UNDERSTANDING THE ROLE OF DNA METHYLTRANSFERASES (DNMTS) IN ZEBRAFISH RETINA REGENERATION

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CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled **"Understanding the Role of DNA methyltransferases (Dnmts) in Zebrafish Retina Regeneration"** submitted by **Ms. Evelyn Abraham (Reg. No. MS11035)** 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. Shravan Kumar Mishra **Dr. Kavita Babu** **Dr.Rajesh Ramachandran**
(Supervisor)

Dated: April 22, 2016

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.

Ms. Evelyn Abraham

(Candidate)

Dated: April 22, 2016

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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CONTENTS

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF GRAPHS

LIST OF TABLES

NOTATIONS

Notation	**Meaning**
Aza	5’-Azacytidine
Zeb	Zebularine
RG	RG108
BrdU	Bromodeoxyuridine
PCNA	Proliferating cell nuclear antigen
dpi	days post injury
mpi	minutes post injury
hpi	hours post injury
RT-PCR	Reverse Transcription PCR
qRT-PCR	Quantitative Real Time PCR
ONL	Outer Nuclear Layer
INL	Inner Nuclear Layer
GCL	Ganglion Cell Layer
dnmt	DNA methyltransferase gene in zebrafish
Dnmt	DNA methyltransferase protein in zebrafish
DNMT	DNA methyltransferase protein in mammals

ABSTRACT

Retinal damage is a serious problem that affects mankind. Unfortunately, there are no feasible solutions available to alleviate this problem. However, lower vertebrates like fishes and frogs mount a very robust regenerative response after retinal damage culminating in functional restoration of vision. Previously published studies reveal hundreds of genes that are up/down regulated post injury. It is probable that most of these genes have CpG islands in their corresponding promoter sequences that are susceptible to DNA methylation events by DNA methyltransferases (Dnmts). Such events modify gene expression epigenetically. Therefore it would be interesting to find out how Dnmts are regulated post-retinal injury during retinal regeneration in zebrafish. If so, then we can also try to understand the pro-proliferative genes whose expression and induction are regulated post injury, by Dnmt mediated methylation events. It is also essential to evaluate the functionality of the identified genes in earlier dedifferentiation of the retina using, cell biological, genetic and pharmacological approaches during regeneration. One can also try to address the question of whether or not Dnmt mediated gene regulation is involved/necessary and sufficient for retina regeneration by trying to block the action of Dnmts using pharmacological inhibitors. Epigenetic mode of silencing like DNA methylation may be required for maintaining various pro-proliferative genes in check in the uninjured condition in the retina. A reversal of this by DNA de-methylation is necessary for initiating the Muller glia de-differentiation necessary for normal regeneration. Later once regeneration is completed, the retinal homeostasis is restored back through epigenetic mechanism of gene silencing. So understanding this hierarchy mediated by Dnmts becomes inevitable for these studies.

This study tries to answer the above questions. Till date, a specific pathway by which Dnmts acts during retina regeneration is not known, however the proposed study might provide us some directions to understand it.

1. INTRODUCTION

In contrast to mammals, the teleost fish like the zebrafish have the ability to regenerate its central nervous system. The zebrafish retina being a part of CNS is found to self-heal resulting in the restoration of its vision[1-3]. The retina is made up of various types of neuronal cell types and glial cells called Muller glia. There are two different cell types that maintain retinal neurogenesis and regeneration. They are the Ciliary Marginal Zone cells; or the classic retinal stem cells and the Muller glia progenitor cells that are induced soon after injury. The Muller glia cells are required for the maintenance of retinal homeostasis[4-5]. There are many different injury paradigms that are standardized and established for delivering specific injury to different components of the retina. e.g.: light lesion model for creating photoreceptor cell damage[11, 12] and needle poke model to injure and activate the Muller glia cells of the retina.

Upon injury, the Muller glia cells near the site of injury gets activated forming Muller glia progenitor cells, to trigger a regenerative response and give rise to and replace all the neuronal components and Muller glia at the site of injury[6-11]. It has been observed that such a regenerative response also restores normal vision in the fish. The exact molecular mechanism of the injury induced retinal regeneration has not been deciphered yet although there are many studies giving us clues in different directions.

The zebrafish retina grows and expands in both size and cell number as the fish grows, unlike the mammalian retina which does not grow. The ability of fishes to mount a robust regenerative response can be because there is no tight regulation over cell proliferation on the expense of regeneration during retinal neurogenesis. In contrast, the mammalian retina hosts a relatively inhibitory environment to adult neurogenesis post injury or embryogenesis, probably to maintain a strict control over cell proliferation to avoid tumorigenesis.[13]

1.1. Anatomy of the Retina: Muller Glia, the major player of regeneration

The three main layers of the vertebrate retina are the outer nuclear layer (ONL), the inner nuclear layer (INL) and the Ganglion cell layer (GCL). The ONL is inner to the retinal pigment epithelium layer and comprises of the photoreceptor cells (rods and cones). The light that traverses to the eye is absorbed by the Retinal Pigment Epithelium (RPE), and then received by the photoreceptor cells which transmit the signal to the Ganglion cells of the GCL through the inter-neurons. The inter-neurons reside in the INL and comprises of the amacrine cells, bipolar cells and horizontal cells. The ganglion cells collect all this information and together all the axonal processes of the ganglion cells join into the optic nerve. The optic nerve takes the information into the brain which processes this information enabling us to see.

The Muller glia is the major glial cell type of the retina. The Muller glia is not restricted to a particular layer of the retina. Rather it spans the ONL, GCL and the INL and contacts all the components of the neural retina via its processes.

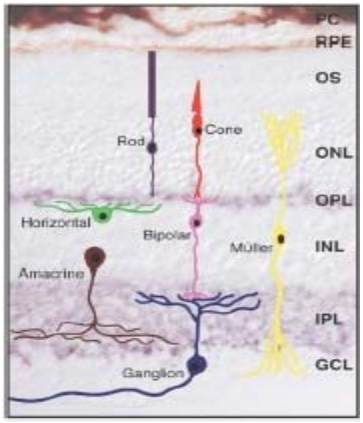


Figure A: Ultra structure of the retina

1.2.The Injury Model for Muller Glia

The retina is poked using a 30 gauge needle from the back side of the eye to create a focal injury. In this scenario, the undamaged and uninjured neighbouring tissue acts as control. This mode of injury damages all neurons and Muller glia cells at the injury spot in contrast to the light lesion model that specifically damages the photoreceptors.

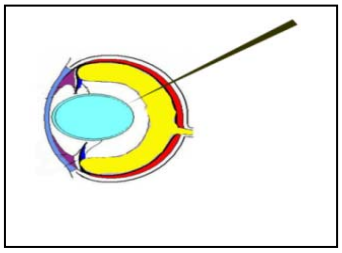


Figure B: Mechanical injury to the retina administered using a 30 G needle

1.3.Mechanism of Muller Glia Induced Retinal Regeneration in Zebrafish

The stab wound at the injury spot damages the Muller glia cells at the lesion, thereby activating them for a reparative response. Upon injury the MG cells near the injury spot gets activated[4-5]. The exact molecular mechanism of this activation step is still not known. There are a few findings which suggest the involvement of growth factors and cytokines like Fgf and Igf to be involved in this process[14-16].

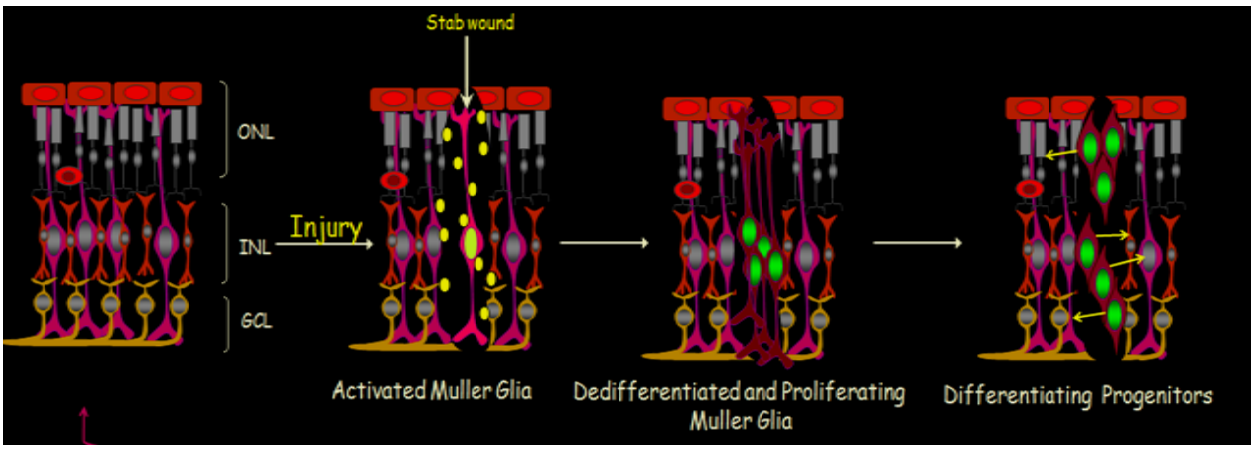


Figure C: Retina Regeneration in zebrafish. Activation of Muller glia in response to injury leads to its de-differentiation to form Muller glia Progenitor Cells (MGPCs) which proliferates and then re-differentiates to give rise to retinal neurons and Muller glia.

Upon activation, the Muller glia cells de-differentiate to go back to their progenitor state. This process begins soon after injury and achieves its peak at around 1 day post injury (dpi) and stays on until the 4th day post injury. At this stage the dedifferentiated Muller glia cells are called Muller glia progenitor cells (MGPC) [17].

At around 2dpi, the next phase called the proliferative phase of regeneration begins. During this phase, the MGPCs undergo rapid proliferation to give rise to many more MGPCs. All of these MGPCs have achieved the capability to give rise to all the neuronal cell types of the retina and the Muller glia. This proliferative phase achieves its peak at around 4 dpi and stays on till 9 dpi.

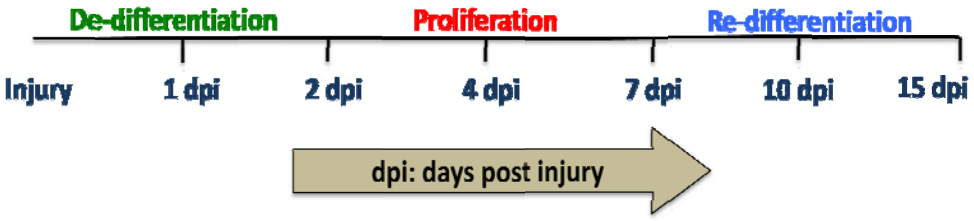
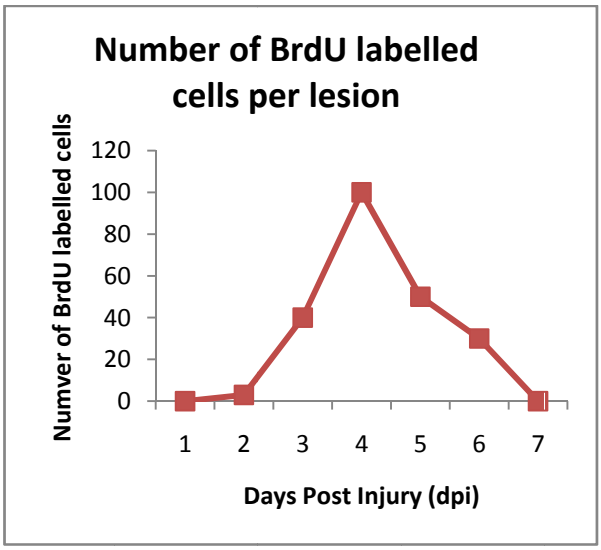


Figure D: Scheme showing the rough timing in days post injury (dpi) of the three phases of retinal regeneration, i.e. de-differentiation, proliferation and re-differentiation.

As mentioned before, the number of BrdU labeled proliferating cells per lesion will be maximum at four days post injury 4 dpi. Given below is a representation of the same.

Figure E: A representation of number of proliferating cells in the retina at the injury spot at different days post injury (dpi). The Muller glia cells after de-differetiation reaches the peak of proliferation at 4 dpi.



After the peak of proliferation, some of the MGPCs, starts to re-differentiate and migrate to different layers of the retina, giving rise to the neuronal cell population and other Muller glial cells. The re-differentiation phase continues until the 15th to 21st dpi thus repopulating the neural and glial components of the retina at the injury spot[6, 7, 10, and 18].

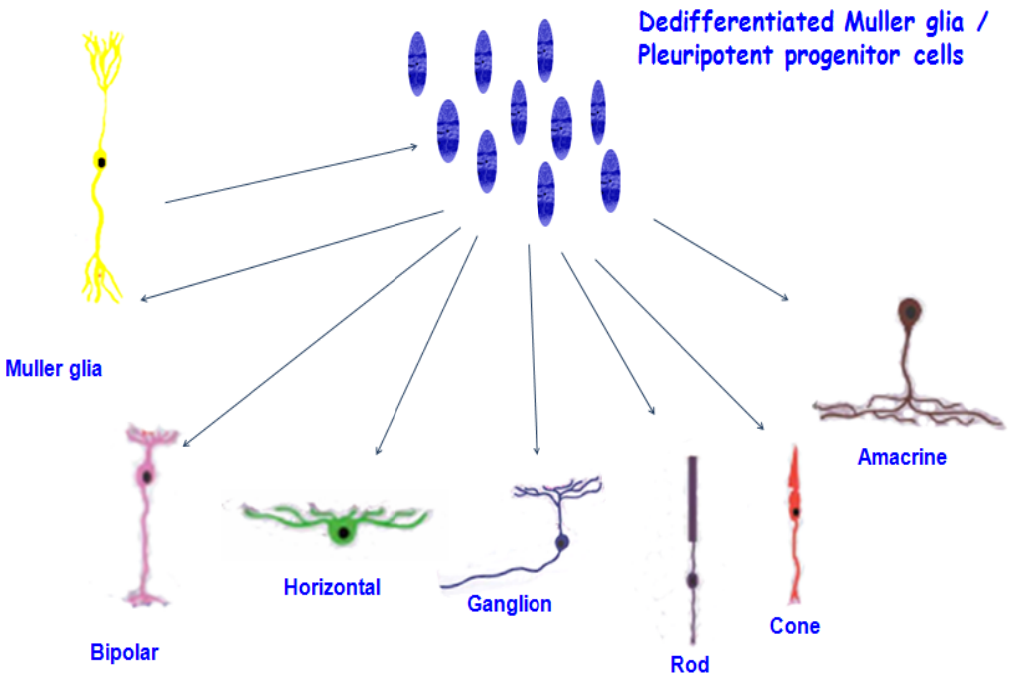


Figure F: De-differentiated and proliferated Muller glia progenitors re-differentiate to give rise to differentiated retinal neurons and Muller glia.

2. OBJECTIVES

- To find out if DNA Methyltransferases (Dnmts) are regulated after retinal injury in zebrafish.

- To find out the regulators of Dnmts and their potential gene targets.

3. BASIC THEORY

3.1. Chapter 1

Epigenetic Gene Regulation During Retina Regeneration in Zebrafish by DNA methyltransferases (Dnmts)

The Histone octamer wrapped around with DNA is called a nucleosome. Several repeating units of nucleosomes together make up the chromatin, which is a dynamic structure. The parts of the chromatin that is subject to active transcription is called the euchromatin and the transcriptionally silenced regions of the chromatin are called the heterochromatin[36].

Some of the prominent examples of epigenetic regulation through biochemical modification are: DNA modifications (methylation), histone modification (methylation, acetylation, sumoylation etc.) and nucleosome remodelling complexes that also interact with the DNA. In addition to this, some RNAs like non-coding RNA are also known to influence chromatin structure *eg.* X Inactivation[20, 21]. Of the above mentioned types of epigenetic modifications, DNA methylation is known to play a key role in maintaining epigenetic states.

Heterochromatin state is associated with intense DNA methylation and histone modification of the nucleosomes that increases the electrostatic interaction between the DNA and histones so that the chromatin becomes inaccessible to transcription factors or DNA binding proteins. In contrast euchromatin is associated with absence of methylated CpG and histone modifications like acetylation of lysine residues of various histones etc that decreases the packing density of chromatin, rendering the DNA more accessible for active transcription. Histone methylation however, can be activating or repressing depending on the position of Lysine that is methylated, proximity to the gene promoter region etc.

The DNA is known to get methylated during and post replication on the cytosine bases. This type of methylation is found only in the cytosine of 5'CpG3' islands and rarely on isolated cytosines[22]. The methylated DNA attracts '5 Methyl Cytosine Binding Proteins' which again condenses the chromatin to silence it. The enzymes that catalyze DNA methylation on the cytosine residue are called DNA methyltransferases.

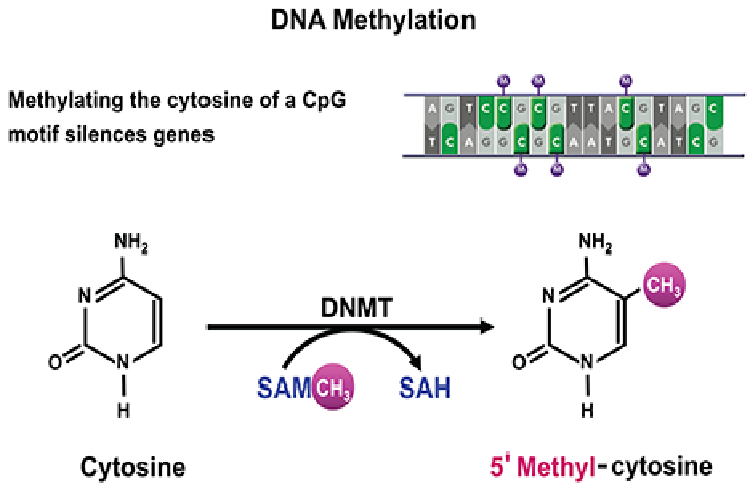


Figure G: Mechanism of action of DNA Methyltransferases. Dnmts catalyse the addition of methyl group to the 5th carbon of cytosine residue in CpG islands of DNA.

Zakhari. S, Alcohol Metabolism and Epigenetics Changes, *Alcohol Research: Current Reviews*, Volume 35, Issue Number 1

There are two classes of DNA methyltransferases based on their function- *denovo* DNA methyltransferases and maintenance methyltransferases.

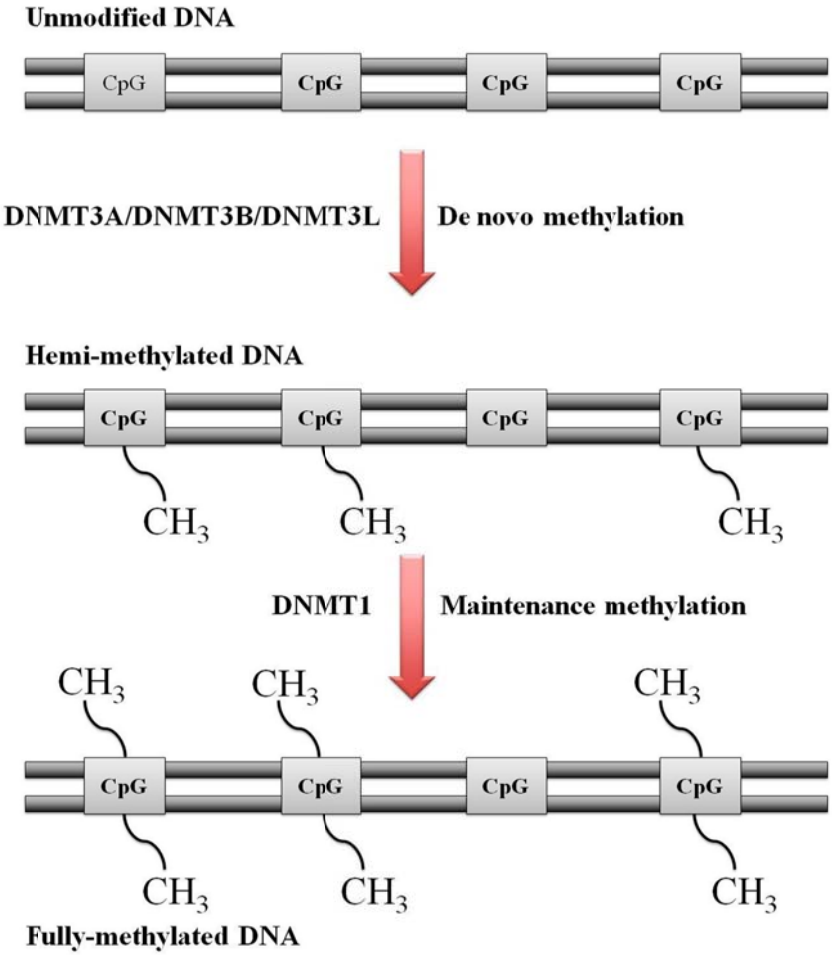


Figure H: Representation of the function of maintenance methyltransferases and de-novo methyltransferases.

Alessandra Maresca et al, Dna Methyltransferase 1 Mutations and Mitochondrial Pathology: Is Mtdna Methylated? Front. Genet., 12 March 2015

After one round of DNA replication the newly synthesized DNA strand will not have the same DNA methylation marks or as that of the template strand and can be in a hemi-methylated state. Since DNA methylation marks should be clonally inherited to maintain a stable methylation pattern after each division, maintenance methyltransferase enzymes come into the picture. *Dnmt1* is known to be the major maintenance DNA methyltransferase enzyme in zebrafish since it shows higher preference for a hemimethylated state than an unmethylated state. In contrast to *Dnmt1*, there are de novo methyl transferases also known in zebrafish that shows higher affinity for unmethylated substrates[23, 24]. The function of such de novo methyl transferases is to silence the viral repeat sequeances and other parasitic element sequences of the genome to keep them in a transcriptionally repressed condition[25, 26, 27].

There are five proteins in mammalian cells that have domains with significant similarity to prokaryotic DNA methyltransferase domains. They are: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. The domain structures of these enzymes are shown in following figure: All these enzymes have an N-terminal regulatory domain and a C-terminal catalytic domain. Thus, it is based on their structure, and its similarities that these DNA methyltransferases can be divided into three families: DNMTl, DNMT2, and DNMT3[28].

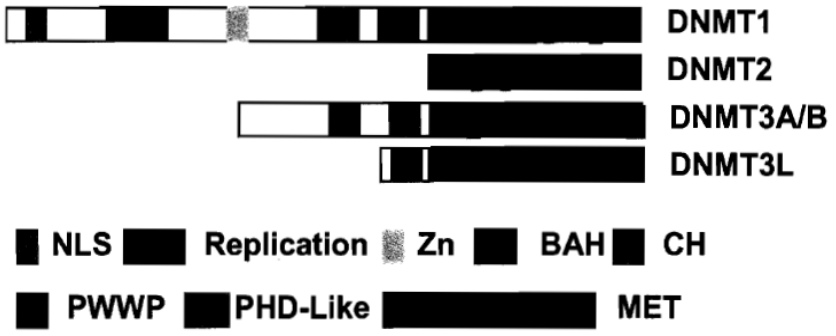


Figure IA: The domain structures of the three families of DNA methyltransferase proteins in mammals are shown.

Kunal Rai, PhD Thesis, University of Utah, 2006

Domain	**Function**
NLS	**Nuclear localization signal** *or* **sequence** (**NLS**) is an amino acid sequence that 'tags' a protein for import into the cell nucleus by nuclear transport[29].
Replication	The **RFTS (replication foci targeting sequence)** domain of Dnmt1 is not only necessary for replication-coupled maintenance DNA methylation, but also protects genome from aberrant DNA methylation[30].

Zn	A **zinc finger** is a small protein structural motif characterized by the coordination of one or more zinc ions in order to stabilize the fold. They function as interaction modules that bind DNA,RNA, proteins, or other small, useful molecules[31].
BAH	**BAH domain** (bromo-adjacent homology) domain acts as a protein-protein interaction module specialised in gene silencing[32].
CH	**Calponin homology domain (or CH domain)** is a family of actin binding domains found in both cytoskeletal proteins and signal transduction proteins[33].
PWWP	PWWP domain is an around 70 amino acids domain that was named after its central core '**Pro-Trp-Trp-Pro**'. The PWWP domain is found in DNA-binding proteins that function as transcription factors regulating developmental processes and in Dnmt3a and Dnmt3b which helps in binding strongly to DNA[34].
PHD-Like	**Plant homeodomain (PHD)** is found mainly in proteins involved in eukaryotic transcription regulation characterized by a conserved Cys4-His-Cys3 zinc finger binding motif. This domain in DNMT3a has been shown to be sufficient to repress transcription, independent of methyltransferase activity[35].
MET	Catalytic domain for the transfer of methyl group to the 5th carbon on cytosine residue in DNA at CpG islands[28].

Table 1: Meaning and functions of various domains found in the three families of DNA methyltransferases in zebrafish

In case of zebrafish there are eight dnmt genes known. They are *dnmt1, dnmt2, dnmt3, dnmt4, dnmt5, dnmt6, dnmt7 and dnmt8.*

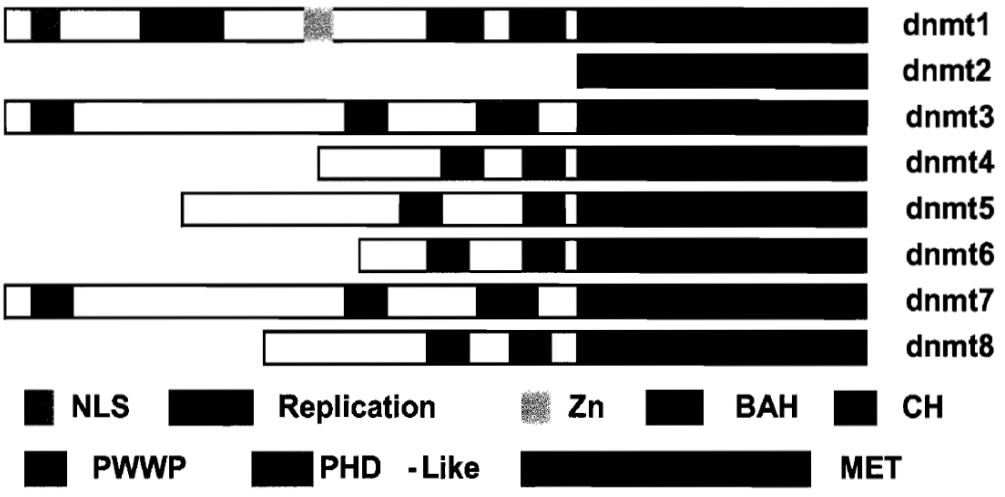


Figure IB: Dnmts in zebrafish. Kunal Rai, PhD Thesis, University of Utah, 2006

Zebrafish harbours all the clear homologs of DNA methyltransferases present in the mammals. Dnmt1 belongs to DNMT1 family and DNMT3 family has three members, DNMT3A, DNMT3B and DNMT3L. The N-terminus of Dnmt1 contains five different domains: nuclear localization signal (NLS) domain, a replication foci targeting domain, a zinc finger domain and two BAH domains of unknown function. Dnmt3, Dnmt4, Dnmt5, Dnmt6, Dnmt7 and Dnmt8 belong to the DNMT3 protein family. In fact, the members of DNMT3 family are duplicated in zebra fish. Dnmt3 and Dnmt7 contain additional domains in their N-termini including a Calponin homology domain[28].

The N terminus of these Dnmt3, Dnmt4, Dnmt5, Dnmt6, Dnmt7 and Dnmt8 enzymes are unrelated to the N-terminus of Dnmt1 which suggests that DNMT1 and DNMT3 family of enzymes might be regulated differently. DNMT3A and DNMT3B additionally have two distinct domains in their N -termini: PHD and PWWP[28].

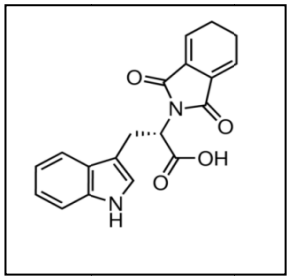
3.1.1. Significance of Dnmts In Retina Regeneration In Zebrafish

Previously published studies reveal hundreds of genes that are up/down regulated post injury. It is probable that most of these genes have CpG islands in their corresponding promoter sequences that is susceptible to DNA methylation events by DNA methyltransferases (Dnmts). Such events modify gene expression epigenetically. Therefore it would be interesting to find out how Dnmts are regulated post-retinal injury during retinal regeneration in zebrafish. If so, then we can also try to understand the pro-proliferative genes whose expression and induction are regulated post injury, by Dnmt mediated methylation events. It is also essential to evaluate the functionality of the identified genes in earlier dedifferentiation of the retina using, cell biological, genetic and pharmacological approaches during regeneration. One can also try to address the question of whether Dnmt mediated gene regulation is necessary and sufficient for retina regeneration by trying to block the action of Dnmts using pharmacological inhibitors. Epigenetic mode of silencing like DNA methylation may be required for maintaining various pro-proliferative genes in check in the uninjured condition in the retina. A reversal of this by DNA de-methylation is necessary for initiating the Muller glia de-differentiation necessary for normal regeneration. Later once regeneration is completed, the retinal homeostasis is restored back through epigenetic mechanism of gene silencing. So understanding this hierarchy mediated by Dnmts becomes inevitable for these studies.

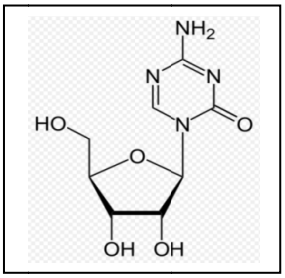
3.2. Chapter 2

Pharmacological Inhibition of DNA methyltransferases – Strategy to study the effect of Dnmt inhibition in Muller Glia De-differentiation and Proliferation following injury

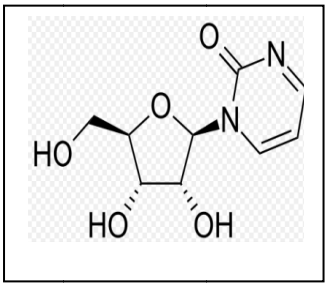
The nucleoside inhibitors 5-azacytidine, 5-azadeoxycytidine, and zebularine after incorporation in DNA, function as suicide substrates for Dnmt enzymes. RG108 have been proposed to inhibit DNA methyltransferases by masking Dnmt target sequences or by blocking the active site of the enzyme[28, 38].



5' Azacytidine (SIGMA)



Zebularine (SIGMA)



RG108 (SIGMA)

DNA methyltransferase (Dnmt) inhibition by enzyme trapping or enzyme blocking: Aza-nucleotides and Zebularine can become incorporated into DNA during replication and then are recognized by Dnmt enzymes. A stable reaction intermediate is formed via the sulfhydryl side chain of the catalytic cysteine residue. Thus, Dnmt is trapped and concomitantly degraded. By this mechanism, cells are depleted of Dnmt protein. Small molecules, such as RG108, can block the catalytic pocket of free Dnmt proteins without the formation of covalent reaction intermediates[28, 37].

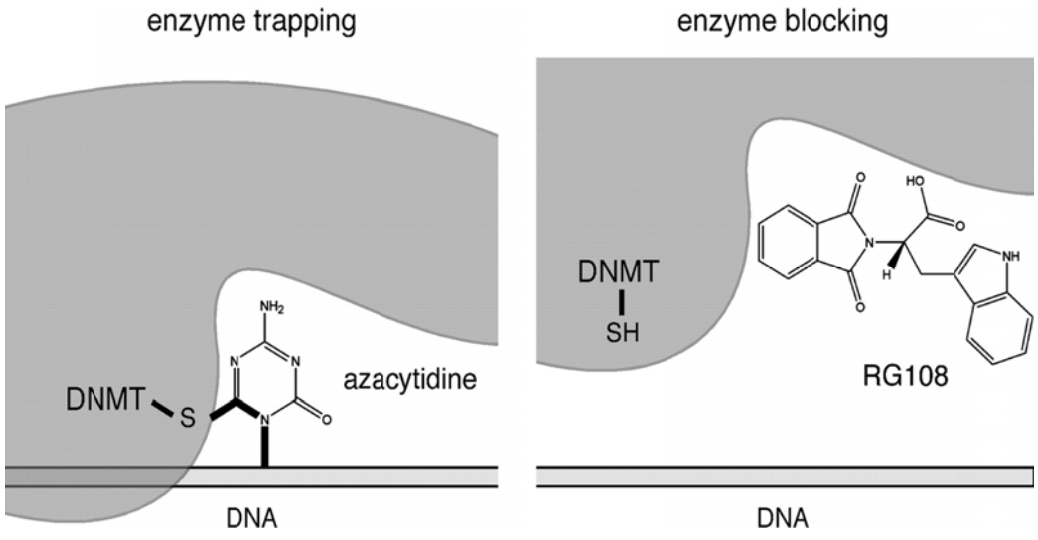


Figure J: Mechanism of action of Dnmt pharmacological blockers: by enzyme trapping and by enzyme blocking[38]

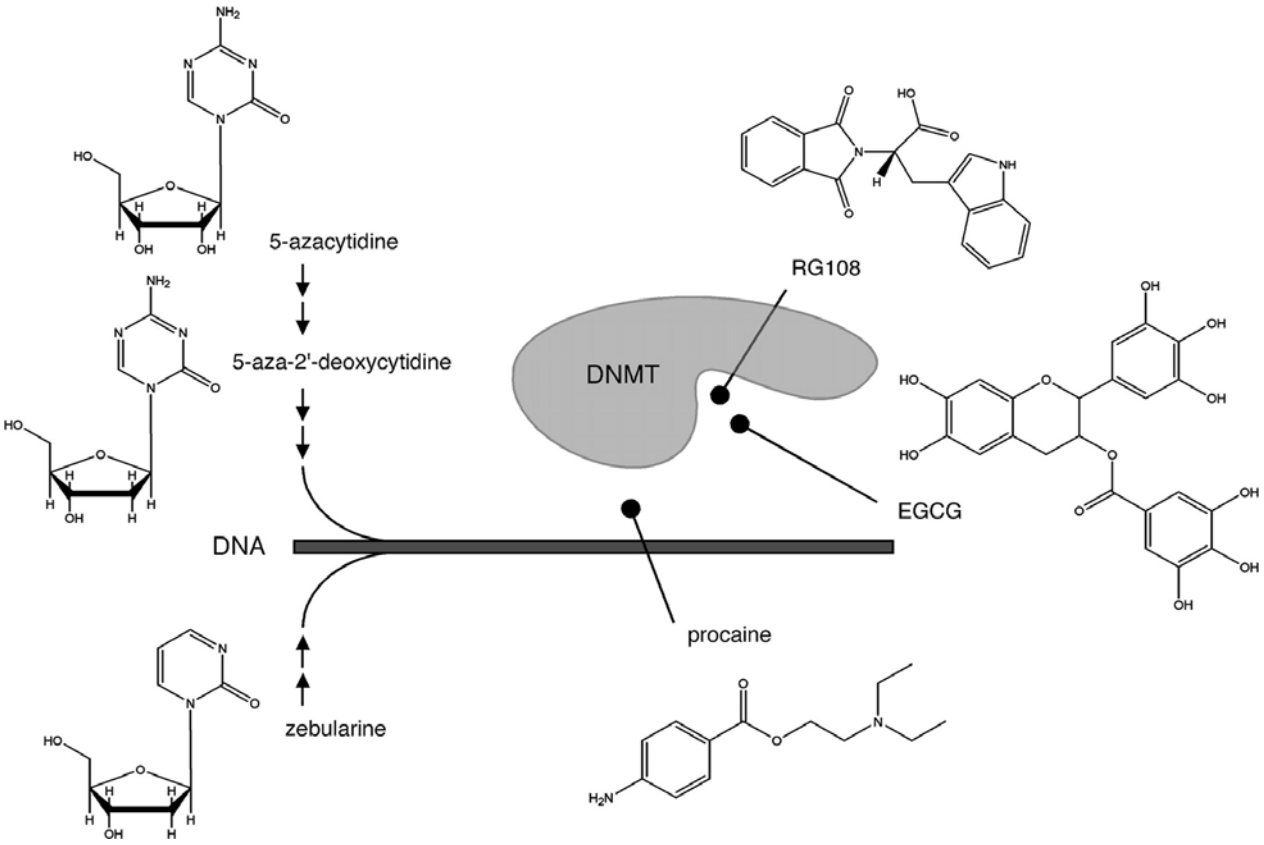


Figure K: DNA methyltransferase (Dnmt) inhibitors and their inhibitory mechanisms[38].

3.3.Chapter 3

Analysis of the cause and fate of the increased number of proliferating Muller glia cells arising in the presence of Dnmt pharmacological blockers at 2dpi and 4dpi in terms of their cell apoptosis at 2 dpi and 4 dpi.

TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is a method for detecting DNA fragmentation that results from apoptotic signalling cascades by labelling the terminal end of nucleic acids.

Terminal deoxynucleotidyl transferase or TdT, an enzyme that catalyzes addition of dUTPs which are secondarily labelled with a marker, for eg., fluorescein, identifies nicks in the DNA. TdT can also label cells that have suffered severe DNA damage[37].

3.4. Chapter 4

Regulation of known pro-proliferative genes- *mmp9* and *ascl1a*, during retina regeneration in the context of pharmacological inhibition of Dnmts

Retina regeneration involves the sequential orchestration of differentially regulated genes which may be regulated by epigenetic mechanisms like DNA methylation/de-methylation. Many genetic factors like Hb-egf, Fgf2, Tgfβ, Tnfα, Ascl1a, Lin-28, Apobec etc are known to play a detrimental role in the earlier induction of regenerative response, further progressed through suppression of let-7 micro RNA, induction of Wnt signalling, necessary for the proliferation of MGPCs. This is followed by the restriction of the regenerative response mediated by the reappearance of let-7 miRNA, delta notch signalling and Insm1a mediated transcription repressive events that are necessary for prevention of tumour formation and restoration of retinal homeostasis. It is possible that these gene regulation events may be happening via an epigenetic mechanism. So, one can also try to address the question of whether or not Dnmt mediated gene regulation is involved/necessary and sufficient for retina regeneration.

Epigenetic mode of silencing like DNA methylation may be required for maintaining various pro-proliferative genes in check in the uninjured condition in the retina. A reversal of this by DNA de-methylation is necessary for initiating the Muller glia de-differentiation necessary for normal regeneration. Later once regeneration is completed, the retinal homeostasis is restored back through epigenetic mechanism of gene silencing. So understanding this hierarchy mediated by Dnmts becomes inevitable

4. RESULTS AND OBSERVATIONS

4.1. Chapter 1

4.1.1. mRNA Expression levels of *dnmt1, dnmt8 and dnmt4* in the uninjured and injured retina at different time points post injury- RT PCR Result

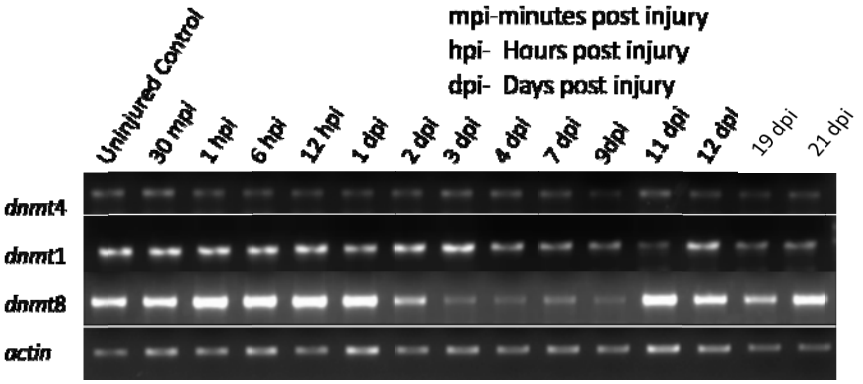


Figure 1: mRNA expression level of *dnmt4, dnmt1* and *dnmt8* at different time points before and after retinal injury. Method: RT-PCR

Observation: *dnmt1* and *dnmt8* mRNA expression level decreases in the whole-retinal tissue at around 4 dpi and 2 dpi respectively. *dnmt8* mRNA level then again increases from 11 dpi to 21 dpi compared to that of uninjured control.

4.1.2. mRNA fold change of various *dnmt* genes at different time points before and after retinal injury- qRT PCR result of whole retina.

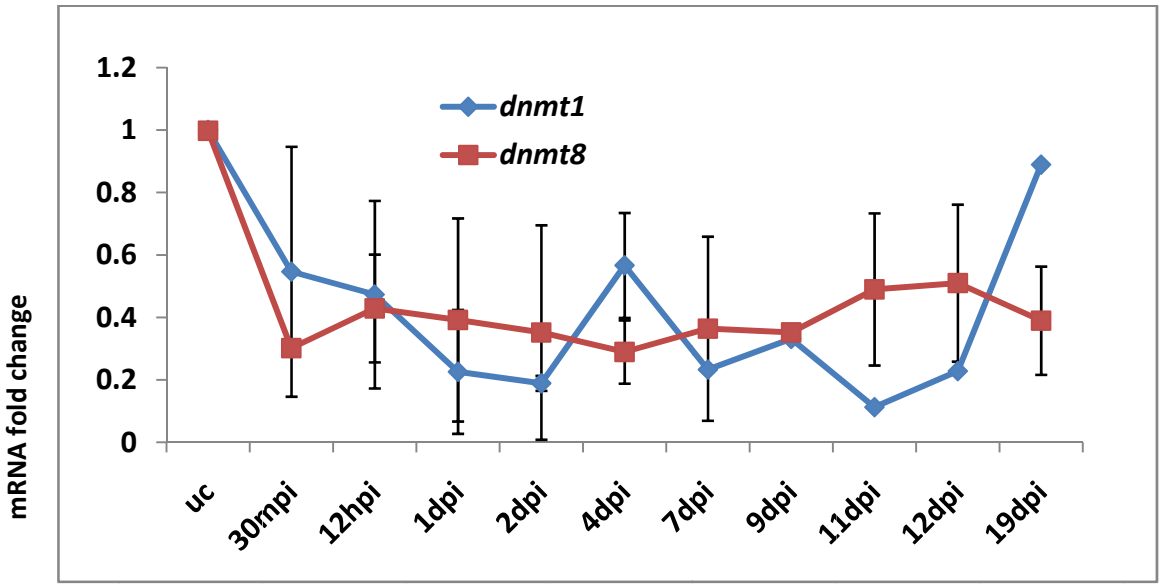


Figure 2A: mRNA fold change of *dnmt1* and *dnmt8* at different time points before and after retinal injury. Method: qRT-PCR

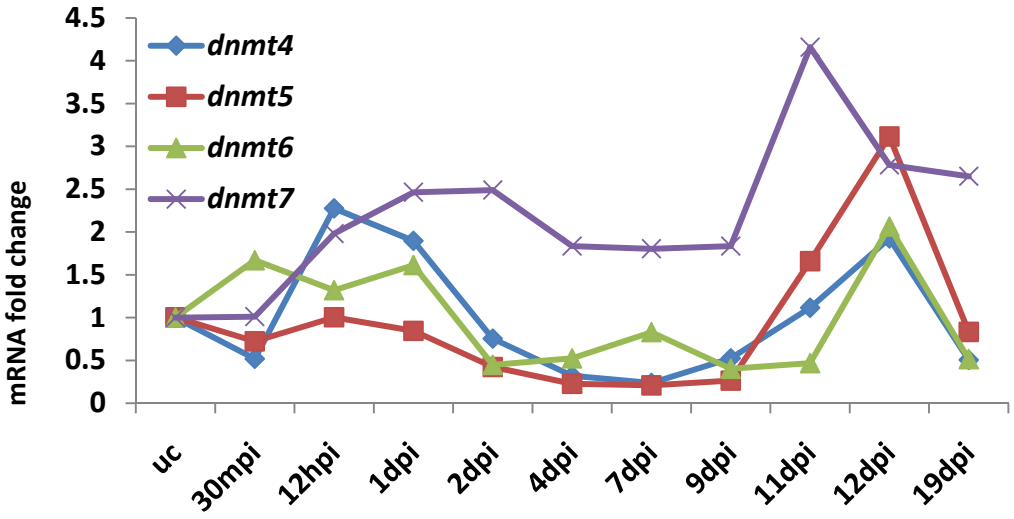


Figure 2B: mRNA fold change of *dnmt4, dntmt5, dnmt6* and *dnmt7* at different time points before and after retinal injury. Method: qRT-PCR

Observation: *dnmt1* and *dnmt8* mRNA expression decreases relative to uninjured control in the injured retina. *dnmt1* seems to be induced at 4dpi slightly but the level does not cross that of the uninjured control. This can be because *dnmt1* is present at basal level pan retinal before and after retinal injury but only preferentially expressed at the injury spot at 4 dpi, the peak of proliferation. *dnmt4*, *dnmt6* and *dnmt7* seems to be induced in the retina after injury whereas *dnmt5* seems to be down-regulated and then induced.

4.1.3. Spatial regulation of *dnmt1*- the major maintenance methyltransferase in zebrafish at different time points before and after retinal injury using *dnmt1* mRNA *in situ* hybridization.

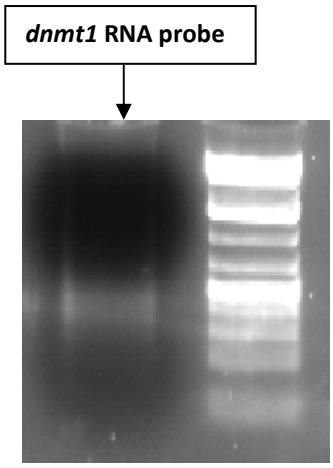


Figure 3A: *dnmt1* RNA Probe that was made and used for subsequent *in situ* hybridization experiments. Size of probe is around 1Kb

***dnmt1* mRNA *in situ* Hybridization Time Course**

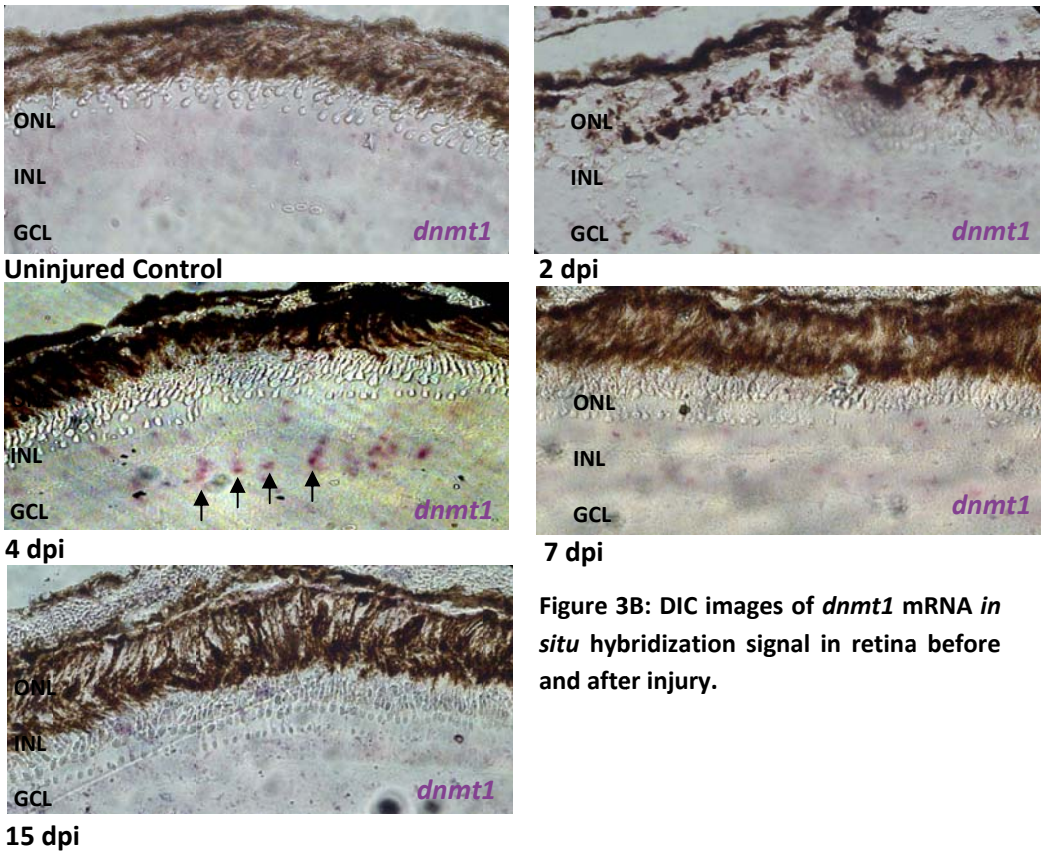


Figure 3B: DIC images of *dnmt1* mRNA *in situ* hybridization signal in retina before and after injury.

Observation: *dnmt1* mRNA *in situ* hybridization result suggests that *dnmt1* is present at a basal level pan-retinal in the uninjured retina. At 2dpi, the de-differentiation phase, a very slight colour reaction seems to develop in the INL nearby the injury spot. At 4 dpi, the peak of proliferation, *dnmt1 in situ* signal is found to preferentially increase at the site of injury in the INL. The Muller glia that responds to injury is about 2% of the total Muller glia cells. At 4 dpi it was observed that the basal signal of *dnmt1* which was present at the areas of the retina excluding the injury spot also decreased compared to uninjured control retina. At 7 dpi, there was no injury site specific expression of *dnmt1* mRNA, it was quite similar to that of uninjured control retina, with a pan- retinal basal level expression of *dnmt1* mRNA. Therefore, this suggests that *dnmt1* mRNA expression is selectively induced at 4 dpi at the injury spot in the retina and in this process, *dnmt1* mRNA level decreases in the neighbouring un- injured regions of the retina. However it is not clear whether it is the proliferating Muller glia at the site of injury that had responded to injury initially that expressed *dnmt1*. To find this out, a BrdU co-immunostaining with *dnmt1* mRNA *in situ* signal was done at 4 dpi, and the cell expressing *dnmt1*, BrdU and both *dnmt1* mRNA and BrdU at 4 dpi at the injury spot was counted and analysed.

4.1.4. *dnmt1* mRNA *in situ* signal and BrdU labeled proliferating cells co-immunostaining at different time points before and after retinal injury

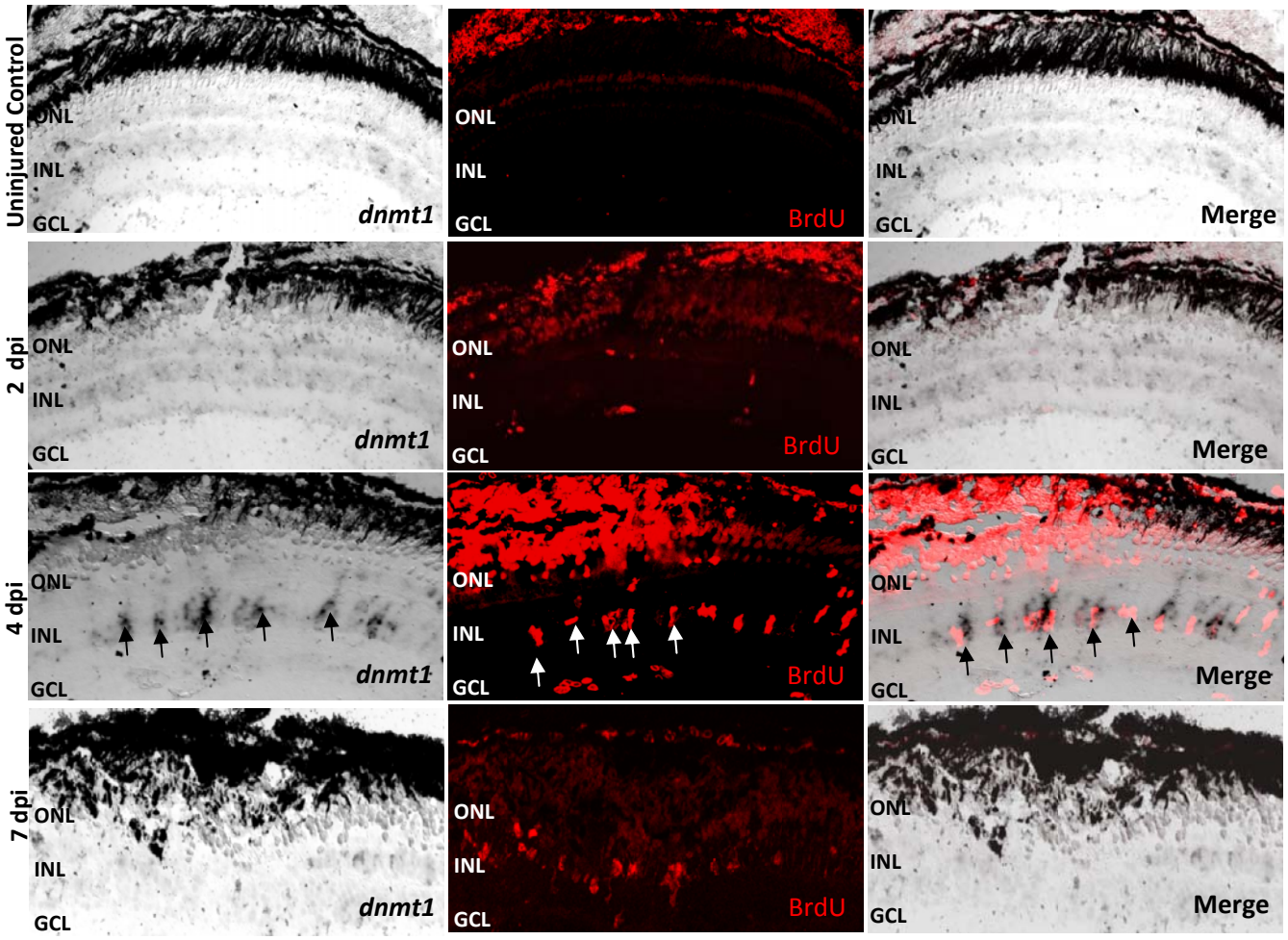
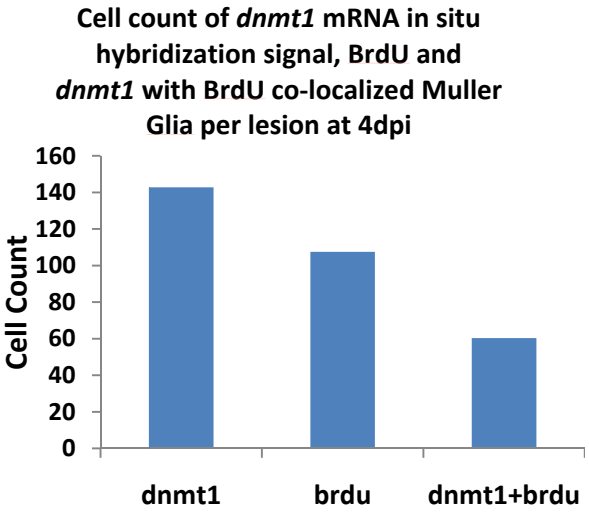


Figure 4A: *dnmt1* mRNA *In situ* hybridization on Uninjured Control, 2 dpi, 4 dpi and 7 dpi retinal sections. *dnmt1* is induced at 4 dpi at the injury spot in the INL and goes down from the other uninjured areas. The BrdU pulse was given for a duration of 6 Hrs before collecting the retinas prior to the time point.

Percentage of BrdU positive cells per lesion co-localizing with *dnmt1* mRNA *in situ* hybridization signal at the site of injury =56.51%



Graph 1: Cell count of *dnmt1* mRNA *In situ* hybridization signal expressing cells, BrdU labelled cells, and *dnmt1 In situ* signal and BrdU co-labelled cells at the site of injury in the 4 dpi retinal section.

4.1.5. *dnmt1* and BrdU do not co-localize completely at 4dpi

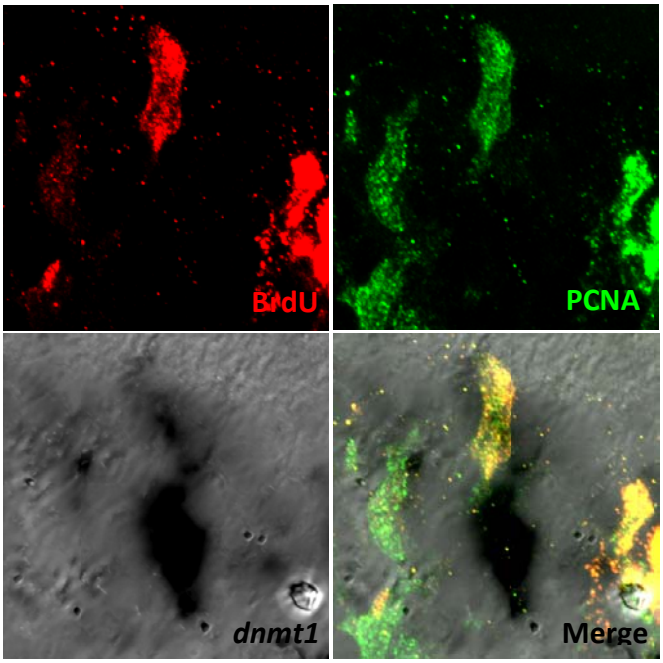


Figure 5: Co-labelling of *dnmt1* mRNA *In situ* signal, BrdU signal and PCNA signal at 4 dpi. Not all *dnmt1* expressing cells have BrdU signal and vice-versa. As shown in the 60X image, those Muller glia that has a high signal of *dnmt1* mRNA has extremely less or no BrdU signal and vice-versa. However, the Muller glia with a faint BrdU signal has a faint *dnmt1* mRNA *In situ* signal and vice-versa.

Observation: It was observed that *dnmt1* mRNA signal and BrdU did not co-localise perfectly at 4 dpi. This can be probably because *dnmt1* is required for cell cycle exit of the proliferating Muller glia cells that had responded to injury. Therefore, Muller glia that express a higher signal of *dnmt1* mRNA and less BrdU signal has probably come out of the cell- cycle and is no longer proliferating. Hence they will not be able to uptake BrdU unlike the cells expressing high BrdU and low *dnmt1* mRNA *in situ* signal that just entered cell cycle and is also proliferating at the site of injury.

4.2. Chapter 2

4.2.1. Dnmt targetting pharmacological blockers do not cause proliferation in the uninjured retina at 4dpi

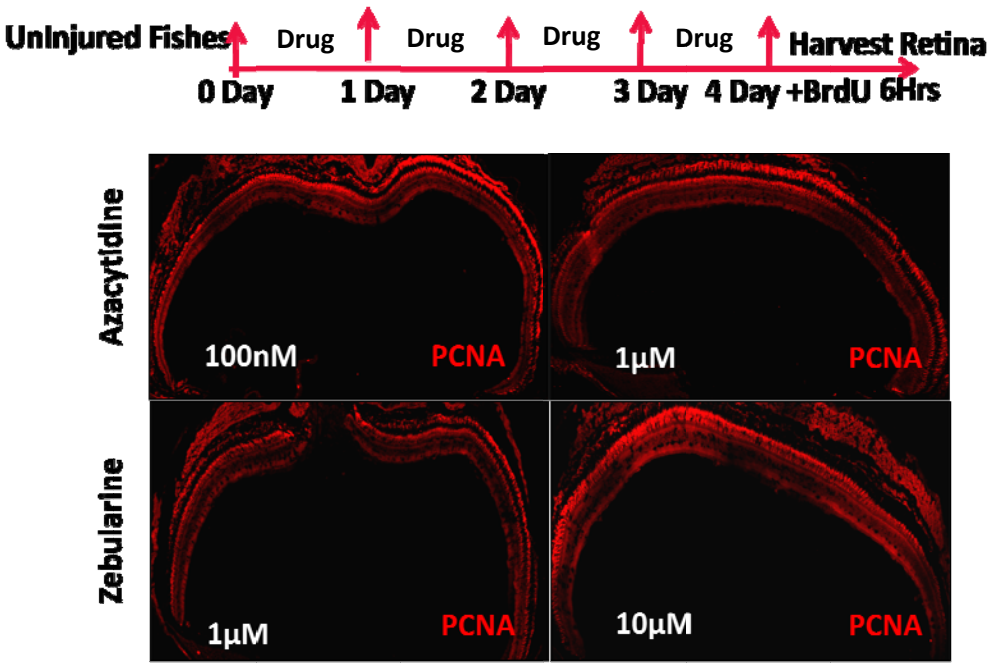
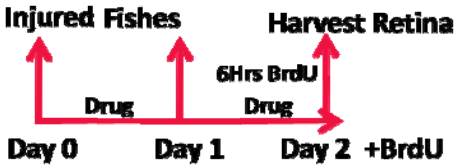


Figure 6: Dnmt pharmacological blockers do not cause fresh proliferation or injury response in the retina in the absence of injury

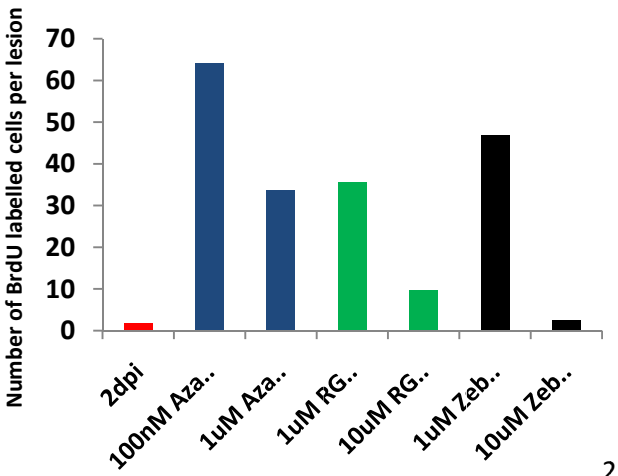
Observation: After continuous dipping of the uninjured fishes in various concentrations of Dnmt specific pharmacological blockers, until the fourth day the retina was isolated and PCNA immune-staining was done. It was seen that there was no induction of freshly proliferating cells in the absence of injury and presence of drug.

4.2.2. Effect of Dnmt inhibition on Muller glia de-differentiation, proliferation and re-differentiation using pharmacological Dnmt blockers.

a. De-differentiation



Graph 2: Injured fishes were treated in Dnmt pharmacological blocker and the retina was isolated at 2 dpi after BrdU pulsing. The number of proliferating cells were counted in both control and experimental condition and compared as shown.



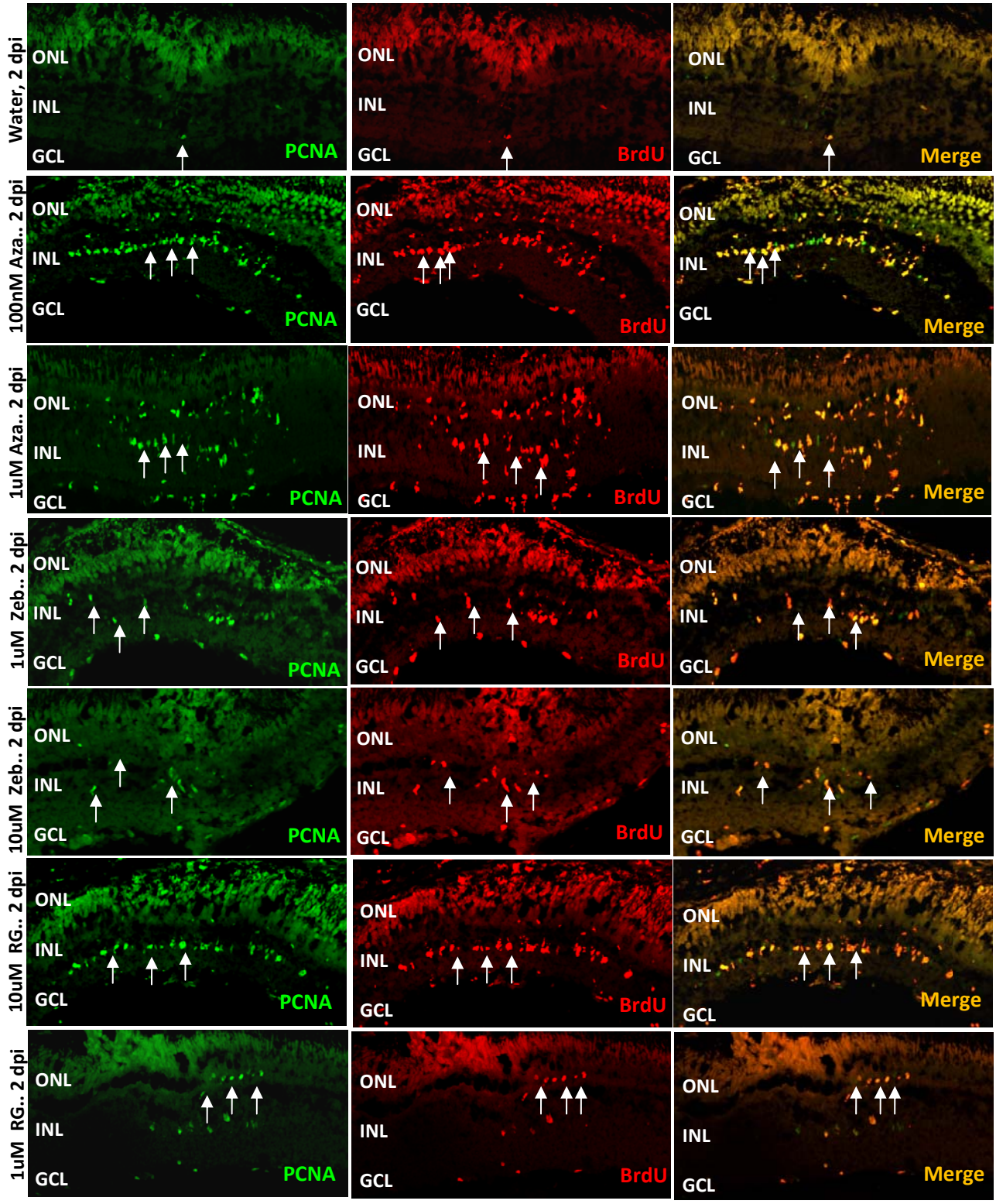
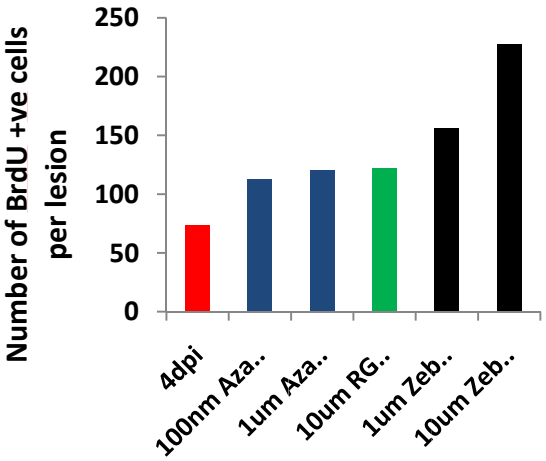


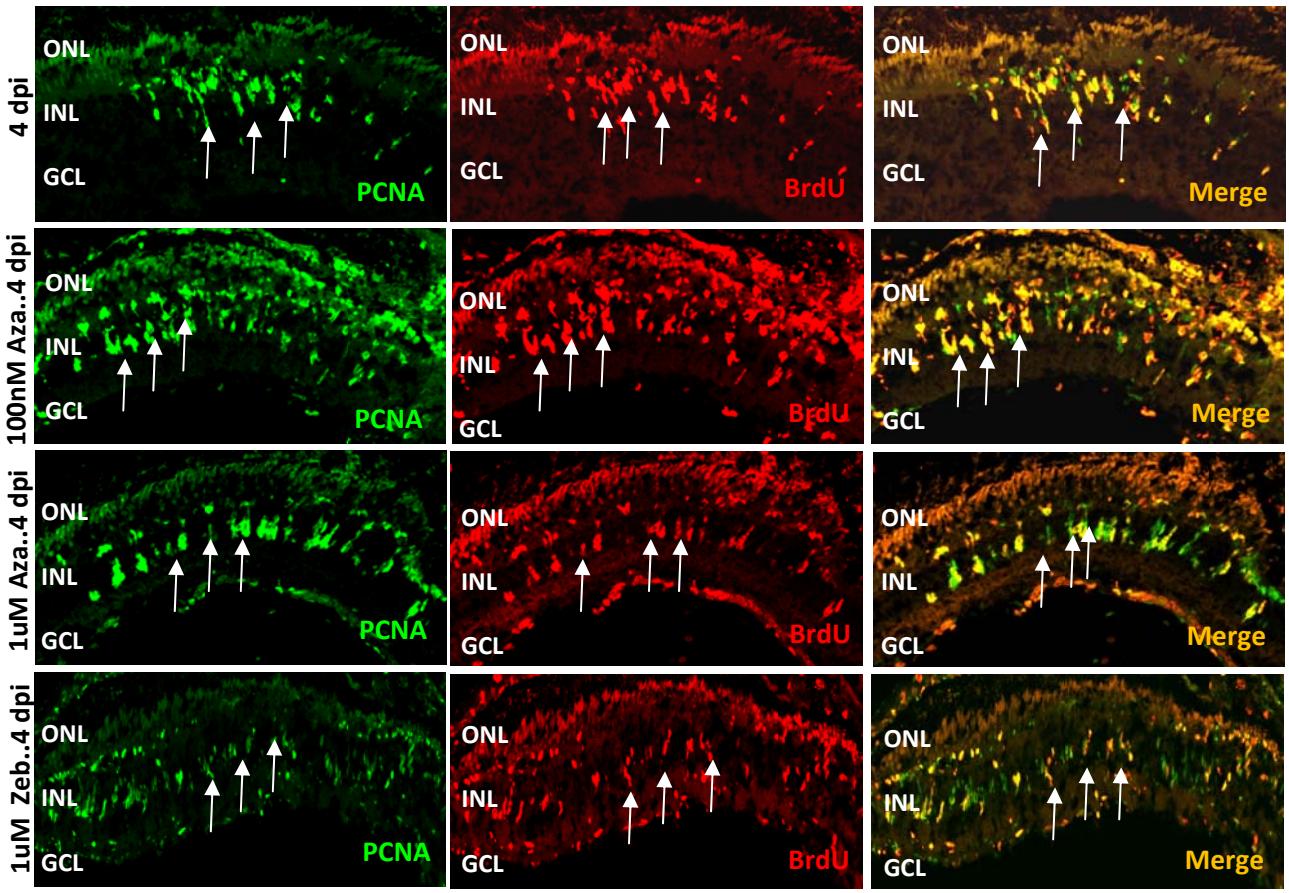
Figure 7: Immuno-staining of PCNA, BrdU and Merge of 2 dpi control and 2 dpi Dnmt pharmacological blocker treated retinas. It is seen that the number and span of proliferating cells at 2 dpi increases with Dnmt blocker treatment compared to control in water.

b. Proliferation

Effect of Dnmt Inhibition on cell proliferation at 4 dpi

Graph 3: Injured fishes were treated in Dnmt pharmacological blocker and the retina was isolated at 4 dpi after BrdU pulsing. The number of proliferating cells were counted in both control and experimental condition and compared as shown.





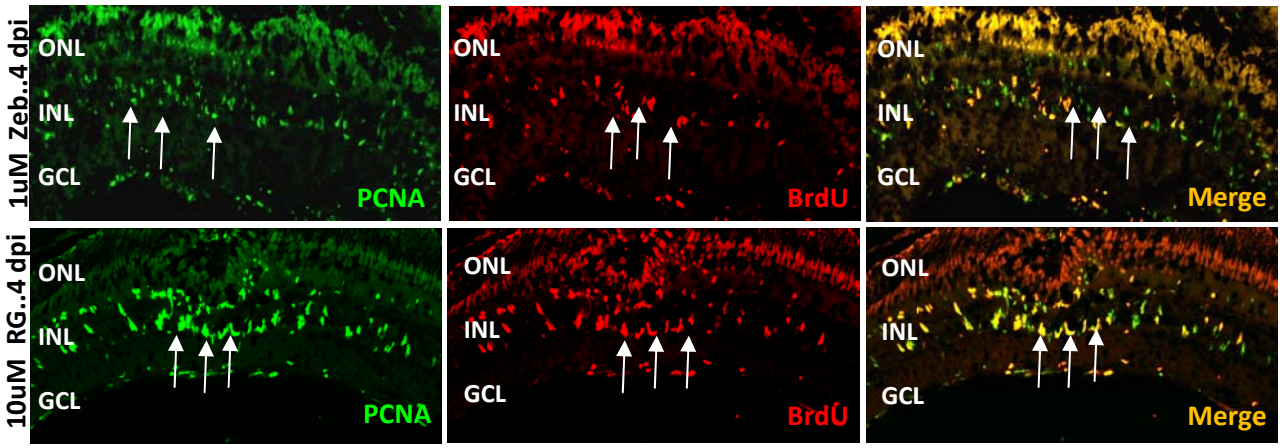


Figure 8: Immuno-staining of PCNA, BrdU and Merge of 4 dpi control and 4 dpi Dnmt pharmacological blocker treated retinas. It is seen that the number and span of proliferating cells at 4 dpi increases with Dnmt blocker treatment compared to control in water.

c. Re-differentiation

Effect of Dnmt Inhibition on Cell Migration at 30 dpi

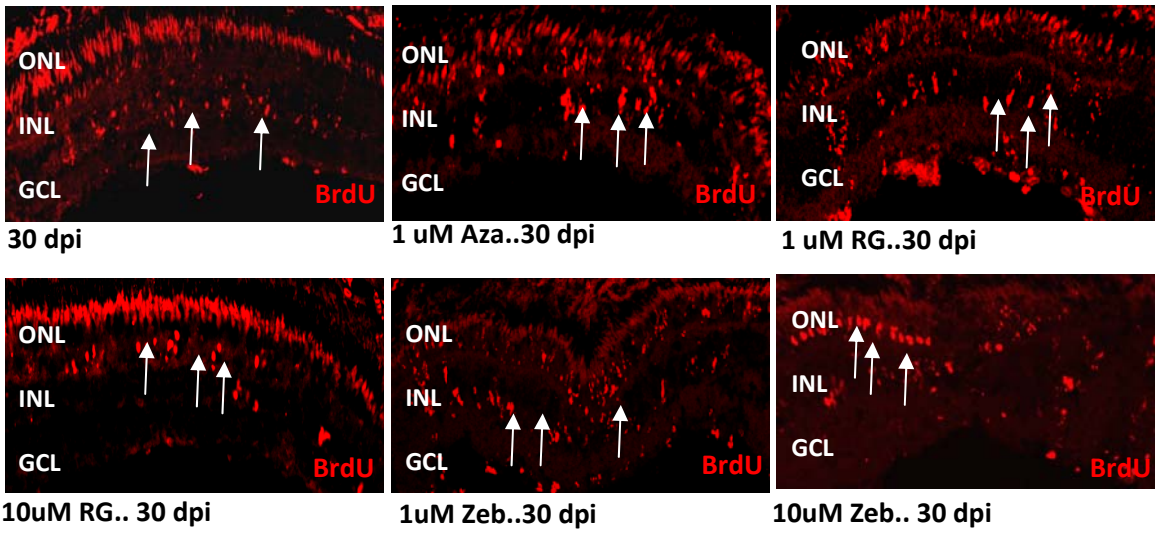
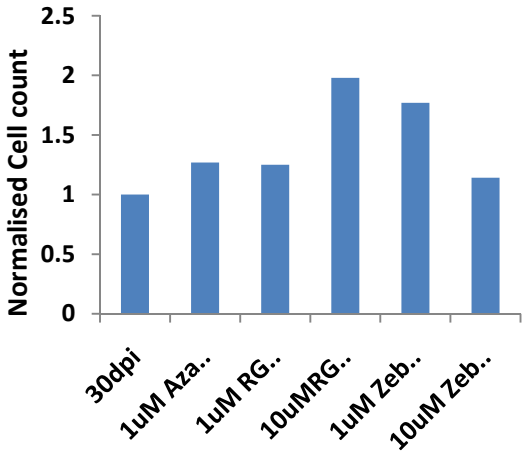
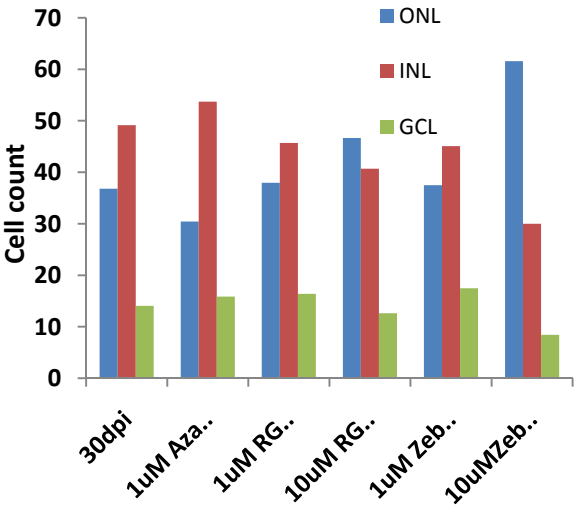


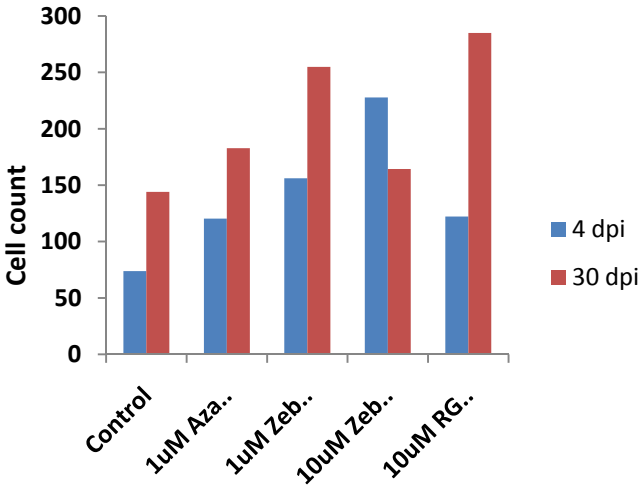
Figure 9: Immuno-staining of BrdU of 30 dpi control and 30 dpi Dnmt pharmacological blocker treated retinas. It is seen that the number and span of proliferating cells at 30 dpi increases with Dnmt blocker treatment compared to control in water.



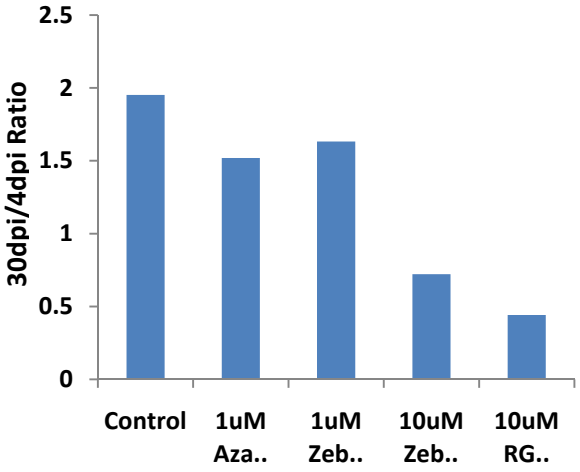
Graph 4: Normalised number of BrdU labelled cells arising from 4 dpi BrdU labelled cells per lesion at 30 dpi



Graph 5 : Number of BrdU labelled cells at 30 dpi migrating to ONL, INL and GCL



Graph 6 : Comparison of the number of BrdU positive cells at 30 dpi control and drug treated to 4 dpi control and drug treated.



Graph 7 : Proportion of BrdU positive cells persisting at 30 dpi compared to 4dpi per lesion in control and drug treated.

Observation:

1) 100 nM and 1 µM Azacytidine acts as a Dnmt blocker and increases the number of proliferating cells after retinal injury at 2 dpi and 4 dpi
2) 1 µM and 10 µM RG108 acts as a Dnmt blocker and increases the number of proliferating cells after retinal injury at 2 dpi and 4 dpi
3) 1 µM and 10 µM Zebularine acts as a Dnmt blocker and increases the number of proliferating cells after retinal injury at 2 dpi and 4 dpi

4) At 30 dpi, a higher number of BrdU positive cells than control was observed in drug treated fishes with no preferential bias to ONL, INL or GCL.
5) However the proportion of BrdU positive cells staying back at 30 dpi to the BrdU positive proliferating cells in 4dpi in Dnmt blocker treated fishes decreased compared to 30dpi control in water.
6) This means that all proliferating cells produced in the presence of drug after injury at 2dpi and 4dpi fail to stay back at 30dpi compared to control

4.3. Chapter 3

4.3.1. Increased number of proliferating Muller glia is not due to increased cell death – TUNEL Assay

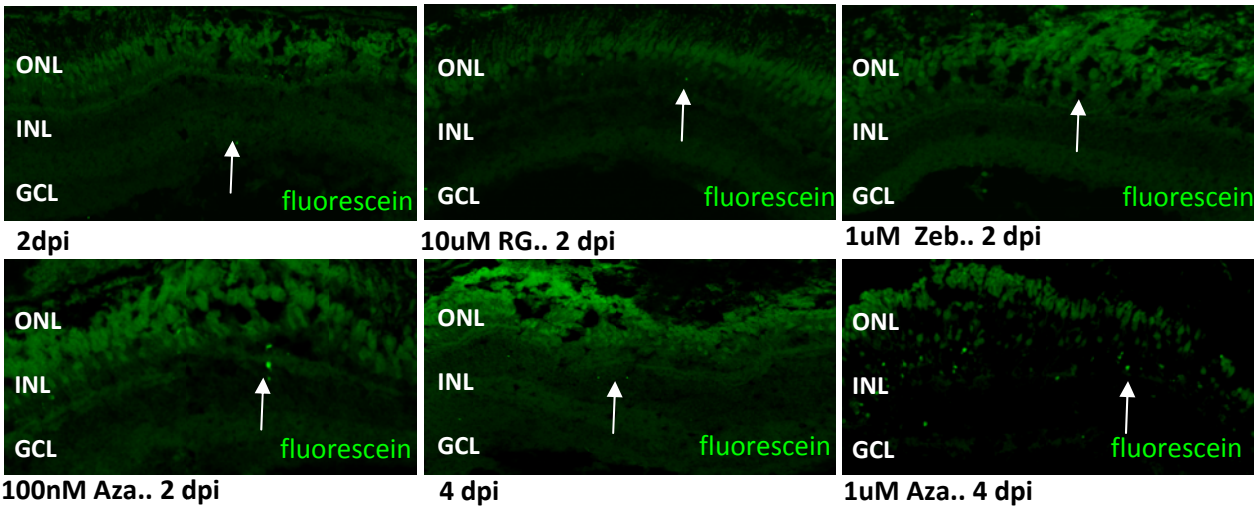


Figure 10: TUNEL Assay to label cells undergoing apoptosis at 2 dpi and 4 dpi in control and Dnmt blocker treated retinas.

Observation:

The increased number of proliferating cells at 2dpi and 4dpi in presence of Dnmt blocker is not due to increased cell death as no significant number of TUNEL positive cells were seen.

4.4. Chapter 4

4.4.1. Regulation of known pro-proliferative genes- *mmp9* and *ascl1a,* during retina regeneration in the context of pharmacological inhibition of Dnmts.

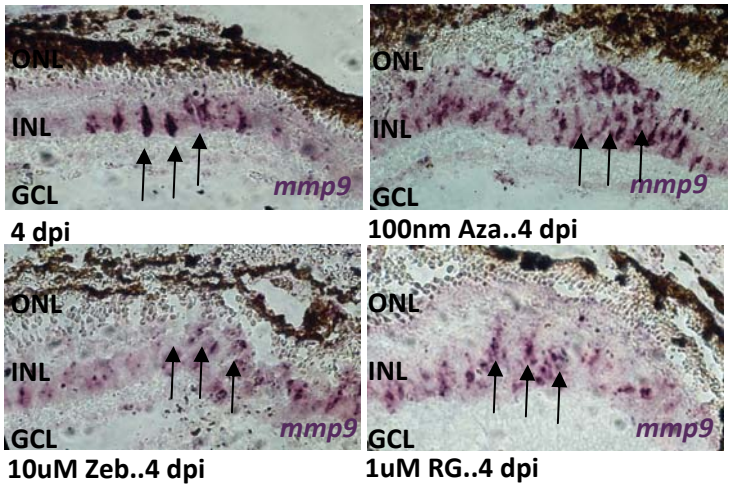


Figure 11 : *mmp9* mRNA *In situ* hybridisation in 4 dpi and Dnmt pharmacological blocker treated retinas at 4 dpi. *mmp9* mRNA signal is expressed in higher number of cells at the injury spot in drug

4.4.2. *mmp9* mRNA *in situ* hybridization co-stained with BrdU at 4 dpi in control and drug treated retinas.

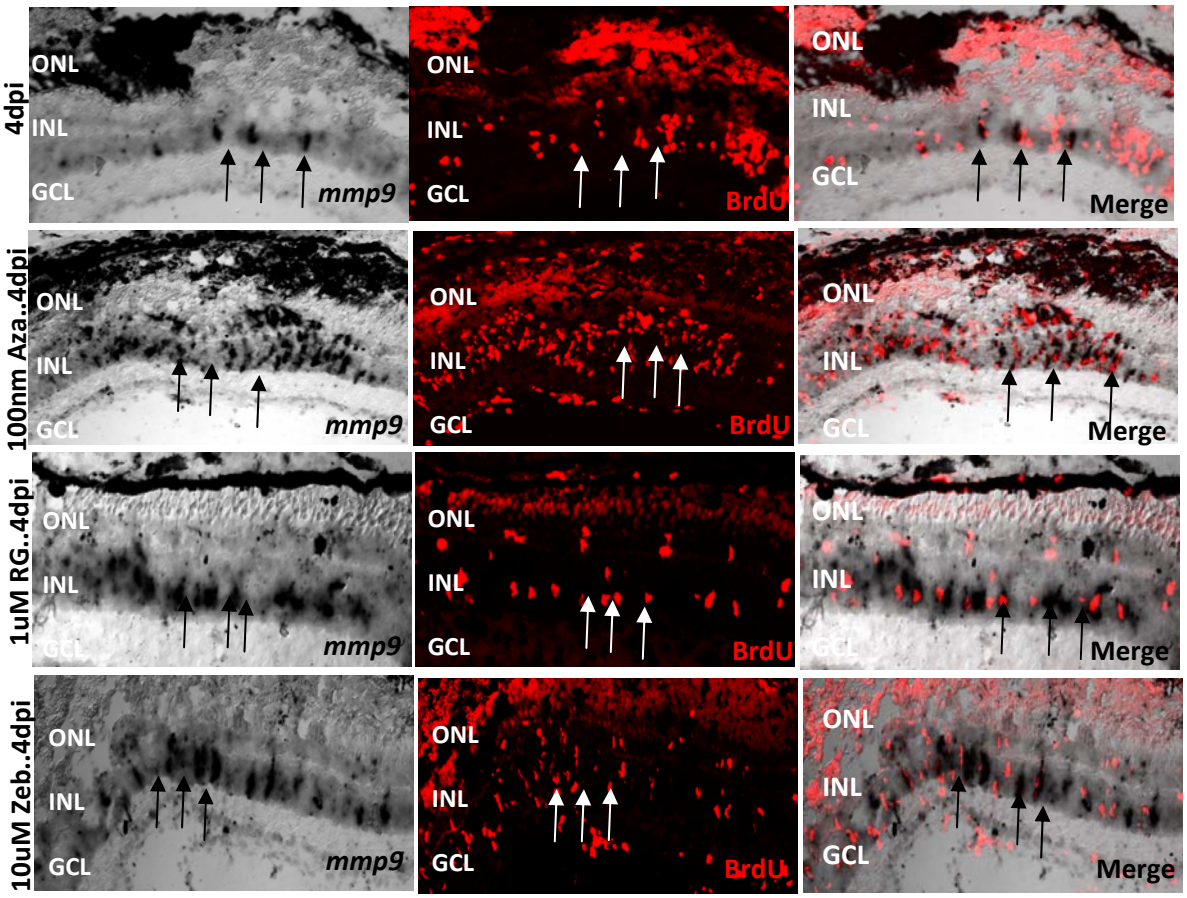
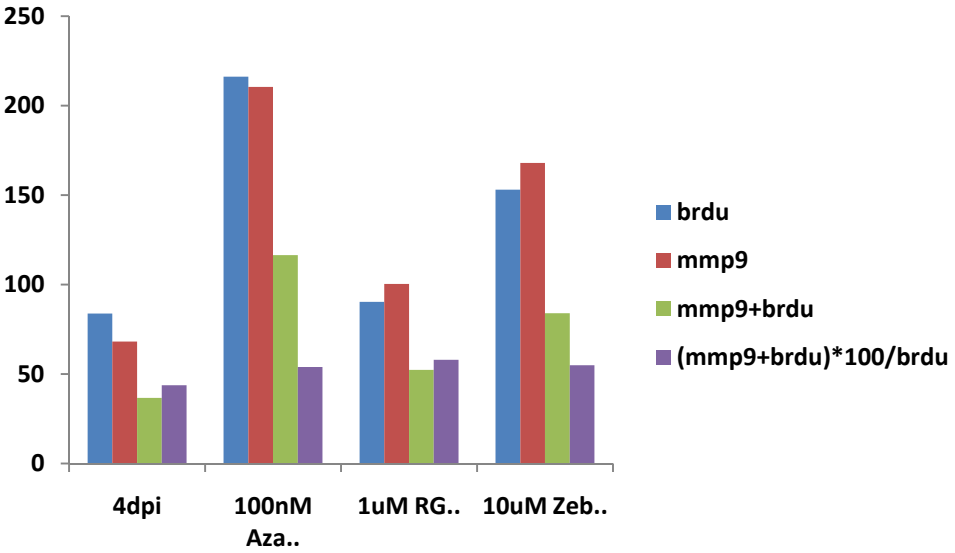


Figure 12: *mmp9* mRNA *In situ* hybridisation in 4 dpi and Dnmt pharmacological blocker treated retinas at 4 dpi co- immunostained with BrdU at 4 dpi.

4.4.3. *mmp9* mRNA *in situ* hybridization on Dnmt inhibitor treated retinas- cell count analysis at 4 dpi, qRT-PCR and RT-PCR analysis at 2dpi



Graph 8: Cell count of total BrdU positive, *mmp9* mRNA *In situ* signal positive, *mmp9* and BrdU co-localised signal positive cells present per lesion at 4dpi in control and Dnmt blocker treated samples. Purple bar indicates percentage of BrdU stained cells at injury spot also expressing *mmp9* mRNA *In situ* signal in respective samples.

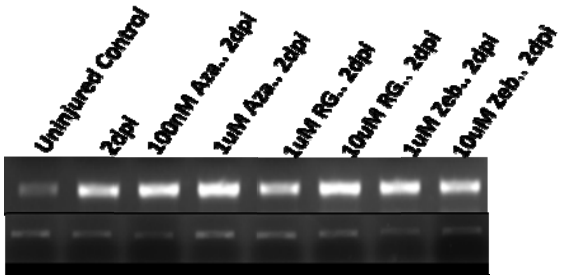
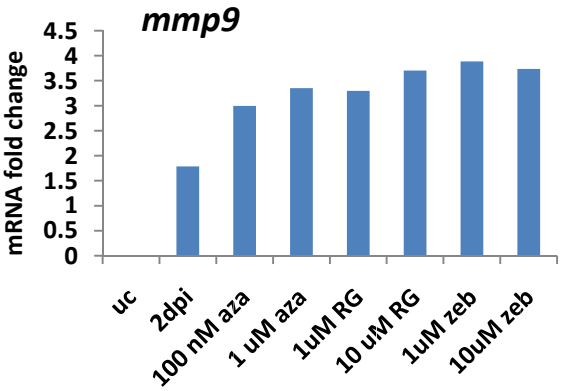
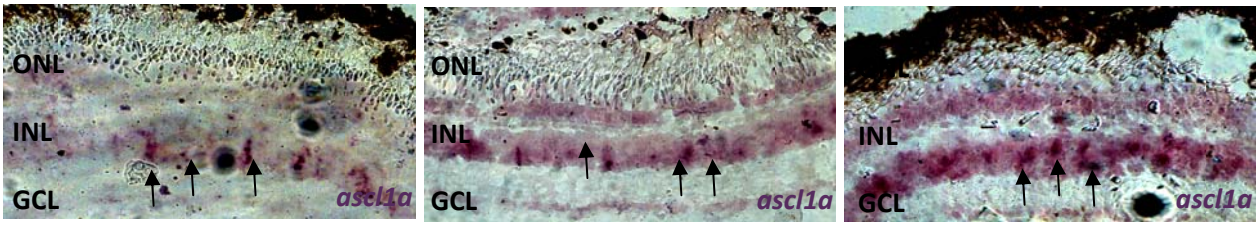


Figure 13: RT-PCR analysis of *mmp9* mRNA expression level in uninjured control, 2 dpi and 2 dpi Dnmt pharmacological blocker treated retinas



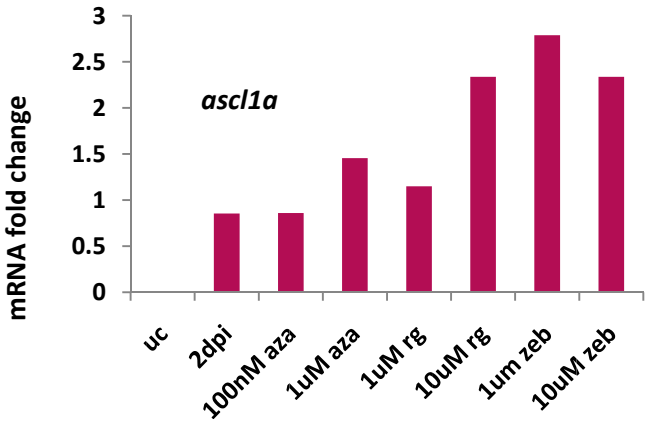
Graph 9: qRT-PCR analysis of *mmp9* mRNA expression level in uninjured control, 2 dpi and 2 dpi Dnmt pharmacological blocker treated retinas

4.4.4. *ascl1a* mRNA *in situ* Hybridization in 4 dpi control and Dnmt blocker treated retina.



4dpi **1uM RG..4dpi** **100nM Aza..4dpi**

Figure 14: *ascl1a* mRNA *in situ* Hybridization on 4 dpi control and Dnmt pharmacological inhibitor treated retinas



Graph 10: qRT-PCR analysis of ascl1a mRNA expression level in uninjured control, 2 dpi and 2 dpi Dnmt pharmacological blocker treated retinas

Observation:

1. It was also seen that some of the known pro-proliferative genes like *ascl1a* and *mmp9* is up-regulated at the site of injury from mRNA *in situ* hybridization and cell counting analysis in Dnmt inhibitor treated retina.
2. This was also confirmed by real time pcr analysis of these genes in the Dnmt blocker treated retinas at 2dpi.

4.4.5. Dnmt1 does not auto-regulate its own expression at 4dpi in Dnmt targeting pharmacological inhibitor treated retinas

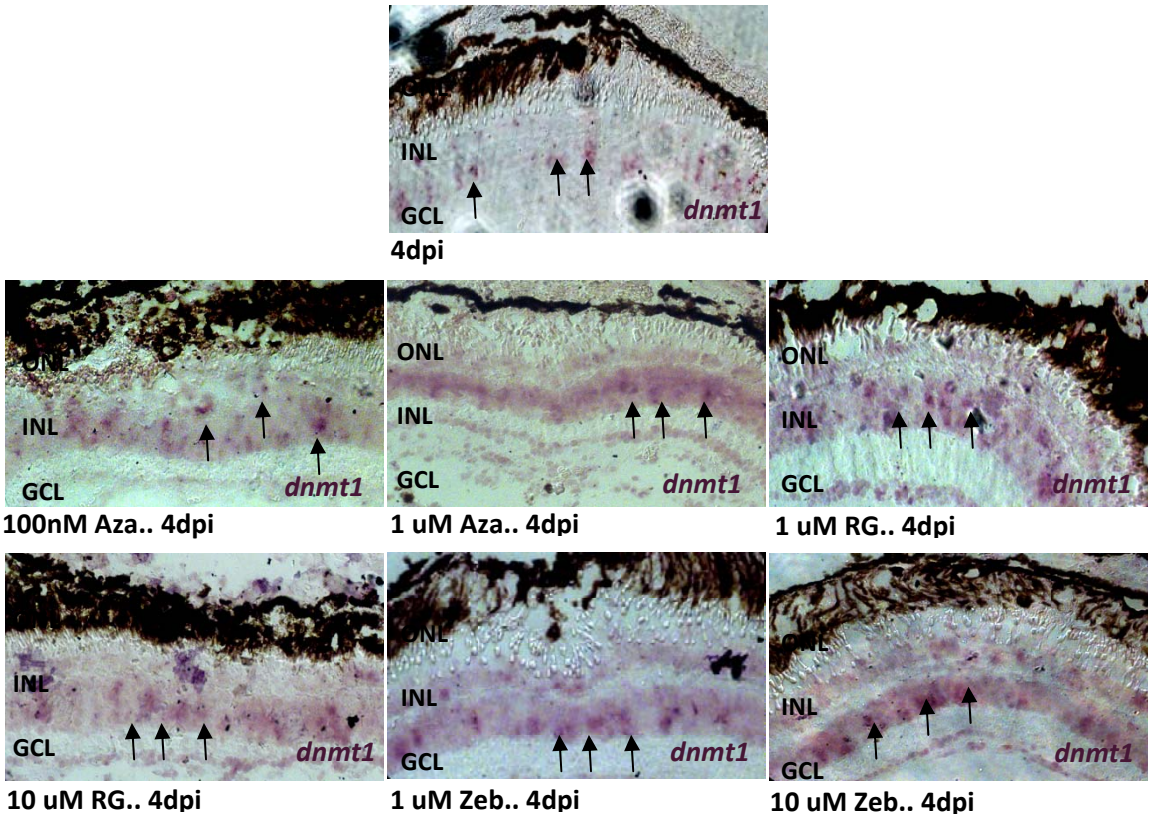


Figure 15 : dnmt1 in situ hybridization on 4 dpi in Dnmt targeting pharmacological inhibitor treated retinas

Observation:

1. *dnmt1* in situ signal is found at 4dpi at the site of injury.
2. *dnmt1* insitu signal is found at 4 dpi in Dnmt targeting pharmacological inhibitor treated retinas also at 4dpi
3. This suggests that Dnmt1 does not auto-regulate its own expression at the site of injury.

5. EXPERIMENTAL TECHNIQUES:

5.1 RNA Isolation:

Fishes were injured and Retina was dissected in PBS at different time points. Isolated Retinal tissue can be kept in Trizol or stored in -80^{o}C and then processed for RNA isolation or immediately after dissection. RNA isolation protocol is as follows:

1) Take 6 retinae (dissected in 1X PBS or 0.85% saline solution) in an MCT containing Trizol reagent (150µl or 200µl) at 4^{o}C.
2) Homogenize the tissue using 200 uL pipette. No tissue clumps should be visible.
3) Add 0.2 volume of Chloroform (40µl)
4) Shake vigorously for 30 seconds.
5) Centrifuge at 12000 rpm at 4^{o}C for 20 minutes.
6) Using a cut tip (it reduces sucking force per unit area) slowly remove the aqueous phase without disturbing the middle inter phase layer (it may otherwise cause genomic DNA contamination).
7) Add 0.6 volume of isopropanol (approximately 40 µl) and store at -80^{o}C overnight or in ice for 10 to 20 minutes.
8) Next day or after 10 to 20 minutes (ice incubation), centrifuge at 12000 rpm at 4^{o}C for 20 minutes.
9) Discard the supernatant.
10) Wash with 200 µl of 70 % EtOH or absolute EtOH. Centrifuged at maximum speed for 10 minutes at 4^{o}C.
11) Dry and dissolve the pellet in deionized H_2O and checked on agarose gel.
12) Stored at -80^{o}C.

5.2. Gel electrophoresis:

1) Weigh 1.5g of agarose and mix it in 100 mL of 1X TAE. Boil and melt it in the microwave so that it completely dissolves and no clumps are visible. When temperature becomes tolerable add 2µL of Ethidium bromide to it and cast the gel in a casting tray.
2) The solidified gel is then placed in the gel electrophoresis tank.
3) 5µl of sample and 2µl of 10X gel loading dye should be taken on a parafilm.
4) The sample dye mixture is then loaded it in the wells and gel electrophoreses is allowed until the dye moves three fourth of the gel.

5) The gel is then observed under UV light and the gel picture also taken for future reference

5.3 cDNA preparation (using superscript III first-strand synthesis system for RT-PCR):

1) Combine the following 0.2- or 0.5mL tube:

- Up to 5µg total RNA: nµL
- 50µM oligo $(dT)_{20}$: 0.5µL
- 50ng/µL random hexamers: 0.5µL
- 10mM dNTP mix: 1µL
- DEPEC-treated water: to 10µL

2) Incubate the tube at 65°C for 5 min, then placed on ice for at least 1 minute.

3) Prepare the following cDNA synthesis mix, and add each component in the indicated order:

- 10X RT buffer - 2µL
- 25mM $MgCl_2$ - 4µL
- 0.1M DTT - 2µL
- RNase out (40unit/µL) - 1µL
- Superscript III Reverse Transcriptase (200U/µL)- 1µL

4) Add 10µL of cDNA synthesis master mix to each RNA mixture, mix gently, and collect by brief centrifugation followed by incubation as follows:

- Oligo$(dT)_{20}$ primer : 50 min at 50°C
- Random hexamer primer : 10 min at 25°C followed by 50 min at 50°C
- Termination of reaction at 85°C for 5 min. Chill on ice.
- cDNA can be stored in -20°C. Or preferably in -80°C.

5.4. Reverse transcription PCR (RT-PCR):

- 20X Buffer – 1.25 µL
- dNTPs – 2.5 µL
- Forward primer - 0.5 µL
- Reverse primer - 0.5 µL
- Template - Adjust µL
- deionized H_2O - 19.35 µL
- Taq - 0.4 µL

- Total - 25µL

This mixture was collected in 0.2- to 0.5mL tube and incubated as follows for all general beta actin standardisation purposes and for checking expression pattern of other dnmts using RT primers.

- 95^{o}C – 15 sec for enzyme activation
- 95^{o}C - 1 min for denaturation
- 60^{o}C– 30 sec for annealing
- 68^{o}C – 30 sec extension
- 72^{o}C – 7 min
- 4^{o} C - infinite time

PCR product was then checked on agarose gel.

5.5. Quantitative Real-Time PCR (qRT-PCR):

Following components should be added to Axygen 0.2- 0.5 mL real time specific tubes and the reaction was set in an already calibrated- Real Time PCR machine.

- SYBr green mix - 5µL
- Primer: Forward- 0.4µL
- Reverse- 0.4µL
- Formamide- 0.4µL
- H_2O- 3.3µL
- Template- 0.5µL

Data was analyzed on excel sheet and graph was plotted.

5.6. TOPO TA cloning:

1) Gene specific band was cut from gel and collected in a 1.5mL MCT
2) Gel extraction protocol was followed.

a. Manual Gel extraction was done.

The band was cut and transferred into a 0.5mL MCT. The gel is then transferred into another MCT having little peices of aluminium foil in it and a small hole at the base created using a fine 30G needle. This setup was placed inside another MCT and taped and centrifuged at 10,000 rpm for 5 minutes. The flow through is then supplemented with an equal volume of PCI(phenyl isoamyl alcohol), mixed and centrifuged at 13ooo rpm for 10 minutes. The

aqueous phase from this step is transferred to another MCT and an equal volume of chloroform is added to it, followed by mixing and centrifuging at 13000 rpm for 10 minutes. Again the supernatant in aqueous phase is taken and transferred to another MCT to which double the volume of Isopropanol and $1/10^{th}$ volume of 10M Ammonium acetate salt solution is added and mixed well. Allow to incubate at -80 degree overnight. The next day this sample is allowed to centrifuge at 4 degree for 30 minutes and the pellet obtained is washed with 200 uL of 70% EtOH, centrifuged for 10 minutes and allowed to dry for about half hour. To the pellet around 20 uL of DEPC water or MilliQ water is added and allowed to dissolve completey. Extracted product (Insert) should be checked on agarose gel for gene specific band.

3) For cloning, following components should be added in a MCT :

- Salt solution : 0.5µL
- TOPO TA vector: 0.3µL
- Insert: 1µL
- H_2O: 1.2µL
- Total: 3µL

5) Transformation:

- Competent cells stored at -80^oC were thawed on ice.
- 3µL of plasmid was added in it, mixed gently (only tapped, should not pipette)
- Incubated on ice for half an hour
- Heat shocked at 42^oC for 45 sec and immediately kept on ice for 5 min'
- Added 1 mL of LB media in it (inside the hood).
- Incubated at 37^oC for 30 min.
- Cells were plated on Amp-resistant LB plates.
- Incubated plates at 37^oC overnight.
- Next day, single colonies were visible.
- Single colonies were streaked on LB agar + IPTG + X-Gal plates. Blue and white colonies were visible next day.
- White colonies were selected and dissolved in 20µL H_2O in a MCT.
- Colonies were mixed well using pipette and centrifuged at 10,000rpm for 10min.

- This solution was added in a culture vial (5mL of LB media). Placed overnight at 37^{o}C for growth.

5.7. Plasmid Isolation:

1) 1.5mL of Overnight incubated culture was centrifuged at 10,000 rpm at room temperature for 2 min (2 times).

2) Add 100µL of prechilled H_2O to the pellet. Pellet was resuspended by gentle vortexing.

3) Add 100µL of freshly prepared lysis buffer in it.

4) Mix by gentle tapping, no vortexing.

5) Boil for 2 min at 100^{o}C.

6) Add 50µL of 1M $MgCl_2$ (to get rid of chromosomal DNA). Tapped and kept in ice for 2 min.

7) Spin it at 12,000 rpm for 2 min at RT.

8) Add 50µL of 3M potassium acetate buffer in it.

9) Tap it immediately and centrifuged at 10,000 rpm for 2 min at RT.

10) Supernatant should be decanted in a fresh MCT and 60µL isopropanol was added in it.

11) Keep on ice for 5 min.

12) Centrifuge at maximum speed for 2 min at RT.

13) 70% EtOH wash was given and pellet dried.

14) Pellet is then dissolved in 50µL of TE/autoclaved DEIONIZEDH_2O. Stored at -20^{o}C

Composition of solutions used for Plasmid isolation:

Lysis buffer: 920µL H_2O, 50µL 20%SDS, 20µL 0.5M EDTA, 10µL 10N NaOH

Potassium acetate buffer: 60mL potassium acetate, 11.5mL glacial acetic acid, 28.5mL H_2O, Total : 100mL (stored at 4^{o}C)

5.8. Plasmid digestion: *dnmt1*-TOPO clone:

1) Following components should be combined in a MCT:
   - i) H_2O - 37 µL
   - ii) buffer- 5 µL
   - iii) DNA- 7 µL

iv) enzyme stuI- 1 µL

Total- 50 µL

2) Incubate at 37°C for 3 hours.
3) Check on agarose gel
4) Manual Gel extraction protocol was followed:

Excise the DNA fragment from an agarose gel using sterile blade/ scalpel. Centrifuged for 1 min at 11,000 rpm and stored at -80°C or immediately used for RNA probe making.

5.9. RNA probe making:

1) Following components should be added in an MCT:
   - Buffer (10X) NEB 3.1- 4µL
   - Template(digested DNA)- 7µL (100-500ng)
   - Dig-UTP- 2µL
   - T7 RNA polymerase- 2µL
   - H_2O- 25µL
   - Total- 40µL

2) Incubate at 37°C for 4 hours.
3) Stop the reaction by adding 0.5M Tris EDTA (4µL).
4) For precipitation add 5M $LiCl_2$ (4µL).
5) To add weight, add 10mg/mL glycogen (2µL).
6) Tap it and add 70µL of absolute EtOH.
7) Tap again and keep in -80°C overnight or one hour.
8) Centrifuge at 4°C for 15 min.
9) 100% EtOH wash should be given (200µL) – and centrifuged for 10 min.
10) Dry and dissolve in 50µL of DEPEC H_2O.
11) Store at -80°C after aliquoting.

5.10. Tissue fixation:

1) Remove lens from eye in 4% PFA in 1X phosphate buffer and eye was kept in a MCT containing 600µL of 4% PFA in 1X phosphate buffer.
2) Mix on rotator for two hours at RT on same day or next day.

3) Pipette out old solution and added 600µL of 5% sucrose in MCT. Mix on rotator for 45 min at RT.

4) Pipette out old solution and added 400µL of 5% and 200µL of 50% sucrose in MCT. Mix on rotator for 45 min at RT.

5) Pipette out old solution and added 300µL of 5% and 300µL of 20% sucrose in MCT. Mix on rotator for 45 min at RT.

6) Pipette out old solution and added 200µL of 5% and 400µL of 20% sucrose in MCT. Mix on rotator for 45 min at RT.

7) Pipette out old solution and added 600µL of 20% sucrose in MCT. Mix on rotator for 45 min at RT.

8) Add 600µL of OCT in it and mixed on rotator for 30 min.

9) Tissue was fixed and block was made using OCT and eye was kept in it.

Composition of solutions used for tissue fixation:

- 4% PFA in 1X Phosphate buffer:
- 2g PFA
- 5mL of 10X phosphate buffer
- Volume made up till 50mL using autoclaved deionized H_2O (stored at -20^oC)

2) 5% sucrose:

- 2.5g sucrose dissolved in 50mL of autoclaved deionized H_2O (stored at 4^oC)

3) 20% sucrose:

- 10g sucrose dissolved in 50mL of autoclaved deionized H_2O (stored at 4^oC)

Fixed tissue should be sectioned using cryosectioner and slides stored at -20^oC, which can be used for *In-Situ* Hybridization, TUNEL Assay or Immunostaining.

5.11. PCNA and BrdU Immunostaining:

1) Slides stored at -20^oC were incubated at 37^oC for 30 min

2) Wash the slides using 1X PBS (twice- 10 min each)

3) Then treat the slides with 2N HCL (should be at 37^oC before hand) for 20 min

4) Wash the slides with 0.1M sodium borate solution (twice- 10 min each)

5) Block the sections using 3% BSA +0.1% Triton in 1X PBS for 30 min.

6) Overlay the slides using anti-Mouse 1^{o} Ab for PCNA (1µL Ab was diluted in 500µL of 1X PBST+1% BSA). In case of BrdU, anti-Rat 1^{o}Ab for BrdU(1 µL Ab was diluted in 500µL of 1X PBST+1% BSA) was used.

7) Keep the slide container at 4^{o}C overnight.

8) Next day slides were washed using PBST (0.1% Triton) (thrice-10 min each).

9) Overlay the slides using anti-Rabbit 2^{o} Ab (0.5µL Ab was diluted in 500µL of 1X PBST+1% BSA).

10) Slides were incubated for 3 hours at RT.

11) Wash slides with PBST (thrice- 10 min each)

12) Wash slides with autoclaved deionized H_2O (thrice-10 min each)

13) Dry for one hour at RT.

14) Slides should be mounted using DABCO and stored at -20^{o}C or immediately proceeded for imaging.

Composition of solutions used for PCNA Immuno-staining:

- 1X PBS: 5mL PBS (from 10X stock)+ 45mL autoclaved MQ
- 1X PBST: 5mL PBS (from 10X stock)+ 45mL autoclaved MQ+ 0.1% Triton(50µL)
- 1X PBST + 3% BSA: 5mL PBS (from 10X stock)+ 45mL autoclaved MQ+ 0.1% Triton(50µL) + 1.5gm BSA
- 1X PBST+ 1%BSA: 5mL PBS (from 10X stock)+ 45mL autoclaved MQ+ 0.1% Triton(50µL)+ 0.5gm BSA
- 0.1M Sodium Borate: 9.53g Sodium Borate dissolved in 500mL of deionized H_2O

5.12. mRNA *in situ* Hybridization:

Day 1: Hybridization:

1) Hydrate the slides in an EtOH series and SSC for 1 min each :
   a. 100% EtOH

b. 95% EtOH
c. 70% EtOH
d. 50% EtOH
e. 2XSSC

2) Incubate slides in Proteinase K solution for 1-5 min at 37^{o}C
   a. Prewarm Proteinase K buffer to 37^{o}C
   b. Add 250µL of 10mg/mL proteinase K
3) Rinse slides briefly in room temp DEPEC water.
4) Rinse slides in 0.1M TEA pH 8.0 for 3 min.
5) Rinse in Acetic anhydride/TEA for 10 min
   a. Add 130µL of acetic anhydride to dry dish.
   b. Add 50mL of TEA
6) Dehydrate the slides in SSC and EtOH series for 1 min each:
   a. 2X SSC
   b. 50% EtOH
   c. 70% EtOH
   d. 95% EtOH
   e. 100% EtOH
7) Slides should be air dried for at least 1 hour at RT.
8) Hybridization solution should be pre warmed at 56^{o}C (200 to 300ng per slide).
9) Probe preparation:
   a. 4µL probe should be added to hybridization solution and mixed.
   b. Boil at 100^{o}C for 5 min.
   c. Cool immediately on ice
10) Add 60µL of Hyb/probe solution to each slide and coverslip with siliconized hybrid slips should be placed.
11) Place slides in humid chamber dampened with 50% formamide/5X SSC and incubate at 56^{o}C overnight.

Composition of solutions used for In-Situ 1st day:

1) 20X SSC
   - Dissolve 87.6g of NaCl in350mL of DEPEC H_2O
   - Add 44.12g sodium citrate
   - Make up final volume to 500mL with DEPEC H_2O.

2) TEA solution
    - 9.3g Triethanolamine (TEA)
    - Make up upto 490mL with DEPEC H_2O
    - Add 12-14 NaOH pellets
    - Adjust pH to 8.0, bring up to 500mL
3) Proteinase K buffer
    - 25mL 1.0 M Tris-HCL
    - 25mL 0.5M EDTA
    - Bring up to 250mL with DEPEC H_2O
4) Hybridization solution (50mL)
    - 3.6mL TEN solution
    - 25mL 100% formamide
    - 10mL 50% Dextran sulphate
    - 5mL 10% RMB blocker
    - 6.4mL DEPEC H_2O
    - Store at -20^{o}C
5) TEN solution
    - 5mL of 1.0M Tris-HCL, pH 7.5
    - 30mL of 5M NaCl
    - 1mL of 0.5M EDTA

Day 2: Post Hybridization

1) Preheat 50% formamide/2X SSC solution to 65^{o}C.
2) Preheat two 50mL RNase buffer washes, one to 37^{o}C and the other to 65^{o}C.
3) Preheat two 2X SSC washes to 37^{o}C.
4) Soak slides with coverslips in 2X SSC for 30 min at RT on shaker table
    a. If the slides do not come off, gently tease them apart from slide with forceps.
5) Rinse slides in 50% formamide/2X SSC solution for 30 min at 65^{o}C.
    a. Gently agitate for the first 5 min.
6) Rinse slides in 2X SSC for 10 min at 37^{o}C (twice).
7) Add 100µL of RNase (10mg/mL) to the 37^{o}C RNase buffer: Incubate slides for 30 min.
8) Wash slides in 65^{o}C RNase buffer for 30 min.

9) Wash slides for 2-3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
    a. Thaw 3mL aliquot
    b. Add 7mL of deionized H_2O.
10) Wash slides in 1X Maleate buffer for 5 min (twice).
11) Incubate slides with 205µL antibody (of choice) diluted in 1X Maleate/0.05% Triton X-100/1% RMB blocker solution overnight at RT.
    a. Add antibody solution to cover

Composition of solutions used for In-situ 2nd day:

1) RNase buffer:0.5M NaCl, 10mM Tris-HCL,1mM EDTA
    - 29.23g NaCl
    - 10mL 1.0M Tris- HCl, pH 7.5
    - 2mL 0.5M EDTA
    - Brought up to 1L with deionized H_2O
2) 1X Maleate/0.05% Triton X-100/1% RMB blocker solution
    - 2mL of 5X Maleate stock
    - 5µL Triton X-100
    - 1mL of 10% RMB blocker
    - Made 3mL aliquots and freezed at -20^{o}C
3) 5X Maleate buffer
    - 8g Maleic acid in 850mL deionized H_2O
    - pH to 7.5 using lots of NaOH pellets
    - Added 43.8g NaCl
    - Brought up to 1L with deionized H_2O

Day 3

1. Wash slides twice with 1X Maleate buffer for 5 minutes.
2. Incubate twice for 5 minutes each in Genius buffer.
3. Add NBT/BCIP, incubated overnight at room temperature in dark.
4. Colour detection: In bright field microscope, slides were visualized at time intervals to check whether reaction worked or not.

6. SUMMARY & CONCLUSIONS

Quantitative PCR of *dnmt1* and *dnmt8* reveals that mRNA expression level decreases in the whole-retinal tissue at around 4 dpi and 2 dpi respectively. *dnmt8* mRNA level then again increases from 11 dpi to 21 dpi compared to that of uninjured control. *dnmt1* and *dnmt8* mRNA expression decreases relative to uninjured control in the injured retina. *dnmt1* seems to be induced at 4dpi slightly but the level does not cross that of the uninjured control. This can be because *dnmt1* is present at basal level pan retinally before and after retinal injury but only preferentially expressed at the injury spot at 4 dpi, the peak of proliferation. *dnmt1* mRNA *in situ* hybridization result suggests that *dnmt1* is present at a basal level pan-retinal in the uninjured retina. At 2dpi, the de-differentiation phase, a very slight color reaction seems to develop in the INL nearby the injury spot indicating a very low expression level of *dnmt1* gene.

At 4 dpi, when the cell proliferation peaks, *dnmt1 in situ* signal is found to preferentially increase at the site of injury in the INL. The Muller glia that responds to injury is about 2% of the total Muller glia cells. At 4 dpi it was observed that the basal signal of *dnmt1* which was present at the areas of the retina excluding the injury spot also decreased compared to uninjured control retina. At 7 dpi, there was no injury site specific expression of *dnmt1* mRNA, it was quite similar to that of uninjured control retina, with a pan- retinal basal level expression of *dnmt1* mRNA. Therefore, this suggests that *dnmt1* mRNA expression is selectively induced at 4 dpi at the injury spot in the retina and in this process, *dnmt1* mRNA level decreases in the neighbouring un- injured regions of the retina. However it is not clear whether it is the proliferating Muller glia at the site of injury that had responded to injury initially that expressed *dnmt1*. To find this out, a BrdU co-immuno-staining with *dnmt1* mRNA *in situ* signal was done at 4 dpi, and the cell expressing *dnmt1*, BrdU and both *dnmt1* mRNA and BrdU at 4 dpi at the injury spot was counted and analysed.. It was observed that *dnmt1* mRNA signal and BrdU did not co-localise perfectly at 4 dpi. This can be because *dnmt1* is required for cell cycle exit of the proliferating Muller glia cells that had responded to injury. Therefore, those Muller glia that express a higher signal of *dnmt1* mRNA and less BrdU signal has probably come out of the cell- cycle and is no longer proliferating. Hence they will not be able to uptake BrdU unlike the cells expressing high BrdU and low *dnmt1* mRNA *in situ* signal that just entered cell cycle and is also proliferating at the site of injury.

After continuous dipping of the uninjured fishes in various concentrations of Dnmt targeting pharmacological blockers, until the fourth day the retina was isolated and PCNA immunostaining was done. It was seen that there was no induction of freshly proliferating cells in the absence of injury but in the presence of drug. However, in the presence of injury, 100 nM and 1 μM Azacytidine acts as a Dnmt blocker and increases the number of proliferating cells after retinal injury at 2 dpi and 4 dpi, 1 μM and 10 μM RG108 acts as a Dnmt blocker and increases the number of proliferating cells after retinal injury at 2 dpi and 4 dpi, 1 μM and 10 μM Zebularine acts as a Dnmt blocker and increases the number of proliferating cells after retinal injury at 2 dpi and 4 dpi. At 30 dpi, a higher number of BrdU positive cells than control was observed in drug treated fishes with no preferential bias to ONL, INL or GCL. However the proportion of BrdU positive cells staying back at 30 dpi to the BrdU positive proliferating cells in 4dpi in Dnmt blocker treated fishes decreased compared to 30dpi control in water. This means that all proliferating cells produced in the presence of drug after injury at 2dpi and 4dpi fail to stay back at 30dpi compared to control. The increased number of proliferating cells at 2dpi and 4dpi in presence of Dnmt blocker is not due to increased cell death as no significant number of tunel positive cells were seen.

It was also seen that some of the known pro-proliferative genes like *ascl1a* and *mmp9* is up-regulated at the site of injury from mRNA *in situ* hybridization and cell counting analysis in Dnmt inhibitor treated retina. This was also confirmed by real time pcr analysis of these genes in the Dnmt blocker treated retinas at 2dpi.

7. CONCLUDING REMARKS

DNA methyltransferases (Dnmts) are regulated post retinal injury in zebrfish as evident from the RT-PCR and qRT-PCR data. *dnmt1* being the only maintenance methyltransferase in zebrafish is induced at the site of injury at 4 dpi in 56.51% of the then proliferating Muller glia, indicating the anti-proliferative nature of *dnmt1*. The lack of complete co-localization of all BrdU positive proliferating and *dnmt1* mRNA expressing Muller glia at 4dpi can be because Dnmt1 is required for cell cycle exit of the proliferating MG cells since cells having intense BrdU signal are lower in the *dnmt1* mRNA level and vice-versa.

Treatment of fishes with pharmacological Dnmt blockers indicates an increased number of proliferating (BrdU positive) cells at 2dpi and 4dpi per lesion. At 30 dpi, a higher number of BrdU positive cells than control was observed in drug treated fishes with no preferential bias to ONL, INL or GCL. However the proportion of BrdU positive cells staying back at 30 dpi to the BrdU positive proliferating cells in 4dpi in Dnmt blocker treated fishes, decreased compared to 30dpi control in water. This means that all proliferating cells produced in the presence of drug after injury at 2dpi and 4dpi fail to stay back at 30dpi compared to control. The increased number of proliferating cells at 2dpi and 4dpi in presence of Dnmt blocker is not due to increased cell death as no significant number of tunel positive cells were seen. Even in the presence of drug, *dnmt1* mRNA is induced at site of injury indicating that *dnmt1* is not auto-regulatory.

It was also seen that some of the known pro-proliferative genes like *ascl1a* and *mmp9* is up-regulated at the site of injury from mRNA *in situ* hybridization and cell counting analysis in Dnmt inhibitor treated retina. This was also confirmed by real time PCR analysis of these genes in the Dnmt blocker treated retinas at 2dpi. This may be because the promoter of these pro-proliferative genes may harbor some potential CpG islands that were subject to the regulation by Dnmts during retinal injury and recovery that regulated and restricted their expression at the injured location. So the increased number of proliferating cells that one finds at 2 dpi and 4 dpi at the injury spot when the fishes were dipped in Dnmt pharmacological inhibitor might be because Dnmts had a role to play in restricting and timing the injury response. So in the absence of the Dnmt mediated regulation, maybe the whole regenerative response got accelerated and showed higher number of proliferating cells at 2 dpi and 4 dpi.

8. FUTURE PERSPECTIVES

We already understand that DNA methyltransferases are regulated post retinal injury. Moreover, in the absence of Dnmt during retinal injury, the number of proliferating cells increase at 2 dpi and 4 dpi compared to control. This can be because the whole regeneration response is accelerated due to absence of Dnmt mediated gene regulation. It will interesting to see what the effect of blocking Dnmt after 2 dpi and then collecting the retinas at 4 dpi will be. The number of proliferating cells in this case will tell us the role of dnmts during active proliferation phase once the dedifferentiation is completed by 2dpi. Next we can try to block Dnmt until 2dpi and let the fish survive until 4 dpi for next two days in water and again collect the retina and see the difference in the number of proliferating cells. We can also injure the fishes and let them survive in water until 4 dpi and then block Dnmts and collect the retina at 7 dpi and see the proliferative response in this case too. Such regime experiments will tell us whether the drug is by itself creating a fresh injury response or not.

One major drawback of using pharmacological inhibitors of Dnmt is that we are never sure of the specificity of the drug and whether it has some off target effects. Therefore to be on the safe side, we can try to knock down individual dnmt genes by using different dnmt gene specific morpholinos and see the effect of dnmt gene knockdown on Muller glia mediated de-differentiation, proliferation and re-differentiation following retinal injury.

Since the fraction of BrdU labelled proliferating cells at 4 dpi that contributes to the re-differentiated and migrated cell population at 30 dpi in the Dnmt blocker treated retinas is lesser than that of 30 dpi control retinas, it is obvious that the portion of the proliferating cells dying after 4 dpi in the Dnmt blocker treated retinas is greater than that of control. It would be interesting to find out when exactly these extra cells are dying during the re-differentiation phase and the reason why they are dying too.

It is possible that the early event of Muller glia mediated de-differentiation may be the after effect of some de-methylation event in some regeneration specific genes. The pre- requisite for DNA Methylation or de- methylation mediated gene regulation is that the promoter and enhancer elements of such genes harbour CpG islands. Therefore, using an already known list of genes that is absolutely essential for regeneration, one can perform an in- silico analysis to select out those genes whose promoter elements harbour such CpG islands. It is possible that such genes may be regulated during retinal regeneration via DNA methylation/ de-methylation mediated epigenetic gene regulation. Such genes are excellent candidates to study the role of

Dnmt. It would be worthwhile to monitor what happens to the expression of these genes in the context of Dnmt inhibition both globally, by using pharmacological inhibitors as well as by specific blockade of specific dnmt genes by using morpholinos. We could block the expression one of the early-induced gene that is regulated through epigenetic mechanism and see if it was important for normal regeneration. Also this could provide information if any of the later induced gene expression got affected because of this initial blocking, that is necessary for normal regeneration.

To test if Muller glia de-differentiation can occur through an early DNA de-methylation event, we can use the drug 3-amino benzamide known to cause DNA methylation and see if the drug mediated DNA hyper methylation could block the early Muller glia de-differentiation event immediately after the retinal injury. We can then compare the effects of DNA hyper-methylation on Muller glia de-differentiation can be reversed or homeostasis re-stored by blocking the DNA methylation event through pharmacological reagents like 5-Azacytidine- and 5-aza-deoxycytidine (5-aza-CdR), which is capable of blocking the maintenance methyl transferase DNMT1, . This way we can understand whether the restoration of homeostasis was through DNA methylation event.

We can also see whether the conditional genetic activation or inactivation of DNA methylation on selected genes can also affect the retina regeneration by functionally evaluate the role of selected CpG island in the retina regeneration events. This would be largely achieved through conditional deletion of the endogenous CpG islands through either CRISPR-CAS system or TALEN mediated DNA deletion *in vivo.*

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10. APPENDIX

Primer Sequences used in this study are:

dnmt1-RT-Fwd	5'ACCTTTGGTGTGCTGCAGGCTGGAC3'
dnmt1-RT-Rev	5'AACCAGGGCACTCATGTCCTTGCAG3'
dnmt1-FL-Fwd	5'CAAAATCGAACTTGAAATGCCTACC3'
dnmt1-FL-Rev	5'ATAAAACATCACATGAATGGCACTGC3'
*dnmt*3-RT-Fwd	5'GACGGACGGTGGTGgttatg3'
*dnmt*3-RT-Rev	5'CTGACAAAAAGCAGCACctgagc3'
*dnmt*3-FL-Fwd	5'catgacagctgctgtcgCTC3'
*dnmt*3-FL-Rev	5'GTCACAGCTCAACATGGGAAAGC3'
*dnmt*4-RT-Fwd	5'caagatgactgccacgGCTG3'
*dnmt*4-RT-Rev	5'ctgttcacactctcatCTGCGG3'
*dnmt*4-FL-Fwd	5'ccgtgttgccaagTTCGG3'
*dnmt*4-FL-Rev	5'accacacattaaggcatcagagtgc3'
*dnmt*5-RT-Fwd	5'ggagtacatcacctgctcagAAACTC3'
*dnmt*5-RT-Rev	5'tggagtctgtctgcagatggc3'
*dnmt*5-FL-Fwd	5'CATGACAGCTGCTGTCGctg3'
*dnmt*5-FL-Rev	5'gctttcccatgttgagctgtgac3'
*dnmt*6-RT-Fwd	5'tgatgggatcgcaacagGGC3'
*dnmt*6-RT-Rev	5'CGACCGGTGCCCTcgtag3'
*dnmt*6-FL-Fwd	5'gtgcactgagatggagagGGTG3'
*dnmt*6-FL-Rev	5'TATAGGGACCAGACGCAGTAAGCG3'
*dnmt*7-RT-Fwd	5'ggagcaatgtcgttcagGTGC3'
*dnmt*7-RT-Rev	5'tcgttcacaggaactggCTCTG3'
*dnmt*7-FL-Fwd	5'TCCTAGGACGGTCCTGGAGC3'
*dnmt*7-FL-Rev	5'attagatgccagtccaatgaggcc3'
*dnmt*8-FL-Fwd	5'caaccatgaccaggactttGAGC3'
*dnmt*8-FL-Rev	5'gaagtgtcctgtggttgaatggtc3'