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Characterization of PIWI⁺ stem cells in *Hydractinia*

*A thesis submitted in partial fulfilment of the
requirements of the National University of Ireland,
Galway for the degree of Doctor of Philosophy*

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Biology

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List of Abbreviations

AGO	Argonaute protein
APS	Ammonium Persulphate
ASW	Artificial Sea Water
BCIP	5-bromo-4-chloro-3' -indolyphosphate
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine Serum Albumin
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
FISH	Fluorescent <i>In Situ</i> Hybridization
hpf	hours post fertilization
IF	Immunofluorescence
LB	Lysogeny Broth
miRNA	microRNA
NBT	Nitro Blue Tetrazolium
PAZ	Piwi, Aronaute and Zwillle
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.1% Tween 20
PBSTx	Phosphate Buffered Saline with 0.3% Triton X-100
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PIWI	P-element Induced Wimpy Testis
piRNA	Piwi interacting RNA
pl	Pico Litre
RISC	RNA induced silencing complex
siRNA	small interacting RNA
SSC	Saline Sodium Citrate
TEA	Triethylamine
TEMED	Tetramethylethylenediamine
TSA	Trichostatin A

TNB

Tris-NaCl-blocking Buffer

WISH

Whole mount *In Situ* Hybridization

Abstract

Hydractinia is a colonial marine invertebrate and a member of the phylum Cnidaria. It represents a very powerful tool for studying basic developmental biology and differentiation due to the presence of a population of stem cells (termed i-cells) capable of giving rise to all somatic and germ cell populations. The genes believed to be involved in the regulation of stem cells and their lineages, such as PIWI, are conserved in *Hydractinia*. PIWI proteins are classical stem and germ cell markers in virtually all studied animals. The *Hydractinia* genome encodes two PIWI genes, *Piwi1* and *Piwi2*. Phylogenetic analysis placed them as orthologues of other cnidarian PIWI proteins. I show that the genes are co-expressed, at least in adults, exclusively detectable in i-cells and germ cells, and that both PIWI proteins are cytoplasmic. *Piwi1* is ubiquitous in early embryos but segregates into embryonic i-cells that remain in the endoderm/gastroderm during later embryonic and larval stages. All *Piwi1*⁺ i-cells migrated to the epidermis during metamorphosis and could only be found in the gastroderm in the context of sexual development. Ectopic expression of *Piwi1*, or its mosaic knockout by CRISPR/Cas9 mediated mutagenesis caused no obvious phenotype. However, transgene expression was silenced in post metamorphic life, and cells with mutated *Piwi1* were eliminated in the animals, suggesting that *Piwi1* does have an essential role in i-cells. Using EdU/BrdU pulse-chase experiments I identified a rare, slow-cycling *Piwi1*⁺ i-cell sub-population that was found exclusively in some areas in the stolons, away from the growing tips. By contrast, all *Piwi1*⁺ i-cells in polyps were fast cycling. Stolonal slow-cycling i-cells did not incorporate EdU for at least 12 days. Injuring the colonies resulted in S-phase entry of these cells within 24 hours, suggesting that slow-cycling i-cells represent a reserve stem cell population that is reactivated under stressful situations. PIWI interacting RNAs (piRNAs) have been identified by small RNA IP by *Piwi1* protein immunoprecipitation, but their sequence analysis is pending. I suggest that *Piwi* genes/proteins in *Hydractinia* are reliable i-cell markers, but i-cells constitute at least two sub-populations with distinct cell cycle characteristics. The developmental potential of these i-cell subsets at single cell level remains to be studied.

Declaration

This thesis has not been submitted in whole, or in part, to this or any other University for any other degree and is, except where otherwise stated, the original work of the author.

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Signed: _____

Emma McMahon, September 2017

Chapter 1. Introduction

1.1 Cnidaria

The phylum Cnidaria is a sister group to all bilaterian animals, having separated from the bilaterian lineage more than 600 million years ago (Technau et al., 2005). It consists of thousands of species of animals found exclusively in aquatic environments. The name Cnidaria comes from the Greek word "cnidos," which means stinging nettle, as their distinguishing feature is a specialized set of cells called cnidocytes that they use mainly for capturing prey. Cnidaria is one of four non-bilaterian phyla often referred to as "basal" animals, the other three being Ctenophora (comb jellies), Porifera (sponges) and Placozoa.

The phylum is composed of two major clades, the anthozoans and the medusozoans (Collins, A.G. 2002). The anthozoans can be further subdivided into two subclasses: Octocorallia (soft corals) and Hexacorallia (sea anemones and stony corals) which form monophyletic groups. The medusozoans are sub-divided into four clades: Hydrozoa (e.g. *Hydra*, *Clytia*, *Hydractinia*), Cubozoa (box jellies), Staurozoa (stalked jellyfish) and Scyphozoa (true jellyfish e.g. *Aurelia*) (Frazao et al., 2012) (Figure 1.1 a.).

Cnidarians develop from two germ layers, the ectoderm and endoderm. This is one feature which distinguishes cnidarians from bilaterian animals, which have a third germ layer, the mesoderm (Marlow et al., 2009, Galliot, 2000). This fundamental developmental difference led to these groups being referred to as diploblasts and triploblasts, respectively (Burton, 2008, Christen et al., 1991). Another distinguishing feature of Cnidarians is that they are mostly radially symmetric, having only one body axis (sea anemones being an exception), whereas the majority of Bilateria possess bilateral symmetry, having two body axes, anterior–posterior and dorsal–ventral (echinoderms being the exception) (Goldstein and Freeman, 1997, Hobmayer et al., 2000, Lee et al., 2007, Petersen and Reddien, 2009). Cnidarian oral-aboral axis has classically been thought to correspond to the anterior posterior axis of higher animals (Goldstein and Freeman, 1997).

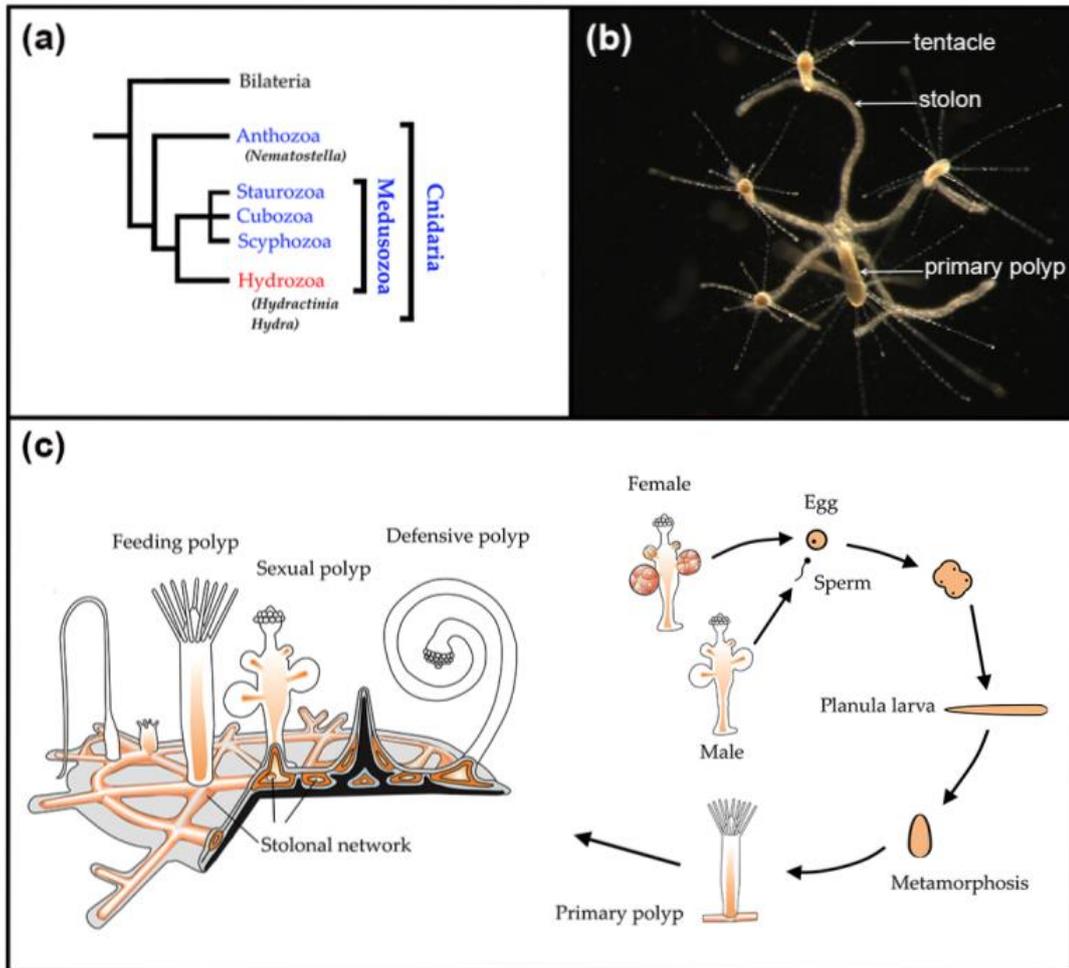


Figure 1.1. Phylogeny, colony structure and life cycle of *Hydractinia* (A) Schematic representation of the phylogenetic relationships of the different cnidarian sub-clades Anthozoa Staurozoa, Cubozoa, Scyphozoa and Hydrozoa. The clade Bilateria is present as an outgroup. (B) A 1 week old *Hydractinia* colony grows through asexual reproduction from the initial primary polyp. Each polyp is connected by a gastrovascular network called a stolon. (C) Illustration of the *Hydractinia* life cycle shows its development from an egg to larva to primary polyp stage until it becomes a mature colony (Gahan et al., 2016).

Considerable variation exists between the life cycles of different Cnidaria clades. All cnidarians are capable of sexual reproduction, while many can also reproduce asexually through colony formation and budding (Collins, 2002, Houliston et al., 2010). Anthozoan cnidarians possess only one adult stage, the polyp, which is also the sexually reproducing one (Technau and Steele, 2011), while a typical medusozoan life cycle is an alternation between a free-living sexual stage called a medusa and a sedentary, asexual polyp (Technau and Steele, 2011). Medusozoan life cycles are most diverse, characterized by multiple, independent losses and gains of the medusa and/or polyp stage over evolutionary time scales (Collins, 2002) (Technau and Steele, 2011, Collins, 2002).

When molecular studies of cnidarians first began, a major goal was to determine whether the genetic toolkit used to construct the bilaterian embryo (represented primarily by the model systems *Drosophila*, *Caenorhabditis elegans*, amphibians, zebrafish and mice) was in place in the common ancestor of cnidarians and bilaterians (Putnam et al., 2007, Schwaiger et al., 2014). A variety of transcription factors, signalling pathway genes and other regulatory elements once thought to be characteristic of bilaterians, and associated with their diverse cell types and more complex morphologies, have now been documented among more ancient clades that lack diverse cell types and complex morphogenesis (Ball et al., 2004, Kortschak et al., 2003). Recent research on cnidarians using molecular methods has revealed that despite their relatively simple anatomies, cnidarians have all major signaling pathways and transcription factor families that are involved in bilaterian development (Putnam et al., 2007, Miller and Ball, 2008). Cnidarians also possess small RNA (miRNA, piRNA, siRNA) mediated gene regulation (Moran et al., 2014) and show an evolutionarily conserved gene regulatory landscape with bilaterians (Schwaiger et al., 2014). The possession of such a complex genetic repertoire coupled with the simple morphology in cnidarians makes this clade a very powerful tool towards understanding animal evolution (Genikhovich and Technau, 2009, Schwaiger et al., 2014).

1.1.1 Cnidarian model organisms

There is growing interest in the use of cnidarians (in particular corals, sea anemones, jellyfish and hydroids) to investigate the evolution of key aspects of animal development, such as the formation of the third germ layer (mesoderm), the nervous system and the generation of bilaterality. The sequencing of the *Nematostella* and *Hydra* genomes, and the establishment of methods for manipulating gene expression, have inspired new research efforts using cnidarians (Technau and Steele, 2011). This section will focus on models that have been utilized in developmental/cell biology. However there is a diverse range of cnidarians available, such as corals and anemones, that are used in the study of environmental science and symbiosis (Hoegh-Guldberg et al., 2007, Mariscal, 1970).

The main models utilized in developmental/cell biology come from the clades Anthozoa and Hydrozoa. Within the Anthozoa clade, many *Acropora* species are studied in order to gain better understanding of the molecular basis of symbiosis and responses to environmental changes (Shinzato et al., 2011). The anthozoan *Nematostella vectensis* has become popular as a developmental model due to its ease of culture, experimental tractability, established molecular techniques and conserved gene content with vertebrates (Genikhovich and Technau, 2009, Technau and Steele, 2011).

The three main models within the *Hydrozoa* clade are *Hydra* and *Hydractinia*. *Clytia hemisphaerica* is another cnidarian model with recent advances made in the field of developmental biology and on the evolution of key features of animal body plans (Houliston et al., 2010). This animal offers many advantages due to its simple morphology, its ease of culturing both sexually and asexually in the laboratory, the access it provides to medusae, and its tractability as an experimental system (Houliston et al., 2010, Technau and Steele, 2011)

Hydra and *Hydractinia* are well established as models for developmental biology, immunity, stem cell and regenerative biology (Frank et al., 2001, Plickert et al.,

2012, Lohmann et al., 1999b). *Hydra* used to be the predominant cnidarian model. However, its predominantly asexual mode of reproduction, and the inaccessibility of its embryos, which develop within an opaque cyst, led developmental biologists to seek additional cnidarian model organisms (Tomczyk et al., 2015).

Along with their key phylogenetic position, conserved gene content and tractability, cnidarians possess unique features that include remarkable regenerative powers and longevity, making them interesting research subjects for studying regeneration and ageing (Bradshaw et al., 2015, Gahan et al., 2016, Plickert et al., 2012, Martínez and Bridge, 2012). Cnidarian stem cells were the first to be studied in any animal by August Weismann (Weismann, 1883). These stem cell populations enable hydrozoans to regenerate all tissue types and they are generally immune to tumorigenesis (Plickert et al., 2012, Domazet-Loso et al., 2014). It is likely that many of the unique biological features of cnidarians depend on chromatin packaging properties and epigenetic regulation. Recent investigations on histones in *Hydractinia* by Török et al has revealed 19 histones, including canonical histones and several histone variants such as a rare replication-dependent H3.3, a female germ cell-specific H2A.X and an unusual set of five H2B variants, four of which are male germ cell-specific. This study also confirmed previous studies that failed to find protamines in hydrozoans (Török et al., 2016).

1.1.2 *Hydractinia* as a model organism

Hydractinia has been a model organism for over a century (Weismann, 1883) and has grown in popularity as a research model in the past few decades (Plickert et al., 2012). Researchers mainly focus on developmental biology, allorecognition and comparative genomics (Künzel et al., 2010, Mokady and Buss, 1996). There are two species of *Hydractinia* mainly used as model organisms in lab research. The European species, *Hydractinia echinata*, has predominated in developmental studies while its North American cousin, *H. symbiolongicarpus*, has been well established as a model of allorecognition (Frank et al., 2001, Karadge et al., 2015, Mokady and Buss, 1996). The use of the two species is a result of the geographical location of the researchers rather than that of any biological difference (Mokady and Buss, 1996). In fact, these two similar species were not established as different until 1989 (Buss and Yund, 1989). The Frank lab previously worked solely on the species *Hydractinia echinata*, but in recent years has also established *H. symbiolongicarpus* in the lab to address developmental biology questions due to its ease of use in the lab in comparison to *H. echinata*. The work described in this thesis is a combination of both species, therefore the animal will be referred to as *Hydractinia*.

Hydractinia is a colony forming marine invertebrate that can be found encrusting the shells of hermit crabs. Adult colonies consist of genetically-identical individuals with two distinct compartments: the stolon and the polyp (Figure 1.1B-C). Each colony consists of four types of specialized polyps: feeding, sexual (male or female) and defensive polyps (Figure 1.1C). The most predominant polyp type is the feeding polyp, also called gastrozoid. The feeding polyp is the basic unit of adult *Hydractinia* colony with a long body column and a head consisting of a mouth and a ring of tentacles. The tentacles are used to capture prey and insert it into the mouth of the feeding polyp. These tentacles are equipped with stinging cells which are used to inject paralyzing venom into the target organism. The adult colony can also be either male or female which would consist of male or female gonozoids or sexual polyps (Figure 1.1C). The gonads are reduced medusae filled with either sperm or oocytes. Hermaphrodite colonies are quite rare and their presence most likely results from chimerism following fusion of allocompatible male and female colonies. There are

also two types of defensive polyps called dactylozooids and tentaculozooids (Figure 1.1C). These polyps have reduced tentacles and usually possess a large number of stinging cells. All of the polyps in a colony are connected by a gastrovascular network of stolons which are attached to the substrate and secrete a protective chitin layer (Müller and Leitz, 2002) .

Hydractinia has a relatively short life cycle that covers many developmental stages (Figure 1.1C) (Millane et al., 2011). The ease of access during embryogenesis and each life stage makes this hydrozoan an attractive experimental model for studying development. Sexual polyps release eggs and sperm on a daily basis and the process of spawning is triggered by light (Frank et al., 2001). Photic stimuli triggers meiosis in maturing oocytes and causes the rupture of gonadal walls. The gametes are released into the surrounding seawater, where fertilization occurs. While *Hydractinia* gastrulation occurs in an apolar fashion, the embryo is strictly polarized which is in striking contrast with other model cnidarians such as *Nematostella*, *Clytia* and *Podocoryna* (Plickert et al., 2012). The fertilized eggs undergo a series of divisions to produce a morula and then a gastrula. Within 48-72 hours the embryo elongates and forms a planula larvae which is capable of metamorphosis (Kraus et al., 2014). In the wild, larvae settle down onto the shells of the hermit crabs and metamorphosis into primary polyps is induced by a bacterial film on the shells. Metamorphosis can be stimulated in the lab by the addition of caesium chloride to the water (Müller & Buchal 1973). Cs^+ ions depolarize GLWamide containing neurons, causing them to release this neurohormone that acts as the internal metamorphosis inducer (Müller & Leitz 2002 Frank et al., 2001). The process of metamorphosis takes about 24 hours and gives rise to a single feeding polyp, which is termed a primary polyp, with a number of stolons. The stolons form the gastrovascular network through which food particles are passed between members of one colony. The stolons grow and give rise to new feeding polyps (Figure 1.1B). This mode of asexual reproduction occurs for a number of months before sexual reproduction commences with the budding of sexual polyps. From this point on the two modes of reproduction occur simultaneously (Frank et al., 2001, Plickert et al., 2012).

Hydractinia, like all other cnidarians, is a diploblastic animal and following gastrulation consists of only two germ layers: ectoderm and endoderm. The layers give rise to the adult epithelial layers called the epidermis and the gastrodermis, respectively (Figure 1.2). Both epithelial layers are separated by a layer called the mesoglea, an extracellular matrix which supports the soft polyp body. Along with epithelial cells, *Hydractinia* also contain gland cells, neural cells (neurons and nematocytes or stinging cells) (Plickert et al., 2012) and stem cells (Millane et al., 2011) (Figure 1.2). Gland cells are secretory cells, which are found in the gastrodermal layer and secrete enzymes or hormones used for food digestion and other biological processes (Figure 1.2). Nematocytes and neurons belong to the neuronal

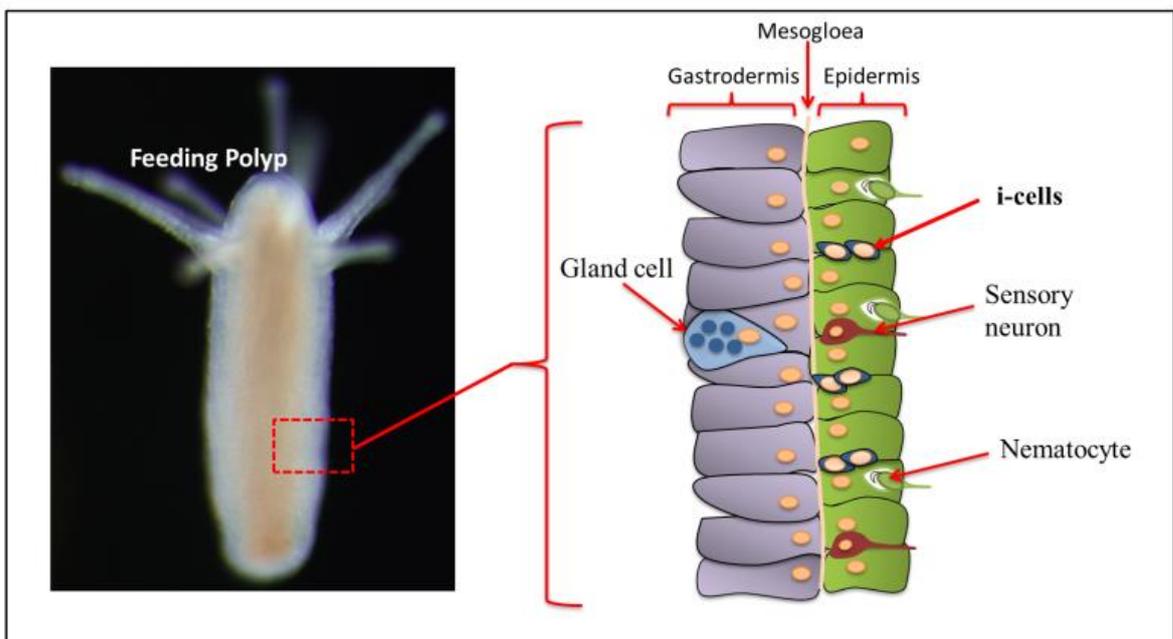


Figure 1.2. Illustration of the diploblastic layers and different cell types in the body wall of an adult *Hydractinia* feeding polyp. The gastrodermis contains cells such as gland cells while the Epidermis contains sensory neurons, Nematocytes and i-cells. The gastrodermis and epidermis layers are separated by an extracellular matrix layer called the mesoglea.

lineage, the progenitors of which are known as nematoblasts and neuroblasts, respectively. The differentiated products of nematoblasts, nematocytes (also called stinging cells or cnidocytes), are a distinct cell type in cnidarians. Nematocytes

contain a post-Golgi capsule, called nematocysts, which can discharge explosively to inject venom into prey and paralyze it or to act as a defense mechanism against predators (Kurz et al., 1991).

Hydractinia contain a population of stem cells that are termed interstitial cells (i-cells) due to their location in the interstitial spaces between epidermal epithelial cells (Figure 1.2). i-cells are relatively small, ~10 μm in size, with a large nucleus and can appear as single or doublets of cells. In *Hydractinia*, i-cells are found in the stolon and in the body column (Figure 1.3). It is believed that hydroids do not sequester a germline during embryonic development but that both germ cells and somatic cells continuously differentiate from i-cells during adult life (Bosch and David, 1987). I-cells have been predominantly studied in the cnidarian *Hydra*. It is already known that they are generally highly proliferative and migratory cells that express genes associated with bilaterian stem and germ cells. I-cells in *Hydra* have also been shown to be multipotent, giving rise to cell types such as gland cells, neural cells and germ cells, but not epithelial cells (Bosch and David, 1987). *Hydractinia* i-cells, however, are thought to be pluripotent and can give rise to all cell types including epithelial cells (Figure 1.4E) (Künzel et al., 2010, Müller et al., 2004).

The lineage potential of *Hydractinia* i-cells has not been studied clonally (Künzel et al., 2010). Only recently are we gaining further insight into the differences between the populations of i-cells present in cnidarians. In *Hydra* it has been shown that there is a population of quiescent i-cells which can be re-activated during regeneration (Govindasamy et al., 2014). It has also been shown that the neoblast population (stem cell) in planarians consists of many sub-populations, each with limited differentiation capacity (Scimone et al., 2014). Since i-cells are pluripotent as a population and also give rise to germ cells, they express typical germline markers such as *Vasa*, *Pumilio* and *Piwi* which can be used as efficient i-cell markers in *Hydractinia* (Figure 1.4A) (Bradshaw et al., 2015). It is not yet clear how the population of i-cells behave in an adult colony, what percentage of these i-cells remain as true pluripotent cells or if there are any differences between the location and expression of i-cell genes in *Hydractinia* (i.e. between feeding polyp i-cells and stolon i-cells) (Figure 1.3).

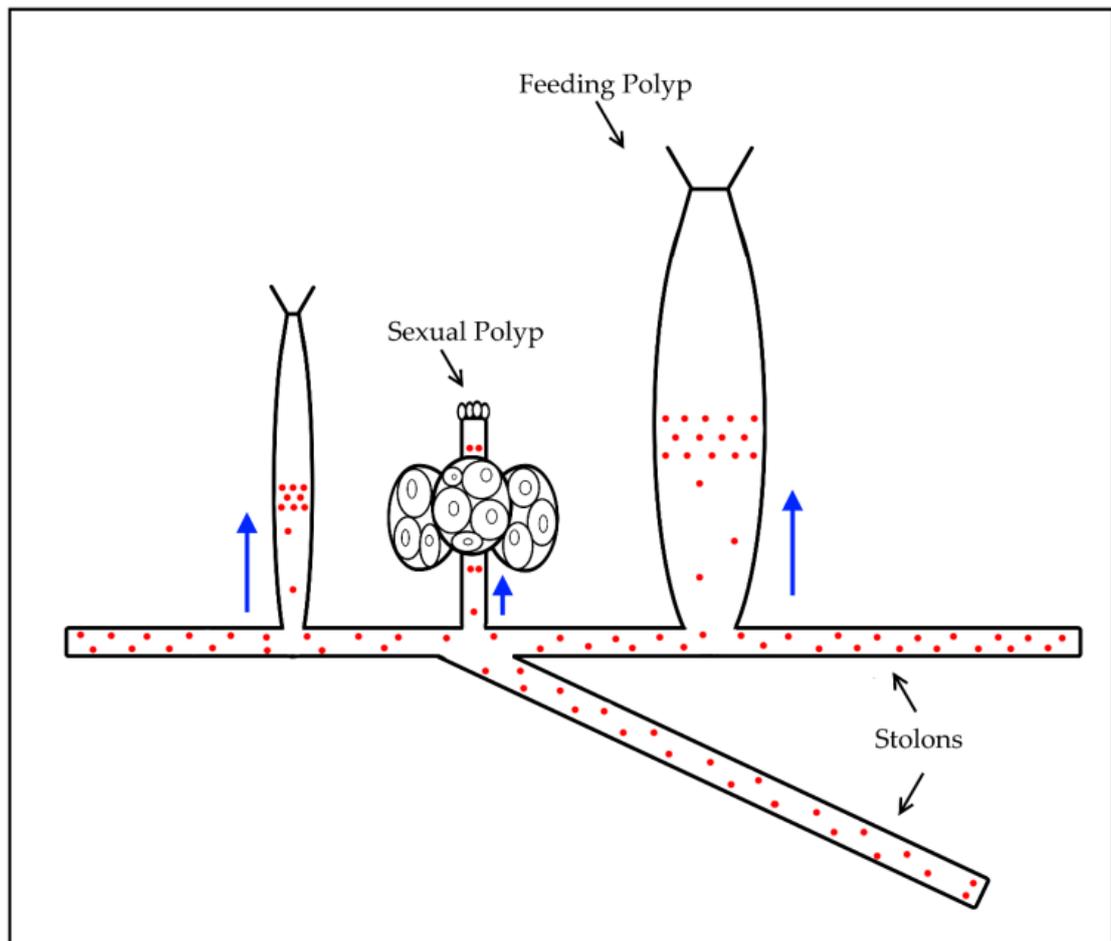


Figure 1.3. Distribution of i-cells in a *Hydractinia* colony. I-cells (red) are found in greatest number in stolons where they migrate into developing feeding and sexual polyps. In feeding polyps they form a 'band' in the aboral region of the polyp (Bradshaw et al., 2015).

Hydractinia is an ideal model to study regeneration due to the presence of a pluripotent stem cell population capable of giving rise to all somatic lineages and germ cells (Gahan et al., 2016){Chin, 2009 #2467}. It has recently been shown to demonstrate both oral and aboral regeneration, i.e. a polyp can regenerate a decapitated head and an isolated polyp can regenerate a colony (Bradshaw et al., 2015). The two processes occur via different mechanisms; decapitation results in the recruitment of i-cells to form a proliferative blastema (Figure 1.4B), whereas the isolated polyp undergoes a transformative process where the entire polyp becomes

stolon tissue before budding polyps, and re-establishing a sexually mature colony (Bradshaw et al., 2015).

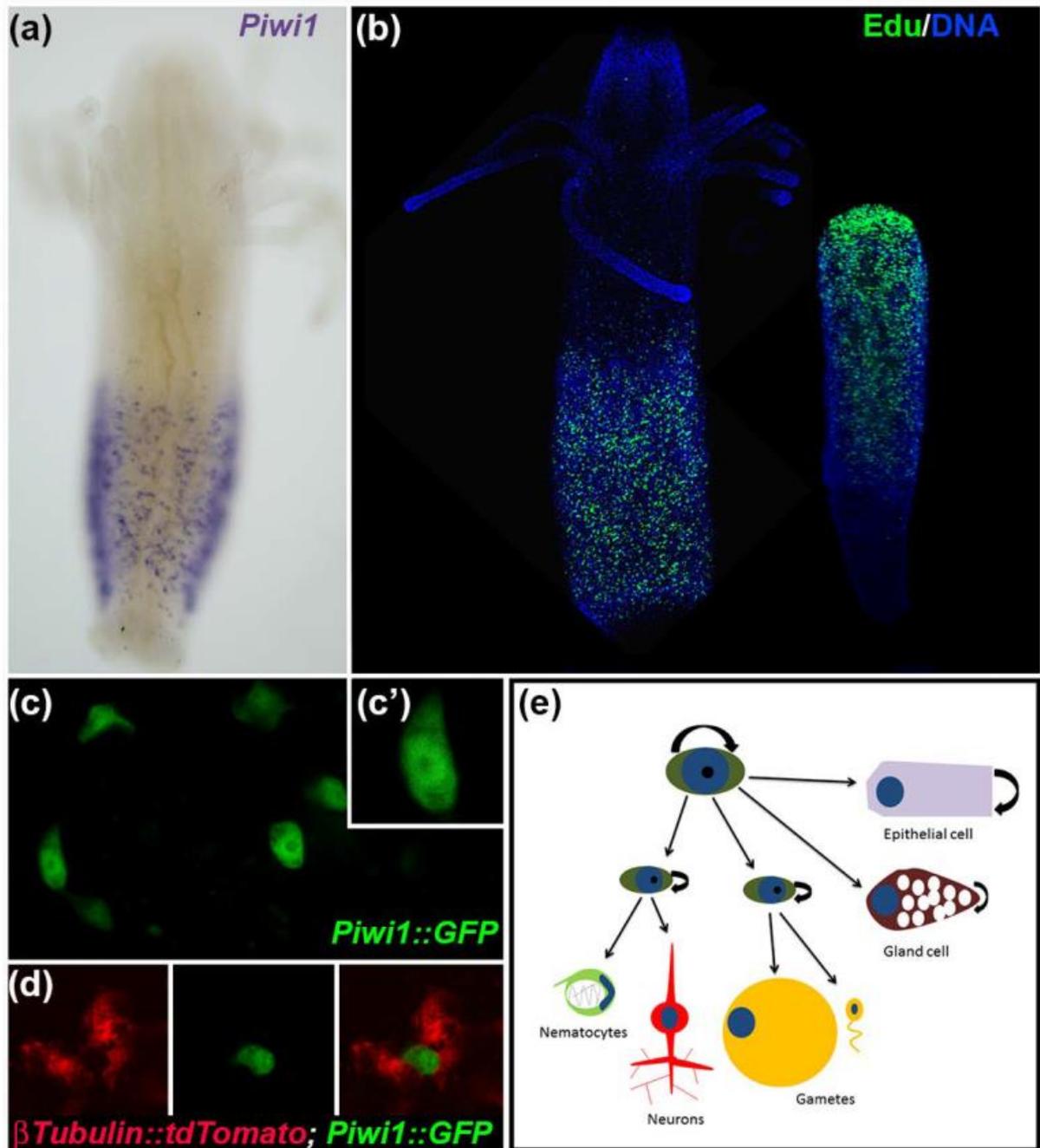


Figure 1.4. i-cells in *Hydractinia*. (A) *Piwi1* in-situ hybridization on an adult polyp. (B) EdU staining on polyps before (left) and 24 hours post decapitation (right). (C, C') GFP⁺ cells in a *Piwi1::GFP* transgenic reporter animal. (D) GFP⁺ i-cell (green) in the interstitial spaces between epithelial cells (red) in a *Piwi1::GFP*,

Btubulin::tdTomato double transgenic stolon. (E) Hypothetical stem cell hierarchy in *Hydractinia* (Gahan et al., 2016).

1.2. Stem cells

Stem cells are defined as undifferentiated cells that have the potential to self-renew and differentiate into at least one specialized cell type. They may serve as an internal repair system, dividing essentially without limit to replenish other cells in living multicellular organisms. When a stem cell divides, each daughter cell has the potential either to remain a stem cell or become another type of cell with a more specialized function. The term “stem cell” was first used by Weismann in 1883 (Weismann, 1883) while working on *Hydractinia* to describe migratory precursors of germ cells. In 1909 the Russian histologist, Alexander Maksimov, suggested the existence of hematopoietic stem cells and their role as common progenitors in the blood (Svendsen and Ebert, 2008). They are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity (Henon, 2003). Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the gut, stem cells regularly divide to repair and replace worn out or damaged tissues in the intestinal lining (van der Flier and Clevers, 2009). In other organs, such as the heart, however, stem cells only divide under special conditions such as ischemic heart failure (Beltrami et al., 2003, Urbanek et al., 2005).

1.2.1 Stem cell potency

The capacity to differentiate into specialized cell types and be able to give rise to mature cell types is referred to as potency. There are five main types of stem cell which have the ability to give rise to other cell types. The first and most potent type of stem cells are known as totipotent stem cells, then pluripotent, multipotent, oligopotent and finally unipotent (Figure 1.5) (Jaenisch and Young, 2008). Totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism. Zygotes and blastomeres of fertilized eggs are the only cells considered to be totipotent cells (Figure 1.5). Totipotent stem cells give rise to somatic stem/progenitor cells and primitive germ-line stem cells (Weissman, 2000), and this totipotency is lost after just a few embryonic divisions. Pluripotent stem cells are the descendants of totipotent cells and can differentiate into nearly all cells, i.e. cells derived from any of the three germ layers (endodermal, ectodermal and mesodermal) and into germ cells (Mitalipov and Wolf, 2009). These pluripotent cells are characterized by self-renewal and a differentiation potential for all cell types of the adult organism, Embryonic Stem Cells come under this category (Pera et al., 2000, Thomson et al., 1998). Human pluripotent stem cells are invaluable for *in vitro* studies of aspects of human embryogenesis. These stem cells have the potential to make any differentiated cell in the body. However, a single cell or a conglomerate of pluripotent cells cannot develop into a fetal or adult animal because they lack the potential to organize into an embryo (Mitalipov and Wolf, 2009). After gastrulation only sequestered germ cells retain their pluripotency and all three germ layers' cells become multipotent. Multipotent stem cells can differentiate to a number of cells, but only those of a closely related family of cells. For example, the bone marrow contains multipotent stem cells that give rise to all the cells of the blood but not to other types of cells. Adult hematopoietic stem cells are multipotent cells that can differentiate into several types of blood cell types like lymphocytes, monocytes, neutrophils, red blood cells etc., but cannot differentiate into, for example, brain cells, bone cells or other non-blood cell types. Multipotent cells are found in many, but not all human tissues. Multipotent cells have been found in adipose tissue (Zuk et al., 2002) cardiac cells (Beltrami et al., 2003) and bone marrow. One example of a multipotent stem cell is the mesenchymal stem cell (MSCs). MSCs are found in

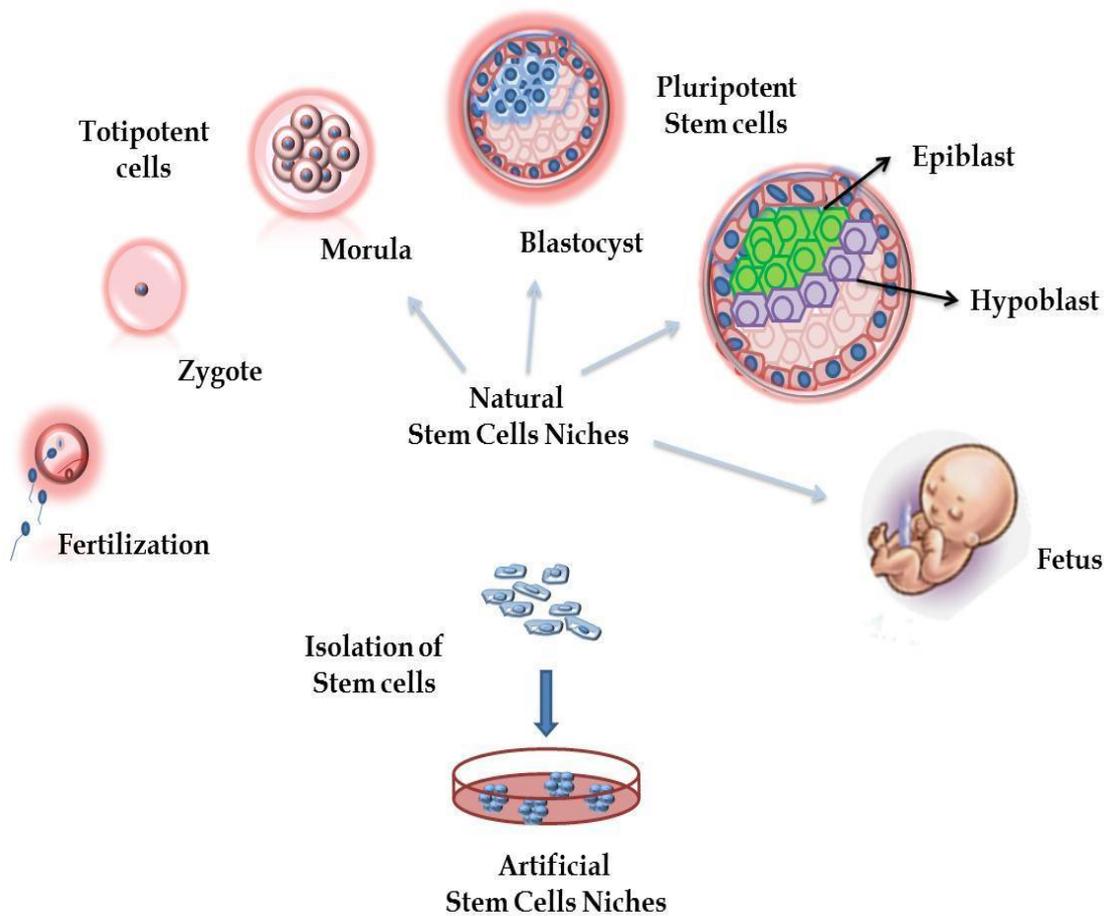


Fig. 1.5. Schematic representation of the changes in differentiation potential along mammalian development. Cells at the morula stage are totipotent and have the highest differentiation potential. As the morula develops and the cells specialize they move from a totipotent state to a pluripotent, multipotent, oligopotent (such as myeloid cells) and unipotent cells (such as liver hepatocytes). Terminally differentiated cells states such as neurons and muscle cells have no differentiation potential (Bruzauskaite et al., 2016).

the bone marrow, adipose tissue and they have been shown to differentiate into osteoblasts, chondrocytes, and adipocytes (Uccelli et al., 2008). Oligopotent stem cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells (Shostak, 2006). The corneal epithelium is a squamous epithelium that is constantly renewing and is oligopotent (Majo et al., 2008). At the bottom of the cell potency

hierarchy are the unipotent stem cells. Unipotent cells are undifferentiated, but can differentiate into only one differentiated cell type and have the property of self-renewal, which distinguishes them from non-stem cells. Such unipotent cells include muscle stem cells and hepatocytes (liver cells). Most epithelial tissues self-renew throughout adult life due to the presence of unipotent progenitor cells (Blanpain et al., 2007). After a unipotent stem cell becomes terminally differentiated, it stops proliferating and generally has a short lifespan (Shostak, 2006).

1.2.2 Maintenance of stemness

Asymmetric division is the main process studied by which stem cells divide to generate the diversity of cell types that populate adult organisms, while also simultaneously self-renewing. Asymmetric division has been found in virtually all developing systems where stem cells need to simultaneously proliferate and generate differentiated cells (Canelles, 2011). When people think of the term stem cells they immediately think of their ability to produce differentiated progeny and their ability to self-renew. It was once mainly believed that stem cells only divided through asymmetric division i.e. cell division leads to one stem cell and one committed cell. However, this state of self-renewal or so called “maintenance of stemness” is not a universal trait of a stem cell, as they have also been shown to carry out symmetrical division (Morrison and Kimble, 2006). Symmetric divisions are where mitosis results in either two stem cells or two mature cells. Symmetric stem cell divisions predominate specifically during growth responses, for example intestinal crypt stem cells exhibit both symmetric and asymmetric stem cell division within this specialized niche in intestinal crypts (van der Flier and Clevers, 2009, Fuchs et al., 2004, Potten and Loeffler, 1990). The microenvironment in which the stem cells are located defines the outcome of division, and the factors present within this microenvironment that define self-renewal is called the stem cell niche. Hence, the number of stem cells are limited by the space in the niche, such that cells that are placed away from the niche differentiate. This niche environment is ideal and essential for maintaining this phenotype, and the factors that maintain this environment are diluted as cells move away (Booth and Potten, 2000). This ideal environment in which a stem cell resides is known as a niche. The ability of stem

cells to carry out maintenance of stemness relies heavily upon the niche in which they reside. Direct cell–cell contact with cellular determinants in the niche has been shown to play an essential role in maintaining stemness (Fuchs et al., 2004). Identification of the molecular interactions between stem cells and their niche has led to an understanding of the mechanisms that control the self-renewal of stem cells. Ultimately, molecular signals triggered by adhesion and junction complexes are responsible for the adoption of specific cell fates (Suda et al., 2005). Asymmetric division is not necessary for stem-cell identity but rather is a tool that stem cells can use to maintain appropriate numbers of progeny and to maintain a healthy population of the stem cells themselves (Morrison and Kimble, 2006).

Studies have shown that in order to maintain a state of pluripotency, stem cells must express a high level of pluripotency genes while suppressing genes which promote cellular differentiation (de Cuevas and Matunis, 2011). The induction and maintenance of pluripotency in mammals has been shown to rely mainly on the expression of the transcription factors *Oct4*, *Sox2*, *c-Myc* and *Klf4* (Takahashi and Yamanaka, 2006). *Oct4* is a major regulator during embryogenesis in mice specifying the inner cell mass while counteracting differentiation (Nichols et al., 1998, Avilion et al., 2003). The expression level of this gene needs to be tightly regulated and act together with the other transcriptional modulators in order to keep stem cell identity (Niwa et al. 2000). The discovery of these essential transcription factors lead to the ability of reprogramming mouse fibroblast cells into induced pluripotent stem cells (iPSC).

1.2.3 Advance in stem cell research

Until recently, scientists primarily worked with two kinds of stem cells from mammals: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells. Embryonic stem cells (ESCs) were first derived, from early mouse embryos, more than 30 years ago, in 1981 (Evans and Kaufman, 1981). The detailed study of the biology of mouse stem cells led to the discovery, in 1998, of a method to derive stem cells from human embryos (human embryonic stem cells (hESC)) and grow them in the laboratory (Thomson et al., 1998). The successful isolation of hESC lines derived from human blastocysts was one of the first major breakthroughs in the field of stem cell biology and enabled the culturing *in vitro* of human embryonic stem cells (Thomson et al., 1998). In 2006, researchers made another breakthrough by identifying conditions that would allow some specialized adult cells to be "reprogrammed" epigenetically to assume a stem cell-like state. This new type of stem cell, called induced pluripotent stem cells (iPSCs) was created by the forced expression of just four transcription factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) already identified as essential for maintaining pluripotency. These four transcription factors were forced expressed using retroviral vectors in mouse embryonic or adult fibroblasts and have become known as the Yamanaka factors (Takahashi and Yamanaka, 2006). hESCs and iPS cells share many characteristics, including the ability become the cells of all organs and tissues (Yu et al., 2007), but they are not identical as it has been shown that iPS cells retain a gene expression signature reflecting their tissue of origin (Chin et al., 2009). iPS cells are a powerful method for creating patient- and disease-specific cell lines for research. These are not adult stem cells, but rather reprogrammed cells with pluripotent capabilities. Using epigenetic reprogramming with protein transcription factors, pluripotent stem cells equivalent to embryonic stem cells have been derived from human adult skin tissue (Toma et al., 2001). Frozen blood samples could now be used as a source of induced pluripotent stem cells (Staerk et al., 2010), opening a new avenue for obtaining the valued cells. The methods available for reprogramming iPSCs are constantly improving with lentiviral/retroviral transfection of cells becoming less popular due to viral integration into the genome of iPS cells leading to issues when brought to therapeutic use (Park et al., 2016). The search for ways to induce pluripotency

without incurring genetic change has thus become the focus of intense research effort. The use of episomal vectors and the Sendai virus vectors have become common practice when reprogramming fibroblasts due to the reduced effects of possible aneuploidy and random integration into the genome (Okita et al., 2008, Nakanishi and Otsu, 2012). These methods however still provide limitations due to the risk of genomic recombination or insertional mutagenesis present with otherwise safe DNA transfection-based methodologies. These transfected cells often require up to two weeks of repeat administration of transient vectors before iPSCs are derived (Fusaki et al., 2009). To overcome these issues recent researchers began to focus on non-integrative approaches for reprogramming cell fate based on administration of modified mRNA encoding the four canonical Yamanaka factors, *KLF4*, *c-MYC*, *OCT4*, and *SOX2*. Once synthesized and transfected into the cytoplasm, cells were able to overcome the innate antiviral response of genome integration (Warren et al., 2010).

The committed stem and progenitor cells isolated from adult species have several advantages as compared with hESC in terms of their possible therapeutic application for tissue regeneration. First, committed stem and progenitor cells such as HSCs can be transplanted autologously without immunologic consequences (Mohamadnejad et al., 2007). As these cells are multipotent, they are already determined or committed to cell types for targeted organs therefore making them a better source to reconstitute target organs *in vivo*. Some controversial ethical issues about isolation and use of hESC exist. Bone marrow stroma provides the microenvironment for hematopoiesis by supporting proliferation and differentiation of hematopoietic stem cells and is also the source of non-hematopoietic, mesenchymal stem cells (MSCs) (Ding et al., 1999). MSCs share certain characteristics of stem cells, including the capability to differentiate in culture into osteoblasts, chondrocytes and adipocytes (Anthony and Link, 2014). Genetic-engineered MSCs have been shown to serve as an effective vehicle for the replacement of genes responsible for deficiencies in circulating proteins, for example, reports of a therapeutic approach for severe osteogenesis imperfecta by transfecting human MSCs with a gene for factor type I collagen (Horwitz et al., 1999). The ability to induce MSC differentiation in specific tissues by using gene transfer techniques reveals the therapeutic possibility of treating a

variety of human diseases (Asahara et al., 2000, Abdallah and Kassem, 2008, García-Castro et al., 2008, Uccelli et al., 2008).

1.2.4 The Germline Multipotency Program (GMP)

The set of genes *vasa*, *nanos* and *piwi*, operate in both multipotent precursors and in the germline. These genes have been found to be expressed in tissues and cells considered to be somatic, such as planarian neoblasts (Guo et al., 2006, Reddien et al., 2005) the mesodermal posterior growth zone (MPGZ) in polychaetes (Giani et al., 2011, Rebscher et al., 2007) the interstitial stem cells (I-cells) of hydrozoan cnidarians (Mochizuki et al., 2001, Mochizuki et al., 2000, Rebscher et al., 2008) and the small micromere lineage in echinoderm embryos (Juliano et al., 2006). Collectively these findings have led to the proposal of a conserved germline multipotency program (Juliano et al., 2010) which operates in the germline and in somatic multipotent cells (Agata et al., 2006). The molecular regulation of the germline is best understood in a handful of animals' such as *C. elegans*, *Drosophila melanogaster* and the mouse.

The same set of genes appears both to specify and maintain PGCs during embryonic germline segregation and to maintain long-term multipotent progenitor cells in animals that segregate their germline after embryogenesis. Extensive functional studies in chordates and ecdysozoans have begun to identify members of this gene set, including *vasa*, *nanos* and *piwi* (Juliano et al., 2010). Loss-of-function studies demonstrate that these genes are required initially to specify the germline and/or to maintain adult GSCs in ecdysozoans and chordates. The molecular functions of these genes are under investigation and identification of their expression patterns and functions outside of the chordates and ecdysozoans demonstrate the persistence of these genes in PGCs, GSCs and multipotent progenitors (Juliano et al., 2010).

Several potential GMP members have distinct functions outside of multipotent and germline cells. For example, *Drosophila piwi* is expressed in the somatic cells of the ovary, where it is required in a non-cell autonomous manner for GSC maintenance (Cox et al., 1998). The jellyfish *Podocoryne carnea* exhibits a low level

of *piwi* expression in all somatic cells, and this expression increases upon transdifferentiation, implicating *piwi* function in reprogramming (Seipel et al., 2003). Human CD34⁺ hematopoietic stem cells are *piwi* positive, and lose *piwi* as they differentiate (Sharma et al., 2001). *Nanos2* also has functions outside of germline cells. For example, it was initially discovered as an early patterning gene in *Drosophila*, and *Nanos1* is expressed in the mouse brain (Haraguchi et al., 2003; Irish et al., 1989). The dramatic loss of many adult tissues when *nanos* is knocked down in the sea urchin and the snail, and the loss of regenerative ability when *piwi* is knocked down in planarians, favors the idea that the GMP is important in maintaining multipotency.

Data available from sea urchins, sponges, cnidarians and several lophotrochozoans, demonstrate genes traditionally classified as 'germline genes' have a broad role in establishing and maintaining multipotency (Juliano et al., 2010). The specification and maintenance of potency in all of the cell types that express genes, such as *vasa*, *nanos* and *piwi*, probably shares a common underlying mechanism of regulatory control. PGCs and multipotent progenitor cells appear to be sister cell types, each realizing their developmental potential differently, but still closely linked by a common regulatory program. A comparison of mechanisms of germline segregation across animal taxa and in animals that use varied developmental strategies will allow us to uncover the crucial and ancient parts of this regulatory program. More work is being carried out on less well-studied organisms, such as the sea urchin, sea star, planarian, *Hydra*, *Hydractinia* and sea anemone, which are amenable to experimental investigation (Chera et al., 2006, Ettensohn et al., 2004) (Fischer and Dorresteijn, 2004, Genikhovich and Technau, 2009, Lohmann et al., 1999a, Rabinowitz et al., 2008, Sanchez Alvarado and Newmark, 1999). Furthermore, *piwi* and *vasa* overexpression is found in somatic and human germline cancers (Hashimoto et al., 2008, Lee et al., 2006, Liu et al., 2006, Qiao et al., 2002). Given these observations, the molecular pathways that are uncovered in sea urchin, lophotrochozoan and cnidarian multipotent cells as a result of such studies may be of broader relevance.

1.3 Argonaute Proteins

The Argonaute protein family (AGO) was first identified in plants, and members are defined by the presence of PAZ (Piwi-Argonaute-Zwille) and PIWI domains (Bohmert et al., 1998). AGO proteins are highly conserved between species and many organisms encode multiple members of the family. The AGO protein family can be divided into the Ago subfamily and the Piwi subfamily. In most organisms investigated, which include *Drosophila*, the zebrafish and the mouse, the expression of Piwi proteins is restricted to the germ line, where they bind Piwi-interacting RNAs (piRNAs). In contrast, Ago proteins are ubiquitously expressed in many organisms and they bind to siRNA and miRNAs (Höck and Meister, 2008).

All AGO proteins share two main structural features: the PAZ domain and the PIWI domain. Studies on isolated PAZ domains from different organisms revealed that this domain contains a specific binding pocket that anchors the characteristic two-nucleotide 3' overhang that results from digestion of RNAs by RNase III (a step in the processing of small RNAs (Lingel et al., 2004, Lingel et al., 2003, Yan et al., 2004). PIWI domains show extensive homology to RNase H (Rivas et al., 2005, Song et al., 2004, Parker et al., 2004). Indeed, biochemical *in vitro* studies of Argonaute proteins from *Arabidopsis thaliana*, *D. melanogaster* and various mammals have shown that some are endonucleases, and these are often referred to as 'slicers'. In humans, only Ago2 has slicer activity, and a catalytic triad consisting of Asp597, Asp669 and His807 has been identified in this protein (Parker et al., 2004, Song et al., 2004).

Structural analysis of the sole AGO protein in the archaeon *Archaeoglobus fulgidus* revealed a third functionally important domain that resides between the PAZ and PIWI domains and is termed the MID domain. This domain binds the characteristic 5' phosphates of small RNAs and thus anchors small RNAs onto the AGO protein (Frank et al., 2010, Parker et al., 2005). Furthermore, the MID domain has been implicated in protein-protein interactions: AGO interactors such as Tas3 in *S. pombe* form a so-called 'Ago hook' that binds the MID domain of Ago proteins

(Till et al., 2007). This domain binds the characteristic 5' phosphates of small RNAs and thus anchors small RNAs onto the Ago protein (Parker et al., 2005)

Members of the Argonaute protein family have been implicated in both transcriptional and post-transcriptional gene silencing. Small RNAs such as short interfering RNAs (siRNAs), microRNAs (miRNAs) or Piwi-interacting RNAs (piRNAs) are anchored into specific binding pockets and guide Argonaute proteins to target mRNA molecules for silencing or destruction. In mammals, these small RNAs guide the RNA-induced silencing complex (RISC) to perfectly complementary target sites in mRNAs, where endonucleolytically active AGO proteins cleave the RNA (Hutvagner and Zamore, 2002, Martinez et al., 2002, Mani and Juliano, 2013){Martinez, 2002 #2551;Hutvagner, 2002 #2552}. A detailed characterization of the biological roles of AGO proteins in conjunction with their associated small RNAs will not only help us to understand gene-silencing mechanisms but might also lead to a better understanding of numerous diseases.

1.3.1 PIWI proteins

The ability of stem cells to renew and produce progeny is critical for the development of a wide variety of tissues in organisms such as insects and mammals. The fruit fly *Drosophila melanogaster* has long been used as a model organism for the study of mechanisms conserved among diverse developmental systems (Jennings, 2011, Singh and Irvine, 2012). Work in *Drosophila* has given an insight into the proteins and small non-coding RNAs involved in maintaining germline stem cells (Brennecke et al., 2007, Stefani and Slack, 2008). One of the most interesting findings is that the asymmetric division of these germline stem cells is controlled by a protein called PIWI (P-element induced wimpy testis), which belongs to the Argonaute protein (AGO) family, and this protein is only expressed in gonadal cells (Lin and Spradling, 1997b). The PIWI family is a subclass of the Argonaute gene/protein family that are well-conserved proteins of approximately ~100 kDa and are defined by three major protein domains: the PAZ, MID and PIWI domains (Hutvagner and Simard, 2008, Okamura et al., 2004) (Figure 1.6). The PAZ domain binds to the 3' end of the non-coding RNA, the MID domain binds to the 5' end of the small non-coding RNA and the PIWI domain contains an RNase H fold responsible for the slicer activity. (Cerutti et al., 2000, Carmell et al., 2002b, Kim, 2006). The Argonaute protein family plays a central role in RNA silencing processes, as essential components of the RNA-induced silencing complex (RISC). RISC is responsible for the gene silencing phenomenon known as RNA interference (RNAi) (Hutvagner and Simard, 2008). Further work on this gene discovered in *Drosophila* has identified it as an essential stem cell gene in other diverse organisms such as *Caenorhabditis elegans*, humans and *Arabidopsis* (Cox et al., 1998). Any reference to all protein and gene non-specific families will be denoted by the capitalized PIWI in this thesis, while any reference to the specific proteins and genes will be reflected by the nomenclature denoted by that species.

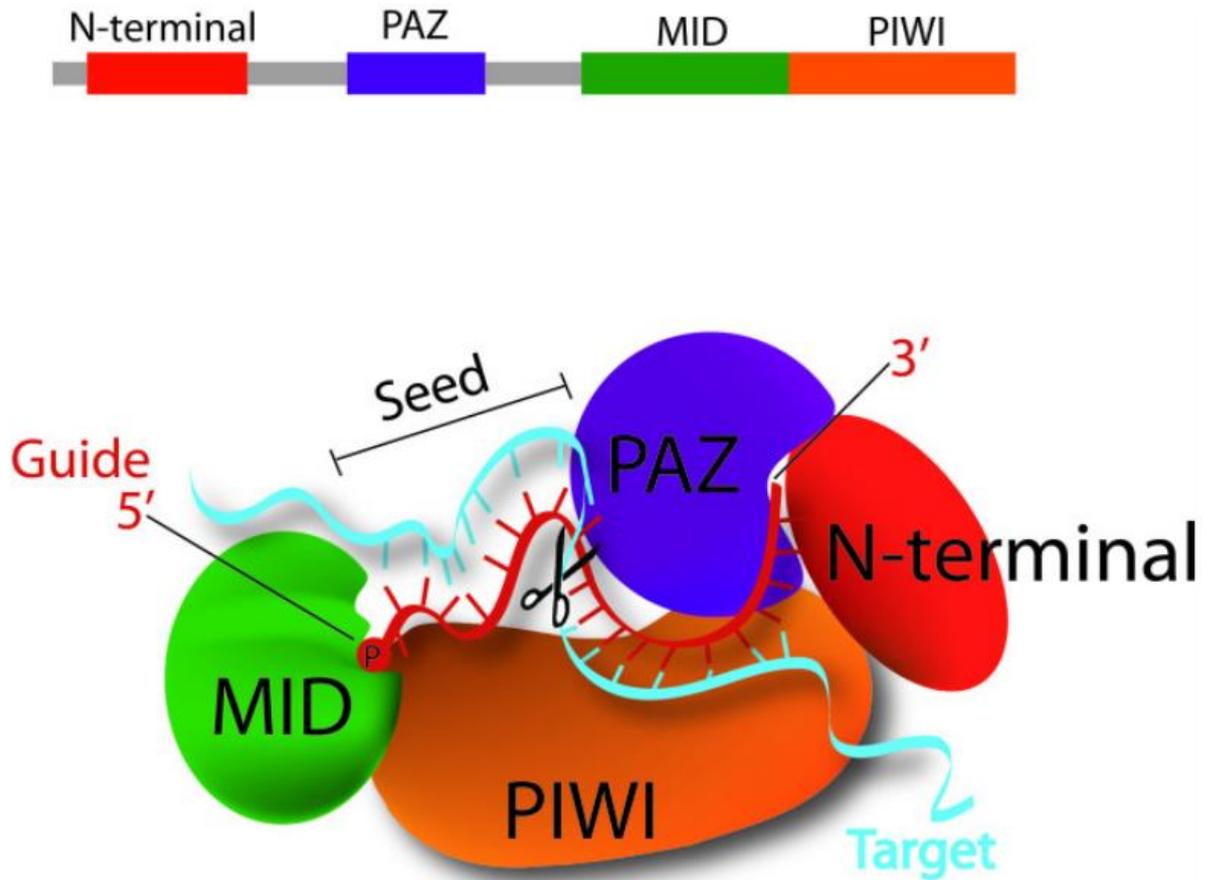


Figure 1.6. Argonaute2 protein domains and RISC complex in *Drosophila*. The terminal 5' monophosphate group of the guide strand tucks in between the MID and PIWI domains of *Drosophila* Argonaute-2 (Ago2). Meanwhile, the PAZ domain has a hydrophobic pocket that specifically recognizes the guide-strand's 3' dinucleotide overhang. This positioning opens up siRNA guide nucleotides 2-8, the "seed region," for base pairing with complementary target mRNA. Base pairing at nucleotides 10-11 correctly orients the scissile phosphate between these two for cleavage by Ago2's PIWI domain, which houses the protein's "slicer" activity (Jinek and Doudna, 2009).

1.3.2 Known PIWI functions

PIWI proteins play important roles in stem cell self-renewal, spermatogenesis, transposon and RNA silencing, translational regulation, and chromatin remodeling in various organisms (Juliano et al., 2011). Homologues of the *Piwi* gene have been identified in mice (Miwi, Mili and Miwi2) *C. elegans* (*prg-1* and *prg-2*), zebrafish (*ziwi* and *zili*), *Drosophila* (Piwi, Aubergine (Aub) and Argonaute 3(Ago3)) and several other organisms (Mani and Juliano, 2013), but not in bacteria or yeast, suggesting that Piwi has a stem cell-related function existing only in multicellular organisms (Lin and Spradling, 1997a). The expression of PIWI proteins is enriched in the germline of many organisms such as *Drosophila*, mice, zebrafish and *Caenorhabditis elegans*. PIWI mutations in mice, *Drosophila*, and zebrafish commonly cause defects in gametogenesis indicating evolutionarily conserved essential roles for PIWI proteins in germline development (Cox et al., 1998, Houwing et al., 2007a, Carmell et al., 2007). Knockdown of both *C. elegans* PIWI proteins (*prg-1* and *prg-2*) leads to decreased germ-cell proliferation (Cox et al., 1998). In zebrafish the knockdown of the PIWI proteins *ziwi* and *zili* gives rise to the loss of germ cells in both males and females (Houwing et al., 2008, Houwing et al., 2007a). *Piwi* expression is also found in hematopoietic stem cells in humans, mesenchymal stem cells in mice, and somatic stem cells in cnidarians and ctenophores (Sharma et al., 2001, Wu et al., 2010, Alié et al., 2011, Seipel et al., 2003, Juliano et al., 2014). Detailed analyses have been largely confined to the function of the PIWI–piRNA pathway in the germline and the gonadal somatic cells in a few model bilaterians, with a focus on transposon silencing (Aravin et al., 2007a, Carmell et al., 2007, Houwing et al., 2007a). PIWI proteins are also involved in embryogenesis and germline specification. In *Drosophila*, PIWI proteins are required early in embryogenesis for patterning and pole cell formation. Both Piwi and Aub are essential for the formation of pole cells in *Drosophila* (Megosh et al., 2006, Harris and Macdonald, 2001). More recently, increasing lines of evidence indicate that PIWI protein and piRNAs function not only in the germline, but also in somatic tissues (Malone et al., 2009).

1.3.3 PIWI-interacting RNAs

PIWI-interacting RNAs (piRNAs) are a class of small RNAs that are 24–31 nucleotides in length. They associate with PIWI proteins to form effector complexes known as piRNA-induced silencing complexes (piRISC), which repress transposons (Tes) via transcriptional or posttranscriptional mechanisms and maintain germline genome integrity (Slotkin, R. K et al., 2007). In addition to having a role in transposon silencing, piRNAs function in the regulation of genes in a number of phylogenetically diverse organisms. Several studies simultaneously reported the identification of Piwi-interacting RNAs from mouse and rat germ cells (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006). These piRNAs have emerged as an extremely complex population of small RNAs that are highly enriched in the germline tissues of the majority of metazoans analysed to date (Juliano et al., 2011). Piwi-piRNA pathways are known to play roles in the fertility of diverse animal species, as evidenced by the fertility defects in mutants lacking Piwi. In *Drosophila* and mice, mutations of piRNA pathway genes results in defects in spermatogenic stem cell maintenance and a reduction of germ cells in the adult stage (Cox et al., 2000, Grivna et al., 2006, Kuramochi-Miyagawa et al., 2004).

TEs are autonomous pieces of DNA that replicate and insert into the genome and thus have the potential to introduce detrimental DNA damage and compromising genomic integrity. The regulation of mobile sequences by piRNAs canonically involves endonucleolytic cleavage ('slicing') of the target sequence after complementary base-pair recognition through the piRISC. In the germline, this process prevents accumulation of changes in the genome of the next generation and represents the most thoroughly understood aspect of piRNA biology. This function may be conserved in mice as loss of Miwi2, a mouse Piwi homolog, leads to germline stem cell and meiotic defects correlated with increased transposon activity (Carmell et al., 2007). Studies in many model systems now point to a similar conserved function (O'Donnell and Boeke, 2007). In the germ cells of *mili* or *miwi2* knockout mice, transposons are dramatically upregulated (Aravin et al., 2007b). In zebrafish, piRNAs are mainly derived from transposon sequences

(Houwing et al., 2007a) and in *Hydra*, piRNA profiling analyses have suggested that transposons are the targets of the piRNA pathway (Juliano et al., 2014, Lim et al., 2014). These findings indicate that the piRNA pathway has a conserved function in silencing transposons across species.

Recent studies on nuclear *Drosophila* Piwi have also suggested that piRNAs are both necessary and sufficient to recruit Piwi and epigenetic factors to specific genomic loci, resulting in the silencing of genes (Pal-Bhadra et al., 2004). The interplay between Piwi, HP1, and H3K9me shown in *Drosophila* has also been demonstrated in *C. elegans* where the Piwi ortholog, PRG-1, can initiate transgenerational gene silencing in the germline by regulating H3K9me, HP1, and histone methyltransferases (Shirayama et al., 2012).

PIWI protein and piRNAs have also been shown to function in somatic tissues. In *Drosophila*, maternal PIWI proteins are essential for the maintenance of chromatin structure and cell cycle progression during early embryogenesis (Mani et al., 2014). In *Hydra*, piRNAs are mainly mapped to non-transposon genes and Hywi is essential for somatic epithelial lineages (Juliano et al., 2014). In planaria SMEDWI-2 (*S. mediterranea* Piwi2) and SMEDWI-3 (*S. mediterranea* Piwi3), are needed for the maintenance of neoblasts (Palakodeti et al., 2008, Reddien et al., 2005).

1.3.4 piRNA biogenesis and function

piRNAs were first discovered through work carried out on the model organism *Drosophila*. In 2001, it was revealed that the testes expressed small RNAs derived from the *Su(Ste)* locus target and silence *Stellate* transcripts, thereby enabling proper spermiogenesis (Aravin et al., 2001). These piRNAs have since been identified in both vertebrates and invertebrates, and although biogenesis and modes of action do vary somewhat between species, the bias for a 5' uridine and a 3' termini of the piRNA that is 2'-O-methylated is common. (Aravin et al., 2007a, Juliano et al., 2011) (Figure 1.6). However the other known small RNAs, siRNAs and miRNAs, are always antisense to their targets. This feature has also been confirmed to exist in zebrafish, *C. elegans*, mice and rats (Juliano et al., 2011). This 3' modification is a 2'-O-methylation has been suggested to increase piRNA stability (Kurth and Mochizuki, 2009, Kawamura et al., 2008, Kawaoka et al., 2011). A significant number of piRNAs identified in zebrafish and *Drosophila* contain adenine at their tenth position, and this has been interpreted as a possible a conserved biosynthetic mechanism across species (Frank et al., 2010, Mohn et al., 2015). In flies, it is shown that PIWI and Aub bind to maternally deposited piRNAs (primary piRNAs). This complex, in turn, binds to the transcripts produced by retrotransposons and cleaves a transcript generating a sense piRNA (secondary piRNAs) that binds to Ago3 (Figure 1.7). The cycle continues as the Ago3-piRNA complex binds to the retrotransposon transcript, generating another set of anti-sense piRNAs and leading to the silencing of retrotransposons within the germline, thus preventing them from inserting elsewhere in the genome. This data ultimately led to the formation of the ping-pong piRNA biogenesis pathway (Brennecke et al., 2007). Ping-pong signatures have been identified in very basal animals such as sponges and cnidarians, pointing to the existence of the ping-pong cycle already in the early branches of metazoans (Burroughs et al., 2014, Kawaoka et al., 2011).

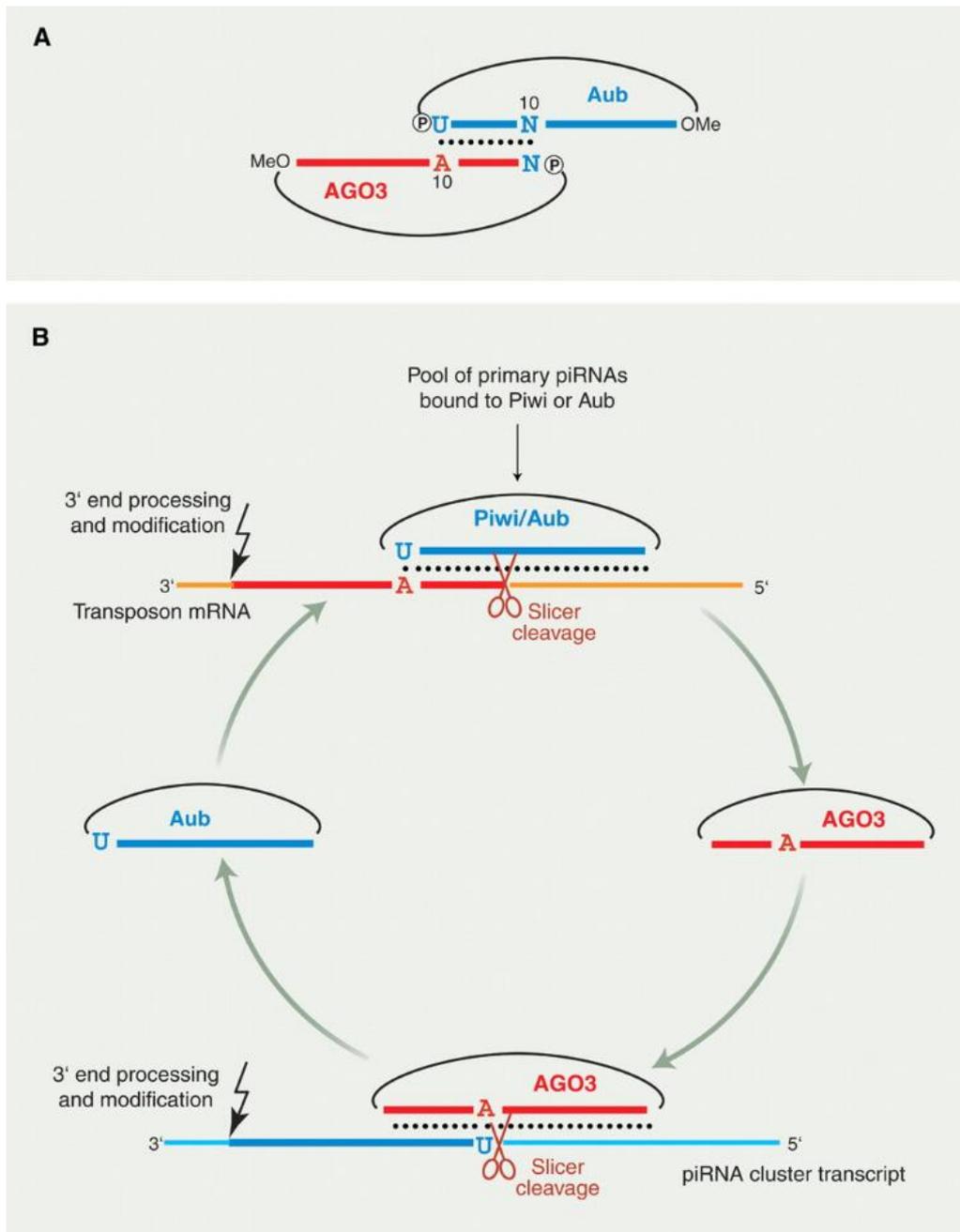


Figure 1.7. Properties and biogenesis of piRNAs. This figure gives a simplistic outline of the biogenesis of piRNAs in *Drosophila*. (A) Features of Aub- and Ago3-associated piRNAs in *Drosophila*. Indicated is the 5' U bias in Aub-bound piRNAs, the 10A bias in Ago3-bound piRNAs, the 5' phosphate, and the 3' O-methylation. (B) Ping-Pong model of piRNA biogenesis in *Drosophila*. Primary piRNAs are generated by an unknown mechanism and/or are maternally deposited. Those with a target are specifically amplified via a Slicer-dependent loop involving Ago3 and Aub (Aravin et al., 2007a).

1.3.5 Ping Pong biogenesis

Two distinct versions of the pathway exist: one genetically encoded that produces primary silencing triggers, capable of detecting and keeping resident mobile elements in check or required for switching off genic transcripts after meiosis (Carmell et al., 2007, Brennecke et al., 2007) and an adaptive one that is used to specifically amplify piRNAs and repress active transposons (Aravin et al., 2008). The latter is now known as the ping-pong cycle and piRNAs produced via this route are known as secondary piRNAs (Figure 1.7). The factors involved in the transcription of piRNA clusters and its regulation remain elusive. The current model proposes that the 5' ends of piRNAs are determined prior to loading onto PIWI proteins. These primary piRNAs have a bias towards a 5' terminal uridine (U), also known as 1U bias, whereas other primary piRNAs feature an adenine at position 10 (10A bias) and no 5' bias (Aravin et al., 2007a, Wang et al., 2014). However, the 3' ends of piRNAs harbor extra bases, which need to be trimmed upon association with PIWI proteins (Kawaoka et al. 2011; Vourekas et al. 2012). The lengths of mature piRNAs are determined during this step, largely depending on the sizes of the PIWI proteins. Thus, piRNAs associated with individual PIWIs show a similar but distinct size distribution (Siomi et al. 2011). The factors responsible for the 3' trimming remain elusive. Upon maturation, the 3' ends of piRNAs are 2'-O-methylated by Hen1/Pimet, which is associated with PIWI proteins (Horwich et al. 2007; Saito et al. 2007).

1.3.6 Non transposon functions of the PIWI-piRNA pathway

In *Drosophila*, maternal PIWI proteins are essential for the maintenance of chromatin structure and cell cycle progression during early embryogenesis—the first phase of somatic development (Mani et al., 2014). Moreover, *Drosophila* Piwi binds to chromosomes in somatic tissues such as salivary glands and is responsible for epigenetic effects at the binding sites (Yin and Lin, 2007). In *Hydra*, piRNAs are mapped to transposons and non-transposon genes, and Hywi is shown to be essential for somatic epithelial lineages (Juliano et al., 2014). In planarians, the PIWI proteins, SMEDWI-2 and SMEDWI-3, are needed for the maintenance of neoblasts, the

pluripotent adult stem cells for tissue regeneration (Palakodeti et al., 2008). A similar mechanism has also been identified in mouse hippocampus, in which piRNAs may target non-transposon genes to control spine shape (Zuo et al., 2016). Mouse Mili and piRNAs are also found to be expressed in adult mouse mesenchymal stem cells (Ku and Lin, 2014). In human, HIWI (human PIWI protein) is expressed in CD34(+) hematopoietic progenitor cells (Sharma et al., 2001). Furthermore, a large number of studies have reported that PIWI proteins are upregulated in cancer cells and tissues, for example, the human *Hili* gene is expressed in prostate, breast, gastrointestinal, ovarian, and endometrial cancers, while the human *Hiwi* is expressed in seminomas, and gastric, uterine cervical, breast, ovarian, and endometrial cancers (Suzuki et al., 2012, Tan et al., 2015). Studies have also confirmed that piRNAs are detected in human cancer cells (Cheng et al., 2011, Huang et al., 2013). These results indicate that cancer development may be linked to PIWI proteins and the piRNA pathway. In *Drosophila*, a study has demonstrated that ectopic expression of piRNA pathway genes contributes to the growth and development of malignant brain tumor growth (Janic et al., 2010). Inactivation of the germline genes *Vasa*, *Piwi*, or *Aub* suppressed malignant tumor growth, demonstrating that germline traits are necessary for tumor growth, at least in *Drosophila* (Wei et al., 1994). Although the functions of PIWI proteins and piRNAs in the somatic tissues remain largely unclear, these findings have pointed out that the PIWI–piRNA pathway exerts a broader-than-expected role beyond the germline.

1.3.7 Piwi-piRNA pathway in *Hydractinia*

Two *Piwi* homologues exist in *Hydractinia* which are called *Piwi1* and *Piwi2*. They are orthologs of the *Podocoryna carnea* gene *Cniwi* (*Piwi1*), and the *Hydra* genes *Hywi* and *Hyli*, respectively, which have already been shown to (Seipel et al., 2004) play important roles in somatic stem cell (Juliano et al., 2014). *Hywi*- and *Hyli*-associated piRNAs also show strong ping-pong signature recorded in many other organisms. *Podocoryna* and *Hydra* are the lowest eukaryotes in which PIWI proteins and piRNAs (only in *Hydra*) have been identified so far. Given that only AGO protein is present in fission yeast *S. pombe* but not baker's yeast *S. cerevisiae*, it is likely that the separation between AGO and PIWI occurred in multicellular

eukaryotes with germ cells (i.e. plants and animals). The function of PIWI proteins in *Hydractinia* is currently unknown, but based on its expression pattern it may have an evolutionarily conserved role in stem cell function and germ cell lineage maintenance.

1.3.8 Current research in Piwi-piRNA

Current understanding of PIWI protein function has primarily been the result of loss-of-function studies carried out in mice, *D. melanogaster*, *C. elegans* and zebrafish. These studies have indicated that the PIWI-piRNA system is involved in germline development, primarily spermatogenesis and maintenance of germline and somatic stem cells (Juliano et al., 2011, Thomson and Lin, 2009). Further studies have indicated that the piRNA-PIWI signaling pathway serves a crucial role in transposon repression, epigenetic regulation and translation control (Juliano et al., 2011). The epigenetic role of PIWI proteins in germ and stem cell regulation has been the subject of study in a number of organisms: In *Drosophila* mutants lacking *PIWI* genes, the inhibition of germline stem cell renewal and depletion of gametes in males and females were observed (Carmell et al., 2002a, Williams and Rubin, 2002). Homozygous *Miwi*, *Mili* and *Miwi2* knockout male mice exhibited arrested spermatogenesis, apoptosis of germ cells and decreased testis size (Carmell et al., 2007, Deng and Lin, 2002, Kuramochi-Miyagawa et al., 2004). PIWI-piRNA complexes are involved in maintaining genomic integrity in germline stem cells and have been demonstrated to be critical for silencing transposon regions in the genome by clustering at these elements and by methylating DNA (Siddiqi and Matushansky, 2012) by nuclear, but not cytoplasmic, PIWI proteins.

PIWI proteins have been widely studied in humans and have received a lot of attention in the field of cancer (Tan et al., 2015, Litwin et al., 2017). The expression of the human PIWI protein PIWIL1 has been described primarily in germ cells and hematopoietic stem cells (Sharma et al., 2001). Several lines of evidence have indicated that the human PIWI proteins PIWIL1 and PIWIL2 are aberrantly expressed in various types of cancer (Qiao et al., 2002, Tan et al., 2015). Preliminary studies also suggest that overexpression and ectopic expression of PIWIL1 is

associated with several types of tumor (Qiao et al., 2002, Taubert et al., 2007). Primarily on the basis of immunohistochemical studies, the increased expression of PIWIL1 has been detected in humans in breast (Cao et al., 2016, Huang et al., 2013, Wang et al., 2013) esophageal (He et al., 2009) pancreatic (Grochola et al., 2008) and gastric (Wang et al., 2012) carcinomas. In the majority of cases, increased levels of PIWIL1 were markedly associated with an advanced histological tumor grade, advanced clinical stage and a poorer clinical outcome for patients. piRNAs have not been extensively studied in cancer; however, a limited number of preliminary studies suggest that piRNAs are altered in cancer. For example, a specific piRNA-651 has been demonstrated to be aberrantly overexpressed in various tumors compared with in wild-type tissues (Cheng et al., 2011).

Our knowledge is constantly and rapidly growing in the field of PIWI-piRNA research in diverse model organisms. Through the use of the basal cnidarian model *Hydractinia*, my project aims to gain further insight into the characterization of the PIWI genes *Piwil* and *Piwi2*, and the cells that express them. This novel investigation will provide important information regarding ancestral features of these genes that evolved before the split between Cnidaria and Bilateria.

1.4 Hypothesis and aims of this project

It is well established that PIWI proteins play important roles in regulating genomic integrity and maintaining germline stem cells via transposon silencing through interaction with specific piRNAs. It is emerging that this regulation can occur in somatic stem cells of diverse organisms (Juliano et al., 2011). Somatic stem cells exist in higher vertebrate models too; however, the role of PIWI proteins is mainly documented in germline stem cells. *Hydractinia* possess a pluripotent stem cell population called i-cells that give rise to all somatic lineages and germ cells. *Piwi1* is already shown to be expressed in i-cells in an adult feeding polyp (Bradshaw et al., 2015, Gahan et al., 2016) (Figure 1.4A). However, the expression profile of this gene in other developmental stages of *Hydractinia* along with *Piwi2* remains largely unknown. How these specific populations of stem cells in different tissues in *Hydractinia* behave, what roles they play in *Hydractinia* development and maintenance, and whether they have specific piRNAs devoted to germ/somatic cell regulation are also open questions. I hypothesize that PIWI is a highly conserved stem cell protein within *Hydractinia* that displays unique stem cell maintenance properties in its somatic stem cells, not seen in other higher organisms.

The specific aims of my PhD are to:

- (1) Study in detail the expression pattern of both *Piwi* genes in all life stages and tissue types of *Hydractinia*.
- (2) Understand the role of PIWI proteins through misexpression techniques.
- (3) Identify the specific PIWI-piRNAs in *Hydractinia*.
- (4) Study the cell cycle characteristics of PIWI⁺ stem cells.

Chapter 2. Materials and methods

2.1. Animal culture and manipulation

2.1.1 Animal culture

Hydractinia echinata colonies, growing on hermit crab shells, were collected from Galway bay or from Roscoff marine station France (<http://www.sb-roscoff.fr/>). Animals were maintained under a 14:10 dark light cycle in artificial sea water (ASW, Red Sea). Spawning of gametes occurs approximately one hour after the onset of light. Animals were fed five days a week, four days on a diet of one day old *Artemia nauplii* and ground oyster on day five. Once animals spawn, the fertilized embryos develop at room temperature. When embryos reach three day old planula larvae, they can be induced to metamorphose. This process occurs naturally on the hermit crab shell due to the presence of a bacterial film but this can be mimicked by incubating the larvae in a 4:1 solution of ASW and 580 mM CsCl for three-four hours. These larvae are then washed in ASW and left on glass slides or petri dish to complete metamorphosis.

For regeneration experiments, colonies were anesthetized in 4% MgCl₂ in ASW. Polyps were cut from shells and subsequently decapitated by cutting directly below the tentacle buds. Animals were kept aside in ASW for experimental analysis.

2.1.2 Microinjection

Fertilized embryos were placed on plankton netting (100 µm in diameter), which was glued to the bottom of a petri dish. 1-2 cell stage embryos were injected with 100-200 pico litres (pl) of plasmid construct (concentration of 3-5 µg/µl in nuclease free H₂O). Needles for injection were prepared from glass capillaries (Narishige CD-1 1x90 mm) and prepared on a pulling machine set as follows: heat 560, pull 70, vel 75, time 150. For CRISPR injection concentration see section 2.5.5.

2.2 Cellular staining

2.2.1 Whole mount RNA *in situ* hybridization (WISH)

Polyps were anesthetized before being cut from the colony (see section 2.1.1) for at least 30 minutes then fixed immediately in 4% PFA in HEPES(100 mM HEPES, 4 mM MgSO₄, 140 mM NaCl) at 4°C overnight (ON) or for 1hour at room temperature (RT). DEPC water and PBS were used when making up all solutions that are used from this point onwards. The 4% PFA in HEPES was removed and the animals were washed three times in 0.1% Tween20 in PBS (136 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) (PBST) for 10 minutes. Animals were be bleached by incubation in increasing concentrations of methanol. This makes them more translucent. 100% MeOH is stored in the fume hood. It is diluted in PBS. Animals are placed in 25% MeOH for five minutes followed by 50% MeOH for five minutes, then 75% MeOH for 5 minutes and finally 100% MeOH. For WISH, 15 minutes in 100% MeOH is sufficient but for Fluorescent in-situ Hybridization (FISH), 1 hour was better. The animals can also be frozen at this stage and thawed at a later stage. The animals were rehydrated slowly by washing in decreasing concentrations of MeOH. First in 75% MeOH for five minutes, then in 50% MeOH for five minutes and in 25% MeOH for five minutes. The animals were washed three times in PBST for five minutes to remove any residual methanol. Following the third wash the animals are again placed in PBST and heat treated at 95°C for 20 minutes. The animals are washed once with 1X Triethylamine (TEA) for five minutes, once in 0.06% acetic acid in 1X TEA for five minutes and once 0.12% acetic acid in 1XTEA for five minutes each. They are then washed in PBST for five minutes. Animals are post fixed by placing in 4% PFA in PBS for 20 minutes. They are then washed three times with 0.3% Triton X-100 in PBS (PBSTx) for five minutes each. The animals are placed in blocking solution of 2 mg/ml yeast tRNA in PBSTx for 10 minutes. An equal volume of hybridization buffer mix (50% deionized formamide, 5x SSC, 0.1 mg/ml yeast tRNA, 0.1 mg/ml Heparin, 0.1% Tween 20) is added to the blocking solution already on the animals and left to incubate for 10 minutes. This solution was then discarded and replaced with 1 ml of hybridisation buffer mix. Pre hybridization was carried out ON rocking at a hybridization temperature of 50°C.

The probes were added to the hybridisation mix at a concentration of 40ng/ml and heated to a temperature of 70°C before adding to the samples. Hybridization was left ON rocking at a temperature of 55°C. The probes are removed and washed once in hybridisation mix for 5 minutes at 55°C. The animals were washed for 1 hour at 55 °C in solution 1 (50% deionized formamide, 2x SSC, 0.1% Triton X-100). Solution 1 was discarded and replaced to wash with solution 2 (2X SSC, 0.1% Triton). Samples were then incubated for 15 minutes at 55°C. Solution 2 was discarded and replaced with solution 3 (0.2X SSC, 0.1% Triton) and incubated at room temperature for 15 minutes. This washed was repeated once (i.e. animals are washed in solution 3 twice). The samples were then washed in PBSTx three times, for five minutes each. The PBSTx was discarded and replaced with 3% (bovine serum albumin) BSA in PBSTx for 1 hour. Samples were incubated in 1:2000 antibody (Anti-DIG AP, Roche Cat. No.11093274910) in 3% BSA/PBSTx ON at 4°C. The antibody solution was removed and the animals were washed three times in PBSTx for 20 minutes each. The samples were removed from 4°C and the solution discarded. They were washed three times for five minutes each in AP buffer (0.1 M NaCl, 0.1 M Tris-HCl pH9.5, 50 mM MgCl₂, 0.1% Tween 20) which was made up fresh every time and sterile filtered. Samples were stained in NBT/BCIP solution (225 µg/ml NBT, 175 µg/ml BCIP in AP buffer) and staining was monitored under a light microscope. Once staining was completed, the samples were washed three times in 10 mM EDTA in PBSTx and mounted in 90% glycerol.

2.2.2 Double fluorescent whole mount RNA *in situ* hybridization

The beginning of this protocol is identical to that of the WISH protocol. Once the third wash in solution 3 is finished and samples are washed in PBSTx, the protocol changes over to suit fluorescent detection.

Incubate in peroxidase quenching buffer (2 H₂O₂%, 1X PBS) buffer for 60 minutes at RT. Incubate the specimen with blocking reagent TNB buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent FP1012) for 60 minutes at RT. Label the cells or tissue with primary antibody in blocking buffer (1:500) diluted in blocking reagent ON at 4°C. Incubate for gene that is more highly expressed first. Make sure to use Anti POD Ab fragments (Roche Cat. No. 11207733910). Only one antibody

can be used at a time and antibodies must be different i.e. DIG and FITC for respective DIG and FITC probes. Incubate for the gene that is more highly expressed first. Rinse the samples three times with PBSTx for five min each. A tyramide working solution was prepared by diluting the tyramide stock solution 1:100 in amplification buffer/0.0015% H₂O₂ just prior to labelling. 1 ul dye to 100 ul of the amplification buffer (Provided with TSA kit, Perkin and Elmer NEL702001KT). Leave to develop in the dye for an hour at RT in the dark. Rinse the cells or tissue three times with PBS triton for 20 min each.

For second antibody (i.e. FITC labelled), quench in H₂O₂ again for 1 hour. block specimen in TNB blocking buffer for an hour. Add second antibody at a concentration of 1/500 in TNB buffer to specimen and leave ON. Wash extensively for the morning eight times for five minutes each. Add a different colour dye to the sample and leave to develop for half an hour. Wash extensively again for 2 hours then leave ON in PBSTx and mount in fluoromount the next day. Note: It is important to include controls when quenching to be sure the first antibody does not continue to stain after adding the second tyramide dye solution.

When performing double fluorescent WISH and IF, after the final was in the first tyramide dye solution, the sample was blocked in 3% BSA, PBSTx for 1 hour at RT. Antibody was then be added ON (concentration of antibodies listed in table 2.1) at 4°C. Normal IF (section 2.3.3) protocol for completion of staining of the second gene was then carried out.

2.2.3 Immunofluorescence (IF)

Animals were anesthetized in a 4% MgCl₂ in ASW solution for at least 30 min prior to fixation in 4% PFA/PBS for 30 mins with rocking. The 4% PFA/PBS solution is discarded and samples are then washed 3 times, 10 minutes each, in PBSTx. Animals were the blocked in 3% BSA/PBSTx solution for 30 minutes at RT before primary antibody (see table 2.1 for dilutions) was added and incubated ON in 3%BSA/PBSTx solution at 4°C. Samples were then washed 3 times for ten minutes each with PBSTx with rocking at RT. Following this blocking in a solution of 3%BSA, 5% goat serum in PBSTx was carried out for 30 minutes at RT. Secondary antibody incubation was

then carried out at RT for 1 hour (see table 2.2 for concentrations) in a solution of 3% BSA, 5% goat serum in PBSTx. Animals were then washed 3 times in PBSTx for 10 minutes each followed by a 20 minute incubation in 10 ng/ μ l of Hoechst 33258 (25 mg/ml, Sigma B2883) to stain nuclear DNA. This was followed by 3 washes in PBSTx for 5 minutes each. Samples were then mounted onto glass slide using fluoromount (Sigma F4680).

2.2.4 EdU staining

EdU staining was performed with Click-iT® EdU Alexa Fluor 594 HCS Assay (Invitrogen, cat no.C10339). Solutions were prepared according to manufacturer's recommendations from the manual and the protocol employed is outlined below. Animals were fixed in 4% PFA/PBS for 30 minutes at RT. Samples were then washed twice in 0.5% PBSTx and left in this solution for 20 minutes to permeabilize samples. Permeabilization solution was removed and samples washed twice for 10 minutes each with 3% BSA, 0.5% PBSTx solution. In the meantime prepare Click-iT® reaction cocktail according to manufacturer's instructions. Remove the BSA solution and add reaction cocktail for 1 hour protected from the light. Remove reaction cocktail and wash 5 times, 5 minutes each in 3% BSA/ 0.5% PBSTx. Perform standard IF or DNA labelling of samples according to protocols.

2.2.5 BrdU staining

Animals were fixed in 4% PFA/PBS for 30 minutes at RT. Samples were washed three times for five minutes each in 0.5% PBSTx. The samples were treated with 2 M HCl in PBS for 30 minutes. These were then washed three times for five minutes in 0.5% PBSTx solution. Incubation of the samples in 3% BSA, 0.5% PBSTx solution was carried out for an hour. The anti-BrdU primary antibody (Sigma: B5002) used at a dilution of 1/500 in 3% BSA was added to samples and left to incubate overnight at 4°C. The following day the samples were washed three times in 0.5% PBSTx for five minutes each. The samples were incubated for one hour in a solution of 0.5% PBSTx/0.3% BSA/5% goat serum. The appropriate secondary was added to the samples in the same solution and left to incubate for one hour at room temperature

concealed from light. These were washed three times in 0.5% PBSTx for five minutes each and DNA staining of nuclei was carried out (See immunofluorescence protocol section 2.2.3) before samples were mounted onto slides for imaging.

2.3 Molecular techniques

2.3.1 RNA extraction and cDNA synthesis

For RNA extraction animals were lysed in 500 µl-1ml of TRizol (ThermoFisher Scientific 155996018) with vortexing or maceration using a pestle. 1/5th the volume of chloroform was then added and the tubes were mixed vigorously. This was centrifuged at full speed for 5 minutes at 4°C and the upper aqueous layer was transferred into a new tube. An equal volume of ethanol was added to and it was processed through the RNeasy Mini Kit (Qiagen 74106) following the manufacturers guidelines. Synthesis of cDNA was carried out on 1 µg of total RNA extract using the Omniscript reverse transcriptase kit (Qiagen 205111). Either random primers (MyBIO C1181) or Oligo (dT) 15 primer (C1101) were used as primers in the reaction, depending on the application of the synthesized cDNA.

2.3.2 Genomic DNA extraction

For general genomic DNA extraction 200-300 adult polyps were used. These were cut and washed once in genomic lysis buffer (100 mM Tris HCl pH8, 1% w/v SDS, 50 mM EDTA). 200 ul of genomic DNA lysis buffer was then added and tissue was macerated using a plastic pestle (cleaned with bleach, ethanol and ddH₂O). A further 800 ul of genomic DNA lysis buffer was added to bring the volume to 1ml and 2ul each of RNaseA (ThermoFisher Scientific ER0531) and RNaseT1 (ThermoFisher Scientific ER0541) was added to the tubes. The tubes were left to incubate for 1 hour at 37°C in a water bath. Following this 8 µl of ProteinaseK (Stock 25 mg/ml) was added to the tube and incubated for 2-3 hours at 50°C or until tissue has been fully digested. Genomic DNA was extracted using phenol/chloroform clean up. Equal volumes of phenol and chloroform were added to the tube and mixed vigorously. This was centrifuged at max speed for 6 minutes at RT. The upper clear aqueous phase was removed and transferred to a new tube. Equal volumes of chloroform was added to the tube and mixed vigorously. This was centrifuged again at max speed for 6 minutes. The upper aqueous phase was removed again and transferred to a new tube. 1/10th the volume of 5 M NaCl was added to the tube along with 2.5 volumes of

100% ethanol. This was centrifuged at max speed for five minutes and supernatant removed. The pellet of genomic DNA was washed with 500 μ l of 70% ethanol and centrifuged again at full speed for five minutes. This washing step was repeated twice more. Ethanol was aspirated and the pellet was left to air dry at RT. It was then re-suspended in nuclease free H₂O and concentration measured on the nanodrop.

2.3.3 PCR and gel clean up

PCR was carried out using either MyTaq polymerase (Bioline BIO-21105) or Phusion high fidelity DNA polymerase (ThermoFisher Scientific F530) according to manufacturer's instructions. MyTaq polymerase was used mainly for standard PCR reactions such as gene amplification and bacterial colony testing whereas Phusion was utilised for all cloning based PCRs. All PCRS were run on 0.5-1% agarose gels at 100V for 25-30 minutes. Gel extraction of the desired bands, or clean-up of PCR product was carried out using the NucleoSpin gel and PCR clean up kit (Macherey-Nagel).

2.3.4 Restriction digest based cloning

For restriction based cloning, the plasmid backbone and template inserts were generated by PCR using primers listed in section 2.8 with overhanging restrictions sites and extracted according to protocol above (2.3.3). Restriction digestion was typically carried out using approximately 100 ng of backbone DNA with either equimolar or three times molar excess of insert in a 20 μ l volume reaction. The reaction mixture contained 1 μ l of per restriction enzyme used and 1 X dilution of appropriate buffer. Digestion was carried out for an hour at 37°C in a water bath and sub sequentially heat inactivated (according to manufacturer's instructions). Ligation was next carried out by adding 2.3 μ l of T4 DNA ligase buffer and 0.7 μ l of T4 DNA ligase enzyme (ThermoFisher Scientific EL0011) to each tube and left to incubate for one hour at room temperature. Ligated products were then transformed into chemically competent E.coli (see section 2.3.7. below) and colony PCRs using MyTaq and sequencing of mini plasmid extracts (see section 2.3.9.) was carried out to test for positive clones.

2.3.5 LB agar and bacteria plates

40 g of LB agar was dissolved in 1 litre of deionized water and autoclaved. LB agar was allowed to cool below 50°C and appropriate antibiotic was added at required concentration. Once mixed the LB agar was poured carefully into petri dishes in a sterile manner. The dishes were left to cool and solidify for five minutes. Once set they were turned upside down, wrapped in parafilm and stored in the fridge for use.

2.3.6 Chemically competent bacteria

Chemically competent XL1 Blue E.coli bacteria were generated by the following protocol. LB agar and LB broth must be autoclaved and unopened before use. The molten agar was opened under a bacteria culture hood and used to make 3 agar plates without any antibiotic added. LB Broth without any antibiotic added was opened under a bacteria culture hood and 200 µl of broth was aliquoted into an Eppendorf. Fresh bacteria stock sample was taken from the -80°C freezer, stabbed with a clean pipette tip and mixed with the 200 µl of broth. This was then spread on an agar plate and left ON at 37°C along with appropriate control plates. The following day, a single colony was selected under the culture fume hood and grown in 5ml of LB broth without any antibiotic added and left to grow on at 37 °C shaking. This was used the following day to inoculate a 300ml culture without any antibiotic. This was left to grow for about 2~3 hours until an OD600 of ~0.4 was reached. The bacteria were then transferred to pre cooled 50ml tubes and centrifuged at 3500 RPM for 10 minutes at 4°C. The supernatant was discarded and pellets were re-suspended in sterile 0.1 M CaCl₂ followed by incubation on ice for 10 minutes. These were centrifuged again at 3500 RPM for 10 minutes at 4°C. The supernatant was discarded again and the pellets were dissolved in 1ml each of sterile 0.1 M CaCl₂/14% glycerol. These were aliquoted into pre cooled 1.5 ml eppendorfs and stored at -80°C.

2.3.7 Bacterial transformation

Transformation of chemically competent XL1 Blue E.coli was carried out by adding either 1µl of plasmid or 20 µl of ligation product to 50 µl of bacteria. This was incubated at 42°C in a water bath for 90 seconds exactly then removed and placed immediately on ice for 5 minutes. 150 µl of LB broth was then added to the tube and left to recover on a rocker at 37°C for half an hour before being plated onto prewarmed LB agar plates containing appropriate antibiotics (100 µg/ml carbenicillin, 50 µg/ml kanamycin).

2.3.8 Gibson assembly

For Gibson assembly both insert and backbone were prepared by PCR and gel clean up as described above (Section 2.3.3). PCR primers were designed to give a 20-30 bp overlap on both 5' and 3' ends between the insert and backbone (Section 2.8). The Gibson assembly reaction was carried out using ~100 ng of backbone and 2 times the molar excess of insert. These were added to 10 µl of 2x mastermix (NEBuilder Hifi DNA assembly mastermix E2621) and nuclease free H₂O to make a final reaction volume of 20µl. In the case of sgRNA cloning (see section 2.7.2.) 10 µl reactions were carried out. The Gibson reaction was the incubated at 50°C for 15 minutes and transformed into bacteria as described above (Section 2.3.7.). Colonies were screened for insert was colony PCR or by sequencing (Eurofins genomic sequencing).

For cloning of the *Piwi1* coding region into the *βtub::GFP::Actin* vector for *Piwi1* ectopic expression, *Piwi1* was amplified using the primers (section 2.8) Betatubulinpiwi1gibsonfwd and Betatubulinpiwi1gibsonrv. These primers contained overhanging base pairs to allow gibbon assembly of the *Piwi1* coding sequence into the vector backbone. The backbone was opened by PCR using primers BetatubulinPromRv and LigDvectorGFPfus+polyA (section 2.8). GFPSeqFusRev primer was used for sequencing to confirm insertion of sequence into the vector. *βtub::Piwi1::GFP::Actin* vector base pair sequence can be found in Supplemental S1.

For unexplained reasons, it was not possible to PCR the entire *Piwi1* coding sequence by standard cloning PCR. Therefore I designed and ordered synthetic gene blocks called gBlocks® Gene Fragments from Integrated DNA Technologies (IDT) (See section 2.9)

2.3.9 Plasmid extraction

For small preparation colonies were grown overnight in 5 ml LB broth containing the appropriate antibiotic at 37°C. The GenElute mini prep kit (Sigma PLN350) was used for plasmid extraction as per manufacturer's instructions. For larger extraction colonies were grown ON in 300 ml of LB broth with appropriate antibiotic added at 37°C. Bacteria were then separated into 50 ml tubes and spun for 5 minute at 6000g. The supernatant was discarded and pellets were re-suspended in 10ml of resuspension buffer (50 mM glucose, 25 mM Tris-HCl pH8, and 10 mM EDTA pH8). 15ml of lysis buffer (0.2 M NaOH, 1% SDS) was added and the solution was mixed by gently inverting the tube. This was left at RT for 5 minutes before 20 ml of neutralization buffer (5 M potassium acetate, 11.5% glacial acetic acid) was added. The tube was incubated on ice for 5 minutes before centrifugation at full speed for 15 minutes at 4°C. The supernatant was filtered into fresh 50 ml tubes, 0.6 volumes of isopropanol was added and tubes were placed in the -20 °C freezer for 20 minutes. Following this the tubes were then centrifuged at full speed for 15 minutes at 4°C. The resulting pellet was washed with 70% ethanol and centrifuged again at full speed for 10 minutes at 4°C. The ethanol was aspirated and pellet was allowed to air dry at RT before being dissolved in 1ml of nuclease free H₂O containing 2 µl each of RNase A (ThermoFisher Scientific ER0531) and RNaseT1(ThermoFisher Scientific ER0541) and incubated at 37 °C for an hour. Subsequently 125 µl of 5 M NaCl, 125 µl 10% SDS and 2 µl Proteinase K (25 mg/ml stock) was added and incubated for a further 2 hours at 55°C. After incubation, the plasmid was extracted using standard phenol/chloroform extraction protocol. One volume of phenol and chloroform were added to the tubes and centrifuged at full speed for 5 minutes at RT. The upper aqueous layer is transferred into a new tube and an equal volume of chloroform is added and shaken vigorously before centrifugation at full speed again for 5 minutes at RT. The upper aqueous phase was removed and 1/5th the volume of 2.5 M KCL

and 2.5 volumes of 100% ethanol was added. The pellet was collected by centrifugation at full speed for 10 minutes at RT, or if the pellet was large enough it was spooled on a 200µl pipette tip, washed several times in 70% ethanol and re-suspended in nuclease free H₂O.

2.3.10 WISH probe synthesis

Primers were designed to amplify the desired fragment of a gene with T7 and SP6 promoter sequences on the forward and reverse primers respectively. The polymerase MyTaq was used in the amplification process of the desired fragment. The fragment was run on a gel to ensure the correct size amplicon was achieved and extracted (see protocol PCR and clean up). This template DNA was used for the synthesis of RNA probes. 20 µl reactions were carried out containing 500 ng-1µg of template DNA, 0.5 µl Ribolock (ThermoFisher Scientific EP0131), 1 µl DIG labelled NTPs (Roche Cat. No. 11277073910), 1 µl T7 RNA polymerase (ThermoFisher Scientific EP0111) or 1 µl SP6 polymerase (ThermoFisher Scientific EP0131) and x µl nuclease free H₂O. The mixture was incubated overnight at 37°C in a water bath. The following day 1 µl RNase free DNase1 (ThermoFisher Scientific EN0521) was added to each and this was incubated for 30 minutes at 37°C. An equal volume of 12M Lithium Chloride was then added and the tubes were incubated for 20 minutes at -20°C. The tubes were then centrifuged at 4°C for 15 minutes at full speed. The pellet was washed once with 70% ethanol and left to air dry at room temperature. The pellet was finally re-suspended in nuclease free H₂O. Probes were stored at a final concentration of 40 ng/µl in 50% hybridisation buffer (see protocol 2.2.1.) in nuclease free H₂O.

2.4 Protein expression and purification for antibody production

2.4.1 Cloning

Primers were designed with a 6X His Tag sequence on the forward primer of the N terminal region of the protein (section 2.8). Cloning of the protein of interest was carried out according to restriction based cloning protocol (Section 2.3.4.) into a pET 3a expression plasmid in E.coli. Plasmid extraction was carried out according to protocol. Plasmid extract was transformed into Rosetta DE3 pLysS bacteria (bacterial transformation protocol) and plated onto LB agar plates with the antibiotic carbenicillin (100 µg/ml) ON.

2.4.2 Protein induction

The next day colonies were picked and added to 300 ml of LB broth with the antibiotics carbenicillin used at final concentration of 100 µg/ml and chloramphenicol at a final concentration 34 µg/ml (stock 34 mg/ml stored in ethanol). This was left to rock at 37°C until for 3-4 hours until bacteria reached on OD of 0.6-0.8. 1ml of media was taken and centrifuged at full speed for 10 min. The supernatant was discarded and pellet kept as control. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the broth at a final concentration of 0.4 mM (1 M stocks kept at -20°C) to induce protein expression. 1 ml aliquots were taken every hour for 4 hours, each aliquot was centrifuged and pellet stored at -20°C for western analysis. Whole 300 ml broth was finally spun down in 50 ml tubes at 6000g for 10 minutes at 4°C. The supernatant was removed and pellets stored at -20°C until western analysis of pellets from induction time points was carried out (western blotting and comassie staining protocol).

To confirm the levels of protein expression to purification, 1 ml of induced aliquots and 1 of uninduced aliquots collected in 1.5 ml micro centrifuge tube were collected and centrifuged at 11,000 g for 1 minute. The supernatant was removed and the cell pellet was lysed by re-suspending in 200 µl of 1X protein gel buffer and heated to 95

°C for 5 minutes. 8 µl of the cell lysate was then run on SDS PAGE and examined by coomassie staining.

2.4.3 Protein extraction

Once an induction of protein of interest was confirmed, bacterial pellets in 50 ml tubes were extracted using fresh pre cooled native extraction buffer (0.1 M Tris HCl pH8, 10% glycerol, 0.5 M NaCl, 1% NP-40, 0.1 mM PMSF, 1 mM DTT). Approximately 30 ml of buffer was added for a ~1 g pellet. The pellet was pipetted up and down several times using a serological pipette. Pellet solution was kept on ice and sonicated.

2.4.4 Sonication

The pellet was sonicated using the following set up; time: 2 minutes, amplitude: 40, pulse on: 30 seconds, pulse off: 30 seconds, repeated 4 times until. Time of sonication can be increased or decreased to achieve lump free solution.

2.4.5 Soluble versus insoluble protein expression test

At this point it was critical to remove a 1 ml aliquot of sonicated pellet/buffer solution to test if the protein of interest is soluble or insoluble. Centrifuge the aliquot at full speed for ten minutes at RT. Transfer the supernatant to a new 1.5 ml eppendorf and keep. The pellet was resuspended in 1 ml of native extraction buffer. An aliquot from each tube containing supernatant and pellet (~ 20 µl) was boiled in 2X SDS loading buffer and standard western blotting protocol was carried out. The gel was then stained using the coomassie staining protocol. The remainder of the sonicated material is centrifuged at full speed for 10 minutes at 4°C. The supernatant was transferred to new 50ml tube and stored at 4°C while the pellet was stored at -20°C until protein solubility of protein was identified. Protein in the supernatant indicates a soluble protein whereas protein in the pellet indicates an insoluble protein.

2.4.6 Soluble protein purification

If the protein of interest was soluble, the supernatant was then incubated in a slurry of Ni-NTA Agarose beads (Qiagen Cat No./ID: 30210) with 1 ml of slurry added per 50 ml of supernatant protein solution. This was incubated on a roller at 4°C for 1 hour before being passed through poly prep chromatography columns (BIO-RAD Cat# 731-1550). Beads were washed in 50ml of buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 200 µM Imidazole) and eluted in 10ml (10 individual 1.5ml eppendorfs were collected of flow through) of elution buffer (50 mM Tris-HCl pH7.5, 50 mM NaCl, 500 mM Imidazole). An aliquot of supernatant (native buffer), buffer and elution buffer (1-5 aliquots) flow through was analysed by western and stained using the comassie staining protocol to ensure purified protein of correct size was eluted in final elution buffer.

2.4.7 Insoluble protein purification

If the protein of interest was insoluble, the supernatant can be discarded and pellet only was kept at -20°C until ready for extraction. The pellet was thawed in an air incubator at 37°C until completely thawed (~10-15 min). 30 ml of buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM Na-EDTA, pH 8.0, 1 mM Benzamidine (added fresh) and 5 mM Beta-mercaptoethanol (added fresh)) was chilled on ice before use and added to the pellet to solubilize the proteins. The solution was chilled on ice before use. The pellet (kept on ice) was fully suspended by pipetting in the buffer before proceeding to sonication. The sonicator was set up with the following settings: –Time: Two minutes

Amplitude: 40 seconds

Pulse on: 30 seconds

Pulse off: 30 seconds

This was repeated for a total of 4 times until the pellet was fully dissolved. Samples were kept on ice during sonication to prevent overheating of the protein. The suspension was centrifuged immediately in a precooled 4°C centrifuge for 15 minutes at 11,000g. The supernatant was discarded and the pellet was re-suspended in the wash buffer again. 5ml pipette tips were used to help break up the pellet into

small pieces. The pellet was centrifuged again and the supernatant was discarded. The wash step was repeated twice more with TW buffer (wash buffer with 1% (v/v) Triton X-100) added and left to centrifuge at 4°C for 30 minutes each at 11,000g. The pellet was washed twice more in wash buffer and centrifuged at 4°C centrifuge for 15 minutes at 11,000g.

The supernatant was discarded and the pellet was then fully resuspended in 0.5ml of DMSO at room temperature for 30 minutes on a roller. The insoluble protein was further solubilized by the addition of 5 ml of unfolding buffer (7 M Guanidium-HCl, 20 mM Tris-HCl, pH7.5, 10 mM DTT (freshly added)) and incubated on a roller for one hour at room temperature. The samples were then centrifuged at 4°C for 20 minutes at 11,000 g. The supernatant containing solubilized protein was collected and further purified by cation exchange chromatography with Ni-NTA agarose beads (see below).

2.4.8 Antibody Production

Final purified protein extractions were shipped to Eurogentec in Belgium for immunization into host animals. The pre immune serum and final antigen aliquots were received from the company for use in all future experiments.

2.4.9 Protein extraction from *Hydractinia* tissue

Animals must be starved for 2 days prior to protein extraction. To ensure a good yield of protein ~5 mg of tissue per 300 µl of RIPA buffer (150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH8) was used for protein extraction. Complete EDTA-free Protease inhibitor cocktail tablets (Roche, Cat. No. 04693132001) are added fresh to the RIPA buffer before use at final concentration of 1x. Animal tissue was homogenized on ice for several by use of a pestle until all tissue was lysed. Samples were then centrifuged at full speed for 20min at 4 degrees. Tubes were removed from centrifuge and placed on ice. Supernatant was aspirated and placed into pre chilled tube while the pellet is discarded. Protein samples were

measured using the Pierce BCA Protein Assay Kit according to manufacturer's instructions. Protein samples were subsequently stored at -80°C.

2.5 Protein detection

2.5.1 Western blotting

Running

Protein was extracted as outlined in protocol above (Protein extraction). Samples were boiled at 100°C in 2x SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue, 20% glycerol) with 200 mM 2-mercaptoethanol added fresh before use. Samples were centrifuged at full speed for 10 minutes at RT. Gels were prepared as listed below for the separating and stacking layers.

For a 10ml 15% separating gel

30% Acrylamide/Bisacrylamide (37.5:1)	3.75 ml
2x separating buffer (0.5 M Tris-HCl pH 8.8, 10% SDS)	5 ml
H ₂ O	1.25 ml
10% Ammonium Persulfate (APS)	0.065 ml
TEMED	0.007 ml

For a 10ml 5% stacking gel

30% Acrylamide/Bisacrylamide (37.5:1)	1.7 ml
4x stacking buffer (0.5 M Tris-HCl pH 6.8, 10% SDS)	1.35 ml
H ₂ O	6.8 ml
10% Ammonium Persulfate (APS)	0.100 ml
TEMED	0.010 ml

5X Sample buffer (loading buffer)

SDS	10% w/v
Dithiothreitol, or beta-mercapto-ethanol	10 mM
Glycerol	20 % (v/v)
Tris-HCl, pH 6.8	0.2 M
Bromophenolblue	0.05% (w/v)

Gels were set for 30 min between 2 glass slides then inserted into the electrophoresis system tank. Running buffer 1X (192 mM Glycine, 25 mM Tris base, 0.1% SDS) in deionized water was added to the system. Protein samples were added to the set gels along with a protein ladder marker (Page ruler Prestained protein ladder ThermoFisher Scientific, Cat. No. 26619). Gels were run at 100 V for ~ 2 hours or until protein ladder dye reached the bottom of the gel.

Transfer

Gels were removed from glass slides and soaked briefly in 1X transfer buffer (192mM glycine, 25mM Tris Base) in deionized water that was pre cooled for at least 2 hours at 4°C. Gels were sandwiched between pre-soaked sponges and filter paper on both sides. The transfer membrane was carefully placed directly on top of the gel in the negative to positive direction to allow the protein to move off the gel and transfer to the membrane. All these components were locked into a casket and placed into the tank system submerged in 1X transfer buffer. The unit was run at 100 V for ~2 hours. The casket was taken apart and membrane removed from the sandwich.

Antibody staining

The membrane was washed once in PBST (PBS buffer, 0.1% Tween 20). The membrane was then left to block for 1 hour in a suitable blocking buffer (3% BSA or 3% semi-skimmed milk in PBST). Antibody was then incubated with membrane overnight (ON) at 4°C at desired concentration (Piwi1 at 1:5000, Piwi2 at 1:500). The following day the membrane was washed three times for five minutes each in PBST. Secondary HRP antibody was added in suitable blocking buffer at 1:10000 dilution for one hour at RT. Again the membranes were washed 3-5 times

for five minutes each in PBST. A 1:1 dilution of Pierce™ ECL Western Blotting Substrate solution (Cat. No. 32106) was added directly to membrane and incubated protected from light for one minute. The membrane was then immediately imaged using a cooled CCD Camera.

2.5.2 Comassie staining

Protein samples were prepared and run as described in western blotting running protocol (Section 2.5.1.). Once finished the gel was boiled with comassie stain (0.1% Comassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) in a microwave for 30 seconds to one minute (until bubbling) and left to rock for five minutes at room temperature. The comassie stain was poured off and de-staining solution (40% methanol and 10% glacial acetic acid) was added to the gel. This was boiled as before until bubbles appeared and left to rock for 5-10 minutes at room temperature. De-staining solution was changed every 20 minutes until protein bands were visible.

2.5.3 Immunoprecipitation

Extract protein from ~500 mg of polyp tissue in IP buffer, (20 mM Tris HCl pH8, 137 mM NaCl, 10% glycerol, 1% Nonident P-40 (NP-40), 2 mM EDTA) with protease inhibitor cocktail added fresh before use, using homogenization (as described in protein extraction protocol 2.4.9) to get at least 1 mg/ml protein. Carry out protein extraction on ice. Centrifuge at full speed for 10 minutes at 4°C. Aspirate the supernatant and transfer to new tube while discarding the pellet. Place supernatant immediately on ice and measure concentration of protein. Wash protein beads (Agarose protein A beads Roche-PROTAA-RO used in all IP protocols) 3 times in fresh IP buffer at 2000g for 2 minutes. Discard last wash solution and add 50-100 µl of protein beads to freshly extracted protein sample. Incubate beads and protein for 1 hour at 4°C rocking to allow pre clearing of beads. Centrifuge protein and bead solution for 2 minutes at 2000g. Aspirate off pre cleared protein supernatant and discard beads. Protein was then separated (~500 µl each) into two tubes, and an extra ~20 µl frozen immediately at -80°C and kept for input control for

RNA IP protocol. Tubes were incubated ON at 4°C with one tube containing 2 µg of Piwi1 antibody and the control tube containing 2 µg of IgG control. The following day (day 2) 50-100 µl of protein beads (washed 3 times in fresh IP buffer and centrifuged at 2000g for 1 min) was added to each tube and left to incubate for 2 hours at 4°C. Tubes were centrifuged again at 2000g for 1 min at 4°C. Supernatant was discarded and beads were washed 3 more times in fresh IP buffer with 5 minutes rocking followed by centrifugation at 2000g for 1 minute at 4°C. Supernatant was discarded and beads (along with input control) were boiled at 100°C for 10 min in 2X SDS loading buffer, followed by centrifugation at full speed for 10 minutes at RT. Samples were loaded directly onto western for analysis. See western blotting protocol above for details (section 2.5.1.).

2.5.4 RNA immunoprecipitation

When carrying out RNA IP protocol, protein from tissue samples must be extracted using fresh RNA free RIP buffer (150 mM KCL, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP-40) with 100 U/ml of RNase inhibitor SUPERase•in™ (ThermoFisher Scientific, Cat. No AM2694) and protease inhibitor cocktails added fresh each time. RIP buffer is prepared with DEPC treated water and filter tips used at all times. Proceed with normal IP protocol up until last wash of protein beads from day two. Discard the supernatant and add 0.5-1ml of TRizol to beads directly. Leave samples at RT for five minutes. Next add 125-250µl of chloroform and shake the tubes vigorously for about 10 seconds. Leave the tubes at RT for five minutes. Centrifuge the tubes at full speed for five minutes at 4°C. At this point there will be three layers in each tube. Aspirate and transfer the top aqueous layer to a new tube while discarding the other two layers. Proceed directly to RNA clean up by adding 1 volume of 1 isopropanol to the aqueous phase and mix gently. Leave tubes at room temperature for five minutes. Centrifuge the tubes at full speed for 20 min, or if a low yield is expected, centrifuge them for 30 min at 4°C. Place samples on ice. There should be a pellet barely visible at the base of each tube. Pour off the isopropanol and add 1 ml 75% ethanol in DEPC treated H₂O. Mix gently and re-centrifuge at full speed for 5 min at 4°C Pour off the ethanol and let the pellets air-dry. This is a critical step; if the pellets dry out too much, the RNA crystallizes and is very difficult

to resolubilize. If not enough of the ethanol evaporates, this also prevents the RNA from going into solution. To quicken the evaporation, centrifuge the tubes briefly to force remaining fluid on the side of the tube to the bottom, then pipette off as much of the ethanol as is feasible. The best time to add DEPC treated water to the RNA pellet is when there is only a tiny meniscus of solution left around the pellet itself. Approximately 10–20 μ l (depending on yield) of DEPC treated water was added to the RNA pellet.). RNA concentration was measure on the Qubit 2.0 fluorometer.

2.5.5 Denaturing polyacrylamide gel

This protocol describes the separation of small RNAs from total RNA on a polyacrylamide gel. It is based on the typical 7M urea 15% polyacrylamide gel electrophoresis ('denaturing' PAGE) but includes a highly denaturing formaldehyde based incubation step prior to RNA loading.

15% denaturing acrylamide gel [1X TBE, 7 M urea, 15% acrylamide (19:1 acryl:bis-acryl)].

for 15ml	Component
6.3 g	Urea (high quality, e.g. Ambion cat #9900)
1.5 ml	10X TBE
5.6 ml	40% Acrylamide (19 acryl:1 bis-acryl, e.g. Ambion cat #9022)
to 15 ml	distilled deionized water

Stir at room temperature until the urea is completely dissolved, then add:

75 μ l	10% ammonium persulfate
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15 μ l	TEMED
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Mix briefly after adding the last two ingredients, which will catalyze polymerization, then pour the gel into a prepared mold immediately.

10X TBE: Tris-Borate-EDTA Buffer; Ambion Cat #9862–9864

Pre-run the gel it for 30 min at 100 V. Mix 5–50 μ g of total RNA with an equal volume of RNA Loading Buffer II and heat for 5 min at 95°C. Heat micro RNA ladder (NEB N2102S) for 5 min at 95°C. Load the gel and run at 50 V until the leading dye travels about 4–5 cm down the gel. Increase the voltage to 150 V and run until dye travels to the bottom of the gel. Stain gel with Syberold 1 in 20000 dilution in 1X TBE for 10 minutes. Image using a UV light source.

2.6 Microscopy and cell counting

Confocal microscopy was carried out using the Olympus fluoview 1000 with an inverted 1X71 microscope.

Cell counting was carried out using ImageJ software. For double positive $\text{EdU}^+/\text{Piwi1}^+$ cells and $\text{EdU}^-/\text{Piwi1}^+$ cells, confocal z-stacks were counted manually without projecting in adult feeding polyps. Cell counting in stolons of primary polyps was restricted to areas of the stolon away from stolon tips, budding polyps or newly formed polyps to avoid already active proliferating cells. Double $\text{EdU}^+/\text{Piwi1}^+$ positive cells and $\text{EdU}^-/\text{Piwi1}^+$ cells were counted in a similar way to the stolon's of primary colonies (~3 weeks old). All cell counting experiments are shown as data from two replicates as the health of the animals was often compromised during long exposure to EdU.

2.7 CRISPR/Cas9 mediated mutagenesis

2.7.1 sgRNA design

sgRNAs were designed using the sgRNA design tool from the Broad institute. (<http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design-v1>)

sgRNAs were chosen based on a score around or above 0.5 (1 being the most optimal score) from their software as well as their location within the genomic DNA. Chosen guides were then Blasted and only used if no significant matches were found that also had an adjacent 5' NGG 3' PAM site.

2.7.2 sgRNA cloning

Overlapping primers were ordered (as listed in section 2.8 below) containing 19bp sgRNA site and overhangs for Gibson assembly into the sgRNA cloning vector (<http://www.addgene.org/41824/>). Primers were resuspended at 1 µg/µl and were annealed and extended using Phusion polymerase. A 50 µl mix containing 1 µg of each primer, 5 µl of 2 mM dNTPs, 10 µl of 5X HF Phusion buffer, 0.5 µl of Phusion enzyme and 32.5 µl of nuclease free H₂O. The reaction was carried out in the PCR machine under the following programme: 98°C for 30 seconds, 50°C for 30 seconds, 72°C for 10 minutes and hold at 4°C. The cloning vector was digested using AF1II (NEB R0520) for 1 hour at 37°C and the linearized product was run on a 0.5% gel and extracted (Section 2.3.3.). Gibson assembly was then used to ligate the annealed primers into the backbone using the protocol described above (Section 2.3.8.). The assembled product was transformed into bacteria and colonies were picked and grown in 5ml of LB broth (50µg/ml Kanamycin) ON and a mini plasmid extraction was carried out as described above (Section 2.3.9.). Colonies were then sequenced using a T7 primer to verify the presence of the sgRNA in the plasmid.

2.7.3 sgRNA synthesis

Guide RNA template was produced from the plasmids generated above by PCR with Phusion polymerase using a guide specific forward primer and T7gRNARv. Cycling conditions were 98°C for 30 seconds, 39 cycles of 98°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute. PCRs were then run on a 1% agarose gel and extracted according to gel extraction protocol as above (Section 2.3.3.). The sgRNAs were produced from the extracted template using the HiScribe T7 high yield RNA synthesis kit (NEB 2040) using the manufacturer's protocol. Briefly a 20µl reaction was prepared containing 1 µg of template DNA, 2 µl each of ATP, GTP, UTP, CTP, 10x buffer, T7 polymerase solution and x µl of nuclease free H₂O. This was incubated at 37°C in a water bath for 1 hour. Following incubation 10 µl of 12 M LiCl was added to each and the tubes were placed at -20°C for 20 minutes before centrifugation at full speed for five minutes. Pellets were washed with 70% ethanol, centrifuged again at full speed for five minutes and left to air dry at RT. Pellets were then re-suspended in nuclease free H₂O.

2.7.4 sgRNA *in vitro* testing

For *in-vitro* testing of the sgRNA, genomic DNA fragments were amplified using Phusion polymerase PCR. Primers used were designed at least 1kb away from the cut site in the 3' and 5' direction. *In-vitro* testing was carried out using the following mixture: 300ng Cas9 (PNA BIO CP02), 250ng sgRNA, 100ng genomic DNA PCR fragment, 2x NEB buffer 3 (NEB B70003S), 2µl BSA, xµl nuclease free H₂O to final volume of 20µl. Cas9 alone with no sgRNA was used as negative control. Samples were incubated for 1 hour at 37°C then run on a 1% agarose gel.

2.7.5 sgRNA injection and testing

For injection the sgRNA were prepared at a final concentration of 250 ng/µl with 2x NEB buffer 3. This was aliquoted for single use as 1 µl aliquots and stored in the -80°C freezer. Cas9 protein (PNA BIO CP02) was re-suspended to a final concentration of 2µg/µl and stored for single use as 1µl aliquots in the -80°C freezer.

PiwiFw2T7	5' GATCATAATACGACTCACTATAGGGCAGCATA CCGCGTGACGAAG 3'
PiwiRv2Sp6	5' TAGCAATTTAGGTGACACTATAGAACCTTAGCGC CACCTGTGC 3'
CniwiForT7	5'GATCATAATACGACTCACTATAGGGAGAGTTGATTT CACAATCGGTTAGAC 3'
CniwiRevSp6	5' AGTGCATTTAGGTGACACTATAGAAGTGTACT ACTACTAACTACTGGTTATTT 3'
HeVASAforT7	5 'TAATACGACTCACTATAGGGAGAAGGTTCAA GTGGTTGCCATTT 3'
HeVASArevSP6	5' ATTTAGGTGACACTATAGAAGAGTACTGCCAAC TTACCAAT 3'
Ncol1-T7fw	5' GATCATAATACGACTCACTATAGGGACGTCCAGGA CCACCAGGAGTA 3'
Ncol1-Sp6 rev	5 'TAGCAATTTAGGTGACACTATAGAACTGGGCA ACAGTATTGTGGACAAGA 3'

Primers for cloning Piwi1 ectopic expression construct

Betatubulinpiwi1gibsonfwd	5' GTGTTGTAGCACGA ACTATATAATTTCAA TATGACAGGTAGAGCGAGAGGAAGATC 3'
Betatubulinpiwi1gibsonrv	5' CATT TTTTCTACCGGGGCTGCAGCGGCCG CTAAATAATAAAGACGATCGGCCAATTGTC GAGC 3'
BetatubulinPromRv	5' ATTATATAGTTCGTGCTACAACACCTA GT 3'
LigDvectorGFPfus+polyA	5' AAAAAAGCGGCCGCTGCAGCCCCGGT 3'
GFP-TAGstop-rev	5' CTATTTGTATAGTTCATCCATGCCAT 3'
GFPSeqFusRev	5' TTGCATCACCTTCACCCTCTCC 3'

Primers for sgRNA cloning

PiwigRNA1fwd	5' TTTCTTGGCTTTATATATCTTGTGGAAAGGA CGAAACAC CGCAAGCAGATCAAACAACAG 3'
PiwigRNA1rev	5' GACTAGCCTTATTTAACTTGCTATTTCTAGCT CTAAAACCTGTTGTTTGATCTGCTTGC 3'
PiwigRNA2fwd	5' TTTCTTGGCTTTATATATCTTGTGGAAAGGACG AAACACCGAAGCAGATCAAACAACAGA 3'
PiwigRNA2rev	5' GACTAGCCTTATTTAACTTGCTATTTCTAGC TCTAAAAC TCTGTTGTTTGATCTGCTTC 3'
PiwigRNA3fwd	5' TTTCTTGGCTTTATATATCTTGTGGAAAGGACGA AACACCGTTGATCCGGACATTCAAAG 3'
PiwigRNA3rev	5' GACTAGCCTTATTTAACTTGCTATTTCTAGCTC TAAAACCTTTGAATGTCCGGATCAAC 3'
PiwigRNA4fwd	5' TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAA ACACCGATAGCTTCACAAAAGCTGA 3'
PiwigRNA4rev	5' GACTAGCCTTATTTAACTTGCTATTTCTAGCT CTAAAAC TCAGCTTTTGTGAAGCTATC 3'
PiwigRNA5fwd	5' TTTCTTGGCTTTATATATCTTGTGGAAAGGACGA AACACCGTTGGTTCGTGAGATCAAAG 3'
PiwigRNA5rev	5' GACTAGCCTTATTTAACTTGCTATTTCTAG CTCTAAAACCTTTTGATCTCACGACCAAC 3'
PiwigRNA6fwd	5' TTTCTTGGCTTTATATATCTTGTGGAAAGGACGA AACACCGCATACGAAATAAGTCCAGC 3'
PiwigRNA6rev	5' GACTAGCCTTATTTAACTTGCTATTTCTAG CTCTAAAACGCTGGACTTATTTTCGTATGC 3'
PiwigRNA7fwd	5' TTTCTTGGCTTTATATATCTTGTGGAAAGGACGA AACACCGATTCTTTAGCCCTGCCAGC 3'
PiwigRNA7rev	5' GACTAGCCTTATTTAACTTGCTATTTCTA GCTCTAAAACGCTGGCAGGGCTAAAGAATC 3'
PiwigRNA8fwd	5' TTTCTTGGCTTTATATATCTTGTGGAAAGGACGA AACACCGGATCTGTACAGTTGTCCTG 3'
PiwigRNA8rev	5' GACTAGCCTTATTTAACTTGCTATTTCTAGC TCTAAAACCAGGACA ACTGTACAGATCC 3'

Primers for sgRNA template synthesis

PiwigRNA1 T7	5' GGATCCTAATACGACTCACTATAGCAAGCAGATCAAACAACAG3'
PiwigRNA2 T7	5' GGATCCTAATACGACTCACTATAGAAGCAGATCAAACAACA GA 3'
PiwigRNA3 T7	5' GGATCCTAATACGACTCACTATAGTTGATCCGGACATTCAAAG 3'
PiwigRNA4 T7	5' GGATCCTAATACGACTCACTATAGATAGCTTCACAAAAGCTGA 3'
PiwigRNA5 T7	5' GGATCCTAATACGACTCACTATAGTTGGTCGTGAGATCAAAG 3'
PiwigRNA6 T7	5' GGATCCTAATACGACTCACTATAGCATACGAAATAAGTCCA GC 3'
PiwigRNA7 T7	5' GGATCCTAATACGACTCACTATAGATTCTTTAGCCCTGCCAGC 3'
PiwigRNA8 T7	5' GGATCCTAATACGACTCACTATAGGATCTGTACAGTTGTCCTG 3'

Primers for sgRNA testing

PiwiGenomicFwdKO	5' GTGACACGAGTGGGTGTTGTAATAAG 3'
PiwiGenomicRvKO	5' GGTGCTCATTTCAGTCACGAACAAC 3'
GenomicPiwi1RvKO	5' AACAATGAAAGGTGAAATTTGTCCTGTGC 3'
GenomicPiwi1fwdKO2	5' GCATCTGGGTCAGACAATGAGG 3'
GenomicPiwi1FwdKO 3	5' GTTGTAGGAGTGCAGGAA 3'
GenomicPiwi1rvKO3	5' CTCGCTATCCTATTCTGTTTTTG 3'
GenomicPiwi1fwdKO4	5' AAGACACGCTCATCATGGGTC 3'

2.9 Synthesized *Piwi1* gene fragment

Piwi1 g-block

Sequence 1

GGATCCATGACAGGTAGAGCGAGAGGAAGATCTAGGGGGAGAGGTA
AACCAAGTCAAGAAGCACCACGACCTGGTGGCGGAAATGCTCCACCA
CCTCAAGCAAGTACTGGTGGCAGGGGGAGATCAAGAGGTTCTGCACC
ACAGCCTACACAGCCCCAAAATCAGCAGTTGCTCCTCAGAGTGCAGT
AGCAGCTGCTCCTGCTGAGCAAATGGCAAAGTTAAATGTGCAGGAGG
AAAGGCCACAGGAAAGAAGGAGACGGCGGCTAGGTGAAGAAGCTAA
CATTTTGAGGTTGCGTCAAGCAGATCAAACAACAGAGGGCACAGGTG
GCGCTAAGGTTGATATTGTGGCCAATTTTTTCAGAGTCGCAAGATTGC
CCAATTTTACAGGATTGCACCAGTATAATGTTAGTTTTGATCCGGACAT
TCAAAGTGGAAGATTAATAATCCGCATTGTTGCACAATCTAGATGATAT
TCTTGGCACTGTGCACTGTTTTGATGGCATGACAATGTTTTTGCCCAA
AAATTGCCTGATCAAGAAACAGTTCGGACTGTTACTACAAGAACGGGC
AATGACATCACTGTCAAGGTTACTTACACTAATTTGGTTCCACAAAAC
TCACCCTCTGTGGTTCAATTGATGAATATTTTATTCAAAAAACAATTGA
AGAAAATTTCAATGCAGCTGATTGGTTCGAAATTATTTTAATCCATCAA
AAAAAATTGATATTCCTGCTCACAAAATGCAAGTATGGCCTGGCATCT
CAACATCCATTTTGCAGTATGAACAAGATGTGATGTTGTGTGCTGATG
TTCAGCATAAGGTTTTACGACAGCAGACAGTGTTGGAATTTTTGTACG
AGTTGTATGGTTCGGTGGACCAAAAAATGTTTTACAATATTGCTGCAA
AGAAATTGATTGGTGAAATTGTCCTGACCAGATACAACAATAAGACAT
ATCGAATTGATGATATTGATTGGGAGAAACACCCAAGTATAGCTTCA
CAAAGCTGATGGCAGTACAATAACTTTCAAAGCATATTACGAAGCAG
TTTATGAAAGAAAAGTCGCTGATGATAATCAGCCACTTTTGATCTCAC
GACCAAAAGAAAAGGACAAGAAGAGAGGAATGTCTGGACCTATTTTA
TTGCTGCCAGAATTTTGTTCATCACTGGATTATCGGATGAAATCAGA
AATAATTATTCTGTAATGAAGGATTTGAATCAACATACGAAAATAAGT
CCAGCTGGCAGGGCTAAAGAATTGGAAAGTTTTATGAATGATCTGTAC
AGTTGTCCTGAGGCAGCAAATGAATTGGCTCAATGGAACATGGCTTTT
GAAAAAATCTACTTAAAATGACTGGACGTTCTCTTCCAGCTGAGGCA

ATGTCTCAAAGAAATGCCAGATTCTCATACAAAATACAAGAAGCTGAT
TGGTCAAGAGAGACACGTGGAAAGGAATTGATATCTTCAGTTCACATG
TCTAACTGGGTTTTGTT

Piwi g-block

Sequence 2

TTCAGTTCACATGTCTAACTGGGTTTTGTTATACACAGCTCGTGACAAC
AACATTGCAAATGACTTTAAACAAACCCTTCAAAGGGTTTGTGGTCCA
ATGGGTATGAATGTAGCCGATCCTCACATGTGTCGTTTAAATAACGAT
AGTGCTCAGACATACTCCATGACTCTCAAGGAGCAAATTCAACGAGAT
ACACAGATTGTTGTGTGTATCGTTCCTAATAACCGTAAAGATCGGTAT
GATGCTATTAAGAAATTATGCTGTGTGGAAAGACCAGTCCCATCTCAA
GTTGTAGTTAGCCGTA CTTTGAGTAAAAAGAACATGTTAATGTCCGTG
TGTACCAAGATTGGCATT CAGTTAAATTGTAAGTTAGGAGGCGAAGTA
TGGGCAGTTGACATACCTTTAAATAAATTGATGGTGATTGGATTTGAC
GTTTACCACGACTCTGCTACTAAGGGCAAGTCCATTGGTGGGTTTGTTG
CCAGCACTAATAAGCATCTTACACGATATTATTCGTGCATTACGTCAC
AAACATCTCATATGGAAATATGTGATCAGCTCAAAGTCTGCATGACCA
ATGCGCTAAAGAAATTCCACGAAATTAACGATGGCTTACCCGACAAGA
TTATTGTCTATCGAGATGGTGTCGGTGATGGACAGATTCGTGACGTTTA
TGAGCACGAGCTTCCACAGTTAAAGTCTGCTTTTGCAACTGTTGCGCC
AAATTACAATCCAAAATTTGCAATGTTAATTGTGAAGAAAAGGATTTT
TACGCGATTGTTGTTAGCTTCCGGTGGACAGTATTCAAATCCTCCTCCC
GGAAGTGTGTTGACACTGTGGTAACACGTCCGGAGTGGTACGACTTT
TTGTTGATTT CACAATCGGTCAGACAAGGTACGGTCACTCCGACGCAT
TATAACGTGATTAACGACACA ACTGGCTTAAAACCGGATCACTTCCAA
CGTTTAACTACAACTTTGTCATTTGTATTACAATTGGCCGGGCACCA
TTCGCGTTCCTGCACCTTGT CAGTACGCTCATAAGTTAGCCTTCTTAGT
TGGTCAGTCGATCCACACGGAGCCTGCTCGACAATTGGCCGATCGTCT
TTATTATTTAGCGGCCGC

Overlapping sequences for gibson assembly in red.

2.10 Accession numbers for PIWI proteins

The following accession numbers were taken from GenBank and used in the phylogenetic analysis and protein alignment of known PIWI proteins.

Model Organism	Ascension No.
<u><i>Hydra Vulgaris:</i></u>	
<i>Hywi</i>	BAN82535
<i>Hyli</i>	BAN82534
<u><i>Schmidtea mediterranea:</i></u>	
<i>Smedwi-1</i>	ABB77337.1
<i>Smedwi-2</i>	ABB777338.1
<i>Smedwi-3</i>	ACC97187.1
<u><i>Drosophila melanogaster:</i></u>	
Piwi	AGL81535.1
Ago3	AB026294
AUB	AGA18946.1
<u><i>Danio rerio:</i></u>	
Ziwi	AAL57170.1
Zili	ACH96370.1
<u><i>Mus musculus:</i></u>	
Miwi	BAA93705.1
Miwi2	AAN75583.1
Mili	BAA93706.1
<u><i>Strongylocentrotus purpuratus:</i></u>	
Seawi	NP_999765.1
Seali	NP_001107667.1

Chapter 3: *Hydractinia* PIWI genes and the proteins they encode: sequence analysis, phylogeny, and expression pattern

3.1 Introduction

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These functional products can be either in the form of non-protein coding gene products such as piRNA, or as proteins. Gene expression is important in all known living organisms to drive the biological processes necessary for life.

Protein-coding gene expression is determined by the two biological processes transcription and translation. Transcription involves the synthesis of mRNA from the DNA sequence encoding a particular gene (Krieg and Melton, 1984). The process of translation involves the produced transcript being decoded by ribosomal RNA to form a functional amino acid product (Kaczanowska and Rydén-Aulin, 2007) (Figure 3.1). Gene expression is the most fundamental level at which a genotype gives rise to a phenotype. The expression of genes in cells is highly variable due to each cell having at least one copy of a genome (Perou et al., 1999). Cells of a specific type, such as stem cells, express at high levels genes that work to maintain stemness (Ramalho-Santos et al., 2002), whereas differentiated cell types express lineage-specific genes (Schuldiner et al., 2000). Regulation of gene expression by controlling transcription or translation is vital for cells to maintain homeostasis (Hetz, 2012).

There are many methods available to study gene expression patterns in cells, divided between techniques that monitor mRNA level and those that identify proteins directly. Furthermore, different techniques of gene expression analyses may provide spatial, temporal, and quantitative information, depending on the method used. The most commonly used techniques are RT-qPCR, northern blotting, western blotting, whole mount RNA *in situ* hybridization (WISH) and immunofluorescence (IF). This project employed mainly WISH and IF to study the expression patterns of

Hydractinia Piwi1 and *Piwi2* mRNA and protein, respectively, providing spatial and temporal accounts,

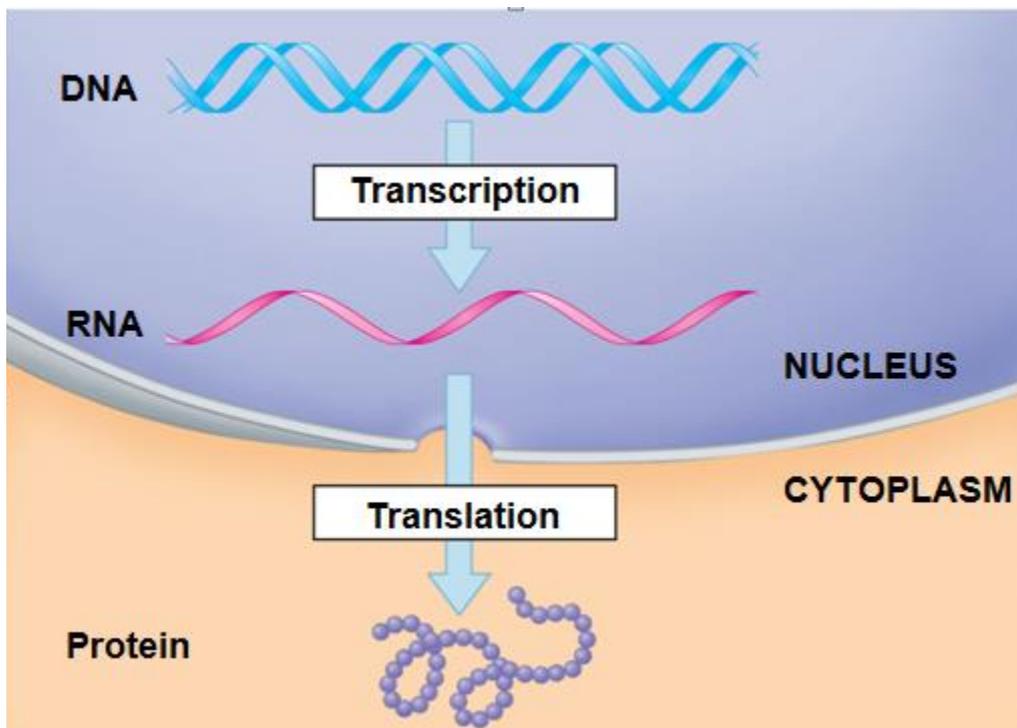


Figure 3.1. Schematic representation of the transcription and translation processes in a eukaryotic cell (Source: http://classconnection.s3.amazonaws.com/333/flashcards/903333/png/transcription_-_translation1320698154407.png).

but only semi-quantitative information. Prior to WISH experiments, RNA probes were generated as described earlier in the materials and methods section 2.3.10. The WISH protocol was carried out for most of the *Hydractinia* life stages. IF analysis was also carried out to detect *Piwi1* and *Piwi2* protein expression by utilizing antibodies raised against the *Hydractinia* *Piwi1* and *Piwi2* N terminal protein regions (Primer sequences section 2.8, Nucleotide sequence Appendix A6-7). A detailed description of the antibody generation protocol can be found in section 2.4. Both these methods of WISH and IF were combined in several different life stages to check for co-localization of mRNA and protein.

3.2 Aims

Understanding the roles of PIWI proteins in *Hydractinia* requires a comprehensive analysis of the sequence, phylogeny, and spatial and temporal expression pattern of these genes/proteins, in order to provide the basis for functional studies. Hence, the aim of this chapter of the thesis is to provide this information, including determining the expression pattern of both PIWI genes' mRNA and protein in all life stages.

3.3 Phylogeny, sequence analysis and PIWI antibody generation

The Piwi gene sequences (*Piwi1* and *Piwi2*) were identified in *Hydractinia* by blasting the cnidarian *Hydra vulgaris* orthologue Piwi gene sequences (*Hywi* and *Hyli*) against our *Hydractinia echinata* transcriptome server found at the following link that is, by the time this dissertation was written, only accessible from within the university's network: http://europa.it.nuigalway.ie/wwwblast/blast/blast_link.cgi. Phylogenetic analysis was carried out on the *Hydractinia* specific Piwi protein sequences identified (Figure 3.2, Appendix A2-5). These sequences contained the conserved PAZ and PIWI domains (Appendix A2,4). These domains are a distinctive feature of PIWI proteins across diverse organisms (Juliano et al., 2011). Piwi amino acid sequence alignment of diverse organisms displays the conservation of these PAZ and PIWI domains (Figure 3.3).

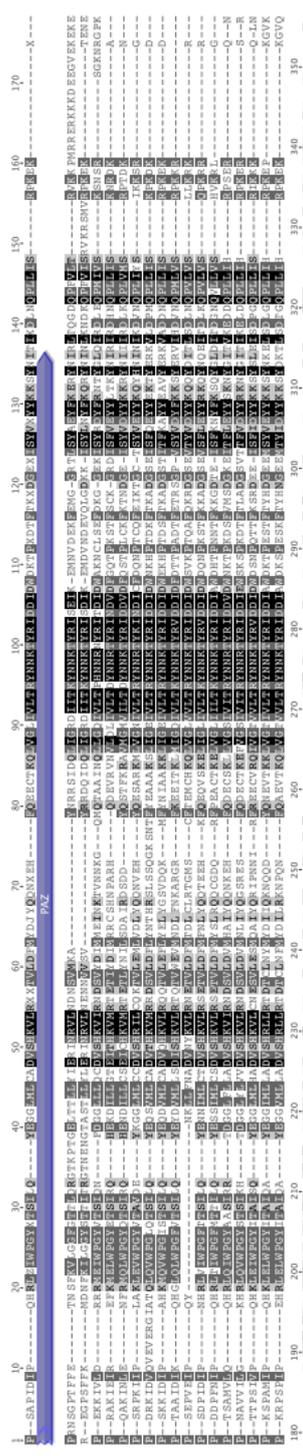
3.3.1 Phylogeny and sequence analysis

An alignment of full-length Piwi and Argonaute proteins was made with MUSCLE (Edgar, 2004). Prottest v 3 (Darriba et al. 2011) was run on the alignment to estimate the empirical model of amino acid substitution that fit the data best. The LG + I + F + Γ (gamma) model had the overall best fit. Maximum likelihood analyses were performed with the MPI version of RAxML v7.2.8 (RAXMLHPC-MPI) (Stamatakis, 2006). We conducted two independent searches with a total of 100 randomized maximum parsimony starting trees. We compared the likelihood values among all result trees. One hundred bootstrapped trees were computed and applied to the best result tree. The midpoint rooted, bootstrapped best result tree was visualized with FigTree v1.3.1. PIWI domain protein sequences from various animals, representing all PIWI and AGO families, were downloaded from GenBank.

Piwi1 and Piwi2 sequences were aligned with other PIWI proteins belonging to different species, in order to analyze the degree of similarity to the *Hydractinia* homolog (Figure 3.3). The Blast tool from the website of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov>) was used to find regions of local similarity between sequences. The alignment of chosen sequences was performed with the use of the bioinformatic software platform Geneious. PAZ and PIWI domains were identified as conserved components of the *Hydractinia* PIWI proteins when compared to all other species analysed.

Alignment of the *Hydractinia* protein sequence with its orthologues showed that all known PIWI domains are conserved in *Hydractinia*. Phylogenetic analysis placed Piwi1 and Piwi2 in distinct clusters that also included other cnidarian and bilaterian PIWI genes, suggesting that the duplication of the two genes from an ancestral PIWI gene predated the split between Cnidaria and Bilateria. AGO genes formed a sister group to PIWI. *Hydractinias* key phylogenetic position as a basal metazoan with a high level of gene conservation makes it a suitable model to study aspects of PIWI stem cell biology.

Figure 3.2. Phylogenetic analysis of PIWI and Argonaute proteins in many species. The Maximum Likelihood (ML) tree was computed from an alignment of PIWI protein sequences. Numbers associated with branches correspond to ML bootstraps (100 replicates). Species names are abbreviated as follows: Aq, *Amphimedon queenslandica*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Ef, *Ephydatia fluviatilis*; He, *Hydractinia echinata*; Hs, *Homo sapiens*; Hv, *Hydra vulgaris*; Mm, *Mus musculus*; Nv, *Nematostella vectensis*; Pc, *Podocryne Carnea*; Sm, *Schmidtea mediterranea*; Sp, *Strongylocentrotus purpuratus*.



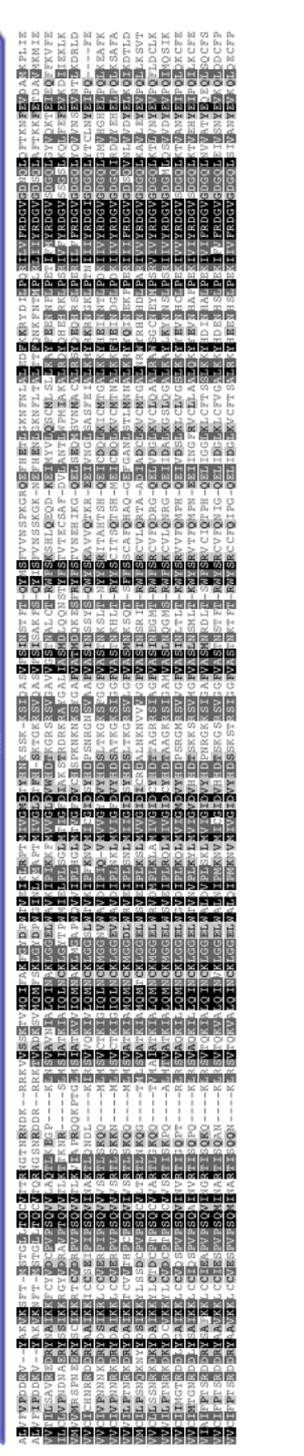
Consensus

1. smcdw2
 2. smcdw1
 3. smcdw3
 4. Dm-pw1
 5. Dm-auberg1
 6. Dm-ago3
 7. hwy
 8. He-pw1
 9. seawi
 10. mw1
 11. miwi
 12. zwi
 13. mli
 14. zili
 15. seali
 16. hyl
 17. He-pw2



Consensus

1. smcdw2
 2. smcdw1
 3. smcdw3
 4. Dm-pw1
 5. Dm-auberg1
 6. Dm-ago3
 7. hwy
 8. He-pw1
 9. seawi
 10. mw1
 11. miwi
 12. zwi
 13. mli
 14. zili
 15. seali
 16. hyl
 17. He-pw2



Consensus

1. smcdw2
 2. smcdw1
 3. smcdw3
 4. Dm-pw1
 5. Dm-auberg1
 6. Dm-ago3
 7. hwy
 8. He-pw1
 9. seawi
 10. mw1
 11. miwi
 12. zwi
 13. mli
 14. zili
 15. seali
 16. hyl
 17. He-pw2

Figure 3.3. PIWI protein alignment sequences of conserved PAZ and PIWI domains in different organisms. Sequence alignment of PAZ and PIWI domains from the PIWI proteins of the following organisms: **1-3** *Schmidtea mediterranea*, **4-6** *Drosophila melanogaster*, **7,16** *Hydra vulgaris*, **8,17** *Hydractinia echinata* **9,15** *Strongylocentrotus purpuratus* (Sea urchin), **10,11,13** *Mus musculus* (Mouse), **12,14** *Danio rerio* (Zebrafish).

3.3.2 PIWI antibody production

The use of tools such as *mRNA in situ* hybridization to study gene expression is a widely common technique. *In situ* hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA, RNA strand (i.e., probe) to localize a specific mRNA species in a portion or section of tissue (*in situ*), or, if the tissue is small enough (e.g., plant seeds, *Drosophila embryos*), in the entire tissue (whole mount ISH (WISH)), in cells, and in circulating tumor cells (CTCs). This is distinct from immunohistochemistry, which usually localizes proteins. *In situ* hybridization is used to reveal the location of specific nucleic acid sequences on chromosomes or in tissues, a crucial step for understanding the organization, regulation, and function of genes. It is a powerful technique for identifying specific mRNA species within individual cells providing insights into physiological processes and disease pathogenesis. However, *in situ* hybridization requires that many steps be taken with precise optimization for each tissue examined and for each probe used. In order to preserve the target mRNA within tissues, it is often required that crosslinking fixatives (such as formaldehyde) be used. This technique allowed for the analysis of Piwi genes in *Hydractinia* by identifying their specific mRNAs within cells. For this project to give a complete picture of PIWI proteins, and their localization compared to the PIWI mRNAs, the protein itself needed to be targeted by a method called immunofluorescence.

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope. This technique uses the specificity of antibodies to their target antigen to target specific fluorescent dyes to biomolecules within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. The specific region an antibody recognizes on an antigen is called an epitope (Mandrell et al., 1988). Antibodies for these particular PIWI proteins did not exist for *Hydractinia* previously, therefore I cloned, expressed and purified partial sequences of Piwi1 and Piwi2 antigens (Section 2.4). The Piwi1 antigen was an insoluble protein whereas Piwi2 was soluble.

These antigens were sent to Eurogentec that were in charge of the immunization of selected hosts and collection of final serum. Piwi1 antigen was administered into two Rabbits and Piwi2 was administered into two Guinea pigs. The final bleed serum from these animals gave the final Piwi1 anti-rabbit and Piwi2 anti-guinea pig antibodies. These antibodies were tested by western blotting and detected to clear single bands in the regions of 95kDa for Piwi1 and 99kDa for Piwi2 as predicted (Figure 3.4B). These antibodies were utilized for all immunofluorescence, western blotting analysis and immunoprecipitation (IP) protocols experiments listed in this thesis. The anti-Piwi1 antibody was much more efficient compared to Piwi2 antiserum. The reasons for this are unknown, but could be related to the differences in solubility of these proteins or the host used for immunization. Therefore, I utilized the Piwi1 antiserum for the majority of my experimental analysis after expression profile of both genes was complete. Pre-immune sera was used as a secondary control for all immunofluorescence experiments.

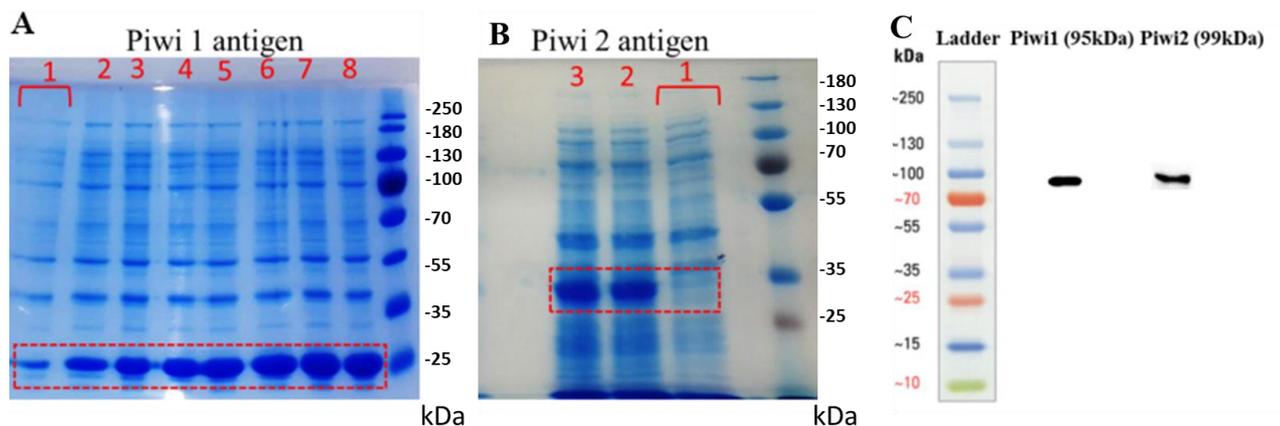


Figure 3.4: (A) Coomassie stained polyacrylamide gel (15%) of Piwi1 antigen tested before and after induction of protein synthesis. Lane 1 on the Piwi1 gel indicates the proteins from the un-induced cells, lanes label 2-8 indicate the protein expression at every 30 min interval (2 = 30mins, 3 = 1 hour, 4 = 1.5 hours, 5 = 2 hours, 6 = 2.5 hours, 7 = 3 hours and 8 = 3.5 hours) until 3.5 hours post IPTG induction. The red dashed box at the bottom of the gel shows the increasing level of overexpression of the protein from left to right with an expected molecular size band of 23kDa. (B) The Piwi2 antigen gel shows similar increases in protein expression post IPTG addition. The lane labelled with 1 shows un-induced protein, label 2 and 3 shows the induced protein with lane 2 = 2 hours and lane 3=4 hours post induction. The red dashed box

at the bottom of the gel shows the increasing level of overexpression of the protein from right to left with an expected molecular size band of 33kDa. (C) Western blot analysis of the purified antibodies Piwi1 and Piwi2 was carried out against total *Hydractinia* protein extract. The purified Piwi1 antibody gave the expected full size of the protein which was 95 kDa and the purified Piwi2 antibody gave the expected full size of the protein which was 99 kDa.

3.4 Piwi1 protein and mRNA expression profile

3.4.1 Embryo

Piwi1 protein was first detected in fertilized eggs and 4 hours post fertilization (hpf) of *Hydractinia* by IF (Figure 3.5A-B), and was also present in later stages of embryonic development from 4 -24 hpf (Figures 3.5C and 3.6A-C). Its expression was ubiquitous until the 10-16 hpf stage. Around 16 hpf Piwi1 protein was no longer ubiquitous and was in fewer cells clustered around one pole of the embryo, presumably the oral end (Figure 3.6B). This is based on studies in *Clytia*, where *ChePiwi* mRNA accumulates at the oral end of the gastrulating larva (Leclère et al., 2012). By 22-24 hpf Piwi1 protein was detected in very few cells in the endoderm of the embryo (Figure 3.5C and 3.6C).

3.4.2 Larva

In the planula larva stage about 48-72 hpf, Piwi1 protein expression analysis revealed the presence of a higher number of Piwi1⁺ cells within the gastroderm/endoderm (Figure 3.7A). *Piwi1* mRNA expression was also found to be present within cells in the gastroderm in 48hpf planula larva (Figure 3.8A). These Piwi1⁺ cells were detected as single cells mainly in the prospective oral regions of the planula larva (Figure 3.7A and 3.8A). Piwi1 protein was exclusively cytoplasmic in all stages studied.

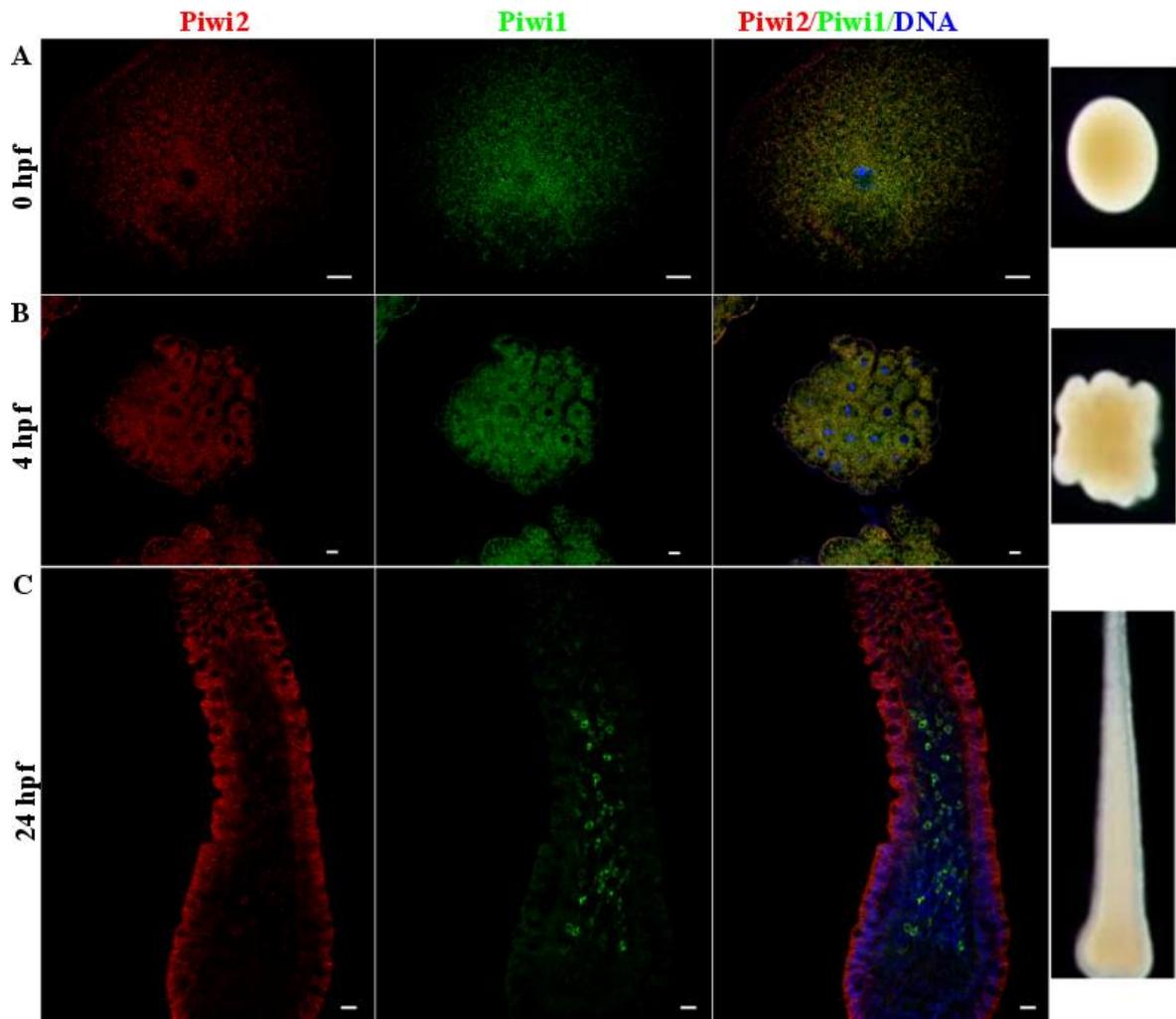


Figure 3.5 Piwi1 and Piwi2 protein localization during embryogenesis. (A) Fertilized egg showing co-localization of both PIWI proteins. (B) The expression of both proteins remains ubiquitous and identical up to 4 hpf. (C) By 24 hpf both proteins remain expressed in some cells of the gastroderm. Piwi2 antibody signal (red) in ectoderm of 24 hpf larva is non-specific. Controls were carried out using pre immune serum from the host animals (rabbit and guinea pig, respectively). Bright field images, representative of the shape of *Hydractinia* embryos and during the different stages of development are present on the right for images A-C. Scale bar equals 10 μm .

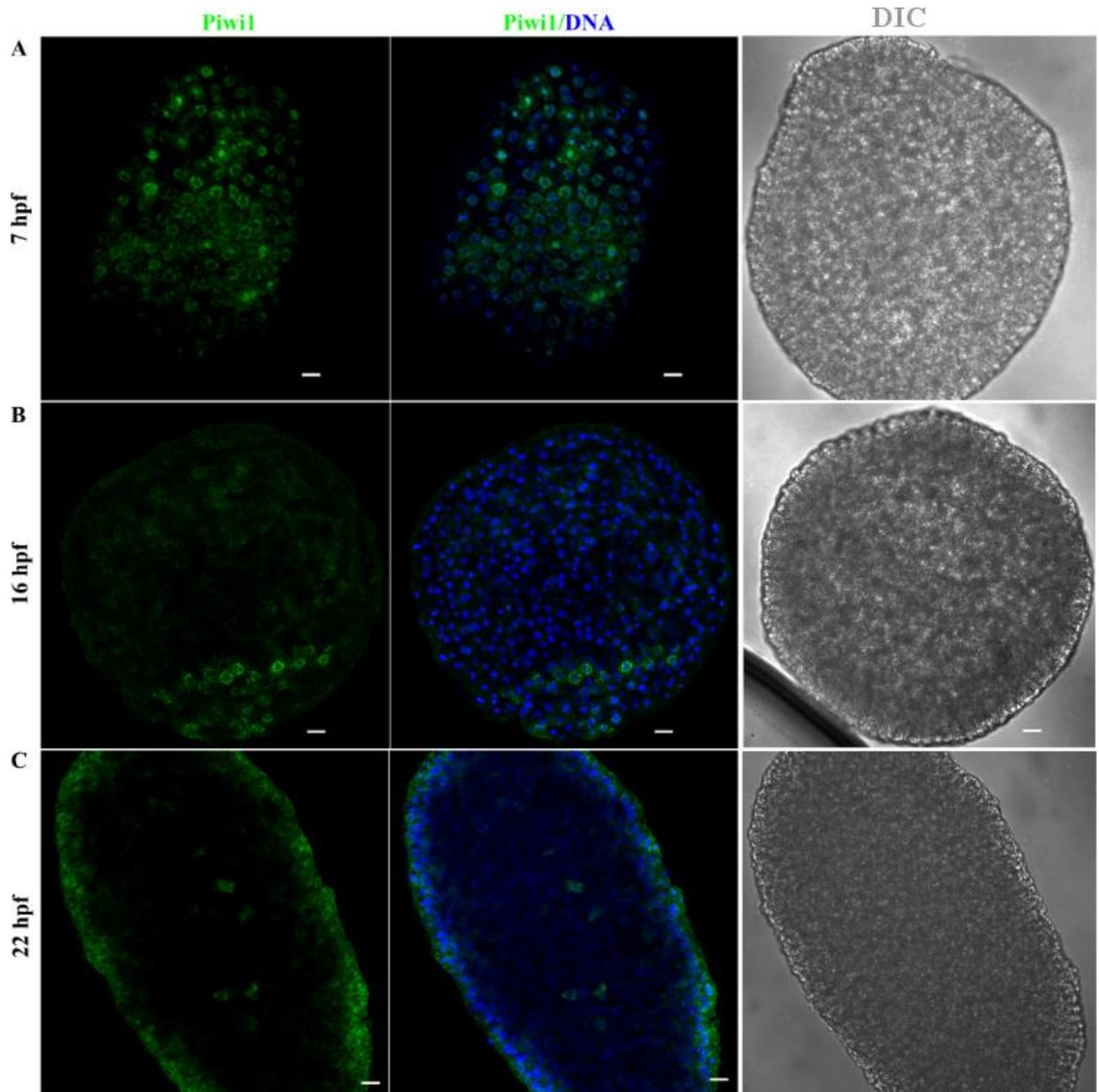


Figure 3.6 Piwil protein localization during 7, 16 and 22 hpf. (A) Piwil continues to be nearly ubiquitous at 7 hpf. (B) Piwil⁺ cells appear to segregate to one gastrodermal pole in *Hydractinia* embryos around the 16 hpf stage of development. (C) By 22 hpf the number of Piwil⁺ cells have decreased within the gastroderm (Signal in the ectoderm is non-specific). Controls were carried out using pre immune serum from the host animals (rabbit and guinea pig, respectively). DIC images of each stage of embryo development are present to the right of each image from A-C. Scale bar equals 10 μ m.

3.4.3 Primary polyp

Larvae were induced to metamorphose into primary polyps at 72 hpf. Metamorphosis is naturally induced by a bacterial film covering the shell of a hermit crab. In the lab, metamorphosis is induced by incubating animals in 100 mM CsCl in seawater (see section 2.1.1.). Upon induction, the larvae contract and settle on virtually any surface (e.g. glass slide) with their anterior (future aboral) pole directed down, and develops into the animals stolons. The tail (i.e., the larval posterior end) contracts and becomes the future feeding polyp's head (also known as the oral end). The 24 hour metamorphosed animal consists of a polyp body column with a mouth and a ring of tentacles at its oral side and mostly 2-3 stolons attached to the substratum at the aboral pole.

Using IF, high numbers of Piwi1⁺ i-cells were detected in young colonies (Figure 3.9A). High resolution images confirmed Piwi1 to be cytoplasmic, like in embryos, i-cell abundance in stolons was higher than in polyps' body columns (Figure 3.6B). Piwi1⁺ cells were detected in large clusters and as single cells, exclusively in the interstitial spaces of the epidermis (Figure 3.9B). Hence, it is clear that Piwi1⁺ cells have changed their position from gastrodermal to epidermal sometime during metamorphosis (Figure 3.7A-C). These Piwi1⁺ cells are cytoplasmic and average around 10µm in size, depending on their activity and location within the animal.

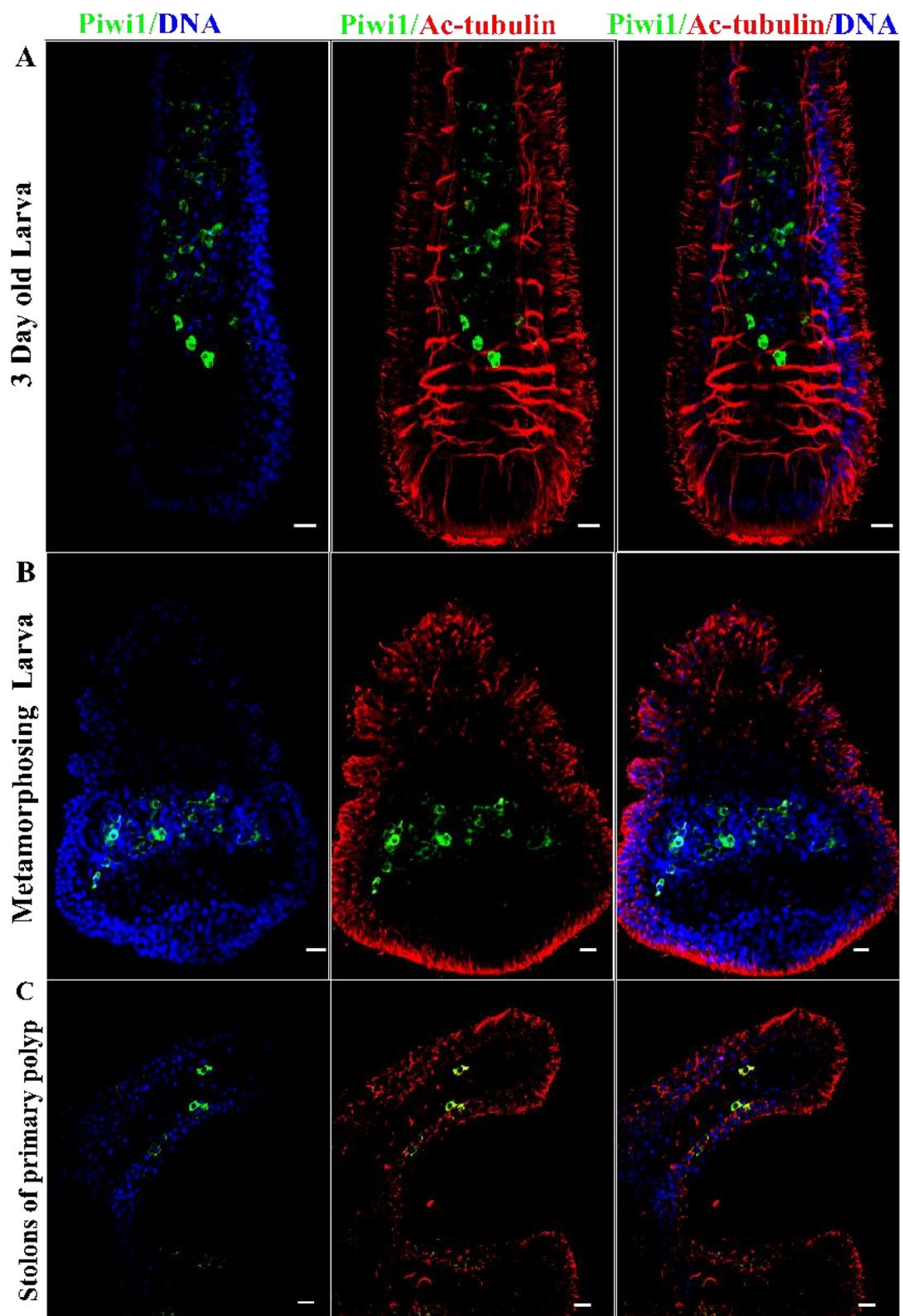




Figure 3.7. Piwi1 protein in late larva, early metamorphosis, and primary polyp stages. (A) 72 hpf larva showing Piwi1⁺ cells in the gastroderm. (B) Larva induced by CsCl to metamorphose showing Piwi1⁺ cells still in the gastroderm. (C) Piwi1⁺ cells are now present in epidermal cells of primary polyp stolon. Figures A', B' and C' represent the bright field images of figures A-C respectively. Scale bar equals 10 μ m.

3.4.4 Feeding polyp

Piwi1 mRNA expression was previously analyzed in the adult feeding polyp life stage of *Hydractinia* (Bradshaw et al., 2015). This expression pattern was confirmed by IF utilizing the Piwi1 antibody (Figure 3.11A). At this stage, the animal has matured and many more Piwi1⁺ cells could be seen in the body column of feeding polyps in a band region known to be highly proliferative (Bradshaw et al., 2015). Higher magnification of these cells revealed different size cells and some clusters of cells within the polyp (Figure 3.10). Piwi1 protein, however, was generally absent from the head and tentacles of adult polyps (Figure 3.11A).

3.4.5 Male and female sexual polyps

PIWI proteins are a known germline specific genes in many other organism , so it was unsurprising to find high levels of both *Piwi1* mRNA expression (Figure 3.8B-C) and Piwi1 protein expression (Figure 3.11B-C) in the developing male and female gonads. There were high levels of both Piwi1 protein and *Piwi1* mRNA detected in spermatocytes within developing gonads and in spermatogonia migrating

through the body column into forming gonads (Figure 3.8B'and 3.11B). *Piwi1*⁺ cells were also detected in the population of i-cells present in the epidermis of male gonads (Figure 3.11B).

The same expression analysis of female sexual gonads was carried out and similar patterns of *Piwi1* gene and protein expression were observed. *Piwi1* mRNA was also detected in high levels in female gonads (Figure 3.8C,C'). In female sexual polyps *Piwi1* was detected in both oocytes within developing gonads and in oocytes migrating through the body column in the direction of forming gonads (Figure 3.11C). As seen in males, *Piwi1*⁺ cells were also found in the body column of females that were either i-cells or early committed oogonia (Figure 3.11C). All WISH and IF experiments carried out on the different *Hydractinia* life stages were performed with sense probe and secondary only antibody controls, respectively. No staining was observed in either control experiments indicating the specificity of the probes used in WISH and antibodies generated for IF.

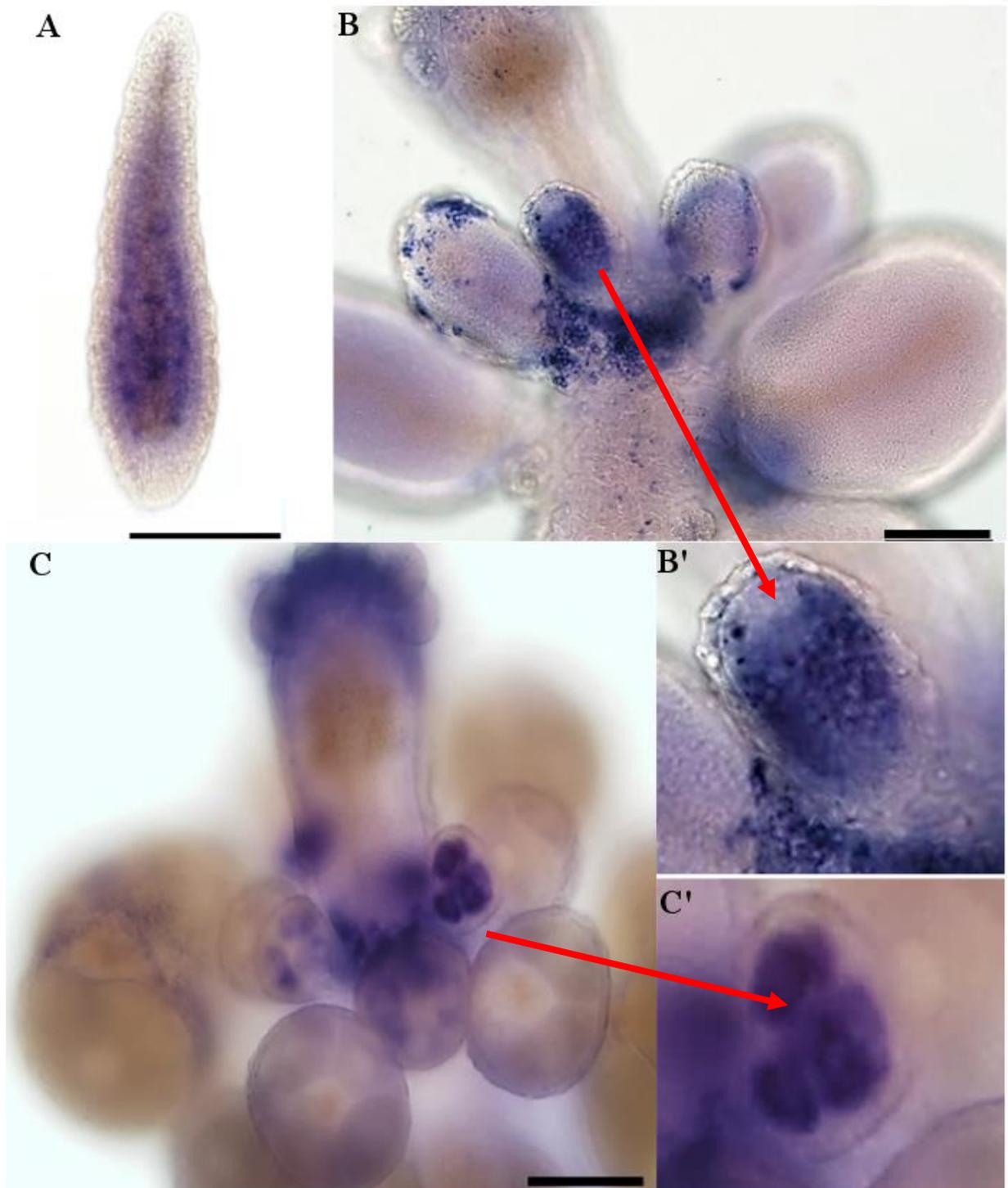


Figure 3.8. *Piwil* mRNA expression by *in situ* hybridization. (A) Expression of *Piwil* in the gastroderm of a 48 hpf larva. (B) Expression of *Piwil* in the gastroderm and the red arrow points to (B') higher magnification showing gametes of a male gonad. (C) Expression of *Piwil* in the female gonad in the gastroderm and a red arrow points to (C') higher magnification showing gametes of a female gonad. Scale bar equals 100 μm .

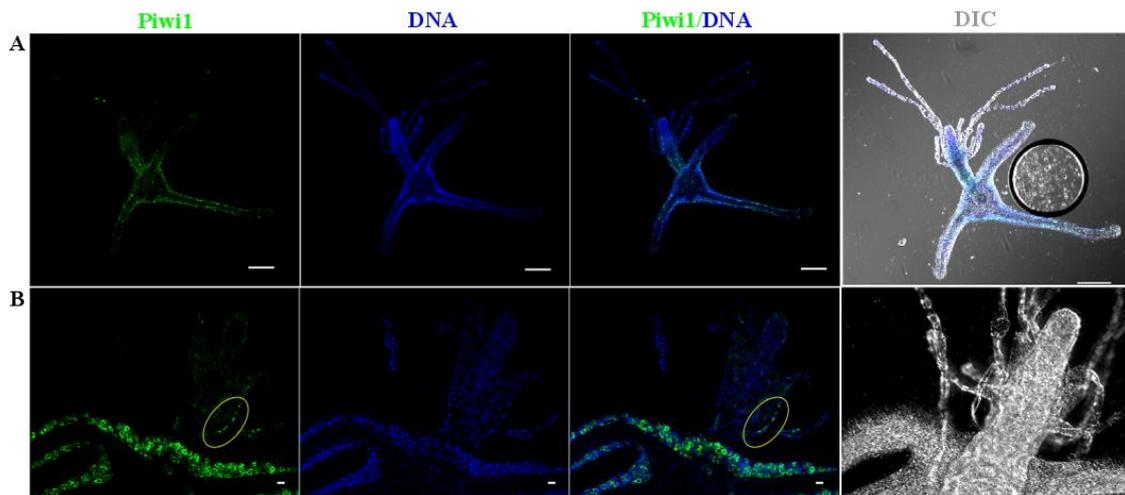


Figure 3.9 Piwi1 staining in primary polyps. (A) 10X magnification of a primary polyp showing Piwi1 staining (green) in the epidermal cells of the stolons. (B) Close up of Primary polyp shows large clusters of Piwi1⁺ cells in stolons (green) and few migratory cells in the polyp body column (yellow circle). The DIC images to the right show the structure and shape of a primary polyp colony. Scale bar equals 10 μ m.

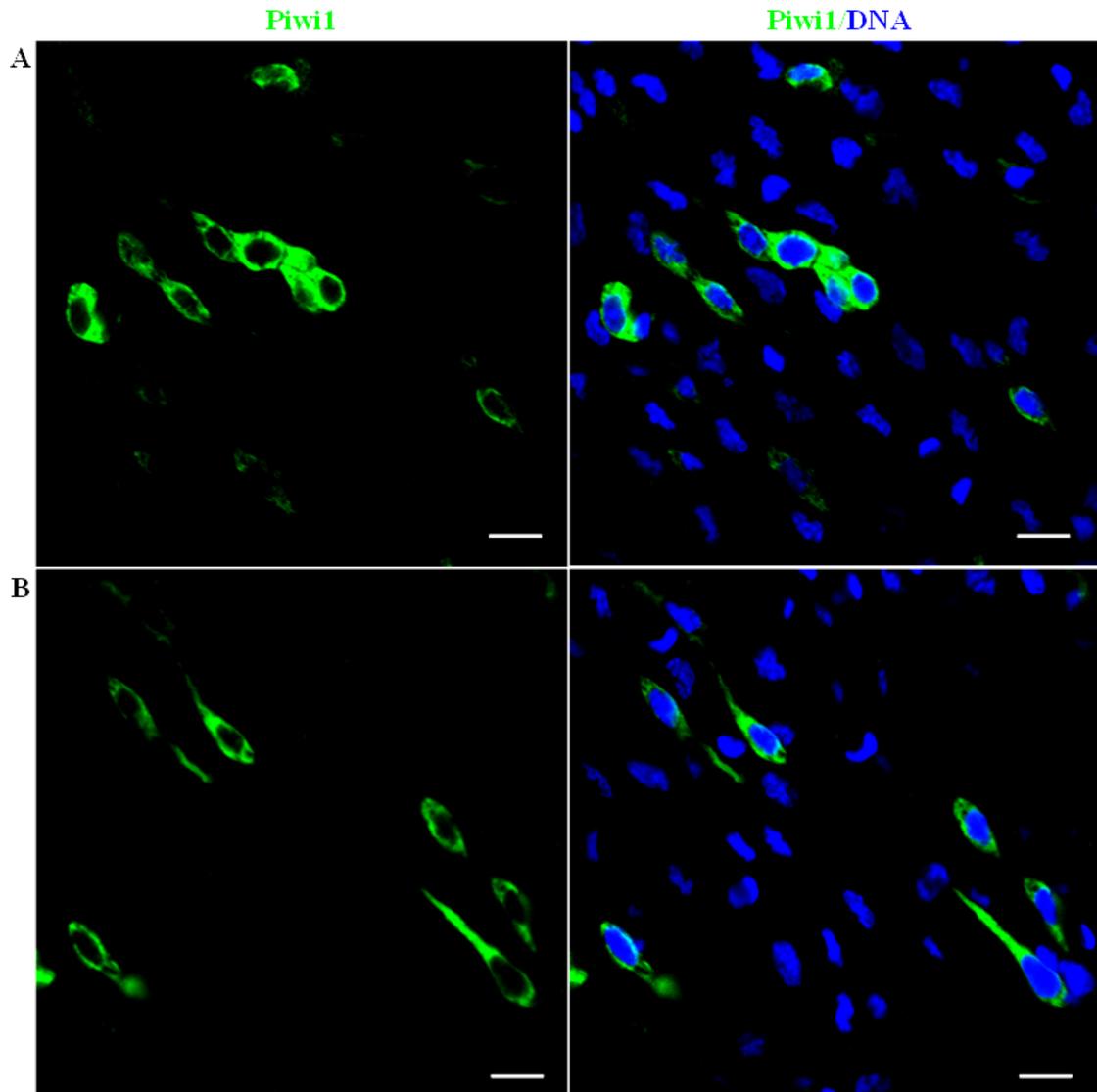


Figure 3.10. Piwi1⁺ cells in adult feeding polyps. (A) 60X magnification of adult feeding polyps' demonstrates the presence of clusters of Piwi1⁺ cells (green) with cytoplasmic staining as seen in the Piwi1/DNA merge. (B) Single Piwi1⁺ cells were also present in the adult feeding polyp. Scale bar equals 10 μ m.

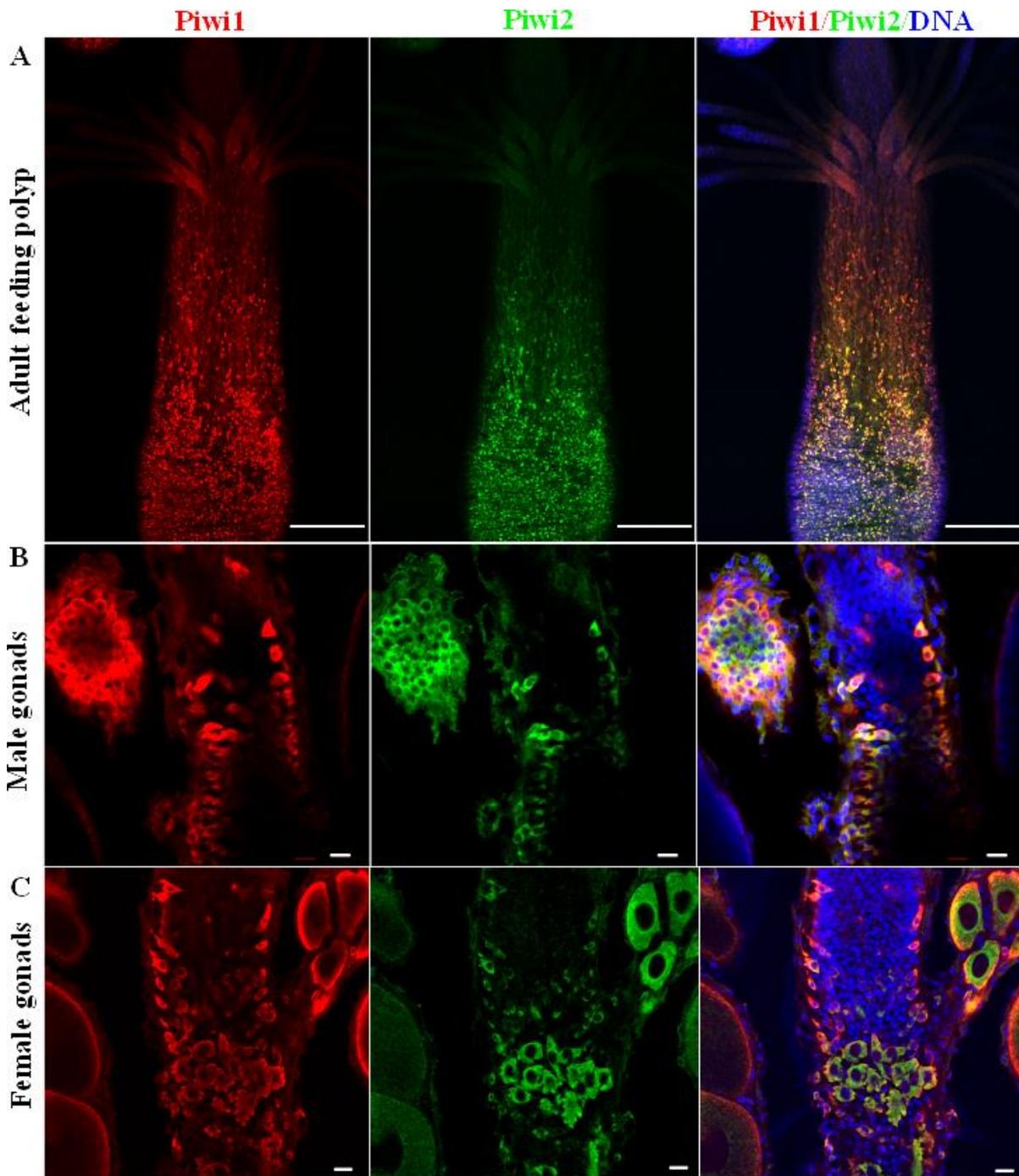


Figure 3.11. Piwi1 and Piwi2 protein co-expression analysis in adult polyps. (A) Adult feeding polyps showing co-expression of Piwi1 (red) and Piwi2 (green) in i-cells in the proliferative region of the body column. (B) Piwi1 (red) and Piwi2 (green) proteins are co-expressed within i-cells and germ cells in the male gonads and (C) female gonads as can be seen with the yellow colour in the merge images on the right. Scale bar equals 10 μ m.

3.5 *Piwi1* mRNA and Piwi1 protein co-localization analysis

In order to examine whether *Piwi1* regulation is different on the mRNA level compared to the protein level, fluorescent *in situ* hybridization (Section 2.2.2.) and IF (Section 2.2.3.) staining protocols were carried out on feeding polyps, male and female gonads (Figure 3.12). These experiments show that *Piwi1* protein and *Piwi1* mRNA are localized within the same cells in all studied life stages.

3.6 *Piwi2* protein and mRNA colocalisation

In order to get a complete picture of how *Piwi* genes are expressed in *Hydractinia* I looked at the expression pattern of the second PIWI gene *Piwi2*. PIWI orthologous proteins present in many other organisms have been shown to be either co-expressed and/or show dynamic expression profile at different stages of development (Juliano et al., 2011, Di Giacomo et al., 2013). To analyze *Piwi2* expression and compare it to *Piwi1*, I generated an antibody against the *Piwi2* N-terminus, as described previously in section 2.4. Expression of *Piwi2* mRNA was also studied in several life stages of *Hydractinia* using specific probes designed against its mRNA and compared it to the *Piwi2* protein expression profile.

3.6.1 Embryo

Piwi2 protein is also detected in fertilized eggs and continues to be expressed ubiquitously throughout cell division similar to *Piwi1* in 0 and 4 hpf embryos (Figure 3.5A-B). *Piwi2* expression pattern is ubiquitous within the gastroderm during embryonic development, matching closely to *Piwi1* expression in embryonic development 24 hpf (Figure 3.5C).

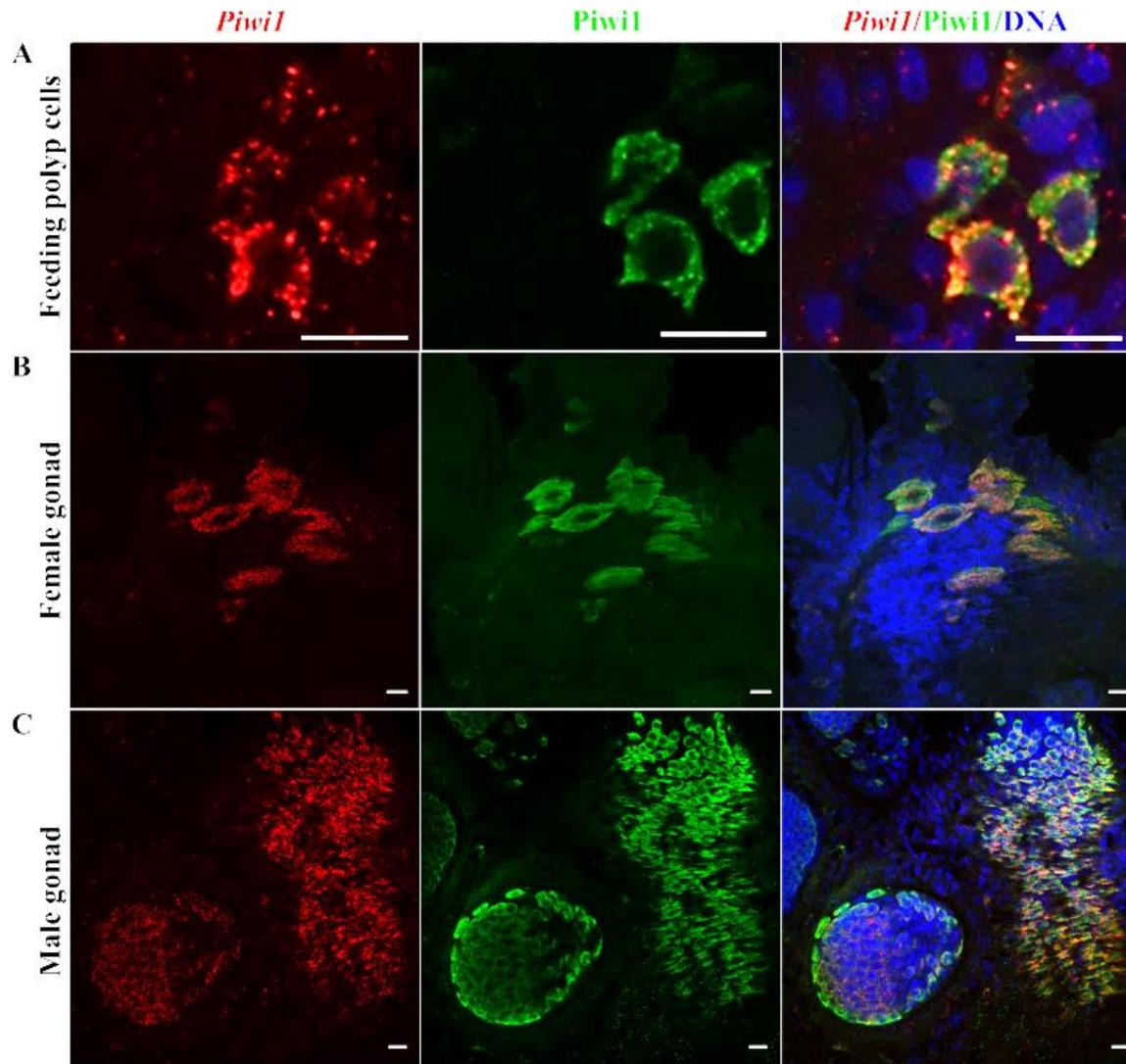


Figure 3.12. *Piwil* mRNA and *Piwil* protein expression analysis. (A) Double FISH and IF close up showing both *Piwil* mRNA (red) and protein (green) are co-expressed in i-cells in feeding polyps. (B-C) *Piwil* mRNA (red) and protein (green) are also co-expressed in female and male gonad germ and stem cells, as can be seen with the yellow colour in the merge images on the right. Scale bar equals 10 μ m.

3.6.2 Larva

In the early larva stage of 48 hpf, *Piwi2* mRNA expression was also found to be present within cells in the gastroderm (Figure 3.14A). These *Piwi2*⁺ cells were mainly detected as single cells and located below the anterior pole (future aboral region) of the larvae.

3.6.3 Feeding polyp

At this stage of development, the polyp has matured and many more *Piwi2*⁺ cells can be seen in the body column of feeding polyps by IF in the band region known to be highly proliferative within *Hydractinia* (Bradshaw et al., 2015) (Figure 3.11A). *Piwi1* and *Piwi2* proteins are co-expressed within the same cells in adult feeding polyps (Figure 3.11A). *Piwi2* mRNA expression was also analyzed in adult feeding polyps (Figure 3.14) and shares the same expression profile as *Piwi1* mRNA as previously characterized (Bradshaw et al., 2015).

3.6.4 Male and female

As mentioned previously, the expression of *Piwi* proteins are enriched in the germline of many animals. Therefore, it was unsurprising to find a high level of *Piwi2* mRNA expression within male and female gonads (Figure 3.13B-C). This corresponds to *Piwi1* expression in male and female gonads (Figure 3.8B-C). The *Piwi2* protein expression profile also matches closely to that of *Piwi1* protein (Figure 3.5 and 3.11B-C). In males there were high levels of *Piwi2* mRNA detected in spermatogonia within developing gonads and in spermatogonia migrating through the body column into forming gonads (Figure 3.13B') as shown with *Piwi1* expression. *Piwi2*⁺ cells were also detected in i-cells present in the epidermis of male gonads (Figure 3.11B). This population will give rise to both gametes and to specialized somatic cells such as epithelia and neurons/nematocytes.

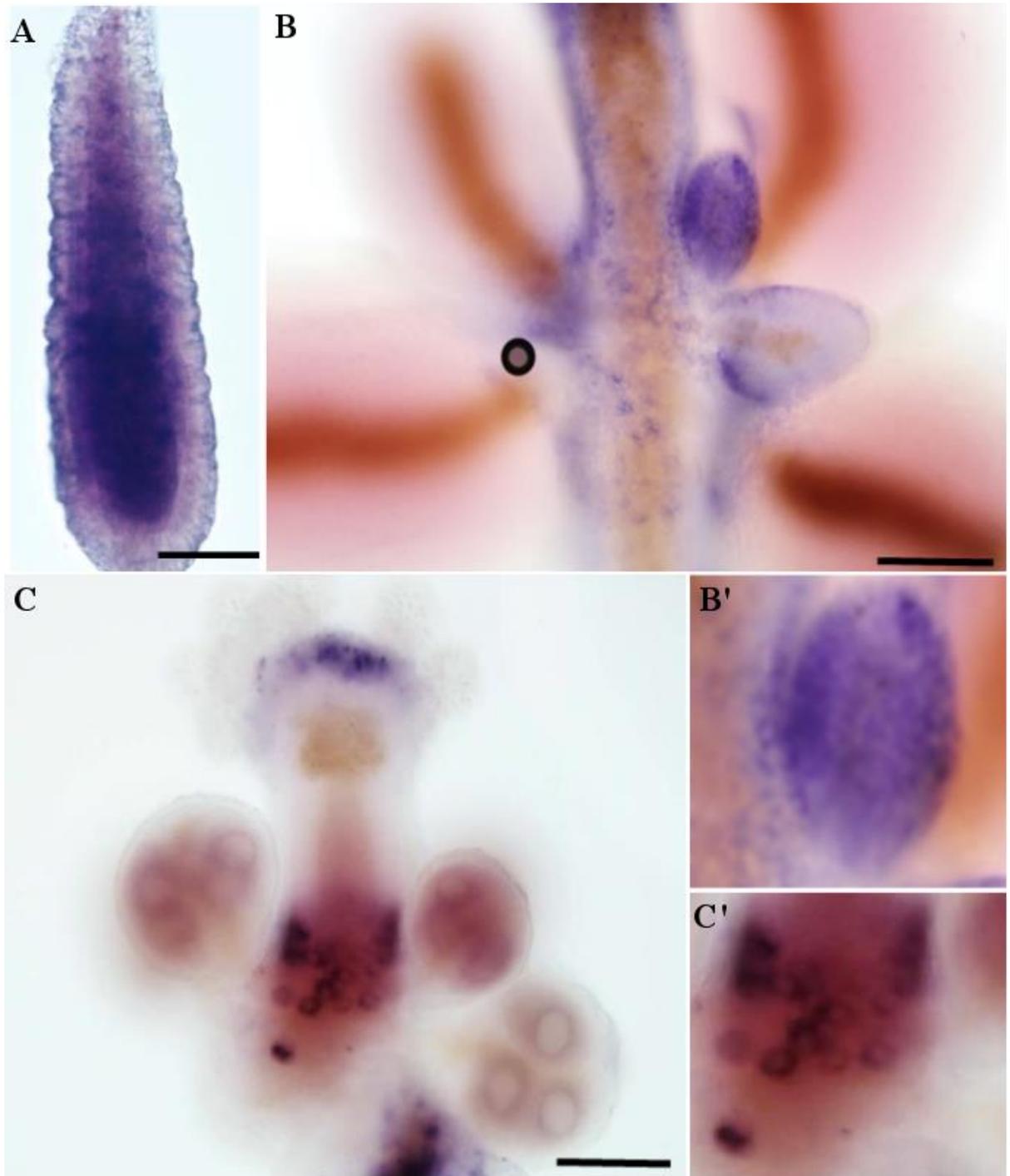


Figure 3.13. *Piwi2* mRNA expression by *in situ* hybridization. (A) *Piwi2* Expression in the gastroderm of a 48 hpf larva. (B) Expression in the epidermis and (B') higher magnification showing gametes of a male gonad. (C) Expression in the female gonad in the epidermis and (C') higher magnification showing gametes of a female gonad. Scale bar equals 100 μ m.

The same expression analysis was carried out in female sexual gonads and similar patterns of *Piwi2* gene expression were observed (Figure 3.13C,C'). In female sexual polyps *Piwi2* protein was detected in both oocytes within developing gonads and in oocytes migrating through the body column in the direction of forming gonads (Figure 3.11C). As seen in males, *Piwi2*⁺ cells were also found in the body column of females in i-cells that were not developing oocytes. *Piwi2* is also a cytoplasmic protein within male and female gonads and epidermal stem cells of *Hydractinia* (Figure 3.11B-C).

All WISH and IF experiments carried out on the different *Hydractinia* life stages were performed with sense RNA probes and secondary only antibody controls. Only non-specific secondary antibody staining was observed in the control IF experiments of embryos and larva in particular.

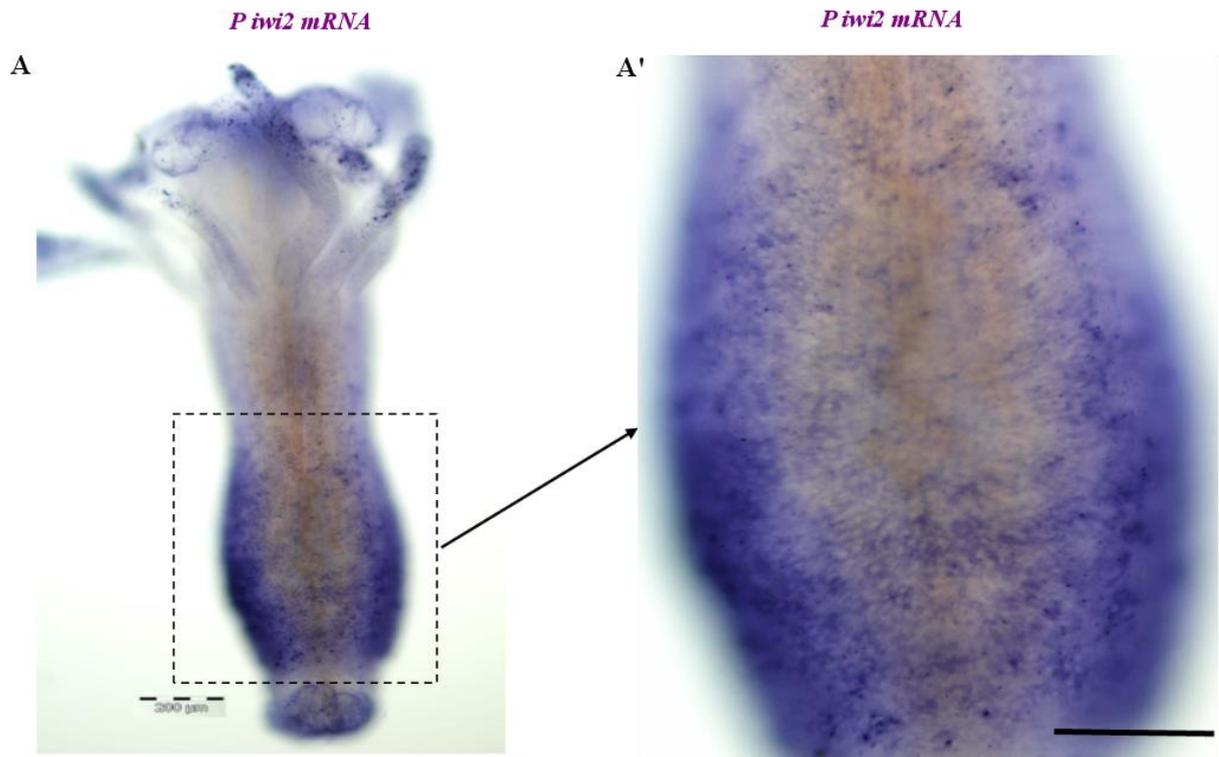


Figure 3.14. (A) *Piwi2* mRNA in adult feeding polyp. (A') Close up of adult feeding polyp showing cytoplasmic *Piwi2* mRNA expression in the body column. Staining present in head is background due to the sense staining. Scale bar equals 100 µm.

3.7 Piwi1 protein and *Piwi2* mRNA co-expression analysis

I have already shown that Piwi1 protein and *Piwi1* mRNA are co-expressed within the same cells in germ cells (Figure 3.12). I have also shown that both PIWI proteins are co-expressed within the same cells (Figure 3.5 and 3.11). There is a poor correlation between mRNA and its relevant protein level. It's often better to check both levels rather than rely on one alone. It can be reasonable to see a high protein level accompanied with high mRNA level, but the protein concentration is affected by many steps in transcription and translation as well as degradation. Basing conclusions on mRNA levels only should be strictly avoided as mRNA levels are often not reflected in protein levels. Even Protein levels do not tell us a lot, since their activity heavily depends on post-translational modifications. In order to test if *Piwi2* mRNA is co-expressed with PIWI proteins I carried out another double fluorescent *in-situ* hybridization of *Piwi2* mRNA with the Piwi1 protein (IF). Since both PIWI proteins are shown to share a similar expression profile (Figure 3.7 and 3.11), I chose to utilize the Piwi1 antibody as it was much more efficient and reliable antibody for the detection of PIWI protein.

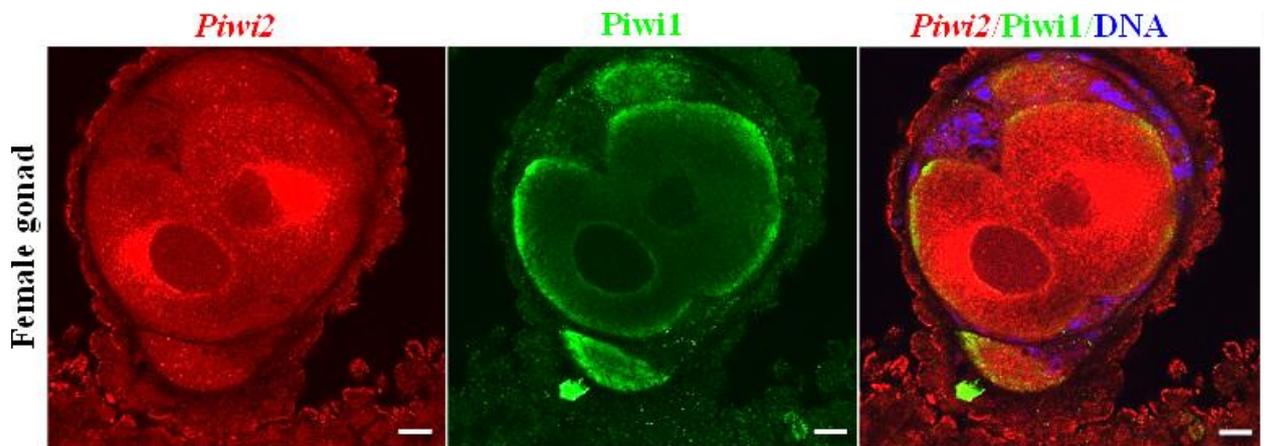


Figure 3.15. Piwi1 protein (green) and *Piwi2* mRNA (red) are co-expressed in the same cell types of a female gonad tissue in *Hydractinia*. Scale bar equals 10 μ m.

3.8 Piwi1 and Piwi2 protein co-expression analysis

I have been able to show the separate expression patterns of Piwi1 and Piwi2 protein in several different life stages of *Hydractinia* development. I therefore wanted to prove further that these two proteins share conserved roles by carrying out double IF staining on different *Hydractinia* life stages to show these two proteins co-localize to the same cells (Figures 3.5 and 3.11). While there were some issues with background signal in the Piwi2 antibody, it appears that both proteins share the same expression location (why not conclusively co-localized in all stages) in the cytoplasm of cells in all of the life stages tested in *Hydractinia*.

3.9 Piwi1 protein expression during metamorphosis

The expression pattern analysis of protein and mRNA for both *Piwi* genes show that they are co-expressed within the same cells. Therefore I wanted to utilize the Piwi antibodies to further characterize the dynamics of PIWI protein expression while undergoing metamorphosis. During metamorphosis, which lasts for only 24 hours, the larva undergoes major changes in order to become a primary polyp. During this period, $Piwi^+$ i-cells change their location from the gastroderm during embryonic/larvae development to the epidermis where they will stay during adult life (Figures 3.5-3.7). In order to fully characterize these $Piwi1^+$ cells during this stage of development I carried out IF on larva using the Piwi1 antibody at several stages after they had been induced to metamorphose in CsCl. 4 different time points post CsCl incubation were chosen to analyze Piwi1 protein expression (3 hours, 5 hours, 8 hours and 12 hours) (Figure 3.16A-D). Epidermal regions were previously defined by DIC, phalloidin and density of DAPI-stained nuclei in metamorphosing larva (Figure 3.17). Images taken at these time points prove Piwi1 expression changes gradually over a 12 hour period from gastroderm to ectoderm in metamorphosing larva.

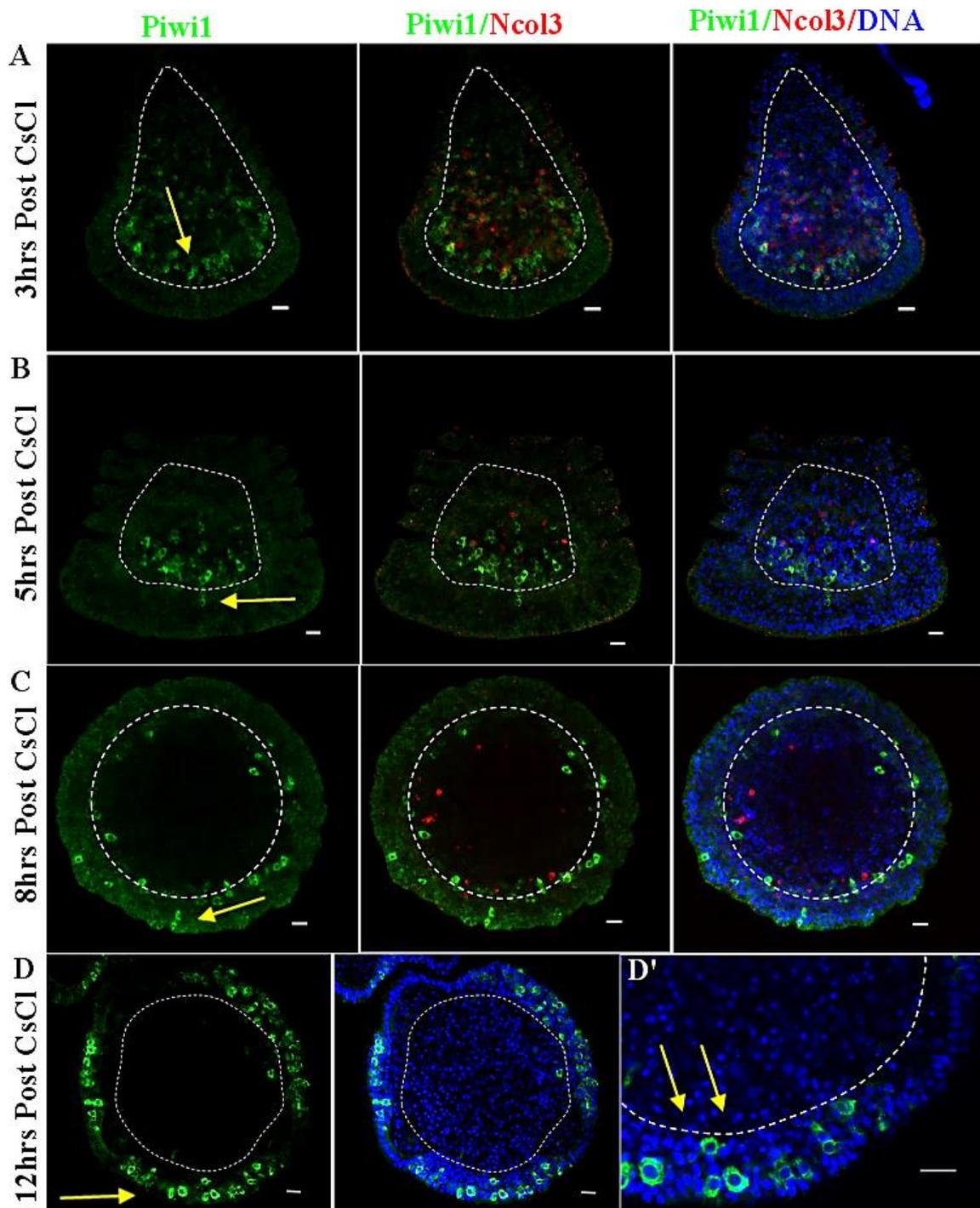


Figure 3.16. Expression of Piwi1 and Ncol3 during larva metamorphosis into a primary polyp. Ncol3 (a minicollagen present in the nematocyst capsule at all stages of maturation) was utilized during this experiment as a control as it remains in the gastroderm of metamorphosing larva. (A) 3 hours post CsCl, Piwi1⁺ i-cells are located exclusively in the gastroderm of larva, indicated by yellow arrow. Ncol3 (red) is also present in the gastroderm. (B) 5 hours post CsCl, some Piwi1⁺ i-cells

appearing in the epidermis of the metamorphosing animal (yellow arrow), while the majority of $Piwi1^+$ i-cells still remain gastrodermal. $Ncol3$ remains in the gastroderm. **(C)** By 8 hours post CsCL many $Piwi1^+$ cells are present in the epidermis. $Ncol3^+$ cells remain in the gastroderm. **(D)** At the 12 hour mark post CsCl incubation, $Piwi1^+$ cells are exclusively epidermal. Dashed lines indicate the mesoglea, the boundary between the gastroderm and epidermis. Scale bar equals 10 μm .

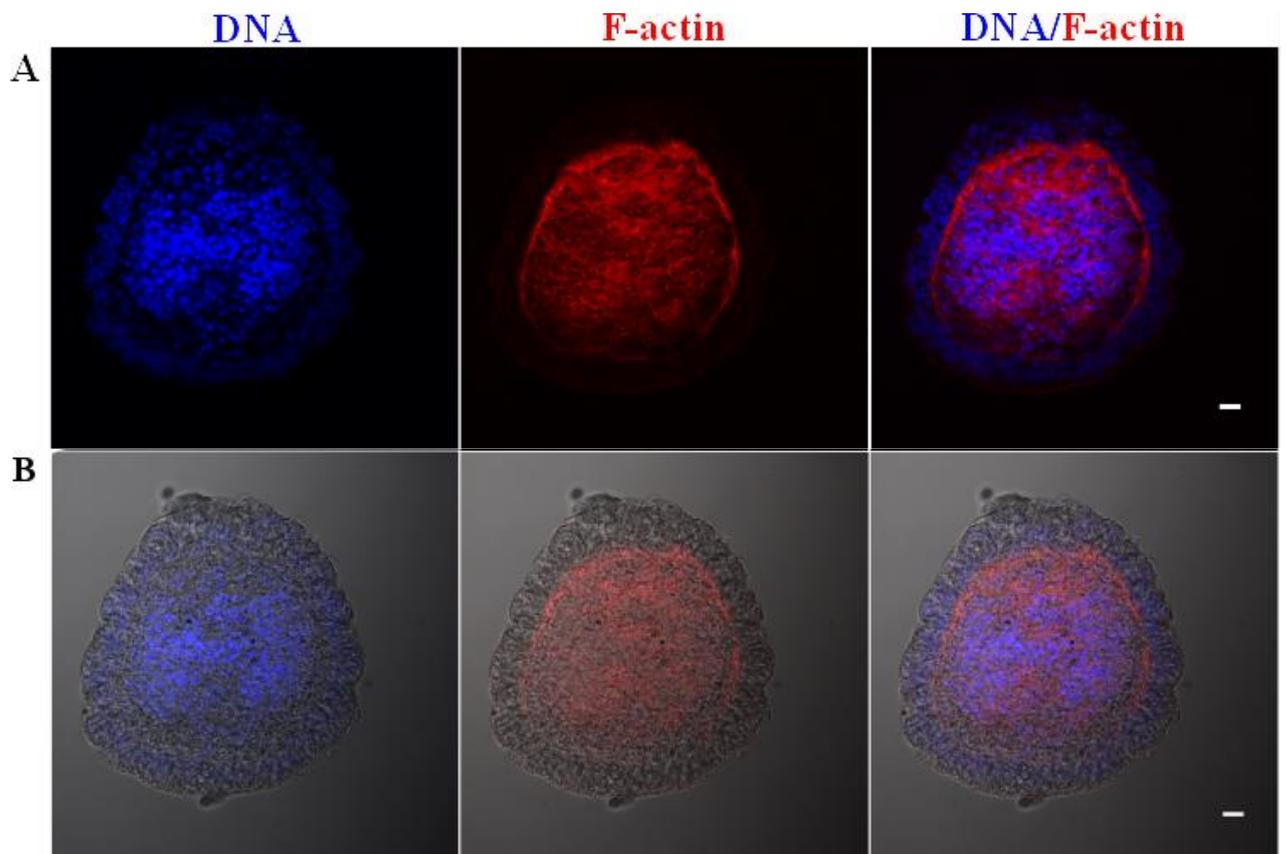


Figure 3.17 IF and DIC images in metamorphosing larva. (A) DNA and F-actin labelling mark separate regions of the larva endoderm and ectoderm. **(B)** DIC images show the F-actin staining circling the boundary of the visible mesoglea layer between the endoderm and ectoderm layers. Scale bar equals 10 μm .

3.10 Piwi1 protein and Vasa co-expression analysis

As both PIWI gene expression patterns overlap in the same cells in all *Hydractinia* life stages, I decided to carry out further co-expression analysis using the Piwi1 protein only. First I wanted to look at other known stem cell markers in *Hydractinia* to further verify the *Piwi* genes as stem cell markers. To do this another known stem cell gene called *Vasa* was used (Rebscher et al., 2008).

The *Vasa* gene is essential for germ cell development in many animals and member of the conserved germline multipotency program ((Juliano et al., 2010)) and has been shown to be restricted to interstitial stem cells (i-cells) and germ cells of *Hydractinia* (Rebscher et al., 2008). *Vasa* was originally (Juliano et al., 2010) identified in *Drosophila melanogaster* as a maternal-effect gene required for the formation of the abdominal segments and for germ-cell specification (Schüpbach and Wieschaus, 1986, Raz, 2000). *Hydractinia Vasa* is shown to be expressed in i-cells of larva, and its expression shifts from gastrodermal to epidermal during metamorphosis (Rebscher et al., 2008), similar to the two Piwi proteins already shown.

To study *Vasa*/PIWI co-expression, FISH (*Vasa*) and IF (Piwi1) was performed to compare expression patterns in female gonads (Figure 3.18A). It is clear from this co-expression analysis that the two genes were co-localized at least in germ cells in *Hydractinia* (Figure 3.18A).

3.11 *Piwi1* protein and *Ncol1* co-expression analysis

I next wanted to analyse the expression pattern of the *Piwi* genes together with differentiated stem cell populations. In order to display the differences in expression of stem cells genes to genes of stem cell progeny, a gene which is expressed exclusively in nematoblasts, i.e., nematocyte progenitors, was chosen. *Ncol1* belongs to the group of minicollagen proteins that form part of nematocyst capsules (David et al., 2008). These nematocysts are an intracellular organelle found in nematocytes of all Cnidaria and are used for capture of prey and defense. The organelle is formed in a post-Golgi vacuole in differentiating nematoblasts (David et al., 2008, Holstein, 1981). *Ncol1* expressing cells are post mitotic i-cell progeny that are morphologically nearly indistinguishable from i-cells. As they differentiate they acquire the typical nematocyst capsule and crescent-shaped nucleus. It was therefore interesting to analyse the expression pattern of the *Piwi1* gene together with *Ncol1*.

To carry out this experiment FISH (*Ncol1*) and IF (*Piwi1*) was utilized to compare the two expression patterns in adult feeding polyps (Figure 3.18B). It is obvious from this co-expression analysis that the two genes never co-localize to the same cells in *Hydractinia* (Figure 3.18B).

3.12 *Piwi1* and *Ncol3* co-expression analysis

As mentioned previously, I wanted to characterize the expression of *Piwi* genes together with differentiated progeny. Having already look at *Ncol1* gene expression, the transitional phase from stem cell to nematocytes, I went on to further look at differentiated cell types such as nematocysts in their final stages of maturation into nematocytes. To do this I utilized the antibody *Ncol3* (gift from Suat Ozbek). *Ncol3* is present in the nematocyst capsule at all stages of maturation, but the epitope for the antibody is only recognizable in the later stages prior to final maturation (David et al., 2008, Tursch et al., 2016). Double IF was carried out to characterize the expression of both proteins in adult feeding polyps (Figure 3.18C). As expected, neither of the proteins were co-expressed within the same cell types, similar to the *Ncol1* expression analysis (Figure 3.18C).

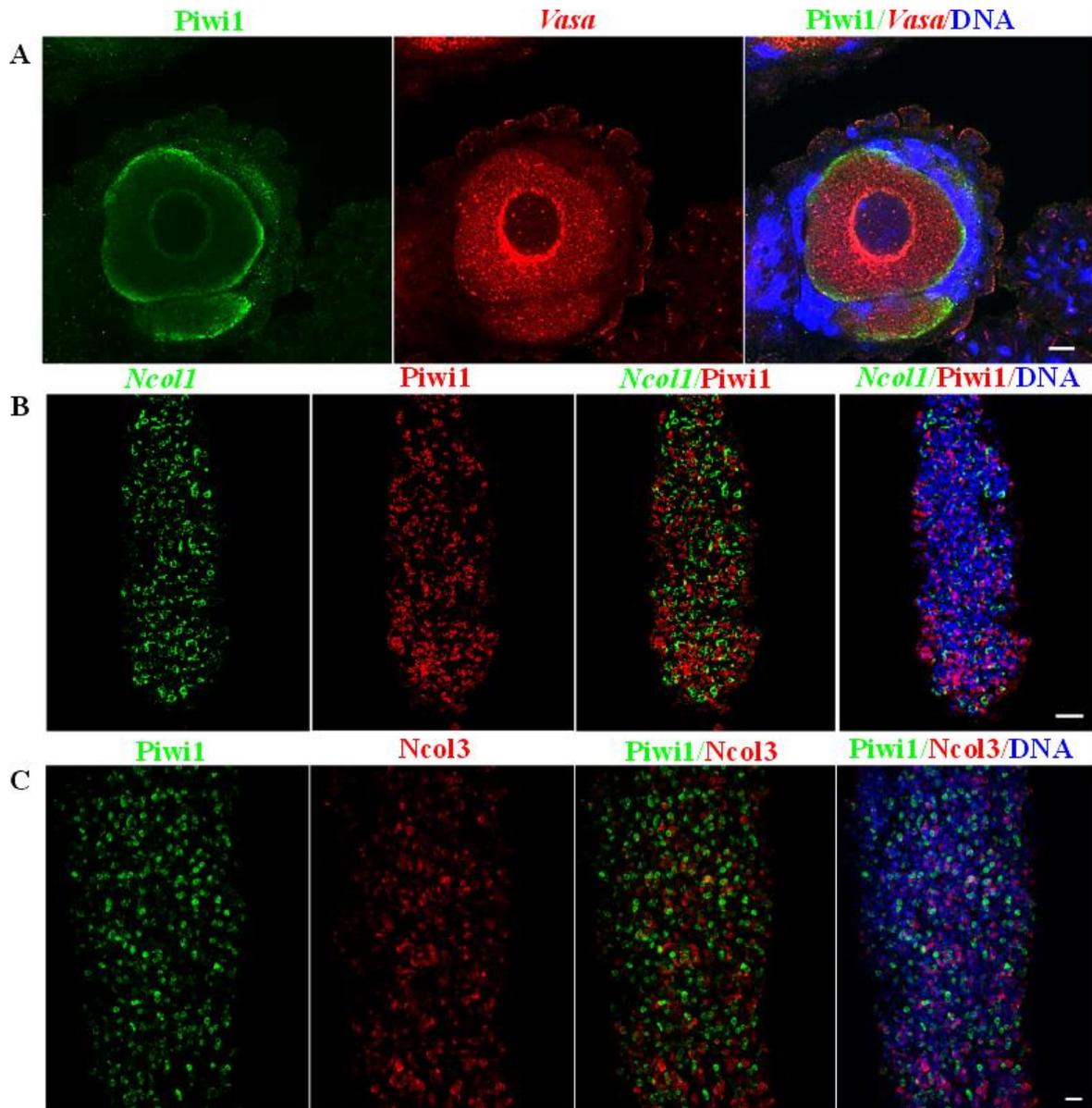


Figure 3.18. Expression analysis of Piwi1 protein with other markers. (A) Piwi1 (green) and *Vasa* (red), another stem cell marker, expression patterns are co-expressed in the same cell types in female gonads. (B) Piwi1 (green) and *Ncol1* (red), a differentiated cell type, expression patterns does not overlap in adult feeding polyp body columns. (C) Piwi1 (green) and *Ncol3* (red) expression patterns does not overlap in adult feeding polyps. Scale bar equals 10 μm .

3.13 Summary

Phylogenetic analysis placed *Hydractinia Piwi1* and *Piwi2* close to both bilaterians such as mice and humans along with other non-bilaterians such as *Aurila aurita*, *Hydra* and planarians. The presence of these *Piwi* genes in cnidarians, in particular *Hydractinia*, suggests that it is ancestral, and that these genes might fulfill a similar role in stem cells across animals.

Until now, the expression profile of PIWI proteins and *Piwi* mRNA was not well studied in *Hydractinia*, except for the *Piwi1* mRNA already recorded by Bradshaw et al. (2015) in adult feeding polyps (Figure 1.4A). Whole mount *in-situ* hybridization is a powerful tool for the detection of gene expression in developmental biology. Utilizing this tool *Piwi1* mRNA was characterized successfully in several stages of development along with *Piwi2* mRNA. Both genes exhibited identical expression profiles in the life stages analyzed. To further confirm the expression patterns of the 2 PIWI genes, two antibodies were generated, targeted against the N-terminal regions of the *Piwi1* and *Piwi2* proteins. The expression profile of both these proteins were confined to the same cell types in all life stages of *Hydractinia* development. The *Piwi1* antibody generated was superior to the *Piwi2* antibody, therefore it was utilized as the main antibody in all following experiments since it co-localizes with *Piwi2* in every stage analyzed.

It is clear from this detailed analysis that *Piwi1*⁺ cells have a dynamic position from early embryos to adult hood. *Piwi1* expression in embryos quickly changes from ubiquitous to endodermal and polar in embryos around 16 hpf (Figure 3.6). These endodermal cytoplasmic cells are present in low numbers by 22 hpf, but by late larva stage (3 days old) they have re-established a large number of *Piwi1*⁺ cells (Figure 3.7A). These cells continue to change their position throughout metamorphosis and gradually migrate to the epidermis of newly forming polyp (Figure 3.7C, 3.9, 3.11 & 3.16). Finally these cytoplasmic *Piwi*⁺ proteins appeared to reside in the epidermal i-cells of adult polyps and gonads, and in developing germ cells of male and female polyps (Figure 3.11). Studies on PIWI proteins in many bilaterian organisms such as mice, flies, and zebrafish, demonstrate high levels of PIWI protein expression specifically in the germ cells of these animals (Lin and Spradling, 1997a, Houwing et

al., 2007a, Carmell et al., 2007). It is clear that *Hydractinia* not only shares this typical characteristic of PIWI protein expression in germ cells, but also possess a unique separate population of stem cell (termed i-cells) that are Piwi⁺, and are capable of giving rise not only to germ cells, but also to somatic lineages during embryonic development and adult life.

Double IF and FISH analysis confirmed both PIWI proteins and mRNA are co-expressed in every developmental stage tested. These double staining experiments also showed that another known stem/germ cell protein, namely Vasa, is expressed in the same cell types as Piwi1 (Figure 3.18A). This co-expression has also been seen in other animals (Tan C-H et al., 2002). More interestingly, double staining of Piwi1 with the early and late nematoblast markers Ncol1 and Ncol3, respectively, demonstrated the loss of any stem cell markers present in these differentiating neural cell types (Figure 3.18B-C).

Chapter 4: Misexpression of Piwi1 in *Hydractinia*

4.1 Introduction

PIWI proteins in *Hydractinia* contain the highly conserved PAZ and Mid domains which together bind small RNAs (Song et al., 2004). The Piwi domain resembles RNase H and is capable of cleaving target RNAs (Okamura et al., 2004). The biological function of the known PIWI proteins, Piwi1 and Piwi2, remains unclear in our model organism as few attempts have been made to carry out targeted genetic approaches such as gain and loss of function studies.

4.2 Ectopic expression in *Hydractinia*

In *Hydractinia*, ectopic expression of desired protein can be achieved in two ways. The first method to do this is by synthesizing polyadenylated mRNAs *in-vitro* and injecting them into zygotes. This results in the ubiquitous expression of the desired protein in a transient manner and can be visualized by the expression of a fluorescent protein such as GFP, if the coding sequence of which is included in the injected mRNA. The second method to achieve ectopic expression of proteins in *Hydractinia* is via the generation of transgenic animals which express a gene under a promoter that is different from the endogenous gene's control elements. This leads to long term but mosaic expression in the animals, since the integration of the transgene does not occur in all cells, and requires breeding the animals to the next generation to obtain non-mosaic expression. This technique has been applied successfully in *Hydractinia* to study patterning and cellular differentiation (Duffy et al., 2012, Millane et al., 2011, Kanska and Frank, 2013). These transgenic tools have been further developed and improved in recent years to include the development of stable ubiquitous expression of GFP in all cell types along with use of more fluorescent proteins such as tdTomato and a photo convertible protein called Kaede (Figure 1.4C-D) (Gahan et al., 2016).

4.3 Loss of function

In *Hydractinia* knockdown/knockout has been achieved by using double stranded RNA-mediated interference (RNAi) induced knockdown. *Hydractinia* cells take up soluble molecules from their environment, including nucleic acids allowing soaking treatment rather than injection. The idea behind RNAi is to degrade the messenger RNA before it can get to the ribosomes, therefore preventing translation. It was first identified in *Caenorhabditis elegans* in 1998 (Fire et al., 1998). The process of RNAi is a complex cellular machinery and is distinct from antisense-mediated hybridization to RNA and interfering with translation. The actual silencing of the mRNA occurs when short fragments of double stranded RNA activate endogenous ribonucleases that target and break up homologous mRNA (Agrawal et al., 2003). It is a natural process of gene silencing in the organism, which is thought to be a defensive mechanism against invading RNA viruses. RNAi has been successfully introduced into the animal by soaking (Duffy et al., 2010) and also by microinjection (Plickert et al., 2004). This soaking technique has been utilized in knocking down Piwi1 during regeneration of *Hydractinia* (Bradshaw et al., 2015) and has shown that Piwi1 is necessary for head regeneration.

The first loss of function study on a PIWI protein was carried out in *Drosophila* where it was shown to be essential in germline stem cell maintenance (Lin and Spradling, 1997a). Further studies have gone on to demonstrate its role in the maintenance of germline stem cells through transposon silencing in models such as mice and zebrafish (Carmell et al., 2007, Houwing et al., 2007a, Aravin et al., 2007a, Juliano et al., 2011). Gene loss of function has also been utilized to study PIWI protein function in the sea urchin *Strongylocentrotus purpuratus* through the use of morpholino oligonucleotide injection (Yajima et al., 2014). This study demonstrated that knockdown of the Piwi homologue in *S. purpuratus* (Seawi) reduced the specific localization of Seawi protein to primordial germ cells and altered the expression of other germ line markers such as Vasa, consistent with a role of Seawi in germ cells. In cnidarians, the first, and so far only, study on PIWI proteins was carried out in the hydrozoan *Hydra*. These authors have demonstrated through knockdown studies by RNAi of *hywi* (orthologue to *Hydractinia Piwi1*) that it is an essential gene in the epithelium of *Hydra* (Juliano et al., 2014).

4.3.1 CRISPR/Cas9 technology

Until recently, gene knockout studies have not been possible in non-established model organisms due to the need for laborious establishment of animal specific approaches. The development of a powerful genetic manipulation tool called CRISPR/Cas9 mediated mutagenesis has allowed researchers to carry out genome editing with ease in virtually any model organism. Clustered regularly interspaced short palindromic repeats (CRISPR) provide acquired immunity in bacteria against viruses and plasmids (Horvath and Barrangou, 2010). CRISPR-associated protein (Cas9) is an endonuclease found in bacteria that uses a guide sequence within an RNA duplex, tracrRNA:crRNA, to form complementary base pairs with DNA target sequences. This enables Cas9 to introduce a site-specific double-strand breaks in the DNA in a sequence specific manner (Bhaya et al., 2011, Horvath and Barrangou, 2010). The two RNA molecules can be fused to generate a chimeric single guide RNA (sgRNA) that supports Cas9 cleavage of DNA substrates. The 20-bp *guide sequence* at the 5' end of the sgRNA directly determines the sequence cleaved by Cas9, by forming Watson–Crick base pairs with the DNA target. In addition to this base-pairing interaction, Cas9 must interact with a *protospacer-adjacent motif* (PAM) on the target DNA molecule. The PAM sequence NGG is found directly downstream of the target sequence in the genomic DNA, on the non-target strand and is recognized by Cas9 (Dickinson and Goldstein, 2016). The technique was first applied in cnidarians by the Gibson lab where they showed its utility in *Nematostella* (Ikmi et al., 2014a) and has been subsequently utilized for functional studies by the Technau lab, also on *Nematostella* (Kraus et al., 2016). This technique has also recently been successively applied in loss of function studies in *Hydractinia* (Gahan et al., 2017) by the Frank lab. In the context of loss of function studies CRISPR/Cas9 can be utilized to cause mutations within any particular gene. This formation of mutations, which assuming a frameshift has been generated, can result in a complete loss of function in the affected allele.

4.4 Aims

The aim of this part of the thesis was to study the role of the Piwi1 protein in *Hydractinia* using three genetic approaches:

1. To analyze the effect of *Piwi1* ectopic expression in mature f0 and f1 generation *Hydractinia* colonies.
2. To study the effect of mutations in Piwi1⁺ cells via CRISPR/Cas9 mediated mutagenesis.
3. Piwi1 knockdown through morpholino injection.

4.5 Results - Piwi1 Ectopic expression

In order to study *Piwi1* ectopic expression the entire *Piwi1* coding region was cloned into the $\beta tub::GFP::Actin$ vector using Gibson assembly (section 2.3.8). The coding region of the *Piwi1* gene was very difficult to PCR from cDNA; therefore, it was synthesized as a gBlocks[®] Gene Fragment (section 2.9). This resulted in a Piwi1-GFP fusion protein under the regulation of the β -tubulin promoter, and Actin1 3' untranslated region (Figure 4.1A). This construct is known to be epithelial-specific in *Hydractinia* in our lab (Gahan et al., 2017). Hence, the construct was designed to drive Piwi1 expression vector in epithelial cells, a population of cells where Piwi1 is not normally found (Figure 4.1C). This expression vector was injected into zygotes. The $\beta tub::GFP::Actin$ plasmid containing only GFP was injected as a control. Larva expressing Piwi1 had no obvious phenotype 3 days after injection. Transgenic, GFP⁺ larva from Piwi1 ectopic expression and control injections were selected and metamorphosed. Following metamorphosis, 100% of control injected larva metamorphosed normally and retained their GFP expression (Figure 4.2A).

There was a distinct change in the animals injected with the Piwi1 ectopic construct after metamorphosis. While 100% of the animals were competent for metamorphosis, all of them lost their transgene expression within one to two days post metamorphosis as manifested by fading of the GFP fluorescence in primary polyps (Figure 4.2A). This could either result from the selective death of transgenic cells, or by epigenetic silencing of the transgene. To identify which scenario was true, we first used a pharmacological approach to alter the chromatin structure of the animal's cells. Trichostatin A (TSA), a class I and II histone deacetylases (HDAC) inhibitor (Yoshida et al., 1990), can be used to alter gene expression by interfering with the removal of acetyl groups from histones and therefore altering the ability of transcription factors to access the DNA (Yoshida et al., 1995). TSA has been utilized in our lab to reverse the differentiated state of neurons (Flici et al. 2017), and, similar to my study, reactivate epigenetically silenced transgenes (A. Török, personal communication). TSA was administered at a concentration of 10 μ M to both GFP and Piwi1:GFP transgenic colonies for two consecutive days. The GFP only control colonies displayed little change in GFP fluorescence (Figure 4.2A). The Piwi1:GFP

transgenic colonies, by contrast, showed reactivation of *Piwi1*:GFP expression in a few cells in a tentacle of a primary polyp (Figure 4.2A). These animals remained otherwise phenotypically normal and grew into large colonies that eventually became sexually mature (Figure 4.2B).

The reactivation of the transgene following TSA treatment suggested that these animals were transgenic, but silenced the transgene epigenetically, hence losing GFP fluorescence. To further prove it, genomic DNA was extracted from feeding polyps from two colonies and subject to PCR analysis to see whether the transgene was indeed present in their genome. PCR was carried out using primers that spanned the region from the 5' UTR to 3'UTR of the *βtub::Piwi1::GFP::Actin* construct (Figure 4.3A). Results show that the *Piwi1*:GFP injected colony still contained the transgene, while the GFP only control PCR showed the expected size without the *Piwi1* coding sequence (Figure 4.3B), since GFP control animals do not contain the *Piwi1* coding sequence which is ~2600bp in length. The size of the GFP coding sequence is ~750bp, plus the regions of the 5' and 3' UTRs gave the expected band size after PCR amplification (Figure 4.3). This data indicates that *Piwi1* ectopic expression in epithelial cell population results in the epigenetic silencing of the injected plasmid, indicated by the loss of GFP signal.

After about three months of growth, a *Piwi1*:GFP transgenic (but with a silenced transgene) colony reached sexual maturity and started budding female gonads. These gonads spawned fertile eggs, and following fertilization gave rise to a F1 generation non-mosaic *Piwi1*:GFP transgenic embryos (Figure 4.4A). At 24 hours post fertilization, a small subset of these embryos were ubiquitously fluorescent, providing the final proof that the transgene was present but silent in the post metamorphic F0 animals. The F1 animals carried the transgene in all their cells as expected (Figure 4.4). They continued to fluoresce until they form a 3 day old planula larva. These larva were able to undergo metamorphosis and formed normal colonies but GFP fluorescence faded away within 4 weeks (Figure 4.4).

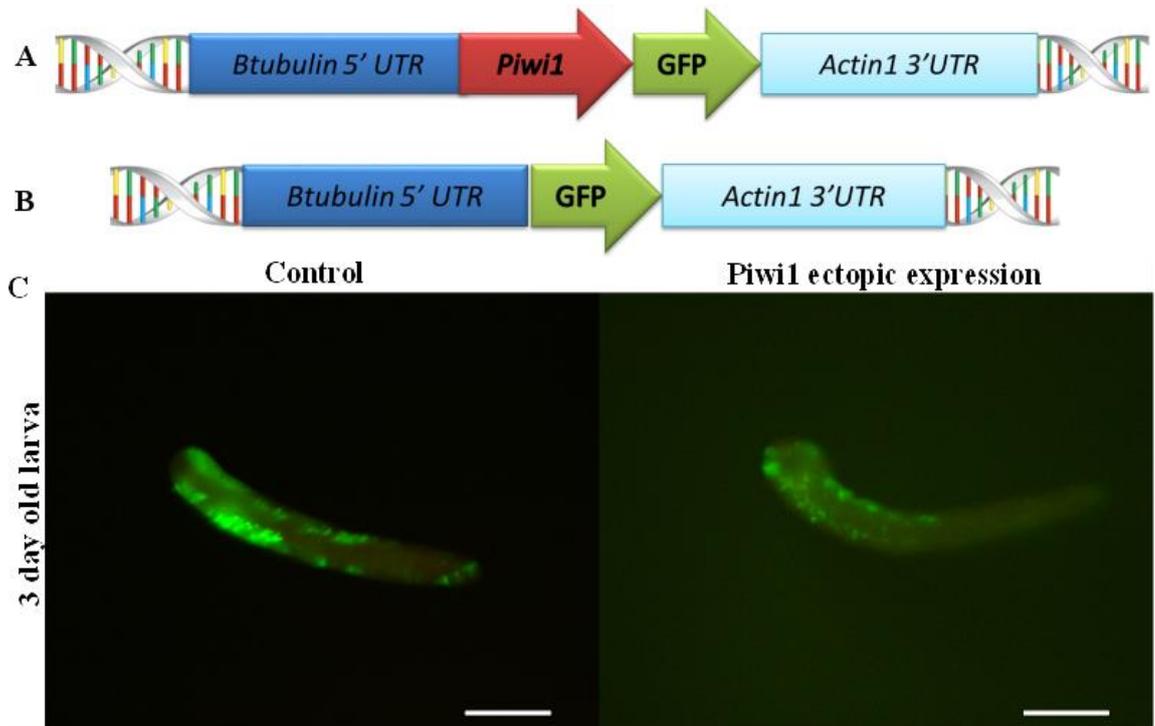


Figure 4.1. Piwi1 ectopic expression plasmid injection. (A) Structure of the Piwi1 ectopic expression construct. (B) Structure of the control plasmid. (C) Larva from both injections were positive for GFP expression. Intensity of GFP fluorescence in transgenic cells is higher in the control Larva. Scale bars indicate 1000 μ m.

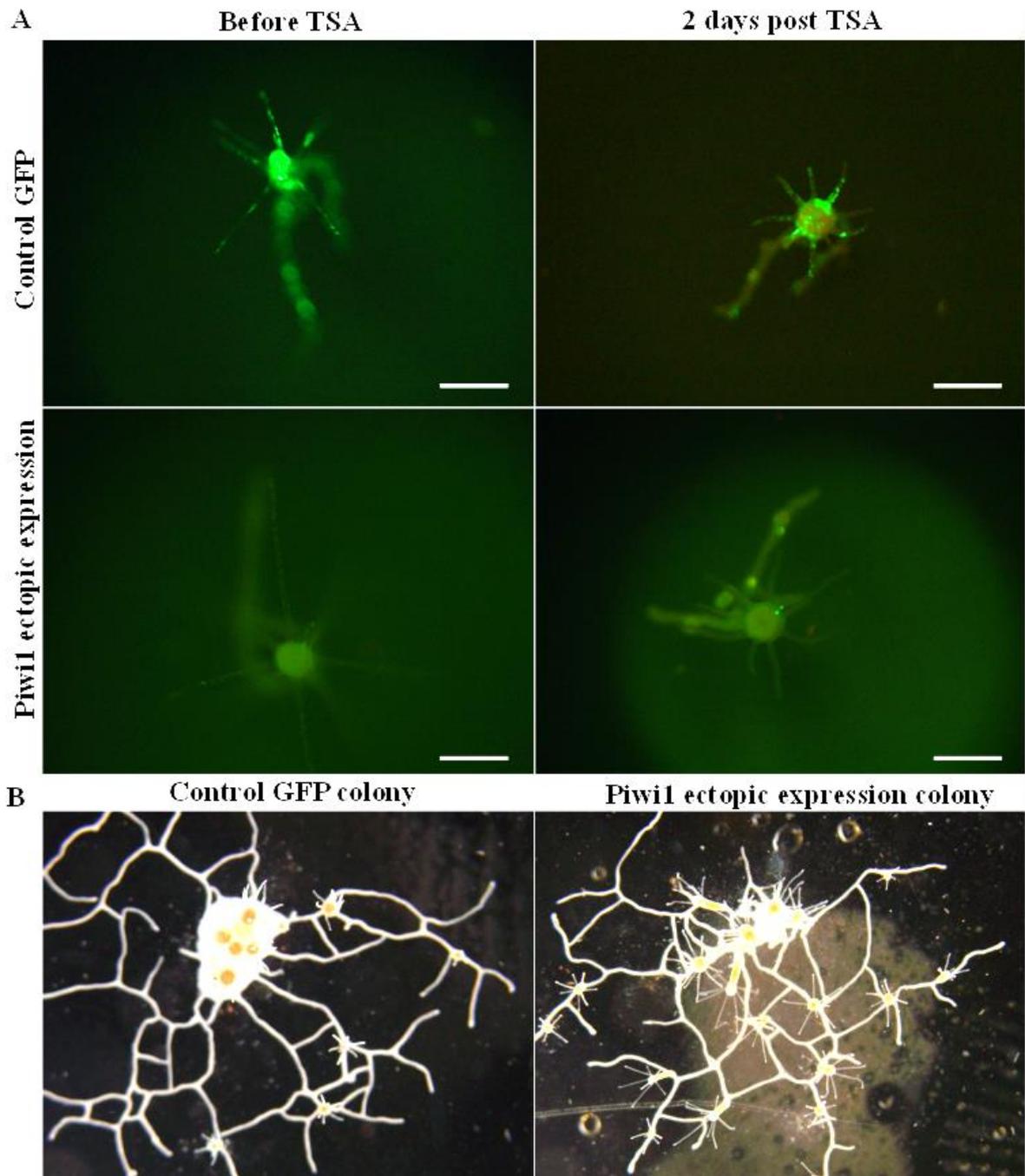


Figure 4.2. (A) Control GFP primary colony retains similar levels of GFP expression before and 2 days after TSA incubation. The Piwi1 ectopic expression colony shows very slight increase of GFP⁺ cells in the head 2 days after TSA incubation. (B) Both the control and Piwi1 ectopic expressing colonies were capable of growing into large normally functioning colonies. Only the GFP control colony maintained its GFP expression. The Piwi1 ectopic expression colony lost its expression again after 2weeks. Scale bars indicate 1000 μ m.

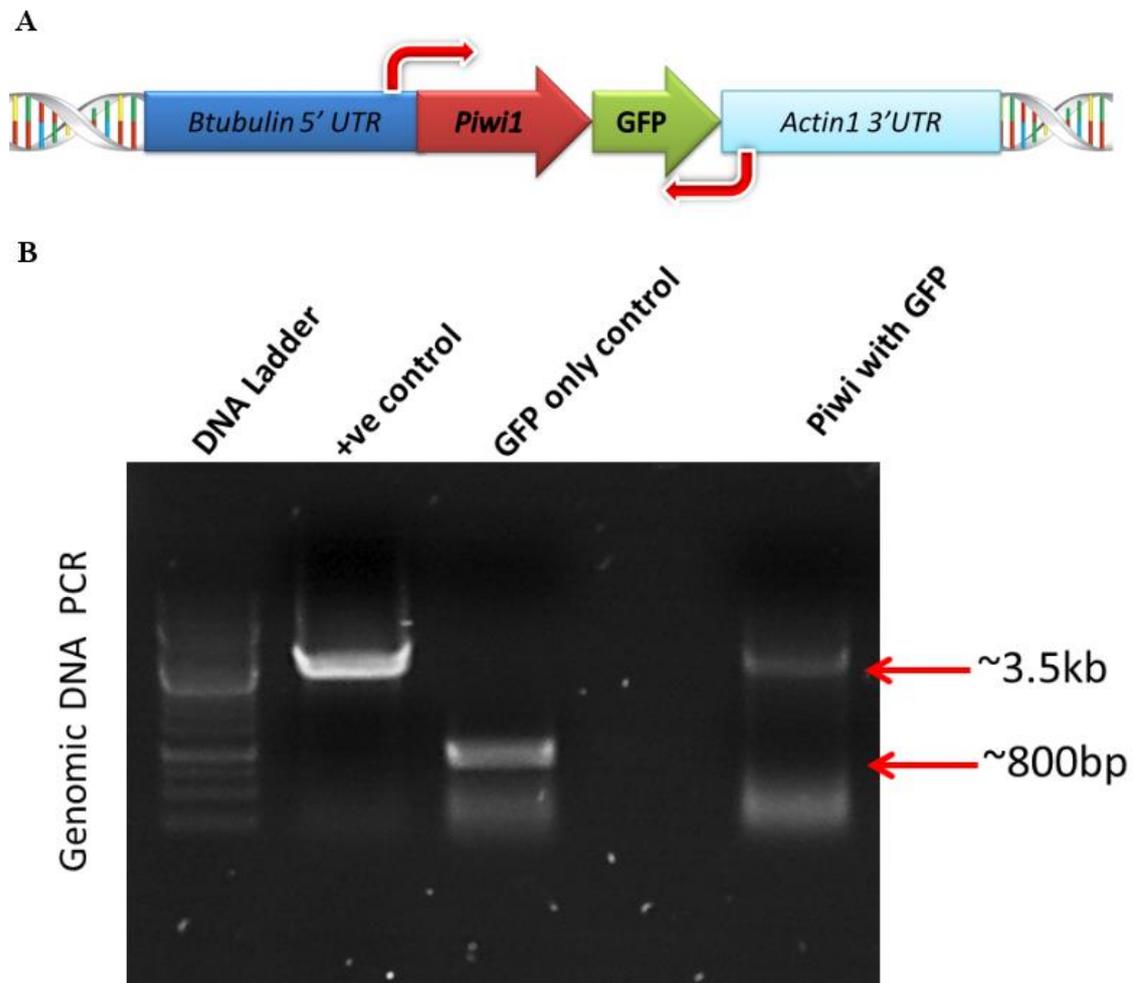


Figure 4.3. Integration testing of the Piwi1 ectopic expressing and control colonies. (A) Red arrows on the schematic of the Piwi1 ectopic expression plasmid indicate the 5' and 3' regions of the plasmid which were amplified by PCR from DNA of control GFP and Piwi1:GFP transgenic animals. (B) Positive (+ve) control lane was the Piwi1 ectopic expression plasmid which gave a band size of 3.5kb; GFP only control animals gave an 800 bp band size and Piwi1:GFP ectopic expression animals gave 3.5 kb band size.

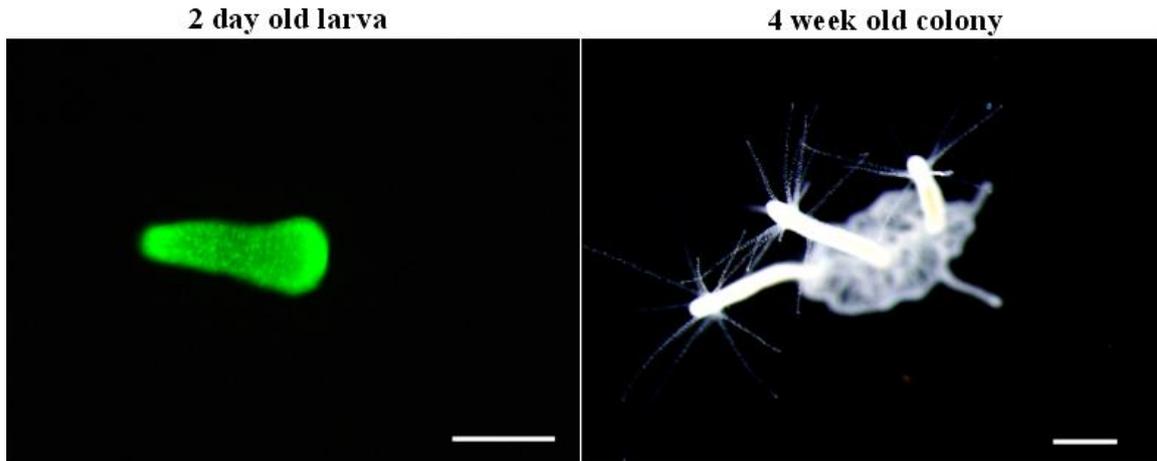


Figure 4.4. Images taken of G1 generation Piwil ectopic animal. Piwil (green) is ubiquitously expressed in a 2 day old larva. The animal is competent to metamorphose into a primary polyp and forms a small colony within 4 weeks. The animal has lost its GFP expression by this time point however, but functions normally and is capable of becoming a sexually mature colony if left to thrive in the appropriate conditions. Scale bar equals 100 μ m.

4.5.1 *Piwi1* mRNA overexpression

As already demonstrated with the generation of integration of the *Piwi1* ectopic expression vector, mosaic transgenic larvae were generated that metamorphosed and grew into adult colonies. These contained the plasmid integrated into their genomes (Figure 4.2B) but kept it epigenetically silenced in F0 animals post metamorphosis. In embryos of the following generation (i.e. F1), the transgene was turned back on, but only during embryonic development and the larval stage. Post metamorphosis the transgene was silenced again. Hence, this approach was not useful for analysing the function of *Piwi1* in adult animals.

Piwi1 is naturally only expressed in i-cells and germ cells in larvae and the adult animal, but appears ubiquitously in early development (Fig 3.2A). I hypothesized that overexpression in early embryogenesis could generate a phenotype that will instruct about *Piwi1* function. Injecting mRNA was the only method that would result in the ubiquitous (albeit transient) gene overexpression without the necessity to generate transgenic animals, bypassing the epigenetic silencing of the transgene. These embryos would express the injected RNA for a finite period of time, usually several days. This tool would be useful for me in studying the function of *Piwi1* during embryonic development and perhaps a few days post metamorphosis as they would be expressed in a ubiquitous manner throughout as visualised by GFP. As this method of overexpression was not established in *Hydractinia*, the generation and injection of GFP mRNA had to be carried out initially. The vector backbone (CS2+) containing a T7 promoter was kindly donated by Dr. Nick Riddiford from Dr. Gerhard Schlosser's lab. The vector map can be found in Appendix B. Codon optimized GFP for *Hydractinia* was generated by PCR with *Eco*R1 and *Bam*H1 restriction sites along with appropriate spacers and a short section of the beta-tubulin 5' UTR. Injection of synthesized *GFP* mRNA resulted in ubiquitous expression in larva (Figure 4.5A), and subsequently disappeared by 2-3 days post metamorphosis. Unfortunately injection of *Piwi1::GFP* mRNA did not give rise to the ubiquitous overexpression of *Piwi1* in embryos for an unknown reason.

4.6 Piwi1 loss of function – CRISPR/Cas9

In order to analyze the effects of Piwi1 loss of function in *Hydractinia*, CRISPR/Cas9 mediated mutagenesis was utilized. This technique had already been applied successfully to carry out Notch loss of function where it was shown that several sgRNAs are much more efficient for *in-vivo* mutagenesis compared to one sgRNA (Gahan et al., 2017). The sgRNAs were designed and targeted against exons two, four and five of the *Piwi1* gene (Figure 4.6A), which were located upstream of the conserved Paz and Piwi coding domains. Out of eight sgRNAs designed and cloned, three sgRNAs proved to be very efficient at cutting template genomic DNA *in-vitro* (Figure 4.6B) and were used for *in-vivo* experiments. These 3 sgRNAs are referred to as sgRNA-1, sgRNA-2 and sgRNA-3.

In order to assess the efficiency of mutagenesis *in-vivo*, all 3 guides were injected together with Cas-9 into zygotes and I extracted genomic DNA from pooled embryos 5 hours post fertilization. Genomic DNA was extracted also from Cas-9 only injected embryos as the control. The DNA from the sgRNA and control embryos was sequenced using primers upstream the sgRNA cut sites (Figure 4.7). When sequencing was carried out on animals that were left to develop into 3 day old larva, no mutations were ever observed and these animals were always capable of metamorphosing into a healthy primary polyp (data not shown).

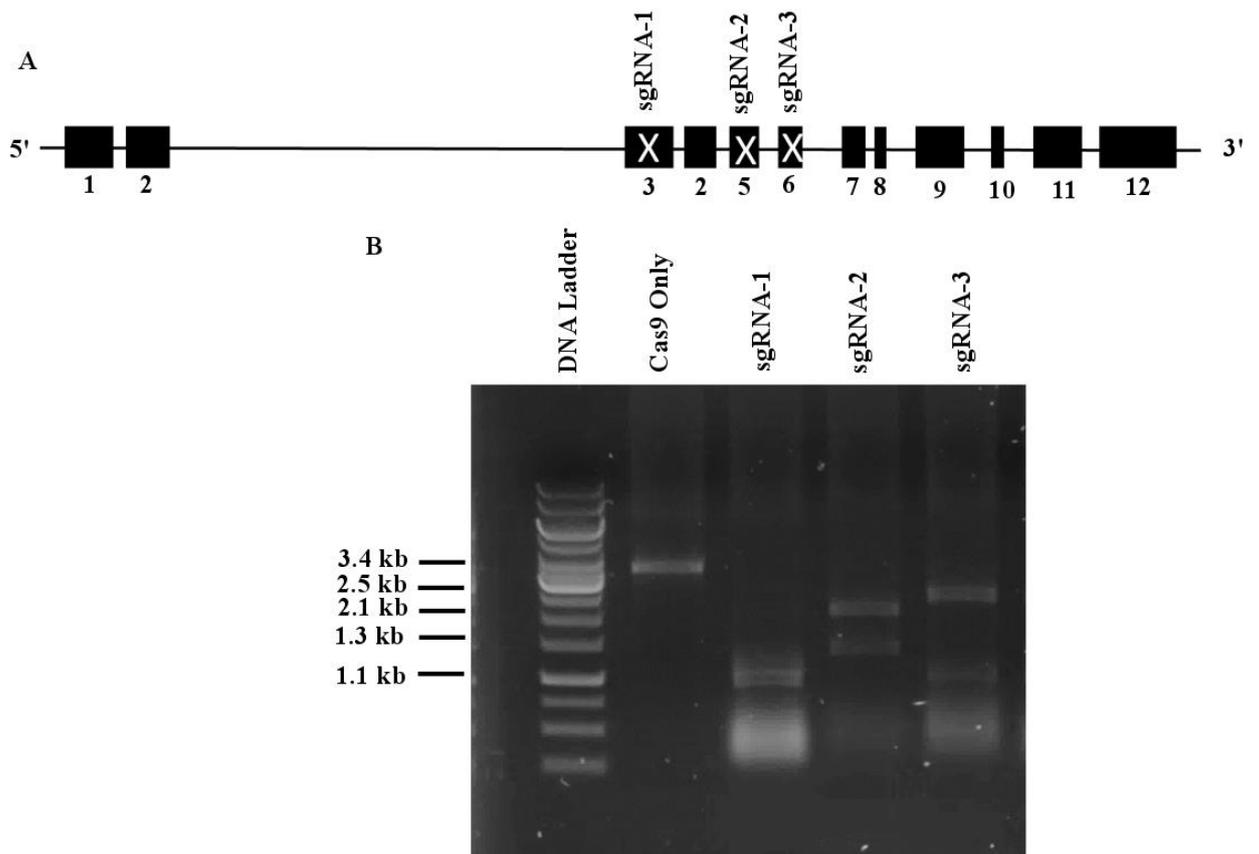


Figure 4.6. *In-vitro* testing of sgRNAs. (A) Schematic of the *Piwil* genomic DNA locus. sgRNAs were designed to target exons 3, 5 and 6 as indicated by the X. (B) *In-vitro* cutting of genomic *Piwil* DNA template with sgRNAs-1, 2 and 3. Cas9 only control shows no cutting of template DNA.

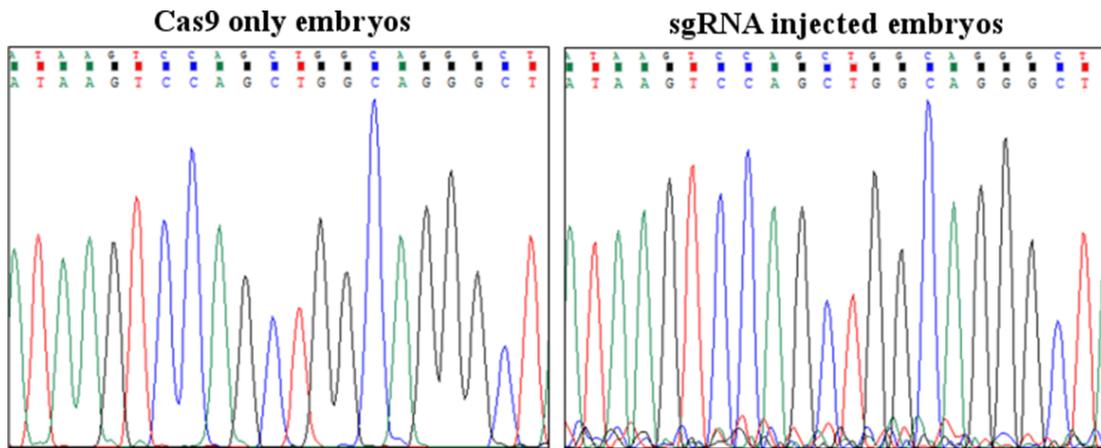


Figure 4.7. CRISPR-Cas9 mutagenesis of *Piwil*. Sequencing chromatogram of the *Piwil* locus around the area recognised by the *Piwil* sgRNA. DNA was extracted from fifty embryos injected with Cas9 only (left panel) and fifty embryos injected with Cas9 plus sgRNA (right panel). Note sequence variability in the Cas9+sgRNA chromatogram, indicating multiple mutations, comparing with the clean sequence of the Cas9 only injected embryos.

4.6.1 *Piwil* Morpholino

Another method to try and assess the effect of *Piwi* loss of function was through the use of translational blocking morpholinos targeted specifically against *Piwil*. *Piwil* morpholino was designed and synthesized by Gentoools and injected at a concentration of 0.5 mM along with a non-coding control morpholino at the same concentration into single cell stage embryos. Animals were allowed to develop in ASW for 2-3 days. No obvious phenotype was observed between the control and *Piwil* morpholino injected animals after these periods. Protein was subsequently extracted from 30 larva that were 2 and 3 days old after being injected with *Piwil* and control morpholinos. Western blot analysis was carried out on the protein extracts to compare the levels of *Piwil* protein between the *Piwil* and control morpholino injected animals. There was no significant change in *Piwil* protein levels at 2 and 3 days post injection in the *Piwil* morphants comparing to the control. Beta-Actin was used as a loading control (Figure 4.8). This chapter shows that this morpholino at the concentration of 0.5 mM is not effective enough to perturb the translation of *Piwil* protein in *Hydractinia* embryos and larvae.

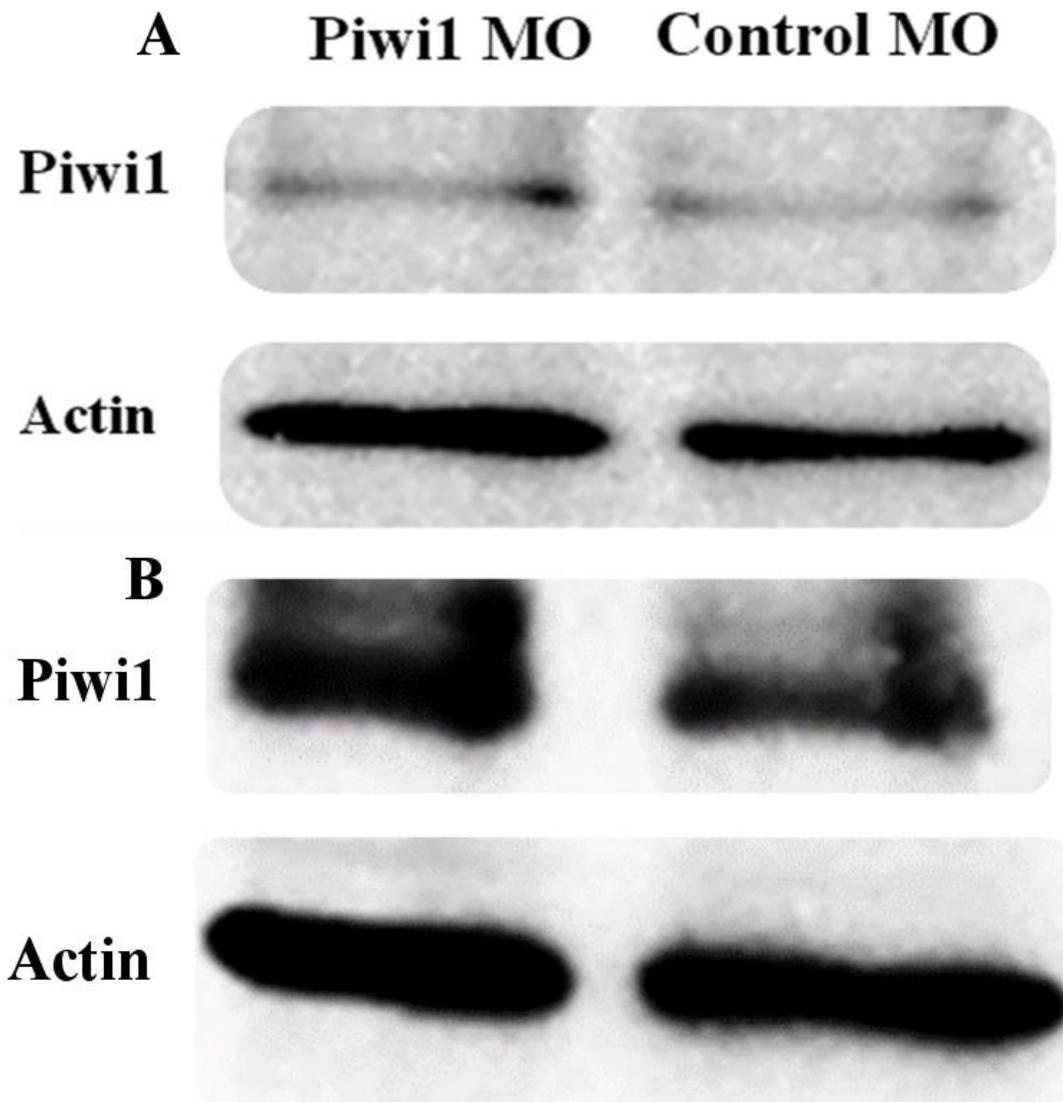


Figure 4.8. Western blot analysis of Morpholino (MO) injections. (A) Analysis of protein extracted from 2 day old larva injected with Piwi1 MO and control MO using the Piwi1 antibody revealed no difference in Piwi1 protein levels. Actin was used as a loading control. (B) Analysis of protein extracted from 3 day old larva injected with Piwi1 MO and control MO using the Piwi1 antibody revealed no reduction in Piwi1 protein levels in the Piwi1 morphant. Actin was used as a loading control. Westerns were analyzed by ImageJ to determine accurate intensities of protein bands.

4.7 Summary

Piwi1 misexpression did not yield any significant results thus far. Ectopic expression gave rise to F0 animals that did not display any obvious phenotype. These F0 animals lost their GFP expression likely through epigenetic silencing, since inhibition of HDAC activity partly restored transgene expression. The colonies formed grew to sexual maturity and gave rise to F1 animals that ubiquitously expressed the *Piwi1::GFP* transgene as seen by GFP⁺ cells in 2 day old larva (Figure 4.4). These F1 animals were able to metamorphose and grow a colony (Figure 4.4); however, they also lost their GFP expression 2-3 weeks after metamorphosis.

CRISPR/Cas9 genome editing technology was utilized in my project to mutate the *Piwi1* genomic coding regions. Targeted mutagenesis of the *Piwi1* gene was carried out by 3 specific guide RNAs that were tested successfully *in vitro* (Figure 4.6B). They also proved to be efficient at cutting *in vivo* when DNA was extracted and sequenced from 5 hour old embryos injected with the guides (Figure 4.7A, B). Sequencing on 2 day old larva injected with the same three guides did not detect any mutations.

4.8 Future experiments

The generation of these *Piwi1* overexpressing f1 animals opens up a whole new avenue of future study. These progeny express the transgene in a stable ubiquitous manner, thus giving rise to more extensive PIWI protein study in the long-term, along with the analysis of other stem cell markers that may be affected such as VASA and SoxBI. These animals could also be used in RNA seq analysis experiments to identify possible transcripts that may be affected by the ectopic expression of *Piwi1*.

The results of *Piwi1* mutation via CRISPR/Cas9 and translational blocking by morpholino injections were preliminary, therefore repeat experiments would be required before any major conclusions should be drawn. Should the experiments be replicated successfully and null phenotypes observed, further loss of function through the use of *Piwi2* mutagenesis together with *Piwi1* may be a possible area for experimentation.

Finally, further experiments involving the CRISPR/Cas9 technology such as endogenously tagging the gene of interest could also be carried out. This would generate a more stable and reliable lineage marker for *Piwi1*, as compared to the conventional and random method of plasmid integration. This method is currently optimized in the Frank lab and will provide a much more reliable source of lineage tracing. $Piwi1^+$ GFP⁺ tagged cells could be easily and reliably sorted by Fluorescence activated cell sorting(FACS) and collected for RNA extraction and sequencing. This would allow us to identify particular transcripts associated with the *Piwi1* gene and open up avenues for other sequencing of $Piwi1^+$ cells after knockdown experiments.

Chapter 5: Analysis of Piwi1 associated piRNAs

5.1 Introduction

Small RNAs are known to be major regulatory factors of gene expression through many different mechanisms, for example through the regulation of transposon activity (Aravin et al., 2007a). As such, significant percentages of piRNAs from *Drosophila*, *C. elegans*, mice and zebrafish are derived from intergenic regions containing transposons and repetitive sequences (Batista et al., 2008, Houwing et al., 2007a, Malone et al., 2009, Wu et al., 2010). The types of transposons vary dramatically in vertebrate species, ranging from a few to over a hundred (Malone and Hannon, 2009). In *Drosophila*, there are approximately 150 different TE types, which have different expression, replication, and mobilization strategies. The regulation of transposons by piRNAs in diverse organisms has been well studied and is proven to act as regulators of genomic integrity, primarily in the germline (Juliano et al., 2011). What is interesting is that the reports on animals which are widely separated on the phylogenetic tree of life show that piRNAs were also found outside the gonadal tissues. Some of these piRNAs map to TE sequences, prompting the hypothesis that genomes may have co-opted the TE-derived piRNA system for their own regulation (Sarkar et al., 2017). There is also emerging evidence that the piRNA pathway can play roles beyond TE silencing and are involved in diverse cellular processes from mRNA regulation to development or genome rearrangement (Peng and Lin, 2013). The Piwi-piRNA pathway can have different modes of regulation depending on its proteins localization. *Drosophila* Piwi is mainly nuclear, giving rise to epigenetic marks on chromatin (Le Thomas et al., 2013). Several PIWI proteins are also localized exclusively in the cytoplasm, such as Aub and Ago3 in *Drosophila* and Mili and Miwi in mice (Harris and Macdonald, 2001, Deng and Lin, 2002, Brennecke et al., 2007). This suggests that similar to miRNAs and the AGO family proteins, Piwi-piRNA complexes also play a role in posttranscriptional regulation (Grivna et al., 2006, Kim, 2006, Meister, 2013).

Piwi genes have also been identified in representative species of several non-bilaterian phyla suggesting that PIWI proteins were present in the last common

ancestor of all bilaterians (Alié et al., 2011); (Denker et al., 2008). The best studied metazoan with respect to PIWI is the cnidarian *Hydra*, which also shares cytoplasmic localization of both its PIWI proteins, Hywi and Hyli (Juliano et al., 2014). These non-bilaterian animals have been shown to contain primary piRNAs, and piRNAs with signatures typical of ping pong piRNA synthesis (Berezikov, 2011, Grimson et al., 2008, Moran et al., 2014). The majority of studies carried out to date on PIWI protein function comes from *Drosophila* and mouse, yet it still remains unclear how they maintain GSCs. Non-bilaterians already share a suite of germline genes (e.g. *Vasa*, *Nanos*) in addition to PIWI with higher animals; therefore, it is likely that the molecular functions of PIWI proteins in these stem cells will be conserved (Juliano et al., 2010). The study of non-bilaterians offers new insights into the functions of PIWI proteins in stem cells, and may uncover the most conserved functions of these proteins in stem cells. So far studies on basal metazoans have mainly been restricted to expression analyses; however, cnidarian models such as *Hydra* and *Nematostella* have developed very efficient methods to study gene function (Wittlieb et al., 2006, Ikmi et al., 2014b). A new and emerging cnidarian model, *Hydractinia*, in terms of functional tools available, provides researchers with another opportunity to study Piwi-piRNA functions in these non-bilaterian models (Gahan et al., 2017, Plickert et al., 2012, Rebscher et al., 2008, Török et al., 2016).

5.2 Aims

The aim of this chapter of the thesis was to identify the piRNA signature of Piwi1 and their specific targets in *Hydractinia*. To do this the following experiments were carried out:

1. Extracted piRNAs bound to Piwi1 protein.
2. Carried out small RNA seq analysis of these piRNAs and mapped them to the *Hydractinia* genome to identify target transcripts, and to identify typical features of the piRNA biogenesis pathway in the samples sequenced.

5.3 Piwi1 RNA immunoprecipitation

In order to test for the presence of piRNAs in *Hydractinia* using the Piwi1 antibody, piRNAs were extracted according to the protocol given in section 2.5.4 from a mix of male, female and feeding polyp protein extractions. The quality of this RNA was then validated by running on a denaturing polyacrylamide gel (section 2.5.5). The result of this gel demonstrated that the Piwi1 antibody was capable of binding to the Piwi1 protein which in turn is bound to piRNAs in *Hydractinia* (Figure 5.1).

To increase the yield of piRNAs for small RNA sequencing, three replicates containing a mix of male and feeding polyp protein were extracted from three different *Hydractinia* male colonies. The RNA IP protocol was carried out again up until the step where the RNA was pelleted in isopropanol, washed in 75% ethanol and it was then stored in the -80°C in 100% ethanol until shipping to NIH for library construction and small RNA seq analysis. Samples were shipped over dry ice as pellets rather than diluted in H₂O to prevent any loss or degradation of the sensitive piRNAs.

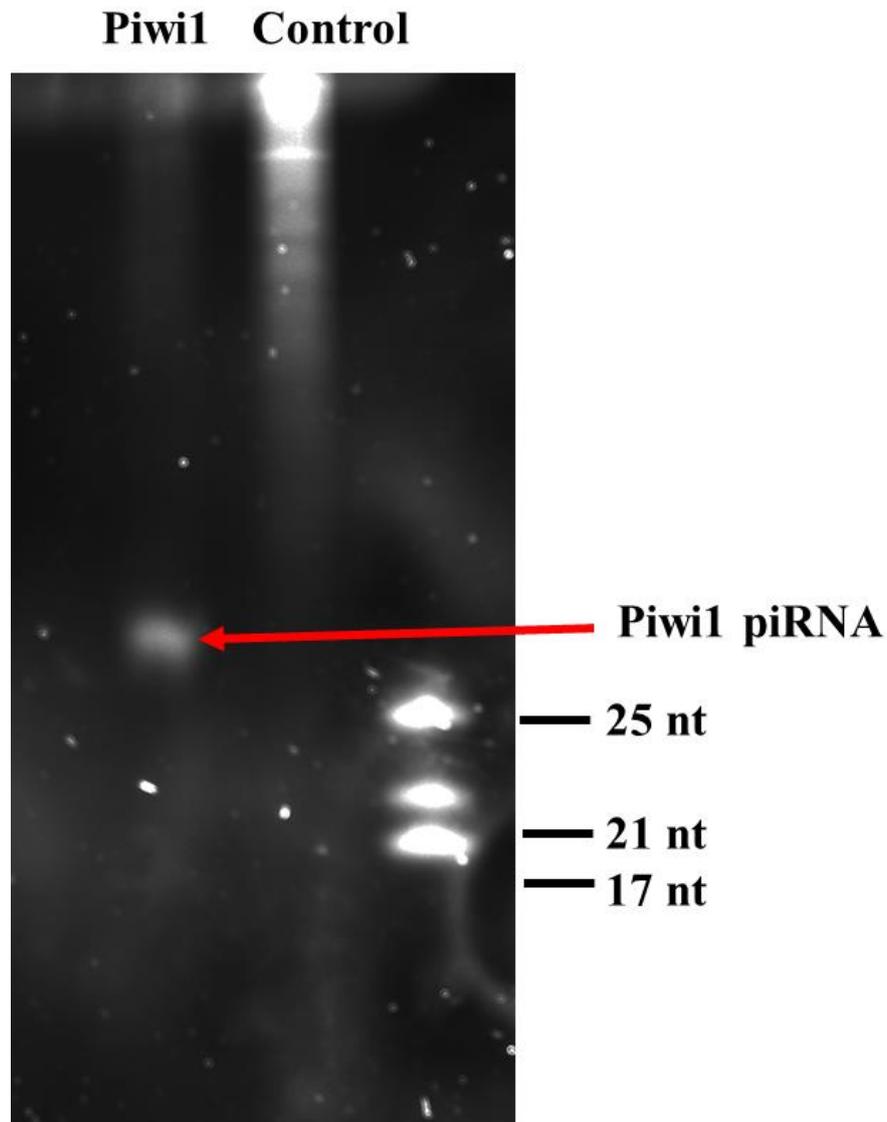


Figure 5.1. Validation of *Hydractinia* piRNAs. A 15% Denaturing polyacrylamide gel showing the quality and size of extracted Piwi1 associated piRNAs in protein mixture of Male and feeding polyps in *Hydractinia* compared to IgG control. The lane labelled Piwi1 contains RNA extracted from male gonads which was immunoprecipitated using the Piwi1 antibody. The faint band detected just above the 25 nucleotide (nt) ladder band (red arrow) demonstrated the success of the RNA immunoprecipitation protocol. No bands were seen in this region for the control RNA, only whole RNAs were detected at the top of the gel as seen by the bright region on the control lane.

5.4 piRNA seq results

All piRNA sample analysis on Piwi1 associated piRNAs was carried out by Alice Young and Christy Schnitzler in the NIH. Out of the three replicates sent for sequencing, two samples contained sufficient RNA to carry out sequencing..

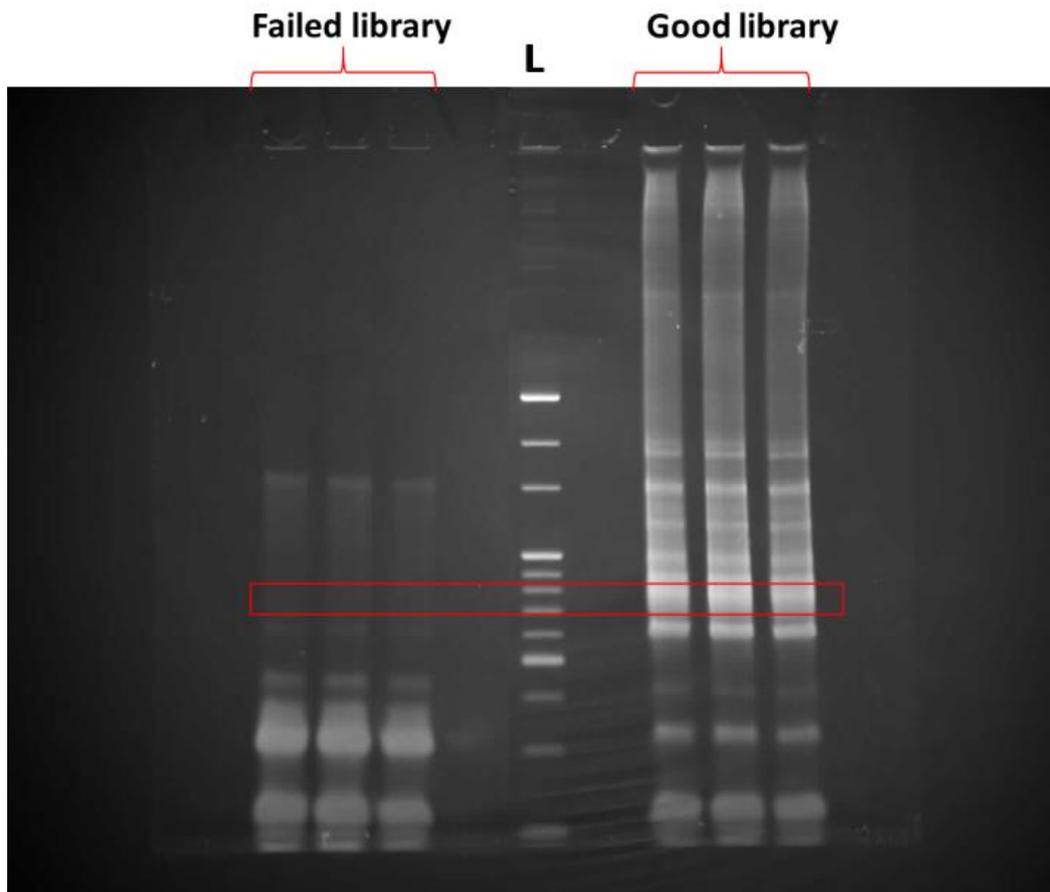


Figure 5.2 Size-selection Gel for piRNA Libraries. Libraries were constructed out of the piRNA samples that were sent to the NIH. The letter L indicates the small RNA ladder used to compare against the failed and good library. These gels show the difference between a sample that didn't work (Failed Library) and one that gave a large amount of small RNA (Good library). *Hydractinia* specific piRNAs present are indicated in the region outlined by the red box.

5.5 Summary

My results demonstrate that there are indeed a population of small RNAs present in *Hydractinia* that bind specifically to the Piwi1 protein. These small RNAs were stable enough to be shipped to the NIH for small library construction and two out of the three samples contained enough data to continue towards sequence analysis (Figure 5.2). Unfortunately the sequence analysis of these small RNAs and the bioinformatics could not be completed in time before completing this dissertation. The analysis of these sequences would include mapping of the reads to the *Hydractinia* genome and transcriptome to identify which genes they target. The results of my thesis suggest that there are no nuclear PIWI's in *Hydractinia*, therefore we don't expect to find targets in non-transcribed loci.

5.6 Future experiments

Once the sequence analysis has been carried out on the piRNAs, two major questions remain: first, do *Hydractinia* piRNAs target also protein coding genes or primarily transposons? It would be interesting to study by qPCR if Piwi1 knockdown causes up regulation of transposons. This could be carried out in any life stage of *Hydractinia* development. It would also be interesting to see if the same would happen to protein coding gene targets (if there are any) if they are unregulated. Second, is the piRNA pool the same in i-cells and germ cells? Finally, what would a Piwi2 RNA IP yield? The antibodies I generated in the course of my thesis work, and the RNA IP protocol I optimized will be instrumental to addressing these questions by others in the future.

Chapter 6: Analysis of i-cell proliferative behavior

6.1 Introduction

Results from the previous gene expression analysis carried out on the two PIWI genes clearly demonstrate their significance as stem cell (i.e. i-cell) marker genes in *Hydractinia*. This is not surprising given the role and expression pattern of PIWI genes in other animals (Juliano et al. 2010). However, treating the PIWI⁺ cells as a single, homogenous population is probably an oversimplification. As shown in planarian stem cell, known as neoblasts, PIWI expressing cells constitute several sub-populations with different developmental potency including pluripotent cells and committed progeny (van Wolfswinkel et al. 2014). *Hydractinia* i-cells have traditionally been regarded a single, pluripotent population (Müller et al. 2004), largely due to the lack of specific markers for i-cell subsets and their proliferative behavior. Hence, the next follow on work in this thesis aims to understand the rate of proliferation of Piwi1⁺ stem cells and thereby provide first evidence for their population complexity in *Hydractinia*. To analyse the rate of proliferation within this population of stem cells, I used two thymidine analogs to label cells in S-phase: EdU (5-ethynyl-2'-deoxyuridine) (Chehrehasa et al., 2009), and BrdU (5-bromo-2'-deoxyuridine (BrdU) (Nowakowski et al., 1989). Both molecules are incorporated into newly synthesized DNA if present during S-phase. Their detection mode is, however, different, enabling double labeling of two consecutive S-phases in one cell. I also used a third cell cycle marker, phosphorylated histone 3 (pH3), which is present in mitotic cells.

EdU detection is based on a copper catalyzed covalent reaction between an alkyne contained in the EdU and an azide contained in the Alexa Fluor® dye. BrdU is detected by an antibody. The EdU and BrdU procedures are described in sections 2.2.4 and 2.2.5. pH3 is also detected by an antibody. Piwi1 staining was carried out according to Immunofluorescence protocol in section 2.2.3.

6.2 Aims

Given the lack of work on *Hydractinia* i-cell population complexity and proliferative behavior, the overall aim of this chapter was to analyze the cell cycle characteristics of Piwi1⁺ stem cells in different *Hydractinia* tissue compartments. To carry out these cell cycle experiments, analysis was carried out on the Piwi1⁺ stem cell populations in adult feeding polyps during head regeneration, on whole adult feeding polyp colonies and on young colonies and their stolons. Specifically, I analyzed:

1. The recruitment of Piwi1⁺ stem cells during regeneration of feeding polyps and their behaviors in the subsequent day.
2. The rate of proliferation of Piwi1⁺ stem cells using short and long pulse labelling.
3. The presence of very slow cycling Piwi1⁺ stem cell populations.

6.3 Piwi1⁺ cell proliferation during regeneration

Previous work by Bradshaw et al (2015) has shown that proliferative, Piwi1⁺ i-cells migrate from the polyp lower body column to the injury site to form a blastema. However, while demonstrating that these i-cells proliferate during migration and after homing to the stump, this study did not analyze the cell cycle characteristics of Piwi1⁺ i-cells and, hence, I performed EdU pulse chase analysis to study this population of stem cells.

An EdU assay was used (Section 2.2.4) to measure the effect of decapitation on the location and proliferation of Piwi1⁺ cells during regeneration. Feeding polyps were cut from a colony and decapitated at different time points. Before fixation, polyps were incubated in an EdU/ASW solution for 40 minutes. Non decapitated controls from the same colony were also incubated for 40 minutes in an EdU/ASW solution and fixed immediately after to compare the proliferation rates of the populations of Piwi1⁺ stem cells between intact (non-regenerating) and regenerating polyps at different time points (Figure 6.1).

EdU⁺ cells were mainly found in the aboral band region of intact, non-regenerating feeding polyps with very few EdU⁺ present in the head and tentacle region of these intact polyps (Figure 6.1 A). Piwi1⁺ stem cells exhibit the same pattern, as previously described (Bradshaw et al. 2015), with high number of cells present in the aboral band region and very low number in the in head and tentacle region of intact polyps (Figure 3.11A). A considerable change in this pattern was observed at the time point of 24 hours post decapitation (hpd). The number of EdU⁺ cells increased overall and many of them shifted from the aboral band to the newly formed blastema at the regenerating tip of the feeding polyp (Figure 6.1B). There was also an observable increase in the expression of Piwi1⁺ cells within the regenerating tip forming a blastema in this figure. All EdU⁺ cells were also Piwi1⁺ within the blastema. At 48 hpd a new mouth and tentacles formed and the number of EdU⁺ cells present in the regenerating tip remained high, while Piwi1⁺ cells in the regenerating tip declined (Figure 6.1C), suggesting that proliferative cells in the regenerating head 48 hpd were i-cell progeny. Previous published work on proliferation studies during

regeneration in *Hydractinia* have shown that EdU⁺ cells are recruited to the site of blastema formation from the aboral region due to migration and not cellular dedifferentiation (Bradshaw et al., 2015). My study is congruent with Bradshaw et al.'s (2015) results, showing that Piwi1⁺ cells are recruited to the blastema. It also provides new information about the dynamics of i-cells recruitment, proliferation and differentiation during regeneration.

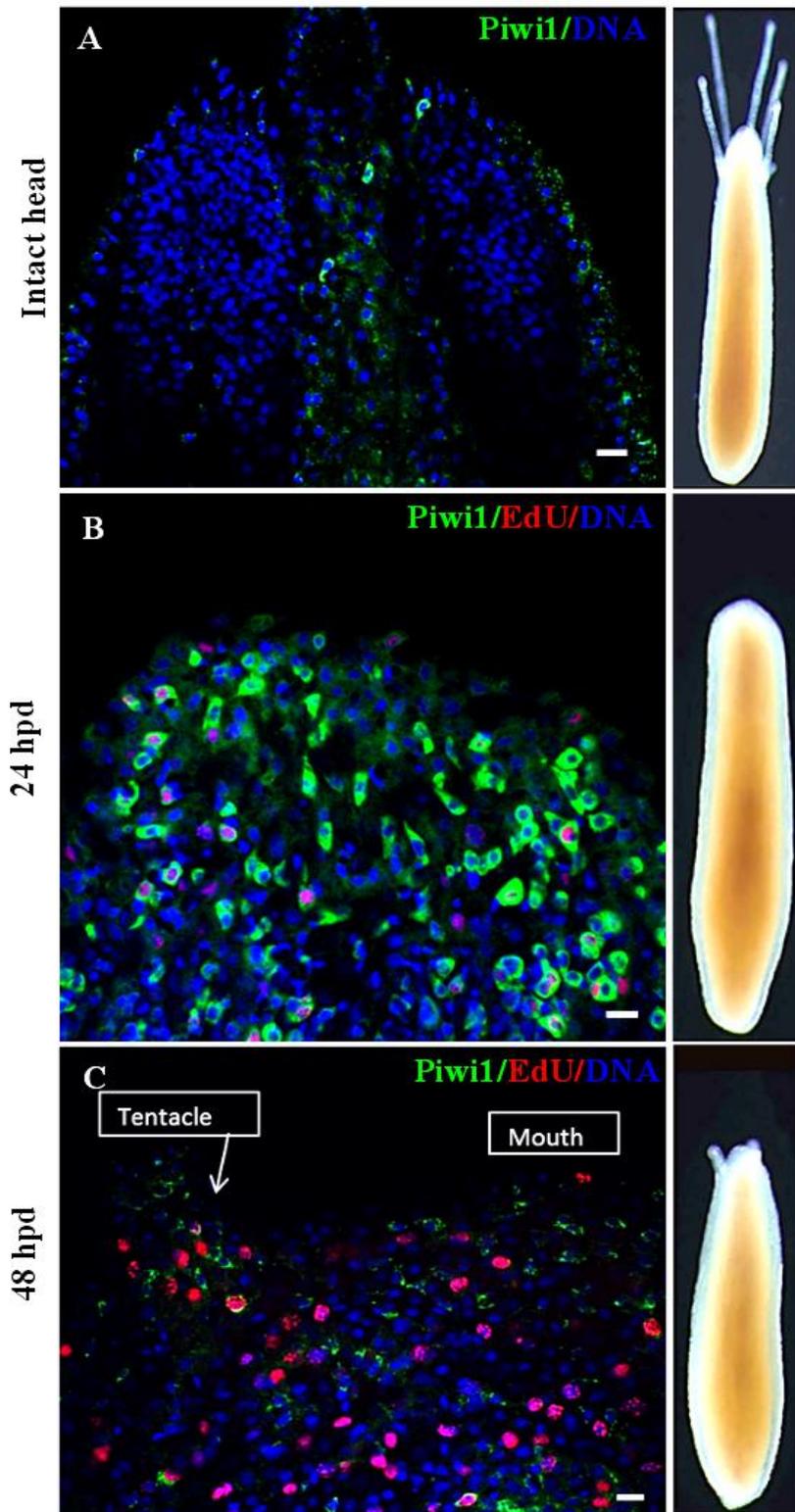


Figure 6.1. Recruitment of $Piwi1^+$ cells during regeneration. (A) Intact head. (B) 24 hpd. $Piwi1^+/EdU^+$ cells in newly formed blastema. Only $Piwi^+$ cells proliferate at this early regeneration stage. (C) 48 hpd. Mouth and tentacles have formed. $Piwi1^+$ cells became scarce and, in contrast to earlier stage, most cycling cells are now $Piwi1^-$. Scale bar equals 10 μm .

6.4 Piwi1⁺ i-cells in adult polyps are fast cycling

In the freshwater, solitary cnidarian, *Hydra*, i-cells proliferate continuously with a cell cycle time of 24-30 hours (Campbell & David 1974). A previous study in *Hydractinia* (Bradshaw et al. 2015) has shown that at least some Piwi1⁺ i-cells proliferate, but did not provide information about their cycling time. To provide a better understanding on i-cell proliferative behavior in *Hydractinia* adult feeding polyps, an EdU pulse-chase experiment was carried out, which involved a whole week of EdU incubation followed by five weeks chasing, as outlined in Figure 6.2. Polyps were cut from a colony at specific time points, fixed immediately and stained for EdU and Piwi1 according to specific protocols (Section 2.2.3. and 2.2.4.). Cell counting was carried out as described in section 2.6 on double positive, Piwi1⁺/EdU⁺, and Piwi1⁺/EdU⁻ cell populations. As cells replicate, they give rise to two daughter cells. Cells are capable of incorporating EdU during S-phase. By utilizing this technique, I was able to analyze the rate of proliferation of Piwi1⁺ cells through EdU pulse chase experiments as each time these Piwi1⁺ stem cells go through S phase, the EdU signal incorporated will become diluted by a factor of two. Once all Piwi1⁺ no longer display an EdU signal, it becomes obvious that cell has undergone many rounds of replication.

At time point Week 0, >400 cells were counted and approximately 99% of all Piwi1⁺ cells were also EdU⁺ (Figure 6.3A). At time point Week 1 a decline in the number of double Piwi1⁺/EdU⁺ from 99% to 65% (Week 1) was observed. However, those cells had only a partial (i.e. dotted) EdU signal, indicating they had gone through S-phase at least once during that week (the signal being diluted), consistent with the nearly 100% EdU positive cells after one week of continuous incubation in EdU. In the following weeks the number and intensity of EdU positive cells further declined (Figure 6.4). By the end of this pulse chase experiment all Piwi1⁺ cells are EdU⁻, indicating that they proliferated several times during this period. Interestingly, few (<0.01%) Piwi1⁺/EdU⁻ cells that were seen at this time point were restricted to the base of the feeding polyp close to the polyp-stolon boundary (Figure 6.5). As a result, these non-cycling cells were only found when polyps were cut very close to the stolon. The lack of

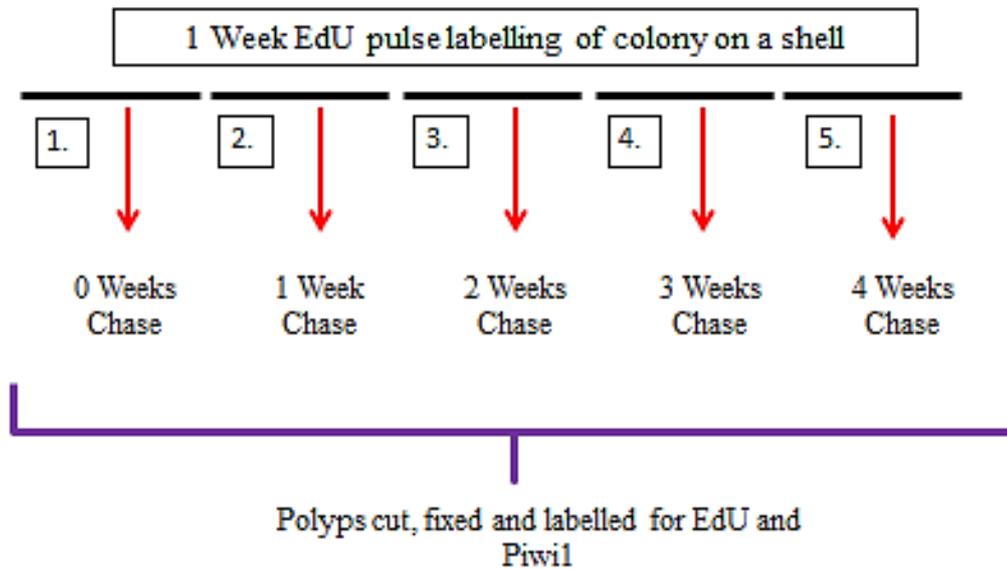


Figure 6.2. Schematic representation of the experimental set up for 1 week EdU pulse labelling of feeding polyps. A whole colony was incubated in a solution of EdU in ASW for 1 week. Removal of the colony from the EdU solution is marked by the number 1. at 0 Weeks chase time point. EdU retaining was chased at each of the time points indicated by numbers the numbers 1.-5. After each week post EdU, polyps were cut, fixed and stained for EdU and Piwi1.

Piwi1⁺/EdU⁻ cells after 1 week pulse in EdU outside of this region, it was clear these cells must arise from the stolonal network of *Hydractinia*.

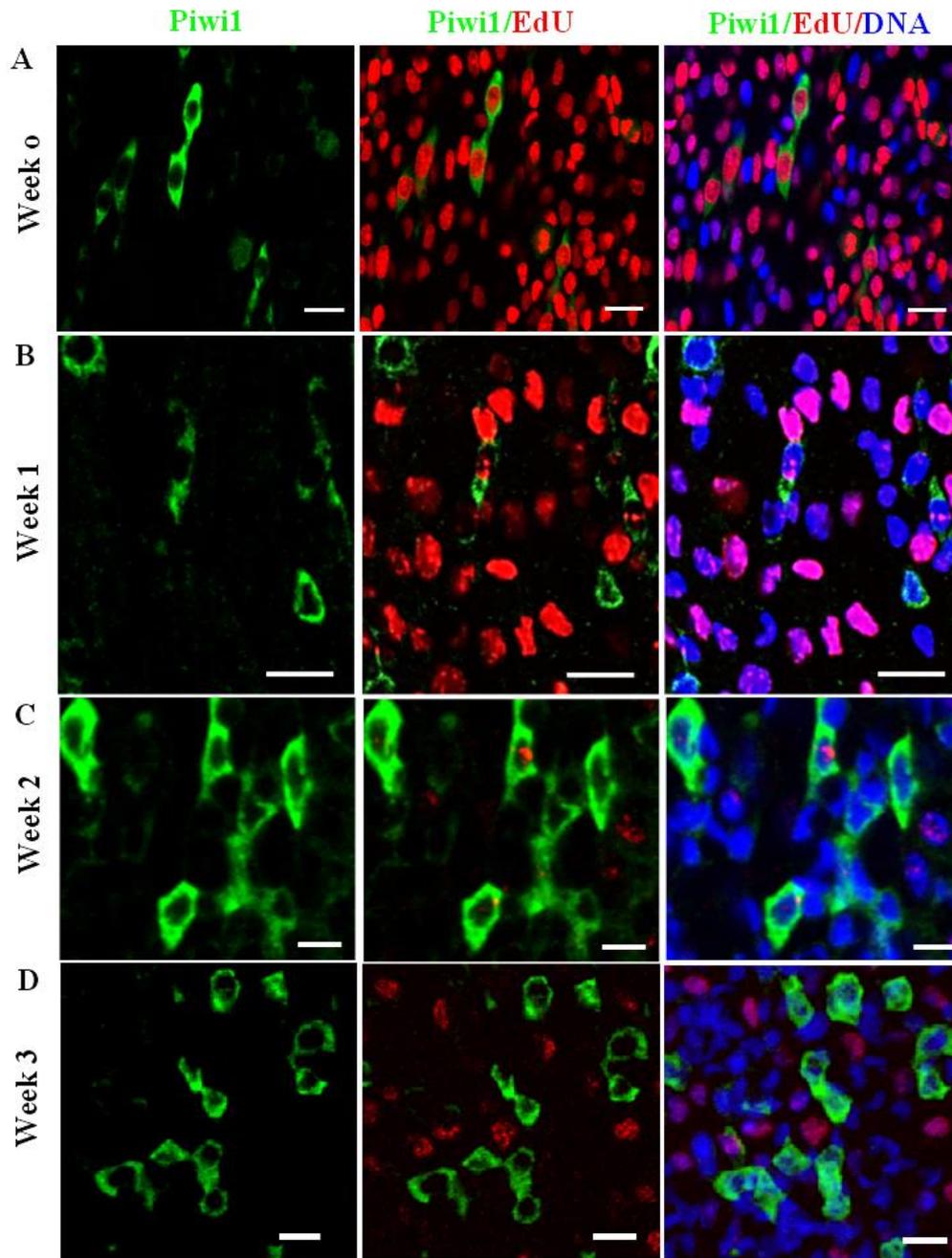


Figure 6.3. EdU incorporation and retention in $Piwi1^+$ cells after 1 week continuous EdU labeling and 3 weeks chase in adult feeding polyps. DNA in cells was labelled with Hoechst (blue) to ensure all cells imaged were viable. **(A)** At 0 weeks chase the whole nucleus is stained. **(B)** EdU signal becomes diluted from 1 weeks chase onwards. **(C)** By 2 weeks chase and **(D)** 3 weeks chase there are few to zero cells with EdU staining remaining in the nucleus in the absence of EdU. Scale bar equals 10 μ m.

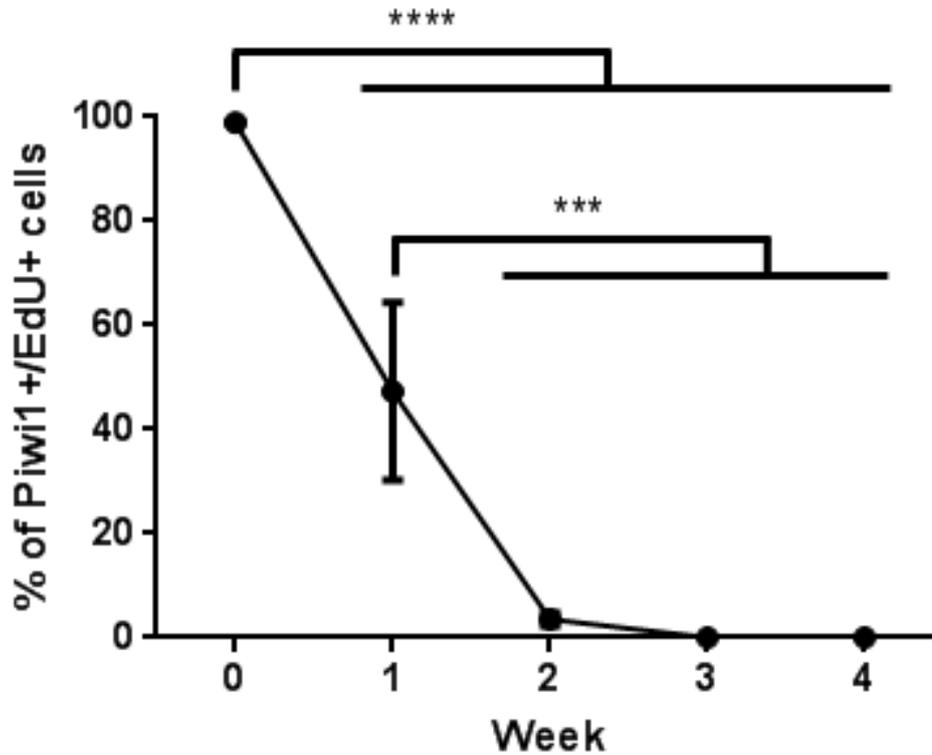


Figure 6.4. EdU retention by Piwi1⁺ cells following 1 week continuous EdU labeling over 4 weeks chase. One way Anova analysis with a Tukeys post test revealed statistical significance between Week 0 chase and all other time points. Statistical significance was also detected between Week 1 and all subsequent time points. **** = p-value \leq 0.0001. *** = p-value \leq 0.001.

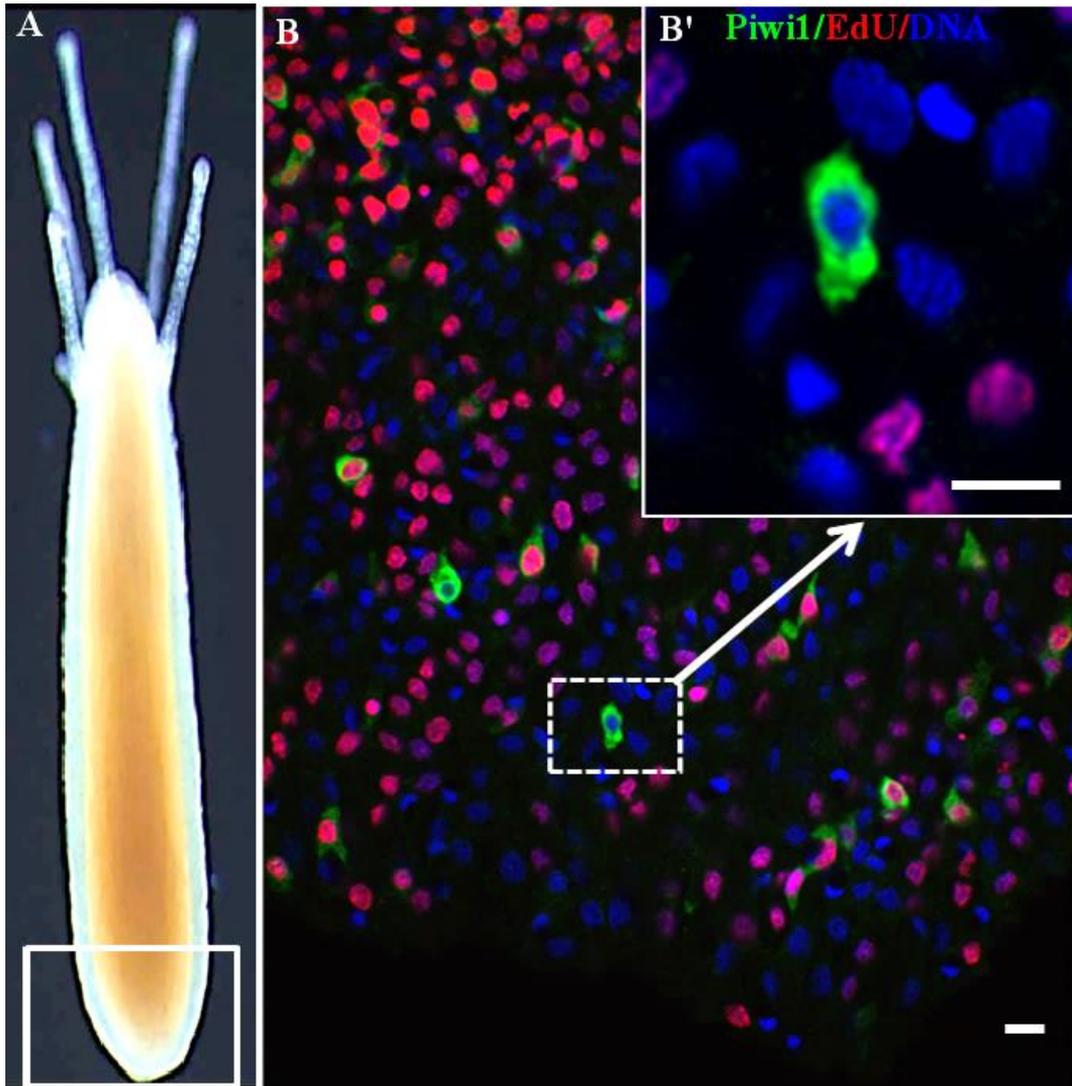


Figure 6.5. $Piwi1^+/Edu^-$ i-cells at the base of a feeding polyp after 1 week EdU pulse. (A) Feeding polyp after being cut from an adult colony. White box at the base of the polyp indicates the region that was analyzed by confocal microscopy. (B) $Piwi1$ (green) and EdU (red) staining showing the base of a feeding polyp. (B') Close up of $Piwi1^+/Edu^-$ cell. DNA is stained blue. Scale bar equals 10 μm .

6.5 Piwi1⁺ cell proliferation during 2-4 days pulse in EdU

It was clear after 1 week of EdU incubation, followed by 4 weeks chase of an adult *Hydractinia* colony that virtually all Piwi1⁺ cells in feeding polyps go through S-phase at least once per week. I have also shown with a very simple EdU incubation and double IF experiment that Piwi1⁺ cells undergo mitosis within 16hrs after S phase (Figure 6.6). To gain further insight into how fast these cells cycle, I decided to narrow down this window of EdU exposure to only 2 and 4 days. For this, adult *Hydractinia* colonies were incubated in 10 mM of EdU for 2 and 4 days. Polyps were then cut from the colony, fixed immediately and stained for Piwi1 and EdU.

There was a high number of double positive Piwi1⁺/EdU⁺ cells in animals exposed to only 2 and 4 days of EdU (Figure 6.7). Of over 400 Piwi1⁺ cells counted in 2 day EdU exposed animals, only 8.8% of these were Piwi1⁺/EdU⁻ (Figure 6.7C). While this number may be higher than the 1 week EdU pulse chase experiment at Week 0 chase (Figure 6.4), most of these Piwi1⁺/EdU⁻ cells were captured at the base of the feeding polyps cut from colonies. After 4 days incubation of a colony in EdU the number of Piwi1⁺/EdU⁻ cells dropped further to 1.4% (over 400 Piwi1⁺ cells counted). This matches closely to the data recorded of Piwi1⁺/EdU⁻ cells from 1 full week EdU incubation, 0 weeks chase (Figure 6.4). The accuracy of cutting polyps equally from colonies affected the number of Piwi1⁺/EdU⁻ counted at the base of polyps, therefore this variable was uncontrollable.

It is clear from these experiments that the vast majority of Piwi1⁺ i-cells in the body column of *Hydractinia* feeding polyps are continuously cycling, with a cycle time of less than two days. Some slow cycling (or non-cycling) cells were detected in all experiments, but only in the very aboral region of feeding polyps, close to the stolon boundary. Angle and position when cutting feeding polyps from colonies gave rise to some variation in numbers of these slow cycling Piwi1⁺/EdU⁻ cells. After extensive analysis, however, these numbers of Piwi1⁺/EdU⁻ cells remained consistently low compared to the overall population of Piwi1⁺/EdU⁺ to effectively exclude them from having any significant presence within the body column of a feeding polyp.

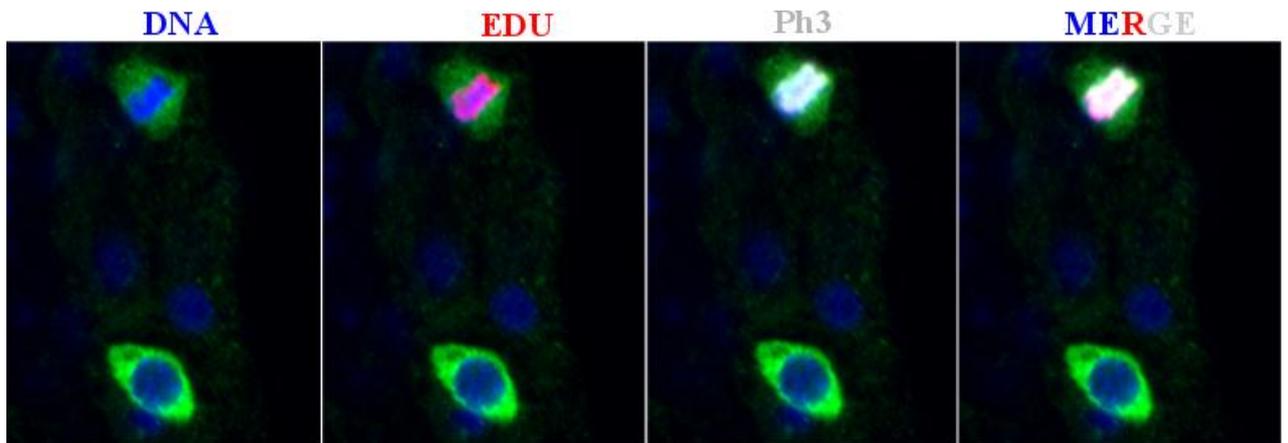


Figure 6.6. Piwi1⁺/Edu⁺/Ph3⁺ i-cells 16hrs after EdU pulse labelling. A Sub population of Piwi1⁺ cells (green) display mitotic activity (Ph3 staining, white) 16hrs after S-phase labelling with EdU (red) as seen in the merge image. Scale bar equals 10 μ m.

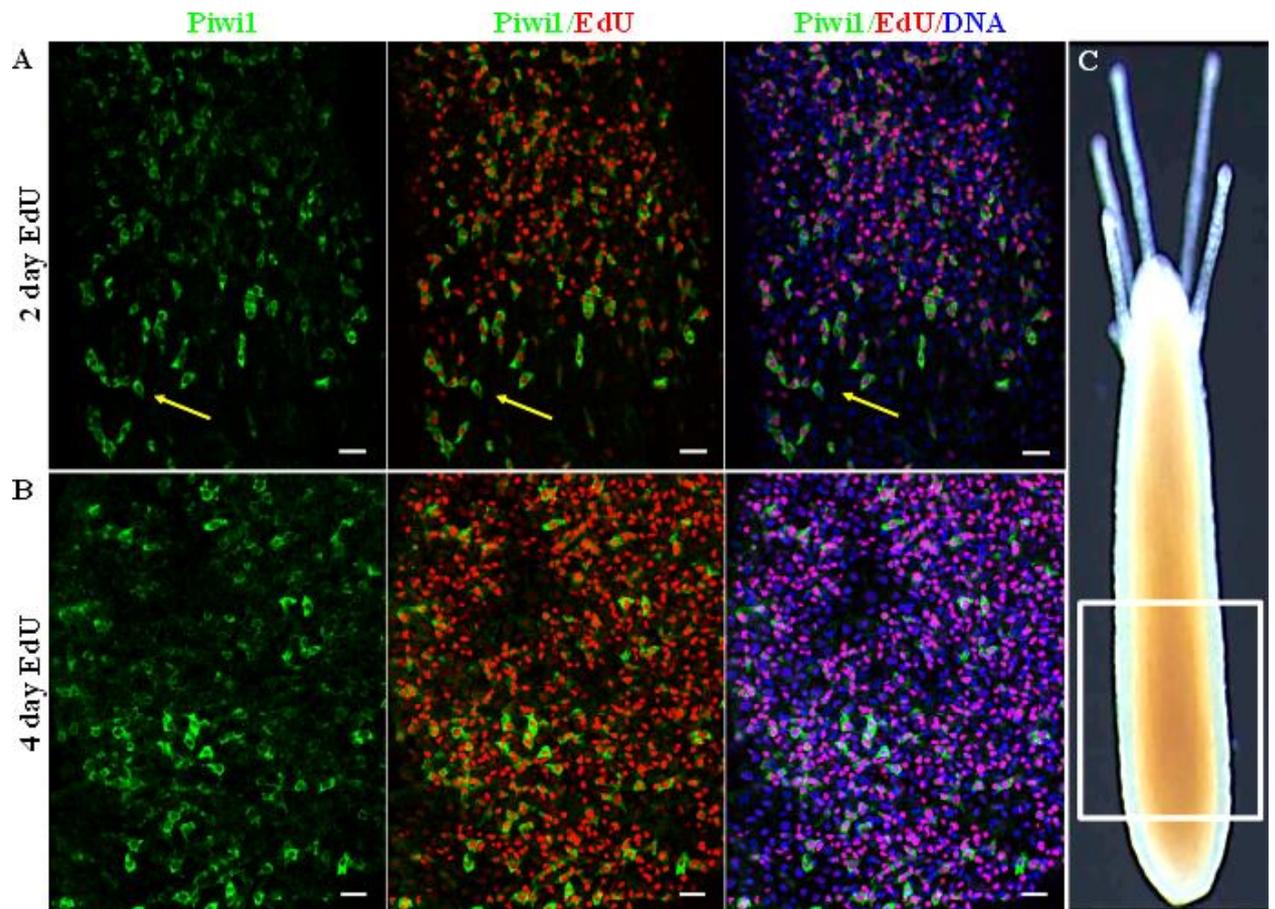


Figure 6.7. 2 and 4 day EdU incubation of *Hydractinia* colonies. (A) Piwil (green) and EdU (red) staining in adult feeding polyps incubated for 2 full days in EdU. Yellow arrows indicate Piwil⁺/EdU⁻ cells detected at base of feeding polyp cut from colony. (B) Piwil and EdU staining in adult feeding polyps incubated for 4 full days in EdU. (C) Representative figure showing region of feeding polyp analyzed for 2 and 4 day EdU incubation experiments. Scale bar equals 10 μ m.

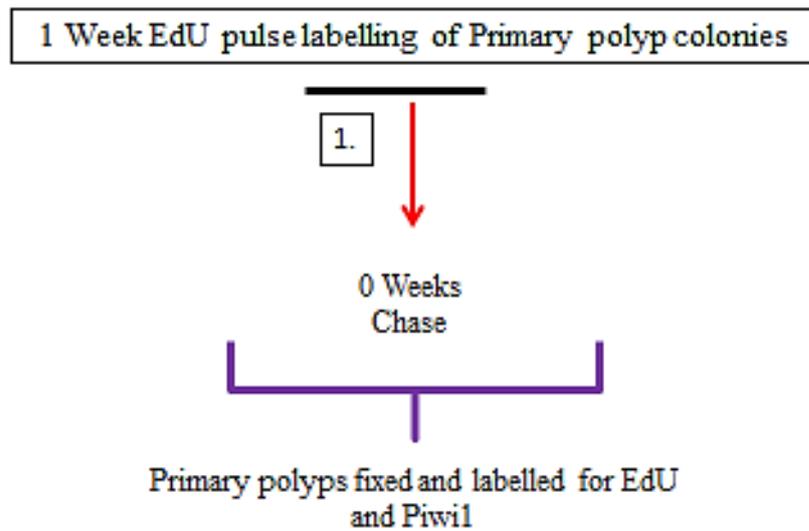
6.6 Slow cycling Piwi1⁺ i-cells in stolons

The previous experiments on adult feeding polyps indicated clearly that almost all Piwi1⁺ cells present in the body column away from the stolon boundary are fast cycling (Figures 6.4 and 6.7). However, as already noted, a small percentage of Piwi1⁺/EdU⁻ stem cells were detected near the base of the feeding polyps, directly near the stolon boundary. This led me to hypothesize that slow cycling i-cells reside primarily in the stolon compartment. To test this hypothesis, I performed cell cycle analysis of Piwi1⁺ i-cells within the stolon network of *Hydractinia*.

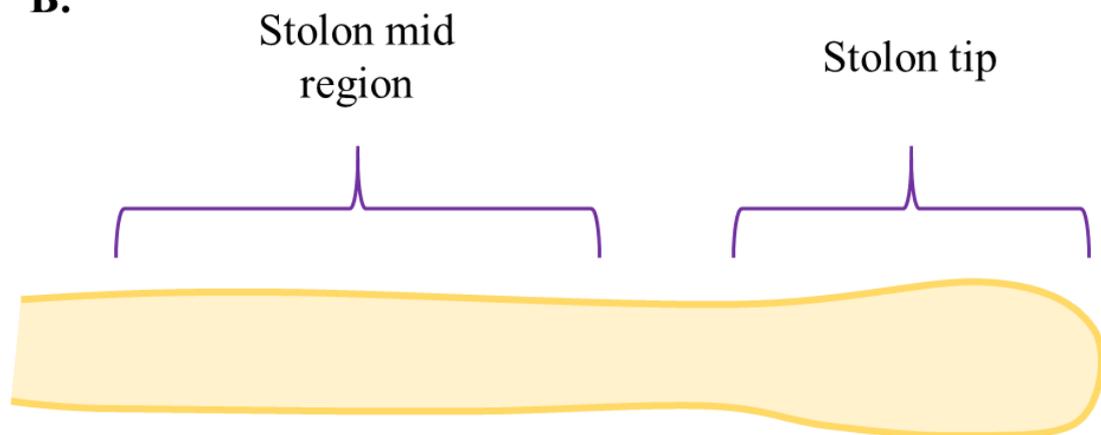
To carry out this experiment another EdU pulse treatment was performed where 1 week post metamorphosis colonies were incubated in 10 mM EdU followed by immediate fixation as outlined in Figure 6.8. EdU and Piwi1 labelling was carried out according to the protocols described in Sections 2.2.3 and 2.2.4, respectively.

At time point Week 0 (i.e., immediately following EdU incubation), stolons were fixed and analyzed for Piwi1⁺/EdU⁺ and Piwi1⁺/EdU⁻ cell populations, similar to the previous 1 week EdU pulse chase experiments in adult feeding polyps (Section 6.4), according to the stolon cell counting protocol (Section 2.6). While close to the growing tips of stolons all Piwi1⁺ i-cells were also EdU⁺ (Fig 6.9A,C), similar to feeding polyps, in mid stolon regions, 35% of these cells were EdU⁻ (Fig. 6.9B,D). In contrast to the amount of fast cycling i-cells in polyps, the analysis of stolons revealed the majority of cells to be EdU⁺ (Figure 6.9C). Comparable results were also obtained following a 12 day of continuous exposure to EdU (Figure 6.10), clearly indicating the presence of slow cycling Piwi1⁺ i-cells in the stolons of *Hydractinia*, in strong contact to feeding polyps that harbored only fast cycling i-cells. The few non-cycling i-cells near the polyp's base, close to the stolon boundary, is consistent with these data.

A.



B.



Stolon

Figure 6.8 (A) Schematic representation of the experimental set up for 1 week EdU pulse labelling of 1 week old primary polyps. 1 week old primary polyps grown on glass slides were incubated in a solution of EdU and ASW for 1 week. Removal of the primary polyps from EdU and ASW solution is marked by 1 at 0 Weeks chase time point. The primary polyp colonies were immediately fixed and labelled at this time point for EdU and Piwi1. **(B)** Schematic representation of the regions of a stolons analyzed during the EdU pulse experiments on polyp colonies. Stolon mid regions and stolon tips were the areas chosen to analyze the number of Piwi1⁺ cells undergoing proliferation.

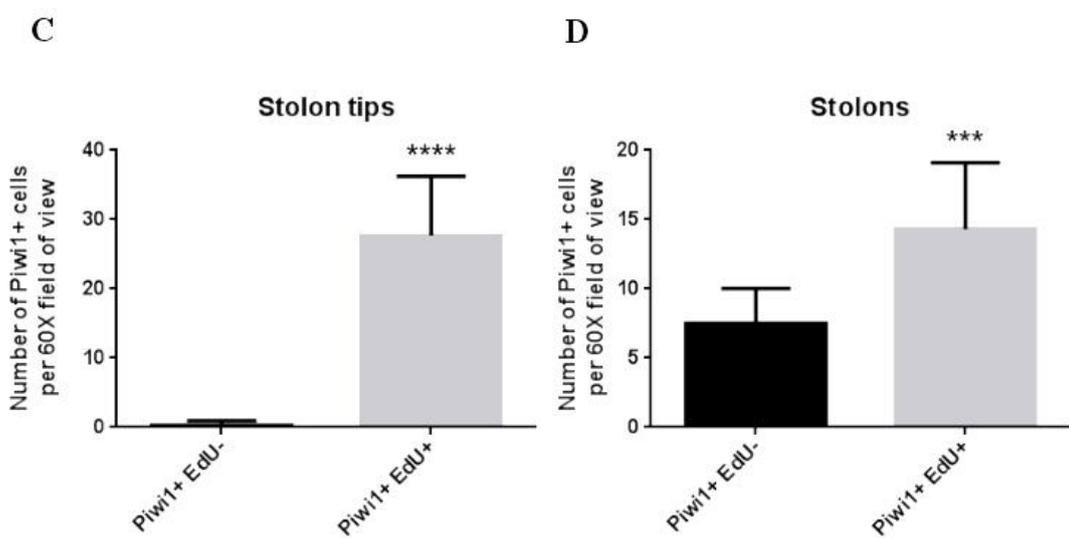
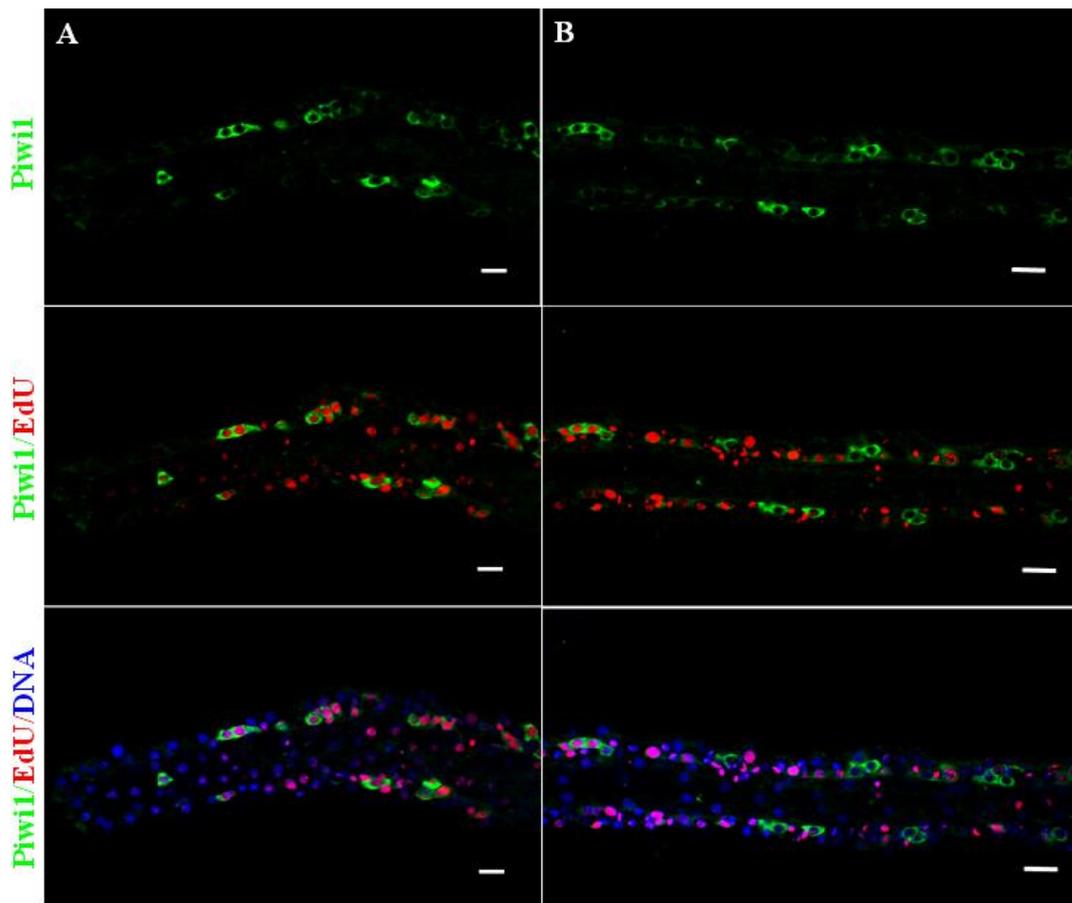


Figure 6.9. Analysis of EdU incorporation by Piwi1⁺ cells in stolons after one week of continuous EdU exposure. DNA of cells is labelled with Hoechst (blue) to show cells imaged are viable. **(A)** Piwi1 and EdU staining in stolon tips (see figure 6.8 for stolon tips region) shows there is a regular population of cycling cells present as almost all Piwi1⁺ cells (green) imaged are labelled with EdU (red) after a week of EdU pulse indicating they are undergoing active proliferation. **(B)** Piwi1 and EdU

staining in the mid stolon section (see figure 6.8 for mid stolon region) reveals a decline in the number of proliferating Piwi1+ as many are now seen to be EdU-. (C) Graphs demonstrating the number of Piwi1+ cells in stolon tips that are EdU+ or EdU-. (D) Graphs demonstrating number of Piwi1+ cells in stolon that are EdU+ or EdU-. N=10 for Unpaired t-tests in Figures C-D. **** = p-value \leq 0.0001. *** = p-value \leq 0.001. Scale bar equals 10 μ m.

6.7. Slow cycling i-cells re-enter the cell cycle following injury

My results suggest the presence of a population of Piwi1⁺ cells in the mid regions of stolons that do not go through S-phase for at least 12 days under normal conditions. This population could represent either slow cycling/quiescent, or post mitotic/senescent cells. Slow cycling/quiescent cells could serve as a cellular reserve to be used under stressful situations. To gain insight into the nature of these, previously undescribed, cells, I incubated colonies in EdU for 12 days and their stolons were then injured by inflicting cuts using a blade. The control group consisted of colonies incubated for the same period of time in EdU but were not subjected to injury or stress. All the colonies were then placed into a solution of BrdU for a further full 24 hours pulse. Colonies were removed and immediately fixed by the end of the 24 hours BrdU exposure. Animals were stained for Piwi1, EdU and BrdU and cells were imaged using confocal microscopy (Figure 6.10A). Piwi1⁺/BrdU⁺/EdU⁻ cells were detected in stolons of injured animals 24 hours post injury (Figure 6.10A'). As expected, the numbers of Piwi1⁺/BrdU⁻/EdU⁻ cells was higher in uninjured colonies (Figure 6.10B), compared to uninjured ones (Figure 6.10C). These experiments were difficult to standardize due to the high variability of Piwi+ cell distribution over the entire stolon (see also previous section). Therefore, mid stolon regions were analyzed.

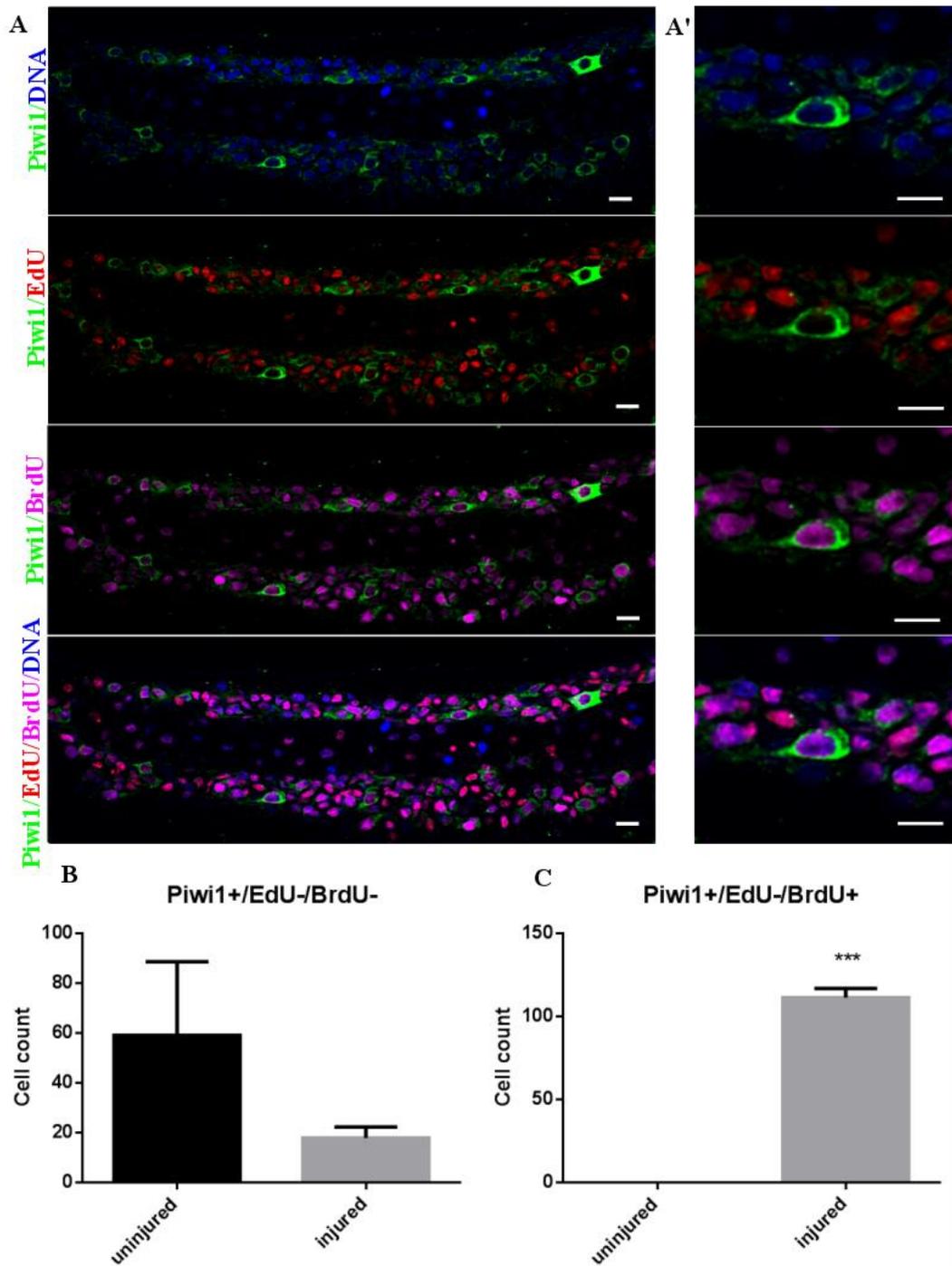


Figure 6.10. 12 day EdU pulse in stolons, followed immediately by 24 hour pulse with BrdU after injury in mid stolon regions. (A) Analysis of EdU (red) and BrdU (pink) staining in Piwi1⁺ cell (green) population in injured stolons (see figure 6.8 for representation of mid stolon regions analyzed). Nuclei are labelled with Hoechst (blue). A merge of all 4 colours results in EdU and BrdU colours overlapping producing a higher intensity of fluorescence. **(A')** Close up showing Piwi1⁺ cells in

an injured stolon section, (confirmed viable due to present of DNA (blue) in nuclei, shows they are lacking EdU staining after 12 day EdU pulse but are capable of re entry into the cell cycle after injury as seen by BrdU (pink) staining. Uninjured stolons did not show any BrdU only positive cells (images not shown). **(B-C)** Statistical analysis of Piwi1⁺ cells stained for EdU and BrdU in uninjured vs. injured stolons is shown in graph format. It is clear that uninjured stolons contain more non-cycling or quiescent stem cells compared to the injured stolons (B). The injured stolons show a significant increase in the number of Piwi1⁺/EdU⁻/BrdU⁺ cells after injury. It's likely these cells are recruited in response to the stress induced upon injury. *** = p-value \leq 0.001. Scale bar equals 10 μ m.

6.8 Summary

The aim of this chapter was to assess the cell cycle characteristics of Piwi1⁺ stem cells (i-cells) in *Hydractinia*. It was previously known that Piwi1⁺ i-cells are present in a region of *Hydractinia* known to be a region of high proliferative activity (Bradshaw et al., 2015). In order to determine if Piwi1⁺ stem cells undergo regular cell division, their proliferative activity was analyzed during regeneration and homeostasis through long term EdU pulse chase experiments.

Piwi1⁺ i-cell proliferation was assessed and it was shown that these cells cycle in the proliferative band region, visualized by a short pulses of EdU, but are absent in the intact head. Following decapitation these cells are recruited to the blastema and proliferate during 24 hpd. However, Piwi1⁺ i-cells disappear progressively once a functional head has formed from the blastema, leaving behind transiently proliferating progeny, consistent with Piwi1 being a reliable i-cell marker.

My work also showed that i-cells in the polyp are fast cycling, while the stolonial i-cell population also include slow cycling ones. A small population of Piwi1⁺/EdU⁻ i-cells identified at the base of adult feeding polyps, close to the stolon boundary, probably originated from the stolonial network, where slow cycling i-cells are common. Finally it was shown that a subpopulation of slow cycling/quiescent Piwi1⁺ cells in the stolon are capable of cell cycle re-entry in response to injury.

6.9 Future experiments

Further analysis is required on the slow cycling Piwi1⁺ cells in the stolons. Repeat experimentation and counting on young colonies to provide more replicates and statistics is the next step. Along with these, parameters such as increase in incubation time with EdU before BrdU injury could be carried out to help support the hypothesis that Piwi1⁺ are quiescent cells that can re-enter a proliferative state even after long periods of time.

Chapter 7. Discussion

7.1 Phylogenetic analysis of PIWI genes reveals conservation of shared domains

Sequence analysis of the *Hydractinia* PIWI proteins also revealed that both Piwi1 and Piwi2 contain PAZ, PIWI, and MID domains, which are well known to be a characteristic of PIWI proteins. PIWI genes are known from four cnidarians, *Hydra*, *Hydractinia*, *Podocoryne* and *Nematostella* (Bradshaw et al., 2015, Praher et al., 2017, Juliano et al., 2014, Seipel et al., 2004). These cnidarians represent the two major clades in this phylum (Medusozoa and Anthozoa, respectively). Cnidarians have two PIWI genes each, but only one has been described in *Podocoryna*, and the *Nematostella* genome encodes a PIWI pseudogene (referred to as Nv-Piwi3 on figure 3.2). Phylogenetic analysis clustered the cnidarian genes in two distinct branches within the PIWI family. One, including the *Hydractinia* Piwi1, *Hydra* Hywi, *Podocoryna* Cniwi, and *Nematostella* Piwi1 and Piwi3 (the pseudogene). The other branch included *Hydractinia* Piwi1, *Hydra* Hyli, and *Nematostella* Piwi2 suggesting that the last common cnidarian ancestor already had at least two PIWI genes. The Argonaute proteins analysed were chosen as an outgroup protein and were shown to cluster with their orthologous counterparts as a sister clade to PIWI proteins. *Hydractinias* key phylogenetic position as a basal metazoan together with its tractability and amenability to functional studies make it a suitable model to study the role of PIWI proteins in stem cells.

7.2. PIWI genes are germ and stem cell markers in *Hydractinia*

Up until now the expression profile of the PIWI proteins and mRNAs had not been fully analyzed in different developmental stages of *Hydractinia*. The results of Chapter 3 confirm the expression of both *Piwi1* and *Piwi2* genes in stem and germ cells. As other clonal animals, *Hydractinia* does not sequester a germline and its stem cells, known as i-cells, give rise to somatic lineages and germ cells continuously in adult life. The expression of germ cell genes such as PIWI, which functions by silencing transposons, is consistent with the necessity to maintain high genomic

stability in cells that will give rise to the indefinitely growing somatic tissues, as well as the next generation through sexual reproduction. Indeed, it has been suggested that *Vasa*, *Nanos* and *Piwi* constitute a conserved germline multipotency program in animals without an embryonically sequestered germline (Juliano et al., 2010).

7.3. Expression pattern of PIWI proteins during development

Immunostaining using anti-Piwi1 and Piwi2 antibodies, generated in this study, revealed that both *Hydractinia* PIWI proteins are cytoplasmic, and no evidence was found for nuclear PIWI in *Hydractinia*. This is similar to the cnidarian *Hydra*, whose PIWI proteins (*Hywi* and *Hyli*: orthologs of *Hydractinia* Piwi1 and Piwi2, respectively) are also cytoplasmic. Many other organisms studied, however, display both nuclear and cytoplasmic PIWI localization. The subcellular localization of PIWI proteins is related to their respective function. Cytoplasmic PIWIs act post-transcriptionally to silence retrotransposons and possibly also endogenous mRNAs. Nuclear PIWIs, by contrast, act epigenetically by recruiting chromatin modifiers to specific loci, identified by their bound piRNAs. If cnidarian PIWIs are indeed exclusively cytoplasmic, it would suggest that the epigenetic function of PIWI has either been lost in the cnidarian lineage, or de novo acquired in bilaterians.

In *Hydractinia*, I have shown that PIWI proteins are maternal and ubiquitous in early cleaving embryos (Figure 3.5A,B). Post gastrulation, Piwi1⁺ cells were segregated to the internal cell mass, which is generally considered equivalent to endoderm in other animals (Figure 3.5C and Figure 3.7A). However, *Hydractinia* gastrulation is unusual, proceeding through apolar delamination (Kraus et al. 2014), and the identity of cell layers is therefore unclear. In particular, gastrulation in bilaterians involves not only morphogenetic processes, but also lineage commitment taken by embryonic pluripotent cells (becoming either ectoderm, endoderm or mesoderm). In this context, maintaining pluripotent cells in adult *Hydractinia* indicates that at least some of the animal's cells, i.e. the i-cells, do not gastrulate in embryogenesis, but rather, continuously throughout life. De-coupling morphogenesis from cellular commitment in the description of gastrulation would facilitate placing unusually gastrulating animals in the correct context.

Piwi1⁺ i-cells maintained their position in the internal mesenchymal cell mass through late embryogenesis and larval stage. This expression pattern is consistent with previous work on *Hydractinia* and other hydrozoans, where all germline multipotent program genes, as well as early neurogenesis genes, are restricted to the 'endoderm' (Flici et al. 2017; Gahan et al. 2016; Gahan et al. 2017; Leclère et al. 2012; Kanska & Frank 2013).

During metamorphosis, all i-cells migrated to the interstitial spaces of the epidermis, a phenomenon well documented in hydrozoans (Martin and Archer, 1986, Martin, 1990, Gahan et al., 2016, Gahan et al., 2017). However, this is the first study showing that these cells are PIWI⁺. In all other post metamorphic stages, PIWI expressing i-cells were exclusively restricted to interstitial spaces between epithelial epidermal cells. There was one exception to this rule: germ-committed i-cells crossed the mesoglea and migrated to the gastroderm. Hence, in sexual polyps, two distinct populations of PIWI⁺ cells could be discerned: 'normal' i-cells, which were epidermal, and germ-committed ones, including differentiated germ cells, which were gastrodermal.

One marked difference between PIWI expression in *Hydra* vs. *Hydractinia* is the lack of epithelial PIWI in the latter. In *Hydra*, PIWI genes are also expressed in epithelial cells, which in this animal, constitute a self-renewing lineage, distinct from i-cells and their derivatives. In *Hydractinia*, PIWI expression (both mRNA and protein) was below detection level in epithelial cells. This is consistent with the presumed difference of i-cell developmental potency between the two hydrozoans, where in *Hydractinia*, i-cells have been shown to give rise to epithelial cells as well. Hence, the restriction of PIWI expression in *Hydractinia* to i-cells is consistent with epithelial cells in this animal not being stem cells (they derive from i-cells) and not requiring extra mechanisms to maintain genome stability, in contrast to the likely pluripotent i-cells. Differentiation markers, like minicollagens (*Ncol1* and *Ncol3*) were not co-expressed with Piwi1, as expected.

Overall, the known role played by PIWI proteins in reinforcing genome stability, which is likely conserved in *Hydractinia*, together with their expression in morphologically identified i-cells, and co-expression with other germline

multipotency genes like *Vasa* (Fig 3.1.8B, C), make a strong case for both *Hydractinia* PIWIs to be exclusive stem/germ cell markers.

7.4 *Piwi1* ectopic expression and knockdown in *Hydractinia*

Piwi ectopic expression has been previously characterized in *Drosophila* to cause over production in the number of Germ stem cells (GSCs) (Cox et al., 2000). It is also shown in humans that testicular seminomas display increased expression of the human PIWI gene *HIWI*, suggesting its involvement in germ cell proliferation and maintenance and that *HIWI* over expression may cause malignant germline development (Qiao et al., 2002). These studies mainly focus on the impact of over expression in germline stem cells (GSCs). In *Hydractinia*, it is known that i-cells differentiate into both germline cells and somatic cells (Gahan et al., 2016, Plickert et al., 2012). PIWI protein manipulation is commonly carried out through the use of RNAi, CRISPR/Cas9, morpholinos and genetic mutations in models such as *Hydra*, *Drosophila* and mice (Carmell et al., 2007, Deng and Lin, 2002, Kalmykova et al., 2005, Han et al., 2014, Juliano et al., 2014). These studies demonstrate a conserved function of PIWI proteins in germline stem cells, along with a somatic function as studied in *Hydra* (Juliano et al., 2014). Here I examine PIWI gain and loss of function in *Hydractinia* through the use of transgenic ectopic expression, CRISPR/Cas9 and morpholino injection strategies.

Piwi1 misexpression did not yield any significant results thus far. Utilizing a transgenic *Piwi1* ectopic expression construct, I have shown that *Piwi1::GFP* ectopic expression gives rise to mosaic GFP⁺ larva that silenced the *Piwi1::GFP* transgene post metamorphosis. Ectopic expressing embryos and larvae appeared normally and gave rise to F1 generation animals. The resultant GFP⁺ F1 generation reactivated the transgene in embryogenesis but capable of forming a normally appearing colony and showed no obvious defects. Interestingly, also these animals lost transgene expression post metamorphosis possibly through epigenetic or posttranslational silencing, reactivating its expression only in embryos and larvae of the next generation. Transgene silencing is a known phenomenon in our lab, seen in some, but not all, transgenes. Why only some are silenced while others remain expressed is

unknown. It could be related to the site of transgene integration; this, however, is unlikely, since loss of expression is gene rather than experiment specific. Alternatively, it is possible that animals silence transgene based on their sequence, which is consistent with our experience in the lab. The mechanisms underlying transgene silencing is interesting but beyond the scope of my work. The only clear conclusion that can be drawn from the ectopic expression experiments is that *Piwi1* does not cause catastrophic damage in epithelial cells that normally do not express it. Further analysis is required on these newly established F1 animals to fully assess if *Piwi1* ectopic expression has any effects on germ and somatic cell types.

The loss of PIWI protein function through mutagenesis in other animals has shown a conserved role of PIWI in maintaining GSCs and fertility (Carmell et al., 2007, Deng and Lin, 2002, Gou et al., Batista et al., 2008, Houwing et al., 2008). Here, CRISPR/Cas9 mutagenesis was utilized to analyze the effect of *Piwi1* loss of function in *Hydractinia*. Mutagenesis of *Piwi1* was detectable only in early embryos, with wild type cells dominating at later stages, suggesting that *Piwi1* mutated cells were disadvantaged and were subsequently taken over by wild type ones over time. This result may suggest an essential role for *Piwi1* in i-cells. *Hydractinia*'s regenerative ability allows the replacement of mutated cells by wild type ones, resulting in normal colonies. .

Morpholino injection for *Piwi* mRNA targeted knockdown has been previously utilized in sea urchins on its PIWI protein, Sp-seawi. Morpholino knockdown of Sp-seawi diminished PGC-specific localization of Seawi proteins, and altered expression of other germ line markers such as Vasa (Yajima et al., 2014) A morpholino oligo designed to translationally silence *Piwi1* failed as no detectable decrease in *Piwi1* protein compared to the control morpholino injected was observed. Thus no further analysis was carried on these injected larva for lack of time, but further experiments utilizing different concentrations of morpholino could be performed by others.

7.5 piRNAs associated with Piwi1 protein

Thus far my research has been focused solely on the Piwi proteins and genes in *Hydractinia*. During my work I immunoprecipitated small RNAs bound to the Piwi1 proteins. As mentioned previously, both Piwi proteins displayed equal patterns of expression, but only the Piwi1 antibody was suitable for RNA-IP. I successfully isolated piRNAs bound to Piwi1 protein which fell within the expected size range (Figure 5.1). Seq analysis would identify the targets of these piRNAs and has yet to be completed; therefore, I could not include these results in my thesis. However these results will provide unique insights into the biology of Piwi1/piRNAs in *Hydractinia*.

7.6 Discovery of a quiescent/slow-cycling i-cell population in *Hydractinia*

The goal of this chapter was to assess the cell cycle characteristics of Piwi1⁺ i-cells. In the freshwater polyp, *Hydra*, i-cells are traditionally regarded as a single, fast cycling population with a 24-30 hour cycle time (Schenkelaars et al., 2017, Buzgariu et al., 2014). A recent study (Govindasami et al. 2014) has shown that some of *Hydra's* cells, including epithelial stem cells and i-cells, are slow cycling and can re-enter the cell cycle upon injury.

In *Hydractinia*, the i-cell population had already been identified through the use of whole mount *in-situ* hybridization for several stem cell gene markers in the feeding polyp (Bradshaw et al., 2015, Gahan et al., 2016) and in the gastrovascular stolon network (Müller et al., 2004), but their cycling behavior is unknown. These stem cells are often referred to as pluripotent, because at a population level, they give rise to all somatic and germ cells (Plickert et al., 2012). A distinctive feature of stem cells is their capacity to self-renew and maintain stemness (Weissman et al., 2001). Revealing the cell cycle characteristics of *Hydractinia* i-cells is of prime interest.

In order to address this knowledge gap, the cell proliferative abilities of i-cells was studied through the use of S phase markers EdU and BrdU, and the mitosis marker phospho-H3. The results obtained demonstrated that these Piwi1⁺ i-cells represent two different populations of i-cells, dependent on their location within the *Hydractinia* colony. Piwi1⁺ i-cells in the polyp body column were almost exclusively fast cycling cells, and could be recruited to sites of injury (Figure 6.1). By contrast, Piwi1⁺ i-cells identified in the stolon tissues displayed dynamic levels of cell cycle abilities, from fast cycling, similar to the i-cells identified in polyps' body columns, to slow cycling cells. Preliminary data from EdU/BrdU pulse experiments suggests these cells are a reserve population of quiescent stem cells capable of undergoing proliferation in response to injury (Figure 6.10), but otherwise maintaining quiescence to reduce replication dependent genotoxicity.

In *Caenorhabditis elegans*, all cells in the adult body are 'post-mitotic' and no longer able to proliferate (Pekovic and Hutchison, 2008). However, in higher multicellular organisms with prolonged lifespans, such as mammals, quiescent cells are fundamental to forming tissues capable of renewal and regeneration, which is critical for the tissue homeostasis of the adult body (Cheung and Rando, 2013, Legesse-Miller et al., 2012). Cellular quiescence also provides protection against stress and toxicities, which is especially important for long-lived cells, such as stem cells (Cheung and Rando, 2013). *In vivo* transgenic work in *Hydractinia* has also demonstrated that these stem cells are recruited in response to stress such as injury, supporting the pluripotency abilities of these cells (Bradshaw et al., 2015, Gahan et al., 2016). No slow cycling stem cells (neoblasts) have been identified in planarians.

The presence of cells that remain undivided for several cell cycles suggests that stem cells in *Hydractinia* share at least one characteristic of cell cycle regulation with mammalian stem cells, namely the ability to retain stem cells in a slow-cycling state. A key question now is to understand the molecular mechanisms that regulate this process and determine if slow-cycling stem cells in *Hydractinia* differ from the rest of the i-cells in terms of their ability to self-renew or differentiate, i.e. whether they convert to 'normal' i-cells following injury. To conclude, my studies show that *Hydractinia* possesses a population of slow-cycling stem cells that re-enter the cell cycle during regeneration, a characteristic it shares with mammalian quiescent stem cells. These studies show that the ability to maintain stem cell populations with heterogeneity in cell cycle characteristics arose early in the evolution of multicellular organisms.

7.7 Concluding remarks

The study of stem cells in invertebrates like *Hydractinia* provides a fresh insight into stem cell biology in general, and into specific questions such as the maintenance of genomic stability over many cycles of replication, a function fulfilled by PIWI proteins. *Hydractinia* is special in several aspects: first, it is clonal and colonial. Post metamorphic growth generates unlimited genetic copies of the primary polyp (i.e. clonal growth), which also maintain tissue continuity (i.e. colonial growth). This life form is facilitated by a population of migratory stem cells (i-cells), which must be able to provide progenitors to any lineage. The reason for this is that remote areas must be able to develop any structure, somatic or sexual. Hence, parsimony suggests that a single, pluripotent population is superior to multiple, lineage restricted ones. Hence, the absence of one specific lineage in a remote area in the colony could impede the establishment of essential structures. Other interesting features of *Hydractinia* include its enormous growth plasticity, and apparent resistance to aging and carcinogenesis, both probably directly related to this i-cell population.

Current studies of the Piwi-piRNA pathway have revealed its diverse biological molecular functions, including germline specification, gametogenesis, stem cell maintenance, epigenetic programming, transposon silencing and posttranscriptional regulation of mRNAs (Aravin et al., 2007a, Cox et al., 1998, Cox et al., 2000, Deng and Lin, 2002, Gou et al., Grimson et al., 2008, Grivna et al., 2006, Houwing et al., 2007b, Juliano et al., 2014, Kalmykova et al., 2005, O'Donnell and Boeke, 2007, Peng and Lin, 2013). Among these the most extensively is its role in repressing TEs in the germline (Saito and Siomi, 2010). However despite these reports, the Piwi-piRNA pathway still remains mysterious in many ways. For example, it is not clear how the de-silencing of transposons might be connected to the various developmental phenotypes observed for *piwi* mutants, such as GSC loss and spermatogenic arrest in mice, germ-cell death in zebrafish, and inhibition of stem cell function in planarians (Carmell et al., 2007, Deng and Lin, 2002, Houwing et al., 2007a, Harris and Macdonald, 2001). There is also evidence that this pathway regulates nontransposon genes post-transcriptionally in *Drosophila*, such as the targeting of maternal genes for deadenylation (Rouget et al., 2010). Understanding

the full regulatory capabilities of the Piwi-piRNA pathway will be essential for understanding how it regulates stem cells and other developmental functions. My research has provided a focused insight into *Hydractinia* stem cells through the study of this class of proteins

My work, although not providing the ultimate proof for pluripotency of single i-cells, does suggest that PIWI genes/proteins are reliable stem cell markers in *Hydractinia*. A *Piwil* reporter line, established in the lab, could be used to transplant a single, fluorescent i-cell to a recipient colony. Also, the antibodies I generated will be instrumental in many future studies, e.g., piRNA analysis, which is ongoing. Piwi-piRNA pathway is required for maintaining stem cells in the animal germline and possibly in a wider variety of stem cells including humans (Cox et al., 2000, Sharma et al., 2001). Understanding the full regulatory capabilities of the Piwi-piRNA pathway will be essential to uncovering how it regulates stem cells and other developmental functions.

I also found that the overexpression through transgenesis or knockout via CRISPR/Cas9 of the *Piwil* gene resulted in the loss of transgenic and mutated cells, possibly due to the animals shutting the transgene down. This preliminary data points to different regulatory mechanisms involved in *Hydractinia* during gain and loss of function of *Piwil* via mutagenesis and ectopic expression. *Piwil*⁺ i-cells appear to be able to self regulate themselves in response to genetic manipulations. This in turn allows the animal to develop normally and grow into mature colonies.

Hydractinia, like many other clonal organisms (Buss et al., 1999), does not sequester a germline. Rather, a populations of stem cells termed i-cells provide progenitors for somatic and germ cells (Bosch and David, 1987). *Piwil* is one of the known markers of this i-cell population, which give rise to all somatic and germline lineages (Gahan et al., 2016). The discovery of slow cycling i-cells opens up additional studies on how they differ from the fast cycling ones; a previous study (Bradshaw et al 2015) has demonstrated that polyp i-cells can generate a new colony, including stolons, suggesting that the two populations do not differ in their developmental potency. Possibly, fast and slow cycling i-cells represent two states of the same cell, rather than being distinct cell types.

Are i-cells a primitive trait in hydrozoans, or a de novo innovation? To date, no i-cells have been discovered in Anthozoan, the sister group to Medusozoa to which hydrozoans belong. On the other hand, planarians, an unrelated group of bilaterians, possess stem cells (called neoblasts) that are strikingly similar to hydrozoan i-cells (Scimone et al., 2014). Understanding how PIWI proteins regulate gene expression in this basal metazoan is key to understanding the regulation of both germline and stem cell identity, maintenance and differentiation. My thesis work has provided the tools and preliminary data required to gain this understanding in a lower metazoan invertebrate model. Studies on PIWI proteins from diverse taxa has demonstrated they have a conserved stem cell function. *Hydractinia* indeed share this conservation within diverse taxa as shown by phylogenetic analysis, however future work on the regulatory mechanisms of these proteins will give further insights into the developmental biology and functional role of these genes in a simple but diverse organism.

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Appendix A

1. Piwil ectopic expression vector sequence

Piwil sequence: [gnl|blast|comp47335_c0_seq1_len=3375_path=\[3354:0-7024058:703-3374\]](http://gnl|blast|comp47335_c0_seq1_len=3375_path=[3354:0-7024058:703-3374])

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Piwil coding sequence

GFP coding sequence

NotI restriction site

2. Piwi1 protein sequence

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PNPVLADRLYYL

PAZ Domain- Green

Piwi Domain- Yellow

3. Piwi2 nucleotide sequence

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GGACATCTCACAACACACTAGAGTGACACCTAACCAAAGAAAACAAGCC
ATGTCAAAATTTATACAAAATATTTACAGTAAAGCAGAAGCAGTCGAAG

AGTTGCGTTCGTGGGGATTGGAAGTGGACAAAGATTTAATGAAGATGGA
AGGACGTTTATTACCACCCGAGAAAATTATACTTGGAAGTAAAACAATTA
TGTGTAACGATCAAGCTGACTGGGGTAGAGAGGCCACACGTGAACAAGT
CATCAGTGCAGTTGATATCAATAATTGGATGGTTGTCTGTACCAAACGTG
ATCAGCAAAAAGCAATGGATTTTGTACAAACCATGAAACAATGTTGTCC
ACAAATGGGAATACAGTGTGCGGACCCATCCACGTTTGCTATTAGAGACG
ATCGTACAGAATCTTACCTGCGTGCTATCAGGGAAAACCTTAAATCCGCAA
GTTCAATGTGTGGTGTGTATATCCCAACTTCAAGAGATGATCGATATGC
TGCTGTGAAAAAGTTATGTTGTGTGCGAGTCACCAGTCCCCTCACAGATGA
TAAACGCTAGAACAATATCGCAACAAAACAACTTCGAAGCGTAACACA
AAAAGTCGCACTTCAAATCAATTGTAAATTAGGTGGAGAGTTGTGGGCGT
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GATTCATCTAAATCTACACGATCGATCGGAGGTTTTGTGGCGAGTACAAA
TAAAACCTTCACTCGCTGGTACTCGCGATGTTGTTTTCAAATACCAGGCC
AGGAGTTGATCGATGGTTTTAAAAGTGTGTTTACCAGCGCACTGCGAAAA
TATCACGAGGAAAATCATTGCTTCCCGAAAAAATCATTGTGTTCCGAGA
TGGTGTAGGCGATGGCCAACTCAGTATAGTTGCTAACTACGAAGTGAAA
CAACTTCAAGAATGCTTTCCATTACTTGGTGAATCATAACGAACCTAACT
CTCGGTTATCGTTGTGCAGAAACGAATCTCTCAAAGAATCTTTGCTGTGC
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TCAATAACTAGATGTGATTGGGATGATTTCTTTTTGGTGAGTCAGCATGTT
CGTCAAGGGACGGTGACGCCACACATTACGTTGTTGTGCATGACACTAC
TGGAATGAAAGCTGATCATTTTCAAAGGTTGTCTTATAAACTTACGCATT
TGTATTACAATTGGCCTGGAACGATCCGTGTGCCCGCTCCATGCCAGTAC
GCTCACAACTTGCATTCTTGGTTGGTCAGTCACTACACAAGGCTCCAGC
ATTGGAAGTGCAGATCGATTGTTCTTTTTATAA

4. Piwi2 Protein sequence

MAGLGRGGRGALLLKALETMPKTPGHSQNGQSPTPSGGRPPSTTDQASSQST
TSASSTASSRPPMMMGRGLLAQKLAEQLKSKSPPPGEQKSPSPTAEKLEEKK
RTTPPTASPPPCTSPSLPTDAKREPSPPVKELEQMTVTSMKNLEKGSAGTPNP
MCVNFIFGKSKKEAAFQYSVSFNPPIESKNMRFGMLNEHRDIIGKTKAFDGST
LFLPNRLPEPQMKLQSKRITDGATIELTLTLVKVLDPTQCIQLYNIVLRRIMKL
LEMSQVGRYYYDPKRPSPIPEHRLELWPGYITAIQAYEGGVMLLADVSHRLL
RTD **TCLNFMYDILRKNPQNFQAEVTKQLVGTVVLTRYNNKTYRIDDIAWDK**
SPESKFTYHNGEEMTYIDYKKS YDKTLSDTGQPLLIHRPKEKKG VQKQKGP
LEVICLIPELCSMTGLTDAIRSDFRVMKDISQHTRVTPNQRKQAMSKFIQNIYS
KAEAVEELRSWGLELDKDLMKMEGRLLPPEKIILGSKTIMCNDQADWGREA
TREQVISAVDINNWMVVCTKRDDQKAMDFVQTMKQCCPQMGIQCRDPSTF
AIRDDRTESYLRAIRENLNPQVQ **CVVCIFPTSRDDRYAAVKKLCCVESPVPSQ**
MINARTISQQNKLRSVTQKVALQINCKLGGELWALQIPMKNVMIVGIDVYH
DSSKSTRSIGGFVASTNKTFTRWYSRCCFQIPGQELIDGLKVCFTSALRKYHE
ENHSLPEKIIVFRDGVGDGQLSIVANYEVKQLQECFPLLGESYEPKLSVIVVQ
KRISQRIFAVQGSNLDNPKPGSVLDHSITRCDWDDFFLVSQHVRQGTVTPT
HYVVVHDTTGMKADHFQRLSYKLTHTLYYNWPGTIRVPAPCQYAHKLAFLV
GQSLHKAPALELSDRLFFL

PAZ Domain- **Green**

Piwi Domain- **Yellow**

5. Piwi1 transcript used for annotation

>HSymbio_Piwi1_mRNA

GTAAACCAAGTCAAGAAGCACCAGAGTTGATGAAAGTTCGTTTGGAGAG
TAGACGCGTTTAGGTTTTAGAAAAATTATGACAGGTAGAGCGAGAGGA
AGATCTAGGGGGAGAGGTAAACCAAGTCAAGAAGCACCACGACCTGGTG
GCGGAAATGCTCCACCACCTCAAGCAAGTACTGGTGGCAGGGGGAGATC
AAGAGGTTCTGCACCACAGCCTACACAGCCCCAAAAATCAGCAGTTGCC
CCTCAGAGTGCAGTAGCAGCTGCTCCTGCTGAGCAAATGGCAAAGTTAA

ATGTGCAGGAGGAAAGGCCACAGGAAAGAAGGAGACGGCGGCTAGGTG
AAGAAGCTAACATTTTGGAGGTTGCGTCAAACAGACCAAACAACAGAGGG
CACAGGTGGCGCTAAGGTTGATATTGTGGCCAATTTTTTCAGAGTCGCAA
GATTGCCCAATTCACAGGATTGCACCAGTATAATGTTAGTTTTGATCCA
GACATTCAAAGTGGAAGATTA AAAATCCGCATTGTTGCACAATCTAGATGA
TATTCTTGGCACTGTGCACTGTTTTGATGGCATGACAATGTTTTTGCCCAA
AAAGTTGCCTGATCAAGAAACAGTTCGAACTGTTACTACAAGAACAGGC
AATGACATCACTGTCAAGGTTACTTACACTAATTTGGTTCCACAAAACCTC
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AAATTTCAATGCAACTAATTGGTCGAAATTATTTAATCCATCAAAAAAG
ATTGATATTCCTGCTCACAAAATGCAAGTATGGCCTGGCATCTAACATC
CATTTTGCAGTATGAACAAGATGTGATGTTGTGTGCTGATGTT CAGCATA
AGGTTTTACGACAGCAGACAGTGTGGAATTTTTGTACGAGTTGTATGGT
TCGGTGGACCAAAAAATGTTTTACAATATTGCTGCAAAGAAATTGATTGG
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TACAATAACTTTCAAAGCATATTACGAAGCAGTTTATGAAAGAAAAGTTG
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GAAGAGAGGAATGTCTGGACCTATTTTATTGCTGCCAGAATTTTGTTCAA
TTACTGGATTATCGGATGAAATCAGAAATAATTATTCTGTAATGAAGGAT
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ATCTTCAGTTCACATGTCTAACTGGGTTTTGTTATACACAGCTCGTGACAA
CAACATTGCAAATGACTTTAAACAAACCCTTCAAAGGGTTTGTGGTCCAA
TGGGTATGAATGTAGCCGATCCTCACATGTGTCGTTTAAATAACGATAGT
GCTCAGACATACTCCATGACTCTGAAGGAGCAAATTCAACGAGATACAC
AGATTGTTGTGTGTATCGTTCCTAATAACCGTAAAGATCGGTATGATGCT
ATTAAGAAATTATGCTGTGTGGAAAGACCAGTCCCATCTCAAGTTGTAGT
TAGCCGTACTTTGAGTAAAAAGAACATGTTAATGTCCGTGTGTACCAAGA
TTGGCATT CAGTTAAATTGTAAGTTAGGAGGCGAAGTATGGGCAGTTGAC
ATACCTTTAAATAAATTGATGGTGATTGGATTTGACGTTTACCACGACTC

TGCTACTAAGGGCAAGTCCATTGGTGGGTTTGTGTTGCCAGCACTAATAAGC
ATCTTACACGATATTATTCGTGCATTACGTCACAAACATCTCATATGGAA
ATATGTGATCAGCTCAAAGTCTGCATGACCAATGCGCTAAAGAAATTCCA
CGAAATTAACGATGGCTTACCCGACAAGATTATTGTCTATCGAGATGGTG
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GGCAGTATTCAAATCCTCCTCCCGGAACTGTTGTTGACACTGTGGTAACA
CGTCCAGAGTGGTACGACTTTTTGTTGATTTACAATCGGTCAGACAAGG
TACGGTCACTCCGACGCATTATAACGTGATTAACGACACAACCTGGCTTAA
AACCGGATCACTTCCAACGTTTAACTTACAACTTTGTCATTTGTATTACA
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TTAGCCTTCTTAGTTGGTCAGTCGATCCACACGGAGCCTGCTCGACAATT
GGCCGATCGTCTTTATTATTTATAAGCAGCTGCGCGTTGTTTACGTGGAG
GTTGTTTTGCGCTATGACTTCTAAAACTGAAAAGTATTGTTTATATTGTT
TATATTGGCGTTTATATTTTTAACAATAACCAGTAGTAGTAGTATTTTAT
TTTAAAACGATTCATATAGGTTGTATTTTCTTGTGTTTCGAGACTTTTTTTA
GCTATATAAGCTTTTTCTTGGGTTATTTTTGACATAGTTTACAAAGATTGC
TTAATCTTACCTGTGTATGTTTCATGCAATGTCGTCAAGATATTCACCTTGT
TAGCAGTTGCTTGTGTAATCATTTTTTCCCTAATCATTTCACGAAGAGCA
AATGTAAAGAGTTTGCAAATCTGTTTTAGATTTTCGTTTTGAGATGCGGGT
GTAAAAAATGTTTTTAGCAAAGGATTTTTGTTCTTTTTTATATTTTTGTTA
ATTTTCATATTTTTTCGAGAAAAGTGATCACACA

6. Piwi1 N term protein sequence with His Tag

CATATG**CACCATCACCATCACCA**CATGACAGGTAGAGCGAGAGGAAGA
TCTAGGGGGAGAGGTAAACCAAGTCAAGAAGCACACGACCTGGTGGCG
GAAATGCTCCACCACCTCAAGCAAGTACTGGTGGCAGGGGGAGATCAAG
AGGTTCTGCACCACAGCCTACACAGCCCCAAAATCAGCAGTTGCTCCTC
AGAGTGCAGTAGCAGCTGCTCCTGCTGAGCAAATGGCAAAGTTAAATGT
GCAGGAGGAAAGGCCACAGGAAAGAAGGAGACGGCGGCTAGGTGAAGA

AGCTAACATTTTGGAGGTTGCGTCAAGCAGATCAAACAACAGAGGGCACA
GGTGGCGCTAAGGTTGATATTGTGGCCAATTTTTTCAGAGTCGCAAGATT
GCCCAATTTTACAGGATTGCACCAGTATAATGTTAGTTTTGATCCGGACA
TTCAAAGTGGAAGATTA AAAATCCGCATTGTTGCACAATCTAGATGATATT
CTTGGCACTGTGCACTGTTTTGATGGCATGACAATGTTTTTGCCCAAAA
ATTGCCTGATCAAGAAACAGTTCGGACTGTTACTACAAGAACGGGCAAT
GACATCACTGTCAAGGTTACTTACACTAATTTGGTTCCA

(His tag sequence in red)

7. Piwi2 N term protein sequence

CATATGCATCATCATCATCACATGGCAGGTCTTGGCCGGGGAGGGCG
TGGAGCTTTGTTGCTTAAGGCGTTGGAACTCCTATGAAAACCCCTGGTC
ATTCCCAGAATGGACAGTCACCAACACCATCAGGAGGTCGACCTCCTTCA
ACTACAGATCAAGCATCAAGTCAGTCAACTACATCAGCTTCATCAACAGC
CTCTTCTCGTCTCCAATGATGATGGGCAGAGGTTTACTAGCACAAAAAT
TAGCAGAACAGCTTAAATCCAAATCTCCTCCACCTGGGGAGCAAAAGTCT
CCATCACCCACAGCTGAGAAGTTGGAAGAGAAAAAAGAACAACCTCCTC
CAACAGCTTCACCGCCACCTTGTACTTCTCCAAGTTTACCTACAGATGCA
AAACGAGAGCCATCACCACTGTAAAAGAATTGGAACAAATGACAGTGA
CATCAAGGAAAAATTTGGAGAAGGGGAGTGCTGGTACACCAAATCCTAT
GTGTGTAAACTTTATTGGCTTTAAAAGCAAGAAAGAAGCAGCATTCCAAT
ACAGCGTGTCTTTAACCACCTATAGAAAGCAAAAATATGCGTTTTGGA
ATGTTGAATGAGCATCGAGATATCATAGGAAAAACAAAAGCTTTTGATG
GTTCAACGTTGTTTCTTCCAATCGTCTACCTGAACCGCAAATGAAATTA
CAAAGCAAAAGAATTACTGATGGCGCCACAATCGTAGGGATCC

(His tag sequence in red)

Appendix B

GFP::mRNA sequence

TAATACGACTCACTATAGTTCTAGAGGCTCGAGAGGCCTTGAATTCCTAT
ATAATTTCAATGCGGCCGCTGCAGCCCCGGTAGAAAAAATGAGTAAAGG
AGAAGA ACTTTTCACTGGAGTTGTCCCAATTCTTGTGTAATTAGATGGTG
ATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGC
AACATACGGAAA ACTTACCCTTAAATTTATTTGCACTACTGGAAA ACTAC
CTGTTCCATGGCCAACACTTGTCACTACTTTTCTGTTATGGTGTTC AATGCT
TTTCAAGATACCCAGATCATATGAAACGGCATGACTTTTTTCAAGAGTGCC
ATGCCC GAAGGTTATGTACAGGAAAGAACTATATTTTTTCAAAGATGACG
GGA ACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGT
AATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTC
TTGGACACAAATTGGAATACA ACTATAACTCACACAATGTATACATCATG
GCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAA AATTAGACACA
ACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATAC
TCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCA
CACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGT
CCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAAC
TATACAAATAGGAGCTCACACTTTTTCGTTTTTAGGATCC

CS2+ vector map

