Deciphering the Role of Developing Brain Homeobox Genes During Zebrafish Retina Regeneration

Kranti Yuvaraj Karande

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

Indian Institute of Science Education and Research Mohali
April 2015

Certificate of Examination

This is to certify that the dissertation titled "**Deciphering the Role of Developing Brain Homeobox Genes During Zebra fish Retina Regeneration** " submitted by **Ms. Kranti Yuvaraj Karande** (Reg. No. MS10078) for the partial ful- filment 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. Shashi Bhushan Pandit Dr. Kuljeet Singh Sandhu Dr.Rajesh Ramachandran

(Supervisor)

Dated: 24th April, 2015

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.

Miss. Kranti Yuvaraj Karande

(Reg.No. MS10078)

Dated : April 24th, 2015

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 have taken efforts in this thesis project. However, it would not have been possible without the kind support and help of many individuals and IISER Mohali. I would like to extend my sincere thanks to all of them.

I am highly indebted to Dr.Rajesh Ramachandran, IISER Mohali for his guidance and constant supervision as well as for providing necessary information regarding the project and also for his support in completing the project. I am equally grateful to IISER Mohali for making me part of it and providing all kind of facilities. With much appreciation, I would like to mention the help and support that I have received from my lab members. Last but not the least I would like to thank my friends and parents who helped me a lot besides their busy schedule and gave me moral support.

Miss. Kranti Yuvaraj Karande

(Reg.No.MS10078)

List of Figures and Photographs

Notation

Ascl1a-	Achaete-Scute homolog1a
BSA –	Bouveine Serum Albumin
cDNA-	complementary Deoxyribose Nucleic Acid
DAPT -	(*N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethylester
Dbx-	Developing Brain Homeobox genes
DEPC-	DiEthylPyrocarbonate
Dig-UTP-	Digoxigenin- Uridine Tri Phosphate
DNA-	Deoxyribose Nucleic Acid
dNTP -	deoxyribose Nucleotide Tri Phosphate
Dkk-	Dickkopf
DTT-	Dithrithreitol
EDTA-	Ethylene Diamine Tetraacetic Acid
EtBr -	Ethidium Bromide
EtOH-	Ethyl Alcohol
HB-EGF-	Heparin Binding- Epidermal Growth Factor
HCl -	Hydrochloric Acid
H_2O-	Water
IPTG -	Isopropyl β-D-1 thyogalactopyranoside
Insm1a-	Insulinoma associated 1a
KCl-	Potassium Cholride
$LiCl_2$-	Lithium Chloride
MG-	Muller Glia
$MgCl_2$-	Magnesium Chloride
mL-	Milli Litre
NaCl-	Sodium Chloride
$NaH_{2P}O_4$-	Sodium phosphate
NaOH-	Sodium Hydroxide
PBS-	Phosphate Buffer Saline
PBST-	Phosphate Buffer Saline Triton
RT -	Reverse Transcription
SDS-	Sodium Dodecyl Sulphate
SSC-	Saline Sodium Citrate
TAE-	Tris Acetate EDTA
Oligo dT-	Oligo deoxythymine
rpm-	rotations per minute
RNA-	Ribose Nucleic Acid
XAV939-	3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl) phenyl]-4H-thiopyrano[4,3-d]pyrimidin-4-one $C_{14}H_{11}F_3N_2OS$
µg-	micrograms

Contents

Abstract

In contrast to mammals, Zebra fish shows complete retinal regeneration in response to injury. Muller Glia cells play a very critical role in the process of Retina regeneration in Zebra fish. Process of retinal regeneration gets completed in three stages: Dedifferentiation, Proliferation, and Re differentiation. Here I report that, Developing Brain Homeobox Genes are robustly expressed in Ganglion cell layer, Horizontal cells in INL and Muller Glia of regenerating retina also, playing a role in the process of regeneration. I also report that, dbx expression might be regulated by Wnt-β catenin signalling pathway.

Chapter 1

1. Introduction

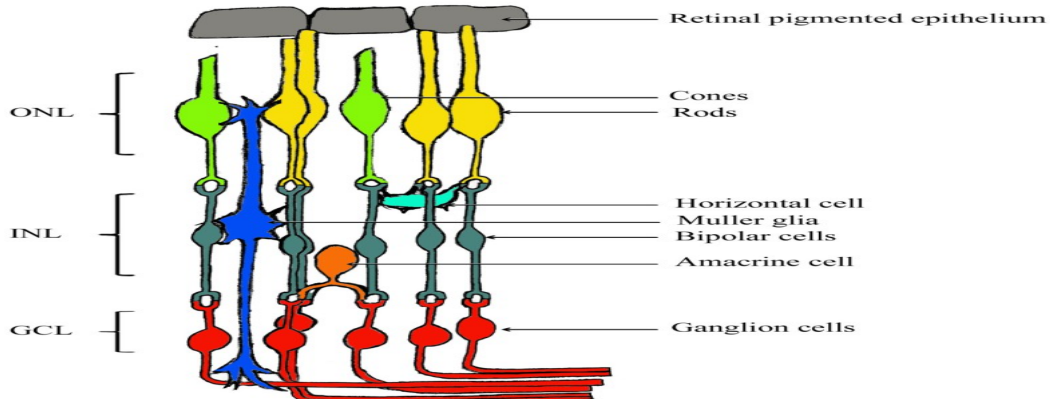
1.1 Retina regeneration study using Zebra fish as a model organism

Vision is one among our precious five senses. Loss of vision can affect our life to an extreme extent. Zebra fish show complete retinal regeneration in response to injury. This regenerative response relies mainly on dedifferentiation and proliferation taking place in Muller Glia cells. The structure of Human Retina and Zebra fish retina are very much similar. In mammals, Muller Glia response to retinal injury is insufficient for repairing damaged retina (b). The main focus of our lab is to decipher the secrets behind Zebra fish Retina regeneration and applying it for curing human retinal disorders.

Zebra fish (*Danio rerio*) is a tropical fresh water fish. It is a popular aquarium fish native to Himalayan region. The name is derived from the zebra stripes present on the body. Their life span is 4 to 5 years. Males are Torpedo shaped and females have big whitish belly (*Wikipedia*).

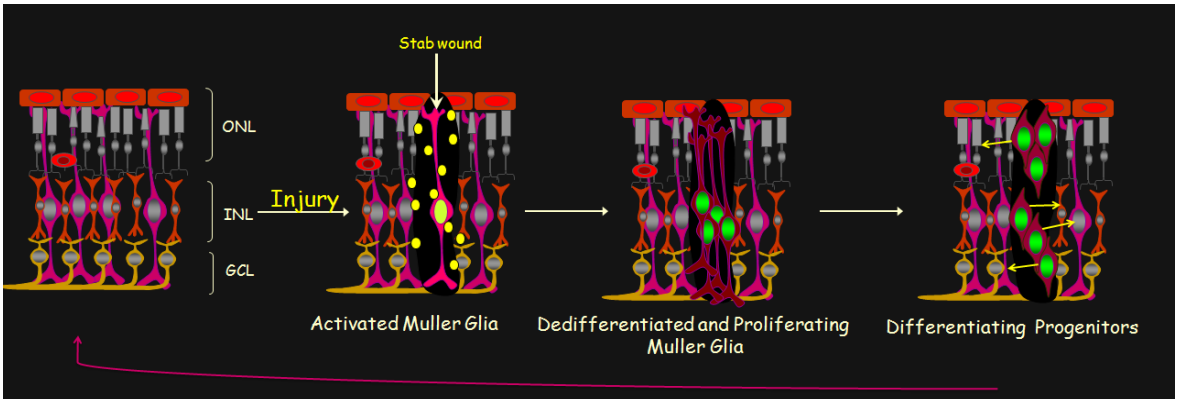
Kingdom: Animalia
Phylum: Chordata
Class: Actynopterygii
Order: Cypriniformes
Family: Cyprinidae
Genus: Danio *(wikipedia)*

1.2 Structure of Zebra fish retina and retina regeneration overview:



Zebra fish retina is divided into three sections: ONL, INL and GCL. ONL consists of photoreceptor cells. Rod cells can function in less intense light and cone cells are important for colour vision and eye colour sensitivity. Horizontal cells are interconnecting neurons, their cell bodies lie in INL. Their function is to regulate the input from photoreceptor cells. Bipolar cells are present in between of Photoreceptor and Ganglion cells. Their function is to transform information. Amacrine cells are inhibitory inter neurons in the Retina. Ganglion cells receive visual information from Rods and cones (*Wikipedia*).

Muller Glia cells span the whole retinal layer, their nucleus lies in the INL. These are one type of retinal glial cells, functioning as supporting cells. Their major function is to maintain retinal extracellular environment, regulating K+ levels, Neurotransmitter uptake etc. (*Google*).

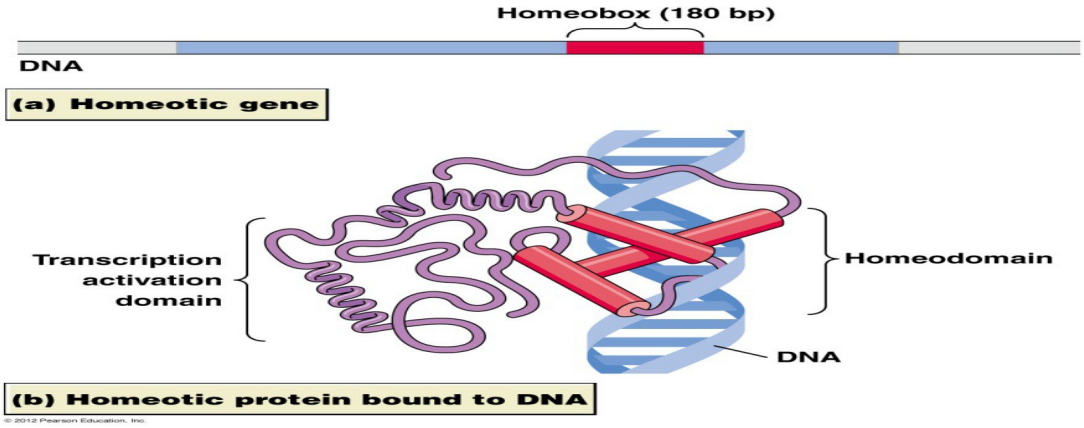


Retinal injury can be done by mechanical, chemical and intense light method. I have used mechanical method, giving six pokes in retina using thirty gauge size insulin syringe. In response to this injury Muller Glia cells get activated and start dedifferentiating. Then

they proliferate and rise in number. Then they differentiate giving rise to progenitors acting as stem cells and giving rise to different kind of retinal cells.

1.3 Developing Brain Homeobox genes:

A Homeobox gene is a DNA sequence (around 180 base pairs) present within genes majorly involved in patterns of anatomical development. Homeotic genes contain a domain (known as a homeo domain) when expressed as a protein can bind to DNA sequence, thus acting as a transcription factor and regulating the cascade of other genes *(Google)*.



dbx1a and dbx1b are duplicate orthologs of amniote dbx1. dbx2 is related but not ortholog of amniote dbx2. Developing Brain Homeobox genes are reported to be playing an important role in Central Nervous System development in Mouse and Chick. dbx genes were first isolated from 13.5 days mouse embryo and their expression was reported to be spatially restricted in forebrain and spinal (f). It is known that Hedgehog signalling is not required for the induction or maintenance of dbx genes. Retinoic acid signalling pathway is not essential for dbx induction (c). dbx genes play a critical role in CNS development and retina being part of CNS it might be playing some role in retina rgeneration.

1.4 Literature Review:

Ascl1a, a proneural transcription factor is essential for Muller Glia dedifferentiation and regeneration of retina (h). Lin-28 is a pleuripotency factor whose expression is dependent on Ascl1a. let-7 micro-RNA expression is suppressed by Lin-28 and this pathway is having critical role in dedifferentiation of Muller Glia during retina regeneration (h). Wnt

β-catenin signalling pathway controls the proliferation of Muller Glia derived progenitor cells (k). Insm1a suppress Ascl1a expression along with its own expression. Induction of Ascl1a suppresses expression of Wnt inhibitor Dkk, thus activating Wnt signalling essential for Muller Glia dedifferentiation (l).

HB-EGF is induced in Muller Glia cells residing near the site of injury. HB-EGF stimulates the formation of Muller Glia derived progenitor cells in uninjured retina. HB-EGF lies upstream of Wnt signalling pathway that controls proliferation (n). Apobec 2a and 2b are genes associated with DNA demethylation.Ascl1a, Apobec2a and 2b are essential for optic nerve regeneration. Induction of Apobec2b requires Ascl1a but it is independent of lin-28 (g).

2. Experimental methods:

2.1 Injury and Dissecting Retina:

1) Zebrafish was anesthetized using Tricane methanesulfonate.
2) Then fish was kept on sponge bed. Retina was injured using 30 Gauge needle and stainless steel forceps.
3) Eye was taken in a sterile petri plate containing 1X PBS, retina was dissected under bright field microscope.

Composition of chemicals used:

1) Tricane methanosulfonate (10X stock)

200mg tricane methanosulfonate in 90mL deionized H_2O
Mixed 0.14g Tris base in 2mL of H_2O and added drop-wise to tricaine solution until pH reaches till 7.
Made up the volume to 100mL with deionized H_2O.

2) 10X Phosphate Buffered Saline

2.76g of $NaH_2PO_4.H_2O$, 11.36g of Na_2HPO_4, 87.6g of NaCl

And 1.87g of KCl in 800mL of deionized H_2O. Volume makeup till 1L.

2.2 Drug injections :

1) Zebrafish was anesthetized using Tricane methanesulfonate.
2) Then fish was kept on sponge bed. Retina was injured using 30 Gauge needle and drugs (DAPT and XAV939) were injected using 10µL Hamilton syringe (rinsed with deionized H_2O).
3) Eye was taken in a sterile petri plate containing 1X PBS and retina was dissected.

2.3 Microscopy:

1) Bright field microscope (Zeiss) was used for dissecting retina and visualizing *in situ* hybridization signal.
2)Confocal microscope (Nikon) was used for imaging.

2.4 Primer designing:

1) Dbx1- Forward CTCGCGTCAACAACACGAAATGCTTC
Reverse GAAAACGTGGTGCTTCTGTTTGGCACGGTT
2) Dbx1b RT- Forward CAGAGCATCATATTTCCCAG
Reverse GGAATTTCGCCATTTCATCCTTCGG

3. Experimental techniques:

3.1 RNA isolation:

Fishes were injured and Retina was dissected in PBS at different time points. Isolated Retinal tissue was kept in Trizol and stored at -80°C and then processed for RNA isolation or immediately after dissection, RNA isolation protocol was followed:

1) Took 4-6 retinae (dissected in 1X PBS or 0.85% saline solution) in a MCT
2) containing TRI reagent (150µl or 200µl) at 4°C.
3) Homogenized the tissue using insulin syringe. No tissue clumps should be visible.
4) Added 0.2 volume of Chloroform (40µl)
5) Mixed for 15-20 min on rotator (No vortexing)
6) Centrifuged at 12000 rpm at 4°C for 20 minutes.

7) Using cut tip (it reduces sucking force per unit area) slowly removed aqueous phase without disturbing the middle inter phase layer (it may otherwise cause DNA contamination.

8) Added 0.6 volume of isopropanol (approximately 40 µl) and kept at -80°C overnight or in ice for 10 to 20 minutes.

9) Next day or after 10 to 20 minutes (ice incubation), centrifuged at 12000 rpm at 4°C for 20 minutes.

10) Discarded the supernatant.

11) Washed with 200 µl of 70 % EtOH or absolute EtOH. Centrifuged at maximum speed for 10 minutes at 4°C.

12) Dried and dissolved it in deionized H_2O and checked on agarose gel.

13) stored at -80°C.

3.2 Gel electrophoresis (UVP-Biotek)

1) Wiped hands with 70 % ethanol. Wiped table, casting tray and comb with 70 % ethanol. I weighed 1.5g of agarose and mixed it in 100mL of 1 XTAE. Melted it. When temperature became tolerable added 2µL of Ethidium bromide in it. A small casting tray was taken. Sealed it from both sides using cellotape. Inserted comb in the notches and poured the agarose gel in the tray. When gel got solidified removed the comb and thus 16- welled gel is prepared. Then removed cellotapes from both sides of the plate
2) Casting tray was placed in RNA gel electrophoresis tank.
3) 5µl of RNA sample and 2µl of 10X RNA loading dye was taken on a parafilm.
4) Then loaded it in the wells and electrophoresed the gel till dye moves three fourth of the gel.
5) Then kept the gel in gel doc and observed the gel on PC.

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

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

i) Up to 5µg total RNA - nµL

ii) 50µM oligo $(dT)_{20}$ – 0.5µL

iii) 50ng/µL random hexamers – 0.5µL

iv) 10mM dNTP mix- 1µL

v) DEPEC-treated water – to 10µL

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

3) Prepared the following cDNA synthesis mix, adding each component in the indicated order:

i) 10X RT buffer - 2µL

ii) 25mM $MgCl_2$ - 4µL

iii) 0.1M DTT - 2µL

iv) RNase out (40U/µL) - 1µL

v) Superscript III RT (200U/µL)- 1µL

4) Added 10µL of cDNA synthesis mix to each RNA mixture, mixed gently, and collected by brief centrifugation. Incubated as follows:

Oligo$(dT)_{20}$ primed : 50 min at 50°C

Random hexamer primed : 10 min at 25°C followed by 50 min at 50°C

5) Terminated the reaction at 85°C for 5 min. Chilled on ice.

6) cDNA synthesis reaction was stored at -20°C.

3.4 Reverse transcription PCR :

1) Buffer – 2.5µL

2) dNTPs – 2.5µL

3) Forward primer - 1µL

4) Reverse primer - 1µL

5) Template - 1µL

6) deionized H_2O - 16µL

7) Taq - 1µL

Total - 25µL

This mixture was collected in 0.2- to 0.5mL tube and incubated as follows:

95°C – 15 sec for enzyme activation

95°C - 1 min for denaturation

57°C– 1 min for annealing

72°C – 1 min extension

72°C – 10 min

4° C - infinite time

PCR product was then checked on agarose gel.

3.5 Quantitative Real-Time PCR:

Following components were added in axygen 0.2- 0.5 mL real time specific tubes:

i) SYBr green mix - 5µL

ii) Primer: Forward- 0.4µL

Reverse- 0.4µL

iii) Formamide- 0.4µL

iv) H_2O- 3.3µL

v) Template- 0.5µL

Data was analyzed on excel sheet and graph was plotted.

3.6 TOPO TA cloning:

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

Gel extraction was done using Nucleo-pore kit:

i) Excised the DNA fragment from an agarose gel using sterile blade/ scalpel. For each 100mg of agarose gel 200µL of buffer SET was added.

ii) Samples were loaded onto the Sure Extract Spin PCR/gel extraction placed in a collection tube(2mL). Centrifuged for 1 min at 11,000 rpm. Discarded flow-through and placed the column back into the collection tube.

iii) Added 700µL buffer SET3 to the Sure Extract Spin PCR/gel extraction

iv) Repeated step3. Discarded flow- through and placed the column back into the column. Centrifuged for 1 min at 11,000 rpm. Discarded flow -through and placed the column back into collection tube.

v) Centrifuged for 2 min at 11,000 rpm to remove buffer SET3 completely. Made sure that the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.

vi) Added 15-50µL of DEPEC H_2O to Sure Extract Spin PCR/gel extraction column placed in fresh MCT and allowed it to stand for 5 min. Centrifuged for

1 min at 11,000 rpm and stored at -80°C or immediately used for cloning.

3) Extracted product (Insert) was checked on agarose gel for gene specific band.

4) For cloning, following components were added in a MCT :

i) Salt solution :	0.5µL
ii) TOPO TA vector:	0.3µL
iii) Insert:	1µL
iv) H_2O:	1.2µL
Total:	3µL

5) Transformation:

i) Competent cells stored at -80°C were thawed on ice.

ii) 3µL of plasmid was added in it, mixed gently (only tapped, should not pipette)

iii) Incubated on ice for half an hour

iv) Heat shocked at 42°C for 45 sec. Without shaking immediately kept on ice for 5 min'

v) Added 1 mL of LB media in it (inside the hood).

vi) Incubated at 37°C for 30 min.

vii) Cells were plated on Amp-resistant LB plates.

viii) Incubated plates at 37°C overnight.

ix) Next day, single colonies were visible.

x) Single colonies were streaked on LB agar+ IPTG+ X-Gal plates. Blue and white colonies were visible next day.

xi) White colonies were selected and dissolved in 20µL H_2O in a MCT.

xii) Colonies were mixed well using pipette and centrifuged at 10,000rpm for 10min.

xiii) This solution was added in a culture vial (5mL of LB media). Placed overnight at 37°C for growth.

3.7 Plasmid isolation:

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

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

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

4) Mixed by gentle tapping, no vortexing.

5) Boiled for 2 min at 100°C.

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

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

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

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

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

11) Kept in ice for 5 min.

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

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

14) Pellet was dissolved in 50µL of TE/autoclaved DEIONIZEDH_2O. Stored at -20°C

Composition of solutions used for Plasmid isolation:

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

2) Potassium acetate buffer: 60mL potassium acetate
11.5mL glacial acetic acid
28.5mL H_2O
Total : 100mL (stored at 4°C)

3.8 Plasmid digestion :

1) Following components were added in a MCT:

i) H_2O - 30µL
ii) buffer- 5µL
iii) DNA- 10µL
iv) enzyme EcoRV- 5µL
Total- 50µL

2) Incubated at 37°C for 3 hours.

3) Checked on agarose gel

4) Gel extraction protocol was followed:

5) Gel extraction was done using Nucleo-pore kit:

i) Excised the DNA fragment from an agarose gel using sterile blade/ scalpel. For each 100mg of agarose gel 200µL of buffer SET was added.

ii) Samples were loaded onto the Sure Extract Spin PCR/gel extraction placed in a collection tube(2mL). Centrifuged for 1 min at 11,000 rpm. Discarded flow-through and placed the column back into the collection tube.

iii) Added 700µL buffer SET3 to the Sure Extract Spin PCR/gel extraction column. Centrifuged for 1 min at 11,000 rpm. Discarded flow -through and placed the column back into the collection tube.

iv) Repeated step3. Discarded flow- through and placed the column back into the collection tube.

v) Centrifuged for 2 min at 11,000 rpm to remove buffer SET3 completely. Made sure that the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.

vi) Added 15-50µL of DEPEC H_2O to Sure Extract Spin PCR/gel extraction column placed in fresh MCT and allowed it to stand for 5 min. Centrifuged for 1 min at 11,000 rpm and stored at -80^{o}C or immediately used for RNA probe making.

3.9 RNA probe making:

1) Following components were added in a MCT:

i) Buffer (10X) NEB 3.1-	4µL
ii) Template(digested DNA)-	7µL (100-500ng)
iii) Dig-UTP-	2µL
iv) SP6 RNA polymerase-	2µL
v) H_2O-	25µL
Total-	40µL

2) Incubated at 37^{o}C for 4 hours.

3) Stopped the reaction by adding 0.5M Tris EDTA (4µL).

4) For precipitation added 5M $LiCl_2$ (4µL).

5) To add weight, added 10mg/mL glycogen (2µL).

6) Tapped it and added 70µL of absolute EtOH.

7) Tapped again and kept in -80^{o}C overnight or one hour.

8) Centrifuged at 4^{o}C for 15 min.

9) 100%EtOH wash was given (200µL) - centrifuged for 10 min.

10) Dried and dissolved in 50µL of DEPEC H_2O.

11) Stored at -80°C by aliquoting.

3.10 Tissue fixation:

1) Removed 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) Mixed on rotator for two hours at RT on same day or next day.
3) Pipetted out old solution and added 600µL of 5% sucrose in MCT. Mixed on rotator for 45 min at RT.
4) Pipetted out old solution and added 400µL of 5% and 200µL of 50% sucrose in MCT. Mixed on rotator for 45 min at RT.
5) Pipetted out old solution and added 300µL of 5% and 300µL of 20% sucrose in MCT. Mixed on rotator for 45 min at RT.
6) Pipetted out old solution and added 200µL of 5% and 400µL of 20% sucrose in MCT. Mixed on rotator for 45 min at RT.
7) Pipetted out old solution and added 600µL of 20% sucrose in MCT. Mixed on rotator for 45 min at RT.
8) MCT was kept at 4°C for 45 min.
9) Added 600µL of OCT in it and mixed on rotator for 30 min.
10) Tissue was fixed and block was made using OCT and eye was kept in it.

Composition of solutions used for tissue fixation:

1) 4% PFA in 1X Phosphate buffer:
   2g PFA
   5mL of 10X phosphate buffer
   Volume makeup till 50mL using autoclaved deionized H_2O (stored at -20°C)
2) 5% sucrose:
   2.5g sucrose dissolved in 50mL of autoclaved deionized H_2O (stored at 4°C
3) 20% sucrose:
   10g sucrose dissolved in 50mL of autoclaved deionized H_2O (stored at 4°C)

Fixed tissue was sectioned using cryosectioner and stored at -20°C, followed by In-Situ Hybridization or Immunostaining.

3.11 PCNA Immunostaining :

1) Slides stored at -20°C were incubated at 37°C for 30 min.
2) Washed the slides using 1X PBS (twice- 10 min each).
3) Then treated the slides with 2N HCL (should be at 37°C before hand) for 20 min.
4) Washed the slides with 0.1M sodium borate solution (twice- 10 min each).
5) Blocked the sections using 3% BSA +0.1% Triton in 1X PBS for 30 min.
6) Overlayed the slides using anti-Mouse 1° Ab (1µL Ab was diluted in 500µL of 1X PBST+1% BSA).
7) Kept the slide container at 4°C overnight.
8) Next day slides were washed using PBST (0.1% Triton) (thrice-10 min each).
9) Overlayed the slides using anti-Rabbit 2° Ab (0.5µL Ab was diluted in 500µL of 1X PBST+1% BSA).
10) Slides were incubated for 3 hours at RT.
11) Washed slides with PBST (thrice- 10 min each).
12) Washed slides with autoclaved DEIONIZEDH_2O (thrice-10 min each)
13) Dried for one hour at RT.
14) Slides were mounted using DABCO and stored at -20°C or immediately proceeded for imaging.

Composition of solutions used for PCNA Immunostaining:

1) 1X PBS

   5mL PBS (from 10X stock)+ 45mL autoclaved MQ

2) 1X PBST

   5mL PBS (from 10X stock)+ 45mL autoclaved MQ+ 0.1% Triton(50µL)

3) 1X PBST + 3% BSA

   5mL PBS (from 10X stock)+ 45mL autoclaved MQ+ 0.1% Triton(50µL) + 1.5gm BSA

4) 1X PBST+ 1%BSA

   5mL PBS (from 10X stock)+ 45mL autoclaved MQ+ 0.1% Triton(50µL) + 0.5gm BSA

5) 0.1M Sodium Borate

9.53g Sodium Borate dissolved in 500mL of deionized H_2O

3.12 mRNA *in situ* Hybridization:

Day 1

Hybridization:

1) Hydrated 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) Incubated slides in Proteinase K solution for 1-5 min at 37°C
    a. Prewarmed Proteinase K buffer to 37°C
    b. Added 250µL of 10mg/mL proteinase K
3) Rinsed slides briefly in room temp DEPEC water.
4) Rinsed slides in 0.1M TEA pH 8.0 for 3 min.
5) Rinsed in Acetic anhydride/TEA for 10 min
    a. Added 130µL of acetic anhydride to dry dish.
    b. Added 50mL of TEA
6) Dehydrated 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 were air dried for at least 1 hour at RT.
8) Hybridization solution was pre warmed at 56°C (200 to 300ng per slide).
9) Probe preparation:

a. 4µL probe was added to hybridization solution and mixed.

b. Boiled at 100°C for 5 min.

c. Cooled immediately on ice

10) Added 60µL of Hyb/probe solution to each slide and coverslip with siliconized hybrid slips was placed.

11) Placed slides in humid chamber dampened with 50% formamide/5X SSC and incubated at 56°C overnight.

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

1) 20X SSC

Dissolved 87.6g of NaCl in350mL of DEPEC H_2O

Added 44.12g sodium citrate

Brought to final volume of 500mL with DEPEC H_2O.

2) TEA solution

9.3g Triethanolamine (TEA)

Brought upto 490mL with DEPEC H_2O

Added 12-14 NaOH pellets

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

Stored at -20°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) Preheated 50% formamide/2X SSC solution to 65°C.
2) Preheated two 50mL RNase buffer washes, one to 37°C and the other to 65°C.
3) Preheated two 2X SSC washes to 37°C.
4) Soaked slides with coverslips in 2X SSC for 30 min at RT on shaker table
   a. If the slides do not come off, gently teased them apart from slide with forceps.
5) Rinsed slides in 50% formamide/2X SSC solution for 30 min at 65°C.
   a. Gently agitated for the first 5 min.
6) Rinsed slides in 2X SSC for 10 min at 37°C (twice).
7) Added 100µL of RNase (10mg/mL) to the 37°C RNase buffer: Incubated slides for 30 min.
8) Washed slides in 65°C RNase buffer for 30 min.
9) Washed slides for 2-3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
   a. Thawed 3mL aliquot
   b. Added 7mL of deionized H_2O.
10) Washed slides in 1X Maleate buffer for 5 min (twice).
11) Incubated slides with 205µL antibody (of choice) diluted in 1X Maleate/0.05% Triton X-100/1% RMB blocker solution overnight at RT.
   a. Added 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°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. Washed slides twice with 1X Maleate buffer for 5 minutes.
2. Incubated twice for 5 minutes each in Genius buffer.
3. Added NBT/BCIP, incubated overnight at room temperature in dark.

Colour detection: In bright field microscope, slides were visualized at time intervals to Check whether reaction worked or not.

Chapter 2

1. Results

1.1 Time course and Semi-quantitative real time PCR of dbx1b:

From qualitative data,

a. dbx1b was getting induced at 1dpi, expression was not observed in uninjured retina.
b. Expression of dbx1b was observed to be raised till 6dpi and then it was decreased.

From quantitative data,

a. dbx1b expression was highest at 4dpi (peak point of proliferation).
b. One phase graph.

1.2 Dbx1b cloned in TOPO vector and RNA probe gel:

1.3 *mRNA in situ* hybridization of dbx1b:

At pre-proliferative stage (2dpi), dbx1b expression was observed in GCL and INL (punctuated expression). In contrast, uninjured retina shows low expression. After PCNA

immune staining proliferating cells were marked with antibody. Co-localization represented PCNA positive cells with dbx1b expression.

At proliferative stage (4dpi), dbx1b was robustly expressed in GCL and INL (punctuated expression. The number of Muller glia cells were high in 4dpi as compared to 2dpi. dbx1b expression was also prominent in 4dpi proliferating cells.

At post proliferative stage (7dpi), dbx1b was robustly expressed in GCL and INL. Proliferating cells were less in number and dbx1b expression was not visualized in proliferating cells.

1.4 Drug treatment results:

Immuno histochemistry to confirm DAPT (Notch signalling blocker) and XAV 939 (Wnt signalling blocker) drugs were working properly.

In DAPT (40µM) treated retinae, area of proliferation was getting widened (away from injury site) and in XAV 939(100µM) treated retinae, proliferating cells were decreasing in number.

Drug treatment at 4dpi (proliferation):

At 4dpi, after blocking notch signalling dbx1b expression was increasing slightly. After blocking Wnt signalling, dbx1b expression was getting down regulated. dbx1b expression might be directly or indirectly regulated by Wnt- signalling during retina regeneration.

Drug treatment at 7dpi (post-proliferation):

At 7dpi, after blocking notch signalling there is no difference in dbx1b expression. After blocking Wnt-signalling, dbx1b expression is increasing at 7dpi. dbx1b expression might be regulated directly or indirectly by Wnt-signalling during retina regeneration

1.5 Dbx1b expression after c-Myc morpholino injection:

At 2dpi, dbx1b expression was getting upregulated after c-Myc morpholino injection.
c- Myc is downstream of Wnt-β catenin signalling pathway. Dbx1b might be regulated directly or indirectly by Wnt-signalling.

Discussion and Conclusion:

dbx1b is induced at 1dpi and expression is highest at 4dpi (peak point of proliferation), it is robustly expressed in GCL and INL. dbx1b might be regulated by Wnt-β catenin signalling pathway. dbx1b gene expression profile has to be checked by knocking

down other genes. dbx1a gene localization and time course has to be checked. hybridization of dbx1b will be repeated.

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