Molecular basis behind the cross-talk between Polycomb Repressor Complex 2 (PRC2) and Histone deacetylases (Hdacs) during Zebrafish Retina Regeneration

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CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled "**Molecular basis behind the cross-talk between Polycomb Repressor Complex 2 (PRC2) and Histone deacetylases (Hdacs) during Zebrafish Retina Regeneration**" submitted by **Ms. K. Kemdina Christina** (Reg. No. MS14043) for the partial fulfillment of BS-MS dual degree programme 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 is accepted.

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Dated : April 26, 2019

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 acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

K. Kemdina Christina
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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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ABBREVIATIONS

1. MG-Müller Glia
2. MGPC-Müller Glia Derived Progenitor cell
3. R-Rods
4. C-Cones
5. A-Amacrine cells
6. BP-Bipolar cells
7. H-Horizontal cells
8. GC-Ganglion cells
9. ONL-Outer nuclear layer
10. INL-inner nuclear layer
11. GCL-Ganglion cell layer
12. Ezh2- Enhancer of zeste homologue zebrafish protein
13. *ezh2-* Enhancer of zeste homologue zebrafish mRNA
14. Hdac- Histone deacetylase protein
15. *Hdac-* Histone deacetylase mRNA
16. TSA-Trichostatin A
17. VPA-Valproic acid
18. BrdU-Bromodeoxyuridine
19. PCNA-Proliferating Cell Nuclear Layer
20. MO-Morpholino-modified antisense oligonucleotides
21. dpi-days post injury
22. Ascl1a - Achaete-scute homolog 1 zebrafish protein
23. *ascl1a - Achaete*-scute homolog 1 zebrafish mRNA

24. Mmp9-Matrix metallo protease 9 zebrafish protein
25. *mmp9*- Matrix metallo protease 9 zebrafish mRNA
26. Co-IP- Co-immunoprecipitation
27. RT-PCR-Reverse Transcription PCR
28. qPCR-Quantitative PCR
29. SUZ12 - Suppressor of zeste 12
30. EED-Embryonic ectoderm development
31. SAM- S-adenosyl methionine

ABSTRACT

Diseased or damaged human retina fails to restore its lost vision despite considerable efforts and significant therapeutic advances. On the other hand, Zebrafish, a teleost fish, is an excellent model to study the molecular mechanisms of regeneration in tissues like retina because it demonstrates a robust regenerative response following injury. Müller glia responds to retinal injury and disease by changing their morphology, biochemistry, and physiology[3]. Further, they undergo a reprogramming event that enables regeneration. This requires changes in gene and protein expression. So, to identify the molecular players that confer regenerative capacities to non-regenerative species is the key relevance. Studies in various types of cancers have revealed that there is a collaboration between epigenetic modifiers like PRC2 and Hdacs. Here, in my work, we have explored the functional links of epigenetic modifiers like Ezh2 and Hdacs during retina regeneration. We have found that both Ezh2 and Hdacs affect the expression levels of each other during retina regeneration in zebrafish. Some regeneration associated genes like *mmp9, notch1a, insm1a, ascl1a, her4.1* were also found to be regulated by the combined blockade of Ezh2 and Hdacs. Earlier reports have also shown that EZH2 and HDACs-mediated epigenetic modifications contribute to constitutive activation of Wnt/b-catenin signaling which is significantly associated with abnormal cell growth. So, here we also investigated the effect of combinational blockades of Ezh2 and Hdacs on MGPCs proliferation mediated through Wnt/ β-catenin signaling pathway during zebrafish retina regeneration.

CHAPTER 1: INTRODUCTION

Visual impairment is a common ailment afflicting the human population. Unfortunately, diseased or damaged human retina fails to restore its lost vision despite the advances. However, Zebrafish (*Danio rerio*), a teleost fish have the potential and ability to regenerate and restore visual function after injury[4]. This capability is contrasted in mammalian species by the formation of glial scars. Zebrafish shows a robust tendency for the regeneration of the Central Nervous System (CNS). The retina, a layer of tissue which lies at the back of the eye is a simple and accessible part of the CNS. Hence, the retina is used for studying CNS regeneration. In Zebrafish, retina regeneration depends mostly on the dedifferentiation of Müller Glia, the dominant glial cell type in the retina. Upon retinal injury, Müller glia responds to it by re-entering the cell cycle and hence, generates multipotent progenitors that regenerate damaged cells. First, it produces progenitor cells with stem cell-like properties; this further proliferates and migrate to various cell layers and differentiate into different neuronal cell types and MG itself. This acquisition of stem cell-like properties (MG-derived progenitor cells) by Müller glia is accomplished with the help of various contributing factors and changes in gene expression. One such consideration is the epigenetic modifiers like Ezh2 (core catalytic component of Polycomb Repressive Complex), Hdacs (Histone deacetylases), Dnmts (DNA methyltransferases). Understanding the molecular basis underlying retinal regeneration in zebrafish could be used to design strategies to push restoration of vision in the mammalian retina.

1.1 RETINAL ARCHITECTURE, INJURY, AND REGENERATION

1.1.1 Zebrafish Retinal Architecture

The vertebrate retina consists of three distinct nuclear layers, the outer nuclear layer (ONL), the inner nuclear layer (INL) and the ganglion cell layer (GCL). The ONL comprises of rods (R) and cones (C) photoreceptor cells. The ONL senses light and transmits this information to ganglion cells (GC) in GCL via INL. INL consists of interneurons (bipolar cells, amacrine cells, horizontal cells). One glial cell type and six neuronal cell types occur throughout these layers. Müller Glia are the only glial cell type to span all the retinal cell layers. This construction allows them to interact with neighboring neurons; hence, it helps in the transfer of molecules across the various retinal

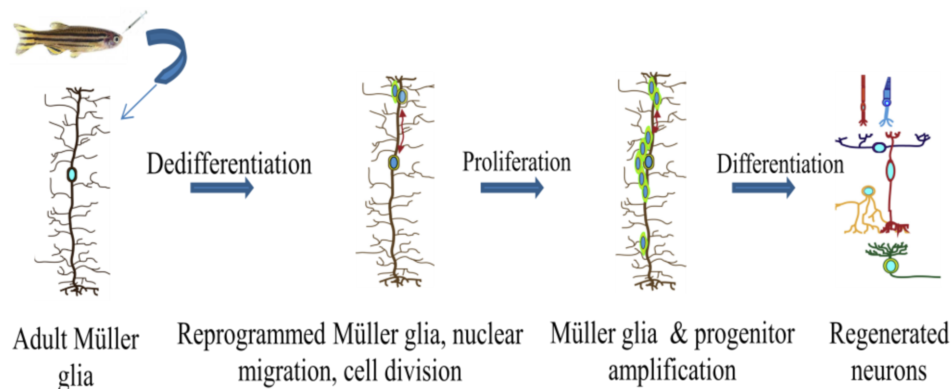
layer. Also, it monitors retinal homeostasis and contributes to retinal structure and function[1]. MG play an active role in regeneration.

1.1.2 Regeneration mechanism

Müller Glia responds to retinal injury; injury can be mechanical (using needle poke)[5], toxic chemicals or toxic genes[6], intense or UV light[7] and laser ablation[8]. Upon sensing damage to the retina, MGs undergo a gliotic response (overexpression of tubulin proteins like glial fibrillary acidic protein (Gfap) and undergo hypertrophy). The gliotic response is accompanied by a reprogramming event allowing MGs to have stem cell-like properties (Figure 1.1.2).

Müller Glia-driven retinal regeneration involves three main steps:

i. Dedifferentiation: upon injury, Müller Glia cells attain stem cell-like properties
ii. Proliferation: Interkinetic nuclear migration to the ONL and asymmetric cell division
iii. Re-differentiation: Migration of progenitors to various retinal layers and differentiate into different neuronal cell types



Reference: Wan J, Goldman D. (2016) Curr Opin Genet Dev. 40:41-47

Figure 1.1.1: Injury response by Müller glia. Upon injury, adult muller glia undergoes reprogramming events to acquire stem cell-like properties. This further proliferates and migrate to different cell layers and form different retinal cell types.

This process involves a large scale of changes in protein and gene expression. Injuries activate multiple signaling cascade. Signaling pathways like glycogen synthase kinase 3β (Gsk3β)–β-catenin, Notch, Mapk–Erk and Jak-Stat signaling are shown to be crucial for

genomic reprogramming during retina regeneration in zebrafish. Among them, β-catenin activation occurs in MGPCs. Wnt expression and β-catenin stabilize in MGPCs upon retinal injury. β-catenin, a multiprotein, links changes in Wnt signaling on the gene expression[1].

Retina regeneration also gets regulated by the suppression of pathways that drive Müller glial cell differentiation and quiescence. For example, *let-7* (miRNA) signaling and Dkk signaling are inhibitory pathways which help MG in a quiescent[13,14]. Notch signaling is also known to have an inhibitory effect during retina regeneration in zebrafish. But unlike other inhibitory pathways (i.e., suppressed after injury), Notch signaling induces its target gene such as the *her4.1* following injury. The *her4.1* inhibits hb-egf which then leads to MG dedifferentiation and proliferation as it activates genes like *ascl1a*[10].

Genes like *ascl1a* (achaete-scute homolog 1), *insm1a* (insulinoma-associated 1a) are the leading players that promote proliferation and suppresses differentiation. Ascl1a stimulates *lin-28* expression and *lin-28* further suppresses *let-7* miRNA (associated with differentiation)[9]. Acsl1a regulates insm1a (a transcriptional repressor) expression, and it also monitors the Wnt signaling pathway by inhibiting dkk (dickkopf, Wnt inhibitor) expression and hence activate Wnt expression, which results in Muller glia reprogramming and progenitor formation[9,16].

1.2 EPIGENETIC MODIFICATIONS

Although it is not very clear until now. Epigenetic modifications are also known to share some feature in regulating chromatin alteration and gene expression during retina regeneration in zebrafish. The study of changes in expression of a gene without a change in a DNA sequence is called epigenetics. Epigenetic modifications can be DNA methylation, RNA-associated gene silencing, and histone modification.

DNA methylation is the process that adds a methyl group to the cytosine nucleotide in the DNA sequence called CpG islands (C next to G). The enzymes called DNA methyltransferases catalyzed the said process. They add methyl groups to these sites causing changes in their structure, thereby, restricting access of transcription factors to the gene promoter and hence silences the gene.

RNA-associated gene silencing can be caused by siRNA, miRNA and other noncoding RNAs. Such RNAs bind to the complementary mRNA transcript and destroy them or prevent translation.

1.2.1 Histone modifications

Histones are alkaline proteins (positively charged) that associate with DNA (negatively charged) to create nucleosomes. So, each nucleosome is made up of DNA wrapped around a 'histone octamer.' Each histone octamer consists of pairs of the basic, positively charged histone proteins namely H2A, H2B, H3, and H4. This positive charge helps to bind the negatively charged DNA thereby condensing it to form a compact chromatin.

Histone modifications is a post-translational modification to histone protein. The modification includes methylation, acetylation, sumoylation, ubiquitylation, and phosphorylation; these post-translational modifications are made on the histone proteins by epigenetic modifiers to alter the expression of genes. Such covalent modifications cause an alteration in the chromatin structure hence further regulate gene expressions. Modifications in histone are known to play a role in diverse biological processes such as transcriptional activation or transcriptional inactivation, DNA damage or repair and chromosome packaging. Therefore investigating the epigenetic regulation of cellular processes would give an insight into the development of histone modifying targeted drugs. My work focuses mainly on exploring the functional links of epigenetic modifiers like Histone deacetylases (Hdacs), and a histone methyltransferase, Ezh2 -core catalytic component of Polycomb Repressive Complex 2 (PRC2)- during zebrafish retina regeneration.

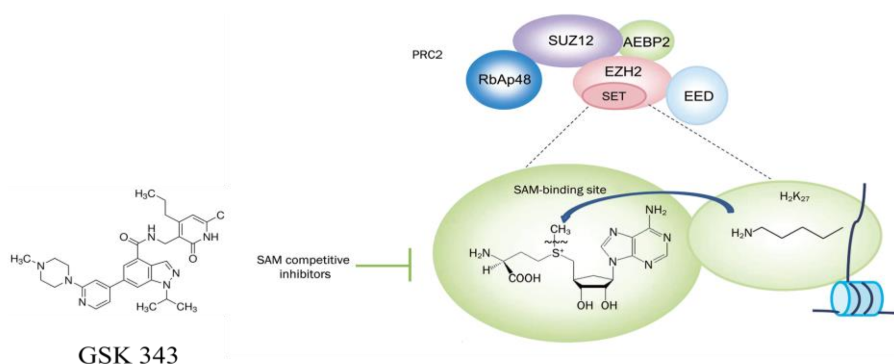
1.2.2 Histone methyltransferases

In Histone methylation, a methyl group is transferred from S-adenosyl methionine (SAM) substrate to lysine/arginine residue of histone tails by histone methyltransferases (HMT). Different HMTs modify different lysine and arginine residues. For example, in mammalian cells, on Histone 3 (H3) at lysine 4 (H3-K4), the histone methyltransferases that catalyze methylation are SET1, SET7/9, Ash1, ALL-1, MLL, ALR, Trx, and SMYD3. ESET, G9a, SUV39-h1, SUV39-h2, SETDB1, Dim-5, and Eu-HMTase are histone methyltransferases that catalyze methylation of histone H3 at lysine 9 (H3-K9) in mammalian cells. Histone methyltransferases such as G9a and polycomb group enzymes like Ezh2 catalyzed the methylation of histone H3 at lysine 27 (H3-K27) in mammalian

cells[16]. H3-K9 as well as H3-K27 methylation mediates heterochromatin formation and also participates in silencing gene expression at euchromatic sites. Both H3-K9 and H3-K27 methylation participates in the formation of compact chromatin and in silencing gene expression. The global increase in H3-K27 is shown to be involved in cancer progression.

Polycomb Repressive Complex 2 (PRC2) is a chromatin modifier which is conserved from Drosophila to mammals. It represses the target genes by trimethylating histone H3 at lysine 27 (H3K27me3)[17,18]. PRC2 complex includes four components: Ezh1/2 (Enhancer of zeste homologue1/2), SUZ12 (Suppressor of zeste 12), EED (Embryonic ectoderm development) and RbAp46/48 (also known as RBBP7/4) and AEBP2. Ezh2 is the core catalytic component of the PRC2 complex; its C terminal SET domain catalyzes the methyltransferase activity[19]. Ezh2 contains the catalytic activity of its own but acquires the activity upon interacting with the non-catalytic subunits EED (contains WD repeats) and SUZ12 (contains zinc finger). AEBP2 stabilizes the PRC2 complex while RbAp46/48 isn't known to have any direct effect on the enzymatic activity of PRC2.

Pharmacological inhibitors like GSK343 and UNC1999 are used to study the enzymatic function of Ezh2. GSK343 is a SAM (S-adenosyl-l-methionine) competitive inhibitor. SAM is a universal methyl donor[20]. GSK343 goes and bind to the SAM-binding site in PRC2 complex, and it further prevents the removal of a methyl group from SAM and hence no methyl group is being transferred to H3K27 (Figure 1.2.2).



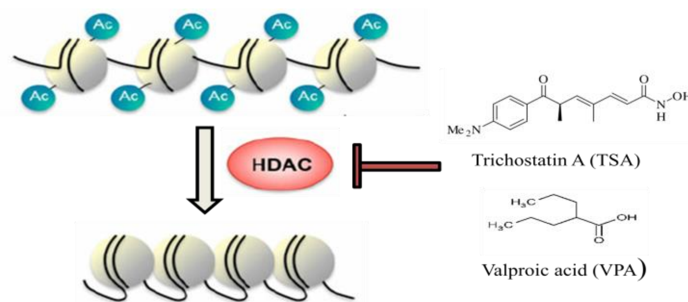
Reference: Tan JZ, Yan Y, Wang XX, Jiang Y, Xu He. (2013) Acta Pharmacol Sin. 35:161-74

Figure 1.2.2: PRC2 composition and Ezh2 inhibitor. Subunits of the PRC2 complex and the mode of action GSK343 on SAM complex.

1.2.3 Histone deacetylases (HDACs)

Histone deacetylases (Hdacs) removes acetyl from acetylated histone lysine residue. And hence, it leads to alteration of chromatin structure and represses gene transcription. Based on the sequence similarity, a classical mammalian HDAC family comprises of four classes namely Class I, Class II, Class III or sirtuins and Class IV. Class I HDACs includes HDAC1, 2, 3 and 8 and are found exclusively in the nucleus.They are involved in cell proliferation and survival. Class II HDACs (HDAC4, 5, 6, 7, 9, 10) can shuttle between nucleus and cytoplasm in response to certain signals. Class IV contains only HDAC11 which resides predominantly in the nucleus[21,22]. HDAC2 is formed from a recent HDAC1 duplication; hence zebrafish lacks HDAC2[22]. All HDACs require Zn2+ for their deacetylase activity, except for Class III HDACs (requires NAD+ as a cofactor).

Trichostatin A (TSA) and Valproic acid (VPA) is used for blocking HDACs activity. TSA inhibits the catalytic activity of Class I and II HDACs but not sirtuins. The molecule inserts itself in the catalytic pocket and chelates the cofactor Zn2+ needed for deacetylation[23,24] (Figure 1.2.3a). While valproic acid inhibits Class I HDACs (HDACs1,2,3,8) and Class IIa HDACs (HDACs 4, 5, 7 and 9), valproic acid inhibits HDAC activity by binding to the catalytic center of HDACs[25] .



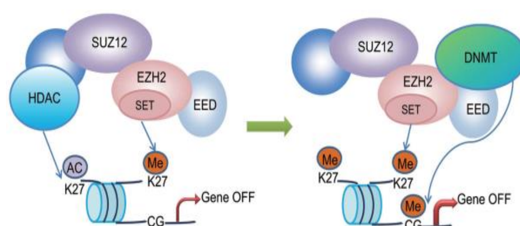
Reference: Schneider A. *et.al.*, (2013) Neurotherapeutics. 10(4):568-88.

Figure 1.2.3: Hdacs and their inhibitors. This image shows that when the acetyl group is removed from the histone proteins by Hdacs, the target gene condenses.The activity of HDACs is inhibited by Trichostatin A (TSA) and Valproic acid (VPA).

1.2.3 Links between HDACs and PRC2

Various reports have shown that the PRC2 complex silences the gene expression in aid of other epigenetic modifiers like Dnmts (DNA methyltransferases) and Hdacs (Histone deacetylases). It has been shown that Ezh2, PRC2 core complex monitors CpG methylation through physical contact with Dnmts. Ezh2 serves as a recruitment platform for Dnmts[26]. There are also reports in human cancer which shows that Hdacs interacts with Ezh2 and some other PRC2 protein, EED[27].

The possible interaction for the epigenetic modifier can be such that Ezh2 trimethylates lysine 27 of histone 3 (H3K27me3) and then silences the target genes. And if the lysine 27 of histone 3 is Pre-acetylated, then Hdacs will come into the picture. Hdacs will remove the acetyl group from pre-acetylated lysine 27 of Histone 3, and hence by removing the acetyl group, it provides a room for Ezh2 to bring in more of the methyl group to further silence the target gene. PRC2 can also recruit Dnmts for more permanent silencing of the target gene (Figure 1.2.4).



Reference: Tan JZ, Yan Y, Wang XX, Jiang Y, Xu He. (2013) Acta Pharmacol Sin. 35:161-74

Figure 1.2.4: Functional links between PRC2, HDAC, and DNMT. Hdacs removes the acetyl group from pre-acetylated histone protein thus creating a room for Ezh2 to facilitate methylation. This further recruits DNMT for permanent silencing of the target genes.

In non-small-cell lung cancer cells, cell proliferation goes down when Ezh2 is inhibited. It has also shown that PRC2 (overexpressed in cancer cells), requires the activity of Hdacs for transcriptional silencing. The combined blockade of Ezh2 and Hdacs synergistically suppressed cell proliferation.

From all this knowledge we wanted to explore if this follows the same during retina regeneration in zebrafish. So my work deals with the crosstalk between Ezh2 and Hdacs. And we have found out that there is a functional link between Ezh2 and Hdacs during retina regeneration in zebrafish. The epigenetic modifiers, Ezh2, and Hdacs both affect each other expression level. And also in the combined blockade of Ezh2 and Hdacs, we have shown that various regeneration associated genes are being regulated.

1.2.5 Ezh2, Hdacs in Wnt/ β-catenin signaling pathway

β-catenin is a multifunctional protein that, in collaboration with other family protein it links changes in Wnt and cadherin signaling on the cell surface to gene expression[28]. Reports have shown that EZH2 and HDACs-mediated epigenetic modifications contribute to constitutive activation of Wnt/β -catenin signaling which is significantly associated with abnormal cell growth. So, here we also investigated the effect of combinational blockades of Ezh2 and Hdacs on MGPCs proliferation mediated through Wnt/ β-catenin signaling pathway. We have shown that the expression level of β-catenin is regulated in the combined blockade of Ezh2 and Hdacs.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal maintenance

- Zebrafish are maintained in an automated water circulation system.
- 14 hrs of light and 10 hrs of dark
- Temperature: 27°C
- pH: physiological pH

2.2. Retina dissection for RNA isolation/ western blotting

1. Anesthetized zebrafish using Tricaine methanesulfonate.
2. Retina were injured using 30 gauge needle.
3. At desired time after injury, eyes were dissected using steel forceps and needle. Dissections were carried out in 1X PBS (Phosphate Buffered Saline) for harvesting retina. Lens were removed for isolating RNA and retina was dissected out. If the retinae are to be used for RNA isolation, they are stored in Trizol, and if it is to be used for western blotting, they are suspended in Laemmli buffer in -80°C.

2.3. Microscopy

1. Bright field microscope (Zeiss) was used for dissecting retina.
2. Confocal microscope (Nikon) was used for imaging.

2.4 Tissue fixation and sectioning

1. The eyes whose lenses were removed were taken and put it in 4% PFA at 4°C overnight for tissue fixation.
2. Next day, serial washes of the fixed tissue was given at RT for 45mins each on a rotor:

   1mL of 5% sucrose

   800μL of 5% sucrose and 400μL of 20% sucrose

500µL of 5% sucrose and 500µL of 20% sucrose

400µL of 5% sucrose and 800µL of 20% sucrose

1mL of 20% sucrose.

3. 500µl of OCT is added and rotated it for 30 min.
4. Embedded the tissue in OCT in small cubes made from aluminum foil, and then it is frozen at -80ºC until sectioning.
5. The blocks are sectioned in cryostats (12µm thickness) and then the sections are collected on super frost plus slides and it is dried overnight and then stored in minus 20ºC.

   The composition of solutions used are:

a) 4% PFA in 1X Phosphate buffer (made DEPC water) :
   - 2g PFA
   - 5mL of 10X phosphate buffer
   - Make up the volume to 50mL with DEPC water.
   - Dissolve it by keeping in 65ºC and constant shaking
b) 5% sucrose:
   - Dissolve 2.5g sucrose in 50mL of autoclaved water. Store at -20ºC.
c) 20% sucrose:
   - Dissolve 10g sucrose in 50mL of autoclaved water. Store at -20ºC.

2.5 RNA isolation

1. Dissected retinae were collected in 200µL of Trizol in MCT.
2. They were homogenized properly using a pipette.
3. 0.2 volume (40µL) of chloroform was added.
4. The solution was shakened gently for 15-20 seconds.
5. The MCT was kept still for 5 minutes at room temperature (RT).
6. Centrifuged for 10 minutes at 8,000rpm at 4°C.
7. Using a cut tip, 20µL of the aqueous layer was transferred into fresh MCT.
8. Equal amount of Isopropanol was added.
9. Stored at -80°C overnight (or in ice for 20 minutes).
10. Centrifuged at 13,000 rpm, 4°C for 10 minutes.
11. The supernatant was discarded, and 80% ethanol wash was given.
12. Pellet was dried completely at RT and then, eluted in DEPC treated water.

13. Eluted RNA was checked on 1% agarose gel by gel electrophoresis and then,
14. stored it in -80°C.

2.6 cDNA synthesis

(Kit used – RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher)

1. Following reagents were added into a sterile tube:
    a. Template RNA 2.5 µL
    b. Primer (Oligo (dT)18 +Random Hexamer) 0.25+0.25 µL

2. The contents were mixed gently and were incubated at 65°C for 5 minutes. Then, the tubes were transferred back on the ice.

3. Following components were added in the indicated order:

5X Reaction Buffer	1µL
RiboLock Rnase Inhibitor	0.25µL
10mM dNTP Mix	0.5µL
Revert-Aid M-Mul VRT	0.5µL
Total volume	5µL

4. The contents were mixed, centrifuged briefly and were incubated at following temperatures:

    - 5 minutes at 25°C
    - 60 minutes at 42°C
    - 5 minutes at 70°C

5. The cDNA was then diluted with autoclaved Milli-Q water and stored at -80°C.

2.7 Reverse transcription-Polymerase chain reaction (RT PCR) using Taq Polymerase

1. The reaction mixture (10 µL volume)

10X buffer	1 µL
2.5mM dNTPs	0.5µL
Primers (forward + reverse)	0.2µL
Taq polymerase	0.2µL
Template	(as per standardized volume)
Water	Rest

2. Reaction Parameters

Enzyme activation	95°C for 2 min
DNA denaturation	95°C for 15 sec
Primer annealing	62°C for 30 sec
Elongation	68°C for 30 sec
Final elongation	72°C for 5 min
Infinite hold	4°C

3. PCR products were then checked on 1.5% agarose gel by electrophoresis

2.8 Quantitative PCR (qRT-PCR)

qPCR is carried out using KOD SYBR qPCR Master Mix (pure gene)

The reaction mixture (5µl reaction):

Master mix	2.5µl
Primers(F+R)	0.25 µl
Template	0.25 µl
MQ water	2 µl

Analyze the data on an excel sheet and plot the graph.

2.9 Plasmid isolation

1. 5mL of culture was centrifuged at 13400rpm for 2 min at RT
2. The supernatant was discarded and the pellet was dissolved in 100µL of autoclaved MQ.
3. 100µL of freshly prepared lysis buffer was added and gently tapped. For 1mL of lysis buffer, add 50µL of 20% SDS solution, 20µL of 0.5M EDTA and 10µL of 10N NaOH in 910µL of water.
4. The samples were boiled at 100°C for 2 minutes (until the solution becomes clear).
5. 50µL of 0.5M MgCl2 was added. Tapped and kept on ice for 2 minutes.
   For 100mL, 60mL of potassium acetate, 11.5mL glacial acetic acid, and 28.5mL H_2O were mixed. Stored at 4°C.
6. Immediately tapped and centrifuged at 13400rpm for 2 min, RT.
7. The supernatant was transferred into another MCT containing 600µL of Isopropanol.
8. Kept on ice for 5 min.
9. Centrifuged at 13400rpm for 2min, RT.
10. 70% ethanol wash and the pellet was dried completely
11. Pellet was dissolved in 50µL of autoclaved MQ.
12. Stored at -20°C.

2.10 Plasmids and restriction digestion for probe making

1. Following components were added in a reaction mixture:

Plasmid	5 µL
MQ water	3.5µL
10X buffer	1µL

(Depending on the restriction enzymes used)

- Restriction enzymes - 0.5µl

Enzymes used:

her4.1 –XhoI (SP6 polymerase)

mmp9- NotI (SP6 polymerase)

ascl1a- XhoI (SP6 polymerase)

2. Incubate at 37°C for 1 hour
3. It is checked on an agarose gel to confirm their digested fragments size and to determine the orientation of the insert.
4. Large scale digestion is made with suitable restriction enzymes depending on the orientation, to linearise the plasmid using the above enzymes.
5. The following components are added in an MCTs:

Plasmid	35 µL
10X buffer	4µL
Enzymes	1µL

6. Incubated at 37ºC for 3 hours or overnight.
7. Loaded the digested products on an agarose gel and the linearized DNA fragments are excised from agarose gel.
8. The gel is extracted and purified.

2.11 RNA probe reaction

1. Following reaction was set up:

RNA Polymerase buffer	1 µL
Template DNA (linearised plasmid	500ng to 1µg (7µL)
Dig /Fl	0.5µL
RNA polymerase	0.5µL
Nuclease-free water	0 µL
Total	10µL

2. Incubated at 37°C for 4 hours.
3. Added following:

0.5M Tris-EDTA	1µL
5M LiCl	1µL
10mg/mL glycogen	0.5µl
Absolute ethanol	17.5µL

4. Tapped to mix well, kept at -80°C overnight for precipitation.
5. Centrifuged at 4°C for 30 min at13,000 rpm.
6. The supernatant is discarded and it is washed with 70% ethanol (200µL) and then centrifuged for 10 min at 13,000rpm, 4°C.
7. Dried and dissolved the pellet in 10µL of DEPC treated water.
8. Validated the probe on an agarose gel
9. Stored at -80°C.

2.12 Immunostaining

Day 1

1. Slides were taken out from -20°C and dried at 37°C for 1 hour.
2. 1X PBS washes for 10minutes, twice.
3. Fixed tissue with 4% PFA solution for 20minutes (This step is done only in the case of MO slides).
4. Treated the slides with 2N HCl (preheated to 37°C) for 20 min.

   (*Use Sodium Citrate for epitope retrieval while performing beta-catenin immunostaining).
5. Washed the slides with 0.1M Sodium borate solution twice for 10 minutes each.
6. Blocked the sections using 6% BSA in 1X PBST (1XPBS + 0.1% Triton X) for atleast 30minutes.
7. Overlaid the slides with primary antibody of choice (PCNA, BrdU, β-catenin or Hdac1), 500µL per slide (Antibody is diluted in 1:500 ratio in 1% BSA in 1XPBST).
8. Incubated the slides at 4°C overnight

   * To perform immuno-histochemistry using a beta-catenin antibody, heat

mediated antigen retrieval is done. For this, 10mM of Sodium Citrate (2mL in 200mL of MilliQ-water) is preheated for about 20 minutes till it starts boiling. And then, slides are kept in this boiling Sodium Citrate for 20 minutes.

DAY 2

1. Washed slides with 1X PBST, 3 times for 10 minutes each.
2. Overlaid the slides with desired secondary antibody, 500µL per slide (Antibody is diluted in 1:1000ratio in 1% BSA-1X PBST solution).
3. Incubated slides for 3 hours at RT.
4. Washed slides with 1X PBST 3 times for 10 min each.
5. Washed slides with autoclaved MilliQ-water 3 times for 10 min each.
6. Dried slides for 1 hour at RT.
7. Mounted slides in DABCO and left at RT in the dark overnight.
8. Stored slides at -20°C.

2.13 Western Blotting

Sample preparation

- Retinae were dissected and collected in 2X Laemlli Buffer and homogenized properly.(2X Laemlli Buffer – 4mL of 10% SDS + 2mL of Glycerol + 1.2mL of 1M Tris-HCl (pH 6.8) + 2.8mL of MilliQ-water + 0.02% of Bromophenol blue. Store at 4°).
- Brief vortexing and ice incubation was given for 10 times.
- Centrifuged at 5,000 rpm for 10 minutes.
- Transferred supernatant to a fresh MCT.
- Heated the sample at 100°C for 10 minutes.
- Stored in -80°C

Day 1

1. Resolving gel was casted.
   (12% Resolving gel –2.5mL Resolving Buffer + 4mL 30% Acrylamide 3.3mL MilliQ-water + 100µL 10% SDS + 100µL 10% Ammonium Persulfate + 6µL TEMED)
2. Stacking gel was casted.

(Stacking gel – 625µL Stacking Buffer + 667µL 30% Acrylamide + 3603µL MilliQwater + 50µL 10% SDS + 50µL 10% Ammonium Persulfate + 5µL TEMED)

3. Samples were taken out from -80°C and thawed.
4. Samples were loaded on the gel along with Protein Ladder.
5. The gel was run for 3 hours at 80V.
6. The transfer was set up using PVDF membrane for 70 minutes at 70V. (PVDF membrane was charged with methanol for 1 minute and then washed with MilliQ-water).
7. Blot was then blocked in 10% skim milk for 1 hour.
8. 0.01% PBST (For 200mL of 1X PBST, add 200µL of TWEEN20) washes were given for 15minutes, four times.
9. Incubated blot in the primary antibody of choice overnight at 4°C.

Day2

10. 0.01% PBST washes were given for 15 minutes each, four times.
11. Incubated blot in secondary antibody for 1 hour at RT.
12. 0.01% PBST washes were given for 15 minutes each, four times.
13. Blot was then developed in ImageQuant LAS4000.

2.14 mRNA *in situ* hybridization on cryosections

Day 1

1. The slides were taken out from -20°C and dried by keeping them at 37°C for 2 hours.
2. Hydrated the slides in the following sequence for 1 minute each :
   a. 100% EtOH
   b. 95% EtOH
   c. 70% EtOH
   d. 50% EtOH
   e. 2XSSC
3. Incubated slides in Proteinase K solution for 5 minutes at 37°C .
   (Proteinase K Buffer: 5mL of 1M Tris-HCl (pH = 8) + 5mL 0.5M EDTA + 40mL of DEPC H2O.)

a. Pre warmed Proteinase K buffer to 37°C.
b. Added 160µL of 10mg/mL proteinase K.

4. Rinsed slides briefly in room temp DEPC water (2-3minutes).
5. Rinsed slides in 0.1M TEA pH 8.0 for 3 minutes.
   (0.1M TEA: 0.93g of Triethanolamine (TEA) + 50mL of DEPC H2O + 173µL of 10N NaOH (for adjusting pH = 8)
6. Rinsed in Acetic anhydride/TEA for 10 minutes.
   Added 130µL of acetic anhydride to 50mL of 0.1M TEA
7. Dehydrated the slides in 2X SSC and EtOH series for 1 minute each:
   a. 2X SSC
   b. 50% EtOH
   c. 70% EtOH
   d. 95% EtOH
   e. 100% EtOH
8. Air dried the slides for at least 1 hour at RT.
9. Pre-warmed the hybridization solution at 56°C (300µL per slide).
   (Hybridization solution (50mL): 3.6 mL TEN solution+25mL 100% Formamide+10mL 50% Dextran Sulphate+5mL 10% RMB blocker+6.4mL of DEPC H_2O.)
10. Probe preparation:
    a. Added probe to hybridization solution and mixed (as per concentration of the probe).
    b. Boiled probe and hybridization solution mix at 100°C for 10 minutes.
    c. Kept immediately on ice for 2-3minutes.
11. Added 300µL of Hyb/probe solution to each slide and coverslip with siliconized hybrid slips.
12. Placed 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

13. Preheated 50% formamide/2X SSC solution to 65°C.
14. Preheated two 50mL RNase buffer washes, one to 37°C and the other to 65°C.
15. Preheated two 2X SSC washes to 37°C.
16. Soaked slides with coverslips in 2X SSC for 30 minutes at RT on a shaker table.
17. If the coverslips do not come off, gently teased them apart from the slide with forceps.
18. Rinsed slides in 50% Formamide/2X SSC solution for 30 minutes at 65°C.
19. Gently agitated for the first 5 minutes.
20. Rinsed slides in 2X SSC for 10 min at 37°C (twice).
21. Added 100μL of RNase (10mg/mL) to the 37°C RNase buffer, incubated slides for 30 minutes.
22. Washed slides in 65°C RNase buffer for 30 minutes.
23. Washed slides for 2-3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RM Blocker solution at RT
24. Washed slides in 1X Maleate buffer for 5 minutes (twice).
25. Incubated slides with 500μL antibody (Anti-DIG/Anti-FL) diluted in 1X Maleate/ 0.05% Triton X-100/1% RMB blocker solution (1:2500 dilution) overnight at RT. (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.)

Day 3

26. Washed slides twice with 1X Maleate buffer for 5 minutes.
27. Washed 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)
28. Added 500μL of NBT/BCIP dissolved in Genius buffer (1:50 dilution), incubated at room temperature in dark for a color reaction.
29. Color detection: In a bright field microscope.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Combined effects of Ezh2 and Hdacs could not rescue the effect of Hdacs blockade

It has been shown that there exists crosstalk between PRC2 and Hdacs during retina regeneration in zebrafish (done in the lab by senior MS student). Having this knowledge, we wanted to explore more to study the links between Ezh2 and Hdacs. So, first of all, we checked the effects of the combined blockade of Ezh2 and Hdacs during zebrafish retina regeneration. To study this, Ezh2 and Hdacs were blocked simultaneously by GSK and TSA respectively. And these effects were checked at 4dpi -In zebrafish, the proliferation of these MGPCs occurs from 2dpi to 6dpi, with peak proliferation at 4dpi. After 6dpi, differentiation of MGPCs to different retinal cell types take place-. Proliferating cell number in 4dpi retinae was obtained by counting PCNA (Proliferating Cell Nuclear Antigen) positive cells. And from the PCNA positive cell counts, we could see that the cell proliferation in the combined blockade showed a reduction in cell proliferation as compared to the 4dpi control ones. The cell proliferation in the combined blockade was lesser than Ezh2 blockade while slightly higher than the Hdac blockade retinae. This suggests that the decrease in cell proliferation in Hdac blockade could not be rescued even after the simultaneous blockade of Ezh2 and Hdacs (Figure 3.1a,b). To validate this, we performed a similar experiment, but now, valproic acid is used instead of TSA to block Hdacs. And it also shows a similar trend (Figure 3.1c,d).

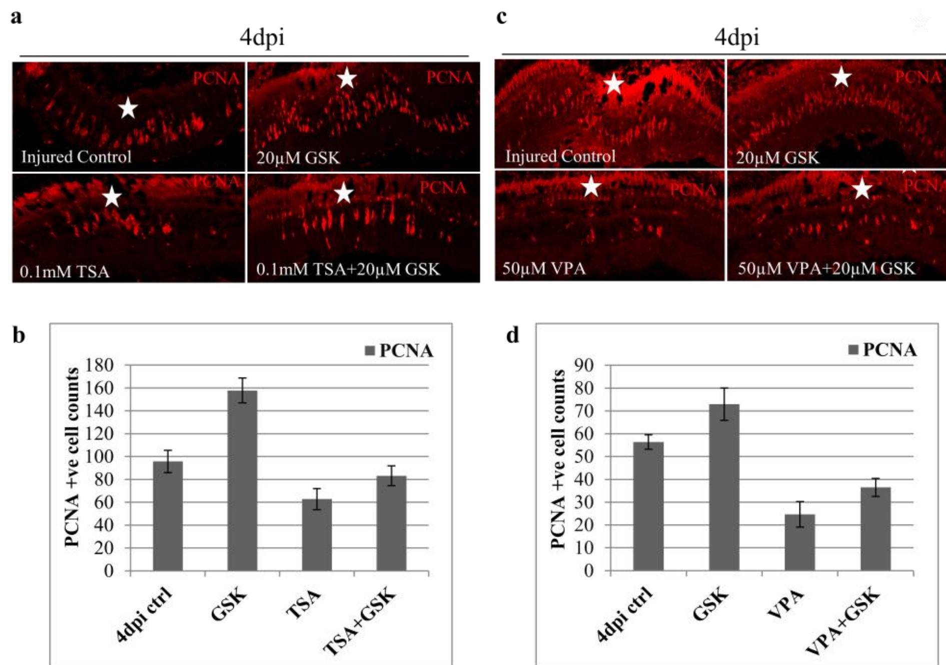


Figure 3.1:Effect of combined blockade of Ezh2 and Hdacs. (a,c) Immunostaining against PCNA. IF microscopy showed an increase in cell number upon Ezh2 blockade as quantified in b and d respectively.

3.2 Combined blockade of Ezh2 and Hdacs regulates the expression levels of various Regeneration-associated genes

So as to see why the above is not being rescued even after the simultaneous blockade, we targeted a few retinae Regeneration-associated genes like *mmp9, ascl1a,* and *her4.1*. mRNA *in situ* hybridization is performed to check the mRNA expression level. The levels of *mmp9* increase in Hdacs blockade while its level decreases in Ezh2 blockade as compared to the control ones. And in the double blockade, the mRNA level was close to that of the control ones suggesting that it is under the regulation of Ezh2 and Hdacs. This data was as expected because *mmp9* is a gene excluded from proliferation. Ascl1a is a proneural transcription factor. Upon injury Mycb (Pro-proliferative gene) induces expression of Ascl1a, a known activator of *lin28* in MGPCs. Hence, it is highly associated with cell proliferation. *ascl1a* levels increase in Ezh2 blockade which correlates with the increase in cell proliferation and in Hdacs blockade its expression level was comparable to the control ones. In the combined blockade *ascl1a* levels was found to increase as compared to the control ones. Activation of *insm1a* represses *ascl1a* in regenerating retina (RT-PCR data). As we can see from the RT-PCR data when *ascl1a* level increases *insm1a* level goes down. Her4.1 is an effector of Delta-Notch signaling. It

is a well-known regeneration-associated repressor gene. So, when there is less cell proliferation (Hdacs blockade) the expression level of *her4.1* was found to increase as compared to control ones. And in the combined blockade, the level of expression was in between Ezh2 blockade ones and Hdacs blockade. So, these genes are being regulated in the combined blockade of Ezh2 and Hdacs (Figure 3.2c). This was validated in RT-PCR (Figure 3.2a) and qPCR (Figure 3.2b).

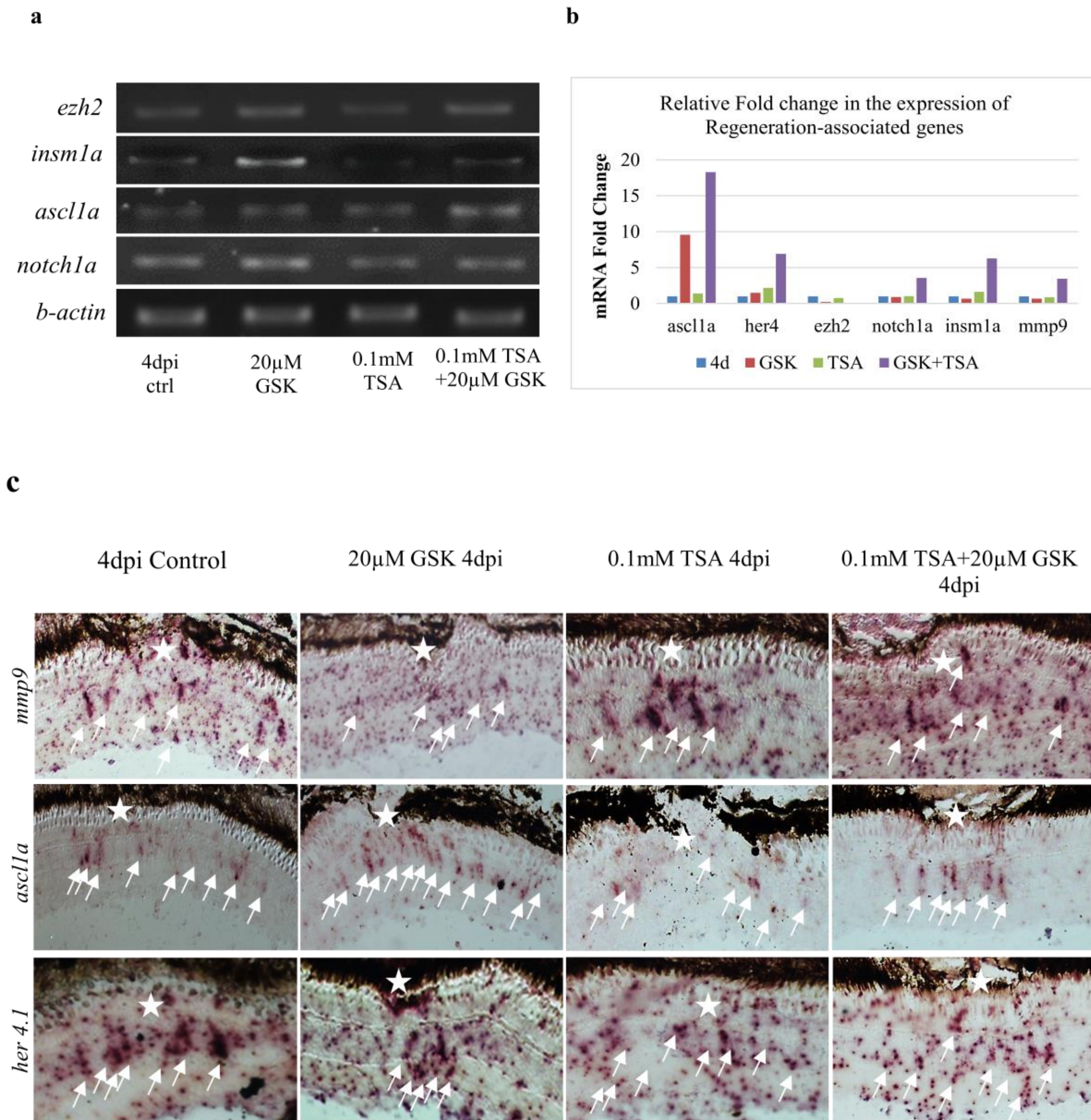


Figure 3.2: Regulation of regeneration-associated genes by combined blockade of Ezh2 and Hdacs. (a) RT-PCR data shows that the regeneration associated genes are regulated upon the combined blockade, the expression level of *ascl1a* goes down when the *insm1a* level goes up.(b) qPCR data also validate the previous data. (c) mRNA expression profiles on targeted regeneration-associated gene like *mmp9, ascl1a, her4.1* in the combined blockade. *ascl1a* expression level goes up when there is a high number of cell proliferation.

3.3 *hdac1* knockdown leads to a reduction in the expression levels of Ezh2

To see the interplay between Ezh2 and Hdacs,we specifically knocked down *hdac1* With morpholino tagged with lissamine. Morpholino (MO) is a modified antisense oligonucleotide which will go and bind to specific mRNA of interest and hence blocks the translation of RNA transcript. From the immunostaining data, the proliferating cells marked by PCNA was found in the control ones but there was nil/negligible amount of cell proliferation in the experimental ones (*hdac*1 knockdown). This suggests that there may be interplay between the two (Figure 3.3). To validate further the following experiment is performed.

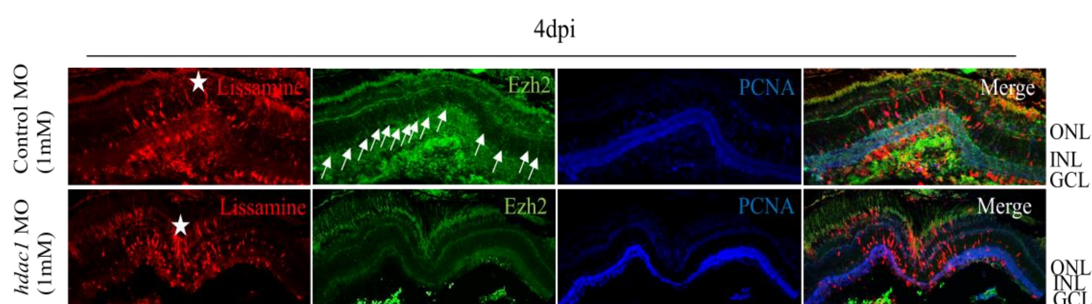


Figure 3.3: MG proliferation reduces after *hdac1* knockdown at 4dpi. Immunostaining profile shows nil/negligible number of cell proliferation when *hdac1* was knockdown. MO is in red because of lissamine.while PCNA is in green.

3.4 Blockade of Hdacs reduces the level of epigenetic mark H3K27me3

When Hdac is blocked, the levels of Ezh2 goes down (This work was done in the lab by a senior MS student). In her work, it has been shown that when Hdacs is blocked in a concentration-dependent manner viz 0.1µM TSA and 0.5µM TSA, it shows that the level of Ezh2 goes down with decrease function of Hdacs (Figure 3.4a).So, I checked the levels of Ezh2 epigenetic mark H3K27me3. Hdacs was blocked in two different concentrations viz. 0.1µM TSA and 0.5µM TSA and the retinae were harvested on 4dpi. We also found that the epigenetic marks created by Ezh2 levels were lesser in 0.5µM TSA as compared to 0.1µM TSA treated ones (Figure 3.4b). the level of Ezh2 and H3K27me3 reduction could be due to reduced cell proliferation in TSA treatment. So, from all of this, we can

conclude that there is an interplay between Ezh2 and Hdacs during retina regeneration in zebrafish. And both Ezh2 and Hdacs affect each other expression levels.

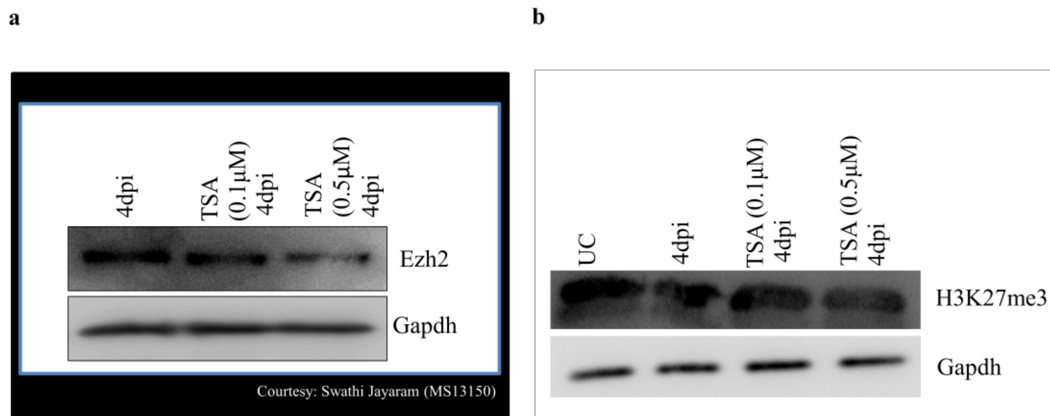


Figure 3.4: Effect of Hdacs blockade on the levels of Ezh2 and its epigenetic marker H3K27me3. Western Blot analysis showed a decrease in the level of Ezh2 (a) as well as its epigenetic mark H3K27m (b), upon Hdacs blockade in a concentration-dependent manner.

3.5 Hdacs are functional in the initial phase of regeneration while Ezh2 in the later phase

It was shown that in sequential blocking of Hdacs and Ezh2 , it leads to a significant increase in cell proliferation (Done in the lab by a senior MS student) (Figure 3.5a). Here, in the dedifferentiation phase (0-2dpi), Hdacs was blocked using 0.1µM TSA and Ezh2 was blocked in the proliferating phase (2-4dpi) using 20µM GSK. And the cell counts of the PCNA positive cells were found to be highest in this as compared to 0.1µM TSA/H2O, H2O/20µM GSK, and H2O/ H2O treated ones (Figure 3.5a,b). So, to check if the functions of Hdacs and Ezh2 are phase dependent, the experiment done earlier was reversed. Here, Ezh2 was blocked in the dedifferentiation phase (0-2dpi) using 20µM GSK and in the proliferating phase (2-4dpi), Hdacs was blocked using 0.1µM TSA. But in this case, the number of cell proliferation was close to the control ones. so we can confer that the functional Hdacs present in the pre-proliferative phase could be helping Ezh2 blockade to exert its effect on cell proliferation. Also implies that maybe Hdacs are functional in the differentiation phase and Ezh2 in the later phase in zebrafish retina regeneration.

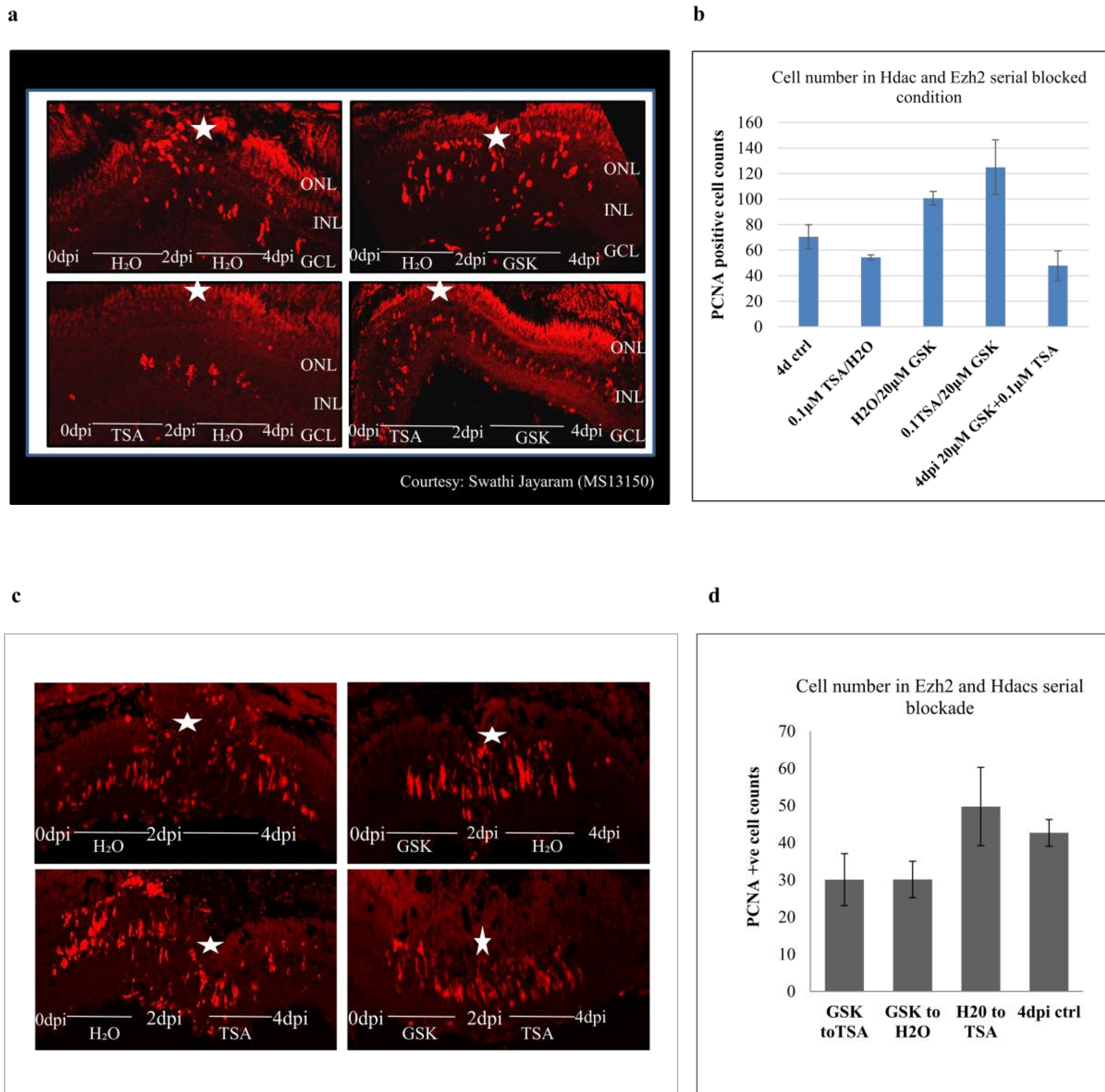


Figure 3.5: Functions of Hdacs and Ezh2 are phase dependent. The immunostaining profile shows that when Hdacs is blocked in the differentiation phase (0-2dpi) and when Ezh2 is blocked in the proliferation phase (2-4dpi) the cell number increases as compared to the control ones (a) which were quantified in (b). Here, the experiment done earlier was reversed (c). Ezh2 was blocked in the dedifferentiation phase and Hdacs in the proliferating phase. In this case, the number of cell proliferation was close to the control ones as observed in (c) and (d). So, the function of Hdacs and Ezh2 may be phase dependent.

3.6 Rescued cells are viable and have the potential to form various retinal cell types

Next, we checked if the rescued cells in the sequential blockade were still viable at 20dpi and if it still has the potential to form retinal cell types to restore its vision. To study this we injured the retina on 0dpi and treated with TSA/water on 0-2dpi (differentiation phase) and then on 2-4dpi (proliferating phase) the fish were treated with GSK/water, and on 5-10dpi it was kept in water. On the 3,4,5 dpi, BrdU pulse was given, BrdU will go and incorporate into newly replicating DNA. The retina was harvested on 20 dpi followed by co-immunostaining. The staining data showed that the rescued cells marked by BrdU were still viable until the 20dpi. And , it has also formed various retinal cell types like the Muller Glia marked by GS marker, Amacrine cells marked by HuD marker and bipolar cells marked by PKC marker (Figure 3.6).

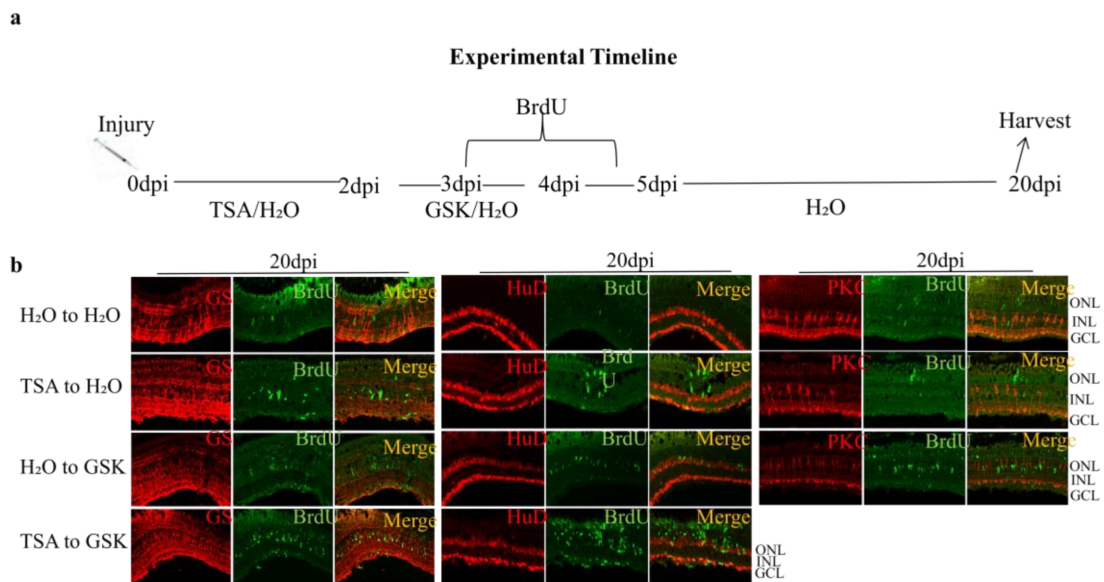


Figure 3.6: Fate of rescued cells after serial blockade of Hdacs and Ezh2. Schematics of experimental timeline (a) followed by co-immunostaining (b) where the rescued cells are marked by BrdU. Various retinal cell types like the Muller Glia marked by GS marker, Amacrine cells marked by HuD marker and bipolar cells marked by PKC marker was observed on 20dpi.

3.7 Effect of the combined blockade of Ezh2 and Hdacs on proliferating MGPCs is mediated by β-catenin

To check the effect of the combined blockade of Ezh2 and Hdacs on MGPCs mediated by β-catenin. We performed immunostaining and western blotting to check β-catenin levels in the combined blockade. From the data, we found out that the β-catenin level was highest in GSK treated ones (high cell proliferation). And the level of β-catenin was shown to decrease in TSA treated ones. In the combined treatment the level was in between GSK treated ones and TSA treated ones. this result supports the first experiment (3.1) as β-catenin is always shown to be associated with cell proliferation. This confers that the effect of Hdacs blockade could not be rescued by simultaneous Ezh2 and Hdacs blockade.

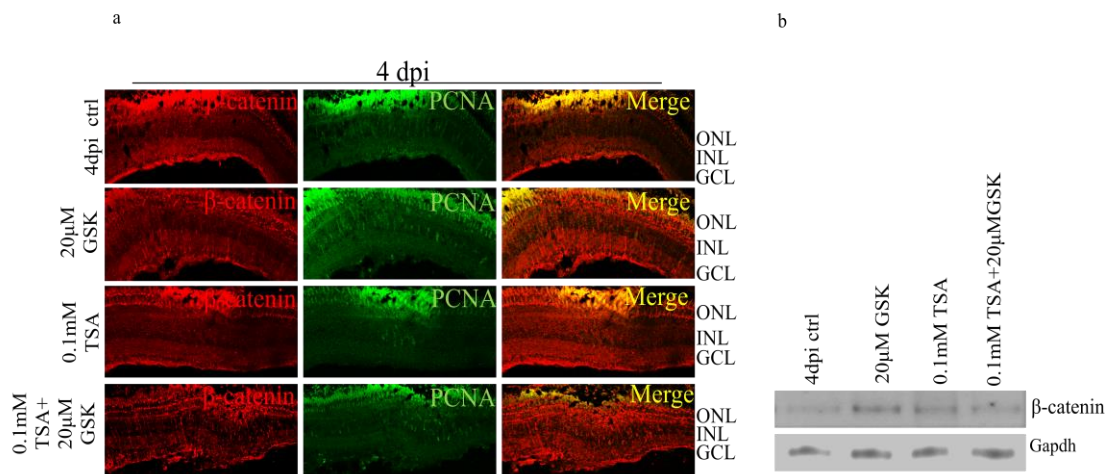


Figure 3.7: Effect of the combined blockade of Ezh2 and Hdacs on proliferating MGPCs is mediated by β-catenin: Immunostaining (a) as well as Western Blot (b) analysis, showed a similar trend. In both the cases, β-catenin levels are found to be low in the combined Ezh2 and Hdacs blockade suggestive of failure in the rescue of number of proliferating MGPCs.

CONCLUSION

This study uncovers the interplay between epigenetic modifiers like PRC2 complex and Ezh2 and also Ezh2 and Hdacs-mediated epigenetic modifications through Wnt/ β-catenin signaling pathway. We conclude that there exist functional links between PRC2 complex core component Ezh2 and Hdacs. we have found out that the decrease in cell proliferation in Hdacs blockade could not be rescued by the simultaneous Ezh2, Hdacs blockade. Also, various regeneration associated genes are being regulated in the combined blockade of Ezh2 and Hdacs as compared to the control ones. in this study, we have found out that both Ezh2 and Hdacs affect each other expression levels. As the Hdacs level goes down the expression level of Ezh2 also goes down. This could be due to a reduction in cell proliferation when Hdacs is blocked. We also found out that Hdacs is functional in the differentiation phase and Ezh2 in the proliferation phase during retina regeneration in zebrafish. In the sequential blocking of Hdacs followed by Ezh2 blockade, the cell proliferation was quite high suggesting that the functional Hdacs in the pre-proliferation phase could be aiding Ezh2 blockade to exert its effect on cell proliferation.

The rescued cells in the sequential blocking are viable and are able to form various retinal cell to restore the lost vision.

We also investigated the effect of combinational blockades of Ezh2 and Hdacs on MGPCs proliferation mediated through Wnt/β-catenin signaling pathway. We have shown that the expression level of β-catenin is regulated in the combined blockade of Ezh2 and Hdacs.

FUTURE PERSPECTIVES

This study gives some preliminary insights into the epigenetic aspects of retina regeneration in zebrafish. My thesis focuses mainly on how functional links of PRC2 and Hdacs are being regulated during retina regeneration in zebrafish. Further more experiments are required to confirmed current results. Some of the experiments that could be done are FISH and co-immunostaining of Ezh2 and Hdac1 to check their localization in the Muller glial cells. Target more of retina regeneration associated genes and see if it is being regulated. Co-IP of Ezh2 and Hdac1 during various phases of retina regeneration so as to check the dynamics of physical interaction of Ezh2 and Hdac1. We could also study the regulation of PRC2 subunits during retina regeneration.

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PRIMER AND MORPHOLINO LIST

Mentioned sequences are in 5’-3’ direction

Table 1: Sequence of hdac1 and control MO

hdac1 MO	TGTTCCTTGAGAACTCAGCGCCATT
Control MO	CCTCTTACCTCAGTTACAATTTATA

Table2: List of RT and FL primer used. RT-Reverse transcription; FL- Full Length

ascl1a FL Fwd	ATGGACATCACCGCCAAGATGGAAATAAGCG
ascl1a FL Rev	TCAAAACCAGTTGGTGAAGTCCAGGAGCTC
ascl1a RT Fwd	ATCTCCCAAAACTACTCTAATGACATGAACTCTAT
ascl1a RT Rev	CAAGCGAGTGCTGATATTTTTAAGTTTCCTTTTAC
her4.1 RT Fwd	GCTGATATCCTGGAGATGACG
her4.1 RT Rev	GACTGTGGGCTGGAGTGTGTT
insm1a RT Fwd	CCAAGAAAGCCAAAGCCATGCGGAAGC
insm1a RT Rev	TTATTGCTTTCCGCGCTCTGCTTGGGTTTG
mmp9 RT Fwd	GGAGAAAACTTCTGGAGACTTG
mmp9 RT Rev	CACTGAAGAGAAACGGTTTCC
β-actin RT Fwd	GCAGAAGGAGATCACATCCCTGGC
β-actin RT Rev	CATTGCCGTCACCTTCACCGTTC