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Identifying Smed-Tbx20 as a Pattern Regulator During Planarian Tissue Regeneration

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IDENTIFYING *SMED-TBX20* AS A PATTERN REGULATOR DURING PLANARIAN TISSUE REGENERATION

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Title: Identifying *Smed-Tbx20* as a Pattern Regulator During Planarian Tissue Regeneration

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The inability to repair aging or damaged tissues is a strong contributor to disease. The freshwater planarian flatworm, containing a pluripotent stem cell population called as Neoblasts, has an incredible capacity to regenerate every cell type in its body, making it an excellent model system to unlock the pathways governing injury-induced tissue repair and maintenance. My study explores the role of a transcription factor, Tbx20, as a patterning regulator. I have cloned whole sequence of the planarian tbx20 gene and have performed RNA-mediated genetic interference to knockdown its function during regeneration. Regenerating fragments exhibit an abnormal posterior indented blastema where medial tissues fail to regenerate and posterior curling forming into a cone. I propose that tbx20 coordinates medial-lateral and Dorsal-ventral axes as well as have sensitivity to anterior-posterior axis during regeneration. My studies on tbx20 function will provide information on how axial patterning in the planarian regulate tissue regeneration.

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CHAPTER 1

INTRODUCTION

Regenerative medicine is currently a principle field of biomedical research. Regeneration is a restorative process of continuous tissue repair and maintenance followed by injury or age-related damage (Bely & Nyberg, 2010). Unlike lower animals, vertebrates have limited capacity to regenerate and repair tissue following injury. Regeneration requires a complex cascade of events to develop spatially proximate yet functionally distinct adult tissues, which can be achieved through stem cell migration and proliferation. Stem cells are undifferentiated cells with a prolonged self-renewal capacity and capability to differentiate into multiple cell types. Stem cell-based therapies have unlocked a domain of novel avenues with the potential to successfully treat human diseases like neurodegenerative disease viz. Alzheimer's disease; diabetes, cardiovascular disease as well as cancer (Lindvall et al., 2004; Rennert et al., 2012).

Planarians are free living, freshwater flatworms that have been explored as an emerging model system to study stem cell-based regeneration for the last few decades. The ability for the planarian to regenerate is centered around a stem cell population, called neoblasts. Unlike other animals, planarians are capable of regenerating into all tissue types including the nervous system, a system with very little regenerative capacity in higher vertebrates (Rink, 2013). *Schmidtea mediterranea* has become the most popular species to study as it has an extraordinary tissue plasticity with a stable diploid genome structure which has already been sequenced (Robb et al., 2007). Moreover, advanced molecular biology techniques such as fluorescent immunohistochemistry, *in situ* hybridization methods to explore gene expression, RNA interference (RNAi) methods to disrupt gene function, cDNA collections along with stem cell markers make it possible to assess gene function in whole-body regeneration (Alvarado, 2006).

Purpose of Study

Planarians are triploblastic metazoans with bilateral symmetry possessing three main body axes namely Antero-Posterior (A/P), Medial-Lateral (M/L) and Dorsal-Ventral (D/V). Spatial organization of tissues along a specific axis must be established during regeneration, which is achieved through sending positional information to stem cells. In the planarian, A/P polarity is, at least partially, regulated through Wnt signaling in a β-catenin-dependent fashion. Wnts are secreted glycoproteins bind to a cell-surface receptor to initiate an intracellular cascade Members of the Wnt pathway, both playing activating or inhibiting roles, exhibit spatial-specific expression patterns along the A/P axis. The TGFβ signaling pathway regulates the M/L and D/V axes and members of the pathway show expression patterns that reflect these roles. In addition to this,the axon-guidance proteins, *Slit*/*Netrin*, also play crucial roles in midline formation during M/L patterning. Some evidence suggests that the Wnt pathway contributes to M/L patterning in a β-catenin-independent manner. As expression of these patterning regulators were observed as gradients across each axis, classic morphogen-based models have been invoked in the planarian whereby morphogen concentration determines the position, and therefore fate, of a neoblast during regeneration (Adell et al., 2010; Reddien, 2011; Cebrià, & Newmark, 2005).

Tbx20 is a T-box family gene belonging to the *Tbx1* subfamily. T-box genes are a group of developmental control genes first identified in mice as *Brachyury* or the *T* locus, which is required for posterior axial development (Halpern et al., 1993; Schulte-Merker et al., 1994). *Tbx20* plays crucial roles in cardiac morphogenesis in mammals through chamber patterning and differentiation and by maintaining a transcription factor network with Nkx2.5, Gata4 and Isl1 in the heart field (Singh et al., 2005). *Tbx20* coordinates patterning and differentiation of motor neurons emanating from the ventral portion of the neural tube and is controlled by a graded Shh

signaling system in mice (Takeuchi et al., 2005). *Tbx20*'s orchestration of nervous system development is conserved from nematodes to human (Pocock et al., 2008). However, the planarian literature significantly lacks characterization of T-Box family genes. Although *Tbx20* is present in Platyhelminthes (Sebé-Pedrós & Ruiz-Trillo, 2017), no study has been found to characterize a functional role for *Tbx20* in any flatworm. In 2011, Martín-Durán and Romero showed that *Tbx20* was expressed in ventral nerve cords and at the periphery of the body in *S. polychroa*, which suggests potential roles in nervous system development and tissue patterning. Further study is required to verify function of *Tbx20* in mutants.

Significance of Study

The primary objective of this study was to examine a role for *tbx20* function in planarian tissue regeneration through RNA interference (RNAi)–based knockdown of gene activity. Literature supports the significance of *tbx20* as a developmental control gene in a variety of tissues, including the nervous system and the heart in a wide variety of model systems. The results of my study will open a path to better understand how multiple axes are coordinated in a highly regenerative model organism.

Abbreviations

%	Percentage
°C	Degree Centigrade
µl	Microliter
µg	Microgram
cDNA	Complementary Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
bp	Base pair
ng	Nanogram
PCR	Polymerase chain reaction

CHAPTER 2

REVIEW OF LITERATURE

Planarian and Regeneration

Planarians belong to phylum Platyhelminthes under the class Turbellaria. Planarian flatworms can be characterized by a triploblastic body plan without distinct segmentation. A significant range of different regeneration capabilities exists between planarian species. Although in some species regeneration is absent, freshwater planarians possess a high regeneration capacity (Brøndsted, 1955). A unique population of adult stem cells is at the center of attraction for researchers for some time. The freshwater planarian is an excellent model system used to reveal facets of stem cell behavior and to better understand why some organisms, like humans, have lost the ability to regenerate complex tissues (Rink, 2013). In 1898, Thomas Hunt Morgan demonstrated the planarian's remarkable capacity to regenerate into fully functional animals even from miniscule fragments in *Planaria maculata*. He coined many terms that we use today to describe aspects of embryonic development and tissue behavior during regeneration. For example, the phenomenon of remodeling pre-existing tissues to form a new functional organ in the absence of new cell production was referred to as "Morphalaxis" (Morgan, 1900).

Regeneration can be defined as a restorative process followed by a traumatic injury or age-related damage of cells or tissue, and continuous renewal to form a new fully functional organ or the whole animal. Regeneration might have co-evolved with multicellularity in early animals. However, restriction or loss of regenerative ability is evident throughout the animal kingdom (Bely & Nyberg, 2010). Regenerative capacity is randomly distributed throughout the animal kingdom with various degrees (Alvarado, 2012). Regeneration occurs at various levels of

biological organizations, namely nerve cell regeneration in anurans, epidermal tissue regeneration in humans, tail regeneration in lizards, limb regeneration in salamanders, heart regeneration in zebrafish, head and tentacle regeneration in hydra, posterior segment regeneration in annelids, fin regeneration in teleost fish, spinal cord regeneration in axolotls and entire body regeneration in planarians (Bely & Nyberg, 2010; Brockes & Kumar, 2008). Planarian stem cell-based regeneration of the entire body, regardless of tissue type, makes them an exciting model system to study the molecular basis of regeneration.

Schmidtea mediterranea is the planarian used for my study and most popular model species for regenerative studies throughout the world. The abundance of neoblasts residing within the loosely packed parenchyma surrounding the internal tissues provide the capacity to complete regeneration within weeks. *S. mediterranea* is a stable diploid (2n = 8) with a genome size of 4.8×10^8 bp (Alvarado & Kang, 2005). Alvarado (2012) described *S. mediterranea* as an ideal model system, because of possessing a stable genome with four pairs of chromosomes with small genome size which is comparable to first four human chromosomes. The *S. mediterranea* genome has been sequenced which not only opened the flood gate for regeneration and stem cell research but also provided a toolkit to expand studies involving evolutionary biology and comparative genomics (Robb et al., 2007). *S.* mediterranea demonstrates both asexual and sexual strains allowing for the collective study of reproduction, embryogenesis, and regeneration. Lastly, it is possible to distinctly visualize different organ systems and cell types with tissue specific histological markers.

Contribution of Neoblasts

Neoblasts are small undifferentiated somatic stem cells residing within the parenchyma. Neoblasts exhibit a large nucleus containing highly decondensed chromatin and a scant

basophilic cytoplasm. Neoblasts make up nearly 25% of the total planarian cell population and are capable of differentiating into 20-30 cell types in the planarian (Alvarado, 2012; Newmark & Alvarado, 2002). Neoblasts can be destroyed upon exposure to gamma irradiation, which precludes cell proliferation (Bardeen & Baetjer, 1904).

A regeneration blastema appears quickly after amputation, as a thin layer of unpigmented tissue adjacent to the injury site. Since, no sign of mitosis, using immunofluorescent markers of M-phase, can be detected within blastemal cells, it is believed that the blastema is constructed by migrating daughter progeny cells of mitotic neoblasts from the preexisting tissue (Saló & Baguñà, 1984). Although minimum neoblast mobilization can be observed without injury, physical perturbation is required for neoblast migration to the wound site. Neoblasts are also capable of integrating multiple wound signals followed by mobilizing and occupying the physically damaged tissue to attain local tissue homeostasis by stem cell niche repopulation (Guedelhoefer & Alvarado, 2012). Neoblasts, which are near the wound migrate at a faster rate than those that are distant. However, the cumulative mitosis from neoblasts within the post-blastema area promotes blastema growth (Saló & Baguna, 1989). The molecular signals governing neoblast migration towards the wound are yet to be unveiled.

Several transcription factors specify the lineage of neoblast progeny. For example, *FoxA* is required for the fate of pharynx tissue, *pax3/7* for *DBH+* ventral midline neurons, and *FoxD* is required for anterior pole regeneration (Scimone et al., 2014a; Scimone et el., 2014b).

Pattern Formation in the Planarian

Bilateral metazoans possess two imaginary polar axes of symmetry perpendicular to each other which resembles a Cartesian coordinate system (Figure 12.). In planarian flatworm, three types of body axes exist. The primary body axis referred to as anterior-posterior (AP) axis, which

is longitudinal, parallel to the direction of movement and visualized as the brain at the anterior point. Two secondary body axes are the dorsal-ventral (D/V), and medial-lateral (M/L) axes, which are perpendicular to the A/P body axis. (Niehrs, 2010).

Planarians are tripoblastic, have a simple brain structure with two cephalic ganglia, connected to two eye-spots, two ventral nerve cords that run along the A/P axis, musculature and cilia for locomotion, a close ended digestive system with a centrally located pharynx, and a variety other cell types. Unlike higher vertebrates, body axis regulation or patterning in the planarian is not restricted to early embryonic development. Asexual reproduction and regeneration both require maintenance of all body axes in the adult worm (Niehrs, 2010; Reddien, 2011).

Studies in other model systems, such as the *Drosophila* fruit fly, have unveiled a great deal of information regarding how axes are patterned. These pathways have conserved roles across Bilateria. Although recent studies have highlighted the roles specific pathways in patterning in the planarian, our understanding of how these pathways are coordinated and how they initiate position-specific gene networks is poor. Investigating these components of the planarian system would reveal key contributors of stem cell behavior and how their fate is specified within an integrated coordinate system.

Preserving Anterior-Posterior (AP) Axis During Regeneration

Wnt/β-catenin pathway is largely responsible for maintaining A/P polarity. Wnt mutations were first described in *Drosphila* as causing wingless phenotypes (Sharma & Chopra, 1976). Wnt proteins are secreted glycoproteins, having a conserved role in growth and patterning during embryogenesis as well as in adult organisms across the animal kingdom (Basler, 2006; Gordon & Nusse, 2006). The Dishevelled protein is an upstream component of the Wnt signaling

pathway and its behavior is affected by acivation of frizzled proteins. Frizzled proteins are transmembrane proteins acting as receptors for the Wnt ligand. Activation of frizzled by Wnt results in an accumulation of β-catenin levels, causing it to migrate to the nucleus to directly target gene expression. Dishevelled acts as a positive regulator of β-catenin levels. Glycogen Synthase Kinase 3 (GSK3) and adenomatous polyposis coli (APC) are well known negative regulators of β-catenin. This complex phosphorylates β-catenin and targets it for degradation, thus suppressing Wnt-responsive gene expression (Gurley et al., 2008; Nusse & Varmus, 2012).

RNAi targeting *β-catenin* showed regeneration of anterior head structures on both anterior- and posterior-facing wounds in planarians. Furthermore, the regeneration of ectopic photoreceptors in several peripheral wounds demonstrated a radial like hypercephalized planarian. APC RNAi inversely produces tails in both anterior- and posterior-facing wounds, resulting in failure to regenerate head structures (Gurley et al., 2008 β-catenin acts on the posterior facing wounds to develop posterior structures rather determining anterior-specific structures, and that polarity of the A/P axis is controlled by the various Wnt genes expressed at different location (Petersen & Reddien, 2008). More recent experiments showed that wound-induced *wntP-1* expression controls regeneration polarity which is independent of β-catenin (Petersen & Reddien, 2009). In 2015, Sureda-Gómez et al. described four Wnt proteins as "Posterior Wnts", namely *Smed-wnt1*, *Smed-wnt11-1, Smed-wnt11-2* and *Smed-wnt11-5,* which are expressed in pharyngeal-to-tail regions in a nested manner.

Preserving Medial-Lateral (ML) Axis During Regeneration

Like with the A/P axis, M/L polarity is tightly regulated during planarian regeneration. Several signaling pathways, likely working together, are involved in maintaining the M/L axis. One of the key components is the Slit/Netrin repulsion–attraction neuron-guidance system. Slit is

a secreted glycoprotein containing leucine-rich repeat domains (Ypsilanti et al., 2010) expressed along the midline as well as near the anterior commissure of the regenerating cephalic ganglia in the planarian. *Slit* RNAi results in collapse of the CNS at the midline, development of ectopic neural tissues in midline regions, and defects in the posterior gut, which collectively substantiate *Slit*'s requirement for M/L patterning (Cebrià et al., 2007). Netrins are secreted molecules that guide axons during development. NetR RNAi in the planarian gives rise to laterally extended cephalic ganglia, disorganization in anterior regenerating ventral nerve cords (VNCs), and a lack of normal growth in posteriorly regenerating VNCs. Hence, these two axon guidance pathways are required for maintaining neural architecture during regeneration and also the M/L axis (Cebrià & Newmark, 2005).

Secondly, Bone Morphogenetic Proteins (BMPs) are a group of extracellular signaling molecules which belong to the transforming growth factor β (TGF-β) superfamily. This signaling pathway plays a crucial role in M/L patterning. BMP4 RNAi in the planarian showed indented blastemas from trunk amputated fragments lacking anterior and posterior tissues (Molina et al., 2007). RNAi designed to target *Smad*1 which is a cytoplasmic activator of BMP transcriptional output, gives rise to indented blastemas on both anterior- and posterior-facing wounds. BMP signaling pathway is associated midline formation because *bmp4-1* expression was observed in asymmetric fragment lacking a midline prior to blastema formation. Since midline formation has an important role in stabilizing bilateral symmetry, it is deducible that BMP signaling is also necessary for M/L patterning during regeneration (Reddien et al., 2007)

Finally, the Wnt pathway is also involved in M/L patterning. Wnt5 restricts lateral expansion of the nervous system and midline tissues, including the pharynx and *slit* expressing cells, and restricts expression of *slit*. Thus, Wnt5 and *slit* negatively regulate each other's

expression to maintain M/L patterning. *Wnt11-2* expression is observed in the tail and along the midline (Gurley et al., 2010).

Preserving Dorsal-Ventral (DV) Axis During Regeneration

D/V polarity is regulated by the BMP pathway during planarian regeneration. BMP and *smad1* RNAi gives rise to indented blastemas in anterior- and posterior-facing wounds, with small dorsal outgrowths, duplication of the D/V boundary (Molina et al., 2007). Another BMP family protein Admp (antidorsalizing morphogenetic protein) is also required for D/V patterning as it acts in a feedback loop with *bmp4*, by promoting *bmp4* expression. High *bmp4* levels inhibit Admp (Gaviño & Reddien, 2011). Noggins are well known BMP inhibitors and RNAi of noggin showed dorsalization of the ventral sides in planarian (Molina et al., 2011).

Significance of Morphogen Gradients to Axis Maintenance

Morphogens are chemical substances controls cell differentiation in a concentration-dependent manner (Turing, 1952). High morphogen concentration at one end of regenerating planarian and low concentration at another end generates patterns on a molecular level which is collectively called a gradient. Canonical Wnt pathways through activation of β-Catenin acts as a tail organizer, *β-catenin* RNAi provides low levels of Wnt signals giving the anterior organizer optimum condition to generate anterior identities (head). β-Catenin is often highest in concentration near the tail region with a gradual decrease in concentration towards the anterior pole (Meinhardt, 2009). Large groups of Wnts have been mostly expressed in the posterior region (Sureda-Gómez et al., 2015) suggesting that Wnt signaling has a posterior to anterior gradient. *bmp4* expression on the dorsal side of the body is highest in medial tissues while tapering off towards the lateral sides (Orii et al., 1998) (Figure 12.). Slit expression at the midline is also observed as a gradient. This gradient-based expression supports the classic view of a

morphogen role in patterning several axes within the planarian (Reddien, 2011; Cebrià et al., 2007).

Role of *Tbx20* Across the Animal Kingdom

In 1994, Bollag et al. first coined the term T-box as a conserved polypeptide domain homologous to *brachyury* (*T*) in zebrafish and *optomotor-blind* (*omb*) gene in *Drosophila*. T-box (*Tbx*) family genes are developmental control genes, encoding transcription factors which can identify specific DNA binding sites of target genes and have highly conserved roles in patterning and organogenesis (Kispert, 1995). The T-box family is divided into five subfamilies, *Tbx1*, *Tbx2*, *Tbx6* and *Tbr1*. *Tbx20* belongs to the *Tbx1* subfamily (Naiche et al., 2005)

In 2000, Ahn et al. first isolated *Tbx20* after successful cloning and expression analysis in zebrafish. Meins et al. (2000) first identified human *Tbx20* which is an ortholog *H15* in *Drosophila* and *Tbx-12* in *C. elegans*. *HI5* and *midline* (*mid*) are the *Tbx20* homologs in *Drosophila* which are required for cardiac patterning and differentiation (Reim, 2005). Mouse *Tbx20* was isolated from embryonic heart and expressed in post-mitotic motor neurons (Kraus et al., 2001). *Tbx20* plays crucial roles in cardiac morphogenesis in mammals through chamber patterning and differentiation and by maintaining a transcription factor network with Nkx2.5, Gata4 and Isl1 in the heart field. *Tbx2* is well known to prevent chamber formation and *Bmp2* positively regulates *Tbx2* expression. *Tbx20* mediates repression of *Tbx2*, leading to a chamber molecular program and regionally restricts expression of *Bmp2* (Singh et al., 2005). *Tbx20* directly controls D/V patterning in the heart by turning a linear heart tube with A/P polarity into a differentiated chamber along the D/V axis. Adult heart integrity and contractile function also requires *Tbx20*. Further data supports that *Tbx20* can dose-dependently regulate tissue-specific

transcription which collectively associates with human congenital heart defects and cardiomyopathies (Takeuchi et al., 2005; Stennard et al., 2005)

Development of motor neurons requires patterning cues to achieve complex innervation into various types of muscles. *Tbx20* controls the patterning and differentiation of motor neurons and is controlled by Shh signaling in mice (Takeuchi et al., 2005). *Tbx20* expression has been found in migrating branchiomotor/visceromotor (BM/VM) neurons within the mouse hindbrain during neuronal circuit formation and is a downstream target of *Hoxb1* which controls neuron specification (Guthrie, 2007). In 2006, Song et al. showed *Tbx20* regulates migration of several neurons and controls planar cell polarity in facial neurons. The *mid* gene in *Drosophila* directly controls Netrin/Frazzled and the Slit/ROBO pathways which are key axon guidance systems, thus coordinates between two attractants and repellants, integrating a balanced signal for axonal growth (Liu et al., 2009). *Tbx20* is required for proper cranial motor neuron positioning in zebrafish. In C. elegans, the ortholog of *Tbx20, mab9,* is required for axon guidance of motor neurons. Human *Tbx20* can functionally replace the zebrafish *Tbx20* and *mab9*. Above data highlights the conserved role for *Tbx20* in development and patterning of the nervous system in a wide variety of model organisms (Pocock et al., 2008).

The *Tbx20* homologs, *H15* and *mid,* act as selector genes downstream of Wnt signaling in *Drosophila* to determine ventral leg axis while *dpp* represses *H15* and *mid* leading to dorsal fate (Brook, 2010). *Tbx20* is also a downstream target of BMP10 during cardiac ventricular wall development in mice (Zhang et al., 2011). Taken together, Tbx20 has role in many types of developmental patterning, development of the nervous system and heart, along with formation of posterior structures and has an important regulatory relationship with the Wnt and BMP

signaling pathways. More than three decades of literature exist to support these roles in multiple systems. However, no study has been done unveiling the role of *Tbx20* in planarian.

CHAPTER 3

MATERIALS AND METHODS

Animals

The asexual CIW4 strain of *Schmidtea mediterranea* was used for my experiments. Animals were kept in planarian water at 21°C and fed pureed calf liver once a week. Feeding schedule was changed prior to experiments. 3-5 mm sized animals were chosen to conduct experimental procedures.

Isolation and Cloning of *S. mediterranea Tbx20* Gene Sequence

The cloning procedure was started with RNA extraction from the 20 planarians using Ambion's ToTally RNA kit followed by cDNA synthesis using Ambion's RETROscript kit. *Tbx20* gene-specific primers were designed using a gene sequence from the SmedGD genome database (Robb et al., 2007). The gene specific primers (Table 1.) were used in a standard PCR amplification protocol and verified using gel electrophoresis.

Table 1

Description of Tbx20 *Gene Specific Primers*

Primer name	Nucleobase Chain	Length
Smed-Tbx20.5 FWD	CCGGAATCGATGGATCTGAGAATG	24
Smed-Tbx20.5 REV	CACGACTGGAGATTGTTGGGATAG	24
Smed-Tbx20.6 FWD	CGACGACAACGATTCCTCGAATA	23
Smed-Tbx20.6 REV	GATCTCGCAATTTGGGCAAGAAG	23
Smed-Tbx20.7 FWD	CGAAACGACTAATGTCAGCGAAAG	24
Smed-Tbx20.7 REV	GTGTCAGTCCAGACCGGATAAA	22
Smed-Tbx20.8 FWD	CGAAACGACTAATGTCAGCGAAAG	24
Smed-Tbx20.8 REV	GTGTCAGTCCAGACCGGATAAA	22
Smed-Tbx20.9 FWD	CGGAATCGATGGATCTGAGAATGG	24
Smed-Tbx20.9 REV	TAGACTGGGTCAGATCGTCTCTTG	24
Smed-Tbx20.10 FWD	GACAGGTGTTGACGGTTTGTTTC	23
Smed-Tbx20.10 REV	ACATTGGAGAGGATGTGATTGGG	23
Smed-Tbx20.11 FWD	TCCTCGAATAAGCCGACAGATTG	23

Smed-Tbx20.11 REV	TCGGATTCGACGCTATTCTATGC	23
Smed-Tbx20.12 FWD	GACGACGACATCAATTCTCAATGC	24
Smed-Tbx20.12 REV	GATCTCGCAATTTGGGCAAGAAG	23

PCR products were purified using a PURELink PCR and Gel purification kit and ligated into a pGEM-T Easy Vector (Promega) (Figure 1.) with flanking T7 and SP6 promoter sequences. Then bacteria were transformed with the ligation reaction and grown overnight at 37°C. White colonies (Figure 3.) were chosen for colony PCR and electrophoretic analysis.

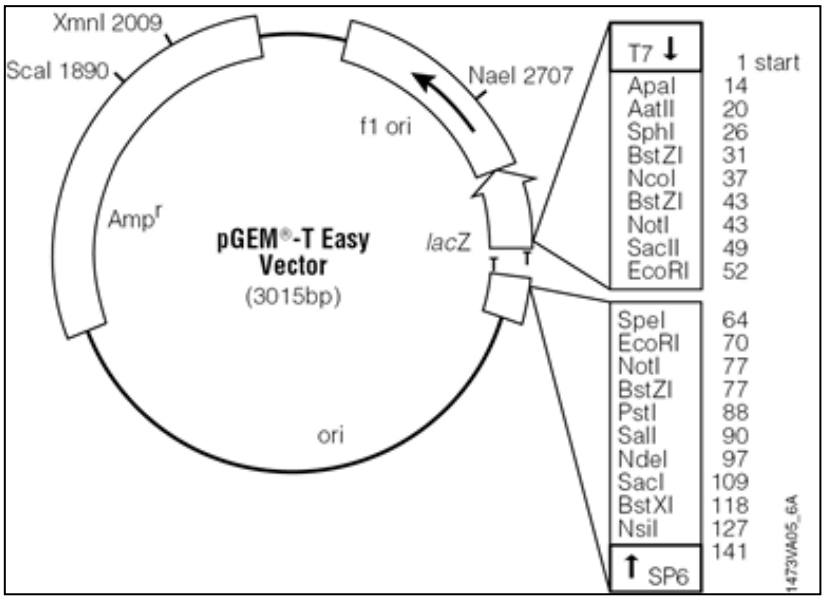

Figure 1. Structure of pGEM-T Easy Vector with flanking T7 and SP6 promotor sequence.

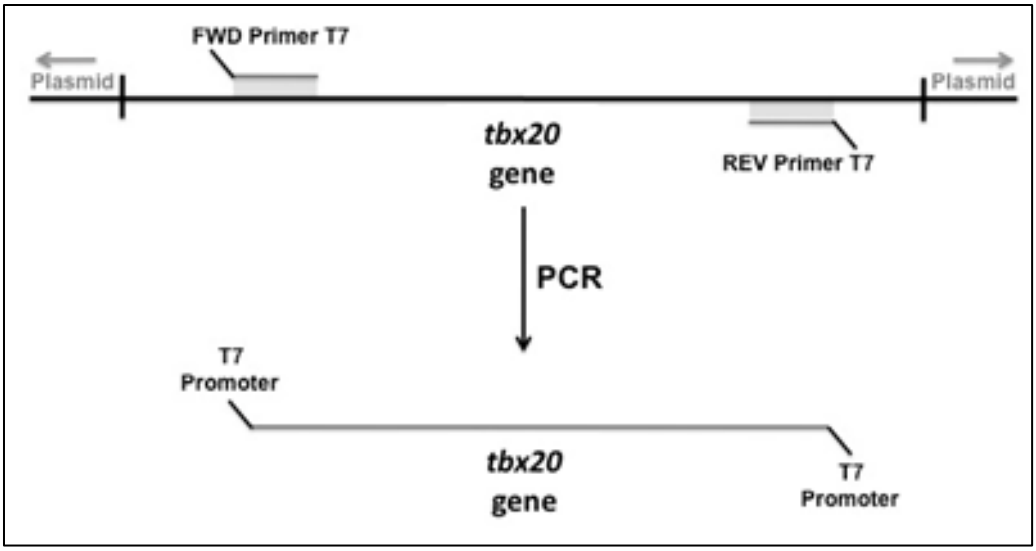

Figure 2. Isolation and cloning of Tbx20 gene sequence: Ligation of T7 promoter.

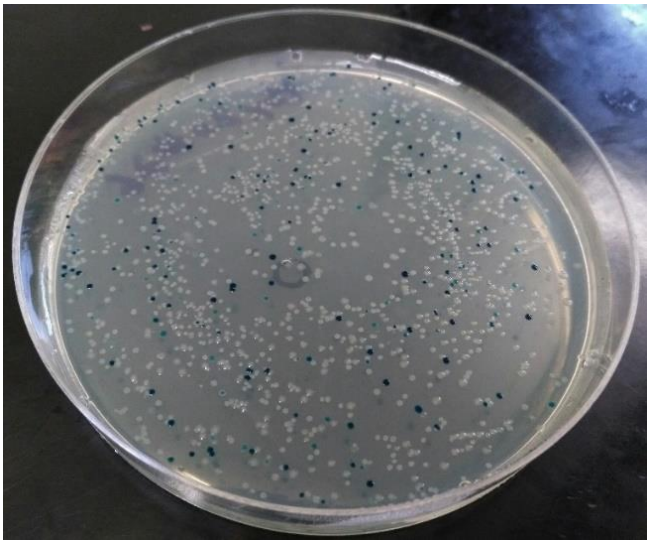

Figure 3. Blue-White Colony.

Colonies giving rise to an amplification product were chosen for plasmid isolation. Plasmids were purified using Wizard Promega plasmid mini-prep kit. The product was sequence verified by GENEWIZ, INC (Figure 2.).

Construction of *Tbx20* dsRNA for RNAi-Mediated Knockdown

The DNA template for a subsequent *in vitro* transcription reaction was constructed by using primers that bind to upstream and downstream regions of the cloned *Tbx20* sequence. These primers also contain T7 promoter sequences so that resulting PCR products contain flanking T7 promoters that bind to a T7 polymerase in an *in vitro* transcription reaction.

In vitro transcription of *Tbx20* to synthesis double stranded RNA (dsRNA) was conducted with a T7 Megascript kit from Invitrogen with T7 promoter sequences (Table 2.). A PCR reaction was performed according to a standard protocol (Table 3.).

Table 2

Description of T7 Promotor Sequences

Sequence name	Nucleobase Chain	Length
Tbx20.2F T7	TAATACGACTCACTATAGGGTG TCGCAATGGACATCATCCCAGT	44
Tbx20.3R T7	TAATACGACTCACTATAGGGGCGGT AACGGCGATGAAAATCGTT	44
Smed-Tbx20.5Forward T7	TAATACGACTCACTATAGGGTGT CACCGAATTTACCGGAACCG	44
Smed-Tbx20.5Reverse T7	TAATACGACTCACTATAGGGATA TCCATGGCGGTCCATGACGTT	44
Smed-Tbx20.5 FWD T7	TAATACGACTCACTATAGGGCC GGAATCGATGGATCTGAGAATG	44
Smed-Tbx20.5 REV T7	TAATACGACTCACTATAGGGCA CGACTGGAGATTGTTGGGATAG	44
Smed-Tbx20.7 FWD T7	TAATACGACTCACTATAGGGCG AAACGACTAATGTCAGCGAAAG	44
Smed-Tbx20.7 REV T7	TAATACGACTCACTATAGGGGT GTCAGTCCAGACCGGATAAA	42
Smed-Tbx20.8 FWD T7	TAATACGACTCACTATAGGGCGA AACGACTAATGTCAGCGAAAG	44
Smed-Tbx20.8 REV T7	TAATACGACTCACTATAGGGGTG TCAGTCCAGACCGGATAAA	42

Table 3

Composition of PCR Mixture for In Vitro Transcription

2µl	10X Reaction Buffer
2µl	ATP Solution
2µl	CTP Solution
2µl	GTP Solution
2µl	UTP Solution
1.65 µl	DNA Template
6.35µl	RNase-free water
2µl	Enzyme

The mixture was incubated at 37ºC for 4 hours. Then 1µl of DNase was added to the mixture and incubated at 37ºC for 15 minutes to degrade the DNA template. Lithium chloride

precipitation was performed to purify the RNA by adding 3x the volume of RNase-free water and 3x the volume of LiCl precipitation solution. The mixture was kept at -20ºC for 30-60 min and then centrifuged at 4ºC for 30 minutes at max speed followed by resuspension of the RNA pellet in 40µl of RNase-free water. Annealing of ssRNA to form dsRNA was done in a PCR tube in a thermal cycler for the following tapered temperature steps viz. 95ºC for 7 minutes, 75ºC for 5 minutes, 50ºC for 5 minutes and 25ºC for 10 minutes (Figure 5.). The product was stored at -20ºC.

Performing *Tbx20* RNA Interference (RNAi)

Gel electrophoresis was performed on a 1% agarose gel in TAE buffer to compare the mobility shifts between annealed and unannealed transcription products (Figure 4.). The concentration of dsRNA was determined by using NanoDrop Spectrophotometer.

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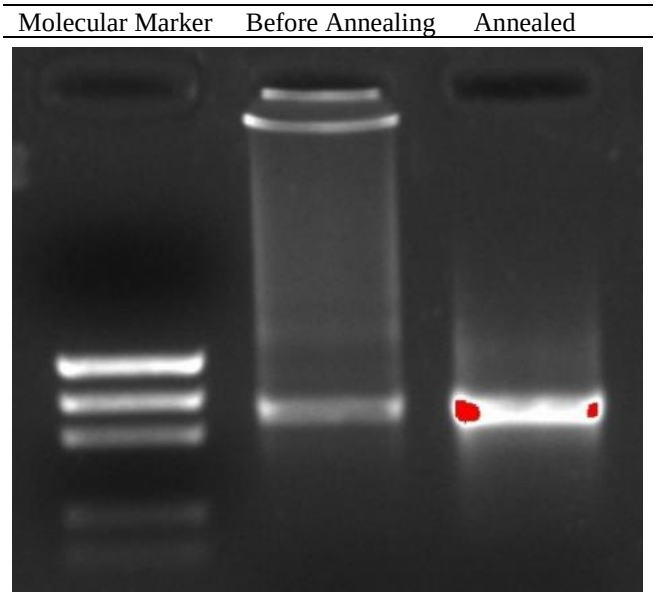


Figure 4. Agarose Gel electrophoresis of ds-RNA. Smeared lane (Before Annealing), Size verified band of Tbx20 ds-RNA (Annealed).

5 animals were chosen for control (water + pureed calf liver) and 5 were subject to dsRNA feeding for *Tbx20* (1.5µg + pureed calf liver). RNAi was accomplished by feeding ds-RNA combined with 10µl of pureed calf liver to the animals. Control animals were fed 10µl

pureed calf liver with RNase free water. All animals were fed on days 0, 2, and 4. Animals were amputated transversely into three segments (head, trunk, and tail) on day 7. Then the animals allowed to regenerate until scoring on 20th day from the initial feeding day (Figure 6.).

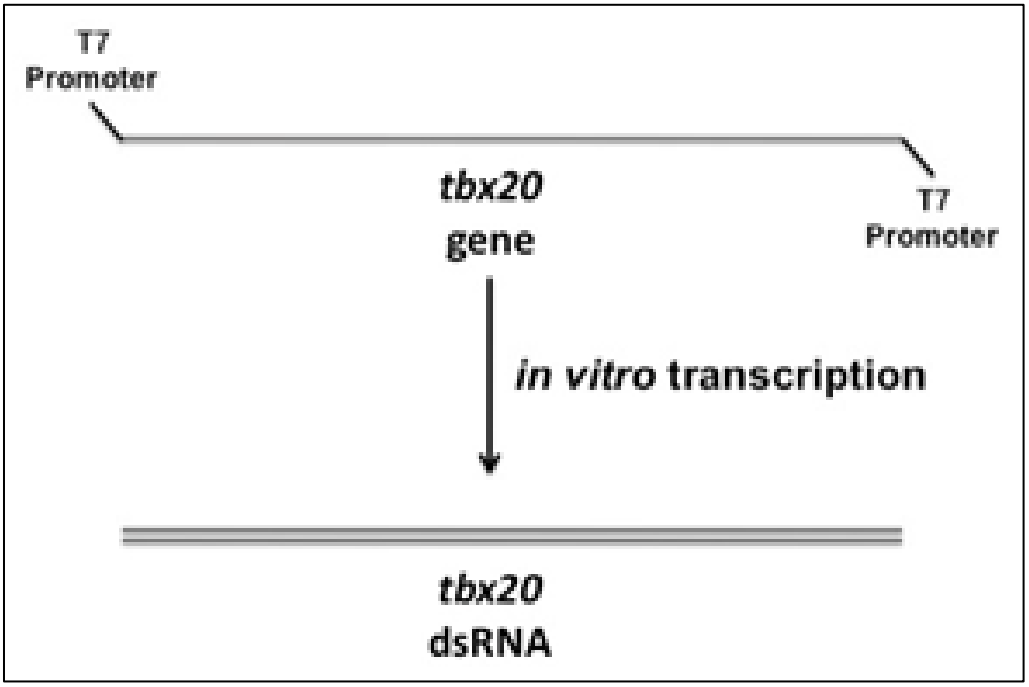


Figure 5. In vitro transcription to obtain Tbx20 ds-RNA from cDNA template

In a repeat experiment, a similar feeding/amputation/scoring schedule was performed with two different concentration. Based on our observations, in our initial experiments, suggesting that *Tbx20* loss might be more sensitive near the posterior regions of the planarian, I performed an amputation experiment using precision amputations using a Peltier cooler platform to slow animal movement. Animals were amputated transversely into five segments namely, head, pre-pharyngeal, pharyngeal, post-pharyngeal and tail regions. 5 control animals and 5

Tbx20 dsRNA-fed animals were amputated and each A/P fragment was isolated from each other prior to regeneration and scoring on 20th day from the initial feeding day (Figure 7.).

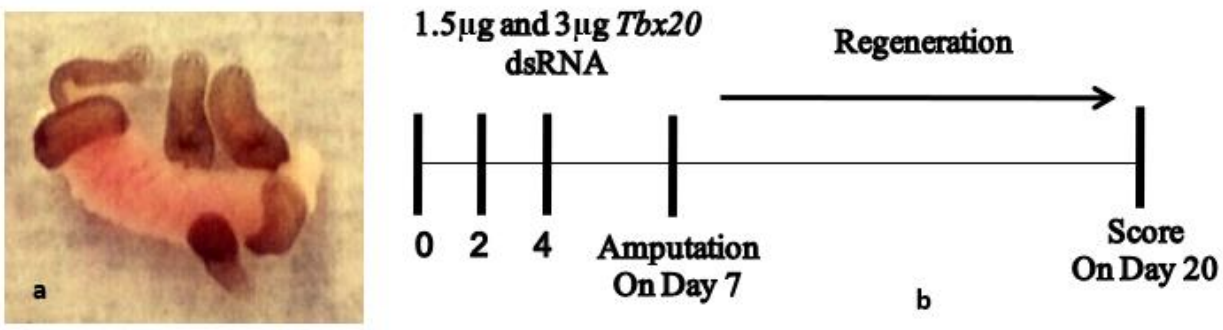


Figure 6. Performing RNAi. a. Animal feeding b. Feeding and amputation schedule

In each experiment, animals were not fed after amputation and allowed to grow at 21 °C temperature. The animals were started to observe from 7 days post amputations and regeneration status was scored on the 20th day.

Figure 7. Five Amputation planes and regeneration of animals.

CHAPTER 4

RESULTS

Preliminary Experiment

The research was initiated with a 380bp long gene fragment of *Tbx20* which was previously cloned in the laboratory. The sequence represented the middle portion of the gene. The DNA template was engineered to contain flanking T7 promoter sequence through PCR. PCR products were size verified by gel electrophoresis and yielded an approximately 410bp fragment. *In vitro* transcription was performed with T7 polymerase to make dsRNA. RNAi was conducted by feeding the animals followed by amputations in a procedure mentioned earlier. Regeneration status of the animals has been recorded and *Tbx20* (RNAi) animals had no obvious regeneration abnormality. Microscopic imaging was used to visualize regeneration and the presence of an unpigmented blastema and anterior photoreceptors was used to indicate regeneration capacity. Cumulative data were collected after two experiments (Table 4.).

Table 4

Result of Preliminary Experiment

N= 10	**Control**	***Tbx20*(RNAi)**
Head	0/10	0/10
Trunk	0/10	0/10
Tail	0/10	0/10

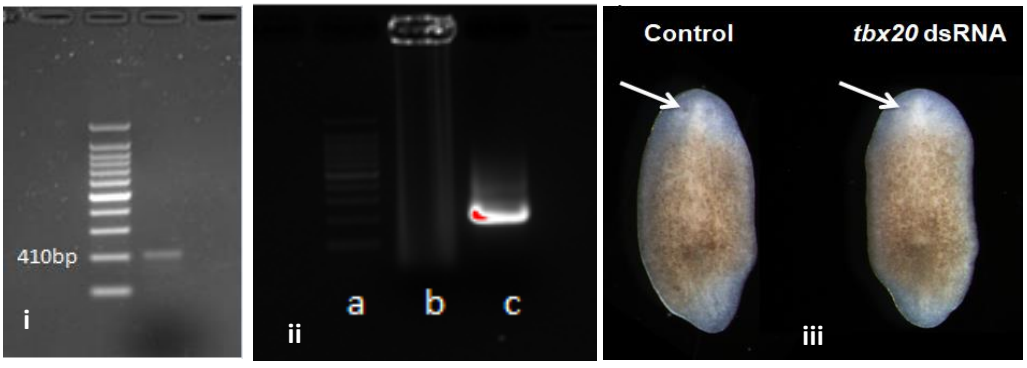


Figure 8. Preliminary experiment. Size verification of Tbx20 with T7 promoter by Gel Electrophoresis yields 410 bp fragment. ii. Gel electrophoresis of ds-RNA (a) Molecular marker (b) smeared lane before annealing (No band) (c) Size verified dsRNA band After annealed iii. No significant regenerative defects in Tbx20 RNAi compared to

Tbx20 Gene Sequence Analysis

Preliminary experiments using a relatively small 380bp previously cloned fragment did not implicate *Tbx20* in planarian tissue regeneration. Suspecting that the fragment was not sufficient for knocking down *Tbx20* function, I set out to isolate several larger gene fragments, and use these for RNAi-based knockdown. Three different fragments of *Tbx20* were cloned *Tbx20.5, Tbx20.7* and *Tbx20.8* which collectively spanned the entire *Tbx20* open reading frame.

The *Tbx20* gene sequence was converted to an amino acid and blasted against several other genomes across the animal kingdom to compare similarity. The relationship of cloned *S. mediterranea Tbx20* with other *Tbx20* homologs were collected (Table 5).

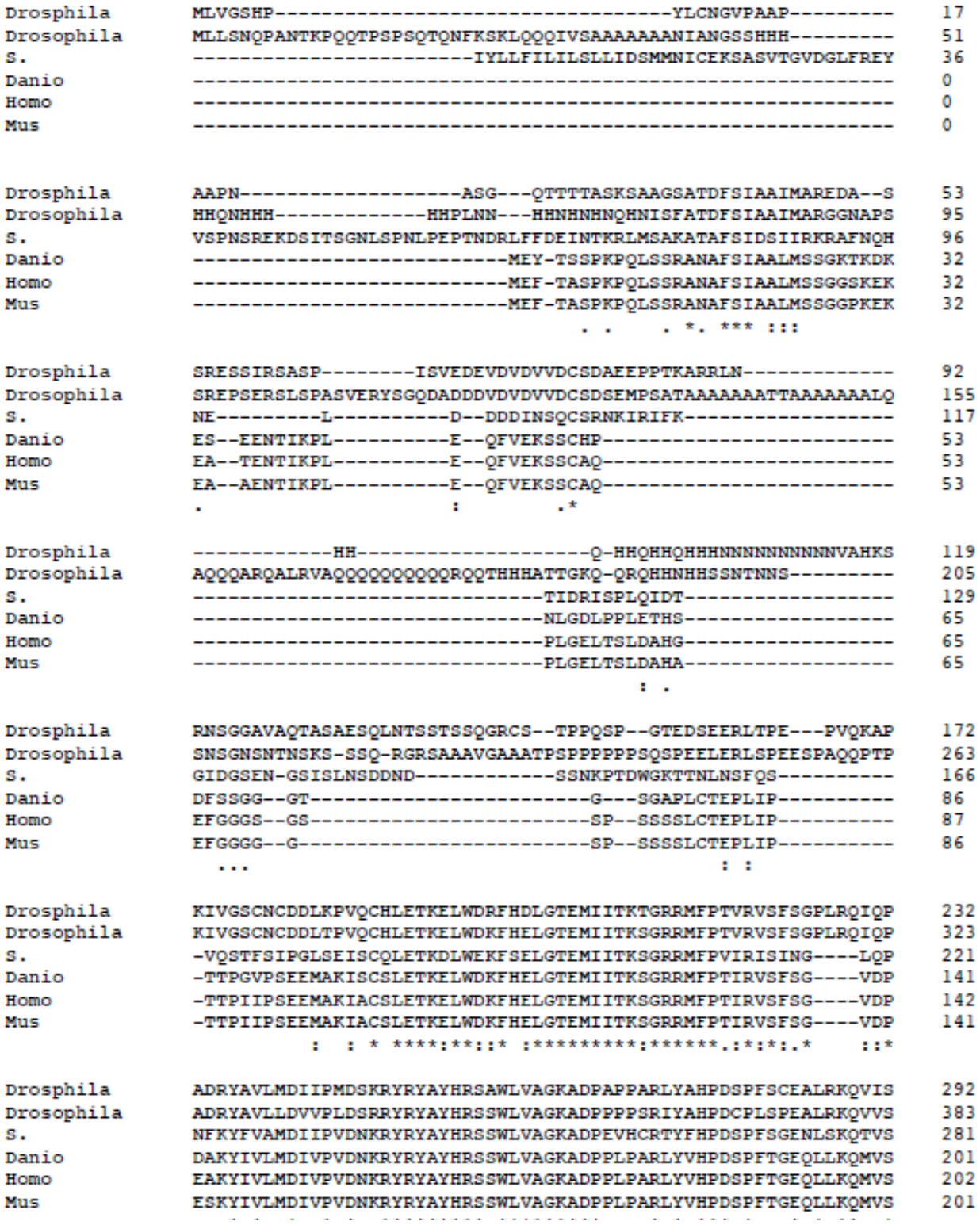
Table 5

Tbx20 Gene Sequence Analysis

Description	**% Identical**	**Accession**
T-box transcription factor TBX20 [Danio rerio]	50%	NP_571581.2
T-box transcription factor TBX15 isoform X1 [Danio rerio]	56%	XP_021334274.1
T-box transcription factor TBX20 isoform 1 [Homo sapiens]	67%	NP_001071121.1
T-box transcription factor TBX20 isoform 2 [Homo sapiens]	69%	NP_001159692.1
T-box transcription factor TBX20 isoform a [Mus musculus]	67%	NP_919239.1
T-box transcription factor TBX20 isoform b [Mus musculus]	69%	NP_065242.1
midline, isoform A [Drosophila melanogaster]	54%	NP_608927.2
H15, isoform A [Drosophila melanogaster]	58%	NP_001285627.1

Sequence analysis reveals that the cloned *S. med Tbx20* was highly similar with other *Tbx20* homologs in different model systems. The *S. med* T-box DNA binding domain, in the central portion of the gene sequence, exhibits the highest level of similarity between other homologs. 5’ end of the *S. med Tbx20* was highly similar to *Tbx15* in a few vertebrates. *Tbx15* is

not present in Platyhelminthes (Sebé-Pedrós & Ruiz-Trillo, 2017). The 3' end of the *S. med* *Tbx20* gene is highly similar to the 3' end of the zebrafish homolog.



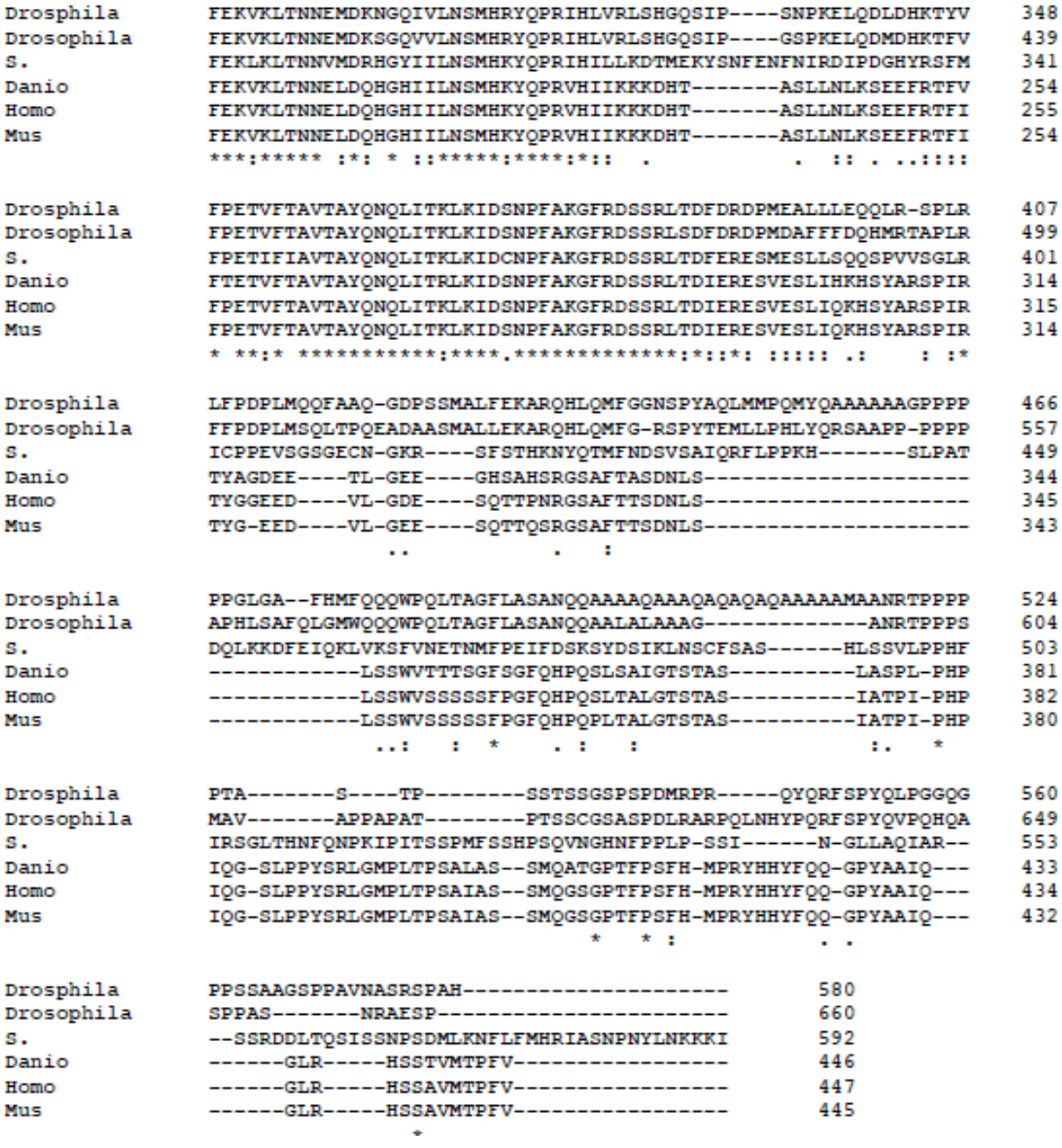


Figure 9. Sequence analysis of Smed-*Tbx20* with other genomes

Tbx20 dsRNA-Feeding Results in a Regeneration Abnormality

As the 380bp fragment used in the preliminary experiment was positioned to the middle of the *Tbx20* gene and didn't show any obvious regeneration phenotype, this time *Tbx20.5* and *Tbx20.8* was used to produce dsRNA. dsRNA-feeding was performed as mentioned earlier with

both types of gene fragments. *Tbx20.8* dsRNA animals did not exhibit any obvious regeneration defect. However, *Tbx20.5* dsRNA-fed animals showed a remarkable phenotype. Trunk fragments were not able to regenerate the tip of the tail and had indented posterior-facing blastemas. Head and tail fragments regenerated normally with control-sized blastemas and photoreceptors where expected. Three of the four animals exhibited indented posterior-facing blastemas. The obtained data were collected after complete regeneration of the animals (Table 6.).

Table 6

Result of Tbx20 RNAi

N= 4	**Control**	***Tbx20.8* (RNAi)**	***Tbx20.5* (RNAi)**
Head	0/4	0/4	0/4
Trunk	0/4	0/4	3/4
Tail	0/4	0/4	0/4

Note: 2.5/4 number of trunk fragments showed posterior indentation as a regeneration defect compared to control, Head and tail fragments regenerate normally

To further test regional specificity on a finer scale, animals were amputated into 5 fragments along the A/P axis, head, pre-pharyngeal, pharyngeal, post-pharyngeal and tail segments following *Tbx20.5* dsRNA feeding. Two different concentrations (1.5µg and 3µg) were used to identify the degree of sensitivity. The indentation of the posterior-facing wound was consistent with lower concentration (1.5µg) of ds-RNA only in the post-pharyngeal fragments. Four of the five post-pharyngeal fragments exhibited indentation at posterior-facing wounds. One of the post-pharyngeal fragments formed a more severe cone-shaped regenerate. Moreover, the higher dsRNA concentration (3µg) resulted death solely within the post-pharyngeal fragment. Three of the five fragments with this high concentration showed no regeneration and died eventually. The remaining four fragments along the A/P regenerated normally (Table 7.). Microscopic imaging was obtained for both the controls and RNAi animals with phenotype (Figure 10.).

Table 7

Final result for Tbx20.5 RNAi

N= 5	**Control**	***1.5 µg Tbx20.5* (RNAi)**	***3µg Tbx20.5* (RNAi)**
Head	0/5	0/5	0/5
Pre-pharyngeal	0/5	0/5	0/5
Pharyngeal	0/5	0/5	0/5
Post-pharyngeal	0/5	3/5 (Phenotype)	3/5 (Died)
Tail	0/5	0/5	0/4

Note: 3/5 number of post-pharyngeal fragments showed posterior indentation along with one of them with cone structure as regeneration defects compared to control (1.5 µg Tbx20.5), 3/5 post-pharyngeal fragments died (3µg Tbx20.5), Head, pre-pharyngeal, pharyngeal and tail fragments regenerate normally

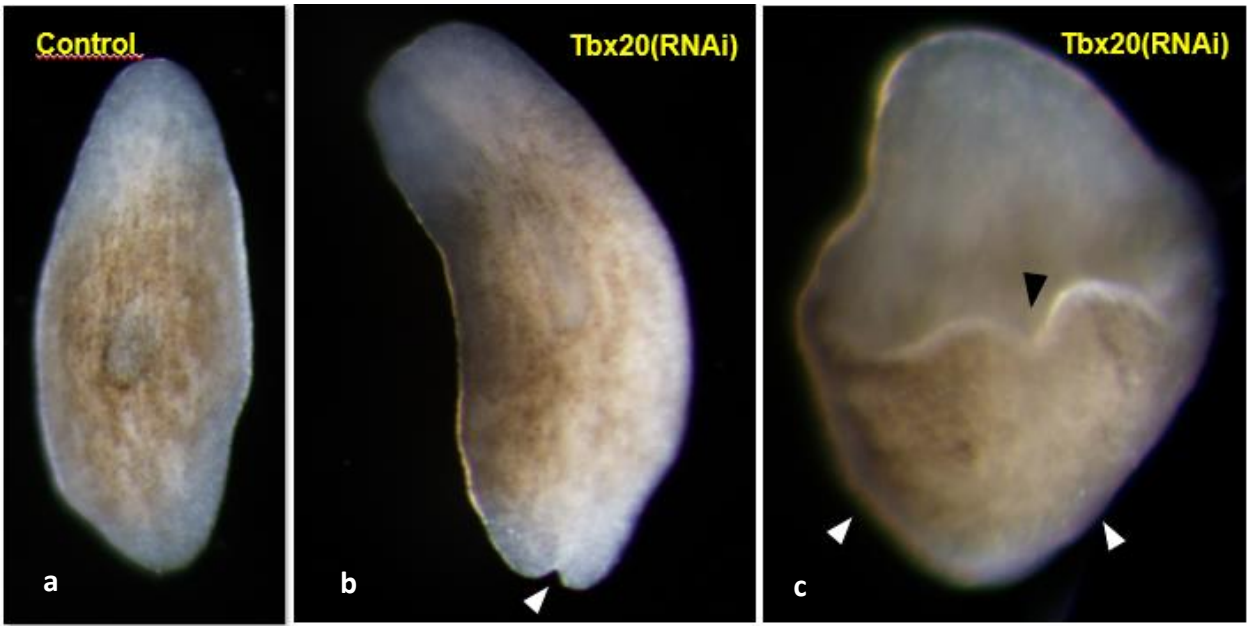


Figure 10. Regeneration defects with RNAi. a. Control b. *Tbx20* RNAi posterior indentation c. *Tbx20* RNAi posterior indentation with curling into cone formation.

CGGGATTGTCACCGAATTTACCGGAACCGACAAATGATCGACTGTTTTTCGATGAGATCAACA
CGAAACGACTAATGTCAGCGAAAGCCACCGCGTTTTCAATCGATTCAATAATTCGAAAACGAGCATTC
AATCAGCACAATGAACTTGATGACGACGACATCAATTCTCAATGCTCTCGAAACAAAATTCGGATTTT
CAAAACGATTGATCGAATTTCACCGCTTCAAATCGATACCGGAATCGATGGATCTGAGAATGGATCGA
TTTCGTTGAATTCCGACGACAACGATTCCTCGAATAAGCCGACAGATTGGGGAAAAACAACGAATCTG
AATTCCTTTCAATCGGTTCAGTCGACCTTTTCAATTCCGGGTTTATCGGAAATTTCCTGCCAGTTGGAA
ACAAAAGATTTGTGGGAGAAATTTTCCGAACTGGGCACTGAAATGATTATTACAAAATCTGGAAGACG
AATGTTTCCGGTCATTAGGATTTCCATCAATGGATTGCAGCCGAATTTTAAATATTTTGTCGCAATGGA
CATCATCCCAGTGGATAATAAACGTTATCGGTACGCATATCACAGATCGTCTTGGTTGGTTGCTGGTAA
AGCCGATCCTGAAGTTCATTGTCGAACGTATTTCCATCCTGATTCTCCGTTTTCTGGAGAAAATCTTTC
AAAACAAACCGTTTCCTTTGAAAAACTTAAACTGACAAACAACGTCATGGACCGCCATGGATATA

Figure 11. *Tbx20.5* gene sequence

CHAPTER 5

DISCUSSION, FUTURE RESEARCH

Discussion

Approximately a century ago, *Brachyury* (Greek for 'short tail'), or *T* (tail), mutation was introduced to researchers contributing discovery of a new family of developmental genes called T-box. T-box family members are transcription factors sharing a similar 200 amino acid DNA binding domain. Heterozygous *T* mutants showed *no tail(ntl)* phenotype in zebrafish and is required for posterior notochord differentiation and maintaining axial patterning (Schulte-Merker et al., 1994; Smith, 1997). *Tbx20* belongs to the ancestral Tbx1/15/20 class (Sebé-Pedrós & Ruiz-Trillo, 2017). It has been shown in this study that *S. med Tbx20* has high similarity with homologues in other model animals. Literature suggests that *Tbx20* is required for axial patterning in notochord differentiation, development of posterior body parts, patterning information during heart development, and development of the nervous system (Pocock et al., 2008; Schulte-Merker et al., 1994; Takeuchi et al., 2005; Singh et al., 2005).

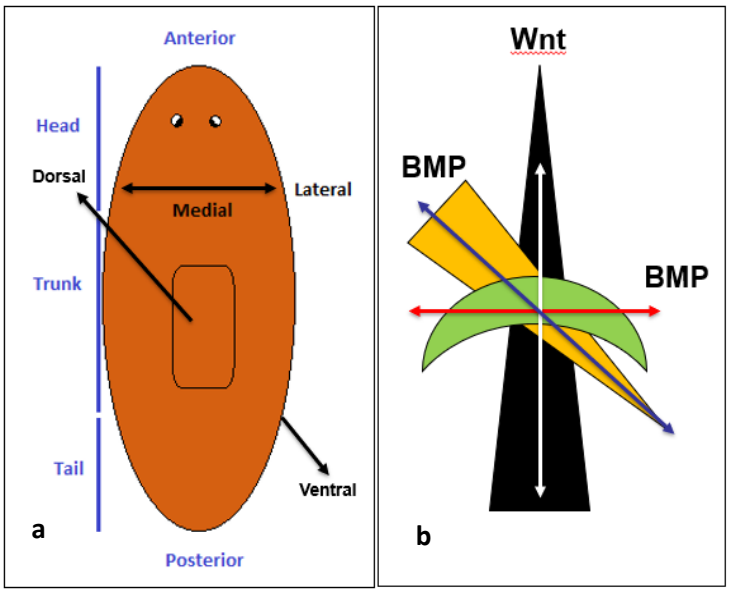


Figure 12. Patterning in Planarian. a Axial organization in planarian b. Gradient of morphogens along with Three axes: Wnt shows posterior to anterior gradient. BMP shows dorsal to ventral and medial to lateral gradient.

S. med Tbx20 RNAi study resulted in posterior indentation in post-pharyngeal fragments. Another result shows an additional posterior curling on the dorsal side of the animal and lacking normal regeneration. Literature reveals other similar RNAi phenotypes, namely those observed with *S. med BMP* RNAi and *S. med Smad1* RNAi treatment showed indented anterior and posterior blastemas from the trunk fragment suggesting a perturbation of D/V axis formation (Molina et al., 2007). *S. med bmp4-1* RNAi, *smedolloid-1* and *S. med smad4-1* RNAi exhibited similar indented blastemas at anterior- and posterior-facing wounds which was the result of a perturbation of M/L regeneration (Reddien et al., 2007). These similar phenotypes to those observed in *Tbx20* dsRNA-fed planarians suggest that *Tbx20* contributes to D/V patterning through some interaction with the BMP signaling pathway. Perhaps *Tbx20* lies downstream of the pathway. Moreover, although *S.med Wnt-11-2* RNAi did not show an indented blastema, the converged and fused gastrovascular cavity along with collapse of the ventral nerve cords at the midline (Gurley, 2010) suggests that Tbx20 contributes to M/L patterning through some interaction with the Wnt signaling pathway. As Wnt ligands are present in most posterior tissues, models invoking a connection between Tbx20 an Wnt signals are exciting. This places Tbx20 at a very interesting location between BMP and Wnt pathways in order to coordinate two axes within a Cartesian coordinate type of patterning system.

Tbx20 RNAi resulted in regeneration abnormality regarding patterning of the animal body. Posterior indentation represents impairment of medial-lateral axis formation. Cone formation further demonstrates medial-lateral patterning problem along with impairment with dorsal-ventral axis formation. Lack of dorsal regeneration made the animal to curl on the dorsal side as the ventral side was growing through regeneration. Defects were consistently

found in the post-pharyngeal regions indicates that Tbx20 is required for posterior identity as well as pattern formation. (Figure 10.).

In 2010, Brook showed that ventral Wingless (Wg), a Wnt family member in *Drosophila* diffuses to activate *H15* and *mid* genes to control ventral identities. *H15* and *mid, Tbx20* homologs, act as ventral selector genes along with downstream targets of Wg signals. My results suggest that *Tbx20* might be a downstream target of a Wnt family protein to determine M/L patterning. Zhang et al. (2011) showed that BMP10 upregulates *Tbx20* expression through a conserved *Smad* binding domain in the *Tbx20* promoter proximal region, which is required for ventricular chamber formation during heart development in mice. My results suggest that *Tbx20* might be a downstream target of BMP signaling to determine M/L patterning.

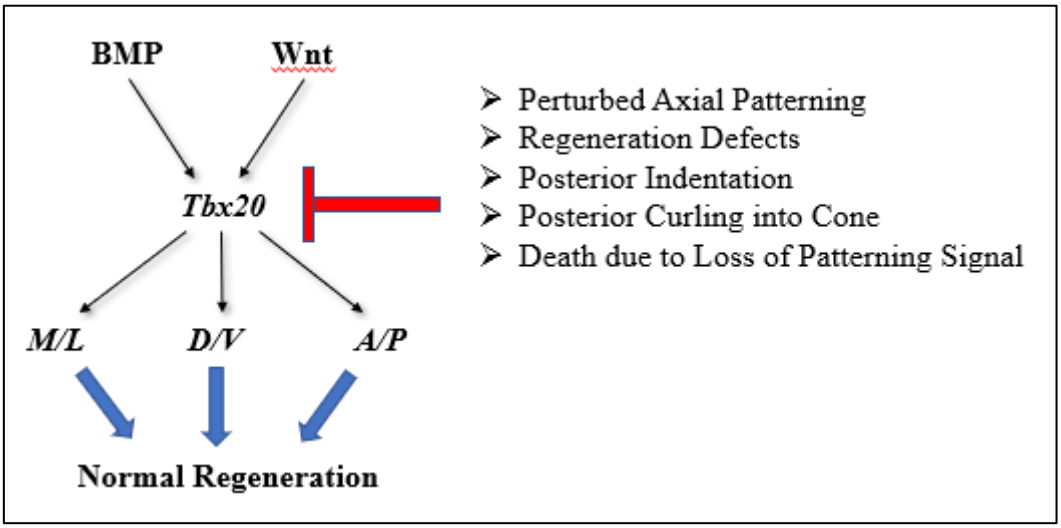


Figure 13. Proposed role of *Tbx20* in axial patterning during planarian regeneration.

In planarians, RNAi analysis of *BMP*, *Tbx20*, and *Smad* produce similar phenotypes. In *Tbx20 RNAi* animals, the formation of a posterior indentation might suggest that BMP works through activation of *Tbx20* for M/L patterning. Additionally, *Wnt-11-2* is required to form a normal posterior midline, at least observed through markers of internal tissues. *Tbx20* dsRNA-fed animals lack the formation of a normal posterior midline as they were not able to develop the

tip of the tail. Further in-depth immunohistochemical analysis is required to reveal whether *S. med Tbx20* dsRNA-fed animals have converged and fused gastrovascular system along with a collapse of the ventral nerve cords. Although not definitive, this will begin to address whether *Tbx20* is a downstream target of *Wnt-11-2* signals (Figure 13.)

It can be postulated that, like in mice, *Tbx20* is a downstream target of BMPs in planarian, it can further suggest that the posterior curling of the animals forming a cone-shaped amputation fragment resulted from lack of development of dorsal identities due to a failure, perhaps, of carrying out BMP signaling. Higher concentrations of *Tbx20* dsRNA leads to death of the post-pharyngeal fragment. It might be a possibility that the death is caused by lack of coordination in axal patterning and not providing enough directional information to stem cells to migrate for proliferation. Since *Tbx20* regulates posterior patterning across animal kingdom, *S. med Tbx20* dsRNA feeding resulted in abnormal posterior regeneration. Taken together, my results suggest that *Tbx20* regulates posterior axial patterning in planarian. Furthermore, Wnt and *Tbx20* double RNAi analysis has the potential to reveal whether Tbx20 and Wnt signals cooperate somehow during these events. For example, as loss of Wnt signals give rise to heads at both anterior- and posterior-facing wounds, I would expect loss of Tbx20 function in these two-headed fragments to not produce indented blastemas at posterior-facing wounds. The inverse experiment is also very exciting. For example, as ectopic Wnt signals in the anterior region of the planarian give rise to tails at both anterior- and posterior-facing wounds, I would expect loss of Tbx20 function in these two-tailed fragments to produce indented blastemas at both anterior- and posterior-facing wounds. I predict these outcomes because my hypothesis is that posterior regions of the planarian are most sensitive to the loss of Tbx20 function. Together, these future experiments will further examine whether Tbx20 function in mediating the M/L axis is under the

control of the A/P patterning system. This model is exciting because it begins to coordinate two important axes (A/P and M/L), and potentially a third (D/V), within the planarian through a transcription factor (Tbx20), and potentially a conserved transcriptional complex (Tbx20/Nkx2.5/Gata4).

Future Research

Tbx20 is a transcription factor of T-Box gene family which are primarily developmental control genes. The role of *Tbx20* has been studied in various phylum. However, it has never been explored in flatworms. Initially, an *in-situ* hybridization of *Smed-Tbx20* will reveal the expression pattern of the gene which might correlate the role in posterior regeneration. RT-PCR analysis will be required to quantify the loss of mRNA after RNAi experiments. Moreover, Wnt/*Tbx20* double RNAi or APC/*Tbx20* double RNAi could address the antero-posterior sensitivity of *Smed-Tbx20.* Additionally, fluorescent antibody imaging techniques can determine any regeneration defects of the organ systems inside the animal. This study shows that *Tbx20* is required for normal regeneration in the planarian which is the groundwork to unlock further components of patterning and stem cell fate during tissue regeneration in the planarian and in higher organisms.

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