Understanding the role of multifaceted transcription factor Yin-Yang1 during zebrafish retina regeneration

A thesis submitted by

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Declaration

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

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

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

Date **Place** **Mansi Chaudhary**

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

"A world where there is no donor organ shortage. Where victims of spinal cord injuries can walk, where weakened hearts are replaced. This is the long-term promise of regenerative medicine"
(NIH, 2006).

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This thesis, which contains only the fruitful outcomes, is just a small part of the long roller-coaster ride of my PhD journey which started with so many dreams in the eyes to start my voyage as a researcher. If I were to summarize my PhD journey, quote from Albert Einstein would suffice to explain, "If we knew what it was we were doing, it would not be called as research, would it?". During our entire tenure as a PhD student, we keep trying, failing and again trying until we get the result, which may or may not even fit our hypothesis. But now as I write my thesis I feel that all the hard work, pain, heartbreaks from the failed experiments are paid off. There were some golden moments during the PhD, which gave immense happiness and sense of small victories, which kept my morals high and motivated to reach to a point where I could summarize my work in the form of this thesis. I, as a PhD student, despite facing lots of failure, gathered immense experience, not only on the scientific part but also as a life lesson which have made me a better human being altogether. All these years has changed me as a scholar because today when I stand on the other side I have the confidence of pursuing my career in this field and also instilled in me some traits such as being patience, persistence and focussed. No thesis is ever complete without the endless support of so many people and therefore can't be complete without expressing gratitude towards them.

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Contents

List of Publications

1. Gupta, Shivangi, Poonam Sharma, **Mansi Chaudhary**, Sharanya Premraj, Simran Kaur, Vijithkumar Vijayan, Manas Geeta Arun, Nagaraj Guru Prasad, and Rajesh Ramachandran. "Pten associates with important gene regulatory network to fine-tune Müller glia-mediated zebrafish retina regeneration." *Glia* (2023).

2. Poonam Sharma, Shivangi Gupta[#], **Mansi Chaudhary**[#], Soumitra Mitra,[#], Bindia Chawla, Mohammad Anwar Khursheed, Navnoor Kaur Saran, and Rajesh Ramachandran[©] (2020). Biphasic role of Tgf-β signaling during Müller glia reprograming and retina regeneration in zebrafish. ***iScience***. 23 (2): 100817. DOI: https://doi.10.1016/j.isci.2019. 100817

3. Poonam Sharma, Shivangi Gupta[#], **Mansi Chaudhary**[#], Soumitra Mitra,[#], Bindia Chawla, Mohammad Anwar Khursheed, and Rajesh Ramachandran[©] (2019). Oct4 mediates Müller glia reprograming and cell cycle-exit during retina regeneration in zebrafish. ***Life Science Alliance*** 2 (5): 1-21.

4. Soumitra Mitra, Poonam Sharma[#], SimranKaur[#], Mohammad Anwar Khursheed, Shivangi Gupta, Akshai Janardhana Kurup, **Mansi Chaudhary**, and Rajesh Ramachandran[©] (2019). Dual regulation of *lin28a* by Myc is necessary during zebrafish retina regeneration. ***Journal of Cell Biology*** 218: 489-507

5. Soumitra Mitra, Poonam Sharma[#], SimranKaur[#], Mohammad Anwar Khursheed Shivangi Gupta, Riya Ahuja, Akshai Janardhana Kurup, **Mansi Chaudhary**, and Rajesh Ramachandran[©] (2018). Histone Deacetylase-Mediated Muller Glia Reprograming Through Her4.1-Lin28a axis is Essential for Retina Regeneration in Zebrafish. ***iScience*** 7: 68-84. DOI: https://doi.org/10.1016/j.isci.2018.08.008.

6. Simran Kaur, Shivangi Gupta[#], **Mansi Chaudhary**[#], Mohammad Anwar Khursheed Soumitra Mitra, Akshai Janardhana Kurup and Rajesh Ramachandran[©] (2018). *let-7* MicroRNA-Mediated Regulation of Shh Signaling and the Gene Regulatory Network Is Essential for Retina Regeneration. ***Cell Reports*** 23: 1409-1423.

Note: Manuscript for this thesis is currently under preparation.

Thesis Abstract

Globally, 7.7 million population suffer from glaucoma and 3.9 million from diabetic retinopathy worldwide as per World Health Organization 2021. Damage to the retina in such pathological conditions often leads to complete vision loss or blindness in mammals. In contrast to mammals, Teleosts such as zebrafish can regenerate their damaged retina and completely restore their lost vision. In response to injury, Müller glial cells of the retina undergo reprograming event and a series of signalling events starts, which bring about major epigenetic and molecular changes in the cells. These changes involve transcription factors, growth factors, cytokines, signalling pathways and epigenetic factors.

Yin-Yang1(YY1) is a ubiquitous protein first discovered in 1991 and named differently by three independent groups. YY1 as a transcription factor plays a pivotal role during embryogenesis, development and normal homeostasis. It can regulate various genes by binding onto its promoter and can act as an activator or repressor, owing to its ability to choose the binding partner. Yy1 can interact with co-activators such as, HATs (histone acetyltransferase) and help open up the chromatin. Also, it can bind to co-repressors like Hdacs and Ezh2 and thus aids in forming heterochromatin. Yy1 also promotes enhancer-promoter interaction and forms TADs, a function similar to CTCF. Besides, differential binding partners, Yy1 carry out plethora of function because of myriads of post translational modification it undergoes.

In our study, we explored the role of Yy1 during retina regeneration. We found that Yy1 acts as a pro-proliferative molecule, as knockdown of Yy1 leads to a decrease in the number of proliferating Müller glia while the overexpression of Yy1 increased proliferation. We also found that Yy1 positively regulates Lin28a expression, which in turn downregulates *let-7a* and causes an increases in the levels of Ascl1a, master regulator of retina regeneration. We further explored Notch signalling in Yy1 knockdown condition and found that Notch signalling indeed

gets affected, which is also reflected in our RNA-seq data. Her4.1, an effector gene of Notch signalling, is downregulated by Yy1, which in turn upregulates Mmp9.

Yy1 regulates BMP signalling, which is an important signalling during development. Previously, it was shown that BMP signalling is pro-proliferative in chick retina regeneration. We also found out that inhibition of BMP signalling leads to a decrease in proliferation in Yy1 dependent manner. Besides having an independent role as a pro-proliferative transcription factor, Yy1 acts synergistically with the BAF complex, a chromatin remodeler, to regulate many regeneration-associated genes.

In this study, we also report that the function of Yy1 is dependent on its acetylation and subsequent timely deacetylation to evoke a successful regenerative response as acetylated-mimetic mutation of Yy1, surprisingly, caused a reduction in the number of proliferating MGPCs.

Taken together, our study sheds light on the pro-proliferative role of Yy1 during retina regeneration and opens up new avenues to explore the therapeutic potential of Yy1.

Thesis Synopsis

Introduction and Literature Review

Living organisms have the ability to take sensory cues from the environment and process that information in the brain. Traditionally identified five types of senses are vision, smell, taste, hear and touch. Various organs are involved in this process, which gathers information, turns the signal into the form that brain can readily understand and necessary actions are taken. One such organ involved in visual sensing is the retina, which converts the light signal into electrical signals. These signals are transduced by the retinal neurons through the optic nerve to the visual cortex of the brain. Visual impairment is a great loss for organisms, such as humans as they cannot heal the damaged retina. Retina gets damaged in numerous pathological conditions such as diabetic retinopathy, age-related macular degeneration and glaucoma. It cannot be regenerated due to the involvement of a complex network of signalling pathways and connections of the neurons. Restoration of the correct connection is a tedious task in the complex system, such as central nervous system (CNS), in higher organisms.

Regeneration refers to the complete restoration of the structure and function of the damaged organ or tissue. On an evolutionary timescale, higher organisms such as humans have lost the regeneration potential of most of the organs, while some of the primitive and lower organisms still possess this capability due to a much simpler system. Since the time of Trembley, regeneration biologists have been trying to find clues from the organisms that can regenerate and serve as a model to unravel the mystery of regeneration. Zebrafish is one such organism, which serves as an excellent model to study the mechanism of regeneration as it can regenerate almost all its organs, including the heart, liver, kidney, fin, retina and even axon.

Studying the molecular mechanism underlying retina regeneration will not only provide us the treatment for some of the diseases which leads to complete loss of vision, but also helps us

study CNS regeneration since the retina is the most accessible part of the central nervous system. Moreover, Zebrafish shares significant structural homology with the human retina and 80% of its genome is similar to that of humans. Retina comprises three retinal layer, namely the outer nuclear layer, the inner nuclear layer and the ganglion cell layer and contains six neuronal subtypes and one type of glial cells called Müller glial cells.

In normal conditions, extensions of these glial cells span all the retinal cell layers and help in maintaining the normal homeostasis of the retina as they are involved in various processes such as ion exchange and neurotransmitter recycling. Upon injury, the Müller glia cells sense the cues secreted by the neighbouring dying cells and undergo reprograming in the adult fish retina. In this process, numerous cytokines; growth factors; immune responsive genes; signalling pathways (Delta-Notch signalling, Wnt signalling, Sonic hedgehog signalling, TGF-β signalling and Pten-Akt pathway); transcription factors, some of which are well-known pluripotency factors such as Ascl1a, Lin28a, Oct4, Sox2, Kfl4 and c-Myc; and epigenetic factors such as APOBEC proteins and Hdacs, are involved. Despite this knowledge, retina regeneration remains an enigmatic process and the search for the missing cues continues.

Yin Yang1 (Yy1) is the multifaceted transcription factor that acts as an activator and repressor depending on the coactivators or corepressors it recruits onto the promoters. Functionality of Yy1 also depends on the post-translational modification (acetylation, methylation, phosphorylation, ubiquitination and SUMOylation) it undergoes. Besides this, YY1 is also known to interact with many epigenetic modifiers, such as EZH2, HDACs, HATs and the non-coding RNAs and recruit them onto the site and modulate the chromatin state. It is a ubiquitous transcription factor and regulates numerous genes associated with the cell cycle, cell proliferation, differentiation and genes involved in maintaining the normal homeostasis of the cell. Besides this, Yy1 has been associated with tumorigenesis as it can regulate many oncogenes and tumor suppressors. Involvement of Yy1 has been well-elucidated in embryonic

stem cells, cancer stem cells and skeletal regeneration. Therefore, in our study, we decided to explore the regulatory role of Yy1 during retina regeneration.

Results

Chapter 1: Yy1 is indispensable for Müller glia reprograming and proliferation

In Zebrafish, Yy1 is encoded by two genes, *yy1a* and *yy1b*, which share almost 88% of sequence similarity. We confirmed the presence of *yy1a* and *yy1b* in the developing eye, brain and the notochord, by performing mRNA *in-situ* hybridization in the 24 hpf (hours post fertilization) and 48hpf embryos. We then checked the expression of both the genes by performing time course analysis post retinal injury and reported a decline of both the genes' expression around 1dpi and 2dpi and levels again started rising when the proliferation was at its peak, at 4dpi. We also observed an exclusion of *yy1a* and *yy1b* from the proliferating cells. We then confirmed this result at the protein level as well, by immunostaining and there also Yy1 was excluded from the proliferating cells. Since Yy1 is ubiquitously present in all the cells of the three retinal layers and proliferating Müller glia cells have low representation, we did not observe any drastic decline in its protein level during time course analysis by western blot. We then checked the effect of Yy1 knockdown on the number of proliferating Muller glia derived progenitor cells (MGPCs). We adopted morpholino (MO) mediated knockdown approach in which we injected and electroporated MO into the retina and observed a drastic decline in the number of proliferating cells in the combined knockdown of *yy1a* and *yy1b* as compared to their individual knockdown. We then checked whether we get opposite result if we overexpress Yy1 in the retina. In the combined overexpression of Yy1a and Yy1b, there was an increase in the number of proliferating cells, thus confirming the pro-proliferative role of Yy1 during the retina regeneration. We also checked if we could rescue the effect of

morpholino mediated knockdown by overexpressing the mRNA with mutated MO binding sites in knockdown background and found that indeed the knockdown phenotype could be rescued. We did lineage tracing to confirm if these increased number of cell in the Yy1 overexpressed conditions are viable. These increased number of $BrdU^{+}$ cells were surviving and could indeed give rise to all the retinal cell type when traced up to 23 days post injury (dpi). We made a transgenic line with *gfap* promoter driving YY1-GFP tagged with FLAG. When we injured this line we found pan-retinal increase in the number of proliferating MGPCs.

We then questioned if mere overexpression of Yy1 is sufficient to reprogram the Müller glia to adopt stem cell like fate. We overexpressed both *yy1a* and *yy1b* in the uninjured retina without disturbing the retina and harvested on 4^{th} days post transfection. Surprisingly, we did not find any proliferating cell marked with BrdU or PCNA, and thus indicating that injury induced early signalling events, such as Shh signalling, TGFβ signalling, cFos-AP1 signalling are necessary for the Yy1 to function as a pro-proliferative factor and induce the downstream signalling pathways and the molecules.

Chapter 2: Yy1 dependent gene regulatory network is essential for retina regeneration.

Previous result confirmed the importance of Yy1 during retina regeneration. Next, we moved onto dissecting out the Yy1-mediated gene regulatory network underlying retina regeneration. We started with looking at the expression level of many regeneration associated genes (RAGs) in the *yy1a* and *yy1b* combined knockdown condition and found that indeed they were getting regulated. Some of them were getting upregulated while some got down regulated. Interestingly, the mRNA levels of *ascl1a* were getting upregulated while the protein levels were going down. On the other hand, another important regeneration associated molecule

Lin28a showed downregulation at both mRNA as well as protein levels. From the literature, we knew that a well-known differentiation associated miRNA *let-7a* is being negatively regulated by Lin28a, which in turn negatively regulates many other RAGs such as *ascl1a, lin28a, c-myc, oct4* and *sox2.* We checked its level in the *yy1a* and *yy1b* knockdown condition and found that indeed *let-7a* levels were going up. From the ChIP and luciferase assay we confirmed that both *ascl1a* and *lin28a* are directly getting regulated by Yy1. Therefore, we can conclude that knockdown of Yy1 resulted in the decrease in the Lin28a levels which in turn removed the repression on *let-7a* miRNA. Increased *let-7a* in turn stopped the translation of *ascl1a* and thus reduction in the number of proliferating cells.

From the whole retina RNA-seq analysis in the *yy1* knockdown condition, we found that there is a downregulation of Notch signalling pathway. We checked the expression of various notch signalling components and found there was indeed a decrease in the expression of *notch1a*, *notch1b*, *delta b* and *delta c* while increase in the *delta a*. On the other hand there was increase in the differentiation associated *notch3* and *dll4* in the *yy1a* and *yy1b* knockdown condition. We also checked the levels of *her4.1*, a known effector gene of the Notch signalling. We observed that levels of *her4.1* went up in the knockdown condition which again justifies the cause of decrease in the proliferation in the *yy1* knockdown. Her4.1 mediated downregulation of *lin28a* is also an explanation for the reduced proliferation. Another very important molecule, Mmp9, which is a pre requisite for the reprograming, was also downregulated. The possible reason could be the downregulation of Notch signalling, increased Her4.1 levels, which in turn downregulated *mmp9*, as *mmp9* is the direct target of Her4.1. Next we checked the levels of pSmad3, member of TGFβ signalling, and found that its levels were high in the *yy1* knockdown. This could also reason the decrease in the levels of *mmp9* in the knockdown condition, pSmad3 negatively regulates *mmp9* by directly binding onto the TIE elements on *mmp9* promoter.

As discussed previously, Yy1 regulates myriad genes, both positively as well as negatively. Its activity is affected by the post translational modifications it undergoes. The central region contains a glycine-lysine rich region. HATs can acetylate the lysine residues for the full repressive activity of Yy1 while Hdacs can deacetylate these residues. We found that on overexpressing the *yy1a* and *yy1b* mRNA with neutral mutation and the acetylated mimetic mutation, there was a decrease in the proliferation in contrast to the wild type unmutated mRNA, indicating that timely acetylation as well as deacetylation is necessary for Yy1 to fulfil its repressive as well as activation role.

In the whole retina RNA-seq analysis, we also found the down-regulation of BMP signalling components in the *yy1a* and *yy1b* knockdown condition. BMP signalling has been reported to be a developmentally important gene. We found the decrease in the proliferation upon pharmacological inhibition of BMP signalling. We checked the level of well-known effector gene of BMP signalling, *id1* and found it to be downregulated. We also found the exclusion of *id1* from the proliferating cells.

Chapter 3: Yy1 and its interplay with the chromatin remodeler BAF complex to regulate retina regeneration.

Yy1 is known to have an important epigenetic regulatory role. It interacts with many epigenetic modifiers, such as HATs, HDACs, PRC2 family of proteins, DNMTs, MBD family proteins, which constitutes both the reader and writers of these epigenetic modification. The epigenetic modification helps in the regulation of expression of genes by modulating the chromatin structure. Other than these chromatin modifiers, one such class is the ATP dependent chromatin remodelers which utilizes ATP to either open the chromatin or condense it by sliding the nucleosome around which DNA is tightly packed. Pharmacological inhibition of BAF complex post retinal injury resulted in the decline in the number of proliferating MGPCs. We also found

that Yy1 and BAF complex do not regulate each other, instead they work synergistically to regulate the Müller glia reprograming and thus the proliferation, because the blockade of BAF complex along with *yy1* knockdown had more drastic decline in the number of MGPCs as compared to the their individual blockade . Interestingly, we found that Yy1 can also regulate proliferation independent of BAF complex because overexpression of *yy1* in the BAF complex inhibited background lead to an increase in the number of MGPC as compared to BAF complex inhibited alone, albeit less than the *yy1* overexpressed condition. We dissected out that the certain RAGs, such as Ascl1a and Lin28a are upregulated in such a scenario which increase the proliferation.

Discussion

Our study identifies the novel role of a transcription factor during zebrafish retina regeneration. Yy1 has a pro-proliferative role and regulates number of regeneration associated genes such as Ascl1a, Lin28a, Sox2, Oct4 and Zic2b, both negatively and positively and thus justifying its name. Yy1 positively regulates the proliferation through Ascl1a-Lin28a-*let-7a* regulatory axis. It also upregulates Notch signalling, whose role during the regeneration has been well elucidated. Many of the Delta notch signalling components are positively affected by the Yy1 and some components such as *notch3* and *dll4*, which are associated with the differentiation are downregulated. Yy1 negatively regulate a well-known repressor and an effector gene of notch signalling, Her4.1, which is known to restrict the zone of proliferation. Her 4.1 in turn negatively affects Mmp9, a molecule which is a pre-requisite for reprograming. Therefore, indirectly or directly Yy1 positively regulates the expression of Mmp9. Yy1 also positively regulates one of the developmentally important signalling, BMP signalling, whose role during zebrafish retina regeneration was not explored. It increases the expression of one of the target genes of BMP signalling known as *id1* and also some of the R-Smads of BMP signalling such

as *smad1* and *smad8.* Acetylation and subsequent timely deacetylation of Yy1 is also an important aspect for the pro-proliferative role of Yy1 because being a multifaceted, ubiquitous transcription factor it has to work as repressor for some genes while as an activator for the other. This role of Yy1 is highly governed by the post translation modification it undergoes at the right time.

Finally, we also found that YY1 works synergistically with the BAF complex and also has an independent role to regulate the proliferation during the course of zebrafish retina regeneration.

Abbreviations

AP-1	Activator protein 1
Ascl1a	Achaete-Scute Complex-Like 1a
BrdU	5-Bromo-2'-Deoxyuridine
BAF	Brahma associated factor
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
ChIP	Chromatin Immunoprecipitation
CoIP	Co-Immunoprecipitation
CMZ	Ciliary marginal zone
CNS	Central nervous system
DAMP	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
dpi	Days post injury
Dig	Digoxigenin
EdU	5-ethynyl-20-deoxyuridine
EMT	Epithelial to mesenchymal transition
FACS	Fluorescence-Activated Cell Sorting
FL	Fluorescein
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
HAT	Histone acetyl transferase

HDAC	Histone deacetylases
HMT	Histone methyl transferases
Her4.1	Hairy related 4, tandem duplicate 1
Hey1	Hairy/enhancer-of-split related with YRPW motif protein 1
hpf	Hours post fertilization
hpi	Hours post injury
ID1	Inhibitor of differentiation 1
IL6	Interleukin-6
INL	Inner nuclear layer
Insm1a	Insulinoma-Associated 1a
INO80	INOsitol requiring 80
ISWI	Imitation switch
LINC RNA	Long intervening/intergenic noncoding RNAs
MG	Müller glia
MGPCs	Müller glia derived progenitor cells
MMP	Matrix metallopeptidase
MO	Morpholino
mpi	Minutes post injury
mTOR	Mammalian target of rapamycin
NMDA	N-methyl-D-aspartate receptor
NuRD	Nucleosome Remodeling and Deacetylase

Nog3	Noggin3
ONL	Outer nuclear layer
PAMP	Pathogen associated molecular pattern
PBS	Phosphate Buffer Saline
PCAF	Acetyltransferase p300/CBP Associated Factor
PCNA	Proliferating cell nuclear antigen
PNS	Peripheral nervous system
PRC	Polycomb repressive complex
PTEN	Phosphatase and TENsin homolog deleted on chromosome 10
RAGs	Regeneration associated genes
RGC	Retinal ganglion cells
RPE	Retinal pigmented epithelial
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Shh	Sonic hedge hog
SMAD	Suppressor of Mothers against Decapentaplegic
SWI/SNF	SWItch/Sucrose Non-Fermentable
TGFβ	Transforming growth factor beta
TNF	Tumor necrosis factor
TSA	Trichostatin A
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Section 1

Introduction and Review of Literature

1.1 Historical roots of regeneration biology

The Fascination of studying regeneration has a long and rich history dating back to Greek mythology. The term 'regeneration' has been so alluring for biologist ever since the beginning of research since it is the revivification of development in later life. In one of the tales, Prometheus, known for stealing fire from gods and giving it to humanity, was cursed that eagle would eat his liver every night and it would regenerate again, which meant that this punishment would continue for eternity. This continued for thirteen generation and ended only Hercules killed eagle. In another story of multiheaded monster Hydra who could regenerate all but one head every time it was chopped. Hercules killed hydra by chopping his head one by one and eventually crushing the final one (Goss, 2013).

An Indian physician, Sushruta, performed thew first ever skin grafting to reconstruct the nose in the 6th century BC (Champaneria, Workman, & Gupta, 2014).

In the year 1740, Abraham Trembley's discovery of regeneration in hydra created a sensation and laid the foundation of the field of regeneration biology. While working as a tutor to William Benedict's sons, Trembley came across a strange water polyp called a hydra. Hydra was discovered long back in 1704 by Antony van Leeuwenhoek, but was left unnoticed until Trembley became curious to know about the enigmatic identity of hydra. To discover whether hydra is a plant or animal, he cut hydra into two parts and noticed that each piece regenerated back the old part. This observation made him think that hydra could be a plant, but he also noticed its locomotory which convinced him that it could be an animal too. He performed several exciting experiments, such as:

1. When hydra's head was cut off along with its tentacles, it formed a new head.
2. When it was cut lengthwise into several pieces, each formed a complete hydra.
3. In one of his most exciting experiments, he turned hydra inside out and found that layers transformed themselves into the other one.

These experiments were later repeated by Réaumur and similar observations were noted. Later his friend Charles Bonnet (1941), confirmed a similar phenomenon in an earthworm also. He proposed that there could be two sources of regeneration. Either new skin forms from the 'Filament gelatineux', i.e., elongation of fibers, or it could have occurred from the pre-existing germ enclosed in a 'Button'. He found that when a worm is cut into two or more pieces, each grows anteriorly to form head and posteriorly to form the tail region. He also noticed that the newly formed head or tail region, if cut again, can regenerate and thus concluded that number of times any part can regenerate depend upon its liability to respond to any injury. He also believed that the fluids in the body contain the information of the organ needed to form at the cut site in the form of nutrients.

In the years 1782 and 1784, Lazzaro Spallanzani published two memoirs on regeneration in snail head. This created a huge sensation amongst the scientific community as was created by Trembley years ago. He found that this small animal could survive after decapitation and regenerate almost all of the lost parts such as eyes, mouth, teeth and horns. Spallanzani's discovery gave rise to debate over the theory of preformation and the theory of preexistence. The theory of preformation states that "development is the mere mechanical growth of a miniature preformed in parent organism" while the theory of preexistence states that " the parents do not produce germ of preformed parts, rather it is created by God at the beginning and is conserved in that state until the moment of its development. Charles Bonnet and Spallanzani believed that the theory of preexistence not only applies to the generation but also to regeneration as well. Today also, Spallanzani 'Prodromo di un–Opera Sopra la Riproduzioni Animal', published in the year 1768, is the foundation for the field of regeneration biology (Spatlanzani, 1768).

Spallanzani found that Salamanders can regenerate their lost tail, upper and lower jaws, part of the eye, toes, and entire legs. Regeneration is more profound in the younger stages and the

ability to regenerate declines in the adult stage. Nearly all the species of salamanders can regenerate but the smaller species are more capable of regenerating in adulthood than the larger ones. He also noticed that the rate at which the amputated organ regenerates depends on feeding habits.

In the 19th century, the concept of epimorphic regeneration was studied in great depth. In 1885, Paul Fraisse, doctor of medicine and philosophy, established the taxonomy of regenerative capacity. He found that an animal's regenerative capabilities are linked with an animal's health, age, and the season. He also expressed certain concerns related to regeneration. He believed that the regeneration process is an exception to *Omnis nucleus e Nucleo* i.e., every nucleus arises from the preexisting nucleus. He could not rule out the possibility that the formation of free cell and free nucleus at the epithelial edge exists during regeneration. Also, he believed that direct cell division or amitosis occurs during the regeneration instead of regular mitotic division.

The 20th century was viewed as the era that focused on finding the reason behind epimorphic regeneration, which paved the way for the field of regenerative medicine. During this century, people started studying embryology and development, and transplantation biology, tissue engineering, and regenerative medicine slowly began to become integral part of it. Although development biology has emerged from the regeneration biology field, during the early 20th century, experimentalist biologists inclined towards development biology and regenerative medicine became part of this field with few groups working on it.

In 1901, Thomas Hunt Morgan published his work on regeneration, distinguishing between the morphallaxis and epimorphosis. In morphallaxis, the new tissue forms from the remodeling of the pre-existing tissue, while in epimorphosis, the new tissue is formed from the cell proliferation. In his book *Regeneration,* he summarized the work on regeneration, including that of Trembley, Bonnet, Réaumur, and Spallanzani. He also studied the external factors and

internal factors that influence the regeneration capability of any animal or plant. Some of the external factors that he talked about in this book include the effect of temperature, light, feeding habits, and diet, the effect of gravity on plant growth, contact with the solid body in the external environment and chemical composition of the environment such as salt concentration and dissolved oxygen. Besides external factors, Morgan, in his book, also discussed about the internal factors that may affect regenerative ability, such as whether the organism is cut, i.e. laterally or obliquely, the number of segments that are removed and the amount of material available for the regeneration, the influence of the old part on the developing part to resemble exactly to the older one. He also emphasizes, in his book, the fact that organs that are more likely to be injured are the ones possessing the greater capacity to regenerate. On the contrary, Thomas discusses the regenerative capability of many internal organs, such as the lens, liver, kidney and salivary glands of many vertebrates, which are not often liable to injury but still have the immense potential to regenerate.

Morgan rejected Weismann's theory of preformation as the basis of regeneration. Weismann believed that certain latent cells are present in the body at different locations, carrying information in the form of determinants that are present on the chromosomes. These cells are predominantly available at those sites which are more prone to injury. On the other hand, Morgan provided evidences to prove that the new cells are formed from the preexisting cells. Weismann believed that these latent cells are different from that of the germ cells, and thus regeneration is different from that development. At the same time, Morgan was convinced that study on regeneration can be extrapolated to the developmental studies. Morgan was also not convinced with the Weismann's idea that the power of regeneration decreases phylogenetically as the organisms become more complex. According to Morgan, if Weismann's statement were true, then complex organ, such as eye would not have regenerated in many higher vertebrates like salamanders (Esposito, 2013). Morgan also rejected the Roux- Weisman hypothesis of

qualitative cell division, which asserts that cellular material distributes unequally during the cell division. Roux experimented with frog's egg in which, after the first cleavage, he killed one blastomere by pricking with the hot needle. The uninjured part developed into half of the embryo while the injured part remained connected to it but did not divide as the uninjured part. Later, this uninjured part developed by the process called as "post-generation" by Roux. He described three kinds of methods by which reorganization takes place. Firstly, the new cells are formed in the injured part by the process of cellulation. Nuclei formed from the sheared chromatin get surrounded by the protoplasm of the injured part and thus form the cells. Other way is that cells from the ends of the uninjured part migrates to the undeveloped part and starts the process of cellulation. Secondly, if the protoplasm is injured severely, cellulation does not take place instead, the part slowly changes to become like the neighboring cells and eventually becomes part of it. In the third way of reorganization, ectodermal cells soon cover the surface of the injured part. After reorganization and cellulation, cells slowly give rise to three layers and eventually develop into new half, thus making complete embryo.

Hans Adolf Eduard Driesch disagreed with Roux's mosaic theory of development, which states that cell fate is determined from the two-cell stage and that there is an unequal division of cellular material at each cell division. He firmly believed that a cell's developmental fate is not determined or fixed at such an early stage and can regulate as per the changing environmental condition.

Later Morgan deviated from his initial research of regeneration and moved on to study the chromosomal theory of heredity. His work on regeneration laid the foundation and motivated many researchers to do breakthrough research in the field of developmental biology and regeneration (T.H. Morgan, 1901).

1.2 Regeneration: An Overview and Comparison with wound healing

Regeneration is a complex phenomenon of restoring the morphology and functionality of a tissue or an organ completely, thus recreating the lost and damaged part. It is an essential phenomenon, and as stated by Richard G Goss there is no life without regeneration (Goss, 2013).In nature, there is a perfect balance and control over the limits of regeneration. Regeneration without limits would have led to immortality, which is against the law of nature, and there would have been no need for reproduction. Purpose of regeneration is not to attain immortality but to make the survival and maintenance of organisms easier by gaining an advantage to recover any injury. An organism with the ability of regeneration can either grow the whole organism when cut into pieces or may grow lost, cut or wounded organs or appendages. The regenerative ability differs across the entire animal kingdom, with lower organisms having more regenerative capability than the higher organisms such as mammals.

Sometimes, certain tissues are constantly renewed, like the epithelial lining of the skin, hair follicles, feathers, molting, intestinal lining, renewal of endometrial lining in mammalian females after menstruation, oocyte regeneration in fish and amphibians and antler regeneration. These all are the case of physiological regeneration in which lost and worn out cells are replenished in the body (Thomas Hunt Morgan, 1901). Such kind of regeneration occurs in standard condition for maintaining normal homeostasis of the body, and not in response to any injury. There is a highly controlled mechanism for the physiological regeneration, which decides the extent to which it can happens whenever needed.

Reparative regeneration, on the other hand, aims to restore the normal function and physiology of any organ or tissue either partially or entirely, drawing parallels to the development. Signal for the induction for reparative regeneration are received from insult, damage or trauma to any organ (Iismaa et al., 2018).

There are different ways in which reparative regeneration can be achieved i.e.,

1. *Epimorphosis:* In this type of regeneration, differentiated tissue undergoes dedifferentiation, proliferates, and gives rise to new tissue whenever required after an injury or trauma. Epimorphosis occurs either via the formation of blastema or without blastema formation by the process of compensatory regeneration. During blastema formation, preexisting progenitor cells are recruited to the site of injury or a particular cell type in the vicinity dedifferentiates. They then start proliferating to form a mass of undifferentiated cells and dedifferentiate to replace the lost tissue. Fin regeneration in zebrafish and limb regeneration in axolotl occurs via formation of blastema.
2. *Compensatory regeneration:* In this type of regeneration, there is no formation of blastema, instead, differentiated cells at the site of injury proliferates to replace the lost or damaged tissue. For example, the liver regenerates through a compensatory mechanism in human beings.
3. *Morphallaxis:* In this regeneration mode, remaining tissue reorganizes or rearranges itself to give rise to the lost part post injury. Regeneration without proliferation is characteristic of this type of regeneration. Regeneration in the hydra is a classic example of morphallactic regeneration. This type of regeneration occurs in animals through enlargement of preexisting cells, and there is no increment in the number of cells. Such kind of regeneration occurs in hydra, if the injury is far away from the mid gastric region. It does not involve intestinal stem cells instead, ectodermal and endodermal cells come together to help in the wound closure, followed by cellular rearrangement to culminate into fully regenerated tissue (Reddy, Gungi, & Unni, 2019).

1.2.1 Wound Healing: more favorable process in human beings

Unlike lower organism, human beings mostly favor wound healing in comparison to regeneration. In response to injury, human beings have the natural ability to heal the wound instead of regeneration, which is the more expensive process in terms of energy. If the injury is superficial, wound healing completely restores tissue functionality, but in the case of deep cuts, it often leads to scar formation and loss of functionality (Atala, Irvine, Moses, & Shaunak, 2010).

Wound healing involves three different yet overlapping phases; Inflammation, proliferation, and remodeling. Upon injury, the cascade of molecular events takes place to start the healing process. First amongst them is the hemostatic phase which aims to control the bleeding, which is done by the process of blood clotting involving platelets and various proteins in the plasma and thus leading to the formation of fibrin clot. Platelets also helps in recruiting the immune cells to the site of injury and releases many factors which acts as chemokine to attract various cells to initiate the repair process.

1. *Inflammation:* Once a blood clot is formed, inflammatory phase starts in which the dying cells release many cytokines, the necrotic cells display the damage-associated molecular patterns (DAMPs), and bacterial cells display pathogen-associated molecular patterns (PAMPs). The injury-induced macrophages remove the cell debris. Neutrophils are attracted to the injury site in response to many interleukins and TNF-alpha, which also engulfs the necrotic tissue and pathogen and releases many cytokines and growth factors. These factors help prepare for the next phase of wound healing by recruiting fibroblasts cells and epithelial cells (H. N. Wilkinson & M. J. Hardman, 2020).
2. *Proliferation:* In the proliferative phase of wound healing, myriads of cells such as keratinocytes, macrophages, fibroblast, and endothelial cells come at the site of injury

and help in the wound closure and angiogenesis. Upon injury, keratinocytes on the edges undergo epithelial-to-mesenchymal transition (EMT) in response to changed mechanical tensions, cytokines, and growth factors. The activated state of keratinocytes is maintained by many pro-inflammatory signals such as IL6 and TNFα. In such a state, keratinocytes are hyperproliferative. They adopt migratory properties and migrate to form epithelial cells, and the process is called re-epithelialization. In this process only the basal keratinocytes proliferate, not the terminally differentiated cells (Rousselle, Braye, & Dayan, 2019). Hair follicle cells start proliferating to cover the wound and fulfill the cellular need. Fibroblast cells help in the degradation of temporarily formed fibrin-rich matrix by releasing MMPs and replacing it with fibronectin, collagen and proteoglycans. During the proliferative phase, new blood vessels are formed to meet the metabolic demands for wound closure and healing. Endothelial cells re-sent at the tip of capillaries receive signals such as hypoxia-induced factors (HIFs) and cyclooxygenase-2 (COX2) along with other pro-angiogenic growth factors. The activated endothelial cells then degrade the ECM by releasing factors such as MMP2. Microvascular endothelial cells then proliferate and sprout out of the basement membrane, migrate into the wound, and fuse together to form the new tubules. Many factors such as VEGF, PDGF, TNFα, and TGFβ play crucial roles during the process of angiogenesis (J. Li, Chen, & Kirsner, 2007).

3. *Matrix remodeling*: The major player of ECM remodeling is the fibroblast cells which helps in the initial deposition of fibrin clot and later replace it with mature collagen fibers. In the uninjured state, collagen type I are present in the majority, but post injury, during the granulation step, collagen type III are found in abundance which are slowly replaced by collagen type I, which provides more tensile strength to the scar. During the entire process of wound healing, collagens are formed and degraded, and thus

MMPs are known to play a significant role which helps in cleaving the collagens during this process. As opposed to the uninjured skin dermis, in which collagens are oriented in the form of a basket, in the injured scar, they are arranged in parallel orientation (Holly N Wilkinson & Matthew J Hardman, 2020).

1.2.2 Limited regeneration capabilities of higher vertebrates including humans

The capability of regeneration decreases as the complexity of the organism increases. In primitive organisms such as hydra and planaria, asexual reproduction and regeneration were highly coincidental. Evolutionarily it is believed that molecular events associated with the regeneration would have embarked for asexual reproduction, and eventually, it has been adopted as a method to survive any injury. Notably, regeneration ability is higher in the organism having diploblastic organization than in triploblastic ones (Sanchez Alvarado, 2000). The striking difference in the regenerative potential across different phyla can be either due to the presence of stem cells or how easily any terminally differentiated cell reprograms itself and attains a pluripotent state upon any trauma or insult. Thus, the competency of certain organs or tissue to regenerate not only differs amongst different phyla, which have genetic differences but also amongst different species owing to differences in the gene regulatory mechanism and at the epigenetic level (Poss, 2010). Hydra, an invertebrate that belongs to the planariidae family, can renew itself even from the tiniest of its parts. Human beings, on the other hand, which belongs to higher vertebrate, have lost most of their regenerating capacity, and it's limited to certain tissues such as skin (Wong, Levi, Rajadas, Longaker, & Gurtner, 2012); the liver which can even regenerate from one-fourth of its part, where hepatocytes

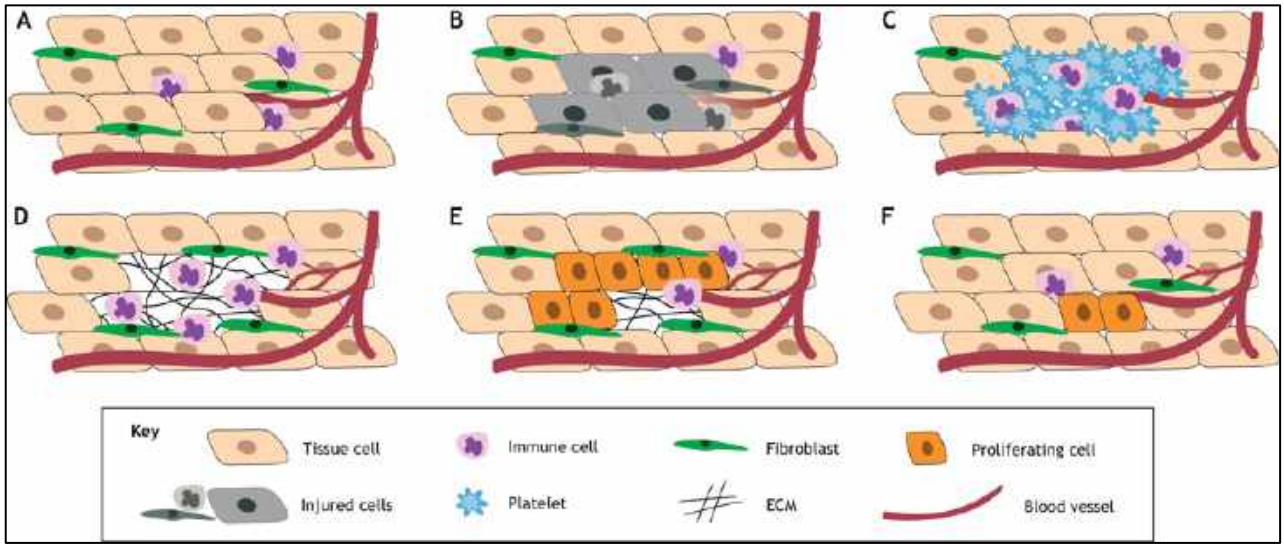


Figure 1.1 Phases of wound healing (adapted from **"**Model systems for regeneration: Zebrafish", doi:10.1242/dev.167692)

proliferate to form the lost mass (Fausto, 2000); and endometrium lining which replenishes itself after each menstrual cycle (Maruyama & Yoshimura, 2008).

There can be numerous reasons for the loss of regenerative potential in various species and also in certain organs of any given species, namely:

1. *Absence or loss of functionality of regeneration-associated genes (RAGs)*: Numerous evidences have reported the absence of some genes in the poorly regenerating species compared to the ones with high regenerative potential. Prod1, which is known to play role in limb regeneration, is exclusively associated with salamander (Garza-Garcia, Driscoll, & Brockes, 2010). Another possibility is that a gene, which is phylogenetically conserved, gets activated only after an insult or injury in highly regenerating animals. During fin regeneration in zebrafish JunB protein gets phosphorylated by JNK kinases, but this phosphorylation site is absent in mammals and *Xenopus laevis,* indicating that this phosphorylation was necessary for the functionality of this gene ((Ishida, Nakajima, Kudo, & Kawakami, 2010) (Kallunki, Deng, Hibi, & Karin, 1996). Furthermore, gene expression is also largely under the control of epigenetic codes comprising of post-translation modification on histones, chromatin remodelers, non-coding RNAs, to name a few. One reason for lost regenerative capability in higher vertebrates could be due the altered epigenetic regulation.
2. *Growth and aging:* It is a well-known fact that with ageing, the regenerative ability decreases substantially, as observed in *Xenopus laevis* (Yokoyama et al., 2000), embryonic mice which can renew digit tips (Borgens, 1982), and human children, which can grow lost finger tips (Illingworth, 1974), only if the nail bed is preserved during amputation. The reason for more regenerative capability in juveniles could be due to the factors which are needed for regeneration. These factors are readily available since the organism is still in the growth phase, while in adults, they had remained

quiescent for a long time. In contrast to this, in animals such as salamanders and zebrafish, which possess remarkable regenerative potential, these developmentally important factors are present in a substantial amount throughout their lifetime (Poss, 2010). Ageing often leads to the decline in the homeostatic renewal of hematopoietic stem cells (X. Li, X. Zeng, et al., 2020), as well injury-induced skeletal muscle regeneration (Mancinelli & Intini, 2023). There is a decline in the stem cell pool as the organism age. Number of factors are responsible for this age-related decline, such as DNA damage due to UV radiation, telomere shortening and accumulation of reactive oxygen species; alteration in the epigenetic factors; mitochondrial dysfunction, and microenvironment factors such as hormones and metabolic factors (A. S. Ahmed, Sheng, Wasnik, Baylink, & Lau, 2017).

3. *Evaluation of cost: benefit ratio of regeneration:* The need of regeneration depends on the risk associated with the tissue loss and the cost: benefit ratio of regeneration. In animals such as lizards, where autotomy and then replenishment of lost tail is associated with the escape mechanism from the predator, regeneration is essential to increase the life span of lizard. On the other hand, in animals in which, loss of a particular tissue does not bring any risk to life but only affects their physiological function such as locomotion, feeding, and reproduction. Typically, regeneration is an energetically expensive process and takes energy from body's normal functions such as growth and development. In complex organism such as humans, this process puts an extra burden as the regeneration mechanism involves more complex pathways and immune responses.

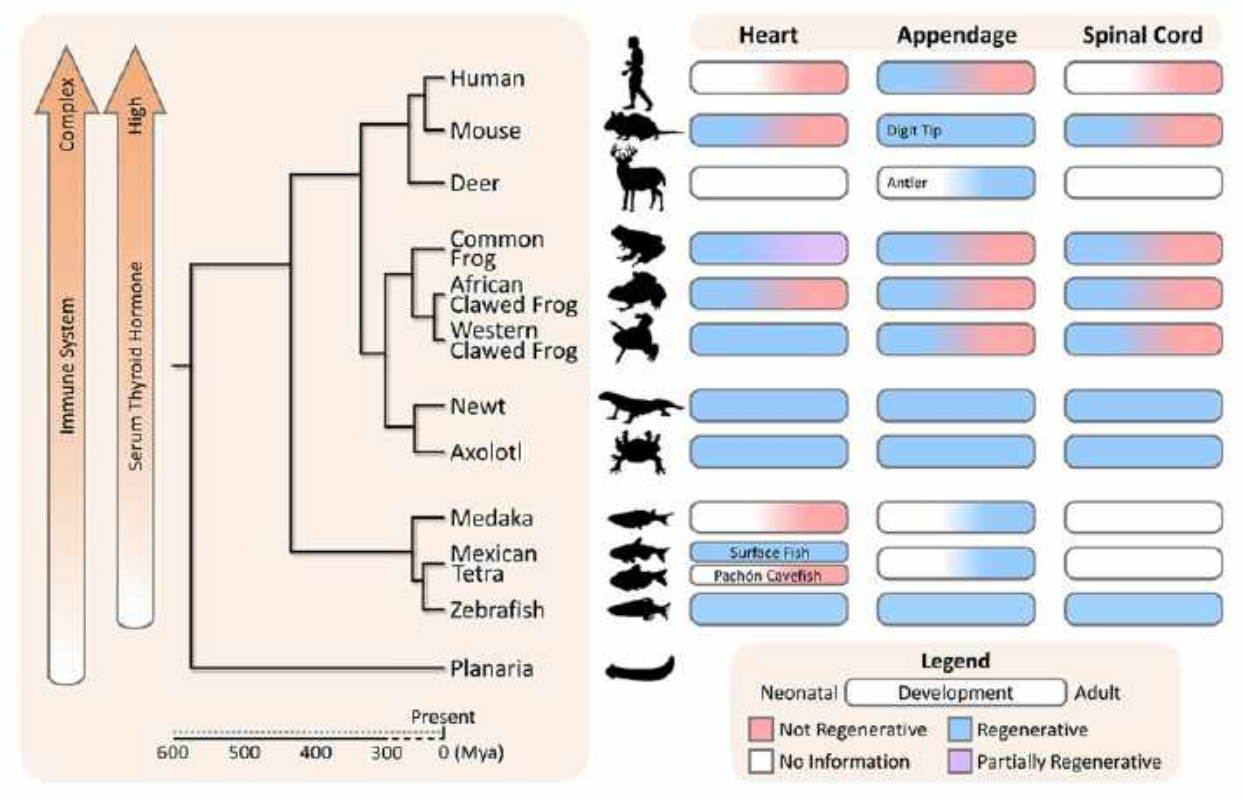


Figure 1.2 Loss in the regenerative capability across different species during evolution (adapted from "Genetic, epigenetic, and post-transcriptional basis of divergent tissue regenerative capacities among vertebrates", doi: 10.1002/ggn2.10042)

1.3 Regenerative potential of the Central Nervous System (CNS) versus Peripheral nervous system (PNS)

The nervous system is the most complex system of the body, comprising neurons and glial cells, which coordinate with each other to control the behavior and all the higher-order functions of the body. Since the time Santiago Ramón y Cajal (1852- 1934) pioneered the study of the brain and the neurons and laid the foundation of neuroscience, the nervous system has always remained a very fascinating subject to be explored in great depth (Ramón y Cajal, 1928).

The nervous system is classified in two parts:

1. The central nervous system, which comprises of brain and the spinal cord
2. The peripheral nervous system contains nerves that branches off from the spinal cord and carry signals to the entire body.

The primary cells of the nervous system are the neuron or the nerve cell. A neuron has a unique structure having dendrites that takes information from the other neuron in the form of a neurotransmitter, a cell body, and the axon, which carries the information as an electrical signal and transmits to the other neuron in the form of a chemical signal. Nervous system also contains non-neuronal cells called glial cells, which give structural support to the neurons, form the myelin sheath for the fast transfer of the signals, and helps in repairing wear and tear of neurons. Bundles of axons, which makes the nerves, helps in communication throughout the body.

Numerous evidences have shown that PNS has the remarkable property of regenerating damaged neurons post injury, as compared to CNS across all the phyla. Schwann cell, which covers the axons in the PNS, are absent in the CNS. Schwann cells, upon injury, change their role of producing the myelin sheath and transdifferentiate to adopt the function of clearing the debris. Studies show that such a reprograming event is controlled by genes such as *c-jun* (Jessen & Mirsky, 2016). Animals such as lizards and salamanders have a striking ability to restore

even complete movement ability post limb amputation (Varadarajan, Hunyara, Hamilton, Kolodkin, & Huberman, 2022). Mammals also have the strength to regenerate axons in PNS and restore the functional abilities in young populations more efficiently than the adults. There could be numerous factors responsible for this disparity. In the adult population, clearing of the debris is not that efficient as it is in the young mammals, which could reason the slow regeneration in adult population (Kang & Lichtman, 2013). Also, the movement of trophic factors responsible for healing and regeneration is slower in adults. Another reason for the non-successful regeneration is that the insult or lesion to the CNS often leads to scar formation, and called as dystrophic endbulbs, due to the deposition of ECM substance such as chondroitin sulphate proteoglycans (Silver & Miller, 2004). The presence of the inhibitory factor such as Semaphorin A, Ephrin b2, Slit proteins (Silver & Miller, 2004), NogoA, myelin-associated glycoproteins, and neuronal cyclic AMPs (Hoffman, 2010) impedes the growth of axons in the central nervous system. Seminal work done by Aguayo and colleagues found that CNS neurons, which generally do not regenerate, if grafted in the permissive environment of PNS, could grow their axons for long distances (Richardson, McGuinness, & Aguayo, 1980) (Richardson, Issa, & Aguayo, 1984).

The Spinal cord and retina are the two most accessible parts of the central nervous system and thus serves as the most valuable models for finding the cues for central nervous system regeneration. The brain and the spinal cord exchange information to and fro via a bunch of neurons. Sensory signals come to the spinal cord via dorsal root ganglia neurons, and brain sends the signal to the body via motor neurons. In the literature, various reports identify different factors and signaling molecules responsible for the axon regeneration after spinal cord injury. In one seminal work, the deletion of *Pten* led to the regeneration of corticospinal neurons post-injury (K. Liu et al., 2010). Co-deletion of *Pten* and *Socs3* (a negative regulator of JAK-STAT3 signaling) led to the sprouting of uninjured axon to innervate denervated spinal

neurons (D. Jin et al., 2015). Inhibition of Hdac1 (Finelli, Wong, & Zou, 2013) and overexpression of *Pcaf* (Puttagunta et al., 2014) help promote dorsal column sensory axon regeneration post-spinal cord injury. Numerous strategies have been suggested for complete functional restoration after spinal cord injury, such as promoting sprouting in the remaining uninjured neuron; transplantation of fibroblast, oligodendrocytes progenitor cells, mesenchymal stem cells, and neuronal stem cell to promote axonal growth by providing a supportive environment, trophic factors, and immune system modulation; and finally the growth of the neuron beyond the injury by creating a conducive environment for axonal growth even in adults (Zheng & Tuszynski, 2023).

Glaucoma is a neurodegenerative disease that leads to irreparable damage to the retinal ganglion cells (RGC) and optic nerve because RGC are unable to repair its damaged axon like any other neuron of CNS. In the past few years, many therapeutic strategies have been explored to promote axon regeneration post-injury and re-establishing the connection with neurons. Few drugs, such as taxol (Hellal et al., 2011)and epithilone-B (Ruschel et al., 2015), have been tested to induce axon regeneration by inducing microtubule polymerization. Interestingly, RGC is composed of different types of ganglionic cells, and different subtypes responds differently to injury as well as various therapies. The intravitreal injection of Sox11 by AAV in the mouse retina promoted the axon regeneration of non-α RGCs in contrast to α RGCs. When mouse RGCs are subjected to axotomy, levels of PTEN decreases dramatically in the αRGCs due to activation of mTOR pathway; and expression of osteopontin and IGF-1 (Duan et al., 2015).

Moreover, the upregulation of regeneration associated genes (RAGs) during CNS axon regeneration is not as much as it is in PNS regeneration, which may account for the limited regeneration ability of CNS in mammals.

1.4 Retina regeneration overview

Visual impairment is one of the leading and fastest-growing medical problem worldwide. At least 2.2 billion people suffer from vision-related problem globally, according to Worldwide Organization. In at least 1 billion people, the problem of visual impairment could have been prevented by adopting healthy lifestyle. The major problems which lead to blindness or visual impairment include:

1. *Age related macular degeneration (AMD):* In this condition there is a loss of central vision due to damaged macula, which is the central region of the retina and mainly concerned with straight, sharp vision. It is further classified into dry AMD and wet AMD. Hall mark for AMD is the formation of drusen, which is the deposition of an extracellular matrix consisting mainly of proteins, lipids, small double RNAs, and lipofuscin below retinal pigmented epithelium (RPE) (Crabb et al., 2002). The primary cause of AMD is the genetic mutation in genes like Complement factor H, C2, C3 and TLRs. They all are related to the immune system of the body (Z. B. Jin et al., 2019).
2. *Cataract:* Cataract is another age-related condition in which the patient experiences blur or hazy vision, reduced intensity of colors, difficulty seeing at night, and change in the refractive index of the eye lens. A mature eye lens comprises 90% of crystallin proteins. These α, β and $\boldsymbol{\gamma}$, when arranged in an orderly fashion, are soluble and give the lens a transparent appearance. As the age progresses, these protein starts coagulating due to exposure to heavy metals or UV, diabetes and genetic predisposition, and making the lens opaque (Moreau & King, 2012).
3. *Diabetic retinopathy*: In diabetic people, high blood sugar may cause tiny blood vessels in the retina to rupture and bleed. Over a period of time, scar forms and new blood vessels start to form, which is called proliferative diabetic retinopathy. The fluid

accumulation in the eye leads to blurred vision, floaters formation, difficulty seeing at night and eventually total loss of vision (Z. Yang, Tan, Shao, Wong, & Li, 2022).

4. *Glaucoma:* Glaucoma is the optic neuropathy in which retinal ganglionic cells are damaged and thus leads optic nerve degeneration. It happens when the balance between the secretion of aqueous humor and its drainage is disturbed due to a blockade called as open-angle glaucoma or closed-angle glaucoma. This can cause pressure on the back side of the eye and, thus affect retinal ganglion cells and disturbing the transport of trophic factors from the brain stem to the ganglion cells (Weinreb, Aung, & Medeiros, 2014).
5. *Retinitis pigmentosa:* It is the rare genetic condition (autosomal recessive, X linked) in which the retina gets damaged subsequently causing vision loss. Children born with this initially have trouble going in the dark and eventually lose their side vision (Hamel, 2006).
6. *Uncorrected refractive error*

Many eye-related condition can be prevented, such as eye infection, injury, trauma, poor nutrition (vitamin A and protein deficiency), certain wrong medication, cultural practices such as applying kohl. Treatments are available for few visions related problems such as discomfort, painful inflammation, and refractive index errors can be resolved by using spectacles and surgical treatment of cataract.

Visual impairment, which is caused due to death of retinal cells, is generally irreparable in the case of higher vertebrates, including human beings. These include diabetic retinopathy, AMD, glaucoma, and retinitis pigmentosa.

1.4.1 Neuroanatomy of retina

The retina is the innermost layer of the eye that lies in the posterior portion of the eyeball and extends up to ora Serrata, the junction between the retina and ciliary body, in the anterior region of the eye. It is the most accessible part of the central nervous system (CNS), derived from the diencephalon, that can be viewed and studied easily. Compared to the CNS, the retina consists of a smaller number of neurons with distinctive stereotypical positions and morphologies. All the cells are arranged into distinct layers, and the dendritic field size is small. Also, the retina become isolated from the CNS early during development, making it an excellent model to study the structure and function of CNS. The retina helps process the light signals into a 3D image construction (Mahabadi & Al Khalili, 2023).

Retina consists of three primary retinal cell layers, namely:

1. Ganglionic cell layer
2. Inner nuclear layer
3. Outer nuclear layer

These three layers contain mainly six neuronal and one major glial cell type, namely:

1. Rods: Rod cells make up almost 95% of the photoreceptors in humans. Rods are sensitive to low light intensity and can even detect a single photon. They help in creating the black and white vision. Rods are absent in the central fovea region and have the highest density in the marginal area of the retina. Although their sensitivity is high, signal transduction speed is low compared to cones. Photon signals from multiple rods converge into a single retinal ganglion. They use glutamate as the neurotransmitter and synapse onto second-order glutamatergic bipolar neurons in the outer plexiform layer (Lamb, 2016) (Hoon, Okawa, Della Santina, & Wong, 2014).
2. Cones: Cones are present in fewer number than the other photoreceptor neuron, rods cells. Cones are less sensitive to light but detect lights of different wavelengths i.e., red,

green, and blue, and are more concentrated in the central region called macula, which also includes the fovea. The fovea consists of red and green cone photoreceptors in the ratio of 2:1, while blue cone cells are present in the periphery of macula (Lamb, 2016) (Hoon et al., 2014).

3. Bipolar cells: Bipolar cells help in the signal transduction from the photoreceptors to the ganglionic cell. They can be divided into two major classes, i.e., rods bipolar cells and cones bipolar cells. They are further subdivided into ON bipolar (depolarized in response to light) and OFF bipolar (hyperpolarized in the absent of light). Rod bipolars are always ON while CONEs can be either ON or OFF because cones develop photopic vision, which can differentiate into colors, provide fine details, and can even sense movement (Euler, Haverkamp, Schubert, & Baden, 2014) (Hoon et al., 2014).
4. Horizontal cells: Horizontal cells are primarily present in the inner nuclear layer towards the outer side, and provide GABAnergic inhibitory signals to the bipolar cells. They help in enhancing the contrast and differentiating between the two points. They surround the bipolar cells making more contact with the dendrites of ON cone bipolar cells than OFF cone bipolar cells (Deniz et al., 2011).
5. Amacrine cells: They help detect various shades and light movement in a particular direction. They have both inhibitory and excitatory functions and release GABA or glycine neurotransmitters. They transmit signals from the bipolar cells to the ganglion cells (Masland, 2012).
6. Ganglion cells: Retinal ganglion cells are the neurons that bridges the retina and the brain. It takes visual inputs from the bipolar cells and the amacrine cells and transmits them to the visual center of the brain through the optic nerve (axons of ganglion cells together forms the optic nerve) (Hoon et al., 2014).

7. Müller glia cells: They are major glial cells of the retina and help maintain the normal homeostasis of the retina. They span all the retinal layer, and their processes make contact with all the neurons, blood vessels and vitreous to exchange ions, water, nutrients and waste products. In response to any threat to the retina, reactive Müller glia become neuroprotective and helps in the survival of other neurons. They also act as an optical fibre to guide light from the outside to the photoreceptor cells (Reichenbach & Bringmann, 2013).

The outer nuclear layer is separated from the inner nuclear layer by the *outer plexiform layer*, and the inner nuclear layer from the ganglionic cell layer by *inner plexiform layer*. At the outer plexiform layer visual signal splits into two channel information, one for detecting lighter objects and one for detecting darker objects from the background. Also, a new pathway is created to create a contrast for simultaneous visualization of any object. In the inner plexiform layer, connection forms between the axons of bipolar cells and dendrites of the ganglion cell layer. The synapses forms between the amacrine processes and bipolar axon; and between the amacrine processes and the ganglion cell bodies or dendrites. This layer helps detect the motion and changes in the brightness, contrast, and hue (Remington, 2012).

Although there is a structural and functional similarity in the retina across various species, but there is different specialization in the circuit. There is a difference in the composition of photoreceptors. For instance, a mouse has two kinds of cones i.e., that express opsin sensitive to short wavelength and the one which expresses both is sensitive to short and middle wavelength. On the other hand, the zebrafish retina has four types of cones with sensitivity to short (blue), middle (green), long(red), and the one sensitive to UV light. There is only one horizontal cell type in the mouse while there are four types in the case of zebrafish.

Arrangement of cone photoreceptors is different in different organism. In mouse, they are arranged in quasi-regualr mosaic while in zebrafish they are neatly arranged in a row, forming a crystalline mosaic.

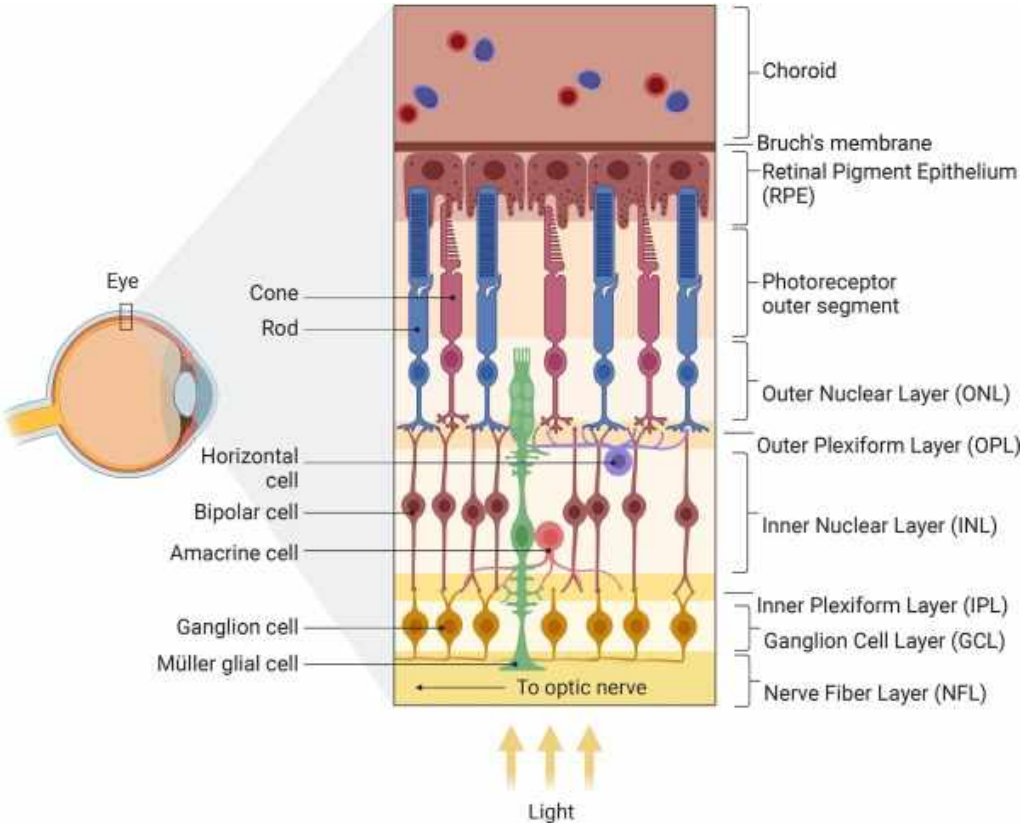


Figure 1.3 Neuroanatomy of the retina (adapted from "Neuropsychological and Neurophysiological Mechanisms behind Flickering Light Stimulus Processing", doi:10.3390/biology11121720)

1.4.2 Model systems for studying retina regeneration

Regenerative ability varies greatly amongst the vertebrates, with lower vertebrates possessing high regenerative capacity compared to mammals. In order to solve the mystery of regeneration in the higher vertebrates such as human, cold-blooded organisms such as newts, salamander and fishes serves as an excellent model organism. These classical organisms provide us with the cues that are being lost during evolution and with the increase organism's complexity. Hydra, zebrafish, salamander, axolotl, and embryonic chick are a few of the organism widely used to study regeneration of various organs. These organisms are easy to maintain and propagate in the laboratory. Amongst all, teleost fish such as zebrafish has the most remarkable property to regenerate, while it is acutely limited in birds and mammals. Besides having differences in the molecular mechanism, these organisms also differ greatly in the source of regenerating cell population such as RPE (Retinal pigmented epithelium), ciliary marginal zone (peripheral part of the retina) and the Müller glia cells (nuclei residing in the inner nuclear layer and its processes spanning all the three retinal layers) (Hamon, Roger, Yang, & Perron, 2016).

1.4.2.1 Retina Regeneration in mammals

Unlike fishes and amphibians, mammals are known not to self-repair or regenerate their damaged retina. In humans, retinal cell death due to diseases or injury typically leads to complete vision loss. Müller glia cells in the adult retinae of mammals remain quiescent but become reactive on injury and undergo reactive gliosis. To a certain extent reactive gliosis have neuroprotective function because of release of antioxidants and neurotrophic factors. In such a condition, there is a change in the morphology of the Müller glia, upregulation of certain regeneration-associated markers, dedifferentiation, nuclear migration and proliferation. Although, till this stage, the regeneration mechanism seems similar to those of Zebrafish and

amphibians, these proliferated cells do not undergo neurogenesis. Reactive gliosis leads to the formation of glial scar, which negatively affects the neuronal functions

Currently, many therapeutic approaches are employed to delay the loss of neurons in the degenerating retina, such as delivering the trophic factors having neuroprotective, anti-apoptotic, or anti-inflammatory role. Recently, in patients with Leber's congenital amaurosis and retinitis pigmentosa, the RPE65 gene was delivered to retinal pigmented epithelium using AAV- mediated gene delivery (Trapani & Auricchio, 2018). Although gene therapy is quite promising, it might not be effective in case of neuronal death due to traumatic injury or in case of diseases such as glaucoma where the gene etiology is complex and still unknown (Hamon et al., 2016). Cell replacement therapy is another area of research that involves replacing the damaged retinal cell with healthy donor cells, such as RPE, retinal progenitor cells and primary photoreceptors (Santos-Ferreira, Borsch, & Ader, 2016) (MacLaren et al., 2006).

For years, numerous research focussed on identifying the missing gaps between the Zebrafish and the mammalian gene expression pattern, which is responsible for the disparity in the regeneration potential between the two. The proneural transcription factor, Ascl1a, was identified as the key factor responsible for the regenerative potential in Zebrafish (Ramachandran, Fausett, & Goldman, 2010) (Fausett, Gumerson, & Goldman, 2008). Ascl1 is not upregulated in the adult but the young mice post retinal injury (Loffler, Schafer, Volkner, Holdt, & Karl, 2015). Forced expression of ASCL1 in adult mouse Müller glia cell culture *in-vitro* helped in their reprograming and eliciting a neurogenic potential. Also overexpression of Ascl1 helped in chromatin remodeling from the repressive to active configuration (Pollak et al., 2013). In 2017, Jorstad et al., showed that forced expression of ASCL1 along with HDAC inhibition using Trichostatin-A can help in the trans-differentiation of MG to interneurons (Jorstad et al., 2017). Also, it was seen that the promoters of pluripotent genes such as ASCL1, LIN28, SOX2, OCT4 and HB-EGF are hypomethylated, indicating that the Müller glial cell

has the stem cell potential (Salman, McClements, & MacLaren, 2021). Activation of Wnt/β-catenin signaling along with fusion with hematopoietic stem and progenitor cell can help reprogram Müller glia in the mouse retina (Sanges et al., 2013).

1.4.2.2 Retina regeneration in birds

Retina regeneration in the embryonic chick is well documented, but extremely limited in the post-hack chicken (Coulombre & Coulombre, 1965) (P. F. Hitchcock & Raymond, 1992). The source of newly generated cells in the embryonic chick is the Retinal pigmented epithelium (RPE) (P. Hitchcock, Ochocinska, Sieh, & Otteson, 2004). RPE cells dedifferentiate, lose pigment, divide, and transdifferentiates into neuronal cells. RPE cells get stimulation from the damaged retina through fibroblast growth factor (FGF) to induce trans-differentiation. Although regeneration is possible in embryonic chick, it still requires exogenous growth factors such as FGF and Insulin. Other growth factors such as Sonic hedgehog (Shh) and bone morphogenetic protein (BMP) can also induce retina regeneration from the ciliary marginal zone (Haynes, Gutierrez, Aycinena, Tsonis, & Del Rio-Tsonis, 2007) (Spence, Aycinena, & Del Rio-Tsonis, 2007). Complement component 3 stimulates retina regeneration by increasing IL-6, a pro-inflammatory cytokine that increases the regeneration-associated genes (Haynes et al., 2013). In the post-natal chick, pigmented cells at the periphery have the capability to proliferate. These cells express transcription factors such as Pax6 and Mitf and are required for trans-differentiation. However, whether the proliferating cell in the ciliary zone form retinal neurons is still unknown. Neurotoxic injury to the adult chick retina causes Müller glia to proliferate, induces expression of neurogenic markers such as Ascl1, Foxn4 and Notch, but have limited capacity to differentiate into neurons (Wilken & Reh, 2016). Post retinal damage using NMDA, Müller glia proliferates and transiently starts expressing neurofilament for 2-4 days post toxin treatment. They also express CASH-1, Pax6 and Chx10 transcription factors,

which are expressed in the embryonic chick. The newly formed cell gets distributed to the inner and outer nuclear layers. Most of them remain undifferentiated, while few attain Müller glia fate and very few get differentiated into retinal neurons (Fischer & Reh, 2001).

1.4.2.3 Retina regeneration in amphibians

Amphibians such as *Xenopus* possess remarkable regenerative ability, but are not easily manipulative like Zebrafish. Earlier it was thought that anuran amphibians possess regenerative ability only in the larval stages but not in the adult stages (F. Lombardo, 1969). Later, it was discovered that this ability was also present in the adult *Xenopus* post-surgical retina removal (Yoshii, Ueda, Okamoto, & Araki, 2007). Xenopus was developed as a model organism that mainly relied on the mechanical injury method, such as partial retina removal. Subsequently various transgenic models were also developed to conditionally ablate the retinal cells (D. C. Lee, Hamm, & Moritz, 2013). In *Xenopus,* CMZ cells are multipotent and can generate all retinal cell types (Wetts, Serbedzija, & Fraser, 1989). In *Xenopus tropicalis* entire retina can be regenerated from the CMZ (Miyake & Araki, 2014), while in *Rana pipiens,* only specific cells are regenerated from the CMZ (Reh, 1987).

In a urodele amphibian, newt, surgical removal of the retina results in the proliferation of RPE cells and subsequent replenishment of retinal neurons and RPE. On the other hand, in *Xenopus laevis* if retina is removed, retaining the retinal vascular membrane (RVM), some RPE cells detach, migrate to RVM, proliferates and forms neuroepithelium layer which give rise to all retinal neurons and glial cells (Ail & Perron, 2017).

The regenerative potential of the Müller glia was unknown in amphibians. Retinal damage using retinectomy or targeted ablation of retinal cells using a transgenic model showed that Müller glia cells are major cells to contribute to central retina regeneration. This shows the diversity in the source for retinal regeneration where RPE, CMZ and Müller glia cells

contribute to retinal regeneration. There is a striking difference between the two species of *Xenopus* in the regeneration process. In the case of *Xenopus laevis,* Müller glia response to the injury is more in the adulthood as compared to *Xenopus tropicalis* (Langhe et al., 2017).

Very little information is available for molecular mechanism involved in the retina regeneration in amphibians. It was shown that upregulation of FGF2 along with IGF-1 in the choroid post-retinal excision is essential for the trans-differentiation of RPE cells (Araki, 2007).

Therefore, the information about complete retinal regeneration in amphibians can provide missing links about the molecular mechanism in mammals.

1.4.2.4 Retina regeneration in fishes

Teleost fish (goldfish, zebrafish, flounder and African cichlid) has emerged as an excellent model to study regeneration mechanism as this process is well present in adult life as well (Braisted, Essman, & Raymond, 1994) (F Lombardo, 1968). In response to injury, the retina regenerates fully and restores complete vision. Persistent neurogenesis in fish is due to continuous growth and an increase in the size of an eye as well. In goldfish, during its lifetime, the eye grows to around 6 folds in area. To compensate for this increase, retina increase its size by hypertrophy, generation of new neurons and enlargement of the existing differentiated neurons.

The primary source of new cell in the growing retina of fish is the ciliary marginal zone (CMZ) cells. As the retina grows, the cells in the CMZ proliferates and newly formed daughter cells move and differentiates into all retinal neurons and replenish its own pool (Otteson & Hitchcock, 2003).

Earlier it was thought that post retinal excision, CMZ cells were the major contributor in tissue repair and healing. But there was no evidence that these CMZ cells migrate to repair the central retina. It was observed that in all types of injury, the bulk of proliferation is observed in the

ONL and thus, rod precursor was thought to be a likely source of regeneration (P. F. Hitchcock, Lindsey Myhr, Easter, Mangione-Smith, & Jones, 1992) (Raymond, Reifler, & Rivlin, 1988). Additionally, a radially elongated cluster of cells was also observed in the INL. Later, these cells were identified as Müller glia cells, stem cell-like cells present in the INL. In goldfish, Müller glia cells respond to the injury and incorporates BrdU which is indicative of active proliferation (Braisted et al., 1994). Majority of the initial research on retina regeneration was done in Goldfish, but now zebrafish has emerged as a more robust model organism to study regeneration mechanism due to its short life cycle, easy manipulation, high fecundity, fully sequenced genome and genetic similarity to human beings. Another teleost, medaka, shows limited regenerative potential in the retina. The Müller glia cells in medaka proliferates but do not reprogram and give rise to only photoreceptor cells (Lust & Wittbrodt, 2018).

1.4.2.4 Zebrafish: a successful model system for regeneration biology

Zebrafish (*Danio rerio*) has emerged to be the most widely used model organism to study the development and regeneration process. It was initially discovered by Charles W. Creaser as a suitable model for studying embryology (Varga, 2018). Later, George Streisinger developed the technique to create homozygous mutants through gamma rays irradiation to study nervous system abnormalities (Streisinger, Walker, Dower, Knauber, & Singer, 1981). He was regarded as the father of Zebrafish Genetics. Zebrafish are 2-5 cm long freshwater teleost fish of the cyprinidae family, native to South Asia, mainly in the Ganges and Brahmaputra River basins. Zebrafish are capable of regenerating almost all of its body parts and therefore used in regeneration studies of the spinal cord (Mokalled et al., 2016) (Cigliola, Becker, & Poss, 2020), retina (Wan & Goldman, 2016) (Powell, Cornblath, Elsaeidi, Wan, & Goldman, 2016), brain (Kizil, Kaslin, Kroehne, & Brand, 2012), heart (Poss, Wilson, & Keating, 2002), fin (Pfefferli & Jazwinska, 2015), liver (Jagtap et al., 2020) (Khaliq et al., 2018) and hair cells (lateral line)

(Jiang, Romero-Carvajal, Haug, Seidel, & Piotrowski, 2014). Zebrafish have many advantages, making it a suitable model for experimental biology, such as high fecundity, short generation time, transparent embryos to see the development as well as the defect in response to treatment, and are easily manipulative.

Compared to other model for regeneration, genome editing and transgenesis are well developed tools in case of zebrafish, which has made loss-of-function studies easier.

Some of the most widely used genetic tools in zebrafish research are:

1. *Reporter line*: Transgenic reporter lines are used for particular tissue or cell labeling and consist of promoter or enhancer driving a fluorescent proteins such GFP or mCherry (Moro et al., 2013).
2. *CreER^{T2}/loxP system*: These transgenic lines are used for lineage tracing and temporal or conditional gene overexpression studies (Langenau et al., 2005).
3. *Heat shock promoter lines*: Used for conditionally overexpressing any gene in response to temperature and does not require any chemical (Adam, Bartfai, Lele, Krone, & Orban, 2000) (Shoji & Sato-Maeda, 2008).
4. *TALEN*: Used for genome editing and creating mutant lines to study function of any particular gene (P. Huang, Xiao, Tong, Lin, & Zhang, 2016).
5. *CRISPER/Cas9*: Recently developed targeted genome editing tools using site-specific sgRNA (Jao, Wente, & Chen, 2013).

Apart from this, *in-vivo* morpholino (by Gene Tools) mediated gene knockdown strategy is widely used in the zebrafish community to study the function of any particular gene. These morpholinos are electroporated into the tissue or injected into the bloodstream and at single-cell staged embryos (Nasevicius & Ekker, 2000).

The possibility of doing live imaging at the embryonic stage and later in certain organs has made zebrafish an advantageous model organism to study developmental stages and

regeneration process. The larvae of zebrafish are transparent up to days of development. Until this stage, light or fluorescent-based microscopy can be easily done. In the adult zebrafish, *in-vivo* imaging can be difficult due to development of the pigment but regeneration of skin or fin can be done.

Zebrafish are an appropriate system for small molecule and drug screening. Effect of any drug can be easily seen in embryo as morphological defects can be visibly seen. To dissect out the molecular pathways involved in regeneration, many pharmacological inhibitors are used. Multiple small molecules can be tested to identify the ones with pro- or anti-proliferative roles (Zon & Peterson, 2005).

Different injury model have been established to study regeneration, often aimed at mimicking the natural injury or pathology of a human condition. Injury can be mechanical such as excision of tissue, stab wound or cryoinjury; chemical based; physical such as high intensity light or heat and genetic ablation of particular cell type such as NTR based ablation of cells. Sometimes, the efficiency with which the cells are replenished also depends on the type of injury. (Gemberling, Bailey, Hyde, & Poss, 2013).

Depending on the nature of the tissue, mode of injury and degree of insult, the source of newly generated cells differs.

i. The proliferation of the pre-existing cell, either through dedifferentiation and then redifferentiation (For instance, heart and retina regeneration) or cells starts proliferating without dedifferentiation (for instance, liver regeneration) to replace the lost or damaged tissue.
ii. Formation of blastema, containing a mass of undifferentiated cells, which is formed by

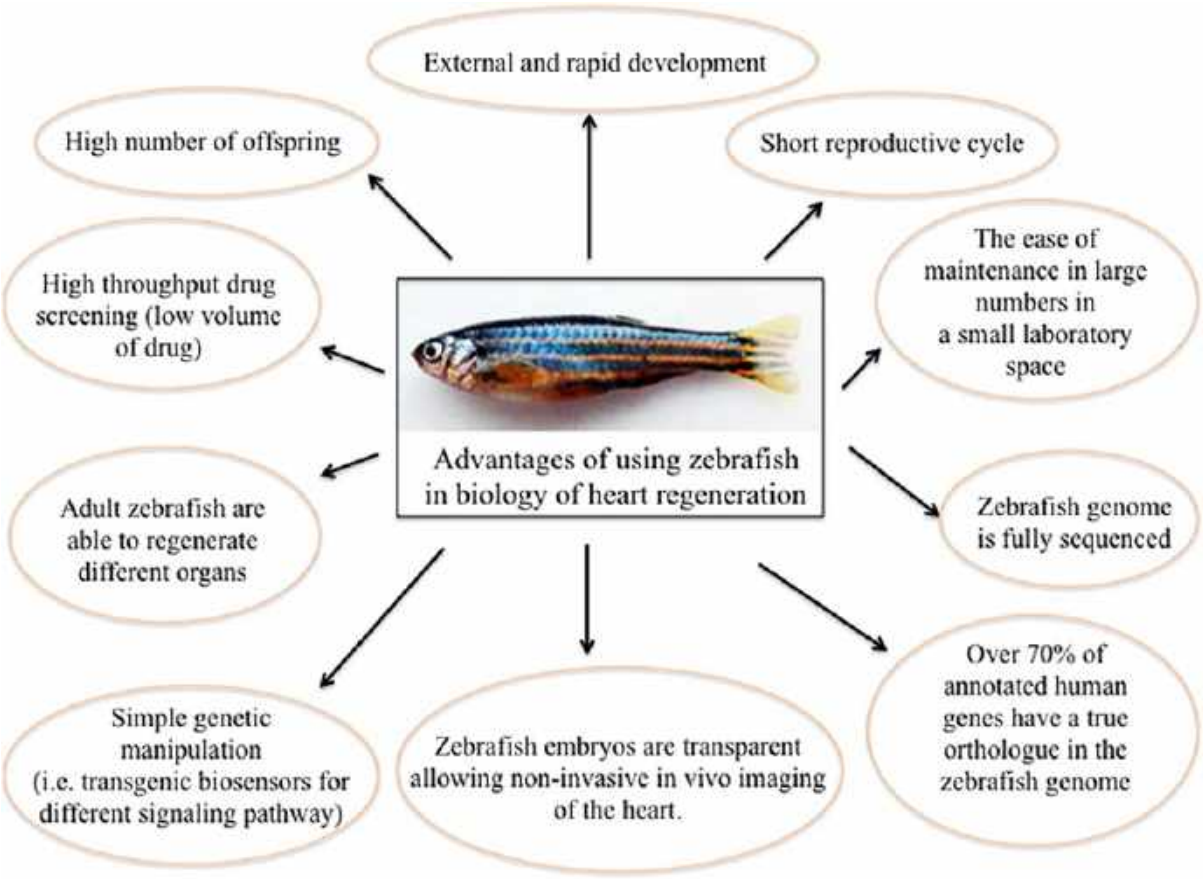


Figure 1.4 Zebrafish as a model organism to study regeneration. (Adapted from "Zebrafish as a Smart Model to Understand Regeneration After Heart Injury: How Fish Could Help Humans", doi:10.3389/fcvm.2019.00107).

a series of steps. First of all, post-amputation, the surface of the injured tissue gets covered with a layer of epithelial cells. Fibroblast cells then accumulate at the injury site and start multiplying. These multiplied fibroblast cells then rearrange within the injured tissue ultimately culminating into formation of regenerated tissue.

iii. Regeneration via trans-differentiation of pre-existing cells to form new cells. For example, in liver regeneration the epithelial cells proliferates and transdifferentiate to the hepatocytes.

In conclusion, zebrafish has proven to be the fascinating model organism to study the regeneration mechanism to solve the mystery of the missing regenerative potential of many human organs'.

1.5 Zebrafish retina regeneration

Unlike mammals, zebrafish is an excellent model system to study retina regeneration as it can regenerate fully functional retina post injury. Thus, it can act as a disease model to understand the pathophysiology of many diseases associated with the retina, such as macular degeneration, retinitis pigmentosa, glaucoma, and diabetic retinopathies. It helps us dig out the cues lacking in the mammals because 84 percent of genes associated with diseases are similar to Zebrafish. Injury to the CNS leads to irreversible damage in mammals but leads to functional restoration in the case of Zebrafish. Retina lies at the back of the eye and is the most accessible part of the central nervous system consisting of neurons and the glial cell types. Therefore, exploring the regeneration mechanism in Zebrafish will help extrapolate the knowledge to the mammalian system to provide cure for many retinal defects and CNS regeneration. Different injury paradigm has been established to mimic the natural injured conditions and study regeneration. Various injury mechanisms can damage the whole retina, particular layer or any cell type.

Types of injury methods are:

i. *Mechanical*: It includes three types of injury methods, namely needle poke injury using a 30 gauge needle, cryoinjury and surgical excision of retina using a micro knife. Mechanical injury generally damages all retinal layers.

ii. *Chemical*: Various chemicals are used that damages different retinal cell types.

- ATP or $CoCl_2$ - photoreceptors and ganglion cell layer
- NMDA (N-methyl D-aspartate)- ganglion cell layer
- Ouabain- all retinal cell layer
- 6-OHDA (6-hydoxy dopamine)- Dopaminergic neurons
- Tunicamycin – photoreceptors

iii. *Light induced*: Retina is exposed to high intensity in the range of 400-1400 nm using a tungsten halogen lamp or metal halide lamp

iv. *Genetic ablation*: Transgenic lines have cell specific promoters driving nitro-reductase enzyme which converts metronidazole into DNA cross-linking agent. This leads to the death of those cell expressing nitro reductase. This technique is used for conditional ablation of specific cell spatially or temporally.

Post-retinal injury, a cascade of molecular regenerate the damaged retina. These include: signalling from the dying cell, dedifferentiation of Müller glia (MG) to form Müller glia-derived progenitor cells (MGPCs), proliferation and migration to different retinal layers, and redifferentiation to form neuronal cells.

1.5.1 Signaling from the dying neurons

We use the needle poke injury model to study the retina regeneration in zebrafish. Retina is injured from the back of the eye using a 30 gauge needle. Dying neurons from the retinal injury release molecular signals which are sensed by the neighbouring neurons. There can be two modes of action of these signalling molecules; the positive-regulation or negative-regulation

models. As per the positive regulation model, regeneration-inducing signals are released by the dying cells, which are sensed by the cell, which will undergo transformation. In contrast to this, according to the negative-regulation model, some inhibitory factors are present in the undamaged retina. Upon injury their expression decreases, relieving the inhibition on the regeneration (Gorsuch & Hyde, 2014). Wnt signalling molecules such as *wnt4a* and *wnt8b* increases post injury and act as a positive signals from dying neurons. Expression of Wnt inhibitors, *dkk1b, dkk2, dkk3* and *dk4* decreased rapidly following injury. Therefore, the agonist and antagonist of Wnt signaling provide us with the negative and positive regulation model (Gorsuch & Hyde, 2014). Another signaling molecule, TNFα is released from the dying neurons and sensed by the Müller glia. Intravitreal injection and electroporation of morpholino against TNFα in the retina prior to light damage leads to a reduction in the number of proliferating Müller glia (Nelson et al., 2013). TNFα is known to play role in the inflammatory response. It activates the microglia and helps in the infiltration of the leucocytes.

Soon after the injury, inflammation is initiated by Damage associated molecular pattern (DAMPs) molecules. These DAMP molecule when binds to pattern recognition receptors (PRRs) leads to the activation of NF-kB, MAPKs, p38, JNK and interferon α. Activation of these molecules further leads to the activation of transcription factors such as cyclic AMP response element binding protein (CREB), AP-1 and induction of pro-inflammatory mediators such as adhesion molecules and extracellular matrix proteins. They help in recruiting the immune cells to the site of injury. Cytokines that are released by the immune cells can be classified as pro- or anti- inflammatory. Pro-inflammatory cytokines includes interleukin1β, IL6 and TNFα while anti-inflammatory cytokines are IL4, IL10, IL11, IL13 and TGFβ (Nagashima & Hitchcock, 2021). ADP, which is released by dying neurons as a consequence of ouabain treatment, act as a paracrine signal to initiate the signaling cascade (Battista, Ricatti, Pafundo, Gautier, & Faillace, 2009).

Microglia cells play a very crucial role during this process. After cell death, microglia adopt amoeboid morphology and migrates to the site of cell death. There they engulf the apoptotic cells, and also there is upregulation of inflammatory factors, such as *il-6, interleukin 1β, tnf- β, granulin1, 2and a and lectin.* Co-ablation of both rod photoreceptor and microglia lead to reduced proliferation of Müller glia and subsequent reduction in the rod cells regeneration (White et al., 2017).

1.5.2 Reprograming and proliferation of Müller Glia

Müller glia is the dominant glial cells present in the retina. Their nuclei reside in the INL, and their processes span all three retinal layers. The tangential processes are also present, which reaches to the extracellular spaces between the neurons. In the normal state, they provide structural integrity to the retina and maintain normal homeostasis. It is involved in glucose metabolism and waste removal in the retina; and helps in the formation and maintenance of the blood-retinal barrier. Müller glia helps protect the neurons by releasing the neurotrophic factors, uptake and degradation of glutamate, an excitotoxin, and release of glutathione, an antioxidant (Bringmann et al., 2006).

In a healthy retina, Müller glia stays quiescent and does not undergo reprograming. There are several mechanisms involved in maintaining this state. Neurotransmitter, GABA is shown to be play a crucial role in this process. Photoreceptors release glutamate, which is received by the horizontal cell, which in turn produces GABA. Müller glia sense GABA in the extracellular milieu and remains quiescent. Whenever there is an injury, photoreceptors no longer produce GABA and this decrease in its level is again sensed by Müller glia, which then undergoes reprograming (Rao, Didiano, & Patton, 2017). Lee et al., showed that Tgfb3 helps maintain the quiescent state of Müller glia via non-canonical Tgf β signalling (M. S. Lee, Wan, & Goldman, 2020). Another study showed that Notch3 and Delta B helps maintain the quiescent

state of Müller glia and knockdown of which, further enhances proliferation in the damaged retina (L. J. Campbell et al., 2021).

Injury to the retina brings large-scale changes in the gene expression that helps Müller glia to acquire stem cell-like characteristics. These changes in the gene expression lead to the reprograming of the Müller glial cell. Their nuclei migrate from INL to the ONL, where asymmetric division takes place and then they return back to the INL. This process is called Interkinetic Nuclear Migration (INM). This asymmetric division generates retinal progenitors, capable of giving rise to all major retinal cell types (Lahne & Hyde, 2016).

1.5.2.1. Signal transduction pathways involved during Müller glia reprograming

Many signaling and epigenetic modification have been elucidated to play a vital role during reprograming, dedifferentiation, proliferation and redifferentiation process. JAK/Stat3 signaling is restricted to MGPC in the injured retina and also directly regulates the expression of *ascl1a.* Also, cytokines such as IL6, IL11 and Leptins synergize to regulate the Jak/Stat3 signaling to initiate the proliferation of Müller glia (Zhao et al., 2014) (Nelson et al., 2012). In the avian retina, inhibition of gp130/Jak/stat signalling suppressed the Müller glia cell proliferation (Todd, Squires, Suarez, & Fischer, 2016). In response to injury, factors such as Insulin, IGF-1 and FGF signaling components are required for Müller glia proliferation. Moreover, these factors are capable of stimulating proliferation in the Müller glia even in the uninjured condition. These factors then stimulates the signaling pathways such as Mapk/Erk, PI3K, β-catenin and Jak/Stat, which are required for the reprograming and proliferation of Müller glia (Wan, Zhao, Vojtek, & Goldman, 2014).

Notch signaling is known to regulate cell fate by lateral specification, which means that one cell's fate influences other cells' fate. Notch 3, which is present in the Müller glia of

undamaged retina, is downregulated post retinal injury and the expression of delta b and dll4, which was expressed in the neighbouring neurons in the undamaged retina, also decreased (L. J. Campbell et al., 2021). Repressing Notch an overexpression of TNF α is sufficient to stimulate Müller glia to enter into the proliferative phase without retinal damage (Conner, Ackerman, Lahne, Hobgood, & Hyde, 2014). Delta-Notch signalling helps limit the zone of proliferation because Her4.1 get induced in the neighbouring cell of MGPCs (Mitra et al., 2018). Therefore, notch signalling is one of the key regulatory signalling which helps to decide whether Müller glia has to remain in quiescent state or proliferative state.

Transcription factors, Ascl1a and Lin28a have emerged as an essential player during the reprograming of Müller glia in zebrafish. Both of them are induced very early post-retinal injury. Ascl1a regulates the expression of Lin28a, which further suppresses the expression of *let-7* microRNA. The *let-7* represses the expression of regeneration-associated genes such as *myc, oct4, ascl1a and lin28a* (Ramachandran et al*., 2010).* Inhibition of Notch signalling, along with overexpression of Ascl1a and Lin28a, could stimulate Müller glia to proliferate (Elsaeidi et al., 2018). Ascl1a has been shown to inhibit the expression of Wnt signalling inhibitor, GSK3-β, and positively regulates the expression of Wnt4a (Ramachandran, Zhao, & Goldman, 2011). Role of other pluripotent transcription factors such as cMyc, Oct4 and Sox2 has also been well studied in zebrafish as well as other species. Mycb is expressed within half an hour post retinal injury and downregulation of which leads to decrease in the number of proliferating Müller glia derived progenitor cells (MGPCs). Mycb also regulates the expression of Lin28a through Ascl1a in Müller glia and acts as a repressor for Lin28a in the neighbouring cells, upon interaction with Hdac (Mitra et al., 2019). Oct4 is also induced pan-retinally post injury, with a peak of its expression at 16hpi (hours post injury). Oct4 expression is essential for the Müller glia reprograming as it regulates the expression of several regeneration associated genes such as Ascl1a, Lin28, Sox2, Zebs and many mi-RNAs (Sharma et al., 2019). In mice, failure of de-

differentiation of Müller glia has been attributed to the Oct4 methylation and silencing (Reyes-Aguirre & Lamas, 2016). Morpholino-mediated knockdown of Sox2 resulted in the decline in the number of proliferating MGPCs and reduction in the levels of *ascl1a and lin28a* but not the *stat3* (Gorsuch et al., 2017).

TGFβ signalling has a crucial role during development as well as regeneration. It has been shown to regulate cell proliferation and differentiation (Massague, 2012). TGFβ signalling can act through canonical or non-canonical pathways to regulate the expression of its downstream effector genes. In zebrafish, *tgfβ3* is induced as early as 1 hour post-injury and thus positively regulates the retina regeneration upon regulation of *junb* and *mycb* (Conedera et al., 2021). Another report showed the biphasic role of TGFβ signalling. In the proliferative phase, it regulates the expression of several regeneration-associated genes such as Ascl1a, Lin28a, Sox2 and Oct4, while helps in the cell cycle exit during the later phase of regeneration (Sharma et al., 2020). In contrast to above-mentioned report, Lee et al., reported the anti-proliferative role of TGFβ signalling involving Tgfb3 and pSmad3 (M. S. Lee et al., 2020). Tappeiner et al., also reported that the inhibition of TGFβ signalling SB431542 resulted in increased cell proliferation (Tappeiner et al., 2016). However, the contrasting role of TGFβ signalling in the different conditions is still unclear.

There is a well-established role of Shh signalling during the development and absence of which causes developmental anomalies such as cyclopia. Shh signalling has been shown to positively regulate retina regeneration and is essential for the expression of many regeneration associated genes such as *ascl1, lin28a, foxn4 and zic2b*. In turn, components of Shh signalling are under tight regulation of *let-7* miRNA, which is regulated by Lin28a (Kaur et al., 2018). Intraocular injection of recombinant SHH protein also leads to increased MGPCs formation and more differentiation into ganglion and amacrine cells (Thomas, Morgan, Dolinski, & Thummel,

2018). Purmorphamine, an activator of Shh signalling, helped in the transdifferentiation of Müller glial cells to rod like photoreceptors (Gu, Wang, Zhang, Zhang, & Zhou, 2017).

The role of Hippo signalling is well-studied in the context of regeneration of various organs in zebrafish (Riley, Feng, & Hansen, 2022). Knockdown of *yap1* or its pharmacological inhibition leads to reduced Müller glia cell proliferation (Hoang et al., 2020). Yap is involved in Müller glia reprograming through the Ascl1a-Lin28a-*let7* axis (Lourenco, Brandao, Borbinha, Gorgulho, & Jacinto, 2021). The two main components of Yap signalling, Yap and Tead, are present in the Müller glia of the adult retina and their expression is increased following photoreceptor loss (Hamon et al., 2017).

Macrophage/Microglia induced inflammation leads to the upregulation of mTOR post retinal injury. It is also essential for the Müller glia dedifferentiation and regulates many key regeneration-associated genes, cell cycle regulators and cytokines (Zhang et al., 2020). The mTor upregulates the expression of *il34* and *mmp9*, which in turn recruits the immune responsive microglia/macrophages to the site of injury during RPE regeneration (F. Lu, Leach, & Gross, 2022). Another report showed that Pten regulates retina regeneration through the Akt-mTORC2 pathway and MMP9/Notch signalling (Gupta et al., 2023).

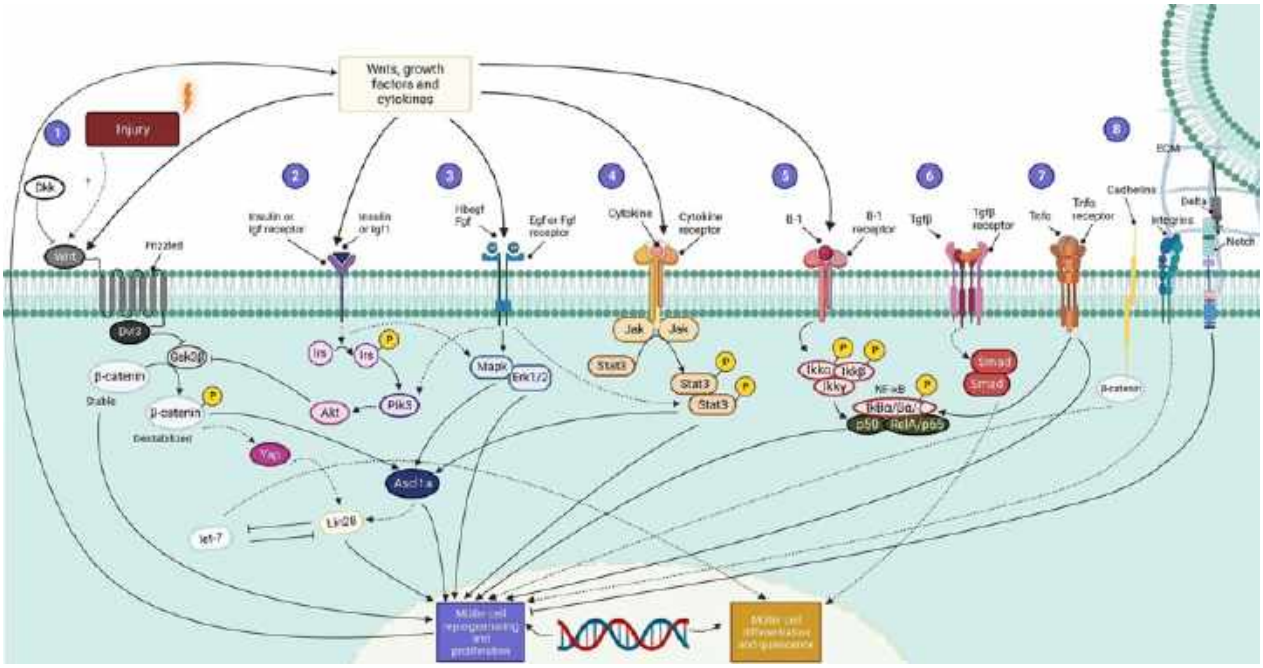


Figure 1.5 Molecules and signaling pathways involved during retina regeneration. (Adapted from "Zebrafish as a Smart Model to Understand Regeneration After Heart Injury: How Fish Could Help Humans", doi:10.3389/fcvm.2019.00107).

1.5.2.2. Role of epigenetic factors during retina regeneration

Gene expression does not merely depend upon the regulatory interplay of transcription factors, epigenetic reprograming also has a crucial role to play. It changes the gene expression without altering the DNA sequence. Epigenetic modifiers alters the chromatin structure through histone modification, DNA methylation and interaction with the non-coding RNAs. Many proteins which are involved in reading, writing and erasing these epigenetic modification have been studied so far, such as PRC2, PRC1, DNMT, TrxG proteins, MBD proteins, TET, NuRD complex and KDMs. Apart from histone modifiers and DNA methylases, ATP-dependent chromatin remodelers such as SWI/SNF, ISWI INO80 family proteins; CHD and HMG proteins regulate the accessibility of chromatin and thus the gene expression.

The two basic states of chromatin are: Euchromatin, associated with active transcription; and Heterochromatin, associated with inactive transcription. Histone modification that best characterizes euchromatin are H3K27ac, H3K4ac, H3K4me3 and H3K36me3. Histone acetylation is catalyzed by HATs and deacetylation is done by HDACs. On the other hand, heterochromatin is characterized by H3K27me3 and H3K9me2/3. Histone methylation are done by HMTases and KDMs remove methylation.

DNA is methylated at 5-methylcytosine at C-phosphate-G (CpG) islands and most of these modifications are done by DNMTs and removed by APOBEC and AIDs.

During retina regeneration, Apobec2a and Apobec2b are induced after injury and their knockdown leads to decreased formation of MGPCs. Ascl1a regulates Apobecs in Lin28a independent manner (Powell, Elsaeidi, & Goldman, 2012). Methylation status of key regeneration-associated genes remained unchanged in Müller glia and MGPCs and was found to be hypomethylated. Surprisingly, promoters of these genes were found to be hypomethylated in mice too. Methylation landscape also changed during the regeneration. During the dedifferentiation phase, demethylation was predominant, but during the proliferative phase it

shifted to *de-novo* methylation (Powell, Grant, Cornblath, & Goldman, 2013). Overexpression of ASCL1 in mouse retina caused a reduction in the repressive mark H3K27me3 and an increase in the activation mark H3K27Ac on the promoters of its target genes such as Dll3, Dll1, Hes6, and Hes5 (Pollak et al., 2013). Inhibition of Hdac1 reduces the number of proliferating MGPCs in the regenerating zebrafish retina and Hdac is differentially regulated in various phases of regeneration (Mitra et al., 2018). Dot1l, a H3K79 methyltransferase is upregulated in the proliferating MGPCs. Dot1l is the direct target of *mir-216a,* and its suppression is necessary for the Müller glia reprograming and proliferation (Kara et al., 2019). One of the benchmark study showed that upon injury the retina with NMDA, along with overexpression of ASCL1 and histone deacetylase inhibitor, led to the increased potential of Müller glia to give rise to new neurons post-retinal injury. ATAC-seq also revealed that the histone deacetylase inhibitor also increased the chromatin accessibility at essential neural genes increased (Jorstad et al., 2017).

1.5.2.3. Role of miRNAs during retina regeneration

The involvement of miRNA during retina regeneration was first shown by Ramachandran et al. in which role of *let7* miRNA was elucidated. Lin28a negatively regulates the level of *let7* miRNA which in turn suppresses the expression of many key regeneration-associated genes including Ascl1a and also Shh signalling components (Ramachandran et al., 2010) (Kaur et al., 2018).

Downregulation of miRNA-203 was found to be essential for successful retina regeneration. Pax6b is the potential target of miRNA-203 and for expression of Pax6b, miRNA-203 must be repressed (K. Rajaram, Harding, Hyde, & Patton, 2014). The miR-124-9-9* overexpression enhanced the effect of Ascl1a overexpression in reprograming the Müller glia into retinal progenitor cells (Wohl & Reh, 2016). The *miR-216a* is considered the gatekeeper miRNA as

it helps hold the Müller glia into quiescent state. Down-regulation of *miR-216* is essential for the expression of Dot1l, discussed above (Kara et al., 2019). TGFβ signalling negatively affects the expression of *miR-200a*/*miR-200b* and *miR-143*/*miR-145* during retina regeneration. The *miR-200* family is known to target the Zeb and *miR-143*/*miR-145* targets the transcription factors such as Oct4, Sox2 and Klf4 (Sharma et al., 2020).

1.6. Multi-functional transcription factor Yin-Yang1

Yin yang1 (YY1) is a ubiquitous transcription factor which is involved in embryogenesis, differentiation, proliferation and replication. Depending on various governing factors, it can activate and repress gene expression. It was discovered in 1991 as a transcription factor that binds to the P5 promoter of adeno-associated virus and represses its transcription and was named NF-E1. It repressed the transcription in the absence of E1A and activated in its presence. One study named it UCRBP, as it binds upstream of Moloney Murine Leukemia Virus (MuLV) and represses its activity. Another study, recognized it as protein δ, which binds to the downstream elements of key ribosomal proteins L30 and L32 (Shi, Lee, & Galvin, 1997).

Several studies highlight the importance of Yy1 during embryonic development and organogenesis. Mutation in the *pho* gene, which is the Drosophila counterpart of Yy1, led to the homeotic transformation due to the misexpression of homeotic genes (Girton & Jeon, 1994). Knockdown of Yy1 in Xenopus resulted in antero-posterior axial patterning defects (Kwon & Chung, 2003). Deletion of Y*y1* resulted in peri-implantation lethality in mice, while heterozygous mutation had a little delay in the development (Donohoe et al., 1999). In zebrafish, the knockdown of *yy1* can lead to an abnormal heart and brain (Shiu, Huang, Hung, Wu, & Hong, 2016). YY1 acts as a trans factor for transcriptional regulation as it can bind to many sites in the imprinted control region of many genes such as *Peg3, Gnas* and *Xist/Tsix* (J. Kim & Kim, 2008) (H. He, Ye, Perera, & Kim, 2017). Apart from this, YY1 is known to load

long non-coding RNA, *Xist,* onto X-chromosome owing to its capability to interact with RNA and DNA (Jeon & Lee, 2011). YY1 is known to regulate the process of apoptosis by interacting with Hdm2 and mediating the ubiquitination of p53 and its subsequent degradation (Sui et al., 2004).

1.6.1. Association of YY1 with cancer

The role of YY1 has been well elucidated in cancer and its level of expression is altered in different kinds of cancer. Justifying its name, it can act as a tumor suppressor or a promoter and therefore have a paradoxical role in tumorigenesis. It interacts with different factors and regulates the expression of many genes in the context of type of cancer and its interaction with its binding partner. It chooses various co-activators and repressors to regulate the expression of multiple genes and non-coding RNA. Epithelial to mesenchymal transition (EMT) is an essential process during the metastasis of cancer cells and therefore, immobile cancer cells adopt the mobile mesenchymal cell's fate and migrate to other organs. Loss of E-cadherin is the key hallmark of EMT (Theys et al., 2011) (Na, Schecterson, Mendonsa, & Gumbiner, 2020). Bonavida et al., have shown that Yy1 is the key regulator of EMT via impaired NF-κB/Snail/YY1/RKIP/PTEN pathway (Bonavida & Baritaki, 2011). It also regulates cYY1, which is highly upregulated in many cancer types, such as breast cancer, prostate cancer, gliomas, cervical cancer, liver cancer and lung cancer. Surprisingly, in the case of breast cancer, YY1 can help both in cancer suppression and progression. YY1 interacts with AP2 to activate oncogene Erbb2 and thus increases tumor invasiveness (Allouche et al., 2008). It also inhibits the expression of p27, a cell cycle inhibitor, and thus helps in tumor progression (M. Wan et al., 2012). Another group showed that FAM3C activates YY1, which in turn activates HSF expression necessary for breast cancer cell proliferation and migration through Akt1-cyclinD1 pathway or Tgf β signaling (W. Yang et al., 2019). On the other hand, Lee et al., have shown

that YY1 positively regulates BRCA1, which is a tumor suppressor. Thus, there are high levels of YY1 in the normal breast tissue and its levels decrease in the breast cancer tissue, thus relieving the suppression on BRCA1 (M. H. Lee et al., 2012). YY1 also has a repressive function on LINC00152 which in turn represses the PTEN, a tumor suppressor. Thus, YY1/LINC00152/PTEN axis plays an important role in the suppression of triple negative breast cancer (Shen, Zhong, Yu, Zhao, & Huang, 2019). Many chemotherapeutic drugs, such as mitomycin and taxol, reduces the expression of YY1. Reduced levels of YY1 lift the repression on FEN1 expression, which helps in DNA repair during the replication, thus leading to drug resistance and tumor progression (J. Wang et al., 2015).

Various groups have reported the role of YY1 in Pancreatic ductal adenocarcinoma cell (PDAC) proliferation. Mutation of KRAS genes has been linked to almost 90% of the pancreatic cancer. It has been reported that KRAS genes positively regulate the expression of YY1 through inflammatory NF- κB signalling. YY1 in turn represses miR-489, which downregulates the expression of ADAM9 and MMP7 and thus inhibiting the cancer cells metastasis (Yuan et al., 2017). Yy1 has been shown to promote PDAC proliferation by increasing the mitochondrial OXPHOS gene expression and thus enhances the nucleotide availability in the mitochondria (B. Li et al., 2022). In one of the study, Ge et al., found that levels of miR-548t-5p were less in the pancreatic cancer tissue as compared to the nearby tissue and that overexpression of miR-548t-5p significantly reduced the proliferation, migration and invasiveness of pancreatic cancer cells. They also found that Yy1 directly and positively regulates the expression of miR-548t-5p, which in turn negatively regulates the expression of CXCL11 (Ge et al., 2020). YY1 is known to directly regulate the expression of CDKN3 (cyclin-dependent kinase inhibitor 3), which forms a complex with Mdm2-p53 to inhibit the expression of p21, thus inhibiting the progression of pancreatic cancer (D. Liu et al., 2018). Overexpression of YY1 increases the levels of pro-apoptotic gene Bax, which translocates to

the mitochondria with subsequent release of cytochrome c and activation of caspase (J. J. Zhang et al., 2016).

The expression level of YY1 is upregulated in lung cancer and is mediated by signaling pathways such as NF- κB and P13K/Akt pathway. Yy1 directly binds to the promoter of LINC01089 and inhibits its transcription. HPDG gene, which is the direct target of LINC01089, also gets downregulated and relieves its repression on STAT3/AKT pathway. Thus, YY1/ LINC01089/HPDG axis promotes lung cancer progression (R. Yang, Liu, Cao, & Shi, 2022). Another study found that YY1 also positively regulates the levels of lncRNA-PVT1 (long noncoding RNA plasmacytoma variant translocation 1). Elevated levels of YY1 in the lung cancer tissue increase lncRNA MCM3AP antisense RNA 1 (MCM3AP-AS1) and accelerate lung cancer cell proliferation, metastasis, and invasion via YY1/MCM3AP-AS1/miR-340-5p/KPNA4 axis (X. Li, Yu, & Yang, 2020). Besides these, there are a few more lncRNA that are regulated by YY1 and play role in lung cancer progression, such as PLIC11 and ZFPM2-AS1. In non-small-cell lung cancer (NSCLC), USP21, a deubiquitinase, stabilizes the YY1 by preventing its degradation and promotes the progression of NSCLC, which leads to elevated levels of lncRNA SNHG16 (Xu et al., 2020).

Cervical cancer is mainly caused by the Human papilloma virus (HPV1) infection. Numerous reports suggest the pro-cancerous role of YY1 in cervical cancer cell proliferation, metastasis ,and invasion. YY1 interacts with SUZ12 and recruits a polycomb group onto the promoter of CEBPD (CCAAT/enhancer-binding protein δ), which is a tumor suppressor, and silences it (Ko, Hsu, Shen, Chang, & Wang, 2008). Wang et al., reported an increased expression of YY1, HSP16E and decreases level of E-cadherins, which is associated with tumorous growth of cervical epithelial (W. Wang et al., 2018). YY1 binds to the long control region of viral genes and controls the expression of E6 and E7. YY1, by binding to these site present in the promoter of HPV-16, interferes with the binding of AP1 proteins and represses the transcription of E6

and E7. This viral protein helps in the proliferation of host cells and in hijacking the host replication machinery. HPV infection starts in the undifferentiated epithelial cells where the expression of Yy1 is high. Expression of E6 and E7 is restricted due to the establishment of CTCF and Yy1-mediated loop formation and recruitment of the polycomb repressor group, PRC1 and PRC2. When these undifferentiated cells start differentiating, levels of YY1 decrease and thus, the repression poised by recruitment of the repressor group is lifted and thereby allowing the expression of viral E6 and E7 protein and hijacking of host-cell DNA replication machinery (Pentland et al., 2018). It has been reported by many groups that mutation in the binding sites of YY1 in the LCR region of the HPV-16 viral genome, helps in the uncontrolled expression of E6 and E7 proteins (X. P. Dong, Stubenrauch, Beyer-Finkler, & Pfister, 1994) (May et al., 1994) (Y. B. Kim et al., 2005).

It has been reported that YY1 levels increase in the case of prostate cancer as well. YY1 inhibits the expression of the XAF1 gene, a tumor suppressor, by binding onto its promoter and recruiting HDAC1. It also interacts with EZH2 and inhibits the expression of tumor suppressor miRNA-146a. HOXB13 is expressed in androgen receptor-positive normal prostate cells and is highly downregulated in case of prostate cancer. It was found that its promoter contains YY1 binding site and in the tumorous condition, YY1 binds onto its promoter, recruits HDAC4, and thus silences it (Ren et al., 2009). YY1 transcriptionally represses the hnRNPM, protein which helps inhibit of migration and invasion of cancer cells, thus promotes EMT (T. Yang et al., 2019).

Besides the above mentioned, role of YY1 has been well explored in hepatocellular carcinoma (HCC) and numerous reports confirm its role in the tumor progression in the liver. Levels of YY1 are high in the HCC cells and it binds to the promoter of HDAC1 and increases its expression, thus rendering cancerous cells insensitive to HDAC inhibitors (S. Dong et al., 2017). In another study, it was reported that high levels of YY1 recruits EZH2 to mediate the

H3K27me3 and cause the downregulation of many tumor-suppressive miRNA (Tsang et al., 2016). According to an interesting study, Twist-YY1-p300 forms a phase-separated complex at the super-enhancer of miR-9 and promotes the malignancy of the HCC cells (Meng et al., 2023). YY1 also recruits the SUZ12 of polycomb repressive complex and DNA methylation machinery on the promoter of the CCAAT/enhancer-binding protein delta (CEBPD) gene, which is a tumor suppressor and silences its expression (Ko et al., 2008).

1.6.2. Role of YY1 in stem cells and regeneration

YY1 being a versatile and ubiquitous transcription factor, performs myriads of functions controlling normal metabolic function, controlling cell cycle, epigenetic modification and nuclear reprograming. All these properties make it an essential factor during the process of regeneration.

It was found that YY1 acts as a transcription repressor for HRS/SRp40, an Arg-Ser-rich domain-containing protein, which is a delayed early gene during liver regeneration (Du, Leu, Peng, & Taub, 1998). Another study reported the role of chromatin architecture in axon regeneration in mice and overexpression of Ctcf along with Yy1 or E2f2 leads increased proliferation during axon regeneration (Avraham et al., 2022).

The Functional role of YY1 has been well studied by various groups during the muscle regeneration post injury. Chen et al., reported that YY1 knockout in satellite cells, which are the muscle stem cells, leads to complete loss of injury-induced repair. YY1 regulates satellite cell reprograming by stabilizing the Hif1a gene, which in turn helps synthesize glycolytic genes necessary for reprograming (F. Chen et al., 2019). Knockdown of YY1 supresses the skeletal muscle regeneration and Yy1, in turn, is being regulated by miR-34c. miR34-c suppresses the expression of *yy1* and thus impeding myoblast proliferation and regeneration (M. Wang et al., 2017). Lee et al., also studied the role of YY1 during the myogenic differentiation of mice.

They reported that YY1 is directly regulated by PHF20 (PHD finger protein 20) and levels of both YY1 and PHF20 decrease during myogenic differentiation indicating its role during injury-induced muscle regeneration in mice (H. Lee et al., 2020). During skeletal myogenesis, genome-wide ChIP seq analysis identified several lincRNAs (large intergenic non-coding RNAs) with the YY1 binding site. One such lincRNA identified was Yam-1, which was positively regulated by YY1. The Yam-1 gets downregulated during differentiation, reducing the expression of miR-715, thus lifting the repression from Wnt-7b and causing myoblast differentiation to myotubes (L. Lu et al., 2013). Linc-RNA YY1, which is synthesized from the promoter of YY1, interacts with YY1 through its middle domain and sequesters YY1-EZH2 complex from the promoter of the target genes and removes the repression. Therefore, Linc-RNA YY1 is a pro-myogenic factor during muscle regeneration (L. Zhou et al., 2015).

YY1 has been shown to play a critical role in the homeostasis of intestinal stem cells as well. Perekatt et al., have demonstrated that conditional deletion of *yy1* in $Lgr5^{+}$leads to increased proliferation of $Lgr5^{+}$ stem cells and movement of these cells from the crypt to the villi. Still, these cells are not sustained for a longer time and ultimately lead to apoptosis. They found that loss of *yy1* lead to the downregulation of mitochondrial complex I components genes such as HSP60 and TFAM and an increase in the levels of cell-cycle progression and RNA processing genes (Perekatt et al., 2014).

Lu et al., in their study, reported that YY1 controls the hematopoietic stem cells (HSCs) renewal and quiescence as it regulates many genes which are required for cell cycle progression, such as c-Kit, which is an important factor for regulating HSC proliferation and quiescence. This function of YY1 is independent of the REPO domain/ Polycomb group (PcG) interacting domain, which is required for early B-cell development (Z. Lu et al., 2018). YY1 is being negatively regulated by miR-29a, which then promotes the differentiation of vascular smooth muscle cells from embryonic stem cells (ESCs) (M. Jin et al., 2016). Epigenetic

modification, such as DNA methylation or histone acetylation/deacetylation, is the key mechanism to regulate the cell fate of the stem cells. Mesenchymal stem cells (MSC) can differentiate into osteocytes or osteoblast, adipocytes and can also adopt chondrogenic cell fate. Aoyama et al., found that the YY1 negatively regulates the expression of *ChM-I(Chondromudulin-1)*, which is a cartilage-specific gene, by recruiting repressive machinery such as Hdacs (Aoyama et al., 2010). On the other hand, YY1 interacts with Hdac9c as a co-activator to increase the expression of p38. Hdac9c is unique and, unlike Hdac1 and Hdac2, does not possess the catalytic domain thus, cannot deacetylate histones. It can work as co-activator upon interaction with YY1. Also, Hdac9c is highly expressed in the osteoblast cells. Therefore, YY1, upon interaction with Hdac9c, commits the MSCs to adopt the osteogenic lineage by upregulating p38, which in turn increases the activity of many osteoblast-specific transcription factors such as DLX5, RUNX2 and OSX (Y. H. Chen et al., 2018).

Interestingly, YY1 is also required for maintaining the stemness of embryonic stem cells (ESC), which are derived from the inner cell mass at the blastocyst stage of development. Three regulatory module maintains the pluripotency of ESC, namely, the Core (comprising of Oct4, Sox2 and Nanog), PRC and Myc modules. The core components occupy the promoters of majority of developmentally important transcription factors and regulate key signalling pathways such as TGFβ and signalling pathway (Boyer et al., 2005). In the PRC module, those genes are involved which are regulated by the Polycomb group members such as Suz12, Eed, Phc1, and Rnf2 (Martinez-Ruiz, Morales-Sanchez, & Pacheco-Hernandez, 2021). Myc module involves genes that are the targets of seven genes, namely Myc, Max, nMyc, Dmap1, E2F1, E2F4, and Zfx (J. Kim et al., 2010). Pietro et al., have shown that YY1 has PcG independent role in ESC. It was found that besides being repressive upon interaction with PcG group, YY1 can bind to the promoter of genes highly expressed in ESC. It binds to the promoter in co-ordination with the Myc-module-related transcription factors and controls the expression of

these highly expressed genes (Vella, Barozzi, Cuomo, Bonaldi, & Pasini, 2012). Earlier, it was shown that YY1 dynamically regulates the expression of OCT4, SOX2, BMI1 and NANOG during cancer stem cells and thus is a part of the core complex during the tumor progression and metastasis (Kaufhold, Garban, & Bonavida, 2016). Wang et al., have shown that YY1 regulates the pluripotency of the embryonic stem cells by interacting with the BAF complex and becomes part of the OCT4-mediated core pluripotency network (J. Wang et al., 2018).

As per Beagan et al., During neural lineage commitment from the pluripotent stem cells, CTCF occupancy is lost from many enhancer-gene interactions of many pluripotent genes. Instead, YY1 helps in the loop formation to promote the enhancer-promoter interaction of neuronal progenitor-specific genes. They also reported that these YY1-mediated loops are often present within the larger loops formed by constitutively present CTCF (Beagan et al., 2017).

1.6.3. Structure of YY1

YY1 is highly conserved amongst many species and belongs to GLI-Krüppel class protein which contains four C2H2 types zinc finger domains at the C-terminal for its binding to the DNA. Human YY1 cDNA codes for 414 amino acids with a predicted molecular weight of around 44kDa, while Zebrafish Yy1 gene consists of 1074 bp, corresponding to 357 amino acids.

N-terminal of human YY1 contains two acidic regions, one from amino acids (aa) 1-54 and the other from aa 80-154 and rich in glutamic acid and aspartic acid. Due to the highly acidic amino acids, N-terminal domain helps in the electrostatic interaction with proteins having positive charge (Deng, Cao, Wan, & Sui, 2010). Between the two acidic cluster, there is a stretch of histidines at aa 70-80. Albeit its role is still unclear, it is believed that when YY1 functions as a transcription factor, this histidine stretch neutralizes the acidic amino acids of the activation domain. Some studies even indicate that, histidine stretch makes YY1 an intrinsically

disordered protein, which allows it to form a phase-separated droplet inside the nucleus and make large complex for enhancer-promoter interaction consisting of co-activators such as EP300, MED4, BRD4 and RNA polymerase II (W. Wang et al., 2022). Interestingly, histidine stretch is absent in zebrafish and therefore, it still remains enigmatic whether Yy1 forms phase separated droplets in nucleus to form activation compartment, as seen in case of human where YY1 is seen as puncta inside the cells (W. Wang et al., 2022). The N-terminus of YY1 has very little secondary structure and a nearly absent tertiary structure, making this terminal less compact (Gorecki et al., 2015). The GS (Glycine/Serine) rich region present in between 154-169 helps in providing the flexibility to the protein. The GK (Glycine/Lysine) rich region present in between 170-200, and the REPO domain is one of the repressive domains of YY1. The REPO domain helps in recruiting the PcG and causing the repression of the target gene by methylating the histone3 at the Lys27 position (Wilkinson, Park, & Atchison, 2006). The REPO domain is also known as the oncogenic protein binding region due to its ability to bind to proteins such as EZH2, AKT and MDM2 (Qiao et al., 2022). The second repressive region lies at the C-terminal of YY1 (aa298-414), which consists of four C2H2-type zinc finger motifs and helps in its binding to the DNA. Each finger contains 2 cysteines which bind to the Zn ion, and out of four, Zn finger 2 and 3 are responsible for carrying out major biological functions, followed by Zn finger 1 and 4 (K. Chen et al., 2019). The co-crystal structure of YY1 with adeno-associated virus (AAV) initiator element (AAV P5) has shown that all four zinc finger motifs are required for their binding onto DNA. These motifs bind to the major groove of the DNA, recognizing the consensus binding site for YY1 (Houbaviy, Usheva, Shenk, & Burley, 1996). The first finger makes single base contact, while the other three zinc fingers make multiple contact with bases. The amino acids which make this interaction are mostly the positively charged amino acids such as lysine, arginine, and histidine.

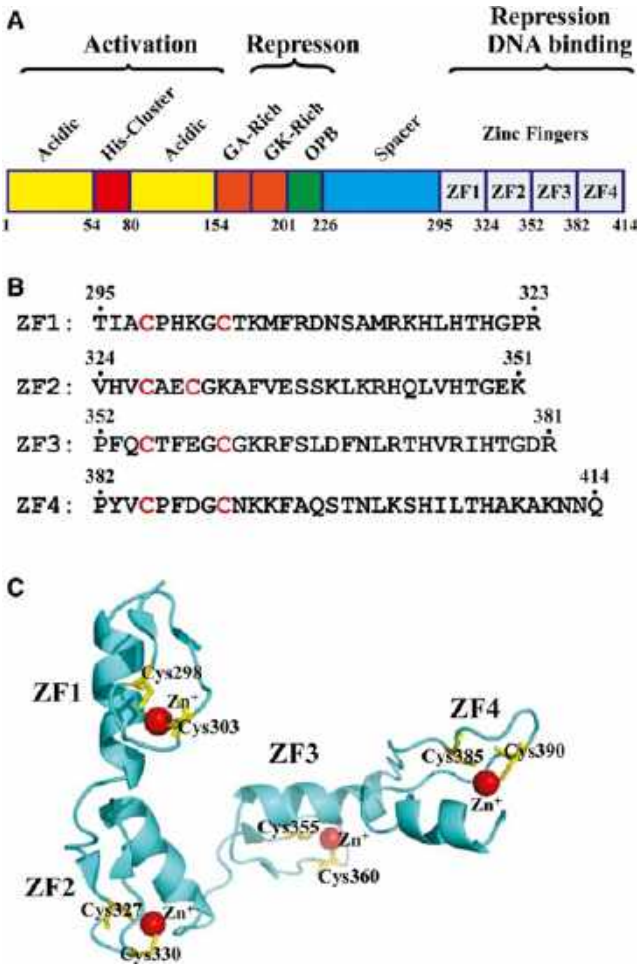


Figure 1.6 Domain structure prediction of Human YY1. (Adapted from "Functional analysis of YY1 zinc fingers through cysteine mutagenesis", doi: 10.1002/1873-3468.13431).

Earlier, it was shown that YY1 binds onto the promoter of *c fos* and bends it to make contact with the other proteins and regulate the expression of cFOS (Natesan & Gilman, 1993). YY1 recognizes the evolutionarily conserved consensus sequence 5'-(C/g/a)(G/t)(C/t/a)CATN(T/a)T/g/c)-3', which is found in most of the promoters and the enhancer, and binds onto it through its C-terminal zinc finger containing domain (Hyde-DeRuyscher, Jennings, & Shenk, 1995) (Jd Kim & Kim, 2009). YY1 consensus site is present in the regulatory region of almost 7% of vertebrates and 24% of the viral promoters.

1.6.4. Regulation of transcription by YY1

The most perplexing feature of YY1 is that it can act as a transcriptional repressor and activator. Till now many research groups have tried to resolve this mystery by collecting the evidences and dissecting out the mechanism of action of YY1 accounting for its dual name, as suggested by its name as well. The two main mechanism by which YY1 functions in a context-dependent manner to act as an activator or a repressor, includes post-translational modification of YY1 or binding with the co-factors such as co-activators or co-repressors (Verheul, van Hijfte, Perenthaler, & Barakat, 2020).

Proteins that are recruited by YY1 are the determining factors for YY1 to execute activation or inhibition function on the promoters of the target genes. It can recruits many co-activators such as E1A, p300, CBP, PCAF, YY1AP (YY1 associated factor 1) and PRMT (Protein arginine methyltransferase) and co-repressor such as HDACs, EZH2, SMAD family members and DNMTs.

It has been shown that YY1 forms a complex with p300 and HDAC3 and inhibits the expression of c-Myc. Later, it was found that the viral protein E1A relieves this repression by interacting with YY1 and dissociating the YY1-p300-HDAC3 complex (Riggs et al., 1993). Similarly, YY1 represses the expression *c-Fos,* a proto-oncogene, by forming the complex with

ATF-CREB. The protein E1A converts YY1 from being repressive to an activator of c-FOS expression (Q. Zhou & Engel, 1995). In the presence of HIF 1α (Hypoxia-inducible factor 1-alpha), YY1 activates the expression of VEGF (Vascular endothelial growth factor), which promotes angiogenesis (de Nigris et al., 2010).

YY1 can recruit co-factors with histone acetyltransferase activity, such as p300 or CBP, which helps in the opening of the chromatin for better exposure of DNA for binding (J. S. Lee et al., 1995). Lee et al., in their study, showed that physical interaction between YY1 and E1A is mediated by p300. Also, in the same study, it was hypothesized that despite the fact that although YY1 has the bi-partite activation domain in the N-terminal, it acts as an repressor. They hypothesized that upon interaction with other proteins, there is a structural change in the YY1 upon interaction with proteins such as E1A, which then unmasks the activation domain at the N-terminal (J. S. Lee et al., 1995).

YY1 has been shown to initiate transcription, as the binding site for YY1 can act as an initiator element at which it can bind to TFIIB (General transcription factor IIB) and stabilize its interaction with DNA, and then recruits RNA polymerase II at the site. Therefore, YY1, TFIIB, and RNA pol II can initiate basal transcription from the supercoiled DNA even in the absence of TATA-box binding protein (Usheva & Shenk, 1994, 1996). It has also been seen that YY1 binds to the Inr region (spanning the transcription start site) and acts as an co-activator for the transcription factor SpI which binds to the TATA motif. A physical interaction exists between the first one and a half zinc fingers of YY1 and the SpI, which raises the question of whether all the four zinc finger domains of YY1 are involved in the repression. (J. S. Lee, Galvin, & Shi, 1993). The cis-regulatory elements of the chick *tinman* homolog *Nkx2.5* contains three cardiac activating regions. The third CAR, termed as CAR3, has an adjacent binding site for SMAD1/4 and YY1, which constituting the minimal BMP response element. Lee et al., have

shown that physical interaction between SMAD1/4 and N-terminal of YY1 is required to induce CAR3 (K. H. Lee, Evans, Ruan, & Lassar, 2004).

YY1 can act as a transcriptional repressor by interfering with the transcriptional activators, either by directly binding to the DNA or without binding. Promoter of *c-Fos* contains YY1 binding site in between the cyclic AMP response element and TATA-box. So, when YY1 binds there, it interferes with the activity of CRE element and represses the transcription of *c-fos.* It can also directly interact with the CREB (Cyclic AMP response element binding protein) and mediates the transcriptional repression of *c-Fos* (Galvin & Shi, 1997) (Shi et al., 1997). Yy1 can also modulate TGF-β signalling by interacting with the SMAD7, an antagonist for TGF-β signalling as it blocks the DNA binding site for R-SMADS-SMAD4 complex (Yan et al., 2014).

There is an overlapping binding site of YY1 with mammary gland factor (MGF) on the *β-casein* promoter. It competes with the MGF from binding onto the promoter and thus causes transcriptional repression. But in the case of lactation, when the concentration of MGF is high, YY1 is replaced by MGF, thus relieving the repression on the *β-casein* promoter (Shi et al., 1997).

Epigenetics have changed the perspective of decoding information from the genome. Epigenetic processes keep a check on the expression of genes and ensure that the information encoded by the genetic codes is delivered at the right time and in the right cell. Epigenetics modification compartmentalizes the nucleus into either heterochromatin (transcriptionally silent) or euchromatin (transcriptionally active) regions. There are numerous players which are involved in the mechanism of epigenetic modifications, which can be broadly classified in three categories:

1. DNA methylation machinery involving DNMTs, MBD proteins and MeCP2.

2. Histone modifications wherein histones tails are post-translationally modified by acetylation, methylation, SUMOylation, ubiquitination and ADP-ribosylation. Many enzymes are involved in this process, such as Histone acetyltransferases (HATs), HDACs (Histone deacetylases) and Polycomb groups of proteins involving PRC1 and PRC2 complexes.
3. Positioning of nucleosomes: Packaging of DNA around nucleosomes not only compacts the DNA inside the nucleus but also regulates the transcription of any genes. Displacement of nucleosomes is essential for the chromosomes to get exposed and get accessible for the transcriptional machinery to assemble. The positioning of the nucleosome is being affected by the DNA methylation status, post-translational modifications of histones and histone variants that are incorporated in the nucleus apart from the core nucleosome subunits, which carry out specialized functions such as DNA repair, tissue-specific functions, chromosomes segregation, to name a few.

Besides, its role as a general transcription factor, YY1 can physically interact with many chromatin modifiers and recruit them to specific chromatin loci. YY1 interacts with the PcG group through its REPO domain and recruits it to the DNA and methylates histone 3 at Lysine 27, causing transcriptional repression (Atchison, Ghias, Wilkinson, Bonini, & Atchison, 2003) (Wilkinson et al., 2006). YY1 also interacts with both HDACs (Glenn, Wang, Chen, Nishimoto, & Gardner, 2009) (Sankar et al., 2008) (X. Wang et al., 2008) (Aoyama et al., 2010) as well as HATs (J. S. Lee et al., 1995), thus helping in the deacetylation and acetylation, respectively. Besides this, YY1 also recruits Histone methyl transferases (HMTs) such as, PRMT1, which methylates Histone 4 at Arg 2, 17 and 26 (Rezai-Zadeh et al., 2003).

The activity of proteins is regulated by post translational modifications as it can affect its function, subcellular localization and interaction with the co-activators and repressors. The

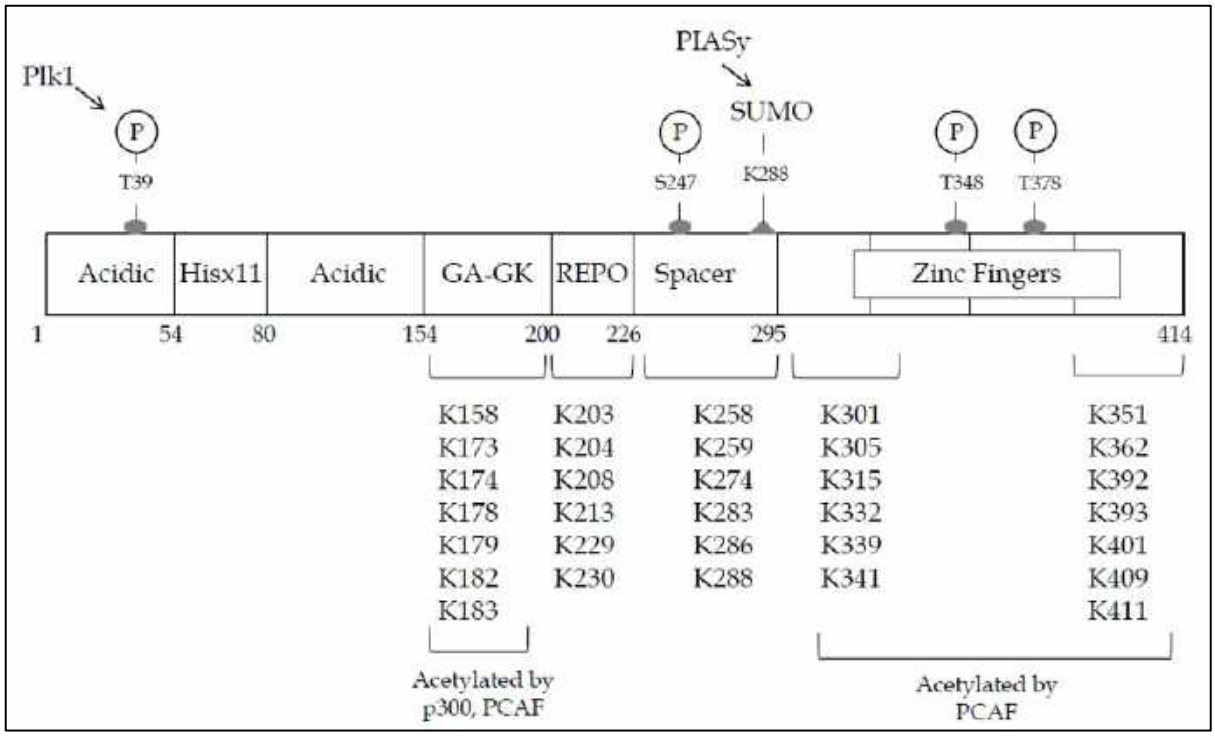


Figure 1.7 Yy1 undergoes various post translational modification by interacting with different proteins. (Adapted from "The Function of YY1 and Its Oncogenic Role in Prostate Cancer", doi: 10.5772/53091).

sequence of YY1 contains 32 lysine residues, which are the most favoured amino acid residues for the post-translational modification such as, acetylation, methylation, SUMOylation or ubiquitination. Most of the lysines are present in the middle or the C-terminal regions of human YY1 (Stovall & Sui, 2013).

YY1 interacts with p300 and PCAF and recruits them to acetylate histone, but both of them can also acetylate YY1 in the central region rich in glycine and lysine (aa 171-200), which renders YY1 as a repressor and HDACs comes to interact with it and deacetylate YY1 to relieve it from its repressor activity. Apart from this, PCAF can acetylate its C-terminal, which decreases the DNA binding ability of YY1. Although acetylation of C-terminal cannot be removed by HDACs, but it makes the interaction between YY1 and HDACs more stable (Yao, Yang, & Seto, 2001).

Yy1 gets methylated by SET7/9 at two lysine residues K173 and K411. Mutating both these residues to Arginine leads to the attenuation in the binding ability of YY1 to its consensus binding motif. These mutation also led to the slow growth of the cells as compared to the wild-type control indicating the role of methylation in the functioning of YY1 (W. J. Zhang et al., 2016).

Phosphorylation of YY1 at the threonine residues at position 348 and 378, which are present in the linker region between zinc finger 2-3 and zinc finger 3-4, decreases the DNA binding ability of YY1 and thus leads to its deactivation during mitosis (Rizkallah & Hurt, 2009). Polo-like kinase phosphorylates YY1 at threonine39 during the G2/M transition during the cell cycle (Rizkallah, Alexander, Kassardjian, Luscher, & Hurt, 2011). Casein kinase II α constitutively phosphorylates YY1 at the ser118 in the transactivation domain, preventing its cleavage by caspase7 during apoptosis (Riman et al., 2012). Yy1 has been shown to get phosphorylated at the Ser184 by Aurora B during G2/M stage when the expression of Aurora B is high (Kassardjian et al., 2012). Aurora A kinase phosphorylates at ser 365 present in zinc finger 3,

which also affects its DNA binding ability leading to its inactivation during mitosis (Alexander & Rizkallah, 2017). Wang and Hoff, showed that Src family kinases phosphorylates YY1 at different site depending on the expression of Src kinases (G. Z. Wang & Goff, 2015).

Proteins undergo degradation via the ubiquitin-proteasome degradation pathway. They get polyubiquitinated at the lysine residues and finally degraded into small peptides. The process involves three enzymes, namely, ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2 and ubiquitin ligase E3. Smurf2 is an E3 ubiquitin ligase that targets YY1 for the degradation, and therefore it has been shown to regulate the transcriptional activity of YY1. They further characterized the PPxY motif at amino acids 248–251 of YY1, which interacts with the Smurf2 (Jeong, Lee, Yum, Yeo, & Lee, 2014) (Ramkumar et al., 2013) (Fu et al., 2021). Besides ubiquitination, YY1 also undergoes SUMOylation, in which the C-terminal of SUMOs (small ubiquitin-related modifiers) form an isopeptide bond with the ε-amino group of a lysine residue. PIASy, SUMO E3 ligase, SUMOylates YY1 at the residue lysine 288 and modulates its function (Deng, Wan, & Sui, 2007).

Section 2

Materials and methods

2.1 Animal maintenance and breeding

Zebrafish is the model organism which was used in our study. Wild type and transgenic animals were kept in the controlled system with a temperature of 25-28°C and pH of 7. Along with it light-dark period of 14-10 hours was maintained to regulate the circadian rhythm. Both wild type and transgenic embryos were obtained from the natural way of breeding and maintained at the similar condition in an incubator.

Adult fish were fed with the prawn feed twice a day and both adults and embryos were fed with artemia, which are crustaceans and are cultured in the lab itself. Use of zebrafish for all the experiments were approved by the ethical committee of our institute.

2.2 Retinal Injury and dissection

Zebrafish which were taken for the experiments were nearly 6months to 2 years old. For one set of experiments, all the fish were taken from the same age group and same size to maintain the uniformity in the experiments.

In our lab, for all the experiments, we adopted mechanical based injury model. Animal was anesthetized using tricaine and once it was in a subconscious state, the retina was injured from the back of the eyeball using a 30 gauge needle . For most of the sections based experiments, 4 pokes were given in all the four quadrants and for the rest of the other experiments 6- 12 pokes were given and again kept in water for revival. Same treatment was given to both the control as well the treated fish.

As per the experimental regime and requirement, dissections were performed. For immunostaining, mRNA in situ hybridization and DNA FISH, BrdU(5mM)and EdU (10mM) pulsing was done to label the actively proliferating cell, 5 hours prior to harvest.

While doing dissections, fish were anesthetized and then the eye was pulled from the eye socket and kept in a petri plate containing solutions in which dissections were performed. For

preparing cryoprotected blocks, eyes were dissected as well as kept in 4% PFA overnight at 4°C. For preparing samples for RNA and protein, dissections were performed in 1X PBS and retinal tissues were either suspended in TRIzol and 2X Laemmli buffer, respectively, and stored at -80°C until sample preparation.

2.3 Drug delivery, Morpholino injection and electroporation

In our study following drugs were used, DETANONOate (Sigma), PFI3 (Sigma), T5224 (Sigma), K02288 (Sigma), SB431542 (Sigma), TSA (Apex bio), DAPT (Sigma) and SB3CT (Sigma). Drugs were either delivered directly into the vitreous of the eye from one of the injury spots made during the injury using a 30 gauge Hamilton syringe or fish were dipped in various concentrations of drug. While injecting drugs in the vitreous, the concentration should be 10 times more as compared to the one used for dipping.

In our study, we had used lissamine tagged morpholinos (MO) to knock down genes of interest. Morpholino for the following genes were used in this study and their sequences are given below:

yy1a MO - CCATTCTTGGCTTTCTTGCTTTCCG

yy1b MO - TCTCCCGGACGCCATCGTTAA

Three different concentrations of morpholino were used in all sets of experiments. Fish were anesthetized and injury was done as explained previously. Morpholino was injected from one of the injury spots using Hamilton syringe and then electroporated at 70V, 5 pulses of 50 milliseconds each. Electroporation aids the entry of positively charged morpholino into the retina.

2.4 Overexpression and rescue experiments

Overexpression of any protein was done by transfecting the mRNA of that particular gene into the retina with the help of Lipofectamine (Invitrogen) and aided by electroporation. The mRNA was made by *in-vitro* transcription using following protocol:

1. Gene of interest was cloned into pCS2+ vector under SP6 promoter.
2. Vector was linearized at the 3'end after the SV40 poly A signal and purified either by manual or gel extraction kit.
3. Purified linearized plasmid was then used for *in-vitro* transcription of the mRNA using the mMessage machine kit from Invitrogen using the manufacturer's protocol (mentioned below).
4. After 2 hrs, 1 µl of the reaction was checked on gel and if mRNA of required size appeared on gel, it was precipitated overnight and next day dissolved in Nuclease free water (NFW) and stored in -80°C until further use.

For overexpression experiments, a mixture was prepared that was injected into the vitreous of the eye. The mixture comprises (a) Equal volume of mRNA in a particular concentration and 2X HBSS (b) Equal volume of 2X HBSS and Lipofectamine. Both of them were allowed to rest for 5 minutes at RT and then mixed together dropwise and allowed to stand at RT for another 30 minutes. After 30 minutes, mixture was injected from one of the injury spot and electroporated, as described previously.

For the rescue experiment plasmid containing coding sequences of Yy1a and Yy1b were mutated so that MO does not bind to it. The mRNA with the silent mutations were then made using the protocol mentioned above. Mixture for the transfection was also prepared in the same way and MO was mixed with it.

2.5 Cryopreservation of the eye and cryo-sectioning

The injured eye or the experimental eye with lens removed was stored in the 4% PFA overnight in 4 degrees and after the incubation, 4% PFA was removed and tissue was cryoprotected with a series of sucrose washes in the following order for the period of 45 minutes each

1. 1ml of 5% sucrose
2. 400µl 5%sucrose and 800µl 20% sucrose
3. 500µl 5%sucrose and 500µl 20% sucrose
4. 400µl 5%sucrose and 800µl 20% sucrose
5. 1ml of 20% sucrose

This series of sucrose washes was followed by addition of 500 µl of OCT in the last 20% sucrose and rotating it for another 30 minutes. The eyes were then embedded in a mould containing 3/4th OCT and immediately kept in -80°C for freezing. Once frozen, thin sections of 8-12uM are taken on Fisherbrand Superfrost Plus Microscope slides in Leica cryostat machine CM3050S with optimum chamber temperature of -20°C. Slides were dried overnight in the dark and then stored in -20°C until further use for immunostaining and mRNA in situ hybridization.

2.6 Immunostaining on retinal section

1. Slides were taken from -20°C or directly after overnight drying and used for immunostaining.
2. Slides were washed 3X for 10 minutes each with 1X PBS. Meanwhile 1N HCl was prepared from 12N HCl and prewarmed in 37 for at least 20 minutes.
3. Epitope retrieval step- This step was done only for protein for which epitope retrieval was required. If not, this step was skipped and proceeded to step5. There are 2 different methods for epitope retrieval which are used in our study and described below

- HCl treatment- Slides were incubated for 20 minutes in prewarmed 2N HCl in 37. 2N HCl prepared from 12N HCl by adding 8ml 12N HCl in 42ml water.
- Boiling in 10mM Sodium citrate- Slides were boiled in 10mM Sodium Citrate (pH6.5) with 0.1% TritonX-100 for 20 minutes.

4. After epitope retrieval neutralization was done by 2 washes of 0.1M Sodium citrate for 10 minutes each.
5. Subsequently, slides were blocked with 6%BSA-PBST for 45 minutes to 2 hours, depending on the nature of antibody.
6. Slides were washed once with 1X PBST for 5 minutes and then overlaid with 500µl of primary antibody made in 1% BSA-PBST and kept in 4 overnight in a humidified chamber.
7. Next day, after an overnight incubation with primary antibody, slides were given 3 washes with 1X PBST, 10 minutes each.
8. Slides were then overlaid with Alexa fluor labelled secondary antibody and incubated for 90 minutes at RT or overnight at 4°C.
9. After incubation with secondary antibody, slides were washed 3 times with 1X PBST.
10. Signals were checked after the last wash and if successful, slides were washed 2 times with water and dried for 20 minutes.
11. Slides were cover-slipped with the help of DABCO and dried overnight at RT in the dark.
12. Slides were imaged with Nikon A1 Confocal Imaging System and cells were counted manually.

Special note:

1. In case sections contain morpholino, slides have to be fixed with 4% PFA for 15 min before proceeding with the epitope retrieval.

2. In some experiments where co-localization is seen between two proteins, reference nuclear staining is done with DAPI or Hoechst and washed 3 times with 1X PBS and then proceed with the water wash and the cover slipping.
3. In cases where proliferating cells are labelled with EdU, staining for the same has to be done which is explained in detail in the next section.

2.6.1 Edu staining after Immunostaining

1. Signals were fixed after immunostaining with ice cold 4% PFA for 15 minutes prior to EdU staining.
2. Slides were again blocked with 3% BSA-PBST for 30 minutes.
3. Blocking solution was removed and slides were overlaid with 100µl of EdU solution and slides were cover-slipped.
4. After 30 minutes of incubation in dark, coverslips were removed by overlaying 3% BSA-PBST in between the slide and the coverslip.
5. Slides were again washed with 3% BSA-PBST and signal was checked.
6. Slides were washed with water twice, dried and cover-slipped.

2.6.2 DAPI staining

1. Slides were overlaid with 0.5ug/ml (prepared from 10mg/ml DAPI solution) DAPI solution and kept for 1minute in dark.
2. Solution was removed and slides were washed thrice with 1X PBS solution.

2.7 mRNA In situ hybridization protocol

1. Slides were rehydrated in sequential ethanol washes for 1 minute each in the following order:

100% ethanol

100% ethanol
95% ethanol
70% ethanol
50% ethanol
2X SSC

2. Slides were dipped in a prewarmed proteinase K buffer for 6minutes (for 12µM sections) at 37°C (160 µl of Proteinase K (10mg/ml) was added just before putting slides into it).
3. Slides were then rinsed in DEPC water for 1 minute.
4. Slides were rinsed in 0.1M TEA solution (pH8.5) for 3 minutes at RT.
5. After this, slides were kept in a coplin jar containing 0.1M TEA with 130µl of Acetic anhydride for 10 minutes.
6. Slides were again rinsed in 2X SSC for 1 minute, followed by dehydration with series of alcohol in following order

   50% ethanol
   70% ethanol
   95% ethanol
   100% ethanol
   100% ethanol
7. Slides were air dried for 1hour in a clean isolated space.
8. Meanwhile, RNA probe was kept for thawing on ice and hybridization solution was kept for pre warming at 56°C.
9. In an MCT containing prewarmed 300µl of hybridisation solution, 300-500ng of RNA probe was added and boiled for 10 minutes with gentle flicking in between.
10. Once the slides were air dried properly, hybridization mix was poured onto the slide dropwise, covered with the hybrislip and kept in a humidified chamber made with 5x SSC/ 50% Formamide.

11. The humidified chamber containing the slide was then kept in 56°C for overnight incubation.

Reagent used on day1 :

- 20X SSC was made by mixing 8.76gm NaCl in 35ml DEPC water. Then 4.412gm of Sodium citrate was added and volume was made upto 50ml.
- Proteinase K

  1M Tris Cl pH 8.0 5ml
  0.5M EDTA. 5ml
  Make the volume upto 50ml
- TEA solution was made by mixing 0.93gm of Triethanolamine hydrochloride and pH 8 was set by adding 10N NaOH.
- TEN solution

  1.0M Tris-HCl (pH 7.5) 5ml
  5M NaCl 30ml
  0.5M EDTA. 1ml
- 5X Maleate was made by mixing 2.9gm of Maleic acid in 42.5ml DEPC H_2O, pH 7.5 was set using 10N NaOH. After this, 2.19gm of NaCl was added and final volume was made upto 50ml.
- 10% RMB blocker
- Hybridization solution

  TEN solution. 3.6ml
  10% RMB blocker. 5 ml
  50% Dextran Sulphate. 10 ml
  100% Formamide. 25 ml
  DEPC H_2O 6.4 ml

Day 2

1. Slides were rinsed in 2X SSC at RT for 30 minutes with continuous rotation and hybrislips were gently teased apart.
2. Then the slides were transferred to 2X SSC/ 50% Formamide solution at 65°C for 30 min with gentle intermittent shaking for at least 5 minutes.
3. After 30 minutes, slides were washed twice with 2X SSC for 10 minutes each, at 37°C.
4. Slides were then given RNase treatment by adding 100µl of 10mg/ml RNase enzyme to the prewarmed RNase buffer at 37°C and incubating slides for 30 minutes.
5. Slides were then rinsed in RNase buffer for 30 minutes at 65°C in order to inactivate the enzyme.
6. Slides were overlaid with 1X Maleate/0.05% TritonX-100/1% RMB Blocker Solution for 3 hours.
7. Once blocking is done, slides were incubated with 500 µl of Alkaline phosphatase labelled primary antibody Anti Dig or Anti FL, overnight at room temperature.

Reagent used on day2 :

- RNase Buffer

  5M NaCl 5ml
  1M Tris Cl pH7.5. 500µl
  0.5M EDTA 100µl
  Volume was made upto 50ml

- 1X Maleate/0.05% Triton/ 1% RMB blocker solution

  5X Maleate stock 2ml
  Triton X-100 5µl
  10% RMB blocker. 1ml
  DEPC H_2O 7ml

Day3

1. Post primary antibody incubation, slides were washed 3 times with 1X maleate buffer, 10 minutes each.
2. Following 1X maleate washes, slides were given 3 washes with Genius Buffer, 10 minutes each.
3. Slides were then overlaid with 500µl of NBT/BCIP substrate (made in Genius buffer in 1:50 ratio) until the colorimetric reaction takes place.

Reagent used on day2 :

- Genius Buffer

  1M Tris Cl pH9.5 5ml
  5M NaCl 1ml
  0.5M $MgCl_2$ 5ml
  Volume was made upto 50ml

2.8 TUNEL assay

1. Slides were washed 3 times with 1X PBS to remove the OCT.

2. Slides were overlaid with 4% PFA for 20 minutes, in case the sections contain morpholino, and then washed with 1X PBS.

3. The sections were then permeabilized with prewarmed trypsin at 37°C for 5 minutes.
4. Post permeabilization, the slides were overlaid with 50µl of labelling solution containing mixture 45µl Label solution (Fluorescein-dUTP) and 5µl Enzyme Solution (TdT) and kept at 37C for 1 hour after cover slipping.
5. Slides were washed thrice with 1X PBS and checked for signals for TUNNEL positive cells.
6. Slides were dried and cover slipped.

2.9 RNA ISOLATION PROTOCOL

1. Tissue suspended in 200µl TRIzol was thawed and homogenized by pipetting up and down several times until it became clear.
2. It was then allowed to rest for 5 minutes at room temperature.
3. After 5 minutes, 40µl of chloroform was added and shaken vigorously 15-20 times until they mix completely, followed by centrifugation at 10000 rcf for 10 minutes at 4°C.
4. Post centrifugation, nearly 30-40 µl of upper phase was taken with a cut tip in fresh MCT. At this point, carefully take the supernatant without disturbing the middle layer as it can lead to genomic DNA contamination.
5. Added equal amount of chilled isopropanol and mixed vigorously. Kept for overnight precipitation.
6. Following overnight precipitation, solution was centrifuged at 10000 rcf for 30-40 minutes.
7. Supernatant was discarded and 2 washes with 80% ethanol was given to the pellet, air dried for 10 minutes and dissolved in 12 µl of nuclease free water.
8. Checked 1 µl of the dissolved RNA on 2% agarose gel and stored in -80°C until further use.

2.10 cDNA synthesis

1. Following reagents were mixed into a PCR tube on the ice:

Template RNA.	Upto 5µg of mRNA
Primer oligo dT	0.125µl
Primer Random Hexamer	0.125µl
Nuclease free water	make the final volume upto 3µl

2. PCR tubes were mixed gently, centrifuged briefly and incubated at 65°C for 5 minutes.
3. PCR tubes were kept on ice immediately.
4. To each PCR tube, following components were added in following order

5X Reaction Buffer	1µl
Ribo Lock RNase inhibitor	0.25µl
10mM dNTP mix	0.5µl
RT enzyme	0.25µl

5. PCR tube was flicked gently and centrifuged briefly.
6. cDNA was prepared as per following PCR program

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

7. The prepared cDNA was diluted in the nuclease free water in the ratio of 1:4 and subsequently used for RT-PCR or quantitative PCR.
8. Remaining cDNA can be stored at -80°C.

2.11 Reverse transcriptase polymerase chain reaction (RT-PCR)

The required gene of interest was amplified from the cDNA using specific primers and following reaction mixture was made

Component	**Amount**
20x PCR buffer	0.5µl
2.5mM dNTP	1.0µl
10pM Forward primer	0.1µl
10pM Reverse primer.	0.1µl
Template	x µl
Taq polymerase	0.1µl
Nuclease free water	make the volume upto 10µl

Reaction was set according to following program in thermocycler

Step	**Temperature**	**Duration**
Initial denaturation	95°C	2minutes

Denaturation	95°C	20seconds	25-40 cycles
Annealing	55-60°C	30seconds	
Extension	72°C	1min/kb	
Final extension	72°C	5minutes	
Hold	4°C	∞	

2.12 Quantitative PCR (qPCR)

qPCR was performed to find out the fold change of genes in different treatment and time points. Following reaction was set and amplification was done in Applied Biosystems QuantStudio3 qPCR machine.

Components	**Amount**
2X PowerUP SYBR mix	2.5µl
10pM Forward primer	0.1µl
10pM Reverse primer	0.1µl
Template	x µl
Milli Q	make final volume upto 5µl

Result was analysed in MS Excel using by ΔΔCt method.

2.13 Western blotting Assay

Sample preparation

1. Retinae were suspended in an appropriate amount of 2X Laemmli buffer depending on the number of retinae pooled and homogenized using a clean piston until the solution was clear.

- Composition of 2X Laemmli buffer

  4% SDS

  20% Glycerol

  10% β-mercaptoethanol

  0.004 % Bromophenol blue

  0.125M Tris HCl

2. The sample was subjected to intermittent pulse vortexing for and incubation on ice for a total period of 20 minutes.
3. Sample was then boiled at 100°C for 10 minutes for denaturation of protein and then stored in 80 until further used or used directly.

Polyacrylamide gel electrophoresis

1. The casting apparatus was set and 10% of Resolving gel was poured in about 3/4th of the plate length. 200 µl of 50% isopropanol was overlaid on top to make it uniform.

- Composition of 10% resolving gel

Components	**Amount**
Resolving Buffer (1.5M Tris HCl, pH 8.8)	2.5ml
30% Acrylamide	3.33ml
Milli Q water	3.97ml
10% SDS	100µl
10% APS	100µl
TEMED	6µl

2. Resolving gel was allowed to solidify and once solidified isopropanol was removed and cleaned with milli Q.
3. Stacking gel was poured over the resolving gel and combs were inserted in between the glass plates so that wells are formed in the stacking gel.

- Composition of stacking gel

Components	**Amount**
Stacking Buffer (1M Tris HCl, pH 6.8)	625µl
30% Acrylamide	625µl
Milli Q water	3.603ml
10% SDS	50µl
10% APS	50µl

TEMED	5µl

4. Once stacking was solidified, combs were removed, cleaned with water and placed in an electrophoretic tank containing 1X SDS running buffer.

- Composition of 10X running buffer (pH 8.3)

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

5. 1X running buffer was filled in between the 2 pairs of glass plates so that wells are submerged into it.
6. Samples were loaded in the wells along with the reference ladder and electrophoresed at 90V.
7. Once the dye front reached to the bottom of glass plates, electrophoresis was stopped and subjected to transfer.

Transfer of protein from polyacrylamide gel to the PVDF membrane

1. Gel was taken out from the cast and given 3 water washes and 1 wash with the transfer buffer.

- Composition of 10X running buffer (pH 8.3)

Components	**Amount**
Methanol	200ml
Tris base	3g
Glycine	14.4g
MilliQ	Up to 1000ml

2. PVDF membrane on which protein needed to be transferred was charged using methanol for 2-3 minutes, followed by 2 water washes and 1 wash with the transfer buffer.

3. Gel was placed in contact with the PVDF membrane and sandwiched between the blotting membrane and transfer was done in the transfer buffer at 70V for 90 minutes.

Post transfer treatment

1. PVDF membrane was blocked with 10% skimmed milk made in 0.05% PBST for 1 hour or sometimes overnight, depending on the type of antibody to be used in the subsequent step.
2. Membrane was given 3 washes with 0.05% PBST for 10 minutes each.
3. Membrane was then incubated overnight with primary antibody diluted in either 0.05% PBST, 10% skim milk or 5% BSA-PBST.
4. Post primary antibody incubation, membrane was washed with 0.05% PBST for 10 minutes each.
5. Blots were incubated with secondary antibody anti-mouse or anti-rabbit which are HRP conjugated.
6. Blots were washed 3X with 0.3% PBST for 10 minutes each.
7. Post washes blots were developed by the Chemiluminescence method of Image Quant LAS4000.

2.14 Genomic DNA isolation

1. Tissue was suspended in 200 µl of TEN buffer in an MCT.
2. Tissue was homogenized with a clean piston after adding 1% Sodium dodecyl sulphate (SDS) till it becomes clear.
3. Proteinase K(10mg/ml) was added to a final concentration of 100-200ug/ml, mixed gently and kept at 37°C in a water bath for 1-2 hours.
4. After incubation, equal volume of Phenol:Chloroform:Isoamyl Alcohol (PCI) was added and mixed gently by inverting the tube.
5. Mixture was centrifuged at 6000rpm for 5 minutes at RT.

6. Upper layer was carefully taken with a cut tip and collected in a fresh MCT.
7. To this aqueous phase, 1/10th volume of 3M Sodium Acetate was added and mixed gently by inverting the tubes.
8. Added double volume of Isopropanol or equal volume of Ethanol from the side of the walls and invert mixed gently till there is a thread like formation was seen in the solution.
9. Thread like mesh is gently spooled out from the solution and placed in 70% ethanol to give it a wash.
10. Centrifuged at 6000rpm for 10 minutes to pellet down genomic DNA.
11. Pellet was dried completely and dissolved in 100µl of Nuclease free water. Do not tap and leave at 4C for complete dissolution.

2.15 Cloning of gene CDS and promoters

Manual method of plasmid isolation

1. 1.5ml of culture was taken in an MCT and centrifuged at 13400rpm for 2 minutes.
2. Supernatant was discarded carefully and the pellet was resuspended in 100µl of water.
3. To the bacterial suspension, 100µl of lysis buffer was added and mixed gently by invert mixing.
4. Bacterial suspension along with the lysis buffer was boiled at100C for 5minutes or till the time solution became clear.
5. Once the solution became clear, 50µl of 0.5M MgCl2 was added and mixed gently by inverting the tube and kept on ice for 2minutes.
6. MCTs were centrifuged at RT for 2minutes at 13400 rpm.
7. Without disturbing the pellet, 50µl of 3N Potassium Acetate was added from the side of the walls and invert mixing gently.
8. MCTs were centrifuged for 2 minutes at 13400 rpm.

9. Supernatant was transferred to the fresh MCT and 600µl of Isopropanol was added to it.
10. The solution was mixed vigorously, kept on ice for 5 minutes for precipitation and centrifuged at 13400rpm for 2 mins.
11. Pellet was washed with 70% ethanol and dried for 15-20 minutes.
12. Pellet was then dissolved in 50µl nuclease free water.

Amplification of gene or promoter of interest

The CDS of genes, to be cloned in any particular vector, were amplified from 24hpf cDNA and promoters were amplified from the genomic DNA using specific primers. List of primers is given in the appendix. Amplification was done using GO Taq long PCR master mix as per the following reaction:

Component	**Amount**
GoTaq Long PCR Master Mix (2X)	25 µl
10pM Forward primer.	0.5µl
10pM Reverse primer	0.5µl
Template	x µl
Nuclease free water	make the volume up to 50µl

Amplified product was cleaned up using Nuclear-pore Sure Extract PCR Clean-up/ Gel Extraction Kit (Cat.#NP-36107) or manual gel extraction protocol.

Restriction Digestion

The restriction digestion reaction was set as per following reaction mixture:

Component	**Amount**
10x Reaction buffer	5 µl
DNA (plasmid or purified PCR product)	30 µl
Restriction enzyme	1.2 µl
Nuclease free water	make the final volume up to 50µl

The prepared reaction mixture was kept in the water bath at the optimum temperature for the enzyme for 5-6 hours. After restriction digestion, sample is loaded onto 0.8% agarose gel along with the reference ladder and required band of appropriate size is cut after complete resolution and later DNA is extracted using manual gel extraction protocol.

Manual gel extraction

The excised band from 0.8% agarose gel is subjected to manual extraction as per following protocol:

1. Column was prepared by filling cut aluminium foil into the MCT with a hole at the A bottom. This MCT was placed in another MCT which was the collection tube.
2. The excised piece of gel was placed the MCT containing aluminium foil and spun at 13400rpm for 3 minutes.
3. Flow through collected in the collection tube was mixed with equal volume of PCI, mixed vigorously and centrifuged at 13400 rpm for 10 minutes.
4. After centrifugation , upper aqueous layer was taken in fresh MCT with the help of cut tip.
5. Equal volume of chloroform was added to the collected upper aqueous layer, mixed vigorously and centrifuged at 13400 rpm for 10 minutes.
6. Again, the supernatant was collected in fresh MCT with the help of cut tip.
7. DNA was precipitated by adding 7N Ammonium acetate (final concentration of 3N), 100% molecular biology grade ethanol (final concentration of 70%) and 1µl of 10mg/ml glycogen.
8. All the components in the MCT were mixed by tapping vigorously and kept in -80°C for overnight.
9. Post overnight incubation, solution was spun at 13400rpm for 30 minutes at 4°C.
10. Supernatant was discarded and pellet was washed twice with 70% ethanol.

11. Pellet was dried and then dissolved in required volume of nuclease free water.

Ligation

Ligation reaction was set up by adding following components:

Component	Amount
10X Reaction Buffer	2 µl
Vector	50ng
Insert	x ng (insert: vector ratio = 4:1)
T4 DNA Ligase	1 µl
Nuclease free water	make the volume up to 20 µl

The following reaction was kept at 16°C for overnight incubation.

Transformation

1. Ultracompetent DH5-Alpha cells were thawed on ice.
2. Once thawed, 5 µl of ligated product was added to the component cell, mixed by gently tapping and kept on ice for 30 minutes.
3. After 30 minutes, heat shock was given at 42°C for 75 seconds and again kept on ice for 5 minutes.
4. Cells were revived by adding 1 ml of LB media and kept for shaking at 37°C.
5. After 1 hour of incubation, cells were pelleted at 4000rpm for 4 minutes.
6. Pelleted cells were redissolved in 100 µl and plated on pre warmed LB-Amp or LB-Kan plate and kept in 37°C incubator.

Screening of transformed cells

Transformed colonies were screened by different methods such as colony PCR, PCI screening, and restriction digestion. Positive clones were then confirmed by Sanger sequencing.

Glycerol stock of positive clones were made and stored at -80°C.

Site directed mutagenesis (SDM)

1. Following reaction was set up for SDM:

Component	Amount
Plasmid DNA	1 µl
10pm Forward primer	1.25 µl
10pM Reverse primer	1.25 µl
Promega Long PCR Master Mix	12.5 µl
Nuclease free water	make up final volume up to 25 µl

The above reaction was set up in 2 different PCR tube and were subjected to following PCR program.

Step	Temperature	Duration	
Initial denaturation	93°C	2minutes	
Denaturation	93°C	30seconds	18 cycles
Annealing	55°C	50seconds	
Extension	72°C	1min/kb	
Final extension	72°C	20minutes	
Hold	4°C	∞	

2. Along with the experimental, control reaction was also set in which no master mix was added.
3. Post PCR, reactions in both the experimental PCR tubes were combined and clean up was done using Nucleo-pore SureExtract PCR Clean-up/Gel Extraction Kit (Cat.#NP-36107).
4. DpnI treatment was given to both the experimental as well as the control PCR product for 1 hour at 37°C
5. After DpnI treatment, 15 µl of the reaction was transformed in DH5-Apha cells.
6. If there were no colonies or very less colonies in control as compared to experimental SDM, at least 5 colonies were picked up from the experimental plate, inoculated and isolated plasmid were sent for Sanger sequencing for confirmation of positive SDM.

2.16 *In-vitro* transcription for RNA probe synthesis

1. Plasmid DNA containing gene of interest was linearized at the 5'prime end of gene such that anti sense copy of probe could be synthesized.
2. Linearized plasmid was purified by manual gel extraction protocol and concentration was recorded.
3. RNA probe reaction was set as follows in an MCT

Component	**Amount**
10X RNA reaction Buffer	4 µl
Digoxigenin or Fluorescein RNA labelling mix	2 µl
Linearized DNA	1µg
RiboLock RNase Inhibitor	0.5 µl
SP6/T7/T3 RNA Polymerase	2 µl
DEPC water	make the volume up to 40 µl

4. The reaction was kept for incubation at 37°C for 4 hours.
5. After 4 hours of incubation, 1 µl of reaction was checked on 1% of agarose gel.
6. The reaction was stopped by keeping on ice and precipitation by adding following components

Component	**Amount**
Tris-EDTA Buffer (pH8.0)	4 µl
LiCl	4 µl
Glycogen (10mg/ml)	2 µl
100% Ethanol	70 µl

7. All the components were mixed properly by gently tapping and kept at -80°C overnight for precipitation.
8. Next day, it was centrifuged at 13400rpm for 30 minutes.
9. Pellet was given 2 washes with 80% ethanol, dried and the dissolved in required volume of DEPC water. Dissolved probe is kept in -80°C for long term storage.

2.17 *In-vitro* mRNA synthesis for overexpression

1. Plasmid containing gene of interest was linearized at the 3' end of the gene.
2. Linearized plasmid was run on 1%agarose gel and purified by manual gel extraction protocol.
3. *In-vitro* transcription reaction was set by adding following components of mMESSAGE mMACHINE® Kit (Invitrogen AM1340):

Component	**Amount**
Linearized plasmid	0.1-1µg
10X Reaction Buffer	2 µl
2X NTP/CAP	10 µl
Enzyme Mix	2 µl
Nuclease free water	make the volume up to 20 µl

4. All the components were mixed by gentle tapping and incubated at 37°C for 2 hours.
5. After 2 hours, 1 µl of TURBO DNaseI was added to the reaction and kept for 15 minutes at 37°C
6. Post incubation, 1 µl of the reaction was checked on gel and then proceeded for precipitation.
7. The mRNA was precipitated by adding following components:

Component	**Amount**
Nuclease free water	30 µl
LiCl	30 µl
100% ethanol	80 µl

8. All the components were mixed gently by tapping and then kept overnight at -20°C for precipitation.
9. The reaction was centrifuged at 13400rpm for 30 minutes at 4°C.
10. Pellet was given 2 washes with 80% ethanol, dried and dissolved in the required volume of nuclease free water.

11. Once pellet was dissolved properly, concentration was measured using nanodrop and mRNA was kept in -80°C for long term storage.

2.18 Co-Immunopreciptation (co-IP)

1. Retinae were dissected in 1X PBS and 100mM PMSF was added to it to a final concentration of 1mM.
2. Dissected retinae were collected in 500C µl C-100 buffer with 1X PI and 1mM PMSF.

- Composition of C-100 buffer

Component	**Amount**
1M HEPES (pH7.6)	2ml
0.5M EDTA	20µl
0.5M $MgCl_2$	150µl
1M KCl	5ml
100% Glycerol	20%
100% Tweem-20	0.02%
Milli Q	up to 50ml

3. It was stored at -80°C for 2-3 hours and then thawed by immediately plunging into the water.
4. Retinae were lysed by pipetting and vortexing 2-3 times at a very slow speed.
5. Once lysed, remaining 500 µl of C-100 buffer was added.
6. Solution was centrifuged at 10,000rpm for 10 minutes at 4°C.
7. Meanwhile, 20 µl magnetic beads (Protein A or Protein G) were taken in a fresh MCT and storage solution was removed with the help of magnetic rack.
8. Beads were washed 3X with 1X PBST (0.1% Tween-20) and finally suspended in 200 µl of 1X PBST.

9. 2 µl of antibody (IgG or protein specific antibody) was added to it and rotated for 10 minutes at RT.
10. After centrifugation (step6), supernatant was collect in fresh MCT and 100 µl of input was saved.
11. Remaining supernatant was divided into 2 MCTs. In one of the MCTs beads bound to IgG were added and to the other beads bound to protein specific antibody were added.
12. They were then incubated for 4 hours at 4°C with rotation (10rpm).
13. After 4 hours of incubation, beads were collected with help of magnetic rack and washed 5X with C-100 buffer at 4°C.
14. Post washing, beads were boiled in 2X Lamemli buffer at 95°C for 10 minutes.

2.19 Chromatin Immunoprecipitation (ChIP)

1. Retinae were dissected out and suspended in 200µl 1X PBS/ 1mM PMSF/ 1X PI cocktail.
2. Tissue were pipetted up and down to make single cell suspension.
3. Cells were immediately crosslinked by adding formaldehyde to a final concentration of 1%vol/vol, gently tapped and rotated at 10rpm for 10 minutes.
4. Glycine was added to the final concentration of 0.125M, to quench the formaldehyde, tapped gently and incubated on ice for 5 minutes.
5. The tube was centrifuged at 1725rpm for 10 minutes at 4°C to sediment the cell. Supernatant was removed carefully with the help of 1ml pipette.
6. To the pelleted cell, 500 µl PBS/PMSF/PI was added and cells were resuspended by gentle tapping.
7. Tubes were centrifuged at 1725rpm for 5 minutes at 4°C and supernatant was discarded.

8. Cells were resuspended in another 500 µl PBS/PMSF/PI, tapped gently and centrifuged at 1725rpm. Dried pellet can be stored at -80°C or proceeded further.
9. Nuclei lysis buffer was added to a total volume of 600 µl, mixed gently by rubbing in between the hand and keeping intermittently on ice. Do not tap or vortex.

- Compostion of Nuclear lysis Buffer

Component	**Final concentration**
1M tris HCL (pH7.5)	500mM
0.5M EDTA (pH8.0)	10mM
20% SDS	1%

10. Cells were sonicated for 110cycles*30 seconds and 45 seconds pause at 4°C in Q Sonica as per the manufacturer's protocol.
11. After sonication, sample was centrifuged at 13400rpm for 10 minutes at 4°C. Supernatant was removed and placed in a clean 1.5ml tube.
12. From the supernatant, 100 µl input control was saved and stored at -80°C.
13. From the remaining supernatant, 2 µl was run on gel to check the sonication efficiency.
14. The sample was diluted with IP dilution buffer/PMSF/PI to make the volume upto 1ml, mixed well and spun down.

- Composition of IP dilution Buffer

Component	**Final concentration**
1M tris HCL (pH7.5)	16.7mM
0.5M EDTA (pH8.0)	1.2mM
5M NaCl	167mM
20% SDS	0.01%
100% Triton X-100	1.1%

15. Sample was pre cleared by adding 10 µl of Protein Agarose A/G (dynabeads) slurry to the sample and incubated for 2 hours at 4°C with rotation at 10rpm.

16. After 2 hours, beads were captured by placing the tubes in the chilled magnetic rack. Supernatant was collected in the fresh MCT.
17. Antibody was added to the supernatant to the final concentration 2µg/ml and rotated overnight at 4°C.
18. Post overnight incubation, 20 µl of Protein Agarose A/G beads were added and rotated for 2 hours at 4°C.
19. Beads bound with antibody were captured by placing in the chilled magnetic rack.
20. Supernatant was discarded and 500µl of ice cold IP dilution/PI/PMSF was added and rotated at 40rpm for 4 minutes at 4°C. This step was repeated twice.
21. Supernatant was discarded and beads were washed in wash buffer/PMSF/PI cocktail with rotation at 40rpm for 4 minutes at 4°C. Again repeated this step.
22. Supernatant was removed and 500µl of TE buffer was added and incubated on rotator for 4 minutes.
23. Supernatant was discarded and 150µl of ChIP elution buffer (20mM Tris-HCl pH7.5, 5mM NaCl, 1%SDS, 50 ug/ml proteinase K. Proteinase K and SDS were added just before use) was added to the added and incubated at 65°C on thermomixer for 2 hours at 1300rpm.
24. Beads were captured in the magnetic rack and eluate was transferred to a clean MCT.
25. Beads were again incubated with 150µl of ChIP elution buffer and incubated on thermomixer for 15 minutes.
26. Both the eluate were pooled and 200µl of ChIP elution buffer was added to it making total volume up to 500µl.
27. Proteinase K was added to input to a final concentration of 2mg/ml and incubated at 68°C, 1300rpm on a thermomixer for 2 hours.

28. Equal volume of Phenol: chloroform: Isoamyl alcohol was added to both input as well as pull down sample, mixed by vortexing and centrifuged at 13400 rpm for 10 minutes. Same step was repeated with chloroform.
29. Aqueous phase was precipitated by adding 1µl of glycerol, 1/10th volume of sodium Acetate, and twice the volume of Ethanol and incubated overnight.
30. Post overnight incubation, DNA was precipitated by centrifugation at 13400 rpm for 30minutes at 4°C.
31. Supernatant was discarded and pellet was washed twice with 70% ethanol, dried and dissolved in 5µl of nuclease free water

2.20 Fluorescence assisted cell sorting (FACS)

1. Retinae were dissected and suspended in 500 µl of L15 media.
2. Hyaluronidase was added at a final concentration of 1mg/ml and incubated at RT for 15 minutes.
3. Hyaluronidase was washed off using L15 media and this was repeated twice. Centrifugation was done at 1000 rpm for 1 minutes.
4. Retinae were resuspended in 500 µl of L15 media.
5. Trypsin was added to a concentration of 0.01% v/v, incubated at RT for 15 minutes and pipetted up and down intermittently for making single cell suspension.
6. Cell were sorted in BD FACS ARIA sorter.

2.21 Raising antibody against Zic2b in mice

1. Zic2b CDS of Zebrafish was cloned in the pET22B expression vector.

2. Protein was expressed in the BL-21strain of E.Coli and the Zic2b protein with the His-tag was purified using Ni-NTA beads.
3. Purified protein was mixed with the Freund's complete adjuvant (Sigma) in 1:1 ratio and suspension was made by vortexing overnight.
4. This mixture was then injected beneath the skin in mice and left for the antibody to develop.
5. After 1 week, purified protein was mixed with the Freund's incomplete adjuvant (Sigma) in 1:1 ratio and again this mixture was injected in the mice as a booster dose.
6. We collected the blood from the mice and mice was euthanized.
7. Blood was kept at 37°C for 10 minute and then centrifuged at 5000rpm and supernatant containing the antibody was collected in the separate tube, aliquoted and stored at -80°C.
8. Specificity of the Anti-Zic2b antibody was checked by checking the levels of Zic2b protein in Zic2b knockdown condition.

2.22 Whole retina RNA-seq Data analysis

Using bulk RNA-seq gene expression profile in *yy1a* and *yy1b* MO injected retinae was compared to control MO injected retinae at 4dpi. Briefly raw reads generated were quality trimmed and adapter sequences were aliped using Trimmomatic (Bolger, Lohse, & Usadel, 2014). Fast QC (Babraham Institute) (Andrews, 2010) was used for visualizing read quality. For subsequent analysis, data was uploaded to the Galaxy web platform, and public server at usegalaxy.org was used to analyse the data ("The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update," 2022). The filtered reads were mapped to GRCz11/danRer11 genome assembly using STAR (RNA STAR) (Dobin et al., 2013). The number of read mapping to each feature was quantified using HTseq-count (L. Wang, Wang,

& Li, 2012). FPKM (fragments per kilobase of exon per million) values were calculated using raw counts of genes. Fold change in expression was calculated as compared to the control sample. Genes with >1.5-fold change were considered for subsequent analysis. Gene Ontology (GO) enrichment analysis was performed using R package “cluster profiler” (Yu, Wang, Han, & He, 2012).

2.23 Luciferase Assay

1. Promoters of respective genes are cloned into pEL promoter.
2. mRNA was transcribed *in-vitro* and diluted in various concentration. Similarly, different concentration of MOs was also prepared.
3. Following mixture is injected into single-celled stage embryos:

Components	**Amount**
mRNA/MO	125ng and 250ng/ 125-250mM
Renilla mRNA.	2-5ng/µl
pEL plasmid	20-50ng/µl

4. Embryos were kept at 28°C for 24 hrs and then collected in a test tube and snap frozen.
5. Snap frozen embryos were thawed and lysed in 1X lysis buffer provided in the kit.
6. The entire volume of the lysate was aliquoted in different MCTs.
7. In the luminometer, on one side luciferase substrate is added and on the other side renilla substrate.
8. Multiple readings were taken and ratio of luciferase to renilla is calculated to see the normalized fold change in the promoter activity in different treatments.

Section 3

Results

Chapter 1

Regulation of *yy1a* and *yy1b* is essential for Müller Glia de-differentiation and proliferation during retina regeneration

3.1.1 Yy1 is required for the development of zebrafish embryos.

Zebrafish have two paralogous copies of the transcription factor Yin-yang1, Yy1a and Yy1b. Yy1a is 357 aa long protein, coded by the reverse strand of chromosome 17, while Yy1b contains 354 aa, transcribed from the reverse strand of chromosome 20. Yy1a and Yy1b shares 88% sequence similarities as analysed by NCBI BLASTP tool (Basic Local Alignment Search Tool) (Fig 3.1.1.1.A). We compared the protein sequence of Yy1a and Yy1b with human YY1 (Verheul et al., 2020) (K. Chen et al., 2019) and predicted the domains. Both Yy1a and b contains the transactivation domain from amino acid 1-114 but do not have the poly-histidine stretch. Surprisingly, Glycine-Serine rich region is present in Yy1b, but is absent in Yy1a. The Glycine-lysine region and the REPO domains, part of the central repressive region, are well conserved in both Yy1a and Yy1b. Zebrafish Yy1a and YY1b also contain four zinc finger domain at the C-terminal, which is highly conserved and show sequence identity with human YY1 (Fig 3.1.1.1.A).

We checked the expression of *yy1a* and *yy1b* in 24 and 48hpf (hours post-injury) embryo by mRNA *in-situ* hybridization. We found that both the isoforms are expressed in the notochord (notochord develops into brain and spinal cord) (Fig 3.1.1.2A,B). Then, we questioned if Yy1 is essential for development. For this, we injected morpholino (MO) against both *yy1a* and *yy1b*, which blocked the translation (Nasevicius & Ekker, 2000) and found that at the concentration of 0.5mM, there was a very high lethality and embryos did not survive. At a concentration of 0.25mM, the embryo showed crooked morphology and eyes did not develop properly; and they did not survive for long (Figure 3.1.1.3.A). Therefore, the results suggest that Yy1 is important for the development of the eyes and nervous system in the zebrafish embryos, and the downregulation of these protein leads to developmental defects.

Previous studies have revealed that the localization of YY1 is dependent on the cell cycle and switch in response to the cell-cycle related signalling. YY1 is predominantly nuclear at the

G1/S boundary and early S phase but transported to the cytoplasm in the mid-S phase (Palko, Bass, Beyrouthy, & Hurt, 2004). At G1/S transition, YY1 nuclear localization coincides with upregulation of replication-dependent histone gene H3.2 (Eliassen, Baldwin, Sikorski, & Hurt, 1998). At the G2/M transition, it is again nuclear but goes off from the nucleus, into the cytoplasm from prophase to anaphase and again becomes nuclear at the telophase.

We were also intrigued to know the subcellular localization of Yy1 in zebrafish. We injected *in-vitro* transcribed mRNA of *yy1a-gfp* and *yy1b-gfp* at the single-celled stage and then fixed the embryo at 24hpf, cryoprotected and sectioned. We then immunostained for GFP and saw its localization with respect to DAPI. We found that predominantly Yy1-GFP was present outside the nucleus, while in some cells it was inside the nucleus (Figure 3.1.1.3.B). Our observation matches the previous findings because in embryos most of the cells are in the dividing phase as the embryo grows. Therefore, according to the phase of the cell-cycle in which the cell is, the localization of Yy1 changes.

A.

```
Yy1a  MASGETLYIEADGSEMPAEIVELHEIEVETIETTVVGGDDDEHQ----PMIALQPLVTDD  56
      MASGETLYIEA+G+ +PAE+VEL EIEVETIETTV+GG+D++       MIALQPL T D
Yy1b  MASGETLYIEAEGAAVPAELVELQEIEVETIETTVIGGEDEDEDEEEPAMIALQPLHTGD  60

Yy1a  PNHVNHQEVILVQTREEVVGCDDSDLHADDSFEDQILIPVPVPVAEEEYIEQTLVTVSGK  116
              VIL+QTREEVVG +D++L  D +FE+QILIPVP P A+ +YI QTL+TV+G+
Yy1b  ----GEPGVILLQTREEVVG-EDAELRGD-AFEEQILIPVPTPGADAQYIGQTLLTVAGR  114

Yy1a  NPSGRMKKGGGSG-KRVVKKSFLNSAEASGRKWEQKQVQIKTLEGEFSVTMWASDDKKDV  175
      +  GR  +   +G K+  KKS+L++AE+SGRKWEQKQVQIKTLEGEFSVTMWASDDKK++
Yy1b  SSGGRAGRRAAAGGKKTGKKSYLSAAESSGRKWEQKQVQIKTLEGEFSVTMWASDDKKEL  174

Yy1a  DHETVVEEQIIGENSPPDYSEYMTGKKLPPGGIPGIDLSDPKQLAEFARMKPRKIKEDDS  235
      +HE  VEE IIGENSPPDYSEYMTGKKLPPGGIPGIDLSDPKQLAEFARMKP+KIKEDD+
Yy1b  EHE--VEEHIIGENSPPDYSEYMTGKKLPPGGIPGIDLSDPKQLAEFARMKPKKIKEDDA  232

Yy1a  PRTIACPHKGCTKMFRDNSAMRKHLHTHGPRVHVCAECGKAFVESSKLKRHQLVHTGEKP  295
      PRTIACPHKGC+KMFRDNSAMRKHLHTHGPRVHVCAECGKAFVESSKLKRHQLVHTGEKP
Yy1b  PRTIACPHKGCSKMFRDNSAMRKHLHTHGPRVHVCAECGKAFVESSKLKRHQLVHTGEKP  292

Yy1a  FQCTFEGCGKRFSLDFNLRTHVRIHTGDRPYVCPFDGCNKKFAQSTNLKSHILTHAKAKN  355
      FQCTFEGCGKRFSLDFNLRTHVRIHTGDRPYVCPFDGCNKKFAQSTNLKSHILTHAKAKN
Yy1b  FQCTFEGCGKRFSLDFNLRTHVRIHTGDRPYVCPFDGCNKKFAQSTNLKSHILTHAKAKN  352

Yy1a  NQ  357
      NQ
Yy1b  NQ  354
```

Figure 3.1.1.1.A NCBI protein BLAST analysis shows sequence similarity between zebrafish Yy1a and Yy1b

A.

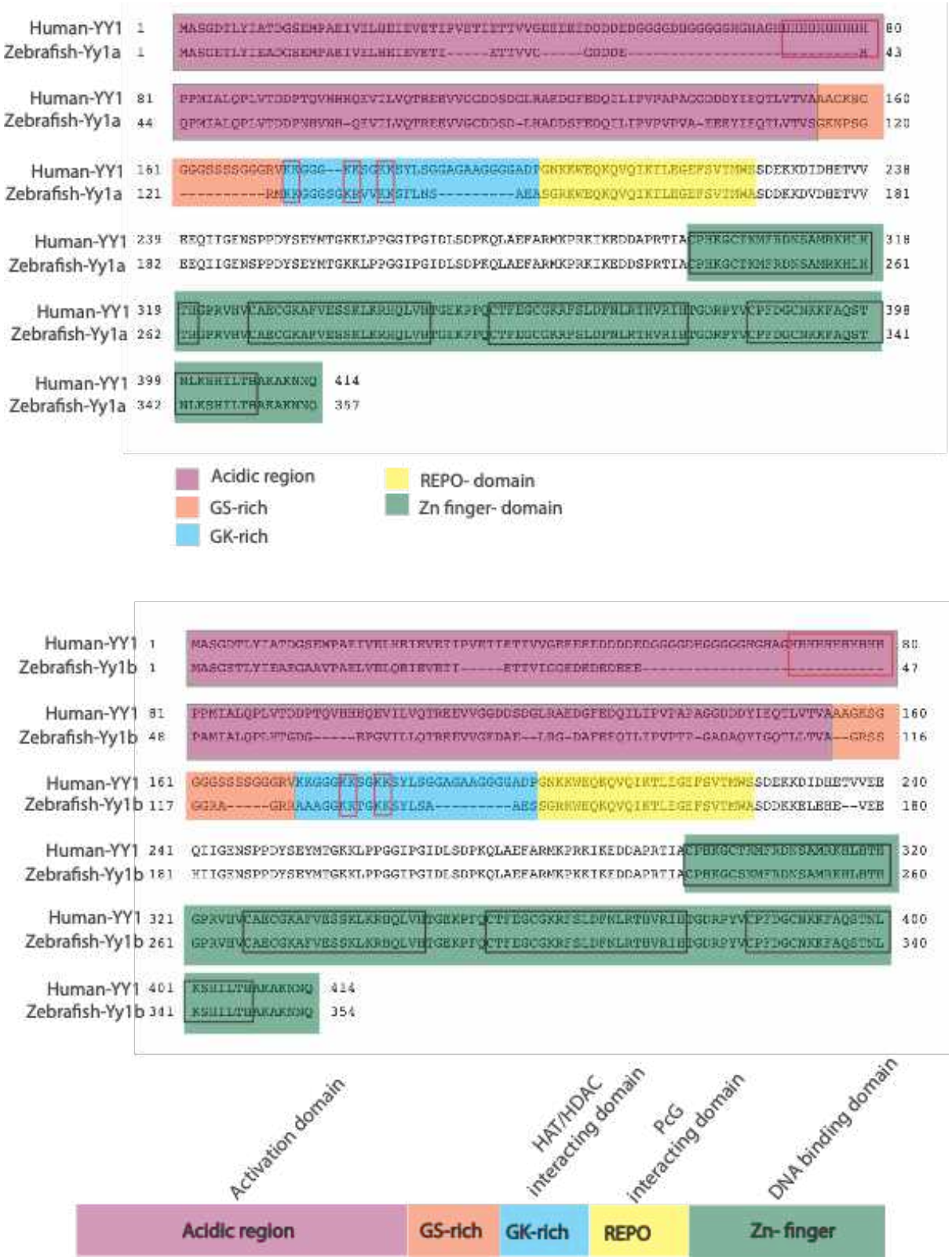
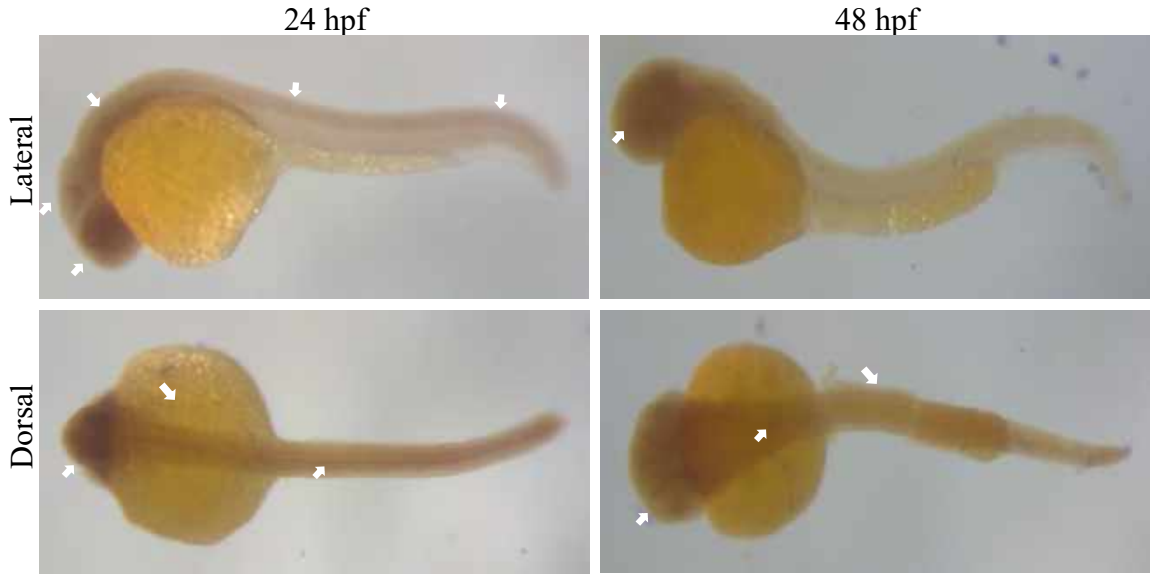


Figure 3.1.1.1.A NCBI protein BLAST analysis between Human and Zebrafish YY1, shows conservation of domains in both the species.

A.



B.

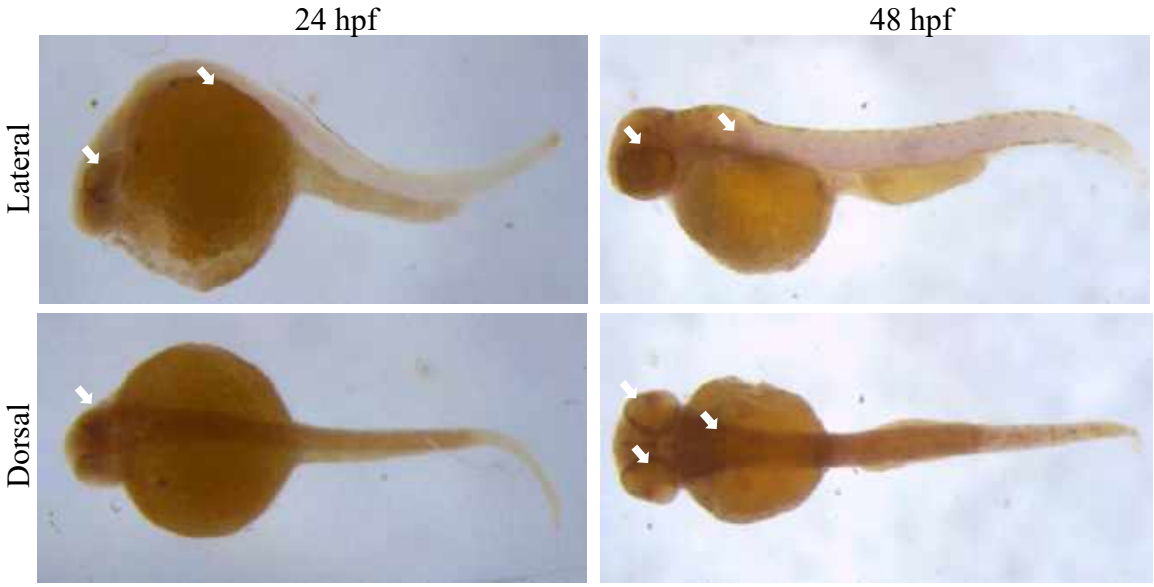
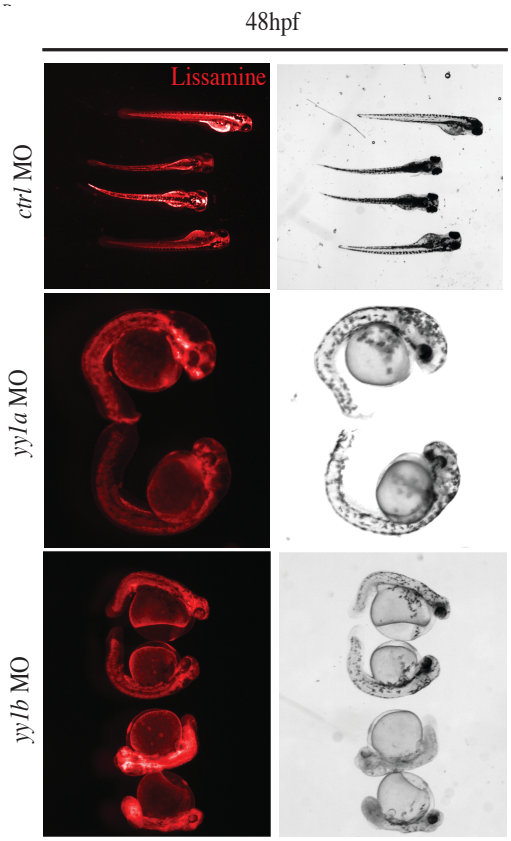


Figure 3.1.1.2. mRNA *in-situ* hybridization done at 24 and 48hpf embryos shows the expression of *yy1a* **(A)** and *yy1b* **(B). Arrow marks the *in-situ* signals.**

A.



B.

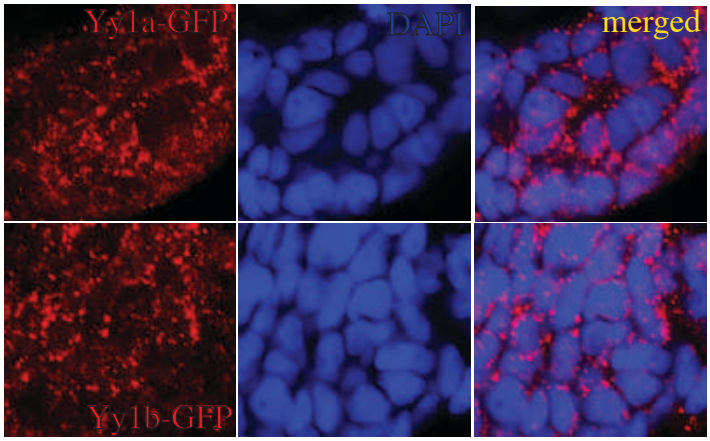


Figure 3.1.1.3. Effect of *yy1a* and *yy1b* knockdown on the zebrafish embryos and cellular localization of *yy1a* and *yy1b*. (A) Fluorescence microscopy images of 48hpf zebrafish embryos shows crooked morphology and underdevelopment in both *yy1a* and *yy1b* knockdown, separately. **(B)** Confocal microscopy images of embryonic sections at 60X shows subcellular localization of Yy1a-GFP and Yy1b-GFP (red) and DAPI marks the nuclei (blue). Scale bar is 10μM.

3.1.2 Yy1 is regulated during the course of retina regeneration and remains excluded from the actively proliferating MGPCs.

Yy1 is known to be a ubiquitous protein that is known to regulate many biological processes in the body. During the development of zebrafish embryo, we found that *yy1* is highly expressed in the retina. It is well known fact that regeneration recapitulates development as many factors, which are very crucial for the development, also gets activated during regeneration. Therefore, we tried to explore the role of Yy1 during retina regeneration post-retinal injury.

We injured the zebrafish retina and harvested it at different time points post-injury to assess the mRNA levels both temporally and spatially. We found that both *yy1a* and *yy1b* are regulated post-retinal injury. Soon after injury, their levels remained the same as uninjured control (UC) but showed a decline at around 12hpi (hours post injury) -2dpi (days post injury), which is considered as the de-differentiation phase and their levels again started to rise 4dpi onwards, which is a peak of the proliferative phase and beginning of the re-differentiation phase (Figure 3.1.2.1.A). We then qualitatively checked the expression of both *yy1a* and *yy1b* by mRNA *in-situ* hybridization on cryosections at UC, 1dpi, 2dpi, 4dpi and 8dpi and found that in the uninjured state, *yy1* is ubiquitously expressed throughout the retina in all the retinal layers. At 1dpi and 2dpi, there was a global decline in the transcript levels, while it began to rise at 4dpi and levels are almost comparable to UC (Figure 3.1.2.1.B). This result was confirmed by cell count analysis as the number of proliferating cells having *yy1* expression also declined in 2dpi and 4dpi retina as compared to the UC (Figure 3.1.2.2).

We quantitatively confirm this result by employing a transgenic line *tuba1016*:GFP to compare *yy1a* and *yy1b* levels in the proliferating and non-proliferating cells. The *tuba1016*:GFP contains 1.7kb of α1-tubulin promoter, exon 1, and the first intron driving the expression of GFP. The 1016bp of α1-tubulin promoter contains the E-box, binding site for the Ascl1a, it is

expressed in the CNS (Fausett & Goldman, 2006) during embryonic stages. Since, it marks the actively proliferating Müller glia cells post retinal injury (Kaur et al., 2018) (Gupta et al., 2023), we sorted the GFP^+ and GFP^- cells from the retinae of *tuba1016*:GFP fish at 4dpi using FACS (fluorescence-assisted cell sorting), extracted RNA and compared *yy1a* and *yy1b* levels. We found that indeed the levels of both genes were less in the GFP^+ fraction as compared to the GFP^- fraction (Figure 3.1.2.3).

We next checked the protein levels of Yy1 both spatially and temporally at different time points post-injury. Immunofluorescence assay of Yy1 revealed that throughout the regeneration regime, Yy1 was present in the entire retina and being a nuclear protein mostly it overlaps with DAPI staining (Figure 3.1.2.3.A). The zoomed images of the injury spot at 2dpi, 4dpi and 6dpi show that at 2dpi, Yy1 is still present minimally in the proliferating nuclei marked with BrdU, but at 4dpi and 6dpi, it completely goes off from the proliferating nuclei (Figure 3.1.2.3.B). Our results indicate that the presence of Yy1 initially is required for initiating the MG cells to start reprograming and enter the cell cycle. But as discussed in the previous result, during the active division or when the cells are in the mitotic phase Yy1 goes off from the nuclei and sits in the cytoplasm. We then checked the protein levels of Yy1 in the whole retinal lysate at different time points post-injury and found that there was no significant change in the protein levels (Figure 3.1.2.4.A). This could be either due to the lower representation of the MGPC in the total retina or YY1 is still present in its basal level and has only moved to the cytoplasm.

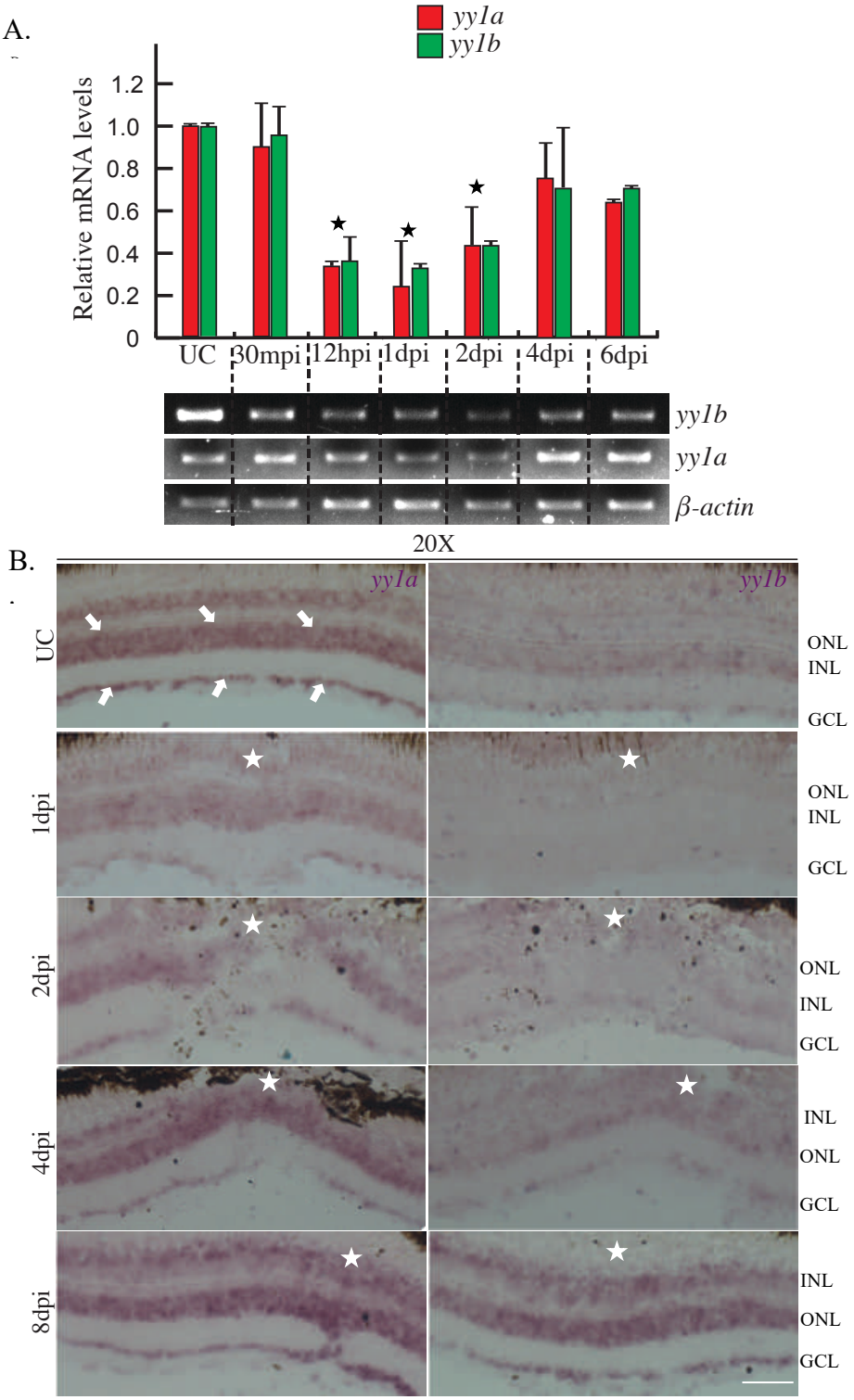


Figure 3.1.2.1. Spatial expression of *yy1a* and *yy1b*. (A) qPCR and RT-PCR analysis shows time course of *yy1a* and *yy1b* during retina regeneration and (B) mRNA *in-situ* hybridization shows spatial expression pattern of both the gene. White Star marks the injury site and arrow represents the *in-situ* signals. Scale bar represents 10μM in B. $p<0.0001$ in A.

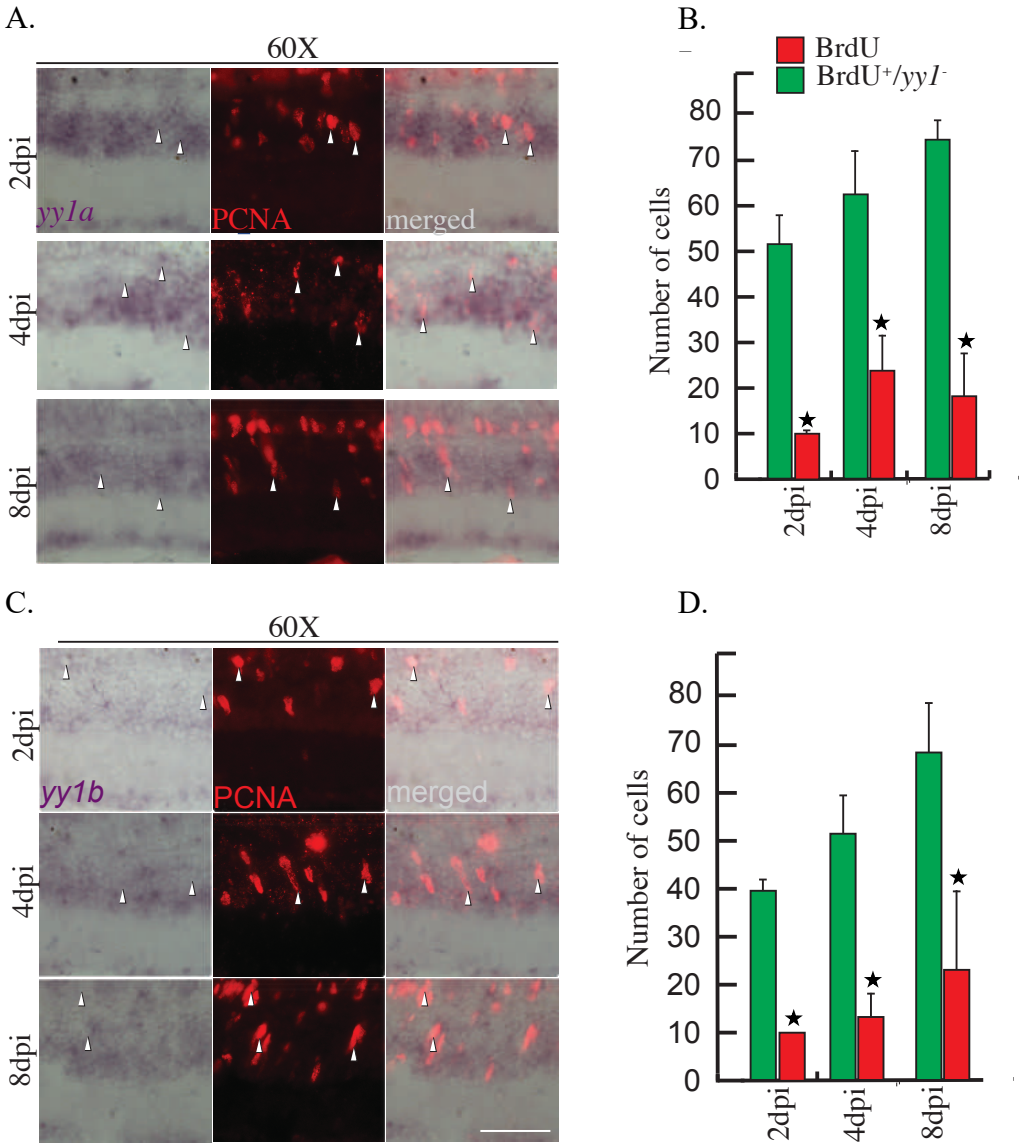


Figure 3.1.2.2. *yy1a* and *yy1b* are excluded from the proliferating cells. 60X images of mRNA *in-situ* hybridization at 2dpi, 4dpi and 8dpi shows exclusion of *yy1a* and *yy1b* from the proliferating cells marked with PCNA (A and C), which are quantified in (B and D). Arrow marks the $BrdU^+$ cells which are devoid of *in-situ* signals. Scale bar represents 10µM. $p<0.05$ in B and D.

A.

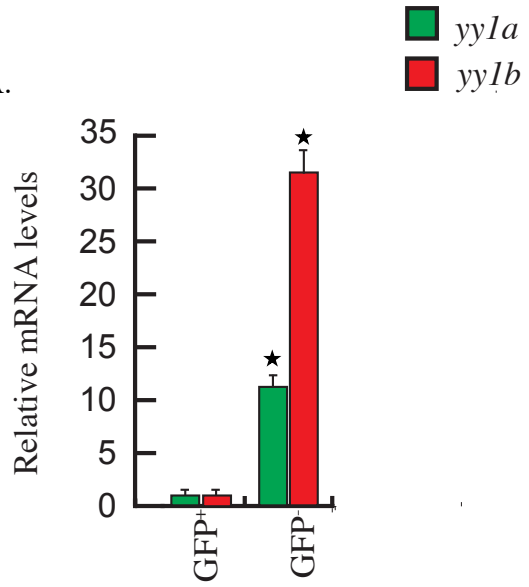
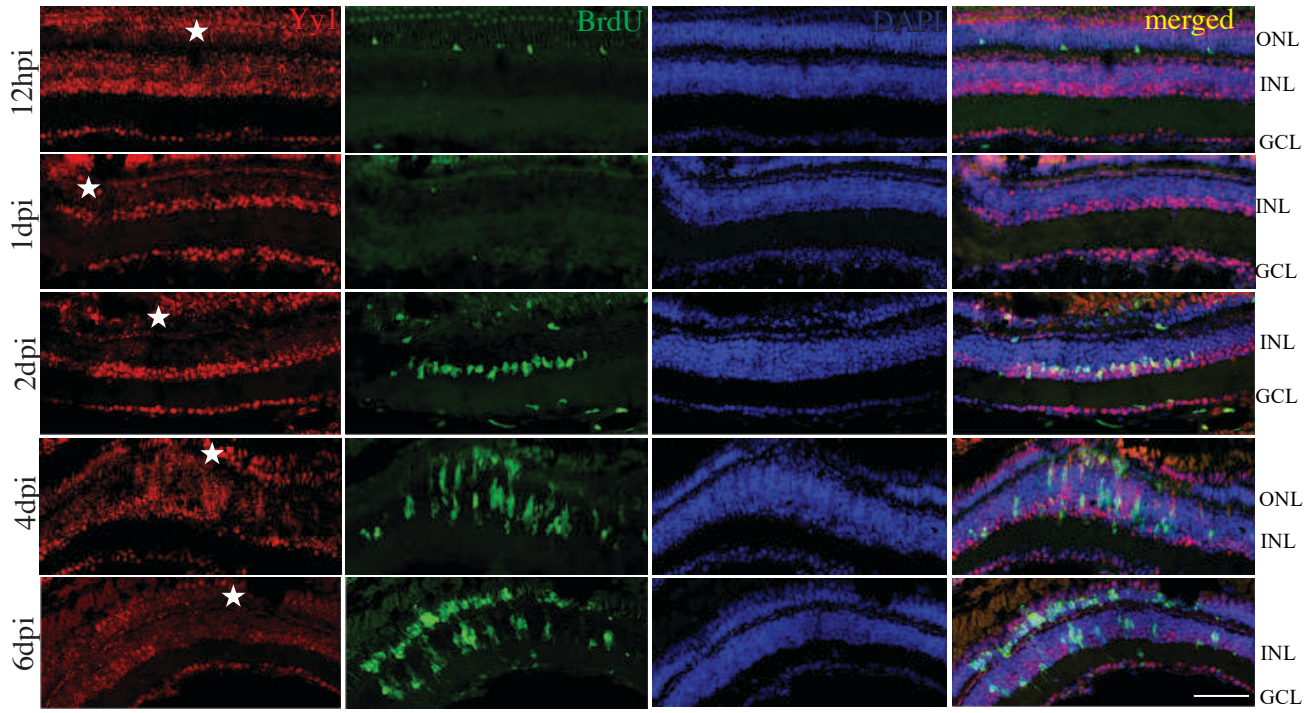


Figure 3.1.2.3. *yy1a* and *yy1b* are excluded from the proliferating cells. (A) qPCR analysis shows that the *yy1a* and *yy1b* are expressed more in the GFP$^-$ fraction of the FACS sorted cells from the retina of *1016 tuba:GFP* transgenic line. P<0.005.

A.



B.

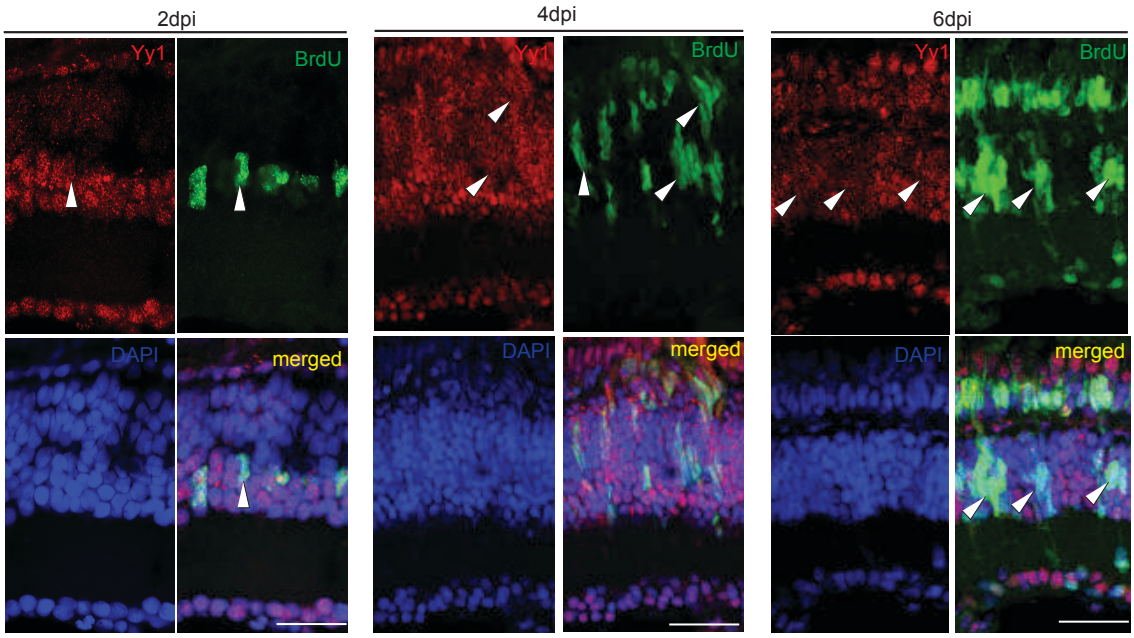


Figure 3.1.2.3. (A) Immunostaining shows pan retinal expression of Yy1 (marked in red) at different time points post retinal injury. **(B)** 60X images at 2dpi, 4dpi and 6dpi shows the exclusion of Yy1 from the $BrdU^{+}$ cells. BrdU marks the proliferating cells and DAPI marks the nucleus. White star marks the injury site. Arrow head marks the $BrdU^{+}$ and $Yy1^{-}$. Scale bar represents 10µM.

A.

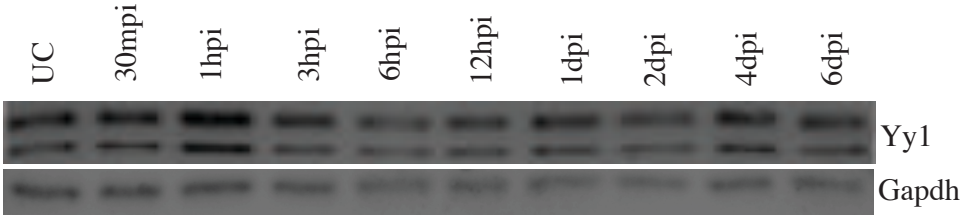


Figure 3.1.2.4. (A) Western blot analysis of whole retinal lysates at different time points retinal post injury shows no significant change. mpi is minutes post-injury.

3.1.3 Yy1 is essential for the reprograming and proliferation of Müller glial cells

The literature survey gave us a clue on the role of Yy1 in embryonic development, cancer and regeneration, such as muscle cell and axon regeneration. We also know that both *yy1a* and *yy1b* were getting regulated during the course of retina regeneration. Therefore, we sought to explore further its role in the Müller glia reprograming and proliferation and tweaked with the expression and function of Yy1 using two approaches.

1. Translation of *yy1a* and *yy1b* was blocked using lissamine-tagged morpholinos (MOs) targeted to bind to their translational start site (Thummel, Bailey, & Hyde, 2011). Morpholinos are the modified oligos that binds to the complementary RNA and block its translation or its splicing. Lissamine attached to it has a positive charge and helps direct the MOs to enter the cells. MOs are stable and remain there for couple of days and knock-down the expression of gene (Nasevicius & Ekker, 2000).
2. We blocked the translation and DNA binding ability of Yy1 using the pharmacological inhibitor DETANONOate, which is the NO oxide donor and S-nitrosylates YY1 at the cysteine residue and inhibits its DNA binding ability (Hongo et al., 2005) (Garban & Bonavida, 2001).

To check the role of Yy1 during retina regeneration, we injected the MO against *yy1a* and *yy1b* into the vitreous of the retina at different concentrations (0.25mM, 0.5mM and 1mM) at the time of injury. We electroporated immediately to block the translation of both these genes so that their effect on the dedifferentiation and proliferation can be seen. At 4dpi, we gave BrdU pulse to the fish and after 5 hours harvested the eye, fixed and cryoprotected it for immunostaining (Figure 3.1.3.1.A). We saw that there was a significant decline in the number of proliferating MGPCs (Müller glia derived progenitor cells), which are marked by BrdU (Bromodeoxyuridine, is a thymidine analogue that gets incorporated in DNA during the S-

phase of the cell cycle) and PCNA (Proliferating cell nuclear antigen), as compared to the control retinae (Figure 3.1.3.1.B). Knockdown of *yy1b* did not cause a significant reduction in the $BrdU^+$ and $PCNA^+$ cells as compared to the *yy1a* knockdown retinae (Figure 3.1.3.1.C). To see if Yy1 and Yy1b have an independent role or have a synergistic effect, we did a combined knockdown of *yy1a* and *yy1b* and found a more pronounced impact on the number of proliferating MGPCs at 4dpi (Figure 3.1.3.1.D).

Further, checked the role of Yy1 in the later phase of regeneration to see if it helps the MGPCs to exit the cell cycle. For this, we designed an experiment in which we injured the retina and injected the MOs into the vitreous but did not electroporate it. At 4dpi, we pulsed the fish with BrdU and then electroporated the eye so that MO enters the retina in the later stage of regeneration, i.e. when the redifferentiation starts (Figure 3.1.3.2.A). At 9dpi, we gave EdU pulse, which is another analogue of thymidine, and eyes were harvested after 5hours. We observed that the number of $BrdU^+$ cells remained same in the combined knockdown of *yy1a* and *yy1b* as compared to control but the number of EdU^+ cells that were $BrdU^+$ also declined significantly and there were no new EdU^+ cells (Figure 3.1.3.2.B). Our results shows that knockdown of *yy1* in the late phase resulted in the early exit from the cell cycle indicating its role in the MGPCs proliferation and does not have any role in the cell cycle exit (Figure 3.1.3.2.C).

Further, we used a pharmacological inhibitor for Yy1, DETANONOate, to see its effect on the proliferation. We injured the fish and dipped in the drug at different concentrations for 4 days. We harvested the eye at 4dpi after the BrdU

pulse for 5 hours (Figure 3.1.3.3.A). In concurrence with the result obtained in the knockdown of *yy1a* and *yy1b,* there was a dose-dependent decline in the number of MGPCs as seen with PCNA and BrdU staining at 4dpi (Figure 3.1.3.3.B), which was quantified also (Figure 3.1.3.3.C).

We also employed PHH3 (Phospho Histone 3) staining to see the effect of DETANONOate on the mitotically active cells. PHH3 stains the condensed chromatin at the anaphase/ telophase boundary, and thus it is a more accurate determinant of the proliferating cells (J. Y. Kim et al., 2017). We observed a drastic decline of $PHH3^{+}$ cells at the highest concentration of the drug used, thus confirming the importance of Yy1 in the proliferation of MGPCs (Figure 3.1.3.4.A), which was quantified also (Figure 3.1.3.4.B). We also ensured that the drug to inhibit the function of Yy1 did not result in apoptosis, we performed Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. This technique detects DNA fragmentation during apoptosis, wherein the TdT enzyme binds to the 3' end of the single-stranded DNA and add random labelled nucleotides which can be detected (King, 2007). There was no significant increase in the number of apoptotic cells in the treatment with DETANONOate (Figure 3.1.3.4.C) and thus confirming that the functional inhibition of Yy1 not only decreases the proliferating cells but also does not lead to cell death.

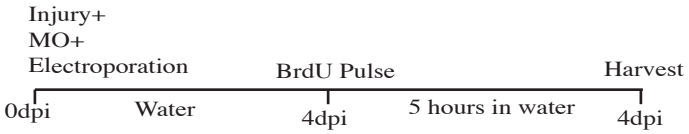
Next, we questioned whether overexpression of Yy1 has an opposite or similar effect as that of knockdown. We made mRNA of *yy1a* and *yy1b* by *in-vitro* transcription and injected it at different concentrations, individually and in combination, at the time of injury (Sharma & Ramachandran, 2019). We followed the same regime as that in knockdown and harvested the eye at 4dpi (Figure 3.1.3.5.A). We found that the overexpression of both *yy1a* and *yy1b* individually caused an increase in the number of MGPC as compared to the control *gfp* mRNA but this effect was more profound in case of *yy1a* overexpression (Figure 3.1.3.5.B), which was quantified also (Figure 3.1.3.5.C). In the combined overexpression, we found an increase in the number of proliferating cells marked by BrdU and PCNA (Figure 3.1.3.6).

To validate the efficacy of the MO and to see if the effect of *yy1* knockdown could be redeemed, we performed a rescue experiment. For this, we did silent point mutation in the *yy1a* MO and *yy1b* MO binding site by Site-directed mutagenesis (SDM) in the plasmid containing *yy1a* and

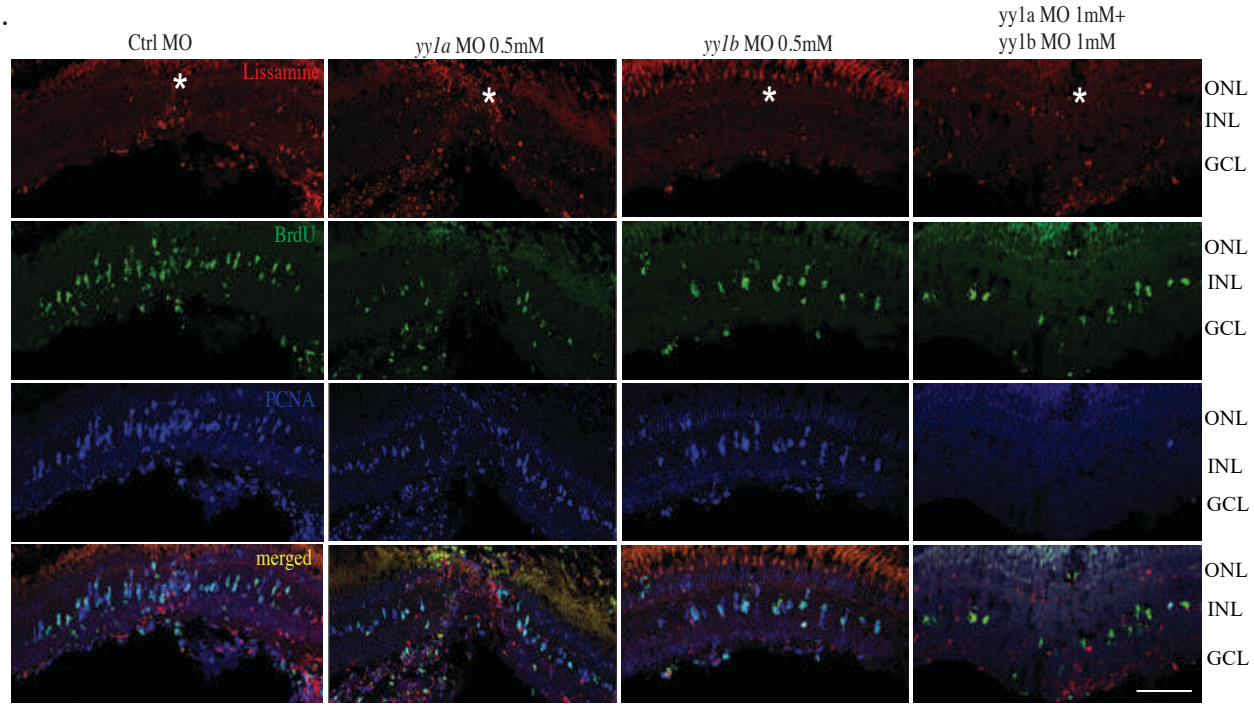
yy1b CDS (pCS2+ -*yy1a* and pCS2+ -*yy1b*). We then *in-vitro* transcribed the mRNA from the mutated construct and co-injected *yy1a* MO or *yy1b* MO along with the respective MO binding site mutated RNA into the retina and observed the effect at the 4dpi. We found that the number of MGPCs in the rescue experiment were almost similar to that of the control, wherein *control (ctl)* MO was injected with *gfp* mRNA in both the conditions (Figure 3.1.3.7.A), which was quantified also (Figure 3.1.3.7.B).

We also made a transgenic line in which *gfap* promoter drives the expression of 2X FLAG-Yy1a-2X FLAG-GFP (Figure 3.1.3.8.A). GFAP is a Müller glial cell marker. Upon injury in the retinae of this transgenic line, we found that there was pan retinal increase in the number of proliferating cells marked by EdU (Figure 3.1.3.7.B). This again proves the pro-proliferative role of Yy1 during retina regeneration.

A.



B.



C.

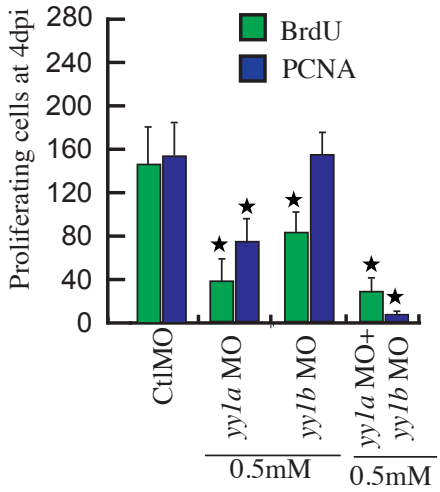


Figure 3.1.3.1. Knockdown of *yy1a* and *yy1b* results in decrease in the proliferation. (A) An experimental timeline showing, injury, electroporation, BrdU pulsing and harvesting. **(B)** Retina injured and electroporated with lissamine tagged MO (red) showed decline in $BrdU^{+}$ (Green) and $PCNA^{+}$ (blue) cells, which are quantified in (C). Star marks the injury site. Scale bar represents 10µM. $p<0.0001$ in (C).

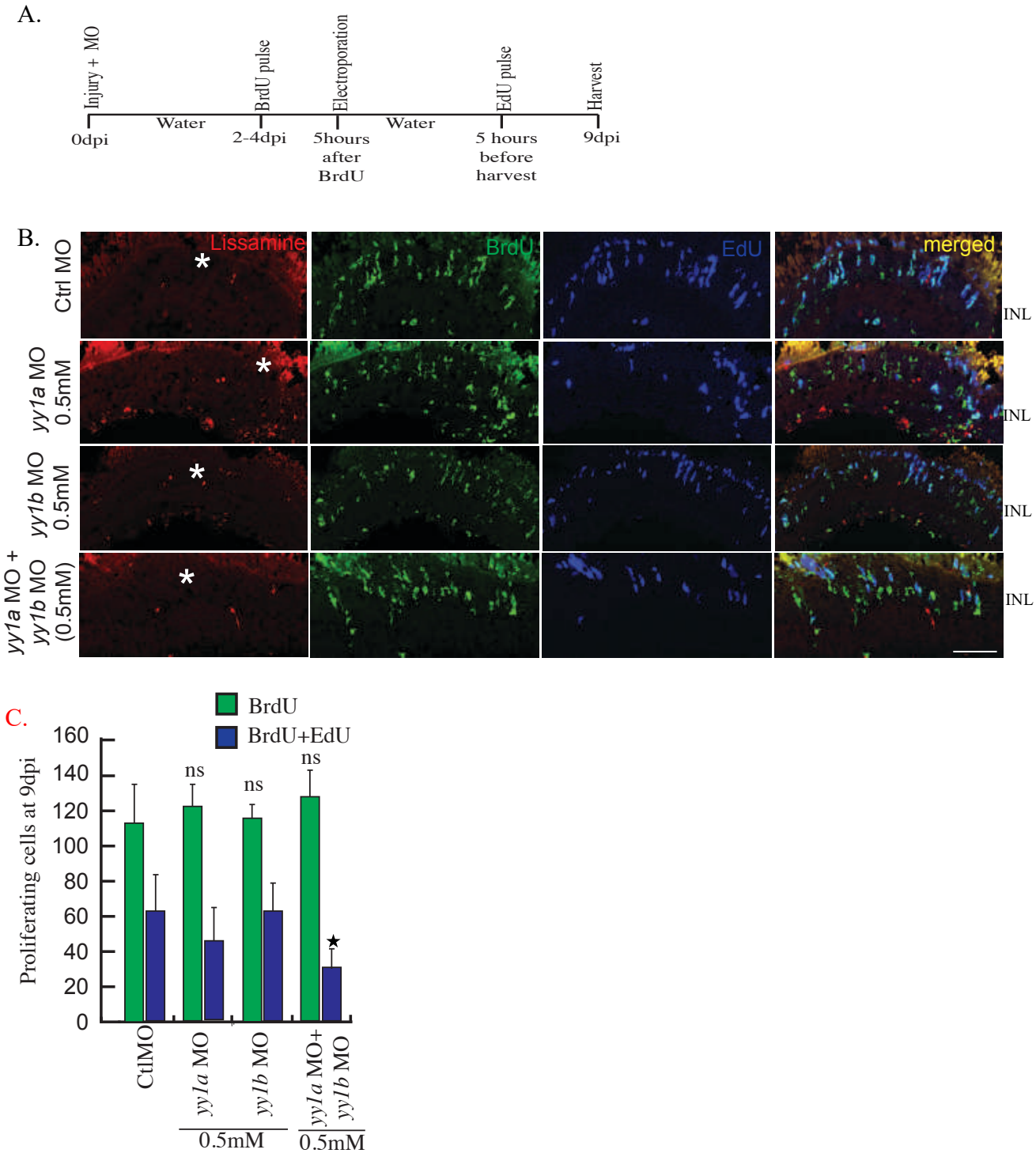


Figure 3.1.3.2. Knockdown of *yy1a* and *yy1b* results in decrease in the proliferation. (A) An experimental timeline showing, injury, electroporation, BrdU and EdU pulsing and harvesting. **(B)** Retina injured and electroporated with lissamine tagged MO (red) during late phase showed decline in $BrdU^+$ (Green) + $PCNA^+$ (blue) cells, which are quantified in **(C).** White star marks the injury site. Scale bar represents 10 µM in B. $p<0.005$. ns is nonsignificant

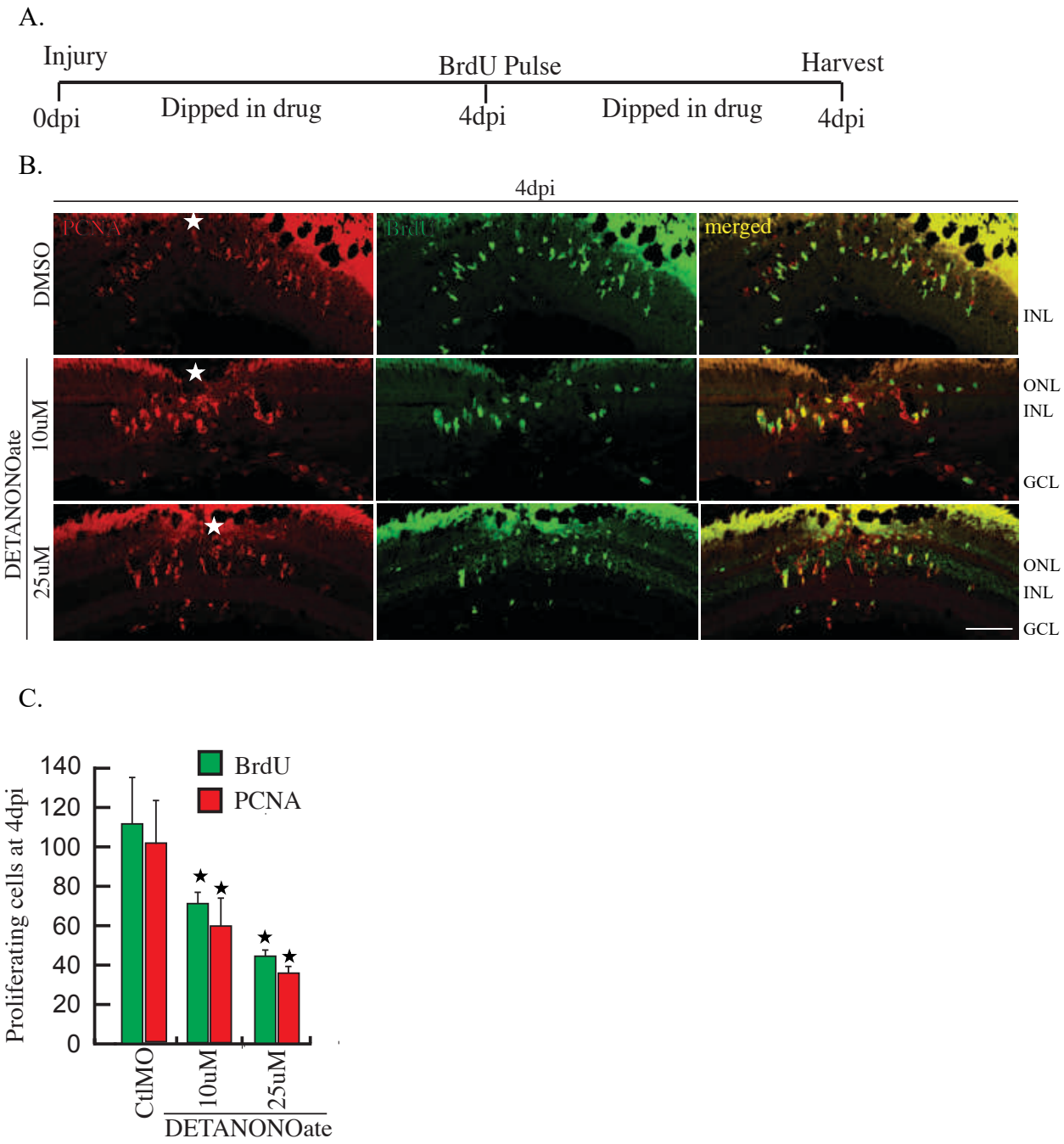


Figure 3.1.3.3. Pharmacological inhibitor of Yy1, DETANONOate, results in decrease in the proliferation. (A) An experimental timeline showing, injury, dipping in the drug, BrdU pulsing and harvesting. **(B)** Confocal microscopy images of retinal sections from fish dipped in DETANONOATE shows decrease in the number of proliferating cells marked by PCNA (red) and BrdU(green), which are quantified in **(C).** Star marks the injury site in B. Scale bar represents10 μM. $p<0.0001$.

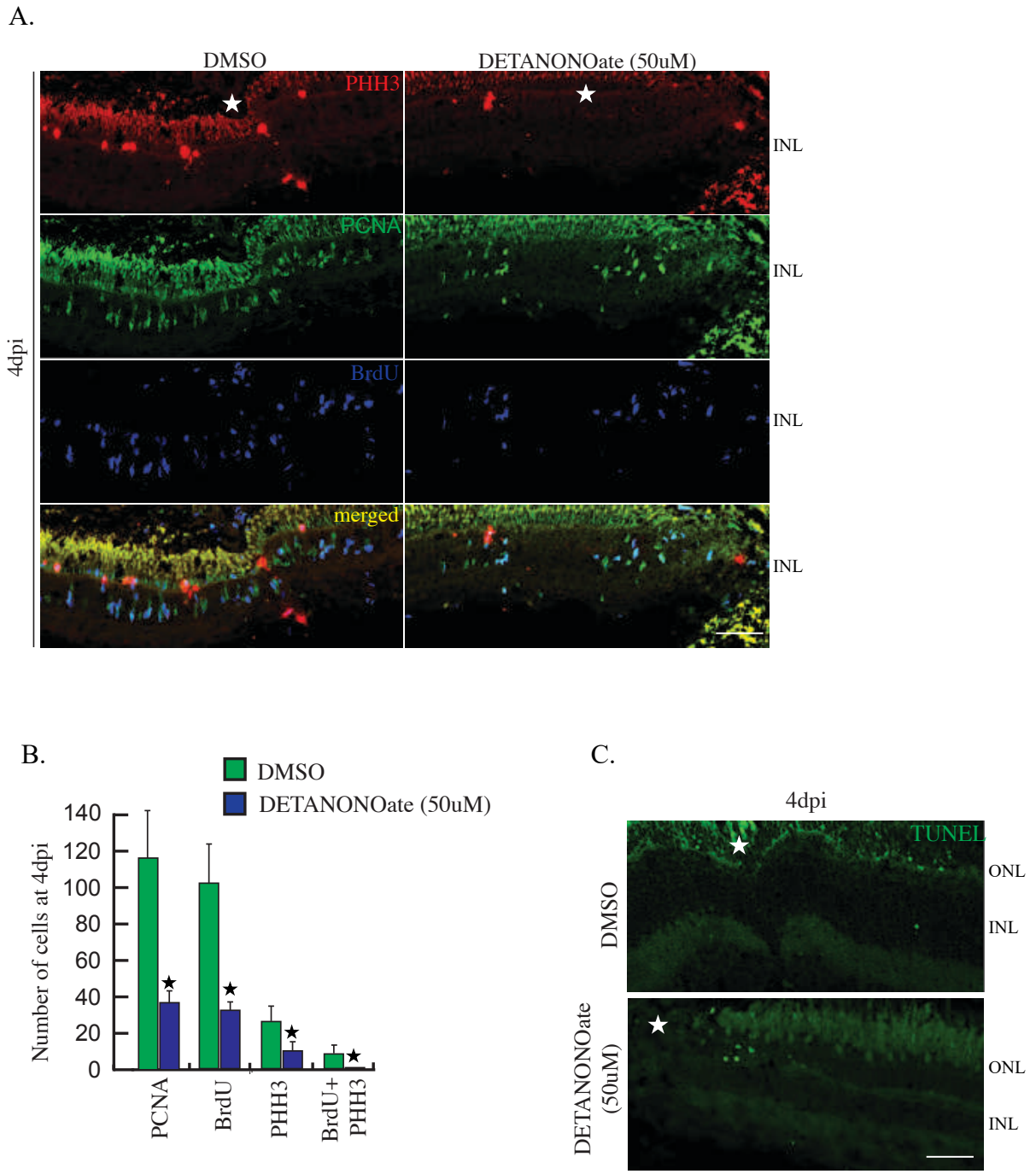


Figure 3.1.3.4. DETANONOate results in decrease in the actively mitotic cell number. (A) Confocal microscopy images showed decrease in the number of actively mitotic cells, marked by PHH3 (red) and colocalized with BrdU (green) and PCNA (blue), which is quantified in **(B). (C)** Confocal microscopy images of retinal sections from fish dipped in DETANONOATE did not show any significant number of TUNEL^{+} cells (green). White star marks the injury spot. Scale bar is 10μM in A and C. $p<0.05$

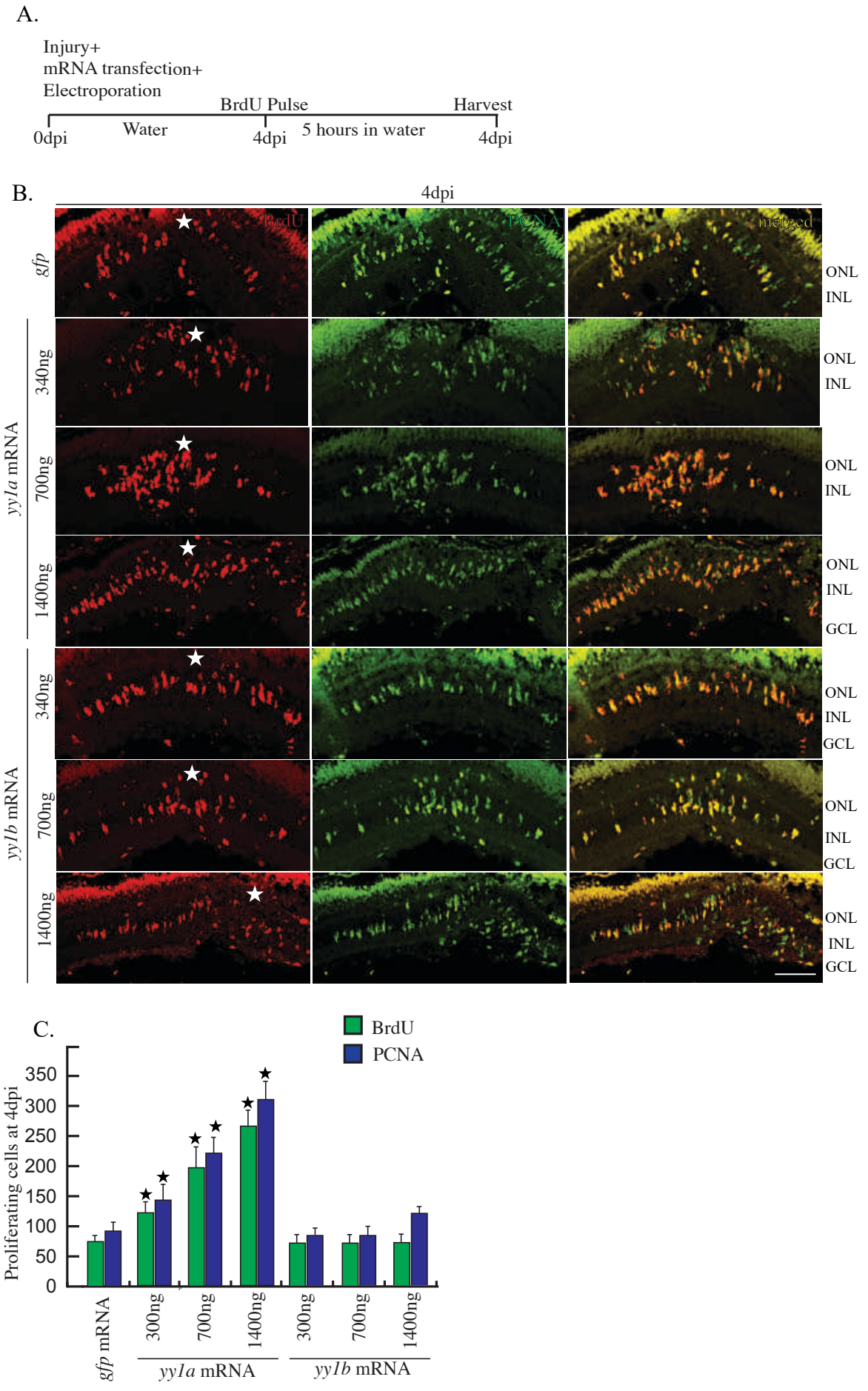

A.
Injury+
mRNA transfection+
Electroporation
BrdU Pulse
Harvest
0dpi
Water
4dpi
5 hours in water
4dpi
B.
4dpi
BrdU
PCNA
merged
gfp
yy1a mRNA
340ng
700ng
1400ng
yy1b mRNA
340ng
700ng
1400ng
ONL
INL
GCL
C.
BrdU
PCNA
Proliferating cells at 4dpi
0
50
100
150
200
250
300
350
gfp mRNA
300ng
700ng
1400ng
yy1a mRNA
300ng
700ng
1400ng
yy1b mRNA

Figure 3.1.3.5. Overexpression of *yy1a* and *yy1b* resulted in the increase in the number of proliferating cells. (A) Experimental timeline showing injury, mRNA transfection, BrdU pulsing and harvest at 4dpi. **(B)** Confocal microscopy images transfected with the *yy1a and yy1b* mRNA individually led to a concentration dependent increase in the number of MGPCs marked with BrdU (red) and PCNA (green), which is quantified in **(C).** Star marks the injury site. Scale bar represents 10μM in B. $p<0.0001$.

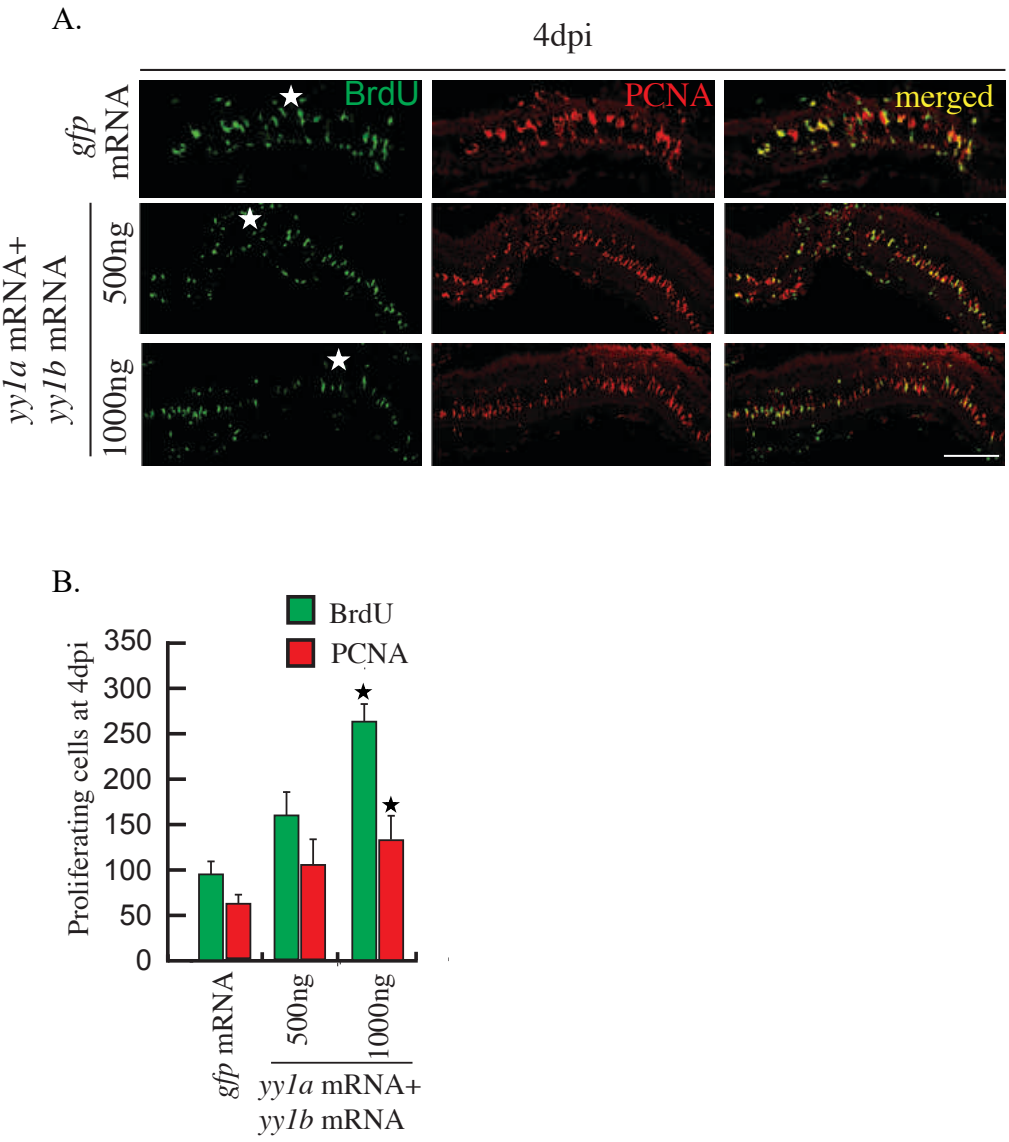
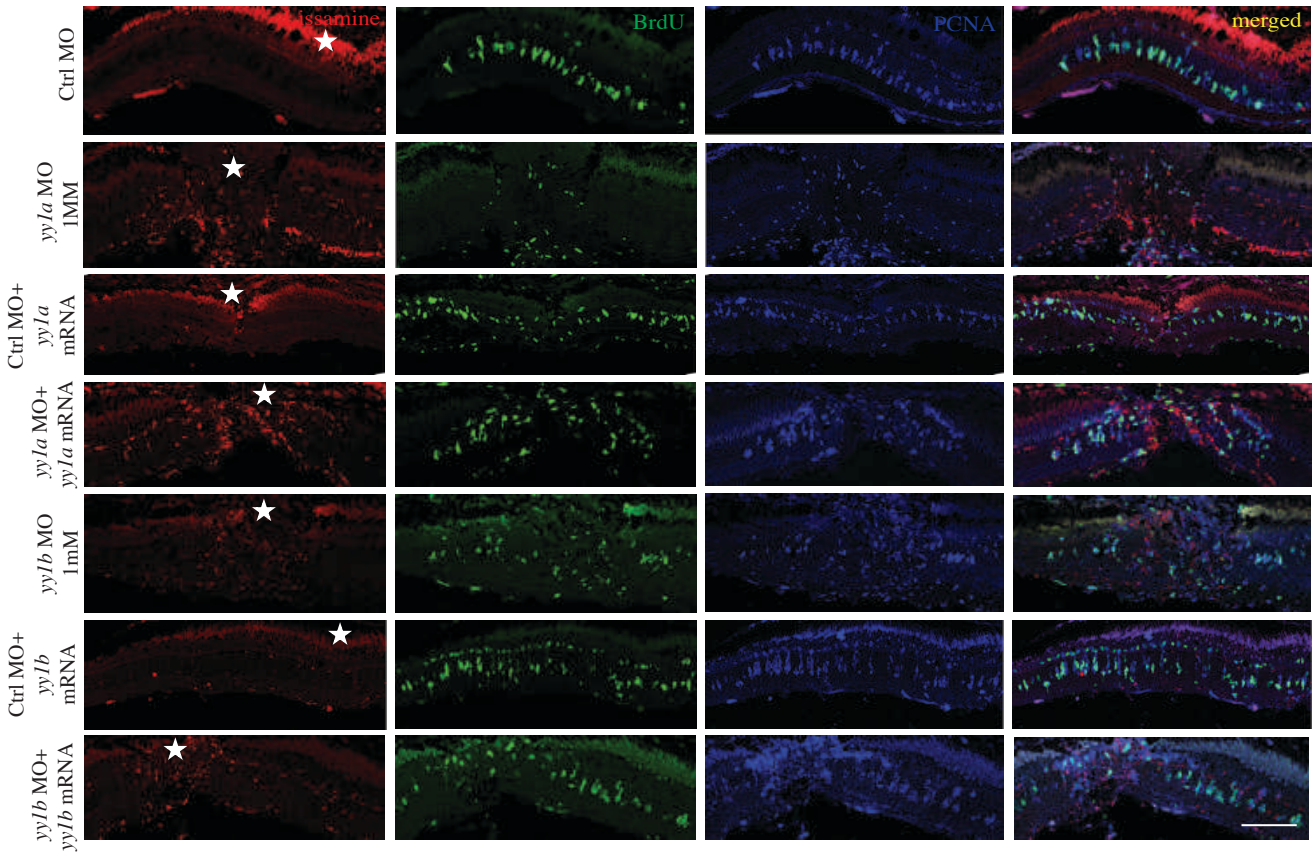


Figure 3.1.3.6. Combined Overexpression of *yy1a* and *yy1b* also resulted in the increase in the number of MGPCs. (A) Confocal microscopy images of retinal section also showed a marked increase in the number of MGPCs marked with BrdU (green) and PCNA (red), which is quantified in **(B).** White star marks the injury site. Scale bar represents 10μM. $p<0.005$ in B.

A.



B.

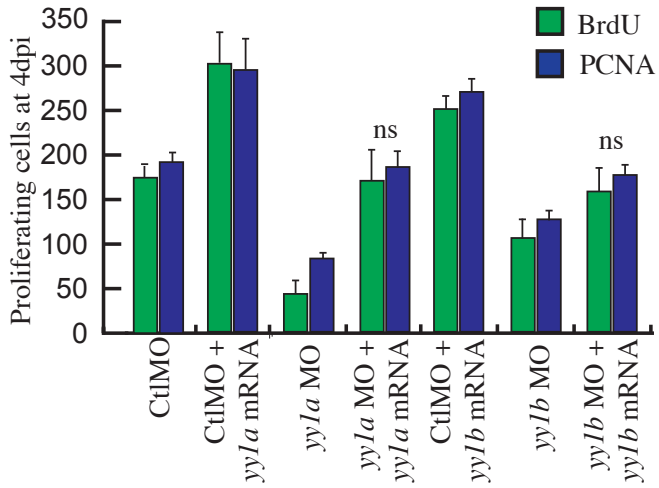


Figure 3.1.3.7. Mutated mRNA for MO binding site could rescue the effect of *yy1a* and *yy1b* knockdown. (A) Confocal microscopy images of retinal section also showed a marked increase in the number of MGPCs marked with BrdU (green) and PCNA (red), which is quantified in **(B).** White star marks the injury site. Scale bar represents 10μM. ns is non-significant w.r.t ctl MO.

A.

B.

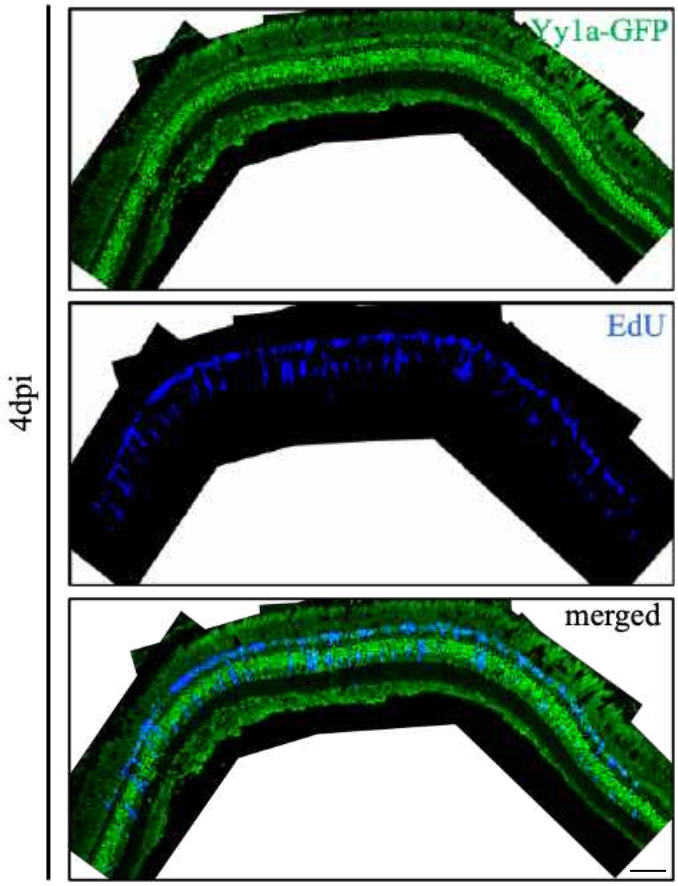


Figure 3.1.3.8. Transgenic line for overexpressing Yy1 in the Müller glial cells. (A) Diagrammatic representation of *gfap* promoter driving 2x-FLAG-Yy1a-2X-GFP cassette cloned in pTAL vector **(B).** Confocal microscopy images of retinal sections of *gfap:* 2X-FLAG-2X-Yy1a-GFP transgenic line, at 4dpi, shows pan retinal increase in the number of EdU^{+} cells. Scale bar represents 10µM in B.

3.1.4 Lineage tracing of newly formed cells in Yy1 overexpressed conditions

Successful regeneration culminates in the structural and functional restoration of lost or damaged organs. Injury-induced reprograming of Müller glia cells to form MGPCs, which have the stem cell-like property, and subsequent proliferation, must eventually lead to re-differentiation to form all the retinal cell types ,thus restoring the retina's homeostasis. It takes about 20-30 days for the retina to completely restore its vision.

As discussed earlier, the overexpression of Yy1 leads to an increase in the proliferating MGPCs. So, we questioned whether these increased numbers of $BrdU^+$ cells have the capacity to survive or they eventually die. For this, we injured the retina and transfected the retina with the *yy1a* and *yy1b mRNA* and labelled the proliferating cells with BrdU on 3, 4 and 5^{th} day and harvested the eyes on the 23^{rd} day (Figure 3.1.4.1.A). We found that they were indeed alive even on 23^{rd} day and the number of $BrdU^+$ cells were still present in more number than the *gfp* mRNA transfected retina (Figure 3.1.4.1.B), which was quantified also (Figure 3.1.4.1.C). To see if these increased number of cells in the Yy1 overexpressed condition could re-differentiate into retinal subtypes, we performed cell type-specific staining on the slides with serial sections. We found that these $BrdU^+$ cells could form various retinal subtypes at 23^{rd} dpi, such as Protein kinase C (PKC), amacrine cells with HuC/D as the marker and also glutamine synthetase (GS) positive cells, which is the marker for the Müller glia (Figure 3.1.4.1.D). Therefore, our result indicates that Yy1, when overexpressed, not only helps in proliferation but also these increased number of cells are functionally viable and being multipotent, they re-differentiates into major retinal cell types.

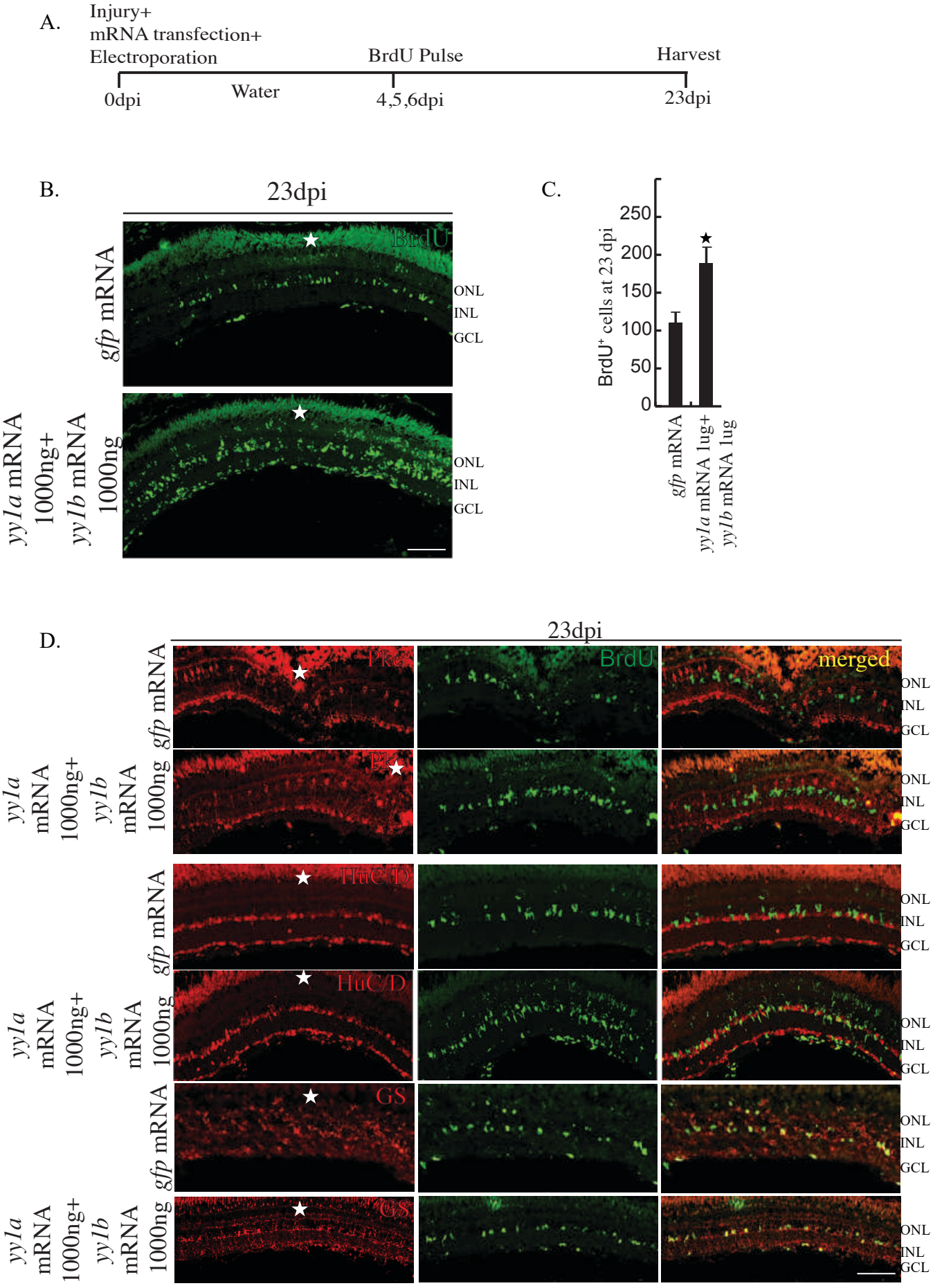

A.
Injury+
mRNA transfection+
Electroporation
BrdU Pulse
Harvest
0dpi
Water
4,5,6dpi
23dpi
B.
23dpi
gfp mRNA
yy1a mRNA 1000ng+ yy1b mRNA 1000ng
BrdU
ONL
INL
GCL
C.
BrdU+ cells at 23 dpi
250
200
150
100
50
0
gfp mRNA
yy1a mRNA 1ug+ yy1b mRNA 1ug
D.
23dpi
PKC
BrdU
merged
HuC/D
GS

Figure 3.1.4.1. Increased number of MGPCs in overexpressed *yy1* condition, were viable up to 23 dpi also. (A) Experimental timeline describes the injury and mRNA transfection at 0dpi, BrdU pulse at 4,5,6 dpi and harvest at 23dpi. **(B)** Confocal microscopy images of retinal sections showed that increased number of $BrdU^+$ cells were viable up to 23dpi as well, which were quantified in **(C).** Confocal microscopy images of retinal section shows that these viable MGPCs were able to form different retinal cell types, where PKC marks the amacrine cells, HuC/D marks the bipolar cells and GS marks the Müller glia cells. White star marks the injury site. Scale bar represents 10μM. $p<0.0001$.

3.1.5 Injury is needed for the Yy1 to function as a pro-proliferative factor.

Since we saw that overexpression of Yy1 leads to increased proliferation of MGPCs we were intrigued to find whether mere overexpression of Yy1 is sufficient to cause reprograming of the Müller glia cell. For this, we injected the invitro transcribed mRNA of *yy1a* and *yy1b* and injected into the retina from the cornea without disturbing the retina. At 4dpi, we did BrdU pulsing and harvested the eye to see the effect in the proliferation. We did not see $BrdU^+$ and $PCNA^+$ cells in the uninjured, *yy1* overexpressed retina (Figure 3.1.5.1.A). Our results, confirm that initial signalling pathways need to be activated to provide initial clues to Yy1 to initiate the reprograming events to form MGPCs and thus proliferation of these MGPCs.

Some early signalling pathways initiated soon after injury are c-Fos-AP1 pathway, TGF-β signalling and Shh-signalling. We individually blocked all these signalling, using specific blockers, and looked at the expression pattern of Yy1. We blocked TGF-β signalling using SB431542, which inhibits the receptors ALK 4,5,7 of TGF-β without affecting the BMP signalling (Inman et al., 2002). Our lab's previous finding has discussed about the pro-proliferative role of TGF- β signalling and that it is induced soon after injury as early as 3hpi (Sharma et al., 2020). On blocking TGF- β signalling, we observed a decline in the levels of Yy1 (Figure 3.1.5.1.B). We then checked what happens to the levels if we overexpress TGF-β and as expected, there was an increase in the YY1 levels (Figure 3.1.5.1.C).

Another signalling pathway which is induced even by15mpi (minutes post injury) is the cfos-AP1 signalling. The c-Fos is an immediate early gene which is known to positively regulate Müller glia proliferation (Todd & Fischer, 2015) (W. A. Campbell et al., 2023) (Gupta et al., 2023) and it is expressed soon after injury in response to extracellular stimuli, such as neuronal growth factors, changes in the calcium levels and neurotransmitters. Upon inhibiting this signalling there was a reduction in the levels of Yy1 at 2dpi (Figure 3.1.5.1.D)

Sonic hedgehog signalling (Shh signalling) is another very early induced signalling which comes up as early as 6 hrs post-injury (Kaur et al., 2018). Inhibiting Shh signalling using a pharmacological inhibitor, cyclopamine, caused a decline in the levels of Yy1 at 2dpi (Figure 3.1.5.1.E).

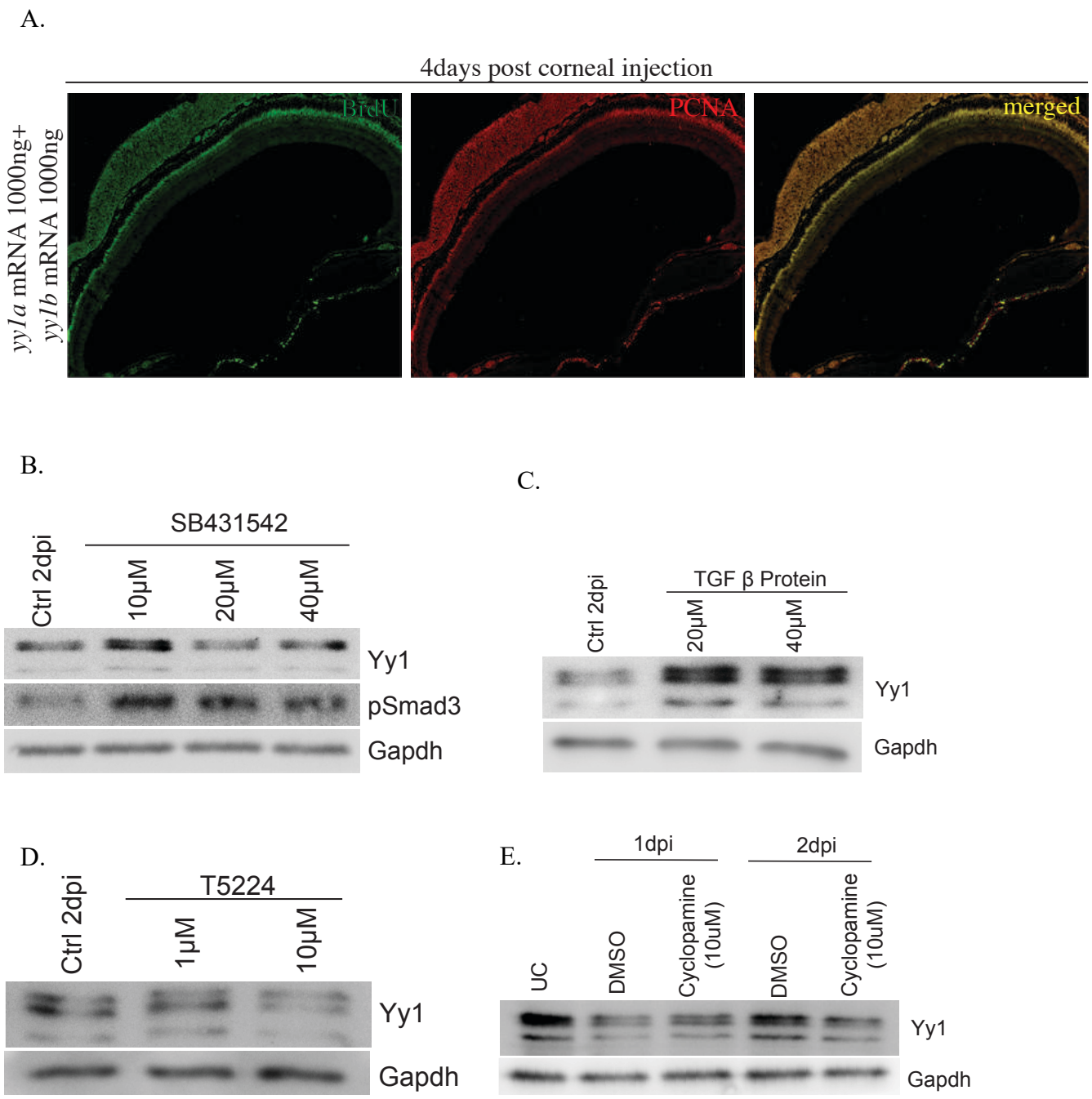


Figure 3.1.5.1. Injury induced early signalling events are necessary for the Yy1-mediated response of the proliferation. (A) Confocal microscopy images shows no proliferation upon intracorneal injection of *yy1a* and *yy1b* mRNA. **(B-E)** Western blot analysis shows decline in the levels of Yy1 upon treatment with SB431542 **(B),** cyclopamine **(E)** and T5224 **(D)**, at 2dpi while increase in its level with the treatment of TGF-β protein **(C).**

Chapter 2

Yy1-mediated gene regulatory network during retina regeneration is important to evoke successful and controlled proliferation

3.2.1 Yy1 is auto-regulated during retina regeneration

The time course analysis of Yy1 showed a decline at the mRNA levels at around 1 and 2dpi (both *yy1a* and *yy1b*) and again came up at around 4dpi though excluded from the proliferating MGPC's nuclei. This decline happens during the de-differentiation phase when Müller glial cells are undergoing reprograming and adopting stem cell-like fate. Despite this, we conclude from our previous results that Yy1 has a pro-proliferative role during retina regeneration.

Moreover, in the above result, we found that pro-proliferative early signalling events positively regulated Yy1. We were amazed to see that these blockade of these early signalling pathways, which decreases the proliferation, there should have been increase in the levels of *yy1a* and *yy1b* because from the transcript level in the time course, we believe that dedifferentiation phase is associated with the downregulation of Yy1. At 2dpi, when the transcript levels of *yy1a* and *yy1b* are low, the protein levels do not change significantly. This prompted us to see if Yy1 regulates the transcription of its own gene during retina regeneration.

We found from the literature that the 1st exon and 1st intron of Yy1 is well conserved amongst many species, including humans, zebrafish and mouse (J. D. Kim, Yu, & Kim, 2009). We looked into the first intron of zebrafish *yy1a* and found six putative Yy1 binding sites (Figure 3.2.1.1.A). We made primers spanning these sites and performed ChIP using Anti-YY1 antibody and found that indeed Yy1 binds to these sites. We then looked at what happens to the binding of Yy1 when we decrease its level either by knockdown or by disrupting the DNA binding ability of Yy1 using the drug, DETANONOate. We found that Yy1 binding is almost completely lost in *yy1* knockdown and remained the same during the DETANONOate (Figure 3.2.1.1.B). We confirmed this loss of binding in the knockdown condition quantitatively by qPCR as well (Figure 3.2.1.1.C). We also checked the transcripts levels of Yy1 in the *yy1* knockdown condition and found that levels of both *yy1a* and *yy1b* decrease in the knockdown condition indicating that it is positively regulated through its binding site (Figure 3.2.1.1.D).

Yy1 helps in maintain the homeostasis of its gene, and when the transcript levels go down during the dedifferentiation phase, it upregulates its expression to maintain the constant basal level of Yy1, which is needed for the regeneration to happen.

This could also be the reason for our previous result that blocking the pro-proliferative early signalling pathways not only decreased the transcripts levels of *yy1a* and *yy1b,* but it also led to the decrease in its protein levels, which could be reason for the reduction in the proliferation of MGPCs.

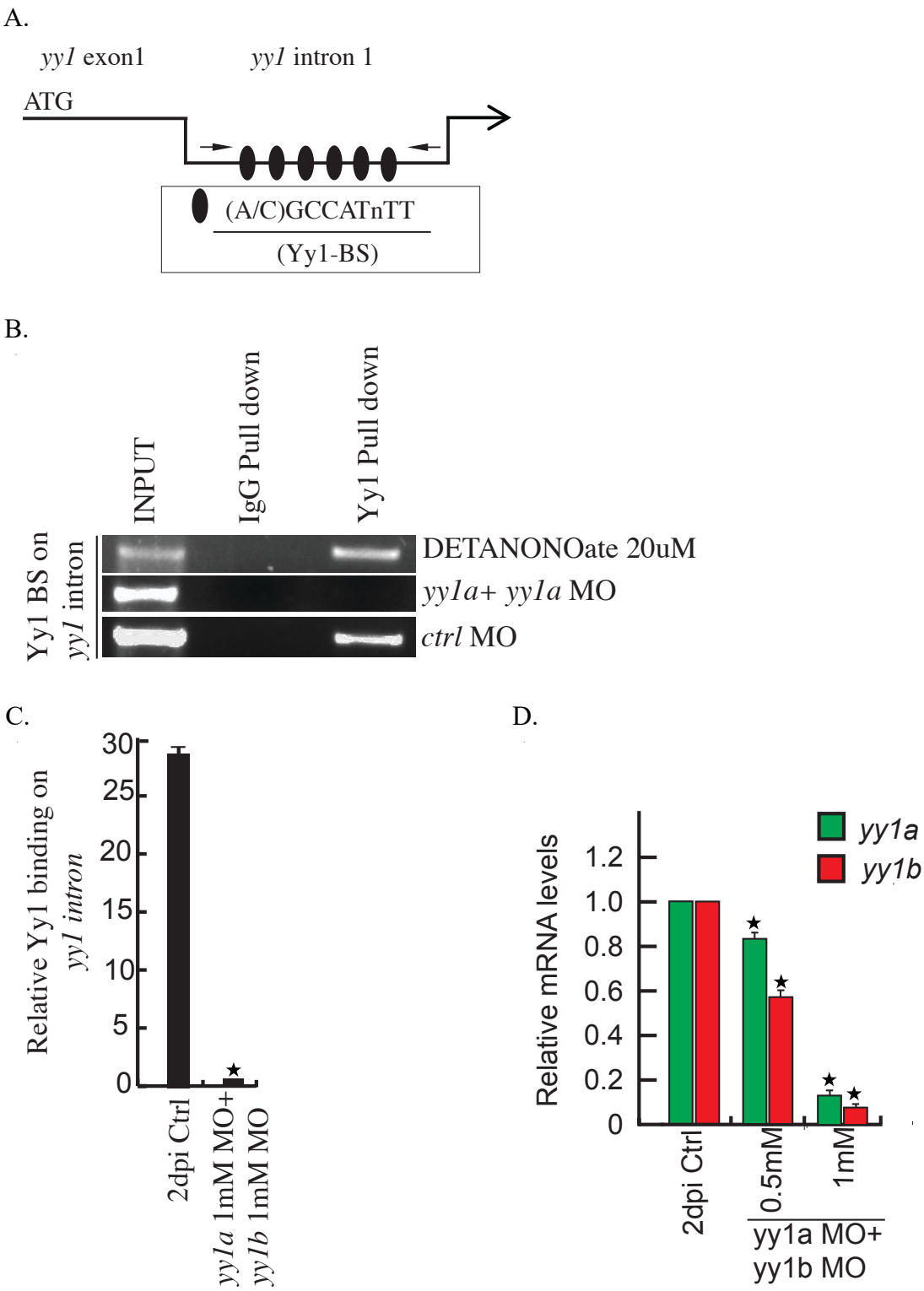


Figure 3.2.1.1. Yy1 is autoregulated through its own binding sites in the first intron. (A) Diagrammatic representation of *yy1a* 1st introns reveals six putative Yy1 binding site. **(B)** ChIP assay done at 2dpi confirms the physical binding of Yy1 on the its own intron and this binding is lost in the knockdown condition. **(C)** qPCR analysis further confirms the loss in the Yy1 binding in the *yy1a* and *yy1b* knockdown condition. **(D)** qPCR analysis shows that Yy1 positively regulates its own levels. P< 0.0001 in C and D.

3.2.2 Yy1 regulates Müller glia reprograming through Ascl1a-Lin28a-*let-7a* axis

Ascl1a is considered the master regulator of retina regeneration, while Lin28a is also a major regulator. Both are drastically induced very early post-retinal injury and are necessary for the de-differentiation of Müller glia to form retinal progenitor cells called MGPCs (Ramachandran et al., 2010). They are known to regulate many signalling pathways and regeneration associated genes (Kaur et al., 2018) (Ramachandran et al., 2011) (Mitra et al., 2019). In mice, it has been reported, that forced expression of ASCL1 along with HDAC1 inhibition can cause Müller glial cells to undergo reprograming (Jorstad et al., 2017).

Knockdown of Yy1 caused a decline in the number of proliferating cell in the retina. We decided to dwell deeper into the mechanism underlying this decline. We checked the expression of numerous RAGs in the combined knockdown of *yy1a* and *yy1b* and found that some of them were upregulated, such as *ascl1a, zic2b, sox2 and her4.1,* while some were downregulated, such as *lin28a, mmp9, hdac1,insm1a* and *rb1* (Figure 3.2.2.1.A). We also performed western blot assay of some regeneration-associated genes in the knockdown condition and overexpression of *yy1a* and *yy1b* (Figure 3.2.2.2.A,B). There also we found the dose-dependent increase or decrease in their levels. Looking closely the transcript levels of *ascl1a* and *lin28a,* surprisingly, we found the dose-dependent increase in the transcript level of *ascl1a* while *lin28a* showed a decline (Figure 3.2.2.1.A). Then we looked at the levels of Ascl1a and Lin28a in the western blot assay and found a dose-dependent decrease in their expression in the knockdown of *yy1a* and *yy1b* at 2dpi (Figure 3.2.2.2.A). Furthermore, we wanted to confirm whether *ascl1a* and *lin28a* are directly or indirectly regulated by Yy1. We analyzed the promoter sequences of *ascl1a* (Figure 3.2.2.3.A) and *lin28a* (Figure 3.2.2.4.A) and found a putative binding site. We then performed ChIP to confirm the physical binding and found that indeed Yy1 occupied those sites, thus confirming the direct regulation of both

these genes by Yy1 (Figure 3.2.2.3.B, Figure 3.2.2.4.B). To further confirm this, we performed a luciferase assay in which we injected *ascl1a:gfp-luciferase* and *lin28a:gfp-luciferase* reporter construct along with the *yy1a and yy1b* MOs and their respective *in-vitro* transcribed mRNAs, in separate experiments, in embryos. There was a dose-dependent decrease in the promoter activity of *ascl1a* in the knockdown condition (Figure 3.2.2.3.C), while the opposite was seen in the case of mRNA (Figure 3.2.2.3.D). Similarly, *lin28a* also showed a decline in the promoter activity in the *yy1a* knockdown (Figure 3.2.2.4.C) and an increase in the overexpression condition (Figure 3.2.2.4.D). Ascl1a regulates the expression of Lin28a by directly binding onto its promoter. Lin28a was discovered as an RNA-binding protein and later, its role as a transcription factor was discovered.

Knockdown of *lin28a* leads to decreased proliferation during retina regeneration and is known to be regulated by both Ascl1a (Ramachandran et al., 2011) and Myc b (Mitra et al., 2019). Numerous studies from developmental biology (Yermalovich et al., 2019), embryonic stem cells and even cancer biology have reported that Lin28 inhibits the processing of the *pre-let7* family of transcription factors and stimulates their degradation. Further, *let-7a* is a well-known differentiation marker, which helps in neuronal stem cell differentiation and neuronal differentiation during CNS development (Bateman et al., 2011) (Rybak et al., 2008). Therefore, we were intrigued to find the levels of *let-7a* in the absence of *yy1a* and *yy1b.* We found that in the combined knockdown of *yy1a* and *yy1b,* there was a drastic increase in the levels of *let7a* miRNA (Figure 3.2.2.2.D).

Taken together, upon *yy1a* and *yy1b* knockdown, levels of Lin28a are low and thus, the repression of the *let-7a* is lifted, leading to the exorbitant increase in its level. The transcription of *ascl1a* and *lin28a* is in turn, being regulated by *let-7a*. This could explain the increased transcript levels of *ascl1a* but its reduced translation and hence the protein levels.

Surprisingly, we also found that the levels of Oct4 and Zic2b increased in the combined knockdown of *yy1a* and *yy1b* while decreased in the overexpression. We looked into the promoter sequences of *oct4* (Figure 3.2.2.5.A) and *zic2b* (Figure 3.2.2.5.D) and found putative binding sites and ChIP assay confirmed that, indeed Yy1 is bound onto their promoters and regulates their expression (Figure 3.2.2.5.B,E). We confirmed these results by performing luciferase assay in which we injected *oct4:gfp-luciferase* (Figure 3.2.2.5.C) and *zic2b:gfp-luciferase* (Figure 3.2.2.5.F) into the embryos, separately, along with *yy1a* and *yy1b* MO and found that they are negatively regulated by Yy1.

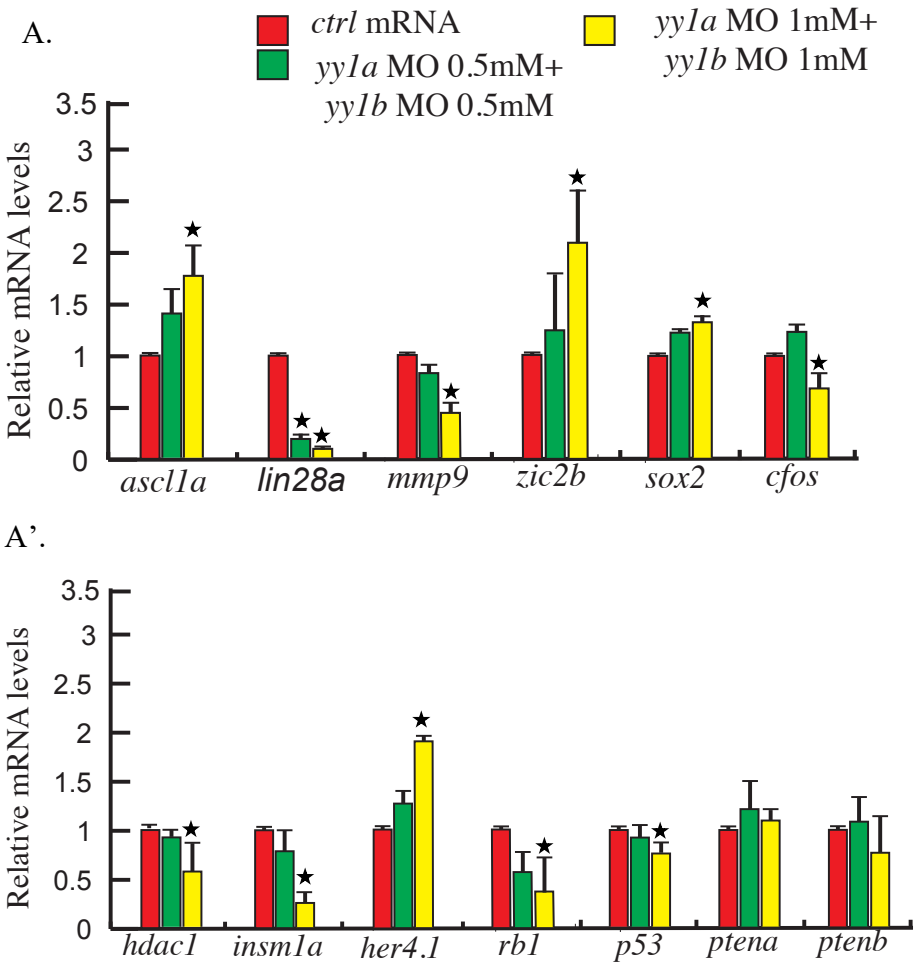


Figure 3.2.2.1. Regulation of RAGs through Yy1 during retina regeneration. (A) qPCR analysis shows the regulation of many RAGs, *ascl1a, lin28a, mmp9, zic2b, sox2, cfos* (upper) and *hdac1, insm1a, her4.1, rb1, p53, ptena, ptenb* (lower), in the *yy1a* and *yy1b* knockdown. $p<0.05$ in A and A'.

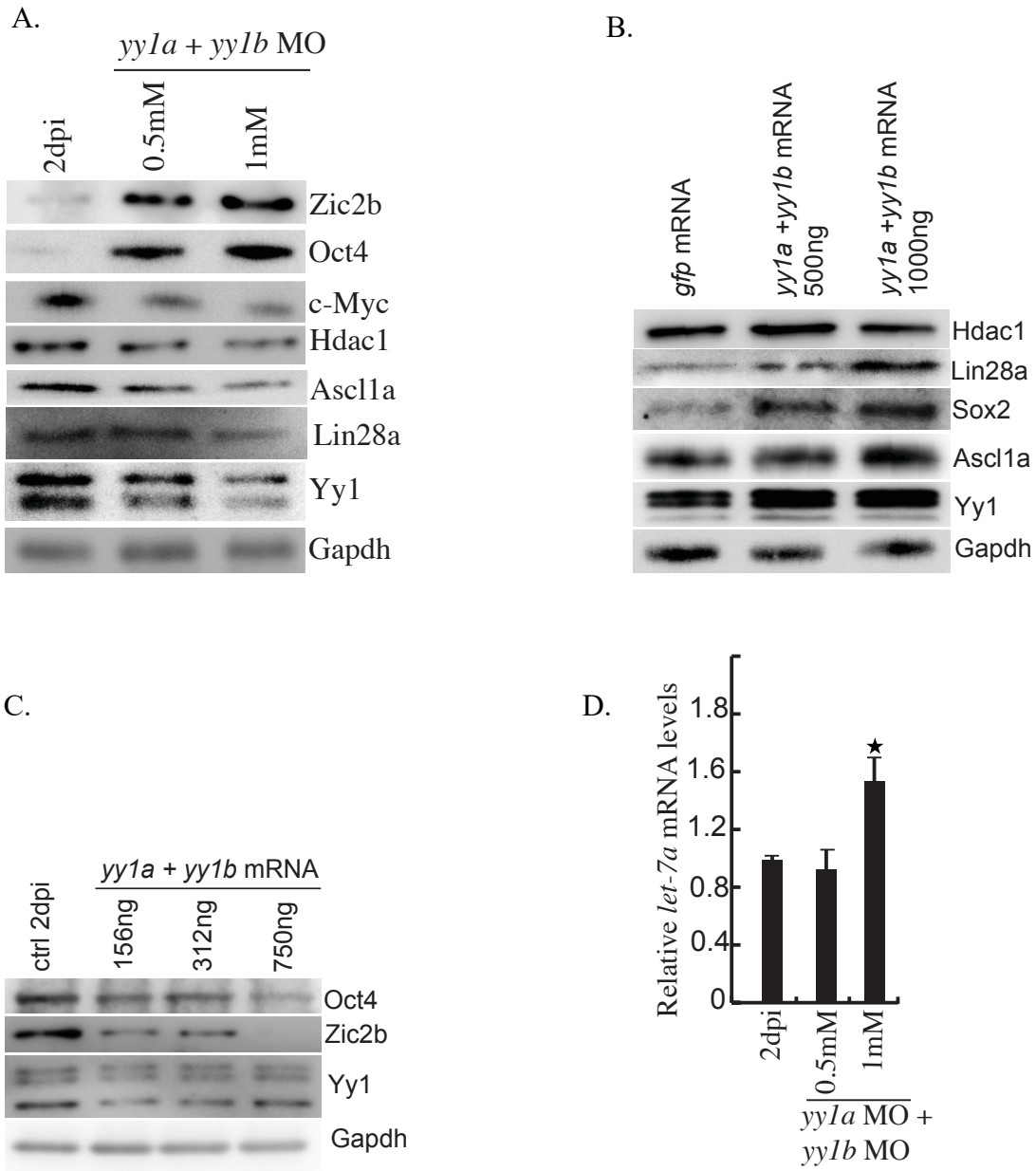


Figure 3.2.2.2. Regulation of RAGs through Yy1 during retina regeneration. (A) Western blot analysis of few RAGs shows regulation in *yy1* knockdown condition. Zic2b and Oct4 shows upregulation while c-Myc, Hdac1, Ascl1a and Lin28a showed downregulation. **(B-C)** Western blot analysis shows the opposite regulation of RAGs in the Yy1 overexpressed conditions, except Hdac1. **(D)** qPCR analysis shows the increase in the levels of miRNA *let-7a* in the knockdown of *yy1a* and *yy1b.* $p<0.05$ in (D).

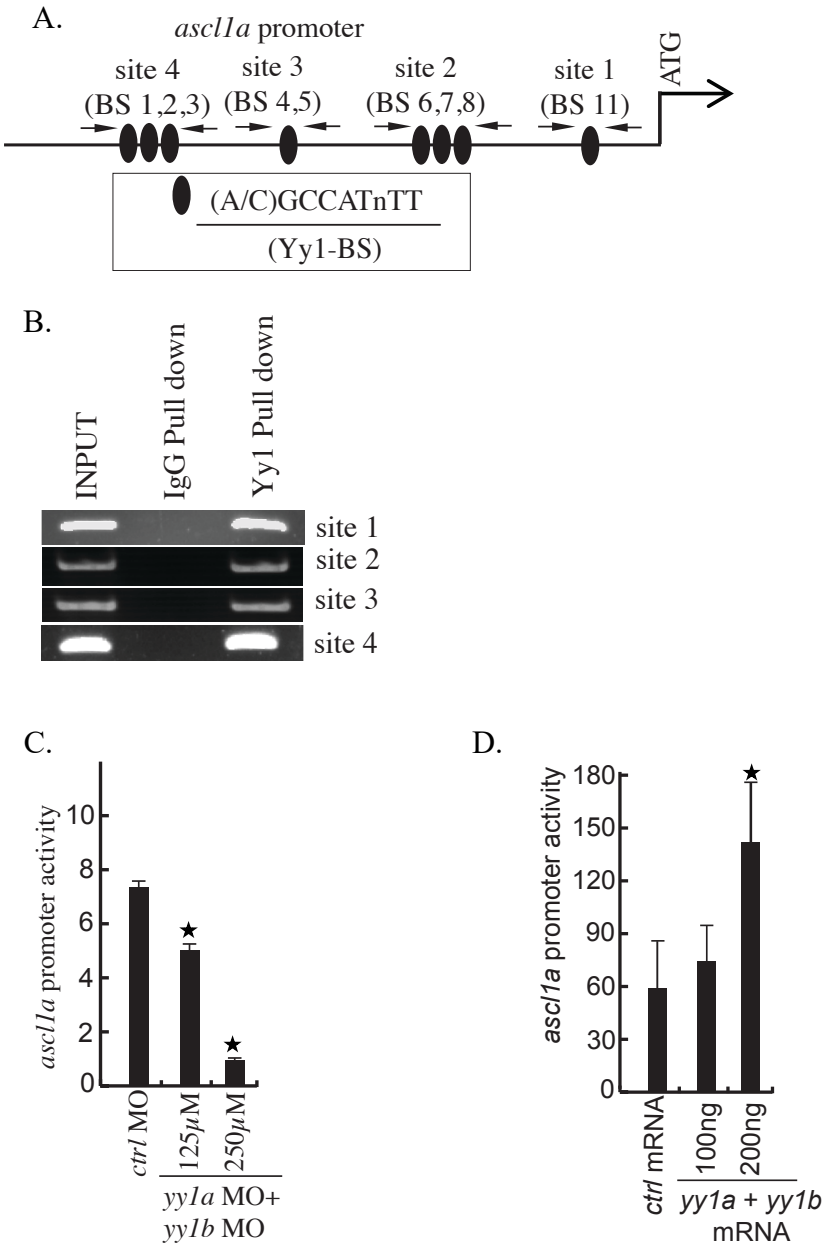


Figure 3.2.2.3. Yy1 directly regulates the expression of *ascl1a*. (A) Diagrammatic representation of *ascl1a* promoter reveals four putative Yy1 binding site. **(B)** ChIP assay done at 2dpi confirms the physical binding of Yy1 at four of these sites. **(C-D)** Luciferase assay done in the 24hpf embryos injected with pEL:*ascl1a-gfp* plasmid along with *yy1a* and *yy1b* MO **(C)** and *yy1a* and *yy1b* mRNA, shows decrease in the *ascl1a* promoter activity in **(C)** and increase in **(D).** $p<0.0001$ in C and <0.05 in D.

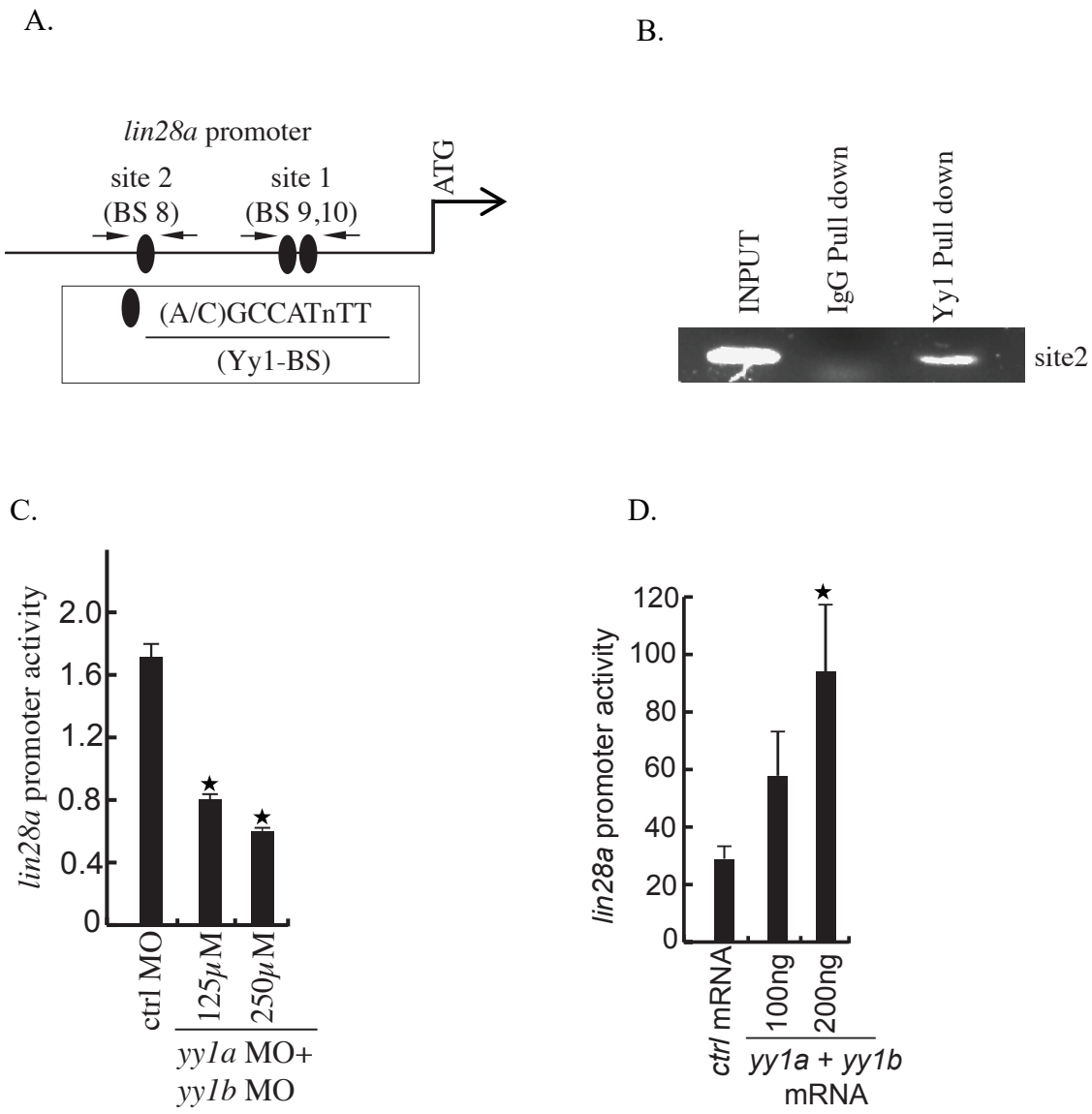


Figure 3.2.2.4. Yy1 directly regulates the expression of *lin28a*. **(A)** Diagrammatic representation of *lin28a* promoter reveals two putative Yy1 binding site. **(B)** ChIP assay done at 2dpi confirms the physical binding of Yy1 at one of these sites. **(C-D)** Luciferase assay done in the 24hpf embryos injected with pEL:*lin28a-gfp* plasmid along with *yy1a* and *yy1b* MO **(C)** and *yy1a* and *yy1b* mRNA, shows decrease in the *ascl1a* promoter activity in **(C)** and increase in **(D).** $p<0.0001$ in C and D.

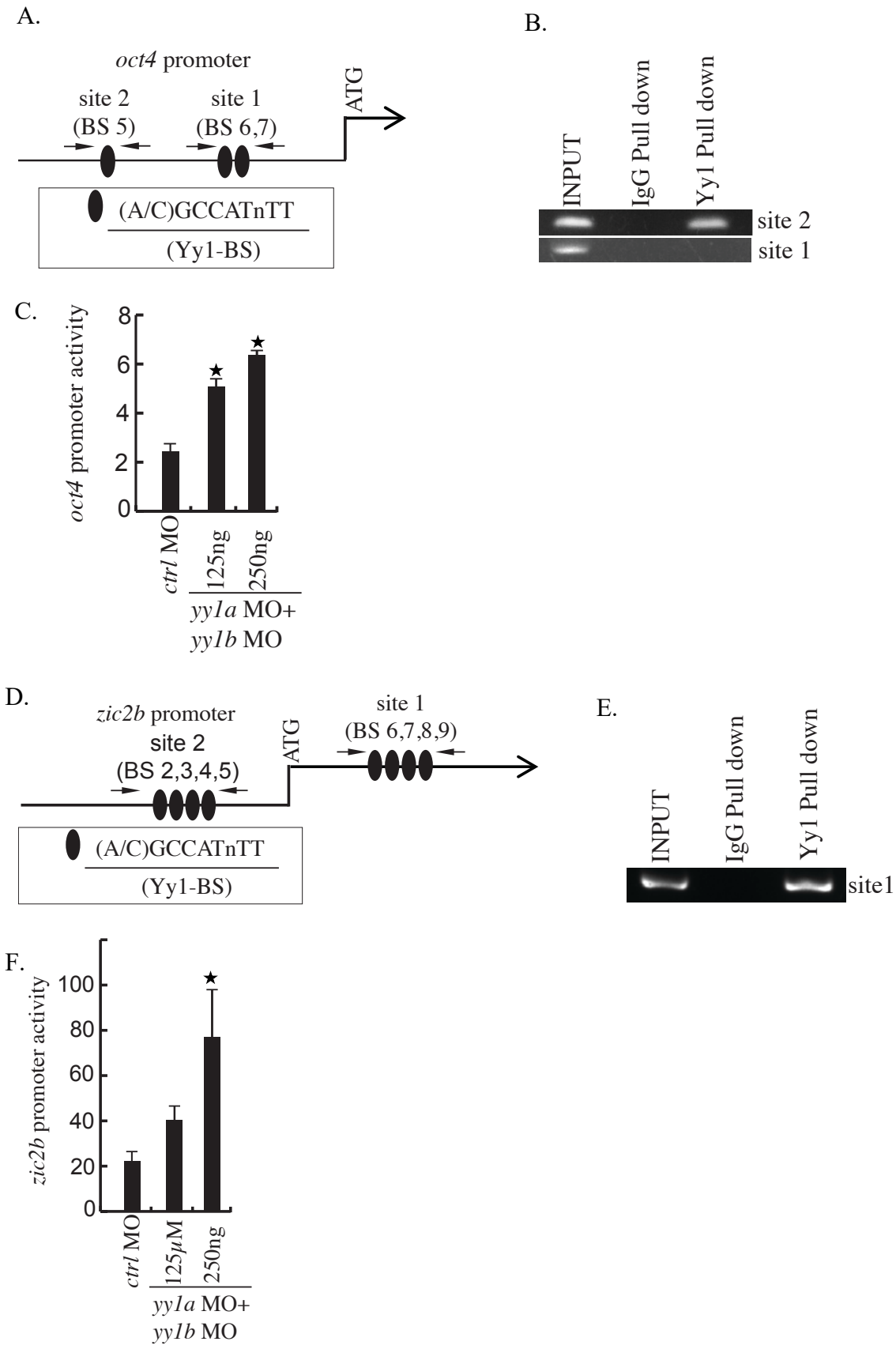

A.
oct4 promoter
site 2
(BS 5)
site 1
(BS 6,7)
ATG
(A/C)GCCATnTT
(Yy1-BS)
B.
INPUT
IgG Pull down
Yy1 Pull down
site 2
site 1
C.
oct4 promoter activity
8
6
4
2
0
ctrl MO
125ng
250ng
yy1a MO+
yy1b MO
D.
zic2b promoter
site 2
(BS 2,3,4,5)
ATG
site 1
(BS 6,7,8,9)
(A/C)GCCATnTT
(Yy1-BS)
E.
INPUT
IgG Pull down
Yy1 Pull down
site1
F.
zic2b promoter activity
100
80
60
40
20
0
ctrl MO
125μM
250ng
yy1a MO+
yy1b MO

Figure 3.2.2.5. Yy1 directly regulates the expression of *oct4* and *zic2b*. **(A)** Diagrammatic representation of *oct4* promoter reveals two putative Yy1 binding site. **(B)** ChIP assay done at 2dpi confirms the physical binding of Yy1 at one of these sites. **(C)** Luciferase assay done in the 24hpf embryos injected with pEL:*oct4-gfp* plasmid along with *yy1a* and *yy1b* MO shows increase in the *oct4* promoter activity. **(D)** Diagrammatic representation of *zic2b* promoter and 1st exon reveals two putative Yy1 binding site. **(E)** ChIP assay done at 2dpi confirms the physical binding of Yy1 at one of these sites. **(F)** Luciferase assay done in the 24hpf embryos injected with pEL:*oct4-gfp* plasmid along with *yy1a* and *yy1b* MO shows increase in the *oct4* promoter activity**.** $p<0.0001$ in C and F.

3.2.3 Yy1 regulates Notch signalling during retina regeneration

Notch signalling is known to play a crucial role in maintaining retinal progenitors and specification of Müller glia, as seen in *Xenopus* (Dorsky, Rapaport, & Harris, 1995), fish and mice(Lindsell, Boulter, diSibio, Gossler, & Weinmaster, 1996) (Furukawa, Mukherjee, Bao, Morrow, & Cepko, 2000). It has been shown in zebrafish that inhibition of Notch signalling leads to abnormal retinal architecture due to failure in the formation of Müller glial cells, which provide a main scaffold for the retinal architecture (Bernardos, Lentz, Wolfe, & Raymond, 2005). Dynamic Notch signalling is required for the development of retinal neurons and Müller glia (Mills & Goldman, 2017). Zebrafish has 5 family of Delta and Delta liked ligand, namely DeltaA, DeltaB, DeltaC, DeltaD, and Delta-like4 (Dll4); and four notch receptors Notch1a, Notch1b, Notch2, and Notch3. There is also a spatial dynamic regulation of notch signalling components during the development. Delta genes and Notch1 are expressed in the central retina, jagged genes in the ciliary marginal zone and Notch2 in the RPE. Notch signalling influences the cell fate by lateral specification i.e., the fate of one cell influences the fate of the other cell.

Regeneration is a process that is believed to recapitulate developmental programs. Differential spatial and temporal regulation of notch signalling and its interaction with the other signalling pathways are required during retina regeneration. Notch signalling helps in keeping a check on the proliferative zone at the site of injury. Sahu et al., have shown that Dll4/Delta B- Notch3 interaction is needed to keep the Müller glia into the quiescent state in the uninjured retina through *hey1* and *id2b,* which decreases chromatin accessibility and helps in keeping the zone of proliferation into limits (Sahu, Devi, Jui, & Goldman, 2021). However, the expression of *notch1a, notch1b, delta a, delta b, delta c* is rapidly induced post-retinal injury, which shows that notch signalling is necessary to maintain quiescence and limit the zone of proliferation

through Notch 3 while helping in Müller glia proliferation through these genes (L. J. Campbell et al., 2021).

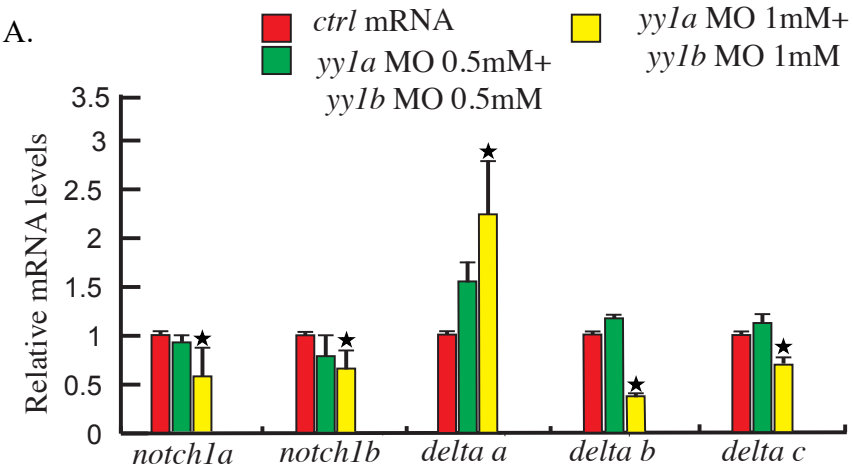
We checked the expression of various components of Notch signalling in the *yy1a and yy1b* in a knockdown condition and found that *notch1a, notch1b, delta b* and *delta c* showed a decline in their expression levels while there was an increase in the levels of *delta a* (Figure 3.2.3.1.A). Notch signalling is a paracrine signalling, wherein notch signalling dependent genes are regulated in the notch receptor-expressing cell while the neighbouring cells expresses the ligand. Moreover, ligand has a negative feedback loop in the signal receiving cells. Therefore, from our observation, we can hypothesize, that the dedifferentiating Müller glia cells are decreased in the knockdown condition, and henceforth, the expression of Notch1a is decreased. Even if the neighbouring cell express Delta a ligand and signals the Müller glial cells at the injury site, they will not undergo reprograming and subsequent proliferation due to the absence of Notch1a.

Furthermore, we explored the levels of *notch3* and *dll4* in the knockdown condition and observed that their levels go up in this scenario (Figure 3.2.3.1.B). Increase in their level again confirms the reduced reprograming of Müller glial cells at the injury site.

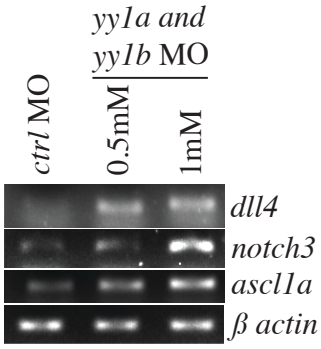
Next, we looked at the expression status of Her4.1, a well-studied effector gene of Delta-Notch signalling. Previous studies have reported that the forced expression of *nicd (notch intracellular domain)* upregulates the levels of *her4.1* and repression of Delta-Notch signalling using a pharmacological inhibitor, DAPT, leads to the reduced expression of *her4.1*(Wan, Ramachandran, & Goldman, 2012). It helps in keeping a check on the zone of proliferation. Moreover, previous studies have reported that the knockdown of *her4.1* leads to an increased proliferation (Mitra et al., 2018). We found that levels of *her4.1* went up by almost two folds in the *yy1a* and *yy1b* knockdown background (Figure 3.2.3.1.C).

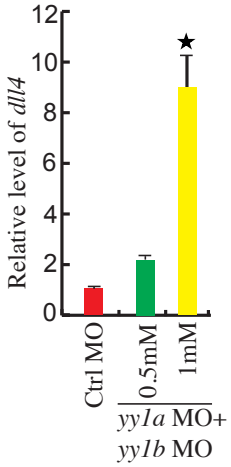
As discussed previously, levels of Lin28a go down in *yy1a* and *yy1b* knockdown condition, a hallmark for reduced proliferation and Yy1 can directly regulate its expression by binding onto its promoter. Interestingly, Her4.1 can also negatively regulate the expression of Lin28a by binding onto its promoter. Since levels of Her4.1 are high in the *yy1* knockdown, this could also be the reason for the reduced levels of *lin28a* in *yy1* knockdown condition.

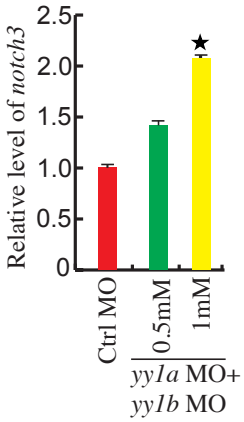
A.


ctrl mRNA
yy1a MO 0.5mM+
yy1b MO 0.5mM
yy1a MO 1mM+
yy1b MO 1mM
Relative mRNA levels
3.5
3
2.5
2
1.5
1
0.5
0
notch1a
notch1b
delta a
delta b
delta c

B.


yy1a and
yy1b MO
ctrl MO
0.5mM
1mM
dll4
notch3
ascl1a
ß actin


Relative level of dll4
12
10
8
6
4
2
0
Ctrl MO
0.5mM
1mM
yy1a MO+
yy1b MO


Relative level of notch3
2.5
2.0
1.5
1.0
0.5
0
Ctrl MO
0.5mM
1mM
yy1a MO+
yy1b MO

C.

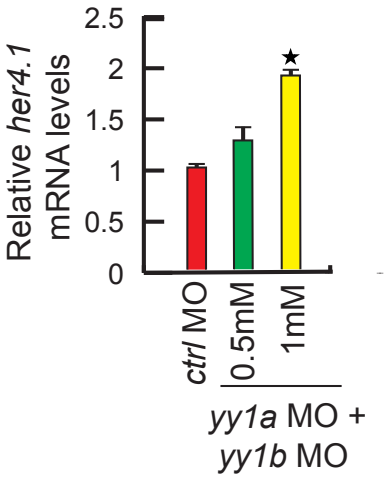

Relative her4.1
mRNA levels
2.5
2
1.5
1
0.5
0
ctrl MO
0.5mM
1mM
yy1a MO +
yy1b MO

Figure 3.2.3.1. Yy1 regulates notch signalling during retina regeneration. (A) qPCR analysis of notch signalling components in *yy1a* and *yy1b* knockdown condition at 2dpi, shows downregulation of *notch1a, notch1b, delta b* and *delta c,* and upregulation of *delta a*. **(B)** RT-PCR and qPCR analysis shows upregulation of *dll4* and *notch3* in *yy1a* and *yy1b* knockdown condition at 2dpi. **(C)** qPCR analysis shows upregulation of *her4.1* in *yy1a* and *yy1b* knockdown condition at 2dpi. $P<0.05$ in A and $P<0.005$ in B and C

3.2.4 Yy1 regulates the Mmp9 expression through Delta-Notch and TGFβ signalling interplay.

Matrix metalloproteinases (MMPs) are the Zn2+ dependent class of endopeptidase which helps in tissue remodelling. They were discovered for their role in the metamorphosis of tadpoles as an enzyme that can degrade tail's collagen. It degrades the extracellular matrix (ECM), as it cleaves all the proteinaceous components of the ECM. They have a role in various biological processes, such as wound healing and inflammation, as well as the pathophysiology of diseases such as rheumatoid arthritis and cancer. They cleave the structural components of ECM and make space for the cells to migrate and thus help in Epithelial-Mesenchymal transition. They can also activate as well as modify certain signalling molecules, such as insulin growth factor, from their latent phase resulting in the change in cell proliferation and differentiation (Page-McCaw, Ewald, & Werb, 2007) (Malemud, 2017) (H. Huang, 2018).

MMP9 is the most studied MMP, which belongs to the gelatinases family of MMPs, causing degradation of type IV, V, XI and XVI collagens and gelatin during tissue remodelling. It is mostly expressed in the central and peripheral nervous system and is associated with the pathophysiology of several diseases, such as neurodegenerative disorders (Reinhard, Razak, & Ethell, 2015). MMP9 degrades the ECM and scar formed by glial cells and collagen deposit to make way for the axon to grow and make a successful connections, thus promoting axon regeneration post optic nerve injury (Z. Ahmed et al., 2005). During retina regeneration, it is induced pan-retinally during early stages of regeneration (Kaur et al., 2018). It regulates the expression of many regeneration associated genes (RAGs) and many signalling pathways (Sharma et al., 2020) (Gupta et al., 2023) (Silva et al., 2020).

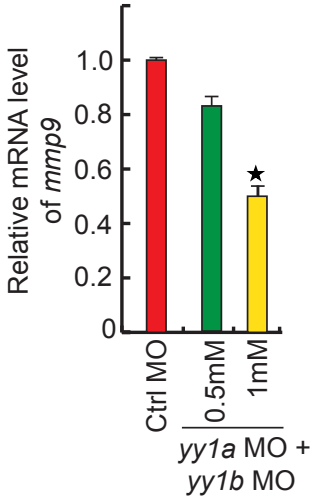
Owing to its importance in reprograming during retina regeneration, we checked the transcript levels of *mmp9* in the *yy1a* and *yy1b* knockdown condition at 2dpi and found that its levels reduced to half as compared to the control MO (Figure 3.2.4.1.A). We then confirmed this

result by doing mRNA *in-situ* hybridization using a probe against the *mmp9* mRNA at 4dpi and found less number of cells with the *mmp9* expression at the injury site (Figure 3.2.4.1.B). We then employed a transgenic reporter line containing the *mmp9* promoter driving the expression of GFP. We confirmed its expression in the embryos and found it to be expressed in the entire brain and the notochord (Figure 3.2.4.2.A). We then injected the MO against *yy1a* and *yy1b* in combination and observed less number of GFP+ cells as compared to the *control* MO-injected retina (Figure 3.2.4.2.B).

A previous paper from our lab reported that Mmp2 and Mmp9 activates the latent TGFβ in the ECM and in turn, TGFβ signalling induced pSmad3 negatively regulates the expression of *mmp9* by directly binding onto the TIE elements present on its promoter (Sharma et al., 2020). Hence, to see if Yy1 regulates the expression of *mmp9* through pSmad3, we checked the levels of pSmad3 in the *yy1* knockdown condition and observed an increase in the levels of *smad3a* but not *smad3b* (Figure 3.2.4.3.A), and also increase at the protein levels (Figure 3.2.4.3.B), which justifies our hypothesis that reduced level of Yy1 upregulates pSmad3 which in turn downregulates *mmp9* and hence the reduced proliferation is seen in *yy1* knockdown.

Another reason for the low levels of *mmp9* could also be due to high levels of *her4.1* in *yy1a* and *yy1b* knockdown condition because Her4.1 negatively regulate the levels of *mmp9* by directly binding onto its promoter (Kaur et al., 2018).

A.



B.

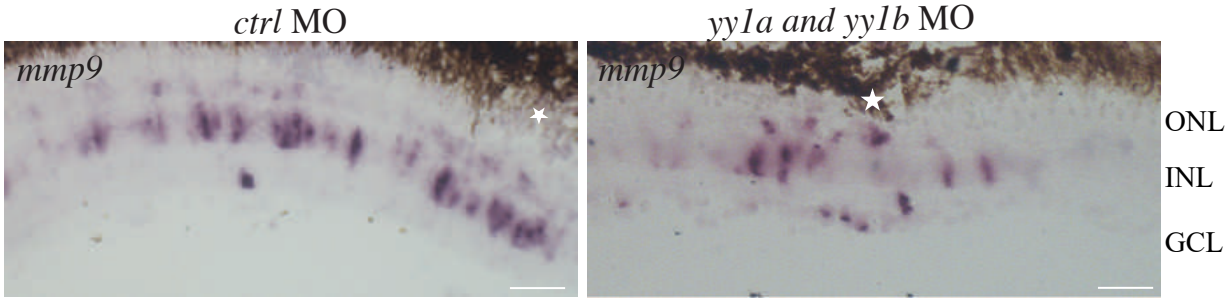
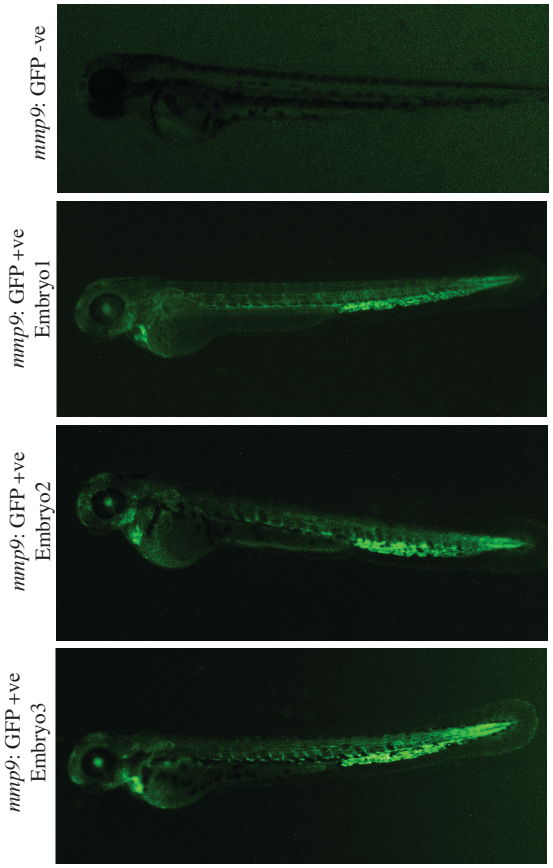


Figure 3.2.4.1. Yy1 regulates the expression of *mmp9* during retina regeneration. (A) qPCR analysis shows upregulation of *mmp9* in *yy1a* and *yy1b* knockdown condition at 2dpi. **(B)** mRNA *in-situ* hybridization done on retinal section at 4dpi, shows decreased expression of *mmp9* in *yy1a* and *yy1b* knockdown condition. Scale bar represents 10µM. $p<0.005$ in A.

A.



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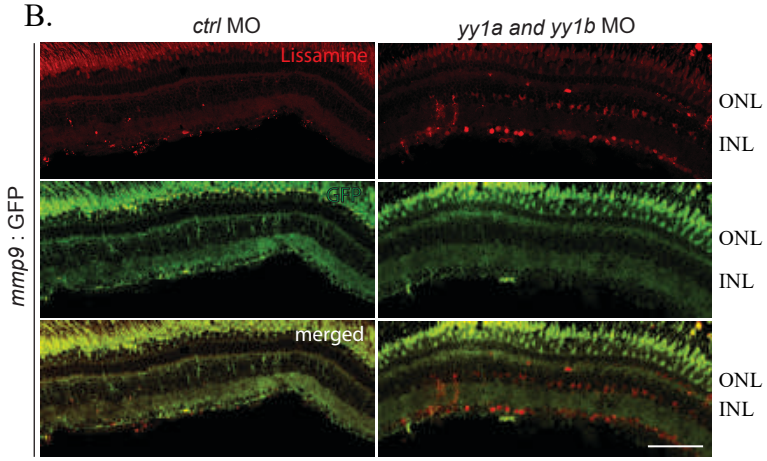
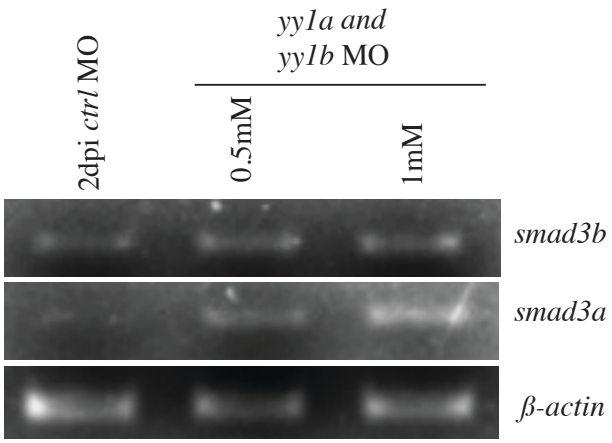


Figure 3.2.4.2. Yy1 regulates the expression of *mmp9* during retina regeneration as seen using *mmp9:*GFP transgenic fish. (A) Fluorescence microscopy images of *mmp9:*GFP transgenic embryos showing GFP expression at 48hpf. **(B)** Confocal microscopy images of retinal section of *mmp9:*GFP transgenic fish showed decreased number of GFP^{+} cells. Scale bar represents 10μM.

A.



B.

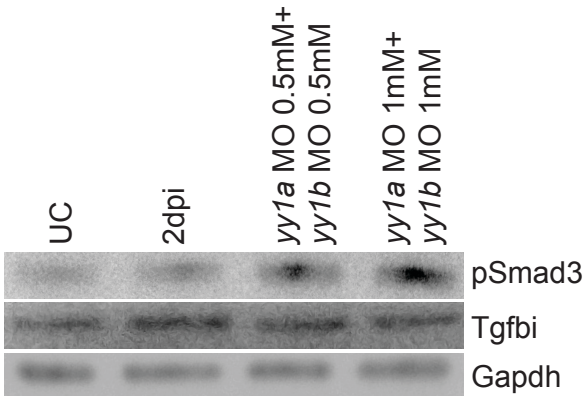


Figure 3.2.4.3. Yy1 regulates the expression Smad3a and Smad3b (A) RT-PCR analysis shows upregulation of *smad3a* and *smad3b* in *yy1* knockdown condition. **(B)** RT-PCR analysis shows upregulation of pSmad3 in *yy1* knockdown condition.

3.2.5 Hdac1 regulates the function of Yy1 by deacetylation in a timely manner for successful regeneration

Histone deacetylases (Hdacs) are the group of proteins that catalyse the deacetylation of the acetylated lysine residues of histones and non-histone protein. They are part of complexes that helps in chromatin remodelling and decrease the accessibility of the transcription factors. They are also known to interact with the transcription factors and amend their activity directly. They can regulate the expression of many cell cycle regulators and, therefore have a role in cell cycle progression and arrest, differentiation and cancer. Epigenetic modifications are critical for the spatial and temporal regulation of the expression of genes. These epigenetic modifications involve DNA modifications, such as methylation, and histones modification, such as acetylation, methylation and phosphorylation. Histones are acetylated by HATs, which are the writers of the modification, while deacetylation is done by HDACs, which are the erasers of the modification (G. Li, Tian, & Zhu, 2020) (Lombardi, Cole, Dowling, & Christianson, 2011). Besides, these HDACs can deacetylate other proteins and regulate their cellular functions and pathophysiology in diseases such as cancer. These include certain transcription factors such as E2F/Rb, p53, STAT3, Smad7, and T-cell transcription factor (TCF); and proteins, such as Tubulin, Importin, Androgen receptors (AR) and MyoD (Peng & Seto, 2011) (Chan, Krstic-Demonacos, Smith, Demonacos, & La Thangue, 2001) (Choudhary et al., 2009)
(Glozak, Sengupta, Zhang, & Seto, 2005).

Yy1a and Yy1b both contain a GK-rich region, which interacts with Hats and Hdacs and gets acetylated or deacetylated at the lysine residues in this region. Acetylation at the central region increases the repressive activity of Yy1, while deacetylation relieves this repression. Moreover, binding the Hdac1 to the acetylated lysine residues at the C- terminal increases the interaction of Hdac1 and Yy1, and thus increased the DNA binding ability of Yy1 (Yao et al., 2001). We first checked if pharmacological inhibition of Hdacs affect the levels *yy1a* and *yy1b* in the

uninjured state and we found that Hdacs indeed positively regulates the expression of both *yy1a* and *yy1b* (Figure 3.2.5.1.A). Co-immunoprecipitation also confirmed the physical binding between Yy1 and Hdac1 (Figure 3.2.5.1.B). Yy1a contains six lysine residues, while Yy1b contains four lysine residues in GK rich region. We mutated these lysine residues to alanine to create a neutral mutation, which cannot be acetylated and lysine to glutamine to create an acetylated mimetic mutation (Figure 3.2.5.1.C). We made these point mutations in Yy1a and Yy1b coding sequences. Then, mRNA was synthesized by *in-vitro* transcription for both the neutral mutation (referred to as KA) and the acetylated mutation and (referred to as KQ) transfected in the retina along with un-mutated mRNA as a control (referred to as wild type, WT) and checked the proliferation at 4dpi. To our surprise, we found a decreased number of proliferating cells marked by PCNA (Figure 3.2.5.2.A), in both the conditions which were quantified also (Figure 3.2.5.2.B). Furthermore, we confirmed our results by dipping the fish in the Hdacs blocker, TSA. In the presence of TSA, HDAC's functional activity will be lost and it will mimic the condition of acetylated mimetic mutation, which cannot be deacetylated by Hdacs. We injected the *yy1a* and *yy1b* mRNA, dipped the fish in water and observed similar results. The number of proliferating cells was more in Yy1 overexpressed condition and less in both TSA and Yy1 overexpression along with dipping in TSA (Figure 3.2.5.3.A), which was quantified (Figure 3.2.5.2.B).These results show that although acetylation is necessary for the activity of Yy1 to act as a pro-proliferative factor, its timely deacetylation is also necessary for the function to happen. That's why we saw less proliferation in both scenarios, one in which acetylation is blocked and the other in which acetylation is happening, but Hdacs could not do deacetylation. We then looked at the expression of some of the regeneration-associated genes in both experiments. We found that there is an increase in the levels of Oct4 and Zic2b, while there was a decrease in the levels of Lin28a in the neutral mutation and the phosphomimetic mutation, which could reason the reduction in the proliferation (Figure 3.3.2.2.C). In the other

experiment, where we overexpressed Yy1 and inhibited Hdacs, using TSA, despite an increase in the levels of Ascl1a, Lin28a, and a decrease in the level of Zic2b, still the proliferation is less (Figure 3.2.5.3). This could be because Yy1 is a multifunctional transcription factor and deacetylation of Yy1 is needed to regulate some other important genes required for the proliferation to happen.

Interestingly, Yy1 is regulated in a fashion similar to Hdac1 (Mitra et al., 2018), both of them are downregulated post-retinal injury while their protein levels remain unchanged in the whole retinal lysate. Knockdown of either *yy1* or *hdac1* showed reduced proliferation. Therefore, we anticipated that they both regulate each other. We checked the expression levels of *hdac1* in the *yy1a* and *yy1b* knockdown condition and found a decline of almost 30% in its level both at the protein level (Figure 3.2.5.3.A) as well as mRNA level (Figure 3.2.5.3.B). Next, we checked the occupancy of H3K9me3 around the transcription start site (TSS) of *hdac1*. We saw an expected increase in the occupancy of H3K9me3, indicative of heterochromatin formation, in the *yy1* knockdown condition (Figure 3.2.5.3.C).

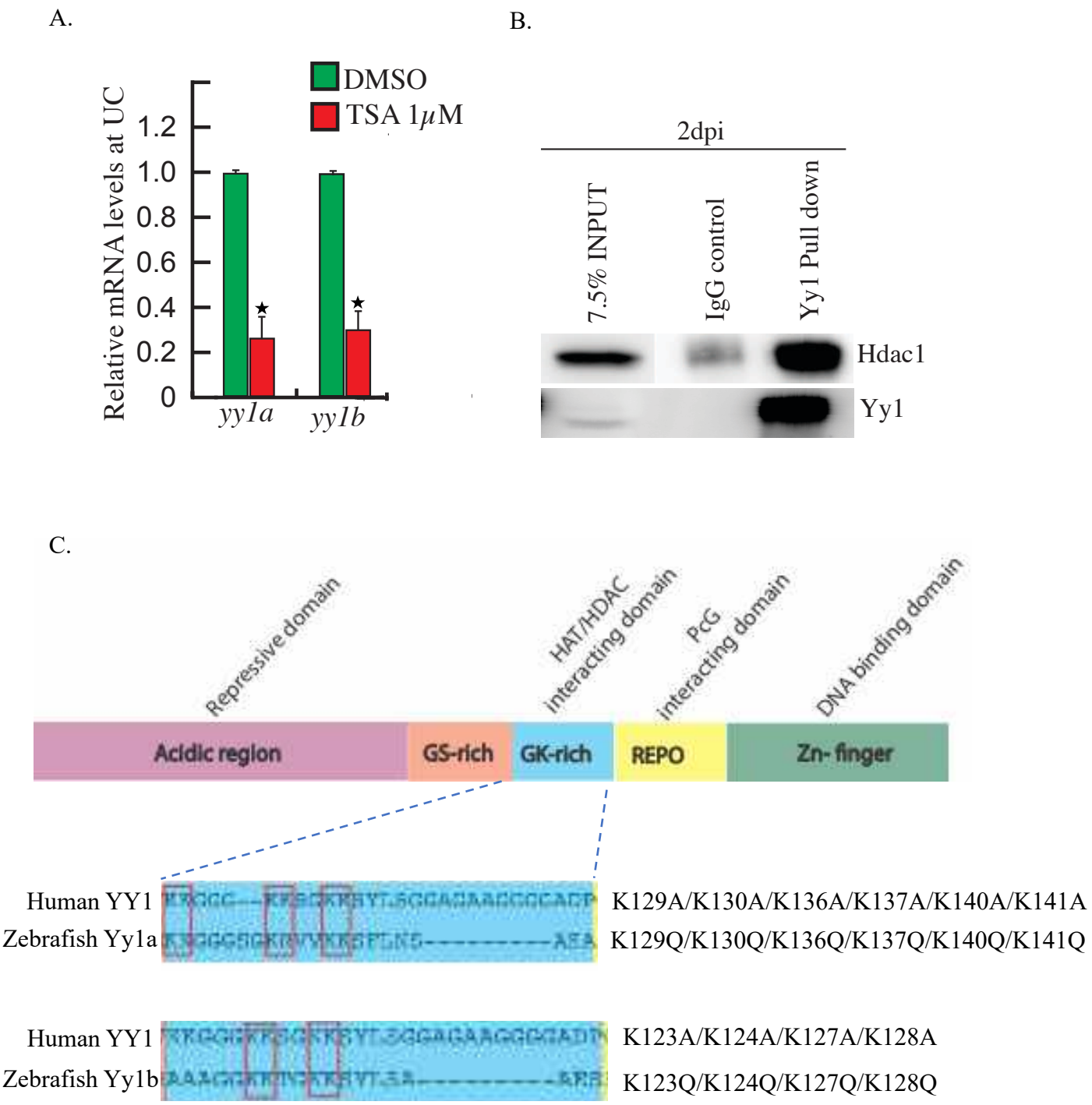


Figure 3.2.5.1. Regulation of Yy1 through Hdacs (A) qPCR analysis shows the decline in the levels of *yy1a* and *yy1b* in the TSA treated retinae**. (B)** Western blot assay done at 2dpi reveals the physical interaction between Yy1 and Hdac1. **(C)** Diagrammatic representation of domain structure reveals six lysine residues in Yy1a and four in Yy1b in their GK rich region, which is the HAT/Hdacs interacting domain. Site directed mutagenesis, changed lysine residues, in both Yy1a and Yy1b, to alanine to make neutral mutant and to glutamine to create acetylated mimetic mutant. P<0.0001 in A

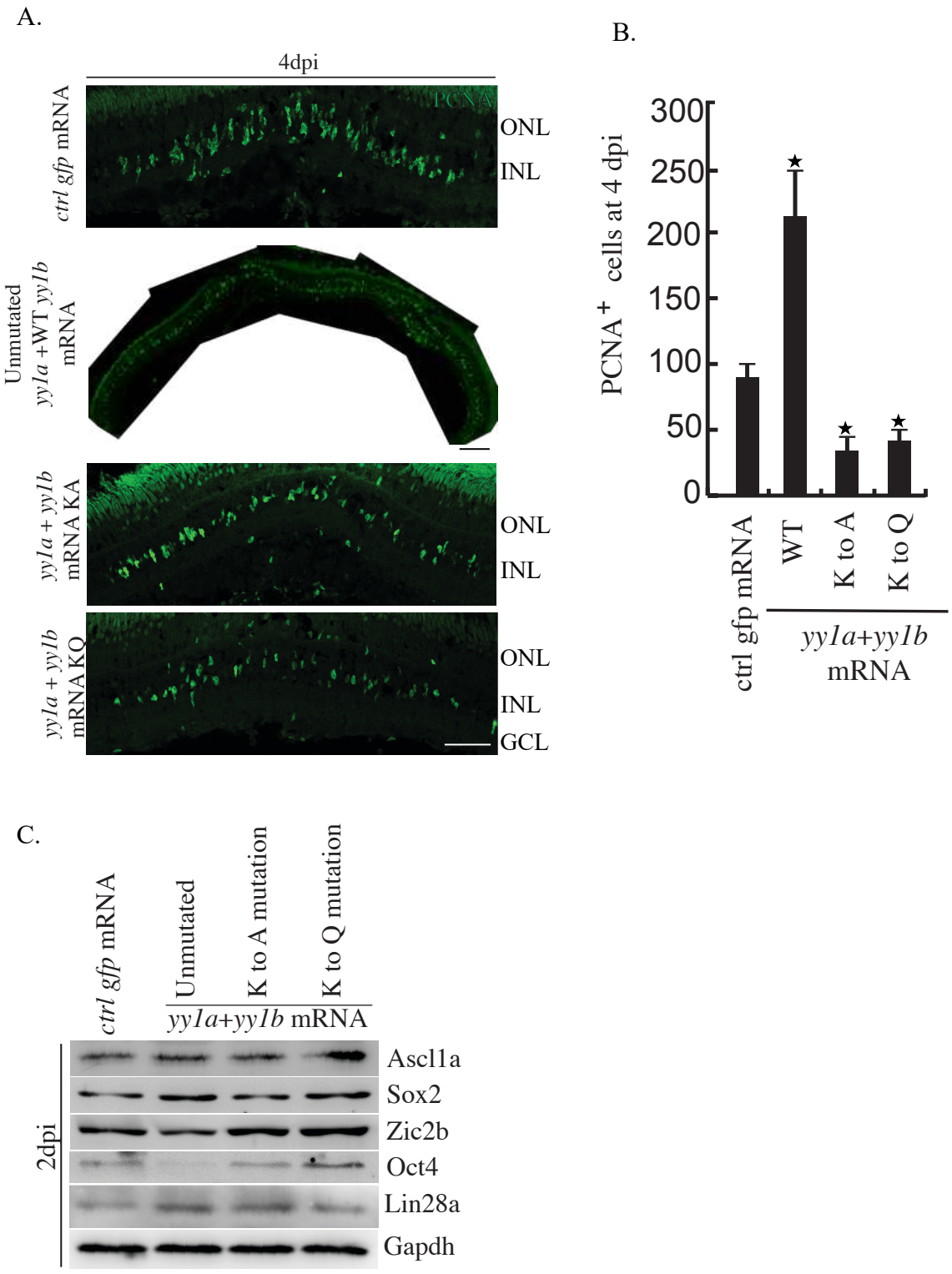


Figure 3.2.5.2. Yy1 regulates proliferation of MGPCs through its acetylation and deacetylation. (A) Confocal microscopy images of retinal section, at 4dpi, shows the decrease in the number of proliferating cells marked with PCNA in both neutral mutation and acetylated mutation, which is quantified in **(B). (C)** Western blot assay done at 2dpi, shows the regulation of few RAGs in the overexpression of unmutated Yy1, neutral mutated Yy1 and acetylated mutated Yy1. P<0.0001

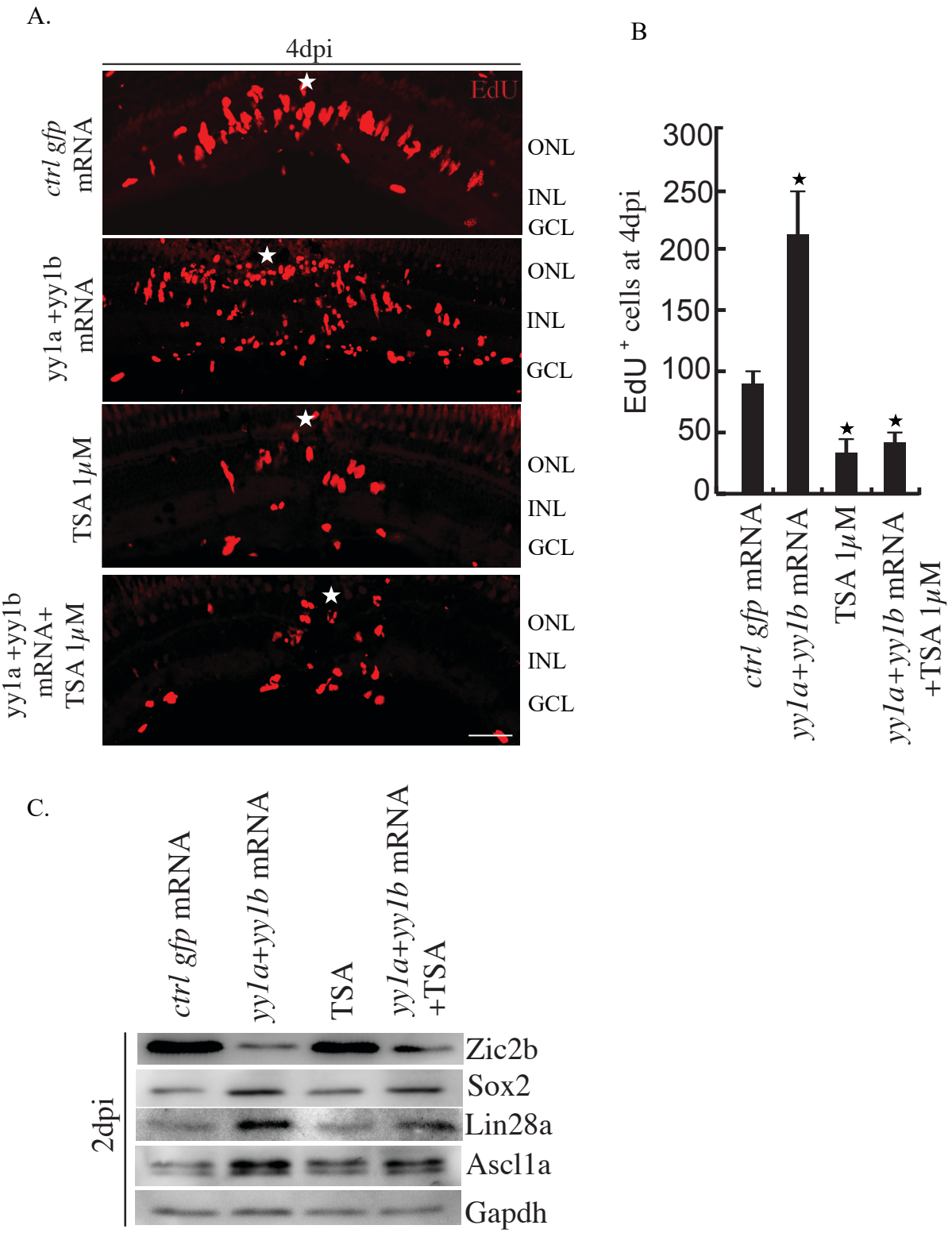


Figure 3.2.5.3. Yy1 regulates proliferation of MGPCs through its interaction with the Hdacs (A) Confocal microscopy images of retinal section at 4dpi shows the decrease in the number of proliferating cells marked with PCNA in TSA as well as overexpression of Yy1 along with TSA treatment, as compared to Yy1 overexpression alone, which is quantified in **(B). (C)** Western blot assay done at 2dpi, shows the regulation of few RAGs in Yy1 overexpression, Hdacs inhibition using TSA and Yy1 overexpression along with TSA treatment. P<0.005 in B.

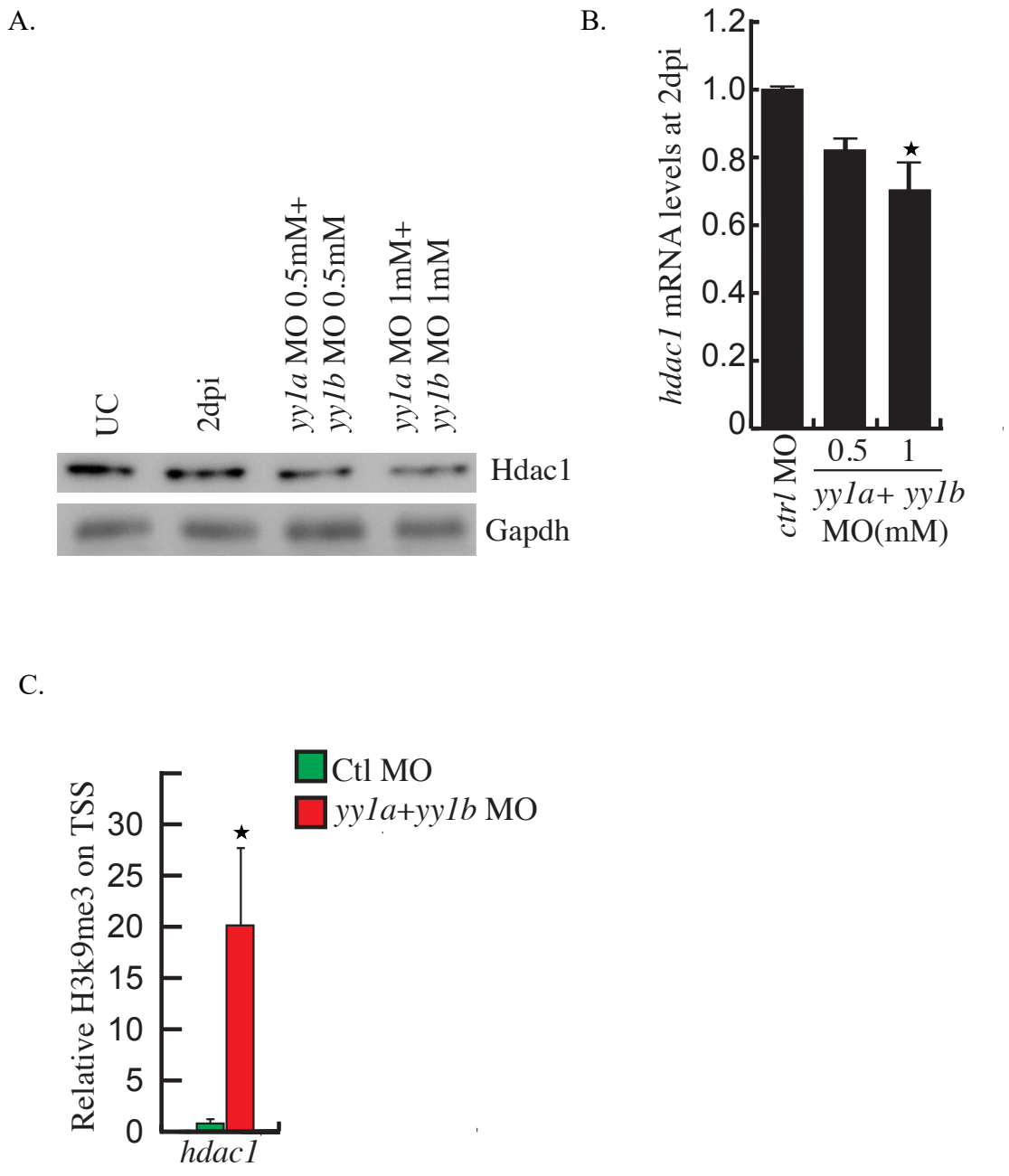


Figure 3.2.5.3. Yy1 regulates the expression of Hdac1 during retina regeneration. (A) Western blot analysis shows a decline in the level of Hdac1 in the knockdown condition of *yy1a* and *yy1b*. **(B)** qPCR analysis also shows a decline in the level of *hdac1* mRNA in the knockdown condition of *yy1a* and *yy1b*. **(C)** qPCR analysis reveals the increase in the occupancy of H3K9me3 on the TSS of *hdac1*. P<0.05 in B and C.

3.2.6 BMP signalling regulation by Yy1 is essential for the retina regeneration in zebrafish

BMPs (Bone morphogenetic proteins) are the members of the TGF-β family of proteins. It transmits its signal by binding the BMP ligand to the heterodimer of type1 and type2 serine/threonine kinase receptors. Upon BMP binding, constitutively phosphorylated type2 receptor trans-phosphorylates type1 receptor by forming a heterodimer. Type 2 receptor then phosphorylates R-Smads (Smad1/5/8), which associate with co-Smad (Smad4) and translocate to the nucleus and bind on the promoters along with co-activators and co-repressor of the effector genes. BMP signalling plays an important role in the development of the heart, kidney, eye, bones, cartilage and muscles (R. N. Wang et al., 2014) (Salazar, Gamer, & Rosen, 2016) (Miyazono, Kamiya, & Morikawa, 2010).

BMP signalling has been reported as important for the maintenance of stem cell niches. It has been reported that BMP signalling helps in repairing craniofacial bone defects (G. Chen et al., 2020). It promotes the proliferation of blastemal stem cells and skeletal and connective tissue differentiation during caudal fin regeneration in the *Poecilia latipinna* (S. Rajaram, Patel, Uggini, Desai, & Balakrishnan, 2017). BMP signalling, involving BMP 2, 6, 7, 9, is required for the differentiation of osteoblast from the mesenchymal stem cell precursor. BMP signalling plays a pro-proliferative role during chick retina regeneration and activates SMADs, which in turn activates FGF signalling via MAPK (Haynes et al., 2007). The BMP4/SMAD1/5/8 signalling is required for the proliferating MGPCs in the avian retina (Todd, Palazzo, Squires, Mendonca, & Fischer, 2017). There is an upregulation of BMP2/4/7 and SMAD1/5/8 in the mouse retina in the post-retinal damage by NMDA leading to the upregulation of Id1 (Inhibitor of differentiation) (Ueki & Reh, 2012).

We performed whole retina RNA sequencing (RNA-seq) in the combined knockdown of *yy1a* and *yy1b,* at 2dpi and found that many genes associated with cellular proliferation, neuronal development, differentiation and wound healing are downregulated Figure 3.2.6.1.

GO-pathway analysis also revealed that along with notch signalling, the BMP signalling pathway is down-regulated in the knockdown of *yy1a* and *yy1b.* We, therefore, first checked the effect of blocked BMP signalling on the proliferation of Müller glia cells in the injured Zebrafish retina. We used a small molecule inhibitor of BMP signalling, K02288, which competitively binds to the BMP ligand and further inhibits the phosphorylation of SMAD1/5/8. It selectively blocks BMP signalling without affecting the TGF-β signalling pathway. In zebrafish, blocking of BMP signalling using K02288 induces dorsalization in the embryos (Sanvitale et al., 2013) (Sanchez-Duffhues, Williams, Goumans, Heldin, & Ten Dijke, 2020). We injured the retina of fish, dipped into the drug in varying concentrations and harvested it at 4dpi. We found dose-dependent decrease in the number of proliferating cells, marked by PCNA, in the BMP signalling blocked condition (Figure 3.2.6.2.A), which was also quantified (Figure 3.2.6.2.B). Further, we quantified the levels of *id1* mRNA. Id1, inhibitor of differentiation, is the known direct target of BMP signalling. There was a decline in the *id1 levels* in the increasing concentration of K02288 (Figure 3.2.6.2.C).

We were intrigued to know if BMP signalling is regulated by Yy1. We checked the expression of *smad1,5 and 8* in the knockdown background and saw a decline in the levels of *smad1* and *smad8* while there was an increase in the levels of *smad5* (Figure 3.2.6.3.A). Next, we knockdown *yy1a* and *yy1b* and checked the changes in the transcript levels of *id1* and *nog3,* gene coding for Noggin3. The Noggin3 is an antagonist for the BMP signalling. It binds to the BMP ligands and prevents them from binding to their receptor, thus inhibiting the BMP signalling (McMahon et al., 1998). There was a decrease in the levels of *id1* by almost 80% (Figure 3.2.6.3.B), while there was a significant increase in the levels of *nog3* (Figure

3.2.6.3.C). We confirmed our result by doing mRNA *in-situ* hybridization of *id1* and found a reduction in its levels (Figure 3.2.6.4.A). Also high magnification image of *id1 in-situ* showed that *id1* is present mainly in the cells adjacent to the proliferating cells marked by EdU (Figure 3.2.6.4.B).

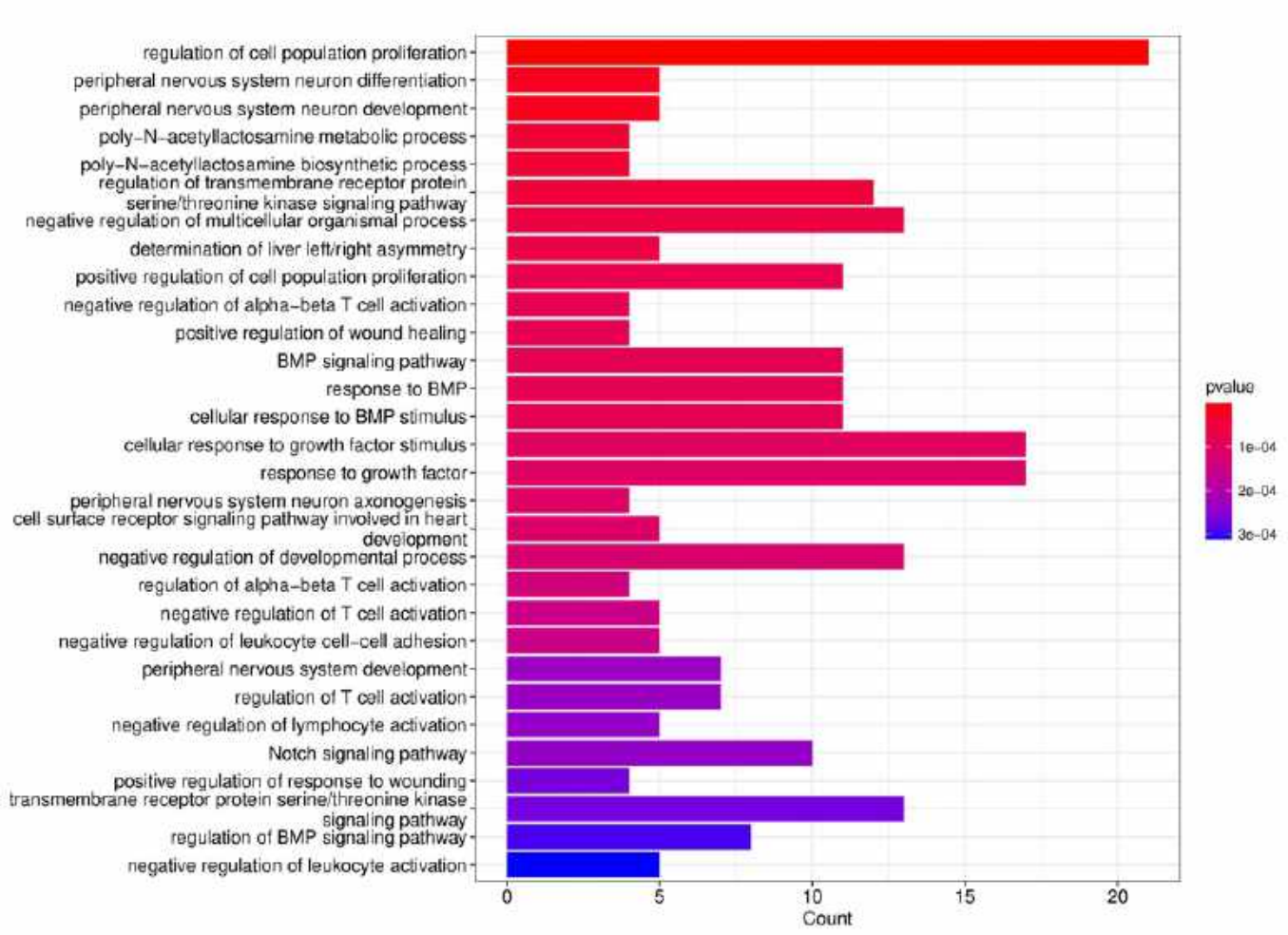


Figure 3.2.6.1. GO function analysis of data from the whole retina RNA seq done in the Knockdown of *yy1a* and *yy1b,* as compared to the control. Whole retina RNA-seq analysis reveals the down regulation of many genes related to cell proliferation, neuronal development and differentiation, wound healing, response to growth factors, Notch signalling and BMP signalling.

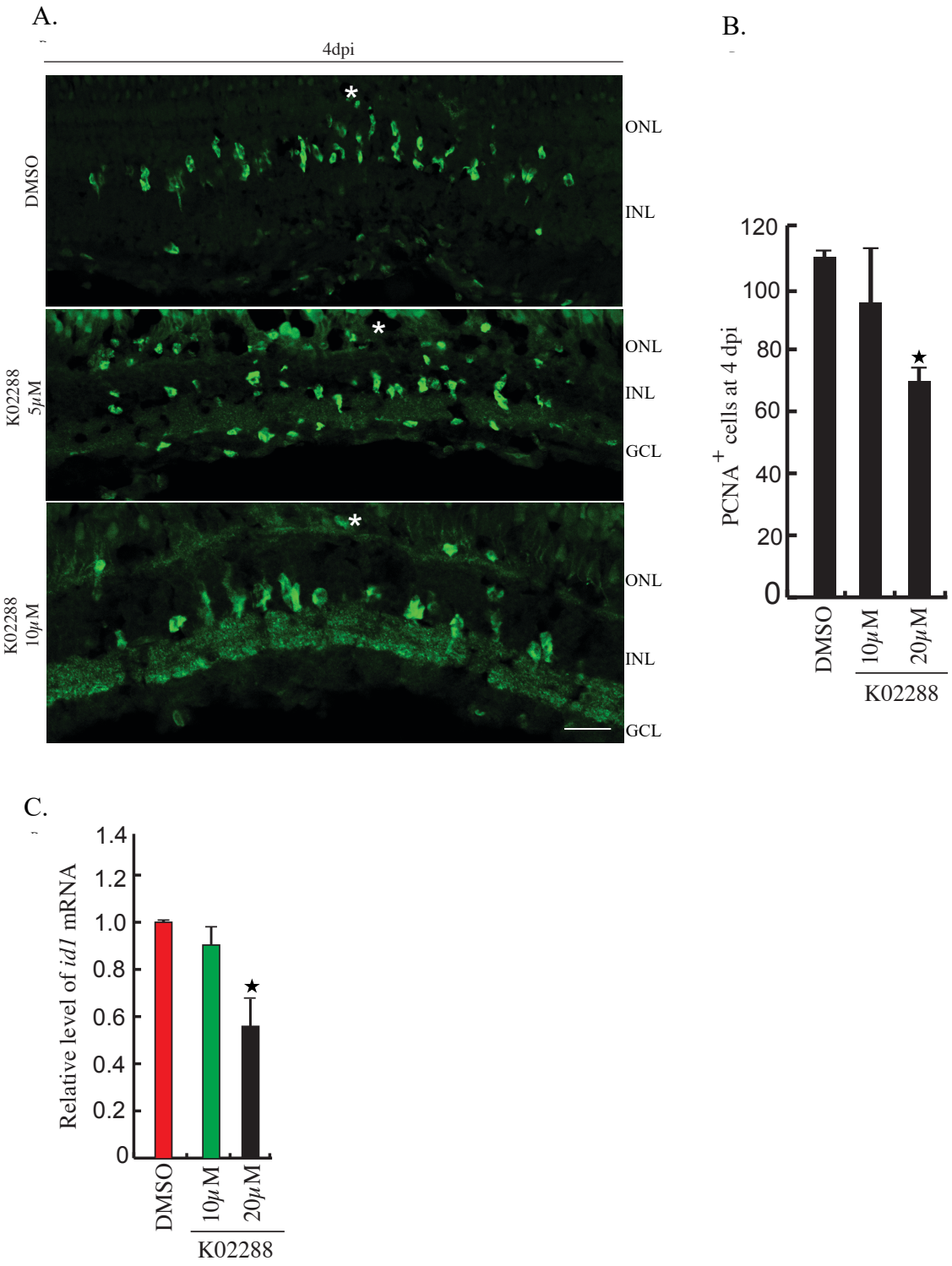


Figure 3.2.6.2. BMP signalling is essential during retina regeneration. (A) Confocal microscopy images of the retinal section shows a decline in the number of proliferating MGPCs, marked with PCNA, on the pharmacological inhibition of BMP signalling using K02288, which is also quantified in **(B)**. **(C)** qPCR analysis shows a decline in the mRNA levels of *id1,* one of the direct targets of BMP signalling, in the K02288 treatment. Star marks the injury site, Scale bar represents 10μM, $p<0.05$ in C.

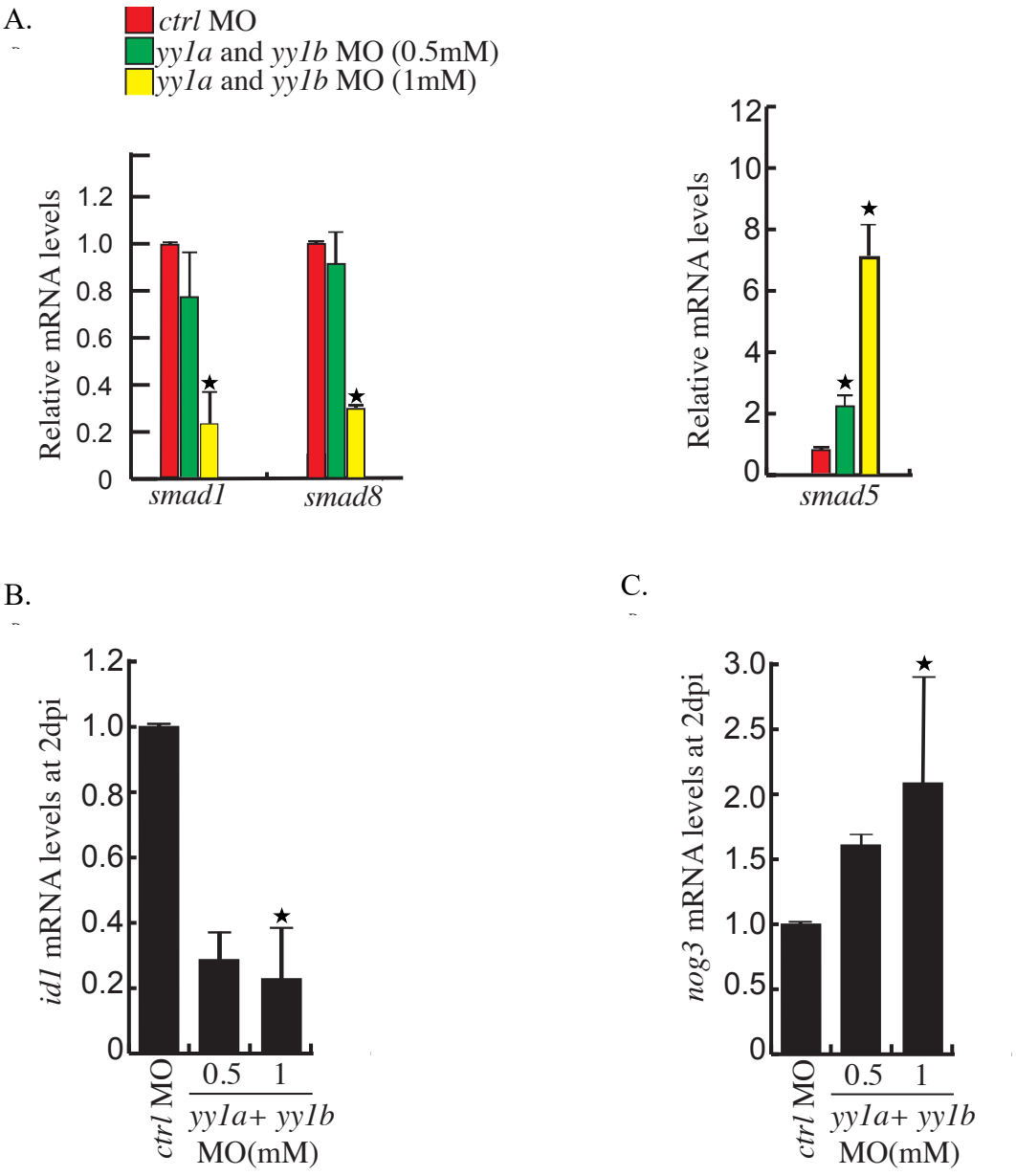


Figure 3.2.6.3. Yy1 regulates the BMP signalling during retina regeneration. (A) qPCR analysis done at 2dpi in the knockdown condition of *yy1a* and *yy1b* reveals decline in the levels of *smad1* and *smad8* while increase in the levels of *smad5*. **(B)**. qPCR analysis shows a decline in the mRNA levels of *id1,* one of the direct targets of BMP signalling, in the knockdown of *yy1a* and *yy1b*. **(C)** qPCR analysis shows a increase in the mRNA levels of *nog3,* an antagonist of BMP signalling, in the knockdown of *yy1a* and *yy1b*. $p<0.05$ in A-C. $p<0.005$ A-C and $p<0.05$ in C.

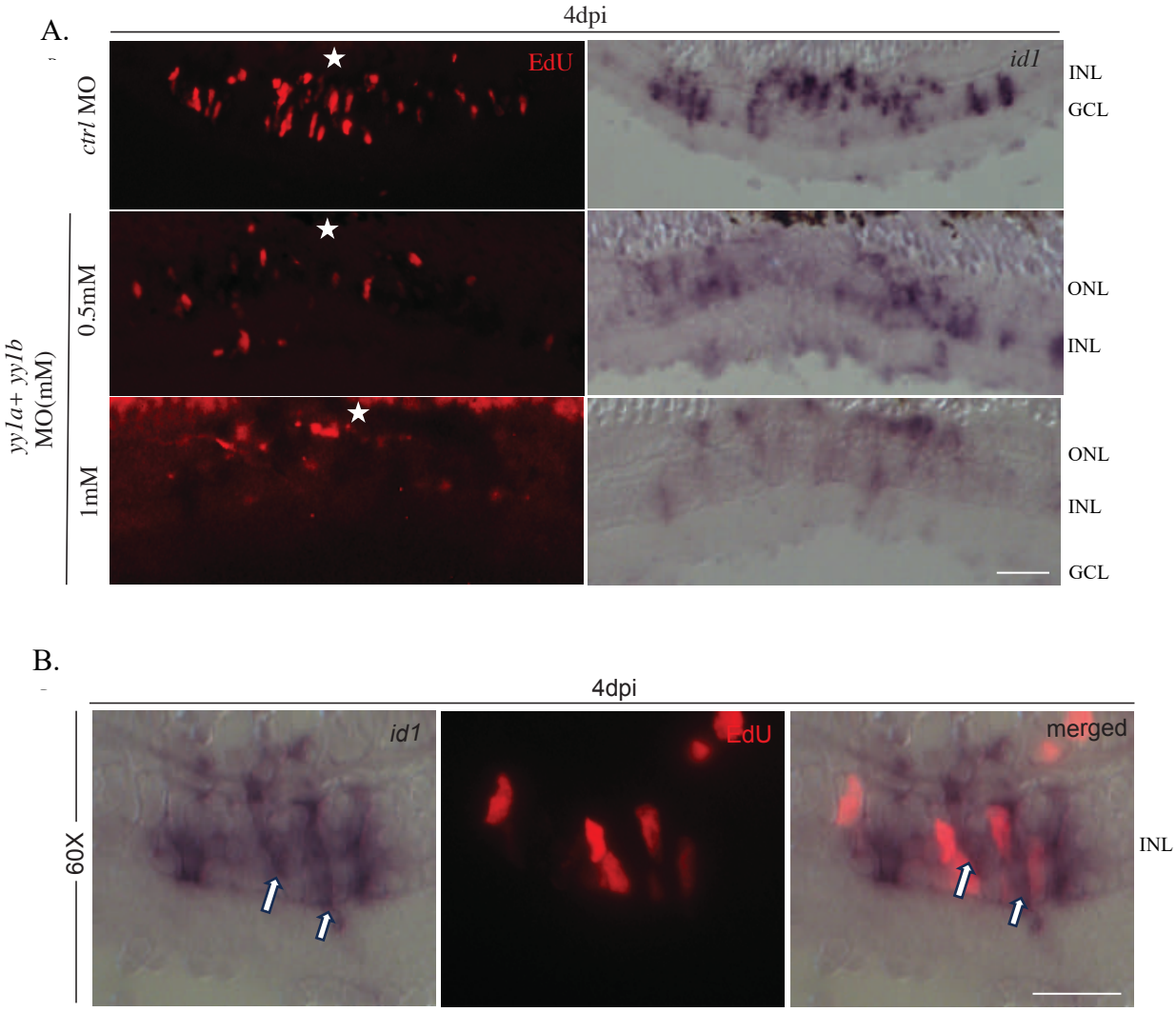


Figure 3.2.6.4. Yy1 regulates the expression of *id1* during retina regeneration. (A) mRNA *in-situ* hybridisation shows the localization of *id1* at the injury site and its decline in the *yy1a* and *yy1b* knockdown condition . **(B)** Zoomed image of *id1* shows that it is expressed in the neighbouring cells of MGPCs, at 4dpi. Star marks the injury site and scale bar represents 10µM. Arrow marks the in-situ signals in the cells adhacent to $BrdU^{+}$ cells.

Chapter 3

Yy1 and its interplay with the chromatin remodeler BAF complex to regulate retina regeneration.

3.3.1 BAF complex is essential for the zebrafish retina regeneration

The DNA is too long to accommodate into the nucleus and folds itself to compress the DNA into the nucleus. This DNA compression happens due to the interaction between the histones and DNA. DNA wraps around the histone proteins, which come together to form the nucleosomes. This brings about transcription repression as DNA becomes inaccessible to the transcription factors and the RNA polymerases. The interaction has to be relaxed during the process of replication, transcription, repair and recombination and access to the DNA is coordinated by the movement of DNA, transcription factors, histone-modifying enzymes (methylation, acetylation, ubiquitination and phosphorylation) and a set of chromatin remodelers. Chromatin remodelers are the class of enzymes with specialized roles in the assembly of chromatin, access to the DNA transcriptional machinery and exchange of histone variants for nucleosome editing. All the chromatin remodelers share some unique features, such as

1. Histone modification recognizing domain
2. An ATPase domain for ATP hydrolysis
3. Proteins for regulating the ATPase domain
4. proteins for interaction with the other transcription factors and the chromatin

Based on the unique flanking sequence and their function, they are classified into four families, which are conserved from yeast to humans.

1. ISWI family of chromatin remodelling complexes: ISWI family (Imitation switch) is responsible for the assembly and proper spacing of the nucleosome and, therefore, essential for the higher-order chromatin assembly. It utilizes DNA-dependent ATPase activity to place the nucleosome at regular intervals properly. During replication, ISWI chromatin remodelers are used extensively because chromatin must be arranged in a compact fashion. During this process, histones are deposited randomly on the naked DNA. These are then

positioned properly by the ISWI family of proteins by the process named nucleosome spacing.

2. CHD family of remodelers: The two main characteristics of the CHD family are the SNF2-kind ATPase domain in the central region and tandemly arranged chromodomains at the N-terminal. They recognize methylated histone as well as phosphorylation of HP1protein. Certain CHD proteins, such as Chd1, helps in the unwinding of DNA around the nucleosome and thus promote transcription. On the other hand, CHD4 is a part of NuRD complex (Nucleosome Remodeling Deacetylase complex) and has a role in the repression of certain genes involved in differentiation during lineage specification (Farnung, Ochmann, & Cramer, 2020)
3. INO80 family remodelers: The members of INO80 family remodelers are involved in the processes such as transcription, replication and DNA repair. They utilize ATP to reposition the nucleosome and correctly position them at +1 and -1 relative to the TSS. In collaboration with the ISWI, it positions the nucleosome downstream to the TSS. INO80 can also exchange the canonical H2A.Z with H2A, which enhances the accessibility of promoters for the transcription factor. INO80 complex interaction with the RNA polymerase II, helps in the eviction of H2A.Z for the smooth passage of RNA polymerases II through +1 nucleosome (Poli, Gasser, & Papamichos-Chronakis, 2017).
4. SWI/SNF (Switching defective/ sucrose nonfermenting) family: The ATP-dependent SWI/SNF complex or BAF complex was discovered in yeast and is indispensable for the developmental processes. Mutations in the BAF complex are even associated with many neurodevelopmental disorders, such as schizophrenia as well certain intellectual disorders, such as autism. Almost 20 % of cancer have been linked to mutations in anyone of the subunits of the BAF complex (Kadoch & Crabtree, 2015) (Wu, 2012) (Sokpor, Xie, Rosenbusch, & Tuoc, 2017). It comprises up to 15 subunits which are encoded by 29 genes.

These subunits are arranged in three different combinations to modulate the spatiotemporal expression of tissue-specific genes (Ho, Lloyd, & Bao, 2019). These are: canonical BAF (cBAF), non-canonical BAF (ncBAF) and PBAF (poly-bromo associated factor). SMARCA2 or SMARCA4 is the main catalytic subunit in all three subtypes of BAF complex. The rest of the subunits have the role in assembly of the complex, maintaining the structure and targeting to various regions of chromatin (Mashtalir et al., 2018). Unlike other ATP-dependent chromatin remodelers, SWI/SNF complex makes direct contact with nucleosome and sandwiches it from the three sides. The base, comprising of ARID1A and SMARCC, forms the primary scaffold for the assembly of BAF complex; the ATPase motor is at the top, which helps in the sliding movement of the nucleosome and the ARP (actin related protein) and connects both the modules (S. He et al., 2020) (Mashtalir et al., 2018). BAF-complex enhances the formation of iPS cells from the fibroblast cells, even in the absence of c-Myc, by enhancing the activity of Oct4 (Singhal et al., 2010). SWI/SNF complex interacts with EZH2 to control H3K27 methylation and with p300 for H3K27ac at the enhancer essential for the lineage specification (Alver et al., 2017). The BAF complex is important for the development of forebrain and regulates the proliferation, differentiation and survival of neural progenitors (Narayanan et al., 2015). BAF60c, a subunit of neural progenitor specific stem cells, is important during retinal development and its overexpression keeps the progenitor cell in the proliferative phase. Also, BAF60c is expressed in the proliferating Müller glia post-retinal damage (Lamba, Hayes, Karl, & Reh, 2008).

3.3.2 BAF complex is essential for the zebrafish retina regeneration

To decipher the role of SWI/SNF complex or BAF complex during retina regeneration, we adopted the pharmacological inhibition strategy using the drug PFI3. A small molecule inhibitor, PFI3, binds to the bromodomain of SMARCA2 (Brahma, BRM) or SMARCA4 (Brahma-related gene 1, BRG1), which recognizes the acetylation mark and blocks the binding of the BAF complex on the chromatin. Prior to the injury, we dipped the fish into the drug for 2 days to ensure that the functioning of BAF complex was inhibited even at the time of injury. After two days, we injured the retina using a 30 gauge needle and kept the fish again in the drug in different concentrations. We harvested the retina at 4dpi after 5 hours of BrdU pulse and checked the proliferation status. We found a decrease in the number of proliferating cells, marked by BrdU, in a concentration-dependent manner (Figure 3.3.2.1.A), which was quantified also (Figure 3.3.2.1.B). We then performed a time course analysis of four of its subunits: *arid1aa, arid1ab*, *smarca2* and *smarca4* by q-RT PCR analysis. We chose just four of the subunits because the BAF complex consists of many proteins. The Arid genes are the ones that helps in the assembly of the BAF complex, while Smarca2 and Smarca4 are the two catalytic subunits of the BAF complex. We found that levels of all these subunits are regulated during retina regeneration. It decreases around 1dpi and 2dpi, in a similar fashion to *yy1a* and *yy1b* and then again increases at 4dpi, which is the peak of proliferation (Figure 3.3.2.1.C).

We also checked the levels of various regeneration-associated genes in the BAF complex inhibition and found that their expression is regulated by the BAF complex (Figure 3.3.2.2.B). We were interested to see if Yy1 and BAF remodelling complex regulate each other. We checked the levels of *yy1a* and *yy1b* in BAF complex inhibition and there was no significant change in the levels of both the genes (Figure 3.3.2.2.B). We then did reverse and checked the expression of *arid1aa* and *smarca4* in the *yy1a* and *yy1b* combined knockdown down condition

and interestingly, we observed a slight increase in their levels. Thus, confirming that they are not getting regulated by each other (Figure 3.3.2.2.C).

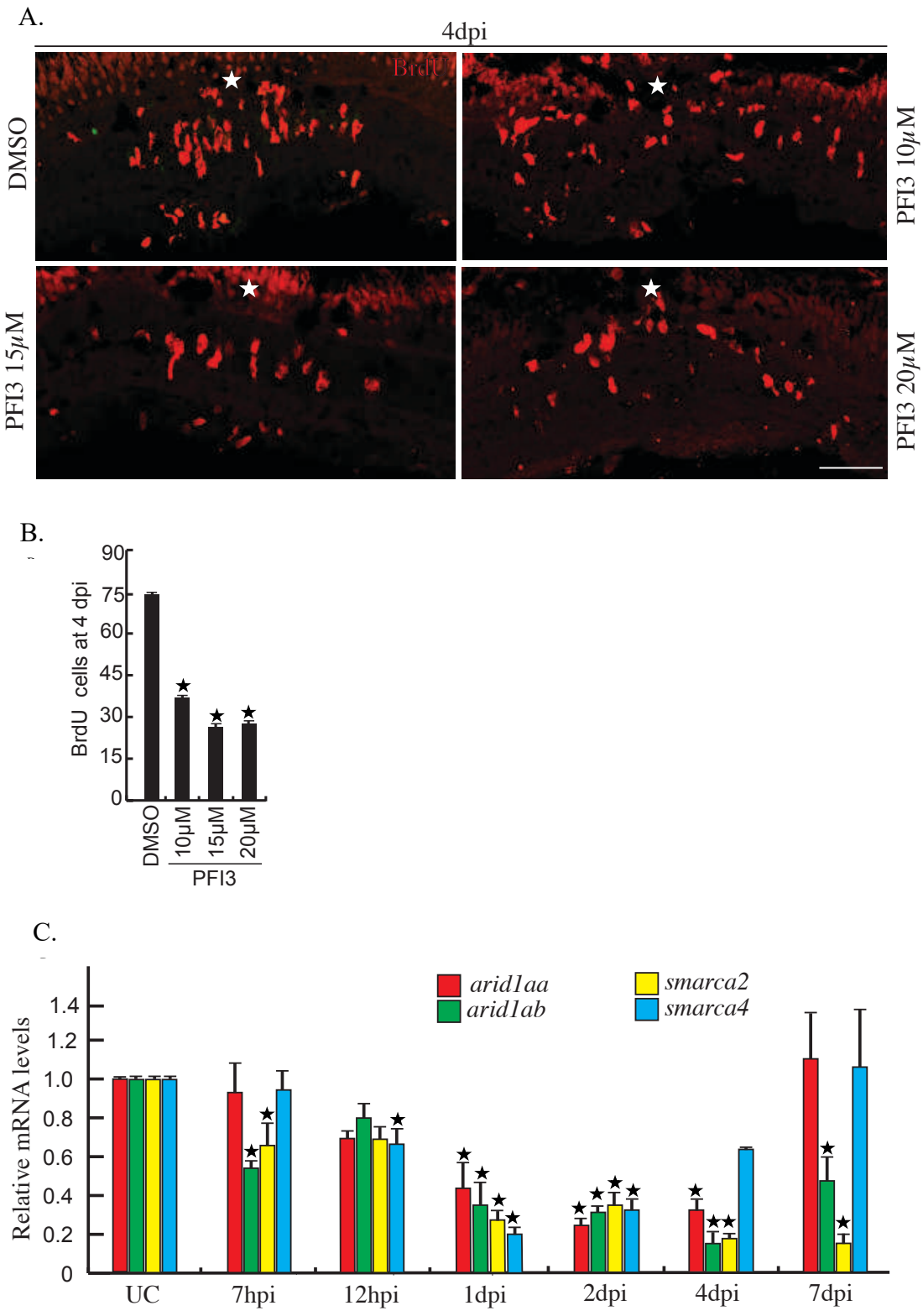


Figure 3.3.2.1. Components BAF complex are regulated and are essential for retina regeneration. (A) Confocal microscopy images of retinal section shows reduction in the number of MGPCs in the BAF complex inhibited condition using the drug PFI3, which was quantified in **(B). (C)** Time course analysis of four subunits of BAF complex, *arid1aa, arid1ab, smarca2* and *smarca4,* shows their regulation during retina regeneration. P<0.0001 in B and C.

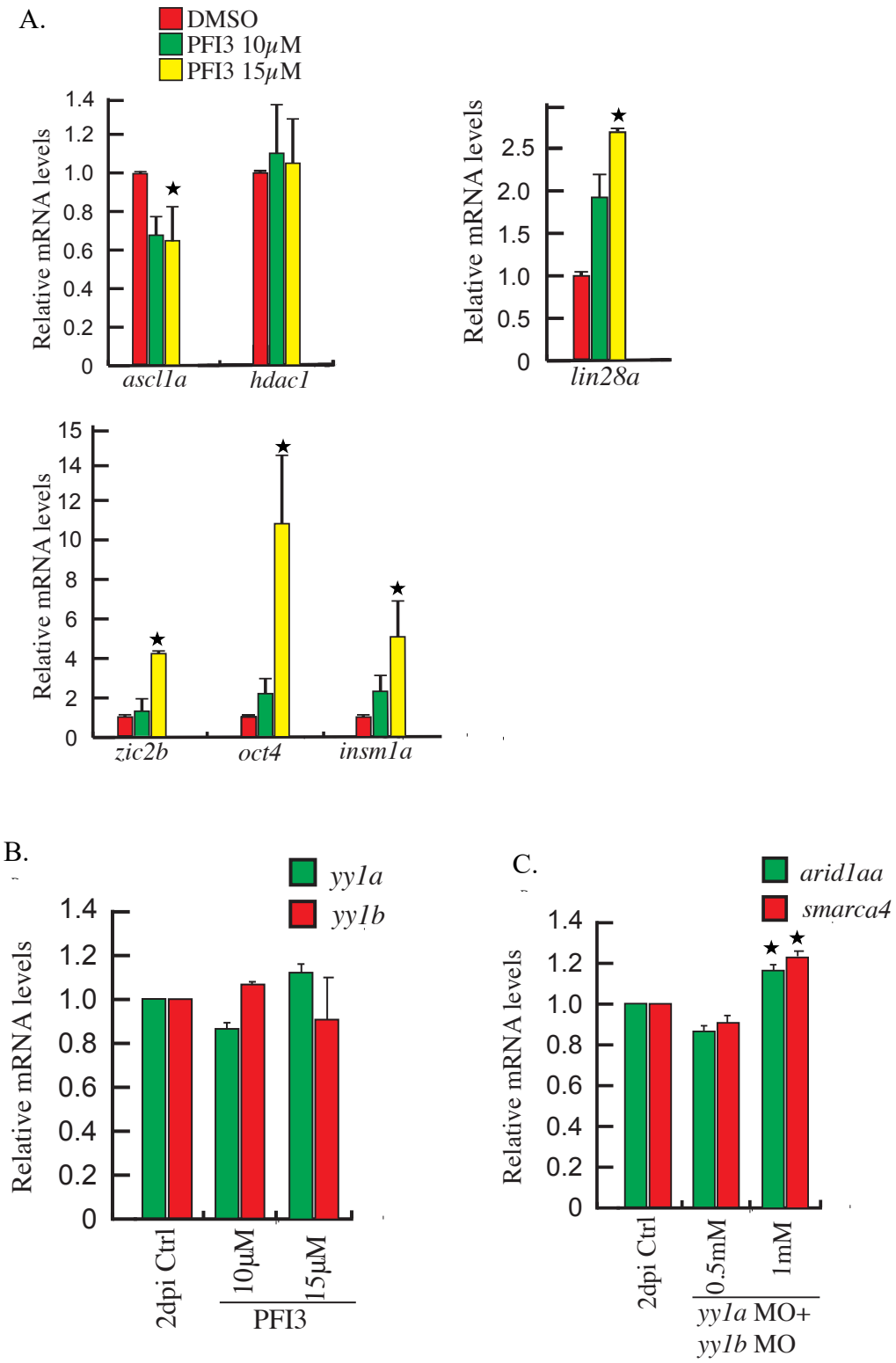
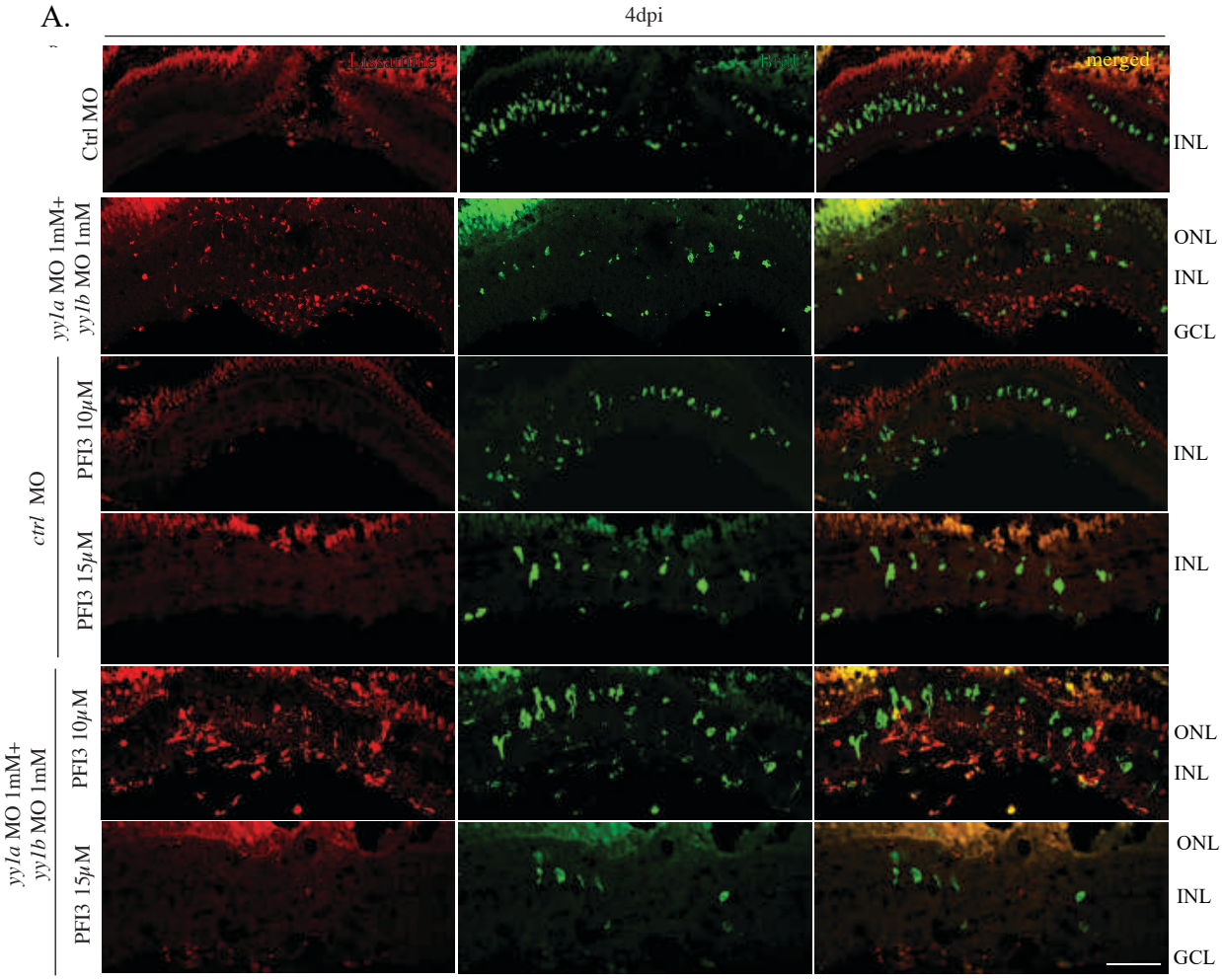
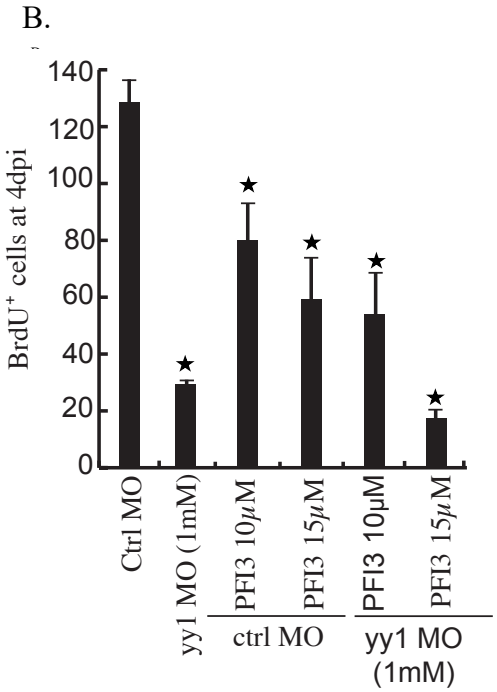


Figure 3.3.2.2. Components BAF complex are regulated and are essential for retina regeneration. (A) qPCR analysis of RAGs, *ascl1a, lin28a, hdac1, zic2b, oct4* and *insm1a* in the treatment with PFI3, at 2dpi. **(B)** qPCR analysis shows that *yy1a* and *yy1b* are not regulated in PFI3 treatment**. (C)** qPCR analysis shows that *arid1aa* and *smarca4* are not regulated in the *yy1a* and *yy1b* knockdown**.** p<0.05 in A and C.

3.3.3 Yy1 and BAF complex synergistically regulate the MGPCs proliferation

YY1 interacts with one of the subunits of BAF complex, SMARCA4, and regulates the transcription of many pluripotency factors such as OCT4, NANOG, n-MYC, etc. Thus, the enrichment of YY1 at the promoter and enhancer of the pluripotency-associated marker genes, along with BAF and OCT4, promotes proliferation in the mouse embryonic stem cells (J. Wang et al., 2018). We also speculated if Yy1 and BAF complex works together to regulate the expression of regeneration-associated genes and thus the reprograming of the Müller glial cell to form the MGPCs. For this, we injected MO against *yy1a* and *yy1b* and dipped the fish in the water containing the drug PFI3, a pharmacological inhibitor for BAF complex. We found that in such a scenario, the number of proliferating cells marked by BrdU was less than that of *yy1a* and *yy1b* knockdown and BAF complex inhibition alone (Figure 3.3.3.1A), which was quantified also (Figure 3.3.3.1.B). We then looked into the expression of few of the RAGs Ascl1a, Lin28a, Oct4 and Zic2b by western blot assay. We found that the expression of the key regeneration-associated genes is regulated in such a scenario and found that the expression of Ascl1a and Lin28a decreases more than the *yy1* knockdown and BAF complex alone, which could be the reason for the decrease in the proliferation. Interestingly, expression of Zic2b, which increases in the knockdown condition, have more profound decrease in the combined knockdown of *yy1* and BAF complex inhibition (Figure 3.3.3.1.C).


A.
4dpi
merged
Ctrl MO
yy1a MO 1mM+
yy1b MO 1mM
PFI3 10μM
PFI3 15μM
ctrl MO
yy1 MO
INL
ONL
GCL


B.
BrdU+ cells at 4dpi
140
120
100
80
60
40
20
0
Ctrl MO
yy1 MO (1mM)
PFI3 10μM
PFI3 15μM
PFI3 10μM
PFI3 15μM
ctrl MO
yy1 MO
(1mM)

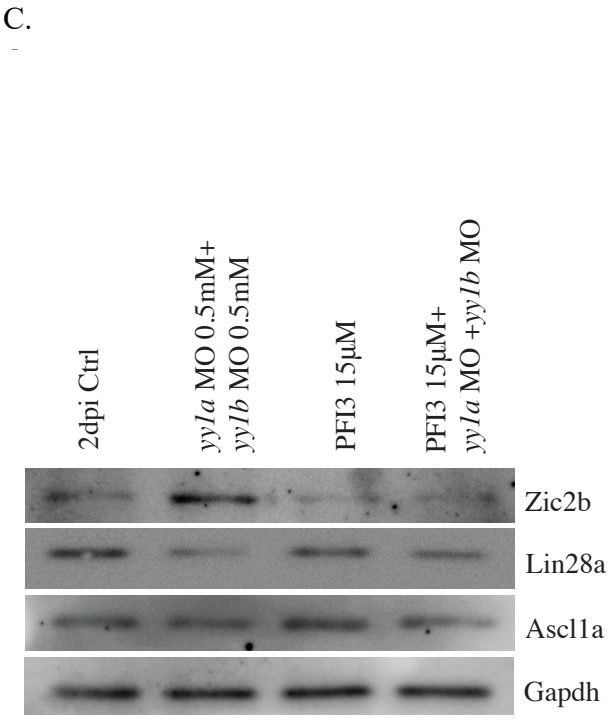

C.
2dpi Ctrl
yy1a MO 0.5mM+
yy1b MO 0.5mM
PFI3 15μM
PFI3 15μM+
yy1a MO +yy1b MO
Zic2b
Lin28a
Ascl1a
Gapdh

Figure 3.3.3.1. Yy1 knockdown and BAF complex inhibition synergistically regulates MGPCs proliferation. (A) Confocal microscopy images shows the more drastic decline in the MGPCs proliferation in the combined treatment of *yy1* MO and PFI3, as compared to their individual treatment and the 4dpi control. Proliferating cells are marked with BrdU (green) and Lissamine tagged MO in red. **(B)** Quantification of the proliferating cell in the experiment mentioned in panel A. **(C)** Western blot analysis shows the regulation of RAGs in the knockdown of *yy1a* and *yy1b* along with PFI3 treatment. **(C)** Western blot analysis shows the regulation of RAGs in the knockdown of *yy1a* and *yy1b* along with PFI3 treatment. Scale bar represents 10μM. $p<0.0001$ in B.

3.3.4 Yy1 can also regulate MGPCs proliferation independent of BAF.

Yy1 interacts with different proteins and complexes to execute its function in different scenarios and regulate various genes differently. Originally, Yy1 was identified as a repressor because it recruits PcG proteins on the chromatin and represses gene expression. It has also been reported to interact with the Myc module and act as an activator for the pluripotency genes. The above result shows that Yy1 can synergistically regulate the MGPCs proliferation along with BAF complex. But, we also speculated if it could regulate proliferation independent of the BAF complex.

For this, we again dipped the fish in the uninjured state in the drug, PFI3, for two days, injured the retina and transfected the *yy1a and yy1b* mRNA into the retina. Retina was harvested on 4dpi after giving 4 hours of BrdU pulse. We observed that in the Yy1 overexpressed condition along with the BAF complex inhibition, there is an increased proliferating cell number as compared to the BAF complex inhibition alone, but still less that of Yy1 overexpression alone (Figure 3.3.4.4.A). The number of MGPCs marked by BrdU was almost equal to that of the 4dpi control (Figure 3.3.4.4.B). This gives us a clue that despite the inhibition of the BAF complex function, Yy1 overexpression could increase the proliferating MGPCs number, indicating that Yy1 can indeed increase the proliferation independent of BAF. However, BAF complex along with Yy1 can enhance the effect on the proliferation.

In this scenario, we then checked few of the regeneration-associated genes, such as *ascl1a, lin28a, oct4, sox2, mmp9, her4.1, hdac1* and *insm1a.* The expression of key RAGs, such as *ascl1a, lin28a* and *sox2* were also seen to increase when Yy1 was overexpressed along with BAF inhibition, which could reason the rescue of proliferation in such a condition. Interestingly, levels of well-known transcriptional repressors *her4.1* and *insm1a* also showed an increase to keep a check on the proliferation (Figure 3.3.4.5).

Next, we wanted to see if these increased number of cells, in the Yy1 overexpressed condition along with the BAF inhibition, survived or died by apoptosis. For this, we injured the retina, after giving the BrdU pulse, we harvested the retina on 20dpi and checked the number of $BrdU^+$ cells. We observed that these cells were indeed survived up until 20dpi (Figure 3.3.4.3.A), and this was quantified in (Figure 3.3.4.3.A).

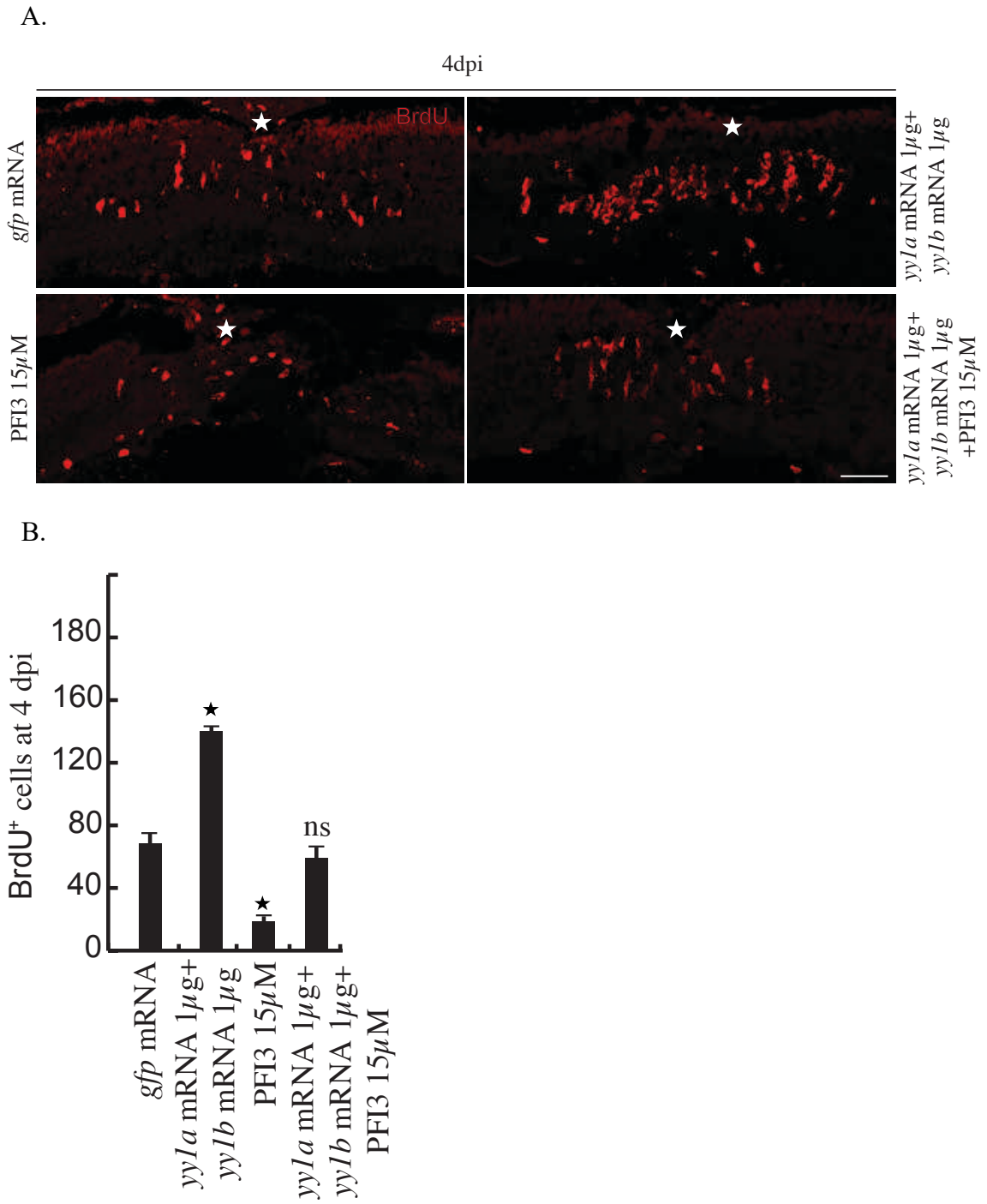


Figure 3.3.4.1. Yy1 can also regulate proliferation independent of BAF complex (A) Confocal microscopy images shows that number of proliferating cells in retina overexpressed with *yy1a* and *yy1b* along with BAF complex inhibition are more as compared to the BAF inhibition alone but less as compared to Yy1 overexpressed condition, which was quantified in **(B).** Scale bar represents 10μM. $p<0.0001$in B. ns represents non-significant.

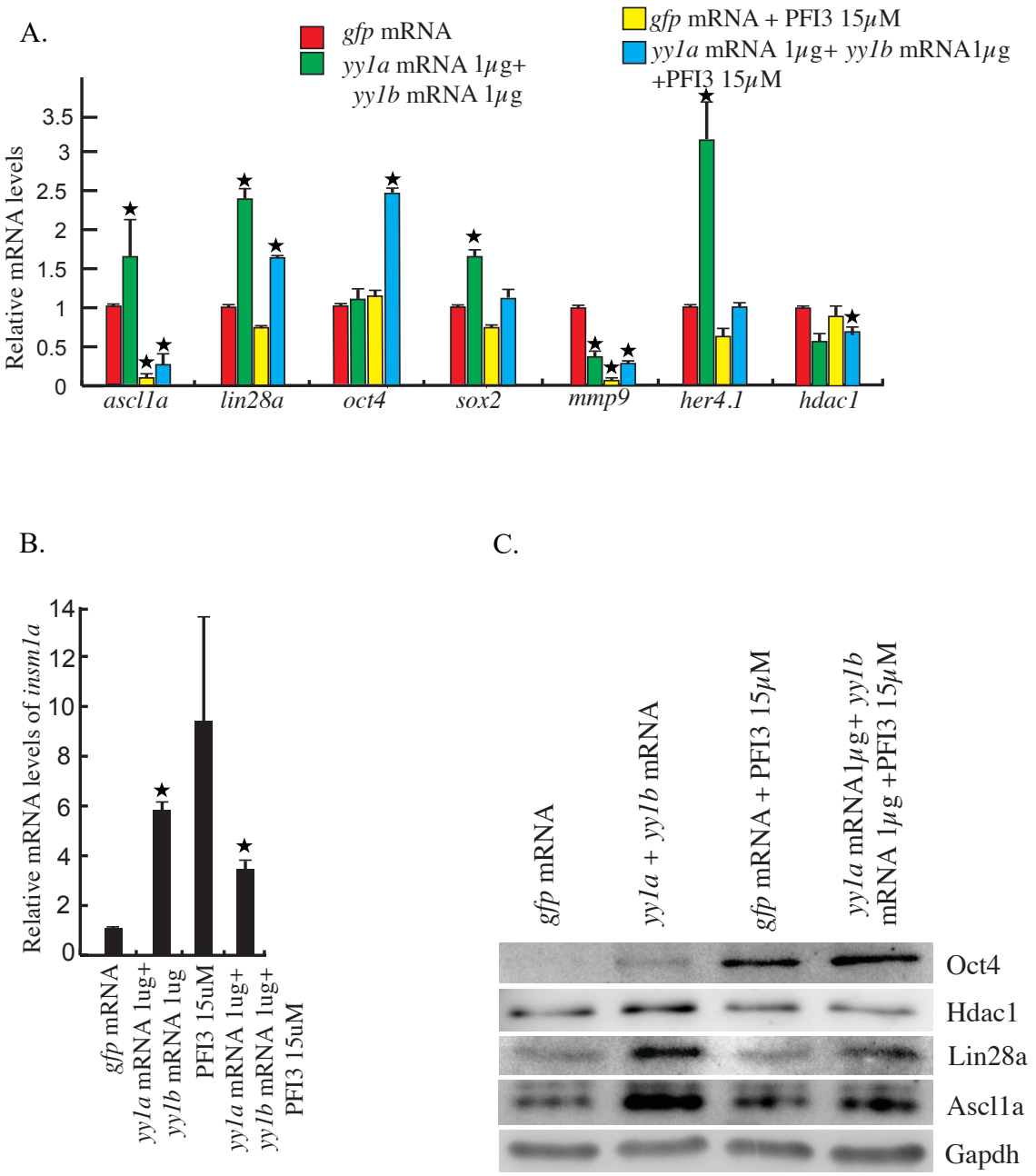


Figure 3.3.4.2. qPCR analysis shows the regulation of many regeneration associated genes such as, *ascl1a, lin28a, oct4, sox2, mmp9, hdac1* **(A)** *and insm1a* **(B),** in an experiment with Yy1 overexpression and BAF complex inhibition. **(C)** Western blot analysis shows the regulation of RAGs in the experiment mention in the panel A of Figure 3.3.2.5. $p<0.05$ in A.

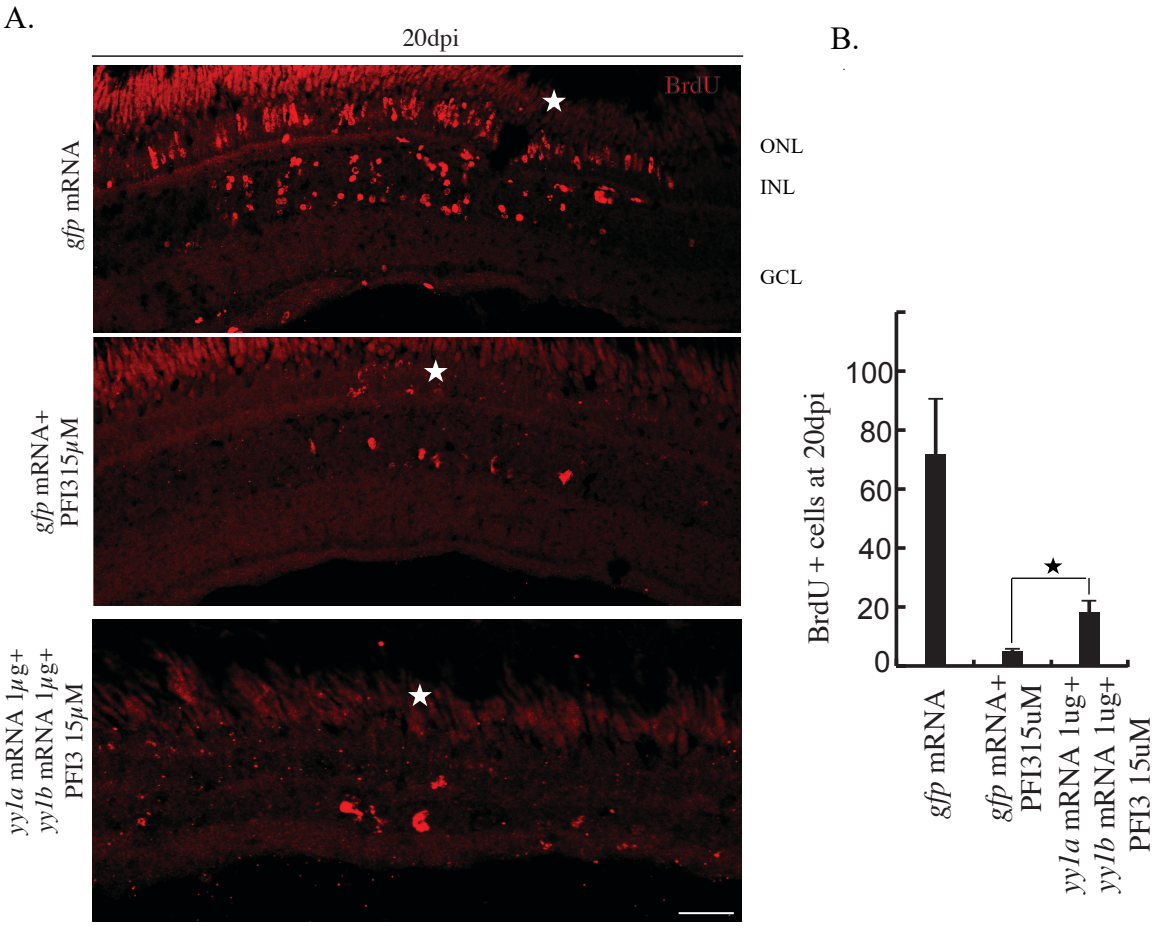


Figure 3.3.4.3. Proliferating MGPCs in the retina with Yy1 overexpressed condition along with BAF inhibition were viable even at 20dpi (A) Confocal microscopy images of the retinal section shows the number of surviving $BrdU^+$ cells, which was quantified in **(B)**. $p<0.05$ in B.

Section 4

Discussion

In the teleost such as zebrafish, Müller glia reprograming is essential to spontaneously heal and restore the retina's normal functioning. After receiving signals from the nearby environment, they reprogram themselves dedifferentiates, proliferates and eventually re-differentiates to form all the major retinal cell types including Müller glia. To date, much has been studied about the roles of many genetic and epigenetic factors involved in either one or two phase of regeneration or all. Stringent regulation of gene activation and repression in a timely manner is prerequisite for the highly elegant yet complex process of regeneration. Co-ordinated expression of genes, which can induce in response to injury and then subsequently evoke a genes regulatory cascade, also requires the involvement of many epigenetic factors. Since regeneration involves recapitulating the developmental pathways, certain part of highly condensed chromatin, which was silenced for long, must be exposed and activated for expression in response to injury. Epigenetic factors are the key players which are involved in changing the accessibility of chromatin during the expression of certain genes. These modifications which helps in changing DNA accessibility, involves DNA modification, Histone modification and ATP-dependent chromatin remodelling. The role of few of the epigenetic factors, such as DNMTs, Apobec2a, Apobec2b and Hdacs, have been well-studied during retina regeneration (Luz-Madrigal et al., 2020) (Powell et al., 2013) (Si et al., 2023). It has been known that YinYang1 (YY1) can interact with many of the epigenetic modifiers, such as HATs, Hdacs and Ezh2, which are recruited on the promoter of key pluripotency genes during the development as well as in the iPSCs.

In our study, we have tried to unravel the role of two genes of Yy1 present in the zebrafish,Yy1a and Yy1b, during retina regeneration. Yy1 has been previously reported to have a pro-proliferative effect in embryonic stem cells and have pro-tumorigenic function. In a similar line, our study also places Yy1 as a pro-proliferative factor, the absence of which causes a reduction in the reprograming efficiency of Müller glia cells. On the other hand, its abundance

tends to increase the proliferation. Yy1 is a ubiquitous transcription factor in all the retinal layers and all the retinal cell subtypes. Despite this, we observed that there were almost 70% of $BrdU^{+}$ cells which were devoid of *yy1a and yy1b* expression giving us a clue that during the proliferative phase, its absence from the proliferating cell while the presence in the neighbouring cell is essential for the expression of pro-proliferative genes. While looking at the protein levels, we found that there was no appreciable change in the levels of Yy1 throughout the regeneration regime, but spatially, we could see that most of the proliferating cells were not having the expression of Yy1. There could be two reasons for this, first, maybe Yy1 is absent from the proliferating cell and since MGPCs number in the whole retina is only 2% percent, we could not appreciate that change in the western blot analysis with whole retina lysate. Secondly, it's been known that Yy1 shuttles between the cytoplasm and the nucleus during different phase of mitosis. It is present in the nucleus during G1/S boundary and during G2/M boundary but it is mostly cytoplasmic during the mitotic phase. Although this hypothesis needs more experimental clarification. Since at 4dpi, MGPCs are in the dividing phase, we speculated that may be Yy1 is in the cytoplasm instead of nuclear and that's why we did not see significant co-localization of Yy1 with the BrdU. From the time course analysis we also assume that presence of Yy1 in the Müller glia is essential for the reprograming to happen. Since, Yy1 can directly bind to the promoter of genes such as *ascl1a, lin28a, oct4, zic2b* and *sox2* and regulate their expression. Interestingly, we found that in the *yy1* knockdown condition there is an increase in the expression of *ascl1a,* while upon overexpression we found that there is an increase in its expression, which is also reflected in the luciferase assay as well. All these observation, tells us that although Yy1 positively regulates Ascl1a, but in the knockdown condition also increased level of *ascl1a* transcripts indicate that *ascl1a* is not solely regulated by Yy1. Moreover, there is also an upregulation of *let-7a* miRNA in the *yy1* knockdown condition, which could be the reason for the decrease in the levels of Ascl1a protein levels.

Interestingly, we observe that Yy1 negatively regulates Oct4 and Zic2b, which has a pro-proliferative role during the early phases of regeneration. This could be due to the fact that many pluripotency factors, such as Oct4, has a concentration dependent activity. Oct4 when present in a excess amount, can be anti-proliferative due to its interaction with repressors like Hdacs. Therefore, the presence of Yy1 is essential to keep a check on the levels of Oct4 during the initial phase when it is expressed pan-retinally to initiate the de-differentiation process.

Yy1, a multifaceted transcription factor, regulates myriads of genes and signalling pathways and this was also reflected in our RNA-seq data done in the *yy1* knockdown condition. We found that two developmentally important signalling pathways, Notch signalling and BMP signalling were downregulated. Previously, various studies have reported the role of Notch signalling during retina regeneration. We also report the differential regulation of Yy1 on various Notch signalling components. Upregulation of *notch3 and dll4* in the *yy1* knockdown indicates that the absence of Yy1 leads to the failure of Müller glia to dedifferentiate.

The increase in the levels of Her4.1, a well-known effector gene of notch signalling, in the knockdown could reason the decrease in the proliferating cell number at 4dpi. Surprisingly, in the overexpression of Yy1 also, levels of *her4.1* goes up to put a curb on the uncontrolled proliferation. This also exemplifies the systematic and controlled regulatory mechanisms that have been programmed during the retina regeneration in zebrafish.

BMP signalling has been previously shown to be pro-proliferative during chick retina regeneration and is also a developmentally important pathway. In our study, we also report the pro-proliferative role of BMP signalling as the absence causes significant decline in the number of proliferating MGPCs. In line with the RNA-seq data, Yy1 positively regulates the expression of two of the R-Smads genes, *smad1* and *smad8*, and also *id1*, which is a well-known direct target of BMP signalling and an early response gene. It will be interesting to know if the R-

Smads of BMP signalling physically interact to activate or repress the RAGs involved during the regeneration cascade.

HDACs are one of the key epigenetic regulators. Primarily they work on histone deacetylation, making them more compact and thus repressing transcription. Besides modifying histones, HATs and HDACs can also acetylate and deacetylate, respectively, other non-histone proteins. Our study, found that Yy1 interacts with Hdacs and gets deacetylated in the central domain to start its activation function. In the absence of Hdacs activity, either the presence of TSA or acetylated mimetic mutant mRNA of Yy1, the full potential of Yy1 to function as pro-proliferative factor is lost, which ultimately leads to decline of the number of proliferating MGPCs. This also sheds light upon the fact that function of Yy1 to act as an activator or repressor is dependent on the timely post-translational modification it undergoes.

Yy1 has been reported to interact with ATP dependent chromatin remodelers, such as INO80 and SWI/SNF complex, besides its interaction with the chromatin modifiers. But very limited knowledge on the role of this complex in the regeneration context was available. Therefore, we started to explore its importance during retina regeneration and we found that indeed , it plays a crucial role as a pro-proliferative factor. Also it acts synergistically with Yy1 to regulate the proliferation and the expression of regeneration-associated genes. Apart from this, Yy1 can have function independent of BAF, which was reflected from our data in which we overexpressed Yy1 along with the BAF inhibition. The result indicated that in such a scenario, overexpressed Yy1 could increase proliferation to some extent even in the absence of a functional BAF complex, probably through other pathways, which needs to be explored further.

Taken together, our study sheds light on the novel role of Yy1 in the field of retina regeneration and gives us more strong evidence that, indeed, it is a multifaceted transcription factor, which

can interact with numerous proteins and can regulate the expression of many proteins, both negatively as well as positively.

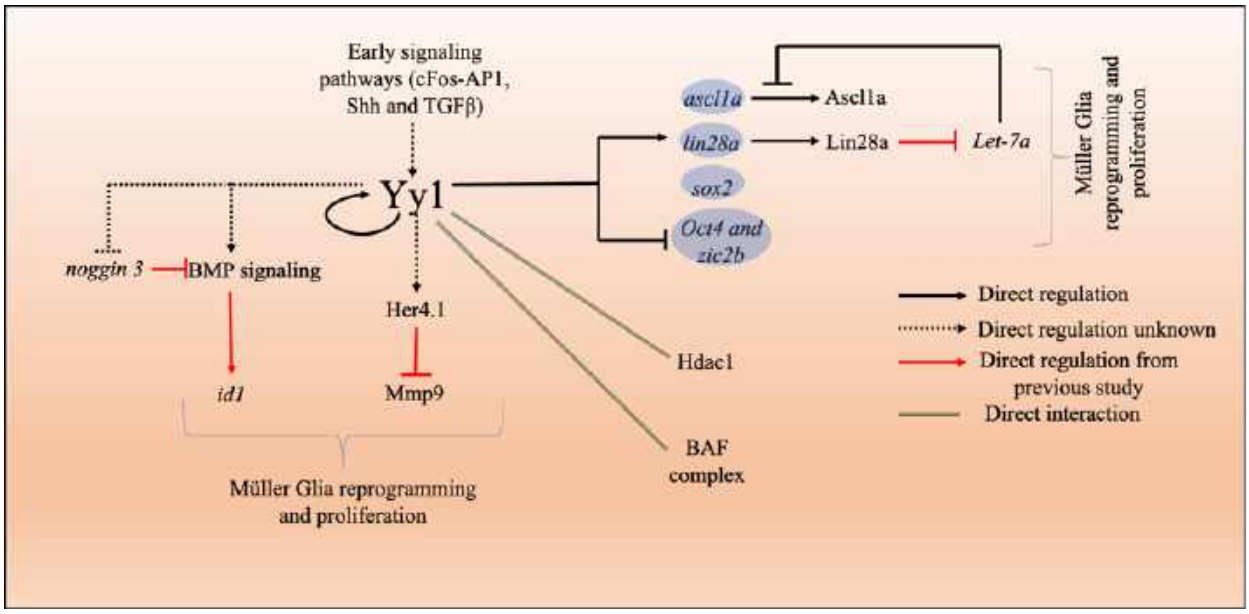


Figure 4.1. Model demonstrating the involvement of Yy1 in the gene regulatory network during zebrafish retina regeneration

Section 5

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Appendix 1:

List of primers

Cloning primers	**Ensembl ID**	Sequence (5'-3')
BamhI-yy1a-fwd	**ENSDARG00000042796**	ATGCTAGCGGATCCACCATGGCGTCGGGCGAGACACTG
XhoI-yy1a-rev		ATGCTAGCCTCGAGTCACTGATTGTTCTTAGCTTTCGCGT
BamhI-yy1b-fwd	**ENSDARG00000027978**	ATGCTAGCGGATCCACCATGGCGTCCGGCGAGACGCTGTAC
XhoI-yy1a-fwd		ATGCTAGCCTCGAGTCACTGGTTGTTTTTGGCTTTGGCGTGTGT
XhoI_gfap pro_fwd		ATGCTAGCCTCGAGTGGAACGGGAGATCAACTGCATG
HindIII_gfap pro_rev		ATGCTAGCAAGCTTGAGTGCGGGTGAACTCTGGGTTTTTATAG
HindIII_FLAG_yy1aCDS_fwd	**ENSDARG00000042796**	ATGCTAGCAAGCTTACCATGGACTACAAGGACGACGATGACAAGGACTACAAGGACGACGATGACAAGATGGCGTCGGGCGAGACACTGTACATC
MluI_FLAG2X_yy1a CDS_rev		GCTGCCACGCGTCTTGTCATCGTCGTCCTTGTAGTCCTTGTCATCGTCGTCCTTGTAGTCCTGATTGTTCTTAGCTTTCGCGTGTGTC
BamhI_zic2b CDS_fwd	**ENSDART00000054066.5**	ATGGTAATGGATCCACCATGCCCAGAGGATTTTTAGTCAAG
HindIII_zic2b CDS_rev		ATGCTAGCAAGCTTAACGTACCACTCGTTAAAATTGGACG
p2a removal_r		GCTCCTCGCCCTTGCTCACCATCTGATTGTTCTTAGCTTTCGCG
p2a removal_f		GAAAGCTAAGAACAATCAGATGGTGAGCAAGGGc
Mutation primers		
yy1a _lysine to alanine_f		CTGGTAGGATGGCGGCAGGAGGAGGCAGTGGGGCTGCAGTGGTCGCAGCGAGCTTCCTAAAC
yy1a_lysine to Alanine_R		GTTTAGGAAGCTCGCTGCGACCACTGCAGCCCCACTGCCTCCTCCTGCCGCCATCCTACCAG

yy1a_lysine to glutamine_F		CTGGTAGGATGGAGGAAGGAGGAGGCAGTGG GGAAGAAGTGGTCGAAGAGAGCTTCCTAAAC
yy1a cds_lysine to glutamine_R		GTTTAGGAAGCTCTCTTCGACCACTTCTTCCC CACTGCCTCCTCCTTCCTCCATCCTACCAG
YY1B_lysine to alanine_F		CTCAGATCTCGAGTTGCCTTCAGAATAAATCA CTAATGTCC
yy1b_lysine to alanine_R		CCGCGGGCCCGGATCCCTTCGCCAGCTGAAA GGCACTTCAGTCG
yy1b_lysine to glutamine_f		GCAGCGGGAGGCGAGGAAACCGGCGAGGAG AGTTACTTGAG
yy1b_lysine to glutamine_r		CTCAAGTAACTCTCCTCGCCGGTTTCCTCGCC TCCCGCTGC
yy1b MO BS Mutation_F		GATCCACCATGGCATCAGGAGAGACGCTGTA
yy1b MO BS Mutation_R		TACAGCGTCTCTCCTGATGCCATGGTGGATC
qPCR Primers		
actin-RT-F	**ENSDARG00 000037746**	GCAGAAGGAGATCACATCCCTGGC
actin-RT-R		CATTGCCGTCACCTTCACCGTTC
yy1a_RT_fwd	**ENSDARG00 000042796**	CAGCTGGCAGAGTTTGCCAGAATGA
yy1a_RT_rev		TTCCACATCCCTCAAATGTGCACTGGA
yy1b_RT_fwd	**ENSDARG00 000027978**	GTCACCATGTGGGCGTCGGATGAT
yy1b_RTnew_rev		CCCTGAACATTTTACTGCAGCCCTTAT
ascl1a RT Fwd	**ENSDARG00 000038386**	ATCTCCCAAAACTACTCTAATGACATGAACTC TAT
ascl1a RT Rev		CAAGCGAGTGCTGATATTTTTAAGTTTCCTTT TAC
lin28a RT Fwd	**ENSDARG00 000016999**	TAACGTGCGGATGGGCTTCGGATTTCTGTC
lin28a RT Rev		ATTGGGTCCTCCACAGTTGAAGCATCGATC
her4.1 RT fwd	**ENSDARG00 000056732**	GCTGATATCCTGGAGATGACG
her4.1 RT rev		GACTGTGGGCTGGAGTGTGTT
insm1a RT Fwd	**ENSDARG00 000091756**	CCAAGAAAGCCAAAGCCATGCGGAAGC
insm1a RT Rev		TTATTGCTTTCCGCGCTCTGCTTGGGTTTG
hdac1 RT_Fwd	**ENSDARG00 000015427**	GACAGCACCATTCCTAATGAGCTCC
hdac1 RT_ Rev		TATCGTGAGCACGAATGGAGATGCG
sox2-RT-F	**ENSDARG00 000102318**	GAAAAACAGCCCGGACCGCATCAAGAGACC

sox2-RT-R		GTCTTGGTTTTCCTCCGGGGTCTGTATTTG
mmp9-RT-F	**ENSDARG00000104474**	GGAGAAAACTTCTGGAGACTTG
mmp9-RT-R		CACTGAAGAGAAACGGTTTCC
oct4-RT-F	**ENSDARG00000037859**	AGATAACGCACATATCCGATGATCTAGGCCTG
oct4-RT-R		TGCGGGTGAGCATGCATGAATTGAGACATTG
zic2b_RT_fwd		ATCCGAGTGCACACGGGAGAGAAAC
zic2b_RT_rev		TGGAGCCTGATCCTCGTGAACCTTC
rb1_RT_F	**ENSDARG00000058557**	GATCGATGGACAAGACAAATATGC
rb1_RT_R		CTGATGAACTGCTTCACACTC
tp53_RT_F		GGGACATCATTAATGATGAGGAG
tp53_RT_R		CAGGTCCGGTGAATAAGTG
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
dll4_rt_fwd	**ENSDARG00000070425**	CCAAACCTGGAGAGTGTGTATG
dll4_rt_rev		GTGTGCAAAAGTTCAAATCTTGG
notch3_RT_F	**ENSDARG00000052139**	GCACAGGGAGTTTTCCAGATTC
notch3_RT_R		AACAGCGGAGTCTCCTCCTTG
smad1_RT_f	**ENSDARG00000027199**	GCCCTTTTCAGATGCCAGAAAC
smad1_RT_r		TAGGCCACAGGATGAACATCTG
smad5_RT_f	**ENSDARG00000037238**	GAACATGCCCAGAGGGGATG

smad5_RT_r		CCCAGGCAGAAGCGATTTTTG
smad8/9_RT_f	**ENSDARG00000021938**	CTGACGGCTCCACAAAGAGAC
smad8_RT_r		TGGAGTTGCGGTTGACGTTG
id1_RT_f	**ENSDARG00000040764**	GACGAGCAGATGACCATGTTTC
id1_RT_r		GGTCATCTGAACAGCCGTTCTC
nog3_RT_f	**ENSDARG00000053528**	AATCCTCCAGAGGACAAGCAC
nog3_RT_r		CACTTTCAAATAACGGGGCCAG
smarca2_RT_f	**ENSDARG00000008904**	GCAAGAGAGAGAATACCGACTTC
smarca2_RT_r		GAAGAATGCTGTTGAGATACTCCTG
smarca4_RT_F	**ENSDARG00000104339**	AGCAGATCCTGGCCAAGATCC
smarca4_RT_R		CACACTTGATGACATACTCCACCTTC
arid1aa_Rt_F	**ENSDARG00000101710**	CCCCTGGGTAGAAACCAGACTC
arid1aa_RT_R		TGGTAAGGAGTGCCTGTCTGTC
arid1ab_rt_f new	**ENSDARG00000101891**	TCGGTGGTATGAGAAAAGCGG
arid1ab_rt_r new		CGGTCTCTTCTGTGTGTAATTCGG
fos ab/cfos_RT_F	**ENSDARG00000031683**	CGCTCAACCAGACTCAGGAGTTC
Fosab/cfos_RT_R		TCAAGCTGGTCAGTTTCAGCTTG
mRNA *in-situ* hybridization Primers		
yy1a_insitu_f		CATTTGAGGGATGTGGAAAGCG
yy1a_insitu_T3_r		AATTAACCCTCACTAAAGGGCTGAACGACATCTCATTCTCC
yy1b_insitu_f		GTTTGAGGGCTGTGGGAAACG

yy1b_insitu_T3_r		AATTAACCCTCACTAAAGGAAGAAGAGCACG CTGCAAAC
id1_insitu_f		GACATGAACAGCTGCTACAGC
id1_insitu_t3_rev		AATTAACCCTCACTAAAGGCTTTGACCGAGA GGGAAATGC
ChIP Primers		
Yy1 BS on yy1 intron_F		GACATCGCATCAAGTGCTTTGTTC
Yy1 BS on yy1 intron_R		GCGGTAACATTTGTTTTTAGGAGACG
Yy1BS123_ascl1 a pro_f		GTTATTCAACATGTGCGTCCG
Yy1BS123_ascl1 a pro_r		CATCTGCAGGTGTTAAACGGG
Yy1BS 45_ascl1a pro_f		CACTATTGACCTACCCTTACACC
Yy1BS 45_ascl1a pro_r		CTTTTCGTTCCATAGAGGGTTC
Yy1BS 678_ascl1a pro_f		CACCATGGTGCTATATGTCAGC
Yy1BS 678_ascl1a pro_r		CAATAAAGGTTCTTGGCAGTTCC
Yy1BS11_ascl1a pro_f		GGACTCCTAGACATGCACCG
Yy1BS11_ascl1a pro_r		GCTCCTGCCACTGAGTAAAC
Yy1 BS8_lin28a pro_F		CACAATCGTCTGTGTAATAACTGG
Yy1BS8_lin28a_ R		ACACCACATAGTGGATGGACA
Yy1BS9,10_lin28 a_F		GCCCATTCAATGATCGTTTTTG
Yy1 BS9,10_lin28a_R		TACTACTTTAACGACGCCAGG
Yy1_BS5_oct4 pro_F		TATTGCATGCTGTCTTCTGGG
Yy1_BS5_oct4 pro_R		CTTCACGCTGCTTGTTGAATC
Yy1BS67_oct4 pro_F		CAACAGCAGCAGACTGCAG
Yy1_BS67_oct4 pro_R		TTTGGGCTGCGTAAAGTCG
Yy1 BS2345_zic2b pro_F		GTGAGTCTCAGATGTTTCAATTGAG
Yy1 BS2345_zic2b pro_R		AAGGAAGAGGGAGGACATGAG

Yy1BS6789_zic2b pro_F		TCGTGGTGATGTTACTGGACG
Yy1BS6789_zic2b pro_R		GAAAACCTCACCGGGTAATCCAAG
Yy1BS12_sox2 pro_f		TGGCCCTTTACAAGAGCGAAC
Yy1BS12_sox2 pro_r		CATGATCCATAGCGTCCAGTC
Yy1BS6_sox2 pro_f		CGGCTTATTCACGCATTATTTTGG
Yy1BS6_sox2 pro_6		GTTTGTCGCTGACCAATCAGAG
Yy1BS8_sox2 pro_f		GATGGAAACCGAGCTGAAGC
Yy1BS8_sox2 pro_r		CTTCATCAGGGTCTTGGTTTTC
Yy1BS9_sox2 pro_f		CAACCAGAGGATGGACAGCTA
Yy1 BS9 _sox2 pro_r		CTCGAACTAGACTCGGACTTG