Regulation of Yy1a and Yy1b during Zebrafish Retina Regeneration

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A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science.

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Indian Institute of Science Education and Research, Mohali
April 2019

CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled "**Regulation of Yy1a and Yy1b during zebrafish Retina Regeneration**" submitted by **Ms. Sushmita Nahar** (Reg. No. MS14167) for the partial fulfilment of BS-MS dual degree program of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Rajesh Ramachandran for giving me the opportunity to work on a project of my interest. I thank him for his constant guidance and invaluable support.

I thank Ms. Mansi Chaudhary, for guiding me throughout the project. I wouldn't have made it through without her directions, support and encouragement. I thank other lab members too, Shivangi, Dr. Poonam, Soumitra Mitra, Anwar, Ashish, Sharanya, Pooja Shukla for being really good friends to me and helping me throughout the project. I would like to thanks my batchmates Kemdina, Komal, Navnoor and also thank zebrafish for their sacrifice.

I would like to thank all my friends for making 5 years wonderful experience and encouraging me to perform better. I also thank my family for the constant motivation, having an unwavering belief in me, and bearing my expenses.

Finally, I would like to thank DBS, IISER Mohali and INSPIRE-DST for facilities and financial support.

DECLARATION

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rajesh Ramachandran at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a 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 acknowledgement 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.

Sushmita Nahar
(Candidate)

Dated: April 25, 2019

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

Dr. Rajesh Ramachandran
(Supervisor)

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NOTATIONS

- MG: Müller Glia
- RP-Retinitis pigementosa
- MG-Muller Glia
- MGPC-Muller glia derived progenitor cell
- R-Rods
- C-Cones
- A-Amacrine cells
- BP-Bipolar cells
- H-Horizontal cells
- GC-Ganglion cells
- ONL: Outer Nuclear Layer.
- INL: Inner Nuclear Layer
- GCL: Ganglion Cell Layer
- RT-PCR: Reverse Transcription PCR
- dpi: days post injury
- hpi: hours post injury
- UC: Uninjured Control
- Hdac: Histone deacetylase protein in zebrafish
- Yy1: Ying Yang 1 geneor transcript in zebrafish
- *Yy1*: Ying Yang protein
- CBN- Cucurbitacine
- Cyclo- Cyclopamine

ABSTRACT

Retinal cell loss after an injury is an inevitable problem in mammals which ultimately leads to blindness. In contrast, teleost fish such as zebrafish (Danio rerio) has a remarkable tendency to regenerate its damaged tissue and reestablish function. Upon retinal damage, they mount a robust response to generate their lost retinal cell types, restoring vision. Müller glial is the major players in retina regeneration. They respond to injury by changing their physiology, morphology, and biochemistry. They undergo phases of dedifferentiation, proliferation, and finally re-differentiation, all of which requires changes in gene expression. Although many signaling cascades and regulatory pathways have been identified to play roles at different stages of retina regeneration, chromatin remodeling, which is also one of the ways for transcriptional regulation of genome, is not well studied in the case of zebrafish retina regeneration. Signaling pathways like Notch, Mapk–Erk, Jak-Stat pathway, etc. have been shown to be turned on in the phases post-retinal injury. Roles of many signaling pathways, genetic and epigenetic factors that are involved in this process have been studied so far.

However, Yin Yang 1 (YY1) which is known to play a fundamental role in normal biologic processes such as embryogenesis, differentiation, replication, and cellular proliferation, still remains enigmatic during the regeneration process. At early time points during retina regeneration the expression levels of Yy1 goes down indicating that it is crucial for pre-proliferative phase. So, here we show the regulation of Yy1 by inhibiting the signaling that gets activated at early time points such as Jak/stat3, Notch signaling and Sonic hedgehog. We found all these three signaling regulating Yy1 expression levels. We have shown that Yy1a and Yy1b function predominantly during the de-differentiation phase to keep a check on proliferation.

As a preliminary result, we found that Inhibition of Jak/stat3 signaling and sonic hedgehog is positively controlled on Yy1 and inhibition of Notch signaling is negatively controlled by Yy1.This study might help provide a new direction for the regulation of regeneration process.

CHAPTER 1: INTRODUCTION

Billions of people suffer from diseases such as glaucoma, diabetic retinopathy, macular degeneration, etc, which can lead to loss of sight. Several approaches are used to restore the vision; the best way out to this problem is to develop a reparative strategy so that retina could heal itself after injury. Mammals have a limited capacity for regeneration of organs and tissues such as, skin, liver and skeletal muscles. Mammals cannot regenerate systems, like Central Nervous System (CNS)[1]. Teleost fish such as zebrafish, on the other hand, show incredible tendency for regeneration of CNS. Unlike mammals, Zebrafish (*Danio rerio*) have the capacity to regenerate their retina post injury and restore the sight. Zebrafish shares 70 percent genetic similarity with humans. Upon injury, Müller glia cells, which are the only glial cell types in retina, contribute to retina regeneration. Zebrafish Müller glia undergoes reprogramming, enters cell cycle and acquires stem cell characteristics that enable them to generate progenitors for retinal repair. Upon being insulted with an injury the MG re-enter cell cycle to acquire stem cell like properties forming Muller glia derived progenitor cells (MGPCs), and thus they proliferate and re-differentiate into various neuronal cell types and MG itself, that make up the retina. Therefore, unlocking the secrets of MG's reprogramming into stem cell like state will shed light on the possible ways with which we can tweak the mammalian retina to regenerate, to restore vision.

1.1 Zebrafish Retinal Architecture

Zebrafish retina is mainly divided into three layers Outer Nuclear Layer (ONL), inner nuclear layer (INL) and Ganglion cell layer (GCL). ONL consists of photoreceptor cells (rods and cones) which receive light. INL consists of interneuron Horizontal cells, Amacrine cells, and bipolar cells which help in transmitting information from ONL to GCL. GCL contains ganglion cells which transfer the incoming information to the brain through optic nerve. The only glial cell type present in the retina is Müller Glia. Their cell body lies in INL,

but their processes span all the retinal layers. Since the MG is well positioned it aids in the transfer of a lot of molecules across various retinal cells[2]. They help in releasing trophic factors, neurotransmitters and controlling ionic balance in the extracellular space. Apart from maintaining homeostasis they phagocytose parts of photoreceptors present near the outer epithelial layer and help in recycling retinal chromophores used in photo detection by photoreceptors[2].

1.2 Injury response by Müller Glia

Müller Glia response to various injuries such as mechanical (using needle poke), toxic chemicals or toxic genes, bright or UV light and, laser ablation. Upon sensing damage to the retina, MGs undergo a gliotic response where they overexpress tubulin proteins like Gfap[3]. Soon the gliotic response gets converted to a regenerative one when the MGs get reprogrammed to have stem cell-like properties. The process of regeneration moves through three main stages:

- **Dedifferentiation**: MG cells acquire stem-cell like properties after injury;
- **Proliferation**: a population of proliferating multipotent cells is generated; and
- **Re-differentiation**: the cells migrate and differentiate into neuronal cell types.

Some of the early signaling events in zebrafish that follow a mechanical injury are glycogen synthase kinase 3β (Gsk3β)–β-catenin, Notch, Mapk–Erk and Jak-Stat signaling pathways. Upon zebrafish retinal injury Wnt signaling gets triggered to induce reprogramming and proliferation of MG. wnt expression and β-catenin stabilization takes place in the MGPCs[2, 3]. The *let-7* miRNA signaling and Dkk signaling are two inhibitory pathways that are necessary to maintain a quiescent state of MG. Notch signaling also has an inhibitory role which suppresses the number of MG in which injury response is triggered which is evident by an increase in the expression of her4 a target of Notch signaling. One of the main players that suppress differentiation programs and promote proliferation is the pro-neural transcription factor *Ascl1a* (Achaete-scute homolog 1)[3]. *Ascl1*a stimulates *lin-28* expression which

suppresses *let-7* miRNA (associated with differentiation) expression and also stimulates *lin-28* expression.

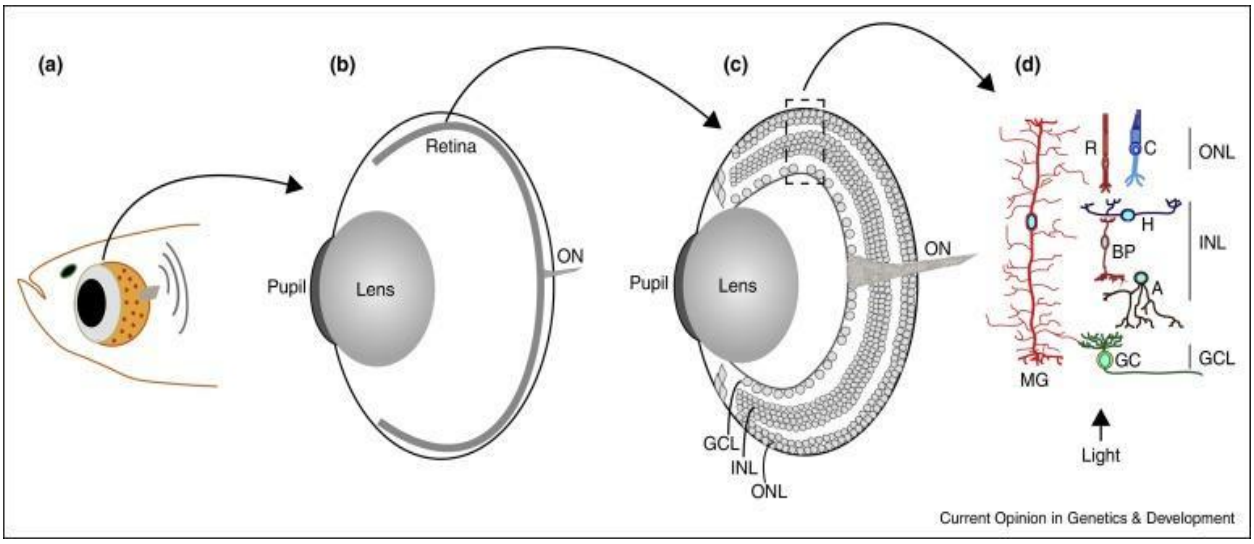


Fig1.1: Retinal Anatomy. Retina consist of ONL- Outer nuclear layer (photoreceptors rods and con cells), INL- Inner nuclear layer (Bipolar cells, Horizontal cells and Amacrine cells), GCL- ganglion cell layer consist of ganglion cells. is Müller Glia cell body lies in INL, but their processes span all the retinal layers.
Reference: Jin Wan and Daniel Goldman, *Science direct*,2017

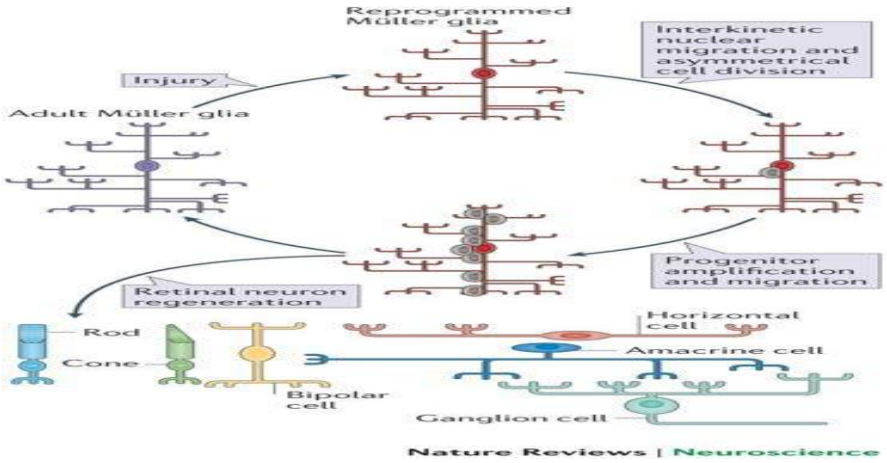


Fig 1.2: Regeneration Mechanism. After reprogramming, MG cells migrate to ONL and undergo asymmetrical cell division at OLM generating multipotent progenitors. These progenitors migrate to all retinal layers, exit cell cycle and form different cell types, regenerating retina. Interkinetic nuclear migration is migration of injury-responsive MG to ONL for division and then back to INL;
Reference: Daniel Goldman Review, july 2014 Müller glial cell reprogramming and retina regeneration.

As mentioned above several genetic and epigenetic factors have been known to play a crucial role during retina regeneration. Ying Yang (YY1) is a ubiquitously distributed transcription factor belonging to the GLI-Krüppel class of zinc finger proteins. The protein is involved in

regulating the different number of promoters. YY1 may direct Hdacs and Hats to a promoter to repress or activate any gene. YY1 also promotes enhancer-promoter chromatin loops by forming dimers and promoting DNA interactions. YY1 is known to have played an essential role in biologic processes such as embryogenesis, differentiation, replication, and cellular proliferation. Since expression and function of YY1 are known to be intimately associated with progression through phases of the cell cycle, the physiologic significance of YY1 activity has recently been applied to models of tumor biology[3].

1.3 Discovery of Yy1

Initially, Yy1 was cloned and characterized simultaneously by two independent groups, Shi et al. (1991) and Park and Atchison (1991) who were inspired by the original observation by Berns and Bohenzky (1987) and Chang et al. (1989). While investigating the adeno-associated virus (AAV) P5 promoter region and its activation by E1A gene products, using systematic deletion analysis of the P5 promoter, Chang et al. (1989) identified two elements associated with basal and E1A-induced P5 activity [3.] (1) the R1–R2 region, a tandem repeat sequence of 10 base pairs, and (2) a binding site for the major late transcription factor (MLTF). Both elements had a negative effect in the absence of E1A oncoprotein but converted to transcriptional activators in its presence. They theorized that the two trans-activators acted in concert to stimulate the P5 promoter and induce transcriptional activation in the presence of E1A. Simultaneous deletion of both elements reduced P5 promoter activity 25-fold, raising the possibility of the presence of the dual-acting transcriptional factor Yy1[4].

1.4 Biochemical and Crystal structure and cellular localization of Yy1

The Yy1 protein contains four C2H2-type zinc-finger motifs. The function of Yy1 protein as an activator or repressor depends on its binding structure. Within the zinc finger motif and glycine-rich residue two other domains contributing to its repression The N-terminus region, however, acts as a potent activation domain. This region is followed by 11 consecutive histidine residues and a glycine rich domain[4]. The role of this sequence remains elusive. For

transcriptional regulation and control, cellular localization of transcription factors to the nuclear matrix is essential. Gal-4-tagged Yy1 fusion proteins construct expressed in Hela cells and human Saos-2-osteosarcoma cells analyzed which reveals the C-terminal domain as the chief constituent involved in high-affinity efficient targeting of Yy1 to the nuclear matrix[10]. In association into the nucleus, the N-terminal domain of the protein permits a low-affinity that suggests the importance of the C-terminus in nuclear localization as well as transcriptional repression[10]. Yy1 nuclear localization and activity significantly increased in G1/S phase, followed by increased cytoplasmic localization in the late S phase with increased DNA-binding activity of Yy1 and Yy1-dependent histone genes[4].

Fig 1.3: The protein contains four C_2H_2-type zinc-finger motifs with two specific domains that characterize its function as an activator or repressor. Transcriptional repression is known to occur at the C-terminus (aa 298–397) directed by a promoter rich in GAL-4-binding sites. The N-terminus (aa 43–53), acts as a potent activation domain. Evidence that the zinc-fingers and glycine-rich regions of YY1 are instrumental in YY1 repression has been provided by deletional experiments of both regions, which render the protein incapable of transcriptional repression.

Reference: S Gordon, G Akopyan Review oncogene (2006), Transcription factor YY1: Structure, function and therapeutic implication in cancer biology.

CHAPTER 2: Materials and Methods

2.1 Retina dissection

1. Zebrafish was anesthetized using Tricaine methaneosulphate.

2. By using 30 gauge needle and stainless steel forceps retina was injured.

3. At a desired time after injury, eyes were dissected using forceps and needles.

4. Dissection were carried out either in 1XPBS (phosphate buffered saline) for RNA isolation or 4% PFA (paraformaldehyde) for tissue fixation. Lens were removed both for isolating RNA as well as tissue fixation.

2.2 Microscopy

Bright field microscope used for dissecting retina.

Confocal microscope (Nikon) was used for fluorescence imaging and its bright field mode was used for imaging in-situ slides.

2.3 RNA isolation

1. Dissected retina was collected in 200µl of Trizol in MCT.

2. Using 200µl pipette homogenize the tissue completely so that no tissues clumps are visible. Keep it at room temperature for 5 minutes.

3. 0.2 volume (40µl) of chloroform was added and mixed it gently by inverting the MCT 10-12 times.

4. Centrifuge at 10,000 rcf for 10 minutes at 4°C.

5. Using a cut tip collect aqueous phase 30µl without disturbing the middle layer phase, collected it in fresh MCT.

6. Equal volume of isoproponal was added and kept at -80°C for overnight precipitation.

7. Then centrifuge it at 10,000 rcf for 20 minutes.

8. Discard the supernatant and wash the pellet with 80% ethanol (200µl), centrifuge at 7600rcf for 10 minutes.

9. Dry the pellet at room temperature and dissolved in DEPEC treated water.

10. Eluted RNA was checked on 1% agarose gel and then stored it in -80°C. If the isolated RNA is free from any genomic contamination then it further proceed for cDNA synthesis.

2.4 cDNA synthesis

(Thermo Scientific Revert Aid First Stand cDNA Synthesis kit)

For 5µl reaction add the following reagents in a PCR tube

Template RNA	2.75µL
Oligo dT + random hexamers	0.25+0.25µL

Mixed it gently and incubate it at 65°C for 5 minutes and then put it back on ice immediately.

Following reagents were added in the indicated order

5X Reaction Buffer	1µL
RiboLock RNase inhibitor	0.25µL
10mM dNTPs	0.5µL
RevertAid M-MuLV RT	0.25µL
Total volume	5µL

Mixed the reaction properly, spin it down and were incubated at the following temperature

25°C	5minutes
42°C	60minutes
70°C	5minutes
4°C	infinite hold

Then cDNA was diluted in Nuclease free water (NFW) and stored in -80°C.

2.5 Reverse transcription polymerase chain reaction (RT PCR) using Taq polymerase

Dilute the synthesized cDNA by 1:8 dilution (1µL cDNA + 7µL NFW).

Set reaction for 10µL

20X buffer	- 0.5µl
dNTP	-1µl
primers (Forward+Reverse)	-0.2µl
template cDNA	-0.5µl (as per standardized volume)
MQ water	-7.7µl
Taq polymerase	-0.1µl

Cycling condition as following

95°C for 2 min	Enzyme activation
95°C for 20 sec	DNA denaturation
62°C for 30 sec	Primer annealing
68°C for 30 sec	Elongation
72°C for 7 min	Final elongation
4°C	Infinite hold

Put 25 cycles

PCR product was then checked by 1% agarose gel electrophoresis.

2.6. Tissue Fixation

1. Lens was removed from an eye in 4%PFA (4%PFA in 1X Phosphate Buffer). The eye was incubated in a MCT containing 4%PFA solution at 4°C overnight for fixation.

2. Next day serial sucrose washes was given each for 45 minutes at room temperature:

1. 5% Sucrose	1mL
2. 5% Sucrose + 20% Sucrose	800µL + 400µL
3. 5% Sucrose + 20% Sucrose	500µL + 500µL
4. 5% Sucrose + 20% Sucrose	400µL + 800µL
5. 20% Sucrose	1mL

3. 500µL of OCT was added into the existing solution and mixed on rotor for 30 min.

4. Then made a small cube of aluminum foil and embed the OCT in that cube and embedded samples kept frozen at -80°C.

5. Then fixed tissue was sectioned using cryostat (12µm thickness) and collects the sections on super frost plus slides and dried overnight and then stored in -20°C.

Composition of solutions used:

1. 4% PFA in 1X Phosphate buffer (made DEPC water).

PFA 2g

10X phosphate buffer 5mL

Make up the volume to 50mL with DEPC water.

Dissolve it by keeping in 65^{o}C and constant shaking.

2. 5% sucrose:

Dissolve 2.5g sucrose in 50mL of autoclaved water. Store at -20°C.

3. 20% sucrose:

Dissolve 10g sucrose in 50mL of autoclaved water. Store at -20°C.

2.7 Immunostaining

DAY 1

1. Slides were taken out from -20ºC and dried them in 37ºC for half an hour.

2. Wash the slides three times with 1xPBS, 10 min each by overlaying over the slides.

3. Then put the slides in 2N HCL/ 0.1mM Sodium Citrate (boiled at 100oC) for 20min.

4. Wash the slides twice with Sodium borate (pH 8.5, 0.1M) for 10 min each.

5. Block the tissues using 3% BSA in 1X PBST (1XPBS + 0.1%Triton X) for 1 hour.

6. Overlay the 1^{o} Antibody (500µL per slide) diluted in 1%BSA /PBST (1:1000) over the slides after blocking and keep it in 4ºC overnight.

DAY 2

7. Wash the slides twice for 10 min each with 1xPBST.

8. Overlay 2^{0} antibody diluted in 1%BSA in 1xPBST (1:1000) and keep in RT for overnight.

DAY 3

9. Wash the slides thrice with PBST for 10 min each.

10. Wash the slides twice with water and let it dry for 1 hour.

11. Then mount the dried slides with DABCO.

12. Take the image of the section under confocal microscope.

2.8 mRNA *in situ* Hybridization

Day 1

1. The slides were taken out from -20°C and dried by keeping them at 37°C for 3 hour.
Hydrated the slides in following sequence for 1 min each.

1. 100% EtOH

2. 95% EtOH

3. 70% EtOH

4. 50% EtOH

5. 2XSSC

2. Incubated slides in Proteinase K solution for 5 min at 37°C

Proteinase K buffer : 5mL of 1M Tris-HCl (pH = 8) + 5mL 0.5M EDTA + 40mL of DEPC water.

- Pre warmed Proteinase K buffer at 37°C.
- Added 160µL of 10mg/mL proteinase K.

3. Rinsed slides briefly in room temp DEPC water (2-3mins).

4. Rinsed slides in 0.1M TEA pH 8.0 for 3 min.

0.1M TEA: 0.93g of Triethanolamine (TEA) + 50mL of DEPC H2O + 173µL of 10N NaOH (for adjusting pH = 8)

5. Rinsed in Acetic anhydride/TEA for 10 min

- Added 130µL of acetic anhydride to 50mL of 0.1M TEA

6. Dehydrated the slides in 2XSSC and EtOH series for 1 min each:

1. 2X SSC

2. 50% EtOH

3. 70% EtOH

4. 95% EtOH

5. 100% EtOH

7. Dried the slides for at least 1 hour at RT.

8. Pre warmed the hybridization solution at 56°C (300µL per slide).

- Hybridization Solution (50mL): 3.6mL TEN solution + 25mL 100% Formamide + 10mL 50% Dextran Sulphate + 5mL 10% RMB blocker + 6.4mL of DEPC H2O.
- **Probe preparation:**

1. Add probe to hybridization solution and mixed (as per concentration of the probe).
2. Boil probe and hybridization solution mix at 100°C for 10 min.
3. Keep immediately on ice for 2-3mins.
4. Added 300µL of probe solution to each slide and cover slip with siliconized hybrid slips.
5. Place slides in humid chamber dampened with 50% Formamide/5X SSC and incubate at 56°C overnight.

20X SSC Stock (500mL):

- 87.6g of NaCl in 350mL of DEPC H2O + 44.12g of sodium citrate + Rest DEPC water to bring final volume to 500mL.

TEN Solution (3.6mL):

- 500µL of 1M Tris-HCl (pH-7.5) + 3mL of 5M NaCl + 1mL 0.5M EDTA.

10% RMB Blocker (50ml):

- 10% RMB blocker added to Maleic acid (100mM maleic acid + 250mM NaCl), heated and pH set to 7.5 to dissolve.

Day 2

- Preheat 50% formamide/2X SSC solution to 65°C.
- Preheat two 50mL RNase buffer washes, one to 37°C and the other to 65°C.

RNase buffer (50mL): 5mL of 5M NaCl + 500µL of Tris-HCl (pH 7.5) + 100µL of 0.5M EDTA + Rest H2O.

- Preheat two 2X SSC washes to 37°C.

1. Soak slides with cover slips in 2X SSC for 30 min at RT on shaker table , If the coverslips do not come off, gently teased them apart from slide with forceps.

2. Rinse slides in 50% Formamide/2X SSC solution for 30 min at 65°C.
3. Gently agitate for the first 5 min.
4. Rinse slides in 2X SSC for 10 min at 37°C (twice).
5. Add 100µL of RNase (10mg/mL) to the 37°C RNase buffer, Incubate slides for 30 min.
6. Wash slides in 65°C RNase buffer for 30 min.
7. Wash slides for 2-3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
8. Wash slides in 1X Maleate buffer for 5 min (twice).
9. Incubate slides with 500µL antibody (Anti-DIG) diluted in 1X Maleate/0.05% Triton X-100/1% RMB blocker solution (1:2500 dilution) overnight at RT.

Day 3

1. Wash slides twice with 1X Maleate buffer for 5 minutes.

- 5X Maleate Buffer (1L): 58g of Maleic acid in 850 mL of H2O, pH to 7.5 using NaOH, 43.8g of NaCl + Rest water to bring volume up to 1L.

2. Wash in Genius buffer twice for 5 minutes each.

- Genius Buffer (50mL): 5mL of 1M Tris-HCl (pH = 9.5) + 1mL of 5M NaCl + 5mL of MgCl2

3. Added 500µL of NBT/BCIP dissolved in Genius buffer (1:50 dilution), incubated at room temperature in dark for color reaction.

4. Colour detection: In bright field microscope.

2.9. Plasmid Isolation

1. 5mL of culture was centrifuged at 10,000rpm for 2 min, RT.
2. The supernatant was discarded and pellet was dissolved in 100µL of autoclaved MQ.

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

- For 1mL of lysis buffer, added 50µL of 20% SDS solution, 20µL of 0.5M EDTA and 10µL of 10N NaOH in 920µL of water.

4. The samples were boiled at 100°C for 2 minutes (till the solution becomes clear).
5. 50µL of 1M MgCl2 was added, tapped and kept on ice for 2 minutes.
6. Centrifuged at 10,000rpm for 2 min, RT.
7. 50µL of 3M potassium acetate buffer was added.

- For 100mL, 60mL of potassium acetate, 11.5mL glacial acetic acid and 28.5mL H_2O were mixed. Stored at 4°C.

8. Tapped immediately and centrifuged at 10,000rpm for 2 min, RT.
9. Supernatant was transferred into another MCT containing 600µL of Isopropanol.
10. Kept on ice for 5 min.
11. Centrifuged at 13,000rpm for 2min, RT.
12. 70% ethanol wash and pellet was dried completely.
13. Pellet was dissolved in 50µL of autoclaved MQ.
14. Stored at -20°C.

2.10. RNA Probe Synthesis

Add the following components to set up a reaction:

1. Buffer (10X) 1µL
2. Digested Template 1µL (100-200ng)
3. DIG-UTP 0.5µL

4. Enzyme (T7 or SP6) 0.5µL

5. DEPC treated MQ 7µL

Total 10µL

Incubated at 37°C for 4 hours.

After the incubation added following components:

1. 0.5M Tris-EDTA 1µL

2. 5M LiCl 1µL

3. 10mg/mL glycogen 0.5µL

4. Absolute ethanol 17.5µL

1. Tapped to mixed well, kept at -80°C overnight for precipitation.

2. Centrifuged at 4°C for 30 min at 14, 000 rpm.

3. Washed with 80% ethanol (200µL) and centrifuged for 10 min at 14,000rpm, 4°C.

4. Dried and dissolved the pellet in 15µL of DEPC treated water.

5. Checked the probe on agarose gel

6. Stored at -80°C.

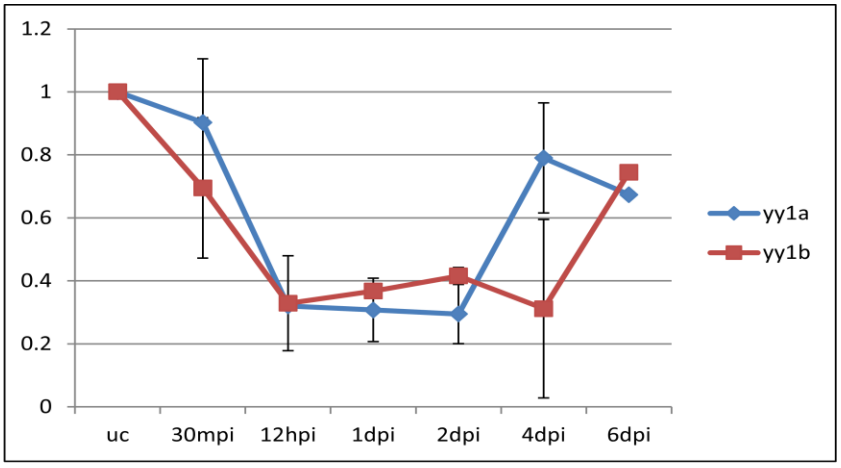
Following enzyme was used for linearization:

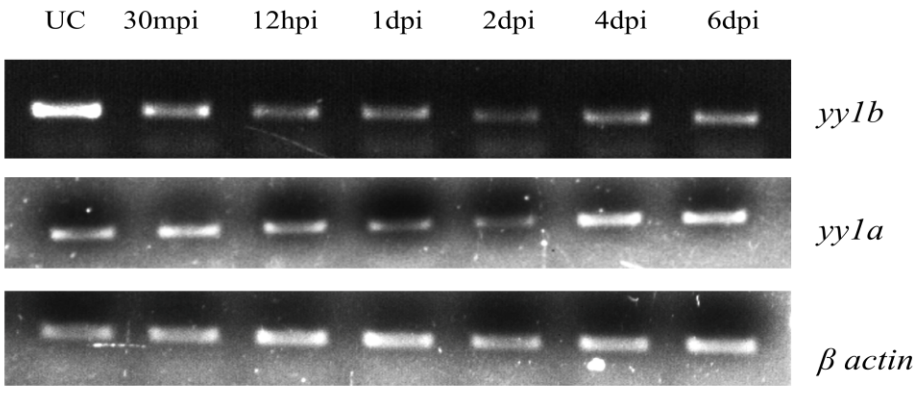
YY1a and yy1b: Bamh1

CHAPTER 3: EXPERIMENTS AND RESULTS

3.1 Temporal expression pattern of *yy1a* and *yy1b*

As a first step to understand the regulation of yy1 at different time points during retina regeneration, regulation of *yy1a* and *yy1b* at various time points post-retinal injury was checked. This time course is already done in the lab by a senior Ph.D. student. To investigate the temporal regulation of *yy1a* and *yy1b* fish were injured at 0dpi. The retina was dissected and mRNA was isolated for RT-PCR and Q-PCR at required time points. We observed a fluctuating expression pattern of *yy1a* and *yy1b* where it decreases drastically at 1dpi and 2dpi de-differentiation phase after injury and then it starts increases at 4dpi, indicating the role of *yy1a* and *yy1b* in pre-proliferative stages of regeneration.





mpi: minutes post injury ; hpi:hours post injury; dpi: days post injury

Fig3.1: mRNA expression level of *yy1a* and *yy1b* at different time course, method RT-PCR and Q-PCR.

3.2 Inhibition of Jak/Stat3 signaling

Just after the injury cytokines acting via Jak/Stat signaling stimulate MG reprogramming and retina regeneration. We check the expression pattern of Yy1a and Yy1b by inhibiting Jak/Stat3 signaling using the pharmacological inhibitor cucurbitacin which inhibits the jak phosphorylation and stat dimmerization did not form so it cannot enter to the nucleus and start the regulation of targeting genes.

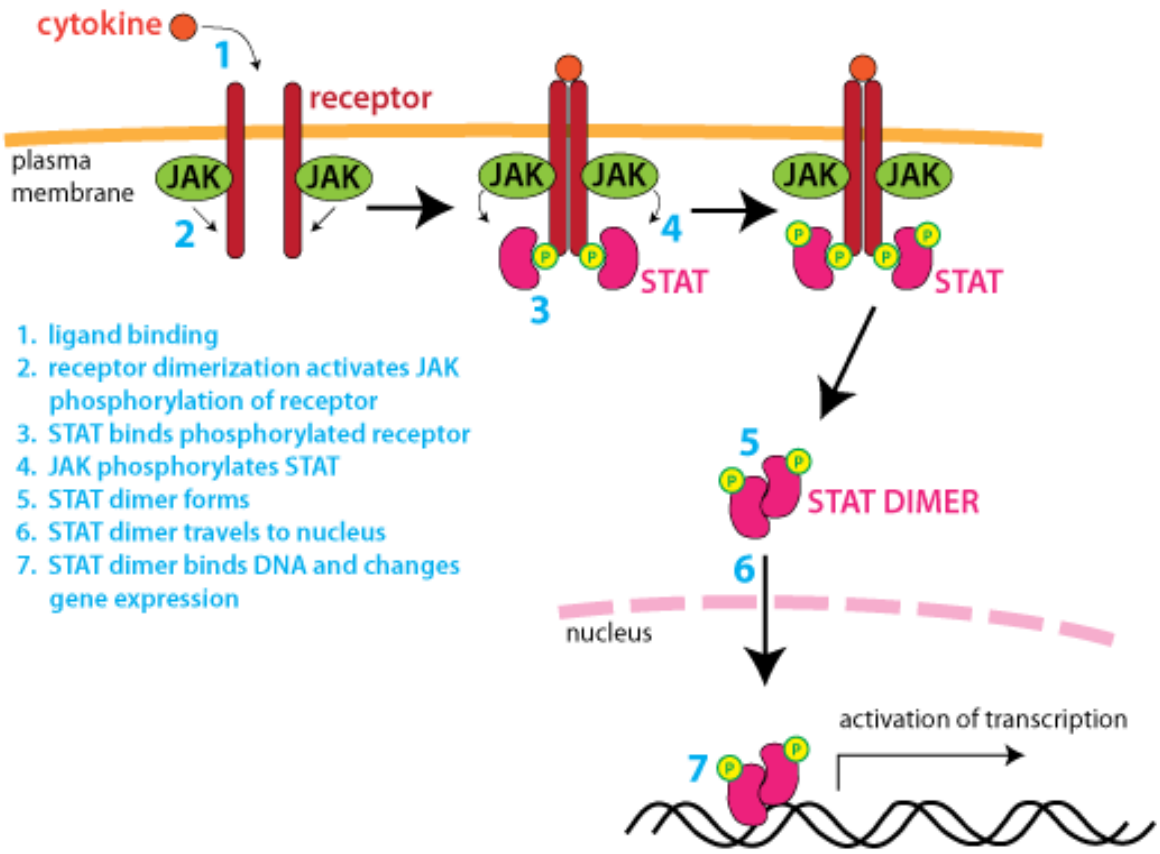


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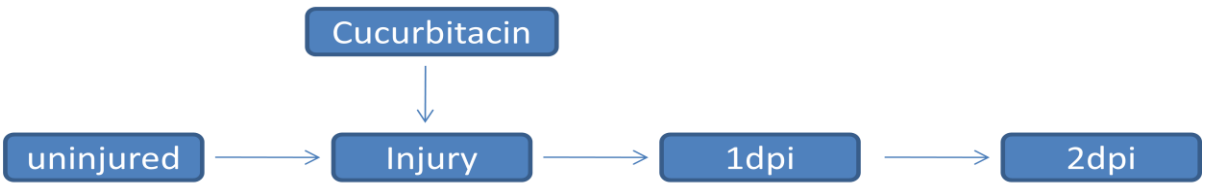
Fig 3.2 Inhibition of Jak/stat3 signalimg

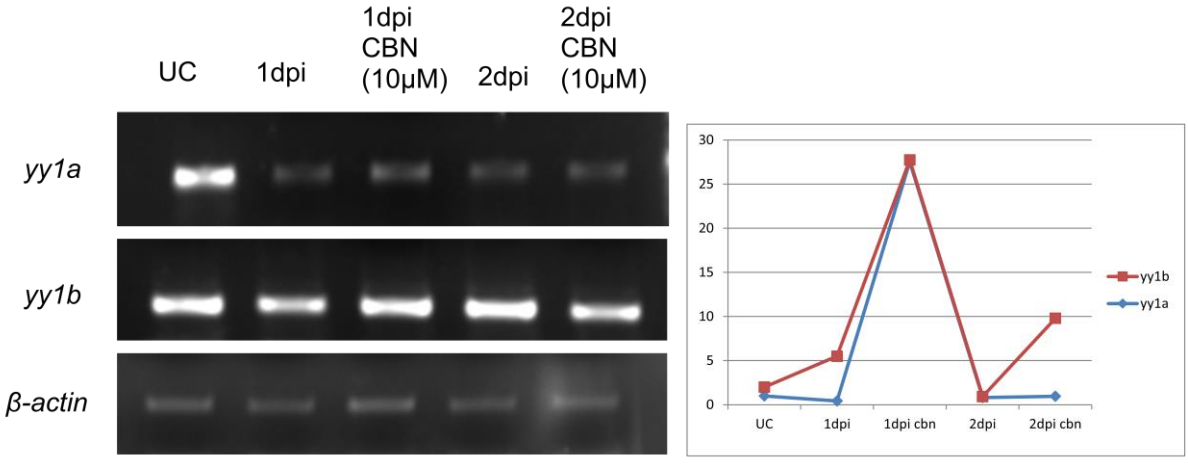


3.2.1Inhibiting Jak/stat3 signaling increases the level of Yy1a

As we observed that yy1a and yy1b expression level decreases at 1dpi and 2dpi, so we decided to check the signaling which plays a role just after the injury. To study this, the effect of the inhibition of the jak/stat3 signaling on the expressions levels of *yy1a* and *yy1b* and on the protein levels ofyy1, using cucurbitacin (10μM) was checked. For this experiment, we injected the drug in the eye of fish at the time of injury. The mRNA levels of *yy1a* and *yy1b* were checked using RT-PCR and Q-PCR and spatial expressions checked by in situ hybridization. The protein levels were analyzed using western blotting and immunochemistry. This revealed that an expression level of *yy1a* increases at 1dpi and 2dpi drug-treated retina as compared to control the genes which play roles in proliferation is upregulated.

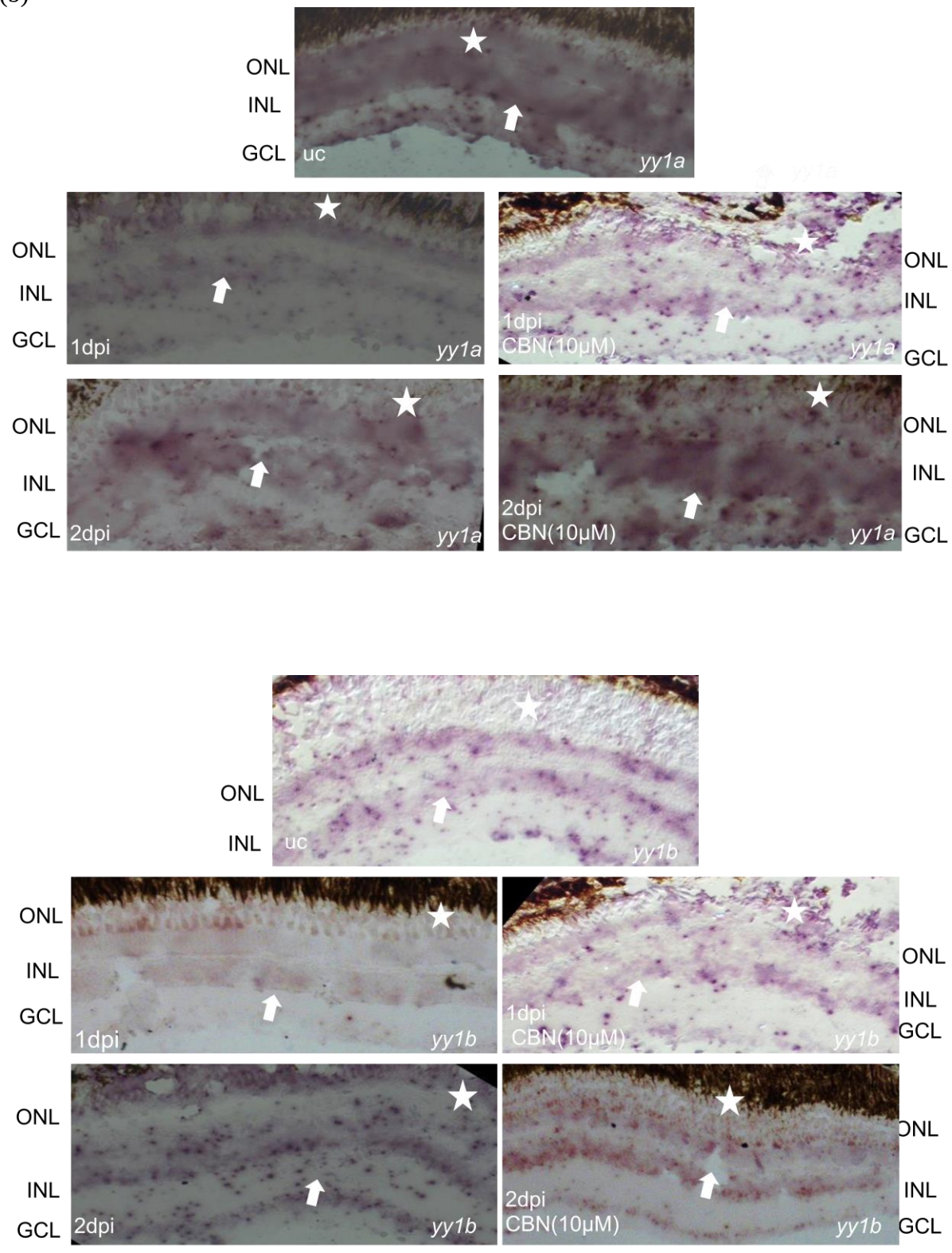
3.2.2 Inhibition of Jak/stat signaling has no effects on the level of Yy1b

Yy1b temporal level also checked at 1dpi and 2dpi by doing RT-PCR and spatial expression by *in situ* hybridization. The protein levels are analyzed using western blotting and immunochemistry. This revealed that inhibition of jak/stat3 by cucurbitacin does not show any effects on *yy1b* expression levels.(a)

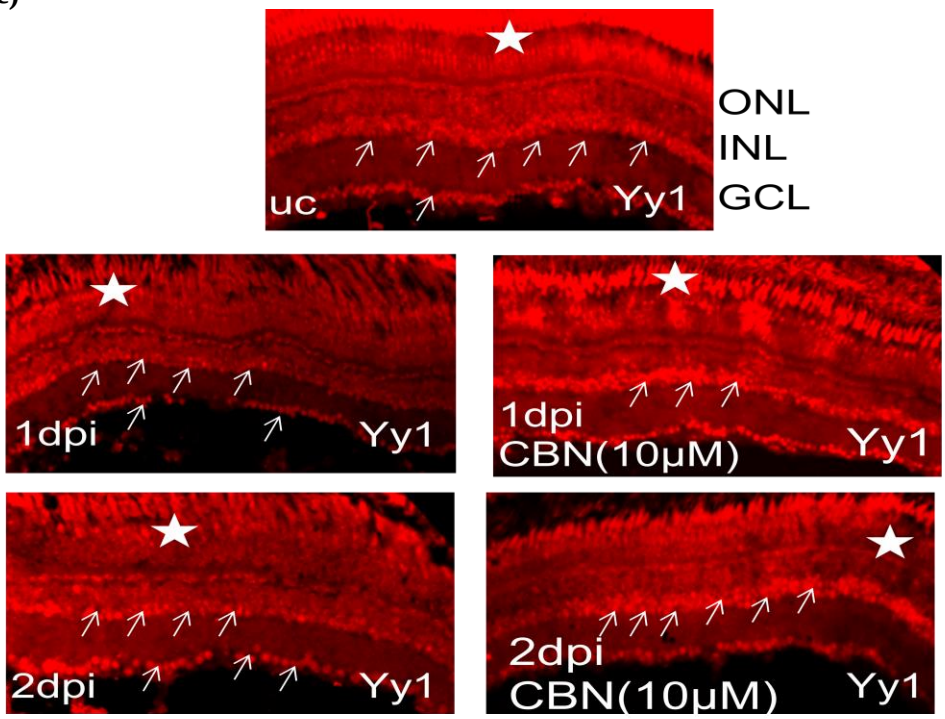


yy1a: yin yang1a *yy1b:yin yang1b* *CBN*: Cucurbitacin

(b)



(c)



(d)

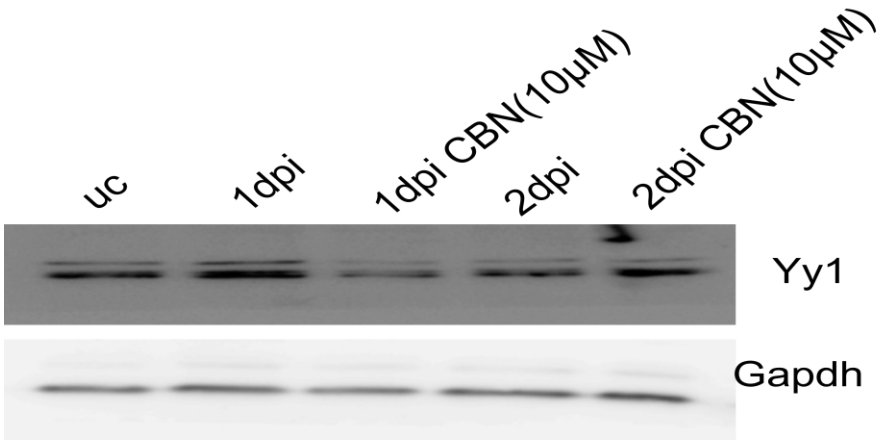
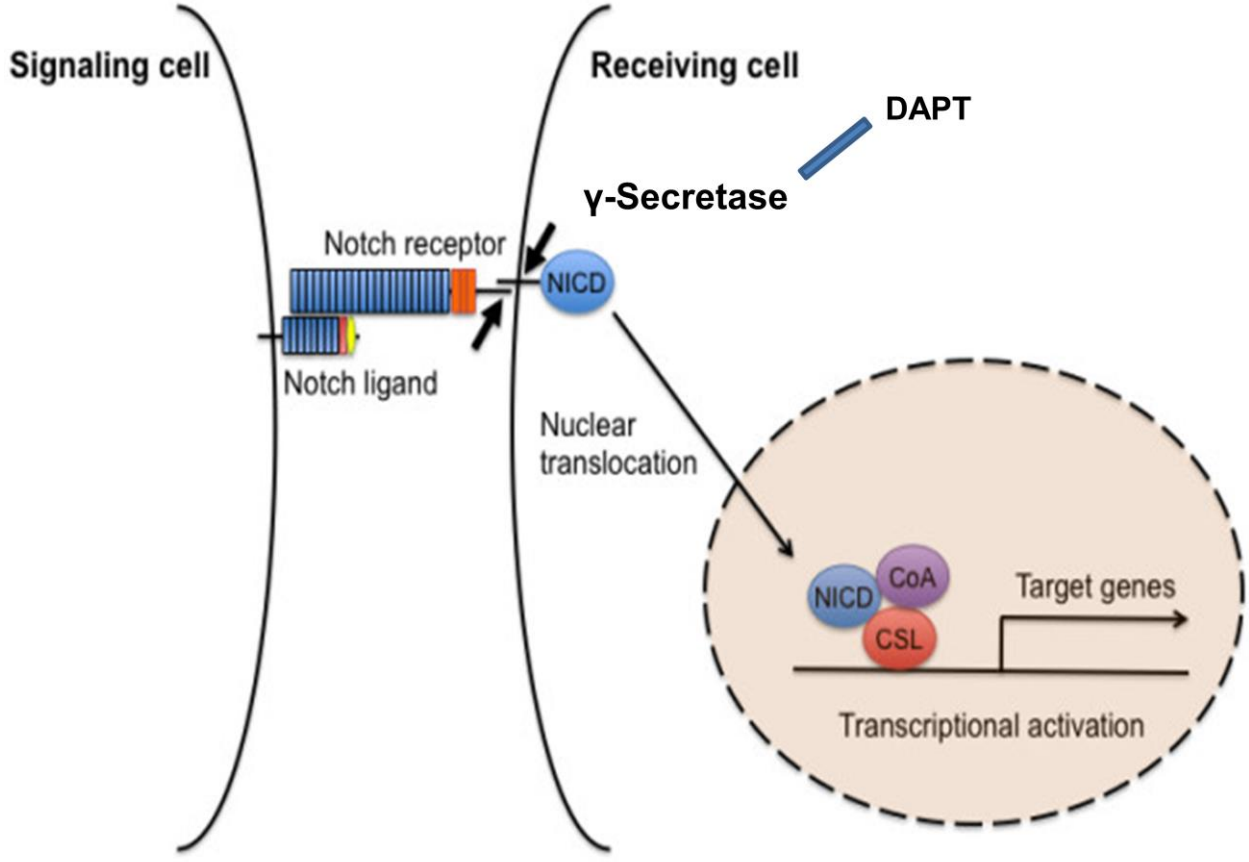


Fig 3.2.1: Inhibition of Jak/Stat3 using cucurbitacin, mRNA expression level of *yy1a* and *yy1b* (a)RT-PCR and QPCR (b)spatial expression level of *yy1a* and *yy1b in situ hybedisation* arrows shows the signals and star point shows the injury spot, (c) protein levels of Yy1 immunochemistry and(d) western blotting. Showing that proetiens levels increases in jak/stat blockase condition using cucurbitacin(10μM).

3.3 Inhibition of notch signaling

Notch signaling is important for retinal progenitor cell maintenance and MG specification during development, and its manipulation may be critical for allowing MG to re-enter the cell cycle and regenerate neurons in adults.

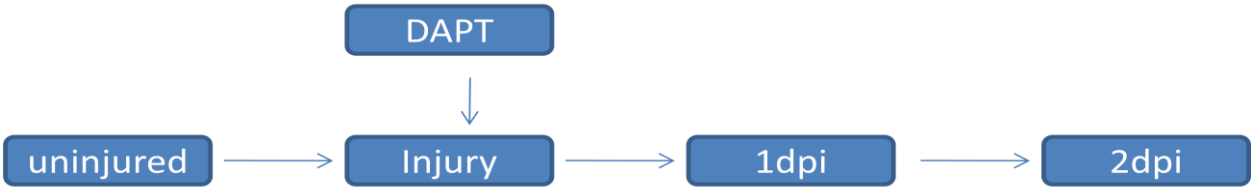
We check the expression pattern of Yy1a and Yy1b by inhibiting this signaling. We inhibit the Notch signaling using pharmacological drug inhibitor DAPT: γsecertase inhibitor, which binds to γsecertase and inhibits the cleavage of NICD complex so it cannot enter the nucleus and activates the Notch-regulated target genes.



FathiHalaweishh,Phcog.Net,2018

FIG 3.3 Notch signaling pathway

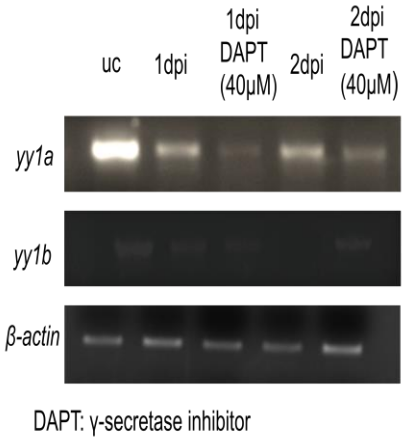
3.3.1 Inhibiting Notch signaling decreases the levels of Yy1a



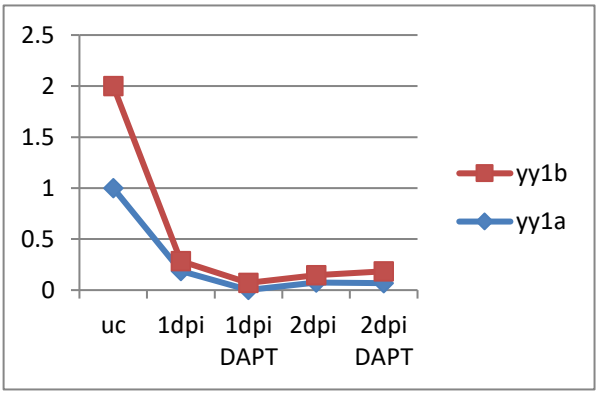
To check the regulation of Yy1 by inhibiting Notch signaling, we used pharmacological drug inhibitor DAPT: γsecertase inhibitor. At the time of injury we inject DAPT (40μM) in the vitreous of zebrafish eye, then harvest eye at 1dpi and 2dpi. The mRNA levels by inhibiting Notch signaling is checked by RT-PCR and Q-PCR and spatial expression checked by mRNA *in situ* hybridization. We observed that expression levels of *yy1a* downregulated by inhibiting Notch signaling. The protein levels checked by immunochemistry and western blotting which also shows the same results expression levels of Yy1 goes down.

3.3.2 Inhibition of notch signaling decreases the levels of Yy1b.

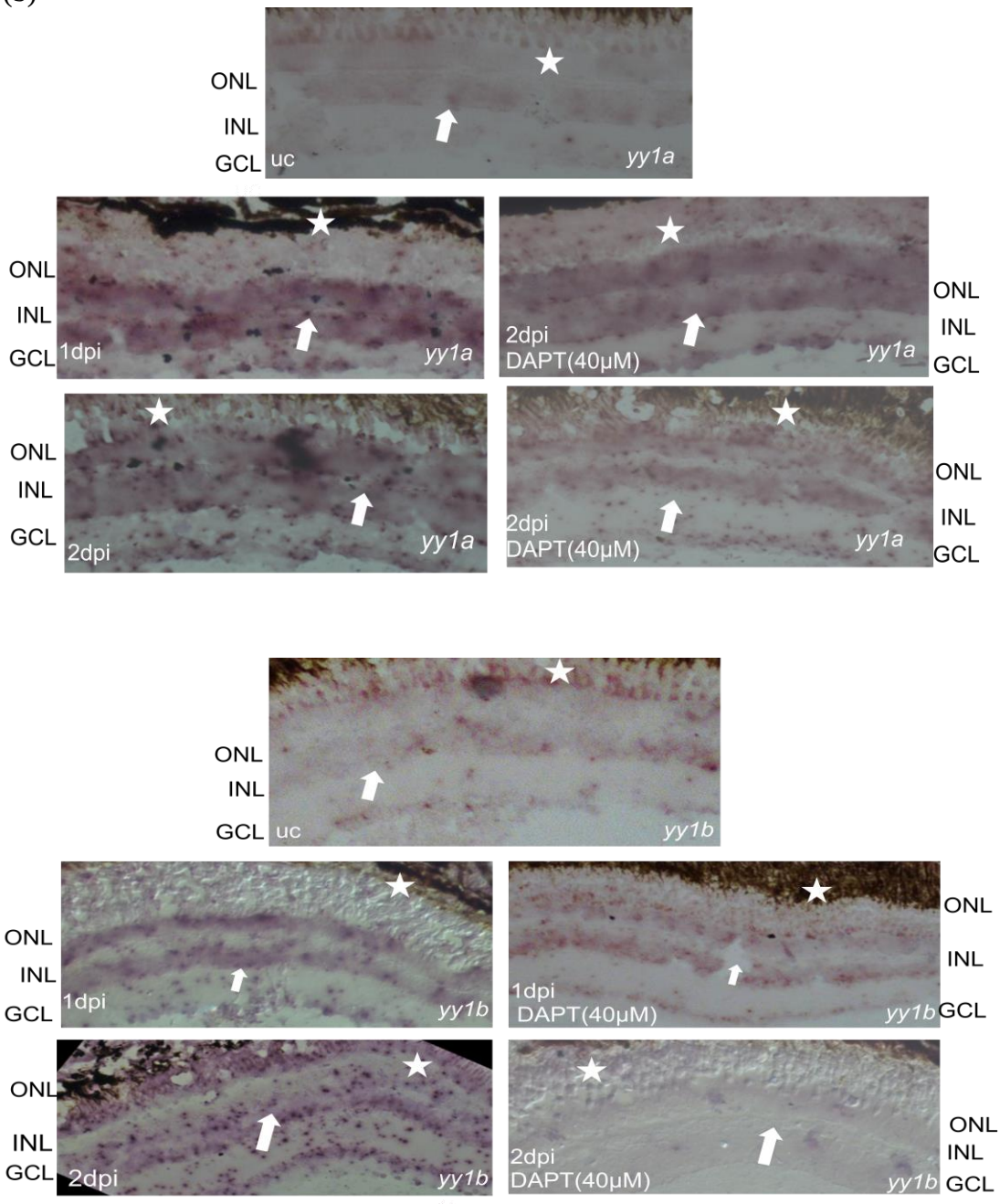
Yy1b temporal level also checked at 1dpi and 2dpi by doing RT-PCR and spatial expression by *in situ* hybridization. The protein levels are analyzed using western blotting and immunochemistry. This revealed that inhibition of Notch by DAPT decreases the Yy1b expression levels.

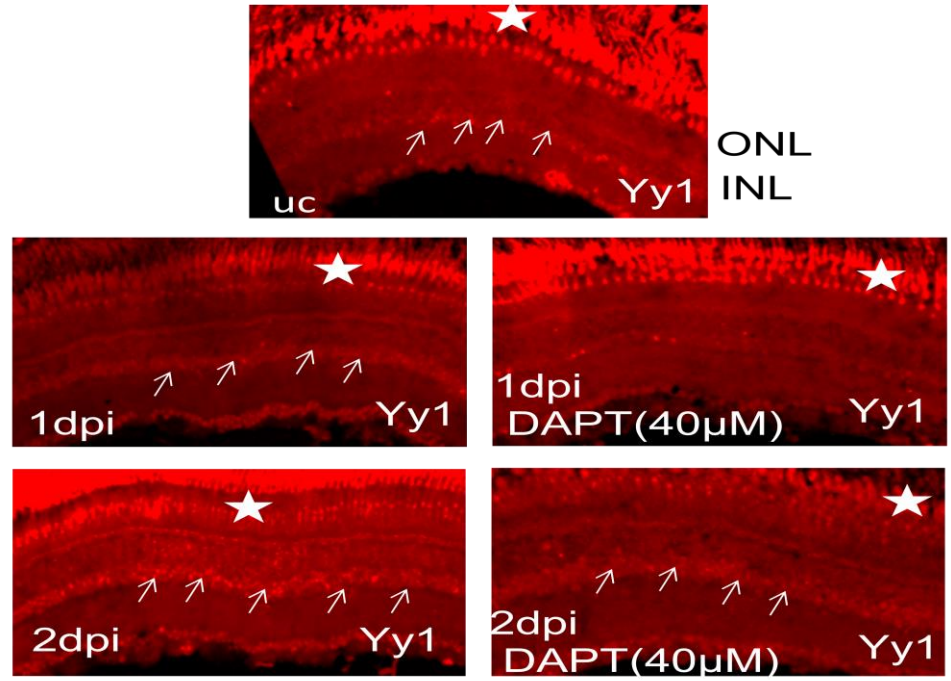


DAPT: γ-secretase inhibitor



(b)





(c)

(d)

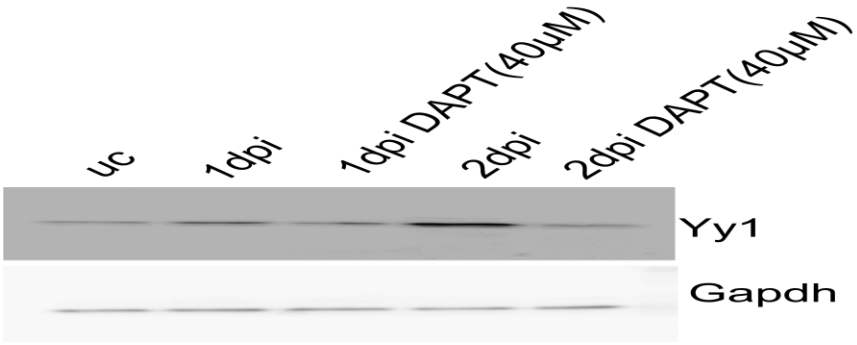


Fig 3.3.1: Inhibition of Notch signaling using DAPT, mRNA expression level of *yy1a* and *yy1b* (a)RT-PCR and QPCR (b)spatial expression level of *yy1a* and *yy1b in situ* hybridisation arrows shows the signals and star point shows the injury spot, (c) protein levels of Yy1 immunochemistry and (d) western blotting. Showing that proetien levels increases in Notch signaling blockade condition using DAPT (40μM).

3.4 Inhibition of Sonic hedgehog signaling

Sonic hedgehog (Shh) during regeneration of retina in zebrafish regulates the percentage of Müller glial cells that re-enter the cell cycle after the injury. Activation of Shh resulted in an increase in a number of differentiated amacrine and ganglion cells in the fully regenerated retina. We check the expression pattern of Yy1a and Yy1b by inhibiting this signaling using cyclopamine which inhibited SMO**.**

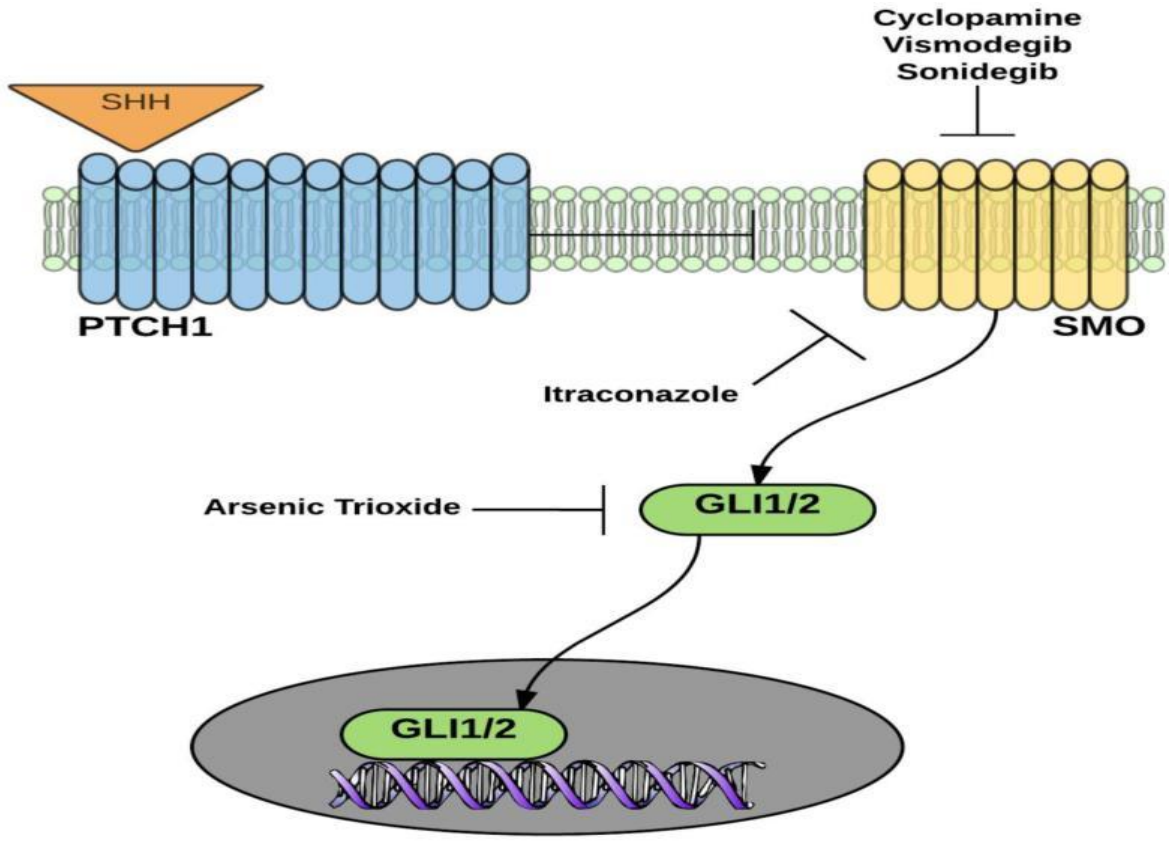
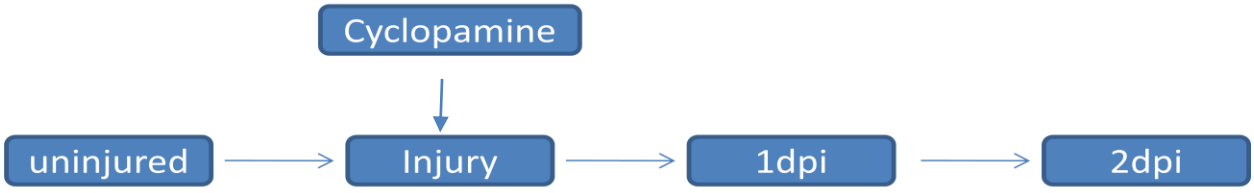


Fig 3.4 Sonic hedgehog signaling pathway

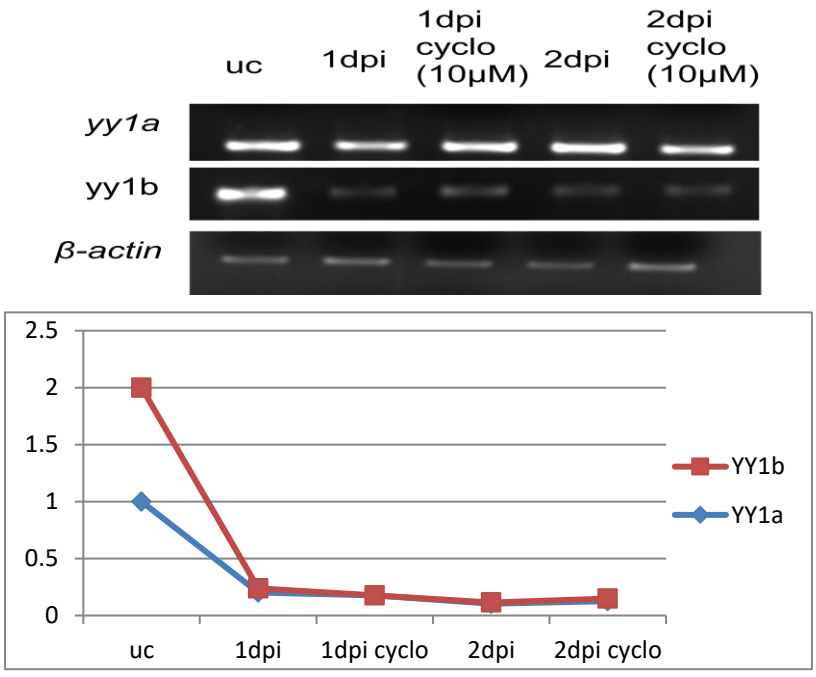


3.4.1 Inhibition of Sonic hedgehog signaling increases the levels of Yy1a.

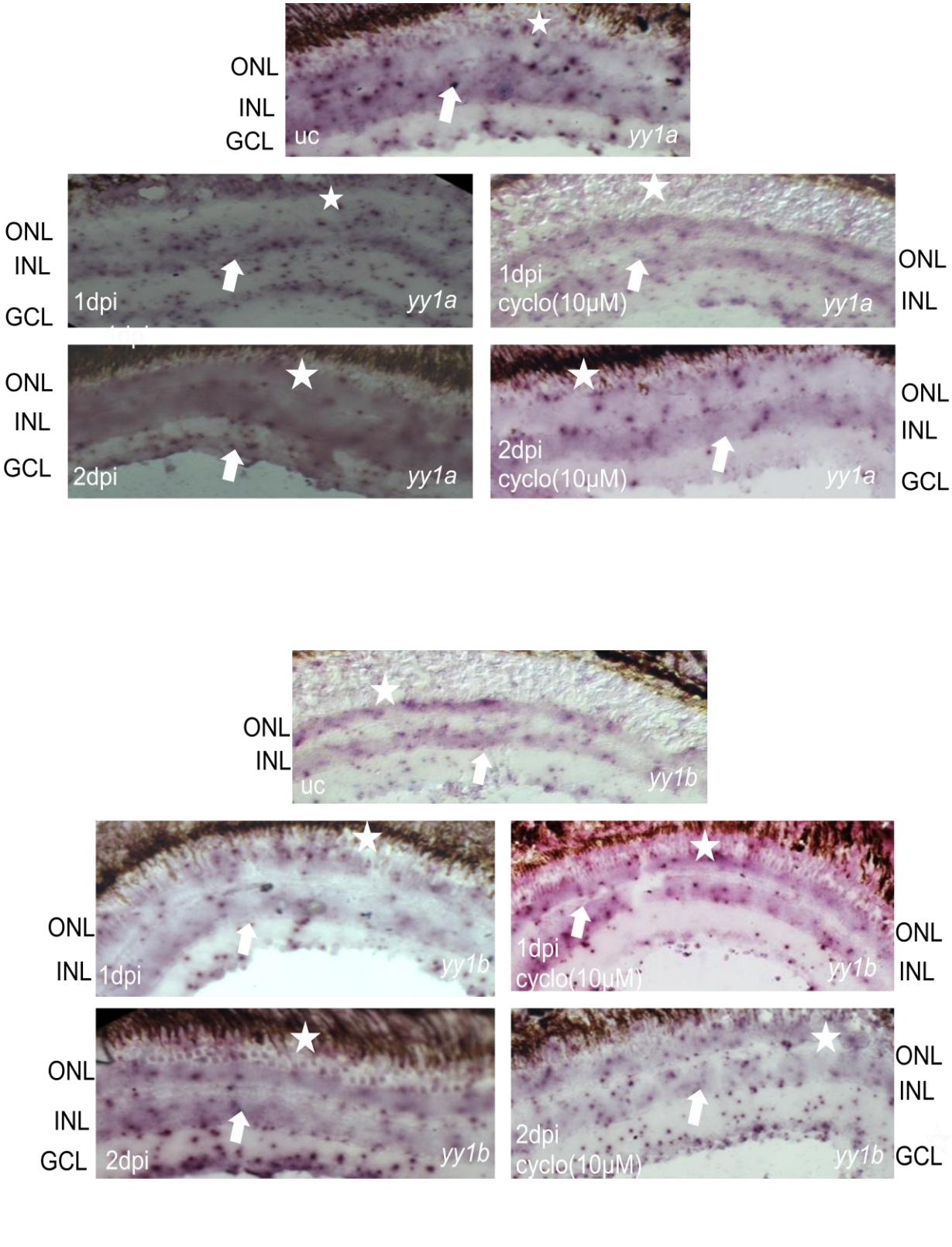
To study this, the effect of the inhibition of the Sonic hedgehog signaling on the expressions levels of *yy*1a and *yy1b* and on the protein levels of Yy1, using the pharmacological inhibitor Cyclopamine (10µM), was checked. For this experiment, we injected the drug in fish eye at the time of injury. The mRNA levels of yy1a and yy1b were checked using RT-PCR and Q-PCR and spatial expressions checked by in situ hybridization. The protein levels were analyzed using western blotting and immunochemistry. This revealed that the expression level of Yy1a increases at 1dpi and 2dpi drug-treated retina as compared to control the genes which play roles in de-differentiation phase is upregulated**.**

3.4.2 Inhibition of Sonic hedgehog signaling increases the level of Yy1b.

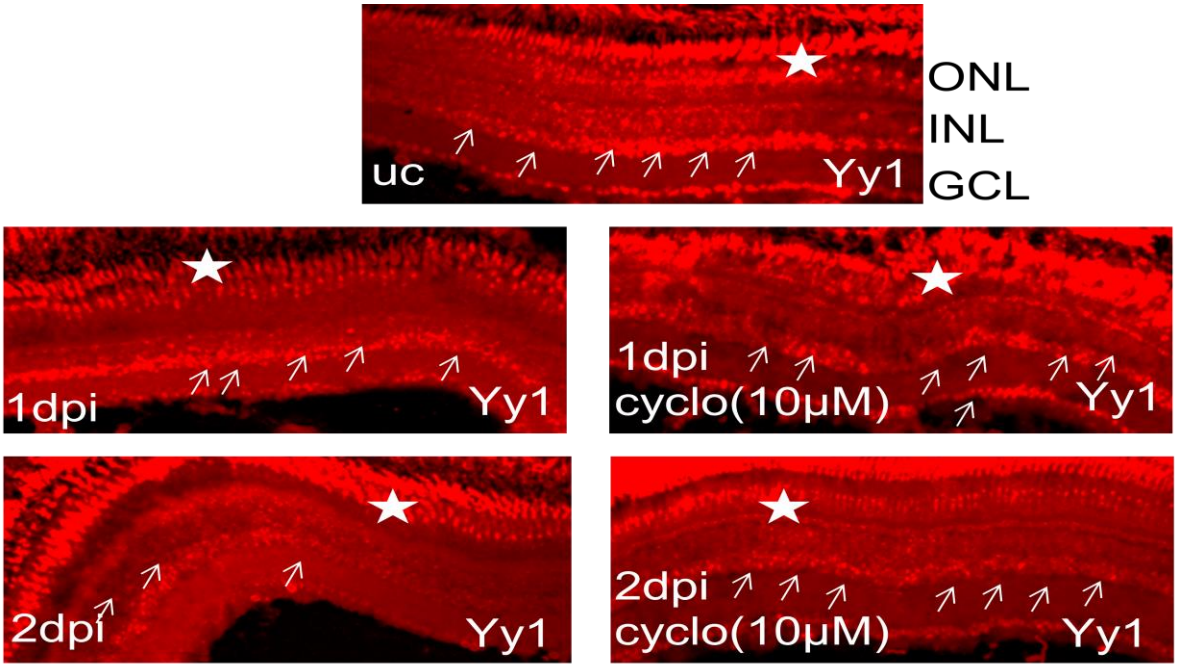
Yy1b temporal level also checked at 1dpi and 2dpi by doing RT-PCR and spatial expression by *in situ* hybridization. The protein levels are analyzed using western blotting and immunochemistry. This revealed that inhibition of Sonic hedgehog using Cyclopamine increases the expression levels of Yy1b.



(b)


ONL
INL
GCL
uc
yy1a
1dpi
yy1a
1dpi
cyclo(10μM)
yy1a
2dpi
yy1a
2dpi
cyclo(10μM)
yy1a
yy1b
1dpi
cyclo(10μM)
2dpi
cyclo(10μM)

(c)



(d)

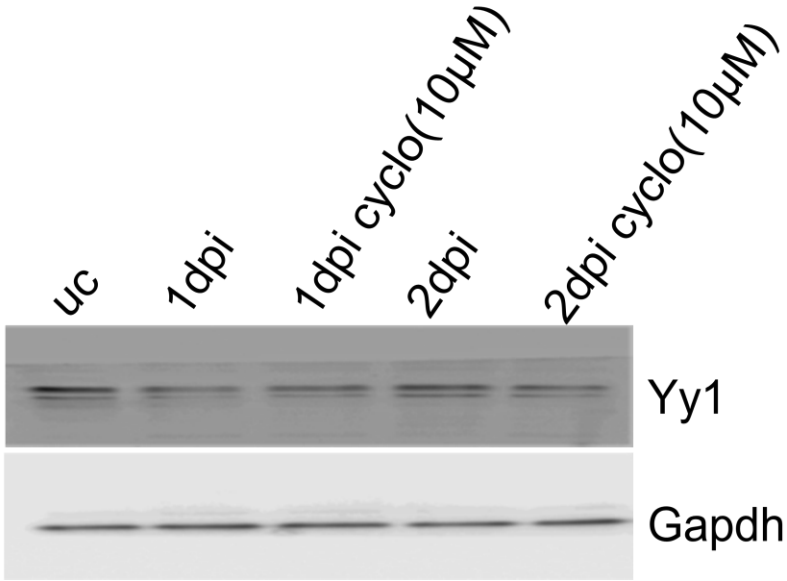


FIG 3.4.1: Inhibition of Sonic hedgehog signaling using cyclopamine (a) mRNA expression level of *yy1*a and *yy1b* RT- PCR and Q- PCR,(b)mRNA spatial pattern of *yy1a* and *yy1b* respectively using *in situ* hyberdization technique,(c) protein levels of Yy1 in immunostainting and western bloting.

CHAPTER 4: CONCLUSIONS

From this study, on understanding At early time points expression levels of *yy1a* and *yy1b* decreases which indicate that it plays a crucial role in the dedifferentiation phase.

- Inhibition of Jak/Stat3 increases the Yy1 expression. We could find that *yy1a* and *yy1b levels* increase significantly in Jak/Stat3 inhibited condition using cucurbitacin the beginning of dedifferentiation phase 1dpi, 2dpi which sheds light on its role during this phase.

- Inhibition of Notch signaling down regulates the Yy1 expression, we could find that *yy1a* and *yy1b* levels decrease significantly at the beginning of dedifferentiation phase 1dpi, 2dpi in Notch signaling inhibited using DAPT which sheds light on its role during this phase.

- Inhibition of Sonic hedgehog increases the level of Yy1. We could find that *yy1a* and *yy1b* levels increase significantly in Shh inhibited using cyclopamine condition the beginning of dedifferentiation phase 1dpi, 2dpi which sheds light on its role during this phase.

FUTURE PROSPECTS

- Elucidation of direct and indirect regulation of Yy1 by Jak-Stat signaling, Notch signaling and Sonic hedgehog signaling.
- Confirmation of these results by doing morpholino based gene knockdown approach.
- To check the regulation by double inhibition of these signaling.

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PRIMERS USED

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RT_*bactin*_rev	CATTGCCGTCACCTTCACCGTTC
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Yy1a_rev _XhoI	ATGCTAGCCTCGAGTCACTGATTGTTCTTAGCTTTCGCGT
Yy1b_fwd_BamHI	ATGCTAGCGGATCCACCATGGCGTCCGGCGAGACTGTAC
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