Role of FUSE binding protein in planarian stem cell maintenance

A thesis submitted towards partial fulfilment of

BS-MS Dual Degree Programme

By

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Under the guidance of

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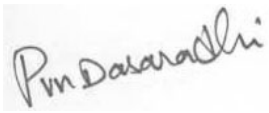
Certificate

This is to certify that this dissertation entitled "**Role of FUSE binding protein planarian stem cell maintenance**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by Viraj Doddihal at Institute for Stem cell and regenerative medicine (inStem), Bengaluru under the supervision of Dr. Dasaradhi Palakodeti, Designation, inStem, Bengaluru during the academic year 2014-2015.



Name and signature of the student

Viraj Doddihal

Supervisor

Dr. Dasaradhi Palakodeti

Date: 25.03.2105

Place: Bengaluru

Declaration

I hereby declare that the matter embodied in the report entitled "**Role of FUSE binding protein planarian stem cell maintenance**" are the results of the investigations carried out by me at Institute for Stem cell and regenerative Medicine (inStem), Bengaluru, under the supervision of Dr. Dasaradhi Palakodeti and the same has not been submitted elsewhere for any other degree.

Name and signature of the student

Viraj Doddihal

Date: 25.03.2015

Place: Bengaluru

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Abstract

The extraordinary ability of the planarians to regenerate lost body parts is dependent on the population of adult stem cells called neoblasts. RNA binding proteins are essential for neoblast maintenance and differentiation. The study here shows that FUSE binding protein (FBP), a RNA binding protein with KH domains is required for planarian regeneration and homeostasis. dsRNA mediated RNAi was employed to deplete *Smed-fbp* in the planarian *Schmidtea meditarranea*. Upon *Smed-fbp* RNAi there was initial blastema formation which did not develop and regressed during the course of regeneration. In homeostatic worms *Smed-fbp* RNAi resulted in animal curling and lesion formation on the dorsal surface. Neoblast proliferation was reduced upon *Smed-fbp* knockdown. The number of cells in G2/M phase was reduced, but the transcript level for *Smed-pcna* was upregulated. So here we hypothesize that *Smed-fbp* is essential for neoblast to exist G1 or S phase and make a transition to G2/M phase of the cell cycle.

Chapter 1

Introduction

1.1.1. Regeneration

Regeneration can be simply defined as the regeneration of lost body parts (Bely and Nyberg, 2009). Regeneration can occur at different levels, from whole organismal level to the cellular level (Fig 1). Across the metazoans, individuals have the ability to regenerate to different extents. Abraham Trembley's extensive work on *Hydra* regeneration in early 1700s and Morgan's work on planarians has shown that these organisms can regenerate even from a small piece of tissue (Dinsmore, 1991). On the other hand few organisms like nematodes, leeches and birds are largely incapable of regeneration (Bely and Nyberg, 2009). In an organism, regeneration can be limited to a part as in lizards. Lizards can only regenerate lost tails, but not limbs (Alibardi and Toni, 2005). Similarly, annelids can regenerate a lost tail but not a head (Bely, 2006). The question of why few organisms can regenerate and why most cannot regenerate still remains a mystery. However with the development of new model systems for regeneration molecular mechanisms involved in regeneration are being solved. Planarian *Schimdtea meditarranea* has come back as one the of exciting model systems to study regeneration (Sanchez Alvarado, 2006).

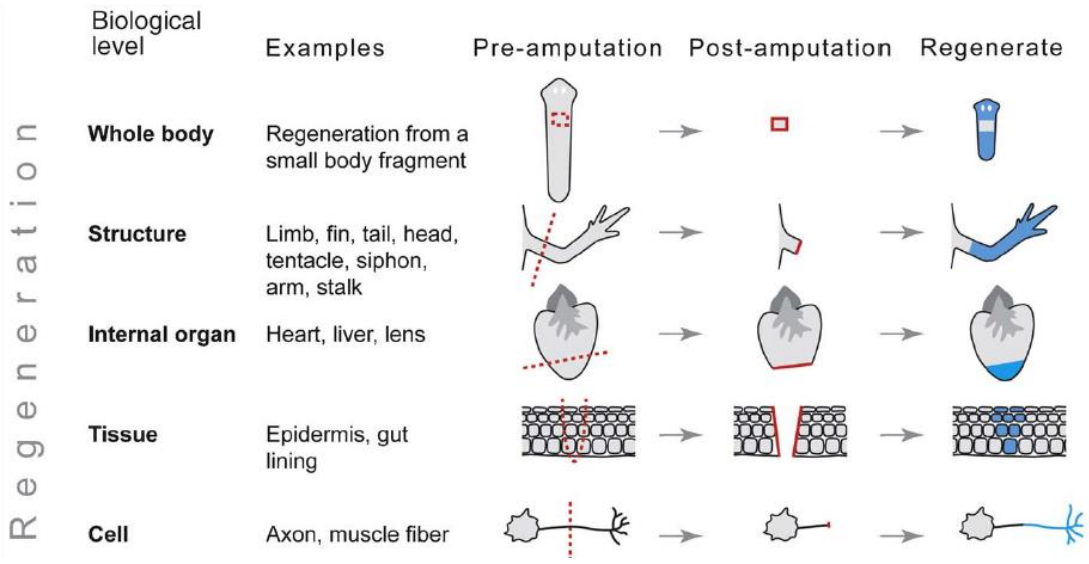

Fig 1.1: Regeneration in nature occurs at different extents. An organism can regenerate completely from a small fragment whereas few others can regenerate only few structures or tissues. Dotted red lines indicate plane of amputation, solid red lines indicate wound surfaces and blue surfaces are the regenerated parts (Adapted from Bely and Nyberg, 2009).

1.1.2. Planarian – *Schmidtea meditarranea*

Planarians are free living, bilaterally symmetric, triploblastic animals belonging to the class of Turbellaria, phylum Platyhelminthes. Planarians are placed in the group lophotrochozoa, which along with ecdysozoa and deuterostomia complete bilateria. Schmidtea meditarranea belongs to the family of Tricalde, which derives its name from three branched digestive system. Planarians have a mouth on its ventral side which acts as an opening for taking in and egestion of food. Planarians are acoelomate, unsegmented, dorso-ventrally flattened animals. Gas exchange occurs via diffusion through the skin (Hyman 1951). Planarians are the first bilaterians to have a central nervous system, consisting to two bi-lobed cephalic ganglia. Two ventral nerve cords connect the cephalic ganglia and run from anterior to posterior end. The ventral nerve cords are connected by commissural neurons (Cebria and Newmark, 2002, Hyman 1951). Excretory system consists of a network of flame cells which are connected by ciliary ducts. Sexually hermaphrodite planarians reproduce by cross-fertilization. Asexual planarians reproduce by transverse fission (Baguna et al., 1999).

Regeneration in planarians is because of the adult pluripotent stem cells called ‘neoblasts’ (Reddien and Sanchez Alvarado, 2004, Wagner et al., 2011). Neoblasts resemble embryonic stem cells and are sensitive to radiation. Neoblasts are small cells with scanty cytoplasm and a large nucleus characterized by chromatoid bodies at the periphery of the nuclear membrane. Chromatoid bodies are centres of ribonucleoprotein complexes involved in RNA processing. Chromatoid bodies are necessary for germ cell maintenance (Betchaku 1967, Hayashi et al., 2006). Neoblasts are defined as the only dividing cells in planarians and are spread all over the body except anterior to eyes and in the pharynx (Morgan 1898, Reddien and Sanchez Alvarado, 2004). Planarians when cut regenerate in a span of 7-10 days. When amputated, the wound closes within 30 minutes. The epithelial cells surrounding the wound on both the dorsal and ventral surface lose their morphologies and extend to cover the wound (Baguna et al., 1994, Newmark and Sanchez Alvarado, 1998). Neoblasts peak their proliferation from 6-12 hours post amputation. This round of proliferation occurs all throughout the body. 48 hours-72 hours post amputation is the second round of neoblast proliferation which occurs

only close to the wound (Wenemoser et al., 2011). Starting from second or third day of regeneration a colourless tissue is formed at the site of amputation. This colourless tissue is called blastema, which consists of neoblast progenitors and differentiated cells. Blastema develops and over the course of seven days gives rise to lost tissues (Fig 2). Neoblast proliferation and blastema constitute the epimorphosis part of the regeneration. Later part of the regeneration involves a great deal of repatterning of tissues, which constitutes the morphallaxis part of regeneration (Newmark and Sanchez Alvarado, 2002, Reddien et al., 2005).

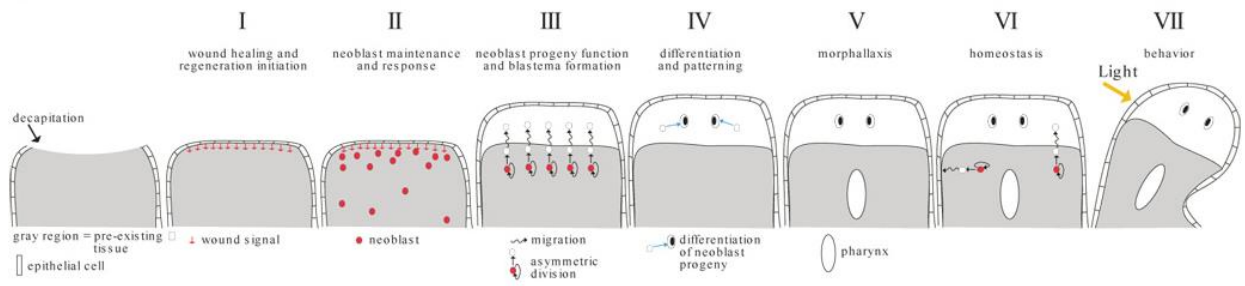

Fig 1.2: Planarian regeneration timeline. Post amputation wound healing occurs within 30 minutes. This is followed with two peaks of neoblasts proliferation, the global prilferation at 6-12 hours and the local proliferation at 2-3 days post amputation. Blastema develops replacing lost tissues and tissue re-patterns according to the size of the worm (Reddien et al., 2005)

1.2.1 RNA binding proteins (RBPs)

In a eukaryotic cell transcription and translation occur in two different compartments. This gives an opportunity for an additional layer of regulation; where in the synthesis of protein is regulated post-transcriptionally. Post-transcriptional modification involves alternative splicing, mRNA editing, polyadenylation. RNA binding proteins (RBPs) mediate these post-transcriptional modifications. In addition to post-transcriptional modifications, RBPs also play a role in mRNA export, localization, turnover and translation (Glisovic et al., 2008).

Post-transcriptional regulation of mRNA is crucial for proper development, germ line formation and neuronal plasticity (Rouhana et al., 2010). Post-transcriptional control of maternal mRNA is critical during early embryo development. A RBP ADAR is protein involved in mRNA editing is necessary for survival. Its mutation in flies leads

to neuronal dysfunction and in mice it is embryonic lethal (Hartner et al., 2004, Palladino et al., 2000, Wang et al., 2004).

In planarian regeneration and homeostasis, RBPs play a key role in maintaining neoblasts and in their differentiation. RBPs Smedwi-2 and Smedwi-3 are known to be essential for planarian stem cell maintenance (Reddien et al., 2005, Palakodeti et al., 2008) and so are Smed-bruli and Smed-tudor (Guo et al., 2006, Salvetti et al., 2005). Vasa-1, a RBP with DEAD-box domain is necessary for both neoblast proliferation and differentiation (Wagner et al., 2012). Smed-khd-1 a RBP with KH domain and Smed-cip29 are required for neoblast proliferation (Wagner et al., 2012). Neoblasts are enriched for RNA binding proteins and other translational regulators, suggesting that neoblasts are under heavy post transcriptional regulation (Eisenhoffer et al., 2008, Rossi et al., 2007, Shibata et al., 1999, Yoshia-Kashikawa et al., 2007).

A similar study performed in the lab showed that the RNA binding proteins are enriched in X1 population. X1, X2 and Xins population were isolated based on the nuclear to cytoplasmic ratio using FACS. Transcriptome analysis of these three cell population revealed that X1 population, which is the proliferating stem cell population enriched for RNA binding proteins. The study identified that 60% of the RBPs are enriched in neoblasts (Fig 3). Approximately 5% of the neoblast enriched RBPs had K-homology domain. This project aimed at understanding the role of one of these KH-domain proteins called FUSE binding protein in planrarian stem cell function.

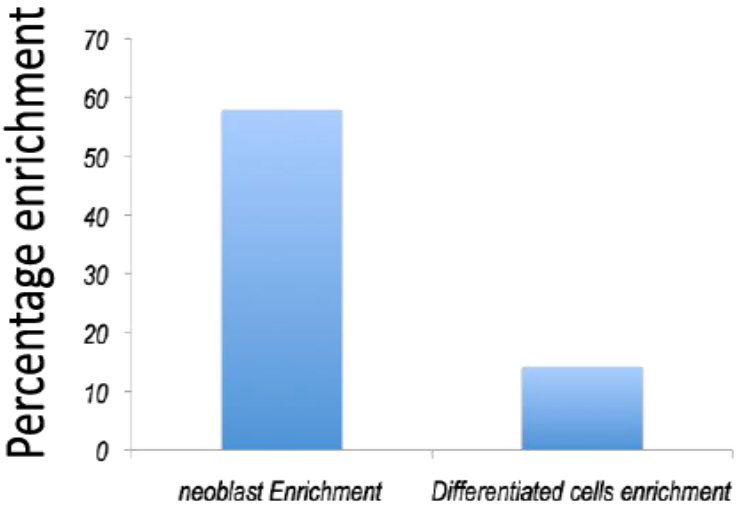

Fig 1.3: Neoblasts are enriched for RNA binding protein. Proteins which were expressed at least two folds more in neoblasts were considered to be enriched in neoblasts.

1.2.2. FUSE binding protein (FBP)

Fuse binding protein (FBP) was first discovered as a protein necessary for maximum expression of *c-myc* in undifferentiated leukaemia cells (Avigan et. al., 1990). FBP functions in far upstream element (FUSE) dependent manner in enhancing the transcription of *c-myc* (Duncan et. al., 1994). FUSE is a DNA segment 1.7kb upstream of P2 start site of *c-myc* gene. Apart from its classical function of c-myc regulator FBP also functions as RNA binding protein regulating translation and stability of several mRNA (Zhang and Chen, 2013). FBP regulate cell proliferation, differentiation, migration and cell death with its transcriptional and post transcriptional functions (Zhang and Chen, 2013). Since FBP is a positive regulator of *c-myc* it is thought to be playing a positive role in carcinogenesis. It is highly expressed in most cancers (Bazar et. al., 1995, Davis-Smith et. al., 1996). FBP is highly expressed in embryonic stem cells and induced pluripotent stem cells and its expression dies down as the cells differentiate (Kim et. al., 2012).

FUSE binding protein is a single strand DNA/RNA binding protein with four K-homology (KH) domains (Duncan et. al., 1996). K-homology domain was named after the protein heterogeneous ribonuclear protein K (Siomi et. al., 1991). Few other well- known proteins with KH domain are fragile X mental retardation protein, fragile X related protein. KH domain consists approximately of 70 amino acids. A cleft in the KH domain recognizes four unpaired bases. To increase the specificity of binding, proteins usually have multiple KH domains. KH motifs are found in two versions in nature (Grishin, 2001). KH-1 motif found in eukaryotes and KH-2 motif found in prokaryotes both have minimal KH motif in linear sequence but the folds of secondary structures are different. The beta strands in KH-1 motif are all antiparallel and are abutted by three alpha helices, whereas in KH-2 motif the β1 and β2 strands are parallel and β’ strand is antiparallel to both (Valverde et. al., 2008). The four KH domains constitute the central domain of FBP. The C-terminal domain hosts tyrosine motifs, which are necessary for activation of TFIIH (Liu et. al., 2001). The N-terminal domain represses the activity of C-terminal domain (Duncan et. al., 1996).

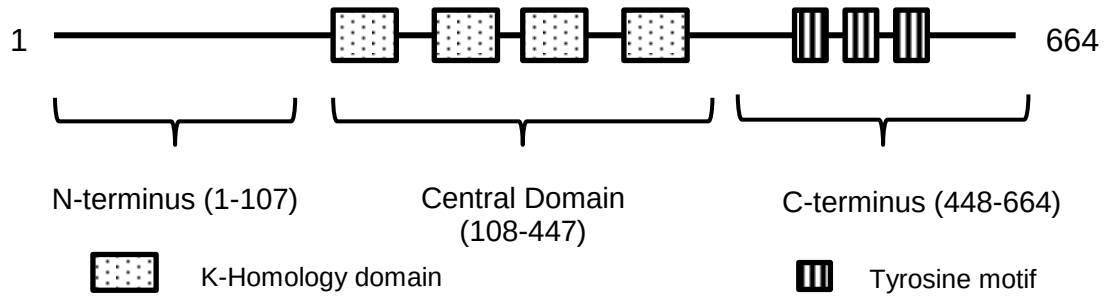

Fig 1.4: Schematic representation of human FBP-1 protein: First 107 amino acids form the N-terminus which suppresses the activity of C-terminal domain. Amino acids 448 to 664 form C-terminal domain with activator tyrosine motifs. Central domain with KH motifs binds ssDNA and RNA. (Modified from Zhang and Chen, 2013)

MYC regulates cell proliferation, growth, differentiation and apoptosis through its targets which constitute 10-15% of genes. Not only increased expression of MYC causes pathological conditions, in quite a few cancers MYC levels are decreased (Chung and Levens, 2005). MYC deficiency during development is known to reduce body size in flies and mice (Davis et. al., 1993, Trumpp et al., 2001). Body size of mice can be predictably manipulated by regulating MYC expression using a combination of hypomorphic, null and wild type alleles, (Chung and Levens, 2005). Cell expressing high levels of MYC induce apoptosis in cells expressing lesser MYC, which if happened in a carcinogenesis results in neoplasia (Chung and Levens, 2005). Hence, it is of great importance to maintain uniform expression of MYC.

Transcription at *c-myc* is activated by conventional transcription factors. When the paused polymerase is released it generates a negative torque causing the melting of FUSE. FBP binds to single stranded FUSE and activates TFIIH resulting in the maximum expression of *c-myc*. FBP interacting repressor is recruited to FUSE causing the shutdown of *c-myc* transcription (Liu et. al., 2006). The FUSE/FBP/FIR thus acts as modulators for *c-myc* expression with FBP being the positive modulator and FIR being the negative modulator. FBP knockdown extinguishes *c-myc* expression and arrests cell proliferation. In osteosarcoma cells, FBP knockdown resulted in decreased proliferation but did not affect cell viability (He et al., 2000).

FBP is also a RNA binding protein which regulates mRNA stability and translation. FBP binds to 3'-UTR of growth associated protein 43 (GAP43) and targets it for degradation. GAP43 is critical for axonal growth and regeneration (Irwin et al., 1997).

p21 a cyclin dependent kinase inhibitor (CKI) regulates G1 to S phase transition of cell cycle. Increased levels of p21 results in G1 phase arrest (Harper et al., 1993). 3'-UTR of p21 mRNA has three AU-rich elements (ARE). FBP binds to p21 ARE and targets it for degradation. In Hep38 cells FBP knockdown increased p21 expression (Rabenhorst et al., 2009).

p27 a CKI expressed in differentiated cells and during stress regulates G1 to S phase transition. p27 inhibits the activity of cyclinE-CDK2 or cyclinD-CDK4 complexes and thus hold cells in G1 phase (Polyak et al., 1994). 5' UTR of p27 mRNA harbours an internal ribosome entry site (IERS). FBP is known to bind to p27 IERS and facilitate its translation (Zheng and Miskimins, 2011).

Nucleophosmin is a dictator of cell proliferation and genomic stability. Nucleophosmin is responsible for hyperactivity of mTOR thus promoting cell proliferation. FBP binds to 3' UTR of *nucleophosmin* mRNA and repressed its translation (Olanich et al., 2010). In addition to these FBP suppresses Japanese encephalitis virus translation by binding to its 5'-UTR (Chien et al., 2011). On the other hand, FBP enhances the replication of Hepatitis C virus by binding to its poly-U tract at 3'-UTR (Zhang et al., 2008).

In this study, the aim was to understand the role of FBP in planarian stem cell function. FBP knockdowns were performed for the purpose and its role in the context of planarian regeneration was investigated. Whole mount *in situ* hybridization was performed to determine the expression pattern of FBP in the animal.

Chapter 2

Materials and methods

2.1. Materials

2.1.1. Planarians

Sexual hermaphrodite strains of planarian *Schmidtea mediterranea* (Zayas et. al., 2005) were maintained as described (Cebria and Newmark, 2005). Animals were starved for a week before using for any experiment. 8-10 mm worms were used for RNAi and 2-3 mm long animals were used for *in situ* hybridizations.

2.1.2. Antibodies

Primary antibodies

Name	Dilution used	Source
Mouse anti-H3P S10	1:100	Abcam
Sheep anti-DIG-POD	1:1000	Roche
Sheep anti-DIG-AP	1:2500	Roche

Secondary antibodies

Name	Dilution used	Source
Anti- Mouse- 488 nm	1:400	Invitrogen
Anti-Mouse-568 nm	1:400	Invitrogen

2.1.3. Planarian water

Planarian water	1.6mM NaCl
(1X Montjuich salt)	1mM $CaCl_2$
	1mM $MgSO_4$
	0.1mM $MgCl_2$
	0.1mM KCl
	1.2mm $NaHCO_3$

2.1.4. Buffers and solutions

5% NAC solution	500mg N-acetyl cysteine
	10ml 1XPBS
PBSTx	0.3% TritonX-100
	1X PBS
Reduction solution	50mM DTT
	1% NP-40
	0.5% SDS
	1X PBS
Proteinase K solution	2µg/µl Proteinase –K
	0.1 % SDS
	1X PBSTx
Carnoy's	6ml Ethanol
	3ml Chloroform
	1ml Glacial acetic acid
Methanol bleaching solution	6% H_2O_2

	100% Methanol
Formamide bleach solution	1.2% H_2O_2
	5% non-deionized formamide
	0.5x SSC
Formaldehyde fixative	4% formaldehyde
	1X PBSTx
Prehybridization buffer (Pre Hyb)	50% Deionized formamide
	25% 20X SSC
	1% Tween 20
	1mg/ml Yeast torula RNA
	DEPC treated water was used to make up the buffer.
Hybridization buffer (Hyb)	Pre Hyb
	5% Dextran sulphate
	Riboprobe (1:400 to 1:1000 diluted)
MABT, pH 7.5	0.1M Maleic acid
	0.15M NaCl
	0.1% Tween 20
AP Buffer	0.1M Tris pH 9.5
	0.1M NaCl
	0.05M $MgCl_2$
	0.1% Tween 20
	2% Poly-vinyl alcohol

Ca/Mg free media (CMF)	3.5 mM NaH_2PO_4
	13.6 mM NaCl
	16mM KCl
	9.5mM $NaHCO_3$
	1.33mM Glucose
	15mM HEPES
	pH 7.3
CMFB	CMF
	0.5% BSA

2.2. Methods

2.2.1. Animal husbandry

Planarians were maintained in planarian water in dark at 20°C. Animals were fed with homogenized beef liver extract twice a week. Planarians were starved for a week before using for experiments.

2.2.2. γ- Irradiation

A blood irradiator (BRIT 2000) with a Cobalt source for gamma rays was used to lethally irradiate planarians. Planarians were given 90 Gy (9000 rads) of radiation and were maintained in petri dishes with planarian water for 3 days before fixing them for experiments.

2.2.3. RNA isolation

RNA was isolated from worms using Trizol, a well-established method to isolate total RNA from tissues and cells. Animals were crushed and homogenized in Trizol. To this $1/5^{th}$ volume of chloroform was added and the samples were shaken vigorously before incubating on ice for 15 minutes. Samples were then centrifuged at 14000 rpm for 30 minutes at 4°C. Aqueous phase was transferred to a fresh RNAse free tube and an equal volume of chilled iso-propanol was added. Samples were

incubated on ice for 10 minutes. RNA was precipitated by centrifugation at 14000 rpm for 30 minutes at 4°C. RNA pellets were then washed with ice cold 70% ethanol and centrifuged at 14000 rpm for 10 minutes at 4°C. RNA pellets were air dried and dissolved in nuclease free water. RNA samples were stored at -80°C.

2.2.4. First strand cDNA synthesis

Invitrogen Superscript II reverse transcriptase was used for cDNA synthesis. 500ng-1000ng of RNA was used to make cDNA. RNA mix contained 1µg of RNA, 2µl of random hexamers, 1µl of 10mM dNTP mix and was made up to 12µl with nuclease free water (NFW). RNA mix was denatured at 65°C for 5 minutes and was chilled on ice immediately for 5 minutes. To this, buffer mix was added and incubated at 25°C for 2 minutes. The buffer mix contained:

5X first strand synthesis buffer - 4µl

0.1 M DTT - 2µl

RNAse out (40U/µl) – 0.5µl

1µl of Superscript II reverse transcriptase was added to the cDNA mix and was incubated at 25°C for 10 minutes. First strand synthesis was carried out at 42°C for 50 minutes. Reverse transcriptase was inactivated at 70°C for 15 minutes.

2.2.5. Amplification of genes from cDNA and cloning

Amplification of genes

Gene specific primers were designed using software Primer3 (http://primer3.ut.ee/). Takara LaTaq polymerase was used to amplify genes from cDNA. The PCR was carried out as follows:

10X PCR Buffer - 5µl

dNTP mixture(2.5 mM each) – 4µl

Primer mix (10µM each) - 4µl

cDNA - 1µl

Enzyme (5U/µl) – 0.3µl

NFW – make up to 50µl

Cloning

Amplified products were cloned into PCR TOPO II. PCR TOPO II vector has both SP6 and T7 promoters which can be used to synthesize antisense RNA strands. PCR product and the linearized vector were ligated at a molar of ratio of 3:1. Ligation reaction was for 1 hour at room temperature using the T4 DNA ligase provided along with the kit. Ligation product was transformed into DH5α competent cells. Bacterial culture was plated and grown overnight on LB Agar plates with Kanamycin (50µg/ml), IPTG(100mM) and X-gal(40mg/ml). Plasmids obtained from white colonies were sequenced.

2.2.6. Rapid amplification of cDNA ends (RACE)

RACE was performed to obtain the complete sequence of the gene of interest. RACE kit supplied by Ambion Life Technologies was used for the purpose. For 3' RACE, cDNA was synthesized using the 3' RACE adapter. Two rounds of nested PCR were carried out using the cDNA to obtain the 3' end of the gene. The program used for thermocycler was:

1. 94°C for 3 minutes
2. 94°C for 30 seconds
3. 60°C for 30 seconds
4. 72°C for 2:30 minutes
5. Back to step 2- 35 times
6. 72°C for 5 minutes

2.2.7. *In-vitro* transcription

In-vitro transcriptions reactions were performed to obtain either double stranded RNA (dsRNA) or antisense RNA riboprobe. PCR product with T7 promoter overhangs at both the ends was used as a template for dsRNA synthesis. Linearized PCR TOPO II with gene of interest cloned was used as template for riboprobe synthesis.

dsRNA synthesis

Ambion megascript RNAi kit was used to synthesize dsRNA. The reaction was carried out as suggest by the providers. The reaction was carried out for 12 hours at 37°C. This was followed by DNAse and RNAse treatment at 37°C for an hour. The dsRNA was purified using the filter cartridge provided with the kit. dsRNA was visualized by gel electrophoresis on an agarose gel.

Riboprobes synthesis

A gene cloned in PCR TOPO II is sandwiched between SP6 and T7 promoter sequences. Depending on the orientation of integration of the gene into the vector one of the enzymes were used to synthesize riboprobes. Accordingly the plasmid was linearized away from the promoter site of the enzyme to be used for transcription. During RNA synthesis labelled UTPs were used which were later detected by antibodies. Usually DIG/Fluroscein labelled UTPs were used during *in vitro* transcription.

Linearized plasmid - 1µg

10X transcription buffer- 2µl

10X DIG/Fluroscein labelling mix- 2µl

RNAse out - 1µl

T7/SP6 polymerase (20U/µl) - 2µl

NFW – to make up to 20µl

The above reaction was carried out in a micro-centrifuge tube at 37°C for 6 hours. This was followed by DNAse treatment for 45-60 minutes at 37°C. Riboprobe was purified by either precipitation or Biorad Micro Bio-Spin P30 gel columns, Tris buffer. When using the Biorad columns, protocol provided by the supplier was utilized. On the other hand for precipitation of riboprobe absolute ethanol was used. The precipitation procedure was:

Riboprobe – X µl

4M LiCl – 1/10th the volume

Absolute ethanol – 3 times the volume

Mix the solution and incubate at -80°C for an hour. The sample was then centrifuged at 14000 rpm for 30 minutes at 4°C to obtain the pellet. Pellet was washed with chilled 70% ethanol prepared with RNAse free water. The pellet was dissolved in NFW and stored at -20°C.

2.2.8. RNA interference

RNA interference was achieved by injecting planarians with dsRNA using a Drummond Scientific Nanoject Injector. Three injections of 69nl of dsRNA of concentration 2µg/ul were given to planarians for 3 consecutive days. Second round of injections as mentioned above were given after a gap of 4 days. A day after the second round of injections, animals were amputated and allowed to regenerate. Phenotypes were scored at the end of 9 days of regeneration, only if a significant number of animals showed phenotype. If there was no phenotype observed then animals were injected with dsRNA as mentioned earlier for 3 consecutive days and amputated on the following day. In case of homeostatic worms dsRNA was injected 3 consecutive days a week for 4 weeks.

2.2.9. Whole mount *in-situ* hybridizations

Planarians of length 2-3 mm were used for *in-situ* hybridizations. A week starved planarians were transferred to 2ml Eppendorf tubes or 15 ml Falcon tubes. Planarian water was replaced with 5% NAC for 10 minutes. Animals were fixed in 4% formaldehyde for 20 minutes. Fixative was replaced and worms were rinsed with PBSTx. Prewarmed reduction solution was added and the reaction was carried out at 37°C for 10 minutes. Post reduction worms were rinsed with PBSTx. PBSTx was replaced with 50% methanol for 5 minutes. 50% methanol was replaced with 100% methanol for 5 minutes and then worms were stored in -20°C for an hour or till further use in 100% methanol. Animals were rehydrated with 50% methanol and PBSTx for 5 minutes each. PBSTx was replaced with 1x SSC for 5 minutes. Formamide-bleach solution was added and worms were bleached under direct light

for 1.5-2 hours. Bleach solution was replaced with 1X SSC for 5minutes. Worms were then rinsed twice in PBSTx for 5-10 minutes. PBSTx was replaced with Proteinase K (2µg/µl) solution for 10 minutes. Animals were post fixed in 4% formaldehyde. Fixative was replaced and worms were rinsed twice with PBSTx for 5 minutes. This was followed with PBSTx:Pre Hyb::1:1 wash for 10 minutes. 1:1 wash was replaced with Pre Hyb for 2 hours at 56°C. Riboprobes were heat denatured in Hyb solution at 65°C for minutes. Pre Hyb was replaced with Hyb buffere containing desired riboprobes (usually diluted 1:500) and hybridization was done at 56°C for more than 16 hours. Post hybridization worms were washed twice each in Pre Hyb, Pre Hyb: 2X SSCx (1:1), 2X SSCx and 0.2x SSCx for 30 minutes each at 56°C. Worms were then washed three times with MABT at room temperature for 10 minutes each. Animals were incubated in blocking solution (5% horse serum + 0.5% Roche Western Blocking Reagent in MABT) for 2 hours at room temperature. Appropriate antibody solution made in blocking solution was added to the worms and incubated overnight at 4°C. Animals were then washed more than 6 times in MABT for 20 minutes each.

For NBT/BCIP detection animals were washed in AP buffer for 10 minutes. NBT/BCIP was added in AP buffer and signal was developed in dark. Time taken for signal development varied from 30 minutes to 2 hours. Development reaction was stopped by washing twice in PBSTx for 10 minutes. Animals were then fixed in 4% formaldehyde for 20 minutes. Background was cleared by washing animals in 100% ethanol for 20-30 minutes. Animals were then washed with 50% ethanol for 5 minutes. Worms were stored in 80% glycerol at 4°C.

For developing tyramide signal, animals were incubated with fluorescent conjugated tyramide in TSA buffer with H_2O_2 for 30-45 minutes in dark. Post development worms were washed 6 times in PBSTw for 20 minutes each. Peroxidase activity was killed by incubating worms in 200mM sodium azide solution made in PBSTw. Animals were post fixed with 4% formaldehyde. Worms were washed overnight in PBSTw and were mounted in Mowiol.

2.2.10. Whole mount immunofluorescence assay

Planarians were transferred to 2ml Eppendorf tubes or 15ml Falcon tubes and treated with 2% HCl on ice to remove mucus. Animals were fixed in Carnoy's fixative for 2.5-3 hours at room temperature. Worms were rinsed with 100% methanol and then stored in -20°C for at least 1 hour or till further use. Animals were bleached in 6%H_2O_2 in methanol solution for 4-6 hours to get rid of the pigment. Post bleaching animals were serially rehydrated with 75%, 50% and 25% methanol. 25% methanol was replaced with PBSTx. Each of the rehydration steps was for 5 minutes on rocker. Worms were blocked using 10% horse serum in PBSTx for 4-6 hours. Primary antibody solution was made in blocking solution and incubated overnight at room temperature or at 4°C depending on the antibody. The next day worms were washed 6-7 times with PBSTx for 20 minutes each. A fluorophore conjugated secondary antibody was added to the wells and worms were incubated overnight at room temperature. Post-secondary antibody incubation, worms were washed 6-7 times in PBSTx. Worms counterstained with DAPI were mounted in Mowiol.

2.2.11. Macerates preparation and flow cytometry

To obtain single cell suspension from planarians, animals were washed with cold CMFB. After removing the liquid the animals were cut into fine pieces in petri dish placed on ice. These fine pieces were transferred to a tube and washed three times with CMF at 4°C. To release cells from the tissue, cut pieces were treated with 0.25% Trypsin for 20 minutes. Trypsinized cell suspension was passed through a 40 micron filter (BD Falcon Cell Strainer). Filtered cell suspension was washed in CMFB to stop trypsin activity. After 3 washes with CMFB, cells were resuspended in required volume of CMFB.

Planarian cell populations can be differentiated on the nuclear to cytoplasmic ratio. Dividing stem cell populations (X1) has higher DNA content and lesser cytoplasm, whereas the stem cells in G1 phase and progenitors cells (X2) have lesser DNA and cytoplasm. Both X1 and X2 cell populations are irradiation sensitive. Terminally differentiated, irradiation insensitive cells (Xins) have higher cytoplasm and less DNA (Hayashi et. al., 2006).

Cell suspension was incubated with cytoplasm stain Calcein and DNA binding stain Hoechst 3342 for an hour in dark at room temperature. Propidium iodide stain was added just before analysis to detect dead cells. Using forward scattering and side scattering small debry was removed. Cells less in PI and high in Calcein were selected. X1, X2 and Xins populations were gated from Hoechst vs. Calcein plot. All experiments were performed in duplicates. Cells were analysed on Becton and Dickenson FACS Aria cell sorter.

2.2.12. Quantitative PCR (q-PCR)

q-PCR was carried out in (machine). Sybr Green PCR master mix supplied by Applied Biosystems was used for the purpose. Different dilutions of cDNA were tried before settling on one for the following reactions. q-PCR was carried out in triplicates with each reaction of 10µl. The reaction master mix was prepared as follows:

1. 2X Sybr Green PCR master mix – 17.5µl
2. cDNA - 2µl
3. Primer mix (2.5mM) - 7µl
4. NFW – 8.5µl

Fold change in gene expression was calculated using $\Delta\Delta$Ct with Actin as the control gene.

2.2.13. Imaging and data analysis

Images were acquired on Zeiss LSM 700 and were analysed using Image J. H3P positive cells were counted in grids on the animals. From the count obtained H3P positive cells per sq. millimetre area was calculated. Statistical analysis was done on obtained data in Microsoft Excel (v. 2007). Both H3P positive cell count and data obtained from q-PCR was analysed using two tailed Student's t-test.

2.2.14. Phylogenetic analysis

Phylogenetic tree was built using MacVector. A multiple sequence alignment of homologues across metazoans was made. Neighbour joining method was used to build the tree with 500 boot strappings.

Chapter 3

Results

3.1. Phylogenetic analysis of Smed-FBP

FUSE binding protein in humans has four K-homology domains. The four KH domains in the central part of the protein are followed by tyrosine motifs at the C-terminal end (Zhang and Chen, 2013). Smed-FBP has three KH domains and no tyrosine motif was discovered in the C-terminal domain of the protein.

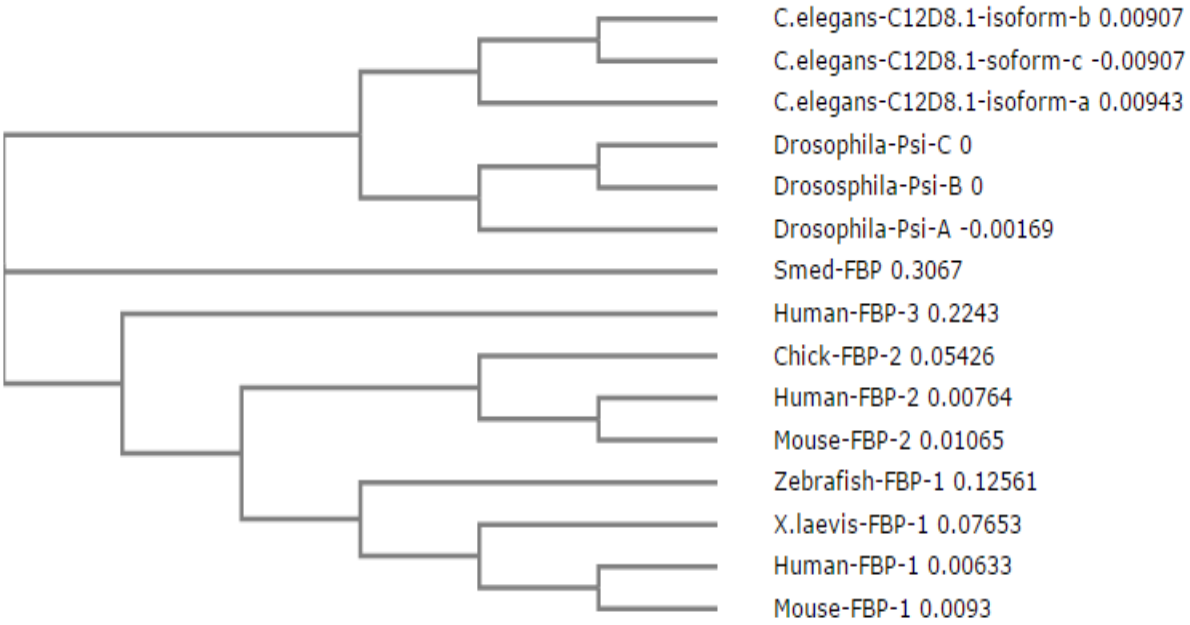

Fig 3.1.1.A phylogenetic tree showing the relationship among the FBPs found in Human, Mouse, Xenopus, Zebrafish, Chick, Fly and C.elegans. A multiple sequence alignment was created out of the protein sequences and neighbour joining method was used to build the tree.

3.2. Expression pattern of *smed-fbp*

Transcriptome sequencing of X1, X2 and Xins populations show that smed-fbp is enriched in X1. X1 population consists of dividing neoblasts. *Smedwi-1* is a marker for neoblasts. *Smedwi-1* expression is seen in the mesenchyme and the region in front of the eyes and the pharynx is free of its expression. *smed-fbp* expression

pattern is similar to *Smedwi-1* expression. *smed-fbp* expression is mesenchymal (Fig 3.2.1). Irradiation is known to kill dividing neoblasts and germ cells. To determine if *smed-fbp* is expressed in neoblasts or germ cells semi-quantitative RT-PCR was performed with RNA isolated from both sexual and asexual worms which were irradiated with 90Gy of γ-radiation. Upon irradiation there was reduction in *smed-fbp* levels.

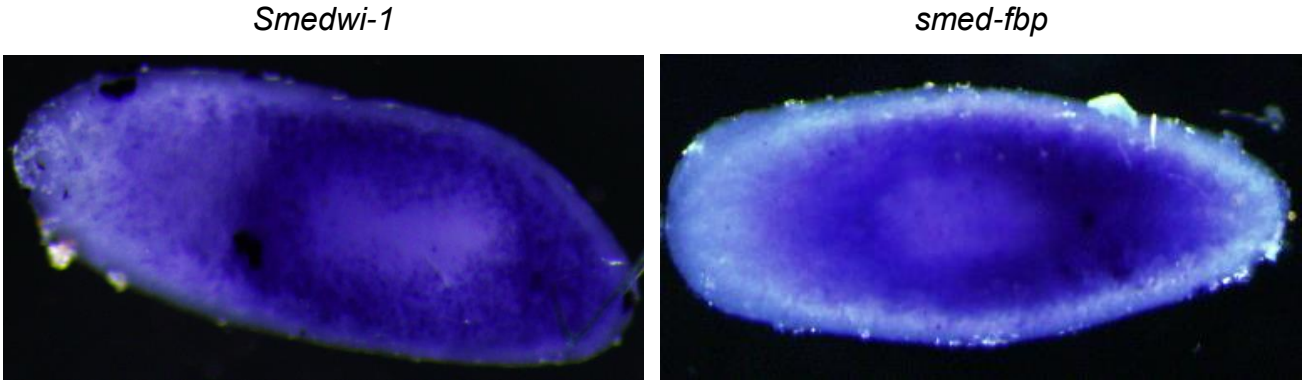

Fig 3.2.1: *smed-fbp* expression pattern. Whole mount *in-situ* hybridizations showing dorsal surface. Anterior of the animal is to left.

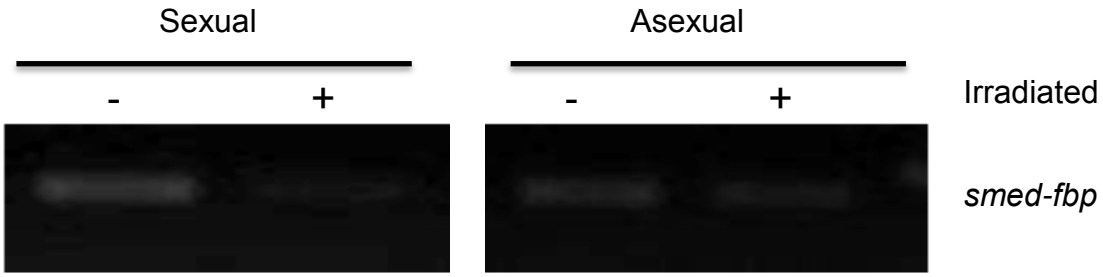

Fig 3.2.2: *smed-fbp* expression is diminished after irradiation. RNA isolated from both sexual and asexual strains with or without irradiation were used to perform the PCR. PCR was carried out for 28 cycles

3.3. Smed-FBP is essential for regeneration and homeostasis

To understand the role of Smed-FBP in regeneration and homeostasis *smed-fbp* was depleted using RNAi. dsRNA was delivered to the worms through injections. dsRNA generated from *gfp* sequence was used as a control. dsRNA injection schedule was carried as mentioned in section 2.2.8 (Fig 3.3.1). dsRNA at a concentration of 2µg/ul was injected into the worms.

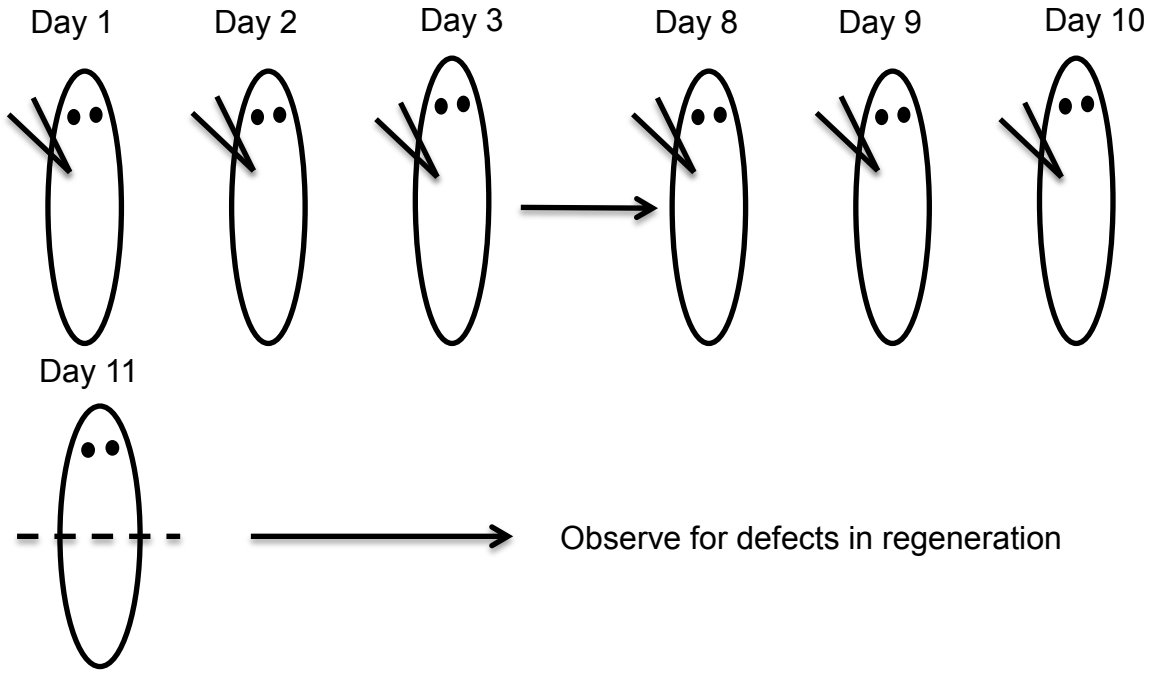

Fig 3.3.1: Methodology followed to set up RNAi. Worms were injected with dsRNA as depicted above. During first round of regeneration animals showed defects.

Worms injected with *gfp* dsRNA had all regenerated completely by 7 days. On the other hand *fbp* RNAi animals did not regenerate. Blastema on 3rd day of regeneration was similar to that of the controls. But during the course of regeneration the blastema didn't grow and develop. It was reabsorbed and by 10th day of regeneration there was no observable blastema. This was true with both the head and the tail blastema. On 12th or 13th day of regeneration indentations were observed at the site of amputation. Animals lysed and died by 20 days. The penetrance for RNAi was 100% (20/20).

Fig 3.3.2: *smed-fbp* RNAi affects posterior regeneration. Anterior side of the worms are on the top. Arrows point to tail blastema in control animals which is not developed in RNAi animals. dpa is days post amputation.

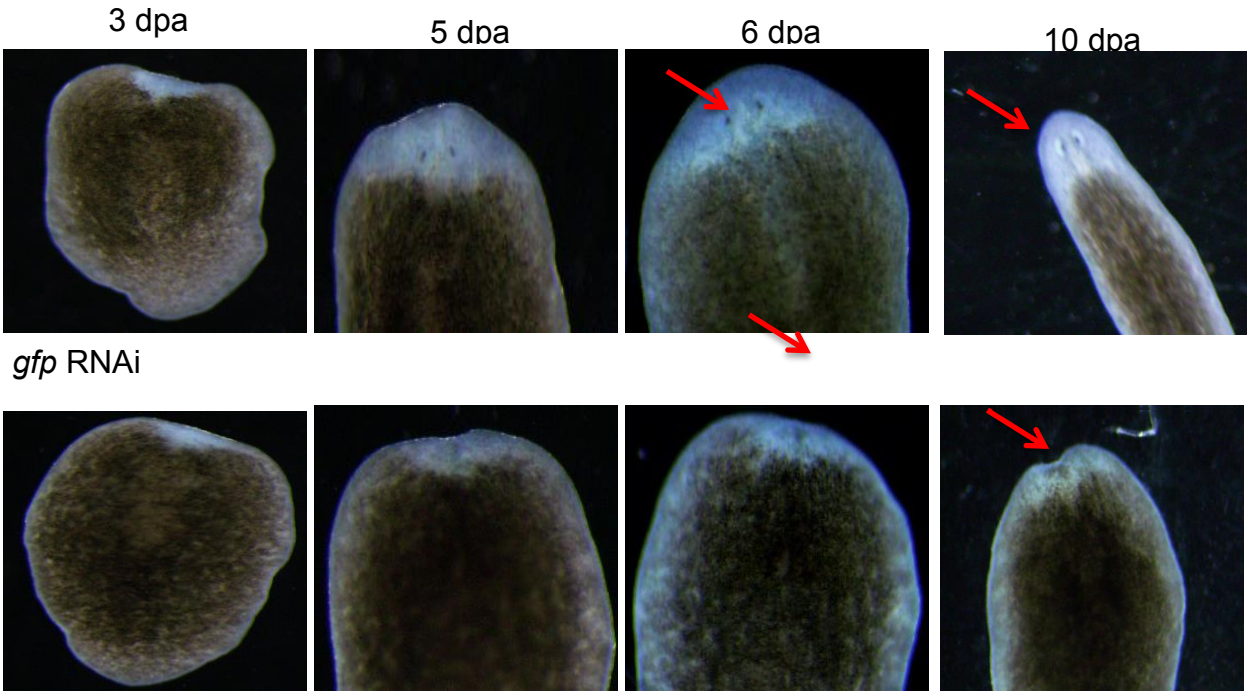

Fig 3.3.3: *smed-fbp* RNAi affects anterior regeneration. Anterior side of the worms are on the top. Arrows point to blastema in control animals which is not developed in RNAi animals. dpa is days post amputation.

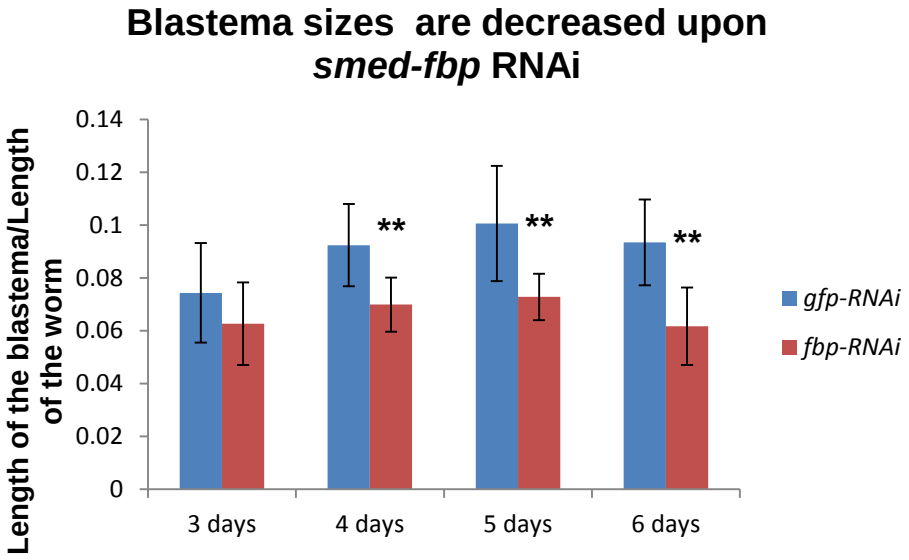

Fig: 3.3.4: *smed-fbp* affects blastema growth. Blastema length was measured at different time points and was normalized with the length of the worm. ** indicates p-value< 0.05. Days indicate days post amputation. Error bars indicate 1 standard deviation.

To understand the importance of Smed-FBP in homeostasis, dsRNA was injected to worms for three consecutive days a week for four weeks. By 25th day of RNAi most animals started to show phenotype. Animals were curled and had lesions close to the eyes on the dorsal surface. By 28th to 30th day the animals had lysed and dead.

Fig 3.3.5: Smed-FBP is essential for homeostasis. Anterior side of the animal is to the left. Arrow points to the lesions observed on the RNAi animals.

3.4. *Smed-fbp* RNAi affects neoblast proliferation

FBP is a known regulator of *myc* in humans, which regulates cell cycle (He et al., 2000). FBP regulates cell proliferation independent of *myc* as well. FBP regulates the translation of p27 and mRNA stability of p21 thus regulating cell progression from G1 to S phase (Zhang and Chen, 2013). Along with this, we had observed that the blastema was not developing. We hypothesized that this may be either because of decreased neoblast proliferation or decreased differentiation of neoblasts. To check neoblast proliferation whole mount immunofluorescence for phosphorylated histone H3 (H3PS10) was performed. H3PS10 marks dividing cells starting from G2/M transition. In *Smed-fbp* RNAi animals neoblast proliferation was significantly reduced by 3 days post amputation (p-value <0.1). The scenario in 9 days regenerating *Smed-fbp* RNAi animals was similar (p-value <0.05).

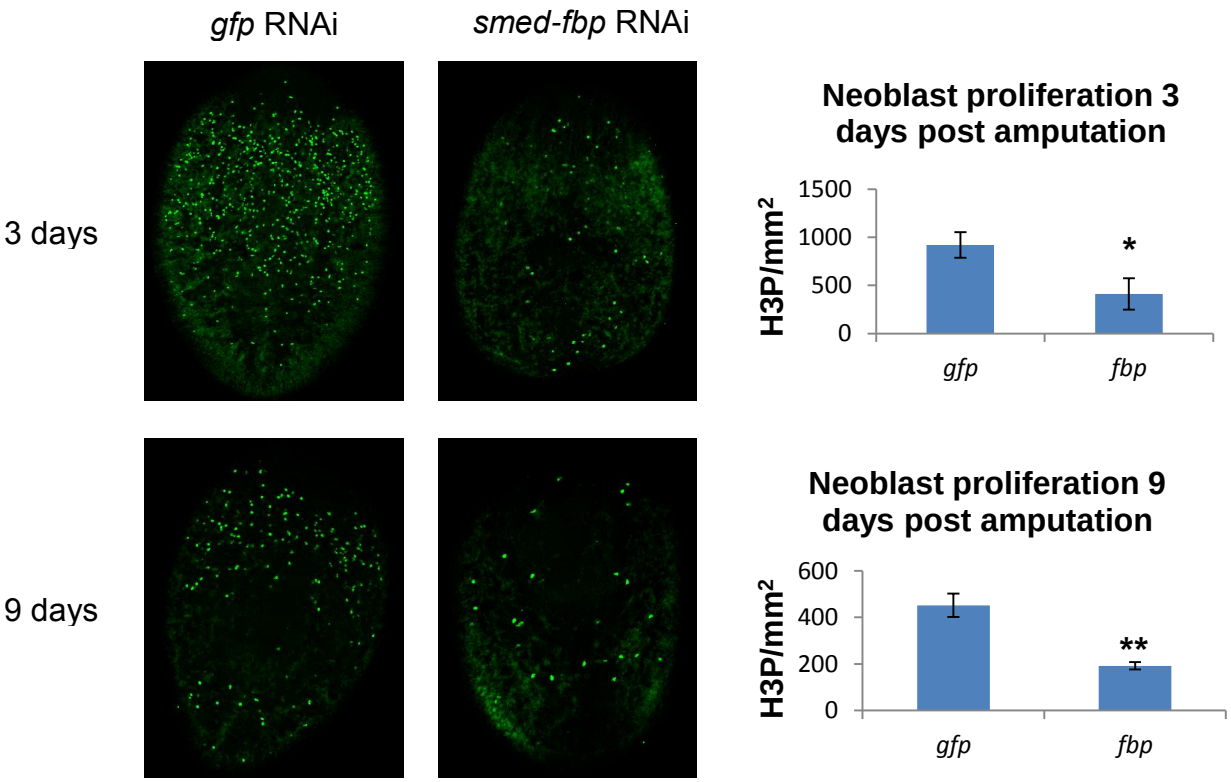

Fig 3.4.1: Cell proliferation analysis during regeneration in *smed-fbp* RNAi.
Maximum intensity projections of the images obtained on confocal with anterior end to the top. Error bars indicate standard error in mean (S.E.M). * indicates p-value<0.1 and ** indicates p-value<0.05.

Smed-fbp knock down efficiency was checked by means of q-PCR. RNA isoloated from 9 day regenerating worms was used for the purpose. In addition to that expression levels of few category markers were determined. A neoblast and its journey through differentiation can determined few markers which are particular to a stage of neoblast and its differentiation. Category 1 marks for neoblasts and *Smedwi-1* is one of them. Similarly there are category 2, 3 and 4 markers for neoblast progenitors, early and late progenitor cells respectively. Expression levels of these category markers were determined to understand the effect of *Smed-fbp* RNAi on neoblast differentiation (Fig 3.4.2). q-PCR data shows that PCNA levels are increased upon *Smed-fbp* knock down and few other category markers are downregulated.

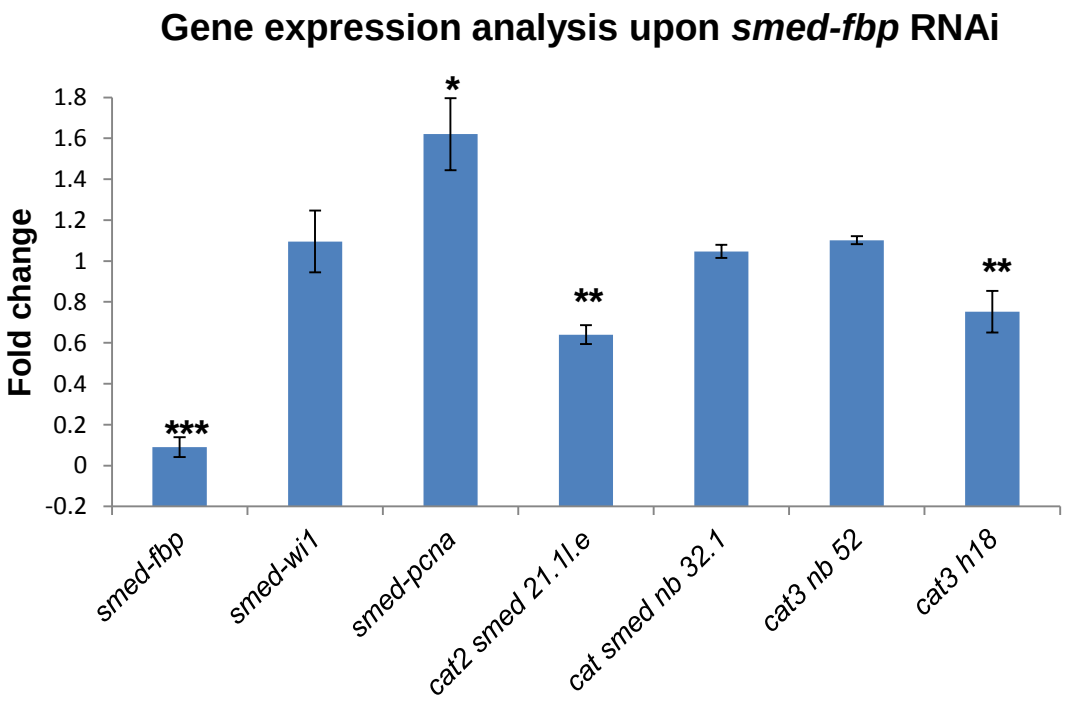

Fig 3.4.2: Effect of *smed-fbp* RNAi on neoblast proliferation and differentiation. q-PCR analysis performed using RNA isolated from 9 day regenerating animals. * indicates p-value < 0.1, ** indicates p-value <0.05 and *** indicates p-value < 0.01. Error bars indicate S.E.M.

Smedwi-1 levels were tried to further analyse with whole mount fluorescence *in situ* hybridizations. The *Smedwi-1* staining in knockdown animals looked similar to

staining in control worms, but it was hard to quantitate this data since counting cells was difficult (Fig 3.4.3).

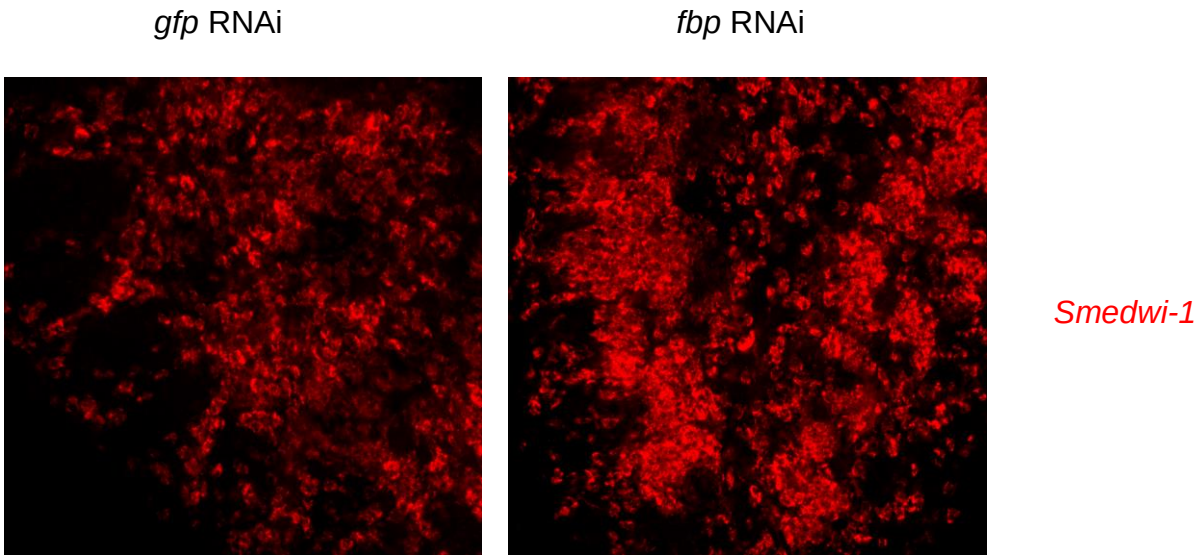

Fig 3.4.3.Whole mount fluorescence *in situ* hybridization for *Smedwi-1* in 9 days regenerating animals.

3.5. Analysis of X1, X2 and Xins cell populations using flow cytometer

Planarian cells can be classified into three populations based on nuclear to cytoplasmic ratio and irradiation sensitivity (Reddien et al., 2005, Hayashi et al., 2006). Animals showing phenotypes were collected at different time points and were processed for flow cytometry. Samples processed for X1, X2 and Xins population analysis were stained with Hoescht 3342, a DNA binding dye and Calcein, a cytoplasmic dye. Dead cells were avoided in the analysis by using propidium iodide. X1, X2 and Xins populations were analysed at 6 days, 10 days and 12 days post amputation. Each sample was analysed in duplicates. The X1, X2 and Xins population dynamics observed were not conclusive. Extensive analysis of cell populations at more time points with triplicates has to be tried out before we can conclude anything about the dynamics of the cell populations upon *Smed-fbp* knockdown.

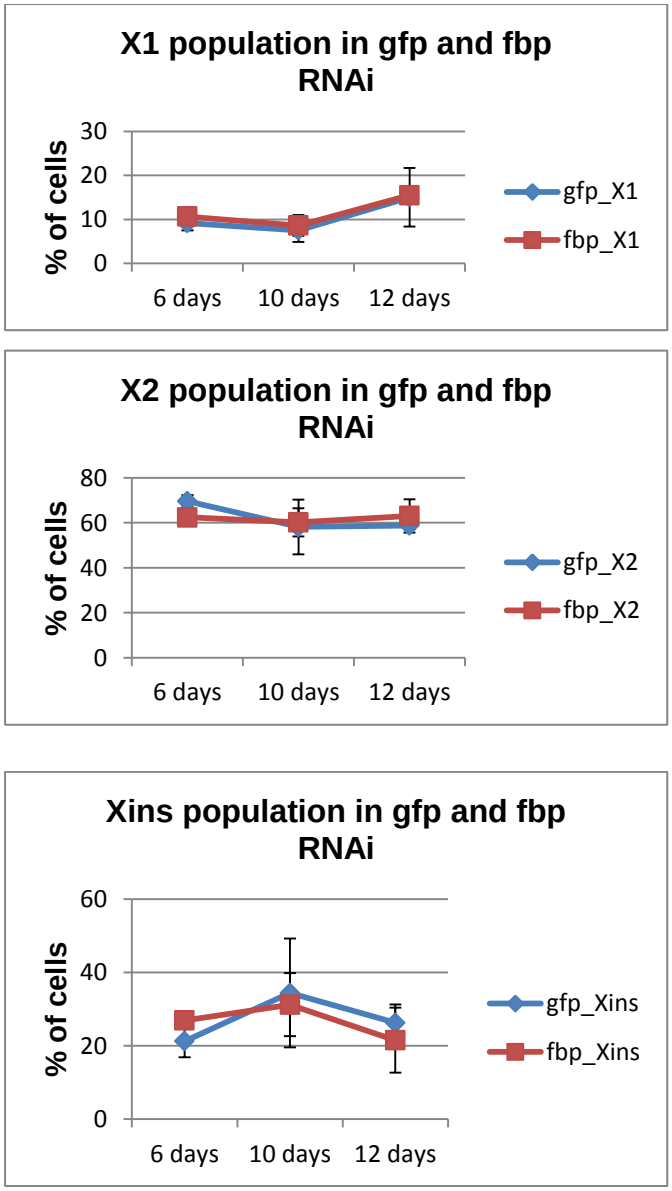

Fig 3.5.1.Analysis of X1, X2 and Xins populations. Animals showing phenotype were analysed at the times indicated using flow cytometer. Error bars indicate standard deviation. (Days – days post amputation)

Chapter 4

Discussion

RNA binding proteins are necessary for stem cell maintenance and differentiation. In planarians RBPs are required for maintenance of neoblast population and differentiation (Reddien et al, 2005, Palakodeti et al., 2008, Wagner et al, 2012). transcriptome sequencing study done in the lab to comprehensively analyse RBPs in neoblast population showed that ~60% of the RBPs are enriched in stem cells. In the current study, we indentified FUSE binding protein (FBP) to be one of the RBPs enriched in neoblasts. The goal of this project was to understand the role of FBP in planarian regeneration and homeostasis.

FBPs are characterized by 4 KH domains in the central domain of the protein and transcription activating tyrosine motifs at the C-terminus. Smed-FBP has 3 KH-domains in the central domain and no tyrosine motifs were found at the C-terminal end. One of the FBP family members in C.elegans has a glutamine-rich activating motif in its C-terminus (Davis-Smyth et al., 1996, Courey and Tijan, 1988). Smed-FBP does not house the glutamine rich activating motif as well. Smed-FBP may be using a different motif for transcription activation in planarians which is still unknown.

Smed-fbp expression was mostly observed in the mesenchyme. The staining was intense in the regions posterior to pharynx and was sparsely distributed in the anterior part of the animal. Like *Smedwi-1* expression pattern *Smed-fbp* was absent from pharynx, region in front of the eyes and in the periphery (Fig 3.2.1). *Smed-fbp* expression was dramatically reduced upon irradiation suggesting its expression mostly in the neoblast population. Since low levels of *Smed-fbp* expression was also observed after Irradiation, we presume that *Smed-fbp* might also be expressed in the progenitor populations. FISH colocalization studies with *Smed-fbp* and progenitor markers are to be performed to confirm its expression in differentiating cells.

Smed-fbp RNAi animals were unable to grow and develop blastema during regeneration. This defect was seen both in anterior and posterior wounds (Fig 3.3.2 and Fig 3.3.3). Blastema sizes in the knockdown worms were quite comparable to control worms until 3 days post amputation (dpa). But we observed decrease in the blastema size from 4 dpa and the size of the blastema decreased significantly by 10

dpa. There can be two reasons for blastema not being developed during regeneration: 1) *Smed-fbp* RNAi affects neoblast proliferation hence there are no new cells to form blastema or 2) *Smed-fbp* RNAi affects differentiation of neoblasts. Since blastema is made up of progenitor and differentiating cells, depletion of differentiated cells because of *Smed-fbp* RNAi may lead to no blastema formation (Reddien and Sanchez Alvarado, 2004). *Smed-fbp RNAi* animals in homeostasis showed defects after 4 weeks dsRNA injection. The animals curled along the dorsal body and developed lesions on dorsal surface close to the photoreceptors by day 24 (Fig 3.3.4). Curling and lesions on the dorsal surface are characteristic phenotype in planarians observed when they are depleted of stem cells (Reddien et al., 2005).

Neoblast proliferation observed using H3PS10 antibody which marks cells in G2/M phase was decreased upon *Smed-fbp* RNAi (Fig 3.4.1). This result suggests that Smed-FBP is essential for neoblast proliferation. Interestingly the levels of *Smed-pcna*, which marks the cells in S-phase are increased and the *Smedwi-1* expression, which marks the overall neoblast population remains unaffected (Fig 3.4.2). This suggests that the neoblasts number itself may not be changing upon knockdown, but the neoblasts are stuck in G1 or S phase of the cell cycle without being able to transit into G2 phase.

In addition to being necessary for neoblast proliferation, Smed-FBP may be required for differentiation. Expression levels of few of the category markers remained unaffected by knockdown whereas expression of few other category markers had significantly reduced. This may be because there are not enough neoblasts which could go down the path of differentiation or it might be that Smed-FBP is necessary for differentiation of neoblasts into certain lineages. Since the *Smedwi-1* levels representing the overall stem cell population are unaffected the decreased expression of differentiation markers suggests the necessity of Smed-FBP in differentiation.

FBP is a known activator of *c-myc* (Duncan et al., 1994). MYC is known to control proliferation, growth, differentiation and apoptosis (Chung and Levens, 2005). Depletion of FBP in cancerous cells decrease MYC expression and thus leads to elongation of cell cycle without affecting cell viability (He et al., 2000). Smed-FBP may also be acting in a similar way via MYC, but surprisingly we were unable to

identify *c-myc* like gene in the planarian genome. FBP can control cell cycle independent of MYC through p27 and p21, the two cyclin dependant kinase inhibitors (CKI) which regulate cell cycle switch from G1 to S-phase and are post-transcriptionally regulated by FBP (Zhang and Chen, 2013). If there is no MYC like protein in planarians then may be Smed-FBP controls cell cycle via post transcriptional regulation of these CKIs or other undiscovered targets. Transcriptome sequencing and pull down studies using Smed-FBP specific antibody will help in identifying direct and indirect targets of Smed-FBP.

One of the factors to decide the fate of neoblast is its postion in the animal which is defined by anterior-posterior (AP) axis maintained by b-catenin (Iglesias et al., 2008), dorsal-ventral (DV) axis controlled by BMP/ADMP (Gavino and Reddien, 2011). Since Smed-FBP is understood to be essential for differentiation, it may be possible that Smed-FBP is playing a role in maintenance of these axes. Hence it will be interesting to look at polarity markers during the course of regeneration.

To summarize, this study shows that Smed-FBP is necessary for planarian regeneration and homeostasis. Smed-FBP is necessary for neoblast proliferation and differentiation. We hypothesize that the knockdown of *Smed-fbp* results in G1 or S-phase arrest of the neoblasts thus affecting blastema growth.

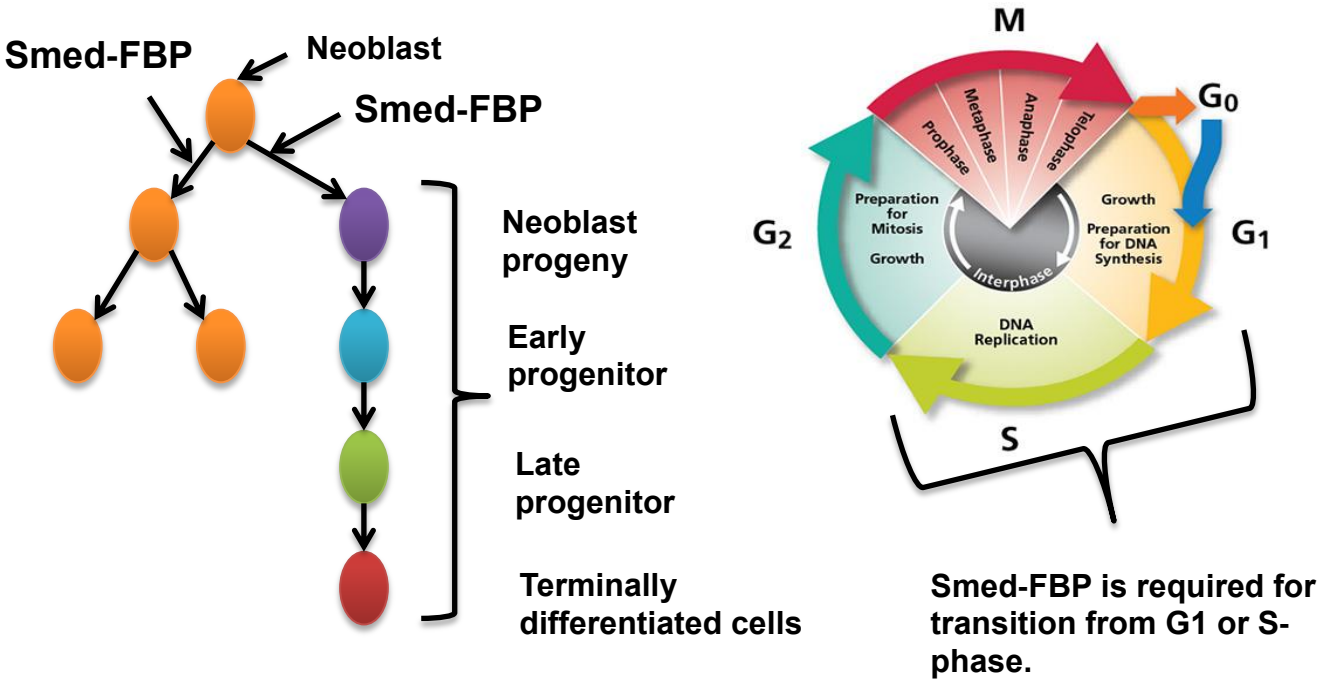

Fig 4.1: Smed-FBP is necessary for neoblast proliferation. Depletion of Smed-FBP arrests neoblasts in G1 or S phase of the cell cycle. Image credits- BDBiosciences.

References

Alibardi, L., and Toni, M. (2005). Wound keratins in the regenerating epidermis of lizard suggest that the wound reaction is similar in the tail and limb. Journal Of Experimental Zoology Part A: Comparative Experimental Biology *303A*, 845-860.

Avigan, M., Strober, B., and Levens, D. (1990). A Far Upstream Element Stimulates c-myc Expression in Undifferentiated Leukemia Cells. The Journal Of Biological Chemistry *265*, 18538-18545.

Baguna, J., Salo, E., Romero, R., Garcia-Fernandez, J., Bueno, D., Munoz-Marmol, A., and Bayascas-Ramirez, J. (1994). Regeneration and pattern formation in planarians: cells, molecules and genes. Zoological Science 781-795.

Bazar, L., Harris, V., Sunitha, I., Hartmann, D., and Avigan, M. (1995). A transactivator of c-myc is coordinately regulated with the proto-oncogene during cellular growth. Oncogene *10*, 2229-2238.

Bely, A., and Nyberg, K. (2010). Evolution of animal regeneration: re-emergence of a field. Trends In Ecology & Evolution *25*, 161-170.

Bely, A. (2006). Distribution of segment regeneration ability in the Annelida. Integrative And Comparative Biology *46*, 508-518.

Betchaku, T. (1967). Isolation of planarian neoblasts and their behaviorin Vitro with some aspects of the mechanism of the formation of regeneration blastema. J. Exp. Zool. *164*, 407-433.

Cebria, F., and Newmark, P. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. Development *132*, 3691-3703.

Cebria, F., Kudome, T., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., and Agata, K. (2002). The expression of neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system. Mechanisms Of Development *116*, 199-204.

Chien, H., Liao, C., and Lin, Y. (2011). FUSE Binding Protein 1 Interacts with Untranslated Regions of Japanese Encephalitis Virus RNA and Negatively Regulates Viral Replication. Journal Of Virology *85*, 4698-4706.

Chung, H., and Levens, D. (2005). c-myc Expression: Keep the Noise Down!. Molecules And Cells *20*, 157-166.

Courey, A., and Tjian, R. (1988). Analysis of Sp1 in vivo reveals mutiple transcriptional domains, including a novel glutamine-rich activation motif. Cell *55*, 887-898.

Davis, A., Wims, M., Spotts, G., Hann, S., and Bradley, A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes & Development *7*, 671-682.

Dinsmore, C. (1991). A History of Regeneration Research: Milestones in the Evolution of a Science (Cambridge University Press).

Duncan, R., Bazar, L., Michelotti, G., Tomonaga, T., Krutzsch, H., Avigan, M., and Levens, D. (1994). A sequence-specific, single-strand binding protein activates the far upstream element of c-myc and defines a new DNA-binding motif. Genes & Development *8*, 465-480.

Duncan, R., Collins, I., Tomonaga, T., Zhang, T., and Levens, D. (1996). A Unique Transactivation Sequence Motif Is Found in the Carboxyl- Terminal Domain of the Single-Strand-Binding Protein FBP. Molecular And Cellular Biology *15*, 2274-2282.

Eisenhoffer, G., Kang, H., and Alvarado, A. (2008). Molecular Analysis of Stem Cells and Their Descendants during Cell Turnover and Regeneration in the Planarian Schmidtea mediterranea. Cell Stem Cell *3*, 327-339.

Gaviño, M., and Reddien, P. (2011). A Bmp/Admp Regulatory Circuit Controls Maintenance and Regeneration of Dorsal-Ventral Polarity in Planarians. Current Biology *21*, 294-299.

Gaviño, M., and Reddien, P. (2011). A Bmp/Admp Regulatory Circuit Controls Maintenance and Regeneration of Dorsal-Ventral Polarity in Planarians. Current Biology *21*, 294-299.

Glisovic, T., Bachorik, J., Yong, J., and Dreyfuss, G. (2008). RNA-binding proteins and post-transcriptional gene regulation. FEBS Letters *582*, 1977-1986.

Grishin, N. (2001). KH domain: one motif, two folds. Nucleic Acids Research *29*, 638-643.

Guo, T., Peters, A., and Newmark, P. (2006). A bruno-like Gene Is Required for Stem Cell Maintenance in Planarians. Developmental Cell *11*, 159-169.

Guo, T., Peters, A., and Newmark, P. (2006). A bruno-like Gene Is Required for Stem Cell Maintenance in Planarians. Developmental Cell *11*, 159-169.

Hartner, J. (2003). Liver Disintegration in the Mouse Embryo Caused by Deficiency in the RNA-editing Enzyme ADAR1. Journal Of Biological Chemistry *279*, 4894-4902.

Hayashi, T., Asami, M., Higuchi, S., Shibata, N., and Agata, K. (2006). Isolation of planarian X-ray-sensitive stem cells by fluorescence-activated cell sorting. Development, Growth And Differentiation *48*, 371-380.

He, L., Liu, J., Collins, I., Sanford, S., O'Connell, B., Benham, C., and Levens, D. (2000). Loss of FBP function arrests cellular proliferation and extinguishes c-myc expression. The EMBO Journal *19*, 1034-1044.

Iglesias, M., Gomez-Skarmeta, J., Salo, E., and Adell, T. (2008). Silencing of Smed-catenin1 generates radial-like hypercephalized planarians. Development *135*, 1215-1221.

Irwin, N., Baekelandt, V., Goritchenko, L., and Benowitz, L. (1997). Identification of two proteins that bind to a pyrimidine-rich sequence in the 3'-untranslated region of GAP-43 mRNA. Nucleic Acids Research *25*, 1281-1288.

Kim, S., Kim, M., Jung, H., Kim, W., Kwon, S., Son, M., Jang, I., Choi, J., Park, S., and Park, B. et al. (2012). Comparative Proteomic Analysis of Human Somatic Cells, Induced Pluripotent Stem Cells, and Embryonic Stem Cells. Stem Cells And Development *21*, 1272-1286.

King, R., and Newmark, P. (2013). In situ hybridization protocol for enhanced detection of gene expression in the planarian Schmidtea mediterranea. BMC Dev Biol *13*, 8.

Liu, J., Akoulitchev, S., Weber, A., Ge, H., Chuikov, S., Libutti, D., Wang, X., Conaway, J., Harris, C., and Conaway, R. et al. (2001). Defective Interplay of Activators and Repressors with TFIIH in Xeroderma Pigmentosum. Cell *104*, 353-363.

Liu, J., Kouzine, F., Nie, Z., Chung, H., Elisha-Feil, Z., Weber, A., Zhao, K., and Levens, D. (2006). The FUSE/FBP/FIR/TFIIH system is a molecular machine programming a pulse of c-myc expression. The EMBO Journal *25*, 2119-2130.

Morgan, T. (1898). Experimental studies of the regeneration of Planaria maculata. Arch. Entw. Mech. Org. *7*, 364-397.

Newmark, P., and Alvarado, A. (2002). Not your father's planarian: A classical model enters the era of functional genomics. Nat. Rev. Genet. *3*, 210-219.

Newmark, P., and Alvarado, A. (1998). The use of planarians to dissect the molecular basis of metazoan regeneration. Wound Repair Regen *6*, S-413-S-420.

Olanich, M., Moss, B., Piwnica-Worms, D., Townsend, R., and Weber, J. (2010). Identification of FUSE-binding protein 1 as a regulatory mRNA-binding protein that represses nucleophosmin translation. Oncogene *30*, 77-86.

Palakodeti, D., Smielewska, M., Lu, Y., Yeo, G., and Graveley, B. (2008). The PIWI proteins SMEDWI-2 and SMEDWI-3 are required for stem cell function and piRNA expression in planarians. RNA *14*, 1174-1186.

Palladino, M., Keegan, L., O'Connell, M., and Reenan, R. (2000). A-to-I Pre-mRNA Editing in Drosophila Is Primarily Involved in Adult Nervous System Function and Integrity. Cell *102*, 437-449.

Pearson, B., and Alvarado, A. (2009). A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. Development *137*, 213-221.

Pearson, B., Eisenhoffer, G., Gurley, K., Rink, J., Miller, D., and Sánchez Alvarado, A. (2009). Formaldehyde-based whole-mount in situ hybridization method for planarians. Developmental Dynamics *238*, 443-450.

Polyak, K., Lee, M., Erdjument-Bromage, H., Koff, A., Roberts, J., Tempst, P., and Massagué, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell *78*, 59-66.

Rabenhorst, U., Beinoraviciute-Kellner, R., Brezniceanu, M., Joos, S., Devens, F., Lichter, P., Rieker, R., Trojan, J., Chung, H., and Levens, D. et al. (2009). Overexpression of the far upstream element binding protein 1 in hepatocellular carcinoma is required for tumor growth. Hepatology *50*, 1121-1129.

Reddien, P., and Alvarado, A. (2004). Fundamentals of planarian regeneration. Annu. Rev. Cell Dev. Biol. *20*, 725-757.

REDDIEN, P., BERMANGE, A., MURFITT, K., JENNINGS, J., and SANCHEZALVARADO, A. (2005). Identification of Genes Needed for Regeneration, Stem Cell Function, and Tissue Homeostasis by Systematic Gene Perturbation in Planaria. Developmental Cell *8*, 635-649.

Reddien, P., Oviedo, N., Jennings, J., Jenkin, J., and Alvarado, A. (2005). SMEDWI-2 Is a PIWI-Like Protein That Regulates Planarian Stem Cells. Science *310*, 1327-1330.

Rossi, L., Salvetti, A., Marincola, F., Lena, A., Deri, P., Mannini, L., Batistoni, R., Wang, E., and Gremigni, V. (2007). Deciphering the molecular machinery of stem cells: a look at the neoblast gene expression profile. Genome Biol *8*, R62.

Rouhana, L., Shibata, N., Nishimura, O., and Agata, K. (2010). Different requirements for conserved post-transcriptional regulators in planarian regeneration and stem cell maintenance. Developmental Biology *341*, 429-443.

Salvetti, A., Rossi, L., Lena, A., Batistoni, R., Deri, P., Rainaldi, G., Locci, M., Evangelista, M., and Gremigni, V. (2005). DjPum, a homologue of Drosophila Pumilio, is essential to planarian stem cell maintenance. Development *132*, 1863-1874.

Shibata, N., Umesono, Y., Orii, H., Sakurai, T., Watanabe, K., and Agata, K. (1999). Expression ofvasa(vas)-Related Genes in Germline Cells and Totipotent Somatic Stem Cells of Planarians. Developmental Biology *206*, 73-87.

Siomi, H., Matunis, M., Michael, W., and Dreyfuss, G. (1993). The pre-mRNA binding K protein contains a novel evolutionary conserved motif. Nucl Acids Res *21*, 1193-1198.

Siomi, H., Siomi, M., Nussbaum, R., and Dreyfuss, G. (1993). The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. Cell *74*, 291-298.

Trumpp, A., Refaeli, Y., Oskarsson, T., Gasser, S., Murphy, M., Martin, G., and Bishop, J. (2001). c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature *414*, 768-773.

Valverde, R., Edwards, L., and Regan, L. (2008). Structure and function of KH domains. FEBS Journal*275*, 2712-2726.

Wade Harper, J., Adami, G., Wei, N., Keyomarsi, K., and Elledge, S. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-816.

Wagner, D., Ho, J., and Reddien, P. (2012). Genetic Regulators of a Pluripotent Adult Stem Cell System in Planarians Identified by RNAi and Clonal Analysis. Cell Stem Cell *10*, 299-311.

Wagner, D., Wang, I., and Reddien, P. (2011). Clonogenic Neoblasts Are Pluripotent Adult Stem Cells That Underlie Planarian Regeneration. Science *332*, 811-816.

Wang, Q. (2003). Stress-induced Apoptosis Associated with Null Mutation of ADAR1 RNA Editing Deaminase Gene. Journal Of Biological Chemistry *279*, 4952-4961.

Wenemoser, D., and Reddien, P. (2010). Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. Developmental Biology *344*, 979-991.

Yoshida-Kashikawa, M., Shibata, N., Takechi, K., and Agata, K. (2007). DjCBC-1, a conserved DEAD box RNA helicase of the RCK/p54/Me31B family, is a component

of RNA-protein complexes in planarian stem cells and neurons. Developmental Dynamics *236*, 3436-3450.

Zhang, J., and Chen, Q. (2012). Far upstream element binding protein 1: a commander of transcription, translation and beyond. Oncogene *32*, 2907-2916.

Zhang, Z., Harris, D., and Pandey, V. (2008). The FUSE Binding Protein Is a Cellular Factor Required for Efficient Replication of Hepatitis C Virus. Journal Of Virology *82*, 5761-5773.

Zheng, Y., and Miskimins, W. (2011). Far upstream element binding protein 1 activates translation of p27Kip1 mRNA through its internal ribosomal entry site. The International Journal Of Biochemistry & Cell Biology *43*, 1641-1648.