"Do not go where the path may lead, go instead where there is no path and leave a trail."

– Ralph Waldo Emerson

University of Alberta

Evolution of the sponge body plan: Wnt and the development of polarity in freshwater sponges

by

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Dedicated to my friends and family who helped me see the light at the end of the tunnel.

Abstract

Body polarity is a fundamental aspect of all multicellular organisms. Metazoans – animals – are monophyletic, but is body polarity homologous among all phyla? Sponges are considered to have branched off first from other animals and therefore studies of polarity formation in the simple sponge body plan may hold key clues to fundamental metazoan characteristics like polarity. In this thesis, I studied the role of Wnt signaling in patterning the body axis of freshwater sponges.

Lithium chloride caused the formation of extra oscula, the excurrent opening of the sponge canal system and transplant experiments showed the osculum can induce canal growth, similar to other animal tissue organizers.

Phylogenetic analysis of sponge Wnt genes showed they are distinct from other animal Wnts and while I found no clear expression patterns by *in situ* hybridization, RNAi knockdown of *gsk3* causes multiple oscula in the sponge. Attempts to express freshwater sponge *wnts* in *Xenopus* were unsuccessful.

Sponges have indirect development through a non-feeding larva. Fates of larval cells in the adult sponge were followed using fluorescent probes. Ciliated cells surrounding the larva become choanocyte chambers in the juvenile, confirming the idea that inner and outer tissue layers are reversed with respect to other animals. Fluorescent labeling of the posterior pole of the larva revealed that this region becomes the osculum linking previously shown reports of *wnt* expression at the posterior pole with formation of the osculum and Wnt signaling. Taken together, the results here suggest that Wnt signaling in early metazoans played a role in the evolution of animal polarity.

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The undertaking of this thesis could not have been accomplished without the support of a number of people. I'd like to take this opportunity to thank those who have been involved, from start to finish.

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List of Symbols, Nomenclature, and Abbreviations

General symbols

(r)DNA	(ribosomal) deoxyribonucleic acid
dph	days post hatch
GRN	gene regulatory networks
(q) or (RT-) PCR	(quantitative) or (reverse transcriptase) polymerase chain reaction
PON	pre-oscular node
(m) or (ds)RNA(i)	(messenger) or (double stranded) ribonucleic acid (interference)
SAR	Stramenopiles, Alveolates and Rhizaria
SEM	scanning electron microscope/microscopy

Soltions/Chemicals

AP	alsterpaullone
BIO	(2'Z,3'E)-6-bromoindirubin-3'-oxime
CMFDA	5-chloromethylfluorescein diacetate
diI	1, 1'-dioctadecyl-3,3,3',3'-tetramthylindocarbocyanine perchlorate
DMSO	dimethyl sulfoxide
FLW	filtered lake water
LiCl	lithium chloride
NMDG-Cl	N-methyl d-glucamine chloride

gene/Protein names

apc/APC	adenomatous polyposis coli
axin/axin	axis inhibition factor
dkk/dkk	dickkopf
dsh/Dsh	dishevelled
fz/Fz	frizzled
gro/Gro	groucho
gsk3/GSK3	glycogen synthase kinase 3
lrp/LRP	lipoprotein-receptor-related
sfrp/sfrp	secreted frizzled protein
tcf/TCF	T-cell factor
wnt/Wnt	wingless/integrated

Chapter One:

A GENERAL INTRODUCTION

1.1 ANIMAL BODY PLANS AND DEVELOPMENT

1.1.1 Metazoan body plans

The body plan (sometimes referred to as *bauplan*) refers to a set of morphological features possessed by a group of animals that share common descent (Woodger 1945). Arguably, it is this principle upon which modern evolutionary developmental biology relies (Willmore 2012), but what exactly is meant by our modern definition of a body plan?

The concept of a body plan is straightforward: a list of characteristics shared by all members of the phylum (Nielsen 2003). While originally the term was meant to describe some idealized archetype that those animals aspired towards, observations over the nineteenth century changed how the body plan was viewed (reviewed in Willmore 2012). For instance, Karl Ernst von Baer noted the importance of embryonic development of an animal in determining its final adult body plan (1828). Von Baer's four laws of development put into context the concept of the body plan, ontogeny and phylogeny as follows:

1. General characteristics develop before specialized characteristics
2. Less general structures form after more general structures, until finally the most specialized structure forms
3. Embryos progressively diverge from embryos of other groups over the course of development
4. Embryos of higher animals resemble embryos, not adults, of lower animals

This set of laws inspired Darwin's observations of embryos and his thoughts on recapitulation theory (1859): descent from a common ancestor resulted in

shared body plans. The most extreme views on recapitulation came from Ernst Haeckel's biogenetic law, "*ontogeny recapitulates phylogeny*" (1874). Unfortunately this extreme view was called into question soon after and it was discovered that Haeckel's drawings of vertebrate embryos, which he used to support the biogenetic law, were inaccurate; drawings of some embryos were embellished so they would appear more similar to each other (Richardson et al. 1997). Modern-day observations on a wide range of vertebrate embryos attempted to find whether the tailbud stage even mildly resembled what Haeckel had drawn. The variability in vertebrate embryos was so great, that authors were forced to conclude that Haeckel's drawings were generalized amniote embryos, and made to fit into his own law (Richardson et al. 1997). Although the recapitulation theory in the sense that Haeckel believed has been shown to be false, von Baer's laws still generally hold and we understand that development does reflect the evolutionary history – in terms of phylogenetic relationships – of animals. While original body plan concepts were used to understand vertebrate evolution, molecular techniques made it possible to extend body plan homology to the bilaterians using comparative gene expression and function (e.g. Slack et al. 1993). By using a similar approach and comparisons to the earliest branching groups of animals, it is possible to find the universal features that define all metazoan body plans (see sections 1.1.2 and 1.1.3 below).

Body plans are limited in their capacity for vast change by constraints which restrict variability in a trait, thereby limiting the number of possible body plans (Hall 1999 as cited by Willmore 2012). Related to this is the idea of evolvability: removal of constraints will lead to an increase in variability, and thus more choices for selection to act upon. Interplay between the two is thought to have contributed to the relatively fast origin and stability of body plans in the Cambrian (Willmore 2012). Reidl (1977) formulated the burden concept in which certain structures within a body plan are more integrated (or functionally important) than others; highly integrated structures will evolve very slowly (high constraint), while less integrated features are free to evolve and change. In other words, a particular trait has a given burden, which gives it a certain balance between

evolvability and constraint. When new features arise from a 'low-burden' part of the body plan, the total burden of the rest of the body plan increases (Reidl 1977). The new part or trait evolved in the context of the old body plan, and thus depends on what used to be low burden traits (Wagner and Laubichler 2004). Therefore, new traits arise, free of burden, but as evolution proceeds they become more and more entrenched eventually becoming fixed features with high burdens.

1.1.2 A modern view of body plan evolution - GRNs

Body plans are not only built by way of development; genetic programs control development to build the structures of the body from general to complex (e.g. Slack et al. 1993, Willmore 2012). This synthesis is the most complete definition of a body plan, and it can be used to test hypotheses of homology (Valentine and Hamilton 1998). Much of the work of Eric Davidson at UC Berkeley has focused on gene regulatory networks (GRNs) in developing animals, especially sea urchins. These networks have been tracked through intensive work searching for the smaller circuits that make up the whole network responsible for building a body plan. There are four modules within the network ranging from highly conserved and inflexible to highly variable and quickly evolving: kernels, plug-ins, input/output switches and gene batteries (Davidson and Erwin 2006). This work was supported independently by measuring the rates of evolution in genes at each of the different levels; kernels were slowly evolving, while gene batteries evolved the fastest of all groups (He and Deem 2010). Their results also showed that kernel genes tended to be expressed earlier than plug-ins and input/output genes following von Baer's first law and showing that kernel genes may be more constrained, and possibly phylogenetically older.

Our current concept of the body plan, then, is one that encompasses a particular set of morphological traits that are present at some point during the life of an organism. These traits develop by the action of genes at differing levels within a hierarchical network whereby slowly evolving ancient genes build a framework upon which quickly evolving genes can make morphological novelties. In particular, it is these slowly evolving ancient genes that will provide

insights into early metazoan evolution and fundamental metazoan body plan characteristics.

One of the earliest traits to evolve in the last common ancestor of metazoans was body polarity; it appears in all metazoan groups at some stage of development. It is plausible that slowly evolving, ancient genes had a role in polarity in early evolution, a role that still exists today. But what evolutionary advantage is gained by having a polarized body plan?

1.1.3 Body polarity in animals

Polarity is present in animals in one form or another, at some stage of development: animal-vegetal, anterior-posterior, dorsal-ventral and oral-aboral. Animal body polarity is set up early in development, and acts as the template upon which further differentiation can occur – either developmentally or evolutionarily. Therefore, the generation of polarity was integral in the evolution and diversification of the Metazoa.

We have come to understand the generation of animal body polarity through the discovery that *Hox* genes are expressed along the anterior-posterior axis of many vertebrates and invertebrates gave rise to the idea that a "Hox code" may represent a universal code for animal body plans (Fig. 1-1). Slack et al. (1993) defined this pattern as the zootype, and hypothesized that this was the defining character of all animals. This as well as other early genetic research connected together the idea of morphology and genes.

In basal metazoans, however, the zootype hypothesis becomes less useful. In cnidarians, the only *Hox* genes present in the genome are the anterior and posterior groups but not group 3 and central *Hox* genes; the validity of these arguments are under debate (reviewed in Finnerty & Martindale 1999; Ferrier and Holland 2001; Kamm et al. 2006; Ryan et al. 2007). The expression of these genes also does not shed much light on the problem; oral and aboral pole expression is inconsistent depending on the species examined (reviewed in Martindale 2005). This fact leads to difficulty in determining whether the anterior-posterior axis is homologous to the oral-aboral axis. Furthermore, the complete lack of true *Hox* genes in Porifera, Ctenophora and Placozoa bars the

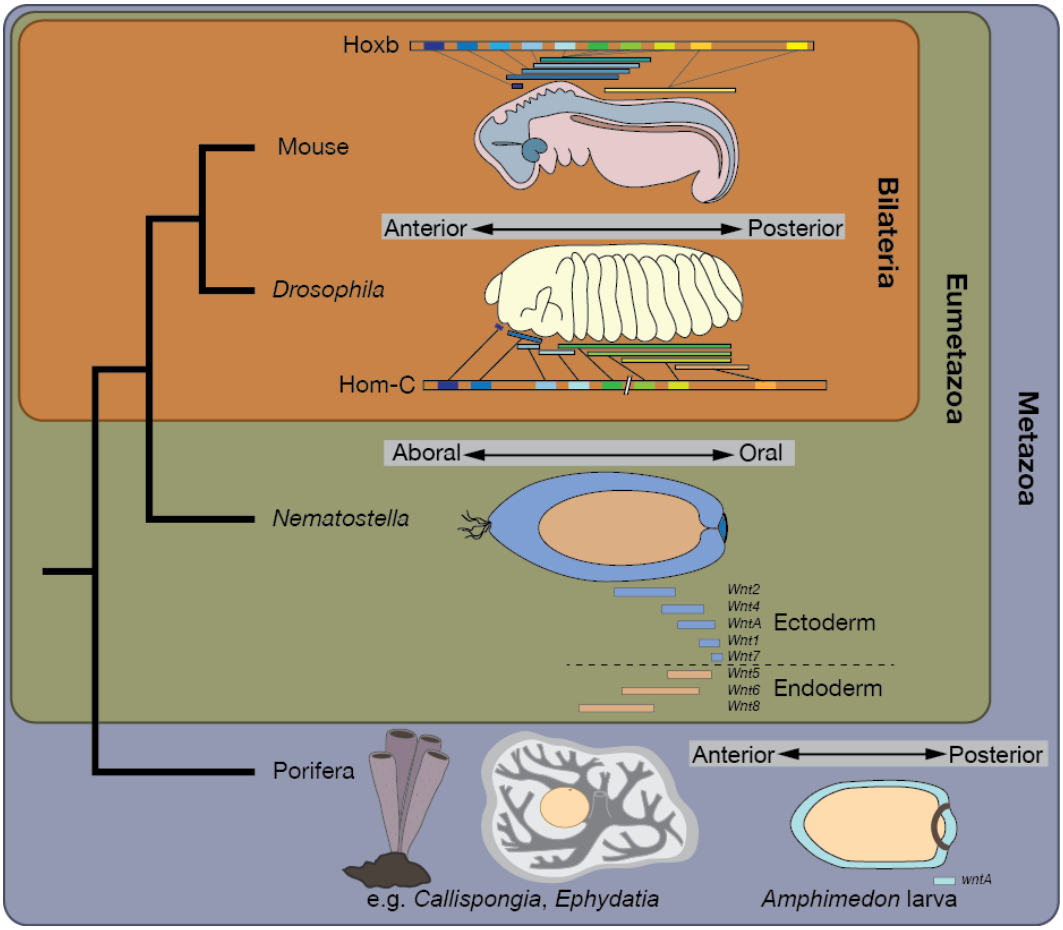


Figure 1-1: Molecular biology of axial patterning in the Metazoa.
Expression of *Hox* genes along the anterior-posterior axis of many bilaterians was originally thought to be universal to all animals. However, expression of *wnt* genes in a similar pattern in the cnidarian, *Nematostella vectensis*, point to a role for Wnt signaling in axis specification and patterning in early metazoan evolution. In sponges, it is still unclear what drives formation of an axis but in the larvae of *Amphimedon queenslandica* a *wnt* gene is expressed at the posterior pole. Mouse and *Drosophila* embryos redrawn from Veraksa et al. (2000); *Nematostella* larva redrawn from Kusserow et al. (2005)

universal metazoan application of the zootype concept.

In 2005, Kusserow et al. showed that both a striking diversity and expression pattern of several *wnt* genes in *Nematostella* hinted towards this signaling pathway being the ancestral mechanism for patterning the oral-aboral axis (see also, Lee et al. 2006). A suite of functional studies also shows that the canonical Wnt pathway seems to be working the same way in these animals as it does in others. Treatment of *Xenopus* embryos with lithium chloride (LiCl), which mimics Wnt overexpression, cause tadpoles to become dorsalized, such that there is an overall gain of dorsal and neural tissue, and a loss of ventral and posterior tissue (Klein and Melton 1996). In *Nematostella*, treatment with LiCl shows an increase in oral tissue specification, where the hypostome becomes enlarged, but head structures (tentacles) do not form (Wikramanayake et al. 2003). Intriguingly, alsterpaullone (a more potent and specific inhibitor than LiCl) cause multiplication of first the head organizer, and then tentacles over the entire body column (Broun et al. 2005 and Philipp et al. 2009). Furthermore, the use of Wnt proteins from mouse, and functional work with *Hydra* dkk1/2/4 (inhibitor of Wnts) suggest that the pathway is functioning in cnidarians as it does in other animals (Guder et al. 2005 and Lengfeld et al. 2009). The fact that most basal node groups lack true *Hox* genes and the evidence showing Wnt signaling as being important for polarity in all animals points to an early requirement of Wnt signaling in the evolution of metazoan body plans (Fig. 1-1).

1.1.4 The molecular developmental toolkit and the evolution of development

Animal model systems have revealed that animals repeatedly use the same proteins throughout development in different contexts to pattern their bodies – this set of proteins is often referred to as the metazoan developmental toolkit. Transcription factors act within the nucleus of a cell to up- or downregulate different genes by directly binding to DNA leading to the specification of that cell during development. While transcription factors act locally, within the cell in which they are expressed, they are often controlled by events outside the cell. Signaling molecules are released from other cells and can affect only those cells that express receptors to them; the receiving cell must be competent to receive

that specific signal. In general, when the signaling molecule becomes bound to the cell surface receptor, a number of downstream intracellular events occur that result in the activation or de-activation of transcription factors (Gilbert 2010, p. 85). In this way, extracellular events can affect the patterning of a developing embryo such that regionalization and specialization of tissues occurs.

There are several examples of conserved signaling pathways throughout metazoans that have a role in development: fibroblast growth factor (FGF), hedgehog (Hh), wingless/integrated (Wnt), transforming growth factor beta (TGF-β and bone morphogenetic proteins, BMPs) and others. When individual signaling pathways are activated or interact with one another at different times and places during development, the resulting adult will differ. Depending on the context of the signal the developmental and thus morphological outcome will differ; this is the basis for the generation of diversity. What we know about these signaling cascasdes is taken from vertebrate or invertebrate model systems, and hypotheses are then tested in non-model animals. In this way we can reconstruct whether a particular trait is homologous or a result of convergent evolution.

During development, these pathways play a number of roles, both during early development, in general patterning and also in later development to form the specializations of different animal groups. For example, FGF signaling is involved in a number of events during early development of vertebrates, such as cell movements during gastrulation, anterior-posterior patterning, neural induction and mesoderm and endoderm formation and patterning (reviewed in Böttcher and Niehrs 2005). However later in development FGF8, in concert with *Hox* genes, BMPs and Sonic hedgehog (Shh), is required for limb development (Lewandoski et al. 2000).

There are several families of TGF-βs, including the BMP family, and these are involved in a number of events in the early developing embryo, especially morphogenesis and dorsal-ventral patterning (reviewed in De Robertis and Kuroda 2004). As the name suggests, BMPs were discovered for their role in bone development in vertebrates but they have since been shown to be involved in

cellular events – division, apoptosis, migration and differentiation (reviewed in Hogan 1996).

Genetic studies in *Drosophila melanogaster* led to the discovery of *hh* and *wingless* (*wnt*) and identified their roles in the development of segment polarity (Nusslein-Volhard and Weischaus 1980). Since then both genes have been discovered to be involved in many processes throughout development in many animals. For example, Hh signaling plays a role in proliferation, morphogenesis, cell fate specification and embryonic patterning (reviewed in McMahon et al. 2003).

Wnt has a conserved role in the early development of polarity in a large number of metazoan embryos but Wnts are involved, like the other signaling pathways, in a large number of developmental events. In addition to segment polarity, they are involved in stem cell maintenance (reviewed in Nusse et al. 2008), cell fate specification (e.g. Logan et al. 1999) and in tooth development (e.g. Chen et al. 2009) among other processes. Wnt signaling also comes in a variety of forms at the molecular level (Planar Cell Polarity, Wnt/Calcium signaling), but body polarity in animals is governed by canonical Wnt signaling and will be focused on here (Fig. 1-2; Croce and McClay 2006). When no Wnt ligand is present, cytoplasmic β-catenin is targeted for degradation by a complex made up of axis inhibition factor (axin), glycogen synthase kinase 3 (GSK3) and adenomatous polyposis coli (APC) and other proteins. When Wnt binds the extracellular region of a frizzled receptor and its co-recptor lipoprotein receptor-related (LRP), the intracellular cascade is activated – disheveled (Dsh) is phosphorylated, and deactivates the β-catenin degradation complex. β-catenin is free to enter the nucleus, and bind and activate the transcription factor T-cell factor/lymphoid enhancer-binding factor (TCF/LEF). The result is transcription of Wnt target genes, eventually resulting in changes, for example differentiation, within the cell. This pathway is used very early in development, often as early as the zygote stage, to begin to set up embryonic polarity. Animals that look quite different as adults, a frog, a sea urchin, and a sea anemone actually use similar mechanisms when their axes are established during very early development.

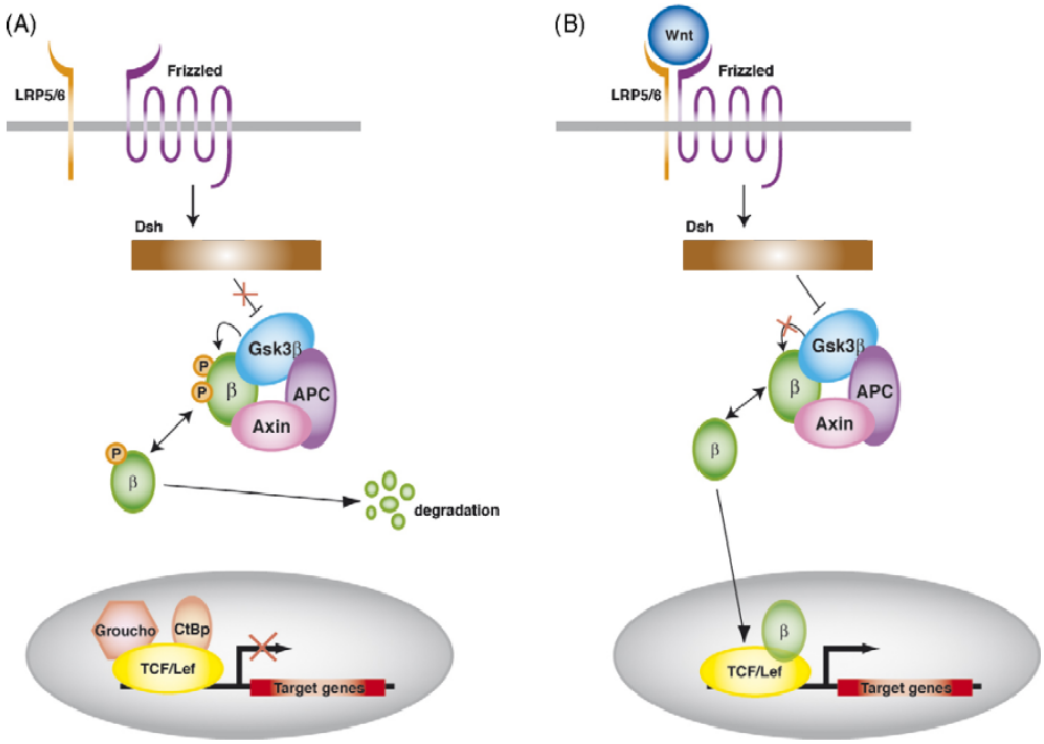


Figure 1-2: An illustration of Wnt signaling, in the absence (left) or presence (right) of the Wnt ligand.
(A) When no Wnt ligand is bound to its receptors, β-catenin is targeted for degradation, and the TCF/LEF transcription factor is repressed. (B) However, when Wnt is bound, Dsh is activated and prevents β-catenin destruction, facilitating its role in transcription within the nucleus. Figure modified from Croce and McClay (2006). See text for details.

Xenopus laevis oocytes are already polarized, as is easily seen with the naked eye; the animal half is pigmented and dark, the vegetal pole contains lighter coloured yolk. However, the animal-vegetal axis does not develop into the main axis in the frog embryo, tadpole and adult. When the oocyte is fertilized it undergoes cortical rotation, which shifts the contents of the cytoplasm at the cortex of the fertilized egg by 30° (Vincent et al. 1986). Many subsequent studies revealed that this rotation resulted in the movement of Dsh to one side of the embryo resulting in β-catenin accumulation in the cytoplasm and entrance into nuclei in that region (reviewed in Weaver and Kimelman 2004). Downstream targets of Wnt signaling are then transcribed, and in this way the embryo generates polarity at what eventually becomes the dorsal side, and the posterior end of the embryo. In the sea urchin and even in pre-bilaterian lineages such as cnidarians, a similar mechanism involving maternally expressed Dsh and regional stabilization of β-catenin can be found (Logan et al. 1999; Weitzel et al. 2004; Lee et al. 2007). The conservation of this mechanism and the widespread role of Wnts in creating polarity during development suggests early evolution of this pathway.

In the last common ancestor and other early metazoans, did these pathways exist? The sequencing of transcriptomes and genomes of an array of basally branching metazoans as well as outgroups of Metazoa – choanoflagellates (*Monosiga*, *Salpingoeca*), mesomycetozoans (*Capsaspora*) and other opisthokonts (e.g. fungi) – can help us find out when these important pathways arose. In addition, knowledge of what the genome of the last common ancestor of metazoans was like can help us to evaluate what such an animal looked like as well.

1.2 THE EVOLUTION OF METAZOAN MULTICELLULARITY AND COMPLEXITY

The transition from single- to multi-celled organisms has occurred repeatedly throughout evolutionary history and across both eukaryotes and prokaryotes (reviewed in Grosberg and Strathmann 2007). The central focus of research on the

origins of multicellularity is the advantages multicellularity confers on an organism: larger size, specialization and division of labour, and better dispersal (Hall and Hallgrimsson 2008, p.149).

Among the eukaryotes multicellularity has evolved in several major clades: Archaeplastida, Excavata, SAR (Stramenopiles, Alveolates and Rhizaria), Amoebozoa and Opisthokonta. In the opisthokonts, multicellularity has arisen in the genus *Fonticula*, in the Fungi and in the Animals (Parfrey and Lahr 2013). The animals were formerly divided into two subkingdoms, Metazoa and Parazoa, reflecting the perceived evolutionary separation between sponges and other animals. Parazoa, although occasionally still used in modern literature, is an outdated term; Metazoa describes all multicellular animals sharing a common ancestor (as opposed to "Protozoa", the unicellular animals). Ribosomal RNA and other molecular data indicates that the metazoans are monophyletic and that the choanoflagellates are the sister group of animals; both groups are within the Opisthokonta (Fig. 1-3; e.g. Carr et al. 2008).

The ancestor of all animals is referred to as Urmetazoa, a form that gave rise to the enormous diversity of animal body plans. I am interested in how the metazoan lineage came about, and what allowed the generation of diversity we see within this Kingdom.

1.2.1 Paleontological evidence of animal origins

Animal fossil evidence is useful when investigating the origins of animals, and perhaps offers clues to what early metazoans were like. The multicellular origins of animals probably occurred between 1 Ga to 750 Ma (*gigaannum*, or billion years ago and *megaannum*, or million years ago) (Nielsen and Parker 2010; Wang et al. 2010). Although the record does not extend all the way back through this time period, below is a summary of the fossil history of animals (Fig. 1-4).

The Ediacaran period (635 to 542 Ma) is characterized by fossils of dubious classifications including frond-like forms and flat, crawling organisms. These have been interpreted both as belonging to different extant metazoan phyla, 'failed animal experiments' and even extinct, multicellular non-animal eukaryotes

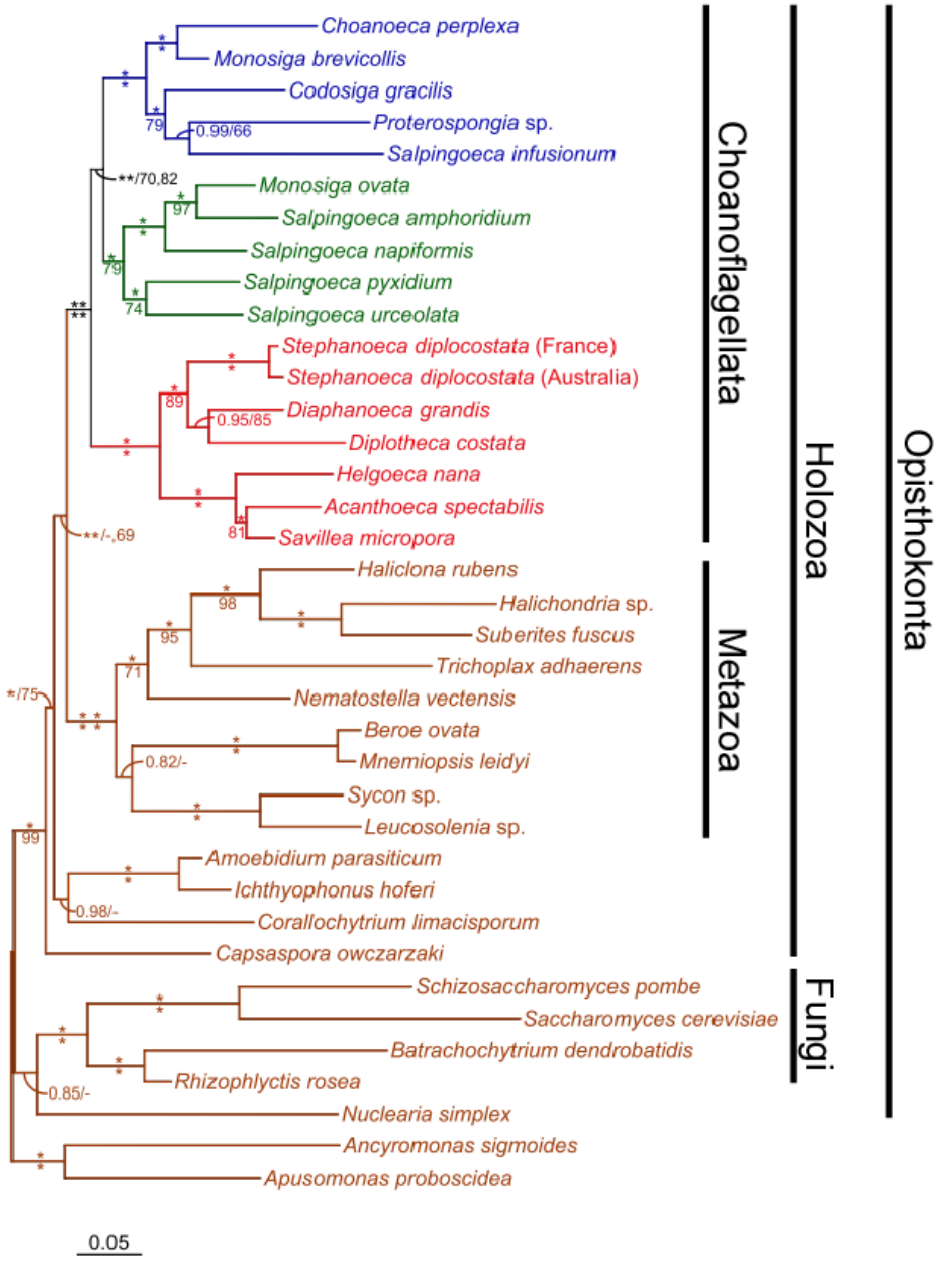


Figure 1-3: Phylogenetic tree representing the relationships among the Opistokonta (modified from Carr et al. 2008).
Bayesian tree generated using a concatenated 4-gene dataset under the CAT + I + Γ model. A similar tree was obtained using maximum likelihood under the GTRCAT and support values are given here. Support values indicated are Bayesian posterior probabilities/maximum likelihood bootstrap support. * indicates maximum support (1.0/100).

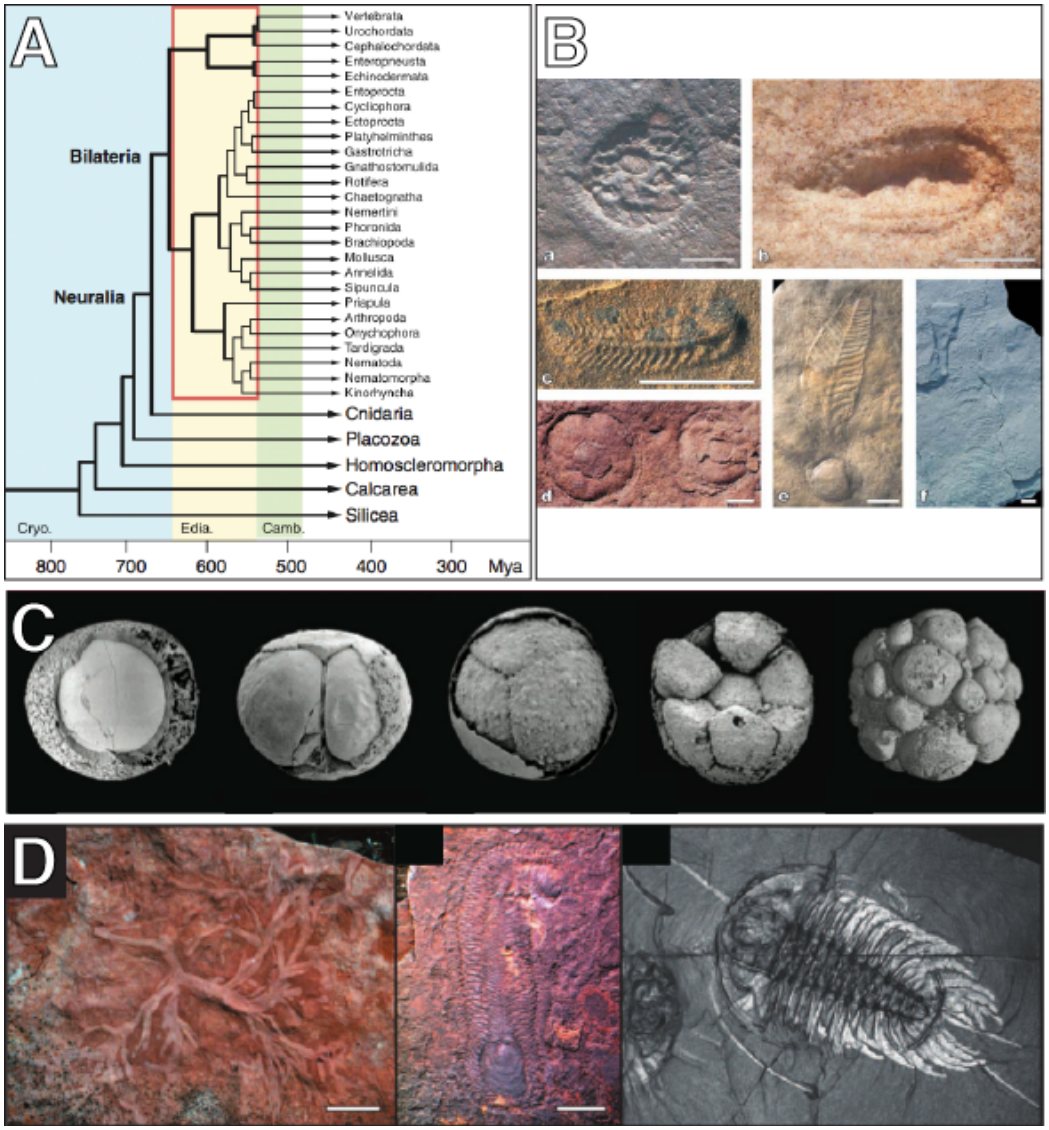


Figure 1-4: Fossil evidence of early Metazoa.
(A) Estimated divergence times of different metazoan phyla (modified from Nielsen and Parker 2010). (B) Examples of Ediacaran fossils (modified from Narbonne 2005). Clock wise from top left: *Palaeophragmodictyon*, *Kimberella*, *Yelovichnus*, *Charniodiscus*, *Medusinites* and *Spriggina*. (C) Examples of Doushantuo fossil embryos from China showing different supposed cleavage stages (modified from Butterfield 2011). (D) Examples of fossils from the Cambrian (modified from Erwin et al. 2011). Left to right: a complex burrow (trace fossil), *Halkiera* and *Olenoides*.

(Fig. 1-4; Narbonne 2005). For example, *Kimberella* is suggested to be a primitive mollusc, having mollusc body plan features such as a radula and a foot (Fig. 1-4B; Narbonne 2005). While almost all fossils from this time period have been designated the "Ediacaran biota", it has been shown to be a mixture of several types of organisms (reviewed in MacGabhann 2013).

Microfossils from the Doushantuo formation of southern China (635-550 Ma) have remarkable preservation comparable to Burgess Shale fauna (Cambrian) from ~100 million years younger (Xiao et al. 1998). Small fossils found at the Weng'an site in this formation represent possible early metazoans; remarkably both internal and external morphology greatly resemble cleavage stages of metazoan embryos (Fig. 1-4; Xiao et al. 1998). However, several alternative suggestions have been put forth regarding their affinities. Bailey et al. (2007a) proposed that these microfossils were actually sulphide-oxidizing bacteria undergoing size-reducing divisions due to stressful conditions, but these claims were heavily questioned (see Xiao et al. 2007, and rebuttal Bailey et al. 2007b). Huldtgren et al. (2011) performed 3D tomography, and internal nuclear structure suggests similarities with encysting protists. The pattern of cell division, termed palintomic, implies a *Volvox*-like algal colony (Xue et al. 1995; Butterfield 2011). Regardless of whether these organisms were truly metazoan embryos, *Vernanimalcula guizhouena* from the Doushantuo formation (approximately 580 Ma) has several features of a bilateral, triploblastic organism: an anterior-posterior axis with bilateral symmetry, a digestive tract sitting between two coeloms, and clear differentiation of ectoderm, endoderm and mesoderm (Chen et al. 2004). This means bilaterians originated prior to the Cambrian Explosion, a scenario supported by molecular clock data (Peterson et al. 2008).

Among the earliest records of animal life on the planet are the fossil and chemical remains of sponges. Love et al. (2009) detected the biomarker compound 24-isopropylcholestane, which has only ever been detected from demosponges, from the Cryogenian, at least 635 Ma to a maximum of 751 Ma based on uranium-lead dating. Sponge spicules have also been found in the record, but these were much more recent (560 Ma; Gehling and Rigby 1996).

Claims of older fossil evidence of sponges are dubious and subject to some speculation and incorrect assumptions. Branching structures in fossils from 660 Ma were interpreted as canals of a sponge (Maloof et al. 2010), however the shape and size of these canals would not allow a sponge to pump water effectively. Brain et al. (2012) describes a porous organism – *Otavia antiqua gen. et sp. nov.* – from 750 Ma and interprets this as a sponge fossil. The age and form of this fossil, however, makes these claims somewhat doubtful.

Although the fossil record has numerous examples of interesting animal forms early on in their evolutionary past, conditions for fossilization were rarely met for the soft-bodied organisms that probably dominated during animal origins. Thus, we turn to a synthesis of hypothetical scenarios and current molecular developmental evidence to reconstruct the Urmetazoa.

1.2.2 Competing theories on the Urmetazoa

Several theories regarding the beginnings of animal multicellularity have been proposed over the last 150 years. When Charles Darwin published *On the Origin of Species* (1859) he awakened a renewed interest in embryology and its relationship with evolution. Several workers in the late nineteenth century through to the present have formulated theories on the ancestral state of the Metazoa, along with several ideas on animal classification and relationships; some of these are outlined below.

Ernst Haeckel proposed the first theory on the Urmetazoa – *Gastraea* – in 1874. Observations of animal embryos led him to believe that developmental stages were a reflection of evolutionary history; "*ontogeny recapitulates phylogeny*". Animal embryos seemed to go through certain stages early in development, which he thought mirrored stages in early animal evolution: a single cell, a morula (cleavage stages), a hollow blastula, and finally a gastrula formed by invagination at one point on the blastula (Haeckel 1874; Fig. 1-5). The gastrula, or *Gastraea*, was composed of two germ layers, outer ectoderm and inner endoderm that lined a gastric cavity or gut, and the process of its formation is termed gastrulation. Haeckel used his investigations of calcareous sponge development to support his arguments, though he did not observe an invagination

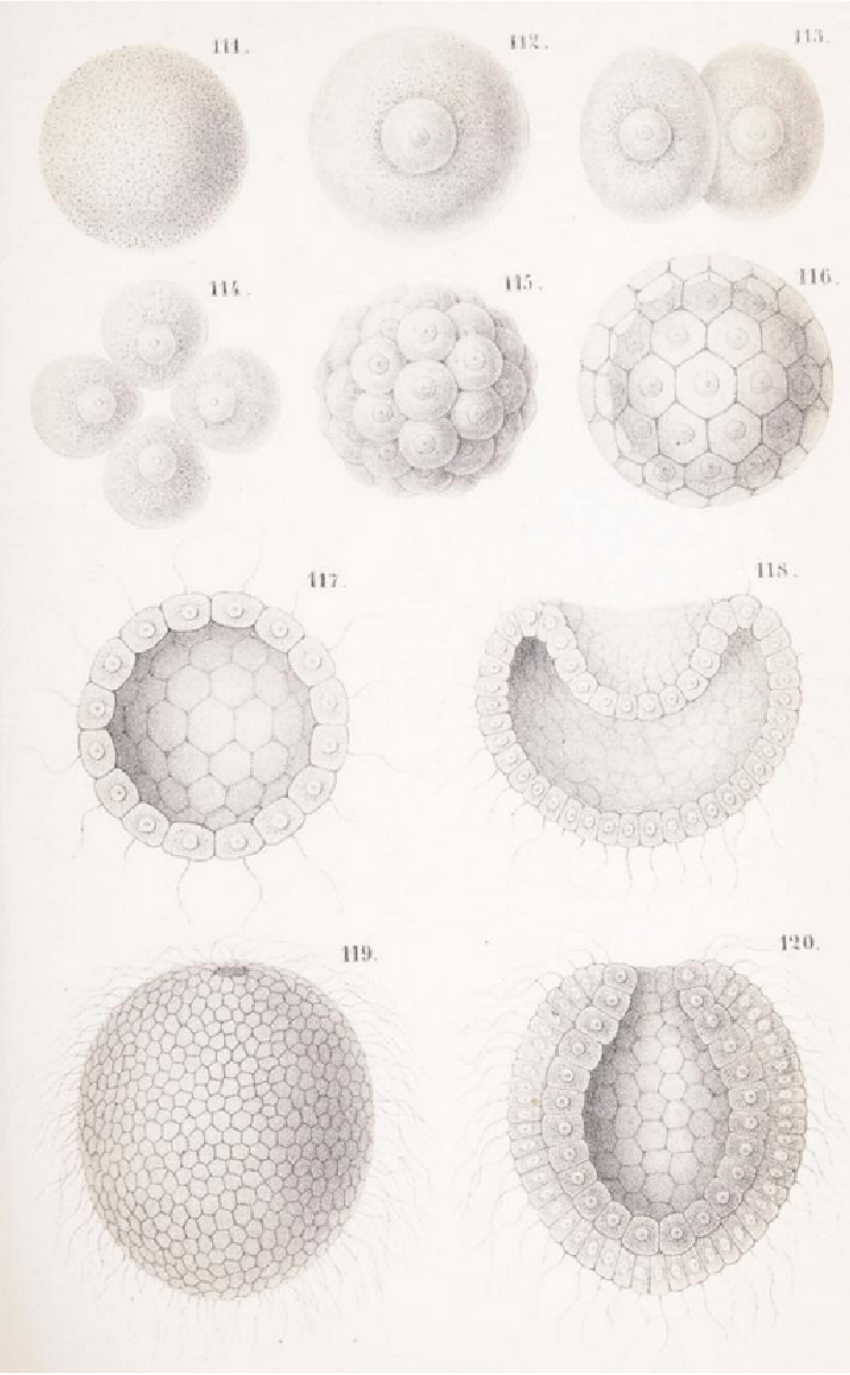


Figure 1-5: The *Gastraea* Theory (from Haeckel 1877).
This figure outlines the progression from single cell to hypothetical colonial stages. The blastula-like stage developed cilia for locomotion, and the gastrula-like stage invaginates to form a primordial gut. In its final form, *Gastraea* is composed of two layers; an endoderm and ectoderm.

stage in these embryos (Haeckel 1873). This stage was later documented, but there is no clear link between invagination and gastrulation; instead it is suggested that the function of gastrulation was originally to create tissue layers (Hammer 1908; Leys and Eerkes-Medrano 2005).

The most common movement involved in gastrulation, however, is not invagination – both Lankester (1877) and Metschnikoff (1887) suggested scenarios that took this fact into consideration. The *Planula* theory states that a blastula underwent delamination to give rise to a bilayered animal (Lankester 1877). A gastric cavity was created by the separation of cells to make an opening into the center of this bilayered animal. Metschnikoff (1887) stressed ingression of cells into the center of the blastula in a succession of steps creating a solid stereoblastula in his *Phagocytella* theory (Metschnikoff 1887). The discovery of *Proterospongia*, a colonial choanoflagellate with an extracellular matrix-like outer structure seemed to lend support to this theory (Kent 1880). This theory incorporated emerging evidence of intracellular digestion among metazoans, as opposed to enzymatic digestion that requires a cavity.

The method of layer and gastric cavity formation in the ancestral metazoan in the *Placula* Theory was quite different from other ideas of the time. A flat, single layer of protozoan cells divided to create a second layer (Bütschli 1884). The discovery of *Trichoplax* – a flat, plate-like animal made of two outer layers and one sandwiched between – offered support for this theory (Schulze 1883). Finally, this flat organism curved upward, forming a gastrula shape (Bütschli 1884). Over a century later phylogenetic evidence suggested that *Trichoplax* (Placozoa) was the earliest branching animal phylum (Schierwater et al. 2009). This was supposedly supported by expression of *Trox2* around the edge of *Trichoplax*, at the point where the top layer bends into the bottom layer. Schierwater et al. (2008) proposed that upward bending of the *Placula* became extreme to the point that an oral-aboral axis was formed, as in Bütschli's view. In cnidarians *Cnox-1* and *3* are expressed at the oral pole in the endoderm and ectoderm respectively, which corresponds to the edge of *Trichoplax* (unpublished results reported in Schierwater et al. 2009). While Bütschli thought the sponges were derived in a

separate lineage, it is now well established that they are not; modern attempts at reviving this theory do not take this into account, and are not considered further.

Hadži's (1963) theorized that the ancestor of metazoans arose from multinucleate unicellular eukaryotes. Within a *Paramecium*-like cell micronuclei gradually became separated by membranes, giving rise to a multicellular worm-like organism (pp. 309-313; Fig. 1-6). This theory has been rejected due to the lack of biological, ontogenetic and morphological support, but current molecular evidence also rejects this scenario, supporting a much more recently evolving position of these flatworms (e.g. Hejnol et al 2009; Pick et al 2010).

The Synzoospore hypothesis was first suggested by Zakhvatkin in 1949, and suggested a very different scenario for early animal evolution. Recently it has been re-examined in light of the fact that opisthokont protists with multicellular life stages never resemble a blastula or gastrula (Mihkailov et al. 2009). Traditionally, most workers took on the view that the urmetazoan was composed of a single cell type, which later differentiated and specialized. These recent authors claim that spatial differentiation of cells already existed when the first animals arose, i.e. multicellularity had essentially already evolved (Mikhailov et al. 2009). In this scenario, a blastula arises from a synzoospore, which is described as similar to morula, but arising from palintomy of a zygote. The blastula then gives rise to either a solid parenchymula (similar to Metschnikoff's *Phagocytella*), or a gastrula. These two lineages then gave rise to sponges and eumetazoans, respectively. Importantly, this idea suggests that multicellularity began as an aggregative event, and not via an embryogenesis-like process as it has always been assumed. However, evidence suggests that colonial choanoflagellates such as *Salpingoeca rosetta*, for example, arise via cell division and not aggregation (Fairclough et al. 2010).

In light of emerging molecular data indicating both the choanoflagellate outgroup of Metazoa and possible sponge paraphyly (see section 1.3 below), Nielsen (2008) proposed the *Choanoblastaea* theory, a newer version of the colonial *Gastraea* theory (Fig. 1-7). Beginning with the idea of a specialized colony of choanoflagellates Nielsen derives the *Choanoblastaea*: a hollow animal

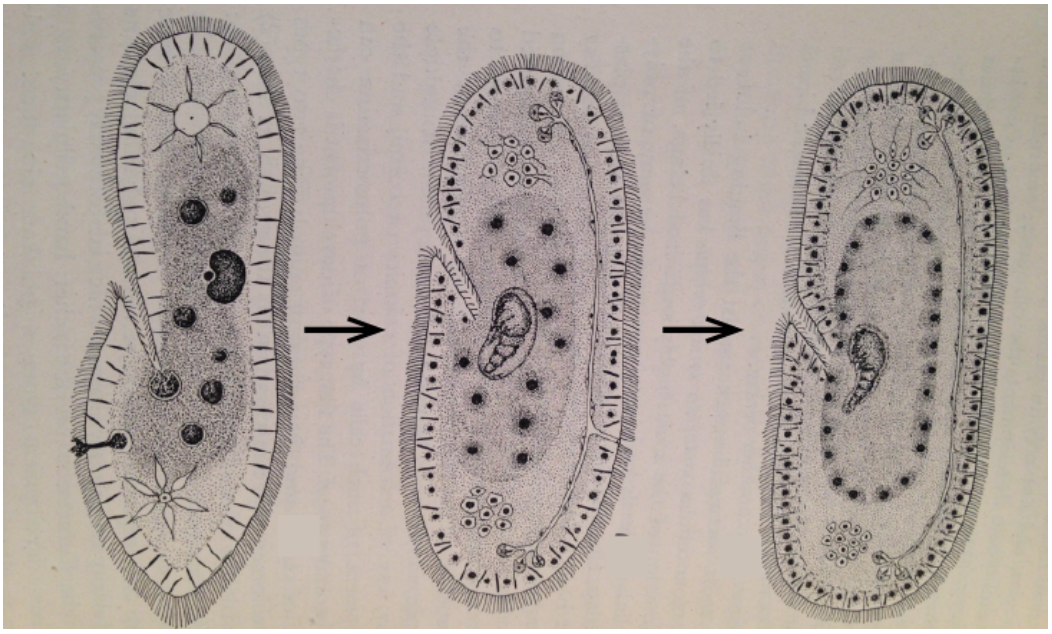


Figure 1-6: Hadži's Cellularization theory (modified from Hadži 1963). Here he outlines how a single celled protist, similar to *Paramecium*, could have developed a multicellular condition.

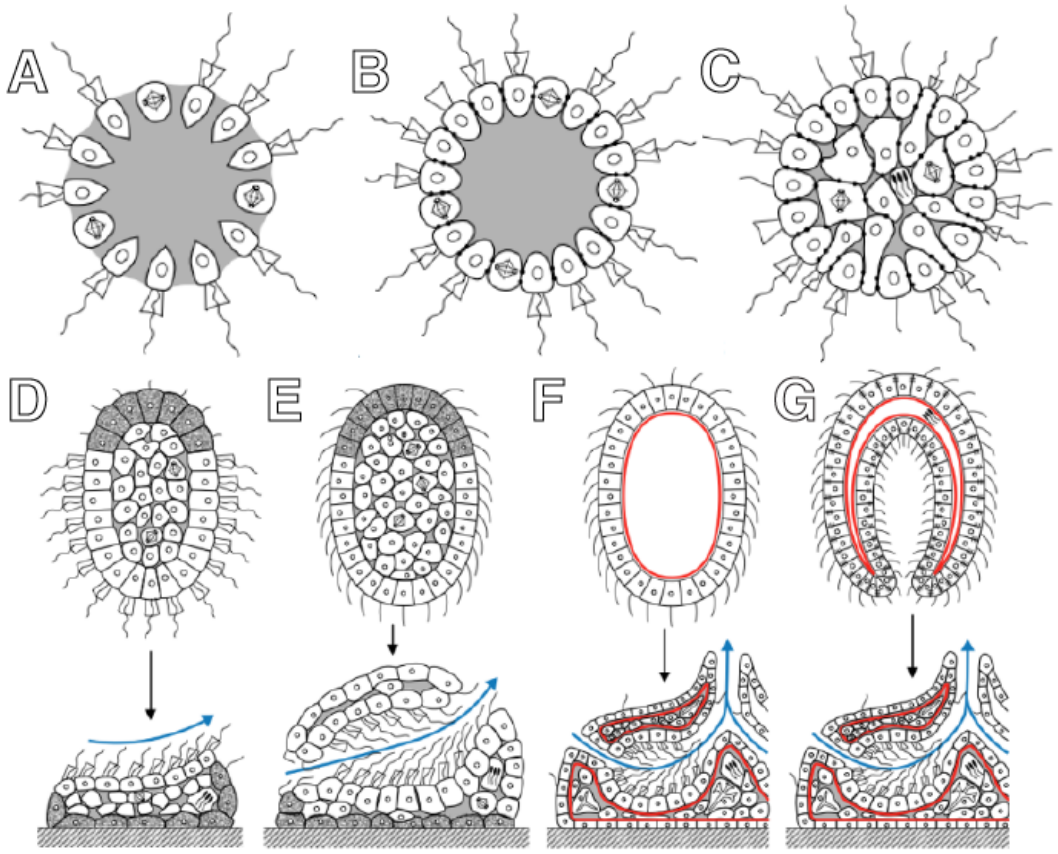


Figure 1-7: Nielsen's *Choanoblastaea* theory (modified from Nielsen 2008). Hypothetical origins of animals from a colonial choanoflagellate according to Nielsen (2008). (A) A colonial choanoflagellate. (B) The *Choanoblastaea*, with a sealed epithelium, surrounding an extracellular matrix. (C) The advanced *Choanoblastaea*, with differentiated cells, including a germ lineage, entering the middle layer. (D) A later stage with polarity, which gave rise to early sponges and the bentho-pelagic lifestyle. Blue arrows depict the direction of water flow over collar cells. (E) Loss of the collared cells in the larva, and settlement into an advanced sponge. (F) Homoscleromorph sponge larva with basement membrane (red). (G) Hypothetical homoscleromorph-like ancestor to Eumetazoa. The larva at this stage has undergone an invagination, giving rise to 2 cell layers as in Haeckel's classic theory.

with a sealed epithelium composed of collar cells pointing outward. The advancement of this animal to a more complex, solid form containing cells with various functions including reproduction is reminiscent of Metschnikoff's *Phagocytella*. Eventually, the advanced choanoblastaea would have acquired polarity, leading to the ability to settle out of the water column, and establishing the bentho-pelagic lifestyle. According to this view, the first sponges were very simply constructed, having an outer layer of choanocytes arranged in a groove for feeding, and a solid composition of various cells internally. Eventually, this groove became overarched, forming the more familiar tube shaped sponge. The larval stage by this point has entirely lost its collars and is instead propelled by cilia. Nielsen then speculates a separate origin of a new lineage of sponges – the homoscleromorphs – and at some point a homoscleromorph-like larval ancestor underwent gastrulation via invagination: the *Gastraea* and the Eumetazoa arise.

To date, the most generally well-accepted theory on the origin of metazoans is Nielsen's version of the colonial theory. A recent review by Richter and King (2013) proposes a similar hypothetical urmetazoan with a genome based on comparative genomics between several unicellular opisthokont and multicellular animal genomes. The close relationship of animals with this group and the striking similarities between choanoflagellates themselves and the feeding cells of sponges – choanocytes – provides the most compelling story of the evolution of the metazoans thus far.

1.3 EVOLUTIONARY RELATIONSHIPS OF BASAL METAZOANS

1.3.1 Traditional relationships and morphology

Relationships between animals have been solidified with molecular data in recent years, though certain relationships remain unresolved depending on specifics of each analysis (data type and methodology). It is surprising how similar new phylogenies are to historical accounts of animal relationships. For example, Aristotle's *scala naturae* (*History of Animals*, Book VIII) arranged Nature from simple (inanimate matter) to complex (man). In general, throughout

history those animals that appeared ‘simple’ were considered to be at the base of the evolutionary tree of animals, as they lacked the more complex characteristics of later evolving animals. Hadži (1953; 1963) was adamant on exclusion of the sponges from the main lineage of animals due to their unique organization, insisting on use of the term “Parazoa” (Sollas 1884), meaning ‘beside animals’. Despite the establishment of metazoans as a monophyletic group, this name still appears in some modern literature. For example, Dondua and Kostyuchenko (2013) use the term Parazoa because they interpret the absence of certain genes and characteristics in sponges as non-homology of body plans. This term is, however, outdated and only reinforces the idea that animals included in the group (Porifera, Placozoa) are somehow not true animals.

When the first molecular phylogenies were performed using, typically, 18S rDNA sequences the basal position of sponges was unchallenged (e.g. Cavalier-Smith et al. 1996; Peterson and Eernisse 2001). However, the recent change in the way genetic information is used to gain insight on the question of metazoan relationships has caused some changes in how some researchers look at these animals.

1.3.2 Phylogenomics and competing hypotheses for basal node groups

In 2008, Dunn et al. used phylogenomics – the use of many concatenated gene sequences – to examine bilaterian relationships. The genes used in this analysis were carefully selected for having slower rates of evolution, and the technique results in a balanced and accurate phylogenetic hypothesis, similar to taking a molecular “average”. The aims of Dunn et al. (2008) were to take a closer look at the relationships among bilaterians, using the basal metazoans to root the analysis and for robust taxon sampling, but the most intriguing finding from that work was the position of ctenophores at the root of the animal tree. This finding had never been postulated previously since ctenophores appear to have a much more advanced body plan than that of sponges; that is, they possessed nerves, guts and muscle.

Others have attempted to either replicate (e.g. Hejnol et al. 2009) or refute this hyporthesis (e.g. Phillippe et al. 2009). However, Pick et al. (2010) were able to

show that the same dataset used in Dunn et al. (2008) could be reanalysed using different parameters and a more traditional result was obtained: that of a basal position of sponges amongst the metazoans. The ctenophore-first hypothesis has not been strongly supported in many studies since 2009, and Nosenko et al. (2013) demonstrated that they could generate the result with high support values by using non-ribosomal (or quickly evolving) sequences in the analysis as opposed to ribosomal or a combined ribosomal/non-ribosomal dataset. The choice of genes and outgroup selected for inclusion in these analyses was made with great care in order to avoid biases that skew relationships of groups sensitive to long branch attraction, along with keeping saturation low and choice of outgroup. The recent publication of the genome sequence of the ctenophore, *Mnemiopsis leidyi*, shows gene absences that might suggest the ctenophores branched off from other animals first (Ryan et al. 2013). While their chosen phylogenetic analyses seem to highly support this, this result is not consistent with their own supplemental data, and this relationship between ctenophores and other animals is still not widely accepted.

It is generally still accepted that sponges are the most ancient animal lineage, and as such represent a useful model in which to study the early evolution of metazoan characteristics. The most highly supported trees from Nosenko et al. (2013) are the ribosomal and non-ribosomal trees (Fig. 1-8A and B, respectively). Thus far a basal position for sponges is the most well supported topology (Fig. 1-8A).

1.3.3 Relationships of classes within the Porifera

Nosenko et al. (2013) recovered both monophyletic and paraphyletic sponges. They conclude that if long branch attraction is taken into account, monophyly of sponges is the most well supported topology (Fig. 1-8). Critically, it is important to realize that whether monophyletic or paraphyletic, whether the metazoan ancestor was sponge-like or not, the basal position of sponges within the monophyletic Metazoa means that this group likely carried over some of the characteristics of the last common ancestor of all animals. Their inclusion in the comparative analysis of the evolution of animal body plans is essential in the

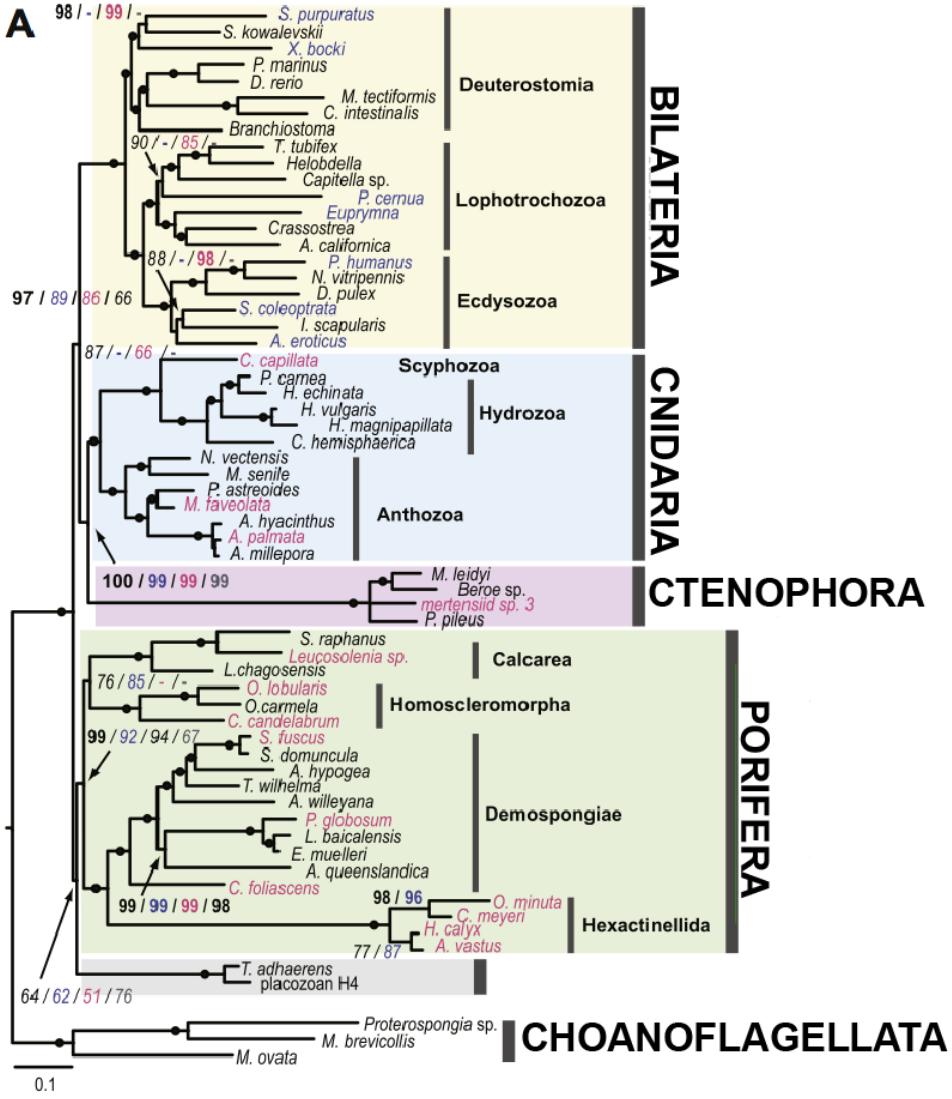
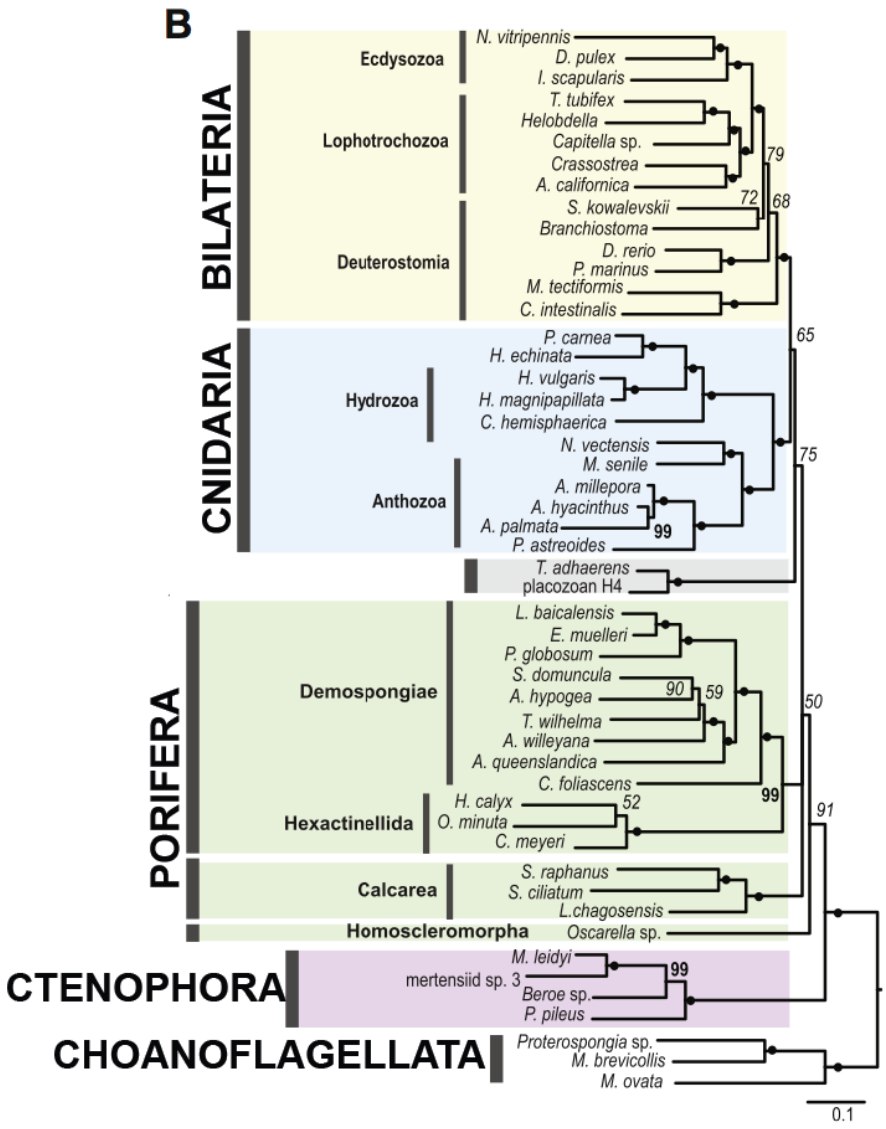


Figure 1-8: Current views on phylogenetic relationships among metazoans (modified from Nosenko et al. 2013).
(A) Bayesian consensus tree of the ribosomal dataset resulting in a basal Porifera+Placozoa, where Porifera is also monophyletic. (B) Bayesian consensus tree of the non-ribosomal data results in a basal Ctenophora and paraphyletic sponges. Support values shown are posterior probabilities for different taxon subsets in multiple analyses, and solid circles indicate 100% support for that node. Both analyses were conducted using the CAT + Γ model.


B
BILATERIA
Ecdysozoa
N. vitripennis
D. pulex
I. scapularis
Lophotrochozoa
T. tubifex
Helobdella
Capitella sp.
Crassostrea
A. californica
Deuterostomia
S. kowalevskii
Branchiostoma
D. rerio
P. marinus
M. tectiformis
C. intestinalis
CNIDARIA
Hydrozoa
P. carnea
H. echinata
H. vulgaris
H. magnipapillata
C. hemisphaerica
Anthozoa
N. vectensis
M. senile
A. millepora
A. hyacinthus
A. palmata
P. astreoides
T. adhaerens
placozoan H4
PORIFERA
Demospongiae
L. baicalensis
E. muelleri
P. globosum
S. domuncula
A. hypogea
T. wilhelma
A. willeyana
A. queenslandica
C. foliascens
Hexactinellida
H. calyx
O. minuta
C. meyeri
Calcarea
S. raphanus
S. ciliatum
L.chagosensis
Homoscleromorpha
Oscarella sp.
CTENOPHORA
M. leidyi
mertensiid sp. 3
Beroe sp.
P. pileus
CHOANOFLAGELLATA
Proterospongia sp.
M. brevicollis
M. ovata
79
72
68
65
75
90
59
50
99
52
99
91
99
0.1

understanding of the nature of the metazoan ancestor.

The morphology of the sponge traditionally unites all groups of sponges into a phylum. Their body plans consist of aquiferous systems for feeding that terminate in an osculum (with the exception of carnivorous sponges e.g. *Asbestopluma*), and their bodies are laid over a framework of spongin, siliceous or calcareous spicules (Simpson 1984; Gazave et al. 2012). Four sponge classes are currently recognized – Hexactinellida, Demospongiae, Homoscleromorpha and Calcarea – and each class was originally separated based primarily on form and composition of the spicules, and molecular data agree with this delineation (Simpson 1984; Nosenko et al. 2013). In fact, molecular data have recently pointed to the possibility that sponges are paraphyletic, with a clade containing hexactinellids and demosponges, and calcareous and homoscleromorph sponges more closely related to other metazoans.

The use of traditional cladistics in combination with newer molecular methods to determine animal relationships resulted in the generation of a tree showing the possible paraphyly of the sponges (Zrzavý et al. 1998). Despite poor taxon sampling from within the Porifera (no homoscleromorphs were included here), the authors recommended the separation of sponges into two subphyla: the Silicispongea and Calcispongea. Borchiellini et al. (2001) followed up with an 18S rDNA analysis, with several new sequences from the three recognized sponge classes and using Neighbor-joining and Maximum Parsimony methods. Their results supported those of Zrzavý et al. (1998) and placed the calcareous sponges in a separate clade, with high support, that was more closely related to other basal metazoans. The authors recognized that a phylogeny with two separate but basally branching clades of sponge phyla implied a sponge-like ancestor for all Metazoa.

Next, a more thorough analysis of the demosponges using new 18S and 28S sequences showed, with high support, that the Homoscleromorpha should not be included within the class Demospongiae, but there was no resolution as to relationships between demosponges, homoscleromorphs, cnidarians and ctenophores (Borchiellini et al. 2004). Using improved taxon sampling from

within the sponges and a new, protein coding gene dataset, Sperling et al. (2007) found that the homoscleropmorphs were most strongly united with eumetazoans, strengthening the hypothesis that sponges are paraphyletic. They also found that calcareous sponges were not falling into a monophyteic clade with demosponges, a finding supported by Manuel et al. (2003), and that they too perhaps represented a separate sponge lineage.

After further improvement of taxon sampling and inclusion of alternative hypothesis testing, Sperling et al. (2009) proposed that the name Epitheliozoa (Ax 1996) include the Homoscleromorpha within the clade containing Eumetazoa + Placozoa to reflect his strongly supported results. In contrast to this finding, Philippe et al. (2009) used phylogenomic methods with a much-improved protein-coding gene dataset to again recover a traditional, monophyletic Porifera, though taxon sampling within the Porifera was necessarily lower due to missing data. It is clear that homoscleromorph sponges do not belong within the class Demospongiae, and in 2012 the Homoscleropmorpha was put forward as a fourth class of sponges although their position relative to the Eumetazoa is still under debate (Gazave et al. 2012).

1.4 THESIS OBJECTIVES AND OUTLINE

Broadly, this thesis aims to strengthen the understanding of the use of conserved patterning mechanisms in the development of animal body plans. I aimed to test whether body polarity, as it exists in other animals, is a homologous feature of all animals and whether comparable pathways control its development.

The adult sponge body plan does not necessarily have any outward symmetry as other animals do, though they are sometimes considered to be radially symmetric about their apical-basal axis. They consist of relatively few cell types that support a filter feeding lifestyle in an aqueous environment. Flat, plate-like pinacocytes line the outer surface, inner epithelia to form canals, and basal side to attach to the substrate. Interspersed among the pinacocytes are porocytes that form the ostia or incurrent entrances to the aquiferous canal system. Incurrent canals lead into chambers full of choanocytes – the feeding cells – and excurrent

canals exit towards the excurrent opening, the osculum. There are also sclerocytes that produce skeletal elements upon which the body grows, and archaeocytes that act as both a stem cell population and as macrophages that clean up cellular debris inside the body. Primarily, sponge body plans are centered on their aquiferous or canal system, which is composed of ostia or pores on the surface of the sponge, incurrent canals, choanocyte chambers, excurrent canals and the atrium and osculum. Water flows through the animal while it filter feeds, in that order.

I chose to study the evolution of body plans and polarity of sponges because of their basal phylogenetic position. Coupled with evidence that they may be paraphyletic, and thus the metazoan ancestor likely sponge-like, they represent an excellent group in which to study questions of ancient metazoans and polarity. Even if sponges are unequivocally shown to be monophyletic or even not the most basal animal group, they can be used for comparative studies among basally branching groups for a well-rounded approach to questions about the early evolution of the Metazoa. I aimed to test whether sponges use Wnt signaling to pattern their body axis, and whether polarity in the larva of sponges carries over to the adult body plan.

At the time I began this thesis, Adamska et al. (2007) had recently shown that a *wnt* gene was expressed at the posterior pole of the larva of *Amphimedon queenslandica*, suggesting that Wnt signaling had a role in establishing polarity in sponges. The work in chapter 2 began as a means of testing the effects of lithium chloride, a well-known pharmacological reagent that disrupts development in many animals, on hatched sponges. We rediscovered work done by Hans Mergner (1964, 1966) that suggested the osculum caused changes in the underlying canals, acting as an inducing tissue. This led to the hypothesis that the osculum had organizer-like properties. I was able to draw parallels with development of another basal metazoan, the cnidarian *Nematostella vectensis*. This work and the work of others (e.g. Adamska et al. 2007; Lapébie et al. 2009) spurred the hypothesis that Wnt was involved in the development of the aquiferous sytem of sponges, and that this may be linked to polarity in the sponge body plan. A version of chapter 2 has been published (Windsor and Leys 2010).

During the first few years of this research, molecular techniques were becoming more widely developed in sponge model systems and it became possible to more directly test molecular hypotheses. In chapter 3 I took advantage of molecular technologies to test the function of Wnts and Wnt pathway components in the sponge, I used a combination of RNAi knockdown and a heterologous expression system (*Xenopus laevis*) to determine whether sponge Wnts functioned as other metazoan Wnts, and whether GSK3 is likely a part of this pathway in the sponge. In this work I also include a bioinformatic analysis of Wnt signaling components of recently sequenced transcriptomes available to our laboratory. While this work followed up on some interesting functional questions, it did not address questions pertaining to the development of polarity in relation to Wnt. This work is collaboration between myself and Drs. William Gillis and Gerald Thomsen (Stony Brook University, Stony Brook, NY), Dr. April Hill (University of Richmond, Richmond, VA) and Dr. Ana Riesgo (Universitat de Barcelona, Spain).

The data comprising chapters 2 and 3 deals primarily with the role of Wnt signaling in sponges hatched from gemmules – that is, sponges that go directly from stem cell-like cells to an essentially adult body plan. In order to link that adult body plan to that of the larva, I became interested in cell fates and the retention of body polarity in the larva of freshwater sponges. In chapter 4, I describe the fates of different populations of cells in the larva of *Eunapius fragilis* following metamorphosis. I examine cell fates at the anterior and posterior poles, and whether larval tissues are retained in the adult sponge; the latter experiments allow me to discuss the problem of gastrulation in sponges. In addition, I analyse the expression of *wnt* mRNAs in swimming larvae to detect whether they might be involved in patterning the anterior-posterior axis as observed in *Amphimedon queenslandica* (Adamska et al. 2007). While providing insight into the development of polarity and gastrulation in these animals, it also provides a map of the freshwater sponge larva for future developmental work on these animals.

In chapter 5, I explore potential directions for future research and evaluate the success of this thesis in addressing some important historical questions.

In addition, I present 4 appendices: additional figures for chapters 2 and 3 are shown in appendices 1 and 2, respectively. Appendix 3 is a collaborative work on T-box transcription factors in 2 sponge species, *Ephydatia muelleri* and *Halichondria bowerbanki*. The lead author was an undergraduate at the University of Richmond with Dr. April Hill, and I contributed sequence data by performing RACE PCR for the phylogenetic analysis. This work was published in *Genes Development and Evolution* (Holstien et al. 2010).

Appendix 4 is another collaborative effort between Dr. Ana Riesgo (Universitat de Barcelona, Spain), Dr. Gonzalo Giribet (Harvard, Boston, MA), Nathan Farrar (PhD student, University of Alberta), myself and Dr. Sally Leys (University of Alberta); it in press with *Molecular Biology and Evolution* due out in 2014. This is a large-scale study comparing the transcriptomes of 8 sponge species spanning all four sponge classes. I conducted searches for genes involved in developmental signaling and wrote the sections of the results and discussion on this topic with aid from Nathan Farrar.

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Chapter Two:

WNT SIGNALING AND INDUCTION IN THE SPONGE AQUIFEROUS SYSTEM: EVIDENCE FOR AN ANCIENT ORIGIN OF THE ORGANIZER[1]

2.1 INTRODUCTION

Body polarity evolved early in metazoan history and was presumably important for coordination of activities such as feeding and excretion. Polarity was therefore a modification to the body plan of colonial protists that provided a template upon which differentiation of more complex animals could evolve.

Polarity can be defined as the property of having two distinct ends, each of which has specific features. Metazoans have anterior-posterior, dorsal-ventral, oral-aboral and embryonic animal-vegetal axes. Although we typically think of these axes as indicators of anterior and posterior ends, they are set up earlier in development before those defining structures have formed. The most familiar axis, anterior-posterior polarity seen in bilaterians from polychaetes to chordates, is patterned by *Hox* gene activity and co-linear expression (Holland and Garcia-Fernandez 1996; Irvine and Martindale 2001; reviewed in Martindale 2005). Even in cnidarians *Hox* and *ProtoHox* genes are expressed at the oral or aboral pole of the polyp of *Hydra*, in the developing scyphozoan polyp *Podocoryne carnea*, and the developing anemone *Nematostella vectensis* suggesting these may correspond to anterior and posterior poles respectively (Gauchat et al. 2000; Yanze et al. 2001; Finnerty et al. 2003; Finnerty et al. 2004). However an even more striking pattern is seen in the expression of *wnt* genes in *Nematostella*, where nine out of fourteen *wnt* genes are expressed in overlapping fields from the oral pole towards the aboral pole (Kusserow et al. 2005; Lee et al. 2006). This suggests that at the base of the metazoan tree Wnts, and not *Hox*, may be the key players in the evolution of body polarity. However the Wnt/β-catenin signaling pathway is also involved in cellular and tissue differentiation and morphogenesis in a myriad of

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animal phyla which makes deciphering whether Wnt has an ancestral role in axis formation difficult (e.g. Nusslein-Volhard and Weischaus 1980; Cui et al. 1995; Cadigan and Nusse 1997; Logan et al. 1999; Prud'homme et al. 2003; Kusserow et al. 2005; Henry et al. 2008).

The main components of canonical or Wnt/β-catenin signaling are the Wnt ligand, its receptor Frizzled (Fz), downstream effector Dishevelled (Dsh), negative regulator GSK-3β, and transcription factor β-catenin (see Logan and Nusse 2004 and Gordon and Nusse 2006 for reviews). Several pharmacological agents are known to act as inhibitors of GSK-3β thereby activating or upregulating the canonical Wnt pathway. Lithium chloride (LiCl) has been widely used in animals from cnidarians to frogs, and although its action cannot be said to be completely specific to GSK-3β the phenotype generated has multiple axes and increased cellular differentiation (Klein and Melton 1996; Stambolic et al. 1996; Hedgepeth et al. 1997; Wikramanayake et al. 2003). Alsterpaullone (AP) and BIO are more specific to GSK-3β and so may be more useful in determining the role of canonical Wnt signaling (Broun et al. 2005; Meijer et al. 2003; Lapébie et al. 2009). Other drugs that inhibit Wnt signaling (such as the β-catenin inhibitor ZTM 000990) are promising tools for further dissection of the pathway (Philipp et al. 2009).

Genes from the Wnt pathway have been found in cnidarians, ctenophores, placozoans and in three sponges studied so far. Generally, within cnidarians, the Wnts have been shown to control germ layer segregation (Wikramanayake et al. 2003), axial specification (Broun et al. 2005) and axial patterning (Hobmayer et al. 2000; Philipp et al. 2009). Although Wnt pathway genes are known to be present within the genomes of placozoans and ctenophores, their functions have not yet been tested (Srivastava et al. 2008; K. Pang, Kewalo Marine Laboratories, personal communication). A pertinent question then, is whether Wnt pathway components are present in and act similarly in the sponges.

Sponges are classically thought to be the earliest branching group of metazoans, forming a monophyletic clade based on a unique and derived morphology, having branched off first from other metazoans and then

diversifying (Hyman 1940). A recent molecular study supports this view (Philippe et al. 2009) while others support a paraphyletic Porifera (Zrzavý et al. 1998; Medina et al. 2001; Borchiellini et al. 2001; Borchiellini et al. 2004; Sperling et al. 2007 and Sperling et al. 2009). A paraphyletic Porifera suggests that a sponge-like ancestor would have existed at some time in animal history. While some would argue that a monophyletic Porifera suggests the opposite, it is important to note that monophyly of the sponges does not refute the hypothesis that a sponge-like metazoan ancestor gave rise to other animals since this view gives us *no* indication of what the ancestor looked like. A sponge feeds using the aquiferous system (spongocoel), which consists of incurrent ostia (pores) and canals, a series of choanocyte chambers (feeding epithelium), neatly branched excurrent canals and an osculum (chimney, or vent). As the excurrent opening to the unidirectional aquiferous system, the osculum effectively polarizes the sponge and defines its body axis (Fig. 2-1E). The likelihood of a sponge-like ancestor in either phylogenetic scenario (monophyly or paraphyly) is reflected in the fact that sponges are represented very early in the fossil record before any other metazoans (Steiner et al. 1993; Gehling and Rigby 1996). It is possible that many sponge lineages were present and eventually one lineage gave rise to the first animals with a true gut, the Eugastraea (Cnidaria + Bilateria) (e.g., Cavalier-Smith 2006). Expression and activity of *wnt* and other genes in sponges may therefore be particularly informative in the question of the evolution of body plans organized around a gut.

Within the sponges only two species have so far been studied in terms of *wnt* expression and function but at very different stages of development and morphogenesis. Lapébie et al. (2009) showed that two *wnt* genes were expressed around the ostia (incurrent openings) and on the surface of the sponge *Oscarella lobularis*. Using BIO, a drug that inhibits GSK-3β and therefore results in improper pan-Wnt signaling, the authors found an increase in the number of ostia on the surface of the sponge and concluded that the role of Wnt in that sponge was in epithelial morphogenesis. During development of the larva in *Amphimedon*

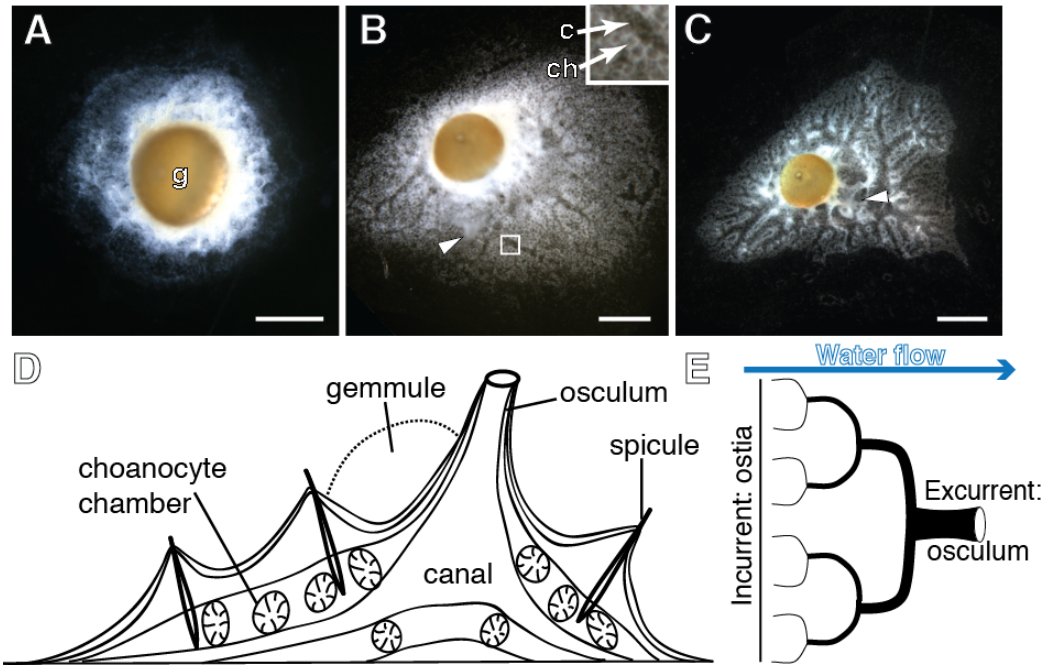

Figure 2-1: Normal development of *Ephydatia muelleri* from the gemmule at (A) 1 day post hatching (dph), (B) 3 dph, and (C) 5 dph.
By 3 dph the sponge has well-organized canals (c) coming from choanocyte chambers (ch) (see inset of boxed area), and a single osculum (arrowhead) which persists as the sponge grows. (D-E) Schematic of the anatomy of the sponge and the polarity of the aquiferous system. Scale bars = 500µm.

queenslandica wntA mRNA is expressed at the posterior pole (Adamska et al. 2007; see also Adamska et al. 2010). Curiously, this is the region that is considered to form the osculum of the juvenile sponge, not the ostia (Sollas 1888; Leys and Degnan 2002), but as the larva has neither ostia nor oscula the role of Wnt in adult morphogenesis cannot be said.

Considering that the genome of *A. queenslandica* as well as ESTs from *Oscarella carmela* contain transcripts that have been identified as Wnt pathway components based on sequence similarity (*frizzled*, *dishevelled*, *GSK-3β*, *β-catenin* and several others) a good hypothesis is that *wnt* controls aspects of polarity in sponges just as it does in other animals (Nichols et al. 2006; Sakaraya et al. 2007; Adamska et al. 2010; Srivastava et al. 2010). We have identified both *wnt* and Wnt pathway genes (*dsh*, *GSK-3β*) in the freshwater sponge *Ephydatia muelleri.* Since expression patterns are difficult to interpret we have taken a pharmacological approach and apply these together with transplant experiments to examine the potential role of Wnt in sponge morphogenesis. Our results show that sponges develop multiple oscula and few or no canals when treated with LiCl and AP respectively. These results suggest Wnt signaling is involved in the formation of the aquiferous system. Mergner (1964, 1966) first hypothesized that the osculum induced development of the aquiferous system. We have repeated his experiments using fluorescent tracers and confirm that the tissue transplanted from the osculum induces canals to grow towards it. These results support the role of Wnt in signaling between canals and the osculum to determine polarity in a freshwater sponge.

2.2 METHODS

2.2.1 Collection and rearing of sponges

Adult individuals of *Ephydatia muelleri* containing gemmules were collected in December to January in Frederick Lake, British Columbia near the Bamfield Marine Sciences Centre. These were returned to the laboratory at the University of Alberta in Edmonton, Alberta and kept at 4˚C. Water was periodically aerated.

Gemmules were removed from the adult spicule skeleton by gentle rubbing on corduroy fabric followed by manual separation and sterilization in 1% hydrogen peroxide. Cleaned gemmules were maintained at 4˚C. To culture, #1 22 mm x 22 mm glass coverslips were flame sterilized and placed into 5 cm diameter Petri dishes. Dishes were filled with culture medium (according to treatment, see below) and individual gemmules were placed on the coverslips in the dark at room temperature (20-22˚C). Typically sponges hatched 1-2 days after plating.

2.2.2 Lithium chloride and alsterpaullone treatment

All treatments were made up in M-medium (1:10 dilution of 10x stock: 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM $NaHCO_3$, 0.05 mM KCl and 0.25 mM Na_2SiO_3; Funayama et al. 2005). Gemmules were hatched in 0.85 mM lithium chloride (LiCl, Fisher Scientific, Ottawa, ON) or 0.25 μM alsterpaullone (Sigma, St. Louis, MO) solution in M-medium for the duration of the experiment. To control for the presence of ions in solution for the LiCl treatment, we used N-methyl-D-glucamine-chloride (NMDG-Cl, Sigma, St. Louis, MO), a large organic ion, in equal or greater concentration to LiCl. Alsterpaullone was initially dissolved in 100% DMSO, and control treatments were 0.003% DMSO in 1x M-medium.

Sponges were treated for the duration of the experiment and were imaged daily using Northern Eclipse software with a Q-Imaging camera mounted on an Olympus SZX12 stereomicroscope. Solutions were exchanged every two days.

2.2.3 Transplants

Sponges were stained in 5 μM carboxymethyl fluorescein diacetate (CMFDA, Invitrogen, OR) for 30 minutes. Oscula and dermal tissue of stained sponges were removed using forceps, rinsed twice for 20 minutes in M-medium, and placed on an unstained host sponge. After one day fluorescence was imaged using a 2.5x lens on a Zeiss Axioskop microscope. Images were processed using Adobe Photoshop. For some experiments, multiple oscula were transplanted to host sponges and imaged prior to and 12 hr after their attachment without moving the dish from the microscope stage.

2.2.4 Scanning electron microscopy

Sponges treated with LiCl and untreated animals were fixed by direct immersion in a cocktail fixative and processed for scanning electron microscopy as described previously (Elliott and Leys 2007). Images were captured on a JEOL 6301F Field Emission SEM and processed using Adobe Photoshop.

2.2.5 RT-PCR

Tissue from 10 individual sponges (cultured in M-medium) was flash frozen in liquid nitrogen at 1 to 5 days after hatching from a gemmule. RNA was extracted from the tissue using the Epicentre RNA Purification System (Epicentre Biotechnologies, Madison, WI) with minor modifications from the manufacturer's protocol; details are available upon request. cDNA was synthesized using the Superscript III First Strand cDNA synthesis kit for RT-PCR (Invitrogen, Burlington, ON) and RT-PCR was performed using the GoTaqFlexi kit (Promega, Madison, WI) according to the manufacturer's specifications. PCR cycling parameters were carried out as follows: initial denaturation at 94°C for 3 minutes, followed by 31 cycles of (94°C 30 sec, 50°C 30 sec, 72°C 30 sec), and final extension time of 3 minutes at 72°C. Samples were loaded into a 1.4% agarose gel. Primer sequences are available upon request.

2.3 RESULTS

2.3.1 Sponge development from the gemmule

Gemmules hatched consistently after 1-2 days at room temperature in M-medium. Totipotent cells move out of the micropyle, a small hole in the surface of the gemmule, and attach to the substrate (Simpson and Fell 1974). One day after hatching (1 dph) cells begin to mass around the gemmule and spread outward still in an undifferentiated state (Fig. 2-1A). Within another two days, at 3 dph (Fig. 2-1B), the young sponge has a fully developed aquiferous (canal) system and excurrent osculum. A typical sponge is shown at 5 dph (Fig. 2-1C) and a cross-sectional diagram (Fig. 2-1D) shows how the tissues are arranged as a system of filters (choanocyte chambers) and canals that empty through the excurrent pole

(osculum) of the aquiferous system. Sponge body polarity is represented by the unidirectionality of the aquiferous system, shown schematically in Fig. 2-1E.

2.3.2 Pharmacological treatment causes disruption of the aquiferous system

We treated developing sponge gemmules with known Glycogen-synthase-kinase- (GSK-) 3β inhibitors lithium chloride (LiCl) and alsterpaullone (AP) (Klein & Melton 1996; Stambolic et al. 1996; Leost et al. 2000). Different concentrations of each drug were tested until a distinct and unusual phenotype was readily seen.

Both LiCl and AP treatments delayed the development of sponges and caused problems with the entire aquiferous system. Whereas excurrent canals of an untreated control sponge were obvious in light microscopy (Fig. 2-2A) in LiCl or AP treated individuals canals were not evident (Fig. 2-2B, C). Incurrent pores (ostia) were visible and abundant in untreated individuals viewed by scanning electron microscopy (Fig. 2-2D), while sponges treated with LiCl had few ostia (Fig. 2-2E) and AP treated animals had none (Fig. 2-2F).

Choanocyte chambers constitute the feeding epithelium of the sponge and are typically dense, semi-spherical structures with well-organized collar microvilli and flagella (Fig. 2-2G). Chambers from animals treated with LiCl (Fig. 2-2H) were malformed and disorganized, suggesting that these animals would not be able to feed efficiently. Choanocytes themselves became rounder in LiCl treated animals when compared to untreated (Fig. 2-2I), and mean values for length to width ratios were found to be significantly different (Mann-Whitney rank sum test; $p < 0.001$; $n = 35$).

LiCl and AP treatment also caused the formation of ectopic oscula. In LiCl animals up to 6 oscula formed (mean of 2) over 5 days of development (Fig. 2-3A and B). AP treatments were slightly more potent, and animals had a mean of 3 oscula per individual with a maximum of 12. Oscula of LiCl treated sponges were small and often deformed, and those of AP treated animals were often difficult to see because of their minute size. Oscula in treated animals usually arose from the periphery of the sponge, whereas in normal animals the osculum was typically central. Controls associated with each treatment (NMDG-Cl or DMSO) developed

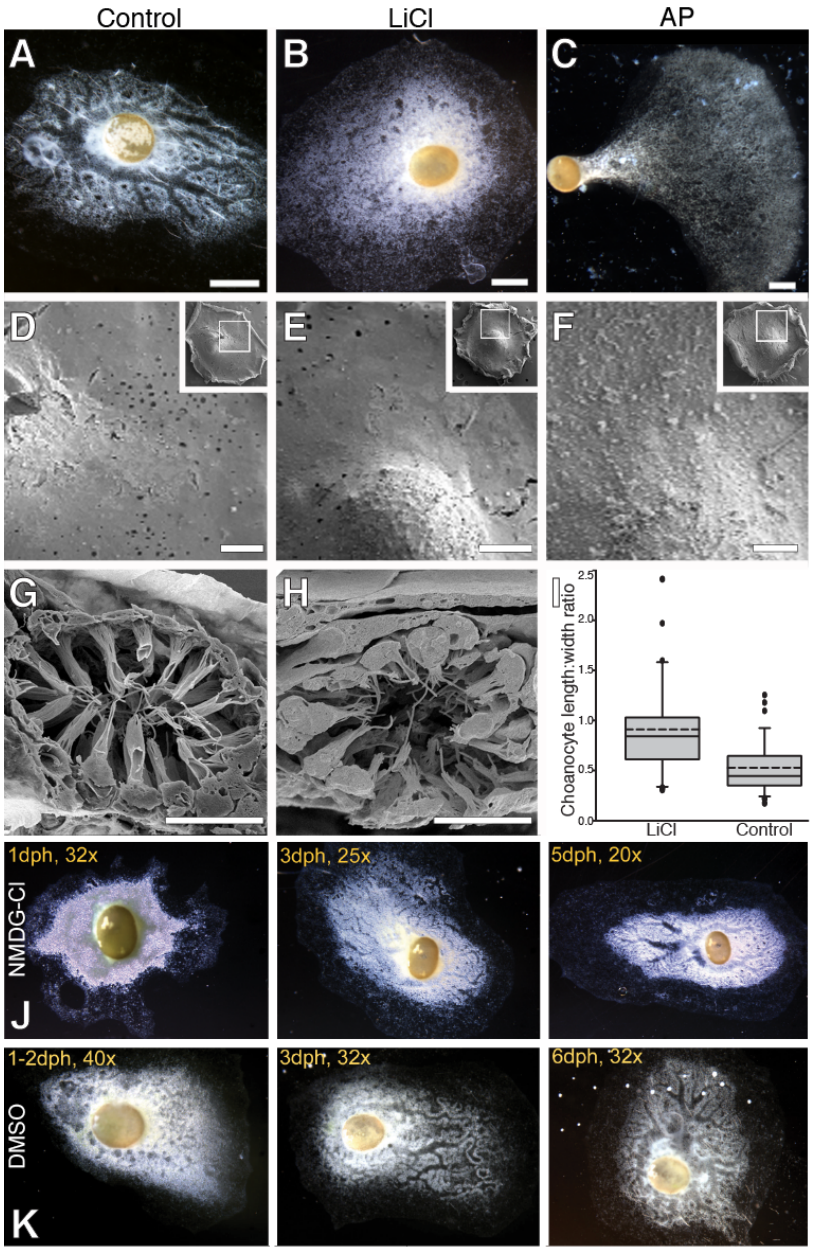

Figure 2-2: The effect on the aquiferous system of treatment of sponges with LiCl and AP (light [A-C], [J, K] and scanning electron microscopy [D-H]). Canals are prominent in normal sponges (A), but reduced in LiCl- (B) and absent in AP- (C) treated sponges. Ostia (incurrent pores, arrows) were abundant on the surface of normal sponges (D), but there were very few on LiCl- and none on AP- (E-F) treated sponges. Choanocytes in chambers of normal sponges were squat with a long collar (G), and in LiCl treated animals choanocytes were round with short collars (H). (I) Box plot of choanocyte dimensions (n = 35). Dots indicate outliers, solid line - mode, dashed line - mean; significance at $p < 0.001$. (J), (K) Control treatments for LiCl and AP (NMDG-Cl and DMSO respectively) at 1, 3 and 6 dph. Sponges developed normally with dichotomously branching canals and a single osculum. Scale bars A-C, 500µm; D-F= 200µm; G,H, 10µm.

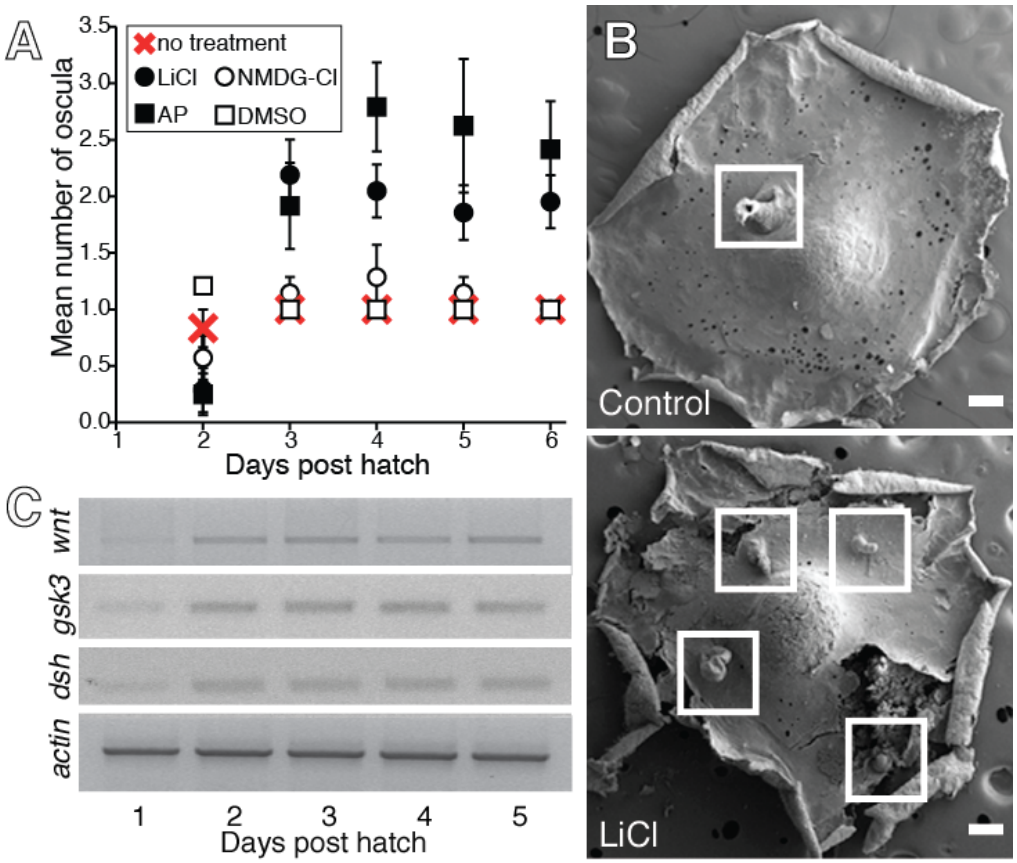

Figure 2-3: Effect of LiCl and AP on osculum formation in *Ephydatia muelleri*.
(A) Plot showing the mean number of oscula counted in each treatment from 2-6dph. Sponges in LiCl and AP formed up to 12 oscula with a mean of 2 and 3 oscula respectively. Control sponges in NMDG-Cl and DMSO formed a single osculum on average. (B) Scanning electron micrographs of LiCl and untreated sponges; oscula are indicated with boxes. (C) *EmWnt*, *Emdsh* and *Emgsk3* expression are correlated with formation of the aquiferous system in untreated sponges (control, *Emactin*).

with normally branching canals and a single osculum (Fig. 2-2J, K; 2-3A).

We isolated a single *wnt* orthologue from *Ephydatia mulleri* (GenBank Accession # HM363029) using degenerate PCR, which we have named *EmWnt* (see Appendix A1-1). During normal development, *wnt* is upregulated at 2 dph when the aquiferous system is being formed, and maintained at a low expression level relative to the actin control (Fig. 2-3C).

Complete loss, malformation or disorganization of ostia, choanocyte chambers, canals and oscula suggested to us that perhaps the aquiferous system was not functioning properly. To test this, we pipetted 1μm diameter fluorescent latex beads on to the surface of untreated and LiCl treated sponges to observe whether sponges were capable of taking up particulates in the medium. Within 5 minutes, untreated sponges became filled with beads, indicating their ability to feed properly (Fig. 2-4A). LiCl treated individuals took up none or only a small number of beads, suggesting that only certain regions of the aquiferous system were able to function (Fig. 2-4B).

2.3.3 Transplanted oscula will reattach and draw canals towards themselves

Sponge oscula detached easily from the sponge when pinched and pulled firmly from their base, and when transplanted onto the dermal tissues of other sponges of the same gemmule batch, they attached and became linked to the excurrent canal system. Within 24 hrs of attaching one transplanted osculum became the new primary vent of the sponge. Canals were reorganized to vent out of the new osculum, causing the host osculum to regress and lose association with the excurrent canal system (Fig. 2-5A and B). Stained dermal tissues (dermal membrane, with associated mesohyl and endopinacoderm layers) placed intact onto host sponges were integrated into the host's dermal tissues (Fig. 2-5C) and never formed a new osculum on the host sponge. Transplants of oscula from other genera (*Spongilla lacustris*) failed to induce a change in the aquiferous system. Detached oscula left on their own rounded up into spheres that lived for several weeks but never re-differentiated into a new sponge.

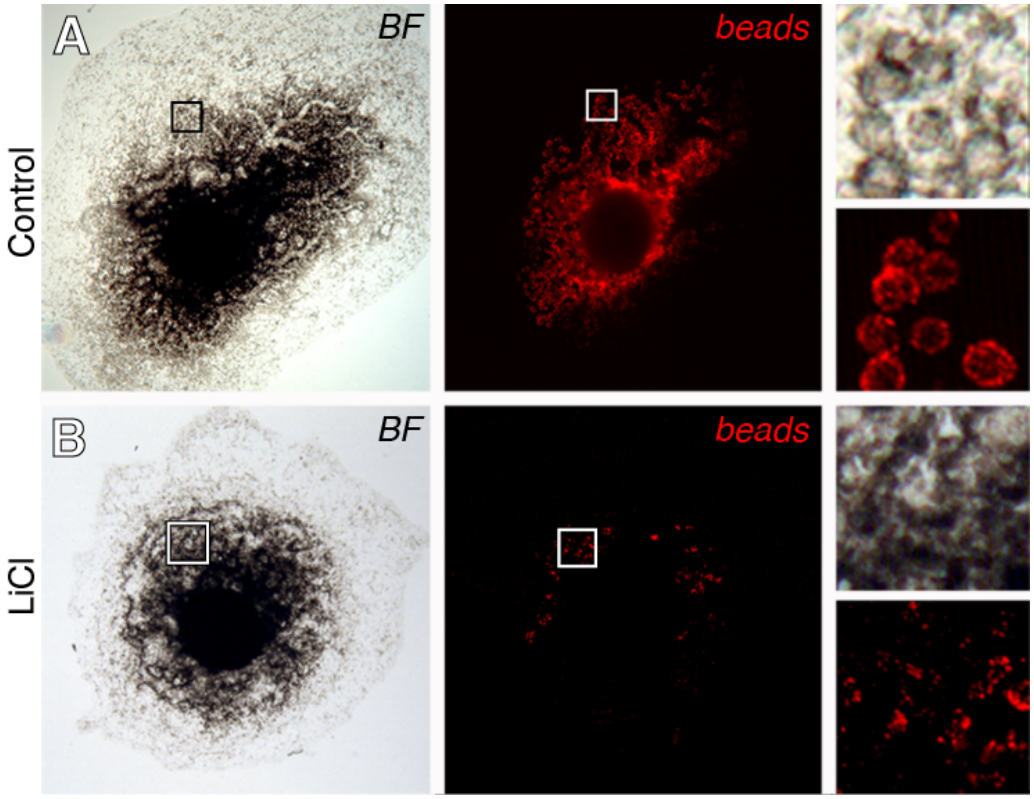

Figure 2-4: Aquiferous system function in normal (A) and lithium treated (B) sponges.
Sponges were fed 1 µm rhodamine-conjugated fluorescent beads over a period of five minutes and then imaged with bright field (BF) and epifluorescence (beads). Insets show boxed areas, enlarged to show beads either in spherical chambers (A) or not (B).

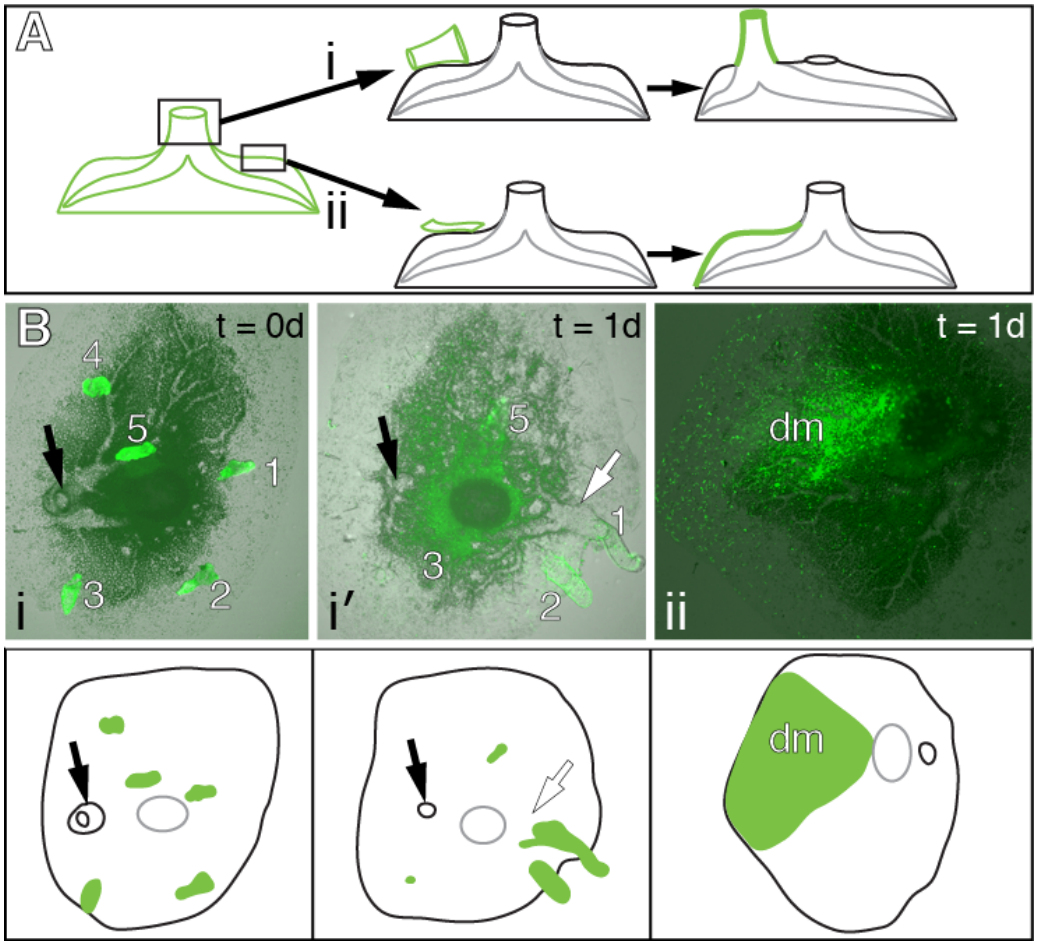

Figure 2-5: Transplanted oscula can induce new canal formation.
(A) Schematic showing the experimental design in which CMFDA-labeled oscula and dermal tissue were placed on host sponges. (B): Fluorescence images and diagrams of five CMFDA-labeled oscula transplanted onto a host sponge prior to and 24 hours after attachment. The original host osculum (black arrow) has regressed while one of the new oscula (1; white arrow) now vents all the water from the sponge. (C) Dermal tissue from a CMFDA-labeled sponge attached to and formed new a new dermal membrane (outer surface) on the host; arrow indicates the osculum of the host sponge.

2.4 DISCUSSION

Our underlying interest is in understanding how polarity is defined in sponges and whether the mechanisms for determining polarity are similar (homologous) to those mechanisms used in other animals. Therefore we asked whether *wnt* genes regulate polarity during development in sponges as they do in other animals. We found that treatment of sponges with pharmacological agents that activate canonical Wnt signaling and affect polarity during the development of other animals generates a phenotype with multiple ectopic oscula and an ineffective canal system. We also found that a transplanted osculum is capable of inducing a host sponge to rearrange canals to vent to the new osculum. Together these results indicate that Wnt signaling is involved in formation of the aquiferous system of the sponge, and suggest that feedback from tissues at the location of osculum formation is involved in maintenance of the polarity of the aquiferous system. The exact role that Wnt plays, and which tissues secrete a Wnt signal are still unknown.

2.4.1 The aquiferous system and sponge polarity

Lithium chloride (LiCl) and alsterpaullone are compounds that inhibit GSK-3β from targeting β-catenin for degradation, as would normally occur when Wnt ligand is bound to its receptor during functioning of the canonical Wnt pathway (Klein and Melton 1996; Stambolic et al. 1996; Leost et al. 2000). Early experiments showed that treatment of developing sea urchin embryos with LiCl caused an increase in "entomesoderm" (endoderm + mesoderm) at the expense of the ectoderm, and thus lithium salts were considered a vegetalizing agent (Herbst 1892). We now know that genes normally expressed only in endomesoderm precursors are upregulated in cells treated with LiCl (for example, Livingston and Wilt 1989). LiCl has been used experimentally on developing embryos of amphibians (Klein and Melton 1996), molluscs (Crawford 2003), annelids (Devriès 1976) and cnidarians (Wikramanayake et al. 2003), and in most of these cases the result was that the endomesoderm became expanded at the expense of ectoderm, indicating vegetalization of the developing animal. In *Nematostella vectensis*, LiCl treated gastrulae exhibited an elongated body with an abnormally

large hypostome, no tentacles and no ectodermal pharyngeal tissue (Wikramanayake et al. 2003). This expansion is mirrored in alsterpaullone-treated hydra in a slightly different way; instead of just an increase in the size of the hypostome ("head") organizer, authors observed a multiplication of the hypostome over the entire body column, such that tentacles were also formed (Broun et al. 2005). This means that the amount of ectoderm was not reduced as much as it normally is with LiCl treatment, but any ectopic production of the hypostome indicates that a second axis is beginning to form.

We have shown that the sponge body axis is equivalent to the polarity of its aquiferous system, since the body plan of a sponge revolves around proper formation of the canals and osculum in order to feed and function normally. When treated with LiCl or alsterpaullone sponges develop abnormally, excessively producing oscula resulting in a chaotic, disorganized and non-functioning canal system. This is similar to observations from other animals described above since the osculum, or at least its precursor, what we term the pre-oscular node (PON), is multiplied, and the entire axis of the sponge is reorganized and duplicated. Furthermore, the lack of differentiation may help to explain the small size of the oscula and lack of well-developed canals in LiCl treated sponges. The flatter appearance of these sponges may also be a result of decreased ability for cellular respiration in LiCl treated animals, as shown in sea urchin embryos (Lindahl 1933 as cited in Runnström 1935).

In *Nematostella vectensis* the striking diversity and pattern of *wnt* gene expression suggests that the role of Wnts in determining animal axial polarity extends deeper in evolutionary time than previously thought (Kusserow et al. 2005; Lee et al. 2006). *wnt* is expressed at the posterior pole of the larval sponge *Amphimedon queenslandica*, a region that has been suggested to give rise to the osculum (Sollas 1888; Leys and Degnan 2002), pointing to an even earlier origin for the role of Wnt signaling. This further supports our hypothesis that the PON has Wnt-controlled axis-inducing properties. In the absence of an effective β-catenin antibody or GFP transgene technology for sponges, it has not yet been possible to test whether this protein's localization is affected by LiCl or

alsterpaullone treatment. Expression of two *wnt* genes in *Oscarella lobularis* suggest that the canonical β-catenin pathway is involved in differentiation of the epithelium in adult tissues (Lapébie et al. 2009); however the authors did not test the role of these *wnt* genes during development of *O. lobularis*.

There are several explanations for the different phenotypes in the different sponges. Adamska et al. (2007) and Lapébie et al. (2009) focus on vastly different times during development and the role of Wnt may be specified by temporal factors. During embryonic development of *A. queenslandica* Wnt signaling may define the body axis, whereas in *O. lobularis* it may act to re-pattern tissues of the adult. Alternatively, since the ostia in *O. lobularis* are formed by invagination of a layer of cells and in other demosponges they form from a single cell, the porocyte, it is possible that in the homoscleromorphs, like *O. lobularis*, Wnt signaling has been co-opted into a role in epithelial patterning. However, if Homoscleromorpha merits Class or even Phylum designation due to features such as a basement membrane with Type IV collagen (Boute et al. 1996) which they share with eumetazoans, (Sperling et al. 2007; Sperling et al. 2009), the role of canonical Wnt signaling in epithelial patterning may be a feature of morphogenesis in more derived animals including the homoscleromorphs. Nevertheless, it is possible that BIO treatment increases canonical Wnt signaling leading to an increase in non-canonical Wnt signaling and causing excess epithelial patterning. A similar phenotype occurs in AP treated hydra, resulting in tentacles all over the body column (Broun et al. 2005; Philipp et al. 2009).

In all these experiments it must be remembered that sponges have few phenotypes (changes to the gross morphology) that can be readily observed and analyzed, and therefore the interpretation of phenotypes is challenging and should be carried out with caution. It is an intriguing and likely possibility that Wnt signaling has multiple roles in the sponge, supporting the growing body of evidence that the Porifera, although an old lineage, is less simple than most would consider.

2.4.2 Inductive abilities of the osculum

The inductive properties of the osculum are similar to those seen in transplanted organizer regions of other animals (e.g. dorsal blastopore lip of ampibians; Spemann and Mangold 1924). Kraus et al. (2007) showed that transplantion of the blastopore lip of the gastrula stage of the anemone *Nematostella vectensis* is also able to induce formation of a secondary axis, including a new blastopore and gut.

Our results indicate that osculum transplants between members of different genera (*Ephydatia* and *Spongilla*) do not result in induction, confirming self-nonself recognition is well established in the Porifera (e.g. Fernandez-Busquets and Burger 1999). Similar intergeneric experiments have not been done with cnidarians, but this result is interesting given that Spemann and Mangold's (1924) original experiments showed salamander congeners were able to induce changes in both species. It would be interesting to determine if different species within the genus *Ephydatia* have the same potential.

Sponges are simple animals without digestive tracts, nervous systems or regionalization of the body into organs (Hyman 1940). Most also consider the group to lack body polarity and symmetry, although Manuel (2009) gives an interesting discussion on this and suggests several types of polarity and symmetry that different groups of sponges may have. If sponges are truly asymmetrical and lack polarity other than apical-basal, it follows that they would not need to have an inductive organizer region during development responsible for setting up a body plan. Our results clearly indicate that this is not the case, as we have shown that if the main body axis is not initially set up correctly, sponges do not differentiate or function properly.

2.4.3 Potential role of Wnt in the aquiferous system

We hypothesize that formation of the axis of the sponge, the aquiferous system, is under the control of the canonical Wnt/β-catenin pathway. This pathway is known to be involved in axis formation and polarity in myriad animal phyla (e.g. Kusserow et al. 2005; Prud'homme et al. 2003; Henry et al. 2008; review, Cadigan and Nusse 1997; Nusslein-Volhard and Weischaus 1980; Logan

et al. 1999; Cui et al. 1995). *EmWnt* expression is upregulated at 2 dph when the aquiferous system forms. Canals form gradually by the complicated fusion of pockets of empty space beneath the dermal tissues. At the point when the canals finally coalesce, the osculum forms on the dermal tissue (see Figure 3-3). Our data show that artificially increasing canonical Wnt signal via the use of GSK-3β inhibitors disrupts the process of aquiferous system formation. The role of Wnt itself in this process remains untested, however several possibilities are evident. Wnt could be secreted from a pre-determined area of the dermal tissue, the pre-oscular node, and this signal could be responsible for drawing the developing canals toward itself, thereby joining up all elements of the aquiferous system. An increase in Wnt signaling would therefore cause canals to be drawn to several locations on the dermal tissue instead of only one, and cause the massive disorganization that we observe.

Alternatively, the lining of the forming canals could secrete Wnt into the luminal space of the canal-forming pockets. As canals fuse with each other, the signal may be amplified until a threshold is reached and the dermal tissues form an osculum. Treatment with GSK-3β inhibitors would thus cause an increase in the number of oscula, and consequently oscula that join up randomly with canals all over the sponge. In either scenario, there appears to be signaling in both directions – between the canals and the osculum – in order to properly form the aquiferous system.

The mounting evidence that Wnt is broadly required for early embryonic axis specification as well as the presence of multiple *wnt* genes in basal phyla implies that this pathway played a critical role in the evolution of multicellular animals. Polarity in the first animals was very likely directly related to food uptake requirements, similar to the situation in sponges.

2.4.4 Concluding remarks

We propose that all metazoans, including sponges, share a common molecular mechanism that is responsible for creating axial polarity of the body plan – the canonical Wnt pathway. Porifera, the sponges, are the oldest branching group of animals (Philippe et al. 2009). We have shown that the adult body axis is similar

to that of other extant Metazoa, supporting the idea that the adult body plan of a sponge-like ancestor may have given rise to early animals with true guts, the Eugastraea.

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Chapter Three:

THE ROLE OF WNT SIGNALING IN THE DEVELOPMENT OF THE CANAL SYSTEM AND POLARITY IN A SPONGE[2]

3.1 INTRODUCTION

Multicellularity has arisen many times in different lineages, and in each instance cellular differentiation and formation of a polarized organism has been intrinsic to the process (reviewed in Kirk 2005). During the evolution of multicellular animals in particular, body polarity appears to have arisen together with regionalization and this presumably led to the generation of organismal complexity and the diversification of animals.

The ancestor of multicellular animals must already have had the tools to form a polarized body, as implied by presence of some genes for toolkit developmental pathways (e.g. a hedgehog precursor, *hedgling*) in pre-metazoan eukaryotes (King et al. 2008; Sebé-Pedrós et al. 2011; Fairclough et al. 2013). One of the notable absences in unicellular eukaryotes is that of Wnt genes and ligands. Now recognized as one of the most widely used signaling proteins in animal development, Wnt was first discovered for its role in *Drosophila* wing development and later for its role in segment polarity (Sharma and Chopra 1976; Nüsslein-Volhard and Weischaus 1980). The canonical Wnt/β-catenin pathway has been shown to be involved in setting up axial polarity during early development in a great diversity of animal phyla. Perhaps the best understood and now classical example is in *Xenopus laevis*, where initial cortical rotation following fertilization places Dsh proteins into the presumptive dorsal side (e.g. Miller et al. 1999) allowing β-catenin to be moved into the nucleus to transcribe Wnt target genes. This is where the region now termed the Spemann organizer is

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[2] *This chapter is the result of a collaborative effort with Drs. William Gillis and Gerald Thomsen at StonyBrook University, Stony Brook, NY; Dr. Ana Riesgo at Universitat de Barcelona, Barcelona, Spain; and Dr. April Hill at the University of Richmond, Richmond, VA:* ***Windsor, P.J****., Gillis, W., Posfai, D., Hill, A., Riesgo, A., Thomsen, G.H., and Leys, S.P.*

specified and where gastrulation and the generation of bodily complexity begins (reviewed in de Robertis et al. 2000). An organizer can be defined as a region – even a single cell – with inductive activity that results in organization of the body during development, especially with respect to axis formation. The organizer was thought to be a vertebrate-specific invention until a single *wnt* from hydra was identified and found to have a role in specifying axial polarity and in organizer activity during development in that animal (Broun and Bode 2002). But cnidarians, seemingly simple animals, were not the first multicellular animals to evolve.

Sponges have long been considered the most ancient animal group, branching off first from other metazoans. In the last years two hypotheses have been proposed for sponge relationships; one showing a monophyletic (Philippe et al. 2009; Pick et al. 2010; Wörheide et al. 2012; Nosenko et al. 2013) the other a paraphyletic (Borchiellini et al. 2001, 2004; Sperling et al. 2007, 2009) Porifera. The latter hypothesis further implies that a sponge-like animal gave rise to all other animals.

The discovery in the mid-1990s (Degnan et al. 1995) of a potential Porifera *hox* gene prompted explorations of polarity genes in sponges (e.g. Coutinho et al. 1998, 2003; Richelle-Maurer et al. 1998; Richelle-Maurer and Van de Vyver 1999; Nikko et al. 2001; Hill et al. 2004). When it was discovered that cnidarians had a full complement of Wnts whose expression patterns suggested a role in the oral-aboral polarity gradient (Kusserow et al. 2005; Lee et al. 2006; Lengfeld et al. 2009), the focus on axial polarity in basal metazoans shifted from *hox* genes to Wnt signaling. In cnidarians GSK3 inhibitors cause the formation of multiple or enlarged head regions in two different cnidarians (LiCl in hydra, Hassel et al. 1993; LiCl in *Nematostella*, Wikramanayake et al. 2003; alsterpaullone in hydra, Broun et al., 2005; alsterpaullone in *Nematostella*, Guder et al. 2006; Philipp et al. 2009; alsterpaullone and LiCl in *Hydractinia*, Müller et al. 2007). Generation of the first sponge genome sequence (Degnan et al. 2008; Leys et al. 2008; Srivastava et al. 2010) revealed the presence of the full Wnt pathway in sponges, and polarized expression of one *wnt* gene (*AquwntA*) and one TGF-β in the

developing demosponge larva (Adamska et al. 2007, 2010). Curiously few other genes in the Wnt pathway showed polarized expression in the larva, and none were studied at metamorphosis when the adult body plan arises. Two other studies have since suggested canonical Wnt signaling is involved in formation of the sponge feeding canals. Treatments with GSK3 inhibitors caused the formation of ectopic oscula (the vent of the aquiferous system) in the freshwater demosponge *Ephydatia muelleri* (Windsor and Leys 2010) and more ostia (the water intake pores) to form in the homoscleromorph sponge *Oscarella lobularis; OlowntII* was also specifically expressed at the newly forming ostia (Lapébie et al. 2009). Together, these data suggest that Wnt is in some manner involved in the formation of polarity in a sponge – either swimming polarity in the larva and/or the polarity of the unidirectional aquiferous canal (feeding) system.

Wnt is not found in pre-metazoans (Holstein 2012). If sponges are the most basal branch of metazoans, then understanding how Wnt functions in sponges can help clarify what Wnt signaling was used for in the early evolution of animals, as well as provide insight into how sponge body plans are patterned. Here we show that Wnt pathway genes are present across sponge groups, and confirm that the critical downstream canonical Wnt signaling regulator GSK3, is involved in osculum patterning using gene knockdown by RNAi.

3.2 METHODS

3.2.1 Transcriptomic and phylogenetic analysis

Transcriptomes of ten sponge species covering all 4 Porifera Classes (Table 3-1) were searched for components of Wnt signaling, with a focus on canonical signaling as in Adamska et al. (2010) using both BLAST and HMMer approaches (Eddy 1998).

Transcriptome datasets were obtained using purified mRNA for cDNA library synthesis using the TruSeq Sample prep kit (Illumina, Inc.) following the

Table 3-1: List of species used in transcriptome searches. Includes other taxonomic identifiers and the source of the data sets.

Species	Class	Order	Family	G group
Aphrocallistes vastus	Hexactinellida	Haplosclerida	Aphrocallistidae	-
Ephydatia muelleri	Demospongiae	Haplosclerida	Spongillidae	G3/G4*
Eunapius fragilis				
Spongilla lacustris				
Petrosia fisciformis			Petrosiidae	G3
Pseudospongosorites suberitoides		Hadromerida	Suberitidae	G4
Ircinia fasciculata		Dictyoceratida	Ircinidae	G1
Chondrilla nucula		Chondrosida	Chondrillidae	G2
Corticium candelabrum	Homoscleromorpha	Homosclerophorida	Plakinidae	-
Sycon coactum	Calcarea	Leucosolenida	Sycettidae	-

* Which group depends on the details of phylogenetic anaylsis. Spongillidae considered G3 from Sperling et al. (2009), and G4 from Borchiellini et al. (2004).

manufacturer's instructions and with details on extraction and sample preparation in (Riesgo et al. 2012). Libraries were diluted to 7-10 nM to be run up to 100 bp paired-end in a Illumina HiSeq 2000 at the Bauer Center of the Faculty of Arts and Sciences at Harvard University (http://sysbio.harvard.edu/csb/resources/instrumentation/instrumentation.html) and LC Sciences (http://www.lcsciences.com/). Filtering of reads based on *Phred* quality scores was performed with CLC Genomics Workbench 5.0 (CLCbio) and datasets were *de novo* assembled with either CLC Genomics Workbench 5.0 with default parameters or Trinity (http://trinityrnaseq.sourceforge.net/).

Sequences of proteins from sponges and other basal metazoans were collected from GenBank on NCBI, aligned using PSI-COFFEE (Kemena and Notredame 2009; Di Tommaso et al. 2011) or MUSCLE (Edgar 2004). Alignments were manually checked for errors and used to create HMM profiles for searching translated transcriptomes. All contigs with an E value of ≥1e-05 were considered homologous. Identity and relationship was confirmed by BLASTP searches against GenBank. Any negatives were double checked by BLASTP searches against translated transcriptome data using BLAST+ (Camacho et al. 2009). The Conserved Domain Database on NCBI was used to identify positions of conserved domains and domain architectures to further assess homology (http://www.ncbi.nlm.nih.gov/cdd). The 3D structure of various Wnt proteins was predicted using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) for comparison and aligned using PROMALS 3D (Pei et al. 2008).

Collected sequence data for phylogenetic analysis is provided in Appendix A2-1. Proteins were aligned in Mafft (Katoh and Standley 2013) and trimmed using the least stringent conditions in GBlocks (Talavera and Castresana 2007) or removing positions missing in more than 85% of taxa in MEGA5 (Tamura et al. 2011). After manually checking alignments for error, trees were constructed using MPI RAxML (Stamatakis 2006) with a gamma distribution and LG substitution model with 500 bootstrap pseudoreplicates.

We also performed a PhyloBayes analysis (Lartillot and Philippe 2004, 2006; Lartillot et al. 2007, 2009) with the same datasets with 2 independent chains, sampling every 100 generations until the splits frequency reached 0.3 (total of 1 600 000 generations). The combination of trees that gave the best overall support values was used to create a consensus tree.

3.2.2 Gene Expression

Expression of Wnt pathway genes was studied in 2 and 5 days post hatch (dph) sponges grown from gemmules of *Ephydatia mulleri* (Demospongiae; Haplosclerida; Spongillidae; Spongillina) using *in situ* hybridization. Sponges were grown to appropriate age in M-medium (0.1 mM $CaCl_2$, 0.05 mM $MgSO_4$, 0.05 mM $NaHCO_3$, 0.005 mM KCl and 0.025 mM Na_2SiO_3; Rasmont 1961) and fixed in 4% paraformaldehyde (PFA) in ¼ Holtfreter's solution (¼ HS; 875 mg NaCl, 12.5 mg KCl, 25 mg $CaCl_2$, 50 mg $NaHCO_3$ in 1 L; Spiegel, 1955) with 0.03% glutaraldehyde overnight at 4°C. Sponges were rinsed in ¼ HS, dehydrated to 100% ethanol and stored at -80°C until ready to use. Tissue was rehydrated and permeabilized with 5 µg/mL Proteinase K for 1-2 minutes, and post fixed in 4% PFA in PBS with 0.1% Tween-20 before hybridization.

Genes were cloned into the pGEM-T vector (Promega) such that reverse transcription using the T7 promoter would generate an antisense probe. This was used as a template to amplify the target sequence with a specific forward primer and T7 primer, and the product was gel extracted using the MinElute Gel Extraction kit (Qiagen). Reverse transcription was carried out using the 10x RNA-DIG or 10x RNA-biotin labelling mix and T7 RNA Polymerase (Roche). The product was precipitated with lithium chloride and ethanol and stored at -20°C containing 1 unit RNase OUT (Invtrogen), and blots were performed to confirm probe detection abilities and determine concentration ranges for hybridization.

3.2.3 Hybridization, Detection and Imaging

Probes were hybridized to sponge tissue for 16-72 hours at 55°C in hybridization buffer (50% formamide, 5x SSC, 50 µg/mL heparin salts, 100 µg/mL Torula yeast tRNA, 5x Denhardt's Solution and 0.1% Tween-20). Post

hybridization washes of 20 minutes each were performed at hybridization temperature using post-hybridization solution (50% formamide, 5x SSC and 0.1% Tween-20) in 2x SSC, pH 4.5, at a ratio of 3:1, 1:1 and 1:3 respectively, with a final set of 3 x 20 minute wash in 2x SSC pH 4.5. Detection of probes was performed using alkaline phosphatase (AP) conjugated anti-DIG antibody (1:200; Roche), or POD conjugated anti-DIG antibody (1:200; Roche) or streptavidin (1:1000; Roche) with Alexa 488 or 594 tyramide reactions. All antibody incubations were performed overnight at 4°C on a shaker. Tissue was rinsed in maleic acid buffer (10 x 30 minutes), and colour reactions were performed in the dark at room temperature from 3 hours to overnight depending on the probes used. Specimens were labelled with Hoechst 33342 for nuclei, and mounted in Mowiol. The protocol in its entirety is included in Appendix A2-2

We imaged slides on a Zeiss Axioskop 2plus compound microscope equipped for epifluorescence. Images were processed using Northern Eclipse (Empix) and Adobe Photoshop CS5.

3.2.4 RNAi

To generate RNAi, partial T7-tailed primers to the 5' end of the gene of interest *EmuGSK3*; control = *EmuSilicateinM2*) were used to amplify the gene fragment (see Appendix A2-3). This fragment was re-amplified using full length T7 sequence so that the T7 promoter site was added to both ends of the template, then purified to remove primer dimers and non-specific products using the MinElute PCR Purification Kit (Qiagen). Double stranded (ds) RNA was synthesized using the RiboMAX Express RNAi System (Promega) and precipitated to remove debris and unincorporated ribonucleotides. RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo-Scientific).

Gemmules were hatched in 1x M-medium as described in Windsor and Leys (2010) in 12-well dishes. Shortly after hatching 10 µg/mL dsRNA was added to the cultures and fresh solution was exchanged every 24 hours. Scoring and imaging were carried out simultaneously. Resulting osculum count data were found not to be normally distributed and had unequal variances, so we used the

Kruskal-Wallis method to test for significant differences in the datasets, and confirmed using a Chi Square test. Finally, the Dunn test was used to determine which treatments differed significantly from each other (Dunn 1964).

Sponges were stored in RNA Later (Invitrogen) and frozen at -80°C, and RNA was extracted using the RNeasy kit (Qiagen). Knockdown of GSK3 was confirmed with qPCR, using *EmuEf1α* as a control (Rivera et al. 2011).

3.2.5 Xenopus mRNA injections

Three Wnt genes from freshwater sponges (*wntA*, *wntB* and *wntC*) were assessed for their functionality in a vertebrate by injecting full-length mRNAs into 8-cell *Xenopus laevis* embryos. We amplified *wntA* from *Spongilla lacustris* (*SlawntA*) and *wntB* and *wntC* from *Ephydatia muelleri* (*EmuwntB, EmuwntC*) using gene specific primers (see Appendix A2-3).

The genes were subcloned into pCS2+, pCS2+myc-tag vectors using XhoI/XbaI sites (*SlawntA*, *EmuwntC*) or XhoI alone (*EmuwntB*) in the polylinker region of each plasmid. The constructs were linearized using NotI, Phenol/Chloroform extracted and transcribed using the AmpliCap TM SP6 High Yield Message Maker Kit (Epicentre) according to the manufacturer's instructions. Poly-adenine tailing reactions were performed to increase the stability and longevity of mRNA using the Poly (A) Polymerase Tailing Kit (Epicentre). mRNAs were precipitated with ammonium acetate, resuspended in water and stored at -80°C or used immediately.

Female *Xenopus laevis* were primed 16-18 hours prior to egg extraction by injection with 500U Human Chorionic Gonadotropin. Males were anaesthetized with MS222 and the testes were removed and kept in 1x MMR and gentamycin at 4°C. Frog eggs were squeezed into a dry glass dish (for adherence), and a small portion of dissected testes were added to 1ml 0.1x MMR, lightly dounced with a pestle and added to eggs. After 1 hour 0.1x MMR was exchanged with freshly prepared 3% cysteine to de-jelly the eggs, and returned to 0.1x MMR for injecting.

We injected mRNA dilutions at the 8 to 16-cell stage into the ventral, vegetal blastomeres near the midline using a PLI-100 Pressure Injector (Harvard

Apparatus). Following injections, embryos were recovered in 0.5x MMR for 4 hours to overnight, raised at 18°C in 0.1x MMR and scored and imaged for phenotype at neurula and tadpole stages.

3.3 RESULTS

3.3.1 Transcriptomic analysis of Wnt/β-catenin pathway components in 10 sponge species

We searched the transcriptomes of 10 species of sponge from all 4 Classes for Wnt/β-catenin pathway components using a combination of HMM profile and BLAST searches. Most members of this pathway were detected in all sponges. The domain structure and architecture for each gene was similar to that described for *Amphimedon queenslandica* (shown schematically in Fig. 3-1A; Adamska et al., 2010). We found that the axin homologues lacked the residues critical for β-catenin binding, as for *A. queenslandica* (Adamska et al. 2010) (Appendix A2-4). Dickkopf-like genes were present in the transcriptomes of the demosponge *Petrosia fisiciformis*, calcareous sponge *Sycon coactum* and homoscleromorph *Corticium candelabrum* despite its reported absence from the genome of *A. queenslandica* (Adamska et al. 2010). Although the sponge dkk amino acid sequence has low amino acid similarity the nearest blast hits were dkks from other metazoans (Appendix A2-5).

We found Wnt genes in all sponges with the noteworthy exception of the glass sponge *Aphrocallistes vastus*. We could only identify 1-3 Wnts in the demosponges (*Ephydatia muelleri*, *Spongilla lacustris*, *Eunapius fragilis*, *Ircinia fasciculata*, *Chondrilla nucula*, *Petrosia ficiformis* and *Pseudospongosorites suberitoides*), but we found 5 Wnts in the homoscleromorph *Corticium candelabrum* and a remarkable 13 in the calcareous sponge *Sycon coactum*. All sponge Wnts were similar to Wnts from other metazoans in terms of sequence, with 23-24 conserved cysteine residues at specific positions along the protein and a conserved RWNC motif (some with small differences, see Appendix A2-6).

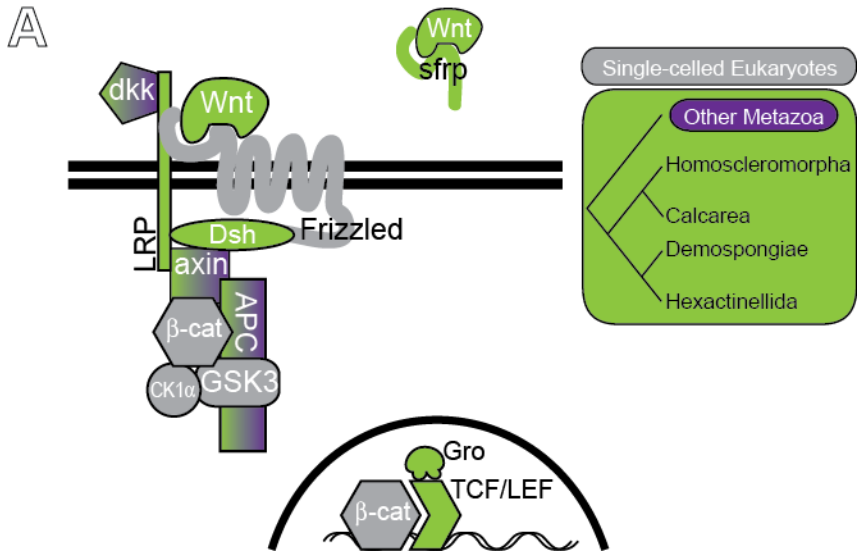
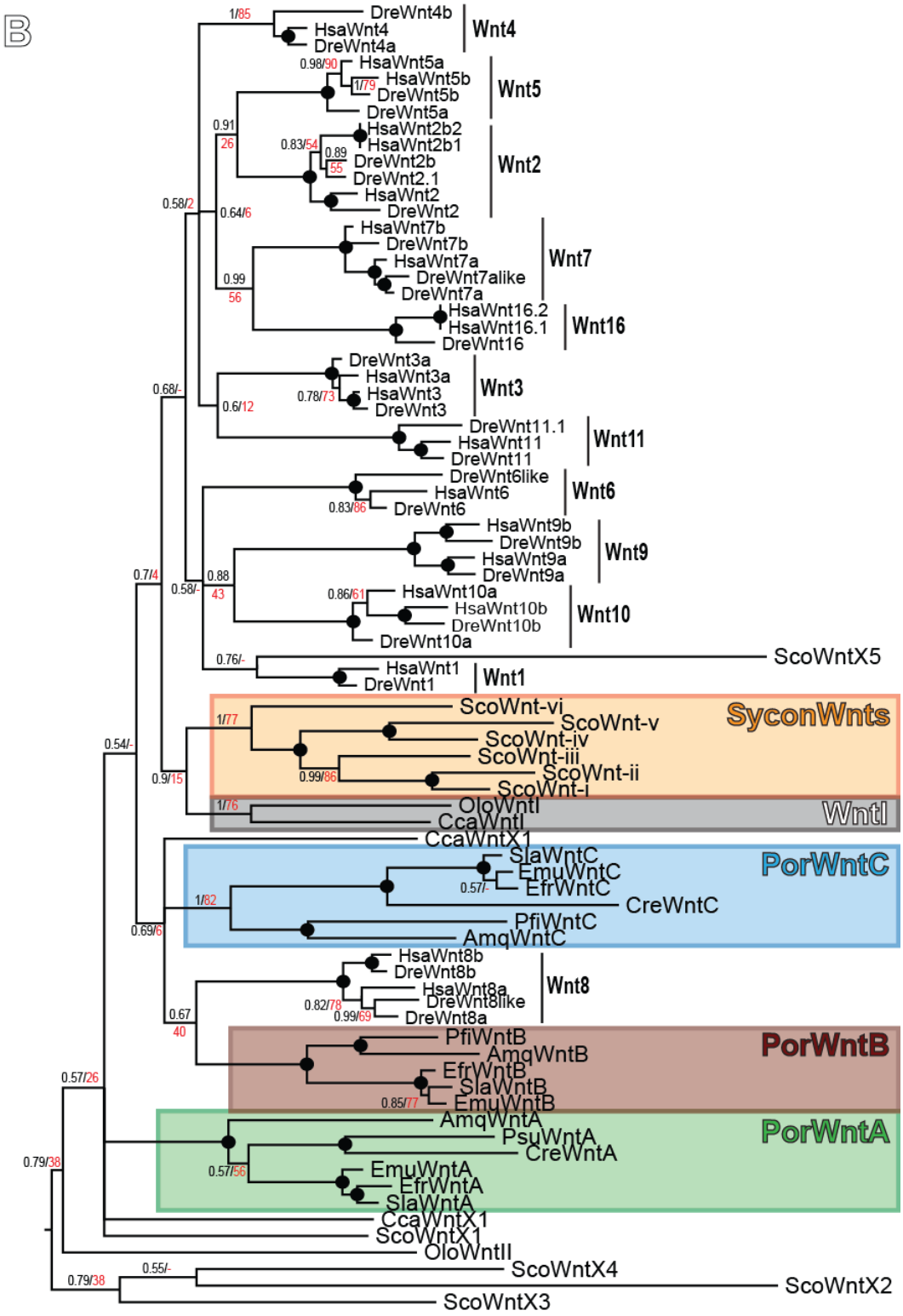


Figure 3-1: Wnt pathway components in sponges.
(A) Diagram of Wnt pathway components indicating when during the evolution of metazoans different proteins arose; proteins of ancient (gray), metazoan (green), and eumetazoan (purple) origin. (B) Consensus tree of Wnt relationships from vertebrates and sponges from RAxML analysis with 500 bootstrap pseudoreplicates and PhyloBayes analysis over 1,600,000 generations. Support values indicated are Posterior probability/bootstrap support (red), or a closed circle (●) for nodes with posterior probabilities of 0.95-1 and bootstrap support of 90-100. (C) Unrooted circular phylogenetic tree (RAxML) of sponge Wnts. Bootstrap support values below 50 were removed, and support of 90-100 is denoted with a closed circle (●). Taxon abbreviations: Aqu, *Amphimedon queenslandica*; Ava, *Aphrocallistes vastus*; Cca, *Corticium candelabrum*; Cre, *Crella elegans*; Emu, *Ephydatia muelleri*; Efr, *Eunapius fragilis*; Ifa, *Ircinia fasciculata*; Olo, *Oscarella lobularis*; Pfi, *Petrosia ficiformis*; Psu, *Pseudospongosorites suberitoides*; Sla, *Spongilla lacustris*; Sco, *Sycon coactum*; Dre, *Danio rerio*; Hsa, *Homo sapiens*.


B
DreWnt4b
HsaWnt4
DreWnt4a
Wnt4
HsaWnt5a
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Wnt5
HsaWnt2b2
HsaWnt2b1
DreWnt2b
DreWnt2.1
HsaWnt2
DreWnt2
Wnt2
HsaWnt7b
DreWnt7b
HsaWnt7a
DreWnt7alike
DreWnt7a
Wnt7
HsaWnt16.2
HsaWnt16.1
DreWnt16
Wnt16
DreWnt3a
HsaWnt3a
HsaWnt3
DreWnt3
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DreWnt11.1
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DreWnt11
Wnt11
DreWnt6like
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DreWnt6
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HsaWnt9b
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HsaWnt9a
DreWnt9a
Wnt9
HsaWnt10a
HsaWnt10b
DreWnt10b
DreWnt10a
Wnt10
ScoWntX5
HsaWnt1
DreWnt1
Wnt1
ScoWnt-vi
ScoWnt-v
ScoWnt-iv
ScoWnt-iii
ScoWnt-ii
ScoWnt-i
SyconWnts
OloWntI
CcaWntI
WntI
CcaWntX1
SlaWntC
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EfrWntC
CreWntC
PfiWntC
AmqWntC
PorWntC
HsaWnt8b
DreWnt8b
HsaWnt8a
DreWnt8like
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SlaWntB
EmuWntB
PorWntB
AmqWntA
PsuWntA
CreWntA
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EfrWntA
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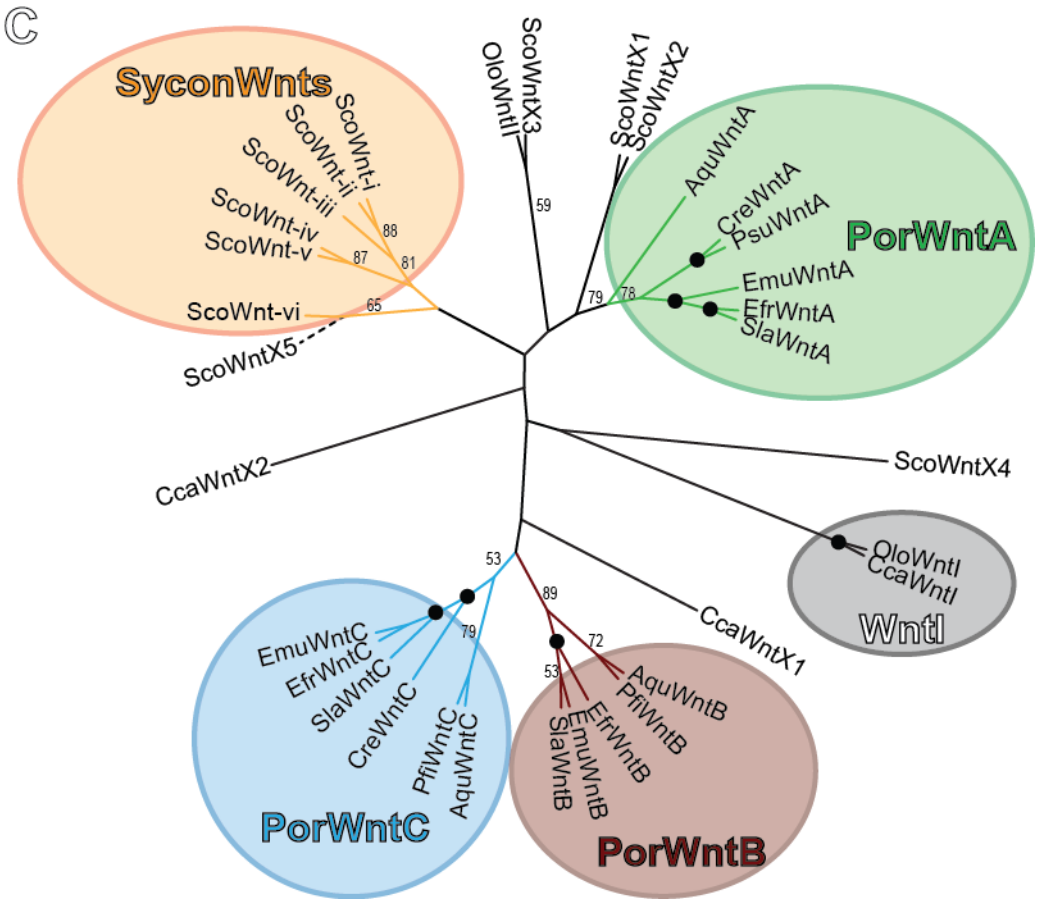
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SyconWnts
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ScoWnt-iv
ScoWnt-v
ScoWnt-vi
ScoWntX5
88
87
81
65
OloWntI
ScoWntX3
59
ScoWntX1
ScoWntX2
AquWntA
CreWntA
PsuWntA
EmuWntA
EfrWntA
SlaWntA
PorWntA
79
78
CcaWntX2
ScoWntX4
OloWntI
CcaWntI
WntI
53
89
72
53
79
CcaWntX1
EmuWntC
EfrWntC
SlaWntC
CreWntC
PflWntC
AquWntC
PorWntC
AquWntB
PflWntB
EfrWntB
EmuWntB
SlaWntB
PorWntB

3.3.2 Sponge Wnt Relationships

Sponge Wnts, with the notable exception of a few Wnts from the calcareous sponge *Sycon coactum* (Calcarea) and the homoscleromorphs *Oscarella lobularis* and *Corticium candelabrum,* largely form groups independent of other metazoan Wnt subfamilies. Calcareous and homoscleromorph sponge Wnts were generally less affiliated with either other sponge Wnt or metazoan Wnt subfamilies, perhaps reflecting a large degree of divergence. Some of these sequences groups with most eumetazoan Wnt subfamilies rather than with sponge Wnts but with very low support (Fig. 3-1B). Vertebrate Wnt sequences fall with high support into distinct, well-defined families, however backbone support throughout the tree was weak and thus no conclusions can be made about the branching order of Wnt subfamilies and the relationships between them. Sponge subfamilies PorWntA-C (named according to Adamska et al. 2010) were found in all analyses. We also recovered the WntI subfamily containing both a *Corticium candelabrum* sequence and the previously published *Oscarella lobularis* sequence, OloWntI with moderate support (1.0/76). The calcareous sponge *Sycon coactum* appears to have undergone multiple lineage specific duplications of Wnts, forming a subfamily of *Sycon*-specific Wnts (SyconWnts). Seven of the thirteen sequences from *Sycon coactum* were fragmentary, and thus were not included in phylogenetic analysis. Those that were are tentatively named ScoWnt-i to ScoWnt-vi.

In order to confirm the placement of sponge Wnts within sponge-specific subfamilies, we conducted a phylogenetic analysis of only sponge Wnts in an unrooted ML tree (Fig. 3-1C). We consistently recovered the 3 subfamilies, as above, PorWntA, PorWntB and PorWntC plus WntI and SyconWnts. Table 3-2 lists members of each family with associated GenBank accession number or transcriptome contig number. Depending on the combination of sponge Wnt sequences included in each tree, PorWntA, B and C subfamilies were supported by bootstrap values ranging from 67-100, 58-98, and 17-70, respectively. Appendix A2-7 contains the final alignment and the PhyloBayes and RaxML trees used to generate the consensus tree in Fig. 3-1B, and Appendix A2-8 contains the final alignment used for the tree shown in Fig. 3-1C.

Table 3-2: Members of each PorWnt subfamily. Groupings were decided by several phylogenetic analyses and are taken from the consensus tree shown in Figure 3-1B and C

PorWntA		PorWntB		PorWntC		SyconWnts		Unaffiliated	
AquWntA	ABX90060	AquWntB	ADO16564	AquWntC	ADO16565	ScoWnt-i	Contig22889*	ScoWntX1	Contig5782
CreWntA	Contig66998	PfiWntB	Contig51648	PfiWntC	Contig10714	ScoWnt-ii	Contig17541	ScoWntX2	Contig26375
PsuWntA	Contig252	EmuWntB	ADM13617	CreWntC	Contig73781	ScoWnt-iii	Contig14145	ScoWntX3	Contig57224
EmuWntA	Contig16045*	SlaWntB	Contig12305*	EmuWntC	Contig23736*	ScoWnt-iv	Contig12941	ScoWntX4	Contig29445
SlaWntA	Contig4731*	EfrWntB	Contig28264	SlaWntC	Contig31918	ScoWnt-v	Contig16518	ScoWntX5	Contig18533*†
EfrWntA	Contig21562			EfrWntC	Contig14524	ScoWnt-vi	Contig38625	CcaWntX1	Contig400
								CcaWntX2	Contig9097
								CcaWntI	Contig2959
								OloWntI	ACS36175
								OloWntII	ACS36174

Listed contigs from transcriptomes reported in Riesgo et al. (in prep) for *Spongilla lacustris* (Sla), and *Sycon coactum* (Sco) are unmarked, while * denotes contigs from transcriptome by S. Leys (unpublished data).

† In sponge-only RAxML analysis, this sequence inconsistently grouped within and without the SyconWnts clade.

3.3.3 Comparison of Predicted 3D Structure

The predicted structure of one sponge Wnt, EmuWntB, was compared with canonical Wnts from *Xenopus laevis*, *Nematostella vectensis*, and *Mus musculus* (XWnt8, NveWnt1 and MmuWnt3) and a non-canonical Wnt from *Xenopus laevis* (XWnt11b), highlighting functional regions and their conservation as recently defined (Fig. 3-2; Bazan et al. 2012; Janda et al. 2012). The overall structure can be compared to a right hand with the finger and thumb grasping forward, a structure seen in both canonical and non-canonical Wnts (Fig. 3-2). Bazan et al. (2012) predicted 3 areas to be involved in Wnt tertiary structure and binding between Wnt ligands and receptors. First, within hairpin 2 (Fig. 3-2, inset 1) a conserved serine residue (Ser187 in *X. laevis*) is present in each species examined. Next, a "linker" region of varying lengths between the two Wnt domains (D1 and D2) is flanked by a set of conserved residues: AXXL/V/M…L/MIF/YXXXS/T (Fig. 3-2, inset 2). Finally, the same authors proposed that an interaction with the LRP receptor occurred at a convex epitope within the D2 domain, and on either side of αH. This site is not conserved in PorWntB subfamily proteins, but is conserved in PorWntA and C subfamilies (Fig. 3-2, inset 3). For the full alignment including all sponge sequences against *Xenopus laevis* Wnt8, see Appendix A2-9.

3.3.4 Wnt pathway gene expression

To assess the role of Wnt we studied the expression patterns of Wnt/β-catenin pathway genes (3 *wnt*s, 3 *frizzled*s, 1 *dishevelled*, 1 *GSK3*, 1 *β-catenin*, and 1 *tcf/lef*) in 2 and 5 dph juveniles of *Ephydatia muelleri* using both alkaline phosphatase (AP) and fluorophore (FISH) based *in situ* hybridization detection methods.

The first cells to emerge from the hatched gemmule form the epithelia that attach the sponge to the substrate and the outer surface (Fig. 3-3A). At 2 dph totipotent cells called archaeocytes fill the space – the mesohyl – between the two epithelial layers and move around the now empty gemmule husk. Between 2 and 3 dph epithelial-lined lacunae move and shift within the cell mass, eventually

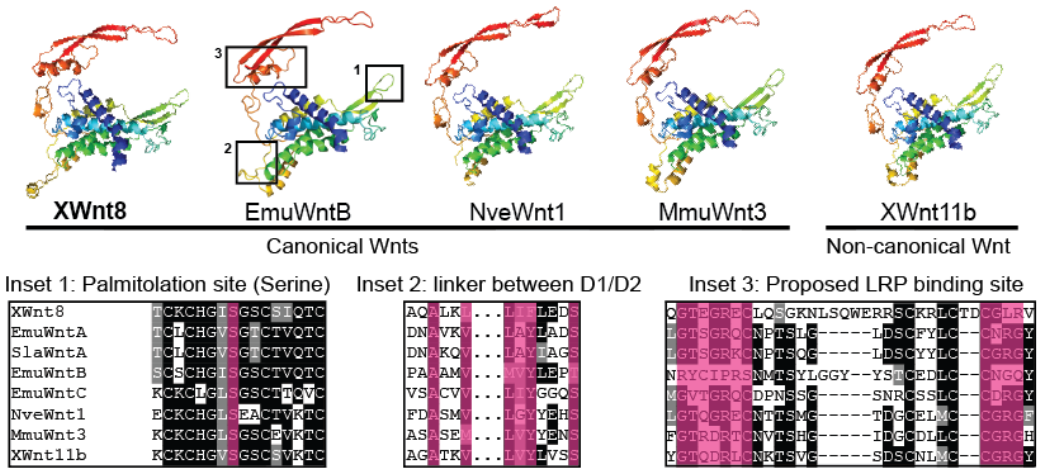

Figure 3-2: Predicted 3D structures of Wnt proteins constructed with Phyre 2 (Kelley and Sternberg, 2009)
XWnt8, *Xenopus laevis* Wnt8, NP_001081637; EmuWntB, *Ephydatia muelleri* Wnt ADM13617; NveWnt1, *Nematostella vectensis* Wnt1, AAT00640; MmuWnt3, *Mus musculus* Wnt3, NP_033547; XWnt11b, *Xenopus laevis* Wnt11b, NP_001084327; additional sponge sequence contig numbers are given in Table 3-2). Inset 1, conserved serine palmitoylation site; inset 2, linker region between Wnt domains D1 and D2; and inset 3, proposed LRP binding site (Bazan et al., 2012). Important residues are coloured magenta over the alignments modified with BOXSHADE. A full alignment is provided in Appendix A2-9.

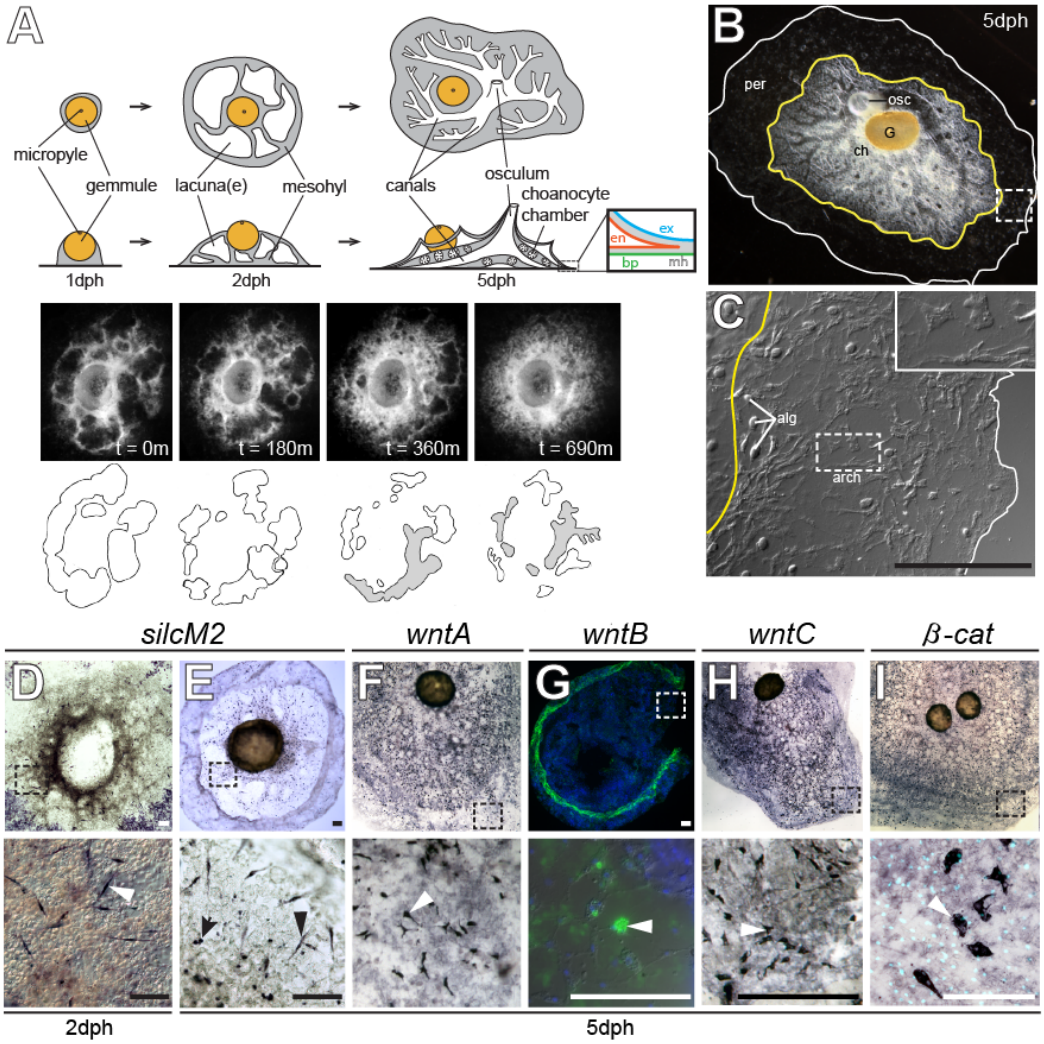

Figure 3-3: Wnt pathway gene expression in *Ephydatia muelleri*.
(A) Schematic of development of the sponge from the gemmule. Inset shows tissue layers: ex, exopinacoderm; en, endopinacoderm; bp, basopinacoderm; mh, mesohyl (middle layer), shaded gray. dph = days post hatch. Formation of the canal system is shown below, showing merging of lacunae to form the canal system within 11.5 hrs (= 690 min) of hatching. (B) Whole sponge at 5 dph showing regions of the body including the gemmule, G, osculum, osc, choanosome, ch with choanocyte chambers and canals and bordered in yellow – and the peripheral region, per, shown by the white border. (C) Cells in the peripheral region shown by DIC. Inset shows amoeboid archaeocytes, arch. (D, E) *silicatein M2* (positive control) *in situ* hybridization in 2 dph (D) and 5 dph (E) sponges (whole mount top; inset bottom). Sclerocytes are spindle-like in shape (arrowhead). (F-I) Expression of *EmuwntA*, *EmuwntB*, *EmuwntC*, and *b-catenin* mRNA in archaeocytes of the peripheral region. (D-F) and (H, I) NBT/BCIP, (G) Fluroescent *in situ* hybridization with *wntB* label in green and nuclei in blue. Scales: (D-H) 100 µm, (I) 50 µm.

fusing with one another to become canals; choanocyte chambers connect with them (Fig. 3B; Wintermann 1951). The osculum arises first as a small bump or raised portion of the sponge surface, the exopinacoderm, and as it grows to full height (approximately 100-200 μm) the excurrent canals join to it and lastly to the incurrent canals and ostia (incurrent holes on the surface of the sponge; Wintermann 1951). By 5 dph a fully functional, pumping sponge has developed. Under stereomicroscopy feeding chambers (choanosome) appear white, and the empty gemmule husk is yellow (Fig 3-3C). Canals branch dichotomously throughout the choanosome and coalesce at the osculum. The outer epithelial layers of the sponge, sometimes referred to as the tent, extends out over the edge of the choanosome forming a transparent window at the periphery of the sponge to the basal epithelium below. In that region (Fig. 3-3C, inset close-up of boxed area) archaeocytes can be seen in live animals crawling in the collagenous middle layer or mesohyl between the exo-, endo- and basopinacoderms (see Fig. 3-3A, boxed area and inset). We found that all gene expression patterns occurred in single, scattered cells rather than regions of the sponge. This finding is not surprising given that the sponge consists of identical sets of choanocyte chambers interspersed with a cellular mesohyl throughout the body; the only regional localization of tissues is the branching aquiferous system itself with incurrent openings (ostia) and excurrent vent (osculum).

As a positive control for *in situ* hybridization we used *silicatein M2*, a gene expressed in sclerocytes in the growing sponge, as previously described in (Mohriet al. 2008). At 2 and 5 dph *silicatein M2* was expressed in sclerocytes, which can be identified by their elongate shape (Fig. 3-3D and E), but never in amoeboid cells at the periphery as above. In 5 dph sponges however, *silicatein M2* also labelled other cells throughout the choanosome (Fig. 3-3E). In contrast, *wnt* genes were only detected in cells of sponges with complete aquiferous systems at 5 dph. In these sponges *wntA*, *B* and *C* were expressed in amoeboid cells in the mesohyl at the periphery of the sponge, and throughout the choanosome (Fig. 3-3F, G and H). Double *in situ* hybridizations did not reveal any convincing co-localization patterns (Appendix A2-10). *β-catenin* was

expressed in a similar population of cells along the periphery of 5dph sponges (Fig. 3-3I), however these cells lacked the thin filopodial-like projections seen in cells expressing *wnt*. In all cases *wnts* and *β-catenin* were expressed in only a subset of (not all) archaeocytes at the periphery (Fig. 3-3I). Other Wnt pathway genes (*frizzled*s, *dishevelled*, *GSK3*, and *tcf/lef*) did not show an informative expression pattern even in 5 dph sponges (Appendix A2-10).

We also found that in all sponges with a fully developed aquiferous system (5 dph) every gene (including non-sponge controls such as *Danio rerio hemoglobin* and *engrailed* as well as sense probe controls) labelled a region adjacent to choanocyte chambers (Appendix A2-10). The probe did not coincide with Hoechst (nuclei) and therefore is not cellular. Our current understanding is that degenerating algae in freshwater sponges capture and harbor RNA in the label somehow; this nonspecific labelling never occurred in 2 dph sponges without an aquiferous system, and algae are rarely evident in 2 dph sponges. Additionally, no probe negative controls showed no discernable label, therefore the effect was not due to a lack of blocking endogenous alkaline phoshatases or peroxidase activity (Appendix A2-10).

3.3.5 RNAi knockdown of GSK3

To test whether Wnt/β-catenin signaling controls the development of the aquiferous system in *E. muelleri* we used double stranded (ds) RNA to knock down GSK3. *GSK3* dsRNA treated sponges developed 2-3 oscula, and canals radiated from the centre of the sponge in an irregular branching pattern (Fig. 3-4A). Untreated sponges and sponges treated with control dsRNA (*silicatein M2*) developed a normal canal system with bifurcating canals and typically with only one osculum (Fig. 3-4B, C), although occasionally 2 were observed.

Because sponges hatched at different times, and because previous experiments had shown that extra oscula generated by LiCl treatment are eventually resorbed (Windsor and Leys 2010 and chapter 2), we counted oscula several times over the 48-hour treatment and recorded the maximum number of oscula observed (Fig. 3-4D). Overall, the data showed significant differences in the number of oscula (Kruskal-Wallis test: test statistic = 19.430, df = 2, $p < 0.0005$; Chi-Square test:

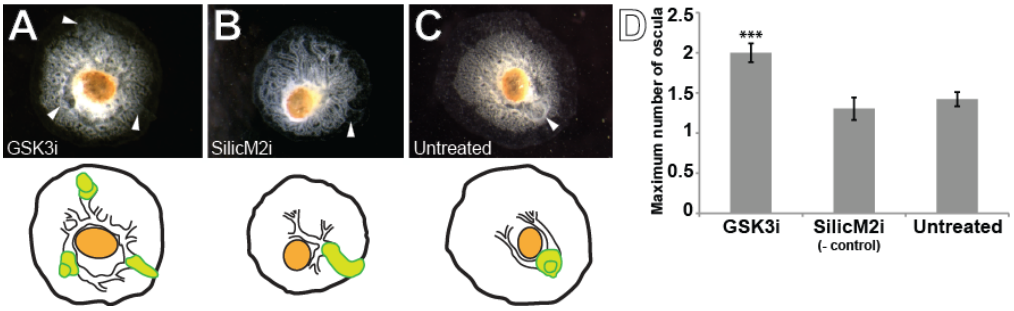

Figure 3-4: GSK3 knockdown with dsRNA causes multiple oscula.
(A) Treatment with 10 µg/ml *GSK3* dsRNA causes multiple oscula (arrowheads) to arise. (B) *silicateinM2* dsRNA treated sponges (negative control) develop with a single osculum. (C) Untreated sponges grown in 1x M-medium alone typically develop 1 osculum. Lower panels show illustrations of each result outlining canals in black and oscula in green. (D) The mean maximum number of oscula for each treatment. *** indicates $p < 0.005$ (Dunn test); difference between controls was not significant ($p > 0.05$). Bars = standard error.

critical value = 23.73, $\chi^2 = 9.488$, $\alpha = 0.05$, v = 4, $p < 0.01$). The maximum number of oscula in *GSK3* dsRNA treated sponges was twice that in both untreated and *silicatein M2* dsRNA treated sponges (Dunn test: n = 85, $p \leq 0.005$, k = 3). Controls, untreated (n = 76) and *silicatein M2* dsRNA treated (n = 33) sponges were not significantly different (Dunn test: $p > 0.05$). A 28% knockdown of *EmuGSK3* was confirmed by qPCR, using *EmuEf1α* as a control (Appendix A2-11).

3.3.6 Heterologous expression of sponge wnts in Xenopus laevis

If sponge Wnt proteins are able to trigger canonical Wnt/β-catenin signaling in the frog *Xenopus laevis*, it is further plausible that canonical signaling also occurs in the sponge. To test this we individually injected three sponge *wnt* mRNAs into frog embryos between the 8 and 16-cell stages and scored for a double axis phenotype. We tried a range of dosages, from 500 pg to 4 ng, to test the potency of sponge *wnts*. We injected *Xwnt8* (5 pg) as a positive control, which consistently gave double axes (Fig. 3-5A), and our negative controls were *mCherry* injected and uninjected embryos.

Sponge *wnt* injections never yielded double axes, and no other obvious phenotypes arose, including those involved in convergence and extension movements associated with non-canonical Wnt signaling (Fig. 3-5A).

Finally, we injected animal caps at the 2-cell stage with a high dosage (4 ng in each blastomere = 8 ng total) of each sponge *wnt* to test whether XWnt8 target genes were expressed, and thus whether organizer activities were being induced. Q-PCR showed no induction of organizer genes was observed for any of the sponge *wnts* injected (Fig. 3-5B).

3.4 DISCUSSION

Functional evidence of canonical Wnt/β-catenin signaling as it is known in other animals is lacking in sponges. Genes for the necessary components of Wnt signaling are present in all 4 classes of sponge, and many of these show a high degree of sequence conservation. Furthermore inhibition of GSK3 in the sponge shows that it plays a role in the development of the osculum, interpreted

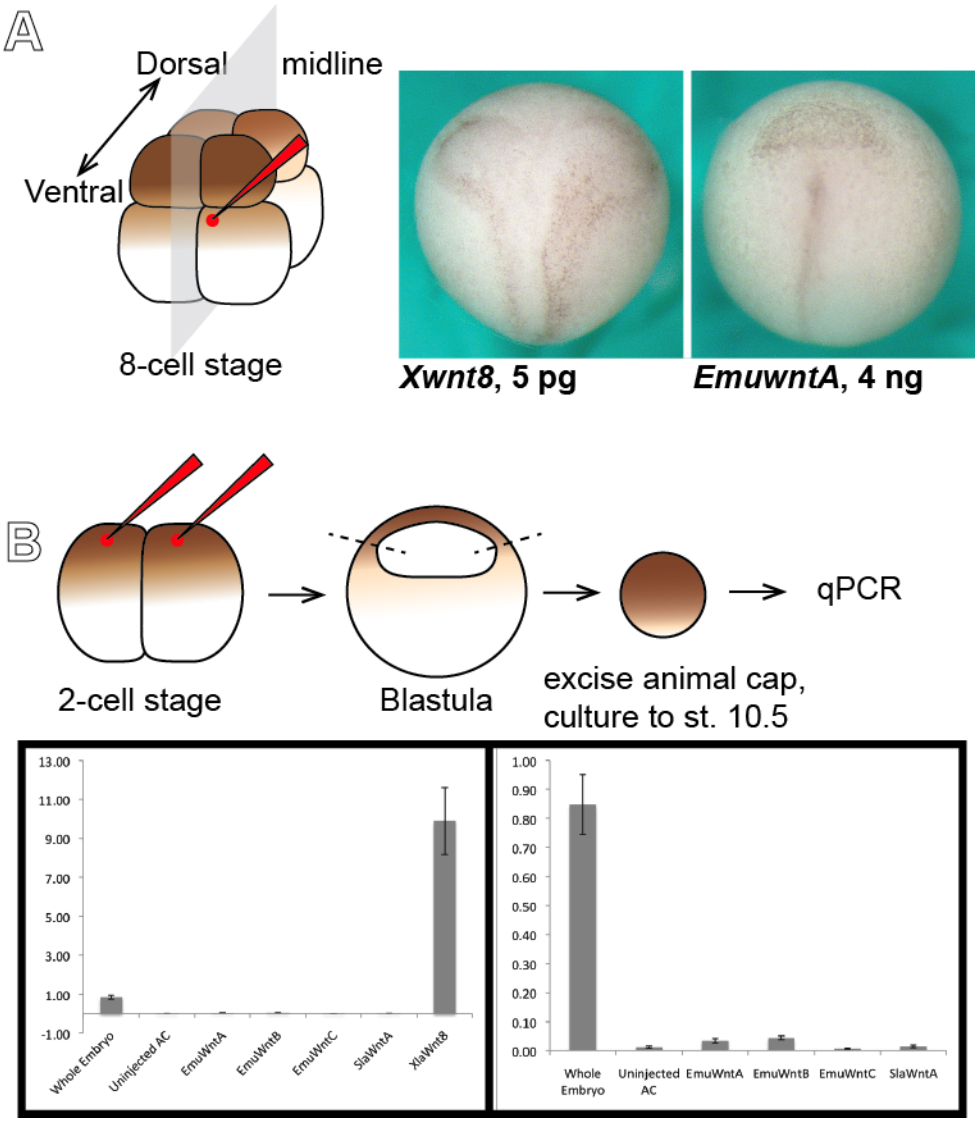

Figure 3-5: Heterologous expression of freshwater sponge *wnt* mRNA in *Xenopus laevis* embryos.
(A) Injection scheme showing precise location of injections for phenotype scoring. *Xwnt8* (5 pg) positive controls consistently gave double axes (n = 36/42) while *EmuwntA* (4 ng) never showed this phenotype, instead giving consistent single axes (n = 0/40). (B) Diagram of animal cap assay and qPCR experiments, with relative expression levels on the y-axis. *Xwnt8* caused induction of the canonical Wnt target *siamois* at ~10 fold higher than in whole embryos (left). When compared to whole embryos and uninjected animal caps (AC), none of the four injected sponge wnts were able to significant increases in expression of siamois. Values were normalized to the positive control, ODC.

previously as the organizer of its body axis (Windsor and Leys 2010 and chapter 2). The inability of sponge Wnts to trigger canonical Wnt signaling in *Xenopus* was unexpected. Aquiferous canal polarity can be thought of as allowing the sponge to capture its food by pumping water through itself directionally in the same way that the long axis of a frog allows it to swim and capture its prey. Our results confirm the role of GSK3 in the development of polarity and further support the notion that canonical Wnt signaling has an ancient, metazoan role in axis formation.

3.4.1 Conservation of Wnt pathway components in sponges

In *Dictyostelium discoideum* a set of proteins, including GSKA and aardvark – homologues to GSK3β and β-catenin, respectively – interact in a manner similar to Wnt signaling in metazoans (Harwood 2009), so finding Wnt pathway genes in the transcriptomes of sponges is not entirely surprising. Sponges are the earliest branching group of animals to have evolved Wnt signaling complete with Wnt ligands, however differences in amino acid sequences suggest that interactions of the ligands with the receptors may differ among metazoans. For example, the predicted 3D structure and alignment of functional regions show that sponge Wnts look like other metazoan Wnts, but it has yet to be demonstrated whether Wnts in sponges directly interact with sponge Frizzled receptors, although the presence of the conserved S187 residue supports this interaction. Conversely, the PorWntB subfamily lacks the predicted LRP interaction site on XWnt8 (Bazan et al., 2012), and therefore it is expected that they interact differently with LRP if at all. It is interesting to note that none of the sponge axins we found have the β-catenin binding site that is required for canonical signaling in bilaterians (Xing et al. 2003). It may be that the cytoplasmic regulation of β-catenin involves only GSK3 as suggested previously (Adamska et al. 2010). The absence of a functional axin, however, is not specific to sponges. The ctenophore *Mnemiopsis leydei* appears to lack axin altogether (Pang et al. 2010), while the axin identified from *Nematostella vectensis* also lacks the β-catenin binding residues (reported in Adamska et al. 2010).

Sponge Wnts fall into 3 sponge specific subfamilies (PorWntA, B and C) with moderate support, reinforcing the idea that sponges diverged before the Wnts split into eumetazoan subfamilies. Within sponges Wnts also underwent duplication events, and this is particularly clear in the calcareous sponge *Sycon coactum* where a surprisingly large complement of *wnt* genes was found. It was a startling discovery that cnidarians have many Wnts (Kusserow et al. 2005; Lengfeld et al. 2009). In these animals Wnts are known to be involved in axial polarity and patterning (Philipp et al. 2009; Marlow et al. 2013). Our phylogenetic analysis suggests *Sycon wnts* are not clearly associated with cnidarian and other metazoan Wnt subfamilies, and certainly the 'need' for that many Wnts in a so-called simple animal is intriguing. At least one *Sycon wnt* is expressed in cells at the tip of the osculum in *Sycon ciliatum* (M. Adamska as reported in Manuel and Forêt 2012), but it is unclear what the others might be used for, and as with all sponges, functional work with the very short-lived larvae and juveniles with dense spicule skeletons is difficult to carry out. One approach would be to investigate whether all calcareous sponges also have this number of Wnts, or other developmentally important proteins, and determine if expression patterns vary among the class since Calcarea is the only group of sponges with three stereotypical aquiferous systems (ascon – single tubes, sycon – chambers off a single tube, and leucon – many branched chambers off canals, as in demosponges). Recently Fortunato *et al*. (Fortunato et al. 2012) found that two other calcareous sponge species, *Sycon ciliatum* and *Leucosolenia complicata*, have multiple *Sox* genes, in contrast to the single *Sox* known from the demosponge *Amphimedon*. The presence of at least 3 Wnts in sponges leads to the intriguing possibility that there is specificity in the role that each Wnt plays during the development of the sponge.

Whereas dickkopf was absent from *Amphimedon* (Adamska et al. 2010) we found one fragment of *dkk* in the demosponge *Petrosia* and longer *dkk* sequences in both *Sycon* and *Corticium*. It is unclear at this time whether these findings are functionally significant in terms of Wnt pathway regulation in sponges, but as genomes become available for the more basally branching metazoans the story of the evolution of negative Wnt pathway regulators may become clearer.

3.4.2 Expression of wnt in the freshwater sponge

It was difficult to interpret expression of *wnt* genes due to the lack of regional pattern and the lack of knowledge of cell function – specifically archaeocytes and more generally, other mesohyl cells in sponges. The three *wnt* genes and *β-catenin* are expressed in archaeocytes in the mesohyl at the periphery of the sponge. These cells appear to be actively crawling, with cytoplasmic extensions reaching out in different directions. Funayama *et al.* found a similar expression pattern for *wnt* in *Ephydatia fluviatilis* (as reported in Manuel and Forêt 2012), where it was suggested they are involved in organizing the positions of spicules. We found no specific co-localization with spicules in our preparations. Alternatively they may be involved in seeking out directions in which the sponge can grow and spread, as this species is an encrusting sponge. Wintermann described two types of crawling archaeocytes, those with and those without filopodia (Wintermann 1951), and these two types respectively express *wnt* and *β-catenin* in *Ephydatia muelleri*.

How does one relate the polarity of an aquiferous feeding system in a juvenile sponge to that of a larva, which is a planuloid ciliated propagule with anterior-posterior swimming polarity (Leys and Degnan 2001)? In *Amphimedon* larvae *AquwntA* is expressed at the posterior pole of the larva, and is juxtaposed with *tgf-β* expression, which covers the whole anterior portion of the larva (Adamska et al. 2007, 2010, 2011). Although no expression studies have been done in *Amphimedon* juveniles, histological and ultrastructural studies show that the posterior pole of the larva forms the osculum in the juvenile (Leys and Degnan 2002). Whereas sponge larvae are often difficult to obtain in sufficient numbers for expression studies, the gemmule-hatched sponge has the advantage of being grown at any time in the laboratory. Gemmules are also polarized by the position of cells in the gemmule husk and the timing of their exit at hatching (Höhr 1977), and therefore polarity could be studied at an even earlier stage to verify whether polarity genes play a role in organizing cells in this way in the gemmule and determine whether this correlates to embryo development and larval formation.

Our findings that a non-specific label is common around choanocyte chambers in 5 dph sponges are important because it suggests algal symbionts can harbor RNA probes in sponges. Since identification of cell types is difficult in all sponges, and many sponges have algal or cyanobacterial symbionts, these findings show that care must be taken with interpretation of regions showing strong labelling within the choanosome wherever symbionts are present.

3.4.3 Functional testing of sponge genes

RNAi knockdown of *GSK3* caused the formation of multiple oscula in *Ephydatia muelleri*, showing that GSK3 is promotes osculum formation in the freshwater sponge. This result confirms the specificity of the result obtained by the inhibition of GSK3 by LiCl and alsterpaullone treatment (Windsor and Leys 2010 and chapter 2), strengthening the hypothesis that canonical Wnt signaling not only takes place but also functions in body polarity development in the sponge.

Sponge Wnts are not capable of creating a double axis in the frog embryo. This perhaps reflects the inability of sponge Wnts to bind to the *Xenopus* targeted receptor. Injection of XWnt8 requires only 5 pg to generate a double axis (Sokol et al. 1991), and 500 pg of *wnt1* mRNA from *Nematostella vectensis* was required to obtain complete axis duplication (Rigo-Watermeier et al. 2012). We used a range from 500 pg to 4 ng, a relatively large amount of mRNA, and still saw no effect, including non-canonical effects such as convergence and extension defects. It is possible that more divergent Wnt proteins from sponges, for example, simply cannot bind to any frizzled and LRP receptors in *Xenopus* or perhaps other technical reasons (competitive inhibition). Several cnidarian *wnts* did not show canonical function in heterologous expression (Rigo-Watermeier et al. 2012) and perhaps this also reflects the divergence of these proteins at important interaction sites. To test this, constructs combining most of the sponge Wnt with the proposed LRP binding site from *Xenopus* could be injected to test for double axes and Wnt target gene induction. It is also possible that the signal sequence of the sponge Wnt proteins are not recognized by *Xenopus* cells (see Appendix A2-8), meaning that the sponge Wnt protein is never actually released into the

extracellular space preventing it from triggering signaling. It is possible to replace the sponge signal sequence with that from *Xenopus laevis* to determine whether export of sponge Wnts in the *Xenopus* embryo is preventing induction of a second axis.

3.4.4 Does Wnt have a function in sponges?

Our phylogenetic anaylsis did not shed light on the deeper relationships between Wnt subfamiles, however the presence of distinct sponge Wnt subfamiles suggests that Wnts diversified separately in sponges and in eumetazoans. General diversification of Wnts perhaps indicates that a single, ancient Wnt-like molecule played a role in the development of an ancestral metazoan. The fact that sponge Wnts have diversified suggests that these proteins were functionally important in this lineage. However the separate diversification of Wnts in sponges and eumetazoans might shed light on the inability of the freshwater sponge Wnts to promote axis formation in *Xenopus laevis*. If sponge Wnts are dissimilar enough to eumetazoan Wnts, they may not be recognized by receptors. Until a clearer picture of Wnt subfamily relationships is obtained there remains doubt as to the evolutionary origin and diversification of Wnts.

One interesting avenue to explore is the possibility that other sponge Wnts, such as those from the calcareous sponge *Sycon coactum* or the homoscleromorph *Oscarella lobularis*, might be able to trigger canonical signaling in heterologous assays. These sequences tended not to group within the demosponge subfamilies of Wnts, and thus testing these may yield different results. Especially in the case of Sycon Wnts, where duplications seem to have occurred several times within that lineage, there are several possibilities for testing. Unfortunately, phylogenetic analysis is unhelpful in determining potential candidates for heterologous expression but sequence similarity and identity from alignments may illuminate the possibilities. Expression data may also help in narrowing the search for candidate canonical triggers, for example by using those that are expressed at either the posterior pole of a larva or the osculum of the adult (M. Adamska as cited in Manuel and Forêt 2012).

Alternatively, Wnt signaling in the sponge may occur through a mechanism in which GSK3 promotes osculum development in a Wnt-independent manner similar to what is seen in the single celled eukaryote, *Dictyostelium* (Harwood 2009). Yet another possibility is that a novel mechanism involving Wnt-like signaling patterns the unique sponge body plan. Previous work done on sponge oscula suggests, however, that a secreted or cell-surface molecule has the capability of causing plasticity in the canal system (Mergner 1964, 1966; Windsor and Leys 2010 and chapter 2), so our work here highlights a significant black box in the roles of signaling pathways in sponges. Our results also illustrate that there are important questions regarding functional homology in light of the increasingly reported molecular similarities between the genomes of sponges and other animals (e.g. Nichols et al. 2006; Harcet et al. 2010; Srivastava et al. 2010).

It is striking that Wnts are not found outside of the animal kingdom, but non-metazoan multicellular organisms use other mechanisms to dictate organization and communication between cells in a population such that they can function as a whole (e.g., Miller and Kirk 1999; Green et al. 2010; Fairclough et al. 2013). During the transition from unicellular to multicellular animals it is clear that there was pressure for body organization, in some form. Though Wnt singalin molecules are generally present, a lack of heterologous expression and promotion of second axis formation might suggest that sponge Wnts cannot function in canonical signaling. However, their diversification separate from other Wnts implies that they are perhaps too widely spaced evolutionarily to bind receptors in that system. Additionally, the involvement of GSK3 in osculum formation has been confirmed, and still points to a role in canonical Wnt signaling in the development in the sponge aquiferous system.

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Chapter Four:

TRACING CELL IDENTITY THROUGH METAMORPHOSIS IN A FRESHWATER SPONGE LARVA

4.1 INTRODUCTION

The recent publication of two sponge genome sequences and several transcriptomes has dramatically increased our understanding of the molecular complexity that must have been present in the first metazoans (e.g. Nichols et al. 2006; Srivastava et al. 2010; Harcet et al. 2010; Conaco et al. 2012; Riesgo et al., *in revision* and Appendix 4). Gene expression studies in sponge larvae have begun to complement genomic data. For example, expression of *wnt*, *hedgling*, and *tgf-b* genes suggests that there may be a link between polarity in sponge larval body plans and other animal larvae (e.g. Adamska et al. 2007; Adamska et al. 2011). Determination of homology versus convergent evolution of particular structures or features of metazoan embryos is generally aided by gene expression and functional data. This has led to a reliance on molecular data to provide information on the homology of developmental processes and structures in sponges at the expense of interest in morphology and embryology. Yet how gene expression patterns in sponge larvae can be equated to those of other animals is still unclear because so little is still understood about cell type and function in sponge larvae and adults. Given the paucity of morphological data on the transition of sponge larvae through to adults, it is unclear whether gene expression data alone can help determine whether a sponge-like adult or a sponge larva gave rise to other animal groups.

Ernst Haeckel's interest in determining homology among metazoans, especially with respect to anterior-posterior polarity, germ layers and the feeding epithelium began with his studies of sponge embryology (Haeckel 1873, 1874). The terms gastrula and gastrulation were coined from observations of calcareous sponge development (Haeckel 1873). From this he postulated an ancestral metazoan with the shape of a gastrula stage embryo with two layers: the ectoderm that gave rise to outer tissues, and the endoderm that formed a digestive

epithelium (Haeckel 1874). Germ layer theory was heavily debated during the late ninteenth century when Delage (1892) observed the internalization of the ciliated outer layer, and its differentiation into the feeding epithelium in sponge juveniles – choanocytes – showing that germ layers in sponges were reversed when compared to other animals. For nearly two centuries two fundamental questions have been studied: whether sponges undergo gastrulation following the formation of embryonic germ layers, and whether positional information (ectoderm versus ectoderm) is retained through metamorphosis.

Libbie Hyman (1940) defined sponges as having a cellular grade organization with cells cooperating little with each other in the functioning of the animal. This has been the primary textbook view of sponges through most of the twentieth century. However, modern work suggests that sponges have sealing epithelia (Leys et al. 2009; Adams et al. 2010; Leys and Riesgo 2012), and a mechanism of formation of polarity of adults and larvae (Bavestrello et al. 1998; Leys and Degnan 2002; Wiens et al. 2006; Adamska et al. 2007, 2010, 2011; Lapebie et al. 2009, Windsor and Leys 2010). Genomic data may yet reveal the presence of unexpected genes; for example, genes encoding a potential dkk, a Wnt inhibitor, have been found in transcriptomes of some sponges though absent from the genome of *Amphimedon queenslandica* (Srivastava et al. 2010; Riesgo et al. *in revision* and Appendix 4; Windsor et al. *in prep* and Chapter 3). Molecular research in sponge adults and larvae continues to push our understanding of the complexity of sponges, and their relationship to other animals (Adamska et al. 2011). Gene expression studies during development in sponges are becoming more common, and functional studies are on the rise especially with the progress in RNAi technology in sponges (Rivera et al. 2011). Unfortunately our grasp of cell types and fates during sponge development and metamorphosis is limited so the connections we can make between gene expression, function and cell types are restricted. A synthesis of insights on cell types, fates, functions, and the gene expression can help to clarify the relationship between the sponge body plan and the rest of the Metazoa.

Freshwater sponges of the family Spongillidae are a tractable model system for work in sponge body plan evolution. They do not release gametes and instead brood fertilized eggs, embryos and larvae so collection of several stages simultaneously is possible (e.g. Saller and Weissenfels 1985). The larvae are released slowly and already have several differentiated cell types: sclerocytes, choanocytes, pinacocytes and collencytes. Gene expression and function studies can benefit from this early differentiation, perhaps leading to the ability to develop molecular markers for particular cell lineages. Species within this family are also developmentally very similar, so each species need not necessarily be widely available and we have generated transcriptomes for 3 species of spongillids for future comparative studies (Windsor et al. *in prep* and chapter 3). The habit of brooding limits our capacity to experiment on early cleavage stages that would normally be the target of cell fate experiments, and we have thus used the larvae of *Eunapius fragilis* for our experiments. The larvae are fairly large with a clear anterior-posterior axis and are appropriate for classical developmental manipulations such as bisection (see Fig. 4-1).

To help bridge the gap in understanding between larval and adult morphology and gene expression, we revisited the question of whether regionalization and differentiation of cells and polarity of the freshwater sponge larva is retained in the settled juvenile. If a restricted region or cell type of the larva is retained, forming a specific region or cell type in the juvenile, we can begin to formulate hypotheses about the relationship between that cell type and similar cell types of other animals. These hypotheses could then be tested with gene expression and function studies. We used live cell labeling to trace cell populations through metamorphosis, larval bisection experiments various immunolabeling techniques and electron microscopy to examine fates of larval regions in the metamorphosed juvenile sponge.

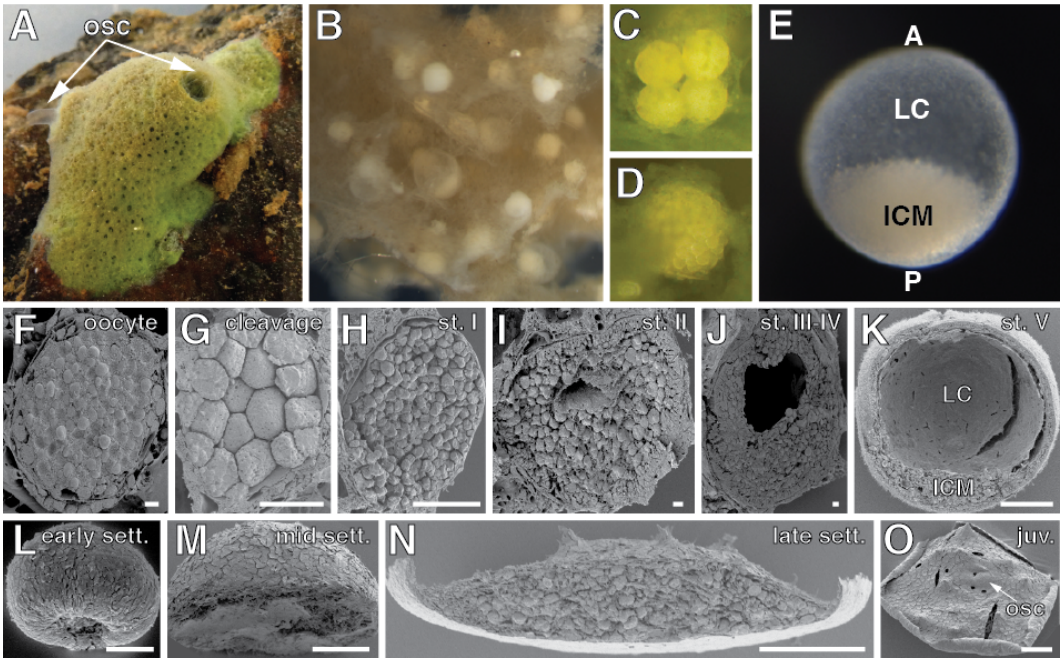

Figure 4-1: Overview of development in *Eunapius fragilis*.
(A) Adult with large excurrent oscula (osc). (B) Close up of sponge tissue showing numerous embryos and larvae of various stages. (C) 4-cell stage and (D) blastula in live tissue. (E) Swimming stage V larva, with inner cell mass (ICM) and large larval cavity (LC). The anterior posterior axis (A, P) is determined by swimming direction. (F) - (O) Stage series showing progression from oocyte through larval stages, settling and metamorphosis. Scales: 10 µm in (F), (I) and (J); 100 µm in (G), (H), (K)-(O).

4.2 METHODS

4.2.1 Collection, Rearing and Observation

Adult specimens of *Eunapius fragilis* were collected in of July 2007, 2008, 2012 and 2013 from Frederick Lake, British Columbia, near the Bamfield Marine Sciences Centre. Sponges were kept in a large volume of fresh unfiltered lake water, refreshed every 1-2 days, at 15-18°C.

Larvae were released throughout the day and night and collected from the surface of the water in which the adults were kept using a flashlight and a glass pipette, and transferred to 0.22 µm filtered lake water (FLW). In some cases, larvae were imaged over time using a Nikon Coolpix digital camera mounted on an Zeiss SZX12 Axioskop stereomicroscope to record general observations. Images were processed (cropped and resized) and assembled using Adobe Photoshop and Illustrator.

4.2.2 Cell Labeling and Larval Manipulation

In order to trace the fate of different cell types in *Eunapius* larvae, we used two fluorescent dyes: Cell Tracker Green (5-chloromethylfluorescein diacetate, CMFDA; stock 1mM in dimethyl sulfoxide [DMSO]; Life Technologies) and DiI (1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate; stock 25 mg/mL in DMSO; Life Technologies). For injection experiments, larvae were immobilized by capillary action in a petri dish next to a glass slide and injections were performed using the FemtoJet® microinjector and and the InjectMan® NI 2 micromanipulator (Eppendorf).

In one set of experiments, we soaked newly released swimming larvae in 10 µM CMFDA in FLW for 30 minutes, rinsed in FLW and cultured these for 24-48 hrs before fixing. We also injected larval cavities with approximately 100 pL 200 µM CMFDA or 2 mg/mL DiI (prepared as described below) using an Eppendorf FemtoJet Automatic Injection setup (Eppendorf Canada) in order to track the fate of the pinacocytes lining the cavity. We used DiI to label more restricted regions of the larva to see whether positional information of particular cells at the anterior or posterior poles could be retained through metamorphosis. DiI was injected at a

concentration of 2 mg/ml in made up in DMSO according to Adamska and Degnan (2008). Labeled sponges were cultured to the juvenile stage (24-48 hrs) and fixed for fluorescence imaging.

Swimming larvae were collected before settlement and immobilized as described above. Larvae were bisected into anterior and posterior hemispheres. These were fixed immediately and 48 hours after cutting for electron and fluorescence microscopy.

4.2.3 Fixation and Microscopy

We fixed specimens for electron microscopy at several stages throughout development, as well as bisected larvae in 1% OsO_4 in 3M sodium acetate (pH 6.4) with 10% w/v sucrose as described in Elliott and Leys (2007). After 5 hours to over night, specimens were rinsed in distilled water, and dehydrated for storage and processing. Adults and larvae were desilicified in 4% hydrofluoric acid v/v in 70% ethanol, and dehydrated to 100%. Fully dehydrated specimens were either freeze-fractured in liquid nitrogen or left whole then critical point dried using a BAL-TEC CPD 030 and finally mounted on stubs for imaging on a JEOL 6301F field emission scanning electron microscope.

We processed samples for fluorescence microscopy by fixation in 4% paraformaldehyde plus 0.3% glutaraldehyde in phosphate buffered saline (PBS). Counterstains and immunolabeling were used for further morphological analysis.

Labels used for each specimen differed depending on the type of experiment, and which dyes would be compatible. Specimens were transferred to 1:100 anti-mouse acetylated α-tubulin primary antibody (Developmental Studies Hybridoma Bank) in PBS containing 0.1% Triton-X 100 (PBTx) and 10% goat serum. All incubations were done over night at 4°C with gentle shaking, followed by rinsing the next day unless otherwise noted. Next, specimens were placed in 1:100 Alexa 594-conjugated goat-anti-mouse secondary antibody (Life Technologies) in PBTx plus 10% goat serum. The actin cytoskeleton was labeled using 1:30 Bodipy-fluorescein phallacidin (Life Technologies) in PBTx containing 10% bovine serum albumin (BSA). Finally, nuclei were labeled with 1:1000 Hoechst 33342 for 10 minutes in PBTx at room temperature, and mounted with Mowiol

mounting medium. DiI labeled specimens were permeabilized using PBS + 0.3% Tween-20, as described by Lukas et al. (1998). Specimens were imaged with a Q-Imaging Cam mounted on an Olympus SZX12 Axioskop with epifluorescence, using the Northern Eclipse imaging software package.

4.2.4 In situ hybridization

Homologs of 3 *wnt* genes in *Eunapius fragilis* were identified as described elsewhere (Windsor et al. *in prep* and Chapter 3). Phylogenetic analysis confirmed their identities and placed each in the one of three main sponge specific Wnt subfamilies, PorWntA, B and C (Windsor et al. *in prep* and Chapter 3).

Fragments between 4-600 bp of each gene was independently isolated by PCR using the following primer sets: *EfrWntA* fwd 5'-TGGTGGAGCTTATCGGTTTC-3' and rev 5'-CTGCACTCATGAAGGAGTAGAC-3'; *EfrWntB* fwd 5'-CGCACTGGTGAACCTTCATA-3' and rev 5'-GTAGTCCTCACGGTCACAAAC-3'; and *EfrWntC* fwd 5'-GTCGGGAGCAGCAGCATAAAGAA-3' and rev 5'-GTTATCTGGGTCTGGACGTAAC-3'. I also attempted to isolate a fragment of *silicateinM2* as a positive control, but PCR amplification was unsuccessful after several attempts at optimization of PCR conditions. Fragments were cloned into the pGEM-T vector (Promega) for storage and as a template for probe synthesis. Probe synthesis and *in situ* hybridization experiments were carried out on larvae from *Eunapius fragilis*, as described (Windsor et al. *in prep* and Chapter 3).

4.3 RESULTS

4.3.1 Overview of current knowledge of development and metamorphosis in freshwater sponges

Reproductive adult specimens of *Eunapius fragilis* can be detected quickly by simple dissection using forceps and a stereomicroscope (Fig. 4-1A, B). Embryos are brooded throughout the mesohyl, each contained within a follicle epithelium. Oocytes in this species are ovoid, and roughly 150-200 μm wide, a size that

remains fairly consistent throughout embryonic and larval development (Fig. 4-1F). Saller and Weissenfels (1985) and Saller (1988) described oogenesis and cleavage and found that in other freshwater spongillids (*Spongilla lacustris* and *Ephydatia fluviatilis*) cleavage was total and unequal to equal, with no discernable pattern of cleavage. The few early embryos we found in adult tissue freeze fractures in *E. fragilis* suggest that cleavage in this species is total and equal (Fig. 4-1C and G), however serial sections through multiple cleavage stages would help to resolve this issue. After a series of cell divisions, a solid blastula results (Fig. 4-1D).

Larval development proceeds through 5 stages as defined by Harrison and Cowden (1975) (Figure 4-1E, H-K); these stages are used with minor revisions based on Saller and Weissenfels (1985; Table 4-1). When the settled larva is released, it enters the water column until settlement followed by metamorphosis (Fig. 4-1L-O). Larval stages primarily reflect the level of differentiation of the development of 3 regions of the larva: the outer layer micromeres, the larval cavity, and the inner macromeres. Differentiation of flattened micromeres at the periphery of the embryo completely surround more centrally located macromeres is indicative of a stage I larva (Table 4-1, Fig. 4-1H and 4-2A). This differs slightly from the staging in Harrison and Cowden (1975) in that they refer to the solid blastula as the first larval stage but do not mention the flattening of the micromeres; the transition to a larval stage I from cleavage shall be delineated by this differentiation. Macromeres at this stage are undifferentiated, and are covered with numerous projections.

In the stage II larva, a larval cavity begins to appear towards the anterior end of the larva as defined by its future swimming direction (Fig. 4-1I and 4-2B. Formation of the larval cavity occurs by differentiation of certain macromeres into pinacocytes, followed by expansion of this space and further differentiation of pinacocytes (Evans 1899). This feature is the first visual indicator of larval polarity to develop since cleavage patterns are unclear after the first three or four divisions. In the stage III larva, the larval cavity continues to expand in size, while the micromeres begin to differentiate into a columnar epithelial layer uniformly

Table 4-1: Staging of freshwater sponge larvae

Stage	Morphology
I	Flattened micromeres surrounding undifferentiated macromeres
II	Appearance of anterior larval cavity; beginning of micromere shape change to columnar
III	Micromeres become columnar; some differentiation of macromeres (sclerocytes, canals, choanocytes)
IV	Micromeres develop cilia; larval cavity now takes up over half the larva
V	Larva released; macromeres fully differentiated

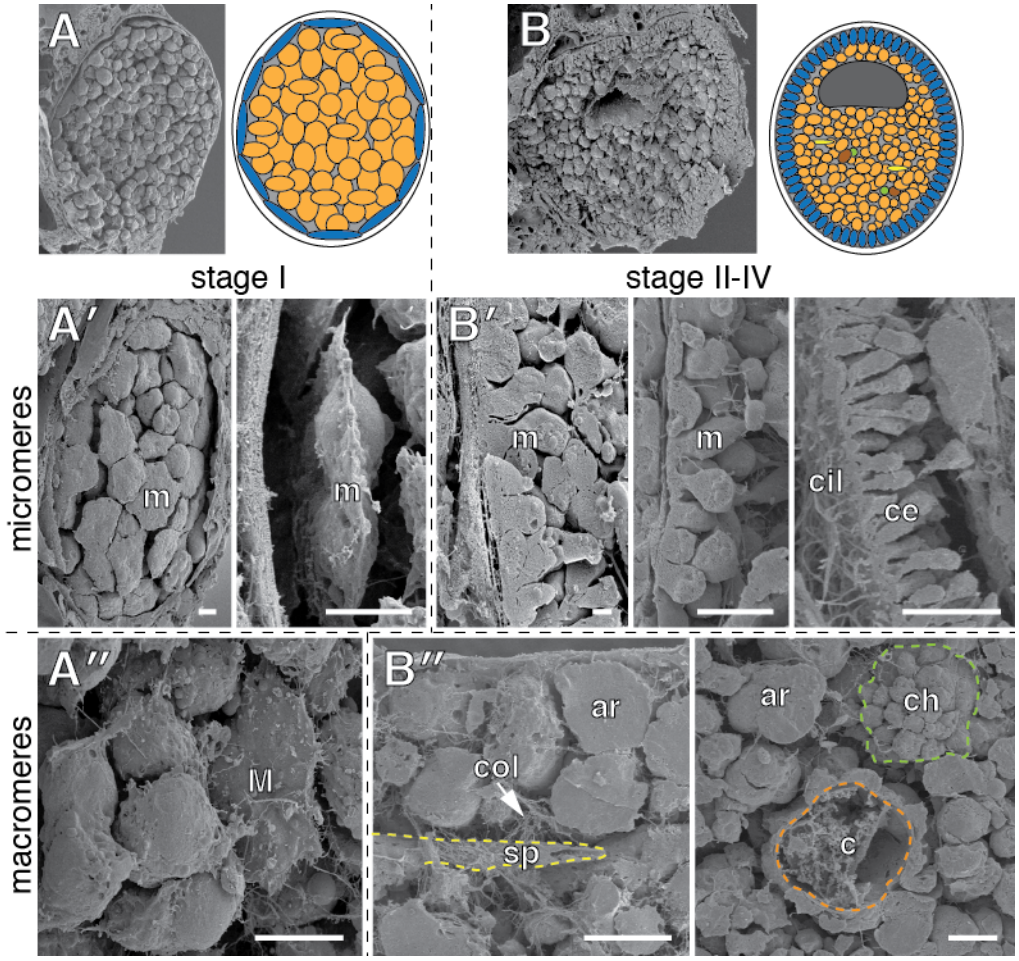

Figure 4-2: Early larval development of micromeres and macromeres. (A) SEM of stage I larva, diagram shows the differentiation of micromeres (blue) and macromeres (orange). (A′) flattening of outer layer micromeres (m), top (left) and side (right) views. (A″) Close up of macromeres (M) showing undifferentiated but active cells. (B) SEM of stage II larva showing further differentiation of micromeres and macromeres. Macromeres have begun differentiating into choanocytes (green), canals (brown) and sclerocytes (yellow). (B′) Formation of columnar epithelium in stage II-III larvae (left, middle) and ciliated epithelium (ce, cil) in stage IV larvae (right). (B″) Details of macromere differentiation, with outlines showing location of differentiated cells: collagen, col; choanocytes, ch; and canals, c (a spicule shaft suggests that sclerocytes are present to make spicules, sp). Archaeocytes, ar, are also present. Scales: 2 µm in (B′, left); 10 µm in all other panels.

surrounding the entire larva (Fig. 4-2B′). We were unable to find a young larva with both flattened micromeres and a larval cavity. We therefore suggest that stage II larvae be characterized both by the formation of a larval cavity and by the change in micromere shape from flattened to columnar. Development of cilia on the outer layer micromeres signifies a stage IV larva (Table 4-1 and Fig. 4-2B′).

When the larvae reach stage V, they are released from the parent sponge, and were thus mostly found as free-swimming larvae, as described (Harrison and Cowden 1975). We noted that the cilia on the surface of the swimming larva were polarized, arising on the posterior edge of the cell (Fig. 4-3A, A′). Monociliated cells derived from micromeres cover the larva completely, and are underlaid by a layer of large, amoeboid cells (Fig. 4-3A″-i). These become the future exopinacoderm, covering the outside of the sponge juvenile. A network of collagen is found just beneath the amoeboid cells and abutting the basal side of the pinacocytes lining the larval cavity, which extend processes that grip the layer of collagen tightly (Fig. 4-3A″-ii). Choanocyte chambers and aquiferous canals are well differentiated in these larvae and in some cases fully developed choanocyte chambers can be seen opening into the larval cavity (Fig. 4-3B-i, ii). This suggests that the larval cavity may become a part of the aquiferous canal system of the sponge, as suggested previously (e.g. Wielspütz and Saller 1990). Sclerocytes have now produced several spicules that will act as a scaffold during settling (Fig. 4-3B′).

Settling of the larva occurs toward the anterior of the larva, but not always directly on the anterior pole, as observed by earlier workers (Evans, 1899; Brien and Meewis 1938). As the larva settles, ciliated cells rapidly disappear, giving way to the amoeboid cells from underneath, which become the exopinacocytes (Fig. 4-4A, A′). The settling sponge spreads outward, becoming flatter and canals expand (Fig. 4-4B). Larger excurrent canals join together where the aquiferous canal system will empty and where the osculum forms (Fig. 4-4B′). The juvenile stage is reached between 16 and 36 hours after settling, with a fully differentiated aquiferous system (Fig. 4-4C-C″).

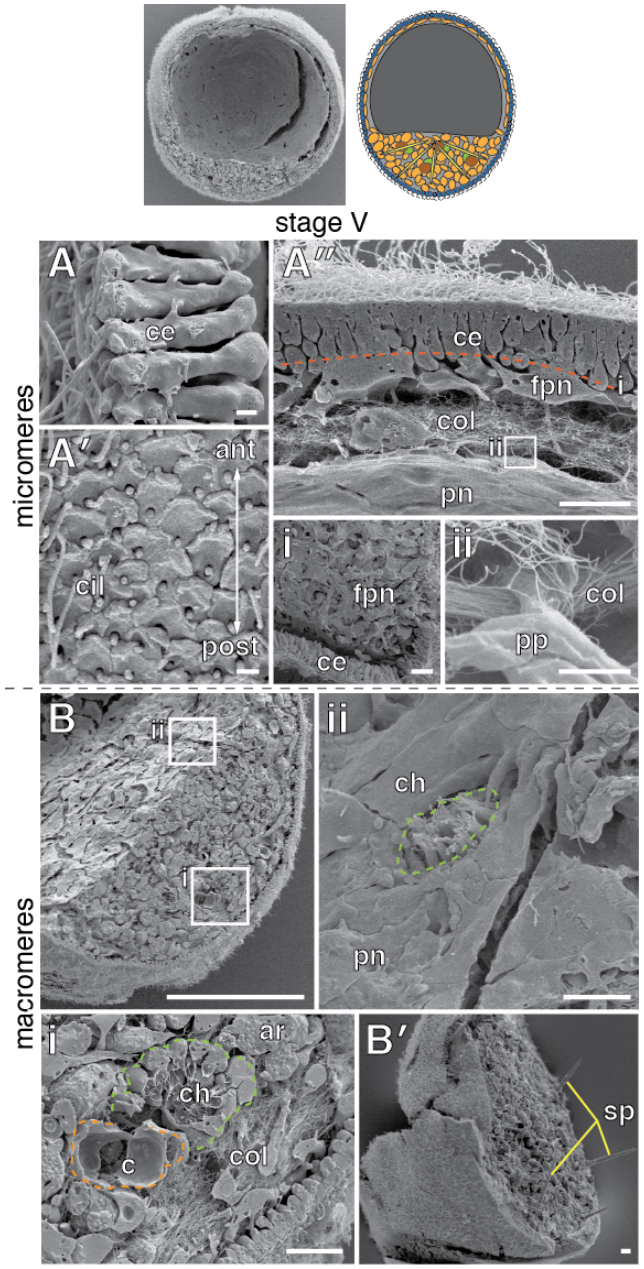

Figure 4-3: Characteristics of the stage V swimming larva.
(A, A′) The fully differentiated ciliated epithelium (ce), showing polarization of cilia (cil) on the posterior (post) side of each epithelial cell. (A″) Layered structure of the larval epithelium. Ciliated cells over lie amoeboid cells that are the future pinacoderm (fpn; inset i shows a view of the amoeboid cells with the ciliated cells removed), and a bed of collagen (col) that directly backs the pinacocytes (pn) lining the larval cavity. Inset ii shows pseudopodia of the pinacocytes tightly gripping the collagen. (B) Inner cell mass at the posterior end. Inset i shows the fully differentiated canals (c), choanocyte chambers (ch) and undifferentiated archaeocytes. (B′) shows the presence of large, well developed spicules in stage V larvae. Scales: 1 µm in (A), (A′) and (A″ ii); 10 µm in (A), (A″ i), (B i, ii) and (B′); 100 µm in (B).

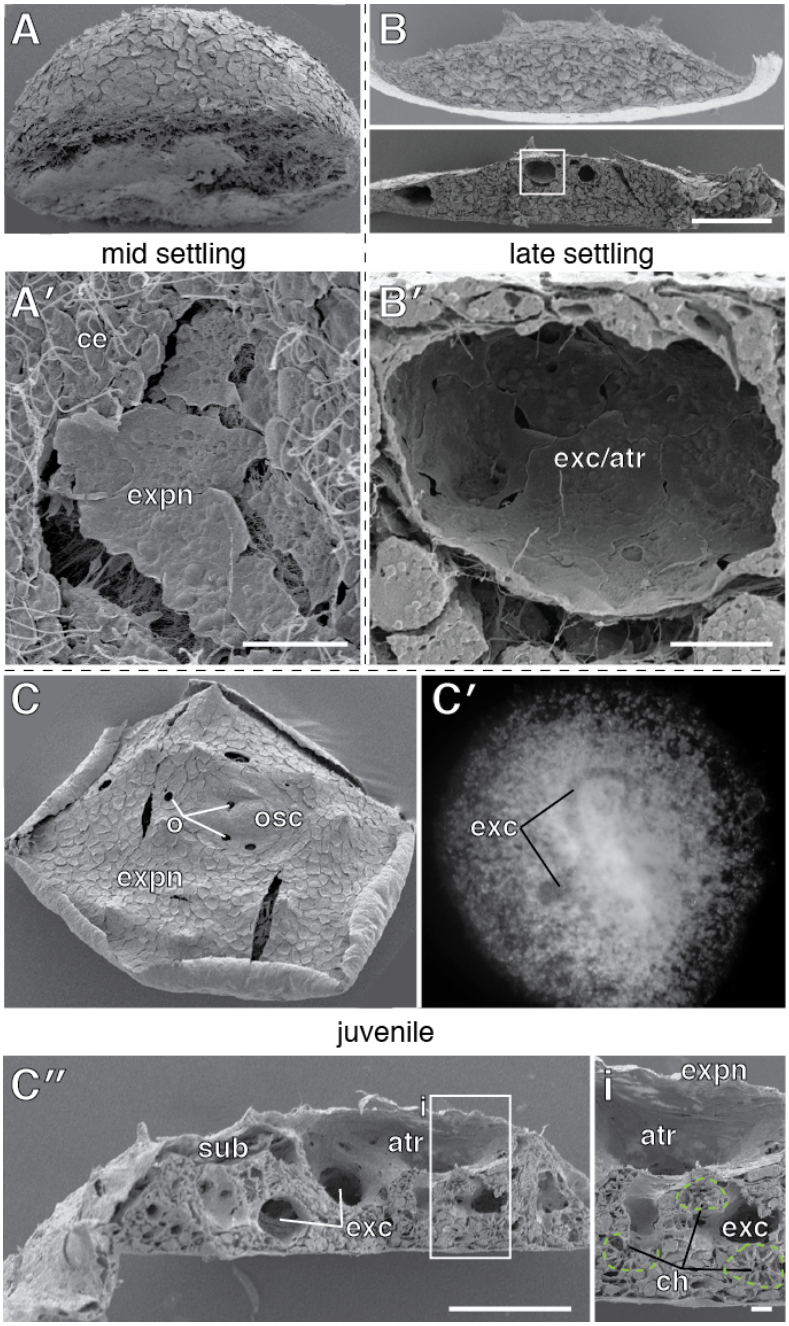

Figure 4-4: Settling and metamorphosis of *Eunapius fragilis*.
(A) Mid-settling stage sponge with pinacocytes covering apical side. (A′) Amoeboid cells emerging from beneath the ciliated epithelium to become exopinacocytes (expn) in a slightly earlier stage. (B) Late settling stages without (top) and with (bottom) visible excurrent canals (exc), some of which will become the atrium (atr) – the future site of the osculum (B′). (C-C″) Juvenile sponge showing fully developed aquiferous system complete with (C) ostia (o), and osculum (osc), (C′, C″) excurrent canals (exc), (C″) subdermal space (sub), choanocyte chambers (ch) and atrium. Scales: 10 µm in (A), (B) and (C″ i); 100 µm in (B) and (C″).

4.3.2 New observations: Fate of the larval cavity pinacocytes

Our timelapse data show that the larval cavity may become incorporated into the canals of the aquiferous system, but we were limited by the opacity of the tissue in this species as it develops (Fig. 4-5A). We were also unable to identify the larval cavity in freeze fractures (SEM) of settling sponges; serial sectioning of settling sponges at several stages is required to confirm that the larval cavity is not lost completely.

To help clarify this, we attempted to trace the fate of the larval cavity pinacocytes by injection of CMFDA and DiI into the cavity, and allowing larvae to settle and develop into fully differentiated juveniles (Fig. 4-5B). We found a complete absence of labeled cells in the juvenile with either dye. Although specific volumes were carefully monitored during injection we could not prevent leakage and thus dilution and nonspecific labeling from occurring.

4.3.3 Revisiting the fate of larval ciliated cells and choanocyte ontogeny

To test whether ciliated cells were retained and reused in the juvenile sponge following metamorphosis, we traced this cell lineage using Cell Tracker Green (CMFDA). In Fig. 4-6, we included drawings of comparable structures from Delage (1892) to show that the process of recycling ciliated cells was documented early on. In settling sponges that had lost the ciliated layer, CMFDA was found in clusters surrounding small, compact nuclei, as indicated by the bright Hoechst staining (Fig. 4-6B). These small nuclei surrounded one larger and more diffuse nucleus, appearing as if a larger cell had engulfed many smaller cells, and generally appear at the edge of the sponge. For simplicity, we will refer to these as polynuclear groups after Delage (1892). In juveniles labeled regions were found in dense groups throughout the sponge, as well as in chaonocyte chambers (Fig. 4-6C, D). Cells in Fig. 4-6C perhaps represent a later stage than shown in Fig. 4-6B. Delage (1892) and Evans (1899) documented this, describing the "splitting off" of individual choanocytes from the larger cell. I was not able to observe these processes in detail; sectioning and transmission electron microscopy will be required to further characterize these events.

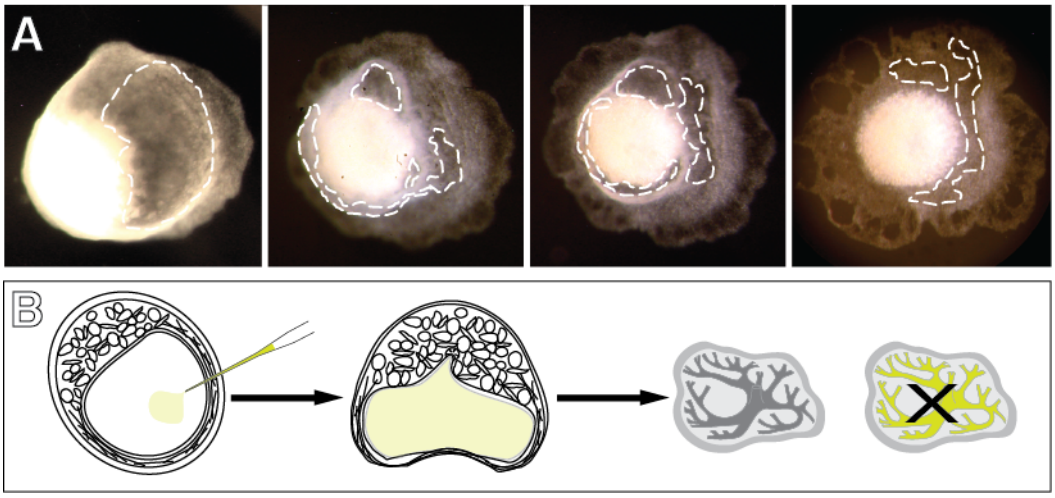

Figure 4-5: Fate of the larval cavity during settlement of *Eunapius fragilis*. (A) Timelapse of settling larva over 1.5 hours with outlines (dotted white line) showing possible continuation of cavity as canals of the juvenile. (B) Schematic showing injection of the larval cavity with CMFDA tracer dye to follow the fate of larval cavity pinacocytes. Injections never yielded juveniles with labeled cells.

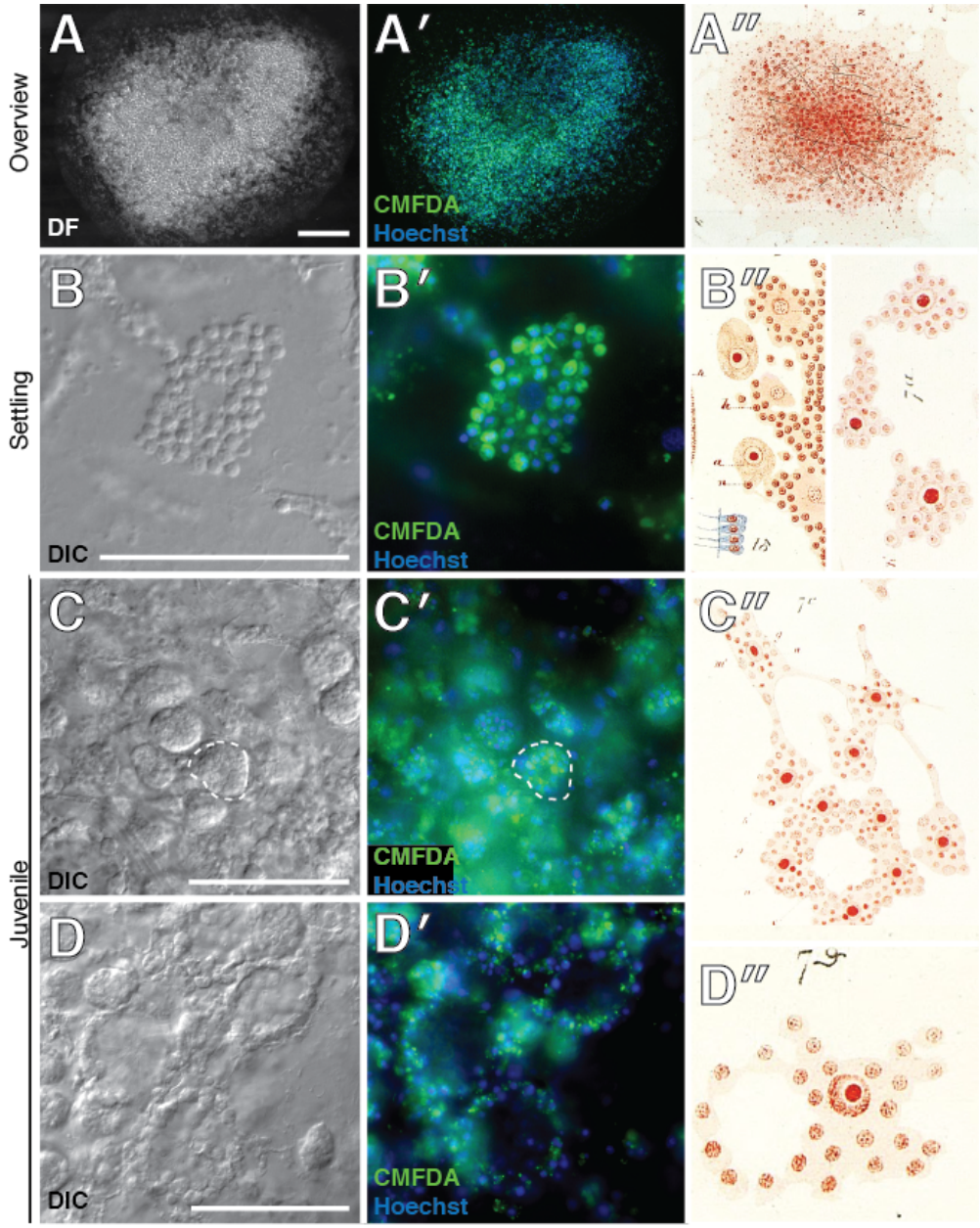

Figure 4-6: Fate of the ciliated outer layer of *Eunapius fragilis*.
(A, A′) DIC and fluorescence view of a late settling stage sponge with CMFDA label (green) and nuclei (blue). (A″) Early drawing of similar stage. (B, B′) An archaeocyte-like cell that has engulfed many smaller ciliated cells, each with its own nucleus. (B″) Drawings of the consumption of ciliated cells by archaeocytes with nucleolated nuclei. The cytoplasm of these cells appears to be completely taken over. (C, C′) Later stage juvenile with CMFDA labeled cells all over the sponge, in almost all cell types. (C″) Cells were drawn as becoming more rounded in some cases, and forming clusters. (D, D′) Some choanocytes retain some CMFDA label, as do many cells around the chambers. (D″) Drawing of supposed formation of a choanocyte chamber. Drawings in (A″), (B″), (C″) and (D″) taken from Delage (1892). Scales: 200 µm in (A); 50 µm in (B)-(D).

4.3.4 Anterior and Posterior cell labeling

We wanted to know whether cell fates differed in cells arising from the anterior or posterior poles, so we labeled cells at either end of the larva using the lipophilic tracer dye, DiI. Labeling at either the posterior or anterior end of the larva resulted in labeled archaeocytes and polynuclear groups in almost every individual observed (Fig. 4-7A, B). These cells appear to have the same morphology as the cells described above (see Fig. 4-6B). Finally, although less common, both sclerocytes and choanocytes were also occasionally labeled in both anterior and posterior cell labeling experiments (Fig. 4A, B insets).

In a few specimens, where the osculum was visible, we found that the osculum was labeled in those individuals tagged at the posterior pole, and this was never observed in anteriorly labeled larvae (Fig. 4-7C, D).

4.3.5 Competence of larval hemispheres

To test the competence of the anterior and posterior hemispheres of the larva of *Eunapius fragilis*, we bisected larvae at the more posterior edge of the larval cavity such that the anterior half contained only larval cavity cells, and the posterior half only limited larval cavity cells – if any – and inner cell mass. We performed the same experiments in a pilot study on *Spongilla lacustris* in July 2011. In this case, anterior halves closed up into ciliated balls that swam continuously for several days, while posterior halves settled and became normal juvenile sponges (Fig. 4-8).

The bisection scheme and label legend are shown in Fig. 4-9A. Uncut larvae from *E. fragilis* settled relatively quickly after being released from the parent sponge (30-60 minutes), while in *S. lacustris* they took somewhat longer (up to several hours). Once the juvenile stage has been reached roughly 24 hours after settling in *E. fragilis*, typical sponge functional structures could be observed, including aquiferous canals, choanocyte chambers and sclerocytes (Fig 4-9B). The scenario in *Spongilla lacustris* was likely comparable, but was not analyzed in this way due to the small number of larvae available.

In *E. fragilis*, anterior hemispheres settled soon after cutting (within 30 minutes), and flattened out into a thin sheet of tissue (Fig. 4-9C). This settled half

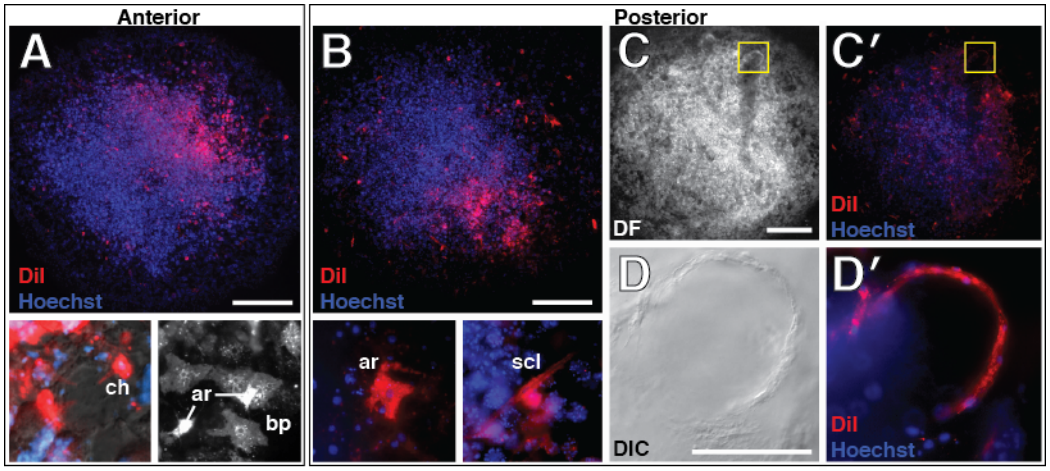

Figure 4-7: Fate of cells at the anterior and posterior poles of the *Eunapius fragilis* larva.
(A, B) Cells labeled at the anterior (A) and posterior (B) pole give rise to choanocytes (ch), archaeocytes (ar), basopinacocytes (bp), and sclerocytes (scl). (C, D) Only cells at the posterior pole are fated to become cells of the osculum. Scales: 200 µm in (A)-(C); 50 µm in (D).

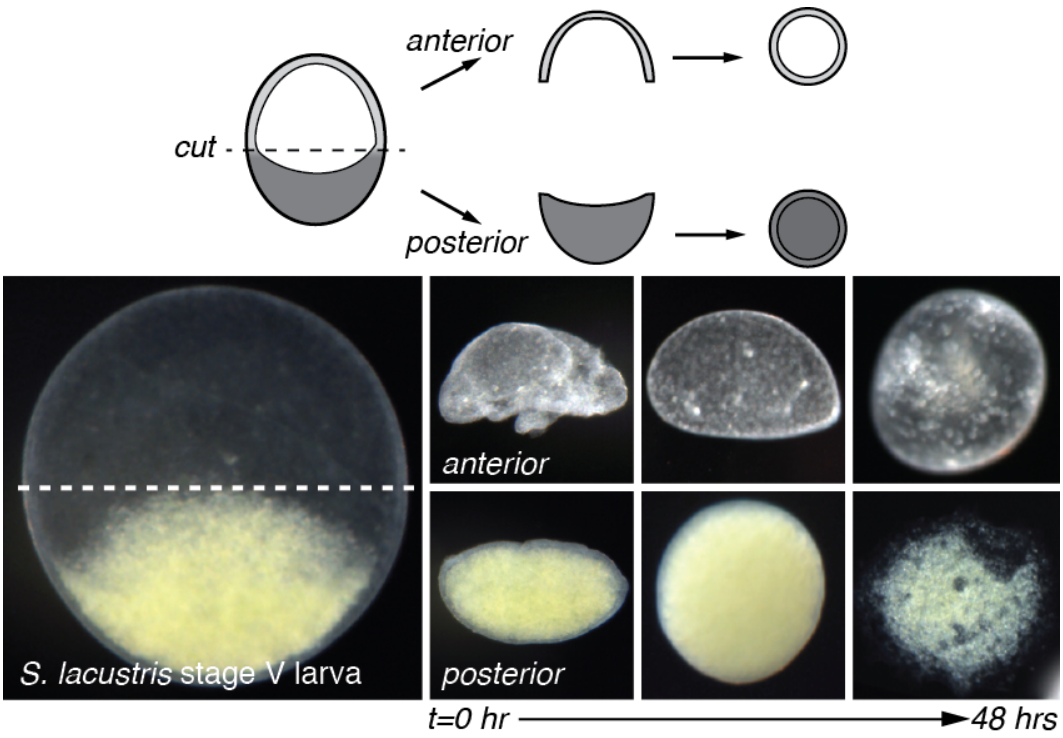

Figure 4-8: Pilot bisection experiment in *Spongilla lacustris* larvae.
Free swimming larvae were bisected and cultured as indicated in the diagram (top). After 24, and 48 hours respectively, anterior halves healed and closed into a rounded ciliated ball that did not settle, while posterior halves settled and developed into normal sponges.

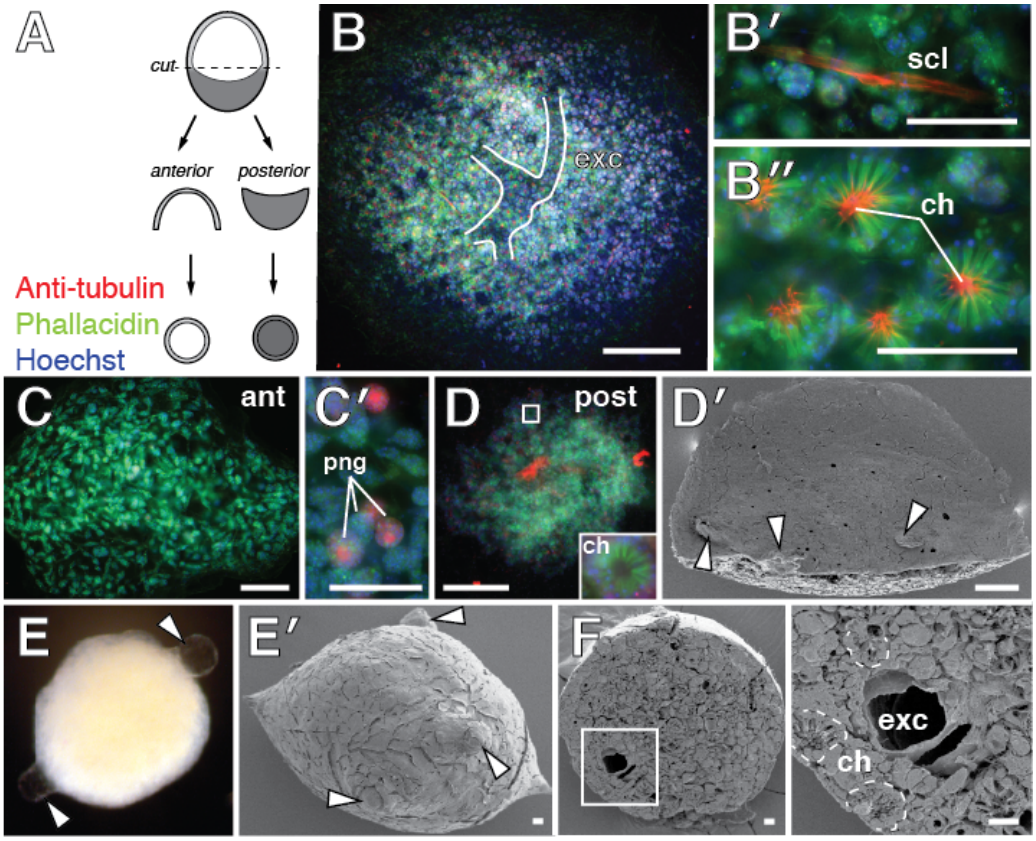

Figure 4-9: Bisection experiment in *Eunapius fragilis*.
(A) Scheme for bisection and immunolabel legend. (B) Normal sponge showing excurrent canals (exc), sclerocytes (scl, B′) and choanocyte chambers (ch, B″). (C) Anterior halves settled and flattened but did not differentiate; close up showing polynuclear groups (png). (D) Posterior half, settled, with differentiated aquiferous system with choanocyte chambers. (D′) Settled posterior halves had multiple oscula (arrowheads). (E, E′) Posterior halves, unsettled live and in SEM, respectively. These also developed multiple oscula. (F, inset) Fractured unsettled posterior half complete with large excurrent canals and choanocyte chambers. Scales: 200 µm in (B) and (D); 100 µm in (C) and (D′); 50 µm in (B′), (B″) and (C′); 10 µm in (E′) and (F) and inset.

was made exclusively of polynuclear groups containing the engulfed ciliated cells, and little else (Fig. 4-9C′). No choanocyte chambers or canals were observed in these animals.

There were two outcomes when posterior halves of *E. fragilis* were allowed to develop. Some individuals settled, and appeared to have normal aquiferous systems (Fig. 4-9D). These individuals often developed multiple oscula (Fig. 4-9D′). Most individuals, however, failed to settle, simply continuing to swim until they were fixed at 2 days after cutting (Fig. 4-9E). Oddly these developed multiple oscula as well. Freeze fracture and SEM revealed an internal structure containing essentially an inner cell mass complete with canals, choanocyte chamabers, sclerocytes and archaeocytes (Fig. 4-9F and inset).

4.3.6 Wnt expression in Eunapius fragilis larvae

In order to determine if *wnt* genes were expressed differentially in the larva of *Eunapius fragilis*, we performed *in situ* hybridization using probes against these three genes. After three attempts at optimizing conditions, none of the *wnt* genes from *E. fragilis – wntA, B* or *C* – were detected in any cells of the larva in whole mount.

4.4 DISCUSSION

To address the current gaps in our understanding of sponge developmental models, we traced the fates of different cell populations in the larva of *Eunapius fragilis* through metamorphosis. Although further study of metamorphic processes will be required to fully understand the development of this animal, gene expression studies will benefit from this early work on cell types and cell fates. A solid understanding of cellular interactions and cell fates in larvae, with fine-tuning of molecular techniques including RNAi (e.g. Rivera et al. 2011), and overexpression (Pfannkuchen and Brümmer 2009) can provide a solid foundation for evo-devo work in sponge model systems.

4.4.1 The larval cavity: beginnings of a juvenile aquiferous system

Although the larval cavity was thought to become a part of the canal system, as suggested by Saller (1988), and Wielspütz and Saller (1990), it had never been directly shown. In fact, in some cases, they refer to the larval cavity being "obliterated", suggesting that the larval cavity completely disappears in the juvenile (Evans 1899).

While our timelapse results suggest that the larval cavity goes on to form the excurrent canals of the juvenile, cell lineage tracing failed to show CMFDA labeled canal endopinacocytes in the juvenile. This implies that the larval cavity disappears and that pinacocytes lining the cavity do not become the endopinacocytes of the aquiferous system consistent with older literature. Other possibilities are that the pinacocytes are too thin and do not retain the label clearly. It is interesting that a larva with such a large degree of differentiation would fail to reuse the larval cavity pinacocytes in the juvenile; presumably these cells differentiate *de novo* from a separate population.

Some limitations of the methodology could also have resulted in these seemingly contradictory findings. One limitation was the diffusability of CMFDA, coupled with the healing time for the cells of the larva. It is entirely possible that the dye injected into the cavity leaked out through the hole created by microinjection; in this case it would diffuse out of the cavity and become diluted, resulting in a failure of larval cavity cells in taking up the dye. We tried to circumvent this by injecting DiI with the expectation that piercing the cells of the larval cavity would injure them, allowing DiI to enter and label those cells. These experiments, however, also failed to produce any labeled pinacocytes in the juvenile.

4.4.2 Loss of the ciliated cells: cell layers are inverted

There are essentially two views on the origin of chaonocyte chambers in freshwater sponge larvae. First, and consistent with what is known from other sponges, is that the ciliated epithelium of the larva is internalized and ciliated cells dedifferentiate and redifferentiate into choanocytes (Delage 1982, Evans 1899). However, since the first studies of freshwater sponge embryogenesis in the mid to

late nineteenth century, this question has been revisited a number of times, and the consensus is that the ciliated cells are either phagocytised or discarded and do not go on to form choanocytes or any other sponge cell lineage in the juvenile (Brien and Meewis 1938; Saller and Weissenfels 1985; Saller 1988; Wielspütz and Saller 1990). Despite efforts throughout the years to clarify this question, the evidence from the sections provided in these previous studies is not unequivocally convincing. During settling of the larva, I observed cells being sloughed at the periphery of the settling edge, something that has not been suggested previously.

We found that the ciliated cells are indeed internalized by archaeocytes of the settling sponge, confirming the findings of several authors from other freshwater sponge species (Delage 1892; Evans 1899; Brien and Meewis 1938; Saller and Weissenfels 1985; Saller 1988; Wielspütz and Saller 1990). Counter to some of these authors, however, we found that choanocytes were derived from the ciliated outer layer of the larva, reviving the idea of inversion of the germ layers relative to other metazoans. While early workers were divided on this issue (reviewed in Evans 1899), later authors adopted the view that ciliated cells were phagocytized, not being carried over into the juvenile because they were unneeded; larvae already contained young choanocyte chambers (Brien and Meewis 1938; Saller and Weissenfels 1985). Saller (1988) and Wielspütz and Saller (1990) observed something slightly different: the amoeboid cells that underlie the ciliated epithelium rather than archaocytes phagocytize them before differentiating into pinacocytes. We offer the first instance of lineage tracing using a vital dye in these cells in freshwater sponges.

Part of the reason for the lack of clarity on this issue is that this process is extremely difficult to visualize; it is astounding that early authors were able to pick out details such as the polynuclear groups and other structures that occur in the more dense areas of settling sponges (for example, see Fig. 4-5C). More modern authors used diagrams to explain stages rather than providing the original micrographs so readers are dependent on their interpretation (e.g. Saller 1988; Wielspütz and Saller 1990). The current work includes several images using different techniques in order to help alleviate this.

4.4.3 Differing potentials of the anterior and posterior hemispheres

Although the history of experimental embryology goes back to the mid nineteenth century, they have rarely been performed in sponge larvae. In general, sponge cell fates have been described through simple observation rather than via an experimental approach. Borojevic (1966) tested the fates of different regions of the larva of *Mycale contarenii* by excising regions and culturing them separately. He found that the central part of the larva, containing mainly archaeocytes and collencytes, could produce a fully functional sponge in 3-4 days. The ciliated outer layer, however, primarily formed a large number of choanocyte chambers but was missing the remaining components of an aquiferous system. Thus, in his study the fate of cells depended on the region of the larva that they came from to an extent, except where many archaeocytes were present.

Here we showed that in the parenchymella larva of the freshwater sponge, *Eunapius fragilis*, different regions of the larva also have differing potentials to an extent. The anterior hemisphere is required for larval settlement, as suggested by the failure of posterior hemispheres alone to settle. Although a few of these did settle and become relatively normal sponges, there is the possibility that enough cells from the anterior half remained, allowing settlement and subsequent development. Those posterior hemispheres that did not settle were able to form the aquiferous system structures that are present in juveniles – choanocyte chambers, canals, and oscula. But since the anterior hemispheres lacked larval choanocytes and other cell types within the inner cell mass in the posterior half, it was unable to form any elements of the aquiferous system despite its ability to settle. Therefore, the posterior hemisphere containing already differentiated cell types and the majority of undifferentiated archaeocytes is required for formation of the basic functional unit of the sponge: the aquiferous sytem. Without the competence of cells from the anterior hemisphere to settle, however, the posterior cannot do so and thus remains a swimming sponge, with a fully differentiated aquiferous system.

Interestingly, the situation is reversed in *Spongilla lacustris*, where the anterior hemisphere becomes a ciliated ball that will continue to swim, and the

posterior half settles and becomes a normal juvenile. The small number of available larvae limited the extent of experimentation in *S. lacustris*, but it would be interesting to investigate this striking species-specific difference.

Our labeling experiments also showed that the osculum appears to arise from the posterior pole of the larva, something suggested by work done in *Amphimedon queenslandica* as well (Leys and Degnan 2002). The link between the osculum and the posterior pole becomes more interesting in light of *wnt* gene expression at the posterior pole of *A. queenslandica* larvae (Adamska et al. 2007, 2010). Recently it was also reported that *wnt* genes were also expressed in the posterior of the larva of *Sycon cilatum*, as well as the tip of the osculum in the adult of the same species and *Halisarca dujardini* (Adamska et al. and Borsienko et al.: *abstracts*, Ninth World Sponge Conference 2013). Windsor and Leys (2010) and chapter 2 showed that osculum formation could be induced in another freshwater sponge – *Ephydatia muelleri* – using LiCl, a compound known for its ability to mimic canonical Wnt signaling in other animals.

We found no evidence of *wnt* expression in *E. fragilis* larvae. If there truly is an absence of *wnt* expression in the posterior of these larvae, it is possible that expression begins later, for example, when the sponge begins to settle and loses its ciliated epithelium. Once the ciliated epithelium is lost, and amoeboid cells differentiate into exopinacocytes, which overlay a very thin layer of mesohyl and endopinacocytes. If *wnt* was expressed in the exopinacocytes, it could cause further differentiation of endopinacocytes into osculum pinacocytes, which have paired cilia thought to be involved in sensing flow in the sponge (Ludeman et al. *in press*). However, whether this can be attributed to a true lack of expression is, however, debatable since the *in situ* hybridization technique is not well developed for freshwater sponges, and especially this little-studied species. One way to resolve this question would be to perform quantitative PCR comparing several stages – swimming larva through juvenile – and even in either anterior or posterior halves. The connection between the posterior pole of sponge larvae, the osculum, and *wnt* is intriguing, and could help to resolve the origins of polarity in the Metazoa.

4.4.4 Concluding remarks and future research

We have begun to build a fate map for the larva of the freshwater sponge, *Eunapius fragilis*. These investigations have highlighted several areas that are lacking in our current understanding of a system that has been studied for so long including the fate of the ciliated cells and the larval cavity. Further study of the cells of the larva, especially those of the inner cell mass, will help to complete the fate map. However, due to the limitations of cell labeling techniques, this may need to rely on molecular innovations that allow us to identify cell types and their origin in the larva. For example, driving the expression of fluorescent markers tagged with particular molecular markers would reveal which cells arise from where, and how they move and differentiate during development.

We also showed evidence that ciliated cells are brought into the sponge to become choanocytes of the juvenile. Consistent with what occurs in many sponges, this supports the idea that germ layers of all sponges are inverted; the outer layer of the larva is actually the inner layer of the juvenile and adult. Our results here challenge other recent studies in this respect (e.g. Saller and Weissenfels 1985; Saller 1988; Wielspütz and Saller 1990), and it is certainly worth revisiting.

Perhaps the most interesting avenue would be to trace cell lineages from an early cleavage stage. The brooding habit of these and other sponges prevents simple lineage tracing experiments, but some sponges spawn eggs and sperm, so development proceeds outside the parent. One example is *Tetilla japonica*, whose development is direct (Watanabe 1978). Direct developers are a tractable system for cell fate experiments since the adult form is achieved without passing through larval stages, which can look very different from the adult.

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Chapter Five:

GENERAL DISCUSSION AND DIRECTIONS FOR FUTURE RESEARCH

5.1 SUMMARY

The primary interest underlying the work presented in this thesis concerns the form of the Urmetazoa – the first multicellular animal – a question that goes back to the mid-nineteenth century. Early animal life may have consisted of unicellular organisms that occasionally came together due to environmental conditions. However the condition of complex (animal) multicellularity requires differentiation and specialization of cell types (Nedelcu 2012). Due to shared microenvironmental conditions, it is plausible that cells in a particular region of a colony would become differentiated and later specialized, in effect causing polarization in a hypothetical animal. Even in unicellular and colonial opisthokonts polarity can be observed (e.g. *Dictyostelium* colonies, Dickinson et al. 2012), but the importance of polarity in the early evolution of animals cannot be underestimated. Structures associated with the role of a cell type in one region – at one pole – could be selected for if they provided some advantage, for example, the tentacles of *Hydra* surrounding its mouth.

Unfortunately, the study of these ancient hypothetical events is impossible and known colonial unicellular eukaryotes are still very derived within their clades; and it is possible that the mechanisms they use for forming aggregations and multicellular structures are largely specific to them. However, in the choanoflagellate, *Salpingoeca rosetta*, the colonial condition arises through a set of semi-synchronous mitotic divisions, and could be similar to primitive cleavage divisions (Fairlcough et al. 2010). Evidence of an incomplete and Wnt-independent signaling mechanism in *Dictyostelium* points to the possibility that this pathway may be conserved in organizing colonial and multicellular behaviour in ancient eukaryotes (Harwood 2009). Coupled with the fact that Wnts are involved in primary body axis formation and maintenance in cnidarians such as *Hydra* and *Nematostella*, this signaling pathway makes an excellent candidate for

the study of early body plan organization (Kusserow et al. 2005; Lengfeld et al. 2009). Clearly there are some fundamental shared aspects to the evolution of multicellularity in eukaryotes, and comparative research across many groups of organisms, including unicellular relatives and other basal metazoans, is needed to understand the foundations of animal multicellularity and evolution. Interest in basally branching metazoan groups (Porifera, Cnidaria, Ctenophora and Placozoa) has helped us understand many of the fundamental aspects of what it means to be an animal. This thesis aimed to examine whether the canonical Wnt signaling pathway is a conserved mechanism for generating polarity in freshwater sponges.

In this work I have proposed that the body axis of an adult sponge does not refer to its apical-basal axis, but to the aquiferous system, along which the animal functions. Work in chapter 2 suggests that the osculum – the terminus of the aquiferous system – has inductive capabilities and can draw canal growth toward itself. This phenomenon is reminiscent of the organizer of vertebrates, a region of a developing embryo that has the ability to induce the formation of a new head – a new individual. When Wnt signaling is mimicked, new oscula form suggesting that Wnt signaling is sufficient for the formation of the osculum, and thus polarity of the sponge.

Following this I searched the transcriptomes of sponges from all four Classes (Hexactinellida, Demospongiae, Calcarea and Homoscleromorpha) for components of Wnt signaling. When this thesis began very little was known about Wnt in sponges, and it was even occasionally suggested that Wnt was altogether absent in this Phylum. The work presented here and the work of others greatly enriched our collective knowledge of Wnt signaling in sponges, ranging from surveys to expression and functional studies (Adamska et al. 2007, 2010; Lapébie et al., 2009; Windsor and Leys 2010 and chapters 2 and 3 of this thesis). Functional work described in chapter 3 suggests that some aspects of Wnt signaling may function as in other animals – for example, GSK3 RNAi knockdown – but others may not. The fact that sponge Wnts cannot trigger canonical signaling in *Xenopus laevis* does not shed light on whether these proteins are controlling axis formation in the sponge, and further work is needed.

Finally, in chapter 4, I identified cell fates of the larva of the freshwater sponge to in an attempt to alleviate difficulties in interpreting gene expression data. Although this connection has ultimately been unsuccessful, I identified some gaps in our current knowledge of how these particular sponges develop. A lack of understanding of the cell types and what their specific functions are in the sponge presents a significant hindrance for the understanding of gene expression patterns, but also to understanding the process of development itself.

In this final chapter of this thesis I will present some of the challenges in working with sponges and suggest interesting avenues for future research.

5.2 ELUCIDATING SPONGE CELL TYPES AND FUNTIONS: DIFFICULTIES IN WORKING WITH SPONGES

Although there are advantages to working with freshwater sponges such as *Ephydatia mulleri* gemmules, and *Spongilla lacustris* and *Eunapius fragilis* embryos and larvae, there are also some practical limitations that affect our ability to work with these animals on a large scale and in a developmental context, as in *C. elegans* or *D. rerio*, for example. Some of the reasons for this are biological. Asexual reproduction via gemmule production occurs in the winter months, allowing long-term storage and laboratory culture for short-term experimentation. However, sexual reproduction occurs only during a few weeks during the summer months, limiting the number of possible live experiments. One advantage that allows model systems to be successful is that they can be induced to breed at any time, allowing fine-tuning of techniques and experiments. While it is possible that long term culturing of sponges may reveal similar capabilities on certain sponge species, sponges require huge volumes of water and often do not thrive in the laboratory. Previous graduate student, Glen Elliott, made attempts to culture adult sponges, but was unsuccessful.

Developmental studies are often complemented by genetics, which helps to elucidate gene function. Genetic models are typically easy to culture, though time ranges for experiments can range greatly (e.g. for a fish versus a mouse). They are also diploid, making assessments of crossover events and Mendelian ratios and

thus genetic evaluation more predictable. Certain sponges, like the freshwater sponges, are relatively easy to culture and keep in the laboratory. Since we cannot induce freshwater sponges to breed however, genetic studies in these animals remain impossible. One sponge, *Tetilla japonica*, spawns eggs and sperm and it is theoretically possible to breed these selectively (Watanabe 1978). However since breeding only occurs once per year this would make genetic experiments greatly impractical. Even if we could perform simple genetics, the assessment of phenotypes in a sponge is subjective at best and at this time there are no characterized mutants; the only phenotypes that we are aware of are the results of pharmacological and molecular manipulation. One way to discover mutants is to create recombinant inbred lines (RILs), and continuously inbreed sponges to produce recessive traits that are easy to score. Then certain interesting mutants can be chosen for more detailed study.

In terms of organismal complexity, sponges are considered to be the simplest animals; they do not have nerves, muscles, a digestive tract nor any organs or organ systems (Hyman 1940, p. 284). There are also a limited number of cell types, some of which are easily distinguishable from each other morphologically: pinacocytes, choanocytes, sclerocytes, and archaeocytes (Bergquist 1978, pp. 52-74; Simpson 1984, pp. 6-7). I, and others, have shown that the expression of certain genes is found not in a region or population of cells, but in individual cells of the gemmule-hatched sponge (Mohri et al. 2008; Funayama et al. 2010; chapter 3 of this thesis). Since double labeling *in situ* hybridization experiments did not show any co-expression of several Wnt pathway genes and not all archaeocytes expressed a given gene, I suspect that cryptic subtypes of archaeocytes exist. They may be morphologically very similar, making distinction difficult at the whole mount level. The full complement of genes expressed in each cell, however, may provide insight into the function or fate of that particular cell. Funayama et al. (2010) described gene expression profiles for archaeocytes, the sponge stem cell population, whereby the stem cell lineage marker, *piwi*, was co-expressed with certain other genes which were only found to be expressed in restricted cell types. For example co-expression of *piwi* and *annexin* signifies an archaeocyte fated to

become a choanoblast, which then proliferates and differentiates to give rise to a choanocyte chamber. By using gene co-expression patterns, it was possible to suggest which genes may commit a stem cell to a particular lineage. The caveat to this, of course, is that we know nothing of potentially earlier steps toward this differentiation. This represents an excellent way to begin dissecting the roles of different cells of the sponge in terms of generating a set of molecular markers.

It is not new that sponges possess an impressive repertoire of genes involved in development of complex body plans of eumetazoans. It is, however, striking to think of the potential functions and roles of all these genes in these animals. An example is the finding that calcareous sponges have surprisingly large complements of both Wnts and Sox transcription factors (Wnts as reported by Manuel and Forêt, 2012 and Windsor et al. *in prep* and chapter 3 of this work; Sox from Fortunato et al., 2012). Understanding the roles that all these genes play will be difficult, but interesting, to uncover.

5.3 POTENTIAL ROLES FOR WNT IN THE SPONGE

The demonstration that Wnt plays a role in formation of the aquiferous system or body axis of the freshwater sponge is supported by work showing that *wnt* is expressed at posterior pole of larvae in *Amphimedon queenslandica* (Adamska et al., 2007). Further, Leys and Degnan (2002) suggested that in the same species the osculum arose from the posterior pole as well. My own work suggests that the osculum arises from posterior pole in the freshwater sponge, *Eunapius fragilis*. The connection between the osculum, the posterior pole and *wnt* expression certainly deserves more attention.

Wnt signaling is involved in axis formation in all metazoan embryos studied so far and in particular, seems to be associated with the posterior pole of many animals (Fig. 5-1). The planula larva of *Nematostella vectensis* settles on its anterior pole; the posterior pole becomes the oral pole of the settled polyp, where the mouth and tentacles develop. Some *wnt* genes in *Hydra* and *N. vectensis* are expressed in and around the hypostome, the area surrounding the mouth (Kusserow et al., 2005; Lengfeld et al., 2009). The hypostome, or head region, of

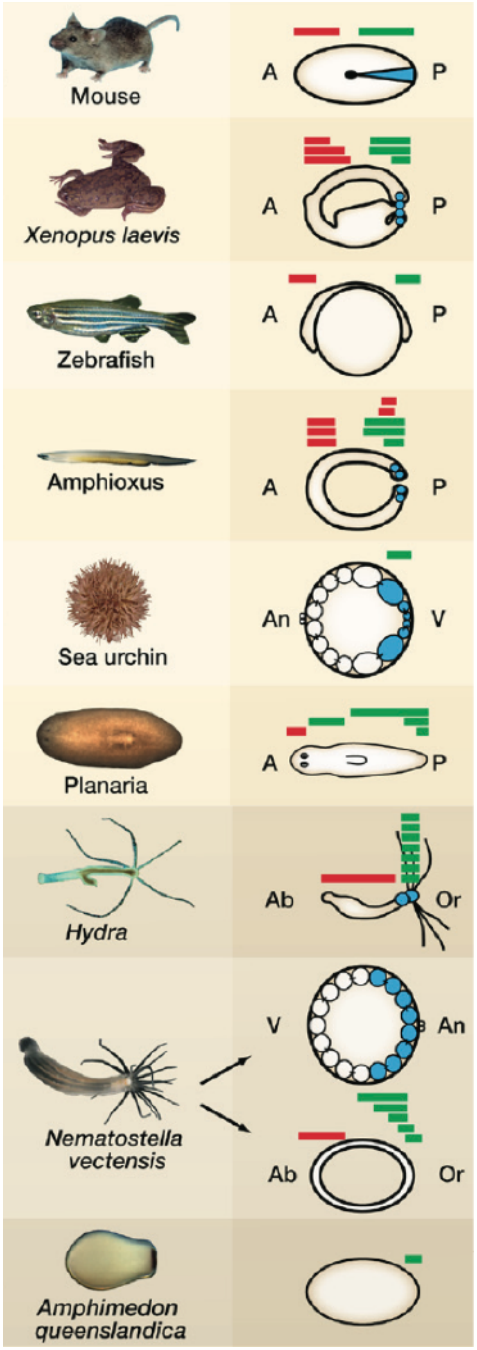

Figure 5-1: Association of Wnt signaling and the posterior pole.
Throughout the animal kingdom, *wnt* genes (represented by green bars) are expressed at the posterior pole or in the posterior region of the developing or adult animal. This is often associated with expression of a Wnt antagonist in the anterior (red bars). During early development, nuclear localization of β-catenin (blue) also tends to occur posteriorly. Abbreviations: A, anterior; P, posterior; An, animal; V, vegetal; Ab, aboral; Or, oral. Figure modified from Petersen and Reddien (2009).

Hydra and the blastopore lip of *N. vectensis* have also been suggested to have organizer-like properties, and Wnt signaling is thought to be involved in this (Broun et al. 2005; Kraus et al. 2007). As a result, it has been postulated that the posterior/oral pole of cnidarians is homologous to the posterior of bilaterians (Petersen and Reddien 2009). It follows that *wnt* expression in the posterior pole of sponge larvae and perhaps even the osculum of adult sponges could mean that the posterior poles of all animal larvae are homologous. Although I was unable to find expression of any *wnt* gene in the osculum of *Ephydatia mulleri* hatchlings or the posterior pole of *Eunpaius fragilis* larvae, others have reported the expression of a *wnt* gene in the osculum of the calcareous sponge, *Sycon ciliatum* (M. Adamska as reported in Manuel and Forêt 2012; Adamska et al. and Borisenko et al., *abstracts*, Ninth World Sponge Conference 2013). It remains to be seen whether the posterior/osculum/Wnt theme is consistent throughout all sponge groups.

An individual sponge can be defined as an osculum and associated structures and canals (Minchin 1900; Hyman 1940, p. 312). In this way, an encrusting sponge with several oscula emerging from its surface may be seen as a colony of individuals not unlike colonial hydrozoans. Evidence of *wnt* expression in the posterior and perhaps the osculum suggests that growth of an encrusting sponge that results in the formation of new oscula is a process similar to clonal reproduction. For example, Wnt signaling promotes oral fates in *Hydractinia echinata* (Müller et al. 2007; Duffy et al. 2010); this could be involved in the production of colonies in this species. In the case of *Hydra* the expression of seven different *wnt* genes is required for bud formation, and *HyWnt2* is specific to early budding stages only (Lengfeld et al. 2009). If a spike in *wnt* expression can trigger formation of a new head organizer and polyp in *Hydra* and also colony formation in *Hydractinia*, perhaps a spike in *wnt* expression at the edge of an encrusting sponge can trigger the formation of a new osculum with new canals and choanocyte chambers that empty into it. This of course is based on some speculations, but it is possible that this direction of research can help to establish the true definition of an individual sponge.

5.4 THE GENOMICS ERA AND THE FUTURE OF SPONGE RESEARCH

At the time this thesis began, the only genomic information about a sponge to have been published was an EST study from *Oscarella carmela*, and the forthcoming genome from *Amphimedon queenslandica* (Nichols et al. 2006; Srivastava et al. 2010). Since that time, the declining costs of generating transcriptomes and genomes has permitted many research groups to begin sequencing their basal metazoan of choice. As of now, many published and unpublished transcriptomes are available from unicellular relatives of metazoans (http://www.broadinstitute.org/index.html) to various sponges and other basal metazoans (reviewed in Richter and King 2013). The ability to analyze data at such an astonishing rate (high-throughput) has helped us to understand that molecularly, there are not as many differences between sponges and other animals as previously thought; the concept that more genes means more complex has been disregarded.

In chapter 2 of this thesis, I used pharmacological treatments to mimic overexpression of Wnt by inhibiting GSK3 using lithium chloride and alsterpaullone. While these drugs have been shown to affect Wnt signaling in other animals, the possibility that non-specific effects might be causing phenotypic changes always exists. For example, Lindahl (1933, as cited in Runnström 1935) showed that lithium treated sea urchin embryos had a decreased ability to perform cellular respiration. Although it is well established that lithium chloride acts as a vegetalizing agent affecting the Wnt pathway throughout metazoans, it is possible that in sponges, where single cells are quite dynamic and independent, an effect on cellular respiration may cause phenotypic changes in the body plan in order to cope with reduced oxygen availability.

In sponge biology progress has been made in the areas of genetic and molecular manipulation, and I employed one of these techniques – RNAi – in chapter 3 of this thesis. To ensure that the effect seen in lithium treated sponges was due to Wnt pathway disruption, I inhibited GSK3 mRNA by treatment with double stranded (ds) RNA. Sponges grown in dsRNA grew more oscula than untreated sponges, confirming that the effect of lithium seemed to be specific to

GSK3 in the sponge. This was distinct from other phenotypes caused by different dsRNAs: *actin*, *PaxB* and *Six1/2* (Rivera et al. 2011, 2013).

Further analytic tools have been developed, including analysis of gene expression in sponges using *in situ* hybridization (Larroux et al., 2008), in addition to standard tools (e.g. quantitative PCR). Unfortunately, this technique may not be an ideal one for unequivocally showing expression because of nonspecific labelling as I showed in chapter 3. It is perhaps of interest to focus more on functional analysis of a gene rather than on describing its expression (though this should not be ignored). In addition to RNAi technology, advances in creating expression constructs that work in different sponge species have been made. Pfannkuchen and Brümmer (2009) used the cytomegalovirus (CMV) universal promoter and drove expression of DsRed2 fluorescent marker in cells of *Spongilla lacustris*. If this technology can be developed, it will be possible to analyze the roles of genes in the absence of forward genetics; use RNAi to knock down gene function, and overexpression vectors to knock in gene function.

The direction of current and future work in sponges should be toward understanding both molecular aspects of sponge biology and development, and their involvement in the roles of different cell types through developmental stages and in the adult. Only through a synthesis of these important areas can we truly begin to understand homology between sponges and other animals.

5.5 CONSERVATION OF POLARITY AND GERM LAYERS IN THE METAZOA

In light of the work presented here, there is evidence that polarity and perhaps germ layers are conserved throughout metazoan evolution. Investigations of the functional role of Wnt in the sponge and the work of others on *wnt* expression have shown a connection between the posterior pole and the osculum of the sponge (Adamska et al., 2007, 2010, 2011; Windsor and Leys 2010; Adamska et al. and Borisenko et al., *abstracts*, Ninth World Sponge Conference 2013). In cnidarians *Nematostella* and *Hydra*, Wnt patterns the oral pole, which is thought to correspond to the posterior of other animals (Petersen and Reddien 2009; Duffy

2011). The blastopore arises from the posterior end of the *Nematostella* larva. Is it then possible that the osculum, which arises from the posterior of the sponge larva and expresses *wnt* (Adamska et al. 2007), could also be homologous to the posterior pole of *Nematostella*? Furthermore, because of the relationship between Wnt and the posterior in metazoans (Fig. 5-1), could posterior poles of larvae in sponges and cnidarians and embryos of bilaterians be homologous? It is unclear whether the germ layers of sponges are homologus to those in other animals, but if this is true we must consider gastrulation in sponges as well. Although this line of research is still in its beginnings, future work could reveal homology between the posterior pole of all animals, accomplishing what Haeckel set out to do over a century ago.

5.6 REFERENCES:

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Appendix 1: Supplemental Material for Chapter 2

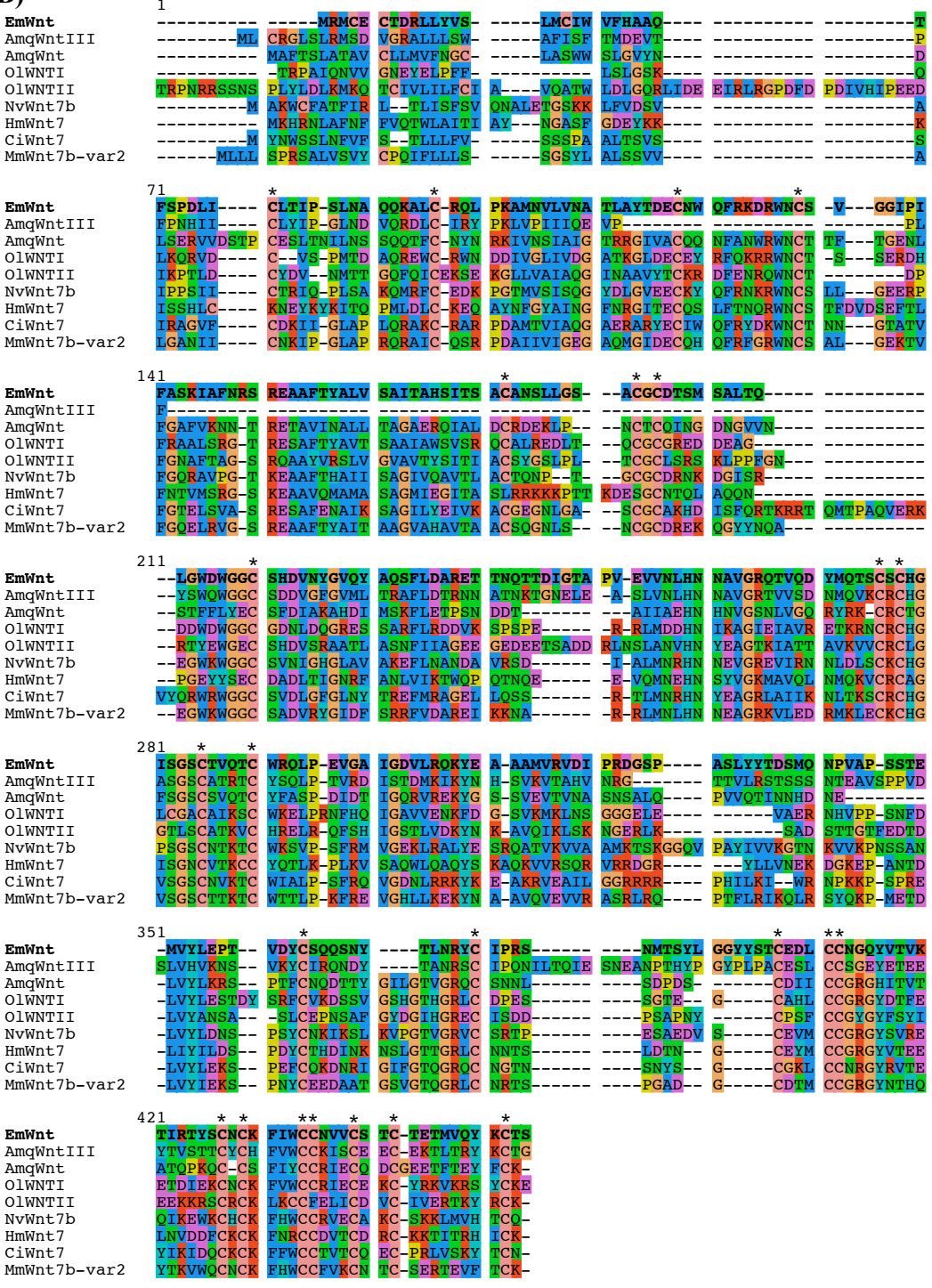
A1.1: *EPHYDATIA MUELLERI* WNT SEQUENCE AND ALIGNMENT

(A) Nucleotide and amino acid sequence for *EmWnt*. Conserved cysteine, C, residues are highlighted in dark blue with white text. (B) Alignment of EmWnt protein sequence with other metazoan Wnts. The alignment was perfomed in SeaView v.4 using the muscle algorithm. 24 conserved C residues (marked by *) characteristic of Wnts are found in the *Ephydatia muelleri* protein (in bold, top line), firmly placing it within the Wnt superfamily. Wnt7 family proteins were primarily selected for the alignment since EmWnt aligns most closely with this family in BLAST searches (http://blast.ncbi.nlm.nih.gov/). Accession numbers and species used are as follows: EmWnt (*Ephydatia muelleri*; HM363029), *AmqWntIII (Amphimedon queenslandica*; GQ144650), *AmqWnt* (EU285557), *OlWNTI (Oscarella lobularis*; GQ144646), *OlWNTII* (GQ144647), *NvWnt7b (Nematostella vectensis*; AY725204), *HmWnt7 (Hydra magnapapillata*; AB426121), *CiWnt7 (Ciona intestinalis*; XM_002127998) and MmWnt7b-var2 (*Mus musculus*; NM_001163634).

A)

```
1    atgcgcatgtgcgagtgtacagatcggctcctatatgtaagctta
     M  R  M  C  E  C  T  D  R  L  L  Y  V  S  L
46   atgtgcatctgggtatttcacgccgcccaaaccttctcgcccgac
     M  C  I  W  V  F  H  A  A  Q  T  F  S  P  D
91   ctgatctgcctcacgattcccagcctgaacgcacagcagaaagcg
     L  I  C  L  T  I  P  S  L  N  A  Q  Q  K  A
136  ctatgcagacagctgcccaaagccatgaacgtgttggtcaacgcg
     L  C  R  Q  L  P  K  A  M  N  V  L  V  N  A
181  acgctcgcatacacagacgagtgcaattggcagtttcgcaaggat
     T  L  A  Y  T  D  E  C  N  W  Q  F  R  K  D
226  cgctggaattgttcggtgggagggatacccatctttgccagcaag
     R  W  N  C  S  V  G  G  I  P  I  F  A  S  K
271  atcgccttcaacaggtccagagaggcagcattcacttacgcactg
     I  A  F  N  R  S  R  E  A  A  F  T  Y  A  L
316  gtgtcggccattactgcacacagcatcaccagtgcctgtgcaaat
     V  S  A  I  T  A  H  S  I  T  S  A  C  A  N
361  agccttttgggttctgcctgcggctgtgatacttccatgagcgca
     S  L  L  G  S  A  C  G  C  D  T  S  M  S  A
406  ctcacacagctggggtgggactggggagggtgcagtcacgacgtg
     L  T  Q  L  G  W  D  W  G  G  C  S  H  D  V
451  aactatggtgtccagtacgctcaatcgttcctcgatgcaagggaa
     N  Y  G  V  Q  Y  A  Q  S  F  L  D  A  R  E
496  acaaccaatcagacaactgatattggaacagcaccagtggaagtt
     T  T  N  Q  T  T  D  I  G  T  A  P  V  E  V
541  gtgaatctgcacaacaacgcagtcggtagacaaactgtccaggac
     V  N  L  H  N  N  A  V  G  R  Q  T  V  Q  D
586  tacatgcagacatcttgcagctgtcatggtatctcagggtcttgc
     Y  M  Q  T  S  C  S  C  H  G  I  S  G  S  C
631  acagtgcagacttgttggcgacagttaccagaggtaggtgcgatt
     T  V  Q  T  C  W  R  Q  L  P  E  V  G  A  I
676  ggagacgtgctccgacagaagtatgaggcagcagccatggtccga
     G  D  V  L  R  Q  K  Y  E  A  A  A  M  V  R
721  gttgacattcccagggacggaagccccgcctccttgtactataca
     V  D  I  P  R  D  G  S  P  A  S  L  Y  Y  T
766  gattctatgcagaaccctgtggccccatcaagcacagagatggtc
     D  S  M  Q  N  P  V  A  P  S  S  T  E  M  V
811  tatttggagcccactgtggactactgctcccagcagtctaactac
     Y  L  E  P  T  V  D  Y  C  S  Q  Q  S  N  Y
856  acattgaacaggtactgcattccacgctccaacatgactagctat
     T  L  N  R  Y  C  I  P  R  S  N  M  T  S  Y
901  ctcggaggatattattcaacatgcgaagatctttgttgcaatgga
     L  G  G  Y  Y  S  T  C  E  D  L  C  C  N  G
946  cagtatgtcactgtgaagacaattagaacttattcatgcaactgc
     Q  Y  V  T  V  K  T  I  R  T  Y  S  C  N  C
991  aagttcatttggtgctgcaatgtagtgtgcagtacttgcacagag
     K  F  I  W  C  C  N  V  V  C  S  T  C  T  E
1036 acaatggtacaatacaagtgtactagctaa 1065
     T  M  V  Q  Y  K  C  T  S  *
```

B)


EmWnt
AmqWntIII
AmqWnt
OlWNTI
OlWNTII
NvWnt7b
HmWnt7
CiWnt7
MmWnt7b-var2

Appendix 2: Supplemental Material for Chapter 3

A2.1: SEQUENCE ACCESSION, CONTIG NUMBERS AND SPECIES CODES

Wnt Sequence GenBank accessions numbers and contig numbers from the reference transcriptome for *Crella elegans* (Pérez-Porro et al. 2013) (http://datadryad.org/resource/doi:10.5061/dryad.50dc6) and publicly available sequence data used in phylogenetic analysis.

Aqu	Amphimedon queenslandica	
	AquWntA	ABX90060
	AquWntB	ADO16064
	AquWntC	ADO16565)
Cre	*Crella elegans* [1]	
	CreWntA	contig66998
	CreWntC	contig73781
Emu	*Ephydatia muelleri*	
	EmuWntB	ADM13617
Olo	*Oscarella lobularis*	
	OloWntIACS36174	
	OloWntII	ACS36175
Dre	*Danio rerio*	
	DreWnt1	NP_001188327
	DreWnt2	NP_571025
	DreWnt2b	NP_001037809
	DreWnt2.1	NP_878296
	DreWnt3	NP_001108024
	DreWnt3a	NP_001007186
	DreWnt4a	NP_001035477
	DreWnt4b	NP_571575
	DreWnt5a	NP_001073303
	DreWnt5b	NP_571012
	DreWnt6	XP_003199237
	DreWnt6like	XP_002662357
	DreWnt7a	NP_001020711
	DreWnt7alike	XP_696514
	DreWnt7b	XP_691878
	DreWnt8a	NP_571021
	DreWnt8b	NP_571034
	DreWnt8like	NP_00108637
	DreWnt9a	NP_001038828
	DreWnt9b	NP_001131132
	DreWnt10a	NP_571055
	DreWnt10b	NP_835737
	DreWnt11	NP_571151
	DreWnt11.1	NP_001138276
	DreWnt16	NP_001093516
Hsa	*Homo sapiens*	

HsaWnt1	NP_005421
HsaWnt2	NP_003382
HsaWnt2b1	NP_004176
HsaWnt2b2	NP_078613
HsaWnt3	NP_110380
HsaWnt3a	NP_149122
HsaWnt4	NP_110388
HsaWnt5a	NP_003383
HsaWnt5b	NP_110402
HsaWnt6	NP_006513
HsaWnt7a	NP_004616
HsaWnt7b	NP_478679
HsaWnt8a	NP_490645
HsaWnt8b	NP_003384
HsaWnt9a	NP_003386
HsaWnt9b	NP_003387
HsaWnt10a	NP_079492
HsaWnt10b	NP_003385
HsaWnt11	NP_004617
HsaWnt16.1	NP_476509
HsaWnt16.2	NP_057171

A2.2: IN SITU HYBRIDIZATION PROTOCOL

Preparation:
Sponges are fixed with 4% paraformaldehyde in 1/4 HS, overnight, washed once in 1/4 HS, then dehydrated with 25% EtOH in 1/4 HS, 50% EtOH in 1/4 HS, 75% EtOH in 1/4 HS and 100% EtOH. Can be stored at –80°C until ready to use.

Pretreatment:

- Transfer coverslips to 6-well dish. Use 1 ml for each wash. Rehydrate through (~2-5min each):
  100% EtOH → 75% EtOH in PBTw → 50% EtOH in PBTw → 25% EtOH in PBTw → PBTw
- 3x2min PBTw
- 1min Proteinase K treatment (0.3 ul ProK 100 mg/ml stock in 6 ml PBTw)
- 2x5min glycine 2 mg/ml in PBTw to stop Prot K (240 ul glycine 100 mg/ml in 12 ml PBTw)
- 3x5min in PBTw
- Post fix in 3.7% PF plus 0.3% glut at room temp for 1 hr. (For 6 ml: 1.2 ml 20% PF + 22.5 ul 8% glutaraldehyde + 4.8 ml PBTw)
- 3x10min PBTw

Pre-hyb:

- Replace wash with HB at room temp for 10 min
- Replace again with fresh, pre-warmed (55°C) HB, and pre-hyb for 1-2 hours at 55°C

Hyb:

- Dilute probe to a final concentration 10-0.05 ng/µl in HB. [10 µl of probe (50ng/µl) in 490µl hybe solution]. Stock probe should be stored at 50ng/µl in HB at -20ºC).
- Denature probe by boiling for 10 minutes at 80-90 °C (OR if using already diluted probes equilibrate to 68°C before use). Remove HB and quickly add probe. Hybridize for 16-72 hrs.

Post-hyb washes:

- Prepare PHB solution of 50% formamide/5 X SSC with Tw20 and 2 X SSC pH4.5 (5ml of 20x SSC pH4.5 in 50ml DEPC water)
- Make fresh wash solutions: PHB1 (75% PHB:25% 2xSSC), PHB2 (50% PHB:50% 2x SSC), PHB3 (25% PHB:75%2x SSC)
- Recover probe and store at –20°C. May be used multiple times.
- 30 minutes in HB at 55 ºC
- 20 min PHB1
- 20 min PHB2
- 20 min PHB3
- 3 x 20 min 2 X SSC “

- 10 minutes in 2X SSC: MAB (1:1) at RT
- 3x5min in MAB at RT
- Block in Blocking buffer (diluted 1:10 in MAB) for 1 hr at RT on rocker
- Incubate in Anti-Dig-AP Fab fragments (diluted in blocking buffer to 1:5000) O/N at 4 ºC.

- Several long washes (at least 5x30min, sometimes to O/N) in MAB
- 10min in fresh $MgCl_2$-free AP Buffer
- 2x5 minutes in fresh AP Buffer
- Add substrate solution: 4.5 µl of NBT + 3.5 µl of BCIP in 1 ml AP buffer, incubate in the dark at RT for hours to days. Usually placed at 4°C if left O/N to develop.
- Wash 2-3x in PBTw to stop
- Post fix in 4% Paraformaldehyde in PBT (1 hr or O/N)
- Wash 3x in PBS and dehydrate to 70%

Solutions

¼ Holtfreter Solution (1/4HS; buffer for freshwater embryos):
875 mg NaCl, 12.5 mg KCl, 25 mg CaCl2, 50 mg NaHCO3 fill to 1.0 L with dH_2O (1L) Store at room temperature

10X PBS: 18.6 mM NaH2PO4 (2.56g NaH2PO4-H2O per liter dH_2O)
84.1 mM Na2HPO4 (11.94 Na2HPO4 per liter dH_2O)
1750 mM NaCl (102.2g NaCl per liter dH_2O)
Mix phosphates in about 800ml of dH_20 for a 1L volume. Check the pH, it should be 7.4 ±0.4. If it is more than 0.4 off, start over. Otherwise adjust pH to 7.4 with NaOH or HCl. Add the NaCl and the rest of the H_2O.

PBTw: 1x PBS + 0.1% Tween 20 detergent
(100ml 10 X PBS + 895ml dH20, DEPC treat/autoclave; when cool add 5 ml 20% Tween)

DEPC H_2O: 0.5% DEPC (0.5ml in 1L); shake the bottles at 250rpm at 37°C O/N, autoclave

20X SSC: for 1L (0.3M Sodium citrate + 3M NaCl)
175.3g NaCl
88.2g Sodium Citrate, dihydrate (CH6H5Na3-2H20)
pH to 7.0 and sterilize by autoclaving
*Dilute 1:10 for 2X

Hybridization Buffer (HB; 50 ml)	add	final
formamide	25 mL	50% formamide
20X SSC pH 4.5	12.5 mL	5x SSC pH 4.5
20 mg/mL heparin	0.125 mL	50 µg/ml
20%Tween-20	0.25 mL	0.1%
100X Denhart's	0.5 ml	5X
10 mg/ml yeast tRNA	0.25 ml	100 µg/ml
dH_2O to 50 mL		

*Store at -20oC

Post Hyb Buffer (PHB; 50mL)	add	final
100% formamide	25 mL	50%
20x SSC pH 4.5	12.5 mL	5X
20% Tween-20 Tween-20	0.25 mL	0.1%
DEPC H_2O to 50 mL		

*Store at -20°C

Maleic acid buffer (MAB; 500mL)	add	final
1M maleic acid	50 mL	0. 1M maleic acid
5M NaCl	15 mL	0.15 M

DEPC H_2O to 500mL pH to 7.5 (it will be close to 1.5 when you start; use NaOH pellets)
*Just before use add 0.1% Tween-20

10x Blocking Buffer
Dissolve 10g blocking reagent (10% w/v; Roche) in ~80ml MAB (for final volume of 100ml), shaking and heated (can microwave)
Autoclave
*keep blocking reagent in aliquots at -20°C

AP Reaction Buffer (50mL)	add	final
1M MgCl2	250ul	5mM
1M NaCl	5mL	100mM
1M Tris pH 9.5*	5mL	100mM
20% Tween-20	250ul	0.1%
DEPC H_20 to 50mL		

*Make just prior to use – solution sitting at room temp for a few hours will not work as well for the reaction. This recipe is for AP reaction buffer pH 9.5 used for BCIP/NBT reactions.

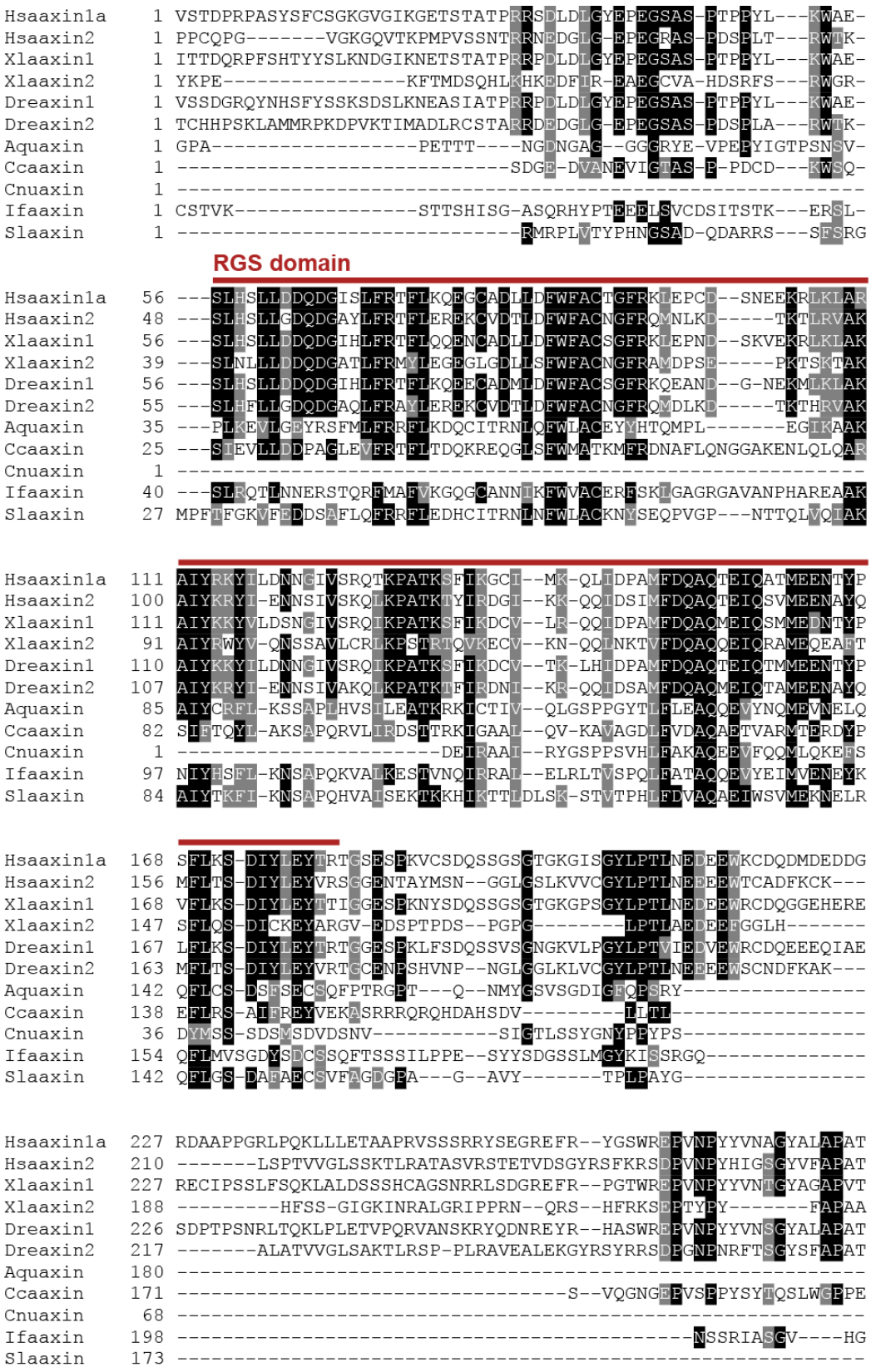
A2.3: PRIMERS FOR AMPLIFICATION OF RNAi AND mRNA INJECTION EXPERIMENTS

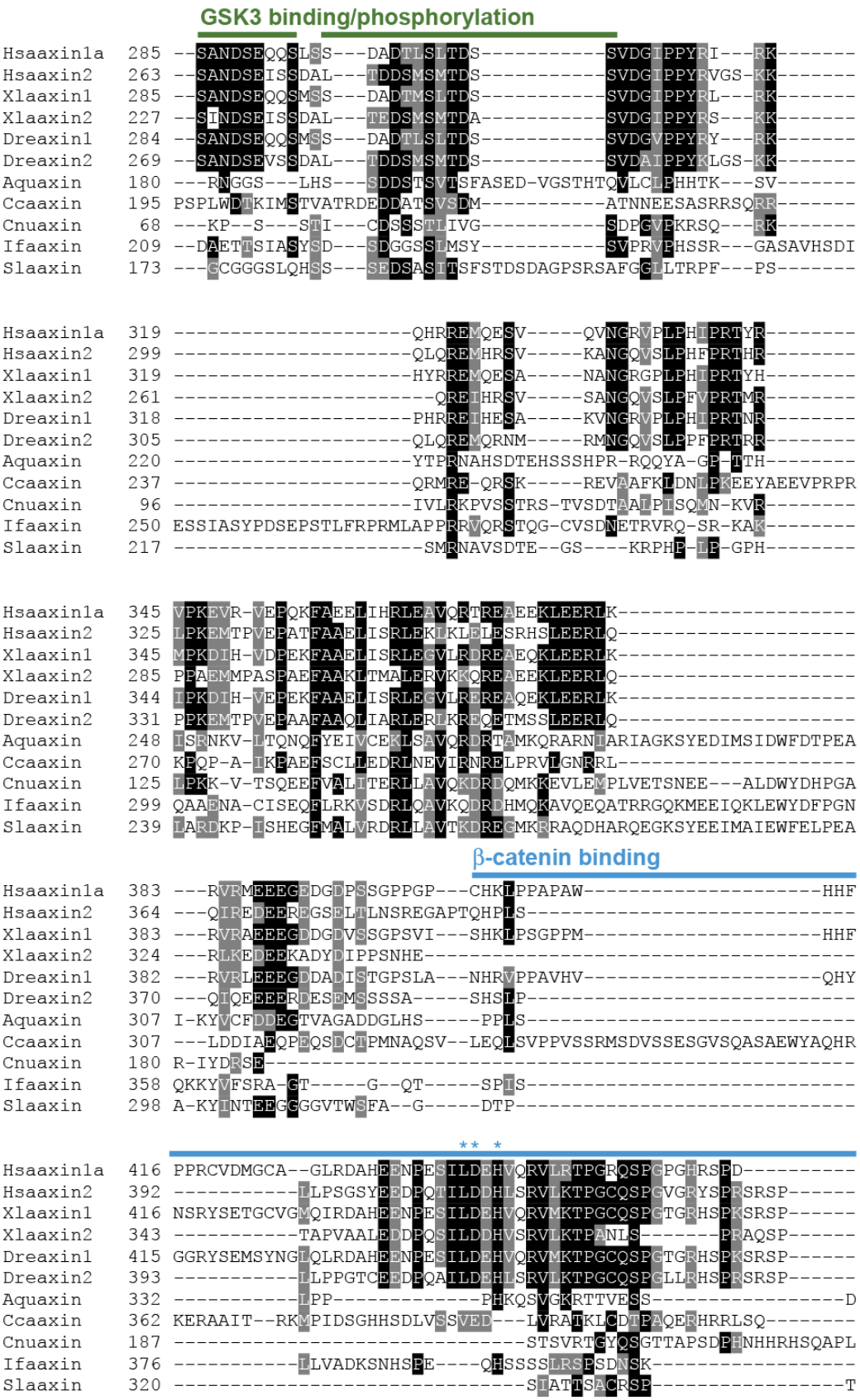
Primers used to amplify sequences for RNAi and mRNA injection experiments. Colour coding: red = T7 partial or full sequence; blue = XhoI restriction cut site; green = XbaI restriction cut site; and orange = forced mismatch in primer to prevent primer secondary structure (no change to amino acid sequence in EmuWntB).

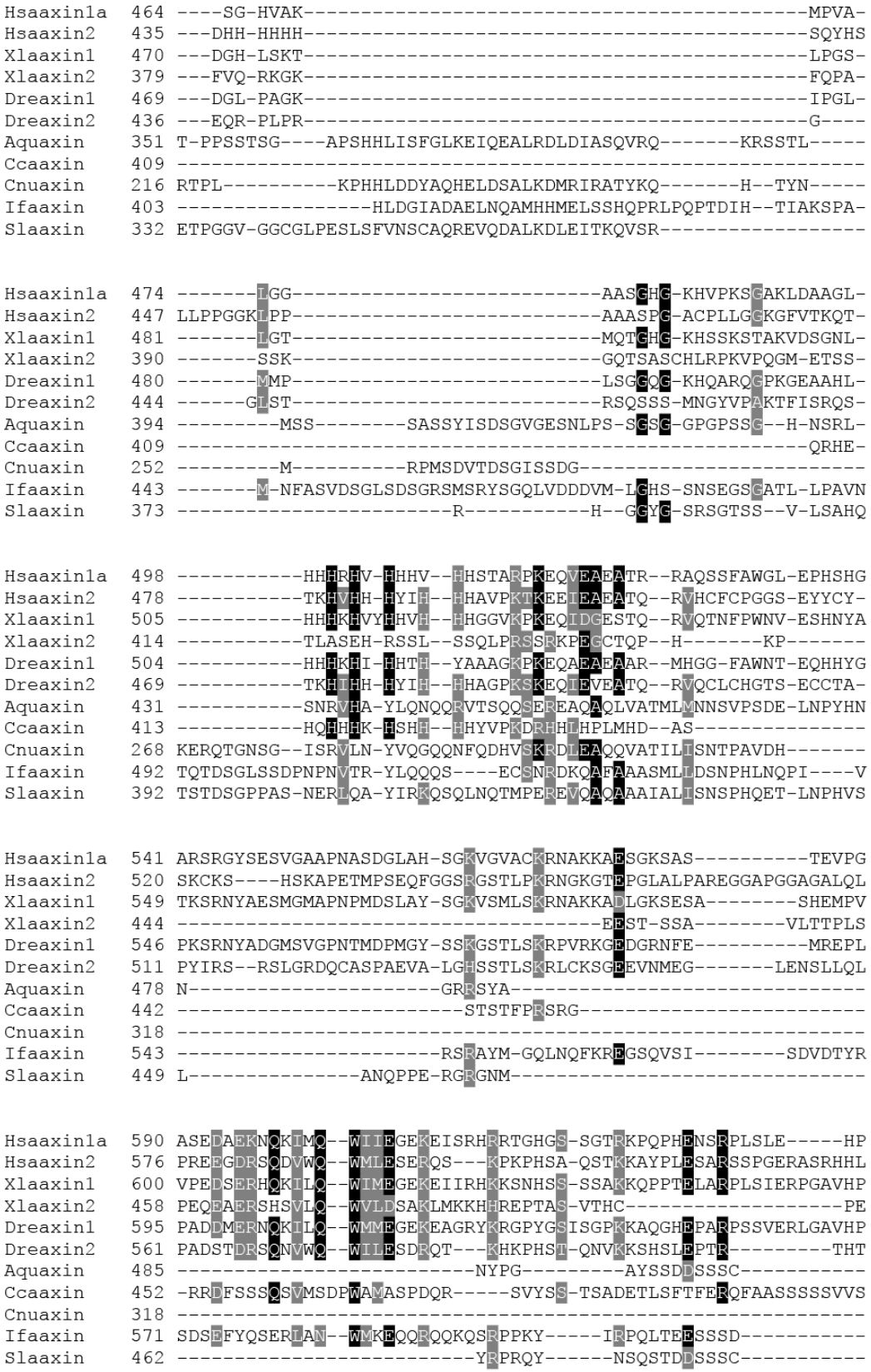
Purpose	Gene	Primer name	Sequence (5' to 3')	Notes
RNAi	*GSK3, Ephydatia mulleri*	iEmuGSK3 F	CGACTCACTATAGGGCCGTCACGTAACAGGACTAC	Partial T7 sequence
		iEmuGSK3 R	CGACTCACTATAGGGAGACAACCCCAAATGATCCA	Partial T7 sequence
	silicateinM2, Ephydatia muelleri	iEmuSilcM2 F	CGACTCACTATAGGGGGAGAGACATGCCATTTGGT	Partial T7 sequence
		iEmuSilcM2 R	CGACTCACTATAGGGCTGGACACCTTTCGGAGCTA	Partial T7 sequence
	-	T7 full	ATAGAATTCTCTAGAAGCTTAATA**CGACTCACTATAGGG**	T7 promoter sequence
Xenopus expression	*wntA, Spongilla lacustris*	injSlawntA F	GTTCTTCTCGAGATGGACAAAAGAGCTGTGCAGGAGC	XhoI restriction site
		injSlawntA R	TCATCATCTAGATGAGGTGTTGTGTGAAACTGG	XbaI restriction site
	wntB, Ephydatia muelleri	injEmuwntB F	GTTCTTCTCGAGATGCGaATGTGCGAGTGTA	XhoI restriction site, forced mismatch
		injEmuwntB R	TCATCACTCGAGAACACAGAGCATTCACATTAGC	XhoI restriction site
	wntC, Ephydatia muelleri	injEmuwntC F	GTTCTTCTCGAGATGGAAAGGTCTGCTTCTACCGTCTTCT CGTGCCTTTCG	XhoI restriction site
		injEmuwntC R	TCATCATCTAGAGGCTCAACCACATTGACACA	XbaI restriction site

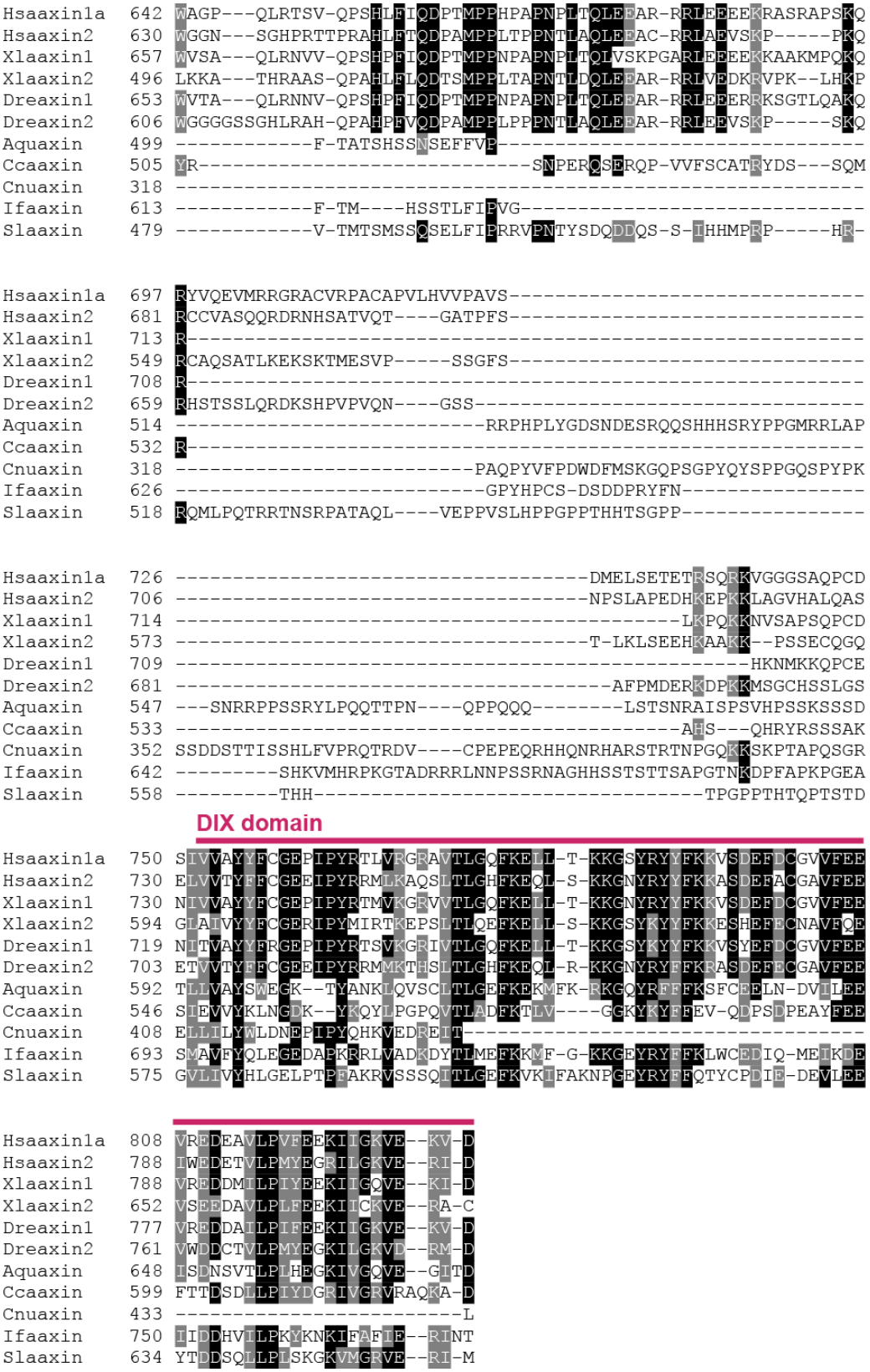
A2.4: AXIN PROTEIN ALIGNMENT

Alignment of axin proteins from human, *Xenopus*, and *Danio* with sponge axins. The RGS and DIX domains are indicated with dark red and magenta, respectively. The GSK3 and β-catenin binding sites (green and blue) are not well conserved in sponges. * Indicates residues known to be required for β-catenin - axin interactions (Xing et al. 2003).


RGS domain


GSK3 binding/phosphorylation
β-catenin binding
Hsaaxin1a 285 --SANDSEQQSLSS---DADTLSLTDS----------SVDGIPPYRI---RK-------
Hsaaxin2 263 --SANDSEISSDAL---TDDSMSMTDS----------SVDGIPPYRVGS-KK-------
Xlaaxin1 285 --SANDSEQQSMSS---DADTMSLTDS----------SVDGIPPYRL---RK-------
Xlaaxin2 227 --SINDSEISSDAL---TEDSMSMTDA----------SVDGIPPYRS---KK-------
Dreaxin1 284 --SANDSEQQSMSS---DADTLSLTDS----------SVDGVPPYRY---RK-------
Dreaxin2 269 --SANDSEVSSDAL---TDDSMSMTDS----------SVDAIPPYKLGS-KK-------
Aquaxin 180 ---RNGGS---LHS---SDDSTSVTSFASED-VGSTHTQVLCLPHHTK---SV-------
Ccaaxin 195 PSPLWDTKIMSTVATRDEDDATSVSDM----------ATNNEESASRRSQRR-------
Cnuaxin 68 ---KP--S---STI---CDSSSTLIVG----------SDPGVPKRSQ---RK-------
Ifaaxin 209 --DAETTSIASYSD---SDGGSSLMSY----------SVPRVPHSSR---GASAVHSDI
Slaaxin 173 ---GCGGGSLQHSS---SEDSASITSFSTDSDAGPSRSAFGGLLTRPF---PS-------
Hsaaxin1a 319 ----------QHRREMQESV-----QVNGRVPLPHIPRTYR--------
Hsaaxin2 299 ----------QLQREMHRSV-----KANGQVSLPHFPRTHR--------
Xlaaxin1 319 ----------HYRREMQESA-----NANGRGPLPHIPRTYH--------
Xlaaxin2 261 ----------QREIHRSV-----SANGQVSLPFVPRTMR--------
Dreaxin1 318 ----------PHRREIHESA-----KVNGRVPLPHIPRTNR--------
Dreaxin2 305 ----------QLQREMQRNM-----RMNGQVSLPPFPRTRR--------
Aquaxin 220 ----------YTPRNAHSDTEHSSSHPR-RQQYA-GPFTTH--------
Ccaaxin 237 ----------QRMRE-QRSK-----REVAAFKLDNLPKEEYAEEVPRPR
Cnuaxin 96 ----------IVLRKPVSSTRS-TVSDTAALPISQMN-KVR--------
Ifaaxin 250 ESSIASYPDSEPSTLFRPRMLAPPRRVQRSTQG-CVSDNETRVRQ-SR-KAK--------
Slaaxin 217 ----------SMRNAVSDTE--GS----KRPHP-LP-GPH--------
Hsaaxin1a 345 VPKEVR-VEPQKFAEELIHRLEAVQRTREAEEKLEERLK----------
Hsaaxin2 325 LPKEMTPVEPATFAAELISRLEKLKLELESRHSLEERLQ----------
Xlaaxin1 345 MPKDIH-VDPEKFAAELISRLEGVLRDREAEQKLEERLK----------
Xlaaxin2 285 PPAEMMPASPAEFAAKLTMALERVKKQREAEEKLEERLQ----------
Dreaxin1 344 IPKDIH-VEPEKFAAELISRLEGVLREREAQEKLEERLK----------
Dreaxin2 331 PPKEMTPVEPAAFAAQLIARLERLKREQETMSSLEERLQ----------
Aquaxin 248 ISFNKV-LTQNQFYEIVCEKLSAVQRDRTAMKQRARNIARIAGKSYEDIMSIDWFDTPEA
Ccaaxin 270 KPQP-A-IKPAEFSCLLEDRLNEVIRNRELPRVLGNRRL----------
Cnuaxin 125 LPKK-V-TSQEEFVALITERLLAVQKDRDQMKKEVLEMPLVETSNEE---ALDWYDHPGA
Ifaaxin 299 QAAENA-CISEQFLRKVSDRLQAVKQDRDHMQKAVQEQATRRGQKMEEIQKLEWYDFPGN
Slaaxin 239 LARDKP-ISHEGFMALVRDRLLAVTKDREGMKRRAQDHARQEGKSYEEIMAIEWFELPEA
Hsaaxin1a 383 ---RVRMEEEGEDGDPSSGPPGP---CHKLPPAPAW----------HHF
Hsaaxin2 364 ---QIREDEEREGSELTLNSREGAPTQHPLS----------
Xlaaxin1 383 ---RVRAEEEGDDGDVSSGPSVI---SHKLPSGPPM----------HHF
Xlaaxin2 324 ---RLKEDEEKADYDIPPSNHE----------
Dreaxin1 382 ---RVRLEEEGDDADISTGPSLA---NHRVPPAVHV----------QHY
Dreaxin2 370 ---QIQEEEERDESEMSSSSA-----SHSLP----------
Aquaxin 307 I-KYVCFDDEGTVAGADDGLHS-----PPLS----------
Ccaaxin 307 ---LDDIAEQPEQSDCTPMNAQSV--LEQLSVPPVSSRMSDVSSESGVSQASAEWYAQHR
Cnuaxin 180 R-IYDRSE----------
Ifaaxin 358 QKKYVFSRA-GT-----G--QT-----SPIS----------
Slaaxin 298 A-KYINTEEGGGGVTWSFA--G-----DTP----------
Hsaaxin1a 416 PPRCVDMGCA--GLRDAHEENPESILDEHVQRVLRTPGRQSPGPGHRSPD----------
Hsaaxin2 392 ----------LLPSGSYEEDPQTILDDHLSRVLKTPGCQSPGVGRYSPFSRSP------
Xlaaxin1 416 NSRYSETGCVGMQIRDAHEENPESILDEHVQRVMKTPGCQSPGTGRHSPKSRSP------
Xlaaxin2 343 ----------TAPVAALEDDPQSILDDHVSRVLKTPANLS-------PFAQSP------
Dreaxin1 415 GGRYSEMSYNGLQLRDAHEENPESILDEHVQRVMKTPGCQSPGTGRHSPKSRSP------
Dreaxin2 393 ----------LLPPGTCEEDPQAILDEHLSRVLKTPGCQSPGLLRHSPRSRSP------
Aquaxin 332 ----------LPP----------PHKQSVGKRTTVESS----------D
Ccaaxin 362 KERAAIT--RKMPIDSGHHSDLVSSVED---LVRATKLCDTPAQERHRRLSQ--------
Cnuaxin 187 ----------STSVRTGYQSGTTAPSDPFNHHRHSQAPL
Ifaaxin 376 ----------LLVADKSNHSPE----QHSSSSLRSPSDNSK----------
Slaaxin 320 ----------STATTSACRSP----------T

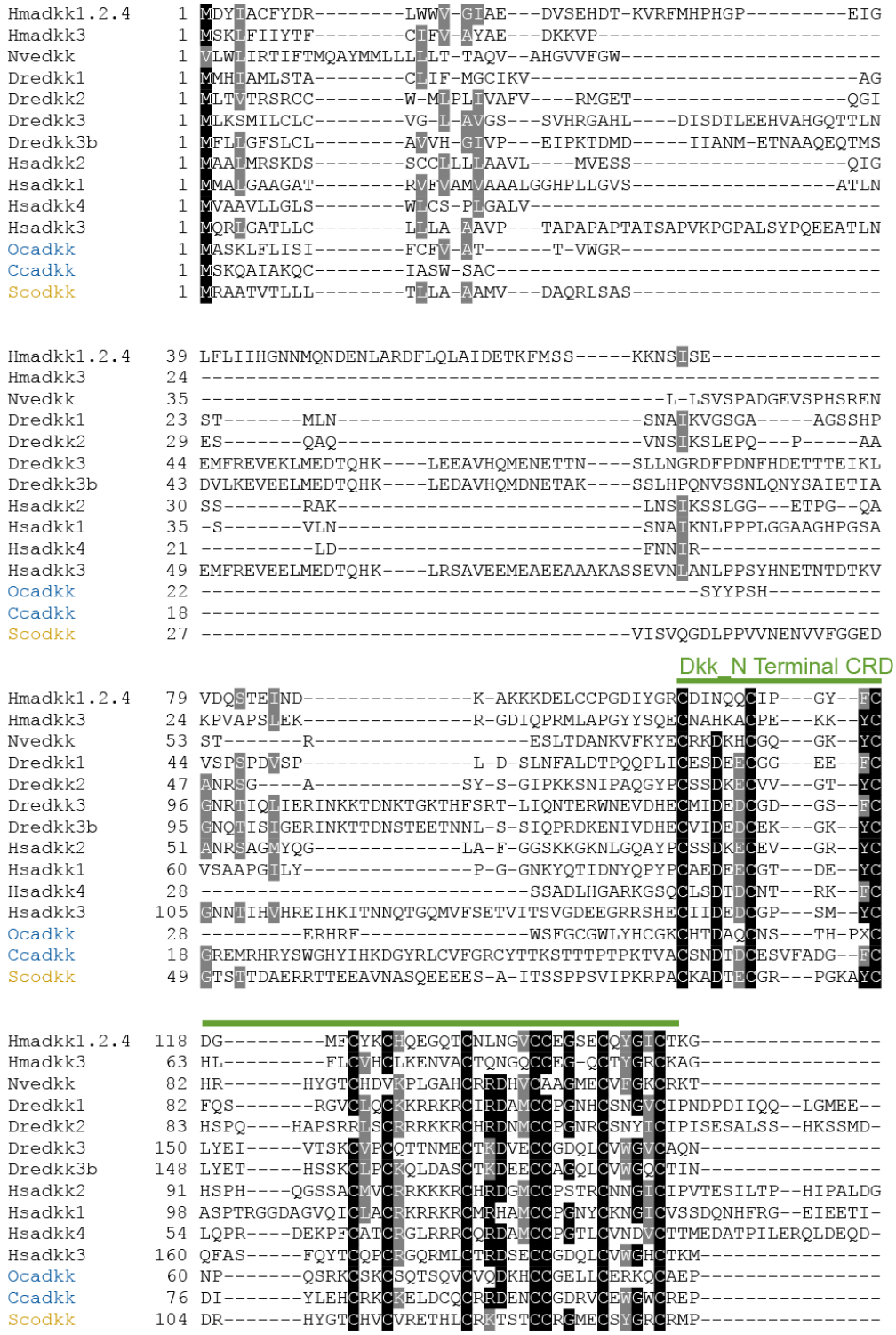

Hsaaxin1a 464 ----SG-HVAK----------MPVA-
Hsaaxin2 435 ---DHH-HHHH----------SQYHS
Xlaaxin1 470 ---DGH-LSKT----------LPGS-
Xlaaxin2 379 ---FVQ-RKGK----------FQPA-
Dreaxin1 469 ---DGL-PAGK----------IPGL-
Dreaxin2 436 ---EQR-PLPR----------G----
Aquaxin 351 T-PPSSTSG----APSHHLISFGLKEIQEALRDLDIASQVRQ-------KRSSTL-----
Ccaaxin 409 ----------
Cnuaxin 216 RTPL----------KPHHLDDYAQHELDSALKDMRIRATYKQ-------H--TYN-----
Ifaaxin 403 ----------HLDGIADAELNQAMHHMELSSHQPRLPQPTDIH--TIAKSPA-
Slaaxin 332 ETPGGV-GGCGLPESLSFVNSCAQREVQDALKDLEITKQVSR----------
Hsaaxin1a 474 -------LGG----------AASGHG-KHVPKSGAKLDAAGL-
Hsaaxin2 447 LLPPGGKLPP----------AAASPG-ACPLLGGKGFVTKQT-
Xlaaxin1 481 -------LGT----------MQTGHG-KHSSKSTAKVDSGNL-
Xlaaxin2 390 -------SSK----------GQTSASCHLRPKVPQGM-ETSS-
Dreaxin1 480 -------MMP----------LSGGQG-KHQARQGPKGEAAHL-
Dreaxin2 444 ------GLST----------RSQSSS-MNGYVPAKTFISRQS-
Aquaxin 394 ---------MSS--------SASSYISDSGVGESNLPS-SGSG-GPGPSSG--H-NSRL-
Ccaaxin 409 ----------QRHE-
Cnuaxin 252 ---------M----------RPMSDVTDSGISSDG----------
Ifaaxin 443 -------M-NFASVDSGLSDSGRSMSRYSGQLVDDDVM-LGHS-SNSEGSGATL-LPAVN
Slaaxin 373 ----------R----------H--GGYG-SRSGTSS--V-LSAHQ
Hsaaxin1a 498 ----------HHHRHV-HHHV--HHSTARPKEQVEAEATR--RAQSSFAWGL-EPHSHG
Hsaaxin2 478 ----------TKHVHH-HYIH--HHAVPKTKEEIEAEATQ--RVHCFCPGGS-EYYCY-
Xlaaxin1 505 ----------HHHKHVYHHVH--HHGGVKPKEQIDGESTQ--RVQTNFPWNV-ESHNYA
Xlaaxin2 414 ----------TLASEH-RSSL--SSQLPRSSRKPEGCTQP--H--------KP-------
Dreaxin1 504 ----------HHHKHI-HHTH--YAAAGKPKEQAEAEAAR--MHGG-FAWNT-EQHHYG
Dreaxin2 469 ----------TKHIHH-HYIH--HHAGPKSKEQIEVEATQ--RVQCLCHGTS-ECCTA-
Aquaxin 431 ----------SNRVHA-YLQNQQRVTSQQSEREAQAQLVATMLMNNSVPSDE-LNPYHN
Ccaaxin 413 ----------HQHHHK-HSHH--HHYVPKDRHHLHPLMHD--AS----------
Cnuaxin 268 KERQTGNSG--ISRVLN-YVQGQQNFQDHVSKRDLEAQQVATILISNTPAVDH-------
Ifaaxin 492 TQTDSGLSSDPNPNVTR-YLQQQS----ECSNRDKQAFAAASMLLDSNPHLNQPI----V
Slaaxin 392 TSTDSGPPAS-NERLQA-YIRKQSQLNQTMPEREVQAQAAAIALISNSPHQET-LNPHVS
Hsaaxin1a 541 ARSRGYSESVGAAPNASDGLAH-SGKVGVACKRNAKKAESGKSAS----------TEVPG
Hsaaxin2 520 SKCKS----HSKAPETMPSEQFGGSRGSTLPKRNGKGTEPGLALPAREGGAPGGAGALQL
Xlaaxin1 549 TKSRNYAESMGMAPNPMDSLAY-SGKVSMLSKRNAKKADLGKSESA--------SHEMPV
Xlaaxin2 444 ----------EEST-SSA--------VLTTPLS
Dreaxin1 546 PKSRNYADGMSVGPNTMDPMGY-SSKGSTLSKRPVRKGEDGRNFE----------MREPL
Dreaxin2 511 PYIRS--RSLGRDQCASPAEVA-LGHSSTLSKRLCKSGEEVNMEG-------LENSLLQL
Aquaxin 478 N----------GRRSYA----------
Ccaaxin 442 ----------STSTFPRSRG----------
Cnuaxin 318 ----------
Ifaaxin 543 ----------RSRAYM-GQLNQFKREGSQVSI--------SDVDTYR
Slaaxin 449 L----------ANQPPE-RGRGNM----------
Hsaaxin1a 590 ASEDAEKNQKIMQ--WIIEGEKEISRHRRTGHGS-SGTRKPQPHENSRPLSLE-----HP
Hsaaxin2 576 PREEGDRSQDVWQ--WMLESERQS---KPKPHSA-QSTKKAYPLESARSSPGERASRHHL
Xlaaxin1 600 VPELSERHQKILQ--WIMEGEKEIIRHKKSNHSS-SSAKKQPPTELARPLSIERPGAVHP
Xlaaxin2 458 PEQEAERSHSVLQ--WVLDSAKLMKKHHREPTAS-VTHC----------PE
Dreaxin1 595 PADLMERNQKILQ--WMMEGEKEAGRYKRGPYGSISGPKKAQGHEPARPSSVERLGAVHP
Dreaxin2 561 PADSTDRSQNVWQ--WILESDRQT---KHKPHST-QNVKKSHSLEPTR---------THT
Aquaxin 485 ----------NYPG---------AYSSDDSSSC----------
Ccaaxin 452 -RRDFSSSQSVMSDPWAMASPDQR-----SVYSS-TSADETLSFTFERQFAASSSSSVVS
Cnuaxin 318 ----------
Ifaaxin 571 SDSEFYQSERLAN--WMKEQQRQQKQSRPPKY-----IRPQLTEESSSD----------
Slaaxin 462 ----------YRPRQY------NSQSTDDSSSC----------

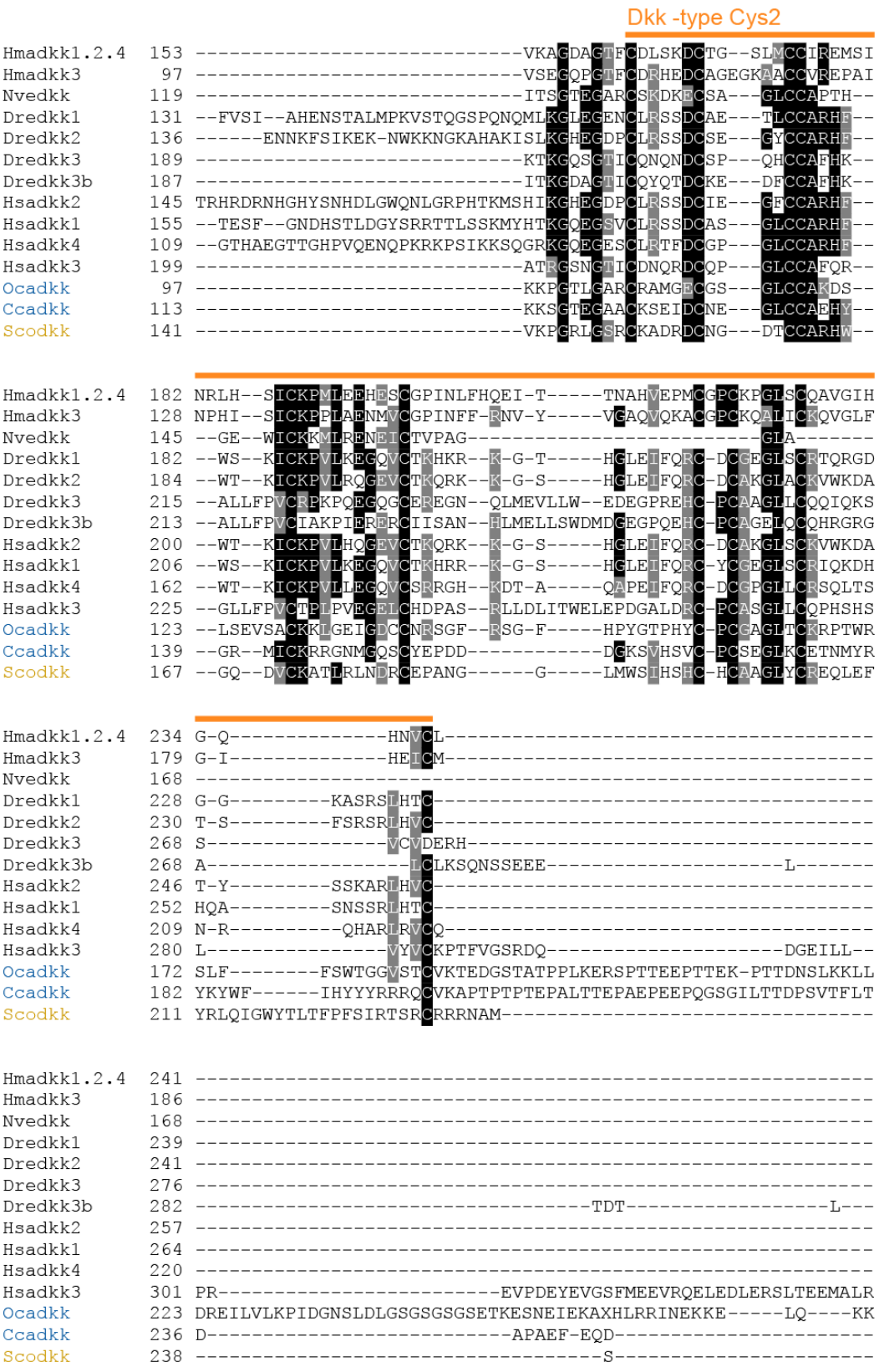

DIX domain

A2.5: SPONGE DKK SEQUENCE ALIGNMENT AND PHYLOGENETIC TREE

Analysis of dkk proteins from sponge. (A) Alignment of dkks from sponge and other metazoans. Two dkk cysteine-rich domains (green, orange) are present in sponges. (B) Partial dkk fragment from *Petrosia ficisformis*. (C) Maximum likelihood analysis does not place the sponge sequences in their own subfamily, but instead with other metazoan dkks with low support.

A


Hmadkk1.2.4
Hmadkk3
Nvedkk
Dredkk1
Dredkk2
Dredkk3
Dredkk3b
Hsadkk2
Hsadkk1
Hsadkk4
Hsadkk3
Ocadkk
Ccadkk
Scodkk
Dkk_N Terminal CRD


Dkk -type Cys2
Hmadkk1.2.4 153 ----------VKAGDAGTFCDLSKDCTG--SLMCCIREMSI
Hmadkk3 97 ----------VSEGQPGTFCDRHEDCAGEGKAACCVREPAI
Nvedkk 119 ----------ITSGTEGARCSKDKECSA---GLCCAPTH--
Dredkk1 131 --FVSI--AHENSTALMPKVSTQGSPQNQMLKGLEGENCLRSSDCAE---TLCCARHF--
Dredkk2 136 ------ENNKFSIKEK-NWKKNGKAHAKISLKGHEGDPCLRSSDCSE---GYCCARHF--
Dredkk3 189 ----------KTKGQSGTICQNQNDCSP---QHCCAEHK--
Dredkk3b 187 ----------ITKGDAGTICQYQTDCKE---DFCCAEHK--
Hsadkk2 145 TRHRDRNHGHYSNHDLGWQNLGRPHTKMSHIKGHEGDPCLRSSDCIE---GECCARHF--
Hsadkk1 155 --TESF--GNDHSTLDGYSRRTTLSSKMYHTKGQEGSVCLRSSDCAS---GLCCARHF--
Hsadkk4 109 --GTHAEGTTGHPVQENQPKRKPSIKKSQGRKGQEGESCLRTFDCGP---GLCCARHF--
Hsadkk3 199 ----------ATRGSNGTICDNQRDCQP---GLCCAFQR--
Ocadkk 97 ----------KKPGTLGARCRAMGECGS---GLCCAKDS--
Ccadkk 113 ----------KKSGTEGAACKSEIDCNE---GLCCAEHY--
Scodkk 141 ----------VKPGRLGSRCKADRDCNG---DTCCARHW--
Hmadkk1.2.4 182 NRLH--SICKPMLEEHESCGPINLFHQEI-T-----TNAHVEPMCGPCKPGLSCQAVGIH
Hmadkk3 128 NPHI--SICKPPLAENMVCGPINFF-RNV-Y-----VGAQVQKACGPCKQALICKQVGLF
Nvedkk 145 --GE--WICKKMLRENEICTVPAG----------GLA-------
Dredkk1 182 --WS--KICKPVLKEGQVCTKHKR--K-G-T-----HGLEIFQRC-DCGEGLSCRTQRGD
Dredkk2 184 --WT--KICKPVLRQGEVCTKQRK--K-G-S-----HGLEIFQRC-DCAKGLACKVWKDA
Dredkk3 215 --ALLFPVCRPKPQEGQGCEREGN--QLMEVLLW--EDEGPREHC-PCAAGLLCQQIQKS
Dredkk3b 213 --ALLFPVCIAKPIERFRCIISAN--HLMELLSWDMDGEGPQEHC-PCAGELQCQHRGRG
Hsadkk2 200 --WT--KICKPVLHQGEVCTKQRK--K-G-S-----HGLEIFQRC-DCAKGLSCKVWKDA
Hsadkk1 206 --WS--KICKPVLKEGQVCTKHRR--K-G-S-----HGLEIFQRC-YCGEGLSCFIQKDH
Hsadkk4 162 --WT--KICKPVLLEGQVCSERGH--KDT-A-----QAPEIFQRC-DCGPGLLCRSQLTS
Hsadkk3 225 --GLLFPVCTPLPVEGELCHDPAS--RLLDLITWELEPDGALDRC-PCASGLLCQPHSHS
Ocadkk 123 --LSEVSACKKLGEIGDCCNESGF--RSG-F-----HPYGTPHYC-PCCAGLTCKRPTWR
Ccadkk 139 --GR--MICKRRGNMGQSCYEPDD----------DGKSVHSVC-PCSEGLKCETNMYR
Scodkk 167 --GQ--DVCKATLRLNDRCEPANG------G-----LMWSIHSHC-HCAAGLYCREQLEF
Hmadkk1.2.4 234 G-Q----------HNVCL----------
Hmadkk3 179 G-I----------HEICM----------
Nvedkk 168 ----------
Dredkk1 228 G-G---------KASRSIHTC----------
Dredkk2 230 T-S---------FSRSRLHVC----------
Dredkk3 268 S----------VCVDERH----------
Dredkk3b 268 A----------LCLKSQNSSEEE----------L-------
Hsadkk2 246 T-Y---------SSKARIHVC----------
Hsadkk1 252 HQA---------SNSSRIHTC----------
Hsadkk4 209 N-R---------QHARLRVCQ----------
Hsadkk3 280 L----------VYVCKPTFVGSRDQ----------DGEILL--
Ocadkk 172 SLF--------FSWTGGVSTCVKTEDGSTATPPLKERSPTTEEPTTEK-PTTDNSLKKLL
Ccadkk 182 YKYWF------IHYYYRRRQCVKAPTPTPTEPALTTEPAEPEEPQGSGILTTDPSVTFLT
Scodkk 211 YRLQIGWYTLTFPFSIRTSRCRRRNAM----------
Hmadkk1.2.4 241 ----------
Hmadkk3 186 ----------
Nvedkk 168 ----------
Dredkk1 239 ----------
Dredkk2 241 ----------
Dredkk3 276 ----------
Dredkk3b 282 ----------TDT----------L---
Hsadkk2 257 ----------
Hsadkk1 264 ----------
Hsadkk4 220 ----------
Hsadkk3 301 PR----------EVPDEYEVGSFMEEVRQELEDLERSLTEEMALR
Ocadkk 223 DREILVLKPIDGNSLDLGSGSGSGSETKESNEIEKAXHLRRINEKKE-----LQ----KK
Ccadkk 236 D----------APAEF-EQD----------
Scodkk 238 ----------S----------

```
Hmadkk1.2.4   241 ----------RVKDS
Hmadkk3       186 --------KEDDKKK
Nvedkk        168 ----------YSVNH
Dredkk1       239 ------------QRH
Dredkk2       241 ------------QRI
Dredkk3       276 -------ASGEGNED
Dredkk3b      286 -------YSEIDYIV
Hsadkk2       257 ------------QKI
Hsadkk1       264 ------------QRH
Hsadkk4       220 ----------KIEKL
Hsadkk3       336 EPAAAAAALLGGEEI
Ocadkk        274 RDKKREKTRKLEKPQ
Ccadkk        245 ----SLNRARKKKKN
Scodkk        239 ----QTTAIQPTPMF
```

B

```
>Petrosia_contig_29967
MMKGSNIFDIMLHQHPSTVHCKKPCKPGRYCDTYLGKCLKQLKEGSTCIMKDQCVDNLQCVWGKC
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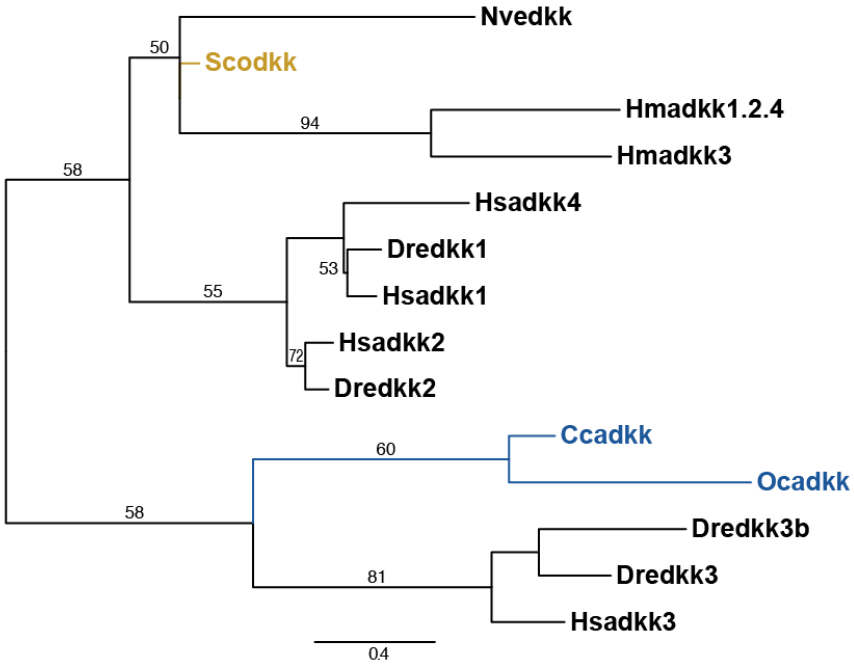
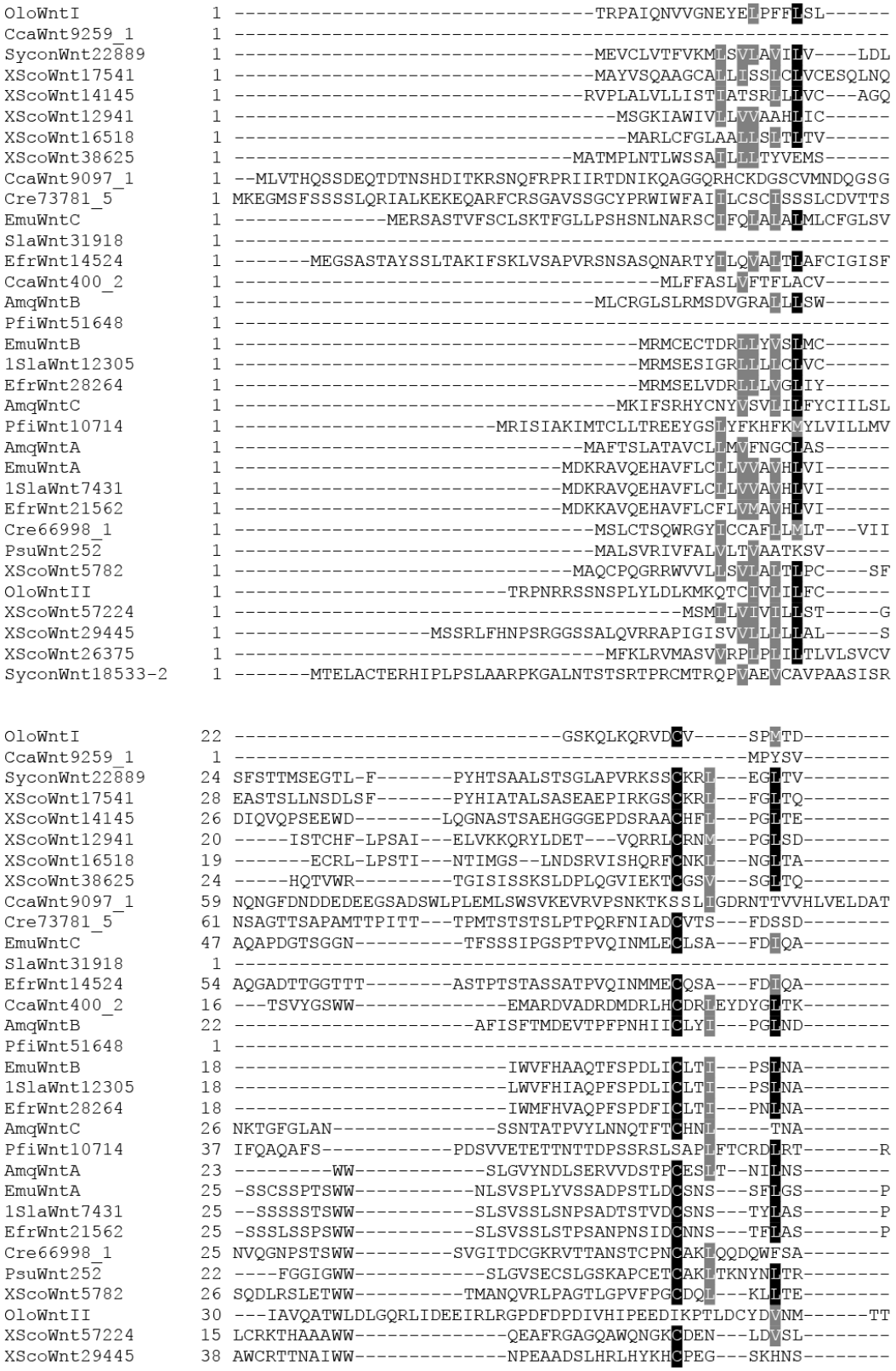
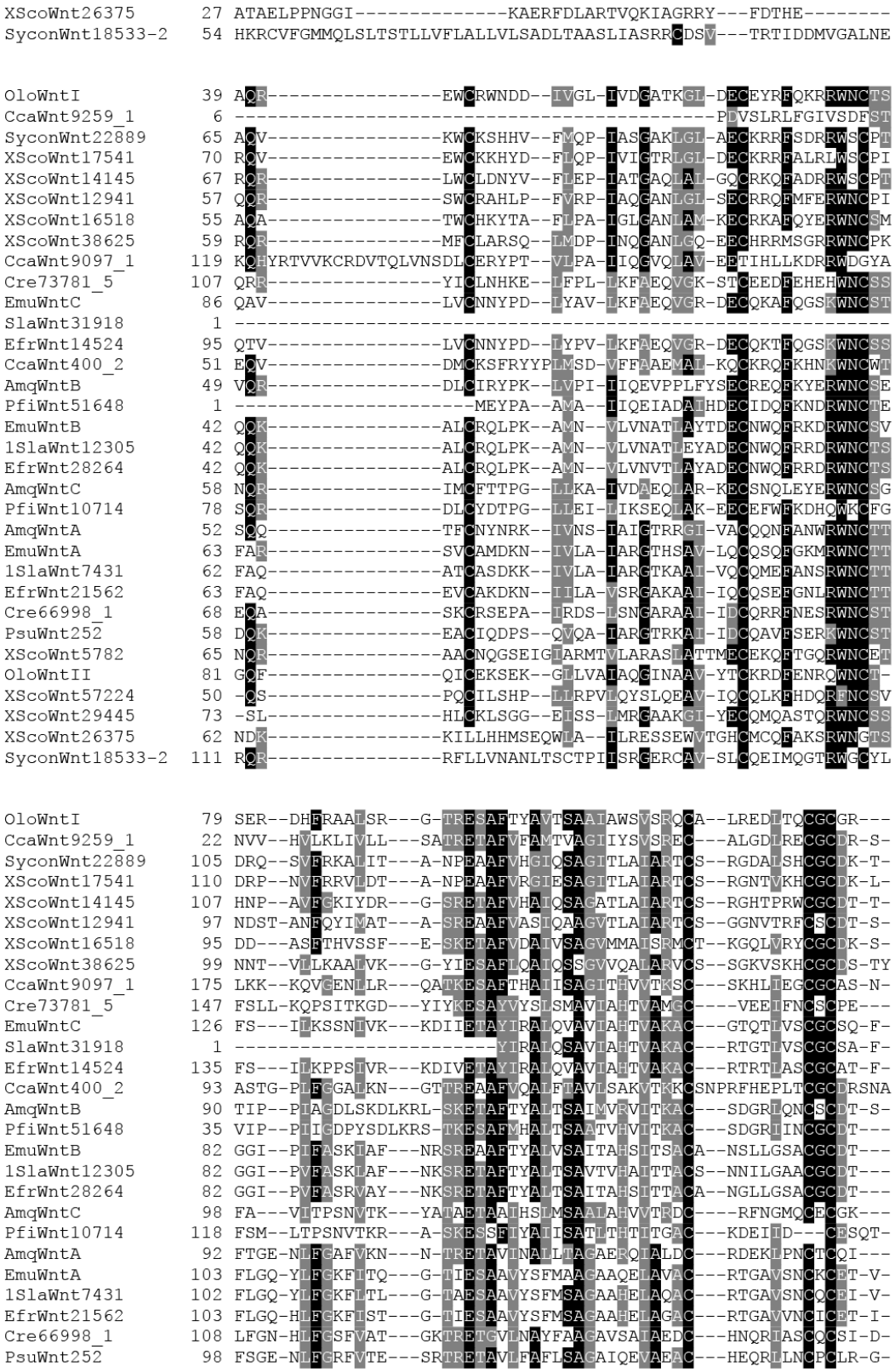
C



A2.6: ALIGNMENT OF FULL-LENGTH SPONGE WNTS

Muscle alignment of Wnt sequences from sponge transcriptomes, shading indicates amino acid similarity (gray) or identity in at least 50% of sequences.


OloWntI 1 ----------TRPAIQNVVGNEYELPFFLSL------
CcaWnt9259_1 1 ----------
SyconWnt22889 1 ----------MEVCLVTFVKMLSVLAVILV----LDL
XScoWnt17541 1 ----------MAYVSQAAGCALLISSLCLVCESQLNQ
XScoWnt14145 1 ----------RVPLALVLLISTLATSRLLLVC---AGQ
XScoWnt12941 1 ----------MSGKIAWIVLLVVAAHLIC------
XScoWnt16518 1 ----------MARLCFGLAALLSLTLTV------
XScoWnt38625 1 ----------MATMPLNTLWSSALLLLTYVEMS------
CcaWnt9097_1 1 --MLVTHQSSDEQTDTNSHDITKRSNQFRPRIIRTDNIKQAGGQRHCKDGSCVMNDQGSG
Cre73781_5 1 MKEGMSFSSSSLQRIALKEKEQARFCRSGAVSSGCYPRWIWFAILCSCISSSLCDVTTS
EmuWntC 1 ----------MERSASTVFSCLSKTFGLLPSHSNLNARSCIFQLALALMLCFGLSV
SlaWnt31918 1 ----------
EfrWnt14524 1 -------MEGSASTAYSSLTAKIFSKLVSAPVRSNSASQNARTYILQVALTLAFCIGISF
CcaWnt400_2 1 ----------MLFFASLVFTFLACV------
AmqWntB 1 ----------MLCRGLSLRMSDVGRALLLSW------
PfiWnt51648 1 ----------
EmuWntB 1 ----------MRMCECTDRLLYVSLMC------
1SlaWnt12305 1 ----------MRMSESIGRLLLLCLVC------
EfrWnt28264 1 ----------MRMSELVDRLLLVGLIY------
AmqWntC 1 ----------MKIFSRHYCNYVSVLILFYCIILSL
PfiWnt10714 1 ----------MRISIAKIMTCLLTREEYGSLYFKHFKVYLVILLMV
AmqWntA 1 ----------MAFTSLATAVCLLMVFNGCLAS------
EmuWntA 1 ----------MDKRAVQEHAVFLCLLVVAVHLVI------
1SlaWnt7431 1 ----------MDKRAVQEHAVFLCLLVVAVHLVI------
EfrWnt21562 1 ----------MDKKAVQEHAVFLCFLVMAVHLVI------
Cre66998_1 1 ----------MSLCTSQWRGYICCAFLLMLT---VII
PsuWnt252 1 ----------MALSVRIVFALVLTVAATKSV------
XScoWnt5782 1 ----------MAQCPQGRRWVVLLSVLALTLPC----SF
OloWntII 1 ----------TRPNRRSSNSPLYLDLKMKQTCIVLILFC------
XScoWnt57224 1 ----------MSMLLVIVILLIST-----G
XScoWnt29445 1 ----------MSSRLFHNPSRGGSSALQVRRAPIGISVVLLLLLAL-----S
XScoWnt26375 1 ----------MFKLRVMASVVRPLPLILTLVLSVCV
SyconWnt18533-2 1 -------MTELACTERHIPLPSLAARPKGALNTSTSRTPRCMTRQPVAEVCAVPAASISR
OloWntI 22 ----------GSKQLKQRVDCV-----SPMTD--------
CcaWnt9259_1 1 ----------MPYSV--------
SyconWnt22889 24 SFSTTMSEGTL-F-------PYHTSAALSTSGLAPVRKSSCKRL---EGLTV--------
XScoWnt17541 28 EASTSLLNSDLSF-------PYHIATALSASEAEPIRKGSCKRL---FGLTQ--------
XScoWnt14145 26 DIQVQPSEEWD--------LQGNASTSAEHGGGEPDSRAACHFL---PGLTE--------
XScoWnt12941 20 -----ISTCHF-LPSAI---ELVKKQRYLDET---VQRRLCRNM---PGLSD--------
XScoWnt16518 19 -------ECRL-LPSTI---NTIMGS--LNDSRVISHQRFCNKL---NGLTA--------
XScoWnt38625 24 -----HQTVWR---------TGISISSKSLDPLQGVIEKTCGSV---SGLTQ--------
CcaWnt9097_1 59 NQNGFDNDDEDEEGSADSWLPLEMLSWSVKEVRVPSNKTKSSLIGDRNTTVVHLVELDAT
Cre73781_5 61 NSAGTTSAPAMTTPITT---TPMTSTSTSLPTPQRFNIADCVTS---FDSSD--------
EmuWntC 47 AQAPDGTSGGN----------TFSSSIPGSPTPVQINMLECLSA---FDIQA--------
SlaWnt31918 1 ----------
EfrWnt14524 54 AQGADTTGGTTT--------ASTPTSTASSATPVQINMMECQSA---FDIQA--------
CcaWnt400_2 16 ---TSVYGSWW----------EMARDVADRDMDRLHCDRIEYDYGLTK--------
AmqWntB 22 ----------AFISFTMDEVTPFPNHIICLYI---PGLND--------
PfiWnt51648 1 ----------
EmuWntB 18 ----------IWVFHAAQTFSPDLICLTI---PSLNA--------
1SlaWnt12305 18 ----------LWVFHIAQPFSPDLICLTI---PSLNA--------
EfrWnt28264 18 ----------IWMFHVAQPFSPDFICLTI---PNLNA--------
AmqWntC 26 NKTGFGLAN----------SSNTATPVYLNNQTFTCHNL-----TNA--------
PfiWnt10714 37 IFQAQAFS----------PDSVVETETTNTTDPSSRSLSAPLFTCRDLRT-------R
AmqWntA 23 ---------WW----------SLGVYNDLSERVVDSTPCESLT--NILNS--------
EmuWntA 25 -SSCSSPTSWW----------NLSVSPLYVSSADPSTLDCSNS---SFLGS-------P
1SlaWnt7431 25 --SSSSSTSWW----------SLSVSSLSNPSADTSTVDCSNS---TYLAS-------P
EfrWnt21562 25 -SSSLSSPSWW----------SLSVSSLSTPSANPNSIDCNNS---TFLAS-------P
Cre66998_1 25 NVQGNPSTSWW---------SVGITDCGKRVTTANSTCPNCAKLQQDQWFSA--------
PsuWnt252 22 ----FGGIGWW----------SLGVSECSLGSKAPCETCAKLTKNYNLTR---------
XScoWnt5782 26 SQDLRSLETWW----------TMANQVRLPAGTLGPVFPGCDQL---KLLTE--------
OloWntII 30 ---IAVQATWLDLGQRLIDEEIRLRGPDFDPDIVHIPEEDIKPTLDCYDVNM------TT
XScoWnt57224 15 LCRKTHAAAWW----------QEAFRGAGQAWQNGKCDEN---LDVSL--------
XScoWnt29445 38 AWCRTTNAIWW----------NPEAADSLHRLHYKHCPEG---SKHNS--------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OloWntI 39 AQR----------EWCRWNDD--IVGL-IVDGATKGI-DECEYRFQKRRWNCTS
CcaWnt9259_1 6 ----------PLVSLRLFGIVSDFST
SyconWnt22889 65 AQV----------KWCKSHHV--FMQP-IASGAKLGI-AECKRRFSDRRWSCPT
XScoWnt17541 70 RQV----------EWCKKHYD--FLQP-IVIGTRLGL-DECKRRFALRLWSCPI
XScoWnt14145 67 RQR----------LWCLDNYV--FLEP-IATGAQLAI-GQCRKQFADRRWSCPT
XScoWnt12941 57 QQR----------SWCRAHLP--FVRP-IAQGANLGI-SECRRQFMFERWNCPI
XScoWnt16518 55 AQA----------TWCHKYTA--FLPA-IGLGANLAM-KECRKAFQYERWNCSM
XScoWnt38625 59 RQR----------MFCLARSQ--LMDP-INQGANLGQ-EECHRRMSGRRWNCPK
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Cre73781_5 107 QRR----------YICLNHKE--IFPL-LKFAEQVCK-STCEEDFEHEHWNCSS
EmuWntC 86 QAV----------LVCNNYPD--LYAV-LKFAEQVGR-DECQKAFQGSKWNCST
SlaWnt31918 1 ----------
EfrWnt14524 95 QTV----------LVCNNYPD--LYPV-LKFAEQVGR-DECQKTFQGSKWNCSS
CcaWnt400_2 51 EQV----------DMCKSFRYYPLMSD-VFFAAEMAI-KQCKRQFKHNKWNCWT
AmqWntB 49 VQR----------DLCIRYPK--LVPI-IIQEVPPLFYSECREQFKYERWNCSE
PfiWnt51648 1 ----------MEYPA--AMA--IIQEIADAIHDECIDQFKNDRWNCTE
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1SlaWnt12305 42 QQK----------ALCRQLPK--AMN--VLVNATLEYADECNWQFRRDRWNCTS
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XScoWnt26375 62 NDK----------KILLHHMSEQWLA--ILRESSEWVTGHCMCQFAKSRWNGTS
SyconWnt18533-2 111 RQR----------RFLLVNANLTSCTPIISRGERCAV-SLCQEIMQGTRWGCYL
OloWntI 79 SER--DHFRAALSR---G-TRESAFTYAVTSAAIAWSVSRQCA--LREDLTQCGCGR---
CcaWnt9259_1 22 NVV--HVLKLIVLL---SATRETAFVFAMTVAGIIYSVSREC---ALGDLRECGCDR-S-
SyconWnt22889 105 DRQ--SVFRKALIT---A-NPEAAFVHGIQSAGITLAIARTCS--RGDALSHCGCDK-T-
XScoWnt17541 110 DRP--NVFRRVLDT---A-NPEAAFVRGIESAGITLAIARTCS--RGNTVKHCGCDK-L-
XScoWnt14145 107 HNP--AVFGKIYDR---G-SRETAFVHAIQSAGATLAIARTCS--RGHTPRWCGCDT-T-
XScoWnt12941 97 NDST-ANFQYIMAT---A-SREAAFVASIQAAGVTLAIARTCS--GGNVTRFCSCDT-S-
XScoWnt16518 95 DD---ASFTHVSSF---E-SKETAFVDAIVSAGVMMAISRMCT--KGQLVRYCGCDK-S-
XScoWnt38625 99 NNT--VLLKAALVK---G-YIESAFLQAIQSSGVVQALARVCS--SGKVSKHCGCDS-TY
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SlaWnt31918 1 ----------YIRALQSAVIAHTVAKAC---RTGTLVSCGCSA-F-
EfrWnt14524 135 FS---ILKPPSIVR---KDIVETAYIRALQVAVIAHTVAKAC---RTRTLASCGCAT-F-
CcaWnt400_2 93 ASTG-PLFGGALKN---GTTREAAFVQAIFTAVLSAKVTKKCSNPRFHEPLTCGCDRSNA
AmqWntB 90 TIP--PIAGDLSKDLKRL-SKETAFTYALTSAIMVRVITKAC---SDGRLQNCSCDT-S-
PfiWnt51648 35 VIP--PIIGDPYSDLKRS-TKESAFMHALTSAATVHVITKAC---SDGRIINCGCDT---
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1SlaWnt12305 82 GGI--PVFASKLAF---NKSRETAFTYALTSAVTVHAITTACS--NNILGAACGCDT---
EfrWnt28264 82 GGI--PVFASRVAY---NKSRETAFTYALTSAITAHSITTACA--NGLLGSACGCDT---
AmqWntC 98 FA---VITPSNVTK---YATAETAAIHSLMSAALAHVVTRDC----RFNGMQCECGK---
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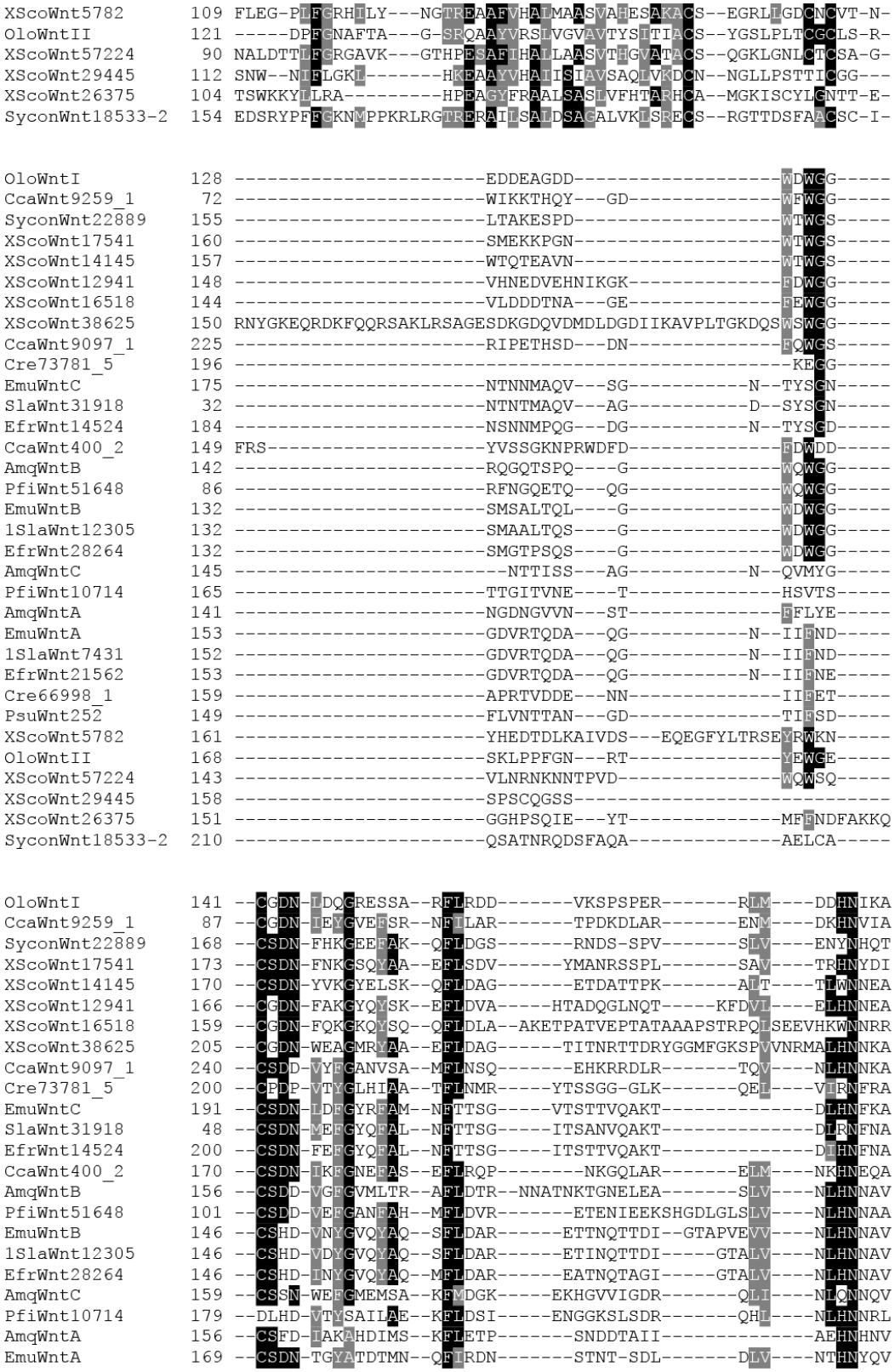
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XScoWnt29445 112 SNW--NIFLGKL-------HKEAAYVHAIISIAVSAQLVKDCN--NGLLPSTTICGG---
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SyconWnt18533-2 154 EDSRYPFFGKNMPPKRLRGTRERAILSALDSAGALVKLSRECS--RGTTDSFAACSC-I-
OloWntI 128 ----------EDDEAGDD----------WDWGG-----
CcaWnt9259_1 72 ----------WIKKTHQY---GD----------WFWGG-----
SyconWnt22889 155 ----------LTAKESPD----------WTWGS-----
XScoWnt17541 160 ----------SMEKKPGN----------WTWGS-----
XScoWnt14145 157 ----------WTQTEAVN----------WTWGS-----
XScoWnt12941 148 ----------VHNEDVEHNIKGK----------FDWGG-----
XScoWnt16518 144 ----------VLDDDTNA---GE----------FEWGG-----
XScoWnt38625 150 RNYGKEQRDKFQQRSAKLRSAGESDKGDQVDMDLDGDIIKAVPLTGKDQSWSWGG-----
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EmuWntC 175 ----------NTNNMAQV---SG----------N--TYSGN-----
SlaWnt31918 32 ----------NTNTMAQV---AG----------D--SYSGN-----
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PfiWnt51648 86 ----------RFNGQETQ---QG----------WQWGG-----
EmuWntB 132 ----------SMSALTQL----G----------WDWGG-----
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EfrWnt28264 132 ----------SMGTPSQS----G----------WDWGG-----
AmqWntC 145 ----------NTTISS---AG----------N--QVMYG-----
PfiWnt10714 165 ----------TTGITVNE----T----------HSVTS-----
AmqWntA 141 ----------NGDNGVVN---ST----------FFLYE-----
EmuWntA 153 ----------GDVRTQDA---QG----------N--IIFND-----
1SlaWnt7431 152 ----------GDVRTQDA---QG----------N--IIFND-----
EfrWnt21562 153 ----------GDVRTQDA---QG----------N--IIFNE-----
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PsuWnt252 149 ----------FLVNTTAN---GD----------TIFSD-----
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OloWntII 168 ----------SKLPPFGN---RT----------YEWGE-----
XScoWnt57224 143 ----------VLNRNKNNTPVD----------WQWSQ-----
XScoWnt29445 158 ----------SPSCQGSS----------
XScoWnt26375 151 ----------GGHPSQIE---YT----------MFFNDFAKKQ
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CcaWnt9259_1 87 --CGDN-IEYGVEFSR--NFTLAR-------TPDKDLAR-------ENM----DKHNVIA
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XScoWnt17541 173 --CSDN-FNKGSQYAA--EFLSDV------YMANRSSPL-------SAV----TRHNYDI
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SlaWnt31918 48 --CSDN-VEFGYQFAL--NFTTSG-----ITSANVQAKT----------DIRNFNA
EfrWnt14524 200 --CSDN-FEFGYQFAL--NFTTSG-----ITSTTVQAKT----------DIHNFNA
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AmqWntB 156 --CSDD-VGFGVMLTR--AFLDTR--NNATNKTGNELEA-------SLV----NLHNNAV
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1SlaWnt12305 146 --CSHD-VDYGVQYAQ--SFLDAR------ETINQTTDI-----GTALV----NLHNNAV
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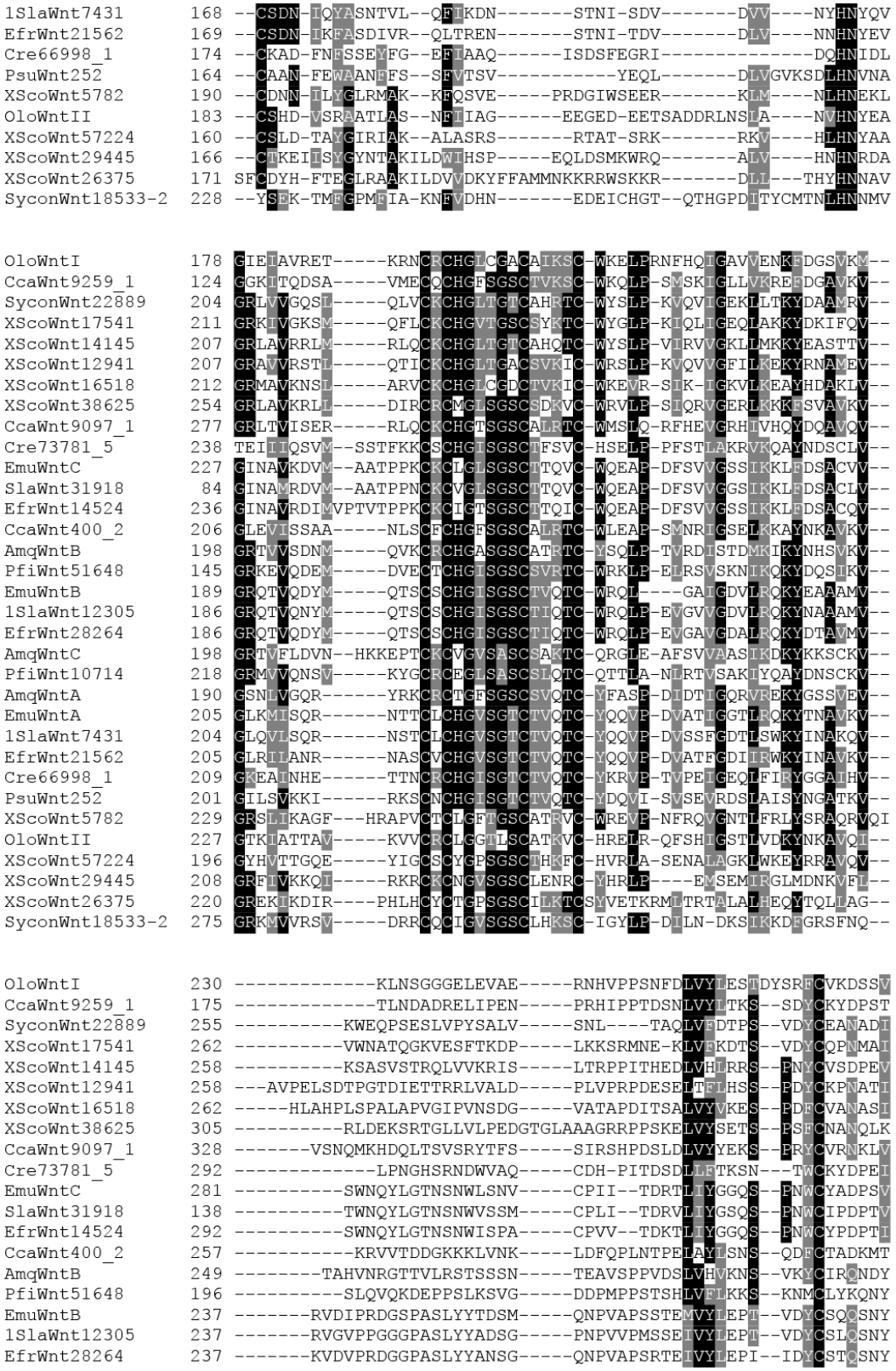
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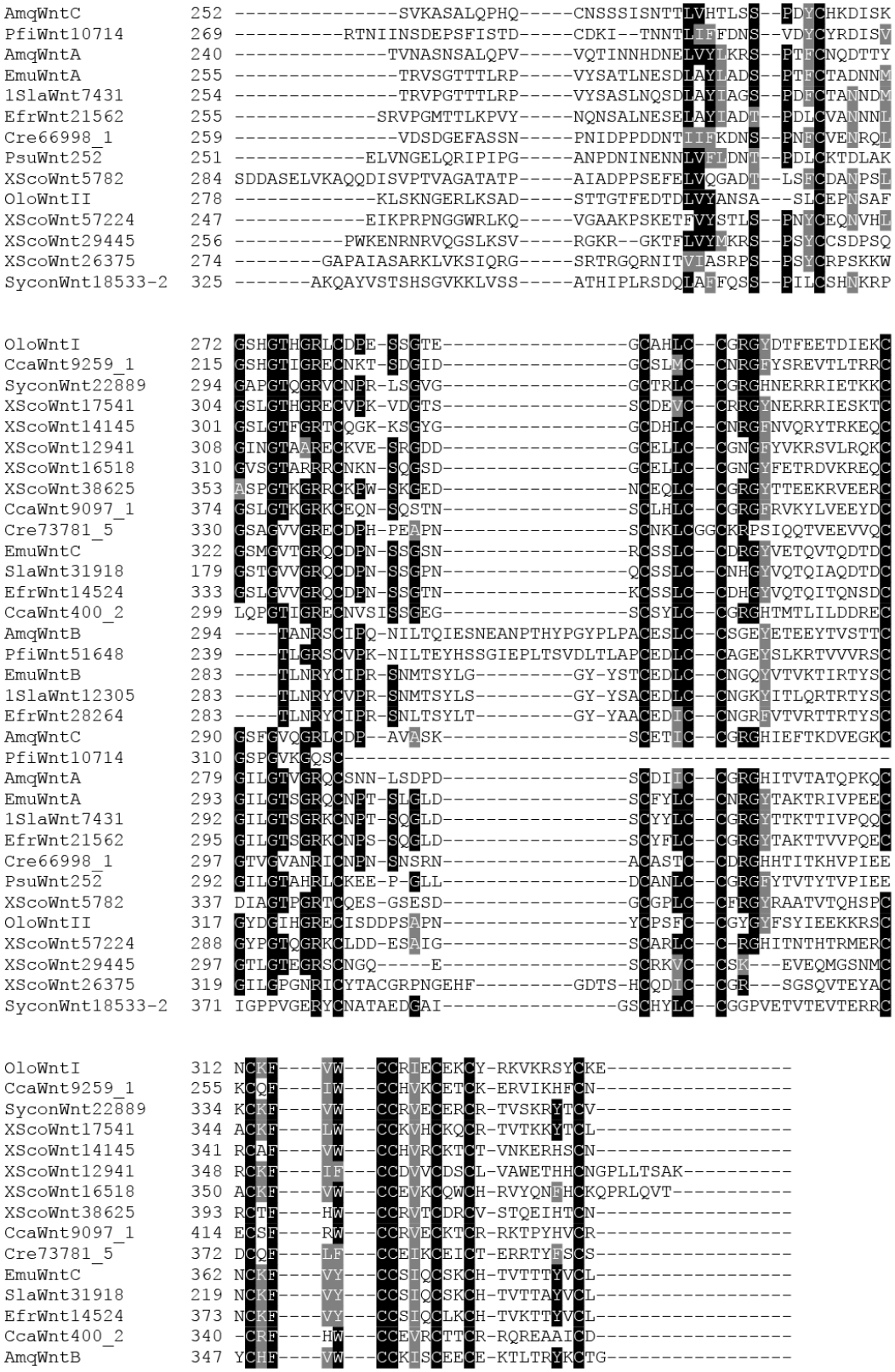
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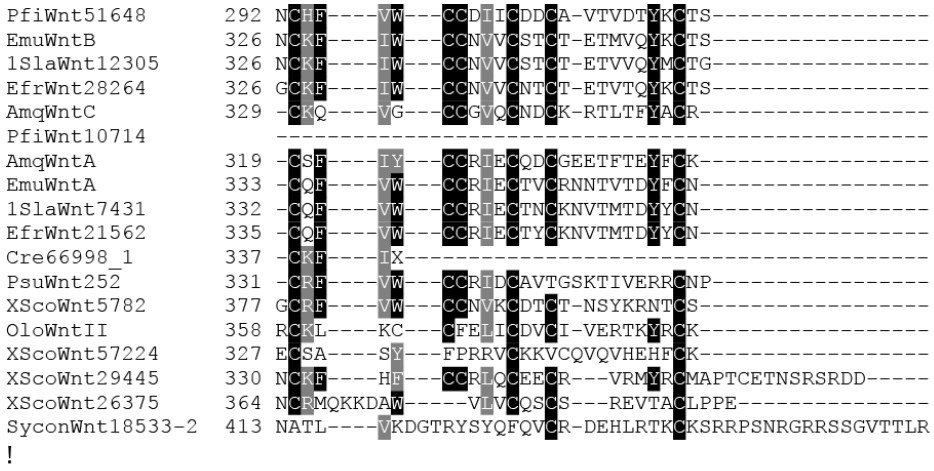
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XScoWnt5782 377 GCRF----VW---CCNVKCDTCT-NSYKRNTCS----------
OloWntII 358 RCKL----KC---CFELICDVCI-VERTKYRCK----------
XScoWnt57224 327 ECSA----SY---FPRRVCKKVCQVQVHEHFCK----------
XScoWnt29445 330 NCKF----HF---CCRIQCEECR---VRMYRCMAPTCETNSRSRDD-----
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SyconWnt18533-2 413 NATL----VKDGTRYSYQFQVCR-DEHLRTKCKSRRPSNRGRRSSGVTTLR
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A2.7: ALIGNMENT AND RAW TREES USED FOR GENERATING WNT PHYLOGENETIC TREE

(A) MEGA trimmed alignment used for the phylogenetic tree shown in Fig. 3-1B, including vertebrate and sponge Wnts. (B) PhyloBayes tree after 1,200,000 generations. Support values are posterior probabilities. (C) RAxML tree with 500 bootstrap replicates.

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OloWntI          T----------NVQLKRVDCVSMTDAQREWCRWNDDIVGLIVDGATKGLDECEYRFQKRR
CcaWntI          M----------VP---VSLRLFIVS-------------------------------DFS
DreWnt10b        MIVTALLSPATLKPNAVCLRLALTKKQMRLCVRSPDVTASALQGIQVAIHECQHQLRDQR
HsaWnt10b        MLLFALCSRA-LKANTVCLTLSLSKRQLGLCLRNPDVTASALQGLHIAVHECQHQLRDQR
DreWnt10a        MFLFLFCSLAALKANTVCLTLPLTKKQLDVCMRNPDVTASAIQGIQIAIHECQHQFRGHR
HsaWnt10a        M-LWLLFFLLLLRANTVCLTLPLSRRQMEVCVRHPDVAASAIQGIQIAIHECQHQFRDQR
DreWnt4a         MRSLMLFLALSLADEETCEKLRLIQRQVQICKRNVEVMDAVRRGAQLAIDECQYQFRNRR
HsaWnt4          MRSLLLVFAVSLAEEETCEKLKLIQRQVQMCKRNLEVMDSVRRGAQLAIEECQYQFRNRR
DreWnt4b         MRLLLLLWAALLAGAEPCGRLRLSPGQVGVCRARGEVMESVRKASEMVIEECQHQFRNRR
DreWnt5a         MIITCVFFMLILAGQPLCSQLSLSKGQKKLCQLYQDHMQYIGEGAKTGIRECQHQFRHRR
DreWnt5b         MAVTIVCNSQLLAAQPLCSQLTLSQGQRKLCQLYQDHMVYIGEGAKTGIKECQYQFRQRR
HsaWnt5b         MTAALSSWAQLLAAQPVCSQLPLSPGQRKLCQLYQEHMAYIGEGAKTGIKECQHQFRQRR
HsaWnt5a         MALAFFSFAQVLGAQPLCSQLALSQGQKKLCHLYQDHMQYIGEGAKTGIKECQYQFRHRR
DreWnt7a         MRKTRWIFHILIGASIICNKIPLAPRQRTICQSRPDAIIVIGEGAQMGINECQFQFKNGR
DreWnt7alike     MRKTRWMFHILMGASIICNKIPLAPRQRIICQSRPDAIIVIGEGAQMGINECQFQFKHGR
HsaWnt7a         MRKARCLGHLLIGASIICNKIPLAPRQRAICQSRPDAIIVIGEGSQMGLDECQFQFRNGR
HsaWnt7b         MRNFKWIFYVLLGANIICNKIPLAPRQRAICQSRPDAIIVIGEGAQMGINECQYQFRFGR
DreWnt7b         MRSISCGALLVLTANIICNKIPLAPRQRAICQSRPDAIIIGEGAQLGINECQYQFRYGR
DreWnt3a         MFLFFCGLTRMLATQPMCSSIPLVPKQLRFCRNYVEIMPSVAEGVKIGIQECQHQFRGRR
HsaWnt3a         MFL-LCSLKQLLASQPLCASIPLVPKQLRFCRNYVEIMPSVAEGIKIGIQECQHQFRGRR
DreWnt3          MLMCWFSSSRLLASQPLCGSIPLVPKQLRFCRNYIEIMPSVAEGVKLGIQECQHQFRGRR
HsaWnt3          MLLGLLGGTRLLASQPLCGSIPLVPKQLRFCRNYIEIMPSVAEGVKLGIQECQHQFRGRR
DreWnt2          MFYLVAICWFSMGSQVMCDNIPLINKQRQLCRQHPKVMQAIGAGIKNWIGECQHQFRTHR
DreWnt2b         MCALFLLLILPIGARVICDNIPLVNKQRQLCQKYPDIMQSIGGGAKEWIRECQYQFRHHR
DreWnt2.1        MLLFLLLLMFPIGARVICDNIPLVNKQRQLCQRHPDLMQSIGQGAKEWIRECQHQFRHHR
HsaWnt2b2        MCLLLLLLTLAIGARVICDNIPLVSRQRQLCQRYPDIMRSVGEGAREWIRECQHQFRHHR
HsaWnt2b1        MCLQ-------IGARVICDNIPLVSRQRQLCQRYPDIMRSVGEGAREWIRECQHQFRHHR
HsaWnt2          MLWLLLLTWLPMRSRVMCDNVPLVSSQRQLCHRHPDVMRAISQGVAEWTAECQHQFRQHR
DreWnt1          MLLVSLTGTGVIVVQLLDPSLALSRRQRKLIRQNPGILHAIAAGLHTAIKECKWQFRNRR
HsaWnt1          MLALALPAALAIVLQLLEPSLQLSRKQRRLIRQNPGILHSVSGGLQSAVRECKWQFRNRR
DreWnt16         MHLFIWLSVYLLGEKLGCAHLPLSHKQKELCARKPHLLPSVKEGARLGITECQTQFRHER
HsaWnt16.1       -ALWALLVLFYLGEKLGCANLPLNSRQKELCKRKPYLLPSIREGARLGIQECGSQFRHER
HsaWnt16.2       MSLW-------LGEKLGCANLPLNSRQKELCKRKPYLLPSIREGARLGIQECGSQFRHER
DreWnt6          MACYPSHISYTVGPNSICRKTKLAGKQAELCQTQPEIVNEVAKGAKLGVRECQYQFRFRR
HsaWnt6          MLLLPAHVG--VGPTSICRKARLAGRQAELCQAEPEVVAELARGARLGVRECQFQFRFRR
DreWnt6like      V----------NGPNSICRKTRLAGRHTDLCQSQPEIIQEVAKGARLGIRECQHQFHNHR
DreWnt11         MLCLTFLLLSQLSKTQHCKTLPLVSSQAQLCRSNLELMQTIIQAAREVKKVCQKTFTDMR
HsaWnt11         MALLALALQTVLSQTQHCKQLELVSAQVQLCRSNLELMHTVVHAAREVMKACRRAFADMR
DreWnt11.1       MLLFTSLSVIPLTQTHHCKLLDLVPDQQQLCKRNLELMHSIVRAARLTKSACTSSFSDMR
ScoWnt-i         VLSVAVILVLDFPRKSSCKRLELTVAQVKWCKSHHVFMQPIASGAKLGLAECKRRFSDRR
ScoWnt-ii        YLLISLCLVCEFPRKGSCKRLFLTQRQVEWCKKHYDFLQPIVIGTRLGLDECKRRFALRL
ScoWnt-iii       LIATRLLLVCGLQSRAACHFLPLTERQRLWCLDNYVFLEPIATGAQLALGQCRKQFADRR
ScoWnt-iv        MLLVAAHLIC-LPQRRLCRNMPLSDQQRSWCRAHLPFVRPIAQGANLGLSECRRQFMFER
ScoWnt-v         MGLALLSLTL-LPHQRFCNKLNLTAAQATWCHKYTAFLPAIGLGANLAMKECRKAFQYER
ScoWnt-vi        MSSALLLTYVMISIEKTCGSVSLTQRQRMFCLARSQLMDPINQGANLGQEECHRRMSGRR
DreWnt9b         MCPLLIALCILLTAHLQCEQMTLTRRQKRLCRREPGLAETLRESVRLSLLECRYQFRNER
HsaWnt9b         MLALGLCLLAPLTAHLQCDLLKLSRRQKQLCRREPGLAETLRDAAHLGLLECQFQFRHER
DreWnt9a         MFTIIVHLISPLTSHYLCDRLKLEKKQRRMC-----------------------------
HsaWnt9a         MAAFLTLLLALLTAHYACDRLKLERKQRRMCRRDPGVAETLVEAVSMSALECQFQFRFER
DreWnt8a         MFASVMSICCI-----------VNNFLMTGPKAYLAYTSSVQAGAQSGIEECKHQFAWDR
DreWnt8like      MWAFFPIWDKI-----------MNNLLITGPKAYLTYANSVRVGAQSGIHECKHQFAWDR
HsaWnt8a         MWA-ALGICCA-----------VNNFLITGPKAYLTYTTSVALGAQSGIEECKFQFAWER
DreWnt8b         MVYYAFILMAM-----------VNNFLMTGPKAYLIYSSSVAAGAQSGIEECKYQFAWDR
HsaWnt8b         MPSVICLFTCL-----------VNNFLMTGPKAYLIYSSSVAAGAQSGIEECKYQFAWDR
AmqWntB          -RGLLRMSDVRSWNHIICLYIPLNDVQRDLCIRYPKLVPIIQEVPPLFYSECREQFKYER
PfiWntB          -----------------------------MEYPAAMAIIQEIADAIHDECIDQFKNDR
EmuWntB          ----MRMCECDVSPDLICLTIPLNAQQKALCRQLPKAMNVLVNATLAYTDECNWQFRKDR
SlaWntB          ----MRMSESGLCPDLICLTIPLNAQQKALCRQLPKAMNVLVNATLEYADECNWQFRRDR
EfrWntB          ----MRMSELDVGPDFICLTIPLNAQQKALCRQLPKAMNVLVNVTLAYADECNWQFRRDR
CcaWnt9097_1     MLPLMLSWSVEDRRTVKCRDVTLVN--SDLCERYPTVLPAIIQGVQLAVEETIHLLKDRR
Cre73781_5       MFAILCSCISSMTFNIDCVTSFSSDQRRYICLNHKELFPLLKFAEQVGKSTCEEDFEHEH
EmuWntC          MLALLMLCFGSDGINMECLSAFIQAQAVLVCNNYPDLYAVLKFAEQVGRDECQKAFQGSK
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SlaWnt31918     ------------------------------------------------------------
EfrWnt14524     MVALLAFCIGSDTINMECQSAFIQAQTVLVCNNYPDLYPVLKFAEQVGRDECQKTFQGSK
CcaWnt400_2     MASLFTFLACTMAMDRHCDRLELTKEQVDMCKRYYPLMSDVFFAAEMALKQCKRQFKHNK
AmqWntA         ML----MVFNCLG--SPCESLTINSSQQTFCNYNRKIVNSIAIGTRRGIVACQQNFANWR
EmuWntA         MLVVVHLVISCLSPSTDCSNSSLSPFARSVCAMDKNIVLAIARGTHSAVLQCQSQFGKMR
1SlaWnt7431     MLVVVHLVISSLSTSTDCSNSTLSPFAQATCASDKKIVLAIARGTKAAIVQCQMEFANSR
EfrWnt21562     MLVMVHLVISSLSPNSDCNNSTLSPFAQEVCAKDKNIILAVSRGAKAAIIQCQSEFGNLR
Cre66998_1      MLLMTVIINVGVG-ANTCPNCALSAEQASKCRSEPAIRDSLSNGARAAIIDCQRRFNESR
PsuWnt252       MALVTVAATKVLG-SKPCETCALTRDQKEACIQDPSQVQAIARGTRKAIIDCQAVFSERK
AmqWntC         MVSVILFYCILLANQTTCHNLT-NANQRIMCFTTPGLLKAIVDAEQLARKECSNQLEYER
PfiWnt10714     MLVILMVIFQQETAPLTCRDLR-TRSQRDLCYDTPGLLEILIKSEQLAKEECEFWFKDHQ
XScoWnt5782     MALTPCSFSQLMAPVFGCDQLKLTENQRAACNQGSEIMTVLARASLATTMECEKQFTGQR
XScoWnt57224    MIVILSTGLCKEAWQNKCDENLVSLQSPQCILSHPLLRPVLQYSLQEAVIQCQLKFHDQR
OloWntII        TKQTIVLILFILGKPTDCYDVNMTTGQFQICESEKGLLVAIAQGINAAVYTCKRDFENRQ
XScoWnt29445    MLLLLALSAWRPELHYHCPEGS-HNSSLHLCKLSGGEISSLMRGAAKGIYECQMQASTQR
SyconWnt18533   MPVAVCAVPASSTVTRIDDMVGLNERQRRFLLVNANLTPIISRGERCAVSLCQEIMQGTR
XScoWnt26375    MPLITLVLSVVLAAGRYFDTHE-NDKKILLHHMSEQWLAILRESSEWVTGHCMCQFAKSR


OloWntI         WNCTSSRHFRAALSRGTRESAFTYAVTSAAIAWSVSRQCALRELTQCGCGREDD-GDDWD
CcaWnt9259_1     TNVVHV-LKLIVLLSATRETAFVFAMTVAGIIYSVSRECALGDLRECGCDRSWIKYGDWF
DreWnt10b       WNCSSLEHQSAILNRGFRESAFSLSLLAAGVVHSVASACSLGKLRGCGCEAKRRLQDTWE
HsaWnt10b       WNCSALEHHSAILKRGFRESAFSFSMLAAGVMHAVATACSLGKLVSCGCGWKGSGQDTWE
DreWnt10a       WNCSSLEYESVVFSRGFRESAFAYAIAAAGVVHAVSNACAMGKLKACGCDEKRRGQDSWE
HsaWnt10a       WNCSSLEYESPIFSRGFRESAFAYAIAAAGVVHAVSNACALGKLKACGCDASRRGQDSWE
DreWnt4a        WNCSTLEVFGKVVTQGTREAAFVYAISAASVAFAVTRACSSGELDKCGCDRNVHGPEGFQ
HsaWnt4         WNCSTLDVFGKVVTQGTREAAFVYAISSAGVAFAVTRACSSGELEKCGCDRTVHGPQGFQ
DreWnt4b        WNCSTTRVFGRVMNQGTREAAFVHALSSAAVAVAVTRGCSRGELERCGCDRKVRGPEGFQ
DreWnt5a        WNCSTVDVLGRVMHIGSRESAFAFAISAAGVLHAVSRACREGALSSCGCSRASRPPRDWL
DreWnt5b        WNCSTVDVFGRVMHIGSRETAFTYAVSAAGVVNAVSRACREGELSTCGCSRAARPPRDWL
HsaWnt5b        WNCSTADVFGRVMQIGSRETAFTHAVSAAGVVNAISRACREGELSTCGCSRTARPPRDWL
HsaWnt5a        WNCSTVDVFGRVMQIGSRETAFTYAVSAAGVVNAMSRACREGELSTCGCSRAARPPRDWL
DreWnt7a        WNCSALGVFGKELKVGSKEAAFTYAIIAAGVAHAITAACTQGTLSGCGCDKEKQGEEGWK
DreWnt7alike    WNCSALGVFGKELKVGSKEAAFMYAIIAAGVAHAITSACTRGNLSECSCDKDKQGDNGWK
HsaWnt7a        WNCSALGVFGKELKVGSREAAFTYAIIAAGVAHAITAACTQGNLSDCGCDKEKQGDEGWK
HsaWnt7b        WNCSALGVFGQELRVGSREAAFTYAITAAGVAHAVTAACSQGNLSNCGCDREKQGAEGWK
DreWnt7b        WNCSALGVFGQELRVGSKEAAFTYAITAAGVAHAVTAACSQGNLSHCGCDREKQGEEGWK
DreWnt3a        WNCTTIDIFGPVLDKATRESAFVHAIASAGVAFXVTRACTEGSATICGCDSRRKGGEGWK
HsaWnt3a        WNCTTVDIFGPVLDKATRESAFVHAIASAGVAFAVTRSCAEGTAAICGCSSRHQGGKGWK
DreWnt3         WNCTTIDIFGPVLDKATRESAFVHAIASAGVAFAVTRSCAEGTSTMCGCDSHHKGGEGWK
HsaWnt3         WNCTTIDIFGPVLDKATRESAFVHAIASAGVAFAVTRSCAEGTSTICGCDSHHKGGEGWK
DreWnt2         WNCNTMRLFGRLLHRSSREAAFVYAISSAGMVYTLTRACSQGELENCSCDPGKKGKGAFD
DreWnt2b        WNCSALRVFGRVIQRSSREAAFVYAISSAGVVFAITRACSQGELKACNCDPQKRGRGEFD
DreWnt2.1       WNCSTLRVFGRVMLRSSREAAFVYAISSAGVVYAITRACSQGELKICSCDSQRRGDGDFD
HsaWnt2b2       WNCTTLRVFGRVMLRSSREAAFVYAISSAGVVHAITRACSQGELSVCSCDPYTRGRGDFD
HsaWnt2b1       WNCTTLRVFGRVMLRSSREAAFVYAISSAGVVHAITRACSQGELSVCSCDPYTRGRGDFD
HsaWnt2         WNCNTLRLFGRVLLRSSRESAFVYAISSAGVVFAITRACSQGEVKSCSCDPKKMGKGIFD
DreWnt1         WNCPTTHVFGKIVNRGCRETAFVFAITSAGVTHAVARSCSEGAIESCTCDYRRRGGPDWH
HsaWnt1         WNCPTAPLFGKIVNRGCRETAFIFAITSAGVTHSVARSCSEGSIESCTCDYRRRGGPDWH
DreWnt16        WNCSTRRVFGYELTSGTKETAFIHAVMAAGLVHAVTRSCSAGNMTECSCDTSLLGTEGWH
HsaWnt16.1      WNCMITALFGYELSSGTKETAFIYAVMAAGLVHSVTRSCSAGNMTECSCDTTLQNSEGWH
HsaWnt16.2      WNCMITALFGYELSSGTKETAFIYAVMAAGLVHSVTRSCSAGNMTECSCDTTLQNSEGWH
DreWnt6         WNCTSQKYFGKILQQDIRETAFVYAITAAGVTHAVTQACSMGELLQCGCEATRSRGVKWE
HsaWnt6         WNCSSHKAFGRILQQDIRETAFVFAITAAGASHAVTQACSMGELLQCGCQAPRGRSAAWE
DreWnt6like     WNCTSQRNLAKILQQDIRETAFVYAVTAAGVMHAVTQACSQGALPQCGCVTLQSSDWHWE
DreWnt11        WNCSSIDKFLPDLERGTRESAFVYALSAAAISHTIARACTSGDLRLCSCGPIPGEEPGYR
HsaWnt11        WNCSSIENYLLDLERGTRESAFVYALSAAAISHAIARACTSGDLPGCSCGPVPGEGPGNR
DreWnt11.1      WNCSSIEHFTPDLAKGTREAAFVFSLAAAVVSHAIARACASGDLPSCSCAAMPSEAPDFR
SyconWnt22889   WSCPTDRVFRKALITANPEAAFVHGIQSAGITLAIARTCSRGDLSHCGCDKTLTASPDWT
XScoWnt17541    WSCPIDRVFRRVLDTANPEAAFVRGIESAGITLAIARTCSRGNVKHCGCDKLSMEPGNWT
XScoWnt14145    WSCPTHNVFGKIYDRGSRETAFVHAIQSAGATLAIARTCSRGHPRWCGCDTTWTQAVNWT
XScoWnt12941    WNCPINDNFQYIMATASREAAFVASIQAAGVTLAIARTCSGGNTRFCSCDTSVHNKGKFD
XScoWnt16518    WNCSMDDSFTHVSSFESKETAFVDAIVSAGVMMAISRMCTKGQVRYCGCDKSVLDAGEFE
XScoWnt38625    WNCPK-NLLKAALVKGYIESAFLQAIQSSGVVQALARVCSSGKSKHCGCDSTYRNDQSWS
DreWnt9b        WNCSMD--RGSLLKRGFKETAFLLAVSSAALSHALAKACSSGRMERCTCDDSPGLREAWQ
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HsaWnt9b        WNCSLE--RMGLLKRGFKETAFLYAVSSAALTHTLARACSAGRMERCTCDDSPGLRQAWQ
DreWnt9a        ------RYRANILKRGFKETAFLYAISSAGLTHAMAKACSAGRMERCTCDEAPDLRKAWQ
HsaWnt9a        WNCTLERYRASLLKRGFKETAFLYAISSAGLTHALAKACSAGRMERCTCDEAPDLREAWQ
DreWnt8a        WNCPESASTHKGLRSATRETAFVHAISAAGVMYTLTKNCSMGDFENCGCDDSKIGGRGWV
DreWnt8like     WNCPDTASTHKGLRSATRESSFVHAISAAGVMYTLTRNCSLGDLNECGCDSSRNGGRGWL
HsaWnt8a        WNCPENASTHNRLRSATRETSFIHAISSAGVMYIITKNCSMGDFENCGCDGSNNGGHGWI
DreWnt8b        WKCPERASTHSGLRSANRETAFFHAISSAGVMYTLTRNCSLGDFDNCGCDDTRNGGQGWL
HsaWnt8b        WNCPERASSHGGLRSANRETAFVHAISSAGVMYTLTRNCSLGDFDNCGCDDSRNGGQGWL
AmqWntB         WNCSETIDLSKDLKRLSKETAFTYALTSAIMVRVITKACSDGRLQNCSCDTSRQGPQGWQ
PfiWnt51648     WNCTEVIDPYSDLKRSTKESAFMHALTSAATVHVITKACSDGRIINCGCDTRFNGQQGWQ
EmuWntB         WNCSVGGSKIA--FNRSREAAFTYALVSAITAHSITSACANSLLSACGCDTSMSAQLGWD
1SlaWnt12305    WNCTSGGSKLA--FNKSRETAFTYALTSAVTVHAITTACSNNILAACGCDTSMAAQSGWD
EfrWnt28264     WNCTSGGSRVA--YNKSRETAFTYALTSAITAHSITTACANGLLSACGCDTSMGTQSGWD
CcaWnt9097_1    WDGYALKVGENLLRQATKESAFTHAIISAGITHVVTKSCSKHLIEGCGCASNRIPDDNFQ
Cre73781_5      WNCSSFLKQPSITKGDYIESAYVYSLSMAVIAHTVAMGC-VEEIFNCSCPE--------K
EmuWntC         WNCSTFIKSSNIVKKDIIETAYIRALQVAVIAHTVAKACGTQTLVSCGCSQFNTNSGNTY
SlaWnt31918     ----------------------YIRALQSAVIAHTVAKACRTGTLVSCGCSAFNTNAGDSY
EfrWnt14524     WNCSSFIKPPSIVRKDIVETAYIRALQVAVIAHTVAKACRTRTLASCGCATFNSNDGNTY
CcaWnt400_2     WNCWTASLFGGALKGTTREAAFVQALFTAVLSAKVTKKCSNPRFHTCGCDRSNAFDFDFD
AmqWntA         WNCTTFTLFGAFVKNNTRETAVINALLTAGAERQIALDCRDEKLPNCTCQINGDN--TFF
EmuWntA         WNCTTFLLFGKFITQGTIESAAVYSFMAAGAAQELAVACRTGAVSNCKCETVGDVQGNII
1SlaWnt7431     WNCTTFLLFGKFLTLGTAESAAVYSFMSAGAAHELAQACRTGAVSNCQCEIVGDVQGNII
EfrWnt21562     WNCTTFLLFGKFISTGTIESAAVYSFMSAGAAHELAGACRTGAVVNCICETIGDVQGNII
Cre66998_1      WNCSTLFLFGSFVATGTRETGVLNAYFAAGAVSAIAEDCHNQRIASCQC-SIDAPENNII
PsuWnt252       WNCSTFSLFGRFVTESTRETAVLFAFLSAGAIQEVAEACHEQRLLNCPC-LRGFLNGDTI
AmqWntC         WNCSGFVTPSNVTKYATAETAAIHSLMSAALAHVVTRDCRFNGM-QCECGKNTTIAGNQV
PfiWnt10714     WKCFGFMTPSNVTKRASKESSFIYAIISATLTHTITGACKDEII-DCESQTTTGINETHS
XScoWnt5782     WNCETFLLFGRILYNGTREAAFVHALMAASVAHESAKACSEGRLGDCNCVTNYHERSEYR
XScoWnt57224    FNCSVNLLFGRAVKGTHPESAFIHALLAASVTHGVATACSQGKLNLCTCSAGVLNPVDWQ
OloWntII        WNCTD--PFGNAFTAGSRQAAYVRSLVGVAVTYSITIACSYGSLLTCGCLSRSKLNRTYE
XScoWnt29445    WNCSSS-NWNIFLGKLHKEAAYVHAIISIAVSAQLVKDCNNGLLPICGGSPSCQG-----
SyconWnt18533   WGCYLESFFGKNMPRGTRERAILSALDSAGALVKLSRECSRGTTDACSCIQSATNAQAAE
XScoWnt26375    WNGTST-SWKKYLLRAHPEAGYFRAALSASLVFHTARHCAMGKI-SCYLGNTTEGDFAKK


OloWntI         WGGCGDNLDQGRESSARFLRDDVKSPPERRLMDDHNIKAGIEIAVRETKRNCRCHGLCGA
CcaWnt9259_1     WGGCGDNIEYGVEFSRNFILARTPDKLARENMDKHNVIAGGKITQDSAVMECQCHGFSGS
DreWnt10b       WGGCSHDIRFGVRFSRDWLDSRGSPRDIHARTRIHNNRVGRQVVTDNMRRKCKCHGTSGS
HsaWnt10b       WGGCNHDMDFGEKFSRDFLDSREAPRDIQARMRIHNNRVGRQVVTENLKRKCKCHGTSGS
DreWnt10a       WGGCSPNVEYGERFSKDFLDSRETYRDIHSRMRLHNNRVGRQVVVDHMRRKCKCHGTSGS
HsaWnt10a       WGGCSPDMGFGERFSKDFLDSREPHRDIHARMRLHNNRVGRQAVMENMRRKCKCHGTSGS
DreWnt4a        WSGCSDNIAYGVAFSQSFVDIRERSKSNRALMNLHNNEAGRKAILNHMRVECKCHGVSGS
HsaWnt4         WSGCSDNIAYGVAFSQSFVDVRERSKSSRALMNLHNNEAGRKAILTHMRVECKCHGVSGS
DreWnt4b        WSGCSDNLSYGVAFSQTFVDEPERAKSGRPLMNIHNNEAGRKAILHNMQVECKCHGVSGS
DreWnt5a        WGGCGDNLNYGYRFSREFVDAREREKSARQMMNLHNNEAGRRIVSDLADVSCKCHGVSGS
DreWnt5b        WGGCGDNVNYGYRFAREFVDAREREKHARTLMNLQNNEAGRMAVYNLANVACKCHGVSGS
HsaWnt5b        WGGCGDNVEYGYRFAKEFVDAREREKQGRVLMNLQNNEAGRRAVYKMADVACKCHGVSGS
HsaWnt5a        WGGCGDNIDYGYRFAKEFVDARERERSARILMNLHNNEAGRRTVYNLADVACKCHGVSGS
DreWnt7a        WGGCSADIRYGLSFSKVFLDAREIKQNARTLMNLHNNEVGRKILEKNMRLECKCHGVSGS
DreWnt7alike    WGGCSADVRYGLGFSKVFMDAKEIKHSARTLMNLHNNEVGRKVLERNMRLECKCHGVSGS
HsaWnt7a        WGGCSADIRYGIGFAKVFVDAREIKQNARTLMNLHNNEAGRKILEENMKLECKCHGVSGS
HsaWnt7b        WGGCSADVRYGIDFSRRFVDAREIKKNARRLMNLHNNEAGRKVLEDRMQLECKCHGVSGS
DreWnt7b        WGGCSADVKYGVEFSRRFVDAREIKKNARRLMNLHNNEAGRKVLEERMKLECKCHGVSGS
DreWnt3a        WGGCSEDVEFGSMVSREFADARENRPDARSAMNRHNNEAGRSSITDHMYLKCKCHGLSGS
HsaWnt3a        WGGCSEDIEFGGMVSREFADARENRPDARSAMNRHNNEAGRQAIASHMHLKCKCHGLSGS
DreWnt3         WGGCSEDAEFGVLVSREFADARENRPDARSAMNRHNNEAGRMTILENMHLRCKCHGLSGS
HsaWnt3         WGGCSEDADFGVLVSREFADARENRPDARSAMNKHNNEAGRTTILDHMHLKCKCHGLSGS
DreWnt2         WGGCSDHVDHAIKFTQVFIDAKERKEDARALMNLHNNRAGRKAVKRFMNLECKCHGVSGS
DreWnt2b        WGGCSDNINYGIKFAKAFIDAKERTVDARALMNLHNNRCGRMAVKRFMKLECKCHGVSGS
DreWnt2.1       WGGCSDNINYGIKFAKAFVDARERMVDARALMNLHNNRCGRMAVKRFMKTECKCHGVSGS
HsaWnt2b2       WGGCSDNIHYGVRFAKAFVDAKEKRLDARALMNLHNNRCGRTAVRRFLKLECKCHGVSGS
HsaWnt2b1       WGGCSDNIHYGVRFAKAFVDAKEKRLDARALMNLHNNRCGRTAVRRFLKLECKCHGVSGS
HsaWnt2         WGGCSDNIDYGIKFARAFVDAKERKGDARALMNLHNNRAGRKAVKRFLKQECKCHGVSGS
DreWnt1         WGGCSDNVEFGRMFGREFVDSSERGRDLRYLTNLHNNEAGRMTVASEMQQECKCHGMSGS
HsaWnt1         WGGCSDNIDFGRLFGREFVDSGEKGRDLRFLMNLHNNEAGRTTVFSEMRQECKCHGMSGS
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DreWnt16        WGGCSDDIAFGTSFSRRFIDSAAKNTEALLIMKQHNSEAGRQAVAKTMLTDCRCHGVSGS
HsaWnt16.1      WGGCSDDVQYGMWFSRKFLDFPIGNTKVLLAMNLHNNEAGRQAVAKLMSVDCRCHGVSGS
HsaWnt16.2      WGGCSDDVQYGMWFSRKFLDFPIGNTKVLLAMNLHNNEAGRQAVAKLMSVDCRCHGVSGS
DreWnt6         WGGCGDDVEFGYEKSKQFMDARRRKGDIRTLIDLHNNEAGRLAVKNYMRTECKCHGLSGS
HsaWnt6         WGGCGDDVDFGDEKSRLFMDARHKRGDIRALVQLHNNEAGRLAVRSHTRTECKCHGLSGS
DreWnt6like     WGGCGDDVDFGYEKSRQFMDIRQRKGDIRSLIDLHNNEAGRVAIQIQMRTECKCHGLSGS
DreWnt11        WGGCADNIHYGLLMGSKFSDAPMKMKHANKLMHLHNSEVGRQALRDALVMKCKCHGVSGS
HsaWnt11        WGGCADNLSYGLLMGAKFSDAPMKV-QANKLMRLHNSEVGRQALRASLEMKCKCHGVSGS
DreWnt11.1      WGGCGDNLRYGLQMGSAFSDAPIRN-QAFRLMQLHNNAVGRQVLMDSLEMKCKCHGVSGS
SyconWnt22889   WGSCSDNFHKGEEFAKQFLDG--SRNSPVSLVENYNHQTGRLVVGQSLQLVCKCHGLTGT
XScoWnt17541    WGSCSDNFNKGSQYAAEFLSDVYMANSPLSAVTRHNYDIGRKIVGKSMQFLCKCHGVTGS
XScoWnt14145    WGSCSDNYVKGYELSKQFLDA-GETDTPKALTTLWNNEAGRLAVRRLMRLQCKCHGLTGT
XScoWnt12941    WGGCGDNFAKGYQYSKEFLDVAHTADTKFDVLELHNNEAGRAVVRSTLQTICKCHGLTGA
XScoWnt16518    WGGCGDNFQKGKQYSQQFLDLAATATTRPQLEEVHNNRRGRMAVKNSLARVCKCHGLCGD
XScoWnt38625    WGGCGDNWEAGMRYAAEFLDAGTTDRPVVNRMALHNNKAGRLAVKRLLDIRCRCMGLSGS
DreWnt9b        WGVCGDNLKYSTKFLKKFLGQKRVSKDLRAQIDAHNINVGIRAVKSGLKTTCKCHGVSGS
HsaWnt9b        WGVCGDNLKYSTKFLSNFLGSKRGNKDLRARADAHNTHVGIKAVKSGLRTTCKCHGVSGS
DreWnt9a        WGGCGDNLKYSNKFVKDFLG-KRSNKDLRARIDMHNSNVGMKVIKTGVETTCKCHGVSGS
HsaWnt9a        WGGCGDNLKYSSKFVKEFLG-RRSSKDLRARVDFHNNLVGVKVIKAGVETTCKCHGVSGS
DreWnt8a        WGGCSDNVNFGDRIAKLFVDALENGHDSRAAVNLHNNEAGRLAVKATLKRTCKCHGLSGS
DreWnt8like     WGGCSDNVDFGERISKQFVDALETGQDARAAVNLHNNEAGRLAVKATMKRICRCHGMSES
HsaWnt8a        WGGCSDNVEFGERISKLFVDSLEKGKDARALMNLHNNRAGRLAVRATMKRTCKCHGISGS
DreWnt8b        WGGCSDNVGFGEVISKQFVDALETGQDARAAMNLHNNEVGRKAVKGTMQRTCKCHGVSGS
HsaWnt8b        WGGCSDNVGFGEAISKQFVDALETGQDARAAMNLHNNEAGRKAVKGTMKRTCKCHGVSGS
AmqWntB         WGGCSDDVGFGVMLTRAFLDTR--NNLEASLVNLHNNAVGRTVVSDNMQVKCRCHGASGS
PfiWnt51648     WGGCSDDVEFGANFAHMFLDVRETENLGLSLVNLHNNAAGRKEVQDEMDVECTCHGISGS
EmuWntB         WGGCSHDVNYGVQYAQSFLDARETTNIGTAVVNLHNNAVGRQTVQDYMQTSCSCHGISGS
1SlaWnt12305    WGGCSHDVDYGVQYAQSFLDARETINIGTALVNLHNNAVGRQTVQNYMQTSCSCHGISGS
EfrWnt28264     WGGCSHDINYGVQYAQMFLDAREATNIGTALVNLHNNAVGRQTVQDYMQTSCSCHGISGS
CcaWnt9097_1    WGSCSDDVYFGANVSAMFLNSQEHKRDLRTQVNLHNNKAGRLTVISERRLQCKCHGTSGS
Cre73781_5      EGGCPDPVTYGLHIAATFLNMRYTSSGGGLKQELRNFRATEIIIQSVMFKKCSCHGISGS
EmuWntC         SGNCSDNLDFGYRFAMNFTTSGVTSTTVQAKTDLHNFKAGINAVKDVMPPKCKCLGLSGS
SlaWnt31918     SGNCSDNMEFGYQFALNFTTSGITSANVQAKTDLRNFNAGINAMRDVMPPNCKCVGLSGS
EfrWnt14524     SGDCSDNFEFGYQFALNFTTSGITSTTVQAKTDIHNFNAGINAVRDIMPPKCKCIGTSGS
CcaWnt400_2     WDDCSDNIKFGNEFASEFLRQPNKGQLARELMNKHNEQAGLEVISSAANLSCFCHGFSGS
AmqWntA         LYECSFDIAKAHDIMSKFLETPSND-DT-AIIAEHNHNVGSNLVGQR-YRKCRCTGFSGS
EmuWntA         FNDCSDNTGYATDTMNQFIRDNSTNTDL-DLVNTHNYQVGLKMISQR-NTTCLCHGVSGT
1SlaWnt7431     FNDCSDNIQYASNTVLQFIKDNSTNIDV-DVVNYHNYQVGLQVLSQR-NSTCLCHGVSGT
EfrWnt21562     FNECSDNIKFASDIVRQLTRENSTNIDV-DLVNNHNYEVGLRILANR-NASCVCHGVSGT
Cre66998_1      FETCKADFNFSSEYFGEFIAAQIS--SFEGRIDQHNIDLGKEAINHE-TTNCRCHGISGT
PsuWnt252       FSDCAANFEWAANFFSSFVTSVYEQLLVGVKSDLHNVNAGILSVKKI-RKSCNCHGISGT
AmqWntC         MYGCSSNWEFGMEMSAKFMDGKEKHGGDRQLINLQNNQVGRTVVNHKKEPTCKCVGVSAS
PfiWnt10714     VTSDLHDVTYSAILAEKFLDSIENGGSDRQHLNLHNNRLGRMVVQNSVKYGCRCEGLSAS
XScoWnt5782     WKNCDNNILYGLRMAKKFQSVEPRDGEERKLMNLHNEKLGRSLIKAGFAPVCTCLGFTGS
XScoWnt57224    WSQCSLDTAYGIRIAKALASRSRTATSRKRKVHLHNYAAGYHVTTGQEYIGCSCYGPSGS
OloWntII        WGECSHDVSRAATLASNFIIAGEEGERLNSLANVHNYEAGTKIATTAVKVVCRCLGGTLS
XScoWnt29445    -SSCTKEISYGYNTAKDWIHSPEQLDWRQALVHNHNRDAGRFIVKKQIRKRCKCNGVSGS
SyconWnt18533   LCAYSEKTMFGPMFAKNFVDHNEHGPITYCMTNLHNNMVGRKMVVRSVDRRCQCIGVSGS
XScoWnt26375    QSFCDYHFTEGLRAAKDVVDKYFFAMKKRDLTHYHNNAVGREKIKDIRHLHCYCTGPSGS


OloWntI         CAIKSCWKELPNFHQIGAVVENKFDGSVKMKLNSGGGELEVAERNHVPPSNFDLVYLEST
CcaWnt9259_1     CTVKSCWKQLPSMSKIGLLVKREFDGAVKVTLNDADRELIPENPRHIPPTDSNLVYLTKS
DreWnt10b       CQFKTCWYVSPEFRLVGSLLREKFLTAIFINSQNKNNGFNSRTGGRRRSISRELVYFEKS
HsaWnt10b       CQFKTCWRAAPEFRAVGAALRERLGRAIFIDTHNRNSGFQPRL--RPRRLSGELVYFEKS
DreWnt10a       CQLKTCWQVTPEFRTVGSLLKERFNVATLIKAHNRNTGVENAHHTRRRANINDLVYFEKS
HsaWnt10a       CQLKTCWQVTPEFRTVGALLRSRFHRATLIRPHNRNGGLEPGPAGRRRASPADLVYFEKS
DreWnt4a        CEVKTCWKAMPPFRKVGNVIKEKFDGATEVELRKVGTTLVPRNSQFKPHTDEDLVYLDPS
HsaWnt4         CEVKTCWRAVPPFRQVGHALKEKFDGATEVEPRRVGSSLVPRNAQFKPHTDEDLVYLEPS
DreWnt4b        CELRTCWKVMPPFRRVGAVLKEHFDGATEVRLTRVGSRLLPRDPQVKPPATRDLVYLAPS
DreWnt5a        CSLKTCWLQLADFRKVGDVLKEKYDSAAAMRMNGRGK-LVQMHSKFSPPSGQDLLYLQPS
DreWnt5b        CSLKTCWLQLADFRRVGEFLKEKYDSAAAMRINRRGK-LELVNNRFNPPTGEDLVYIDPS
HsaWnt5b        CSLKTCWLQLAEFRKVGDRLKEKYDSAAAMRVTRKGR-LELVNSRFTQPTPEDLVYVDPS
HsaWnt5a        CSLKTCWLQLADFRKVGDALKEKYDSAAAMRLNSRGK-LVQVNSRFNSPTTQDLVYIDPS
DreWnt7a        CTTKTCWTTLPKFRQLGYILKERYNHAVHVEPVRASRNKVKKPYSYRKPMDTDLVYIEKS
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DreWnt7alike    CATRTCWTTLPKFRELGYILKDKYTSAIHVEPVKATRHKIKKPYSYRKPMDTDLVYIEKS
HsaWnt7a        CTTKTCWTTLPQFRELGYVLKDKYNEAVHVEPVRASRNKIKKPLSYRKPMDTDLVYIEKS
HsaWnt7b        CTTKTCWTTLPKFREVGHLLKEKYNAAVQVEVVRASRLRIKQLRSYQKPMETDLVYIEKS
DreWnt7b        CTTKTCWTTLPKFREIGYVLKERYTTALEVEAVRATRFRLKQSRGYIKPTDTDLVFLERS
DreWnt3a        CEVKTCWWSQPDFRVIGDYMKDKYDSASEMVVEKHRESLRPKYPYYKPPTETDLVYYESS
HsaWnt3a        CEVKTCWWSQPDFRAIGDFLKDKYDSASEMVVEKHRESLRPRYTYFKVPTERDLVYYEAS
DreWnt3         CEVKTCWWAQPDFRLLGDYLKDKYDSASEMVVEKHRESLRAKYAFFKHPTERDLVYYEGS
HsaWnt3         CEVKTCWWAQPDFRAIGDFLKDKYDSASEMVVEKHRESLRAKYSLFKPPTERDLVYYENS
DreWnt2         CNVRTCWLAMADFRQTGDYLRKKYNNAIQVVMNQYGTGFTSAYRMLKRPNKNDLVYFEDS
DreWnt2b        CTLRTCWLAMSDFRKTGDYLRKKYNGAIEVTMNQDGTGFTVANKDFRKATKNDLVYFENS
DreWnt2.1       CALRTCWLAMPDFRRTGDYLRKKYNAAVEVMMNQDGTGFMVADRDYKRTTKNDLVYIENS
HsaWnt2b2       CTLRTCWRALSDFRRTGDYLRRRYDGAVQVMATQDGANFTAARQGYRRATRTDLVYFDNS
HsaWnt2b1       CTLRTCWRALSDFRRTGDYLRRRYDGAVQVMATQDGANFTAARQGYRRATRTDLVYFDNS
HsaWnt2         CTLRTCWLAMADFRKTGDYLWRKYNGAIQVVMNQDGTGFTVANERFKKPTKNDLVYFENS
DreWnt1         CTVRTCWMRLPSFRLVGDYLKDRFDGASRVVYANKGSNLEPENPAHKLPSSRDLVYFEKS
HsaWnt1         CTVRTCWMRLPTLRAVGDVLRDRFDGASRVLYGNRGSNLEPEDPAHKPPSPHDLVYFEKS
DreWnt16        CAVKTCWRTMAAFERVGAYLKDRYETSVHVVDRSKRKVRKDKEQRHVPITKDELIFFNKS
HsaWnt16.1      CAVKTCWKTMSSFEKIGHLLKDKYENSIQISDKTKRKMRREKDQRKIPIHKDDLLYVNKS
HsaWnt16.2      CAVKTCWKTMSSFEKIGHLLKDKYENSIQISDKTKRKMRREKDQRKIPIHKDDLLYVNKS
DreWnt6         CTLRTCWKKMPHFREVGDRLLERFNGASKVMGGNDGKTLIPVGQNIKPPDKQDLIYSAES
HsaWnt6         CALRTCWQKLPPFREVGARLLERFHGASRVMGTNDGKALLPAVRTLKPPGRADLLYAADS
DreWnt6like     CTLRSCWKKMPLFRQVGDQLMQSFHTAVRVMGGNDGKSLVSIDPDAPPLDANVLIYSAES
DreWnt11        CSIRTCWRGLLDLKDIAIDLKTKYLSATKVVHRPMGTRLVPKDIDIRPVRENELVYLQSS
HsaWnt11        CSIRTCWKGLQELQDVAADLKTRYLSATKVVHRPMGTRLVPKDLDIRPVKDSELVYLQSS
DreWnt11.1      CSVKTCWKGLQDISTISADLKSKYLSATKVIPRQIGTRLVPREMEVRPVGENELVYLVSS
SyconWnt22889   CAHRTCWYSLPKVQVIGEKLLTKYDAAMRVK-----WES---ESL-VSNLTAQLVFDTPS
XScoWnt17541    CSYKTCWYGLPKIQLIGEQLAKKYDKIFQVV-----WNQGKVESF-KSRMNEKLVFKDTS
XScoWnt14145    CAHQTCWYSLPVIRVVGKLLMKKYEASTTVKSASVSTRVVKRISL----THEDLVHLRRS
XScoWnt12941    CSVKICWRSLPKVQVVGFILKEKYRNAMEVAVPE--LSPGTDIETPRPD-ESELTFLHSS
XScoWnt16518    CTVKICWKEVRSIK-IGKVLKEAYHDAKLV---H--LALSPALAPTAPDITSALVYVKES
XScoWnt38625    CSDKVCWRVLPSIQRVGERLKKKFSVAVKVRLDEKSRTVLPEDGTGRRPPSKELVYSETS
DreWnt9b        CAVRTCWKQLSPFQDTGHLLKYRYDTAVRVHSVTNSATE-TELAG---PRPTELVFLEES
HsaWnt9b        CAVRTCWKQLSPFRETGQVLKLRYDSAVKVSSATNEALR-LELWAL-APRSGDLVYMEDS
DreWnt9a        CTVQTCWRQLAPFHEIGKQLKQRYETSVKVASSTNEATE-GEISQD-IPRTPDLLHIEDS
HsaWnt9a        CTVRTCWRQLAPFHEVGKHLKHKYETALKVGSTTNEAAEAGAISPDPLPRTPELVHLDDS
DreWnt8a        CSIQTCWMQLADFRDIGSYLKIKHDQARKLEMDKIRMRRGAIADTFSSVARTELIFMEDS
DreWnt8like     CTMQTCWMQLADFREIGNYLKVKHDQAQKLEMDKRRMRRVTMTDAFGSIARTELIYLEDS
HsaWnt8a        CSIQTCWLQLAEFREMGDYLKAKYDQALKIEMDKRQLRHWVPAEAFLPSAEAELIFLEES
DreWnt8b        CTTQTCWLQLPEFREVGNYLKEKYHRAVKVDLLR---GRGAIAETFNSISRKELVHLEDS
HsaWnt8b        CTTQTCWLQLPEFREVGAHLKEKYHAALKVDLLQ---GRGAIADTFRSISTRELVHLEDS
AmqWntB         CATRTCYSQLPTVRDISTDMKIKYNHSVKVTAHVNRG-TSSSNTEAVSPPVDSLVHVKNS
PfiWnt51648     CSVRTCWRKLPELRSVSKNIKQKYDQSIKVSLQVQKD-LKSVGDDPMPPSTSHLVFLKKS
EmuWntB         CTVQTCWRQL---GAIGDVLRQKYEAAAMVRVDIPRDGYTDSMQNPVAPSSTEMVYLEPT
1SlaWnt12305    CTIQTCWRQLPEVGVVGDVLRQKYNAAAMVRVGVPPGGYADSGPNPVVPMSSEIVYLEPT
EfrWnt28264     CTIQTCWRQLPEVGAVGDALRQKYDTAVMVKVDVPRDGYANSGQNPVAPSRTEIVYLEPI
CcaWnt9097_1    CALRTCWMSLQRFHEVGRHIVHQYDQAVQVVSNQMKHDLTSVSRYRSHPDSLDLVYYEKS
Cre73781_5      CTFSVCHSELPPFSTLAKRVKQAYNDSCLVLPNGH--SNDWVAQCDHPITDSDLLF-TKS
EmuWntC         CTTQVCWQEAPDFSVVGSSIKKLFDSACVVSWNQYLGTSNWLSNVCPIITDRTLIYGGQS
SlaWnt31918     CTTQVCWQEAPDFSVVGGSIKKLFDSACLVTWNQYLGTSNWVSSMCPLITDRVLIYGSQS
EfrWnt14524     CTTQICWQEAPDFSVVGSSIKKLFDSACQVSWNQYLGTSNWISPACPVVTDKTLIYGGQS
CcaWnt400_2     CALRTCWLEAPSMNRIGSELKKAYNKAVKVKRVVTDDGKLVNKLDFQPLNTPELAYLSNS
AmqWntA         CSVQTCYFASPDIDTIGQRVREKYGSSVEVTVNASNSAQPVV--QTINNHDNELVYLKRS
EmuWntA         CTVQTCYQQVPDVATIGGTLRQKYTNAVKVTRVSGTTTRPVY--SATLN-ESDLAYLADS
1SlaWnt7431     CTVQTCYQQVPDVSSFGDTLSWKYINAKQVTRVPGTTTRPVY--SASLN-QSDLAYIAGS
EfrWnt21562     CTVQTCYQQVPDVATFGDIIRWKYINAVKVSRVPGMTTKPVYNQNSALN-ESELAYIADT
Cre66998_1      CTVQTCYKRVPTVPEIGEQLFIRYGGAIHVVDSDGEFA---SNPNIDPPDDNTIIFKDNS
PsuWnt252       CTVQTCYDQVISVSEVRDSLAISYNGATKVELVNGELQIPIPGANPDNINENNLVFLDNT
AmqWntC         CSAKTCQRGLEAFSVVAASIKDKYKKSCKVS---VKASLQPHQCNSSSISNTTLVHTLSS
PfiWnt10714     CSLQTCQTTLANLRTVSAKIYQAYDNSCKVRTNIINSDPSFISTDCDKITNNTLIFFDNS
XScoWnt5782     CATRVCWREVPNFRQVGNTLFRLYSRAQRVQISDDASEPTVAGATADPPSEFELVQGADT
XScoWnt57224    CTHKFCHVRLASENALAGKLWKEYRRAVQVEIKPRPNGWRLKQVGAAKPSKETFVYSTLS
OloWntII        CATKVCHRELRQFSHIGSTLVDKYNKAVQIKLSKNGERLKSADSTTGTFEDTDLVY-ANS
XScoWnt29445    CLENRCYHRLPEMSEMIRGL---MDNKVFLPWKENRNRQGSLKSVRGKRGKTFLVYMKRS
SyconWnt18533   CLHKSCIGYLPDIKSIKKDFGRSFNQAKQAYVSTSHSGKLVSSATHIPLRSDQLAFFQSS
XScoWnt26375    CILKTCSYVETRMTRTALALHEQYTQLLAGGAPAIASASIQRGSRTRGQRNITVIASRPS
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OloWntI          SRFCVKDSSVGSHGTHGRLCDPESSGTEGCAHLCCGRGYDTFEETDIEKCNCKFVWCCRI
CcaWnt9259_1     SDYCKYDPSTGSHGTIGRECNKTSDGIDGCSLMCCNRGFYSREVTLTRRCKCQFIWCCHV
DreWnt10b        PDFCDREPAVDSLGTQGRICNKSSPGMDGCGSLCCGRGHNILKQARSERCHCRFHWCCYV
HsaWnt10b        PDFCERDPTMGSPGTRGRACNKTSRLLDGCGSLCCGRGHNVLRQTRVERCHCRFHWCCYV
DreWnt10a        PDFCERDLGSDSAGTQGRICNKTSQGMDNCESLCCGRGHNILQQTRSERCNCKFHWCCYV
HsaWnt10a        PDFCEREPRLDSAGTVGRLCNKSSAGSDGCGSMCCGRGHNILRQTRSERCHCRFHWCCFV
DreWnt4a         PDFCEHDPRTGIMGTAGRFCNKTSKAIDGCELMCCGRGFHTEEVEVVDRCSCKFHWCCYV
HsaWnt4          PDFCEQDMRSGVLGTRGRTCNKTSKAIDGCELLCCGRGFHTAQVELAERCSCKFHWCCFV
DreWnt4b         PDFCRLDPDNGIPGTAGRRCNGTSRAPDGCELLCCGPGFRAGRAEVVQRCSCKFSWCCSV
DreWnt5a         PDYCIRNSSSGSLGTQGRLCNKTSEGMDGCALMCCGRGYDQYKAELVERCHCKFHWCCYV
DreWnt5b         PDYCLRNETTGSLGTQGRLCNKTSEGMDGCELMCCGRGYDQFKTYKHERCHCKFHWCCYV
HsaWnt5b         PDYCLRNESTGSLGTQGRLCNKTSEGMDGCELMCCGRGYNQFKSVQVERCHCKFHWCCFV
HsaWnt5a         PDYCVRNESTGSLGTQGRLCNKTSEGMDGCELMCCGRGYDQFKTVQTERCHCKFHWCCYV
DreWnt7a         PNYCEADPVTGSMGTQGRICNKTAQHTNGCDLMCCGRGYNTHQYSRVWQCNCKFLWCCYV
DreWnt7alike     PNYCEADLRSGSIGTQGRVCNKTMHHPNGCDLMCCGRGYNTHQYSRVWQCNCKFFWCCYV
HsaWnt7a         PNYCEEDPVTGSVGTQGRACNKTAPQASGCDLMCCGRGYNTHQYARVWQCNCKFHWCCYV
HsaWnt7b         PNYCEEDAATGSVGTQGRLCNRTSPGADGCDTMCCGRGYNTHQYTKVWQCNCKFHWCCFV
DreWnt7b         PNYCEEDTVTGSAGTRGRLCNHTSPLTDGCNLMCCGRGHNTHQYTRVWQCNCKFQWCCFV
DreWnt3a         PNFCEPNPETGSFGTRDRTCNLTSHGIDGCDLLCCGRGHNTRTEKRKEKCHCIFHWCCYV
HsaWnt3a         PNFCEPNPETGSFGTRDRTCNVSSHGIDGCDLLCCGRGHNARAERRREKCRCVFHWCCYV
DreWnt3          PNFCEPNPETGSFGTRDRACNVSSHGIEGCDLLCCGRGHNTRTEKRKEKCHCIFHWCCYV
HsaWnt3          PNFCEPNPETGSFGTRDRTCNVTSHGIDGCDLLCCGRGHNTRTEKRKEKCHCIFHWCCYV
DreWnt2          PDYCIWDHESGSVGTGGRVCNRTSRGTDSCEVMCCGRGYDTSRVSRTTKCECKFQWCCAV
DreWnt2b         PDYCLMDKTAGSLGTAGRVCNKTSRGTDGCEVMCCGRGYDTTRSKRITKCECKFKWCCTV
DreWnt2.1        PDYCLMDRSAGSLGTSGRVCNKSSRGMDGCEIMCCGRGYDTTRVNRMTKCECKFKWCCAV
HsaWnt2b2        PDYCVLDKAAGSLGTAGRVCSKTSKGTDGCEIMCCGRGYDTTRVTRVTQCECKFHWCCAV
HsaWnt2b1        PDYCVLDKAAGSLGTAGRVCSKTSKGTDGCEIMCCGRGYDTTRVTRVTQCECKFHWCCAV
HsaWnt2          PDYCIRDREAGSLGTAGRVCNLTSRGMDSCEVMCCGRGYDTSHVTRMTKCGCKFHWCCAV
DreWnt1          PNFCSYNGKTGTHGTSGRTCNSSSPALDGCELLCCGRGYKTRMEQVTERCHCTFHWCCHV
HsaWnt1          PNFCTYSGRLGTAGTAGRACNSSSPALDGCELLCCGRGHRTRTQRVTERCNCTFHWCCHV
DreWnt16         PNYCLEDRRLGVTGTRGRKCNRTSAGPDGCNLLCCGRGYNTHVVRHVERCECKFVWCCYV
HsaWnt16.1       PNYCVEDKKLGIPGTQGRECNRTSEGADGCNLLCCGRGYNTHVVRHVERCECKFIWCCYV
HsaWnt16.2       PNYCVEDKKLGIPGTQGRECNRTSEGADGCNLLCCGRGYNTHVVRHVERCECKFIWCCYV
DreWnt6          PDFCLPNRKTGSLGTRGRTCNSTALDVSGCDLLCCERGHRDETVVLEENCLCRFHWCCVV
HsaWnt6          PDFCAPNRRTGSPGTRGRACNSSAPDLSGCDLLCCGRGHRQESVQLEENCLCRFHWCCVV
DreWnt6like      PDFCKANHRSGTEGTGGRACNRTETGPGGCDSLCCGNGFADFTVEEEENCECRFHWCCEV
DreWnt11         PDYCMKNDKLGSFGTQDRQCNKTSSGSDSCDLMCCGRGYNPYTERVVERCHCKYHWCCYV
HsaWnt11         PDFCMKNEKVGSHGTQDRQCNKTSNGSDSCDLMCCGRGYNPYTDRVVERCHCKYHWCCYV
DreWnt11.1       PDYCTQNAKQGSLGTTDRQCNKTASGSESCGLMCCGRGYNAYTEVLVERCQCKYHWCCYV
SyconWnt22889    VDYCEANADIGAPGTQGRVCNPRLSGVGGCTRLCCGRGHNERRRIETKKCKCKFVWCCRV
XScoWnt17541     VDYCQPNMAIGSLGTHGRECVPKVDGTSSCDEVCCRRGYNERRRIESKTCACKFLWCCKV
XScoWnt14145     PNYCVSDPEVGSLGTFGRTCQGKKSGYGGCDHLCCNRGFNVQRYTRKEQCRCAFVWCCHV
XScoWnt12941     PDYCKPNATIGINGTAARECKVESRGDDGCELLCCGNGFYVKRSVLRQKCRCKFIFCCDV
XScoWnt16518     PDFCVANASIGVSGTARRRCNKNSQGSDGCELLCCGNGYFETRDVKREQCACKFVWCCEV
XScoWnt38625     PSFCNANQLKASPGTKGRRCKPWSKGEDNCEQLCCGRGYTTEEKRVEERCRCTFHWCCRV
DreWnt9b         PSFCRPSR--YSPGTAGRPCSKDTS----CSSLCCGRGYNTALRLTTLSCHCQVRWCCHV
HsaWnt9b         PSFCRPSK--YSPGTAGRVCSREAS----CSSLCCGRGYDTQSRLVAFSCHCQVQWCCYV
DreWnt9a         PSFCRPSK--YSAGTLARKCYKDKN----CEAICCGRGHNTQSRVVTRPCQCQVRWCCYV
HsaWnt9a         PSFCLAGR--FSPGTAGRRCHREKN----CESICCGRGHNTQSRVVTRPCQCQVRWCCYV
DreWnt8a         PDYCVKNLSMGLHGTEGRECLQSGKNLSSCRRLCCGLKVEERRIETVSSCNCKFHWCCTV
DreWnt8like      PDYCAKNLSLGLPGTEGRECVQHGESLSSCRRLCCGLRVEERRTEVVSSCNCKFHWCCTV
HsaWnt8a         PDYCTCNSSLGIYGTEGRECLQNSHNTSSCGRLCCGLQVEERKTEVISSCNCKFQWCCTV
DreWnt8b         PDYCLENRTLGLPGTEGRECLRKGKNLSTCKRLCCGLAVEERRAETVSSCNCKFHWCCAV
HsaWnt8b         PDYCLENKTLGLLGTEGRECLRRGRALGSCRRLCCGLAVEERRAETVSSCNCKFHWCCAV
AmqWntB          VKYCIRQNDY----TANRSCIPQNILTQACESLCCSGEYETEEYTVSTTCYCHFVWCCKI
PfiWnt51648      KNMCLYKQNY----TLGRSCVPKNILTEPCEDLCCAGEYSLKRTVVVRSCNCHFVWCCDI
EmuWntB          VDYCSQQSNY----TLNRYCIPRSNMTSTCEDLCCNGQYVTVKTIRTYSCNCKFIWCCNV
1SlaWnt12305     VDYCSLQSNY----TLNRYCVPRSNMTSACEDLCCNGKYITLQRTRTYSCNCKFIWCCNV
EfrWnt28264      IDYCSTQSNY----TLNRYCIPRSNLTSACEDICCNGRFVTVRTTRTYSCGCKFIWCCNV
CcaWnt9097_1     PRYCVRNKLVGSLGTKGRKCEQNSQSTNSCLHLCCGRGFRVKYLVEEYDCECSFRWCCRV
Cre73781_5       NTWCKYDPEIGSAGVVGRECDPHPEAPNSCNKLCCKRPSIQQTVEEVVQCDCQFLFCCEI
EmuWntC          PNWCYADPSVGSMGVTGRQCDPNSSGSNRCSSLCCDRGYVETQVTQDTDCNCKFVYCCSI
SlaWnt31918      PNWCIPDPTVGSTGVVGRQCDPNSSGPNQCSSLCCNHGYVQTQIAQDTDCNCKFVYCCSI
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EfrWnt14524     PNWCYPDPTIGSLGVVGRQCDPNSSGTNKCSSLCCDHGYVQTQITQNSDCNCKFVYCCSI
CcaWnt400_2     QDFCTADKMTLQPGTIGRECNVSSSGEGSCSYLCCGRGHTMTLILDDREC-CRFHWCCEV
AmqWntA         PTFCNQDTTYGILGTVGRQCSNNLSDPDSCDIICCGRGHITVTATQPKQC-CSFIYCCRI
EmuWntA         PTFCTADNNMGILGTSGRQCNPTSLGLDSCFYLCCNRGYTAKTRIVPEEC-CQFVWCCRI
1SlaWnt7431     PDFCTANNDMGILGTSGRKCNPTSQGLDSCYYLCCGRGYTTKTTIVPQQC-CQFVWCCRI
EfrWnt21562     PDLCVANNNLGILGTSGRKCNPSSQGLDSCYFLCCGRGYTAKTTVVPQEC-CQFVWCCRI
Cre66998_1      PNFCVENRQLGTVGVANRICNPNSNSRNACASTCCDRGHHTITKHVPIEE-CKFI-----
PsuWnt252       PDLCKTDLAKGILGTAHRLCKEEPGLLD-CANLCCGRGFYTVTYTVPIEE-CRFVWCCRI
AmqWntC         PDYCHKDISKGSFGVQGRLCDP-AVASKSCETICCGRGHIEFTKDVEGKC-CKQVGCCGV
PfiWnt10714     VDYCYRDISVGSPGVKGQSC---------------------------------------
XScoWnt5782     LSFCDANPSLDIAGTPGRTCQESGSESDGCGPLCCFRGYRAATVTQHSPCGCRFVWCCNV
XScoWnt57224    PNYCEQNVHLGYPGTQGRKCLDDESAIGSCARLCC-RGHITNTHTRMERCECSASYFPRR
OloWntII        ASLCEPNSAFGYDGIHGRECISDPSAPNYCPSFCCGYGYFSYIEEKKRSCRCKLKCCFEL
XScoWnt29445    PSYCCSDPSQGTLGTEGRSCN----GQESCRKVCCSK---EVEQMGSNMCNCKFHFCCRL
SyconWnt18533   PILCSHNKRPIGPPVGERYCNATEDAIGSCHYLCCGGPVETVTEVTERRCNATLVTRYSY
XScoWnt26375    PSYCRPSKKWGILGPGNRICYTACGRPNHCQDICCGRS---GSQVTEYACNCRMQDAWVL


OloWntI         ECEKCYRKVKRSYCK
CcaWnt9259_1    KCETCKERVIKHFCN
DreWnt10b       LCEECKVTEWVNVCK
HsaWnt10b       LCDECKVTEWVNVCK
DreWnt10a       VCEECRITEWVSVCK
HsaWnt10a       VCEECRITEWVSVCK
DreWnt4a        KCKQCRKMVEMHTCR
HsaWnt4         KCRQCQRLVELHTCR
DreWnt4b        RCQQCKNTVLIHTCR
DreWnt5a        RCKRCSSIVDQYVCK
DreWnt5b        KCKRCTSLVDQFVCK
HsaWnt5b        RCKKCTEIVDQYICK
HsaWnt5a        KCKKCTEIVDQFVCK
DreWnt7a        KCNTCSERTEVYTCK
DreWnt7alike    KCNTCSERTEVYTCK
HsaWnt7a        KCNTCSERTEMYTCK
HsaWnt7b        KCNTCSERTEVFTCK
DreWnt7b        KCNTCSEKTEVFTCK
DreWnt3a        SCQECTRVYDVHTCK
HsaWnt3a        SCQECTRVYDVHTCK
DreWnt3         SCQECVRVYDVHTCK
HsaWnt3         SCQECIRIYDVHTCK
DreWnt2         HCRDCQEEVDVHTCK
DreWnt2b        ECKDCEEAVDIHTCK
DreWnt2.1       ECRDCEETVDVHTCK
HsaWnt2b2       RCKECRNTVDVHTCK
HsaWnt2b1       RCKECRNTVDVHTCK
HsaWnt2         RCQDCLEALDVHTCK
DreWnt1         SCLNCTSTQTVHQCL
HsaWnt1         SCRNCTHTRVLHECL
DreWnt16        RCRRCETMNDMHTCK
HsaWnt16.1      RCRRCESMTDVHTCK
HsaWnt16.2      RCRRCESMTDVHTCK
DreWnt6         QCKKCLVRKELSLCQ
HsaWnt6         QCHRCRVRKELSLCL
DreWnt6like     QCQTCSQRKNVSLCL
DreWnt11        TCKKCDKTVEKYVCK
HsaWnt11        TCRRCERTVERYVCK
DreWnt11.1      SCKTCKRTVERYVCK
SyconWnt22889   ECERCRTVSKRYTCV
XScoWnt17541    HCKQCRTVTKKYTCL
XScoWnt14145    RCKTCTVNKERHSCN
XScoWnt12941    VCDSCLVAWETHHCN
XScoWnt16518    KCQWCHRVYQNFHCK
XScoWnt38625    TCDRCVSTQEIHTCN
DreWnt9b        ECQTCLREEEVYTCK
HsaWnt9b        ECQQCVQEELVYTCK
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DreWnt9a        ECKQCTQKEEVYTCK
HsaWnt9a        ECRQCTQREEVYTCK
DreWnt8a        KCETCTQTVTRYFCA
DreWnt8like     KCENCSQVTVKHVCT
HsaWnt8a        KCDQCRHVVSKYYCA
DreWnt8b        KCEQCRKTVTKYYCV
HsaWnt8b        RCEQCRRRVTKYFC-
AmqWntB         SCEECEKTLTRYKCT
PfiWnt51648     ICDDCAVTVDTYKCT
EmuWntB         VCSTCTETMVQYKCT
1SlaWnt12305    VCSTCTETVVQYMCT
EfrWnt28264     VCNTCTETVTQYKCT
CcaWnt9097_1    ECKTCRRKTPYHVCR
Cre73781_5      KCEICTERRTYFSCS
EmuWntC         QCSKCHTVTTTYVCL
SlaWnt31918     QCSKCHTVTTAYVCL
EfrWnt14524     QCLKCHTVKTTYVCL
CcaWnt400_2     RCTTCRRQREAAICD
AmqWntA         ECQDCGEETTEYFCK
EmuWntA         ECTVCRNNTTDYFCN
1SlaWnt7431     ECTNCKNVTTDYYCN
EfrWnt21562     ECTYCKNVTTDYYCN
Cre66998_1      ---------------
PsuWnt252       DCAVTGSKTVERRCN
AmqWntC         QCNDCKRTLTFYACR
PfiWnt10714     ---------------
XScoWnt5782     KCDTCTNSYKRNTCS
XScoWnt57224    VCKKCQVQVHEHFCK
OloWntII        ICDVCIVERTKYRCK
XScoWnt29445    QCEECRV--RMYRCM
SyconWnt18533   QFQVCRDEHLRTKCK
XScoWnt26375    VCQSCSREV--TACL
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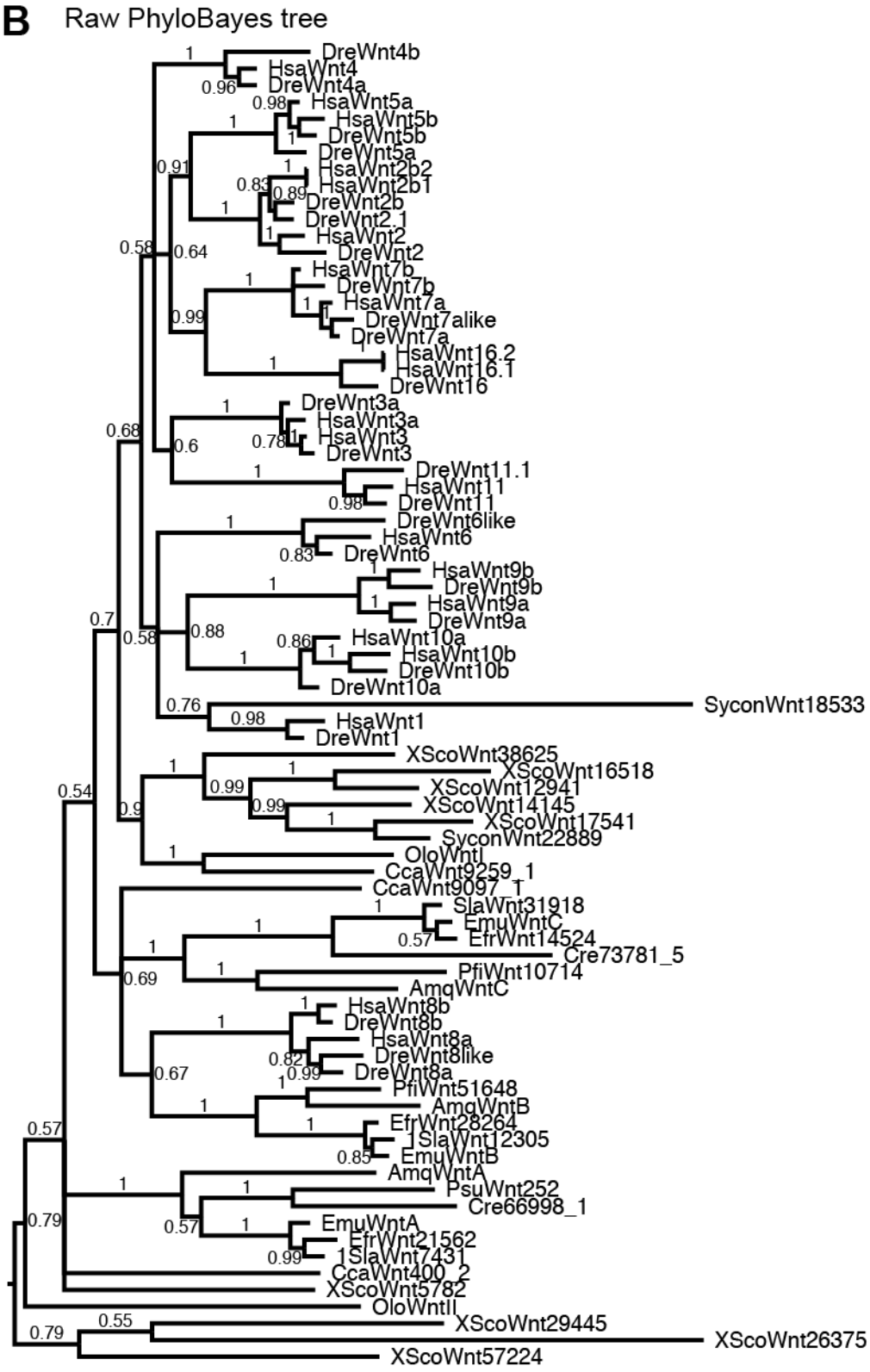
B
Raw PhyloBayes tree
DreWnt4b
HsaWnt4
DreWnt4a
HsaWnt5a
HsaWnt5b
DreWnt5b
DreWnt5a
HsaWnt2b2
HsaWnt2b1
DreWnt2b
DreWnt2.1
HsaWnt2
DreWnt2
HsaWnt7b
DreWnt7b
HsaWnt7a
DreWnt7alike
DreWnt7a
HsaWnt16.2
HsaWnt16.1
DreWnt16
DreWnt3a
HsaWnt3a
HsaWnt3
DreWnt3
DreWnt11.1
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HsaWnt9a
DreWnt9a
HsaWnt10a
HsaWnt10b
DreWnt10b
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SyconWnt18533
HsaWnt1
DreWnt1
XScoWnt38625
XScoWnt16518
XScoWnt12941
XScoWnt14145
XScoWnt17541
SyconWnt22889
OloWntI
CcaWnt9259_1
CcaWnt9097_1
SlaWnt31918
EmuWntC
EfrWnt14524
Cre73781_5
PfiWnt10714
AmqWntC
HsaWnt8b
DreWnt8b
HsaWnt8a
DreWnt8like
DreWnt8a
PfiWnt51648
AmqWntB
EfrWnt28264
1SlaWnt12305
EmuWntB
AmqWntA
PsuWnt252
Cre66998_1
EmuWntA
EfrWnt21562
1SlaWnt7431
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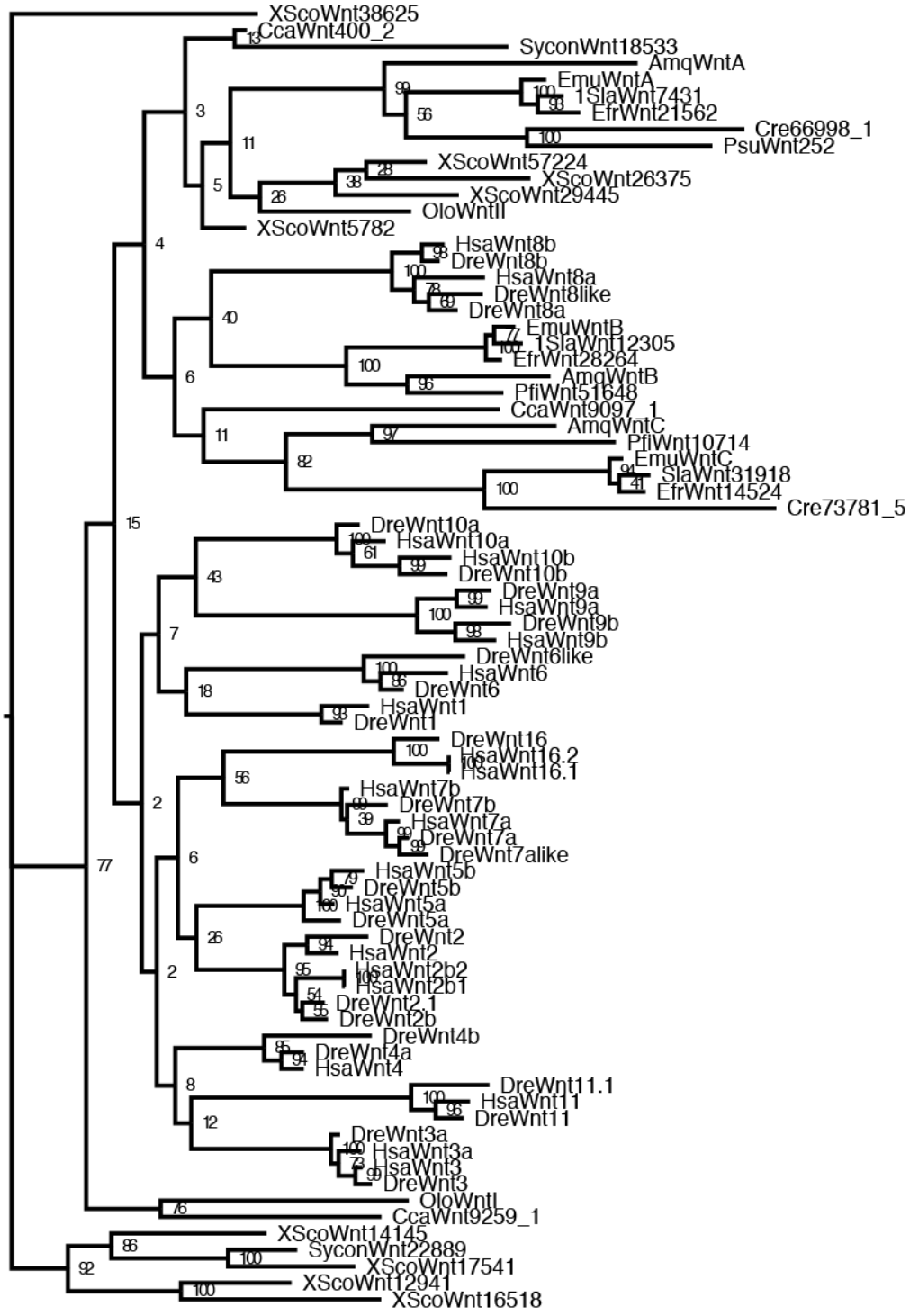
XScoWnt5782
OloWntII
XScoWnt29445
XScoWnt26375
XScoWnt57224

C Raw RAxML tree



A2.8: TRIMMED ALIGNMENT USED FOR GENERATING SPONGE WNT PHYLOGENETIC TREE

GBlocks trimmed alignment of sponge Wnt sequences used for the phylogenetic tree shown in Fig. 3-1C.

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OloWntI           QREWCIVDGATKGLDECEYRFQKRRWNCESAFTYAVTSAAIAWSVSRQCCGDNLDQGSAR
CcaWnt9259_1      -----VSDFSTNVVHVLKL---------ETAFVFAMTVAGIIYSVSRECCGDNIEYGSRN
SyconWnt22889     QVKWCIASGAKLGLAECKRRFSDRRWSCEAAFVHGIQSAGITLAIARTCCSDNFHKGAKQ
XScoWnt17541      QVEWCIVIGTRLGLDECKRRFALRLWSCEAAFVRGIESAGITLAIARTCCSDNFNKGAAE
XScoWnt14145      QRLWCIATGAQLALGQCRKQFADRRWSCETAFVHAIQSAGATLAIARTCCSDNYVKGSKQ
XScoWnt12941      QRSWCIAQGANLGLSECRRQFMFERWNCEAAFVASIQAAGVTLAIARTCCGDNFAKGSKE
XScoWnt16518      QATWCIGLGANLAMKECRKAFQYERWNCETAFVDAIVSAGVMMAISRMCCGDNFQKGSQQ
XScoWnt38625      QRMFCINQGANLGQEECHRRMSGRRWNCESAFLQAIQSSGVVQALARVCCGDNWEAGAAE
CcaWnt9097_1      D--LCIIQGVQLAVEETIHLLKDRRWDGESAFTHAIISAGITHVVTKSCCSDDVYFGSAM
Cre73781_5        RRYICLKFAEQVGKSTCEEDFEHEHWNCESAYVYSLSMAVIAHTVAMGCCPDPVTYGAAT
EmuWntC           AVLVCLKFAEQVGRDECQKAFQGSKWNCETAYIRALQVAVIAHTVAKACCSDNLDFGAMN
SlaWnt31918       ------------------------------YIRALQSAVIAHTVAKACCSDNMEFGALN
EfrWnt14524       TVLVCLKFAEQVGRDECQKTFQGSKWNCETAYIRALQVAVIAHTVAKACCSDNFEFGALN
CcaWnt400_2       QVDMCVFFAAEMALKQCKRQFKHNKWNCEAAFVQALFTAVLSAKVTKKCCSDNIKFGASE
AmqWntB           QRDLCIQEVPPLFYSECREQFKYERWNCETAFTYALTSAIMVRVITKACCSDDVGFGTRA
PfiWnt51648       -----IQEIADAIHDECIDQFKNDRWNCESAFMHALTSAATVHVITKACCSDDVEFGAHM
EmuWntB           QKALCLVNATLAYTDECNWQFRKDRWNCEAAFTYALVSAITAHSITSACCSHDVNYGAQS
1SlaWnt12305      QKALCLVNATLEYADECNWQFRRDRWNCETAFTYALTSAVTVHAITTACCSHDVDYGAQS
EfrWnt28264       QKALCLVNVTLAYADECNWQFRRDRWNCETAFTYALTSAITAHSITTACCSHDINYGAQM
AmqWntC           QRIMCIVDAEQLARKECSNQLEYERWNCETAAIHSLMSAALAHVVTRDCCSSNWEFGSAK
PfiWnt10714       QRDLCLIKSEQLAKEECEFWFKDHQWKCESSFIYAIISATLTHTITGACDLHDVTYSAEK
AmqWntA           QQTFCIAIGTRRGIVACQQNFANWRWNCETAVINALLTAGAERQIALDCCSFDIAKAMSK
EmuWntA           ARSVCIARGTHSAVLQCQSQFGKMRWNCESAAVYSFMAAGAAQELAVACCSDNTGYAMNQ
1SlaWnt7431       AQATCIARGTKAAIVQCQMEFANSRWNCESAAVYSFMSAGAAHELAQACCSDNIQYAVLQ
EfrWnt21562       AQEVCVSRGAKAAIIQCQSEFGNLRWNCESAAVYSFMSAGAAHELAGACCSDNIKFAVRQ
Cre66998_1        QASKCLSNGARAAIIDCQRRFNESRWNCETGVLNAYFAAGAVSAIAEDCCKADFNFSFGE
PsuWnt252         QKEACIARGTRKAIIDCQAVFSERKWNCETAVLFAFLSAGAIQEVAEACCAANFEWAFSS
XScoWnt5782       QRAACLARASLATTMECEKQFTGQRWNCEAAFVHALMAASVAHESAKACCDNNILYGAKK
OloWntII          QFQICIAQGINAAVYTCKRDFENRQWNCQAAYVRSLVGVAVTYSITIACCSHDVSRAASN
XScoWnt57224      SPQCILQYSLQEAVIQCQLKFHDQRFNCESAFIHALLAASVTHGVATACCSLDTAYGAKA
XScoWnt29445      SLHLCLMRGAAKGIYECQMQASTQRWNCEAAYVHAIISIAVSAQLVKDCCTKEIISYTAK
XScoWnt26375      KILLHLRESSEWVTGHCMCQFAKSRWNGEAGYFRAALSASLVFHTARHCCDYHFTEGAAK
SyconWnt18533-2   QRRFLISRGERCAVSLCQEIMQGTRWGCERAILSALDSAGALVKLSRECYSEKTMFGAKN


OloWntI           FLNIKAGICRCHGLCGACAIKSCWKELPGAVVENKFDGSVKMYSRFCVKDSSVGSHGTHG
CcaWnt9259_1      FINVIAGGCQCHGFSGSCTVKSCWKQLPGLLVKREFDGAVKVSSDYCKYDPSTGSHGTIG
SyconWnt22889     FLNHQTGRCKCHGLTGTCAHRTCWYSLPGEKLLTKYDAAMRVSVDYCEANADIGAPGTQG
XScoWnt17541      FLNYDIGRCKCHGVTGSCSYKTCWYGLPGEQLAKKYDKIFQVSVDYCQPNMAIGSLGTHG
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XScoWnt12941      FLNNEAGRCKCHGLTGACSVKICWRSLPGFILKEKYRNAMEVSPDYCKPNATIGINGTAA
XScoWnt16518      FLNNRRGRCKCHGLCGDCTVKICWKEVRGKVLKEAYHDAKLVSPDFCVANASIGVSGTAR
XScoWnt38625      FLNNKAGRCRCMGLSGSCSDKVCWRVLPGERLKKKFSVAVKVSPSFCNANQLKASPGTKG
CcaWnt9097_1      FLNNKAGRCKCHGTSGSCALRTCWMSLQGRHIVHQYDQAVQVSPRYCVRNKLVGSLGTKG
Cre73781_5        FLNFRATECSCHGISGSCTFSVCHSELPAKRVKQAYNDSCLVSNTWCKYDPEIGSAGVVG
EmuWntC           FTNFKAGICKCLGLSGSCTTQVCWQEAPGSSIKKLFDSACVVSPNWCYADPSVGSMGVTG
SlaWnt31918       FTNFNAGICKCVGLSGSCTTQVCWQEAPGGSIKKLFDSACLVSPNWCIPDPTVGSTGVVG
EfrWnt14524       FTNFNAGICKCIGTSGSCTTQICWQEAPGSSIKKLFDSACQVSPNWCYPDPTIGSLGVVG
CcaWnt400_2       FLNEQAGLCFCHGFSGSCALRTCWLEAPGSELKKAYNKAVKVSQDFCTADKMTLQPGTIG
AmqWntB           FLNNAVGRCRCHGASGSCATRTCYSQLPSTDMKIKYNHSVKVSVKYCIRQNDY----TAN
PfiWnt51648       FLNNAAGRCTCHGISGSCSVRTCWRKLPSKNIKQKYDQSIKVSKNMCLYKQNY----TLG
EmuWntB           FLNNAVGRCSCHGISGSCTVQTCWRQL-GDVLRQKYEAAAMVTVDYCSQQSNY----TLN
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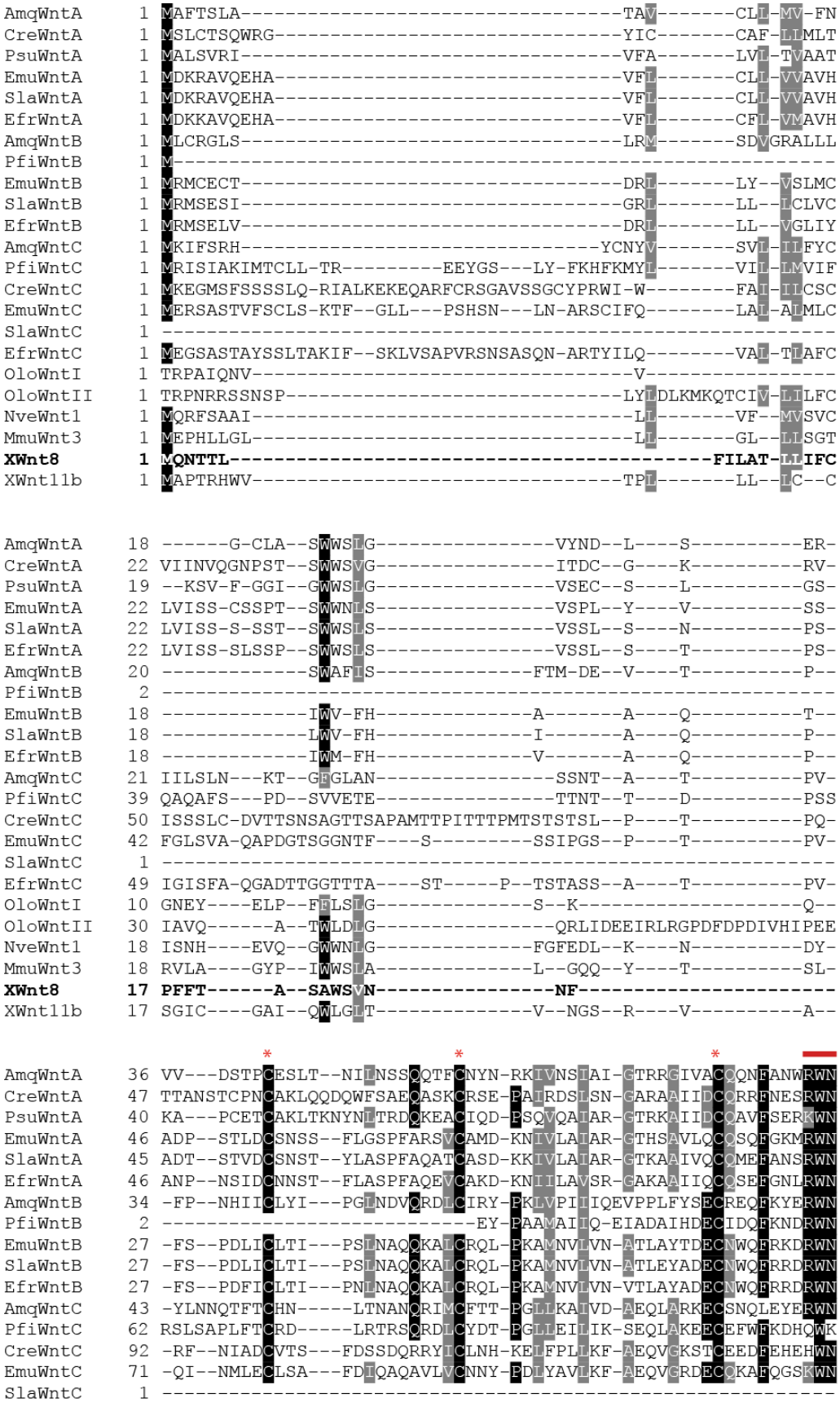
1SlaWnt12305      FLNNAVGRCSCHGISGSCTIQTCWRQLPGDVLRQKYNAAAMVTVDYCSLQSNY----TLN
EfrWnt28264       FLNNAVGRCSCHGISGSCTIQTCWRQLPGDALRQKYDTAVMVIIDYCSTQSNY----TLN
AmqWntC           FMNNQVGRCKCVGVSASCSAKTCQRGLEAASIKDKYKKSCKVSPDYCHKDISKGSFGVQG
PfiWnt10714        FLNNRLGRCRCEGLSASCSLQTCQTTLASAKIYQAYDNSCKVSVDYCYRDISVGSPGVKG
AmqWntA           FLNHNVGSCRCTGFSGSCSVQTCYFASPGQRVREKYGSSVEVSPTFCNQDTTYGILGTVG
EmuWntA           FINYQVGLCLCHGVSGTCTVQTCYQQVPGGTLRQKYTNAVKVSPTFCTADNNMGILGTSG
1SlaWnt7431       FINYQVGLCLCHGVSGTCTVQTCYQQVPGDTLSWKYINAKQVSPDFCTANNDMGILGTSG
EfrWnt21562       LTNYEVGLCVCHGVSGTCTVQTCYQQVPGDIIRWKYINAVKVTPDLCVANNNLGILGTSG
Cre66998_1        FINIDLGKCRCHGISGTCTVQTCYKRVPGEQLFIRYGGAIHVSPNFCVENRQLGTVGVAN
PsuWnt252         FVNVNAGICNCHGISGTCTVQTCYDQVIRDSLAISYNGATKVTPDLCKTDLAKGILGTAH
XScoWnt5782       FQNEKLGRCTCLGFTGSCATRVCWREVPGNTLFRLYSRAQRVTLSFCDANPSLDIAGTPG
OloWntII          FINYEAGTCRCLGGTLSCATKVCHRELRGSTLVDKYNKAVQISASLCEPNSAFGYDGIHG
XScoWnt57224      LANYAAGYCSCYGPSGSCTHKFCHVRLAAGKLWKEYRRAVQVSPNYCEQNVHLGYPGTQG
XScoWnt29445      ILNRDAGRCKCNGVSGSCLENRCYHRLP---IRGLMDNKVFLSPSYCCSDPSQGTLGTEG
XScoWnt26375      ILNNAVGRCYCTGPSGSCILKTCYVETKALALHEQYTQLLAGSPSYCRPSKKWGILGPGN
SyconWnt18533-2   FVNNMVGRCQCIGVSGSCLHKSCIGYLPKKDFGRSFNQAKQASPILCSHNKRPIGPPVGE


OloWntI           RLCDPCAHLCCNCKFWCCRIEC
CcaWnt9259_1      RECNKCSLMCCKCQFWCCHVKC
SyconWnt22889     RVCNPCTRLCCKCKFWCCRVEC
XScoWnt17541      RECVPCDEVCCACKFWCCKVHC
XScoWnt14145      RTCQGCDHLCCRCAFWCCHVRC
XScoWnt12941      RECKVCELLCCRCKFFCCDVVC
XScoWnt16518      RRCNKCELLCCACKFWCCEVKC
XScoWnt38625      RRCKPCEQLCCRCTFWCCRVTC
CcaWnt9097_1      RKCEQCLHLCCECSFWCCRVEC
Cre73781_5        RECDPCNKLCCDCQFFCCEIKC
EmuWntC           RQCDPCSSLCCNCKFYCCSIQC
SlaWnt31918       RQCDPCSSLCCNCKFYCCSIQC
EfrWnt14524       RQCDPCSSLCCNCKFYCCSIQC
CcaWnt400_2       RECNVCSYLCC-CRFWCCEVRC
AmqWntB           RSCIPCESLCCYCHFWCCKISC
PfiWnt51648       RSCVPCEDLCCNCHFWCCDIIC
EmuWntB           RYCIPCEDLCCNCKFWCCNVVC
1SlaWnt12305      RYCVPCEDLCCNCKFWCCNVVC
EfrWnt28264       RYCIPCEDICCGCKFWCCNVVC
AmqWntC           RLCDPCETICC-CKQGCCGVQC
PfiWnt10714       QSC--------------------
AmqWntA           RQCSNCDIICC-CSFYCCRIEC
EmuWntA           RQCNPCFYLCC-CQFWCCRIEC
1SlaWnt7431       RKCNPCYYLCC-CQFWCCRIEC
EfrWnt21562       RKCNPCYFLCC-CQFWCCRIEC
Cre66998_1        RICNPCASTCE-CKF-------
PsuWnt252         RLCKECANLCE-CRFWCCRIDC
XScoWnt5782       RTCQECGPLCCGCRFWCCNVKC
OloWntII          RECISCPSFCCRCKLCCFELIC
XScoWnt57224      RKCLDCARLCCECSAYFPRRVC
XScoWnt29445      RSCNGCRKVCCNCKFFCCRLQC
XScoWnt26375      RICYTCQDICCNCRMWV--LVC
SyconWnt18533-2   RYCNACHYLCCNATLTRYSYQF
```

A2.9: STRUCTURAL ALIGNMENT OF WNTS

Structural alignment of sponge Wnts against XWnt8 (bold) and other metazoan Wnts, as in Fig. 3-2, built by PROMALS 3D (Pei et al. 2008). Conserved cysteine residues are indicated with * and the conserved RWNC motif is overlaid with a red line. The inset boxed areas from Fig. 3-2 are shown in 1, blue palmitoylation site; 2, gold linker region; and 3, green predicted LRP binding sites. Particularly important residues are marked with an arrow.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AmqWntC
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CreWntC
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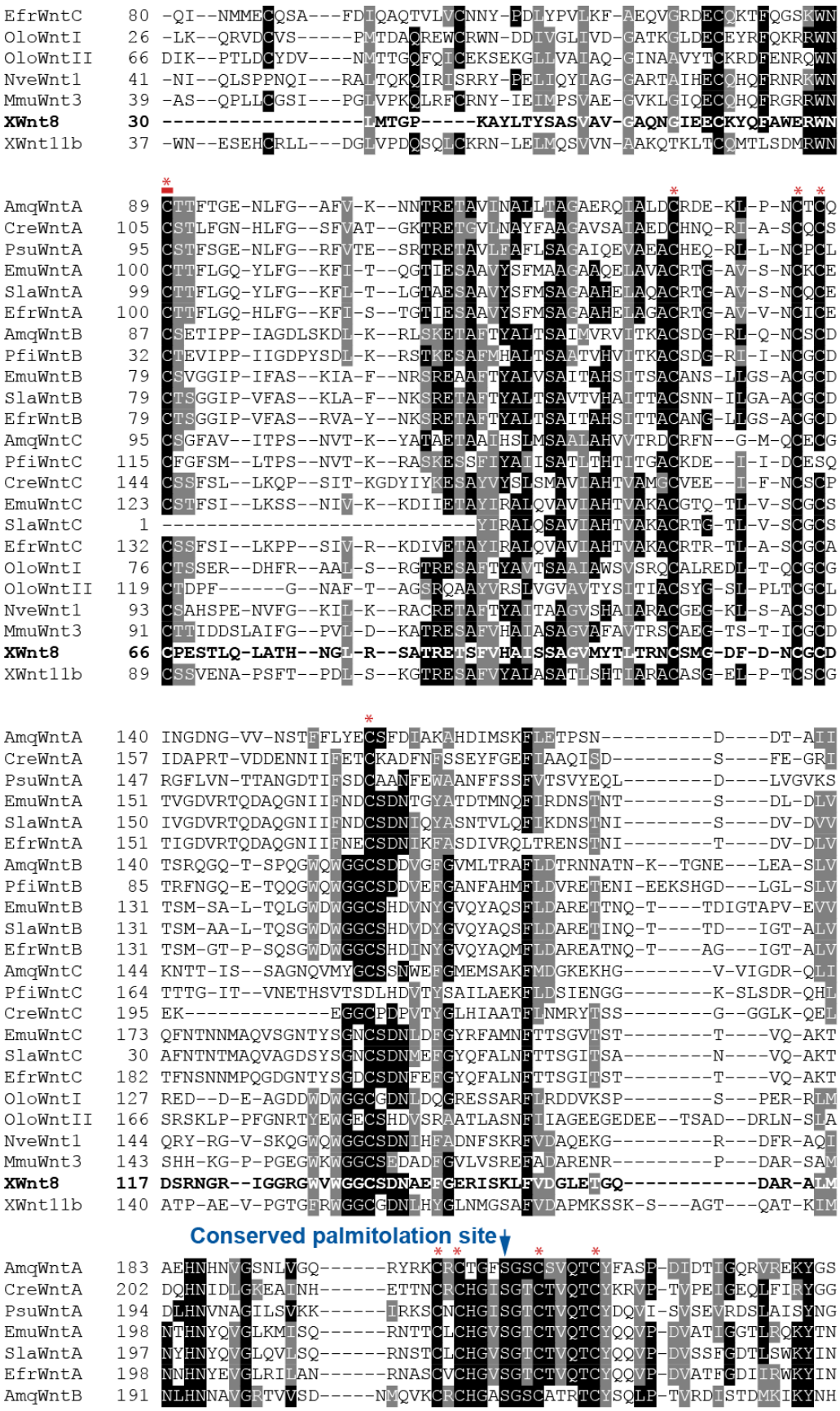
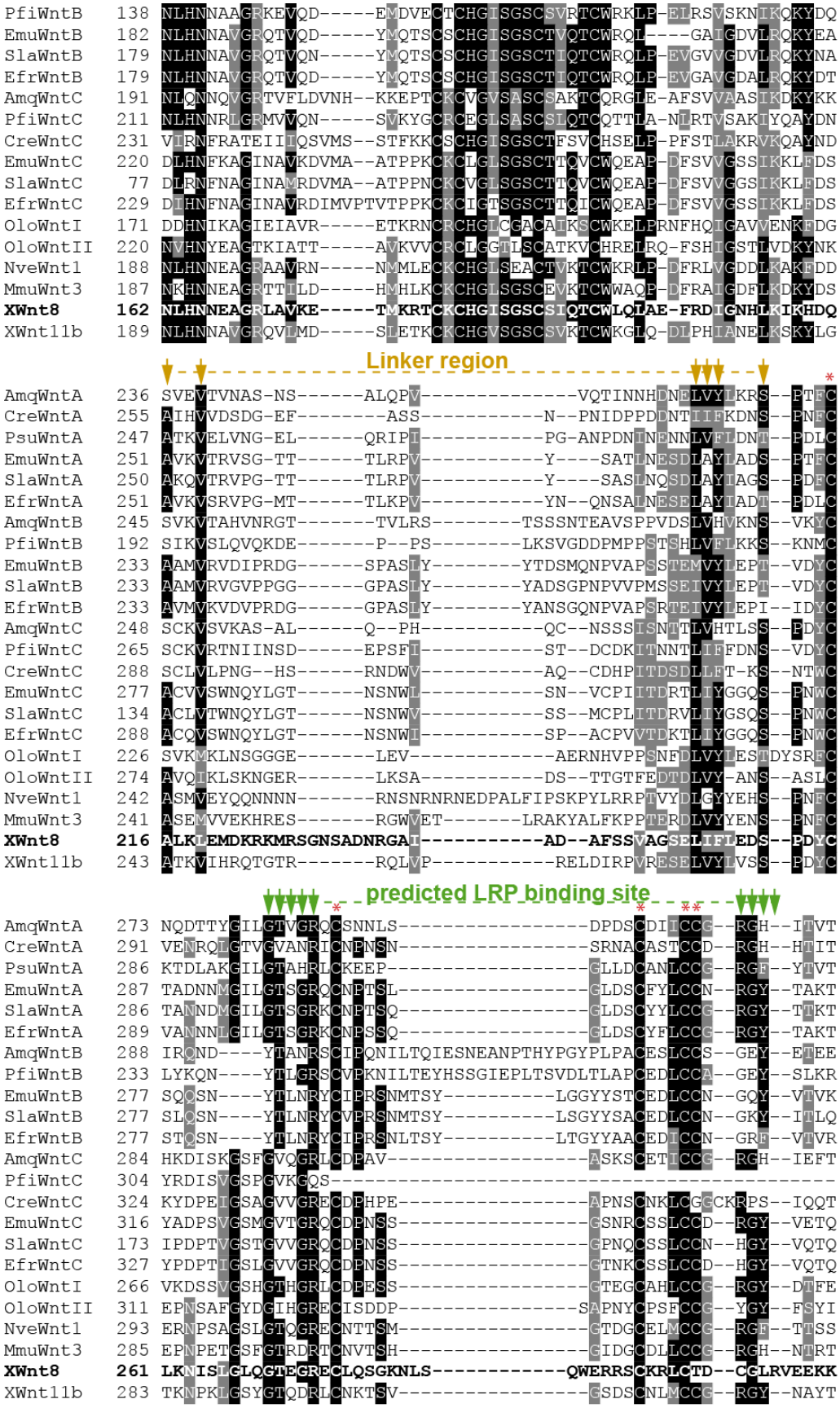
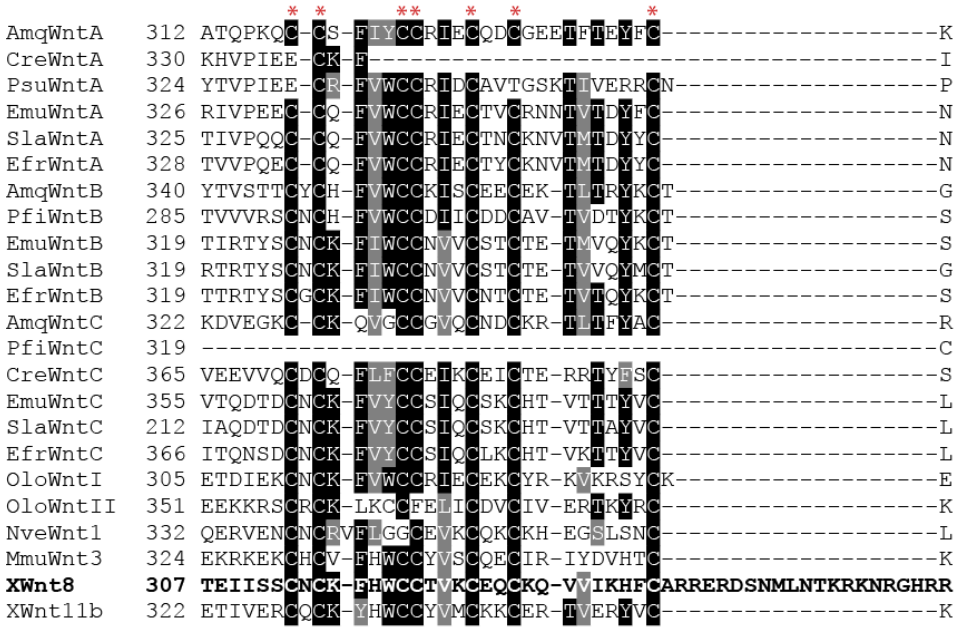

Conserved palmitolation site


Linker region
predicted LRP binding site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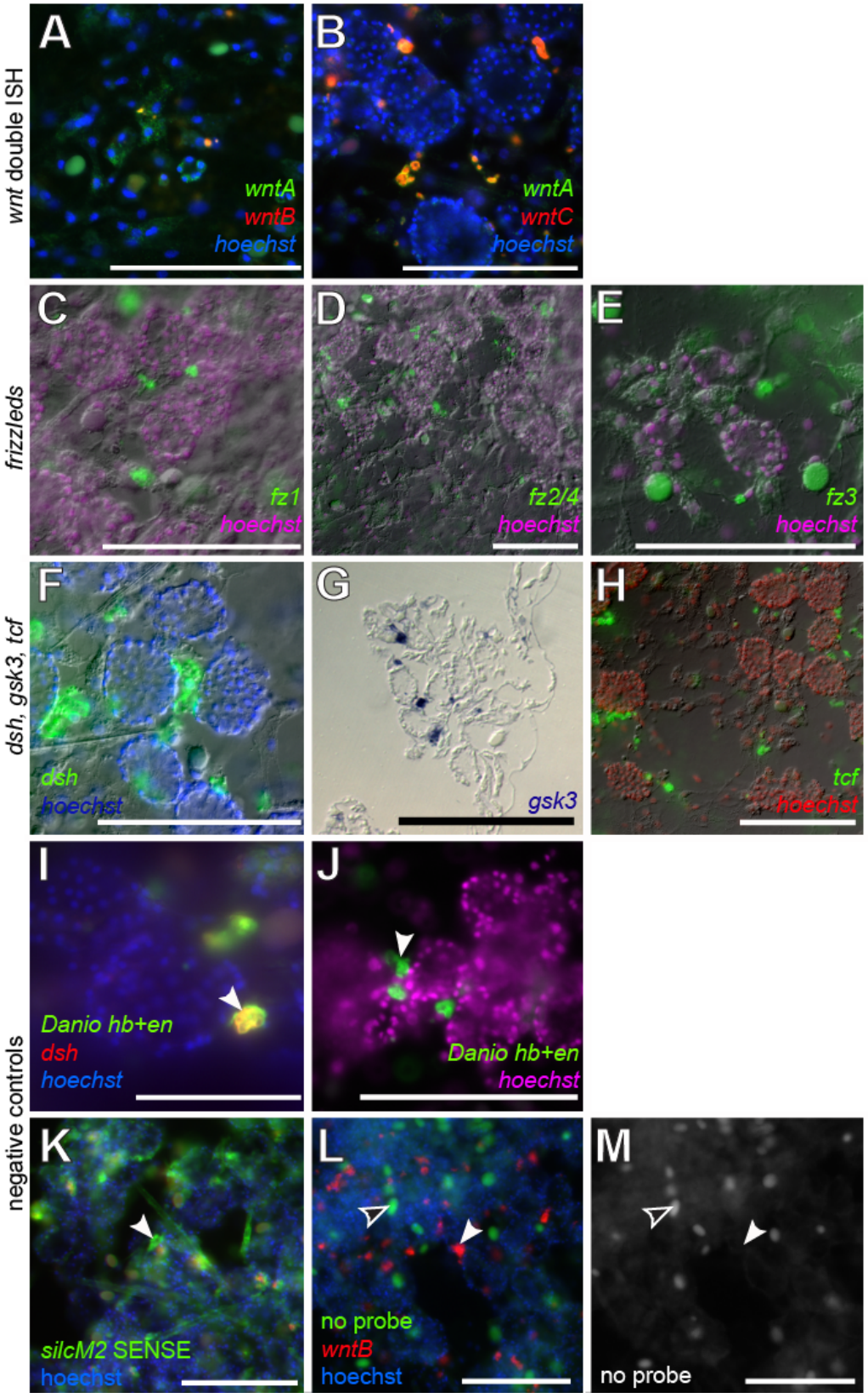
CreWntA 330 KHVPIEE-CK-F----------I
PsuWntA 324 YTVPIEE-CR-FVWCCRIDCAVTGSKTTVERRCN----------P
EmuWntA 326 RIVPEEC-CQ-FVWCCRIECTVCRNNTVTDYFC----------N
SlaWntA 325 TIVPQQC-CQ-FVWCCRIECTNCKNVTMTDYYC----------N
EfrWntA 328 TVVPQEC-CQ-FVWCCRIECTYCKNVTMTDYYC----------N
AmqWntB 340 YTVSTTCYCH-FVWCCKISCEECEK-TLTRYKCT----------G
PfiWntB 285 TVVVRSCNCH-FVWCCDIICDDCAV-TVDTYKCT----------S
EmuWntB 319 TIRTYSCNCK-FIWCCNVVCSTCTE-TVVQYKCT----------S
SlaWntB 319 RTRTYSCNCK-FIWCCNVVCSTCTE-TVVQYMCT----------G
EfrWntB 319 TTRTYSCGCK-FIWCCNVVCNTCTE-TVTQYKCT----------S
AmqWntC 322 KDVEGKC-CK-QVGCCGVQCNDCKR-TLTFYAC----------R
PfiWntC 319 ----------C
CreWntC 365 VEEVVQCDCQ-FLFCCEIKCEICTE-RRTYESC----------S
EmuWntC 355 VTQDTDCNCK-FVYCCSIQCSKCHT-VTTTYVC----------L
SlaWntC 212 IAQDTDCNCK-FVYCCSIQCSKCHT-VTTAYVC----------L
EfrWntC 366 ITQNSDCNCK-FVYCCSIQCLKCHT-VKTTYVC----------L
OloWntI 305 ETDIEKCNCK-FVWCCRIECEKCYR-KVKRSYCK----------E
OloWntII 351 EEKKRSCRCK-LKCCFELICDVCIV-ERTKYRC----------K
NveWntl 332 QERVENCNCRVFLGGCEVKCQKCKH-EGSLSNC----------L
MmuWnt3 324 EKRKEKCHCV-FHWCCYVSCQECIR-IYDVHTC----------K
XWnt8 307 TEIISSCNCK-FHWCCTVKCEQCKQ-VVIKHFCARRERDSNMLNTKRKNRGHRR
XWnt11b 322 ETIVERCQCK-YHWCCYVMCKKCER-TVERYVC----------K

A2.10: DOUBLE IN SITU HYBRIDIZATION EXPERIMENTS AND EXPRESSION OF OTHER WNT PATHWAY GENES

Expression of double in situ hybridizations (ISH), other Wnt pathway genes and negative controls in 5 dph sponges. (A) *wntA* and *B* double ISH in the peripheral region of the sponge. No convincing colocalization patterns were observed, as was the case for *wntA* and *C* experiments, (B). (C)-(E) *frizzled* expression in the choanosome shows no clear pattern or identifiable, unique cellular localization (note: the 3 frizzled sequences are named for convenience here, rather than for phylogenetic reasons). (F)-(H), *dsh*, *gsk3* (in section) and *tcf* expression, respectively, was seen only within the choanosome next to choanocyte chambers in what appears to be algal cell remnants. (I), negative control using a mixture of *Danio rerio* probes to *hemoglobin* and *engrailed* (kindly provided by Dr. Andrew Waskiewicz, University of Alberta), co-labeled with *dsh*, showing the same pattern as seen in all ISH experiments with all genes at 5 dph and older. (J) shows the same *Danio* probes without *dsh*, labelling the same region next to chambers. (K) sense probe negative control with unexpected similar labeling in the choanosome. (L), (M) no probe control showing lack of staining as expected, except in autofluorescent algal cells (black arrowhead). Scale bars in all panels = 100µm, except (I) = 50µm.


wnt double ISH
A
wntA
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hoechst
B
wntA
wntC
hoechst
frizzleds
C
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D
fz2/4
hoechst
E
fz3
hoechst
dsh, gsk3, tcf
F
dsh
hoechst
G
gsk3
H
tcf
hoechst
negative controls
I
Danio hb+en
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J
Danio hb+en
hoechst
K
silcM2 SENSE
hoechst
L
no probe
wntB
hoechst
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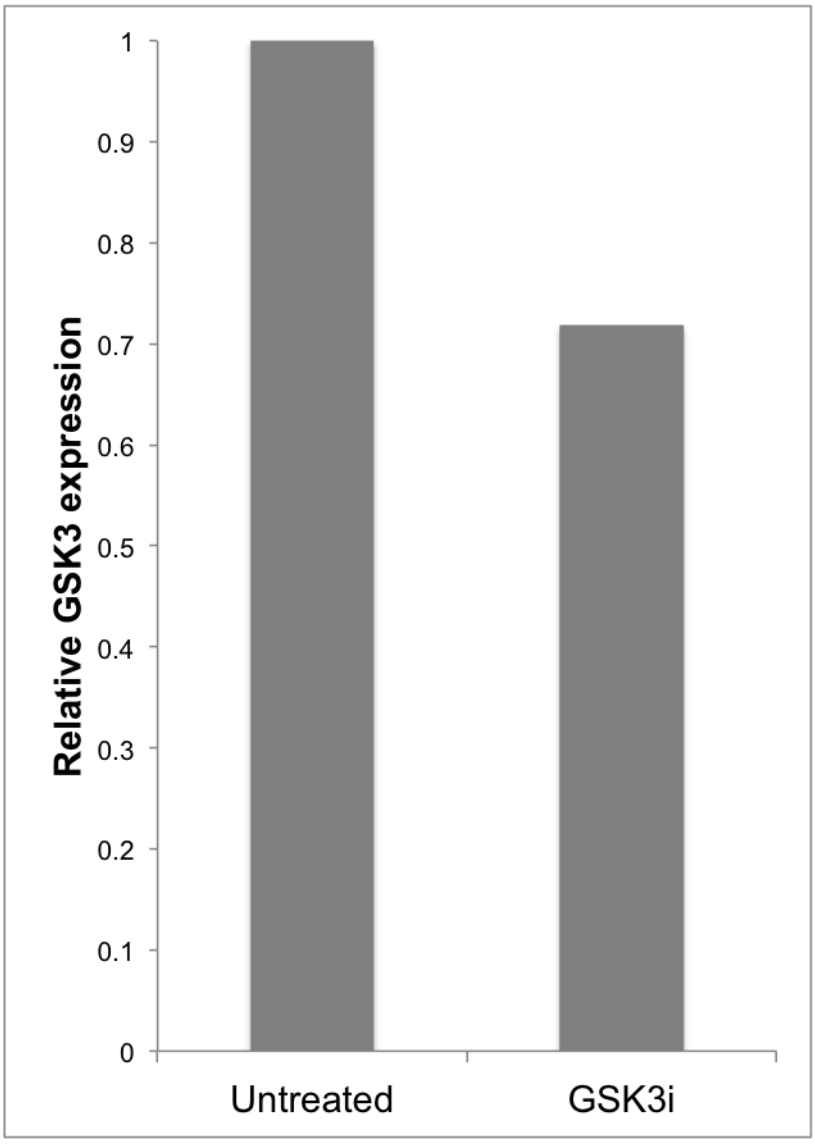
no probe

A2.11: RELATIVE EXPRESSION OF GSK3 AFTER RNAi KNOCKDOWN

Confirmation of *gsk3* knockdown by qPCR. Relative expression levels of *gsk3* in untreated versus dsRNA treated sponges (GSK3i). Expression levels normalized to *Ef1-α*.



Appendix 3:

EXPANSION, DIVERSIFICATION, AND EXPRESSION OF T-BOX FAMILY GENES IN PORIFERA[3]

A3.1 INTRODUCTION

The earliest diverging metazoans are an important group of organisms to study if we are to understand the evolutionary history and emergence of animal body plans. Though the relationships of the basal metazoan taxa (i.e., Porifera, Cnidaria, Ctenophora, and Placozoa) are still uncertain from a phylogenetic perspective (e.g., see Sperling et al., 2009), our most current understanding of metazoan phylogenomics places sponges at the base of the animal lineage (e.g. Srivastava et al. 2008; Sperling et al. 2009; Pick et al. 2010). Thus, the phylogenetic position of the Porifera as a metazoan outgroup to all other animals makes it a crucial group for exploring hypotheses regarding early animal history. Furthermore, sponges possess important body plan features that include both shared and derived characteristics when compared to other animals. Among the shared metazoan characteristics, one finds polarized body plans in larvae and adult sponges, as well as differentiated epithelia and sensory cells. Traits found in sponges but not the rest of the Metazoa include a canal system for pumping water built with choanocytes in the choanosome. Thus, sponges provide the only opportunity for comparative studies directed at 1) understanding the genetic regulatory components that were in place prior to the advent of nerves, complex tissues, and complex body plans (e.g. Simpson 1984; Larroux et al. 2006; Nichols et al. 2006; Sempere et al. 2006), and 2) how important components of the genetic regulatory machinery were utilized in an animal lineage with simple tissues and body plans.

Recent analyses of the genetic toolkit present in sponges and its regulation of development have yielded important insights regarding the early evolution of

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[3] *This appendix has been published: Holstien, K., Rivera, A., **Windsor, P.**, Ding, S., Leys, S.P., Hill, M., and Hill, A. 2010. Dev Genes Evol 220: 251-262.*

animals (e.g. Larroux et al. 2008; Sakarya et al. 2007). An important family of genes known to play crucial roles in a wide variety of developmental processes across the animal kingdom is the T-box family. This evolutionarily ancient class of transcription factors contains a large DNA-binding domain, and T-box proteins exhibit a strong conservation of DNA binding functions among family members (Naiche et al. 2005). The eponymous T-box family member, *T (Brachyury*), is instrumental in vertebrate mesodermal formation and notochord differentiation (Herrmann et al. 1990; Holland et al. 1995). Other members of the T-box family play roles in endodermal and ectodermal specification in vertebrates, function in extraembryonic tissues, gastrulation and patterning of embryonic mesoderm, cardiogenesis, limb patterning, craniofacial development, pituitary cell fate and T-cell differentiation (reviewed in Naiche et al. 2005). Given the expansive roles of T-box genes it is not surprising that the gene family is quite large with 18 members in mammals (Wilson and Conlon 2003). The biologically important roles for T-box family members have also been demonstrated in a variety of triploblastic model systems other than the vertebrates. Some genes, like *Brachyury*, have homologous functions across bilaterians in processes involving morphogenetic movements, while other family members demonstrate species-specific roles (reviewed in Wilson and Conlon 2003; Showell et al. 2004). Comparative studies indicate that the gene family includes five evolutionarily related subfamilies designated as *Brachyury/T*, *Tbx1* (including *Tbx1/10, 15/18/22, 20*), *Tbx2* (including *Tbx2/3, 4/5*), *Tbx6*, and *Tbr1* (including *Tbr1*, *Tbx21* and *Eomes/Tbr2*) (Papaioannou 2001).

T-box orthologs have been found in all of the basal metazoans including cnidarians (Technau and Bode 1999; Spring et al. 2002; Scholz and Technau 2003), ctenophores (Martinelli and Spring 2005; Yamada et al. 2007), placozoans (Martinelli and Spring, 2003), and sponges (Adell et al. 2003; Manuel et al. 2004; Larroux et al. 2006; Larroux et al. 2008). In the Porifera, T-box family members have been found in all major sponge lineages including the demosponges, calcareous sponges, and hexactinellids. T-box genes were not found, however, in the genome of the choanoflagellate *Monosiga brevicollis* (King et al. 2008), a

unicellular protist belonging to a lineage that shares ancestry with the animal lineage. This finding supported a long held view that T-box genes arose in the common ancestor of metazoans. Recently, however, putative T-box genes were detected in sequences from the genomes of the unicellular amoeba opisthokont, *Capsaspora owczarzaki* which is a close relative of multicellular animals and fungi (Broad Institute Sequence Database), and the mesomycetozoean *Amoebidium parasiticum* (Mikhailov et al. 2009). These findings reveal that T-box-like genes were lost in the choanoflagellate lineage, and evolved early in the opisthokont lineage long before multicellularity evolved.

Among the basal metazoans, roles for T-box genes have been elucidated only partially. The most in depth study of T-box function in a diploblast is from the ctenophore *Mnemiopsis leidyi*. Expression analysis of T-box genes during gastrulation and early organogenesis in this organism showed that all five ctenophore T-box family members exhibited distinct expression patterns during gastrulation and that some members are also expressed during the formation of the mouth, presumptive mesendoderm, sensory organs, and the tentacular system (Yamada et al. 2007). Further studies demonstrated that the *M. leidyi Brachyury* ortholog (*MlBra*) is expressed in ectodermal cells around the site of gastrulation and in cells derived from the blastopore; it is also involved in regulating morphogenetic movements involved with gastrulation as determined by morpholino oligonucleotide knockdown (Yamada et al. 2010). Using *Xenopus* embryos it was shown that *Xbra* and *MlBra* are functionally interchangeable, thus showing that the primitive role of *Brachyury* is to regulate morphogenetic movements involved in the blastopore (Yamada et al. 2010). In the Hydrozoa, the Hydra *Brachyury* ortholog, *HyBra1*, is expressed in endodermal cells of the head and plays a role in head formation (Technau and Bode 1999). In the anthozoan *Nematostella vectensis*, the *Brachyury* ortholog is expressed around the blastopore (Scholz and Technau 2003), and another *Brachyury* gene is expressed at the site of ingression in early jellyfish gastrula (Spring et al. 2002). In placozoans, two T-box genes show distinct expression patterns with the *Brachyury*-like ortholog

expressed in potential outgrowth zones while the *Tbx2/3* ortholog is expressed at the periphery of attached animals (Martinelli and Spring 2003).

Adult expression patterns of T-box proteins have been reported for only one species of demosponge, *Suberites domuncula* (Adell and Müller, 2005). In *S. domuncula*, a purported *Brachyury* ortholog is expressed in early sponge cell cultures ('pre-primmorphs'), when cell-cell and cell-matrix interactions are being established, and also in adherent primmorphs during a stage of tissue reorganization (Adell and Müller 2005). The latter finding may suggest a role for this *Brachyury*-like gene in morphogenetic movements through regulation of cell adhesion and migration. A second *Suberites Tbx* gene (*SdTbx2*), a likely *Tbx4/5* ortholog, is expressed during the first day of sponge cell culture and in isolated cells of the mesohyl of adult sponges suggesting possible roles in cell identity determination (Adell and Müller 2005). Expression of T-box proteins has so far not been studied during sponge embryogenesis or metamorphosis of larvae into adults, when ganstrulation-like movements may occur (Leys 2004).

The relatively limited body of data regarding the structure and function of the T-box class of transcription factors among poriferan and diploblast animals underscores why it is important that additional studies be conducted to elucidate the early evolution of this gene family. Here, we report T-box family members from degenerate PCR surveys in both a freshwater and marine sponge (*Halichondria sp.* and *Ephydatia muelleri*). We compare these sequences with T-box genes from the genomes of *Amphimedon queenslandica*, the homoscleromorph sponge *Oscarella carmela*, and other known sponge T-box genes, to illustrate that T-box gene duplication in Porifera may be more extensive than was previously believed. We show that some T-box family members appear to have been lost in certain sponge lineages. We also present *in situ* hybridization data on expression patterns during larval development in a marine sponge (*Halichondria sp.*). These findings show that T-box family members have distinct yet overlapping expression profiles, and they reveal patterns of expression at the anterior and posterior ends, as well as along the larval midline in swimming sponge larvae for two T-box family members.

A3.2 METHODS

A3.2.1 Collection and rearing of sponges

Halichondria sponges were collected from the Chesapeake Bay at Virginia Institute of Marine Science, Gloucester Point, Virginia. In the laboratory, sponges were reared in re-circulating seawater aquaria (all water was replaced weekly) or were used immediately. For larval collection, individual sponges were placed in beakers containing filtered, sterile seawater. The mother sponges were allowed to naturally release larvae into the water column. Newly released, mature larvae of *Halichondria* were easily collected as they swim toward the surface in slow spirals and congregate at the air-water interface. Larvae were washed several times in filtered, sterile seawater and were grown in 24-well plates (≈50 larvae per well) in filtered, sterile seawater, which was replaced daily.

For the purposes of this experiment, we defined four basic developmental stages: free-swimming larvae, skating larvae, attached larvae and settled/spreading tissue. Free-swimming larvae were periodically collected from the mother sponge over the course of a day; since no larvae were present when the mother sponge was initially placed in the beaker, we considered all larvae collected during the day to belong to the 0-8 hours post-release age cohort. Before settlement, the larvae of this species stop moving, sink to the bottom of the container, lose the characteristic free-swimming morphology, and skate or crawl along the substrate. We call this stage "skating" since larvae look as if they are gliding along the bottom surface. After the skating stage, larvae form an attachment to the substrate and cannot be removed without force; we define this stage "attachment". After attachment, the larval cells begin "spreading" across the bottom of the well, differentiate, and will eventually develop into juvenile sponges (i.e., rhagons).

Ephydatia muelleri gemmules were collected and harvested from sponges collected from a dam outflow near Griswold, Connecticut, USA (41˚35'4"N, 71˚55'15"W). Gemmules were washed in 3% hydrogen peroxide, and re-washed several times in sterile, cold, 1X Strekal's (Strekal 1974) media and stored at 4^{o}C

in the dark until use. Gemmules were hatched in 1X Strekal's media and grown to developmental stages 0-6 as per Funayama et al. (2005).

A3.2.2 Isolation of Tbx sequences

Halichondria bowerbanki and *Ephydatia muelleri* RNA was isolated from either larvae, reaggregated adult tissue, or hatched gemmules all of which were washed several times in sterile media before RNA was isolated using the RNeasy mini kit (Qiagen) according to manufacture's protocol. cDNA was made using Thermoscript Reverse Transcriptase (Invitrogen) using oligo(dT) and/or random hexamer primers.

Degenerate PCR reactions were performed using primer pairs designed from an alignment of the conserved T-domain from a collection of T-box genes isolated in other animals. The following primer combinations were used in all possible combinations including nesting when appropriate: GRRMFP, NP(Y/F)AKAF, TAYQNE, NEMIVT or (T/N)EMI(V/I)TK , FG(S/A)HWM. cDNA from *Halichondria sp*. from pooled larval stages and from reaggregated adult tissue or cDNA from *Ephydatia* isolated from gemmules hatched and harvested across several developmental stages served as template. Annealing temperatures ranged from 40^{o}C to 55^{o}C. All resulting PCR products of expected sizes were excised and cloned using the TOPO TA Cloning Kit (Invitrogen). Clones were sequenced using the SequiTherm EXCEL II kit (Epicenter) on a LiCor DNA Sequencing System or using an ABI sequencer. Sequences for each gene were extended (when possible) in the 5‘ and 3‘ directions using RACE (SMART RACE, Clontech). Genbank accession numbers are given in Supplemental Fig. A3-S1 where sponge sequences are highlighted according to classes.

A3.2.3 Phylogenetic Analyses

Bilaterian species were chosen based on the availability of whole-genome protein models. Sequences from two deuterostomes, *Homo sapiens (Hs)* and *Strongylocentrotus purpuratus (Sp)*, and two ecdysozoans, *Caenorhabditis*

elegans (Ce) and *Drosophila melanogaster (Dm)*, were obtained by using BLAST searching of the NCBI genome databases. Sequences from two lophotrochozoans, *Capitella telata (Ct)* and *Lottia gigantea (Lg)*, were obtained by using BLAST searching of the JGI genome databases (JGI, unpublished data) as was placozoan sequence from *Trichoplax adhaerans (Ta)* (Srivastava 2008), choanoflagellate sequence from *Monosiga brevicollis (Mb)* (King 2008), and cnidarian sequence from *Nematostella vectensis (Nv)* (Putnam 2007). Sequence from a second cnidarian species, *Hydra magnipapillata (Hm)*, was obtained from NCBI. A single sponge genome, *Amphimedon queenslandica (Aq)*, was searched using BLAST at the Trace Archive draft genome downloaded from NCBI. Four opisthokont genomes, *Allomyces macrogynus (Ama), Capsaspora owczarzaki (Co), Spizellomyces punctatus (Spun),* and *Proterospongia sp (Ps),* were searched using BLAST at the Broad Institute website (http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html). Additional cnidarian, ctenophore, and poriferan sequences were obtained from the published literature (Bielen et al. 2007; Yamada et al. 2007; Martinelli and Spring 2005; Adell et al. 2003; Spring et al. 2002) or using the methods described above. EST sequences from *Acropora mellifera (Am)*, a cnidarian coral, were also used and were obtained by BLAST searches of a larval EST database (http://sequoia.ucmerced.edu/SymBioSys/) and genomic sequences from *Oscarella carmela (Oc*) were obtained via BLAST searches by Dr. Scott Nichols (unpublished data).

Alignments were performed using Muscle (Edgar 2004) implemented in Seaview using the default settings. Sequences were trimmed to contain only the Tbox regions. Gblocks was run under a variety of conditions and yielded subsets of the sites we used in our phylogenetic analysis (Dereeper et al. 2008). We employed both Maximum Likelihood (ML) and Bayesian approaches in our phylogenetic analyses. A model of sequence evolution was determined from aligned sequences using ProtTest (v1.2.6, Abascal et al. 2005). PhyML (Guindon and Gascuel 2003) was used for ML analysis with 500 bootstrap replicates; in PhyML gaps are treated as ambiguous characters. All parameters were optimized

based on empirical data. Bayesian analysis, as implemented in Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001), was performed until the average standard deviation of split frequencies achieved stationarity (n = 2,000,000 generations). We used four independent chains in our analyses. The gamma distribution for among site substitution rates was approximated using four rate categories with a proportion of invariable sites. The first 25% of the samples were discarded as burn-in. All branches with less than 60% support were collapsed in the ML tree shown (Fig. A3-1).

A3.2.5 Expression analyses

Tissues from the various developmental stages were either stored in RNAlater (Ambion) overnight and then placed at -80^{o}C for subsequent RNA isolation or fixed for *in situ* hybridization. RNA was isolated using the RNAeasy Mini Kit (Qiagen) and treated on column with DNase I to limit contaminating genomic DNA. For RT reactions, 200 ng of RNA was reverse transcribed using the Thermoscript RT kit (Invitrogen) and subsequent PCR reactions were first carried out using Platinum Taq DNA polymerase (Invitrogen) to test gene specific primers and RT reactions. SYBR Green chemistry and the Chromo4 (BioRad) were used for qRT-PCR using cycling conditions of: 94^{o}C for 3 min followed by 30s 94^{o}C, 30s 55-61^{o}C, 1 min 72^{o}C for 35 cycles. Gene specific primers were used to amplify isolated *Halichondria* Tbx genes for profiling expression by qRT-PCR across developmental stages. In each case, controls were performed to ensure expression levels were from cDNA and not contaminating genomic DNA and that each primer pair only amplified the target Tbx gene (as determined by testing each primer pair on plasmids for each Tbx gene). Actin was picked to standardize the amount of expression calculated for *Tbx* genes at each developmental stage because it has often been utilized in other systems (including cnidarians) for this purpose **(**McCurley and Callard 2008, Rodriguez-Lanetty et al., 2008; Yüzbaşıoğlu et al., 2010**).** Nonetheless, we used qRT-PCR to compare actin mRNA levels at each developmental stage compared to total RNA amounts and though some degree of variability was observed, the expression stability

across larval developmental stages was high. Further, we have also used Ef1α (Siah et al., 2008; Curtis et al., 2010) as a standardization control for some of these genes as well, and do not see differences in the relative expression profiles. For each gene, data from two different batches of RNA was assessed and all PCR reactions were performed with two replicates.

To determine mRNA distribution in larvae we used a protocol similar to that described in Hill et al. (2010), but adapted for the larvae. Briefly, sponge tissues were fixed overnight in 4% paraformaldehyde, 0.02% glutaraldehyde in sterile seawater and then transferred into ascending concentrations of methanol, and stored in 100% methanol at -80°C. Fixed tissues were rehydrated through a methanol and PTw (1X PBS containing 0.1% Tween-20) series. Tissues were prepared for prehybridization through three washes in 1X PTw and one wash of 1X PTw containing Proteinase K (1 μg/mL) at 37 °C. (Alternatively, in some cases, tissues were washed for 30 min in detergent solution (1% SDS, 0.5% Tween, 50 mM Tris-HCl pH 7.5, 1 mM EDTA and 150 mM NaCl) followed by six washes in PTw with no differences observed). The tissues were then re-fixed in 4% paraformaldehyde in PBS, washed twice with 0.1 M TEA buffer (pH 8) and then treated once in 0.1 M TEA containing 0.25% acetic anhydride, followed by two washes in 1X PTw. Tissue was processed to a 1:1 solution of hybridization buffer (50% formamide, 5X SSC, 50 mg/ml heparin, 0.25% Tween-20, 1% SDS, 100 μg/ml sheared salmon sperm DNA; pH 5) and PTw. Tissue was then prehybridized in hybridization solution at 60°C for at least 3 hours. All probes were labeled using the Dig RNA labeling kit (Roche). After overnight hybridization at 45-60°C, tissue was washed 7 times in hybridization solution at 60°C and gradually processed to room temperature through half washes in TBST and hybridization solution. Alternatively, after overnight hybridization, tissue was washed three times in 2X SSC, twice in 1X SSC, and once in TBST or NTE buffer containing 20 ug/mL RNAse A at 37 °C, followed by two washes of 0.1X SSC at 37 °C. After a several washes in TBST, tissue was incubated in TBST containing 1% BSA to block nonspecific binding of antibody. Anti-Dig alkaline phosphatase antibody (Roche) was diluted 1:3000 in TBST with BSA and larvae

were incubated overnight in this solution at 4°C. Larvae were washed 5 times in TBST and and then processed for staining using NBT and BCIP in AP reaction buffer. After staining, embryos were then cleared in 80% glycerol/PBS before imaging. For further validation of the in situ staining patterns, for some genes (*TbxA* and *Tbx*) two different probes were utilized that were directed to different portions of the genes. In these cases, no differences in staining patterns were observed. For *TbxA*, one probe was labeled for position 697-928 nt which included the 3' portion of the T-domain. The alternate *TbxA* probe was 1,527 nt in length and corresponded to the entire *TbxA* mRNA sequence including 3' UTR. For *Tbx4/5*, one probe included a 428 nt coding region containing the majority of the T-domain and the alternate probe was a 3' RACE product that included a 195 bp overlap with the first probe (from forward 5' primer: GCGGTATGGGAGAAGCAGCTGAT) and extending into the 3' end of the clone.

A3.3 RESULTS AND DISCUSSION

A3.3.1 T-box gene families in basal metazoans: divergent evolution

While the relationships between many T-box sub-families remain ambiguous, several families, along with new members of those families, have high support in our phylogenetic analysis (Fig. A3-1). The clear sub-families, supported by both Bayesian and Maximum Likelihood analysis, include bilaterian-specific groups, Cnidaria+Bilateria-specific groups, Porifera+Cnidaria+Bilateria-groups, and Porifera-specific groups (Fig. A3-1). The sponge-specific groups include a demosponge-specific *TbxPor* clade and a demosponge-specific clade within the *Tbx4/5* group (a homoscleromorph *Tbx4/5* sequence is not included in that clade). Also supported are a demosponge specific lineage we call *TbxA*, and a demosponge specific lineage designated as *TbxC/D*. Finally, there is another group identified in Figure A3-1, *TbxA/B/C/D/E*, that is supported by the ML tree, but not by the Bayesian tree. It is intriguing that this group includes protist, poriferan, and placozoan *Tbx* genes. These sequences in the *TbxA/B/C/D/E* group

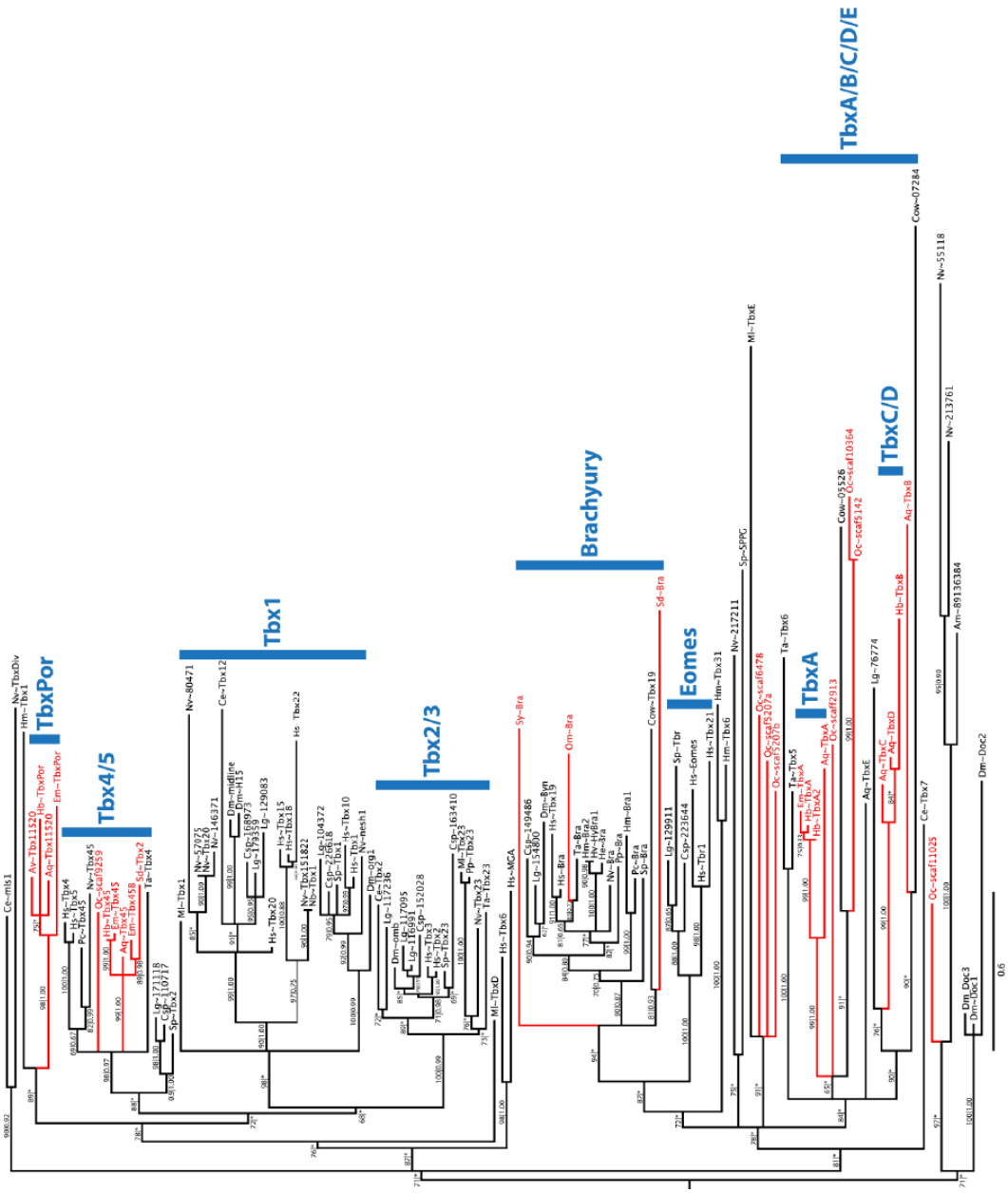

Figure A3-1: Unrooted ML phylogenetic tree of the T-box gene family. The T-box phylogeny was evaluated with both maximum likelihood (ML) and Bayesian methodologies. Bootstrap support values and posterior probabilities are shown in the numerator and denominator respectively. The topology shown includes clades with >60% support. Major families are identified with blue bars, sponge genes are identified in red. Accession numbers for each of the entries are provided in the supplementary files.

may represent ancestral *Tbx* genes. However, this is a region of the tree that could suffer from long-branch attraction issues (Felsenstein 2004). Additional sequences from other protists and basally branching animals would be necessary to address this issue, and the hypothesis that these *TbxA/B/C/D/E* genes are ancestral and not lineage specific duplications.

Given that some T-box family clades do not contain poriferan representatives and that the sponges appear to contain unique T-box families, we propose that the Urmetazoan had at least one T-box gene that underwent several rounds of independent duplication and divergence after the sponges split from other metazoans. While synteny studies remain to be performed, our phylogenetic analyses suggest that a proto-T-box gene evolved before the advent of multicellularity since we find three T-boxes in the genome of a unicellular opisthokont amoeba, *Capsaspora owczarzaki* (50-60% similarity to *Homo sapiens* T-brain T-box). This kind of large-scale duplication and divergence has been observed repeatedly for many eumetazoan "toolkit" genes (e.g. Larroux et al. 2008; Yamada et al. 2007). However, the presence of T-box genes in the protistan genome could be the result of lateral gene transfer, though we believe common ancestry is the most parsimonious explanation of the data.

Our analysis recovered three demosponge-specific *Tbx* clades. The *TbxPor*, *TbxA*, and *TbxC/D* clades have strong support in both Bayesian and Maximum Likelihood analysis. They are comprised of T-box sequences from four, three, and two sponge species respectively, and each clade contains two of the orders in the Demospongiae (Haploclerida and Halichondrida). Additionally, our analysis indicates that there are a number of divergent T-box genes in Porifera (e.g., *AqTbxE* and several putative *Oscarella carmela* T-box sequences) that are not associated with any specific T-box clades. Another sponge sequence, *AqTbxB* groups with the amoeboid *C. owcsarzaki* with weak support.

While the sponge-specific groups contain only demosponge sequences (i.e., they lack representatives from the Calcarea or Hexactinellida), it is unclear whether this is due to demosponge-specific duplications or because of the extremely small amount of sampling that has been done in Porifera. However,

with two poriferan genomes (*Amphimedon queenslandica* and *Oscarella carmela* (S. Nichols, unpublished data), and our exploration using degenerate PCR in *H. bowerbanki* and *E. muelleri,* we can make inferences about the evolution of *Tbx* genes in sponges. For example, it is clear that the non-bilaterian *Tbx* clades contain a large number of sponge members. This, and the presence of several sponge-specific groups, is suggestive of extensive duplication and divergence of *Tbx* genes specific in the sponge lineage.

Tbx1 subfamily (Tbx1/10, 15/18/22, 20)

The *Tbx1* subfamily contains bilaterian, cnidarian, and ctenophore members. This subfamily has no sponge members, making it likely that it arose after sponges split from the rest of Metazoa. The *Tbx-1/10* clade groups with the *Tbx-15/18/20/22* clade, a clade with Bilateria+Cnidaria genes. These groups were likely present in the bilaterian/cnidarian ancestor, but not in the Urmetazoan unless Tbx1 was lost in the sponge lineage. The evolutionary history of the ctenophore (*M. leidyi) Tbx1* gene is unclear and since there is no ctenophore genome sequenced and the position of ctenophores relative to bilaterians and cnidarians is debated, there may be additional ctenophore *Tbx1* subfamily members. Our *Tbx1/10* group is consistent with results from previous studies (Larroux et al. 2008; Yamada et al. 2007). In those studies, sponge sequences *AvTbx1/15/20* and *AqTbx1/15/20* were placed in the *Tbx1* subfamily. In our analysis, a separate poriferan-specific clade, that we call *TbxPor*, has strong support. Whether this clade is part of the *Tbx1* subfamily or a distinct Tbx lineage will require greater resolution.

Tbx2 subfamily (Tbx2/3, Tbx4/5)

The *Tbx2/3/4/5* subfamily genes are most often known for their demonstrated roles in heart and eye development and in the evolution of developmental programs involved in appendage outgrowth and patterning across the vertebrates (reviewed in Papaioannou 2001; Horton et al. 2008). While our ML analysis (but not Bayesian) supports the grouping of Tbx2/3 with Tbx1, though with low

bootstrap support, synteny analyses across Metazoa suggest that Tbx2/3 and Tbx4/5 are more closely related (see below). However, a fuller understanding of the true evolutionary history of the origins and order of appearance of these gene families await additional data from more species. By our analysis *Tbx2/3* clade has high support and comprises genes from bilaterian, cnidarian, placozoan, and ctenophore species (Fig. A3-1). Sponges are not represented in the *Tbx2/3* group. The Tbx-4/5 sub-family is one of only two large clades in our phylogeny with representatives from Porifera, Placozoa, Cnidaria, and Bilateria (Fig. A3-1). We recovered a single clade containing all known sponge *Tbx4/5* members (Fig. A3-1). Interestingly, while *A. queenslandica* and *O. carmela* have single *Tbx4/5* representatives, there are two *E. muelleri Tbx4/5* genes, possibly representing a duplication within the spongillid sponge lineage. The lack of a ctenophore *Tbx4/5* may be due to incomplete sampling or gene loss in this lineage (it is known that *Tbx4/5* has also been lost in two major bilaterian lineages (Horton et al. 2008)).

Close linkage of the *Tbx2/3* to the *Tbx4/5* genes has been reported across chordates (except ascidians) and cephalochordates (Horton et al. 2008), and recently it was shown that the *Nematostella* genome contains a *Tbx2/3* and *Tbx4/5* gene in the same orientation within 20 kb of each other (Yamada et al. 2007). It has thus been proposed that a duplication of an ancestral *Tbx2/3/4/5* locus pre-dated the divergence of modern diploblasts and triploblasts (Horton et al. 2008; Yamada et al. 2007). There are two possible historical explanations for the distribution of *Tbx2* subfamily genes as indicated by our data. First, the *Tbx2/3* gene may have been lost in the poriferan lineage. This would imply that duplication of the *Tbx2/3/4/5* ancestral gene occurred before the sponges and Metazoa diverged; alternatively, that duplication event occurred after sponges and Metazoa split. The problem with this interpretation of the data is that there is strong support for sponge *Tbx4/5* genes in our phylogeny. Either convergent evolution pushed the sponge lineage toward the diploblast/triploblast *Tbx4/5*-like gene sequence, or the ancestral *Tbx2/3/4/5* gene was *Tbx4/5*-like.

Brachyury/T subfamily

The *Brachyury* clade is the only large group with representatives from all phyla sampled, including an amoeba sequence from *C. owczarzaki.* As *Brachyury* is typically associated with mesoderm development, gastruation, and morphogenic movements, we were surprised to recover *Brachyury*-like sequences from a unicellular organism. Placozoan, ctenophore, and cnidarian sequences also fell into this clade as well as sequences from 3 sponges - *Oopsacas minuta* (*OmBra* – a hexactinellid), *Suberites domuncula* (*SdBra* - a demosponge) and *Sycon raphanus* (SyBra - calcareous sponge). Of particular relevance to this point, it should be noted that neither the *Amphimedon queenslandica* genome or the *Oscarella carmela* genome possess a gene in the *Brachyury* clade, nor did we recover a *Brachyury*-like gene from *Halichondria sp.* or *Ephydatia muelleri* despite numerous attempts with *Tbx* and *Brachyury*-specific primers. Several explanations of this pattern are possible. It is possible that the *Brachyury*-like gene was lost in some sponge lineages. Alternatively, sponges may lack a true *Brachyury* gene, and those sponges that do fall in this group may have converged on similar signature sequences.

To further distinguish between these possibilities, we examined key residues in the T-box regions of proteins falling within the *Brachyury* clade (See Supplemental Figure A3-S2). While the placozoan, ctenophore, and cnidarian sequences appear to represent true *Brachyury* proteins, inspection of the sequence alignments reveals that sponge and protist sequences are likely not true *Brachyurys*. *Brachyury* is the most well studied Tbx gene family and, as such, functionally and phylogenetically important residues have been elucidated. Among putative DNA and protein binding residues (~30 total (Müller and Herrmann 1997)), there are two *Bra*-specific residues that differentiate *Brachyury* genes from other closely related T-box family members (excepting *S. purpuratus*). These are a diagnostic Lys106, involved in DNA-binding specificity (rev. in Wilson and Conlon 2002), and Met-42, potentially involved in dimerization (Müller and Herrmann 1997) (all numbering is based on the *Drosophila Byn* T-box protein sequence starting at LDDRELW). A third residue,

Asn-85, is found in all canonical *Brachyury* and *Eomes/Tbx* genes recovered in our analysis, excepting *S. purpuratus*. This residue is a potential synapomorphy of the Brachyury + Eomes clade and is not found in protist or *Sy-Bra*.

Both the protist and *SyBra* lack Lys106 and have the canonical T-box Arginine at this position (note that the Eomes group has an Asparagine at this position, unlike most other Tbox groups). Of the two sponges included in the *Brachyury* clade we recovered (Fig. A3-1), the known *OmBra* sequence is extremely short and lacks much of the T-box domain. Of 30 residues directly involved in DNA binding and protein-protein interactions, 11 have not yet been elucidated from the *OmBra* sequence. The third sponge sequence, *SdBra* does not have the Met-42. Interestingly, the functionally important Alanine at position 171 in *Brachyury* genes is also found in the *TbxPor* group. This residue is known to be involved in DNA-binding (Muller and Hermann 1997), and is typically replaced with a Guanine in other T-box genes. Since *Brachyury* and *TbxPor* are not sister groups, it seems likely that they converged on this Alanine, especially due to the fact that it has functional significance. The potential convergence in T-box sequence is likely due to strong selective pressures on these sites given the constraints of DNA-binding and dimerization. This may also be responsible for the difficulties encountered in resolving branching orders among the T-box families (i.e. the high degree of polytomy) and the presence of non-*Bra* genes in the *Brachyury* sub-family. The presence of T-box genes but absence of a *Brachyury* homolog in some sponges may be important to our understanding how sponges form tissues without undergoing the typical or 'conventional' morphogenetic movements seen in gastrulation in other animals (e.g., Stern 2004). Whereas sponge embryos show cellular differentiation and form layers made of distinct cell types during early development, a feeding epithelium (equivalent of a gut) is only formed at metamorphosis (Leys 2004). T-box's may therefore be involved in directing morphogenetic events involved in the formation of polarity during larval development and at metamorphosis.

A3.3.2 Distinct larval expression profiles of Poriferan T-box genes

We used real time RT-PCR to assess relative levels of expression for four *Halichondria Tbx* genes. For each gene, we determined expression levels during settlement and attachment relative to free-swimming larvae, across four larval developmental stages (Fig. A3-2). The first stage examined was free-swimming larvae, which consisted of a pool of larvae that were collected from the top of the water column between 0-24 hours post larval release. Though these larvae are positively phototactic throughout the free-swimming period (which typically lasts 48 hours but can continue past 72 hours), they enter a stage where they swim at or near the bottom of the dish where they may temporarily settle. This behavior has been described in a variety of sponges (see Simpson 1984) as 'creeping,' 'crawling,' or 'preattachment' and may involve cilia cell-substratum interactions. We call this stage 'skating' since the larval behavior looks more like a gliding movement than a creep or a crawl in *Halichondria*. In fact, the larvae are often observed to be spinning on their axis as they glide along the bottom of the dish. During the skating stage, larvae may resume swimming near the bottom of the dish if a pulse of water current is applied near their site of contact, but they resume skating quickly after the disturbance. The next stage we collected at included larvae that were attached to the dish and clearly had basopinacocyte formation that adhered the larvae to the substrate. Finally, we collected larvae that had begun the metamorphosis process with proliferative (archaeocyte) cells that were 'spreading' across the surface of the dish. In this study, we did not follow development through metamorphosis to the juvenile rhagon stage because of a parasite that preys on the juveniles that we could not eliminate from the cultures without also compromising the sponge's development. Future studies using another species of demosponge (*Ephydatia muelleri*) will be aimed at examining the role of orthologs to these *Tbx* genes during metamorphosis and adult sponge function.

The *TbxA* gene showed the highest expression levels during the "skating" stage relative to free-swimming and had very low expression in spreading larvae. *TbxC/D*, *Tbx4/5*, and *TbxPor* all showed the greatest expression levels at larval

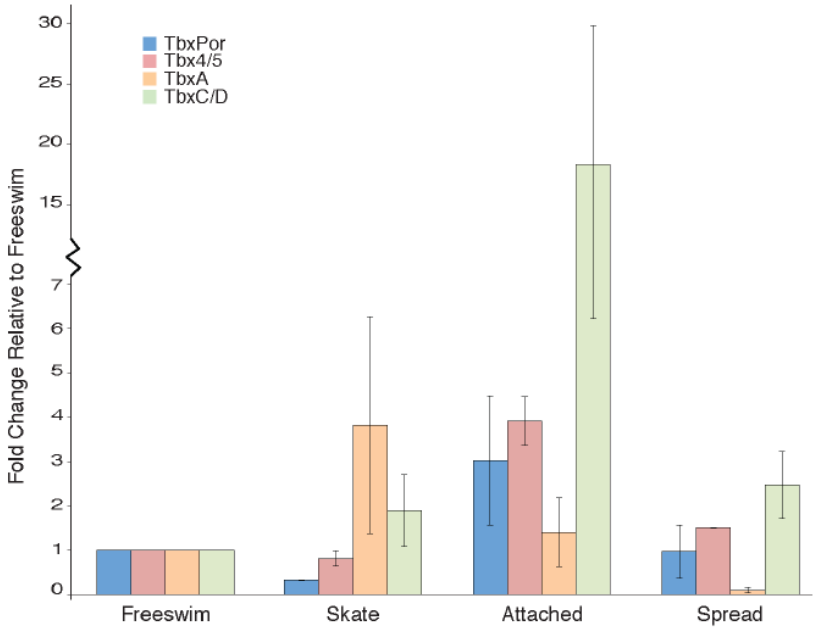

Figure A3-2: qRT-PCR analysis of T-box gene expression during larval developmental stages of *Halichondria.*
Gene expression levels are plotted relative to free-swimming larvae and normalized to actin expression levels at each developmental stage. Y-axis denotes relative levels of expression.

attachment to the substrate. For *Tbx4/5*, expression during larval attachment is more than two-fold higher than at the spreading stage and is more than three-fold higher than free-swimming or skating stages. *TbxC/D* expression at attachment is up to six-fold higher than all other developmental stages. The overall expression profile for *TbxPor* and *Tbx4/5* are similar with attachment as the highest expression level, spreading as the next highest, followed by free-swimming larval expression and skating as the lowest level of expression. We did not assay expression levels of the *TbxA2* gene since it was discovered much later in our study, (during RACE of TbxA) and we would not have been able to compare qRT-PCR data directly with the other genes since each gene was analyzed from multiple matched sets of RNA/cDNA at each developmental stage. Nonetheless, it is clear from the expression profiles for *TbxA*, *TbxC/D*, *Tbx4/5* and *TbxPor* that there has been some level of functional divergence of these *Tbx* genes. The different levels of expression over several developmental stages suggests that the sponge T-box genes have undergone divergence in their cis-regulatory regions, at least. This is likely tied to some functional divergence, which might be as simple as a split in the timing of deployment or as complex as a completely novel function for one of the duplicates.

To identify patterns of expression and possibly suggest functions, the expression of sponge T-box genes were also assayed by whole mount in situ hybridization to newly released sponge free-swimming larvae (Fig. A3-3). These larvae are about 250-300 μm long, and have a ciliated columnar epithelium (CE). Immediately inside the CE is a sub-epithelial layer of cells that surrounds a large spicule-containing inner cell mass (ICM). At maturity, the posterior pole of the larvae will have longer cilia (~36 μm in length compared to ~12 μm around the rest of the surface (see Fell and Jacob 1979). Since all five *Halichondria* T-box genes were expressed in 0-24 hour free-swimming larvae, we chose this stage for initial analysis. Given that expression profiles for genes in this species of larvae have not been reported, we include a supplemental figure (Supp. Fig. A3-S3) showing positive controls for expression of actin which hybridizes in all cells and the *BarBsh* gene which has previously been reported to be expressed in the inner

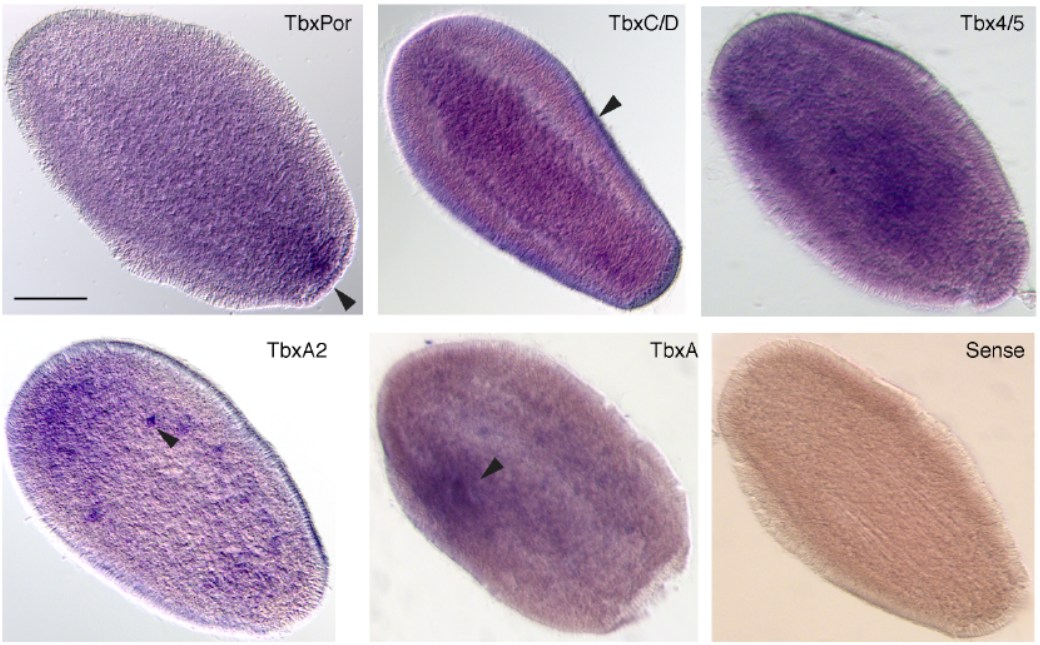

Figure A3-3: Expression of T-box genes in early stage free-swimming *Halichondria* larvae with whole-mount in situ hybridization
Anterior poles of larva are oriented at the top left of each panel. The sense probe for *HbTbx 4/5* is shown, other sense probes also exhibited no staining. *HbTbxPor* is highly expressed at the poster pole as indicated by black arrow. *HbTbxC/D* is expressed in the columnar epithelial layer (CE, black arrow) as well as throughout the subepithelial layer (SE) that is directly beneath the CE and also in the inner cell mass (ICM). *HbTbx4/5* is expressed throughout the ICM and SE, but not in the CE. *HbTbxA2* is expressed at the anterior end of the larvae and in cells around the inner cell mass (black arrow). *HbTbxA* reveals an asymmetric expression pattern on one side of the larvae as indicated by black arrow.

cell mass of *Amphimedon queenslandica* larvae (Larroux et al. 2006). We also observe expression of *BarBsh* exclusively in the inner cell mass of *Halichondria* larvae thus illustrating that the expression patterns we observe in this study have been validated with positive and negative controls (Supp. Fig. A3-S3).

Each T-box gene exhibited a distinct pattern of expression, though some of the expression patterns (e.g., *Tbx4/5* and *TbxC/D*) were less pronounced. The *TbxPor* gene seemed to be most concentrated at the posterior end and spread toward the anterior within the inner cell mass. *Tbx4/5* staining is nearly ubiquitous throughout the inner cell mass and subepithelial layer at this stage, however, it does not seem to be expressed in the columnar epithelium. *TbxC/D* staining is evident in all cells (though most concentrated in the inner cell mass), including the ciliated columnar epithelium and it is the only T-box gene identified that is evidently expressed in these cells (see arrow). The *TbxA* gene has an interesting expression domain that is concentrated on one lateral side of the larvae (see arrow, Fig. A3-3). This apparently asymmetrical pattern of expression in symmetrical larvae is enigmatic. Finally, *TbxA2* has an expression pattern that includes a concentration of staining at the anterior end with several small foci (see arrow) of cells around the outside of the inner cell mass (Fig. A3-3). These data support the hypothesis that the T-box genes have diverged in the poriferan lineage and may perform sub-functionalized their roles in larval development.

To further investigate the potential polarity of expression observed for the *TbxPor* gene, we examined expression in later stage free-swimming larvae. These larvae have a pronounced ciliated posterior pole and can often be seen making connections with the substrate at this end. Interestingly, we see distinct expression of *TbxPor* (Fig. A3-4,A-C) at both the anterior and posterior poles of the larvae. The expression at the posterior pole is most concentrated at the far posterior and less concentrated in more anterior cells as was seen in the earlier larvae (Fig. A3-3). At the anterior pole, there are distinct cells, mostly along the subepithelial layer that express *TbxPor*. The expression in the subepithelial layer extends around the lateral sides of the larvae as well. We also examined expression for *Tbx4/5* in late free-swimming larvae. Like *TbxPor*, expression is most

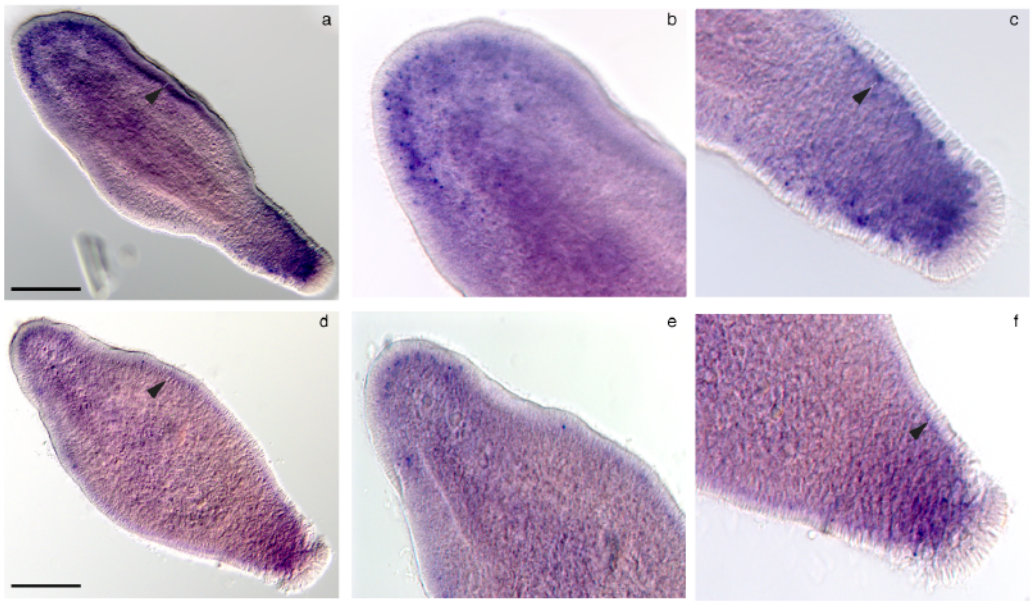

Fig. A3-4 Expression of *HbTbx4/5* and *HbTbxPor* in late stage free-swimming *Halichondria* larvae by whole-mount in situ hybridization
Anterior poles of larvae are oriented at the top left. B and E are higher magnifications of the anterior poles and C and F are higher magnifications of the posterior poles. A-C. *HbTbxPor* expression is most concentrated at the anterior and posterior pole of the larvae with expression extending along the midline, mostly in the sub-epithelial cell layer (black arrow). D-F. *HbTbx4/5* expression is also seen at the anterior and posterior poles with expression extending along the midline in the sub-epithelial layer (black arrow).

concentrated at both the posterior highly ciliated pole and also at the anterior pole. Furthermore, expression is also observed around the lateral sides of the larvae in the subepithelial layer. These interesting patterns of expression in free-swimming larvae may suggest some involvement of *TbxPor* and *Tbx4/5* in establishing or maintaining axial polarity in these early metazoans. Additionally, the location of staining along the lateral sides of the larvae is in cells that look quite similar to the "flask" cells observed in the subepithelial layer of *Amphimedon queenslandica* larvae. These cells have been shown to express a variety of post-synaptic orthologs and have been suggested to be evolutionary intermediates to neurons (Sakarya et al. 2007). We are currently developing gain and loss of function methods in our lab that will help us test whether or not either of these *Tbx* genes play roles in larval axis formation, settlement, metamorphosis, or other aspects of development. Given the conserved roles that some T-box family members have played over the course of evolution, investigating the roles of poriferan T-box genes during early development and during metamorphosis or formation of the adult body plan is particularly relevant.

A3.4 SUPPLEMENTAL FIGURES

Figure A3-S1: GenBank Accession numbers for Phylogenetic tree shown in Figure A3-1

Short Name	Accession Number	Species
Am~89136384	N/A	Acropora mellifera
Aq~Tbx11520	Supp Fig 8; Larroux et al., 2008	Amphimedon queenslandica
Aq~Tbx45	Supp Fig 8; Larroux et al., 2008	Amphimedon queenslandica
Aq~TbxA	ACA04753	Amphimedon queenslandica
Aq~TbxB	ACA04754	Amphimedon queenslandica
Aq~TbxC	Supp Fig 8; Larroux et al., 2008	Amphimedon queenslandica
Aq~TbxD	Supp Fig 8; Larroux et al., 2008	Amphimedon queenslandica
Aq~TbxE	Supp Fig 8; Larroux et al., 2008	Amphimedon queenslandica
Av~Tbx11520	CAE45764	Axinella verrucosa
Ce~mls1	NP_498640	Caenorhabditis elegans
Ce~Tbx12	AAB37243_1	Caenorhabditis elegans
Ce~Tbx2	NP_498088_1	Caenorhabditis elegans
Ce~Tbx7	NP_498313_1	Caenorhabditis elegans
Cow~05526	C.owCAOG_05526	Capsaspora owczarzaki
Cow~07284	C.owCAOG_07284_TranscriptCAOG	Capsaspora owczarzaki
Cow~TBX19	C.owCAOG_05512.1_TBX19	Capsaspora owczarzaki
Csp~110717	Csp~110717_e_gw1_78_45_1	Capitella telata
Csp~149486	Csp~149486_estExt_Genewise1_C	Capitella telata
Csp~152028	Csp~152028_estExt_Genewise1_C	Capitella telata
Csp~163410	Csp~163410_estExt_Genewise1_C	Capitella telata
Csp~168973	Csp~168973_estExt_Genewise1Plu	Capitella telata
Csp~223644	Csp~223644_estExt_fgenesh1_pg_	Capitella telata
Csp~226618	Csp~226618_estExt_fgenesh1_pg_	Capitella telata
Dm~Byn	NP_524031_1	Drosophila melanogaster
Dm~Doc1	BAA87864_1	Drosophila melanogaster
Dm~Doc2	NP_648282_1	Drosophila melanogaster
Dm~Doc3	NP_648280_1	Drosophila melanogaster
Dm~H15	NP_608926_2	Drosophila melanogaster
Dm~midline	NP_608927_2	Drosophila melanogaster
Dm~omb	NP_525070_2	Drosophila melanogaster
Dm~org1	NP_511085	Drosophila melanogaster
Em~Tbx45	HM778023	Ephydatia mulleri
Em~Tbx45B	HM778024	Ephydatia mulleri
Em~TbxA	HM778026	Ephydatia mulleri
Em~TbxPor	HM778025	Ephydatia mulleri
Hb~Tbx45	HM778027	Halichondria bowerbanki

Hb~TbxA	HM778029	Halichondria bowerbanki
Hb~TbxA2	HM778030	Halichondria bowerbanki
Hb~TbxB	HM778031	Halichondria bowerbanki
Hb~TbxPor	HM778028	Halichondria bowerbanki
He~Bra	AAL26836_1	Hydractinia echinata
Hm~Bra1	XP_002154016_1	Hydra magnipapillata
Hm~Bra2	XP_002164912_1	Hydra magnipapillata
Hm~Tbx1	XP_002153954_1	Hydra magnipapillata
Hm~Tbx31	XP_002167350_1	Hydra magnipapillata
Hm~Tbx6	XP_002167463_1	Hydra magnipapillata
Hs~Bra	NP_003172.1	Homo sapiens
Hs~Eomes	CAB37939_1	Homo sapiens
Hs~MGA	NP_001074010_1	Homo sapiens
Hs~Tbr1	NP_006584_1	Homo sapiens
Hs~Tbx1	NP_542377_1	Homo sapiens
Hs~Tbx2	NP_005985_3	Homo sapiens
Hs~Tbx3	AAD50989_2	Homo sapiens
Hs~Tbx4	AAI44063_1	Homo sapiens
Hs~Tbx5	NP_000183_2	Homo sapiens
Hs~Tbx6	NP_004599_2	Homo sapiens
Hs~Tbx10	NP_005986_2	Homo sapiens
Hs~Tbx15	NP_689593_2	Homo sapiens
Hs~Tbx18	NP_001073977_1	Homo sapiens
Hs~Tbx19	CAI22639_1	Homo sapiens
Hs~Tbx20	NP_001071121_1	Homo sapiens
Hs~Tbx21	NP_037483_1	Homo sapiens
Hs~Tbx22	NP_058650_1	Homo sapiens
Hv~HyBra1	AY366371	Hydra vulgaris
Lg~104372	Lg~104372_e_gw1_2_972_1	Lottia gigantea
Lg~116991	Lg~116991_e_gw1_25_6_1	Lottia gigantean
Lg~117095	Lg~117095_e_gw1_25_31_1	Lottia gigantean
Lg~117236	Lg~117236_e_gw1_25_117_1	Lottia gigantean
Lg~129083	Lg~129083e_gw1_63_220_1	Lottia gigantean
Lg~129911	Lg~129911_e_gw1_67_191_1	Lottia gigantean
Lg~154800	Lg~154800_fgenesh2_pg_C_sca_60	Lottia gigantean
Lg~171118	Lg~171118_fgenesh2_pg_C_sca_10	Lottia gigantean
Lg~179359	Lg~179359_fgenesh2_pm_C_sca_63	Lottia gigantean
Lg~76774	Lg~76774gw1_19_257_1	Lottia gigantea
Ml~Tbx1	DQ988138_ABL68079_1	Mnemiopsis leidyi
Ml~Tbx23	DQ988139_ABL68080_1	Mnemiopsis leidyi
Ml~TbxD	DQ988140_ABL68081_1	Mnemiopsis leidyi
Ml~TbxE	DQ988141_ABL68082_1	Mnemiopsis leidyi
Nb~Tbx1	Nb_tbx1fgenesh1_pg_scaffold_20	Mnemiopsis leidyi
Nv~213761	XP_001626870	Nematostella victensis
Nv~55118	gw_103_143_1	Nematostella victensis

Nv~146371	Nv_e_gw_146_37_1_XP_001629268_	Nematostella victensis
Nv~217211	Nv_e_gw_217_21_1_XP_001626767_	Nematostella victensis
Nv~57075	Nv~57075_gw_518_27_1	Nematostella victensis
Nv~80471	Nv_e_gw_80_47_1_XP_001632874_1	Nematostella victensis
Nv~Bra	Nv~Bra_AAO27886_2	Nematostella victensis
Nv~nesh1	Nv_fgenesh1_pg_scaffold_203000	Nematostella victensis
Nv~Tbx151822	Nv_e_gw_19_61_1Tbx15/18/22_XP_	Nematostella victensis
Nv~Tbx20	Nv_e_gw_146_31_1Tbx20_XP_00162	Nematostella victensis
Nv~Tbx23	Nv_e_gw_65_117_1Tbx2/3_XP_0016	Nematostella victensis
Nv~Tbx45	Nv_estExt_gwp_C_650150Tbx4/5_X	Nematostella victensis
Nv~TbxDiv	Nv_e_gw_75_204_1TbxDivergent_X	Nematostella victensis
Oc~scaf10364	scaffold10364 98.9*	Oscarella carmela*
Oc~scaf11025	scaffold11025 94.4*	Oscarella carmela*
Oc~scaf5142	scaffold5142 145.0*	Oscarella carmela*
Oc~scaf5207a	scaffold5207 96.1*	Oscarella carmela*
Oc~scaf5207b	scaffold5207 96.1*	Oscarella carmela*
Oc~scaf6478	scaffold6478 115.4*	Oscarella carmela*
Oc~scaf9259	scaffold9252 125.0*	Oscarella carmela*
Oc~scaff2913	scaffold2913 114.8*	Oscarella carmela*
Om~Bra	AY626265	Oopsacus minuta
Pc~Bra	CAD21521_1	Podocoryne carnea
Pc~Tbx45	CAE45765_1	Podocoryne carnea
Pp~Bra	CAE45766_1	Pleurobrachia pileus
Pp~Tbx23	CAE45769_1	Pleurobrachia pileus
Sd~Bra	CAD66614	Suberites domuncula
Sd~Tbx2	CAD66613	Suberites domuncula
Sp~Bra	XP_782140_2	Strogylocentrotus purpuratus
Sp~SPPG	S.puSPPG_06782	Strogylocentrotus purpuratus
Sp~Tbr	XP_791266_1	Strogylocentrotus purpuratus
Sp~Tbx1	XP_790408_2	Strogylocentrotus purpuratus
Sp~Tbx2	XP_797010_2	Strogylocentrotus purpuratus
Sp~Tbx23	NP_001123280_1	Strogylocentrotus purpuratus
Sy~Bra	AAU95752	Sycon raphanus
Ta~Bra	CAD70269_1	Trichoplax adhaerans
Ta~Tbx23	CAD70270_1	Trichoplax adhaerans
Ta~Tbx4	30221e_gw1_12_130_1	Trichoplax adhaerans
Ta~Tbx5	53394fgeneshTA2_pg_C_s	Trichoplax adhaerans
Ta~Tbx6	17171gw1_24_148_1	Trichoplax adhaerans

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Brachyury clade
Nv~Bra1                    KRRMRKPDSP NFGKNKSVTA YINPFA-KAF Q
He~Bra                     KRRMRKPDSP NFGKNKSVTA YINPFA-KAF H
Hs~Bra                     KRRMRKPDSP NFGKNKSVTA YINPFA-KAF H
Hv~Bra1                    KRRMRKPDSP NFGKNKSVTA YINPFA-KAF H
Ta~Bra                     KRRMRKPDSP NFAKNKSVTA YINPFA-KAF Q
Pc~Bra                     KRRMRKPDSP NFGKNKSVTA YINPFA-KAF Q
Hs~Tbx19                   --RMRKPDSP NFGKNKSVTA YINPFA-KAF L
Dm~Byn                     KRRMRKPESP NFGKNKSVTA YINPFA-KAF T
Csp~149486                 KRRMRKPDSP NFGKNKSVTA YINPFA-KAF P
Lg~154800                  KRRMRKPDSP NFGKNKSVTA YINPFA-KAF Q
Hm~Bra1                    KRRMRKPDSP NFGKNKSVTA YINPFA-KAF Q
Hm~Bra2                    KRRMRKPDSP NFGKNKSVTA YINPFA-KAF H
Pp~Bra                     KRRMRKPDSP NFGKNKSVTA YINPFA-KAF K
Om~Bra                     ---MRKPDSP HFGKNKSVTA YIN------- -
Cow~TBX19                  RRNLRKPDSP KSGKNRSVTA YINPFA-KAF S
Sp~SPPG_06782              KRCLKKSSAT QSGKNRSHTH YKNPHA-KGF A
Sd~Bra                     KRRTRRPSSP STGKNKSLTA YINPFA-KAF T
Sy~Bra                     RRRMRRPDSP HYGKNRSETA YINPYA-KAF V

Eomes clade
Hs~Tbr1                    KRRHHRPDSP NTGKNNSVTA YINPFA-KGF T
Hs~Tbx21                   KRRHHRPDSP NTGKNNSVTA YINPFA-KGF T
Hs~Eomes                   KRRHHRPESP NTGKNNSVTA YINPFA-KGF -
Sp~Tbr                     KRRQQKPDSP SNGKNHSLTA YINPFA-KGF P
Csp~223644                 KRRHHKPDSP NTGKNNSVTA YINPFA-KGF K
Lg~129911                  KRRQHKPDSP NTGKNNSVTA YINPFA-KGF -
                              42       85  106
```

Figure A3-S2: Selected Eomes and Brachyury functional residues
Functional residues after Wilson and Conlon (2002) include DNA binding and protein binding residues. Colored residues indicate Brachyury or Eomes clade specific sequences that differ between sponge/protist and other animals. Red indicates that a residue is found in all non-sponge animal Brachyury sequences. Blue indicates deviations from this. Two residues, 42 and 106 (see text for numbering convention), are Bra-specific. A third residue, 85, is identical in all non-sponge animal Bra/Eomes sequences, excepting *S. purpuratus*.

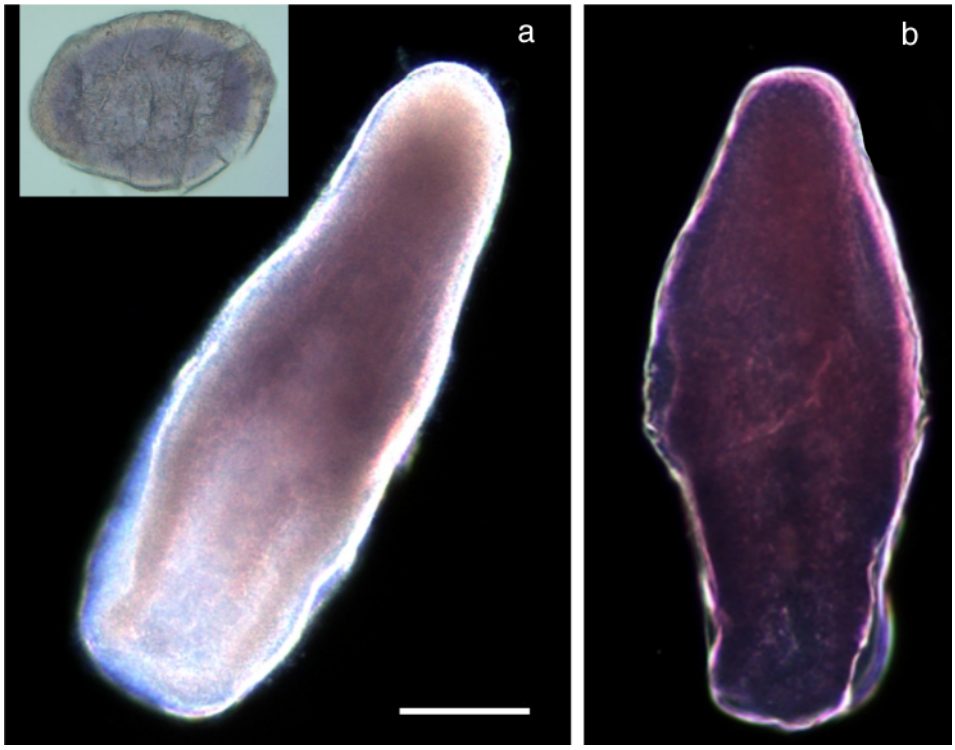

Figure A3-S3: In situ hybridization to illustrate positive controls
a) Hb Bar/Bsh probe with cross-section shown in inset where hybridization is specific to inner cell mass. b) Hb Actin probe hybridizes throughout larvae.

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Appendix 4:

THE ANALYSIS OF EIGHT TRANSCRIPTOMES FROM ALL PORIFERA CLASSES REVEALS SURPRISING GENETIC COMPLEXITY IN SPONGES[4]

A4.1 INTRODUCTION

Despite a plethora of genomic data now available current metazoan phylogeny is still in flux, especially with respect to the basal-most branching phyla Porifera, Ctenophora, Cnidaria and Placozoa. Their branching order is however, fundamental for understanding the early evolution of animal features such as tissues and epithelia, nerves and coordination, immune recognition, and propagation of the germ lineage. Traditional markers such as 18S rRNA tend to place sponges as the sister lineage to the rest of metazoans (e.g., Medina et al. 2001; Zrzavý et al. 2005), and while recent hypotheses using transcriptomic and genomic data from ctenophores have challenged this view (e.g., Dunn et al. 2008; Hejnol et al. 2009; Ryan et al. 2013), the outcome depends on the type of model and parameters used in analysis of these data sets. Other analyses of the same data either confirm that sponges are the sister lineage to the rest of animals (Pick et al. 2010; Philippe et al. 2011), or fail to resolve this dichotomy (Nosenko et al. 2013).

It is commonly considered that morphological complexity in animals is acquired over evolutionary time (McShea 1996). Sponges are morphologically simple in comparison to ctenophores, which possess complex structures such as gonads, nerves and muscles, structures that are not known at all in sponges. The absence of certain homeodomains in both ctenophores and sponges led Ryan et al. (2010) to suggest an early branching of ctenophores and sponges prior to placozoans, cnidarians, and bilaterians. In this way perhaps complex structures may have appeared in two branches of early evolving animals almost

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simultaneously, one in ctenophores and the other giving rise to the rest of animals. Alternatively, sponges might be derived, and having specialized for a filter-feeding lifestyle, become morphologically simplified by losing ancestral cell types. Therefore, the molecular basis to create complex structures might be still present in sponges even though in structure they appear simple.

While sponges might appear morphologically 'simple', analysis of two sponge genomes and transcriptomes, *Amphimedon queenslandica* (Class Demospongiae) and *Oscarella carmela* (Class Homoscleromorpha) revealed a remarkable molecular complexity (Nichols et al. 2006, 2012; Srivastava et al. 2010; Conaco et al. 2012). The morphology of *Amphimedon queenslandica* corresponds perfectly well to the textbook view of a sponge: a massive body with branching aquiferous canals lined by a single cell layer – the pinacoderm – and enclosing very few cell types many of which are pluripotent (Simpson, 1984). There are however, over 8,500 species of sponge currently recognized with an estimated 12-18,000 more to be described (Appeltans et al. 2012). These live in diverse habitats – from the abyssal deep sea to freshwater lakes and rivers – and have contended with changes caused by uptake of symbionts, infection, changing temperature, salinity and food abundance over at least 600 million years (Conway Morris et al. 2000; Jackson et al. 2007). Some are carnivorous (Vacelet and Boury-Esnault, 1995) and many different ways of forming a skeleton, from calcium carbonate, aragonite, to spongin and silica, exist (Simpson 1984; Jackson et al. 2007). Within the context of this extraordinary sponge diversity, the genomes and now transcriptomes of *Amphimedon* and *Oscarella* are informative, but by no means conclusive in terms of providing the absolute gene complement of sponges.

To provide a wider framework for understanding the molecular complexity of sponges, we sequenced the transcriptomes eight sponge species covering all four currently recognized poriferan classes (*Aphrocallistes vastus*, Class Hexactinellida; *Chondrilla nucula*, *Ircinia fasciculata*, *Petrosia ficiformis*, *Spongilla lacustris*, and *Pseudospongosorites suberitoides* Class Demospongiae; *Sycon coactum*, Class Calcarea; *Corticium candelabrum*, Class Homoscleromorpha). These species represent for the most part sponges that have

been well-studied in other contexts (cell biology, ecology, physiology), and for which quality starting material could be obtained. We analyzed protein families and Gene Ontologies, and specifically screened each transcriptome for the presence of genes involved in signaling, neuronal and ionic conduction, epithelia, immunity, and reproduction.

A4.2 METHODS

A4.2.1 Sample collection

We collected tissue samples from 8 sponge species, belonging to the four currently recognized classes (Fig. A4-1): *Aphrocallistes vastus* (Hexactinellida), *Sycon coactum* (Calcarea), *Ircinia fasciculata*, *Chondrilla nucula*, *Petrosia ficiformis*, *Spongilla lacustris*, *Pseudospongosorites suberitoides* (Demospongiae), and *Corticium candelabrum* (Homoscleromorpha). Collecting information is provided in Supplementary File A4-S1. Hereafter we refer to each animal by its genus to ease readability.

A4.2.2 Sample preparation

In order to avoid contamination from epibionts, prior to fixation tissues were cleaned carefully using a stereomicroscope. A piece of sponge tissue was removed with razor blades that were rinsed in RNAseZap® (Ambion, Texas, US). All procedures were carried out on ice and quickly to avoid RNA degeneration. Tissues were either flash-frozen in liquid nitrogen and stored at -80ºC or they were immersed in at least 10 volumes of RNA*later*® at 4ºC for 1 hour, incubated overnight at -20 ºC, and subsequently stored in the same buffer at -80ºC until RNA was extracted (sometimes samples placed in RNA*later* were transported back to the laboratory at room temperature, where they were stored at -80ºC). See Supplementary File A4-S1 for details. The amount of tissue used depended on the extent of the spicule skeleton: in most cases 20 to 80 mg of tissue was used but for *Petrosia* and *Aphrocallistes*, 200 mg was needed due to the large silica skeleton (see Riesgo et al. 2012a).

Figure A4-1: Phylogenetic relationships in the phylum Porifera and images of each sponge used in this study.
The monophyly of sponges and their sister-group relationship to Placozoa and "Eumetazoa" are shown. "Eumetazoa" in this tree contains Cnidaria, Ctenophora, and Bilateria. B. *Aphrocallistes vastus*, *Spongilla lacustris*, *Petrosia ficiformis*, *Pseudospongosorites suberitoides*, *Ircinia fasciculata*, *Chondrilla nucula*, *Sycon coactum*, and *Corticium candelabrum.* (Image authors are provided in the acknowledgements.) Colored names indicate their phylogenetic affiliation in Figure 1A. The species *Amphimedon queenslandica* and *Oscarella carmela*, used for gene comparisons, belong to Demopospongiae and Homoscleromorpha respectively.

A4.2.3 mRNA extractions

Two different methods of RNA extraction were used: 1) total RNA extraction followed by mRNA purification for Corticium and 2) direct mRNA extraction for all other species. Protocols used for both extraction types are available elsewhere (Riesgo et al. 2012a, b). Total RNA from Aphrocallistes was extracted using the Norgen Biotek Animal Tissue RNA Purification Kit (Norgen Biotek, Thorold, ON, Canada). Quantity and quality (purity and integrity) of mRNA were assessed by three different methods, reported in Riesgo et al. (2012a) and shown in Supplementary Table A4-S1.

A4.2.4 Next-Generation Sequencing

For all sponges except *Aphrocallistes*, next-generation sequencing was performed using Illumina GAII and HiSeq2000 (Illumina, Inc., San Diego, California, USA) platforms at the FAS Center for Systems Biology at Harvard University. mRNA concentrations between 20 and 79.9 ng/µL (Table 1) were used for cDNA synthesis with the TruSeq RNA sample preparation kit (Illumina, Inc.), as described previously (Riesgo et al. 2012a,b). cDNA was ligated to homemade adapters in one sample of *Petrosia* (5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GGT T-3'), whereas ds cDNA was ligated to Illumina adapters in the rest of species. Size-selected cDNA fragments of around 300 bp excised from a 2% agarose gel were amplified using Illumina PCR Primers for Paired-End reads (Illumina, Inc.) and 18 cycles of the PCR program 98 ºC-30 s, 98 ºC-10 s, 65 ºC-30 s, 72 ºC-30 s, followed by an extension step of 5 min at 72 ºC.

The concentration of the cDNA libraries was measured with the QubiT® dsDNA High Sensitivity (HS) Assay Kit using the QubiT® Fluorometer (Invitrogen, Carlsbad, California, USA). The quality of the library and of size selection were checked using the "HS DNA assay" in a DNA chip for Agilent Bioanalyzer 2100 (Agilent Technologies, California, USA). cDNA libraries were considered successful when the final concentration was higher than 1 ng/µL and the Bioanalyzer profile was optimal (see Riesgo et al. 2012a). Successful libraries were brought to 10 nM or 7nM depending on the initial concentration prior to

sequencing. The paired-end reads had lengths of 100 or 150 bp, depending on availability of sequencers (Illumina GAIIx or HiSeq).

The *Aphrocallistes* transcriptome was prepared by LC Sciences (http://www.lcsciences.com/) using 1 µg of total RNA for polyA tail selection of the mRNA (Supplementary File A4-S1). Library preparation was performed using also the TruSeq RNA sample preparation kit (Illumina,, Inc.) following the manufacturer's instructions and the sequencing of a 9 nM library performed on HiSeq2000 with paired-end 100 nt reads also by LC Sciences (Texas).

A4.2.5 Sequence assembly

Thinning and trimming for the raw reads was done with CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark). Thinning refers to discarding of nucleotides and/or entire reads based on quality parameters. It was performed using either 0.05 or 0.005 as the limit, based on *Phred* quality scores, and resulting quality of the thinned reads was visualized in FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). After thinning, only those terminal bases with a *Phred* quality score under 30 were trimmed (where a *Phred* score of 30 corresponds to a probability of 10^{-3} of incorrect base calling), producing sequences of unequal size. Reads were re-screened to check for presence of adapter or primer sequences using FastQC, and if present, adapters or primers were removed using with CLC Genomics Workbench 5.1.

De novo assemblies with all datasets thinned and/or trimmed were performed with CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark), or Trinity [http://trinityrnaseq.sourceforge.net/]. Global alignments for the *de novo* assemblies were used with the following parameters: mismatch cost=2; insertion cost=3; deletion cost=3; length fraction=0.5; similarity=0.8; and randomly assigning the non-specific matches. Best *k*-mer length was estimated by the software.

A4.2.6 Sequence Annotation

For each species, two methods were used for annotation: a more global assignment of gene ontology using BLAST and a more specific assignment of

domain by HMMer. For Blast, contigs shorter than 300 bp were removed, as very few of these short contigs retrieved results for Gene Ontology assignments (see Riesgo et al. 2012b). The remaining contigs were independently mapped against three different selections of the non-redundant (*nr)* NCBI database (all Metazoan proteins in *nr*, all Bacterial proteins in *nr*, and all Protozoan proteins in *nr*) using the blastx program of the BLAST suite. All searches were conducted with BLAST+ (Atschul et al. 1990; Camacho et al. 2009) using an e-value cut-off of 1e-5. We used the output file from the blast against Metazoa which contained the best hits and Blast2GO v2.5.0 (Conesa et al. 2005) to retrieve the Gene Ontology (GO) terms and their parents associated with the top BLAST hit for each sequence. Searches for specific genes were carried out using HMMer hidden Markov models using Interproscan tools (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and either HMM profiles present in the PFAM Protein families database or HMMerbuilds generated specifically using sequences downloaded from NCBI.

To estimate the complexity of the complements of genes involved in different pathways, independently from the general BLAST results, we selected gene targets from conserved developmental signaling pathways, and genes associated with post-synaptic signaling, germ lineage and reproduction, adhesion, and innate immune regulation. We retrieved at least three different sequences of the selected protein targets from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) from a range of metazoan groups to use them for searches in our transcriptome datasets. These sequences were aligned using either T-COFFEE (Notredame et al. 2000), MAFFT (Katoh et al. 2005), or MUSCLE (Edgar 2004) depending on the level of conservation of the protein, and the alignments used to create HMM profiles for each protein of interest. HMMER searches were performed against all 8 transcriptomes, translated into all 6 reading frames. We selected only the hits with the maximum similarity (cutoff of 1e-05; which varied greatly between groups), and checked each open reading frame with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). A similar approach was performed using the software CLC Genomics Workbench 5.0, selecting three protein

sequences from other metazoans and blasting them using the local BLAST suite plug-in with each contig list as the targeted database. Each predicted protein sequence was reverse blasted against the database *nr* in NCBI using the blastp and DELTA-BLAST programs (http://blast.ncbi.nlm.nih.gov/) and the domain structure checked with SMART (http://smart.embl-heidelberg.de/) using HMMER, PFAM domain, and internal repeats searching. To avoid the bias in the detection of genes derived from the use of cnidarian or bilaterian protein queries, each time we found a target gene in the transcriptomes, we added the sequence to the list of protein queries to improve the searches.

We also confirmed the presence/absence of the same set of conserved developmental signaling pathways, and neuronal signaling, germ lineage and reproduction, adhesion, and innate immune regulation in three different unicellular eukaryote species (*Capsaspora owzarczaki*, *Monosiga brevicollis*, and *Salpingoeca rosetta*) to determine whether the appearance of the genes in sponges was a novel acquisition or a feature shared with these unicellular organisms. We used the same gene targets and searched in the genomes with the blastp engine implemented in the Broad institute website using default setttings (http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html). We also used searched the *Amphimedon queenslandica* genome and *Oscarella carmela* draft genome (Nichols et al. 2006, 2012; Srivastava et al. 2010) to confirm the presence/absence of each genes at the genomic level.

We performed 3D reconstructions of the translated sequences of the targeted genes using PHYRE2 for protein fold recognition (Kelley and Sternberg, 2009).

Sequences obtained in this study are available in Supplementary File A4-S5 and also deposited under the Bioproject accession numbers in Genbank: *Aphrocallistes vastus* (PRJNA225584), *Ircinia fasciculata* (PRJNA225586), *Chondrilla nucula* (PRJNA225590), *Petrosia ficiformis* (PRJNA162901), *Spongilla lacustris* (PRJNA225591), *Pseudospongosorites suberitoides* (PRJNA225580), *Sycon coactum* (PRJNA162899), and *Corticium candelabrum* (PRJNA162903).

A4.2.7 Phylogenetic analyses

For each of the selected genes, independent protein alignments were built using MUSCLE implemented in SEAVIEW 4.3.0 (Gouy et al. 2010) and MAFFT (Katoh et al. 2002) with default parameters. For the maximum likelihood phylogenetic analysis of the protein sequences we used RAxML (Stamatakis 2006) with the LG model and an estimated gamma shape parameter and 500 independent searches. Nodal support was estimated via the rapid bootstrap algorithm (1000 replicates) using the WAG-CAT model (Stamatakis et al. 2008). Bootstrap resampling frequencies were then mapped onto the optimal tree from the independent searches.

A4.3 RESULTS

A4.3.1 Sequence assembly and annotation

The cDNA libraries rendered between 38,866,233 reads for *Pseudospongosorites* and 234,585,429 for *Spongilla* of which, between 67% in *Spongilla* and 87% in *Aphrocallistes* of the reads after the thinning process were used for the assemblies (Supplementary Table A4-S1). The assembly of each species produced between 10 Mb and 65 Mb of assembled contigs in all species (Supplementary Table A4-S1).

The average length of the contigs was close to 500 bp in all datasets (Supplementary Table A4-S1), with the transcriptomes of *Aphrocallistes*, *Sycon*, *Petrosia* and *Chondrilla* showing the greatest N50 values (N50 is a weighted median statistic such that 50% of the entire assembly is contained in contigs equal to or larger than this value in bp). The *Corticium* transcriptome had a large number of short contigs that resulted in low N50 values (Supplementary Table A4-S1). The average coverage per contig was 190 reads for the transcriptomes of all species in the study (Supplementary Table A4-S1).

Sponges host a great number of symbionts (mainly bacteria) within their tissues that are impossible to remove prior to cDNA construction. In order to assess the percentage of sequences that can be assigned to metazoans bacteria

and/or protozoans, we compared the results of each independent BLAST analysis against separate databases containing metazoan, bacterial and protozoan proteins (Supplementary File A4-S2A), and found that most contigs in all datasets returned hits from Metazoa. For the BLAST against the Bacteria database, *Corticium* had the highest number of contigs with hits not found in Metazoa and Protozoa (Supplementary File A4-S2A). Only *Sycon* showed unique hits against Protozoa, while the other species produced both protozoan and metazoan hits (Supplementary File A4-S2A).

Among those sequences that blasted to metazoans (more than 60% of the contigs assembled for *Aphrocallistes*; between 40-50% in the demosponges; and around 20-30% in *Sycon* and *Corticium*), most hits were assigned to sponges (mostly *Amphimedon*) for the demosponges, and to bilaterians in the case of *Aphrocallistes, Sycon*, and *Corticium* (see Supplementary File A4-S2B).

Regardless of the potential different physiological states of the sponges when collected, the percentage of sequences with assigned gene ontology (GO) terms was similar for the ontology categories 'biological function', 'molecular function', and 'cellular component' for all datasets (Supplementary File A4-S2C), allowing comparisons at that level. It is important to note however, that the total number of GO terms retrieved for each dataset was very different, with 32,604 in *Corticium* and only 6,501 in *Petrosia* (Supplementary File A4-S2C). For all datasets, in the GO category 'biological process', the primary metabolic process was the most abundant term, in the 'molecular function' category, catalytic activity was most common, and in 'cellular component', macromolecular complex was most abundant (Supplementary File A4-S2C).

A4.3.2 Protein families in Porifera

We analyzed the number of protein families (Pfams) in each sponge dataset and found the highest number of Pfams in *Corticium* (50,798) and the lowest in *Pseudospongosorites* (10,137). The number of Pfams in *Corticium* could be either due to the high number of symbionts reported in the transcriptomic dataset (Supplementary File A4-S2D-E) or an enrichment of certain domains in this particular species. We found very similar abundances for all protein families in

Aphrocallistes, Chondrilla, Ircinia, Spongilla, Petrosia, and *Pseudospongosorites*, and a different profile in *Sycon* and *Corticium* (Supplementary File A4-S4A). We obtained the functions for all the most abundant protein families and grouped them under the following categories: 'signaling', 'cell adhesion', 'immune system and metabolism' and 'structural/cytoskeletal', and those which showed more than one main function were grouped under 'multiple functions' (Supplementary File A4-S2D-E). Again, in most cases, the number of protein families was higher in *Corticium* and *Sycon* than in the other species. The families showing the larger differences in the 'cell adhesion' category were I-set and Laminin_EGF, where both *Sycon* and *Corticium* had higher values. For example, the family MAM was only in *Sycon* and *Corticium*. For the category 'metabolism and differentiation', *Corticium* had a much larger complement of protein kinases (Pkinase) and sulfatases.

A4.3.3 Targeted Gene Study

We used the transcriptome datasets to search for specific genes in pathways related to the acquisition of morphological complexity in metazoans. Absences of genes in any of the species studied here should be interpreted with caution given that transcriptomes convey expressed transcripts. To confirm the presence/absence of all the genes at the genomic level, we carried out the same surveys of the *Amphimedon queenslandica* genome and *Oscarella carmela* draft genome (Nichols et al. 2006, 2012; Srivastava et al. 2010)

A4.3.4 Developmental Toolkit Genes

Developmental signaling pathways have been considered a hallmark of metazoan complexity. While most components of the major metazoan signaling pathways are present in sponges, some key absences have been noted (Nichols et al. 2006). We specifically examined the Hedgehog, Wnt, TGF-β and Notch-Delta pathways. Most components of the Hedgehog (Hh) signaling pathway were present in all sponge classes (Fig. A4-2A). Since Hh proteins are composed of an N-terminal signaling domain and a C-terminal Hint domain, similarity to Hh may have simply reflected the presence of one of these domains. We therefore

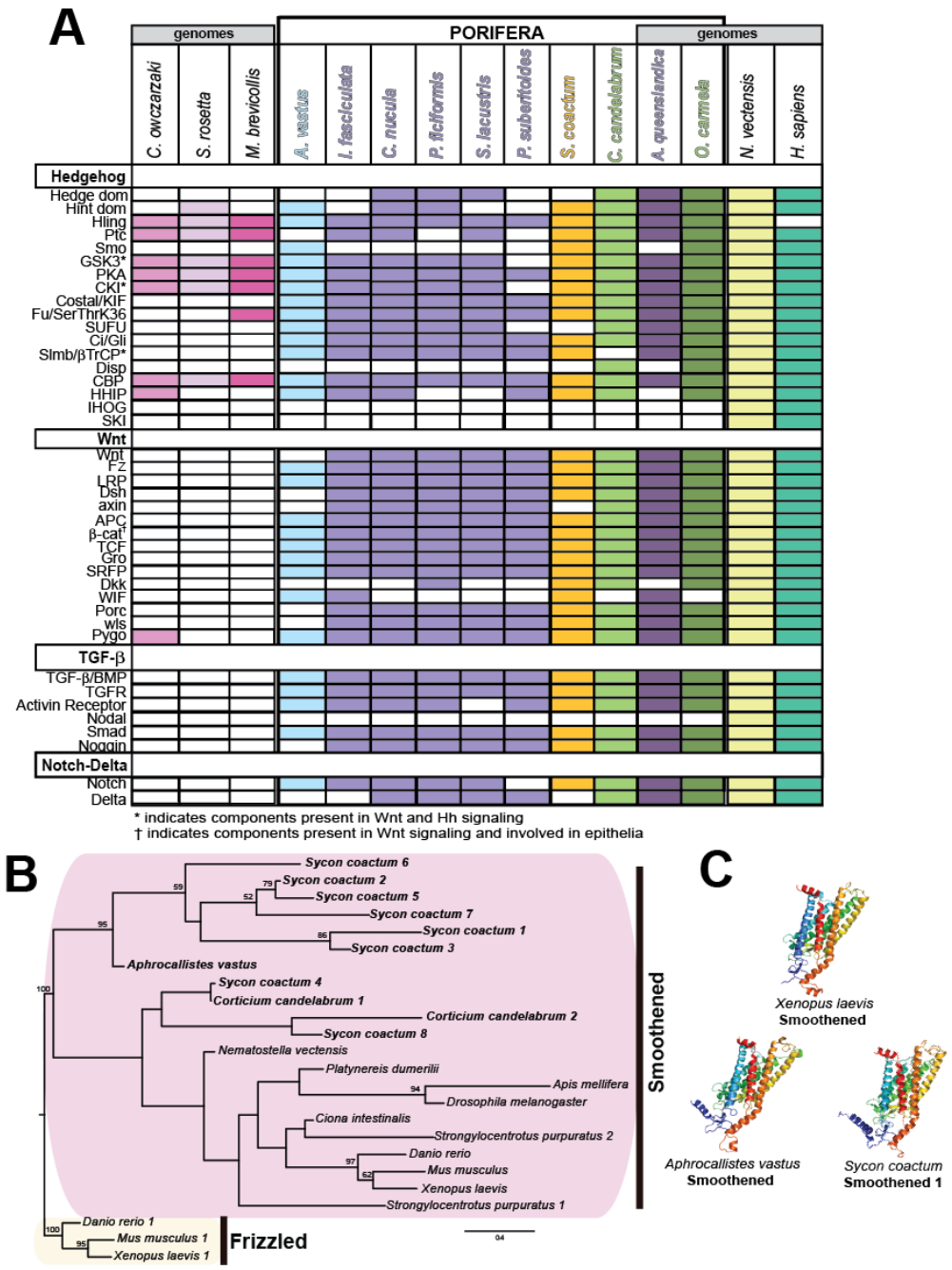


Figure A4-2: Signaling molecules identified in Porifera.
A. Presence of the genes belonging to the signaling pathways for Hedgehog, Wnt, TGF-β, and Notch-Delta. B. Evolutionary relationships of sponge Smoothened proteins determined with Maximum Likelihood analysis. Bootstrap support is shown for nodes greater than 50% of 500 pseudoreplicates. Accession numbers of sequences used to construct the phylogenetic tree are available in Supplementary Table 4. C. Three-dimensional reconstructions of the Smoothened proteins in two distant sponges (*Aphrocallistes vastus* and *Sycon coactum*) and the vertebrate *Xenopus laevis*.

included one row for sequences containing the hedge domain and a row for Hint domains. We found no instances in which both a hedge and a hint domain were in the same sequence, and no true hedgehog proteins were found in any sponge. Lastly, Hedgling (Hling) proteins were identified by the whole or partial presence of the domain architecture identified in Adamska et al. (2007b), as well as simple blast similarity. Importantly, in *Aphrocallistes*, *Corticium* and *Sycon* we also found *smoothened*, a component of the Hedgehog pathway that was thought to be absent from sponges (Nichols et al. 2006; Adamska et al. 2007b; Srivastava et al. 2010; Ingham et al. 2011). Phylogenetic analysis indicates that the sponge sequences lie within the smoothened family, and do not cluster together with closely related *frizzled* genes, and predicted 3D structure supports this finding (Fig. A4-2B, C).

Wnts and other Wnt pathway components identified in *Amphimedon queenslandica* (Adamska et al. 2007a, 2010), *Suberites domuncula* (Adell et al. 2003, 2007), *Lubomirskia baikalensis* (Harcet et al. 2010), *Ephydatia muelleri* (Windsor and Leys 2010) and *Oscarella carmela* (Nichols et al. 2006; Lapebie et al. 2009) were also found in our transcriptomes (Fig. A4-2). Of note is the possible absence of *Wnt* in *Aphrocallistes*, despite the presence of other *Wnt* signaling components in that sponge.

Our findings confirm and expand the presence of TGF signaling components in all four sponge classes. We found TGF family ligands as well as TGF family receptors and/or activin receptors in all 8 sponge transcriptomes. Whereas the TGF-β ligand antagonist *noggin* and the downstream effectors *SMADs* were found in all species, *nodal* was not identified in any of the transcriptomes (Fig. A4-2).

Homologs of *notch* and *delta* were found in all four sponge classes (Fig. A4-2) as had been reported previously for *Oscarella carmela* and *Amphimedon queenslandica* (Nichols et al. 2006; Richards and Degnan 2012). Some of the sponge sequences showed characteristics aligning them more closely with *jagged* than *delta*, but phylogenetic analysis confirmed that they cluster with *delta* of other sponges (not shown).

A4.3.5 Neuronal signaling: Post Synaptic Densities (PSD) and neurotransmission

Genes associated with postsynaptic densities and signaling via neurotransmitters are shown in Figure A4-3. Perhaps unsurprisingly we found the same general set of genes in all 8 transcriptomes as shown previously for *Amphimedon queenslandica* (Sakarya et al. 2007). Several genes however, were not found in some sponges, and importantly we were unable to identify some genes previously described from *Amphimedon* in any of the 8 transcriptomes. For example, *citron* was not found in *Pseudospongosorites* and *cortactin* was not found in *Petrosia* or *Ircinia*, while *homer* was absent from *Petrosia*. Given the presence of these genes in most of the demosponges we studied, it is likely that the variability reflects the fact that these are transcriptomes (only expressed genes are detected) rather than genomes (where all genes are detected).

Our findings are also broadly consistent with gene absences reported from the *Amphimedon* genome (Sakarya et al. 2007; in supplementary material), although in contrast to Alié and Manuel (2010) we did not find a true *Shaker-type K+* channel in any sponge nor could we find *neurolignin* or *stargazin* in any of the eight transcriptomes (as reported for *Amphimedon* by Sakaraya et al. 2007). We did find a sequence that blasted to a Kv subfamily-A type, which is characterized as 'shaker-like' in *Corticium*, and genes with sequence similarity to shaker-like Kv channel were also found in the three unicellular eukaryote genomes, but it does not necessarily imply Kv channels are present and further characterization is required.

One significant finding that differs from both previous reports on sponge PSD genes was the presence of an *ionotropic glutamate receptor* (iGluR) in *Corticium*, *Sycon*, and *Iricinia*. The iGluRs present in *Corticium* and *Ircinia* appear AMPA-like possessing a Q/R site and all three sponges appear to possess most of the pore motif, SYTANLAAF. Phylogenetic analysis confirmed these channels group with other metazoan iGluRs (Fig. A4-3 and Supplementary File A4-S3).

We attempted to identify core components of the catecholamine signaling pathway (adrenaline, noradrenaline, epinephrine, etc.; Fig. A4-3). Curiously,

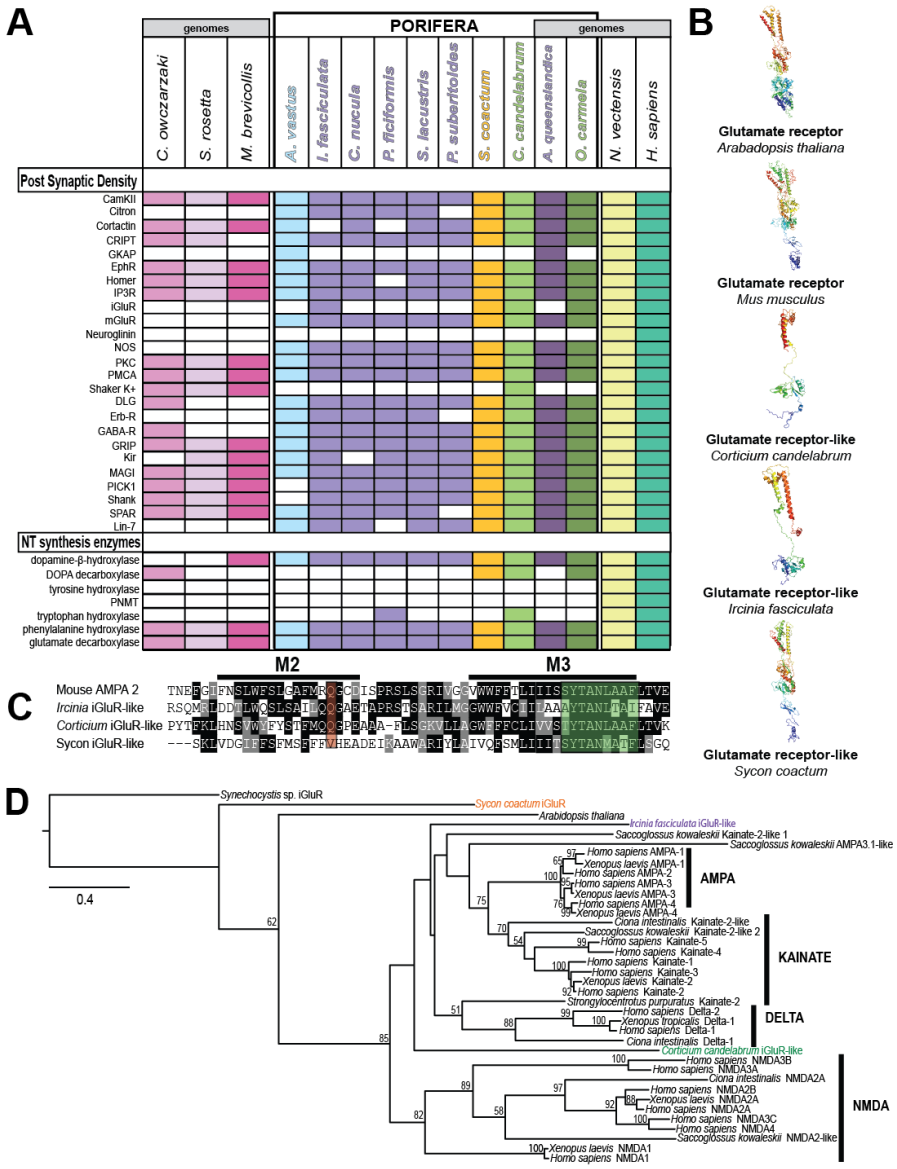


Figure A4-3: Post-synaptic density molecules identified in Porifera.
A. Presence of genes comprising the PSD and those involved in synaptic neurotransmission in metazoans. B. Three-dimensional reconstructions of the ionotropic glutamate receptors in the plant *Arabidopsis thaliana*, the vertebrate *Mus musculus,* and three sponges, *Ircinia fasciculata, Corticium candelabrum,* and *Sycon coactum.* C. Alignment of the ionotropic glutamate receptor proteins from the mouse (*Mus musculus*; AMPA type) and the three sponges *Ircinia fasciculata, Corticium candelabrum,* and *Sycon coactum.* D. Evolutionary relationships between ionotropic glutamate receptors found in bacteria, plants, and metazoans determined with Maximum Likelihood analysis. Bootstrap support is shown for nodes greater than 50% of 500 pseudoreplicates. Accession numbers of sequences used to construct the phylogenetic tree are available in Supplementary Table 4. Colored sequences are matched to the sponges in Figure 1A.

while we found pieces of the biosynthesis pathway in the transcriptomes we were unable to show the full pathway in any single sponge species, and some components were missing from all species. For example dopamine-β-hydroxylase, which catalyzes the reaction of dopamine to norepinephrine, was identified in all transcriptomes, yet *DOPA decarboxylase*, which produces dopamine from L-DOPA could only be identified in *Sycon* and *Corticium*. Furthermore, while gene prediction suggests that the *Amphimedon* genome encodes a *tyrosine hydroxylase* (GI:340369773) an enzyme that catalyzes the reaction of tyrosine to L-DOPA, this gene was not identified in any of the eight sponge transcriptomes studied here, yet *phenylalanine hydroxylase*, the gene that encodes for a protein that catalyzes the synthesis of tyrosine from phenylalanine was identified in all eight transcriptomes. Finally, adrenergic receptors were identified, but subtypes including a *dopamine receptor* were not. Therefore, while components of the catecholamine signaling pathway are present among the four classes of sponges, a more complete picture of this pathway could not be constructed even from data from all of the new transcriptomes. Nevertheless, we did find *glutamate decarboxylase* (which carries out synthesis of GABA from glutamate), which supports previous reports that show both glutamate and GABA are physiologically active in demosponges (Elliott and Leys 2010).

A4.3.6 Adhesion and epithelia

Genes involved in maintenance of epithelial polarity, in adhesion to other cells and to a basal matrix, and genes involved in secretion of a basement membrane have previously been considered indicative of evidence of tissue-level differentiation in some sponges and not others. We found this an ideal opportunity to survey the transcriptomes for the same genes studied previously (Nichols et al. 2007; Fahey and Degnan 2010). Our findings are summarized in Figure A4-4. Unsurprisingly, we found most of the polarity genes *Par3*, *Par6*, *Lgl*, *scribble*, and *disks large* (Fig. A4-4). We had difficulty identifying strict homologs of *Patj*, previously identified in *Amphimedon* (Fahey and Degnan 2010), but in contrast to previous work, we found good evidence for *stardust/Pals*, and the ligand of *stardust*, *crumbs*, in all sponge transcriptomes. In terms of adhesion, we found

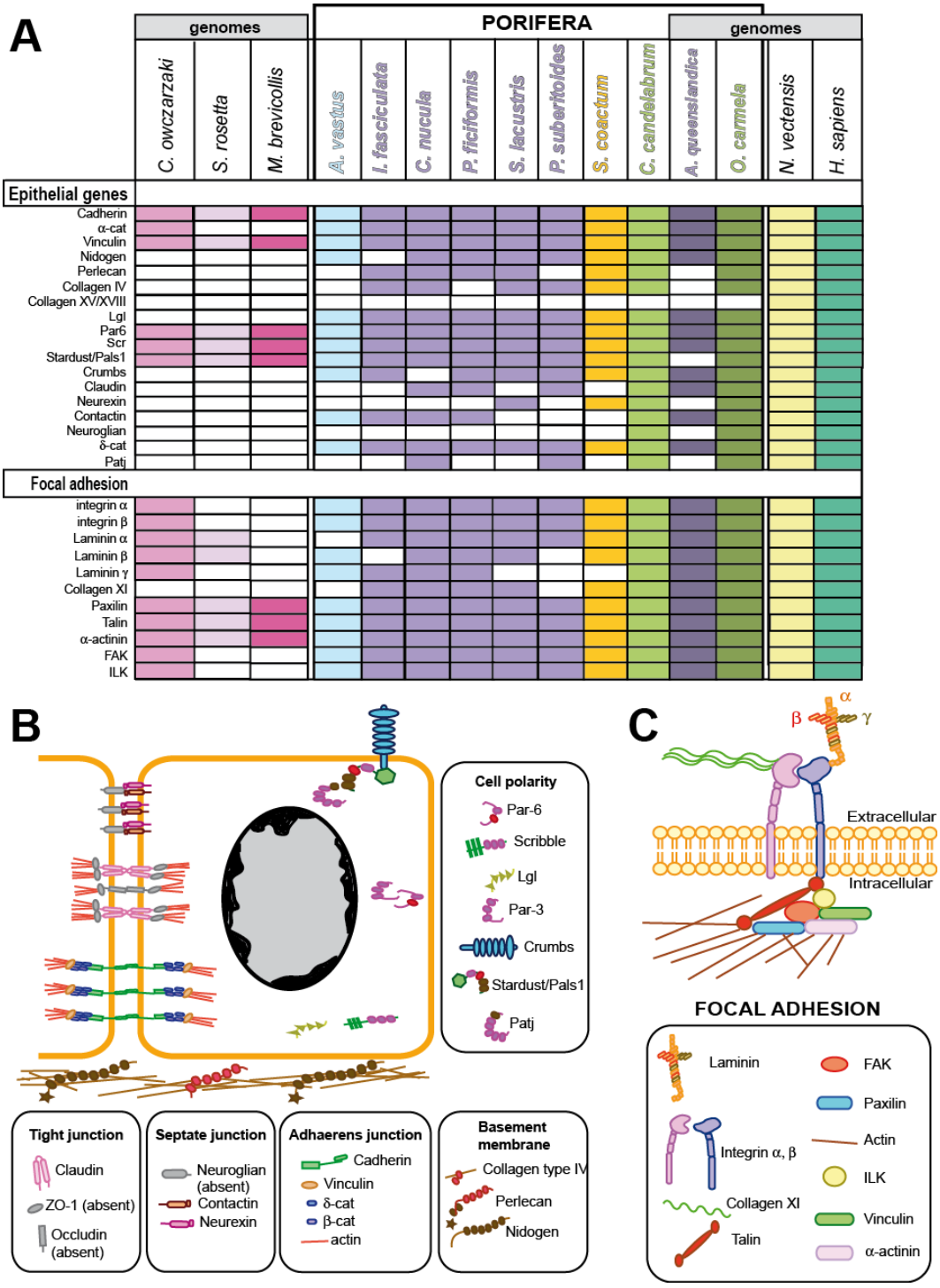


Figure A4-4: Cell adhesion, focal adhesion and epithelial development molecules identified in Porifera.

A. Presence of the genes involved in cell adhesion, epithelia formation, and focal adhesion. B. Schematic depicting the cell adhesion, polarity signaling, and basement membrane proteins found in metazoans. C. Schematic depicting focal adhesion molecules found in the cell membranes of a metazoan.

protocadherin in all sponges, and the components typically associated with *cadherin* adhesion (*β-catenin*, *alpha-catenin*, *p120 catenin*, and *vinculin*) were all present. What was unusual in comparison with earlier surveys of the *Amphimedon* genome was the presence of homologs of *claudin* in the three demosponges (*Spongilla*, *Pseudospongosorites,* and *Chondrilla*) as well as in the homoscleromorph *Corticium* and in the calcareous sponge *Sycon* (Fig. A4-4)

Also in contrast to previous work, we found homologs of important components of basement membrane genes, including *type IV collagen* in *Spongilla, Ircinia, Chondrilla, Sycon and Corticium* (the latter two were shown previously by Leys and Riesgo 2011). *Perlecan* and *nidogen* – molecules that connect the cell membrane to the protein type IV collagen – were also found in all except two of the demosponge transcriptomes (*nidogen* was not identified in *Ircinia*, and *perlecan* was not found in *Pseudospongosorites*). The genes for Laminins, which play a fundamental role in basement membrane assemblage as well as focal adhesion to the extracellular matrix, are composed of three non-identical chains, alpha, beta, and gamma, whose specific functions depend on the tissue in which they are present (Fig. A4-4). The three chains (alpha, beta, and gamma) were only found in *Corticium* and *Chondrilla* (Fig. A4-4), whereas in the other sponge transcriptomes we found only two of the chains, or just one in the case of *Pseudospongosorites* (Fig. A4-4).

Adhesion of cells to the surrounding extracellular matrix together with their stimulation by growth factors are key features that help cells to survive, proliferate, differentiate, or migrate in all animals (Turner 2000; Labouesse and Georges-Labouesse 2003). Cell adhesion is enabled via transmembrane *integrins* and their coupling with extracellular components such as *collagen* and *laminins* as well as their anchoring to *actin* through several protein components such as *focal adhesion kinase*, *paxillin*, *talin*, *integrin-linked kinase*, and *vinculin*. We found all basic components of this mechanism (*focal adhesion kinase, paxilin, talin, integrin alpha and beta, filamin, alpha-actinin*, and *vinculin*) in all eight transcriptomes (Fig. A4-4). In addition, we found the fibrillar *collagen XI*, known

for giving support to connective tissues in mammals, in all species except for *Pseudospongosorites* (Fig. A4-4).

A4.3.7 Innate immunity

While in vertebrates the immune system has a two-tier system consisting of either phagocytic activity or the opsonization and direct lysis of pathogens via the 'complement cascade', basally branching invertebrate phyla typically lack phagocytic activity and only have the ability to detect, contain, and kill pathogens (Miller et al. 2007). The complement cascade has been fully described in three cnidarian species (Miller et al. 2006), but the *Amphimedon* genome has important absences (Srivastava et al. 2010). We focused on the 11 major gene families involved in immunity, as shown in Miller et al. (2007) and Srivastava et al. (2010). We found all of them in almost all eight sponge transcriptomes, with a few exceptions (Fig. A4-5), and significantly, there was only one sequence in the unicellular eukaryote genomes (Fig. A4-5). The *nuclear factor kappa-light-chain-enhancer of activated B cells* (NF kB), the *interleukin receptor-associated kinase 1/4* (IRAK 1/4), *TGF-β activated kinase* (TAK-1), the *TNF receptor-associated factors* (TRAF), and the *interferon regulatory factor* (IRF), were found in all species of sponges and the latter was also found in *Capsaspora* (Fig. A4-5). The *Toll/interleukin 1 receptor 2* (TLR2) was found in *Ircinia, Petrosia,* and *Corticium* (Fig. A4-5). In contrast, the *myeloid differentiation primary response 8* (MyD88) gene was found in all sponges except *Sycon* (Fig. A4-5). α_2-*macroglobulin* (A2M) is an evolutionarily conserved element of the innate immune system whose best-characterized function is the clearance of active proteases from the tissue in many animals (Armstrong and Quigley 1999); it is thought to be absent in *Amphimedon* (Srivastava et al. 2010), but we found *A2M* in all the transcriptomes. Similarly, the *mannose-binding lectin associated serine protease* (MASP), which is responsible for activation of the *lectin* complement pathway (Iwaki et al. 2011) was not found in *Amphimedon* (Srivastava et al. 2010) but was found in the *Corticium* transcriptome (Fig. A4-5).

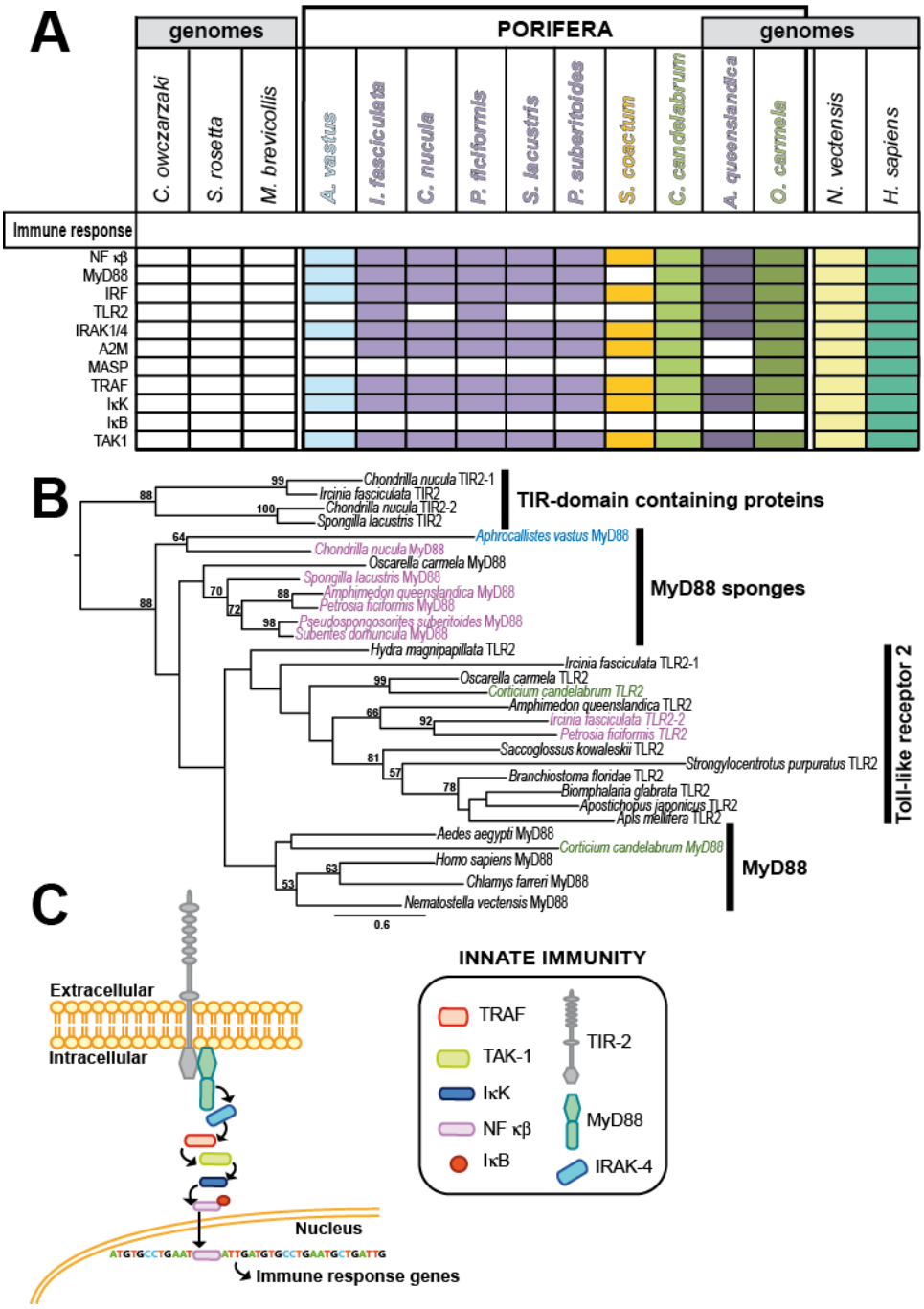


Figure A4-5: Innate immunity molecules identified in Porifera.
A. Presence of the genes involved in the innate immune response in metazoans. B. Evolutionary relationships of sponge *MyD88* and *Toll-like receptors* determined with Maximum Likelihood analysis. Bootstrap support is shown for nodes greater than 50% of 500 pseudoreplicates. Colored sequences are matched to the sponges in Figure 1A. Accession numbers of sequences used to construct the phylogenetic tree are available in Supplementary Table A4-S4. C. Schematic depicting innate immunity molecules in the cell membrane of a metazoan.

A4.3.8 Reproductive machinery: germ line, sex determination, pheromones, and vitellogenesis

The ability of differentiated cells to dedifferentiate into dedicated reproductive cell populations (gametes) is exclusive to multicellular animals. Whether these cells are segregated early in the development of the individual, or are continually transformed from undifferentiated cells varies among animal phyla. In sponges, it appears that the mechanism of gamete determination is triggered by environmental cues and involves somatic cell differentiation into gametes (see Riesgo and Maldonado 2008). Well-known germ line machinery exists in metazoans (Ewen-Campen et al. 2010), even though some of the genes may be involved in maintaining totipotency and not specifically in germ line determination (Juliano and Wessel, 2010). Knowing what genetic machinery used for germ line (and eventually gamete) specification and sex determination (Miller et al. 2003) exists in sponges can shed light in the evolution of reproduction in metazoans. Of the 20 genes known to be involved in determination of the germ line, we found eleven (with some exceptions) in sponges (Fig. A4-6). The genes *germ cell-less* and *pumilio* were not present in *Aphrocallistes* and *Pseudospongosorites*, and *boule* was not found in *Chondrilla* (Fig. A4-6).

For sex determination, all metazoans investigated use *Dmrt* genes, which work as tissue-specific developmental regulators that integrate information about sex, position, and time to direct narrow populations of cells toward male or female fates (Kopp 2012). The sex determination gene *DMRT1* was found exclusively in *Corticium,* while *FEM-1* (a gene involved in gamete specification that appears broadly in metazoans; e.g., Mckeown and Madigan 1992) was found in all sponge transcriptomes (Fig. A4-6). Another important event genetically and environmentally regulated after gametogenesis is gamete release, which is usually synchronized using pheromones in marine invertebrates (Hardege and Bentley 1997; Painter et al. 1998; Counihan et al. 2001). Even though it has been suggested that pheromones may synchronize gamete release in *Neofibularia nolitangere* (Hoppe and Reichert 1987), it is only very recently that the presence

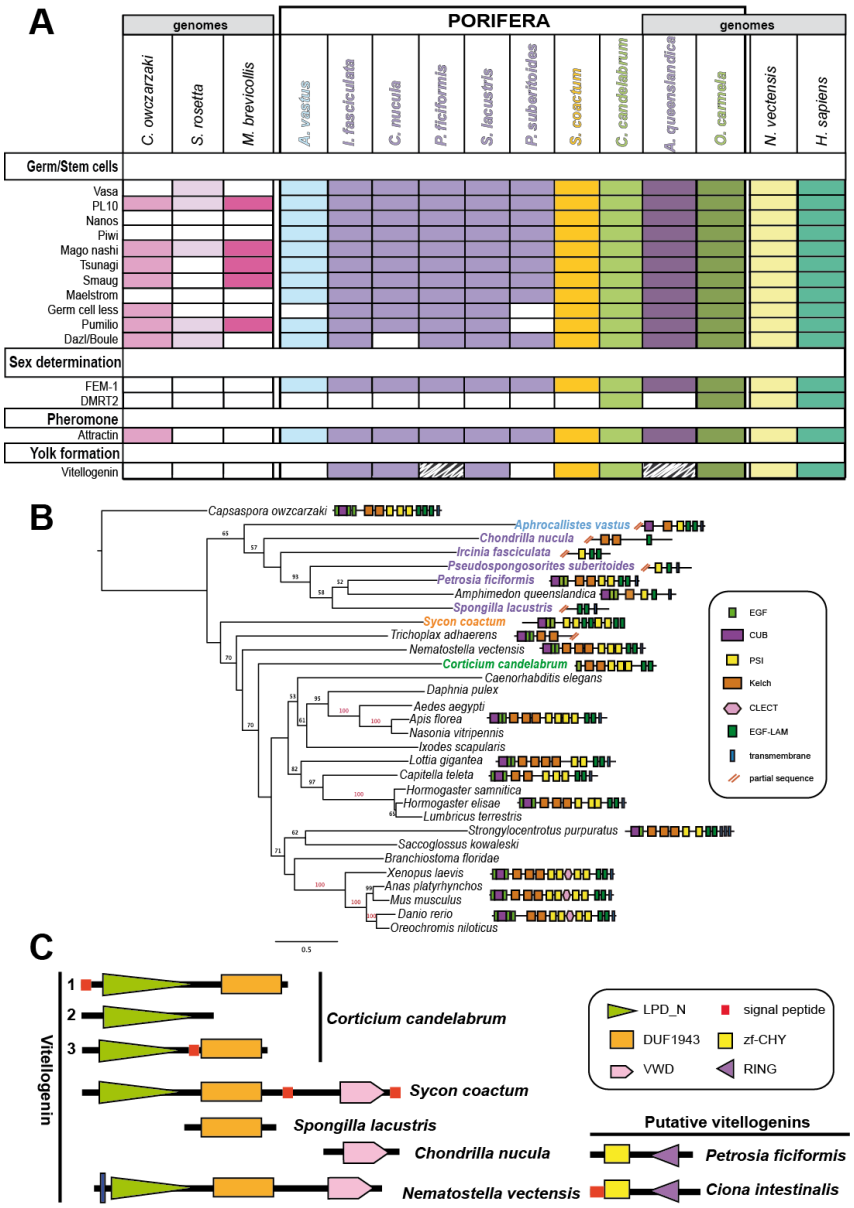


Figure 6. Reproductive, sex determination, pheromone, and vitellogenesis genes identified in Porifera.

A. Presence of the genes involved in germ line and sex determination, pheromone communication, and vitellogenesis in metazoans. Hashmarks denote the presence of a sequence with similarity to vitellogenin-like proteins that are highly divergent from vitellogenin. B. Evolutionary relationships of the sponge pheromone precursor protein attractin as determined with Maximum Likelihood analysis. Bootstrap support is shown for nodes greater than 50% of 500 pseudoreplicates. Colored sequences are matched to the sponges in Figure 1A. Accession numbers of sequences used to construct the phylogenetic tree are available in Supplementary Table 4 C. Protein schematic illustrating the domain structure of vitellogenin and putative-vitellogenin proteins in sponges, cnidarians, and ascidians.

of a pheromone precursor was shown in a demosponge (Novo et al. 2013). In our study, the transcriptomes from all sponge species contained the precursor of the pheromone *attractin* (Fig. A4-6) and there was a high degree of conservation of amino acid sequence in all sponges except *Sycon*.

Vitellogenesis is also a fundamental reproductive process that occurs during gametogenesis not only in sponges but in all metazoans; it allows embryos and lecithotrophic larvae to survive until they develop feeding structures. The variety of processes converging in the formation of a yolk platelet is remarkable in sponges, as are the various morphologies of yolk (e.g., Riesgo and Maldonado 2009), but the genetic regulation of the yolk formation has been investigated only in bilaterians (e.g., Bownes 1986; Wiley and Wallace 1981). In our datasets, one or several *vitellogenin* genes were also found in all species except for *Aphrocallistes* (Fig. A4-6), even though the sequences were very divergent among species (not shown).

A4.4 DISCUSSION

We searched the transcriptomes of eight sponges for genes that have been considered important for metazoan body organization and function. We also checked for the presence of these genes in three well-referenced unicellular eukaryotic genomes, *Capsaspora owzcarzaki*, *Monosiga brevicollis, and Salpingoeca rosetta,* and confirmed their presence in two other metazoan genomes (the cnidarian *Nematostella vectensis* and the vertebrate *Homo sapiens*). We found that few genes in the sponge transcriptomes were sponge-specific – sponges shared between 20 and 50% of genes with other metazoans, between 6.5 and 32% with other eukaryotes (protozoans), and a moderate number were bacterial (between 6 and 24%) – either from bacterial symbionts in the sponge, or in the water they filter; it is unlikely they arise from horizontal gene transfer, which can be the case in pre-metazoans (Tucker 2012). With respect to Pfam domains, *Corticium* contained the highest number, which is similar to that found in the transcriptomes of annelids and molluscs (45,000 to 59,000) and higher than arthropods (around 35,000) (Riesgo et al., 2013). However, the transcriptome of

Corticium may also contain high numbers of bacterial Pfams due to the abundance of symbionts in its mesohyl. From the genes shared with metazoans, in most of the sponges studied more than 50% were most similar to bilaterian genes. In fact, *Aphrocallistes, Sycon,* and *Corticium* showed less than 25% of similarity between their genes and the sponge genes in the NCBI databases; 75% were more similar to bilaterian genes. The number of annotated genes in the sponge transcriptomes was very similar to that of other non-model organisms that also have very few genetic resources (e.g., Riesgo et al. 2012b; Pérez-Porro et al. 2013). Over 30% of the transcriptomic contigs were not assigned any annotation, which highlights the necessity for a greater effort in sequencing and annotation of sponge genomes and/or transcriptomes.

The complement of genes present in sponges appears far greater than previously understood from the single and now well-studied demosponge species *Amphimedon queenslandica* (Srivastava et al. 2010). The publication of the transcriptome of *Oscarella carmela* suggested that homoscleromorph sponges have a far greater complexity than demosponges (Nichols et al. 2008). Yet the demonstration here of the broad presence of genes in so many functional categories – development, signaling, adhesion, epithelia, immune recognition, and germ-lineage/specification – across Demospongiae, Homoscleromorpha, Calcarea and Hexactinellida shows that sponges are universally much more complex at the molecular level than previously appreciated.

What then defines metazoan complexity? We found a number of genes which previously had been associated with complex structures of metazoans and thought to be absent in sponges. For example we found homologs of *smoothened*, *type IV collagen*, and *iontropic glutamate* receptors in several of the sponges. We found also quite clear differences across the sponge classes – almost all metazoan genes were found in the homoscleromorph *Corticium*; *Sycon* shared many of these, but the sequences in *Sycon* though blasting with high e-value to the same genes, and folding to the same proposed 3D structure, always had highly divergent amino acid sequences. Interestingly we also found many of these homologs in the glass sponge *Aphrocallistes*. Some of these differences could be due to variation in

transcriptome quality (coverage and length of contigs – the most complete transcriptomes were those of *Petrosia, Corticium, Sycon,* and *Aphrocallistes*), but they may also reflect differences in the length of time that the sponge classes have been separated, and in their level of tissue/functional complexity. The fact that sequences found in *Corticium, Sycon,* and *Aphrocallistes* were more similar to bilaterian sequences than to sponge sequences in the databases supports the notion that compared to other sponge groups Demospongiae, broadly speaking, have diverged significantly from other metazoans.

A4.4.1 Gene searches

We looked for a total of 127 genes involved in development, neuronal and epithelial signaling, immunity and reproduction. Out of those 127, 100 (78% of the genes) were already identified in the *Amphimedon* genome (Srivastava et al. 2010). In our study, we found 119 genes (18 more than in the *Amphimedon* genome) that were thought to be absent in sponges, mainly in *Corticium* and *Sycon* (see below), which brings the percentage of genes shared by sponges and other metazoans in the pathways studied here to 93%.

A4.4.2 Developmental Toolkit Genes

In unicellular opisthokonts such as *Monosiga* and *Salpingoeca* components of the conserved metazoan toolkit signaling pathways are mostly absent (King et al. 2008; Fairclough et al. 2013); our survey of the genome of *Capsaspora* largely agreed with that finding and confirmed the apparent absence of signaling pathway components outside Metazoa. Notably, however, in *Dictyostelium*, the signaling system involved in forming fruiting bodies contains several elements considered to be critical for Wnt signaling: *Fz receptors*, *GSK3β*, and *β-catenin* homologs (called *GSKA* and *aardvark* in this organism) and a *dkk* (Guder et al. 2006; Harwood, 2008). Interactions between some members of the Wnt pathway therefore may be predate the origin of metazoans. We found that sponges possess many metazoan toolkit signaling genes in the Wnt, Hedgehog, TGF-β and Notch-Delta pathways, and our study of TGF-β and Notch-Delta signaling gave results generally consistent with what is already know from *Amphimedon* (Richards et al.

2008; Richards and Degnan 2009, 2012; Srivastava et al. 2010).

The lack of a Hedgehog ligand is unsurprising as it is missing from the genome of *Amphimedon* (Nichols et al. 2006; Adamska et al. 2007b; Srivastava et al. 2010). It was found in expressed sequence tags (ESTs) from *Oscarella carmela* (Nichols et al. 2006), but the fragment did not contain all diagnostic domains and the characteristic domain structure of a full-length *hedgehog*, therefore these data should be considered cautiously (see Matus et al. 2008 and Ingham et al. 2011 for further discussion). Interestingly homologs of the *smoothened* gene in *Aphrocallistes*, *Sycon* and *Corticium* were found and these new findings push the origin of Smoothened genes further back in metazoan evolution. The lack of *smoothened* in demosponge transcriptomes further supports the idea that the demosponges may have lost certain genes. Furthermore, this trend highlights the need for genomic data from a wider variety of basal branching metazoans – especially sponges – to allow a more complete assessment of the origins of signaling pathways and other characteristic metazoan genes.

A4.4.3 Neuronal signaling

Sponges lack conventional neuronal signaling systems and so it is intriguing that molecules of the protein-rich post-synaptic density (PSD) have been characterized in *Amphimedon* (Sakarya et al. 2007; Alié and Manuel 2010; Srivastava et al. 2010). We found that PSD genes are present in all 4 classes of poriferans and there is little variation among species, demonstrating that PSD genes were present in the poriferan ancestor. Other genes known to be involved in the development of nervous systems in metazoans (neuralians sensu Nielsen 2010) have been identified in the *Amphimedon* genome, such as *elav- mushashi-like* RNA binding genes, neural transcription factors like *Notch*, *Delta*, and *BHLH* (Richards et al. 2008; Gazave et al. 2009; Richards and Degnan 2012). These genes are also widely expressed in the sponge transcriptomes we studied. Molecules involved in signaling (e.g., *G-coupled receptors* (*GPCRs*)), and neuroendocrine secretion are known in part from the *Amphimedon* genome (Srivastava et al. 2010), but clearly this is where sponges vary in complexity.

We found the first evidence of a rapid, ionic-based receptor in sponges, the *ionotropic glutamate receptors* (*iGluRs*). Both *Corticium* and *Ircinia* transcriptomes have sequences with good similarity to vertebrate *iGluRs*, and we found a similar although more divergent sequence in the calcareous sponge *Sycon*. This pattern of divergence was noted in many genes identified in *Sycon*, an observation that may simply indicate an accelerated rate of evolutionary change in that lineage. In contrast to sponges, in the unicellular eukaryote *Capsaspora* only slightly more than half the PSD genes were present. In general, a small number of structural elements of the PSD that in other metazoans lie deeper within the cell can be found in unicellular eukaryotes, while sponges seem to possess a larger set of PSD genes, notably with an increase in the presence of receptor and signaling molecules. Our results are consistent with the scenario summarized by Ryan and Grant (2009), in which the PSD evolves by adding complexity through the addition of channels and receptors while leaving the underlying scaffolding largely intact. The post synaptic density – as a structure - therefore appears to be a characteristic of neuralians.

In contrast, components of classical neurotransmitter synthesis pathways do not seem to form a coherent group, but rather are scattered throughout the different sponge transcriptomes. The presence of genes encoding for enzymes known to be important components of neurotransmitter synthesis, yet lacking the full synthesis pathway could be viewed as evidence of gene loss. An alternate explanation however, is that some enzymes associated with neurotransmitter synthesis are involved in the production of secondary metabolites in the sponge, rather than classical neurotransmitters to be used for signalling. For example, the gene *DOPA decarboxylase*, which codes for an enzyme involved in the synthesis of both dopamine and serotonin, was found in both *Corticium* and *Sycon* yet neither type of receptor was unambiguously identified in any transcriptome. However, a number of serotonin-derived alkaloids have been identified from the demosponges *Hyrtios erectus* and *Hyrtios reticulatus* (Salmoun *et al*., 2002). This may explain why serotonin has been visualized in sponge tissue and why some synthetic enzymes are present, yet why no clear functional role for the

neurotransmitter has been demonstrated (Lentz 1966; Emson, 1966; Weyrer *et al*., 1999; *cf*. Ellwanger and Nickel, 2006); we were also unable to find sequences for a serotonin receptor in the genomes of *Amphimedon* and *Oscarella*. However, the ubiquitous presence of other enzymes such as *glutamate decarboxylase* among all the sponges is consistent with the demonstrated physiological roles for GABA in sponge physiology (Elliott and Leys 2010). Full genomes showing the concrete absence of any molecules, and careful physiological assessments of neurotransmitter effects on sponges, are needed to fully appreciate the roles these enzymes and molecules might play in sponge physiology and behavior.

It is somewhat surprising not to find sequences for voltage gated potassium channels (Kv) in most of the sponges. Until the genomes of these animals can be surveyed it is difficult to draw conclusions about these absences; however such a consistent absence in most of the sponge transcriptomes could also be an indication of the lack of the need in sponges for rapid changes in membrane potential, typically mediated by Kv channels. The next obvious step will be to experimentally characterize the *shaker-like* sequences we found in the unicellular eukaryotes, and in *Corticium*.

A4.4.4 Adhesion and epithelia

Epithelia are complex and highly versatile structures and are one of the unifying characters of multicellular organisms. Even aggregates of unicells form epithelial-like characters such as adherens junctions, for example when *Dictyostelium* amoebae congress to form fruiting bodies (Dickinson et al. 2011, 2012). Adherens-junctions probably provide support in the raised structure formed of clones of cells. But the full complement of epithelial characters requires proteins that allow adhesion, sealing, polarity and stability. When these features were assessed for *Amphimedon* (Fahey and Degnan 2010), it was determined that sponges possessed genes allowing polarized epithelia, but lacked the essential conventional molecules typically thought to seal the epithelium from the environment or to stabilize it by attachment to a basement membrane. These conclusions were thought to support the absence of morphological structures for occlusion or a basement membrane in most sponges. In contrast, where these

genes were found in *Oscarella*, their presence was justified by ultrastructure showing a typical basement membrane, although it was not considered what this structure would be needed for in a homoscleromorph sponge and not in a demosponge or calcareous sponge.

We found transcripts for genes with homology to *claudin* – involved in sealing the spaces between cells in deuterostomes – and surprisingly *type IV collagens* and other basement membrane genes such as *nidogen* and *perlecan*, which attach *type IV collagen* to the cell's plasma membrane, were present in nearly all sponges. Overall there was no pattern of presence/absence of these genes across the sponge classes that might provide a hint as to the lineage of evolution. The collagen type IV amino acid sequence from *Spongilla* was the most divergent, which might reflect the recent radiation of sponges into freshwater and the challenges of that environment. Indeed the changes involved in the marine-freshwater transition would be exciting to revisit with a survey of ion channels and transport molecules in addition to sealing of epithelia.

A4.4.5 Innate Immunity

Immune genes evolve at an extraordinarily rapid pace, which makes it difficult to draw up hypotheses about their evolution (Hughes 1997; Hibino et al. 2006). Presumably the pace of mutation is driven by intense selection in the interplay between host and pathogen. As a consequence, finding immune gene homologues with standard molecular strategies and inferring primitive states is a difficult task. The *Amphimedon* genome encodes several molecules involved in innate immunity including *Nod-like* and *Toll-like receptors*, *IRAK*, *MyD88*, *IRF*, and *IKK* (Srivastava et al. 2010); many 'immune' molecules however, seem to be largely "eumetazoan" acquisitions (i.e., found in Cnidaria, Ctenophora, and Bilateria). In contrast, we found the most complete molecular machinery involved in sponge immune response to date, finding all genes involved in the innate immune response pathway described in basal invertebrates (Miller et al. 2007). Also, two genes previously described as "eumetazoan" acquisitions, *alpha-2 macroglobulin* and *mannose-binding lectin associated serine protease*, were found in sponges, even though they were absent in *Amphimedon* (Srivastava et al.

2010). While *A2M* was found in all sponges classes, *MASP* was only in *Corticium*. None of the components from the selected innate immunity response toolkit was present in any of the three unicellular eukaryotes, as also known from the study of Song et al. (2012). Therefore, our results indicate an ancient origin of the innate immune response in metazoan evolution, which predated the separation of sponges and other metazoans.

A4.4.6 Reproductive machinery

In sexually reproducing animals, germ cells are the source of gametes in the adult (Lin 1997). Germ cells carry the hereditary information for the next generation, thus their segregation and protection from a somatic cell fate is essential for animal development and evolution (Buss 1988; Saffman and Lasko 1999; Wylie 1999; Raz 2000). Modern studies identify primordial germ cells more often by the localization of the products of germ-specific genes (Extavour and Akam 2003; Ewen-Campen et al. 2010). Very recently *vasa*, *nanos, piwi, and PL10* genes were isolated from sponges (Mochizuki et al. 2000, 2001; Funayama et al. 2010; Srivastava et al. 2010), but whether the complete germ line machinery is present in all sponge classes was not investigated. We found that all classes of sponges possess all the genes reported necessary for germ cell determination (note that those specific to *Drosophila* (e.g., *oskar*) were not found). Interestingly, even though sex has not been reported for the three unicellular eukaryotes surveyed here, we found germ line markers in their genomes (*PL10*, *mago nashi*, *smaug*, *pumilio*, *germ cell-less*, and *boule*). The presence of germ line genes in unicellular eukaryotes seems to support the alternative suggestion for the function of these genes: it may be that germ line markers originated in multipotent cells, where they maintain multipotency, and were subsequently co-opted by more-specialized, embryonic germ cells to determine their germ fate (Juliano and Wessel 2010). Whether or not these genes are used by sponge cells to maintain multipotency or determine their germ fate remains unknown, but the fact that they possess the complete molecular program for the germ line specification could indicate its potential role in germ line determination, but should be evaluated more closely for their role in determining a germ cell lineage in metazoans.

Sex determination in metazoans involves a wide array of solutions, from splicing-based mechanisms in insects to endocrine regulation in mammals (Kopp 2012). However, the occurrence of the sex determining factors *Dmrt* in all metazoans investigated has emerged as a common theme in sexual dimorphism. The main function of *Dmrt* genes in the gonads of metazoans is to promote male-specific and repress female-specific differentiation (Kopp 2012). Interestingly, we only found an ortholog of *Dmrt1* in *Corticium*, which is a hermaphroditic organism with a remarkable similarity in the gametogenic process with that of other metazoans, for instance in the continuous and asynchronous production of sperm in the cysts (Riesgo et al. 2007). Likewise, a *Dmrt*1 ortholog is expressed in the hermaphroditic *Acropora millepora* during sexual reproduction (Miller et al. 2003). Therefore, our results could suggest that the sex determination mechanisms involving *Dmrt* genes evolved prior the divergence of Porifera from the rest of metazoans.

Communication via semiochemicals such as pheromones occurs in water by either "sniffing" or by contact chemoreception (Wyatt 2003). The peptide Attractin from *Aplysia* was the first water-borne sex pheromone characterized in invertebrates (Painter et al. 1998); and the full length protein Attractin, which has been found expressed in gonads of mammals (Li et al. 2009) was recently reported in several metazoans including a demosponge (Novo et al. 2013); although the pheromone features were not corroborated. We found that the gene *attractin* was expressed in all classes of sponges, even though it was not originally found in the *Amphimedon* genome. In *Amphimedon*, the gene characterized as *Fanconi anemia group I protein-like* exhibited the highest similarity to *attractin*. Although the potential role of *attractin* on the synchronization of gamete release in sponges remains uninvestigated, it opens the possibility of further research in this novel area.

Vitellogenesis in sponges produces two types of yolk platelets, homogeneous (mainly proteinaceous) and heterogeneous (lipidic and proteinaceous) (see Simpson 1984; Riesgo and Maldonado 2009 for reviews). The participation of autosynthetic and heterosynthetic (through nurse cells) mechanisms has been

described for several species, but the protein precursor has never been characterized in sponges. We found two types of yolk precursors in our sponge transcriptomes, one *vitellogenin* gene in *Ircinia*, *Chondrilla*, *Spongilla*, *Sycon*, and *Corticium*, and a *vitellogenin-like* gene in *Petrosia*. Whether the different genes are involved in the formation of multiple yolk platelets as in *Xenopus* (Wiley and Wallace 1981) needs further study.

A4.4.7 Conclusions

This is the first study to survey a wide set of metazoan-specific genes in-depth across all four sponge classes. It provides texture to the question of which molecules might have been present in early animal groups, and more importantly provides the framework for posing new hypotheses on the evolution of multicellularity and animal complexity.

One of the remarkable outcomes of this work is the understanding that most metazoan genes, or the greater complement of genes involved in complex gene pathways, are present in all sponge groups, including genes supposedly absent in the genome of the demosponge *Amphimedon*. Importantly, there are very few key absences (mainly these concern rapid signaling molecules), but overall transcriptomic datasets proved useful to detect complex molecular machineries, even though they are known to contain genes expressed only at a given time.

Determining gene function in sponges is the next challenge. Usually function of genes in an organism is inferred by comparing to gene function known from other animals. For example, *occludins* are known to seal the epithelium at its apical surface in mice, therefore the presence of *occludins* is taken to imply that sponge epithelia seal. Only one functional study has shown that sponge epithelia are sealed against the outside milieu (Adams et al. 2010), but the species in which this work was carried out, *Spongilla lacustris,* does not appear to possess *occludins* in our transcriptome. In *Spongilla*, therefore, sealing function could be the work of other as yet unspecified molecules.

Here we show that the great majority of metazoan genes are present in all sponge groups, but sponges do not have conventional structures, behavior, or even mechanisms of development. So either the genes we know from other animals

have a different function in sponges, and were co-opted later in the evolution of metazoans for the function we are familiar with, as suggested for adhesion molecules in unicellular eukaryotes (Sebé-Pedrós et al. 2010), or the structure has been lost in sponges. In this sense, the recent publication of the genome of *Mnemiopsis leydi* (Ryan et al. 2013), which places ctenophores as the sister group to all other animals, suggests that structures such as nerves might have been present in the metazoan ancestor and were secondarily lost in placozoans and sponges. Another alternative is that sponge genes might carry out a similar function as they do in other animals, but we do not understand that function because we do not recognize the morphology of such a different structure yet.

Our comparative transcriptomic analysis strengthens the view that sponge complexity as revealed by their molecular toolkit is poorly reflected in their morphology, as it has also been shown inplacozoans (Srivastava et al. 2008). Quite interestingly, our data provides an indication that demosponges have diverged substantially from other classes of Porifera, and highlights the strong similarity of genes in calcareous, homoscleromorph and even hexactinellid sponges with those in other metazoans. Our data also show that a number of genes are present in calcareous and homoscleromorph sponges but absent in the Silicea (hexactinellids and demosponges). Both the greater number of genes in Calcarea and Homoscleromorpha and the similarity of those sequences in those two species lend support to the suggested sister relationship of these two groups shown in recent phylogenetic studies (e.g., Nosenko et al. 2013; Ryan et al. 2013). Taken together the overall view given by patterns shown in gene presence and absence across the 4 sponge classes supports the idea that sponges are monophyletic with Demospongiae + Hexactinellida and Calcarea + Homoscleromorpha forming sister groups, a hypothesis that is in agreement with the latest phylogenomic analysis using several sponge taxa (Nosenko et al. 2013). One noteworthy observation provided by the data presented by the publication of the ctenophore genome is the remarkable similarity in the gene absences in the post-synaptic density and signaling pathways in the ctenophore genome (Ryan et al. 2013) and our study. These absences (and their significance) deserve more attention given

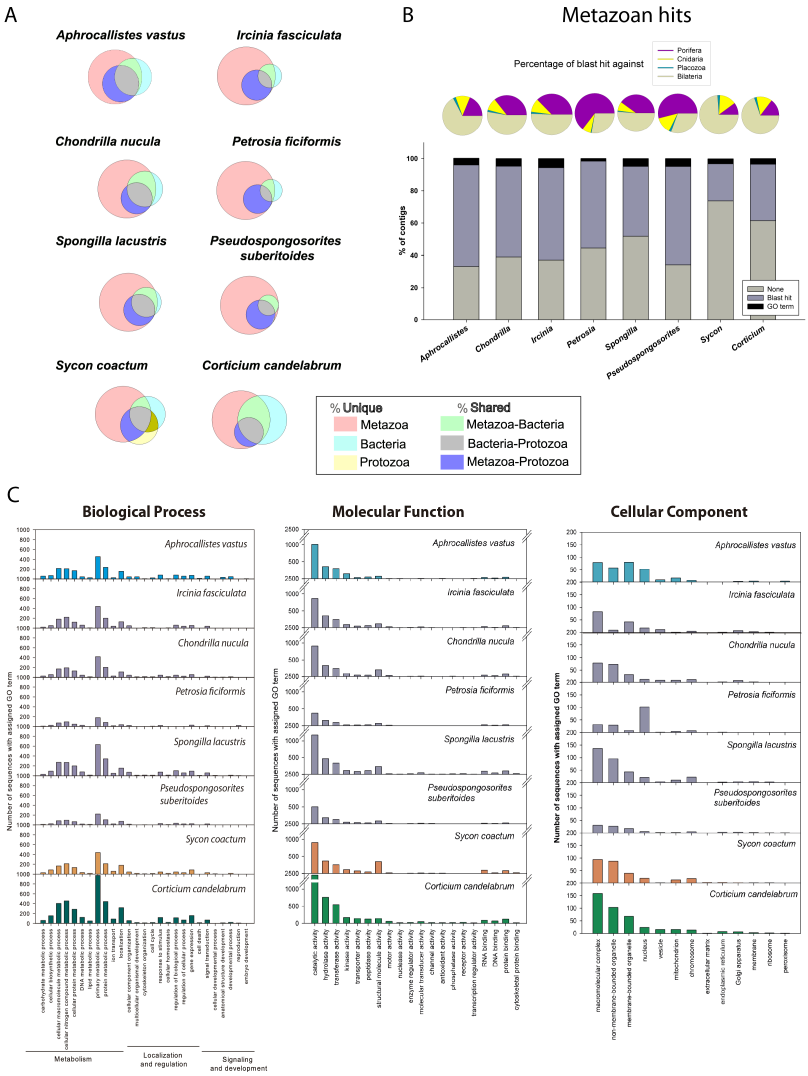
that they may support the placement of ctenophores at the base of the metazoan tree.

A4.5 SUPPLEMENTAL FILES

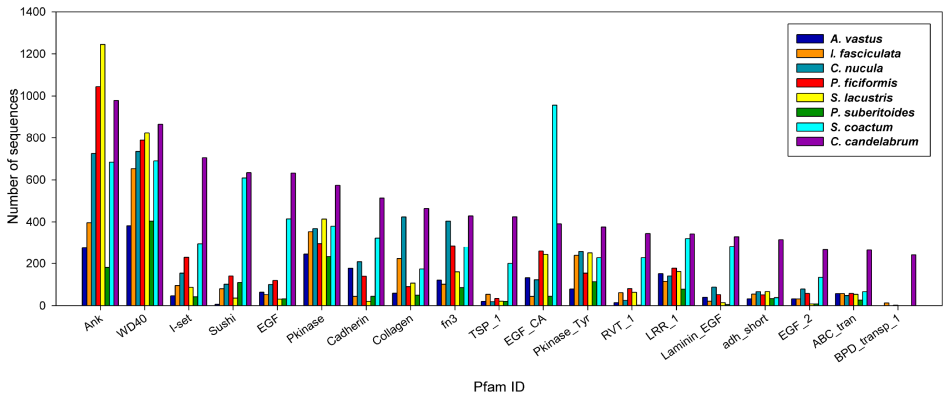
Table A4-S1: Collection details, mRNA concentration, and basic statistics on the transcriptomes of each sponge species.

Species name	Year of collection	Location	N samples sequenced	Fixation	cc mRNA (ng/ul)	A260/280	N reads BT	Avg. Read Length	N reads used	N contigs	N bases (Mb)	Avg. L Contigs (bp)	N Contigs >1Kb	max contig length (bp)	N50	Avg. Coverage/ base	N Contigs <300 bp (removed)	Predicted metazoan proteins
Aphrocallistes vastus	2009	Fraser Ridge, Vancouver Island, Canada	2	LN2	6.7	2.09												
					6.6	2.18	90,190,158	100.00	78,154,171	46,987	64.60	1,375	22,483	24,685	8,881	121.00	25,792	28,243
Sycon coactum	2009	Barnfield, Vancouver Island, Canada	2	LN2	79.9	2.20												
					16.9	2.07	86,167,952	98.89	64,809,007	41,571	23.5	565	5,636	10,613	782	187.42	24,071	19,062
Ircinia fasciculata	2011	Blanes, Catalonia, Spain	1	LN2	20.4	1.84	85,395,382	95.80	60,904,622	34,868	17.5	502	3,422	11,482	598	202.12	14,924	16,898
Pseudospongosorites suberitoides	2012	Gulf of Mexico, US	1	LN2	49.7	2.17	125,668,773	84.26	89,053,669	20,295	28.10	567	6,547	17,037	802	202.21	6,958	11,536
Chondrilla nucula	2012	Bocas del Toro, Panama	1	RNAlater	29.9	2.20	234,585,429	88.82	159,447,843	56,696	29.10	514	6,243	8,078	603	341.69	17,098	21,229
Petrosia ficiformis	2009	Blanes, Catalonia, Spain	2	LN2	62.5	2.17												
					40.7	2.22	38,866,233	98.67	31,568,871	49507	10.3	510	1,931	7,596	604	202.35	21,865	20,152
Spongilla lacustris	2009	Frederick Lake, Vancouver Island, Canada	2	LN2	20.6	2.07												
					74	2.22	152,127,057	90.79	115,070,661	70,220	48.30	687	12,930	63,759	1,138	164.01	21,269	15,025
Corticium candelabrum	2009-2011	Blanes, Catalonia, Spain	3	RNAlater	42.5	2.24												
				RNAlater	20	2.38												
				LN2	20.3	2.36	139,201,494	108.78	96,670,783	141,629	65.17	452	9,991	16,849	474	108.50	64,938	41,146

Figure A4-S2: Affiliation of BLAST hits, annotation Gene Ontology (GO) and Protein family (Pfam) assignments of contig sequences of sponge transcriptome datasets.
A. Percentage of BLAST hits against Metazoan, Bacterial, and Protozoan protein databases and the percentage of contigs with hits shared between more than one database. B. Percentage of contigs without a BLAST hit, and with a BLAST hit against the Metazoan database coupled with a Gene Ontology (GO) annotation. For the hits against the Metazoan database, pie charts illustrate the percentages with highest similarity to Porifera, Cnidaria, Placozoa, and Bilateria for each sponge species. C. Gene Ontology (GO) assignments for each transcriptome under the Biological Process, Molecular Function, and Cellular Component categories. D. Protein families (Pfam) retrieved from the transcriptomes of each sponge species. E. Protein families (Pfam) retrieved from the transcriptomes of each sponge species grouped by these functions: cell adhesion, signaling, immune system, metabolism, structural/cytoskeletal, and multiple functions.



D


Number of sequences
1400
1200
1000
800
600
400
200
0
A. vastus
I. fasciculata
C. nucula
P. ficiformis
S. lacustris
P. suberitoides
S. coactum
C. candelabrum
Ank
WD40
I-set
Sushi
EGF
Pkinase
Cadherin
Collagen
fn3
TSP_1
EGF_CA
Pkinase_Tyr
RVT_1
LRR_1
Laminin_EGF
adh_short
EGF_2
ABC_tran
BPD_transp_1
Pfam ID

E

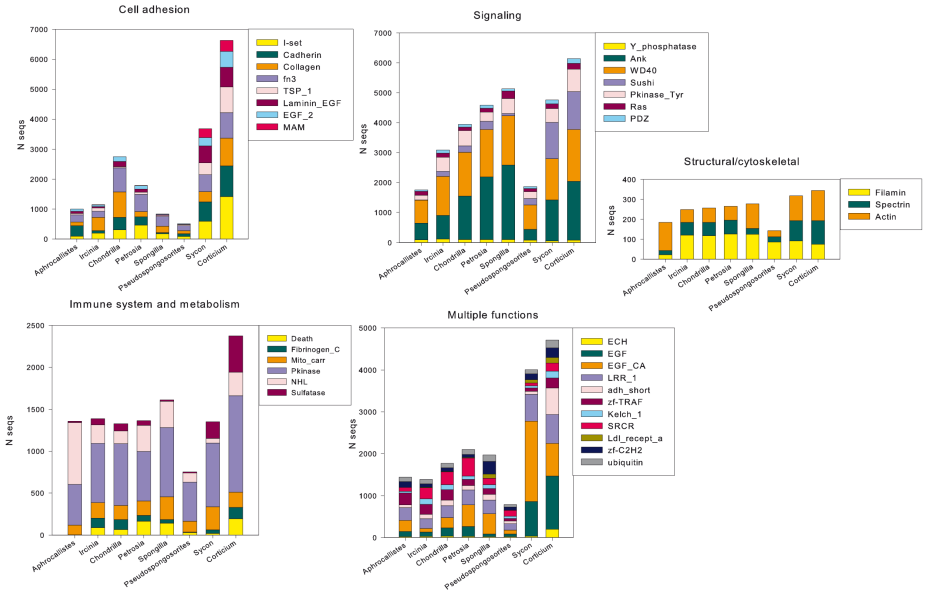

Cell adhesion
I-set
Cadherin
Collagen
fn3
TSP_1
Laminin_EGF
EGF_2
MAM
Signaling
Y_phosphatase
Ank
WD40
Sushi
Pkinase_Tyr
Ras
PDZ
Structural/cytoskeletal
Filamin
Spectrin
Actin
Immune system and metabolism
Death
Fibrinogen_C
Mito_carr
Pkinase
NHL
Sulfatase
Multiple functions
ECH
EGF
EGF_CA
LRR_1
adh_short
zf-TRAF
Kelch_1
SRCR
Ldl_recept_a
zf-C2H2
ubiquitin
N seqs
Aphrocallistes
Ircinia
Chondrilla
Petrosia
Spongilla
Pseudospongosorites
Sycon
Corticium

Figure A4-S3: Evolutionary relationships between *ionotropic glutamate receptors* (iGluR) found in bacteria and metazoans determined with Maximum Likelihood analysis.

A. Phylogenetic reconstruction without non-metazoan outgroups. B. Phylogenetic reconstruction without bacterial *glutamate receptors*. C. Phylogenetic reconstruction with bacterial *glutamate receptors* and excluding the iGluR found in *Sycon coactum*. Bootstrap support is shown where nodes are greater than 50% of 500 pseudoreplicates.

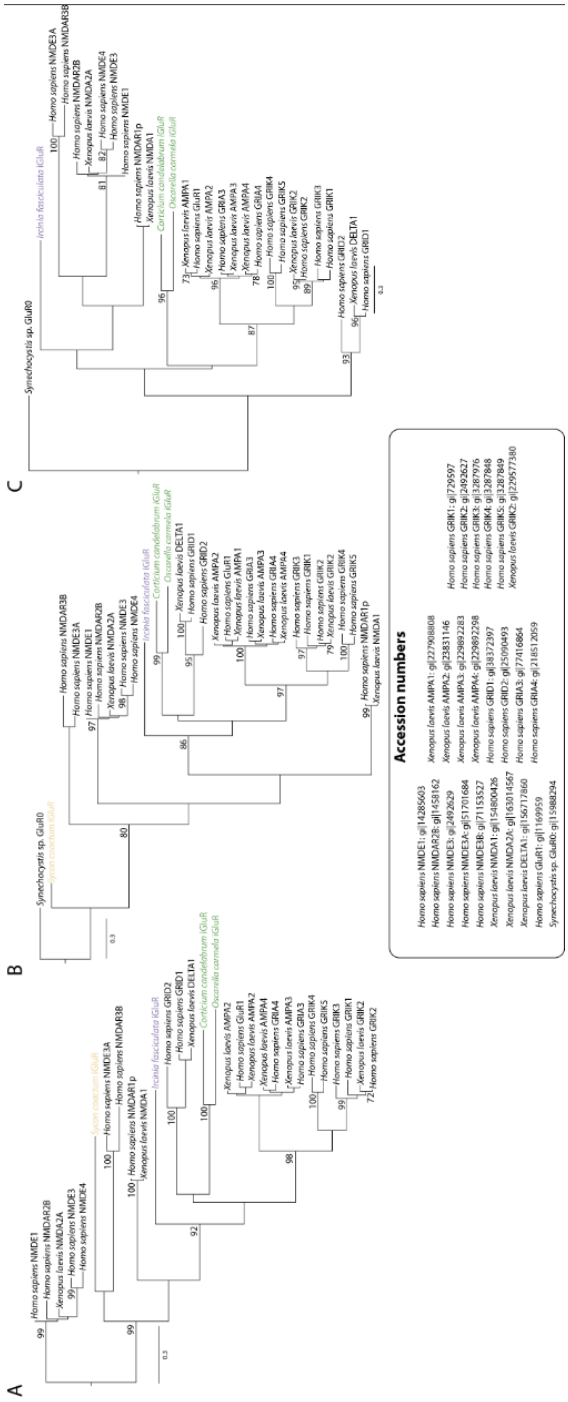


Table A4-S4: Accession numbers from GenBank for all amino acid sequences used in each of the phylogenetic analyses in Figures 3-6.

Accession numbers

Signalling Genes	Smoothened tree
	Smoothened
Mus musculus	NP_795970.3
Danio rerio	NP_571102.1
Drosophila melanogaster	NP_523443.1
Xenopus laevis	NP_001128704.1
Ciona intestinalis	NP_001071819.1
Nematostella vectensis	XP_001632182.1
Strongylocentrotus purpuratus	XP_781487.3/XP_799079.2
Platynereis dumerilii	ADK38671.1
Apis mellifera	XP_395373.3
	Frizzled
Danio rerio	NP_001124086.1
Mus musculus	EDL14633.1
Xenopus laevis	NP_001079207.1
Immunity Genes	**MyD88 tree**
	MyD88
Amphimedon queenslandica	ADR78337
Suberites domuncula	CAL36105
Aedes aegypti	EAT40501
Homo sapiens	AAB49967
Chlamys farreri	ABB76627
Nematostella vectensis	Nemve5.187.1
	TOLL-LIKE RECEPTOR 2
Amphimedon queenslandica	ADR78339
Hydra magnipapillata	ABE26988
Saccoglossus kowaleskii	XP_002741808
Strongylocentrotus purpuratus	NP_999671
Branchiostoma floridae	EEN46716
Biomphalaria glabrata	AGB93809
Apostichopus japonicus	AFV38972
Apis mellifera	AGM20107

PSD Genes	iGluR tree
	iGluR-kainate 2
Saccoglossus kowaleskii	XP_002730360.1/XP_002739876.1
Strongylocentrotus purpuratus	XP_787239.3
Homo sapiens	Q13002.1
Arabidopsis thaliana	AAR27949
Xenopus laevis	NP_001153158.1
Ciona intestinalis	XP_002120626.1
	iGluR-AMPA1
Saccoglossus kowaleskii	XP_002731595.1
Homo sapiens	P42261
Xenopus laevis	NP_001153151.1
	iGluR-AMPA2
Homo sapiens	P42262.3
	iGluR-AMPA3
Homo sapiens	P42263.2
Xenopus laevis	NP_001153154.1
	iGluR-AMPA4
Homo sapiens	P48058.2
Xenopus laevis	NP_001153157.1
	iGluR-kainate 5
Homo sapiens	Q16478.1
	iGluR-kainate 4
Homo sapiens	Q16099.1
	iGluR-kainate 1
Homo sapiens	P39086.1
	iGluR-kainate 3
Homo sapiens	Q13003.2
	Delta-2
Homo sapiens	O43424.1
	Delta-1
Ciona intestinalis	XP_002120434
Xenopus tropicalis	NP_001096470
	NMDA-1
Homo sapiens	AAB67724
Xenopus laevis	NP_001081616.1
	NMDA-2
Saccoglossus kowaleskii	XP_002733261.1
Homo sapiens	O60391.2/XP_489725.1/Q13224.3/Q12879.1
Xenopus laevis	NP_001106367.1
Ciona intestinalis	XP_004226517.1
	NMDA-3
Homo sapiens	Q14957.1

Reproductive Genes	Attractin
Capsaspora owzarzaki	CAOG_07456.2
Nematostella vectensis	XP_001628937.1
Trichoplax adhaerens	XP_002114607.1
Caenorhabditis elegans	AAK14396.1
Daphnia pulex	EFX85858.1
Aedes aegypti	XP_001652702.1
Apis florea	XP_003695980.1
Nasonia vitripennis	XP_001606408.2
Ixodes scapularis	EEC09897.1
Lottia gigantea	jgi|Lotgi1|108840
Capitella teleta	jgi|Capca1|219601
Lumbricus rubellus	pers.com.
Hormogaster elisae	GAHS00000000
Hormogaster samnitica	GAHR00000000
Saccoglossus kowaleskii	XP_002742338.1
Branchiostoma floridae	XP_002601127.1
Strongylocentrotus purpurat	XP_003724782.1
Xenopus tropicalis	XP_002941104.1
Danio rerio	XP_001331928.4
Oreochromis niloticus	XP_005453430.1
Anas platyrhynchos	XP_005022046.1
Mus musculus	EDL01792.1

Figure A4-S5: Nucleotide and amino acid sequences in fasta format of all genes found within this study in the eight sponge transcriptome datasets.
(Text file not included due to length)

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