Development of transgenic and genome editing tools to study the role of Notch signalling in *Hydractinia echinata*

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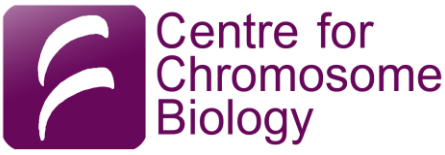

Table of contents

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List of abbreviations

ASW	Artificial Sea Water
AP	Alkaline Phosphatase
BCIP	5-bromo-4-chloro-3’-indolyphosphate
BSA	Bovine Serum Albumin
DAPI	4',6-diamidino-2-phenylindole
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycinet-butylester
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
HPF	Hours Post Fertilisation
HR	Homologous Recombination
LB	Lysogeny Broth
NBT	Nitro Blue Tetrazolium
NICD	Notch IntraCellular Domain
NLS	Nuclear Localisation Signal
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.1% Tween 20
PBSTE	Phosphate Buffered Saline with 0.3% Triton X-100 and 10 mM EDTA
PBSTx	Phosphate Buffered Saline with 0.3% Triton X-100
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
SSC	Saline Sodium Citrate
TEA	Triethylamine
TM	Transmembrane
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
WISH	Whole mount RNA *In Situ* Hybridisation

Abstract

Hydractinia is a colony forming marine invertebrate and a member of the phylum Cnidaria. Members of this genus have been models for developmental biologists for over a century and feature many characters of evolutionary significance, most notably a stem cell system called i-cells, the evolution of which is not well understood. *Hydractinia* is still a relatively new genetic model organism and so nearly any work involves the development of new tools. Here I present novel transgenesis tools in *Hydractinia* and discuss the implications of the successes and failures of this work on future work in *Hydractinia*. In particular I present, for the first time, genome editing via CRISPR-Cas9 in *Hydractinia*. Using this tool as well as classical pharmacological inhibition experiments I analysed the role of Notch signalling in *Hydractinia*. I show that Notch signalling plays a role in tentacle patterning, a function conserved across studied cnidarians. Additionally, I show a role for Notch signalling in nematocyte differentiation in *Hydractinia*, again a function which is conserved among cnidarians. Most interestingly, I show that Notch signalling is not required for early nervous system development or neural commitment in *Hydractinia*, a feature which apparently emerged within hydrozoans, as anthozoans and most studied metazoans appear to use Notch signalling in a similar manner during neurogenesis. I further discuss this loss in terms of the evolution of i-cells in hydrozoans and hypothesise that loss of Notch signalling during neurogenesis would have been a major event which facilitated the evolution of i-cells by removing the requirement for neural progenitors to emerge and exist in an epithelial context.

Declaration

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

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Signed: _____

James Gahan, September 2016

Chapter 1. Introduction

1.1. Cnidaria

The phylum Cnidaria is the sister to group to all bilatarian animals. It is one of four phyla often referred to as “basal" or "early diverging" animals; the other three being Ctenophora (comb jellies), Porifera (sponges) and Placozoa. Although the evolutionary position of the other three groups is controversial and has been the subject of much debate (Ryan et al., 2013, Moroz et al., 2014, Borowiec et al., 2015, Pisani et al., 2015, Whelan et al., 2015, Halanych et al., 2016, Pisani et al., 2016) the placement of the Cnidaria as the sister group to the Bilateria is well supported (Hejnol et al., 2009, Pick et al., 2010).

The Cnidaria are broadly divided into two clades: the Anthozoa and the Medusozoa (Bridge et al., 1995). The anthozoans are further sub-grouped into two monophyletic clades: Hexacorallia (e.g. anemones and scleractinians) and Octocorallia (e.g. soft corals). The medusozoans have more complex sub-divisions. There are considered to be four clades within the Medusozoa: Staurozoa (stalked jellyfish), Scyphozoa (true jellyfish e.g. *Aurelia*), Cubozoa (Box jellyfish), which form a monophyletic group, and Hydrozoa (*Hydra*, *Clytia*, *Hydractinia*) (Zapata et al., 2015) (Fig. 1.1A). Also of interest are the myxozoans which are now placed within the Cnidaria as a sister group to medusozoans along with *Polypodium*. Both *Polypodium* and the myxozoans are parasitic and have undergone various degrees of gene loss, most notably in the Myxozoa (Chang et al., 2015).

Almost all cnidarian life cycles contain a polyp stage and a planula larva stage (Technau and Steele, 2011). Medusozoans, as the name suggests, are characterised by the additional presence of a medusa although not all medusozoans contain a medusa stage and it may have been lost and gained several times in different lineages, independently (Govindarajan et al., 2006). Cnidarians exhibit various life history modalities. Most are capable of asexual reproduction; some grow as colonies while others exist as solitary polyps (Technau and Steele, 2011).

Although cnidarians exhibit relatively simple morphology, they are known to have a much more complex repertoire of protein coding genes than initially hypothesised (Kortschak et al., 2003). Many genomes and transcriptomes are now available for

species across the phylum. These data sets show clearly that all major signalling pathways and transcription factor families involved in bilatarian development are conserved within the Cnidaria (Putnam et al., 2007, Chapman et al., 2010, Baumgarten et al., 2015). In addition cnidarians possess all genes necessary for small RNA (miRNA, piRNA, siRNA) mediated gene regulation (Moran et al., 2013a, Moran et al., 2014) and exhibit a bilaterian like gene regulatory landscape (Schwaiger et al., 2014). This complex genetic repertoire, in the face of seemly simple body plans makes cnidarian biology an exciting avenue to understand early animal evolution. However, although it is true that the basic body plan and morphology of cnidarians is simple, that is not to say they do not exhibit exiting and complex evolutionary novelties. Cubozoan jellyfish, for example, evolved an eye independently of bilaterians (Piatigorsky and Kozmik, 2004). Most notably, the cnidarians are characterised by the almost ubiquitous presence of a cnidocyst, a highly complex evolutionary innovation that is the main defining character of the phylum (discussed in detail in later sections) (Babonis and Martindale, 2014).

1.1.1. Cnidarian model organisms

Cnidarian models have been used for many decades across many different disciplines. Although I will focus here on models in developmental/cell biology many cnidarians are used as model for symbiosis and environmental science including corals and anemones such as *Aiptasia* (Baumgarten et al., 2015).

Within the Anthozoa two major models have been used namely *Acropora* species and *Nematostella vectensis* although *Nematostella* has predominated in recent years due to its experimental accessibility (Technau and Steele, 2011, Layden et al., 2016b). Within the Hydrozoa both *Hydra* and *Hydractinia* have been established models for developmental biology, immunity, stem cell and regenerative biology (Frank et al., 2001, Chapman et al., 2010). Other models such as *Clytia hemespherica* and *Podocoryne carnea* have also been utilised (Technau and Steele, 2011).

Although *Hydra* has been classically the vastly predominant cnidarian model organism its use has been hampered in recent years as it reproduces sexually only rarely and is not accessible during embryonic development. Many modern genetic techniques, with the exception of transgenesis, established in other cnidarians, have

lagged behind in *Hydra*. *Nematostella*, on the other hand, has undergone a massive rise with large numbers of researchers turning to the model due to its ease of culture as well as the presence of established techniques (Layden et al., 2016b). *Nematostella* researchers have led the way in terms of the implementation of genetic interference tools most notable genome editing via CRISPR/Cas9 (Ikmi et al., 2014).

Nematostella has predominantly been studied during development as, until recently, it was only possible to perform gain and loss of function experiments during embryogenesis. *Hydractinia*, therefore, fills a niche as a medusozoan model which can be manipulated during embryogenesis and used in a comparative manner along with *Nematostella.*

1.1.2. *Hydractinia* as a model organism

Hydractinia has been a model organism for over a century (Weismann, 1883). It has had a small but productive community of researchers for many decades, now working principally on developmental and stem cell biology, allorecognition as well as comparative genomics. The European species, *Hydractinia echinata*, has predominated in developmental studies while its North American cousin *Hydractinia symbiolongicarpus* has been well established as a model for allorecognition (Frank et al., 2001, Mokady and Buss, 1996, Rosa et al., 2010, Karadge et al., 2015). The use of two species is a result of the geographical location of researchers rather that any biological difference. In fact until 1989 (Buss and Yund, 1989) it was not yet established that they were different species.

Hydractinia is a colony forming member of the class Hydrozoa. Its adult form grows mainly on the shells of hermit crabs. The adult colony consists of two distinct compartments: the stolon and the polyp (Fig. 1.1B-C). There are several polyp types which facilitate a division of labour. The most predominant polyp type is the gastrozooid or feeding polyp. Each colony is either male or female and therefore contains male or female gonozoids or sexual polyps. Additionally there are two defensive polyp types called tentaculozooids and dactylozooids (Frank et al., 2001, Plickert et al., 2012) (Fig. 1.1C).

The feeding polyp, the basic unit of most adult cnidarians, has a long cylindrical body column and a “head” consisting of a hypostome or mouth and tentacles. All the

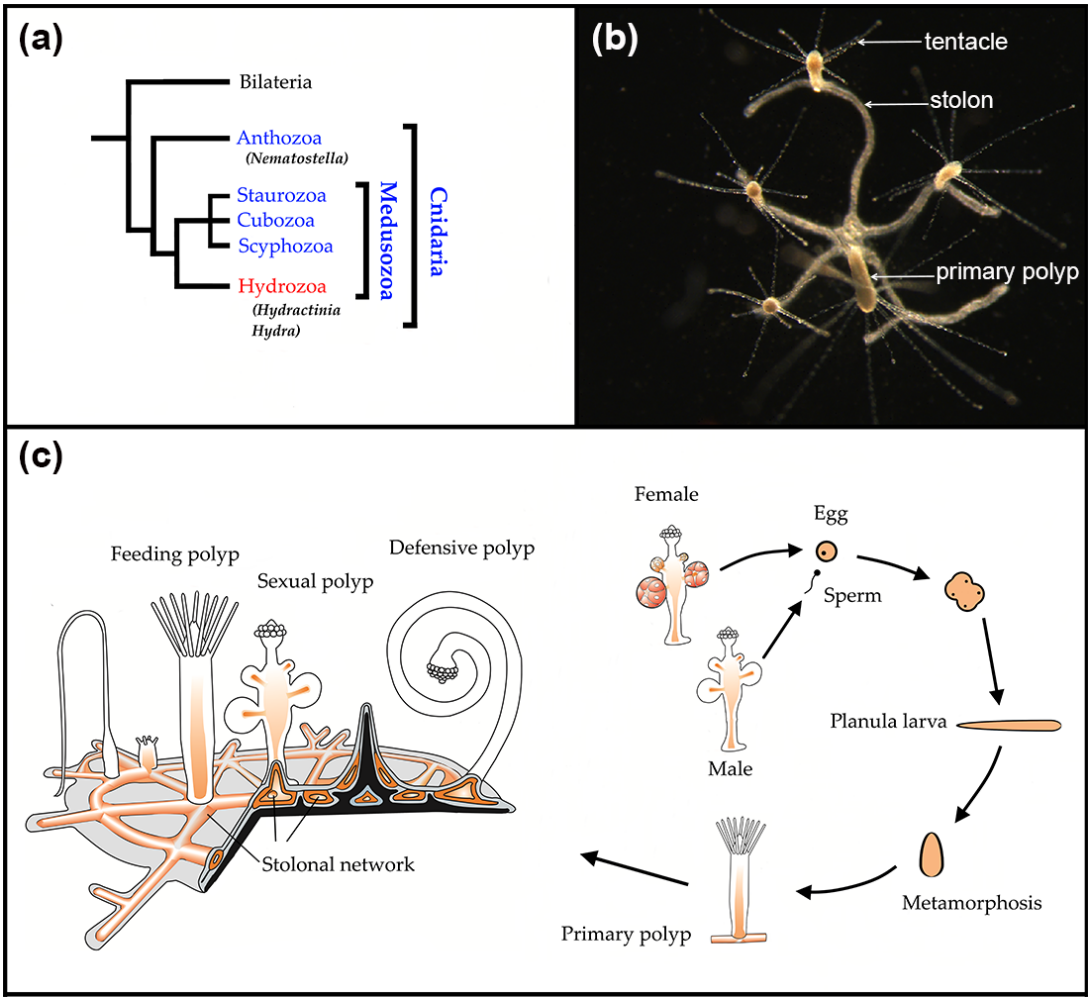


Figure 1.1: *Hydractinia echinata*. (A) Schematic representation of the phylogneetic relationships of different cnidarian clades. (B) 1 week old *Hydractinia echinata* colony. (C) Illustration of the *Hydractinia* life cycle and colony structure (Gahan et al., 2016).

polyps are connected by a gastrovascular network of stolons which are attached to the substrate and secrete a protective chitin layer.

Hydractinia reproduces both sexually and asexually (Fig. 1.1C). Eggs and sperm are released into the water column by sexual polyps and fertilisation occurs shortly thereafter. The embryo then undergoes a number of reductive cleavages to produce a transient blastula. Gastrulation in *Hydractinia* is atypical and involves an apolar delamination. Gastrulation produces a pre-planula which then elongates along the oral-aboral axis and after 48-72 hours a planula larva is formed which is competent to metamorphose (Kraus et al., 2014). Metamorphosis in the wild in induced by bacteria but can be stimulated in the lab by the addition of caesium chloride (Frank et al., 2001). Metamorphosis takes about 24 hours and gives rise to a single feeding

polyp with a number of stolons, the primary polyp. These stolons grow and give rise to new feeding polyps (Fig. 1.1B). This mode of asexual reproduction occurs for a number of months before sexual reproduction commences. From this point onwards the two modes of reproduction occur simultaneously (Frank et al., 2001, Plickert et al., 2012).

Hydractinia, like all cnidarians, are diploblastic animals and following gastrulation consist of only two germ layers: ectoderm and endoderm (sometimes referred to an endo-mesoderm). The adult animals contains two epithelial layers the epidermis and gastrodermis. These layers are sometimes referred to as the ectoderm and endoderm, respectively, but this is incorrect as, at least in hydrozoans, both layers contain cells derived from both germ layers. In addition to epithelial cells *Hydractinia* also contains gland cells, neural cells (neurons and nematocytes or stinging cells) and their progenitors (Plickert et al., 2012). The composition and structure of the nervous system is discussed in detail below.

Hydractinia, like other hydrozoans, contains a population of stem/progenitor cells called i-cells (Müller et al., 2004) (Fig. 1.2). I-cells have been most extensively studied in *Hydra*. I-cells are relatively small cells with large nuclear to cytoplasmic ratios and prominent nucleoli (Fig. 1.2C, D). I-cells are commonly highly proliferative, migratory and express genes associated with bilatarian stem cells and germ cells such as *Piwi* genes (Fig. 1.2A). I-cells in *Hydra* are multipotent, giving rise to gland cells, neural cells and germ cells, but not to epithelial cells (David and Murphy, 1977, Bosch and David, 1987). I-cells in *Hydractinia*, by contrast, are thought to be pluripotent and can additionally give rise to epithelial cells (Kunzel et al., 2010, Müller et al., 2004) (Fig. 1.2E). Importantly, the lineage potential of *Hydractinia* i-cells has not been studied clonally. In *Hydra* there is extensive evidence for i-cell heterogeneity including cells with limited proliferative capacity which are committed to the neuronal lineage (Hager and David, 1997, Holstein and David, 1986). Based on gene expression analysis there are likely bipotent i-cells which can give rise to both neurons and nematocytes (Miljkovic-Licina et al., 2007). There are also reports of i-cells committed to the germ cell lineage (Littlefield and Bode, 1986, Nishimiya-Fujisawa and Sugiyama, 1993, Littlefield, 1991). Most intriguingly, it was recently reported that there is a population of quiescent i-cells in *Hydra* which can be re-activated during regeneration (Govindasamy et al., 2014).

Similarly, the neoblast population in planarians, once thought to be homogenous, is now known to contain many sub-populations with limited differentiation capacity (Scimone et al., 2014, van Wolfswinkel et al., 2014). Although it has not been shown experimentally it is parsimonious to think the i-cell population in *Hydractinia* may be similar, with the pluripotent cells representing a minority of the total population and the remainder of the cells constituting either transit amplifying or long term committed, lineage specific progenitors (Gahan et al., 2016).

Hydractinia is capable of regenerating both distally and proximally, i.e. a polyp can regenerate a decapitated head and isolated polyps can regenerate a colony. The two modes of regeneration however, occur through staggeringly different mechanisms. Following decapitation and wound closure head regeneration proceeds with the recruitment of i-cells to the prospective head and formation of a proliferative blastema (Fig. 1.2B). The proliferation within the blastema is essential for regeneration. On the other hand an isolated polyp undergoes a transformative process where the entire polyp transforms into stolon tissue before budding polyps and re-establishing a colony (Bradshaw et al., 2015).

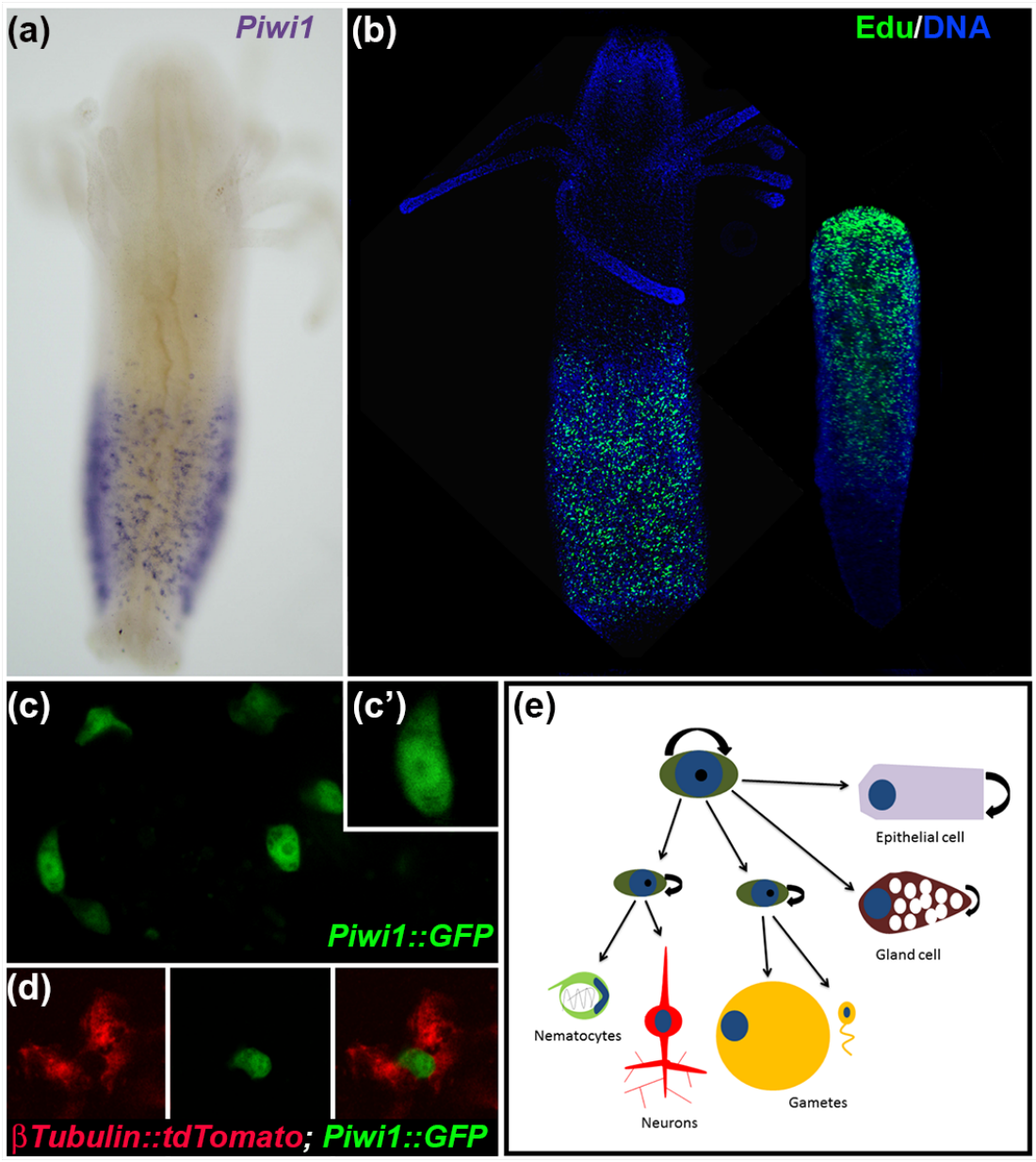


Figure 1.2: I-cells. (A) *Piwi1* in-situ hybridisation on an adult polyp. (B) Edu staining on polyps before and 24 hours post decapitation. (C, C'). GFP^{+} i-cells in a *Piwi1*::*GFP* transgenic animal. (D) GFP+ i-cell in the interstitial spaces between epithelial cells in a *Piwi1*::*GFP, Btubulin*::*tdTomato* transgenic stolon. (E) Hypothetical stem cell hierarchy in *Hydractinia* (Gahan et al., 2016).

1.2. The evolution of neurogenesis

The evolution of the nervous system in animals has been an intensively studied area for many decades. Many questions prevail: How did neurons first arise and what was their ancestral function? How were the earliest nervous systems organised? How did a "simple" early nervous system evolve to give rise to the complex central nervous system (CNS) of vertebrates? Here I will primarily address the evolution of early or developmental neurogenesis with a particular focus on early branching phyla as this is most pertinent to this study.

The origin of the nervous system is controversial. Cnidarians have neurons organised as a nerve net, as do ctenophores. However, sponges and placazoans have no neurons altogether (Galliot et al., 2009, Galliot and Quiquand, 2011, Marlow and Arendt, 2014, Moroz et al., 2014). In past years, when ctenophores and cnidarians were thought to be sister groups in a single phylum (the Coelenterata), it was thought that the nervous system evolved in the coelenterate-bilaterian last common ancestor. The recent placement of ctenophores as the earliest branching animal phyla complicates the discussion, raising two alternative hypotheses: The first is that a nervous system was already present in the common ancestor of all extant animals and was secondarily lost in both the Porifera and Placozoa. The second is that nervous systems evolved in Ctenophora and in the cnidarian-bilaterian ancestor, independently (Ryan et al., 2013, Ryan, 2014, Marlow and Arendt, 2014, Moroz et al., 2014, Moroz, 2015, Moroz and Kohn, 2016).

Regardless of the origin of the ctenophore nervous system it is widely accepted that cnidarian and bilaterian nervous systems are homologous. This positions cnidarians as reference, or outgroup, for uncovering the structure and molecular determinants of the nervous system of the common bilatarian ancestor (urbilatarian). How was the nervous system organised? What genes and pathways were involved in patterning and cell commitment? Using comparative research between cnidarians and bilaterians we can address these questions.

1.2.1. The cnidarian nervous system

1.2.1.1. Organisation of the nervous system

As mentioned above the cnidarian nervous system consists of two broad categories of cells: neurons and cnidocytes (stinging cells), with each category encompassing several cell types. This should be taken into consideration when discussing the organisation and structure of the nervous system as cnidocytes are also intrinsically connected into the neuronal network (Westfall et al., 1971, Westfall, 2004).

In all cnidarians the nervous system consists of both epithelial sensory cells and ganglionic neurons which are located basally and project neurites along the mesoglea (Fautin and Mariscal, 1991, Lesh-Laurie and Suchy, 1991, Thomas and Edwards, 1991). Within these two broad categories many neural subtypes can be identified, based on expression of different marker genes (Nakanishi et al., 2011, Marlow et al., 2009, Kelava et al., 2015). Commonly used among these are neuropeptides, like RFamide and GLWamide, as well as neurotransmitters such as GABA (Marlow et al., 2009, Galliot et al., 2009, Galliot and Quiquand, 2011, Kelava et al., 2015). It is generally considered that the cnidarian nervous system is organised as a nerve net, generally lacking centralisation (Hejnol and Rentzsch, 2015, Galliot et al., 2009). However, some degree of centralisation can be argued in medusa stages, particularly in Cubozoa (Mackie, 2004, Satterlie, 2011). In many cnidarians there are regional differences in the concentration of neurons. In hydrozoans both larval and adult life stages have different neuronal subtypes located either orally or aborally (Plickert et al., 2003, Kanska and Frank, 2013, Piraino et al., 2011).

In *Hydra* it has been shown that, independent of marker gene expression, the concentration of total neurons is higher both in the oral and aboral poles, i.e. in the head and foot (Koizumi et al., 1990, Koizumi, 2007). In the anthozoan *Nematostella* many studies using specific markers have found regional difference in neuron concentration. However, more recent works using broader neural markers in transgenic reporter lines reported a rather ubiquitous neuronal distribution along the polyp oral-aboral axis (Layden et al., 2016b, Marlow et al., 2009, Watanabe et al., 2014, Kelava et al., 2015).

Cnidarian neurons, in all classes, are known to utilise both neuropeptides as well as classical chemical neuro-transmission (Kass-Simon and Pierobon, 2007). Key to

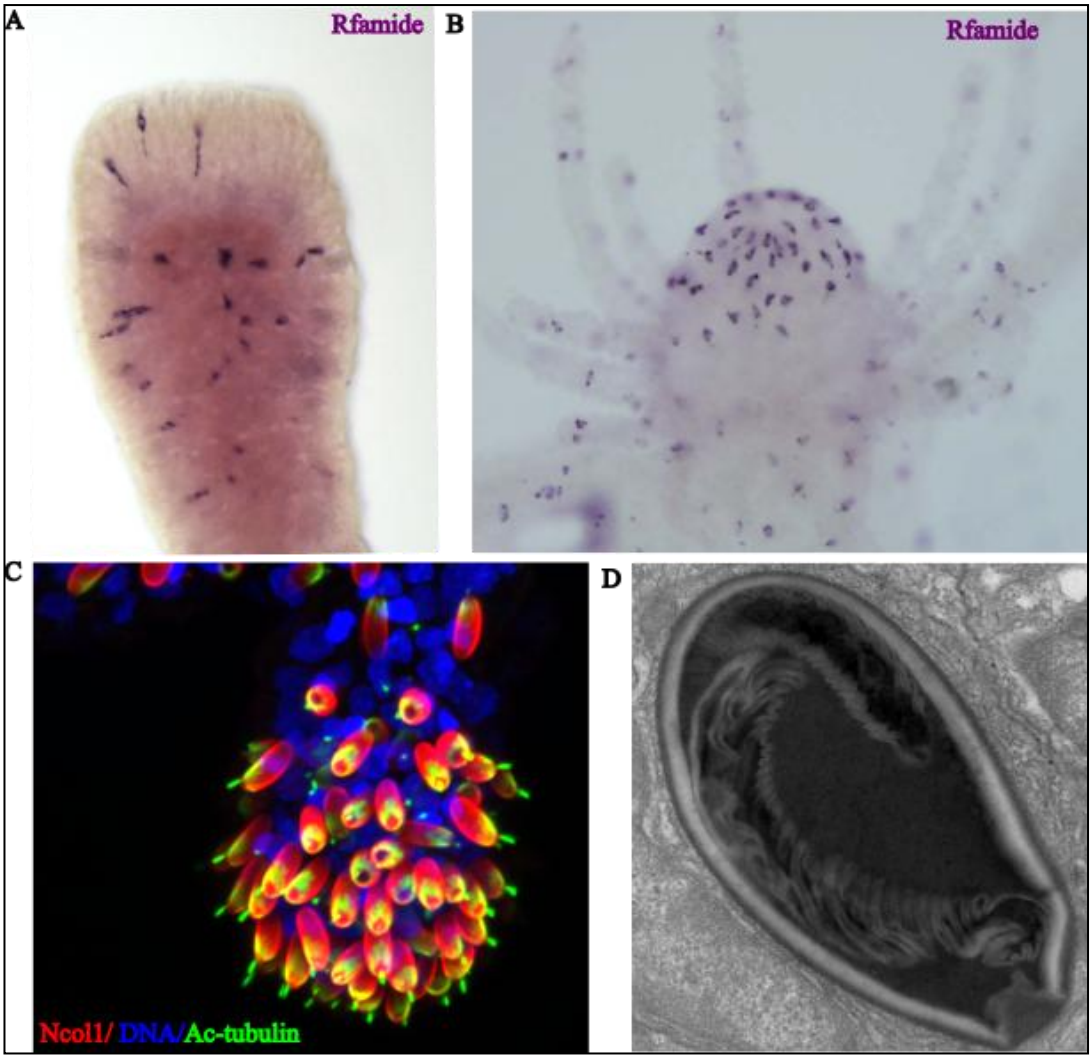


Figure 1.3: Neural cells in *Hydractinia*. (A) *Rfamide*$^{+}$ cells, shown by ISH, in the aboral domain of the planula larva. (B) Three day old primary polyp showing the *Rfamide*$^{+}$ oral nerve ring (ISH). (C) Regenerating tentacle showing nematocyst capsules (red), and cnidocils (green). (D) TEM image of a developing nematocyst (K. Thompson unpublished).

understanding the structure of cnidarian nerve nets is the question of neural polarity. It is still not clear whether truly polarized neurons occur in cnidarians (Rolls and Jegla, 2015). There is ample evidence for bi-directional chemical synapses (Anderson, 1985, Anderson and Grünert, 1988) but other evidence suggest the presence of polarised synapses (Westfall et al., 1971, Anderson and Spencer, 1989). Certain complex neural functions, particularly in medusae, would also suggest the presence of neuronal polarity (Rolls and Jegla, 2015, Satterlie, 2011).

The nervous system of *Hydractinia* first appears around 24 hours post fertilisation (hpf), although it should be noted that detailed studies with neural progenitor markers are lacking. Electron microscopy shows the first appearance of nematoblasts within the larval endoderm at this stage (Kraus et al., 2014). In addition, the first reported expression of RFamide and GLWamide occurs at 24 and 28 hpf, respectively (Plickert et al., 2003). The majority of GLWamide and RFamide positive neurons are located aborally in the larva (Schmich et al., 1998a) (Fig. 1.3A). During metamorphosis neuronal cell death occurs and this then leads to the reformation, at least partially de-novo, of the nervous system of the primary polyp (Seipp et al., 2010, Seipp et al., 2006). GLWamide positive neurons first appear in the gastrodermis in the oral part of the primary polyp and later in the oral epidermis, below the tentacle line (Schmich et al., 1998b). RFamide positive neurons are concentrated as an oral nerve ring in the epidermis of adult polyps (Kanska and Frank, 2013) (Fig. 1.3B). Although Rfamide positive neurons are not located in the stolons, GLWamide positive neurons are sometimes seen at the polyp-stolon boundary where they form a ring (Plickert et al., 2003).

1.2.1.2. Cnidocytes

Cnidocytes are a neural cell type present only in cnidarians and represent an evolutionary novelty (Babonis and Martindale, 2014, Brinkmann et al., 1996, Miljkovic-Licina et al., 2007). They are mechanosensory cells with both sensory and effector roles (Pantin, 1942), and are utilised primarily for prey capture and defence. There are many types of cnidocytes which can be clade specific. These include nematocytes (found across the Cnidaria), spirocytes (which are found only in anthozoans) and ptychocytes (found in tube building anemones) (Özbek et al., 2009). Nematocytes are the most abundant type of cnidocyte with >30 different types (Östman, 2000). There also appears to be an expansion of nematocyte complexity within the hydrozoan lineage in comparison to other clades (Kass-Simon and Scappaticci, 2002). Nematocytes are the most diverse and widely studied subtype of cnidocyte and therefore I will focus from here specifically on nematocytes. All cnidocytes have a specialised organelle called a cnidocyst or nematocyst in nematocytes. In addition, myxozoans, although highly reduced, contain structures called polar capsules which are now thought to be homologous to cnidocytes (Shpirer et al., 2014).

Cnidocytes form a large percentage of the cells found in adult cnidarians. Hydrozoan nematocytes are derived from i-cells (David and Gierer, 1974, Bosch and David, 1987, David and Murphy, 1977, Miljkovic-Licina et al., 2007, Denker et al., 2008b). In *Hydra* they account for 25% of the total cells with 40% of the total cells in some stage of nematocyte differentiation (David and Gierer, 1974). Committed cells form clusters which proliferate and differentiate synchronously, a process which is facilitated by the presence of cytoplasmic bridges (Fawcett et al., 1959). In *Hydractinia* such clusters do not occur (unpublished data). In *Hydra*, nematogenesis occurs uniformly along the body column whereas in *Hydractinia* early nematogenesis is restricted to the base of the body column (Kanska and Frank, 2013, Beckmann and Özbek, 2012). Similarly, in *Clytia* tentacle bulbs, different phases of nematocyte differentiation are spatially separated (Denker et al., 2008b). Some evidence suggests that *Nematostella* nematogenesis occurs at the site where a nematocyte is needed (Babonis and Martindale, 2014). The majority of nematocytes are found in the adult tentacle (Fig. 1.3C).

The mature nematocyst consists of a capsule with an invaginated internal tubule, often covered with spines (Fig. 1.3D). The nematocyst is capable of explosive exocytosis (Holstein and Tardent, 1984), reaching huge speeds and expelling both its contents as well as the tubule which can be used to harpoon prey or to cause harm in the context of defence or competition. Firing is thought to involve both mechanical and chemical stimuli (Kass-Simon and Scappaticci, 2002). The nematocyst is connected to an extracellular facing cnidocil (Hausmann and Holstein, 1985a, Hausmann and Holstein, 1985b) (Fig. 1.3C). The cnidocil consists of a modified cilium surrounded by a symmetric ring of short microvilli and an asymmetric ring of stereocilia (Hausmann and Holstein, 1985a). This structure is similar to the arrangement found in hair cells in the vertebrate lateral line and ear (Hausmann and Holstein, 1985b) and is thought to be crucial for the mechanical component of sensing.

The nematocyst develops from a specialised post-Golgi vesicle (Özbek et al., 2009). It consists of two components: a capsule and a tubule. The development of the nematocyst has been studied widely on the biochemical level and a detailed picture is beginning to emerge. Components of the nematocyst are shuttled through the secretory pathway to the growing vesicle. The process of tubulation occurs later and

is spread into 4 stages. It begins with an apical protrusion of the growing vesicle. This then extends via the addition of new vesicles, supported by microtubules. The tubule then undergoes a process of invagination followed by further maturation and compaction (Özbek et al., 2009, Adamczyk et al., 2010, Hwang et al., 2010, Beckmann and Özbek, 2012). Full maturation of the capsule wall only proceeds after tubule invagination (Engel et al., 2001).

The first and best studied components of the nematocyst were the minicollagens (Kurz et al., 1991, David and Gaub, 1994, Lenhoff et al., 1957, David et al., 2008). This family consists of many unusually short collagen related proteins found not only in nematocysts but also spirocysts (Zenkert et al., 2011) and possibly in polar capsules (Shpirer et al., 2014). Three of these proteins Ncol1 (nematocyst collagen 1), Ncol3 and Ncol4 have been localised to the capsule wall in one or more cnidarian species (Engel et al., 2001, Zenkert et al., 2011, Beckmann and Özbek, 2012) (Fig. 1.3C). They have been hypothesized to provide tensile strength to the capsule, allowing for the speed of discharge (David and Gaub, 1994). As discharge involves radical changes in nematocyst volume (Holstein and Tardent, 1984) it was hypothesized that an elastic component was necessary within the capsule and this was recently shown to be the silk like protein cnidoin (Balasubramanian et al., 2012, Beckmann et al., 2015). Another minicollagen, Ncol15, so far only known to be present in *Hydra*, forms part of the tubule (Adamczyk et al., 2008). Interestingly, both Ncol15 and another tubule component, spinalin, are found in large globular aggregates within the developing matrix during tubule elongation and are only added to the tubule following invagination (Koch et al., 1998, Adamczyk et al., 2008). Evidence suggests that lectin type proteins may form scaffolds on the developing tubule and be important for timing and assembly of tubule components (Özbek, 2011). A non sulphated chondroitin was shown to act as a scaffold during the early stages of tubule formation (Yamada et al., 2007, Adamczyk et al., 2010). Multiple splice variants of the lectin like protein Nematogalectin are also located along the tubule (Hwang et al., 2010) and a lectin like domain containing protein, NOWA, is also expressed in nematocytes during capsule development in *Hydra* (Engel et al., 2002).

The evolution of the nematocyte has long interested researchers, particularly the acquisition of the genes involved in nematocyst formation. Although there are

examples of prokaryotic contributions via lateral gene transfer (Denker et al., 2008a), an endosymbiont origin of nematocysts has been discredited by proteome analysis (Balasubramanian et al., 2012). Proteomic and transcriptomic analyses of nematocysts and nematocytes, respectively, have revealed that many genes involved in nematocyst formation are cnidarian specific (Moran et al., 2013b, Milde et al., 2009, Hwang et al., 2007, Balasubramanian et al., 2012) and have complex genomic organisation and splicing (Milde et al., 2009). Based on proteomic analysis it is hypothesised that nematocytes have evolved from a venom secreting cell (Balasubramanian et al., 2012).

1.2.1.3. Neural development

In most studied animals the nervous system derives from the ectodermal lineage. In cnidarians, however, neural cells either arise from both ectoderm and endoderm, as is the case in some anthozoans (Marlow et al., 2009, Nakanishi et al., 2011), or only from the endoderm, as in hydrozoans (Galliot et al., 2009). In fact, in hydrozoan cnidarians, as well as in acoels and planarians, neural cells arise from migratory non-ectodermal cells (Hartenstein and Stollewerk, 2015). In some animals, such as *Nematostella* and hemichordates, the entire ectoderm is primed for a neural fate, while in vertebrates a specifically defined portion of ectoderm, termed neuroectoderm, gives rise to the central nervous system (Hartenstein and Stollewerk, 2015). In *Hydractinia* the first neural progenitors (Kraus et al., 2014) as well as expression of neural progenitor genes (Flici, unpublished, Kanska and Frank, 2013) (unpublished results) begins within the endoderm, suggesting that the nervous system is endodermal in origin, as previously suggested to be the case for all hydrozoans, but lineage tracing has not been performed yet.

Neural development has, until recently, only been addressed in hydrozoans. In *Hydra* and *Hydractinia*, both neurons and nematocytes are derived from i-cells (Bosch and David, 1987, Müller et al., 2004, Khalturin et al., 2007, Kunzel et al., 2010). A neuron and nematocyte specific i-cell has been suggested to exist in *Hydra* based on gene expression data (Miljkovic-Licina et al., 2007), but its long-term self-renewal capability is unknown. Recent lineage tracing data in *Nematostella* has shown that both neurons and cnidocytes derive from a common pool of proliferating *NvSoxB(2)* positive cells (Richards and Rentzsch, 2014). It is, however, not known if this

population is heterogeneous, i.e. containing separate neural and cnidocyte progenitors, or if it contains multipotent cells giving rise to both neurons and nematocytes.

1.2.2. Molecular control of neurogenesis

The molecular pathways controlling neurogenesis in cnidarians is perhaps the most interesting avenue of study. Does the cnidarian nervous system arise through differentiation pathways controlled by the same mechanisms as in bilatarians, or are there major differences? Is the cnidarian nervous system pattern by conserved or divergent mechanisms? Are there major differences in the molecular regulation of neurogenesis within different cnidarian clades? Below I summarise what is known to date about the molecular mechanisms at play during cnidarian neurogenesis.

1.2.2.1. Role of secreted signalling factors

Several groups of secreted signalling molecules play conserved roles in patterning the bilatarian nervous system, most notably components of the BMP and Wnt signalling pathways as well as other growth factors (Pera et al., 2014).

The BMP signalling pathway is involved in patterning the embryonic nervous system of both protostome and deuterostome bilatarians. The role of BMP signalling in neural induction is best studied in *Xenopus* where secreted BMP ligands act to suppress neural induction and complex interplay between expressed ligands and antagonists pattern the embryonic nervous system (Pera et al., 2014, De Robertis, 2009, Ozair et al., 2013, De Robertis, 2008). In cnidarians, the BMP signalling pathway has only been studied functionally in *Nematostella*. BMP signalling has been shown to play a role in patterning in *Nematostella*, specifically of the directive axis. However, modulation of BMP signalling does not affect the embryonic nervous system. Inhibition of BMP signalling does affect later larval neurogenesis in a dose dependent manner but the extent to which this is due to more general patterning defects rather than directly effecting neurogenesis is unclear (Saina et al., 2009, Watanabe et al., 2014, Layden et al., 2016b).

Canonical Wnt signalling is involved in many facets of both embryonic and adult neurogenesis. It is involved in patterning the nervous system through modulation of BMP signalling as well as through BMP independent mechanisms (Baker et al.,

1999, Patapoutian and Reichardt, 2000, Megason and McMahon, 2002). In addition, Wnt signalling plays various roles in the maintenance of neural progenitor identity, proliferation and differentiation in vertebrates (Megason and McMahon, 2002, Hirabayashi et al., 2004, Valenta et al., 2011, Zechner et al., 2003). This function of Wnt signalling is also conserved in protostomes (Demilly et al., 2013). In *Hydractinia*, ectopic activation of Wnt signalling leads to an increase in RFamide positive neurons and nematocysts in the primary polyp (Teo et al., 2006). Similarly, disruption of Wnt signalling in *Nematostella* leads to a decrease in neural gene expression and ectopic activation of Wnt signalling leads to increased expression of neural genes (Marlow et al., 2013, Watanabe et al., 2014). As is the case with BMP signalling, however, it is unclear whether these effects are direct or due to more broad patterning defects. Modulation of Wnt signalling affects the size or the oral domain in both *Hydractinia* and *Nematostella* and given the concentration of neurons at the oral pole this may be the cause of the increased number of neurons. In addition, activation of canonical Wnt signalling causes a major increase in i-cell proliferation in *Hydractinia* and this could also lead indirectly to an increase number of differentiated progeny, i.e. neural cells. In *Hydra* it has been proposed that expression of a Wnt antagonist, *Dickkopf*, could down regulate Wnt signalling in the body column and allow neuronal differentiation to occur (Guder et al., 2006).

In addition, MAPK signalling has many known roles in neurogenesis across phyla (Hurtado and De Robertis, 2007, Ozair et al., 2013) and recent evidence suggests that it may be required for neural induction in *Nematostella* (Layden et al., 2016a).

1.2.2.2. Neurogenic transcription factors

It is impossible to discuss the molecular regulation of neurogenesis without discussing the role of conserved transcription factor families in both the maintenance and establishment of neural identity and neural differentiation. Foremost among these are the SoxB family and the proneural basic helix-loop-helix (bHLH) proteins.

The Sox family constitutes a group of evolutionarily conserved HMG-box proteins which are divided into many subfamilies. The SoxB family is the best studied subfamily with regards to its role in neurogenesis. However, proteins in other subfamilies also have various roles in the nervous system (Guth and Wegner, 2008). The SoxB family in bilatarians, particularly in vertebrates, can be broadly divided

into 2 groups: the SoxB1 genes and the SoxB2 genes. In pre-bilaterian lineages, on the other hand, it has been shown that many species, particularly cnidarians, have multiple SoxB genes that are not orthologous with specific bilaterian SoxB genes. Furthermore, even the relationships between individual cnidarian SoxB genes is not clear, suggesting lineage-specific ramification of Sox genes (Schnitzler et al., 2014, Jager et al., 2006, Shinzato et al., 2008, Jager et al., 2011). In vertebrates, SoxB1 genes are involved in the maintenance of neural progenitor cells and act to inhibit neural differentiation (Holmberg et al., 2008, Graham et al., 2003, Bylund et al., 2003). SoxB2 genes, on the other hand, have been shown to promote neural differentiation, counteracting the effect of SoxB1 genes (Sandberg et al., 2005).

Sox genes have been studies in a number of cnidarian species although functional studies exist in just two, *Nematostella* (Richards and Rentzsch, 2014, Richards and Rentzsch, 2015) and *Hydractinia* (Flici, unpublished). In *Clytia*, SoxB genes, as well as other Sox genes, are expressed in putative neural progenitors (Jager et al., 2011). In *Nematostella*, two SoxB genes have been studied functionally. *NvSoxB2a* knockdown leads to a block in oral neurogenesis but does not lead to an aberrant effect on *Elav* positive neurons suggesting it plays a role in a specific subset of neurons (Watanabe et al., 2014). Another SoxB gene, *NvSoxB(2)*, has been shown to be expressed in neural progenitors and its knockdown leads to a loss of both neurons and cnidocytes (Richards and Rentzsch, 2014, Richards and Rentzsch, 2015). In *Hydractinia*, knockdown of two SoxB genes, *SoxBI* and *SoxBII*, has been shown to reduce the expression of neural markers in adult polyps during homeostasis and regeneration, and overexpression of *SoxBII* during embryogenesis leads to an increased number of neurons, nematocytes and ectopic nematoblasts in the ectoderm. *SoxBI* ectopic expression is embryonic lethal (Flici, unpublished). All these studies show that SoxB genes play roles in the development and maintenance of cnidarian nervous systems although it is as yet unclear how these roles relate to the roles of SoxB genes in bilaterians.

The proneural bHLH genes are a large family of genes with many roles in the nervous system (Bertrand et al., 2002). Most notably they promote neural differentiation, partially by inhibiting the expression of SoxB1 genes (Bylund et al., 2003) and promoting SoxB2 gene expression (Sandberg et al., 2005). One such class of genes are the *Achaete-scute* homologs *(Ash)*. There are 4 *Ash* homologs in

Nematostella, and one, *NvAshA*, has been studied functionally. Knockdown of *NvAshA* leads to a loss of neural markers whereas overexpression leads to an increase in neural marker gene expression but only within their normal domains (Layden et al., 2012). It has also been shown that *NvAshA* targets represent genes expressed in multiple neural subtypes (Layden et al., 2016a). Interestingly, although *NvAshA* is co-expressed with *NvSoxB(2)* in some cells, it is not expressed in proliferating cells and it is also co-expressed with later neural markers (Richards and Rentzsch, 2015, Layden et al., 2012). This suggests that *NvAshA* may act at a later stage of neurogenesis than SoxB genes, at least in *Nematostella*. One *Ash* homolog has also been studied in *Hydra* where it has been shown to be expressed in putative neural progenitors as well as differentiated neural cells (Hayakawa et al., 2004, Grens et al., 1995). An *Ash* homolog has also been reported to be expressed in progenitor type cells in *Podocoryne* (Seipel et al., 2004) and *Hydractinia* (Kanska, 2012).

Another proneural bHLH gene, related to Atonal like genes, *NvAth-like (NvArp3)* has also been shown to be important in *Nematostella* neurogenesis. It is expressed in a sub-population of proliferating, *NvSoxB(2)* positive cells and is never co-expressed with *NvAshA* suggesting that it may be involved in an early stage of neurogenesis (Richards and Rentzsch, 2015). Additionally, knockdown experiments confirmed that it is required for nervous system development (Richards and Rentzsch, 2015, Watanabe et al., 2014)

1.3. The Notch signalling pathway

The Notch signalling pathway is a juxtacrine signalling system which is conserved across animal phyla (Gazave et al., 2009). Both the Notch receptor and ligand are single pass transmembrane proteins, and their interaction induces the release of the Notch intracellular domain (NICD). The NICD then translocates to the nucleus and interacts with a transcription factor from the CSL family (CBF1/RBP-J, Su (H), Lag-1) family and induces activation of target gene expression such as the Hes (Hairy/enhancer of spit) family of transcription factors. The physiological roles of Notch signalling are diverse and it plays roles in cell fate and commitment, differentiation, proliferation, apoptosis and migration. Due to the rather unusual nature of the Notch pathway, where both ligand and receptor are membrane bound, effective signal transduction requires direct cell-cell contact.

1.3.1. Mechanistic insights

1.3.1.1. Receptor activation

The Notch receptor is a single pass transmembrane protein. It is translated as a single open reading frame and is subsequently proteolytically cleaved by Furin-like convertases (S1 cleavage site) and then re-assembled as a heterodimer consisting of the NICD and TMECD (transmembrane and extracellular domain). It is then transported to the cell membrane (Kopan and Ilagan, 2009). With some exceptions, most invertebrates have a single copy of the Notch receptor, while vertebrates tend to have multiple receptors (Gazave et al., 2009). All Notch receptors have a number of conserved domains. The extracellular domain contains a variable number of EGF repeats which are important for ligand binding. It also contains a negative regulatory region which consists in part of LNR domains. The intracellular domain contains a RAM domain for interaction with CSL proteins, ankyrin repeats, transactivation domain, nuclear localisation signal and a PEST domain which is required to control the stability of the released NICD (Kopan and Ilagan, 2009, Guruharsha et al., 2012).

The Notch ligands fall into two distinct families: Delta-like and Jagged/Serrate. Both are single pass transmembrane proteins but have slightly different domain architectures. Both ligand types have a number of EGF repeats in their extracellular domain as well as a characteristic DSL domain. Both of these are required for

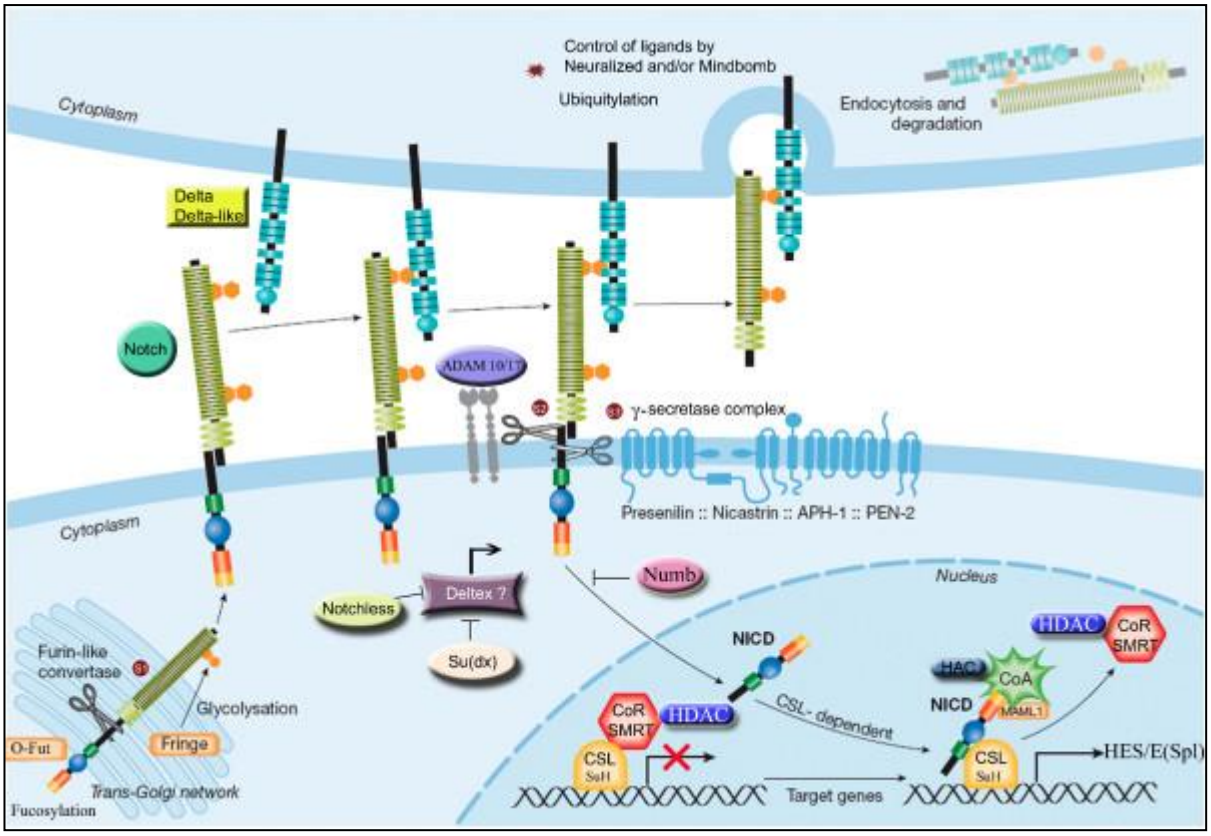


Figure 1.4: Schematic representation of the canonical Notch signalling pathway (Radtke et al., 2005).

receptor binding. In addition Jagged proteins have a cysteine rich domain N-terminal to the transmembrane domain (Kopan and Ilagan, 2009).

Upon ligand-receptor binding, the ligand is ubiquitinylated on its intracellular domain. This leads to endocytosis of the ligand and this is essential for receptor activation. Binding and endocytosis of the ligand cause a mechanical force to be applied to the receptor. This leads to the exposure and cleavage of an ADAM metalloprotease cleavage site on the receptor (S2 cleavage site) in a process known as ectodomain shedding (Gordon et al., 2015). Once the extracellular domain had been cleaved the receptor is available for cleave by the γ-secretase complex (S3 cleavage site). This leads to release of the NICD into the cytoplasm (Selkoe and Wolfe, 2007).

1.3.1.2. Nuclear function

Once the NICD is cleaved by γ-secretase it translocates to the nucleus where it interacts with a CSL transcription factor: RBP-J in mammals, Su(H) in *Drosophila*

and Lag-1 in *C.elegans*. CSL transcription factors have been shown to act as transcriptional repressors in the absence of NICD. In mammals, RBP-J has been shown to form complexes containing SMRT MINT/SHART and CtBP, while in *Drosophila* the co-repressor Hairless leads to recruitment of Groucho and CtBP (Bray, 2006). It is unclear if the gene repressive function of CSL proteins is a general mechanism or only evident in specific cases (Bray, 2006). It is also unclear whether CSL proteins remain bound to DNA in the absence of NICD. Recent evidence suggests that in the absence of NICD RBP-J is dynamically interacting with is binding sites and that NICD may stabilise this interaction (Castel et al., 2013).

In all cases studied, binding of NICD to CSL proteins leads to recruitment of transcriptional activator proteins of the mastermind family. Mastermind proteins, Maml, contain a single recognisable mastermind domain. Formation of the NICD-CSL-Maml complex occurs in a stepwise fashion with NICD first binding CSL. This occurs either on the DNA or is immediately followed by recruitment of the complex to DNA. This then leads to the recruitment of Maml and then further recruitment of co-activators (Kovall, 2008). Further factors are either recruited by interaction directly with NICD such as Skip or via interaction with Maml such as p300 (Bray, 2006).

How Notch signalling achieves specificity in terms of target genes has been the subject of much speculation. It is known that in different cellular contexts the same Notch receptor can activate different sets of target genes. Because of this apparent dependence on cellular context it has been proposed that NICD-CSL can only bind to sites where the DNA is accessible and the possible targets change based on their local chromatin organisation in different cellular contexts (Skalska et al., 2015). There is, however, evidence that Notch can act as a pioneering factor and lead to de-novo chromatin re-organisation (Hass et al., 2015). NICD-CSL complexes have also been shown to bind co-operatively to DNA with other transcription factors and the presence or absence of these factors may affect the target specificity (Hass et al., 2015, Skalska et al., 2015).

1.3.2. Physiological functions of Notch

Notch signalling plays important and diverse roles in the development of virtually all animals studied in different tissue and cellular contexts. Activation of the pathway

can lead to differentiation, proliferation or apoptosis depending on the context. Due to the diverse roles played by Notch signalling I will only summarise a number of relevant examples here. I will briefly outline the role of Notch signalling in stem-cell progeny communication, in the nervous system and then finally I will discuss the current state of the art regarding the roles of Notch signalling in cnidarians.

1.3.2.1. Notch signalling in stem cell-progeny communication

Notch signalling plays many roles in the context of stem cell biology and has been studied across many systems and conserved themes have begun to emerge. Of particular interest are studies on mouse and *Drosophila* intestinal stem cells (ISCs). In the mouse, Notch signalling is required to maintain the crypt stem cell identity but is also involved in binary cell fate choices during later differentiation (VanDussen et al., 2012, Fre et al., 2005, van Es et al., 2005). Work carried out on *Drosophila* has also implicated Notch in intestinal stem cell biology. It has been known for a long period that Notch signalling controls differentiation of the ISC progeny in *Drosophila* (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006). This was shown to be controlled by differential expression of Notch ligands on ISCs which then led to differential Notch activation in their progeny. This differential activation of Notch was critical in modulating cell fate decision within the progeny (Ohlstein and Spradling, 2007). More recent evidence has unearthed even more interesting regulation of this system by Notch, showing that ISCs also require a Notch signal from their progeny in order to maintain identity and multipotency making the signalling in this system bidirectional (Guo and Ohlstein, 2015).

In addition to ISCs, recent evidence from mouse basal airway stem cells has also highlighted important aspects of the pathway. In mice the basal airway stem cells give rise to transit amplifying secretory cells which then differentiate into ciliated cells (Tata et al., 2013). Notch signalling from the stem cells to the transit amplifying cells is critical to maintain their transit amplification and repress differentiation into secretory cells (Pardo-Saganta et al., 2015).

Taken together these examples, as well as many others, highlight the diverse and often multi-faceted roles that Notch signalling can play in the regulation of hierarchical stem cell differentiation systems. Not only can Notch signalling regulate binary cell fate decisions but stem cells can transmit differentiation cues via Notch

signalling to control daughter cell fate and indeed daughter cells can utilise Notch signalling to control stem cell maintenance.

1.3.2.2. Notch signalling in the nervous system

The function of Notch signalling in the nervous system is considered to be a well conserved characteristic of the nervous system and a role for Notch signalling in the nervous system of the last common ancestor of cnidarians and bilaterians is well established (Hartenstein and Stollewerk, 2015). The phenotypic consequence of a premature loss of Notch signalling or its target genes during early neurogenesis represents the so-called 'neurogenic' phenotype, i.e. an increase in neural cells. This phenotype has been shown in both protostome and deuterostome bilaterians as well as in the cnidarian *Nematostella*; however the mechanistic basis of this phenotype is quite divergent.

In *Drosophila* the first neurons are derived from the delamination of neuroectodermal cells to form neuroblasts. These neuroblasts first appear as a group of cells called a proneural cluster, each with equivalent competence and ability to give rise to either non-neural ectoderm or to form a neuroblast. These cells all express proneural genes and also initially all express both Notch and the ligand Delta. Through a not fully understand mechanism, but thought to be triggered stochastically, one of the cells in the cluster comes to express higher levels of Delta which then activates Notch in the surrounding cells. Notch then activates expression of *E(spl)* genes which downregulate proneural gene expression leading to these cells adopting a non-neural fate. In addition *E(spl)* genes downregulate Delta expression thus establishing a feedback loop to maintain the system. This process in known as lateral inhibition (Jan and Jan, 1994, Hartenstein and Wodarz, 2013). Notch loss of function phenotypes thus lead to the adoption of a neural fate by all cells in the proneural cluster and a resulting increase in neural cells (Lehmann et al., 1983).

In other protostome lineages the Notch signalling pathway also plays roles in cell fate decisions in the early neuroectoderm and subsequent neurogenesis (Ungerer et al., 2012, Gold et al., 2009). In spiders, for example Notch signalling, acting via lateral inhibition, selects groups of cells, rather than single cells like in Drosophila, which will adopt a neural fate and subsequently delaminate (Stollewerk, 2002).

In vertebrates Notch signalling also acts to repress neural differentiation but in this case Notch acts within the neural progenitor/stem cells where it again acts to repress expression of proneural genes and maintain the progenitor cell fate (Louvi and Artavanis-Tsakonas, 2006). Importantly mutations leading to a loss of Notch signalling in vertebrates also lead to an increase in neural cells due to premature differentiation of the neural progenitor cells (Chitnis et al., 1995, Chitnis and Kintner, 1996, Henrique et al., 1997). Again in other deuterostome lineages Notch signalling also plays significant roles in neurogenesis (Lu et al., 2012, Hudson et al., 2007).

Hence, in both flies and vertebrates Notch signalling acts by repressing proneural genes (Bertrand et al., 2002). These data led to the idea that Notch signalling was part of an evolutionarily conserved 'neural gene cassette' in the bilaterian ancestor. More recent evidence from *Nematostella* (discussed in detail below) has pushed the origin of this mechanism back to the last common cnidarian-bilaterian ancestor (Hartenstein and Stollewerk, 2015).

1.3.2.3. Notch signalling in cnidarians

Given the varied roles of Notch signalling in bilaterian development it is unsurprising that it has been studied in the two major cnidarian models: *Nematostella* and *Hydra*. A focus of these studies has been on two topics: neurogenesis and patterning. Although some similarities have emerged, there are many conflicting results in the literature leading to an unclear picture of the ancestral functions of Notch signalling.

The first experiments on Notch signalling in cnidarians were carried out on the nervous system of adult *Hydra*. DAPT, a γ-secretase inhibitor, was used and showed clearly that neuronal differentiation was unaffected by Notch inhibition. Commitment of cells to the nematocyte lineage and their proliferation was also unaffected but later differentiation events were severely blocked, specifically capsule maturation (Käsbauer et al., 2007, Khalturin et al., 2007). It was also shown that differentiating nematocytes undergo apoptosis in response to DAPT (Khalturin et al., 2007). Although it is interesting that Notch has no apparent effect on neuronal differentiation or commitment of neural cells, these studeis were performed using

only pharmacological approaches and exclusively in the adult context; they did not address a developmental role for Notch signalling in the nervous system.

Work done on *Nematostella*, using DAPT as well as genetic interference, showed that inhibition of Notch signalling leads to an increase in the numbers of neurons during embryogenesis (Marlow et al., 2012, Richards and Rentzsch, 2015, Layden and Martindale, 2014). In addition, early work showed a decrease in cnidocytes (Marlow et al., 2012) but later work using both DAPT and genetic methods showed a clear increase in the number of differentiating cnidocytes (Layden and Martindale, 2014, Richards and Rentzsch, 2015). Like in *Hydra*, DAPT also led to a block in capsule maturation and late nematocyte differentiation but this has not been confirmed genetically (Richards and Rentzsch, 2015). Together, these studies show a bilaterian like phenotype of Notch inhibition on the *Nematostella*, but not *Hydra*, nervous system, i.e. an increase in the numbers of neural cells.

Along with work on the nervous system, other work in *Hydra* has suggested a role for Notch signalling in polyp budding and tentacle morphogenesis with DAPT leading to a block in tentacle formation (Münder et al., 2010, Prexl et al., 2011, Münder et al., 2013). Additionally, in *Nematostella* DAPT also leads to defects in tentacle formation although the exact phenotypes varies from fused tentacles (Marlow et al., 2012), through block in tentacle elongation (Fritz et al., 2013) during development, to a complete block in tentacle formation in regeneration (DuBuc et al., 2014).

The mechanism through which Notch signalling is acting in cnidarians is not yet fully understood. *Su(H)* morpholino or dominant negative overexpression mimicked at least some DAPT phenotypes (Marlow et al., 2012), consistent with Notch signalling acting through a canonical pathway with Su(H). The downstream targets of Notch signalling are also unclear. In bilaterian systems Notch often works through *Hes* genes, particularly in the nervous system where these act to repress proneural *Ash* genes (Bertrand et al., 2002, Hartenstein and Wodarz, 2013, Louvi and Artavanis-Tsakonas, 2006). In *Nematostella* inhibition and ectopic activation of Notch signalling lead to an increase or decrease in *AshA* expression, respectively. It is not, however, clear if inhibition of Notch signalling leads to a decrease in expression of *Hes* genes with conflicting reports coming from two different papers

(Richards and Rentzsch, 2015, Layden and Martindale, 2014). It is also unclear what the role of *Hes* genes is in the *Nematostella* nervous system and indeed it is unknown whether cnidarian Hes proteins can regulate *Ash* genes. During tentacle patterning Notch inhibition has also been shown to downregulate expression of *Hes* genes in both *Hydra* and *Nematostella* (Münder et al., 2010, Münder et al., 2013, Fritz et al., 2013).

Despite the conflicting reports on Notch signalling a number of central themes emerge from the literature. Firstly, Notch signalling is required in *Nematostella* neurogenesis and its inhibition has a bilaterian-like neurogenic phenotype but it is uncertain whether Notch signalling acts canonically through *Hes* and *Ash* genes to regulate neurogenesis. This suggests a deep conservation of the role of Notch in bilaterian and cnidarian neural development. The lack of embryonic data in *Hydra* makes a comparison difficult but the adult situation suggests that this function of Notch may have been lost in *Hydra* or possibly earlier in related groups. Secondly, a role for Notch signalling during tentacle patterning appears to be conserved in both *Hydra* and *Nematostella*. Although this has yet to been shown genetically it is backed up by expression data. Finally γ-secretase inhibition leads to a severe defect in capsule maturation in both animals but again this has yet to be confirmed by genetic studies.

Chapter 2. Materials and Methods

2.1. Animal culture and manipulation

2.1.1. Animal culture

Hydractinia echinata colonies, growing on hermit crab shells, were sampled from Galway bay or Roscoff Marine Station (http://www.sb-roscoff.fr/). Animals were maintained under a 14:10 light dark cycles in artificial seawater (ASW). Spawning occurs approximately 1 hour after light induction. Animals were fed three times a week with one-day old *Artemia* nauplii supplemented with dried egg powder twice weekly with ground oyster or fish. Embryos were developed at room temperature or in a 17°C incubator. Larvae were induced to metamorphose by incubation in a solution of 116 mM CsCl in ASW for ~4 hours, then washed numerous times in ASW and left to settle on the desired substrate.

For regeneration experiments colonies were anesthetised in 4% $MgCl_2$ in ASW and polyps subsequently removed from the colony. Individual polyps were decapitated by transverse cutting below the tentacles, washed several times in ASW and kept at room temperature or at 17°C.

2.1.2. DAPT treatment

DAPT **(**N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycinet-butylester**)** (Abcam ab120633) was dissolved in DMSO to a stock concentration of 10 mM or 0.1 mM and aliquots were stored at -20°C. Immediately before use the stock solution was diluted 1:1000 in ASW. Animals were incubated in DAPT in the dark and the solution was changed at least every 24 hours. The number of animals in each dish was kept low (no more that ten polyps in a 35 mm Petri dish). DMSO was used as a control for all experiments.

2.1.3. Microinjection

Microinjection was carried out using 1 cell stage *Hydractinia* embryos. Embryos were immobilised on 100 um plankton netting attached to the bottom of a 35mm Petri dish lid. Injection needles were produced from glass capillaries (Narishige GD-1 1x90 mm) using a pulling machine. Plasmids were injected at 2-5 ug/ul in nuclease free H_20. For CRISPR injection concentrations see section 2.5.5.

2.2. Cellular staining

2.2.1. Whole mount RNA in-situ hybridisation (WISH)

Polyps were anaesthetised for 30 minutes in 4% $MgCl_2$ in 50% ASW/ 50% H_2O. They were then fixed in 4% paraformaldehyde (PFA) in HEPES buffer (100 mM HEPES, 4 mM $MgSO_4$, 140 mM NaCl) for one hour at room temperature or overnight at 4°C. Animals were then washed three times for five minutes in 0.1% TWEEN20 in PBS (136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) (PBST). Animals were then dehydrated through a series of 25%, 50%, 75% and 100% methanol with each step incubated for ten minutes. Animals were then stored at -20°C for later use or used immediately. Animals were rehydrated through a reverse series of methanol, as above, followed by three washes in PBST for five minutes each. This was followed by incubation for 15 minutes at 95°C in PBST. Animals were then washed once with 1x triethylamine (TEA), 0.06% acetic acid in 1X TEA, 0.12% acetic acid in 1X TEA and PBST for five minutes each. Animals were then fixed for 20 minutes in 4% PFA in PBS followed by three washes in 0.3% Triton X-100 in PBS (PBSTx) for five minutes each. Blocking was then carried in 2 mg/ml yeast tRNA for ten minutes. An equal volume of hybridisation buffer (50% deionised formamide, 0.1 mg/ml yeast tRNA, 0.1 mg/ml Heparin, 5x SCC, 0.1% Tween 20) was then added and incubated for a further ten minutes. Pre-hybridisation was then carried out overnight at 50°C in hybridisation buffer. Probes were diluted to a concentration of 40 ng/ml in hybridisation buffer and hybridisation was carried out overnight at 50°C. Following hybridisation animals were washed once in hybridisation buffer at 50°C for five minutes followed by one hour in wash one (50% formamide, 2x SCC, 0.1% TWEEN 20) at 50°C, 15 minutes in wash two (2x SCC, 0.1% TWEEN 20) at 50°C, 15 minutes in wash three (0.2x SCC, 0.1% TWEEN 20) at 50°C and 15 minutes in wash three at room temperature. This was followed by three washes for five minutes in PBSTx and then one hour blocking in 3% bovine serum albumin (BSA) in PBSTx. Animals were incubated in 1:2000 antibody (Anti-DIG AP, Roche) in 3% BSA/PBSTx overnight at 4°C followed by thee washes with PBSTx. Three washes in alkaline phosphatase (AP) buffer (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCL_2$, 0.1% Tween 20) were then performed prior to staining. Animals were stained in NBT/BCIP solution (225 ug/ml NBT, 175 ug/ml BCIP in AP buffer) and staining was monitored under a

microscope. Once staining was completed animals were washed three times in 10 mM EDTA in PBSTx and mounted in 90% glycerol.

2.2.2. Immunofluorescence (IF)

Prior to fixation adult stages were anesthetised in 4% $MgCl_2$ in 50% ASW/ 50% H_2O. Animals were fixed in 4% PFA in PBS for 20-60 minutes at room temperature or overnight at 4°C. This was followed by three washes for 10 minutes in PBSTx. For larva an additional wash overnight at 4°C was carried out. Animals were then blocked in 3% BSA in PBSTx for one hour at room temperature before primary antibody (see table below for dilutions) incubation in 3% BSA in PBSTx overnight at 4°C. Animals were then washed four times in PBSTx for at least 10 minutes each. An additional overnight wash in PBSTx was carried out on larva. Following this, blocking was carried out in 5% goat serum, 3% BSA in PBSTx and then secondary antibody incubation was carried out at room temperature for two hours at a concentration of 1:500 in 5% goat serum, 3% BSA in PBSTx. Animals were then washed once for 20 minutes in 10ng/µl Hoechst 33258 (25mg/ml, Sigma B2883) in PBSTx followed by three washes with PBSTx. Animals were mounted in Fluoromount (Sigma F4680).

For Ncol3 stained larva the protocol was the same until after the secondary antibody washes. Animals were then incubated in 1% v/v cyanine 3 amplification reagent in TSA Plus amplification diluent (PerkinElmer, NEL753001KT) for 30 minutes at room temperature. If another antibody was used then the secondary antibody was only applied after the TSA reaction.

2.2.3. Nematocyte staining using a modified DAPI protocol (Szczepanek et al., 2002)

Animals were anesthetised, if necessary, for 30 minutes in 4% $MgCl_2$ in 50% ASW/ 50% H_20. Fixation was carried out for 30 minutes at room temperatures in 4% PFA in PBS supplemented Triton X-100 to a final concentration of 0.3% and EDTA to a final concentration of 10 mM (PBSTE). Following fixation animals were washed three times in PBSTE and then stained overnight in 1:200 DAPI (10 mg/ml) in deionised water. After staining animals were washed three times in PBSTE and mounted in Fluoromount (Sigma F4680). To combine with antibody staining the IF

protocol was carried out as above (Section 2.2.2) except EDTA was added to all solutions to a final concentration of 10 mM and DAPI staining was carried out after the secondary antibody.

2.2.4. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

TUNEL was carried out using the In situ BrdU-Red DNA Fragmentation (TUNEL) Assay Kit (ab66110) from Abcam using the manufacturer's instructions with a number of modifications. All solutions except PBSTx are provided in the kit. Briefly, animals were fixed for one hour in 4% PFA in PBS at room temperature and washed three times for five minutes with PBSTx. Animals were washed twice for five minutes with wash buffer and then incubated for one hour in DNA staining solution (10 µl 5x TdT reaction buffer, 0.75 µl TdT, 8 µl Br-dUTP, 32.25 µl H_2O) at 37°C. Animals were then washed three times for ten minutes in PBSTx and incubated for two hours in antibody staining solution (195µl Rinse buffer, 5µl antibody). Following antibody animals were incubated for 20 minutes in t-AAD/RNAse solution, incubated for 20 minutes PBSTx containing 1:2000 Hoechst (10 mg/ml) and then washed three times with PBSTx before mounting with fluomount.

2.3. Molecular techniques

2.3.1. PCR and gel clean up

PCR was carried out using either Phusion high fidelity DNA polymerase (Thermo Scientific F530) or MyTaq polymerase (Bioline BIO-21105) as per the manufacturer's instructions. Phusion was utilised for all cloning PCRs and MyTaq was used for routine PCR. All PCR reactions were ran on 0.5-1% agarose gels at 100 V for 25-30 minutes and gel extraction was carried out using the NucleoSpin gel and PCR clean up kit (Macherey-Nagel).

2.3.2. Restriction digest based cloning

For restriction digest based cloning, backbone and inserts were generated by PCR using primers with overhanging restriction sites and extracted (see section 2.3.1). Primers used are listed below. Restriction digestion was then carried out using 100ng backbone with either equimolar amounts or three times molar excess of insert in 20 µl reaction volume containing 1 µl of each restriction enzyme and 1x appropriate reaction buffer. Digestion was carried out overnight at 37°C followed by heat inactivation (as per manufacturer's instructions). Once the reactions had cooled 2.3 µl T4 DNA ligase buffer and 0.7 µl T4 DNA ligase (ThermoFisher Scientific EL0011) were added to each tube and they were incubated at room temperature for 30-60 minutes. They were then transformed into chemically competent E.coli (see section 2.3.3) and colony PCRs were carried out to test for positive clones using MyTaq.

For cloning of the *β-tubulin* 3' genomic region into the *βtub*::*GFP*::*Act* vector the backbone was amplified with GFPrevSac1 and TermfwdPac1 and the genomic region with βtubtermfwdSac1 and βtubtermrevSac1 from *Hydractinia* genomic DNA and the enzymes Sac1 (ThermoFisher Scientific ER0431) and Pac1 (ThermoFisher Scientific ER2201) were used. PBigfor primer was used for sequencing to check the cloning.

For cloning *DsRed2* and *mOrange* in place of *GFP* in the *βtub*::*GFP*::*βtub* vector the backbone was amplified with βtubpromrevNot1 and βtubtermfwdSac1, *DsRed2* was amplified with DsRed2fwdNot1 and DsRed2revSac1 from a plasmid provided by Rob Steele and *mOrange* with mOrangefwdNot1 and mOrangerevSac1 from a

plasmid provide by Fabian Rentzsch. The enzymes Not1 (ThermoFisher Scientific ER0591) and Sac1 (ThermoFisher Scientific ER0431) were used. The gene specific forward and reverse primer were used for sequencing to check insertion.

To clone *tdTomato* and *Kaede* they were first codon optimised and synthesized commercially (Eurofins Genomics), using a *Hydractinia* codon usage table (Kazusa database). They were then amplified from the plasmid they were provided in using the following primers: tdTomatofwdNot1 and tdTomatorevPac1 for *tdTomato*, KaedefwdNot1 and KaederevSac1 for *Kaede*. The backbone was produced form the *βtub*::*GFP*::*βtub* vector using the primers βtubpromrevNot1 and βtubtermfwdSac1. The restriction enzymes Not1 (ThermoFisher Scientific ER0591) and Sac1 (ThermoFisher Scientific ER0431) were used. The gene specific forward and reverse primer were used for sequencing to check insertion.

The double *βtub*::*GFP*::*βtub*, *Piwi1*::*GFP*::*Piwi1* plasmid was generated by first generating the backbone from the *βtub*::*GFP*::*βtub* plasmid with βtubtermrevPac1 and TerminatorfwdKpn1. The insert was generated from the *Piwi1*::*GFP*::*Piwi1* plasmid with Piwi1termrevPac1 and Piwi1promfwdKpn1. The restriction enzymes Kpn1 (ThermoFisher Scientific ER2201) and Pac1(ThermoFisher Scientific ER0521) were used.

In order to clone the *βtub*::*CyclinB-GFP*::*βtub* plasmid he CyclinB N-terminal fragment was generated by PCR with primers CyclinBfwdNot1 and CyclinBrevNot1. The *βtub*::*GFP*::*βtub* plasmid and PCR product were digested with Not1 (ThermoFisher Scientific ER0591) and gel extracted as above (Section 2.3.1). Ligation was performed as above and transformed into chemically competent bacteria (see section 2.3.3). Correct insertion was shown by sequencing with GFPseqfusrev primer.

2.3.3. Bacterial transformation

Chemically competent XL1 Blue E.coli were generated by first inoculating LB agar plates with stock XL1 blue bacteria and incubating overnight at 37°C. Following overnight growth a single colony was grown overnight in 5 ml LB broth and this was used the following day to inoculate a 300 ml culture. This was then incubated shaking at 37°C until an OD600 of ~0.4 was reached. The bacteria were then

transferred to pre-cooled 50ml tubes on ice for 10 minutes and centrifuged at 3500 RPM for 15 minutes at 4°C. The supernatant was discarded and the pellets were each resuspended in 5ml of ice cold, sterile 0.1 M $CaCl_2$ followed by incubation on ice for ten minutes. These were again centrifuged at 3500 RPM for 10 minutes at 4°C and the supernatant discarded. The pellets were finally dissolved in 1ml each of 0.1 M $CaCl_2$/ 14% glycerol, aliquoted and stored at -80°C.

For transformation 1 µl plasmid or 20 µl ligation reaction was added to 50 µl of competent cells on ice an incubated for 10 minutes. This was then incubated at 42°C for 90 seconds in a water bath and placed immediately in ice for five minutes. 150 µl LB broth was then added to the tubes and they were incubated rocking at 37°C for 30 minutes before being plated on LB agar plates containing appropriate antibiotic (100 µg/ml carbenicillin 50 µg/ml kanamycin).

2.3.4. Gibson assembly

For Gibson assembly both insert and backbone were prepared by PCR and gel clean up as above (Section 2.3.1). PCR primers were designed to give 20-30 bp overlap on both the 5' and 3' ends between the insert and backbone. The reaction was carried out using ~100ng backbone and 2x molar excess of insert. This was added to 10 µl 2x mastermix (NEBuilder Hifi DNA assembly mastermix, E2621) and nuclease free H_2O to make a final reaction volume of 20 µl. In the case of sgRNA cloning (see section 2.5.2) 10 µl reactions were carried out. The reaction was incubated at 50°C for 20 minutes before being transformed into bacteria as above (Section 2.3.3). Colonies were screened by colony PCR or sequencing.

2.3.5. Plasmid extraction

For small preparations colonies were grown overnight in 5 ml LB broth containing appropriate antibiotic at 37°C. The GenElute miniprep kit (Sigma PLN350) was used as per the manufacturer's instructions. For larger extractions colonies were grown overnight in 300 ml LB broth containing appropriate antibiotic. Suspensions were then centrifuged for five minutes at 6000 g and the resulting pellets resuspended in 10 ml resuspension buffer (25 mM Tris-HCl pH 8, 10 mM glucose, 10 mM EDTA). 15 ml lysis buffer (0.2 M NaOH, 1% SDS) was then added and the solution was gently mixed and left at room temperature for five minutes before 20 ml of

neutralisation buffer (3 M CH_3CO_2K, 11.5% v/v acetic acid) was added. Tubes were then incubated on ice for five minutes before being centrifuged at full speed for 15 minutes at 4°C. The resulting supernatant was filtered into fresh 50 ml tubes and 0.5 volumes of isopropanol was added to each before being placed at -20°C for 20 minutes. Following this the tubes were centrifuged at full speed for 15 minutes at 4°C and the resulting pellet was washed once with 70% ethanol before being allowed to dry at room temperature. The pellet was dissolved in 1 ml nuclease free H_2O containing 2 µl each RnaseA (ThermoFisher Scientific EN0531) and RnaseT1 (ThermoFisher Scientific ER0541) and incubated at 37°C. After 1 hour 125 µl 5 M NaCl, 125 µl 10% SDS and 2 µl Proteinase K (stock: 25ml/ml) were added and incubated for a further two hours at 55°C. After incubation the plasmid was extracted using a standard phenol:cholorform extraction protocol. Briefly, one volume each of phenol (pH 8) and chloroform were added and vigorously mixed. The sample was then centrifuged at full speed for five minutes and the upper aqueous layer was retained. An equal volume of chloroform was then added to this and it was again mixed vigorously, centrifuged at full speed and the aqueous layer retained. The plasmid was precipitated by adding $1/5^{th}$ the volume of 2.5 M KCl and 2.5 volumes of 100% ethanol. The pellet was collected by centrifugation at full speed for five minutes or if a large precipitate was formed it was spooled on a 200 µl tip, washed several times with 70% ethanol and resuspended in nuclease free H_2O.

2.3.6. Genomic DNA extraction

For general genomic DNA extraction adult polyps were used. 100-300 polyps were cut and washed once in genomic lysis buffer (100 mM Tris-HCl pH8, 1% w/v SDS, 50 mM EDTA). Animals were then placed in ~300 µl genomic lysis buffer and the tissue homogenised using a plastic pestle. The volume was then brought to 1 ml with genomic lysis buffer, 2 µl each RnaseA (ThermoFisher Scientific ER0531) and RnaseT1 (ThermoFisher Scientific ER0541) were added and the tubes incubated at 37°C in the water bath for 1 hour. Following this 2 µl Proteinase K (25 mg/ml stock) was added and incubated for a further 2 hours at 50°C. The genomic DNA was extracted using Phenol: Chloroform (as in plasmid extraction, replacing KCl with $1/10^{th}$ the volume of NaCl).

For CRISPR experiments (see section 2.5.5) genomic DNA was extracted with NucleoSpin Tissue kit (Macherey-Nagel) using the manufacturer's protocol except elution was carried out in 20 µl nuclease free H_20, preheated to 50°C.

2.3.7. RNA extraction and cDNA synthesis

For RNA extraction animals were first lysed thoroughly in 500µl-1ml Trizol (ThermoFisher Scientific 15596018) with vortexing. $1/5^{th}$ the volume of chloroform was then added and the tubes mixed vigorously. This was centrifuged at full speed for five minutes and the upper aqueous phase removed and retained. An equal volume of ethanol was then added to this and it was processed through the RNeasy Mini kit (Qiagen 74106) following the manufacturers guidelines.

cDNA synthesis was carried out using the Omniscript RT kit (Qiagen 205111). Either random primers (MyBIO C1181) or Oligo(dT) 15 primer (MyBIO C1101) were used as primers.

2.3.8. WISH probe synthesis

Primers were designed to amplify the desired fragment of a gene with T7 and SP6 promoter sequences on the forward and reverse primer respectively. MyTaq was used to amplify the fragment and it was ran on an agarose gel and extracted (see section 2.3.1). This DNA was used as template for probe synthesis. 20 µl reactions were carried out containing 500 ng-1 µg template DNA, 0.5 µl Ribolock (ThermoFisher Scientific EO0381), 1 µl DIG labelled NTPs (Roche), 1 µl T7 RNA polymerase (ThermoFisher Scientific EP0111) or 1 µl SP6 polymerase (ThermoFisher Scientific EP0131) and x µl nuclease free H_2O to 20 µl. This was incubated overnight at 37°C in the oven. The next day 1 µl RNase free DNase1 (ThermoFisher Scientific EN0521) was added to each and this was incubated for a further 30 minutes at 37°C. An equal volume of 12 M LiCl was then added and the tubes incubated at -20°C for 20 minutes before being centrifuged at full speed. The pellet was washed once with 70% ethanol before being dried at room temperature and resuspended in nuclease free water. Probes were stored at a final concentration of 40 ng/µl in 50% hybridisation buffer (see above) in nuclease free water. The Ncol1 and Rfamide probes used were the same as those previously reported (Kanska and Frank, 2013).

2.4. Microscopy and cell counting.

Fluorescence microscopy was carried out using the Olympus BX51 compound microscope. Confocal microscopy was carried out on either an Olympus fluoview 1000 with an inverted IX71 microscope or Andor revolution spinning disc confocal (Yokagawa CSU_{22}). Devonvlution microscopy was performed on a Delta Vision microscope. Image analysis was carried out in all cases using ImageJ.

Cell counting was carried out using ImageJ cell counting feature. For GLWamide positive neurons confocal z-stacks were counted manually, without projecting. For DAPI stained nematocytes images were taken at the surface of the larva. These were then manually counted. All cell counting experiments are shown as data from a single replicate as the variation between batches of larva precludes the combination for the data, although in all cases similar results were seen in at least 3 independent replicates. Data is shown +/- standard deviation and p values were calculated using students T-test.

2.5. CRISPR-Cas9 mediated mutagenesis

2.5.1. sgRNA design

sgRNAs were designed using the sgRNA design tool form the Broad institute (http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design-v1). sgRNAs were chosen based on their score from the software as well as their position in the genomic DNA. Chosen guides were blasted and only used if no significant matches were found that also had an adjacent 5' NGG 3' PAM site.

2.5.2. sgRNA cloning

Overlapping primers were ordered (as below) containing the 19 bp sgRNA site and overhangs for Gibson assembly into the sgRNA cloning vector (http://www.addgene.org/41824/). Primers were annealed and extended using Phusion polymerase. Each 50µl reaction mix contained 1ug of each primer, 0.5 µl Phusion polymerase, 10 µl 5x HF buffer, 1 µl 10 mM dNTPs and nuclease free H_2O to 50 µl. Reaction was carried out in a PCR machine with the following conditions: 98°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute. The plasmid was digested with AflII (NEB R0520) and the linearised product was ran on a 0.5% agarose gel and extracted (Section 2.3.1). Gibson assembly was used to ligate the annealed primers into the backbone as above (Section 2.3.4). Colonies were then grown in 5ml LB broth (50µg/ml kanamycin) overnight at 37°C and a plasmid extraction was carried out as above (Section 2.3.5). Colonies were sequenced with T7 primer to ensure the guide RNA sequence was correct.

2.5.3. sgRNA synthesis

Guide RNA template was produced from the plasmids generated above by PCR with Phusion polymerase using a guide specific primer and T7gRNArev. Cycling conditions were 98°C for 30 seconds; 39 cycles of 98°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute. PCRs were then ran on an agarose gel and bands were gel extracted as above (Section 2.3.1). sgRNAs were produced from the extracted template using the HiScribe T7 high yield RNA synthesis kit (NEB E2040) using the manufacturers protocol. Briefly 20 µl reactions were prepared containing 1 ug template DNA, 2 µl each ATP, CTP, GTP, UTP, 10x buffer and T7 polymerase solution and x µl nuclease free H_2O to 20 µl. This was incubated at 37°C in a water

bath for one hour. Following incubation 10 µl 12 M LiCl was added to each and the tubes were placed at -20°C for 20 minutes before being centrifuged at full speed for five minutes. Pellets were then washed with 70% ethanol, centrifuged for five minutes and then allow to dry for five minutes at room temperature. Pellets were resuspended in nuclease free H_2O.

2.5.4. sgRNA *in vitro* testing

For in-vitro testing of the sgRNAs genomic fragments were produced by Phusion PCR using primers at least 1 kb away from the cut site in the 3' and 5' direction and gel extracted. In-vitro testing was carried out using the following reaction mixture: 300 ng Cas9 (PNA Bio CP02), 250 ng sgRNA, 100 ng PCR fragment, 2µl NEB buffer 3 (NEB B7003S), 2 µl 10x BSA , x µl nuclease free H_20 to 20µl. Cas9 alone was used as a negative control. Samples were incubated at 37°C for one hour and were run on a 1% agarose gel.

2.5.5. Injection and testing

For injection the sgRNA were mixed to a final concentration of 250 ng/ul each along with 2x NEB buffer 3. This was then aliquoted into single use, 1 µl aliquots. Cas9 protein (PNA Bio CP02) was resuspended to a final concentration of 2ug/µl and aliquoted into single use 1µl aliquots. Prior to injection 1µl sgRNA mix was added to 1µl Cas9 and the solution heated to 37°C for 5-10 minutes. As a control 1µl water was added to 1µl Cas9 and this was also heated. Microinjection was performed as above (Section 2.1.3).

For genotyping larva or primary polyps were pooled and genomic DNA extracted using the NucleoSpin Tissue kit (Macherey-Nagel). PCR was performed using the primers Notchgfrag1fwdNot1 and Notchgfrag3revPac1. For cloning *βtub*::*GFP*::*βtub* and insert were digested with Not1 and Pac1 and cloning was performed as above (Section 2.3.2). Positive colonies were determined by colony PCR. For sequencing Notchgfrag2fwd and NotchGfrag3revpac1 were used.

2.6. Sequence identification and manipulation

Blast searches were carried out using an in house transcriptome generated from multiple life stages and assembled using Trinity (Liam Doonan, unpublished) and an in house genome generated using SMRT sequencing (Christine Schnitzler, unpublished). Transcripts were translated using expasy translation tool (http://web.expasy.org/translate/) and the longest open reading frame from each transcript was used to annotate the protein.

Protein annotation was carried out using blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), pFam (http://pfam.xfam.org/) and interpro (https://www.ebi.ac.uk/interpro/). Nuclear localisation sequences (NLS) were analysed using cNLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Kosugi et al., 2009). Signal peptides were predicter using SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). Transmembrane domains were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001). PEST domains were predicted using epestfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/help/epestfind).

Table. 2.1. Primary antibodies.

Antibody	Source	Host species	Dilution
Anti-PhosphoHistone H3(S10)	Abcam ab5176	Rabbit	1:1000
Anti-Ncol3	Suat Ozbek	Guinea pig	1:500
Anti-Acetylated Tubulin	Sigma AldrichT7451	Mouse	1:1000
Anti-RFamide	Gunther Plickert	Rabbit	1:1000
Anti-Ncol1 N-CRD	Suat Ozbek	Guinea pig	1:1000
Anti-GLWamide	Thomas Leitz	Rabbit	1:500

Table. 2.2. Secondary antibodies.

Antibody	Source	Host species	Dilution
Anti-Rabbit 488	Invitrogen A11008	Goat	1:500
Anti-Guinea pig 594	Abcam ab150188	Goat	1:500
Anti-Mouse 594	Abcam Ab150116	Goat	1:500
Anti-Mouse 488	Invitrogen A11029	Goat	1:500
Anti-Guinea pig HRP	Abcam ab6906	Goat	1:500

Table. 2.3. Primer sequences

Primers for cloning	
GFPrevSac1	5’AAAAAGAGCTCCTATTTGTATAGTTCATCCATGCCATG3’
TermfwdPac1	5’AAAAATTAATTAACGTACGGGCCCTTTCGTCT3’
Btubtermfwdsac1	5’AAAAAAGAGCTCACACTTTTCGTTTTAATTTATAATTTTAAGTTTTGC 3’
Btubtermrevpac1	5’ AAAAAATTAATTAAGTGAGCCATAGGAGC3’
PBigfor	5’ TAAAAATAGGCGTATCACGAGGCCC3’
BtubpromrevNot1	5’AAAAAGCGGCCGCATTGAAATTATATAGTTCGTGCTACAACACC 3’
DsRed2fwdNot1	5’AAAAAAGCGGCCGCATGGCTTCATCAGAAAATGTTATTACAG3’
DsRed2revSac1	5’AAAAAAGAGCTCCTATAAAAATAAATGATGTCTACCTTCTGTTCTTTC3’
mOrangefwdNot1	5’AAAAAAGCGGCCGCATGGTGAGCAAGGGCG 3’
mOrangerevSac1	5’AAAAAAGAGCTCTCAGGAGAGCACACACTTG 3’
tdTomatofwdNot1	5’ AAAAAAGCGGCCGCATGACTTCCAAAGGTGAAGAGG 3’
tdTomatorevSac1	5’AAAAAAGAGCTCTTATTTGTACAGTTCGTCCATTCCG 3’
KaedefwdNot1	5’AAAAAAGCGGCCGCATGAGTCTGATAAAACCGGAAATGAAG 3’
KaederevSac1	5’ AAAAAAGAGCTCTTATTTGACATTGTCAGGTAAACCTGA 3’
TerminatorfwdKpn1	5’ AAAAAAGGTACCGGGCCCTTTCGTCTCG3’
Piwi1termrevPac1	5’ AAAAATTAATTAAGAAGGCTTACGCTAGTGTGAATTAG 3’
Piwi1promfwdKpn1	5’ AAAAAAGGTACCGGTCTATAGACATGTTTAATTTGCCC3’
CyclinBfwdNot1	5’ AAAAAAGCGGCCGCATGGCAGCGATACCTCG3’
CyclinBrevNot1	5’ AAAAAAGCGGCCGCTCTCTCTGCTTTTCGCATATGTC3’
GFPseqfusrev	5’ TTGCATCACCTTCACCCTCTCC 3’

Primers for sgRNA cloning	
NotchgRNA_1_fwd	5’TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTACAAGGCGGTGTACAAAC3’
NotchgRNA_1_rev	5’GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTTGTACACCGCCTTGTAC3’
NotchgRNA_2_fwd	5’TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGATCCATTACCACTACAAGG3’
NotchgRNA_2_rev	5’GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCTTGTAGTGGTAATGGATC3’
NotchgRNA_3_fwd	5’TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCATTTGCCTTTGTAAACCT3’
NotchgRNA_3_rev	5’GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTTTACAAAGGCAAATGC3’
NotchgRNA_4_fwd	5’TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCAGTAACATTTGTACGAGG3’
NotchgRNA_4_rev	5’GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCTCGTACAAATGTTACTGC3’
NotchgRNA_5_fwd	5’TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCTGTCTTGCCGCAGTCGAA3’
NotchgRNA_5_rev	5’GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTTCGACTGCGGCAAGACAGC3’
NotchgRNA_6_fwd	5’TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGAAGCCTGTAACTATGACAA 3’
NotchgRNA_6_rev	5’GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTTGTCATAGTTACAGGCTTC3’
NotchgRNA_7_fwd	5’TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGTTTGGTGCAATTCACCCA3’
NotchgRNA_7_rev	5’GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTGGGTGAATTGCACCAAACC3’
Primers for sgRNA template synthesis	
T7gRNArev	5’AAAAGCACCGACTCGGTGCC3’
JG_NtchgRNA_1_T7	5’GGATCCTAATACGACTCACTATAGTACAAGGCGGTGTAAAAC3’
JG_NotchgRNA_2_T7	5’GGATCCTAATACGACTCACTATAGATCCATTACCACTACAAGG3’
JG_NotchgRNA_3_	5’GGATCCTAATACGACTCACTATAGCATTTGCCTTTGTAAA

T7	CCT3’
JG_NotchgRNA_4_T7	5’GGATCCTAATACGACTCACTATAGCAGTAACATTTGTACGAGG3’
JG_NotchgRNA_5_T7	5’GGATCCTAATACGACTCACTATAGCTGTCTTGCCGCAGTCGAA3’
JG_NotchgRNA_6_T7	5’GGATCCTAATACGACTCACTATAGAAGCCTGTAACTATGACAA3’
JG_NotchgRNA_7_T7	5’GGATCCTAATACGACTCACTATAGGTTTGGTGCAATTCACCCA3’
Primers for sgRNA testing	
Notchlongfragfwd	5’ GCCGGTAAACTAGTCGATC 3’
Notchlongfragrev	5’ GGTCGTGTGAAAATGGATAATAAC 3’
Notchgfrag1fwdNot1	5’AAAAAAGCGGCCGCGCTTCATAATTATTGTATTATTCAATCTATTTTGTAGTATTG3’
Notchgfrag2fwd	5’AAAAAATTAATTAACCCACTTGCATTTAGTCTCAGAAAAAC 3’
Notchgfrag3revPac1	5’AAAAAATTAATTAACCCACTTGCATTTAGTCTCAGAAAAAC 3′

Chapter 3. Generation of transgenic tools in *Hydractinia echinata*

3.1. Introduction

The use of transgenic technologies is at the core of modern biology. All major model organisms are amenable to sophisticated transgenic techniques. These technologies allow the generation of reporter lines to study gene expression patterns, misexpression of genes with temporal and/or spatial control, as well as generation of knockout and knock-in organisms (Gama Sosa et al., 2010). Many of the technologies for generating transgenics in established model organisms have been built up over long periods of time. The establishment and refinement of transgenic technologies is of upmost importance to improve the usefulness of any emerging model organism.

Within cnidarian model organisms transgenesis is currently possible in the genera *Hydra*, *Nematostella* and *Hydractinia*. The Bosch lab were the first to generate a transgenic cnidarian (Wittlieb et al., 2006). They, and others, have subsequently generated many different transgenic *Hydra* lines and these have been used to study gene expression, cell migration and lineage potential, to carry out gain and loss of function experiments, and as a tool to enable sorting of specific cell populations (Khalturin et al., 2007, Anton-Erxleben et al., 2009, Gee et al., 2010, Siebert et al., 2008, Boehm et al., 2013, Bridge et al., 2010, Juliano et al., 2014b, Hemmrich et al., 2012, Khalturin et al., 2008, Franzenburg et al., 2012). The *Nematostella* research community have also had success with the utilisation of transgenic animals and there have now been many well characterised transgenics lines. The first was a muscle specific reporter line generated by the Technau lab (Renfer et al., 2010) and many neural reporters lines generated by the Rentzsch (Richards and Rentzsch, 2014, Nakanishi et al., 2011) and Martindale labs (Layden et al., 2016a). Subsequently, genome engineering techniques as well as inducible expression systems have been established by the Gibson lab (Ikmi et al., 2014).

Transgenic tools were first used in *Hydractinia* to show the developmental potential of migratory cells (Kunzel et al., 2010) and have subsequently been used for gain of function approaches and to study cell migration (Duffy et al., 2012, Bradshaw et al.,

2015, Kanska and Frank, 2013, Millane et al., 2011, Flici, unpublished, Gahan et al., 2016). The improvement of transgenic tools in *Hydractinia* would not only allow for more detailed gain and loss of function approaches but also for more elegant studies on the properties of i-cells and other, interesting cell types.

Intriguingly, the techniques used to generate transgenic animals differ slightly between cnidarian species. In *Hydra* and *Hydractinia*, transgenics can be generated by injecting plasmid DNA into 1-2 cell stage embryos and this is then presumably randomly incorporated into the genome (Kunzel et al., 2010, Wittlieb et al., 2006, Juliano et al., 2014a). In *Nematostella,* this is not possible and instead the insertion cassette is flanked with two I-Sce1 meganuclease cleavage sites and then co-injected with the meganuclease (Renfer et al., 2010). It is assumed that the meganuclease cuts the genome at yet unknown points and allows for integration of the cassette. Unfortunately no study on any cnidarian to date has looked at the integration site or copy number of inserted transgenes although it was shown in the case of the muscle specific nematostella line that integration was at a single site in the genome (Renfer et al., 2010) and single integration sites have also been shown in *Hydra* (Wittlieb et al., 2006, Dana et al., 2012).

3.2. Aims

The aim of this part of my work was to generate new and improve existing transgenic tools in *Hydractinia* including:

1. Development of a ubiquitous reporter.
2. Optimisation of new fluorescent proteins.
3. Generation of “double” transgenic animals carrying both ubiquitous and stem cell specific reporters.
4. Developed transgenic tools to monitor proliferation and cell fate in-vivo

3.3. Results

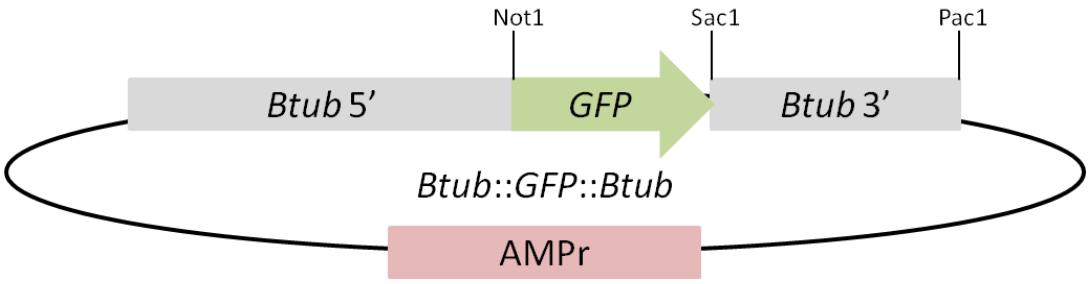


Fig. 3.1: Schematic of the *β-tub*::*GFP*::*β-tub* plasmid.

Previously reported transgenic lines generated using *Actin1*, *ActinII*, *EF1alpha* and β-*tubulin* reporter constructs had failed to drive robust expression in all cell types in adult *Hydractinia* in the hands of the Frank lab (unpublished data). Due to the success of the use of the 3' genomic region of the *Piwi1* gene to generate robust and faithful reporter expression in the adult (Bradshaw et al., 2015) I decided to clone the 3' region of the β-*tubulin* gene to replace the current *ActinII* 3' in the *β-tubulin* reporter plasmid (Flici, unpublished). The plasmid was cloned using a PCR and restriction digest strategy and the resulting plasmid is shown in Fig. 1. This plasmid, known henceforth as *β-tub*::*GFP*::*β-tub* includes GFP downstream of a ~4.5 kb genomic region 5' of the *β-tubulin* start codon and followed by 1.1 kb of genomic sequence 3' of the *β-tubulin* stop codon. This plasmid was used to successfully generate transgenic animals which were analysed by confocal microscopy and GFP expression was seen in all differentiated cell types including epithelial cells, gland cells, neurons, nematoblasts, nematocytes and also putative nematosomes in the gastric cavity in at least some animals (Fig. 3.2). Although this construct is capable of driving expression of GFP in all cell types of the adult, different individuals expressed GFP in different subtypes of cells but all expressed GFP in epithelial cells. In fact many animals had expression restricted only to epithelial cells.

In order to generate more complex transgenics it is essential to utilise multiple fluorescent proteins. To date only enhanced GFP (henceforth GFP) has been used in *Hydractinia*. To this end I cloned and tested a number of red fluorescent proteins in *Hydractinia*. I first cloned both *DsRed2*, which is used in *Hydra* (Dana et al., 2012), as well as *mOrange*, which is used in *Nematostella* (Richards and Rentzsch, 2014, Nakanishi et al., 2011), obtained from Rob Stelle and Fabian Rentzsch, respectively.

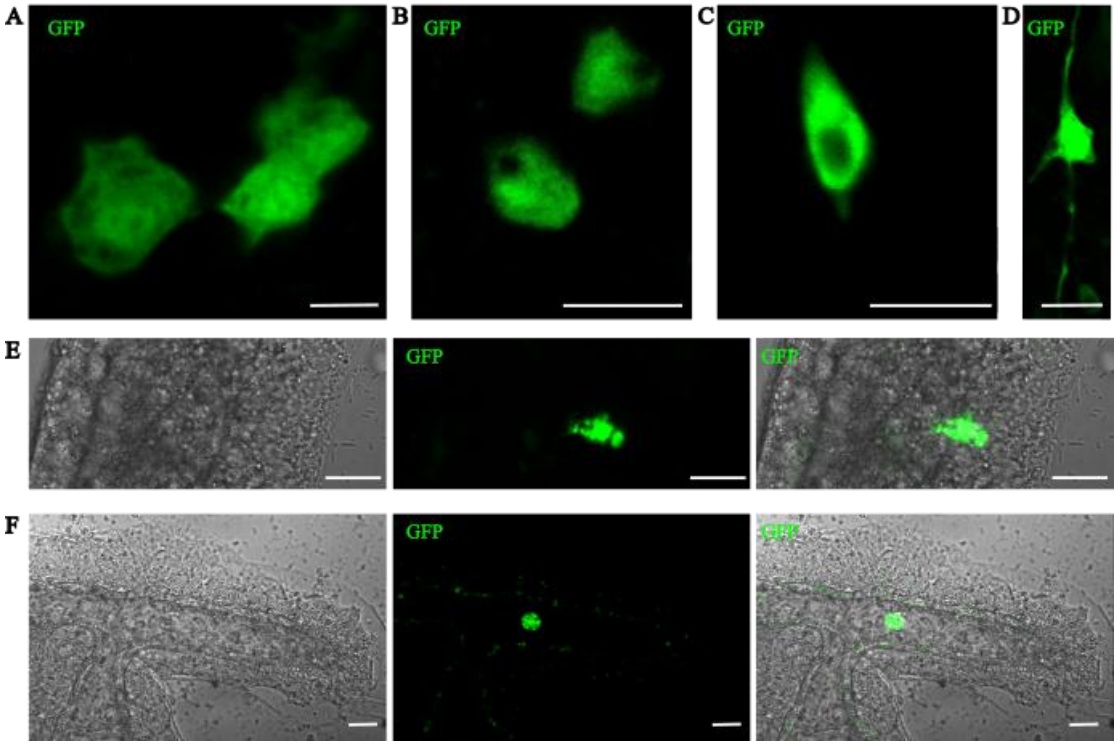


Fig. 3.2: Analysis of *β-tub*::*GFP*::*β-tub* transgenics. Confocal images of live *β-tub*::*GFP*::*β-tub* animals showing epithelial cells (**A**), nematoblasts (**B**), mature nematocyte (**C**), neuron (**D**), gland cell (**E**) and putative nematosomes (**F**). Scale bar: 10μm.

Both genes were cloned into the *β-tub*::*GFP*::*β-tub* plasmid replacing *GFP* to generate *β-tub*::*DsRed2*::*β-tub* and *β-tub*::*mOrange*::*β-tub* plasmids. Although many injections were performed no mOrange^{+} transgenics were ever generated. Utilising the *βtub*::*DsRed2*::*βtub* plasmid I was able to successfully obtain dsRed^{+} animals. These animals, however, tended to have weak fluorescence and this tended to fade over time (Fig. 3.3A). An analysis of the codon usage in the three genes, *GFP*, *DsRed2*, and *mOrange* (Table 3.1), shows that only *GFP* has an AT richness close to that of endogenous *Hydractinia* genes (61.2% compared to 61.34% transcriptome wide, 63.14% across core eukaryotic genes mapping approach (CEGMA) genes: Liam Doonan unpublished data) while *mOrange* has a much lower AT richness (37.8%) (Table 3.1). The *DsRed2* gene used was codon optimised for use in *Hydra* (Dana et al., 2012) and so has a much higher AT richness (69.2%) than that of *Hydractinia*. In order to overcome any possible problems arising from this I codon optimised the *tdTomato* coding sequence according to the *Hydractinia* codon usage

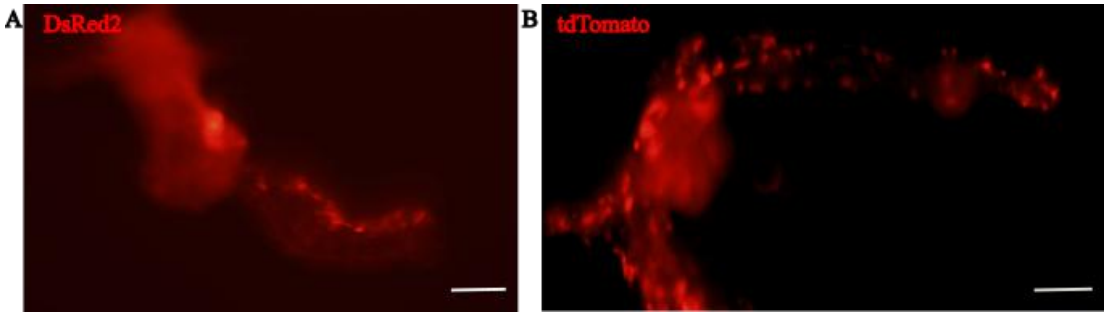


Fig. 3.3: Red fluorescent proteins in *Hydractinia*. Live images of transgenic *β-tub*::*DsRed2*::*β-tub* (**A**) and *β-tub*::*tdTomato*::*β-tub* (**B**) animals. Scale bar: approx. 50μm.

Table 3.1: AT content analysis of fluorescent reporter genes.

	% AT content
Average *Hydractinia* transcriptome	61.43
Average *Hydractinia* CEGMA genes	63.14
GFP	61.2
tdTomato	58.7
mOrange	37.8
DsRed2	69.2

table (obtained from the Kazusa database) and ordered gene-synthesis commercially (Eurofins Genomics). The sequence can be found in Appendix A. I cloned the fragment as described above to generate the *β-tub*::*tdTomato*::*β-tub* plasmid. Injection of this plasmid yielded transgenic animals with bright fluorescence which did not fade over time (Fig. 3.3B).

In order to study cell differentiation in *Hydractinia* in-vivo I aimed to generate a transgenic line expressing GFP under the *Piwi1* promoter and tdTomato under the *β-tubulin* promoter. In order to do this I cloned the entire *Piwi1*::*GFP*::*Piwi1* expression cassette (Bradshaw et al., 2015) into the *β-tub*::*tdTomato*::*β-tub* plasmid

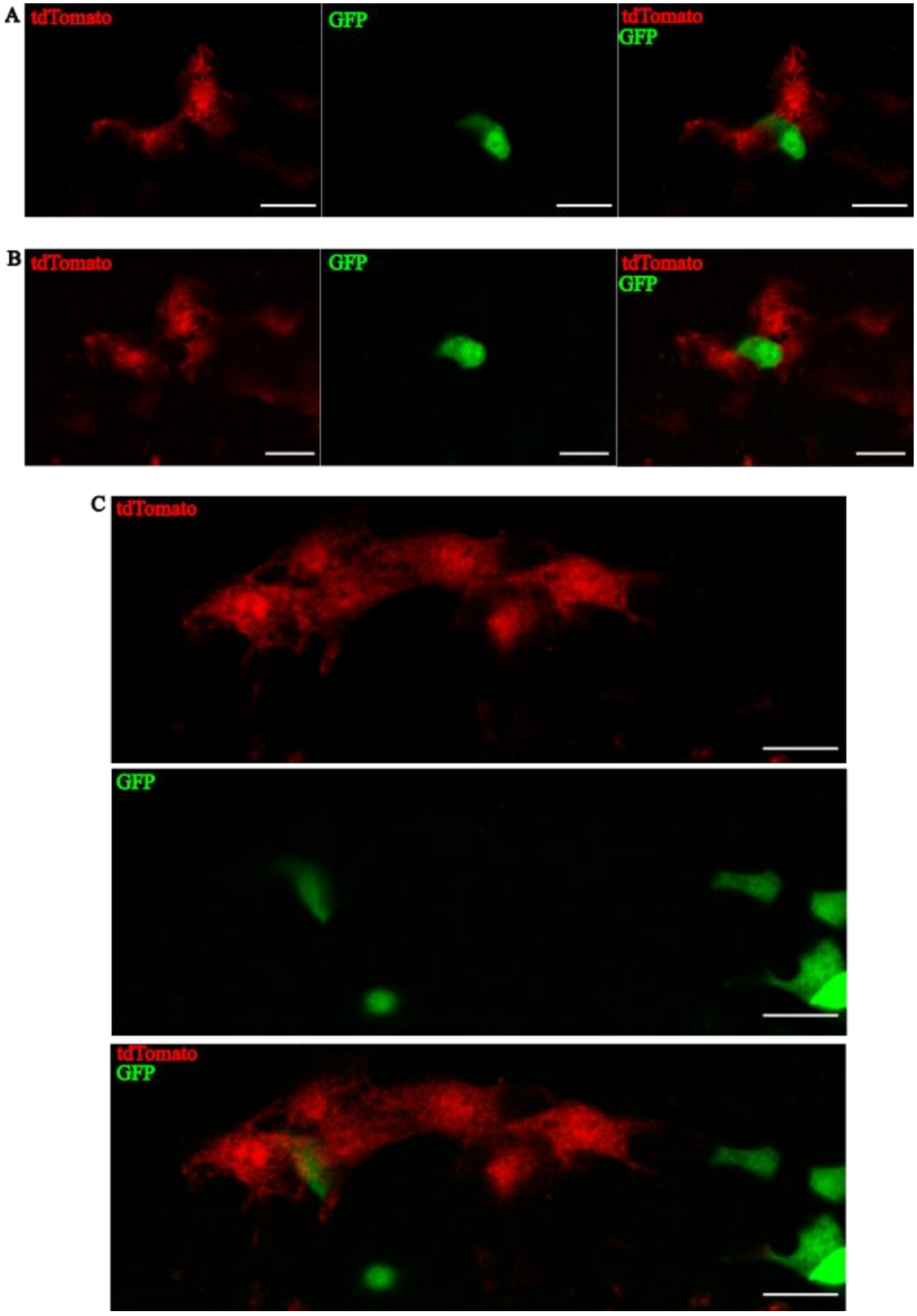

A
tdTomato
GFP
tdTomato
GFP
B
tdTomato
GFP
tdTomato
GFP
C
tdTomato
GFP
tdTomato
GFP

Fig. 3.4

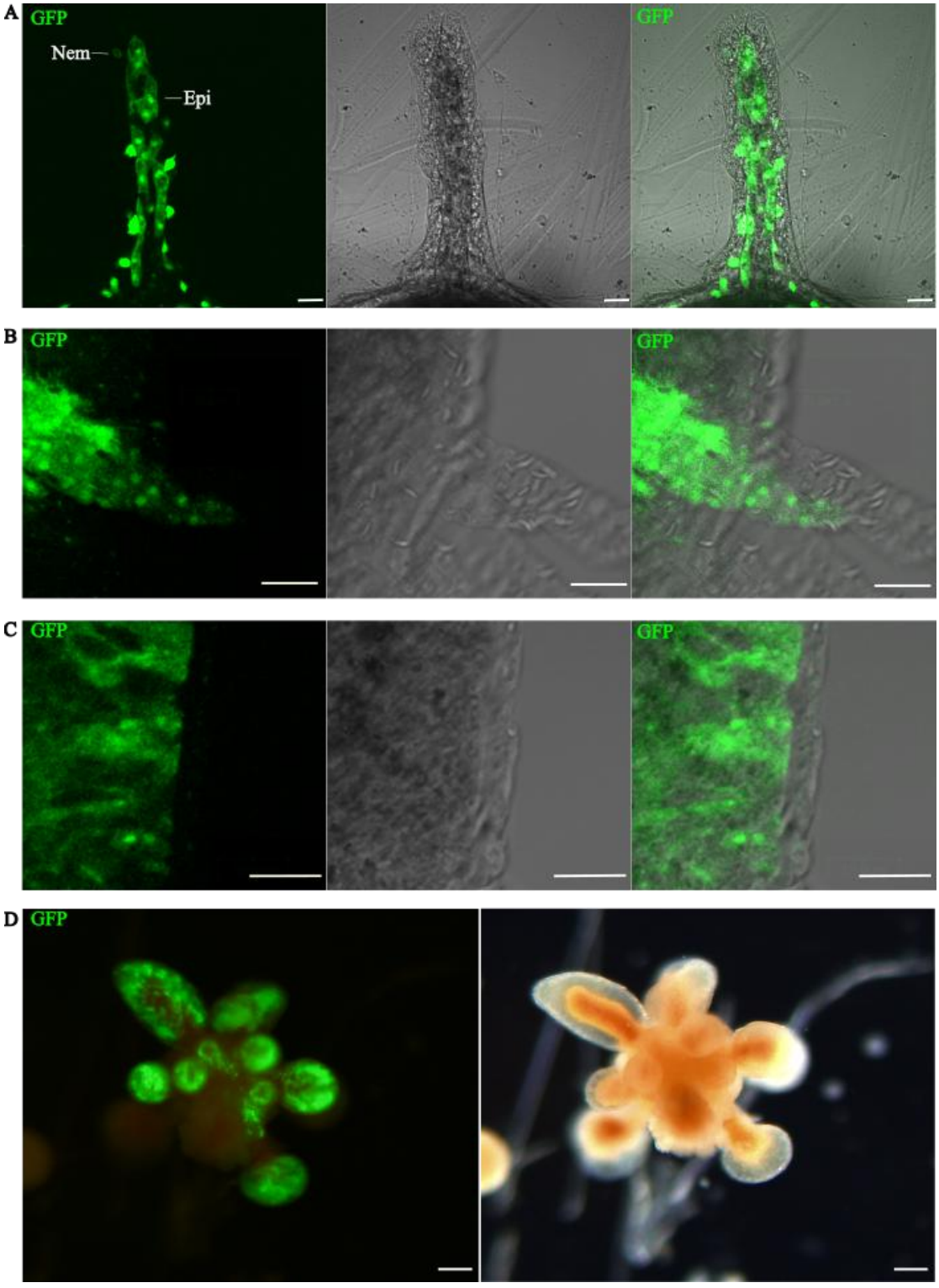


Fig. 3.5

Fig. 3.4: Double transgenic animals. Live confocal analysis of animals generate using the *β-tub*::*tdTomato*::*β-tub*, *Piwi1*::*GFP*::*Piwi1* double plasmid. All images show putative *Piwi1*$^{+}$ i-cells in green residing within the interstitial spaces between epithelial cells. Scale bar: 10μm

Fig. 3.5: Double transgenic animals after several weeks of growth. (**A**) Live confocal analysis of *β-tub*::*tdTomato*::*β-tub*, *Piwi1*::*GFP*::*Piwi1* animal shows expression of GFP in epithelial cells and nematocytes in the stolons. (**B**, **C**) Confocal imaging of fixed polyps from the same animals show GFP in epidermal epithelial cells in the tentacle (**B**) and gastrodermal epithelial cells in the polyp (**C**). (**D**) A mature male sexual polyp from this animal shows bright GFP expression in developing gonads. Nem=nematocyte, Epi=Epithelial cell. Scale bar: 10μm.

so the two reporters were on opposite strands with the 3' regions at the intersection. This plasmid was injected into *Hydractinia* embryos and many lines were generated which expressed tdTomato and not GFP. A single animals was produced which expressed both fluorescent proteins stably. Analysis of this transgenic individual was carried out using both in-vivo confocal (on stolons) as well as analysis of GFP in fixed polyp. In the young post metamorphosis animals the expression of GFP was restricted to i-cells (Fig. 3.4) as previously reported for *Piwi1*::*GFP*::*Piwi1* animals (Bradshaw et al., 2015). In-situ hybridisation as well as antibody staining with a custom-made anti-Piwi1 antibody has confirmed that the endogenous *Piwi1* gene is exclusively expressed in i-cells and germ cells (Bradshaw et al., 2015)(E. McMahon, unpublished data). In my double transgenic animal the expression of tdTomato was restricted to epithelial cells as is common with *β-tub*::*reporter*::*β-tub* constructs (Fig. 3.4). Interestingly, however, as the animals got older, GFP, driven by the *Piwi1* promoter, was no longer restricted to i-cells but was also expressed in other cell types such as epithelial cells and nematocytes in the stolons (Fig. 3.5A), and epidermal and gastrodermal epithelial cells in the polyp and tentacles (Fig. 3.5B,C). This has also been seen in older animals carrying only the *Piwi1*::*GFP*::*Piwi1* cassette (Brian Bradshaw unpublished data). Interestingly, the two transgenes were never co-expressed, i.e. GFP^{+} cells never expressed tdTomato. This suggests two separate integrations with each reporter cassette being expressed in cells with a different insertion event. The plausibility of this hypothesis is supported by other experiments where I co-injected the *β-tub*::*GFP*::*β-tub* plasmid along with the *βtub*::*tdTomato*::*βtub* plasmid and obtained animals expressing GFP in some cells

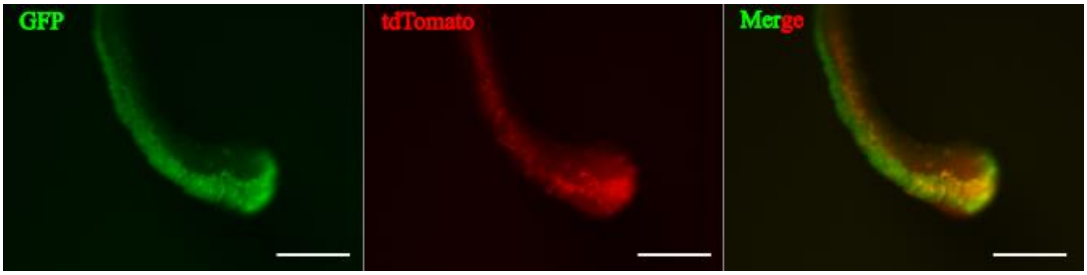


Fig. 3.6: Double transgenic animals generated by co-injections. Live fluorescent images of animals generated form co-injection of *β-tub*::*tdTomato*::*β-tub* and *β-tub*::*GFP*::*β-tub* plasmid.

and tdTomato in others. Interestingly, there were also cells which expressed both proteins suggesting the possibility of multiple integrations or plasmid concatenation (Fig. 3.6).

The double transgenic animal was bred to sexual maturity and turned out to be a male although no offspring were ever generated from this animal. Although by this point the expression of GFP had spread to *Piwi1*$^{-}$ cells there was still a very strong GFP expression in the gonads of the animal, matching what has been seen with mRNA in-situ hybridization and antibody staining (Fig. 3.5D).

Along with generating double transgenics I generated additional transgenic tools for use in *Hydractinia*. I first wanted to test the possible usage of a photoconvertible protein which could be utilized for single cell tracking. For this purpose I chose Kaede, a green to red photoconvertible protein where photoconversion is induced by UV illumination (Ando et al., 2002). This protein has previously been used in *Nematostella* (Amiel et al., 2015). The *Kaede* gene was codon optimised, synthesized and cloned to replace *GFP* in the *β-tub*::*GFP*::*β-tub* plasmid using PCR and restriction digest to generate a *β-tub*::*Kaede*::*β-tub* plasmid. The Kaede sequence can be found in AppendixA. This plasmid was used to generate transgenic animals. These animals displayed strong fluorescent signal, mostly in the green channel, but also weakly in red (Fig. 3.7A). I then tested the conversion by exposing 24 hpf pre-planula to UV illumination for 10 minutes. This resulted in efficient conversion of the green fluorescence to red (Fig. 3.7A, B). These animals were then monitored over the course of 5 days and through metamorphosis (Fig. 3.7B-D). The red fluorescent was maintained for up to 5 days. The return of the green fluorescence was apparent after approximately 24 hours reflecting the synthesis of new Kaede

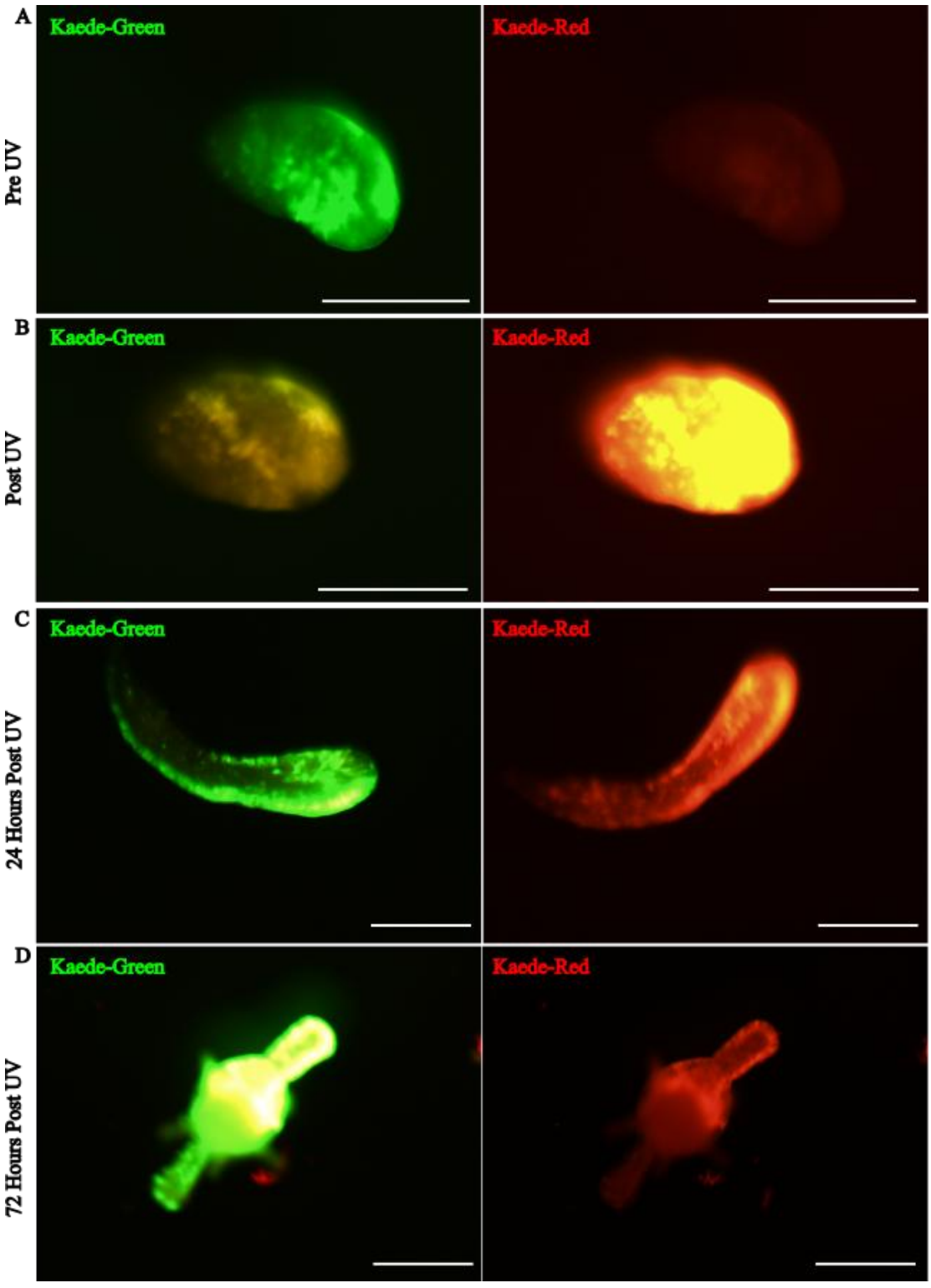

A
Kaede-Green
Kaede-Red
Pre UV
B
Kaede-Green
Kaede-Red
Post UV
C
Kaede-Green
Kaede-Red
24 Hours Post UV
D
Kaede-Green
Kaede-Red
72 Hours Post UV

Fig. 3.7

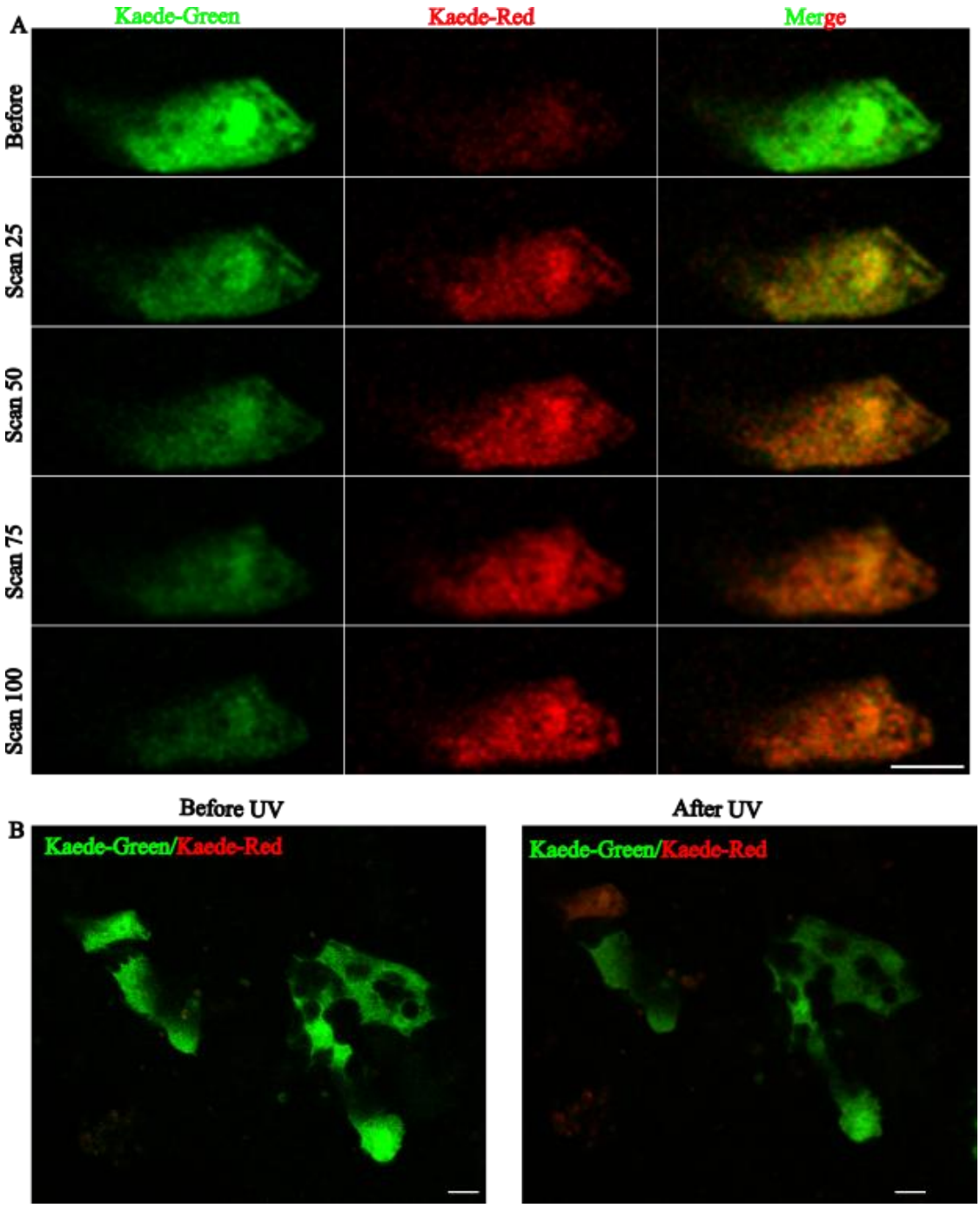

A
Kaede-Green
Kaede-Red
Merge
Before
Scan 25
Scan 50
Scan 75
Scan 100
B
Before UV
After UV
Kaede-Green/Kaede-Red
Kaede-Green/Kaede-Red

Fig. 3.8

Fig. 3.7: Kaede photoconvertible protein in transgenic embryos. (**A**, **B**) Kaede fluorescent signal in 24 hour embryos in the green and red channels before (**A**) and after (**B**) photoconversion. (**C**, **D**) The same animals imaged 24 hours post conversion (**C**) and 72 hours post conversion/24 hours post metamorphosis (**D**). Scale bar: 200μm

Fig. 3.8: Photoconversion of Kaede in single cells. (**A**) A single epithelial cell in the stolon expressing Kaede were scanned 100 times at fast speed at 10% power with the UV laser and images are shown after 0, 25, 50, 75 and 100 scans in both the red and the green channel. (**B**) Live confocal analysis pre and post conversion shows a single labelled cell. Scale bar: 20μm

protein (Fig. 3.7C). I then generated primary polyps carrying the *β-tub*::*Kaede*::*β-tub* transgene. After metamorphosis the expression was not as strong as in the embryo. Utilising the FV1000 confocal microscope I exposed single cells to the UV laser for 100 scans at fast speed on 10% laser power. This resulted in conversion of the green fluorescence to red in these single cells (Fig. 3.8A). The contrast between the converted and un-converted cells was mostly evident when the green and red channels were merged (Fig. 3.8A-B). These cells were distinguishable for up to 3 days.

Finally I aimed to generate a system to monitor individual cells proliferating in live animals which would allow detailed analysis of cell cycle characteristics of individual cells both during homeostasis and during regeneration. To this aim I utilised a previously published system which uses a CyclinB N-terminus GFP fusion protein which is actively degraded during G1 and G0, and reaches peak levels at G2-M phases of the cell cycle (Klochendler et al., 2012). I cloned 390 bp (corresponding to 130 amino acids) of the *Hydractinia* CyclinB N-terminus, into the *β-tub*::*GFP*::*β-tub* plasmid generating the *β-tub*::*CyclinB-GFP*::*β-tub* plasmid. This portion of CyclinB contains the destruction box which is remarkably similar to the destruction box of the sea urchin *Arbacia punctulata* and contains the conserved RXXL motif (Fig. 3.9A). I utilised this plasmid to generate transgenic animals which were analysed at primary polyp stage. The animals were in-vivo imaged for 16 hours using deconvolution microscopy. Fig. 3.9B Shows an example of an epithelial cell which is CyclinB-GFP^{+} and proceeds through mitosis before losing the GFP signal.

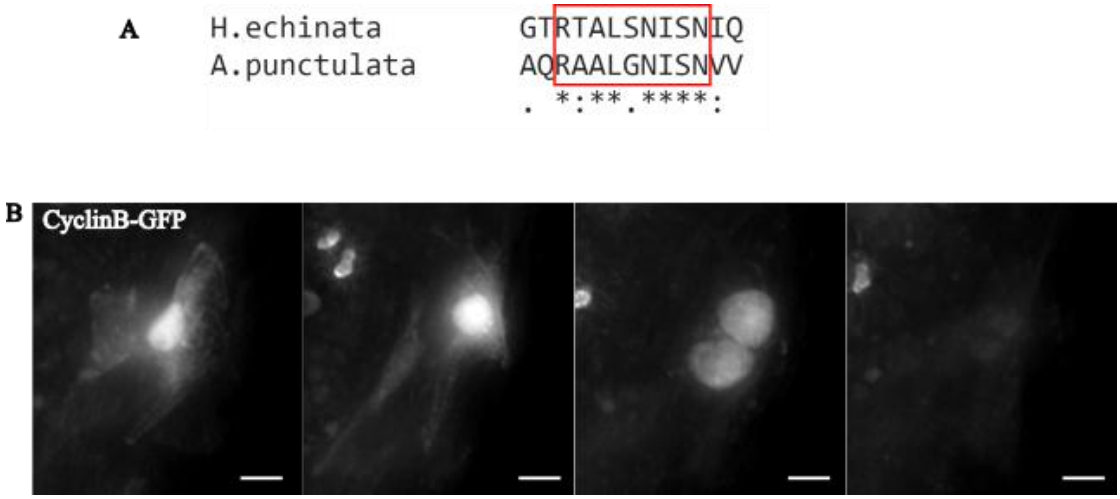


Fig. 3.9: Proliferation marking transgenics. (**A**) Alignment of the *Hydractinia* and *A. punctulata* CyclinB proteins shows a conserved destruction box (Red outline). (**B**) Deconvolution timelapse imaging showing a single CyclinB-GFP+ proliferating epithelial cell. Images were taken every 30 minutes. Scale bar: 10μm.

3.4. Summary

Here I aimed to generate new and improve existing transgenic tools in *Hydractinia echinata.* Currently many of the major questions in the *Hydractinia* community are unanswerable due to a lack of necessary tools. These questions include those concerned with the developmental potential of individual i-cells in *Hydractinia* as well as the relative proliferative contribution of different cell types during homeostasis and regeneration.

Two prerequisites for generating more sophisticated tools in *Hydractinia* are a ubiquitous overexpression system as well as additional fluorescent reporter proteins. Although individual animals expressed GFP in different cell lineages, collectively I have shown that the *β-tubulin* promoter in combination with its 3' genomic region is capable of driving expression in most cell types. I have also shown that the codon-optimised *tdTomato* transgene works efficiently in *Hydractinia*, in contrast to the *mOrange* and *dsRed2* genes utilised in *Nematostella* and *Hydra*, respectively, which both had a *Hydractinia* atypical codon usage. This suggests that strict codon optimisation is required to express transgenes in *Hydractinia* cells and is consistent with the narrow range of AT richness across the animal's genes.

Next I generated double transgenic animals expressing GFP under the *Piwi1* promoter and tdTomato under the control of the *β-tubulin* promoter. The resulting animal generated from this construct initially expressed GFP in putative $Piwi1^{+}$ i-cells but over time the expression of GFP became broader and in older animals GFP was expressed in many $Piwi^{-}$ cells. The cause of this is unknown but it is easy to speculate that this may be due to integration dependent problems with the transgene. Furthermore, epithelial cells expressing GFP never co-expressed tdTomato possibly due to two separate integrations. The feasibility of this scenario is shown by co-injection of two *β-tubulin* plasmids driving GFP and tdTomato, respectively, where it is obvious that independent integrations can happen in the same animal.

Finally I have shown the development of two additional tools to allow the study of cell proliferation and cell fate in *Hydractinia*. I have shown that the Kaede photoconvertible protein and the previously published CyclinB-GFP proliferation marking constructs both work in *Hydractinia*.

These tools, in combination or separately, will be useful for a broad array of approaches including lineage and proliferation tracking and will allow researchers to ask more detailed and difficult questions in *Hydractinia*. With the development of these tools, the major remaining bottleneck impeding the generation of complex transgenic *Hydractinia* lines is the generation of stable, faithful reporter lines. The ”leakiness” of the *Piwi1* and other reporter constructs developed in the Frank lab (unpublished data) remains a problem. The emergence of CRISPR tools in cnidarians and their utilisation in *Hydractinia* (shown in chapter 6) may allow these issues to be overcome.

Chapter 4. The Notch signalling pathway in *Hydractinia echinata*

4.1. Introduction

Notch signalling is an evolutionarily conserved juxtacrine signalling pathway which is thought to have been present in the ancestor of all extant metazoans (Gazave et al., 2009). Notch signalling is utilised throughout animal development in multiple contexts, requiring signalling between neighbouring cells, including boundary formation and patterning, neural development and stem cell-progeny communication as well as in numerous other contexts. Understanding the Notch pathway is therefore essential to understanding the development of animals.

The core pathway consists of three principal components: Notch receptors, DSL (Delta/ Serrate/ Lag-2) ligands and CSL (CBF1/ Su(H)/ Lag1) family transcription factors. Notch ligands belong to one of two evolutionarily conserved families: Delta-like and Jagged-like (Gazave et al., 2009). Notch receptors and ligands are single pass transmembrane proteins and receptor activation occurs between two adjacent cells, one expressing the ligand, the other the receptor, requiring direct cell-cell contact. Upon receptor activation the Notch intracellular domain (NICD) is cleaved and can translocate to the nucleus where it physically interacts with CSL transcription factors. NICD acts as a transcriptional co-activator and activates expression of CSL bound target genes. This is achieved through the formation of a complex with a protein known as Mastermind which acts to recruit chromatin modifiers such as p300 (Bray, 2006, Kopan and Ilagan, 2009, Ntziachristos et al., 2014).

Notch signalling has been studied in both the hydrozoan *Hydra* (Käsbauer et al., 2007, Münder et al., 2010, Prexl et al., 2011, Münder et al., 2013) and the anthozoan *Nematostella* (Fritz et al., 2013, Marlow et al., 2012, Richards and Rentzsch, 2015, Layden and Martindale, 2014). In *Hydra* genes encoding a receptor, *HyNotch* (Käsbauer et al., 2007, Gazave et al., 2009), and a canonical ligand, *HyJagged* (Prexl et al., 2011, Gazave et al., 2009), have been identified. In *Nematostella*, in addition to a single gene encoding the receptor, *NvNotch* (Marlow et al., 2012, Gazave et al., 2009), two ligands have also been identified- one Delta–like, *NvDelta,* and one

Jagged-like (Marlow et al., 2012, Layden and Martindale, 2014, Gazave et al., 2009). A gene encoding a CSL transcription factor has also been described in *Nematostella*, *NvSu(H)*, perturbation of which mimics inhibition of Notch signalling (Marlow et al., 2012, Gazave et al., 2009). Together, the current evidence suggests that the core components of the Notch signalling pathway predate the cnidarian-bilaterian ancestor and that these components have been conserved in both major cnidarian lineages, the Medusozoa and Anthozoa (Gazave et al., 2009)

4.2. Aims

Here I aimed to characterise the components of the Notch signalling pathway as a first step towards understanding Notch function in *Hydractinia*. Specifically I aim to do the following:

1. Utilise BLAST searches into in house transcriptomic/genomic databases to determine the presence/absence and number of core pathway components in *Hydractinia*.
2. Analyse the domain architecture of the pathway components using available online tools.
3. Determine the temporal/spatial expression of *Notch* in *Hydractinia*

4.3. Results

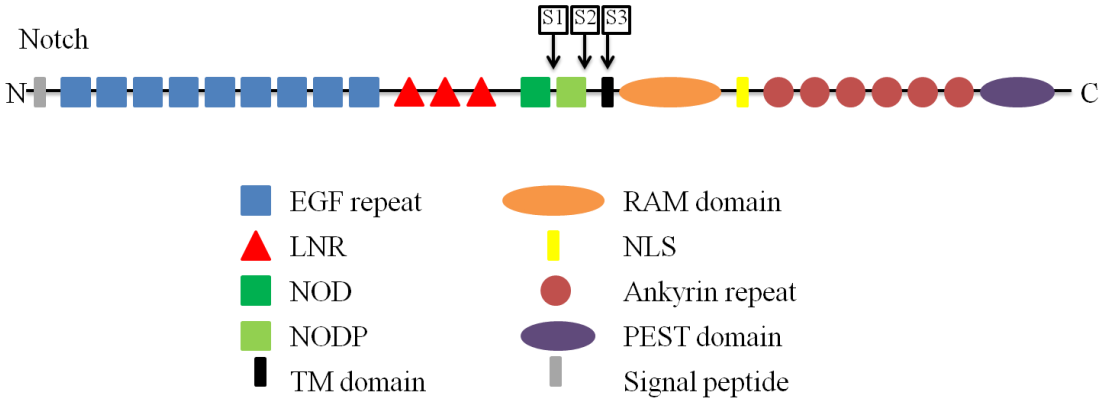


Fig. 4.1. Domain architecture of the *Hydractinia* Notch receptor.

I first searched for putative Notch receptors in *Hydractinia* by utilising tBLASTn searches with Notch receptor sequences, obtained from NCBI, from *Hydra*, *Nematostella*, *Drosophila* and human Notch1 into an in-house transcriptome representing multiple life stages of *Hydractinia echinata* (L. Doonan unpublished). Due to the presence of various common domains within the Notch protein sequence several hits were found and these were filtered using Pfam and pBLAST to identify the domain architecture. Only one gene (hereafter referred to as *Notch*)(transcript sequence can be found in AppendixB) was found to encode a protein containing all the required domains to classify it as a Notch receptor as defined by Gazave and colleagues (Gazave et al., 2009). Pfam, pBlast and Interpro were used to further define the domain architecture of Notch. Signal peptides were predicted using SignalP, transmembrane domains using TMHMM, nuclear localisation signals (NLS) using NLS-mapper, and PEST domains were predicted using PEST-find (Full details in the Materials and Methods, Chapter 2). Fig. 4.1 shows the full domain architecture of *Hydractinia* Notch as evaluated by the above methods. Notch contains nine EGF repeats in its extracellular domain. In addition the extracellular domain contains three LNR domains as well as a NOD and NODP domain. A transmembrane domain is present and the intracellular domain consists of an N terminal NLS, six ankyrin repeat domains and a C-terminal PEST domain. I also predicted S1 (furin convertase), S2 (Adam metalloprotease) and S3 (Presenelin) cleavage sites based on homology to known cleavage sites in other Notch proteins. A RAM (RBP-jk associated molecule) domain could also be predicted based on the

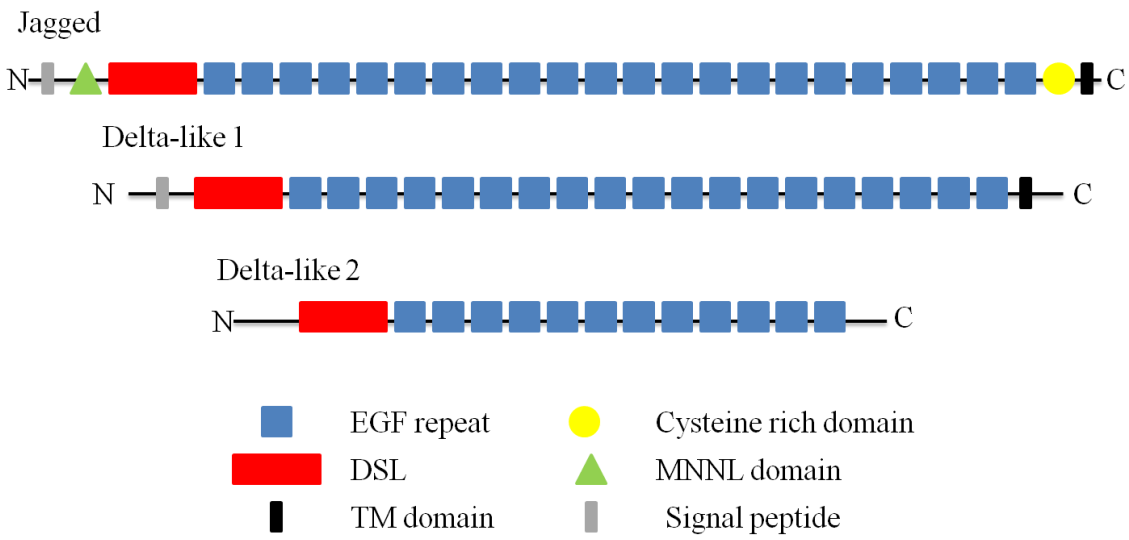


Fig. 4.2: Schematic of the domain architecture of the three *Hydractinia* Notch ligands.

presence of conserved amino acids known to be involved in association with CSL transcription factors (Wilson and Kovall, 2006).

Next, I searched for Notch ligands in *Hydractinia*. In general there are 2 broad families of ligands: Delta like and Jagged like (Gazave et al., 2009). Blast searches were carried out, as above, using NvDelta from *Nematostella*, HyJagged from *Hydra* as well as *Drosophila* Delta and Human Delta1 and Jagged1. Three putative ligands were found in the transcriptome based on the presence both a DSL domain and EGF repeats and analysed as above (transcript sequences can be found in AppendixB). In addition many DSL containing proteins lacking EGF repeats were also found. This has been reported previously in *Nematostella* (Gazave et al., 2009, Marlow et al., 2012) but the function of these genes is unknown. Fig. 4.2 shows schematics of all three proteins. All three contained a characteristic N-terminal DSL domain followed by a varying number of EGF repeats. Delta-like and Jagged-like ligands can be distinguished from each other by the presence of cysteine rich domain N-terminal to the transmembrane domain only in Jagged-like proteins (Gazave et al., 2009). One of the three contained such a domain and, hence, is hereafter refered to as Jagged. This protein also contains an MNNL domain at the N-terminus as well as an N-terminal signal peptide. The transcript encoding for the second ligand, hereafter referred to as Delta-like1, contained both a signal peptide as well as a transmembrane domain in addition to its DSL domain and EGF repeats. The third protein, hereafter referred to as Delta-like2, also contained a DSL domain followed by a number of EGF repeats.

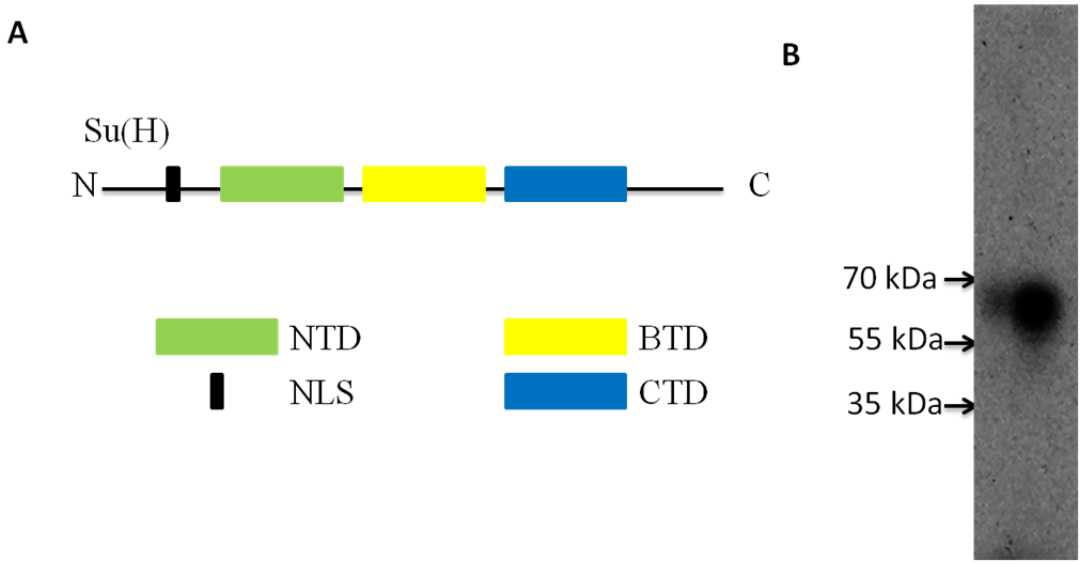


Fig. 4.3: *Hydractinia* Su(H). (**A**) Schematic showing the domain structure of *Hydractinia* Su(H). (**B**) Western blot using anti-Su(H) on whole *Hydractinia* polyp protein extracts.

No evidence was found for an MNNL domain, cysteine rich domain, transmembrane domain or signal peptide. As both Jagged and Delta-like 2 appeared to be lacking a transmembrane domain which would be necessary in the context of a Notch ligand I analysed the genomic sequences corresponding to these genes. Using BLAST, I searched for both genes in the draft *Hydractinia* genome and extracted the contigs containing the respective genes. I then utilised Augustus to build gene models for each contig and analysed the proteins generated for both. In the case of Jagged, a clear TM domain was found to be present. In the case of Delta-like2 this gene model showed no additional domains to those found in the transcript.

In order to search for CSL transcription factors in *Hydractinia* I utilised the same methods as above, using tBLASTn of *Nematostella* Su(H), *Drosophila* Su(H) and human RBP-jk sequences against the *Hydractinia* transcriptome database. Only one CSL transcription factor was found, hereafter known as Su(H) (Fig. 4.3A) (transcript can be found in AppendixB). This protein contains the three diagnostic domains characterising CSL transcription factors: An N-terminal Lag1-like DNA binding domain (NTD), a central B-trefoil domain (BTD) and a C-terminal immunoglobulin-like fold domain (CTD) (Kovall, 2007). This protein had a strong sequence homology with *Drosophila* Su(H) in the C-terminal region. A polyclonal antibody against this region of the *Drosophila* protein is commercially available and was tested in both western blot and immunofluorescence. Although no signal was

detected for immunofluorescence, a single band was present on a western blot at the predicted size of *Hydractinia* Su(H) (approx ~65 kDa) using whole polyp protein extract (Fig. 4.3B).

Finally I searched for a mastermind homolog in *Hydractinia*. I performed blast searches with human and *Drosophila* Mastermind but was unable to find any *Mastermind*-like sequence encoded by the *Hydractinia* transcriptome or genome. In addition, using the NCBI database and online genomic resources, I was able to find a Mastermind homolog in the anthozoans *Nematostella*, *Acropora* and *Aiptasia* but not in *Hydra* or any other hydrozoan.

In order to try to ascertain the biological function of the Notch pathway in *Hydractinia* I aimed to analyse the expression pattern of *Notch*. However, despite many attempted experiments I was unable to obtain *in situ* hybridisation data for *Notch* at either larva or polyp stage. Speculating that its mRNA levels are below detection by *in situ* hybridization I obtained temporal transcriptome data from P. Schiffer (KCL) and C. Scnitzler (NIH), taken from independent experiments (Fig. 4.4). These data showed that *Notch* is expressed across all life stages but, as predicted, at relatively low levels. I then compared *Notch* mRNA levels with *Wnt5a* expression since the latter is known to be undetectable by *in-situ* hybridisation at larva and polyp stages (Hensel, 2013, Hensel et al., 2014, Stumpf et al., 2010). Data provided by C. Schnitzler showed that *Notch mRNA* levels are lower than *Wnt5a*'s (Fig. 4.4B).

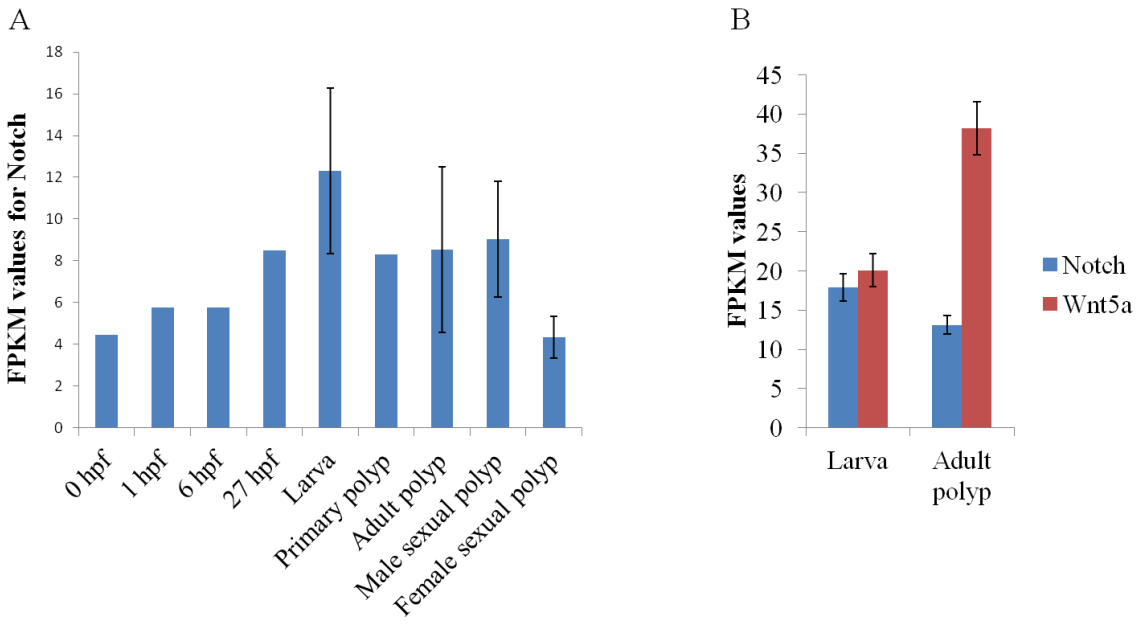


Fig. 4.4: Temporal, quantitative *Notch* expression in *Hydractinia*. (**A**) FPKM values for *Notch* across different life stages (Phillip Schiffer unpublished). (**B**) Comparison of expression of *Notch* and *Wnt5A* in 48hpf larva and adult polyps (Christine Schnitzler unpublished). Error bars, where present, represent standard deviation of FPKM values. Values lacking error bars represent data from single replicates.

4.4. Summary

The *Hydractinia* genome encodes all core components of the Notch signalling pathway, including a single *Notch* receptor, three putative ligands and a single CSL type transcription factor. This is in contrast to a previous report claiming that hydrozoans do not have a bona fide Notch receptor containing all diagnostic domains (Ryan et al., 2013). This discrepancy is due to the use of incorrect gene models in the Ryan et al. analysis. The presence of a single Notch receptor is common in most studied invertebrates with some exceptions. The presence of three ligands, two Delta-like and one Jagged-like, also substantiates previous reports that the divergence of these two ligand families predates the cnidarian-bilaterian ancestor (Gazave et al., 2009). Although only one ligand (*HyJagged*) has been reported in *Hydra*, two have been reported in *Nematostella*, and with the addition of the present data it is parsimonious that *Delta-like* has been lost in the *Hydra* lineage. One *Hydractinia* ligand, Delta-like2 appears to be missing a transmembrane domain. This is also the case for many Delta-like ligands in other organisms (Gazave et al., 2009) but whether this gene is truly missing a domain or is simply misassembled is unknown.

The presence of a single CSL type transcription factor is also typical of all animals studied. The apparent loss of *Mastermind* is, however, more intriguing. As Mastermind has been shown to be recruited by the Notch-CSL complex in all animals studied it is generally considered a part of the core signalling pathway (Kopan and Ilagan, 2009, Bray, 2006, Hass et al., 2015). This is not, however, the first report of loss of Mastermind and it appears to have been independently lost many times throughout evolution (Gazave et al., 2009) which may undermine current thinking of Mastermind as an essential player in the pathway. Studies on the transcriptional activation complex formed on Notch target genes in animals missing *Mastermind* would be necessary to fully evaluate the effect of its loss. Interestingly a synthetic peptide which blocks recruitment of Mastermind to the NICD-CSL complex has been shown to block Notch signalling in *Hydra* (Münder et al., 2013). It is possible that the hydrozoan Mastermind is simply very derived and therefore cannot be found using Blast searches, or that another protein has replaced

mastermind function and binds to the same site on the NICD-CSL complex. Further studies are needed to clarify this point.

I also show here that the *Notch* gene is expressed in all life stage of *Hydractinia*. This data alone is not indicative of function since spatial information is lacking. Low expression levels shown by RNAseq data can be indicative of two scenarios: either few cells express the gene at high levels, or alternatively, the gene is broadly expressed at low levels. If the former were the case, one would expect the gene to be readily detectable by *in-situ* hybridisation. Given that I could not obtain an expression pattern by *in-situ* hybridisation I favour a scenario in which *Notch* is expressed at low levels in many cells. No definitive evidence is yet available to support this.

Chapter 5. Inhibition of γ-secretase in *Hydractinia*

5.1. Introduction

Studies on *Nematostella* and *Hydra* have revealed roles for Notch signalling in both neurogenesis and patterning, particularly tentacle patterning (Richards and Rentzsch, 2015, Käsbauer et al., 2007, Münder et al., 2010, Prexl et al., 2011, Münder et al., 2013, Marlow et al., 2012, Layden and Martindale, 2014, Khalturin et al., 2007). One of the main tools utilised in these studies is DAPT **(**N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a γ-secretase inhibitor (Dovey et al., 2001).

γ-secretase is a multi-protein complex consisting of Presenelins (catalytic subunit), Nicastrin, Aph1 and Pen-2 and acts as an aspartyl protease (Zhang et al., 2013). γ-secretase mediates cleavage of transmembrane proteins to release an intracellular fragment. The most well known targets of γ-secretase are Notch receptors and amyloid-β precursor protein (APP) (Small et al., 2010, Selkoe and Kopan, 2003).

The γ-secretase complex is required for activation of the Notch signalling pathway. Ligand binding to the Notch receptor leads to the exposure of a cleavage site on the extracellular part of Notch, adjacent to the transmembrane domain. This is then cleaved by ADAM metalloproteases, a process known as ectodomain shedding. Once the extracellular domain has been cleaved the Notch receptor is then available for cleavage by γ-secretase. Cleavage by γ-secretase leads to release of the Notch intracellular domain (NICD) into the cytoplasm and subsequent downstream activation of Notch target genes (Kopan and Ilagan, 2009). Notch ligands are also single pass transmembrane proteins and are processed in a similar manner and can be substrates for γ-secretase (LaVoie and Selkoe, 2003).

Many studies have shown that γ-secretase inhibition by DAPT mimics Notch loss of function and it is clear that inhibition of γ-secretase can block activation of Notch signalling by preventing release of NICD and its translocation to the nucleus (Geling et al., 2002, Micchelli et al., 2003). This was confirmed in *Hydra* where DAPT could block a constitutively active form of HyNotch where the extracellular domain was removed allowing it to be automatically cleaved by γ-secretase (Käsbauer et al.,

2007). Although it is clear that DAPT is capable of effectively blocking Notch signalising it is also clear that γ-secretase has many other substrates (Haapasalo and Kovacs, 2011) and therefore it cannot be assumed that a phenotype caused by DAPT treatment is linked to Notch signalling without confirmation by genetic studies.

5.2. Aims

Here I aimed to study the effect of γ-secretase inhibition by DAPT in *Hydractinia* in order to gain an understanding of the potential roles played by Notch signalling during embryogenesis, development and regeneration. Specifically I wanted to:

1. Study the effect of DAPT on adult and embryonic neurogenesis.
2. Evaluate the effect of DAPT on development and patterning.
3. Analyse the effect of DAPT treatment on head regeneration.

5.3. Results

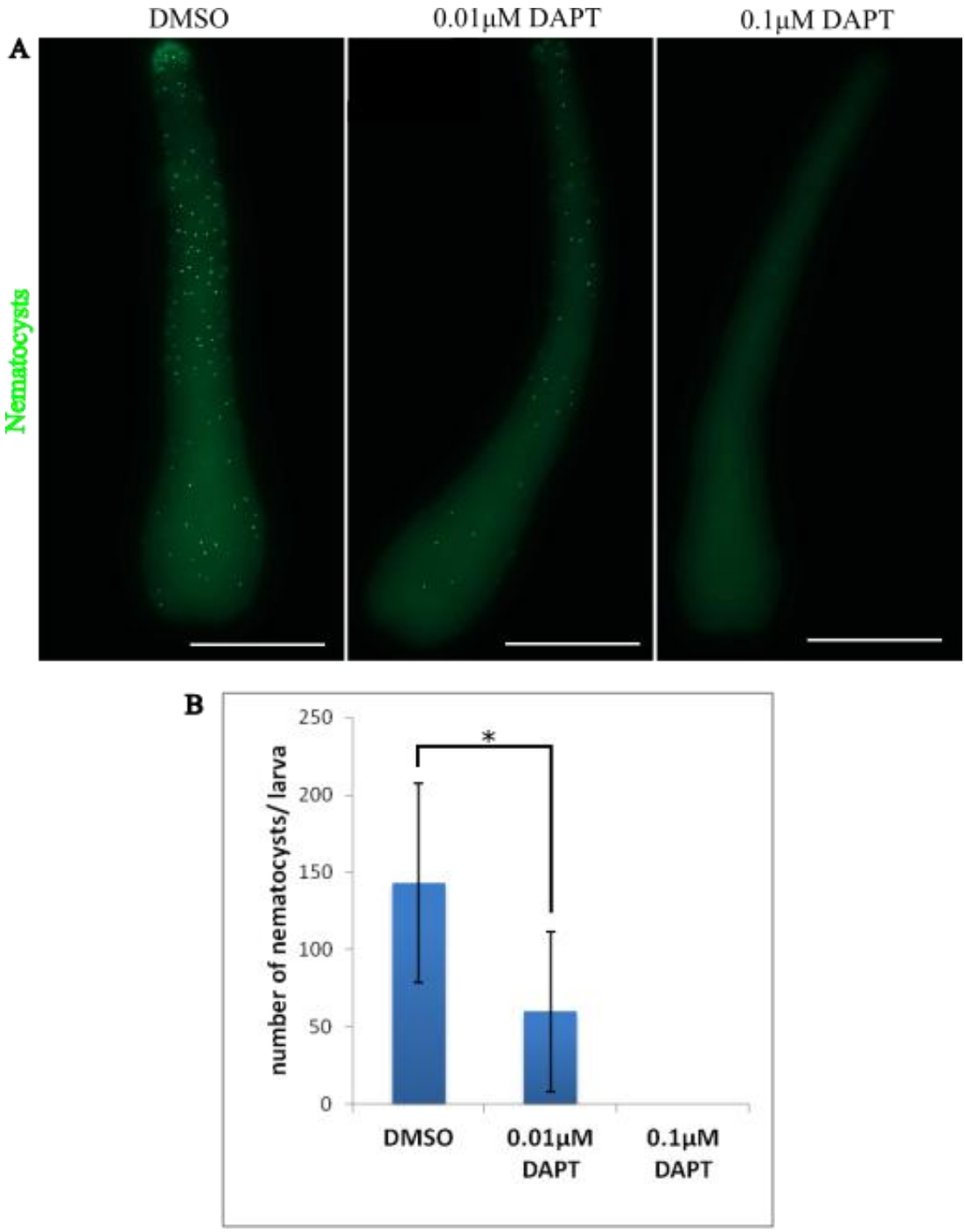


Fig. 5.1. DAPT treatment blocks nematogenesis. (**A**) Embryos were treated for 48 hours with DAPT show a dose dependent decrease in poly-γ-glutamate^{+} nematocysts. (**B**) Quantification of poly-γ-glutamate^{+} nematocysts. Data represent three independent biological replicates. Scale bar: 200 µm. * $p<0.05$

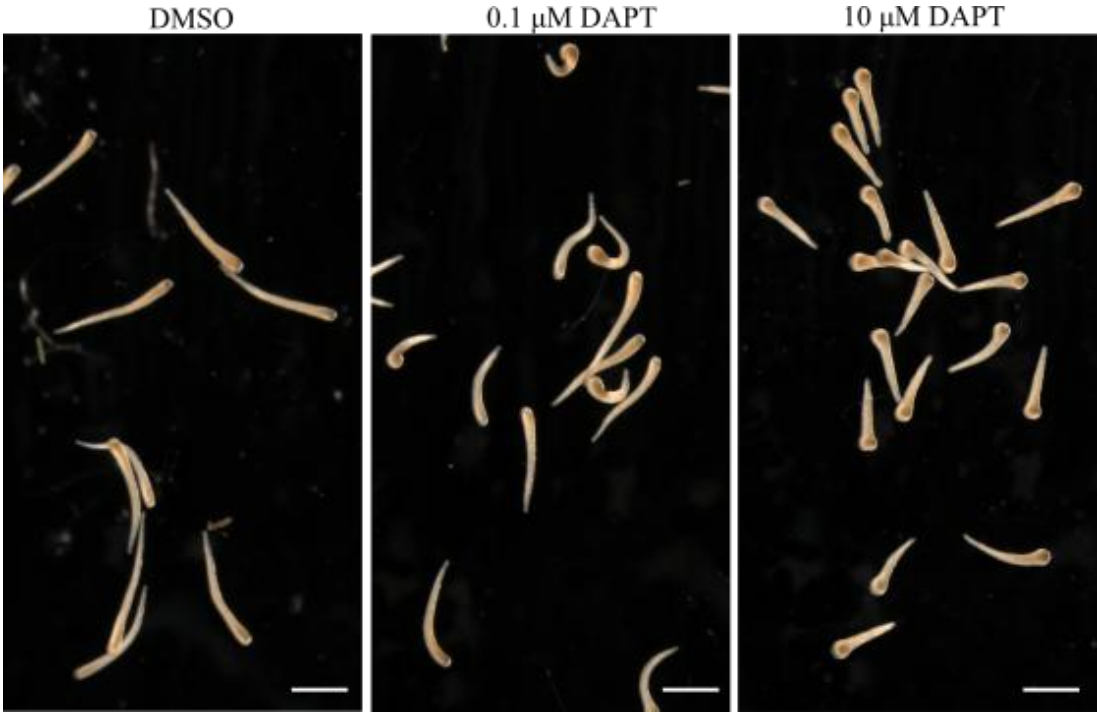


Fig. 5.2. DAPT causes a growth defect during embryogenesis. Animals treated with 10 μM DAPT from 2-72 hpf are shorter than treatments at lower DAPT concentrations or controls. Scale bar: 500 μm.

In order to characterise the effect of γ-secretase inhibition on *Hydractinia* development I began by treating animals during embryogenesis. I used a concentration gradient from 0.01 μM to 10 μM DAPT and treated animals from 1-2 to 48 hours post fertilisation (hpf). I then analysed the number of nematocysts in animals using a modified DAPI staining protocol (Szczepanek et al., 2002) which stains for poly-γ-glutamate which accumulates in nematocysts during maturation. At a concentration of 0.1 μM DAPT there was a complete loss of mature poly-γ-glutamate^{+} nematocysts. This confirms previous studies done in *Nematostella* (Marlow et al., 2012, Richards and Rentzsch, 2015) and *Hydra* (Käsbauer et al., 2007, Khalturin et al., 2007). The effect of DAPT was dose dependent with 0.01 μM treatments resulting in a reduction but not complete loss of capsules (Fig. 5.1). In addition to this phenotype, higher concentrations of DAPT, 10 μM, resulted in shorter larva comparing to control animals treated with DMSO only or animals treated with 0.1 μM DAPT (Fig. 5.2). Because of the two different effects at different concentrations I used 0.1 μM and 10 μM DAPT for all other experiments on embryos.

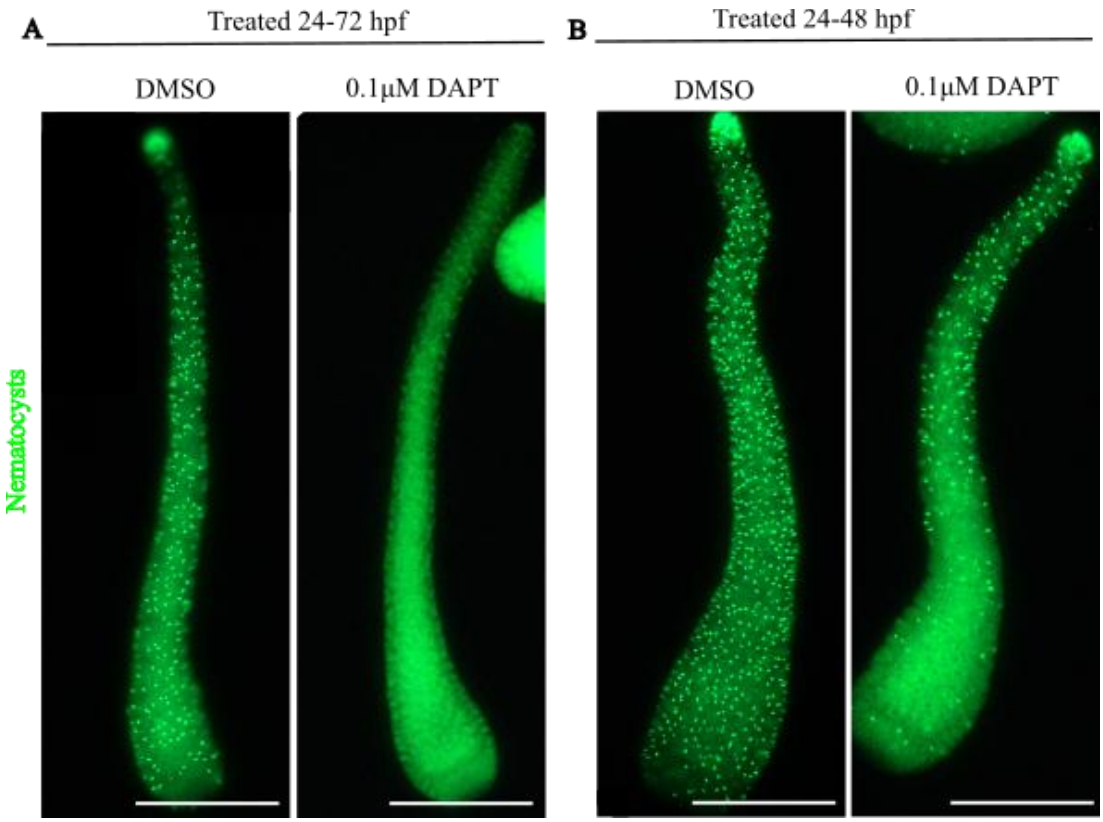


Fig. 5.3. DAPT treatment is reversible. (**A**) Animal treated from 24-72 hpf with 0.1 µM DAPT shows a complete loss of poly-γ-glutamate $^{+}$ nematocysts. (**B**) Removal of DAPT leads to a return of poly-γ-glutamate^{+} nematocysts after 24 hours, predominantly in the oral region. Scale bar: 200 µm.

In order to further characterise the effect of DAPT on nematocyst development I started treatments at different time points during embryonic development. I found that starting treatment at 24 hpf gave the same phenotype and that this phenotype persisted for up to 72 hours, indicating a complete block as opposed to a delay in nematogenesis (Fig. 5.3A). To test the reversibility of this phenotype, I treated animals from 28-48 hpf, at which point they were poly-γ-glutamate^{+} nematocyst free. I then terminated the treatment and left animals to further develop in seawater for a further 24 hours. This resulted in return of poly-γ-glutamate^{+} nematocysts in the larva; however, most of these were restricted to the oral end where the larva is continuously growing while the aboral end of the larva appeared to have relatively few nematocysts (Fig. 5.3B). Together, this data indicates that DAPT has a profound effect on the number of mature nematocysts.

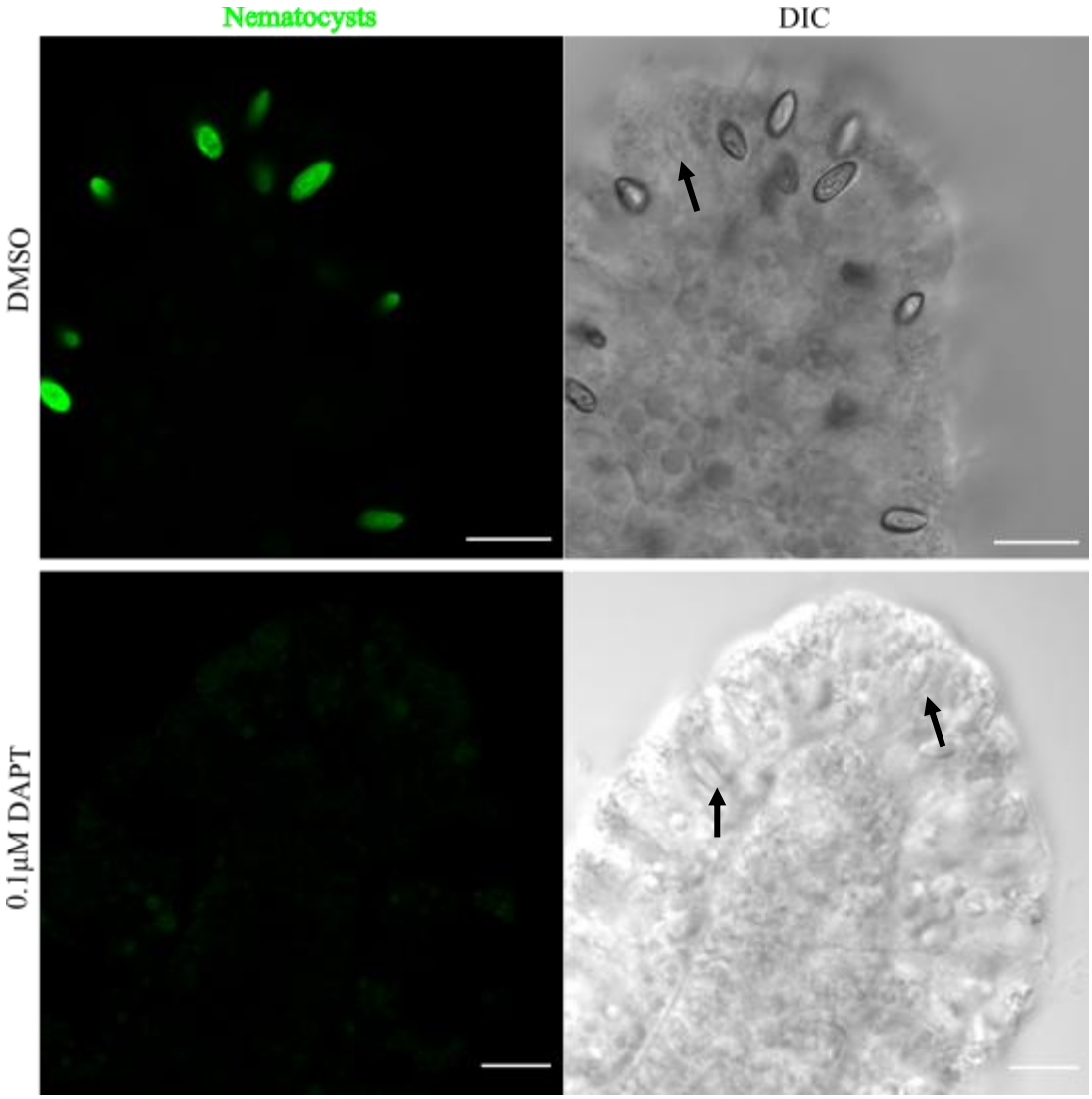


Fig. 5.4. DAPT treatment leads to a block in epidermal nematogenesis. DAPT treated animals have no poly-γ-glutamate^{+} nematocysts in the tail but do have immature poly-γ-glutamate^{-} nematocysts in the epidermis (arrows). Scale bar: 10 µm.

I next aimed to assess the stage of nematogenesis affected by DAPT treatment. For this I performed confocal DIC analysis on 0.1 µM DAPT treated and control larva. As shown above, this treatment resulted in the complete elimination of poly-γ-glutamate^{+} namtocysts. However, DIC imaging revealed immature poly-γ-glutamate^{-} capsules in the epidermis of these animals (Fig. 5.4). To confirm this I stained animals with anti-Ncol3 antibody. Although Ncol3 is present in the nematocyst capsule at all stages of maturation the epitope is only recognisable by the antibody during early stages prior to final maturation due to the large scale condensation of

the capsule wall during maturation (Zenkert et al., 2011, Tursch et al., 2016). Analysis of 0.1 μM DAPT treated larva showed that early nematogenesis, occurring

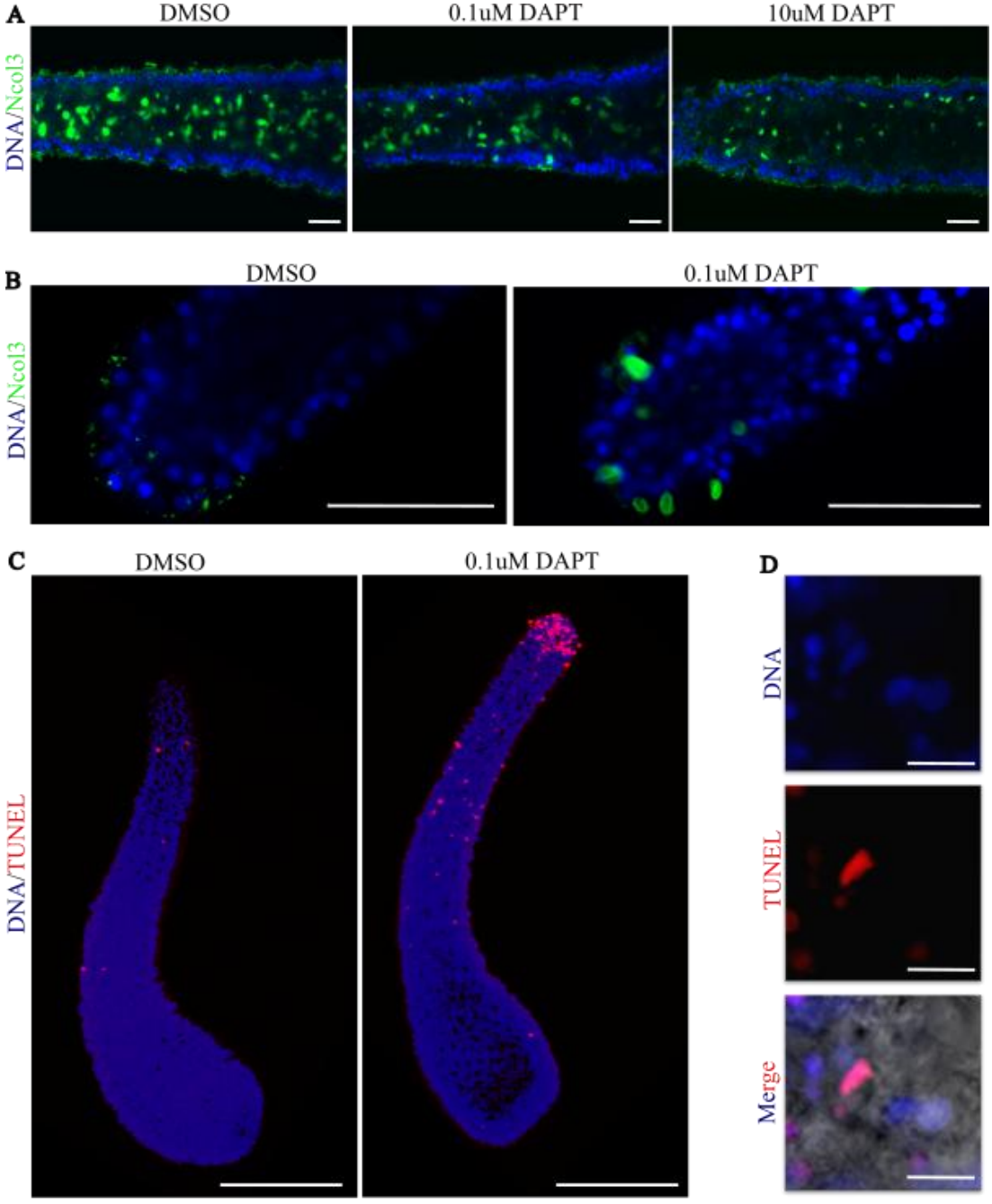


Fig. 5.5. DAPT blocks capsule maturation and causes nematocytes to undergo apoptosis. (**A**,**B**) Ncol3 staining of embryos treated from 0-48 hpf with DMSO, 0.1 DAPT or 10um DAPT. showing the endoderm (A) and oral epidermis (B). (**C**) TUNEL staining of larvae treated from 0-48hpf with either DMSO or 0.1u DAPT. (**D**) Close up of the oral part of a larva treated from 0-48hpf with 0.1 μM DAPT. Scale bar: 10 μm (A, D), 50 μm (B) 200 μm (C).

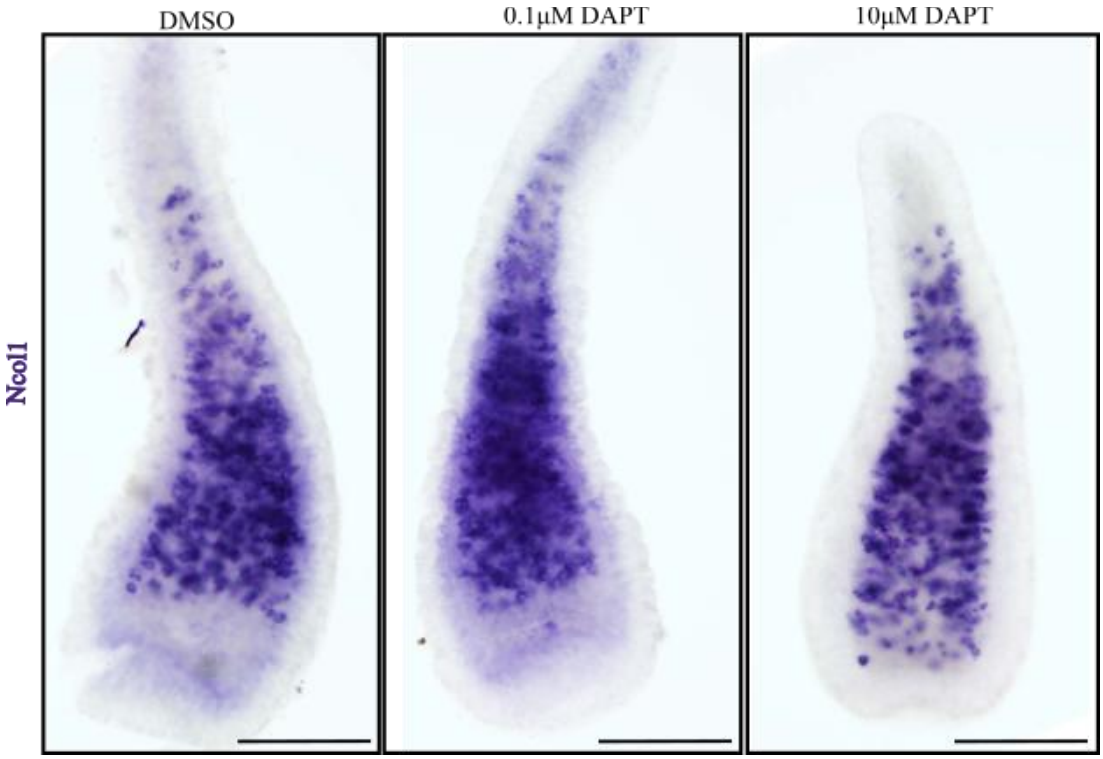


Fig. 5.6: DAPT treatment does not affect *Ncol1*$^{+}$ nematoblasts. Embryos were treated from 1-48 hpf with DMSO, 0.1 μM DAPT or 10 μM DAPT. At 48hpf they were stained for *Ncol1* by in situ hybridisation. Scale bar: 100 μm.

within the larval endoderm, was unaffected (Fig. 5.5A). Analysis of the oral end of the larva, however, revealed a build up of immature Ncol3^{+} nematocysts in the epidermis (Fig. 5.5B). 10μM DAPT treatment led to an even more profound defect on nematogenesis and developing nematocysts within the gastrodermis appeared to be drastically reduced and the staining was no longer in capsule like structure but rather more punctate (Fig. 5.5A), indicating an earlier arrest of capsule development. Finally, using TUNEL staining I could show that apoptotic cells accumulate in the same region where immature capsules are building up in 0.1 μM DAPT treated animals (Fig. 5.5C). Some of these TUNEL^{+} nuclei localised adjacent to developing nematocysts, further suggesting that these cells are late differentiating nematoblasts (Fig. 5.5D). Taken together, DAPT has a dose dependent effect on nematocyst maturation. Lower concentrations lead to a block in late stage maturation, following migration into the epidermis, while higher concentrations lead to a block in early capsule formation.

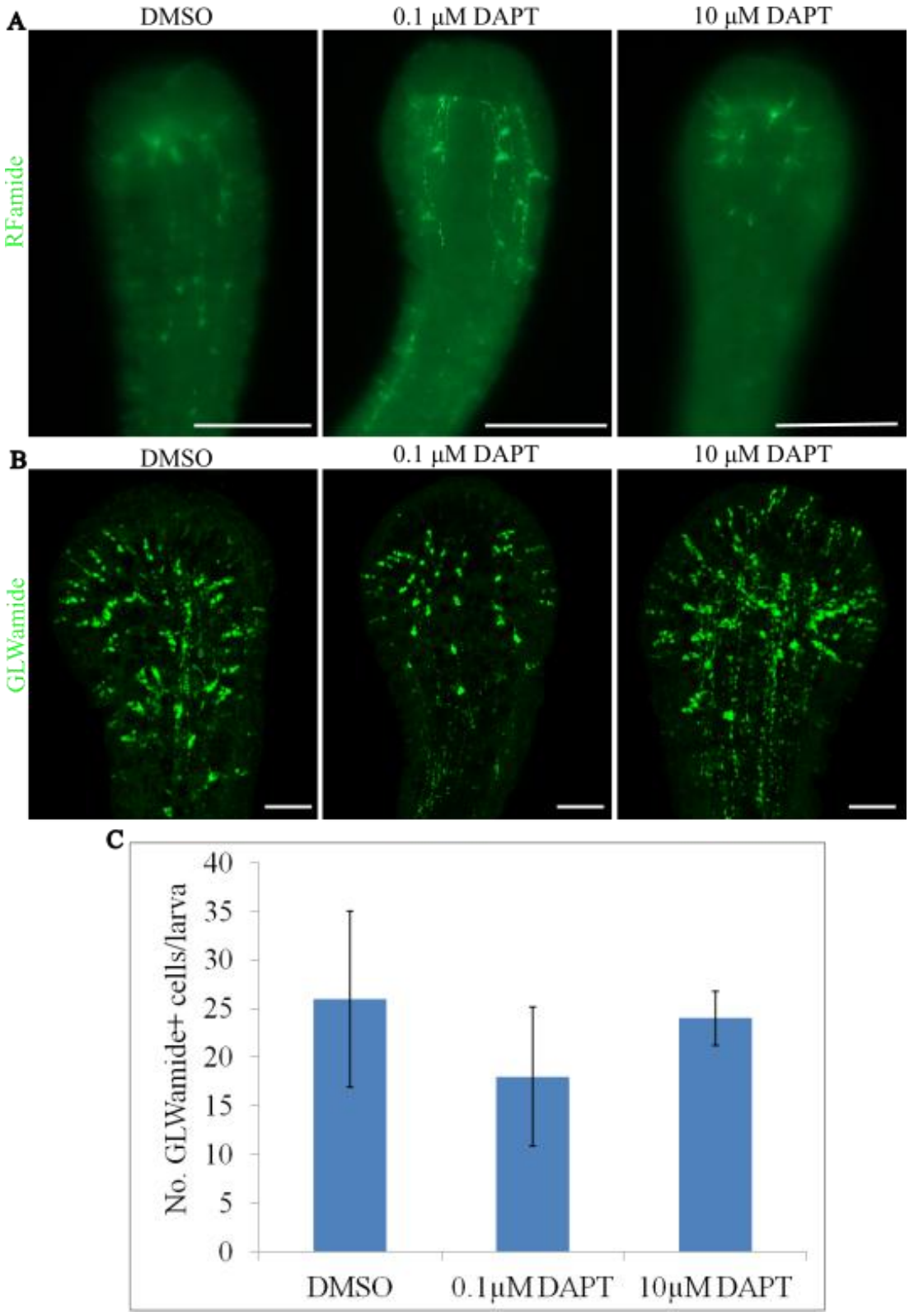


Fig. 5.7. DAPT does not affect embryonic neurogenesis. (**A-B**) Animals treated 0.1uM DAPT or 10uM DAPT show no obvious defects on RFamide (A) or GLWamide (B) positive neurons in the larva. (**C**) Quantification of GLW positive neurons.. Representative of three independent biological replicates

As Notch signalling inhibits neuronal differentiation in many animals, including anthozoan cnidarians (Layden and Martindale, 2014, Richards and Rentzsch, 2015, Marlow et al., 2012), I looked at markers of committed and mature neural cells in DAPT treated animals. I first looked at the effect of DAPT on *Ncol1*, a post mitotic nematoblast marker, by *in situ* hybridisation. No apparent effect of DAPT treatment at any concentration on *Ncol1* expression could be observed, despite the pronounced defects on later stages of nematocyte differentiation (Fig. 5.6). I then went on to look at two different neuronal populations, RFamide$^+$ neurons and GLWamide$^+$ neurons, by immunofluorescence. Consistent with previous work done on *Hydra* (Käsbauer et al., 2007, Khalturin et al., 2007), there was no visible defect on either neuronal population in DAPT treated animals at any concentration tested (Fig. 5.7A,B). Counting of GLWamide+ neurons revealed no statistically significant difference in the number of cells between DAPT treated larva and controls (Fig. 5.7B). This data indicates that DAPT treatment does not affect commitment of neural cells or neuronal differentiation.

Given the defects seen during embryogenesis I wanted to extent my studies to adult life stages, beginning with metamorphosis. Animals were treated with DAPT after metamorphosis induction with caesium chloride and were stained for neural markers two-three days later. Ten μM DAPT treatment led to a strong defect in tentacle morphogenesis in primary polyps (Fig. 5.8A,B). There was also a drastic reduction in the number of poly-γ-glutamate$^+$ nematocysts (Fig. 5.8A). Lower, 0.1uM, DAPT concentration had no effect on either of these parameters (Fig. 5.8B, data not shown). Interestingly, although tentacle morphogenesis was blocked following 10 μM DAPT treatment, these animals still formed a normal oral nerve ring as shown by anti-acetylated tubulin immunofluorescence (Fig. 5.8A) and *in situ* hybridisation for *Rfamide precursor* (Fig. 5.8C). This indicates a similar effect of γ-secretase inhibition in the adult nervous system with neurogenesis being unaffected but nematogenesis being blocked as well as a role for γ-secretase in tentacle patterning.

I then analysed the effect of DAPT treatment on adult polyps during both tissue homeostasis and regeneration, first looking at nematogenesis. As adult polyps already have large number of mature, poly-γ-glutamate$^+$, nematocysts I treated polyps for five days with 10 μM DAPT along with daily feeding to deplete mature nematocysts. After five days there was a drastic reduction in the number of poly-γ-

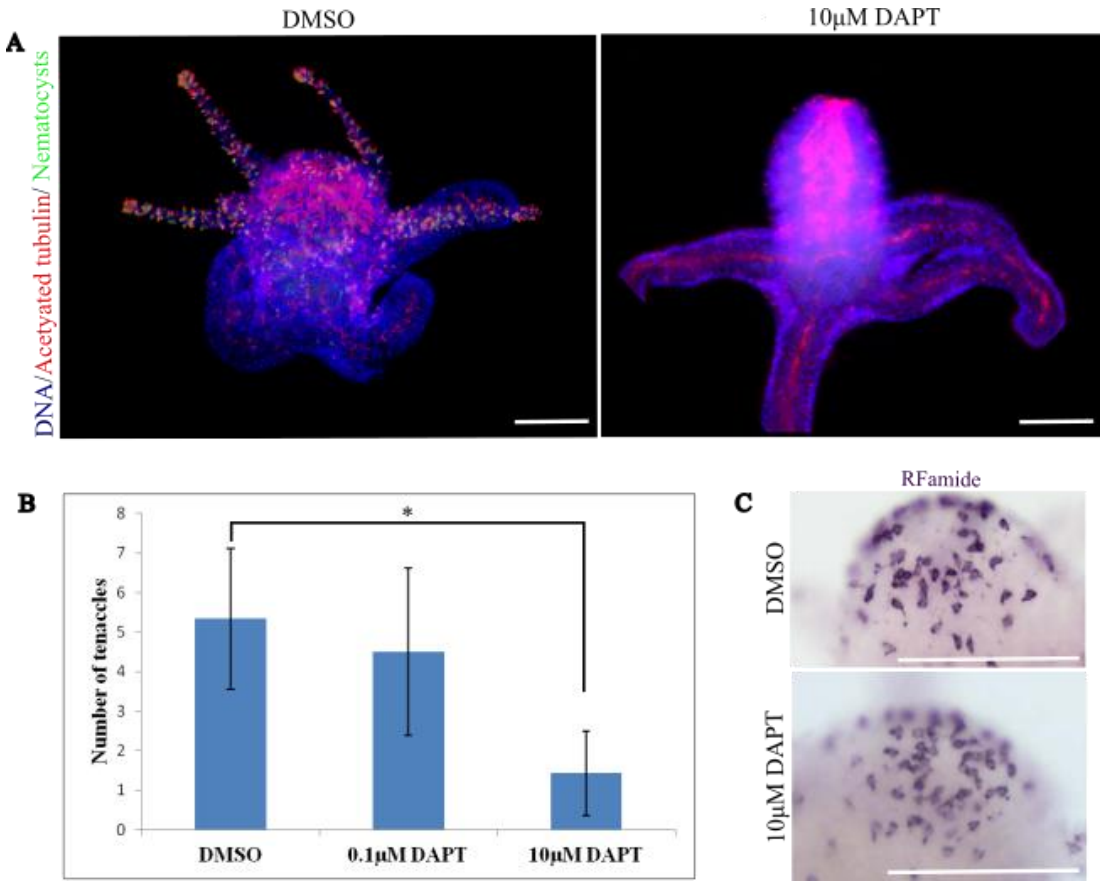


Fig. 5.8. DAPT blocks tentacle formation but not oral neurogenesis during metamorphosis. (**A**) Animals were treated with wither DMSO or 10 µM DAPT after induction of metamorphosis for two-three days and then stained with DAPI to visualize nematocysts (Green) and for acetylated tubulin (Red). (**B**) Counting of tentacles shows that 10 µM but not 0.1 µM DAPT leads to a reduction in tentacle number. (**C**) In situ hybridisation for *Rfamide* confirms the presence of an oral nerve ring in DAPT treated animals. *p<0.0001. Scale bars: 100 µm.

glutamate $^{+}$ nematocysts (Fig. 5.8A), consistent with other life stages. To analyse the stage of nematogenesis affected I treated animals with 10 µM DAPT for two days and stained for Ncol3. In control animals the anti-Ncol3 antibody stained distinct developing nematocyst capsules. In 10 µM DAPT treated animals, on the other hand, few capsule like structures were visible and Ncol3 staining appeared fragmented and punctuate (Fig. 5.9B). This is strikingly similar to the effects of DAPT reported in other cnidarians on capsule maturation (Käsbauer et al., 2007, Marlow et al., 2012, Richards and Rentzsch, 2015) and that seen during embryogenesis (Fig. 5.5A). In addition I looked at *Ncol1* expression by *in situ* hybridisation. Here, different to what was observed in larva, there is a large decrease in *Ncol1* staining in DAPT treated

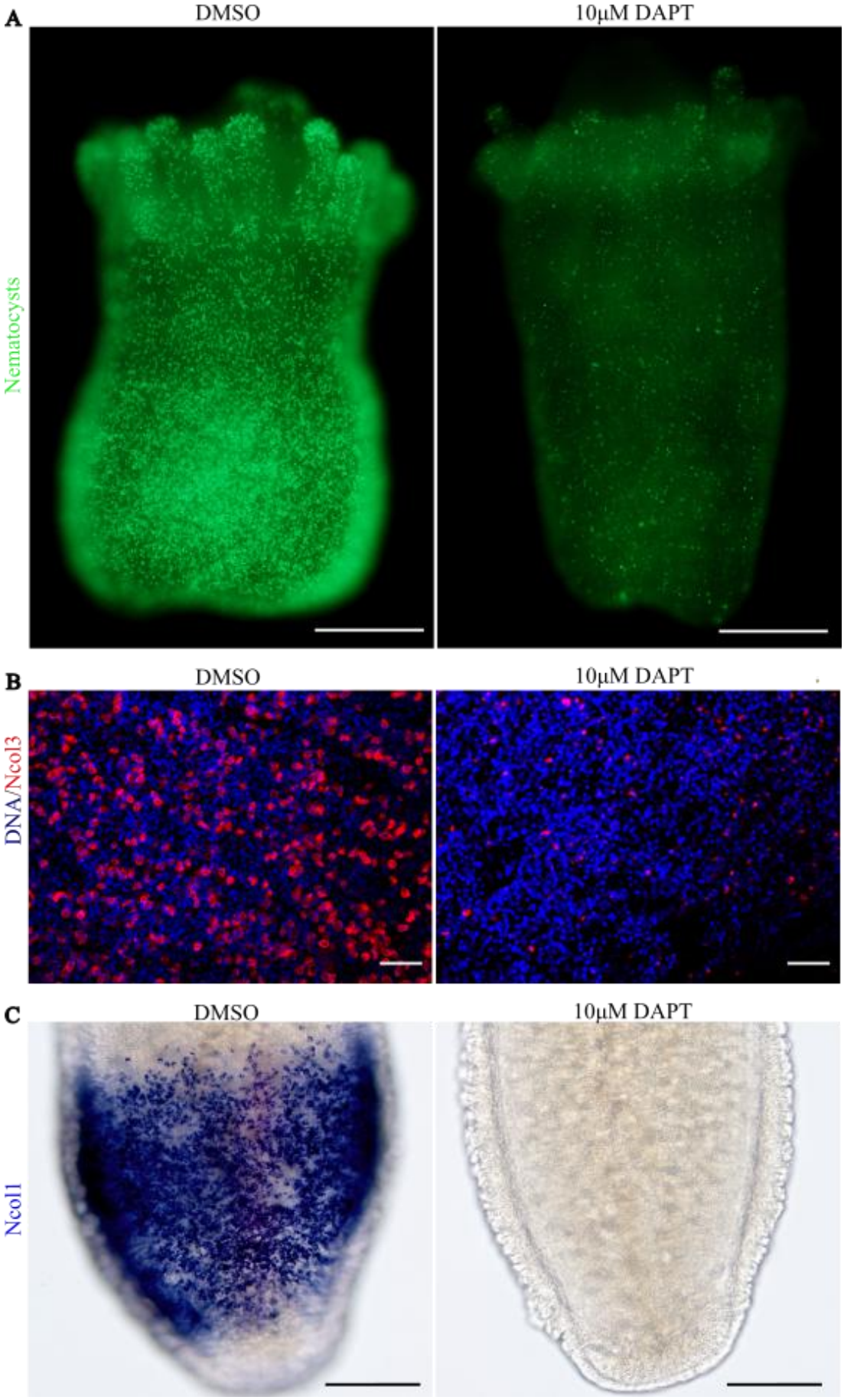

A
DMSO
10μM DAPT
Nematocysts
B
DMSO
10μM DAPT
DNA/Ncol3
C
DMSO
10μM DAPT
Ncol1

Fig. 5.9

Fig. 5.9. DAPT blocks nematogenesis in adult polyps. (**A**) Polyps treated by DAPT for five days and fed every day have a reduction in $DAPI^+$ nematocysts. (**B**) Polyps treated for 24 hours with DAPT show a loss of maturing Ncol3+ nematocysts. (**C**) Treatment with 10 μM DAPT leads to a reduction in *Ncol1*$^+$ nematoblasts. Scale bar: 200 μm (A,C) 10 μm (B).

animals (Fig. 5.9C). The effect on *Ncol1* expression was variable between a reduction and a complete loss of staining. I also performed this staining during regeneration and I also saw a decrease in *Ncol1* staining in DAPT treated animals as well as a decrease in developing Ncol3+ capsules (Fig. 5.10).

Next, to assess the effect of DAPT on head regeneration I decapitated animals and treated them with 10 μM DAPT starting immediately after decapitation. *Hydractinia* polyps can regenerate a functional head within 72 hours post decapitation including a newly formed oral nervous system (Bradshaw et al., 2015). Like in primary polyps, DAPT treatment led to a drastic reduction in the number of tentacles formed (Fig. 5.11A). Strikingly however, the hypostome appears to be intact in these animals (Fig. 5.11). Additionally, staining for acetylated-tubulin showed an intact, regenerated oral nerve net (Fig. 5.11B). DAPI staining also showed mature nematocysts in the regenerated mouth region but whether these matured during the DAPT treatment is unclear. Together this data indicates that DAPT has similar effects on neurogenesis and tentacle formation during development, adult homeostasis and regeneration, but the effect on *Ncol1+* cells is different in polyps.

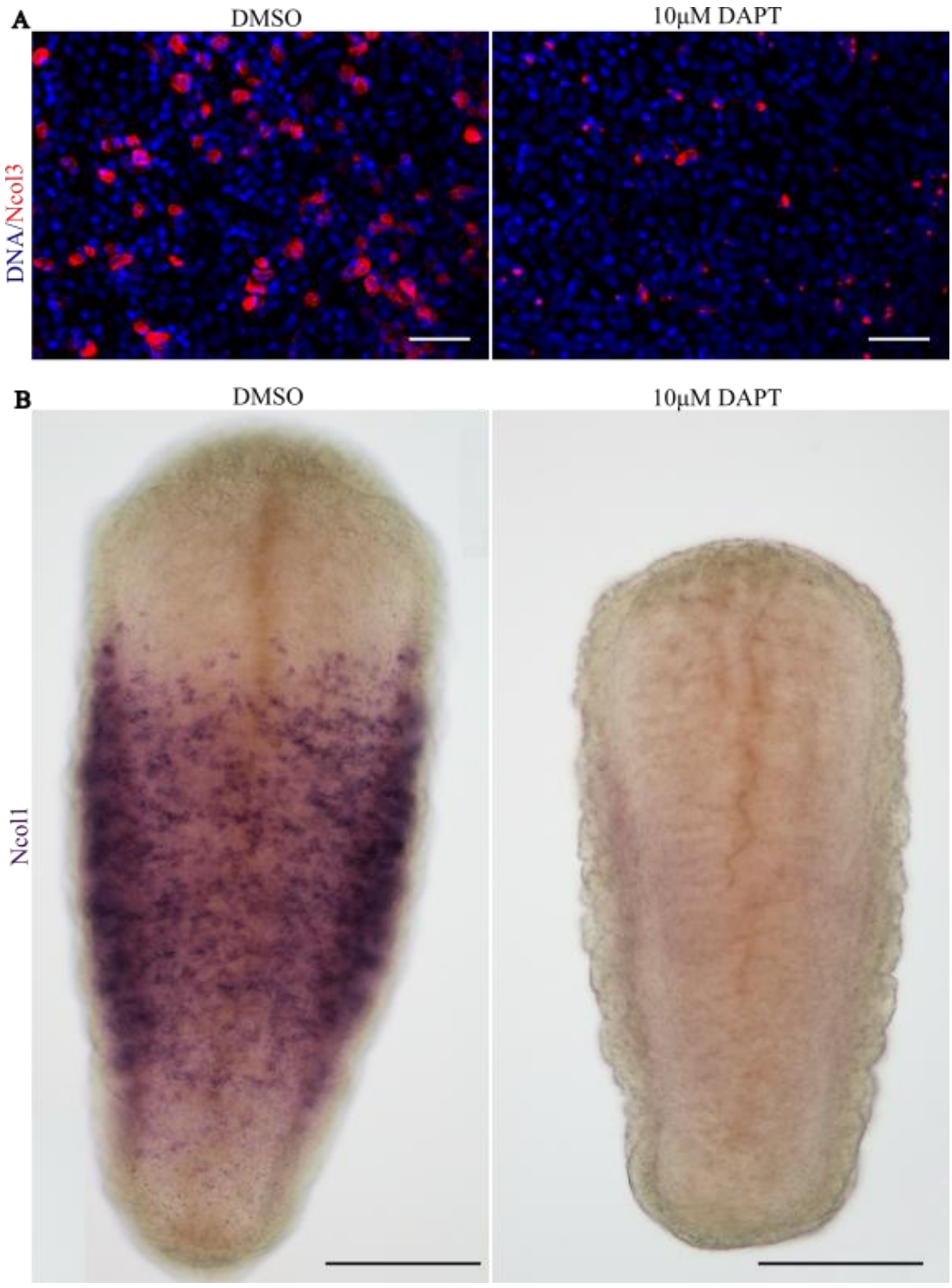


Fig. 5.10. DAPT blocks nematogenesis during regeneration. (**A**,**B**) Animals were decapitated and allow to regenerate in wither DMSO or 10 μM DAPT. After 24 hours animals were stained for Ncol3(A) or *Ncol1*(B). Scale bar 10 μm (A), 200 μm (B).

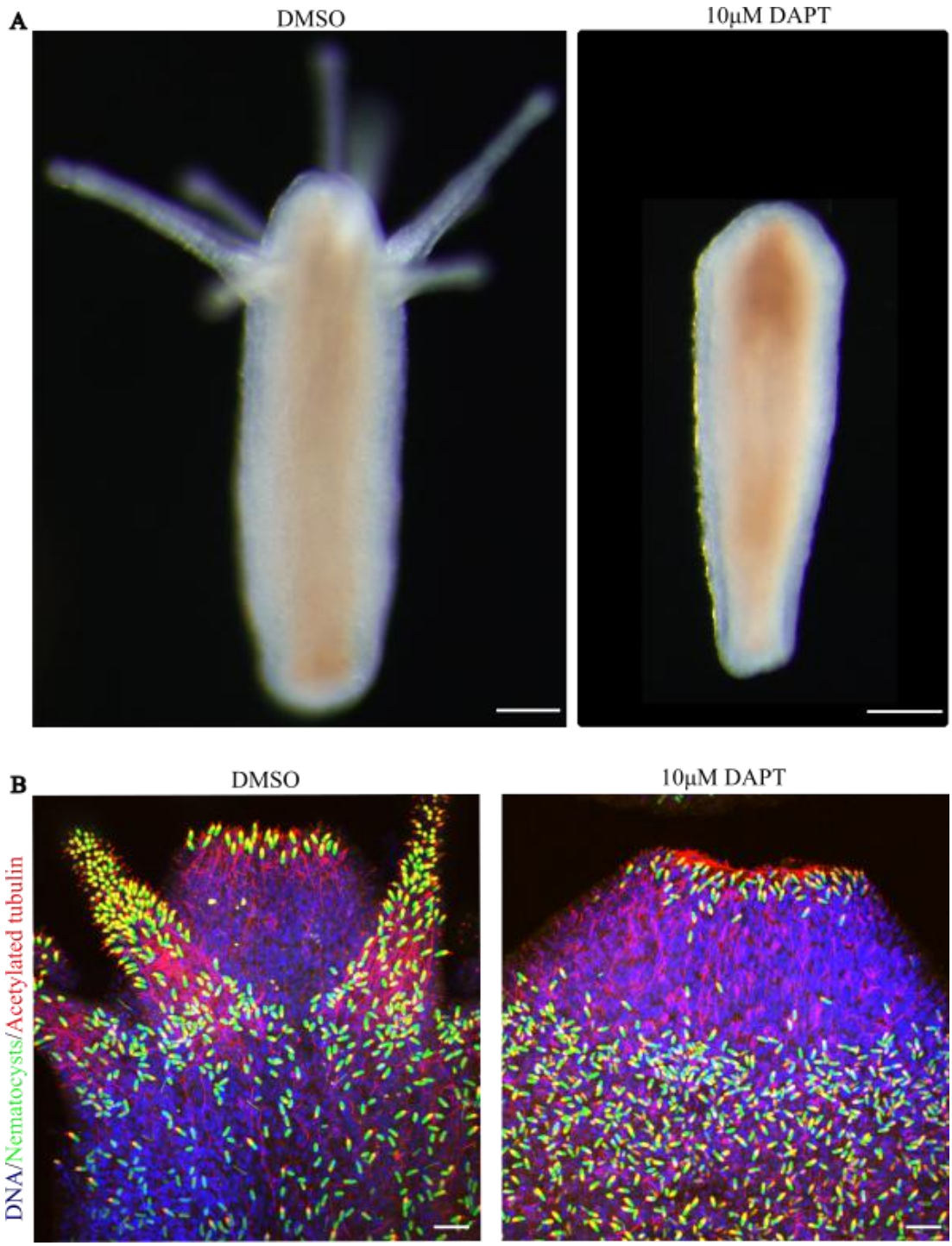


Fig. 5.11. DAPT blocks tentacle regeneration. (**A**) Animals decapitated and and treated by DAPT failed to regenerate tentacles. (**B**) Staining for $DAPI^{+}$ nematocysts and acetylated tubulin shows a regenerated oral nervous system in DAPT treated polyps. Scale bar: 200 μm (A), 10 μm (B).

5.4. Summary

DAPT has been used in both *Nematostella* and *Hydra*, as well as in many bilaterian models, as a powerful tool to block Notch signalling (Käsbauer et al., 2007, Münder et al., 2010, Münder et al., 2013, Richards and Rentzsch, 2015, Marlow et al., 2012, Geling et al., 2002, Micchelli et al., 2003, Khalturin et al., 2007). Here I compare the effect of DAPT treatment observed during development, tissue homeostasis and regeneration in *Hydractinia* with the phenotypes previously reported in *Nematostella* and *Hydra*.

A role for Notch signalling in embryonic neurogenesis is considered an evolutionarily basal and conserved component of animal nervous systems (Hartenstein and Stollewerk, 2015). Here I have shown that DAPT treatment does not affect commitment and neuronal differentiation in the early *Hydractinia* nervous system. DAPT treatment did not affect the number of cells committed to either neuronal or nematocyte fate although the treatment did appear to block late stages of nematocyst formation. This is in contrast to the situation in *Nematostella* and bilaterians where γ-secretase inhibition leads to a striking increase in the number of neural cells.

γ-secretase inhibition leads to a strong and penetrant effect on nematocyst formation. Like in *Hydra* and *Nematostella*, DAPT causes a loss of mature capsules. The phenotype was dose dependent in the embryo. At lower concentration nematocyst formation in the endoderm is unaffected but the latter stages of differentiation that occur in the epidermis were blocked. This led to apoptosis of developing nematoblasts. In contrast, at higher concentration, 10 μM, capsule formation was blocked at an earlier stage and capsule proteins, although expressed, do not form capsules. In adult polyps the same phenotype was observed, with capsule proteins not forming nematocysts. In contrast, however, the number of *Ncol1*$^{+}$ cells was decreased while in the larva the number was unaffected. The reason for this difference is unknown, but it may be tied to differences in the process or timing of nematogenesis in adults vs embryos. For example, early stages of nematogenesis occur in the larval endoderm and later stages in the epidermis while in adults all stages of nematogenesis occur in the epidermis.

The final major effect of γ-secretase inhibition is a block in tentacle formation. This was seen in both the context of metamorphosis and regeneration. During regeneration, although tentacle formation is blocked, a normal hypostome and oral nervous system were formed. This effect on tentacle patterning appears to be due to a conserved role of γ-secretase as it has been reported in both *Hydra* and *Nematostella* also (Münder et al., 2013, DuBuc et al., 2014, Fritz et al., 2013).

It appears that, as γ-secretase inhibition has no effect on the early nervous system, that a role for Notch signalling in this process had been lost in hydrozoans. The observed effects of DAPT, namely a block in nematocyte differentiation and a block in tentacle formation, appear to be conserved within the cnidaria. However, whether these phenotypes are related to Notch signalling is yet unknown in the absence of genetic evidence.

Chapter 6. Genetic manipulation reveals key roles for Notch signalling in *Hydractinia*

6.1. Introduction

The core components of the Notch signalling pathway are conserved in *Hydractinia* (Chapter 1); however, the functional roles played by the pathway are unknown. Focusing on patterning and neurogenesis, γ-secretase inhibition via DAPT has illuminated several potential roles and interesting losses of function (Chapter 5). DAPT had no effect on the commitment of neural cells which is in striking contrast to observations in bilaterians and indeed in *Nematostella* (Richards and Rentzsch, 2015, Marlow et al., 2012, Layden and Martindale, 2014), but is similar to results from another hydrozoan, *Hydra* (Käsbauer et al., 2007, Khalturin et al., 2007). Despite this, several potential roles from Notch signalling emerged from γ-secretase inhibition. Firstly, DAPT blocks nematocyte differentiation as it does in other cnidarians (Marlow et al., 2012, Khalturin et al., 2007, Käsbauer et al., 2007). Secondly, DAPT inhibits tentacle formation, again a phenotype seen in both *Nematostella* and *Hydra* (Münder et al., 2013, Fritz et al., 2013, Käsbauer et al., 2007, DuBuc et al., 2014). In order to ascertain if these phenotypes are Notch related, more targeted genetic approaches are necessary, i.e. gain and loss of function studies.

Gain of function studies on the Notch signalling pathway are usually achieved using an overexpression of the NICD. Expression of this mimics constitutively active Notch signalling and essentially leads to ligand independent Notch activity. This strategy has been successfully applied in *Nematostella* where NICD expression via mRNA injection generated neural phenotypes opposite to those seen in DAPT treated embryos (Layden and Martindale, 2014). In *Hydractinia*, overexpression can be achieved in two ways. Firstly polyadenylated mRNAs can be synthesised in-vitro and injected into 1 cell stage zygotes (E. McMahon unpublished). This leads to ubiquitous overexpression of the desired protein but in a transient manner. The second option is to generate transgenic animals which express the gene under a specific promoter. This leads to long term but mosaic expression in the animal. The transgenic approach has been applied in *Hydractinia* successfully to study patterning

and cellular differentiation (Millane et al., 2011, Duffy et al., 2012, Kanska and Frank, 2013).

Gene loss of function has also been utilised to study Notch signalling in *Nematostella*, primarily through morpholino oligonucleotide injection (Layden and Martindale, 2014, Richards and Rentzsch, 2015). These studies have shown that, like DAPT, knockdown of *NvNotch* or *NvDelta* recapitulates the neurogenic phenotype seen in DAPT treated animals. Morpholinos against *NvSu(H)* and mRNA injection of a dominant negative form of NvSu(H) lead to a loss of capsules, also similar to DAPT treatment (Marlow et al., 2012). Problems with a morpholino based approach are that it is transient and leads to only partial knockdown. Indeed *NvNotch* morpholinos lead to a weaker effect on gene expression than DAPT (Richards and Rentzsch, 2015). Because of this it is possible that dose dependent effects of Notch signalling may be obscured.

Until recently gene knockout studies have not been possible in non established model organisms due to the necessity for complex genome editing approaches. The advent of the use of CRISPR-Cas9 mediated genome editing has changes this and allowed researchers to apply mutagenesis strategies in virtually any organism.

The CRISPR-Cas9 system is an RNA guided genome defence system found in prokaryotes (Horvath and Barrangou, 2010). In its native context the system is used to recognise and cleave foreign DNA via the induction of double strand breaks. Spacer RNAs with sequence specificity are utilised by the Cas9 enzyme to direct cutting in a sequence specific manner. In recent years a number of labs modified the system for use in human cells where double stranded breaks could be target in specific genomic locations leading of mutations via indel formation during non-homologous end-joining or the introduction of foreign DNA by taking advantage of homologous recombination (Jinek et al., 2013, Cong et al., 2013, Mali et al., 2013). The technique has been rapidly adapted and is now utilised in many animals and is becoming a commonly used technique in developmental biology and evo-devo research. The technique was first applied in cnidarians by the Gibson lab where they showed its utility in *Nematostella* (Ikmi et al., 2014) and has been subsequently utilised for functional studies by the Technau lab, also on *Nematostella* (Kraus et al., 2016). It has also been reported that CRISPR-Cas9 has been established in *Clytia* but

no experimental data has yet been published (Leclère et al., 2016). In the context of loss of function studies CRISPR-Cas9 can be used to generate mutations in a gene thereby leading to the formation of loss of function alleles. This is beneficial for a number of reasons. Firstly the effects are permanent, and secondly the mutations, assuming a frameshift is generated, lead to complete loss of function in the affected allele. Utilising CRISPR-Cas9 in F0 animals, however, has the added disadvantage of being mosaic, similar to transgenic overexpression.

6.2. Aims

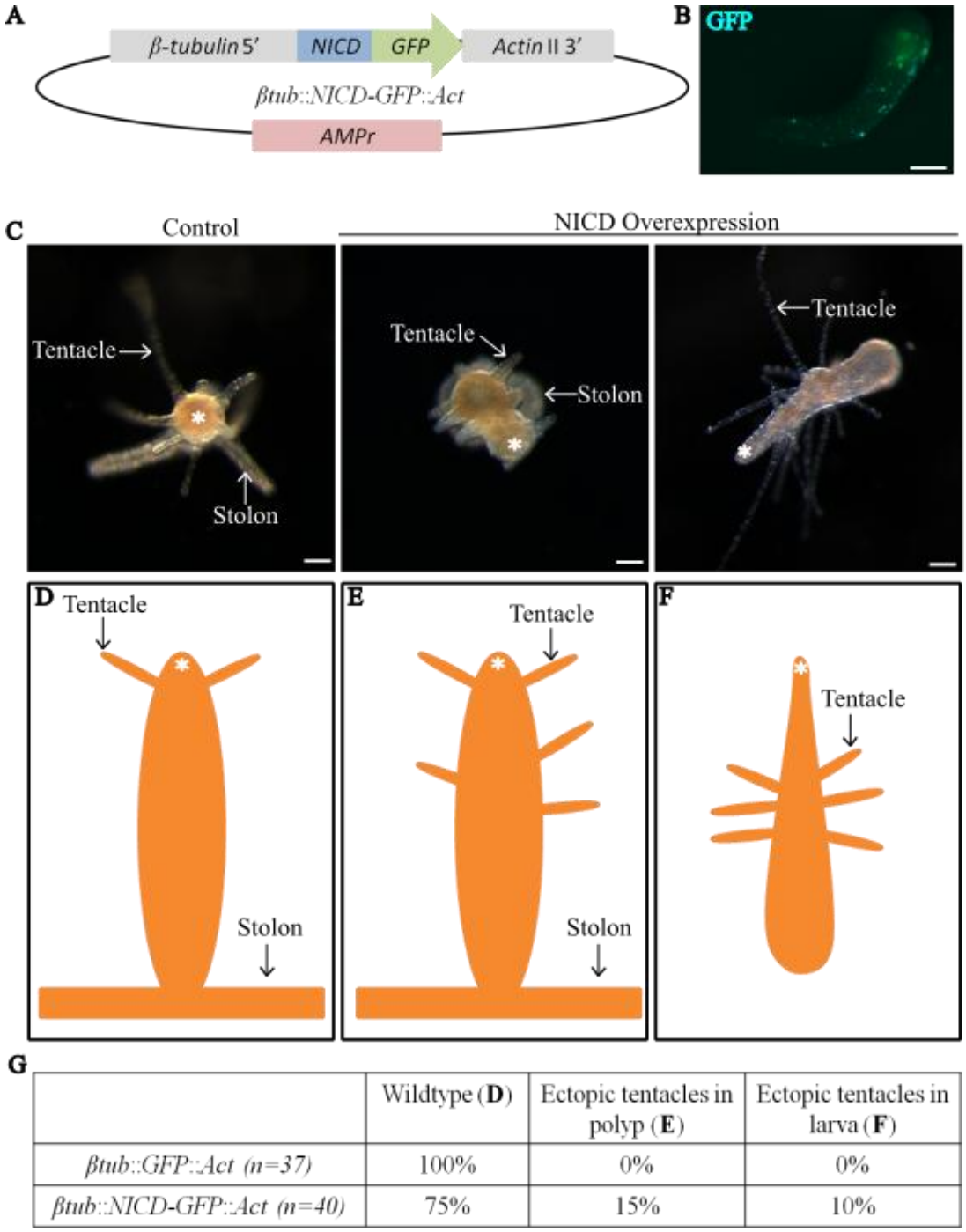
The aim of this part of my work was to study the role of Notch signalling in *Hydractinia* using direct genetic approaches. Specifically:

1. To evaluate the role of Notch signalling during metamorphosis and tentacle patterning.
2. To study the role of Notch signalling in nematocyte differentiation.

6.3. Results

In order to ectopically express the *Hydractinia* NICD I cloned the entire intracellular domain of *Notch* from cDNA into the *βtub*::*GFP*::*Act* vector (see Chapter 1) to generate an NICD-GFP fusion protein under the regulation of the *β-tubulin* promoter (Fig. 6.1A). In my hands this expression vector drove expression exclusively in epithelial cells post-metamorphosis and in many cell types during embryogenesis. This plasmid was then injected into 1 cell stage embryos and a plasmid containing only GFP was used as a control. Larva obtained from injection of the overexpression construct had exclusively nuclear GFP (Fig. 6.1B) as opposed to both nuclear and membrane bound GFP seen in GFP only animals. Larva expressing NICD had no discernible phenotype. Transgenic, GFP^{+}, larva from NICD overexpression and control injections were selected and metamorphosed. Following metamorphosis 100% (37/37) of control animals had metamorphosed normally and developed a ring of tentacles surrounding their hypostome (Fig. 6.1C, D, G). In those animals overexpressing NICD on the other hand two distinct phenotypes became apparent. Fifteen percent of them (6/40) completed metamorphosis, as indicated by the presence of stolons. However, these animals had ectopic tentacles along the body column of the polyp (Fig. 6.1C, E, G). In addition, in 10% of animals (4/40), metamorphosis was not completed and the resulting larva had large number of ectopic tentacles along the length of the larva (Fig. 6.1C, F, G). These animals never recovered and eventually died. This data shows that ectopic activation of Notch signalling can induce the formation of ectopic tentacle but only post induction of metamorphosis.

Next, I wanted to analyse the effect of Notch loss of function in *Hydractinia* using CRISPR-Cas9 mediated mutagenesis. I designed sgRNAs targeted against exons five and eight of the *Notch* gene (Fig. 6.2A), which are located upstream the NICD. I then used these sgRNAs in an in-vitro Cas9 assay with a PCR product spanning 3 kb of the *Notch* genomic locus containing cut sites for all sgRNAs. Four of the sgRNAs (hereafter guides sgRNA-A, sgRNA-B, sgRNA-C, and sgRNA-D) could efficiently guide Cas9 to cut in-vitro (Fig. 6.2B) and so were used for further experiments. In order to assess the efficiency of mutagenesis in-vivo I injected Cas9 along with all 4 guides and extracted genomic DNA from pooled larva (Fig. 6.3A). PCR across the



G

	Wildtype (**D**)	Ectopic tentacles in polyp (**E**)	Ectopic tentacles in larva (**F**)
βtub::GFP::Act (n=37)	100%	0%	0%
βtub::NICD-GFP::Act (n=40)	75%	15%	10%

Fig. 6.1. NICD overexpression leads to ectopic tentacle post metamorphosis. (**A**) Schematic representation of the NICD overexpression plasmid. (**B**) Transgenic larva expressing NICD-GFP. (**C**) Representative images showing control and NICD overexpression animals. (**D-F**) Cartoons representative of wildtype (D) and NICD overexpression animals (E,F). (**G**) The numbers of animals displaying ectopic tentacles following NICD overexpression. Scale bar: 75 μm.

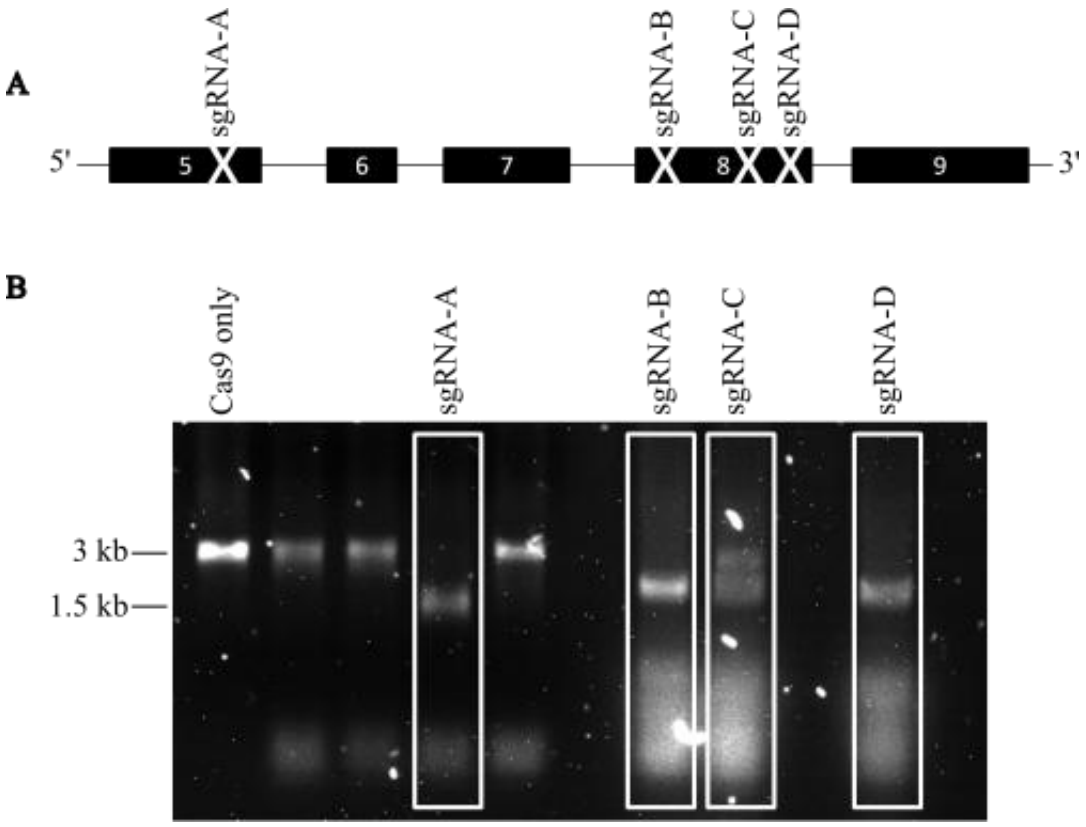
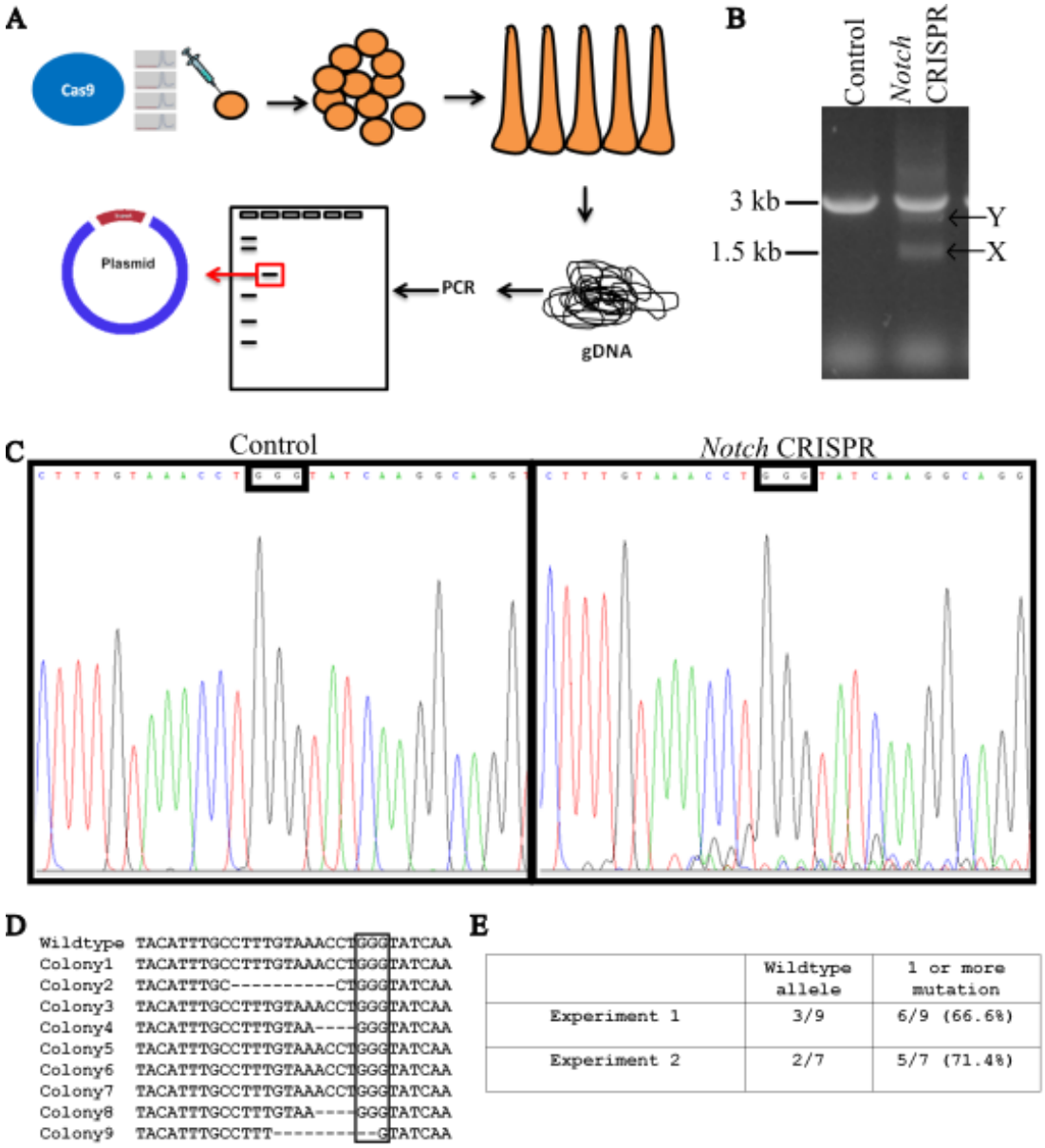


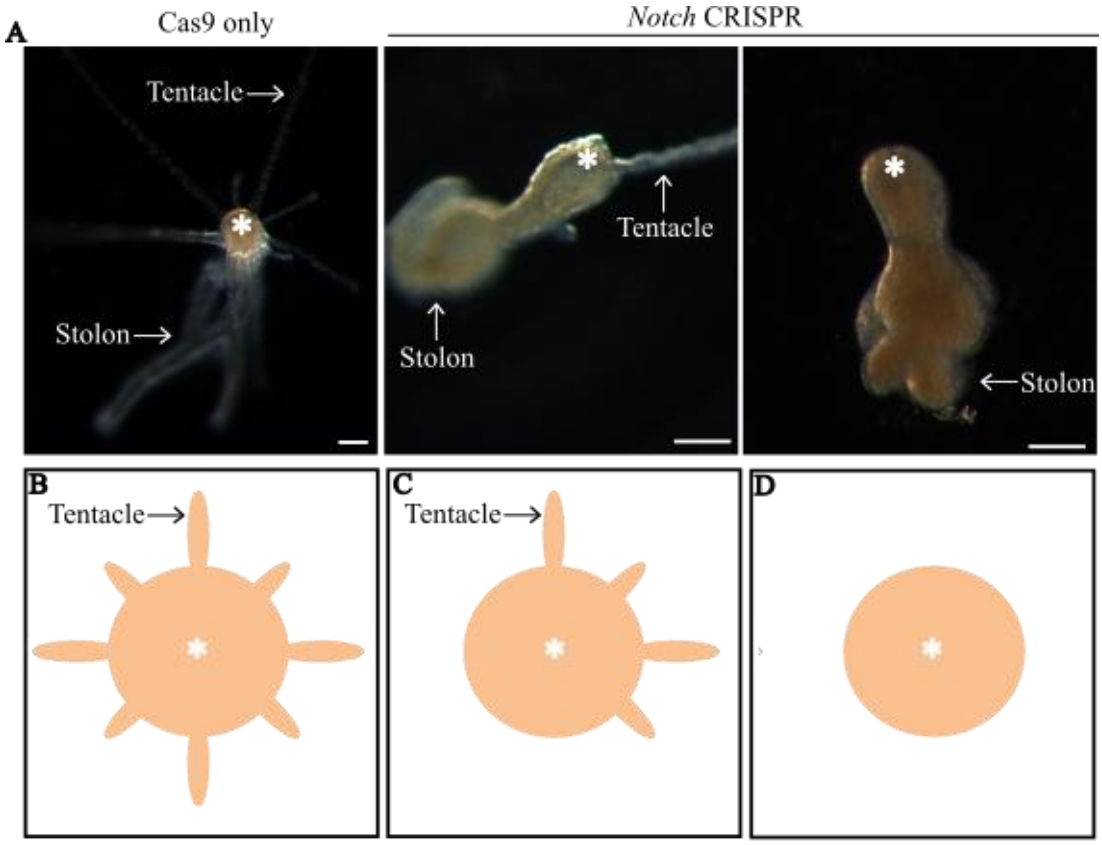
Fig. 6.2. In-vitro testing of Notch sgRNAs. (**A**) Schematic representation of exons 5-9 of the *Notch* genomic locus showing the target sits of sgRNAs A-D. (**B**) An *in vitro* Cas9 assay shows sgRNAs A-D can effectively guide cutting of a PCR amplified genomic fragment from the *Notch* locus (white boxes) but Cas9 only does not cut (lane1). Other lanes represent sgRNAs that were unable to guide cutting *in vitro*.

region containing the sgRNAs showed that a majority of alleles within the population had a size similar to the wildtype allele. However there were also a number of additional bands present in the sgRNA injected animals. One band (labelled X on Fig. 6.3B) in the PCR is at approximately 1.5kb, representative of the expected size if guide sgRNA-A and any of sgRNA-B, sgRNA-C or sgRNA-D cut simultaneously and generated a deletion between them (Fig. 6.3B). Sequencing confirmed that this was in fact the case. Another band (labelled Y) was present just below the wild-type size and could represent a large deletion at a single cut site (Fig. 6.3B). In order to assess the number of mutations in the normal sized allele I first extracted the band, including the band just below 3kb. Sanger sequencing revealed the presence of additional sequences in the injected but not control sample (Fig.



	Wildtype allele	1 or more mutation
Experiment 1	3/9	6/9 (66.6%)
Experiment 2	2/7	5/7 (71.4%)

Fig. 6.3. *In vivo* testing of *Notch* sgRNAs. (**A**) Schematic showing the experimental plan for testing sgRNAs *in vivo*. (**B**) PCR of the *Notch* locus from wildtype or *Notch* sgRNA injected animals shows the presence of an additional bands (X and Y) in the injected animals. (**C**) Chromatogram trace files from Sanger sequencing of the extracted "upper band" in (B) show the presence of multiple sequences resulting from cutting guides by sgRNA-A in the injected but not control samples. The Pam site in highlighted with a black box. (**D**) Sequences from 9 colonies showing mutations resulting from Cas9 cutting guided by sgRNA-A. PAM site is highlighted by a black box. (**E**) Results from 2 independent experiments showing the number of "normal size" alleles carrying a mutation resulting from one or more sgRNA guiding cutting.



E

	Wildtype	Abnormal tentacles
Cas9 only	25/26	1/26
Notch CRISPR	13/30	17/30

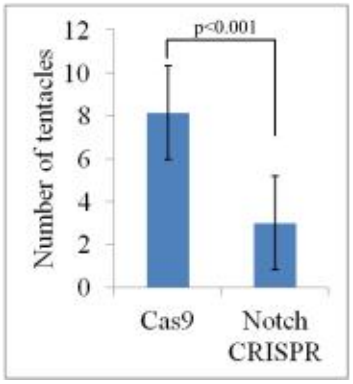


Fig. 6.4. Notch is required for tentacle formation during metamorphosis. (**A**) Representative images showing animals from control and Notch CRISPR injections post metamorphosis. (**B-D**) Schematic oral view representations of phenotypes observed in Notch CRISPR animals ranging from normal tentacles (B) to partial (C) or complete (D) loss of tentacles. (**E**) Table showing the number of animals with abnormal tentacles in Cas9 and Notch CRISPR injected animals. (**F**) Quantification of tentacle number in Cas9 only and Notch CRISPR animals. Data is representative of three independent replicates. * denotes the mouth. Scale bar: 75 μm.

6.3C). I then cloned this PCR product into a plasmid and sequenced across all the sgRNA sites. Fig. 6.3D shows the results obtained from one experiment looking at the sgRNA-A cut site. In two experiments, 66.6% (6/9) and 71.4% (5/7) of the sequenced clones, respectively, contained a mutation at at least one sgRNA cutting site (Fig. 6.3E). In one clone a large ~200 base pair deletion was found flanking an sgRNA cut site, providing further evidence that band Y represents large deletions resulting from resection following cutting by a single sgRNA. This data shows that, using 4 sgRNAs in combination, it is possible to mutate ~70% of *Notch* alleles in a mosaic fashion. This number is an underestimation of the total mutation rates since the alleles with large deletions were not included in the quantification. Since this approach generated such a large rate of mutation in the F0 injected larva I proceeded to use this injection strategy to study the phenotype of *Notch* loss of function.

Larva injected with *Notch* sgRNAs (hereafter referred to as *Notch* CRISPR injected animals) did not display any obvious defects and appeared similar to the Cas9-only injected animals. I then went on to metamorphose these animals. Like in the NICD overexpressing animals, phenotypes only became apparent post metamorphosis. In Cas9 only injections almost all (25/26) animals developed normal tentacles (Fig 6.4A, B, E). *Notch* CRISPR injected animals, by contrast, displayed a spectrum of tentacle defects. These defects ranged from the presence of tentacles on only one side of the primary polyp (Fig. 6.4A, C) to a complete loss of tentacles (Fig. 6.4A, D). In total, 17/30 *Notch* CRISPR injected animals had some form of tentacle morphological abnormality (Fig. 6.4E) and this caused a significant reduction in overall tentacle number (Fig. 6.4F). Hence *Notch* is necessary for tentacle patterning.

Finally I analysed the number of poly-γ-glutamate positive nematocysts in *Notch* CRISPR animals. Animals were injected and stained with DAPI as planula larva. Animals were then assigned to groups based on the number of nematocysts counted (Fig. 6.5). Across three biological replicates there was an increase in the number of animals fitting into the lower groups in Notch CRISPR injections compared to Cas9 only injected controls.

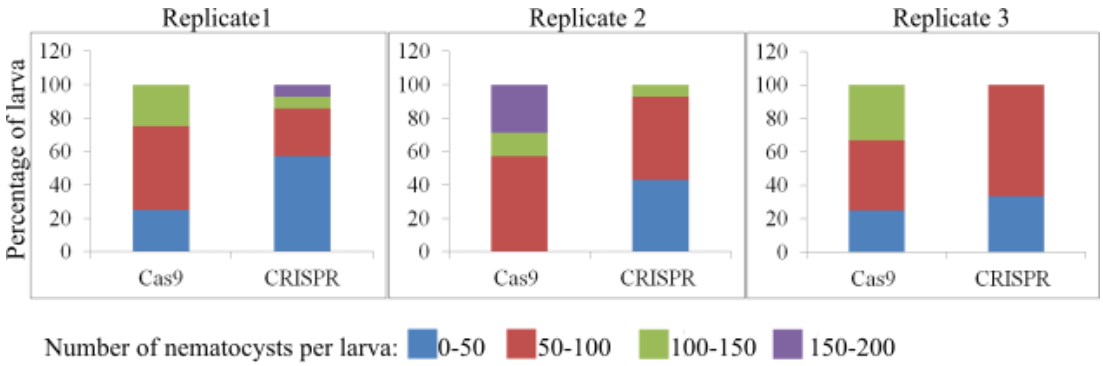


Fig. 6.5. *Notch* plays a role in nematogenesis. Notch CRISPR and Cas9 only control animals were stained with DAPI for nematocytes and assigned to one of 4 groups based on the number of nematocysts. In three independent experiments the Notch CRISPR led to more animals fitting into the lower categories.

6.4. Summary

Experimental manipulation of the Notch signalling pathway is often achieved by pharmacologically inhibiting γ-secretase, an essential component of Notch receptor activation, using DAPT. This strategy has been employed in *Nematostella* and *Hydra* (Käsbauer et al., 2007, Münder et al., 2010, Münder et al., 2013, Khalturin et al., 2007, Richards and Rentzsch, 2015, Marlow et al., 2012, Layden and Martindale, 2014, Fritz et al., 2013). Only in the anthozoan, *Nematostella*, however, have phenotypes resulting from γ-secretase inhibition been confirmed genetically and even there only in a neurogenic context (Marlow et al., 2012, Layden and Martindale, 2014, Richards and Rentzsch, 2015). I have shown that γ-secretase inhibition leads to two major phenotypes in *Hydractinia*: a block of nematocyte differentiation without changing their progenitors numbers, and a block in tentacle formation. Here I have confirmed using both genetic gain and loss of function approaches that these two phenotypes are *Notch* dependent.

Using a transgenic NICD overexpression system I have shown that activation of the Notch signalling pathway leads to ectopic tentacle formation post-metamorphosis induction but not in un-induced larva. I have also shown, using CRISPR-Cas9 mediated mutagenesis that mutation of *Notch* leads to a loss of tentacles, essentially phenocopying γ-secretase inhibition.

The loss of mature nematocysts/nematocytes upon DAPT treatment has been observed in both *Hydra* and *Nematostella* (Richards and Rentzsch, 2015, Käsbauer et al., 2007, Marlow et al., 2012, Khalturin et al., 2007). Genetic interference experiments in *Nematostella*, however, have given contradictory results with regard to whether this phenotype is Notch dependent or is simply a Notch unrelated side effect of γ-secretase inhibition (Marlow et al., 2012, Richards and Rentzsch, 2015, Layden and Martindale, 2014). Here, using the CRISPR-Cas9 system, I have confirmed that loss of *Notch* indeed leads to a reduction in the numbers of mature nematocysts. The reduction seen was not statistically significant but this may be due to a combination of the large variability in nematocyst number in different larva in combination with the mosaic nature of the CRISPR-Cas9 technique.

From this data I can conclude that both major DAPT phenotype, loss of nematocytes and loss of tentacles, are in fact mediated by γ-secretase's role in the Notch signalling pathway and that *Notch* is essential for differentiation of nematocysts and for tentacle formation.

Chapter 7. Discussion

7.1. Transgenesis in *Hydractinia*

Hydractinia transgenesis is in its infancy but specific advancements have added to the utility of using *Hydractinia* transgenics in research, many of them first described here. I have shown the development of a ubiquitous reporter as well as double reporter lines along with the first use of new fluorescent reporter proteins in *Hydractinia*, i.e. tdTomato and Kaede (see Chapter 3). Most interesting, however, are not the technical successes of this work, but rather the mechanisms underlying the failures. Throughout the work on developing transgenic *Hydractinia* it has become increasingly obvious that yet unknowm mechanisms act on these transgenes in the animal's genome. Although it is clear that injected plasmids become integrated in the genome of hydrozoans (Wittlieb et al., 2006, Dana et al., 2012), my work suggests that the integration context is a key factor determining its expression; the case of the *β-tub*:*GFP*:*β-tub* animals is a good example. I have shown that the plasmid is in principle capable of driving expression of GFP in all cell types of the adult, and so the promoter must contain all necessary regulatory elements for ubiquitous expression (Fig. 3.2). In the majority of cases, however, this construct drove GFP expression exclusively or preferentially in epithelial cells and only in the minority of animals was GFP expression seen in other cell types. How is this possible? What is the difference between these animals underlying different expression patterns by the very same construct? I suggest two alternative mechanisms to explain these phenomena: Firstly, differences in the copy number of integrated plasmids; secondly the integration sites of the plasmid. Both of these potential variables could give rise to different expression patterns in different animals. In plants it is well established that multi-copy integrations are correlated with increased silencing of transgenes (Kohli et al., 2006, Matzke et al., 1994) and I have shown that it is possible for multiple plasmids to co-integrate (Fig. 3.6). More so, however, positional effects have been linked to silencing of transgenes (Matzke and Matzke, 1998, al-Shawi et al., 1990, Haruyama et al., 2009) where often the chromatin context of the integration site leads to repression of expression, e.g. when the transgene is inserted into heterochromatin. It is also possible that there are alternative explanations. It has been shown that even at specific locations transgenes

can be silenced in only some but not all cases (Day et al., 2000) and that the orientation can lead to silencing (Feng et al., 2001). These may be of potential relevance as silencing of the *β-tubulin* transgenes as well as other constructs, not reported here, seems to be more pervasive in non-epithelial cells. This may have many reasons. For example, it is possible that epithelial cells have a different propensity to recognise and silence foreign DNA than other cell types, or that major genomic regions which are silenced in non-epithelial cells are not maintained in this silent conformation in epithelial cells. Further analysis is necessary to understand the underlying causes of transgene silencing in *Hydractinia*.

Although the *β-tubulin* transgenic animals appear to display variable silencing patterns, the opposite appears to be the case with *Piwi1* reporter transgenics. In this case the transgene begins with a restricted expression pattern which matches the known expression of *Piwi1* in i-cells (Fig. 3.4). Over time, however, the transgene became leaky and began to be expressed in other cell types, notably epithelial cell (Fig. 3.5). The expression of the *Piwi1* gene has been extensity studied on both the RNA and Protein level in *Hydractinia* and is well established to be specifically expressed in i-cells and germ cells (E. McMahon, unpublished)(Bradshaw et al., 2015). The idea that a transgene may have 'leaky' expression is known in many systems and is usually explained by the transgene integrating near some positive regulatory element which then affects the transgene (Haruyama et al., 2009). Although possible, in this case it is unlikely as both the transgenic animal described here as well as several others (B. Bradshaw, unpublished) showed this same phenomenon and no long-term faithful *Piwi1* reporter has ever been made. Also this phenomenon has been seen with other reporter lines (E. Chrysostomou, unpublished). This would also not explain how the transgene only becomes leaky over time. The causes of this are therefore intriguing but may be linked to the silencing discussed above. In the *Piwi1* case, it is possible that the endogenous *Piwi1* gene requires a specific chromatin context to become silenced in i-cell progeny. In randomly integrated *Piwi1* transgenes this context is lost, leading to precocious expression. Why then does this only happen over time? It is possible that initial silencing of the *Piwi1* gene requires only proximal regulatory elements within the transgenic cassette but that long term silencing requires specific, context dependent

inputs from more distal elements absent in the transgene, leading to precocious expression.

It is now, however, possible that random integration transgenesis will become redundant. In this thesis I have shown, for the first time, the use of CRISPR-Cas9 mediated mutagenesis in *Hydractinia* (See Chapter 6). I have shown here that the use of CRISPR-Cas9 leads to mutation in >70% of alleles when utilising 4 sgRNAs (Fig. 6.3). The efficiency of using only one sgRNA resulted in approximately 50% of alleles being mutated (data not shown) in a mosaic animal. Relevant to this discussion is the ability to utilise CRSIPR-Cas9 for targeted insertion of DNA into the genome by taking advantage of homologous recombination (HR). Although I have not shown this here, it is likely that with such high cutting rates that insertion of DNA by HR will also be readily possible and indeed has already been utilised in *Nematostella* (Ikmi et al., 2014). This would allow for the targeting of transgenes to a single locus which would dramatically improve the consistency between individual experiments (Roberts et al., 2014) . This could revolutionise *Hydractinia* research as CRISPR-Cas9 can be used to endogenously tag genes and therefore will show not only the expression of that gene, *in vivo*, but also the localisation and levels of the endogenous protein, allowing for more detailed analysis of gene expression and function. This will also allow for detailed analysis of protein biology in *Hydractinia* which is currently limited by the low number of antibodies available for experiments.

7.2. Tentacle patterning and the Notch signalling pathway

I have shown here that inhibition of γ-secretase via DAPT disrupts tentacle formation both during metamorphosis and also during regeneration (Fig. 5.8, 5.11). This has also been shown in *Nematostella* and *Hydra* (Fritz et al., 2013, Münder et al., 2013, DuBuc et al., 2014, Marlow et al., 2012). In *Hydra* in particular, this process has been well studied and it is clear that γ-secretase inhibition acts by repressing NICD cleavage that, in turn, inhibits Hes mediated tentacle patterning (Münder et al., 2013, Käsbauer et al., 2007). Although many pharmacological experiments have been carried out, including in the present work, to date there has been no genetic evidence to link Notch signalling to tentacle patterning. I have shown here, for the first time, that NICD overexpression leads to the development of ectopic tentacles (Fig. 6.1), and CRISPR-Cas9 mediated Notch loss of function leads to defective tentacle patterning (Fig. 6.4). These lines of evidence, together with the DAPT experiments, show clearly that Notch signalling is required for tentacle formation.

Ectopic expression of NICD led to ectopic tentacles only post metamorphosis. Transgenic larva did not have any visible defects. This indicates that NICD is not sufficient to drive the formation of tentacles and that a signal initiated during metamorphosis is necessary to generate a competence to respond to Notch signalling. The nature of this signal is unknown. Given the emerging evidence, particularly in *Hydra* (Münder et al., 2013), it is likely that Notch signalling is playing a role in the separation of tentacle from non-tentacle fate within the tentacle zone during head formation. This tentacle zone is established by an as yet unkown gradient which is initiated during metamorphosis. It is not yet clear whether Notch responsive cells are prospective tentacle cells or non-tentacle ones. This is a classical example of Notch signalling mediating binary cell fate decisions, i.e. creating a sharp boundary.

In addition to its role in tentacle patterning in *Hydra*, Notch signalling is also required for head regeneration. This has been hypothesised to occur due to a role for Notch in separating hypostome from tentacle zone cells (Münder et al., 2013). In *Hydractinia*, on the other hand, I have shown that DAPT does not block formation of the hypostome, even in cases where tentacles are completely absent (Fig. 5.11). This can be clearly seen in the nervous system where an oral nerve net (consisting of

Rfamide^{+} neurons), present in the hypostome, forms normally in DAPT treated animals, and an oral ring of nematocytes at the tip of the hypostome also forms correctly (Fig. 5.8, 5.11). It is therefore possible that the regeneration of the *Hydra* head may differ from that of *Hydractinia*. Indeed, this is not the first noted difference as *Hydra*, unlike *Hydractinia*, can regenerate a head in the absence of proliferation (Bradshaw et al., 2015, Park et al., 1970). However, it should be noted that the regeneration of the nervous system was not analysed in *Hydra* treated with DAPT.

7.3. The role of Notch signalling in neurogenesis

7.3.1. The origin of cell types in cnidarians

The emergence of the nervous system was a key event in animal evolution. A nervous system is present in all animal phyla, with the exception of placazoans and sponges. It is also clear now that the developmental origins of the nervous system are the same in the majority of studied animals including bilaterians and anthozoan cnidarians. Almost invariably, neural cells in these taxa arise from the embryonic ectoderm (Hartenstein and Stollewerk, 2015). In some animals (e.g. hemichordates), the entire ectoderm has the capacity to give rise to neurons while in others a specialised region of the ectoderm (i.e. the neuroectoderm) achieves neurogenic potential. These ectodermal cells give rise to neural progenitor cells which then proliferate and go on to differentiate into neurons and, in bilaterians, glial cells. If we focus more closely on cnidarians, one major difference is already obvious in some anthozoans where endodermal cells also give rise to neural progenitor cells, a unique situation (Richards and Rentzsch, 2014, Marlow et al., 2009, Nakanishi et al., 2011). It is the hydrozoan cnidarians, however, that have an even more unique system whereby exclusively the endoderm gives rise to neural cells. This occurs via i-cells, a hydrozoan specific stem cell which give rise not only to all neural cells (Galliot et al., 2009, Gahan et al., 2016) but also to other somatic and germ cells (Gahan et al. 2016).

It is worth at this point discussing the ectoderm-endoderm division in cnidarians. Cnidarians are diploblastic. Following gastrulation, only two germ layers arise: an outer ectoderm and an inner endoderm; no mesoderm sensu stricto forms in these animals. The endoderm is often also referred to as endomesoderm in *Nematostella* as it appears to have properties of the bilaterian endoderm and mesoderm and may be the evolutionary origin of both germ layers in bilaterians (Martindale et al., 2004). The mechanisms of gastrulation vary widely between different cnidarians with all major forms of gastrulation being represented in the phylum. Notably however invagination appears to be the prominent mode of gastrulation in anthozoans while it is not seen in hydrozoans; the latter are the most diverse in terms of gastrulation modalities (Byrum and Martindale, 2004). The process of gastrulation has been extensively studied in *Nematostella* where both morphological and molecular aspects

are well understood and it involves many conserved processes (Wikramanayake et al., 2003, Martindale et al., 2004, Matus et al., 2006, Matus et al., 2007). *Nematostella* gastrulates primarily by invagination (Magie et al., 2007) wih some possible contribution from immigration (Kraus and Technau, 2006). In hydrozoans gastrulation can be a widely different process in different species for example polar delamination in *Clytia* and apolar delamination in *Hydractinia* (Plickert et al., 2012, Kraus et al., 2014).

The endo-mesoderm of *Nematostella* is not, in all aspects, the same as its bilaterian equivalent. Strikingly this germ layer is capable of giving rise to neural progenitor cells, normally a feature restricted to the ectodermal lineage (Nakanishi et al., 2011, Marlow et al., 2009, Hartenstein and Stollewerk, 2015). In the case of hydrozoans the divide between ecto and endoderm has become even more blurred. The hydrozoan nervous system is exclusively derived from endoderm, as are germ cells, gland cells and, in *Hydractinia* at least, epithelial cells in the adult (Plickert et al., 2012, Müller et al., 2004). This process is achieved via a hydrozoan specific cell type, the interstitial cell or i-cell. I-cells are present within the embryonic endoderm but migrate, during metamorphosis, to reside in the interstitial space of epithelial cells in the adult epidermis (Rebscher et al., 2008). I-cells are segregated during gastrulation (Leclère et al., 2012).

So what is the fate of the embryonic ectoderm in hydrozoans? This question has never been analysed by lineage tracing but if i-cells give rise to all cells in the adult then over time the adult will in fact become derived exclusively from the endoderm with no ectodermal contribution. Is there, however, another explanation for what we see in hydrozoans? Have hydrozoans moved germ layer decisions from a specific point in time and space and made it a cell autonomous process? It is possible to think about evolution of i-cells as a move away from gastrulation defined ectoderm/endoderm to a more flexible system. By sequestering a population of cells early in development, keeping them uncommitted and self-renewing to give rise to any cell type life long, hydrozoans, in fact, gastrulate continuously during their lives. Hence, hydrozoan evolution included a separation of the morphological process of gastrulation from germ layer decision-making which are linked processes in other animals. This hypothesis requires detailed lineage tracing studies to be put to the test, and my work on transgenesis/genome editing in *Hydractinia* lays the foundation for

these exciting investigations. *Hydractinia* is without doubt the most suitable hydrozoan to carry out this work.

Is this system hydrozoan specific or do other animals use a similar process? The most obvious candidates to look at are planarians. Like hydrozoans, planarians have a population of cells called neoblasts which also give rise to all cells throughout the life of the animals and at least a sub-population of these cells are pluripotent and can give rise to all cells, i.e. cells from all germ layers (Wagner et al., 2011). These cells are therefore making germ layer commitments throughout the life of the animal as well, and in fact in this case neoblasts must make commitments to all three classical bilaterian germ layers, ectoderm, endoderm and mesoderm. Since most work on planarians has been conducted on non-sexually reproducing strains it is still unclear how and when clonogneic neoblasts appear in planarian ontogeny. In addition, in the mouse bipotent progenitors capable of giving rise to both neuroectoderm and mesoderm persist post gastrulation and therefore even in the mouse not all germ layer decisions are linked to gastrulation (Tzouanacou et al., 2009).

7.3.2. Loss of Notch signalling and the evolution of the i-cell

The evolution of i-cells has not been well studied despite extensive studies on their biology, particularly in *Hydra* (Gahan et al., 2016). In the context of this study it is important to note that i-cell evolution involved a very fundamental change in the way in which neurogenesis occurs. In other animals, anthozoans included, neural progenitor cells arise from epithelial cells of the ectoderm, and are themselves epithelial in nature (Richards and Rentzsch, 2014, Hartenstein and Stollewerk, 2015). It is important that these cells remain epithelial in order for Notch signalling to play a role in this process. What is more important than the epithelial nature of these cells per se is that the requirement for Notch makes them sedentary. Notch signalling establishes complex feedback loops between neighbouring cells in order to mediate lineage decisions and to select neural from non-neural cells, or direct cells to neural differentiation vs. maintenance of progenitors. This is a feature of neurogenesis seen across all bilaterian lineages (Ungerer et al., 2012, Gold et al., 2009, Stollewerk, 2002, Hartenstein and Stollewerk, 2015, Hudson et al., 2007, Lu et al., 2012, Chitnis et al., 1995, Chitnis and Kintner, 1996, Jan and Jan, 1994, Henrique et al., 1997, Wakeham et al., 1997, Louvi and Artavanis-Tsakonas, 2006).

This would not be possible in a dynamic population of migratory cells such as i-cells where any stochastic changes between neighbouring cells could not be converted into stable systems as cells would move away from each other. I-cells are mesenchymal in nature and therefore are free to move. In *Hydractinia* it is clear that neural differentiation begins in the endoderm prior to the presence of any epithelialisation of the tissue (Kraus et al., 2014, Kanska and Frank, 2013). It is therefore probable that any role for Notch signalling in this process would have acted as a constraint to migration and therefore evolution of i-cells.

Here I have shown that, indeed, Notch signalling does not play a similar role in commitment of neural progenitors, unlike in other animals. Disruption of Notch signalling via γ-secretase inhibition had no effect on the number of neurons or their committed progenitors in the larva (Fig. 5.7) and also did not affect the formation of the oral nerve net during metamorphosis or regeneration (Fig. 5.8, 5.11). It also has had no effect on the commitment of cells to the nematocyte linage (Fig. 5.6). This is despite the fact that DAPT treatment could recapitulate other confirmed Notch loss of function phenotypes, i.e. the loss of tentacles (Fig. 5.8, 5.11) and mature nematocysts (Fig. 5.1). This is further supported by work in *Hydra* where neurogenesis was shown to be unaffected by DAPT (Käsbauer et al., 2007, Khalturin et al., 2007) .Apparently, the role for Notch signalling in the early nervous system of hydrozoans has been lost. This is clear as the role of Notch signalling has been shown to be a conserved part of bilaterians and basal cnidarian neurogenesis (Marlow et al., 2012, Hartenstein and Stollewerk, 2015, Richards and Rentzsch, 2015, Layden and Martindale, 2014).

I therefore hypothesize that the removal of a requirement for Notch signalling has been a key event in the evolution of i-cells. Hydrozoans have moved from a situation where neurogenesis occurs in a Notch dependent epithelial context to a Notch independent mesenchymal context (Fig. 7.1). Lifting of this epithelial constraint would have allowed for the evolution of the i-cell system in hydrozoans, perhaps a key event in the evolutionary history of this lineage.

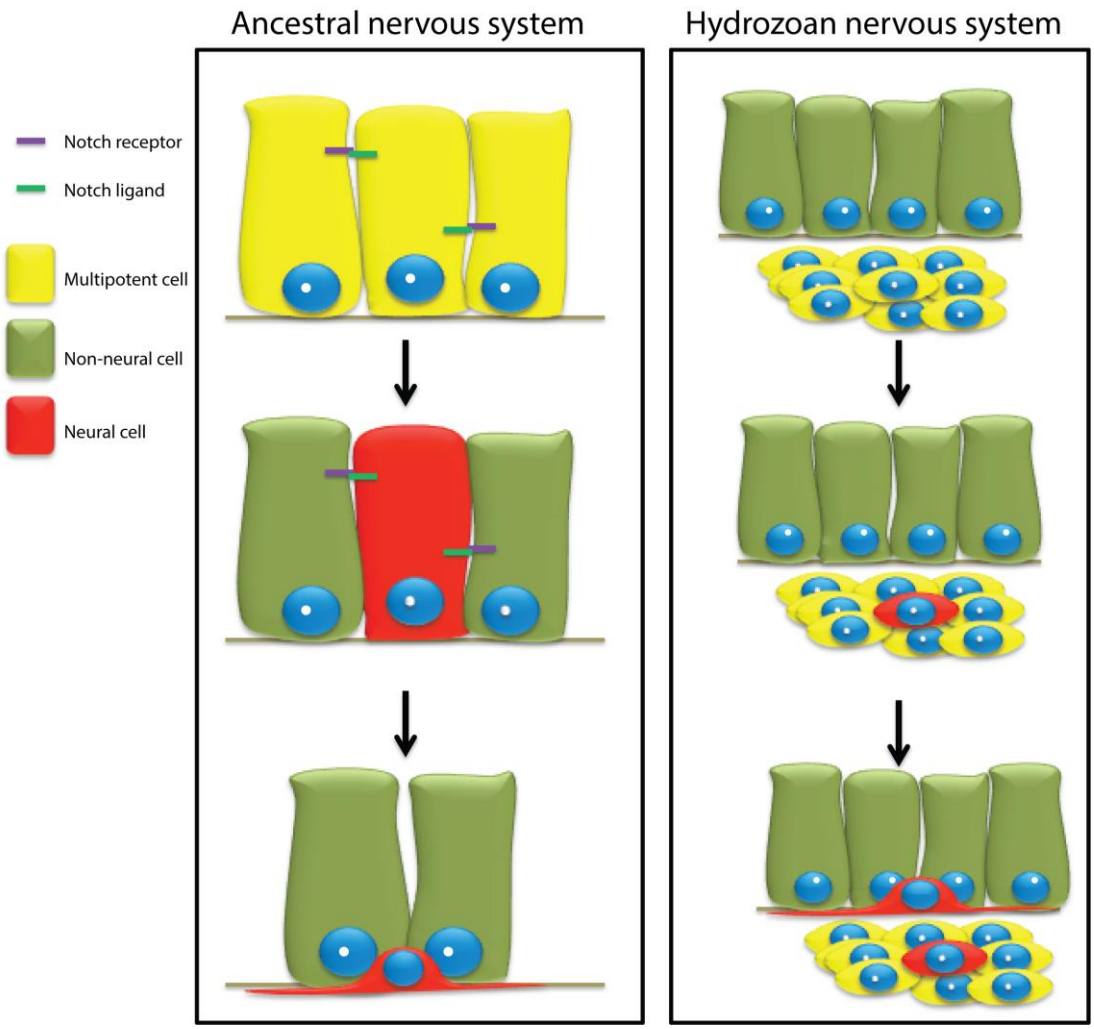


Fig. 7.1. Comparison of the putative ancestral mode of neurogenesis and its derived counterpart in hydrozoans. In most studied animals, neural progenitors are segregated from epithelial cells via lateral inhibition mediated by Notch signalling. In hydrozoans, neural progenitors arise from Notch independent, mesenchymal endodermal cells (i-cells).

7.4. Notch signalling plays a conserved role in nematocyte differentiation

The inhibition of γ-secretase has been shown to block the differentiation of nematocytes in both *Nematostella* and *Hydra* (Käsbauer et al., 2007, Marlow et al., 2012, Richards and Rentzsch, 2015). This block appears to occur during capsule formation and leads to either a failure to form capsules or the breakdown of formed ones. Genetic evidence for this is only present in *Nematostella* where a Morpholino against *NvSu(H)* or injection of mRNA encoding a dominant negative form of NvSu(H) can recapitulate this phenotype. Morpholino against *NvNotch* or *NvDelta* was later shown to lead to an increase in neural cells, like DAPT, but also to increase the number of nematoblasts based on minicollagen expression. Reciprocal results were seen when the pathway was over activated by ectopic NICD expression (Layden and Martindale, 2014, Richards and Rentzsch, 2015). This is consistent with the idea that Notch signalling plays a classical role in inhibiting neural differentiation, with inhibition of the pathway leading to an increase in all neural cells. However, in cnidarians Notch signalling is also essential for the differentiation of nematocytes from committed nematoblasts. A similar situation is also seen in bilaterians where Notch signalling represses precocious neural differentiation but is simultaneously required for the differentiation of glial cells (Lundkvist and Lendahl, 2001, Grandbarbe et al., 2003).

I have shown that DAPT can inhibit nematogenesis also in *Hydractinia*; furthermore, this effect was dose dependent (Fig. 5.1, 5.2-5.6). High concentrations of DAPT lead to phenotypes similar to those seen in *Nematostella* whereby capsule formation is blocked. Exposure to lower DAPT concentrations during embryogenesis, on the other hand, lead to a block in the later stages of nematocyte differentiation when the capsule has already formed but before becoming poly-γ-glutamate positive. CRISPR-Cas9 mediated mutagenesis of the *Notch* gene led to a decrease in poly-γ-glutamate positive nematocysts, thus confirming that this effect of DAPT is *Notch* dependent (Fig. 6.5). In combination with published data, this is consistent with Notch signalling having a role in nematogenesis, but adds the observation that this role is dose dependent. This dose dependence could represent the same defect with different severities. Alternatively, Notch signalling may act at two different stages

during nematogenesis with the later stage being more sensitive to loss of Notch signalling than the early one. Without further experimentation it is impossible to distinguish between these hypotheses.

Interestingly, in adults, this dose dependency was not seen. Lower DAPT concentrations that completely blocked poly-γ-glutamate positive capsules in embryos had no visible effect in polyps; only higher concentrations could block nematogenesis. In addition, DAPT affected the number of *Ncol1*$^{+}$ cells in adults but had no effect on these cells in embryos. The cause of this anomaly is unknown but may be linked to the differential localisation of nematogenesis in embryos and adults. In embryos the early stages of nematogenesis, including all stages expressing minicollagens, occur within the endoderm before the cells migrate into the epidermis to undergo the final stages of maturation. In adults, on the other hand, early and late stages of nematogenesis occur within the epidermis. This difference may lead to a differential requirement for inputs from Notch signalling at different stages of differentiation. An alternative explanation to this difference could be related to a feedback mechanism from mature nematocytes that regulates early nematogenesis in polyps but not in embryos. Blocking final nematocyte maturation would result in an excess of nematoblasts that signal early progenitors to exit the cell cycle. No experimental evidence to support this hypothesis is available, however.

7.5. Conclusion

7.5.1. Future perspectives

The work presented here sets up a number of interesting avenues not only for advancing the *Hydractinia* model system, but also for research on Notch signalling in general and the evolution of new cell types. The establishment of transgenic reporters in *Hydractinia* appeared to be complicated by specific aspects of hydrozoan biology, possibly related to chromatin status. These may not, however, be only negative but could mark a useful path to study the role of chromatin context in controlling gene expression. With the genome of *Hydractinia* nearing completion it is now possible to tackle such questions at a high resolution. In addition, CRISPR-Cas9 technology will allow for targeted manipulation of genomic sites. Together with the tractability of *Hydractinia* and the access it provides to all life stages this route of study could potentially unveil novel methods of gene regulation.

The finding that the role of Notch signalling in early neurogenesis is absent in *Hydractinia* is the most exiting outcome of this thesis. My hypothesis that the loss of Notch signalling removed an evolutionary constraint and allowed for the evolution of migratory stem cells – the well known i-cells in hydrozoans – has broad implications. First, the evolution of i-cells has never been addressed previously; second, this sequence of events is one of only few known examples of emergences of novel cell types in animal evolution. A number of open questions still outstand. They include the embryonic origin of i-cells, their heterogeneity and the molecular events underlying their commitment. A basic or pluripotent sub-population, similar to the clonogenic neoblast of planarians (Wagner et al., 2011), is a likely possiblity that remains to be demonstrated at single cell level. These experiments would also reveal further interesting losses and gain of function which contributed to the evolution of this lineage.

Finally, although this thesis devoted a lot of work to the function of Notch signalling in *Hydractinia*, many questions maintain. They include the detailed temporal and spatial expression pattern of *Notch*, its mechanistic role in nematocyte differentiation, and what function does it fulfill in tentacle patterning. The tools and techniques developed over the course of this study will facilitate addressing these broadly relevant questions using *Hydractinia* as a model organism.

7.5.2. Summary

Notch signalling is considered a conserved process regulating neurogenesis since the emergence of the first nervous system, and indeed plays related roles across studies animals in embryonic neurogenesis. I have shown that this function has been lost in *Hydractinia echinata*, and likely in hydrozoans in general, while other functions of Notch signalling, i.e. regulation of tentacle patterning and nematogenesis, are conserved among cnidarians. Furthermore I hypothesise that this loss led to the lifting of a migration constraint on stem cells in stem hydrozoans which opened the way for the evolution of i-cells, the enigmatic stem cell population of this clade. This opens up a new area of investigation, i.e. what conditions were necessary for i-cell evolution and how did these cells subsequently emerge. Finally, I have shown several new transgenic and genome editing tools in *Hydractinia* which will allow for these questions to be addresses in the future.

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Appendix A-Codon optimised genes

tdTomato:

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ATGACTTCCAAAGGTGAAGAGGTTATCAAAGAATTTATGCGTTTCAAAGTTCGAATGGAAGGGAGTATGAATGGACATGAGTTTG
AGATAGAAGGAGAAGGGGAAGGTAGACCCTATGAAGGCACTCAAACGGCTAAGTTAAAAGTTACAAAGGGAGGTCCACTGCCATT
TGCTTGGGATATTTTGTCTCCTCAATCATGTATGGTTCGAAAGCATACGTTAAACACCCTGCAGATATTCCGGACTATAAGAAGT
TATCGTTTCCAGAAGGTTTTAAATGGGAACGCGTTATGAACTTCGAAGATGGTGGGTTAGTAACAGTAACACAGGATAGCAGCTT
GCAAGATGGTACGTTAATCTACAAAGTGAAAATGAGAGGCACCAATTTTCCACCAGATGGACCAGTCATGCAGAAGAAAACTATG
GGCTGGGAAGCAAGCACCGAAAGGCTATATCCTAGGGATGGTGTTTTAAAAGGAGAGATCCATCAAGCTTTGAAACTAAAAGACG
GTGGCCACTATTTGGTGGAATTTAAGACCATATACATGGCTAAGAAACCGGTACAGTTACCGGGATATTATTACGTGGATACTAA
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GATTTAAAGTGCGAATGGAGGGCTCAATGAATGGACACGAATTTGAAATTGAAGGGGAGGGTGAAGGACGACCGTACGAAGGAAC
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AAAGCCTATGTCAAACATCCAGCTGATATTCCCGATTACAAGAAACTTAGCTTTCCAGAGGGATTCAAATGGGAACGTGTGATGA
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GGAGTCTTGAAAGGGGAGATACATCAAGCCCTAAAGCTTAAAGATGGTGGCCATTATCTGGTAGAATTTAAAACCATTTATATGG
CGAAAAAACCTGTCCAGTTACCAGGCTACTACTACGTTGACACAAAGTTAGATATTACAAGTCACAATGAAGATTACACTATTGT
TGAACAGTATGAACGCTCTGAAGGTAGACACCACTTATTTCTTTACGGAATGGACGAACTGTACAAATAA
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Kaede:

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AACAACAGCTTTTCACTATGGCAATCGTGTGTTTGCCAAATACCCAGACCATATTCCAGACTACTTTAAGCAGTCATTTCCCAAA
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CACTGAGAAAATGTACTTGAGAGATGGTGTACTTACAGGCGATATTACTATGGCGTTATTGCTTAAAGGCGATGTTCACTATCGT
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TAAGGCATGACAAGGATTACAACGAAGTGAAGTTGTATGAGCATGCTGTTGCACACTCAGGTTTACCTGACAATGTCAAATAA
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Appendix B- Transcripts used for annotation

Notch:

AAAAACTGTACTCTACATGGTGTTGTGTTGTGTATAATCATGTTTTTCGTGTTTTTTTATTTATTTTTTGCCACAACTCCAACAA
CACACGCTTCAACTAATTCAACAAGGTTATGTCTATTGAGATGTGTGAATGGAGGCACTTGCCAGTTTCAAAATGGTACAGCAAT
GTGCAAGTGCAGCATTACTCACACAGGAAATGTATGTCAAACACTTATAAAAGAATTTTGTGGACATCCGAATCCTTGCAAAAAT
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CGCCTTGTAGTGGTAATGGATCAAAGTGTACACTTTTAAGTAATAATCAAACTGGCTGTCAGTGTGAGAAATTGGGCTTTGATGG
TCCAGATTGTAAGAAAGACATTAATGAATGCCTTCGGCACAAAGGCATTTGTGGCCAACATGGTATGTGCAACAACACCATTGGT
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GCACAAGACGAAGTGTCGATCAAAAGCAACCGAAGCTGCGAGTAACAACCACCATTAACAATAAAATGTGCACTGGTGGTTGTTT
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CCTGCTTCGTTATAGTTATGGTTGTAACCGGAAAAAGGACCTATGGTGCCCTCTGGGCGCCAGAAGGCCATAGGCAACACCGGGC
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Jagged:

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Delta1:

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Delta2:

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