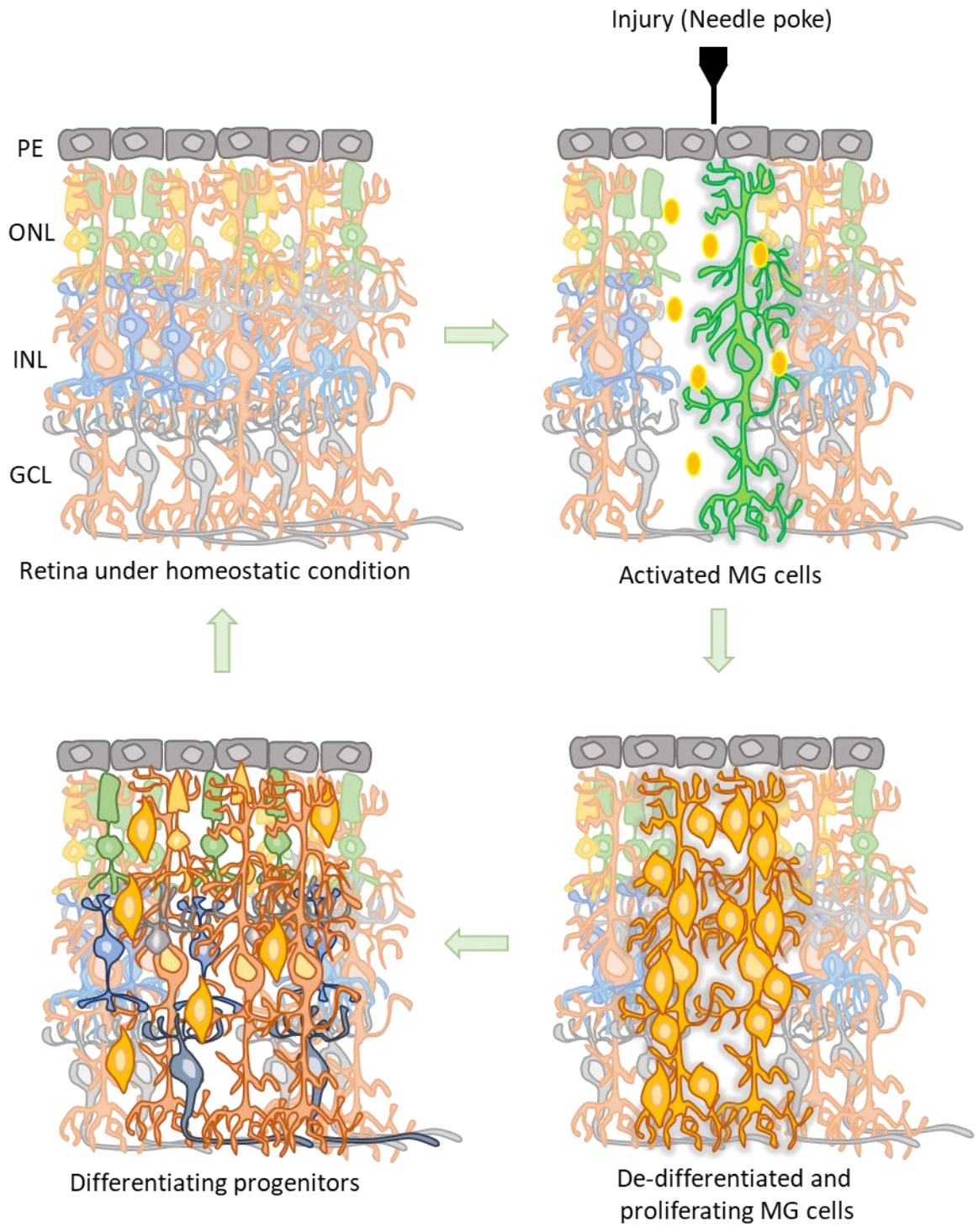


Fig 1.4: Stages of zebrafish retina regeneration.

bringing about the various genetic and epigenetic changes required for MGPCs formation (Ramachandran et al., 2010; Kaur et al., 2018).

Shh and Wnt/β-catenin signalling pathways have been shown to induce Ascl1a (Kaur et al., 2018) which further can induce the expression of Insm1a, a transcriptional repressor which downregulates *dkk1b*, a Wnt inhibitor, thus forming a positive feedback loop (Ramachandran et al., 2011). The significance of Wnt/β-catenin signalling is evident from the fact that β-catenin stabilization through GSK-3β inhibition is sufficient to induce proliferation of MG cells (Ramachandran et al., 2011). Shh signalling has also been shown to directly regulate Lin28a expression through Gli-transcriptional activator (Kaur et al., 2018).

Ascl1a dependent induction of Delta/Notch signalling to restrict the extent of proliferation shows a glimpse of the vastly diverse functionality of Ascl1a in fine-tuning the complex process of retina regeneration. Delta notch signalling has been shown to regulate the number of MGPCs by negatively regulating the expression of Ascl1a (Wan et al., 2012). Insm1a, in addition to help in the activation of Wnt signalling, has also been coaxed to suppression of *hb-egf* expression (Ramachandran et al., 2012), and induction of *p57kip2* through suppression of its inhibitor *bcl11a* (Ramachandran et al., 2012). Genes of the Pax family such as *pax6a* and *pax6b* are also reported to be induced with a crucial role to play during retina regeneration (Thummel et al., 2010). Besides all these genetic factors, a few of the epigenetic modulators and modifiers such as Apobec1, Apobec2 and Dnmts have been associated with MGPCs de-differentiation and proliferation (Powell et al, 2013).

Taken together, all these novel findings not only add significant clarity to the complex process of retina regeneration in zebrafish (Fig 1.5) but also help us comprehend the reasons behind the incapability of mammalian retina to regenerate successfully. Many of these molecules show differential expressions or activities in the piscine and mammalian retina. Such factors provide

us with several possible ways of therapeutic intervention of injured mammalian retina. In fact, Shh-mediated and ASCL1 overexpression-mediated MG proliferation in adult and young mice, respectively, have already emphasized the importance of studying the molecular mechanisms underlying retina regeneration in zebrafish (Wan et al., 2007; Ueki et al., 2015).

1.9 Pluripotency inducing factors and their role in tissue regeneration.

In truest of senses, the induction of pluripotency through forced expression of specific Pluripotency inducing factors (PIFs) is one of biology's most relevant and well-studied fields. The immense importance of the PIFs in transforming somatic cells to induced pluripotent stem cells (iPSCs) was acknowledged worldwide in 2012. One of the major scientific advances that influenced the inception of the field of iPSCs is the identification of these PIFs, otherwise termed as the "Master" transcription factors that can influence the fate of a cell (Yamanaka 2012).

Klf4, Sox2, cMyc, Nanog and Oct4 were subsequently identified as few such factors which upon forced over-expression, can alter the fate of differentiated somatic cells to resemble embryonic stem cells (ESCs) through global genetic reprogramming (Takahashi and Yamanaka 2006, Stadtfeld 2010). Subsequently, such "defined factors" were identified that

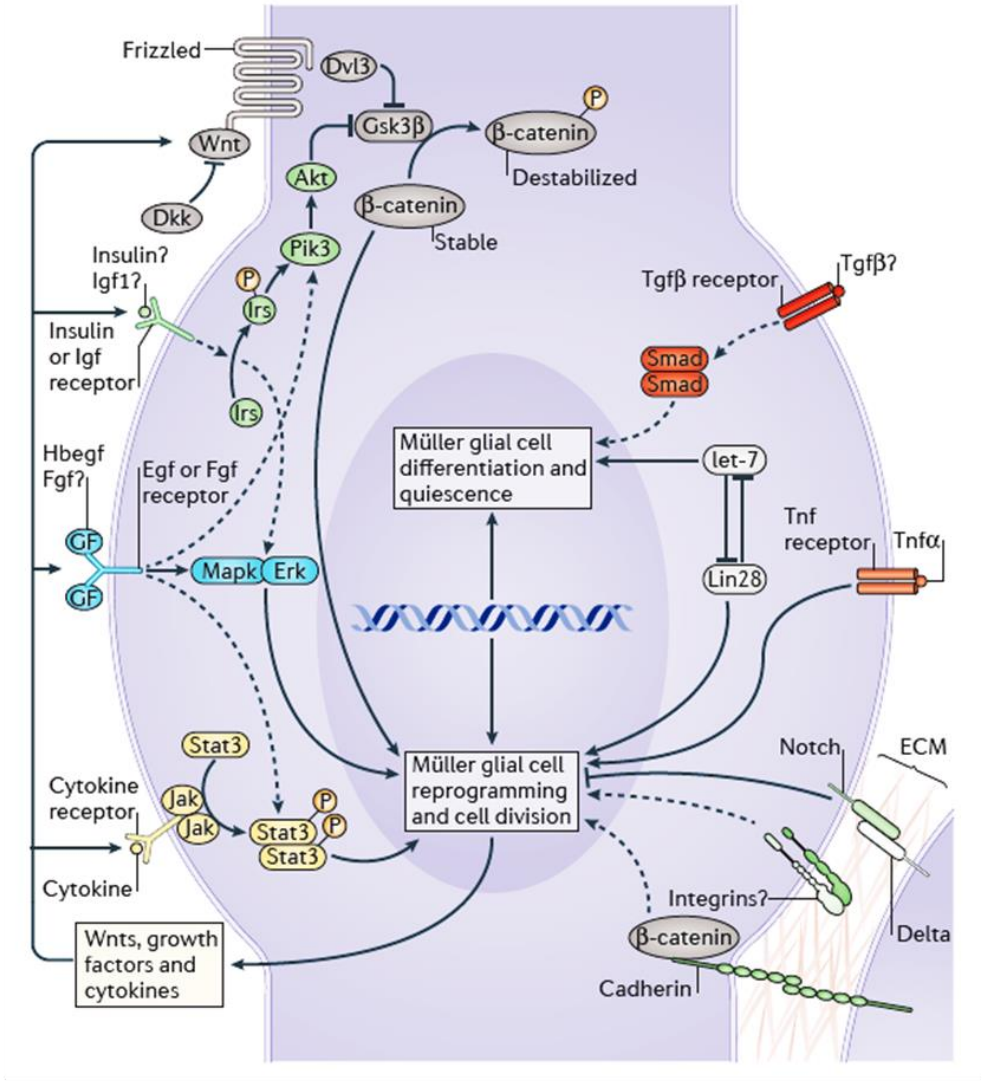


Fig 1.5: Signalling cascade driving de-differentiation and proliferation of MGPCs. Taken from Goldman, 2014.

can induce a specific cell fate such as induction of neural characteristics in fibroblasts by forced expression of *Ascl1*, *Myt1l*, and *Brn2* (Vierbuchen et al., 2010). One of the mechanisms by which these transcription factors orchestrate the process of reprogramming is repression of target genes associated with differentiation by recruiting repressive chromatin-remodelling complexes such as NuRD (Kaji et al, 2006) and Polycomb (Boyer 2006) to their promoters. These iPSCs often show molecular signatures similar to that of an ESC (Maherali et al., 2007; Stadtfeld 2010). Interestingly, the MGPCs, following acute retinal injury also have been reported to express several of these "master" transcription factors as well as ESC marker such as Lin28a (Ramachandran et al., 2010). These similarities, along with abrogation of differentiation-maintaining factors such as *let-7*, necessitates the investigation about the involvement of PIFs in MG de-differentiation and proliferation.

1.9.1 c-Myc

The c-Myc is a transcription factor encoded by the cellular homologue of v-Myc, an oncogene of the avian myelocytomatosis retrovirus (Vennstrom et al., 1982). c-Myc has also been reported to be associated with several human cancers (Dang et al., 1999; Nesbit et al., 1999; Schlagbauer et al., 1999; Henriksson et al., 1996). c-Myc belongs to the same family of proteins that homes L-Myc and N-Myc (Brodeur et al., 1984; Maris, 2010; Nau et al., 1985). Substantial body of evidence have placed this proto-oncogene in crucial cross-talk points between quite a few of the ligand-receptor mediated signalling cascade as Myc can be regulated by a number of these pathways, quite often, directly (Armelin et al., 1984; Kelly et al., 1983) (Fig 1.6). To date, c-Myc has been attributed to a number of diverse functionalities such as differentiation, cell growth, cell cycle progression, cell division and even apoptosis (Cole et al., 1986; Luscher et al., 1990; Luscher et al., 1999; Prendergast et al., 1999). Discovery of the protooncogene c-

Myc and its multifaceted functional ability has in fact given rise to several different streams of related research fields in biology (Fig 1.7). Amongst all of Myc's activities, Myc's contribution in cell cycle progression, and inhibition of cellular differentiation are of most relevance here.

1.9.2 c-Myc's function in cell cycle regulation and cell division.

Several studies have established the mechanisms of Myc's action. In order to function as a transcriptional regulator or activator, to be precise, c-Myc must form a heterodimer with Myc associated protein X (Max) through its carboxy-terminal domain containing a Basic Region/Helix-Loop-Helix/Leucine Zipper (BR/H-L-H/LZ) motif (Pelengaris et al., 2002). Together, this protein complex can bind to promoters of target genes on a specific sequence which is 5'-CACGTG-3' and can transactivate their transcription through the amino-terminal Myc boxes (MBI and MBII) of c-Myc (Pelengaris et al., 2002), promoting cell cycle progression and division. Through a similar mechanism, c-Myc has been associated with upregulation of Cyclin D2 and CDK4, thus preparing the cell for G1-S progression (Steiner et al., 1995; Berns et al., 1997). Some of the interesting studies have shown c-Myc exerting its effect through chromatin modifiers. One such example is the recruitment of Transformation/Transcription domain Associate protein or TRAP, with histone acetyl-transferase (HAT) activity, to regulate the promoter of Cyclin D2 (Bouchard et al., 2001; McMahon, 1998; McMahon, 2000; Amati et al., 2001).

1.9.3 c-Myc's function in inhibition of cellular differentiation

c-Myc's ability to inhibit differentiation does not merely come up from its drive for cell cycle progression (La Roca et al., 1994; Ryan et al., 1997). Except for c-MYC, MAX has been shown to form a heterodimer with a bunch of other similar proteins such as MAD1, MAD3, MAD4, and MXI1 (Pelengaris et al., 2002). MAD/MAX complexes with properties antagonistic to that of MYC/MAX, has been reported to directly associate with SIN3 which further recruit HDAC1 and HDAC2 to target sequence so as to make the promoter sequence unavailable for MYC/MAX complexes (Ayer et al., 1995; Schreiber et al., 1995). Naturally, super-abundant c-MYC can competitively bypass this repressive action of MAD/MAX complexes by building more inducive complex with Max, thus inhibiting differentiation. Apart from that, c-Myc has been also shown to induce the transcription of Lin28, an RNA binding protein that can degrade *let-7* microRNA (Chang et al., 2009).

1.9.4 c-Myc's involvement in tissue regeneration.

Being a "master" transcription factor of supreme status, as discussed in the earlier sections, c-Myc is also one of the most studied molecules with respect to differentiation, cell cycle progression or cell division. Unfortunately, to date, its involvement in tissue regeneration has only been reported for liver and intestinal regeneration, but without any strong characterization (Arora et al., 2000; Coni et al., 1993; Ashton et al., 2010; Feng et al., 2006). It is surprising that in retina regeneration, where all these processes are of vital significance, the involvement of c-Myc is greatly underexplored. In fact, the upregulation of its transcript following a mechanical injury to the fish retina and translational regulation of c-Myc by *let-7* microRNA are the only two available information in the literature (Ramachandran et al, 2010).

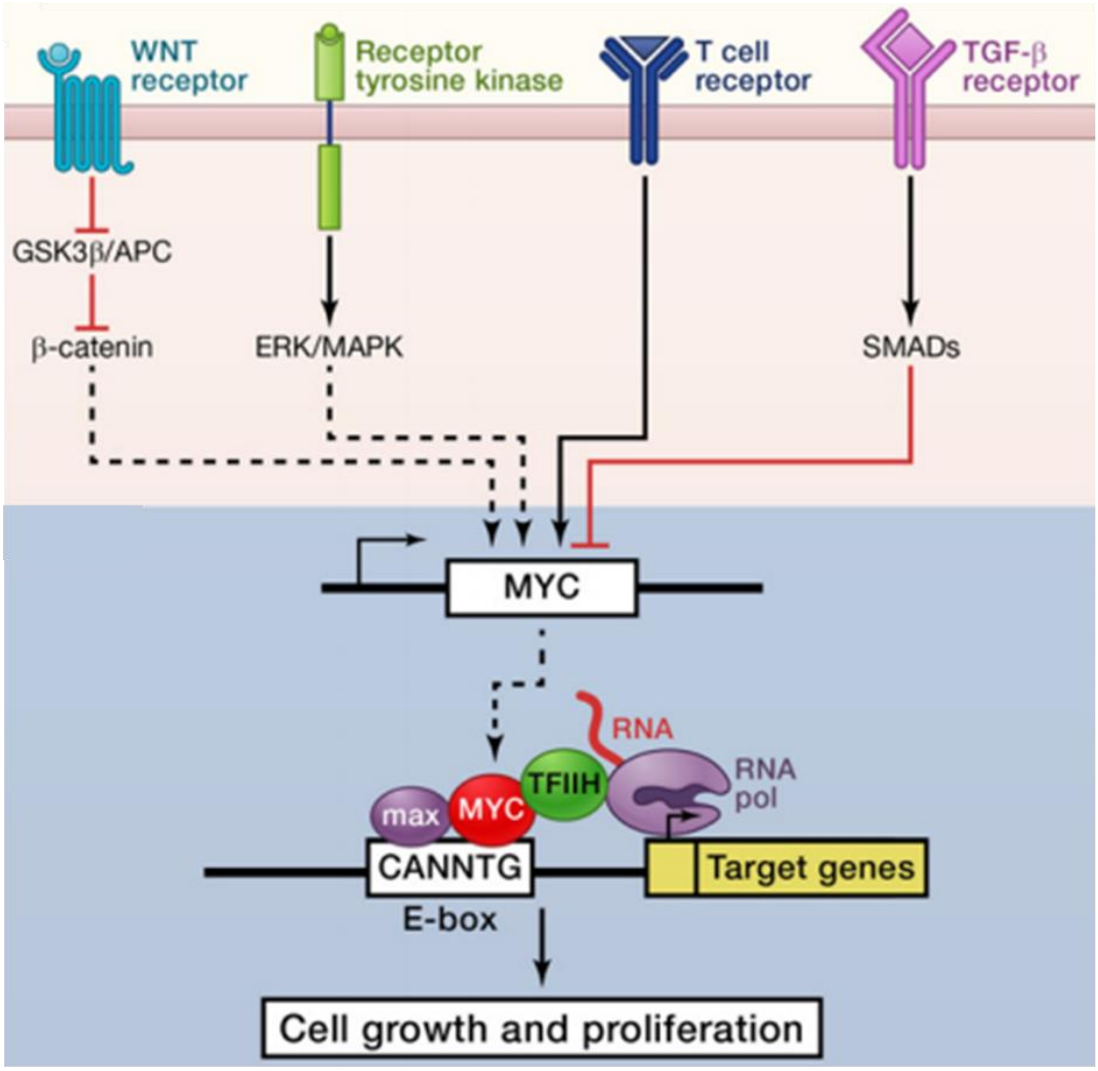


Fig 1.6: Myc as mediator of cell growth and proliferation is regulated by several signalling pathways. Taken from Dang, 2012.

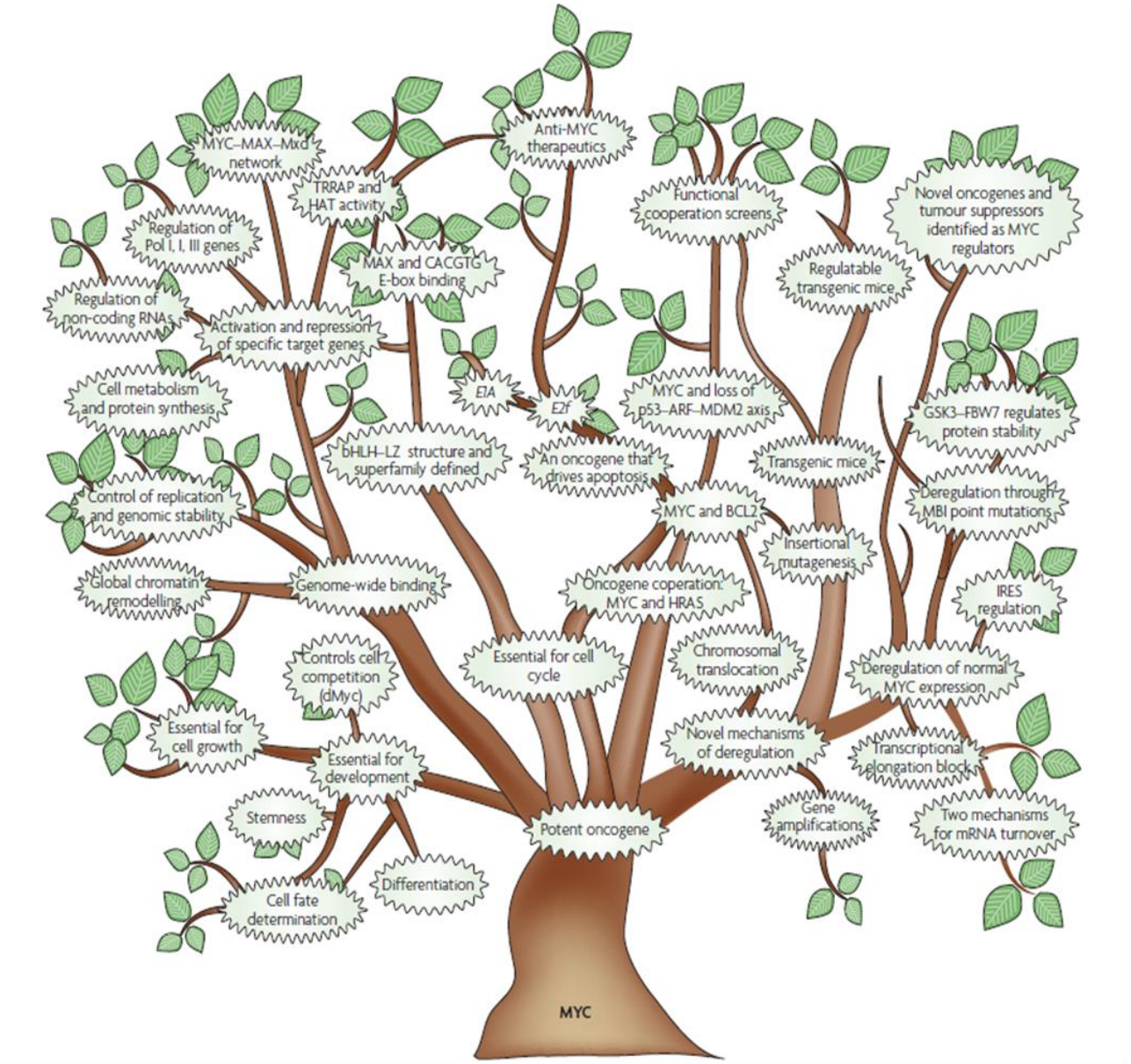


Fig 1.7: The c-Myc tree of knowledge. Various brances of research that originated from c-Myc. Taken from Meyer and Penn, 2008.

1.10 Epigenetic modifiers and their role in retina regeneration.

Epigenetics is the "study of stable genetic modifications that result in changes in gene expression and function without a corresponding alteration in DNA sequence" (Piekarz and Bates, 2009). Histone deacetylases (HDACs), Histone acetyltransferases (HAT) and DNA methyltransferases (DNMTs) are a few examples of such molecules that can regulate the epigenetics and thus are termed as epigenetic modifiers. Such molecules have long been associated with the progression of several human diseases including cancer (Egger et al., 2004; Feinberg, 2007). Recently, along with many of the genetic factors, the importance of studying the epigenetic modulators and modifiers came to prime-light with the involvement of methylation and acetylation (or deacetylation) of the genome in tissue regeneration in various model organisms (Powell et al., 2013, Tseng et al., 2011, Havasi et al., 2013).

Recent studies on regenerating zebrafish retina suggested the involvement of a basic methylation pattern in fish Muller glia rendering them capable of going through a reprogramming event. Intriguing enough, the same kind of methylation pattern was also reported to be present in mammalian retinal cells (Powell et al., 2013). This is particularly of interest as it makes the possibility of mammalian retina regeneration believable at least in terms of similarities of their epigenetic regulations. The transient transcriptional regulation of several components of Pollycomb repressor complex2 (PRC2) such as *Eed* (*embryonic ectodermal development*), *Ezh2* (*enhancer of zeste homolog 2*), and *Suz12* (*suppressor of zeste 12*) along with up-regulation of H3K27 specific de-methylases such as, *UTX* (ubiquitously transcribed tetratricopeptide repeat, X chromosome), and *Jmjd3* (*Jumonji-domain containing protein 3*) during drossophilla imaginal disc regeneration suggested the importance of studying the involvement of epigenetic factors in tissue regeneration (Katsuyama and Paro, 2011). Another important study from the recent past demonstrating the differential methylation of the CpG of

limb-specific *shh* enhancer region in *Xenopus* froglet and adults sheds much light into the importance of the epigenetic regulators during regeneration (Yakushiji et al., 2007). Along with various factors controlling the methylation status in the genome, several factors responsible for acetylation of the chromatin have also been reported in the recent past to be involved in tissue regeneration. One such example is the involvement of HBO1 and JADE1, which bind to each other to promote acetylation, in epithelial cell regeneration (Havasi et al., 2013). Compared to the involvement of methylation and factors regulating methylation in tissue regeneration, the involvement of HDACs is scarcely explored.

1.10.1 Histone deacetylases (HDACs)

Primarily known for catalysing the removal of acetyl group from Lysine residues from histone amino terminals, HDACs are enzymes, that can modify the structure of chromatin (Fig 1.9), thus begetting transcriptional regulation of several relevant genes (Wade, 2001; Ito et al., 2000; Forsberg, 2001; Bolden et al., 2006; Hyunh et al., 2017). This particular property of HDACs that enables them to globally control transcription, has initiated many surveys regarding the roles these molecules might play in a context of cellular differentiation or proliferation (Annemieke et al., 2003). Studies from not so distant past have shown another very interesting aspect of HDACs, which is to interact with several tumour suppressor genes or proto-oncogenes, thus recruiting themselves to regulate transcription of target genes (Annemieke et al., 2003; Cress and Seto, 2000; Timmermann et al., 2001). Higher organisms contain several HDACs in their genome. Till date, a total of 18 different HDACs have been reported to be

functioning in humans, which can be classified based on their structure into four major classes (Kim and Bae, 2011, Bolden et al., 2006) (Fig 1.8). Inhibition of these HDACs has also been

a very popular strategy in the quest for the development of anti-cancer drugs (Kim and Bae, 2011; Shabason, 2010; Ververis et al, 2013; Bolden et al., 2006).

1.10.2 HDACs in tissue regeneration.

Involvement of HDACs is better understood in the context of regeneration when compared to that of c-Myc, though not much. Although several studies have reported involvement of different HDACs in different tissue regeneration, all of them lack any deep investigation about their mechanism. Several HDACs such as, HDAC1, HDAC2, HDAC3, and HDAC6 have been associated with bone and other mineralized tissue regeneration involving osteoblasts, mesenchymal stem cells (MSCs) or dental-derived pluripotent stem cells (DPSCs) (Huynh et al., 2017; Paino et al., 2014; Westendorf et al., 2002; Schroeder et al., 2004; Lee et al., 2006; Razidlo et al., 2010). Another study reported that upregulation of HDACs activity is required and HDAC1 is expressed during the early phases of *Xenopus* tadpole tail regeneration (Tseng et al., 2011; Taylor and Beck, 2012). Further, the authors showed that levels of Notch1 and BMP2, two of the regeneration associated genes in tail regeneration, became aberrant. In a contrasting note, in young Mdx mice, inhibition of HDACs has been shown to facilitate regeneration (Mozetta et al., 2013). Class I HDACs have been reported to be essential for mouse renal regeneration (Tang et al., 2014). But apart from these, the most interesting is some of the HDACs' involvement in the regeneration of the nervous system. Such examples include HDAC1 downregulation during axonal growth in spinal cord injury (Chen and Shifman, 2016; Cho and Cavelli, 2014), induction of HDAC6 post spinal cord injury (Rivieccio et al., 2009), regulation of axon regeneration by HDAC5 (Cho and Cavelli, 2012). In spite of having all these informations about the involvement of HDACs in the regeneration of a wide range of tissues, their involvement in retina regeneration remains greatly underexplored.

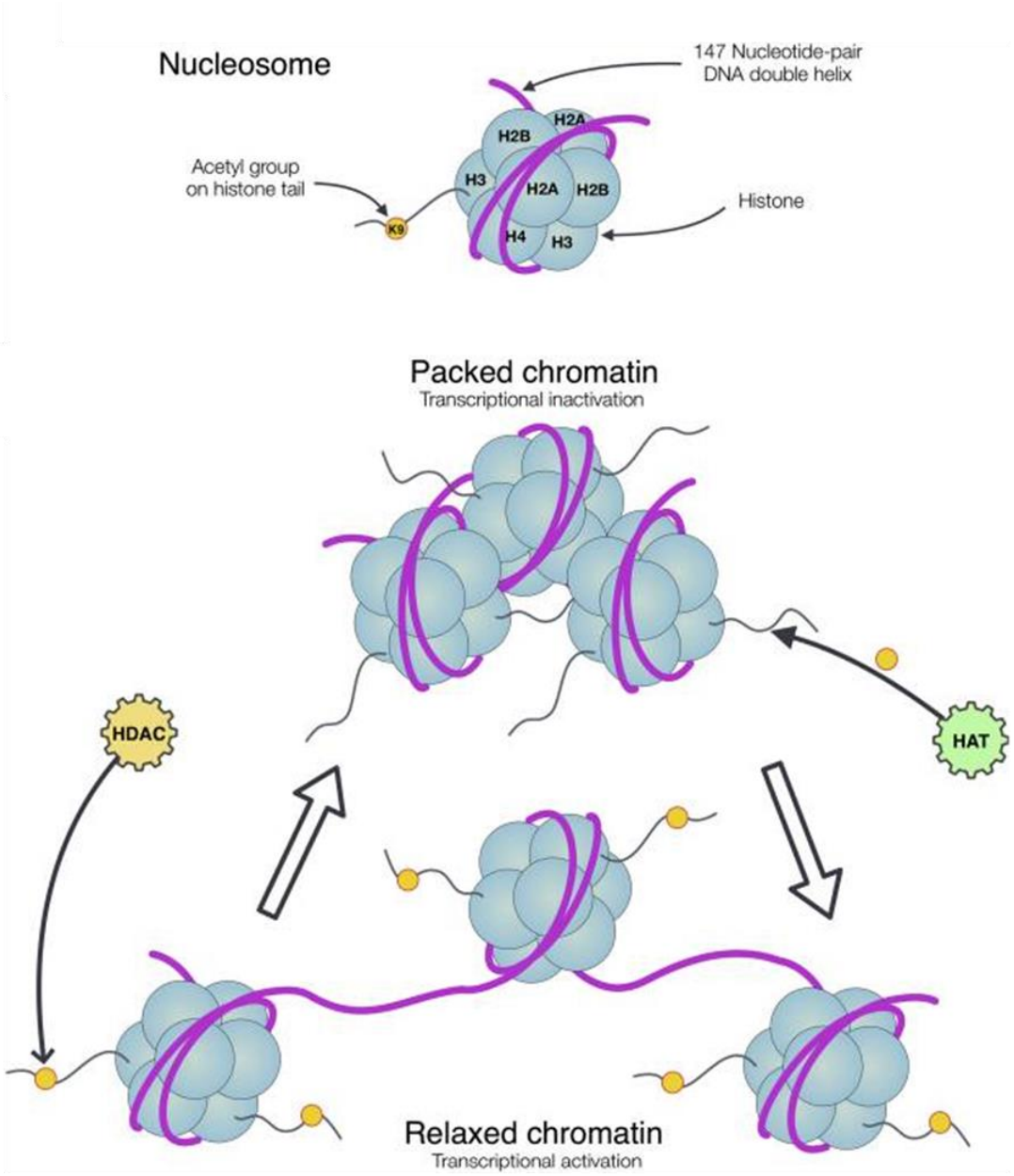


Fig 1.8: HDACSs control transcription by modulating chromatin structure. Taken from Huynh et al., 2017.

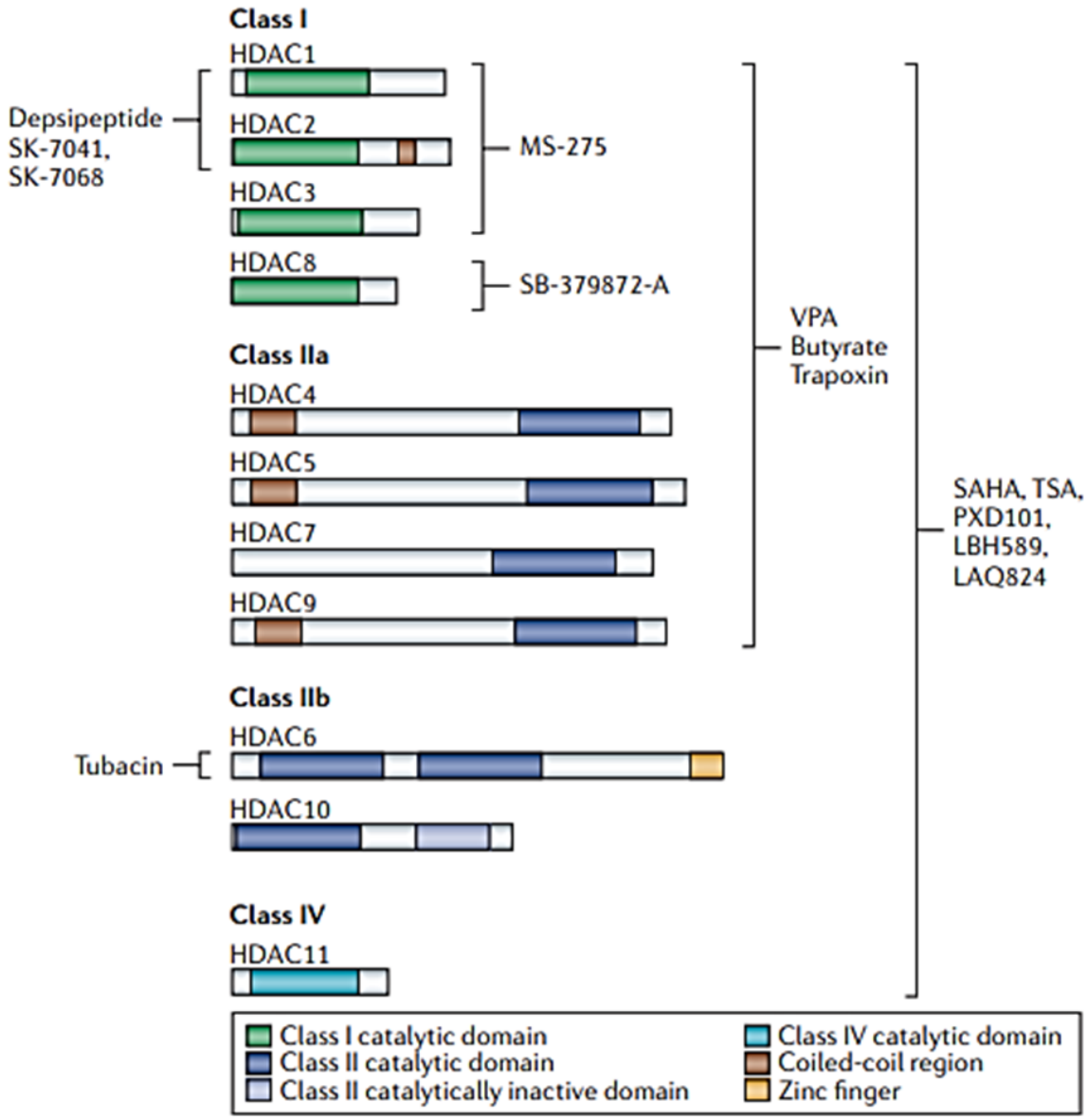


Fig 1.9: Classification of HDACs and their inhibitors. Taken from Bolden et al., 2006.

Section 2

Materials and methods

2.1 Animal maintenance.

Zebrafish were maintained at the temperature between 25-28 ^{o}C with the light/dark cycle of 14h/10h. These fish were fed with prawn feed and live artemia twice a day. The *1016tuba1a*: GFP transgenic fish used in the study was also maintained under the same conditions. For performing injection and luciferase activity assays, fish embryos were obtained by crossing wild-type fish and embryos were further maintained at 28 ^{o}C.

C57BL/6 mice strain was used in this study. They were maintained between 25-28 ^{o}C and were kept in light/dark cycle of 12h/12h with continuous food and water availability. Isoflurane was used to anesthetize these mice, followed by retinal injury using a 30-gauge needle. For harvesting the eyes, mice were euthanized by exposing them to CO_2. All the experiments were approved by the animal ethical committee of IISER Mohali.

2.2 Retinal injury and drugs delivery.

Zebrafish were anesthetized using tricaine followed by retinal injury using a 30- gauge needle. In order to give injury to the retina, the eyeball was slightly pressed from one corner of the eye with the help of a tweezer that makes the back of the opposite corner of the eye to pop out. A 30-gauge needle was inserted through the back of the eye which would result in injuring all retinal layers.

For treatments with various inhibitors of different molecules and signalling pathways, either fish were dipped in micromolar solutions of the drugs or the drugs were injected into the vitreous humor using a Hamilton syringe following injury. The following solutions were prepared to a concentration of 1mM in DMSO or water as required: Trichostatin A ([TSA]

Sigma), Sodium salt of valproic acid ([VPA] Sigma), 10058-F4 (Sigma), N-[N-(3,5-difluorophenylacetyl)-L-alanyl]-S-phenylglycine t-butyl ester ([DAPT] Sigma).

2.3 BrdU pulsing and eye or retina dissection.

Injured control or drug-treated fish were given BrdU pulsing on a day suitable and advantageous for the experiments. If not specified, most of the times, we have harvested eyes from 4dpi retinae with or without any treatment as that day demonstrate a maximum number of BrdU positive cells. For BrdU pulsing, fish were either dipped in 5mM solutions of BrdU for 4 to 5 hours or they received IP injection of 20mM BrdU 4 to 5 hours before anaesthesia.

Following BrdU pulsing, the eye was pulled out completely from the eye socket of an anesthetized fish using a tweezer and was used for further procedures like tissue fixation. For RNA isolation, eyes were pulled out without BrdU pulsing and the retinae were dissected out using sharp surgical tweezers and needles. Retina dissection for RNA isolation was carried out by placing the eye in a solution of chilled 1X PBS.

2.4 RNA isolation.

1. After harvesting the eyes, retinal dissections were performed in the 1X PBS solution.

2. Tissues were suspended in 200 µl of Trizol and were homogenized using a piston or a pipette.

3. Following homogenization, 0.2 volume of chloroform was added, and it was gently mixed 10 to 15 times by inverting the MCT upside down.

4. MCTs were centrifuged at 10,000 rcf for 10 minutes at 4 ^{o}C. The uppermost layer containing the RNA was transferred into new MCTs.

5. An equal volume of isopropanol was added to the tubes and the MCTs were kept on ice for 20 minutes or in -80 ^{o}C freezer for overnight to precipitate the RNA.

6. After precipitation, MCTs were centrifuged at 10,000 rcf for 10 minutes at 4 ^{o}C.

7. Followed by centrifugation, pellets were washed with 80% ethanol, twice.

8. Washed pellets were centrifuged at 7600 rcf for 10 minutes at 4 ^{o}C, followed by air drying and dissolution in 10-20 µl of DEPC water.

Following reagents were used in the process of extracting the whole RNA:

1. 10X Phosphate buffer saline (PBS) solution composition:

    2.76g NaH_2PO_4 x H_2O (monobasic)

    11.36g Na_2HPO4 (dibasic)

    87.6g NaCl

    1.87g KCl

    Bring up to 1 litre with DEPC water

2. Trizol (Sigma)

3. Chloroform (Sigma)

4. Isopropanol (Sigma)

5. Ethanol (Sigma)

6. DEPC water

2.5 Preparation of cDNA.

Following reagents were used for cDNA synthesis:

1. mRNA from retinal tissue/embryonic tissue

2. Thermo scientific RevertAid RT First-strand cDNA synthesis kit

3. PCR Thermocycler

After thawing and mixing the mRNA and the components of the cDNA synthesis kit, they were placed on ice. Further, the following steps were performed:

1. Following components were added into a sterile PCR tube:

Components	**Amount**
Template mRNA	up to 5μg of RNA
Oligo (dT)Primers	1μl
Random Hexamer primers	1μl
Nuclease-free water	up to 12μl

2. PCR tube was incubated at 65 ^{o}C for 5 minutes and was immediately transferred onto ice.

3. After incubating the PCR tube on ice for 2 minutes, following components were added into the mix:

Components	**Amount**
5X reaction buffer	4 μl
RiboLock RNase inhibitor	1 μl
dNTPs Mix	2 μl
RevertAid M-MuLV RT (Enzyme)	1μl

4. Components were gently mixed and centrifuged, and the following program was run on PCR thermocycler.

Temperature	**Time**
25 ^{o}C	5 minutes
42 ^{o}C	60 minutes
70 ^{o}C	5 minutes

5. The cDNA was used for performing RT-PCR (Reverse transcription-PCR) and qPCR, and the rest of it was stored at -20 ^{o}C.

2.6 Primers for cloning.

Several primers were used in this study for performing various full-length cDNA cloning or PCR. These are all listed in Appendix 1.

2.7 Genomic DNA isolation.

Amplification of the promoter elements was done using zebrafish genomic DNA. Reagents required for isolating the genomic DNA were as follows:

1. TEN buffer

Components	**Final concentration**
1M Tris-HCl (pH 8)	100mM
0.5M EDTA	1mM

2. 10% SDS solution

3. Proteinase K (10mg/ml)

4. Phenol: Chloroform: Isoamyl alcohol (PCI)

5. Chloroform

6. Ethanol

7. Ammonium acetate Buffer

8. TE buffer

Components	Final concentration
1M Tris-HCl (pH 8)	100mM
0.5M EDTA	1mM
5M NaCl	150mM

Steps taken for isolating the genomic DNA were as follows:

1. Zebrafish fin was clipped and suspended in 500 µl of TEN buffer.

2. After adding 1% v/v SDS, the fin was homogenized.

3. For degrading proteins that are present in the cell, proteinase K was added to a final concentration of 200 µg/ml and incubated for 4 hours at 37 ^{o}C.

4. Following proteinase K treatment, an equal volume of PCI was added into it and mixed gently by inverting the MCT upside down.

5. The aqueous phase was transferred into a fresh MCT after centrifuging the above MCT at 6000rpm for 5 min at room temperature.

6. An equal volume of chloroform was mixed into the aqueous phase, which was further centrifuged at 12000 rpm for 5 min at room temperature for collecting the upper phase.

7. The upper phase was transferred into the fresh MCT, and 1/10$_{th}$ volume of ammonium acetate buffer was added into it.

8. Further, 2 volume of isopropanol was added and mixed into it by inverting the MCT gently.

9. With the help of needle or tip, the mesh was taken out in a fresh MCT and washed with 70% ethanol at 12000 rpm for 10 min.

10. Following 70% ethanol wash, the pellet was air dried and dissolved in TE buffer.

2.8 PCR (Polymerase chain reaction) Amplification.

Following reagents were added to a PCR tube:

Components	Amount
20X reaction buffer	0.5µl
2.5mM dNTPs	1µl
10 pM Forward primer	0.4µl
10pM Reverse primers	0.4µl
Template	10ng
Taq polymerase	0.5µl
MQ Water	upto10µl

PCR tube containing these reagents were subjected to the following thermo-cycler cycling conditions:

Steps	Temperature	Time
Initial denaturation	93 ^{o}C	1min
Cycling (35-55 cycles)	93 ^{o}C	20sec
	55-62 ^{o}C	1min
	68 ^{o}C	1min/kb
Final extension	72 ^{o}C	5min
storage	4 ^{o}C	Infinite hold

Further, the final PCR product was run on agarose gel electrophoresis for amplification.

All PCR reactions for cloning of full length cDNA and SDMs were carried out with an annealing temperature of 58 ^{o}C with an exception of cDNAs for *insm1a*, *myca* and *mycb* which were carried out in 56 ^{o}C.

2.9 Quantitative PCR (qPCR).

The qPCR was performed in Eppendorf Realplex4 (Mastercycler) Epgradient S machine. Following components were added to each PCR plate well:

Components	**Amount**
KOD SYBR qPCR	
Master Mix (Pure gene)	2.5µl
10 pM Forward primer	0.1µl
10pM Reverse primer	0.1µl
Template 0.25µl	(5ng)
MQ Water Final volume to	5µl

A protocol was run on Eppendorf Realplex4 (Mastercycler) Epgradient S machine, which was followed by analysing the data on excel sheet using the ΔΔCT method. All reactions were done at an annealing temperature of 60 ^{o}C.

2.10 Cloning of full-length cDNAs and promoters for plasmid construction.

The cDNA which was prepared from the total RNA isolated from retinae was used to amplify several cDNAs needed for the study, like *myca*, *mycb*, *ascl1a*, *insm1a*, *lin28a*, *her4.1*, and several *hdac*s. These cDNAs were cloned in a pCS2+ plasmid under the *cmv* promoter by using restriction enzymes or in pCRII-TOPO by using a kit (Invitrogen) for their future use in *in vitro* transcription reaction for making RNA probes which were further to be used in mRNA *in situ* hybridization.

Genes like *ascl1a*, *mycb*, *lin28a*, *hdac1*, *hdac3*, *hdac4*, *hdac5*, *hdac6*, *hdac9* and *nicd* were cloned from complementary DNA amplified from zebrafish retinal RNA at 4 dpi using primer pairs Bam-Ascl1a FL-F and Xho-Ascl1a FL-R (~0.6 kb); Bam-*mycb*-F and Xba-*mycb*-R (~1.2 kb); Bam-*lin28a* FL-F and Xho-*lin28a* FL-R (~0.6 kb). The full details of these primers are listed in table 2.1. Post-digested PCR amplicons were cloned into their respective enzyme sites in pCS2+ plasmid to obtain *CMV:ascl1a*, *CMV:mycb*, and *CMV:lin28a* plasmid constructs.

All *hdac* genes, *hdac1* (~0.8kb), *hdac3* (~1.1kb), *hdac4* (~1.1kb), *hdac5* (~1kb), *hdac6* (~1kb), and *hdac9* (~1kb) were amplified by PCR using primers listed in table 2.1 and cloned in pCRII-TOPO vector using TOPO-TA cloning kit (Invitrogen, catalogue number 45-0640).

The *nicd* mRNA was prepared by *in vitro* transcription of PCR product, specific to *nicd*, using primer pairs T7-HSP M-F and Sv40-R (~2kb) from a clone of *nicd* driven by Hsp70 promoter. This clone, in turn, was made in pTAL plasmid vector by digesting an amplicon of *nicd* obtained using PCR primers Hind2X-flag-NICD-F and MluI NICD-R. The details of these primers are given in table 2.1.

For the confirmation of MO activity, an adaptor having respective MO targeted region for *hdac1* and *her4.1* was cloned in pEGFP-N1 at BamHI and HindIII restriction sites, which append in-frame to GFP reporter.

The promoter of *her4.1*, *lin28a*, *mycb*, and *ascl1a* were amplified from zebrafish genomic DNA using primer pairs listed in table 2.1. The digested PCR amplicons were cloned into a pEL luciferase expression vector to create *gfp-luciferase* reporter constructs driven by respective promoters.

2.11 Plasmid isolation.

1. 5mL of culture was centrifuged at 13,500rpm for 2 min at RT.

2. The supernatant was discarded and the pellet was dissolved in 100µL of autoclaved MQ.

3. 100µL of freshly prepared lysis buffer was added and gently tapped.

    1mL of Lysis Buffer was prepared freshly by adding 50µL of 20% SDS solution, 20µL of 0.5M EDTA and 20µL of 10N NaOH in 910µL of MilliQ-water.

4. The samples were boiled at 100°C for 2 minutes (until the solution becomes clear).

5. 50µL of 1M MgCl2 was added. Tapped and kept on ice for 2 minutes.

6. Centrifuge at 13,00rpm for 2 min at RT.

7. 50µL of 3M Potassium Acetate buffer was added and tapped immediately.

    60mL of 5M Potassium Acetate, 11.5mL glacial acetic acid, and 28.5mL H_2O were mixed to get 100ml of Potassium Acetate Buffer. It was Stored at 4°C).

8. Centrifuged at 10,000rpm for 2 min at RT.

9. The supernatant was transferred to another MCT containing 600µL of Isopropanol.

10. Kept on ice for 5 min.

11. Centrifuged at 13,000rpm for 2min at RT.

12. 70% ethanol wash was given and the pellet was dried completely.

13. Pellet was dissolved in 50µL of autoclaved MQ.

14. Stored at -20°C.

2.12 Ultra-competent cells preparation.

1. 5mL primary culture of *E.coli* DH5α strain was incubated at 37°C overnight

2. Secondary culture (1% of primary culture) was incubated at 18°C till OD_{600} reached the value of 0.6-0.8.

3. The culture was kept on ice for 10-15 minutes.

4. Centrifuged at 2500 g for 10 minutes at 4°C.

5. Pellet was re-suspended in 80 mL of TB buffer. [TB Buffer: 10mM of PIPES + 15mM of $CaCl_2.2H_2O$ + 250mM of KCl + 55mM of $MnCl_2.4H_2O$, pH = 6.8 is set using KOH.].

6. Kept on ice for 10 minutes.

7. Centrifuged at 2500 x g for 10 minutes at 4°C.

8. Pellet re-suspended in 20mL of TB buffer.

9. DMSO is added to a final concentration of 7% (1.4mL+18.6mL of TB buffer).

10. Kept it on ice for 10 minutes.

11. 100µL volumes were aliquoted and stored at -80°C.

2.13 Restriction digestion.

1. Following reagents were added to an MCT:

Components	Amount
DNA	5µg
Restriction digestion buffer (10X)	3µl
Restriction digestion Enzyme	1µl
MQ Water Final volume to	30µl

2. The reaction was mixed and given a short spin/briefly centrifuged.

4. Further, the reaction was incubated at 37 ^{o}C for 3-4 hours or overnight depending on the need.

5. The digested product was run on an agarose gel and isolated using a Nucleopore gel extraction kit.

2.14 The *in vitro* transcription reaction of making RNA probe.

1. Following reagents were added to an MCT:

Components	Amount
DNA	0.5-1µg
Transcription buffer	(10X) 1µl
DIG/Fluorescein RNA labelling mix Solution	0.5µl
RNA polymerase (SP6/T7/T3)	0.5µl
DEPC Water	up to 10µl

2. The reaction was mixed and briefly centrifuged.

3. The reaction was further incubated at 37 ^{o}C for 4 hours.

4. The reaction was stopped by adding 1ul of 0.5M Tris EDTA.

5. Precipitation was performed by adding 1µl of 5M LiCl, 0.5µl of 10mg/ml glycogen and 18µl of 100% ethanol, further this reaction was mixed and kept overnight at -80 ^{o}C.

6. Next day, the reaction mix was centrifuged for 30 minutes at 14,000rpm.

7. Pellet was washed twice with 80% ethanol and centrifuged at 14,000rpm for 15minutes.

8. Further, RNA pellet was air dried and dissolved in 20µl of DEPC water.

2.15 Site-Directed Mutagenesis.

A typical PCR amplification for SDM contained approximately 50 ng of double-stranded DNA, 20 pM of each of the two oligonucleotide primers, 2 µl of 10 mM dNTP (deoxynucleotide triphosphate) mix, 0.2 µl (1 unit) of proofreading thermo polymerase (PCR extender system, 5 Prime) and 2.5 µl of 10× reaction buffer in a reaction volume of 25 µl. Amplification conditions were: 94 °C for 5 min without enzyme, 2 min on ice; Polymerase was then added followed by 30 cycles of denaturation at 93 °C for 30 sec, annealing at 58 °C for 1 min and extension at 68 °C for 10 min. Following PCR, the product was treated with DpnI and mutagenized DNA was purified and electroporated into XL1-Blue cells. Transformed cells were selected on LB (Luria bertani) agar plates containing 50 µl ml^{-1} kanamycin and colonies harbouring mutant sequences were identified by PCR and confirmed by DNA sequencing. water.

2.16 Manual gel extraction.

1. Desired gene band was cut from agarose gel and collected in a MCT containing little pieces of aluminum foil. A small hole was made at the base of MCT using a needle.

2. This MCT was then placed inside another MCT and both of them are taped together.

3. The entire setup was then centrifuged at 10,000rpm for 10 minutes at RT.

4. Equal volume of PCI (Phenol:Chloroform:Isoamyl alcohol) was added into the flow through and mixed properly.

5. Centrifuged at 10,000rpm for 10 minutes at RT.

6. Aqueous layer was carefully taken out and collected in another MCT.

7. Equal amount of Chloroform was added and mixed thoroughly.

8. Centrifuged at 10,000rpm for 10 minutes at RT.

9. Upper aqueous layer was carefully pipetted out and collected in another MCT.

10. Half volume of 7N Ammonium Acetate and twice the volume of Isopropanol was added and mixed properly.

11. The sample was kept at -80°C overnight.

12. Centrifuged at 15,000rpm for 10 minutes at 4°C.

13. 70% ethanol wash (500µL) was given and the pellet was dried completely.

14. The pellet was then eluted in DEPC MQ and checked on 1% agarose gel.

15. Stored at -20°C.

2.17 Morpholino electroporation, mRNA transfection, and knockdown-rescue.

Lissamine-tagged MOs (Gene Tools) of approximately 0.5µl (0.5 to 1.0 mM) volume were injected at the time of injury using a Hamilton syringe of 2µl volume capacity. MO delivery to cells was accomplished through electroporation by placing the positive electrode on the anaesthetised fish's eye that received the MO and by giving 5 electric pulses of 70V with duration of 50 milli second each (Fig 2.1).

Morpholinos used in this study are:

hdac1 MO, 5′-TGTTCCTTGAGAACTCAGCGCCATT- 3′

2- *hdac1* MO, 5'-TTACCCTCCAATTACAGCCTGCGCC-3'

her4.1 MO, 5'-TTGATCCAGTGATTGTAGGAGTCAT-3'

insm1a MO, 5′-ATGCCCCCGGCAAATCCGCATCTCA-3′

myca MO, 5′-AACTCGCACTCACCAGCATTTTGAC-3'

2-*myca* MO, 5′-TTTAACGAATGCCGTTCCAGAATTG -3'

mycb MO, 5′- CCATACTTGAATTCAGCGGCATGGT -3'

2-*mycb* MO, 5′-GAGTGCCGTAGCCGTGGTAAAAGCT-3'

Transfection mixture contained two solutions constituted in equal volumes. (A) 4-5 µg of mRNA mixed with HBSS (Hanks balanced salt solution), (B) Lipofectamine messenger max reagent (Invitrogen, Catalogue number LMRNA001) mixed with HBSS. Both the solutions were allowed to stand at room temperature for 10 minutes and then mixed dropwise followed

by 30 minutes incubation at room temperature. The resultant solution was mixed with morpholino in equal proportion, and 0.5µl of this mixture was used for injection in zebrafish retina followed by electroporation as described earlier.

In vivo rescue experiments were designed for testing the specificity of MO antisense oligos. We did the transfection of zebrafish retina using gene-specific mRNA alongside the MO targeting 5' UTR region of concerned genes or control MO. For confirming the efficient mRNA transfection, GFP mRNA was also delivered by transfection in the control retina, whereas GFP fusion with gene mRNA was used in other sets.

2.18 Promoter activity assay.

Single-cell zebrafish embryos were injected with a total volume of ~1nl solution containing 0.02 pg of *Renilla* luciferase mRNA (normalization), 5 pg of *promoter:gfp-luciferase* vector and 5-10 pg of mRNA or MO as mentioned in the experiments. In the case of inhibitor treatment, embryos were dipped in micromolar solutions of the inhibitors. To further assure the consistency of results, a master mix was made for daily injections, and ~ 300 embryos were injected at the single cell stage. 24 hours later, the embryos were divided into three groups (~ 70 embryos/group) and lysed for promoter activity assay by using dual luciferase reporter assay kit (Promega, catalogue number E1910) and a luminometer.

2.19 Chromatin immunoprecipitation (ChIP) assay.

Day 1 (and possibly 2)

1. Transfer around 15-20 injured retinae into a micro-centrifuge tube with 250 ml PBS containing protease inhibitors (from 100X cocktail stock) and PMSF (from 100X stock, 100mM).
    a. PMSF and protease cocktail should be added just before harvesting
2. Let the retinae sink to the bottom of the tube and remove the PBS with a pipette, leaving 0.5ml buffer on tip of the retinae.
3. Homogenize the retinae with a pestle not to have any tissue clumps.
4. Immediately cross-link the cells by adding formaldehyde to 1% vol/vol final concentration, vortex, and incubate for 10 min at RT with spinning.
5. Add glycine to 0.125M final concentration to quench the formaldehyde. Vortex, place the tube on ice, and incubate for 5min. From this step onward, handling of chromatin is carried out on ice.
6. Centrifuge the tube at 1725rpm for 10min at 4C to sediment the cells and carefully remove and discard the supernatant with a 1ml pipette with the tip cut.
7. Add 500ul PBS/PMSF/protease cocktail solution and resuspend the cells by vertexing. Centrifuge at 1725rpm for 10min at 4C and discard the supernatant.
8. Add another 500ul PBS/PMSF/protease cocktail solution, centrifuge at 1725rpm for 5min at 4C.
9. Remove all of the supernatant. The cells can be stored as a dry pellet @ -80C for several weeks.

10. Add lysis buffer (nuclei lysis buffer) to a total volume of 600ul. Resuspend the pellet by rotation by hand. Be careful not to make any bubbles. Starting with a frozen or a fresh cross-linked cell pellet has no noticeable influence on ChIP efficiency or results.
11. Sonicate on ice each tube for 8X 30 s with 30s pauses between sonication rounds. This is performed with a power of 4.3.
12. Centrifuge at maximum speed for 10min at 4C. Remove the supernatant and place in a clean 1.5ml tube.
13. Use 2ul of the supernatant to measure the concentration on a nanodrop. The concentration for my samples has a A260 ~ 30U and 1000ng/ul nucleic acid concentration.
    a. Use lysis buffer with all of its additives as a blank
14. Dilute samples in IP Dilution buffer/PMSF/protease cocktail solution to 250ng/ul nucleic acid concentration. Aliquot in samples of 250 ul.
15. Mix well and spin down. The diluted chromatin can be stored for several months @ -80C.
16. Add 250ul of ChIP dilution buffer/PMSF/protease cocktail solution to a 250ul diluted chromatin sample.
    a. At this step you can remove a small sample (around 5% of total volume) and store @ -80C as an input sample.
17. Preclear the sample: Add 10ul (.3mg, binding capacity 2.4ug of human IgG) of Protein Agarose G (dynabeads) slurry to the sample and incubate for 2hrs at 4C with agitation by using a rotator in the cold room.
18. Spin the tubes 1-2s to bring down any solution trapped in the lid and capture the beads by placing the tubes in a chilled magnetic rack.

19. Collect the supernatant in a fresh tube and add antibody. Incubate over-night at 4C with rotation.

Day 2

1. Add 20ul (0.6mg, binding capacity of 4.8 ug of human IgG) of Protein Agarose G (dynabeads). Rotate for 2hrs in the cold room.
    a. Prolonged incubation may enhance background.
2. Centrifuge the tubes for 1s and capture the immune complexes by placing the tubes in the chilled magnetic rack.
3. Discard the supernatant, add 500ul of ice-cold IP dilution buffer/PMSF/protease cocktail. Resuspend the complexes by gentle manual agitation and place the tubes on a rotator at 40rpm for 4min at 4C.
4. Repeat step 3.
5. Discard the supernatant, and wash in wash buffer/PMSF/protease cocktail. Resuspend the complexes by gentle manual agitation and place the tubes on a rotator at 40rpm for 4min at 4C.
6. Repeat step 5.
7. Remove the supernatant, add 500 ul of TE buffer, and incubate on rotator for 4min.
8. Remove supernatant.

ChIP Elution (with ProteinaseK treatment)

1. To each chip reaction, add 150ul of ChIP elution buffer: (20mM Tris-HCl pH 7.5, 5mM EDTA, 50mM NaCL, 1%SDS, 50ug/mL proteinase K. SDS and proteinase K should be added just before use). Incubate on thermomixer at 1300rpm for 2 hrs.
2. Spin down, capture the beads in the magnetic rack, and transfer the eluate from the tube to a clean 1.5ml tube.
3. Add 150 ul ChIP elution buffer to the beads. Incubate for 15min on thermomixer as in Step 1.
4. Spin down, capture beads in the magnetic rack, remove the eluate, and pool it with the first eluate from step 2.
5. To the pooled eluate (300ul total volume), add 200ul of ChIP elution buffer.
6. Add proteinase K to 2mg/ml of the input chromatin sample and incubate @ 68C, 1300 rpm, on a thermomixer for 2hrs.
7. Add 500 ul of phenol:chloroform:isoamyl, vortex and centrifuge @ max for 5min. Transfer aqueous phase to a clean 1.5ml tube. Repeat with 500 ul chloroform:isoamyl
8. To the aqueous phase, add 1ul of glycerol, NaAC, EtoH to precipitate.
9. Finish precepitation procedure.

<u>Cell lysis Buffer</u>

- 10mM Tris-HCl (pH 7.5)
- 10mM NaCl
- 0.5% NP-40

<u>Nuclei Lysis Buffer</u>

- 50mM Tis-HCl (pH 7.5)
- 10mM EDTA
- 1% SDS

IP dilution Buffer

- 16.7mM Tis-HCl (pH 7.5)
- 167mM NaCl
- 1.2mM EDTA
- 0.01% SDS
- 1.1% Triton X-100

IP Wash Buffer

- 100mM Tis-HCl (pH 8)
- 500mM LiCl2
- 2mM EDTA
- 1% NP40

Use pH7.5 for Protein G and pH8.0 for protein A. Add protease inhibitors to all lysis buffers before use.

2.20 Tissue fixation and cryo-sectioning.

1. The zebrafish eyes were dissected in 4% paraformaldehyde solution for removing the lens.

2. Eyes were fixed overnight at 4 ^{o}C in 4% paraformaldehyde solution.

3. Fixed eyes were washed with a series of sucrose solutions at varying concentrations for 45 minutes. Series of sucrose solutions used were as follows:

5% Sucrose (1000µl)

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

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

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

20% Sucrose (1000µl)

4. Followed by washing, eyes were further washed with the mixture of 1000µl of 20% Sucrose and 500 µl of OCT (2:1) for 30 minutes.

5. Eyes were embedded into the blocks of OCT and were frozen and stored in -80 ^{o}C.

6. Frozen blocks were taken out from the freezer and using a Lyca cryosectioning machine, 8-10µ retinal sections were taken on poly-lysine coated slides.

2.21 mRNA *in situ* hybridization.

DAY 1:

Reagents used:

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

The 20X SSC solution was prepared by dissolving 87.7g of NaCl in 350ml of DEPC water. Further 44.12g of sodium citrate was dissolved in it and volume was made up to 500ml.

6. Proteinase K buffer

   25ml Tris-HCl

   25ml 0.5M EDTA

   Bring the volume up to 250ml with DEPC water

7. Proteinase K enzyme (10mg/ml)

8. TEA Solution

   Add 9.3g Triethanolamine (TEA) to 490ml water. Add 173µl of 10N NaOH solution to bring the pH to 8.0. Finally, bring the volume up to 500ml with DEPC water.

9. TEN solution

   5ml 1.0M Tris-HCl (pH 7.5)

   30ml 5M NaCl

   1ml 0.5M EDTA

10. Hybridization Solution

    3.6ml TEN solution

    25ml 100% Formamide

    10ml 50% Dextran sulphate

    5ml 10% RMB blocker

    6.4ml DEPC water

    Store at -20^{o}C

Steps taken:

1. Slides were hydrated in a series of ethanol solution for 1 minute each:

100% Ethanol

100% Ethanol

95% Ethanol

70% Ethanol

50% Ethanol

2. Ethanol was washed off by keeping the slides in 2X SSC solution for 1 minute.

3. Further, the slides were incubated in pre-warmed Proteinase K Solution with 10mg/ml Proteinase K (160µl).

4. Slides were rinsed briefly in DEPC water at room temperature.

5. Slides were rinsed with 0.1M TEA solution for 3 minutes.

6. Slides were treated with TEA solution (with 130µl of acetic anhydride) for 10 minutes.

7. Slides were dehydrated with a series of 1 minute each SSC and Ethanol washes:

2X SSC solution

50% Ethanol

70% Ethanol

95% Ethanol

100% Ethanol

100% Ethanol

8. Slides were air dried for at least 1 hour.

9. The hybridization solution was pre-warmed at 56 ^{o}C.

10. For preparing the probe mixture, the probe was boiled for 10 minutes at 100 ^{o}C with 64µl of water. The mixture was immediately kept on ice and hybridization solution was added into it (with a total volume of 300µl for each slide).

11. Added 300µl of probe solution to each of the slides and it was coverslipped using Hybrislips.

12. Slides were kept overnight at 56 ^{o}C in a humidified chamber, which was damped using 50% Formamide/5X SSC.

DAY 2:

Reagents used:

1. 2X SSC solution
2. 50% Formamide/2X SSC solution
3. RNase buffer

   RNase Buffer solution was made by mixing 5ml of 5M NaCl, 500µl of 1M Tris (pH7.5) and 100µl of 0.5M EDTA in DEPC water to bring the final volume to 50ml.

4. 5X Maleate buffer

   Maleate buffer was prepared by dissolving 58g of Maleic acid in 850ml of MQ water, and then pH of this solution was adjusted to 7.5 using NaOH pellets. Further 43.8g of NaCl was dissolved into the solution and volume was made up to 1L using MQ water.

5. 1X Maleate/0.05% Triton/ 1% RMB blocker solution

   This solution was made by mixing 2ml of 5X Maleate stock solution, 5µl of Triton X-100 and 1ml of 10% RMB blocker. 3ml aliquots were made and frozen at -20^{o}C.

Steps taken:

1. Slides along with cover-slips were soaked in 2X SSC solution for 20 min at room temperature on a shaker table.
2. Hybrislips were gently removed, and slides were soaked in pre-warmed 50% Formamide/2X SSC solution for 30 min at 65 ^{o}C. The Coplin jar was gently agitated for the first 5min.

3. Slides were rinsed twice, using 2X SSC solution at 37^{o}C for 10 min each.

4. RNase A (100µl of 10mg/ml) was added into RNase buffer and slides were incubated in this solution for 30 min at 37^{o}C.

5. Slides were washed using RNase buffer for 30 min at 65^{o}C.

6. Slides were incubated with 1X Maleate/0.05% Triton/ 1% RMB blocker solution for 2-3 hours at room temperature.

7. Following incubation, slides were washed twice with 1X Maleate buffer for 5min each at room temperature.

8. After washing, slides were incubated overnight with anti-DIG/anti-FL antibody, which was diluted at 1:2500 dilution in 1X Maleate/0.05% Triton/1% RMB blocker solution.

DAY 3:

Reagents used:

1. 1X Maleate buffer

2. Genius buffer

The genius buffer was made by dissolving 5ml of 1M Tris-HCl (pH9.5), 1ml of 5M NaCl and 5ml of $MgCl_2$ in MQ water to make the volume up to 50ml.

3. NBT/BCIP solution, which was mixed with Genius buffer at 1:50 dilution.

Steps taken:

1. Slides were washed twice with 1x Maleate buffer for 5 min each time.

2. Slides were washed twice with Genius buffer for 5 min each time.

3. Slides were incubated with NBT/BCIP solution in the dark for colour reaction.

4. Signals were detected under the microscope.

Fluorescence mRNA *in situ* hybridization was also performed in the same manner as above; however, on the 2nd day, a tyramide reaction was performed instead of NBT/BCIP color reaction.

2.22 Immuno-fluorescence study.

DAY 1:

Reagents used:

1. 1X PBS (diluted for 10X PBS)

    10X Phosphate buffer saline (PBS) solution composition:

    2.76g $NaH_2PO_4 \times H_2O$ (monobasic)

    11.36g Na_2HPO_4 (dibasic)

    87.6g NaCl

    1.87g KCl

    Bring up to 1 litre with DEPC water

2. 4% Paraformaldehyde (Sigma) solution (made in 1X Phosphate buffer)
3. 2N HCl
4. 0.1M Sodium borate solution (pH8.5)
5. 3% Bovine serum albumin (BSA) in 1X PBST (PBS with 0.1% Triton X)
6. 1% BSA in 1X PBST (1X PBS with 0.1% Triton X)
7. A primary antibody of choice

Steps taken:

1. Slides were washed thrice using 1X PBS, 10 min each.

2. Retinal sections were fixed using 4% PFA solution for 20 min (This step is conducted in the case of MO bearing retinal sections).

3. Slides were treated with 2N HCl (pre-heated at 37 ^{o}C) for 20 min.

4. HCl solution was washed away and neutralized using 0.1M Sodium borate solution. Slides were washed twice with 0.1M Sodium borate solution for 10 min each.

5. Blocking of the retinal sections was carried out by incubating the slides with 3% BSA-PBST solution for 30 min at room temperature.

6. Following blockade, slides were overlaid with primary antibody (in most of the cases the antibody dilution was 1:500, i.e., 1 µl of antibody in 500 µl of 1% BSA-PBST solution), for overnight at 4 ^{o}C.

DAY 2:

Reagents used:

1. 1X PBST
2. Secondary antibody
3. DABCO

Steps taken:

1. Slides were washed thrice with 1X PBST solution for 10 min each.

2. Slides were overlaid with 500µl of the secondary antibody of choice (in most of the cases the secondary antibody dilution was 1:1000, i.e., 1µl of antibody in 500µl of 1% BSA-PBST solution) for 3 hours at room temperature.

3. Following incubation, the slides were washed three times with 1X PBST for 10 min each.

4. Further, the slides were again washed thrice with MQ water for 10 min each.

5. Slides were dried for 30min at room temperature, following which they were mounted using DABCO and stored at -20 ^{o}C.

2.23 Western blotting.

Sample preparation:

1. Retinas were dissected out and immersed in 2X Laemmli buffer.

Components	**Amount**
10% SDS	4ml
Glycerol	2ml
1M Tris-HCl (pH6.8)	1.2ml
Bromophenol blue	0.002gm
MQ water	up to 10ml

2. Samples were vigorously homogenized using a piston.

3. Following homogenizing samples were vortexed briefly and kept on ice intermittently for 20 min.

4. Samples were boiled at 100^{o}C for 10 min and stored at -80 ^{o}C until they were used for performing western blotting experiments.

Following reagents are required for performing Western blotting:

Reagents and tools required

1. Western blotting gel apparatus

2. Resolving buffer (4X)

For preparing this, 18.7g of Tris base was dissolved in 85ml of water, and pH was adjusted to 8.8 with HCl. The final volume was adjusted to 100ml using MQ water.

3. The composition of the 12% resolving gel

Components	**Amount**
Resolving buffer	2.5ml
30% Acrylamide	4ml
MQ water	3.3ml
10% SDS	100µl
Ammonium persulfate	100µl
TEMED	6µl

4. Stacking Buffer (1X)

For preparing this, 12.08g of Tris base was dissolved in 85ml of water, and pH was adjusted to 6.8 with HCl. The final volume was adjusted to 100ml using MQ water.

5. The composition of stacking gel

6. Composition of running buffer (10X) (pH 8.3)

Components	**Amount**
Tris base	30g
Glycine	144g
SDS	10g
MQ water	up to 1000ml

7. PVDF membrane

8. Skimmed milk

9. PBST (PBS with 0.1% TWEEN20)

Steps taken:

1. The resolving gel was made between the glass plates of the assembly.

2. Once resolving gel was solidified, stacking gel was made over it and a gel comb was placed for forming the wells.

3. Once stacking gel got solidified, the comb was removed, and wells were washed using MQ water. Following which the gel assembly was placed in a tank carrying 1X running buffer.

4. Samples were loaded into the wells, and SDS gel electrophoresis was performed for 3 hours at 60V.

5. Protein was transferred from the SDS-Gels to PVDF membranes in the presence of transfer buffer for 1 hour.

6. Once the protein is fully transferred, blots were blocked with 10% skimmed milk-PBST for 1 hour.

7. After blocking, the blots were washed three times using PBST for 10 min each.

8. Blots were incubated overnight with primary antibody at 4 ^{o}C.

9. Next day, the blots were washed thrice with PBST for 10 min each.

10. Blots were incubated with secondary antibody for 1 hour at room temperature.

11. After antibody incubation, blots were washed thrice with PBST for 10 min each.

12. Blots were developed using ImageQuant LAS4000.

Molecular weight of Myca/b: 55 kDa, Ascl1a: 25 kDa, Hdac1: 55 kDa.

2.24 TUNEL assay.

The *in situ* cell death detection kit (Roche) was used for performing terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The steps taken are as follows:

1. Slides were washed twice with 1X PBS for 10 min each.

2. Followed by washing, retinal sections were permeabilized with 1ml of prewarmed trypsin at 37^{o}C for 15 min.

3. Slides were overlaid with the mixture of 45µl of label solution and 5µl of enzyme solution and cover-slipped. This reaction was carried out for 1 hour at 37^{o}C in a humidified chamber in the dark.

4. Slides were washed twice with 1X PBS for 10 min each and were analysed under the microscope for TUNEL+ cells.

2.25 Fluorescence assisted cell sorting (FACS).

1. Retinas were dissected out from the eyes and suspended in 500µl of L15 media.

2. After adding hyaluronidase (1mg/ml), it was incubated for 15 min at room temperature.

3. Hyaluronidase was washed away using L15 media twice and spinning it at 1000rpm for 1 min each.

4. After washing, retinal tissue was suspended in 500µl of L15 media.

5. Tissues were further treated with trypsin (0.01% final concentration in L15 media) for 15 min with intermittent pipetting.

6. Finally, BD FACS ARIA instrument was used for sorting the GFP+ve and GFP-ve cells.

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

Nikon N*i*-E fluorescence microscope assembled with fluorescence optics and Nikon A1 confocal imaging system was used for all the retinal imaging purposes. The PCNA+ and BrdU+ cells were visualized and counted by directly looking at the fluorescence present in the retinal sections. The ISH+ cells were visualized, imaged and counted using the same microscope by looking though the bright field. Every section of the retinal tissue was visualized and counted for fluorescence and ISH signals, and more than three retinae were used for each experiment. The statistical analysis of the data for all the experiments was done using a two-tailed unpaired students' *t*-test. Comparison based studies were analysed 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. The amino acid sequence identity analysis was done using Clustal Omega.

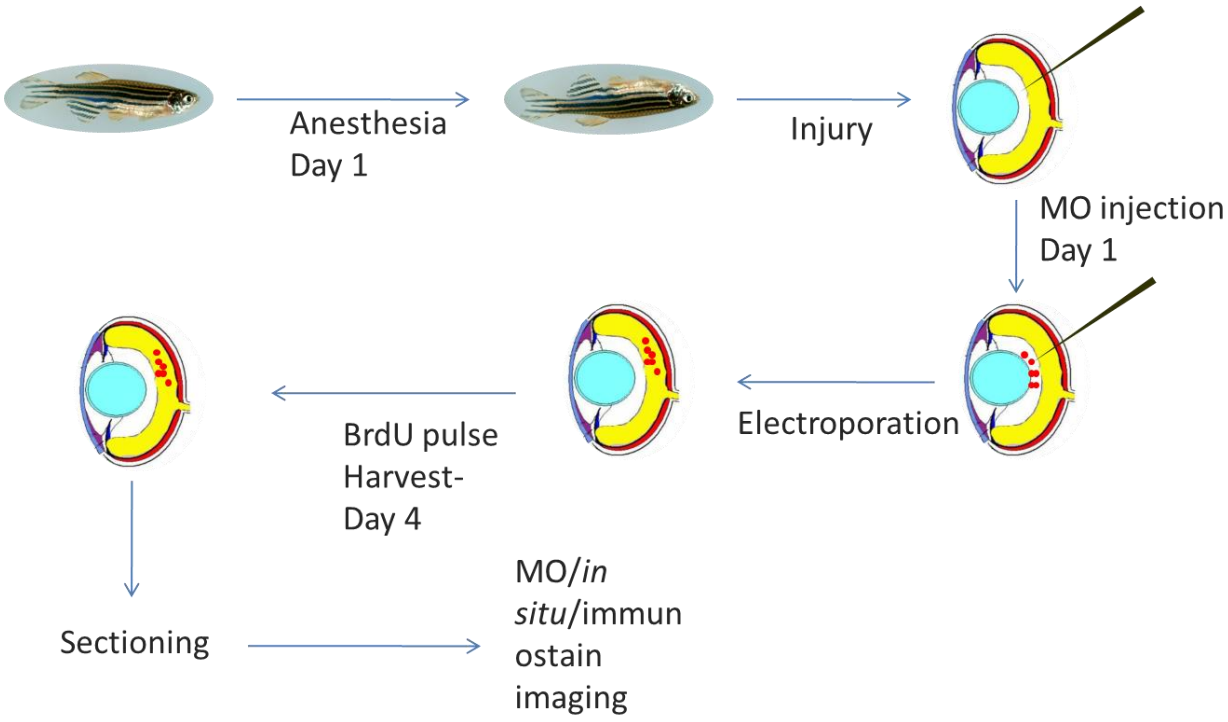


Fig 2.1: Morpholino modified antisense Oligonucleotide (MO) delivery method.

Section 3

Results

Chapter 1

Induction and regulation of *myca* and *mycb* are essential for Muller Glia de-differentiation and retina regeneration

3.1.1 *myca* and *mycb* are induced post retinal injury

Zebrafish have two isoforms of c-Myc: *myca* and *mycb*. Clustal Omega analysis of protein sequences of Myca and Mycb show 76% similarity (Fig 3.1a). Whole retina RNAseq analysis from zebrafish retina suggested both *myca* and *mycb* to be upregulated as early as 12-hour post injury (Fig: 3.1b). This encouraged us to probe for checking the transcript levels of *myca*, as well as *mycb*, at various time points post retinal injury, both temporally and spatially.

Fish were subjected to retinal injury and retinae were harvested for mRNA extraction at various time points post injury (minutes post injury or mpi, hours post injury or hpi and days post injury or dpi) or without injury (uninjured control or UC). These RNA samples were used for first strand cDNA synthesis and subsequently, transcript levels were examined by RT-PCR and semi-quantitative real-time PCR (qRT-PCR) using gene-specific primers. The *myca* and *mycb* both showed upregulated expression at a time point as early as 30 mpi, with their peak of expression spanning predominantly the de-differentiation phase (Fig: 3.1 c, d). The *mycb* was upregulated immediately after injury around 30 mpi; whereas *myca* expression lagged behind. Both of their expression declined around the proliferative phase. The qRT-PCR also showed higher expression levels of *mycb* compared to that of *myca.* We also checked for the expression levels of *max*, a well-established interaction partner of Myc. The mRNA levels of *max* also showed significant induction of expression at an early phase of de-differentiation which is reduced with some amount of wobbling at later time points (Fig 3.2 a, b).

To find out the exact site of expression of *myca* and *mycb*, mRNA *in situ* hybridization (ISH) was carried out on retinal sections at different time points post retinal injury. ISH showed a minimal basal level of expression of both *myca* and *mycb* in UC but a pan-retinal induction of both mRNAs at 12hpi which was reduced and restricted to the injury responsive zone by 2 to

6 dpi (Fig 3.3 a, and, Fig 3.4 a). We also performed PCNA Immuno-fluorescence (IF) on the ISH retinal sections to mark the proliferating MGPCs at 4dpi, when they are proliferating actively. Both *myca* and *mycb* were found to be strongly associated with PCNA positive MGPCs. (Fig 3.3 b, and Fig 3.4 b). Interestingly, we also found strong *myca* and *mycb* expression in the ganglion cell layer (GCL) at 4dpi (Fig 3.3 b, and Fig 3.4 b) suggesting its possible role in repairing the injured GCL or optic nerve injury.

IF study and a closer observation of *myca* and *mycb* positive cells revealed only a subset of *myca* (Fig 3.3 c) or *mycb* (Fig 3.4 c) positive cells co-localized with PCNA positive cells. Around 40% of PCNA positive cells expressed *myca* and *mycb*, whereas 60% of *myca* or *mycb* positive cells were also PCNA positive (Fig 3.3 d and Fig 3.4 d). Spatial expression patterns of *myca*, *mycb*, and *max* were further verified by Fluorescent ISH (FISH) on *tuba1016:gfp* transgenic line which marked dedifferentiating cells at 4dpi with GFP expression under *tuba1016* promoter. FISH showed *myca* was associated with GFP positive cells at 4dpi (Fig 3.5 a). We also performed double mRNA *in situ* hybridization, probing for both *myca* and *mycb* followed by BrdU immunostaining to label proliferating cells and found only a subset of both *myca* and *mycb* positive cells colocalized with BrdU (Fig 3.5 b) which supported our previous finding. Also, *mycb* and *max* FISH in *tuba1016:gfp* transgenic retinae confirmed colocalization of *mycb* and *max* in a subset of GFP positive MGPCs (Fig 3.5 c). Interestingly, *max* exclusively showed complete colocalization with GFP positive MGPCs (Fig 3.5 c).

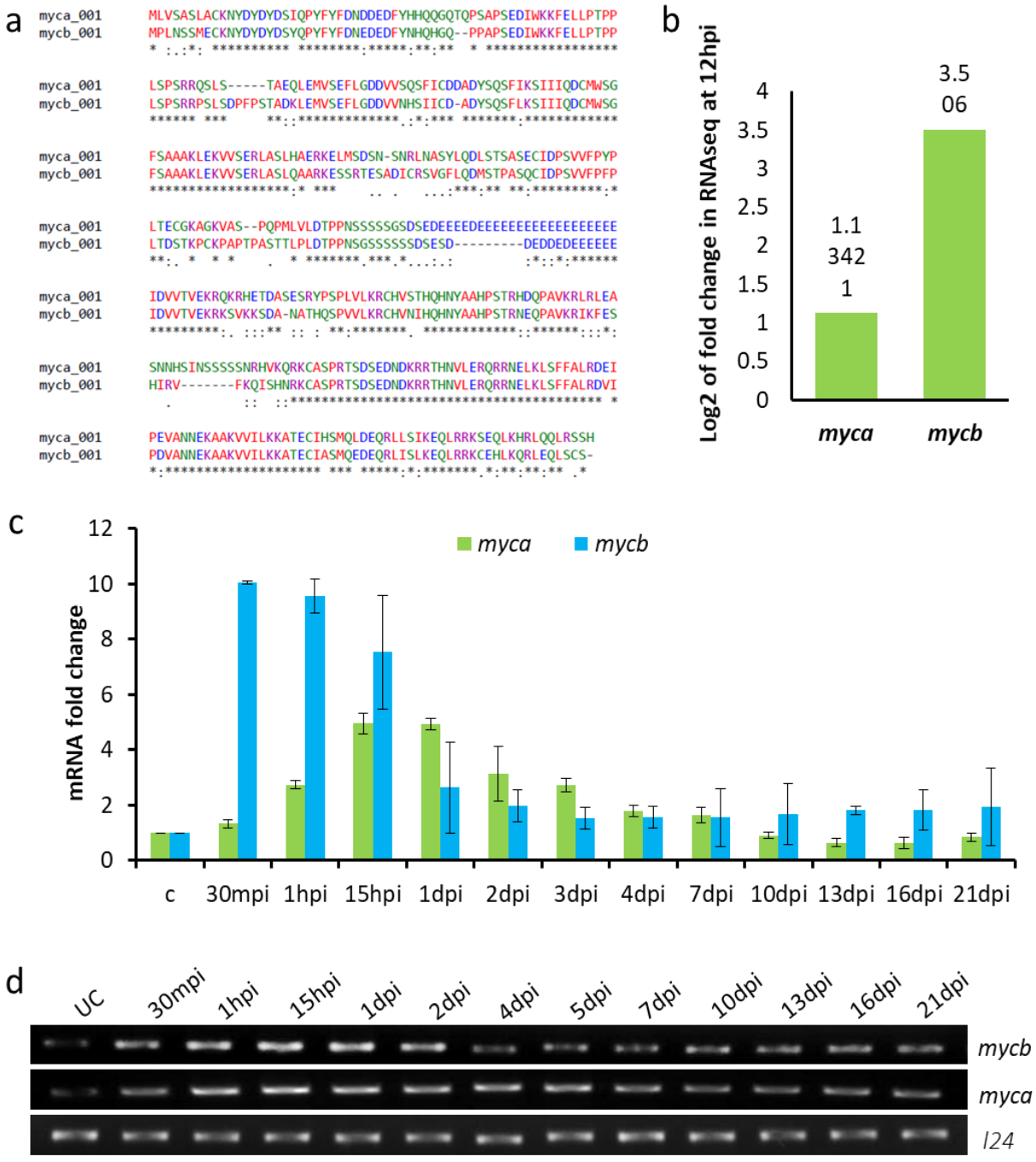


Fig 3.1: upregulation of *myca* and *mycb* after retinal injury. (a) Clustal Omega alignment of *myca* and *mycb* bearing 76% amino acid similarity. (b) *myca* and *mycb* are upregulated at 12hpi in RNAseq analysis from injured whole retina. (c) qRT-PCR of mRNA fold change and (d) RT-PCR of mRNA level of *myca* and *mycb* at various time points post injury shows immediate early upregulation of *myca* and *mycb*. . hpi: hours post injury, dpi: days post injury.the *l24* is internal control.

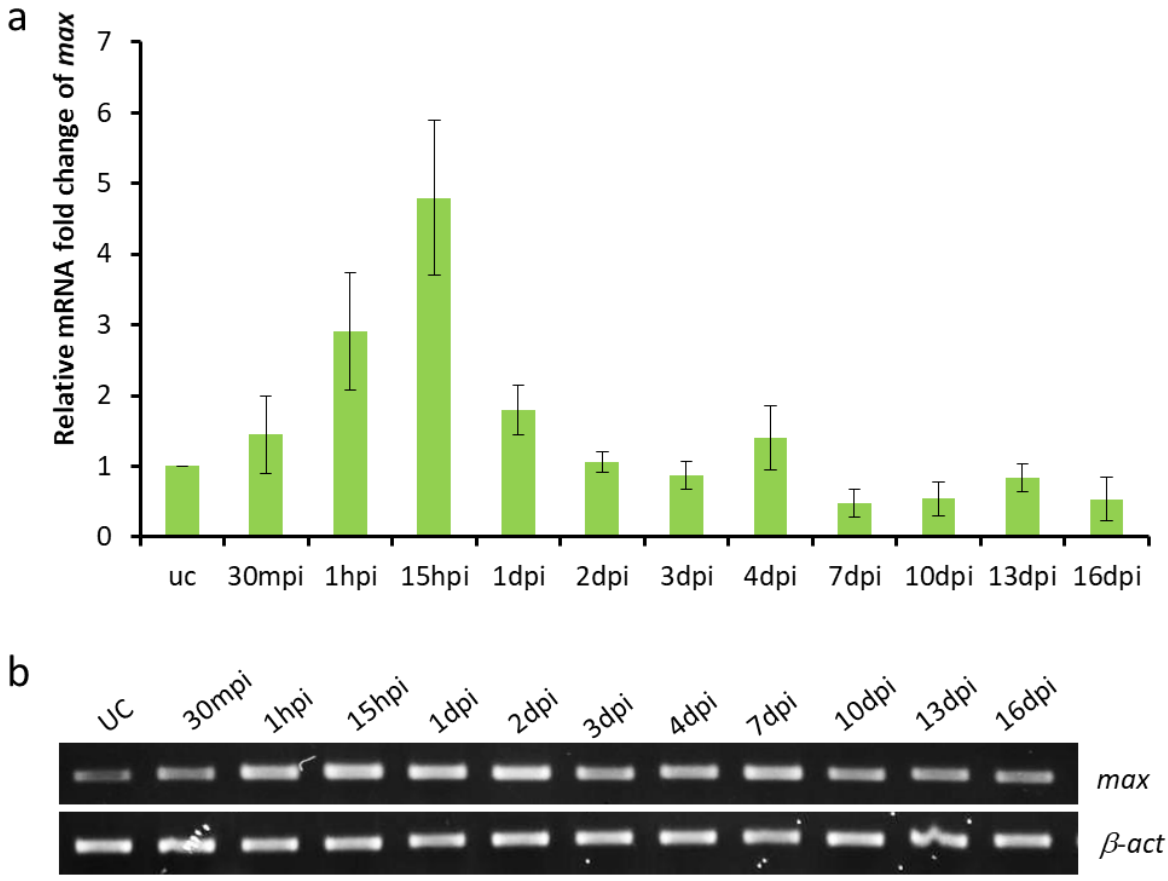


Fig 3.2: upregulation of *max* after retinal injury. (a) qRT-PCR shows upregulation of *max* mRNA at different time points post retinal injury. (b) RT-PCR shows expression pattern of *max* post retinal injury. . hpi: hours post injury, dpi: days post injury. The *b-act* is internal control.

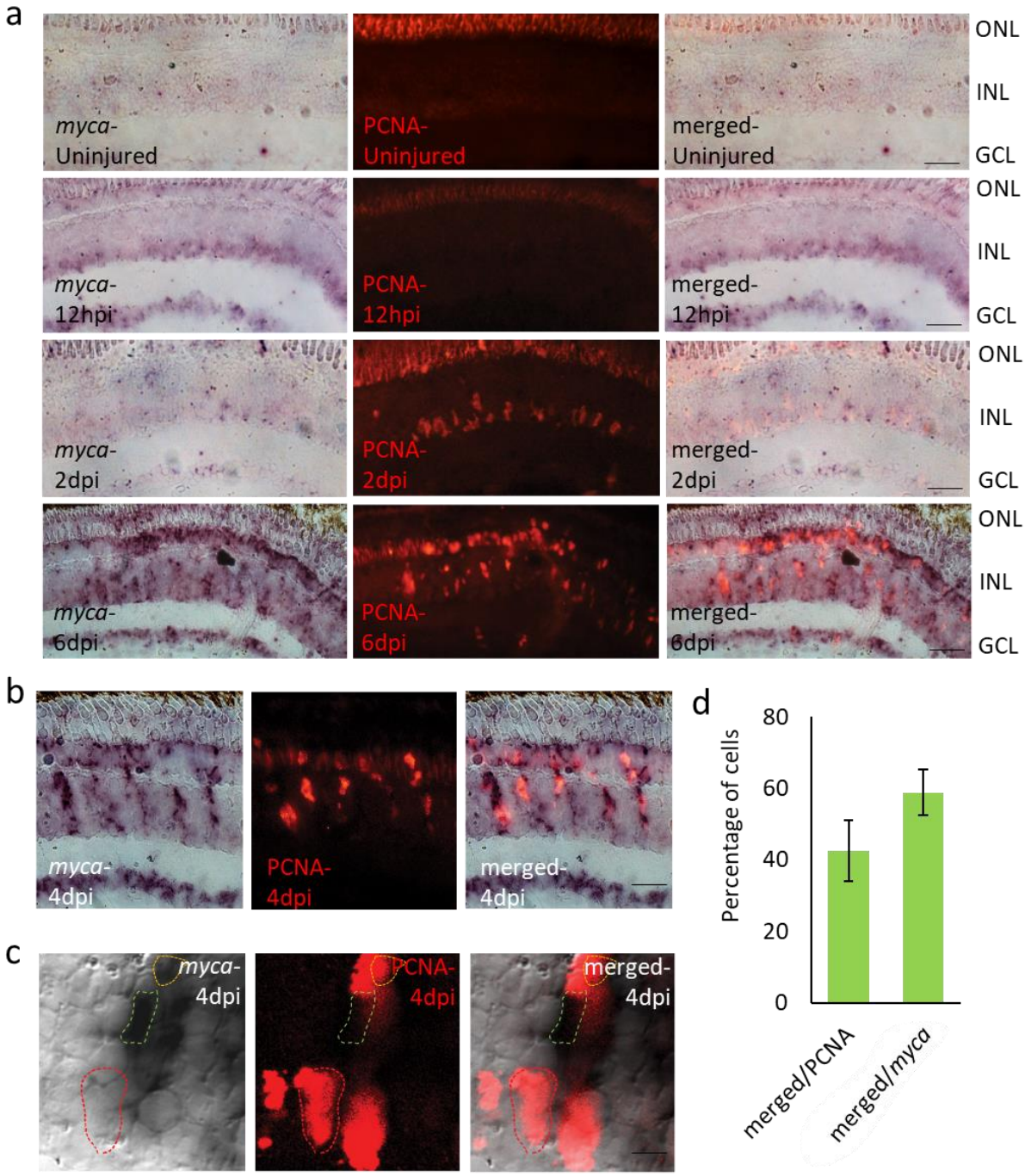


Fig 3.3: spatial expression pattern of *myca* after retinal injury. (a) mRNA *in situ* hybridization (ISH) shows basal expression of *myca* in unijred retina which gets quickly upregulated pan-retinally at 12hpi and finally at around 2 to 6dpi, gets restricted to the injury responsive zone as demarcated by PCNA positive MGPCs. (b) Immunofluorescence (IF) study and mRNA ISH show *myca* expression is closely associated with PCNA positive MGPCs at 4dpi. (c) High resolution confocal microscopy on retinal samples from 4dpi shows *myca* expression stays significantly secluded from PCNA positive MGPCs. Yellow denotes PCNA+/*myca*+ cells, green denotes PCNA-/*myca*+ cells and red denotesPCNA+/*myca*- cells. (d) quantification of the same. hpi: hours post injury, dpi: days post injury. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Scale bar: 30µ in A, 15µ in B and 5µ in C.

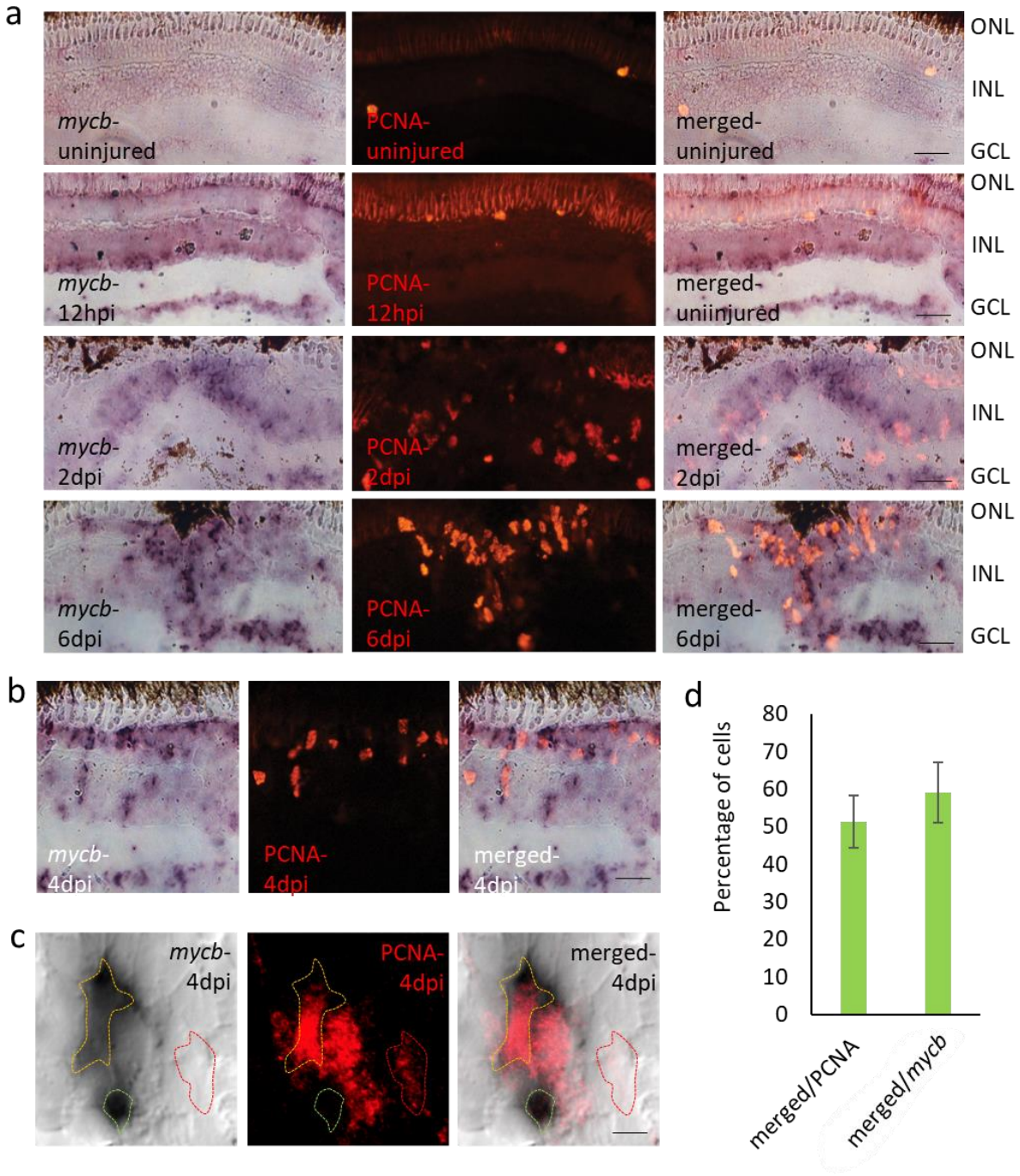


Fig 3.4: spatial expression pattern of *mycb* after retinal injury. (a) mRNA *in situ* hybridization (ISH) shows basal expression of *mycb* in unijred retina which gets quickly upregulated pan-retinally at 12hpi and finally at around 2 to 6dpi, gets restricted to the injury responsive zone as demarcated by PCNA positive MGPCs. (b) Immunofluorescence (IF) study and mRNA ISH show *mycb* expression is closely associated with PCNA positive MGPCs at 4dpi. (c) High resolution confocal microscopy on retinal samples from 4dpi shows *mycb* expression stays significantly secluded from PCNA positive MGPCs. Yellow denotes PCNA+/*myca*+ cells, green denotes PCNA-/*myca*+ cells and red denotesPCNA+/*myca*- cells. (d) quantification of the same. hpi: hours post injury, dpi: days post injury. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Scale bar: 30µ in A, 15µ in B and 5µ in C.

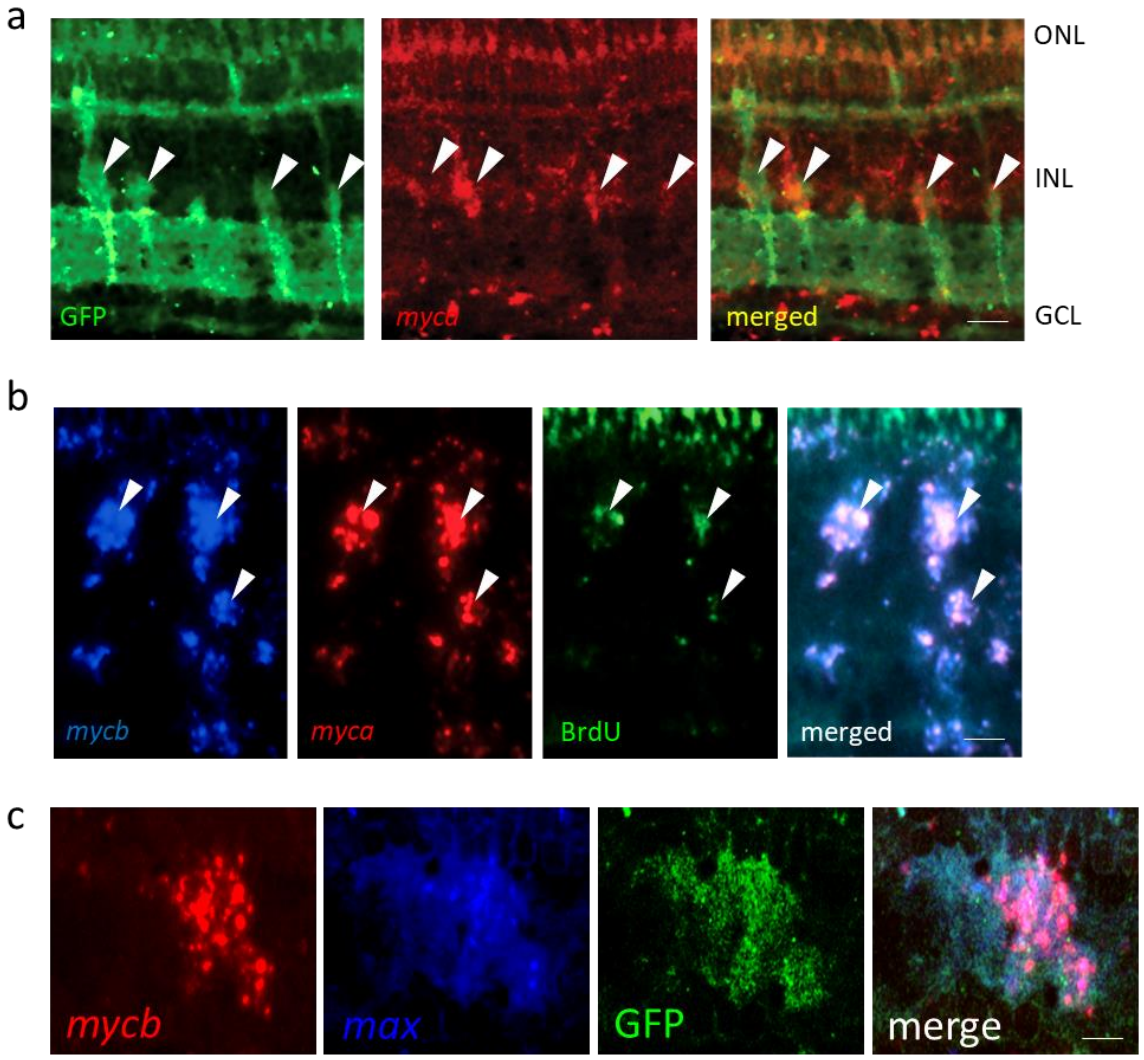


Fig 3.5: spatial expression pattern of *myca*, *mycb* and *max* in injured retina. (a) mRNA ISH of *myca* in 4dpi retina from *tuba1016::gfp* transgenic fish shows GFP positive cells juxtaposed to *myca* positive cells. (b) double FISH shows a subset of *myca* and *mycb* positive cells to be colocalized with BrdU positive proliferating cells at 4dpi. (c) FISH and IF show a subset of *max* positive cells are *mycb* positive and colocalizes with GFP positive MGPCs from *tuba1016:gfp* transgenic fish retina at 4dpi. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Scale bar: 10µ in A, 5µ in B and C.

3.1.2 *myca* and *mycb* induction is essential for Muller Glia de-differentiation.

Furthermore, we sought to check the necessity of *myca* and *mycb* induction in the context of proliferation of Muller Glia derived progenitors (MGPCs). Translation of Myca and Mycb protein from their respective mRNA was blocked by using morpholino modified oligonucleotides (MO). The specificity of these MOs was verified by injecting them into embryos along with GFP mRNA appended with MO binding sites. Both MOs against *myca* and *mycb* significantly brought down GFP expression (Fig 3.6 a, b). The specificity of these MOs was also confirmed by western blotting from a lysate of retinae injected and electroporated with these MOs. Western blot showed the effectiveness of these MOs as protein level was depleted in retinae electroporated with MO against both Myca and Mycb (Fig 3.6 c).

To check the necessity of *myca* or *mycb* with respect to regeneration, these MOs were injected along with retinal injury and were electroporated immediately into the retinae to block protein synthesis at de-differentiation and pro-proliferative stage. Retinae were harvested for BrdU IF at 4dpi after BrdU pulsing for 4-5 hours so as to get a maximum number of BrdU positive cells. Both *myca* and *mycb* MOs significantly diminished proliferative cells in a dose-dependent manner at 4dpi (Fig 3.7 a, b). We further verified by TUNEL assay that these two MOs targeting *myca* and *mycb* did not cause increased apoptosis (Fig 3.8 a, b). So, the reduction in the number of MGPCs with *myca* or *mycb* knockdown is not an effect of increased cell death. Double knockdown of both *myca* and *mycb* recapitulated the same in a greater extent than their single knockdowns (Fig 3.9 a, b). For additional verification of the effectiveness of these MOs, we carried out the same experiment with another set of MOs against *myca* and *mycb*. These 2nd

set of MOs targeting the UTR region of *myca* and *mycb* also resulted in ablated proliferation to a similar extent (Fig 3.10 a, b).

In another set of experiment, MO against *mycb* was injected along with injury but was electroporated at 3dpi and retinae were harvested at 5dpi for BrdU immunostaining (Fig 3.11 a). This experiment was carried out to figure out the necessity of the residual expression of *mycb* at proliferative phase. BrdU IF showed a reduced number of BrdU positive cells in this scenario also (Fig 3.11 b, c) suggesting Mycb's possible role at both de-differentiation and proliferation.

To rescue the blockade on proliferation caused by these MOs, respective mRNAs were transfected into 4dpi retinae along with GFP mRNA (reporter) and the 2nd set of MOs targeting the UTR region or control MO. Transfection of *myca* and *mycb* mRNA rescued the blockade on proliferation successfully (Fig 3.12 a-c).

Further, to prove Myc's involvement in MGPCs proliferation during regeneration, we also used 10058-F4, a small molecule inhibitor of Myc/Max interaction to disrupt their physical interaction. In one set of experiments, fish were kept in solutions of 10058-F4 with different concentrations post retinal injury for 2days and subsequently transferred to water till 4dpi. Retinae were harvested at 4dpi after BrdU pulsing and subsequently, retinal sections were subjected to BrdU IF (Fig 3.13 a, b). Proliferating cell quantification as determined by BrdU IF showed 70% less BrdU positive cells in injured retinae (Fig 3.13 c). In another set of experiments, continuously for 4 days, we dipped the fish in solutions of 10058-F4 with varying concentrations following retinal injury and harvested retinae for BrdU IF after 4-5 hours of BrdU pulsing (Fig 3.14 a). This also resulted in a significant reduction in the number of BrdU positive cells at 4dpi compared to DMSO or injury-only control (Fig 3.4 b, c). Further, to prove that the reduction in the number of MGPCs, caused by inhibition of Myc/Max interaction, is

not an effect of increased cell death, we performed TUNEL assay on sections of 4dpi retina treated with varying concentration of 10058-F4. TUNEL assay shows no significant change in the number of TUNEL positive cells in retinae treated with 10058-F4 compared with control retinae (Fig 3.15 a, b). This suggests that Myc mediated gene regulatory network might be regulating the formation of MGPCs during retina regeneration.

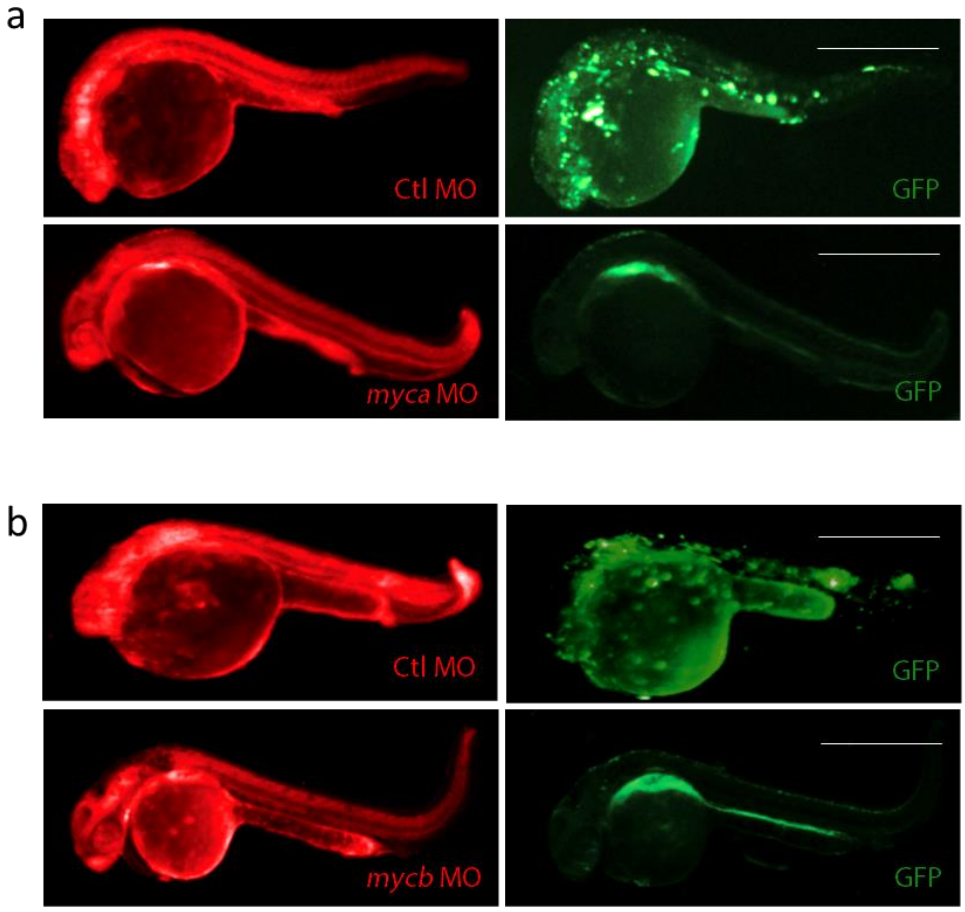


Fig 3.6: Effectiveness of morpholino modified oligoneucleotides against *myca* and *mycb*. (a) embryos injected with control MO and *gfp* mRNA shows GFP expression at 2 days post fertilization, but embryos injected with *myca* MO and *gfp* mRNA fused with MO targeting site do not show GFP expression. (b) same set of experiments with morpholino targeting *mycb*. (c) Western blot analysis of 1dpi retinae electroporated with MOs targeting either *myca*, or *mycb* or both resulted in depletion of Myca/b protein. GS acts as internal control. dpi: days post injury. Scale bar: 1mm.

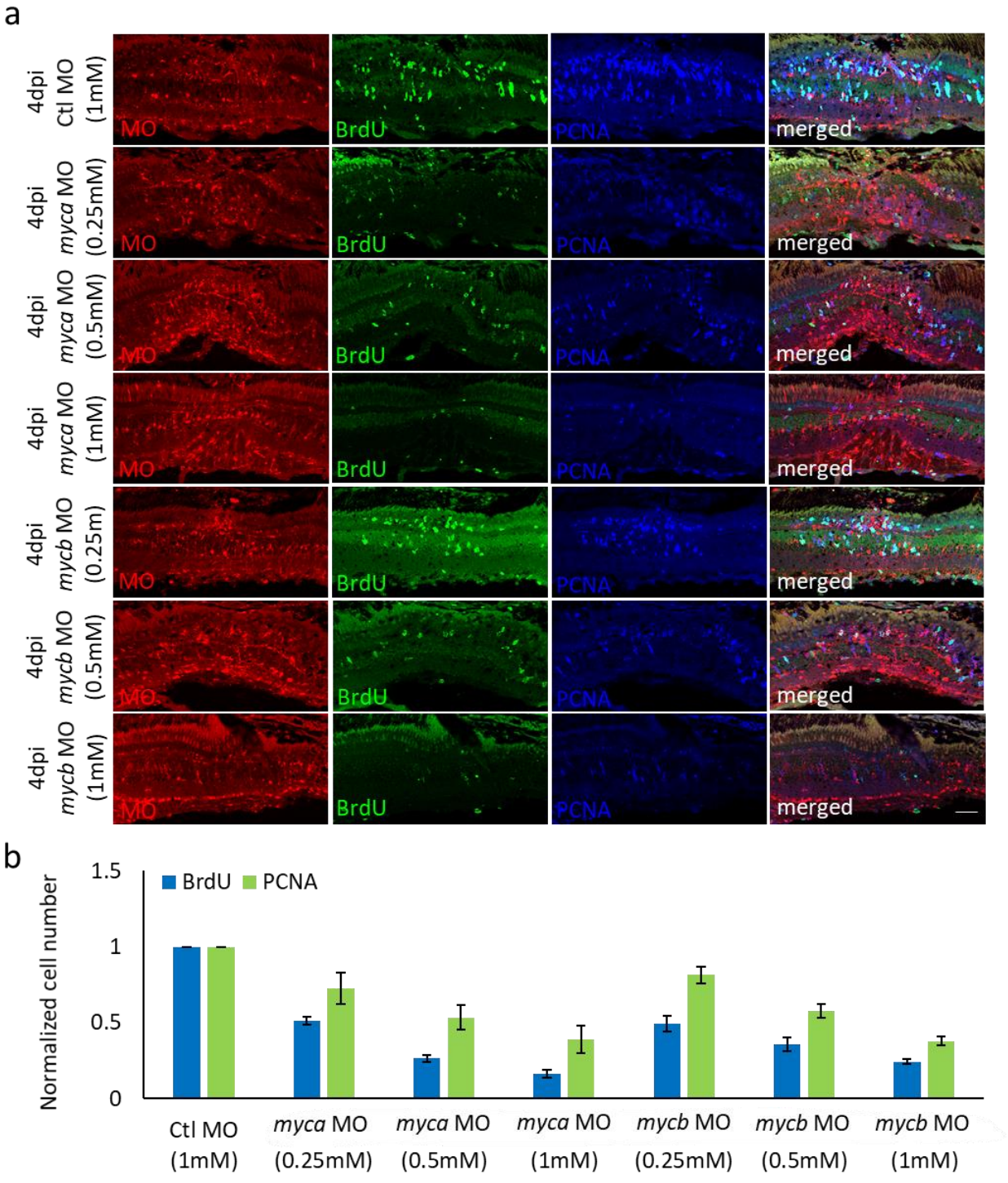


Fig 3.7: knock down of myca and *mycb* resulted in reduced proliferation at 4dpi. (a) BrdU and PCNA immunofluorescence study along three different concentration of MO targeting *myca* and *mycb* separately resulted in a significant decrease in BrdU and PCNA positive cells at 4dpi. Scale bar: 30µ. (b) quantification of the same.

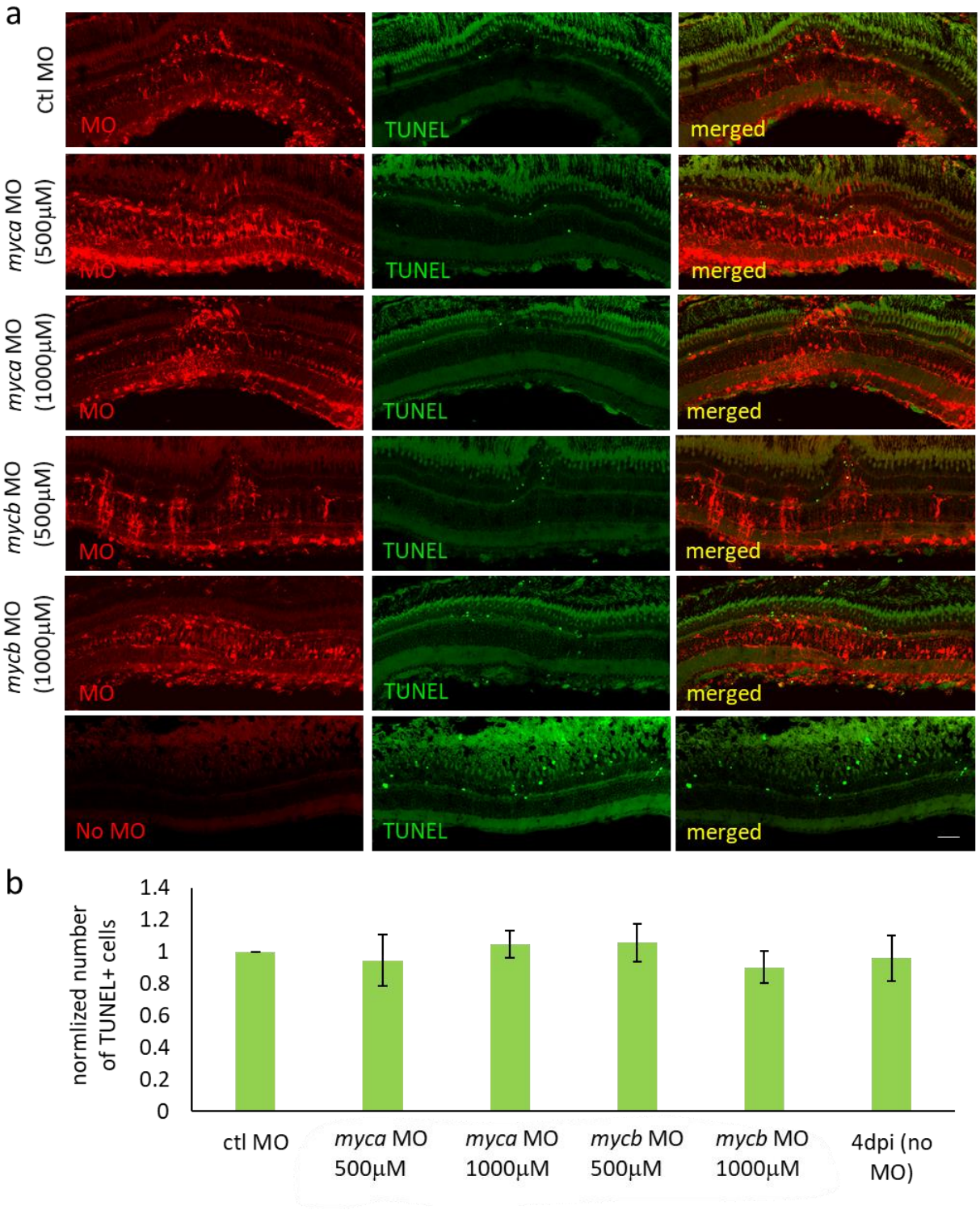


Fig 3.8: electroporation of Mos into the retina does not cause apoptosis (a) confocal microscopy on sections of 4dpi retina which received varying concentration of MOs, targeting both *myca* as well as *mycb,* shows no significant change in the number of TUNEL positive apoptotic cells caused by MOs. Scale bar: 30µ. (b) quantification of the same.

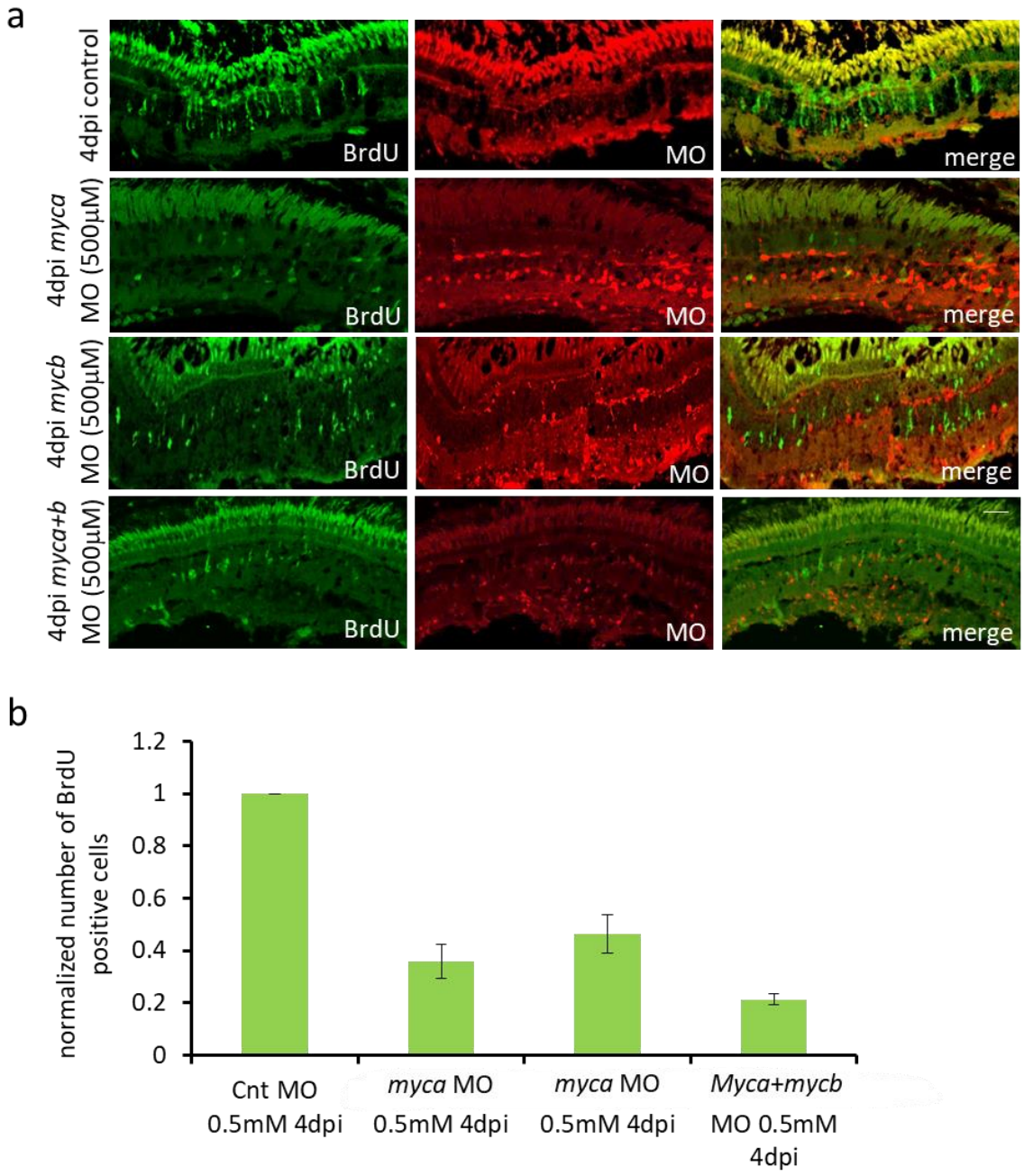


Fig 3.9: Double knock-down of *myca* and *mycb* caused reduction in the number of proliferative MGPCs. (a) confocal microscopy on sections of 4dpi retina electroporated with *myca* and *mycb* targeting MOs separately and together show doble knock-down of *myca* and *mycb* has a greater effect on reducing number of BrdU positive MGPCs compared with their single knock-down. Scale bar: 30µ. (b) quantification of the same.

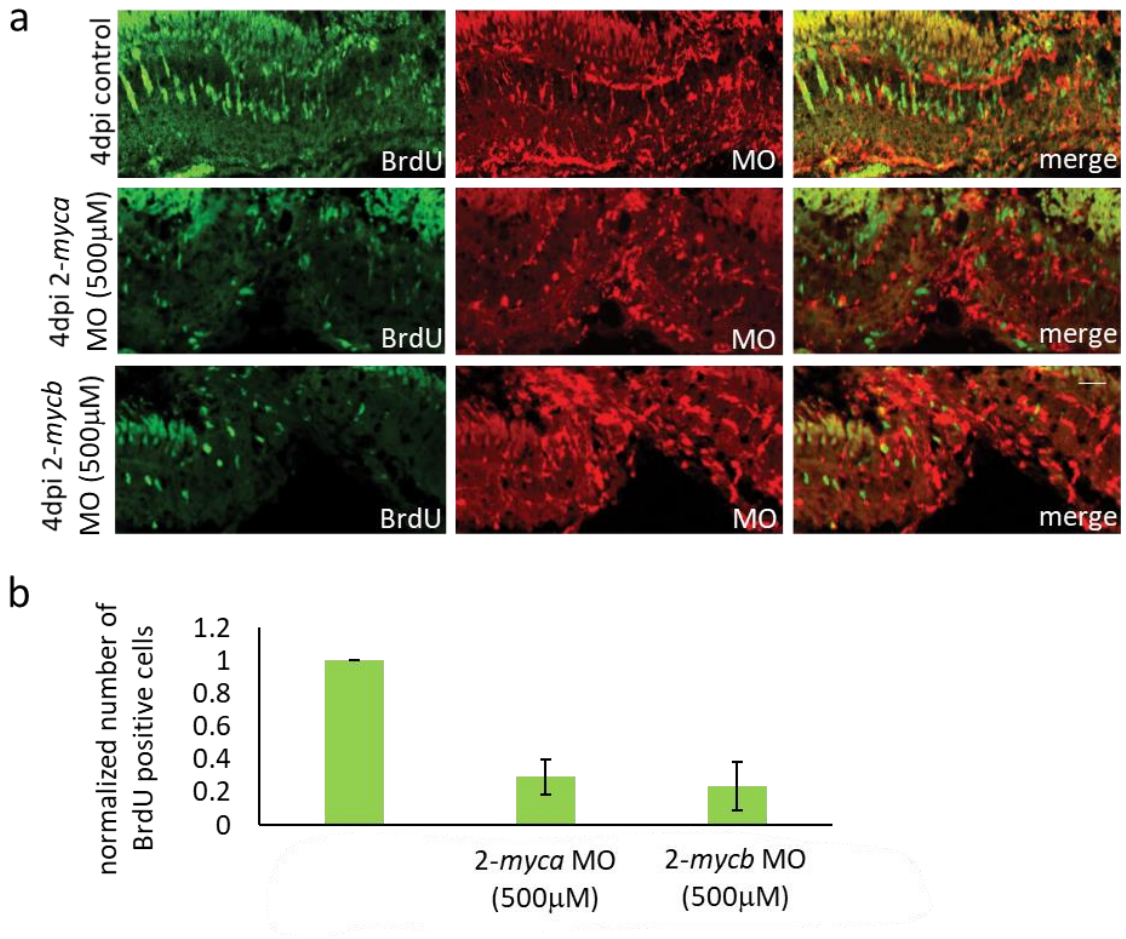


Fig 3.10: Knock-down of *myca* and *mycb* with second set of MOs caused reduction in the number of BrdU positive cells . (a) confocal microscopy on sections of 4dpi retina electroporated with a second set of *myca* and *mycb* targeting MOs separately caused decresed number of BrdU positive MGPCs. Scale bar: 30µ. (b) quantification of the same.

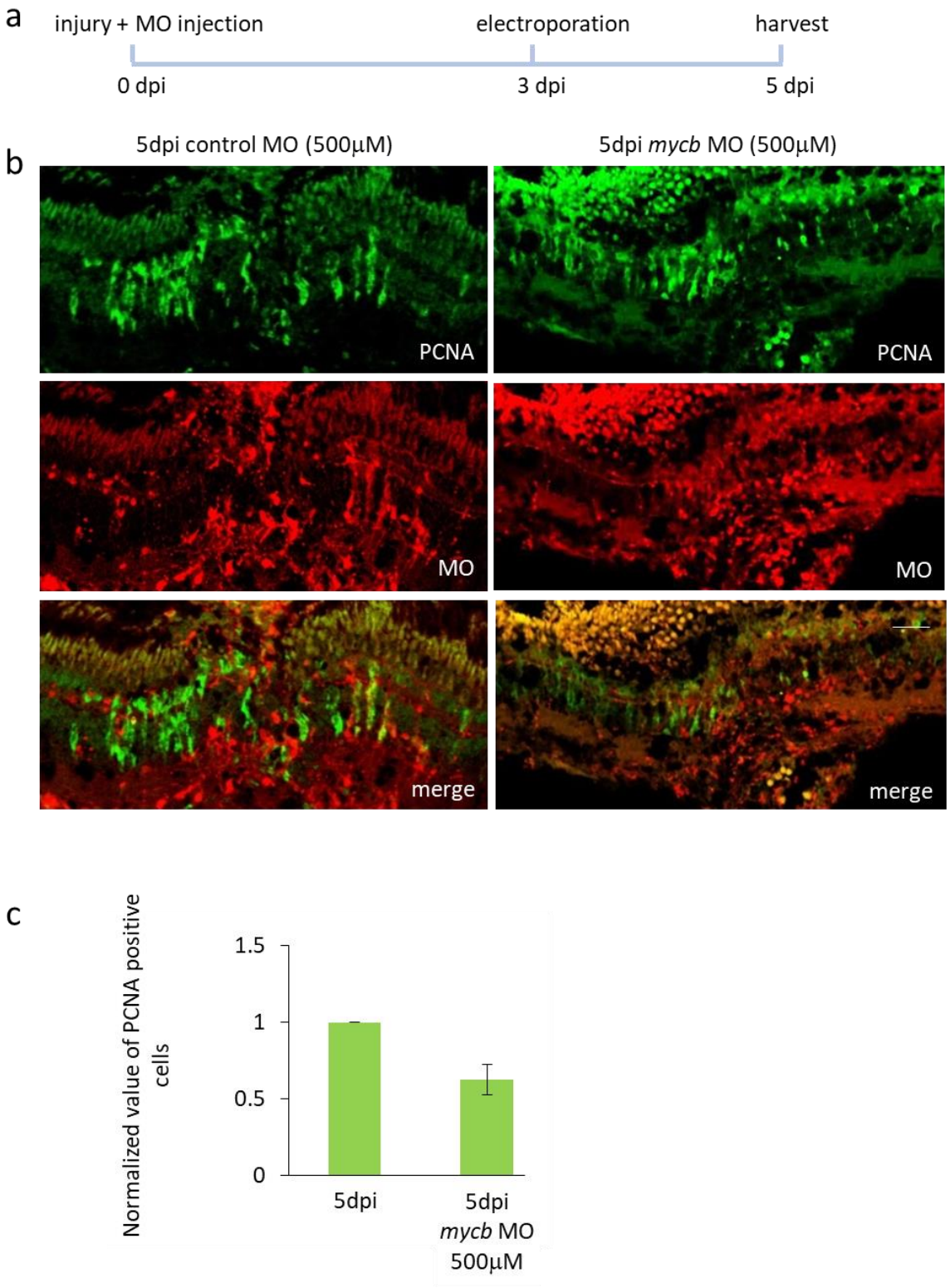


Fig 3.11: Late knock-down of *mycb* caused reduction in the number of BrdU positive cells at 5dpi . (a) experimental time-line for late knock-down of *mycb*. (b) confocal microscopy on sections of 5dpi retina electroporated with *mycb* targeting MO at 3dpi caused decresed number of BrdU positive MGPCs at 5dpi. Scale bar: 30µ. (c) quantification of the same.

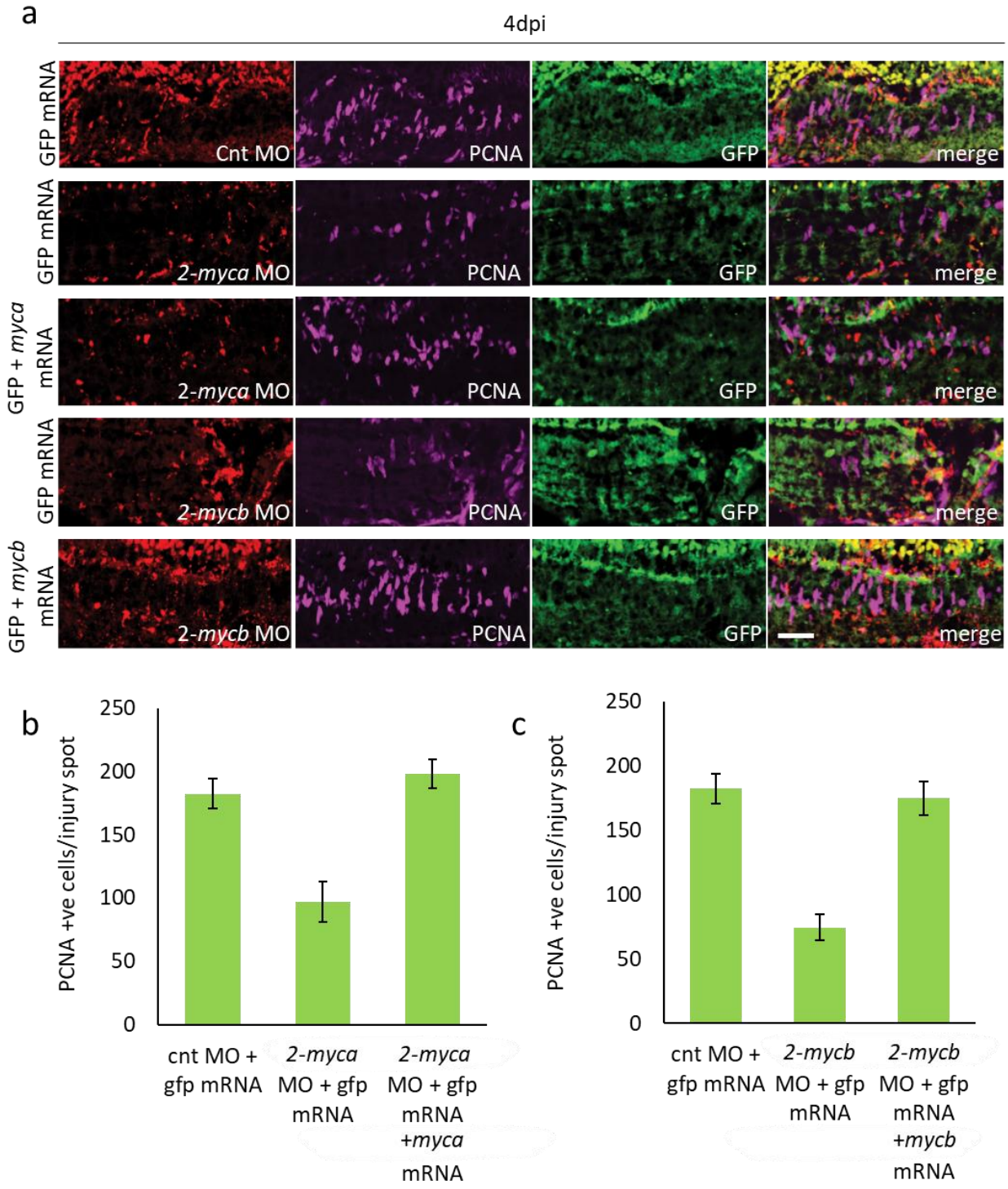


Fig 3.12: The *myca* and *mycb* mRNA could rescue the effect of the MOs targeting endogenous *myca* and *mycb*. (a) confocal microscopy on sections of 4dpi retina shows the reduction in PCNA positive cell number caused by MOs targeting *myca* or *mycb* can be rescued by transfection of their respective mRNA into the retinae. Scale bar: 20µ. (b, c) quantification of rescue experiment from **a** with *myca* mRNA (b) and *mycb* mRNA (c).

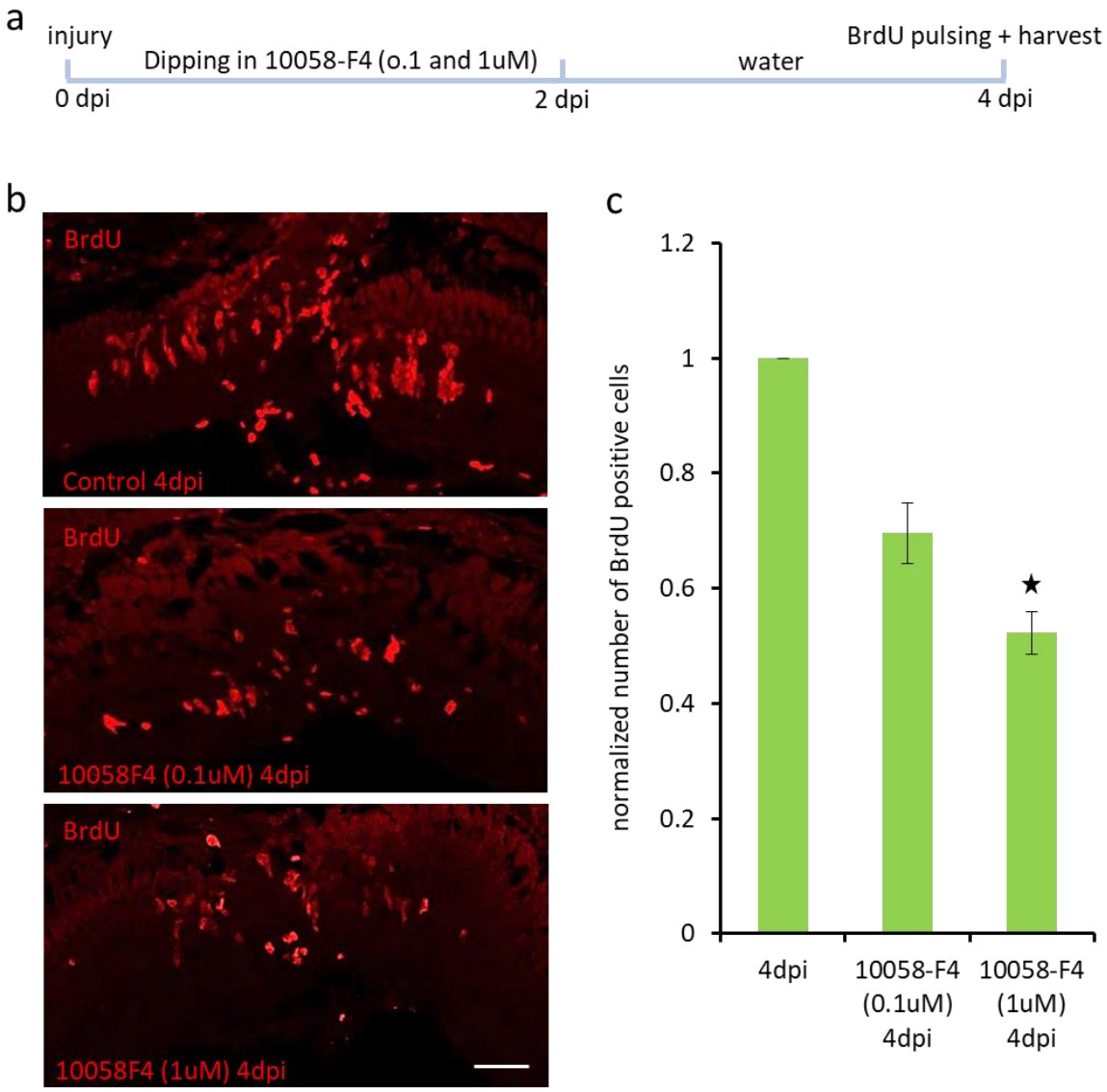


Fig 3.13: Early inhibition of Myc/Max interaction causes reduction in the number of MGPCs. (a) Experimental time-line showing time of injury followed by treatment with Myc/Max inhibitor 10058-F4, transfer to water and harvesting at 4dpi after BrdU pulsing. (b) BrdU IF study shows a dose dependent reduction in the number of BrdU positive cells at 4dpi as an effect of 10058-F4 treatment in the initial two days following retinal injury. Scale bar: 30µ. (c) quantification of the same. $P < 0.002$.

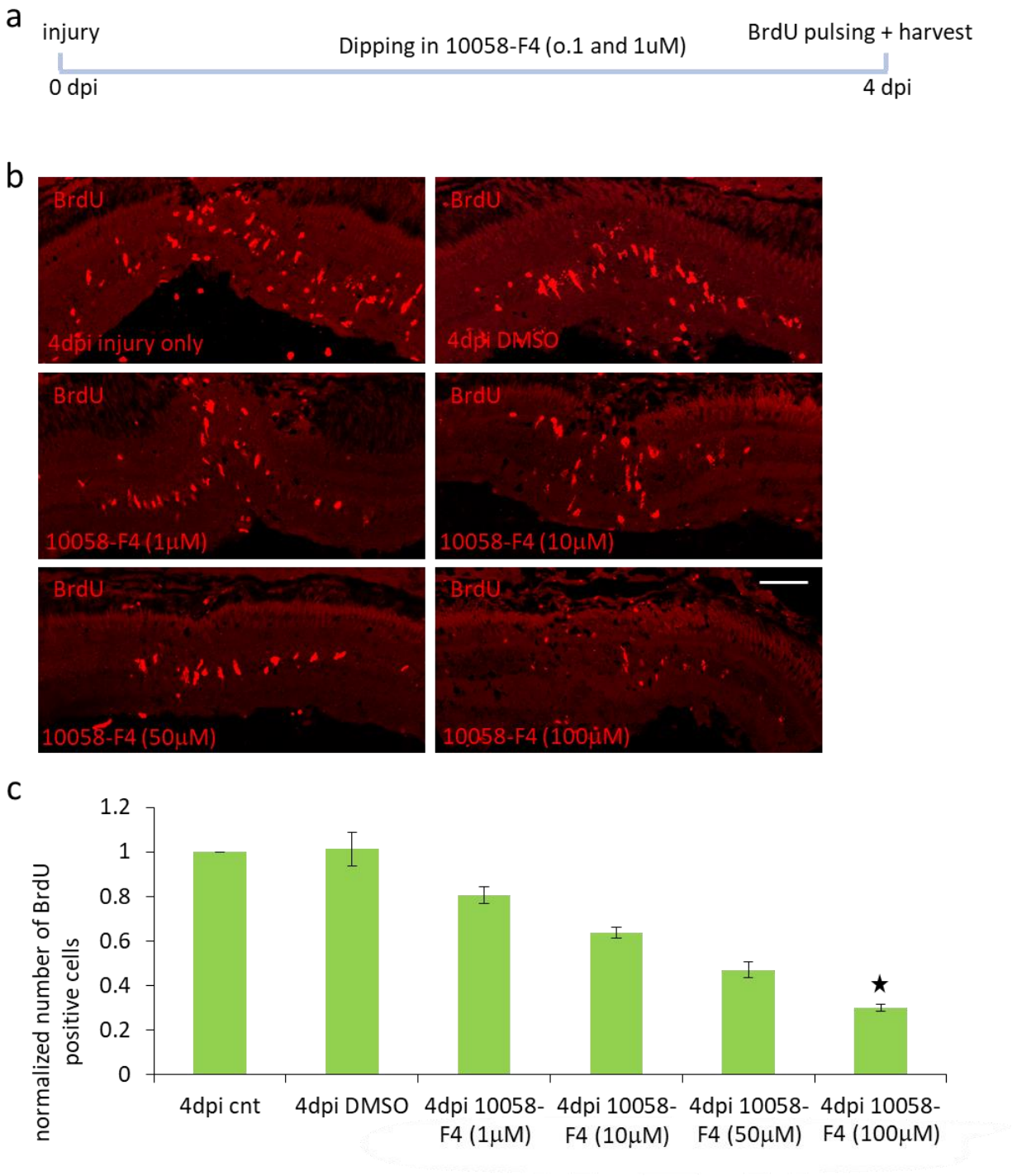


Fig 3.14: Early inhibition of Myc/Max interaction causes reduction in the number of MGPCs. (a) Experimental time-line showing time of injury followed by treatment with Myc/Max inhibitor 10058-F4, and harvesting at 4dpi after BrdU pulsing. (b) BrdU IF study shows a dose dependent reduction in the number of BrdU positive cells at 4dpi as an effect of 10058-F4 treatment. Scale bar: 30µ. (c) quantification of the same. $P<0.02$.

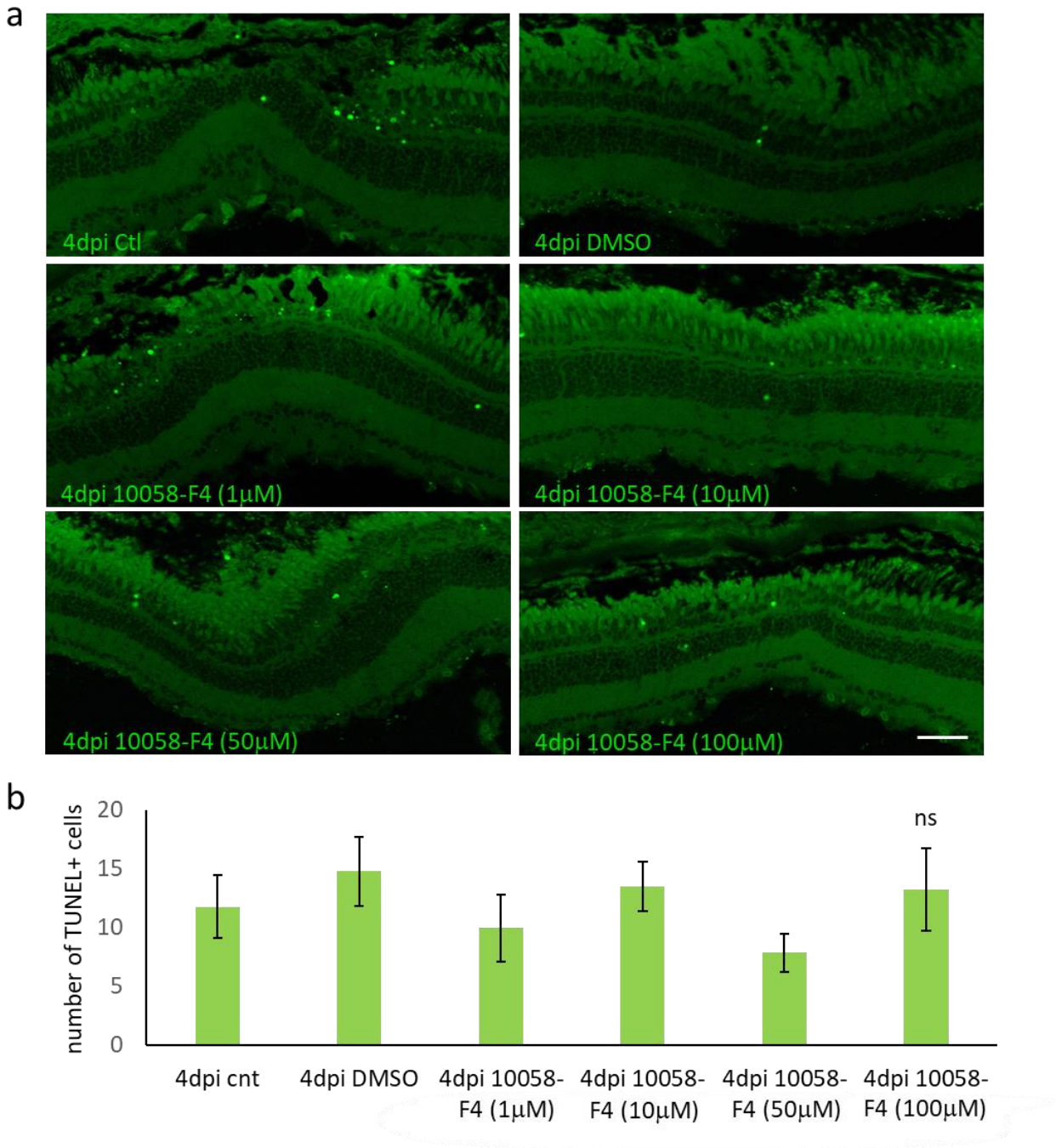


Fig 3.15: Inhibition of Myc/Max interaction with 10058-F4 does not induce cell death. (a) TUNEL assay on retinal sections of 4dpi retinae treated with varying concentration of 10058-F4 shows no significant change in the number of TUNEL positive cells compared to controls. Scale bar: 30µ. (b) quantification of the same. ns: not significant.

3.1.3 Expression of myca and *mycb* is fine-tuned by an Asl1a-Insm1a-Myc regulatory axis during retina regeneration.

To figure out possible up-stream regulators of *mycb*, known regeneration associated genes or signalling pathways were blocked by pharmacological inhibitors (for Wnt and Hedgehog signalling) or MOs (for Ascl1a). The *mycb* was found to be down-regulated in retinae treated with Wnt-signalling inhibitor XAV939 (Fig 3.16 a), and Hedgehog signalling blocker cyclopamine (Fig 3.16 b) as well as in retinae electroporated with Ascl1a targeting MO (Fig 3.16 c, d). Interestingly, both Wnt and Hedgehog signalling have been reported to regulate proliferation by upregulating Ascl1a, a transcriptional activator. So, we further focused our experiments on finding out the regulation of *myca* and *mycb* by Ascl1a. As *ascl1a*, *myca*, and *mycb* all are expressed very early during retina regeneration, we further wanted to check the existence of such a regulation at 8 hpi, where all these mRNAs are induced pan-retinally. Surprisingly, 8hpi retinae electroporated with *ascl1a* targeting MO showed a concentration-dependent increase in the relative fold change of *mycb*, but not *myca* (Fig 3.16 e, f).

To further verify the regulation of *mycb* by Ascl1a, fish embryos were injected with *mycb:gfp-luciferase* plasmid and increasing concentration of Ascl1a targeting MO which showed a dose-dependent reduction in promoter activity (Fig 3.17 a) in support of the previous data. Next, we wanted to check the effect of *ascl1a* mRNA on *mycb* promoter. For this, fish embryos were co-injected with *mycb:gfp-luciferase* and increasing concentration of *ascl1a* mRNA. Surprisingly, this also showed a concentration-dependent decrease in *mycb* promoter activity (Fig 3.17 b). Ascl1a, being a transcriptional activator, cannot possibly downregulate *mycb* promoter activity. So, it must be through some transcriptional repressor like Insm1a, which is induced by Ascl1a. In support of this hypothesis we also found one putative Insm1a binding site on *mycb* promoter

(Fig 3.17 c). To test this hypothesis, *mycb:gfp-luciferase* reporter plasmid was co-injected into fish embryos along with increasing concentration of *insm1a* mRNA which showed a concentration-dependent downregulation of *mycb* promoter activity (Fig 3.17 d). Oppositely, embryos injected with *mycb:gfp-luciferase* and increasing concentration of a MO targeting *insm1a* significantly induced *mycb* promoter activity (Fig 3.17 e). Additionally, to further verify this regulation of *mycb* promoter by Insm1a, we mutated the Insm1a binding site on *mycb* promoter in *mycb:gfp-luciferase* and was co-injected into embryos with *insm1a* MO. As expected, we did not observe the increase in *mycb* promoter activity (Fig 3.17 f). To validate these findings during retina regeneration, we electroporated injured retinae injected with MO against *insm1a*. Compared to 2dpi control, qRT-PCR and RT-PCR revealed retinae electroporated with *insm1a* targeting MO showed increased mRNA levels of both *myca* and *mycb* (Fig 3.17 g, h). These experiments demonstrate the involvement of an Ascl1a-Insm1a regulatory axis to fine-tune the expression of *myca* and *mycb* during zebrafish retina regeneration.

Since Ascl1a is a transcriptional activator and knock-down of Ascl1a resulted in reduced expression of *mycb* at 2dpi, we wanted to check if Ascl1a is physically binding to the *mycb* promoter. For this, we first looked for putative Ascl1a binding site on the *mycb* promoter. The 6kb *mycb* promoter showed three putative Ascl1a binding sites (CAGGTG and CAGCTG). We probed for the first two proximal sites for ChIP assay at 2 and 4dpi (Fig 3.18 a). ChIP assay showed binding of Ascl1a to these sites on mycb promoter at both 2 and 4dpi (Fig 3.18 b). Taken together, our results reveal a novel regulatory mechanism exhibited by the Ascl1a/Insm1a signalling axis which succeeds in restricting the initial pan-retinal expression of *myca* and *mycb* to the injury responsive zone.

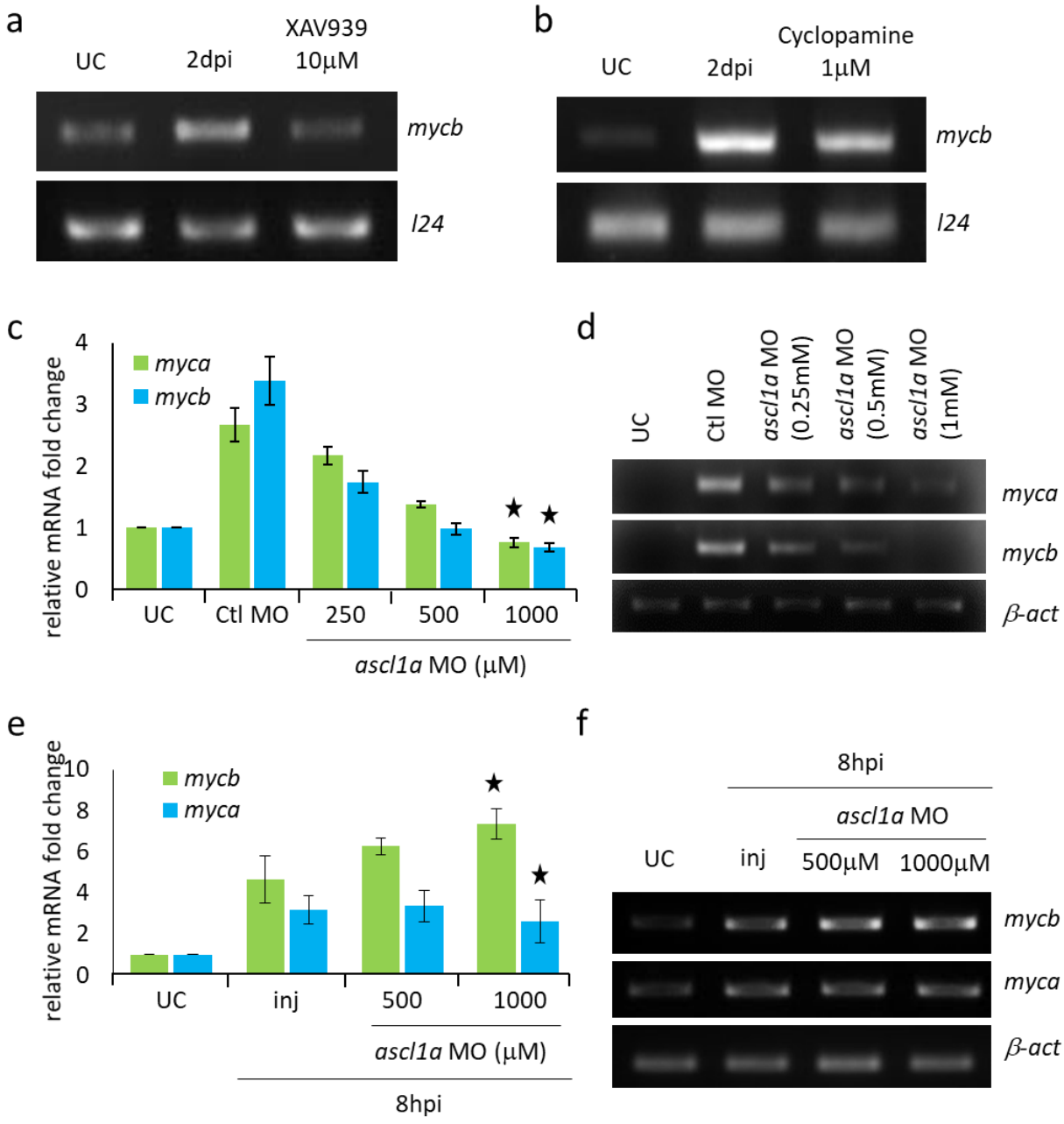


Fig 3.16: Regulation of *myca* and *mycb* by regeneration associated molecules or signalling pathways. (a) RT-PCR shows *mycb* is downregulated at 2dpi retinae with compromised Wnt signalling caused by XAV939. (b) RT-PCR shows inhibiting Hedgehog signalling in 2dpi retinae with cyclopamine down-regulated *mycb*. (c, d) qRT-PCR (c) and RT-PCR (d) show a dose dependent downregulation of *myca* and *mycb* mRNA in 2dpi retinae electroporated with MO targeting *ascl1a* compared with control. (e, f) qRT-PCR (e) and RT-PCR (f) show a concentration dependent upregulation of *myca* and *mycb* in 8hpi retinae electroporated with increasing concentration of *ascl1a* targeting MO. hpi: hours post injury, dpi: days post injury. P<0.001

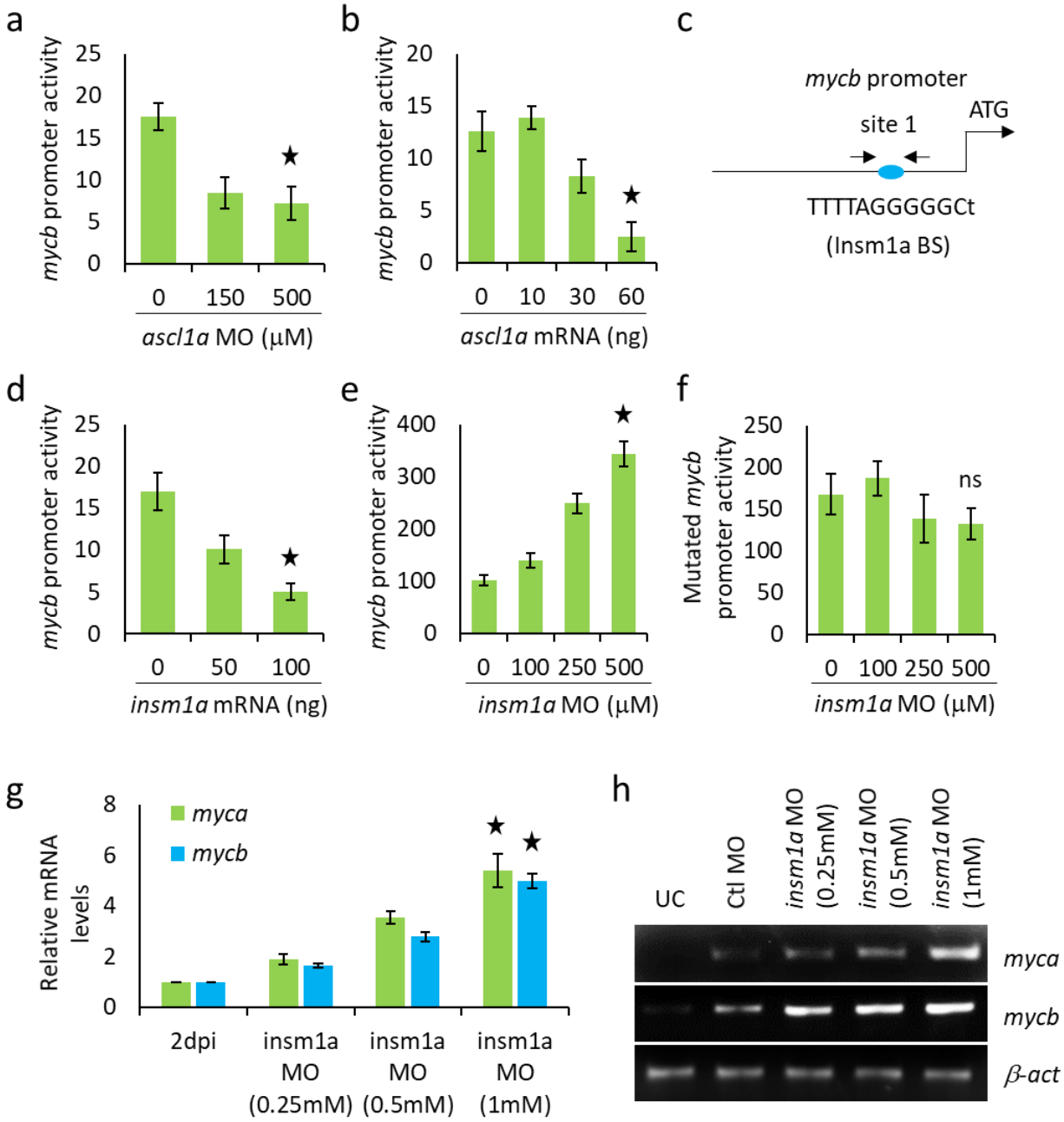


Fig 3.17: Regulation of *myca* and *mycb* by Ascl1a/Insm1a regulatory axis. (a) reduction in *mycb* promoter activity as assayed by luciferase intensity in embryos injected with increasing concentration of *ascl1a* MO. (b) reduction in *mycb* promoter activity as assayed by luciferase intensity in embryos injected with increasing concentration of *ascl1a* mRNA. (c) digramatic representation of *mycb* promoter bearing Insm1a binding site. (d) reduction in *mycb* promoter activity as assayed by luciferase intensity in embryos injected with increasing concentration of *insm1a* mRNA. (e) increase in *mycb* promoter activity as assayed by luciferase intensity in embryos injected with increasing concentration of *insm1a* MO. (f) The *mycb:gfp-luciferase* plasmid with mutated Insm1a binding site injuected into zebrafish embryos along with *insm1a* MO did not show any change in promoter activity. (g, h) qRT-PCR (g) an RT-PCR (h) show an increase in the relative mRNA levels of *myca* and *mycb* in 2dpi retinae electroporated with MO targeting *insm1a*. Arrows indicate primers for ChIP assay in **c**. p<o.oo1. ns: not significant.

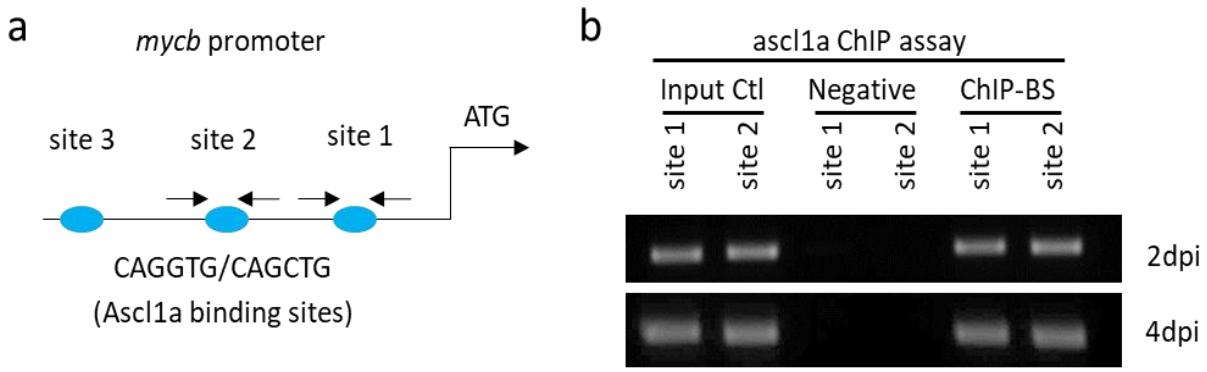


Fig 3.18: Ascl1a directly regulates *mycb* promoter. (a) diagrammatic representation of 6kb *mycb* promoter with three putative Ascl1a binding sites. Arrows indicate primers for ChIP assay. (b) ChIP assay shaos Ascl1a binds to two proximal binding sites on *mycb* promoter.

3.1.4 Regulation of Ascl1a/Insm1a/Myc regulatory axis by c-Myc.

Next, we wanted to check the expression of various regeneration associated genes after either inhibiting Myc/Max interaction or electroporating *mycb* MO in the injured retinae. RNA was isolated from 2dpi retinae treated with 10058-F4 or electroporated with MO against *mycb* and subsequently, cDNA was prepared. qRT-PCR and RT-PCR showed both *mycb* and *ascl1a* mRNA levels to be reduced in a concentration-dependent manner with inhibition of Myc/Max interaction (Fig 3.19 a, b) or knocking down *mycb* with MO (Fig 3.19 c, d). To further verify this auto-regulation of *mycb*, mRNA *in situ* hybridization was carried out on sections of 4dpi retina treated with Myc/Max inhibitor (10058-F4) which showed reduced *mycb* expression (Fig 3.19 e). We reasoned that this might be a result of positive feedback through Ascl1a acting on *mycb* promoter.

Since Mycb is also well characterised as a transcriptional activator, and inhibition or knock-down of mycb resulted in reduced RNA level of *ascl1a*, we wanted to check if Mycb can physically interact with *ascl1a* promoter to regulate its expression. For this to be true, *mycb* and *ascl1a* should be expressed in the same cells. Double FISH of *mycb* and *ascl1a* along with BrdU IF proved that *ascl1a* and a subset of *mycb* positive cells are expressed in BrdU positive proliferating cells (3.20 a). To further validate this regulation, *ascl1a:gfp-luciferase* reporter construct was co-injected in fish embryos along with MO targeting *mycb*. This showed reduced *ascl1a* promoter activity (Fig 3.20 b). The same construct when co-injected along with *mycb* mRNA, showed increased promoter activity (Fig 3.20 c). In support of our hypothesis, 2kb promoter of *ascl1a* showed a putative Myc binding site very closely located near the start codon (Fig 3.20 d). ChIP assay revealed a physical binding of Mycb to the site on the *ascl1a* promoter (Fig 3.20 e).

Next, we wanted to check the expression profile of other regeneration associated genes involved in the same regulatory pathway such as *max*, insm1a, or *myca.* qRT-PCR showed mRNA levels of *insm1a* (Fig 3.21 a-d) levels to be reduced and *max* and *myca* (Fig 3.22 a-d) levels to be increased significantly in Myc inhibited retinae at 2dpi. Taken together, these experimental findings show the existence of a stringent regulation between the genes in the Ascl1a/Insm1a/Myc axis.

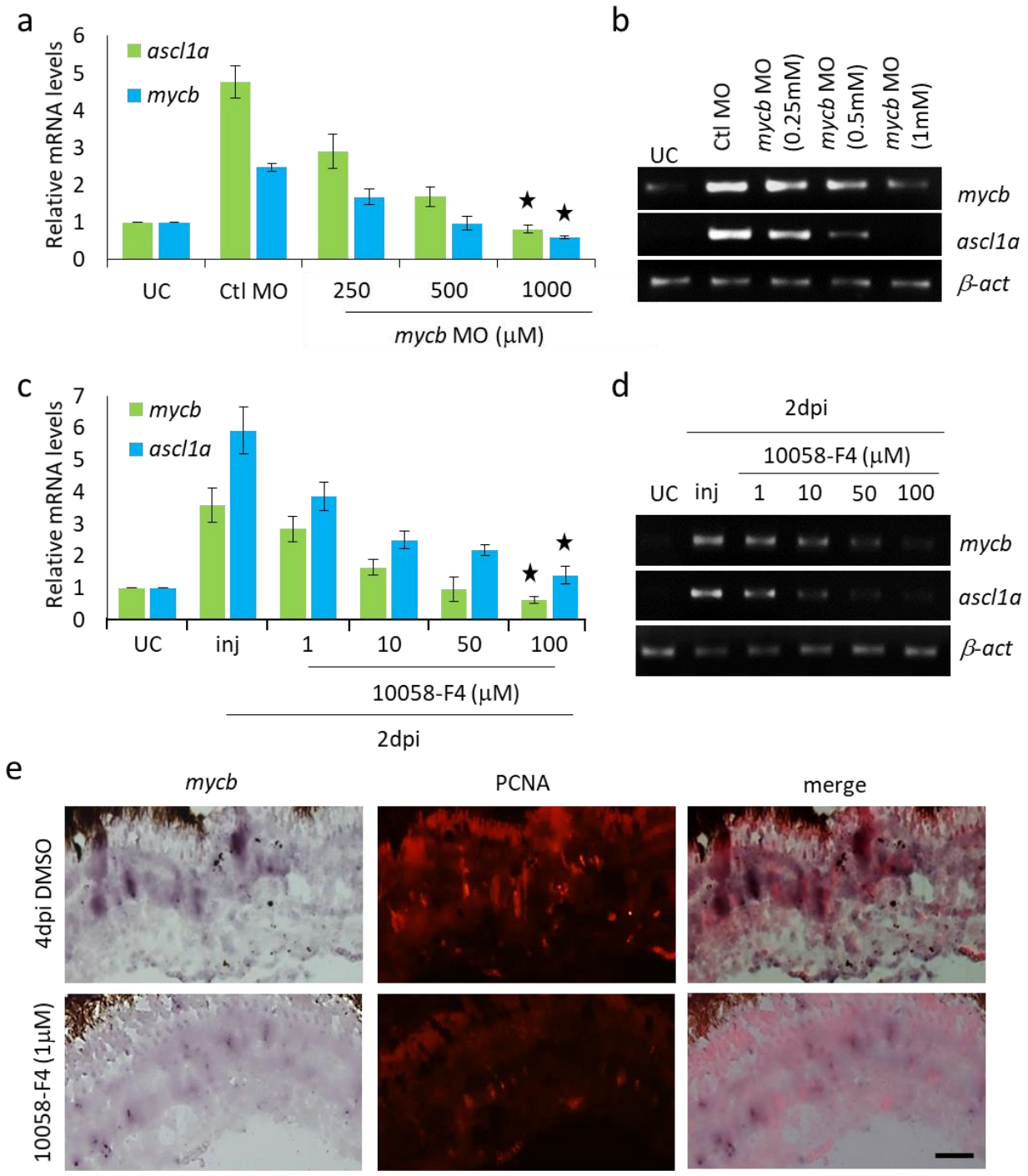


Fig 3.19: Regulation of *mycb* and *ascl1a* by Mycb. (a, b) qRT-PCR (a) and RT-PCR (b) show a dose-dependent downregulation of *mycb* and *ascl1a* mRNAs in 2dpi retinae electroporated with *mycb* targeting MO. (c, d) qRT-PCR (c) and RT-PCR (d) show a dose-dependent downregulation of *mycb* and *ascl1a* mRNAs in 2dpi retinae treated with increasing concentration of 10058-F4. (e) mRNA *in situ* hybridization shows decrease in the number of *mycb* positive cells in 4dpi retina treated with 10058-F4, compared with control. Scale bar 15µ. dpi: days post injury. $P < 0.002$.

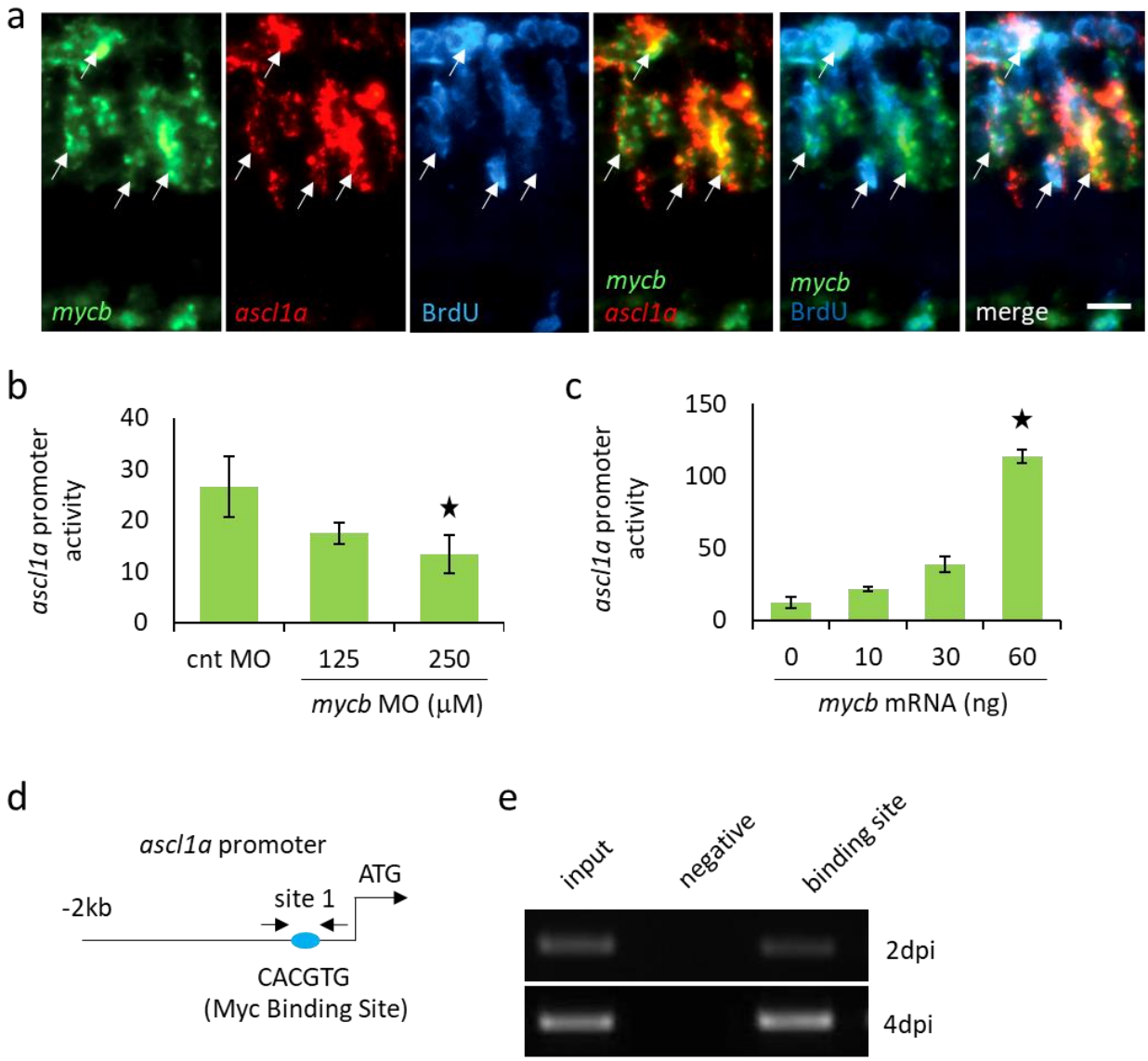


Fig 3.20: Direct regulation of *ascl1a* promoter by Mycb. (a) FISH assay of *ascl1a* and *mycb* followed by BrdU IF shows a subset of *mycb* positive cells colacalized with *ascl1a* positive proliferating cells marked by BrdU in 4dpi retina. Scale bar: 5µ. (b) promoter activity assay shows reduction of *ascl1a* promoter activity in embryos injected with *ascl1a:gfp-luciferase* and increasing concentration of *mycb* MO. (c) promoter activity assay shows induction of *ascl1a* promoter activity in embryos injected with *ascl1a:gfp-luciferase* and increasing concentration of *mycb* mRNA. (d) diagrammatic representation of 2kb *ascl1a* promoter bearing one Myc binding site. Arrows mark primer for ChIP assay (e) ChIP assay on *ascl1a* promoter with anti-Myca/b antibody shows direct binding of Mycb on *ascl1a* promoter.. P<0.02.

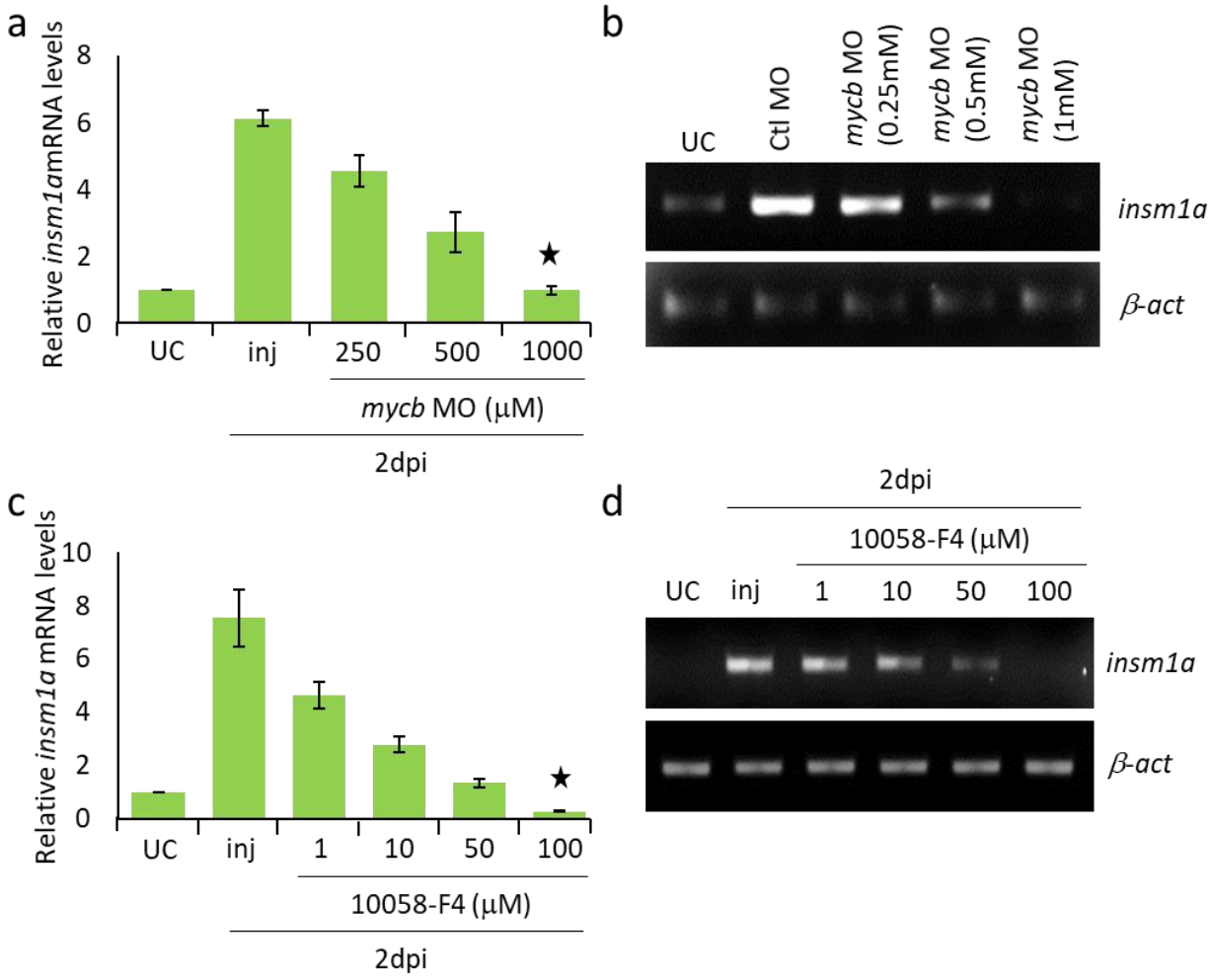


Fig 3.21: Myc regulates *insm1a*. (a, b) qRT-PCR (a) and RT-PCR (b) show a dose-dependent decrease in the relative mRNA fold change of *insm1a* in 2dpi retinae electroporated with increasing concentration of *mycb* targeting MO. (c, d) qRT-PCR (c) and RT-PCR (d) show dose dependent down-regulation of *insm1a* in 2dpi retinae treated with increasing concentration of Myc/Max blocker 10058-F4. $p<0.005$.

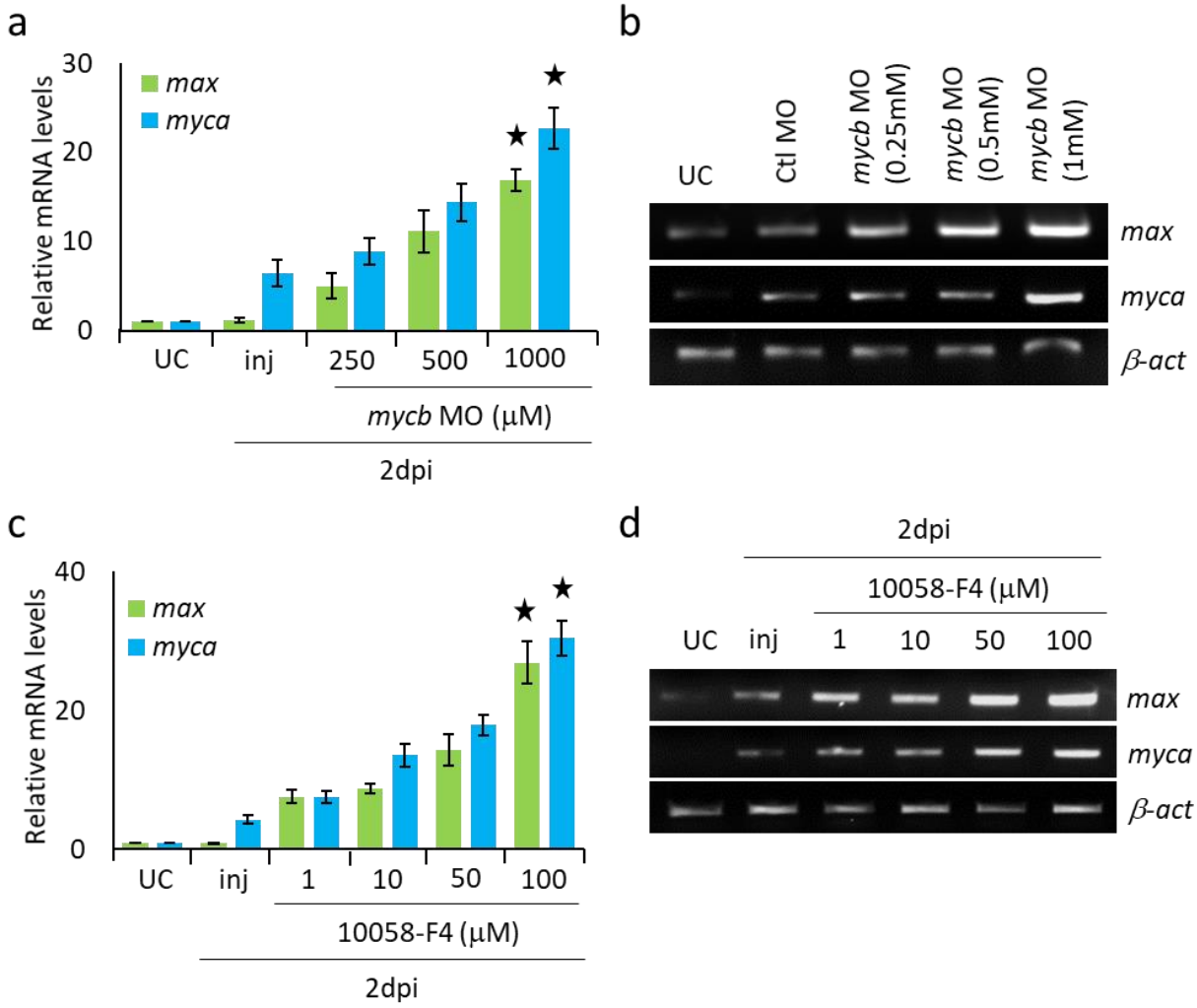


Fig 3.22: Myc regulates *max* and *myca*. (a, b) qRT-PCR (a) and RT-PCR (b) show a dose-dependent decrease in the relative mRNA fold change of *max* and *myca* in 2dpi retinae electroporated with increasing concentration of *mycb* targeting MO. (c, d) qRT-PCR (c) and RT-PCR (d) show dose dependent down-regulation of *max* and *myca* in 2dpi retinae treated with increasing concentration of Myc/Max blocker 10058-F4.

3.1.5 Regulation of *lin28a* and *her4.1* by c-Myc.

Another important regeneration-associated gene is *lin28a* which was previously shown to be induced by Ascl1a in zebrafish retina regeneration. Other studies also have shown *lin28a* to be induced by Myc. But this regulation in zebrafish retina regeneration was not characterised. Double FISH followed by BrdU IF on 4dpi retinae showed *lin28a* positive cells colocalized with only a subset of *mycb* positive cells (Fig 3.23 a). This prompted us for checking the expression level of *lin28a* in retinae either treated with Myc/Max inhibitor or electroporated MO against *mycb*. Surprisingly, in these two scenarios, we found an increased level of *lin28a* at 2dpi when compared with control (Fig 3.23 b-e). Further to validate this result, we performed fluorescent mRNA *in situ* hybridization (FISH) at 4dpi on sections of retina treated with various concentration of 10058-F4. The FISH assay showed that although BrdU positive cell number dropped significantly, expression of *lin28a* became pan retinal (Fig 3.24 a).

These seemingly contradicting results were also verified by carrying out promoter activity assay in embryos injected with *lin28a:gfp-luciferase* and *mycb* MO which resulted in increased *lin28a* promoter activity (Fig 3.25 a). To the much of our surprise, embryos injected with *mycb* mRNA and *lin28a:gfp-luciferase* also showed a concentration-dependent increase in promoter activity which was abrogated upon knocking down Aacl1a (Fig 3.25 b). This suggests that Mycb mediated induction of *lin28a* could be through upregulation of *ascl1a* by Mycb as we already have shown previously. To find out if Mycb binds to *lin28a* promoter directly, we looked for and found two putative Myc binding sites on 3kb upstream promoter region of *lin28a* (Fig 3.25 c). ChIP assay revealed physical binding of Myc to one of the binding sites, which is also proximal to the start codon (Fig 3.25 d). These results suggest Mycb can directly

bind on to the *lin28a* promoter. But, the exact mechanism by which Myc, being a *de facto* transcriptional activator, could down-regulate *lin28a*, still remained unknown.

Next, we wanted to decipher the possible way by which it can act as a repressor on *lin28a* promoter. Previous reports on Myc suggested its possible role as a repressor by collaborating with Hdacs. So, we wanted to check if Mycb physically interacts with Hdac1 and recruits it to *lin28a* promoter. In support of this hypothesis, co-immunoprecipitation with anti-Hdac1 antibody pulled down both Hdac1 and Mycb from injured retinae (Fig 3.25 e). Next, ChIP assay was carried out in 4dpi retinae using anti-Hdac1 antibody to figure out binding of Hdac1 on Mycb binding site on *lin28a* promoter. In this experiment, to amplify the site containing the binding sequence, we used the same set of primers that we used for ChIP of *lin28a* promoter using anti-Mycb antibody. ChIP assay could pull down the same DNA segment that was pulled down by anti-Mycb antibody, suggesting Hdac1 binds *lin28a* promoter on the same site as that of Mycb (Fig 3.25 f). These results suggest the involvement of Hdac1 in Mycb mediated regulation of *lin28a* during zebrafish retina regeneration.

Next, we wanted to check for the existence of Myc mediated regulation of *her4.1*, another regeneration-associated gene, which was previously demonstrated to be upregulated in retina regeneration by Notch signalling. qRT-PCR showed significant upregulation of *her4.1* in both Myc/Max inhibitor-treated retinae and *mycb* MO electroporated retinae at 2dpi, compared to 2dpi control (Fig 3.26 a-d). ISH showed a pan retinal expansion of *her4.1* in 10058-F4 treated 4dpi retinae which otherwise remained restricted to the injury responsive zone in control (Fig 3.27 a). Further, validating this observation, zebrafish embryos, injected with 10058-F4 and *her4.1:gfp-luciferase* reporter construct, showed increased promoter activity as opposed to reduced activity in embryos injected with Notch signalling inhibitor DAPT (Fig 3.28 a). Much like the regulation of *lin28a* by Mycb, *her4*.1 also showed increased mRNA levels in Myc blocked background suggesting a negative regulation of Myc acting on the *her4.1* promoter.

As Myc is a transcriptional activator, it cannot possibly exert an inhibitory effect on the transcription of *her4.1*. This led us to speculate the existence of a regulatory mechanism acting on *her4.1* promoter, similar to that of *lin28a,* which is mediated through the physical interaction of Myc and Hdac1. Validating our hypothesis, we indeed found three putative Myc binding sites on the 5kb promoter region of *her4.1* (Fig 3.28 b). To check if Myc and Hdac1 actually regulate this transcription by binding onto these sites, we pulled down chromatin with both anti-Myc and anti-Hdac1 antibody for ChIP assay from 4dpi retinae and found that Myc could precipitate all of these three sites whereas Hdac1 could do so for two of them (Fig 3.28 c).

Taken together, our data shows the importance of *myca* and *mycb* during retina regeneration which is they orchestrate by regulating a plethora of regeneration associated molecules and signalling pathways. These findings show how finely balanced is the whole process of cellular reprogramming during retina regeneration and probably would pave ways for future therapeutic interventions which aim towards successful retina regeneration in mammals.

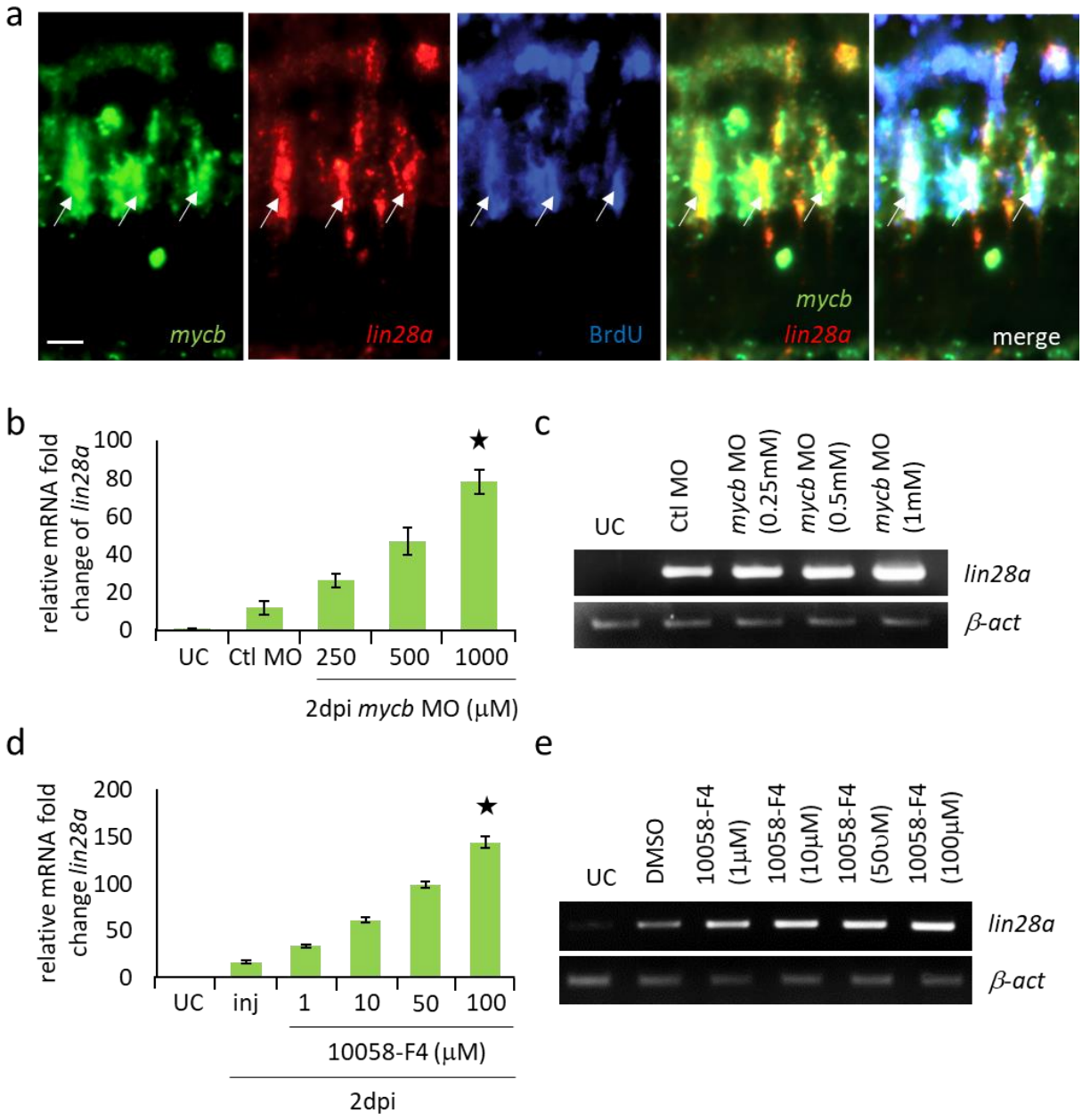


Fig 3.23: Mycb regulates *ln28a*. (a) Double FISH of *mycb* and *lin28a* followed by BrdU IF shows a subset of *mycb* positive cells colocalizes with *lin28a* positive proliferating cells as marked by BrdU. Scale bar is 5µ. (b, c) qRT-PCR (b) and RT-PCR (c) show upregulation of *lin28a* in a dose-dependent manner in 2dpi retinae electroporated with *mycb* targeting MO. (d, e) qRT-PCR (d) and RT-PCR (e) show upregulation of *lin28a* in 2dpi retinae treated with increasing concentration of 10058-F4. $p<0.005$.

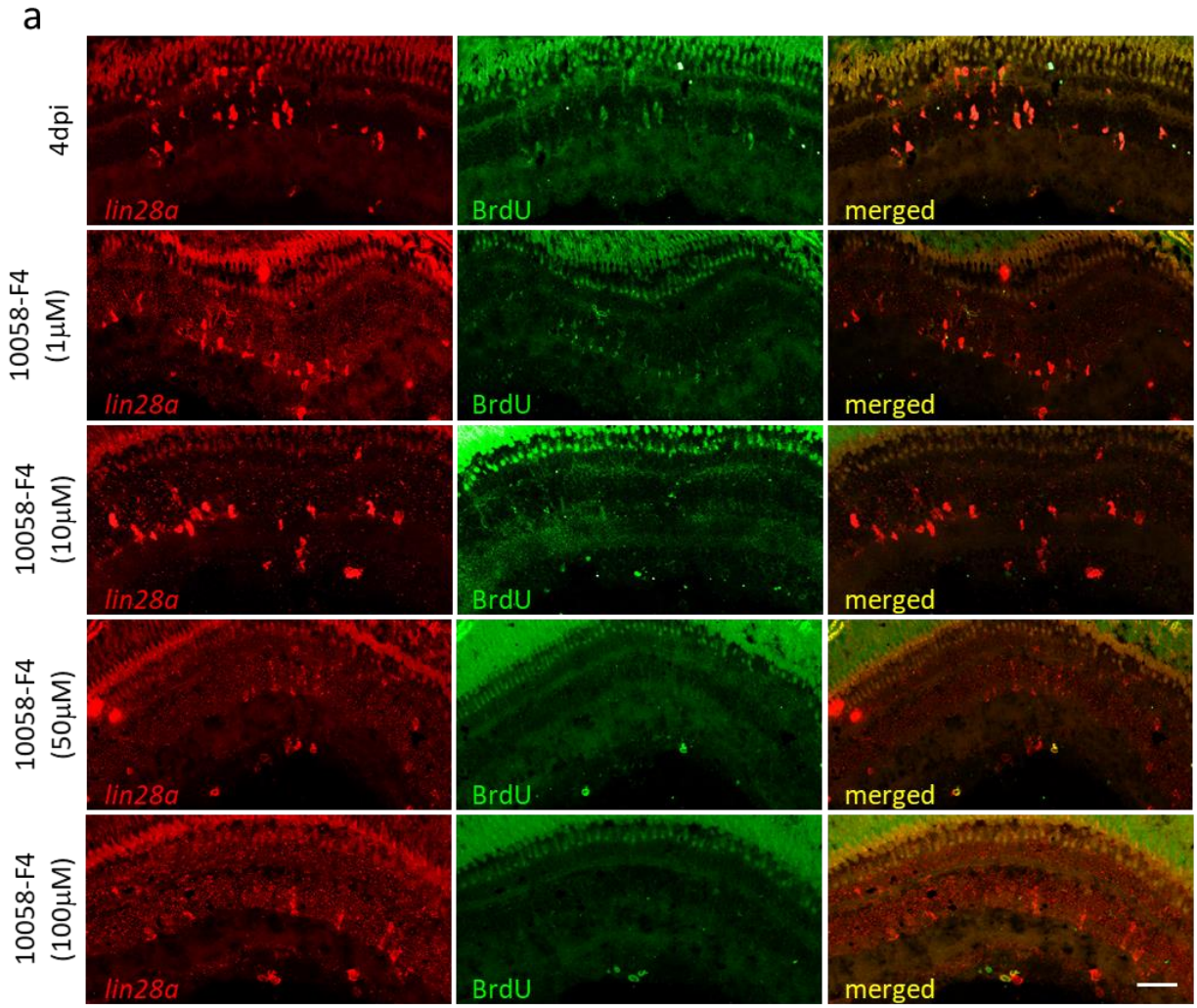


Fig 3.24: Pan-retinal upregulation of *lin28a* in Myc/Max interaction inhibited retinae. (a) FISH assay shows pan-retinal upregulation of *lin28a* mRNA in 4dpi retinae treated with increasing concentration of 10058-F4. Scale bar is 30μ.

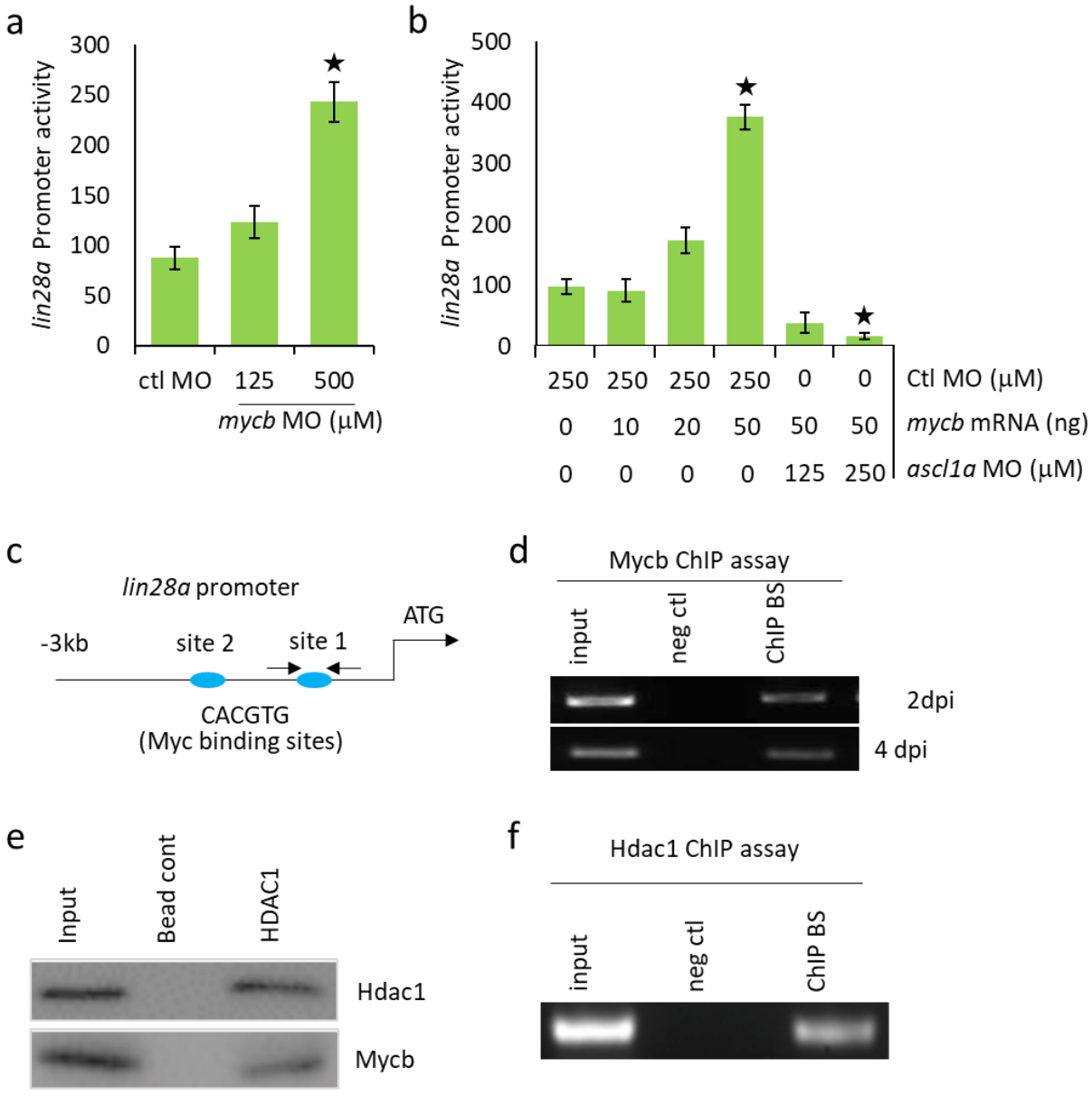


Fig 3.25: Mycb recruits Hdac1 to downregulate *lin28a* promoter. (a) induction of *lin28a* promoter activity in embryos injected with increasing concentration of *Mycb* MO. (b) induction of *lin28a* promoter activity in embryos injected with increasing concentration of *Mycb* mRNA which is abrogated by injecting increasing concentration of *ascl1a* MO. (c) diagrammatic representation of *lin28a* promoter with two putative Myc bnding sites. Primers for ChIP assay were designed for the proximal site as shown by black arrows. (d) ChIP assay with anti-Myc antibidy on *lin28a* promoter shows Mycb directly binds to *lin28a* promoter. (e) co-IP of HDAC1 and Myca/b shows Mycb and Hdac1 are parts of a same protein complex. (f) HDAC1 ChIP assay on Myc binding site on *lin28a* promoter shows Hdac1 is recruited at the Myc binding site on *lin28a* promoter. P<0.002.

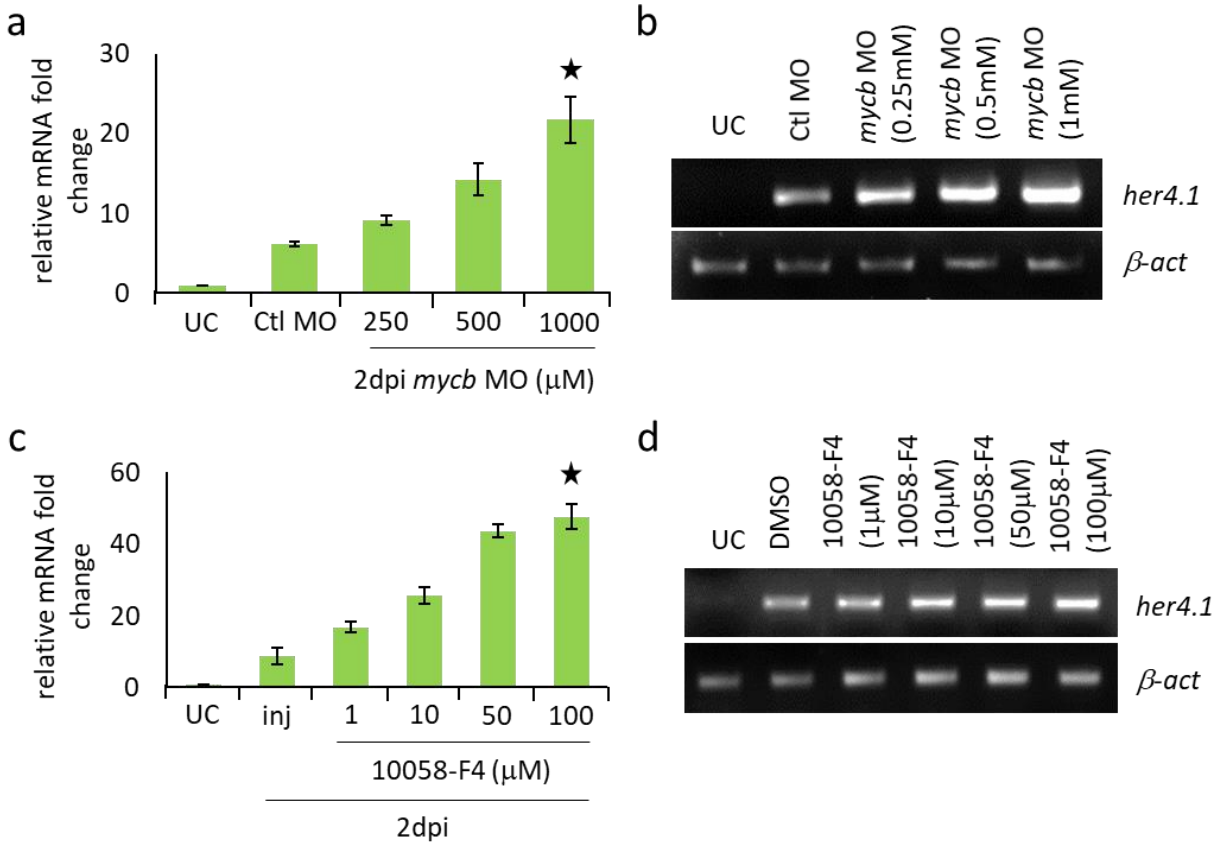


Fig 3.26: Mycb regulates *her4.1*. (a, b) qRT-PCR (a) and RT-PCR (b) show induction of *her4.1* in 2dpi retinae electroporated with various concentration of *mycb* targeting MO. (c, d) qRT-PCR (c) and RT-PCR (d) show upregulation of *her4.1* mRNA in 2dpi retinae treated with Myc/Max blocker 10058-F4. $p<0.001$.

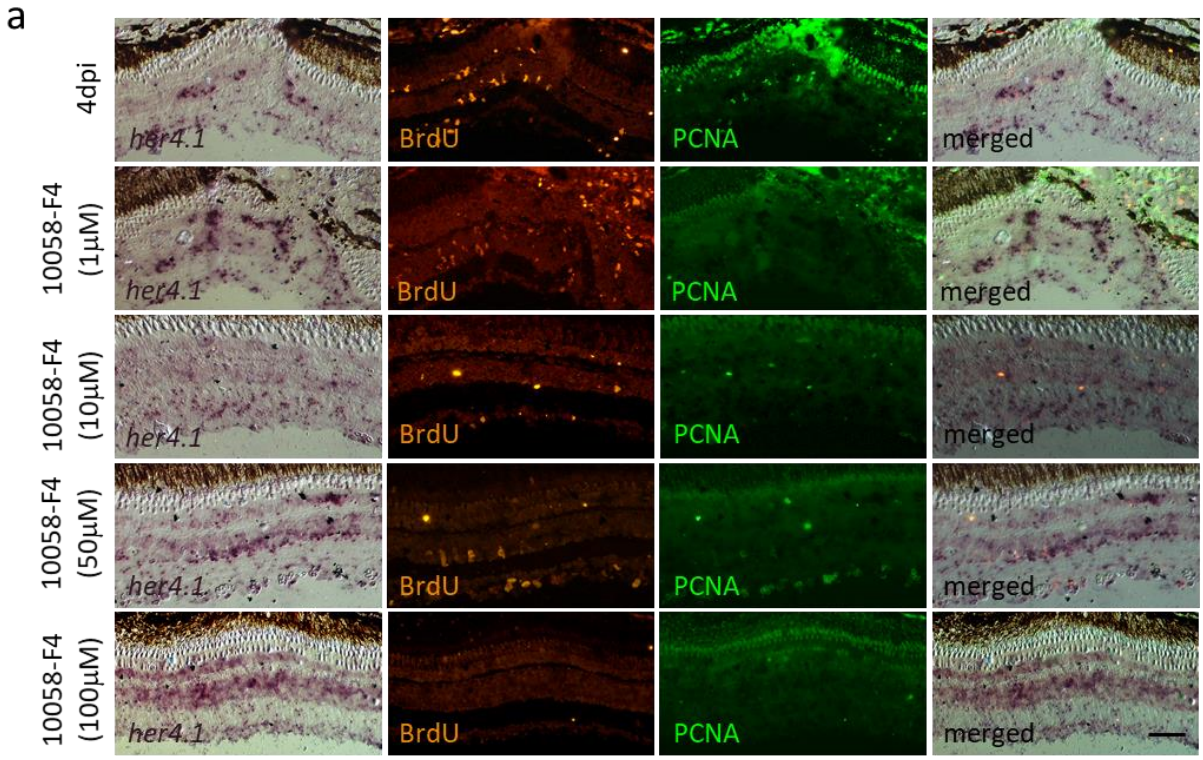


Fig 3.27: Pan-retinal upregulation of *her4.1* in 4dpi retinae with Myc/Max inhibitor. (a) mRNA *in situ* hybridization shows pan-retinal up-regulation of iher4.1 in 4dpi retinae treated with various concentrations of 10058-F4, compared with control. Scale bar: 35µ.

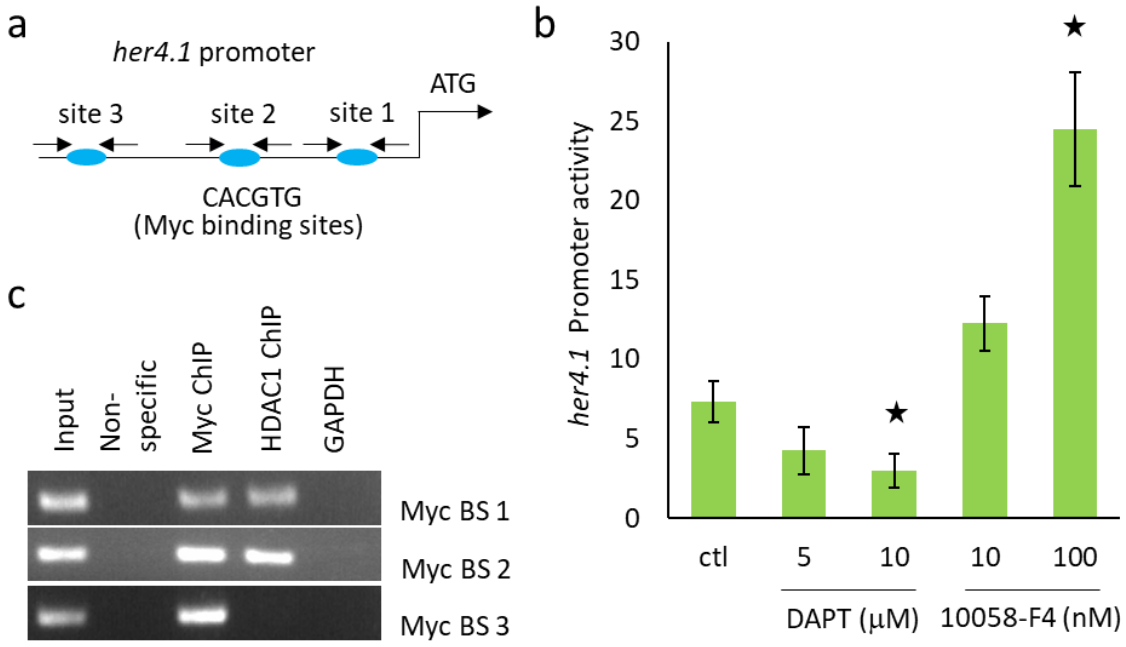


Fig 3.28: Myc recruits Hdac1 to downregulate *her4.1* promoter. (a) mRNA *in situ* hybridization shows pan-retinal up-regulation of iher4.1 in 4dpi retinae treated with various concentrations of 10058-F4, compared with control. (b) promoter activity assay from embryos injected with *her4.1:gfp-luciferase* reporter plasmid and increasing concentration of 110058-F4 shows a dose-dependent induction of *her4.1* promoter activity compared with reduced promoter activity in DAPT treated embryos. $P < 0.01$.

Chapter 2

Regulation of histone deacetylases (Hdacs) is essential for Muller glia de-differentiation and retina regeneration.

3.2.1 Hdac1 is regulated at transcriptional and translational level post retinal injury.

The physical interaction of Mycb and Hdac1 prompted us to check for the regulation of different *hdac*s post injury. We first wanted to check whether *hdac1* is regulated at transcriptional level after retinal injury. For this, retinae were harvested from uninjured and injured fish at various time points and cDNA was prepared to be used for PCR. qRT-PCR showed *hdac1* to be downregulated after retinal injury (Fig 3.29 a). Next, we wanted to check its spatial expression pattern by mRNA *in situ* hybridization in the uninjured retina and various time points after retinal injury. ISH showed a pan retinal expression pattern of *hdac1* in uninjured retinae which got down-regulated and became highly restricted to the injury responsive zone at 4dpi retinal sections (3.29 b). When compared in various time points post retinal injury, we found *hdac1* to be downregulated as soon as 6hpi, which then became restricted in injury responsive zone at 4dpi. Around 6dpi the expression of *hdac1* started to become pan retinal again (Fig 3.29 c). At 4dpi, although expression of *hdac1* seems to be associated with BrdU positive proliferating MGPCs a closer look revealed that only a subset of *hdac1* positive cells is BrdU or PCNA positive and mostly *hdac1* positive cells reside just beside proliferating cells (Fig 3.30 a-c).

We also checked the level of Hdac1 protein by immuno-fluorescence (IF) assay. Interestingly, although at 4dpi, *hdac1* expression is restricted to the injury site, IF of Hdac1 on 4dpi retinal sections revealed Hdac1 protein level is depleted in proliferating cells present in the injury responsive zone (Fig 3.31 a, b). This prompted us to hypothesize that upon injury, Hdac1 protein might be going down from the dedifferentiated cells in order to allow them to undergo proliferation. After the proliferative phase, Hdac1 protein level should again come up in order

to get the cells out of the cell cycle as a prelude to re-differentiation. To check if this hypothesis is true, we performed Hdac1 immunostaining on 2dpi retinal sections along with parallel BrdU immunostaining and found almost full colocalization of BrdU and Hdac1 with very less exclusion of them (Fig 3.32 a, b). Similarly, at 6dpi retinal sections, we also found no exclusion of Hdac1 from BrdU positive cells (Fig 3.33 a, b). This suggests Hdac1 is needed to be absent from proliferative cells.

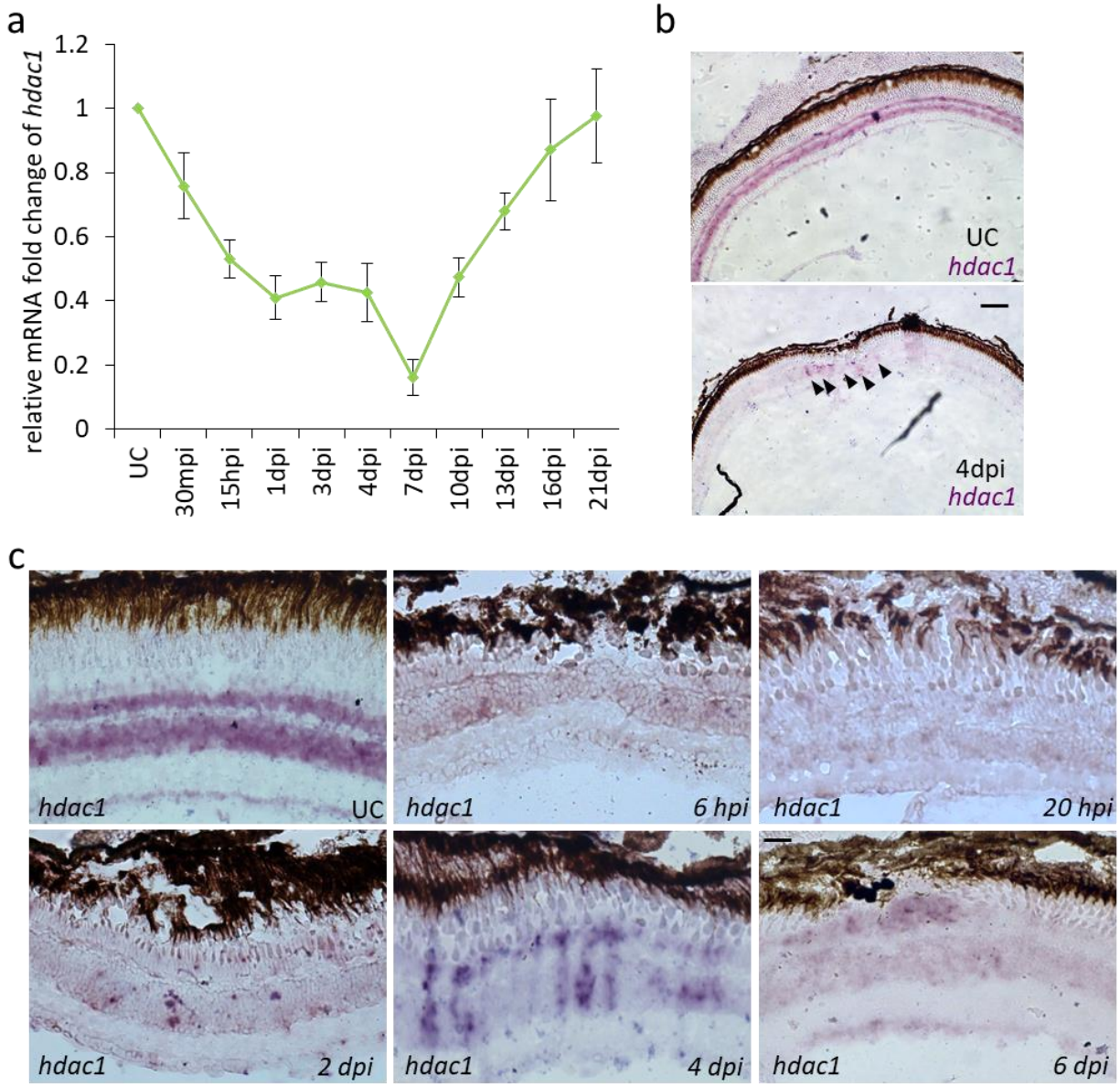


Fig 3.29: temporal and spatial expression pattern of *hdac1* at different time points post retinal injury. (a) qRT-PCR shows temporal expression pattern of *hdac1* at different time points post retinal injury. (b) mRNA ISH of *hdac1* in uninjured and 4dpi retinae shows downregulation of *hdac1*. Scale bar: 70µ. (c) spatial expression pattern of *hdac1* at various time points post retinal injury shown by mRNA ISH. Scale bar: 15µ.

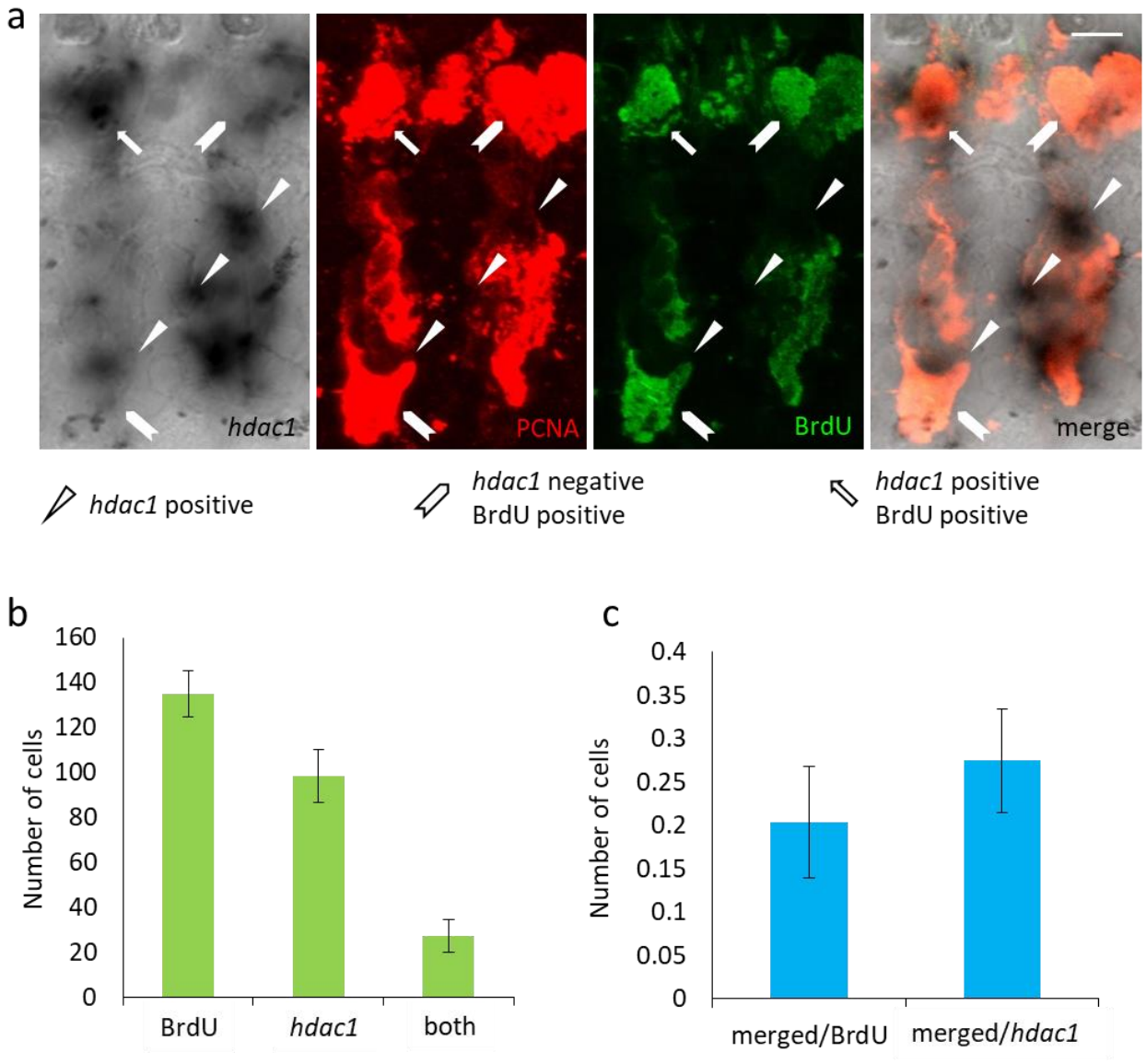


Fig 3.30: The *hdac1* positive cells and proliferating cells shows mutual exclusion at 4dpi. (a) high magnification confocal microscopy image of *hdac1*+ve cells with BrdU and PCNA IF shown mutual exclusion. Scale bar: 5µ. (b, c) quantification of the same.

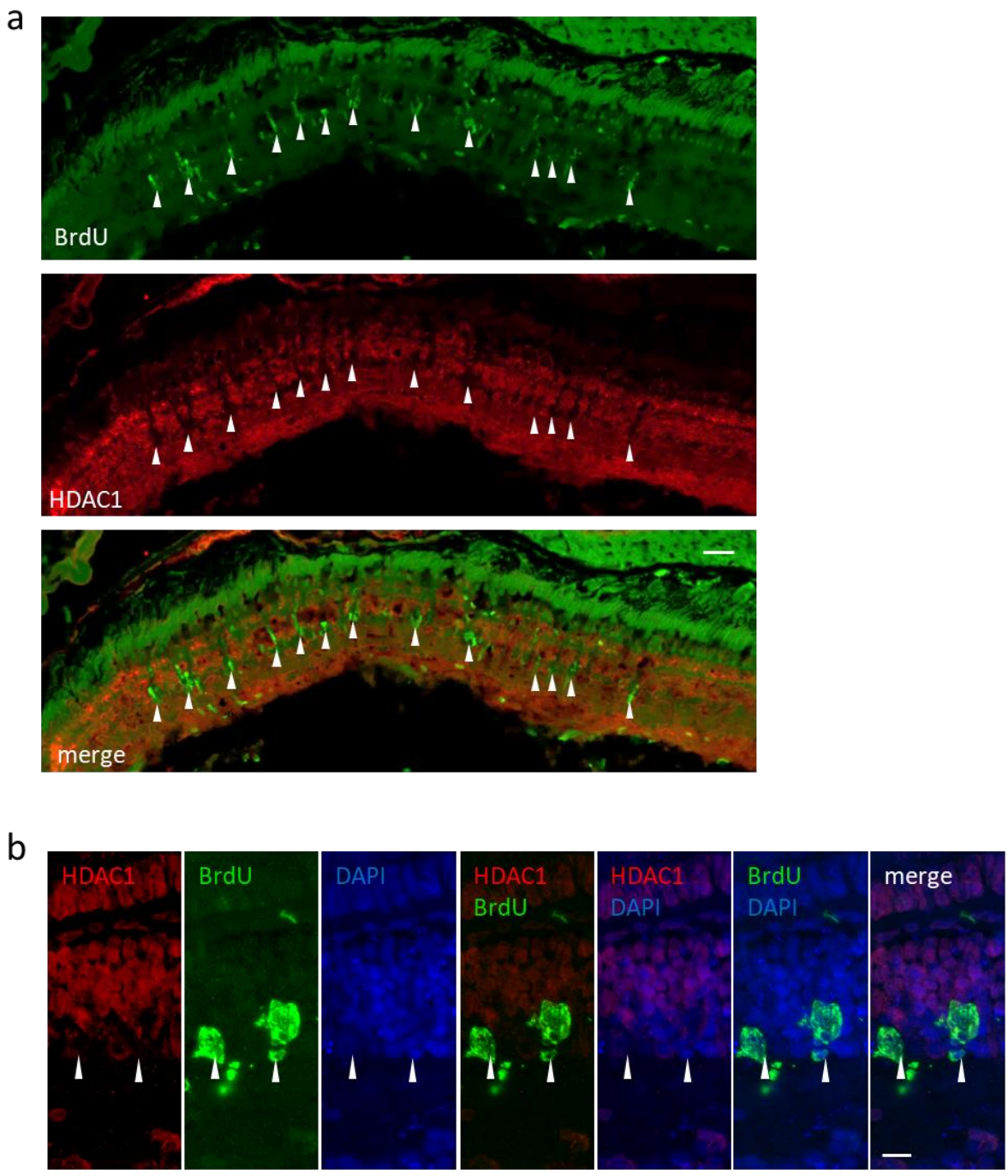


Fig 3.31: HDAC1 IF at 4dpi retinal sections. (a) HDAC1 IF at 4dpi retinal sections shows exclusion of HDAC1 from BrdU positive proliferating cells. Scale bar: 35µ. (b) high magnification confocal microscopy shows mutual exclusion of HDAC1 positive cells and BrdU positive proliferating cells. Scale bar: 5µ.

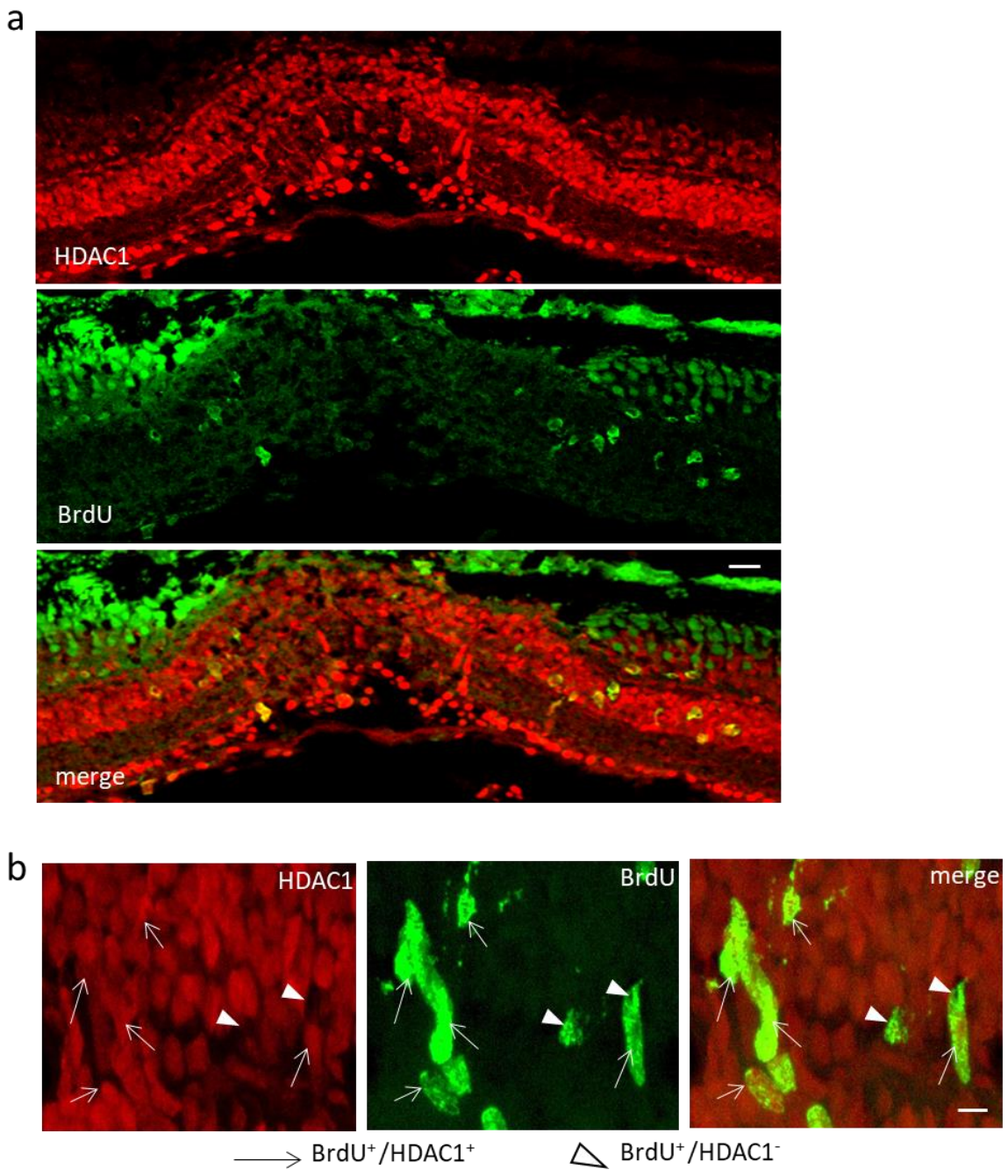


Fig 3.32: HDAC1 IF at 2dpi retinal sections. (a) HDAC1 IF at 2dpi retinal sections shows very less or almost no exclusion of HDAC1 from BrdU positive proliferating cells. Scale bar: 35µ. (b) high magnification confocal microscopy shows very less mutual exclusion (indicated bt arrowheads) of HDAC1 positive cells and BrdU positive proliferating cells. Scale bar: 5µ.

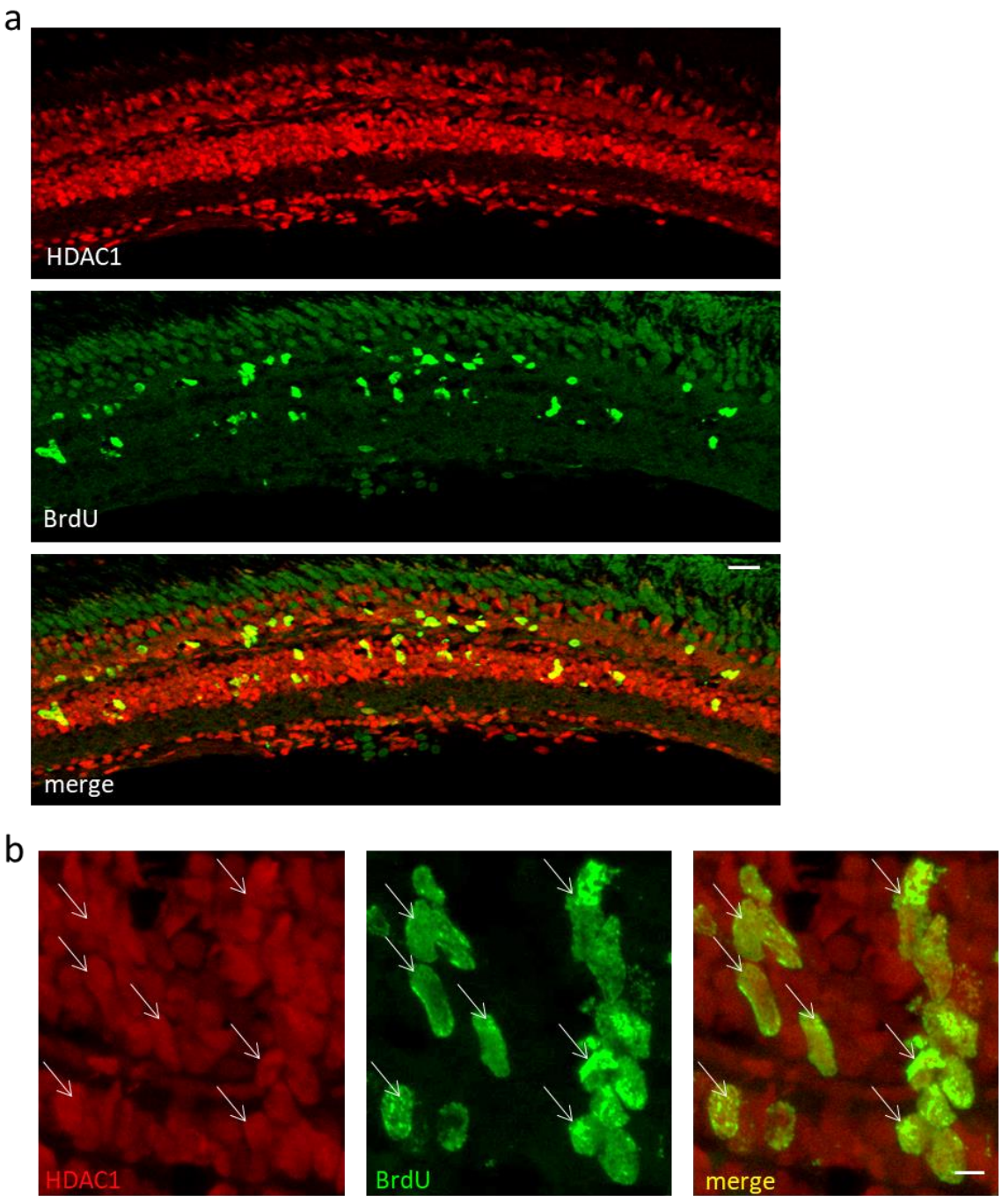


Fig 3.33: HDAC1 IF at 6dpi retinal sections. (a) HDAC1 IF at 2dpi retinal sections shows no exclusion of HDAC1 from BrdU positive proliferating cells. (b) high magnification confocal microscopy shows no mutual exclusion of HDAC1 positive cells and BrdU positive proliferating cells.

3.2.2 Regulation of other Hdacs post retinal injury.

Next, we wanted to check the expression pattern of other *hdac*s. We found that, except for *hdac2*, zebrafish possesses all other *hdac*s that is present in mammalian systems. These can be broadly classified into four classes. Class I hdacs are *hdac1, hdac2*, *hdac4*, and *hdac8*. *hdac4, hdac5, hdac7* and *hdac9* belong to class IIa. Class IIb consists of *hdac6* and *hdac10. hdac11* belongs to class IV (Fig 3.34 a). First, we wanted to check if these *hdac*s are regulated at transcriptional level post retinal injury.

To check the temporal expression pattern of *hdac3* post retinal injury, we performed qRT-PCR from cDNAs prepared from RNA isolated at various time points after injury. In qRT-PCR, *hdac3* showed a significant upregulation post retinal injury when compared to uninjured control (Fig 3.35 a) further to check spatial expression pattern of *hdac3*, mRNA ISH was performed in uninjured as well as injured retinae harvested at various time points post injury. *hdac3* shows a basal level of expression in uninjured retinae, which was induced at injury responsive zone at 6hpi and remained high till 2 and 4dpi. At 4dpi, *hdac3* was upregulated in the injury responsive zone along with its basal level throughout the retina (Fig 3.35 b). A closer look at 4dpi retinal sections bearing *hdac3* positive cells showed only a subset of them to be proliferating (Fig 3.35 c-e) as marked by BrdU and PCNA immunostaining.

We further checked for the temporal expression pattern of hdac4 by qRT-PCR. The *hdac4* mRNA was found to be down-regulated twice during a span of three weeks after injury, namely during the de-differentiation and differentiation phase. The expression of hdac4 in 4dpi seemed similar to that of uninjured retinae (Fig 3.36 a). The spatial expression pattern of *hdac4* in the uninjured and 4dpi retina was also found to be similar (Fig 3.36 b). ISH assay of *hdac4* and

BrdU/PCNA co-localization showed no significant correlation between ISH signal and proliferation in 4dpi retina (Fig 3.36 c-e).

qRT-PCR showed significant upregulation of *hdac5* immediately after retinal injury which was reduced by 1dpi and remained so till 21dpi (Fig 3.37 a). In agreement with this observation, mRNA ISH showed comparable but very low and level of *hdac5* in the uninjured and 4dpi retina (Fig 3.37 b). BrdU immunostaining at 4dpi retina also showed that this low expression of *hdac5* did not have any correlation with proliferation (Fig 3.37 c-e).

The mRNA of *hdac6* showed an interesting upregulation in transcript level after retinal injury (Fig 3.38 a) which was supported by mRNA ISH at 4dpi retinal section (Fig 3.38 b). Interestingly, BrdU immunostaining on 4dpi retinal sections showed a strong negative correlation of proliferation and mRNA signal (Fig 3.38 c-e).

Next, we checked the temporal expression pattern of *hdac9* by qRT-PCR which showed an expression pattern similar to that of *hdac4.* The mRNA of *hdac9* was found to be downregulated twice during the regeneration process and showed similar expression level in 4dpi retinae compared with uninjured retinae (Fig 3.39 a). The mRNA ISH of *hdac9* at uninjured and 4dpi retinae also showed similar levels of its transcript throughout the retina. (Fig 3.39 b). Interestingly BrdU immunostaining and mRNA ISH showed negative correlation between mRNA signal and proliferation (Fig 3.38 c-e).

a

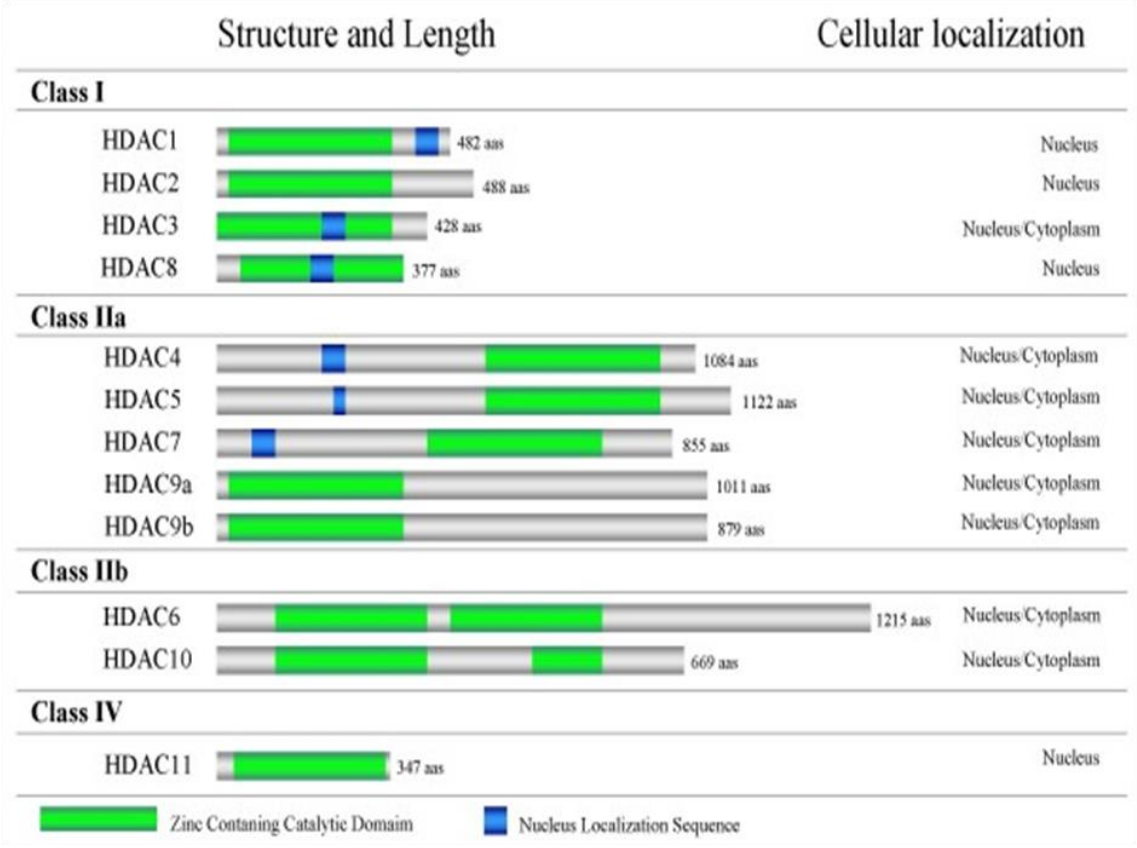


Fig 3.34: Classification of mammalian HDACs. (a) four classes of mammalian HDACs with their domain structures. Taken from Lucio et al., 2008.

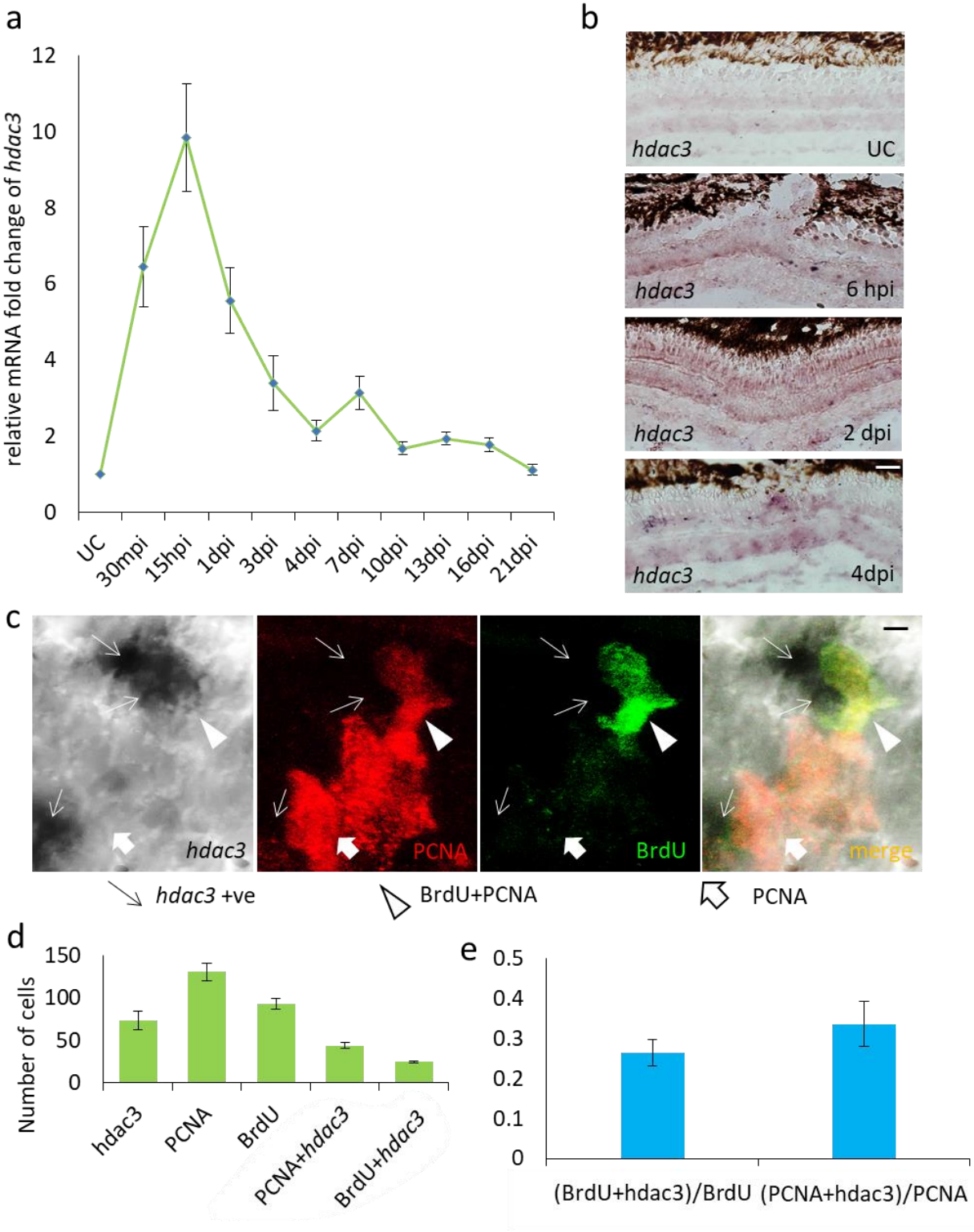


Fig 3.35: Regulation of *hdac3* post retinal injury. (a) qRT-PCR shows upregulation of *hdac3* post retinal injury. (b) spatial expression pattern of *hdac3* as shown by mRNA ISH in retinal sections at different time points post injury. Scale bar: 25µ.(c) high magnification confocal microscopy shows only a subset of *hdac3* positive cells colocalized with proliferating cells. Scale bar: 5µ. (d, e) quantification of the same.

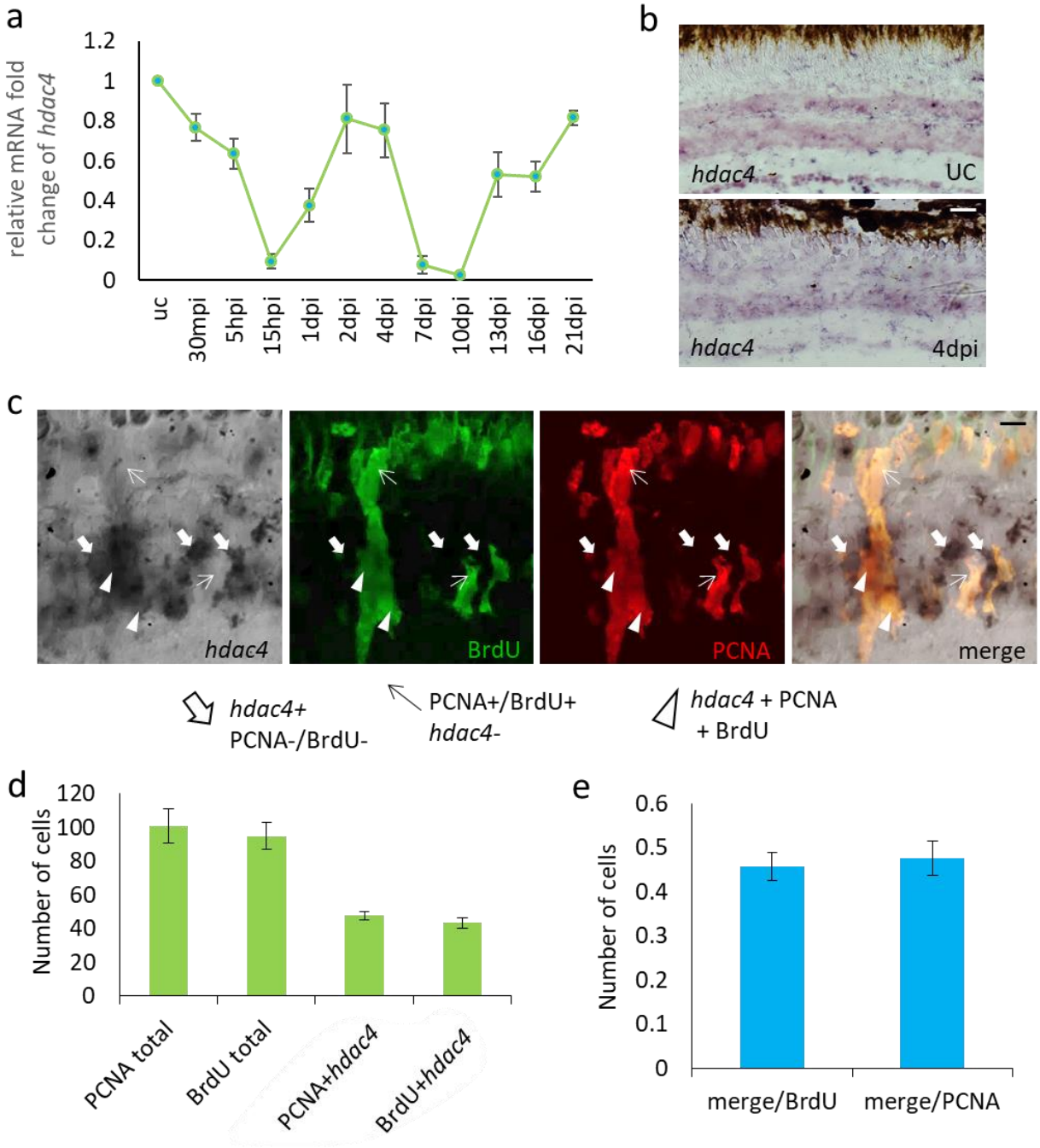


Fig 3.36: Regulation of *hdac4* post retinal injury. (a) qRT-PCR shows upregulation of *hdac4* post retinal injury. (b) spatial expression pattern of *hdac4* as shown by mRNA ISH in uninjured and 4dpi retinal sections. Scale bar: 25µ. (c) high magnification confocal microscopy shows a subset of *hdac4* positive cells colocalized with proliferating cells. Scale bar: 5µ. (d, e) quantification of the same.

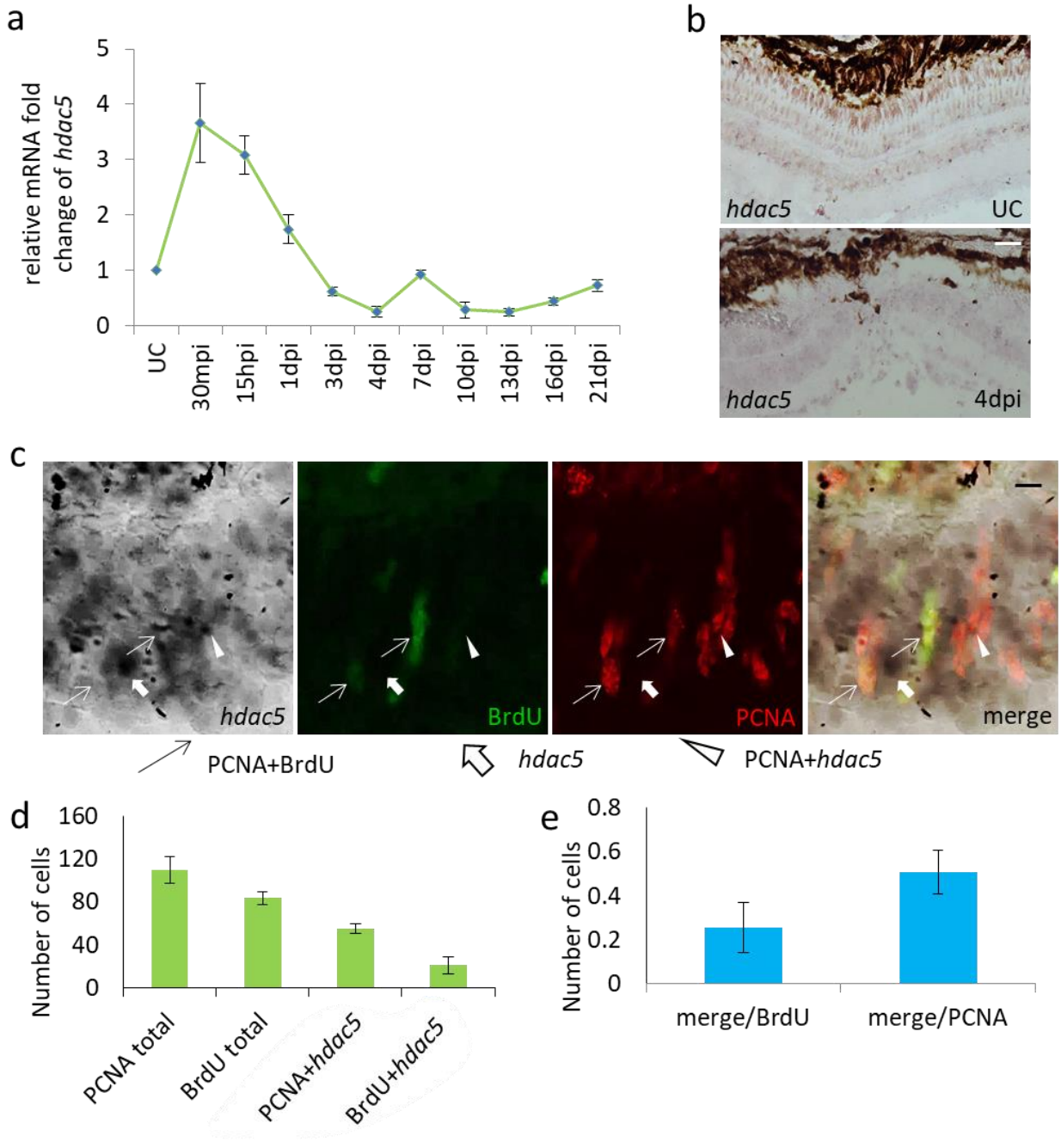


Fig 3.37: Regulation of *hdac5* post retinal injury. (a) qRT-PCR shows upregulation of *hdac5* post retinal injury. (b) spatial expression pattern of *hdac5* as shown by mRNA ISH in uninjured and 4dpi retinal sections. Scale bar: 25µ. (c) high magnification confocal microscopy shows a subset of *hdac5* positive cells colocalized with proliferating cells. Scale bar: 5µ. (d, e) quantification of the same.

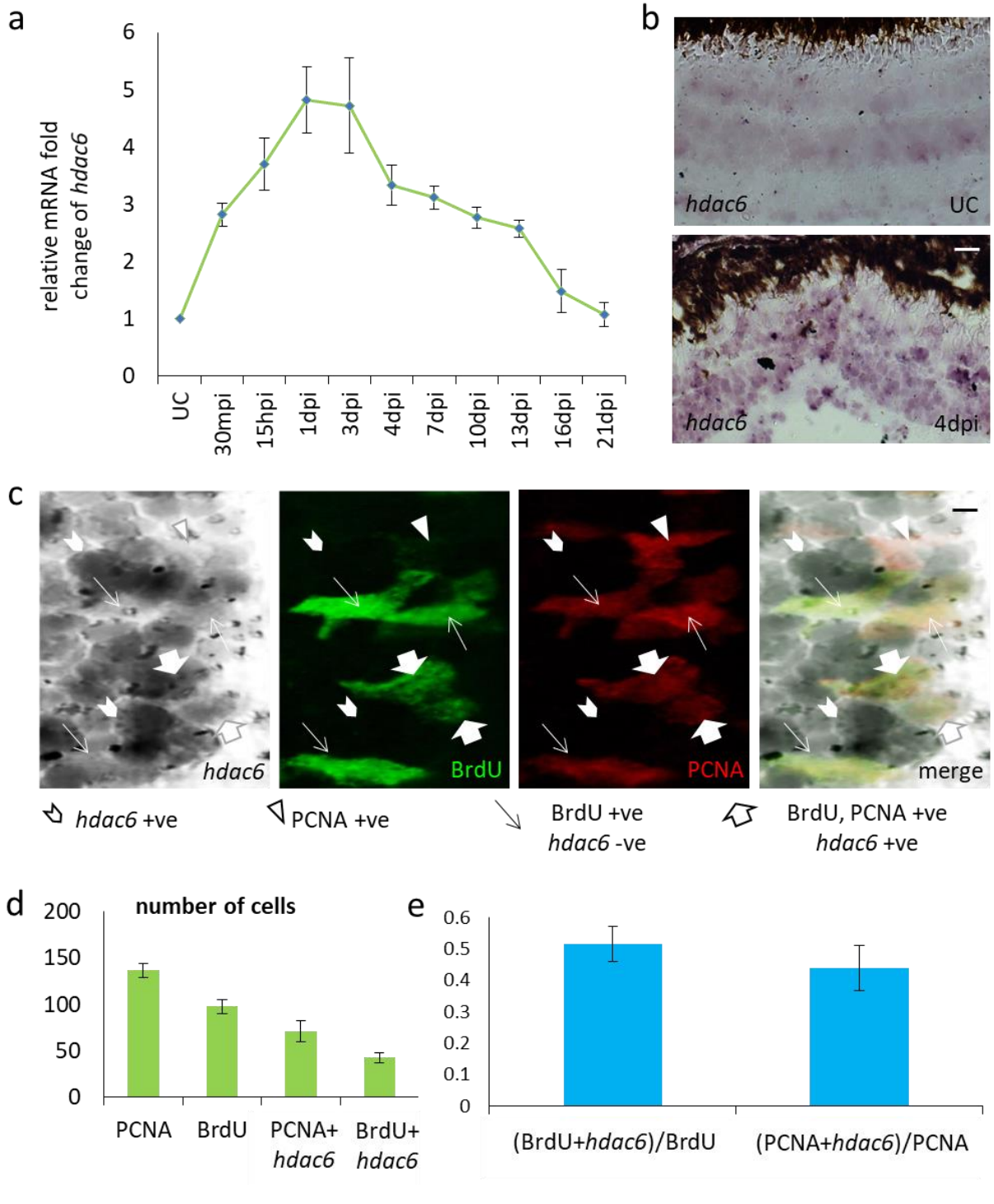


Fig 3.38: Regulation of *hdac6* post retinal injury. (a) qRT-PCR shows upregulation of *hdac6* post retinal injury. (b) spatial expression pattern of *hdac6* as shown by mRNA ISH in uninjured and 4dpi retinal sections. Scale bar: 25µ (c) high magnification confocal microscopy shows a subset of *hdac6* positive cells colocalized with proliferating cells. Scale bar: 5µ. (d, e) quantification of the same.

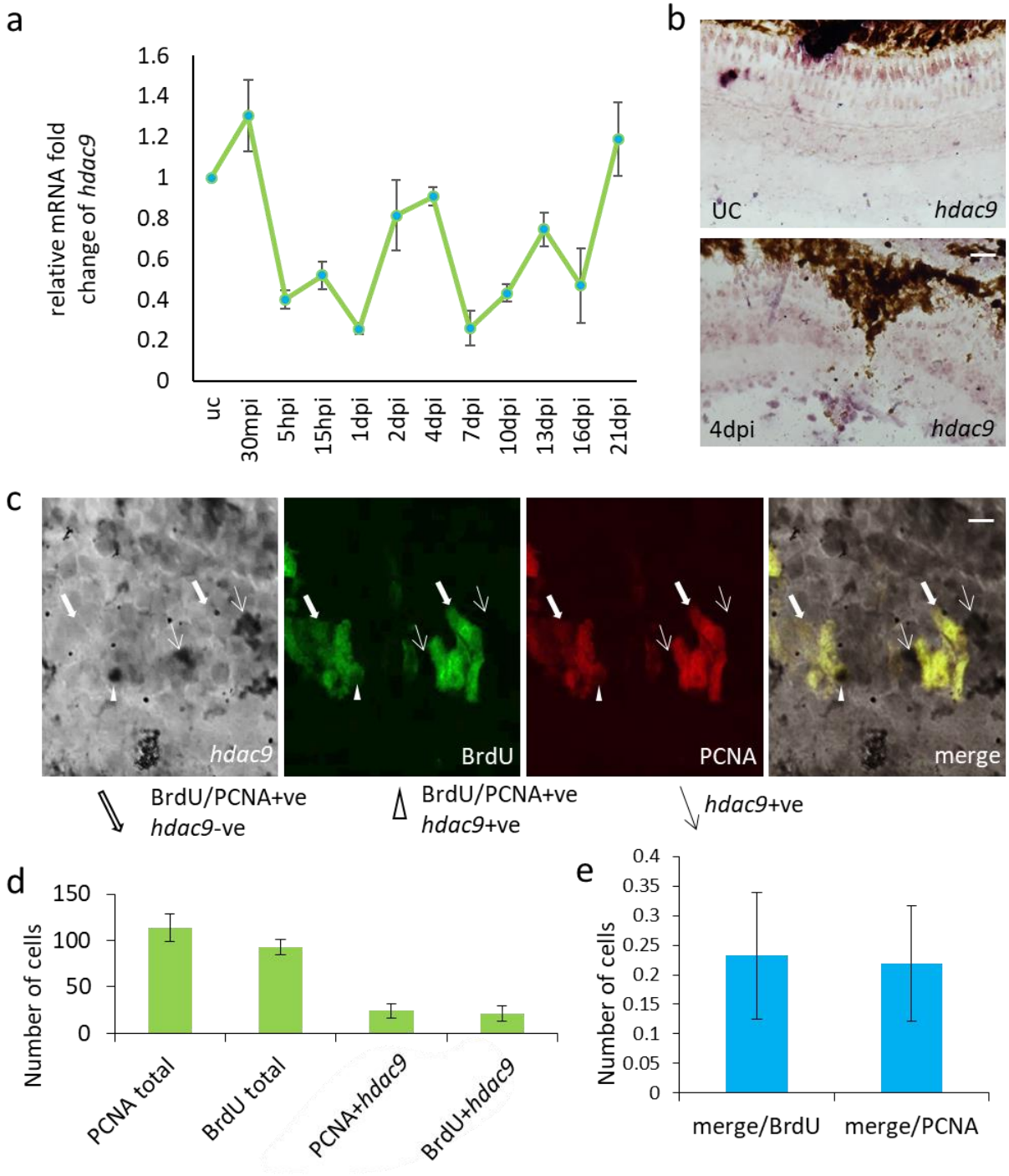


Fig 3.39: Regulation of *hdac9* post retinal injury. (a) qRT-PCR shows upregulation of *hdac9* post retinal injury. (b) spatial expression pattern of *hdac9* as shown by mRNA ISH in uninjured and 4dpi retinal sections. Scale bar: 25µ. (c) high magnification confocal microscopy shows a subset of *hdac9* positive cells colocalized with proliferating cells. Scale bar: 5µ. (d, e) quantification of the same.

3.2.3 Inhibition of HDACs deregulates proliferation during retina regeneration.

Since HDACs are known to downregulate transcriptions by rendering the chromatin unavailable for transcription machinery, we hypothesized; pharmacological inhibition of HDACs might cause *de novo* expression of regeneration-associated genes and subsequently might cause proliferation even without injury. To check this hypothesis, we employed inhibitor-based approach for preventing Hdacs' functionality. Various pharmacological inhibitors of HDACs are available commercially, of which, we chose Trichostatin A (TSA, pan-HDAC inhibitor), Valproic acid (VPA, class I and IIa inhibitor) and Sodium butyrate (NaB, class I and IIa inhibitor) for inhibiting HDACs. First, we checked whether these inhibitors are effective by checking the levels of Histone4 acetylation. For this purpose, we chose to estimate the level of acetylated Histone4 (Ac.H4) after treatment with VPA or TSA in retinae by performing Western blotting. We found an increased level of Ac.H4 in 2dpi retinae treated with either VPA or TSA (Fig 3.40 a). Once sure of the effectiveness of the inhibitors, fish were dipped in solutions of VPA (100μM) and NaB (100μM) for 4 days and uninjured retinae were harvested after 4-5 hours of BrdU pulsing, fixed with 4% PFA, sectioned and subsequently was used for BrdU (Fig 3.40 b). Surprisingly we found no proliferating cells in the inner nuclear layer in uninjured VPA and NaB treated retinae (Fig 3.40 c, d).

Next, we hypothesized that in the injured retina, VPA, TSA or NaB treatment for 4 days post retinal injury would cause increased proliferation at 4dpi. For this Fish were either dipped in VPA (50μM and 100μM) or NaB (100μM and 300μM) or received an intra-vitreous injection of TSA (0.1μM and 1μM), for 4days post retinal injury followed by BrdU pulsing (Fig 3.41 a).

But, much to our surprise, all of them caused a significant concentration-dependent reduction in the number of proliferating cells (Fig 3.41 b-g).

To check, whether the residual proliferating cells at 4dpi can migrate to all retinal cell layers; or in other words, whether these cells retain normal regenerative capacity, fish were dipped into 100μM VPA solutions (experimental set) or water (control) post retinal injury for 4days followed by 4-5 hours of BrdU pulsing for 4^{th} and 5^{th} day to label the residual proliferating cells after treatment with Hdacs' inhibitors. In one set, retinae were harvested at 5^{th} day (Fig 3.42 a). In another set, after BrdU pulsing for 2 days, fish were shifted to water and retinae were harvested at 30^{th} day as at this time, normal proliferating cells are supposed to be migrated to different retinal cell layers (Fig 3.42 c). At 5dpi, we found less number of BrdU positive cells as expected (Fig 3.42 b, e, f). But at 30dpi, although less in number compared to that of control, we found BrdU positive cells in all different retinal cell layers (Fig 3.42 c, e, f) suggesting that these few residual proliferating cells can migrate to different retinal cell layers.

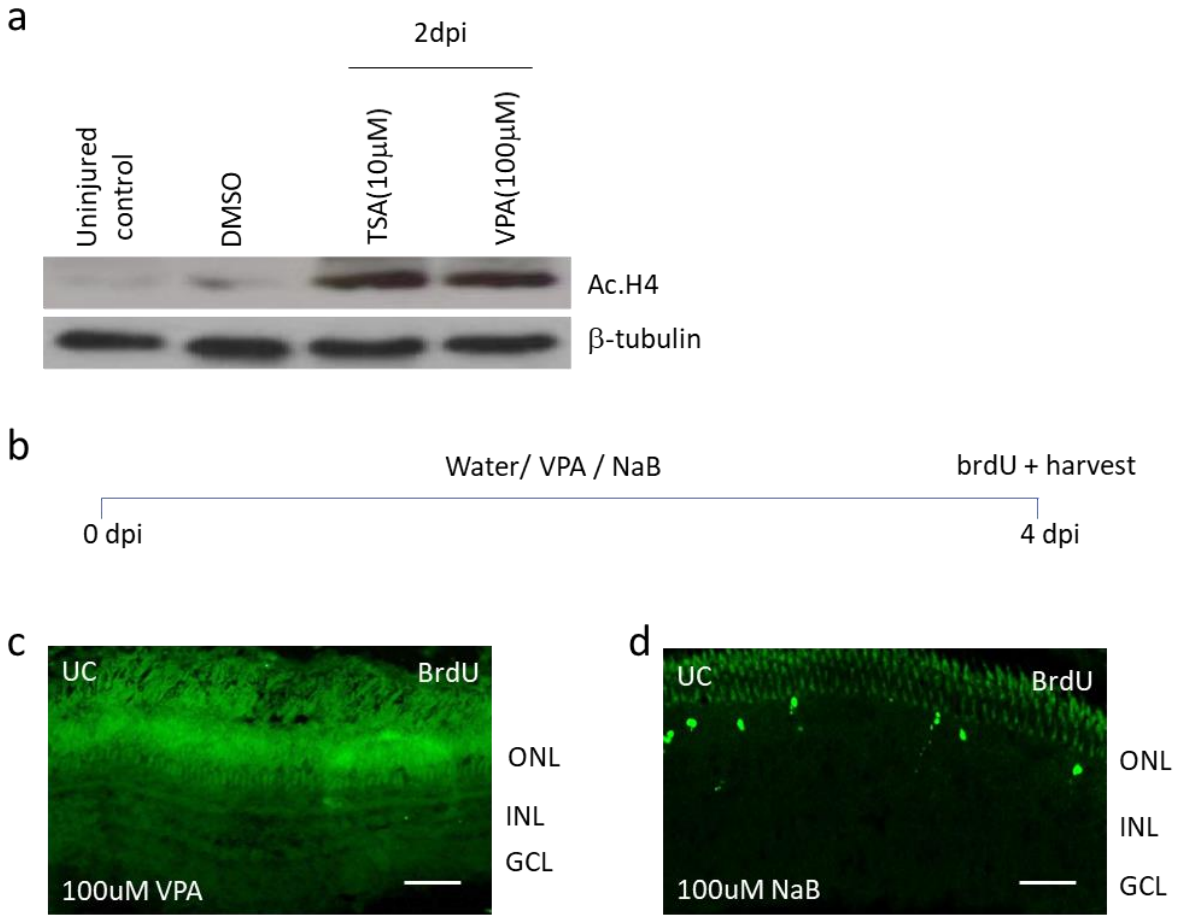


Fig 3.40: Inhibiting HDACs in uninjured retinae does not cause *de novo* proliferation. (a) Western blot analysis shows increased Ac.H4 in TSA and VPA treated 2dpi retinae. (b) experimental time-line demonstrating the treatment of VPA o NaB in uninjured retinae. (c, d) BrdU IF shows no BrdU positive cells in the INL in VPA (c) or NaB (d) treated uninjured retinae. Scale bar: 35μ.

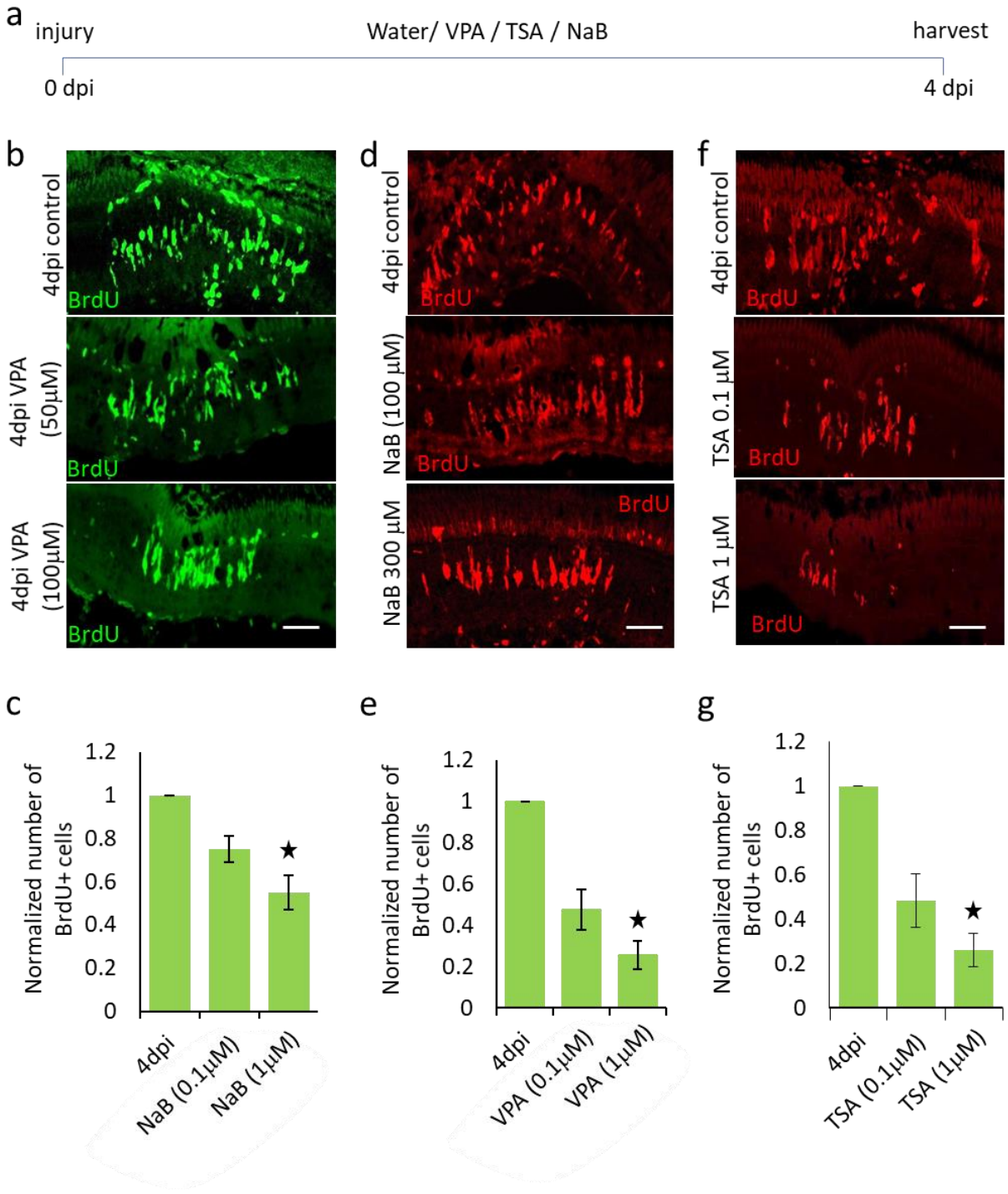


Fig 3.41: Fig 3.41: Continuous inhibition of Hdacs in injured retinae reduces proliferation at 4dpi. (a) experimental regime of treating uninjured fish with VPA or Nab for 4 days. (b, d, f) Nab (b) or VPA (d) or TSA (f) treated retinae after BrdU IF assay shows a significant decrease in the number of BrdU positive cells. (c, e, g) quantification of the same. $P < 0.002$. Scale bar: 35µ in b, d and f.

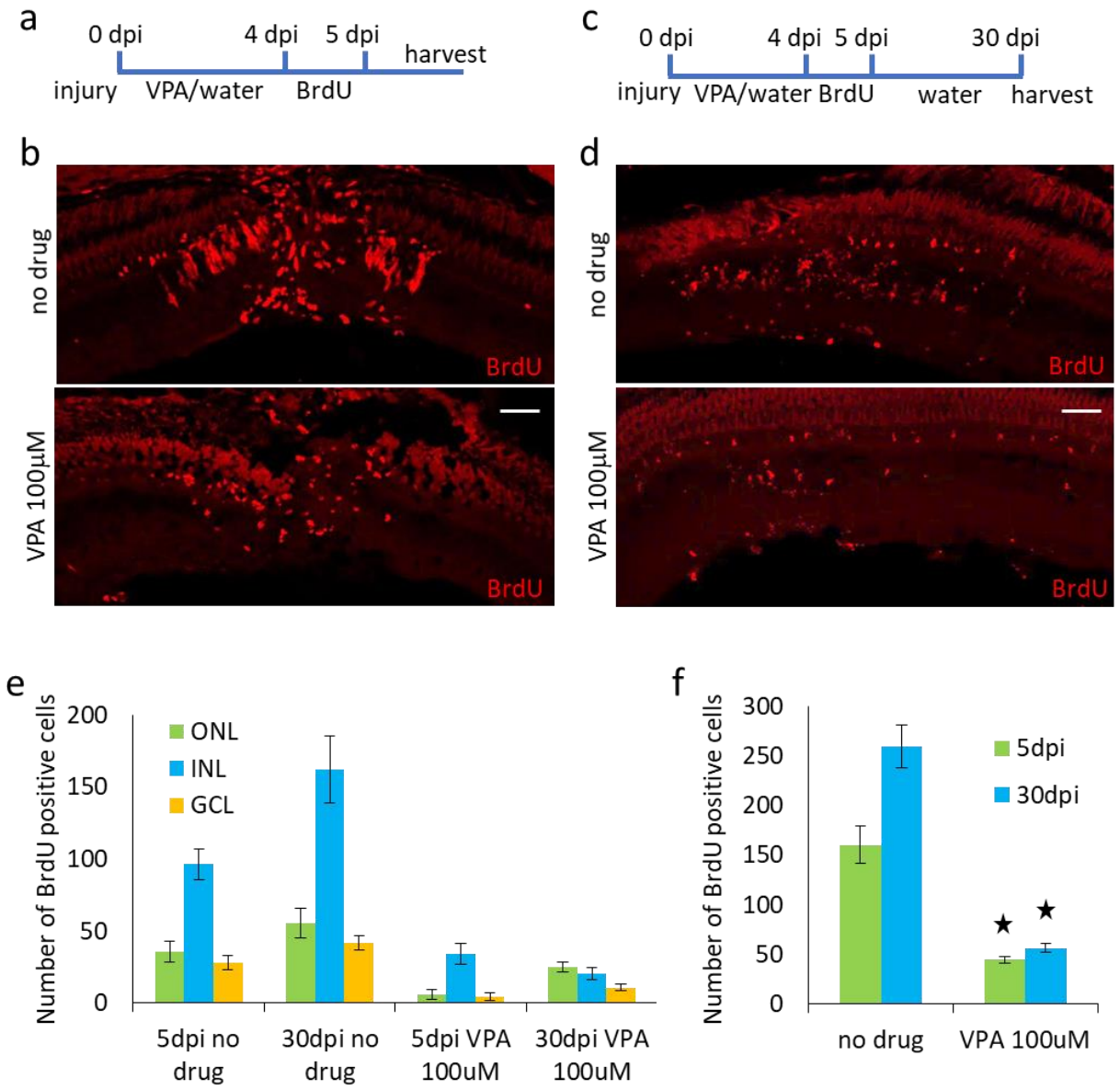


Fig 3.42: HDACs inhibition reduces proliferation at 4dpi and residual proliferating cells migrate to all retinal layers. (a, c) experimental time-lines for checking whether the residual proliferative cells in 4dpi retinae after VPA treatment can migrate to different cell layers. (b. d) BrdU IF in 5dpi (b) and 30 dpi (d) retinal sections that received BrdU as indicated in the experimental regime shows the residual brdU positive cells after VPA treatment in 5dpi retinae can migrate to all retinal cell layers. (e) quantification of BrdU positive cell number in different cell layers at 5 and 30 dpi. (f) quantification of total BrdU positive cell number in all retinal layers at 5dpi and 30dpi. Scale bar: 35µ in b, and d.

3.2.4 Inhibition of HDAC1 deregulates proliferation during retina regeneration.

Next, we wanted to selectively knock down HDAC1 by using MO against it and check its effect on proliferation. For this, at first, the MO efficacy was checked by injecting *hdac1* MO into embryos with *gfp* mRNA appended by MO binding site. MO targeting *hdac1* significantly brought down GFP expression (Fig 3.43 a). Western blotting with anti HDAC1 antibody in 4dpi retina electroporated with *hdac1* MO also showed depleted HDAC1 protein level (Fig 3.43 b).

Fish were injured and simultaneously MO against *hdac1* was injected intravitreally followed by electroporation. At 4dpi, retinae were harvested after 4-5 hours of BrdU pulsing. Surprisingly, BrdU immunostaining revealed a slight but significant increase in the number of proliferating cells (Fig 3.44 a, b). A closer look at proliferating cells revealed a significant subset of proliferating cells also contained lissamine tagged MOs (Fig 3.44 c) suggesting knockdown of HDAC1 facilitates proliferation at 4dpi.

The above observation made us speculate that if *hdac1* knockdown is resulting in increased proliferation at 4dpi, it should also increase proliferation at 2dpi. As compared to 4dpi, proliferation is significantly low in 2dpi retinae, we expected an increased number of BrdU positive cells in retina with knocked down *hdac1*. Fish retinae were injured along with *hdac1* MO electroporation and were harvested at 2dpi. Surprisingly, we found a significantly smaller number of PCNA positive proliferative cells (Fig 3.45 a, b). These observations made us to hypothesize that Hdac1 might have two different kinds of roles to play during retina regeneration. Initially, the presence of Hdac1 protein must be necessary for the induction of

pluripotency during the de-differentiation phase. Later, during 4dpi or the proliferative phase, as revealed by its IF microscopy, Hdac1 must be depleted from MGPCs in order for them to proliferate. This exact scenario might be also functioning when we are knocking down *hdac1* and harvesting the retinae for BrdU IF at 4dpi and get a slightly increased number of MGPCs. These observations suggest that during the proliferative phase, Hdac1 might have an anti-proliferative role to play.

Next, if the observation of a decreased number of proliferative MGPCs in 2dpi retinae electroporated with *hdac1* MO was to be true, then this unfavourable effect of the MO on proliferation should be rescued by overexpressing Hdac1 in injured retinae. For this, fish eyes were injured and simultaneously *hdac1* mRNA lacking the MO binding sites was electroporated with *gfp* mRNA and *hdac1* MO. GFP worked as a reporter to indicate proper delivery of the *hdac1* mRNA and also acted as a control. PCNA immunostaining at 2dpi revealed that the effect of *hdac1* MO on proliferation can actually be rescued by overexpression of Hdac1 (Fig 3.46 a, b).

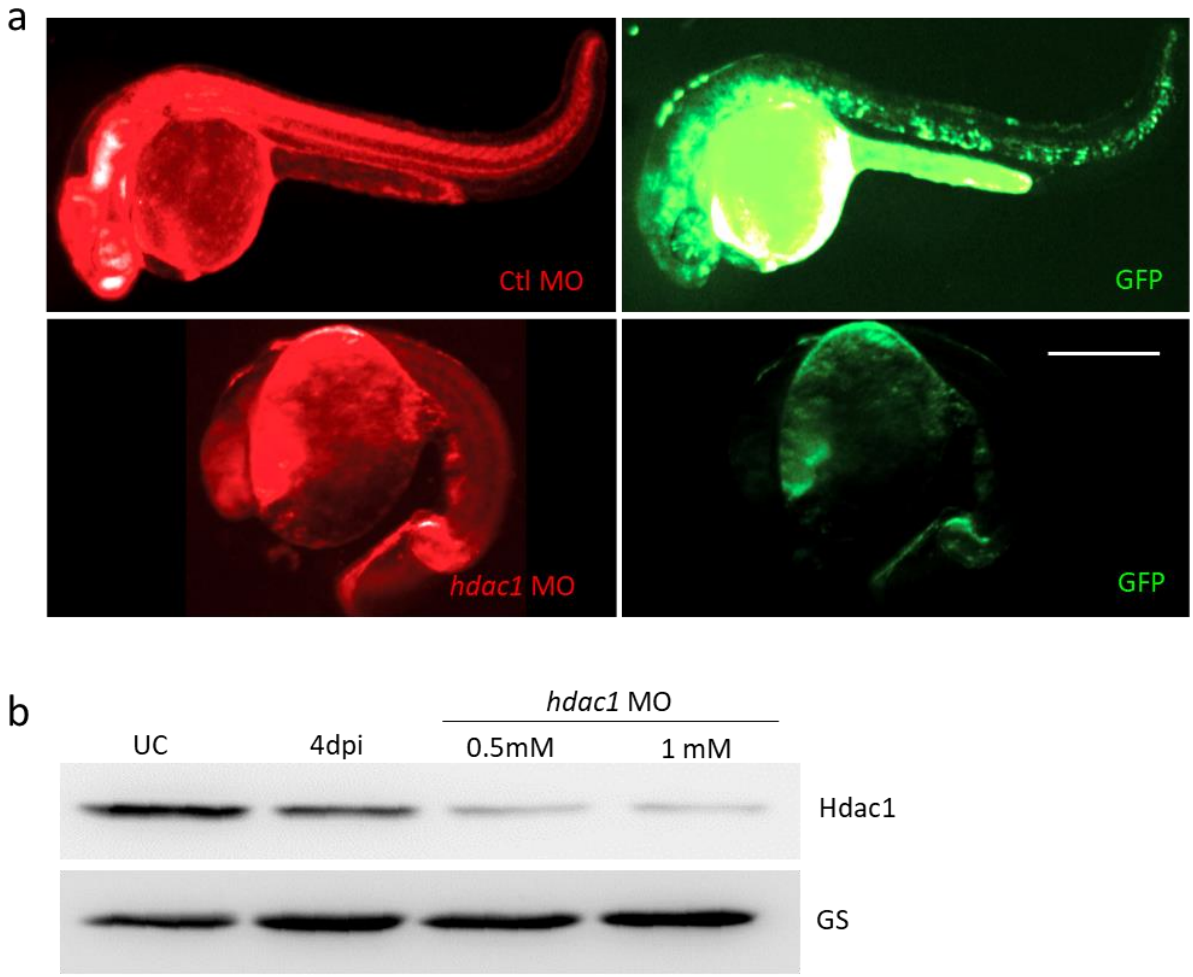


Fig 3.43: Effectiveness of morpholino modified oligoneucleotides against *hdac1*. (a) embryos injected with control MO and *gfp* mRNA shows GFP expression at 2 days post fertilization, but embryos injected with *hdac1* MO and *gfp* mRNA fused with MO targeting site do not show GFP expression. (b) Western blot analysis of 4dpi retinae electroporated with MOs targeting *hdac1* resulted in depletion of HDAC1 protein. GS acts as internal control. Scale bar: 1mm.

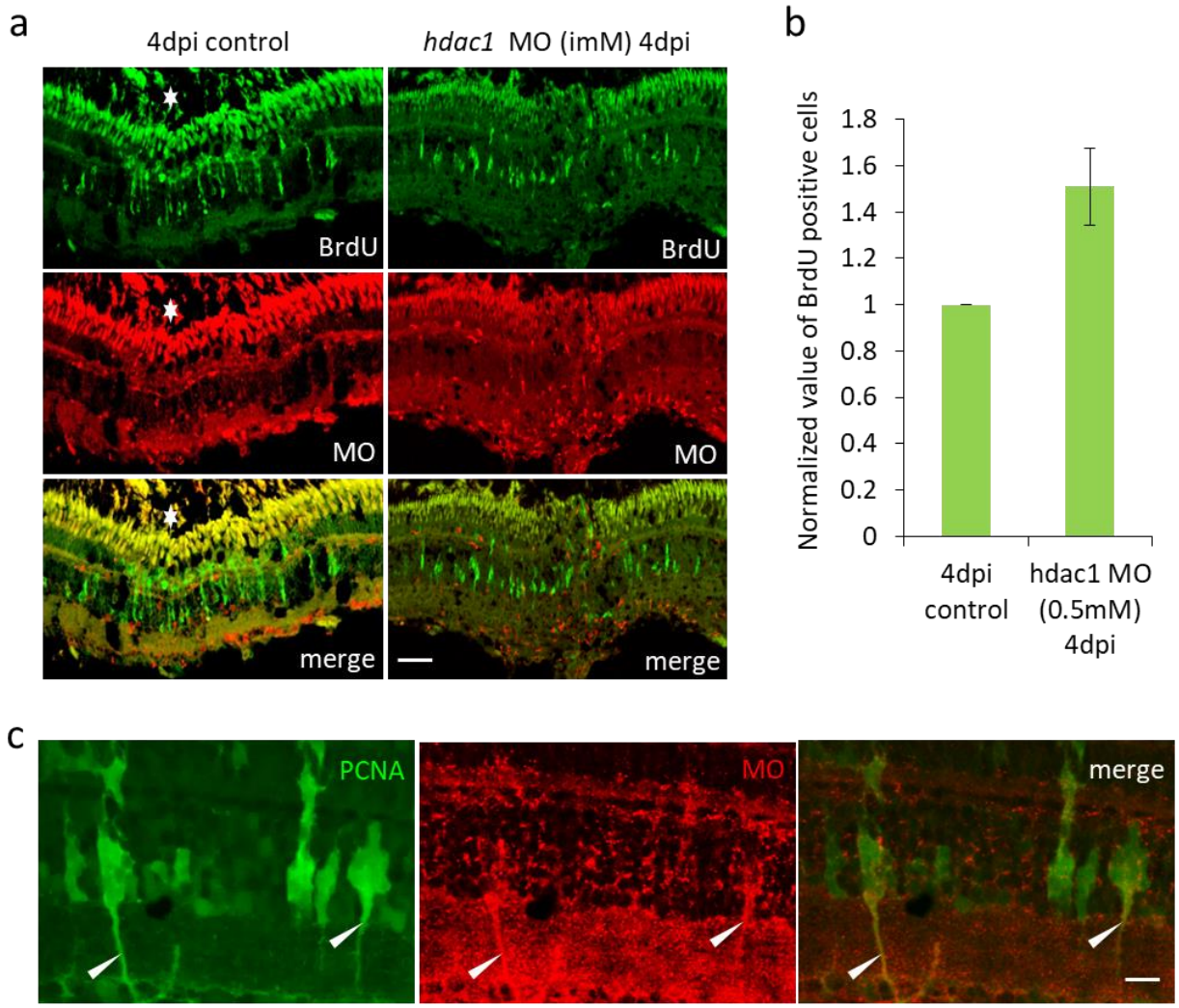


Fig 3.44: HDAC1 knock down increases proliferation in regenerating retina at 4dpi. (a) BrdU IF in 4dpi control MO and *hdac1* MO electroporated retinae. Scale bar 25µ. (b) quantification of BrdU positive cell number in the same. (c) high magnification confocal microscopy shows proliferating cells bearing *hdac1* MO. Scale bar 5µ.

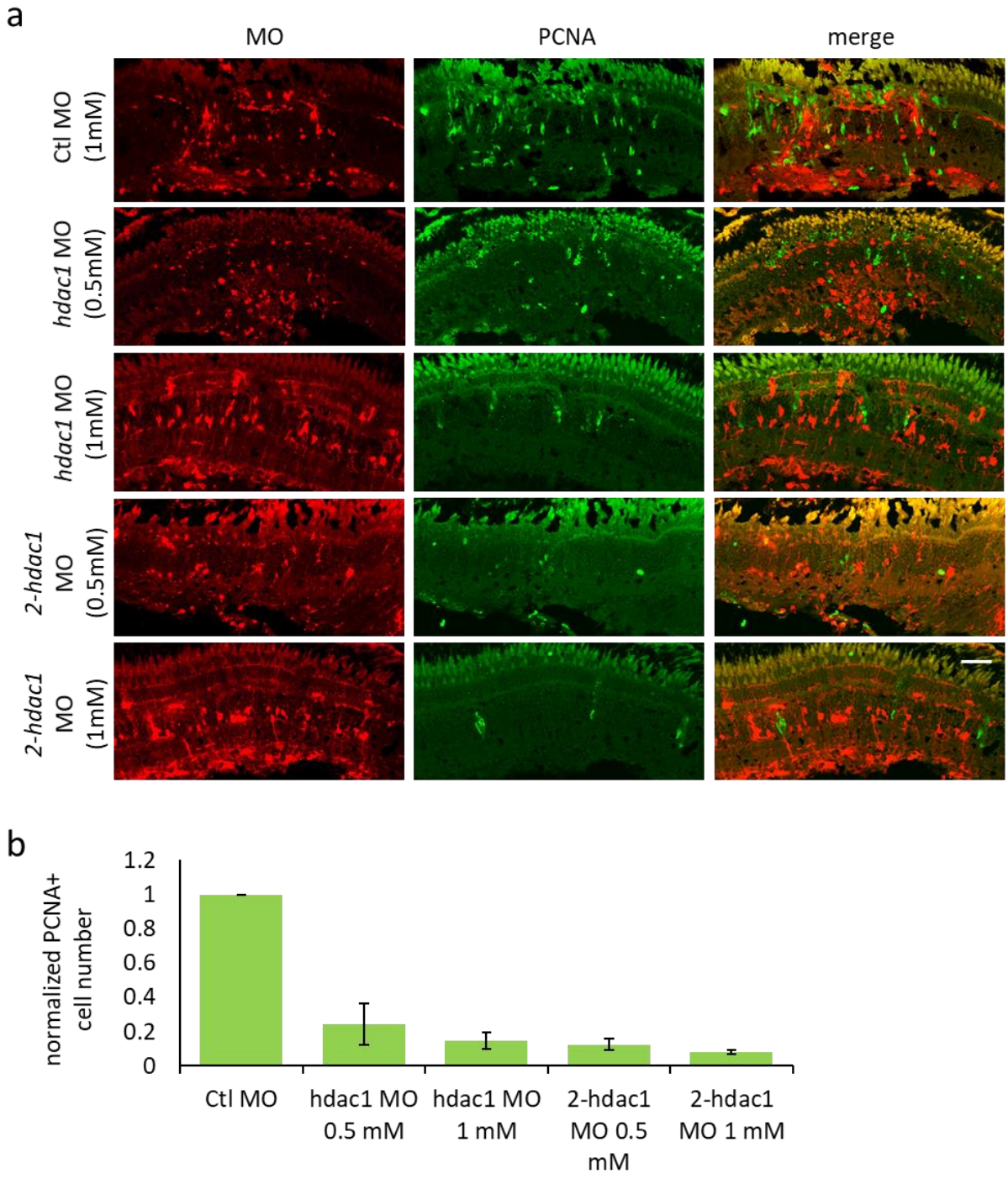


Fig 3.45: HDAC1 knock down decreases proliferation in regenerating retina at 2dpi. (a) BrdU IF in 2dpi retina electroporated with control and *hdac1* MO. Scale bar 20µ. (b) quantification o BrdU positive cell number in the same.

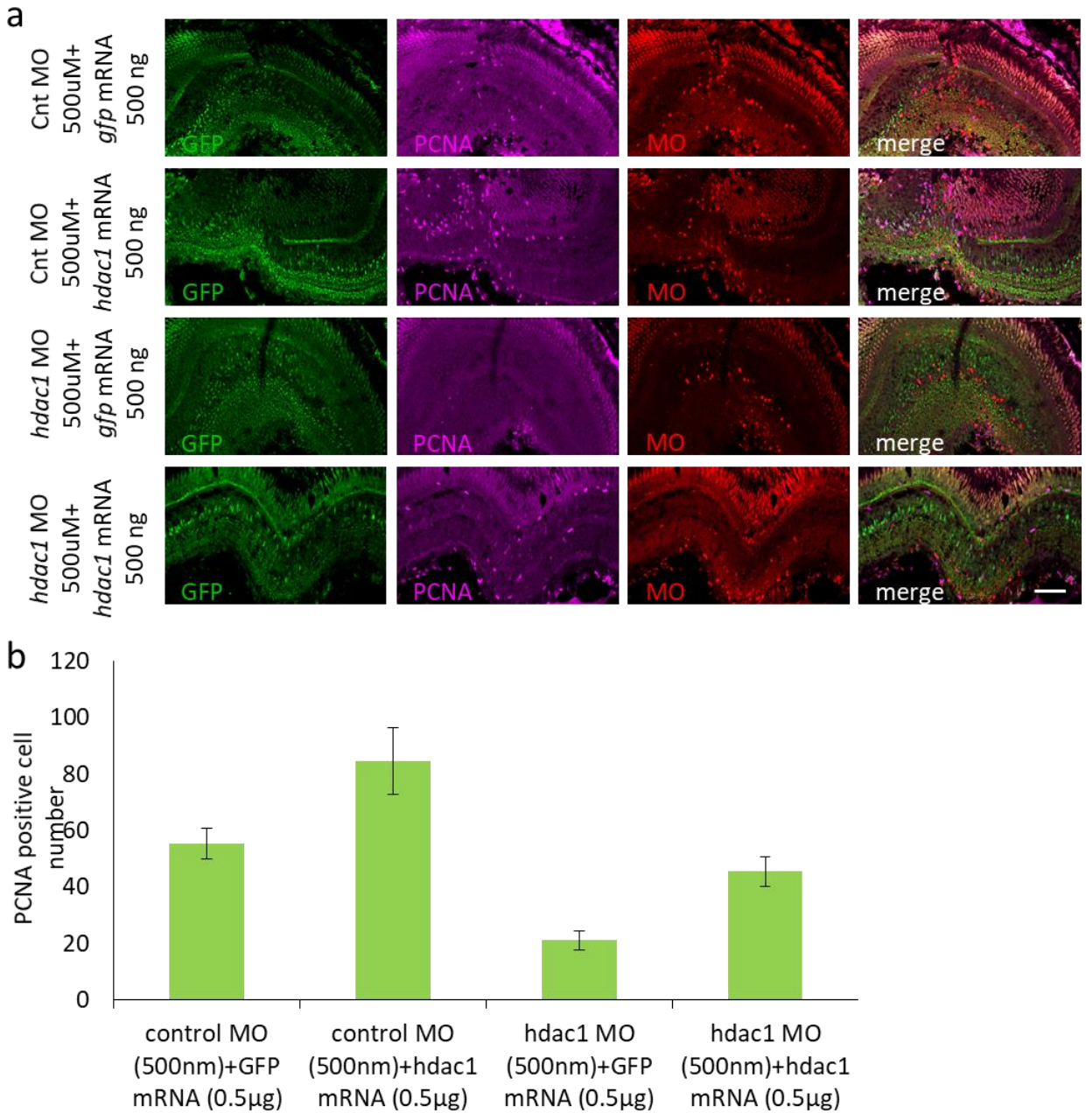


Fig 3.46: mRNA of *hdac1* can rescue the effect of the MO on proliferation. (a) PCNA IF in 2dpi retina electroporated with control and *hdac1* MO along with *gfp* and *hdac1* mRNA showed that Hdac1 overexpression could rescue the blockade on proliferation by *hdac1* MO. Scale bar 35µ. (b) quantification o BrdU positive cell number in the same.

3.2.5 Regulation of HDACs and other regeneration associated genes by HDACs.

To find out the possible causative factors for the reduced proliferation in Hdacs inhibited retinae, we undertook a candidate-based approach. In this background, we wanted to check expression levels of different regeneration associated genes, some of which are already reported to play important roles in retina regeneration. For this, fish were dipped into solutions of VPA, or TSA following retinal injury and harvested at 2dpi for retina dissection and RNA isolation or at 4dpi, after 4-5 hours of BrdU pulsing, for mRNA *in situ* hybridization. qRT-PCR (Fig 3.47 a and c) and RT-PCR (Fig 3.47 b, and d) at 2dpi showed both *ascl1a* and *mycb* to be upregulated significantly in VPA (Fig 3.47 a, b) and TSA (Fig 3.47 c, d) treated retinae in a concentration-dependent manner. This was further re-established by mRNA *in situ* hybridization in 4dpi retinal sections treated with VPA (Fig 3.48 a, b). Apart from these two transcripts, expression status of other regeneration associated genes like *zic2b* (Fig 3.49 a-e) and *mmp9* (Fig 3.50 a-e) was also checked by qRT-PCR, RT-PCR in VPA treated 2dpi retinae and by mRNA ISH in VPA treated 4dpi retinae. Both of them were found to be significantly upregulated at 2 dpi as well as at 4dpi. qRT-PCR and RT-PCR also showed pluripotency genes like *sox2*, *oct4*, *klf*, and morphogens like *shha* were also upregulated significantly (Fig 3.51 a, b).

We next thought, as the injury responsive zone also became super-constricted in HDACs inhibited retinae, and as Notch signalling has been reported to be upregulated to restrict the zone of proliferation around the injury spot, it might be possible that Notch signalling is getting hyper-activated in the HDACs inhibited retinae. As *her4.1* is a downstream target of Notch signalling, we checked its expression level at 20hpi as well as 2dpi in VPA (Fig 3.52 a, b) and

TSA (Fig 3.52 c, d) treated retinae and found it to be significantly upregulated. The mRNA *in situ* hybridization at 4dpi in VPA treated retinae confirmed the same (Fig 3.53 a). This was further verified by *her4.1* promoter activity assay carried out in embryos injected with *her4.1:gfp-luciferase* and increasing concentration of TSA (Fig 3.53 b) We also checked expression levels of other Notch signalling components like *dla, dlb, dlc, dld,* and *notch1a,* and found them to be upregulated significantly (Fig 3.53 c).

If HDACs inhibitor-mediated upregulation of Notch signalling is causing reduced proliferation, then, inhibiting Notch signalling in HDACs inhibited retinae should rescue the normal number of proliferative cells at 4dpi. With this hypothesis, we injected 400uM DAPT (*N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-*S*-phenylglycine-t-butyl ester) a γ-secretase inhibitor (thus inhibits Notch signalling by blocking NICD formation) after injury and dipped the fish in 100uM VPA solution for 4 days. Eyes were harvested for sectioning after 5 hours of BrdU pulsing. As controls, one set of fish was dipped into 100uM VPA following injury. The second set of fish received intravitreal injection of 400uM DAPT following injury and then was transferred to water. The third set of fish were used as 4dpi controls without any inhibitor treatment. All three sets of control fish received 5 hours of BrdU pulsing before euthanized. BrdU immunostaining in these retinal sections showed an increased zone of proliferation and also number of proliferating cells in only DAPT treated retinae, constriction of the same in VPA treated retina but a significant rescue of the length of the proliferative zone in VPA and DAPT co-injected retinae (Fig 3.54 a, b). But surprisingly, although the zone length was rescued significantly, the proliferative cell number did not (Fig 3.54 d). To further confirm the effect of Hdacs inhibition through Delta/Notch signalling, we sought to knock down *her4.1*, a downstream target of Delta/Notch signalling, in TSA treated retinae (Fig 3.55 a). BrdU immunostaining showed significantly increased number of BrdU positive proliferating cells in *her4.1* knocked down retinae as compared to that of the control (Fig 3.55 b, c).

Next, we were interested to check the expression level of another regeneration-associated gene, *lin28a*. Interestingly RT-PCR and qRT-PCR showed significant downregulation of its mRNA level at 2dpi in TSA (Fig 3.56 a, b) and VPA (Fig 3.56 c, d) treated retinae. The mRNA ISH in 4dpi retinae treated with increasing concentration of VPA confirmed the same (Fig 3.57 a, b). As discussed before, Lin28a is a nucleic acid binding protein which binds to *let-7* microRNA and degrades it. Translation of several regeneration-associated genes like *ascl1a*, *mycb* has been reported to be regulated by *let-7* microRNA. So, in VPA or TSA treated retinae, if *lin28a* is downregulated, *let-7* microRNA cannot be degraded. Thus, in spite of higher levels of mRNA, protein levels of Ascl1a and Mycb should not be increased. To test this hypothesis, retinae from VPA and TSA treated fish were harvested for SDS-PAGE and Western blotting. At 2dpi, protein levels of Ascl1a and Mycb, as opposed to their increased transcript levels, were less as compared to control (Fig 3.58 a, b). In fact, when we check the levels of *let-7* microRNA in TSA or VPA treated retinae, we indeed saw a dose-dependent increase in the levels of *let-7* microRNA (Fig 3.58 c, d).

We also checked the expression pattern of another regeneration associated genes *insm1a* in HDACs inhibited retinae. We found concentration-dependent downregulation of *insm1a* in 2dpi retinae treated with increasing concentration of TSA (Fig 3.59 a, b). This result also goes at par with our previous results that showed upregulation of *mycb* in HDACs inhibited retinae. As we have already shown previously that Insm1a downregulates *mycb*, this result suggests HDACs might be downregulating *mycb* through Insm1a.

We have also carried out a whole retina RNA-seq analysis with 4dpi retinal samples treated with VPA along with 4dpi and 12hpi controls (GEO accession: GSE98094). VPA treated 4dpi retina showed several important transcription factors including *mycb* to be upregulated (Appendix 2).

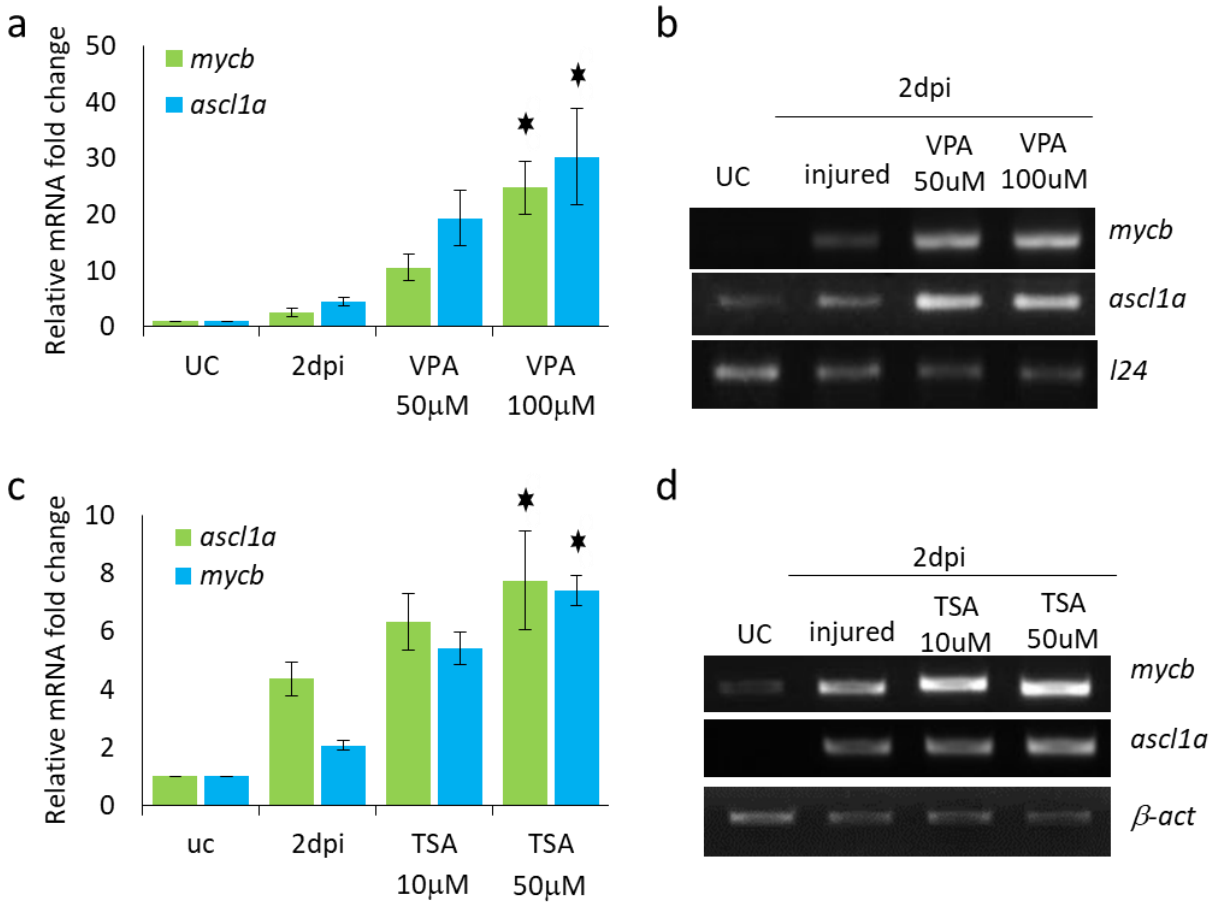


Fig 3.47: Hdacs repress *mycb* and *ascl1a* transcription. (a, b) qRT-PCR (a) and RT-PCR (b) show a concentration-dependent increase in the mRNA levels of *mycb* and *ascl1a* in 2dpi retinae treated with various concentrations of VPA. (c, d) qRT-PCR (c) and RT-PCR (d) show a concentration-dependent increase in the mRNA levels of *mycb* and *ascl1a* in 2dpi retinae treated with various concentrations of TSA.

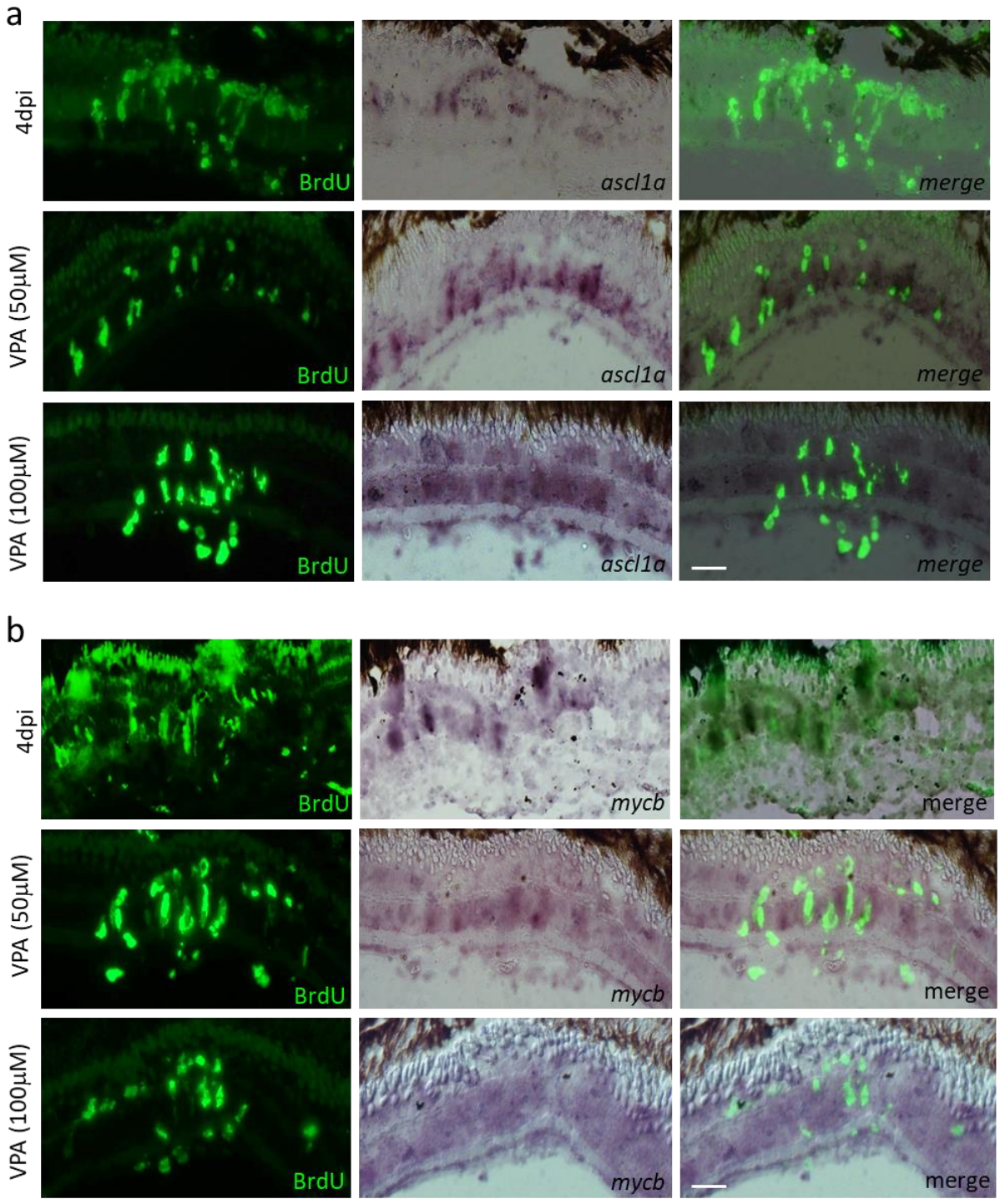


Fig 3.48: Pan-retinal induction of *ascl1a* and *mycb* in 4dpi retinae treated with Hdacs'inhibition. (a, b) mRNA *in situ* hybridization shows a pan retinal induction of *ascl1a* (a) and *mycb* (b) along with a dose-dependent decrease in the number of BrdU positive MGPCs in 4dpi retina treated with VPA, compared with control 4dpi retina, in which the expression is restricted to the injury responsive zone. Scale bar: 20µ in both.

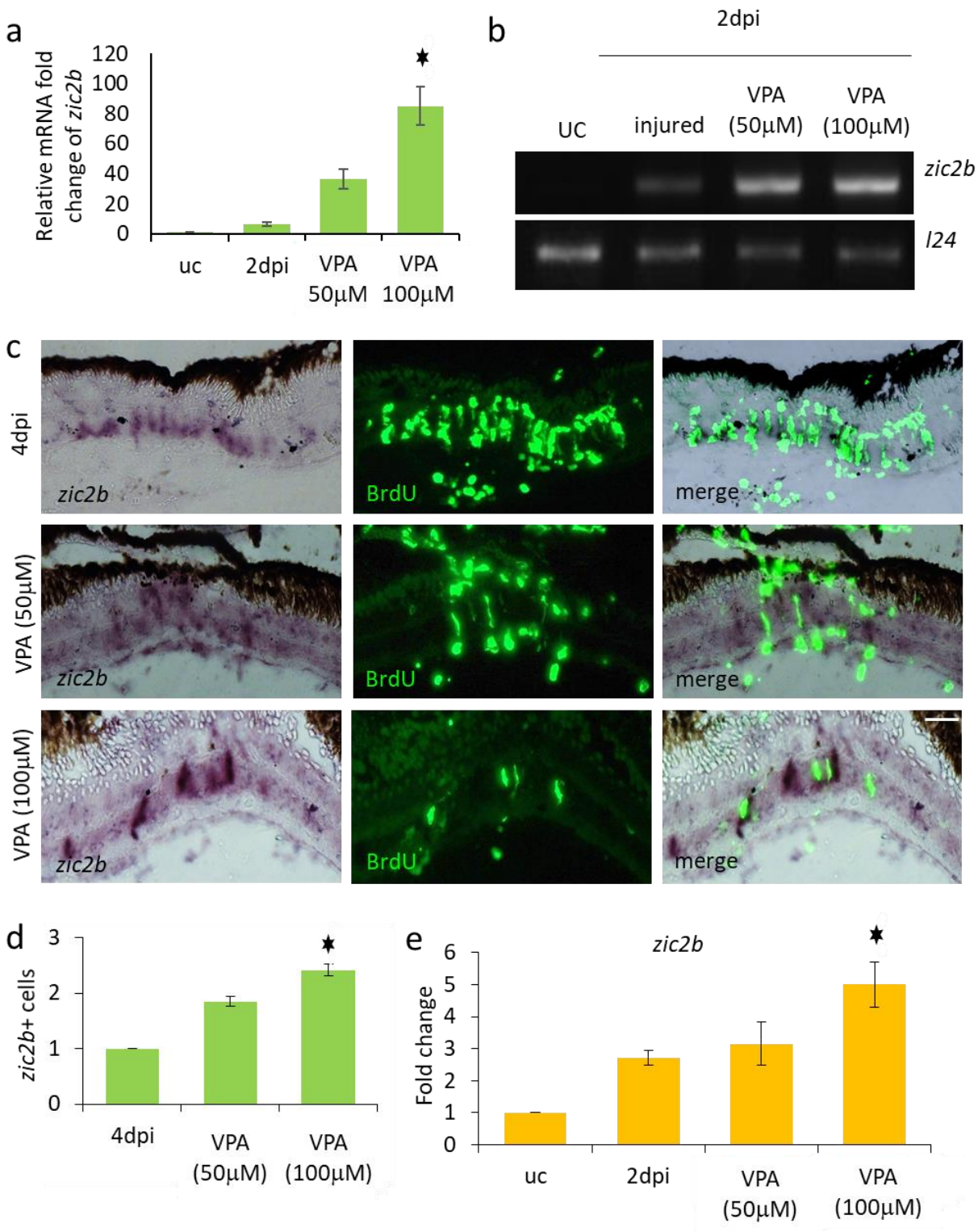


Fig 3.49: Upregulation of *zic2b* in HDACs inhibited retinae. (a) qRT-PCR and (b) RT-PCR shows concentration dependent upregulation of *zic2b* in VPA treated 2dpi retinae. (c) mRNA ISH shows upregulation of *zic2b* in VPA treated 4dpi retinal sections. Scale bar: 20µ. (d) quantification of *zic2b*+ve cells in 4dpi control and 4dpi VPA treated retinal sections. (e) upregulation of *zic2b* in TSA treated 2dpi retinal sections treated with different concentration of TSA. P<0.001.

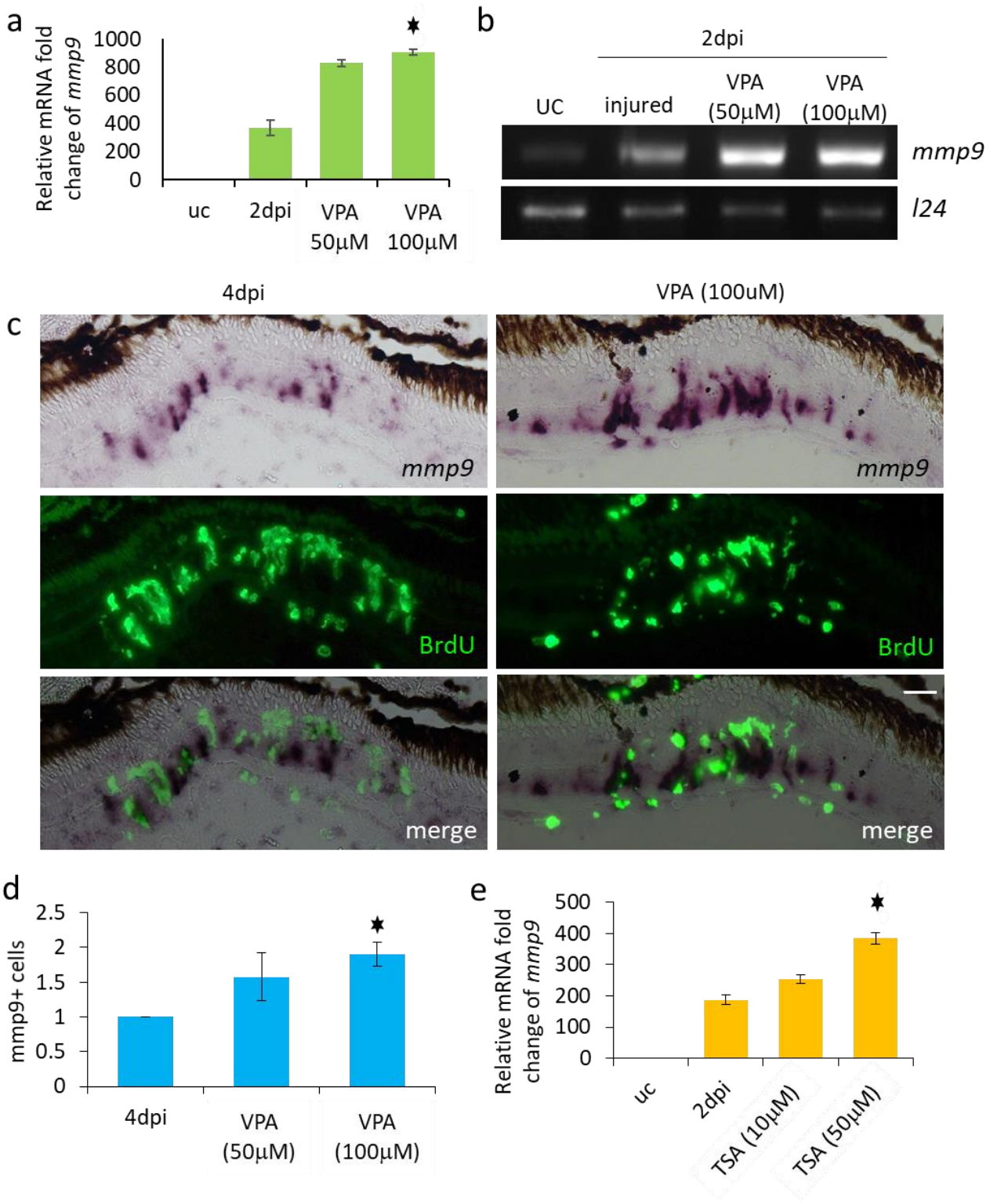


Fig 3.50: Upregulation of *mmp9* in HDACs inhibited retinae. (a) qRT-PCR and (b) RT-PCR shows concentration dependent upregulation of *mmp9* in VPA treated 2dpi retinae. (c) mRNA ISH shows upregulation of *mmp9* in VPA treated 4dpi retinal sections. Scale bar: 20µ. (d) quantification of *mmp9*+ve cells in 4dpi control and 4dpi VPA treated retinal sections. (e) qRT-PCR shows concentration dependent upregulation of *mmp9* in TSA treated 2dpi retinae. P<0.02.

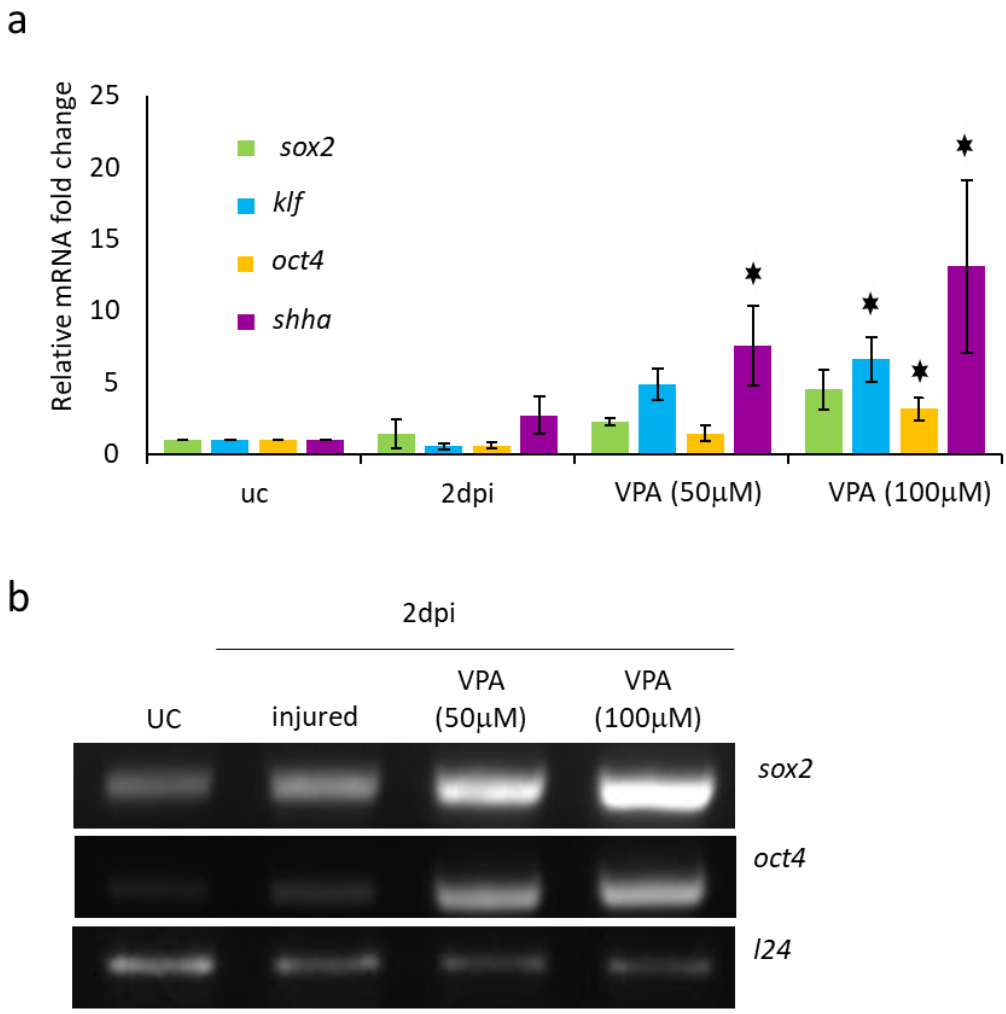


Fig 3.51: Upregulation of different pluripotency factors genes and *shha* in HDACs inhibited retinae. (a) qRT-PCR shows concentration dependent upregulation of pluripotency factors like *sox2*, *klf* and *oct4* and morphogen like *shha* in 2dpi retinae in which HDACs are inhibited by different concentration of VPA. (b) RT-PCR shows concentration dependent upregulation of *sox2* and *oct4* in VPA mediated HDACs inhibited retinae. $P<0.05$.

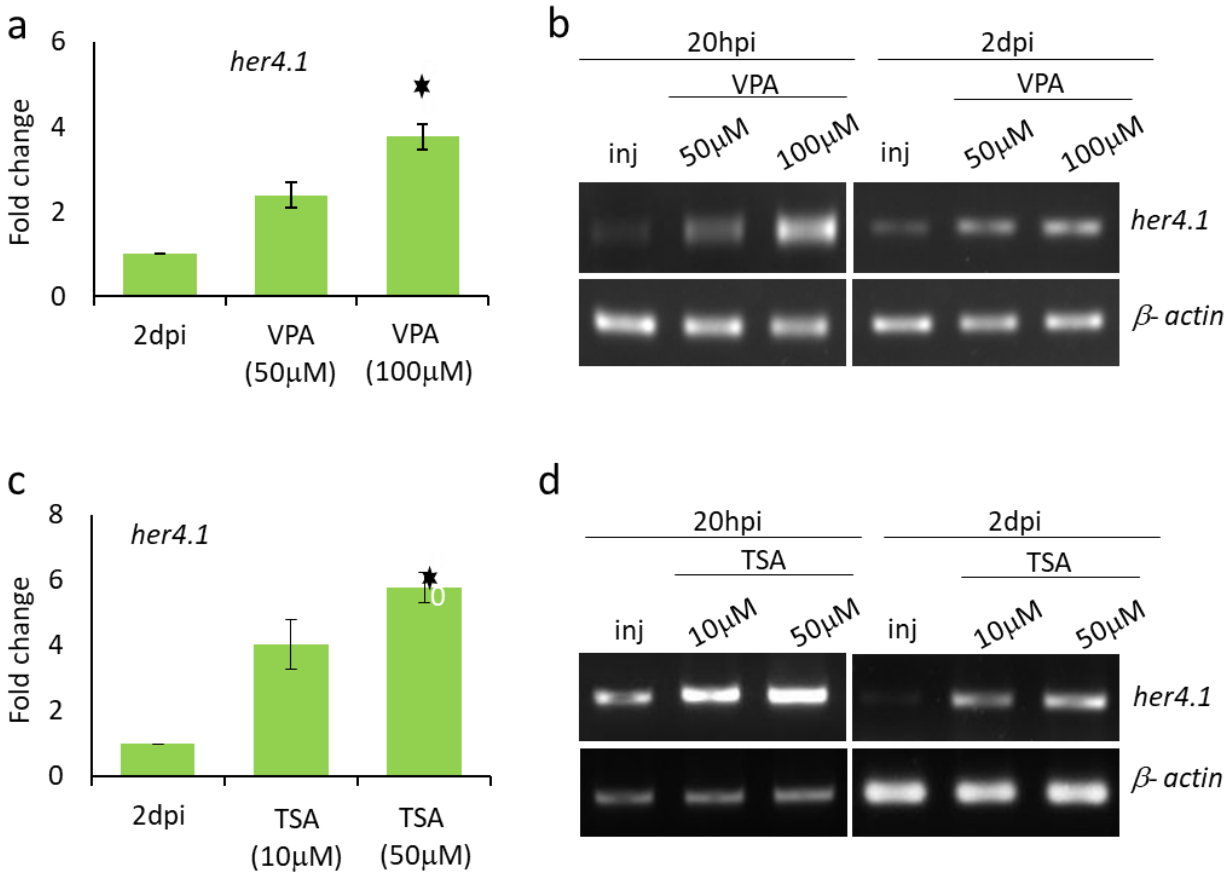


Fig 3.52: Upregulation of *her4.1* in HDACs inhibited retinae. (a) qRT-PCR shows upregulation of *her4.1* in VPA treated 2dpi retinae. (b) RT-PCR shows concentration dependent upregulation of *her4.1* in 20hpi and 2dpi retina treated with VPA. (c) qRT-PCR shows upregulation of *her4.1* in TSA treated 2dpi retinae. (d) RT-PCR shows concentration dependent upregulation of *her4.1* in 20hpi and 2dpi retina treated with TSA.

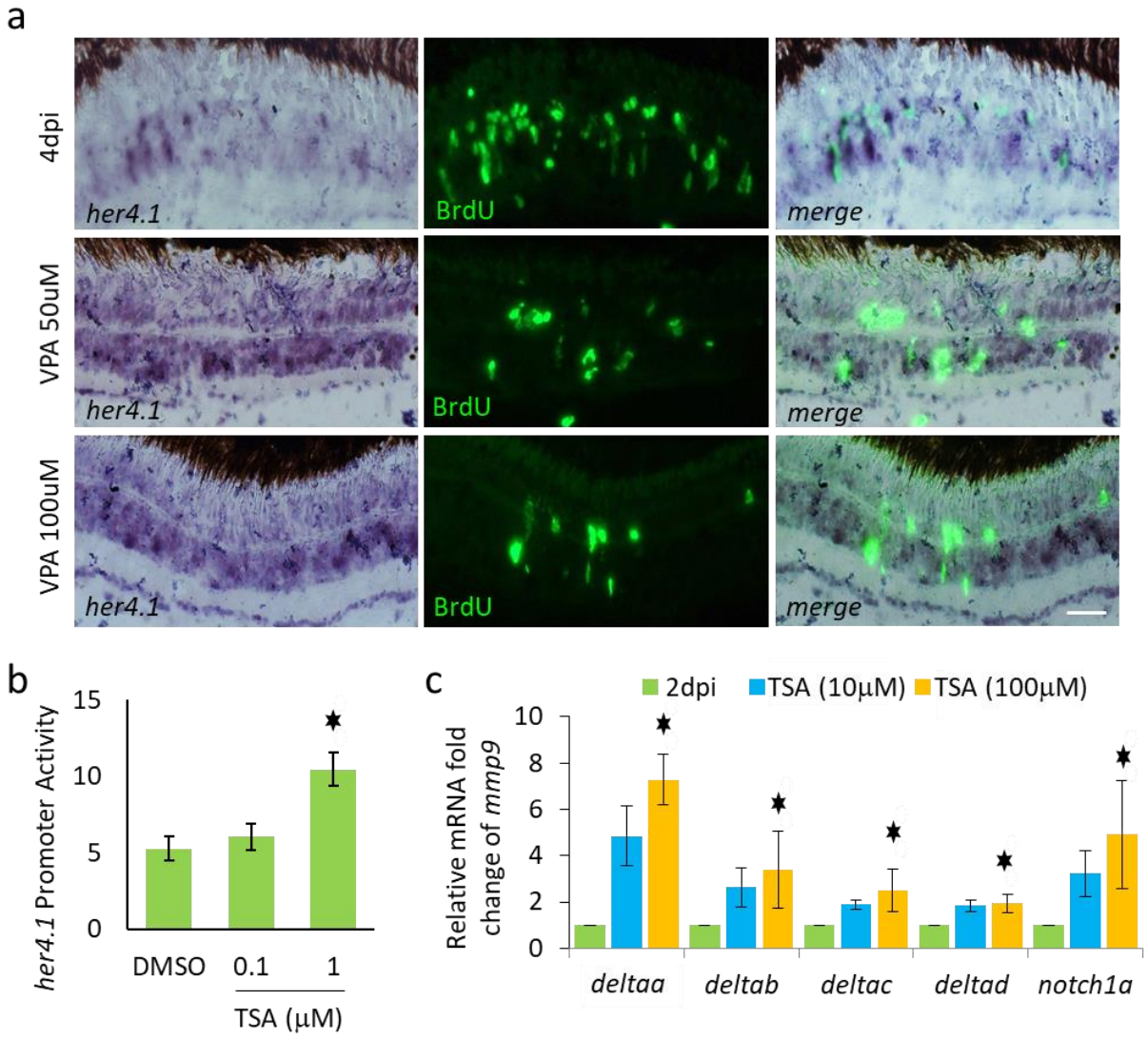


Fig 3.53: Upregulation of Notch signalling components in HDACs inhibited retinae. (a) mRNA ISH shows pan-retinal upregulation of *her4.1* in VPA treated 4dpi retinal sections. Scale bar: 20μ. (b) upregulation of *her4.1* promoter activity by increasing concentration of TSA. (c) transcriptional upregulation of Notch signalling components in 2dpi retinae treated with increasing concentration of TSA, shown by qRT-PCR. (d) RT-PCR shown upregulation of *notch1a*, *dla* and *dlb* in TSA treated 2dpi retinae. P<0.002.

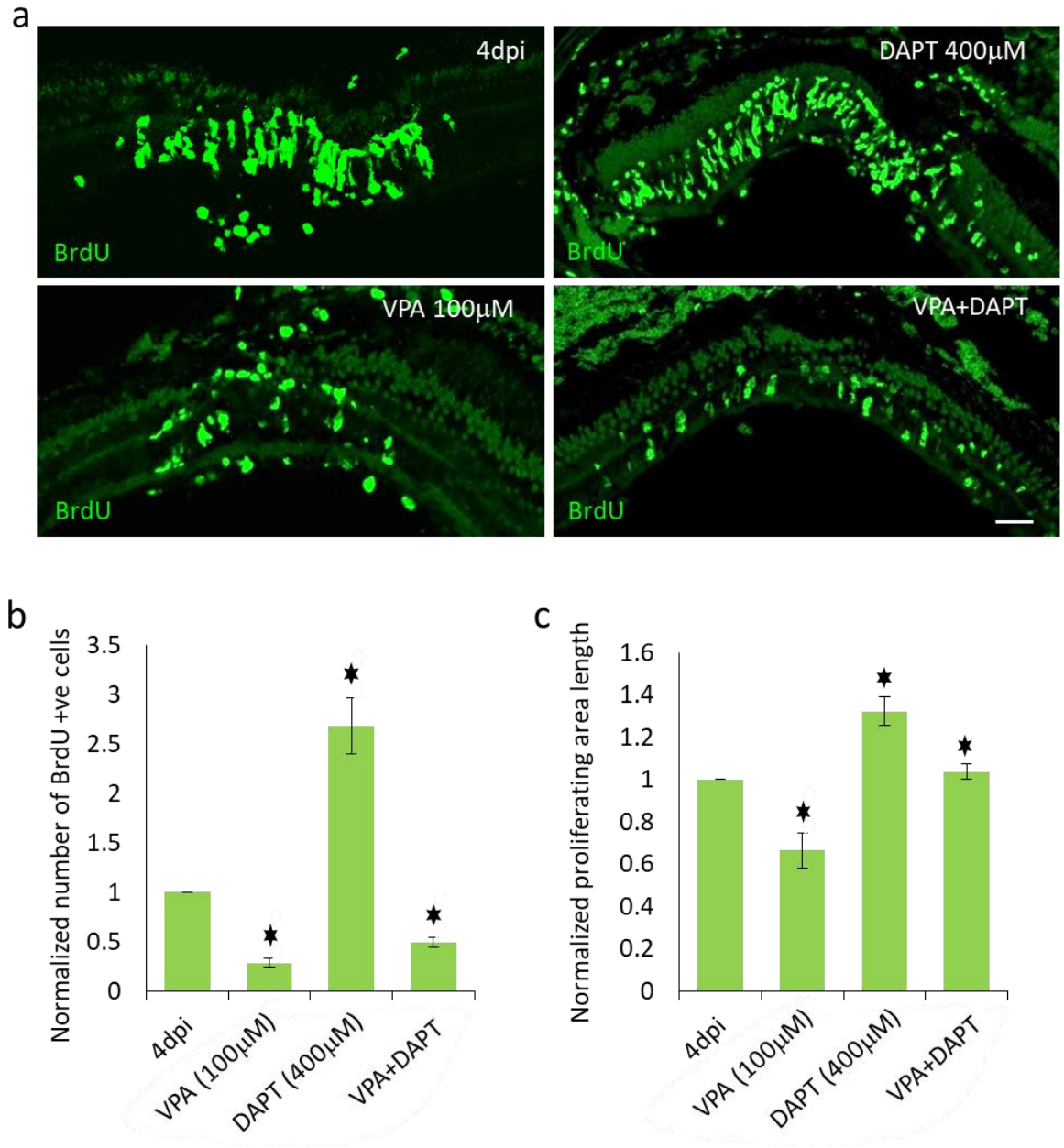


Fig 3.54: Rescue of the length of VPA mediated super-constricted injury responsive zone by blockade of Notch signalling. (a) super-constricted injury responsive zone as seen by inhibiting HDACs by VPA is rescued by blocking Notch signalling by DAPT. Scale bar: 25µ. (b) quantification of BrdU positive proliferating cells in the same. (c) quantification of the length of injury responsive zone in the same experiment. $P<0.001$.

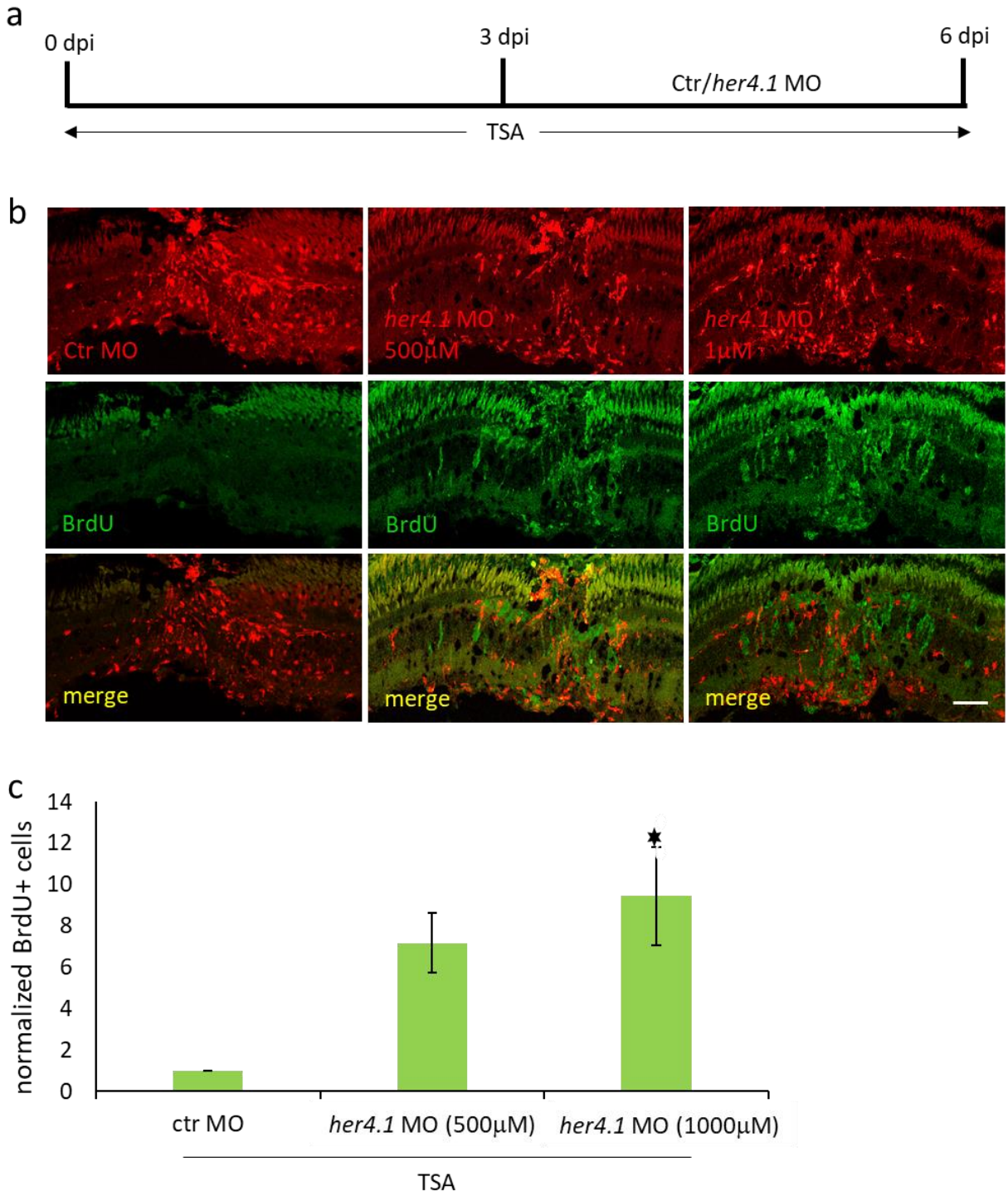


Fig 3.55: Hdacs regulate proliferation through Her4.1. (a) experimental time-line for knocking down *her4.1* in TSA treated retinae. (b) BrdU immunostaining shows increased number of BrdU positive proliferating cell number in TSA treated retinae electroporated with *her4.1* MO. Scale bar: 25µ. (c) quantification of the same. P<0.005.

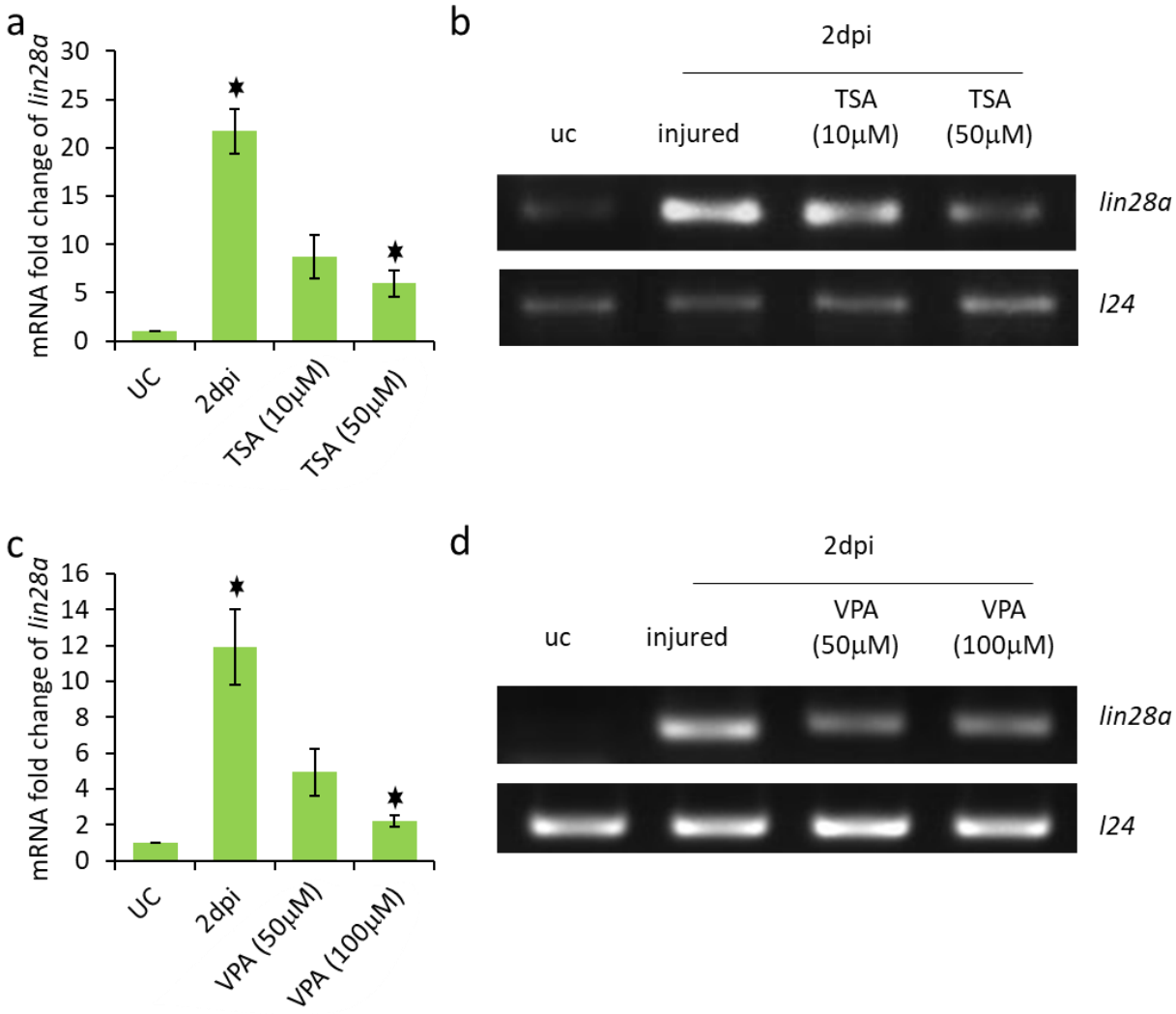


Fig 3.56: Downregulation of *lin28a* Hdacs inhibited retinae at 2dpi. (a, b) qRT-PCR (a) and RT-PCR (b) shows concentration dependent downregulation of *lin28a* in 2dpi retinae treated with different concentration of TSA. (c) qRT-PCR and (d) RT-PCR shows concentration dependent downregulation of *lin28a* in 2dpi retinae treated with different concentration of VPA. P<0.0005.

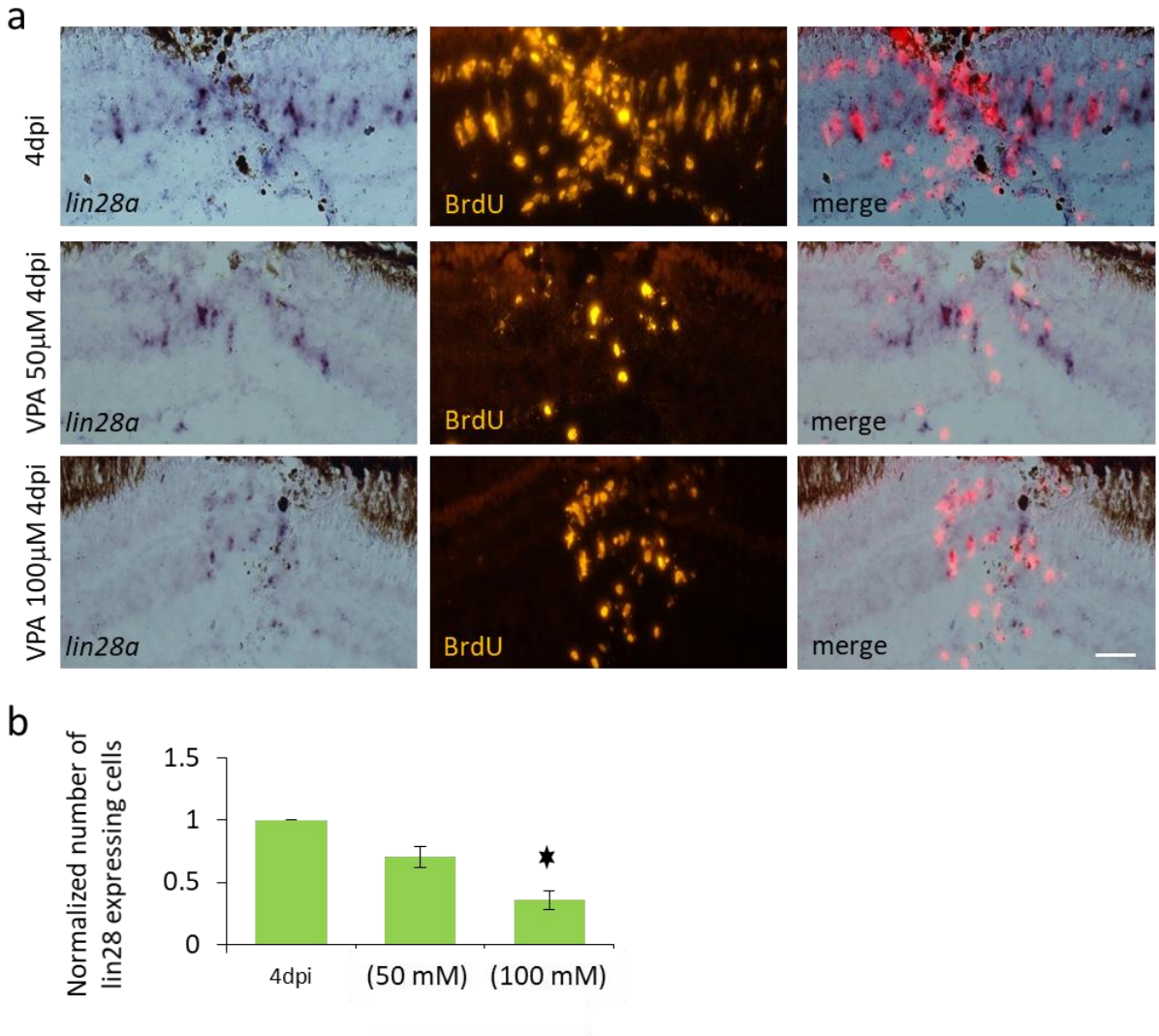


Fig 3.57: Downregulation of *lin28a* Hdacs inhibited retinae at 4dpi. (a) mRNA ISH in 4dpi retina treated with VPA shows downregulation of *lin28a* in a concentration dependent manner. Scale bar: 30µ. (b) quantification of *lin28*+ve cell number in the same experiment. P<0.02.

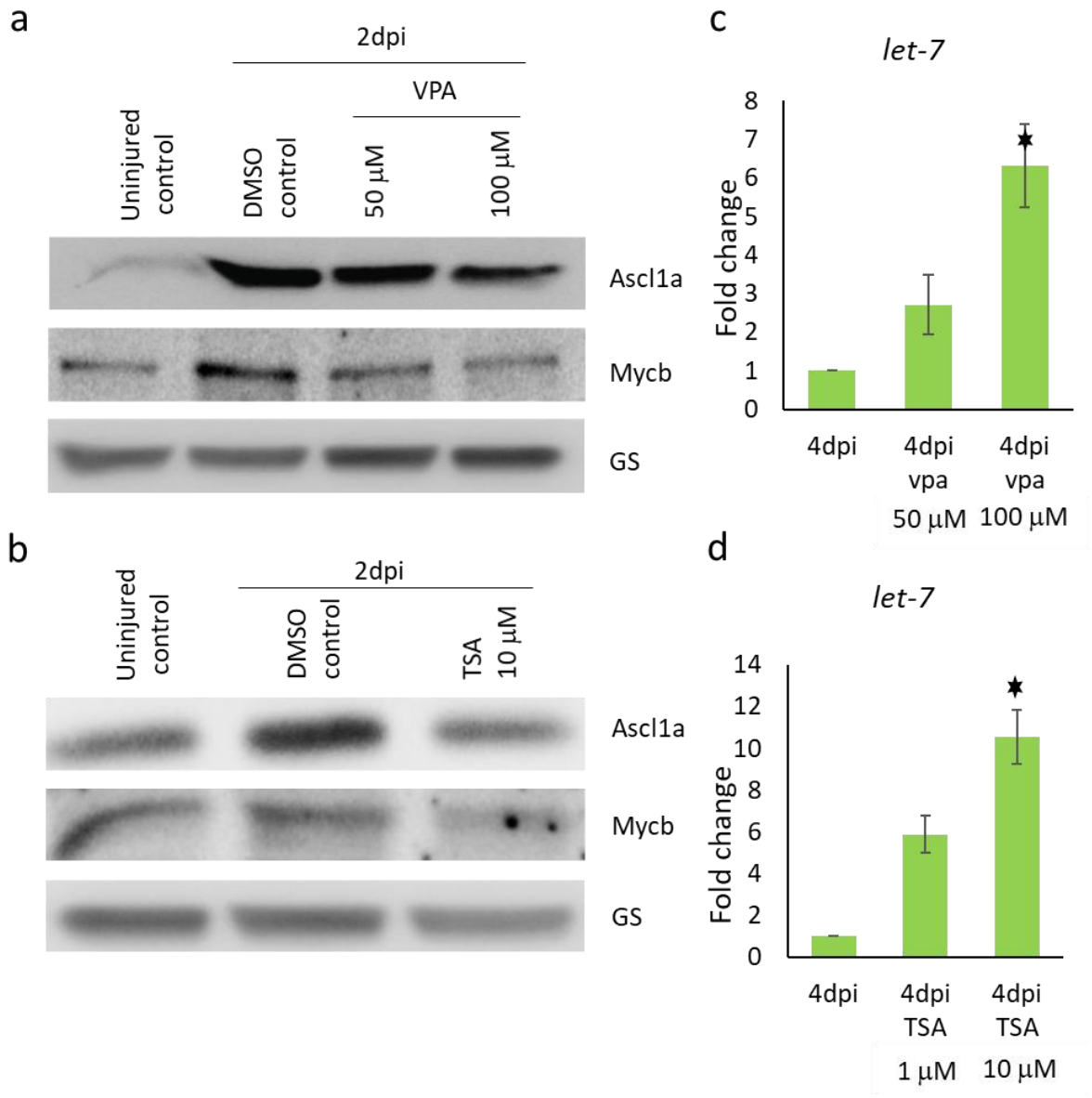


Fig 3.58: Depletion of Ascl1a and Myc protein levels in HDACs inhibited retinae as a result of abundant *let-7* microRNA. (a) Western blotting shows concentration dependent downregulation of Ascl1a and Myc in 2dpi retinae treated with increasing concentration of VPA. (b) Western blotting shows downregulation of Ascl1a and Myc in 2dpi retinae treated with TSA. P<0.002.

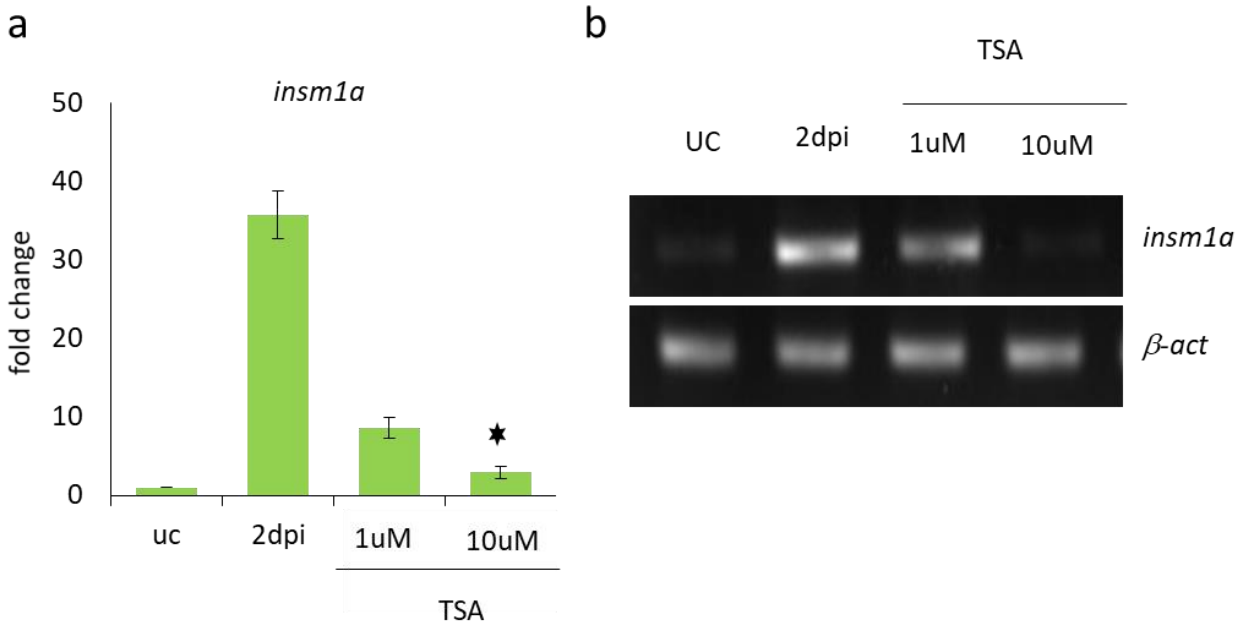


Fig 3.59: Downregulation of *insm1a* in HDACs inhibited retinae. (a) qRT-PCR and (b) RT-PCR shows downregulation of *insm1a* in TSA treated 2dpi retinae. P<0.001.

3.2.6 Regulation of HDACs and other regeneration associated genes by HDAC1.

As we have discussed in 3.2.4, knockdown of HDAC1 had an opposing effect on proliferation at different days following injury. At 4dpi, it caused increased proliferation, whereas, at 2dpi, it caused decreased proliferation. We wanted to find out the reason for this.

We looked for the expression pattern of different regeneration associated genes like Iascl1a, *myca*, *mycb*, *lin28a*, *hdac1*, and *hdac3*. qRT-PCR showed expression of *ascl1a* to be significantly upregulated in retinae electroporated with *hdac1* MO at both 2dpi (Fig 3.60 a) and 4dpi (Fig 3.60 b). This was further confirmed by mRNA ISH in 4dpi retinal sections with control and *hdac1* MO electroporated into them. At 4dpi, *hdac1* MO electroporated retinae showed a pan-retinal induction of *ascl1a* as opposed to its injury restricted expression pattern in 4dpi control MO treated retinae (Fig 3.60 c).

Similarly, *myca* showed significant upregulation in 2dpi *hdac1* MO electroporated retinae as opposed to control in qRT-PCR (Fig 3.61 a). We got similar observation with *mycb* also (Fig 3.61 b) which was further confirmed by mRNA ISH (Fig 3.61 c, d) performed in sections of 2dpi retina which was electroporated with *hdac1* MO. We also found a similar pattern of *mycb* expression in 4dpi retinae electroporated with *hdac1* MO as revealed by both qRT-PCR and mRNA ISH assay (Fig 3.62 a, b).

Next, we checked the expression pattern of *hdac1* itself and *hdac3* in 2dpi retinae electroporated with *hdac1* MO to probe for the existence of any feedback regulatory mechanism. The qRT-PCR showed mRNA of both *hdac1* (Fig 3.63 a) and *hdac3* (Fig 3.63 b) to be significantly downregulated in retinae with *hdac1* knock-down. Similarly, mRNA ISH

also showed a drastic decline in *hdac1* expression in 4dpi retinae electroporated with *hdac1* targeting MO (Fig 3.63 c).

We also found *lin28a* to be significantly downregulated in 2dpi retinae electroporated with *hdac1* MO compared to control as shown by both qRT-PCR (Fig 3.64 a) and mRNA ISH (Fig 3.64 b, c). Surprisingly in 4dpi retinae electroporated with *hdac1* MO, *lin28a* was significantly upregulated in qRT-PCR (Fig 3.65 a) and mRNA ISH (Fig 3.65 b) in the same condition showed a pan-retinal induction of *lin28a*.

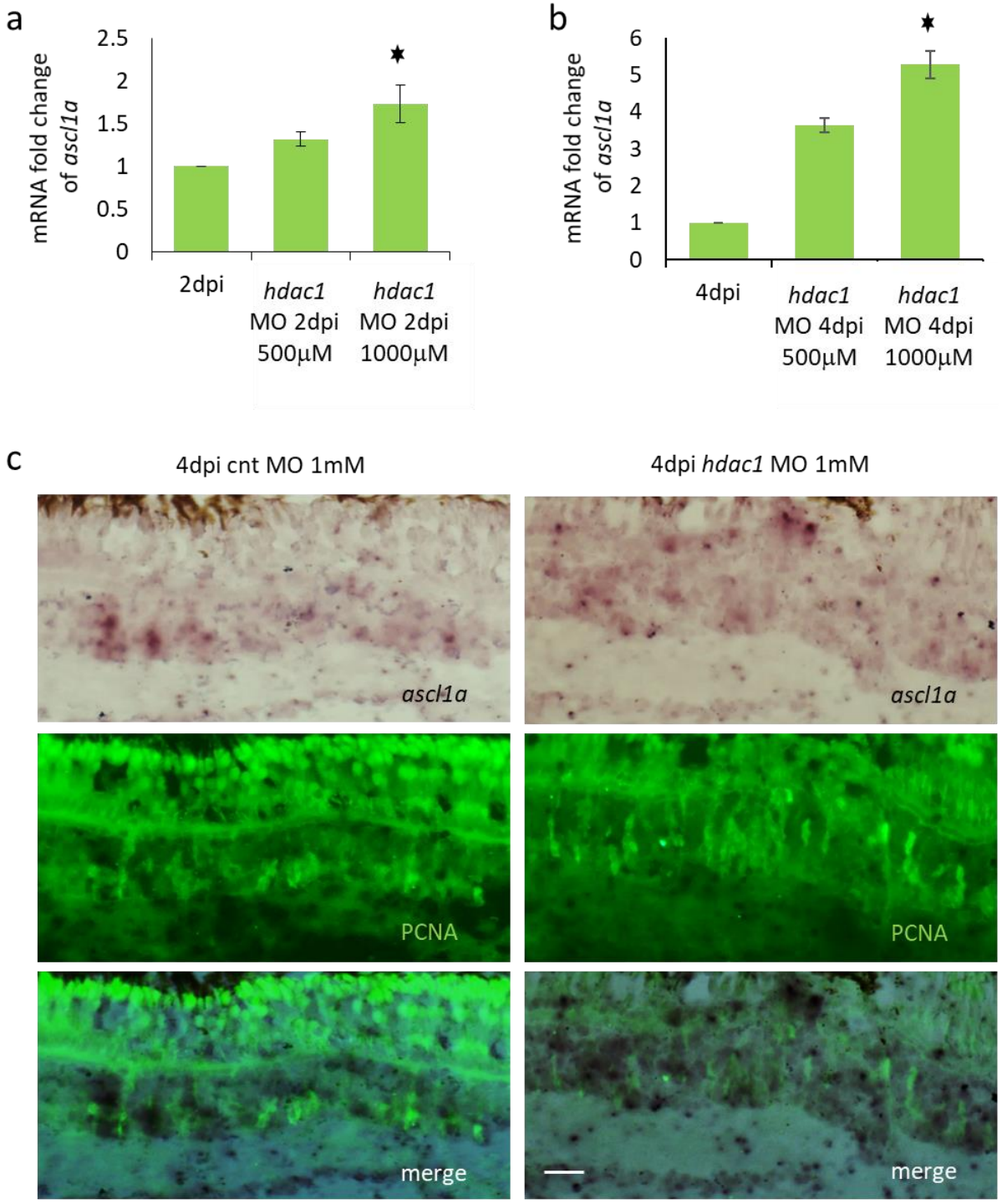


Fig 3.60: Upregulation of *ascl1a* in *hdac1* knocked down retinae. (a) qRT-PCR shows upregulation of *ascl1a* in 2dpi retinae electroporated with increasing concentration of *hdac1* MO. (b) qRT-PCR shows upregulation of *ascl1a* in 4dpi retinae electroporated with increasing concentration of hdac*1* MO. (c) spatial expression pattern of *ascl1a* as shown by mRNA ISH in 2dpi retinae electroporated with *hdac1* MO. $P<0.05$. Scale bar: 20µ.

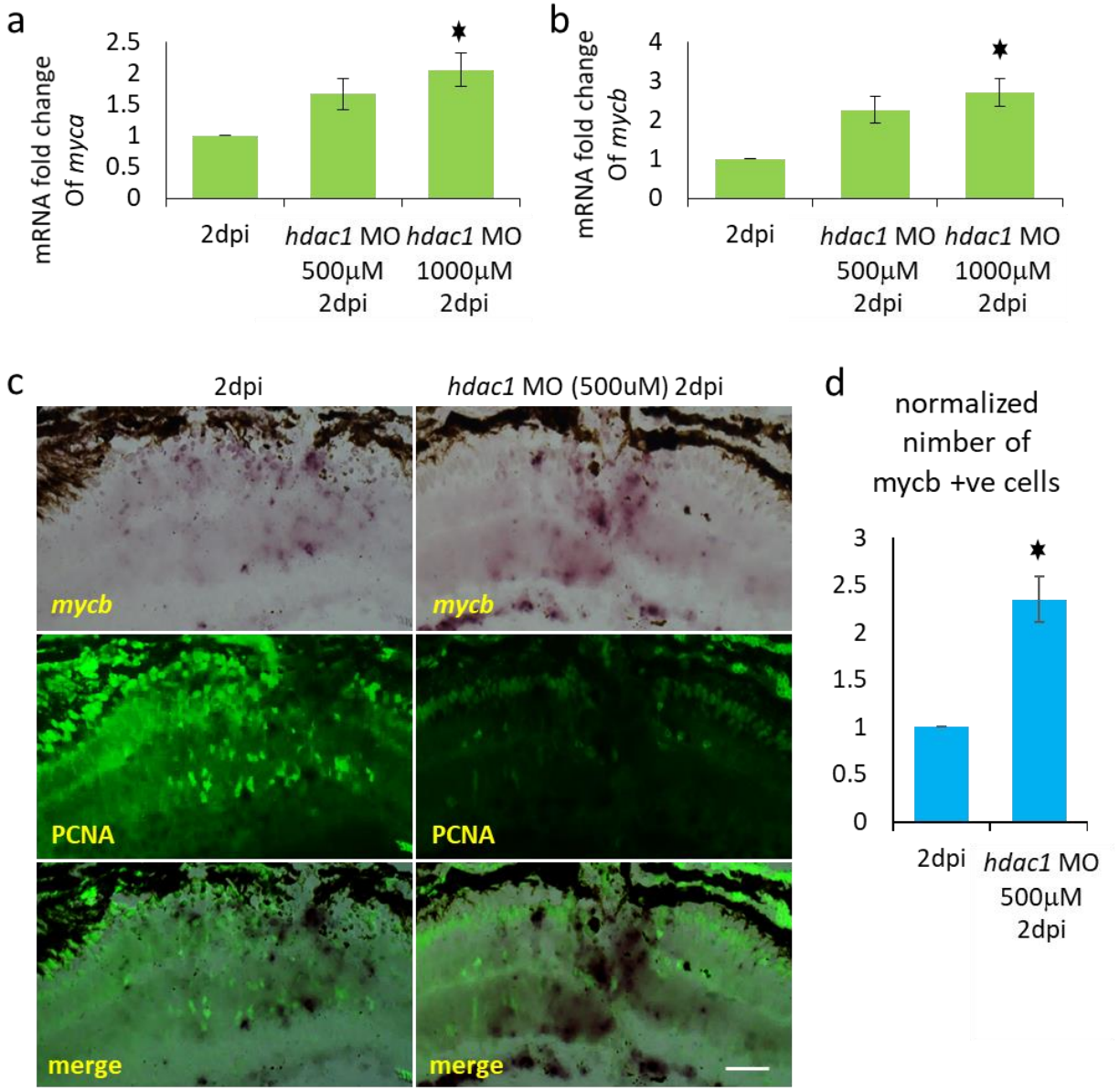


Fig 3.61: Upregulation of *myca* and *mycb* in *hdac1* knocked down retinae at 2dpi. (a, b) qRT-PCR shows upregulation of *myca* and *mycb* in 2dpi retinae electroporated with *hdac1* MO. (c) mRNA ISH of *mycb* in similar background. Scale bar: 20µ. (d) quantification of *mycb* +ve cells in the same. $P<0.05$.

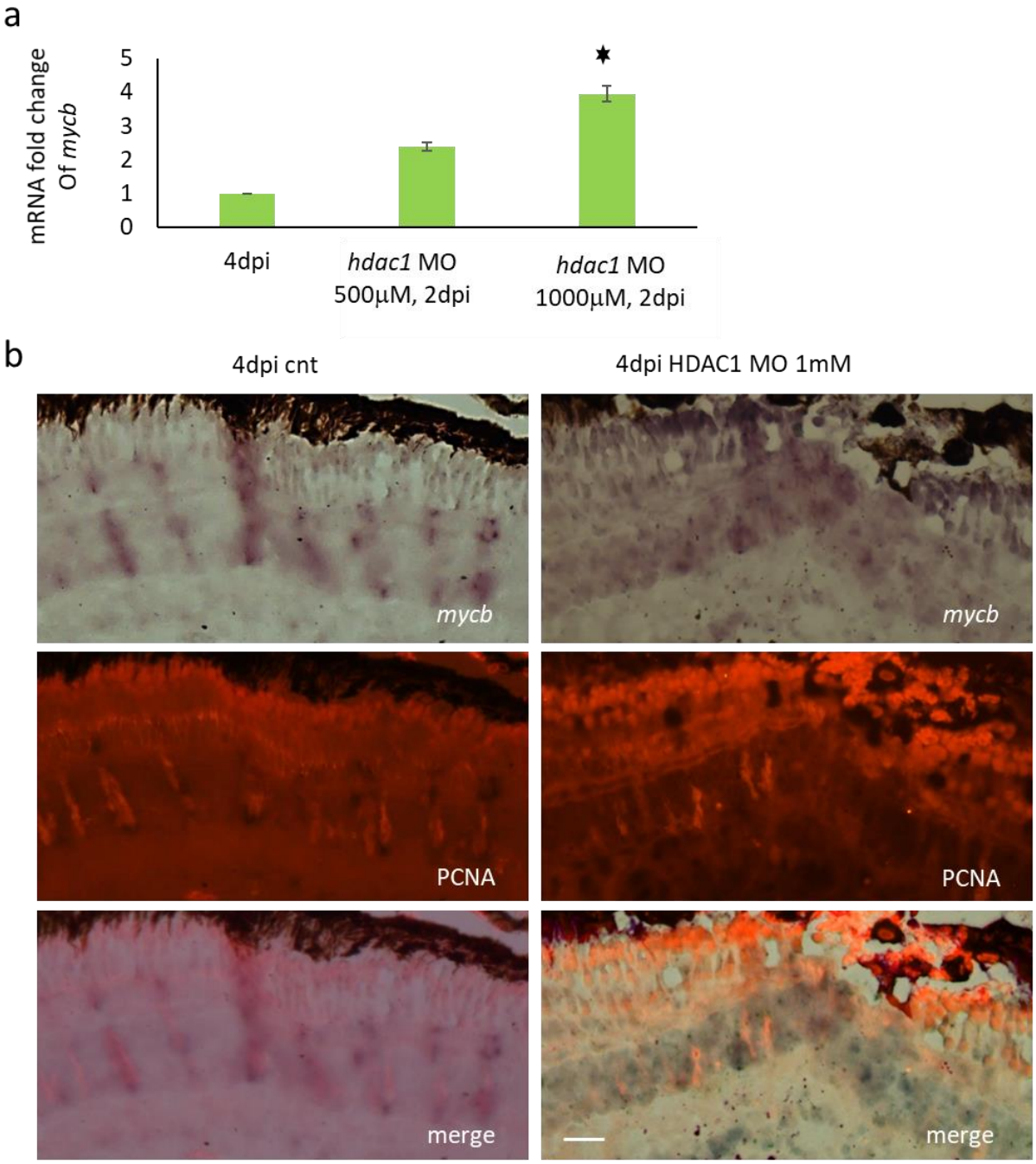


Fig 3.62: Upregulation of *mycb* in *hdac1* knocked down retinae at 4dpi. (a) qRT-PCR shows *mycb* is upregulated in a concentration dependent manner in 4dpi retinae electroporated with *hdac1* MO. (b) mRNA ISH in same background showed upregulatin of *ascl1a*. Scale bar: 20µ. P< 0.001.

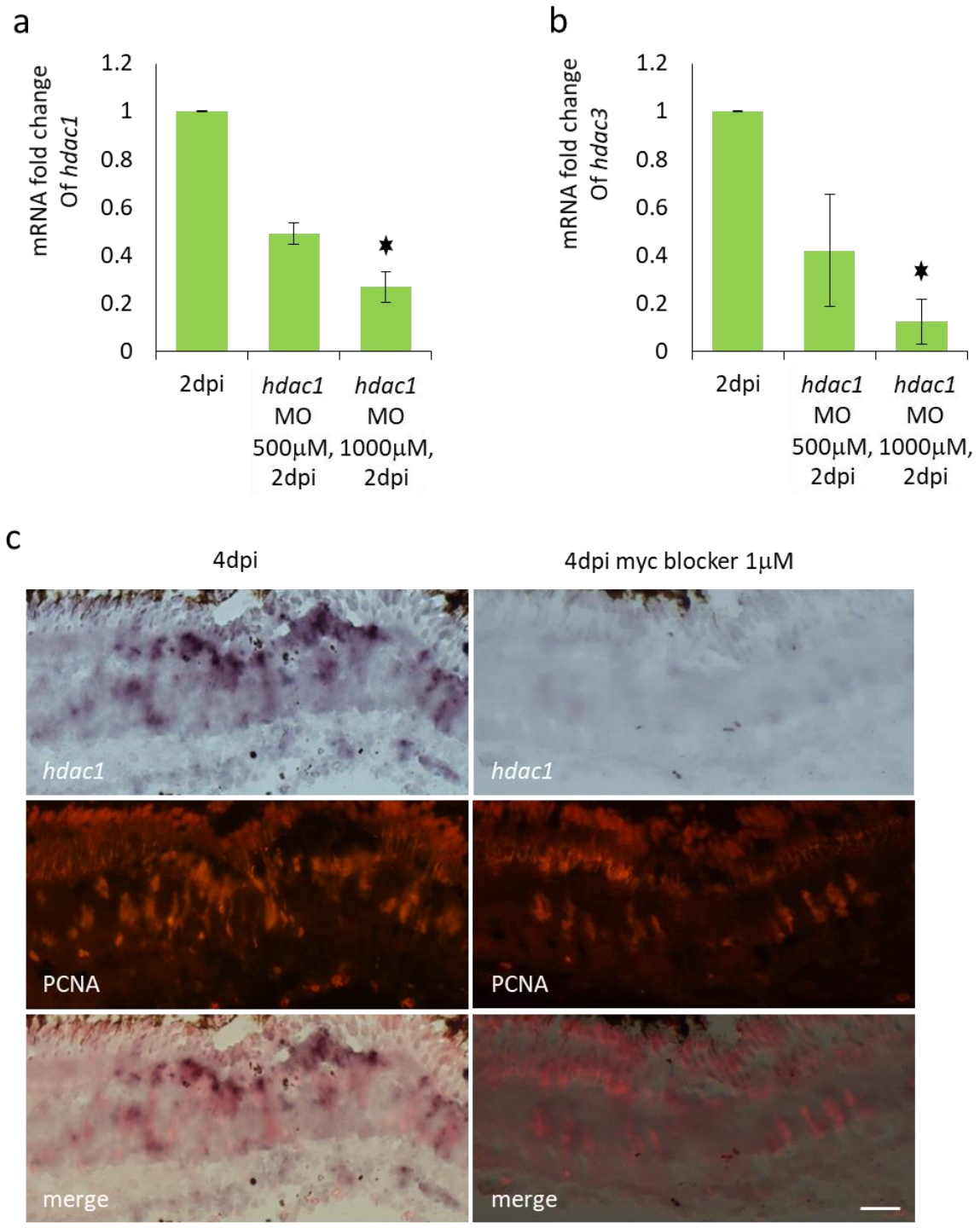


Fig 3.63: Downregulation of *hdac1* and *hdac3* in *hdac1* knocked down retinae at 2dpi. (a) qRT-PCR shows downregulation of *hdac1* in 2dpi retinae electroporated with increasing concentration of *hdac1* MO. (b) mRNA ISH shows drastic decline in *hdac1* level in same condition. (c) quantification of tha same. (d) qRT-PCR shows downregulation of *hdac3* in 2dpi retinae electroporated with increasing concentration of *hdac1* MO. Scale bar: 20µ.

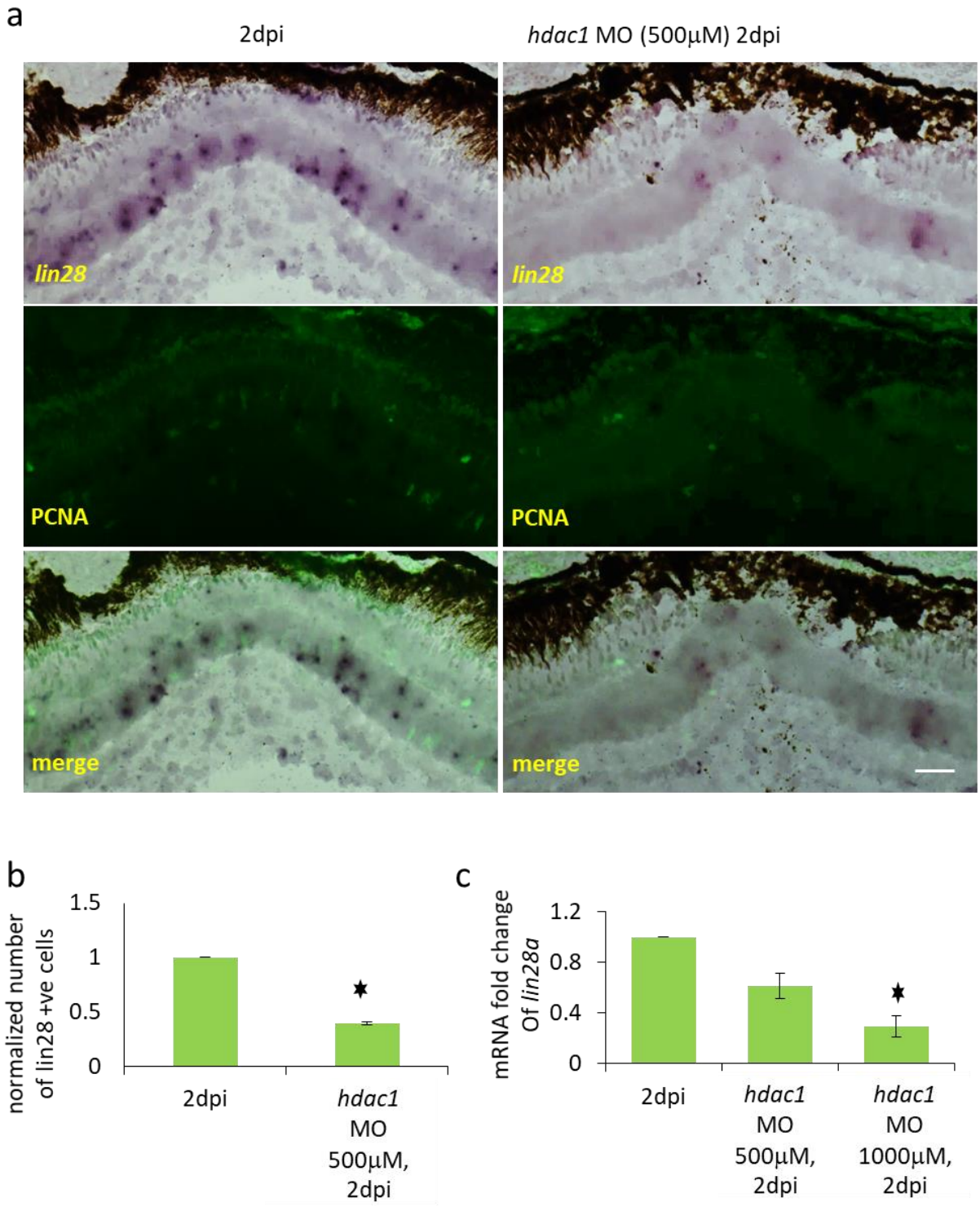


Fig 3.64: Downregulation of *lin28a* in *hdac1* knocked down retinae at 2dpi. (a) mRNA ISH shows significant decline in *lin28a* expression level in same condition. Scale bar: 25µ. (c) quantification of tha same. (c) qRT-PCR shows downregulation of *lin28a* in 2dpi retinae electroporated with increasing concentration of *hdac1* MO. $P<0.05$.

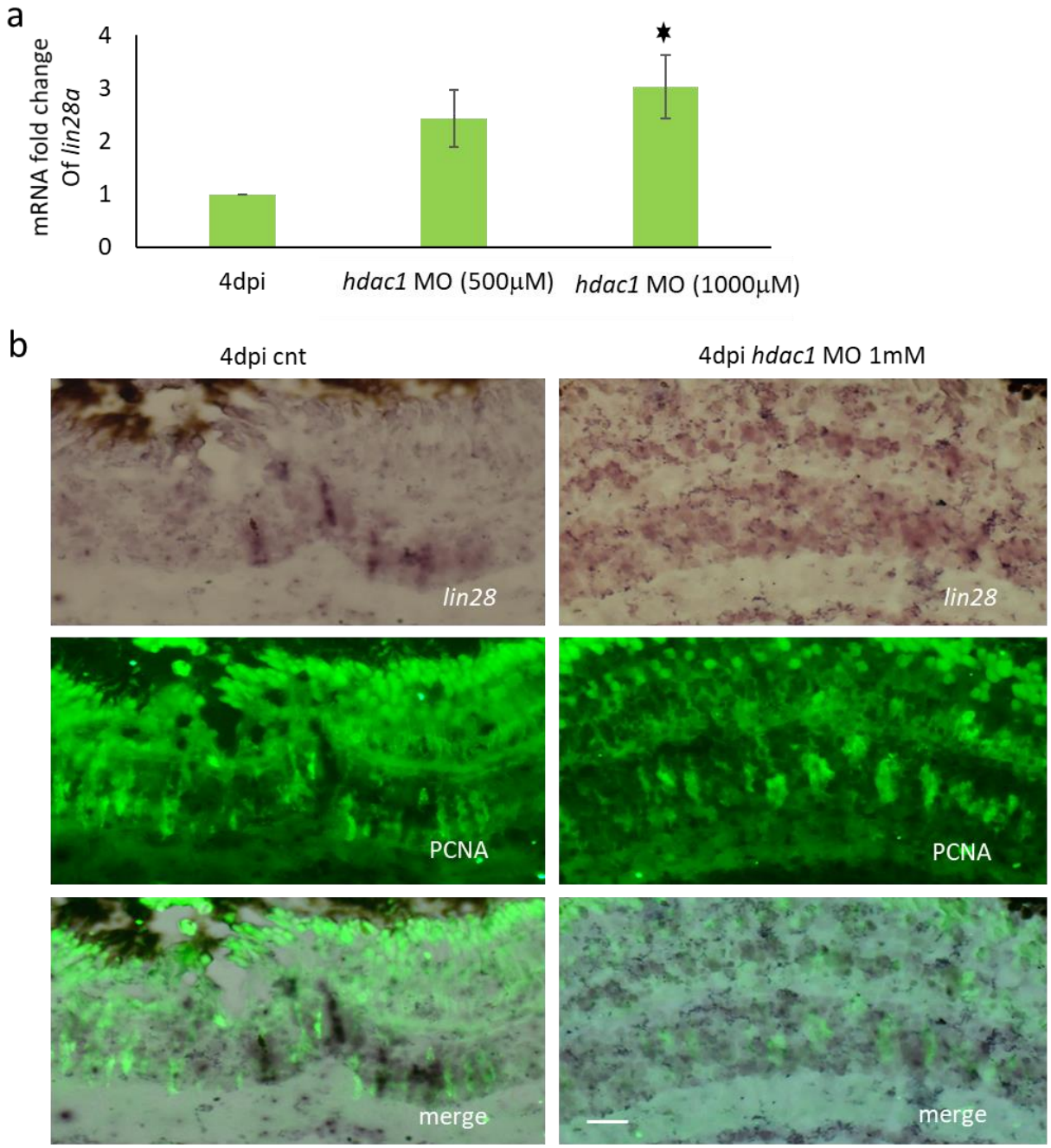


Fig 3.65: Upregulation of *lin28a* in *hdac1* knocked down retinae at 4dpi. (a) qRT-PCR shows upregulation of *lin28a* in 4dpi retinae electroporated with *hdac1* MO. (b) mRNA ISH shown pan retinal induction of *ln28a* in same background compared to injury restricted expression in control. P<0.01. Scale bar: 20µ.

3.2.7 Hdacs inhibitors mediated restriction in proliferation is reversible and might be regulated through Lin28a and Hdacs mediated upregulation of regeneration associated cytokines and hormones.

Some of the cytokines and hormones, such as *il6, il6r, il11a, il11b, il11ra, lepa, lepb, lepr, lifra, lifrb, clcf, crlf,* which are already reported to be upregulated at various time points following retinal injury. Next, by qRT-PCR and RT-PCR, we checked their expression levels in 2dpi retinae treated with increasing concentration of TSA (Fig 3.66 a, b) or VPA (Fig 3.66 c, d), and found them to be significantly downregulated in both these Hdacs inhibited background.

Next, we wanted to check, following injury, if we withdraw the inhibitors after 4 days; whether new cells start entering cell cycle by re-inducing these cytokines. For this, we carried out two sets of experiments. In one set, fish, following retinal injury, were dipped into either VPA (experimental) solution or water (control) for 4days. At 4dpi, they received a 5 hours BrdU pulse and then they were shifted to normal system water and were euthanized at 8dpi (Fig 3.67 a). In another set, fish were given retinal injury followed by dipping them in VPA solution or water for 4 days. In this set, fish were removed to normal water at 5dpi without giving them BrdU pulsing at 4dpi. These fish received a BrdU pulse at 8dpi before euthanisation (Fig 3.67 b). Fish eyes were then, fixed and sectioned for BrdU and PCNA immunostaining. Interestingly, the first set showed very less BrdU positive cells and more PCNA positive cells at 8pi (Fig 3.67 c, e, f). As these fish received BrdU pulsing at 4dpi after continuous VPA treatment for 4 days, a low number of BrdU positive cells were expected. But the increased

number of PCNA positive cells came as a surprise and this must be pertaining to the new cell divisions that started after the withdrawal of VPA. If this was to be believed, then in the second set, in which BrdU pulsing was done at 8dpi, we should get a greater number of BrdU positive cells which will colocalize with PCNA also. As expected, the second set showed nearly all PCNA positive cells were also BrdU positive (Fig 3.67 d, e, f). This suggested VPA mediated blockade of proliferation is reversible and once the VPA mediated blockade of Hdacs is withdrawn, cells can re-enter the cell cycle. We also repeated this experiment with TSA and got similar results (Fig 3.68 a-f).

Next, we wanted to check whether these newly dividing cells possess the capacity to migrate to all retinal cell layers. For this, in one set, when injured fish were transferred to water at 4dpi, they received 5 hours BrdU pulsing for 2 days till 5dpi and were euthanized at 9dpi (Fig 3.69 a). In another set, instead of 9dpi, fish were euthanized at 60dpi so as to make sure all cells get enough time to migrate to all retinal cell layers (Fig 3.69 c). BrdU immunostaining showed punctate post-proliferative BrdU positive cells at control 9dpi retinae. Compared to this, VPA treated retinae showed an increased number of BrdU positive cells with an elongated shape indicative of proliferation (Fig 3.69 b, e, f). At 60 dpi, like that of control, the newly proliferating cells also were found to be migrated to all different retinal layers (Fig 3.69 d-f).

As we have already seen *lin28a* levels goes down in Hdacs inhibited retinae, we wanted to check its level after withdrawal of inhibition. For this, after 4 days of dipping injured fish in VPA solution, they were transferred to normal system water for another 2 days. Retinae were harvested at 6dpi for RNA preparation. Fold change was compared to 2dpi control. qRT-PCR (Fig 3.70 a) and RT-PCR (Fig 3.70 b) showed *lin28a* levels to be upregulated in VPA withdrawn retinae. As *lin28a* is one of the most important key factors in zebrafish retina regeneration, this data explains the reason for new cells entering the cell cycle after the withdrawal of Hdacs inhibition.

We also checked levels of different regeneration associated cytokines and hormones, which we found to be downregulated in continuous TSA treatment, and found them to be also upregulated (Fig 3.70 c, d) after withdrawal of TSA. Cytokines and hormones also were found to be upregulated in VPA withdrawn retinae (Fig 3.71 a, b) as shown by qRT-PCR and RT-PCR.

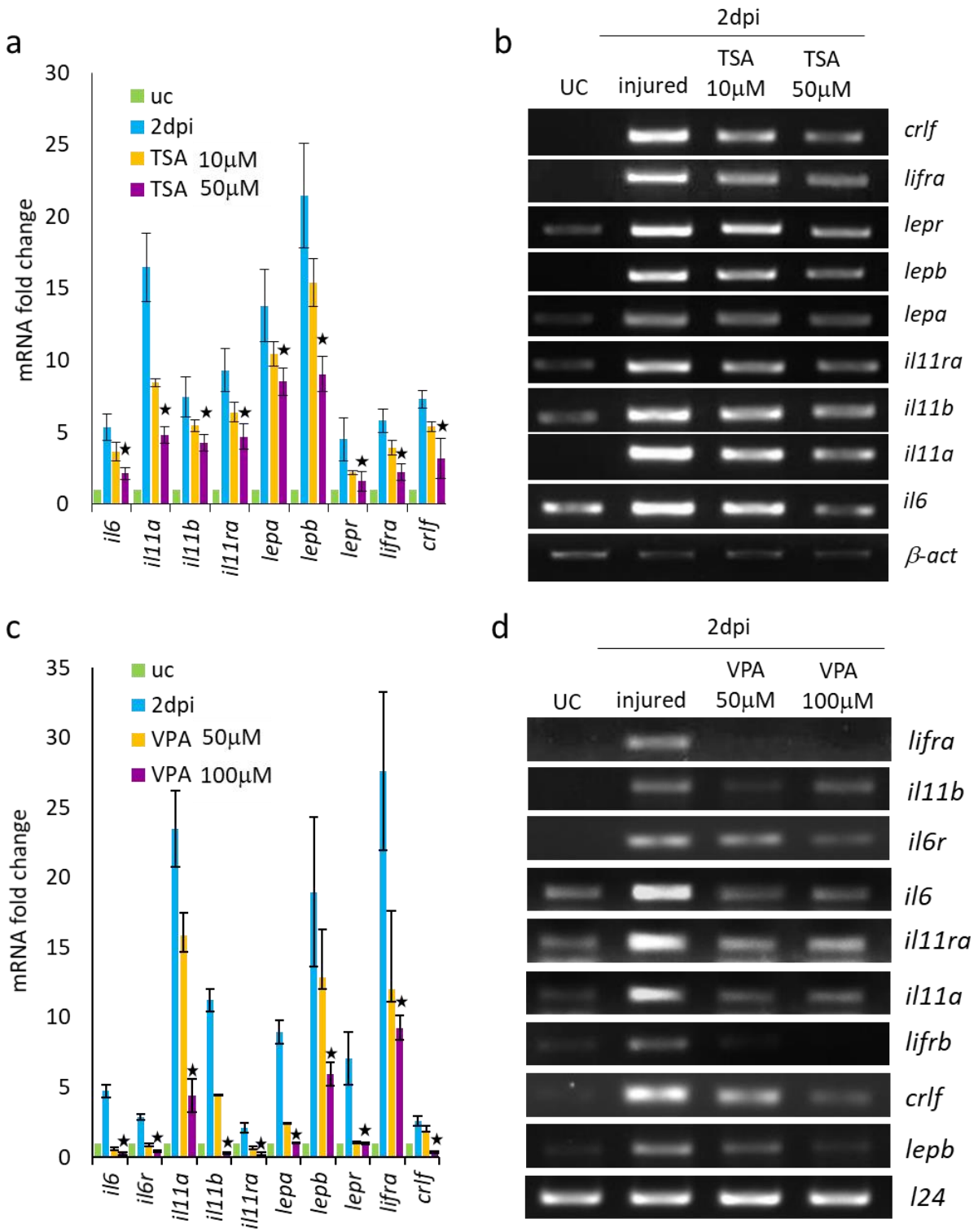


Fig 3.66: Down regulation of regeneration associated cytokines and hormones in 2dpi and 4dpi. (a) qRT-PCR and (b) RT-PCR shows downregulation of different cytokines and hormones in 2dpi retinae treated with increasing concentration of TSA. (c) qRT-PCR and (d) RT-PCR shows similar result in 2dpi retinae treated with increasing concentration of VPA. $P<0.001$.

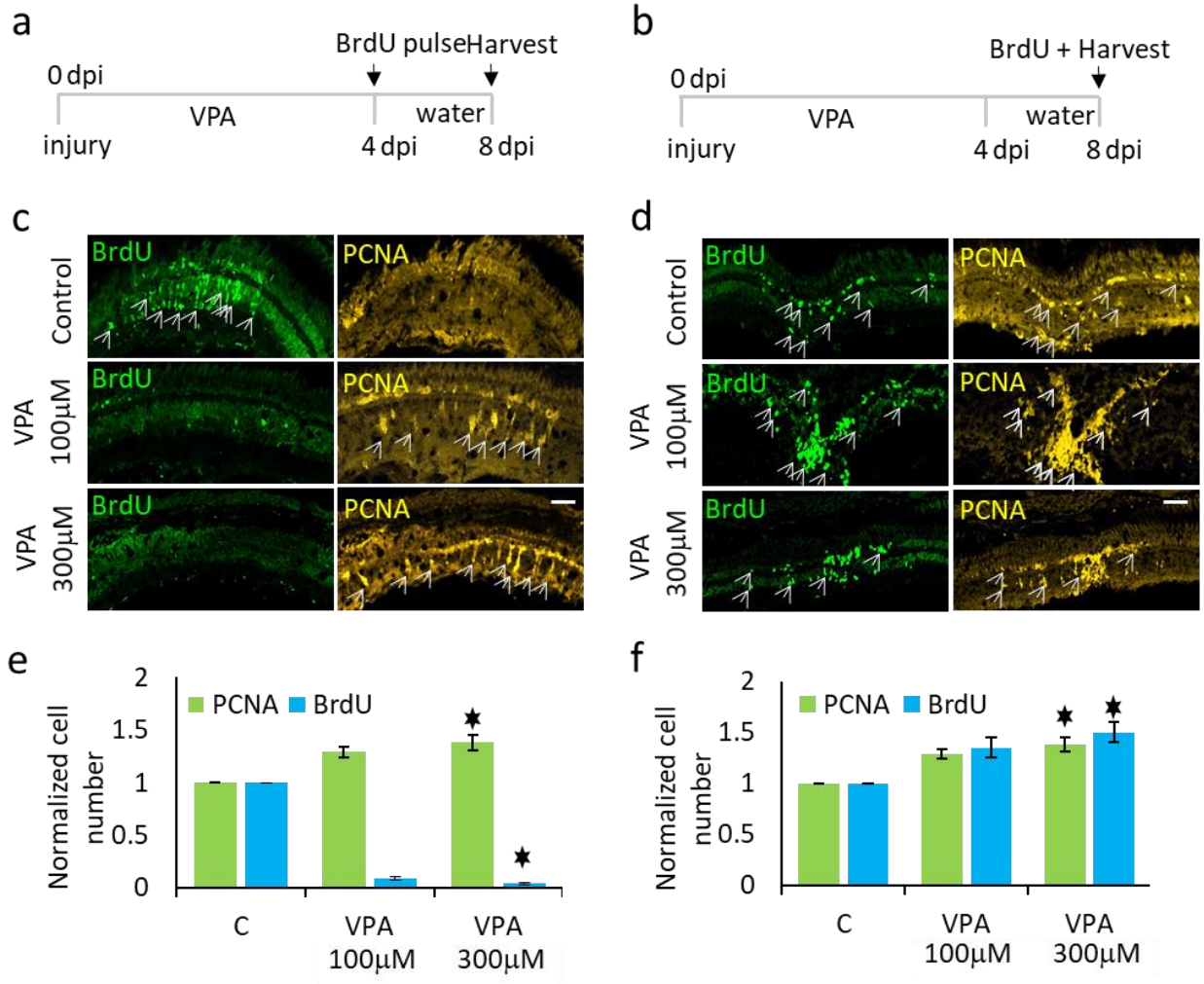


Fig 3.67: VPA mediated blockade of proliferation is reversible. (a) and (b) experimental regime showing days of BrdU pulsing and harvest after VPA withdrawal. (c) BrdU and PCNA IF shows very less BrdU positive cells in VPA treated retinae at 8dpi compared to PCNA posive cell number when BrdU pulsing was done at 4dpi and immediately after withdrawal of VPA. (d) BrdU and PCNA IF shows all PCNA positive cells at 8dpi are BrdU positive when BrdU pulsing was done at 8dpi after withdrawal of VPA at 4dpi. (e) quantification of BrdU and PCNA positive cell number in retinae which received BrdU pulsing at 4dpi. (f) quantification of BrdU and PCNA positive cell number in retinae which received BrdU pulsing at 8dpi. $P< 0.05$. Scale bar: 25µ in c and d.

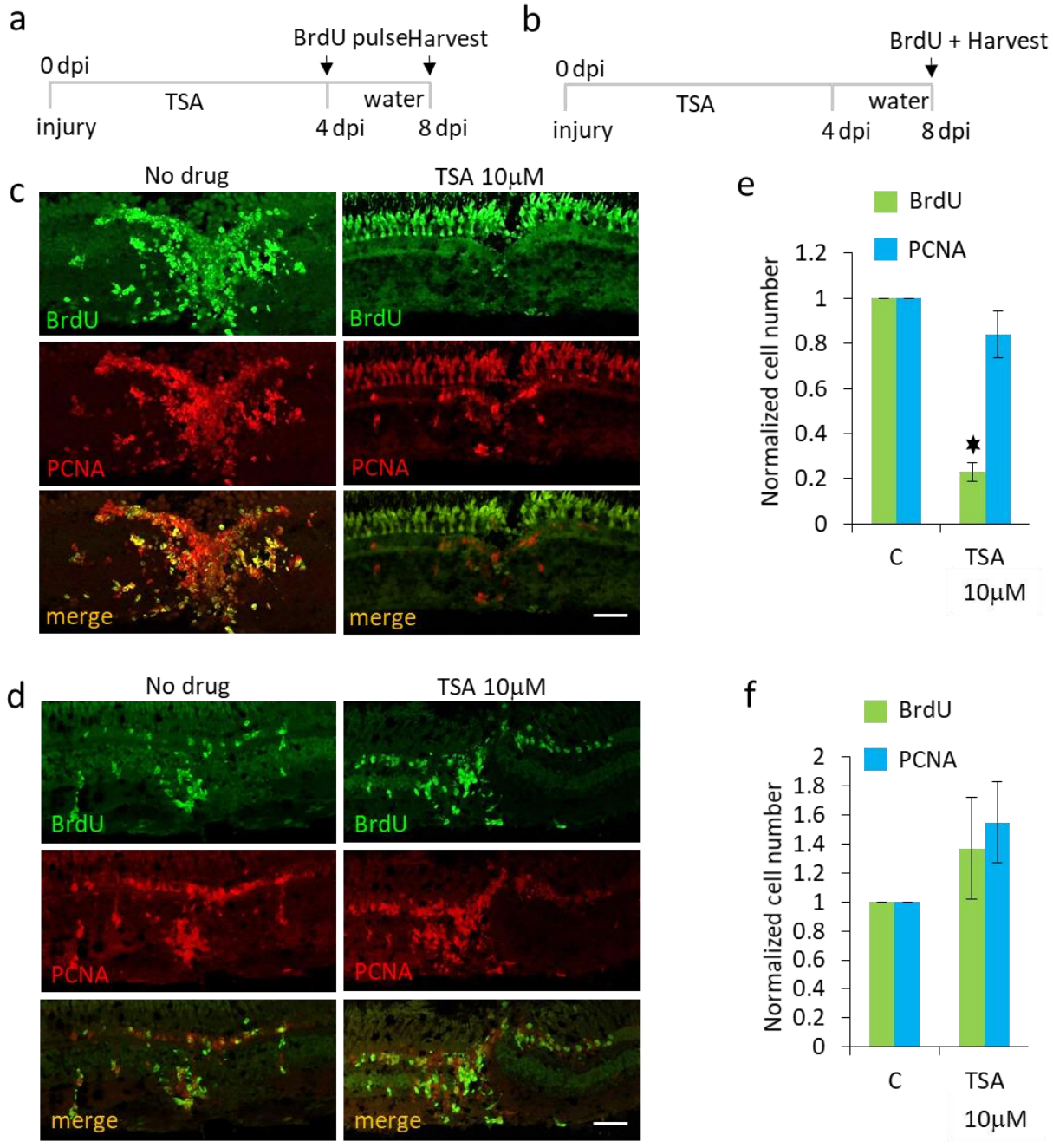


Fig 3.68: TSA mediated blockade of proliferation is reversible. (a) and (b) experimental regime showing days of BrdU pulsing and harvest after TSA withdrawal. (c) BrdU and PCNA IF shows very less BrdU positive cells in TSA treated retinae at 8dpi compared to PCNA posive cell number when BrdU pulsing was done at 4dpi and immediately after withdrawal of TSA. (d) BrdU and PCNA IF shows all PCNA positive cells at 8dpi are BrdU positive when BrdU pulsing was done at 8dpi after withdrawal of TSA at 4dpi. (e) quantification of BrdU and PCNA positive cell number in retinae which received BrdU pulsing at 4dpi. (f) quantification of BrdU and PCNA positive cell number in retinae which received BrdU pulsing at 8dpi. $P<0.05$. Scale bar: 25µ in c and d.

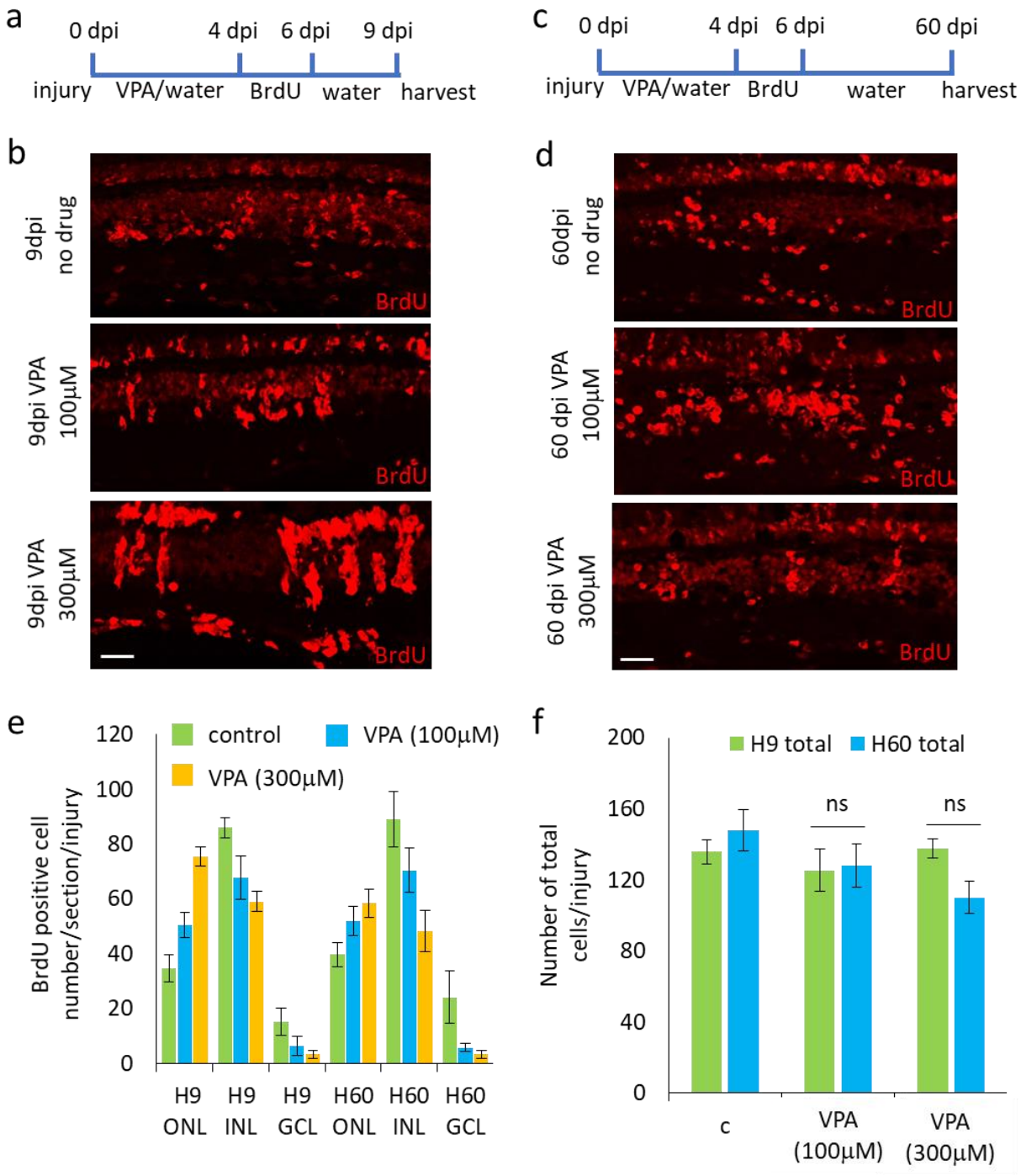


Fig 3.69: Newly proliferating cells after withdrawal of inhibition on HDACs migrate to all cell layers. (a) experimental regime where BrdU pulsing was done for 2 days after VPA withdrawal and retinae were harvested at 9dpi. (b) BrdU IF shows columnar proliferating cells at 9dpi compared to punctate cells in control retina. (c) experimental regime where BrdU pulsing was done for 2 days after VPA withdrawal and retinae were harvested at 60 dpi. (d) BrdU IF shows newly proliferating cells migrated to all retinal cell layers. (e) and (f) quantification of the same. ns:not significant. Scale bar: 20µ in c and d.

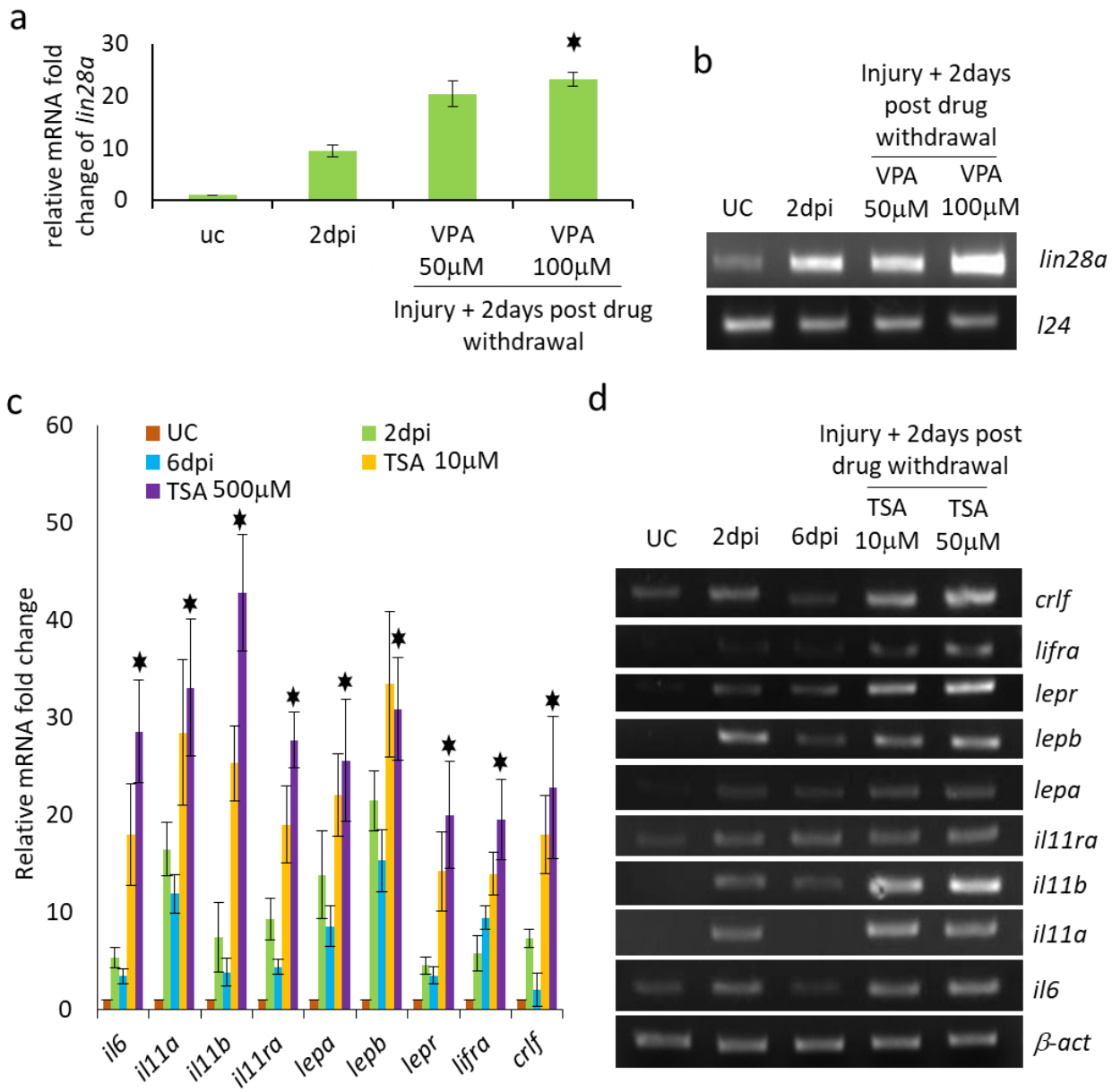


Fig 3.70: Upregulation of *lin28a* and regeneration associated cytokines after withdrawal of inhibition on HDACs. (a, b) qRT-PCR (a) and RT-PCR (b) show upregulation of *lin28a* in injured retinae after 2 days of VPA withdrawal after a treatment of 4 days. (c, d) qRT-PCR (c) and RT-PCR (d) shows upregulation of regeneration associated hormones and cytokines in injured retinae after 2 days of TSA withdrawal after a treatment of 4 days. P<0.002.

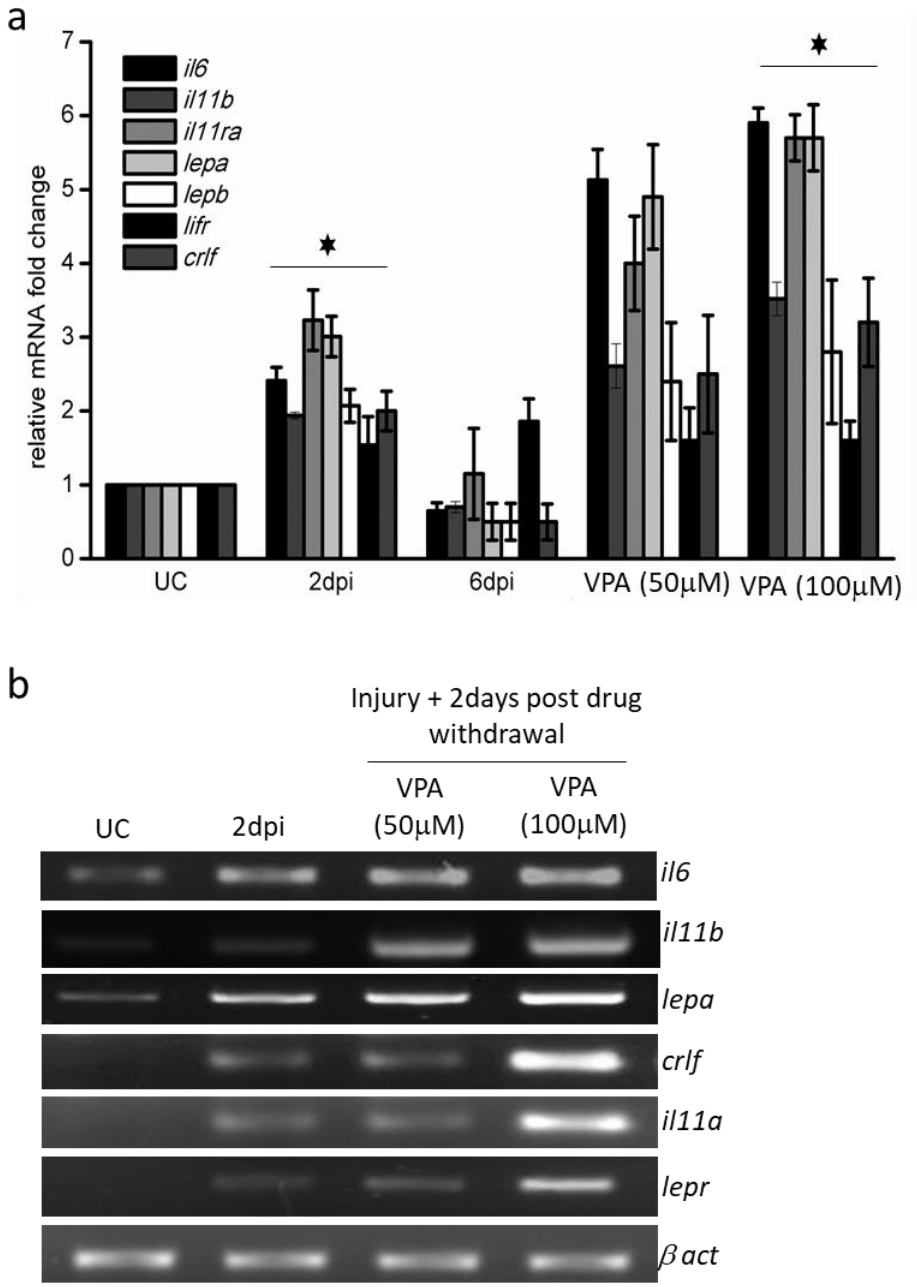


Fig 3.71: Upregulation of regeneration associated hormones and cytokines after withdrawal of VPA. (a) qRT-PCR and (b) RT-PCR shows upregulation of regeneration associated hormones and cytokines in injured retinae after 2 days of VPA withdrawal after a treatment of 4 days. $P<0.002$.

Chapter 3

Delta/Notch signalling restricts de-differentiation and proliferation in the injury responsive zone by suppressing *mycb* and *lin28a* and also mediates the effect of Hdacs.

3.3.1 Delta/Notch signalling regulates the expression of *lin28a* through Her4.1.

According to previous reports, Notch signalling is upregulated post retinal injury to restrict proliferation in the injury responsive zone. Inhibiting Notch signalling results in increased proliferating cells in an expanded zone of proliferation. Over-induction of *ascl1a* and *hb-egfa* have also been shown as a causative factor behind this expansion of proliferation upon blockade of Notch signalling. We further wanted to check the expression levels of other regeneration associated genes like *lin28a.*

To understand whether *lin28a* and Notch signalling components are regulated in same cell, we carried out a double FISH experiment in 4dpi retinae to probe for *lin28a* and *her4.1*, which is a direct read-out of Notch signalling. mRNA FISH showed only a few of the *her4.1* positive cells colocalized with *lin28a* positive cells which are also proliferating as marked by BrdU incorporation (Fig 3.72 a). This mutual exclusive expression pattern of *her4.1* and *lin28a* made us speculate a repressive regulation on *lin28a* enforced by Notch signalling. Also, when we co-injected *lin28a:gfp-luciferase* reporter plasmid and various concentrations of DAPT in zebrafish embryos, we found *lin28a* promoter activity to be increased in a does dependent manner (Fig 3.72 b). We also found an increased level of *lin28a* mRNA in 2dpi retinae treated with increasing concentration of DAPT by qRT-PCR (Fig 3.72 c) and RT-PCR (Fig 3.72 d).

Subsequently, we also found two putative Her/Hes binding sites on *lin28a* promoter (Fig 3.73 a) which suggested a possible direct regulation of *lin28a* by Delta/Notch signalling mediated upregulation of *her4.1*. To Figure out the possibilities, we decided to co-inject zebrafish embryos with *lin28a:gfp-luciferase* reporter and increasing concentration of *notch intra-*

cellular domain mRNA, expecting a decrease in *lin28a* promoter activity exerted by *her4.1* which should be upregulated by *nicd* mRNA.

To check if *nicd* mRNA can induce *her4.1*, we first carried out another luciferase assay with embryos co-injected with *her4.1:gfp-luciferase* and *nicd* mRNA which showed increased *her4.1* promoter activity in a dose-dependent manner (Fig 3.73 b). Luciferase assay carried out in fish embryos with *lin28a:gfp-luciferase* and increasing concentration of *nicd* mRNA showed a dose-dependent decrease in *lin28a* promoter activity (Fig 3.73 c). Had Her4.1 been binding to *lin28a* promoter to suppress its activity, *lin28a:gfp-luciferase* reporter with mutated Her/Hes binding sites should not show the same regulation. For this, we mutated both the Her4.1 binding sites on *lin28a* promoter (Fig 3.73 a) and co-injected this mutated *lin28a:gfp-luciferase* reporter along with *nicd* mRNA which, indeed, did not show downregulation of *lin28a* promoter (Fig 3.73 c). These data suggest Delta/Notch signalling regulate the expression of *lin28a* in regenerating retinae.

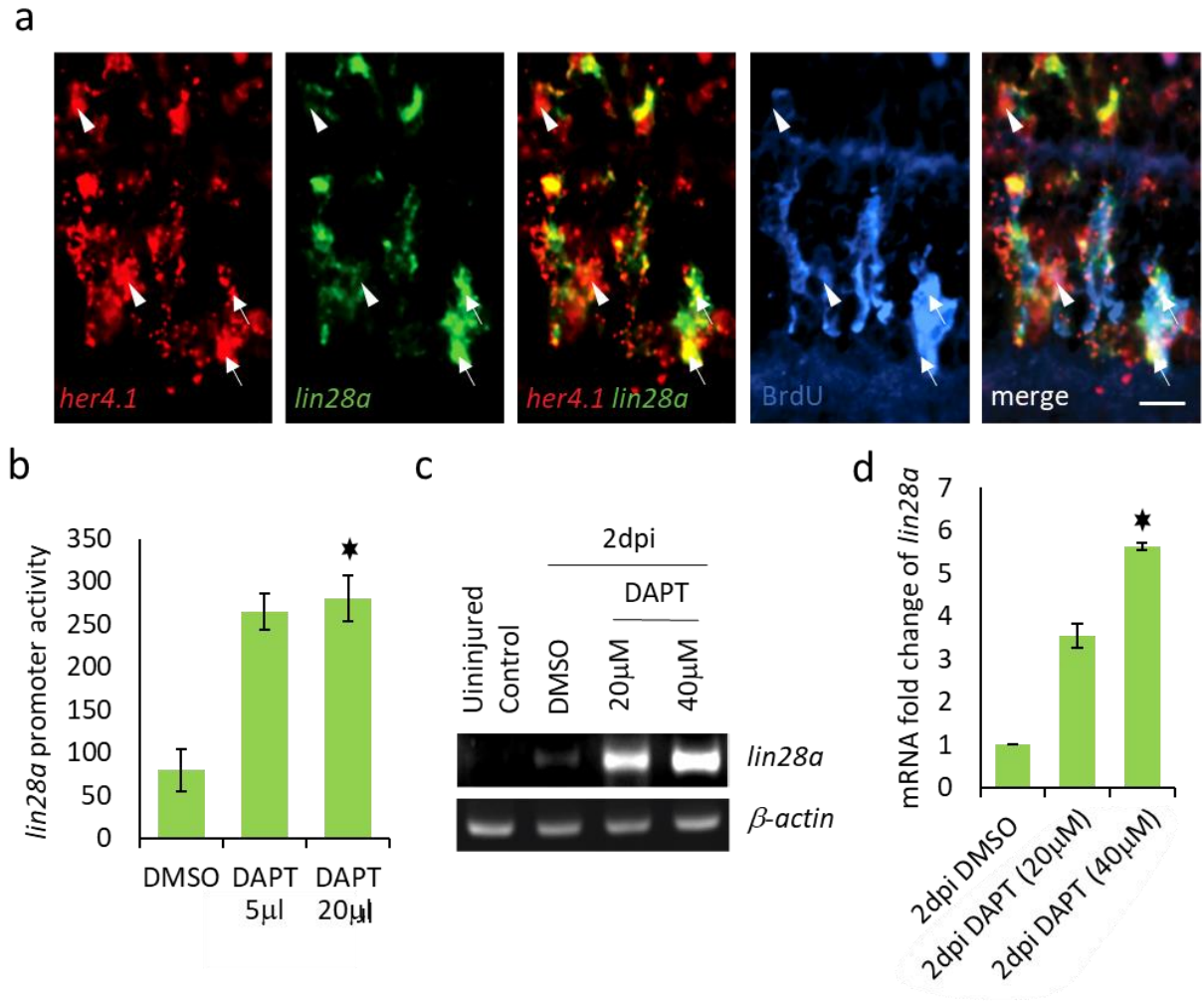


Fig 3.72: Delta/Notch signalling regulates lin28a. (a) double mRNA FISH shows mutual exclusive expression of *lin28a* and *her4.1*. Scale bar 5µ.(b) increase of *lin28a* promoter activity by DAPT mediated blockade of Notch signalling. (c, d) RT-PCR (c) and qRT-PCR (d) shows upregulation of *lin28a* in 2dpi retinae treated with DAPT. P<0.001.

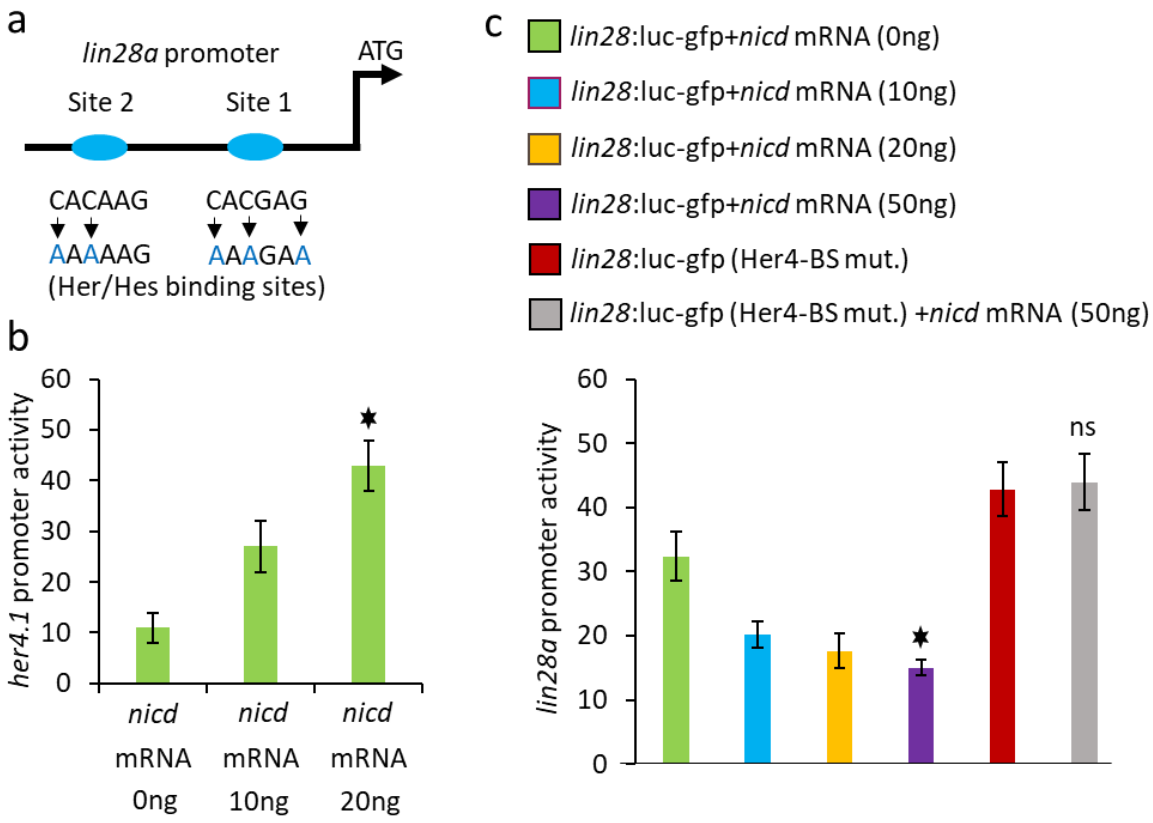


Fig 3.73: Delta/Notch signalling controls the expression of *lin28a* directly through Her4.1. (a) diagrammatic representation of *lin28a* promoter with two putative Her4.1 binding sites. Sites of mutations are also indicated below the wild type sequenc. (b) dose dependent increase in *her4.1* promoter activity by *nicd* mRNA. (c) regulation of wild type and mutated *lin28a* promoter activity by increasing concentration of *nicd* mRNA. P< 0.002. ns: not significant.

3.3.2 Delta-Notch signalling restricts proliferation to the injury responsive zone by suppressing Mycb.

To expound more about the mechanisms by which Delta/Notch signalling restricts the zone of proliferation, we checked the expression pattern of other regeneration associated genes like *ascl1a* and *insm1a*, which has already been reported to be induced post retinal injury. After DAPT treatment, *ascl1a* is also reported to be upregulated to facilitate increased proliferation. Both qRT-PCR and RT-PCR showed *ascl1a* to be upregulated and *insm1a* to be down-regulated in 2dpi retinae treated with DAPT (Fig 3.74 a). Upregulation of *ascl1a* and *insm1a* also supported the observed upregulation of *myca* and *mycb* in a similar background (Fig 3.74 a) as we have already shown that *insm1a* negatively regulates *mycb*.

If Delta/Notch signalling regulates the number of proliferative cells by regulating *mycb*, then in Delta/Notch signalling blocked background, if we block Myc, then the increase in proliferative cell number should be reversed back. To check this hypothesis, we performed BrdU IF in retinal sections treated with wither DAPT alone or in combination with Myc/Max inhibitor 10058-F4 Interestingly, the increase in BrdU positive cells and the expansion of injury responsive zone observed in DAPT treated retinae were reversed back by treating retinae with DAPT along with 10058-F4 or *mycb* MO (Fig 3.74 b, c).

To check if this Myc-inhibitor mediated abatement of increased proliferation seen in Delta/Notch signalling blocked retinae is associated with decreased expression of regeneration-associated genes, we estimated the expression levels of several regeneration-associated genes. RT-PCR (Fig 3.75 a) and qRT-PCR (Fig 3.75 b) showed, indeed, the expression of regeneration associated genes like *myca*, *mycb*, *ascl1a*, *lin28a* or *dld* are decreased significantly in 2dpi

retinae treated with 100-58-F4 alone or in combination with DAPT compared to 2dpi retinae treated with DAPT alone. We also checked Ascl1a and Myca/b protein level by Western blot from whole retinal extract treated with either DAPT, or 10058-F4 or both in combination. As expected, in 2dpi retinae treated with DAPT alone we got higher levels of both Ascl1a and Mycb which got decreased in retinae treated with 10058-F4 alone or in combination with DAPT (Fig 3.75 c).

Also, RT-PCR (Fig 3.75 a) and qRT-PCR (3.75 b) showed *her4.1* to be upregulated which might be another reason for the reduction of the proliferative zone in 2dpi retinae treated with both 10058-F4 and DAPT. This was further accompanied by an increase in the level of acetylated histone 4 (Ac.H4) as shown by IF of Ac.H4 in 2dpi retinae treated with 10058-F4 and DAPT together (Fig 3.75 d).

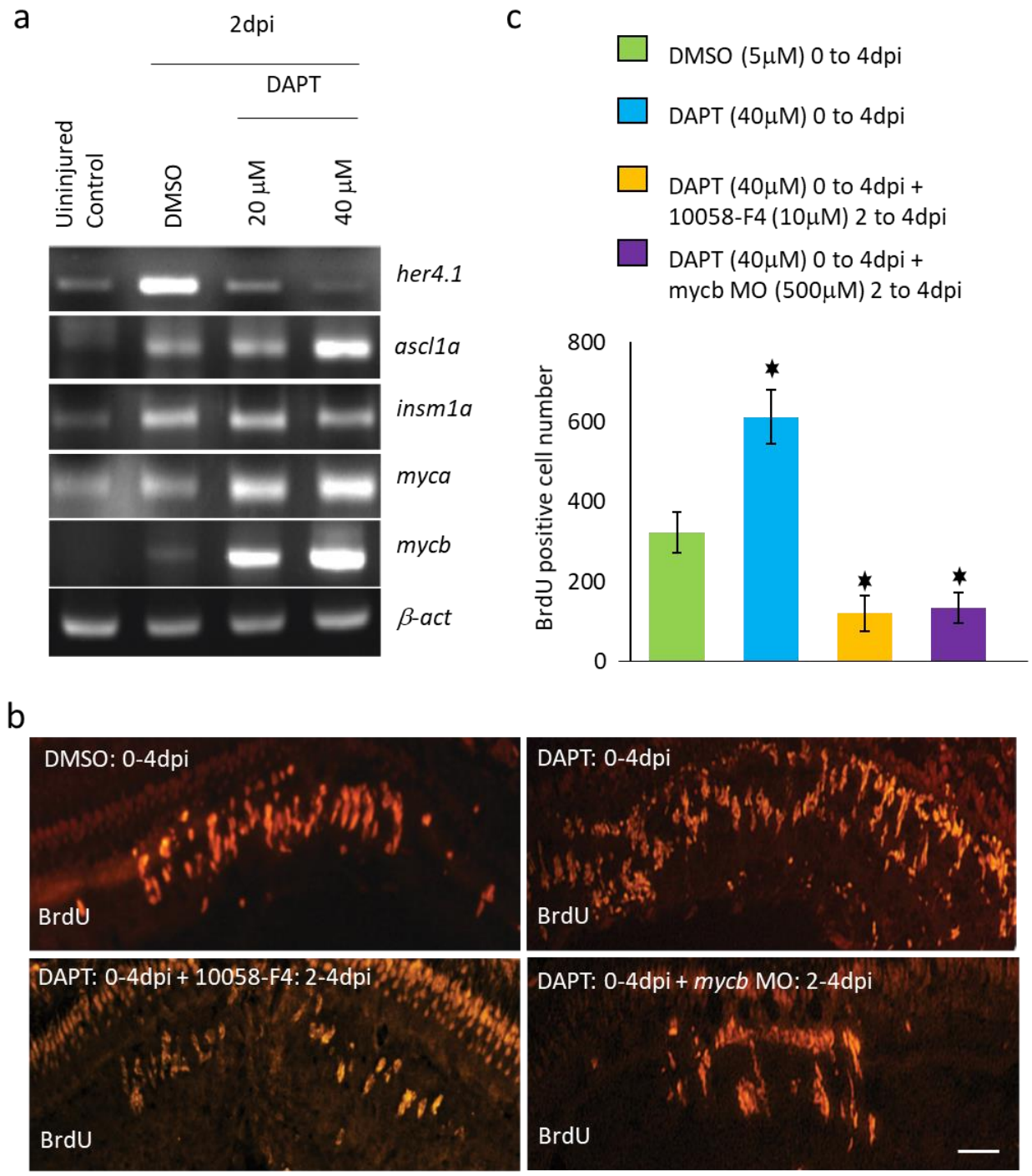


Fig 3.74: Delta/Notch signalling restricts the zone of proliferation by suppressing *mycb*. (a) RT-PCR and (b) qRT-PCR shows mRNA levels of several regeneration associated genes in 2dpi retinae treated with DAPT. (c) BrdU IF shows the expansion of proliferative zone seen in DAPT treated retinae is abated in retinae treated with DAPT in combination with 10058-F4. Scale bar: 30µ. P<0.02.

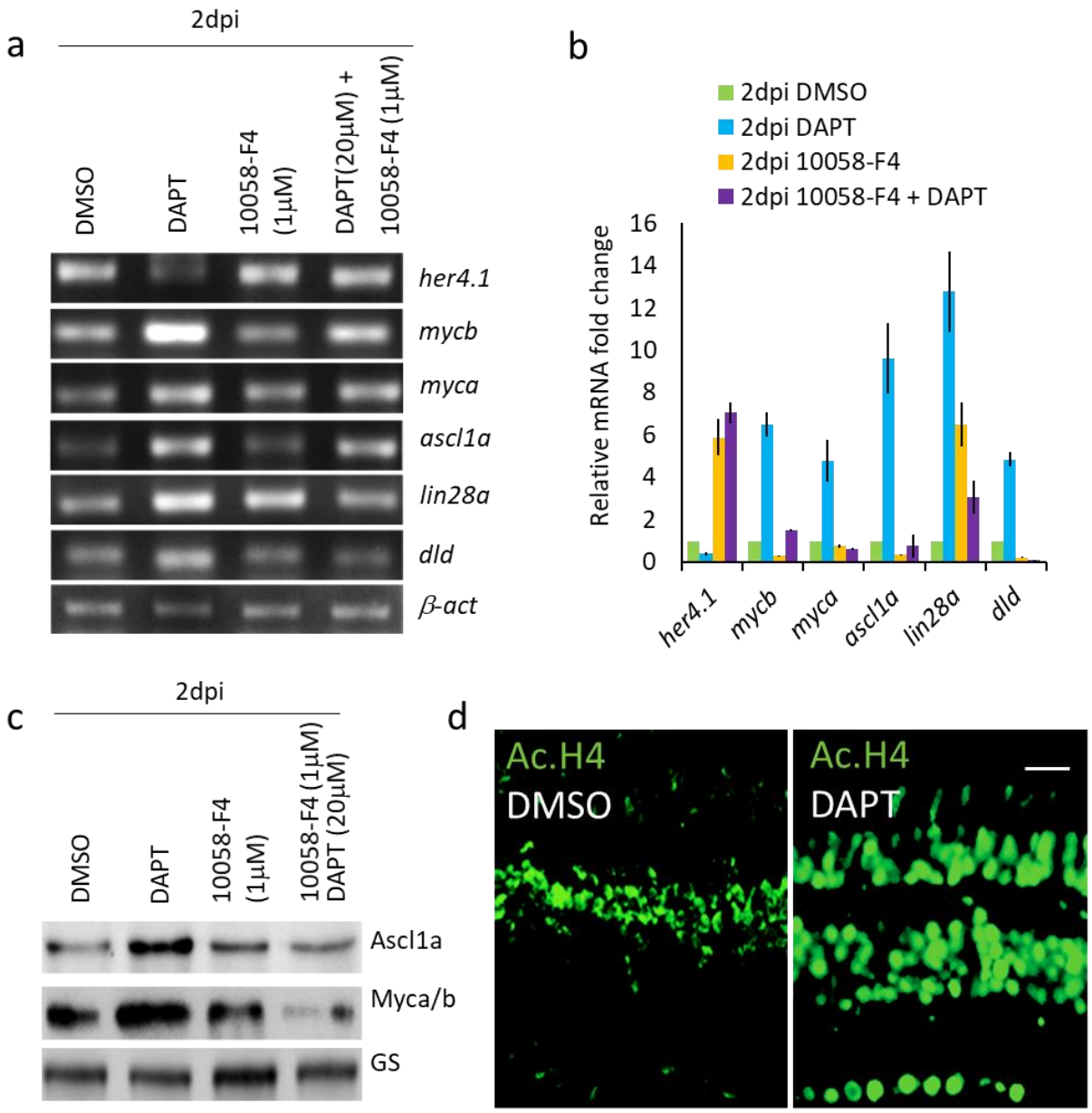


Fig 3.75: Reduction in expression of regeneration associated genes in 2dpi retinae treated with DAPT in combination with 10058-F4. (a) RT-PCR and (b) qRT-PCR shows reduced expression of mRNA of several regeneration-associated genes in 2dpi retinae treated with 10058-F4 alone or in combination with DAPT compared to 2dpi retinae treated with DAPT alone. (c) Western blot of Ascl1a and Mycb in 2dpi retinae treated with DAPT or 10058-F4, separately or in combination. (d) Acetylated Histone 4 (Ac.H4) immunostaining in vehicle (DMSO) and DAPT treated mice retinae. Scale bar: 10µ.

3.3.3 Hdacs regulate retina regeneration by suppressing the regulation of regeneration and regeneration associated genes by Delta/Notch signalling effector Her4.1.

Previous studies have shown a regulatory behaviour of Delta/Notch signalling on the status of proliferation during retina regeneration. One of the downstream targets of Delta/Notch signalling is Her4.1 whose transcription is upregulated during retina regeneration along with various genes of Delta/Notch signalling pathway. Surprisingly, no previous studies have characterized the direct effect of *her4.1* induction on proliferation. As Delta/Notch signalling is known to be upregulated to restrict the proliferation of MGPCs in the injury responsive zone, we hypothesized a similar role for Her4.1. To find out the exact role of Her4.1 during retina regeneration, along with injury, retinae were injected with MO against *her4.1* followed by electroporation at 3dpi and harvested at 6dpi after 4-5 hours of BrdU pulsing (Fig 3.76 a). BrdU immunostaining showed an increased number of BrdU positive cells in *her4.1* knocked down retinae as compared with control MO (Fig 3.76 b, c).

Next, we wanted to find out the mechanism behind the regulation of proliferation by Her4.1. By qRT-PCR, we probed for *ascl1a*, *insm1a*, *lin28a*, *mycb*, *hdac1* and *her4.1* in *her4.1* knocked down retinae and found increased levels of *ascl1a*, *lin28a*, *insm1a*, and *her4.1* knocked down retinae 5dpi retinae following a similar experimental time-line as described previously (Fig 3.76 a). The mRNA levels of *ascl1a*, *insm1a, her4.1*, and *lin28a* were found to be upregulated and that of *hdac1* to be down-regulated in *her4.1* knocked down 5dpi retinae (Fig 3.77 a, b). The unchanged mRNA levels of *mycb* mRNA (Fig 3.77 b) could result as an outcome of a dual regulation by both *ascl1a* and *insm1a* as we have discussed earlier.

As we found increased proliferation in *her4.1* knocked down retinae and increased levels of several regeneration associated genes, we further wanted to check the expression status of various regeneration associated cytokines and hormones which are in general upregulated during regeneration and facilitate proliferation by activating several signalling pathways and transcription factors. qRT-PCR showed increased levels of several cytokines and their receptors like *il6, il6r, il11a, il11b, il11ra, lifrb, crlf1, lepa* and *lepr* (Fig 3.77 c). We also found putative Her/Hes binding sites on the 3kb promoter of several regeneration-associated cytokines, hormones and their receptors (Fig 3.77 d). Taken together, these observations suggest that Her4.1 plays a crucial role in negatively controlling the formation of MGPCs by regulating a plethora of different regeneration associated genes. The exact mechanism behind this feat of Her4.1 remains yet to be looked for.

It is interesting to note that we already showed induction of *her4.1* in Hdacs inhibited retinae in chapter 2. We have also seen that *her4.1* knockdown in TSA treated retinae had increased number of MGPCs in 6dpi as compared to control. In the light of the current results, it seems logical to suggest that the re-entry in cell cycle after withdrawal of Hdacs' inhibition by VPA or TSA, might be regulated through Her4.1, had we been able to find a decrease in the transcript levels of *her4.1* in retinae with TSA withdrawal. Indeed, qRT-PCR showed that *her4.1* level goes down when the inhibition on Hdacs is withdrawn (Fig 3.77 e). This data also supports the down and up-regulation of *lin28a* in TSA-treated and TSA-withdrawn retinae respectively, as we have already shown a direct regulation of *lin28a* promoter by Her4.1 in early sections of this chapter.

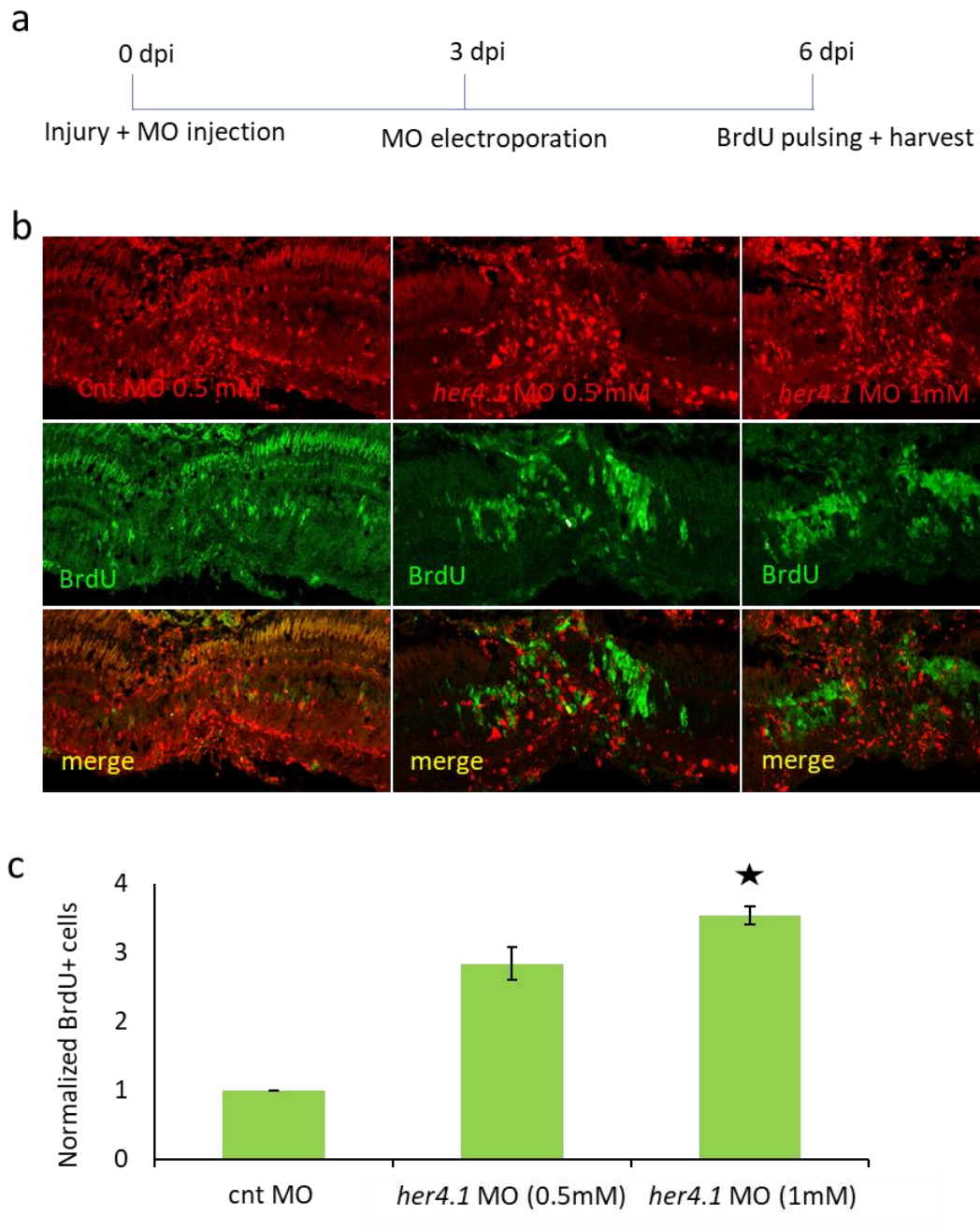


Fig 3.76: Regulation of regeneration by Her4.1. (a) experimental time points for *her4.1* knock down in retinae. (b) BrdU immunostaining shows increased number of BrdU positive proliferating cells in INL in 6dpi retinae electroporated with MO against *her4.1* as compared to control. Scale bar: 35µ. (c) quantification of the same. P< o.oo1.

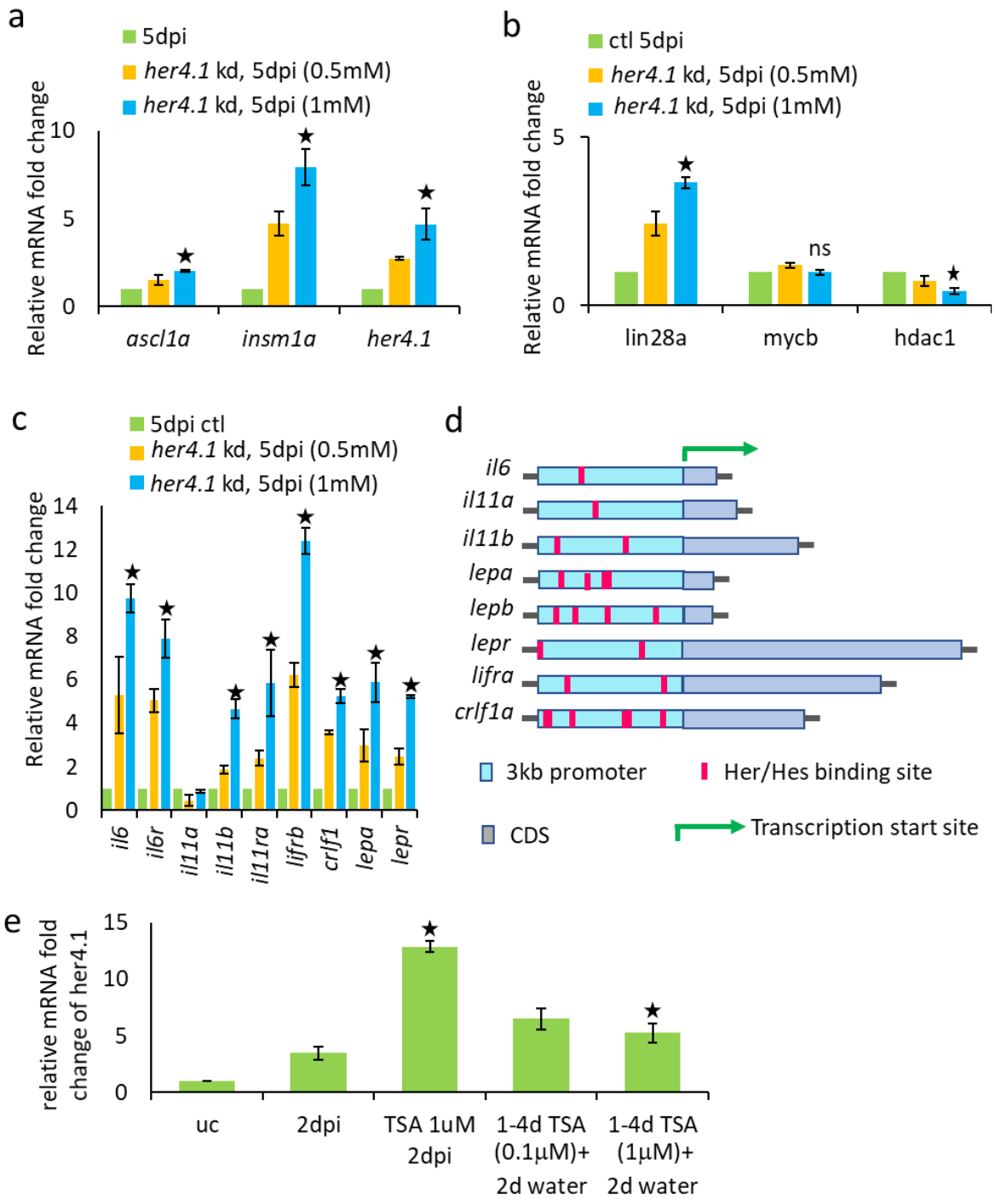


Fig 3.77: Hdacs regulate regeneration by Her4.1 mediated regulation of regeneration associated genes. (a, b) qRT-PCR shows relative mRNA fold change of regeneration associated genes in *her4.1* knocked down retinae. (c) Relative mRNA levels of several cytokines and their receptors in *her4.1* knocked down retinae. (d) diagrammatic representation of 3kb promoter of several cytokines and their receptors bearing putative Her/Hes binding sites. (e) qRT-PCR shows a dose dependent decrease in the mRNA level of *her4.1* in retinae treated with TSA withdrawal regime. P< 0.02.

3.3.4 Regulation of *hdac*s by Myc and Delta/Notch signalling

The increase in Ac.H4 in injured retinae treated with DAPT and 10058-F4 together suggested the possibility of histone acetylation being regulated by Myc and Delta/Notch signalling. Also, since we found different *hdac*s to be regulated in transcriptional level post retinal injury, they must be getting regulated by some of the regeneration associated signalling pathway or genes. These logics prompted us to check expression levels of different *hdac*s in Myc blocked condition. By qRT-PCR and RT-PCR, we found *hdac1, hdac3, hdac5, hdac6,* and *hdac7* to be downregulated in 2dpi retinae treated with increasing concentration of 10058-F4 (Fig 3.78 a, b). this was further verified by mRNA ISH which showed a drastic decline in *hdac1* expression in 4dpi retinae treated with 10058-F4 (Fig 3.78 c, d).

Next, we checked the expression levels of different *hdac*s in retinae treated with Notch signalling inhibitor DAPT. RT-PCR (Fig 3.79 a) and qRT-PCR (Fig 3.79 b) showed *hdac3, hdac5, hdac6* and *hdac8* to be downregulated and *hdac1, hdac4* and *hdac9* to be upregulated in a dose-dependent manner in 2dpi retinae treated with increasing concentration of DAPT.

These data suggest the existence of a strong regulatory network working between Myc, Hdacs and Delta/Notch signalling which ultimately is responsible for the restriction of the proliferative zone in the injury responsive zone.

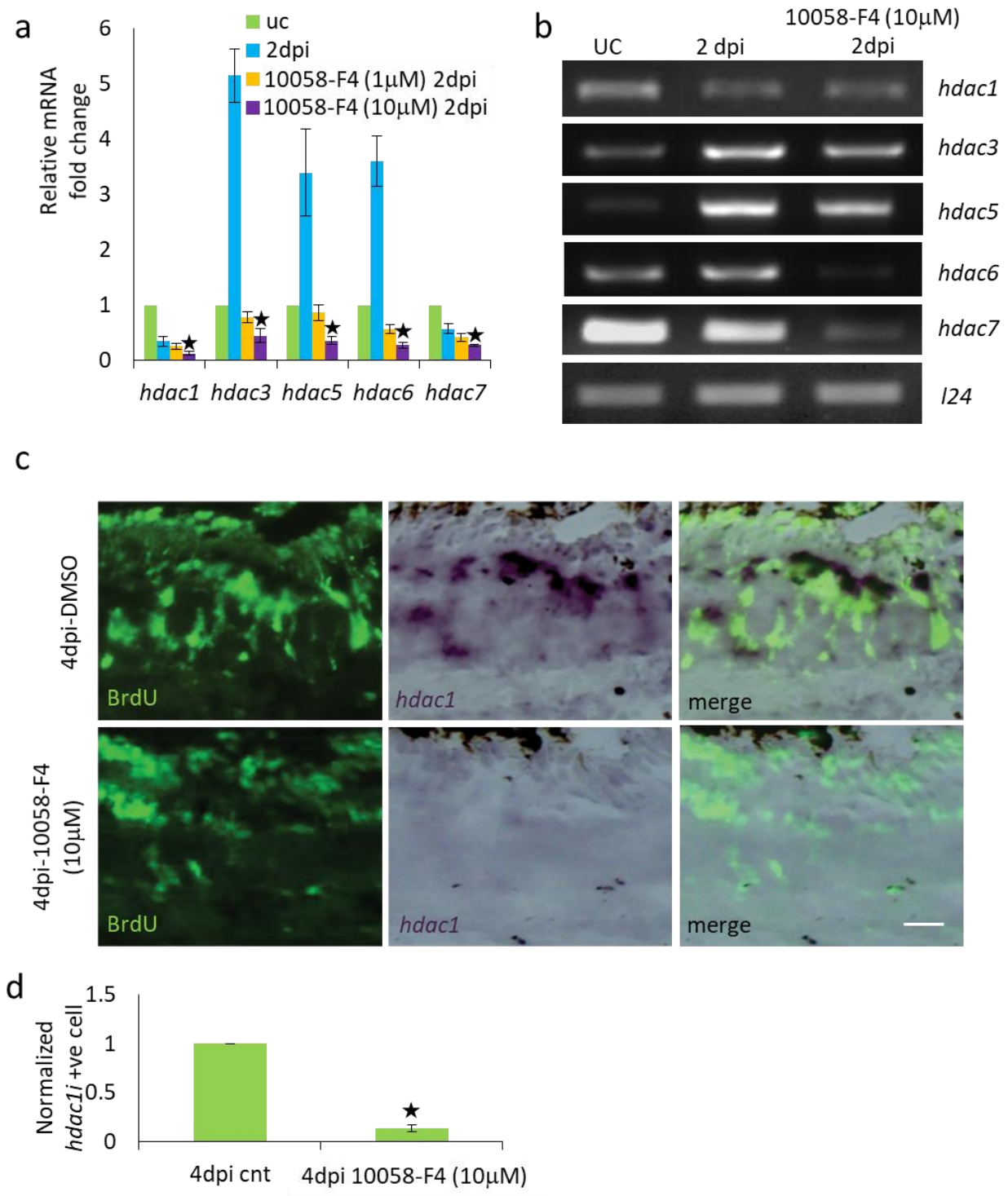


Fig 3.78: Regulation of *hdacs* by Mycb. (a) qRT-PCR and (b) RT-PCR shows downregulation of *hdac1, hdac3, hdac5, hdac6* and *hdac7* in 2dpi retinae treated with increasing concentration of 10058-F4. (c) mRNA ISH shows a drastic decline in *hdac1* expression in 4dpi retinae treated with 10058-F4. Scale bar: 15µ. (d) quantification of the same. P<0.005.

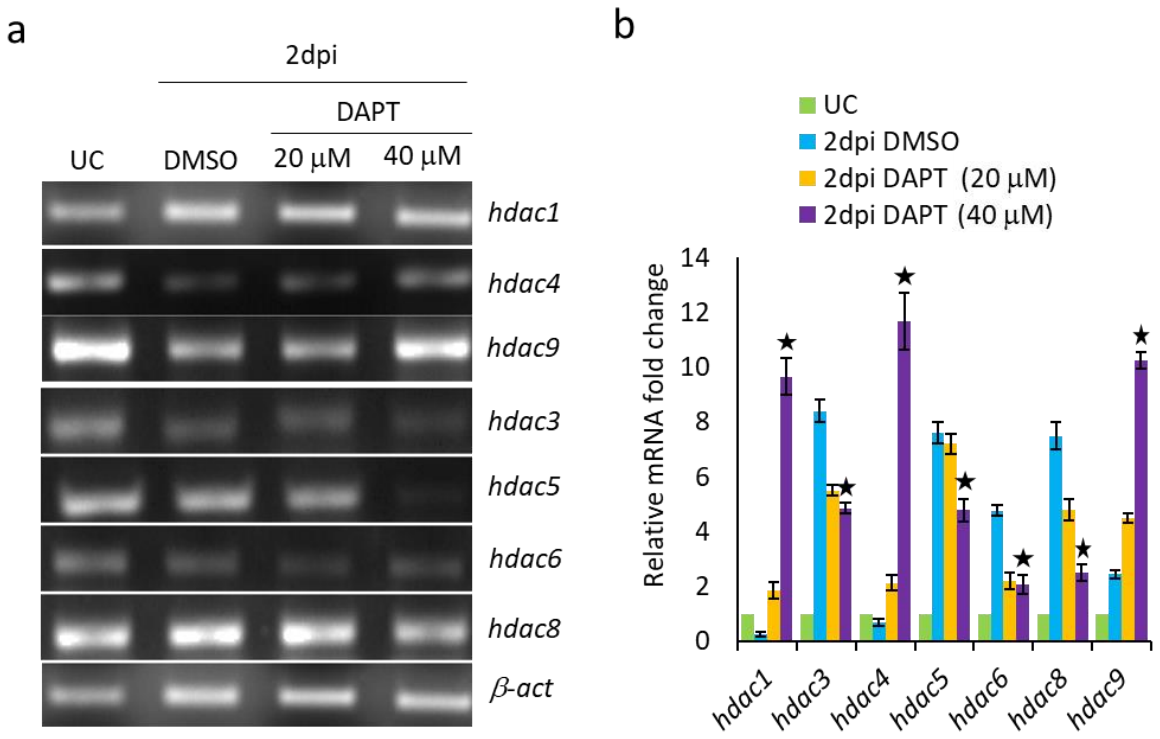


Fig 3.79: Regulation of *hdacs* by notch signalling. (a, b) RT-PCR (a) and qRT-PCR (b) showing regulation of different *hdacs* in 2dpi retinae treated with increasing concentration of DAPT. P<0.05.

Chapter 4

mycb is upregulated in optic nerve regeneration and ganglion cell layer specific induction of *mycb* during retina regeneration regulates Muller glia proliferation.

3.4.1 Mycb is upregulated in ganglion cell layer after optic nerve crush and this ganglion cell layer specific expression of *mycb* regulates proliferation of Muller glia during retina regeneration.

When we performed mRNA ISH at 4dpi retinae for checking the spatial expression pattern of *mycb*, post retinal injury, we observed a huge induction of *mycb* in ganglion cell layer. To find a reason for this apparently superfluous GCL specific *mycb* induction, we came to the following hypothesis. While mechanically injuring the retina with a needle poke, it is unavoidable not to injure the ganglion cell layer. Also, through this injured region of the GCL, many axons must be traversing towards the optic nerve. All these axons will also be inevitably injured as we injure the retina by pushing a needle through it. This injury of the axons in the GCL should be physiologically an equivalent to an injury to the optic nerve which is also capable of regenerating and repair through a genetic programme initiated by the ganglion cells. Several regeneration-associated genes should be regulated in these ganglion cells in that case. The observed induction of *mycb* in the GCL might be reminiscent of the same mechanism suggesting an involvement of *mycb* in optic nerve regeneration.

To check if our hypothesis holds true, the eye was pulled slightly out of the eye-socket and optic nerve was crushed carefully not to damage the blood vessels. After 3 days (3 days post lesion or 3 dpl) these retinae were harvested for RNA preparation and eyes were harvested for mRNA ISH. qRT-PCR (Fig 3.80 a) and RT-PCR (Fig 3.80 b) showed a significantly increased level of *mycb*, which was also supported by mRNA ISH (Fig 3.80 c).

Next, we wanted to check if this GCL specific induction of *mycb* is also contributing to regulate proliferation in the INL, i.e. the proliferation of Muller glial cells in response to retinal injury.

For that, we decided to injure the retina with a needle poke and also crush the optic nerve following the retinal injury and subsequently knocking down Mycb protein expression in GCL by *mycb* MO. To deliver the MO in the GCL, we placed a tiny piece of sponge soaked with *mycb* MO inside the eye socket beneath the optic nerve crush so that the MO can be taken up by the open end of the axons coming from the ganglion cells. Eyes were harvested at 4dpi after 5 hours of BrdU pulsing. Interestingly knocking down Mycb expression in GCL significantly brought down Muller glia proliferation in the INL in response to retinal injury (Fig 3.81 a, b).

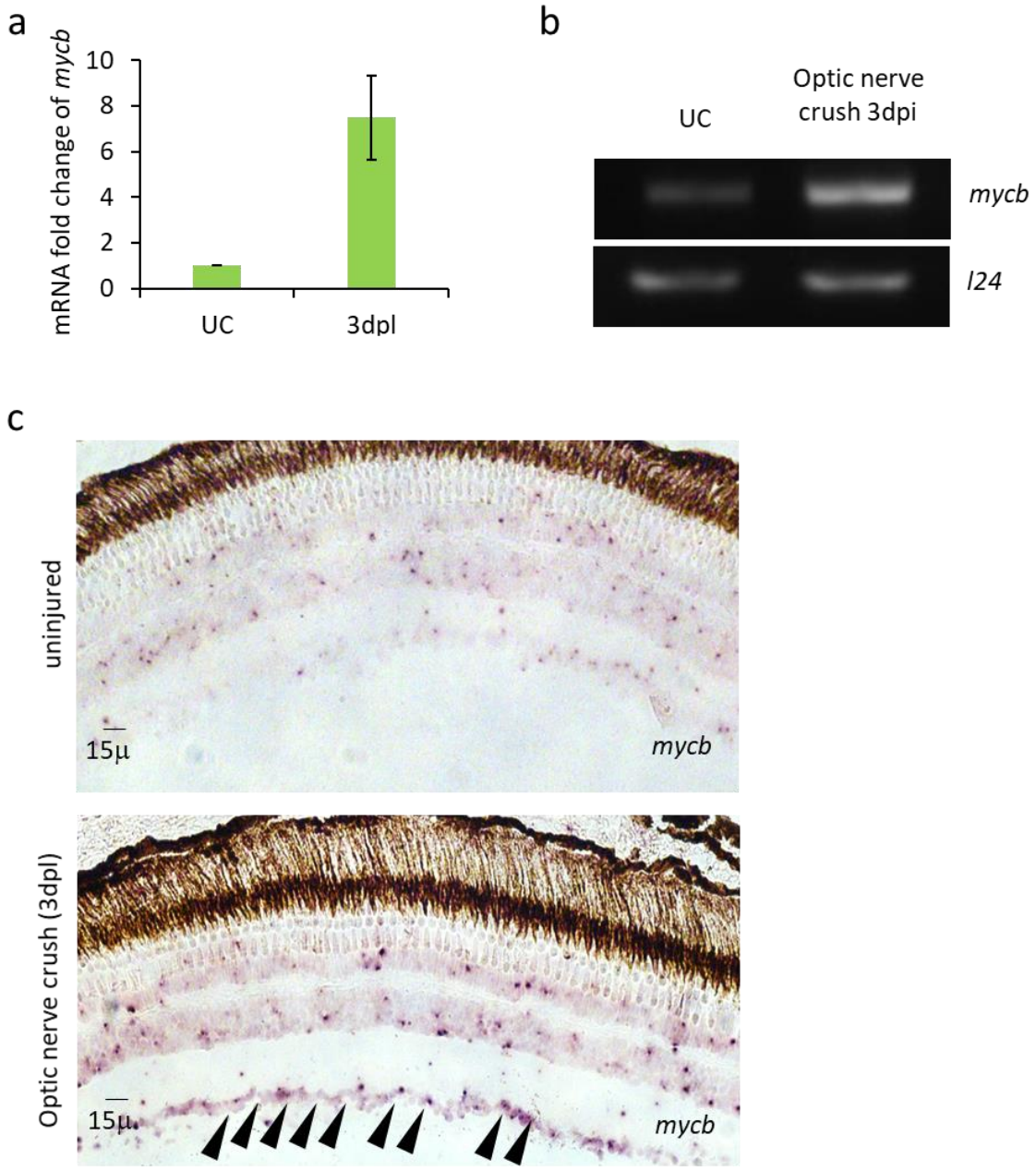


Fig 3.80: The *mycb* is upregulated in GCL during optic nerve regeneration (a) qRT-PCR and (b) RT-PCR shows upregulation of *mycb* post optic nerve crush. (c) mRNA ISH shown induction of *mycb* in GCL post optic nerve crush in 3dpl retinal sections.

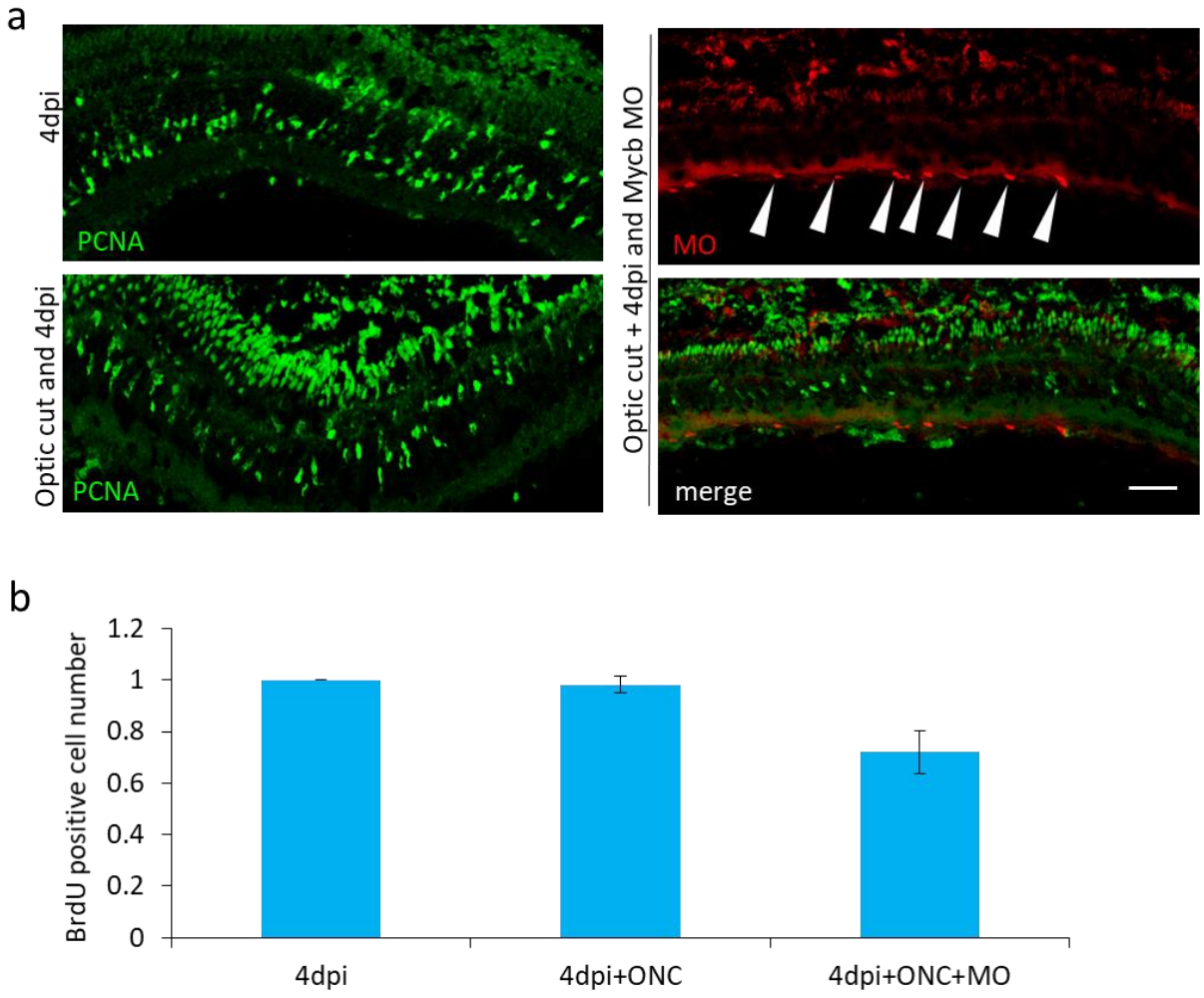


Fig 3.81: The GCL specific induction of *mycb* contributes towards regulating MG proliferation in INL during retina regeneration (a) BrdU IF shows reduction in the number of proliferating cells in INL in retinae which received *mycb* MO in GCL. Scale bar: 30µ. (b) quantification of the same.

Section 4

Discussion

Over the past few years, much has been studied about zebrafish retina regeneration and the involvement of several transcription factors and signalling pathways in different phases of regeneration such as, de-differentiation, proliferation and re-differentiation (Goldman, 2014; Wan and Goldman, 2016). Despite having substantial knowledge about the involvement of transcriptional factors, such as Ascl1a, and Insm1a, in regulating de-differentiation and proliferation of MGPCs, very little is known about the involvement of pluripotency inducing factors such as c-Myc (Ramachandran et al., 2010; Ramachandran et al., 2012).

Beside genetic regulations, reprograming of Muller glial cells to MGPCs also involves epigenetic regulation to alter the gene regulatory network which paves the way towards a successful regenerative response (Powel et al., 2013). Hdacs are a family of epigenetic regulators which are involved in various physiological processes but are poorly characterized during the course of tissue regeneration. Interestingly, c-Myc, a *de facto* transcriptional activator, has been reported in various other tissues and systems to be associated with epigenetic regulations of transcription by regulating different epigenetic regulators like Hdacs (Wang et al., 2014).

The present study places Myca and Mycb, the two zebrafish isoform of c-Myc, as key regulators of retina regeneration. The molecular regulations exerted by c-Myc are diverse, not only as it regulates transcription factors like Ascl1a and Insm1a, but also pertaining to its physical association with Hdac1 to regulate transcription of other regulators like Lin28a and He4.1. This dual mode of regulation is extensively balanced by the fine-tuned regulation of various transcription factors and epigenetic regulators which might be crucial for successful interventions of mammalian retina regenetaion.

4.1: Myca and Mycb are essential and regulate proliferation post retinal injury.

Zebrafish has got two homologues of c-Myc genes: *myca* and *mycb.* Although c-Myc has been studied extensively as a transcriptional activator and pluripotency inducing factor in several developmental pathways, its role as a transcriptional repressor has only been significantly uder-explored. Our study shows Mycs regulate de-differentiation and MGPCs proliferation in zebrafish retina regeneration both as a transcriptional activator and repressor which further is responsible to fine-tune the expression of several other regeneration associated genes in and around the injury responsive zone.

Our study shows immediately post retinal injury, both *myca* and *mycb* are highly upregulated in a pan retinal manner which is restricted to the injury responsive zone by the peak of proliferative phase, i.e. 4 days post injury. Quantifying the number of colocalized *myca* or *mycb* positive cells with PCNA positive proliferating cells revealed that only around 40-50% proliferating cells expressed *myca* or *mycb*. But around 70% of *myca* or *mycb* positive cells had PCNA expression. Most of the *myca* or *mycb* positive cells were juxtaposed to PCNA positive proliferating cells. These cells should be the initial de-differentiated cells which did not enter the cell cycle and are residual of the initial pan retinal expression which becomes restricted to the injury responsive zone by 4dpi. This suggests expression of only *myca* or *mycb* is not sufficient for Muller glial cells to enter the cell cycle. Also, as suggested by the quantification data, we can assume that early de-differentiating Muller glial cells should have had *myca* or *mycb* expression while the newer proliferating cells which de-differentiated later can carry out their function even without expressing *myc*. All these points towards a Myc independent mechanism of de-differentiation and proliferation in function post retinal injury.

We also showed Myca/b to be necessary for fine-tuned regulation of de-differentiation and proliferation as knocking down *myca* and *mycb* alone or in combination or blocking Myc function ablate the number of proliferating cells at 4dpi. Interestingly blocking Myc function by 10058-F4 or knocking down *myca* and/or *mycb* by morpholino modified oligonucleotides (MO) did not completely diminish the number of proliferating cells, suggesting, again about the existence of Myc-independent pathways of de-differentiation and proliferation. Further to prove this hypothesis, lineage tracing experiments using Cre-loxP system, in which the expression of *cre* is driven by *myc* promoter, can be used.

GCL specific induction of *mycb* after optic nerve lesion also suggested a possible role for *mycb* in optic nerve regeneration. Moreover, the GCL specific induction of *mycb* can also influence proliferating Muller glia post retinal injury.

4.2: Hdacs are regulated in transcriptional and translational levels and are necessary for regulating proliferation of Muller Glia derived progenitors.

Similar to our limited knowledge about the involvement of pluripotency factors in zebrafish retina regeneration, not much is known about the epigenetic regulations of the regeneration associated genes. Hdacs being a group of one of the most important and key epigenetic regulators, were of much of our interest. Our study reveals transcriptional regulation of several *hdac*s post retinal injury. One of the most interesting post-injury expression patterns was shown by *hdac1* becomes restricted to the injury responsive zone by 4dpi as compared to its pan-retinal expression pattern in uninjured retinae. Interestingly, this residual expression of *hdac1*

in injury responsive zone at 4dpi also showed significant mutual exclusion with BrdU or PCNA positive proliferating cells. Quantification of *hdac1* positive, BrdU positive and PCNA positive cells in 4dpi retinae from fish which received BrdU pulsing for 5 hours prior to harvest shows *hdac1* positive cells shows more colocalization with PCNA than with BrdU. This is suggestive of a cell-cycle-inhibitor role played by *hdac1* during the later stages of proliferative phase. This also rationalizes the drastic decline of *hdac1* post retinal injury which must be necessary for the Muller Glia derived progenitors proliferate. For the earlier dividing cells, to come out of cell cycle and stop dividing, the expression of *hdac1* must commence again. That is why actively proliferating cells marked by BrdU does not show significant colocalization with *hdac1* as opposed to PCNA positive cells which might be the earlier dividing cells with residual PCNA expression. Although *hdac1* mRNA was only present in injury responsive zone at around 4dpi, interestingly, at the same time Hdac1 protein was found to be completely absent from proliferating cells suggesting the existence of some mechanism by which Hdac1 translation was blocked from *hdac1* mRNA. For future studies, it would be worth looking for any microRNA mediated regulation of *hdac1*.

The mutually exclusive *hdac1* positive cells and BrdU positive cells and the reduced proliferation in 2dpi retinae in which *hdac1* was knocked down suggest *hdac1* can function both as a pro-proliferative and anti-proliferative gene in retina regeneration. Earlier during de-differentiation, *hdac1* might be responsible for upregulating some pro-proliferative regeneration associated genes like *lin28a* as seen in 2dpi retinae electroporated with *hdac1* MO. Later, Hdac1 might be upregulating repressors like Her4.1 thus bringing proliferative cells out of cell cycle. The same is also evident from double FISH experiments which shows colocalization of *hdac1* and *her4.1* but both colocalized with very few BrdU positive cells.

The decrease in cell proliferation seen in retinae electroporated with *hdac1* MO till 2dpi is interestingly in contrast to the increase in proliferation in retinae electroporated with *hdac1*

MO till 4dpi. This again confirms the cell cycle inhibitory function of Hdac1 shown at later times during the end of proliferating phase as cells which received *hdac1* MO also shwed to be PCNA positive. The decrease of proliferation in 2dpi retinae with *hdac1* MO is also similar to the effect of blocking Hdacs with TSA or VPA or NaB. Downregulation of *lin28a* in TSA or VPA treated retinae is in stark contrast with the expression of several other regeneration-associated genes like *ascl1a*, *myca*, *mycb*, *her4.1* which suggested Hdacs or specifically Hdac1 might be responsible for directly regulating *lin28a* in the injury responsive zone. Our data also showed increased *her4.1* in Hdacs inhibited retinae also as a reason for this decreased proliferation. These data, taken together, suggest for Hdacs-mediated regulation of regeneration facilitated by Ascl1a, Myc, Her4.1 and Lin28a.

4.3: Myc acts both as a transcriptional activator and repressor and directly or indirectly regulates several regeneration-associated genes or pathways.

To elucidate the fine-tuned gene regulation during zebrafish retina regeneration, our study aims to unravel the dual role played by Mycb in activating and repressing several other regeneration-associated genes or transcription factors. One of the most interesting findings was the direct binding of Mycb onto the *ascl1a* promoter to induce its expression which in turn directly regulates *mycb*'s expression. This positive feedback loop is central for regulating proliferation of Muller glia derived progenitors as both Ascl1a and Myc knockdown resulted in less proliferation of MGPCs.

Ascl1a has previously been reported to induce the expression of *lin28a* in retina regeneration to regulate de-differentiation. In other non-homologous systems, but not in zebrafish retina regeneration, Myc has also been reported to induce *lin28a* expression in a similar fashion. Interestingly, we found a drastic induction of *lin28a* in Myc blocked retinae or in retinae electroporated with *mycb* MO. Mechanism of this seemingly perplexing behaviour of repressing *lin28a* shown by Mycb which is a *de novo* transcription activator was further investigated to fruition as we found Mycb and Hdac1 are part of the same repressor complex And Mycb recruits Hdac1 to *lin28a* promoter to suppress its expression in non-proliferating cells. Keeping in mind that Hdac1 is completely excluded from the proliferating cells, this repressive behaviour of Mycb would be absent from proliferating Muller glial cells. In these cells, Mycb is more likely to bind directly to *lin28a* promoter and activate it. Another possibility is the induction of *lin28a* by Mycb mediated by Ascl1a. We also checked for the possibility of Her4.1 mediated repression of *lin28a* and found that directly or via Delta/Notch signalling, Mycb can regulate *her4.1* which in turn regulates the level of *lin28a*. All these regulatory cross-talks culminate in a fine-tuned expression of regeneration-associated genes in injury responsive zone.

Our study also expounds more on a previously reported observation of increased proliferation in Delta/Notch signalling blocked retinae is associated with increased expression of *lin28a* and *myca/b*. This increased proliferation is not sustainable in absence of Myc as suggested by the ablation of proliferation in DAPT treated injured retinae which also received Myc/Max inhibitor.

4.4: Mycb is regulated by regeneration associated genes and HDACs to control cell proliferation.

Apart from our finding of Delta/Notch signalling mediated regulation of Mycb and the *vice versa*, we have also shown that Mycb can be regulated by several other regeneration-associated genes like Wnt/β-catenin signalling, and Shh signalling. Promoter activity assay done in zebrafish embryos suggested Insm1a, a transcriptional repressor, induced heavily during retina regeneration also suppresses *mycb*. This regulation might underlie the ablation of *mycb* expression from a pan-retinal expression pattern to the one which is restricted in the injury-responsive zone. Insm1a mediated regulation of Mycb might be the reason for restriction of *mycb* expression in and around the proliferating zone as *insm1a* is also present in non-proliferative cells.

Beside genetic regulation of *myca* and *mycb*, we also show various evidence for its regulation by epigenetic modifiers like Hdacs. In Hdacs blocked injured retinae, we found a profuse increase of *mycb* as well as *myca* mRNA which was not reflected in protein level. We showed this was mediated by downregulation of *lin28a*. Lin28a, an RNA binding protein is responsible for degrading *let-7* microRNA which is responsible for maintaining the differentiated state of a cell. In Hdacs blocked retinae, since *lin28a* level goes down, *let-7* miRNA would be present in abundance. The *let-7* miRNA has previously been shown to regulate translation of several regeneration-associated genes including *ascl1a* and *mycb* and. which justifies the differing levels in their mRNA and protein.

4.5: Hdacs inhibitor-mediated blockade of proliferation is reversible and is regulated through Her4.1 mediated regulation of *lin28a* and cytokines.

The reduced number of proliferative cells in Hdacs blocked retinae was quite contradictory to our hypothesis. Hdacs are principally transcriptional blocker regeneration would require *de novo* transcription of several genes like cytokines, growth factors, cell cycle regulators, pluripotency factors and several other transcription activators or repressors. For all these transcriptions to be regulated, we first hypothesized that upon blockade of Hdacs, these transcriptions should be induced more leading to increased proliferation. But, blocking Hdacs caused reduced proliferation. This reduction of proliferation was also associated with downregulation of mRNA levels of several regeneration-associated cytokines and most importantly *lin28a* and upregulation of *her4.1*. But transcriptions of most of the other regeneration-associated genes like *ascl1a*, *myca*, *mycb* etc. were induced heavily in Hdacs inhibited retinae. This made us to speculate, since all these transcripts would already be present already in Hdacs inhibited retinae, this should render the Muller glia already poised for de-differentiation and withdrawing the inhibition might enable the cells to re-enter the cell cycle. Interestingly, we found the blocked of regeneration after preventing Hdacs to be reversible. This reversal was also associated with re-induction of cytokines and hormones which have previously been reported to be upregulated post retinal injury. We also found *lin28a* to be re-induced in this background. These newly proliferating cells were capable of re-differentiation and migration to all retinal cell layers.

4.6: Conclusion.

Our data, taken together sheds much light on the fine-tuned regulation of several regeneration-associated genes and signalling pathway that have been reported previously but were not linked together. We also show that the previously reported molecules like Ascl1a, Lin28a, cytokines, and Her4.1 are extensively regulated by Myca or Mycb which we report to be upregulated post retinal injury and also by Hdacs, particularly Hdac1, which is downregulated both transcriptionally and translationally in injured retina (Fig 4.1).

This study places Myca and Mycb as key regulators of de-differentiation and proliferation. We investigated its role, both as an activator and repressor, the latter being reported for the first time in retina regeneration.

We show that Wnt/β-catenin signalling or Ascl1a and Insm1a regulate Myc expression which in turn regulates Muller glia derived progenitor cells de-differentiation and proliferation through regulating Ascl1a, Lin28a, Her4.1, and Hdacs. Mycb also contributed towards Her4.1/Lin28a/*let-7* axis mediated restriction of proliferation in the injury-responsive zone.

We also have shown, for the first time in zebrafish retina regeneration, that Hdacs are regulated both transcriptionally and translationally as evident by the presence of its mRNA around the injury responsive zone, but complete degradation of its protein at the same time and site. We have also established that Hdacs are also responsible for fine-tuning the cross-talking network of several regeneration-associated genes. Hdacs also contribute towards the induction of cytokines post retinal injury, an effect which is possibly mediated through Her4.1. Further, our data also shows that the blockade of proliferation upon Hdacs inhibition is reversible and mediated through Her4.1/Lin28a/*let-7* regulatory axis.

Interestingly, we also found a GCL specific induction of *mycb* which might be contributing towards optic nerve regeneration and also to some extent is capable of regulating Muller glia proliferation. This might be mediated through some secretory molecules which can travel the distance from GCL to INL to influence proliferation. Further experiments are needed to be performed to figure out the exact mechanistic dynamics of this regulation as well as for Mycs' involvement in optic nerve regeneration. It would also be interesting to investigate further regulatory mechanisms exerted by Mycs and Hdacs to regulate other pluripotency factors and epigenetic regulators and to figure out the global impact of this regulation with comparative transcriptome and proteome analysis of regenerating zebrafish and mammalian retina. Taking insights from high-throughput studies, further candidate based detailed investigations are important for medical intervention towards successful mammalian retina regeneration.

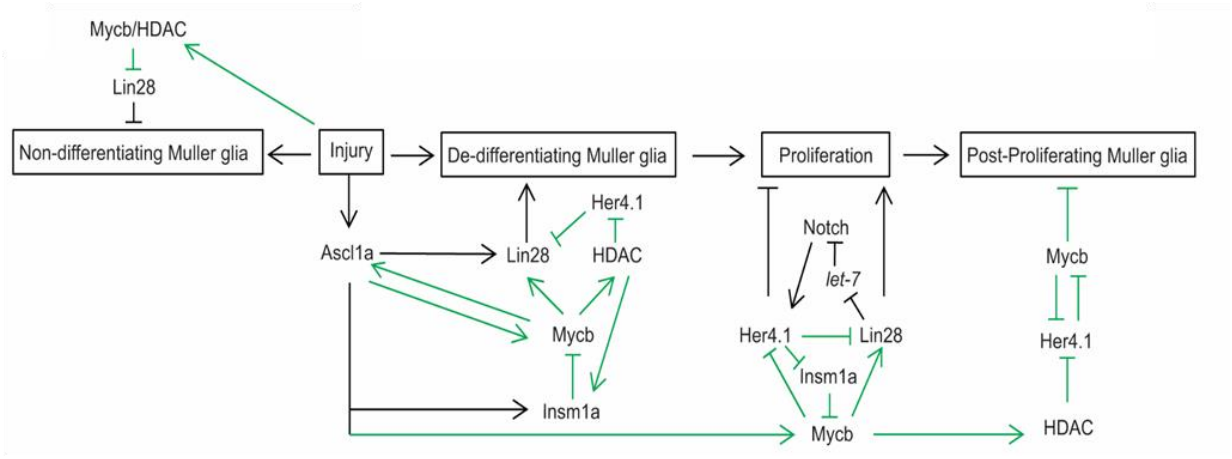


Fig 4.1: A model elucidating the mechanisms of Mycs and Hdacs to regulate different regeneration associated molecules. Green arrows indicate findings from this study. Black arrows show regulations known from previous studies.

Section 5

References

Books

1. A History of Regeneration Research: Milestones in the Evolution of a Science, Charles E. Dinsmore, 1991, Cambridge University Press.
2. Asexual Propagation and Regeneration, M. A. Vorontsova and L. D. Liosner, 1960, Pergamon Press.
3. Muller cells in the healthy and diseased retina, Andreas Reichenbach, Andreas Bringmann, Springer.
4. Principles of Regeneration, Richard J. Goss, 1969, Academic Press.
5. Principles of Regenerative Biology, Bruce M. Carlson, 2007, Academic Press.

Articles

1. Amati, B., Frank, S. R., Donjerkovic, D. & Taubert, S. 2001. Function of the c-Myc oncoprotein in chromatin remodeling and transcription. Biochim. Biophys. Acta 1471, M135–M145.
2. Amy J. Taylor, Caroline W. Beck. 2012. Histone deacetylases are required for amphibian tail and limb regeneration but not development. Mechanisms of Development 129: 208-218.
3. Andrea Havasi, Joseph A. Haegele, Jonathan M Gall, Sherry Blachmon, Takaharu Ichimura, Ramon G. Bonegio, Maria V. Panchenko. 2013. Histone acetyl transferase (HAT) HBO1 and JADE1 in epithelial cell regeneration. The American Journal of Pathology 182 (1): 152-162.
4. Andreas Bringmann and Andreas Reichenbach. 2001, Role of müller cells in retinal degenerations. Frontiers in Bioscience, 6, e77-92.

5. Andreas Bringmann, Ianors Iandiev, Thomas Pannicke, Antje Wurm, Margrit Hollborn, Peter Wiedemann, Neville N. Osborne, Andreas Reichenbach. Cellular signaling and factors involved in Müller cell gliosis: neuroprotective and detrimental effects. Progress in Retinal and eye Research, 2009, 28(6): 423-451.
6. Andreas Bringmann, ThomasPannicke, Jens Grosche, Mike Francke, Peter Wiedemann, Serguei N.Skatchkov, Neville N.Osborne, Andreas Reichenbach. Muller cells in the healthy and diseased retina. 2006, progress in Retinal and Eye Research, 25(4): 397-424.
7. Andreas Reichenbach and Andreas Bringmann. New functions of Muller cells. 2013, Glia, 61(5): 651-678.
8. Andrew P. Feinberg. 2007. Phenotypic plasticity and the epigenetics of human disease. Nature 447: 433–440.
9. Annemieke J. M. DE Ruijter, Albert H. Van Gennip, Huib N. Caron, Stephan Kemp and Andre B. P. Van Kuilenburg. 2003. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. 370: 737–749.
10. Annu Chohan, Usha Singh, Atul Kumar, Jasbir Kaur. Müller stem cell dependent retinal regeneration. 2013, Clinica Chimica Acta, 464: 160–164.
11. Armelin, H.A., Armelin, M.C., Kelly, K., Stewart, T., Leder, P., Cochran, B.H., and Stiles, C.D. 1984. Functional role for c-myc in mitogenic response to platelet-derived growth factor. Nature 310, 655–660.
12. Ashok Kumar, Nazeem Shamsuddin. Retinal Muller Glia Initiate Innate Response to Infectious Stimuli via Toll-Like Receptor Signaling. 2012, PLoS One, 7(1): e29830.
13. Ashok Kumar, Rajeev K Pandey, Lindsay J Miller, Pawan K Singh, and Mamta Kanwar. Muller cells in the healthy and diseased retina. 2013, Crit Rev Immunol, 33(2): 119–135.

14. Ashutosh P.Jadhav, KarinRoesch, Constance L.Cepko. Development and neurogenic potential of Müller glial cells in the vertebrate retina. 2009, Progress in Retinal and Eye Research, 28(4): 249-262.

15. Ayer, D. E., Lawrence, Q. A. & Eisenman, R. N. 1995. Mad–Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell 80, 767–776.

16. Bailey T. J., Fossum S. L., Fimbel S. M., Montgomery J. E., Hyde D.R. 2010. The inhibitor of phagocytosis, O-phospho-L-serine, suppresses Muller glia proliferation and cone cell regeneration in the light-damaged zebrafish retina. Exp Eye Res 9:1–14.

17. Bernhard Lüscher, Lars-Gunnar Larsson. 1999. The basic region/helix-loop-helix/leucine zipper domain of Myc-protooncoproteins: function and regulation. Oncogene 18: 2955–2966.

18. Berns, K., Hijmans, E. M. & Bernards, R. 1997. Repression of c-Myc responsive genes in cycling cells causes G1 arrest through reduction of cyclin E/CDK2 kinase activity. Oncogene 15, 1347–1356.

19. Bil Lin, Steven W. Wang, and Richard H. Masland. Retinal Ganglion Cell Type, Size, and Spacing Can Be Specified Independent of Homotypic Dendritic Contacts. 2004, Neuron, 43: 475-485.

20. Bjorn Vennstrom, Diana Sheiness, Jan Zabielski, and J. Michael Bishop. 1982. Isolation and Characterization of c-myc, a Cellular Homolog of the Oncogene (v-myc) of Avian Myelocytomatosis Virus Strain 29. JOURNAL OF VIROLOGY 42(3): 773-779.

21. Bolden J. E., Peart M. J., Johnstone R. W. 2006. Anticancer activities of Histone deacetylase inhibitors. Nat Rev Drug Discov 5(9):769-84.

22. Bouchard, C. et al. 2001. Regulation of cyclin D2 gene expression by the Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. Genes Dev. 15, 2042–2047.

23. Braisted J. E., Essman T.F., Raymond P.A. 1994. Selective regeneration of photoreceptors in goldfish retina. Development 120:2409– 2419.

24. Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E., and Bishop, J.M. 1984. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224, 1121–1124.

25. Chhetri, J.; Jacobson G.; Gueven N. 2014. Zebrafish—on the move towards ophthalmological research. Eye 28: 367–380.

26. Chi V. Dang. 2012. Myc on the path to cancer. Cell. 149(1): 22-35.

27. Chiara Mozzetta, Silvia Consalvi, Valentina Saccone, Matthew Tierney, Adamo Diamantini, Kathryn J. Mitchell, Giovanna Marazzi, Giovanna Borsellino, Luca Battistini, David Sassoon, Alessandra Sacco, Pier Lorenzo Puri. 2013. Fibroadipogenic progenitors mediate the ability of HDAC inhibitors to promote regeneration in dystrophic muscles of young, but not old Mdx mice. EMBO Molecular Medicine 5: 626-639.

28. Choi R. Y., Engbretson G. A., Solessio E. C., Jones G. A., Coughlin A., Aleksic I., Zuber M. E. 2011. Cone degeneration following rod ablation in a reversible model of retinal degeneration. Invest Ophthalmol Vis Sci 52:364–373.

29. Close J. L., Liu J, Gumuscu B, Reh T. A. 2006. Epidermal growth factor receptor expression regulates proliferation in the postnatal rat retina. Glia 54:94–104.

30. Cole M. D. 1986. The Myc oncogene: its role in transformation and differentiation. Annu Rev Genet 20: 361-84.

31. Coni, P., Simbula, G., de Prati, A. C., Menegazzi, M., Suzuki, H., Sarma, D. S., Ledda-Columbano, G. M. and Columbano, A. 1993. Differences in the steady-state levels of c-*fos*, c-*jun* and c-*myc* messenger RNA during mitogen-induced liver growth and compensatory regeneration. Hepatology, 17: 1109-1116.

32. Conner C, Ackerman KM, Lahne M, Hobgood JS, Hyde DR. 2014. Repressing Notch signaling and expressing TNF are sufficient to mimic retinal regeneration by inducing Muller glial proliferation to generate committed progenitor cells. J Neurosci 34:14403–14419.

33. Cress, W. D. and Seto, E. 2000. Histone deacetylases, transcriptional control, and cancer. J. Cell. Physiol. 184, 1–16.

34. Curtis Powell, Ana R. Grant, Eli Cornblath, and Daniel Goldman. 2013. Analysis of DNA methylation reveals a partial reprogramming of the Müller glia genome during retina regeneration. PNAS 110(49): 19814–19819.

35. Dang, C. V. 1999. c–Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell. Biol. 19, 1–11.

36. Daniel Goldman. Muller glia cell reprogramming and retina regeneration. 2014, Nature Reviews Neuroscience, 15: 431-442.

37. Das A. V., Mallya K. B., Zhao X, Ahmad F, Bhattacharya S, Thoreson W. B., Hegde G. V., Ahmad I. 2006. Neural stem cell properties of Muller glia in the mammalian retina: regulation by Notch and Wnt signaling. Dev Biol, 299:283–302.

38. David F. Razidlo, Tiffany J. Whitney, Michelle E. Casper, Meghan E. McGee-Lawrence, Bridget A. Stensgard, Xiaodong Li, Frank J. Secreto, Sarah K. Knutson, Scott W. Hiebert, Jennifer J. Westendorf. 2010. Histone Deacetylase 3 Depletion in Osteo/ Chondroprogenitor Cells Decreases Bone Density and Increases Marrow Fat. PLoS ONE 5(7): e11492.

39. del Debbio C. B., Balasubramanian S, Parameswaran S, Chaudhuri A, Qiu F, Ahmad I. 2010. Notch and wnt signaling mediated rod photoreceptor regeneration by Muller cells in adult mammalian retina. PLoS One, 5: e12425.

40. Divya Ail and Muriel Perron, 2017. Retinal degeneration and regeneration-lessons from fishes and amphibians. Current Pathobiology Reports, 5(1): 67-78.

41. Donika Gallina, Levi Todd, Andy J. Fischer. A comparative analysis of Müller glia-mediated regeneration in the vertebrate retina. 2013, Experimental Eye Research, 123: 121-130.

42. Feng Li, Yan Xiang, James Potter, Ramani Dinavahi, Chi V. Dang, and Linda A. Lee. 2006. Conditional Deletion of c-myc Does Not Impair Liver Regeneration. Cancer Research 66(11): 5608-12.

43. Fischer A. J., Bongini R. 2010. Turning Muller glia into neural progenitors in the retina. Mol Neurobiol 42:199–209.

44. Fischer A. J., McGuire C. R., Dierks B. D., Reh T. A. 2002. Insulin and fibroblast growth factor 2 activate a neurogenic program in Muller glia of the chicken retina. J Neurosci, 22:9387–9398.

45. Fischer A. J., Reh T.A. 2002. Exogenous growth factors stimulate the regeneration of ganglion cells in the chicken retina. Dev Biol, 251: 367–379.

46. Fischer A. J., Scott M. A., Zelinka C, Sherwood P. 2010. A novel type of glial cell in the retina is stimulated by insulin-like growth factor 1 and may exacerbate damage to neurons and Muller glia. Glia, 58:633–649.

47. Fischer A. J., Scott MA, Ritchey E. R., Sherwood P. 2009a. Mitogen activated protein kinase-signaling regulates the ability of Muller glia to proliferate and protect retinal neurons against excitotoxicity. Glia, 57:1538–1552.

48. Fischer A. J., Zelinka C, Gallina D, Scott M. A., Todd L. 2014. Reactive microglia and macrophage facilitate the formation of Muller glia-derived retinal progenitors. Glia, 62:1608–1628.

49. Forsberg, E. C. and Bresnick, E. H. 2001. Histone acetylation beyond promoters: long-range acetylation patterns in the chromatin world. Bioessays 23, 820–830.

50. Gabrielle H. Ashton, Jennifer P. Morton, Kevin Myant, Toby J. Phesse, Rachel A. Ridgway, Victoria Marsh, Julie A. Wilkins, Dimitris Athineos, Vanesa Muncan, Richard Kemp, Kristi Neufeld, Hans Clevers, Valerie Brunton, Douglas J. Winton, Xiaoyan Wang, Rosalie C. Sears, Alan R. Clarke, Margaret C. Frame, and Owen J. Sansom. 2010. Focal Adhesion Kinase Is Required for Intestinal Regeneration and Tumorigenesis Downstream of Wnt/c-Myc Signaling. Dev Cell 19, 259–269.

51. Gaia Gestri1, Brian A, and Stephan C. F. Neuhauss. The Visual System of Zebrafish and its Use to Model Human Ocular Diseases. 2012, Dev Neurobiol, 72(3): 302–327.

52. Gallina D, Todd L, Fischer A. J. 2014a. A comparative analysis of Muller glia-mediated regeneration in the vertebrate retina. Exp Eye Res, 123:121–130.

53. Gallina D, Zelinka C, Fischer A. J. 2014b. Glucocorticoid receptors in the retina, Muller glia and the formation of Muller glia-derived progenitors. Development, 3340–3351.

54. Geoffrey C. Gurtner, Sabine Werner, Yann Barrandon & Michael T. Longaker. Wound repair and regeneration. 2008, Nature, 453: 314-321.

55. Gerda Egger, Gangning Liang, Ana Aparicio, Peter A. Jones. 2004. Epigenetics in human disease and prospects for epigenetic therapy. Nature 429: 457–463.

56. Grigorian E. N., Poplinskaia V. A. 1999. Discovery of internal sources of the neural retinal regeneration after its detachment in Pleurodeles. II. The radioautography study. Izv Akad Nauk Ser Biol, 583–591.

57. H R Taylor, M L Pezzullo, J E Keeffe. The economic impact and cost of visual impairment in Australia. 2006, Br J Ophthalmol, 2006;90:272–275.

58. Hari Jayaram, Megan F. Jones, Karen Eastlake, Phillippa B. Cottrill, Silke Becker, Joseph Wiseman, Peng T. Khaw, G. Astrid Limb. Transplantation of photoreceptors derived from human Müller glia restore rod function in the P23H rat. 2014, Stem Cells Translational Medicine, 3(3): 323-333.

59. Haynes T, Luz-Madrigal A, Reis E. S., Echeverri Ruiz N. P., GrajalesEsquivel E, Tzekou A, Tsonis P. A., Lambris J. D., Del Rio-Tsonis K. 2013. Complement anaphylatoxin C3a is a potent inducer of embryonic chick retina regeneration. Nat Commun 4:2312.

60. Helga Kolb, Ralph Nelson, Peter Ahnelt and Nicolas Cuenca. Cellular organization of the vertebrate retina. 2001, Prog in Brain Res, 131: 3-26.

61. Henriksson, M. & Luscher, B. 1996. Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv. Cancer Res. 68, 109–182.

62. Hyun-Jung Kim and Suk-Chul Bae. 2011. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. Am J Transl Res 3(2): 166–179.

63. Ito, K., Barnes, P. J. and Adcock, I. M. 2000. Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. Mol. Cell. Biol. 20, 6891–6903.

64. James M. Fadool and John E. Dowling. Retina regeneration in zebrafish. 2008, Prog Retin Eye Res, 27(1): 89-110.

65. Jeremy S. Rabinowitz, Aaron M. Robitaille, Yuliang Wang, Catherine A. Ray, Ryan Thummel, Haiwei Gu, Danijel Djukovic, Daniel Raftery, Jason D. Berndt, and Randall T. Moon. Transcriptomic, proteomic, and metabolomic landscape of positional memory

in the caudal fin of zebrafish. 2017, Proceedings of the National Academy of Sciences, 114 (5) E717-E726.

66. Jian Wang, Abul Elahi, Abidemi Ajidahun, Whalen Clark, Jonathan Hernandez, Alex Achille, Ji-hui Hao, Edward Seto, and David Shibata. 2014. The interplay between histone deacetylases and c-Myc in the transcriptional suppression of HPP1 in colon cancer. Cancer Biology & Therapy 15:9, 1198–1207.

67. Jie Chen and Michael I. Shifman. 2016. The expression of histone deacetylases and the regenerative abilities of spinal-projecting neurons after injury. Neural Regen Res. 11(10): 1577–1578.

68. Jin Wan and Daniel Goldman. Retina regeneration in zebrafish. 2016, Curr. Opin. Genet. Dev. 40, 41–47.

69. Jin Wan, Hua Zheng, Zu-Lin Chen, Hong-Lei Xiao, Zhen-Jue Shen, Guo-Min Zhou. Preferential regeneration of photoreceptor from Mu¨ller glia after retinal degeneration in adult rat. 2008, Vision Research, 48(2): 223-234.

70. Jinhua Tang, Yanli Yan, Ting C. Zhao, Rujun Gong, George Bayliss, Haidong Yan, and Shougang Zhuang. 2014. Class I HDAC activity is required for renal protection and regeneration after acute kidney injury. Am J Physiol Renal Physiol 307: 303–316.

71. Johns P. R. 1982. Formation of photoreceptors in larval and adult goldfish. J Neurosci 2:178–98.

72. Johns P. R., Fernald R. D. 1981. Genesis of rods in teleost fish retina. Nature 293:141–142.

73. Judith Robinson, Ellen A. Schmitt, Ferenc I. Harosit, Richard J. Reecet, and John E. Dowling. Zebrafish ultraviolet visual pigment: absorption spectrum, sequence, and localization. 1993, PNAS, 90: 6009-12.

74. Julia Pollak, Matthew S. Wilken, Yumi Ueki, Kristen E. Cox, Jane M. Sullivan, Russell J. Taylor, Edward M. Levine, Thomas A. Reh. Ascl1 reprograms mouse Müller glia into neurogenic retinal progenitors. 2013, Development, 140(12): 2619–2631.

75. Julian D., Ennis K., Korenbrot J. I. 1998. Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina. J Comp Neurol 394:271–282.

76. Karin Roesch, Ashutosh P Jadhav, Jeffrey M Trimarchi, Michael B Stadler, Botond Roska, Ben B Sun, and Constance L Cepko. The transcriptome of retinal Muller glial cells. 2008, Journal of Comparative Neurology, 509(2): 225-238.

77. Kati Löffler, Patrick Schäfer, Manuela Völkner, Tina Holdt, Mike O. Karl. Age-dependent Müller glia neurogenic competence in the mouse retina. 2015, Glia, 63(10): 1809-1824.

78. Kaur S. Gupta S, Chaudhary M, Khursheed M. A., Mitra S., Kurup A. J., Ramachandran R. 2018. let-7 microRNA mediated regulation of Shh signalling and the gene regulatory network is essential for retina regeneration. Cell Rep. 23: 1409-1423.

79. Kazutoshi Takahashi, Shinya Yamanaka. 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126(4): 663-676.

80. Keisuke Kaji, Isabel Martín Caballero, Ruth MacLeod, Jennifer Nichols, Valerie A. Wilson and Brian Hendrich. 2006. The NuRD component Mbd3 is required for pluripotency of embryonic stem cells. Nature Cell Biology **8**: 285–292.

81. Kelly, K., Cochran, B.H., Stiles, C.D., and Leder, P. (1983). Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35, 603–610.

82. Kenneth D. Poss, Lindsay G. Wilson, Mark T. Keating. Heart regeneration in zebrafish. 2002, Science, 298: 2188-90.

83. Kun Do Rhee, Steven Nusinowitz, Kevin Chao, Fei Yu, Dean Bok, and Xian-Jie Yang. 2013. PNAS 110 (47) E4520-E4529.

84. La Rocca, S. A., Crouch, D. H. & Gillespie, D. A. 1994. c-Myc inhibits myogenic differentiation and myoD expression by a mechanism which can be dissociated from cell transformation. Oncogene 9, 3499–3508.

85. Laurie A. Boyer1, Kathrin Plath, Julia Zeitlinger, Tobias Brambrink, Lea A. Medeiros, Tong Ihn Lee, Stuart S. Levine, Marius Wernig, Adriana Tajonar, Mridula K. Ray, George W. Bell, Arie P. Otte, Miguel Vidal, David K. Gifford, Richard A. Young, and Rudolf Jaenisch. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441: 349–353.

86. Lee, H. W., Suh, J. H., Kim, A. Y., Lee, Y. S., Park, S. Y., Kim, J. B. 2006. Histone deacetylase 1-mediated histone modification regulates osteoblast differentiation. Mol. Endocrinol. 20 (10), 2432–2443.

87. Lenkowski J. R., Raymond P. A. 2014. Muller glia: stem cells for generation and regeneration of retinal neurons in teleost fish. Prog Retin Eye Res 40:94–123.

88. Liu B, Hunter D. J., Rooker S, Chan A, Paulus Y. M., Leucht P, Nusse Y, Nomoto H, Helms J. A. 2013. Wnt signaling promotes Muller cell proliferation and survival after injury. Invest Ophthalmol Vis Sci, 54:444–453.

89. Lombardo F. 1968. La rigenerazione della retina negli adulti di un Teleosteo (Regeneration of the retina in an adult teleost). Accad Lincei-Rend Sci Fis Mat Nat Ser 8:631–635.

90. Luscher B., Eisenman R. N. 1990. New light on Myc and Myb. Genes Dev. 4: 2025-35.

91. Maris, J.M. 2010. Recent advances in neuroblastoma. N. Engl. J. Med. 362, 2202–2211.

92. Mark A. Rivieccio, Camille Brochier, Dianna E. Willis, Breset A. Walker, Melissa A. D'Annibale, Kathryn McLaughlin, Ambreena Siddiq, Alan P. Kozikowski, Samie R. Jaffrey, Jeffery L. Twiss, Rajiv R. Ratana, and Brett Langley. 2009. HDAC6 is a target for protection and regeneration following injury in the nervous system. PNAS 106(46): 19599–19604.

93. Matthias Stadtfeld and Konrad Hochedlinger. 2010. Induced pluripotency: history, mechanisms, and applications. Genes and Development 24:2239-2263.

94. Mayssa H. Mokalled, Chinmoy Patra, Amy L. Dickson, Toyokazu Endo, Didier Y. R. Stainier, Kenneth D. Poss. Injuryinduced ctgfa directs glial bridging and spinal cord regeneration in zebrafish. 2016, Science 354: 630–634.

95. McMahon, S. B., Van Buskirk, H. A., Dugan, K. A., Copeland, T. D. & Cole, M. D. 1998. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 94, 363–374.

96. McMahon, S. B., Wood, M. A. & Cole, M. D. 2000. The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. Mol. Cell. Biol. 20, 556–562.

97. Michael A. Dyer and Constance L. Cepko. Control of Müller glial cell proliferation and activation following retinal injury. 2000, Nature Neuroscience, 3(9):873-80.

98. Mike O. Karl, Susan Hayes, Branden R. Nelson, Kristine Tan, Brian Buckingham, and Thomas A. Reh. Stimulation of neural regeneration in the mouse retina. 2008, PNAS, 105(49): 19507–19512.

99. Mitashov V. I. 1996. Mechanisms of retina regeneration in urodeles. Int J Dev Biol 40:833–844.

100. Muller H. Anatomisch-physiologische untersuchungen uber die Retina bei Menschen und Wirbelthieren. 1857, Z Wiss Zool, 8:1–122.

101. N. Yakushiji, M. Suzuki, A. Satoh, T. Sagai, T. Shiroishi, H. Kobayashi, H. Sasaki, H. Ide, K. Tamura. 2007. Correlation between Shh expression and DNA-methylation status of the limb-specific Shh enhancer region during limb regeneration in amphibians. Dev Biol., 312: 171-182.

102. Nam Cong-Nhat Huynh, Vincent Everts, Ruchanee Salingcarnboriboon Ampornaramveth. 2017. Histone deacetylases and their roles in mineralized tissue regeneration. Bone Reports 7: 33-40.

103. Natalie Meyer and Linda Z. Penn. 2008. Reflecting on 25 years with Myc. Nature 8: 967-990.

104. Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F., and Minna, J.D. 1985. L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318, 69–73.

105. Nazeem Shamsuddin and Ashok Kumar. TLR2 Mediates the Innate Response of Retinal Muller Glia to Staphylococcus aureus. 2011, J Immunol, 186 (12) 7089-7097.

106. Nelson CM, Ackerman KM, O'Hayer P, Bailey TJ, Gorsuch RA, Hyde DR. 2013. Tumor necrosis factor-alpha is produced by dying retinal neurons and is required for Muller glia proliferation during zebrafish retinal regeneration. J Neurosci 33:6524–6539.

107. Nesbit, C. E., Tersak, J. M. & Prochownik, E. V. 1999. MYC oncogenes and human neoplastic disease. Oncogene 18, 3004–3016.

108. Nikos Kyritsis, Caghan Kizil, Sara Zocher, Volker Kroehne, Jan Kaslin, Dorian Freudenreich, Anne Iltzsche, Michael Brand. Acute inflammation initiates the regenerative response in the adult zebrafish brain. 2012, Science, 338: 1353-6.

109. Nimet Maherali, Rupa Sridharan, Wei Xie, Jochen Utikal, Sarah Eminli, Katrin Arnold, Matthias Stadtfeld, Robin Yachechko, Jason Tchieu, Rudolf Jaenisch, Kathrin Plath,

Konrad Hochedlinger. 2007. Directly reprogrammed fibroblasts show global epigenetic remodelling and widespread tissue contribution. Cell Stem Cell 1(1): 55-70.

110. Novikova I. P., Poplinskaia V. A, Aleinikova K. S., Grigorian E. N. 2008. A study of the localization and accumulation of S-phase cells in the retina of newt Pleurodeles waltl after experimental pigment epithelial detachment. Ontogenez 39:143–150.
111. Osakada F, Ooto S, Akagi T, Mandai M, Akaike A, Takahashi M. 2007. Wnt signaling promotes regeneration in the retina of adult mammals. J Neurosci, 27:4210–4219.
112. Otteson D. C., D'Costa A. R., Hitchcock P. F. 2001. Putative stem cells and the lineage of rod photoreceptors in the mature retina of the goldfish. Dev Biol 232:62–76.
113. Otteson D. C., Hitchcock P. F. 2003. Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. Vision Res 43:927–936.
114. Paino, F., La Noce, M., Tirino, V., Naddeo, P., Desiderio, V., Pirozzi, G., De Rosa, A., Laino, L., Altucci, L., Papaccio, G. 2014. Histone deacetylase inhibition with valproic acid downregulates osteocalcin gene expression in human dental pulp stem cells and osteoblasts: evidence for HDAC2 involvement. Stem Cells 32 (1): 279–289.
115. Pamela A.Raymond, Linda K. Barthel, Mary Ellen Rounsifer, Steven A.Sullivan, Jennifer K.Knight. Expression of rod and cone visual pigments in goldfish and zebrafish: a rhodopsin-like gene is expressed in cones. 1993, Neuron, 10: 1161-74.
116. Philip I. Song, Jonathan I. Matsui, and John E. Dowling. Morphological Types and Connectivity of Horizontal Cells Found in the Adult Zebrafish (Danio rerio) Retina. 2008, J Comp Neurol, 506 (2): 328-338.
117. Prendergast G. C. 1999. Mechanism of apoptosis by c-Myc. 18:2967-87.
118. Rajesh Ramachandran, Blake V. Fausett, and Daniel Goldman. Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-

dependent, let-7microRNA signalling pathway. 2010, Nature Cell Biology, 12: 1101–1107.

119. Rajesh Ramachandran, Xiao-Feng Zhao, and Daniel Goldman. 2011. Ascl1a/Dkk/β-catenin signaling pathway is necessary and glycogen synthase kinase-3β inhibition is sufficient for zebrafish retina regeneration. PNAS 108 (38) 15858-15863.
120. Raymond PA, Reifler MJ, Rivlin PK. 1988. Regeneration of goldfish retina: rod precursors are a likely source of regenerated cells. J Neurobiol 19:431–463.
121. Richard H. Masland. The fundamental plan of the retina. 2001, Nat Neurosci, 4(9):877-86.
122. Richard L. Piekarz and Susan E. Bates. 2009. Epigenetic Modifiers: Basic Understanding and Clinical Development. Clin Cancer Res 15(12): 3918-26.
123. Rober E. Marc, and David Cameron. A molecular phenotype atlas of the zebrafish retina. 2002, Journal of Neurocytology, 30, 593–654.
124. Ruth Bejarano-Escobar, Hortensia Sánchez-Calderón, Josué Otero-Arenas, Gervasio Martín-Partido, Javier Francisco-Morcillo. Müller glia and phagocytosis of cell debris in retinal tissue. 2013, Journal of Anatomy, 231: 471-483.
125. Ryan, K. M. & Birnie, G. D. 1997. Cell-cycle progression is not essential for c-Myc to block differentiation. Oncogene 14, 2835–2843.
126. Schlagbauer–Wadl, H. et al. 1999. Influence of increased c–Myc expression on the growth characteristics of human melanoma. J. Invest. Dermatol. 112, 332–336.
127. Schreiber–Agus, N. et al. 1995. An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. Cell 80, 777–786.

128. Schroeder, T. M., Kahler, R.A., Li, X., Westendorf, J. J. 2004. Histone deacetylase 3 interacts with runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. J. Biol. Chem. 279 (40), 41998–42007.

129. Seth Blackshaw, Sanjiv Harpavat, Jeff Trimarchi, Li Cai, Haiyan Huang, Winston P Kuo, Griffin Weber, Kyungjoon Lee, Rebecca E Fraioli, Seo-Hee Cho, Rachel Yung, Elizabeth Asch, Lucila Ohno-Machado, Wing H Wong, Constance L Cepko. Genomic analysis of mouse retinal development. 2004, PLoS Biology, 2(9): E247.

130. Shabason J. E., Tofilon P. J., Camphausen K. 2010. HDAC inhibitors in cancer care. Oncology 24(2):180-5.

131. Shinya Yamanaka. 2012. Induced pluripotent stem cells: past, present, and future. Cell Stem Cell 10(6): 678-684.

132. Sotaro Ooto, Tadamichi Akagi, Ryoichiro Kageyama, Joe Akita, Michiko Mandai, Yoshihito Honda, and Masayo Takahashi. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. 2004, PNAS, 101 (37) 13654-13659.

133. Steiner, P. et al. 1995. Identification of a Myc-dependent step during the formation of active G1 cyclin–cdk complexes. EMBO J. 14, 4814–4826.

134. Stella Pelengaris, Mike Khan, and Gerard Evan. 2002. c-Myc: more than just a matter of life and death. Nature Reviews cancer 2: 764–776 (2002).

135. Stephen Yazulla, Keith M. Studholme. Neurochemical anatomy of the zebrafish retina as determined by immunocytochemistry. 2001, Journal of neurocytology, 30(7): 551-92.

136. Sumeet PalSingh, Jennifer E.Holdway, Kenneth D.Poss. Regeneration of amputated zebrafish fin rays from de novo osteoblasts. 2012, Dev. Cell 22, 879–886.

137. Takeda M, Takamiya A, Jiao J, Cho K, Simon G, Matsuda T, Chen D. F. 2008. alpha-Aminoadipate induces progenitor cell properties of Muller glia in adult mice. Invest Ophthalmol Vis Sci 49:1142–1150.

138. Thomas Vierbuchen, Austin Ostermeier, Zhiping P. Pang, Yuko Kokubu, Thomas C. Sudhof, Marius Wernig. 2010. Direct conversion of fibroblasts to functional neurons by defined factors. Nature **463**: 1035–1041.

139. Thummel R., Kassen S. C., Enright J. M., Nelson C. M., Montgomery J. E., Hyde D. R. Characterization of Müller glia and neuronal progenitors during adult zebrafish retinal regeneration. 2008, Exp Eye Res, 87(5):433-44.

140. Timmermann, S., Lehrmann, H., Polesskaya, A. and Harel-Bellan, A. 2001. Histone acetylation and disease. Cell. Mol. Life Sci. 58, 728–736.

141. Todd L, Fischer A. J. 2015. Hedgehog-signaling stimulates the formation of proliferating Muller glia-derived progenitor cells in the retina. Development, 142:2610–2622.

142. Tomonori Katsuyama, Renato Paro. 2011, Epigenetic reprogramming during tissue regeneration. FEBS Letters, 585 (11): 1617-1624.

143. Travis J.Bailey, David R.Hyde, Kenneth D.Poss. The zebrafish as a model for complex tissue regeneration, Matthew Gemberling. 2013, Trends in Genetics, 29(11): 611-620.

144. Tseng A-S., Carneiro K, Lemire J. M., Levin M. 2011. HDAC Activity Is Required during Xenopus Tail Regeneration. PLoS ONE 6(10): e26382.

145. Tsung-Cheng Chang, Lauren R. Zeitels, Hun-Way Hwang, Raghu R. Chivukula, Erik A. Wentzel, Michael Dews, Jason Jung, Ping Gao, Chi V. Dang, Michael A. Beer, Andrei Thomas-Tikhonenko, and Joshua T. Mendell. 2009. Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. PNAS 106 (9): 3384-3389.

146. V. P. Connaughton and R. Nelson. Axonal stratification patterns and glutamate-gated conductance mechanisms in zebrafish retinal bipolar cells. 2000, Journal of Physiology, 524(1):135-146.

147. Ververis K., Hiong A., Karagiannis T. C., Licciardi P. V. 2013. Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. Biologics 7:47-60.

148. Vikram Arora, Derek C. Knapp, Barbara L. Smith, Mary L. Statdfield, David A. Stein, Muralimohan T. Reddy, Dwight D. Weller and Patrick L. Iversen. 2000. c-*Myc* Antisense Limits Rat Liver Regeneration and Indicates Role for c-*Myc* in Regulating Cytochrome P-450 3A Activity. Journal of Pharmacology and Experimental Therapeutics 292(3): 921-928.

149. Wade, P. A. 2001. Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. Hum. Mol. Genet. 10, 693–698.

150. Wan J, Ramachandran R, Goldman D. 2012. HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration. Dev Cell 22:334–347.

151. Wan J, Zhao X-F, Vojtek A, Goldman D. 2014. Retinal injury, growth factors, and cytokines converge on b-catenin and pStat3 signaling to stimulate retina regeneration. Cell Rep 9:285–297.

152. Wan J, Zheng H, Xiao H-L, She Z-J, Zhou G-M. 2007. Sonic hedgehog promotes stem-cell potential of Muller glia in the mammalian retina. Biochem Biophys Res Commun, 363:347– 354.

153. Ward M. Peterson, Quan Wang, Roumiana Tzekova and Stanley J. Wiegand. 2000. Ciliary Neurotrophic Factor and Stress Stimuli Activate the Jak-STAT Pathway in Retinal Neurons and Glia. JNeurosci 20 (11) 4081-4090.

154. Wells I. Mangrum, John E. Dowling, and Ethan D. Cohen. A morphological classification of ganglion cells in the zebrafish retina. 2002, Visual Neuroscience, 19(6): 767-779.

155. Westendorf, J. J., Zaidi, S.K., Cascino, J. E., Kahler, R., van Wijnen, A. J., Lian, J. B., Yoshida, M., Stein, G. S., Li, X. 2002. Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. Mol. Cell. Biol. 22 (22), 7982–7992.

156. White L.M., Roy S., Gordillo G.M., Kalliainen L.K., Melvin W.S., Ellison E.C., and Sen C.K. Wound healing and regeneration. 2010, Physiology and Medicine, 1:193.

157. Wu D. M., Schneiderman T, Burgett J, Gokhale P, Barthel L, Raymond P. A. 2001. Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. Invest Ophthalmol Vis Sci 42:2115–2124.

158. Wu DM, Schneiderman T, Burgett J, Gokhale P, Barthel L, Raymond PA. Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. 2001, Invest. Ophthalmol. Vis. Sci, 42(9): 2115–2124.

159. Yongcheol Cho, and Valeria Cavalli. 2014. HDAC signalling in neuronal development and axon regeneration. Current Opinion in Neurobiology 27: 118-126.

160. Yongcheol Cho, Valeria Cavalli. 2012. HDAC5 is a novel injury-regulated tubulin deacetylase controlling axon regeneration. The EMBO Journal 31: 3063-3078.

161. Yoshii C, Ueda Y, Okamoto M, Araki M. 2007. Neural retinal regeneration in the anuran amphibian Xenopus laevis post-metamorphosis: transdifferentiation of retinal pigmented epithelium regenerates the neural retina. Dev Biol 303:45–56.

162. Yumi Ueki, Matthew S. Wilken, Kristen E. Cox, Laura Chipman, Nikolas Jorstad, Kristen Sternhagen, Milesa Simic, Kristy Ullom, Masato Nakafuku, and Thomas A.

Reh. Transgenic expression of the proneural transcription factor Ascl1 in Müller glia stimulates retinal regeneration in young mice. 2015, PNAS, 112 (44) 13717-13722.

163. Zhao X-F, Wan J, Powell C, Ramachandran R, Myers MG, Goldman D. 2014a. Leptin and IL-6 family cytokines synergize to stimulate Muller glia reprogramming and retina regeneration. Cell Rep 9:272–284.

Section 6

Appendix

Appendix 1:

List of primers

Cloning primers	**Ensembl ID**	Sequence (5'-3')
Mycb_F_Bam	**ENSDARG00000007241**	ATGCTGAGGGATCCACCATGCCGCTGAATTCAAGTATGGAG
Mycb_R_Xho		ATGGTAGCCTCGAGAGAACAGCTCAACTGCTCGAGTCTC
ascl1a FL Fwd	**ENSDARG00000038386**	ATGGACATCACCGCCAAGATGGAAATAAGCG
ascl1a FL Rev		TCAAAACCAGTTGGTGAAGTCCAGGAGCTC
lin28a FL BamI F	**ENSDARG00000016999**	ATGCTGATGGATCCACCATGCCCCCGGCAAATCCGCATC
Lin28a FL XhoI R		ATGCTGATCTCGAGATCAGTGCTCTCTGGCAGTAAGGGAG
her4.1 FL Fwd	**ENSDARG00000056732**	GAAACTCTACTGACAAACAAGCTG
her4.1 FL Rev		GATGTTGTCCATCTTCGTTTAGTGC
Bam-Insm-F	**ENSDARG00000091756**	ATGGTAATGGATCCACCATGCCCAGAGGATTTTTAGTCAAG
XhoI-Insm-R		ATGGTACTCGAGGCAGGCTGGACGCACCGGCATCTGAAG
hdac1 FL Fwd	**ENSDARG00000015427**	GTGATGAAGAGTTCTCAGACTCTGAGG
hdac1 FL Rev		CTAACAGCAATACAGCAAGCCTTCGCC

hdac3 FL Fwd	**ENSDARG00000037514**	TCACAGACAGGGTCATGACTGTGTCC
hdac3 FL Rev		TAACAAACAGCACTGGTGATCCGCCAC
hdac4 FL Fwd	**ENSDARG00000098349**	CTGTCTCTCCATCGCTATGATGATGG
hdac4 FL Rev		TCTCGAACGTCAGAAGCAAGAATGGCG
hdac5 FL Fwd	**ENSDARG00000075139**	AACTCAGTCGCCATCACTGCCAAACTCC
hdac5 FL Rev		CATGCCTTTGTTGTCCACTTTGGGC
hdac6 FL Fwd	**ENSDARG00000008384**	ACACACACAGAGTTTCAGCAGGCC
hdac6 FL Rev		CATCTCCTTGGCCTGAATTCTTAC
hdac9 FL fwd	**ENSDARG00000056642**	TCACAGTCGAGGTTATTAGGG
hdac9 FL rev		GAACTCCATAGTGCCATGGG
Hind2X flag NICD Fwd		TCAATCATGAAGCTTACCATGGACTACAAGGACGACG ATGACAAGGACTACAAGGACGACGATGACAAGTCCAG GAAGAGGAAGCGGGAACATGGC
MluI NICD Rev		CCGCTGCCACGCGTCTTGAAAGCCTCTGGAATATGGTT C
T7-HSP m F		TAATACGACTCACTATAGCCTTTCATAATGAAACAATT GCAC
Sv40 Rev		GATCATAATCAGCCATACCACATTTG
ChIP and Promoter primers		
lin-28-pro-F		AGATCTCGAGGTTCTCCTTCTCAGAAAATTAAATCTAC ACAC
lin-28-pro-R		ATCCGGGCCCTTTATGATTTAGCCTTCTACTTCTTAAAA AATAAC
Xho-her4.1 pro- F		GGCTGAAGCTCGAGAACAAACAGACCATCAAAATGAA GTGTGAC

Bam-her4.1 pro-R		ATGCGTAGGGATCCTGCTGTGTGTCTTGTGTTCAGTTCTCCG
Xho-insm1a -Pro-F		CTCAGATCTCGAGTTGCCTTCAGAATAAATCACTAATGTCC
Bam-insm1a -Pro-R		CCGCGGGCCCGGATCCCTTCGCCAGCTGAAAGGCACTTCAGTCG
qPCR Primers		
mycb RT Fwd	**ENSDARG00000007241**	AGTAGTGACAGCGAATCCGATGACG
mycb RT Rev		ATGTGGCTCTCGAATTTAATCCGC
ascl1a RT Fwd	**ENSDARG00000038386**	ATCTCCCAAAACTACTCTAATGACATGAACTCTAT
ascl1a RT Rev		CAAGCGAGTGCTGATATTTTTAAGTTTCCTTTTAC
lin28a RT Fwd	**ENSDARG00000016999**	TAACGTGCGGATGGGCTTCGGATTTCTGTC
lin28a RT Rev		ATTGGGTCCTCCACAGTTGAAGCATCGATC
her4.1 RT fwd	**ENSDARG00000056732**	GCTGATATCCTGGAGATGACG
her4.1 RT rev		GACTGTGGGCTGGAGTGTGTT
insm1a RT Fwd	**ENSDARG00000091756**	CCAAGAAAGCCAAAGCCATGCGGAAGC
insm1a RT Rev		TTATTGCTTTCCGCGCTCTGCTTGGGTTTG
hdac1 RT 001 Fwd	**ENSDARG00000015427**	GACAGCACCATTCCTAATGAGCTCC
hdac1 RT 001 Rev		TATCGTGAGCACGAATGGAGATGCG

hdac3 001 RT Fwd	**ENSDARG00000037514**	CCCAGGAACTGGTGACATGTATGAAG
hdac3 001 Rt Rev		ACAAACTCCACACATTCTCCATGTCC
hdac4 201 RT Fwd	**ENSDARG00000098349**	CAAGCTCACGGCCAAATGTTTTGGC
hdac4 201 RT rev		GCAGTGAACGCCAGTATTTACTCTGG
hdac5 201 RT Fwd	**ENSDARG00000075139**	GATCCTCATCATAGACTGGGATATCC
hdac5 201 RT Rev		AATGGGCATCACCACCGTTCTGAA
hdac6 001 RT Fwd	**ENSDARG00000008384**	TGATTCATCAGCCGTGAAGGATCAGG
hdac6 001 RT Rev		ATAACGCCCGCAAAGCACCTTATAGC
hdac7 201 RT Fwd	**ENSDARG00000105179**	AATGGCTTTGCTGTGGTCAGGCCACC
hdac7 201 RT Rev		GATTGTGAAAGCAGTACTGTTGAGC
hdac8 RT Fwd	**ENSDARG00000105159**	GTGTCTAACCTATATACTTGGCTGGG
hdac8 RT Rev		ACATTCTTCAGATTCCCTTTGATGG
hdac9 RT Fwd	**ENSDARG00000056642**	CACCAAAAGATCCTCTCTTCTGGTC
hdac9 RT Rev		GCTCGATGGATTTGGAAAGCATCTTG
hdac10 001 RT Fwd	**ENSDARG00000086458**	AAAGGTGTTCCTAGTCCAGATCTGC
hdac10 001 RT Rev		CCAGCAGATCTTTATCATAGCCCTGC
hdac11 201 RT Fwd	**ENSDARG00000087573**	CCTGAACAGACTTAAGTGGTCTCTGG

hdac11 201 RT Rev		CCTCAACCCTCTCAAAGAGAAACTTG
il6 RT Fwd	**ENSDARG00000102318**	GCTATTCCTGTCTGCTACACTGG
il6 RT Rev		TGAGGAGAGGAGTGCTGATCC
il6r RT Fwd	**ENSDARG00000104474**	TCAGCTCCTGAGACAACTACTGC
il6r RT Rev		AAACGGCATAGTCTGTTTCCC
il11a RT Fwd	**ENSDARG00000037859**	CTCCTCATCGCTGCTTCTCTCG
il11a RT Rev		TTGCGAAGTCACTGGCTCTGC
il11b RT Fwd	**ENSDARG00000058557**	GCTAACAGTGTCGCCTGACTCC
il11b RT Rev		CTGTAGTTCAGTGAGGGCAGGG
il11ra RT Fwd	**ENSDARG00000026736**	GTTGGACTGTTGGTTTTGTTGG
il11ra RT Rev		TGGATTGTGGGTAATGAAGGC
lepa RT Fwd	**ENSDARG00000091085**	TTTCCAGCTCTCCGCTCAACC
lepa RT Rev		CGGCGTATCTGGTCAACATGC
lepb RT Fwd	**ENSDARG00000045548**	CATTGCTCGAACCACCATCAGC
lepb RT Rev		TCTTTATGCACCGGGGTCTCG
lepr RT Fwd	**ENSDARG00000070961**	CAGTACGAGCTGCAATTCAAGG
lepr RT Rev		TAAAATGCGCCAGAAGTCTGG

crlf1a RT Fwd	**ENSDARG00000024365**	GGGATTCTGGGATCTAGGAAAGC
crlf1a RT Rev		TCCTTGAAGAACCTGGTTGCG
clcf RT Fwd	**ENSDARG00000076140**	GAAAGTTGGTCAGGTTGCTGTGC
clcf RT Rev		CATAAGTCCACACGTGTTGCTGC
lifra RT Fwd	**ENSDARG00000098857**	AAGCCGTCTCCACACAGTCTGG
lifra RT Rev		TTCCCCCCATTCTGCTTTCC
dla RT Fwd	**ENSDARG00000010791**	GCGCAGGAAACGTCTGAAAAGTGAC
dla RT Rev		ATCCTGCAGGCCCATTACACCTCAG
dlb RT Fwd	**ENSDARG00000004232**	AAGAATGGCGGCAGTTGTAATGATTTG
dlb RT Rev		AGATCCACACATTCACCACCGTTG
dlc RT Fwd	**ENSDARG00000002336**	GAGCACCTCAAACACCAG
dlc RT Rev		CACCTCCTCCACCCATAA
dld RT Fwd	**ENSDARG00000020219**	AAATGGAGGAAGTTGCACTGATC
dld RT Rev		AAGATCGAGACACTGAGCATCATTC
notch1a RT Fwd	**ENSDARG00000103554**	ACGGATTCACTCCACTGATGATCGCATC
notch1a RT Rev		TCGGTTCCGAATGAGGATCTGGAAG

Appendix 2:

List of transcription factors regulated in the RNA-seq analysis

12hpi upregulated	
ss18	ENSDARG00000002970
irf9	ENSDARG00000016457
stat3	ENSDARG00000022712
klf6a	ENSDARG00000029072
nr1d4a	ENSDARG00000031161
fosab	ENSDARG00000031683
myclb	ENSDARG00000034956
nfkb2	ENSDARG00000038687
cbfb	ENSDARG00000040917
pou3f3a	ENSDARG00000042032
cebpb	ENSDARG00000042725
nfil3	ENSDARG00000042977
jun	ENSDARG00000043531
nr2e3	ENSDARG00000045904
homeza	ENSDARG00000054304
nr1d4b	ENSDARG00000059370
foxo1b	ENSDARG00000061549
atf5a	ENSDARG00000068096
pitx3	ENSDARG00000070069
irx5b	ENSDARG00000074070
junba	ENSDARG00000074378
sall1b	ENSDARG00000075891
mych	ENSDARG00000077473
zgc:113886	ENSDARG00000077712
cers3a	ENSDARG00000078541
cebpd	ENSDARG00000087303
BX510934.1	ENSDARG00000087544

12hpi downregulated	
cbx1b	ENSDARG00000054442
rad54l2	ENSDARG00000063031
rbpms2b	ENSDARG00000079578

LHX3	ENSDARG00000003803
nr1d2a	ENSDARG00000003820
esrrd	ENSDARG00000015064
sox6	ENSDARG00000015536
npas2	ENSDARG00000016536
rorcb	ENSDARG00000017780
foxo3a	ENSDARG00000023058
arntl1b	ENSDARG00000035732
irx4b	ENSDARG00000036051
nr2f2	ENSDARG00000040926
nfya	ENSDARG00000042004
carhsp1	ENSDARG00000053129
rorca	ENSDARG00000057231
srebf1	ENSDARG00000067607
hey1	ENSDARG00000070538
hmga1b	ENSDARG00000070951
sall1a	ENSDARG00000074319
rereb	ENSDARG00000075670
samd11	ENSDARG00000077852

4dpi upregulated	
ezh2	ENSDARG00000010571
rbb4l	ENSDARG00000015208
rbbp4	ENSDARG00000029058
hells	ENSDARG00000057738
actl6a	ENSDARG00000070828
zgc:163040	ENSDARG00000101114
cct4	ENSDARG00000013475
ybx1	ENSDARG00000004757
vsx2	ENSDARG00000005574
atf3	ENSDARG00000007823
sox21b	ENSDARG00000008540
e2f7	ENSDARG00000008986
ascl1b	ENSDARG00000101628
foxn4	ENSDARG00000010591
stat6	ENSDARG00000015902
irf9	ENSDARG00000016457
gli2a	ENSDARG00000025641
hmga1a	ENSDARG00000028335
klf6a	ENSDARG00000029072
hmgb2a	ENSDARG00000029722
hmgb1b	ENSDARG00000030479
mybl1	ENSDARG00000030999
stat2	ENSDARG00000031647

mybl2b	ENSDARG00000032264
myclb	ENSDARG00000034956
pou2f2a	ENSDARG00000019658
zic2b	ENSDARG00000037178
smad5	ENSDARG00000037238
klf2b	ENSDARG00000040432
cbfb	ENSDARG00000040917
olig2	ENSDARG00000040946
cebpb	ENSDARG00000042725
jun	ENSDARG00000043531
nr2e3	ENSDARG00000045904
hmgb2b	ENSDARG00000053990
irf8	ENSDARG00000056407
spi1a	ENSDARG00000067797
atf5a	ENSDARG00000068096
hes2.2	ENSDARG00000068168
atoh7	ENSDARG00000069552
pitx3	ENSDARG00000070069
e2f3	ENSDARG00000070463
sox2	ENSDARG00000070913
sall1b	ENSDARG00000075891
stat1b	ENSDARG00000076182
mych	ENSDARG00000077473
atf5b	ENSDARG00000077785
ZNF217	ENSDARG00000088123
insm1a	ENSDARG00000091756

4dpi downregulated	
jmjd1cb	ENSDARG00000079939
eno1a	ENSDARG00000022456
rad54l2	ENSDARG00000063031
aff4	ENSDARG00000001857
arid2	ENSDARG00000007413
camta1b	ENSDARG00000007824
esrrgb	ENSDARG00000011696
foxn3	ENSDARG00000012833
esrrd	ENSDARG00000015064
sox6	ENSDARG00000015536
tfdp1b	ENSDARG00000016304
nr1d1	ENSDARG00000033160
egr1	ENSDARG00000037421
bhlhe41	ENSDARG00000041691
thrab	ENSDARG00000052654
npas4a	ENSDARG00000055752

srebf1	ENSDARG00000067607
klf9	ENSDARG00000068194
hmga1b	ENSDARG00000070951
cica	ENSDARG00000071150
si:ch73-386h18.1	ENSDARG00000073944
si:dkey-4c15.5	ENSDARG00000074125
hlfa	ENSDARG00000074752
rereb	ENSDARG00000075670
foxj3	ENSDARG00000075774
nfatc3a	ENSDARG00000076297
rerea	ENSDARG00000077353
si:dkey-18a10.3	ENSDARG00000090814

4dpi_VPA upregulated	
tcf7l2	ENSDARG00000004415
ybx1	ENSDARG00000004757
mycb	ENSDARG00000007241
atf3	ENSDARG00000007823
ascl1b	ENSDARG00000101628
stat6	ENSDARG00000015902
tfdp1b	ENSDARG00000016304
irf9	ENSDARG00000016457
klf6a	ENSDARG00000029072
nr1d4a	ENSDARG00000031161
mybl2b	ENSDARG00000032264
myclb	ENSDARG00000034956
nfkb2	ENSDARG00000038687
dmrta2	ENSDARG00000039412
cbfb	ENSDARG00000040917
cebpb	ENSDARG00000042725
nfil3	ENSDARG00000042977
jun	ENSDARG00000043531
tp63	ENSDARG00000044356
nr2e3	ENSDARG00000045904
irf8	ENSDARG00000056407
cers2b	ENSDARG00000058992
nr1d4b	ENSDARG00000059370
spi1a	ENSDARG00000067797
atf5a	ENSDARG00000068096
pitx3	ENSDARG00000070069
sall1b	ENSDARG00000075891
stat1b	ENSDARG00000076182
mych	ENSDARG00000077473
cebpd	ENSDARG00000087303

ss18	ENSDARG00000002970
cct4	ENSDARG00000013475

4dpi_VPA downregulated	
hcfc1b	ENSDARG00000012519
eno1a	ENSDARG00000022456
rad54l2	ENSDARG00000063031
nr1d2a	ENSDARG00000003820
arid2	ENSDARG00000007413
esrrgb	ENSDARG00000011696
esrrd	ENSDARG00000015064
sox6	ENSDARG00000015536
foxo3a	ENSDARG00000023058
klf11a	ENSDARG00000030844
nr1d1	ENSDARG00000033160
irx4b	ENSDARG00000036051
egr1	ENSDARG00000037421
cers6	ENSDARG00000053583
npas4a	ENSDARG00000055752
srebf1	ENSDARG00000067607
hmga1b	ENSDARG00000070951
cica	ENSDARG00000071150
si:ch73-386h18.1	ENSDARG00000073944
rereb	ENSDARG00000075670
nfatc3a	ENSDARG00000076297
rerea	ENSDARG00000077353
si:dkey-18a10.3	ENSDARG00000090814

12hpi_4dpi upregulated	
trib3	ENSDARG00000016200

12hpi_4dpiVPA upregulated	
mycb	ENSDARG00000007241
tfdp1b	ENSDARG00000016304
nr1d4a	ENSDARG00000031161
nfkb2	ENSDARG00000038687
nfil3	ENSDARG00000042977
nr1d4b	ENSDARG00000059370
flna	ENSDARG00000074201
cebpd	ENSDARG00000087303

12hpi_4dpiVPA downregulated	
nr1d2a	ENSDARG00000003820
foxo3a	ENSDARG00000023058
irx4b	ENSDARG00000036051

4dpi_4dpiVPA upregulated	
ybx1	ENSDARG00000004757
stat6	ENSDARG00000015902
mybl2b	ENSDARG00000032264
ssrp1a	ENSDARG00000037397
irf8	ENSDARG00000056407
spi1a	ENSDARG00000067797
stat1b	ENSDARG00000076182
sox11b	ENSDARG00000095743

4dpi_4dpiVPA downregulated	
arid2	ENSDARG00000007413
esrrgb	ENSDARG00000011696
eno1a	ENSDARG00000022456
nr1d1	ENSDARG00000033160
egr1	ENSDARG00000037421
cica	ENSDARG00000071150
si:ch73-386h18.1	ENSDARG00000073944
nfatc3a	ENSDARG00000076297
rerea	ENSDARG00000077353
MAPK8IP1 (1 to many)	ENSDARG00000090326
si:dkey-18a10.3	ENSDARG00000090814
arid1b	ENSDARG00000092503

12hpi_4dpi_4dpiVPA upregulated	
irf9	ENSDARG00000016457
klf6a	ENSDARG00000029072
myclb	ENSDARG00000034956
txnipa	ENSDARG00000036107
cbfb	ENSDARG00000040917
cebpb	ENSDARG00000042725
jun	ENSDARG00000043531

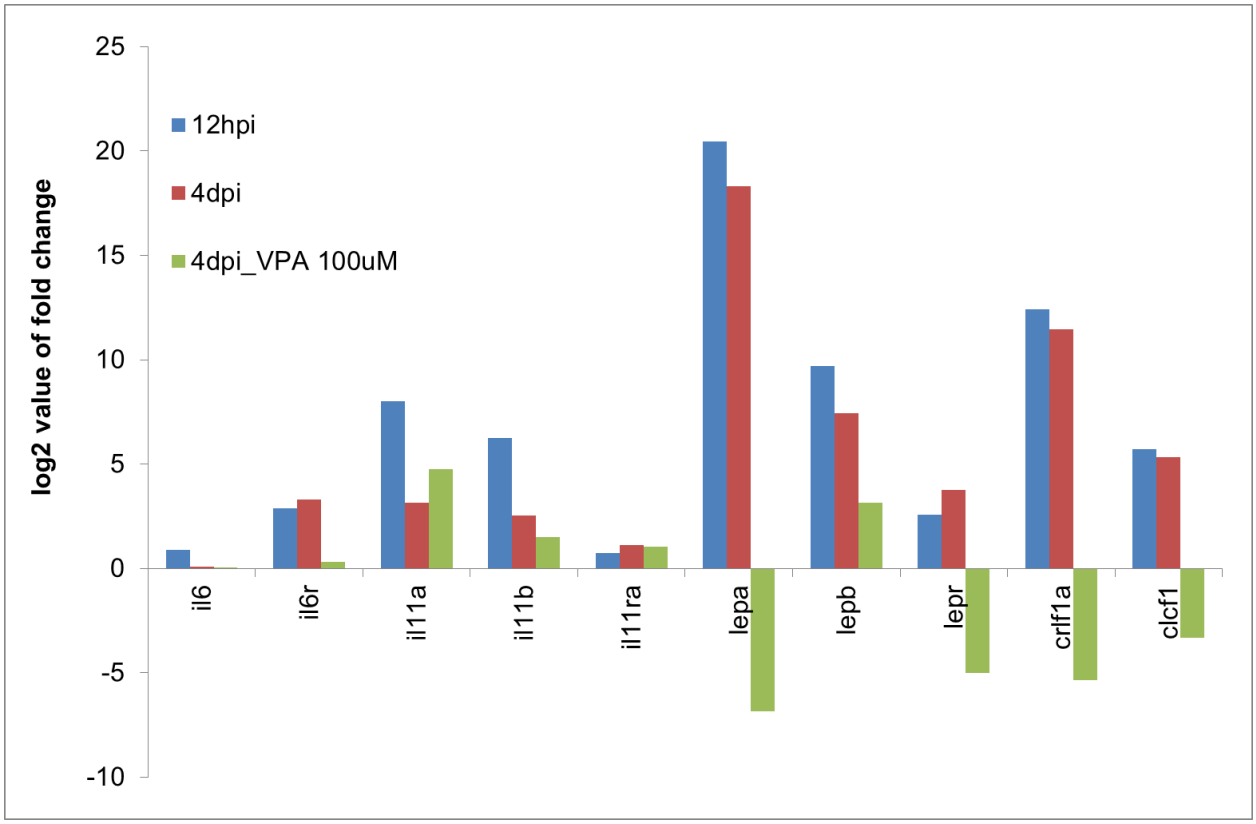
nr2e3	ENSDARG00000045904
atf5a	ENSDARG00000068096
pitx3	ENSDARG00000070069
sall1b	ENSDARG00000075891
mych	ENSDARG00000077473

12hpi_4dpi_4dpiVPA downregulated	
esrrd	ENSDARG00000015064
sox6	ENSDARG00000015536
srebf1	ENSDARG00000067607
hmga1b	ENSDARG00000070951
rereb	ENSDARG00000075670

Appendix 3:

Regulation of cytokines in the RNA-seq analysis

log2 value of fold change from whole retina RNAseq at different time points post injury										
	il6	*il6r*	*il11a*	*il11b*	*il11ra*	*lepa*	*lepb*	*lepr*	*crlf1a*	*clcf1*
12hpi	0.9	2.9	8	6.2656	0.75	20.45	9.69	2.593	12.4237	5.7
4dpi	0.09	3.3	3.164	2.55	1.12	18.3	7.43	3.78	11.44	5.33
4dpi_VPA 100uM	0.00083	0.3135	4.746	1.51792	1.05	-6.85637	3.15146	-5.0201	-5.337	-3.3229



“IS THERE ANYTHING MORE DANGEROUS

THAN DISSATISFIED AND IRRESPONSIBLE GODS

WHO DON’T KNOW WHAT THEY WANT?”

-YUVAL NOAH HARARI, SAPIENS: A BRIEF HISTORY OF HUMANKIND.