Distinct mechanisms underlie oral versus aboral regeneration in the cnidarian hydractinia echinata

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Distinct Mechanisms Underlie Oral Versus Aboral Regeneration in the Cnidarian *Hydractinia echinata*

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Discipline: Zoology  

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Summary of Contents

Distinct mechanisms underlie oral versus aboral regeneration in *Hydractinia echinata*. Head regeneration is preceded by stem cell migration, proliferation and blastema formation. After decapitation stem cells migrate from the aboral band, proliferate and form a blastema. Cell proliferation is essential for head regeneration as regeneration cannot be completed when proliferation is blocked. The i-cell genes *Piwi1*, *Pl10*, *Myc2* and *Vasa* plus the nematoblast differentiation marker *Ncol1* are expressed in or below the blastema during regeneration. RNAi knockdown of these genes revealed that they are not required for blastema development but are required for regeneration.

The generation of a transgenic stem cell reporter animal enabled the tracking, for the first time, of single transgenic cells *in vivo* during *Hydractinia* regeneration. This was a major breakthrough and revealed the migration and proliferation of *Piwi1*+GFP stem cells from the i-cell band to the blastema and the contribution these cells play in regeneration.

Aboral regeneration occurs in a completely different way and time scale. It does not involve blastema formation but rather a transformation of tissue identity from polyp to stolon. It is preceded by the loss of *Wnt3* expression and the anterior-posterior polarity of the polyp. This is then followed by the reabsorption of tentacles and head structures and a change in the restricted distribution of stem cells. Once transformed this new stolon tissue can found a new colony and continue to sexually reproduce being able to contribute to further generations.

These results reveal that *Hydractinia* polyps regenerate by epimorphosis and are capable of regenerating lost tissues in two distinct ways. Cell proliferation and blastema formation are essential in distal regeneration in *Hydractinia* indicating a possible evolutionarily conserved mechanism within the Metazoa.
Declaration of Contribution

I declare that this thesis is all my work and I did not obtain a degree in the National University of Ireland, Galway or elsewhere on the basis of this work.

My Project was funded by The Programme for Research in Third-Level Institutions (PRTLI) and Science Foundation Ireland (SFI)
Chapter 1. Introduction

1.1 Regeneration overview

Regeneration, the renewal or restoration of cells, tissues and organs is a topic that has long fascinated humans. While humans and other mammals are generally poor regenerators, some basal invertebrates have staggering powers of regeneration being able to replace any missing body part (Sanchez Alvarado and Tsonis, 2006; Galliot and Chera, 2010). Regeneration is widespread throughout the animal kingdom with representatives in every phylum capable of regenerating missing body parts (Fig. 1.1.1). Though regeneration is widespread the ability and modes used varies greatly within and between different taxa (Brockes and Kumar, 2008). The rearrangement of pre-existing tissues, the use of adult somatic stem cells and the dedifferentiation or transdifferentiation of cells are all processes employed during regeneration. The use of these modes varies greatly with more than one mode often used within the same animal and different modes often employed in closely related taxa (Sanchez Alvarado and Tsonis, 2006). Thomas H. Morgan originally classified regeneration in the Metazoa into two groups. Morphallaxis - the regeneration or repair of tissue without a significant contribution from cell proliferation and epimorphosis - regeneration which requires proliferation (Morgan, 1901). These two processes have been shown to be not mutually exclusive with different taxa often using a combination of both to regenerate lost tissue (Sanchez Alvarado, 2000).
Fig. 1.1.1. Simplified schematic outlining the distribution and regenerative ability of different metazoa taxa. Red line indicates regenerative ability while the absence of a red line reflects inability to regenerate. Cnidarians, echinoderms and platyhelminthes all contain species that can regenerate complete animals from cut sections. Many Arthropod species, including crustaceans and hemimetabolous insects, can regenerate lost limbs though *D. melanogaster* and *C. elegans* cannot. Vertebrates including salamanders and fish can regenerate lost limbs but most species including humans cannot.
*Hydra* has long been used as an example of an animal that regenerates through morphallaxis (Cummings and Bode, 1984; Bode, 2003) though recent work has cast doubt on it being the only process contributing to head regeneration (Chera et al., 2009). Epimorphosis, regeneration though cell proliferation, can be classified into two distinct groups, blastemal and non-blastemal regeneration. Blastema epimorphosis involves the formation of a specialised structure called a regeneration blastema which can form within hours or days after amputation or injury. This structure is made up of two distinct compartments. An outer cell layer or epidermis which closes and covers the wound site and a second population of cells that proliferate and accumulate beneath this epidermis out of which the regenerating section will develop (Sanchez Alvarado and Tsonis, 2006). The use of a blastema to regenerate and repair lost tissue has been observed in both vertebrate and invertebrate taxa though the exact process of its formation can differ (Fig. 1.1.2.).

![Image of blastema formation in vertebrates and invertebrates. Stem cell proliferation plays a pivotal role in blastema regeneration in invertebrates while both cell-dedifferentiation and stem cell proliferation are key in vertebrates. (Picture taken from (Sanchez Alvarado and Tsonis, 2006)](image-url)
Planarians which regenerate using both morphallaxis and epimorphosis, first close the wound by modifying cells already present at the amputation site. The second population of proliferating cells are derived from the migration and proliferation of resident pluripotent stem cells called neoblasts (Reddien and Sanchez Alvarado, 2004; Guedelhoefer and Sanchez Alvarado, 2012). This source of proliferating cells is in contrast to the cellular dedifferentiation observed in limb regeneration in some vertebrate groups (Sandoval-Guzman et al., 2014).

Non-blastemal epimorphosis does not involve the formation of a blastema, instead, regeneration is completed through processes such as transdifferentiation, dedifferentiation and proliferation of stem cells already present in the damaged tissue (Fig. 1.1.3) (Jopling et al., 2011; Tanaka and Reddien, 2011). Non-blastemal epimorphosis has been observed in lens and retina regeneration in newts (Call et al., 2005) and bone regeneration in humans (Prockop, 1997).

Many invertebrate groups show robust powers of regeneration. The two most widely studied groups are the freshwater cnidarian *Hydra* (See section 1.4 for *Hydra* overview) and the non-parasitic planarian flat worms (Reddien and Sanchez Alvarado, 2004). Both of these groups, though not closely related, are capable of regenerating complete animals from small cut sections while *Hydra* can regenerate whole new animals from re-aggregations of dissociated cells (Gierer A et al., 1972).

Regeneration in the phylum Annelida has been studied for over 100 years (Randolph, 1892). This phylum of segmented worms which includes the oligochaete and polychaete worms is grouped with planarians and others in the superphylum Lophotrochozoa. Though not closely related these two groups do both share many regenerative characteristics. Annelids are capable of both head and tail regeneration and like both planarians and *Hydra* can in some cases regenerate from very small fragments (Bely and Wray, 2001; Bely, 2014).
Differentiation is the process whereby a less specialised cell becomes more specialised. Transdifferentiation is the process where one differentiated somatic cell changes into another differentiated somatic cell without undergoing an intermediate multipotent or pluripotent state. Dedifferentiation is the process where a more specialised cell reverts back to a less specialised state.

The superphylum Ecdysozoa contains the Arthropoda and the much smaller phylum Nematoda among others. The Arthropoda is the largest phylum in the animal kingdom and contains both groups that can regenerate robustly and some that cannot. Among the Arthropoda, many species of crustaceans and insects are capable of replacing lost limbs though *Drosophila*'s poor regenerative ability is well documented (Hopkins, 2001). *C. elegans* a member of the Nematoda also shows remarkably poor regenerative ability (Sanchez Alvarado and Tsonis, 2006).

Echinoderms are capable of regenerating new arms, disc, gut, spines and podia. Moreover, separated sections containing partial tissue of the central disk are capable of regenerating whole new organisms (Candia Carnevali and Bonasoro, 2001). Echinoderms have been shown to use both morphallaxis and epimorphosis during regeneration. The brittle stars and feather stars use epimorphosis, forming a blastema.
through the migration of undifferentiated cells (amoebocytes and coelomocytes) that proliferate and accumulate at the end of the nerve cord. This is in contrast to sea stars and sea urchins which regenerate though morphallaxis using cells derived from existing tissues by differentiation, transdifferentiation or migration (Thorndyke et al., 2001; Carnevali, 2006).

Chordates which include both invertebrate and vertebrate taxa are grouped with the Echinoderms and others in the superphylum Deuterostomia. Though some species of invertebrate chordates are good regenerators most species of vertebrates excluding amphibians and fish are not. The invertebrate chordate Amphioxus is capable of regenerating both anterior and posterior structures including muscles, fins, notochord and neural tube (Somorjai et al., 2012). Other invertebrate chordates including some species of tunicates are also good regenerators. The solitary ascidian *Ciona intestinalis* is capable of siphon regeneration post amputation (Jeffery, 2014b, a) while the colonial species *Botrylloides leachi* can regenerate an entire adult zooid from a small blood vessel fragment (Rinkevich et al., 2007).

With the exception of urodele amphibians and some fish species, vertebrates are mostly poor regenerators. Adult teleost fish are capable of regenerating fins, spinal cord and brain tissue (Poss et al., 2000) while the rainbow fish *Lebistes reticulatus* can regenerate the forebrain within 2 months (Zupanc, 1999; Zupanc, 2006).

Urodele amphibians including both newts and salamanders can regenerate many lost body parts throughout their lives. This includes limbs, tail, lens, retina and heart (Bader and Oberpriller, 1978; Giudice et al., 2008) while the anuran amphibians, like *Xenopus*, lose the ability to regenerate after metamorphosis (Dent, 1962; Lin et al., 2013). Limb regeneration in urodele amphibians is achieved by blastemal regeneration through the use of lineage restricted rather than pluripotent stem cells (Kragl et al., 2009). Whether these blastema cells arise by cellular dedifferentiation or from resident adult stem cells has long been debated. Recent work in this area has shown that in the newt, *Notophthalmus viridescens*, dedifferentiation of cells in the regenerating limb
provide progenitor cells to the blastema, while in the closely related axolotl *Ambystoma mexicanum*, resident stem cells fulfil the same task (Sandoval-Guzman et al., 2014) outlining two very different systems in two closely related taxa. Mammals do not possess the same regenerative capabilities that are observed in other vertebrate groups. Digit tip regeneration, distal of the first phalange has been observed in both mice and young children and is achieved through a regenerative blastema (Illingworth, 1974). In mice this blastema is mediate by fate-restricted progenitor cells that give rise to the new tissue (Lehoczky et al., 2011). Other regenerating tissues in mammals includes skin, intestinal tract epithelium, haematopoietic tissue and gamete primordia (Giudice et al., 2008)
1.2 Stem Cell Differentiation Potential

Stem cells are undifferentiated cells, capable of both self-renewal and differentiation into other specialised cell types (Fig. 1.2) (Bongso and Richards, 2004). The term was first used by Weismann in 1883 (Weismann, 1883) while working on *Hydractinia* to describe migratory precursors of germ cells and later by the Russian histologist Alexander Maksimov to propose the existence of hematopoietic stem cells (Svendsen, 2008). Since then many different types of stem cells have been discovered including the root and shoot meristems of plants and the embryonic and adult stem cells in animals (Bongso and Richards, 2004; Sugimoto et al., 2011). Stem cells play a major role in the regenerative capacity of an organism. The presence or absence of different stem cell types have been shown to directly affect the regenerative potential of an organism throughout its life cycle (Sanders et al., 2006; Tanaka and Reddien, 2011). Because of their undifferentiated state and ability to self-renew, stem cells have been used in both basic and translational research. The most successful use of stem cells in a clinical context is the replacement of bone marrow in cancer patients (Karanes et al., 2008). Though research has been ongoing in many areas including degenerative diseases like Parkinson’s and Alzheimer it has proven less successful (Tabar and Studer, 2014).

Stem cells can be classified by their differentiation potential i.e. their ability to differentiate into different cell types. The common terms used are totipotent, pluripotent, multipotent, oligopotent or unipotent depending on their differentiation potential (Sanders et al., 2006) (Fig. 1.2). Totipotent stem cells have unlimited regenerative ability and can form all cell and tissues types that make up an embryo including extraembryonic tissues. Totipotent cells have the greatest differential potential but are often only present in the very early stages of an organism’s life cycle (Papp and Plath, 2013). In humans, fertilization creates a single totipotent cell, the zygote. During the first few hours after fertilization the zygote itself divides into two identical totipotent cells. At the 16 cell stage the morula differentiates into cells that
will eventually become either the inner cell mass or the outer trophoblast of the blastocysts. This inner cell mass is the source of embryonic stem cells and at this stage is no longer totipotent but pluripotent. Pluripotent stem cells only have the ability to differentiate into germ cells and any cells of the three germ layers ectoderm, mesoderm and endoderm. They do not have the ability to contribute to the extraembryonic tissue or to organize into an embryo (Gilbert, 1997; Mitalipov and Wolf, 2009). As the pluripotent cells of the inner cell mass develop, all cells, excluding the sequestered germ line cells become multipotent. Multipotent stem cells can develop into a limited number of different cell types, usually within the same germ layer (Mitalipov and Wolf, 2009). Multipotent hematopoietic blood cells, which resided in the bone marrow, can differentiate into several different blood cell types but are unable to differentiate into brain, bone or other non-blood cell types. Some of these blood cell types like the lymphoid and myeloid stem cells are both derived from multipotent stem cell but are themselves considered oligopotent stem cells. Oligopotent stem cells are more specialised than multipotent cells and can differentiate into a more limited number of different cell types compared with their precursors. Unipotent stem cells have the lowest differentiation potential of all stem cell types. Unipotent hepatoblasts are capable of self-renewing and differentiating into hepatocytes which constitute the majority of the tissue in the liver (Prockop, 1997; Fernández and de Alarcón, 2013).

The undifferentiated state and self-renewing ability displayed by stem cells is tightly regulated. These processes are controlled by extrinsic signals from the microenvironment which is called the stem cell niche (Zhang and Li, 2005). If these tightly regulated processes are lost it can often lead to uncontrolled cell growth or pre-maturation which can lead to tumours or tissue defects. To maintain a state of pluripotency stem cells express a high level of pluripotency genes while suppressing genes which promote cellular differentiation (Zhang and Li, 2005; de Cuevas and Matunis, 2011; Wagers, 2012). The expression of the transcription factors Oct4, Nanog
and Sox2 has been shown to be vital in maintaining pluripotency. Oct4 is a major regulator during embryogenesis in mice specifying the inner cell mass while counteracting differentiation (Nichols et al., 1998; Wang et al., 2012). The expression level of this gene needs to be tightly regulated. Overexpression of Oct4 triggers differentiation of mouse embryonic stem cells into endoderm and mesoderm while inactivation causes loss of self-renewal and induction of trophectoderm differentiation (Niwa et al., 2000; Ivanova et al., 2006; Wang et al., 2012). These genes work together while incorporating other genes into a complex network that maintains pluripotency. Other important genes playing roles in pluripotent cells include Myc, Klf4 and Lin28 (Papp and Plath, 2013).

The successful isolation of human embryonic stem cell (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). This commitment to fate displayed by stem cells cannot normally be reversed in vivo (Papp and Plath, 2013). However, new technologies have emerged capable of reprogramming somatic cells to pluripotency in vitro. The reversal of somatic cells back to a pluripotent state was first demonstrated using mice fibroblasts. These induced pluripotent stem cells (iPSC) were obtained by forced expression of four transcription factions Oct4, Sox2, Klf4, and c-Myc in mouse adult fibroblasts and highlighted the ability of differentiated cells to be reprogrammed back to an undifferentiated pluripotent state (Takahashi and Yamanaka, 2006). This technique was also successfully used in reprogramming human somatic skin cells into iPSC and highlighted the importance of these four transcription factions Oct4, Sox2, Klf4, and c-Myc in inducing and maintaining pluripotent stem cell properties in different taxa (Yu et al., 2007; Wang et al., 2012). Other research has shown the ability of multipotent cells to be converted into unrelated cell types. This has been demonstrated in the ectopic expression of neural specific transcription factors in mouse fibroblasts converting them into neurons (Vierbuchen et al., 2010) and the ectopic expression of
Sox2 and c-Myc in human umbilical cord blood converting them into neuronal cells (Giorgetti et al., 2012)

Fig. 1.2. Schematic describing the change in differentiation potential found in a typical mammalian stem cell through development. Stem cells at the morula stage are totipotent and have the highest differentiation potential. As the animal develops and the cells specialise they move from a totipotent state to a pluripotent, multipotent and finally terminally differentiated state where the cells have no differentiation potential. (Original image http://healinghandsmassages.com/wp-content/uploads/Stem-cells.jpg)
1.3 Regeneration in Cnidarians

The extraordinary regenerative power of cnidarians has long been known. The first experiments on animal regeneration were completed on the hydroid *Hydra* by the Swiss naturalist Abraham Trembley in 1744 (Trembley, 1744). He experimented by amputating the head and foot of *Hydra* and observed their regenerative response. He also conducted experiments by grafting cut sections of different polyps and made observations on asexual reproduction through budding (Trembley, 1744; Lenhoff, 1988). Other early work on the hydroid *Tubularia* was completed by T.H. Morgan and C.M. Child in the early 1900’s where they studied regeneration and axial patterning. Morgan proposed the existence of “a gradient of material” that decreased in distance from the apical to the basal end of the polyp which enabled pattern formation and development (Morgan and Stevens, 1904; Child, 1907; Morgan, 1908). Morgan and his student Annah Hazen also completed transplantation experiments in *Hydractinia echinata*. They described the presence of polarity and tissue memory after transplantation of cut sections from different polyps. They also described the ability of isolated feeding polyps to regenerate stolons and transform into new colonies thus completing both distal and proximal regeneration (Hazen, 1902). More recently, the transdifferentiation of cells has been reported in the hydrozoan *Podocoryne carnea* where striated muscle cells have been show to transdifferentiate into new cell types (Schmid and Reber-Müller, 1995).
1.4 The *Hydra* Model

*Hydra* has long been the reference point for cnidarian regeneration, all though, recent work describing regeneration in the starlet sea anemone *Nematostella vectensis* has been published (Marlow et al., 2009; Passamanek and Martindale, 2012). *Hydra* is a genus of freshwater hydroids. They are solitary polyps and possess a simple body plan of a head, body column and foot (Fig. 1.4). They reproduce both asexually through budding and sexually, though sexual reproduction is less frequent. Like all cnidarians, *Hydra* consists of two cell layers, the epidermis and gastrodermis which are separated by an extracellular matrix called the mesoglea. They possess a number of different cell types including i-cells, gland cells, neurones, nematocytes and epithelial cells. *Hydra* contains three distinct cell lineages, epidermal and gastrodermal epithelial cells and i-cells. Epithelial cells of the epidermis and gastrodermis proliferate in the body column and are displaced to the extremities being sloughed off at the termini of the tentacles and foot. All other cells in the animal are derived from the multipotent i-cell lineage. A bisected *Hydra* will regenerate the oral and aboral section within three days. Any isolated fragment of the *Hydra* body which includes at least a few hundred epithelial cells can regenerate into a new animal. Animals that are dissociated into a cell suspension can re-aggregate and regenerate into new polyps (Gierer A et al., 1972; Galliot and Schmid, 2002; Holstein et al., 2003; Bode, 2009). *Hydra* has long been used as an example of regeneration by morphallaxis (Sanchez Alvarado and Tsonis, 2006; Poss, 2010; Sugimoto et al., 2011; Technau and Steele, 2011). This was illustrated when decapitated *Hydra* regenerated after cell division was blocked using both γ-irradiation (Hicklin et al., 1975) and hydroxyurea (Cummings and Bode, 1984). Interestingly polyps devoid of i-cells following treatment with hydroxyurea can also regenerate and produce new polyps by asexual budding (Cummings and Bode, 1984). Similar results were also seen in experiments completed on the *Hydra magnipapillata* mutant strain sf-1. These animals lose their i-cells following heat treatment, after which they were still capable of head regeneration (Sugiyama and Fujisawa, 1978).
The dogma of regeneration in *Hydra* strictly by morphallaxis has recently been challenged. It has been shown that after mid-gastric bisection, head regeneration depends on an initial apoptotic response at the wound site. This response in turn triggers a blastema like proliferative zone which is essential for oral regeneration (Chera et al., 2009; Chera et al., 2011). The importance of proliferation in *Nematostella* regeneration has also been demonstrated, as blocking cell division also blocks regeneration, highlighting different regenerative modes that exist in the phylum (Passamananeck and Martindale, 2012).

![Fig. 1.4. (A) Schematic outlining the typical body plan found in *Hydra* showing the position of the head, body column and foot. The head contains both the mouth/anus and tentacles. The body column contains the proliferating epidermal and gastrodermal cells. The proliferating i-cells are located in the interstitial spaces of the epidermal epithelial cells (not shown). (B) Schematic outlining the regenerative ability of a *Hydra* polyp. (a) A polyp cut in half will regenerate a new head at the oral tip of the lower half and a foot on the aboral tip of the upper half. (b) A polyp with both the head and foot amputated will regenerate both. (c) A polyp with both the head and foot amputated and cut longitudinal will close the longitudinal cut first and then regenerate both a head and foot. (d) A single polyp can regenerate from a very small fragment of tissue. Pictures taken from (Bode, 2003)]
1.5 A Closer Look at Cnidarians

The phylum Cnidaria contains over 10,000 species of marine and freshwater invertebrates. They are divided into two major sub-groups the Anthozoa and the Medusozoa (Fig. 1.5 A) (Steele et al., 2011). The class Anthozoa contains the corals, sea pens and the sea anemones. *Nematostella vectensis* and *Acropora millepora* both belong to the Anthozoa and have both been studied extensively. The Medusozoa is divided into four classes, Staurozoa (stalked jellyfish), Cubozoa (box Jellyfish), Scyphozoa (true jellyfish) and the Hydrozoa (Collins, 2009).

The defining shared characteristic of all cnidarians is the nematocyte or stinging cell (Fig. 1.5 B). This cell is unique to cnidarians and is not found in any other group. They are used for defence, prey capture and adhesion. There are over 30 different types of nematocytes found in cnidarians and they can be classified into three distinct groups. Volvents, which release a lasso type string and are used in prey capture, glutinants, which aid surface adhesion and penetrants, which release a harpoon and are used for defence and prey capture. Each nematocyte contains a specialized capsule called a nematocyst. Penetrant nematocysts carry a coiled thread, venom, barb and trigger. These nematocysts can release and discharge venom when physically or chemically stimulated. Nematocytes are found all over the animal but are found in greatest numbers in the tentacles and around the mouth (Holstein et al., 2003).
The life cycle of the Medusozoa is complex with most species being able to reproduce both sexually and asexually. The life cycle usually begins with fertilization of the egg, development of the embryo into a planula larva which in turn develops into a polyp. Some polyps bud medusa, which in those species is the sexually reproducing stage. Some developmental stages are shared between all groups, like the planula larva, while some stages like the adult medusa are present in only selected species (Holstein et al., 2003). Amazingly, it has been reported that the medusa of the hydroid *Turritopsis nutricula* can transform back into a colonial hydroid. This can be done either directly or through a resting period enabling the animal to achieve potential immortality (Piraino et al., 1996).
1.6 *Hydractinia echinata*

*Hydractinia echinata* belong to the class Hydrozoa in the phylum Cnidaria (Fig. 1.5). *Hydractinia* is a clonal marine invertebrate and can be found living on gastropod shells inhabited by hermit crabs (Frank et al., 2001). There are over 30 species of *Hydractinia* worldwide but the majority of research has been conducted on only two species *H. echinata* and *H. symbiolongicarpus*. Both species are found coastally in the north Atlantic, *H. echinata* in northern Europe and *H. symbiolongicarpus* in North America (Frank et al., 2001). Adult colonies are made up of four different types of polyps, feeding polyps (gastrozooids), sexual polyps (gonozooids) and two types of defensive polyps (dactylozooids, tentaculozooids). These polyps are interconnected through a network of gastrovascular tubes called stolons where food and stem cells can be shared among the colony. Colonies are diecious and increase in size by growing new stolons out of which new polyps bud (Frank et al., 2001; Plickert et al., 2012). The *Hydractinia* life cycles starts with the release of male and female gametes from different colonies into the water where fertilization occurs (Fig. 1.6.). The animals spawn daily about 1-2 hour after exposure to light. Embryonic development takes between 2-3 days to complete and at which point the embryos develop into planula larva. These planula larvae are motile but are mouthless so do not feed. Metamorphosis begins when the larva adheres to a gastropod shell. It is induce by bacteria that grow on the shells and takes 24 hours to complete. During metamorphosis the larva transforms into a single feeding polyp with 1-3 short stolons. The primary polyp will grow into a new colony by asexual reproduction and will continue to increase in size indefinitely. The colony will become sexually mature within 2-3 months completing the life cycle (Frank et al., 2001).
Fig. 1.6. The life cycle of *Hydractinia echinata*. After fertilization the embryo develops into a planula larva and then metamorphose into a primary polyp. This polyp will develop into either a male or female adult colony. Female colonies will develop sexual polyps that contain oocytes while male colonies develop sexual polyps that contain sperm.
1.7 *Hydractinia* i-cells

*Hydractinia* possess several different cell types. These include stem cells, several types of nematocytes, gland cells, epidermal and gastrodermal myoepithelial cells and several types of neurons (Frank et al., 2001; Muller et al., 2004a; Plickert et al., 2012). The stem cells of *Hydractinia* were the first to be described in biological literature by Weismann in 1883 (Weismann, 1883; Frank et al., 2009). It was while studying these stem cells that Weismann proposed his germ plasm theory. This was centred on the idea that multicellular animals contain two different groups of cells, “germ cells” that contain and transmit heritable information and somatic cells that carry out ordinary functions (Weismann, 1892). Incidentally it is today thought that hydroids do not sequester a germ line during embryonic development as is seen in other clonal animals. All cell types, both somatic and germ cells are thought to be derived from a population of pluripotent stem cells present throughout the animal’s life (Frank et al., 2009; Millane et al., 2011; Plickert et al., 2012). Stem cells in *Hydractinia* are known as i-cells (interstitial cells). They are undifferentiated and found within the interstitial spaces between epithelial cells. So far i-cells have only been found in hydrozoans and may not be present in anthozoans (Frank et al., 2009). I-cells are about 10 µm in size and generally contain a large nucleus. They are found in large numbers in stolons but also in smaller numbers in polyps (Fig. 1.7). They migrate from stolons into polyps where they form a small population in the aboral region of the polyp. Adult stem cells in *Hydractinia* maintain pluripotency, being able to give rise to germ cells throughout their lifetime (Plickert et al., 2012). Highly conserved RNA binding proteins such as *Vasa, Nanos, Pumilio,* and *Piwi* prevent somatic differentiation and control many other functions in animal germ cells (Juliano et al., 2010; Lehmann, 2012). Because i-cells are pluripotent and also give rise to germ cells, they express these typical germline markers which can be used as efficient i-cell markers in *Hydractinia* (Plickert et al., 2012).
Fig. 1.7. Distribution of i-cells in a typical *Hydractinia* colony. I-cells (red) are found in greatest number in stolons where they migrate into both feeding and sexual polyps. In feeding polyps they form a band in the aboral region of the polyp.

*Hydractinia echinata* is an ideal model to study regeneration. It’s impressive regenerative ability has long been known but is has also been used to study countless different biological processes for many years. Many experimental techniques have already been established including *in situ* hybridisation, Immunostaining, overexpression and knockdown. *Hydractinia* has previously been used to study stem cells and cell fate determination, pattern formation in embryonic and post metamorphic development, regeneration, allore cognition and embryonic development.
1.8 Aims of this Project

To answer the question of why some animals can regenerate robustly and some cannot it is important to understand the fundamental differences that exist between such groups. The basal phylogenetic position and regenerative powers displayed by cnidarians give them a pivotal position for studying the regenerative processes within the Metazoa. The majority of research on regeneration in this phylum has been completed on *Hydra*. It is not known, however, whether this animal’s regenerative mechanisms are employed throughout the phylum. Studying regeneration in another cnidarian can help answer this question while also addressing the phenomenon of why some animals possess such powerful regenerative abilities and others do not.

The specific aims of my project were as follows:

1. Identify the cellular modes employed during both oral and aboral regeneration in *Hydractinia*
2. Study stem cell behaviour *in vivo* during regeneration using a transgenic reporter approach
3. Perform expression and functional studies on a selection of stem cell genes to identify the role they play in regeneration using *in situ* hybridization, immunohistochemistry, irradiation and RNAi knockdown techniques.

The results of my work revealed that *Hydractinia echinata* can regenerate using two distinct methods. Cell proliferation and blastema formation are essential in oral regeneration but not in aboral regeneration. The presence of a blastema during head regeneration would suggest a possible conserved role of this process throughout the Metazoa.
Chapter 2. Materials and Methods

2.1 Animal culture

2.1.1 Feeding and cleaning

- The colonial marine hydroid *Hydractinia echinata* was used as an experimental model in this study. In the wild the animals grow on hermit crab shells and were sampled off the west coast of Ireland.
- Animals were fed 5 days per week with brine shrimp nauplii (*Artemia*) and cleaned daily 6 hours after.
- Animals were kept in filtered seawater tanks and at a constant temperature of 18°C under a 14/10 light-dark time phase.
- Fertilized embryos were collected daily 2 hours after the onset of light.

2.1.2 Metamorphosis

- Metamorphosed was instigated 2-3 days after fertilization by incubation in a 1:4 CsCl 580 mM/seawater solution.
- After incubation the larvae were washed in seawater and transferred to a glass slide or petri dish to complete metamorphosis.
- Metamorphosis took about 24 hours to complete.

2.2 DNA and RNA Protocols

2.2.1 Genomic DNA extraction

- Add 1.5 mL of lysis buffer with freshly added DNase-free RNase to the animals.
- Incubate the tube on a heat block for 1 hour at 37°C.
- Add 15 μl of Proteinase K (Sigma P6556) stock solution to a final concentration of 100 μg/mL.
- Mix the solution by gently inverting the tube.
- Incubate the lysate at 50°C in a hybridization oven with gentle rotation until the solution is clear.
- Cool and divide each sample into two aliquots (~750 μl each).
- Add 750 μl of phenol equilibrated with 0.1 M Tris (pH 8) to each tube.
- Gently mix the two phases for 2 minutes.
- Centrifuge the sample in a centrifuge at 5000 g for 15 minutes at room temperature (RT).
- Use a P-1000 pipette tip with the end cut off to transfer the viscous aqueous phase to a fresh tube.
- Repeat the phenol extraction once.
- Add 750 μl of phenol:chloroform:isoamyl alcohol (25:24:1 [v/v]).
- Extract once with 750 μl of chloroform.
- Transfer the upper aqueous phase to a fresh 2 ml tube and measure the volume.
- Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5x volume of 100% ethanol and mix gently.
- The DNA should immediately form a stripy precipitate in the solution.
- Recover the DNA by centrifugation at 15000 g for 15 minutes at RT.
- A small white pellet should be visible at the bottom of the tube.
- Remove the supernatant and wash with 75% ethanol.
- Centrifuge at 15000 g for 5 minutes.
- Remove the supernatant and wash with 1 ml of 100% ethanol.
- Centrifuge at 15000 g for 5 minutes.
- Remove the ethanol and air-dry the pellet at RT for no more than 10 minutes.
- Dissolve and combine the DNA pellets from the same animal in 200 μL of Nuclease free H2O.
- Check the concentration and quality of the DNA using a spectrophotometer.
- Store the DNA at -20°C.

2.2.2 Total RNA Extraction

- Add 500-100 μl of Trizol (Life Technologies # 10296010) to each sample.
- Homogenised tissue by vortexing and pipetting.
- Incubate at RT for 5 minutes.
- Add 200 μl of chloroform per 1 ml of Trizol and shake vigorously.
- Incubate at RT for 5 minutes.
- Centrifuge at 12000 g for 15 minutes at 4°C.
- Remove aqueous layer and add 125 μl of salt precipitation solution (1:1 1.2 M NaCl and 0.8 M Na-Citrate).
- Add 0.6 volumes of isopropanol and incubated at RT for 10 minutes.
- Centrifuged at 12000 g for 5 minutes at 4°C.
- Remove supernatant and wash the pellet with 70% ethanol.
- Centrifuge at 75000 g for 5 minutes at 4°C.
- Repeat steps 11-12 two more times.
- Re-suspend RNA in nuclease free water.
- Use a Qiagen RNA extraction kit to now clean the RNA using the standard protocol attached.
- Check the concentration on a NanoDrop and test the quality by running a small amount on a MOPS gel.
2.2.3 Formaldehyde denaturing gel for RNA analysis

- RNA analysis was completed using a 1.5 % agarose gel.
- Dissolve 0.75 g of agarose in 36 ml of DEPC H₂O.
- Add 5 ml 10x MOPS buffer and 9 ml of deionised formaldehyde.
- Mix by rotating flask and pour into cast tray.
- Mix RNA sample 1:1 with 2x RNA loading dye.
- Heat RNA samples plus RNA ladder for 10 minutes at 70°C.
- Place on ice for 5 minutes.
- Run sample plus ladder on 1.5% MOPS gel for 30 minutes.
- Photograph under UV.

10x MOPS Buffer

- Dissolve 41.8 g MOPS in 700 ml DEPC H₂O.
- Adjust pH to 7.0 using 2 M NaOH.
- Add 20 ml 1M sodium acetate in DEPC H₂O.
- Add 20 ml of 500 mM EDTA ph8 in DEPC H₂O.
- Bring up to 1 litre with DEPC H₂O.

2.2.4 Reverse Transcriptase cDNA amplification

RT cDNA was synthesised in a 20μl reaction according to the protocol Omniscript RT Kit 205111

2 μl  10X buffer  
1 μl  Oligo dT primer  
2 μl  5 mM dNTPs  
1 μl  RNase inhibitor  
1 μl  Reverse transcriptase  
X μl  1 μg RNA  
Y μl  RNase free H₂O  
20 μl

- Mix reaction and incubate at 37°C for 2 hours. RT cDNA can be stored at -20°C.
2.2.5 Race cDNA synthesis

RACE cDNA was transcribed according to the protocol for Omniscript Reverse Transcriptase (Qiagen cat. no. 205110). 2 µg of RNA was used per reaction and the oligos were designed according to SMARTTM RACE cDNA amplification kit.

5’ RACE cDNA

X µl RNA
1 µl 5’ CDS Oligo (1 µm)
1 µl Smart2
X µl Nuclease free H₂O
10 µl

3’ RACE cDNA

X µl RNA
1 µl 3’ CDS Oligo (1 µm)
X µl Nuclease free H₂O
10 µl

- Incubate reactions at 70°C for 10 minutes.
- Place on ice for 2 minutes.
- 10 µl of master mix was then added to each tube.

Master Mix:
2 µl 10X Buffer
2 µl 5 mM dNTP
1 µl RNase inhibitor.
1 µl Reverse transcriptase
4 µl Nuclease free H₂O
10 µl

- Mix samples and incubate for 2 hours.
- Dilute 10 times with nuclease free H₂O.
- Heat to 72°C for 7 minutes.
2.2.6 PCR

Bioline MyTaq™ DNA Polymerase, 50 µl PCR Reaction Setup.

Hot start:
10 µl 5x MyTaq reaction buffer
X µl RT cDNA (1ng - 1 µg)
2 µl Forward primer (5 µm)
2 µl Reverse primer (5 µm)
10 µl H₂O
39.75 µl

Master Mix:
0.25 µl MyTaq DNA polymerase
10 µl H₂O
10.25 µl

Conditions:
≤ 40x cycles
1 minute Hot start.
1 minute Initial denaturation at 95°C.
15 seconds Denaturation at 95°C.
X°C Annealing depends on primer melting temperature.
X minute Extension depends on template size.

Phusion High-Fidelity DNA Polymerases, 20 µl PCR Reaction Setup.

Hot Start:
1 µl RT cDNA (1ng - 1 µg)
2 µl Forward primer (5 µm)
2 µl Reverse primer (5 µm)
0.4 µl dNTPs (200 µm)
4 µl HF buffer (5X)
9 µl Nuclease free H₂O
18.4 µl

Master Mix:
1.5µl Nuclease free H₂O
0.1µl Phusion polymerase
1.6 µl
Conditions:
≤40x cycles
1 minute 80°C hot start.
98°C Initial denaturation.
30s Denaturation at 98°C.
X°C Annealing depends on primer melting temperature.
X minute Extension depends on template size.

2.2.7 Agarose gel DNA extraction

Gel extraction was performed using Promega Wizard® SV Gel and PCR Clean up system (Cat no.A1120).

- Excise the DNA band using a clean blade.
- Add 10 µl of membrane binding solution per 10 mg of gel.
- Incubate at 55°C until gel slice has dissolved.
- Insert SV® mini column into collection tube.
- Transfer dissolved gel mixture into SV® mini column.
- Incubate at RT for 1 minute.
- Centrifuge at 16000 g for 1 minute.
- Discard flow through.
- Add 700 µl membrane wash solution and centrifuge at 16000g for 1 minute.
- Repeat wash step with 500 µl of wash solution.
- Empty collection tube and centrifuge column for 1 minute to evaporate any remaining wash solution.
- Transfer SV® mini column to clean 1.5 ml tube.
- Add nuclease free H2O and incubate at room temperature for 1 minute.
- Centrifuge at 16000 g for 1 minute.
- Measure concentration on a NanoDrop.

2.2.8 RNA Probes for in situ hybridization

The DNA sequence is transcribed from RT cDNA by PCR using SP6 and T7 attached primers.

4 µl 5x Transcription buffer
0.5 µl RNase inhibitor (Ribolock)
2 µl DTT (0.2M)
500 ng DNA template
2 µl Labelled NTPs
1 µl RNA polymerase (T7, SP6)
X µl RNase free H2O
20 µl
Incubate samples at 37°C overnight.
Add 1 µl of DNase and incubate at 37°C for 30 minutes.
Add 1 µl of 0.2 M EDTA.
Add an equal volume of 12 M LiCl, vortex and incubate at -20°C for 30 minutes.
Centrifuge for 5 minutes at full speed.
Remove supernatant and wash pellet in 70% ethanol.
Centrifuge for 5 minutes at 16000g.
Air dry pellet in the fume hood for no more than 10 minutes.
Suspend pellet in RNase free H2O.
Check the concentration on a NanoDrop and mix 1:1 with in situ hybridization mix.
Run 100 ng on a MOPS gel to test quality.

2.2.9 dsRNA synthesis for RNAi

The DNA sequence is amplified from RT cDNA by PCR using SP6 and T7 attached primers. Two 20 µl reactions, sense and antisense are synthesised for each experiment.

4 µl 5x Transcription Buffer
6 µl NTP mix 25 mM each
1 µl T7 or SP6 polymerase
Y µl (8 µl max) template (100 ng)
X µl Nuclease free H2O
20 µl

Mix the reactions by pipetting and incubate at 37°C overnight.
The next day mix the complementary RNA strands together and heat to 70°C in a water bath for 10 minutes.
Leave the tubes on the desk to cool allowing the strands to anneal.
Add 2 µl of RQ1 RNase free DNase (Promega #M6101) per 40 µl reaction plus 2 µl of 10xRNase solution and incubated for 30 minutes at 37°C.

10x RNase Solution:
20 µl of 10 mg/ml RNase A (Thermoscientific # EN0531)
10 µl 1,000u/µl RNase T1 (Thermoscientific # EN0541)
970 µl T.E buffer.
• Add 10 M ammonium acetate to a final concentration of 1 M.
• Add 2.5 volumes of 100% ethanol to the samples and put on ice for 5 minutes.
• Centrifuge at full speed for 10 min.
• Remove the supernatant and wash the pellets with 75% ethanol
• Centrifuge at full speed for 2 minutes.
• Remove the ethanol and air dry the pellets for no more than 10 minutes.
• Dissolve in 50 µl RNase free H2O and measure the concentration on a nano-drop, aliquot and freeze at -70°C

2.2.10 Bacteria plasmid extraction

• Grow up 250-300 ml of bacteria in LB medium with carbenicillin overnight on a shaker at 37°C.
• Put solution III on ice and cool centrifuge.
• Spin down bacteria at 2000 g at 4°C for 10 minutes.
• Remove the supernatant and add 10 ml of alkaline lysis solution I and vortex and incubate at RT for 5 minutes.

Alkaline Lysis Solution I (autoclave and add 20 mg of lysozyme before use)
25 mM Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)
50 mM Glucose

• Add 20 ml of alkaline lysis solution II and invert 5 times and incubate at RT for 5 minutes.

Alkaline Lysis Solution II (prepare fresh before each use)
0.2 N NaOH
1% SDS

• Add 15 ml of alkaline lysis solution III and invert several times and incubate on ice for 5 minutes.

Alkaline Lysis Solution III (can be made up before and stored in the fridge)
5 M potassium acetate
11.5 ml glacial acetic acid
28.5 ml H2O
- Centrifuge for 10 minutes at 16000g at 4°C.
- Remove supernatant and separate into 2 tubes and add 0.6 volumes of isopropanol and incubate for 20 minutes at -20°C.
- Centrifuge at 7500rpm for 15 minutes.
- Discard the supernatant and re-suspend the pellet in 5 ml of H₂O.
- Add 1 g of ammonium acetate and mix until dissolved.
- Incubate on ice for 20 minutes.
- Precipitate with 2.5x volumes of 100% ethanol.
- Wash pellet in 75% ethanol.
- Dissolve pellet in 1 ml of H₂O.
- Add 2 µl of RNaseA (Thermoscientific #EN0531) plus 1 µl of RNase T1 (Thermoscientific #EN0541).
- Incubate at 37°C for 1 hour.
- Add SDS to a final concentration of 1% and NaCl to a final concentration of 0.5 M.
- Add 1µl proteinase K (Sigma #P6556) and incubate at 55°C for 1 hour.
- Phenol chloroform clean and ethanol precipitate with KCL.

2.2.11 Phenol chloroform clean-up

- Add phenol (pH7) and chloroform 1:1:1 with sample and vortex.
- Spin at 16000g for 2 minutes.
- Remove aqueous solution to a new tube and re-precipitate original supernatant with chloroform 1:1.
- Spin at 16000g for 2 minutes.
- Add 1/20 the volume of 5M KCl and 1 µl of glycogen (Sigma #1767).
- Add 2.5x 100 % ethanol.
- Place in the freezer for 20 minutes.
- Spin for 2 minutes at 16000g.
- Remove supernatant and wash pellet with 200 µl 75% ethanol.
- Spin for 2 minutes at 16000 g and remove supernatant.
- Air dry for no more than 10 minutes and dissolve in Nuclease free H₂O.
2.2.12 DNA ligation

Set up a 10 µl 1:3 vector to insert reaction

- 1 µl 10x T4 DNA ligase buffer
- 1 µl T4 DNA ligase
- X µl Vector 10-50 ng
- X µl Insert 50-500 ng
- X µl H2O

- Incubate for 1 hour at room temperature
- Add 15 µl of H2O and denature of 15 minutes at 65°C
- Put on ice for 5 minutes.

2.2.13 Bacteria transformation

- All work is done aseptically.
- Remove agar plates from the fridge place at 37°C.
- Defrost bacteria stock on ice.
- Add 25 µl of ligation mix to 50 µl of bacteria.
- Gently mix and place on ice for 10 minutes.
- Heat shock at exactly 42°C in water bath for 90 seconds and put back on ice for 5 minutes.
- Add 200 µl LB medium with no carbenicillin and allow to recover at 37°C on the shaker for 45 minutes.
- Spread medium on warm plates and leave to grow overnight at 37°C.

2.2.14 LB agar and bacteria plates

- Dissolve 40 g of LB agar in 1 litre of distilled H2O and autoclave.
- After autoclaving allow to cool to around 50°C and aseptically add carbenicillin to a final concentration of 1 mg per litre.
- Once mixed pour the liquid into the petri dishes in a sterile manner just covering the bottom of the dishes.
- Once set turn upside down and wrap in parafilm.
- Store upside down in the fridge
2.2.15 Restriction enzyme digestion

- 1 µl  10x Enzyme Buffer
- 0.5 µl  Restriction enzyme (1 unit per 1 µg of DNA)
- X µl  DNA (1 µg of DNA)
- X µl  Nuclease free H₂O
- 10 µl

- Incubate reaction for 2 hours at 37°C.
- Inactive by heading to temperature suggested by manufacturer.
- Run 1 µl of mix on 1 % agarose gel to verify digestion was successful.

2.1.16 Dephosphorylation of vector

- Add 1 µl of fast AP and dephosphorylate at 37°C for 1 hour.
- Only used in blunt end cloning.

2.1.17 Phosphorylation of primers

- 2 µl  Stock primer forward and reverse (1 µm)
- 2 µl  10x Reaction buffer A
- 1 µl  T4 Polynucleotide Kinase PNK (Thermoscientific #EK0031)
- 2 µl  ATP 10 mm
- X µl  Nuclease free H₂O

- 20 µl reaction
- Incubate reaction for 1 hour at 37°C
2.2.18 \textit{In Situ} hybridisation protocol

Day 1

- Anesthetize the animals by incubating in 4\% MgCl\textsubscript{2} for 30 minutes.
- Fix animals in 4 \% Formaldehyde/Seawater for 1 minute to stop shrinkage.
- Fix the animals overnight at 4°C in 1 ml 4 \% PFA—Hepes.
- Wash 3 x 10 minutes with PBS Tween.
- Bleach in methanol by washing in 25, 50, 75, 100 \% methanol in DEPC H\textsubscript{2}O for 5 minutes and back down from 100 to 25 \%.
- Wash twice in PBS Tween for 5 minutes.
- Heat treat by incubating at 95°C for 20 minutes in PBST.
- Acetic acid treatment
  - Wash once for 5 minutes in 1 M TEA/PBS
  - Wash once for 5 minutes in 1 M TEA/PBS + Acetic acid (6 \mu l acetic acid + 1 ml 1 M TEA/PBS)
  - Wash once for 5 minutes in 1 M TEA/PBS + Acetic acid (12 \mu l acetic acid + 1 ml 1 M TEA/PBS)
  - Wash once for 5 minutes in PBS.
- Post-fix using 1 ml of 4 \% PFA in PBS at RT for 20 minutes.
- Wash 3 x 5 minutes in 1 ml PBS Tween.
- Pre-absorb the anti-DIG antibody in a 1:1000 dilution in 1 \% BSA/PBS Triton overnight at 4°C with a batch of previously fixed polyps.
- Incubate samples in blocking solution for 30 minutes at RT with rocking (Use 500 \mu l 2 mg tRNA/1 ml PBST per Eppendorf).
- Add 500 \mu l hybridisation mix to the blocking solution (final vol. 1 ml) and rock for 30 minutes at RT.
- Remove solution and add 1 ml of hybridisation mix and incubate overnight between 50°C and 55°C in a hybridisation oven, with rocking.

Day 2

- Pre warm hybridization mix containing 40 ng of RNA probe per 1 ml for 10 minutes at 70°C to denature the probes.
- Place on ice for 5 minutes.
- Remove pre-hybridisation mix from samples and replace with 1 ml of hybridization mix containing 40 ng of sense or antisense RNA probes.
- Hybridise overnight at 50°C to 55°C in the hybridization oven with rocking.
Day 3

- Post-hybridisation washes are completed in the hybridization oven at 2 degrees higher than the hybridization.
- Wash 1: Wash once in hybridization mix for 5 minutes.
- Wash 2 (50 % formamide, 2x SSC, 0.1 % Triton): Wash 1 ml per tube, 1x 1h.
  For 10 ml:
  - 5 ml Deionised formamide
  - 1 ml 20 x SSC
  - 10 µl Triton
  - 4 ml DEPC water

- Wash 3 (2x SSC, 0.1 % Tween): Wash 1 ml per tube, 1x 15 minutes
  For 10 ml:
  - 1 ml 20 x SSC
  - 10 µl Triton
  - 9 ml DEPC water

- Wash 4 (0.2x SSC, 0.1% Tween): Wash 1 ml per tube, 2 x 15 minutes,
  For 10 ml:
  - 100 µl 20 x SSC
  - 10 µl Triton
  - 9.9 ml DEPC water

- Wash 5: 1 x PBS Triton for 5 minutes at RT with rocking
- Block with 1 % BSA in PBS Triton for a minimum of 1 hour at RT with rocking.
- Incubate for 2 hours RT in 1:2000 diluted pre-absorbed anti-DIG-AP conjugated Fab fragments in PBS Triton.
- Wash 4 x 20 minutes 1 ml PBS Triton, RT, rocking. (4ºC overnight in last wash)

Day 4

- Rinse 3 x 5 minutes in freshly made alkaline phosphatase buffer (AP-buffer), 1 ml per tube.
- AP-buffer for 10 ml:
  - 1 ml 1 M NaCl 0.0584g per 1 ml
  - 1 ml 1 M Tris-HCl pH 9.5 0.121g per 1 ml
  - 1 ml 500 mM MgCl2 0.102g per 1 ml
  - 10 µl Tween 20
  - 7 ml DEPC water
• Sterile filter to avoid precipitate.
• Stain with BCIP/NBT staining solution in the dark.
• Stop the reaction by removing the staining solution and washing 2 x 5 minutes in 1 x PBS Triton, 10 mM EDTA.
• 0.1754 g EDTA in 60 ml PBST. The PBST must be pH 7.5 for EDTA to dissolve
• Bleaching, 25, 50, 75, 100 % Ethanol in DECP H2O and back.
• Mount on glass slides in 50-80 µl Glycerol/PBST, 10 mM EDTA (9:1) per slide. Seal with nail polish and store at -20°C.

2.3 Animal maceration

• Anesthetise animals in 4 % MgCl for 30 minutes.
• Cut desired amount of polyps from the colon
• Place directly into maceration mix I for 10 minutes (100 µl per 5 polyps)

  Maceration mix I
  Glycerol/Acetic acid/Seawater (1:1:13)

• Carefully remove all of maceration mix 1 and wash polyps in maceration mix 2

  Maceration mix II
  Glycerol /Acetic acid/Distilled Water (1:1:13).

• Repeat wash step three times to remove any seawater
• After the last wash leave in maceration mix II for 2 hours. (100 µl per 5 polyps)
• After 2 hours pipette mix up and down until any clumps of tissue that remain have been disassociated
• Leave for a further hour.
• Fix polyps by adding 8 % formaldehyde in distilled water to the maceration mix for 30 minutes (100 µl per 5 polyps)
• Transfer maceration mix onto glass slide and let dry overnight in fume hood
• Use a pap pen to draw a hydrophobic circle around the area where the cells are fixed.
• Cells are now ready for EdU or Antibody staining
2.4 Cellular staining

2.4.1 EdU staining of S-Phase Cells

EdU was performed using a Click-iT® EdU Alexa Fluor® 488 Imaging kit (Life Technologies cat # C10337).

The solutions were prepared according to the manufactures manual and the protocol used is outlined below. Fluorescence excitation/emission maxima for Alexa Fluor® 488: 495/519 nm respectively.

- Fix animals in 4 % Formaldehyde/Seawater for 1 minute to stop shrinkage
- Fix animals in 4 % PFA/PBS for 1 hour.
- Wash samples 2x 3 % BSA/ PBS.
- Permeabilize sample by adding 1 mL of 0.5 % PBS/Triton
- Incubate for 20 minutes.
- Prepare 1X Click-iT® EdU buffer additive.
- Prepare Click-iT® reaction cocktail.
- Remove the permeabilization buffer.
- Wash sample 2x 3 % BSA/PBS for 10 minutes.
- Remove the wash solution.
- Add Click-iT® reaction cocktail.
- Rock briefly to insure that the reaction cocktail is distributed evenly.
- Incubate for 30 minutes and protected from light.
- Remove the reaction cocktail.
- Wash 2x 3 % BSA/ PBS for 10 minutes.

2.4.2 DNA fragmentation analysis by TUNEL

DNA Fragmentation was analysed using a Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay kit (Life Technologies cat # C10245). Solutions were prepared according to the manufactures manual. The protocol used is outlined below.

- Fix animals in 4 % Formaldehyde/Seawater for 1 minute to stop shrinkage
- Fix animals in 4 % PFA/PBS for 1 hour.
- Wash 3x5 minutes in 0.25 % Triton.
- Wash 1x20 minutes in 0.25 % Triton.
- Then wash twice with nuclease free H2O.
- Add 100 μl TdT reaction buffer to each sample.
- Incubate for 10 minutes at RT.
- Prepare the TdT reaction cocktail.
• Remove TdT reaction buffer.
• Add 100 μl of the TdT reaction cocktail.
• Incubate for 60 minutes at 37°C.
• Remove the reaction cocktail.
• Wash twice with 3 % BSA in PBS for 5 minutes.
• Prepare the Click-iT® reaction buffer additive
• Prepare the Click-iT® reaction cocktail and mix well by vortexing.
• Add 100 μl of the Click-iT® reaction cocktail.
• Incubate coverslips for 30 minutes at RT and protected from light.
• Remove the Click-iT® reaction cocktail and wash each coverslip with 3 % BSA in 1xPBS for
  5 minutes.

2.4.3 Immunostaining on polyps

• Anesthetize animals in 4 % MgCl for 30 minutes.
• Fix animals in 4 % Formaldehyde/Seawater for 1 minute to stop shrinkage
• Fix in 4 % PFA PBS for 30 minutes at room temperature
• Wash 3x5 minutes in 0.3 % PBS Triton-x100
• Block for 1 hour in in 3 % BSA/PBS
• Incubate sample in primary antibody BSA/PBS overnight at 4°C or for 1 hour at room temperature.
• Wash 3x10 minutes in PBS Triton-x100
• Block for 15 minutes BSA/PBS/5 % Serum
• Incubate for 1 hour in secondary antibody BSA/PBS/Serum
• Wash in 1-2000 Hoechst (Sigma #145333) nuclear stain for 30 minutes.
• Wash 3x10 minutes 0.3 % PBS Triton-x100

2.4.4 Immunostaining on macerated animals

• All wash and blocking steps are performed in a slide staining jar.
• Wash slides in 0.3 % PBS Triton-x100 for 20 minutes.
• Block for 1 hour in 3 % BSA/PBS
• Remove slides from staining jar and dry in fume hood.
• Pipette 200 μl of primary antibody BSA/PBS on to slide carefully insuring the cells are covered
• Incubate overnight at 4°C or for 1 hour at room temperature.
• Wash slides PBS Triton-x100 for 20 minutes.
• Remove slides from staining jar and dry in fume hood.
• Pipette 200 μl of blocking solution BSA/PBS/5 % Serum on to the slide carefully insuring the cells are covered.
• Quickly wash slides and dry in the fume hood
• Incubate for 1 hour in secondary antibody BSA/PBS/Serum and keep in the dark
• Quickly wash slides and dry in the fume hood
• Pipette 200 µl 1-2000 Hoechst (Sigma 145333) nuclear stain and leave for 30 minutes.
• Wash slides in 0.3 % PBS Triton-x100 for 20 minutes.

2.4.5 Antibody list

• Alexa Fluor® 546 Phalloidin (A22283)
• Anti Phopspho H3(PH3) Abcam(ab5176)
• Alexa Fluor® 488 Invitrogen (A-11059)
• Anti-acetylated-tubulin Sigma (T7451)
• Anti- Hiwi antibody was kindly donated by Dr. Celina Juliano, Yale University.

2.5 Microinjection

• Needles for injection were prepared from glass capillaries (Narishige CD-1 1x90 mm) and prepared on a pulling machine (heat 560, pull 70, vel 75, time 150)
• Animals were injected in a petri dish with a 100 µm plankton net attached.
• Embryos were collected 1 - 2 hours after fertilization.
• Embryos were injected at the 1 cell stage with a concentration of 3-5 ng/µl of construct.

2.6 Irradiation protocol

• A caesium 137 gamma irradiator source (14Gy/min) was used to irradiate animals.
• Anesthetize colony in MgCl₂ for 30 minutes.
• Cut polyps from the colony and wash in seawater.
• Separated polyps into two petri dishes, one for irradiation and one not.
• Set desired dosage on the irradiation machine and place polyps inside.
• Once completed anesthetize polys again in MgCl₂ for 30 minutes.
• Decapitate polyps using a sharp blade and wash in seawater.
## 2.7 Primer list

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2.8 Solutions

**DEPC H$_2$O**: 1ml of DEPC per l of double distilled H$_2$O
- Leave to evaporate in fume cupboard overnight and auto-clave the following morning.

**1 x PBS** (Phosphate-buffered Saline)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final Conc.</th>
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<tr>
<td>NaCl</td>
<td>137 mM NaCl</td>
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<tr>
<td>KCl</td>
<td>2.7 mM KCl</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>10 mM Na$_2$HPO$_4$</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2 mM KH$_2$PO$_4$</td>
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</table>

- Dissolve in 800 ml of distilled H$_2$O.
- Adjust the pH to 7.5 with HCl.
- Add H$_2$O to 1 litre and DEPC overnight (1ml per 1L).
- Dispense the solution into aliquots and autoclave.
- Store the buffer at room temperature.

**1 x PBST** (Tween or Triton)

<table>
<thead>
<tr>
<th>Volume</th>
<th>Solution</th>
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<tr>
<td>50ml</td>
<td>1 x PBS pH 7.5</td>
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<tr>
<td>50µl</td>
<td>Tween 20/Triton</td>
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</table>
**4% PFA/Hepes fixing solution**

For 50ml

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<th>Component</th>
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</thead>
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<tr>
<td>50ml</td>
<td>0.1M Hepes pH7.5</td>
<td>1.1955g in 50ml</td>
</tr>
<tr>
<td></td>
<td>2mM MgSO₄</td>
<td>0.0246g per 50ml</td>
</tr>
<tr>
<td></td>
<td>0.42M NaCl</td>
<td>1.227g per 50ml</td>
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- DEPC overnight, autoclave.
- Add 2 g Paraformaldehyde to make 4%.
- Heat to 60°C with stirring till the solution is clear.
- Store at -20°C

**4% PFA/PBS fixing solution**

- 4g PFA in 100mls PBS.
- Heat and add NaOH drops to help dissolve.
- Dissolve on rocker for about an hour.

**In situ hybridisation mix**

For 10ml

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<td>5ml</td>
<td>Deionised Formamide</td>
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<tr>
<td>2.5ml</td>
<td>20 x SSC</td>
<td>5 x SSC</td>
</tr>
<tr>
<td>1ml</td>
<td>1mg/ml Heparin stock</td>
<td>0.1mg/ml</td>
</tr>
<tr>
<td>1ml</td>
<td>1mg/ml tRNA stock solution (store frozen)</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>10μl</td>
<td>Tween 20</td>
<td>0.1%</td>
</tr>
<tr>
<td>0.5ml</td>
<td>DEPC H₂O</td>
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</tr>
</tbody>
</table>

**20 x SSC**

For 1L

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</thead>
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<tr>
<td>175.3g</td>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td>88.2g</td>
<td>Sodium Citrate</td>
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- Put in 800ml water. Adjust pH to 7 with a few drops of 14N HCl.
- Adjust volume to 1L with water.
- Aliquot then autoclave.
**In situ staining solution**

In 1ml of AP buffer

4.5μl 50mg/ml NBT in 70% dimethylformamide

3.5μl 50mg/ml BCIP in 100% dimethylformamide

**Lysis buffer**

500μl Solution D

500μl Phenol pH4 (fume hood)

100μl 2M Na-acetate (add 1.64g to 10mls for stock)

7.7μl beta-mercaptoethanol (in fridge in liquid form)

**Solution D**

Final conc:

To 19.53ml of ddH2O add

16.66g Guanidinium thiocinate (fume hood) 4M

1.17ml 0.75M Sodium citrate (add 2.21g to 10mls for stock) 25mM

1.76ml Sarcosyl (N-lauroyl sarcosine)(liquid form) 0.5%
Chapter 3. Head Regeneration in Hydra Feeding Polyps

3.1 Introduction

To answer the question of why some animals can regenerate robustly and some cannot it is important to understand the fundamental differences that exist between such groups. Whether different taxa evolved their regenerative powers independently or it was present in the last common ancestor of all metazoans and then subsequently lost in some species is unknown. The basal phylogenetic position and regenerative powers displayed by cnidarians give them a pivotal position while studying the regenerative processes within the Metazoa.

The majority of work completed on regeneration in cnidarians has been done on the hydroid Hydra and as such it has become a reference point for regeneration within the phylum. It has long been thought that Hydra regenerates solely by morphallaxis, without cell proliferation or the formation of a blastema. Hydra is one of only a few animals where regeneration without a significant contribution from cell proliferation has been reported. The majority of animals studied regenerate by epimorphosis, through cell proliferation and the formation of a blastema (See Chapter 1).

How widespread the use of morphallaxis is during regeneration in cnidarians is unclear. Recent publication have revealed that cell proliferation actually does play a role in both Hydra (Chera et al., 2011) and Nematostella (Passamaneck and Martindale, 2012) regeneration casting doubt on morphallaxis being the only method employed within the phylum. Whether Hydra regenerates using similar methods employed in Hydra was not known but studying regeneration in this species would help resolve the overall question of cnidarian regeneration and how it relates to systems employed in other metazoan taxa.
3.2 Regeneration Time and Response to Wounding

To characterise *Hydractinia* head regeneration I first removed polyps from a colony by a transverse bisection close to the polyp stolon border. Polyps were then decapitated and left to regenerate in seawater. This first stage of regeneration began with wound healing where the epidermal epithelial cells stretch out and stick together closing the open wounds on each cut section. This took between two and four hours to complete (Fig. 3.2.1 B). The second stage was characterised by a dome like cap which developed at the regenerating tip between 24 and 48 hours post decapitation (HPD) (Fig. 3.2.1 C). This cap then developed into a new mouth and tentacles (Fig. 3.2.1 D). The final stage in head regeneration took between 48 and 72 hours to complete at which stage polyps were again able to catch and ingest food (Fig. 3.2.1 E). This experiment was repeated over 100 times with the same results obtained in each.

![Fig. 3.2.1. Different stages of regeneration in isolated feeding polyps. (A) Intact feeding polyp (Scale 300 µm). (B) Polyp three HPD, wound has closed (C) Polyp 24 HPD, dome like cap present at regenerating tip (D) Polyp 48 HPD, tentacles now visible (E) Polyp 96 HPD, head has fully regenerated.](image)
To further investigate the regenerative capacity of isolated polyps I completed a second series of experiments. This time I made a series of mid-gastric transverse and longitudinal bisections to isolated feeding polyps. A mid-body transverse bisection resulted in a head being generated on the oral tip of the lower half while the wound closed on the aboral tip of the upper half (Fig. 3.2.2 A). Regeneration progressed through the same time and steps as was previously observed in adult polyps. A longitudinal bisection resulted in the polyp closing the longitudinal wound (Fig. 3.2.2 B) and the polyp was capable of feeding again within 8 hours (Fig. 3.2.3). This experiment was repeated over 20 times and the same results were obtained in each.

![Schematic outlining mid-body transverse and longitudinal bisections of isolated feeding polyps.](image)

Fig. 3.2.2: Schematic outlining mid-body transverse and longitudinal bisections of isolated feeding polyps. (A) Mid-body transverse bisection. (B) Longitudinal bisection

![Regenerating polyps after longitudinal bisection](image)

Fig. 3.2.3: Regenerating polyps after longitudinal bisection (A) Polyp eight hours after longitudinal bisection. Wound has closed (Scale 100 µm). (B) Polyp feeding eight hours after longitudinal bisection, Artemia (Arte) visible in mouth.
3.3 Regeneration of the head nervous system

To characterise the nervous system in the head of feeding polyps and its regeneration after decapitation I used an anti-acetylated tubulin antibody to stain neurons and nematocytes (Fig. 3.3). Intact feeding polyps have a concentration of neurons and nematocytes in the head, mouth and tentacles (Fig. 3.3 A-C). After decapitation this nervous system needs to be rebuilt. Polyps four HPD have a small number of neurons or nematocytes at the regenerating tips (Fig. 3.3 D-F). There was a visible increase in neurons and nematocytes 24 HPD in tentacle buds and in the new regenerating head (Fig. 3.3 G-I). This increase continues through to 48 and 72 HPD where the tips of new tentacles and the new regenerated mouth are full of new neurons and nematocytes (Fig. 3.3 J-L). The nervous system is fully functional at this stage as attested by the ability to catch prey and feed again. This experiment was completed 4 times and the same results were obtained in each.
Fig. 3.3. Regeneration of the head nervous system in decapitated polyps stained for Acetylated tubulin (green) F-actin (red) and DNA (Blue). (A) Intact feeding polyp. (B) Close up of neurons around its open mouth. (C) Close up of head showing nematocytes (Nem) and neurons (Neur). Asterisk - head (D) Polyp four HPD. (E) Close up of regenerating section. (F) Close up of regenerating section, few nematocytes and neurons visible. (G) Polyp 24 HPD, regenerating buds now visible. (H) Close up of regenerating oral section. (I) Many neurons and nematocytes now visible (J) Polyp 72 HPD, regeneration now complete. (K) Close up of regenerated section. (L) Many new neurons and nematocytes now visible (Scale 200 µm first column, 40 µm second column, 10 µm third column).
3.4 Cell proliferation during Regeneration

3.4.1 DNA synthesis detection using EdU

EdU (5-ethynyl-2’-deoxyuridine) is a nucleoside analogue of thymidine and is incorporated into DNA during S-phase of the cell cycle. Detection is based on a copper-catalysed covalent reaction between an alkyne contained in the EdU and an azide contained in the Alexa Fluor® dye.

I used an EdU assay as described in protocol 2.4.1 and table 3.4.1.1 to measure the effect of decapitation on cell proliferation. Polyps were removed from the colony and decapitated. They were then fixed at different time points throughout regeneration. Before fixation the polyps were incubated in an EdU/seawater solution for 40 minutes. A number of intact polyps were also treated with the EdU/seawater solution and fixed straight away to compare regenerating and non-regenerating polyps (Table 3.4.1.1).

EdU+ cells were found mainly in an aboral band in non-regenerating polyps (Fig. 3.4.1.2 A) however a small number of cells were sometimes visible in the head. I observed a change in this expression pattern during regeneration. Twenty four HPD a second band of EdU+ cells appeared in the regenerating tip forming a blastema which was not present in non-regenerating polyps (Fig. 3.4.1.2 B). This type of change in S-phase cells is reflective of the regenerating blastema seen in many other animal groups including planarians (Reddien and Sanchez Alvarado, 2004), zebrafish (Poss et al., 2000) and salamanders (Sandoval-Guzman et al., 2014). This experiment was repeated over 20 times with the same results obtained in each.

To investigate what effect removing polyps from the colony had on proliferation I preformed a second EdU experiment. This time I compared intact and regenerating polyps that were both removed from the colony and incubated in an EdU/seawater solution at the same time. I also treated a number of polyps while still attached to the colony to see if removal had any effect on proliferation.
I did not observe any change in the pattern of S-phase cells four or 24 hours after removal from the colony in intact polyps (Fig. 3.4.1.3 B, D). The majority of EdU+ cells were present in the aboral section of the polyp, while regenerating polyps displayed a second band in the regenerating blastema at 24 HPD (Fig. 3.4.1.3 A, C). This shows that the change in S-phase cells was due to decapitation and not to their removal from the colony.

Intact polyps that were treated with EdU while still attached to the colony showed similar patterns as intact polyps that were removed prior to exposure. This showed that removing the polyps did not cause any visible increase in proliferation. Both of these experiments revealed that decapitation not removal from the colony was the cause of the new band of EdU+ cells in the regenerating head.

Table 3.4.1.1. Steps performed in EdU assay.

<table>
<thead>
<tr>
<th>Colony anaesthetised in 4% MgCl₂ for 30 minutes</th>
<th>Polyps cut from colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-decapitated polyps separated from decapitated</td>
<td>Polyps decapitated and left in Seawater for 24 - 48 hours</td>
</tr>
<tr>
<td>Non-decapitated polyps soaked in EdU/Seawater for 40 minutes</td>
<td>Polyps soaked in EdU/Seawater for 40 minutes</td>
</tr>
<tr>
<td>Polyps washed in MgCl₂ for 30 minutes</td>
<td>Polyps fixed in 4% Formaldehyde/Seawater for 1 minute</td>
</tr>
<tr>
<td>Polyps fixed in 4% PFA/Seawater for 1 hour</td>
<td>Polyps washed in PBS/Triton 3x 5 minutes</td>
</tr>
<tr>
<td>Polyps bleached in methanol and stored at -20°C</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.4.1.2 EdU incorporation in intact and regenerating polyps. Green - EdU, Blue - Hoechst (Scale 200 µm) (A) Intact polyps. No blastema present. EdU+ cells found in the aboral band. (B) Polyp 24 HPD. Blastema now present. EdU+ cells found in the aboral band and in the head blastema. (C) Polyp 48 HPD. EdU+ cells still present in the regenerating tip.
Fig. 3.4.1.3 EdU incorporation in regenerating and non-regenerating polyps. Green - EdU, Blue – Hoechst (Scale 200 µm) (A) Four HPD. No blastema present (B) Intact polyp four hours after removal from the colony. No second EdU band present (C) Polyp 24 HPD. Second EdU+ band now present at the regenerating oral tip. (D) Intact polyp 24 hours after removal from the colony. No second EdU+ band present. (E) Intact polyps removed from the colony, treated and fixed. No second EdU+ band present. (F) Intact polyp treated on the colony then removed and fixed. No second EdU+ band present.
3.4.2 EdU Pulse Chase

To investigate whether the new S-phase cells that are present in the regenerating blastema are due to local dedifferentiation or to migration of existing cells from the aboral band, I performed an EdU Pulse/Chase experiment. To create a Pulse/Chase experiment, animals are treated with EdU and then fixed at a later time point. By comparing the chase EdU+ pattern to a non-chase experiment where polyps are fixed directly after labelling it is possible to ascertain any cell movement during the chase time frame. The majority of S-phase cells in non-regenerating polyps are located in the aboral band (Fig. 3.4.1.2 A). If a 24 hour chase experiment is performed and this pattern changes then it would mean that the cells have migrated to this new location having incorporated the EdU 24 hours previous while present in the aboral band. However if no new pattern is observed it would indicate that the cells did not migrate as they are still located in the same position as 24 hours previous when they incorporated the EdU.

For this EdU chase experiment I removed polyps from a colony and incubated them in an EdU/seawater solution for one hour. The polyps were then washed vigorously and all were decapitated at the same time. A number of polyps were then fixed at four, 24, 48 and 72 HPD.

EdU+ cells were almost exclusive expressed in the aboral band in four hour chase polyps (Fig. 3.4.2 A). This expression changed in 24 – 72 hour chase polyps where an increase in EdU+ cells was visible in the blastema (Fig. 3.4.2 B-D). These cells which were now present in the head could only have incorporated the EdU aborally 24 - 48 hours previously. This result would suggest that these new EdU+ cells present in the head 24 HPD are actually a product of cell migration and not of cellular dedifferentiation. This experiment was repeated 5 times with the same results obtained in each.
Fig. 3.4.2. EdU Pulse/Chase experiment (Scale 200 µm). (A) Four hour chase. EdU+ cells in an aboral band. (B) Twenty four hour chase. Increase in EdU+ cells in the regenerating head now visible. (C) Forty eight hour chase. Many more EdU+ cells now present in the regenerating head and aboral band (D) Seventy two hour chase. EdU+ cells still present in the regenerating head and aboral band.
3.4.3 DNA synthesis detection using pH3

EdU labels cells during S-phase and as such is not a true marker of proliferation. In particular, it has been shown in *Hydra* that terminally differentiated epithelial cells are arrested in G2. Hence, counting EdU+ cells probably overestimates the number of cycling cells. To obtain a more realistic view on cell proliferation I used antibodies raised against the mitosis marker Phospho-Histone H3, (hereafter pH3: Abcam ab5176). This anti-pH3 antibody detects endogenous levels of histone H3 only when phosphorylated at serine 10 which is tightly correlated with chromosome condensation during mitosis.

Immunohistochemistry was completed as described in protocol 2.4.3

Non-regenerating polyps expressed pH3 mainly in the aboral region of the polyp with a few single cells in the oral region (Fig. 3.4.3 A). After decapitating, this expression pattern changed. At 24 and 48 HPD pH3+ cells were now present in the regenerating tip (Fig. 3.4.3 C, D). The pattern is consistent with the one observed following EdU labelling (Fig. 3.4.1.2) in decapitated and intact polyps.
Fig. 3.4.3. Cell proliferation in regenerating polyps as detected by anti-pH3 antibody (yellow) Hoechst (blue) (Scale 200 µm). (A) Intact polyp. Proliferation found mainly in the aboral body region. (B) Polyp 24 HPD. Proliferation now in the regenerating oral tip. (C) Polyp 48 HPD. Proliferation still in the regenerating oral region.
3.5 Gene Expression Analysis

3.5.1 Whole Mount *in Situ* Hybridisation

*In Situ* Hybridisation is a very well established technique used to investigate gene expression patterns where a labelled (e.g. Digoxigenin, Fluorescence) antisense probe (DNA or RNA) which is complementary to a target sequence is hybridized to the target sequence *in situ*. An antibody is then used against the labelled probes which is conjugated to an enzyme. This enzyme will itself interact with a dye to cause a colorimetric reaction staining cells where the target mRNA is present (Gilbert, 1997)

Whole mount *in situ* hybridisation was used to analyse the change in gene expression during regeneration for the *Hydractinia* stem cell marker genes *Piwi1, Pl10, Vasa, Myc* and the nematoblast differentiation marker *Ncol1*. Polyps were removed from their colony and either fixed intact or decapitated and then fixed 24 and 48 HPD. This experiment was repeated over 20 times with similar results each time.
3.5.2 Piwi1

Piwi genes (P element induced wimpy testis) belong to the piwi/argonaute family and are characterized by their piwi/paz domains. They are one of the first class of genes that were shown to play a crucial role in stem cell self-renewal and germ line establishment, division and maintenance (Thomson and Lin, 2009). Piwi proteins have also been shown to play a role in RNA interference and transposon silencing through their piwi interacting RNA’s (piRNA) (Juliano et al., 2011; Peng and Lin, 2013). Piwi is expressed in mesenchymal stem cells in mice and in hematopoietic cells in humans. Loss of piwi function has been shown to effect fertility, germline stem cell formation and maintenance in Drosophila melanogaster (Li et al., 2009), Mus musculus (Carmell et al., 2007), Danio rerio (Houwing et al., 2008) and Caenorhabitis elegans (Wang and Reinke, 2008). Piwi proteins are expressed in pluripotent stem cells of planarians, ctenophores, sponges and tunicates and in all stem/progenitor cells in Hydra (Rinkevich et al., 2010; Alie et al., 2011; Millane et al., 2011; Juliano et al., 2014; Lim et al., 2014).

I investigated the expression of Piwi1 in regenerating feeding polyps by in situ hybridization. The primers used to amplify the target sequence were previously designed by Dr Cathriona Millane from the public available partial coding sequence (GenBank: JG772275.1).

Templates for cRNA synthesis were generated by PCR as outlined in protocol 2.2.6 using the primers CniwiFort7, CniwiRevSp6 (Primer list 2.7). An annealing temperature of 50ºC and extension time of 2 minutes was used to produce a 376bp template. This PCR product was then gel extracted according to protocol 2.2.7 and then used as a template to produce both sense and antisense RNA probes according to protocol 2.2.8. In situ hybridisation was then completed according to protocol 2.2.18 at a temperature of 55ºC on intact and regenerating feeding polyps.
Intact feeding polyps expressed \textit{Piwi1} mainly in a band in the aboral region of the polyp (Fig. 3.5.2.1 A, B). Expression is seen in low and high concentrations within different cells. Cells expressing \textit{Piwi1} in a low concentration are found in greater numbers and are usually found forming clusters in the aboral band (Fig. 3.5.2.1 C). Cells expressing \textit{Piwi1} in a high concentration are less numerous and do not form clusters, instead they can be found in pairs or on their own (Fig. 3.5.2.1 C). Both \textit{Piwi1} high and low cells are found in the aboral band before decapitation but after decapitation this expression pattern changes. Firstly at the blastema stage around 24 HPD we see new ectopic expression of \textit{Piwi1} low cells at the regenerating tip and a small number of \textit{Piwi1} high cells between the band and regenerating tip (Fig. 3.5.2.2). After regeneration has completed the expression pattern returns to normal and the \textit{Piwi1} cells are no longer in the oral region but again being expressed in the aboral band (Fig. 3.5.2.3).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{\textit{Piwi1} in situ hybridisation on intact feeding polyps. (A) \textit{Piwi1} expression in intact feeding polyp. Expression is found in the aboral region. Head and tentacles are devoid of expression. (B) Close up of oral region devoid of expression. (C) Close up of aboral region showing both \textit{Piwi1} high and low cells. (D) Sense control. (Scale A, D 200 µm, B, C 10 µm)}
\end{figure}
Fig. 3.5.2.2. *Piwi*1* in situ* hybridisation on regenerating polyp fixed 24 HPD. (A) *Piwi*1+ cells are now found at the regenerating tip with a small number between the oral and aboral regions. (B) Close up of *Piwi*1*+* cells in regenerating head. (C) Close up of a single *Piwi*1 high cell located between the aboral band and regenerating tip. (D) Close up of two *Piwi*1 high cells (Scale A 200 µm, B 20 µm C, D 10 µm)

Fig. 3.5.2.3. *Piwi*1* in situ* hybridisation on regenerating polyp fixed 48 HPD (A) Head regeneration is now complete and *Piwi*1*+* cells are now only found in the aboral band. The regenerated head and tentacles are devoid of *Piwi*1 expressing cells. (B) Close up of regenerated oral section devoid of *Piwi*1*+* cells. (C) Close up of single *Piwi*1 high cell located in the aboral band. (D) Two *Piwi*1 high cells on the border between aboral band and regenerated oral tissue (Scale A 200 µm, B-D 10 µm)
3.5.3 Vasa

Vasa is a RNA binding protein and member of the DEAD-box helicase family. It was first discovered in *Drosophila melanogaster* where it was shown to be essential for germ cell development (Schupbach and Wieschaus, 1986). It is expressed in the germ line stem cells of the female ovaries and in the early stages of spermatogenesis in the male testis in *Drosophila* (Castrillon et al., 2000; Liu et al., 2009; Gustafson and Wessel, 2010). Vasa is required for germ cell specification in zebrafish, *C. elegans, Xenopus, Hydra* and *Drosophila* and is a marker of germ cells from the earliest stages and throughout development (Gustafson and Wessel, 2010; Nishimiya-Fujisawa and Kobayashi, 2012). A similar pattern is seen in humans where *Vasa* is specifically expressed in the germ cell lineage of both males and females (Castrillon et al., 2000). Outside the germline; *Vasa* is expressed in the pluripotent neoblasts of planarians (Shibata et al., 1999), the interstitial stem cells in *Hydra* and *Hydractinia* (Rebscher et al., 2008; Alie et al., 2011; Millane et al., 2011; Plickert et al., 2012), the pluripotent stem cells in ctenophores (Alie et al., 2011) and in the stem cells of the tunicate *Botryllus* (Rosner et al., 2009).

I investigated the expression of *Vasa* in intact and regenerating feeding polyps by *in situ* hybridization. The primers used to generate the probe sequence were designed from the public available coding sequence, GenBank accession No. EF467228.1.

PCR was completed according to protocol 2.2.6 using the primers HeVASAforT7 and HeVASArevSP6 (Primer list 2.7) at an annealing temperature of 50°C and an extension time of 2 minutes, creating a 300 bp fragment. This PCR product was then gel extracted according to protocol 2.2.7 and then used as a template to produce both sense and antisense RNA probes according to protocol 2.2.8. *In situ* hybridisation was then completed according to protocol 2.2.18 at a hybridization temperature of 50°C on intact and regenerating feeding polyps.
Intact feeding polyps expressed *Vasa* mainly in a band in the aboral region of the polyp with little expression seen orally (Fig. 3.5.3.1). During regeneration this expression pattern changes. Firstly at the blastema stage around 24 HPD strong expression of *Vasa*+ cells was now visible at the regenerating tip and in the aboral band (Fig. 3.5.3.2). This oral expression remained until after head regeneration had completed. At this point *Vasa*+ cells were no longer found in the oral region but were again expressed in an aboral band (Fig. 3.5.3.3).

Fig. 3.5.3.1. *Vasa* in situ hybridization on intact feeding polyps. (A) *Vasa* expression is located in a band in the aboral region (B-D) Close up of cells expressing *Vasa* found in the aboral region. (E) Close up of oral region devoid of expression. (E) Sense control (Scale A, F 200 μm, B-E 10 μm)
Fig. 3.5.3.2. Vasa expression in regenerating polyp fixed 24 HPD. (A) Vasa expression is now found in the blastema at the regenerating tip and in cells in the aboral region. (B) Close up of Vasa+ cells in the blastema. (C-D) Vasa+ cells found at the blastema (Scale A 200 µm, B-D 10 µm).

Fig. 3.5.3.3. Vasa expression in a regenerating polyp fixed 48 HPD. (A) Head regeneration is now complete and Vasa expression is now only found in the aboral band. The regenerated head and tentacles are devoid of Vasa+ cells. (B) Close up of regenerated oral section devoid of Vasa expressing cells. (C) Close up of single Vasa+ cell located in the aboral band. (D) Close up of Vasa+ cells in the aboral band (Scale A 200 µm, B-D 10 µm).
3.5.4 **PI10**

*Pl10* belongs to the DEAD box polypeptide 3 (DDX3) subfamily and like *Vasa* contains a DEAD motif which is required for proliferation and maintenance of the germline in many animals (Chang and Liu, 2010). In Mammals *Pl10*, also called DDX, is essential for normal differentiation during spermatogenesis (Rosner et al., 2006) while in *Hydra* it is expressed in the germline, i-cells and nematoblasts (Nishimiya-Fujisawa and Kobayashi, 2012). The pluripotent neoblasts of planarians (Wang et al., 2013) and the stem cells of ctenophores both express *Pl10* (Alie et al., 2011) and it also plays a role in fertility in *Drosophila* (Johnstone et al., 2005).

I investigated the expression of *Pl10* in intact and regenerating feeding polyps by *in situ* hybridisation. The primers used to amplify the target sequence were designed from a partial coding sequence, GenBank accession No. AB048849.1.

PCR was completed using the primers Pl10ForT7, Pl10RevSP6 (Primer list 2.7) as outlined in protocol 2.2.6. An annealing temperature of 50°C and extension time of two minutes was used to produce a 358bp template. This PCR product was then gel extracted according to 2.2.7 and then used as a template to produce both sense and antisense RNA probes according to 2.2.8. *In situ* hybridization was then completed according to 2.2.18 at a temperature of 55°C on intact and regenerating feeding polyps. Intact feeding polyps expressed *Pl10* mainly in a band in the aboral region, similar to the expression pattern seen in *Piwi1* and *Vasa* (Fig. 3.5.4.1). After decapitation this expression pattern changed. Firstly at the blastema stage around 24 hours after decapitation we see new expression of *Pl10* at the regenerating tip (Fig. 3.5.4.2 A, C, D). After regeneration has completed *Pl10* expression is again mainly found in an aboral band (Fig. 3.5.4.2 E) though a small number of *Pl10*+ cells are still visible in the regenerating tentacles and head (Fig. 3.5.4.2 B, E).
Fig. 3.5.4.1. *Pl10* in situ hybridization on intact feeding polyps. (A) *Pl10* expression found in the aboral region. Head and tentacles devoid of expression. (B) Close up of aboral region showing the absent of *Pl10*+ cells (C) Close up of aboral region showing *Pl10* expression (D) Control sense (Scale A, D 200 µm, B, C 10 µm).

Fig. 3.5.4.2. *Pl10* expression in regenerating polyp fixed 24 HPD. (A) Expression now in the blastema structure in the regenerating tip. Cells expressing *Pl10* now present throughout the polyp. (B) Close up of *Pl10*+ cells in a regenerating tentacle fixed 48 HPD. (C) Close up of *Pl10*+ cells found in the aboral region of a polyp fixed 48 HPD. (D) Close up of two cells expressing *Pl10* located at the blastema 24 HPD. (E) *Pl10* expression in a regenerating polyp fixed 48 HPD (Scale A, E 200 µm, B-D 10 µm).
3.5.5 Myc2

C-Myc encodes for the transcription factor Myc which is a bHLH-Zip DNA binding protein. Myc controls many fundamental cellular processes including the cell cycle, growth, proliferation, metabolism, and apoptosis (Simionato et al., 2007). Deregulation of c-myc leads in many cases to tumorigensis and is a hallmark of many cancers (Simionato et al., 2007; Hishida et al., 2011; Zinin et al., 2014). In humans Myc has been shown to regulate fundamental cellular processes like growth, proliferation, metabolism, differentiation and apoptosis (Eisenman, 2001). In Drosophila Myc (dMyc) controls cell growth, cell size and regulates cell competition (Johnston et al., 1999). In cnidarians; Hydra Myc2 is present in i-cells, nematoblast nests and all rapidly proliferating cell types including proliferating gland cells but is not detectable in terminally differentiated nerve cells, nematocytes, or epithelial cells by in situ hybridization (Hartl et al., 2010). It is also a marker for i-cells in Hydractinia (Millane et al., 2011).

I investigated the expression of Hydractinia Myc2 in regenerating feeding polyps by in situ hybridization. The primers used to amplify the target sequence were designed from a complete, public available sequence, GenBank accession No. JF820068.1. PCR was completed using the primers MycInSituFWt7, MycInsituRVsp6 (Primer list 2.7) as outlined in protocol 2.2.6. An annealing temperature of 50ºC and extension time of two minutes was used to produce a 394 bp template. This PCR product was then gel extracted according to 2.2.7 and then used as a template to produce both sense and antisense RNA probes according to 2.2.8. In situ hybridisation was then completed according to 2.2.18 at a temperature of 50ºC on intact and regenerating feeding polyps.
Intact feeding polyps expressed *Myc*-2 exclusively in the epidermal, aboral region. (Fig. 3.5.5.1). During regeneration I observed a change in this expression pattern. Firstly around 24 hours after decapitation during the formation of a blastema *Myc*-2 expression can be found not only in the aboral band but also in the regenerating tip (Fig. 3.5.5.2). This second band of expression is at its strongest during regeneration but decreases as the polyps regenerates (Fig. 3.5.5.3)

Fig. 3.5.5.1. *Myc2 in situ* hybridization on intact feeding polyps. (A) *Myc2* expression is found in the aboral region. (B) Close up of oral region devoid of *Myc2* expression. (C) Close up of aboral region showing *Myc2* expression. (D) Control sense (Scale A, D 100 µm, B, C 10 µm)
Fig. 3.5.5.2. *Myc2* in situ hybridization on polyp 24 HPD. (A) *Myc2* expression in regenerating polyp fixed 24 HPD. *Myc2* expression now in the blastema at the regenerating tip and in cells in the aboral band region. (B) Close up of the blastema showing *Myc2* expression. (C) Close up of *Myc2*+ cells in the blastema. (D) Cells located between the blastema and aboral band showing low levels of expression. (E, F) Close up of *Myc2*+ cells in the aboral band (Scale A 250 µm, B-F 10 µm).

Fig. 3.5.5.3. *Myc2* in situ hybridization on polyps fixed 48 HPD. (A) Head regeneration is now complete and *Myc2* expression is no longer in the regenerated head and is mainly found in an aboral band. (B) Close up of regenerated oral section with low *Myc2* expression. (D) Close up of *Myc2*+ cells in the aboral band. (D) Close up of *Myc2*+ cells located in a growing tentacle (Scale A 250 µm, B-D 10 µm).
3.5.6 Ncol1

*Ncol1* (Nematocyst collagen 1) codes for a minicollagen protein that is expressed exclusive in nematoblasts that are undergoing differentiation into nematocytes. Minicollagens are uniquely short collagen molecules that are major constituents of the nematocyst wall and tubule structures (David et al., 2008). It has been previously shown to be expressed in the epidermal aboral region on intact feeding polyps of *Hydractinia echinata* and in the gastric region and distal peduncle of *Hydra* but never in the head or tentacle region of either animal during normal homeostasis (Ozbek et al., 2002; Kanska and Frank, 2013).

I investigated the expression of *Ncol1* in regenerating feeding polyps by *in situ* hybridization. Primers were previously designed by Dr Justyna Kanska from an EST partial coding sequence, GenBank accession No. JX486117.1.

PCR of this product was completed using an annealing temperature of 50°C and an extension time of two minutes creating a 208 bp fragment. PCR was completed using the primers Ncol1-T7fwd, Ncol1-Sp6rev (Primer list 2.7) as outlined in protocol 2.2.6. This PCR product was then gel extracted according to 2.2.7 and then used as a template to produce both sense and antisense RNA probes according to protocol 2.2.8. *In situ* hybridisation was then completed according to 2.2.18 at a hybridization temperature of 50°C on intact and regenerating feeding polyps.

Intact feeding polyps expressed *Ncol1* exclusively in the epidermal, aboral region. (Fig. 3.5.6.1). During regeneration I observed a change in this expression pattern. Firstly around 24 hours after decapitation during the formation of a blastema *Ncol1* expression can be found not only in the aboral band but also in the regenerating tip (Fig. 3.5.6.2 A, B). This second band of expression is at its strongest during regeneration but decreases as the polyps regenerate (Fig. 3.5.6.3 A)
Fig. 3.5.6.1. Ncol1 in situ hybridization on intact feeding polyps. (A) Ncol1 expression is found exclusively in the aboral region. (B) Close up of single Ncol1+ cells in the aboral band (C) Close up of aboral region showing Ncol1 expression. (D) Control sense (Scale A, D 250 µm, B, C 10 µm).

Fig. 3.5.6.2. Ncol1 in situ hybridization on polyps fixed 24 HPD. (A) Ncol1 expression is now in the blastema at the regenerating tip and in cells in the aboral band. (B) Close up of blastema showing Ncol1 expression. (C) Close up of Ncol1+ cells in the aboral band (D) Close up of area between blastema and the aboral band devoid of Ncol1+ cells (Scale A, D 250 µm, B-D 10 µm).
Fig. 3.5.6.3. *Ncol1* in situ hybridization on polyps fixed 48 HPD. (A) Head regeneration is now complete and *Ncol1* expression is now low in the regenerated head. It is now mainly found in the aboral band. (B) Close up of regenerated head showing low expression of *Ncol1*. (D) Close up of *Ncol1* positive cells in the aboral band. (C, E) Close up of *Ncol1*+ cells in a regenerating tip and growing tentacle (Scale A 250 µm, B-E 10 µm).
3.6 Cell identification by Maceration

Whole polyp maceration was used to identify what cell types were proliferating during regeneration and to see if they differed from non-regenerating polyps. Polyps were macerated into single cells and proliferating cells were then identified using either an EdU assay or pH3 IHC. This method of macerating polyps has been widely used before in Hydra (David, 1973) with some variations as described in protocol 2.3.

3.6.1 Maceration of polyp and EdU labelling

Polyps were removed from a colony, decapitated and then left for 24 hours in seawater. The following day more polyps were removed but were not decapitated. Both sets of polyps, regenerating and intact were then soaked in an EdU/seawater solution for three hours, macerated into single cells and affixed on to glass slides as described in protocol 2.3. EdU fluorescent staining was then completed as described in protocol 2.4.1. Cells were then counterstained using the nuclear DNA stain Hoechst (Sigma 14533) and visualized on a FV1000 Olympus Confocal Scanning Laser microscope.

Epithelial and non-epithelial cells were then counted and the number of EdU positive cells in each group was recorded for both regenerating and intact polyps. Epithelial cells can be identified by their size and shape so any cells that were bigger than 20 µm I grouped as epithelial and any cells below this size I grouped as other cell type. Other cells would include i-cells, nematoblasts, gland cells and other non-epithelial cell types. Pictures of both epithelial and non-epithelial EdU positive cells are shown in Fig. 3.6.1.1.
Fig. 3.6.1.1. EdU+ epithelial and non-epithelial cells labelled by Hoechst (blue) EdU (green) (Scale bars 10 µm). 

(A) EdU+ i-cell and non-EdU+ nematocytes. 

(B) EdU Positive epithelial cell 

(C) EdU+ and EdU- i-cells and an EdU- epithelial cell. 

(D) EdU+ epithelial cell plus 2 non-EdU+ nematocytes. 

(E-G) EdU+ i-cells
A total of 5.4% epithelial and 19.5% non-epithelial EdU positive cells were observed in intact polyps. The numbers were quite similar in regenerating polyps where 5% epithelial and 20% non-epithelial EdU positive cells were observed (Fig. 3.6.1.2). The total cell count numbers are listed in table 3.6.1.3.

![EdU+Cells](chart.png)

Fig. 3.6.1.2. Percentage of EdU+ versus EdU- epithelial cells and EdU+ versus EdU- non-epithelial cells labelled over a 3 hour period in regenerating polyps.

<table>
<thead>
<tr>
<th></th>
<th>Epithelial EdU+</th>
<th>Percentage total</th>
<th>Non-Epithelial EdU+</th>
<th>Non-Epithelial EdU+</th>
<th>Percentage total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>947</td>
<td>52</td>
<td>5.4%</td>
<td>1610</td>
<td>315</td>
</tr>
<tr>
<td>Regenerating</td>
<td>1021</td>
<td>52</td>
<td>5%</td>
<td>1425</td>
<td>292</td>
</tr>
</tbody>
</table>

Table 3.6.1.3. Total EdU +/- cell count numbers for non-regenerating and regenerating polyps.
3.6.2 Maceration and pH3 IHC

Polyps were removed from a colony decapitated and then left for 24 hours in seawater. The following day more polyps were removed but were not decapitated. Both sets of polyps, regenerating and intact were then disassociated into single cells and affixed onto glass slides as described in protocol 2.3. IHC was then performed using an anti pH3 antibody as described in protocol 2.4.4. Cells were then counterstained using the nuclear DNA stain Hoechst and visualized on a FV1000 Olympus Confocal. Pictures were taken of both epithelial and non-epithelial pH3 positive cells and are shown in figures 3.6.2.1 and 3.6.2.2.

Fig. 3.6.2.1. pH3+ epithelial cells stained by Hoechst (blue) and pH3 (red). (A) Single pH3+ epithelial cell (Scale 10 µm) (B) Single pH3+ epithelial cell plus three pH3- nematocytes (Scale 10 µm).
Fig. 3.6.2.2. pH3+ i-cells, Hoechst (blue) and pH3 (red) (Scale 10 µm) (A-E) Single pH3+ i-cell. (F) Final stages of i-cell division. Two nuclei present (G) Final stage of i-cell division. Two new cells are present.
A calculation of the total percentage of pH3 positive versus pH3 negative epithelial and non-epithelial cells was not possible. This is due to the low number of cells in mitosis at any one time so pH3+ cells were much harder to locate. Because of this I counted just pH3 positive cells and recorded how many of which were epithelial and how many were i-cells. This count then gave me the percentage of epithelial versus i-cells during mitosis at any one time.

A total of 2.7% pH3+ epithelial cells were observed in intact polyps while a total of 3.8% pH3+ epithelial cells were observed during regeneration (Fig. 3.6.2.3). As over 95% of pH3+ cells were in fact i-cells it shows that in both regenerating and intact polyps the vast majority of mitotic cells are not epithelial but i-cell. The total cell count numbers are listed in Table 3.6.2.4.

Fig. 3.6.2.3. Total percentage of pH3+ epithelial versus i-cells in regenerating polyps.
Table 3.6.2. Total pH3+ epithelial and non-epithelial cell counts in regenerating and non-regenerating polyps.

<table>
<thead>
<tr>
<th></th>
<th>pH3+</th>
<th>pH3+ Epithelial</th>
<th>Percentage pH3+ epithelial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>217</td>
<td>6</td>
<td>2.7%</td>
</tr>
<tr>
<td>Regenerating</td>
<td>155</td>
<td>6</td>
<td>3.8%</td>
</tr>
</tbody>
</table>

3.7 Piwi1 Antibody staining

Piwi1 IHC was completed on non-regenerating and regenerating polyps to see if the expression pattern matched the pattern seen in Piwi1 *in situ* hybridisation. S-phase cells were also labelled using EdU to see if Piwi1+ cells were proliferating. Polyps were removed from a colony, decapitated and fixed at different time points. A number of polyps were also processed intact to see the expression patterns in non-regenerating polyps. Polyps were treated in EdU/seawater for 40 minutes before fixation. IHC was completed as described in protocol 2.4.3 and EdU staining was completed as described in protocol 2.4.1. Anti Hiwi antibody was kindly donated by Dr. Celina Juliano, Yale University.

Piwi1+ cells were found mainly in the aboral band in non-regenerating polyps (Fig. 3.7.1.1 A) while in polyps 10 hours post decapitation single cells could be seen between this aboral band and the regenerating head (Fig. 3.7.1.1 B). A small number of Piwi1+ cells in regenerating polyps were EdU+ (Fig. 3.7.1.2) but the majority of these cells were not.
Fig. 3.7.1.1. Piwi1 IHC of polyps (Scale 200 μm). (A) Non-regenerating polyp, Piwi1+ cells restricted to the aboral band (White arrow). (B) Regenerating polyp 10 hours post decapitation, single cells present between regenerating head and aboral band (White arrows)
Fig. 3.7.1.2. Piwi1 IHC on regenerating polyp and co-localisation of S-phase cells, anti Piwi1 antibody (green), EdU (red) Hoechst (blue). Scale 10 µm. (A) Piwi1+ IHC and EdU+ cells in regenerating polyp. (B) Close up of Piwi1+/EdU+ cells. (C) Nuclear staining of Piwi1+ cells in figure B
3.8 Blocking Proliferation and Regeneration

3.8.1 Irradiation Blocks Proliferation and Regeneration

To study the requirement of cell proliferation in regeneration I used gamma irradiation to block proliferation and examine the effect. I exposed polyps to a Caesium 137 gamma irradiator source (14Gy/min). Exposure to high levels of irradiation causes DNA damage. Cycling cells are particularly sensitive and undergo death by apoptosis or senescence following irradiation. This method of blocking proliferation is well established and has been used to ablate neoblasts and block regeneration in planarians (Guedelhoefer and Sanchez Alvarado, 2012) and also block proliferation in Hydra (Hicklin et al., 1975).

To establish the correct dosage, polyps were removed from a colony, irradiated at different doses, decapitated and then left to see if they would regenerate (Protocol 2.6). An EdU assay was also completed at different doses to ascertain if the cells were entering S-phase which would lead to proliferation. Regeneration (Fig. 3.8.1.1) and S-phase cells were observed in polyps exposed to doses of 100 and 300 Gy (Fig. 3.8.1.2 C, D). At 500 Gy neither S-phase cells nor full regeneration were observed (Fig. 3.8.1.2 E). A number of polyps did begin to regenerate and build a head at this dosage but this was quickly reabsorbed and all polyps died after 6 to 12 days a phenomenon also observed in planarians (Guedelhoefer and Sanchez Alvarado, 2012). To measure the effect this dose was having on the rebuilding of the head nervous system after decapitation, polyps were stained for acetylated tubulin one week after exposure and decapitation. There was no new visible structure after this exposure (Fig. 3.8.1.2 A) showing the importance of proliferation in building a new head nervous system.
Fig. 3.8.1.1. Regenerative ability of polyps exposed to different levels of irradiation. (A) Control 24 hours after decapitation 0 Gy (Scale 100µm). (B) Polyp exposed to 100Gy. One Week after irradiation and decapitation. Polyp has fully regenerated. (C) Polyp exposed to 300Gy. One Week after irradiation and decapitation. Some regeneration has occurred. (D) Polyp exposed to 500Gy. One Week after irradiation and decapitation. Polyp did not regenerate.

Fig. 3.8.1.2. Analysis of polyps exposed to different levels of irradiation. (A) Polyp exposed to 500 Gy irradiation. Polyp did not regenerate one week after exposure and decapitation. Acetylated tubulin (Green), Hoechst (blue), F actin (Red) (Scale 200µm) (B) Polyp exposed to 0 Gy. 48 hours after decapitation. Green showing EdU positive cells Scale 200µm. (C) EdU+ cells in polyp exposed to 100 Gy (Scale 10µm) (D) EdU+ cell in polyp exposed to 300 Gy (Scale 10µm). (E) No EdU+ cells in polyp exposed to 500 Gy (Scale 10µm).
3.8.2 Loss of Gene Expression Post Irradiation

To investigate what effect irradiation was having on polyps, *in situ* hybridisation was performed using some classical stem cell marker genes *Piwi1*, *Myc2*, *Pl10* and the nematoblast differentiation marker *Ncol1*.

Polyps were removed from the colony exposed to 500 Gy irradiation, decapitated and then fixed at different time points. *In situ* hybridisation was then completed as described 2.2.18 and comparisons were made with irradiated and non-irradiated polyps. In all cases a decrease was seen in the expression levels of the genes tested. About 24 hours after decapitation the expression in the aboral band was very low and there was no second band of expression in the oral section (Fig. 3.8.2).

![Gene expression patterns 24 HPD in non-irradiated and 500 Gy irradiated polyps. (A) Myc2 (B) Ncol1 (C) Piwi1 (D) Pl10](image)

Fig. 3.8.2. Gene expression patterns 24 HPD in non-irradiated and 500 Gy irradiated polyps. (A) Myc2 (B) Ncol1 (C) Piwi1 (D) Pl10
3.8.3 Cell Death Increases Post Irradiation

To investigate the effect of irradiation on cells a TUNEL assay was used to see if cell death increased post irradiation. Polyps were removed from a colony, irradiated, decapitated and fixed at different time points. A TUNEL assay was then completed as describe in protocol 2.4.2. An increase in cell death was observed in irradiated decapitated polyps and this increase was seen throughout the polyps but was concentrated at the oral tip (Fig. 3.8.3). Two hours post irradiation and decapitation TUNEL+ cells were mostly confined to the aboral band. This pattern changed from six hours though to 24 hours (Fig. 3.8.3 B, C). The location of TUNEL+ cells changed from the aboral band to the decapitated oral section. An increase in cell death was also observed in non-irradiated polyps but at a much lower amount (Fig. 3.8.3) which is consistent with work done on Hydra (Galliot and Chera, 2010), planarians (Pellettieri et al., 2010) and Xenopus (Tseng et al., 2007) which showed an increase in cell death during regeneration or as a response to wounding.
Fig. 3.8.3. TUNEL assay on non-irradiated and irradiated polyps. Dotted line showing outline of polyps. Non-irradiated polyp on the left and 500 Gy irradiated on the right (A) Polyp two hours after irradiation and decapitation. TUNEL+ cells in aboral region (Scale 200µm). (B) Polyp six hours after decapitation. TUNEL+ cells in aboral region and a smaller number towards the oral section (C) Polyp 24 hours after decapitation. TUNEL+ cells now in oral section of polyp.
3.8.4 Mitomycin-C blocks Regeneration

Mitomycin C inhibits DNA synthesis and can be used as a method to block proliferation. It forms crosslinks between complementary strands of DNA which prevents the separation of these strands, thus inhibiting DNA replication.

Mitomycin-C was dissolved in methanol so a methanol control was used for these experiments. Polyps were removed from their colony decapitated and then soaked in different concentrations of Mitomycin-C in seawater to establish the lowest concentration needed to block regeneration. A final concentration of 1.5 µm was calculated. BrdU (5-bromo-2’-deoxyuridine) IHC was then performed on polyps as described 2.4.3 to see if any cells were completing DNA synthesis after exposure to the Mitomycin-C. No BrdU positive cells were seen in polyps where proliferation and regeneration was blocked (Fig. 3.8.4). This result along with the results obtained from the irradiation experiments show that after proliferation is blocked, decapitated Hydractinia feeding polyps are unable to regenerate. This is in contrast to results observed in Hydra where polyps are capable of regeneration after proliferation is blocked by either γ-irradiation (Hicklin et al., 1975) or hydroxyurea (Cummings and Bode, 1984).
Fig. 3.8.4. BrdU immunohistochemistry marking cells in S-phase in regenerating polyps treated with Mitomycin-C. (A) Polyp 24 HPD treated with Mitomycin-C. No signs of regeneration (scale 200 µm) (B) Polyp treated with Mitomycin-C, no BrdU positive cells present. (C) Control polyp 24 HPD, treated with Methanol. New tentacles in the head. (D) Control polyp 24 HPD BrdU positive cells present showing cells in S-phase (scale 10 µm).
3.9 RNA interference (RNAi)

3.9.1 Knockdown Delays Regeneration

RNAi is a method of studying the roles of specific genes by inhibiting their expression by sequence specific targeting of their mRNA. Double stranded RNA is designed around a target sequence and introduced into the cell. The target mRNA is then degraded and its effect can then be used to study the knockdown of the target gene (Gilbert, 1997). RNAi was first described by (Fire et al., 1998) where the introduction of dsRNA into *C. elegans* resulted in more efficient gene silencing than was observed in the introduction of either sense or antisense strands alone.

The RNAi pathway is found in many eukaryotes and is initiated by the enzyme Dicer. This enzyme cuts long dsRNA into shorter strands of about 20 nucleotides long called small interfering RNA (siRNA). The siRNAs are then unwound into two single stranded RNA molecules or ssRNA molecules, the passenger strand and the guide strand. The passenger strand is degraded and the guide strand which is complementary to the target mRNA is then targeted and cleaved by a new enzyme complex; the RISC/Argonaute complex (RNA induced silencing complex). As viruses often have dsRNA genomes it is thought that this complex evolved as a means of targeting and preventing viral infections (Gilbert, 1997; Kim and Rossi, 2008).

RNAi has been used as a tool in many animal groups including planarians, *C. elegans*, *Drosophila*, *Hydra* and, *Hydractinia* (Gonczy et al., 2000; Dietzl et al., 2007; Duffy et al., 2010; Millane et al., 2011; Rouhana et al., 2013)

Primers were designed to amplify a sequence of exactly 200bp in length for each of the genes of interest (Primer table 3.8.0.1). These sequences were then amplified by PCR as described in protocol 2.2.6, gel extracted as described in protocol 2.2.7 and dsRNA was made as described in protocol 2.2.9. Control dsRNA was amplified from a pBluescript vector.
Table 3.9.1. Primer list for sequence amplification used during RNAi

<table>
<thead>
<tr>
<th>RNAi Treatment</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>T7pGEM-TRNAifwd2</td>
<td>5'-atcctatactagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td></td>
<td>pGemRNAiT7new</td>
<td>5'-gatcctatactagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td>Ncol1</td>
<td>Ncol1-T7fwd</td>
<td>5'-gatcctatactagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td></td>
<td>Ncol1-Sp6rev</td>
<td>5'-tagcatttaggtacactataagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td>Piwi1</td>
<td>PiwiRNAiT7Fw</td>
<td>5'-gcgtaatactagactcactatatgggacaggactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td></td>
<td>PiwiRNAiSP6Rv</td>
<td>5'-tgctttaggtacactataagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td>PI10</td>
<td>PI10rnaiFWT7</td>
<td>5'-tgctttaggtacactataagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td></td>
<td>PL10rnaiRVsp6</td>
<td>5'-gatcctatactagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td>Vasa</td>
<td>VasaRNAiFWT7</td>
<td>5'-gcgtaatactagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
<tr>
<td></td>
<td>VasaRNAiRVsp6</td>
<td>5'-tgctttaggtacactataagactcactatatgggacaggactataagattacagcag-3'</td>
</tr>
</tbody>
</table>
I used RNAi to study the knockdown of *Piwi1, Vasa, Pl10 and Ncol1* and to see if they played a role during regeneration as each was shown to be expressed in the blastema during head regeneration. About 10 polyps were removed from a colony and decapitated for each treatment. They were then soaked in seawater containing between 20µg and 40µg of dsRNA per ml. The seawater containing the dsRNA was replaced every 24 hours. As different individual colonies were used for each experiment different concentrations had to be used for each experiment. This is due to a slight variation in the amenability of each animal to RNAi. To calculate the optimum concentration for each colony a starting concentration of 40µg per ml was initially used and this was decreased if the controls did not regenerate within three days.

Each experiment was scored in three ways. If an animal regrew tentacles that were at least twice the length than their width the animal was deemed to have regenerated and was given a score of 1. If an animal regrew tentacles that were less than twice the length than their width the animal was deemed to have only reached a tentacle budding stage and were given a score of 0.5. An animal without any tentacles was deemed to have not regenerated and was given a score of 0.

The introduction of dsRNA for each gene was observed to have a clear effect on regeneration (Fig. 3.9.1.2). A chi-square statistical test was used to determine if there was a significant difference between the expected frequencies (the control) and the observed frequencies (the dsRNA treated) in one or more categories (Regeneration, Budding stage and Non-Regeneration). Each treatment showed an effect that was significant at $p < 0.001$ (Table 3.9.1.3).
Fig. 3.9.1.2. Polyps 72 hours after RNAi treatment (A) control (B) Ncol1 (C) Piwi1 (D) Pi10 (E) Vasa. Control RNAi polyp had regenerated after 72 hours while polyps treated with specific target RNAi had not.

Table 3.9.1.3. Polyps response to RNAi treatment (Scores used in Chi-squared test).

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>Regenerated</th>
<th>Budding Stage</th>
<th>Non-Regenerated</th>
<th>Chi-square statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>13</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ncol1</td>
<td>4</td>
<td>7</td>
<td>32</td>
<td>33.9001</td>
<td>&lt; 0.00001</td>
</tr>
<tr>
<td>Piwi1</td>
<td>7</td>
<td>7</td>
<td>25</td>
<td>22.883</td>
<td>&lt; 0.00001</td>
</tr>
<tr>
<td>Pi10</td>
<td>2</td>
<td>14</td>
<td>31</td>
<td>35.1458</td>
<td>&lt; 0.00001</td>
</tr>
<tr>
<td>Vasa</td>
<td>6</td>
<td>6</td>
<td>30</td>
<td>29.5477</td>
<td>&lt; 0.00001</td>
</tr>
</tbody>
</table>
To plot the RNAi treatment results I added up each of the scores calculated in the four RNAi experiments. I then transformed the scores of each experiment into a product of 100 using the formulas \((a+(b/2)=d)\) and \((dx100/(a+b+c)=e)\) (a = regenerated, b = budding, c= fail, d = treatment score, e = total score). The results of experiment #3 are shown in table 3.9.1.4. The standard deviation and mean score were then calculated for all four experiments and the results plotted on a bar chart (Fig. 3.9.1.5).

Table 3.9.1.4. The results of the third RNAi experiment. The response of individual polyps is shown in column a, b and c. The total score for each treatment is shown in the final column.

<table>
<thead>
<tr>
<th>dsRNA</th>
<th># Regenerated</th>
<th># Budding</th>
<th># Fail</th>
<th>Treatment ((a+(b/2)=d))</th>
<th>Calculation ((dx100/(a+b+c)=e))</th>
<th>Total Score ((e))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>7 + 0.5 = 7.5</td>
<td>7.5 x 100 / 10</td>
<td>75</td>
</tr>
<tr>
<td>Ncol1</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>0 + 1.5 = 1.5</td>
<td>1.5 x 100 / 10</td>
<td>15</td>
</tr>
<tr>
<td>Piwi1</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>1 + 0 = 1</td>
<td>1 x 100 / 10</td>
<td>10</td>
</tr>
<tr>
<td>Pl10</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>0 + 1 =</td>
<td>1 x 100 / 10</td>
<td>10</td>
</tr>
<tr>
<td>Vasa</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0 + 0 = 0</td>
<td>0 x 100 / 10</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 3.9.1.5. RNAi treatment of control vs gene specific dsRNA. Mean scores, control – 71.6 N=40, Ncol1 – 18.4 N=43, Piwi1 – 25.6 N=39, Pl10 18.5 N=47, Vasa – 21.3 N=42 (*P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001).
3.9.2 Knockdown Delays Regeneration but Not Proliferation

To check if the RNAi treatment was effecting the formation of a blastema in regenerating polyps I performed an EdU labeling assay on a random selection of polyps from each treatment as described in protocol 2.4.1. I did not observe a difference in the accumulation of EdU positive cells located at the blastema when comparing control versus target RNAi treated polyps (Fig. 3.9.2). Some natural variation was seen in the number of EdU+ cells at the regenerating tip but the appearance of EdU+ cells 24 HPD at the blastema was unaffected.

![Fig. 3.9.2. EdU assay on control v gene-specific RNAi treated polyps 24hr after decapitation. S-phase cells present in blastema of all treated polyps. (A) Control (B) Ncol1 (C) Piwi1 (D) Pi10 (E) Vasa](image-url)
3.10 Summary
Both *Hydra* and *Hydractinia* display a remarkable power of regeneration yet the methods used to regenerate lost tissue appear to be very different among the two taxa. Like *Hydra*, *Hydractinia* polyps can regenerate a new head within three days while also being able to regenerate from small cut sections of polyp (Bode, 2003). While *Hydra* can regenerate after DNA synthesis has been blocked (Hicklin et al., 1975; Cummings and Bode, 1984) this is not the case in *Hydractinia*. Cell proliferation is usually concentrated aborally in intact polyps but 24 hours post decapitation a burst of proliferation occurs at the regenerating oral section forming a blastema. When DNA synthesis is blocked head regeneration does not occur, revealing the essential role it plays during regeneration. The i-cell marker genes Piwi1, Pl10, Myc2 and Vasa plus the nematoblast differentiation marker Ncol1 are all expressed aborally in intact feeding polyps but are all found in or below the blastema in regenerating polyps. RNAi knockdown of these genes during regeneration significantly affects head regeneration though it does not block proliferation or blastema formation resembling results observed in planarians (Reddien et al., 2005). Unlike in *Hydra* (Buzgariu et al., 2014), epithelial cells do not contribute to head regeneration as less than 5% of all mitotic cells in *Hydractinia* were epithelial. Instead, i-cells contribute to the majority of cell proliferation in both regenerating and non-regenerating polyps. EdU pulse chase labelling revealed that cells which had incorporate EdU during S-phase were migrating to the blastema during regeneration.

These results outline the differences in regeneration that exist between *Hydractinia* and *Hydra*. The importance of both cell proliferation and blastema formation during regeneration reveal that *Hydractinia* head regeneration is achieved through epimorphosis. The EdU pulse chase experiment revealed the migration of S-phase cells aborally to the blastema. The ability to track these cells *in vivo* using a transgenic stem cell reporter would be a major tool in revealing what cells were migrating and the role these cells play in head regeneration.
Chapter 4. Tracking stem cell migration using a \textit{Piwi1} Transgenic Reporter line

4.1 Introduction

For head regeneration to occur successfully it appeared that cells were migrating to the head, proliferating and differentiating. The results of the maceration experiments (Chapter 3.5) have shown that over 95\% of proliferating cells were i-cells but it was still to be proven whether they were actually migrating to the site of regeneration or not. To investigate this I decided to create a transgenic i-cell reporter line where cells could be tracked \textit{in vivo}. This would show definitively if i-cells were migrating to the site of regeneration.

A gene promoter is a unique region of DNA upstream of a gene start site on the 5’ end of the sense strand. Promoters can activate or repress transcription of a gene through the binding of transcription factors and can be 100’s to 1000’s of base pairs in length. Transcription is initiated by the attachment of the enzyme RNA polymerase to the promoter site. This is essential for the expression of a gene which is mirrored by its promoter activity (Gagniuc and Ionescu-Tirgoviste, 2012). Gene expression is not always ubiquitous throughout an animal. Some genes like \textit{Ncol1} are only expressed in a specific type of cell (differentiating nematoblasts) (Ozbek et al., 2002; Kanska and Frank, 2013) while some genes like \textit{Actin} and \textit{Tubulin} are expressed in all cells types. By selecting the right gene promoter and inserting a reporter gene like GFP downstream of it, it is possible to study the activity of a gene in a transgenic animal \textit{in vivo} (Ma, 2001), or to follow the fate of a specific cell type that is known to express the reported gene (Renfer et al., 2010).

Transgenic animals in \textit{Hydractinia} are created by introducing a vector containing a cloned expression cassette into embryos using microinjection. After injection this cassette is inserted into the animal’s genome and is transcribed and translated in cells where the promoter activity is active.
The insertion point of the cassette is random and impossible to control. Moreover, the process of insertion into the genome is unknown. There are many problems that can occur through this arbitrary process. After injection the circular plasmid is randomly digested by endogenous endonucleases. These can digest the plasmid at any point disrupting the coding sequence and rendering any successful integration useless. The stability of the integrated cassette can also depend on the insertion point due to the flanking sequences around it. Moreover random insertion into an animal’s genome can cause deleterious problems by disruption of other genes important in development and homeostasis. Because of these issues large amounts of embryos need to be injected and the success rate is relatively low. Even so this method has been previously used to create both overexpression and reporter transgenic animals in Hydractinia (Kunzel et al., 2010; Millane et al., 2011; Kanska and Frank, 2013).

4.2 Piwi1 i-cell Marker

Piwi proteins are expressed in pluripotent stem cells of planarians, ctenophores, sponges and tunicates and in all stem/progenitor cells in Hydra (Rinkevich et al., 2010; Alie et al., 2011; Millane et al., 2011; Juliano et al., 2014; Lim et al., 2014). Piwi homologs have long been used as stem cell makers in planarians where pluripotent neoblasts (planarian stem cells) have been shown to migrate to a site of injury, proliferate and form a blastema of undifferentiated cells (Reddien et al., 2005; Nakagawa et al., 2012).

Through in situ hybridisation I have shown that Piwi1 high cells were present in the regenerating head 24 HPD (Chapter 3.5.2). I have also shown through immunohistochemistry and by marking proliferating cells by EdU that at least a fraction of Piwi1+ cells were proliferating 10 HPD (Chapter 3.7). All of these results plus results previous outlined in other animal groups indicated that Piwi1 would be a good candidate i-cell reporter maker. The Hydractinia pluripotency gene, Polynem (Pln), would also potentially be a suitable marker; however, all attempts to clone its promoter and establish a Pln reporter line were unsuccessful.
4.3 *Piwi1* promoter expression vector

To create a *Piwi1* reporter line I would need to clone the *Piwi1* promoter region followed by GFP and a 3’ untranslated region (UTR) into a plasmid (Fig. 4.3). A gene promoter is found upstream of the gene start codon and contains the promoter regions which are essential in gene transcription. Once cloned this plasmid could be transfected into bacteria, grown up in large numbers and injected into *Hydractinia* embryos. This procedure does not affect the endogenous *Piwi1* expression but the transgene that contains the inserted *Piwi1* promoter would drive GFP expression, highlighting *Piwi1+* cells which would be visible *in vivo* under blue light.

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**Fig. 4.3.** *Piwi1* reporter cassette. GFP is flanked by the *Piwi1* promoter and the *Actin* 3’ UTR.
4.4 Piwi1 promoter expression vector plus the Actin1 3’ UTR

I first cloned the Hydractinia Piwi1 promoter into the plasmid pAct-I:51Act-eGFP. This plasmid was cloned by Dr. Güenter Plickert, University of Köln. This plasmid contained the Hydractinia Actin1 promoter region followed by GFP and then the Hydra Actin 3’ untranslated region (UTR) which had been cloned into a pBluescript backbone (Kunzel et al., 2010) (Fig. 4.4).

![Cassette in pAct-I:51Act-eGFP](image)

**Fig. 4.4.** Cassette contained in the plasmid pAct-I:51Act-eGFP. GFP flanked by the Hydractinia Actin1 promoter and the Hydra 3’ UTR.

At the time I completed this experiment I only had access to a partial EST database and an incomplete genome database. The genome database had large unknown regions while some genes were split between different contigs. Unfortunately the Piwi1 gene was split between different contigs and it contained a large intron about 280 base pairs upstream of the gene start codon. To find the correct start codon I needed to perform RACE PCR.

4.4.1 Piwi1 Promoter (RACE PCR)

Rapid Amplification of cDNA Ends (RACE) is a method that can be used to find the full length DNA sequence of a gene when partial EST or genomic sequences are available. Many RACE protocols are available, but the presence of a trans-spliced leader (TSL) sequence at the 5’ end of mRNAs in some taxa facilitates this procedure in these groups. TSL addition has been shown in some but not all members of the phyla Cnidaria, Nematoda, Platyhelminthes and Chordata, (Nilsen, 1993; Stover and
Steele, 2001; Vandenberghe et al., 2001; Zayas et al., 2005). The exact role TSLs play is still not fully understood. Hydroids including *Hydra* and *Hydractinia* attach a TSL to the majority of their mRNAs. Primers can be designed against this sequence and can be used in combination with a known gene specific primer to amplify the unknown 5’ end of a gene.

PCR was performed as described in protocol 2.2.6. The reverse primer PiwiRv2Sp6 was designed using a known sequence section around 280 bp downstream of the start codon. A primer against the TSL which was previously designed by Dr David Duffy was used as the forward primer (Primer list 2.7). This PCR product was gel extracted as described in protocol 2.2.7 and sequenced (Fig. 4.4.1).

I then aligned the translated protein sequence with the *Piwi1* sequences of two other cnidarian species, *Hydra* and *Podocoryna* (Fig. 4.4.1.2). These three species contained a highly conserved section over the first 11 amino acids and I concluded that this was...
likely to be the correct start codon of the *Hydractinia Piwi1*. This now gave me access to the start site of the *Piwi1* gene and to the promoter which was on the same contig. I analysed all regions upstream on the contig by BLASTX to ensure that the 3’ end of any other genes was not included.

| Hydractinia | MTGRARGRSRG--RGKSARGGGNAAS---TGGRGRSRSATKSAVSAVAAA---MAKN 52 |
| Podocoryna | MTGRARGRGARGDAGGGAAAAGGAAGGRGRRSRRGAATAAAAAAMASR--MAVG 58 |
| Hydra     | MTGRARGRSRG----RGGNDAVGVYAAAGGRGRRSRSVVHAAATAVKVSADMSSADMSKV 53 |

********:::***:***:********:.....:*:|

Hydractinia - VRRRRRRRGARNRADTTTGAGK 74
Podocoryna  RAARRRRRRGARKDVTGTAGAR 81
Hydra       RDRDRRRGGDSHVKTGKVGTG 76

*****::..*:*|

Fig. 4.4.1.2. Alignment of the predicted *Hydractinia Piwi1* protein with its putative orthologues, *Podocoryna Cniwi* and *Hydra Hywi*.

Primers were designed to amplify 2800 bp upstream of the start codon and to remove the *Actin1* promoter from the original plasmid. The Primers Piwi1ProFw1new and Piwipromrevin were used to amplify the promoter (Primer list 2.7). The primers LigDVectorGFP-Fusion and BackboneactRV1 were used to synthesis the pAct-I:51Act-eGFP plasmid backbone and removed the *Actin1* promoter (Primer list 2.7). The cloning was completed using blunt end techniques. Blunt end cloning requires dephosphorylated of the vector to minimise the chance of self-ligation. Ligation requires the presence of a 5’ phosphate group so all primers used were phosphorylated as described in protocol 2.2.17. Both sequences were amplified by PCR using Phusion polymerase as described in protocol 2.2.6. Amplification of the promoter was completed using genomic DNA that was extracted as described in protocol 2.2.1. PCR of the backbone was completed using a stock solution at a concentration of 20 ng/µl of plasmid backbone DNA. All PCR products were then gel extracted as described in protocol 2.2.7.
4.4.2 Cloning the *Piwi1* Promoter

Blunt-end cloning of the promoter was then completed as described below:

The open vector was dephosphorylated as described in protocol 2.1.16 to minimise the chance of self-ligation. Ligation was performed as described in protocol 2.2.11 with the addition of 1 µl of polyethylene glycol (PEG). Bacteria transformation was then performed as described in protocol 2.2.12 using 50 µl of competent *Escherichia coli* XL1-Blue bacteria and grown on agar plates as described in protocol 2.2.14. Colonies were then tested by PCR using the primers Piwi1ProFw1New and GFPSeqFusRev which were located both inside and outside of the insert. This was done to make sure that the ligation was successful and was inserted in the correct direction. Positive colonies were then grown and extracted as described in protocol 2.2.10.

Once cloned the plasmid was sequenced to ensure the cloning worked correctly. After the sequence was verified the plasmid was injected as described in protocol 2.5. Injection of this plasmid produced low numbers of transgenic larva (Fig. 4.4.2.1). Larva were metamorphosed as described in protocol 2.1.2. After metamorphosis all young colonies lost their transgenic cells and were no longer fluorescent (Fig. 4.4.2.2). I continued injecting this plasmid for a number of weeks but the results remained the same. Based on studies showing a role for the 3’ UTR in translation efficiency, localisation and stability of mRNA (Barrett et al., 2012), I decided to replace the *Hydra Actin* 3’ UTR with the *Piwi1* 3’ UTR in the vector with the anticipation that this change would improve the stability of transgenic cells.
Fig. 4.4.2.1. *Piwi*+GFP transgenic larva using the *Hydra Actin* 3’ UTR. (A) Bright field (B) GFP (C) Merger

Fig. 4.4.2.2. *Piwi*+GFP transgenic colony using the *Actin* 3’ UTR. Only one transgenic cell remains after metamorphosis. (A) Bright field (B) GFP (C) Merger
4.5 *Piwi1* promoter expression vector plus the *Piwi1* 3’ UTR

The *Piwi1* 3’ UTR was cloned using sticky end cloning due to problems experienced while using blunt-end cloning techniques. This would make the cloning more efficient due to it being direction specific and it would also aid ligation. The contig that contained the 3’ portion of the *Piwi1* gene also contained a large portion of the 3’ UTR. I designed primers using this contig with the restriction sites *PacI* and *SacI* attached. I also designed primers with the same restriction sites attached to remove the *Hydra Actin* 3’ UTR from the vector. The primers used to amplify the 3’ UTR were Piwi1TerFw1-SAC1 and Piwi1TerRV1-PacI (Primer list 2.7). The primers used to open the backbone and removed the *Actin1* 3’UTR were TerminatorFw-PacI and gfpRv-SAC1 (Primer list 2.7).

Both sequences were PCR amplified using Phusion polymerase as described in protocol 2.2.6. PCR amplification of the promoter was completed using genomic DNA that was extracted as described in protocol 2.2.1 as template. Amplification of the backbone was completed using a plasmid backbone DNA stock solution at a concentration of 20 ng/µl. All PCR products were then Gel extracted as described in protocol 2.2.7. Insert and backbone were then restriction digested as described in protocol 2.2.15. The vector was dephosphorylated as described in protocol 2.2.16.
Sticky end cloning was completed as described below:

Ligation was performed as described in protocol 2.2.12. Bacteria transformation was then performed as described in protocol 2.2.13 using 50 µl of competent *Escherichia coli* XL1-Blue bacteria and grown on agar plates as described in protocol 2.2.14. Colonies were then tested by PCR using the primers LigDVectorGFP-Fusion and PBIGFor which were located on either side of the insert. This was done to ensure that the ligation was successful. Positive colonies were then grown and extracted as described in protocol 2.2.10. They were then sequenced to verify that the cloning was completed successfully. After the sequence was verified the plasmid was injected as described in protocol 2.5.

The new cassette contained GFP flanked by the *Hydractinia Piwi1* promoter and the *Hydractinia Piwi1* 3’ UTR (Fig. 4.5.1). The full vector map is shown in Fig. 4.5.2 and the full nucleotide sequence is listed in Fig. 4.5.3.

![Piwi1 Promoter - GFP - Piwi1 3’ UTR](image)

Fig. 4.5.1. Schematic showing the *Piwi1* promoter, GFP cassette containing the *Piwi1* 3’ UTR
Fig. 4.5.2. Schematic of the Piwi1 promoter expression vector used to create the Piwi1+GFP reported line.

- *Piwi1* Promoter – 2,534 bp
- *NotI* – 5’- GCGGCCGC – 3’
- GFP - 717 bp
- *Sac1* – 5’ – GAGCTC – 3’
- *Piwi1* 3’ UTR – 1120 bp
- *Pac1* – 5’ – TTAATTTA – 3’
- pBluescript backbone, 2,508 including B-lactamase-AMP 861 bp
Fig. 4.5.3. Sequence of Piwi1/GFP reporter line vector. Red = Piwi1 promoter, Green = GFP, Purple = -1 promoter, Black = -3 promoter, Yellow = GFP reporter line, ac1 = ac1 vector, ac2 = ac2 vector.
Injection of this plasmid initially showed no difference in *Hydractinia* larvae when compared to larvae injected with the construct containing the *Hydra Actin* 3’ UTR. After metamorphosis, however, the changes became obvious. Young colonies did not lose their transgenic cells as had happened in animals injected with the plasmid containing the *Hydra Actin* 3’ UTR. As colonies grew they produced new transgenic polyps (Fig. 4.5.4) and stolons (Fig. 4.5.4 – 4.5.6). The distribution of these transgenic cells in stolons and feeding polyps was consistent with the distribution of *Piwi1*+ cells observed by *in situ* hybridisation and immunostaining (Chapter 3). Cell morphology and distribution was observed using confocal microscopy (Fig. 4.5.5 – 4.5.6). Cells in feeding polyps were mostly confined to the aboral band though some cells were seen scattered throughout the polyp. Cells were more evenly distributed throughout the stolonal tissue and were less than 10 µm in diameter reflecting the known size of i-cells (Fig. 4.5.5). As colonies aged the expression pattern in some animals did change. Transgenic epithelial cells appeared and transgenic cells were seen in long tracts through some feeding polyps. *Piwi1* expression is never seen in epithelial cells by either *in situ* hybridisation or immunohistochemistry. The epithelial GFP cells could be explained by a number of different scenarios. Firstly, i-cells could be differentiating into epithelial cells. Transgenic animals were kept in the dark which may have delayed the degradation of GFP after cells were no longer *Piwi1*+ or possibly the promoter was incomplete, lacking some enhancers.
Fig. 4.5.4. *Piwi1-GFP*+ transgenic colonies (Scale bar 500 µm) (A) *Piwi1-GFP*+ expression in stolon (B-F) *Piwi1-GFP*+ expression in polyps of different colonies.
Fig. 4.5.5. Confocal images of Piwi1-GFP+ cells in stolons of transgenic colonies (20x lens, Scale bar 40 μm). (A',B') GFP (A'′, B'′) Merge of GFP and bright field.
Fig. 4.5.6. Confocal images of Piwi1-GFP+ cells in two stolons of transgenic colonies running side by side (60x lens, Scale bar 10 μm). (A’, B’) GFP (A’’, B’’) Merge of GFP and bright field.
4.6 Migration and Proliferation of Piwi1+ cells during regeneration

The next step was to investigate if these Piwi1+ cells were capable of migration during regeneration. Firstly I removed a number of transgenic polyps from a colony and photographed them under both blue light and bright field (Fig. 4.6.1 A). I then cut off the heads and then pictured the polyps every hour to see if Piwi1+ cells were appearing in the head region during regeneration (Fig. 4.6.1 B,C). Piwi1+ cells could be clearly seen in the regenerating head 24 HPD in an area that had been devoid of Piwi1+ before decapitation (Fig. 4.6.1 D). This was strong evidence that Piwi1+ cells were indeed present in the head during the first 24 hours of regeneration. To rule out dedifferentiation as a possible explanation for the appearance of the Piwi1+ cells I would need to be able to follow cells in vivo and track their movement from the aboral band to the regenerating head.

I repeated the regenerating experiment with transgenic polyps and this time created time-lapse movies on both a Deltavision deconvolution microscope and a spinning disk confocal microscope. By taking a picture every five minutes I was able to track individual cells moving from the aboral band post decapitation and migrating to the head (Fig. 4.6.2 A). This work provided evidence of the migration of Piwi1+ cells during regeneration while the results obtained during the EdU pulse chase experiments (Chapter 3) provided evidence that migrating cells were in fact proliferating during regeneration.

I was also able to capture cell proliferation (Fig. 4.6.2 B) during migration. Cells were seen to migrate from the aboral band towards the regenerating head. During migration a cell is clearly seen to stop for 25 minutes. It can then be seen dividing and once this has completed both cells continue migrating towards the regenerating head area.

A more detailed series of pictures showing the transgenic tracking in vivo of cells during migration and cell proliferation is shown in Fig. 4.6.3 and Fig. 4.6.4.
Fig. 4.6.1. Live images of a single *Piwi1*-GFP+ transgenic polyp during regeneration. Merge of bright field and GFP (green) (Scale bar A-C 400 µm, D 150 µm). (A) Isolated intact feeding polyp, *Piwi1*-GFP+ cells in a band below the head. (B) Polyp four HPD, *Piwi1*-GFP+ cells absent from regenerating tip. (C) Polyp 24 HPD, *Piwi1*-GFP+ cells now present in the regenerating tip (D) Close up of regenerating oral tip 24 HPD containing *Piwi1*-GFP+ cells.
Fig. 4.6.2. *In vivo* tracking of migrating *Piwi1*-GFP+ cells during regeneration (scale bar 40 μm). (A’) A single *Piwi1*-GFP+ cell four HPD (green circle). (A’’) The same cell five hours later having migrated towards the regenerating blastema (pictures taken using a Deltavision deconvolution microscope). (B’) A single cell having migrated from the aboral band towards regenerating blastema. (B’’) The same cell divides and both daughter cells continue migration towards the regenerating blastema (pictures taken using a spinning disk confocal microscope).
Fig. 4.6.3. Series of live pictures tracking a single transgenic cell in vivo over a five hour period in a regenerating polyp. White asterisk marks the oral pole, scale bar 20 µm. Pictures taken using a Deltavision deconvolution microscope.
Fig. 4.6.4. Series of live pictures tracking a single transgenic cell *in vivo* over a five hour period in a regenerating polyp. As the cell migrates orally it stops, proliferates and then both cells continue migration. White asterisk marks the oral pole, scale bar 250 μm. Pictures taken using an Olympus spinning disk confocal microscope.
4.7 Summary

The generation of a transgenic stem cell reporter animal was a major breakthrough in my research and enabled, for the first time, the tracking of single transgenic cells in vivo during *Hydractinia* regeneration. The EdU pulse chase experiments revealed the aboral migration of S-phase cells to the blastema but the type of cells and the genes they were expressing was unknown. Piwi proteins are expressed in the pluripotent stem cells of planarians, ctenophores, sponges and tunicates (Rinkevich et al., 2010; Alie et al., 2011; Millane et al., 2011; Lim et al., 2014). This expression of Piwi proteins in pluripotent stem cells allied to the change in Piwi1 expression seen during head regeneration in *Hydractinia* all pointed towards its suitability as a marker in a transgenic stem cell reporter line.

Interestingly the use of an Actin 3′ UTR within the vector resulted in animals losing their GFP expression after metamorphosis. It was only after the Actin 3′ UTR was replaced with the Piwi1 3′ UTR that the true expression patterns in both polyps and stolons was seen. Gene expression in stem cells is known to be tightly regulated (Wagers, 2012) but the reason why the presence of the Actin 3′ UTR resulted in the loss of transgenesis is unclear. One possibility is that the Piwi1 3′ UTR itself plays a role in regulating gene expression and with its inclusion this transgenic insert was recognised as Piwi1, yet without, it was targeted for down regulation.

The ability to follow transgenic cells in vivo is a powerful tool for studying regeneration and development in any animal model. Through the use of this reporter line I was able to show the migration of Piwi1+GFP cells during regeneration from the aboral i-cell band to the blastema. These cells proliferated and were contributing to the regeneration of the polyp. The *Hydractinia* model system, where stem cell behaviour can now be monitored in vivo at single cell resolution, offers amazing new opportunities in the study of regenerative biology.
Chapter 5. Aboral regeneration

5.1 Introduction

The ability of isolated *Hydractinia* polyps to regenerate stolons has been known since the early work of Morgan and Hazen in 1902 (Hazen, 1902). It was thought that only young feeding polyps possessed this regenerative power and that adult feeding polyps obeyed the rule of distal regeneration being able to regenerate new heads but not new stolons (Müller et al., 1986). There are many examples of the phenomenon of distal versus proximal regeneration. It is seen in the regeneration of limbs in axolotl (Kragl et al., 2009) and *Xenopus*, (Tseng et al., 2007) zebrafish tail and fin regeneration (Poss, Shen et al. 2000) and in head regeneration in both planarians (Reddien and Sanchez Alvarado, 2004) and *Hydra* (Galliot and Schmid, 2002; Bode, 2003). The ability of young *Hydractinia* polyps to regenerate stolons (proximally) had been previously described, yet the cellular and molecular mechanisms underlying this capability were not.
5.2 Polyp to Stolon Transformation

Polyps were isolated to study the long-term effect of separation from their colonies. Initially, the polyps healed the cut surface and fed as normal. This continued for a number of weeks where the polyps seemed unaffected by their removal from the colony. After a number of weeks the polyps began to retract their tentacles and they then elongated into long tube-like structures (Fig. 5.2.1 B-D). Tentacle retraction resulted in the polyps not being able to feed. However, they remained responsive to mechanical stimuli. After a period of between 2-5 weeks these tube like structures began to develop new feeding polyps (Fig. 5.2.1 F), and secrete chitin (Fig. 5.2.2 B). Finally, new sexual polyps appeared (Fig. 5.2.2 B, C). These new sexual polyps developed gonophores and spawned fertile gametes (Fig. 5.2.3). This phenomenon was seen in the majority of polyps but the time and sequence of events during transformation varied between different polyps. New polyps both sexual and feeding, plus the secretion of chitin strongly suggest that this new tube like tissue that appears during the polyp’s transformation is in fact stolonal tissue. This experiment was repeated over 100 times.

Fig. 5.2.1 Time course of a single isolated polyp transforming into a stolon and budding new polyps (Scale 400 μm) (A) Single polyp four hours post isolation (B) Polyp 10 days post isolation, tentacles begin to be resorbed (C) Polyp 13 days post isolation, tentacles almost fully resorbed (D) Polyp 18 days post isolation, no tentacles present (E) Polyp 20 days post isolation, polyp now transformed into stolonal tissue (F) Polyp 23 days post isolation, stolonal tissue now budding new polyps.
Fig. 5.2.2. New colonies derived from a single isolated polyp (Scale 400 µm). (A) New colony containing multiple feeding polyps (FP). (B) New colony containing feeding polyps (FP), chitin (CT) and a sexual polyp (SP). (C) New colony containing a feeding polyp (FP) and a sexual polyp (SP).

Fig. 5.2.3. Embryos derived from transformed male colony (Scale 100 µm).
5.3 I-cell Distribution in Colonies

To investigate further whether or not this newly formed tissue was in fact stolonal I performed in situ hybridisation using genes with known stolonal expression patterns and compared them to transformed colonies. I selected the i-cell markers *Piwi1*, *Pl10*, *Myc2*, and *Vasa* plus the early nematoblast differentiation marker *Ncol1* and the oral pole marker *Wnt3*. The genes *Piwi1*, *Pl10*, *Myc2*, *Vasa*, and *Ncol1* are all expressed differently in stolons than in polyps. In stolons, cells expressing these genes are evenly distributed throughout, while in polyps they form a distinct aboral band. The oral pole maker *Wnt3* is not highly expressed in stolons but is concentrated at the oral pole of polyps (Fig. 5.3.1).

Polyps were removed from their colonies, stored in petri dishes containing artificial seawater and fed and cleaned daily. Polyps transformed at different speeds so they were checked daily and when ready, fixed and stored in methanol at -20°C.

Primers for *Wnt3* were designed from the publicly available coding sequence, GenBank: AM279678.1. Templates for cRNA were designed as described in protocol 2.2.6 using the primers Wnt3ForwardPrimerT7, Wnt3ReversePrimerSP6 (Primer list 2.7). I used probes previously designed for *Piwi1*, *Pl10*, *Myc2*, *Vasa* and *Ncol1* as described in Chapter 3.
Fig. 5.3.1 Expression patterns of i-cell markers, Ncol1 and Wnt3 in stolons of young colonies. Animals attached to glass slides and pictures are taken from beneath (Scale 100 µm). (A) Piwi1 (B) Pl10 (C) Vasa (D) Ncol1 (E) Myc2 (F) Wnt3
5.4 Changes in Gene Expression during Polyp Transformation

*Wnt3* is expressed in hydrozoans at the oral tip of polyps and is known to be involved in the formation and maintenance of axis polarity in development and regeneration (Broun et al., 2005; Chera et al., 2009; Duffy et al., 2010; Galliot and Chera, 2010). Decapitation of feeding polyps results in the disappearance of the oral *Wnt3* spot. This *Wnt3* spot reappears within 24 hours after decapitation outlining the re-establishment of the oral pole (Duffy et al., 2010). During polyp to stolon transformation the expression of *Wnt3* is lost from the transforming polyp accompanying or preceding the disappearance of head structures (Fig. 5.4.1 A). *Wnt3* then reappeared at the oral tip of all new feeding polyps that grew from the newly transformed stolons (Fig. 5.4.1 B, C).

Changes were also seen in the expression of each i-cell marker and *Ncol1* consistent with their known expression patterns in stolons. These genes are all expressed in an aboral band in feeding polyps (Chapter 3) but are ubiquitous throughout the stolonal tissue in colonies (Fig. 5.3.1). Isolated polyps first expressed these genes in an aboral band as normal. As the polyps resorbed their tentacles and elongated the expression could be seen throughout the transformed tissue. As new polyps grew the genes re-established their original expression patterns being expressed in bands in new polyps and throughout new stolonal tissue Fig. 5.4.2 - 5.4.6.
Fig. 5.4.1. Loss and gain of polarity highlighted by Wnt3 expression in isolated polyps (Original head location indicated by asterisk, Scale 150 µm). (A) Wnt3 no longer expressed in the head. (B) Original polyp has transformed into stolonal tissue and has lost Wnt3 expression. New polyp expressing Wnt3 is growing from transformed tissue. (C) Two new polys both expressing Wnt3 at their oral tips growing from transformed tissue.
Fig. 5.4.2. Piwi1 expression in transformed polyps shown by in situ hybridisation (Scale 100 µm). (A) Piwi1 expression throughout transformed tissue. (B, C) Newly formed feeding polyps and sexual polyp in transformed colony. (D, E) Close up of individual Piwi1+ cells.
Fig. 5.4.3. Pl10 expression in transformed polyps shown by in situ hybridisation (Scale 100 µm). (A) Isolated polyp during first stages of transformation. Tentacles no longer present. (B) Pl10 expression now throughout transformed tissue. (C) Close up of Pl10+ cells in transformed stolonal tissue. (D, E) Expression pattern in newly formed polyp and stolonal tissue. (F) Close up of Pl10+ cells in transformed stolonal tissue.
Fig. 5.44. *Vasa* expression in transformed polyps shown by *in situ* hybridisation (Scale 100 μm). (A) *Vasa* expression now throughout transformed tissue. (B) Close up of individual *Vasa*+ cells in transformed tissue. (C) Expression pattern in newly formed polyp and stolonal tissue. (D) Close up of individual cells in transformed stolonal tissue. (E) Newly formed chitin in transformed stolonal tissue.
Fig. 5.4.5. *Myc2* expression in transformed polyps shown by *in situ* hybridisation (Scale 100 µm). (A) Isolated polyp during first stages of transformation. Tentacles almost fully absorbed. (B) *Myc2* expression now throughout transformed tissue. (C, D) Expression pattern in newly formed polyps and stolonal tissue. (E, F) Newly formed chitin in transformed stolonal tissue.
Fig. 5.4.6. *Nct1* expression in transformed polyps shown by *in situ* hybridisation (Scale 100 µm). (A-D) Expression pattern in newly formed polyp and stolonal tissue. (E) Newly formed chitin in transformed stolonal tissue. (F-G) Expression pattern in newly formed polyps and stolonal tissue.
5.5 Summary

The ability of isolated *Hydractinia* polyps to regenerate stolons was discovered by Morgan and Hazen in 1902 (Hazen, 1902). It was thought that only young feeding polyps possessed this regenerative power and that adult feeding polyps obeyed the rule of distal regeneration being able to regenerate new heads but not new stolons (Müller et al., 1986). I have shown that all *Hydractinia* polyps possess this regenerative power and any isolated feeding polyp has the ability to regenerate stolons.

Aboral regeneration in isolated *Hydractinia* polyps occurs in a completely different way and time scale to that observed in head regeneration. It does not involve an initial burst of proliferation or blastema formation when removed from the colony, but rather a transformation of tissue identity from polyp to stolon. Polyp to stolon transformation is preceded by the loss of Wnt3 expression and the anterior-posterior polarity of the polyp. This is followed by the reabsorption of tentacles and head structures and a shift in the restricted expression patterns of stem cell genes observed in polyps to the more ubiquitous expression observed in stolons. Once transformed the new stolonal tissue can generate a new colony, producing chitin and new feeding and sexual polyps regaining sexual competence. These results highlight the ability of isolated *Hydractinia* polyps to use two fundamentally distinct mechanisms when regenerating different body parts.
Chapter 6. Discussion

6.1 Introduction

Research into the development and regeneration of cnidarians has continued since the first experiments were completed by Trembley in 1744 (Trembley, 1744; Lenhoff, 1988). The vast majority of this work has been conducted on the hydroid Hydra and as such it is often used as a reference point for all cnidarian regeneration and development. Hydra and Hydractinia are both hydrozoans but many differences exist between the species. Hydractinia is marine, forms colonies, reproduces sexually and i-cells contribute to all cell lineages (Frank et al., 2001). Hydra on the other hand is freshwater, lives as single isolated polyps, has three separate cell lineages and reproduces mostly asexually (Technau and Steele, 2011). The regenerative ability of Hydractinia polyps was revealed through the early work of T.H. Morgan and others (Hazen, 1902) although the cellular and molecular mechanisms underlying this capability remained unclear. Whether head regeneration in Hydractinia was similar to Hydra and achieved through morphallaxis, or whether a different mode was employed was not yet known.
6.2 Head Regeneration in *Hydractinia* Feeding Polyps

The first experiments I completed were to assess the overall regenerative capacity of *Hydractinia* polyps. The initial response to both removal from the colony and decapitation was the same. Wounds were closed by stretching out of the epidermal epithelial cells and closure of both oral and aboral wounds was achieved within 4 to 6 HPD. Next, a dome shaped cap was observed at the regenerating oral tip between 24 to 48 HPD out of which tentacle buds began to grow. This was only observed at the regenerating oral tip and not the aboral. Animals were able to catch food and feed again between 48 and 72 HPD marking the complete regeneration of the decapitated head. Many different bisections were made at different points on the polyps and all cut sections displayed robust powers of regeneration.

This initial response to wounding in *Hydractinia* polyps, where decapitation is followed by wound closure, is similar to the response observed in both *Hydra* (Bode, 2003), planarians (Reddien and Sanchez Alvarado, 2004) and salamanders (Yokoyama, 2008). Decapitated *Hydra* can also regenerate within a similar time frame of three days (Galliot and Schmid, 2002) while planarian regeneration can take up to two weeks (Reddien and Sanchez Alvarado, 2004). Limb regeneration in salamanders is a longer process, taking up to two months depending on age (Yokoyama, 2008). These vastly different time frames for regeneration reflect both the size and complexity of the regenerating limbs or organs found in each animal. Planarians contain a central nervous system (CNS) plus simple eyespots (ocelli) in the head (Reddien and Sanchez Alvarado, 2004) while *Hydra* and *Hydractinia* contain a relatively simple head structure without a CNS (Brusca, 2003). Salamanders on the other hand are unable to regenerate a complete head and CNS although limb and partial brain regeneration is possible (Bader and Oberpriller, 1978). This regeneration in salamanders is quite complex and would include the reconstruction of new nerves.
and blood vessels and in the case of limbs new muscle and bone (Davis et al., 1989; Yokoyama, 2008).

Cnidarians do not possess a CNS (Brusca, 2003), but acetylated tubulin antibody staining did reveal the presence of a simple nervous system within intact feeding polyps similar to the nerve net observed in Nematostella (Marlow et al., 2009). Neurons were observed throughout the Hydractinia polyps but a large concentration of both neurons and nematocytes were found in the head, tentacles and in and around the mouth. For head regeneration to be completed this nervous system needs to be replaced post decapitation. Antibody staining at four, 24, and 48 HPD revealed the reconstruction of this system as the head was being regenerated. The first visible difference in nematocyte and neuron concentration post decapitation began at 24 hours in conjunction with the appearance of the dome shaped structure in the regenerating oral pole. As tentacle buds appeared and grew larger the concentration of neurons and nematocytes also increased enabling the animal to catch and consume prey.
6.3 Cell Proliferation is Essential for Head Regeneration

*Hydra* can regenerate after DNA synthesis has been blocked (Hicklin et al., 1975; Cummings and Bode, 1984) while planarians cannot (Reddien and Sanchez Alvarado, 2004), emphasising the role cell proliferation may or may not play in regeneration between different animals. Marking S-phase cells by EdU and anti pH3 antibodies revealed that the majority of proliferation in intact *Hydractinia* feeding polyps occurs in a band in the aboral region. Decapitation resulted in a second burst of proliferating cells which formed a blastema in the regenerating oral tip of the polyp 24 HPD (Fig. 6.9B). Non-decapitated isolated polyps did not produce a second band of proliferation within the first 48 hours, while no differences were observed in non-decapitated polyps exposed to EdU while attached or isolated from their colonies. This showed that decapitation rather than isolation from the colony was the sole cause of this second band of proliferation.

This clear response to decapitation resembled the regeneration blastema present in regenerating planarians (Reddien and Sanchez Alvarado, 2004), annelids (Bely, 2014), echinoderms (Carnevali, 2006) and vertebrates (Bader and Oberpriller, 1978). Exposure to γ-irradiation or the use of cytostatic drugs such as mitomycin-C and hydroxyurea have all been used to cause cell cycle arrest in both *Hydra* (Hicklin et al., 1975; Cummings and Bode, 1984) and planarians (Reddien and Sanchez Alvarado, 2004; Reddien et al., 2005). *Hydractinia* polyps were not capable of regenerating after DNA synthesis was blocked using either γ- irradiation or mitomycin-C (Fig. 6.3) reflecting result seen in planarians (Nakagawa et al., 2012) but not in *Hydra* where regeneration is possible after proliferation is blocked (Hicklin et al., 1975; Cummings and Bode, 1984).

Two clear results were obtained from these experiments: first, decapitated polyps produced a second band of proliferation at the site of regeneration and secondly, proliferation was essentially to regeneration in *Hydractinia*. The type of cells that were
proliferating and whether their origin at the blastema was a result of migration or trans/de-differentiation of local cells was still unknown.

Fig. 6.3. Irradiation of isolated feeding polyps blocked both proliferation (red dots) and regeneration. After irradiation and decapitation DNA synthesis stops and no blastema forms at the oral tip. Polyps did not regenerate and eventually died 2-3 weeks post decapitation (PD)
6.4 The role of i-cell proliferation during regeneration

All cell types in adult *Hydra* derive from three distinct stem cell populations, epidermal and gastrodermal epithelial and i-cell (Galliot and Schmid, 2002; Holstein et al., 2003). This is not the case in *Hydractinia* where it is thought that i-cells are capable of creating all cell types both epithelial and others (Kunzel et al., 2010; Plickert et al., 2012). *Hydra* epithelial cells have been shown to cycle every 3-4 days. Cells can take between 12 - 15 hours to complete S-phase with about 25% in S-phase at any one time. This is in contrast to i-cells which cycle faster taking between 16 - 27 hours. S-phase lasts between 9 - 13 hours with about 50% of i-cells in S-phase at any one time (Buzgariu et al., 2014). The exact cell cycle length of both epithelial and i-cells in *Hydractinia* is unknown. While using EdU to mark cells in S-phase over a three hour period, I found that a total of 5.4% epithelial and 19.5% non-epithelial (i.e. i-cells, nematoblasts, gland cells) cells were EdU+. The numbers were quite similar in regenerating polyp’s observer 24 HPD. Here 5% epithelial and 20% non-epithelial EdU+ cells were observed showing no real difference in the number or type of cells in S-phase between intact and 24 hour regenerating polyps. *Hydra* cells can take up to 15 hours to complete S-phase. If this is also the case in *Hydractinia*, exposing them to EdU for three hours probably underestimates the number of S-phases cells during regeneration. Polyps would need to be exposed to EdU for longer time periods to get a truer estimation of the amount of epithelial and i-cells that are entering S-phase. Exposing polyps to EdU for longer time frames can cause problems when identifying and counting cells. Cells may begin a second round of proliferation causing an overestimation or cells may differentiate into different cell types, retaining the EdU and causing incorrect identification of proliferating cells. For this experiment I only wanted to observe which cell types were proliferating and contributing to head regeneration and to investigate if the numbers and cell types changed when compared to intact polyps. The majority of EdU+ cells 24HPD were in fact non-epithelial. As this
is the point of blastemal formation and is a crucial time point for the regeneration of a polyp head it would indicate that the majority of the cells contributing to head regeneration are in fact i-cells and not epithelial.

While S-phase in *Hydra* is quite long, mitosis is much quicker and can take as little as 90 minutes. By using an anti pH3 antibody I was able to mark all cells in mitosis both in intact and regenerating polyps 24 HPD. The pH3 antibody marks cells during mitosis and as such there is far less pH3+ cells in a polyp when compared to a three hour EdU exposure. For this experiment I counted all pH3+ cells and recorded how many were epithelial and how many were i-cells. I observed that at any one time over 95% of mitotic cells in intact *Hydractinia* polyps were in fact i-cells and that epithelial cells accounted for as little as 2.7%. Again similar numbers were observed in polyps 24 HPD showing no difference between regenerating and intact polyps. Of the 161 pH3+ cells counted in regenerating polyps only 6 were epithelial cells. These results along with the results obtained during the EdU experiment outline the essential contribution of i-cells to proliferation and head regeneration in *Hydractinia*. They also reveal that unlike in *Hydra*, epithelial cell proliferation is insignificant during head regeneration. That said it is clear that epithelial cells do proliferation but the exact role they play during both regeneration and normal homeostasis remains unknown.
6.5 Gene expression during Regeneration

The i-cell marker gene *Vasa* has previously been shown to be expressed in an aboral band in *Hydractinia* feeding polyps (Rebscher et al., 2008) reflecting the location of S-phase and mitotic cells. *In situ* hybridisation of other known *Hydractinia* stem cell markers *Piwi1, Myc2, Pl10* and the early nematoblast differentiation marker *Ncol1* revealed that they were also expressed in the same area in intact polyps. Decapitation resulted in a change in gene expression patterns reflecting the change observed in proliferating cells. Post decapitation, cells expressing these genes were now found in the oral section of the polyps. The strongest expression was seen 24 HPD at the blastema in the regenerating tip. This second band of expression was only present 24-48 HPD and disappeared at the early stages of tentacle formation. Interestingly, the genes *Myc2* and *Ncol1* both appeared to be expressed within the blastema at the anterior tip while *Pl10* and *Vasa* were both expressed more strongly lower in the blastema or just outside it. *Piwi1* expression was weakest and was never expressed at the anterior tip being generally found in single cells just below the blastema. The different patterns in gene expression may reflect changes in expression during cell differentiation. *Myc2* and *Ncol1* being expressed in cells during late stages of differentiation while *Piwi1* is expressed in early stages.

Double *in situ* hybridisation on pairs of these genes could have revealed a pattern of change in gene expression during differentiation. While many attempts were made I was unable to successfully construct a working protocol to complete double *in situ* hybridisation with these genes.
6.6 Knockdown of Ncol1 plus i-cell genes inhibits regeneration

Exposure to 500 Gy $\gamma$- irradiation in Hydractinia polyps, before decapitation, resulted in an almost total loss of expression of Piwi1, Myc2, Pl10 and Ncol1 throughout the polyp. As previously discussed, exposure at this level also blocks proliferation and regeneration. Similar results are seen in planarians (Reddien et al., 2005) where exposure to high levels of $\gamma$ irradiation also blocks proliferation and regeneration, and results in the down regulation of known neoblast markers including the Piwi homologs Smed-piwi-1, 2 and 3. The use of RNAi as a tool to knockdown genes in Hydractinia is now well established (Duffy et al., 2010; Millane et al., 2011). Knockdown of Piwi1, Vasa, Pl10 and Ncol1 using RNAi all resulted in a significant delay in head regeneration. The formation of a blastema or the distribution of proliferating cells at the regenerating tip, however was unaffected after knockdown. This was shown by EdU labelling and no differences in S-phase cells at the blastema could be observed 24 HPD between control RNAi treated polyps versus Pl10, Piwi1, Vasa and Ncol1 RNAi treated ones.

Hydra contains two Piwi homologs called Hydra PIWI (Hywi) and Hydra PIWI-like (Hyli). Both genes are co-expressed in all Hydra stem/progenitor cells and knockdown of Hywi in epithelial cells has been shown to be lethal (Juliano et al., 2014). Knockdown of Smedwi-2 in planarians results in a similar phenotype seen in Hydractinia where regeneration but not blastema formation is blocked (Reddien et al., 2005). In contrast knockdown of the stem cell gene Sox2 in axolotls does inhibit proliferation. This results in spinal-cord regeneration failure in amputated tails although the mesodermal blastema does form normally (Fei et al., 2014). The tunicate Botrylloides leachi also fails to regenerate after knockdown of a Piwi homolog (Bl-Piwi) (Rinkevich et al., 2010), while knockdown in Drosophila melanogaster (Li et al., 2009), Mus musculus (Carmell et al., 2007), Danio rerio (Houwing et al., 2008) and Caenorhabitis elegans (Wang and Reinke, 2008) all result in sterility.
After decapitation the appearance of cells in the head expressing Ncol1 plus the i-cell markers Piwi1, Pl10, Vasa and Myc-2 outline the important role these genes play in regeneration. Knockdown of these genes significantly delayed head regeneration but did not affect blastema formation or proliferation. This would suggest that these genes might play a role in differentiation rather than proliferation or maintaining an undifferentiated state. Intact feeding polyps normally only express these genes aborally and their presence at the blastema is a direct result of amputation. As Hydractinia i-cells are migratory, the most likely source of these new cells at the blastema is from the migration of stem cells from the aboral band. However, trans/dedifferentiation of cells already present at the blastema, as observed in the closely related hydrozoan Podocoryna carnea (Schmid and Reber-Müller, 1995), could not be ruled out.
6.7 Stem Cell Migration during Regeneration

The tracking of migrating cells was possible through the use of both EdU pulse-chase labelling experiments and the tracking in vivo of transgenic cells. Proliferation in intact feeding polyps is mainly reserved to the aboral band so any EdU uptake at this time point would be attributed to cells located aborally. Cells labelled using EdU prior to decapitation were then visualised at later time points revealing their new position if migrating. The pattern of EdU+ cells four HPD was quite similar to that seen in intact polyps in that it was largely located aborally, but some cells were located more orally. The biggest changes were observed 24-48 HPD where the accumulation of EdU+ cells in the head labelled 24-48 hours previously was clearly revealing their migratory behaviour. This EdU pulse-chase method of tracking cells revealed similar changes in neoblast movement during rostrum (anterior) regeneration in planarians. In intact planarians, neoblast proliferation is restricted to the body column posterior of the brain. After decapitation EdU+ cells were shown to move into the regenerating rostrum using this pulse-chase approach (Dirks et al., 2012).

The establishment of a transgenic stem cell reporter line would enable us to track individual cells and follow any possible migration in vivo. The Hydractinia pluripotency gene, Polynem (Pln), was potentially a suitable marker to use. However, attempts to clone its promoter and establish a Pln reporter line were unsuccessful. In situ hybridisation had revealed that cells expressing Piwi1 were present in the regenerating head 24 HPD while the results obtained by Piwi1 immunohistochemistry in tandem with EdU revealed that at least some Piwi1+ cells were proliferating 10 HPD. The results I previously outlined, the effect of Piwi knockdown in many other animal groups plus the use of Piwi homologs as stem cell makers in planarians (Reddien et al., 2005; Nakagawa et al., 2012) all indicated that it would be a good candidate i-cell marker.
The establishment of a Piwi1+GFP transgenic reporter line was successful. As is consistent with this method of transgenesis, genomic integration of the Piwi1 transgene did not occur in all cells after injection. This in effect means that the animals were mosaics and not all Piwi1+ i-cells expressed GFP. Cells in feeding polyps were mostly confined to the aboral band though some cells were seen scattered throughout the polyp. Cells were more evenly distributed throughout the stolonal tissue and were less than 10 µm in diameter reflecting the known size of i-cells (Frank et al., 2009; Plickert et al., 2012). The distribution of these transgenic cells in stolons and feeding polyps was consistent with the distribution of Piwi1+ cells observed by in situ hybridisation and immunostaining. Post decapitation, this powerful new tool enabled me to track individual Piwi1+ cells in vivo during their migration from the aboral band to the regenerating head. I also observed Piwi1-GFP+ cells proliferating during migration. This process of migrating Piwi+ cells has also been observed during planarian head regeneration where neoblasts migrate to the site of injury after decapitation, proliferate and contribute to the regenerating tissue (Reddien and Sanchez Alvarado, 2004; Reddien et al., 2005; Wenemoser and Reddien, 2010). This data plus the results obtained from the EdU pulse-chase labelling experiment enabled me to confidently conclude that stem cells were migrating from the aboral band to the site of regeneration and contributing to head regeneration in Hydractinia. Although migration was occurring, the possibility that local cell trans/dedifferentiation also contributed to head regeneration could not entirely be ruled out.

Variation in the width of the i-cell band was observed between feeding polyps of different ages and size taken from adult colonies (sexually mature). This variation was observed using both in situ hybridization and EdU experiments. Within smaller and younger polyps, the distance between the oral most boundary point and the head was less obvious than in older and longer polyps. As polyps aged and grew, the boundary position became more pronounced and defined, while the distance between the head and aboral band became greater. During regeneration experiments, the exact point of
decapitation was not always the same due to the natural variation in polyp size and the difficulties that exist while working with such small animals. Because of this the distance between the regenerating head and the i-cell band was not always the same. No differences were observed in the head regeneration time of polyps of different sizes taken from adult colonies which all regenerated within 48 and 72 hours. However, polyps taken from the much younger transgenic colonies regenerated at a much faster rate, often within 24 hours. These transgenic colonies were only a few weeks or months old, sexually immature and the polyps were much smaller than the ones used during the in situ and EdU experiments. This faster rate of regeneration could be due to the smaller distance between the i-cell band and the regenerating head within these polyps. If the migrating i-cells needed to travel a much smaller distance before proliferation and differentiation it may account for the quicker regeneration times observed in these colonies.
6.8 Aboral regeneration: Proximal vers Distal

The ability of isolated *Hydractinia* polyps to regenerate stolons has been known since the early work of Morgan and Hazen in 1902 (Hazen, 1902). It was thought that only young feeding polyps possessed this regenerative power and that adult feeding polyps obeyed the rule of distal regeneration being able to regenerate new heads but not new stolons (Müller et al., 1986). This ability to regenerate stolons was previously described, but the cellular and molecular mechanisms underlying this capability were not.

By isolating adult *Hydractinia* feeding polyps I was able to show that this ability to regenerate stolons is present in both young and adult feeding polyps. Surprisingly, the steps taken during aboral (stolon) regeneration were fundamentally different than the ones observed during head regeneration. Head regeneration in polyps begins post decapitation with the closing of the wound and the migration of stem cells from the aboral band. These stem cells migrate to the regenerating oral tissue where they proliferate and establish a blastema out of which the new head will form. The regeneration of aboral structures was completely different and occurred over a longer time period. Isolated feeding polyps did not regenerate stolons directly and did not appear to generate a blastema at the aboral cut surface within the first 72 hours. Polyps removed from their colony showed no visible change in proliferation patterns using either EdU or an anti pH3 antibody and it was only after decapitation that a blastema was formed at the oral pole. Over time, isolated polyps reabsorbed both tentacles and head and appeared to transform into stolonal tissue. Branches appeared at irregular intervals out of which new feeding and sexual polyps grew. The latter produced fertile gametes. Chitin, which is stolon specific, was also secreted and eventually whole new colonies developed from one transformed feeding polyp.

In cnidarians, the establishment of the anterior posterior axis is thought to involve components of the canonical Wnt signalling pathway (Muller et al., 2004b; Plickert et
Gardiner et al., 2006; Teo et al., 2006). *Hydractinia Wnt3* is an established oral marker in feeding polyps but it is also expressed weakly in the aboral band (Plickert et al., 2006). *In situ* hybridisation of *Wnt* in transforming polyps revealed that they were losing *Wnt3* expression at the oral pole while the tentacles and head were reabsorbed (Fig. 6.8A-B). As new feeding polyps grew out of this transformed tissue *Wnt3* reappeared orally in the new polyps. It has previously been shown that in *Hydractinia*, Wnt signalling promotes oral structures but represses stolons (Duffy et al., 2010). Knockdown of *Wnt3* or *Tcf* in decapitated polyps produced similar phenotypes seen in polyp to stolon transformation but over a much shorter time scales (Duffy et al., 2010). The disappearance of *Wnt3* would suggest that the polyps were losing oral-aboral polarity enabling the change in tissue identity from polyp to stolon. As new polyps appeared, they expressed *Wnt3* orally showing that the oral-aboral polarity and polyp identity had been re-established.

*In situ* hybridisation of the known stem cell markers *Piwi1, Vasa, Pl10* and *Myc2* plus the early nematoblast differentiation marker *Ncol1* also revealed changes in expression patterns as feeding polyps transform into stolonal tissue. These genes are largely restricted to the aboral band in feeding polyps but in stolons they are found equally distributed throughout the tissue. As isolated polyps began transforming the expression pattern of these genes changed and became less restricted, eventually being expressed throughout the transformed tissue. As new polyps grew from this transformed tissue the polyp-type expression pattern was re-established (Fig. 6.8 C).

The polyp-stolon transformation enables isolated feeding polyps to develop new sexually competent colonies. The secretion of chitin and the presence of fertile gametes after transformation highlight the ability of feeding polyps to produce tissue and structures not normally present in feeding polyps. Whether this transformation is enabled by the present of pluripotent i-cells or several lineage restricted progenitors remains unclear but my results show that fundamentally distinct mechanisms exist in *Hydractinia* when regenerating different body parts.
Fig. 6.8. The stages in polyp to colony transformation. (A) Single isolated polyps express Wnt3 orally and stem cell markers aborally in a band. (B) As polyps change, tentacles are reabsorbed, Wnt3 expression is lost in the head and stem cell markers begin being expressed throughout the polyp. (C) New stolon tissue now expressed stem cell markers throughout the new tissue. (D) When new polyps emerge from the transformed tissue they again express Wnt3 orally while restricting stem cell genes aborally in a band.
6.9 Concluding Remarks

Morphallaxis or epimorphosis? The remarkable ability of *Hydra* to regenerate when DNA synthesis is blocked (Hicklin et al., 1975; Cummings and Bode, 1984) is often used as proof of morphallactic regeneration in this animal. The traditional classification of regeneration as being dichotomic; i.e. either through morphallaxis or epimorphosis, is becoming less and less clear (Sanchez Alvarado, 2000). DNA syntheses clearly plays a role in *Hydra* head regeneration (Chera et al., 2011) while both regeneration modes are employed at different points during planarian regeneration (Reddien and Sanchez Alvarado, 2004). *Hydractinia* polyps cannot regenerate after DNA synthesis is blocked. In this sense *Hydractinia* regenerate at least to some extent by epimorphosis, though the use of morphallaxis during the transformation of polyps into stolons cannot be excluded. The differences displayed between oral and aboral regeneration highlight the ability of *Hydractinia* polyps to employ two distinct mechanisms while regenerating different structures (Fig. 6.9). The ability of feeding polyps to regenerate whole new colonies and viable gametes also points to the presence of pluripotent i-cells within feeding polyps.

Cnidarians occupy a pivotal position within the tree of life. As basal invertebrates, they are classified within the Metazoa but excluded from the Bilateria. The use of a blastema in regeneration has long been described in many different taxa throughout the Bilateria (Sanchez Alvarado, 2000). *Hydra*, has been the reference point for cnidarian regeneration for centuries but does not regenerate using a blastema (Bode, 2003). The fact that *Hydractinia*, a cnidarian hydroid as well, uses a blastema to regenerate distal structures is an exciting revelation and would suggest the blastema as an evolutionarily conserved mechanism for regeneration within the Metazoa. Whether or not this is true and these mechanism employed in cnidarians and planarians were lost in *Hydra* and in non-regenerating species is unclear but the fact that many closely related species show vastly different powers of regeneration can
make answering such questions difficult. The idea that such powers would be selected against might seem unlikely while the presence of pluripotent stem cells throughout an animal’s lifetime would initially seem advantageous. These advantages in regeneration may in turn result in other problems related to cancer and uncontrolled cell proliferation. The presence or absence of pluripotent stem cells plus the level of developmental complexity of a species would all seem important to its regenerative potential. It can take up to 3 months for a salamander to regenerate a lost limb (Yokoyama, 2008). Even if complete head regeneration was possible, the fact that the animal would be incapacitated, unable to feed or defend itself for such long periods, would obviously negate any advantage head regeneration might give. Cnidarians and planarians being smaller in size and less complex can regenerate in much shorter times counteracting problems that such long regeneration times displayed in salamanders might produce (Reddien and Sanchez Alvarado, 2004).

Whether the mechanisms employed during metazoan regeneration are evolutionarily conserved or not remains unknown. The uses of a blastema in different groups and the fact that not all animals possess the same powers of regeneration could be explained by convergent evolution and that each group developed the ability independently. Further studies into the underlying and shared mechanisms involved in regeneration within different groups would help unlock these questions. The study of regeneration in a basal species like Hydractinia echinata can help shed light on these questions. The ability to study regeneration at a single cell level in this animal in vivo is an exciting new development and can help unlock some of the most difficult questions related to regeneration in higher animals including humans.
Fig. 6.9. Schematic outlining the regenerative processes and location of proliferating i-cells during *Hydractinia* polyp regeneration. (A) *Hydractinia* colony, outlining the location of i-cells in stolons, feeding and sexual polyps (red dots). (B) Regeneration in an isolated feeding polyp. Following decapitation stem cells migrate to the head where they proliferate and differentiate forming a blastema. After regeneration is complete, i-cell proliferation is again restricted to the aboral band. (C) Aboral regeneration. Isolated feeding polyps first transform into a stolon out of which new feeding and sexual polyps can grow.
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