STUDY OF FACTORS INVOLVED IN CHROMATIN REMODELING DURING ZEBRAFISH RETINA REGENERATION

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MS16076

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Certificate of Examination

This is to certify that the dissertation titled "STUDY OF FACTORS INVOLVED IN CHROMATIN REMODELING DURING ZEBRAFISH RETINA REGENERATION" submitted by Mr NIKHIL C (Reg. No. MS16076) 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.

Dr Sharvan Sehrawat

Dr Shashi Bhushan Pandit

Dr Rajesh Ramachandran

Dated: May 17, 2021

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 bonafide record of original work done by me, and all sources listed within have been detailed in the bibliography.

Nikhil C

Dated: May 17, 2021

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)

Acknowledgement

I want to thank Dr Rajesh Ramachandran, my supervisor/guide, without whom this work would not have been possible. Not only did he guide me through this research, but he is also an excellent life coach who always has proper advice in all aspects of life. I am also highly thankful to Bindia Chawla (di!) for taking the time from her usual research (and marriage) to get me accustomed to the lab and always be there to troubleshoot all my errors. I am incredibly thankful to her for structuring this work. The other lab members, Dr Poonam (di), Dr Shivangi (di), Anwar (Bhaiya), Mansi (di), Ashish (Bhaiya), Sharanya (di), Pooja (di), Kshitiz, Pallavi and Omkar, for making my time at the lab more enjoyable and helping me out through all my troubles.

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Abstract

Chromatin architecture is the structure of the chromatin present inside the nucleus. It is a highly dynamic structure that allows for access to condensed DNA and helps regulate gene expression.

There are two ways in which the structure is altered: Non-histone mediated, and Histone negotiated. The non-histone mediated factors directly interact with the DNA and uncoil by introducing non-invasive breaks to the DNA. The HMG proteins and the Topoisomerases are such factors. HMGs are transcriptional regulators, while the topoisomerases function as transcriptional facilitators and help in DNA damage control. The Histone mediated pathway involves modifying the histone complexes that lead to variable folding of the chromatin. In general, acetylation unpacks and methylation compacts the structure.

Most of the genes that help in development are seen to be highly expressed during proliferation. Drug-induced inhibition of demethylation of histones showed a decrease in proliferating cells. This indicates that changes to the chromatin architecture are imminent for modifications to the cell.

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Notations

RBC – Red Blood Cells

MG – Muller Glia

HPF – Hours Post Fertilization

DPF – Days Post Fertilization

MPI – Minutes Post Injury

HPI – Hours Post Injury

DPI – Days Post Injury

PCNA – Proliferating cell nuclear marker

BrDu – Bromodeoxyuridine

PCR – Polymerase Chain Reaction

RT-PCR – Real-time PCR

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Chapter 1: Introduction

Regeneration (in biological terms) is a highly regulated process of renewal and restoration of cells or tissues. The regeneration method reveals plenty of underlying mechanisms that help in the normal development of the cells/tissues and their adverse effects if expressed otherwise. Regeneration is present in various degrees in all living beings. In humans, that ability is unfortunately restricted to very few cell types. A few examples are the outer epidermal layer (skin), the liver tissue, and Red Blood Cells (RBCs). A few model organisms exist where these regenerative abilities extend beyond these domains, one of which is the zebrafish.

Zebrafish (*Danio rerio*), a teleost fish native to the Southeast Asian region, has been developed into a model organism over the past 50 years. It shares 70% human genes, and almost all human genetic diseases were replicated in the zebrafish. Its fast growth and cheaper maintenance make it an ideal model organism in this study. The transparent embryos make it easy to visualize the developmental stages. The retinal regeneration ability of zebrafish is a quick and observable process. This study has leveraged to understand the effects of the chromosomal architecture altering genes during the process. [1]

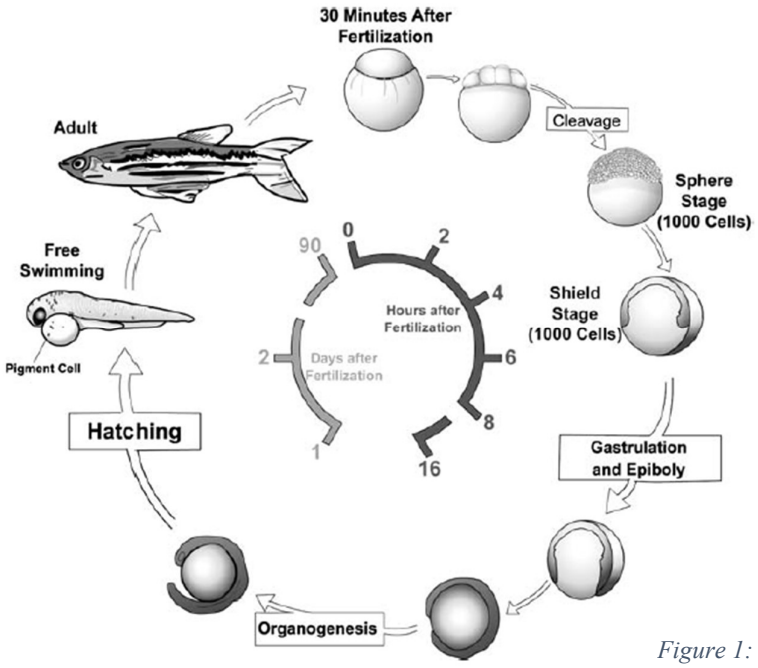


Figure 1: The Life cycle of zebrafish

Zebrafish retinal architecture is very similar to that of the human. The retina is made up of three layers, the outer nuclear layer (ONL), the inner nuclear layer (INL) and the ganglion cell layer (GCL). The ONL is the layer exposed to the light, and it houses the photoreceptor cells. The interneuron cells in the INL transmit the signals from the ONL to the GCL. The GCL has the axon endings, responsible for transmitting signals to the optic nerves that relay them to the brain. Apart from the neuron cells, there are also glial cells present in the retina. A specialized cell type, known as the Muller Glia, spans all the retina layers and is found in all the vertebrates. It attains the regenerative stage only in the zebrafish. Upon mechanical injury spanning all three retina layers, the Muller glia gain stem cell-like characteristic and help regenerate the cells in all the retina layers. Due to genome-wide duplication during speciation of the zebrafish, there are two orthologs for each gene of interest.

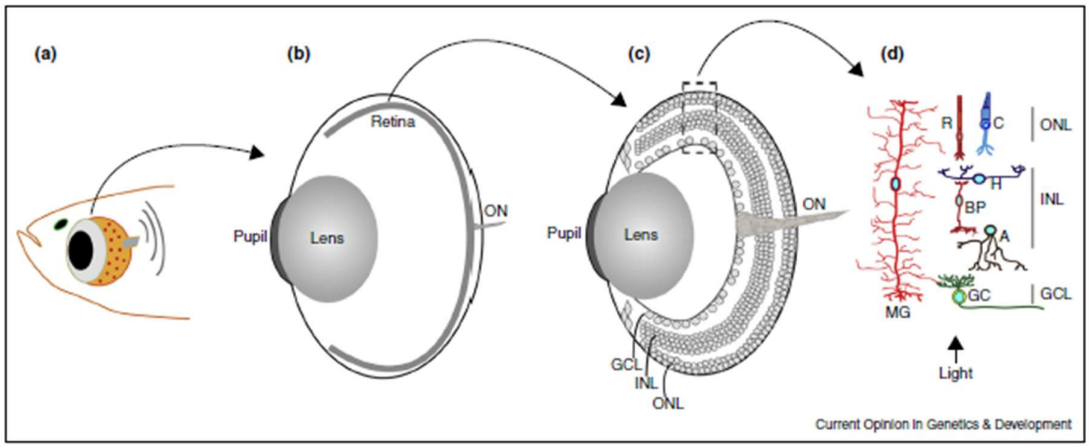


Figure 2: Zebrafish Retina Structure. (a) The location of the eye (b) schematic of cross-sectional view (c) Cell-layers of retina enlarged and focused (d) The different cell types present in the retina[2].

The muller glia temporal variations from de-differentiation to proliferation to re-differentiation have been extensively classified[3]. The 2dpi stage shows the maxima for the number of de-differentiated cells. The 4dpi stage shows the most for the number of proliferative cells, and the 7dpi stage shows the most for the number of differentiated cells. This does not mean that the tissue has wholly regenerated within seven days. It usually takes up to 21 days for the complete process to take place. The time points of 30mpi, 6hpi, 12hpi, 1dpi, 10dpi,14dpi, 18dpi and 21dpi can help in resolving the temporal patterns to a greater extent. For the purpose of this study, proliferation was of utmost importance, so time points until 7dpi were only considered.

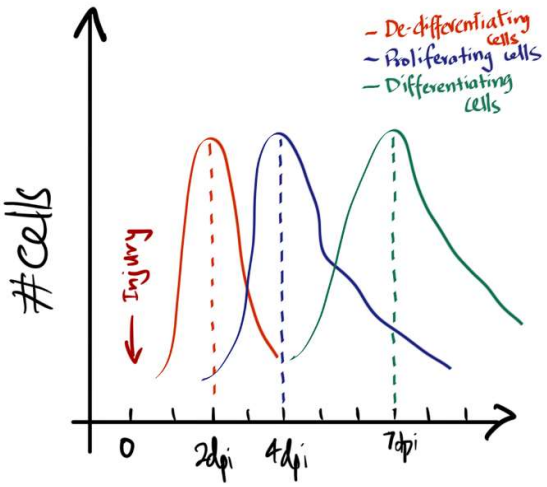


Figure 3: Schematic representation to show the progression of the cells during retina regeneration. At 2dpi, the maximum number of the cells are de-differentiated. At the 4dpi stage, the maximum number of cells are in the proliferative phase, and at the 7dpi stage, the maximum number of cells are undergoing differentiation again. The cells under observation are the Muller glial cells.

Chromosomal architecture is the structure of the chromatin present inside the nucleus of the cell. The structure is dynamic as the genes present in the various folds need to be exposed based on their requirement. This dynamic restructuring can be termed chromatin remodelling. The remodelling is possible through multiple factors regulating the architecture. Histones help with the coiling of the DNA and give the chromatin its structure. Therefore, there are two broad groups of chromatin remodelers, one which alters the structure by interacting with and modifying the histones and directly interacting with the DNA molecules.

There are three major compound groups identified as factors involved in chromatin remodelling: the HMG (High Mobility Group) proteins, Topoisomerases, and the EP300 complex. The HMG proteins and Topoisomerases directly interact with the DNA backbone and alter its structure, while the EP300 Complex is a histone acetyl-transferase. Apart from these genes, other Histone modifying pathways affect the chromosomal structure. These include the histone modifications like H3K4me3, H3K9me3 and H3K27me3. The methylation modification of Histone promotes coiling and therefore enforces the chromatin to be packed more tightly. The tight coiling will suppress the genome expression as the transcription machinery can no longer activate the genes. The H3K4me3 modification is an exception to this belief. Its overexpression leads to the coming together of the enhancer and promoter regions of a gene and leads to increased expression levels. Therefore, depending on the location of the modification, the structure gets altered differently. These modifications are processed through different mechanisms and are therefore easy to study individually.

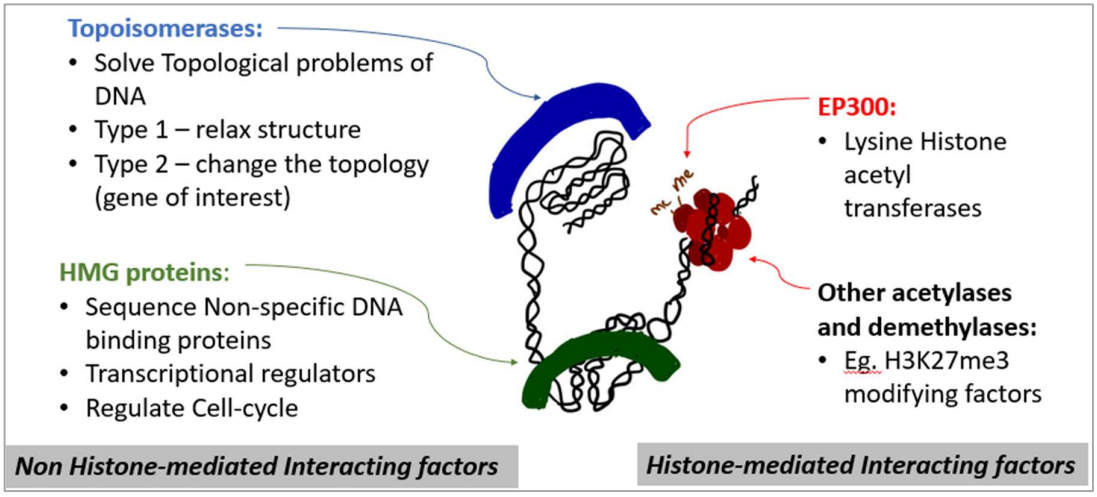


Figure 4: Various factors involved in the reorganization of the chromosomal structure.

HMG Proteins

HMG proteins are a group of proteins that have derived their name from the fact that they are fast-moving proteins on a protein gel. These proteins are small and constitute a wide variety of functions. Their most important functions include the regulation of transcription as well as the cell cycle. They are also known to bind to the DNA in a sequence non-specific manner.

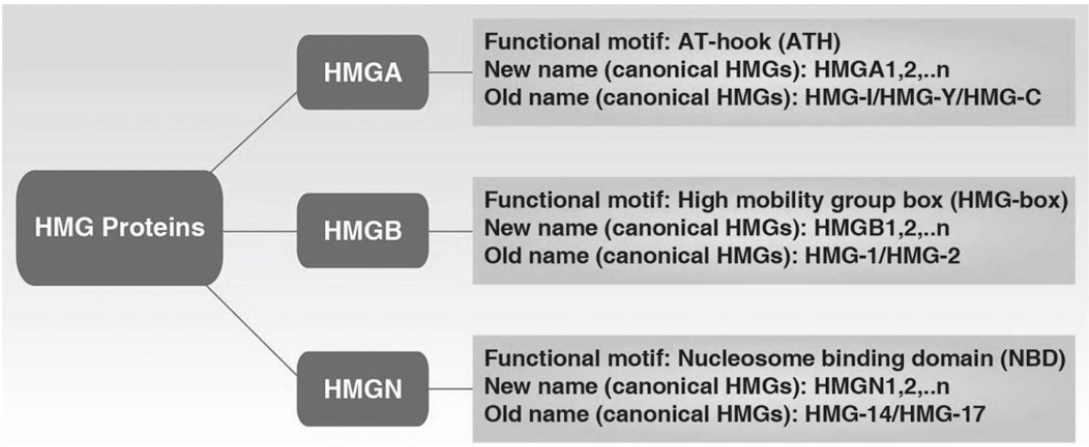


Figure 5: Three types of HMG proteins: HMGA, HMGB and HMGN (based on the new terminology standardized after the 2000s).[4]

The HMGA proteins bind to the AT-hook region of DNA and majorly help in the transcriptional regulation processes. The AT-hook binding capability of the HMGA is also

known to affect the structure of the chromatin. The HMGA proteins[5] are known to be present in abundant quantities during embryogenesis and downregulated upon differentiation. In zebrafish, the *hmga1a* gene is known to be regulated during neurogenesis. The AT-hook binding domains of this protein are similarly spaced to that of the HMGB genes and is therefore expected to affect the architecture.

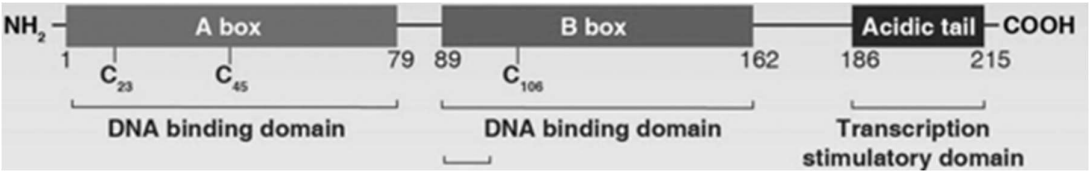


Figure 6: Structure of HMGB proteins.[4]

The HMGB genes form the central group of DNA interacting proteins. Their unique protein structure aids a better binding ability to the DNA, albeit a non-specific binding. The linker histones H4 and H5, along with the HMGB protein, interact with the DNA. The A-box and B-box recognize the DNA structure, and the acidic C-terminal end can regulate the transcriptional process after that. The DNA binding domains of the HMGB protein are not sequence-specific but are regulated by the linker histone molecules. The C-terminal acidic tail is sufficient to induce transcriptional changes. This protein has three different paralogs present in the zebrafish, namely *hmgb1, hmgb2* and *hmgb3*. [6]

During zebrafish embryogenesis, the mRNA expression levels at various time-points post-fertilization revealed that these different forms are varyingly regulated. The *hmgb3b* is wholly lost upon the embryo formation, while the others show varying patterns through the process. Their tissue-wide expression showed that these are majorly expressed in the Central nervous system and the brain of the zebrafish embryo. Therefore, it is a target gene to study for the chromosomal structural changes during retina regeneration.

HMGB1 plays a critical role in neuroregenration. The studies on spinal cord regeneration[7] of the zebrafish have shown that *hmgb1b* get expressed in large amounts in a short time, then their intracellular concentration decreases. The extracellular levels increase, though, and help promote angiogenesis to promote more regeneration at the injured locations. In general, HMGB1 has other significant functions, and it depends on its site in the cell. The intracellular HMGB1 functions as a DNA binding protein and subsequently affects the DNA folding. The extracellular version works as a stress signal and is categorized as DAMPs (Damage associated Molecular Proteins).[4]

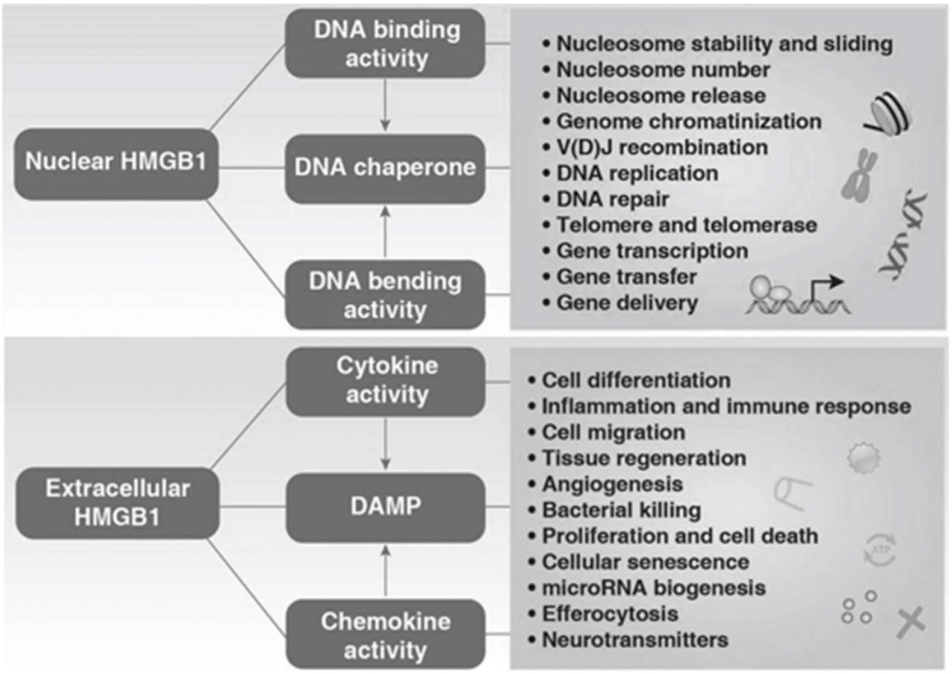


Figure 7: Various functions of HMGB1 protein.[4]

Various deletion experiments showed that the mutation in these genes is almost always detrimental towards embryogenesis, indicating that this gene is necessary for growth and development. Therefore, it can also play an essential role during regeneration.

Topoisomerases

Topoisomerases are intranuclear enzymes that are vital to solving the topological problems of the DNA. Initially, topoisomerases were considered to be histone substitutes that aid in the chromosomal restructuring in bacteria. Still, they also play a significant role in developing eukaryotic organisms. The human genome codes for six types of topoisomerases, namely the TOP1, TOP1mt, TOP2α, TOP2β, TOP3α and TOP3β. They selectively act on DNA and RNA in both nuclei and mitochondria. They repair the DNA structure by forming topoisomerase catalytic complexes (TOPcc), which are the catalytic intermediates. It introduces transient reversible DNA breaks through a transesterification reaction. These breaks their binding to DNA facilitates structural changes. They are ideal therapeutic candidates for anti-cancer drugs as they can promote the repair of damaged DNA structures.[8]

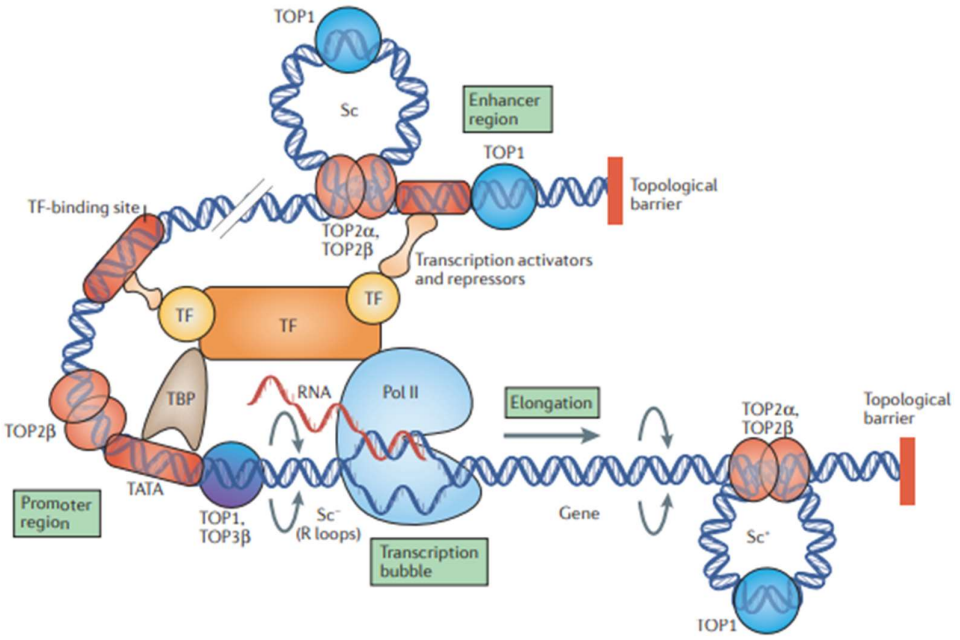


Figure 8: TOP2a and TOP2b actively remove the positive supercoiling hindering the progress of the RNA Pol II, aiding in better transcription. TOP1 moves behind the complex and relaxes the negative supercoils the DNA encounters after the progression of the transcription factors.[8]

Topoisomerase 1 is usually involved in the relaxation of the DNA structure, whereas type 2 are the transcriptional regulators. There are two isoforms of type 2, α and β. Both are 65% identical, only differing in their C-terminal region that contains the localization signals and the phosphorylation sites. *top2α* is mainly found in proliferating cells and helps with the mitotic division of the cell. *top2β* is activated in the post-mitotic cells, where it helps in the expression of long genes that help cell differentiation.

In zebrafish, *top2α* is found to be a maternal transcript[9]. It helps in rapid cell division during embryogenesis. Mutation to this maternal transcript or blockage of the transfer led to adverse side-effects and always led to deformed embryos that couldn't survive beyond three days post-fertilization. The retinal architecture, in particular, was deformed and the various layers were not properly organized. The same study also found that the *top2α* and *top2β* are not functionally redundant. RT-PCR analysis of their expression levels in the embryonic stages revealed that the *top2β* is not expressed until 10 hours post-fertilization. Meanwhile, maternal *top2α* fulfils the necessities.

Therefore, *top2α* and *top2β* are essential to understand the chromosomal structure during regeneration. It is expected that the *top2α* be highly expressed and aid during regeneration,

while the *top2β* helps differentiate the cells into the various subtypes. The C-terminal localization signals can be further studied to check for the location of the genes that are getting activated preferentially during proliferation or in differentiation.

EP300

EP300 is a member of the EP300/CBP family of lysine acetyltransferases (KATs). It plays a vital role in the development and maintenance of cells. Its intrinsic acetyltransferase-catalytic activity as well as its transcriptional coactivator associates its function in a diverse range of physiological processes.[10]

The zebrafish genome has two co-orthologs, *ep300a* and *ep300b*. Interestingly, the KAT domain of the human and zebrafish gene share a slightly higher similarity as compared to the two co-orthologs from zebrafish. The RNA-seq data of zebrafish embryos revealed that the *ep300b* is highly expressed in the brain, more than the other one. Also, the analysis over different time steps during embryogenesis, *ep300b* is always more than *ep300a*. Chemical inhibition of *ep300* in zebrafish leads to malformed embryos, which showed a high mortality rate. It was found that the chemical inhibition leads to increased levels of apoptosis, and subsequent loss of tissue structure, leading to death. Meanwhile, reintroduction of the KAT domain leads to almost complete recovery in the affected embryos. Further, the usage of de-acetylase inhibitors showed a similar result, though the phenotype rescue rate was far less. Thus, *ep300* might play a crucial role in the proliferation of the cells during retina regeneration.

The *ep300* is also known to be the protector of the retina from light-induced retinopathy. It is known to be an upstream regulator of the STAT1 and STAT3 pathways. Upon exposure to bright light, the increase in putative Muller cells has been linked to the EP300. Therefore, it can be related to the treatment of neurodegenerative diseases. It is expected that the EP300 levels improve upon injury. Thereby meaning that EP300 aids in the proliferation of the cells.[11]

Histone Methylation markers

Since the unpacking of DNA by the histone acetylases promotes the proliferation of the cell, the methylation markers become a source of study to figure out the possible locations of unpacking of the DNA. There are many different types of methylation markers present

in the cell, and each has its significance. In general, the methylated sites are considered to be repressed, but there are few exceptions to such cases.

The H3K4me3 is a transcriptional activator as it gets together the enhancer and promoter regions of the gene through folding.[12] The H3K9me3 marker is a heterochromatin marker, and its presence denotes the heterochromatic area in the DNA.[13] The H3K27me3 is considered to be the hallmark of transcriptional repression. Reduction in levels of H3K27me3 is deemed to be a prognostic marker in lung cancer.[14] Due to its ubiquitous nature of repressing the gene expression, it is termed as polycomb repression factor. Thus the complex that promotes the H3K27me3 is termed as Polycomb repression complex (PRC).[14,15]

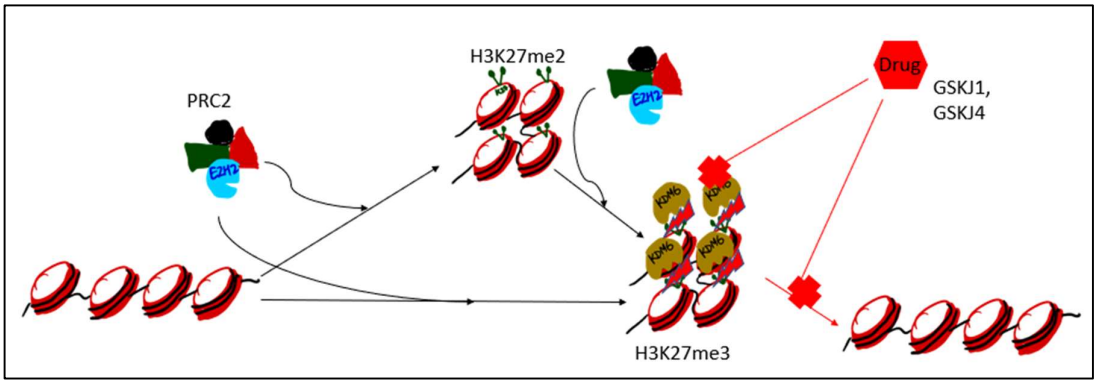


Figure 9: Methylation through PRC2 interaction promotes packing. The demethylase family KDM6 acts on H3K27me3 to unpack the complex. Drugs such as GSKJ1 and GSKJ4 inhibit such demethylases and disallow unpacking.

The inhibition of demethylases leads to more packing of the DNA and, therefore, will result in the reduction of gene expression. This can directly link to a decrease in various genes and thus might lead to a reduction in proliferative or differentiative pathways and hinder the process of retina regeneration.

GSKJ1 and GSKJ4 are drugs that inhibit the demethylases. GSKJ1 interacts explicitly only with the KDM6 family of demethylases, whereas the GSKJ4 is a broad-spectrum inhibitor.[16] These drugs are commercially available and can quickly enter through membranes and hence can be used for chemical inhibition assays.

Chapter 2: Experimental Procedures and Protocols

Maintenance of Zebrafish

Zebrafish tanks:

The integral part of the lab was the maintenance of the zebrafish in a laboratory setup. The lab had an in-house aquarium with tanks to store the fish, the racks to arrange them, and a constantly monitored water flow with multistep micron filters (50 and 100), UV decontamination, PH monitor and biofilms. The setup was maintained at a constant 22^0C. The water was kept in a continuous flow through the individual fish tanks to remove any waste and maintain the oxygen levels. Every day, the main tank used to reuse 40% of the water in the system, while the remaining 60% was freshwater sourced from the building's water supply. There is a periodicity in lighting the aquarium part of the lab. The fish undergo 14-hour light and 10-hour dark periods.

Zebrafish Food:

The zebrafish adult was fed twice a day with commercially available fish feed pellets (highly enriched with essential nutrients). Zebrafish embryos and juvenile fish are fed with crushed food dissolved in water once a day.

Zebrafish Breeding, egg isolation and screening:

The zebrafish, female and male, are selected and grown in separate tanks until the female show enlargement in their abdomen (shows the buildup of eggs). There are individual breeding tanks with perforations big enough to allow for the egg to pass through. They are placed in tanks containing water such that the region above the perforated base creates a shallow body of water. A divider is used to split the tank into 2 part. In one part, three females will be added, and in the other two males. This setup is then kept in the dark overnight.

The following day, the separator is removed, and the setup is placed next to a bright light source to mimic sunlight. The setup can be left for the rest of the day, and the fish can be

separated in the night and then fed. Meanwhile, the eggs are collected in the base of the tank.

The eggs are small and usually concentrated in the side away from light exposure. They can be picked up by a wide-mouthed dropper and transferred to a different tank to be kept in the incubator. Eggs that are translucent or those that seem to contain some contaminants are dead eggs, and they can be selectively discarded. The remaining eggs can be incubated at 18^0C and kept in a bright light source with similar light and dark cycles as those of adults. The age of the embryo is counted from the time they are kept in the incubator.

Embryo screening can be done for certain genetically modified lines of zebrafish (TUBA Lines). At the 24hpf stage, the neurons start expressing intrinsic GFP can be visualized using a fluorescence microscope. The fish are then differentiated into different tanks based on their lines.

Retinal Injuries and isolation

Injuries and isolation of zebrafish retina are done under a binocular dissection scope.

The following need to be kept ready before the process:

1. Tricaine solution (anaesthetic)
   The anaesthetic solution used for the zebrafish is known as Tricaine (usually stored at 4^0C). In 40 ml of water from the system, 8 to 10 drops of Tricaine are dissolved. This solution can be reused multiple times within the same day; if not contaminated, else needs to be remade before every injury.
2. Fish
   Appropriate fish need to be separated into a tank for a quick and easy process.
3. Injury/Dissection apparatus.
   Forceps, Dissection scope, 30-gauge medical-grade needle, small sponge, cold-plate to perform dissection, and dissection solution based on the kind of the dissection. (DEPC water for RNA isolation and PFA for tissue removal for cryosectioning.)

The desk and the apparatus need to be sanitized with 80% ethanol solution before starting with the following procedures.

Injury:

1. Transfer 1 fish into the tricaine solution. Wait for the fish to stop wiggling. NOTE: Overexposure in tricaine solution might kill the fish.
2. Transfer fish onto a wet sponge.
3. Using a 30-gauge needle, poke the underside of the eye such that the needle goes through all three retinal layers. A short finger twist to the needle will make sure that the injury is substantial.
4. For RNA isolation, at least six pokes per eye need to be done to ensure a good cDNA yield for further studies. For tissue fixation, four well-spaced jabs per eye need to be done.
5. The fish needs to be immediately revived and kept for observation for at least 20 minutes to make sure the fish didn't die from the injury or overexposure to Tricaine.

The epithelial cells surrounding the retina undergo a dark pigmentation upon exposure to light. Dark-adaptation of the fish before tissue removal will help in the compactness of the dark epithelial layer and easily removed from the retina. A 4-hour dark adaptation is suggested before the tissue removal.

Tissue-removal for RNA isolation

1. The fish is anaesthetized using tricaine solution, and the eye plucked out of the socket and transferred onto DEPC water on a cold plate.
2. Using forceps, remove the cornea around the eye.
3. The lens is gently removed from the eye to avoid damage to the retina.
4. The silver epithelial layer around the retina is gently removed.
5. The resulting transparent retina is gently transferred into 200µl trizol solution and stored at -80^0C.

Tissue-removal for Cryofixation:

1. The fish is anaesthetized using tricaine solution, and the eye plucked out of the socket and transferred onto 4% PFA (paraformaldehyde solution; CAUTION-carcinogenic).

2. The cornea and optic nerve remove from the outside. The lens is nudged out of the socket.
3. The eye is then transferred into 200µl 4% PFA solution and stored at 4^0C.

RNA isolation, cDNA preparation and PCRs

RNA Isolation:

1. Resuspend tissue in 200µl trizol
2. Lyze/homogenize the tissue using a homogenizer or pipette. Keep in room temperature for 5 minutes.
3. Add 40µl of Chloroform. Invert to mix gently. [DO NOT SHAKE]
4. Centrifuge @ 12,000 RCF for 10 minutes @ 4^0C.
5. Collect the upper interphase [~40µl] into a new MCT and the remaining into a different MCT. Discard the lower interphase.
6. Add 40µl Isopropanol and mix well. Keep in -80^0C overnight for better precipitation.
7. Centrifuge @ 13,500 RPM for 40 minutes at 4^0C.
8. Discard the supernatant and wash the pellet with 80% ethanol (diluted in DEPC water). Centrifuge @13,500 RPM for 10 minutes @4^0C. Repeat the ethanol ash step once or twice more.
9. Dry till ethanol smell disappears.
10. Dissolve in 12µl DEPC water and store at -80^0C.

The RNA samples need to be checked before continuing cDNA preparation. The quality of the solution can be checked on a nanodrop scale. Values between 300-500 µg/ml indicate a good yield of RNA. Further, The samples can be run on 1% agarose gel and smear under UV illumination suggests the presence of RNA. The brighter the smear, the better is the yield.

cDNA Preparation:

1. Prepare the mixture for denaturation based on the following composition:

Table 1: Mixture for denaturation in cDNA prep

	10µl	20µl
Template RNA	5µl (max.)	10µl (max.)
Random primer	0.5 µl	1 µl
Oligo DT primer	0.5 µl	1 µl
NFW		
Total	6 µl	12 µl

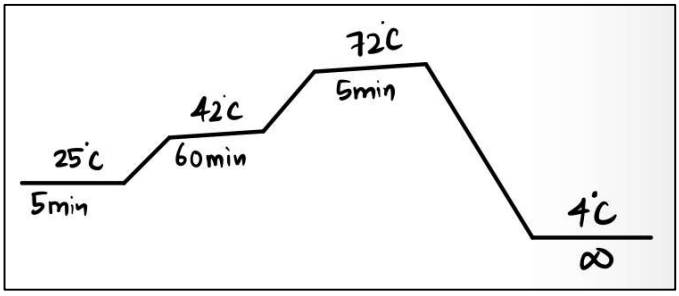
Put for denaturation at 65^0C for 20 minutes, immediately transfer into an ice bucket.

2. Mix the other contents before putting them for PCR.

Table 2: cDNA Reaction mix

	10 µl	20 µl
5X reaction Buffer	2 µl	4 µl
RNAse inhibitor	0.5 µl	1 µl
dNTPs	1 µl	2 µl
RT enzyme	0.5 µl	1 µl
Total	4 µl (+ 6 µl)	8 µl (+ 12µl)

3. Follow the following cycle for PCR reaction.



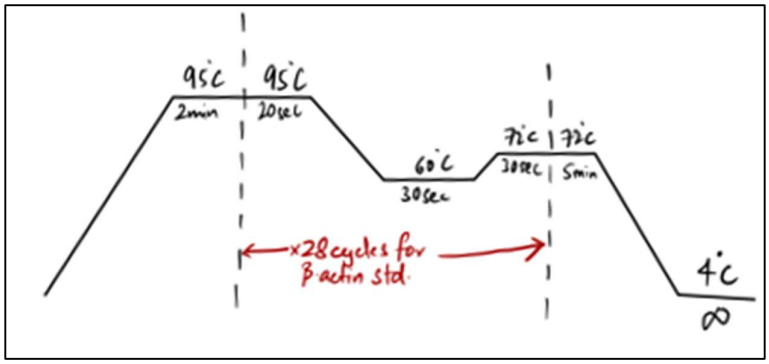
PCR:

1. Add the contents to the mixture in the following ratios:

Table 3: Components of PCR

Template	(0.5 µl)
20X buffer	0.5 µl
2.5 mM dNTPs	1 µl
10mM primers	0.5 µl
Taq Enzyme	0.1 µl
Water	(7.4 µl)
Total	10 µl

2. Run PCR based on the following cycle.



RT-PCR:

The method for RT-PCR is similar to that of the PCR, except that the SYBR mix used in the case of RT-PCR is a master concoction of all the required solutions in PCR. Then the reaction mix for RT-PCR will become as follows:

Table 4: RT-PCR Reaction mix

SYBR Mix	2.5 µl
Primers	0.2 µl
Template	1 µl
Water	1.3 µl
Total	5 µl

The samples are added in 96-well plates and analyzed using the Thermo scientific qPCR machine.

PCR primer design:

1. Identify target gene and acquire sequence from Ensembl genome browser 102.
2. An exonic sequence including a slight overlap area over the 3' UTR region is considered and the sequence copied onto Primer3 web app.
3. Adjust the parameters based on the target fragment required. For PCR, the product length should be a maximum of 200bp. Other aspects of GC content and GC clamp can also be set.
4. Once satisfied with the primer design, place the order for acquiring the primers.

Cryofixation and Immunostaining

Tissue Cryofixation:

1. Keep the isolated eyes in 4% PFA solution overnight @ 4^0C.
2. The following wash steps are done sequentially at room temperature. The MCTs containing the samples are kept on a rotor at 13RPM for 45 minutes per wash. Before each wash, the solution is discarded carefully to not distort the eye samples.
    a. 1000µl of 5% sucrose solution.
    b. 800µl 5% sucrose solution + 400µl 20% sucrose solution.
    c. 600µl 5% sucrose solution + 600µl 20% sucrose solution.
    d. 400µl 5% sucrose solution + 800µl 20% sucrose solution.
    e. 1000µl 20% sucrose solution.
3. 500µl solution removed and 500µl of OCT solution added.
4. A block is made of aluminium foil and filled with OCT. The eyes from the wash are aligned in a single file, such that the dark hemisphere of the eye is pointing upwards.
5. The block is then stored at -80^0C.

Section Preparation:

The sections are made on the cryofixation machine. The temperature inside maintained at a constant -17^{0}C inside. The sections are taken on a charged microscope plate. The sectioning is usually done at 10micron size, but can be adjusted based on requirement.

Immunostaining:

Day 1:

1. Dry slides for 30 minutes in an incubator @ 37^{0}C.
2. Place the slides in a slide box and do three 10 minute PBS washes. Make sure the solution covers the entire slide. After each wash, discard the solution.
3. Meanwhile, pre-warm a solution of 2N HCl [42.5 ml water and 7.5 ml conc. HCl] @ 37^{0}C for 30 minutes.
4. Dip the slides in the 2N HCl and incubate @ 37^{0}C. {Epitope retrieval}
5. EXACTLY 20 minutes later, remove the slides and immediately proceed for borate washes. Place the slides in the slide box and three 10 minute washes with 0.1M borate solution. Make sure the solution covers the entire slide. Discard the solution after every wash.
6. Balance the box at room temperature. Block with 5% BSA solution for a minimum of 1 hour. For best results, leave undisturbed for 3 hours.
7. Transfer the slide box into a 4^{0}C refrigerator and balance the box. Add 500µl of 1:500 diluted primary antibody to each slide and leave it undisturbed overnight [10-12 hours].

Day 2:

1. Collect the primary antibodies and store them in 4^{0}C. Transfer the box to room temperature and do three washes with PBST solution [0.1% Triton-X-100 in 1X PBS] for 10 minutes each.
2. From now onwards, the slides should be handled in comparatively darker environments to avoid photobleaching of secondary antibodies.
3. Balance the box at room temperature and add 500µl of secondary antibody solution. Leave it undisturbed for 3 hours.

4. Collect the antibodies and wash them three times with PBST solution for 10 minutes each.
5. Dry the slide a bit and check for signals under a fluorescence microscope.
6. Dry the slides for 10 to 15 minutes (max) and mount a coverslip onto 40µl DABCO solution.
7. Dry the slides for 24 hours at room temperature, and then store them at -20^{0}C.

The slides are visualized and photographed using the Nikon fluorescence microscope.

Drug treatment

The drug treatment on the fish was done externally, and no drug was injected into the fish. The fish were individually placed in a beaker containing 20ml of water. In the control beaker, no drug is mixed with the water. For different concentrations, the following were the volumes of the drugs mixed with the water.

Table 5: Drug compositions for exposure

GSKJ1	5 µM	7.80 µl in 20 ml
	10 µM	15.58 µl in 20 ml
	15 µM	23.36 µl in 20 ml
GSKJ4	4 µM	6.68 µl in 20 ml
	7 µM	11.70 µl in 20 ml

The drugs are light sensitive, so the beakers were covered in aluminium foil and placed in a dark cabinet. Once in 24 hours, the solutions need to be changed. During the transfer, the fish are isolated to separate beaker and fed. All this while, there should not be mislabeling of the fish.

Chapter 3: Results, Summary and Conclusions

Year-long experiments have culminated in many failures and a few successes revealing some interesting facts about the structural changes during regeneration.

The first aspect of the study was to identify and design primers for the genes of interest. They were ordered from GCC biotech. The following is the list of the genes of interest:

Table 6: Primers used and their sequences

Gene Name	Forward Primer	Reverse Primer
top2α	ACGATGACAATTTGCGAGTG	GATCTCGATGGTGGTGGAGT
top2β	Primer already designed and present	
hmgb1b	CTGTCGTGAGGAGCACAAAA	GCGTTGGGATCCTTAAACCT
hmgb2a	Primers already designed and present	
hmgb3a	CTTACGAAAATGGCGAAAGG	GGCATTGGGATCCTTCTTTT
ep300a	Primers already designed and present	
ep300b	Primers already designed and present	

The PCR reactions were done to test for temporal patterns of genes of interest during the zebrafish retina regeneration. There were multiple problems encountered during the standardization of PCR. Few results were achievable through this; the remaining have been tested using the RT-PCR (Real-time PCR). The RNA samples were extracted successfully, and the cDNA was prepared from that to test for the temporal patterns.

HMGB:

All three genes show higher levels of expression during the proliferation maximum stage of 4dpi, showing that these genes are essential to unpack the proliferation genes. h*mgb1b* and *hmgb2a* show similar patterns of increase in expression as proliferation increases and sharp decrease as proliferation is ending. The rise in levels *hmgb2a* is gradual, whereas the *hmgb1b* dramatically increases during the proliferation. *hmgb3a* shows a different pattern. Its levels are similar during de-differentiation and proliferative stages, meaning that this gene might be involved during the de-differentiation process. Comparing the levels at the 7dpi stage, the *hmgb1b* and *hmgb3a* show lesser than usual expression levels. This might

mean that these genes are detrimental to the differentiation process and are suppressed. Since the HMGB proteins are known to have extracellular functions, it might mean that these genes are quickly translated and transported to the ECM and might help with the decision of the final cell type of the cell.

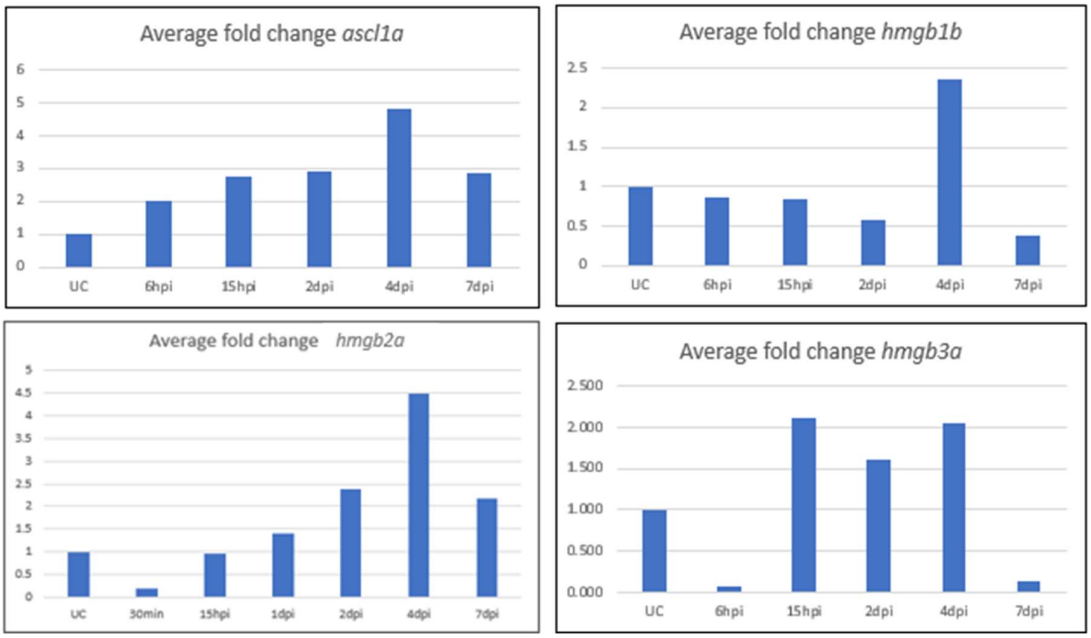


Figure 10: RT-PCR results of hmgb genes

HMGA:

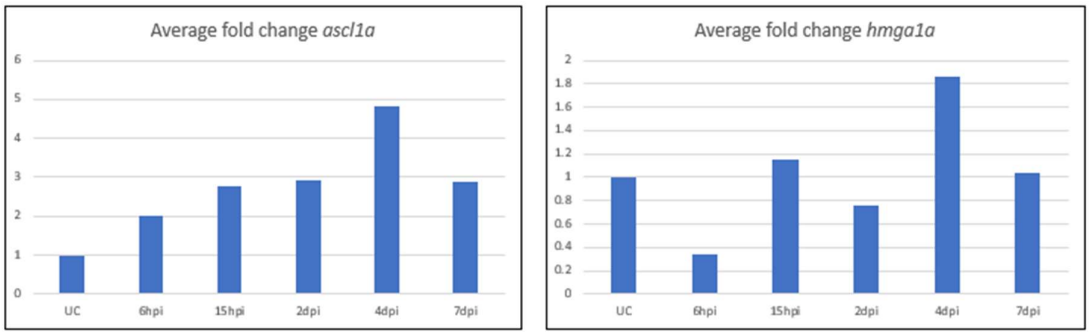


Figure 11: RT-PCR result of hmga gene

The *hmga1a* also shows an increase in the proliferation of the cells. The sudden decrease in the levels soon after injury might be due to response times when the tissue adjusts to the stress. The levels increase at 15hpi, denoting this might initiate the process of de-differentiation, while it is not required during the process (seen as a decrease in expression during 2dpi). The levels are again highly expressed during the proliferation maxima,

showing that it is required for the proliferation to occur. The levels come back to normal soon after this phase passes.

Topoisomerase 2:

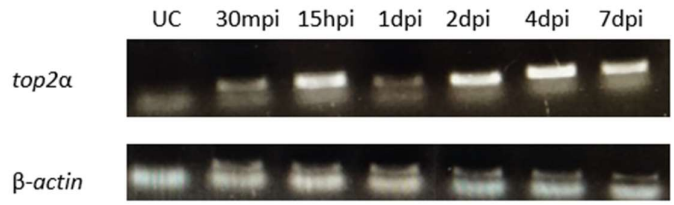


Figure 12: top2α PCR assay

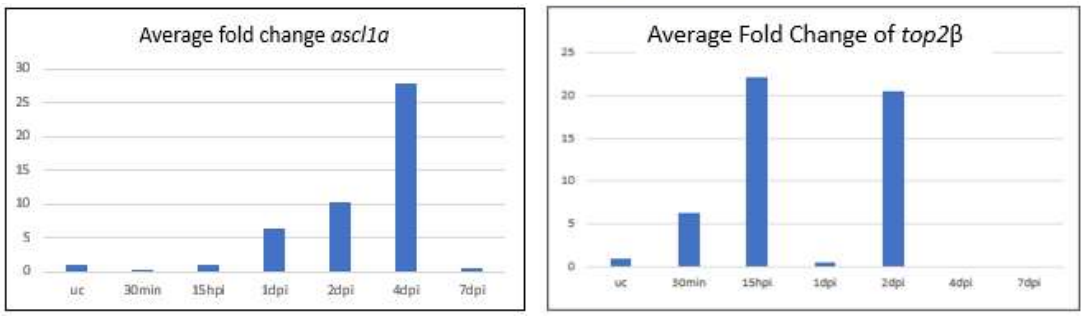


Figure 13: top2β RT PCR result.

RT-PCR results for the *top2a* gene ran into multiple calibration errors, probably arising from the primers designed. The PCR result shows the expression differences.

Both the *top2a* and *top2b* show a sudden increase in levels as a response to the stress of the injury. It might activate the various repair mechanisms in the tissue. The levels decrease drastically at 1dpi, possibly indicating a regulatory mechanism for the cells to initiate the de-differentiation process. The *top2a* levels slowly increase after that and reach a maximum at the 4dpi stage, suggesting that it is necessary for the proliferation of the cells. Meanwhile, the *top2b* levels reach a max at the 2dpi stage and are almost not present during the proliferative and differentiation stages. This shows that *top2b* is necessary for the de-differentiation of the cells and not helpful during proliferative stages.

EP300:

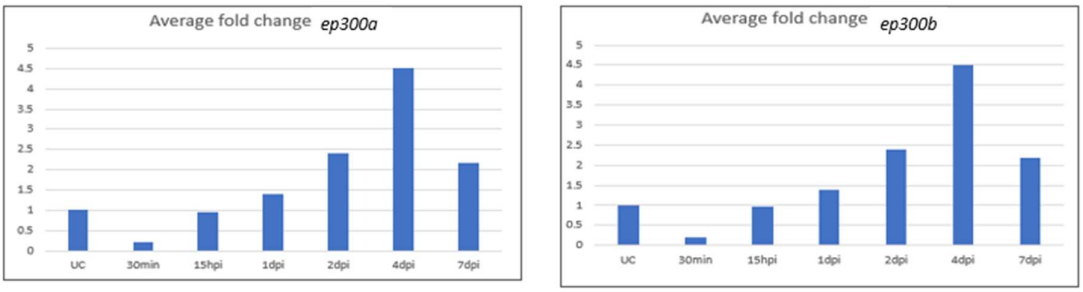


Figure 14:RT PCR analysis of ep300 genes.

ep300a and *ep300b* show a gradual increase and reach a maximum expression value at the 4dpi stage. This indicates that the *ep300* mediated histone acetylation is helping with the unpacking of the chromatin, thereby helping in the expression of the proliferative genes. As the proliferation decreases, the *ep300* levels also decrease. This might show that Histone mediated unpacking of the chromatin is necessary for the proliferation to occur in the cells.

To validate these hypotheses, further experiments need to be done. The tissue wide *in situ* hybridization can reveal the spatial expression differences. Western blot analysis and immunostaining analysis would help show the position and the protein levels that affect the cellular functions. Further, morpholino based knock-out experiments can be conducted to test for the necessity of the genes during the regeneration process.

H3K27me3:

The immunostaining analysis of the 4dpi sample shows the exclusion of the H3K27me3 from the BrdU positive cells (proliferative cells). This indicates that the methylation is lost in the proliferative cells, meaning the chromatin is unpacked to allow for the expression of proliferation promoter genes. But H3K27me3 is a hallmark of transcription repression and

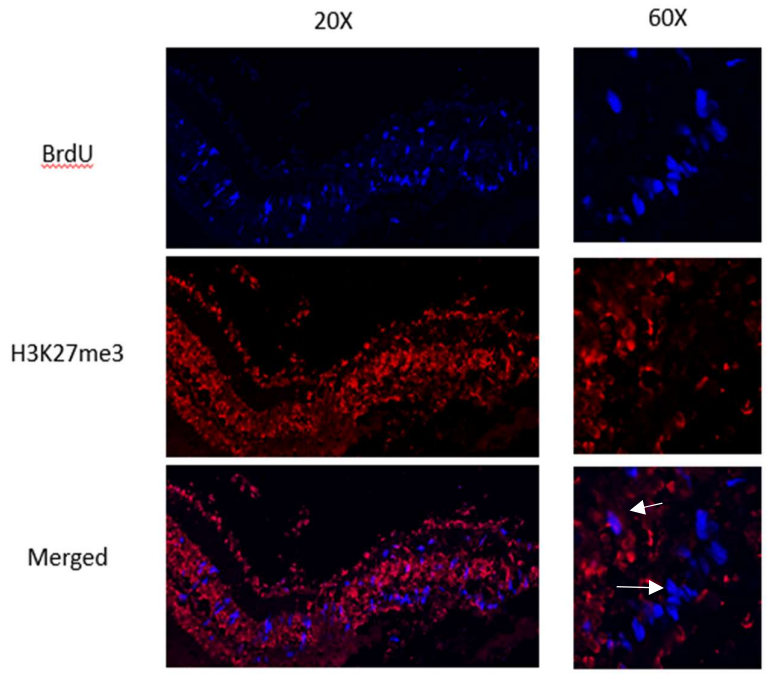


Figure 15: Immunostaining analysis at 4dpi.

cannot be completely absent in the cells. Therefore, it might indicate that the loss of H3K27me3 occurs at a much faster pace than its creation. This can happen in two ways:

1. The methylation of H3K27me3 is hindered by interfering with the promoter factor.
2. The demethylases are enhanced in their activity to overturn the changes.

The first possibility involved another gene, *ezh2,* that helps in forming the various methylation states of H3K27. The role of *ezh2* in regeneration is ongoing research. The second possibility was tested using the commercially available GSKJ1 and GSKJ4 drugs.

GSKJ4 and GSKJ1:

GSKJ4, being a non-specific drug, showed some detrimental results. At higher concentrations, anything above 4micromolar, the fish couldn't survive as the drug hindered other methylation markers and meddled with the cell's normal functioning. Only one fish could survive the 7 micromolar concentration of the drug at the 4dpi stage. 12 to 15 injury spots over three retinal sections were considered for cell counting. There is a slight increase in the number of cells at 7 micromolar concentration

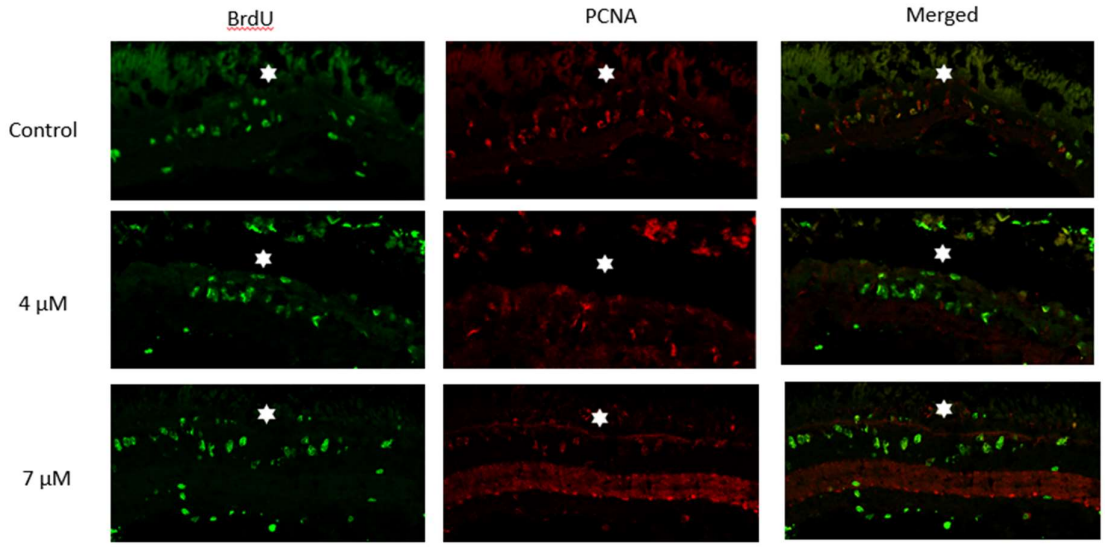


Figure 16: Immunostaining samples of 4dpi eye sample treated with GSKJ4. Stars denote injury spots

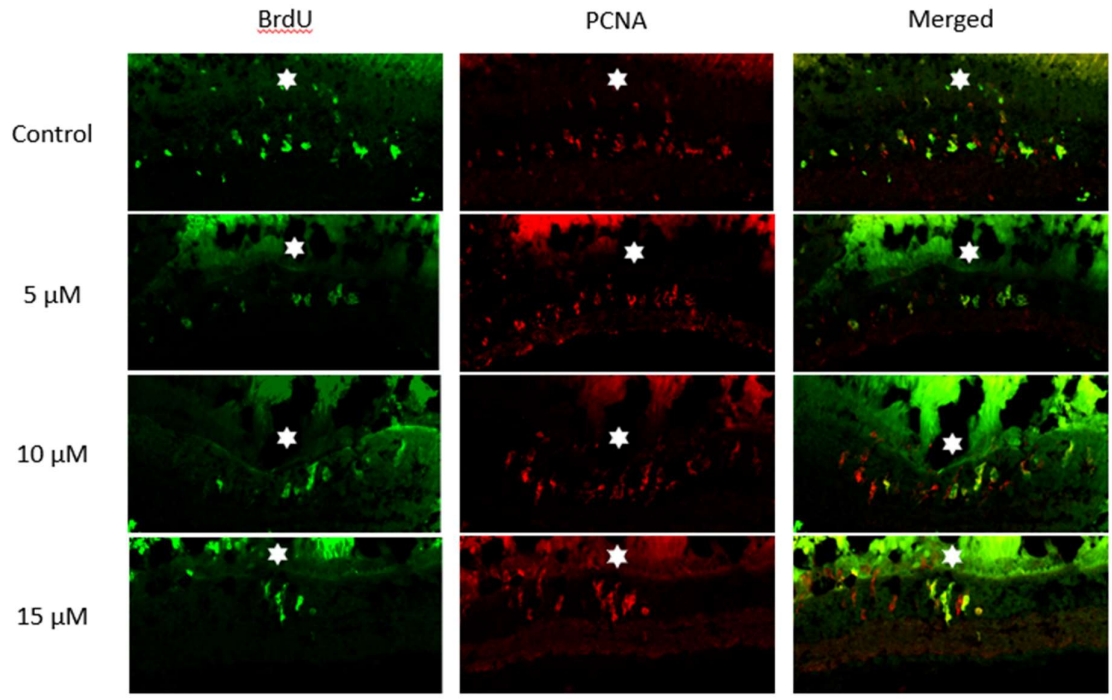


Figure 17: Immunostain Samples of 4dpi treated with GSKJ1. Stars denote injury spots.

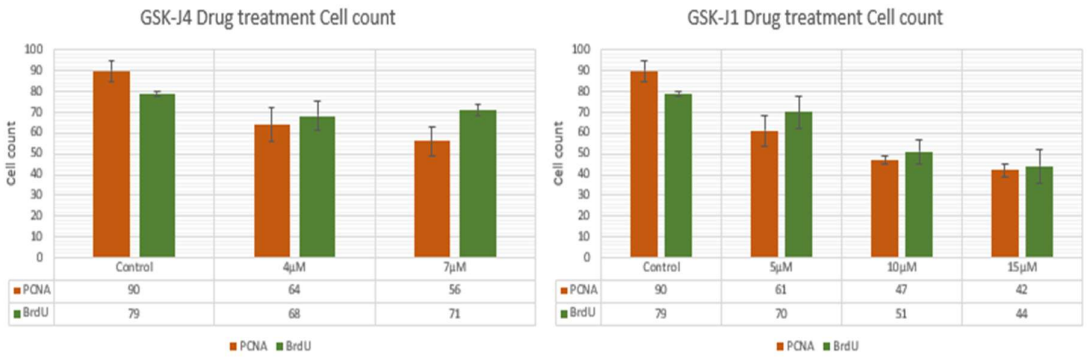


Figure 18: Cell count difference with increase in drug concentration.

GSKJ1, being a more specific drug to demethylation of H3K27me3, is tolerable at higher concentrations. The decrease in cell numbers is very evident from the cell count readings. The cell count at higher concentrations of 10 micromolar and 15 micromolar are not significantly different. This could be due to the ability of the drug to enter the injury spots as well as the quality of the injury. To further validate this result, different individuals need to replicate the conditions to remove the researcher bias.

The inhibition of the demethylation process resulted in more compact chromatin, thereby inactivating the proliferation markers. The expression of proliferative markers is delayed, reducing the number of cells when the proliferation is maximum. But this might only mean that the proliferation is delayed and not wholly hindered. Further to check that the process is delayed, sections from 5dpi, 6dpi, 7dpi, 12dpi, and 21dpi time-points should be isolated, and a cell-counting analysis will reveal whether the proliferation is slowed or has been completely stopped.

Conclusion:

Most genes show a common trend of upregulation during the proliferation stages, denoting that the unpacking of the chromatin is a necessary and essential part of the process. The histone modification is also promoting unpacking and subsequent expression of proliferation promoter genes. The subsequent genes that are activated during the process can be understood through RNAseq analysis and the CHIPseq analysis. The genes identified can then be further studied through knock-down and knock-in experiments

Bibliography

1. D'Costa, A. & Shepherd, I. T. Zebrafish Development and Genetics: Introducing Undergraduates to Developmental Biology and Genetics in a Large Introductory Laboratory Class. *Zebrafish* **6**, 169–177 (2009).
2. Wan, J. & Goldman, D. Retina regeneration in zebrafish. *Curr. Opin. Genet. Dev.* **40**, 41–47 (2016).
3. Goldman, D. Müller glial cell reprogramming and retina regeneration. *Nat. Rev. Neurosci.* **15**, 431–442 (2014).
4. Kang, R. *et al.* HMGB1 in health and disease. *Mol. Aspects Med.* **40**, 1–116 (2014).
5. Vignali, R. & Marracci, S. HMGA Genes and Proteins in Development and Evolution. *Int. J. Mol. Sci.* **21**, 654 (2020).
6. Moleri, S. *et al.* The HMGB protein gene family in zebrafish: Evolution and embryonic expression patterns. *Gene Expr. Patterns* **11**, 3–11 (2011).
7. Fang, P. *et al.* HMGB1 Contributes to Regeneration After Spinal Cord Injury in Adult Zebrafish. *Mol. Neurobiol.* **49**, 472–483 (2014).
8. Pommier, Y., Sun, Y., Huang, S. N. & Nitiss, J. L. Roles of eukaryotic topoisomerases in transcription, replication and genomic stability. *Nat. Rev. Mol. Cell Biol.* **17**, 703–721 (2016).
9. Sapetto-Rebow, B. *et al.* Maternal topoisomerase II alpha, not topoisomerase II beta, enables embryonic development of zebrafish top2a-/- mutants. *BMC Dev. Biol.* **11**, 71 (2011).
10. Babu, A. *et al.* Chemical and genetic rescue of an ep300 knockdown model for Rubinstein Taybi Syndrome in zebrafish. *Biochim. Biophys. Acta BBA - Mol. Basis Dis.* **1864**, 1203–1215 (2018).

11. Kawase, R. *et al.* EP300 Protects from Light-Induced Retinopathy in Zebrafish. *Front. Pharmacol.* **7**, (2016).
12. Liu, X. *et al.* Distinct features of H3K4me3 and H3K27me3 chromatin domains in pre-implantation embryos. *Nature* **537**, 558–562 (2016).
13. Monaghan, L. *et al.* The Emerging Role of H3K9me3 as a Potential Therapeutic Target in Acute Myeloid Leukemia. *Front. Oncol.* **9**, 705 (2019).
14. Cai, Y. *et al.* H3K27me3-rich genomic regions can function as silencers to repress gene expression via chromatin interactions. *Nat. Commun.* **12**, 719 (2021).
15. Biga, P. R., Latimer, M. N., Froehlich, J. M., Gabillard, J.-C. & Seiliez, I. Distribution of H3K27me3, H3K9me3, and H3K4me3 along autophagy-related genes highly expressed in starved zebrafish myotubes. *Biol. Open* **6**, 1720–1725 (2017).
16. Raeisossadati, R. *et al.* Small Molecule GSK-J1 Affects Differentiation of Specific Neuronal Subtypes in Developing Rat Retina. *Mol. Neurobiol.* **56**, 1972–1983 (2019).