Expression Pattern and Function of eIFE4 Isoforms in Planarian Adult Stem Cells

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Abstract

The eukaryotic translation initiation factor 4E (*eIF4E*) is central to translation initiation in eukaryotic cells. Recent studies revealed that there are multiple isoforms of *eIF4E* present in different species, some of which were found to have specific functions other than translation initiation. Furthermore, some were found to be expressed during specific developmental stages and/or in specialized tissue types. Multiple isoforms of *elF4E* have been found in several model organisms. For example, five *eIF4E*s were found in *Caenorhabditis elegans*, three in mammals and sea urchins, two in zebrafish and eight in *Drosophila* (Henderson et al., 2009). Among these species, stem cell specific isoforms were found in mouse, *C. elegans*, and *Drosophila* (Amiri et al., 2001). None of the *eIF4E* isoforms have yet been investigated in planarians which is an ideal model organism for studying stem cell biology *in vivo*.

Planarians are flatworms that belong to the phylum Platyhelminthes. They are known to have a large population of totipotent stem cells called neoblasts which provide the animal with an almost infinite ability to regenerate. Neoblasts are also known to have electron-dense structures called chromatoid bodies. These structures are similar in nature to germ granules which are present in the germ line cells of almost every metazoan (Saffman and Lasko, 1999). Given the close relationship between germ line cells and planarian neoblasts, and in light of the fact that other examined animals show *eIF4E* isoforms that are germ line specific, we speculate that neoblast specific *eIF4E* isoform(s) exist in planarians.

In 2007, Yoshida-Kashikawa and co-workers published a paper discussing RNA-binding proteins in planarian stem cells and neurons (Yoshida-Kashikawa et al., 2007). One of the genes found to be neoblast specific was an *eIF4E* isoform. Based upon this finding, we intended to further investigate and confirm the expression and

function of this neoblast specific *eIF4E* isoform. This isoform will be referred to as *eIF4E-A* for ease of reference in this text.

In the initial research, the neoblast specific expression of *eIF4E-A* was confirmed via performing whole mount *in situ* hybridization (WMISH) on intact and irradiated (stem cell eliminated) worms, and *in situ* hybridization on sexual planarian histological sections. The function of *eIF4E-A* was then studied by RNAi which in some cases resulted in phenotypes that in turn were then studied by immunohistochemistry using a chromatoid body marker. *eIF4E-A* RNAi experiments resulted in a few mild phenotypes despite the confirmation of the neoblast expression pattern of *eIF4E-A*. The knock down of the gene's expression was not enough to cause regeneration aberration, nor did it affect the number or size of the chromatioed bodies. This finding led to the supposition that there might be additional *eIF4E* isoforms that are neoblast specific in planarians.

Genome screening and planarian transcriptome analysis revealed four additional *eIF4E* isoforms. For ease of reading, they are named *eIF4E-B*, *eIF4E-C*, *eIF4E-D*, and *eIF4E-E*. WMISH of the remaining isoforms and irradiation transcriptome data analysis revealed two additional strong candidates for neoblast specificity, namely *eIF4E-B* and *eIF4E-D*. These results suggest that there is more than one isoform specific to neoblasts in planarians working redundantly towards neoblast specific post-transcriptional regulation.

This study contributes to the knowledge as related to the key factors that govern stem cell regulation (eukaryotic translation initiation factors), thus adding value to the understanding of stem cells and regeneration, while contributing to the evolutionary data of gene members in the *eIF4E* family.

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Chapter 1: Introduction

1.1 Cap-Dependent Translation

1.1.1 Eukaryotic Translation Initiation Factors

All eukaryotic cells are almost entirely dependent on cap-dependent translation. After being transcribed, mRNAs go through a modification process which consists of the addition of a 7^{th} methyl group on the 5' end of the mRNA m7GpppX (where X is any nucleotide) creating the cap. The proteins involved in the regulation of the translation initiation process at the cap level are a group of factors called eukaryotic translation initiation factors. These factors mediate the necessary interactions between ribosomal subunits and the mRNA template. Translation initiation occurs at four main steps. Step one is the formation of the 43S preinitiation complex which consists of the small ribosomal subunit 40S, initiation factors, and Met-tRNA. Step two is the recruitment of the 43S preinitiation complex to the 5' end of the mRNA with the help of initiation factors. Step three is the scanning of the mRNA at the 5' untranslated region (UTR) in search of the start codon. Step four is the joining of the large ribosomal subunit 60S to assemble the complete 80S complex (Preiss et al., 2003).

The adhesion of mRNA to the 43S preinitiation complex requires eIF3, poly (A)-binding protein, and eIF4 proteins. The process is instigated when translation initiation factor 4E (*eIF4E*) recognises the cap at the 5' end of the mRNA. The binding increases the affinity of eIF4G to bind to *eIF4E*. eIF4G also binds to PABP which allows it to connect to both mRNA ends after which eIF4G then binds to an RNA helicase called eIF4A. In the process, the RNA helicase then unwinds the secondary structure of the mRNA in the cap proximal region allowing factor eIF3 from the 43S preinitiation complex to bind to eIF4G (Figure 1). This interaction

permits the small ribosomal subunit 40S to scans along the untranslated region (UTR) until it reaches the start codon (AUG). It is at this stage that the large ribosomal subunit is recruited, the complete 80S ribosome is formed and the synthesis of the first peptide bond begins (Rhoads, 2009).

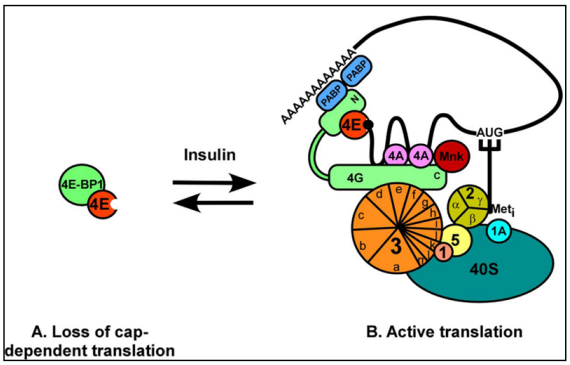

Figure 1: The translational inactive and active states of the *eIF4E*. **A:** The inactive state of *eIF4E* when it is bound to 4E- binding proteins such as BP1 in this example. **B:** The cap binding state of *eIF4E*. *eIF4E* in the translationally active 48S initiation complex, the interactions among eIF3 and eIF4 proteins involved are shown by eIF1, eIF2, eIF3, eIF4A, *eIF4E*, eIF4G, eIF5, Mnk (mitogen-activated protein kinase-interacting kinase), poly(A)-binding protein (*PABP*), mRNA, and the 40S ribosomal subunit.

Figure adapted form (Rhoads, 2009)

1.1.2 Eukaryotic Translation Initiation Factor 4E

Eukaryotic translation initiation factor 4E (*eIF4E*) is key to the translation initiation process. This factor has been found to be highly conserved in all eukaryotic cells from yeast to human. The N and C termini of the protein vary in length and sequence, however, a core region of approximately 170 amino acids that includes the binding domains required for cap recognition is found to be highly conserved in all eukaryotic organisms (Rhoads, 2009).

It was previously believed that *eIF4E* was a universal prototypical protein which was part of the canonical translation initiation process through its interactions with the cap and eIF4G. However, recent studies revealed new *eIF4E* binding partners other than eIF4G which has shed light on new functions. For example, it is now known that when the interaction between *eIF4E* and *eIF4E*-binding protein Maskin (in the presence of short poly(A) tails) takes place, the translation of maternal mRNAs in

Xenopus laevis oocytes is oppressed (Rhoads, 2009). Interestingly, recent studies have also revealed the presence of multiple *eIF4E* family members in almost all eukaryotes. It has also been shown that some of the newly found *eIF4E* family members functioned in specific types of cells/tissues and in some cases at different developmental stages (Goodfellow and Roberts, 2008, Hernandez and Vazquez-Pianzola, 2005, Joshi et al., 2005). For example one of the isoforms in *C.elegans* (IFE-1) is expressed only in germ line cells and its role is highly crucial in spermatogenesis (Amiri et al., 2001).

1.2 *eIF4E* in Model Organisms

As previously discussed, multiple *eIF4E* family members exist in almost all eukaryotic species. In some model organisms, the different family members were studied and classified in details more than others, in the next section I discuss the different isoforms of some of the model organisms. The focus will be on isoforms expressed in the germ line and the roles they play during different temporal and spatial stages of development.

1.2.1 *eIF4E* in *Drosophila*

Similar to many model organisms, subsequent studies in *Drosophila* revealed the presence of several genes encoding different *Drosophila eIF4E* isoforms. The classification of these different isoforms was conducted on the basis of their primary amino acid sequence, functionality, and cap/eIF4G binding capacity (Hernandez et al., 2005).

In total, *Drosophila* has 7 genes encoding 8 different known isoforms. The first gene to be studied among them was the *eIF4E-1* gene. Later, this gene was found to

encode two different isoforms via alternative splicing giving rise to isoforms *eIF4E-1*, and *eIF4E-2*. This gene was later renamed *eIF4E-1,2* (Hernandez et al., 1997; Lavoie et al.,1996). The importance of this gene is determined by its unique and specific isoform product *eIF4E-1*. *eIF4E-1* was found to be exclusive in its association with the germ granules of *Drosophila*'s germ plasm known as the polar granules. Notably, it was also expressed at elevated levels during embryogenesis. Among the remaining genes, namely *eIF4E-3, eIF4E-4, eIF4E-5, eIF4E-6, eIF4E-7 and eIF4E-8*, two additional isoforms (*eIF4E-3* and *eIF4E-8*) were found to be expressed in the embryo. These two, however, were expressed at significantly lower levels than the embryo dominant isoform *eIF4E-1*. Isoform functional experiments concluded that *eIF4E-1* constitutes the bulk of the cap-dependent translation initiation process in the *Drosophila* embryo (Hernandez et al., 2005).

1.2.1.1 Drosophila eIF4E functional properties and classification

Drosophila's *eIF4E*-family members are found to differ from each other in their terminal regions, yet their core amino acid region is highly conserved. Interestingly, the *Drosophila* isoforms are found to have the longest *eIF4E* terminal regions with isoform *eIF4E*-7 being the longest *eIF4E* known in all organisms thus far (Hernandez et al., 2005).

The classification and identification of the *Drosophila* isoforms were based on their core amino acid sequence, cap binding capacity, 4E-binding proteins and eIF4G interactions, and functionality. All of the *Drosophila eIF4E* isoforms were found to bind to the cap. Isoforms *eIF4E-6* and *eIF4E-8*, however, had lower binding capacity likely due to the substitutions of some of their conserved residues by others. Tests performed in regards to the ability of the isoforms to bind to 4E-BPs and eIF4G gave similar results. Isoforms *eIF4E-1*, *2* and *eIF4E-4* were highly capable of binding to

these two proteins. Isoforms *eIF4E-3, eIF4E-5* and *eIF4E-7* did bind, but had lower affinities. No interactions were observed between 4E-BPs and eIF4G and isoforms *eIF4E-6* and *eIF4E-8* as a result of several substitutions in the isoforms primary amino acid sequence (Hernandez et al., 2005).

To examine functionality, the different isoforms were tested for their ability to rescue *eIF4E* knockout yeast strains. Isoforms *eIF4E-1*, *eIF4E-3,* and *eIF4E-7* were able to rescue the mutant yeast strain with high affinity, while *eIF4E-2* and *eIF4E-4* were also able to rescue the mutant yeast strain with somewhat lower, yet significant levels. The remaining isoforms, primarily *eIF4E-5*, *eIF4E-6,* and *eIF4E-8* were found to be non-functioning *in vivo* (Hernandez et al., 2005).

The isoforms were then tested at different developmental stages. Isoforms *eIF4E-1 and eIF4E-2* were found to be expressed throughout the life cycle of the *Drosophila. eIF4E-8* was also found expressed throughout the life cycle but with very low levels of expression, whereas isoforms *eIF4E-3*, *eIF4E-4*, *eIF4E-5*, *eIF4E-6,* and *eIF4E-7* where found to be expressed from the third instar larva stage and onwards. Very weak expression of *eIF4E-8* was found at early embryonic stages, and very low expression levels were found of *eIF4E-3* in late embryonic stages. *In situ* hybridization of *eIF4E-3* and *eIF4E-8* detected weak ubiquitous signal levels in the embryo. Isoform *eIF4E-1* was detected during all the developmental stages at a significantly higher level than all the other isoforms, yet it was also the highest in expression level in embryos; specifically at the early stages of embryonic development (Hernandez et al., 2005).

Mutation experiments of embryo-expressed isoforms were later carried out, with results suggesting that the main cap-recognition activities in embryos relied mainly

on isoform *eIF4E-1*, whereas very little traces of *eIF4E-3* and *eIF4E-8* were observed (Hernandez et al., 2005). Table 1 summarizes the characteristics of the *Drosophila eIF4E* isoforms.

TABLE 1. Classification of *Drosophila* eIF4E isoforms

Isoform	Cap binding capacity	4E-BPs and 4G binding capacity	ability to rescue *eIF4E* mutant yeast strains	Expression in the embryo	Expression at developmental stages
eIF4E-1	Yes	Yes, with high affinity	Yes, with high affinity	Very high levels at early embryonic stages	Detected during all the developmental stages and at a higher level than all the other isoforms
eIF4E-2	Yes	Yes, with high affinity	Yes, with lower affinity	No	Detected during all the developmental stages
eIF4E-3	Yes	Yes, with lower affinity	Yes, with high affinity	Very low levels from late embryos	From the third larva instar onwards
eIF4E-4	Yes	Yes, with high affinity	Yes, with lower affinity	No	
eIF4E-5	Yes	Yes, with lower affinity	No	No	
eIF4E-6	Yes, with lower affinity	No	No	No	At lower levels from the third larva instar onwards
eIF4E-7	Yes	Yes, with lower affinity	Yes, with high affinity	No	
eIF4E-8	Yes, with lower affinity	No	No	Very low levels at early embryonic stages	Detected during all the developmental stages at very low levels

1.2.2 *eIF4E* in *C. elegans*

C. elegans are known to have five isoforms: *eIF4E-1*, *eIF4E-2*, *eIF4E-3*, *eIF4E-4*, and *eIF4E-5*. These isoforms were classified into three different groups based on their cap binding specificity, viability, and amino acid primary sequences. Class A includes *eIF4E-3*, which is the most similar to the canonical *eIF4E* and its knock down affected viability. Class C includes *eIF4E-4* which was found to be dispensable. Class B (which is analogous to the germ line specific *eIF4E* isoforms in *Drosophila*) includes the remaining three isoforms, *eIF4E-1*, *eIF4E-2*, and *eIF4E-5*. All three isoforms were found to be enriched in *C. elegans* germ line (Amiri et al., 2001).

Of all the isoforms enriched in the germ line, *eIF4E-1* was found to be the only *eIF4E* isoform associated with PGL-1, a component of the P-granules in the *C.elegans* germ line (Noble et al., 2008). Despite it being the only isoform associated with the p-granules, the knock down of *eIF4E-1* showed no defects in embryogenesis. It was, however, specifically needed for normal spermatogenesis to take place. Later studies showed that the knock down of the three class B isoforms caused embryonic lethality, which suggested that the functions of *eIF4E-1, eIF4E-2*, and *eIF4E-5* were redundant to each other during embryogenesis. For this reason, all isoforms must be knocked down to cause the defect (Amiri et al., 2001). Table 2 summarizes the *eIF4E* isoforms in *C.elegans*.

TABLE 2. Classification of *C.elegans* eIF4E isoforms

	Class A	Class B			Class C
Isoforms	*eIF4E -3*	*eIF4E -1*	*eIF4E -2*	*eIF4E -5*	*eIF4E -4*
Viability	Essential for viability	Enriched in the germ line: •*eIF4E-1* is essential for spermatogenesis and is associated with the p granules component PGL-1. •All three isoforms function redundantly in embryogenesis and it takes the knock down of all three to effect the process.			Dispensable

1.2.3 Zebrafish *eIF4E* family members

Two isoforms were found in zebrafish, *eIF4E-1A* and *eIF4E-1B* (Robalino et al., 2004). When these isoforms were analysed in terms of their primary amino acid sequences, functionality, and binding capacity to the cap, 4E-BPs and eIF4G, findings determined *eIF4E-1A* to be the universal prototypical *eIF4E* in Zebrafish, thus its presence is vital to its survival. *eIF4E-1A* was found able to bind to the cap 4E-BPs in addition to eIF4G. Furthermore, *eIFE4-1A* was expressed ubiquitously in

zebrafish and was able to rescue the yeast *eIF4E*-defective mutant strain (Robalino et al., 2004).

Despite the fact that the core amino acid sequence of isoform *eIF4E-1B* contains all the conserved binding domain residues needed for interaction, this isoform was unable to bind to neither the cap, E4-BPs, or eIF4G. It was also unable to rescue the *eIF4E*-deprived yeast strain. The role of *eIF4E-1B* is still unknown, however, several EST sequencing experiments show *eIF4E-1B* related proteins to be conserved in many organisms, including human, *Oncorhynchus mykiss, Xenopus laevis, Silurana tropicalis, and Sus scrofa* (Robalino et al., 2004). This suggests that the conservation and multiplicity of this gene is not present in all these animals simply for redundancy. Other experiments show the expression pattern of *eIF4E-1B* to be enriched in the embryo and associated with cytoplasmic and nuclear compartments (Robalino et al., 2004). The functions of this related gene are still unknown, but the findings hitherto suggest it may constitute a specialized function in zebrafish germ line/stem cells, and/or may be utilised during the development and determination of their lineages (Robalino et al., 2004).

1.2.4 *eIF4E* family members in mammals

The accumulation of sequencing projects allowed the grouping of mammalian *eIF4E* isoforms into three different categories based on their primary amino acid sequences, functionality, and pattern of expression. The classes were named *eIF4E-1*, *eIF4E-2* (4EHPor 4E-LP), and *eIF4E-3* as summarized in table 3 *(Joshi et al., 2004)*. The significance of this classification is the indication of some sort of niche for mRNA expression modulation whether in specific tissues, or at different developmental stages thus contradicting the past belief in one universal 4E eukaryotic initiation factor. The outcome of this classification given that certain classes exist in certain

phyla also demonstrates a pattern of evolutionary links. Class *eIF4E-1* is the universal prototypical eukaryotic translation initiation factor found in all eukaryotes and expressed ubiquitously. Class *eIF4E-2* is restricted to metazoans and is also ubiquitously expressed with the highest levels of expression found in the germ line. The third class (*eIF4E-3*) is restricted to chordates and is found to be expressed in specific areas such as the heart, skeletal muscle, lung and the spleen (Joshi et al., 2004).

Affinity to translational repressor proteins (4E-BPs) and eIF4G in addition to cap binding capacity were some of the other characteristics that contributed to the classification. Clear interaction differences are present between *eIF4E-1*, *eIF4E-2,* and *eIF4E-3*. Class *eIF4E-2* does not interact with eIF4G, but does interact with 4E-BPs whilst class *eIF4E-3* does not interact with 4E-BPs but does interact with eIF4G. Class *eIF4E-1* on the other hand does interact with both 4E-BPs and eIF4G. The three classes *eIF4E-1*, *eIF4E-2,* and *eIF4E-3* can all bind to the cap at the 5' end of the mRNA (Table 3). This classification of mammalian *eIF4E* family members demonstrate how each class serves a specialised niche in the recruitment of mRNA and through their different binding abilities post-regulate the critical process of protein synthesis (Joshi et al., 2004).

TABLE 3. Classification of mammalian eIF4E isoforms

The three different eIF4E-family members in mammals	Cap binding capacity	4E-BPs and eIF4G binding capacity	Ability to rescue *eIF4E* mutant yeast strains	Expression location	Found in
eIF4E -1	Yes	Yes	Yes, able to rescue	Ubiquitously expressed	All eukaryotic cells
eIF4E -2 (4EHP, 4E-LP)	Yes	Interacts with 4E-BPs but not eIF4G	Unable to rescue	Ubiquitously expressed with the highest levels in testis	Restricted to metazoans
eIF4E -3	Yes	Interacts with eIF4G but not 4E-BPs	Unable to rescue	Was only detected in heart, skeletal muscle, lung, and spleen.	Restricted to chordates

Only one isoform of *eIF4E* thus far has been explored in planarians, however, before we present the research background of *eIF4E* as related to planaria, an introduction of the planarian specie *Schmidtea mediterranea* is required.

1.3 Freshwater Planarian *Schmidtea mediterranea*

1.3.1 Planarian anatomy and biology

The freshwater planarian *Schmidtea mediterranea* is a free-living, unsegmented, dorsoventrally flattened, bilaterian flatworm (Salo, 2006). They are members of the *Turbellaria* class among the platyhelminthes phyla and have been classified along with the annelides, the molluscs, and others in the Lophotrochozoan clade (Carranza et al., 1997). One of the simplest animals of the *tricladida* order, planarians are bilaterally symmetrical and lack a coelom and an anus. Yet despite their simplicity, these animals occupy an important role in understanding metazoan evolution (Sanchez Alvarado and Kang, 2005).

Planarians have a digestive system made of three dorsally-located branches (one anterior and two posterior) that connect in the middle to the pharynx which is a muscular organ that protrudes from the central ventral part of the animal and serves as the mouth opening (Figure 2B). As mentioned above, planarians lack an anus and a coelom; they also lack a skeletal, respiratory, and a circulatory system. Despite the absence of the later two systems, the oxygen needed for the animal's survival is simply diffused via a network of flame cells dispersed along the body. These flame cells also act as osmoregulators and waste dispensers, filtering waste into the excretory system (Handberg-Thorsager et al., 2008, Salo, 2006). Planarians have a distinctive central nervous system made of bi-lobed cerebral ganglia in the anterior, and two ventral longitudinal nerve cords that connect at the posterior end of the

animal. Nervous plexus spread beneath the muscular tissue and connect to the main nerve cords. Planarians have two photoreceptors and a system of chemoreceptors which connect directly to the brain (Figure 2A). These receptors send instant signals that allow the animal to move or behave in response to the surrounding environment. The animal also has a population of sub-epithelial gland cells that secrete mucous which serves as protection, facilitates locomotion, adhesion, and captures substrates from its surroundings (Handberg-Thorsager et al., 2008, Salo, 2006). The space between the epidermis and the gut branches is called the parenchyma or the mesenchyme and it contains a range of cell types such as pigment cells, muscular cells, and fixed parenchyma cells, in addition to a range of specialized tissue, such as connective tissue. The mesenchyme is also the hub for the totipotent adult stem cells known in planarians as the neoblasts (Handberg-Thorsager et al., 2008, Salo, 2006).

As planarians are hermaphrodites, they can produce asexually by transverse fission and sexually by cross-fertilization. Alternation between the two reproductive methods is determined based on environmental factors and the availability of resources. The neoblasts in the asexual strains give rise to the germ line, and a sexual reproductive system is developed *de novo* once the sexual strain matures and grows to the optimal size (Handberg-Thorsager et al., 2008, Salo, 2006). The reproductive system is made of two ovaries that are located anteroventrally behind the cephalic ganglia. They are connected to the ventral oviduct which runs dorsoventrally along the animal housing numerous yolk glands. The testes also spread dorsoventrally and both the oviduct and the testes lead to the copulatory apparatus which is located at the posterior part of the animal (Figure 2C). Interestingly, when sexual worms de-grow in size due to starvation, the reproductive mature organs shrink. They can, however, grow again once the animal

grows back to optimal size, highlighting the complex plasticity of not only the neoblast cells but also the germ line cells (Handberg-Thorsager et al., 2008, Newmark and Sanchez Alvarado, 2002, Salo, 2006).

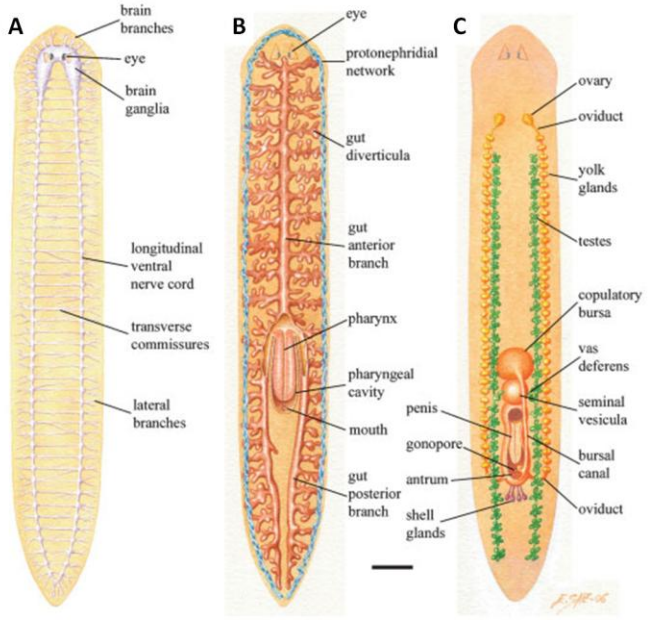

Figure 2: Diagram of the main organ systems in the freshwater planarian *Schmidtea mediterranea*. **A:** The central nervous system consists of a bilobed brain ganglia with eight brain branches each, and two ventro-lateral longitudinal nerve cords which are interconnected by commisural neurons and lateral branches. The eyes (photoreceptors) are located dorsal to the brain. **B:** The digestive system consists of a blind diverticulated gut with one anterior and two posterior branches and a single pharynx in the centre. The excretory system is a dorsal network of canals in which the flame cells occur. **C:** The reproductive system of hermaphrodite *Schmidtea mediterranea* consists of two ventral located ovaries, two dorsolateral lines of testes and viteline glands. The copulatory apparatus which contains a copulatory bursa (were the sperm interchanged during the copula is stored); a penis and seminal vesicular (were the mature sperm is stored); and shell glands that connect to the atrium to produce the polyembrionic eggs or cocoons.

Figure adapted form (Salo, 2006)

1.3.2 Planarian regeneration

Classic regeneration is divided into two types, namely morphallaxis and epimorphosis. The former refers to the replacement of lost tissue through re-modelling and boundary re-establishment of somatic tissue with little growth. *Hydra* regeneration is a classical example of this type of regeneration. The latter refers to regeneration by growth when lost parts are built anew through the production and differentiation of new tissue. Typical examples of this type of regeneration can be seen in amphibian tail and limb regeneration. Notably, the regeneration process in planarians is neither morphallactic nor epimorphic, however, is as an alternative, a mixed model between the two regeneration types (Newmark and Sanchez Alvarado, 2002, Salo, 2006).

In intact planarians, neoblast progeny replace cells lost during the course of physiological cell turnover. When the worm is injured, the neoblasts are stimulated to proliferate and migrate towards the wound epithelium giving rise to the blastema which is a specialized structure composed of an epithelial layer and mesodermally derived undifferentiated cells. It is from this structure that the missing parts of the planarian will be regenerated (Handberg-Thorsager et al., 2008, Salo, 2006).

It is still unclear what exactly stimulates the regeneration process in planarians, however, recent studies suggest that the neoblasts, when injured, receive and respond to a signal issued from the wounded epithelium layer. This signal is believed to be generated in the event that dorsoventral discontinuities are encountered (Handberg-Thorsager et al., 2008, Newmark and Sanchez Alvarado, 2002, Salo, 2006, Wenemoser, 2010).

During the first 30 minutes after the wound is incurred, muscle contractions enable the old dorsal and ventral epidermis to align and an epidermal layer covers the wound. The muscles then relax, and the epidermal layer becomes thinner. Following wound closure, a first mitotic burst takes place mainly in the area of old tissue adjacent to the blastema which is referred to as the postblastema. The newly divided cells responsible for generating the new tissue migrate from the postblastema to the blastema area, where they cease dividing and begin the process of differentiation. Depending on environmental conditions, the planarian regeneration process is normally completed within 2 to 3 weeks which is a phenomenon that has long distinguished planarians (Handberg-Thorsager et al., 2008, Newmark and Sanchez Alvarado, 2002, Salo, 2006).

When a double transverse amputation (pre-and-post phyrangeal) is carried out in planarians, the following three fragments are created:

- A head fragment.
- A middle fragment including the pharynx.
- A tail fragment.

The regeneration process taking place in the middle fragment will be the focus as a result of the fact that this fragment demonstrates regeneration in both the anterior and posterior parts of the animal. With this in mind, immediately after an amputation is carried out, the closure of the anterior and posterior wounds in the middle fragment takes place within the first 30 minutes. Within the first 2 to 3 days, a blastema starts forming in the position of the wound distinguished by its unpigmented appearance. In the anterior part of the animal, the blastema formed starts taking shape and colour. A newly formed head starts developing eyespots by day 4, and by day 5 to 8, the eyespots grow to normal size through the aggregation of newly formed tissue in the blastema. Furthermore, the brain is regenerated and pigments start developing in the newly generated tissue creating it's brown colour. In the posterior part of the central fragment another blastema is formed, and by day 5 to 8 the missing tissue and organs are replaced and the nervous ventral cord and the gut branches are fully developed. Remodelling and re-establishment of boundaries occur to adjust the old tissue and organs to the newly shaped worm. The result is that the amputated planarian is fully regenerated within 17 days with only one difference which is the slight reduction in its size (Handberg-Thorsager et al., 2008).

1.3.3 Neoblasts and chromatoid bodies

The amazing regenerative abilities of planarians are due to the large population of neoblast cells located in their mesenchymal layer. Neoblasts are the adult stem cells of planarians and what is so unique about them is their totipotency (their ability to give rise to all types of cells, including germ line cells) (Handberg-Thorsager et al., 2008). These neoblasts are self maintained and are not only able to replace damaged tissue in amputated animals, but are also able to sustain homeostasis (self renewal) in intact animals. Moreover, planarians have both sexual and asexual reproductive abilities which are also attributed to the plasticity of these neoblasts. Very little is known about the way neoblasts maintain this plasticity, however, the unique presence of electron-dense and membrane-lacking RNA granules (chromatoid bodies) is what separates these regenerative cells from others lacking this feature further down the lineage (Handberg-Thorsager et al., 2008, Newmark and Sanchez Alvarado, 2002).

Neoblasts are round or ovoid cells with a large nucleus to cytoplasm ratio and have little endoplasmic reticulum. The chromatoid body is the main structure diagnostic for neoblasts under the electron microscope. The cytoplasm contains a high level of ribosomes and only a few mitochondria that are sometimes found to be in close proximity with the chromatoid bodies which are almost always found in close association with the pores of the nuclear envelope (Handberg-Thorsager et al., 2008). These structures shrink in size during the differentiation process until they completely disappear in differentiated cells (Coward, 1974, Handberg-Thorsager et al., 2008, Higuchi et al., 2007). Chromatoid bodies are also found present in germ line cells of sexual planarians, further highlighting the similarity between these two types of stem cells (Anderson et al., 2006, Handberg-Thorsager et al., 2008, Newmark and Sanchez Alvarado, 2002).

1.3.4 Neoblast heterogeneous distribution and sub-populations

Planarian neoblast distribution is uneven along the anterior-posterior axis of these worms. While the neoblasts occupy the mesenchymal space, neoblast gradients along the anterior-posterior axis in this tissue layer vary thus suggesting asymmetrical regeneration capacity along the AP axis (Handberg-Thorsager et al., 2008). In *Schmidyea mediterranea,* neoblasts are absent from the pharynx and the anterior tip of the head. The neoblast population is also found to be in constant activity. This has been seen in anti-phosphorylated histone 3 (anti-H3P) labelling experiments which detected mitotic activity of neoblasts and provided evidence of an existing proliferating neoblast population distributed throughout the mesenchyme of the animal (Handberg-Thorsager et al., 2008).

What is interesting about the neoblast population is that not only does the distribution vary in density, but the cell population itself is not homogeneous. Recent cytological studies enabled researchers to classify two types of neoblast cells on the basis of size, chromatoid body number, and chromatin structure. Type A neoblasts (later renamed type 1) are larger in size (9.6- 2.9 μm) with more chromatoid bodies (4.4 ± 2.1) than type B neoblasts (later renamed type 2), which are comparatively smaller in size (size 6.2 ± 1.4 μm) with fewer chromatoid bodies (2.1 ± 0.9). Furthermore, type 1 neoblasts are rich in euchromatin, whereas type 2 neoblasts are rich in heterochromatin (Handberg-Thorsager et al., 2008, Higuchi et al., 2007).

Fluorescence-Activated Cell Sorting (FACS) helped demonstrate that the neoblast population was in fact composed of stem cells at different cell cycle stages, thereby further reflecting the heterogeneity of the neoblast population. The population consisted of stem cells, differentiating cells, and differentiated cells at a ratio of 1:2:6 (Handberg-Thorsager et al., 2008, Hayashi et al., 2006). Planarians irradiated

with the appropriate dose of X-ray or Gamma-rays are not able to regenerate due to the selective elimination of neoblasts (Reddien and Sanchez Alvarado, 2004). FACS illustrated that neoblasts could be categorized into the following three fractions:

- Fraction X1 which is constituted of irradiation-sensitive dividing cells (stem cells).
- Fraction X2 is constituted of irradiation-sensitive non-dividing cells (differentiating cells).
- Fraction XIS which included the remaining cells among the neoblast population, these being differentiated irradiation-insensitive cells (differentiated cells).

1.3.5 Germ line granules, chromatoid bodies, and post-transcriptional control

The similarities between germ line granules and planarian chromatoid bodies lead scientists to believe they serve similar functions. These similarities also support the notion that mRNAs of which the chromatoid body is composed are required for the regulation of regeneration, homeostasis, and differentiation in neoblasts. Moreover, not only do chromatoid bodies contain specific RNAs that are essential for regenerative potential, they are also found to have close associations with RNA-binding proteins and factors that control post-transcriptional regulation (Rouhana et al., 2010). For example, Tudor-related genes have been localized in the germ line granules and were found to be highly conserved in most model organisms. When tudor related genes were investigated in planarians, tudor was found to be associated with the chromatoid body and served an important role in the viability of neoblasts (Solana et al., 2009).

1.3.6 *eIF4E* in planarians

The resembling features between neoblasts and germ line cells, as well as the similar role chromatoid bodies and germ granules play in both stem cell types has lead to the speculation that chromatoid bodies contain mRNAs that are essential for proliferation and differentiation. It is important to note that these mRNAs are regulated at the post-transcriptional level. Similar to germ line cells in other animals, there seem to be families of mRNA binding proteins that are involved in regulating specific mRNAs in planarian neoblasts. It is very likely that these proteins are associated with the chromatoid body. As a consequence of the relation between germ line cells in most animals and planarian neoblasts, the genes and proteins known to be related to germ line control were investigated in planarians. Several planarian EST projects were performed in order to identify mRNA binding proteins that regulate neoblasts. One of these studies revealed a member of the eukaryotic translation initiation factor 4E family which was found expressed exclusively in neoblast cells of asexual planarian strains (Yoshida-Kashikawa et al., 2007).

In 2007, Yoshida and Shibata identified 99 genes from the planarian species *Dugesia japonica* that have mRNA binding domains from the planarian EST project (Yoshida-Kashikawa et al., 2007). They short-listed 60 of these genes which were confirmed to be RNA binding proteins via BLAST analysis. They then further analysed them based on their expression pattern to find out which are expressed in the neoblast. They preformed this analysis by comparing whole mount *in situ* hybridization in normal, irradiated, and regenerating animals. Genes expressed in the neoblast were found distributed in the mesenchymal space of the animal. X-ray irradiation specifically eliminates neoblasts but leaves other types of cells unharmed, hence, genes expressed in the neoblast would be reduced or eliminated post-irradiation. During regeneration, genes expressed in the neoblast show elevated levels of

expression near the amputated regions. Based on the outcome of their WMISH analysis, the genes were classified into four categories namely A, B, C, and D. They then focused on the me31B homologue found in Planarians. This homologue corresponded to 1352HH cDNA which is a fragment encoding a subfamily of DEAD Box RNA helicase members. This subfamily includes *Xenopus* Xp54, *Drosophila* Me31B, and *C.elegans* CGH-1 which were all found to be components of their RNA granules and at certain levels can be involved in post-transcriptional regulation. They named the planarian homologue djCBC-1.

Type A was considered a neoblast-specific group. Following whole mount *in situ* hybridization on intact animals, the genes in this group showed a neoblast specific pattern. Moreover, the expression pattern of the genes belonging to this group decreased post-irradiation and demonstrated elevated levels near the locus of amputation during regeneration (Figure 3A). All RNA binding protein-encoding genes sharing these characteristics constituted potential neoblast-specific genes. For example, *Djpiwi4* (belonging to type A genes) was homologous to *Smedwi1* which is a gene known to be neoblast specific in *S. mediterranea* (Reddin et al., 2005).

Genes categorized in type B were found expressed in the neoblast in addition to the nervous system. Staining was elevated in the mesenchymal space in addition to the brain and nerve cords. When genes in this group were irradiated, the mesenchymal space staining pattern decreased after irradiation, while staining in the brain and nerve cords remained normal. Expression was also detected at high levels near the wound during regeneration (Figure 3B). The majority of the genes (36 out of 60) belonged to type B genes which suggested that the majority of RNA binding proteins are expressed in both neoblasts and neural cells in planarians. For example, *DjCBC-*

1 was found associated with the chromatoid body and was also found to form chromatoid body-like complexes in brain neurons (Yoshida-Kashikawa et al., 2007).

Four genes were found to be expressed exclusively in the nervous system (mainly in the brain), and were categorized as Type C. The expression of these genes was not affected by irradiation and regenerating experiments did not detect expression near the region of the wound (Figure 3C). One of these genes encoded a protein that contained 3 RRMs binding domains and belonged to a family that includes a neural marker in *Xenopus* (Yoshida-Kashikawa et al., 2007).

Figure 3:
The expression patterns of representative genes encoding RNA binding proteins categorized into 4 types, type A to D. **(A–D):** Whole-mount *in situ* hybridization in intact animals. **(A'–D'):** 4 days after X-ray irradiation. **(A", B", and D"):** 3 days after amputation. **(A–A"):** Expression patterns of representative type A gene (*5895HH).* **(B –B"):** Expression pattern of representative type B gene (*1352HH).* **(C,C'):** Expression pattern of *2659HH* gene of type C. **(D,D"):** *0417HH* gene of type D. All of the images are dorsal views.

Figure adapted from (Yoshida-Kashikawa et al., 2007)

Type D genes were expressed elsewhere or ubiquitously in all tissues and organs (Figure 3D). There was no specificity in their expression pattern. Following X-ray irradiation and amputation experiments, the expression remained ubiquitous. None of the expression levels shown by these genes decreased or was eliminated post-irradiation and during regeneration experiments, these genes continued to be expressed ubiquitously. They suggested that the genes belonging to this group are part of housekeeping genes vital for survival. Strong examples of genes found in this group are eIF4A and poly A-binding protein (Yoshida-Kashikawa et al., 2007).

1.3.7 *eIF4E* Type A isoform

Part of the screening analysis done by Yoshida and his co-workers included an *eIF4E* isoform (Table 4) (Yoshida-Kashikawa et al., 2007). They determined that this mRNA binding protein is specific for neoblasts, i.e., categorized as type A. The whole mount *in situ* hybridization analysis in normal, irradiated, and regenerating worms corresponded to a neoblast expression pattern. However, no further analysis was performed in regards to this *eIF4E* isoform. Given the important role *eIF4E* plays in germ line cells in other animals, this gene became an interesting subject of study.

With this in mind, the aim of this study is to examine and confirm the neoblast specificity of the *eIF4E* isoform which is claimed to be selectively expressed in the noblest by Yoshida (Yoshida-Kashikawa et al., 2007). In addition to investigating its functions and/or role in planarian regeneration, it is also the aim of this study to find the total number of *eIF4E* isofroms that exist in planarians and explore the possibility of having additional *eIF4E* isoforms involved in post-transcriptional regulation of regeneration.

TABLE 4. Classification of Planarian EST Clones Including RNA Binding Motif				
Clone ID	Access. no.	Annotation	e-value	Motif
Type A				
00412_HN	AK248078	Polyadenylate-binding protein protin (*D. melanogaster)*	*2.00E-21*	*2 RRMs*
00565_HH	AK248079	SF1 protein, pre-mRNA splicing (*D. melanogaster)*	*4.00E-39*	*KH*
03689_HH	AK248080	Y-Box factor protein (*A. californica)*	*2.00E-20*	*Y-box*
03978_HH	AK248081	Translation eIF4E protein (*A. californica)*	*1.00E-46*	*eIF4E*
05895_HH	AK248082	Tudor protein (*X. laevis)*	*9.00E-03*	*Tudor*
06646_HH	AK248083	Cap binding protein 80 protein (*D. melanogaster)*	*1.00E-26*	—

Table adapted from (Yoshida-Kashikawa et al., 2007)

1.3.8 *eIF4E* and post-transcriptional regulation in planarian neoblasts

While we were conducting this research, an interesting study by Rouhana was published in which the importance of post-transcriptional regulators in maintaining stem cell identity in neoblasts was looked at (Rouhana et al., 2010). They found gene homologues known to germ granules from other organisms in planarians and carried out an analysis of their functions during planarian regeneration. The majority of the genes analysed (49 out of 55 genes) were expressed in the neoblast underscoring the importance of post-transcriptional regulation in planarian stem cells. The 49 homologues were then categorised into 5 different groups by comparing whole mount *in situ* hybridization patterns in wild type animals and animals depleted of neoblasts by X-ray irradiation. The groups were sorted based on the sensitivity of the genes to irradiation. Type A classification consisted of genes that were exclusively expressed in irradiation sensitive cells. The gene specific to our research (neoblast specific *eIF4E-A*), in addition to, a number of other translation initiation factors (eIF-2A, eIF-3A, eIF-4G, and eIF-5A) were categorized as type A genes (Rouhana et al., 2010).

Another translation initiation factor (RNA helicase eIF4a3; a conserved eIF4A1 homologue associated with nonsense-mediated decay and the spliceosome) was categorised as type B which is a class that consisted of genes expressed exclusively in both neoblasts and neurons. These findings projected and confirmed the existence of different eukaryotic initiation factor members in planarians. Moreover, these results highlighted the essential role that neoblast specific translation initiation factors play in planarian regeneration and stem cell maintenance (Rouhana et al., 2010).

The functional analysis of the genes was investigated by RNA interference (RNAi) which is a protocol used to perform selective gene knock down. Followed by a transverse amputation anterior and posterior to the pharynx, the regenerative process was later monitored for any phenotypic abnormalities. Interestingly, the study carried out by Rouhana and co-workers demonstrated that the gene specific to our research (neoblast specific *eIF4E-A*) did not give rise to any phenotype, while other characterized translation initiation factors did. For example, eIF-4A3 RNAi animals failed to form a blastema after amputation and instead underwent lysis in the first two days following amputation. Furthermore, eIF-3A knock down showed limited regeneration, which was reflected by an incomplete formation of the eyes or the formation of a single eye. The result of eIF-3A knock down indicated that neoblasts were able to proliferate and replace lost tissue until the decline in the levels of expression of eIF-3A. They speculated that the RNAi of eIF-3A caused mitotic arrest as observed by the absence of phospho-Histone H3 (a marker of mitotic cells) in the regenerated RNAi animals.

Rouhana expected other translation initiation factors to have a role in neoblast post-transcriptional regulation but argued that the reason these factors did not result in any phenotypic abnormality was due to the incomplete ablation of gene expression by RNAi in their experiments. They also suggested that the absence of observed phenotypes showed by the remaining translation initiation factors might be due to high genetic redundancy (Rouhana et al., 2010).

Chapter 2: Materials and Methods

The following relates to the materials and methods that were used in conducting the experiments.

2.1 Material

- *Holtfreter 5/8:* 38 mM NaCl, 0.413 mM KCl, 0.568 mM $CaCl_2$, 1.5 mM $NaHCO_3$.
- *PBS ×10:* 27 mM KCl, 1.4 mM NaCl, 14.7 mM KH_2PO_4, 88 mM $Na_2HPO_4.12H_2O$, (pH 7.2-7.4).
- *MABx5:* 5x [86.5 mM Maleic acid, 168 mM NaCl, (pH 7.5)].
- *Carnoy:* 60% ethanol/ 30% chloroform/ 10% acetic acid.
- *TPBS:* 0.1% Triton ×100, 2.7 mM KCl, 138 mM NaCl, 1.47 mM KH_2PO_4, 8.8 mM $Na_2HPO_4.12H_2O$.
- *TEA:* 0.1 M Triethanolamine (1.35ml TEA (Sigma) in 100 ml ultra distilled water).
- *Prehybridization:* 50% formamide, 5×SSC, 0.1 mg/ml yeast tRNA, 0.1 mg/ml heparin, 0.1% Tween 20, 10 mM DTT.
- *Hybridization:* Prehybridization Solution + 10% Dextran Sulphate + probe.
- *Hybe solution:* 50% formamide, 5×SSC.
- *Buffer I:* 0.1% Triton ×100 + MABx1.
- *Buffer II:* Buffer I with 1% Blocking Solution For nucleic acid hybridization and detection (Boehringer Mannheim).
- *Blocking solution for in situ in sections*: Maleic buffer + 1% blocking solution.
- *Blocking solution for immunohistochemistry*: 5% skimmed dry milk in PBS, 0.05% Tween20.
- *Washing buffer*: 50% formamide, 5x SSC, 0.1% Tween20.

- *Maleic buffer*: 99.4 mM Maleic acid, 167 mM NaCl, 0.1% Triton x-100,(pH 7.5).
- *Citrate Buffer*: 10 mM citric acid, 0.05% Tween 20, (pH 6.0).
- *TMN:* 0.1 M Tris (pH 9.5), 0.1 M NaCl, 0.05 M $MgCl_2$, 1% Tween 20, 10% PVA (Polyvinyl alcohol, 98-99% hydrolyzed).

2.2 Animals

The organisms used in this research are from a clonal line of an asexual strain of the planarian species *S. mediterranea*. All worms used in the experiments were maintained at 20ºC in tap water treated with activated charcoal and buffered with 0.5 ml/L 1 M $NaHCO_3$. Planarians were fed beef liver as a substitute for the food source of their native environment, and prior to all experiments were starved for at least one week.

2.3 Isolation of *smed-eIF4E* Isoforms

To identify planarian homologues of eukaryotic translation initiation factors 4E (*eIF4E*), we searched a local database of Version 3.1 of the *S. mediterranea* with homologues from different organisms. (Genome Center, Washington University in St Louis, 2010). The resulting gene found was then subjected to blast analysis.

The genes were amplified using primers and a full length planarian cDNA: Reverse-transcribed from planarian RNA extractions. The PCR products were used as a template for the synthesis of digoxigenin-labelled probes and dsRNA as described in the next section. Primer sequences for the primers used are found in Appendix 1.

2.4 Digoxigenin-labeled RNA Probe Synthesis

The DNA template was amplified in a primary PCR using gene specific primers with a linker region. A secondary PCR was performed by using the forward primer of each gene and a universal T7 3' reverse primer (with a linker region attached to a T7 promoter region). The secondary PCR products were purified using PureLink PCR purification kit (Invitrogen). The DNA template was incubated for 2 hours at 37°C with RNA DIG labelling mix (Roche), RNAse out (Invitrogen), T7 RNA polymerase (Roche) and transcription buffer. Distilled water was then added until a total volume of 20µl was reached. After the first incubation, Turbo DNAse (Ambion) and Turbo DNAse buffer were added and samples were incubated at 37°C for another 30 minutes, after which they were transferred to eppendorf tubes that contained water, 4M LiCl, Glycogen, and ethanol and left to precipitate overnight at -20°C. The next day, when the samples were centrifuged at 4°C for 20 minutes, a pellet was observed. The pellets were then washed with 70% ethanol and centrifuged for 5 minutes again at 4°C. The supernatant was discarded; the pellet was air dried for 1-2 minutes and then resuspended with resuspension buffer (Formamide, 1xTE pH 7.5, and tween) and stored at -20 °C. 1 µl of each sample's concentration was checked using a nanodrop and 1 µl was run in 1% agarose gel to confirm the presence and integrity of the probe.

2.5 Double-Stranded RNA Synthesis for RNAi Interference

The DNA template was primary amplified using gene specific primers with linker regions. Secondary PCR was performed with a universal T7 5' forward primer and a universal T7 3' reverse primer (each with the correspondent linker region attached to a T7 promoter region). Secondary PCR products were purified using PureLink PCR purification kit (Invitrogen) and the resulting product was used for *in vitro*

transcription. First, the DNA templates were incubated for 4 hours at 37°C with transcription buffer, RNAse out (Invitrogen), 20 µM rNTPs (Roche) and T7 RNA polymerase (Roche). Distilled water was then added until a total volume of 20µl was reached. After the incubation, turbo DNAse buffer and Turbo DNAse (Ambion) was added and samples were incubated for another 30 min at 37°C. Following the second incubation, STOP solution (1 M NH_4 OAc, 10 mM EDTA, 0.2% SDS) was added in addition to glycogen. Thereafter, samples went through the following phenol/chloroform extraction procedure:

- A phenol/chloroform solution was added,
- The samples were vortexed, then centrifuged for 5 minutes at 4°C with maximum speed,
- The supernatant was then transferred to a fresh tube and chloroform was added,
- The samples were again vortexed and centrifuged for 5 minutes at 4°C with maximum speed,
- The supernatant was transferred to a fresh tube. Cold ethanol was added, the samples were vortexed, and centrifuged at 4°C for 15 minutes,
- Supernatant was discarded and cold 70% ethanol was added, the samples were vortexed, and centrifuged for 10 minutes at 4°C at maximum speed,
- The supernatant was once more discarded, the pellet was left to dry for 2-3 minutes, and then was resuspended with ultra distilled water,
- 0.5 µl of the product was run in a 1% agarose gel, and 0.3 µl was used to check the sample's concentration on a nanodrop.

2.6 Whole Mount *In Situ* Hybridization (WMISH)

The following protocol was used for whole mount *in situ* hybridization (WMISH):

Note: All solutions were kept at the required experiment temperatures prior to use. For example, after Carnoy was made, it was kept in the fridge until its temperature dropped to 4°C before it was used.

Note: steps from the beginning until the acetic anhydride washing steps were performed in falcon tubes.

Note: steps from the beginning until the incubation with proteinase K step were performed under RNAse free conditions i.e. all tubes and solutions were autoclaved or sterile.

2.6.1 WMISH Day 1: Fixation and bleaching

- Worms were washed in fresh planarian water for 1 minute at room temperature to remove the mucus and other substances that might interfere with the reaction.
- The worms were fixed in 2% HCl/5/8 Holfreter for not more than 5 minutes on ice.
- The worms were then fixed again with Carnoy and placed on a shaker for 2 hours at 4°C.
- The worms were also fixed in 100% methanol and placed on a shaker for 1 hour at 4°C.
- The worms were then left overnight (for at least 20 hours) in 8% H_2O_2/ 100% Methanol solution under a white light source, which bleached the worms and eliminated the animal's pigmentation.

2.6.2 WMISH Day 2: Washes and application of probe

- The next morning, the worms underwent a series of ethanol washes, during each wash the samples were placed on a shaker for 30 minutes at 4°C. The ethanol washes began with a 70% ethanol in 5/8 Holtfreter, followed by 50% and 30%, respectively.
- The worms were then washed with TPBS and placed on a shaker for 30 minutes at 4°C. This step prepared the worms for the next proteinase K digestion.
- The worms were incubated with 0.9 u/ml proteinase K/TPBS for not more than 10 minutes at 37°C. This step broke down proteins and enzymes creating pores that facilitated the access of probe to the cell.
- After the digestion step, the worms were washed three times. At each wash, the samples were placed on a shaker for 1 minute at 4°C. This step ensured washing out the proteinase K and facilitated re-fixing the worms after being digested.
- The worms were post-fixed with 4% paraformaldehyde (PFA)/5/8 Holtfreter and placed on a shaker for one hour at 4°C.
- The samples were then washed three times with 5/8 Holtfreter, each time placed on a shaker for at least 1 minute at 4°C (these washes were required to remove any traces of PFA), the samples then received a final wash with 5/8 Holtfreter and placed on a shaker for 1 hour at 4°C.
- In order to reduce background staining, worms were washed with 0.1 M Triethanolamine (TEA) in distilled water four times. During each wash the samples were places on a shaker at room temperature for 15 minutes. Acetic anhydride was added to the third wash (25µl for each 10ml of TEA), and an additional 25µl/10ml of acetic anhydride was added to the fourth and final TEA wash.
- Acetic anhydride was removed by three quick PBS washes followed by two 15 minutes PBS washes, during each wash the samples were placed on a shaker at room temperature**.**

- The worms were poured out of the falcon tubes into a petri dish and then distributed into 1.5 ml eppendorf tubes. 1 ml of prehybridization solution was added to each tube and then the samples were placed on a shaker at 56°C for one hour to incubate.
- Prehybridization solution was then carefully removed and hybridization buffer containing the probe was added. Before this addition, the hybridization solution was heated at 70°C for 10 minutes and then immediately placed on ice. The heating of the probe facilitates the unwinding of any secondary structures of the RNA probe before it is added to the samples. The samples were left to incubate at 56°C for not less than 17 hours (overnight).

2.6.3 WMISH Day 3: Washing of the probe and incubation with anti-digoxigenin-conjugated with alkaline phosphatase antibody (anti-DIG-AP)

- The samples were removed from the eppendorf tubes and distributed into a 6-well plate or nylon mesh columns by applying *hybe* solution. After being distributed, the samples were thoroughly washed with 100% *hybe* solution and placed on a shaker for 10 minutes at 56°C.
- The samples went through a series of stringency washes; during each wash the samples were placed on a shaker for 10 minutes at 56°C.

a. 75% *hybe* sol. +25% (2xSSC; 0.1% Triton X-100)
b. 50% *hybe* sol. +50% (2xSSC; 0.1% Triton X-100)
c. 25% *hybe* sol. +75% (2xSSC; 0.1% Triton X-100)

- The samples were then washed twice with 100% (2x SSC; 0.1% Triton X-100) and twice with (0.2x SSC; 0.1% Triton X-100) consecutively, during each wash the samples were placed on a shaker for 30 minutes at 56°C.

- The samples were then washed with Buffer I, one quick wash followed by two 10 minutes washes; during each wash the samples were placed on a shaker at room temperature.
- Samples were transferred to a 24-well plate, Buffer II was added (as a blocking buffer prior to the antibody incubation), and the samples were placed on a shaker for 1 hour to incubate at room temperature.
- 1:2000 dilution of anti-DIG-AP (Roche) in Buffer II was added and the samples were placed on a shaker to incubate for 3 hours at room temperature.
- Anti-DIG-AP was quickly washed with Buffer I and then samples were again washed twice with Buffer I, during each wash the samples were placed on a shaker for 10 minutes at room temperature.
- The samples were then washed with Buffer I and placed on a shaker at 4°C to incubate overnight.

2.6.4 Day 4: *In situ* development and mounting

- The samples left overnight were washed four times with Buffer I, during each wash the samples were placed on a shaker for 15 minutes at room temperature.
- The buffer was removed, freshly made TMN was added, and samples were placed on a shaker for 5 minutes at room temperature.
- 20µl of NBT/BCIP stock solution (nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche))/ 1 ml of TMN was used to colorimetrically detect the probe hybridization. 1 ml of the developing mix was added to each sample, the 24-well plate was covered and placed on a shaker at room temperature.
- The samples were monitored until the colour reached the desired state. The reaction was stopped using PBS.

- After the reaction was stopped, the samples were washed in PBS, fixed in PFA, washed in a series of ethanol percentages, and finally kept in 70% glycerol/PBS as laid out below:

    a. The samples were washed twice with PBS, during each wash the samples were placed on a shaker for 5 minutes at room temperature.
    b. The samples were fixed in 4% PFA and placed on a shaker for 30 minutes at room temperature, they were then washed twice with PBS; during each wash the samples were placed on a shaker for 5 minutes at room temperature.
    c. The samples were washed with different percentages of ethanol, during each wash the samples were placed on a shaker for 5 minutes at room temperature. The ethanol washes took place in the following order: 30% EtOH/PBS, 50% EtOH/ PBS, 70% EtOH/PBS, 100% EtOH, 70% EtOH/PBS, 50% EtOH/PBS, and 30% EtOH/PBS.
    d. Two more washes were performed with 100% PBS; the samples were placed on a shaker for 5 minutes at room temperature during each wash.
    e. Finally, the samples were kept in 70% glycerol/PBS at 4°C.

- Following this procedure, bright-field pictures with white background were taken on a Zeiss Discovery V8 from CarlZeiss using an AxioCam MRC from CarlZeiss.

2.7 *In situ* Hybridization on Histological Sections

Before introducing this method, the following protocol was used in preparing the slides.

2.7.1 Paraffin embedding

Notes: Paraffin is usually melted during the previous night because it takes a long time for it to melt at the appropriate temperature. Moreover, all materials used for manipulating samples while embedding the next day were kept warm. The embedding procedure takes place in an oven at 60°C; thus, all materials used for embedding (e.g. petri dishes, pasteur pipettes) were placed in advance into an oven at 60° C.

- Fixing and dehydration steps:

  a. The desired worms were fixed in 2% HCl/PBS for 5 minutes on ice.

  b. The worms were fixed in 4% PFA/PBS and placed on a shaker for 4 hours at 4°C.

  c. They were then washed in PBS and placed on a shaker overnight at 4°C.

  d. The next day, a series of four ethanol washes were performed. During each wash the samples were placed on a shaker for 15 minutes at 4°C. These steps dehydrated the worms. The washes were in the following order: 70% ethanol, 96% ethanol, 100% ethanol, and lastly, 100% ethanol.

  e. The worms were then washed with 100% xylene twice. During each wash the samples were placed on a shaker for 7 minutes at room temperature. Xylene is an organic solvent that permits worms to be embedded in paraffin.

- Embedding steps:

  a. The melted paraffin was poured into a petri dish and left in the oven at 60°C.

b. The worms were then dropped into the paraffin carefully and individually.

c. In order to remove traces of xylene, the worms were transferred at least once to a new paraffin plate before they were taken out of the oven and left to solidify for an hour at room temperature. When the plates were ready they were stored at 4-20°C.

- Histological sections preparation steps:

  a. The embedded worms were cut using a Leica microtome and the histological sections were placed on slides containing a layer of water located on a heating block at 37°C. The slides remained on the heating block until dry.

  b. The slides were then stored at 4°C until use.

The following protocol was used for *in situ* hybridization on histological sections:

Note: All solutions were kept at the required experiment temperatures prior to use.

2.7.2 Day 1: *In situ* hybridization on sections

- The slides were prepared as mentioned previously in the paraffin section.
- The slides were placed in a glass container and underwent a series of washes at room temperature to eliminate the paraffin traces:

  a. Two 100% xylene washes, each wash was for 5 minutes.
  b. One 100% ethanol wash for 5 minutes.
  c. One 96% ethanol wash for 5 minutes.
  d. One 70% ethanol wash for 5 minutes.
  e. Two PBS washes, each wash was for 5 minutes.

- The slides were then distributed in 50 ml falcon tubes including citrate buffer and left to incubate for at least 20 minutes in the steam bath at 95°C. This step broke down proteins and enzymes, creating pores that facilitated the access of probe to the cell.
- The slides were then placed back in the glass container and washed twice with PBS. Each wash was for 5 minutes at room temperature.
- They were then washed with 0.2 M HCl/H_2O for 15 minutes to neutralize the sample after the citrate treatment.
- The slides were then fixed in 4%PFA/PBS for 20 minutes at room temperature.
- In order to reduce background staining:

    a. The slides were washed twice with 0.1 M Triethanolamine (TEA) in distilled water. Each wash was for 15 minutes and at room temperature.
    b. Washed once with 0.1 M Triethanolamine (TEA) including acetic anhydride (25µl for each 10ml of TEA) at room temperature for 15 minutes.
    c. And finally washed once with 0.1M Triethanolamine (TEA) including acetic anhydride (50µl for each 10ml of TEA) at room temperature for 15 minutes.

- The slides were then washed twice with PBS. Each wash was for 5 minutes at room temperature.
- Prehybridization buffer was added and the slides were left to incubate in the glass container for one hour at 55°C.
- Hybridization buffer containing the probe was added. Before this addition, the hybridization solution including the probe was incubated at 70°C for 10 minutes

and then immediately placed on ice. The heating of the probe facilitates the unwinding of any secondary structures of the RNA probe before it was added to the samples. Around 100µl was applied to each slide. The slides were covered with a fitted size parafilm, and left to incubate at 55°C in a humidity chamber over night.

2.7.3 Day 2: *In situ* hybridization on sections

- The next day, the slides were transferred back to the glass chamber and washed three times with washing buffer. Each wash was for 15 minutes at 55°C.
- The slides were then washed four times with maleic buffer. Each wash was for 10 minutes at room temperature.
- Blocking solution was applied individually on each slide and the samples were left to incubate for 30 minutes at room temperature.
- 1/500 anti-DIG-AP in blocking solution was applied to the slides individually which were then placed in the humidity chamber for 3 hours at room temperature to incubate.
- The slides were transferred to a glass container and washed four times with maleic buffer. Each wash was for 20 minutes at room temperature.
- The slides were then placed back into the humidity chamber. 20µl of NBT/BCIP stock solution (Roche)/ 1 ml of TMN was used in developing the samples. The TMN mix was applied and the slides were left to incubate in the dark. The slides were monitored until the colour reached the desired state. The reaction was stopped using two washes of PBS.
- The slides were mounted with 70% glycerol/PBS and sealed with transparent nail polish. After the sealing dried out, the samples were stored at 4°C.
- Following this procedure, bright-field pictures with white background were taken on a Zeiss Discovery V8 from CarlZeiss using an AxioCam MRC from CarlZeiss.

2.8 Exposure to Gamma Irradiation

Gamma- irradiation results in the selective elimination of neoblasts (Reddien and Sanchez Alvarado, 2004). Asexual worms were starved for one week prior to experiment and were exposed to a lethal dose of (30 Gy) of gamma irradiation.

2.9 Immunohistochemistry in Histological Sections

The above paraffin embedding protocol was also performed to prepare the slides used in carrying out immunohistochemistry in histological section.

2.9.1 Immunohistochemistry

- Rehydration:

These steps rehydrated the samples by dissolving the paraffin using a series of washes. The slides were placed in a glass chamber where the solutions were carefully poured in. All of the following washes were performed at room temperature:

a. Two xylene washes- each wash was for 7 minutes.

b. Two 100% ethanol washes- each wash was for 7 minutes.

c. One 96% ethanol wash for 10 minutes.

d. One 70% ethanol wash for 10 minutes.

e. One 0.05% Tween-20/ PBS wash for 15 minutes.

- Permeabilization:

a. Citrate buffer was added and the samples were left to incubate for 20 minutes at 95°C. The citrate buffer helped create permeability for the antibody access.

b. The slides were washed twice with 0.05% Tween-20/PBS at room temperature. Each wash was for 5 minutes.

c. Blocking solution was added and the samples were left to incubate at room temperature for 1 hour.

- Primary antibody:

  a. The primary antibody was diluted in blocking solution and applied to the slides individually, the slides were then covered with a fitted size parafilm and left to incubate in a humidity chamber overnight.

  b. The next day, the slides were washed with 0.05% Tween-20/PBS in the glass chamber at room temperature for not less than 6 washes of 10 minutes each.

- Secondary antibody:

  a. The secondary antibody was diluted in blocking solution and applied to the slides individually, the slides were then covered with a fitted size parafilm and left to incubate in a humidity chamber for 4 hours.

  b. The slides were then washed with 0.05% Tween-20/PBS in the glass container at room temperature for at least 6 times for 10 minutes each.

- Hoescht staining:

  a. The slides were again placed in the glass chamber and incubated with Hoesch 1/5000/0.05% Tween-20/PBS for 15 minutes in the dark.

  b. The samples were washed twice with 0.05% Tween-20/PBS at room temperature, each wash was for 5 minutes.

- Mounting:

  a. The slides were dried carefully and PBS/glycerol 1:1 was used to mount the slides. The slides were sealed and stored at 4°-20°C.

- Fluorescence pictures were taken under a Leica MZ16F fluorescence stereomicroscope using a Leica DFC 300Fx camera (Leica Lasertechnik, Heidelberg). Confocal laser scanning microscopy was performed with a LeicaSP2 confocal laser scanning microscope (CLSM) (Leica Lasertechnik, Heidelberg).

2.10 RNA Interference

RNA interference is a protocol used to silence genes post-transcriptionally. The silencing is a result of injecting animals with a dose of dsRNA homologous to the specific mRNA of the desired gene. RNAi is widely used in planarian laboratories around the world to investigate stem cell-specific gene functions. In our RNAi experiments, we followed standard planarian RNAi procedure in which a pulse of 100 nl of 1µg/µl dsRNA was injected into the gut of the animal. Injection was usually performed during three consecutive days to ensure the proper introduction of dsRNA. The dsRNA used was synthesised as described previously, and control animals were injected with distilled water, which does not have an effect on the animals.

2.10.1 Injection protocol

Three rounds of the aforementioned injection protocol were performed. Each round was followed by pre and post-pharyngeal amputations. After each amputation, the worms were left to regenerate for 6 days before the following injection round during which, the injected animals were monitored for phenotypic abnormalities. Photomicrographs of the resulting defects were taken using the AxioCam MRc-digital camera and AxioVision (CarlZeiss).

Chapter 3: Results

3.1 Isolation of *eIF4E* in Planarians

We started our investigation by isolating the *S. mediterranea* orthologue corresponding to the gene described as a neoblast specific *eIF4E* in *D.japonica*, via tblastn searches. The sequence obtained was aligned with known eukaryotic translation initiation factor 4Es in other model organisms (Figure 4).

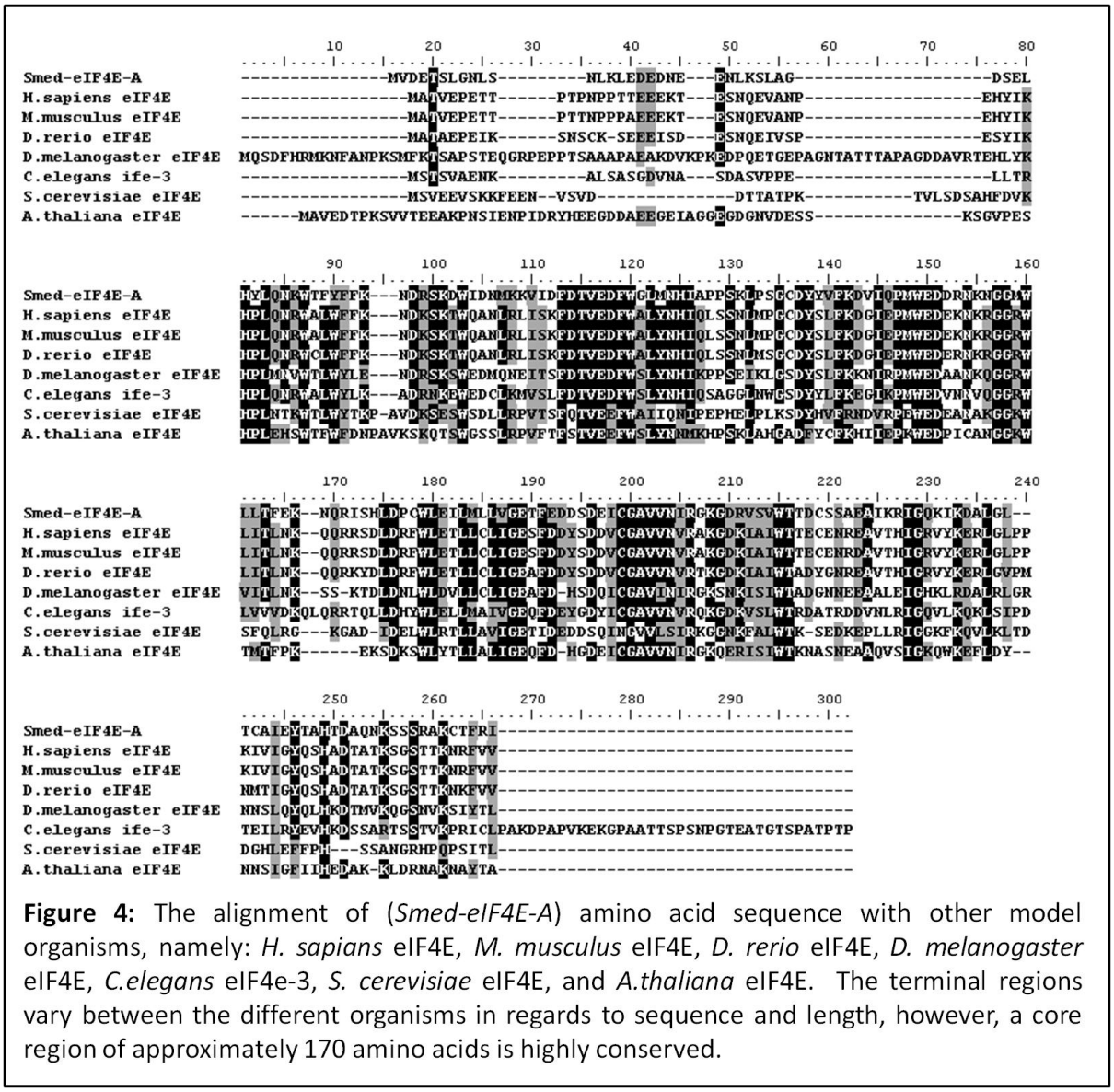

Figure 4: The alignment of (*Smed-eIF4E-A*) amino acid sequence with other model organisms, namely: *H. sapians* eIF4E, *M. musculus* eIF4E, *D. rerio* eIF4E, *D. melanogaster* eIF4E, *C.elegans* eIF4e-3, *S. cerevisiae* eIF4E, and *A.thaliana* eIF4E. The terminal regions vary between the different organisms in regards to sequence and length, however, a core region of approximately 170 amino acids is highly conserved.

The sequence of *smed-eIF4E-A* was found to be similar to that in other model organisms. It varied at the terminal regions in regards to sequence and length. i.e. the terminal regions were not conserved. However, a core region of approximately 170 amino acids was found to be highly conserved. Binding domains required for mRNA and eIFG4/4E binding proteins interaction were found to be preserved as demonstrated in the conserved tryptophan residue (W56, W73, and W102) (Figure 4).

The sequence was then amplified using a full length *S. mediterranea* cDNA template and specifically designed primers (Appendix 1). The resulting amplification gave a clear band when run in a 1% agarose gel. The bioinformatic analysis (carried out by Jordi Solana, 2010), and its amplification by PCR confirmed its existence in the planarian species *S. mediterranea.*

3.2 Whole Mount *In Situ* Hybridization (WMISH) on Intact Animals

Following the verification of the presence of the *eIF4E* homologue in *S. mediterranea,* we wished to investigate the expression pattern of the gene and confirm its neoblast specificity. A specific digoxigenin-labelled probe was synthesized as mentioned previously and used for performing whole mount *in situ* hybridization on intact wild type animals. As a control, a neoblast marker called *histone-2B* (H2B) which is a histone variant expressed only in neoblast cells (Guo et al., 2006), was used in order to structure an objective analysis on the pattern of expression. This WMISH was performed twice with 6 animals in each replicate. The experiment was designed with these controls to ensure the consistency of the expression pattern obtained.

The expression pattern obtained from whole mount *in situ* hybridization of *eIF4E-A* homologue corresponded to a neoblast specific gene. The expression of *smed-eIF4E-A* was observed in a distribution similar to those of neoblast cells, as seen by the level of probe staining distributed thought-out the mesenchymal space and the absence of probe staining in the anterior tip of the head region and the pharynx. A consistent staining for *smed-eIF4E-A* was also observed in other additional areas. For example, expression could be observed in the central nervous system of the animals which was different to that of the neoblast marker gene H2B (Figure 5).

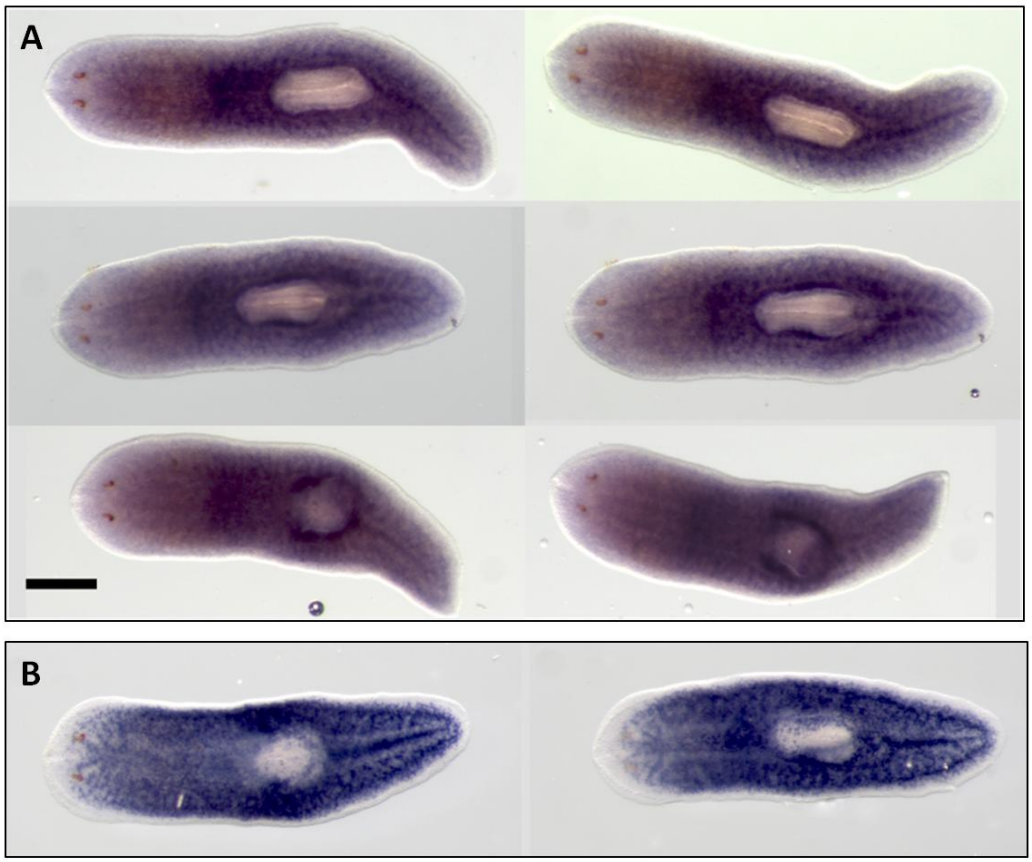

Figure 5: Whole mount *in situ* hybridization on intact wild type *Schmidtea mediterranea* using *eIF4E-A* probe. **A:** Samples showing the stain distribution of *eIF4E-A* in their dorsal and ventral views. The staining is saturated along the mesenchymal space and is absent form the pharynx and the anterior most part of the animals, the expression corresponds to a neoblast specific gene. **B:** A sample of the neoblast specific marker H2B used as control, the staining is filling the mesenchymal space and is absent from the pharynx and the anterior most part of the animal.

3.3 Post Irradiation Decrease in Expression of s*med-eIF4E-A*

In order to confirm that the expression pattern observed for *smed-eIF4E-A* was due to a putative expression in neoblast cells, we decided to conduct whole mount *in situ* hybridization on irradiated intact animals.

As mentioned previously, gamma-irradiation selectively eliminates neoblasts. Therefore, we conducted whole mount *in situ* hybridization on irradiated intact animals to further investigate the neoblast specific pattern obtained from the above experiment. If the *eIF4E-A* isoform was indeed exclusively or preferentially expressed in the neoblasts, its level of expression would decrease with irradiation as a result of eliminating them. Therefore, we irradiated samples of worms with a lethal dose of irradiation (30 Gy) and then fixed each sample at different time points: 24 hours, 3 and 7 days after irradiation. Non-irradiated animals were used for control. H2B was used once again as a control that decreases with irradiation. Furthermore, neural marker *smed-eye53* which does not decrease with irradiation, was used as a control to ensure that the WMISH being performed under irradiation conditions was successful and that irradiation did not affect differentiated cells. The expression of *smed-eIF4E-A* did decrease post-irradiation. However, the expression was not completely eliminated by day 7 which confirmed that the gene was expressed in cells other than neoblasts such as in the central nervous system (Figure 6).

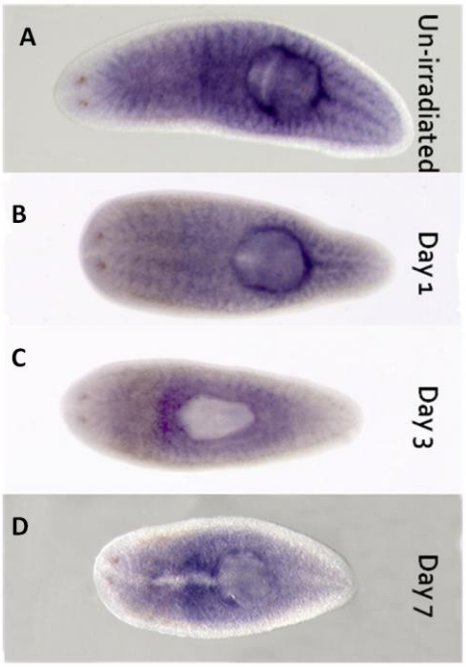

Figure 6: Whole mount *in situ* hybridization on intact irradiated *Schmidtea mediterranea* using *eIF4E-A* probe. **A:** Expression pattern of an un-irradiated animal showing the stain distribution of *eIF4E-A*, the expression corresponds to a neoblast specific gene. **B:** The expression pattern of an irradiated animal after 24 hours, *eIF4E-A* demonstrates a decrease in expression. **C:** The expression pattern of an irradiated animal after three days ,*eIF4E-A* demonstrates further decrease in expression. **D:** The expression pattern of an irradiated animal after 7 days, the expression did not completely disappear which was an indication that in addition to the neoblast other expression areas for *eIF4E-A* are possible.

3.4 Expression of *smed-eIF4E-A* was Found Elevated in Germ Line Cells of Sexual Strains.

Most of the mRNA binding proteins whose genes have been characterised in different planarian species are homologues of genes characterised in the germ line cells of other animals. The similarities between neoblasts and germ line cells in planarians, as well as their exclusive trait of chromatoid body presence, suggested that *smed-eIF4E-A* could also be expressed in planarian germ line cells. We therefore performed *in situ* hybridization on histological sections in sexual planarians to investigate the expression pattern of *smed-eIF4E-A* in planarian germ line cells.

The same anti-DIG-labelled probe (*smed-eIF4E-A*) was used in performing the *in situ* on histological sections of sexual planarians. *Smedwi1*, a neoblast specific marker that also presents elevated levels of expression in the germ line, was used as a positive control.

The resulting *in situ* stainings showed elevated levels of expression in the planarian testes confirming the specific presence of *smed-eIF4E-A* in the planarian germ line (Figure 7). This finding was consistent with the described expression in neoblast cells since the great majority of genes expressed in neoblast cells in planarians are also expressed in the germ line of sexual strains.

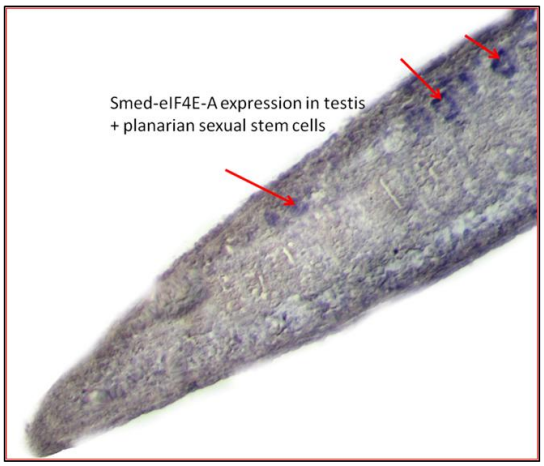

Figure 7: *in situ* hybridization in histological sections of sexual *Schmidtea mediterranea* using *eIF4E-A* probe. The expression is found elevated in the animals germ line cells (testis).

3.5 Three Rounds of RNAi Result in Mild Phenotype

Given that the expression pattern of *smed-eIF4E-A* was similar to neoblast specific genes, elevated in the testes of sexual strains, and had decreased post irradiation (thus indicating neoblast specificity); we investigated the function of *smed-eIF4E-A* during regeneration. In order to carry out this investigation, the expression of *smed-eIF4E-A* was knocked down by RNA interference. The knock down of *smed-eIF4E-A* was performed by injecting animals with a dose of dsRNA followed by regeneration (amputation) experiments. Namely knocking down the expression of *smed-eIF4E-A*, amputating the animals post/pre-pharyngeally, and then observing the regeneration process (Figure 8 and 9).

Knock down of genes expressed in neoblasts can lead to phenotypic effects during the regeneration process. For example, knocking down a gene such as *smedwi2* (a Piwi homologue) results in the complete abrogation of the organism's regenerative power (Reddien et al., 2005). Therefore, we attempted to ascertain whether or not the knock down of *smed-eIF4E-A* led to phenotypic effects during the process of regeneration.

The animals were then subjected to three rounds of injections with dsRNA corresponding to the *smed-eIF4E-A* (control animals were injected with three rounds of distilled water). After each round of injections, the animals were amputated pre/post pharyngeally and left to regenerate for six days.

Preliminary experiments showed the absence of a phenotype after two rounds of injection and regeneration. Injected worms were still able to form a blastema and then fully regenerate and differentiate. Therefore, we performed a third round of injections.

However, even after three rounds of injections and the addition of the amputation stimulus, the majority of the animals were still able to form a small blastema during the first day of amputation (Figure 8). They were also able to undergo complete regeneration and differentiation within 7 days. The vast majority of the samples showed no obvious defects. Notably, only a fraction of the *eIF4E-A* RNAi sample (6 animals out of 30) gave rise to different mild phenotypes (Figure 9).

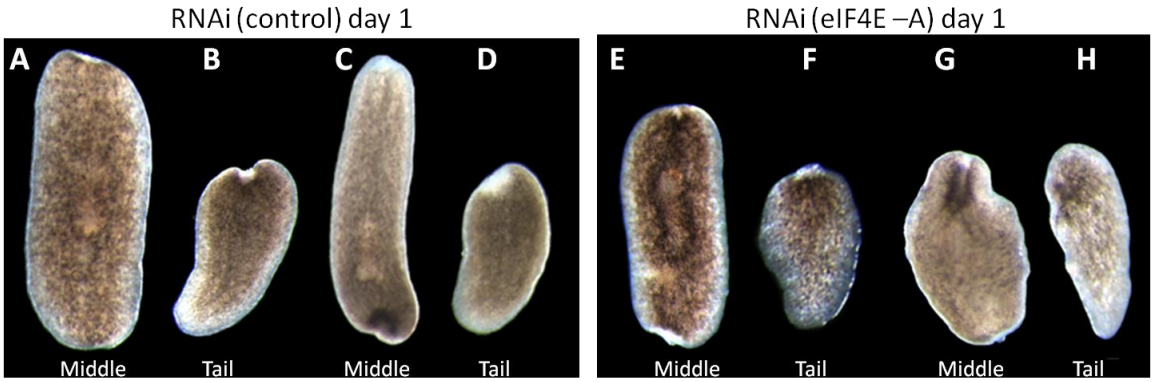

Figure 8: Animals of the RNAi experiments representing the blastema formation during day 1 after three rounds of injections and amputations. *eIF4E-A* RNAi animals were able to initiate a regenerative blastema even after three rounds of injections and amputations. **(A-D):** Control RNAi regenerating pieces . **(A,C):** Middle pieces of control RNAi. **(B,D):** Tail pieces of control RNAi. **(E-H):** *eIF4E-A* RNAi regenerating animals. **(E,G):** Middle pieces of *eIF4E-A* RNAi. **(F,H):** Tail pieces of control RNAi.

The resultant phenotypes were inconsistent. Defects primarily involved changes in the anterior (head) and posterior (tail) regions of *smed-eIF4E-A* RNAi animals with varying degrees of severity. Despite their inconsistencies, the animals shared a single trait in the posterior region. In place of a normal tail, a concave tail posterior had developed with the tip adopting a rectangular shape. Other mutations of the anterior portion were varied and differed in severity. One of the phenotypes observed had only one eye, another had failed to develop eyes (Figure 9). We classified the mild phenotypes into three different groups based on their anterior phenotypic differences (Table 5).

Table 5: *smed-eIF4E-A* RNAi phenotypes

no. = 30	Blastema formation	Normal full regeneration with no phenotype	Phenotype		
Description	All were able to form a blastema on day 1	24 Were able to regenerate normally	4 regenerated with rectangular tail Small eyes	1 regenerated with rectangular tail One eye	1 regenerated with rectangular tail No eyes

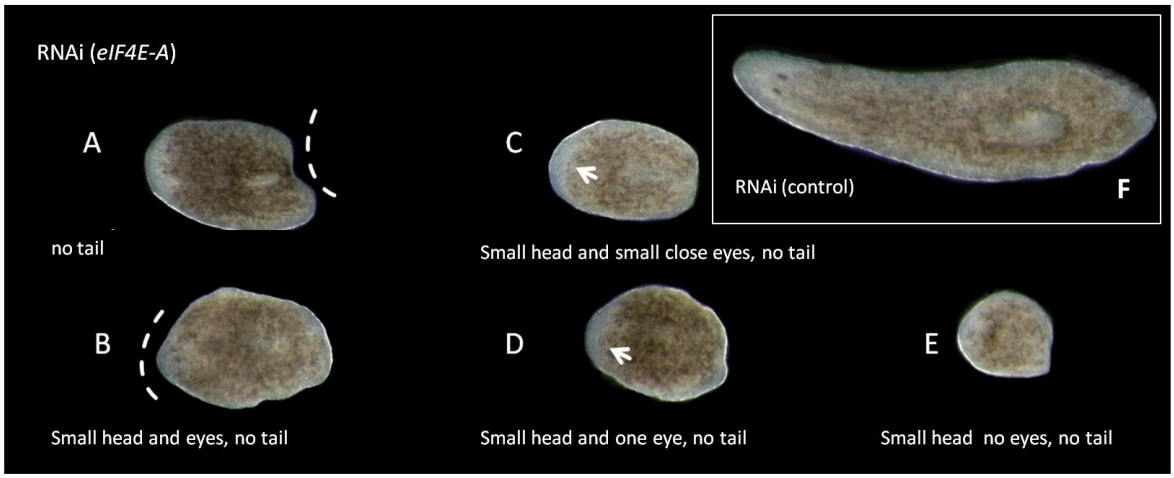

Figure 9: Regenerated animals representing the small fraction of the varied mild phenotypes of *eIF4E-A* RNAi after three rounds of injections and amputations. **(F):** Control RNAi. No phenotypic abnormalities observed. **(A-E):** *eIF4E-A* phenotypes, defects primarily involved changes in the anterior (head) and posterior (tail) regions with varying degrees of severity, shared mark between the phenotypes is the development of a concaved/no tail posterior. **(A):** *eIF4E-A* phenotype; normal head development and concaved/no tail. **(B):** *eIF4E-A* phenotype; small odd shaped head and eyes and concaved/no tail. **(C):** *eIF4E-A* phenotype; small head and small close eyes development and concaved/no tail. **(D):** *eIF4E-A* phenotype; small head and one eye development and concaved/no tail. **(E):** *eIF4E-A* phenotype; small head and no eyes development and concaved/no tail.

3.6 Immunohistochemistry on Histological Section for RNAi Phenotypic Animals Showed No Significant Changes in Chromatoid Body Features

Since *smed-eIF4E-A* was found highly expressed in the neoblasts and germ line cells of *S. mediterranea* and since some germ line specific *eIF4E(*s) in animals are associated with germ granules (Amiri et al., 2001), we expected that a knock down of *smed-eIF4E-A* would have an effect on the chromatoid bodies. We hypothesised that the effect would either be numerical (affecting the number of chromatoid bodies found associated with the nuclear envelope), or proportional (affecting the size of the chromatoid bodies found associated with the nuclear envelope). In order to test this hypothesis, we performed immunohistochemistry on the histological sections of *smed-eIF4E-A* RNAi animals using a chromatoid body marker (*smed-tud-1*) and a nuclear marker (Hoescht) (Figure 10).

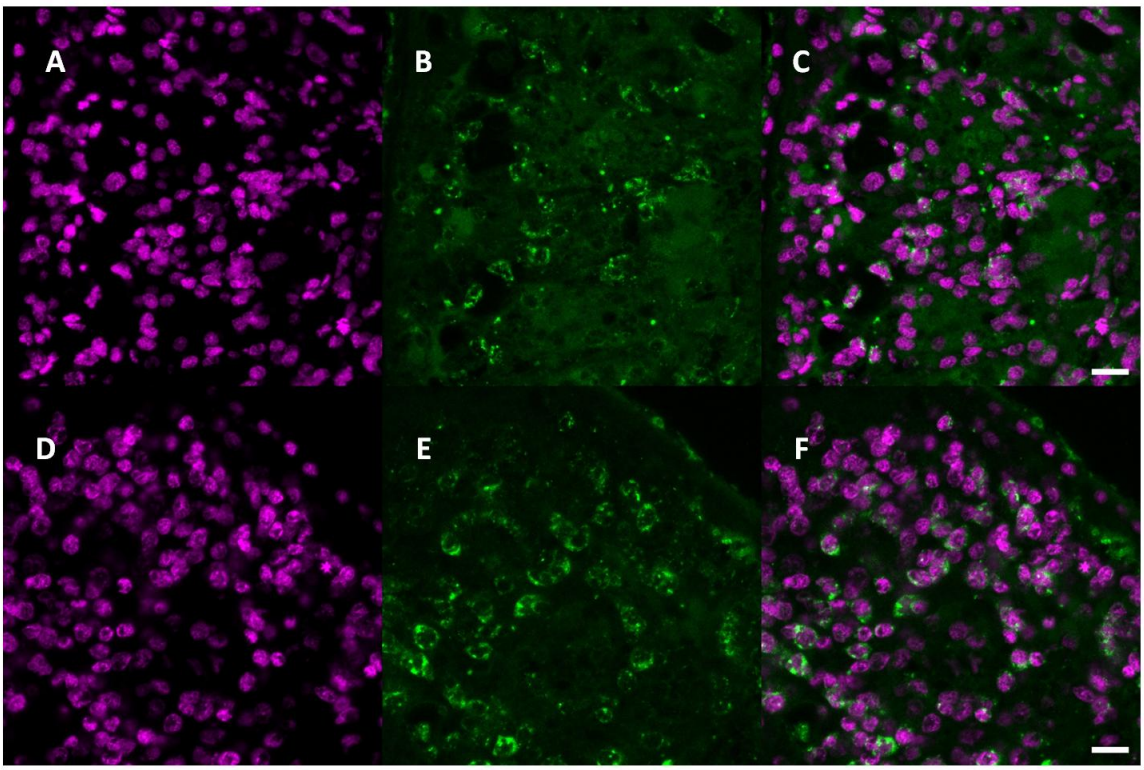

Figure 10: Immunohistochemistry on the histological sections of RNAi animals using a chromatoid body marker (*smed-tud-1*) shown in green and a nuclear marker (Hoescht) shown in magenta. No marked differences in chromatiod bodies were observed between the control and *eIF4E-A* RNAi samples. **(A-C):** *eIF4E-A* RNAi histological sections sample. **(D-F):** Control RNAi histological sections sample. **(A+D):** Nuclear staining (Hoescht). **(B+E):** Chromatoid body staining (*smed-tud-1*). **(C):** Merged A and B. **(F):** Merged D and E.

The magenta staining in A (*eIF4E-A* RNAi sample) and D (control RNAi sample) represents the nuclear Hoescht stating. The green staining in B (*eIF4E-A* RNAi sample) and E (control RNAi sample) represents the chromatoid bodies staining. When comparing control RNAi and *eIF4E-A* RNAi samples by merging the Hoescht staining and *smed-tud-1* images together. We were not able to detect any significant changes, numerical or proportional, in the chromatoid bodies between the two.

3.7 RNAi and Immunohistochemistry Findings Suggest More Than One *eIF4E* Neoblast Isoform is Involved in Planarian Translation Initiation Regulation

Despite the results obtained from the intact and irradiated whole mount *in situ* hybridization experiments (showing neoblast specific staining patterns for *smed-eIF4E-A)*, the RNAi and chromatoid body analysis did not reveal any apparent effects on regeneration. RNAi animals were able to regenerate and even those with phenotypic disorders continued to survive for at least 3 weeks. Furthermore, the knocking down of *smed-eIF4E-A* did not have a significant effect on the number or size of the chromatoid bodies. These observations led to the speculations that there could be more than one *eIF4E* isoform specific for neoblasts in planarians and that these isoforms function redundantly so the animal may maintain normal regenerative capacities. This is similar to the case of class II *eIF4E(*s) in *C. elegans*: *eIF4E* isoforms (*eIF4E-1, eIF4E-2,* and *eIF4E-5*). As previously mentioned in a study involving class II *eIF4E* isoforms in *C. elegans*, no embryonic defects were observed when only one of the isoforms expressed in the germ line was knocked down (isoform *eIF4E-1*, the only isoform associated with the p granules). However, the multiple knock down of the three *eIF4E* isoforms (*eIF4E-1, eIF4E-2*, and *eIF4E-5*) of class II *C. elegans* resulted in embryonic lethality. It required the knock down of all the isoforms found to be expressed at elevated levels in the germ line of *C. elegans* to result in aberration of embryogenesis (Amiri et al., 2001).

To be able to investigate the likelihood of this scenario in planarian neoblasts, we screened the planarian genome for all possible *eIF4E* isoforms with the intention of finding new *eIF4E* isoforms expressed in the neoblast other than *smed-eIF4E-A*. In the beginning of the screening analysis, seven to eight isoforms were found but

when isoforms in the genome were compared to those found in the ongoing transcriptome project, only five of the isoforms predicted were confirmed (Table 6). For ease of reference, we gave each of the isoforms a code (*smed-eIF4E-A, smed-eIF4E-B, smed-eIF4E-C, smed-eIF4E-D, smed-eIF4E- E*).

Table 6: Planarian genome screening and planarian transcriptome analysis revealed five *eIF4E* isoforms in planarians. The below table shows the contigs and transcriptome locations the additional isoforms were found in. The isoforms were given the below codes for ease of reference. Isoforms B to E are the new potential *eIF4E* planarian isoforms.

Transcriptome	Contig	Isoform code
AAA.544ESTABI.19955	563	*smed-eIF4E-**A***
AAA.544ESTABI.8858	548	*smed-eIF4E-**B***
AAA.544ESTABI.17307	3814	*smed-eIF4E-**C***
AAA.544ESTABI.10969	15109	*smed-eIF4E-**D***
AAA.544ESTABI.8361	17395	*smed-eIF4E-**E***

3.8 Planarian *eIF4E* Isoforms' Different Patterns of Expression in Intact Wild Type animals Proposed New Members of *eIF4E* Family

After discovering the above mentioned isoforms, the research then aimed at the patterns of expression of these additional isoforms to determine if there were other *eIF4E* isoforms involved in the neoblast specific post-transcriptional regulation in planarians. In order to carry out this investigation, the four additional isoforms were amplified. Specific isoform digoxigenin-labelled probes were synthesised and whole mount *in situ* hybridization experiments were performed on intact wild type animals. The WMISH(s) carried out revealed different expression patterns for some of the

additional isoforms. There was no success in terms of a clear amplification of the additional isoforms *smed-eIF4E-B* and *smed-eIF4E-D.* This was possibly due to their low level of expression. Despite these difficulties, there was enough evidence to demonstrate that the additional isoforms do reveal obviously different expression patterns. The most interesting marked differences were highlighted in Isoforms *smed-eIF4E-B* and *smed-eIF4E-D.* The former isoform showed a staining pattern with similar characteristics to that of a neoblast specific gene. In addition to other possible areas of expression, the latter isoform showed an elevated expression pattern in the nervous system (Figure 11).

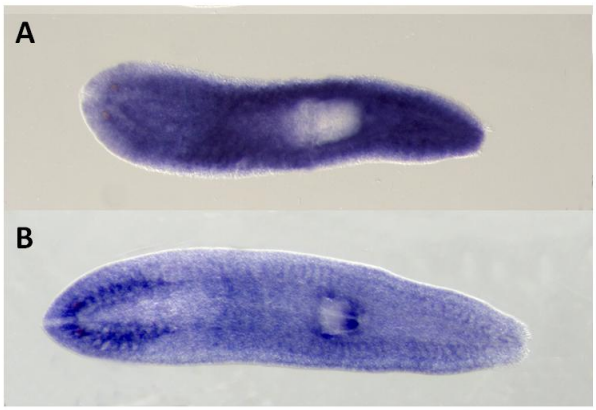

Figure 11: Whole mount *in situ* hybridization on intact *Schmidtea mediterranea* using the additional *eIF4E* probes revealed differences in expression pattern. **A:** Expression pattern of isoform *eIF4E-B* showing similar pattern characteristics to that of a neoblast specific gene (expression is absent form the pharynx and the anterior part of the head). **B:** Expression pattern of *eIF4E-D* showing elevated levels in the nervous system.

The identification of different specialised niches of *S. mediterranea eIF4E* isoforms suggests that there are different family members of *eIF4E(*s) in planarians and that these genes are there to serve specific functions either in different types of tissue or at different developmental stages.

With the expansion of the project and the limited time specific resources at hand to further confirm the expression pattern of the additional neoblast candidates via irradiation experiments, a decision was made to revert to the planarian irradiation

transcriptome data to compare and analyse the preliminary findings to the irradiation data at hand. Interestingly, when looking at all the additional isoforms including the initial isoform in question (*smed-eIF4E-A)*, isoforms *smed-eIF4E-A*, *smed-eIF4E-B*, and *smed-eIF4E-D* were strong candidates for neoblast isoforms. Isoform *smed-eIF4E-A* showed a significant decrease in the level of expression (from 45 pre-irradiation to 26.35 post-irradiation) as had also been demonstrated in the previous irradiation experiment result. Isoform *smed-eIF4E-B* showed a significant decrease (from 865 pre-irradiation to 2.05 post-irradiation) and isoform *smed-eIF4E-D* showed a decrease in the level of expression (from 45 pre-irradiation to 35 post-irradiation) (Table 7).

Table 7: Transcriptome analysis of the different planarian *eIF4E* isoforms

Isoform code	p value	expression pre-irradiation	expression post-irradiation
smedeIF4E ***-A***	*1.08e-3*	*45*	*26.35*
smedeIF4E ***-B***	*3.89e-3*	*865*	*2.05*
smedeIF4E ***-C***	*1.08e-3*	*11.09*	*18.75*
smedeIF4E ***-D***	*0.86*	*45*	*35*
smedeIF4E ***-E***	*No value obtained*	*No value obtained*	*No value obtained*

The results of the irradiation trascriptome analysis revealed two strong neoblast specific candidates: *smed-eIF4E-A and smed-eIF4E-B* and one possible neoblast candidate: *smed-eIF4E-D*. These findings support the notion that more than one isoform functions redundantly to regulate eukaryotic translation initiation in planarian neoblasts.

Chapter 4: Discussion

4.1. *Smed-eIF4E-A*: mRNA Binding Neoblast Specific Gene

The isoform of interest in the beginning of the research (*smed-eIF4E-A*) did indeed correspond to a neoblast specific gene. Our findings consistently showed the elevated expression of *smed-eIF4E-A* in neoblasts and germ line cells, and its expression decrease post-gamma-irradiation which is a trait indicative of neoblast specific genes. We attempted to identify *smed-eIF4E-A*'s functions using RNA interference (RNAi), however, dsRNA injected animals were able to undergo normal regeneration, with the exception of a fraction of the population in which mild anterior and posterior differentiation abnormalities appeared. Upon RNAi knock down of *eIF4E-A*, all animals generated a blastema and continued to regenerate normally after amputation. The small fraction of phenotypic abnormalities that were found, appeared to take place at the differentiation level. The phenotypes regenerated either failed to form photoreceptors or only formed one, similar to what was observed after non-lethal irradiation (Salvetti, Rossi, 2009). Neoblasts were able to undergo mitosis as shown by their ability to form a blastema, however, some phenotypes did not differentiate properly. Moreover, the level of expression directly post-gamma-irradiation, was still detected 7 days post-gamma-irradiation. Neoblasts belonging to this fraction are the differentiated neoblast progeny. Genes functioning at this level are said to be involved in differentiation (Eisenhoffer, Kang, Alvarado, 2008). Control of gene expression via post-transcriptional regulation of mRNAs has been shown to be essential for various aspects of development, cell differentiation, and neural plasticity in other animals (Dever, 2002, Kimble and Crittenden, 2007, Martin et al., 2000). It was therefore hypothesised that *smed4E-A* might be involved in differentiation rather than stem cell maintenance or proliferation. Some phenotypes of other translation initiation factors studied in planarian neoblasts such

as *DjeIF-2a* (Rouhana et al., 2010), did not result in aberration of the regeneration processes but showed abnormalities in their anterior differentiation. The knock down of the *DjeIF-2a* factor resulted in an incomplete formation of the eyes or the formation of only one eye which is similar to what was observed during our RNAi experiments. The phenotypes obtained from these studies and the relation between chromatoid bodies and differentiation suggested the involvement of neoblast specific *eIF4E-A* in the differentiation process of neoblasts. Our RNAi experiments, however, did not result in the complete aberration of differentiation. 24 animals out of a total sample of n=30 underwent full differentiation with complete formation of both eyes and tail. We therefore speculate that the reason differentiation was not completely hindered could be due to the existence of more genes exhibiting a redundant function to that of *eIF4E-A* which is a phenomenon found evident in germ line specific *eIF4E* isoforms in C. *Elegans* (Amiri et al., 2001). Three *eIF4E* isoforms of class II *C. elegans* function redundantly to maintain normal embryogenesis. By knocking down only one, the remaining isoforms are functionally redundant to it and thus compensate for its loss. The involvement of *eIF4E-A* in differentiation is not yet clear and it could be possible that *eIF4E-A* was not completely knocked down by the RNAi experiments. Regardless of these possibilities and given the fact that the animals were subjected to three rounds of RNAi, we find a stronger reasoning in the possibility of having multiple neoblast specific *eIF4E* in planarians. It is this speculation that led us to investigate all possible *eIF4E* isoforms in *Schmidtea mediterranea*.

4.2 *eIF4E-A* and the Chromatoid Bodies in Neoblasts

Chromatoid bodies are found to be essential in the process of neoblast differentiation. They are believed to be mRNA transit granules containing factors and mRNAs

required for neoblast regulation (Shibata et al. 2010). The critical role chromatoid bodies play in differentiation is evident in the gradual disappearance of the chromatoid body and then the complete disappearance in the differentiated neoblast progeny (Coward, 1974, Handberg-Thorsager et al., 2008). Chromatoid bodies show differences in their electron density in terms of size, number, and distribution, depending on the differentiating stage of the neoblast.

When we analysed the state of the chromatoid bodies in our obtained phenotypic animals via immunohistochemistry staining, *eIF4E-A* phenotypic RNAi animals did not show any significant changes in the morphology of their chromatoid bodies. We expected the number and size of the chromatoid bodies to change as a result of affecting the specific translation initiation factor responsible for instigating mRNA translation in neoblast cells, however, this did not take place. The dispensable effect of *eIF4E-A* knockdown on chromatoid bodies also supported the notion that there exist other genes functioning redundantly to *eIF4E-A* in the neoblasts.

4.3 *eIF4E-B* and *eIF4E-D* Candidates for Neoblast Specificity

We expanded the research to look for other neoblast specific *eIF4E* isoforms in planarians, thereby investigating the possibility of *eIF4E* functional redundancy. Several *eIF4E* isoforms in planarians were found by blasting against the planarian genome, but when comparing the results with the ongoing transcriptome local data base, only five isoforms were confirmed. We named these isoforms *eIF4E-A, eIF4E-B, eIF4E-C, eIF4E-D,* and *eIF4E-E*. Whole mount *in situ* hybridization on intact animals revealed different patterns of expression for the *eIF4E* isoforms seen in *eIF4E-B* and *eIF4E-D*. The expression patterns of the planarian *eIF4E* isoforms are still unverified. However, transcriptome analysis using the irradiation data suggested other isoforms

could be expressed in the neoblasts. The expression pattern of isoform *eIF4E-B* decreased drastically (from 865 to 2.05 with p value = 3.89e-3) which was a larger decrease than that seen in the expression pattern of isoform *eIF4E-A,* thus indicating that isoform *eIF4E-B* is a strong potential candidate for neoblast specificity. Isoform *eIF4E-D* did show a slight decrease (from 45 to 35 with p value =0.86) in its level of expression, however, the decrease was relatively low. Since this isoform was found expressed in the nervous system, it may be that it is expressed in the neoblasts as well, but at a low level. Our analysis hitherto strongly suggested *eIF4E-B* as a candidate for neoblast specificity. Furthermore, it suggested the possibility that isoform *eIF4E-D* might also be a redundant candidate with lower neoblast specificity than that of *eIF4E-A* or *eIF4E-B*.

A complete analysis of the planarian *eIF4E* isoforms would be necessary in order to discover the functions of *smed-eIF4E-A*. This analysis would be based on comparing their amino acid primary sequence, their ability to bind (to the cap, eIF4G, and 4E-BPs), in addition to their ability to rescue *eIF4E mutant* yeast strains. Moreover, functional analysis based on multiple knock down of redundant isoforms would be crucial in the investigation as related to the funcitons of *smed-eIF4E-A*.

4.4 Importance of Post-Transcriptional Regulation

Post-transcriptional regulation mechanisms at the level of mRNA localization, mRNA stabilization, and mRNA translation initiation are found to be essential for germ line formation, accurate early development, and the plasticity of regeneration (Dever, 2002, Kimble and Crittenden, 2007, Martin et al., 2000). The striking similarities between planarian stem cells and germ line stem cells (differentiated by the presence of ribonucleoproteins RNP), indicate that totipotency in planarian adult

stem cells might be due to the unique post-transcriptional regulatory mechanisms that are carried out by these RNPs and their associated factors (Rouhana et al., 2010). Polar granules, germ line granules, and mammalian chromatoid bodies present in the germ line of those animals contain mRNA which some studies showed are required for localized processing and future specification (Hernandez et al., 2005). Understanding the regulatory mechanisms of these granules would contribute to the understanding of totipotency and stem cell plasticity (Rouhana et al., 2010). However, the function and regulatory mechanisms of the RNP granules are still poorly understood. Eukaryotic initiation factor 4E has been found to be associated with germ line granules in many organisms (Amiri et al., 2001). Therefore, investigating *eIF4E* isoforms in planarian neoblasts, their functions, and especially their association with the chromatoid body is an area of study with much potential. Not only would this study contribute to the understanding of post-transcriptional regulation in neoblasts, but it would also be useful in discovering new genes involved in regeneration and other stem cell processes by the future targeting of the mRNAs that interact with *eIF4E*.

Chapter 5: Future Work

5.1 Further Classification of *eIF4E* Planarian Members

To be able to achieve our primary goal of investigating the role *eIF4E-A* plays in neoblasts, the identification of the *eIF4E*s performing functions redundant to those of *eIF4E-A* must be achieved. Once neoblast specific isoforms are isolated, multiple RNA interference experiments would facilitate the identification of their functions. Our future work would include whole mount *in situ* hybridization on intact and irradiated worms using newly designed primers in order to optimize the expression pattern identification. We would first investigate the expression pattern in intact animals and then test the neoblast specificity by performing WMISH on irradiated animals. We would again use the three different time points; day 1, day 3, and day 7 to investigate which fraction of neoblast cells the isoforms are functioning in. As a result of the fact that chromatoid body association in most animals is found to be involved in differentiation (along with the belief that these isoforms likely work redundantly), we expect these isoforms to be expressed in differentiating and differentiated neoblast progeny. There is also evidence suggesting that post-transcriptional regulatory factors (which are associated with germ line granules) are linked to differentiation and the development mechanisms common in most metazoans (Hernandez et al., 2005). With this in mind, a similar link could arguably be expected to be found in planarians.

After confirming the expression patterns of all planarian isoforms and identifying expression decreases post irradiation (i.e. those that are potentially expressed in neoblast cells), we will test the functionality of each of the isoforms in *eIF4E*-mutant yeast strains. Yeast is known to have one *eIF4E* isoform, and strains lacking this

gene would not be able to survive. Investigating functionality based on the ability to rescue the yeast strain would help further confirm the functionality of the isoforms in question.

Once the neoblast specific isoforms are confirmed (we expect it to be either isoforms *eIF4E-A* and *eIF4E-B*, or isoforms *eIF4E-A*, *eIF4E-B* and *eIF4E-D*) we would perform single, double, and, if more than two isoforms are involved, multiple RNAi experiments. First, we would test the ability of planarians to regenerate and differentiate by knocking down each isoform individually. Second, we would perform double RNAi on each of the combinations (A and B, A and D, B and D), and finally we would knock down all isoforms expressed in neoblasts (A, B and D simultaneously). The planarians would be amputated and observed after knock down. We would then confirm the knock down of the expression level of the corresponding mRNA by real time PCR. We would also study the effects of the different isoform knock down experiments on the chromatoid bodies of the RNAi animals by performing immunohistochemistry on histological sections. Furthermore, we will also investigate the expression of these genes in germ line cells and will thus perform *in situ* hybridization on histological sections of sexual planarinas to examine the levels of expression in the testes and ovaries.

The findings of the above future work would enable us to achieve a better understanding of *eIF4E* isoforms specific to neoblasts. This is a step that would be essential in understanding planarian regeneration and regeneration in metazoans as a whole. Once these members are classified and better understood, we propose using these mRNA binding factors to investigate their target mRNAs as the next step for future experiments.

Chapter 6: Conclusion

Despite the similarities highlighted in comparative studies between somatic and germ line stem cells, key differences exist in their evolutionary origin, functions, and regulation. Significant functional differences between these two cell types highlight the limitations to the understanding of regenerative biology that would result from investigating the germ line cells (Sanchez Alvarado and Kang, 2005). An example of the functional controversy these two types of stem cells hold is demonstrated in the fact that germ stem cells are restricted to sexual reproduction whereas somatic stem cells are involved in multiple biological processes and functions ranging from asexual reproduction, replacement of lost or damaged tissue, homeostasis, and in some animals, the reproduction of germ stem cells. Some pathways and genes are indeed common between the two; however, the functions of these pathways and genes might differ significantly. Therefore, studying both lineages is important in formulating an objective understanding of animal regeneration processes (Sanchez Alvarado and Kang, 2005).

In recent years, stem cell biology has become one of the most cutting-edge sciences of this century. The lack of accessibility to these cells in other organisms and the difficulty of conducting the research *in vivo* has posed limits to our understanding of the way stem cells work. Newly developed molecular tools such as RNAi and fluorescence-activated cell sorter (FACS) in planarians have enabled this model organism to constitute one of the most important scientific models in understanding stem cell biology (Handberg-Thorsager et al., 2008, Newmark and Sanchez Alvarado, 2002).

A century has passed since planarians were first introduced to the lab as a model organism; however, attention in the field of stem cell biology was focused on other models until just recently. Prior models, such as *C. elegans* and *Drosophila*, were used to study pathways and genes involved in stem cells, but the stem cell population in these models are restricted to the germ line cells. Moreover, none of these models are able to regenerate new body parts thus limiting the ability to investigate adult stem cells, regeneration, and adult stem cell maintenance. The recent development of molecular and analytical tools has brought planarians back into the limelight due to the presence within the organism of a large population of totipotent adult stem cells called neoblasts which are known for their ability to regenerate almost any body part. Not only do these cells allow the animal to rebuild missing body parts, they also allow the animal to replace old or damaged tissue as well as change in size based on food availability and environmental conditions in intact animals (Handberg-Thorsager et al., 2008). Therefore, our model freshwater planarians constituted an ideal model organism in which to study the nature of stem cells *in vivo* (Sanchez Alvarado, 2004). Furthermore, understanding the regenerating mechanisms of this bilateral and almost immortal worm will shed light on the mechanisms of regeneration as they exist in other organisms.

Post-transcriptional regulation is found to be essential in maintaining stem cell totipotency as was recently observed in planarian neoblasts (Rouhana et al., 2010). We therefore attempted to investigate the functions of stem cell specific eukaryotic translation initiation factor 4E found in planarians. A *S. mediterranea eIF4E* named *eIF4E-A* (which was orthologue to a neoblast specific gene in *D. japonica*) was the primary target of our study. This study showed that *smed-eIF4E-A* was found to be expressed at elevated levels in neoblast and germ line cells of *S. mediterranea*, and moreover, that the gene *smed-eIF4E-A* expression decreased post-gamma-

irradiation. Yet despite observations that consistently confirmed neoblast specificity, knock down of *smed-eIF4E-A* did not result in a significant aberration of regeneration. The majority of the RNAi treated animals were able to form a blastema, regenerate, and differentiate completely with only a few resulting in mild phenotypic abnormalities. Furthermore, analysis of the mild phenotypic animals' chromatoid bodies did not reveal any significant changes. Due to these observations, we speculated the presence of other *eIF4E* neoblast isoforms redundant in function to that of the primary gene in question. This phenomenon was discussed earlier as it occurs in *C. elegans* class II germ line specific *eIF4E* isoforms (Amiri et al., 2001). We therefore examined other possible *eIF4E* isoforms specific for neoblasts in planarians. The study hitherto suggested two candidates that could function as neoblast specific genes namely *eIF4E-B* and *eIF4E-D*.

The significance of this research sets a foundational base for understanding the role of *eIF4E* in planarian neoblasts which are the adult stem cells of planarians. Further analysis and functional studies of these *eIF4E* family members would greatly contribute to the understanding of the post-transcriptional regulation mechanism found in almost all metazoans stem cells. It will also help shed light on the control processes of regeneration in terms of discovering new genes that are involved in regeneration via the future targeting of stem cell specific mRNAs. Finally, this research adds to the universal body of knowledge as related to understanding *eIF4E* family members and lastly, may contribute to a better understanding of adult stem cells for future medical applications (Graff et al., 2008).

Chapter 7: References

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Chapter 8: Appendix

Appendix 1: *Smed-eIF4E* Primer Sequences

F primer	
smed-eIF4E-A	ggccgcggTGGTTGATGAAACATCGTTGGGGAAT
smed-eIF4E-B	ggccgcggTGAACCGATCGAGTCTGAAA
smed-eIF4E-C	ggccgcggATGTGGTTCATGTATTATGATAGC
smed-eIF4E-D	ggccgcggGACGATAGCGCAATCAATCA
smed-eIF4E-E	ggccgcggATGTCTGAAGAAAATGAAATTGTAAATTG

R primer	
smed-eIF4E-A	gccccggccGATCAAGGTGGGAAATGCGCTGA
smed-eIF4E-B	gccccggccTCCTTGATACTGTCTGCGTGTT
smed-eIF4E-C	gccccggccTCAATTGCCTATTGAATAAAGAGG
smed-eIF4E-D	gccccggccTCGAATGTAAAATCCGGTGA
smed-eIF4E-E	gccccggccttataattccaataaaggccttcc

UniversalT7_5'_Linker GAGAATTCTAATACGACTCACTATAGGGCCGCGG
UniversalT7_3'_Linker AGGGATCCTAATACGACTCACTATAGGCCCCGGC

Appendix 2: *Smed-eIF4E* Isoforms Alignment

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                        10        20        30        40        50        60        70
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
smed-eIF4E-A   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MVDETSLGNLSNLKLEDEDNEENLKSLAGDSE
smed-eIF4E-B   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MIEPIESEKSLK~~~~
smed-eIF4E-C   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MNEEVILADEEKLHYLN~~~~
smed-eIF4E-D   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MNGYFFNIDDSAINQCLSPYHD~~~
smed-eIF4E-E   MSEENEIVNCHEKENIKFSQVATDESQLNSNCEVPNPLAILPPDSDEVSNSNSIDSKDETAFNYLQ~~~~

                        80        90       100       110       120       130       140
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
smed-eIF4E-A   LHYLQNKWTFYFFKNDRSKDWIDN~MKKVIDFDTVEDFWGLMNHIAPPSKLPSGCDYYVFKDVIQPMWED
smed-eIF4E-B   ~~~~~NSYTYWFSNYDRQNLYDKS~LVEVATFDTLEKFWNIFTSLSYPNELNIHADCLIFKNGIKPLWED
smed-eIF4E-C   ~~~~~DKWCMWFMYYDSNSVWKETNLQQIFEFNTVEEFWGMFYHLKSPMDLKNGRDLSLFKSGIKPVWED
smed-eIF4E-D   ~~~~~RYWRLYIHESNTFGKWSTHLCGSVQNQHQLQKNTPIG~~~SLKNGRNFYCHCSMFVKKYYPDWDF
smed-eIF4E-E   ~~~~~NEWSLWFCKRDSSRDWTES~LHLIEHFNTVEDFWRLIDHITPPFALPTGCDYYLFRRDIKPMWEH

                       150       160       170       180       190       200       210
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
smed-eIF4E-A   DRNKNGGAWLLTFEKNQR~~~~~~~~~~~ISHLDPCWLEILMLLVGETFEDDSD~~~~EICGAVVNIRGK
smed-eIF4E-B   EANINGGKWILRLKKG~~~~~~~~~~~~~~~LSSEIWENLVLSMLTHKFDDENE~~~~~ICGMVVSVRGQ
smed-eIF4E-C   ASNKGGGSWTIHFPRNEN~~~~~~~~~~~RTKLTIIWEEILLFVISDIVDENEEDLNQFICGVAVNIRGK
smed-eIF4E-D   HSFHNGCRWILSLKDQSRKHTFNCKSRNGYFSFEKFWIETGKWLLNMDKYTLTG~~~LGICGMMYTR~~~
smed-eIF4E-E   NTNRCGGSWQVTFKNSTR~~~~~~~~~~~SLLLNRFWENLTMLLVGEGCGSDSE~~~~IVCGGVVNVRKM

                       220       230       240       250       260       270
               ....|....|....|....|....|....|....|....|....|....|....|....|...
smed-eIF4E-A   GDRVSVWTTDCSSAEAIKRIGQKIKDALGLTC~~AIEYTAHTDAQNKSSSRAKCTFRI*~~~~
smed-eIF4E-B   EDILSVWNRSSNNEVVKNKIRESMIRVLQLPYNTVIEYKKHADSIKDSSSFFNTVIPKEIK*
smed-eIF4E-C   VDKISLWIHHQSQKDSILKIGRKLKEWLNYSS~~TLNYYSHDHMKSRTYDS~~~~PLYSIGN*
smed-eIF4E-D   ~NKLSIWMIDQDNFDANIEVGILWRSSLNLLH~~SPDFTFELKALKKPKILTIL*~~~~~~~~
smed-eIF4E-E   TDRLCLWVSNYEDKQQIMRIGRHIKRILEIDF~~PIEFYPHIDVVKNALYSGRGRPLLEL*~~
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