Understanding Cross-Talk Between Polycomb Repressive Complex 2(PRC2) And Histone Deacetylases (Hdacs) During Zebrafish Retina Regeneration

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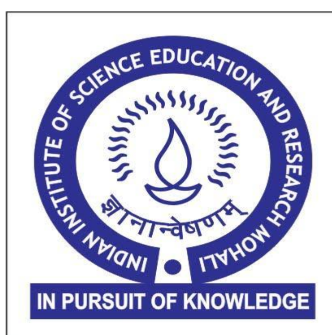

Indian Institute of Science Education and Research Mohali

April 2018

CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled **"Understanding the cross-talk between Polycomb repressive complex 2(PRC2) and Histone deacetylases (Hdacs) during zebrafish retina regeneration"** submitted by Ms. Swathi Jayaram (Reg.No.MS13150) for the partial fulfilment 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 be accepted.

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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.

Swathi Jayaram
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Dated: April 20, 2018

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

1. RP-Retinitis pigementosa
2. MG-Muller Glia
3. MGPC-Muller glia derived progenitor cell
4. R-Rods
5. C-Cones
6. A-Amacrine cells
7. BP-Bipolar cells
8. H-Horizontal cells
9. GC-Ganglion cells
10. ONL-Outer nuclear layer
11. INL-inner nuclear layer
12. GCL-Ganglion cell layer
13. Ezh2- Enhancer of zeste homologue zebrafish protein
14. *ezh2-* Enhancer of zeste homologue zebrafish mRNA
15. Hdac- Histone deactylase protein
16. *Hdac-* Histone deactylase mRNA
17. Gfap - glial fibrillary acidic protein
18. TSA-Trichostatin A
19. dpi-days post injury
20. hpi-hours post injury
21. Ascl1a - Achaete-scute homolog 1 zebrafish protein
22. *ascl1a -* Achaete-scute homolog 1 zebrafish mRNA
23. Mmp9-Matrix metallo protease 9 zebrafish protein
24. *mmp9-* Matrix metallo protease 9 zebrafish mRNA
25. SAM- S-adenosyl methionine
26. ChiP-seq- Chromatin immunoprecipitation- sequencing
27. Co-IP- Co-immunoprecipitation
28. SUZ12 - Suppressor of zeste 12
29. EED-Embryonic ectoderm development

ABSTRACT

"Regeneration—the reactivation of development in later life to restore missing tissues—is so "unhuman" that it has been a source of fascination to humans since the beginnings of biological science."[1]. Planaria, salamanders, zebrafish, African spiny mice etc. are some of the animals that show remarkable ability to regenerate their body parts. Amongst these, zebrafish is one of the best model organisms to explore regeneration because of its ability to regrow almost all damaged body parts like liver, fin, heart and even the central nervous system (CNS). CNS regeneration is almost nil in mammals, therefore a lot of information can be elucidated by studying regeneration of the retina of zebrafish, one of the most accessible part of its CNS. Signalling pathways like Notch, Mapk–Erk, Jak–Stat pathway etc. have been shown to be turned on in the phases post retinal injury. Epigenetics of zebrafish retina regeneration has been studied mainly in terms of DNA methylation and underexplored in terms of histone modifications. In this study we have explored the crosstalk between Polycomb repressive complex 2(PRC2) and Histone deacetylases (Hdacs) during zebrafish retina regeneration. We found that both PRC2 (Ezh2; its catalytic subunit) and Hdacs affect each other's expression levels and that combined pharmacological blockade of Ezh2 and Hdacs reduce cell proliferation compared to control and keep it similar to exclusive Hdac blockade. We have shown that Hdacs function predominantly during the de-differentiation phase to keep a check on proliferation whereas Ezh2 plays its role during the pre-proliferative phase of regeneration. During the pre-proliferative phase Ezh2 controls cell proliferation with the help of functional Hdacs. As a preliminary result, we also report that the effects of Ezh2 and Hdacs on proliferation is mediated by regulating the expression of regeneration associated genes like *ascl1a* and *mmp9* and *ezh2*. Interestingly, at the peak of proliferative phase (4dpi) Ezh2 and Hdac1 were found to physically interact. Hence, this study sheds some light on the roles of two histone modifiers to regulate the proliferative phase of zebrafish retina regeneration.

CHAPTER 1: INTRODUCTION

We appreciate the world around us best using the power of sight. Diseases like retinitis pigmentosa (RP), macular degeneration and accident associated injuries to the retina lead to vision loss. Unfortunately, in mammals regenerative ability is limited to organs like skin, liver and muscles [1] and not extant for the retina. One of the best strategies to circumvent this problem is to find ways to trigger the retina to heal itself. Towards this, zebrafish (*Danio rerio*) stands as a great model organism because of its robust regenerative abilities and similarity of the retinal structure.

Zebrafish is a teleost fish which can regenerate almost all its organs such as the retina, fin, heart, liver and bone. Retina regeneration is mediated mainly through the only glial cell type in all vertebrate retinas, the Muller glia (MG). 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 RETINAL ARCHITECTURE, INJURY AND REGENERATION

1.1.1 Zebrafish Retinal Architecture

The zebrafish retina consists of three nuclear layers, namely outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL). The ONL contains rod (R) and cone (C) photoreceptors which sense light and transmit the information to ganglion cells (GC) in GCL via the INL which houses amacrine (A) cells, bipolar (BP) cells, horizontal (H) cells and Muller Glia (MG). MGs are special cells that extend their processes out to all the layers yet housing the cell body within the INL. Such an anatomy allows these glial cells to monitor the retinal environment to sense any change in the retinal neural homeostasis (Fig 1.1).

Since the MG is well positioned it aids in the transfer of a lot of molecules across various retinal cells. 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. It has also been reported that MGs can act as optical fibres to guide light to the photoreceptors.[2]

1.1.2 Injury response by MG

Injury can be inflicted to the retina by 4 ways namely; mechanical (using needle poke)[3], toxic chemicals or toxic genes[4], bright or UV light [5] and, laser ablation [6] Upon sensing damage to the retina, MGs undergo a gliotic response where they overexpress tubulin proteins like Gfap (glial fibrillary acidic protein) and undergo hypertrophy. 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:

i. **Dedifferentiation** of adult MGs into MGPCs
ii. Interkinetic nuclear migration to the ONL and asymmetric cell division (**proliferation**)
iii. Migration of progenitors to various retinal layers and **redifferentiation** into respective cell types

Some of the early signalling events in zebrafish that follow a mechanical injury are glycogen synthase kinase 3β (Gsk3β)–β-catenin, Notch, Mapk–Erk and Jak–Stat signalling pathways[7,8,9,10]. Upon zebrafish retinal injury Wnt signalling gets triggered to induce reprogramming and proliferation of MG. *wnt* expression and β-catenin stabilisation takes place in the MGPCs.

The let-7 miRNA signalling and Dkk signalling are two inhibitory pathways that is necessary to maintain a quiescent state of MG [11,12] . Notch signalling also has an inhibitory role which suppress the number of MG in which injury response in triggered which is evident by increase in the expression of *her4* a target of Notch signalling.[8] One of the main players that suppress differentiation programs and promote proliferation is the pro-neural transcription factor Ascl1a (Achaete-scute homolog 1). Ascl1a stimulates *lin-28* expression which suppresses *let-7* miRNA (associated with differentiation) expression and also stimulates *lin-28* expression.

1.2 EPIGENETICS AND ITS ARMY

The environment around us like the food we eat, the air quality, the chemicals we are exposed to etc. play a key role in shaping our genetics. These factors exert a control over gene expression through factors other than the DNA per se. The study of changes in expression of a gene due to factors other than modifying the genetic code itself is called

epigenetics. Epigenetic modifications can be grouped into 3 categories: DNA methylation, histone modification and RNA-associated gene silencing.

The process that adds a methyl group to regions in the DNA called CpG islands (C next to G) is called DNA methylation, mediated through the enzymes called DNA methyltransferases that add methyl groups to these sites causing changes in their structure, thereby, making the region inaccessible to the transcription machinery and hence silencing the gene.

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

1.2.1 Histone modifications

If the DNA in each of our cells is stretched, one could travel from the Earth to the sun and back 300 times. Each diploid cell of a human contains nearly 2m long DNA. Yet how is this packed into a micro-meter sized cell? The answer lies in the way the DNA is packed within the nucleus. DNA wraps around proteins called histones, 1.65 times to form structures called nucleosomes. Such nucleosomes make up the chromatin. 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 compacting it (Fig 1.2.1).

Various covalent post translational modifications like methylation, acetylation, sumoylation, ubiquitylation and phosphorylation are made on these histone proteins by epigenetic modifiers to alter the expression of genes it associates with. Such covalent modifications cause a change in the chromatin structure as mentioned to regulate gene expression. My project deals with understanding the role of the writers of histone modifications during the zebrafish retina regeneration namely, histone deacetylase (HDAC), the histone methyl transferase Ezh2 from Polycomb Repressive Complex 2 (PRC2). So let's take a closer look at them.

1.2.2 Histone methyl transferases

Histone methylation is the process of transfer of methyl groups from S-adenosyl methionine (SAM) substrate to lysine or arginine residues of histone tails by histone methyl

transferases (HMT). There exist different HMTs to modify different lysine and arginine residues. SET1, SET7/9, Ash1, ALL-1, MLL, ALR, Trx, and SMYD3 are histone methyltransferases that catalyse methylation of histone H3 at lysine 4 (H3K4) in mammalian cells. Histone 3 at lysine 9 (H3K9) is methylated by ESET, G9a, SUV39-h1, SUV39-h2, SETDB1, Dim-5, and Eu-HMTase. G9a and polycomb group enzymes such as EZH2 are histone methyltransferases that catalyze methylation of histone H3 at lysine 27 (H3-K27) in mammalian cells[13]. Similarly, different histone modifications imply a different state of the chromatin; either active or repressed as shown in Fig 3 [14].

The Polycomb Repressive Complex 2(PRC2) catalyses the trimethylation of H3 on lysine 27 (H3K27me3) in a sequential manner resulting in mono (H3K27me1), di (H3K27me2) and tri methylated (H3K27me3) H3K27[15,16]. The core PRC2 complex, which is conserved from Drosophila to mammals, comprises of 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, of which EZH2 is the catalytic subunit which contains a C terminal SET domain[16,17,18]. Ezh2 contains 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 by binding to all other components of the complex stabilises the PRC2 while RbAp46/48 isn't known to have any direct effect on the enzymatic activity of PRC2.

In order to study the enzymatic function of Ezh2 pharmacological inhibitors like GSK343 and UNC1999 are used. These are SAM competitive inhibitors and bind to the SAM binding site in PRC2 complex thereby preventing removal of methyl group from SAM and transferring to H3K27 as seen in Fig 1.2.2.

1.2.3 Histone deacetylases (HDACs)

HDACs are enzymes that help in the hydrolytic removal of acetyl groups from acetylated histone lysine residues. At least 4 classes of HDACs are known so far in eukaryotes namely Class I, ClassII, Class III or sirtuins and Class IV. Class I includes HDAC1, 2, 3 and 8. Class II are comprised of HDAC 4, 5, 6, 7, 9 and 10. Sirtuins include SIRTs 1-7 and require NAD+ as cofactors for their activity. Class IV HDACs have only one member, namely HDAC11 and all HDACs except for sirtuins require Zn2+ for their activity. HDACs play a crucial role in enhancing apoptosis, cell cycle arrest and differentiation of cancer cells,

therefore targeted as an anticancer drug too[19]. However, in zebrafish *hdac2* isn't present as it is formed by duplication of *hdac1* which didn't happen in fish. Histone deacetylation tightens the bond between DNA and histones and therefore is associated with repression of gene expression.
The pharmacological inhibitor used for blocking HDACs activity is Trichostatin A (TSA). It inhibits 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[20] (Fig.1.2.3).

1.2.4 Crosstalk between HDACs and PRC2

Various reports exist that shows that PRC2 co-operates with other epigenetic modifiers such as DNA methyl transferases (DNMTs) and HDACs to silence gene expression. Vire E.*et al* has shown that EZH2 acts as a recruiting platform for DNMTs and that DNA methylation on EZH2 targeted promoters depend on Ezh2 [21]. Van der Vlag J et al has shown the interaction of EED with HDAC proteins in co-immunoprecipitation assays[2] (Fig 1.2.4).

So the model for possible interactions between these epigenetic modifiers is as follows. If a lysine 27 residue is pre-acetylated then HDACs may remove them and then target genes are repressed by methylating K27 using PRC2. To have a more permanent silencing Ezh2 might recruit DNMTs to methylate and silence that segment of gene (Fig 1.2.4).

Studies in various types of cancers also reveal a collaboration between epigenetic modifiers like PRC2 and HDACs and a crosstalk existing between them. Warren Fiskus *et al*'s work shows that HDAC inhibition leads to decrease in the levels of Ezh2 and associated PRC2 proteins and a decrease in proliferation of human acute myeloid leukaemia cells in culture[23]. Taichi Takashina *et al* has shown that combined inhibition of HDACs and EZH2 as a potential therapeutic target for non-small cell lung cancer (NSCLC), a condition during which there is an over expression of EZH2[24].

Inspired from such studies we wished to explore if there exists any cross talk between PRC2 and HDACs during zebrafish retina regeneration. We have been able to show that there indeed exists a collaboration between the two to regulate one of the most important and preliminary stages of regeneration, that is proliferation and that the presence of HDACs is necessary for PRC2 to exert its effects on proliferation.

CHAPTER 2: MATERIALS AND METHODS

2.1. Retina dissection for RNA isolation/ western blotting

1. Zebrafish was anesthetized using Tricaine methanesulfonate.
2. Retina were injured using 30 gauge needle.
3. At a desired time after injury, eyes were dissected using steel forceps and needle. Dissections were carried out either in 1X PBS (Phosphate Buffered Saline) for harvesting retina or 4% paraformaldehyde (PFA) for tissue fixation. Lens were removed for both isolating RNA/ tissue fixation. If the retinae are to be used for RNA isolation they should be stored in Trizol and if to be used for western blotting they should be suspended in Laemmli buffer in -80◦C.

2.2 Tissue fixation and sectioning

1. Take the eyes whose lenses were removed and put them into 4% PFA in 4ºC overnight.
2. Next day give serial washings of the fixed tissue at RT for 45mins each on a rotor:

   1ml of 5% sucrose

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

   500µl of 5% and 500µl of 20% sucrose

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

   1ml of 20% sucrose.
3. Then add 500µl of OCT and rotate it for 30 min.
4. Then embed the in OCT in small cubes made from aluminium foil and the embedded samples are kept frozen at -80ºC until sectioning.
5. Then section the blocks in cryostats (12µm thickness) and collect the sections on super frost plus slides and dried overnight and then stored in -20ºC.

Composition of solutions used:

1. 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

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.

2.3 RNA isolation

1. Take the stored embryos/dissected retinas from -80°C and thaw it.
2. Homogenise the tissues (use 200µl pipette for retinas and homogeniser in case of embryos) completely so that no tissue clumps are visible. Allow it to settle for 5 min.
3. Add 0.2 volumes (40µl) of chloroform and mix it gently by inverting the MCT up and down ten times.
4. Then centrifuge it at 10000 rcf for 10min at 4°C.
5. Using cut tips collect aqueous phase slowly without disturbing the middle phase layer and put it in fresh MCTs.
6. Add equal volume of Isopropanol and keep it on ice for 5 mins.
7. Then centrifuge it at 10000rcf at 4°C for 10min.
8. Discard the supernatants.
9. Wash it with 80% alcohol (200µl) and centrifuged it at 4°C for 10 min at 7600 rcf.
10. Dry and dissolve the pellet in DEPC treated water and stores it at -80°C.
11. Check whether there is any contamination of genomic DNA in it by using agarose gel electrophoresis (1%gel) and if the isolated RNA is pure and free from any genomic DNA contamination we measure the amount of RNA present by measuring the Optical density using spectrophotometer (nano drop) and proceed further for cDNA synthesis.

2.4 cDNA synthesis (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit)

Set reaction for 5µl reaction

1. Add the following components in a PCR tube:-

   Template RNA – 1.5µl

   Oligo (dT) Primer - 0.25µl

   Random Hexamer - 0.25µl

   Nuclease free water -1µl

2. Mix it properly and put in 65^{0}C for 5min, after this immediately put it in ice for about 2 min.

3. Add the following to the above mixture:-

   5X Reaction Buffer - 1µl

   RiboLock RNase inhibitor - 0.25µl

   10mM dNTP Mix – 0.5µl

   RevertAid M-MuLV RT – 0.25µl

4. Mix the reaction mixture properly. Spin it down briefly and give the following incubation:-

   25°C - 5min

   42°C - 60min

   70 - 5min

   4^{0}C - infinite hold

5. Store the cDNA in -80° C

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

Dilute the synthesised cDNA by 1:4 dilutions (1µl cDNA+ 4µl Milli-Q water) and set reactions for 10µl.

1. 20X buffer- 0.5µl
2. dNTP-1µl
3. forward primer and reverse primer(F+R) -0.4µl
4. template cDNA-0.5µl
5. MQ water -7.1µl
6. Taq polymerase -0.5µl
7. Put cycling conditions as:-

   95°C - 2min

   95°C - 20secs

   60°C - 30secs

   68°C - depending on size of gene (1kb/1min),

   72°C - 7 min

   4°C - Infinite hold

   Put 35 cycles.

8. Check the PCR product by Agarose gel electrophoresis.

2.6 Quantitative PCR (qRT-PCR)

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

We set 5µl reaction:-

1. Master mix- 2.5µl
2. Primers (F+R)-0.25 µl
3. Template -0.25 µl

MQ water-2 µl

Analyse the data on excel sheet and plot the graph.

2.7 Plasmid isolation

1. Take 1.5ml of the overnight incubated cultures in MCTs and centrifuge it at 14,000rpm for 2 min.
2. Discard the supernatant and elute it in 100µl of MQ water and vortex it.
3. Add 100µl of freshly prepared lysis buffer, mix it properly and boil it in 100°C for 2 min.

   Compositions of lysis buffer are:-

   MQ water - 920µl

   20% SDS - 50µl

   0.5M EDTA - 20µl

   10N NaOH - 10µl
4. Add 50µl of 0.5M $MgCl_2$, mix properly and put it in ice for 2 min.
5. Then spin for 2 min at 14,000rpm and add 50µl of 3M potassium acetate buffer in it and mix it properly by inverting the MCT up and down.
6. Spin it again at 14,000 rpm for 2 min.
7. Take the supernatant in a new MCT and add 600µl of Isopropanol to it, mix it properly and put it in ice for 5 min.
8. Spin at 14,000rpm for 2 min.
9. Discard the supernatant and wash the pellet with 70% ethanol.
10. Dry the ethanol and elute it in 50µl of Nuclease free water.

2.8 Plasmids and restriction digestions for probe making

1. Add the following components in an MCTs:

Plasmid - 5µl

MQ water- 3.5µl

10X buffer - 1µl

(Depending on the restriction enzymes used)

Restriction enzymes - 0.5µl

Enzymes used:

ezh2 – Xho I (SP6 polymerase)

mmp9- Not 1(SP6 polymerase)

ascl1a- Xho I (SP6 polymerase)

2. Incubate at 37ºC for 1 hour
3. Check on agarose gel and confirm their digested fragments size and determine the orientation of the insert.
4. Put large scale digestion with suitable restriction enzymes depending on the orientation, to linearise the plasmid using above enzymes.
5. Add the following components in an MCTs:

   Plasmid - 35µl

   10X buffer - 4µl

   Enzymes – 1µl
6. Incubate at 37ºC for 3 hours or overnight.
7. Load the digested products on agarose gel and excise the linearised DNA fragments from agarose gel.
8. Follow the manual gel extraction method as given earlier in TOPO TA cloning protocol.

2.9 RNA probe reaction

1. Add the following component in MCTs.

   Template DNA (linearised plasmid) – 500ng to 1µg (7µl)

   RNA Polymerase buffer - 1µl

   Dig/Fl – 0.5µl

   RNA polymerase – 0.5µl

   Nuclease free water- 0µl

   Total – 10µl

2. Incubate it in 37ºC for 4 hours.
3. Add 1µl of 0.5M Tris EDTA for stopping the reaction
4. Add 1µl of 5M LiCl for precipitation
5. Add 0.5µl of Glycogen and tap it
6. Add 18µl of 100% ethanol, tap it and mix properly and keep it in -80ºC
7. Next day or after one hour centrifuge at 14,000rpm for 15 min
8. Discard the supernatant and wash the pellet with 100% ethanol, centrifuge it at 14,000 rpm for 10 min.
9. Dry the pellet and dissolve it in 15µl of DEPC water and store at -80ºC.

2.10 Immunostaining

1. Take the slides 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 1XPBST for one hour.
6. Overlay the 1° Antibody diluted in 1%BSA /PBST (1:1000) over the slides after blocking and keep it in 4ºC overnight.
7. Next day, we then 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 3 hours.
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.

2.11 mRNA *insitu* hybridisation on cryosections.

Day 1: Hybridisation.

1. Hydrate the slides in following sequence in coplin jar for 1 min each :

    100% EtOH

    95% EtOH

    70% EtOH

    50% EtOH

    2XSSC

2. Incubate slides in Proteinase K solution for 6 min at 37°C

    -Prewarmed Proteinase K buffer to 37°C and add 250µL of 10mg/mL proteinase K

3. Rinse slides briefly in room temp DEPC water for 2 min.
4. Rinse them in 0.1M TEA pH 8.0 for 3 min.
5. Rinse in Acetic anhydride/TEA for 10 min.

    -Add 130µL of acetic anhydride 0.1M TEA.

6. Dehydrate the slides in SSC and ethanol series for 1 min each:

2X SSC

50% EtOH

70% EtOH

95% EtOH

100% EtOH

7. Dry the slides for at least 1 hour at RT.
8. Pre warm the hybridization solution at 56°C.
9. Probe preparation:

-Add probe to hybridization solution and mixed properly and boil probe and hybridization solution mi at 100°C for 10 min. Plunge immediately on ice for 2mins.

10. Add the hybridisation/ probe mix solution to each slide and coverslip with siliconized hybrid slips.
11. Place slides in humid chamber dampened with 50% formamide/5X SSC and incubate at 56°C overnight.

Day 2: Post hybridisation.

1. Preheat the following solutions:
    - 50% formamide/2X SSC solution to 65°C.
    - Two 50mL RNase buffer washes, one to 37°C and the other to 65°C.
    - Two 2X SSC washes to 37°C.
2. Soak slides with cover slips in 2X SSC for 30 min at RT on shaker table.
3. Gently remove the hybrid slip apart from slide with forceps.
4. Rinse slides in 50% formamide/2X SSC solution for 30 min at 65°C, gently agitate for the first 5 min.
5. Rinse slides twice in 2X SSC for 10 min at 37°C.

6. Incubate slides in RNase buffer having 100µl of RNaseA (10mg/mL) in 37°C for 30 min.
7. Then wash slides in 65°C RNase buffer for 30 min.
8. Wash slides for 3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
9. Wash slides twice in 1X Maleate buffer for 5 min.
10. Incubate slides with 300ul antibody (anti-Dig) diluted (1:2500 dilution) in 1X Maleate/0.05% Triton X-100/1% RMB blocker solution overnight at RT.

Day 3

1. Wash slides twice with 1X Maleate buffer for 5 minutes.
2. Wash twice with Genius buffer for 5 minutes each.
3. Add NBT/BCIP, diluted in genius buffer (1:50) and incubate it at room temperature in dark for colour reaction.
4. Detect colour in bright field microscope.

Solution compositions are same with that of whole mount *insitu* hybridisation.

2.12 Microscopy

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

2.13 Co-immunoprecipitation (Co-IP)

Reagents required:

1. 50mM Tris-HCl (pH 7.6)
2. 137mM NaCl
3. 2mM EDTA
4. 0.1% Triton X-100
5. 0.1% SDS
6. 1% PMSF (protease inhibitor)

Procedure:

10 to 12 retinae from injured fish eyes were harvested and whole cell lysate was prepared in lysis buffer containing 50mM Tris-HCl (pH 7.6), 137mM NaCl, 2mM EDTA, 0.1% Triton, 0.1% SDS and 1% PMSF. Whole cell lysate was precleared and incubated with anti

Ezh2 antibody for 4 hours at 4 degree with gentle rotation. Protein A agarose beads were added and incubated for overnight at 4 degree with rotation, washed trice with PBS and was subjected to SDS-PAGE and Western blotting by using anti-Hdac1 antibody.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 *ezh2* is regulated at various time points post retinal injury.

As a first step towards understanding the role of PRC2 and its crosstalk with HDACs during retina regeneration, the regulation of the catalytic subunit; *ezh2,* at various time points post retinal injury was checked. (The time course for various *hdacs* is already done in the lab by a senior Ph.D. student).

The temporal expression profile of *ezh2* was analysed at various time points post injury using RT-PCR and qPCR, which revealed that the mRNA levels of *ezh2* remained somewhat unchanged at initial time points with a slight upregulation at 2dpi (Fig 3.1), indicating the role of *ezh2* in pre-proliferative stages of regeneration.

3.2 Ezh2 blockade results in an increase in *hdac3* and *hdac4*, while *hdac1*/ Hdac1 remains unaffected.

A control over each other's expression could indicate a possible crosstalk existing between PRC2 and HDACs in regulating expression of a common target gene. To study this, the effect of the blockade of the catalytic activity of Ezh2 on the transcript levels of *hdac1, hdac3* and *hdac4* and on the protein levels of Hdac1, using the pharmacological inhibitor GSK343 (20µM), was checked. The mRNA levels of *hdac1, hdac3* and *hdac4* were checked using RT-PCR and qPCR and the protein levels were analysed using western blotting and immunohistochemistry. This revealed that Hdac1 remained constant upon inhibition of Ezh2 activity during the early phase of regeneration (2.5dpi). But the levels of *hdac3* and *hdac4* was observed to increase compared to the control (Fig 3.2).

3.2.1 Hdac1 levels remain unchanged upon Ezh2 blockade at 4dpi.

Hdac1 levels were also checked at the proliferative phase (4dpi), using immunohistochemistry which showed that its expression remained unchanged. While at this phase, proliferation was observed to increase upon Ezh2 blockade as compared to the control, no change in the levels of Hdac1 were found (Fig 3.2.1).

3.3 HDAC blockade results in a decrease in the levels of *ezh2*/Ezh2.

Like the study on the expression of *hdac*s in Ezh2 blockade, transcriptional and translational levels of Ezh2 were analysed upon HDACs blockade. The blockade of HDACs was achieved using TSA in two different concentrations viz. 0.1µM TSA and 0.5µM TSA. The *ezh2* levels were checked at 2dpi using RT-PCR which showed a concentration dependent decrease compared to the control. In the retinal tissue also, *ezh2* levels were less in 0.5µM TSA compared to control seen through mRNA *in situ* hybridisation. The concentration dependent decrease in Ezh2 levels were recapitulated in western blotting also. Immunohistochemistry done at 4dpi at 0.5µM TSA concentration, showed a decrease in Ezh2 levels, with Ezh2 co-localised with the proliferating cells (marked by proliferating cell nuclear antigen (PCNA)). Therefore, the reduction in Ezh2 levels could have been due to a reduction in cell proliferation post treatment with TSA (Fig 3.3).

The above set of experiments suggest that there exists a crosstalk between PRC2 and HDAC1 during the pre-proliferative and proliferative phase of retina regeneration.

3.4 Blockade of Ezh2 and HDACs together showed a reduction in cell proliferation.

Upon obtaining an evidence for the cross-talk of Ezh2 and Hdac1, we tried to check their combinational effects during regeneration. To study this, the functional activities of Ezh2 and Hdacs were blocked together, and the effects of this combined blockade on proliferation was checked at 4dpi. Proliferating cell number in 4dpi retinae was obtained by counting BrdU (bromodeoxyuridine) positive cells. The cell proliferation was significantly less than 4dpi and Ezh2 blocked retinae, while slightly higher than Hdac blocked retinae at 4dpi.

This result implies that the decrease in proliferation observed during Hdac blockade could not be rescued by simultaneous Ezh2 blockade (which generally shows an increase in cell proliferation compared to control) (Fig 3.4).

3.5 The effect of combined blockade on regeneration associated genes such as *ascl1a* and *mmp9* and, *ezh2* itself.

In order to study the effect of combined blockade of Ezh2 and HDACs on regeneration associated genes such as *ascl1a* and *mmp9* we performed mRNA *in situ* hybridisation of the same. The levels of *ascl1a* was found to be increased in HDAC blockade because of downregulation of *insm1a* in HDAC inhibition (data not shown) which is a negative regulator of *ascl1a,* inspite of cell proliferation decrease. In Ezh2 blockade the levels of *ascl1a*

increased compared to control which correlates with the increase in cell proliferation. As expected in the combined blockade the levels of *ascl1a* was found to increase compared to control because in the Ezh2 and Hdacs blockade there is an increase in *ascl1a. mmp9* is a gene which is excluded from proliferating cells. In Hdacs blockade *mmp9* levels increase while in Ezh2 blockade *mmp9* levels decrease as compared to control. In the combined blockade *mmp9* levels become comparable to 4dpi control indicating that the gene is under the regulation of both Ezh2 and Hdacs. Ezh2 co-localises with proliferating cells and hence the *ezh2* levels in the tissue were in accordance with the cell proliferation. It was found to be increased compared to 4dpi control in Ezh2 blockade while decreased in Hdac blockade. In the combined blockade of both Ezh2 and Hdacs *ezh2* levels were less compared to control as similar to the levels in Hdac blockade.

3.6 Sequential blocking of Hdac1 followed by Ezh2 leads to a significant increase in cell proliferation.

In order to understand the phase of regeneration in which Hdacs and Ezh2 exert their effect, a serial block experiment was done, where Hdacs were blocked in the dedifferentiation phase (0-2dpi) using 0.1µM TSA, and then Ezh2 was blocked from the beginning of the proliferative phase (2-4dpi) using 20µM GSK. Firstly, the cell counts of the condition in which Hdacs are blocked exclusively in dedifferentiation phase (0.1µM TSA/H_2O) were similar to the ones with continuous blockade of Hdacs from 0-4dpi. The cell counts of exclusive Ezh2 blockade in the beginning of proliferation (H_2O/20µM GSK) also were similar to the continuous Ezh2 blockade (0-4 dpi). Secondly, in serial blockade where Hdacs are blocked from 0-2dpi followed by Ezh2 blockade from 2-4dpi, the cell proliferation was found to be more than the exclusive Ezh2 blockade in 2-4dpi condition. This increase in cell count above exclusive Ezh2 blockade from the beginning of proliferation, is possibly due to the fact that withdrawal of TSA causes an increase in proliferation by itself and Ezh2 blockade in the pre-proliferative phase enhances this effect.

We compared the combined blocked condition where the cell proliferation decreases to almost that of the count where Hdacs are blocked in dedifferentiation phase (0.1µM TSA/H_2O). One of the difference between serial blockade and combined blockade is that in the pre-proliferative phase the combined block has 0.1µM TSA in addition to 20µM GSK. This implies that when Hdacs are blocked Ezh2 blockade's effect of increased proliferation is not being manifested. In serial block functional Hdacs are present in the pre-proliferative phase which could be aiding Ezh2 blockade to exert its effects on cell proliferation (Fig 3.6).

In a nutshell, functional Hdacs are necessary for Ezh2 to exert its function of regulating proliferation during zebrafish retina regeneration.

3.7 Co-immunoprecipitation (Co-IP) of Ezh2 and Hdac1 reveals physical interaction between these epigenetic modifiers at 4dpi.

In order to confirm the interaction between Ezh2 and Hdac1, a Co-immunoprecipitation assay was performed with 4dpi retinal lysate using anti-Ezh2 antibody. The pulled down lysate contained Hdac1 protein as well, which was observed by doing a western blotting of the same (Fig3.7).

CONCLUSION

From this study on understanding the crosstalk between Hdacs and PRC2 during zebrafish retina regeneration, we could conclude that there indeed exists a crosstalk and interaction between Ezh2; the catalytic subunit of PRC2, and Hdacs. We could find that *ezh2* levels increase significantly at the beginning of pre-proliferative phase (2dpi). We also found that Hdacs and PRC2 (through Ezh2) regulate each other's expression levels. Hdacs aid in increasing the levels of ezh2/Ezh2 at 2dpi/4pi probably due to the fact that cell proliferation decreases upon Hdac blockade. Ezh2 helps in decreasing the levels of *hdac3* and *hdac4* while not causing any change in the levels of *hdac1*. Hdac1 levels weren't affected by Ezh2 even at the protein level in spite of an increase in cell proliferation upon Ezh2 blockade. Upon combined blockade of Ezh2 and Hdacs using the pharmacological inhibitors GSK343 and TSA respectively we found that cell proliferation decreases compared to control and becomes comparable to that of Hdac blockade alone. In the combined blockade *ascl1a* levels go up compared to the control which could be due to the synergistic effect of both Ezh2 and Hdacs blockade (wherein *ascl1a* levels go up), while *ezh2* levels decrease correlating with the cell proliferation. In the combined blockade *mmp9* levels become comparable to the control.
Serial blockade experiments where Hdacs were revealed that Hdacs exert their effects on proliferation during the de-differentiation phase and Ezh2 during the pre-proliferative phase. We could also conclude that functional Hdacs are necessary during the pre-proliferative phase for Ezh2 to perform its task of restricting cell proliferation. Finally, we performed co-immunoprecipitation (Co-IP) of Ezh2 and Hdac1 at 4 dpi (the peak of proliferative phase) and observed that these two epigenetic modifiers physically interact to mediate the effects on proliferation.
In a nutshell, PRC2 complex and Hdacs keep a check on proliferation through regeneration associated genes like *ascl1a, mmp9* and *ezh2*. It needs to be explored if it is the directly interacting complex that regulates these genes' expression levels or if these modifiers separately fine tune their expression.

FUTURE PERSPECTIVE

Epigenetic aspects of retina regeneration with respect to histone modifications remain largely underexplored. My thesis was directed towards this goal and has been through a preliminary study about how PRC2 and Hdacs regulate the proliferative phase of regeneration. A lot more experiments need to be done to have a holistic picture of the game. Some of the key experiments that could be done to take this work forward are as follows. We could check the mRNA levels of the other components like *suz12a, suz12b and eed* during various time points post injury to have an idea about the regulation of the whole PRC2. mRNA *in situ* hybridisation for *hadac1*, *hdac3, hdac4,* on Ezh2 blockade also can be done to see the expression level of these genes on tissue. In the combined blockade and serial blockade regeneration associated genes viz. *mmp9, ascl1a and ezh2* can be quantified using qRT-PCR also. Co-IP of Ezh2-Hdac1 in serial blockade will help us to know the interaction status of these modifiers at different stages prior to proliferation. Most importantly, performing a ChIP-seq for Hdac1 and Ezh2 at 4dpi and at various phases of regeneration will help us find out the genes that are regulated separately by Hdac1, Ezh2 and also in combination.

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PRIMERS USED

RT_*bactin*_fwd	GCAGAAGGAGATCACATCCCTGG
RT_*bactin*_rev	CATTGCCGTCACCTTCACCGTTC
RT_*ezh2*_fwd	CTGCCAGTGTAGCTCAGAATGTCAG
RT_*ezh2*_rev	CTTTGATGAAGATTCCCCACCCGGCC
RT_*hdac1*_fwd	GACAGCACCATTCCTAATGAGCTCC
RT_*hdac1*_rev	TATCGTGAGCACGAATGGAGATGCG
FL_ *hdac1*_fwd	GTGATGAAGAGTTCTCAGACTCTGAGG
FL_ *hdac1*_rev	CTAACAGCAATACAGCAAGCCTTCGCC
RT_*hdac3*_fwd	CCCAGGAACTGGTGACATGTATGAAG
RT_*hdac3*_rev	ACAAACTCCACACATTCTCCATGTCC
FL_*hdac3*_fwd	TCACAGACAGGGTCATGACTGTGTCC
FL_*hdac3*_rev	TAACAAACAGCACTGGTGATCCGCCAC
RT_*hdac4*_fwd	CAAGCTCACGGCCAAATGTTTTGGC
RT_*hdac4*_rev	GCAGTGAACGCCAGTATTTACTCTGG
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FL_*hdac4*_rev	TCTCGAACGTCAGAAGCAAGAATGGCG
FL_Ascl1a_BamH1_ F	AGTCTGCAACCGGATCCACCATGGACATCACCGCCAAGATGGAAATAAG
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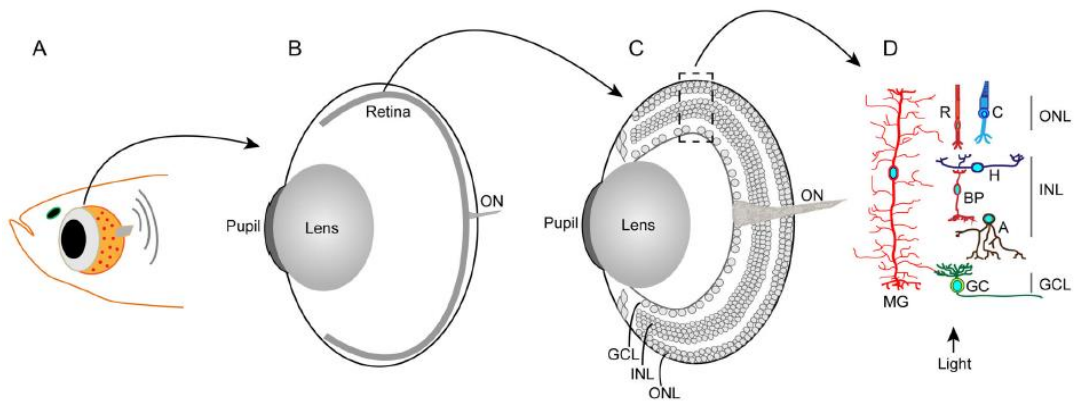


Fig 1.1 Zebrafish retinal anatomy and structure. (a, b, c, d) The image showing zebrafish eye and retinal architecture, with a view spanning all its layers and cells, namely outer nuclear layer (ONL) which contains rod (R) and cone (C) photoreceptors, inner nuclear layer (INL) housing amacrine (A) cells, bipolar (BP) cells, horizontal (H) cells and Muller Glia (MG), and ganglion cell layer (GCL) having Ganglion Cells (GC).

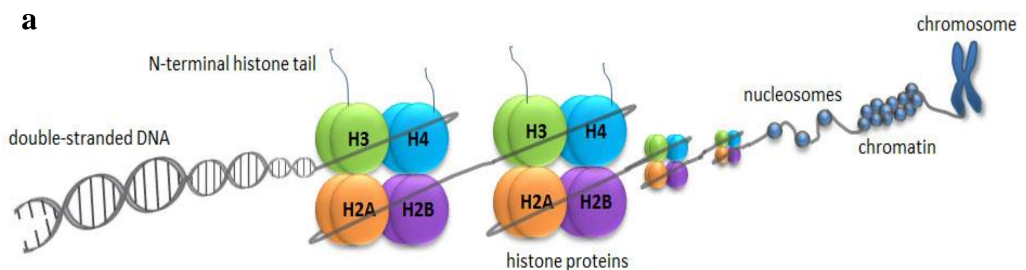


Fig 1.2.1 Chromatin architecture. (a) The image showing chromatin wound around positively charged histone proteins forming nucleosomes, which upon further condensation form a chromosome.

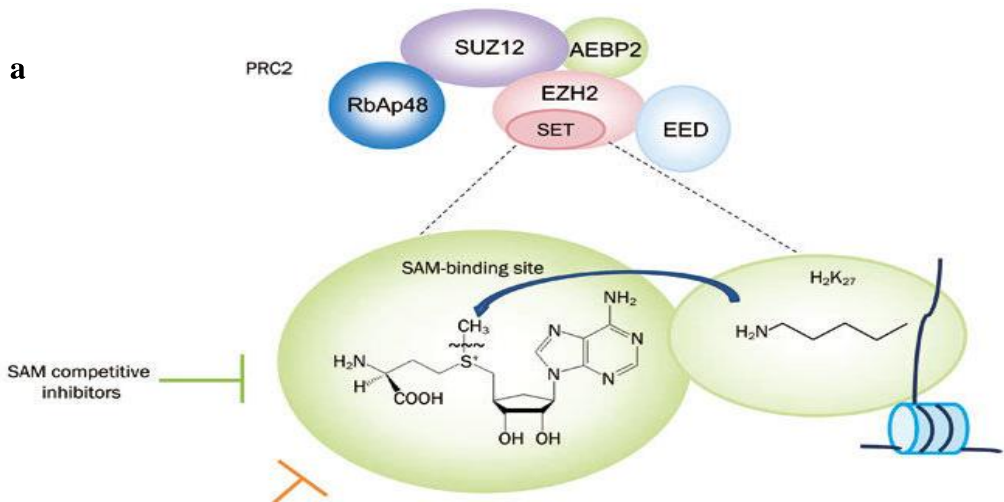


Fig 1.2.2 PRC2 assembly and mechanism of competitive inhibition of catalytic subunit of PRC2. (a) The image showing subunits of PRC2 complex and the mechanism of inhibition of the function of its catalytic subunit EZH2 by SAM competitive inhibitor like GSK343.

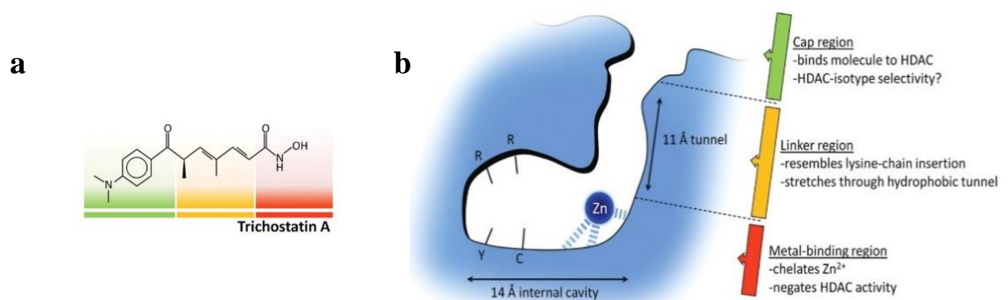


Fig 1.2.3 Trichostatin A mechanism of action. (a) The image showing the chemical structure of Trichostatin A (TSA), a small molecule pharmacological blocker of HDACs. **(b)** TSA inhibits HDACs by chelating Zn2+, which act as cofactors for HDACs.

a

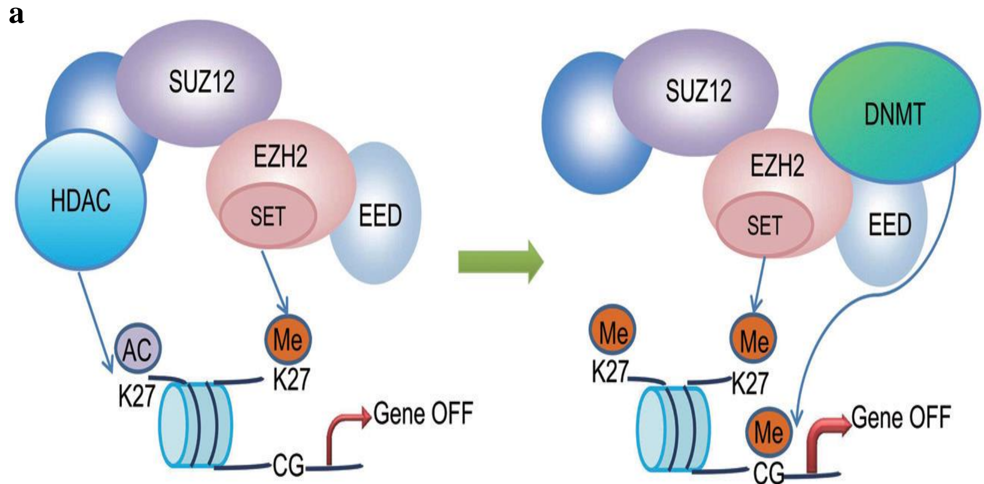
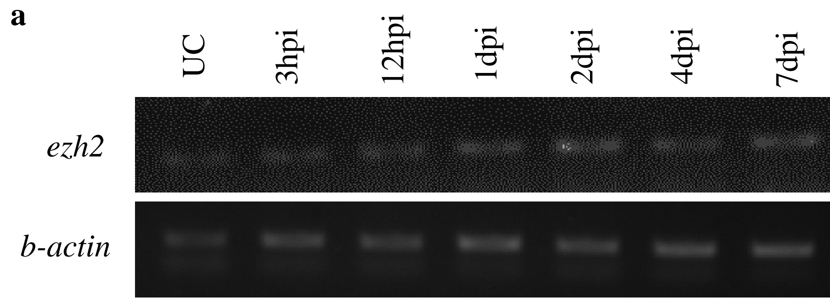


Fig 1.2.4 Crosstalk between PRC2, HDAC and DNMT. **(a)** The image showing the mechanism of recruitment of HDACs on pre-acetylated histones which removes the acetylation mark, which facilitates the appointment and function of methylation by EZH2 subunit of PRC2. Further this EZH2 recruits DNMTs, which methylate CpG islands on DNA.



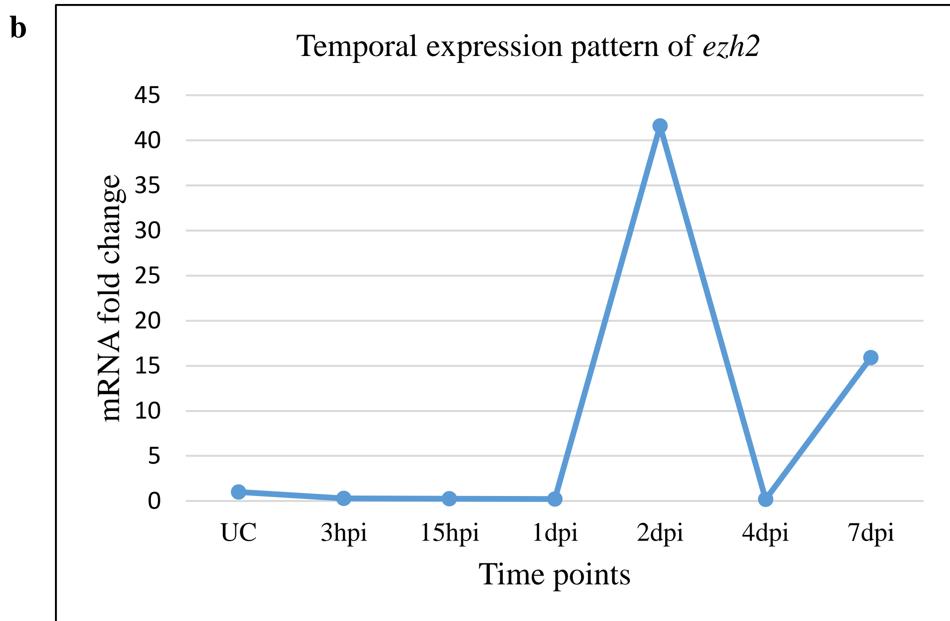
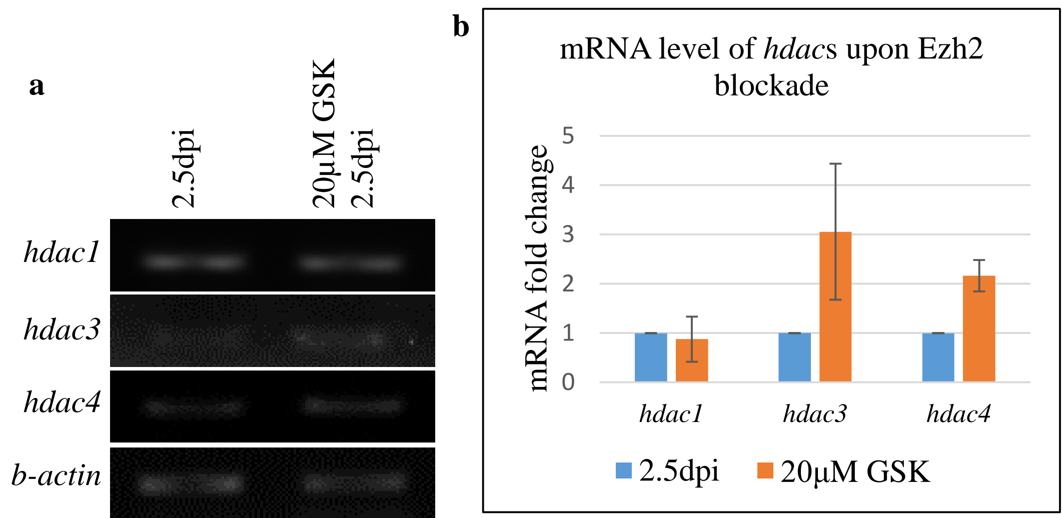
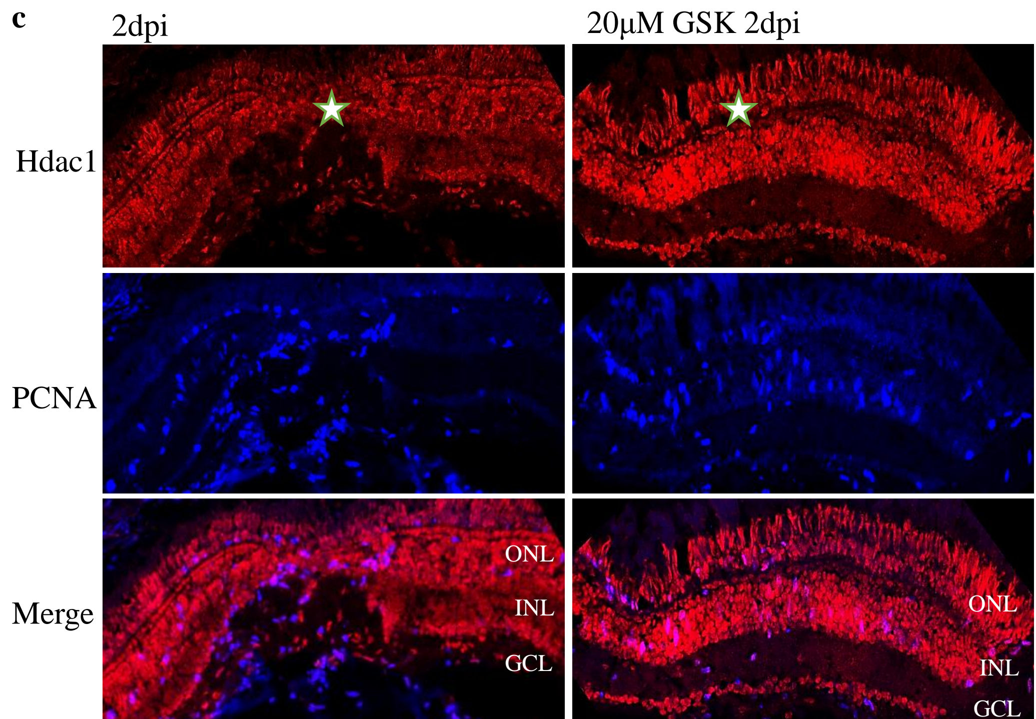


Fig 3.1 mRNA levels of *ezh2* in the injured retina. (a, b) RT-PCR **(a)**, and qPCR **(b)** analysis showed the mRNA levels of *ezh2* at various times post injury in retina..





d

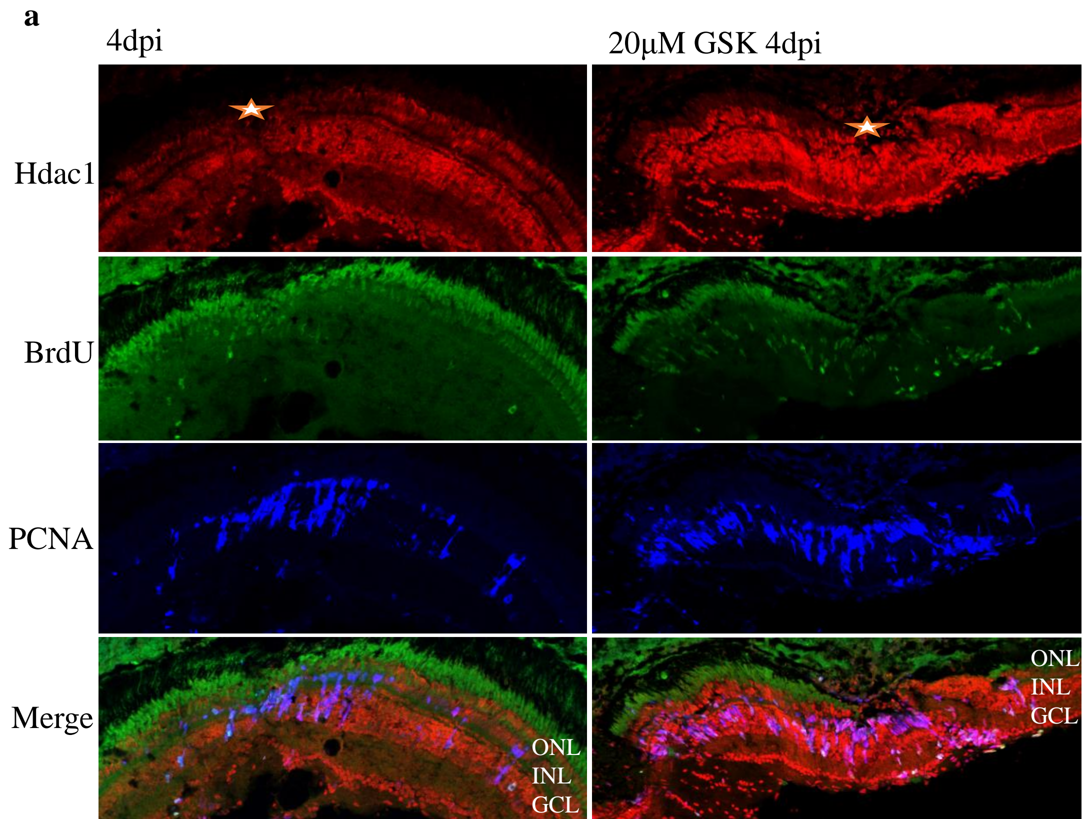
2.5dpi

20µM GSK 2.5dpi

Hdac1

GS

Fig 3.2 Expression of *hdacs* upon Ezh2 blockade in an early time point post injury. (a, b) RT-PCR **(a)**, and qPCR **(b)** analysis showed that the levels of *hdac1* mRNA remained constant, while that of *hdac3* and *hdac4* showed an increase upon Ezh2 blockade mediated by 20uM GSK343, as compared to the control, at early time point of 2.5 days post injury (dpi). **(c, d)** Immunofluorescence (IF) microscopy images at 2dpi **(c)**, and Western Blot analysis at 2.5 dpi **(d)** revealed that the expression of Hdac1 protein remained same upon blockade of Ezh2, even if the number of proliferating cells (marked by PCNA) showed an increase as compared to the control.



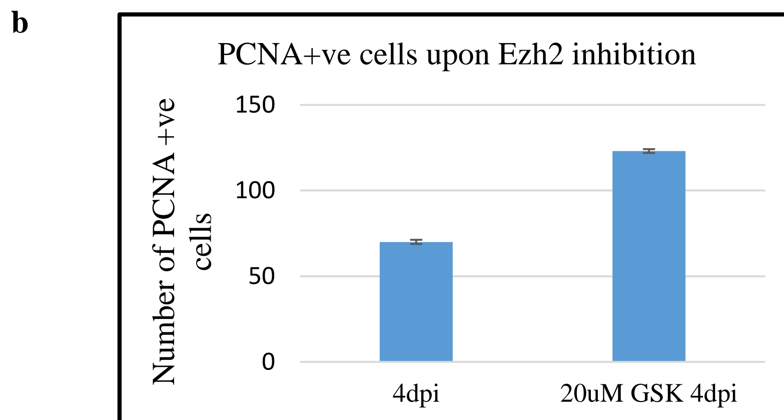


Fig 3.2.1 Expression of *hdacs* upon Ezh2 blockade in 4dpi retina. (a, b) IF microscopy images **(a)** revealed that the expression of Hdac1 protein remained unchanged, upon Ezh2 blockade even at 4dpi condition, irrespective of the increase in the number of PCNA+ cells with respect to the control, as quantified in **(b)**.

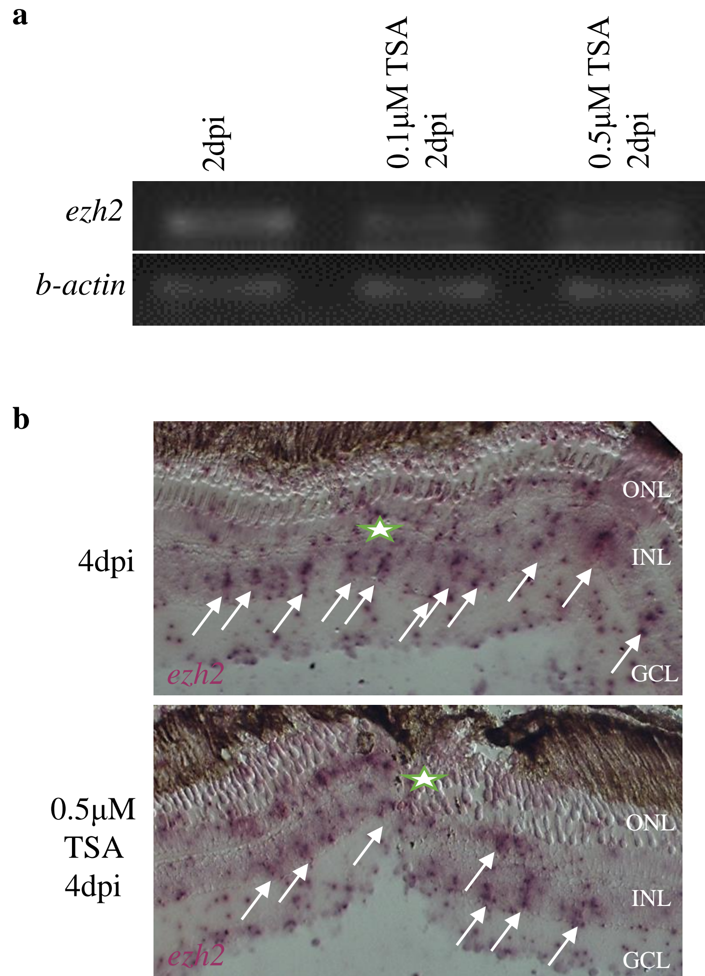
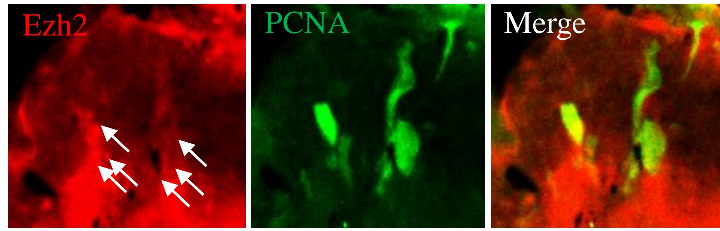
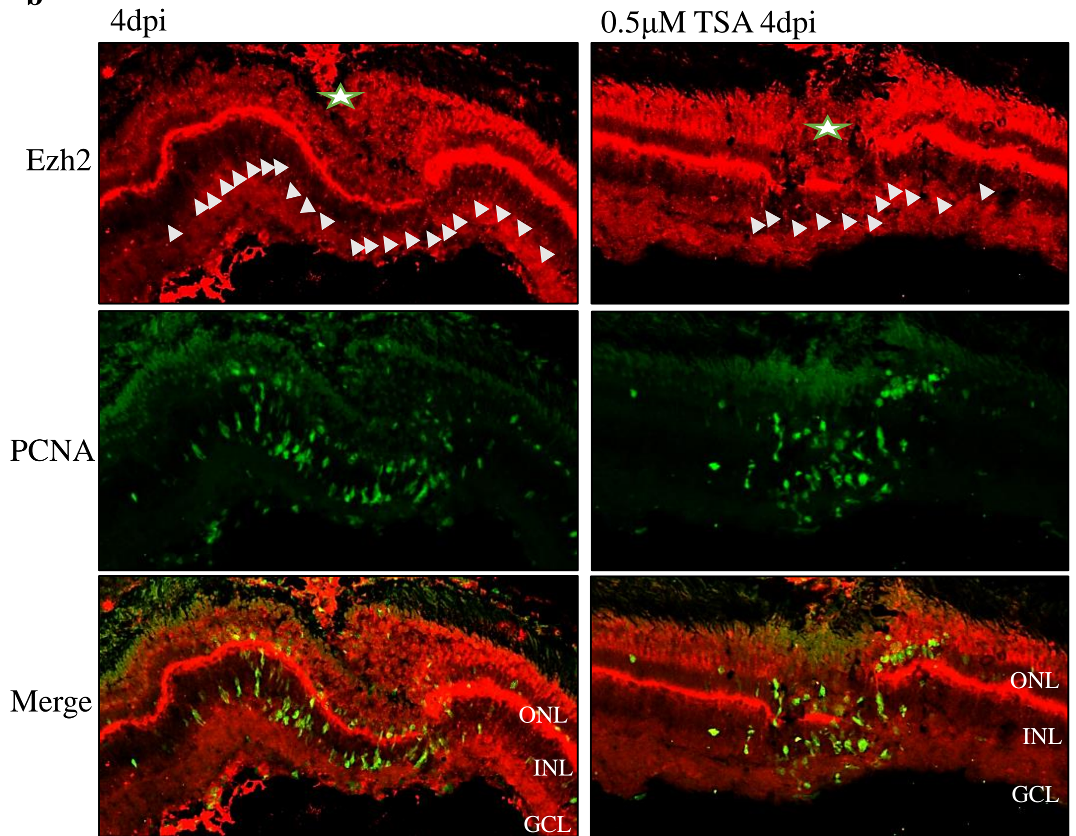


Fig 3.3a Regulation of *ezh2* upon blockade of Hdacs. (a) RT-PCR showed a downregulation of *ezh2* upon blockade of Hdacs by TSA, at 2dpi, in a concentration dependent manner. **(b)** mRNA in situ hybridization (ISH) and Brightfield (BF) microscopy also showed a decrease in the levels of *ezh2* after blocking Hdacs at 4dpi, as compared to the control.

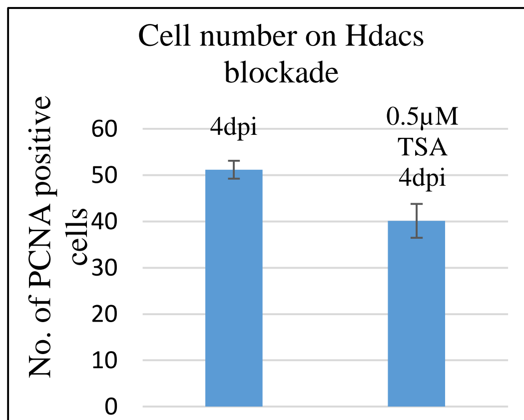
a



b



c



d

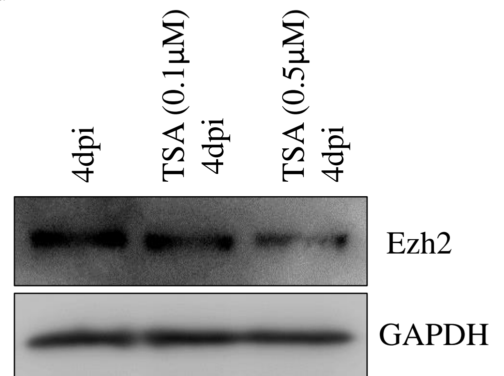
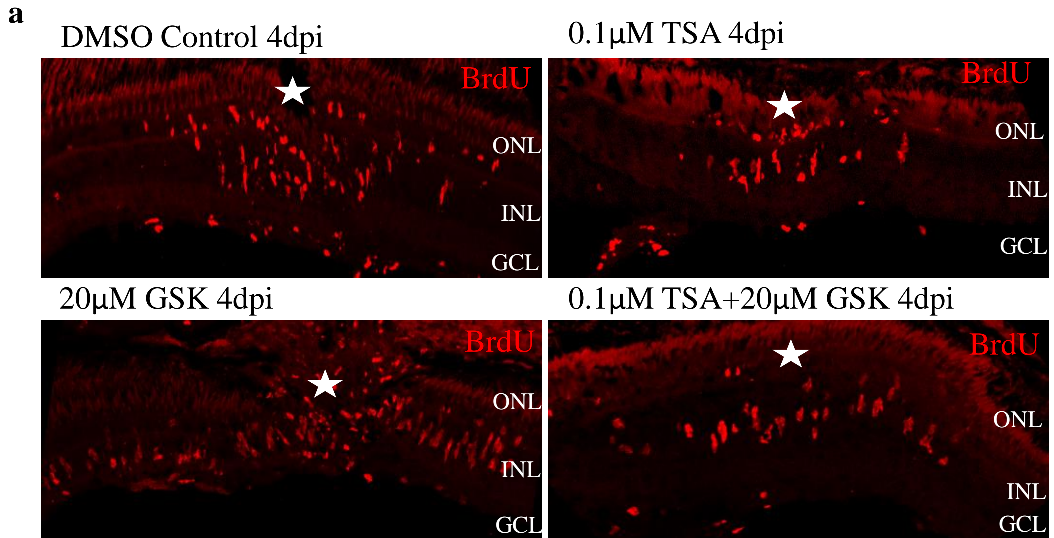


Fig 3.3b Expression of Ezh2 upon blockade of Hdacs. **(a)** High resolution IF microscopy revealed colocalization of Ezh2 expression with PCNA, in proliferating cells at 4dpi. **(b, c)** IF microscopy showed a decrease in the expression of Ezh2 upon TSA treatment in 4dpi retina **(b)**, along with the decrease in the cell number as compared to control, in **(c). (d)** Western Blot analysis at 4dpi showed a decrease in the levels of Ezh2 protein, upon Hdacs' blockade, in a concentration dependent manner.



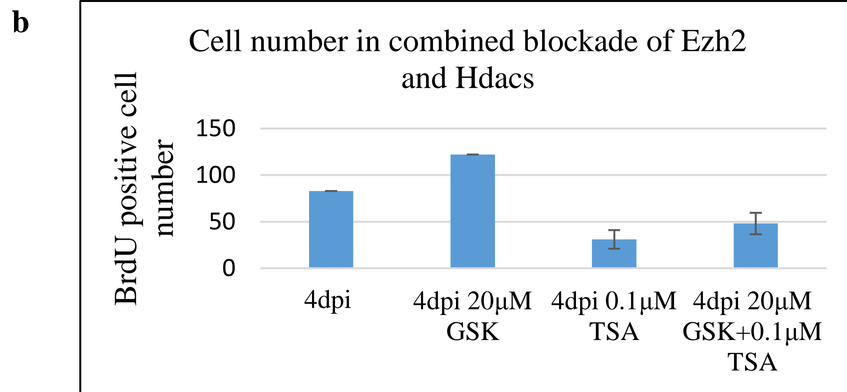


Fig 3.4 Effect of combined blockade of Ezh2 and Hdacs. **(a, b)** IF microscopy showed that the decreased cell number upon Hdacs inhibition is not rescued by the double blocking of Ezh2 and Hdacs at 4dpi **(a)**, as quantified in **(b)**.

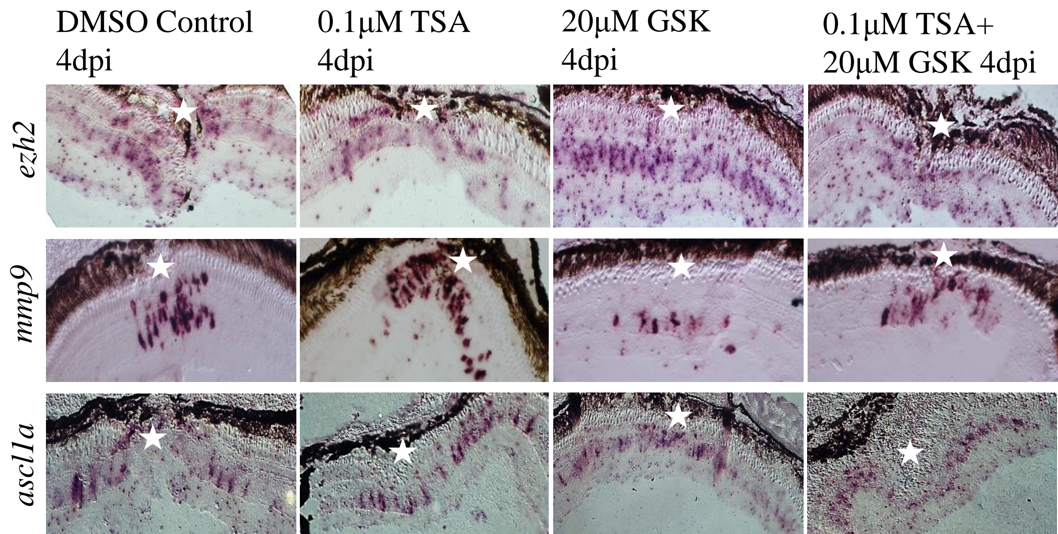
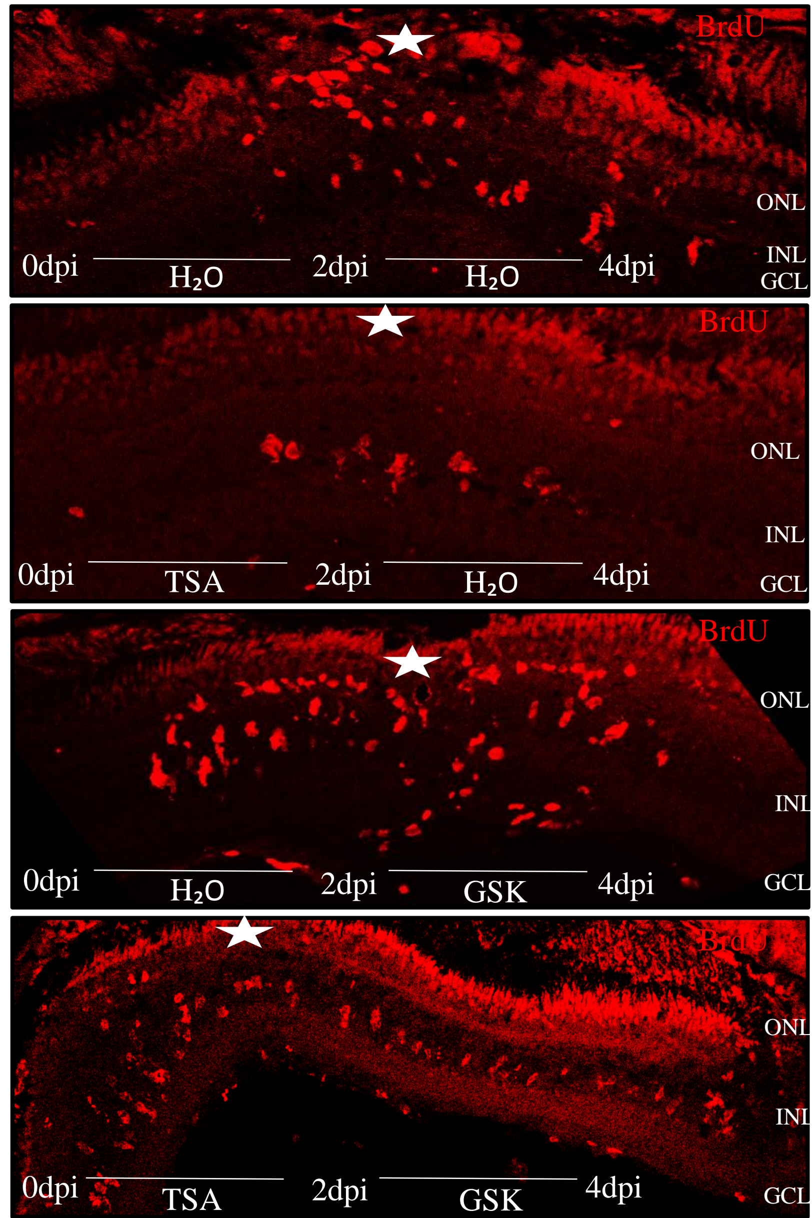


Fig 3.5 Effect of combined blockade of Ezh2 and Hdacs on regeneration associated genes like *ascl1a* and *mmp9* and on *ezh2*. ISH microscopy showed the regulation of various genes like *ascl1a*, *mmp9* and *ezh2* upon combined blockade of Ezh2 and Hdacs function, in 4dpi retina.

a



b

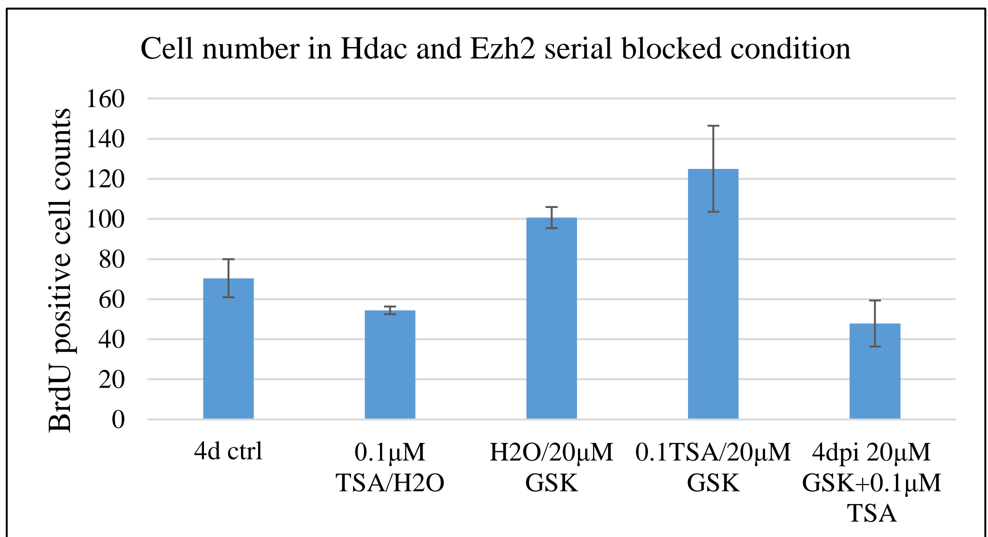


Fig 3.6 Effect of serially blocking Ezh2 and Hdacs in various phases of regeneration. (a, b) IF microscopy showed that upon withdrawal of TSA (given in 0-2dpi) at 2dpi and administering GSK in 2-4dpi, the cell number got increased compared to the cell number in the Hdacs blockade from 0-2dpi **(a)**, which was quantified in **(b).**

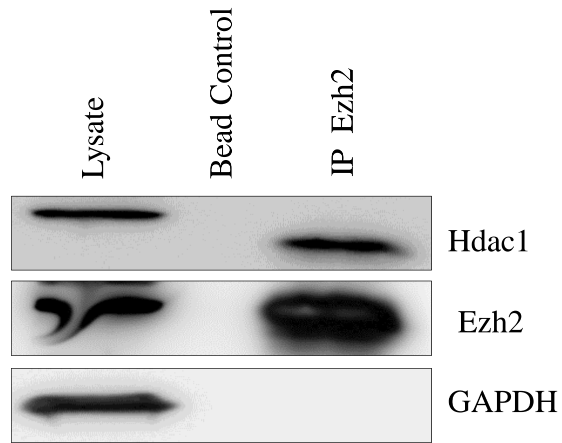


Fig 3.7 Physical interaction between Ezh2 and Hdac1 (a) Co-immunoprecipitation assay (Co-IP) using Ezh2 antibody and 4dpi retinal lysate revealed Ezh2-Hdac1 collaboration during retina regeneration.