Investigation of Exoribonuclease-1 Function in Regulation of Stem Cells during Planarian Regeneration

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Investigation of Exoribonuclease-1 function in regulation of stem cells during planarian regeneration

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

Steven G. Sayson
B.S., Wright State University, 2012

2016
Wright State University
WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

November 20, 2015

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Steven G. Sayson ENTITLED Investigation of Exoribonuclease-1 function in regulation of stem cells during planarian regeneration BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Sayson, Steven G., M.S., Department of Biological Sciences, Wright State University, 2015. Investigation of Exoribonuclease-1 function in regulation of stem cells during planarian regeneration.

Precise regulation of gene expression is crucial for the maintenance of pluripotency and proper differentiation of stem cells. Due to their exceptional capacity for stem cell-driven regeneration, planarians are ideal models to dissect mechanisms regulating gene expression that secure stem cell function in vivo. While mechanisms of transcriptional regulation of gene expression in stem cells have been extensively investigated, less is known about regulation at the post-transcriptional level. Exoribonuclease-1 (XRN-1) is a conserved enzyme in eukaryotes that catalyzes 5’ to 3’ exonucleolytic degradation of RNA. XRN-1 is known to be required for proper regeneration of Dugesia japonica. Here, I validate the requirement of XRN-1 in regeneration of planarian Schmidtea mediterranea and extend the study into identifying the underlying mechanisms that contribute to regeneration-deficiency in animals with reduced xrn-1 mRNA. Expression of S. mediterranea xrn-1 (Smed-xrn-1) in stem cells and in the central nervous system was detected by in situ hybridization. Additionally, targeted disruption of Smed-xrn-1 expression by RNA-interference (RNAi) validated the requirement for XRN-1 function for normal regeneration in S. mediterranea.
Interestingly, planarians subjected to *Smed-xrn-1* RNAi displayed regeneration deficiencies, but retained their mitotic stem cell populations. Assessment of the requirement XRN-1 during differentiation shows interruptions of stem cell specialization into early progenitor cells. Collectively, these results support the hypothesis that XRN-1 function is to degrade transcripts whose sustained presence after *Smed-xrn-1* RNAi impedes proper stem cell behavior during regeneration. However, it is still unclear of what transcriptional changes occur during regeneration deficiency in *Smed-xrn-1* depleted animals and further study is needed to assess the possibility of pleiotropic effects likely due to a broad effect on RNA degradation.
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INTRODUCTION

Gene regulation in stem cells

Stem cells have the unique ability to self-renew and to specialize into multiple cell types (Till et al., 1961; Weissman, 2000). The cell potential to differentiate into unlimited cell types is termed pluripotency. Accurate regulation of gene expression is crucial for the maintenance of pluripotency and proper differentiation of stem cells. For example, three well-characterized transcription factors, Nanog, OCT4, and SOX2, maintain pluripotency and self-renewal by regulating gene expression in mammalian stem cells (Avilion et al., 2003; Choi et al., 2012; Ivanova et al., 2006; Kashyap et al., 2009; Takahashi et al., 2006; Wang et al., 2012). The loss of any one of these transcription factors (Nanog, OCT4, or SOX2) results in the loss of pluripotency, causing stem cells to begin differentiation (Wang et al., 2012). Remarkably, the expression of Nanog, OCT4, and SOX2 in terminally differentiated cell types leads to reestablishment of pluripotency, reverting somatic cells back to an undifferentiated state (Jaenisch et al., 2008; Takahashi et al., 2006).

Post-transcriptional regulation has also been shown to be important in regulating gene expression in stem cells. For example, post-transcriptional regulator Lin-28 regulates pluripotency by repressing the processing of microRNA (miRNA) in mouse embryonic stem cells (Moss et al., 2003;
Stem cell potency is maintained when Lin-28 represses the processing of precursors of the let-7 miRNA family into mature miRNA molecules. The absence of mature let-7 miRNA allows its mRNA targets to be expressed, which contributes to cell pluripotency. In contrast, pluripotency is lost when Lin-28 is absent, since precursors of let-7 miRNA are processed into mature microRNA by Dicer in the cytoplasm (Piskounova et al., 2011). Mature let-7 miRNAs silence the expression of target mRNAs resulting in the loss of pluripotency.

While regulation of gene expression is important at both transcriptional and post-transcriptional levels for maintaining stem cell pluripotency and self-renewal, less is known about regulation at the post-transcriptional level. A better understanding of mRNA regulation in stem cells is paramount in understanding pluripotency and self-renewal. Post-transcriptional regulatory proteins could then be exploited as a powerful tool for identifying genes required maintaining cell potency.

Regeneration and neoblast differentiation

Planarians owe their regenerative capabilities to a large population of neoblasts that comprise 20-30% of their cells (Bagūna et al., 1989). Depletion of neoblasts by irradiation eliminates the regenerative capabilities and loss of normal homeostatic tissue turnover (Bagūna et al., 1989). Neoblasts give rise to all cell types within planarians, such as epithelial, neuronal, and muscle cell types (Scimone et al., 2014). Additionally, neoblast are required for development of the
Following injury in planarians, neoblasts are stimulated to rapidly divide throughout the entire animal (Wenemoser et al., 2010). This proliferation event is followed by migration of the neoblasts to the site of injury (Guedelhoefer et al., 2012). In wounds that result in tissue loss, a second neoblast proliferation event occurs immediate to the injury site (Wenemoser et al., 2010). Neoblasts then begin differentiating to form unpigmented tissue, called the blastema, which ultimately gives rise to regenerated tissue (Baguna et al., 1989).

**Gene regulation in planarian stem cells**

Planarians are non-parasitic flatworms that have the ability to regenerate complete animals from small fragments of tissue due to a population of adult somatic stem cells, called neoblasts (Newmark et al., 2002). Due to their exceptional capacity for stem cell-driven regeneration, planarians are ideal models to dissect mechanisms regulating gene expression that secure stem cell function *in vivo*. Neoblasts differentiate to replace epithelial, muscle, and nervous tissue during homeostatic turnover and regeneration (Baguna et al., 1989; Reddien et al., 2004; Wagner et al., 2011).

Both mammalian and planarian stem cells exhibit similar expression of known pluripotency genes and genes involved in epigenetic regulation (Onal et al., 2012; Tang et al., 2010). Many genes known to be required for pluripotency in human embryonic stem cells are enriched in neoblasts, including direct targets...
of transcriptional regulators OCT3 and NANOG (Onal et al., 2012). Additionally, the expression of post-transcriptional regulators are surprisingly well conserved between mammalian germline stem cells and planarian stem cells (Table 1) (Eisenhoffer et al., 2008; Onal et al., 2012; Resch et al., 2012; Rouhana et al., 2010; Tang et al., 2010). These enriched RNA-binding proteins are involved in RNA splicing, post-transcriptional regulation and translational regulation (Eisenhoffer et al., 2008; Onal et al., 2012; Resch et al., 2012; Rouhana et al., 2010). For example, VASA, a member of the DEAD-box family of RNA helicases, has a biologically conserved function responsible for processing silencing of transposon transcripts (Xiol et al., 2014). Vasa is also expressed in mouse developing germ cells and is required for proper development of male germline cells (Fujiwara et al., 1994; Tanaka et al., 2000). The homolog of Vasa in planarian Schmidtea mediterranea (Smed-vasa-1) has preferential expression in neoblasts and germ cells (Shibata et al., 1999; Wagner et al., 2012). Smed-vasa-1 and its ortholog in Dugesia japonica are required for neoblast-mediated regeneration (Rouhana et al., 2010; Wagner et al., 2012). The RNA-binding protein Pumilio has a biologically conserved role of repressing translation of specific target mRNAs by binding to their 3’ untranslated region (Bernstein et al., 2005; Spassov et al., 2003b). Mouse Pumilio genes are expressed in the developing embryo and in adult hematopoietic stem cells (McKee et al., 2005; Spassov et al., 2003a). Mutations in this gene are lethal in early, preimplantation embryonic development (Zhang et al., 2015). Moreover, human Pumilio-2 gene is expressed in embryonic stem cells and germ cells (Moore et al., 2003).
Table 1. RNA binding proteins enriched in mammalian stem cells and planarian neoblasts.

<table>
<thead>
<tr>
<th>Homolog</th>
<th>Function in other organisms</th>
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| Vasa-1   | Required for development of mouse germ cells (Fujiwara et al., 1994; Tanaka et al., 2000)  
Expressed in human embryonic stem cells (Clark et al., 2004) | |
| PIWI-1   | Required for germ cell development in mice (Deng et al., 2002)                                                                                             | |
| Pumilio  | Expressed in mouse hematopoietic stem cells and germline cells (McKee et al., 2005; Spassov et al., 2003a)  
Required for early, preimplantation embryonic development (Zhang et al., 2015)                     | |
| Bruno-like | Mouse reduced male fertility (Dev et al., 2007)                                                                                                               | |
| DAZL     | Expressed in Human embryonic stem cells (Clark et al., 2004)                                                                                                 | |
The *Pumilio* homolog in *D. japonica* (*DjPum*) has expression restricted to the neoblasts and brain. *DjPum* is required for the maintenance of neoblast populations and regeneration in *D. japonica* (Salvetti et al., 2005).

The similarities between genes enriched in planarian neoblasts and mammalian stem cells demonstrate conserved functions of stem cell genes that can be studied in planarians.

**5’-3’ Exoribonuclease 1 (XRN-1)**

One RNA processing factor of interest is Exoribonuclease 1 (XRN-1), a conserved enzyme in eukaryotes that catalyzes 5’ to 3’ exonucleolytic degradation of mRNA and long non-coding RNA (Figure 1) (Jones et al., 2012; Nagarajan et al., 2013). XRN-1 function occurs after removal of the 5’-cap (Braun et al., 2012). The protective 5’-cap is removed from transcript by a complex composed of Decapping Complex Protein -1 and -2 (DCP1 and DCP2) as well as Enhancer of Decapping Protein-4 (EDC4) (Braun et al., 2012). XRN-1 is then recruited to the uncapped RNA by binding DCP1 and EDC4 of the decapping complex and initiates degradation of transcripts from the uncapped 5’-end (Braun et al., 2012; Nagarajan et al., 2013).

XRN-1 shows strong sequence conservation across eukaryotes, including *Drosophila melanogaster* (Pacman), *Caenorhabditis elegans* (XRN1), *Saccharomyces cerevisiae* (Xrn1p), *Mus musculus* (XRN1), and *Homo sapiens* (XRN1) (Bashkirov et al., 1997; Jones et al., 2012; Larimer et al., 1990; Nagarajan et al., 2013; Till et al., 1998). Mutations of *xrn-1* in *C. elegans* show a
Figure 1. XRN-1 catalyzes 5’ to 3’ exonucleolytic degradation of cellular transcripts. The protective 5’-cap is hydrolyzed by a complex composed of Decapping Complex Protein -1 and -2 (DCP1 and DCP2) as well as Enhancer of Decapping Protein-4 (EDC4). XRN-1 is recruited to the RNA by binding with DCP1 and EDC4. XRN-1 degrades transcripts from the uncapped 5’-end towards the 3’-end.
lethal embryonic defect in ventral enclosure (Newbury, 2004). In *D. melanogaster*, *pacman* mutations cause failure of epithelial sheet closing and decreased fertility (Grima et al., 2008; Zabolotskaya et al., 2008). Furthermore, *pacman* mutations show an increased size of processing bodies in *D. melanogaster* nurse cells (Lin et al., 2008). Processing bodies are distinct foci in eukaryotic cells that contain enzymes that play an important role in mRNA decay and turnover (Kulkarni et al., 2010). The distinct phenotypes from *xrn-1* deficiencies suggest that XRN-1 function is not random, rather it targets specific transcripts for degradation at specific times during development.

**XRN-1 in planarians**

Previous research showed that expression of *Djxrn-1* mRNA is detected in both neoblasts and specialized cell types (Rouhana et al., 2010). *Schmidtea mediterranea* *xrn-1* has a three-fold increased expression in neoblasts over differentiated cells (Onal et al., 2012). *xrn-1* (RNAi) animals failed to regenerate in planarian *D. japonica* (Rouhana et al., 2010). Additionally, *xrn-1* is not required for neoblast proliferation or maintenance in *D. japonica* (Rouhana et al., 2010).

While the function of XRN-1 in the planarian *D. japonica* has been identified, the mechanism of how XRN-1 contributes to proper regeneration has not been revealed. Continuing the investigation of XRN-1 in planarian *S. mediterranea* is beneficial because of the broad tools available for this model organism. This model organism has a sequenced genome and several published transcriptomes (Abril et al., 2010; Robb et al., 2008; Rouhana et al., 2012),
allowing powerful analysis of expression changes and identification of transcripts regulated by XRN-1. Validation of the requirement of XRN-1 during regeneration in *S. mediterranea* and investigation of the events which impede regeneration after *xrn-1* RNAi will provide a better understanding of XRN-1 function and the transcripts it regulates.
SPECIFIC AIMS

The goal of this project was to extend the study of *S. mediterranea* XRN-1 to determine more precisely the event(s) most sensitive to XRN-1 function during planarian regeneration. To accomplish this, the following specific aims were investigated.

*Specific Aim 1:* Validate the expression of *xrn-1* in immature and mature *S. mediterranea*.

*Specific Aim 2:* Determine the requirement of XRN-1 on regeneration of *S. mediterranea* and the stage dependent on XRN-1 function.

*Specific Aim 3:* Identify the requirement of XRN-1 function for germline development in *S. mediterranea*. 
MATERIALS AND METHODS

Planarian maintenance

A sexual line of *S. mediterranea* planarian species was used in all experiments (Zayas et al., 2005). Planarian cultures were maintained in 0.75x Montjüic planarian salts (Cebria et al., 2005). Planarians of 4–6 mm in length were used for regeneration, *in situ* hybridization, and RNAi experiments. Planarians of 8–10 mm in length were used for RNAi experiments during germline analysis. Planarians were starved for at least seven days prior to any procedure.

Amputation

An amputation stage was prepared by placing an aluminum block on ice. Two sheets of Whatman paper moistened with 0.75x Montjüic planarian salts were placed on the aluminum block. Planarians were transferred to the prepared stage. Samples were cut transversely anterior and poster to the pharynx using a clean scalpel. The animals were transferred to 0.75x Montjüic salts and allowed to recover 1 hour. Planarians were cleaned using 0.75x Montjüic salts and monitored for recovery over 7 days at 18°C.

In experiments performed to identify the requirement of *xrn-1* in regeneration, a second amputation was performed to further diminish *xrn-1* mRNA within the planarian.
Irradiation

Irradiation was performed as previously described (Tasaki et al., 2016). A stage was prepared by placing moistened Whatman paper on an aluminum block on ice as described above and planarians were arranged on top of the prepared stage. The stage and ice bucket were transferred into the X-ray cabinet (Hewlett Packard Faxitron) and subjected to 110 kVp for 10 minutes. The animals were transferred to fresh 0.75x salts and allowed to recover for 3 or 4 days. Partially irradiated planarians were protected posterior to the pharynx with a lead plate prior to irradiation.

cDNA constructs

Potential xrn-1 homologs were identified in S. mediterranea using BLAST on Schmidtea mediterranea Genome Database (Robb et al., 2008). Total RNA was extracted from planarians using TRI Reagent (Molecular Research Center, Inc) and reverse transcribed using Superscript IV as directed by manufacturer (Invitrogen). S. mediterranea xrn-1 cDNA sequence was amplified using DreamTaq (Thermo Scientific) and primers [ACGGCGATTACTTTCACCTC] and [ATCGCTGCTATTCAGAGTGG]. The resulting PCR product was then cloned into pJC53.2, a plasmid engineered for dsRNA [T7 – TAATACGACTCTATAGGG] and riboprobe synthesis [SP6 – TACGATTTAGGTGACACTATA; T3 – CAATTACCCTCACTAAAGG] (Collins et al., 2010). The xrn-1 clone was sequenced and confirmed by BLAST.
5’/3’ Rapid amplification of xrn-1 cDNA Ends (RACE)

5’- and 3’-RACE was performed as directed by manufacturer (Clontech). First-strand cDNA synthesis was performed on total RNA and used in 5’- and 3’-RACE PCR reactions using primers [ACTTTAGGACAAAGACCCGGCACAATG-G] and [GCGTTTGTCAATTGGCCGGCCTTTATCG]. Amplified products were cloned into pRACE using the included In-Fusion HD Cloning Kit (Clontech). Successful clones were sequenced by primer walking every 500 bp. Sense and antisense cDNA strands were sequenced using primers [M13F - GTTTTCCCAGTCACGACGTTGTA] and [M13R - CAGAAACAGCTATGACC].

Phylogenetic analysis of XRN-1

*S. mediterranea* xrn-1 cDNA was cloned and sequenced. The predicted *S. mediterranea* XRN-1 protein sequence was aligned with yeast [Saccharomyces cerevisiae; AAA35219.1], nematode [Caenorhabditis elegans; CAC35856.3], fruit fly [Drosophila melanogaster; AAF48958.1], zebrafish [Danio rerio; NP_957327.2], frog [Xenopus tropicalis; XP_002937144.2], human [Homo sapiens; NP_061874.3], and mouse [Mus musculus; EDL20946.1] XRN-1. These sequences were aligned with the XRN-2 protein sequence of planarian [S. mediterranea; predicted], yeast [S. cerevisiae; CAA99240.1], nematode [C. elegans; CAB54449.2], fruit fly [D. melanogaster; AAF52452.2], zebrafish [D. rerio; NP_001001944.2], frog [X. tropicalis; XP_002939479.1], human [H. sapiens; NP_036387.2], and mouse [M. musculus; NP_036047.2]. Alignment
was performed using Clustal Omega and resulting output was used to generate a neighbour-joining tree (Sievers et al., 2011).

**Quantitative polymerase chain reaction (qPCR)**

qPCR was performed using GoTaq 2-Step RT-qPCR as directed by manufacturer (Promega). Each reaction consisted of 40 μl containing 1 μl of cDNA and 10 mM of each primer. \( xrn-1 \) [CGGTTTACCGGCCGACAG] and [TGCTTCTAGGAGCTGTTTTCTTCG]; \( smed-ef-2 \) [CAGCCAGTAGCTTTAAG-CGATGA] and [ACTCTCAACGCTGCTGTCACTTC]; \( smedi-1 \) [CCAAGAAGAG-GAGGTCTCCAAGAAG] and [CACTCGGAGACATAACTGAAACCTC]. The real time qPCR was run on a 7900HT System (Applied Biosystems). The cycling conditions were 1 cycle of polymerase activation/denaturation at 95°C/2 min, followed by 40 two-segment cycles of amplification (95°C/15 sec, 60°C/1 min) where the fluorescence was automatically measured during PCR.

**Northern Blot Analysis**

Riboprobes were synthesized from cDNA PCR templates and *in vitro* transcribed with either T3 or SP6 RNA polymerase (Thermo Scientific). Riboprobes were synthesized with digoxigenin-12-UTP (Roche). Northern blots were performed as previously described by Miller et al. (2012). Total RNA was extracted as previously described from non-amputated, intact RNAi animals and separated on a 1.5% agarose formaldehyde gel. Total RNA was transferred to a charged nylon membrane (Hybond, GE Healthcare). Visualization of \( xrn-1 \) mRNA
was performed utilizing digoxigenin-labeled probes, anti-digoxigenin-alkaline phosphatase antibody (Roche), and chemiluminescence substrates (CDP-STAR, Roche). Luminescent signal from northern blots were visualized with Fuji LAS 4000 chemiluminescent detectors.

**In situ hybridization**

*In situ* hybridization was performed according to a standard protocol for planarians (King et al., 2013; Pearson et al., 2009). Starved planarians were sacrificed in 7.5% N-acetylcysteine for 10 minutes and fixed in 4% formaldehyde for 20 minutes. Animals were bleached with formamide bleaching solution (1.2% H₂O₂, 5% formamide, and 0.5x SSC) under bright white light. Non-specific hybridization was blocked using blocking buffer (5% horse serum and 0.5% Roche Western Blocking Reagent in TNTx) for 2 hours. Digoxigenin-labeled riboprobes (described above for northern blot) were bound with anti-digoxigenin-alkaline phosphatase antibodies. Colorimetric signal was developed with NBT/BCIP (Roche). Samples were cleared and mounted in 80% glycerol.

**Fluorescent In situ hybridization (FISH)**

Riboprobes were synthesized from PCR templates and *in vitro* transcribed with either T3 or SP6 RNA polymerase. Riboprobes were synthesized with either Digoxigenin-12-UTP or Fluorescein-12-UTP (Roche) for double FISH. Starved planarians were sacrificed, fixed, and bleached as described above. Probes were detected using anti-digoxigenin-POD (Roche) or anti-fluorescein-POD (Roche).
FISH was developed with FAM- and TAMRA-tyramides (Thermo Fisher Scientific). Peroxidase reactions were quenched between developments of multiple probes using 100mM sodium azide. Samples were co-labeled with nuclear stain DAPI (Thermo Fisher Scientific). Samples were cleared and mounted in 80% glycerol.

*Immunofluorescence*

Immunofluorescence was performed as described by Forsthoefel et al. (2014). Starved, intact planarians were sacrificed in 2% hydrochloric acid and fixed for 20 minutes in methacarn (6:3:1 methanol:chloroform:acetic acid). Animals were bleached in methanol containing 6% H$_2$O$_2$ overnight under white light, and then washed with PBSTx (PBS + 0.3% Triton-X 100) three times for 5 minutes, and blocked overnight in Forsthoefel blocking buffer (PBSTx, 0.6% BSA, and 0.45% fish gelatin). Planarians were labeled using a primary antibodies for anti-phospho-histone H3 (Ser 10) (Anti-H3Ser$^{10}$P; rabbit) and anti-VC1 (mouse) (Agata et al., 1998; Newmark et al., 2000). Samples were washed a minimum of six times over 8 hours in PBSTx. Animals were exposed to goat anti-rabbit-568 and goat anti-rabbit-488 secondary antibodies (Invitrogen). The samples were co-labeled with DAPI and washed a minimum of eight times over 8 hours. Samples were cleared and mounted in 80% glycerol.
**Imaging and image processing**

Samples were imaged on a Zeiss Axio Zoom.V16 stereo microscope with a Canon EOS Rebel T3 camera and EOS utility, or Olympus FluoView FV1000 confocal microscope running FluoView 2.1.3.10 software. Images were processed using ImageJ 1.48v (Schneider et al., 2012) and Adobe Photoshop CS6. Exposure times were kept constant and adjustments to brightness and contrast were applied uniformly to allow comparison between samples. Quantification of H3Ser\(_{10}\)P(+) cells in RNAi animals were performed by ImageJ cell counting on whole animals. Area of animals were measured by ImageJ analysis.

**RNA interference**

Double stranded RNA (dsRNA) synthesis was performed *in vitro* according to Rouhana et al. (2013) from PCR templates with flanking T7 promoters using T7 polymerase (Thermo Fisher Scientific). Resulting dsRNA was purified using ammonium acetate (2.5M final concentration) and ethanol precipitation. Purified dsRNA was suspended in 50 µL RNase-free water then diluted to 1000 ng/µL.

Animals were fed in excess with 100 ng/µL dsRNA in liver mix (2:1 bovine liver:water) with 1% food coloring. Bacterial gene *ccdB* dsRNA served as negative control. Various schedules of RNAi treatment (5 dsRNA treatment over 21 days, 3 dsRNA treatment over 10 days, and 4 dsRNA treatment over 14 days) were performed to optimize *xrn-1* knockdown phenotype. In the remaining RNAi
experiments, except analysis of the germline, animals were fed dsRNA 5 times over 21 days. RNAi experiments performed on planarians for analysis of the germline were fed dsRNA 6 times over 4 weeks.
RESULTS

One main xrn-1 transcript identified in S. mediterranea

*S. mediterranea* xrn-1 cDNA sequence was cloned and sequenced. Northern blot analysis of xrn-1 revealed one main mRNA transcript, of approximately 5.5 kb, in total RNA from both asexual and sexual strains of *S. mediterranea* (Figure 2). The 5'- and 3'-ends were identified using rapid amplification of cDNA ends (RACE) and sequenced. The 5'-RACE product had a 519 bp overlap with the initial cDNA clone, while the 3'-RACE product had an overlap of 31 bp (Appendix A). The overlapping sequences had 100% identity with zero gaps. Due to the overlap of sequences, it is expected that the 5'-RACE product, the initial cDNA sequence, and the 3'-RACE comprise the entire xrn-1 sequence (Appendix B). This predicted xrn-1 sequence is 4800 bp. The discrepancy between the northern blot analysis and the predicted xrn-1 sequence may be due to the DNA marker used during analysis of RNA in the northern blot.

The predicted coding sequence and architecture of *S. mediterranea* xrn-1 cDNA shows similarity with XRN-1 from other organisms and is distinct from XRN-2 (Figure 3; Appendix C). The XRN-domain, which is responsible for the exonucleolytic activity, shows a 45% sequence identity to human XRN-1; while the structural domains (PAZ, KOW, WH, and SH3-like) show 23%, 46%, 33% and 43% identity, respectively (Figure 4).
Figure 2. One main xrn-1 transcript found in *S. mediterranea*. Northern blot analysis of xrn-1 identified one main transcript (~5,500 nt) from total RNA in both asexual and sexual strains of *S. mediterranea*. Ribosomal RNA (rRNA) shown below for loading control.
Figure 3. Phylogenetic analysis of predicted *S. mediterranea* XRN-1 protein sequence. *S. mediterranea* XRN-1 shows conservation and association with the XRN-1 clade. XRN-2 clade and MUC4 are shown for comparison.
Figure 4. XRN-1 domain architecture is conserved in humans, flatworms, fruitflies, and yeast. Percentage shown indicates identity of respective domain based on predicted amino acid sequence with respect to human XRN1 (Jinek et al., 2011). Illustration drawn using DomainDraw software (Fink et al., 2007).
The identification of one main \textit{xrn-1} transcript in \textit{S. mediterranea} indicates that \textit{xrn-1} is conserved and there are no paralogous gene duplications. Additionally, the similar domain structure suggests that XRN-1 exonucleolytic function may be conserved in \textit{S. mediterranea}.

\textit{xrn-1} transcripts are expressed in neoblasts and specialized cell types.

Colorimetric signal from \textit{xrn-1} mRNA transcripts were detected by \textit{in situ} hybridization in a pattern that resembles the distribution of neoblasts (Figure 5). The presence of \textit{xrn-1} in neoblasts was confirmed by examining the expression profile in intact and irradiated animals. Irradiation causes the loss of all neoblasts, allowing a clearer visualization of non-neoblast signal during \textit{in situ} hybridization. Neoblast marker, \textit{smedwi-1}, was used as positive control for neoblast loss. We observed that after irradiation, depletion of stem cells and the reduction in expression suggest the presence of \textit{xrn-1} in neoblasts (Figure 5). In addition to expression in neoblasts, \textit{xrn-1} shows additional expression resembling the pattern of the central nervous system and intestine. The expression of \textit{xrn-1} in neoblasts and other specialized cell types corroborates with previous experiments by Rouhana et al. (2010) and Onal et al. (2012).

Next, we attempted to measure relative quantities of \textit{xrn-1} in intact and irradiated animals qPCR to confirm the presence of \textit{xrn-1} in neoblasts. It was hypothesized that \textit{xrn-1} abundance would decrease in irradiated animals due the loss of \textit{xrn-1} contained in the depleted neoblast population. Unexpectedly, \textit{xrn-1} mRNA increased in animals 2 and 5 days post irradiation (dpi) in relation to
Figure 5. *xrn-1* is expressed in stem cells and differentiated cell types. Whole-mount *in situ* hybridization analysis of *xrn-1* and *smedwi-1* in normal and irradiated *S. mediterranea*. Animals were irradiated three days prior to fixation for depletion of stem cells (bottom row). *smedwi-1* is a stem cell marker used as positive control for neoblast depletion and visualization of neoblasts (Reddien et al., 2005). Scale bars = 0.5 mm
control (Appendix D). While this result seems to be in conflict with the presence of \textit{xrn-1} in neoblasts, it could also suggest that loss of neoblasts or irradiation stimulates the increase of \textit{xrn-1} in other cell types. Thus, we attempted to validate the expression of \textit{xrn-1} in neoblasts by FISH.

Using a combination of fluorescent \textit{in situ} hybridization and confocal microscopy, planarians were co-labelled with \textit{smedwi-1} and \textit{xrn-1}, and analyzed to confirm the presence of \textit{xrn-1} in neoblasts. Distinct \textit{xrn-1} signals are seen in both \textit{smedwi-1(+) and smedwi-1(-)} cells (Figure 6). The presence of \textit{xrn-1} in both \textit{smedwi-1(+) and smedwi-1(-)} cells indicate that XRN-1 function may be required in various cell types and not exclusive to neoblasts. However, \textit{xrn-1} foci in \textit{smedwi-1(+) cells are significantly increased in number over smedwi-1(-) cells (Two sample T-Test, }p<0.001; Figure 7). These results are not necessarily a qualitative indication of transcript abundance in each cell type. However, these results corroborate with a previous study that posits an enrichment of \textit{xrn-1} in neoblasts over differentiated cell types in \textit{S. mediterranea} (Onal et al., 2012). The presence of \textit{xrn-1} in neoblasts is consistent with our hypothesis that XRN-1 regulates neoblast-mediated regeneration in \textit{S. mediterranea}. 


Figure 6. *xrn-1* mRNA is detected in stem cells. Double fluorescent *in situ* hybridization analysis of *xrn-1* (magenta) and *smedwi-1* (green) reveals coexpression in *S. mediterranea* neoblasts. DAPI (gray) labels DNA in the nuclei of all cells. *smedwi-1* labels neoblasts (Reddien et al., 2005). Cell boundaries shown by increasing the nuclear outline by 40%. Green line, neoblasts and red lines, non-neoblasts. Scale bar = 5 μm.
Figure 7. *xrn-1* FISH signal is enriched in neoblasts over non-neoblasts. Quantification of number of foci from *xrn-1* FISH signal was significantly more abundant in neoblasts in comparison to non-neoblasts. Error bars represent one standard deviation. Two-tailed student t-test (n=168) revealed a *p* value of *p*<0.001.
XRN-1 is required for regeneration.

The function of XRN-1 during regeneration was investigated by double-stranded RNA (dsRNA) mediated RNA interference (RNAi). RNAi is an effective method for disrupting planarian gene function (Sanchez Alvarado et al., 1999).

Planarians were fed bovine liver containing 100ng/μL of in vitro synthesized dsRNA. Animals were fed 5 times over 3 weeks to disrupt accumulation of XRN-1 protein. Then planarians were amputated transversely anterior and posterior to the pharynx, and allowed to recover for 7 days. The regenerated trunk fragments were treated with an additional RNAi feeding followed by another amputation anterior and posterior to the pharynx. Animals were allowed to recover for 7 days, then scored based on the presence of visible photoreceptor pigmentation (complete regeneration) or lack thereof (incomplete regeneration). After 7 days post amputation (dpa), xrn-1(RNAi) planarians showed incomplete regeneration (n= 9/17) while control animals showed complete regeneration (n= 18/18; Figure 8). Successful knockdown of xrn-1 mRNA was confirmed by northern blot analysis (Figure 9), which revealed a readily noticeable but incomplete reduction in xrn-1 mRNA levels. While RNAi treatment decreased xrn-1 transcript abundance, the inability to compare protein concentrations is limiting and one must consider the possibility of prolonged XRN-1 protein stability. Several attempts were made by modify the RNAi feeding schedule and length of feeding to allow for XRN-1 destabilization. However, these efforts resulted in no significant changes in the fraction of animals showing a regeneration-deficient phenotype (Appendix F). The requirement of XRN-1 for proper regeneration of S.
*mediterranea* validates the conservation of function reported by its ortholog in *D. japonica* (Rouhana et al., 2010).

The cellular function of XRN-1 is to degrade uncapped mRNA and non-coding RNA of uncapped transcripts. To investigate if this function is conserved in planarians, total RNA of control and xrn-1 depleted animals was investigated by northern blot. Recently, a study also showed that loss of XRN-1 leads to
Figure 8. *xrn-1* RNAi results in regeneration deficiencies. Planarians subjected to RNAi feedings 3 times over 10 days followed by transverse amputation anterior and posterior to the pharynx, allowed to recover for 7 days, and subjected to one additional RNAi treatment and additional amputation. All control planarians (n=18) had complete regeneration of head and tail regions 7 days post-amputation (dpa). Approximately 47% of *xrn-1(RNAi)* planarians (n=17) displayed incomplete regeneration 7 dpa. Numbers represent planarians with visible photoreceptor pigmentation. Arrow head, photoreceptor. Scale bar = 0.5 mm.
Figure 9. *xrn-1* mRNA levels decrease in *xrn-1(RNAi)* planarians. Northern blot analysis of total RNA from three groups of intact control and *xrn-1(RNAi)* planarians confirm reduction of *xrn-1* mRNA after 5 dsRNA feedings over 3 weeks. Each group contained total RNA extracted from three planarians chosen at random. Ribosomal RNA is shown as loading control.
dsRNA accumulation (Burgess et al., 2015). Initially, an accumulation of what appeared to be \textit{xrn-1} dsRNA was notably abundant in northern blots used to confirm successful knockdown of \textit{xrn-1} mRNA (Appendix E). To test whether this smeared material was dsRNA, total RNAs from control or \textit{xrn-1(RNAi)} animals were analyzed using equal concentrations of \textit{ccdB} (control) and \textit{xrn-1} DIG-labeled probes designed to detect only antisense sequence (presumably from ingested dsRNA) during northern blot analysis. Sense probes allow hybridization to antisense strands contained in the total RNA; the only antisense strands present were exogenously introduced during dsRNA feeding during RNAi treatment. From this analysis, I observed an accumulation of dsRNA in \textit{xrn-1(RNAi)} animals (Figure 10). However, while equal amounts of riboprobes and dsRNA were used for \textit{ccdB} and \textit{xrn-1} sequences, control dsRNA was harder to detect in total RNA for control animals (Figure 10). Additional confirmation of dsRNA accumulation can be obtained by using a third gene for dsRNA feedings during control or \textit{xrn-1} RNAi treatments and detecting the decay of the third gene dsRNA with sense probes in both groups. This will provide a more accurate comparison between control and \textit{xrn-1} depleted animals. Nonetheless, the increased amount of dsRNA seen in \textit{xrn-1} depleted animals suggests that XRN-1 function of RNA degradation is conserved in planarians.
Figure 10. XRN-1 is required for processing of excess dsRNA. Total RNA from triplicate groups of animals treated with 3 feedings of control or xrn-1 dsRNA were subjected to northern blot analysis with a mixture of equal amounts of ccdB (control) and xrn-1 sense DIG-labeled riboprobes. Blot was developed using CDP-Star chemiluminescence substrate (Roche). Higher levels of dsRNA were observed for xrn-1(RNAi) animals in comparison to controls. In vitro synthesized ccdB dsRNA directly loaded to the gel is shown as positive control, ccdB(+). rRNA is shown as loading control.
XRN-1 is required for proper photoreceptor and optic nerve regeneration

Lost tissue is replaced in planarians by stem cell-driven regeneration. Regeneration of the anterior portion of planarians involves development of specialized structures such as epithelial tissue, cephalic ganglia, intestines, and sensory neurons. The posterior region contains tissues such as reproductive organs, intestines, and the pharynx. Planarians contain two photoreceptors connected by optic nerves, which can be observed by immunofluorescence using monoclonal antibody VC-1 (Agata et al., 1998; Sakai et al., 2000). The previous regeneration experiment was based on visible photoreceptor pigmentation (Figure 8). However, the underlying structures of the photoreceptors may be developing prior to visible pigmentation. To confirm that xrn-1(RNAi) planarians resulted in incomplete regeneration, successful photoreceptor and optic nerve regeneration were analyzed in detail using the control and xrn-1(RNAi) animals from the regeneration experiment. Animals were classified in 3 levels of regeneration: 1) full development of the photoreceptors and connecting optic neurons, 2) partial development of the photoreceptors and incomplete non-connecting optic neurons, and 3) no development of the photoreceptors or optic neurons (Figure 11). 10% of animals subjected to xrn-1 RNAi treatment completely failed to develop photoreceptors and optic nerves, while 65% had partial development and 25% had full development of the photoreceptors and optic nerves (Figure 12; n= 20). Control animals showed 100% full regeneration of the photoreceptors and optic neurons (Figure 12; n= 20). The large percentage
of $xm-1$ (RNAi) animals that failed to regenerate normal photoreceptors indicate that XRN-1 is required for proper stem cell-driven regeneration.
Figure 1. XRN-1 is required for proper regeneration of the visual system.

Planarians subjected to control or xrn-1 RNAi treatment (as described in Figure 9). Control planarians showed full development of photoreceptors (PR) and optic nerves (ON). A large fraction of xrn-1(RNAi) planarians displayed incomplete regeneration showing partial or lack of development of the PR and ON. VC-1 antibodies were described by Agata et al. (1998) and obtained as a gift from Hidefumi Orii, University of Hyogo. Scale bar = 0.05 mm
Figure 12. Quantitative analysis of RNAi animals with optic developmental defects. Animals were scored based upon detection of PR and ON regeneration using VC-1 antibodies. All of the control planarians exhibited full development (n=20). In comparison, only 25% of *xrn-1*(RNAi) animals showed full development of PR and connecting ON; 65% of *xrn-1*(RNAi) animals showed partial development seen by the presence of PR but incomplete ON development; 10% of the animals showed no development of PR and ON (n=20).
**XRN-1 is disposable for neoblast proliferation and maintenance**

The regenerative capabilities of *xrn-1(RNAi)* planarians are limited, which could be explained by the loss of neoblasts as has been reported for other regulators of RNA (Bardeen et al., 1904). To elucidate this possibility, we analyzed the distribution of mitotically active neoblasts in control and *xrn-1* depleted animals by performing anti-phosphohistone H3 Ser10 (anti-H3Ser$^{10}$P) whole mount immunohistochemistry (Newmark et al., 2000). Both control *and* *xrn-1(RNAi)* planarians contained mitotic neoblasts after 5 dsRNA treatments over 3 weeks (Figure 13). The presence of mitotic neoblasts indicate that XRN-1 is disposable for cell maintenance and self-renewal. Quantitative analysis of mitotic cells revealed that *xrn-1(RNAi)* planarians had a 23% increase in number of mitotic neoblasts in comparison to control animals (Figure 14; n= 15 animals each). These animals displayed normal neoblast distribution, but their abundance was increased throughout the animal. The increased population of neoblasts in *xrn-1(RNAi)* planarians suggest that neoblasts have accumulated due to the inability to differentiate. However, the accumulation of neoblasts may also be due to XRN-1 absence stimulating an injury-like response of global neoblast proliferation.
Figure 13. *xrn-1*(RNAi) planarians retain mitotically active stem cells. Mitotic stem cells of planarians subjected to 5 feedings of control or *xrn-1* dsRNA over a period of 3 weeks were analyzed by anti-H3Ser\(_{10}\)P whole-mount immunohistochemistry. Scale bar = 0.5 mm (left panels) or 0.1 mm (right panels).
Figure 14. M-phase stem cells are significantly increased in \textit{xrn-1(RNAi)} planarians. Number of H3Ser10P(+) cells per mm$^2$ were quantified from control (n= 15) and \textit{xrn-1(RNAi)} (n= 15) planarians from fluorescent images using ImageJ analysis (Schneider et al., 2012). Error bars represent two times the standard error. $p<0.001$, 2-tailed student t-test.
XRN-1 is first required for proper neoblast dynamics during regeneration

During differentiation, neoblasts specialize into early progenitor cells, to late progenitor cells, and eventually become terminally differentiated cell types. Specific genes are expressed at different stages of differentiation (Eisenhoffer et al., 2008). Neoblasts express transcripts which promote pluripotency and inhibit differentiation (Onal et al., 2012). Additionally, neoblasts preferentially express smedwi-1, which encodes for a protein belonging to the Argonaute/PIWI family (Reddien et al., 2005). In contrast, progenitor cells preferentially express genes that inhibit pluripotency and promote differentiation (Onal et al., 2012). Early division progenitor cells selectively express NB.21.11e, while late division progenitor cells express agat-1 (Figure 15; Eisenhoffer et al., 2008). These transcripts can be used to identify cells in distinct stages throughout the neoblast differentiation process.

During regeneration and homeostatic tissue turnover, neoblasts differentiate into early and late progenitor cells before becoming terminally specialized cell types. Regeneration deficiency seen in xrn-1(RNAi) animals can be explained by XRN-1 function being required at any stage of differentiation (neoblast into early progenitor cell, early progenitor cell into late progenitor cell, or late progenitor cell into specialized cell types). Interruption at any stage will result in phenotypic regeneration deficiency. Based on the work by Guedelhoefer et al. (2012), the differentiation process can visualized by using specific markers that identify neoblasts (smedwi-1), early progenitor cells (NB.21.11e), and late progenitor cells (agat-1).
Figure 15. Neoblasts and progenitor cells display differential expression profiles. During differentiation, neoblasts specialize into early progenitor cells, to late progenitor cells, and eventually become terminally differentiated cell types. *smedwi-1* is preferentially expressed in neoblasts. In contrast, early division progenitor cells selectively express *NB.21.11e*, while late division progenitor cells express *agat-1* (Eisenhoffer et al., 2008). These transcripts can be used to visualize distinct stages throughout the differentiation process using *in situ* hybridization. Scale bar = 0.5mm.
An assay was designed to detect changes in neoblast dynamics and differentiation dependent on XRN-1. To accomplish this, planarians were subjected to partial irradiation. The anterior portion of the animal was selectively irradiated by protecting the region posterior of the pharynx with a lead plate (Figure 16). The neoblasts and their progeny are lost in the exposed region 4 days after irradiation. Planarians were then transversely amputated immediately posterior to the photoreceptors. Animals were allowed to partially regenerate and fixed 6 days post-amputation, analyzed for the presence and location of neoblasts, as well as progenitor cells in the irradiated region.

Migratory neoblasts and early progenitor cells toward the amputation site were significantly decreased in *xrn-1(RNAi)* planarians in comparison to control animals (*p*<0.001 and *p*<0.05, respectively; Figure 17 and 18). There were no significant changes observed in the migration late progenitor cells (Figure 17 and 18). Although the location of neoblasts were different between control and *xrn-1(RNAi)* animals, there was no increase in the number of migratory neoblasts or progenitor cells in the irradiated region (Table 2; Appendix G). One would expect an increase in migratory neoblasts in *xrn-1(RNAi)* planarians since mitotically-active neoblast counts suggest that these could not differentiate (Figure 14).

The reduced migration of neoblasts in *xrn-1* depleted animals indicates an interruption of stem cell dynamics during regeneration. These results suggest that XRN-1 is first required before differentiation into early progenitor cells. It still is plausible that XRN-1 function may be required at later stages; however, interruption of neoblast progression into early progenitor cells would inhibit any
Figure 16. Motility sensitized stem cell dynamics assay. Experimental design to discern the possible neoblast-related events dependent upon XRN-1 function during regeneration. Control and xrn-1(RNAi) planarians will be subject to partial irradiation of the anterior region. After loss of stem cells and progenitor cells (4 days) in the irradiation-exposed region (anterior), animals were amputated immediately posterior to the photoreceptors. Planarians are then allowed to recover for 6 days and analyzed for the location of neoblasts and progenitor cells in irradiated tissue.
Figure 17. XRN-1 is required for proper stem cell dynamics during regeneration. Planarians subjected to stem cell motility assay as shown in Figure 16. Neoblasts (*smedwi-1*), showed reduced presence in irradiated regions of *xrn-1*(RNAi) planarians (top right) in comparison to control planarians (top left). No differences were seen in migration of early progenitor cells (*NB.21.11e*; middle row) and late progenitor cells (*agat-1*; bottom row). Irradiated anterior region is shown to the left of the plane of irradiation (dashed red line). Box (red) = inset shown to right. Scale bar = 0.5 mm.
Figure 18. Location of stem cells and progenitors during stem cell motility assay. Stem cells (smedwi-1) were located significantly closer to their source in xrn-1(RNAi) planarians in comparison to control. No differences were seen in the location of late progenitor cells (agat-1).
Table 2. Quantitative analysis of stem cells and progenitor cells during stem cell motility assay.

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<th>Average number of (+) cells in irradiated region</th>
<th>Average distance from plane of irradiation (mm)</th>
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subsequent stages. This requirement of XRN-1 during neoblast differentiation supports the hypothesis that XRN-1 degrades transcripts that promote normal stem cell dynamics.

*XRN-1 RNAi does not show detectable defects in the development of the male germline*

Neoblasts also give rise to non-somatic cell types and are responsible for development of the germline during sexual maturation. During the development of planarian testes, neoblasts differentiate into germline stem cells. These germline stem cells give rise to spermatogonia. Spermatogonia undergo mitosis to produce 8 spermatocytes. After meiosis, 32 spermatids are formed (Wang et al., 2007). The spermatids elongate and develop into mature sperm. To investigate the first stage of differentiation in non-somatic cells dependent on XRN-1, testes morphology of control and *xrn-1(RNAi)* planarians were analyzed. Anti-H3Ser10P immunohistochemistry and nuclear stain DAPI were used to visualize mitotic cells and DNA, respectively. After 6 dsRNA feedings over 4 weeks, the developmental state of the testes was analyzed. The number of H3Ser10P-positive cells within a cluster can be used to discern between dividing spermatogonia and spermatocytes. Additionally, DAPI staining allowed observation of the gross morphology of the cells that make up the testis, including clear visualization of spermatocytes, spermatids, and spermatozoa.

No obvious differences were observed in *xrn-1* depleted planarians in relation to control animals after 6 dsRNA treatments over 4 weeks. There were
no significant differences between the number of mitotic clusters in control and
xrn-1(RNAi) planarians (Figure 19). Moreover, spermatozoa was present in both
control and xrn-1(RNAi) planarians (Figure 20). The presence of sperm indicates
that neoblasts are able to progress completely through each stage of
differentiation and complete spermatogenesis. These results suggest that normal
levels of XRN-1 are not crucial for normal development of the testes and
spermatogenesis.
Figure 19. XRN-1 is not required for the development of spermatogonia or spermatid development. No significant differences were observed between the number of mitotic clusters within testes of xrn-1(RNAi) planarians and control animals after 6 dsRNA treatments over 4 weeks.
Figure 20. No significant defects in testes development observed after *xrn-1* RNAi. Control and *xrn-1*(RNAi) planarians were analyzed using nuclear stain DAPI to visualize DNA. No detectable morphological differences were seen in the testes of control and *xrn-1*(RNAi) planarians. Spermatozoa was present in both control and *xrn-1*(RNAi) planarians (arrowheads).
DISCUSSION

In this study, planarian *S. mediterranea* was used to investigate the role of XRN-1 on regulating differentiation during neoblast-mediated regeneration. Previous studies show the requirement of XRN-1 in proper regeneration of *D. japonica* (Rouhana et al., 2010). Extending the study of XRN-1 *S. mediterranea* was of importance due to the transcriptomic and genomic tools available in this species. These tools will allow comprehensive analysis of changes in transcript abundance, as well as identification of candidate XRN-1 target genes.

*xrn-1* mRNA expression in neoblasts

Conserved expression of *xrn-1* in neoblasts validated and confirmed at the cellular level using FISH and confocal microscopy.

*XRN-1* dependent regeneration

Validating previous studies in *D. japonica* (Rouhana et al., 2010), *xrn-1* RNAi in *S. mediterranea* resulted in incomplete regeneration. Furthermore, *xrn-1* mRNA was reduced in *xrn-1(RNAi)* planarians as detected by northern blot analysis. While RNAi treatment was successful in reducing transcript abundance, perhaps the incomplete penetrance of *xrn-1* RNAi results in continued, but reduced, presence and function of XRN-1. This may explain the incomplete phenotype of regeneration-deficiency seen in *xrn-1(RNAi)* planarians. The inability to compare protein concentrations is limiting and one must consider the possibility of prolonged XRN-1 protein stability. Decreased XRN-1 protein levels
need to be verified once suitable antibodies can be produced. Another approach is \textit{xrn-1} gene mutations to resolve the issue of lingering XRN-1 protein. However, there is no reliable protocol to produce gene mutations in planarians.

\textit{XRN-1 nucleolytic function in planarians}

XRN-1 functions biologically as an exonuclease, degrading uncapped transcripts from the 5' to 3' end (Nagarajan et al., 2013). Additionally, a recent study showed that XRN-1 regulates the processing and degradation of dsRNA (Burgess et al., 2015). This study confirms the nucleolytic functions of XRN-1 in planarians are conserved as demonstrated by \textit{xrn-1} RNAi resulting in dsRNA accumulation. It is hypothesized that XRN-1 substrates accumulate in neoblasts, which may hinder stem cell dynamics (proliferation, migration, and differentiation). The incomplete regeneration seen in \textit{xrn-1}(RNAi) planarians may be due to the lingering uncapped transcripts binding and retaining regulatory proteins which are needed for proper stem cell dynamics.

However, the accumulation of uncapped transcripts in \textit{xrn-1}(RNAi) animals are merely speculated and have not been confirmed. It is possible that these transcripts are being degraded from the 3’ end by deadenylation-dependent decay. Confirming the presence of these uncapped transcripts will be essential in determining the transcript specificity of XRN-1.
**XRN-1 requirement for proper stem cell dynamics**

The partial irradiation assay supports the hypothesis that XRN-1 is required for proper stem cell dynamics, which demonstrated that depletion of \textit{xrn-1} results in significantly decreased number of migratory neoblasts during regeneration (Figure 17 and 18). However, the migration of neoblasts and progenitor cells seen in our experiments were surprisingly different than the migration pattern shown by Guedelhoefer et al. (2012). In their experiments, migration of progenitor cells were increased over neoblasts. We observed increased migration of neoblasts rather than the progenitor cells. The discrepancy between these results may be a factor of multiple variables. Guedelhoefer et al. (2012) transplanted non-irradiated tissue into irradiated animals. The site of injury in these animals were adjacent to the population of neoblasts and may respond more quickly to injury signaling pathways. This may allow a more rapid and robust response of progenitor cell migration into the irradiated regions of transplant planarians. Analyzing the migration of neoblasts and progenitor cells in neoblast-transplanted, irradiated animals will provide additional evidence that XRN-1 is required for proper stem cell dynamics.

**Requirement of XRN-1 during germline development**

Surprisingly, the requirement of XRN-1 seems disposable for male germline development in planarians. XRN-1/Pacman has been previously shown to be required for gametogenesis in \textit{D. melanogaster} (Nagarajan et al., 2013). Pacman mutations in \textit{D. melanogaster} result in reduced spermatogonia and
sperm. The presence of sperm in *xrn-1* depleted planarians show that neoblast-mediated development of the testes were able to progress completely through mitosis, meiosis, and spermatogenesis. However, these measurements were made qualitatively by gross observation of the testes and were lacking methods to quantify any changes in sperm presence and reduction in fertility. Validation of the results will need to be performed to quantify a reduction of spermatogonia and fertility.

**Conclusion**

Collectively, these results suggest that XRN-1 is required for differentiation into somatic cell types. Since *xrn-1* deficiencies in other organisms result in specific developmental abnormalities, XRN-1 is suggested to degrade specific transcripts, rather than random uncapped transcripts. Therefore, it is hypothesized that uncapped mRNA accumulates in stem cells as result of the lack of XRN-1 nucleolytic function in *xrn-1*(RNAi) planarians and that their presence impede differentiation during regeneration. It is hypothesized that transcripts which promote pluripotency may be increased in *xrn-1*(RNAi) animals, as these transcripts are enriched in stem cells. Future studies seek to identify these transcripts using next generation RNA sequencing and understand the role of these transcripts in maintaining pluripotency.
Appendix A. *xrn-1* clone, 5'- and 3'-RACE product sequences. Sequences of initial *xrn-1* cDNA clone, 5'- and 3'-RACE products. Bolded text indicates overlapping regions between sequences.
Appendix B. Predicted sequence of xrn-1 mRNA. Lines indicate relative overlap of xrn-1 cDNA clone, 5'- and 3'-RACE products and resulting predicted xrn-1 mRNA. Bolded text indicates overlapping regions between sequences.
Appendix C. One XRN-1 ortholog found in *S. mediterranea*. BLAST of human *xrn*-1 in the *S. mediterranea* genome (Robb et al., 2008) found three candidates. Reciprocal BLAST of these candidates found one XRN-1 and two XRN-2 homologs. XRN-1 functions in degradation of cytoplasmic RNA, while XRN-2 functions in degradation of nuclear-restricted RNA (Jones et al., 2012; Nagarajan et al., 2013).

<table>
<thead>
<tr>
<th>Human BLAST results in <em>Smed</em> genome</th>
<th>E Value</th>
<th>Reciprocal BLAST to human</th>
</tr>
</thead>
<tbody>
<tr>
<td>lcl</td>
<td>v31.010275</td>
<td>8e-128</td>
</tr>
<tr>
<td>lcl</td>
<td>v31.000444</td>
<td>4e-80</td>
</tr>
<tr>
<td>lcl</td>
<td>v31.009587</td>
<td>5e-65</td>
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</table>
Appendix D. *xrn-1* mRNA expression increases after neoblast depletion.

Planarians were subject to 110kVp irradiation to deplete neoblasts. Total RNA from control animals or from animals 2 or 5 days post irradiation (dpi) was analyzed using RT-qPCR. Although the average relative abundance of *xrn-1* mRNA increased, there was no significant difference in *xrn-1* mRNA abundance after irradiation. Neoblasts, *smedwi-1*, used as irradiation control. Error bars represent standard error.
Appendix E. RNA accumulation in \textit{xrn(RNAi)} planarians. Total RNA of control or \textit{xrn-1(RNAi)} triplicate groups after 5 feedings over 3 weeks subjected to northern blot analysis using antisense \textit{xrn-1} probes. RNA accumulation observed in \textit{xrn-1(RNAi)} planarians is similar to dsRNA banding pattern.
Appendix F. Optimization of RNAi treatments. No significant differences were seen in various RNAi treatment schedules. Planarians were subjected to RNAi treatment according to table. Animals were transversely amputated anterior and posterior to the pharynx and allowed to regenerate for 7 days. In all treatments, regenerate trunk fragments were fed 1 additional dsRNA treatment followed by amputation. Animals were scored as full regeneration or incomplete regeneration based on presence of photoreceptor pigmentation.

<table>
<thead>
<tr>
<th>Treatment schedule</th>
<th>dsRNA treatment</th>
<th>Full regeneration</th>
<th>Incomplete regeneration</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 feeds over 21 days</td>
<td>control</td>
<td>100%</td>
<td>0%</td>
<td>n=46</td>
</tr>
<tr>
<td></td>
<td>xrn-1 (RNAi)</td>
<td>45%</td>
<td>55%</td>
<td>n=40</td>
</tr>
<tr>
<td>3 feeds over 10 days</td>
<td>control</td>
<td>100%</td>
<td>0%</td>
<td>n=18</td>
</tr>
<tr>
<td></td>
<td>xrn-1 (RNAi)</td>
<td>53%</td>
<td>47%</td>
<td>n=17</td>
</tr>
<tr>
<td>4 feeds over 14 days</td>
<td>control</td>
<td>100%</td>
<td>0%</td>
<td>n=30</td>
</tr>
<tr>
<td></td>
<td>xrn-1 (RNAi)</td>
<td>47%</td>
<td>53%</td>
<td>n=30</td>
</tr>
</tbody>
</table>
Appendix G. Number of migratory of neoblasts and progenitors detected during partial irradiation assay. No significant differences were seen in the number of migratory cells in neoblasts (smedwi-1), early progenitor cells (NB.21.11e), or late progenitor cells (agat-1) in control (n= 5, 6, and 5, respectively) and xrm-1(RNAi) planarians (n= 5, 5, 5, respectively).
LITERATURE CITED


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reveals anatomically restricted expression in the developing mouse brain.


