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An unexpected role for Nanos in cnidarian neural stem cell fate determination

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# Table of Contents

Acknowledgements........................................................................................................6
Summary of contents........................................................................................................7
Declaration of contribution...............................................................................................8

Chapter 1. Introduction....................................................................................................9
  1.1. Stem cells...............................................................................................................9
  1.2. Nanos....................................................................................................................13
  1.3. *Hydractinia echinata* as a model organism.........................................................16
  1.4. Aims of the project...............................................................................................22

Chapter 2. Materials and methods.................................................................................23
  2.1. RNA and DNA protocols......................................................................................23
      2.1.1. Total RNA extraction from animal tissue.......................................................23
      2.1.2. DNase digestion of total RNA.........................................................................25
      2.1.3. Formaldehyde denature gel for RNA analysis.................................................26
      2.1.4. Plasmid DNA extraction from bacteria...........................................................27
      2.1.5. DNA extraction from agarose gel...................................................................29
      2.1.6. dsRNA synthesis for RNAi..............................................................................30
      2.1.7. DNA digestion with restriction enzyme..........................................................31
      2.1.8. DNA clean up and precipitation......................................................................32
      2.1.9. Synthesis of RNA probes for *in situ* hybridization.......................................33
      2.1.10. Single and double whole-mount *in situ* hybridization.................................35
      2.1.11. Fluorescent *in situ* hybridization (FISH).........................................................38
      2.1.12. RACE cDNA synthesis..................................................................................39
      2.1.13. PCR reaction..................................................................................................40
      2.1.14. Sequencing reaction.......................................................................................42
      2.1.15. DNA ligation..................................................................................................43
      2.1.16. Bacteria transformation..................................................................................43
Chapter 3. Gene cloning and analysis

3.1. Introduction
3.2. Gene amplification by RACE PCR
3.3. Nanos2
3.4. Nanos2 promoter
3.5. Nanos1
3.6. Pumilio
3.7. NcoI
3.8. Ash
3.9. RFamide precursor
3.10. Other neural genes
3.11. Summary

Chapter 4. Gene expression analysis

4.1. Introduction
4.2. Nanos2
4.3. Nanos1
4.4. Pumilio
4.5. NcoI
8.2.2. *Nanos2* promoter activity .................................................. 165
8.3. *Nanos2* ectopic expression .................................................. 167
8.4. *Nanos2* knockdown .............................................................. 170
8.5. Suggested mechanism of *Nanos2*-mediated specification of neural cell types .......................................................... 171
8.6. Concluding remarks .............................................................. 175

Chapter 9. References ................................................................. 176
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Summary of contents

The mechanisms of lineage commitment and acquiring specific cell fate remain obscure and require investigating the role of particular genes involved in these complex processes. To address this issue, I studied the role of Nanos in the nervous system of a cnidarian Hydractinia echinata.

Nanos is an mRNA-binding translational repressor, which functions in germ stem cell specification in many species and in posterior patterning in Drosophila. Interestingly, Nanos was also found to be expressed in the nervous system of many animals, although this role is poorly understood. In Drosophila Nanos is important for proper dendrite morphogenesis and for maintaining sodium current and excitability of motoneurons.

While Hydractinia Nanos1 mRNA was detected only in germ cells, Nanos2 was also expressed in cells committed to neural fate, from early progenitors to maturing nematocytes. To gain further insight into the biological functions of Hydractinia Nanos2, I studied this gene by ectopic expression in transgenic animals, and by dsRNA and morpholino-mediated downregulation.

Nanos2 ectopic expression caused severe defects in animal development. This was manifested by formation of supernumerary and ectopic tentacles (i.e. oral structures), which led to complete oralization in severe phenotypes. Further analysis showed that the reason for the abnormal development is a change in the commitment of early embryonic cells, driving them to make more stinging cells (cnidarian specific sensory cells), at the expense of RFamide+ neurons. The excess of stinging cells possibly induced formation of extra tentacles post metamorphosis.

Nanos2 knockdown resulted in reduced proliferation and decrease in the number of stinging cells. Animals also had fewer tentacles following metamorphosis, conversely to Nanos2 ectopic expression phenotype.

Nanos' roles in germ cells development and proliferation seem to be ubiquitous in metazoans, but a role for Nanos in specification of neural fates has not been found so far in any other animal. My data also demonstrate that the use of basal invertebrate models can have implications for basic developmental biology and biomedicine.
Declaration of contribution

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

My project was founded by Science Foundation Ireland (SFI) and Thomas Crawford Hayes Trust Fund.
1. Introduction

1.1. Stem cells

Stem cells are basic biological units of most multicellular organisms. The term “stem cell” was used for the first time by the Russian histologist, Alexander Maksimov, on the 1st of June, 1909 on a meeting of Haematological Society of Berlin (Svendsen and Ebert, 2008). During his lecture, Maksimov suggested the existence of hematopoietic stem cells and their role as common progenitors of cellular elements in the blood. At this time, however, cell biology was poorly understood and the existence of DNA not even discovered yet. Stem cell research expanded only few decades ago and was driven by the scientific and technological progress of the 1960’s. Studying the fundamental properties of stemness (e.g. self-renewal, plasticity, differentiation potency) and stem cell commitment to different lineages became a subject of research worldwide. Increasing interest in stem cell research was especially observed over the last few years. Only in 2006 more than 1100 papers on embryonic stem cells were published, in comparison to 140 papers in 1997 (Svendsen and Ebert, 2008). The breakthrough in stem cell research was brought by the isolation of human embryonic stem cells (hESC) (Thomson et al., 1998), followed by the generation of induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006). Interestingly, iPSCs were created by an overexpression of only four transcription factors: Oct4, Sox2, Klf4 and c-Myc, in mouse embryonic and adult fibroblasts. This experiment resulted in reprogramming of differentiated fibroblasts back to undifferentiated pluripotent stem cells.

The interest in stem cells is due to their remarkable properties to maintain an undifferentiated state and self-renew, as well as to produce progeny of differentiated daughter cells (Svendsen and Ebert, 2008). These stem cell features represent great perspectives for both regenerative medicine and basic developmental biology. Stem cells have been studied in order to use them in therapeutic approaches, e.g. treatment of incurable diseases, like Parkinson’s disease, and in transplantation of somatic tissues or even whole organs (Wang et al., 2006). They are a source of all types of specialized cells, like neurons or myocytes, which makes tissues, organs and eventually the whole multicellular organism (Potten and Loeffler, 1990). Stem cells have the ability to divide both symmetrically and asymmetrically (Morrison and Kimble, 2006). By symmetric division a stem cell produces two copies of itself. This process is called self-renewal and allows stem cells to expand in number during development and regeneration (Morrison and Kimble, 2006). Asymmetric division leads to generation of two different cell types: one stem cell and one committed cell.
that will differentiate into a specialized cell. Early committed cells often possess stem cell properties, like ability to proliferate, maintain stem cell gene expression pattern and morphology, but they have limited differentiation potency. Expression of particular set of lineage-determining transcription factors and repression of stem cell genes eventually lead to the restriction of transcriptional circuits and to the fixation of lineage fate (Hemberger et al., 2009).

To maintain self-renewal properties, pluripotent stem cells must retain the proper balance of stemness-sustaining genes. They usually keep at the high level the expression of pluripotency genes, and repress at the same time the expression of genes promoting cellular differentiation (Sperger et al., 2003; Boyer et al., 2005; Loh et al., 2006). Expression of about 216 genes is enriched in ESCs (Embryonic Stem Cells), many of which are likely to maintain stemness (Ramalho-Santos et al., 2002). Regulation of stem cell pluripotency and differentiation mostly depends on the regulatory circuit of Nanog, Oct4, and Sox2, three core pluripotency transcription factors (Boyer et al., 2005; Kashyap et al., 2009; Ramalho-Santos, 2009). To keep stem cell identity, these genes act together with other transcriptional modulators, creating a complex regulatory network. Other genes are not able to directly control pluripotent state but their expression is very important for retaining some stem cell properties. For example, Myc, Klf4 or LIN28 maintain self-renewal and regulate the timing of cell commitment and differentiation (Segre et al., 1999; Murphy et al., 2005; Darr and Benvenisty, 2009). The role of these genes involves protection of stem cells from premature differentiation and depletion before enough numbers of progenitors are formed. Myc, besides its role in cell survival and proliferation, also plays a role in apoptosis, differentiation, metabolism, ribosomal biogenesis and cell growth (Dang, 1999; Hartl et al., 2010). This gene is an oncogene too, and its upregulated expression commonly leads to tumorigenesis (Collins and Groudine, 1982; Levens, 2010). Other stem cell genes, like Piwi or Nanos control the identity, maturation and migration of germ stem cells (Kobayashi et al., 1996; Tsuda et al., 2003; Wang and Lin, 2004; Megosh et al., 2006). The upregulated expression of stem cell genes, like Oct4, Myc or even Nanos, is often observed in cancer cells (Collins and Groudine, 1982; Monk and Holding, 2001; Janic et al., 2010).

Maintaining stemness also involves posttranslational regulations on RNA and protein level, e.g. inhibition of mRNA translation. However, not only intracellular signals play important role in maintaining stem cell properties, but also extracellular signals are crucial for this process. It is the combinatorial control of gene transcription and stem cell microenvironment that is fundamental to the stem cell state (Chambers and Tomlinson, 2009). Stem cells’ surrounding microenvironments, so called stem cell niches, are anatomic locations which save stem cell populations
from depletion and premature differentiation (Scadden, 2006). Each type of multipotent stem cells have their specific niches, e.g. the germline stem cell niche (Xie and Spradling, 2000; Li and Xie, 2005), vertebrate-specific hematopoietic stem cell niche or cardiovascular stem cell niche (Moore and Lemischka, 2006; Martinez-Agosto et al., 2007).

Stem cell properties are described by their differentiation potential, which varies and defines the ability of stem cells to generate either all cell types, or only lineage-specific cells. On the top of cell potency ladder are totipotent cells, then pluripotent, multipotent and unipotent at the bottom (Mitalipov and Wolf, 2009). Zygotes and blastomers of fertilized eggs are the only cells considered to be the master totipotent cells (Fig. 1.1.1). They are capable to generate all cell types of the conceptus and the adult organism, including extraembryonic membranes, the placenta and germ cells (Hemberger et al., 2009). Totipotency is lost after few more embryonic divisions. Then the differential capacity of cells decreases and at the blastocyst stage cells become pluripotent, e.g Inner Cell Mass cells (ICMc) (Fig. 1.1.1). The pluripotency is defined as the ability of cell to differentiate into any of the three germ layers’ cells (endodermal, ectodermal and mesodermal) and into germ cells. Pluripotent stem cells have restricted differentiation potential and alone cannot create a new adult organism as they lack the ability to contribute to extraembryonic tissue and organize into an embryo (Mitalipov and Wolf, 2009). Blastocyst’s cells lose their pluripotency as the embryo gastrulates. At this stage only sequestered germ cells retain their pluripotent state and all three germ layers’ cells become multipotent (Fig. 1.1.1). Multipotent stem cells generate somatic cells which form all the building blocks of the body. In all three germ layers there are different lineages of multipotent stem cells and each of them have an ability to generate several related cell types. For example, among multipotent mesodermal stem cells lineages are myoblasts or hematopoietic stem cells, which can differentiate into several types of cells, but only muscle- or blood-specific, respectively. Also ectodermal multipotent stem cells, like neuroblasts are not capable to differentiate into mesodermal cells (Fig. 1.1.1). Multipotent stem cells reside in their niches and maintain ability to self-renew or to differentiate into more restricted oligopotent stem cells. Oligopotency represents another degree of stem cell potency and defines the ability of cells to generate very limited number of daughter cell types, e.g. lymphoid or myeloid stem cells of the blood system (Shostak, 2006). At the bottom of the cell potency ladder is the unipotent stem cell. It has the most limited differentiation capacity and has the ability either to self-renew or to give rise to only one cell type, e.g. hepatocytes, which constitute most of the liver tissue. After a unipotent stem cell becomes terminally differentiated, it stops proliferating and has relatively short lifespan (Shostak, 2006).
The mechanism by which stem cells lose their potency and acquire specific fate is very complex and poorly understood. It requires coordinated action of many cell signaling pathways, which switch on or off crucial transcription factors. Organisms need to respond quickly for the demand of different cell types and be able to generate them always on time and in the right place in order to maintain homeostasis. Activation or repression of particular set of genes, coupled with posttranslational modifications, protein regulations, and altered micro-environmental factors eventually lead to the determination of specialized cellular function. Studies on genes involved in stem cell maintenance and differentiation are important for understanding the trigger for cellular specification and cell fate choice.
1.2. Nanos

Studying the genes expressed in stem cells and their daughter cells can give insight into the mechanism of cellular commitment and differentiation. To address this question, I decided to study the role of the germline-specific gene *Nanos* in development of the cnidarian *Hydractinia echinata*, focusing on its regulatory functions in stem cells and their derivatives.

*Nanos* belongs to a family of Zn-finger proteins. It acts as an RNA-binding protein, repressing translation of its target mRNA transcripts (Parisi and Lin, 2000). *Nanos* is a classical germline marker, required for proper differentiation of primordial germ cells (PGCs) into germ cells (Tsuda et al., 2003). Its role in germline involves maintaining germ stem cells self-renewal (Wang and Lin, 2004), by preventing them from premature differentiation, apoptosis and acquiring somatic cell fate (Kobayashi et al., 1996; Hayashi et al., 2004). Nanos was also found to be required for migration of PGCs into the gonad, in both *Drosophila* and mouse, and its knockout results in sterility of both sexes (Kobayashi et al., 1996; Tsuda et al., 2003). Nanos’ roles in germ cells development seem to be well conserved in the animal kingdom.

In addition to its role in germ cells, Nanos was also well characterized as a posterior determinant in *Drosophila melanogaster*. It regulates abdomen formation by blocking translation of *hunchback* (*hb*) mRNA (Wang and Lehmann, 1991). The exact mechanism of *hb* translational repression is shown on Fig. 1.2.1 (Lodish et al., 1995). Nanos molecules diffuse along the embryo and form a gradient with the maximum concentration in the posterior end and the minimum in the anterior end. In all known cases, Nanos acts together with its partner, Pumilio. However, Nanos has no effect on the amount and distribution of Pumilio (*Pum*) protein, or its target - *hunchback* - mRNA (Fig. 1.2.1). In the posterior end, where Nanos gradient is at its maximum, Pumilio recognizes and binds Nanos Response Element (NRE) within the 3’-UTR of *hb* mRNA. 32-nucleotide sequence of NRE contains conserved binding site for Pumilio, called Pumilio Regulatory Element. Once bound, Pumilio recruits Nanos, which binds to *hb* mRNA sequence too. The binding site for Nanos is also within NRE and is called Nanos Regulatory Element. Functional studies indicate that the Nanos Regulatory Element (GUUGU) and the Pumilio Regulatory Element (UGUAXAUA) are necessary for proper functioning of the complex Nanos-Pumilio. These short sequences also seem to be conserved in other mRNA transcripts regulated by these proteins (Murata and Wharton, 1995). After the complex is formed, Brain Tumor (Brat) is recruited resulting in the repression of *hb* mRNA.
transcription. Nanos-mediated mechanism of *hb* translational repression includes both deadenylation of target mRNAs and poly(A)-independent silencing (Chagnovich and Lehmann, 2001). As a consequence, the gradient of Hb protein is formed, with its minimum at the posterior end and the maximum in the anterior, where Nanos concentration is minimal.

Regulation of Hb protein level is critical for proper embryonic patterning of *Drosophila* embryos. However, *hb* has not been identified outside of arthropods, and the similar role of Nanos in AP patterning has not been described in animals.
belonging to different phyla. Possibly, Nanos-mediated *hunchback* translational blockage is an arthropod innovation (Pinnell et al., 2006).

The C-terminal region of Nanos protein is essential for its function (Curtis et al., 1997). This region is well conserved between Nanos homologs from different species and contains Cys-Cys-His-Cys zinc-finger motifs, which are indispensable for Nanos-mediated translational blockage (Fig. 1.2.2) (Hashimoto et al., 2010).

![Fig. 1.2.2. Domain architecture and sequence alignment of Nanos. (A) Domain architecture of two CCHC zinc-finger motifs, ZF1 and ZF2. (B) Sequence alignment of the zinc-finger domains of Nanos proteins from different species: zebrafish (z), mouse (m) and *Drosophila* (dm). The CCHC motifs in ZF1 and ZF2 are highlighted by a pink and purple background, respectively (Hashimoto et al., 2010).](image)

Mammalian genomes encode three Nanos homologs. Two of them, *Nanos2* and *Nanos3*, are exclusively expressed in germ cells (Tsuda et al., 2003). The third homolog, *Nanos1* is expressed in the Central Nervous System (CNS) (Haraguchi et al., 2003). *Drosophila* has a single *nanos* homolog, expressed both in germ cells and in neural cells (Brechbiel and Gavis, 2008). Nanos’ roles in germ cells seem to be conserved among invertebrates and vertebrates, however its role in the nervous system remains poorly understood and very intriguing. *Nanos1* knockout in mouse does not cause any severe defects in mouse development nor does it affect its fertility (Haraguchi et al., 2003). Knockout mice do not have any significant neural deficiencies or behavioral changes. Interestingly, in *Drosophila*, Nanos was shown to be essential for dendrites’ morphogenesis, where together with its canonical partner, Pumilio, elaborates high-order dendritic branching of peripheral neurons (Ye et al., 2004; Brechbiel and Gavis, 2008). Nanos was also found to be essential for Pumilio-mediated regulation of sodium current and excitability in *Drosophila* motoneurons (Muraro et al., 2008). Its ectopic expression was shown to drive malignant brain tumor growth in flies and its knockdown suppressed brain tumourigenesis (Janic et al., 2010). However, the mechanism of Nanos-mediated function in nerve cells still remains unknown and requires further investigation.
1.3. *Hydractinia echinata* as a model organism

*Hydractinia echinata* represents great model for studying basic developmental biology, including questions on the roles of particular genes in development, cell fate determination and regeneration. It has been established as an experimental model quite recently (Frank et al., 2001). *Hydractinia* belongs to the phylum Cnidaria, to the class Hydrozoa that together with Scyphozoa and Cubozoa makes the subphylum Medusozoa (Hinde, 1998). The diversity of Cnidaria – one of the most ancient animal phyla, is shown on Fig. 1.3.1 (Miller and Ball, 2008). It consists of medusozoans and anthozoans.

Fig. 1.3.1. The diversity of the phylum Cnidaria. Cnidarians are divided into four classes: Anthozoa, Hydrozoa, Scyphozoa and Cubozoa. The last three are classified to the subphylum Medusozoa. The picture shows the complexity of their life cycles and differences between them. Among the best described hydrozoans are: *Clytia hemisphaerica* and *Hydra magnipapillata*, scyphozoans: *Aurelia aurita* and anthozoans: *Nematostella vectensis* (Miller and Ball, 2008).
Anthozoans are represented by sessile corals, sea anemones and sea pens. The most used anthozoan genetic models are *Nematostella vectensis* and *Acropora millepora*. Medusozoans have more complex life cycles than anthozoans. Besides the typical planula and polyp stage, which are also found in anthozoans, they additionally have swimming medusa, which represents adult, sexually reproducing form. Example representatives of the class Hydrozoa are *Clytia hemisphaerica*, *Hydra magnipapillata* and *Podocoryne carnea*, of the class Scyphozoa is *Aurelia aurita*, and of the class Cubozoa (box jellyfish) is *Chironex fleckeri* (Fig. 1.3.1).

*Hydractinia echinata* is a marine animal which lives in the European northeastern Atlantic Ocean (Fleming, 1828; McFadden et al., 1984). Its colonies encrust the shells of the hermit crabs and are commonly found on the coasts of Great Britain and Ireland, where these crabs occur (Fig. 1.3.2).

![Fig. 1.3.2. The colony of *Hydractinia echinata* encrusting the shell of a hermit crab. Source: http://www.arkive.org/common-hermit-crab/pagurus-bernhardus/image.](image)

Adult *Hydractinia* colonies consist of genetically-identical individuals and have four types of specialized polyps: feeding, sexual (male and female) and defensive polyps (Fig. 1.3.3) (Cartwright, 2003). Feeding polyps, called gastrozooids, have a smooth and long body column (Fig. 1.3.3A). Their mouth is surrounded by ring of tentacles, which are used to capture prey and insert it into the gastric cavity. Tentacles are usually equipped with stinging cells. Stinging cells inject the venom to the target organism in order to paralyze it. There are two types of defensive polyps: dactylozooids and tentaculozooids (Fig. 1.3.3C and D, respectively). Defensive polyps have reduced tentacles and usually possess large numbers of stinging cells too. Sexual polyps, called gonozooids, have gonads filled with sperm and oocytes. The gonads are reduced medusae. The adult colony can be either male or female (Fig. 1.3.3B, F: female and M: male). Hermaphrodite colonies are quite rare and their presence most likely results from an environmental stress.
Fig. 1.3.3. *Hydractinia echinata* colony. *Upper left:* female colony consists of adult feeding polyps (A) and female sexual polyps (F). *Bottom left:* male colony consists of adult feeding polyps (A) and male sexual polyps (M). *Right:* adult colony consists of four types of zooids (polyps): (A) feeding polyps (gastrozooids), (B) sexual polyps (gonozooids), and two types of defensive polyps: (C) dactylozooids and (D) tentaculozooids (Cartwright, 2003).

*Hydractinia* has a relatively short life cycle that covers many developmental stages (Fig. 1.3.4) (Millane et al., 2011). An easy access to all different life stages makes this hydrozoan an interesting experimental model for studying development.

Fig. 1.3.4. The life cycle of *Hydractinia echinata* (Millane et al., 2011).
Sexual polyps release eggs and sperm on daily basis and the process of spawning is triggered by the light (Millane et al., 2011). Fertilized eggs divide, develop into morula, then gastrula and within two-three days elongate into planula larvae. Larvae settle down on the shells of the hermit crabs and are induced to metamorphose into primary polyps by a bacterial film on the shells (Fig. 1.3.4). Primary polyps spread their stolons (aboral structures), from which new polyps bud. Stolons form a network of gastrovascular canals, through which food particles are passed between the members of one colony. They are also a place of residence for pluripotent stem cells, so called i-cells, from which all specialized cells arise (Millane et al., 2011). Hydractinia colony becomes sexually mature within few months.

*Hydractinia* is a diploblastic animal and, like all other cnidarians, have epidermal and gastrodermal epithelial layers, which are derived from the embryonic ectoderm and endoderm, respectively. Fig. 1.3.5 shows *Hydractinia* adult feeding polyp and a drawing of a typical hydrozoan body wall (Technau and Steele, 2011). Both epithelial layers are separated by the mesoglea, an extracellular matrix, which supports the soft polyp body. The layers are one cell thick and are built from epitheliomuscular cells. In the interstitial spaces between these cells, stem cells and their early derivatives are found, like in the case of *Hydra magnipapillata* (Fig. 1.3.5) (Bode, 1996; Technau and Steele, 2011).

![Fig. 1.3.5. Hydractinia adult feeding polyp and the schematic diagram of a typical hydrozoan body wall. In the interstitial spaces between epitheliomuscular cells of the epidermal layer, duplets of i-cells and clusters of nematoblasts are present. Neurons are found both in the epidermis and in the gastrodermis. Gland cells are secretory cells, which resides in the gastrodermis (Technau and Steele, 2011).](image-url)
Hydractinia is believed to have pluripotent stem cells lifelong, and based on their localization in the interstitial spaces between epitheliomuscular cells, they were named interstitial-cells (i-cells) (Fig. 1.3.6A) (Millane et al., 2011). Hydractinia’s stem cells share many features with their mammalian counterparts, in aspects of their morphology and gene expression pattern. I-cells are about 10 µm in size, have a relatively big nucleus, and usually are single or form duplets of cells (Fig. 1.3.6A). It is believed that hydroids do not sequester a germline and that both germ cells and somatic cells differentiate from i-cells (Bosch and David, 1987). Stem cells are primarily present in stolons and migrate towards the polyp body as their differentiation progresses. In stolons mainly undifferentiated cells are found, whereas in the head of the polyp, almost no i-cells, but mostly specialized cells are present. Besides germ cells, i-cells give rise to all types of differentiated cells including mechanosensory cells (nematocytes), neurons, gland cells and epitheliomuscular cells. Gland cells are secretory cells, which are found in the gastrodermal layer and secrete enzymes or hormones used e.g. for food digestion (Fig. 1.3.5). Nematocytes and neurons belong to the neuronal lineage and Hydractinia possesses few types of each of these cell types. Their progenitors are called nematoblasts and neuroblasts. Nematoblasts are located in the epidermis and usually form clusters of 4, 8, 16 or more cells (Fig. 1.3.5) (Technau and Steele, 2011). Their differentiated products, nematocytes, also called stinging cells or cnidocytes, are very distinct cell types, found only in cnidarians. They are mechanosensory cells and possess very specialized structures, called nematocysts, which distinguish them from all the other cell types. Nematocysts contain a coiled thread and venom. After physical or chemical stimuli this thread is discharged and venom injected into the target organism (Fig. 1.3.6B). Large numbers of stinging cells are present in tentacles, where they serve to paralyze and capture prey or to defend against predators (Kurz et al., 1991; Stachowicz and Lindquist, 2000).

![Fig. 1.3.6.](http://siera104.com/bio/cnid.html)

Fig. 1.3.6. (A) I-cells in Hydractinia’s stolons stained with Gimsa (Millane et al., 2011). (B) Nematocyst’s discharge after stimulation by the prey. Source: http://siera104.com/bio/cnid.html.
So far *Hydractinia* was used as an experimental model for studying many important biological processes, like embryonic development and patterning (Schlawny and Pfennenstiel, 1991; Plickert et al., 1998; Plickert et al., 2006; Müller et al., 2007; Duffy et al., 2010), stem cells and the mechanism of cell fate determination (Plickert et al., 1998; Rebscher et al., 2008; Künzel et al., 2010), apoptosis (Seipp et al., 2001), allore cognition (Toth, 1967; Gild et al., 2003; Cadavid, 2004; Ferrell, 2004; Rosa et al., 2010), and regeneration (Toth, 1967; Frank et al., 2009). Interestingly, most of the genes involved in cell signaling pathways are conserved in *Hydractinia*, so it is also possible to study the evolution of genes’ functions and their roles in development (Frank et al., 2001). Many experimental methods are already well established for this cnidarian model (Duffy et al., 2010; Millane et al., 2011). The following techniques are used for gene manipulation:

a) Overexpression:

- Microinjection of overexpression plasmid: permanent insertion of overexpression cassette into the *Hydractinia* genome. First generation results in mosaic expression and uniform transgenic animals can be obtained following sexual reproduction.

b) Knockdown:

- Microinjection of morpholino allows studying gene knockdown in early *Hydractinia* development. It is possible to order fluorescent and non-fluorescent gene-specific morpholino directed either against the start codon to block mRNA translation or against intro-exon boundary to block splicing.

- Double stranded RNA mediated interference (RNAi) can be conducted by soaking animals in a dsRNA solution. This technique allows studying gene knockdown during all stages of *Hydractinia* development and during regeneration.
1.4. Aims of the project

Understanding the mechanisms of lineage commitment and acquiring specific cell fate requires investigating the role of particular genes involved in these complex processes. To address this issue, I decided to study the role of the Nanos2 gene in neurogenesis. Nanos functions in germ cells development and proliferation seem to be conserved among metazoans, but its role in the nervous system has not been well studied so far. With the use of a basal invertebrate model, the cnidarian Hydractinia echinata, I investigated the role Nanos2 plays in the nervous system.

Using Hydractinia as an experimental model for studying developmental biology gives an opportunity to look at the effect of altered gene expression on the well-being of the whole organism, not only on particular cells. Another advantage of Hydractinia is the simplicity of its culturing and an easy access to the wild type population on the coast of Ireland. Also, work on Hydractinia does not bring about any ethical issues, in contrast to mammalian models. Importantly, studying such an early branching metazoan can provide insight into the evolution of animals and may reveal new roles for genes, which remain cryptic in other systems.

The specific aims of my work were as follows:

1. Identification of Hydractinia Nanos and Pumilio homologs.
2. Studying the expression patterns of these genes in all Hydractinia’s life stages.
3. Studying the role of Nanos2 in Hydractinia development with the use of available gene manipulation techniques.

The results of my work reveal a novel function for Nanos2 in the regulation of neural cell type specification and suggest the mechanism of its action. My data also demonstrate that the use of early branching metazoan models can have implications for basic developmental biology and biomedicine.
2. Materials and methods

2.1. RNA and DNA protocols

2.1.1. Total RNA extraction from animal tissue

I. For small amount of animal tissue Qiagen RNeasy Minikit (cat. no. 74104) was used according to suggested protocol:

1. Disruption and homogenization: 20-30 mg of animal tissue homogenize by vortexing in 600 μl of supplied buffer RLT.
2. Centrifuge for 3 min at full speed and separate the lysate.
3. Add 1 volume of 70% ethanol to the cleared lysate and mix immediately by pipetting.
4. Transfer 700 μl of the sample to an RNeasy spin column placed in a 2 ml collection tube (supplied) and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow-through. Repeat the procedure if volume of the sample is bigger than 700 μl.
5. Add 700 μl of supplied buffer RW1 to the RNeasy spin column and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through.
6. Add 500 μl of buffer RPE to the RNeasy spin column and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow-through.
7. Add 500 μl of buffer RPE to the RNeasy spin column and centrifuge for 2 min at 8000 x g (10,000 rpm). Discard the flow-through.
8. Place the RNeasy spin column in a new 2 ml collection tube (supplied) and centrifuge at full speed for 1 min to eliminate any possible carryover of Buffer RPE.
9. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 μl RNase-free water directly to the spin column membrane and centrifuge for 1 min at 8000 x g (10,000 rpm) to elute the RNA.

II. For large amounts of animal tissue, the following protocol for total RNA extraction was used:

1. Homogenize sample by vortexing in 0.5 ml of lysis buffer (0.5 ml per 50 μg of eggs). Centrifuge and collect supernatant.
2. Add 100 μl of chloroform (per 0.5 ml of supernatant) and vortex sample for 15s. Incubate lysate on ice for 20 min.
3. For phase separation centrifuge sample for 5 min at room temperature and at the maximum speed. RNA is in the upper colorless layer. DNA and proteins are in the lower, pink, phenol-chloroform layer.
4. Transfer upper layer to the new RNAase-free tube and add 500 μl of chloroform, spin 2 min and collect upper aqueous layer.
5. Per 0.25 ml aqueous phase add 125 μl of 1.2M NaCl/0.8M sodium citrate, vortex and add 0.6 volume of ice cold isopropanol (225 μl).
6. Incubate sample on ice for 30 min.
7. Centrifuge sample for 5 min, at maximum speed, at room temperature and then remove supernatant.
8. Add 500-700 μl of 75% ethanol, vortex and spin for 1 min at maximum speed.
9. Remove supernatant and air dry the pellet at room temp for 2-5 min.
10. Add 50 μl of 0.1% SDS. Wait 2-3 hours to dissolve. If still some particles remain undissolved in the solution centrifuge the sample and collect supernatant containing RNA.
11. Add 1 volume (50 μl) of 12M LiCl to precipitate RNA and incubate sample on ice for 20 min.
12. Spin the tube for 5 min, at maximum speed and remove supernatant.
13. Add 500 μl of 70-75% ethanol, vortex and spin for 1 min at room temperature.
14. Remove supernatant and let the pellet air dry, then add 20 μl of RNase-free water to dissolve RNA and measure its concentration on the Nanodrop.

**Lysis buffer**

500 μl solution D
500 μl phenol pH 4.0
100 μl 2M Na-acetate (stock: 1.64 g per 10 ml)
7,7 μl β-mercaptoethanol

**Solution D:**

To 19,53 ml of distilled water add:
16.66 g guanidinium thiocyanate
1,17 ml 0.75 M sodium citrate
   (stock: 2.21g per 10 ml)
1,76 ml sarcosyl
2.1.2. DNase digestion of total RNA

RQ1 RNase-Free DNase, from Promega, cat. no. M6101 was used for DNase digestion.

The DNase digestion reaction was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>x</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>x</td>
</tr>
<tr>
<td>10 x DNase buffer</td>
<td>1µl</td>
</tr>
<tr>
<td>RNase-free DNase</td>
<td>1µl/g</td>
</tr>
<tr>
<td></td>
<td>10µl</td>
</tr>
</tbody>
</table>

1. Incubate reaction at 37°C for 30 min.
2. Add 10 µl of phenol (pH 4.0) and 10 µl of chloroform.
3. Centrifuge sample at the maximum speed for 3 min.
4. Take the upper, aqueous layer and re-extract with equal volume of chloroform.
5. Centrifuge sample for 3 min at the maximum speed.
6. Separate the upper aqueous layer and add 0.1 volume of 3M sodium acetate pH 4.8 and 1 volume of ice-cold isopropanol. Vortex sample and place on ice for 50 min.
7. Centrifuge sample at the maximum speed for 10 min.
8. Remove supernatant and wash the pellet with 400 µl of 75% ice-cold ethanol.
9. Centrifuge sample for 5 min.
10. Air dry the pellet for 1 min and dissolve in 20 µl of RNase-free water.
2.1.3. Formaldehyde denature gel for RNA analysis

1. Mix RNA samples 1:1 with 2 x RNA loading dye.

2. Heat RNA mixtures and 2 µl of RNA ladder to 70°C for 10 min and cool on ice for 2 min. Run RNA samples on 1.5% RNase- free MOPS agarose gel, next to 2 µl of RNA ladder (e.g. RiboRuler™ High Range RNA Ladder, Fermentas, cat. no. SM1821).

3. Gel preparation:
   - 1.5 g agarose
   - 10 ml 10 x MOPS
   - 18 ml deionized formaldehyde
   - 72 ml DEPC-treated water

4. 10 x MOPS electrophoresis buffer:
   - 41.8 g MOPS in 700 ml DEPC-treated water
   - 20 ml 1M Sodium Acetate (in DEPC-treated water)
   - 20 ml 0.5M EDTA (pH 8.0)
2.1.4. Plasmid DNA extraction from bacteria

1. Grow bacteria in 100-500 ml of LB medium with carbanicilin overnight, in shaker at 37°C.
2. Spin bacteria culture at 2000 x g for 10 min at 4°C and remove the supernatant.
3. Re-suspend bacteria pellet by vortexing in 25 ml of Solution I containing 1 granule of lysozyme.

Solution I:
- 50 mM glucose
- 25 mM Tris-Cl (pH 8.0)
- 10 mM EDTA (pH 8.0)

4. Leave minimum 5 min at room temperature.
5. Add 50 ml of Solution II. Mix by gentle inverting the tube. Put the tube on ice.

Alkaline Lysis Solution II:
- 0.2 N NaOH
- 1% SDS

6. Immediately add 37.5 ml of Solution III and vortex.

Alkaline Lysis Solution III:
- 5 M potassium acetate, 60.0 ml
- glacial acetic acid, 11.5 ml
- H₂O, 28.5 ml

7. Leave on ice for 3-5 min, spin next 2 min at full speed, at room temperature.
8. Add 0.6 volume of isopropanol (67.5 ml) and put the sample to the freezer (-20°C ) for 20 min.
9. Spin for 10 min at 7500 rpm at room temperature. Remove supernatant.
10. Dissolve the pellet in 5 ml TE buffer and add 1 g of solid ammonium acetate. Vortex the tube until ammonium is dissolved.
11. Incubate 20 min on ice.
12. Spin 10 min at 7500 rpm.
13. Precipitate supernatant with 2.5 volume of 100% ethanol (12.5 ml).
14. Spin for 10 min at 7500 rpm.
15. Wash the pellet with 10 ml of 75% ethanol, vortex, and spin 10 min.
16. Dissolve the pellet in 100 ul TE buffer with RNAses mix containing 20 µg/ml DNase-free RNase A (pancreatic RNase) and 1000 U/µl RNAase
1. Vortex the solution gently for few seconds and leave at 37°C for minimum 1 h.
17. To 100 µl of DNA in TE-RNAses solution add SDS to the final concentration of 1% and then add NaCl to the concentration of 0.4-0.6 M. Vortex and add 1 µl of proteinase K.
18. Leave for 1 h at 55°C.
19. Add equal volume of phenol.
20. Add equal volume of chloroform.
21. Vortex and centrifuge the mixture for 3 min at the maximum speed.
22. Transfer aqueous upper phase to 1 volume of chloroform, vortex and spin for 1 min.
23. To collected upper phase add 1/5 volume of 10 M ammonium acetate, mix and add 2.5 volume of 100% ethanol.
24. Gently invert the tube and keep at room temperature for 2 min to precipitate DNA. Take forming clumps of DNA to new tube and allow ethanol to evaporate.
25. Dissolve the pellet in 100 µl TE buffer.
26. Add 1/10 volume of 10 M ammonium acetate and mix.
27. Add 2.5 volume of 100% ethanol.
28. Shake gently; take clumps of DNA to new tube containing 1 ml of 70% ethanol.
29. Invert the tube and take clumps with new tip to the new tube, allow ethanol to evaporate and dissolve DNA in 100 µl TE buffer.
30. Add 1/20 volume of 5 M NaCl, vortex and add 2.5 volume of 100% ethanol.
31. Invert tube; take clumps to 1 ml of 70% ethanol.
32. Mix, take clumps to dry tube and allow ethanol to evaporate.
33. Dissolve DNA in 100 µl of TE buffer.
2.1.5. DNA extraction from agarose gel

DNA extraction from agarose gel after gel electrophoresis was performed with QuickClean II Gel Extraction Kit, GeneScript, cat. No. L00418, according to manufacturer’s recommendations:

1. Excise the DNA band from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice. Then add 3 volumes of Binding Buffer II to 1 volume of gel slice (100 mg ≈ 100 µl).
3. Incubate at 55°C for 10 min with occasional vortexing or until the gel slice has been completely dissolved.
4. Add 1 volume of isopropanol to 1 volume of gel and mix.
5. Transfer the sample to supplied spin column, centrifuge at 6,000 × g for 1 min. Discard the flow through. If the sample volume is more than 750 µl, load and centrifuge again using the same column.
6. Add 500 µl Binding Buffer II to the same spin column, centrifuge at 12,000 × g for 30~60 s. Discard all flow through liquid.
7. Add 750 µl Wash Buffer to spin column, centrifuge at 12,000 × g for 30~60 s. Discard all flow through liquid.
8. Centrifuge at 12,000 × g for an additional 1 min and transfer the spin column to a sterile 1.5 ml microcentrifuge tube.
9. Add 30~100 µl of nuclease-free water or TE buffer to the spin column and let it stand for 1 min at room temperature.
10. Centrifuge at 12,000 × g for 1 min to collect DNA.
2.1.6. dsRNA synthesis for RNAi

Prepare two 20 μl reactions for the synthesis of both antisense and sense RNA transcript.

4 μl 5 x Transcription Buffer
6 μl NTP mix 25mM each
x μl DNA template
x μl RNase-free water
2 μl T7 or Sp6 RNA polymerase
20 μl

As a template use digested plasmid or PCR product amplified with primers, to which T7 and Sp6 RNA polymerase promoters were attached.

1. Mix reactions by pipetting, then incubate at 37°C overnight.
2. After incubation mix two reactions together, so the complementary RNA strands can align to each other. Heat up the mixture to 70°C in a water bath for 10 min.
3. Cool down slowly reaction until room temperature reached.
4. Add 1 μl of RQ1 RNase-free DNase and 2 μl of 10 x RNase solution per each 20 μl volume of reaction and incubate for 30 min at 37°C.
5. Add 10 M ammonium acetate to a final concentration of 1 M.
6. Then add 2.5 volumes of 100% ethanol, vortex and leave sample on ice for 5 min.
7. Centrifuge at full speed for 10 min.
8. Remove the supernatant and wash the pellet with 75% ethanol (500 μl). Then spin at full speed for 2 min.
9. Remove the ethanol and air dry the sample (~10 min).
10. Dissolve the pellet in 150 μl H2O.

10 x RNase Solution:
In TE Buffer
- 200 μg/ml RNase A
- 10,000 units/ml RNase T1

20 μl of 10 mg/ml RNase A (Fermentas) and 10 μl 1,000 U/μl RNase T1 (Fermentas) are added to 970 μl TE buffer.

RQ1 RNase-Free DNase, from Promega, cat. no. M6101 was used for DNase digestion.
2.1.7. DNA digestion with restriction enzyme

1. Enzymatic reaction was prepared as follows:

   1 μl 10 x enzyme buffer
   x μl DNA (1 μg)
   0.5 μl restriction enzyme
   x μl nuclease-free water
   10 μl

2. Incubate reaction for 1.5 – 2 h at 37°C.
3. Run 1 μl of reaction mix on 1% agarose gel to verify if digestion was completed.

   For gel electrophoresis mix 1 μl of reaction with 10 μl of nuclease-free water and 2 μl of 6 x DNA gel loading dye. Run prepared samples next to 6 μl of DNA ladder on 1% agarose gel. I used GeneRuler™ 1kb DNA Ladder, Fermentas, cat. no. SM0311.

4. When digestion was completed restriction enzyme was inactivated by heating to 65°C or 80°C for 20 min, according to manufacturer’s recommendations for particular enzyme.
5. Clean digested DNA by following instructions in the protocol 2.1.8, “DNA clean up and precipitation”.
2.1.8. DNA clean up and precipitation

Use the following protocol after performing DNA enzymatic digestion or DNA extraction from the agarose gel.

1. Add phenol pH 7.0 and chloroform in ratio 1:1, then vortex well.
2. Centrifuge at full speed for 2 min.
3. Remove aqueous solution and re-extract the supernatant with equal volume of chloroform.
4. Centrifuge at full speed for 1 min.
5. Transfer aqueous phase of supernatant to a new tube and add 1/20 volume of 5M NaCl and 1 μl of glycogen. Vortex sample and add 2.5 volumes of ethanol (100%).
6. Place the mixture in the freezer for at least 10 min.
7. Centrifuge sample at maximum speed for 5 min.
8. Take off all the supernatant and wash the pellet with 200 μl of 75% ethanol; vortex well.
9. Centrifuge sample for 3 min at the maximum speed, then remove supernatant and air dry the pellet.
10. Dissolve the pellet in 10 μl of nuclease-free water.
2.1.9. Synthesis of RNA probes for *in situ* hybridization

For synthesis of *in situ* hybridization probes the following templates can be used:

- PCR product, amplified with primers, to which T7 or Sp6 RNA polymerase promoters were attached.
- Linearized plasmid containing gene of interest.

In case of the plasmid template, the following restriction enzymes are recommended for plasmid linearization:

**Bluescript:**

1) For T3 promoter: sacI, sacII, notI, xbaI, spel, bamHI, smal, pstI, ecorI, ecorv
2) For T7 promoter: hindIII, claI, SalI, xhoI, apaI, kpnI

Important: no 3’ overhangs should be generated by enzymatic digestion.

**pGEM-T:**

1) For Sp6 promoter: apaI, aatII, sphI, ncoI, sacII,
2) For T7 promoter: spel, notI, pstI, SalI, ndel, sacl, bstxI, nsi

Important: no 3’ overhangs should be generated by enzymatic digestion.

Perform DNA digestion and purification according to protocols 2.1.7 and 2.1.8.

Perform probes’ synthesis according to the following steps:

1. Mix reagents in the following order:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC water</td>
<td>x μl</td>
</tr>
<tr>
<td>Transcription Buffer (5x)</td>
<td>2 μl</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>0,5 μl</td>
</tr>
<tr>
<td>DTT (0,2M)</td>
<td>1 μl</td>
</tr>
<tr>
<td>DNA template (up to 0,5 μg)</td>
<td>y μl</td>
</tr>
<tr>
<td>Labeled NTP mix</td>
<td>1 μl</td>
</tr>
<tr>
<td>T7 or Sp6 RNA polymerase</td>
<td>0,5 μl</td>
</tr>
</tbody>
</table>

10 μl
If performing double *in situ* hybridization, synthetize one probe with Dig-labeled NTPs (DIG RNA Labeling Kit, Roche, cat. no. 11175025910), and the second with Fluorescein-labeled NTPs (Fluorescein RNA Labeling Mix, Roche, cat. no. 11685619910).

2. Incubate reactions at 37°C for 2 h or overnight.
3. Add 1 µl of Exonuclease and incubate for 30 min at 37°C.
4. Add 1 µl RNase-free DNAse and incubate for another 30 min at 37°C.
5. Add 1 µl 0.2 M EDTA to stop the reaction.
6. Measure the volume and add the same amount of LiCl (12M). Mix well and precipitate RNA for 30 min at -20°C in the freezer.
7. Spin the sample for 5 min at maximum speed.
8. Discard supernatant and add 1 ml of 70% ethanol to the pellet, mix well.
9. Spin for 2 min at maximum speed.
10. Discard the supernatant, air dry the pellet and dissolve it in 20-100 µl of nuclease-free water.
11. Verify RNA fragments on formaldehyde denature gel according to the protocol 2.1.3.
12. Mix RNA probes in ratio 1:1 with Hybridization Mix.
13. Follow *in situ* hybridization protocol in 2.1.10.

**Hybridization Mix:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ml Deionized Formamide</td>
<td>50%</td>
</tr>
<tr>
<td>2.5ml 20 x SSC</td>
<td>5 x SSC</td>
</tr>
<tr>
<td>1ml 1mg/ml Heparin stock</td>
<td>0.1mg/ml</td>
</tr>
<tr>
<td>1ml 1mg/ml tRNA stock solution</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>10µl Tween 20</td>
<td>0.1%</td>
</tr>
<tr>
<td>0.5ml DEPC H₂O</td>
<td></td>
</tr>
</tbody>
</table>
2.1.10. Single and double whole-mount *in situ* hybridization

1. Anesthetize animals before fixation by incubating them for 60 min at 18°C in MgCl₂ (4% MgCl₂ dilution in seawater).
2. Fix polyps overnight at 4°C on the shaker, in 1ml 4% PFA-Hepes solution.
3. Wash animals 3 x 10 min with PBS-Tween
4. Wash for 5-10 min in 1 ml of 25% MetOH, then take out solution, add another 1 ml of 50% MetOH, then remove and replace with 75%, at the end put animals in 1ml of 100% for 10 min. Then gradually go down to 75%, then 50% and 25% MetOH.
5. Wash 2 x 5 min in PBSTween.
6. Heat treatment: incubate animals in PBSTween at 95°C for 15 min.
7. Post-fixation: put animals in 1 ml of 4% PFA in PBS at room temperature for 20 min, with rocking, and in the fume hood.
8. Wash 3 x 5 min in 1ml PBS Triton at room temperature, with rocking.
9. Preabsorb anti-DIG antibodies-AP (Anti-Digoxigenin-AP, cat no. 11093274910, Roche) or anti-Fluorescein-AP (Anti-Fluorescin-AP, cat no. 11426338910, Roche) in a 1:1000 dilution in 1% BSA/PBSTriton, overnight at 4°C, with a batch of previously fixed polyps.
10. Incubate the rest of animals in blocking solution for 10 min at room temperature with rocking. Blocking solution consists of 2 mg tRNA / 1ml PBS.
11. Add equal volume of hybridization mix to the blocking solution and rock for another 10 min at room temperature.
12. Remove solution and add 200 µl of hybridization mix / tube or 1ml / petri dish. Pre-hybridize samples by incubating them for 2 h at 50°C in a hybridization oven, with rocking.
13. Hybridization:

   To denature the probes, prewarm 1 ml hybridization mix containing 40 ng of RNA probes for 10 min at 70°C. After 10 minutes put probes on ice. Add probes to the animals and hybridize them overnight at 45-55°C. Hybridization temperature needs to be optimizes empirically for each probe. Hybridize two probes at once, if performing double *in situ* hybridization.

14. Remove RNA probes and wash animals once with hybridization mix, at hybridization temperature of 45-55 °C, for 5min.
15. Wash once in 50% Formamide, 2 x SSC, 0.1% Triton at 45-55 °C for 1h.
16. Wash once in 2x SSC, 0.1%Triton for 15 min at 45-55°C.
17. Wash twice in 0.2x SSC, 0.1% Triton for 15 min at 45-55°C.
18. Wash 1 x PBSTriton for 5 min at room temperature with rocking.
19. Block in 1% BSA in PBSTriton for a minimum of 1 h at room temperature, with rocking.
20. Incubate animals in pre-absorbed Anti-Fluorescein-AP or Anti-DIG-AP antibodies, in 1:2000 dilution in 1% BSA/PBSTriton, for 2 h, at room temperature.
21. Wash 4 x 20 min in 1 ml PBSTriton at room temperature, with rocking.
22. Signal detection for alkaline phosphatase:

   Wash 3 x 5 min in freshly made alkaline phosphatase buffer (AP buffer), 1 ml per tube.

   **AP buffer:**
   
   1 ml 1M NaCl
   1 ml 1M Tris-HCl pH9.5
   1 ml 500mM MgCl2
   10 µl Triton
   7 ml DEPC water

23. Colorimetric Reaction:

   To develop red color staining Sigma Fast Red TR/Naphthol AS-MX, cat. no. F4523 was used according to manufacturer’s recommendations.

   Staining with blue color was developed with the mixture of 4.5 µl NBT and 3.5 µl BCIP / 1 ml of AP buffer.

24. Colorimetric reaction was stopped with 2 x 5 min wash in PBSTriton and samples were mounted.

   If double *in situ* hybridization was performed additional steps for protocol were introduced:

25. Heat animals in PBTriton for 30 min at 65 °C.
26. Wash 2 x 5 min in PBTriton.
27. Wash 1 x 10 min with 100 mM glycine · HCl, pH 2.2/0.1% Tween, at room temperature.
28. Wash samples 4 x 5 min in PBTriton.
30. Mount animals on glass slides with Glycerol/PBST, 10mM EDTA (9:1) per slide and seal cover slips with nail vanish.
Fixing solution
For 50 ml
50 ml 0.1 M Hepes 1.1955 g in 50 ml
pH 7.5
2 mM MgSO₄ 0.0246 g per 50 ml
0.42 M NaCl 1.227 g per 50 ml

Add 50 µl of DEPC, mix well and incubate overnight. Autoclave solution. Then add 2 g Paraformaldehyde to make 4%.

1 x PBS (Phosphate-buffered Saline)
For 1L Final conc.
8 g NaCl 137 mM NaCl
0.2 g KCl 2.7 mM KCl
1.44 g Na₂HPO₄ 10 mM Na₂HPO₄
0.24 g KH₂PO₄ 2 mM KH₂PO₄

Adjust pH to 7.5 with HCl. Add 1ml of DEPC to 1l of PBS, mix well and incubate overnight. Autoclave solution.

1 x PBST

50 ml 1 x PBS pH 7.5
50 µl Tween 20

Hybridization Mix:

Final Conc.
5 ml Deionized Formamide 50 %
2.5 ml 20 x SSC 5 x SSC
1 ml 1mg/ml Heparin stock 0.1 mg/ml
1 ml 1mg/ml tRNA stock solution 100 µg/ml
10 µl Tween 20 0.1 %
0.5 ml DEPC-treated H₂O

DEPC-treated water:
Add 1 ml of DEPC to 1l of distilled water, mix well and incubate overnight. Autoclave water.

20 x SSC
For 1l
175.3 g NaCl
88.2 g Sodium Citrate

Adjust pH to 7.0.
2.1.11. Fluorescent in situ hybridization (FISH)

FISH was performed according to monochromatic in situ hybridization protocol described in 2.1.10, with the following amendments:

1. After hybridization of probes, all washes were done without Tween or Triton.
2. Antibodies were conjugated with Peroxidase, instead of Alkaline Phosphatase.

Antibodies were used in the following dilutions:

- Anti-Fluorescein-POD, cat no. 11426346910 (Roche): 1:50.
- Anti-Digoxigenin-POD, cat no. 11207733910 (Roche): 1:100.

3. As a blocking buffer, TNB Blocking Buffer was used, according to the Perkin Elmer’s recommendations:

- M TRIS-HCl, pH 7.5
- 0.15 M NaCl
- 0.5% Blocking Reagent (available separately, cat. no.: FP1020).

4. For signal detection Perkin Elmer Tyramide Signal Amplification kit was used (TSA Plus Cyanine 3/Fluorescein Kit, cat. no. NEL753001KT). The Fluorophore Amplification Reagents were used in the dilution of 1:50 in the supplied Amplification Buffer.
5. Reaction was stopped by washing the animals 4 x in PBS.
2.1.12. RACE cDNA synthesis

The cDNA was transcribed according to the protocol for Omniscript Reverse Transcriptase (Qiagen cat no. 205110) using oligos designed according to the SMART™ RACE cDNA Amplification kit.

1. Use 2 μg of total RNA as template for the following reactions:

a) 5’ RACE cDNA:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>x μl</td>
</tr>
<tr>
<td>5’CDS oligo</td>
<td>1 μl</td>
</tr>
<tr>
<td>Smart 2</td>
<td>1 μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>x μl</td>
</tr>
<tr>
<td></td>
<td>5 μl</td>
</tr>
</tbody>
</table>

b) 3’ RACE cDNA:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>x μl</td>
</tr>
<tr>
<td>3’CDS oligo</td>
<td>1 μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>x μl</td>
</tr>
<tr>
<td></td>
<td>5 μl</td>
</tr>
</tbody>
</table>

3’CDS oligo: 5’ AAGCAGTGGTTATCAACGCAGAGTAC T₃VN 3’
5’CDS oligo: 5’ T₃VN 3’
Smart 2: 5’ AAGCAGTGGGTATCAACGCAGAGTACGCGGG 3’

2. Incubate reactions at 70°C for 10 min
3. Cool down reactions on ice for 2 min.
4. Add the following solutions to each tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction mix</td>
<td>5 μl</td>
</tr>
<tr>
<td>10 x RT buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>5 mM dNTP</td>
<td>2 μl</td>
</tr>
<tr>
<td>RNase Inhibitor (10 U/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>x μl</td>
</tr>
<tr>
<td>RT (Reverse Transcriptase)</td>
<td>1 μl</td>
</tr>
<tr>
<td></td>
<td>20 μl</td>
</tr>
</tbody>
</table>

5. Mix samples by pipetting and incubate for 1-2 h.
6. Dilute sample 10 x with nuclease-free water.
7. Heat samples to 72°C for 7 min.
2.1.13. PCR reaction

To amplify DNA fragments from cDNA, two types of PCR reactions were performed.

1. For RACE PCR and verification of cloning, GoTaq® DNA Polymerase (Promega, cat no. M3001) was used and reaction was prepared as follows:

   - 2 µl forward primer
   - 2 µl reverse primer
   - 1 µl DNA template/bacteria suspension
   - 8.4 µl nuclease-free water
   - 4 µl 5 x enzyme buffer
   - 0.4 µl dNTP mix 10mM
   - 2 µl MgCl₂ (supplied)
   - 0.2 µl DNA polymerase
   - 20 µl

   Run PCR mixture under the following cycling conditions:

   Initial denaturation (1 cycle):
   - 98°C - 1 min

   Amplification reaction (up to 40 cycles):
   - 95°C - 30 s
   - 50-65°C - 30 s / temperature depends on the melting temperatures of primers
   - 72°C – 1 min /1000 bp

2. For PCR with gene-specific primers, Finnzymes' Phusion™ High-Fidelity DNA Polymerase (cat no. F530S) was used and reaction was prepared as follows:

   - 2 µl forward primer
   - 2 µl reverse primer
   - 1 µl template
   - 10.5 µl nuclease-free water
   - 4 µl 5 x buffer
   - 0.4 µl dNTP mix 10mM
   - 0.1 µl DNA polymerase
   - 20 µl

   Run PCR reaction under the following cycling conditions:
Initial denaturation (1 cycle):
- 98°C - 3 min

Amplification reaction (up to 40 cycles):
- 98°C - 30 s
- 50-65°C-30 s/temperature depends on the melting temperatures of primers
- 72°C – 1 min/1000 bp
2.1.14. Sequencing reaction

1. Sequencing reaction was performed in order to verify DNA fragments amplified in PCR reactions, and was prepared as follows:

- 1 µl primer
- 2 µl sequencing buffer
- 1 µl Big dye sequencing mix
- x µl DNA (50-150ng depending on the length of fragment)
- x µl nuclease-free water
  10 µl

2. Sequencing reaction was run for 40 cycles at the following conditions:

   Initial denaturation (1 cycle):
   - 98°C - 1 min
   Amplification reaction (40 cycles):
   - 95°C - 10 s
   - 50°C - 10 s
   - 60°C - 4 min

3. To stop reaction the following solutions were added:

   - 2.5 µl nuclease-free water
   - 1 µl Ammonium Acetate
   - 1 µl EDTA pH 8.0
   - 0.5 µl glycogen
   - 45 µl 100% ethanol

4. Samples were vortexed and kept at room temperature for 1 min, then centrifuged at maximum speed for 5 min.

5. The supernatant was removed and 200 µl of 75% ethanol was added.

6. Samples were vortexed and centrifuged for 4 min at maximum speed.

7. Supernatant was removed and samples air dried.

8. The pellet was dissolved in 20 µl of nuclease-free water and send for sequencing analysis.
2.1.15. DNA ligation

The ligation reaction was set up as follows:

- x µl of linear vector: 20-100 ng
- x µl of insert in 3:1 excess to vector (100-500 ng)
- 2 µl of 10 x DNA ligase buffer
- 2 µl 50% PEG solution
- 0.2 µl (sticky ends) or 1 µl (blunt ends) of T4 DNA ligase 5 U/µl

20 µl reaction

Ligation conditions:

Blunt end ligation: 1 h, at room temperature or overnight.
Sticky end ligation: 20 min, at room temperature.

2.1.16. Bacteria transformation

1. Warm up agar plates to room temperature.
2. To 50 µl bacteria add 20 µl of ligation product.
3. Keep tube on ice for 5 min.
4. Transfer tube to the shaker at 37°C for 5 min.
5. Put tube on ice immediately after 5 min is over.
6. Add 0.5 ml of LB medium.
7. Put tubes back to the shaker for 30-40 min at 37°C.
8. Transfer bacteria to centrifuge tube and spin 0.5 min at 1,5 rcf.
9. Discard 0.5 ml of supernatant.
10. Re-suspend bacteria pellet in the remaining LB medium by gentle pipetting.
11. Spread bacteria suspension on agar plates.
12. Incubate plates at 37°C overnight.
### 2.1.17. List of primers

For PCR reactions the following primers were used:

<table>
<thead>
<tr>
<th>Gene name/other application</th>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanos2</td>
<td>Nanosfwd-ATG</td>
<td>5'atgtcgtgaagagaatgtagtgatagcta 3'</td>
</tr>
<tr>
<td></td>
<td>NanosrevRNAi</td>
<td>5'aatctgaaaatggaaaaatcactgc 3'</td>
</tr>
<tr>
<td></td>
<td>NanosfwdRNAiT7</td>
<td>5'ggtctcaatagctactataaggagatctgttgatagtgatagct 3'</td>
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<td>NanosrevRNAiSp6</td>
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<td>Nanosrevpro1</td>
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<td></td>
<td>Nanos realtimerev1</td>
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<td>Nanos-RT fwd3</td>
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<td>Splinkerin</td>
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<td>JNK-T7-fwd</td>
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<td>JNK-sp6-rev</td>
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<td></td>
<td>Zip2-T7-fwd</td>
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<td>Nowa-T7-fwd</td>
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<td>Ncol1-T7 fwsd</td>
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<td></td>
<td>Pumilio2nestsrvl</td>
<td>5'aagcttgagacatataaccacgaga 3'</td>
</tr>
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<td>Plasmid preparation</td>
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</tr>
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<td><strong>pGEM-T RNAi Rev</strong></td>
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</tr>
<tr>
<td><strong>pGEM-T RNAi fwd 2</strong></td>
<td>5' cgacaggactataaggactataagg 3'</td>
<td></td>
</tr>
<tr>
<td><strong>T7pGEM-T RNAi fwd2</strong></td>
<td>5' tcataaagcactataaggactgacgtggactataaaagctgactataaaagctgactataaaagctgac 3'</td>
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</tr>
<tr>
<td><strong>GFP-TAGstop-rev</strong></td>
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</tr>
<tr>
<td><strong>T7+SL fwd</strong></td>
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<td><strong>ShortT7+ SL fwd</strong></td>
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<tr>
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<td><strong>5' CDS</strong></td>
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<td><strong>3' CDS</strong></td>
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<td><strong>Smart UPM long</strong></td>
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<table>
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<td><strong>Smart UPM long</strong></td>
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<td><strong>Smart UPM short</strong></td>
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</tr>
</tbody>
</table>
2.2. Cellular staining

2.2.1. Immunohistochemical staining

1. Anesthetize animals using 4% MgCl₂ solution.
2. Fix animals in 4% PFA/PBS under fume hood for 20 min with rocking.
3. Wash 3 x 10 min in PBS.
4. Wash 3 x PBS/Triton 0.1%/BSA (2%) for 5 min.
5. Pre-absorb 2° antibodies, 1:500 dilution in PBS/BSA/Triton/5% serum against 2° antibodies for 30 min, at room temperature, with rocking and in dark.
6. Incubate animals with 1° anti-bodies diluted in PBS/BSA/triton/5% serum, for 1h at room temperature, with rocking.
7. Wash 3 x 10 min in PBS/BSA/Triton/5% serum, with rocking.
8. Remove last wash and add pre-absorbed 2° anti-bodies, 1:500 dilution in PBS/BSA/Triton/5% serum. Incubate animals for 1h, with rocking; keep in dark.
9. Wash 3 x 5 min in PBS/BSA/Triton/5% serum.
10. Mount animals in 90% glycerol/PBS.

1 x PBS for 1l:

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<th>Ingredient</th>
<th>Amount</th>
<th>Final conc:</th>
</tr>
</thead>
<tbody>
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<td>NaCl</td>
<td>8 g</td>
<td>137 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
<td>2.7 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.44 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.24 g</td>
<td>2 mM</td>
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</tbody>
</table>

Adjust pH to 7.5 with HCl. Add 1ml of DEPC, mix well and incubate overnight. Autoclave prepared solution.

4% PFA in PBS:
4 g PFA add to 100 ml PBS.

PBS/ triton (final 0.5%) / BSA (final 2%)/5% serum
To 47 ml PBS add 1 g BSA + 0.25 g Triton + 2.5 ml serum

The nematocyst-specific antibodies, NCol1 Ab (Adamczyk et al., 2010) were a kind gift from Dr Suat Özbek (University of Heidelberg, Germany). Working dilution was 1:500 in PBS/ triton (final 0.5%) / BSA (final 2%)/5% serum.

As secondary antibodies AlexaFluor488 goat anti-rabbit IgG (cat no. A-11012, Invitrogen) were used, at the working dilution of 1:500 in PBS/ triton (final 0.5%) / BSA (final 2%)/5% serum.
2.2.2. DAPI staining of DNA

1. Anaesthetize animals using 4% MgCl₂ solution.
2. Fix animals in 4% PFA/PBS under fume hood for 20 min with rocking.
3. Wash 3 x 10 min in PBS.
4. Incubate animals for 25 min in DAPI solution at room temperature.
5. Wash 3 x 5 min in PBSTriton.
6. Mount animals in 90% glycerol/PBS.

DAPI staining should be performed after IHC staining.

DAPI stock solution: 10 mg/ml.
DAPI working dilution: 1µg/ml (1:10 000 dilution in distilled water).

2.2.3. EdU staining of DNA

EdU staining was performed with Click-iT® EdU Alexa Fluor® 488 HCS Assay (Invitrogen, cat no. C10351), according to manufacturer’s recommendations from manual:

1. Prepare 2 x working solution of EdU (Component A) from the 10 mM stock solution. Add 100 µl 2 x EdU working solution to each tube containing treated animals in 100 µl of complete medium, which results in 1 x EdU (10 µM).
2. Incubate animals for 1h in 1 x EdU solution.
3. Remove solution and wash animals with 100 µl of 4% formaldehyde in PBS for 15 min, at room temperature.
4. Remove fixative and wash animals once with PBS.
5. Remove wash solution. Add 100 µl of 0.1% Triton® X-100 in PBS for 15 min at room temperature.
6. Prepare 1x Click-iT® EdU buffer additive by diluting the 10 X solution 1:10 in deionized water.
7. Remove the permeabilization buffer and wash animals twice with PBS.
8. Remove wash solution and add 100 µl of Click-iT® reaction cocktail. Incubate for 30 min at room temperature, protecting from light.
9. Remove the reaction cocktail and wash animals once with 100 µl of Click-iT® reaction rinse buffer (Component F).
10. Remove the Click-iT® reaction rinse buffer.
11. Perform antibody or DAPI labeling of samples, according to protocols 2.2.1 or 2.2.2, if required.
2.2.4. DAPI staining of nematocysts

DAPI staining of nematocysts (capsules of mature stinging cells) was performed according to the following protocol:

1. Anaesthetize animals using 4% MgCl₂ solution.
2. Fix animals in 4% PFA/PBS/10mM EDTA, overnight, under fume hood, with rocking.
3. Wash 3 x 10 min in PBSTriton.
4. Incubate animals for 60 min in DAPI solution, at room temperature.
5. Wash 3 x 5 min in PBSTriton.
6. Mount animals in 90% glycerol/PBS.

DAPI stock solution: 10 mg/ml.
DAPI working dilution: 50 µg/ml (1:200 dilution in distilled water).

Protocol for DAPI-based nematocysts staining was developed for Hydra (Szczepanek et al., 2002).
2.3. Microinjection

2.3.1. Microinjection

1. For the time of injection animals were placed on the plankton netting (100 μm), which was glued to the bottom of a Petri dish.
2. 1-2 cell embryos were injected with 100-200 pl of overexpression construct (concentration of 4-5 µg/µl).
3. Needles for microinjection were prepared from glass capillaries by the pulling machine. The puller was set up as follows: heat 560 or 660, pull 70, vel 75, time 150.
4. Glass capillaries used for preparing the needles were ordered from Narishige, GD-1 1 x 90 mm (1 mm outer diameter).
2.4. Animal culture

2.4.1. Animal culturing

*Hydractinia echinata* was used as an experimental model. These marine hydroid colonies grow on the shells of the hermit crabs in the coast of Ireland and Great Britain. Colonies were sampled in Galway Bay, Ireland.

Animal culturing took place in the natural sea water, at 19°C, under a 14/10 hour light-dark regime.

Animals were fed with *Artemia* (brine shrimp nauplii) for five days a week, each morning, and washed in the evenings under a jet of water.

Crabs were fed with small pieces of cod fish every morning.

Embryos for experiments were collected about 2 hours after the onset of light.

2.4.2. Animal metamorphosis

1. Metamorphosis of larvae (about 3-5 day old) was induced by a 2.5 hour incubation in 200 mM CsCl solution.
2. 200 mM CsCl solution was prepared by diluting 600 mM stock solution 1:2 in sea water.
3. After incubation, animals were washed three times with sea water and placed on glass slides.
4. Larvae settled down and after 1 day transformed into primary polyps.
3. Gene cloning and analysis

3.1. Introduction

In order to maintain homeostasis, an organism needs to be able to regulate and stabilize its internal environment in all its aspects. Each living system must be capable to balance all live parameters, respond quickly to their fluctuations and normalize them. It is a dynamic process that ensures a well-being of every living creature. One of the parameters, which need to be constantly regulated, is generation and maintenance of populations of different cell types. During embryonic development all cells types must be created in the right place and on time to build a healthy organism. Later on, these cells need to be renewed or replaced as they have a limited lifespan to protect the body from a cellular depletion (Rando, 2006). These mechanisms of cell renewal, commitment and specification of different cell types are poorly understood. They involve complex processes of switching on expression of some genes and turning off the others, but also include posttranscriptional regulations of RNA and posttranslational regulation of proteins (Hemberger et al., 2009). These complex processes determine cell fate and ensure the right timing of cellular differentiation. Understanding the mechanism of acquiring specific cell fate requires investigating the role of genes expressed in particular cell lineages. For example, studying genes expressed in neural cells, from early progenitors to mature neurons, can expand our knowledge on the specification of neural cell types. Gaining the ability of sensing the environment by formation of sophisticated sensory systems in animals is one of the most astonishing phenomena in nature. Studies performed on basic metazoan models, like cnidarians, can provide insight into the evolution of the nervous system and may reveal new roles for neural genes, which remain cryptic in other animals. My project was focused on studying the role of gene called Nanos in Hydractinia echinata neurogenesis.

Nanos is well known as a germline-specific gene, crucial for proper development of germ cells. This role of Nanos is conserved among metazoans, from invertebrates to vertebrates (Tsuda et al., 2003). In arthropods, Nanos have an additional role in Anterior-Posterior patterning (Lehmann and Nusslein-Volhard, 1991). Nanos mutants have defects in abdominal development and also lack germ cell progenitors (Lehmann and Nusslein-Volhard, 1991; Kobayashi et al., 1996), what is consistent with two major Nanos functions. Interestingly, Nanos is also expressed in the nervous system of many animals, although a role for Nanos in neurons has not been well studied so far. Drosophila Nanos was found to be
essential for morphogenesis of high-order dendritic branches in peripheral neurons (Ye et al., 2004). Other research group published that Nanos is also important for Pumilio-mediated regulation of sodium current and excitability of Drosophila motoneurons (Muraro et al., 2008). However, the role of Nanos in the nervous system of other animals remains unknown.

I studied the role of Nanos2 in Hydractinia echinata development with the use of available overexpression and knockdown techniques. In order to do that I had to clone the Nanos2 coding sequence from cDNA. At the time I started my project, only a partial EST database was available. I needed to perform RACE PCR to obtain the full-length coding sequence of Nanos2. Later on, sequencing of Hydractinia genome delivered contigs up to 30 kb in size. From the genome database, the sequences of the second Nanos homolog, Nanos1, was obtained, as well as the Nanos canonical partner Pumilio.

To examine Nanos2 ectopic expression and knockdown phenotypes, I also searched available EST and genome databases for the presence of classical neural markers. I found the sequences of Ash, RFamide, and Ncol1, which I amplified by PCR reactions from cDNA. In situ hybridization probes were later prepared for all obtained genes.

I also performed PCR-based genome walking to find the Nanos2 5′ regulatory sequence in order to look at the promoter activity during Hydractinia development. For this purpose I used splinkered genomic DNA, which was prepared by Dr Reinhardt Heiermann, a former postdoc in the lab.
3.2. Gene amplification by RACE PCR

cDNA for all PCR reactions was synthesized according to the following steps:

1. Total RNA extraction from the animal tissue was performed according to protocol 2.1.1. II.

2. DNase digestion of total RNA was performed according to protocol 2.1.2.

3. RACE cDNAs was synthesized according to protocol 2.1.12.

Available EST and genomic databases were screened for the genes of interest and for found fragments, primers for PCR reactions were designed. Melting temperatures of primers were predicted by the pDRAW32 DNA Analysis Software of AcaClone Software. DNA fragments were amplified according to the protocol for PCR reaction (2.1.13).

At the time I started my project, only a partial EST database was available. From this reason, I had to perform RACE PCR to amplify the Nanos2 and Pumilio full coding sequences. RACE PCR was performed using GoTaq® DNA Polymerase (Promega, cat no. M3001). For amplification of 5’ ends, primers against the trans spliced leader were used (Fig. 3.2.1). A trans spliced leader (TSL) is at the 5’ end of many, but not all, hydroid mRNAs (Bonen, 1993). Not all animals attach TSLs to their mRNAs, but those who do, use it for the majority of their genes (70% in C. elegans) (Blumenthal, 2005). However, the roles TSLs play are still not known. Most Hydractinia transcripts seem to possess SL sequences. From this reason, I tried to amplify the 5’-UTR of Nanos2 and Pumilio, by RACE PCR using TSL primers. Luckily, both genes had TSL sequences at the 5’ end of their transcripts.

Known Hydractinia TSL sequences were aligned by the ClustalW2 software, together with TSL primers, “spliceleadershort” and “spliceleaderlong”, used for PCR reaction and are shown in Fig. 3.2.1.
In order to amplify the 3’-UTR of Nanos2 and Pumilio transcripts by RACE PCR, UPM primers mix was used. The mixture was prepared by adding 1 µl of “Smart UPM long” and 5 µl of “Smart UPM short” to 244 µl of nuclease-free water. For each 3’RACE PCR reaction 2 µl of UPM mix were used as reverse primers. The sequences of the UPM primers are listed in Fig 3.2.2. Primers were designed to match the 3’CDS primer sequence used for 3’RACE cDNA synthesis, according to protocol 2.1.12.

<table>
<thead>
<tr>
<th>Smart UPM long</th>
<th>5’CTAATACGACTCATATAGGGCAAGCAGTGATCAACGCAGAGT3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smart UPM short</td>
<td>5’CTAATACGACTCATATAGGGCAAGCAGTGATCAACGCAGAGT3’</td>
</tr>
</tbody>
</table>

Fig. 3.2.2. Sequences of UPM primers used for RACE PCR.
3.3. Nanos2

In order to study the role of Nanos2 in Hydractinia echinata development, I cloned the full Nanos2 coding sequence from cDNA by RACE PCR. RACE PCR was performed according to description in 3.2.

Few runs of PCR reaction were made until the full coding sequence was amplified. RACE PCR was performed using GoTaq® DNA Polymerase (Promega, cat no. M3001). For all reactions the following cycling conditions applied:

Initial denaturation (1 cycle):
- 98°C - 1 min

Amplification reaction (40 cycles):
- 95°C - 30 s
- 55-60°C - 30 s/temperature depend on the melting temperature of primers
- 72°C – 1 min

For each PCR reaction, obtained DNA fragments were sequenced according to protocol 2.1.14 in order to verify the Nanos2 nucleotide sequence. When the full coding sequence was known, primers for amplification of the entire coding sequence were designed: forward primer starting with an ATG start codon, “Nanosfwd-ATG”, and reverse primer excluding the TAA stop codon, “Nanosrev-TAA” (Fig. 3.3.1). The stop codon was excluded from the reverse primer in order to clone the Nanos2 coding sequence into the overexpression vector, in frame with the GFP coding sequence. Such overexpression construct was later on injected into embryos, to study the effect of Nanos2 ectopic expression on Hydractinia development.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanosfwd-ATG</td>
<td>5' ATGTCGTTGAGTGATGTTATGCTA 3'</td>
</tr>
<tr>
<td>Nanosrev-TAA</td>
<td>5' CATCCTTACCTTCCATGCTGGTT 3'</td>
</tr>
</tbody>
</table>

Fig. 3.3.1. Sequences of primers used to amplify the full coding sequence of Hydractinia Nanos2 homolog.

The Nanos2 full coding sequence encodes 975 bp and was amplified by PCR using Finnzymes' Phusion™ High-Fidelity DNA Polymerase (cat no. F530S) under the following PCR conditions:

Initial denaturation (1 cycle):
- 98°C - 3 min
Amplification reaction (up to 40 cycles):
- 98°C - 30 s
- 60°C - 30 s
- 72°C - 1 min

The PCR product was run on 1% agarose gel to verify the size of obtained fragment (Fig. 3.3.2). GeneRuler™ 1kb DNA Ladder, cat. no. SM0311 (Fermentas) was used as a DNA marker according to manufacturer’s recommendations (Fig. 3.3.2).

The Nanos2 full coding sequence was also verified by sequencing with “Nanosfwd-ATG” and “Nanosrev-TAA” primers. Sequencing reaction was performed according to protocol 2.1.14 under the following conditions:

Initial denaturation (1 cycle):
- 98°C - 1 min

Amplification reaction (40 cycles):
- 95°C - 10 sec
- 50°C - 10 sec
- 60°C - 4 min

The sequencing results are shown in Fig. 3.3.3. Two separate sequences were put together to obtain the full Nanos2 coding sequence. Few bases from the 5’ and 3’ ends of the sequences are missing as common in Sanger sequencing reactions.
Fig. 3.3.3. Sequencing results of Hydractinia Nanos2.
Nanos2 mRNA translates into 324 aa protein (Fig. 3.3.4).

\[
\text{MSLSDVMLQNFMPRNILESDEDELDTWNSLQGSSRSSRSSRSGYPYPTKDASDFSFLQDYFGGLSNLLN}
\text{NVIKISEDNPLLNYTHKLALLQRARAVSWSSADASAAVLSPTDKPOYYNCERNFDSPMSPDFPNKQQ}
\text{LPPMRSLKSMARDHISAYQGQINSRMPPEKVPFLAQPQPSPPQPSSQPPQPLHLHSNTPOYPPRSTLSQ}
\text{VCVFCRNNGESEVSYTSHVLDTECTGRACILPRAYTCPICKANGDGSHTKYCPLNQTRAGVMGQCPP}
\text{AQNHMPPRNQLPPHRPLQFPFPPGPVLPQPRFNQHKGKVRM}
\]

Fig. 3.3.4. Nanos2 amino acid sequence.

Nanos2 sequence was aligned with other Nanos proteins belonging to different species, in order to analyze the degree of similarity to the Hydractinia homolog (Fig. 3.3.5). The Blast tool from the website of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov) was used to find regions of local similarity between sequences. BlastX was used to search protein database using a translated nucleotide query. The alignment of chosen sequences was performed with the use of the online version of ClustalW2.

The sequence analysis revealed that the Nanos2 N-terminal region is divergent from its mammalian homologs (e.g. Homo sapiens), but very similar to cnidarian Nanos2 (e.g. Clytia hemisphaerica) (Fig. 3.3.5). In contrast, the C-terminal region, which includes the putative zinc-finger domain, is well conserved. C-termini of all Nanos-related genes contain conserved Cys-Cys-His-Cys Zn-finger motifs, which are indispensable for their function (Hashimoto et al., 2010). The Nanos2 Zn-finger domain, aligned with human and Clytia Nanos homologs, is highlighted in Fig. 3.3.5.

Fig. 3.3.5. Alignment of Hydractinia Nanos2 with Clytia hemisphaerica Nanos2 and Homo sapiens Nanos1 sequence. Conserved zinc-finger domains are boxed.
Nanos mediates translational blockage by binding to Nanos Response Elements (NRE) on the 3’-UTRs of target transcripts. *Drosophila hb* mRNA has a 32-nucleotide sequence of NRE. It contains a Nanos binding site, called Nanos Regulatory Element (GUUGU), and a Pumilio binding site, called Pumilio Regulatory Element (UGUAXAUA). Functional studies have indicated that both sequences are necessary for proper function of the Nanos/Pumilio/Brat complex and seem to be conserved in other transcripts, regulated by these proteins.

*Hydractinia Nanos2* mRNA was analyzed for the presence of Nanos and Pumilio binding sites (Fig. 3.3.6). A single Pumilio Regulatory Element (UGUACUAU) was identified within the Nanos2 3’-UTR, suggesting that Nanos2 mRNA may be bound by Pumilio. In this case, Pumilio can directly influence Nanos activity by reducing its protein level. Interestingly, *Drosophila* Pumilio was found to reduce the level of Nanos mRNA and suggested to have a potential negative-feedback to protect neurons from Nanos overactivity (Muraro et al., 2008). However, I did not perform further binding and functional studies, so the role of putative Pumilio binding site in *Hydractinia Nanos2* mRNA remains unknown.

![Fig. 3.3.6. The analysis of Nanos2 mRNA sequence for the presence of putative Nanos Regulatory Elements or Pumilio Regulatory Elements. Shortened fragment of the coding sequence is in lowercase. UTRs are in uppercase. Putative Pumilio binding site (UGUACUAU) within 3’-UTR is highlighted by grey background.](image-url)
3.4. Nanos2 promoter

I cloned the Nanos2 promoter in order to study the dynamic changes in its activity during Hydractinia development. At the time I started my project, only partial genome sequence was available, so I had to perform PCR-based genome walking. For this purpose, I used splinkered genomic DNA, which was a gift from a former postdoc in the lab, Dr Reinhardt Heiermann. Dr. Heiermann prepared DNA templates by extracting total DNA from the adult animal tissues and digesting it with 7 restriction enzymes, all in separate reactions: EcoRI, HindIII, XbaI, SalI, Nhe, Xho, SalII. Restriction sites were chosen carefully, based on the rarity of their occurrence in DNA. Digested genomic DNA was later ligated to a splinkered primer, which was cut with the same enzyme. Such prepared DNA was used as a template for PCR reaction. Produced DNA templates were mixtures of DNA fragments which varied in length, depending on the presence of restriction sites within the genome. From this reason only partial fragments of searched promoters may be amplified, depending on the position of restriction recognition sites within their sequences.

For PCR-based genome walking the following conditions applied:

- Reactions were prepared according to protocol 2.1.13.
- 1 µl of splinkered DNA was used as a template.
- DNA was amplified with GoTaq® DNA Polymerase (Promega, cat. no. M3001).
- As forward primers: “Splinkerout” or “Splinkerin” were used (Fig. 3.4.1).
- As a reverse primer: “Nospromoterrev-NotI” gene-specific primer was used (Fig. 3.4.1).
- The reverse primer was designed for the 3’end of Nanos2 5’-UTR and NotI restriction site was artificially added to primer’s 5’ end (Fig. 3.4.1). Later on, the obtained promoter fragment was cut with NotI and inserted into NotI-digested expression vector by sticky-end ligation (chapter 5).

| Nospromoterrev-NotI | 5’ tatggegegecccttmtttagaaaattttttcagaac 3’ |
|Splinkerout          | 5’ egaatctgaacctgctgtagagaa 3’ |
|Splinkerin           | 5’ tgtagagaaatgcttctctec 3’ |

Fig. 3.4.1. Sequences of primers used to amplify Nanos2 promoter.
Cycling conditions for PCR were as follows:

Initial denaturation (1 cycle):
- 98°C - 1 min

Amplification reaction with gene-specific “Nospromoterrev-NotI” primer was carried on for 20 cycles:
- 95°C - 30 s
- 50°C - 30 s
- 72°C - 10 min

After 20 cycles, 1 µl of “Splinkerout” primer was added to the PCR mixture and reaction was run for next 20 cycles under the same cycling conditions. Then 1 µl of each above PCR reaction was used as a template for nested PCR, performed with “Nospromoterrev-NotI” and “Splinkerin” primer. 10 µl of each PCR reaction were analysed on 1% agarose gel. The longest amplified DNA fragments were extracted from the gel (protocol 2.1.5, “DNA extraction from agarose gel”) and analysed by sequencing reaction (protocol 2.1.14).

By genome walking, partial sequence of Nanos2 promoter of 846 bp in size was obtained (Fig. 3.4.2).

The amplified fragment was later on ligated into an expression vector upstream of the GFP coding sequence. Obtained expression construct was microinjected into early embryos and GFP+ cells were traced using microscopy (chapter 5).

Fig. 3.4.2. Agarose gel presenting 846 bp sequence of the Nanos2 promoter (left). GeneRuler™ 1kb DNA Ladder (middle). Nanos2 promoter sequence obtained by PCR-based genome walking (right).
3.5. Nanos1

Many animals underwent partial or full genome duplications during their evolution, resulting in multiple copies of some or all genes. Those duplicated genes became free of selective pressure, could accumulate mutations. In many cases, altered copies of the same gene became functionally diverged from each other. Nanos genes represent good examples of this described situation. Cnidarians have two Nanos homologs, whereas mammals have three, most likely resulting from further duplications. Drosophila has only a single homolog, although additional genes could have been lost during evolution (Lehmann and Nusslein-Volhard, 1991). Interestingly, all mouse Nanos genes have different biological functions, based on their expression pattern and some functional studies. Nanos1 is expressed in the central nervous system, whereas Nanos2 is predominantly expressed in male germ cells and Nanos3 in migrating PGCs of both sexes (Haraguchi et al., 2003; Tsuda et al., 2003).

I searched for other Nanos homologs in Hydractinia in order to study their sequence divergence and expression patterns. By searching available EST and genome databases I identified the second gene, Nanos1.

The full coding sequence of Nanos1 was 708 bp long and was amplified by PCR reaction according to protocol 2.1.13 (Fig. 3.5.1).

```
ATGTTCAAGGCAATGTATAGCAATGTTTGCAGCTACGACTACGAGCTGTTTGGAGTATG
TGTTGATCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
ATGTTCAAGGCAATGTATAGCAATGTTTGCAGCTACGACTACGAGCTGTTTGGAGTATG
TGTTGATCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
CTACGAGCTGTTTGGAGTATG
```

Fig. 3.5.1. The full coding sequence of Hydractinia Nanos1.

The Nanos1 3′-UTR was also analyzed for the presence of Nanos or Pumilo Regulatory Elements. A single Pumilo binding site (UGUAUAUA) was identified, suggesting that Nanos1 mRNA translation, similarly to Nanos2, may be regulated by Pumilio (Fig. 3.5.2).
Fig. 3.5.2. Analysis of Nanos1 mRNA sequence for the presence of putative Nanos and Pumilio Regulatory Elements. A fragment of the coding sequence is shown in lowercase. UTRs are shown in uppercase. Putative Pumilio Regulatory Element (UGUAUAUA) within the 3’-UTR is highlighted in grey.

Nanos1 mRNA sequence encodes a 235 aa protein (Fig. 3.5.3).

Nanos1 sequence was aligned with other Nanos proteins belonging to different species to analyze the degree of similarity (Fig. 3.5.4). The BLAST tool from the website of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov) was used to find regions of similarity between protein sequences. BLASTX was used to search the non-redundant protein database using a translated nucleotide query. The Clytia hemisphaerica Nanos1 and Homo sapiens Nanos1 were chosen for alignment, made by ClustalW2 software. Sequences analysis revealed that the Hydractinia Nanos1 N-terminal region is divergent from mammalian Nanos1 (e.g. Homo sapiens), but similar to cnidarian Nanos1 homologs (e.g. Clytia hemisphaerica) (Fig. 3.5.4). However, the C-terminal region, including the zinc-finger domain is well conserved in both genes. Zn-finger domains are boxed in Fig. 3.5.4.
Fig. 3.5.4. Alignment of *Hydractinia* Nanos1 with *Clytia hemisphaerica* Nanos1 and *Homo sapiens* Nanos1 sequence. Conserved zinc-finger domains are boxed.

The alignment of *Hydractinia* Nanos1 and Nanos2 sequences shows that the two proteins are quite different from each other. The only conserved regions are the Zn-finger domains which are boxed in Fig. 3.5.5.
3.6. Pumilio

Pumilio is a canonical partner of Nanos. A duet of Nanos and Pumilio represses translation of target mRNAs (Murata and Wharton, 1995; Wharton et al., 1998; Parisi and Lin, 2000). It is involved in germline development (Lin and Spradling, 1997; Asaoka-Taguchi et al., 1999) and posterior patterning in *Drosophila* (Murata and Wharton, 1995). Interestingly, *Pumilio*, similarly to *Nanos*, was found to be expressed in the nervous system of many animals. Pumilio is crucial for regulation of morphology, arborization, sodium current and excitability of *Drosophila* motoneurons (Ye et al., 2004; Muraro et al., 2008). Two *Pumilio* genes, *Pum1* and *Pum2*, have been also identified in mammals (Spassov and Jurecic, 2002). *Pum2* mRNA is expressed during neuronal development and its protein is found in discrete particles in the cell body and the dendritic compartment of fully polarized neurons (Vessey et al., 2006). Recently, mouse *Pum2* was found to play key roles in the encompassing neuronal excitability and behavioral response of this animal to environmental challenges (Siemen et al., 2011).

In order to verify, how many *Pumilio* homologs are present in *Hydractinia*, the genome database was searched and a single sequence showing high degree of similarity to mammalian *Pumilio* homologs was found. Other cnidarian species seem to have only one *Pumilio* homolog as well. To find out, if identified gene also has a role in *Hydractinia* nervous system or germ cells development, I had to examine its expression pattern. Comparing *Pumilio* mRNA distribution to *Nanos1* and *Nanos2* in situ hybridization results may show if these genes are co-expressed in the same type of cells. This would indicate if in *Hydractinia* these genes are likely to act together in a form of a canonical Nanos/Pumilio duet. To prepare in situ hybridization probe, *Pumilio* sequence was amplified from cDNA. Few runs of RACE PCR reaction were performed to make sure that the fragment from the genome database covers the full coding sequence. PCR was performed according to protocol 2.1.13. Sequencing reactions of amplified fragments were performed according to protocol 2.1.14. The full coding sequence of Pumilio was amplified with primers: “PumilioATG-startfwd” and “Pumiliostop-TGA-rev” (Fig. 3.6.1).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pumilio ATG -start fwd</td>
<td>5’ atgcgcgacatcatttggcaaca 3’</td>
</tr>
<tr>
<td>Pumilio stop-TGA-rev</td>
<td>5’ aacttctttcaactaataagcttca 3’</td>
</tr>
</tbody>
</table>

Fig. 3.6.1. Sequences of primers used to amplify the full coding sequence of *Hydractinia Pumilio* homolog.
PCR reaction was performed with Finnzymes' Phusion™ High-Fidelity DNA Polymerase (cat no. F530S), under the following cycling conditions:

Initial denaturation (1 cycle):
- 98°C - 1 min

Amplification reaction (40 cycles):
- 98°C - 30 s
- 60°C - 30 s
- 72°C - 4 min

*Pumilio* sequence is 2940 bp in size (Fig. 3.6.2).

```
ATGCCGCACATCTATTTGGCAACAACAAAAATACACCAGTGAGATACAGTTATCAAACAGCAAGGAATATCTATATATGACCAGCTACATATGAATTCATGAACATGGCAAGCTTTGTGCAAGGACATATGACATTTGCAAGAAGGAAAAGGACCAGGTGAGAAGGATAGTGGAAGGTCAAAGTTACTTGAAGATTTTCGTAACAACAGGTTTCTAACCTTCAACTTCCATGATCTGCAAAGACATATAGTTGAGTTTTCTCAAGATCAACATGGCTCTAGATTTATCACAA
```

Fig. 3.6.2. The full coding sequence of the *Hydractinia Pumilio* homolog.
**Pumilio** full coding sequence was verified by sequencing and by gel electrophoresis on 1% agarose gel (Fig. 3.6.3).

Fig. 3.6.3. Agarose gel showing the full sequence of 2940 bp *Hydractinia Pumilio* amplified from cDNA (left). GeneRuler™ 1kb DNA Ladder (right).

**Pumilio** mRNA encodes 979 aa protein (Fig. 3.6.4)

Alignment of *Hydractinia* Pumilio sequence with Pumilio homologs from other animals shows that the C-terminal region of Pumilio is conserved and almost identical to mammalian Pumilio2 homolog (e.g. *Homo sapiens*). Conserved regions are boxed in Fig. 3.6.5. However, the *Hydractinia* Pumilio N-terminal region showed high degree of similarity only to the other cnidarian Pumilio homologs (e.g. *Hydra magnipapillata*).
Fig. 3.6.5. Alignment of Hydractinia Pumilio with Homo sapiens Pumilio2 and Hydra magnipapillata Pumilio. Conserved regions are boxed.
3.7. Ncol1

Ncol1 (minicollagen1) belongs to the group of minicollagen proteins, which are synthesized in nematoblasts that are undergoing differentiation into nematocytes. Minicollagens form part of nematocyst capsules (David et al., 2008). Nematoblasts are precursors of cnidarian-specific stinging cells (nematocytes, cnidae). These cells develop complex secretory structures inside their cytoplasm called nematocysts. Nematocysts are products of post-Golgi vacuoles and consist of a capsule containing an inverted and coiled tubule with spines and venom (Fig. 3.7.1a). Capsules can discharge within few milliseconds after being physically or chemically stimulated. When undischarged, they remain under high osmotic pressure which is built up during synthesis of poly-γ-glutamate in the capsule’s matrix (WEBER, 1989; Szczepanek et al., 2002). The main purpose of discharging capsules is to inject venom into the target organism to capture prey and defend against predators (Tardent, 1995), but some cnidae are used for attachment.

The capsule wall and tubule consists primarily of minicollagens (Fig. 3.7.1b-f) (Kurz et al., 1991; David et al., 2008). Minicollagen mRNAs are highly expressed in differentiating nematoblasts, containing developing capsule. They stop being expressed in maturing stinging cells and fully differentiated nematocytes, because only one capsule is produced by any one nematocyte. Polymerization of Ncol into the wall is organized by Nematocyst Outer Wall Antigen (NOWA) and additional cysteine-rich molecules (Fig. 3.7.1f) (Engel et al., 2002; David et al., 2008). The nematocyst’s wall is stabilized by the intermolecular disulfide bonds (Engel et al., 2001).

All cnidarians have few Minicollagen genes and they seem to be, at least in part, specific for different types of nematocytes. Analysis of Ncol protein sequences shows that all of them contain a central region consisting of 14 to 16 Gly-X-Y triplets, flanked on both sides by Proline-rich amino acid stretches (Kurz et al., 1991). However, N- and C-terminal cysteine-rich domains (CRDs) are different in all of them and are important for their polymerization. This diversification of CRDs was suggested to trigger formation of novel capsule structures and nematocyst types in their early evolution (David et al., 2008).
Fig. 3.7.1. The structure of the nematocyst wall. (a) A mature stenotele nematocyst with internal tubule and spines. (b) Sonication of the nematocyst wall generates aggregates of NOWA protein with small spikes corresponding to minicollagen molecules (Özbek et al., 2004). (c) FESEM (field emission scanning electron microscopy) images of fixed capsules reveal NOWA molecules on the wall’s surface (Özbek et al., 2004). (d) Intact capsules pictured with AFM (atomic force microscopy) showing fibers consisting of minicollagens (Holstein et al., 1994). (e) Recombinant minicollagen molecules spontaneously aligning into fibers (Engel et al., 2001). (f) The schematic structure of minicollagen fibers and of minicollagens/NOWA aggregates (David et al., 2008).

**Hydractinia** EST database was searched for the presence of *Minicollagen1 (Ncoll1)* homolog and a partial, 249 bp sequence was found (Fig. 3.7.2).

![Partial Ncoll1 sequence](image)

Fig. 3.7.2. Partial *Ncoll1* sequence. *Ncoll1* coding sequence is shown in uppercase, 3’-UTR is shown in lowercase.

PCR was performed to amplify the partial *Ncoll1* coding sequence from cDNA, according to protocol 2.1.13. The primers “Ncoll1-T7 fwd” and “Ncoll1-Sp6 rev” were used, to which T7 and Sp6 RNA polymerase promoters were attached, respectively, in order to prepare in situ hybridization probes for this gene (Fig. 3.7.3).
Fig. 3.7.3. Sequences of primers used to amplify the partial coding sequence of *Hydractinia Ncol1*. This fragment was used for preparation of *in situ* hybridization probes.

### Ncol1-T7 fwd
5' gatcataatacgactcactatagggacgtccaggaccaccaggagta 3'

### Ncol1-SP6 rev
5' tagcaatttaggtacactataaggggcaacagtatttgacacaaga 3'

**PCR reaction was performed with Finnzymes' Phusion™ High-Fidelity DNA Polymerase (cat no. F530S) under the following cycling conditions:**

- **Initial denaturation (1 cycle):**
  - 98°C - 1 min

- **Amplification reaction (40 cycles):**
  - 98°C - 30 s
  - 60°C - 30 s
  - 72°C - 45 s

Alignment of the partial *Hydractinia Ncol1* sequence with *Clytia NCol1* shows significant sequence similarities between those two proteins (Fig. 3.7.4).

![Alignment of Hydractinia Ncol1 with Clytia NCol1](image-url)

Fig. 3.7.4. Alignment of *Hydractinia Ncol1* with *Clytia NCol1*. 
Members of the Achaete-scute family (ASH) of proneural genes encode transcriptional activators of the basic helix–loop–helix (bHLH) class that are involved in a neuronal specification and differentiation, both in vertebrates and invertebrates (Negre and Simpson, 2009). In vertebrates, most of Achaete-scute homologs are expressed during neural development, e.g. mouse MASH1 (Guillemot and Joyner, 1993), Xenopus XASH genes (Ferreiro et al., 1994), or chicken CASH1 (Jasoni et al., 1994). Knockout of mouse MASH1 leads to deficiencies in both the central and peripheral nervous systems (Guillemot et al., 1993). Ectopic expression of Xenopus XASH3 in early embryogenesis causes conversion of ectodermal cells to a neural fate (Turner and Weintraub, 1994). Drosophila has four and mouse has two ASH homologs. Cnidarians have a single ASH gene (Grens et al., 1995; Seipel et al., 2004). The Hydra vulgaris Ash homolog, CnASH, shares a high degree of amino acid sequence similarity with both vertebrate and Drosophila Achaete-scute proteins. It was found to be expressed in a subset of the interstitial cells, in differentiating nematocytes (Grens et al., 1995) and in sensory neurons (Hayakawa et al., 2004).

Hydractinia EST database was searched for the presence of an Ash homolog and its full coding sequence was identified (Fig. 3.8.1). Hydractinia Ash is 483 bp in size and encodes a 160 aa protein (Fig. 3.8.2).
A cDNA fragment including the complete Ash coding sequence plus a partial 5' and 3' UTR regions was amplified by PCR in order to prepare in situ hybridization probes for this gene. PCR was performed with “Ash-T7 fwd” and “Ash-Sp6 rev” primers, to which T7 and Sp6 RNA polymerase promoters were attached, respectively (Fig. 3.8.3). PCR was prepared according to protocol 2.1.13.

| Ash-T7-fwd | 5' gatcaataagcagtacataggggeacgattaacaaatgatga 3' |
| Ash-sp6-rev | 5' tagaattaggtagcatagataaagcgaagagaatacttaact 3' |

Fig. 3.8.3. Sequences of primers used to amplify the coding sequence of Hydractinia Ash.

PCR reaction was performed with Finnzymes' Phusion™ High-Fidelity DNA Polymerase (cat no. F530S), under the following cycling conditions:

Initial denaturation (1 cycle):
- 98°C - 1 min

Amplification reaction (40 cycles):
- 98°C - 30 s
- 60°C - 30 s
- 72°C - 1 min

The analysis of Hydractinia Ash protein indicates high degree of sequence similarity with Hydra vulgaris and Homo sapiens homologs (Fig. 3.8.4). The most conserved region covers the basic helix-loop-helix domain.

Fig. 3.8.4. Alignment of Hydractinia Ash with Hydra vulgaris and Homo sapiens ASH1 homologs. The conserved bHLH domains are boxed.

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73
Ash 3’-UTR was also analyzed for the presence of Nanos Regulatory Element or Pumilo Regulatory Element. Interestingly, two putative Pumilio binding sites (UGUAACAUA and UGUAUAUA) were identified, suggesting that Ash mRNA translation may be directly regulated by Pumilio but not necessarily by Nanos1 or Nanos2 (Fig. 3.8.5).

![Pumilio Regulatory Elements in Ash mRNA](image)

Fig. 3.8.5. Pumilio Regulatory Elements in Ash mRNA. A shortened fragment of the coding sequence is shown in lowercase. UTRs are shown in uppercase. Putative Pumilio binding sites (UGUAACAUA and UGUAUAUA) within 3’-UTR are highlighted by grey background.
3.9. RFamide precursor

Neuropeptides of the RFamide family play many important roles in the nervous systems of both vertebrates and invertebrates (Darmer et al., 1991). To date, a total of five RFamide peptide genes have been identified in mammals (Fukusumi et al., 2006). In human, RFamide peptides are involved in the central coordination of neuroendocrine and autonomic responses (Goncharuk et al., 2004). They also participate in the modulation of the cardiovascular system, inducing increased blood pressure (Laguzzi et al., 1996). Furthermore, RFamides were found to be important for food consumption (Bechtold and Luckman, 2007), water balance (Sunter et al., 2001) (Majane and Yang, 1991), adipogenesis (Herrera-Herrera and Salazar-olivo, 2008), and locomotion (Betourne et al., 2010). Additionally, mammalian RFamide peptides, like neuropeptides FF (NPFFs) also participate in the development of opiate tolerance and dependence (Lake et al., 1991), and prolactin-releasing peptides (PrRPs) regulate stress response in the Central Nervous System (Maruyama et al., 2001). In cnidarians, RFamide neuropeptides were found to be important for larval metamorphosis (Katsukura et al., 2003) and migration (Katsukura et al., 2004).

The sequence of the Hydractinia RFamide neuropeptide precursor was taken from GenBank (accession No. CAA66062.1). The coding sequence was 1137 bp in size (Fig. 3.9.1).

```
ATGGTATCATGGCTTTCAAGGCGATCTGTTTGCAGTCATATTTCATACGTCATACGTAACAGCGA
AAGATACAGTAAACTACAGAAAAAAGATACACTACAAACAGAAGAAACGAAATTAGCTAGCCTTCT
GATGATATAAAATCTCGAGAGAAGAATTTCTGACCGCAGCCAAAAAAGGAGTTTGGGCTCAACTG
AAGGGCGGTTTTGGAAGAGAAGGAGAGCCGAGAGAGAGAGATTTGGGCGTGAAGCTGAAACAGT
GGTTAAAAGGACGTTTTGGTAGGGAAAGTGAACAATGGCTAAAAGGGCGTTTTGGGAAGAGAAG
AGTGAGCAGTGGGCGTTTCGGAGGGAGATTTGGGCGTGAAGCTGAAACAGTGGTTAAAAGGACG
CTTTGGGCGTGAAGTGAACAGTGGTAAAAGGACGCTTTGGTGCGAGAATAGGGAATGGCAATTG
GCTGAAGCTGAAACAGTGGTAAAAGGACGCTTTGGTGCGAGAATAGGGAATGGCAATTG
```

Fig. 3.9.1. The full coding sequence of Hydractinia RFamide precursor.
A fragment of the RFamide sequence was amplified by PCR in order to prepare in situ hybridization probes for this gene. For this purpose, “RFamide-T7 fwd” and “RFamide-Sp6 rev” primers were used, with T7 and Sp6 RNA polymerase promoters attached, respectively (Fig. 3.9.2).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFamide-T7fwd</td>
<td>5’ gatcataatacgactcactatagggatgttaatcatggcttcaaaggc 3’</td>
</tr>
<tr>
<td>RFamide-sp6rev</td>
<td>5’ tagcaatttagtgcaactaatagtcctctctcggtgg 3’</td>
</tr>
</tbody>
</table>

Fig. 3.9.2. Sequences of primers used to amplify the coding sequence of Hydractinia RFamide. The fragment was used for preparation of in situ probes.

PCR reaction was performed with Finnzymes’ Phusion™ High-Fidelity DNA Polymerase (cat no. F530S) according to protocol 2.1.13, under the following cycling conditions:

Initial denaturation (1 cycle):
- 98°C - 1 min

Amplification reaction (40 cycles):
- 98°C - 30 s
- 60°C - 30 s
- 72°C – 2 min

The RFamide mRNA sequence encodes a 378 aa precursor protein (Fig. 3.9.3).

MLIMASKAIVFAVIFITSVYVTAKDTGKLQKESHNEETKLASLLDIITAEENSHATEPKEKLDQWLKGRF GREADQWLKGRFGKEAEQWLKGRFGRESEQWLKGRFGRESEQWLKGRFGREAEQWLKGRFGREAEQ WLKGRFGREVEQWLKGRFGREVEQWLKGRFGRENAQWLKGRFGRESEQWLKGRFGRENEQWLKGR FGRENEQWLKGRFGRENEQWLKGRFGREMEQWLKGRFGRESAEILKGFNDVTSQWLKGRFGRAE SNGHRNMRNGREVLPLRRGGRYKEVEEYNKKEVDQWLKGRFGREAEQWLKGRFGREMEQWLKGR FGREAEQWLKGRFGRSTQTESETKSVSNDNTKVETVKKSKIKM

Fig. 3.9.3. Amino acid sequence of Hydractinia RFamide neuropeptide.
3.10. Other neural genes

To find out if other *Hydractinia* neuronal genes have putative Nanos or Pumilio Regulatory Elements I searched available EST database for known neural markers and studied their 3’-UTRs for the presence of Nanos or Pumilio binding sites. I found partial sequences of *Elav3, JNK, Zic2* and nematoblast-specific gene *Nowa*. The conserved family of Elav genes are required for proper differentiation and maintenance of neurons both in vertebrates and invertebrates (Good, 1995; Marlow et al., 2009). JNK was found to be expressed during nematocyte differentiation (Philipp et al., 2005). HyZic homolog was found to be involved in the early nematocyte specification (Lindgens et al., 2004). Nowa, similarly to Neo1, represents a classical marker for developing nematoblasts (Engel et al., 2002).

I also examined the UTRs of genes involved in the Notch signaling pathway, which is known to be involved in neurogenesis in other species (Kageyama et al., 2007; Andersson et al., 2011; Chi et al., 2012). I checked the 3’UTRs of *Hydractinia* putative homologs of *Hes1, Hes2, Botch* and *Dishvelled* for the presence of Nanos or Pumilio binding sites. Hes genes are classical Notch targets. Most of them repress neurogenesis both Notch-dependently and independently; however, some were found to promote neural development (Kageyama et al., 2007). Botch promotes neurogenesis by blocking Notch signaling (Chi et al., 2012).

Out of all examined genes, only the *Hydractinia* homologs of *Hes1, Elav3, Botch* and *Nowa* appeared to have Nanos Regulatory Elements (GUUGU) within their 3’UTRs (Fig. 3.10.1, Fig. 3.10.2, Fig. 3.10.3 and Fig. 3.10.4, respectively). *Hes1* and *Nowa* transcripts were also found to possess Pumilio binding sites (UGUAXAUA) (Fig. 3.10.1 and Fig. 3.10.4, respectively). The summary of above analysis is shown in Fig. 3.10.5.

The presence of Nanos or Pumilio Regulatory Elements within the 3’ UTRs of these neural genes suggests that their translation may be regulated by either or both of these genes. However, no further binding studies were performed to verify this hypothesis.
Fig. 3.10.1. The analysis of Hes1 mRNA sequence for the presence of putative Nanos Regulatory Elements or Pumilio Regulatory Elements. Shortened fragment of the coding sequence is in lowercase. 3'UTR is in uppercase. Putative Pumilio binding sites (UGUAXAUA) within 3'-UTR are highlighted by grey background and putative Nanos binding site (GUUGU) is boxed.

Fig. 3.10.2. The analysis of Elav3 mRNA sequence for the presence of putative Nanos Regulatory Elements or Pumilio Regulatory Elements. Shortened fragment of the coding sequence is in lowercase. 3'UTR is in uppercase. Putative Nanos binding sites (GUUGU) within 3'-UTR are highlighted by grey background.
Fig. 3.10.3. The analysis of Botch mRNA sequence for the presence of putative Nanos Regulatory Elements or Pumilio Regulatory Elements. Shortened fragment of the coding sequence is in lowercase. 3’UTR is in uppercase. Putative Nanos binding site (GUUGU) within 3’-UTR is highlighted by grey background.

Fig. 3.10.4. The analysis of Nowa mRNA sequence for the presence of putative Nanos Regulatory Elements or Pumilio Regulatory Elements. Shortened fragment of the coding sequence is in lowercase. 3’UTR is in uppercase. Putative Pumilio binding site (UGUAXAUA) within 3’-UTR is highlighted by grey background and putative Nanos binding sites (GUUGU) are boxed.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Nanos Regulatory Element</th>
<th>Pumilio Regulatory Element</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pumilio</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nanos2</td>
<td>-</td>
<td>present</td>
</tr>
<tr>
<td>Nanos1</td>
<td>-</td>
<td>present</td>
</tr>
<tr>
<td>RFamide</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Elav3</td>
<td>present</td>
<td>-</td>
</tr>
<tr>
<td>Hes1</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Hes2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JNK</td>
<td>-</td>
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<tr>
<td>Zic2</td>
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<tr>
<td>Ncol1</td>
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<td>-</td>
</tr>
<tr>
<td>Nowa</td>
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<td>present</td>
</tr>
<tr>
<td>Ash</td>
<td>-</td>
<td>present</td>
</tr>
<tr>
<td>Botch</td>
<td>present</td>
<td>-</td>
</tr>
<tr>
<td>Dishvelled</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 3.10.5. Summary of the analysis of few neural markers’ 3’UTRs for the presence of the putative Nanos Regulatory Elements or Pumilio Regulatory Elements.
3.11. Summary

I managed to clone the full sequences of two Hydractinia Nanos homologs: Nanos1 and Nanos2. Thanks to available genome database, I also obtained full sequence of the Nanos canonical partner, Pumilio. All genes were amplified by PCR and the identities of all DNA fragments were verified by gene electrophoresis and sequencing. In order to study their expression patterns in Hydractinia development, in situ hybridization probes were prepared for all of them and the results are described in chapter 4. Amino acid sequences of two Nanos and a single Pumilio protein were analyzed for similarities with their homologs from other species. Alignment was performed with the use of the free BLASTX tool on NCBI's website (http://www.ncbi.nlm.nih.gov). All sequences showed high degree of similarity with both their cnidarian and mammalian homologs.

I also cloned a few neural/nematocyte markers, like Ash, RFamide, and Ncol1, in order to analyze Nanos2 ectopic expression and knockdown phenotypes, as described further in chapters 6 and 7. For these genes in situ hybridization probes were prepared as well. Gene expression patterns of those genes have been studied in most Hydractinia life stages, and are described in chapter 4.

To study Nanos2 promoter activity in Hydractinia development, I cloned the Nanos2 promoter from splinkered DNA using PCR-based genome walking. The Nanos2 promoter was later on ligated into an expression vector and injected into fertilized eggs. Nanos2 promoter studies have been described in chapter 5.

I also analyzed all above genes, as well as few other neural markers, for the presence of putative Nanos and Pumilio Regulatory Elements. The suggested impact of Nanos and Pumilio – mediated regulation of these genes’ translation is proposed in the discussion (chapter 8).
4. Gene expression analysis

4.1. Introduction

The gene expression is determined by the two biological processes, transcription and translation. Transcription involves the synthesis of mRNA from the DNA sequence encoding a particular gene. In the process of translation, the produced transcript is decoded by the ribosome to form a functional amino acid product. Both processes are regulated by many factors to determine the proper amount and the right timing of manufactured proteins (Hames, 1984; Berg et al., 2002). The balance between different proteins dictates the fate of the cell and ensures its proper functioning. Each cell has at least one copy of a genome and potentially is able to express all genes, but in fact only a fraction of them is expressed at any one time. These limitations in gene expressions are specific for different cell types, e.g. stem cells express at high level genes maintaining stemness, whereas differentiated cells express lineage-specific genes.

Transcription begins when the RNA polymerase recognizes and binds the promoter of a particular gene and uses the downstream DNA sequence as a template for the synthesis of a single-stranded RNA (Fig. 4.1.1) (Colman, 1984). The initiation of transcription itself is tightly controlled and involves many regulatory mechanisms. For example, it is dependent on the activity of transcription factors, which bind to promoters’ sequences and repress or initiate the transcription process. A significant role in this process is also played by enhancer sequences. Enhancers provide binding sites for regulatory proteins and blocking access to these sequences can significantly affect the RNA polymerase activity (Berg et al., 2002). Eukaryotic cells also developed few mechanisms of posttranscriptional modifications, which serve as additional checkpoints for the regulation of genes expression (Kozak, 1992; Berg et al., 2002). Eukaryotic transcripts are equipped with introns, which presence distinguishes them from prokaryotic cells. Such transcripts have exons, separated by introns, which do not code for functional proteins, but most likely have stabilizing or regulatory functions. To form a mature mRNA transcript introns need to be spliced out and exons merged together (Fig. 4.1.1). To increase the complexity of transcription reactions even more, eukaryotic mRNAs need to be additionally modified by 5’-capping and 3’-polyadenylation. Such modifications ensure the stability of generated transcripts. In eukaryotic cells, once mRNA is postranscriptionally modified, it is exported outside the nucleus to be used as a template for the synthesis of a functional protein product. However, a wide range of
RNA modulators can block or enhance the actual translation process. Such regulatory mechanisms provide additional control points in case faulty transcription decisions were made. Nanos represents a perfect example of such a regulatory protein. It binds target transcripts and blocks their translation by mediating mRNA deadenylation and other poly(A)-independent silencing (Chagnovich and Lehmann, 2001). The translation process is completed when the transcript is decoded by the ribosome to produce an amino acid product (Fig. 4.1.1) (Kozak, 1999). Even at this point, cells evolved sophisticated mechanisms for regulation of the protein activity. Proteins can be either inhibited or activated by some enzymes, but also degraded by the proteasome, if so required by the cell (Berg et al., 2002).

Fig. 4.1.1. Schematic diagram of the transcription and translation processes in the eukaryotic cell. First, both introns and exons are transcribed from DNA into RNA. By RNA splicing non-coding introns are removed and exons joined together. Spliced mRNA molecule (red) is modified by 3’-polyadenylation and 5’-capping and the mature transcript (mRNA) exported outside the nucleus. In the cytoplasm, mRNA is translated by the ribosomes into a functional protein. Source: www.nature.com/scitable/topicpage/gene-expression.
To be fully functional, all living cells need to respond and adapt to challenges of their constantly changing environment. Regulation of genes expression, by controlling the processes of transcription or translation, is critical for cells to maintain homeostasis.

There are multiple techniques, allowing studying gene expression in cells. Among the most used are RT–qPCR, northern blotting, western blotting, in situ hybridization and immunohistochemistry.

RT-qPCR allows measuring mRNA abundance by the PCR reaction (Nolan et al., 2006). This method is quantitative and very sensitive, although quite limiting. It gives information about the total amount of mRNA in the extracted tissue, but does not inform about its distribution throughout the tissue or amount of particular transcript in single cells.

Northern blotting allows semi-quantitatively measuring of the levels of mRNA (Goda and Minton, 1995; Kevil et al., 1997). By using the northern blotting technique, gene expression levels can be determined during differentiation, morphogenesis, as well as during abnormal or diseased conditions (Liang and Pardee, 1995). For this purpose a sample of RNA is separated by electrophoresis on an agarose gel, blotted onto a membrane and hybridized to a labeled complementary RNA probe. The probe can be radioactively labeled, biotinylated or Digoxigenin labeled. The information from a northern blot can provide insight into the size of the mRNA or the presence of alternative splice variants. However, northern blotting has significant disadvantage that includes inaccurate quantification and lack of cellular resolution, similarly to qPCR (Goda and Minton, 1995). Another limitation of this technique is a relatively low sensitivity and a possible risk of RNA degradation by ubiquitous RNases.

Western blotting is used for protein quantification (Kurien and Scofield, 2006). In this method protein extract is separated on a polyacrylamide gel, transferred to a membrane and bound by the specific antibody. The antibody can be conjugated to a fluorophore or to Horseradish Peroxidase (POD), which is later detected and imaged. Unfortunately, quantification of protein level is not accurate and relies on the intensity of the band on the membrane (Bra et al., 1993).
also very often gives more than one band, depending on the specificity of used antibodies. Produced bands can smear as well, making results difficult to interpret.

**In situ** hybridization (ISH) represents another method used for the analysis of the gene expression patterns (Wilkinson, 1992; Boenisch et al., 2001). This technique allows for the **in situ** localization of cells expressing the gene of interest and the study of spatial organization of these cells in their natural location. It is based on an mRNA detection by hybridization with a labeled probe and can be performed on single cells, tissue sections or even on whole animals, if they are relatively thin and optically translucent (so called whole-mount ISH). This method involves preparation of labeled-RNA probes. Nucleotides used for the probes’ synthesis are usually DIG or Fluorescein-labeled. Fixed tissues or animals are incubated in the antisense probe solution, which is complementary to a specific mRNA. As a control experiment, usually a sense transcript is used, which is not capable to bind the endogenous mRNA. The sample is later incubated in antibodies, which recognize the labeled NTPs of the probe. Antibodies have to be tagged as well and usually for this purpose Digoxigenin or Peroxidase is used, but also biotinylation and radioactive labeling has been employed. However, the last two labeling methods are inefficient or dangerous and rarely applied nowadays. After antibody binding, the staining reaction is induced to detect the endogenous gene transcripts. Available staining methods depend on the type of antibody label used and allow color detection in blue, brown and in red. The results of ISH are examined using microscopy. Recently, fluorescent **in situ** hybridization (FISH) technique was developed and became a very valuable mRNA detection technique. Its principle is very similar to chromogenic ISH, but allows picturing samples by confocal laser scanning microscopy and projecting them in three dimensions. However, FISH has many disadvantages, including high background, difficulty in detection of low-copy genes and instability of the fluorescent signal (Trinh et al., 2007).

**Immunohistochemistry (IHC)** represents another commonly used method of studying the gene expression patterns (Kiernan, 1981; Ramos-Vara, 2005; Taylor and Levenson, 2006). By using IHC it is possible to detect individual protein molecules within single cells. IHC is also used to look at the distribution and localization of differentially expressed proteins in different parts of the examined tissues. The quality of this method relies on the specificity of the primary antibodies, raised against the protein. Primary antibodies are detected by incubation of the tissue with the labeled secondary antibodies. Secondary antibodies can be labeled by a number of ways, e.g. conjugation to peroxidase which can catalyze a color-producing reaction, or an antibody can be tagged to a fluorophore, such as fluorescein or rhodamine. IHC allows also incubating few antibodies at the same
time and detecting few different proteins in the same sample. For this purpose however, all primary antibodies have to be raised in different host animals, to avoid cross-reactivity of the secondary antibodies.

In my project, I applied *in situ* hybridization (ISH) to study the expression patterns of *Hydractinia Nanos1, Nanos2, Pumilio, Ash, RFamide*, and *Ncol1*. Prior to these experiments, all genes were amplified by PCR and synthesized DNA fragments were verified by gel electrophoresis and sequencing (chapter 3). In order to study expression patterns of these genes, *in situ* hybridization probes were prepared as described later in this chapter. The ISH protocol was then performed whole-mount for most of the *Hydractinia* life stages.

I also performed immunohistochemical analysis of the Ncol1 protein expression. For this purpose I used antibodies raised against *Nematostella vectensis NCol1*. Due to the high sequence similarity between the *Nematostella* and *Hydractinia* Ncol1 homologs, cross-reactivity of the antibodies was very good, allowing specific staining of developing nematocyst capsules with low background. The NCol1 nematocyst-specific antibodies (Adamczyk et al., 2010) were a kind gift from Dr Suat Özbek (University of Heidelberg, Germany).
4.2. Nanos2

As a template for the synthesis of the Nanos2 in situ hybridization probes, I used PCR fragment, covering the unconserved region of the gene outside the Zn-finger domain. The total length of the amplified fragment was 700 bp in size and included the entire 5'-UTR and a partial sequence of the 5' end of the coding sequence. For gene amplification I used primers to which T7 and Sp6 RNA polymerase promoters were attached. The PCR product was analyzed on 1% agarose gel by gel electrophoresis. The band of proper size was extracted from the gel according to protocol 2.1.5 and sequenced according to protocol 2.1.14. Probes were later on synthesized according to protocol 2.1.9. As a control, the sense transcript was used. Probes were verified for the correct size and the quality of the transcripts was assessed on a formaldehyde denaturing gel according to protocol 2.1.3.

In situ hybridization experiments with Nanos2 sense and antisense probes were performed for most of the Hydractinia life stages, according to ISH protocol 2.1.10. I also tried to optimize fluorescent in situ hybridization (FISH) for Nanos2, following protocol 2.1.11. Unfortunately, Nanos2 FISH had too much background and detection of the specific staining was almost impossible. However, chromogenic ISH worked very well so I applied this method to reveal Nanos2 mRNA expression pattern throughout Hydractinia development.

Nanos2 transcripts were maternal and first detected in the fertilized egg (Fig. 4.2.1a). Nanos2 expression was enriched at one pole of the embryo (Fig. 4.2.1a,b,d, arrows) and around the nucleus in a nuage fashion (Fig. 4.2.1a, c). This expression pattern was maintained for a few more blastomeric divisions.
Fig. 4.2.1. *Nanos2 in situ* hybridization performed on the embryonic development. (a) Polarized *Nanos2* expression in the fertilized egg. (b) Polarized *Nanos2* expression in the two-cell embryo. (c) *Nanos2* expression in the four-cell embryo. Staining was detected around the nucleus of dividing blastomeres in so called nuage. (d) Polarized expression in the eight-cell embryo. Arrows indicate polarized *Nanos2* expressions in the early embryonic development. Scale bars in (a)-(c) represent 100µm.

In the preplanula stage, about 30 hpf (hour post fertilization), *Nanos2* expression was found in cells present both in the ectoderm and in the endoderm (Fig. 4.2.2a). *Nanos2*+ cells in the endoderm were forming clusters, typical for nematoblasts (Fig. 4.2.2b). In three day old planula larvae, *Nanos2* mRNA continued to be present in both embryonic layers (Fig. 4.2.2c). In the ectoderm of the larva, especially around its head and tail, it was possible to identify *Nanos2* expression in differentiating nematocytes (Fig. 4.2.2d-f). In the endoderm of the larvae, *Nanos2*+ cells were detected in clusters or as single cells, which most likely represented early nematoblasts or committed stem cells, respectively (Fig. 4.2.2g).
Fig. 4.2.2. Nanos2 in situ hybridization performed on the larva. (a) Ectodermal and endodermal expression in 30 hpf preplanula. (b) Higher magnification of Nanos2+ cells in endoderm of preplanula. (c) Expression in three day old larvae. Larva is directed with the head down. (d)-(e) Higher magnification of Nanos2+ differentiating nematocytes in the ectoderm of the planula larva’s head. (f) Higher magnification of Nanos2+ differentiating nematocytes in the ectoderm of the planula larva’s tail. (g) Nanos2 expression in the clusters of nematoblasts in the planula larva’s endoderm. Scale bar in (b), (d)-(g) represents 10 µm.

Nanos2 expression was also examined in the larvae after induction of metamorphosis. Hydractinia embryonic development lasts about two to three days, until reaching competence for metamorphosis into the primary polyps. Metamorphosis is naturally induced by a bacterial film covering the shell of a hermit crab. However, in the laboratory it is possible to induce metamorphosis by incubating animals for two and a half hours in 200 mM CsCl solution. During
metamorphosis, the larva contracts, settles down on the surface (e.g. glass slide) with its anterior part directed down. The tail at the posterior end contracts and the polyp's head develops at this pole. Conversely, the larval anterior end becomes the aboral end of the polyp, which develops stolons. During metamorphosis, many cytological and biochemical changes take place (Leitz, 1993; Plickert et al., 1998). As a consequence, the animal undergoes intensive cell proliferation and differentiation, providing a completely new cellular composition.

Fig. 4.2.3 shows the results of the Nanos2 in situ hybridization performed after induction of metamorphosis. Nanos2 mRNA was strongly expressed in the endoderm of metamorphosing larvae in cells forming nematoblast clusters. Its expression was also found in single cells, which could be either stem cells or maturing nematocytes that already separated from their clusters.

Following metamorphosis larvae transform into the primary polyp. A polyp consists of the body column, which on its oral side has a mouth surrounded by the ring of tentacles. Tentacles are used to capture prey and insert it into the gastric cavity. On the aboral side, polyps develop stolons, which form a network of
gastrovascular canals. Food particles are passed between the members of one colony through these stolons. They are also a place of residence for pluripotent stem cells, so called i-cells.

Nanos2 expression in the primary polyp was abundant in the epidermis of the stolons and the lower part of the body column (Fig. 4.2.4a). Cells expressing Nanos2 in stolons were presumably stem cells, or cells committed to nematoblasts fate (Fig. 4.2.4b). It is also likely that they were a mixture of both these cell types. In the body column of the primary polyps, stained cells were forming clusters typical for early nematoblasts. Also many single cells were expressing Nanos2 mRNA and they could be differentiating nematocytes, which detached from their clusters or migrating stem cells (Fig. 4.2.4c).

Fig. 4.2.4. Nanos2 in situ hybridization performed on the primary polyp. (a) Expression in the epidermis of the stolons and the lower part of the body column. (b) Higher magnification of cells expressing Nanos2 in the stolons. (c) Higher magnification of cells in the body column. Scale bar in (b) and (c) represents 10 µm.
Nanos2 expression pattern was also examined in the adult polyps. In contrast to primary polyps, in adult animals Nanos2 mRNA was detected in the whole polyp body, including head and tentacles (Fig. 4.2.5a).

![Image of in situ hybridization](image)

**Fig. 4.2.5.** Nanos2 in situ hybridization performed on adult feeding polyps. (a) Expression in the epidermis of the body column, head and tentacles. (b) Clusters of putative nematoblasts in the lower part of the body column. (c) Nanos2+ cells in the upper part of the body column, likely maturing nematoblasts. (d) and (e) Differentiating nematocytes in the tentacles. Scale bar in (b), (c), (d) and (e) represents 10 µm.

I could identify at least three distinct cell types expressing Nanos2 in the body column. Throughout the whole epidermis of the polyp body Nanos2 in situ hybridization detected cells, resembling nematoblasts, precursors of cnidarians-specific stinging cells. In the lower part of the body column stained cells were forming clusters, typical for early proliferating progenitors (Fig. 4.2.5b). In the upper part of the body column, Nanos2 was additionally expressed in much bigger cells, which looked like maturing nematoblasts (Fig. 4.2.5c). However, their identity is not...
certain. Strong Nanos2 expression was also found in the head and tentacles. Cells stained in the tentacles were elongated, about 8 µm in size and look like differentiating nematocytes (Fig. 4.2.5d-e).

Nanos2 was also expressed in the developing female and male gonads (Fig. 4.2.6a-e). In female sexual polyps Nanos2 was detected both in oocytes within gonads and in oocytes, which were migrating through the body column in direction of the gonads (Fig. 4.2.6c-e). Interestingly, Nanos2 expression was excluded from the mature gametes, suggesting that it has a role in the early germ cells development.

While the expression pattern of Nanos2 in germ cells is consistent with the general role of Nanos in germ cells development in other animals, Nanos2 mRNA was also detected in cells belonging to the neural lineage, from early nematoblasts to putative differentiating stinging cells. Nematocytes are mechanosensory cells, which share common origin with neurons. Nanos was found to be expressed in the nervous system of many animals so far, although this role of Nanos has not been widely studied.

For all different Hydractinia life stages, I always performed control in situ hybridization with the sense probe. No staining was observed in control experiment, indicating the specificity of an antisense probe (Fig. 4.2.6f).
Fig. 4.2.6. *Nanos2* in situ hybridization performed on the sexual polyps. (a) *Nanos2* expression in developing female gonads. (b) Expression in the developing male gonads. (c) Oocytes expressing *Nanos2*. (d) Developing sperm expressing *Nanos2*. (e) Oocyte migrating through the body column in direction of the gonads. (f) Sense probe showing no staining. Scale bar in (c), (d) represents 50 µm.
4.3. Nanos1

As a template for the synthesis of the Nanos1 in situ hybridization probes, I used a PCR fragment covering the first 600 bp of the gene's coding sequence. The fragment was amplified with “Nanos1ATG-T7-fwd” and “Nanos1-Sp6-rev” primers, to which T7 and Sp6 RNA polymerase promoters’ sequences were attached, respectively (Fig. 4.3.1).

<table>
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<th>DNA Fragment</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Nanos1ATG-T7-fwd</td>
<td>5' gatcataatacgactcactatagggatgttcaaggcaatgataagctggtt 3'</td>
</tr>
<tr>
<td>Nanos1-Sp6-rev</td>
<td>5'tagcaatttaggtacactataagaaggtggtttgttggccttagaat 3'</td>
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Fig. 4.3.1. Sequences of primers used to amplify 600 bp of the Hydractinia Nanos1 coding sequence. Amplified fragment was used for the synthesis of in situ hybridization probes.

DNA fragments were amplified according to the protocol for PCR reaction (2.1.13). PCR product was analyzed by 1% agarose gel electrophoresis. The band of a proper size was extracted from the gel according to protocol 2.1.5 and sequenced according to protocol 2.1.14. Sense and antisense probes were synthesized according to protocol 2.1.9 and verified on the formaldehyde gel according to protocol 2.1.3. In situ hybridization experiments were performed for all Hydractinia life stages, according to protocol 2.1.10.

Interestingly, Nanos1 expression was detected only in germ cells (Fig. 4.3.2) similarly to mouse Nanos2 and Nanos3 (Tsuda et al., 2003). In female sexual polyps Hydractinia Nanos1 was found in oocytes at the very similar developmental stage as Nanos2 expression (Fig. 4.3.2a-b). Hydractinia Nanos1 has not been detected in male germ cells, nor in any other cell types at any other developmental stage, including embryos, larvae, primary polyps and adult feeding polyps (Fig. 4.3.2c). Hence, Hydractinia Nanos1 has a similar expression to mammalian Nanos2 and Nanos3 and shows gender specificity like mammalian Nanos2. However, while mammalian Nanos2 is male specific, Hydractinia Nanos1 is female specific.

I also performed the control in situ hybridization with the sense probe. In each experiment no staining was observed in the control experiment, indicating the specificity of the antisense probe.
Fig. 4.3.2. *Nanos1 in situ* hybridization performed on the sexual polyps. (a) *Nanos2* expression in the developing female gonads. (b) Expression in the developing female gonads of the immature female sexual polyp. (c) Sense probe showing no staining.
4.4. Pumilio

Nanos is known to act together with its canonical partner, Pumilio. Both proteins form a complex, which inhibits translation of the target transcripts. If the role of the complex is conserved in *Hydractinia*, then Nanos and Pumilio have to be expressed in the same cells. In order to verify Pumilio expression pattern and compare it to the Nanos expression, *in situ* hybridization probes for Pumilio were prepared.

As a template for the probes’ synthesis, I used PCR fragment, covering 750 bp of the *Pumilio* coding sequence. The fragment was amplified with “Pumilio-T7-fwd” and “Pumilio-Sp6-rev” primers, to which T7 and Sp6 RNA polymerase promoters’ sequences were attached, respectively (Fig. 4.4.1).

| Pumilio2 T7-fwd | 5’gatcataatacgactcactatagggatcggaggtcatcttcagtctact 3’ |
| Pumilio2 sp6-rev | 5’tagcaatttaggtgacactatagaatacatgtattgtcatttctggtggtat 3’ |

Fig. 4.4.1. Sequences of primers used to amplify 750 bp of *Hydractinia Pumilio* coding sequence. Amplified fragment was used for the synthesis of the *in situ* hybridization probes.

DNA fragment was amplified according to the protocol for PCR reaction (2.1.13). PCR product was analyzed by gel electrophoresis and sequencing. Sense and antisense probes were synthesized according to protocol 2.1.9 and verified on formaldehyde gel according to protocol 2.1.3. *In situ* hybridization experiments were performed for all *Hydractinia* life stages according to protocol 2.1.10.

*In situ* hybridization performed on two-three day old planula larvae detected endodermal *Pumilio* expression found in small clusters of cells, which most likely represented early nematoblasts (Fig. 4.4.2a-b). Its expression was also present in single cells and in cells forming duplets, typical for stem cells or early committed cells. Stained cells were morphologically similar to *Nanos* cells in the larvae.

*In situ* hybridization with the sense probe did not detect any specific staining in the larvae, indicating the specificity of an antisense probe (Fig. 4.4.2c).
Fig. 4.4.2. *Pumilio* in situ hybridization performed on the larvae. (a) Expression in the two-three day old larva. The larva is directed with the head down. (b) *Nanos2* expression in single cells and in small clusters, presumably nematoblasts, in the endoderm of the planula larvae. (c) Sense probe showing no staining. Scale bar represents 50 µm.

*Pumilio* expression pattern in the primary polyps was very similar to *Nanos2* expression as well. *Pumilio* was found in the epidermis of stolons and the lower half of the body column (Fig. 4.4.3a). In the body column, stained cells were forming clusters typical for early nematoblasts. Also many single cells were expressing *Pumilio* and their morphology suggested that they were either stem cells or maturing nematoblasts that already detached from their clusters (Fig. 4.4.3b). Cells expressing *Pumilio* in stolons were presumably nematoblasts as well (Fig. 4.4.3c).

*Pumilio* mRNA was also detected in germ cells (Fig. 4.4.3d). *Pumilio* was expressed in small oocytes of the early developing gonads. Interestingly, its expression was excluded from mature gametes, similarly to both *Nanos1* and *Nanos2*. It suggests that Pumilio may regulate germ cells development together with *Hydractinia* Nanos proteins. This function would be consistent with the general role of the complex Nanos/Pumilio in many animals.
Fig. 4.4.3. *Pumilio in situ* hybridization performed on primary and sexual polyps. (a) Expression in the epidermis of the stolons and the body column of a primary polyp. (b) Higher magnification of *Pumilio*⁺ cells in the body column. (c) Higher magnification of *Pumilio*⁺ cells in the stolons. (d) *Pumilio* expression in oocytes of developing female gonads. Scale bar in (b) and (c) represents 10µm.

*Pumilio* expression pattern was also examined in adult feeding polyps (Fig. 4.4.4a). Surprisingly, *Pumilio* mRNA was detected only in the lower part of the body column in clusters of cells, typical for early nematoblasts (Fig. 4.4.4b). Its expression pattern was only slightly overlapping with Nanos2 expression, which was present throughout the whole polyp body, including head and tentacles. This result suggests that Pumilio may be important only at the early stage of nematogenesis, in which stage it may act with Nanos2. However, regulation of later nematoblasts may
not require Pumilio. Interestingly, Pumilio-independent Nanos function in the nervous system has never been observed in other species before.

*In situ* hybridization with the sense probe did not detect any specific staining at any developmental stage, indicating the specificity of an antisense probe (Fig. 4.4.4c).

Fig. 4.4.4. *Pumilio in situ* hybridization performed on the adult feeding polyps. (a) Expression in the epidermis of the body column. (b) Clusters of cells, presumably nematoblasts, in the lower half of the body column. (c) Sense probe showing no staining. Scale bar in (b) represents 10 µm.
4.5. Ncol1

In order to study Nanos2 ectopic expression and knockdown phenotypes, I optimized in situ hybridization for a few neural/nematoblast markers. One of the examined markers was Ncol1, a nematocyte-specific minicollagen gene.

As a template for the synthesis of Ncol1 probe I used a PCR fragment, covering the last 170 bp of the coding sequence. The fragment was amplified with “Ncol1-T7-fwd” and “Ncol1-Sp6-rev” primers, to which T7 and Sp6 RNA polymerase promoters’ sequences were attached, respectively (Fig. 4.5.1).

```
Ncol1-T7 fwd  5' gatcataatacgacgactatagggacgtccaggaccaccaggagta 3'
Ncol1-Sp6 rev 5' tagcaatttagtgacactatagaactgggcaacagtattgtggacaaga 3'
```

Fig. 4.5.1. Sequences of primers used to amplify 170 bp of the Hydractinia Ncol1 coding sequence. Amplified fragment was used for the synthesis of in situ hybridization probes.

DNA fragment was amplified according to the protocol for PCR reaction (2.1.13). PCR product was analyzed by gel electrophoresis and sequencing. Sense and antisense probes were synthesized according to protocol 2.1.9 and verified by formaldehyde gel electrophoresis according to protocol 2.1.3. In situ hybridization experiments were performed according to protocol 2.1.10.

In situ hybridization performed on two-three day old planula larvae showed abundant Ncol1 expression in the endoderm of the larvae (Fig. 4.5.2a). Ncol1 was detected in post mitotic nematoblasts, which already detached from their clusters and were undergoing development of the nematocysts (Fig. 4.5.2b). Nematocyst is a complex secretory structure inside the cytoplasm of a stinging cell. It is a post-Golgi vacuole and consists of a capsule, containing a coiled tubule with spines and venom. In Ncol1+ cells it was possible to distinguish forming vacuoles within the cells’ cytoplasm.

In situ hybridization with the sense probe did not detect any specific staining in the larvae, indicating the specificity of an antisense probe (Fig. 4.5.2c).
Fig. 4.5.2. *Ncol1* in situ hybridization performed on larvae. (a) Expression in the three day old larvae. The larva is directed with the head down. (b) *Ncol1* expression in nematoblasts, in the endoderm of the planula larvae. (c) Sense probe showing no staining.

*Ncol1* expression in the primary polyps was mostly restricted to the stolons where *Ncoll*-expressing cells were very abundant (Fig. 4.5.3a-b). *Ncoll*+ cells were about 6-7 µm in size and did not form clusters. Only in few animals, some staining was observed in the polyp body column, and presumably marked migrating nematoblasts. In *Ncoll*+ cells, it was possible to distinguish developing vacuoles, which would become capsules. The size of developing vacuoles varied, from very small to one-third of the volume of the cell (Fig. 4.5.3a, insets). Looking at the distribution and morphology of the *Ncoll*+ cells, I could conclude that this minicollagen gene was expressed only in the narrow stage of nematoblasts differentiation. Its expression was found in post mitotic cells, which did not form clusters that are typical for early precursors. *Ncoll*+ nematoblasts had developing vacuoles, but the typical capsules’ structures, like the coiled tubule, were not visible yet. Also, *Ncoll* was not expressed in the upper part of the polyp body and tentacles, where mature stinging cells reside (Fig. 4.5.3a).
Fig. 4.5.3. *Ncol1* in situ hybridization performed on the primary polyp. (a) Expression in the epidermis of the stolons and the body column. Insets show *Ncol1* cells with developing capsules. (b) Higher magnification of nematoblasts in the stolons.

*Ncol1* expression pattern was also examined in the adult feeding polyps (Fig. 4.5.4a). Its mRNA was detected in the epidermis of the body column, in cells undergoing nematocyst capsule development (Fig. 4.5.4b). Similarly to primary polyps, in head and tentacles *Ncol1* cells were not observed.
In situ hybridization with the sense probe did not detect any specific staining at any developmental stage, indicating the specificity of an antisense probe (Fig. 4.5.4c).

4.5.4. *Ncoll* in situ hybridization performed on adult feeding polyps. (a) Expression in the epidermis of the body column. (b) Higher magnification of nematoblasts in the body column. (c) Sense probe showing no staining. Scale bar in (b) represents 10 µm.

Fluorescent in situ hybridization (FISH) was optimized for the *Ncoll* gene as well. FISH was performed according to protocol 2.1.11. Because *Ncoll* is a highly expressed gene in cells, it can be detected by colorimetric reaction of in situ hybridization in a very short time, which is usually less than 10 minutes. From the same reason FISH protocol worked for this gene very well and gave relatively little background (Fig. 4.5.5a).
I also performed immunohistochemical analysis of Ncol1 protein expression according to protocol 2.2.1. For this purpose I used antibodies directed against *Nematostella vectensis* NCol1. Due to the high sequence similarity of the *Nematostella* homolog to *Hydractinia* Ncol1 protein, cross-reactivity of the antibodies was very good, allowing specific staining of developing nematocysts (capsules of stinging cells). I used NCol1 antibody for staining of the larvae and primary polyps (Fig. 4.5.5b-c). NCol1 protein expression pattern was similar to *Ncol1 in situ* hybridization, suggesting that antibodies do no detect mature stinging cells.

The nematocyst-specific antibodies NCol1 (Adamczyk et al., 2010) were a kind gift from Dr Suat Özbek (University of Heidelberg, Germany). To detect NCol1 Ab I used AlexaFluor488 goat anti-rabbit IgG (cat no. A-11012, Invitrogen) as secondary antibodies.

4.5.5. *Ncol1* fluorescent *in situ* hybridization (FISH) and NCol1 IHC staining. (a) FISH performed on the larvae. (b) Anti-NCol1 antibody staining performed on larvae. (c) Anti-NCol1 antibody staining performed on a primary polyp. Scale bar represents 100 µm.
4.6. Ncol1 and Nanos2 co-expression analysis

I performed double in situ hybridization for Nanos2 and Ncol1 to verify if expression of these two genes co-localize to the same cells. In situ hybridizations were performed according to protocol for double ISH in 2.1.10. Nanos2 probe was labeled with Digoxigenin and stained with the mixture of NBT/BCIP to obtain the blue staining, whereas the Ncol1 probe was labeled with Fluorescein and colorimetric reaction was performed with Sigma Fast Red to obtain the red staining.

Double in situ hybridization performed on the larvae confirmed that Ncol1 is expressed only in the larval endoderm, whereas Nanos2 is found in both embryonic layers (Fig. 4.6.1a). In few cases it was possible to detect co-localization of both genes within the endoderm (Fig. 4.6.1b, arrows). However, Nanos2 mostly was not co-expressed with Ncol1, indicating that their expression patterns do not overlap.

4.6.1. Results of double in situ hybridization for Nanos2 and Ncol1. (a) Larvae stained in blue with Nanos2 probe and in red with Ncol1 probe. The larva is directed with the head down. (b) Magnification of larval endodermal cells which were likely co-expressing both genes (arrows). Scale bar in (b) represents 10 µm.

Ncol1 and Nanos2 expression patterns in the primary polyp were mostly restricted to the stolons (Fig. 4.6.2a). Nematoblasts expressing Ncol1 in most cases
were not co-expressing Nanos2, although in few cells co-localization of both genes was detected (Fig. 4.6.2b-d). However, I cannot exclude the possibility that two different cells were laying on the top of each other, giving the illusion of co-localization.

**Fig. 4.6.2.** Double *in situ* hybridization performed for Nanos2 and Ncol1. (a) Primary polyp stained in blue for Nanos2 and in red for Ncol1. (b)–(d) Higher magnification of cells in stolons. Scale bar in (a) represents 100 μm and in (b)-(d) represents 10 μm.

*In situ* hybridization on the adult feeding polyps revealed that both genes have very distinct overall expression patterns (Fig. 4.6.3a). Ncol1 was expressed in nematoblasts in the lower half of the body column. It was found in cells which were undergoing early nematocyst development and was excluded from maturing and fully differentiated stinging cells. Interestingly, Nanos2 was strongly expressed in the upper part of the body column, head and in the tentacles. Its expression was found in cells, which morphologically resembled maturing nematocytes. The half way through the body column, where Ncol1 stopped being expressed, relatively big and single Nanos2+ cells appeared (Fig. 4.6.3b). These cells could have been differentiating nematocytes, which already stopped expressing minicollagen1, although their identity remains unknown. On the boarder of expression, few cells were co-localizing two genes; however, most of them seemed to be either Nanos2+ or Ncol1+ (Fig. 4.6.3c).
In situ hybridization with the sense probe did not detect any specific staining at any developmental stage, indicating the specificity of an antisense probe (Fig. 4.6.3d).

Fig. 4.6.3. Double in situ hybridization for Nanos2 and Ncol1 on adult feeding polyps. (a) Polyp stained in blue with Nanos2 probe and in red with Ncol1 probe. (b) The border of expression of two genes. (c) Magnification of cells in the middle part of the body column. Few cells co-expressing both genes were found, however most of the cells were either Ncol1⁺ or Nanos2⁺. (d) Sense probes showing no staining. Scale bar in (b) and (c) represents 10 μm and in (d) 200 μm.

In Hydractinia nematocyte differentiation progresses towards the head, where mostly mature differentiated cells are present. Nanos2 seemed to be expressed both in progenitor cells in stolons and in maturing nematocytes in the head and tentacles. Nanos2 most likely is present throughout the nematogenesis pathway. Whereas, Ncol1 was expressed only in a narrow stage of nematoblasts development, and was only slightly overlapping with the Nanos2 expression pattern.
4.7. Ash

Ash belongs to the members of the Achaete-scute family (Ash) of proneural genes. It encodes transcriptional activators of the basic helix–loop–helix (bHLH) class that are involved in the neuronal specification and differentiation, both in vertebrates and invertebrates (Negre and Simpson, 2009). Cnidarians have a single Ash gene (Grens et al., 1995; Seipel et al., 2004). Hydra vulgaris Ash homolog, CnASH, was found to be expressed in a subset of the interstitial cells and in differentiating nematocytes (Grens et al., 1995), but also in sensory neurons (Hayakawa et al., 2004).

I amplified the Hydractinia homolog of Ash by PCR reaction, in order to prepare in situ hybridization probes for this gene. I performed ISH protocol on the larvae and on the adult feeding polyps to identify the type of cells expressing Ash. I used Ash probes later on for analyzing the phenotypes of Nanos2 ectopic expression and knockdown.

As a template for the synthesis of Ash probes I used PCR fragment of 520 bp in size, which was covering the entire coding sequence, flanked by the two untranslated regions. The fragment was amplified with “Ash-T7-fwd” and “Ash-Sp6-rev” primers, to which T7 and Sp6 RNA polymerase promoter sequences were attached, respectively (Fig. 4.7.1).

| Ash-T7-fwd | 5’ gatacatataacagacactatatagggtcggaaagttatatagagtaagtaa 3’ |
| Ash-Sp6-rev | 5’ tagcaatttaggtgacactatagaataacagcgaagaaatatttacaatcta 3’ |

Fig. 4.7.1. Sequences of primers used to amplify 520 bp of Hydractinia Ash sequence.

DNA fragment was amplified according to protocol 2.1.13. PCR product was analyzed by gel electrophoresis and sequencing. Sense and antisense probes were synthesized according to protocol 2.1.9 and verified on formaldehyde denaturing gel electrophoresis according to protocol 2.1.3. In situ hybridizations were performed for the larvae and feeding polyps according to protocol 2.1.10.
In situ hybridization performed on two-day old planula larvae showed endodermal Ash expression, presumably in neuroblasts and nematoblasts (Fig. 4.7.2a-b).

In adult feeding polyps Ash mRNA was found in the epidermal layer, in the lower half of the body column and in the tentacles (Fig. 4.7.2c-d). Cells expressing Ash in the polyp were most likely neurons or their precursors, but some could also be nematoblasts (Fig. 4.7.3d). However, stained cells were not very numerous, suggesting that Ash expression is unlikely to be essential for nematocyte development. Nematoblasts and nematocytes are very abundant cell types (Greber et al., 1992) as animals use large numbers of stinging cells every day to capture prey and defend against predators.

Fig. 4.7.2. In situ hybridization with Ash performed on larvae and adult feeding polyps. (a) Expression in the two day old larvae. The larva is directed with the head down. (b) Magnification of Ash expression in the endoderm of the planula larvae. (c) Expression in the epidermis of the adult feeding polyp. (d) Magnification of putative neuroblasts or nematoblasts in the lower half of the body column. Scale bar in (b) represents 10 μm.
4.8. RFamide

RFamide neuropeptides play many important roles in the nervous systems of both vertebrates and invertebrates (Darmer et al., 1991). In cnidarians, RFamide neuropeptides were found to be important for larval metamorphosis (Katsukura et al., 2003) and migration (Katsukura et al., 2004).

I amplified *Hydractinia RFamide* neuropeptide precursor by PCR in order to prepare *in situ* hybridization probes for this gene. I performed ISH on the larvae, primary polyps and adult feeding polyps to identify the type of cells expressing *RFamide*. Later, I used *in situ* probes for this gene to analyze the phenotypes of Nanos2 ectopic expression and knockdown.

As a template for the synthesis of *RFamide* probes I used a PCR fragment, covering the entire coding sequence of 1137 bp. Fragment was amplified with “RFamide-T7-fwd” and “RFamide-Sp6-rev” primers, to which T7 and Sp6 RNA polymerase promoters’ sequences were attached, respectively (Fig. 4.8.1).

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<td>5’ gatcataatacgactcactatagggatgttaatcatggcttcaaaggc 3’</td>
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<tr>
<td>RFamide-Sp6rev</td>
<td>5’ tagcaatttaggtgacactatagaacttaacagtctctttcgtgttg 3’</td>
</tr>
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Fig. 4.8.1. Sequences of primers used to amplify 1137 bp of *Hydractinia RFamide* sequence.

DNA fragment was amplified according to protocol 2.1.13. PCR product was analyzed by gel electrophoresis and sequencing. Sense and antisense probes were synthetized according to protocol 2.1.9 and verified by formaldehyde gel electrophoresis according to protocol 2.1.3. *In situ* hybridizations were performed according to protocol 2.1.10.

*In situ* hybridization performed on two day old planula larvae detected *RFamide*+ cells throughout the ectoderm of the larvae (Fig. 4.8.2a). Antisense probe stained mature neurons, which had long neurites, and they were either unipolar or bipolar (Fig. 4.8.2, insets). In the primary polyp, *RFamide* was detected only in the head, around the hypostome (Fig. 4.8.2b). In the adult feeding polyps, its expression
was found in the ectoderm of the whole body column, including head and tentacles (Fig. 4.8.2c-d). Head and tentacles are usually very abundant in all different types of neurons, as they are important for the coordination of the tentacle movements involved in prey capture and in the opening and closure of the mouth during ingestion.

Fig. 4.8.2. RFamide in situ hybridization. (a) Expression in two-day old larvae. A larva is directed with the head down. Insets show magnification of RFamide+ unipolar and bipolar neurons. (b) Expression around the hypostome of the primary polyp. (c) Expression in the epidermis of the body column, head and tentacles. (d) Higher magnification of RFamide+ neurons in the head of the feeding polyp.
4.9. Summary

By the use of in situ hybridization and immunohistochemistry methods, I studied mRNA expression patterns of two Hydractinia Nanos homologs: Nanos1 and Nanos2, but also the Nanos canonical partner, Pumilio, and few neural markers: Ash, RFamide and Ncol1.

Nanos2 was found to be expressed in germ cells and in cells committed to nematocyte fate from early progenitors to maturing stinging cells. Its expression was detected from early embryonic development throughout all different Hydractinia life stages. In the fertilized eggs and in embryos, during the first blastomeric divisions, Nanos2 expression was polarized. In later embryogenesis and during larval development, it was expressed both in the ectoderm and endoderm. However, following metamorphosis, Nanos2 expression was detected only in the epidermis. This shift in the expression from endoderm to ectoderm post metamorphosis is observed very often for neural markers. Also, neural progenitors usually proliferate in the endoderm of the larvae and possibly migrate to the larval ectoderm while undergoing differentiation. In adults, mature nematocytes and most of the neurons are present only in the epidermis. Nanos2 expression in adult feeding polyps was found throughout the whole polyp body, including head and tentacles, in developing nematoblasts. Nanos2 expression was also examined in the sexual polyps. Its mRNA was detected in both developing male and female germ cells and was excluded from mature gametes.

Interestingly, the second Hydractinia Nanos homolog, Nanos1, was detected only in female germ cells. Its expression was found in oocytes at the very similar developmental stage as Nanos2 expression, but unlike Nanos2 it was not expressed in males.

The expression pattern of the Nanos canonical partner, Pumilio, was also examined in different life stages. In the larva and primary polyp, its expression was very similar to the Nanos2 expression pattern, and found in cells which resembled nematocyte progenitors. Pumilio expression also overlapped with both Nanos1 and Nanos2 expression in developing germ cells. Surprisingly, in adult feeding polyps Pumilio mRNA was found only in the lower half of the body column, presumably in clusters of nematoblasts. Its expression pattern only slightly overlapped with the Nanos2 expression, which was detected throughout the whole polyp body, including head and tentacles.
I also performed *in situ* hybridizations for few neural markers in order to use them for analyzing the phenotypes of Nanos2 ectopic expression and knockdown. *Ncol1* is a classical cnidarian nematoblast marker and it was found in cells undergoing nematocyst development, from the larval stage to the adult polyps. Its expression also shifted from endodermal in early embryogenesis to epidermal in the primary and adult polyps, what is typical for most neural precursors. Additionally, I used immunohistochemical staining to study NCol1 protein expression pattern. Antibodies stained developing nematocysts, capsules of stinging cells. Similarly to *Ncol1 in situ* hybridization, antibody staining was observed in the endoderm of the larvae, suggesting that antibody does not detect mature stinging cells. Also in the primary polyps, staining was observed only in the epidermis of the lower half of the body column, whereas mature nematocytes mostly reside in the head and tentacles.

I also performed *in situ* hybridizations for *Hydractinia Ash*, which was detected in putative neuroblasts and nematoblasts. Its expression shifted from endodermal in early embryogenesis to epidermal in the polyps as well.

*RFamide in situ* hybridization detected mature neurons, which had long neurites, and were either unipolar or bipolar. *RFamide* expression was found in larvae throughout its ectoderm, and in the primary polyps around the hypostome only. In adult polyps, it was found in the epidermis of the whole body column, including head and tentacles, which are usually abundant in all different neural cell types.

*In situ* hybridizations, optimized for *Ash, RFamide* and *Ncol1*, were later on used for an analysis of the Nanos2 ectopic expression and knockdown phenotypes, described in chapter 6 and 7.
5. Nanos2 promoter activity

5.1. Introduction

Gene promoter is a DNA sequence within the genome, which is directing the expression of a particular gene (Hames, 1984; Levine and Tjian, 2003). Each gene has its own unique promoter and all promoters vary in sizes, e.g. some can span few kilobases of the genomic DNA. They occur upstream of the gene coding sequence and are bound by transcription factors, which have an ability to either activate or block the transcription of this gene. Transcription is mediated by RNA polymerase which binds to the promoter’s sequence (Hames, 1984). The promoter’s activity reflects the expression pattern of the gene regulated by this particular promoter. Some genes are expressed in all types of cells, like tubulin, which is crucial for maintaining basic cellular functions. Other genes are expressed only in certain lineages and they are important for acquiring or maintaining of specialized cellular functions. Studying a promoter activity can provide insight into the developmental stage at which an examined gene is active and the types of cells in which it is expressed.

One way to study the promoter activity is to place a reporter gene downstream of the examined promoter (Ma, 2001). As a reporter gene commonly used are Green Fluorescent Protein (GFP) or luciferase. Generated expression construct after microinjection or transfection will drive expression of the reporter gene only in cells and at the developmental stage, in which the promoter of examined gene is active naturally. The promoter activity can be traced in vivo by following the reporter's activity (e.g. fluorescence). Applying such a method allows examining the dynamic changes of its spatiotemporal expression over the whole individual’s lifetime (Ma, 2001). In contrast, in situ hybridization or immunohistochemistry can be performed only on the fixed tissues and allow to visualize only one single time point of the gene expression at a particular developmental stage.

Promoters can direct stable or transient reporter expression. Stable expression is dependant from the insertion of the expression cassette into an animal genome. It allows tracking cells in which the examined promoter is active within the living organisms. Also, by stable expression it is possible to examine the dynamic changes of the spatiotemporal gene expression throughout the individual's development. In Hydractinia, transgenesis is performed by microinjection to embryos and no other
method for introducing new genes was established for this animal model so far (Millane et al., 2011). Unfortunately, the mechanism of gene insertion into an animal genome is poorly understood and difficult to control. Injection is usually very inefficient and only a small percentage of embryos stably express GFP over the entire animal’s lifetime. The vector is usually randomly inserted into the genome and this can cause few additional problems that are likely to decrease the efficiency of expression studies. First, random insertion can disrupt the expression of other genes, which may be important for proper development. Such incorrect insertion can increase the lethality of injected embryos. Furthermore, after injection the circular vector is digested with endogenous endonucleases and the sequence of expression cassette can be shortened so its entire fragment may not be incorporated into a genome. For example, if the GFP reporter sequence is cut somewhere in the middle, it will result in the synthesis of non-functional protein product. From this reason only few out of hundred injected embryos ever become transgenic. The stability of the promoter expression also depends very much on the site of the integration into an animal’s genome. The flanking sequences are very likely to have strong influences on the activity of the promoter and may either increase it or decrease. In order to overcome above difficulties, injections of expression construct need to be performed many times, until enough numbers of transgenic animals are generated. Then hopefully the expression pattern is repetitive and it can be correctly interpreted.

Transient reporter expression does not rely on the insertion of the expression cassette into the genome. It depends only on the amount of introduced vector and the ability of cells to transcribe it. As in the case of the stable expression, a vector is transcribed and translated only within the cells in which promoter is active. However, transient expression is temporal because the introduced plasmid is not integrated into the genome and lacks the elements for replication in the cell. Injected plasmid will be passed to daughter blastomers for few more mitotic divisions, where it might be expressed, but will eventually be diluted and the animal will lose fluorescence.

I studied the GFP expression pattern under the Nanos2 promoter in early Hydractinia development. For this purpose I have created the expression construct which was injected into fertilized eggs and the reporter expression was traced throughout the embryonic development, including preplanula and larvae stages, but also shortly post metamorphosis.
5.2. *Nanos2* promoter expression vector

At the time I started my project no genome database was available. In order to find the *Nanos2* promoter, I had to perform PCR-based genome walking. For this purpose, I used splinkered *Hydractinia* genomic DNA, which was prepared by Dr Reinhardt Heiermann, a former postdoc in the lab. Dr Heiermann prepared this DNA by digesting it with few restriction enzymes: EcoRI, HindIII, XbaI, Sall, Nhe, Xho, SalII. The distribution of restriction sites within genomic DNA determined the lengths of DNA fragments. I amplified the *Nanos2* promoter from prepared DNA samples by a PCR-based genomic walking following a protocol described in chapter 3.4. DNA template digested with EcoRI restriction enzyme delivered the longest fragment of 846 bp in size. Sequencing of the obtained PCR product confirmed it to be upstream of the *Nanos2* coding sequence, but determining if this fragment represents the full promoter sequence can only be done empirically. I therefore cloned it into the expression vector containing the GFP reporter coding sequence. The expression construct was microinjected into early *Hydractinia* embryos and GFP\(^+\) cells were traced in live animals using fluorescence microscopy.

The expression vector, which I used for the promoter studies, consisted of 2100 bp of *Hydractinia Actin1* promoter upstream of 717 bp of the GFP reporter sequence, followed by 707 bp of Hydra Actin1 terminator (Fig. 5.2.1). The whole expression cassette was inserted into the Bluescript\(_{SK}\) backbone of 2164 bp in size, which also included 328 bp of pUC18 sequence. The Bluescript polylinker sequence was removed to minimize the number of restriction sites, but the Ampicillin resistance gene was left intact to allow antibiotic resistance selection of transfected bacteria colonies (Fig. 5.2.1).

In order to study the *Nanos2* promoter activity, I amplified by PCR reaction the whole sequence of the above mentioned vector, excluding the *Actin1* promoter. The fragment was amplified with primers to which *FseI* and *NotI* restriction sites were attached. The 846 bp fragment of the *Nanos2* promoter was also amplified with primers to which these restriction sites were attached. Prior to ligation, PCR products were digested with both enzymes according to the manufacturer’s recommendations (protocol 2.1.7) and DNA was purified (protocol 2.1.8). Digestion of amplified fragments allowed insertion of the promoter by sticky-end ligation. This ligation method is usually much more effective than blunt-end cloning and ensures correct orientation of the ligated fragments.
pUC 18 (328 bp)

Hydra Actin Terminator (707 bp)

GFP (717 bp)

Hydractinia Actin Promoter (~2100 bp)

Bluescript<sub>SK</sub> (2164 bp, without polylinker, LacZ, f1-origin)

Fig. 5.2.1. Schematic drawing of the Actin1 promoter expression vector used for generation of the Nanos2 promoter expression construct.
Ligation was performed according to protocol 2.1.15. The reaction was set up as follows:

- 80 ng of linear expression vector, excluding Actin1 promoter
- 250 ng of Nanos2 promoter (3:1 excess to vector)
- 1 µl of 10 x DNA ligase buffer
- 1 µl 50% PEG solution
- x µl of nuclease-free water
- 1 µl of T4 DNA ligase 5 U/µl

10 µl reaction

Due to the complexity of the performed ligation and relatively big size of ligated fragments, reaction was treated as blunt-end cloning and performed for 1h. Later on 50 µl of competent Escherichia coli C600 culture was used for bacteria transformation according to protocol 2.1.16. After overnight incubation all formed bacteria colonies were tested by PCR for the presence of an insert (protocol 2.1.13). PCR products were verified by gel electrophoresis. Positive clone was selected and plasmid extracted from the bacteria colony according to protocol 2.1.4. The vector was later verified by sequencing according to protocol 2.1.14, to make sure that the promoter insertion was correct and no mutations are present within the vector’s sequence. Possible deletions or nucleotide insertions, e.g. in the GFP sequence could result in the synthesis of a non-functional protein product.

The simplified expression cassette is shown on Fig. 5.2.2. Nanos2 promoter was ligated upstream of the reporter coding sequence (GFP). GFP sequence was followed by the Hydra Actin1 terminator.

![Fig. 5.2.2. Schematic diagram of Nanos2 promoter expression cassette.](image)

The whole Nanos2 promoter expression vector was 4856 bp in size (Fig. 5.2.3). Expression vector was injected in a circular form at the concentration of 1-2 µg/µl according to the protocol for microinjection in 2.3.1. Embryos were injected with 100-300 pl of the expression construct.
5.3. *Nanos2* promoter activity

In order to study *Nanos2* promoter activity, the expression vector described in chapter 5.2 was injected into *Hydractinia* 1-2-cell stage embryos. GFP+ cells appeared for the first time between 10-24 hours post fertilization (hpf) and were visible throughout the larva development (Fig. 5.3.1a-d). Fluorescent cells were found both in the ectoderm and in the endoderm of preplanula (Fig. 5.3.1a) and larvae (Fig. 5.3.1b-c). In the preplanula, the identity of these cells was very difficult to determine. In the larvae, GFP+ ectodermal cells morphologically looked like neurons and nematocytes (Fig. 5.3.1, d). Both mentioned cell types are normally very abundant in the larval ectoderm and they reside in the interstitial spaces between epithelio muscular cells. GFP+ cells were also about 8-10 µm in size and have elongated shape, as neurons or stinging cells do.

To trace reporter expression throughout *Hydractinia* development, larvae were induced to metamorphose into primary polyps (Fig. 5.3.2). In the polyps, GFP+ cells were detected throughout the epidermis of the whole polyp, including stolons, body column, head and tentacles (Fig. 5.3.2a-b). Some of the cells in the head were spindle-shaped and possessed extensions, resembling neurites, suggesting that they were neurons (Fig. 5.3.2c-e, arrows). Other GFP+ cells around the hypostome were found to form a ring-shaped agglomerate of nerve cells (Fig. 5.3.2c). Hydroid polyps are known to possess an agglomeration of a few large ganglion cells with thick processes around their mouth opening (Grimmelikhuijzen, 1985). These cells are involved in the opening and closure of the mouth during ingestion. In the head also different types of fluorescent cells were found. They were round, about 7-8 µm in size and were forming clusters of three to five cells (Fig. 5.3.2d and e, asterisks). Clusters could represent either nematoblasts or some other type of proliferating cells surrounded by their daughter cells. In stolons, fluorescent cells appeared to be spindle-shaped, what is common for *Hydractinia*’s stem cells or some neuronal cell types (Fig. 5.3.2b and f). However, the identity of the GFP+ cells in stolons remains uncertain as migrating cells may sometimes give an impression of being elongated too.

First generation of transgenic animals are mosaics and uniform reporter expression could be obtained only following the sexual reproduction. Unfortunately, the promoter expression was not stable. About two weeks post transfection animals lost all GFP+ cells, long before sexual maturation was reached.
Fig. 5.3.1. GFP reporter expression under 846 bp sequence of the Nanos2 promoter. (a) Expression in 24 hpf preplanula was both ectodermal and endodermal. (b) Expression in two-tree day old larvae. (c) Expression in the larvae was both ectodermal and endodermal. (d) Ectodermal GFP⁺ cells in the larvae looked like neurons or nematocytes. Scale bar in d) represents 10 µm.
Fig. 5.3.2. GFP reporter expression under 846 bp sequence of the Nanos2 promoter. (a) and (b) GFP+ cells were detected throughout the epidermis of the whole polyp, including stolons, body column, head and tentacles. (c) Reporter expression in putative neurons around the mouth opening. (d) Reporter expression in the head. (e) Magnification of GFP+ cells in the head. (f) Higher magnification of GFP+ cells in the stolons. Asterisks point clusters of cells. Arrows point putative neurons. Scale bars in (c)-(f) represent 20 µm.
5.4. Summary

In order to study Nanos2 promoter activity in early Hydractinia development, I cloned partial sequence of the Nanos2 promoter into the expression vector containing GFP reporter sequence. Obtained expression construct was microinjected into 1-2-cell stage embryos and GFP\textsuperscript{+} cells were traced using fluorescence microscopy. Fluorescent cells were found both in the ectoderm and in the endoderm of preplanula and larvae, consistent with Nanos2 in situ hybridization. Ectodermal cells resembled neurons and nematocytes morphologically. After the larvae were induced to metamorphose, reporter expression was detected in the epidermis of the primary polyps, including their stolons, body column, head and tentacles. The identity of cells in the stolons and the body column was very difficult to determine. In the head GFP\textsuperscript{+} cells had a spindle shape and some possessed extensions, resembling neurites. Other fluorescent cells in the hypostome, around the mouth opening, formed a ring, which is reminiscent of the ganglionic nerve ring around hydroid polyps' mouths (Grimmelikhuijzen, 1985). In the head also different types of GFP\textsuperscript{+} cells were found. Some of these cells were round and formed small clusters, which could represent nematoblasts. Others looked like maturing nematocytes. Unfortunately, it was not possible to trace the fate of those cells as well as the further reporter expression pattern in the adult colonies, because few weeks post transfection all animals lost fluorescence.
6. *Nanos2* ectopic expression

6.1. Introduction

The term “ectopic expression” describes the expression of a particular gene in the abnormal cells or tissues within an organism or in cell cultures. Naturally this process can be caused by a disease. However, ectopic expression is also widely applied as a method for studying the biological functions of genes. This method represents the so called gain-of-function evidence in developmental biology, which constitutes the most convincing proof for the role of an examined gene (Gilbert, 1997).

Ectopic expression can be performed by transfecting cells with a transgene, whose transcription is regulated under a particular promoter (Brand et al., 1994). In *Hydractinia*, the method of ectopic gene expression is performed by the microinjection of an overexpression vector into fertilized eggs (Künzel et al., 2010; Millane et al., 2011). Injected constructs consist of a transgene, cloned downstream of a specific gene promoter and upstream of a reporter coding sequence (e.g. GFP). The promoter determines where and when the transgene will be expressed. If the promoter is active in all cell types, then the transgene will be ubiquitously expressed, but if the promoter is active only in epithelial cells, then the transgene will be present only in these cells. Injection of an overexpression construct can result either in the transient or in the stable reporter expression. This method represents a powerful tool for studying the gene functions. However, depending on the gene it can be inefficient and even lethal for the embryos. Additionally, the mechanism of the gene insertion into *Hydractinia* genome by means of transgenesis is poorly understood and hard to control.

There are few other methods for ectopic gene expression, like mRNA injection or various pharmacological treatments (Wikramanayake et al., 2003; Duffy et al., 2010). mRNA injection is a widely used method of transient gene expression. It is based on the insertion of a mature transcript consisting of the coding sequence of examined gene in frame with a reporter sequence. Such transcript is synthetized and postranscriptionally modified by 5’-capping and 3’-poly(A)tailing with the use of available *in vitro* transcription kits. Injection of prepared mRNA allows generating large numbers of transgenic animals in a relatively short time. Usually few hours
post injection the reporter protein undergoes maturation, becomes active and GFP+ cells can be traced using microscopy. Applying this method allows expressing the reporter protein ubiquitously and the effect of this expression can be traced for few days. Unfortunately, fluorescence cannot be traced throughout an animal lifetime and eventually the reporter proteins become diluted following cellular divisions or degraded over time. Unluckily, the attempt to ectopically express genes by mRNA injection into *Hydractinia* embryos was not successful.

Another gene manipulation method is performed by the chemical treatment of living cells or even whole organisms. It is optimized only for few signaling pathways but often works well on many experimental models. For example, the use of azakenpaullone, a small chemical compound, causes ectopic Wnt activation both in vertebrates and invertebrates (Cohen and Goedert, 2004; Plickert et al., 2006; Duffy et al., 2010). However, chemical treatment is established only for few signaling pathways so far and cannot be applied for most of the genes.

I studied the effect of Nanos2 ectopic expression in *Hydractinia* development by microinjection of an expression construct into fertilized eggs. For this purpose, the Nanos2 coding sequence was cloned downstream of the Actin1 promoter and in frame with the GFP reporter sequence. The transgene expression was later on traced using microscopy and phenotypes were examined by a dissecting microscope, *in situ* hybridizations and immunohistochemical stainings.
6.2. *Nanos2* ectopic expression vector

I order to ectopically express *Nanos2* an expression construct was prepared as follows:

- *Nanos2* coding sequence was cloned into the *Actin1* promoter expression vector.

- The *Actin1* promoter expression vector included 2100 bp of *Hydractinia* genomic DNA upstream of the *Actin1* start codon. The complete GFP coding sequence (717 bp) was inserted downstream of the promoter, followed by 707 bp of the *Hydra Actin1* Terminator (Fig. 6.2.1). The entire expression cassette was inserted into the BluescriptSk backbone of 2164 bp in size, and 328 bp of the pUC18 sequence. The Bluescript polylinker was removed to minimize the number of restriction sites but the Ampicillin resistance gene was left intact to allow antibiotic resistance selection of transfected bacteria colonies (Fig. 6.2.1).

- The entire sequence of above mentioned vector was amplified by PCR and later on verified by sequencing according to protocol 2.1.14.

- The *Nanos2* coding sequence was cloned downstream the *Actin1* promoter, and the GFP coding sequence was inserted in frame into the 3’ end of *Nanos2* (Fig. 6.2.2).

- The expression vector had a spacer introduced between the *Nanos2* coding sequence and GFP (Fig. 6.2.1 and Fig. 6.2.2, red color). The role of the spacer is to generate few extra amino acids between the two sequences, in order to allow both fused proteins to fold independently. This should enable two fused, but properly folded, active proteins.

- Ligation was performed blunt-ended according to protocol 2.1.15. The reaction was set up as follows:

  - 80 ng of linear expression vector containing *Actin1* promoter
  - 250 ng of *Nanos2* coding sequence (3:1 excess to vector)
  - 1 µl of 10 x DNA ligase buffer
  - 1 µl 50% PEG solution
  - x µl of nuclease-free water
  - 1µl of T4 DNA ligase 5U/µl

  10 µl reaction, 1h incubation
10 μl of ligation reaction was mixed with 50 μl of competent *Escherichia coli* C600 culture and bacteria transformation was performed according to protocol 2.1.16. After overnight incubation all formed colonies were tested by PCR for the presence of inserted *Nanos2* sequence. PCR was performed according to protocol 2.1.13.

PCR products were verified by gel electrophoresis. Positive clone was selected and plasmid extracted from the bacteria colony according to protocol 2.1.4. The vector was later sequenced according to protocol 2.1.14 to make sure that the insertion was correct and no mutations in the *Nanos2* or GFP reporter sequence were present.

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**Fig. 6.2.1.** Schematic drawing of the *Actin1* promoter expression vector, used for the generation of *Nanos2* ectopic expression construct.

The entire *Nanos2* ectopic expression vector was 6925 bp in size (Fig. 6.2.2).
Fig. 6.2.2. Sequence of the *Nanos2* ectopic expression vector. Black color represents the Bluescript promoter sequence, followed by the *Nanos2* open reading frame, green color shows the GFP reporter sequence, followed by the Bluescript Terminator, and pink shows the pUC18 sequence.
The simplified expression cassette is shown on Fig. 6.2.3. The *Nanos2* promoter was ligated upstream the reporter coding sequence (GFP). The GFP sequence was followed by the *Hydra Actin1* terminator.

![Diagram of expression cassette](image)

Fig. 6.2.3. Schematic diagram of the *Nanos2* ectopic expression cassette.

The vector was injected in a circular form at the concentration of 5-6 µg/µl according to the protocol for microinjection described in 2.3.1. Injection was performed on fertilized eggs, which were put on 100 µm plankton netting, glued to the bottom of a Petri dish to keep them in place when injecting.

*Hydractinia Actin1* promoter is ubiquitously expressed during early embryonic development but post metamorphosis it becomes epithelial-specific (Künzel et al., 2010; Millane et al., 2011). For this reason, ectopic expression of *Nanos2* under this promoter can affect all different cell types of the embryo, but following metamorphosis only epithelio-muscular cells will express the transgene.

First generation of transgenic animals is a mosaic, and only a fraction of cells into which the construct is integrated express the transgene. Uniform expression can only be obtained following the sexual reproduction.

Injection of *Nanos2* ectopic expression vector was not very efficient and resulted in obtaining of only few transgenic animals (Fig. 6.3.1a-b). Embryos with the high percentage of fluorescent cells were deformed, failed to develop into larvae and died. Some of the survived larvae had severe developmental malformations, like ectopic or truncated tails (Fig. 6.3.1c-d). Luckily, few animals which had less transgenic cells survived and were induced to metamorphose into primary polyps.

Fig. 6.3.1. *Nanos2* ectopic expression under *Actin1* promoter. (a) Expression in 24 hpf preplanula was both ectodermal and endodermal. (b) Expression in three day old larvae was ectodermal and endodermal. (c)-(d) Deformed larvae expressing the transgene.
Metamorphosed animals developed a range of phenotypic variations, from mild to severe. The least affected individuals generated an excess of tentacles, which are the oral structures around the polyp’s head (Fig. 6.3.2 and Fig. 6.3.3). Stinging cells are abundant in tentacles and are used to capture prey or defend against the predators. Transgenic animals grew up to twice more tentacles than non-fluorescent polyps. For these calculations, I counted the numbers of tentacles separately for each fluorescent and non-fluorescent polyp within the same colony and estimated their average numbers (Fig. 6.3.2). I chose this calculation method because the first generation of transgenic animals have mosaic expression pattern and the transgene is not expressed in all polyps or in all cells, in which Actin1 promoter is active. As a result transgenic colonies are a mixture of fluorescent and non-fluorescent individuals. Calculations were performed for 19 colonies in total.

<table>
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<tr>
<th>Colony no.</th>
<th>Numbers of tentacles in fluorescent polyps</th>
<th>Numbers of tentacles in non-fluorescent polyps</th>
<th>Average numbers of tentacles in fluorescent polyps</th>
<th>Average numbers of tentacles in non-fluorescent polyps</th>
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<td>19</td>
<td>23</td>
<td>10,10</td>
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Average number of tentacles 14.28421053 9.319444

Average increase % 53.2731979

P value 0.000

Fig. 6.3.2. Average tentacles’ numbers in transgenic polyps versus non-fluorescent polyps.
The increase in the tentacles’ number ranged from 15% up to 130% more in transgenic animals. The average tentacles’ number in non-fluorescent polyps was 9.3, and in fluorescent polyps the average was 14.3. Taken together, the average increase in the number of tentacles was 53.3% in transgenic animals, in comparison to non-transgenic individuals (Fig. 6.3.3). For calculation of tentacle numbers, a statistical significance (P value) was evaluated by the Two-Sample T-Test with the use of Minitab software.

Fig. 6.3.3. Nanos2 ectopic expression phenotype. Transgenic animals (right) grew up to twice more tentacles than non-transgenic polyps (left). View on the animals is from the top. Scale bar represents 100 µm.

Nanos2 ectopic expression caused also formation of hyperplastic stolons early post metamorphosis (Fig. 6.3.4, arrows). Stolons are the aboral structures, which attach an animal to the surface of the hermit crabs shell and also serve as gastrovascular canals to transport food particles between the members of one colony. In *Hydractinia* colonies hyperplastic stolons are produced as a result of allogeneic rejections, when two colonies fight for the space on the same surface. In such situation, large numbers of nematocytes are produced to eliminate the foreign tissues. An accumulation of the stinging cells is observed within the hyperplastic stolons and their appearance can be interdependant. However, transgenic animals were not exposed to a direct contact with other colonies. In some cases, hyperplastic stolons can be produced without allogeneic contact and might either be a genetic mutation (Buss et al., 1985), or simply due to stress. In the absence of obvious stress factors, this phenotype was probably induced by the Nanos2 overexpression, which caused the accumulation of stinging cells within the stolonal tissues. Transgenic animals very often developed these hyperplastic stolons and in some cases this phenotypes appeared together with the growth of the supernumerary tentacles (Fig. 6.3.4).
Nanos2 ectopic expression also caused generation of much stronger phenotypes. Some polyps developed not only supernumerary but also ectopic tentacles (Fig. 6.3.5). Wild type feeding polyps normally have a smooth body column with the head surrounded by a ring of 9 to 11 tentacles. In few transgenic animals these tentacles were growing all over the body column and their growth was not restricted only to the head region, like in case of wild type animals.

Fig. 6.3.4. Nanos2 ectopic expression phenotypes. Transgenic animal (right) developed both hyperplastic stolons (arrows) and supernumerary tentacles. Scale bar represents 100 µm.

Fig. 6.3.5. Nanos2 ectopic expression phenotype. Transgenic animal (right) developed ectopic tentacles (arrows), which were growing all over the body column. Scale bar represents 100 µm.
In the most severe cases, transgenic animals generated enormous amounts of ectopic tentacles and very reduced or no stolons (Fig. 6.3.6). It is possible that the excessive growth of tentacles could inhibit formation of the stolons. In *Hydractinia*, the optimal distance between the head organizer and an aboral end of the polyp need to be generated to allow the growth of the stolons (i.e. aboral structures). Some of the transgenic animals after few days turned into a ball of tentacles. These oralized polyps had functional mouths and were able to feed, however could not set up the colony as they did not possessed stolons (Fig. 6.3.6 c-d).

Fig. 6.3.6. *Nanos2* ectopic expression phenotypes. (a)-(b) Transgenic animals developed numerous ectopic tentacles at the expense of stolons. (c)-(d) Transgenic animals underwent complete oralization.
I calculated the numbers of transgenic animals which developed two different phenotypes post metamorphosis. The first group of animals had no stolons but normal tentacles and the second group had compromised stolonal development and grew ectopic tentacles (Fig. 6.3.7). Statistical analysis showed that only 22% of transgenic animals developed normally, in contrast to 83% of animals injected with the control GFP reporter construct. 11% of transgenic animals had no stolons but were normal otherwise and this number for control experiment reached 6%. However, almost 66% of Nanos2 overexpressing polyps developed the second phenotype, i.e. ectopic tentacles and none or delayed stolons. This phenotype was observed only for 11% of the control animals (Fig. 6.3.7). Interestingly, few animals belonging to the first group developed phenotypes of the second group overtime.

<table>
<thead>
<tr>
<th>GFP reporter expression under Actin1 promoter (control experiment)</th>
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<tbody>
<tr>
<td>No. of animals</td>
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</tr>
<tr>
<td>17</td>
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<tr>
<td>after next 5 days</td>
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<tr>
<td>17</td>
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<tr>
<td>Nanos2 ectopic expression</td>
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<tr>
<td>No. of animals</td>
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<tr>
<td>12</td>
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<tr>
<td>after next 5 days</td>
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<td>9</td>
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</table>

Fig. 6.3.7. Phenotypic variations of transgenic animals post metamorphosis.

I also looked at the effect of Nanos2 ectopic expression on the wellbeing of the adult colonies. Unfortunately, transgenic animals which developed very severe phenotypes did not survive for a very long time, or were not able to form a colony, due to the lack of stolons. As a result only animals, which had mild phenotypes (e.g. supernumerary tentacles), could be cultured longer. Surprisingly, Nanos2 ectopic expression did not cause any significant abnormalities in the growth and morphology of the adult colonies. The new budding polyps did not develop ectopic or supernumerary tentacles. The only visible phenotype was the growth of the large amounts of hyperplastic stolons, which also occurs periodically in wild type animals. Hyperplastic stolons in primary polyps, however, were only observed in transgenic animals. For some animals the increase in the stolonal tissue was so drastic that it inhibited formation of new polyps, causing premature death of the colony.
(Fig.6.3.8). However, I cannot exclude the possibility that the massive increase of the hyperplastic stolons was stress-induced, for example by infection.

![Image](image_url)

Fig. 6.3.8. Nanos2 ectopic expression phenotype in the adult colonies. (a) Wild type colony. (b) Transgenic colony growing large amounts of hyperplastic stolons. (c) Higher magnification of hyperplastic stolons in transgenic animals. View on the colonies is from the top.

The GFP reporter expression was traced throughout the individuals’ development, from the early embryos to the adult colonies (Fig. 6.3.9). Actin1 promoter, under which Nanos2 was ectopically expressed, was ubiquitous during early embryogenesis but in the larvae, as well as post metamorphosis, it appeared to be epithelial-specific (Künzel et al., 2010; Millane et al., 2011). Due to the nature of the promoter used, ectopic expression of Nanos2 could affect all populations of different cell types in the embryo, but only the epithelio-muscular cells in other developmental stages. The ubiquitous expression in the embryo most likely led to the development of severe phenotypes in the young animals. However, Nanos2 expression in the adult transgenic colonies did not cause any strong phenotypic changes, suggesting that this gene has no significant effect in epithelial cells, where it is not expressed naturally.

First generation of transgenic animals results in the mosaic expression and in such case only a fraction of cell expresses the transgene. The uniform expression can only be obtained following the sexual reproduction. Unfortunately, I did not manage to obtain the offspring from transgenic animals. After a year of culturing, they still
did not reach sexual maturity and unluckily due to unknown infection all transgenic colonies died.

Fig. 6.3.9. Nanos2-GFP expression under the Actin1 promoter in adult colonies. (a) Expression in the epidermis of the adult polyps. (b) Expression in the gastrodermis of the adult polyp. (c) Expression in the epithelial cells of tentacles. (d) Expression in the epidermis and gastrodermis of the head. (e) Expression in the gastrodermis of the stolons. View on all the animals is from the top.
6.4. Analysis of *Nanos2* ectopic expression phenotypes

*Nanos2* ectopic expression in *Hydractinia* promoted formation of ectopic tentacles which are oral structures, normally restricted to the head region. Interestingly, similar oralization phenotype was shown already for this animal model as an effect of global Wnt activation (Duffy et al., 2010). A *Hydractinia* polyp, exposed to azakenpaullone treatment, which upregulates Wnt targets, do not form stolons but instead form numerous ectopic tentacles. Some animals can even undergo complete oralization. This suggests that the oralization phenotype, caused by *Nanos2* ectopic expression, may be related with altered Wnt signaling. To test this hypothesis, Wnt3 probes were prepared and *in situ* hybridization performed on transgenic animals according to protocol 2.1.10. Interestingly, Wnt3 expression was not altered and no additional staining was observed within or around the ectopic tentacles, than normal, restricted to the tip of the hypostome (Fig. 6.4.1a-b). If oralization was caused by the general Wnt upregulation, Wnt3 expression would be ubiquitous all over the polyp, like in case of azakenpaullone-treated animals. This is because Wnt3 is a target of the canonical Wnt signaling. However, *Nanos2*-mediated oralization did not change Wnt3 expression pattern. Based on the *in situ* hybridization results, I conclude that in transgenic animals this particular cell signaling pathway has not been affected. Noteworthy, Wnt-independent oralization has never been observed in *Hydractinia* before.

Interestingly, when I performed *Nanos2* *in situ* hybridization on azakenpaullone-treated primary polyps, I saw that upregulation of Wnt signaling by this chemical compound significantly increased *Nanos2* expression (Fig. 6.4.1 d-e). Wnt signaling is known to promote cellular proliferation and these results would suggest that *Nanos2* is downstream of Wnt signaling and is associated with general “stemness” or proliferation.
Fig. 6.4.1. Results of Wnt3 in situ hybridization performed on the transgenic animals. (a) In transgenic animals Wnt3 expression was normal. Wnt3 was found only in the tip of the hypostome and was not detected around the ectopic tentacles. (b) Wnt3 expression in the wild type polyp is restricted only to the tip of the hypostome. (c) Control experiment: polyp did not stain with the Wnt3 sense probe. (d) Azakenpaulone-treated (Wnt-activated) animal showed upregulated Nanos2 expression in comparison to the control experiment (e).

The results of Wnt3 in situ hybridization eliminated the possibility that the upregulation of Wnt signaling caused the formation of ectopic tentacles in transgenic animals. To find out what induces development of this interesting phenotype, I performed additional gene expression analyses to identify the possible abnormalities
in the composition or distribution of different cell types during early development of transgenic animals.

First, I performed immunohistochemical analysis of two day old transgenic larvae with the use of Anti-NCol1 antibody staining (Fig. 6.4.2a). This antibody stains developing nematocysts capsules of cnidarian-specific stinging cells. The Anti-NCol1 antibodies were a kind gift from Dr Suat Özbek (University of Heidelberg) (Adamczyk et al., 2010). They were raised against Hydra NCol1 and due to the high sequence similarities between all cnidarian NCol1 proteins, they cross-reacted with the Hydractinia Ncol1. Antibody staining was performed according to protocol 2.2.1. Interestingly, anti-NCol1 Ab IHC staining performed on transgenic larvae revealed that Nanos2 overexpressing animals have over twice more nematoblasts than the wild type animals (Fig. 6.4.2a-b). This finding was very interesting as Nanos was shown to affect only the numbers of germ cells so far, but never any other cell types. The calculations of NCol1⁺ nematoblasts were performed for three independent experiments. For each experiment, statistical significance (P value) was calculated by the Two-Sample T-Test with the use of Minitab software. The increase in the numbers of nematoblasts in transgenic larvae was ranging from 57% to 183% more than in wild type larvae (Fig. 6.4.2, table).

Animals could gain extra nematoblasts by two possible ways, transdifferentiation or increased proliferation of their precursor stem cells. To find out which scenario was more likely, I performed EdU staining on Nanos2 transgenic larvae (Fig. 6.4.2a). Cells undergoing mitosis were detected with Click-iT® EdU Alexa Fluor® 488 HCS Assay kit according to protocol 2.2.3 and calculated for transgenic and wild type animals. Interestingly, transgenic larvae had over 90% more proliferating cells than the wild type larvae (Fig. 6.4.2b). EdU⁺ nuclei were only rarely observed within close distance from NCol1⁺ developing nematocysts, suggesting that Ncol1⁺ nematoblasts are post mitotic. It is possible that these proliferating cells were actually committed stem cells which contributed to the increase in the nematoblasts’ number. However, no clear double labeling could be performed because EdU stains nuclei, whereas Ncol1 is present only in the developing capsules. Noteworthy, these data are consistent with the Ncol1-Nanos2 double in situ hybridization (chapter 4.6) that showed non-overlapping expression domains between the two genes.

In order to monitor the numbers of mature stinging cells I stained transgenic larvae with DAPI according to protocol 2.2.4 (Fig. 6.4.2a). DAPI-based nematocysts’ staining was developed for Hydra (Szczepanek et al., 2002) and
appeared to work very well in *Hydractinia* as well. Interestingly, the numbers of nematocytes decreased by 45% in transgenic animals, suggesting that *Nanos*2 ectopic expression promotes proliferation of nematoblasts but prevents their differentiation (Fig. 6.4.2b). These results are consistent with the general role of Nanos genes in germ cells, where Nanos maintains the proliferation of PGCs and prevents them from premature differentiation and acquiring somatic fate to ensure their proper development.

<table>
<thead>
<tr>
<th>b)</th>
<th>Wild type</th>
<th>Transgenic</th>
<th>% increase</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-NCol1 Ab – stained larvae</td>
<td>37.5 (n=22)</td>
<td>106.0 (n=6)</td>
<td>182.7</td>
<td>0.005</td>
</tr>
<tr>
<td>37.5 (n=22)</td>
<td>78.7 (n=19)</td>
<td>110</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>52.8 (n=11)</td>
<td>82.7 (n=9)</td>
<td>56.7</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>EdU+ cells in larvae</td>
<td>67.19 (n=19)</td>
<td>128.0 (n=18)</td>
<td>90.5</td>
<td>0.000</td>
</tr>
<tr>
<td>Mature stinging cells in larvae</td>
<td>2-day old 215.2 (n=11)</td>
<td>2-day old 117.6 (n=5)</td>
<td>45.35</td>
<td>0.026</td>
</tr>
<tr>
<td>3-day old 254.6 (n=12)</td>
<td>3-day old 234 (n=6)</td>
<td>8.1</td>
<td>0.690</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6.4.2. (a) Transgenic larvae were compared with the wild type animals (WT) in terms of nematoblasts’ number (Anti-NCol1 Ab), proliferation (EdU) and the numbers of mature stinging cells (DAPI). (b) Summary of above histological analysis. n represents the number of animals used in any particular experiment.

I also performed *RFamide* and *Ash* in situ hybridizations on transgenic two-day old larvae in order to look at the numbers of mature neurons and neuronal precursors, respectively (Fig. 6.4.3). Surprisingly, *Nanos*2 ectopically expressing larvae had over 80% less of both *RFamide*+ and *Ash*+ cells than the wild type animals.
The summary of the numbers of NCol1+ nematoblasts and RFamide+ neurons in the transgenic larvae in comparison to wild type larvae is shown in Fig. 6.4.3c. Interestingly, the more nematoblasts were formed, the less neurons were present in transgenic animals. These proportions were quite constant and suggested that both cell types can differentiate from a single common precursor stem cell.

Fig. 6.4.3. (a) Transgenic larvae were compared with the wild type animals (WT) in terms of the numbers of neuronal precursors (Ash), and mature neurons (RFamide). (b) Summary of above histological analysis. n represents the number of animals used in any particular experiment. (c) Summary of the numbers of NCol1+ nematoblasts and RFamide+ neurons in the transgenic versus wild type larvae. Abbreviations: wild type (WT), 1-day old larvae (1d), 2-day old larvae (2d).

I also looked at the expression of RFamide and NCol1 in transgenic animals post metamorphosis (Fig. 6.4.4a). As in case of the larvae, anti-NCol1 immunohistochemical staining detected more nematoblasts in the Nanos2 overexpressing polyps. However, the increase was much smaller than in earlier developmental stages and reached only 26%. The similar increase was observed in the number of cells undergoing mitosis (~19%) (Fig. 6.4.4b). Interestingly, transgenic primary polyps completely lost RFamide+ neurons, which are primarily present around the hypostome of young polyps (Fig. 6.4.4).
Fig. 6.4.4. (a) Transgenic polyps were compared with the wild type animals (WT) in terms of the numbers of nematoblasts (Anti-NCol1 Ab), mature neurons (RFamide) and proliferation (EdU). (b) Summary of above histological analysis. n represents the number of animals used in any particular experiment.
6.5. Summary

I studied the effect of Nanos2 ectopic expression on Hydractinia development by microinjection of an overexpression vector into fertilized eggs. For this purpose I have created the overexpression construct in which Nanos2 coding sequence was cloned upstream of the GFP reporter sequence and downstream of the Actin$^{l}$ promoter. Due to the nature of the promoter, in the early development Nanos2 was ubiquitously expressed and in later developmental stages expression became epithelial-specific.

Transgenic animals developed a range of phenotypic defects post metamorphosis. The mild phenotypes included supernumerary tentacles and hyperplastic stolons, whereas in severe cases polyps produced multiple ectopic tentacles and had no stolons. Few animals even underwent complete oralization like in the case of global Wnt activation. However, Wnt3 in situ hybridization results revealed that this signaling pathway was not deregulated in transgenic animals.

To find out what induces development of these phenotypes, I performed a series of marker gene expression analyses to identify the possible abnormalities in the composition or distribution of different cell types in transgenic larvae. Interestingly, Nanos2 ectopic expression appeared to strongly affect the numbers of neural cell types during embryogenesis, when the Actin$^{l}$ promoter drove Nanos2 expression in different populations of cell types. I observed a decrease in the numbers of RFamide$^{+}$ and Ash$^{+}$ neurons by over 80% in the transgenic larvae. Moreover, RFamide expression was knockdown completely post metamorphosis. At the same time the numbers of NColl$^{+}$ nematoblasts increased even up to three times in transgenic larvae and surprisingly the numbers of mature stinging cells decreased by 45%. These results suggest that Nanos2 ectopic expression promotes proliferation of nematoblasts but prevents their differentiation. This observation would be consistent with the general role of Nanos in germ cells development in other species, where Nanos is known to maintain PGCs proliferation and inhibit their premature differentiation to ensure their proper development.
7. **Nanos2 knockdown**

**7.1. Introduction**

Gene knockdown and gene overexpression represent two core methods of studying the function of a gene in animal development. Gene knockdown is based on the downregulation of the expression of one or more genes within the organism or single cells. It does not involve complete gene knockout, which role is to replace wild type alleles with the mutant ones, leading to the synthesis of non-functional proteins (Gilbert, 1997). The effect of the gene knockout is permanent and for many genes is lethal when performed on the early embryos. From this reason developmental biologists very often use the knockdown method to determine the function of a gene. However, for some genes both techniques are equally good and can produce similar phenotypes. Gene knockdown techniques target gene transcripts and degrade them or block their translation. They are usually performed by introducing short RNA oligonucleotides with a sequence complementary to target mRNAs. For example, injection or transfection of large amounts of antisense RNA into an organism or cells, which contain the endogenous sense transcripts of the same gene, results in a formation of the double-stranded nucleic acids (Gilbert, 1997). Such dsRNAs are degraded by the enzymes within the cell cytoplasm, resulting in the deletion of a message for protein synthesis.

Another type of sequence-specific targeting of mRNA that leads to inhibition of its translation can be mediated by morpholino antisense oligomers. These synthetic molecules contain six-membered morpholino rings instead of five-membered ribose sugars. Such modification makes them resistant for a nuclease-mediated cleavage. After injection these morpholino oligomers hybridize with the target genes’ transcripts and block their translation or splicing process. This way they can be designed to either bind the translation initiation start codon or the exon-intron boundary.

RNA interference (RNAi) represents another well-established method for targeting genes’ function (Gilbert, 1997). It is based on the introduction of a double-stranded RNA (dsRNA), containing the same sequence as an endogenous mRNA which is meant to be silenced. RNAi was established for many experimental models, from plants and fungi, to cnidarians, arthropods and mammals. It was discovered for the first time in 1998 in *C. elegans* (Fire et al., 1998). Injection of dsRNA into this nematode resulted in a much more efficient gene silencing than the injection of
I have applied both morpholino and dsRNA to downregulate Nanos2 expression in order to study the role of this gene in Hydractinia development.

Fig. 7.1.1. The double-stranded RNA-mediated gene silencing. Introduced dsRNA is recognized and bound by the Dicer complex. Dicer cleaves its sequence into short siRNA fragments (silencing RNA). siRNA are unwound and the antisense strands are bound by the RISC/Argonaute complex. Formed complex binds to the complementary endogenous sense transcript and mediates its degradation (Gilbert, 1997).
7.2. *Nanos2* morpholino-mediated knockdown

*Nanos2* knockdown was performed in *Hydractinia* with the use of two methods, morpholino injection and RNA interference. RNAi technique allows studying gene knockdown during all stages of *Hydractinia* development and during regeneration. Microinjection of morpholino allows studying gene knockdown only in early development. It is possible to order fluorescent and non-fluorescent gene-specific morpholino directed either against the start codon to block mRNA translation or against the intron-exon boundary to block its splicing. I ordered the fluorescent *Nanos2* morpholino, directed against the translation initiation start (Fig. 7.2.1), from Gene Tools, LLC. The sequence of the gene-specific morpholino was “GCATAACATCAGGTCAACATCTT”. For the negative control the fluorescent Standard Control oligos from Gene Tools, LLC were used. The control Morpholino oligo targets a human beta-globin intron mutation that causes beta-thalassemia. This oligo has not been reported to have other targets or generate any phenotypes in any known test system except human beta-thalassemic hematopoetic cells. The sequence of the control morpholino is “CCTTCTACCTAGTTACAATTTATA”.

Gene-specific and control morpholino injection was performed at the concentrations of 0.25 mM and 0.5 mM. Injection of the higher concentration of morpholino resulted in unspecific effects, manifested by the defects in early development and downregulation of all examined neural markers, both in the *Nanos2* morpholino injected and control injected larvae in comparison to wild type.
animals. Injection was performed into 1-2 cell stage *Hydractinia* embryos according to the protocol 2.3.1. The fluorescence was traced using microscopy and injected embryos were separated from non-fluorescent ones soon after injection (Fig. 7.2.2).

![Fig. 7.2.2. Fluorescent Nanos2 morpholino injection. (a) Two-cell stage embryo. (b) Four-cell stage embryo. (c) Morula. (d) Primary polyp. Scale bars represent 200 µm.](Image)

Two-day old *Nanos2* morpholino-injected larvae were analyzed for the number of nematoblasts by the nematocyst-specific anti-NCol1 antibody staining according to protocol 2.2.1 and by the *Ncol1* in situ hybridization performed according to protocol 2.1.10. Conversely to *Nanos2* ectopic expression, gene-specific morpholino injection resulted in a decrease in the number of *Ncol1* cells by an average of 46% in comparison to control morpholino-injected animals (Fig. 7.2.3 and Fig. 7.2.4).
Fig. 7.2.3. *Nanos2* morpholino-injected larvae were compared with the control animals in terms of nematoblasts’ number (Anti-NCol1 Ab staining and *Ncol1* in situ hybridization), the numbers of mature stinging cells (DAPI) and the number of *RFamide* neurons.
In order to monitor the numbers of mature stinging cells I stained transgenic larvae with the nematocyst-specific DAPI staining according to protocol 2.2.4 (Fig. 7.2.3 and Fig. 7.2.4). Three independent experiments revealed that the numbers of nematocytes decreased by 27 to 49% in Nanos2 morpholino-injected larvae in comparison to control experiment. For all calculation of cell numbers the statistical significance (P value) was evaluated by the Two-Sample T-Test with the use of Minitab software.

Interestingly, observed decrease in nematocytes’ number was also observed after Nanos2 ectopic expression. However, in Nanos2 knockdown the reduction of the mature stinging cells’ number resulted from the decrease in the number of their progenitors, unlike in case of Nanos2 ectopic expression, where nematoblasts were most likely prevented from differentiation (Fig. 7.2.3).

Conversely to the ectopic expression experiments, Nanos2 - morpholino mediated translational blockage resulted in an increase in the number of RFamide⁺ neurons by over 35% in comparison to control larvae (Fig. 7.2.3 and Fig. 7.2.4).

<table>
<thead>
<tr>
<th>Larvae</th>
<th>Control morpholino</th>
<th>Nanos2 morpholino</th>
<th>% increase</th>
<th>% decrease</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ncoll⁺ nematoblasts in larvae</strong></td>
<td>O.25mM morpholino: 127.1 (n=6)</td>
<td>O.25mM morpholino: 68.3 (n=6)</td>
<td>46%</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td><strong>DAPI⁺ mature stinging cells in larvae</strong></td>
<td>O.5mM morpholino: 110.1 (n=15)</td>
<td>O.5mM morpholino: 56.1 (n=18)</td>
<td>49</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O.25mM morpholino: 109.3 (n=18)</td>
<td>O.25mM morpholino: 68.5 (n=11)</td>
<td>37.3</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O.25mM morpholino: 139.8 (n=16)</td>
<td>O.25mM morpholino: 102.1 (n=20)</td>
<td>27</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td><strong>RFamide⁺ neurons in larvae</strong></td>
<td>O.5mM morpholino: 28.86 (n=7)</td>
<td>O.5mM morpholino: 39.2 (n=16)</td>
<td>35.8</td>
<td>0.028</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7.2.4. Summary of the analysis of the Nanos2-morpholino mediated knockdown phenotypes on larvae. n represents the number of animals used in any particular experiment.
I metamorphosed morpholino-injected animals to look at the effect of *Nanos2* downregulation post metamorphosis. Conversely to *Nanos2* ectopic expression, morpholino-mediated knockdown decreased the number of tentacles up to 77%. Primary polyps also had 50% less mature stinging cells than the control morpholino-injected animals (Fig. 7.2.5).

<table>
<thead>
<tr>
<th></th>
<th>Control morpholino</th>
<th><em>Nanos2</em> morpholino</th>
<th>% decrease</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Numbers of tentacles in primary polyps</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.087 (n=23)</td>
<td>6.522 (n=23)</td>
<td>28.2</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>7.59 (n=22)</td>
<td>6.29 (n=24)</td>
<td>17.1</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>5.875 (n=32)</td>
<td>1.36 (n=25)</td>
<td>76.85</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td><strong>DAPI+ mature stinging cells in primary polyps</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5mM morpholino: 133.2 (n=29)</td>
<td>0.5mM morpholino: 67 (n=29)</td>
<td>49.7</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7.2.5. *Nanos2* morpholino-knockdown primary polyps were compared with the control animals in terms of tentacles’ number and the numbers of mature stinging cells (DAPI). Scale bars represent 200 µm.
7.3. *Nanos2* dsRNA-mediated knockdown

Gene knockdown can be also performed by RNA Interference (RNAi). In *Hydractinia* this method can be conducted by soaking animals in a dsRNA solution. This technique allows studying gene knockdown during most stages of *Hydractinia* development and during regeneration. However, morpholino-mediated translational blockage is much more efficient for performing gene knockdown during embryogenesis. I applied RNAi method to study the effect of *Nanos2* downregulation during and post metamorphosis.

I synthesized dsRNA of 200 bp in size designed against the unconserved region within the 5'end of the *Nanos2* coding sequence, outside the Zn-finger domain. As a template for the transcription reaction, I used a PCR product amplified according to protocol 2.1.13. Control dsRNA was prepared from 150 bp fragment amplified from the pGEM-T vector for the region unlikely to exist within the *Hydractinia* transcriptome. dsRNA was synthesized according to protocol 2.1.6. RNAi was performed by soaking two-day old larvae for 48 hours in a 100 µg/µl dsRNA solution in sea water, followed by the induction of metamorphosis. The knockdown phenotype has been studied two - three days post metamorphosis.

I performed the analysis of *Nanos2* knockdown phenotypes and categorized animals in four distinct groups: animals which developed neither tentacles nor stolons, polyps which had either stolons or tentacles, and the ones whose development was normal (Fig. 7.3.1). For this experiment I made three separate control treatments, pGEM dsRNA and two seawater controls (SW). About 26% of *Nanos2* knockdown primary polyps were ball-shaped and had neither tentacles nor stolons and this number for pGEM control was 10% and between 9% and 14% for sea water controls (SW) (Fig. 7.3.1). 21.7% of knockdown animals had no tentacles in comparison to 3.5 - 4.5% for SW. In pGEM control none polyps developed such a phenotype. Only 8.6% of knockdown animals did not develop stolons and the statistics for all control treatments were similar in this phenotypic group. 43.5% of *Nanos2* knockdown primary polyps developed normally and this number was above 75% for all control experiments (Fig. 7.3.1). Interestingly, *Nanos2* dsRNA affected mostly the tentacle growth, which is an opposite effect to ectopic expression phenotype, supporting specificity of both experiments.
Fig. 7.3.1. Phenotypic variations in Nanos2 dsRNA-mediated knockdown post metamorphosis. SW is an abbreviation for the control treatment in sea water.

I also performed EdU staining on Nanos2 knockdown primary polyps (Fig. 7.3.2). Cells undergoing mitosis were detected with the Click-iT® EdU Alexa Fluor® 488 HCS Assay kit according to protocol 2.2.3. Nanos2 dsRNA-mediated downregulation resulted in the reduction of the number of proliferating cells by up to 40%.

Fig. 7.3.2. Nanos2 dsRNA-mediated knockdown primary polyps were compared with the control animals in terms of the number of proliferating cells (EdU). SW is an abbreviation for the control treatment in sea water. Scale bars represent 200 µm.
7.4. DAPT treatment and Notch signaling

The phenotype observed after Nanos2 ectopic expression and knockdown suggested that Nanos2 may affect the number of nematocytes by altering Notch signaling. Notch is a transmembrane receptor protein, which controls cellular proliferation, differentiation and apoptotic programs to regulate neurogenesis and dictate the cell fates (Artavanis-Tsakonas et al., 1999; Lai, 2004; Andersson et al., 2011). Notch was found to play many other important roles in animal development and the components of Notch signaling pathway seem to be conserved between invertebrates and vertebrates. Importantly, it was also found to have a role in the specification of neural cell types in many experimental models including cnidarians (KAGEYAMA and Ohtsuka, 1999; Gaiano and Fishell, 2002; Käsbauer et al., 2007; Marlow et al., 2011). The canonical Notch signaling pathway starts with the binding of a Notch ligand, present on a signaling cell, to the Notch receptor on the responding cell. This induces Notch proteolytic cleavage mediated by a metalloprotease and the \( \gamma \)-secretase complex (Fig. 7.4.1) (Schroeter et al., 1998). The biologically active product of this cleavage, Notch Intracellular Domain (NID), is transported from the plasma membrane to the nucleus, where it interacts with the members of the CSL family of DNA-binding transcription factors (Drosophila Suppressor of hairless (Su(H), vertebrate CBF1 and nematode LAG-1). Once bound by NID, these transcription factors activate expression of Notch target genes, like Myc or Hairy enhancer of split (Hes) (Fig. 7.4.1)(Käsbauer et al., 2007). Hes suppresses expression of proneural genes, like Ash or Neurogenin (Ngn), inhibiting neural development. Numb is a known Notch antagonist and mediates its ubiquitination leading to subsequent endocytosis and degradation. Cnidarian genomes seem to contain most of the genes belonging to Notch pathway. Hydra vulgaris putative homologs are indicated in brackets (e.g. HvHES) on the Fig. 7.4.1 (Käsbauer et al., 2007).

DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyler ester) is a \( \gamma \)-secretase substrate, which inhibits Notch signaling by inhibiting \( \gamma \)-secretase-mediated Notch cleavage (Geling et al., 2002). This chemical is commonly used in Notch knockdown studies. Interestingly, in Nematostella vectensis DAPT-mediated blocking of the Notch signaling pathway decreased the number of stinging cells and increased the number of RFamide\(^+\) neurons (Marlow et al., 2011). I observed similar effect after Nanos2 knockdown, and an opposite effect after Nanos2 ectopic expression. Studies performed on Nematostella also showed that DAPT treatment reduces the expression of Notch target genes (Hes) and upregulates expression of Ash homolog, what is consistent with an expected Notch inhibition effect. In Hydra, DAPT treatment caused post-mitotic nematocyte differentiation
defects, but nerve cell differentiation proceeded normally. Also early female germ cell differentiation was observed to be inhibited before exit from mitosis (Käsbauer et al., 2007).

To study possible links between Nanos2 and Notch signaling, I performed DAPT treatment during Hydractinia embryogenesis to block endogenous Notch signaling. DAPT treatment was performed by soaking animals in DAPT solution. DAPT (Sigma, cat no. D5942) was dissolved in DMSO to make a stock solution of 10 mM. Working solutions of 10 nM, 100 nM, 1 µM, 2.5 µM, 5 µM and 10 µM were prepared by diluting the stock in seawater. As a control treatment DMSO solutions were prepared of 0.0001%, 0.001%, 0.01%, 0.025%, 0.05%, and 0.1% in seawater, respectively. DAPT-treated animals were examined in terms of RFamide, Ncol1, Ash, Hes1 and Hes2 expression, but also the number of mature stinging cells was counted. 48 hour long treatment performed on 2 hpf embryos with a high concentration of DAPT caused a significant decrease in the number of nematoblasts (Ncol1+ cells) and a complete elimination of mature stinging cells, verified by DAPI staining (Fig. 7.4.2).

DAPT treatment started at 48 hpf and performed for 24h had milder effect on nematogenesis and resulted only in a 30% decrease in the number of stinging cells (Fig. 7.4.3). For all calculation of cell numbers the statistical significance (P value) was evaluated by the Two-Sample T-Test with the use of Minitab software.
Fig. 7.4.2. 48h long DAPT-treated larvae were compared with the control DMSO-treated animals in terms of numbers of Ncol1+ nematoblasts and mature stinging cells (DAPI).

<table>
<thead>
<tr>
<th></th>
<th>DMSO control</th>
<th>DAPT treatment</th>
<th>% decrease</th>
<th>P value</th>
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</thead>
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<tr>
<td>DAPT+ mature stinging cells in larvae</td>
<td>0.05% DMSO 337 (n=17)</td>
<td>5µM DAPT 225.9 (n=12)</td>
<td>33</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>0.025% DMSO 337 (n=17)</td>
<td>2.5µM DAPT 237.1 (n=21)</td>
<td>29.6</td>
<td>0.010</td>
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Fig. 7.4.3. Statistics on the numbers of mature stinging cells (DAPI) in the 72 hours old larvae treated for 24h with DAPT solution.
DAPT-treated animals were also analyzed for RFamide and Ash expression by in situ hybridization (Fig. 7.4.4). The number of RFamide+ neurons was not significantly changed after treatment, which is an opposite result to studies performed on Nematostella (Marlow et al., 2011). Also Ash expression was downregulated after treatment with all possible concentrations of DAPT, from 100 nM to 10 μM. DAPT concentration below 100 nM showed no difference in the number of Ash+ cells between the treated and the control animals. These results are contrary to studies performed on Nematostella, for which both Ash and RFamide expression was upregulated after DAPT treatment (Marlow et al., 2011).

I also identified putative two Hes genes in Hydractinia draft genome database. Hes are classical Notch targets and are downregulated in DAPT-mediated inhibition of Notch signaling. Expression of both genes was studied by qRT-PCR and, contrary to my expectations, no significant change in the mRNA level was observed in DAPT-treated animals compared to DMSO control.

Fig. 7.4.4. DAPT-treated larvae were compared with the control DMSO-treated animals in terms of the numbers of RFamide+ and Ash+ neurons.
7.5. Summary

Nanos2 role in Hydractinia development was examined by morpholino and dsRNA-mediated knockdown. Morpholino injection allowed studying the effect of Nanos2 downregulation during early embryogenesis. The experiments resulted in a decrease in the numbers of nematoblasts by 46% and mature stinging cells by over 30%. The decrease in the number of mature stinging cells resulted from the decrease in the number of their progenitors. These results are opposite to Nanos2 ectopic expression, confirming the specificity of both studies. The number of RFamide\(^+\) neurons had increased by 35% in knockdown animals. Nanos2 morpholino-injected primary polyps had up to 76% less tentacles and also had 50% less mature stinging cells than control injected animals.

Double strand RNA-mediated interference (RNAi) caused reduction of the tentacle numbers in primary polyps, similarly to morpholino-injected animals. EdU staining revealed that Nanos2 knockdown reduced the number of proliferating cells up to 40% in comparison to control pGEM treatment.

To study possible links between Hydractinia Nanos2 and Notch signaling, I performed DAPT treatment during early embryogenesis to block endogenous Notch. However, the treatment did not fully support the published data on Notch inhibition in the cnidarians Hydra and Nematostella. The decrease in the number of nematoblasts and nematocytes after DAPT treatment was the only result consistent with the studies performed on Nematostella. Expression of RFamide, Ash, Hes1 and Hes2 following DAPT treatment indicated that these genes are not necessarily Notch targets in Hydractinia. From this reason, determining if Nanos2 ectopic expression or knockdown - mediated phenotypes are related with this signaling pathway was not possible.
8. Discussion

In my project I studied the role of the *Nanos2* gene in the nervous system of a cnidarian *Hydractinia echinata*. Nanos functions in germ cells development and proliferation seem to be conserved among metazoans (Kobayashi et al., 1996; Parisi and Lin, 2000; Tsuda et al., 2003), but its role in the nervous system has not been well studied so far (Haraguchi et al., 2003; Ye et al., 2004; Muraro et al., 2008). Using *Hydractinia* as an experimental model gave me an opportunity to look at the effect of altered *Nanos2* expression on the well-being of a whole organism, not only on particular cells. Studies performed on a phylogenetically basal and morphologically simple metazoan model can reveal not only new functions for genes, which remain cryptic in other systems, but also their ancestral role.

Nanos belongs to a family of Zn-finger proteins. It acts as an RNA-binding protein, repressing translation of its target mRNAs (Parisi and Lin, 2000). Nanos is known to be required for germ cell development (Tsuda et al., 2003). Specifically, it maintains Primordial Germ Cells (PGCs) self-renewal (Wang and Lin, 2004), prevents them from premature differentiation, apoptosis and acquiring a somatic fate (Kobayashi et al., 1996; Hayashi et al., 2004). Nanos was also found to be required for migration of PGCs into the gonad and knockout of *nanos* and *Nanos2-3* results in sterility of both sexes in *Drosophila* and mouse, respectively (Kobayashi et al., 1996; Tsuda et al., 2003).

In addition to its role in germ cells, Nanos was also well characterized as a posterior determinant in *Drosophila melanogaster*. It regulates abdomen formation by blocking translation of *hunchback* (*hb*) mRNA (Wang and Lehmann, 1991). However, a similar role for Nanos in AP patterning has not been described outside of arthropods and no *hb* homolog has been identified in animals other than insects. Possibly, Nanos-mediated *hunchback* translational blockage is an arthropod innovation (Pinnell et al., 2006).

Nanos was also found to be expressed in the nervous system of many animals; however, this role for Nanos has not been well studied so far. Mouse *Nanos1* is expressed in the Central Nervous System (CNS) but its knockout in mice does not cause any significant developmental or behavioral defects (Haraguchi et al., 2003). It is possible that the role of Nanos1 in the mouse neural development has been overlooked. Conversely, in *Drosophila* Nanos was shown to be essential for dendrite morphogenesis, where together with its partner Pumilio it elaborates high-
order dendritic branching of peripheral neurons (Ye et al., 2004; Brechbiel and Gavis, 2008). Nanos was also found to be crucial for Pumilio-mediated regulation of sodium current and excitability in *Drosophila* motoneurons (Muraro et al., 2008). Nanos’ role in germ cells seems to be conserved among invertebrates and vertebrates; however, its role in the nervous system remains poorly understood and requires further investigation.

In my studies I identified *Hydractinia Nanos* and *Pumilio* homologs and examined their expression patterns in most developmental life stages. Furthermore, I aimed to study the role of Nanos2 in *Hydractinia* development with the use of available gene overexpression and knockdown methods.
8.1. **Identification of Hydractinia Nanos and Pumilio homologs**

Two Nanos and a single Pumilio gene were identified in *Hydractinia* EST and genomic databases and were amplified by RACE PCR from cDNA. Obtained sequences were verified by gel electrophoresis and sequencing. *Hydractinia* Nanos1 and Nanos2 protein sequences were compared with vertebrate and invertebrate homologs and the alignment revealed high degree of sequence similarity within the conserved Zn-finger domains. Alignment of *Hydractinia* Pumilio sequence with Pumilio homologs from other animals shows that the C-terminal region of Pumilio is conserved and almost identical to Pumilio homologs belonging to other species (e.g. *Homo sapiens*).

I also identified few neural markers in order to use them for analysis of the Nanos2 knockdown and ectopic expression phenotypes. The sequences of the *Hydractinia* homologs of Ncoll, Ash and RFamide neuropeptide precursor were amplified from *Hydractinia* cDNA and verified by sequencing.

The mRNA expression patterns of all above genes were examined by *in situ* hybridization that was performed on most *Hydractinia* life stages to identify the type of cells in which these genes are present. Additionally, the activity of Nanos2 promoter was studied by microinjection of an expression construct, including the putative Nanos2 promoter, into early embryos. The reporter protein was traced using fluorescence microscopy.
8.2. Investigation of gene expression patterns

8.2.1. mRNA expression

*Nanos1* and *Nanos2* transcripts were identified in germ cells, supporting their conserved role in germ cell development. Interestingly, while *Nanos1* expression was detected only in oocytes, *Nanos2* mRNA was also found in developing sperm and cells committed to nematocyte fate, from early progenitors to maturing stinging cells. Nematocytes are mechanosensory cells and belong to the neuronal lineage. *Drosophila* Nanos was shown before to have a role in dendrite morphogenesis and mammalian *Nanos1* was found to be expressed in the nervous system as well. *In situ* hybridization of *Hydractinia* Nanos homologs revealed that their expression patterns are diverse. Similar diversity between *Nanos* homologs is found in mammals, suggesting that *Hydractinia Nanos1* may be functionally similar to the mammalian *Nanos2* and *Nanos3* genes, which are expressed exclusively in germ cells (Tsuda et al., 2003). *Hydractinia Nanos2*, in contrast, may be more alike the third mammalian homolog, *Nanos1*, expressed in the Central Nervous System (Haraguchi et al., 2003). *Drosophila* has only a single *nanos* gene, expressed both in germ cells and in neurons (Brechbiel and Gavis, 2008).

The presence of Nanos homologs is conserved in metazoans. Most described species have at least one homolog of this gene. Cnidarians have two Nanos homologs, which have been described so far in *Hydra magnipapillata*, *Podocoryne carnea*, *Clytia hemisphaerica* and *Nematostella vectensis* (Mochizuki et al., 2000; Torras et al., 2004; Torras and Gonzalez-Crespo, 2005). Studies on cnidarian Nanos genes show that in early embryonic development Nanos expression is polarized, suggesting that Nanos’ role in AP patterning may be ancestral (Torras et al., 2004). However, no *hunchback* homolog was identified in cnidarians so far and this function of Nanos in these early branching animals remains an open question. In later developmental stages, Nanos expression is found in female and male germ cells and interestingly, in the head and tentacles (cnidarians’ oral structures), which are considered to be formed at the posterior end (Torras and Gonzalez-Crespo, 2005). In *Clytia hemisphaerica* one of the Nanos homologs, *CheNanos2*, was found to be expressed in nematoblasts (precursors of cnidarian-specific stinging cells) (Leclère et al., 2012). Stinging cells belong to the neural lineage and the fact that the expression of Nanos is found in the cells of neuronal origin, both in cnidarians and in mammals, indicates that Nanos may be important for their normal development or
function. This suggests that Nanos’ role in the nervous system could have evolved in the common ancestor to cnidarians and bilaterians.

I have also investigated the expression pattern of the canonical Nanos partner – Pumilio. *Hydractinia Pumilio* expression pattern overlapped in germ cells with *Nanos1* and *Nanos2* expression patterns, suggesting that Pumilio’s role in germ cells development may be conserved between all metazoans and may be Nanos-dependent also in *Hydractinia*. However, in the adult feeding polyps, *Pumilio* expression pattern only slightly overlapped with *Nanos2*. *Pumilio* was expressed only in early, but not late nematocyte progenitors like *Nanos2*, which was expressed in both. These results suggest that Pumilio may not always be necessary for Nanos-mediated repression. Pumilio may be important only during germ cells development and at the early stage of nematogenesis, when it is co-expressed with *Nanos2*. However, regulation of later nematoblasts development may be Pumilio-independent. So far Pumilio-independent Nanos function in the nervous system has not been observed in other species.

The expression patterns of *Hydractinia Ncol1*, *Ash* and *RFamide* were also examined by *in situ* hybridization. *Ncol1* was expressed in early nematoblasts. *RFamide* was found in mature neurons and *Ash* in putative neuroblasts and nematoblasts. Expression patterns of these neural markers were consistent with the published data, suggesting that their roles in neurogenesis and nematogenesis are ancestral (Mitgutsch et al., 1999; Katsukura et al., 2003; Hayakawa et al., 2004; David et al., 2008). *In situ* hybridization experiments, optimized for *Ash*, *RFamide* and *Ncol1*, were later on used for analysis of the *Nanos2* ectopic expression and knockdown phenotypes.
8.2.2. Nanos2 promoter activity

I studied the GFP reporter expression under the Nanos2 promoter by microinjection of the expression construct into Hydractinia fertilized eggs. The reporter expression was traced throughout embryonic development, including the preplanula and larva stages, but also post metamorphosis. GFP was found in early nematoblasts and maturing nematocytes, consistent with Nanos2 in situ hybridization. In post metamorphic polyps, GFP+ cells also resembled neurons morphologically. They had a spindle shape and some possessed neurite-like structures (Fig. 5.3.2). Other fluorescent cells in the hypostome, around the mouth opening, formed a ring, which is reminiscent of the ganglionic nerve ring around hydroid polyps’ mouths (Grimmelikhuijzen, 1985). Detection of the reporter expression in neurons was contrary to in situ hybridization results, which revealed Nanos2 expression in putative nematoblasts. It is possible that the amount of mRNA in neurons was below detectable level, but also it is likely that the strong staining of nematoblasts could have camouflaged the weaker staining of neurons. Nanos2 promoter also might have been too short and did not include all regulatory elements of the Nanos2 promoter, necessary to reveal complete reporter expression pattern. Most importantly, Hydractinia Nanos2 is expressed in the nervous system, like homologs of this gene in other animals. Both nematocytes and neurons belong to the neural lineage and most likely differentiate from a common precursor neural stem cell. However, it is likely that in both cell types Nanos2 has different roles.

By studying the Nanos2 promoter activity I hoped to identify all types of cells expressing Nanos2. This would help me to interpret the role this gene plays in Hydractinia development. However, the microinjection was not very efficient and resulted in generation of only few fluorescent animals. Also, I faced many difficulties to identify the GFP+ cells, primarily due to unstable reporter expression post metamorphosis. I hoped that injected expression cassettes will be permanently incorporated into the animal genome to allow me studying cell fate over a longer period. Stable expression would let me trace fluorescent cells in all different Hydractinia life stages. Also, through sexual reproduction, F1 animals could obtain uniform reporter expression and reveal its complete expression pattern. Unluckily, GFP expression dissipated in all animals within 2 weeks post metamorphosis.
The loss of expression could have had few reasons:

- The expression construct might have never been integrated into the genome of injected embryos and was only transiently expressed. Under these circumstances, GFP level was diluted with each cell division until it was no longer visible.

- The construct could have been inserted into a location in the genome where it could not be expressed (e.g. heterochromatin).

- The construct, which included only 800 bp upstream the *Nanos2* coding sequence, could have been too short and did not include all regulatory modules of the *Nanos2* promoter.

- The expression vector containing the *Nanos2* promoter had the *Hydra Actin1* terminator. It is possible that the sequence of *Nanos2* terminator is required for the native *Nanos2* expression.

All above mentioned difficulties made studying the activity of *Nanos2* promoter not very efficient and informative. Possibly, injection of the expression construct containing the full *Nanos2* promoter would bring a more stable reporter expression. When combined with additional immunochemical analysis, it could provide more information on the identity of GFP\(^+\) cells. Nevertheless, the results obtained from these experiments corroborated the expression of *Nanos2* in the *Hydractinia* nervous system, in particular in nematocytes.
8.3. *Nanos2* ectopic expression

*In situ* hybridization experiments revealed that *Nanos1* expression is restricted to germ cells, whereas *Nanos2* is expressed both in germ cells and in cells committed to nematocyte fate, from early progenitors to maturing stinging cells. Nanos function in germ cells development is well known, but the role of Nanos in the nervous system is poorly understood. From this reason I decided to study the role of the *Nanos2* gene in *Hydractinia* neurogenesis by *Nanos2* misexpression, starting with ectopic expression.

*Nanos2* ectopic expression was performed by injection of an overexpression vector into *Hydractinia* fertilized eggs. The construct drove *Nanos2*-GFP expression by the *Hydractinia* Actin1 promoter. The Actin1 promoter is active ubiquitously in early embryonic development and become epithelial-specific following metamorphosis (Künzel et al., 2010; Duffy et al., 2011; Millane et al., 2011). Introducing the transgene caused severe defects in animal development. Many embryos and larvae died before metamorphosis. Whereas few survived animals formed very interesting morphological phenotypes. They generated supernumerary and ectopic tentacles (i.e. oral structures), which led to complete oralization in extreme cases. The increase in the number of tentacles was even 130% more in transgenic versus non-transgenic animals. Many polyps also developed hyperplastic stolons early post metamorphosis, which are usually formed due to accumulation of stinging cells within the stolonal tissues and are created as a response to allogeneic contact. Interestingly, complete oralization observed after *Nanos2* ectopic expression has been reported before in *Hydractinia* as a result of ectopic Wnt activation induced by an azakenpaullone treatment. To test if the generated phenotypes are related with altered Wnt signaling, *Wnt3 in situ* hybridization was performed on transgenic animals. Surprisingly, Wnt signaling was unaltered as no additional staining was observed within or around the ectopic tentacle than normal restricted to the tip of the hypostome (Fig. 6.4.1a). This suggests that Nanos2-mediated excess of tentacles is Wnt-independent.

However, Nanos2 may be a target of Wnt signaling as *Nanos2* expression is upregulated in azakenpaullone-treated primary polyps. Wnt signaling is known to promote cellular proliferation in many animals (Reya et al., 2003; Teo et al., 2006). In *Hydractinia* blocking of GSK-3 with paullones causes an initial burst of proliferation and also induces recruitment of nematocytes and nerve cells from a pool of interstitial stem cells (Teo et al., 2006). Upregulation of *Nanos2* expression
after azakenpaulone treatment suggests that *Nanos2* may be associated with general “stemness” or proliferation. Furthermore, in *Hydractinia* Wnt-induced increase in the number of neural cells does not cause formation of numerous or ectopic tentacles, supporting again the hypothesis that *Nanos2*-induced excess of tentacles is Wnt-independent.

Further histological analysis of transgenic animals revealed that the abnormal development may be caused by an increase in the numbers of nematoblasts (progenitors of stinging cells). Anti-NCol1 antibody and modified nematocyst-specific DAPI stainings performed on larvae showed that *Nanos2* ectopic expression not only increased the number of nematoblasts up to 180% comparing to wild type animals, but also decreased the amount of mature stinging cells by 45%. The number of cells undergoing mitosis was increased by 90%, suggesting that *Nanos2* promotes proliferation of nematoblasts and at the same time blocks their maturation. *Nanos* was shown to play similar role in primordial germ cells, maintaining their proliferation and preventing them from both acquiring somatic fate and premature differentiation into germ cells (Kobayashi et al., 1996; Tsuda et al., 2003; Hayashi et al., 2004). Surprisingly, *Nanos2* ectopic expression also caused significant decrease in the number of both *RFamide*+ neurons and *Ash*+ cells by 87% in transgenic larvae. *RFamide* expressing cells were almost completely lost post metamorphosis.

By comparing the increase in the numbers of *NCol1*+ nematoblasts with the decrease in the numbers of *RFamide*+ neurons in the transgenic larvae, I noticed that the more nematoblasts were formed, the less neurons were present. This observation implies that the proportions between both cell types can be interdependent and these cells may differentiate from the common precursor stem cell. This scenario would not be very surprising, as nematocytes are mechanosensory cells which belong to the neural lineage, like neurons. It is possible that they share a common origin with neurons and arise from the same progenitor cells which, in turn, derive from the pluripotent i-cell population that also gives rise to other somatic and germ cells.

All the above analyses of the phenotypic variations led to the conclusion that the abnormal development of transgenic animals may be caused by a change in the commitment of early embryonic cells, driving them to make more nematoblasts (precursors of stinging cells), at the expense of neurons (Fig. 8.3.1). *Nanos2* ectopic expression also prevented differentiation of these nematoblasts. However, following metamorphosis the *Actin1* promoter stopped being ubiquitous and became epithelial-specific. Then *Nanos2*-mediated repression of differentiation was released, allowing the supernumerary nematoblasts to fully mature and cause an increase in the number
of nematocytes post metamorphosis. In later developmental stages Nanos2 was not expressed anymore in stem cells so it could not affect their fate.

It is possible that these excessive nematocytes induced formation of hyperplastic stolons and ectopic tentacles in transgenic animals. Stolons become hyperplastic, when large amount of stinging cells accumulate in the interstitial spaces of their epidermal epithelial cells. They are usually formed as a result of allogeneic interactions. However, transgenic animals were not in direct contact with foreign, allogeneic colonies. In this situation, hyperplastic stolons were likely to be produced due to an increase in the numbers of stinging cells, induced by Nanos2 ectopic expression.

The excess of nematocytes could also affect the amounts of tentacles formed. Tentacles are normally packed with stinging cells as these are used by the animals in large amounts to capture prey or defend against predators. However, such a correlation between the numbers of tentacles and the numbers of stinging cells has never been described in cnidarians before. In summary, Hydractinia Nanos2 seems to act as a switch between nematogenesis and neurogenesis promoting formation of nematocytes at the expense of neurons.

Fig. 8.3.1. Nanos2-mediated regulation of neural cell fates in Hydractinia nervous system.
8.4. *Nanos2* knockdown

*Nanos2* knockdown was performed to verify the specificity of overexpression studies. To downregulate endogenous *Nanos2* expression I applied RNAi and morpholino designed to degrade the mRNA or to block mRNA translation, respectively. The effect of *Nanos2* downregulation was examined by antibody, modified DAPI staining and by *in situ* hybridizations.

*Nanos2* dsRNA-mediated knockdown resulted in reduced proliferation post metamorphosis. Morpholino-mediated knockdown allowed me to study the effect of *Nanos2* downregulation in the early *Hydractinia* development. *Nanos2* morpholino injection resulted in a decrease in the number of nematoblasts by 46% and mature stinging cells by 30% up to 49%. Interestingly, the decrease in the number of mature stinging cells has been observed in transgenic larvae too. However, in knockdown animals it was caused by a decrease in the number of their progenitors rather than differentiation blockage observed after *Nanos2* ectopic expression. Conversely to ectopic expression studies, *Nanos2* knockdown led to the decrease in the number of RFamide+ mature neurons by 36%, supporting the specificity of both functional studies. Similarly to RNAi animals, metamorphosed morpholino-injected primary polyps had between 30% and 77% less tentacles than control morpholino injected animals. The effect was much more severe when verified early post metamorphosis, as morpholino-mediated knockdown effect fades overtime. Primary polyps also had 50% less stinging cells than control animals, contrary to *Nanos2* ectopic expression.

These results suggest that *Nanos2* downregulation decreases the number of nematoblasts in early development, later on affecting the number of mature stinging cells. Together with the decreasing number of nematocytes, the number of RFamide+ mature neurons was observed to increase. This suggests that both neurons and nematocytes share common neuronal origin and most likely these cell types differentiate from the common precursor stem cell.
8.5. Suggested mechanism of Nanos2-mediated specification of neural cell types

Nanos2 misexpression studies suggest that Nanos2 maintain the balance between different neural cell types. Nanos2 ectopic expression increases proliferation of nematoblasts and blocks their differentiation but at the same time decreases the number of neuroblasts and neurons. Nanos acts as a translational inhibitor binding to the Nanos Response Element (NRE) on the 3'-UTR of target transcripts and inhibiting their translation. NRE contains conserved binding sites for Pumilio, called Pumilio Regulatory Element (UGUAXAUA) and the binding site for Nanos, called Nanos Regulatory Element (GUUGU). Functional studies indicate that both Regulatory Elements are necessary for proper functioning of the complex Nanos-Pumilio. These short sequences also seem to be conserved in other mRNA transcripts regulated by these proteins (Murata and Wharton, 1995). To find out if Hydractinia neuronal genes have putative Nanos or Pumilio binding sites I studied the 3'-UTRs of neural genes like Ash, RFamide, Elav3, JNK, Zic2 and nematocyte genes like Ncol1 and Nowa, for the presence of the conserved Nanos or Pumilio Regulatory Elements.

I also examined the UTRs of genes involved in the Notch signaling pathway, which is known to be involved in neurogenesis in other species (Kageyama et al., 2007; Andersson et al., 2011; Chi et al., 2012). Unfortunately, I failed to optimize Hydractinia Notch targets, because DAPT-mediated Notch inhibition performed on Hydractinia did not support the published data on Notch knockdown in other cnidarian Nematostella vectensis. The decrease in the number of nematoblasts and nematocytes after DAPT treatment was the only result consistent with the studies performed on Nematostella. Nevertheless, I decided to check the 3’UTRs of Hydractinia putative homologs of Hes1, Hes2, Ash, Botch and Dishvelled for the presence of Nanos or Pumilio binding sites.

Out of all examined genes, only the Hydractinia homologs of Hes1, Botch, Elav3 and Nowa appeared to have Nanos Regulatory Elements (GUUGU) within their 3’UTRs (Fig. 8.5.1A). Nowa, similarly to Ncol1, represents a classical marker for developing nematoblasts (Engel et al., 2002). Nowa UTR sequence, besides the Nanos Regulatory Element, was found to contain additionally Pumilio binding sites, suggesting that it may be bound and regulated by the complex of Nanos/Pumilio. This conclusion would be consistent with Nanos2 and Pumilio expression patterns, overlapping in early nematoblasts where Nowa is expressed. The inhibition of Nowa
translation by Nanos2 may prevent nematoblasts from premature differentiation and is likely to affect the number of mature stinging cells in transgenic larvae.

Another potential Nanos target may be Botch. Botch promotes neurogenesis by inhibiting Notch signaling (Chi et al., 2012). By blocking Botch, Nanos2 may indirectly upregulate Notch signaling and decrease Ash expression (Fig. 8.5.1A). The downregulation of the proneural gene Ash would cause the decrease in the number of neuroblasts and neurons, the phenotype actually observed after Nanos2 ectopic expression. DAPT-mediated Notch downregulation also caused a decrease in the number of nematoblasts so most likely Notch upregulation caused by Botch inhibition would result in an increase in the number of stinging cells’ precursors.

*Hydractinia Elav3* was also found to have a putative Nanos Regulatory Element (Fig. 8.5.1A). The conserved family of Elav genes are required for proper differentiation and maintenance of neurons both in vertebrates and invertebrates (Good, 1995; Marlow et al., 2009). Nanos2-mediated translational blockage of Elav transcripts would result in a decrease in the number of neurons. Interestingly, RFamide sequence does not possess conserved Nanos binding sites, suggesting that the observed RFamide knockdown phenotype does not result from a direct Nanos2-mediated blockage of RFamide expression. The fact that within Elav3 and Botch transcripts Pumilio binding sites have not been identified, suggests that the translation of these transcripts may be directly regulated by Nanos2 and may not require Pumilio.

I also identified putative Nanos and Pumilio Regulatory Elements within the Hes1 3’UTRs (Fig. 8.5.1A and B). Hes genes are classical Notch targets. Most of them repress neurogenesis by both Notch-dependent and independent mechanisms; however, some were found to promote neural development (Kageyama et al., 2007). The role of *Hydractinia* Hes1 homolog is not known so the effect of Nanos2-mediated translational blockage of Hes1 is difficult to predict. The presence of additional binding site for Pumilio suggests that the translation of Hes1 transcript can be regulated by the complex Nanos2/Pumilio.
Fig. 8.5.1. Suggested mechanism of Nanos2-mediated regulation of neural cell types. (A) Hes1, Botch, Elav3 and Nowa mRNAs possess putative Nanos binding sites and their translation may be directly regulated by Nanos2. (B) Hes1, Ash, Nowa, Nanos1 and Nanos2 mRNAs possess putative Pumilio binding sites and their translation may be directly regulated by Pumilio or by the Nanos/Pumilio complex.
I also would like to suggest another model for Nanos2-mediated regulation of nematogenesis and neurogenesis, which would be dependent of Pumilio. All putative neural and nematocyte genes or Notch targets were analyzed also for the presence of Pumilio Response Elements. The 3’-UTRs of Hes1, Ash and Nowa transcripts were found to possess single Pumilio binding sites (Fig. 8.5.1B). Nowa and Hes1 were also found to have Nanos binding sites and are likely to be regulated by the Nanos2/Pumilio complex. The possible mechanism of Nowa and Hes1 translational regulation was described above. However, Pumilio-mediated regulation of Ash may be independent of Nanos, as no conserved Nanos Regulatory Element was identified in its 3’-UTR. The mechanism of Ash translational repression does not exclude binding of Nanos2 to Pumilio, because it is likely that Pumilio may form a protein-protein complex with Nanos2 which does not require binding of Nanos2 to the target transcript. Nevertheless, involvement of Nanos2 in Pumilio-mediated translational blockage remains uncertain. Ash translational blockage would result in a decrease in the number of neurons, the phenotype observed after Nanos2 ectopic expression and opposite to the knockdown results.

Analysis of Nanos1 and Nanos2 mRNAs also revealed that these transcripts possess single Pumilio Regulatory Elements. Their presence suggests that translation of these genes may be directly regulated by Pumilio itself or by the Nanos/Pumilio complex. Such regulatory mechanism could have evolved to protect cells from Nanos overactivity and may function as a negative feedback loop as a means of normalizing Nanos level.
8.6. Concluding remarks

Nanos2 expression pattern and misexpression experiments suggest that Hydractinia Nanos2 homolog plays a role in nematocytes differentiation and maturation. Nanos2 most likely acts with its partner, Pumilio, in the early neural cell fate specification. However, Nanos2 – dependent regulation of later nematocyte development may not require Pumilio, a phenomenon that has never been observed in other species before. The results of my studies suggest that Nanos2 protein has few distinct functions:

1. It maintains the proliferation of primordial germ cells and nematoblasts.
2. It is most likely involved in nematoblasts’ maturation and migration and as well protects them from premature differentiation.
3. It plays important role in the specification of stem cells to different neural fates by promoting nematogenesis and suppressing neurogenesis.

Nanos' roles in germ cell development and proliferation seem to be conserved in cnidarians, but regulation of commitment of stem cells to different neural fates has not been documented previously. Nanos2 ectopic expression caused the loss of a balance between the neural cell types through an unknown mechanism which remains to be investigated. Nevertheless, this is the first time Nanos was found to have a role in the specification of neural cell types. My data also demonstrate that the use of early branching metazoan models can have implications for basic developmental biology and biomedicine. Further studies on other species will reveal whether this role is ancient and shared among metazoans, or is a cnidarian innovation.
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