Molecular analysis of the development and function of the apical organ in the sea anemone *Nematostella vectensis.*

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Abstract

The shapes and patterns displayed across the animal kingdom exhibit great diversity, but at the same time similarities in their appearance can be observed. Scientist in the past have studied animal forms based on morphology. In this way, comparison of animal forms has engaged generations of biologists. Nowadays, the study of the evolution of developmental mechanisms, which build these diverse forms of life, is based on molecular methods which enable comparisons across the whole width of animal diversity.

Patterning along the primary body axis is a fundamental event in the life history of every animal. The correct positional information ensures that organs and organ systems develop in the right part of the body. Understanding the molecular mechanisms underlying these events is important for understanding the evolution of animal body forms, and can shed light on two questions that have puzzled evolutionary and developmental biologists for decades: How are animal axes related to each other? And: Are larval forms homologous? Both questions are crucial for revealing the characteristics of the last common ancestor of all animals and the last common ancestor of bilaterians. In the present study we are addressing these questions using the anthozoan *Nematostella vectensis*. This sea anemone is a member of the bilaterian sister clade Cnidaria, and has a biphasic life cycle consisting of a free-swimming larva and a solitary benthic adult.

The ectoderm of the *Nematostella* planula larva exhibits no obvious morphological differences along the body column, except for the oral opening and the aboral apical organ. The apical organ is believed to be a larval sense organ and it plays a crucial yet poorly defined role in the transition from the larval to the juvenile stage. Despite this apparently simple axial morphology, a number of developmental genes are expressed in distinct territories along the primary body axis and these can be used as markers for specific axial regions, and used to assess patterning changes that arise from experimental manipulations. Additionally, patterning events within the developing apical organ occur and can be investigated experimentally.

In this study we examined the interplay of oral and aboral signalling centres in *Nematostella* larvae by gene knockdown experiments as well as overexpression/overactivation experiments. We further explored modulators of the main morphogenetic pathways involved in these

processes, the Wnt and the FGF pathway. Special focus arose on the HSPG (**h**eparan **s**ulfate **p**roteo**g**lycan) Glypican1/2/4/6, and differential sulfation of HS chains, as key modulators.

Our results identify three steps in the establishment and patterning of the primary axis: 1) Activation of oral *and* aboral networks by different levels of β-catenin. 2) A feedback-loop between the transcription factor NvSix3/6 and the Wnt receptor NvFrizzled5/8 (NvFz5/8) at the aboral end of the primary axis to control the size of the aboral territory. 3) Development of position-dependent features, such as the apical organ. We further characterize NvGlypican1/2/4/6 (NvGpc1/2/4/6) and HS chain sulfation as indispensable modulators of steps 2 and 3. Specifically, at gastrula stage, knockdown of *NvGpc1/2/4/6* and inhibition of glycosaminoglycan sulfation phenocopy *NvFz5/8* knockdowns, as they also cause the expansion of aboral marker gene expression and the reduction of oral markers.

By comparing these results to mechanisms in bilaterians, we reveal both evolutionary conservation and plasticity in the aboral/anterior patterning system of metazoans. Coming back to the two questions raised in the beginning, our results are in favour of the homology of the anterior-posterior axis in bilaterians and the oral-aboral axis in cnidarians, with the aboral pole corresponding to the anterior pole. This also provides support for the homology of marine invertebrate larvae, but a final verdict on this matter will require further research in *Nematostella* as well as in a broader range of other marine invertebrates.

1. Introduction

1.1 Cnidaria

Cnidarians are a diverse phylum of marine and freshwater gelatinous animals that include five classes – Anthozoa: corals, sea anemones; Hydrozoa: hyroids, siphonophores; Scyphozoa: jellyfish; Cubozoa, box jellyfish; and Staurozoans, stalked jellyfish; with the latter four forming the subphylum Medusozoa (Steele *et al.*, 2011). Phylogenetically, cnidarians are the sister group to the Bilateria (e.g. vertebrates, insects, worms) (Hejnol *et al.*, 2009; Ryan *et al.*, 2013; Philippe *et al.*, 2011) and therefore comparisons between cnidarian and bilaterian features allow us to infer what the last common ancestor of those two groups (which together comprise almost all animals) might have looked like.

Many cnidarians have complex life cycles including two adult life forms, the polyp (often colonial) and the medusa. However *Nematostella vectensis*, the animal used in our study, belongs to the class Anthozoa, members of which do not possess a medusa stage.

1.1.1 *Nematostella vectensis*

The starlet sea anemone *Nematostella vectensis* Stephenson, 1935 belongs to the family *Edwardsiidae*. Its natural habitat is in brackish water along the North American west coast, however it is now also found along the coast of England (Darling *et al.*, 2004).

Nematostella vectensis has been established as a model organism in recent years (Darling *et al.*, 2005; Tarrant *et al.*, 2014). It has many advantages compared to other cnidarian models, which have other values depending on the evolutionary or developmental question you want to ask. For example studies in *Hydra* are constrained to the adult stage, but are very informative for stem cell evolution. *Nematostella* instead is a suitable model for studies on gene interaction and developmental processes.

The life cycle of *Nematostella vectensis* is shown in Figure 1: *Nematostella* has separate sexes which either release eggs in gelatinous egg packages, or sperm, through the animals only opening. This opening is commonly called the mouth or oral opening, despite its three functions (mouth, anus, gonopore). In the laboratory, spawning can be induced by light and

temperature shifts (Fritzenwanker and Technau, 2002). *Nematostella* can also reproduce asexually through fission (Reitzel *et al.*, 2007).
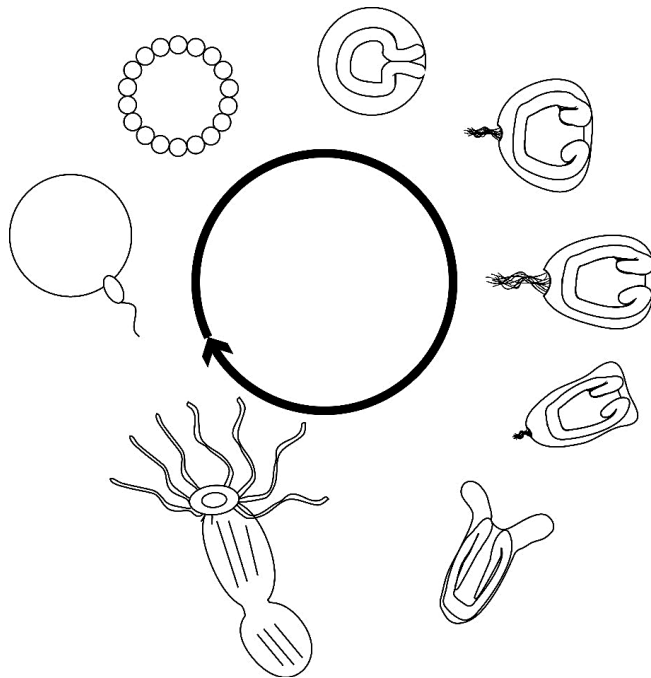
Figure 1: Nematostella vectensis life cycle: Starting on the left clockwise: Egg and sperm; blastula; gastrula; early planula; late planula; tentaclebud stage; primary polyp; adult.

Note: the sketches are not to scale.

Following fertilisation, early cleavage is highly variable but synchronous (Fritzenwanker *et al.*, 2007) leading to the formation of a hollow blastula comprising a single cell layer. Gastrulation occurs via invagination between 18-24 hpf (hours post-fertilisation) and separates the endo- and ectoderm (Kraus and Technau, 2006). The free swimming, ciliated planula larva develops a long ciliary tuft at the aboral pole after 2-3 days. This tuft is considered to be part of a larval sense organ, the "apical organ" which I will discuss further in 1.4.1. Around the oral pole, four tentacle buds form in the late planula, and the larva transforms into a primary polyp. The ciliary tuft starts degenerating (Nakanishi *et al.*, 2012) around the same time that the primary polyp changes its direction of swimming. While the larva swims with the apical organ pointing forward, the polyp changes to pointing the oral pole forward. The primary polyp starts catching prey and grows into an adult form which is sexually mature within 3-6 months depending on animal density and food supply. As a

caveat, all these reported observations are under laboratory conditions, thus, we do not know if this is a true reflection of how the larva/juvenile behaves in nature.

During the last 10-15 years many molecular biological techniques have been established in *Nematostella*, including morpholino mediated gene knockdown (Rentzsch *et al.*, 2008; Magie *et al.*, 2007), transgenesis (Renfer *et al.*, 2010), over- and ectopic expression (Wikramanayake *et al.*, 2003) and in vivo imaging (DuBuc *et al.*, 2014), via microinjection. The genome of *Nematostella* has also been available since 2007 (Putnam *et al.*), and recently the TALEN and CRISPR/Cas9 systems have also been introduced in *Nematostella* (Ikmi *et al.*, 2014), which allows targeted genome editing.

1.2 The larva – more than just a free swimming foetus

Larvae are a developmental stage that occur in a multitude of animals and which can fulfil several purposes, such as dispersal; reduced competition with the adult stage (due to a different habitat); and accumulation of nutrients for the development of the adult form, which decreases the investment on the mothers side (the last two points only in case of feeding larvae). Larvae can bear stage-specific organs which are lost during metamorphosis, famous examples are the tail of the tadpoles of amphibians or larval eyes in annelids. In more dramatic transformations the adult form arises out of 'imaginal discs' or 'imaginal rudiments' and does not look anything like the larva (also called catastrophic metamorphosis); examples here are *Drosophila* or the sea urchin. Free-swimming larvae are very abundant in marine invertebrates and mostly serve the purpose of geographical distribution, as often the adult form is sessile. Marine larvae can therefore mostly be defined as "a posthatching, presettlement stage" (Hadfield, 2011). The larval stage ends with the metamorphosis to either a juvenile or a fully functioning adult stage. Per definition, larvae are clearly separated from juveniles; juveniles are essentially smaller versions of the adult that will go on to grow and mature sexually. However, as I will describe in the following paragraphs, this sharp separation between larva and juvenile is not always that clear and easy to define.

The term metamorphosis has been defined in many different ways, varying from "*major remodelling of the body at a late stage of development*" (Slack, 2013), to various more

elaborate and controversial definitions gathered in (Bishop *et al.*, 2006). Some authors considered to include alternations of generation in algae in the definition (e.g. Jacobs in (Bishop *et al.*, 2006)). In this study, I follow the definition given by Professor Michael Hadfield in the same article. Hadfield simply describes metamorphosis as the transition from a larva to a juvenile in animals. He further defines this transition as changes in morphology, habitat, behaviour and physiology. A larva differs in these parameters from the juvenile, which shares those traits with the adult form, but is not yet sexual mature. Generally, most definitions agree on the following criteria: habitat shift, morphological change, and that the pre-metamorphic stage is postembryonic.

1.2.1 Is the planula of *Nematostella* a true larva?

Cnidarian larvae are ciliated and are called planula larvae. Besides some shared features, there are also notable differences in the larval forms of cnidarians. For example, the apical organ has only been described in anthozoans (Chia and Bickell, 1978) and the scyphozoan *Aurelia* (Yuan *et al.*, 2008; Nakanishi *et al.*, 2008), although in hydrozoans accumulations of sensory cells at the anterior pole (the pole pointing forward during movement) have also been described in several species (Plickert and Schneider, 2004; Piraino *et al.*, 2011; Gajewski *et al.*, 1996). In addition, the gastrulation mode and timing can vary extremely between different cnidarian taxa, as does the extent of metamorphosis, which ranges from catastrophic (including degeneration of the larval nervous system (Seipp *et al.*, 2010)) to almost direct development (see below).

Nematostella has been described as a direct developer (Magie *et al.*, 2007; Reitzel *et al.*, 2006) because the development and expression of most developmental regulatory genes is continuous, and because no dramatic tissue reorganisation occurs during the transition from planula to juvenile stage. Yet, *Nematostella* changes its behaviour (swimming direction and general activity level), its habitat (free swimming to sessile, buried in the sediment in nature), its feeding strategy (although there are indications that planulae can take up dissolved organic material (Smith and Jaeckle, 2013) prey catching and digestion is in the laboratory only observed from primary polyp stage on), and its morphology (degeneration of apical organ, budding of tentacles) during development. Thus we can clearly distinguish between a larval stage and a juvenile primary polyp stage and this transition might well be called a metamorphosis. On the other hand, the gradual continuity of this process resembles

that of a direct developer, and this leaves us with a terminological dilemma. I suggest to use both terms when describing *Nematostella*, calling its mode of development *direct metamorphic* with a true larval stage.

1.2.2 Is the larval stage homologous across the animal kingdom?

It is an ongoing debate whether larval forms are homologous across the animal kingdom or whether they evolved independently. It is widely accepted that insect and vertebrate larvae are the results of gradual intercalation (Minelli, 2010; Suzuki *et al.*, 2008; Wolpert, 1999) in that they have been secondarily added into a direct developing lifestyle and they are therefore not homologous to the so-called primary larva of marine invertebrates.

Whether primary larvae of marine invertebrates are homologous has broad implications for reconstructions of the last common ancestor of all animals and the last common ancestor of bilaterians. Many different evolutionary scenarios around this issue have been reviewed and interpreted by many authors (Arenas-Mena, 2010; Nielsen, 2009; Baguñà *et al.*, 2008; Page, 2009; Sly *et al.*, 2003; Nielsen, 2013). These theories can be mainly divided into scenarios in which the last common ancestor either had a larva-like adult form ("larva-first" or "terminal addition" theory); or was more similar to present adult forms, with direct development and thus modern larval forms have evolved independently multiple times ("adult-first" or "intercalation" theory).

The larva-first theory goes back to Haeckel's Gastraea-theory (Haeckel, 1873) which is part of his idea that ontogeny recapitulates phylogeny, implying that new stages are added terminally. Haeckel's Gastraea-theory received new support from Nielsen's Trochaea-hypothesis. With this, Nielsen proposes that a uniformly ciliated *gastraea* ancestor evolved a ring of specialised cilia around the blastopore to generate the *trochaea*. In a modified version of this theory, the trochaea is the ancestor of all protostomes (Nielsen (1979); (2009; 2013)). Another variation of the larva-first theory is based on maximally indirect developing species and their imaginal rudiments. Peterson *et al.* (1997) proposed a model where 'set-aside cells' within the larval body remain pluripotent and develop into the adult form, while all other larval structures are discarded.

The intercalation theory instead has its roots with Garstang, who opened the field of evolutionary-developmental biology (evo-devo) by stating that ontogeny creates phylogeny instead of recapitulating it (Garstang, 1922). Garstang further put early development and larvae into the focus of natural selection (Garstang, 1929). The intercalation theory argues that characters required for a larval lifestyle are fewer and more simple than characters required for an adult life style, and therefore are more likely to have evolved convergently (Sly *et al.*, 2003; Raff, 2008).

Degnan and Degnan (2006) extended this discussion by taking sponge larvae into consideration, and pointing out that they *gastrulate* (this depends on the definition of the term gastrulation and will be discussed later on), but are non-feeding. They argue that sexual reproduction was already in place at the base of metazoan evolution and that the separation of the embryo from the adult is the initiation to a biphasic lifestyle. Following on from this, natural selection has shaped the embryo in different marine phyla into different larval forms which serve the same purpose and therefore coevolve similar traits (e.g. for existence in the pelagic marine environment). Even if recent experimental data suggests that the cell layers of sponges are not homologous to the eumetazoan germ layers (Nakanishi *et al.*, 2014) (an issue which is still under debate (Hashimshony *et al.*, 2014; Leininger *et al.*, 2014)) and thus sponges may not gastrulate, the idea of convergent larval evolution through natural selection on free swimming embryos is appealing.

It has also been proposed that the larval lifestyle was the driving force of nervous system development and evolution (Jékely, 2011). If this is true, it would be a strong argument either for the "terminal addition" theory or for the existence of a biphasic life cycle in the metazoan ancestor prior to nervous system innovation as otherwise, nervous systems would be non-homologous. Within the Cnidaria+Bilateria this is very unlikely considering the molecular and developmental conservation of their nervous systems (e.g. Ryan *et al.* 2013). However, the recent phylogenetic positioning of ctenophores, suggests either the convergent evolution of nervous systems in ctenophores and the rest of the Eumetazoa, or the loss of a nervous system in sponges (Ryan *et al.*, 2013; Moroz *et al.*, 2014).The phylogeny of early diverging phyla is still under debate and nervous system loss is not considered as unlikely as it used to be. Regarding the proposal, of whether larval lifestyle is a good candidate to be a driving force behind nervous system evolution one should also keep in mind, that sponge larva manage the same basic functions (such as dispersal and sensing of settlement cues) as their eumetazoan 'school mates' without a nervous system (Jékely, 2011). Yet Jékely (2011)

argues that the grade of efficiency increases dramatically by simple neural circuits that enable the reduction of sensory structures and can lead to signal amplification; and modern larva demonstrate examples for these simple circuits which could have been at the base of nervous system evolution.

The discovery of the Hox cluster and its conserved role in patterning along the anterior-posterior (A-P) axis in bilaterians is a landmark in the field of evo-devo (Heffer and Pick, 2013). This finding showed that, despite molecular conservation, morphological diversity could be achieved. The Hox cluster also has implications for the questions addressed in this thesis: It has been shown that the Hox code does not play its canonical role in patterning the A-P axis in many indirectly developing species. This is clearly evident in animals with catastrophic metamorphosis like the sea urchin (Arenas-Mena *et al.*, 2000; Arenas-Mena *et al.*, 1998) and in nemerteans with the pilidium larval type, as shown by Hiebert and Maslakova (2015). In this work they found that *Hox* genes patterned the juvenile's A-P axis, but were not expressed in the pilidium larva (which bears an apical organ). The intercalation theory would argue that this reflects a co-option of parts of the developmental tool-kit into the newly evolved larval form (as discussed by Sly *et al.* (2003) and Arenas-Mena (2010)). Another explanation is that developmental modules were transferred from the ancestral larva to a later stage in taxa with catastrophic metamorphosis; or that Hox co-linearity must have evolved several times (arguably the most unlikely option).
I will discuss the issue of the evolutionary origins of the Hox cluster as the main organizer of patterning along the primary body axis further in 1.3.

In my view the contentious question of homology of marine invertebrate larvae should be addressed within smaller units. We should begin by defining and comparing single larval features, and determining their homology independently, and these data can then in a second step help to resolve the larger question of whether the larval stage as a whole is homologous. Units of interest for such focused analyses could include: the developmental programs used; the establishment of axes and patterning along axes (see 1.3); the larval organs (see 1.4) and the fate of larval tissues; the signalling pathways (see 1.5 and 1.6) and the transcription factors (TF) used to establish these specific features. Since it has become clear that a limited number of developmental pathways and conserved TFs have been co-opted numerous times during evolution, to form many dissimilar structures, these data must be interpreted with care. Nonetheless, by increasing our knowledge of these traits, and their developmental regulation across a wide sample of larval diversity, we may be able to distinguish between

conservation and convergence and in the end this can bring us closer to see the greater picture.

In summary, I want to emphasis my opinion that it is very important to see larvae as more than just free swimming foetuses (although this might be their origin). Larvae are highly specialised life forms which serve important roles in many animals development, in their ecology and their success. And we can learn a lot about the evolution and development of animals by studying them.

1.3 Body axes and axial patterning

The establishment of body axes, and patterning along these axes, is one of the major events in animal development. The primary body axis can be already determined maternally through localised mRNA or protein. Two other mechanisms for the establishment of the primary body axis, which are sometimes correlated, relate to the site of the sperm entry or the position of the polar bodies. In all cases the primary axis is determined prior to gastrulation, in fact gastrulation occurs according to the polarisation of the egg/embryo (summarised e.g. in Martindale (2005); (Martindale and Hejnol, 2009)).

In bilaterians the first body axis to be established is the anterior-posterior (A-P) axis (which can relate to the animal-vegetal axis of the egg), in cnidarians it is the oral-aboral (O-A) axis. The homology of the oral-aboral axis to the anterior-posterior axis is still under debate, although evidence is accumulating that they are indeed homologous (Sinigaglia *et al.*, 2013; Marlow *et al.*, 2013; Petersen and Reddien, 2009). In both clades the establishment and polarisation of the primary body axis is governed by Wnt/β-catenin activity (Martin and Kimelman, 2009; Niehrs, 2010; Plickert *et al.*, 2006; Petersen and Reddien, 2009) and the interplay of posterior Wnt signalling and anterior Wnt inhibition seems to provide positional information along the A-P axis, with this mechanism predating the bilaterian-cnidarian split (Petersen and Reddien, 2009; Wikramanayake *et al.*, 2003).

Discussions of homology relate not only to the axes, but also to the end points of these axes. Some authors favour a homology between the oral pole in cnidarians and the anterior pole in bilaterians (e.g. (Martindale, 2005)) whereas a relatively recent study found strong evidence to homologise the anterior pole of bilaterians with the aboral pole of cnidarians (Sinigaglia *et al.*, 2013). In bilaterians the correct development of anterior structures is governed by a suite

of TFs with Six3 at the top of this cascade (e.g. shown and reviewed in (Lowe *et al.*, 2003; Steinmetz *et al.*, 2010; Posnien *et al.*, 2011). And also the patterning of the aboral pole in *Nematostella* is under control of NvSix3/6 and other TFs involved in bilaterian head patterning (Sinigaglia *et al.*, 2013). One of the crucial functions of Six3 for the development of anterior structures is the repression of Wnt signalling, in turn, Wnt signalling represses Six3 (Lagutin *et al.*, 2003; Wei *et al.*, 2009; Sinigaglia *et al.*, 2013).

The posterior pole in bilaterians and the future oral pole in cnidarians are both well documented Wnt signalling centres (see above). Those who support a homology of the oral pole in cnidarians and anterior in bilaterians explain this discrepancy by suggesting a translocation of the Wnt system from the animal to the vegetal pole in bilaterians – which in turn caused a switch in the site of the gastrulation between cnidarians and bilaterians, but in their view, this does not correlate with a switch of the identity of the pole (Martindale and Hejnol, 2009).

Whichever of these two scenarios of homology of the poles is correct, the patterning along this axis is an entirely different story. Patterning along the anterior-posterior axis in bilaterians is governed by the expression of the Hox-cluster (e.g. (Kulakova *et al.*, 2007; Slack *et al.*, 1993; Niehrs, 2010; Pearson *et al.*, 2005), in that Hox TFs control correct positioning of structures along the previously established axis (Manuel, 2009). To be able to do that, first a posterior Wnt organizer plays a role in driving a posterior growth zone, at least partly by activating Caudal which in turn activates *Hox* genes (Martin and Kimelman, 2009). Posterior growth is not observed outside the Bilateria. In cnidarians the function of *Hox* genes remains unclear. Their expression patterns in *Nematostella* (Finnerty *et al.*, 2004; Ryan *et al.*, 2007) do not suggest a role in patterning along the oral-aboral axis. Their expression is limited to one germ layer and they do not show regionalisation along the primary body axis. In addition *anthox1/HoxF* is co-expressed in a domain with bilaterian head patterning genes, which does not occur in bilaterians (Finnerty *et al.*, 2004; Sinigaglia *et al.*, 2013). Also in the hydrozoan *Clytia hemisphaerica Hox* gene expression does neither correspond to the situation in bilaterians, nor to that in *Nematostella* (Chiori *et al.*, 2009). Probably the co-linearity and patterning function of *Hox* genes is a bilaterian invention (Manuel, 2009; Kamm *et al.*, 2006). It has been proposed that differentially expressed Wnt ligands may serve this role in *Nematostella*, and with respect to this analogy to the 'Hox-code', the term 'Wnt-code' has been used (Guder *et al.*, 2006). Ryan and Baxevanis (2007)

extended this view and suggested that, the *Wnt* genes were the earliest primary body axis patterning genes, and *Hox* genes were later co-opted into this network.

The Wnt-pathway in general and in the current state of knowledge in *Nematostella* will be discussed in 1.6.

Bilaterians establish two more body axis during their development, the dorso-ventral and the left-right axis. It is not clear to date if all cnidarians have a secondary body axis, but certainly anthozoans exhibit a polarity axis orthogonal to the primary axis, the directive axis (reviewed in Manuel, 2009). The directive axis in *Nematostella* is regulated by BMP-signalling, that generates a pSmad1/5/8 gradient (Leclère and Rentzsch, 2014; Saina *et al.*, 2009; Genikhovich *et al.*, 2015). The homology of this axis to the dorso-ventral axis, or the left-right axis of bilaterians is also subject of debate. Interestingly, most of the *Nematostella Hox* genes are asymmetrically expressed along the directive axis, leaving room for speculation that their ancient role as patterning genes may not being linked to a specific developmental axis.

How axes are formed, and how patterning along them is established, is a key consideration to be taken into account when comparing homologous vs. convergently evolved features in both adult and larval forms. Questions of whether these axes are maintained during life-phase transitions and whether the same programs for axial establishment and patterning are deployed when they are re-installed during metamorphosis are of particular importance for understanding the evolution of body forms in marine invertebrates. As discussed in 1.2.2, marine invertebrates with catastrophic metamorphosis do not use the *Hox* genes to pattern their primary body axis, in fact the juvenile primary body axis in these animals can be completely uncoupled from the A-P axis of the larva (Hiebert and Maslakova, 2015; Arenas-Mena, 2010), and therefore also uncoupled from egg polarity.

1.4 Apical organ

The apical organ of some marine invertebrate larvae is a larval-specific feature whose homology and function has inspired many discussions among evolutionary developmental biologists. In the following chapter I will give an overview regarding what is known about

this organ, what the apical organs of different taxa have in common, and I will highlight areas of uncertainty in our knowledge of apical organs which still need to be clarified and also explain, why it does matter.

1.4.1 Definition of the term "apical organ"

The term *apical organ* has mainly been assigned to structures on the basis of a few morphological criteria, including the presence of an apical ciliary tuft or particular cell shapes. This term is also used to describe the statocyst in ctenophores, but this organ is considered to not be homologous to the apical organs of other marine larvae (Martindale and Hejnol, 2009). Other terms often used to describe structures at the apical pole of larvae are: apical sensory organ, cephalic sensory organ, apical ganglion, apical rosette, apical plate, apical tuft, apical ciliary organ and frontal organ. Some of these terms are misleading, as they only refer to a subset of the apical organ, whereas others also include other parts of the anterior pole or the adult nervous system. In Platyhelminthes the term *frontal organ* has been used to avoid suggesting that it is homologous to the apical organs of other spiralians (Rawlinson, 2010; Rawlinson, 2014).

According to "*Invertebrate neurophylogeny: suggested terms and definitions for a neuroanatomical glossary*" (Richter *et al.*, 2010), the apical organ is a sensory organ and as such part of a nervous system. It comprises an apical ciliary tuft and receptor cells and is located at the anterior pole of larvae. There is an ongoing discussion about many aspects of apical organs. The two main ones are: 1) What is its function? 2) Is it homologous in all taxa? In my opinion the above mentioned definition will likely have to be reconsidered, depending on the outcomes of investigations into these questions.

1.4.2 Fragmented knowledge from diverse larva

Apical organs have been described in several taxa, mainly within the two bilaterian clades: Spiralia and Ambulacraria (taxonomy according to Edgecombe *et al.* (2011)) see Figure 2. These taxa show a variety of larval types. In spiralians we find pilidium and trochophore larvae as well as the actinotroch larva of phoronids and the cyphonaute larva of brachiopods (in which the apical organ is not situated at the anterior pole according to the swimming

direction). The deuterostome echinoderms and hemichordates also have different larval types with apical organs, including tornaria and doliolaria larva. And also in some cnidarian planula larvae there is a ciliary structure at the aboral pole, but this has so far only been described in Anthozoa (e.g. (Chia and Koss, 1979; Chia and Bickell, 1978).

The following section gathers our current knowledge on the function, structure and molecular identity of apical organs in different species.

A recently well-studied apical organ is that of the annelid *Platynereis dumerilii*. *Platynereis* early development is highly stereotypic and invariant, which allows techniques like "*whole-body gene expression pattern registration*" in order to achieve a gene expression atlas for *Platynereis* larva (Asadulina *et al.*, 2012); or models to identify cell types based on spatially referenced single-cell expression data (Pettit *et al.*, 2014). Also functional studies to the level of revealing neural circuit and intracellular recordings are possible (Tosches *et al.*, 2014; Jékely and Arendt, 2007; Pettit *et al.*, 2014). From such experiments we can say that the apical organ of *Platynereis* controls swimming behaviour and senses settlement cues, we also know the expression patterns for many transcription factors in the anterior domain, which exhibit similarities and differences to apical organs in other species (Conzelmann *et al.*, 2011; Marlow *et al.*, 2014).

It has been assumed for a long time that the apical organ is used for substrate selection / sensing of settlement cues and subsequently metamorphosis (Chia and Koss, 1979; Chia and Bickell, 1978). Experimentally proof of sensing settlement cues has been found in several species, including certain molluscs and annelids (Conzelmann *et al.*, 2011; Hadfield *et al.*, 2000; Hadfield, 2011). In *Nematostella*, a connection between the apical organ and metamorphosis has also been suggested (Rentzsch *et al.*, 2008). A different proposition has been made in molluscs: The apical organ may actually repress metamorphosis until the right cue is sensed (Voronezhskaya and Khabarova, 2003). Contradictory to this theory, veliger larvae with ablated apical organ cells do not undergo metamorphosis, unless metamorphosis is activated further downstream. Which suggests the opposite: the larva is able to transform without the apical organ, but it needs to sense the external cue (Hadfield *et al.*, 2000). It is of course possible that both is true in different species (due to a few changes in the control of signal transduction from apical organ cells, during the course of evolution).
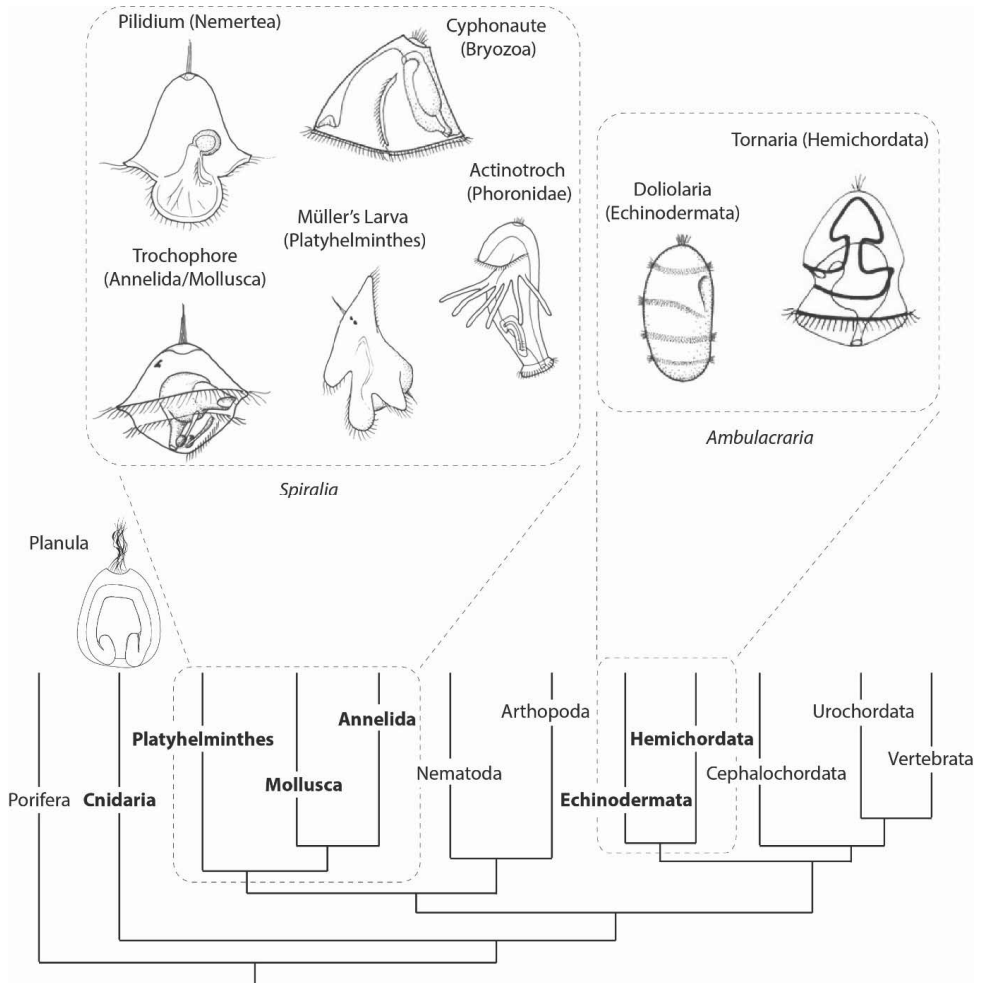
Figure 2: Larvae bearing apical organs mapped on a simplified tree of life.
The sketches show examples of larval forms bearing apical organs / apical tufts, the name of the larval type and the phyla they are found in are indicated on top of each sketch. The tree is a simplification of the animal tree of life only displaying a subset of phyla with (in **bold**) or without larvae with an apical organ. The dotted lines encircle the two bilaterian clades in which apical organs are found, Spiralia and Ambulacraria (sketches for those two groups are taken from http://scaa.usask.ca/gallery/lacalli/tutorial/tutorial_all.php.)

Another shared function of some apical organs seems to be the control of locomotion, based on the innervation of larval ciliary bands by apical organ cells in a number of species. These cells express neuropeptides, and the experimental application of synthetic neuropeptides to these larvae has been shown to impact upon swimming behaviour (Conzelmann *et al.*, 2011; Croll and Dickinson, 2004; Jékely, 2011). In addition to such functions in locomotion and settlement, it has also been proposed, that the apical organ induces the formation of the adult brain in lophotrochozoans (reviewed in Richter *et al.* (2010).

Another often cited similarity of apical organs in different taxa is the presence of serotonergic cells. Within the Ambulacraria, Byrne *et al.* (2007) compared apical organs and found that the serotonergic cells in the apical organs of different taxa "*differ in ontogeny, cellular organization and anatomy*". They also found that apical organs are lost at different time points during larval development within the echinoderms. Regarding function, the serotonergic cells within and outside apical organs have been linked to both the control of ciliary beat and settlement (Byrne *et al.*, 2007).

Serotonergic cells in apical organs have also been described in spiralians including annelids, molluscs, brachiopods, flatworms, and phoronids (Hay-Schmidt, 2000; Kempf *et al.*, 1997; Altenburger and Wanninger, 2010; Wanninger, 2009; Temereva and Wanninger, 2012); with these cells commonly being flask-shaped. In *Nematostella*, no serotonin positive cells have been identified in the apical organ (Marlow *et al.*, 2009), and according to Anctil (2009) there are no serotonin receptors in the *Nematostella* genome.

Besides structural and functional approaches to compare apical organs, a few studies also started to compare the expression of development genes to find similarity and differences between apical organs. Dunn *et al.* (2007) showed that in the sea urchin *Strongylocentrotus purpuratus* the TFs NK2.1 and HNF6 control apical tuft formation through the regulation of ciliary genes. Expression studies in a mollusc by the same authors revealed that these genes are not co-expressed in the apical tuft of the mollusc larva, supporting convergent evolution of apical organs. Also in *Platynereis NK2.1* and *onecut* (the *Platynereis* HNF6 orthologue) are expressed in the anterior domain, but not in the cells forming the apical organ (Marlow *et al.*, 2014). The expression patterns for a range of other transcription factors in the anterior domain have also been described. For example studies in *Nematostella*, sea urchin, *Platynereis* and the hemichordate *Saccoglossus* have shown that orthologs of the TF FoxQ2 are expressed in the anterior/aboral domain of all these species (Sinigaglia *et al.*, 2013;

Yaguchi *et al.*, 2010; Fritzenwanker *et al.*, 2014; Marlow *et al.*, 2014). In sea urchin and *Nematostella,* morpholino mediated knockdown has further revealed a role for FoxQ2 in apical organ development (Sinigaglia *et al.*, 2013; Yaguchi *et al.*, 2010). However, the expression domain of *FoxQ2* differs quite significantly between these species, e.g. in *Nematostella* the apical organ is surrounded by *FoxQ2a* expressing cells, while expression in *Platynereis*, sea urchin and *Saccoglossus*, includes the apical organ cells.

In summary, despite the broad similarities shared by at least a few taxa (e.g. cilary tuft, contra-blastporal position, seretonergic cells, role in locomotion and settlement, expression of certain regulatory genes), on closer examination there are many differences between the specific apical organs of different species. These differences might be the result of evolutionary change acting on homologous structures; alternatively the similarities in apical organs could be explained by convergent evolution due to adaptation to the larval life style. Inspired by the ideas of Garstang (1929) and Degnan and Degnan (2006), the independent evolution of apical organs may have been driven by natural selection, acting similarly on the evolving pelagic larvae of marine invertebrates, which have to serve a similar function (dispersal, settlement and metamorphosis to a benthic adult).

1.4.3 The apical organ of *Nematostella*

In *Nematostella*, the apical organ is first evident in early planula larvae, with the appearance of a tuft of long apical cilia, and an indentation at the aboral pole. The development of the aboral domain is controlled by the transcription factor NvSix3/6 in early stages and in the planula, a negative feedback loop of two antagonistic FGF ligands defines the size of the apical organ (Sinigaglia *et al.*, 2013; Rentzsch *et al.*, 2008) (see also 1.5). The apical organ itself consists of a variety of cell types; two types of gland cells and cells bearing long cilia have been identified through electron microscopy (Nakanishi *et al.*, 2012) (Figure 3).

A microarray analysis comparing animals with an experimentally extended apical organ and animals lacking an apical organ identified 78 genes predominantly or exclusively expressed in apical organ cells in *Nematostella* (Sinigaglia *et al.* 2015). This analysis also revealed that the cells within the apical organ have different expression profiles, with some genes being expressed in only a subset of cells, for example in a ring or in a small dot in the middle of the apical organ region. The genes identified by this analysis provide a good toolkit to facilitate
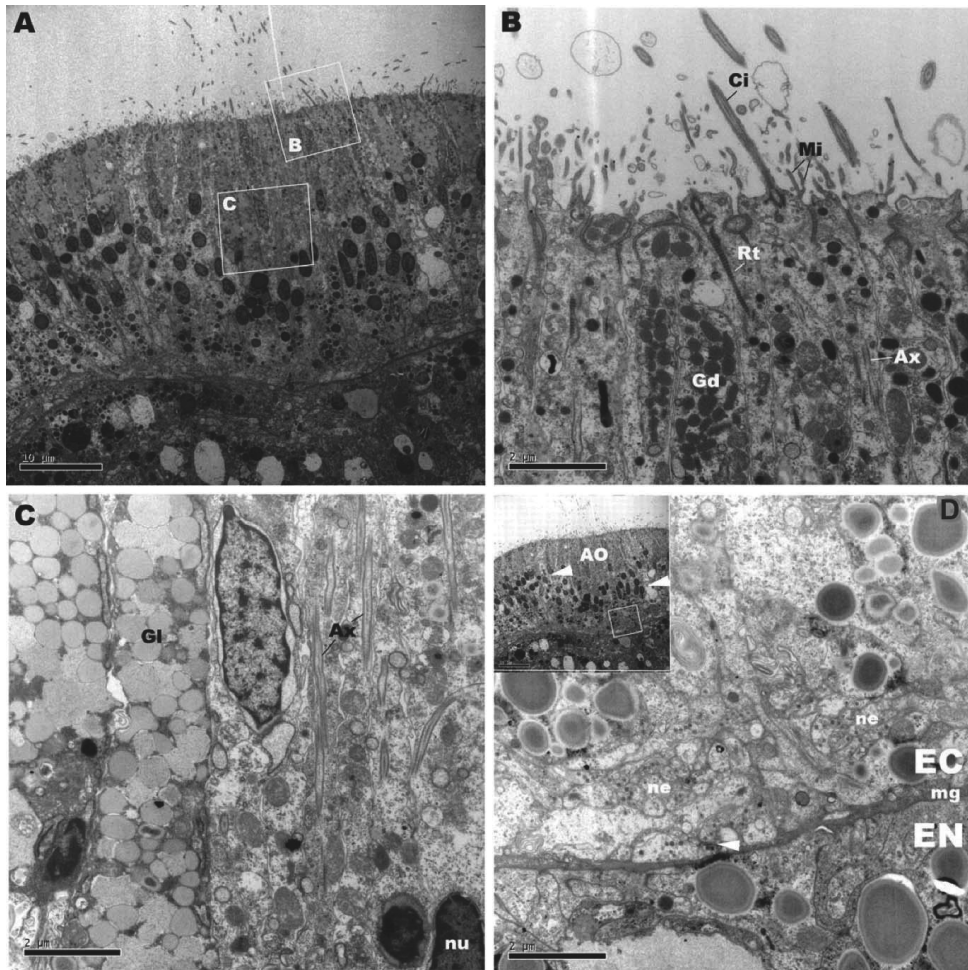
Figure 3: The apical organ of Nematostella vectensis (modified from (Nakanishi *et al.*, 2012)) (**A-D**) Electron microscopy sections of the apical organ at the late planula stage. In addition to the monociliated cells with basally located nuclei and intracellular axonemes with basal extensions, the apical organ contains two types of gland cells, one with electron-dense granules (Gd in B) and the other with electron-lucent granules (Gl in C). Inset in D is the same image as in A. ax, axonemes; ci, cilium; Gd, gland cells with electron-dense granules; Gl, gland cells with electron-lucent granules; Rt, ciliary rootlet; at, apical tuft, mv, microvilli, ne, neurites, mg, mesoglea, EC, ectoderm, EN, endoderm. Scale bars: 10 µm in A; 2 µm in B-D.

comparisons with the molecular fingerprints of apical organ cells across the animal kingdom. In an initial comparison, Sinigaglia *et al.* (2015) found that 12 out of 18 genes they had found to be expressed in the *Nematostella* apical organ cells, were also expressed specifically, or at least found to be enriched, in the apical organ of developing sea urchin larvae.

As described earlier, the apical organs of many taxa are part of the nervous system and serve as sensory organs. Although many studies have tried to find evidence for this in *Nematostella*, no connection to the nervous system has yet been shown (Sinigaglia *et al.*, 2013; Nakanishi *et al.*, 2012; Marlow *et al.*, 2014; Richards and Rentzsch, 2014). These studies include the analysis of two transgenic reporter lines, which mark what is believed to be a large proportion of the nervous system, but neither line shows cells within the apical organ (Richards and Rentzsch, 2014; Nakanishi *et al.*, 2012). In addition, no FMRFamide or GLWamide positive cells have been identified within the ciliated cells of the apical organ (Nakanishi *et al.*, 2012; Marlow *et al.*, 2009). There is however a plexus of neurites underneath the apical organ cells (Figure 3D), which may be innervated by sensory cells within the apical organ. Importantly, some of the apical organ specific genes identified by Sinigaglia et al. (2015) are nervous system related, e.g. two Acetylcholine receptors.

Also, this larval organ plays a yet unresolved but crucial role in the metamorphosis process, since larva lacking it do not undergo metamorphosis (Rentzsch *et al.*, 2008). I discussed the issue of direct development and the terms metamorphosis and larva in *Nematostella* previously (1.2.1).
Contradictory to a function in sensing settlement cues is the observation that *Nematostella* planulae will develop into primary polyps without any known external cue – e.g. even single larvae in sterile artificial sea water will metamorphose into the adult form (Reitzel *et al.*, 2006).

1.4.4 Open questions

From a review of the available literature on the apical organs of metazoans, two strategies to investigate apical organs emerge. One regards the comparison of gene expression patterns at the anterior/aboral pole and the second focuses on examining settlement cues and the connection of apical organs to the nervous system. This second strategy is in general, a more

functional approach. In the light of the question of apical organ homology, both strategies have some disadvantages. If we can show that the anterior pole of bilaterians and the aboral pole of cnidarians (more precise only anthozoans or even only *Nematostella*) are homologous, we still do not know if the larval structures that develop at this pole are homologous. And function – as interesting and important as it is – is not a criterion for homology. It is also important that we consider what we mean by homology. A sound discussion of the term homology has been provided by Sommer (2008) who comes to the conclusion that the definition from Owen (1843) (the same in different animals regardless of function or form) holds still true.

Despite published statements to the contrary (e.g. Marlow *et al.*, 2014), most consider that the homology of apical organs is still not solved. Nor is the question of apical organ function(s). As described earlier, the most commonly accepted definition describes the apical organ as part of the larval nervous system. However in our model *Nematostella*, not only has no connection been shown between the apical tuft cells and the nervous system, but also the *Nematostella* nervous system does not change dramatically during the larval to juvenile transition – yet another reason to describe the development of *Nematostella* as gradual.

If apical organs sense settlement/metamorphic cues, how do larvae sense those which lack an apical organ? Why is it even lost in some species before metamorphosis takes place (e.g. Wanninger and Haszprunar (2003)?

Currently, it is still an open question what the function of the *Nematostella* apical organ is. To answer this, I believe that finding a connection between the apical tuft cells and the nervous system (or convincingly showing a lack thereof) is crucial. If we want to call it a sensory organ, then information must be transmitted from the apical tuft cells to the nervous system; though it is also a possibility that information instead goes towards the gland cells and that the apical organ of *Nematostella* does not initiate metamorphosis, but facilitates it.

Finally, to conclude whether the apical organ, and in a next step larvae in general are homologous and ancestral to cnidarians and bilaterians, we must gain greater insight into the development of this organ in different species. What is its ontogeny and what are the molecular mechanisms underlying its development? Can we distinguish between a homology of the territory it arises from and a homology of the organ itself? Can we rule out co-option in cases where we find molecular similarities? To solve these questions we will

also need to really define and separate the apical organ cells from the surrounding territory and consider these organs at a single cell resolution with regards to cell type homology.

1.5 FGF pathway

Cell to cell signalling is an important mechanism during animal development and one way to accomplish that is by the interaction of secreted proteins and cell surface receptors.

Fibroblast Growth Factors (FGFs) are a class of signalling molecules (most commonly secreted) that act via members of the receptor tyrosine kinase family (FGFRs). There are also intracellular FGFs, which will not be discussed here. FGF signalling plays many crucial roles during the development of most animals (it is also important in homeostasis and in regeneration) (reviewed in (Ornitz and Itoh, 2015; Turner and Grose, 2010). Although *FGF-like* genes (which are evolutionary related to FGFs but have quite divergent sequences) date back to the last common ancestor of animals and choanoflagellates, true FGFs are found only in cnidarians and bilaterians (Bertrand *et al.*, 2014).

FGF ligands bind with high affinity to FGFRs and with lower affinity to **h**eparan **s**ulfate **p**roteo**g**lycans (HSPGs). Interactions between FGF ligands and FGF receptors, and HSPGs or heparin have been investigated on many levels, and the interactions of all three lead to receptor dimerisation and subsequently to intracellular signal transduction (Pellegrini *et al.*, 2000; Goetz and Mohammadi, 2013; Mohammadi *et al.*, 2005). **Figure 4** shows the different branches of the FGF pathway that can be activated downstream of ligand-receptor binding, with the MAPK branch (left panel) being the most common. The downstream targets of the FGF pathway are highly tissue specific and often interactions between the FGF pathway, and other signalling pathways such as the Wnt pathway, take place. For example, FGF signalling can activate the expression of Wnt as well as that of the Wnt inhibitor Dickkopf (Dkk) (e.g. (Venero Galanternik *et al.*, 2015; Ornitz and Itoh, 2015)).
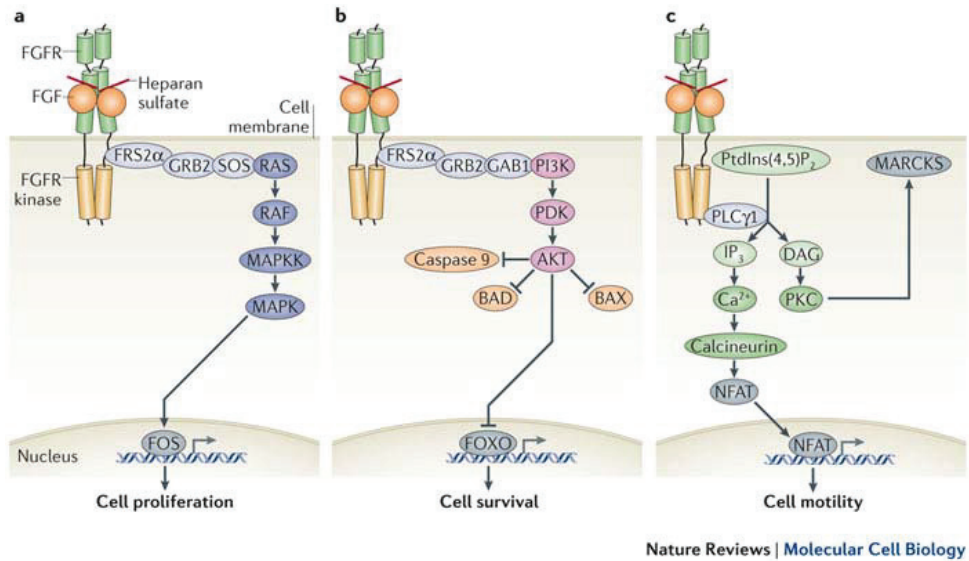
Figure 4: The FGF pathway: Taken from (Goetz and Mohammadi, 2013) Binding of the FGF ligand to the FGFR (enhanced by heparan sulfate) leads to FGFR dimerisation and reciprocal phosphorylation. **a)** The MAP kinase (MAPK) cascade is the most common downstream pathway of FGF signalling and has been shown to be the branch used by NvFGFRa to facilitate apical organ formation (Rentzsch *et al.*, 2008). Transcriptional regulation by this pathway is linked to cell proliferation but can also lead to cell differentiation, cell migration and other cellular responses. **b & c)** Alternative downstream pathways, lead to cell survival **(b)** and cell motility **(c)**. The downstream pathway in **c** has also been shown to interact with the other two branches shown in **a & b**.

1.5.1 The FGF pathway in *Nematostella*

Many FGF ligands (15), two FGF receptors, and one FGF-receptor-like "FGFRlike" (without the tyrosine kinase) have been identified in *Nematostella* and while for several of these the expression patterns are known (Matus *et al.*, 2007; Rentzsch *et al.*, 2008; Bertrand *et al.*, 2009), there is only one study that addressed their functions (Rentzsch *et al.*, 2008). In this study the role of the FGF pathway in the development and maintenance of the apical organ has been investigated. The results suggested that one of the ligands (FGFa2) has an inhibitory function, and competes with the activator ligand FGFa1 for the same receptor (FGFRa). This type of antagonistic ligand-ligand interaction would be a novelty in FGF signalling. The expression dynamics of the two ligands further imply that there are additional modulators involved in this feedback loop: *NvFGFa1* and *NvFGFa2* are first expressed in a

broad aboral domain, which then gets restricted to a smaller spot, but how this restriction is achieved is unclear. The expression ratio of the two genes changes only once during development: *NvFGFa1* is expressed more weakly maternally, but then increases its expression within the first 12 hours of development faster than the expression of *NvFGFa2* increases, and after 24-72 hours *NvFGFa1* is consistently expressed at a slightly higher level than *NvFGFa2* (tested by RT-qPCR; data not shown).

Other FGF pathway members in *Nematostella* with known features include the *FGFRlike* gene, which in the genome is in the vicinity of the FGF ligands *FGF8a* and *FGF8b*, and this may suggest a functional link (Bertrand *et al.*, 2009). The expression patterns of *FGF8a* and *FGF8b* include the pharynx and at later stages domains in the ecto-and endoderm of the apical organ region (Matus *et al.*, 2007), however as the expression of *FGFRlike* is currently unknown we have no evidence to suggest a functional link with these ligands.

NvSprouty is a candidate inhibitor of the FGF pathway and it has an interesting expression pattern, including a domain in the apical organ and in the endoderm directly beneath the apical organ, as well as around the blastopore and pharynx. The gene locus of *Sprouty* is also linked to the loci of *FGF8a*, *FGF8b* and *FGFRlike* (Matus *et al.*, 2007).

Other known modulators of FGF signalling present in the *Nematostella* genome are the aforementioned HSPGs, which will be discussed in 1.7.

In light of the essential role that FGF signalling plays in the development of the *Nematostella* apical organ, it is interesting that in bilaterians, there is no functional data on FGFs in apical organ development. However, the expression of a FGFR at the anterior pole has been reported in sea urchin (Lapraz *et al.*, 2006), *Saccoglossus* (Green *et al.*, 2013) (here in a broad and dynamic domain associated with mesoderm formation, but also in the tuft region there is expression of the ligand *fgf-sk1* (Pani *et al.*, 2012), and in *Platynereis*, where *fgfr1* is expressed in a few cells in the apical domain which are not part of the apical tuft (Marlow *et al.*, 2014).

1.6 Wnt pathway

The FGF pathway discussed in 1.5 is one example of a highly conserved developmental signalling pathway, another is the Wnt pathway, in which Wnts are the secreted signalling proteins and Frizzled is their main receptor in the cell membrane (Hoppler and Nakamura, 2014). There are three well-characterised Wnt-pathways, the canonical Wnt/β-catenin pathway, the non-canonical Wnt/PCP pathway and the Ca+ pathway (Figure 5). Central to each of these pathways is the binding of a Wnt ligand to a Frizzled receptor. I will give a brief summary of the first two pathways below.
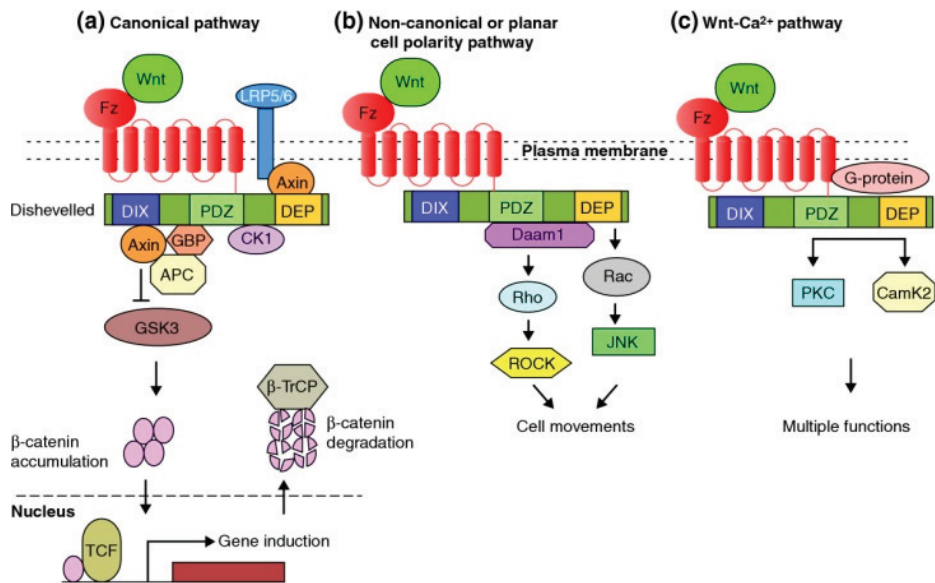
Figure 5: The Wnt pathway: (a) The canonical pathway: signalling through the Frizzled (Fz) and LRP5/6 receptor complex induces the stabilisation of β-catenin via the DIX and PDZ domains of Disheveled (Dsh) and a number of factors including Axin, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). β-catenin translocates into the nucleus and forms a complex with LEF/TCF to mediate transcriptional induction of target genes. β-catenin is then exported from the nucleus and degraded via the proteosomal machinery. **(b)** Non-canonical or planar cell polarity (PCP) signalling: Wnt signalling is transduced through Frizzled, independent of LPR5/6. Utilising the PDZ and DEP domains of Dsh, this pathway mediates cytoskeletal changes through activation of the small GTPases Rho and Rac. **(c)** The Wnt-Ca^{2+} pathway: Wnt signalling via Frizzled mediates activation of heterotrimeric G-proteins, which engage Dsh, phospholipase C (PLC; not shown), calcium-calmodulin kinase 2 (CamK2) and protein kinase C (PKC). Figure taken from (Habas and Dawid, 2005)

1.6.1 The canonical Wnt pathway, or, how to polarise an embryo

The canonical Wnt signalling pathway is one of the most well-studied pathways in animal development. In the canonical pathway, Wnt ligands form a complex with LRP5/6 and Frizzled, which activates an intracellular cascade leading to the inhibition of β-catenin degradation. β-catenin can then enter the nucleus where it acts in a complex with TCF as a transcription factor (Figure 5a; reviewed e.g. in (Habas and Dawid, 2005; Hoppler and Nakamura, 2014).

As described earlier (see 1.3), the canonical Wnt pathway is crucial in the establishment of the anterior-posterior axis in bilaterians and the oral-aboral axis in cnidarians (Wikramanayake *et al.*, 2003; Plickert *et al.*, 2006; Momose *et al.*, 2008). In this role, nuclear β-catenin at the posterior pole is key for the polarisation of the primary body axis and for giving graded positional information along this axis (Petersen and Reddien, 2009).

Outside of Cnidaria+Bilateria, Wnt family members have also been found in all animals including sponges, where they also have been shown to be expressed at the posterior end of larvae (with respect to the swimming direction) (Adamska *et al.*, 2007). Conversely in ctenophores, *Wnts* do not seem to play a role in embryonic axial polarity (Pang *et al.*, 2010), but they may be involved in later patterning events along the axis (Jager *et al.*, 2013).

1.6.2 The non canonical Wnt pathway

The non-canonical or PCP (planar cell polarity) pathway is also activated by Wnt ligands binding to Frizzled receptors, but the downstream cascade is different to the canonical pathway; it involves JNK and leads to rearrangements of the cytoskeleton. These rearrangements can promote cell migration, cell rearrangements such as convergent extension (in this process both the canonical and the PCP pathway are involved), and the direction of cilia growth/beating (Devenport, 2014).

Wnt/PCP signalling has been studied in several cnidarians, revealing a number of different roles for this pathway during cnidarian development. In *Nematostella* it has been proposed that the initial invagination of the gut is dependent on PCP signalling, and that this process is uncoupled from the endodermal gene regulatory network (GRN) which is initiated by canonical Wnt signalling (Kumuregama *et al.*, 2011; Röttinger *et al.*, 2012). Furthermore,

using *Xenopus* embryos as a heterologous system, NvWnt5 and NvWnt11 have been identified to promote the PCP pathway (Rigo-Watermeier *et al.*, 2012).

In the hydrozoan *Clytia hemisphaerica* it has been shown that the Frizzled receptor ChFz1 and ChStrabismus are crucial for planar cell polarity in the ectoderm, leading to both elongation along the oral-aboral axis, and the correct formation and beating of cilia (this is important for directed swimming behaviour) (Momose and Houliston, 2007; Momose *et al.*, 2012). A more recent study came to the conclusion that PCP signalling in *Clytia* is also involved in the regulation of endodermal and post-gastrula gene expression, in contrast to the canonical Wnt pathway that regulates gene expression along the oral-aboral axis in the larva (Lapébie *et al.*, 2014).

1.6.3 What is known about the Wnt pathway in *Nematostella*?

The expression patterns of the twelve *Nematostella* Wnt ligands have been known for many years now (Kusserow *et al.*, 2005), and there have been several studies published that have investigated the role of Wnt signalling in *Nematostella*. These studies have mostly focused on how Wnt signalling regulates the specification of the oral pole, gastrulation events and the endodermal gene regulatory network (Röttinger *et al.*, 2012; Lee *et al.*, 2007; Kumburegama *et al.*, 2011). More recent studies have also linked the Wnt pathway to patterning along the oral-aboral axis, by studying gain-of-function experiments using drug treatments (Sinigaglia *et al.*, 2013; Marlow *et al.*, 2013).

As such, there is a significant body of work describing Wnt functionality in a range of contexts of *Nematostella* development, but little is known about the Frizzled receptors that are essential for transmitting the Wnt signal. Even less is known about the presence and/or function of modulators that may act to fine-tune Wnt signalling, or about the range and ability of the Wnt ligands to diffuse along the cell surface.

1.7 Glypicans

Glypicans (Gpcs) are members of the **h**eparan **s**ulfate subfamily of **p**roteo**g**lycans (HSPGs). Proteoglycans are molecules in which one or more **g**lycos**a**mino**g**lycans (GAGs, long unbranched sugar chains consisting of disaccharide repeats) are covalently linked to a protein core. Glypicans are bound to the cell membrane by a GPI (**g**lycosyl**p**hosphatidyl**i**nositol) anchor. They can function in cell adhesion, cell crosstalk and as co-receptors in almost all the major developmental signalling pathways (Fico *et al.*, 2011; Bishop *et al.*, 2007; Häcker *et al.*, 2005; Filmus *et al.*, 2008; Lin, 2004).

Their large interactome makes HSPGs indispensable during development (Kreuger and Kjellén, 2012). Yet despite this importance, proteoglycans in general get little attention in classical Developmental Biology textbooks; this is probably due to their interactome being almost incomprehensible in size. The following overview is not exhaustive, but it aims to illustrate the myriad of functions that glypicans can play.

In mouse embryos the six *glypicans* are differentially expressed, both spatially and temporally, displaying distinct expression patterns in the developing brain. Their expression domains include important signalling centres which are sources of Wnt, FGF and BMP signals (Luxardi *et al.*, 2007). The significance of this expression is seen in the range of developmental defects that occur upon loss-of function experiments, reviewed in Fico *et al.* (2011).

Capurro *et al.* (2014) showed in cell lines that GPC3 interacts with Wnt ligands and the Frizzled receptor to stimulate signalling, and that binding of GPC3-Wnt leads to endocytosis of the complex, while in contrast GPC6 inhibits Wnt signalling. The interactions of Glypicans and Wnts shall be reviewed further in later sections, in connection to the sulfation state of HS chains.

In *Xenopus* Ohkawara *et al.* (2003) suggested that Xgly4 (a glypican 4 orthologue) acts as a positive regulator in non-canonical Wnt/PCP signalling, leading to convergent extension movements during gastrulation. A similar function has been reported for the zebrafish glypican4 (named knypek in that study) (Topczewski *et al.*, 2001). Meanwhile dynamic expression patterns of all ten zebrafish *glypican* genes during embryonic development have been documented (Gupta and Brand, 2013) and in a recent study Venero Galanternik *et al.*

(2015) could pinpoint signalling pathway modulations by HSPGs to the particular sulfation state of the HS chains (see also 1.7.1 and 1.7.3).

Outside the vertebrate models, in sea urchin, *glypican5* is expressed asymmetrically during development and is controlled by BMP2/4 (Lapraz *et al.*, 2009). Inhibition of HS sulfation in sea urchin led to defects in oral-aboral patterning, which is mediated by Nodal signalling (Bergeron *et al.*, 2011). Note that the oral-aboral axis in sea urchin is the secondary axis, perpendicular to the primary animal-vegetal axis the two axis are linked by a Wnt-FoxQ2-Nodal interplay (Yaguchi *et al.*, 2008).

In *C. elegans* multiple HSPGs are involved in neuron migration and act in parallel. The HS modifications of these HSPGs seem to be dependent on the core protein (Kinnunen, 2014). However, *C. elegans* has unlike other animals only one copy of each core protein coding gene and enzyme involved in HSPG formation, and this may influence the specificity of HS modifications.

The difficulty which arises in studies on glypicans is that they usually have multiple functions, depending on the characteristics of the HS chains they bear. On the other hand, if one only focusses on studying the HS chains, the problem of pinpointing single functions still arises, since the HS chains can be attached to different core-proteins and are highly variably modified. In order to understand the function of glypicans, we therefore always also need to look at the HS chains as well; and in turn, if we want to know the function of HS chains then we need to look at the core-proteins. Only by comparing the insights gained on both sides (core protein + HS chains) can we increase our knowledge about the undeniably important role that HSPGs play in animal development.

1.7.1 Heparan sulfate biosynthetic machinery

HS biosynthesis involves multiple enzymes and takes place in the Golgi, as summarised in **Figure 6** and reviewed for example by Gorsi and Stringer (2007) and Kreuger and Kjellén (2012).

Briefly:
The initial step is the formation of a linkage region to a serine, next to a glycine of the core protein. The linkage region is composed of a tetrasaccharide. The chain is extended by

enzymes of the extostosin (Ext) family. The chain of 50-400 sugar units is than modified by a series of enzymes. First the NDSTs (N-deacetylase/N-sulfotransferase) bifunctional enzymes deacetylate and sulfate the nitrogen. The next step is C5-epimerisation of specific GlcAs (glucuronic acids) and 2-O-sulfation, followed by 6-O-sulfation and finally 3-O-sulfation.

Esko and Selleck (2002) suggested that the modifying enzymes form a so-called "GAGosome complex" in which all these enzymes are linked to each other. This is based on the fact that almost all of the enzymes are transmembrane proteins and they are found in the same region – in the cis/medial Golgi – and because some interactions between the enzymes have also been shown, reviewed in Kreuger and Kjellén (2012). This idea has found more support over the years and it seems to be a likely option although direct proof is still missing (Lindahl, 2014).

The specificity with which HSPGs can act in the different signalling pathways relies on these modifications of the sugar chains. The selectivity of the different HS sulfation motifs and whether there is such a thing as a "sulfation code" remains unclear (Kreuger and Kjellén, 2012; Lindahl, 2014). Modifications can also happen later on, at the cell surface, for example through endosulfatases which can remove non terminal sulfations (reviewed in: (Esko and Selleck, 2002; Bülow and Hobert, 2006; Lamanna *et al.*, 2007)) and will be discussed in 1.7.3.). Also at the cell surface, the enzyme heparanase can chop fragments of the HS chain off. This degradation can potentially lead to small bioactive fragments of HS in the ECM (extracellular matrix); the core protein can then be recycled after being internalised by the cell (Kreuger and Kjellén, 2012).

Another "modification" is the shedding of the whole HSPG from the cell membrane. Although for the past decade it has been believed that Notum enzymes can shed glypicans by cutting their GPI anchors (Kreuger *et al.*, 2004) a recent study showed that this is actually not the case, and that Notum instead acts by deacylation of Wnt ligands, and uses glypicans as a scaffold (Kakugawa *et al.*, 2015). Nevertheless, there are other enzymes such as secreted serine proteases which can shed HSPGs and thereby influence ligand concentrations and/or diffusion, and co-receptor activity (Hou *et al.*, 2007).

As for many aspects of developmental biology, our knowledge of HSPGs is restricted to insights gained in a few model organisms. For HSPGs almost all available data have been

generated in the classical model systems of *Drosophila*, zebrafish and *C. elegans,* as well as human cell lines. There are also a few studies in the sea urchin *Strongylocentrotus purpuratus* (Fujita *et al.*, 2010; Lapraz *et al.*, 2009), but our knowledge in invertebrates remains very limited. Finally, the ability of HS to interact with many different, structurally unrelated ligands, suggests that these interactions are the result of convergent evolution (Lindahl, 2014). This hypothesis makes it very interesting to investigate the state of HSPGs in cnidarians, and to consider whether any of the interactions described in bilaterians already took place in the last common ancestor of cnidarians and bilaterians.
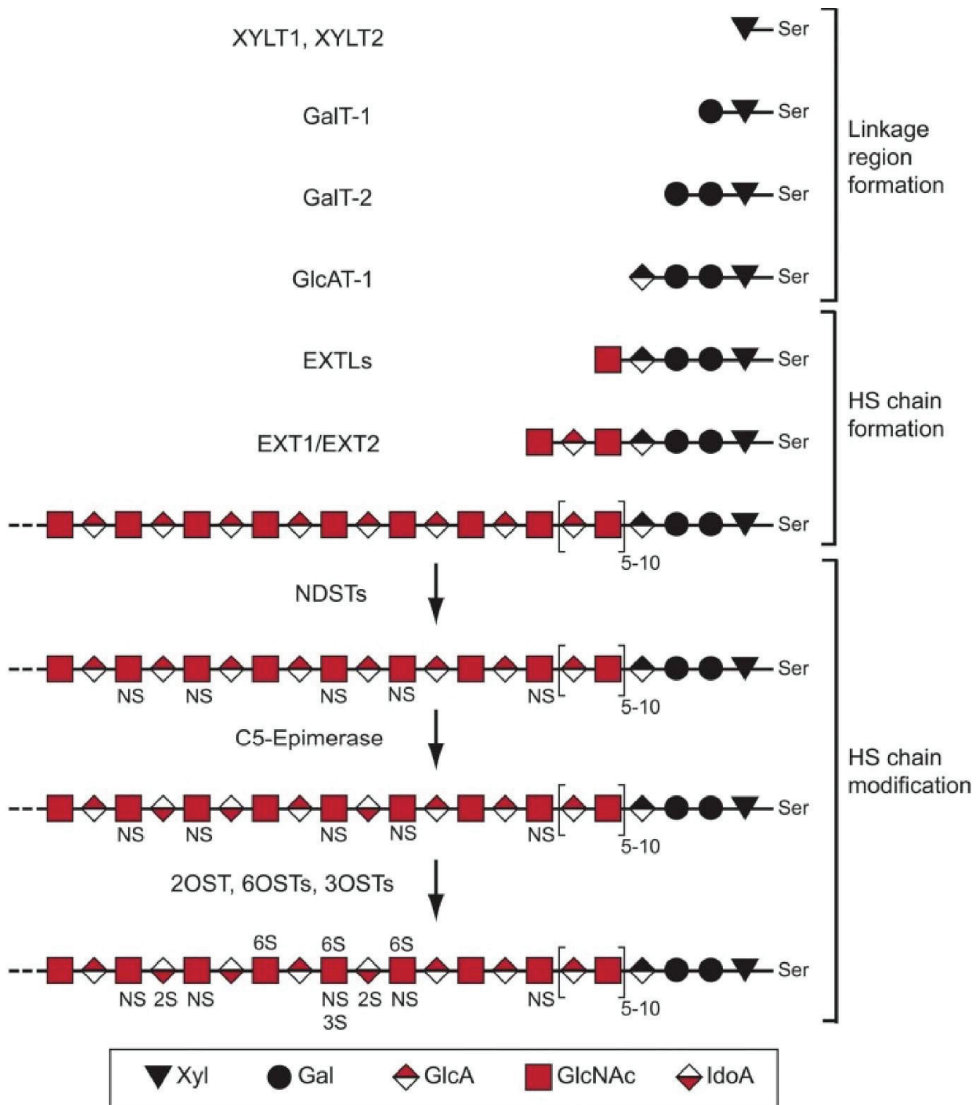
Figure 6: Heparan sulfate biosynthesis: (Figure taken from Kreuger and Kjellén (2012)) HS biosynthesis starts with the formation of a glucuronic acid (GlcA)-galactose (Gal)-galactose-xylose (Xyl) tetrasaccharide linkage region. A glycosyltransferase (EXTL) attaches a N-acetylglucosamine (GlcNAc), this step initiates HS chain formation and is different to chondroitin sulfate synthesis. Extostosins 1 and 2 (EXT1/2) extend the chain by adding GlcA and GlcNAc alternating. The following steps of HS chain modification by sulfotransferases and epimerases are described in the main text.

1.7.2 What is known about HSPG synthesis and interactions in cnidarians?

A previous study revealed that orthologues of HSPG modifying enzymes are present in the *Nematostella* genome (Feta *et al.*, 2009). Interestingly, the number of different members of the enzyme classes varies a lot compared to the mammalian situation, suggesting a degree of subfunctionalisation and convergent evolution. Whereas in mice and humans there is only one 2OST, there are two in *Nematostella*, in contrast there are three 6OSTs and seven 3OSTs in mammals, but only one each in *Nematostella*. Feta *et al.* (2009) also showed that the *Nematostella* heparan sulfate enzyme machinery is used to synthesise a polysaccharide with an unusual structure. This may support a view of HS chains *per se* being conserved modulators of signalling, but that the specificity of HSPG binding may have evolved convergently.

Regarding other potential HSPG interaction partners in cnidarians, there are seven (!) *Notum* genes in *Nematostella* (Lapébie *et al.*, 2014), though currently we only know the expression pattern of one of these; it is expressed at the oral pole. Lapébie *et al.* (2014) identified two *Notums* in *Clytia* and showed that one is active at the aboral pole and one at the oral pole and that both are regulated by Wnt signalling. This expansion of *Notum* genes, and their axial expression domains are interesting points for considering whether HSPGs might contribute to the regulation of the cnidarian primary axis.

As a final note, there have also been some studies in cnidarians which have focused on activities of proteoglycans as scaffolds for nematocyst development in *Hydra* and *Nematostella* (Ozbek *et al.*, 2010; Yamada *et al.*, 2007; Adamczyk *et al.*, 2010; Yamada *et al.*, 2011). The nematocyst is the proteinaceous stinging capsule found within the nematocytes – which are the characteristic stinging cells of cnidarians. However, from these studies it appears that it is mainly chondroitin sulfates, and not heparan sulfates, that contribute to this function.

1.7.3 The endosulfatase Sulf

Various studies have shown that the sulfation or desulfation of HS significantly changes the specificity of ligand binding in the BMP, the FGF and the Wnt signalling pathways (Dhoot

et al., 2001; Kleinschmit *et al.*, 2013; Ai *et al.*, 2003; Venero Galanternik *et al.*, 2015; Wang *et al.*, 2004; Viviano *et al.*, 2004). In these studies, the investigators either inhibited sulfation (mostly by treatment with sodium chlorate – which inhibits PAPS formation; PAPS is the sulfate donor for HS sulfation) or by doing gain- and loss-of-function experiments of the endosulfatase (Sulf) enzyme.

Sulfs are unique sulfatases in that they remove non-terminal sulfations, unlike all other described sulfatases, which are exosulfatases and are involved in glycosaminoglycan degradation (Lamanna *et al.*, 2007). Sulfs are membrane bound, and they act both in the Golgi as well as on the cell surface, and can therefore regulate signalling cell autonomously (Ai *et al.*, 2003). Sulfs remove only 6-O-sulfations and are HS specific (they do not act on chondroitin sulfate), and this has been shown both *in vitro* and *in vivo* (Ai *et al.*, 2003; Lamanna *et al.*, 2006).

The abovementioned studies on the influence of sulfation on developmental signalling pathways revealed that particularly 6-O-sulfation, which is removed by Sulfs, regulates extracellular signalling both positively and negatively in different phyla. For example, Reichsman *et al.* (1996) showed, that desulfation inhibits Wnt signalling in *Drosophila* and that this effect could be rescued by heparin. In contrast in quail, Dhoot *et al.* (2001) showed that desulfation through Sulf activity enhanced Wnt signalling, leading them to the assumption that Wnt ligands bind to HS and need to be released by Sulf to bind to the Frizzled receptor.

A study by Kleinschmit *et al.* (2013) showed that vertebrate and *Drosophila* Sulfs have the same enzymatic activity, but that the outcome is different. In vertebrates Wnt signalling is enhanced, while in *Drosophila* Sulf facilitates Wg degradation. Two additional, recent studies looked at the influence of HS chain sulfation on signalling modulation in different vertebrate models. One showed that in zebrafish, desulfation promotes Wnt only through an indirect effect by inhibiting the FGF pathway (Sulf actually also inhibits FGF signalling in quail (Wang *et al.*, 2004)), which activates the Wnt inhibitor Dickkopf (Dkk) (Venero Galanternik *et al.*, 2015). The second study showed that the effects of Sulf1 on Wnt signalling (both canonical and PCP) are ligand specific (Fellgett *et al.*, 2015).

To summarise, it seems to be a common theme, that on one hand the main players in HSPG synthesis and modification have all been already present in the last common ancestor of

bilaterians and cnidarians, and that they all have maintained similar or even identical functions in modern lineages, but on the other hand, there have been linage specific duplications of different modulators (glypicans, NDSTs, OSTs, Notums, Sulfs). As such, HSPGs have evolved to become a highly flexible, yet nonetheless highly specific system for fine-tuning the major intercellular signalling pathways.

2.   Aim of the study

Axial patterning

Data regarding the establishment of body axes in the starlet sea anemone *Nematostella* are accumulating in recent years. These data have revealed unifying features with the formation of bilaterian axes, as well as cnidarian-specific characters. But what remains unclear are the mechanisms that pattern the animal along these axes. Identifying these mechanisms has large implications for understanding the homology of axes in the Metazoa, and the homology of the organizing centres and structures which appear along, and at the end points, of the axes. With the experiments presented in this thesis, my colleagues and I have aimed to shed light on these open questions.

The presence of an anterior domain defined by a TF cascade headed by Six3/6, and a posterior, β-catenin dependent domain, is well documented in bilaterians and cnidarians. One of the central questions for understanding the mechanisms and the evolution of axial patterning regards how these territories are initially established. This question, however, also has not been addressed in previous studies. In cnidarians, the processes that lead to the formation of these two opposing domains, and whether the anterior and posterior patterning systems interact to maintain the two territories, is unclear. The first study presented in my thesis targeted these early processes and interactions in *Nematostella* using loss and gain of function experiments, including single blastomere injections to cause mosaic gene knockdown. The results and insights obtained by these experiments are presented in paper I.

Apical organ

The unifying feature of my work is the larval apical organ of *Nematostella*, whose development is tightly linked to the development of the anterior pole as a result of axial patterning. As such, a main aim of this work was to look at the apical organ from different angles. How can we understand its development and function better? What cells is it composed of and what is the identity and function of the genes that are active in these cells?

Glypicans and signal pathway evolution

The Wnt and the FGF pathway play important roles in axial patterning and the development of the apical organ respectively. That is why we targeted potential modulators of those two signalling pathways. The main focus was here on a potential role for glypicans as co-factors

in these pathways during *Nematostella vectensis* larval development. We also attempted to connect different functions of glypicans to different modifications of their HS chains.

The following questions formed the basis of this investigation:

What is the role of glypicans in the Wnt pathway during regulation of axial patterning?

What is the role of glypicans in the FGF pathway during apical organ formation?

What role does HS sulfation, especially 6-O-sulfation, play in glypican mediation of Wnt and FGF signalling?

In summary, the studies presented in my thesis report the analysis of two larval features (the primary axis and the apical organ) which can contribute valuable information towards two broad questions in evolutionary developmental biology – namely, the evolution and homology of marine invertebrate larvae; and the evolution and function of signalling pathways and their modulators.

3. List of publications

Paper I

Leclère L., Bause M., Sinigaglia C., Steger J. and Rentzsch F. "β-catenin establishes a *six3/6*
  - *frizzled5/8* feedback loop that patterns the aboral domain of *Nematostella*",

  Development, under review

Paper II

 Bause M., van der Horst R. and Rentzsch F. "*Glypican1/2/4/6* and sulfated
  glycosaminoglycans regulate the patterning of the primary body axis in the cnidarian
  Nematostella vectensis"

   In preparation

4. Summary of results

4.1 Glypicans

Our investigation of *glypican* genes in *Nematostella* revealed that the two major groups of *glypicans* (Gpc1/2/4/6 and Gpc3/5) are likely to have been already present in the cnidarian+bilaterian ancestor (Figure S1 paper II). *Nematostella* possesses two *glypican* genes: *NvGpc3/5* within the Gpc3/5 clade, and *NvGpc1/2/4/6* which is likely a member of the Gpc1/2/4/6 clade. The *Nematostella glypicans* are expressed opposingly to each other during early development, in clearly defined domains along the oral-aboral axis (Figure 1 paper II). The *NvGpc1/2/4/6* domain encompasses the expression domains of *NvFz5/8*, *NvFGFRa*. The uniquely cell-surface localised heparan sulfate modulator *NvSulf* is expressed in a domain which includes the *NvGpc3/5* domain and large parts of the *NvGpc1/2/4/6* domain (Figure 1 paper II). The aboral pole of the ectoderm shows no expression of *NvSulf*, but strong expression of *NvGpc1/2/4/6*.

4.2 Oral-aboral patterning

By undertaking detailed experimental studies of Wnt signalling, and its interactions with other axial patterning components, we uncovered that β-catenin initiates gene expression at the aboral domain of *Nematostella*. In a second step a *six3/6-frizzled5/8* feedback loop establishes to control the size of the aboral domain.

Overexpression of *NvSix3/6* led to the expansion of the aboral domain at the expense of mid-body gene expression at gastrula stage (Figure 1 paper I). Knockdown of *NvSix3/6* on the other hand had the opposite effect in gastrulae, but earlier blastula stages exhibited no patterning defects. This revealed that this TF controls the aboral domain, but does not initiate its development. Instead, we found that initiation of the aboral synexpression group requires β-catenin activity (Figure 2S-U paper I). Single blastomere injections with a β-catenin morpholino inhibited marker gene expression in progenitors of the injected blastomere at the aboral pole. This indicates that aboral nuclear β-catenin activates the aboral network cell autonomously. When β-catenin knockdown animals were treated with Azakenpaullone, thus stabilising the small amounts of cytoplasmic β-catenin remaining, aboral gene expression

was recovered, but not oral marker expression (Figure 2 paper I). These results indicate that β-catenin activates oral and aboral signalling centres in a dose dependent manner. This was further confirmed by RT-qPCR, showing that aboral markers are regulated differently (up and down) by different morpholino concentrations, whereas for oral markers only the level of downregulation varied (Figure 2Q, R paper I).

The Wnt receptor *NvFz5/8* is expressed at the aboral pole, and its expression domain is controlled by Nvβ-catenin and NvSix3/6 (Figure 3 paper I). Nvβ-catenin suppresses *NvFz5/8* expression in the oral half, while NvSix3/6 enhances its expression in the aboral half. Knockdown of *NvFz5/8* showed that the Wnt receptor limits the size of the aboral domain during gastrulation (Figure 4 paper I). This was visible by the shifted expression domains of aboral and midbody marker genes towards the oral pole in *NvFz5/8* knockdown animals. We observed a similar effect upon knockdown of *NvGpc4/6* and also after treatment with the GAG sulfation inhibitor sodium chlorate (Figure 2 paper II).

At planula stage these patterning changes along the oral-aboral axis no longer affected all marker genes (Figure 4X-AA paper I & Figure S5 paper II). This reflects a change in the aboral gene regulatory network (Figure 7 paper I). The central region of the aboral pole exhibited a different expression profile, and undergoes differential expression regulation to the surrounding region of the aboral pole (see also 4.3).

NvFz5/8 knockdown also led to a failure of body elongation (Figure 6 paper I), presumably due to altered cell rearrangement in the ectoderm (Figure S2). This effect was independent of *NvGpc4/6* and GAG sulfation. Knockdown of *NvGpc1/2/4/6* and chlorate treatment did not lead to shortening along the primary axis, also no reduction in rosette formation was observed (data not shown).

It is now clear from our results, and from previous studies, that the oral-aboral axis in *Nematostella* is established by Wnt signalling. In contrast to earlier studies, we also found that Nvβ-catenin has a positive role at the aboral pole, which may be a characteristic that is specific to *Nematostella*.

Subsequent to the establishment of axial polarity by Wnt signalling, the patterning along this axis is controlled by the interaction between two signalling centres, which occupy opposing ends the of axis. A Wnt signalling centre is at the oral end, and at least one of the Wnt ligands signals to the NvFrizzled5/8 receptor that is localised at aboral domain where it

antagonises NvSix3/6 controlled gene expression. Furthermore, we have identified NvGpc4/6 and GAG sulfation as factors that are involved in the signal transduction, and which likely provide fine-tuning to these signalling systems.

4.3 Apical organ

Following on from the investigation of how axial patterning is established in *Nematostella*, we examined the development of the apical organ at the aboral pole.

By using both single and double knockdown experiments, we revealed that *NvFz5/8* regulates the size of the apical organ by interacting with FGF signalling in the aboral domain (Figure 5 paper I). Further, that *NvFz5/8* and *NvGpc4/6* morphants have a reduced apical tuft (Figure 5 paper I & Figure 3 paper II). By using marker genes for subpopulations of cells within the aboral pole, we found that they also exhibit disruption of aboral patterning at planula stage. *NvFoxJ1* and *NvFGFa2* are markers for the central region (and most likely the long ciliary cells); *NvMeprin-like* and *NvTauD* demarcate small ring domains at the outer boundary of the apical organ; and *NvSix3/6* is expressed in a broader ring that surrounds the apical organ (Figure S1 paper I & Figures 4, 5 & S7 paper II).

In *NvGpc4/6* morphants, there is a strong correlation between patterning changes, apical tuft size and morpholino concentration (translated into level of knockdown) (Figure S3 paper II). With increasing severity of phenotype, the expression domains of marker genes shift towards the centre of the aboral pole, either collapsing from a ring to a spot domain, a large spot to a small spot, or being reduced below detection levels (Figure 5 & S3 paper II).

As at gastrula stage, in planulae sodium chlorate treatment also results in patterning defects that are similar to *NvGpc4/6* and *NvFz5/8* knockdowns (Figure 6 paper II). Having said that, there are also undeniable differences in the three phenotypes at this stage: Whereas in *NvGpc4/6* and *NvFz5/8* knockdown the diameter of the *NvMeprin-like* and *NvTauD* domains were generally reduced, they were often expanded after sodium chlorate treatments (compare Figure 5 and Figure 6 paper II).

5. Additional results

5.1 NvTauD::mOrange transient expression

The taurine dehydrogenase NvTauD was initially identified as it was differentially expressed in a microarray analysis that compared animals with an extended, and animals with an absent apical organ; these phenotypes were generated via either *NvFGFa1* or *NvFGFa2* knockdown (Sinigaglia *et al.*, 2015). *NvTauD* expression is not detectable before gastrula stage, when it covers the aboral pole. At planula stage only a circle of cells, that are either located at the outer edge of the apical organ or just surrounding it, expresses *NvTauD* (Sinigaglia *et al.*, 2015).

Due to this striking expression pattern, we selected the promoter of *NvTauD* as a good candidate in our approach to use transgenic animals to study the apical organ in *Nematostella*. We generated a transgenic construct in which a promoter region of *NvTauD* drives the expression of a membrane-bound orange fluorescent protein, mOrange (Shaner *et al.*, 2004).

The predicted start site of *NvTauD* was identified by 5prime RACE; and the upstream genomic DNA from this site was cloned and sequenced. From this we were able to isolate a putative promoter sequence of 2.6 kb, which we cloned in front of mOrange. Transient transgenic animals were produced using the meganuclease method (Renfer *et al.*, 2010), with the construct being injected into fertilised eggs. Transient expression of mOrange was observed after 48 hpf and expression was strongest at six days post-fertilisation.
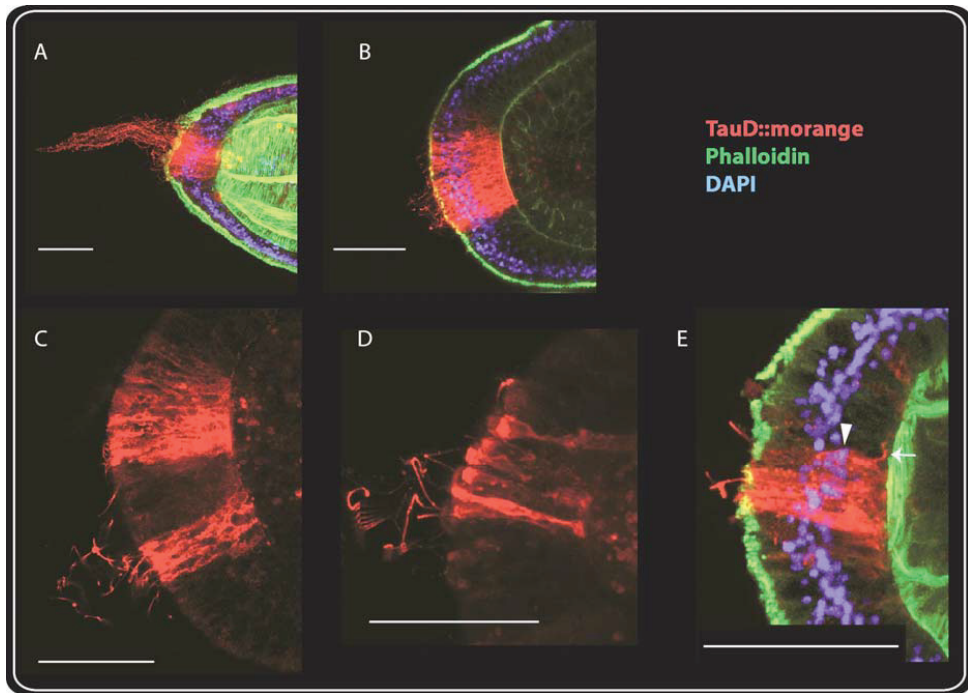
Figure 8: TauD::mOrange F0 animals: A-E) Aboral pole of mOrange positive planulae after injection with a construct containing the NvTauD promoter in front of mOrange. Aboral is to the left. **C-E)** show different cell types; columnar ciliated cells in **C&D**. White arrowhead in **E** marks a short cell with basal extensions (marked with white arrow). Pictures are maximum projections of confocal stacks. mOrange (shown in red)is visualised using anti-DsRed antibodies. F-Actin is stained with phalloidin (green) and nuclei with DAPI (blue). Scale bars = 50μm

Figure 7 shows immunostaining of mOrange positive animals. To aid in visualising NvTauD:mOrange expression, we used cross-reactive antibodies that were raised against the closely related DsRed protein to amplify the signal from mOrange. Transient expression of transgenes is usually mosaic in *Nematostella* (Renfer et al., 2010), and is also not restricted to the endogenous activity domain of the promotor. As such, it is striking that the transient expression of NvTauD::mOrange was tightly constrained. With the exception of a few single cells in other parts of the larvae, only the cells of the apical organ region showed expression of mOrange. Among these were cells bearing the long cilia of the apical tuft (Figure 7A-D). As mOrange is membrane bound, the cilia of these cells were clearly visible. Intriguingly we also identified some smaller mOrange expressing cells with basal extensions, which could be neurites (Figure 7E).

5.2 *NvSulf* knockdown affects aboral patterning

The fact that the region surrounding the apical organ co-expresses *NvSulf*, *NvFz5/8* and *NvGpc1/2/4/6* and the region abbuting more orally co-expresses *NvSulf*, *NvWnt* and *NvGpc1/2/4/6* awoke our interest. If desulfation of HS chains affects patterning, what are the effects of an increase in 6-O-sulfation? – The following section shows preliminary results of NvSulf morpholino injection.
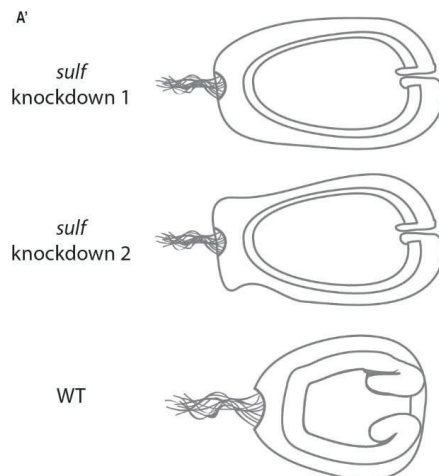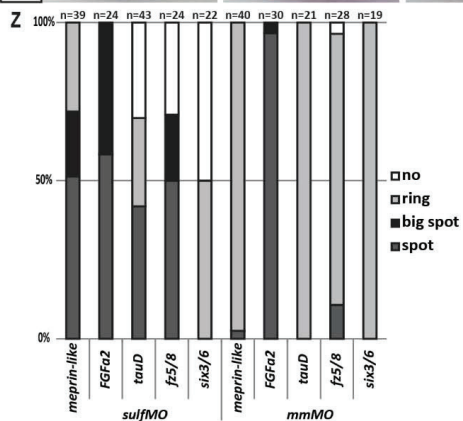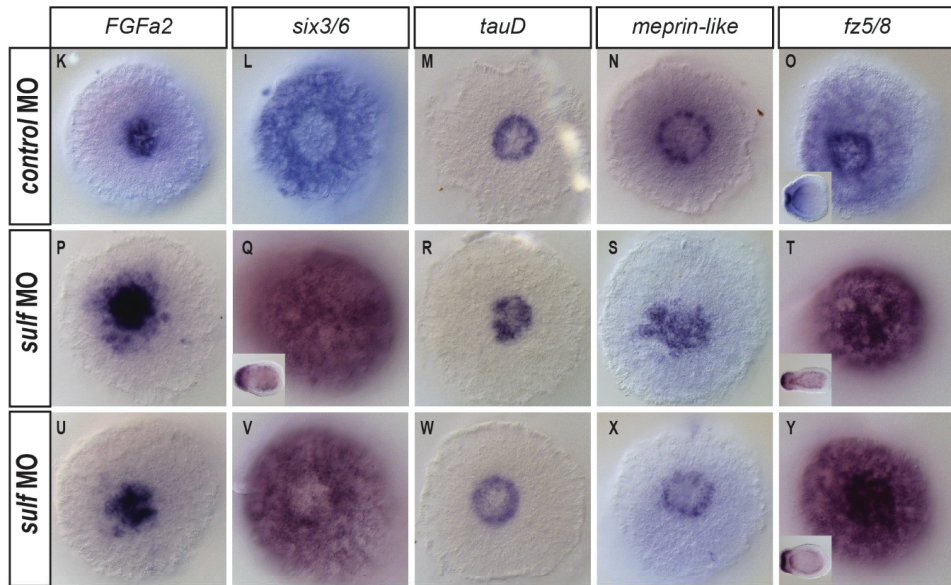
Injection with a *NvSulf* morpholino showed no change in the tested marker gene expression at gastrula stage, indicating that ectodermal oral-aboral patterning depends on 6-O-sulfated HS chains at the aboral pole at this time (Figure 8A-J).

In contrast, at planula stage knockdown of *NvSulf* caused patterning defects similar to *NvGpc4/6* knockdown (Figure 8K-Y). *NvSix3/6* expression showed no gap in 50% of the injected animals and 60-70% of the animals showed changes in *NvMeprin-like* (ring to spot) and *NvTauD* expression (ring to spot or gone). Also *NvFz5/8* expression in the apical organ region was affected, no animal showed the WT or control ring domain, rather, *NvFz5/8* was expressed either in a spot (in 50% the spot covered an enlarged region and in 21% the spot domain corresponded in size to the WT ring domain) or with no difference to the surrounding low level *NvFz5/*8 expression domain (29%) (Figure 8Z).

Morphologically, *NvSulf* knockdown planulae are elongated and almost cylindrical, and in some cases displayed a thickening at the aboral pole (Figure 8A'; inset in 8T).

(next page)

Figure 9 *NvSulf knockdown:* **A-E)** Control morpholino injected animals at gastrula stage (lateral view, oral to the right). **F-J)** SulfMO injected animals at gastrula stage show no obvious change in gene patterning along the oral-aboral axis. **K-L)** Control MO injected planulae, aboral view. **P-Y)** SulfMO injected animals exhibit patterning changes at the aboral pole by planula stage. **Z)** Quantification of patterning phenotypes; "no" represents undetectable expression. **K)** Sketches of phenotypes.

	FGFa2	six3/6	fz5/8	wnt2	fkh
control MO	A	B	C	D	E
sulf MO	F	G	H	I	J

	FGFa2	six3/6	tauD	meprin-like	fz5/8
control MO	K	L	M	N	O
sulf MO	P	Q	R	S	T
sulf MO	U	V	W	X	Y

Z

□ no
▨ ring
■ big spot
■ spot

n=39 n=24 n=43 n=24 n=22   n=40 n=30 n=21 n=28 n=19

meprin-like FGFa2 tauD fz5/8 six3/6   meprin-like FGFa2 tauD fz5/8 six3/6
sulfMO   mmMO

A'

sulf knockdown 1

sulf knockdown 2

WT
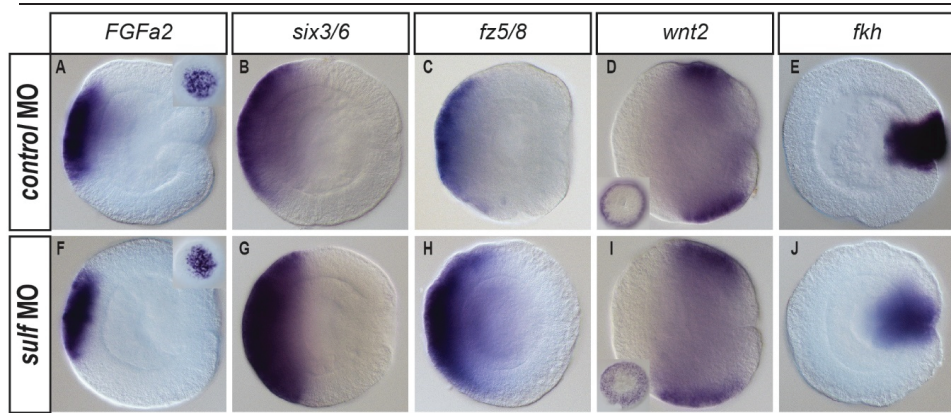
5.3 Glypicans outside the Bilateria

Beyond the Bilateria, very little is known about the distribution of glypicans. Here I make a short analysis of the presence, absence, and molecular features of these molecules in non-bilaterian clades.

The two *Nematostella* glypicans contain 14 conserved cysteine residues, a feature shared by all glypicans found so far (Fico *et al.*, 2011). NvGpc1/2/4/6 also has seven potential GAG attachment sites, consisting of a serine N-terminal to a glycine near the C-terminus (Häcker *et al.*, 2005). NvGpc3/5 is lacking this conserved feature.

Glypican-like genes can be found in the genomes of all investigated early branching metazoans, in the sponges *Amphimedon queenslandica* (2) and *Sycon ciliatum* (3), as well as in the ctenophores *Mneiopsis leidyi* (3) and *Pleurobrachia pileus* (3) and also in *Trichoplax adhaerens* (2). Of the 14 conserved cysteine residues, 5 were shared in all putative glypicans and most were present in all but one or two hypothetical protein sequences. Also the GAG attachment sites are present in most cases. Some of the ctenophore *glypican*-like genes are most likely lineage or species specific duplications. A clear grouping into the two bilaterian glypican groups was not possible.

As an additional note, similar to bilaterians, Nv3OST is the only enzyme of the HS chain modulating enzymes in *Nematostella* which is not membrane bound (data not shown, based on sequence and domain predictions).

6. Discussion

I will herein discuss our findings in the broader picture of the evolution of animal development. The focus will be on the main questions raised in the introduction, namely, how is axial patterning governed in *Nematostella*? What does it tell us about axial patterning evolution? How can we generate data to compare larval traits, in particular the apical organ? And what do HSPGs add to the plate? Please find additional discussion of the experimental work in the discussion sections of the two papers.

6.1 Axial patterning in *Nematostella*

The establishment of polarity and subsequently of body axes is a crucial step in animal development. Our findings in *Nematostella* corroborate an ancient link between β-catenin activity, gastrulation, and the establishment of the primary body axis.

The polarisation of early cleavage stages by β-catenin is widespread in the Metazoa (see 1.3 and 1.6). And also in *Nematostella* localized Dsh and subsequently localized β-catenin has been reported as the driving force that polarises the embryo (Lee *et al.*, 2007). This early polarisation leads to the formation of the primary body axis, the oral-aboral axis. Within this ancient system however, we also found some peculiarities in *Nematostella* that have not been found in other animals.

An oral centre initiated by high β-catenin and an aboral centre initiated by low β-catenin

Evidence accumulating from a number of previous studies supports the existence of two signalling centres in *Nematostella*, at either side of the oral-aboral axis, one controlled by Wnt (posterior) and one controlled by Six3/6 (anterior) (Sinigaglia *et al.*, 2013; Röttinger *et al.*, 2012; Marlow *et al.*, 2013). In the present paper I we have addressed the question how these two centres are initially established, and how they interact.

Prior to our work, endoderm specification in *Nematostella* has been linked to localised nuclear β-catenin (and thus Wnt signalling) at the vegetal pole (Wikramanayake *et al.*, 2003; Lee *et al.*, 2007); in contrast, gastrulation may instead be driven by the PCP branch of the Wnt pathway (Kumburegama *et al.*, 2011). In bilaterians inhibition of β-catenin function

leads to anteriorization through reduction of posterior marker gene expression and expansion of anterior markers (Darras *et al.*, 2011; Wikramanayake *et al.*, 1998).

Our results challenge certain views in the aforementioned studies: First, we found that nuclear β-catenin is not only necessary to induce endodermal fate in *Nematostella*, but also, that low levels of nuclear β-catenin are needed to induce aboral gene expression, as shown by the single blastomere injections. As such, in contrast to the data from bilaterians, we report that β-catenin has a role in activating expression at the aboral/anterior pole. The second controversy is that our results suggest that β-catenin signalling is in fact also involved in gastrulation in *Nematostella*, as β-catenin MO injected animals failed to gastrulate. This suggestion is actually in line with observations made by Kumburegama *et al.* (2011) in their study, but discounted by these authors. They described that cadherin, which binds to β-catenin and reduces its free concentration, also blocks gastrulation events, as previously described in (Wikramanayake *et al.*, 2003).

In an attempt to confirm our hypotheses about β-catenin functioning along the entire oral-aboral axis at the protein level, we conducted β-catenin antibody stainings at early stages in *Nematostella*, using a cross-reactive antibody raised in rabbit (Sigma-Aldrich C2206). From this we observed that nuclear β-catenin was present in a much broader domain then the domain in which Nvβ-catenin-GFP expression was previously reported (data not shown; Wikramanayake *et al.* (2003). However, we considered our results to be inconclusive, in that we could not rule out that a small group of future aboral cells lacked nuclear β-catenin. A custom *Nematostella* β-catenin antibody would be desirable to obtain clarity in this question.

Is the oral half of *Nematostella* patterned by a Wnt code?

In *Nematostella*, Wnt ligands are not only expressed in a staggered manner along the oral-aboral axis, they are also the most likely candidates behind patterning the embryo along this axis. Confirmation or rejection this attractive hypothesis will require single and combined knockout experiments to find out the specific function of each Wnt, as well as binding assays with the different Frizzled receptors. Overexpression studies could also be informative. Studies in heterologous systems may also provide useful data (Rigo-Watermeier *et al.*, 2012), even so the ability of a ligand to activate a receptor in a heterologous system does not necessarily mean that the same downstream pathway is activated by this ligand in its natural environment. Building upon our studies, we would suggest that NvGpc3/5 and NvSulf should also be examined in this context of Wnt ligand functionality, although as NvGpc3/5

is lacking HS attachment sites, it is a less likely candidate for Wnt modulation than NvGpc1/2/4/6. Nonetheless, direct interactions of the glypican core protein with Wnt ligands have been reported in other animals (Yan *et al.*, 2009).

A NvSix3/6 NvFz5/8 feedback loop at the heart of the anterior GRN

It has been previously reported that the aboral GRN of *Nematostella* operates differently at different stages, with different grades of complexity and with different regulatory architecture (Sinigaglia *et al.* (2013) and this study). In the centre of this network at gastrula stage is an interplay between NvSix3/6 and NvFz5/8 to regulate the size of the apical domain. NvSix3/6 activates NvFz5/8, whereas NvFz5/8 inhibits NvSix3/6, and this feedback loop is the heart of the interaction between the oral and aboral signalling centres to control the size of the oral and the aboral domain.

A feedback loop between Six3 and Frizzled at the anterior pole has also been described in sea urchin (Range *et al.*, 2013; Wei *et al.*, 2009). While there is currently no functional data to support this loop also being active in other species, overlapping expression of Six3 and Frizzled orthologs has also been found in *Platynereis*, *Tribolium, Saccoglossus* and vertebrates thus including all major eumetazoan clades (Cnidaria, Lophotrochozoa, Ecdysozoa, Ambulacraria and Chordata) except Ctenophores (Steinmetz *et al.*, 2010; Marlow *et al.*, 2014; Beermann *et al.*, 2011; Posnien *et al.*, 2009; Lowe *et al.*, 2003; Pani *et al.*, 2012; Kim *et al.*, 1998; Seo *et al.*, 1998).

From these combined date a picture is developing of an ancient patterning centre at the anterior pole of the cnidarian+bilaterian ancestor, which not only comprised a TF cascade headed by Six3/6 and inhibiting Wnt signalling (most likely via SFRP and Dkk activation), but also involved positive inputs from Wnt signalling through members of the Fz5/8 family.

NvGpc1/2/4/6 involved in oral-aboral patterning, but not polarisation

Despite the early polarised expression of *NvGpc1/2/4/6* and *NvSulf* (Figure 1 in paper II), both of these genes seem to neither play an important role in the polarisation of the embryo and establishment of the primary axis, nor in gastrulation. Instead, we found them to function in the modulation of patterning along the oral-aboral axis. These are interesting results, as the involvement of glypicans in axial patterning has only been described in very few species. In zebrafish and *Xenopus*, glypican4 regulates non-canonical Wnt signalling and in consequence the elongation of the anterior-posterior body axis by convergent-extension tissue movements (Topczewski *et al.*, 2001; Ohkawara *et al.*, 2003). In sea urchin

a HSPG is involved in patterning along the oral-aboral axis by interacting with Nodal (Bergeron *et al.*, 2011), but this axis is not homologous to the oral-aboral axis of *Nematostella* and there is anyway no Nodal orthologue in *Nematostella*. Finally, glypican5 is asymmetrically expressed in sea urchin and it seems to interact with BMP signalling rather than the Wnt or FGF pathways (Lapraz *et al.*, 2009).

In our work, the almost identical changes in patterning along the oral-aboral axis upon *NvFz5/8* or *NvGpc1/2/4/6* knockdown and sodium chlorate treatment support the idea that NvGpc4/6 (bearing 6-O-sulfated HS chains) is a modulator of Wnt signalling, via interactions with NvFz5/8. The two most likely scenarios for how NvGpc4/6 could modulate Wnt signalling are discussed in paper II, namely, that NvGpc1/2/4/6 could function as a NvFz5/8 co-receptor and / or a function in Wnt diffusion across the cell surface (Figure 7 paper II). Here, I would like to expand upon this discussion by suggesting variations of these models and other mechanisms that are also possible:

1. In a variation of the second model, NvGpc1/2/4/6 could have a dual role in the diffusion of Wnts depending on its sulfation state. Meaning that HS chains which have been 6-O-desulfated in the oral 2/3 of the ectoderm (due to the presence of *NvSulf* in this domain), would be unable to bind Wnt, allowing diffusion. At the anterior pole, the 6-O-sulfated HS chains could bind and accumulate Wnts, increasing the possibility to bind to NvFz5/8.
The role of 6-O-sulfated HS at the aboral pole, and of the sulfatase NvSulf in axial patterning, will be discussed further in 6.3

2. Enzymes of the Notum family have long been believed to release glypicans from the cell membrane by cutting their GPI anchor, though recently it was instead found that they act by deacytilating Wnt ligands and probably use glypicans as a scaffold to do so (Kakugawa *et al.*, 2015). The presence of seven Notum genes in the *Nematostella* genome (Lapébie *et al.*, 2014) 12 Wnt ligands, but only two glypican genes, suggests that Wnt regulation via Notum might be ligand specific, but not glypican specific. Thus we can imagine a scenario in which different Notums along the oral-aboral axis could modulate Wnt signalling in a staggered and / or ligand specific manner. Unfortunately we currently only know the expression of one *Notum* in *Nematostella*, which is expressed at the oral pole (data not shown), it would therefore be very interesting to investigate the expression of the other 6 *Notum* orthologs.

3. An indirect effect of NvGpc1/2/4/6 on Wnt signalling through another pathway is also possible, like that which was described by (Venero Galanternik *et al.*, 2015). In this study they observed upregulation of Wnt signalling in animals treated with sodium chlorate. They further found that sulfation of HSPGs is crucial for FGF signalling by enabling HSPGs to limit ligand diffusion. The FGF pathway activates Dkk transcription, which in turn inhibits Wnt signalling. They revealed that the effect of the HSPGs on Wnt signalling was a secondary effect in this context.

A detailed survey of the expression dynamics of all genes involved in the regulation of Wnt signalling and HS synthesis is desirable to identify temporal and spatial co-expression and test for interactions.

6.2 Apical organ patterning and evolution

Discussing the literature

Regarding gene expression, Marlow *et al.* (2014) came to the conclusion that larval body patterning and apical organs are conserved in Cnidaria and Bilateria. The authors described similar expression of transcription factors at the anterior pole of different species, which they interpreted as proof for common heritage of marine invertebrate larvae. Nonetheless, many of these transcription factors in fact show very different patterns and degrees of co-expression within the apical domains of the different species; e.g. most of the TFs are only expressed in a few cells in *Platynereis* but are in much broader and more overlapping patterns in *Nematostella*. For example, *NvIrx* and *NvHoxF* are co-expressed in the apical organ in *Nematostella*, but *PdIrx* and *PdHox1* have no overlap in *Platynereis*; *NvFoxQ2* expression only surrounds the apical organ in *Nematostella*, but is expressed in the ciliary cells in *Platynereis*. These patterns could indicate that conserved apical organ cell types became more subfunctionalised during the course of evolution in different branches of the animal tree, but they could also reflect the recruitment of TFs with similar functions, within the evolution of similar structures. In the end, a TF is a switch, and such regulatory elements can be replaced and exchanged. And finally, it is important to remember that the homology of the anterior pole does not necessarily mean that the apical organs which develop within this domain are themselves also homologous.

Regarding function, a connection between Wamides, settlement, and neuropeptide expressing cells at the aboral pole, has been described in *Platynereis* and in hydrozoan larvae (Conzelmann *et al.*, 2013; Gajewski *et al.*, 1996). Also the scyphozoan *Aurelia* exhibits FRFamide and Taurine (which has been shown to inhibit metamorphosis in *Hydractenia echinata* (Berking, 1988)) positive cells in the apical organ (Nakanishi *et al.*, 2008). It is interesting to compare the features of the apical regions of larvae from different cnidarian lineages. We can see that hydrozoans lack an apical ciliary tuft, yet they possess neuropeptide expressing cells at the aboral pole and their settlement response is sensitive to Taurine and Wamides. In contrast, *Nematostella* has a cilary tuft, but no roles for amides in the transition from larva to polyp has been observed (though it is interesting in this context that the Taurine dehydrogenase, NvTauD, is expressed in the apical organ). There are two possible conclusions from this: The first is that hydrozoans and *Nematostella* reduced different parts of an ancestral cnidarian apical organ which consisted of both ciliary cells and chemosensory-neurosecretory cells, and functioned in settlement via Wamides. The second option is that different cell types or cellular structures were recruited to the aboral pole in the different cnidarian groups and that in bilaterians all these different features got combined in intercalated larvae.

Looking at the scattered data about apical organs available in the literature, it is evident that these studies are mainly concentrated on only a few species. As such, there is a risk of over-extrapolation from these few species, to the entire Bilateria/Metazoa. Especially in the light of the Trochaea theory, there appears to sometimes be a tendency to treat the *Platynereis* larva as representative of the LCA (last common ancestor) of bilaterians. This is clearly problematic, as *Platynereis* is a modern species, nested deeply within the phylum Annelida (Weigert *et al.*, 2014). As such, it is important that we continue to explore the vast diversity of marine invertebrate larvae that is out there, particularly regarding questions of larval and life history homologies.

NvFz5/8, NvGpc1/2/4/6 and 6-O-sulfated HS chains play a role in patterning the apical organ

Using *NvMeprin-like* and *NvTauD* as marker genes, we observed the activities of two distinct patterning mechanisms; one which regulates the size of the apical domain and a second that regulates whether the centre of this domain is clear of expression or not. Both of these mechanisms were affected by *NvFz5/8* and *NvGpc1/2/4/6* knockdown, as well as by sulfation inhibition. Nonetheless, the correlation between these three phenotypes was weaker

at the planula stage, in comparison to the strong correlation we saw in the effects of each treatment on the earlier axial patterning events. This difference could be due to one or all of these players also performing a second independent function at the aboral pole, or through secondary effects due to a function that is unrelated to the shared patterning role. For example, NvFz5/8 knockdown seems to be involved in body elongation, which is not observed for the other two treatments, thus, changes in the size of the apical domain in NvFz5/8 morphants could be an indirect effect of this change in body shape.

The expansion of the aboral domain upon *NvFz5/8* knockdown at gastrula stage only led to a slight enlargement of expression domains at planula stage for the markers that indicate the central region of the aboral pole (Figure S1 paper I and Figure 5 paper II). Similarly, sodium chlorate treatment also translated into enlarged expression domains in the apical organ domain, especially when the treatment started early, suggesting that these effects are due to an early patterning role of NvFz5/8.
In contrast in *NvGpc1/2/4/6* knockdowns the size of the spot or remaining ring domains was reduced instead of enlarged, thus suggesting a second function independent of NvFz5/8 and 6-O-sulfation of HS, or conversely, that NvGpc1/2/4/6 is not involved in a second function NvFz5/8 and sulfation of HS may have (e.g. a different HSPG could be involved).

All three knockdowns showed an increase in the aboral expression domain of the inhibitory ligand NvFGFa2. However, the apical tuft was reduced in diameter in *NvFz5/8* and *NvGpc1/2/4/6* knockdowns only (Figure 5 paper I; Figure 3 paper II). This suggests that HS chains may have additional effects on FGF signalling, and that they may be required to modulate the inhibitory action of the NvFGFa2 ligand, after the establishment of patterning at the aboral pole.

As a final point, it is also worth mentioning, that the apical organ is a transitory structure and its determination appears to be unstable. We know for example that maintenance of the apical organ requires constant FGF signalling (Rentzsch *et al.*, 2008). We also know through cutting experiments (Fritzenwanker *et al.*, 2007) that the aboral half is able to maintain the apical organ without the oral half, indeed it also appears to be expanded; this implies the apical organ experiences constant negative regulation from the oral pole. To take this observation a step further, I speculate that cell fates in the larval ectoderm are dependent on persistent signals from various sources, and that interfering with NvFz5/8, NvGpc4/6 or its HS chains can alter these signals and thus affect cellular identities along the oral-aboral axis.

In conclusion, studies aiming to characterise the regulation of apical organ formation in other animals, including testing a role for Wnt signalling and for glypican fine-tuning would be desirable. Such studies could shed fresh light on issues of apical organ and larva homology, as well as providing further insight into the evolution of these pathways and the evolution of their modulation, regardless of apical organ and larva homology.

Transgenic approach

One of the intended aims of this study was to create transgenic lines in which a fluorescent reporter is driven by a promoter of apical organ specific genes. Towards this goal, the F0 expression of the NvTauD::mOrange construct has been described in 5.1. Despite the challenges in generating an F1 generation of transgenic animals, this approach should not be discarded. In particular, with the rise of technologies such as correlative fluorescent microscopy, and serial section transmission electron microscopy, transgenic lines for different marker genes, that show expression in only a subset of apical organ cells, would be immensely helpful for identifying different cell types (see Figure 7E). They would also help to answer the question of whether the gene expression domains we observe around the apical pole are partly overlapping or abutting. Such lines would also make it easier to assess perturbations in the patterning and cellular composition of the apical organ (e.g. is a knockdown phenotype representing cell type loss, or cell dispersal away from the apical pole?).

The transgenic reporter constructs can also be used to examine subcellular localisation of proteins of interest – by cloning gene CDSs between the promoter and reporter sequences. For example, it would be very informative to assess the localisation of the acetylcholine receptors that were identified as expressed in the apical domain of *Nematostella* by Sinigaglia et al. (2015). Most importantly – are they located in the vicinity of the neural plexus that lies beneath the aboral pole?

FGF-pathway modulators

It is noteworthy that both NvFGFa1 and NvFGFa2 have the conserved glycine box with all 5 HS binding sites, but the FGF receptor NvFGFRa is lacking an acidic box, which has been shown to be important in receptor HSPG interactions (Sakaguchi *et al.*, 1999). This evolutionary conservation in FGF ligands which are otherwise quite divergent in sequence is likely to have a functional basis. But it also does not necessarily indicate that these ligands interact with HS chains bound to NvGpc4/6, they could also be interacting with other

HSPGs. It is noteworthy that in *Xenopus,* glypican4 has been shown to not only regulate Wnt signalling during axis elongation, but is also required by FGF signalling for proper dorsoventral forebrain patterning (Galli *et al.*, 2003). Our results suggest that future work in *Nematostella* could also reveal such a dual function for NvGpc1/2/4/6, and in both species a focus on determining if this duality is facilitated by differential HS modifications would be important.

In addition to the glypicans we also considered the roles of other known FGF pathway modulators such as FGFRlike and Sprouty (see 1.5.1). Knockdown experiments did not reveal a function of either of these genes in apical organ development. However, a negative result is always difficult to interpret and we can not rule out entirely that they still may play a role in modulating FGF signalling in the apical organ. NvSprouty knockdown via morpholino caused severe defects in gastrulation and endoderm formation (data not shown). As the general development of these morphants was so impaired, we considered this method to not be feasible for investigating whether *NvSprouty* has a role in the later development of the apical organ. Morpholinos against FGFRlike did not show a strong morphological phenotype at any stage of larval development. We observed in the primary polyp that FGFRlike knockdown animals had only three instead of four tentacles, and one of these appeared to be larger, or else was the product of tentacle bud fusion. Interestingly, in *Hydra FGFRL1* is expressed between the tentacles (Ellen Lange and Monika Hassel personal communication).

There are 15 *FGF* ligands in the *Nematostella* genome and at least two more of them are expressed in the apical organ (Matus *et al.*, 2007; Rentzsch *et al.*, 2008; Sinigaglia *et al.*, 2015). As there is only a single FGF receptor expressed in this region (NvFGFRa), competition or parallel signalling of these FGF ligands is more than likely and this issue should be addressed in future experiments.

6.3 Just scratching the (cell-)surface

Our experiments on glypicans and on the sulfation of HS chains have given us some interesting insights into the roles that modulators on the cell surface can play during developmental patterning events. They have also provoked many new questions, and

revealed to us that our understanding of these complex mechanisms for fine-tuning signalling pathways is still very rudimental.

The post-synthetic HS modulator Sulf

It has been already highlighted that 6-O-sulfation is the only sulfate moiety to be regulated post-synthetically - indicating its importance (Lamanna *et al.*, 2007), and contrasting to the view that it is only the number of HS sulfations that influences ligand binding, regardless of sulfation position.

Within the *Nematostella* ectoderm, the observation that the only domain free of *NvSulf* expression correlates with the domain of highest *NvFrizzled5/8* and *NvFGFRa* expression domains (Figure X in paper II), makes it tempting to speculate about interactions between these molecules and HSPGs. The similarities in patterning changes upon knockdown of *NvFz5/8* and *NvGpc1/2/4/6* and after desulfation through sodium chlorate treatment are also very striking. Together, these data would support a model in which 6-O-sulfation of HS chains bound to NvGpc1/2/4/6 positively regulates Wnt signalling through the NvFz5/8 receptor (Figure 7A paper II).

We can also conclude from our experiments that the sulfation state of HS chains within the apical organ affects not only one or more of the signalling pathways acting in that region, but also, that the diffusion of signals arising more orally must be differentially regulated by sulfated and desulfated sugar chains (revealed by *NvSulf* knockdown, see Figure 7). The orally expressed Wnt ligands and the NvFz5/8 receptor are among the most likely candidates here, as we can be quite certain that they interact with the 6-O sulfated HSPG NvGpc4/6 at gastrula stage. Varying the timing of the sodium chlorate treatments also revealed that most of the patterning disruptions are due to effects within the first 48 h; some even within the first 24 h. Prior to gastrula stage the pattern formation of achieving a ring or spot fate is set, although the ring genes are still expressed broadly at 48 hpf (see Figure 6 in paper II). The effects relating more to the size of the resulting domain were caused by treatments starting prior or at 24 h. The temporal expression dynamics of some enzymes involved in the sulfation of HS chains support this idea. Corresponding to the time at which the embryos were most responsive to the sodium chlorate treatment, *Nv6OST* (HS 6-O-sulfotransferase), *Nv3OST* and *NvSulf* are highly upregulated (from 8-24hpf) while the other *OST* and *NDST* genes are more uniformly expressed during development in *Nematostella* (Feta *et al.*, 2009).

These early (pre-patterning events) were also impaired by *NvSulf* knockdown. Although no patterning effect on marker genes at gastrula stage was visible, the knockdown resulted in less than 30% of the animals expressing the later markers *NvMeprin-like* and *NvTauD* in a ring, which the sodium treatment revealed to be due to early pre-patterning events.

Saha and Schaffer (2006) discovered that a reduction of Sonic hedgehog (Shh) diffusion paradoxically resulted in an increase in the range of Shh activity. As reviewed in Lander (2007) this may in fact be a general feature of morphogen diffusion, making terms like positive or negative regulator problematic, as the same molecule may have both functions, depending on how close to the source it acts. In our case, this mechanism could explain similarities between artificial desulfation (sodium chlorate treatment) and loss of desulfation activity (*NvSulf* knockdown – these experiments need further confirmation and additional controls). One scenario is that if glypicans bearing a HS chain with 6-O-sulfation create a critical Wnt concentration at the aboral pole, than 6-O-sulfation in the oral half could lower that concentration and affect signalling in a similar way desulfation does.

In vertebrates there are two *Sulf* genes as a result of the whole genome duplication, and these genes seem to have subfunctionalised, despite having the same substrate specificity *in vitro* (Lamanna *et al.*, 2007). In fish there has been at least one further gene duplication of the *Sulf2* gene and subfunctionalisation of the orthologues compared to mammalian *Sulf2* has been reported (Gorsi *et al.*, 2010). These expansions of HSPG modifying enzymes underpin the ability of HSPGs to subtly change their modulating role in a tissue- or even a cell-specific manner. This provides another regulatory dimension to understanding how a handful of signalling pathways has been co-opted and redeployed to drive developmental diversity during the course of evolution. Key to this understanding could be the modulation of signalling activity on the cell surface. The most recent findings in zebrafish on the Wnt/FGF interplay (Venero Galanternik *et al.*, 2015) and in *Drosophila* on Notum activity (Kakugawa *et al.*, 2015) show that many older interpretations of HSPG functions and modifications need to be reconsidered.

Our findings together with previous studies suggest a complex interaction of Wnt and FGF signalling through a variety of modulators on the cell surface. HSPGs play a crucial role in this modulation through their ability to bind numerous ligands, receptors and enzymes.

HSPGs in early branching phyla

Glypicans and HSPGs in general seem to be a metazoan invention (Filmus *et al.*, 2008; Pei and Grishin, 2012). Preliminary data suggest that all metazoans possess glypicans and the HS synthesis machinery (see 5.3). If their ancient function was in signalling modulation or in a more general contribution to the ECM remains unclear, although the cysteine rich domain (CRD) of glypicans exhibits sequence and structure similarities to CRDs in Frizzled receptors and Hedgehog-interacting proteins (Pei and Grishin, 2012). The evolution of intracellular communication and of the ECM were most likely linked, and HSPGs are obvious candidates to have also have played an early role in the emergence of either or both of these metazoan innovations.

The evolution of the modulation of cell communication

The modulation of extracellular signalling is highly complex, and accordingly, very difficult to investigate. Specifically with regard to the glypicans, we are not looking at a defined structure, we are looking at a system. A system to tinker with morphogens. A modular system which consists of several units that can be put together in different ways; and we can observe that different branches of the tree of animal life have increased the complexity of this system by expanding the variety of different units. Ranging from the core glypican protein, to the enzymes involved in HS chain formation and modification, up to third party interactors like the Notums, all these components have increased in number, in different proportions in different genomes. Irrespective of these differential expansions, the final result seems to be the same, i.e. a system that can modulate specific morphogens on the cell surface, and that can change this specificity by changing different units of its own structure.

6.4 On the homology of larvae

In certain species, such as some sea urchins or members of the Pilidophora (nemerteans with a pilidium larva), the juvenile develops inside the larva like an alien form of life or almost like the larva is a free swimming uterus, providing the juvenile with nutrition and ensuring dispersal. It is difficult to understand how the larva and the juvenile from such species could be homologous to species in which the larva develops more gradually into the adult form. For example, can the anterior pole of a larva which degenerates after metamorphosis be homologous to that of a larva in which the larval anterior pole transforms into the anterior

pole of the adult? If yes, does it mean that the adult form of the former can not be homologous to the adult of the later? Such are the types of questions which require further research before issues of larval homology can be answered. It should also be mentioned here, that it is possible to find examples of both homologous larvae, and intercalated larvae, occurring at different levels of evolutionary distance across the metazoan tree. The point of interest is to understand and identify how deeply in the metazoan tree larval homology can be found. Although, this picture might still be blurred by the occurrence of secondary larvae co-opting ancient larval traits.

Cell types

Our work in *Nematostella* has shown that the activation and patterning of apical organ specific gene expression is much more complicated than simply the deployment of a binary (on/off) NvFGFa1 driven expression cassette. Future work should aim to link the 78 apical organ specific genes recovered by Sinigaglia *et al.*, (2015) to the different cell types within the *Nematostella* apical organ (as well as potentially finding more cell types) and then compare this cell-type specific expression data to that which is known in other phyla. Notably, the range of expression phenotypes achieved by experimental manipulations, and the different regulation of marker genes (Figures 4-6 & S7 paper II & Figure S1 Paper I), suggest that the apical organ consists of different cell types, which are differentially regulated by Wnt and FGF signalling.

I discussed in 6.1 the study by Marlow *et al.* (2014), in which they identified nine transcription factors which are expressed in similar ways in all larval apical domains. They argued that convergent evolution of such a pattern would be highly unlikely, and that these domains were homologous. But a close examination of these data reveals that these nine TFs are not always co-expressed in the same cells, and whether their expression domain is part of the apical organ or whether it lies in the surrounding anterior domain also differs. Furthermore, as it is known that these TFs control each other's expression; then it is not necessary that nine parallel convergent events needed to have taken place to generate this pattern. Instead, just one or two co-options of TFs located at the top of a GRN or regulatory cascade would have been sufficient to end up with similar co-expression domains. Thus while the expression data of Marlow et al., (2014) does provide support for homology of larval apical domains, it is not indisputable evidence.

Axis

The identification of larval traits that have been transferred into adults, or *vice versa,* can provide important information towards identifying which *bauplan* of the two life stages within a species is the more ancient, and which life stages we should compare between species/clades. An interesting issue to consider in this context is axial patterning. Primary axial patterning occurs once in the life history of animals without catastrophic metamorphosis, but twice in those who undergo this dramatic transition. Of these two patterning events, we can ask which is the ancestral, and which was either transferred from another life stage, or evolved independently (for example by co-opting patterning systems initially used in other developmental processes)?

The larval stage in insects is considered to be an intercalation product. Yet we find homology in patterning and development to embryonic and larval stages of other phyla. It has been proposed that heterochronic shifts of conserved modules are the driving force behind the evolution of holometabolous insects from hemimetabolous ancestors (Mito *et al.*, 2010). So if the insect larva co-opted or transferred developmental modules from the embryonic or juvenile stages, is that also possible in marine larvae?

Based on current data, axial patterning by Wnts is presumably the ancient patterning system for the primary body axis, with the Hox-code being later added to the system in the bilaterian lineage. Does this insight favour either the intercalation or the terminal addition theory? I described in the introduction that the Hox-code has been used by the intercalation theorists (e.g. Sly *et al.*, 2003) as an example for a tool taken out of the adults kit and lent to the novel larval stage to build its body. In favour of this theory are the findings that *Hox* genes are not used to pattern the larvae of species with catastrophic metamorphosis (e.g. pilidium (Hiebert and Maslakova, 2015) and reviewed for sea urchin and some trochophores in (Arenas-Mena, 2010)). The larvae of at least some of those species are instead patterned using the ancient Wnt system, and in a second axial patterning event, the (almost independently) developing juvenile uses the Hox-code.

However if the catastrophic metamorphic type of development is the ancestral state, then more direct developing species would have transferred the later mode of patterning to the larva, maybe driving direct development by this change. If larvae are homologous, a likely scenario is that after the split of cnidarians and bilaterians, the Hox-code got integrated into the patterning along the primary axis. In some clades a change to dramatic metamorphosis either caused a secondary decoupling of the Wnt driven patterning and the Hox-code in a

simplified larval body, or the other way round, this simplification led to a loss of the canonical Hox-code.

Summary

Are marine invertebrate larvae homologous? The findings of similarities, both in apical domain development and in axial patterning hint in that direction. Reasonable doubt comes from the indisputable differences (expression profiles and structure of the apical organs; different axial patterning systems). Figure 9 provides a summary of the strategies I followed to address this question.

As I suggested in the introduction, breaking the broad question of larval homology into smaller questions could help us to understand homology issues. Further we should consider whether the scope of marine larvae we are comparing among may be too wide, with regard to evolutionary time; maybe we should first identify the similarities and differences within closely related clades, to distinguish the derived and the ancient features at more recent nodes within the metazoan tree. Finally, it is possible that comparing marine larvae in more detail to the interpolated larvae of insects and vertebrates, may help us to better understand how convergent evolution and co-option can act on larval forms, and we can then use this information to identify the 'noise' generated by these processes in the ocean of marine larval diversity.
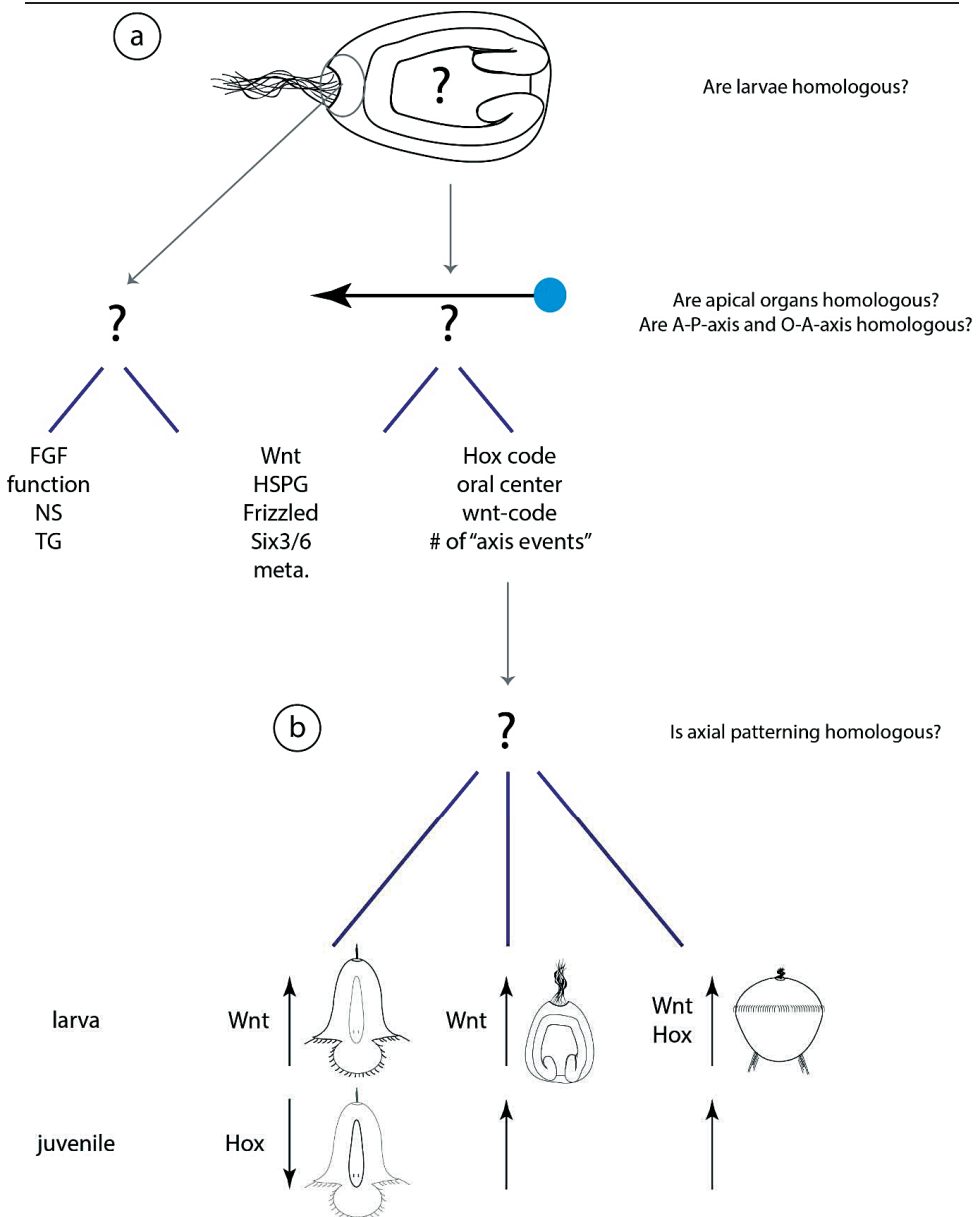
Figure 10 Graphical summary: a) To answer the question of whether marine invertebrate larvae are homologous and how developmental patterning processes evolved, we need to break the question up into smaller units, focusing on describing larval developmental traits in each species, such as the apical organ or axial properties. In *Nematostella*, the apical organ and axial patterning are connected by Wnt signaling, HSPG modulation, Fz5/8 – Six3/6 feedback and metamorphosis (meta.). The apical organ is also governed by FGF signaling and its potential function as part of the nervous system (NS) could be revealed by transgenic lines (TG). Blastoporal / oral Wnt signaling (blue dot) is a unifying and crucial feature in the establishment of the primary body axis in cnidarians and bilaterians. **b)** Patterning along the primary axis involves the Hox-code in bilaterians with gradual development; in phyla with catastrophic metamorphosis the *Hox* genes are only involved the establishment of the axes of the juvenile. Illustrated by pilidium (with juvenile inside), planula and trochophore larvae (left to right).

6.5 Concluding remarks

Our results identify three key steps for the establishment and patterning of the primary axis in *Nematostella*:

1) Activation of oral *and* aboral networks by different levels of β-catenin.
2) A feedback-loop between the transcription factor NvSix3/6 and the Wnt receptor NvFrizzled5/8 at the aboral end of the primary axis to control the size of the aboral territory.
3) Development of position dependent features, such as the apical organ.

It is very likely that Wnt signalling is the evolutionarily oldest mechanism for establishing a polarised axis. Further, that an antagonistic centre at the opposite pole of this Wnt axis is a conserved feature shared by at least cnidarians and bilaterians. However, the patterning along this axis has undergone significant change over the course of evolution.

We further characterise the HSPG Glypican1/2/4/6 and differential sulfation of HS chains as indispensable modulators of steps 2 and 3. HSPGs offer a multitude of structural diversity that can be controlled by many other factors. This diversity empowers them to be dynamic moderators of cell communication, and allows them the flexibility to interact with signalling pathways in both conserved and novel manners.

The question of the homology of apical organs and of marine invertebrate larvae in general requires further research.

7. Source of data

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