# Chitin the Good Fight – Identification and Description of Chitin and Its Genes in Cnidaria

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Abstract

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This dissertation explores broad aspects of chitin biology in Cnidaria, with the aim of incorporating glycobiology with evolution and development. Chitin is the second-most abundant biological polymer on earth and is most commonly known in metazoans as a structural component of arthropod exoskeletons. This work seeks to determine whether chitin is more broadly distributed within early-diverging metazoans than previously believed, and whether it has novel non-structural applications in cnidarians. The Cnidaria (i.e., medusae, corals, sea anemones, cubomedusae, myxozoans) comprises over 11,000 described species exhibiting highly diverse morphologies, developmental programs, and ecological niches. Chapter 1 explores the distribution of *chitin synthase* (CHS) genes across Cnidaria. These genes are present in all classes and are expressed in life stages or taxa that do not have any reported chitinous structures. To further elucidate the biology of chitin in cnidarian soft tissues, in Chapters 2 and 3 I focus on the model sea anemone *Nematostella vectensis*, which has three *chitin synthase* genes – each with a unique suite of domains. Adult and developmental data show that the *CHS* genes are differentially expressed in *Nematostella* tissues, indicating that the chitin molecule has an integral role in the biology of this species. Disruption of genomic sequences for *chitin synthase* genes results in disorganized morphology in larvae and polyps,

and a lack of spirocyst development. Histochemical labeling shows that chitin is distributed widely in endodermal and ectodermal tissues, and that it comprises the tubule of spirocysts. In the Appendix, I discuss a putative *CHS* gene cloned from the ctenophore species *Pleurobrachia bachei*. Chitin histochemical labeling localizes to a portion of the tentacle bulbs in both *P. bachei* and *Mnemiopsis leidyi*. Spacial gene expression of the putative *Pb-CHS* also localizes to the tentacle bulb. Understanding how early-diverging phyla like Cnidaria employ chitin may provide new insight into hitherto unknown functions of polysaccharides in animals as well as their role in the evolution of biological novelty.

**Appendix and Table of Contents**

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If you have made it this far, I'd like to extend my appreciation to you as well.

## 0.1 Introduction

*Chitin and its Significance in Nature*

Chitin is an unbranched long-chain glycopolymer composed of anhydro-2-acetamido-2-deoxy-D-glucose (GlcNAc [β-1,4-linked N-acetyl-D-glucosamine). Each *N*-acetylglucosamine molecule is attached in a β-1,4 linkage (Figure 0.1). Chitin is considered to be the second-most prevalent biomolecule on earth, after cellulose, and is present across a wide array of eukaryotic taxa, from fungi to animals to microalgae (Kim 2011; Zakrzewski et al 2014; Martinez et al 2016). Chitin is similar in structure to cellulose, except that it contains an acetyl-amino group rather than a hydroxyl in the second carbon position (Figure 0.1). Chitin fibrils are composed of multiple individual adjacent chitin fibers, and the acetyl-amino group likely facilitates hydrogen bonding between the individual chitin chains, increasing its tactile strength (Sawada et al 2012; Zhu et al 2016).

The word “chitin” is derived from a Greek word for covering “chiton” or “χιτών”. The term was given to the substance by Auguste Odier in the early 1800s in his description of the composition of insect exoskeletons (Odier 1823). Three types of chitin “forms” are found in nature – α-chitin (the most common form) is made of antiparallel chitin chains, β-chitin is composed of parallel chains, and the rare γ-chitin alternates two parallel chains with an antiparallel chain. It has been proposed that γ-chitin may be a mixture of α-chitin and β-chitin (Roberts 1992; Souza et al 2011). Arthropod exoskeletons, marine sponges, and fungal cell walls contain α-chitin, while squid pens, and diatom skeletons are composed of β-chitin (Kaya et al 2015; Zhu et al 2016). The *Loligo* squid has been described as synthesizing all three forms of chitin in different tissues: the hard beak is composed of α-chitin, its internal skeletal structure – the gladius or pen – is composed of β-chitin, and γ-chitin lines the stomach walls (Rudall and Kenchington 1973).

As evidenced by the overachieving squid, a variety of animal taxa produce chitin in a large diversity of structures. Though widely occurring throughout Metazoa, chitin formation has been

studied primarily in arthropods where it is found in its well-known mineralized form, the exoskeleton (reviewed in Hyman, 1966). Chitin is an important component in mollusk shells, present in both in the calcareous outer layer and the nacre layer (also known as mother of pearl) of bivalves and gastropods, in radulas (Peters 1972), and in the beaks of cephalopods (Zheng et al 2016; Furuhashi et al 2009). Fossilized chitinous material has been recovered from 500 mya demosponge poriferans (Ehrlich et al 2015), and potentially from 200 mya gastropod egg shells (Wysokowski et al 2014).

Chitin has also been described in a broad array of hard anatomical structures in other invertebrates: annelid chaetae (Ruppert et al., 2004), some coral endoskeletons (Bo et al., 2012b), and recently discovered in the holdfasts and tissues of all classes of sponge (Ehrlich et al 2007a; Ehrlich et al 2007b; Ehrlich et al 2018). Chitin is found in soft tissues in nudibranch gastropods as well, though its function is unknown (Martin et al 2007). It was shown in recent years that chitin, long assumed to be absent from vertebrates, is endogenously produced in fishes and amphibians (though not in amniotes) (Tang et al 2015). Intreguingly, vertebrate chitin is used nonstructurally (not in skeletal or bony structures), demonstrating that this glycopolymer can be utilized in more diverse ways than previously thought.

Chitin is the most abundant polymer in the marine environment (Souza et al 2011). It is estimated that copepods (small marine crustaceans) alone produce billions of tons of chitin (Yu et al 1991). Chitinolytic bacteria (bacteria that digest or degrade chitin) are responsible for the majority of chitin degradation in the oceans, whether free-living in the plankton or associated with gut flora of animals (Keyhani and Roseman 1999; Li et al 2016). Chitin is a major component of “marine snow”, as digested food particles, arthropod molts, and dead chitinous organisms, that slowly sink through the water column from biologically productive shallow waters into the deep ocean. Sediment cores from the deep ocean contain little chitin, indicating that most of it is ingested or taken up by organisms rather than settling out (Alldredge and Gotschalk 1990; Souza

et al 2011). Chitin is thus a significant source of carbon in deep sea ecosystems and is involved in marine carbon and nitrogen cycling.

The organismal distribution of chitin in diverse lineages, tissues, and cell types (cellular and extracellular chitin), and evolutionary conservation of chitin synthase genes present across metazoan taxa suggest important roles for chitin in physiology, anatomy, and development in animals.

*Chitin Biology: Synthesis and Metabolism*

The molecular pathway of chitin synthesis is broadly conserved across taxa as divergent as fungi and arthropods. The classical chitin synthesis pathway in fungi and insects involves approximately a dozen reactions beginning with a glycogen substrate, which is subsequently modified until a UDP-GlcNAc is formed (Merzendorfer 2011). Chitin synthase (CHS) is the final catalytic enzyme in this biosynthetic pathway. Chitin synthases are normally localized to cell membranes, where several transmembrane domains create a pore through which the newly synthesized/growing chitin molecule is secreted extracellularly. The enzymatically active region of the CHS enzyme is the glycosyl transferase domain. It is at this site in the protein that UDP-GlcNac units are sequentially added to the elongating chitin chain. Chitin synthases contain glycosyltransferase family-2 domains; this family also includes enzymes that assemble glucose- and mannose-derived molecules. Glycosyltransferases work by transferring a monosaccharide residue from an activated nucleotide sugar donor to a specific receiving molecule, thereby forming glycosidic bonds (Breton et al 2006). The amino acid sequence of the chitin synthase glycosyl transferase domain is highly conserved across evolutionary time (Zakrzewski et al 2014).

Much of what is known about intracellular synthesis and trafficking of chitin synthase enzymes comes from extensive research in fungi such as yeast (Duran et al 1975; Yarden and Yanofsky 1991; Bowman and Free 2006) and insects (reviewed in Zhu et al 2016). Up to seven families of *chitin synthases* have been discovered in fungi (Martinez et al 2016; Zakrzewski et al

2014), where homologous *chitin synthases* contain a diversity of protein domains and have been shown to have specific functions. It has been proposed that the last common Opisthokonta ancestor possessed four *CHS* orthologs, and that the ancestral metazoan genome contained one *CHS* gene (Goncalves et al 2016). In fungi, evolution and diversification of CHS genes with unique domains correlates with evolution of body plans and adaptation to diverse niches (Liu et al 2017), and some fungi species possess up to fifteen *chitin synthase* orthologs in their genomes (Goncalves et al 2016). Metazoan chitin synthases fall into two categories – CHS Type I, or CHS Type II (Zakrzewski et al 2014), as defined by conserved domains and phylogenetic analyses. CHS Type I enzymes contain sterile alpha motifs (SAMs); this type of domain is involved in a diversity of protein-protein, membrane lipid, and RNA interactions. Its function in animal chitin synthase enzymes has not been well described. Type II chitin synthases lack SAMs, and some orthologs contain myosin motor domains (Figure 0.3).

*Chitinases and Chitobiases*

Chitinases belong to a class of enzymes that specifically cleave glycosidic bonds in glycans – glycoside hydrolases (GHs). The term “glycan” is similar to “polysaccharide”; both are defined as compounds composed of a large number of monosaccharides that are linked glycosidically - a type of covalent bond that links a sugar to some other molecule (IUPAC – Gold Book: http://goldbook.iupac.org/html/G/G02645.html). Chitin is an example of a glycan (composed of β-1,4-linked N-acetyl-D-glucosamine). Organisms that do not have chitinous structures or that do not synthesize chitin still commonly possess multiple chitinase enzymes. A vast array of taxa – from bacteria to plants to humans – produce chitinases. These enzymes serve a variety of functions; chitinases are involved in immunity, digestion, and environmental nutrient cycling (Fredericksen et al 2013). In organisms that possess chitinous structures (for example, fungi and insects), chitinases are also utilized in the construction, modeling, and maintenance of chitin-containing body parts.

Chitinases come in two flavors – exochitinases and endochitinases. Endochitinases can cleave chitin molecules at any position in the chitin chain, producing multimers. Exochitinases work by either cleaving the terminal-most two acetylglucosamines in the chitin chain (di-acetylchitobioses) or by splitting multimers of a cleaved chitin molecule. Exo- and endochitinases can act in differing capacities in different life stages of animals (Dravid et al 2015). Chitinases have been tentatively divided into groups based on sequence homology and which family of glycosyl hydrolase they contain. For example, animal chitinases commonly contain GH family 18 (Patil et al 2000). In the chitin degradation process, chitinases break up chitin into oligosaccharides and the enzyme chitobiase then cleaves these into monomers. Chitobiases have a similar catalytic domain structure to the glycosyl hydrolase domain of chitinases (Avila et al 2011).

*Chitin Binding Domains*

Chitin binding domains (CBDs) are extracellular protein domains about 30-60 amino acids in length that are rich in cysteine residues (Suetake et al 2000; Shen and Jacobs-Lorena 1999). These residues form a series of disulfide bridges and are highly conserved across kingdoms, as they likely contribute to stabilization of the CBD-containing protein. It has been proposed, however, that this domain similarity is a result of convergent evolution in distantly-related taxa (Shen and Jacobs-Lorena 1999; Suetake et al 2000). Many organisms – from plants to bacteria to dikont protists to animals – possess proteins that contain CBDs, such as mucins, peritrophins (insect membrane proteins), and chitinases. It has been shown that some proteins containing CBDs have an antimicrobial role in plants and metazoans (Kawabata et al 1996; Suetake et al 2000). Charged amino acid residues and aromatic amino acids are thought to bind polysaccharides, including chitin and cellulose (Shen and Jacobs-Lorena 1999).

*Chitin synthesis inhibitors and effects on marine ecosystems*

Because common agricultural pests such as insects, roundworms (nematodes), and fungi have prominent morphological structures (e.g. cell wall, or cuticle) that are largely composed of chitin, proteins in chitin metabolic pathways are often targeted for development of pesticides. Benzophenylurea (BPU) family compounds are widely utilized, and include drugs such as diflubenzuron, triflumeron, and lufenuron. These drugs have been shown to inhibit chitin synthesis in animals and disrupt reproduction and larval development in terrestrial species – *Ascaris* nematodes, *Drosophila*, and mosquito (Fetterer et al 1989; Wilson and Cryan 1997; Belinato et al 2009). The antifungal compound Polyoxin-D may act against the chitin synthase enzyme as a competitive inhibitor with UDP-GlcNAc molecules, due to its structural similarity with these building blocks of chitin (Bartnicki-Garcia and Lippman 1972). Although effects of many pesticides targeting ecdysozoan taxa (arthropods, nematodes) have been well-studied, the molecular mechanisms through which they act are unknown (Meyer et al 2013; Zhu et al 2016), and it is unclear which proteins in the chitin production pathway are affected (Van Leeuwen et al 2012).

Many chitin synthesis-inhibiting drugs, such as those applied in terrestrial or aquatic environments as pesticides, are not designed to specifically target any particular taxon (Van Leeuwen et al 2012), and can conceivably have detrimental effects on non-target species. In aquatic environments, BPU class insecticides are used in the aquaculture industry to target lice, parasitic nematodes, and harmful algae (Langford et al 2014), though its effects on the broader aquatic community are not well understood. An improved understanding of the presence and roles of chitin in metazoans other than Ecdysozoan parasites may help facilitate development of specific chitin synthase-targeted pesticides, and could potentially mitigate harmful effects on the broader ecosystem.

*Applications of Chitin: Biotech and Medicine*

Chitin and its derivatives are employed in the manufacturing of biology-inspired materials that are valued for their tactile strengths (Fernandez and Ingber, 2014; Muzzarelli, 2011). Crustacean exoskeletons and fungal cell walls (e.g. yeast waste from the fermentation process) are major sources of chitin collected for industrial or medical uses (Kumar 2000). Pure chitin is nontoxic, has antimicrobial properties, is biodegradable, and is a target for novel materials engineering (Rinaudo 2006; Wysokowski et al 2015). Compounds that are complexed with chitin have been deployed in manufacturing to remove heavy metal-containing pollutants such as silver thiosulfate (Songkroah et al 2004) and as filters for toxic runoff from mining activities (Westholm et al 2014). Chitin is also thermally stable, with degradation beginning at 200°C (Koll et al 1991; reviewed in Wysokowski et al 2015).

Chitinous fibers and thin films are widely used as bandages or tissue dressings. Chitin has been deployed in mammalian wound healing and tissue regeneration studies as a scaffold for growing cells, as well as for hemostatic bandages, drug delivery systems (as micro syringes or pill capsule casings), and as surgical sutures (Kostopoulos et al., 2001; Kumar, 2000). The molecule chitosan, which is synthesized by removing N-acetyl groups from chitin, has strong antimicrobial characteristics – chitosan is positively charged and binds to bacterial cell walls, hindering bacterial growth (Zou et al 2015). Advancement of chitin materials science and expansion of its applications in medicine can be informed by the uses of this glycomolecule in diverse animal taxa. Throughout Metazoan, chitin is involved in the construction of both hard (e.g. tough crab claws) or flexible structures (e.g. squid pens) and can be complexed with a diversity of other biomaterials and minerals.

*Cnidarians*

Cnidaria is a phylum of aquatic (marine and freshwater) animals classically described as diploblastic, with endo-mesodermal and ectodermal tissue layers, radial symmetry, and a superficially “simple” body plan (but see Martindale et al 2004, Ball et al 2007) (Figure 0.4A). With

fossil records dating to the Ediacaran, cnidarians are one of the earliest-diverging animal phyla (Zapata et al 2015). The phylum's body plans diversified early, with the fossil record showing examples of multiple clades present as far back as the pre-Cambrian (Xiao et al 2000; Zapata et al 2015). Cnidarians have been widely accepted as the sister clade to Bilateria (Chen et al 2002; Dunn et al 2014; Pisani et al 2015; Whalen et al 2015; Whelan et al 2017). Recent morphological and molecular analyses have shown that some cnidarian taxa display bilateral symmetry (Kayal et al 2017). Their evolutionary placement makes this clade critical for understanding both bilaterian evolution and the emergence of animals.

A lack of cephalization (cnidarians have a "nerve net"), possession of a single body cavity, few tissue layers, and absence of major organ systems (e.g. excretory or circulatory tissues) has traditionally categorized cnidarians as having primitive body plans (Brusca et al 2016). However, genomic and developmental studies over the last two decades have revealed that cnidarians possess an unexpectedly sophisticated suite of genes involved in developmental patterning. Cnidarians are the only non-bilaterian phylum that possesses *bona fide Hox* genes (Finnerty and Martindale 1999; Ryan et al 2007; Dubuc et al 2012; Rentzsch and Holstein 2018), though the homology of the cnidarian *Hox* or *Hox*-like suite of homeobox genes to bilaterian Hox clusters remains contentious (Amemiya and Wagner 2006; Reddy et al 2015).

All members of Cnidaria possess an evolutionarily unique cell type – the cnidocyte. These cells synthesize explosive one-time-use organelles called cnidocysts. These organelles broadly cover the epidermis and pharynx of most cnidarians, and are utilized in defense, movement, pray capture, and sometimes in the construction of protective coatings (e.g. the ptychocysts of cerianthid anemones). A cnidocyst consists of a long, thread-like tubule wound tightly within a capsule comprised of collagen. The eversion or "firing" of the cnidocyst can be initiated by mechanical, electrical, or chemical stimuli, and, in some cases, light (Barnes 1967; Platchetzki et al 2012). Firing of cnidocysts is one of the fastest known biological processes, with tubule eversion taking only 700 nanoseconds in some taxa (Anderson and Bouchard 2009; Ozbek et al 2009).

Nematocysts come in a diversity of morphologies, can be bristling with spines, and many contain neurotoxins. These organelles give cnidarians their well-known stinging abilities, penetrating the dermis of prey, predators, or conspecific competitors like a harpoon. Spirocysts are a type of cnidocyst specific to anthozoans; these cnidocysts have very long tubules that lack spines or toxins and are used to snarl prey. About 30 varieties of cnidae have been described (Östman, 2000). Many types of cnidae are clade-specific and are often used as a taxonomic marker (Figure 0.4B). Nematocysts are being explored as a potential drug delivery vector for transdermal injections of pharmaceuticals (Tal et al 2014).

The diversity of cnidarian life histories and body plans – from individual polyps to large colonies to free-swimming medusae to worm-like parasites – make this group an intriguing, complex system for which to study the emergence of novel structures and early metazoan evolution. Divergent taxa within Cnidaria possess chitinous structures, but the presence of chitin throughout the phylum is largely unknown – particularly in species that lack obvious skeletal structures. In Chapter 1, I explore the distribution of *chitin synthase* (CHS) genes across Cnidaria using genomic and transcriptomic data and find that these genes are present in all classes. Intriguingly, transcriptomic data show that *CHS* is expressed in life stages of cnidarians that do not have any reported chitinous structures. Chitin histochemistry reveals that chitin is present in the soft tissues of diverse cnidarian medusae. To explore the biology of chitin in cnidarian soft tissues, I focus on the model sea anemone *Nematostella vectensis* in Chapters 2 and 3. Paradoxically, although this soft-bodied species has no obvious chitinous structures, its genome contains three *CHS* genes. Each of the *N. vectensis chitin synthase* genes has a unique protein domain signature, suggesting diverse roles for the chitin-producing enzyme. Adult and developmental data show that the three *CHS* genes are differentially and widely expressed in *Nematostella* tissues, indicating that the chitin molecule has an integral role in the biology of this species. Guide RNA/Cas9 disruption of genomic DNA for *chitin synthase* genes results in

disorganized morphology in larvae and polyps, and a lack of spirocyst development. Chitin labeling shows that chitin itself is distributed widely in endodermal and ectodermal tissues, and that it comprises the tubule of spirocysts. When *Nematostella chitin synthase* genes are disrupted with guide-RNAs/Caspase9, larvae and polyps exhibit broad morphological defects; in triple knock-downs, the polyps also lack spirocysts. I putatively identify the presence of chitin in two ctenophore species – *Pleurobrachia bachei* and *Mnemiopsis leidyi* (Appendix I). Chitin histochemical labeling localizes to a portion of the tentacle bulbs in both species. Although a partial *CHS* gene was cloned from *P. bachei*, no *chitin synthase* genes could be identified in any available ctenophore genome or transcriptome.

Finally, in addition to providing fundamental knowledge on the biology of an ancient and abundant polymer, our understanding of how early-diverging phyla like Cnidaria utilize chitin may provide new insight and opportunities for developing novel biotechnologies.

## 0.2 Introduction Figures

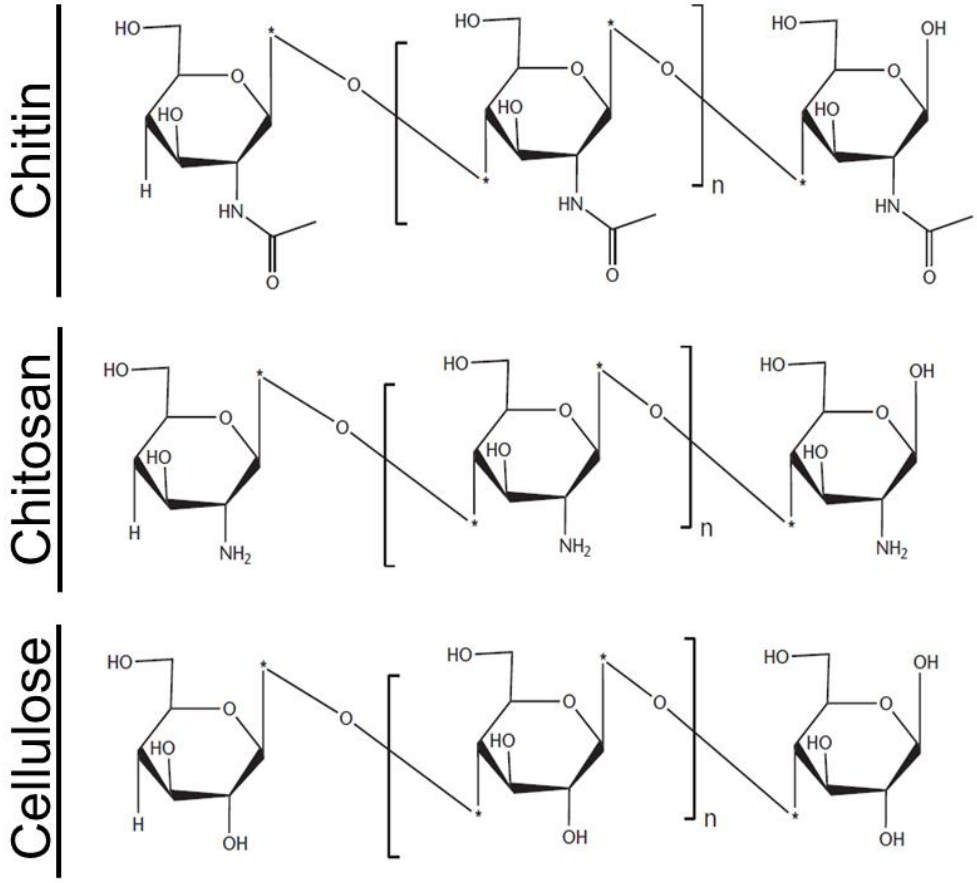


**Figure 0.1 – Comparison of chitin, chitosan, and cellulose molecules.** Chitosan is synthesized by the deacetylation of chitin molecules and is water soluble. Cellulose is another important polysaccharide found widely in nature – mostly in plants – with glucose linkages similar to the glucosamine units that form chitin – both contain β-(1→4)-linkages.

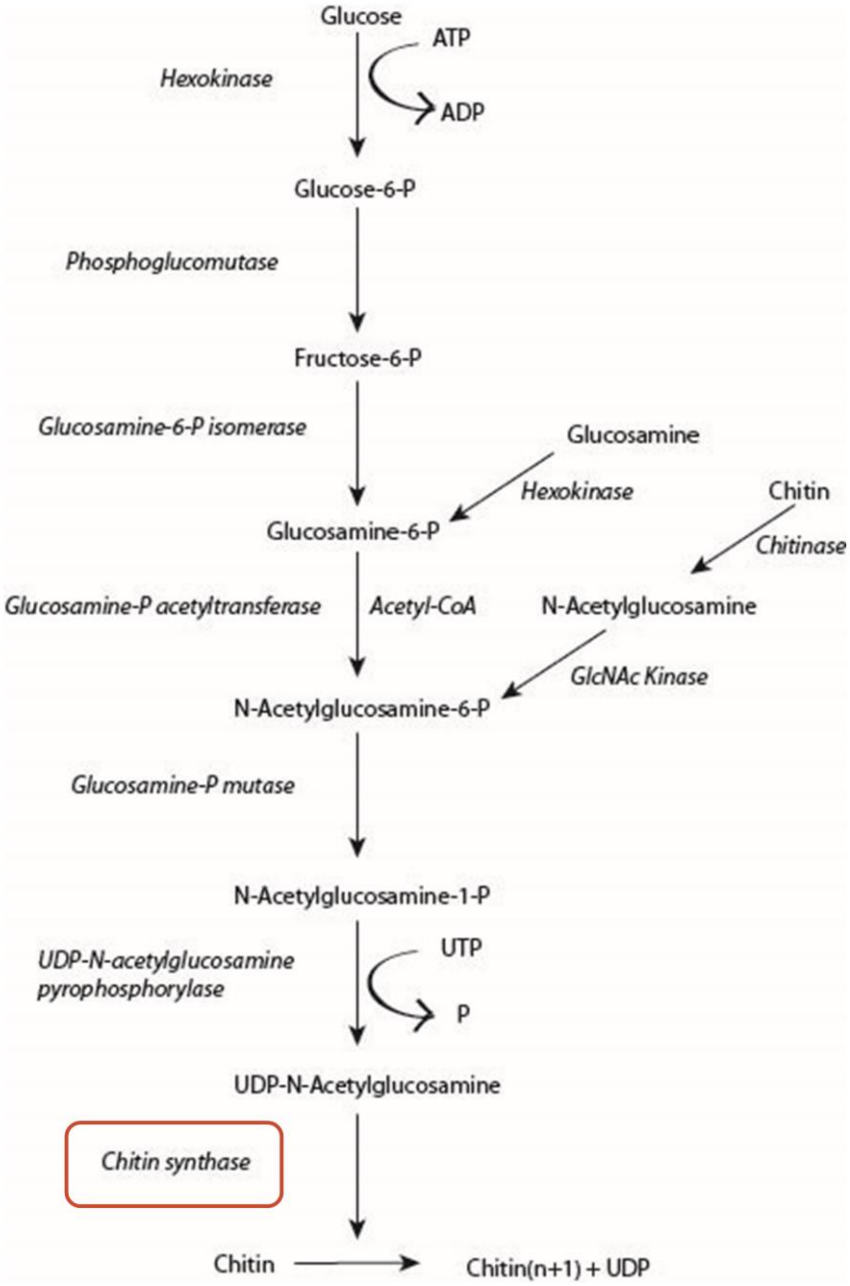


**Figure 0.2 – Example of enzymatic reactions involved in chitin production in yeast (*Saccaromyces cerevisiae*).** Chitin assembly with the enzyme chitin synthase is the final step in the process. Chitin synthase sequentially adds N-acetyl-D-glucosamine units to the growing chitin chain, forming glycosidic bonds. The enzyme chitin synthase (red box) carries out the final step (red box). Modified from "Chitin: Fulfilling a Biomaterials Promise" by Eugene Khor.

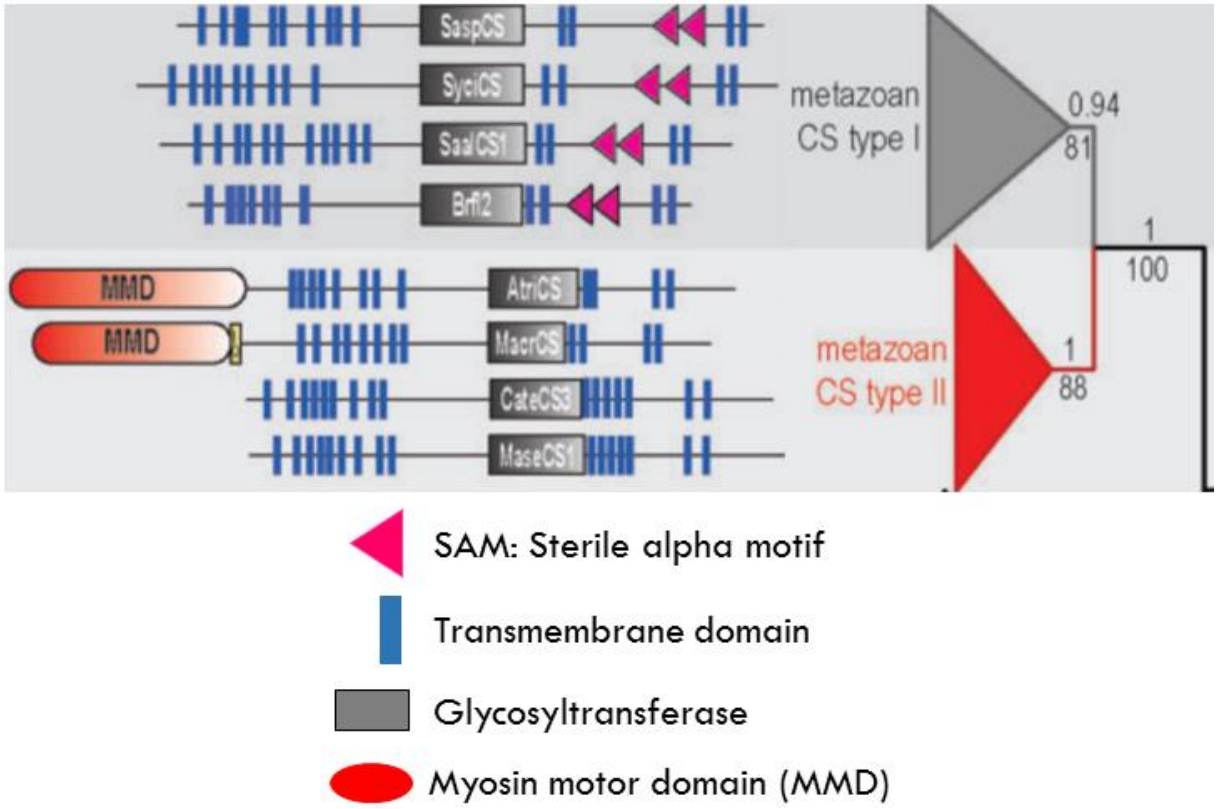


**Figure 0.3: Examples of domains present in some metazoan and choanoflagellate *chitin synthases* – modified from Zakrzewski *et al*. 2014.** Blue rectangles represent predicted transmembrane domains, gray boxes represent the glycosyl transferase region (chitin synthase domains), red ovals are myosin motor domains (MMD), and pink triangles represent sterile alpha motifs (SAMs).

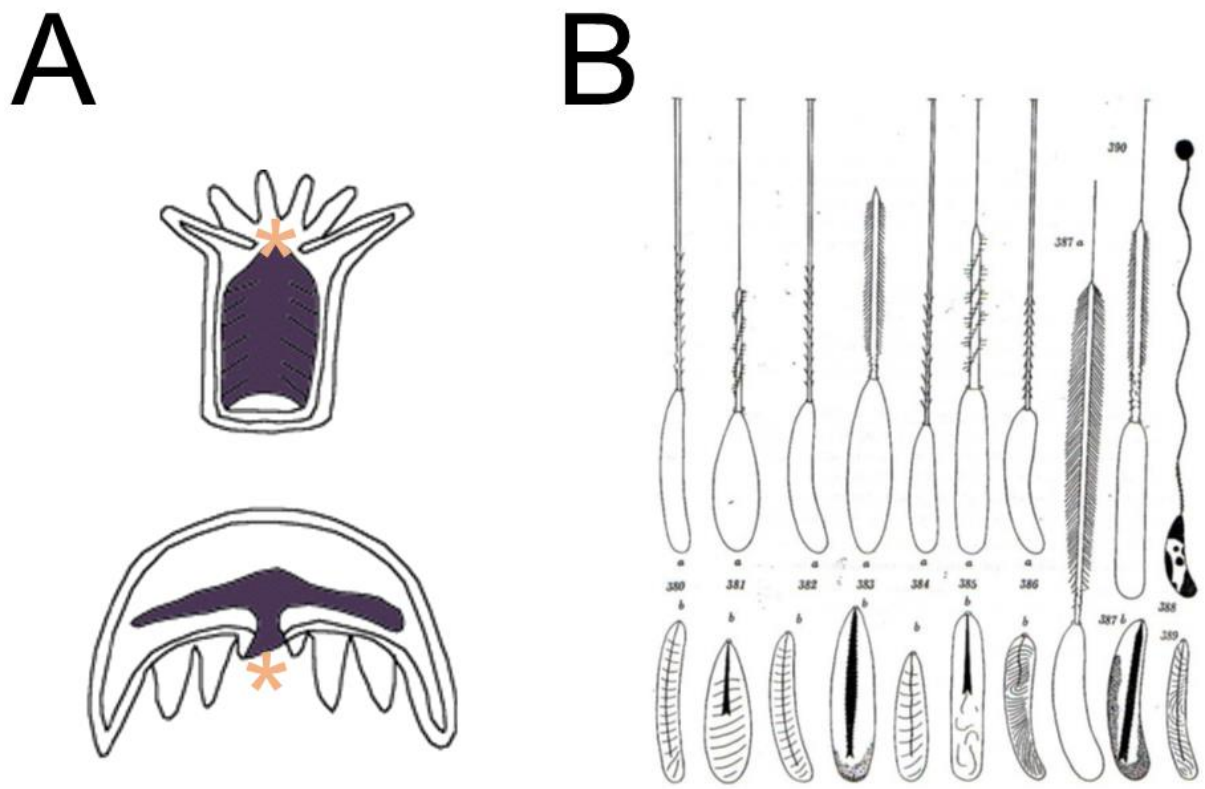


**Figure 0.4 – Cnidarian morphology and diversity of cnidae.** A.) Simplified stereotypical body plans cnidarians: polyps (top) and medusae (bottom). Orange asterisk marks the site of the mouth. Shaded region represents the gastric cavity. B.) Examples of everted (tubular harpoon ejected – “fired”) and uneverted (unfired) cnidae of actinarian anthozoans, showing diverse morphologies. K. V. A. Handl 1:1, Plate IV.

## 0.3 General Methods

Abbreviations:

**CBD** – chitin binding domain; **PBS** – phosphate-buffered saline; **PFA** – paraformaldehyde; **PTw** – PBS + 0.2% Tween-20; **RT** – room temperature; **1/3 SW or FSW** – 11 parts per thousand (ppt) seawater or 0.2µm filter sterilized seawater

### Animal Collection, Care, and Spawning

*Nematostella vectensis* was collected locally (Washington State Permit: VANDEPAS 15-025) from the Duwamish basin. Animals were kept in 1/3 SW at room temperature in the dark and fed enriched brine shrimp twice per week, with regular water changes to prevent media fouling. Spawning was initiated and zygotes collected following standard protocols (Stefanik *et al* 2013; detailed in Chapter 3).

### Fixation and Storage

Embryos or larvae were fixed at specific timepoints with a 90 second incubation in 4% PFA (VWR) + 0.2% glutaraldehyde (Sigma-Aldrich) in FSW, followed by a 1 hour incubation with 4% PFA in PTw. Adult animals were fixed in either 4% PFA or in Lavdovzky's Fix (ethanol:formaldehyde:acetic acid:ddH2O; 50:10:4:36) for several hours at 4°C. Fixation solutions were freshly made. Samples were thoroughly washed in PTw and stored in methanol at -20°C.

### Description of chitin binding domain peptide probe

To detect the presence and distribution of the chitin molecule, our laboratory utilizes a tagged probe that includes a chitin binding domain (CBD) from a chitinase of *Bacillus circulans*. This peptide probe has been employed to detect and label chitin in an array of animal taxa, including squid, insects, and nematodes (Zhang et al 2005; Mandel et al 2012; JayaNandanan et al 2014; Tang et al 2015). A complete preparation protocol for the probe may be found in Tang *et al*. 2015.

**Sample embedding and tissue sectioning**

Cnidarian tissues were washed in PBS (3 x 10 minutes) and equilibrated in 15% sucrose in PBS for three hours at room temperature then in 15% sucrose/7.5% gelatin in PBS at 37°C for three hours. Samples were then infiltrated with 20% gelatin in PBS overnight at 37°C and embedded in fresh 20% gelatin in PBS using plastic molds. Embedded samples were mounted onto a cryotome chuck with Tissue-Tek O.C.T. (optimum cutting temperature) compound (VWR) and frozen in liquid nitrogen. Samples were allowed to equilibrate to cryotome temperature (-17°C) for one hour prior to sectioning. Embedded samples were sectioned at ~7 µm on a Cryostat cryotome, and mounted on charged Superfrost Plus slides (VWR). Slides were allowed to sit at room temperature for 24 hours prior to further processing.

**Chitin Histochemistry Protocol**

*Whole adults, embryos, and larvae*

Animals were gradually rehydrated from methanol into PTw. All washes and incubations were performed on a rotating platform. Tissues were permeabilized with 0.5% Triton X-100 in PBS (three 15-minute washes) and blocked in Protein-Free T20 (TBS) blocking buffer (Thermo Scientific) for one hour at RT or overnight at 4°C. Slides were then incubated with CBD-546 (1:40) and DAPI (1:1000) (4′,6-diamidino-2-phenylindole; Sigma-Aldrich) in TBS blocking buffer overnight at 4°C. Samples were thoroughly washed in PTw, mounted in VectaShield (Vector Laboratories), and imaged.

*Tissue sections*

Sections were de-gelatinized with 0.3% gelatin in 50% ethanol PBS (a.k.a. gelatin glue) for 15-30 minutes at room temperature in a glass slide holder. Slides were then washed for 5 minutes in PBS and rinsed briefly in water. Slides were allowed to air dry completely prior to staining.

Sections were permeabilized in 0.2% Triton X-100 in PBS (three 5 minute washes) and blocked in Pierce Protein-Free T20 (TBS) blocking buffer (Thermo Scientific) for 30 minutes. Slides were incubated with CBD-546 (1:50) and DAPI (1:1000) (4′,6-diamidino-2-phenylindole; Sigma-Aldrich) in blocking buffer overnight at 4°C. Slides were gently rinsed in several PBS washes (taking care not to disturb tissue sections), mounted in VectaShield (Vector Laboratories), and imaged.

**Nucleic Acid Isolation and cDNA synthesis**

*RNA isolation and cDNA synthesis*

Total RNA was prepared from 50–100 mg of whole adult *Nematostella* homogenized in TRIzol (Invitrogen) utilizing a Bullet Blender (Next Advance) and 0.5mm stainless steel beads (Next Advance). Resulting total RNA was then isolated and purified using the PureLink RNA Mini kit (Ambion), and treated with DNase I (Ambion) to eliminate potential genomic contamination. RNA was reverse-transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen) to generate cDNA. The cDNA was then used as template for PCR to amplify target genes using the primers listed in Table 1.

*DNA isolation*

Genomic DNA was extracted as previously described (Ikmi et al 2014; Servetnick et al 2017). In brief, individual embryos (24-hpf, one embryo per DNA extraction reaction) were transferred into PCR tubes. Embryos were incubated in DNA extraction buffer (10mM Tris-HCl pH 8, 50mM KCl, 0.3% Tween 20, 0.3% NP40, 1mM EDTA, 0.5 µg/µl Proteinase K) at 55°C for 3 hours with occasional vortexing. The proteinase K was inactivated by incubating tubes at 98° for 5 minutes.

***In situ* hybridization Protocol**

*Nematostella vectensis* embryos, larvae, and adults were processed for *in situ* hybridization as previously described (Wolenski et al 2013; Pang and Martindale 2004; Servetnick et al 2017) with

some modifications. Samples were digested with Proteinase-K for varying times, depending on developmental stage. Younger embryos had shorter incubations with proteinase-K because their tissues are more delicate than older larvae or adults.

Table M1: Proteinase-K incubation times for *in situ* samples

| Embryo stage: | 24h | 48h | 72h | 96h | 120h | 144h$^{+}$ | Adults |
|---|---|---|---|---|---|---|---|
| Digest time (min): | 7 | 10 | 15 | 18 | 22 | 25 | 33 |

**Chitinase Treatment**

To specifically remove chitin from tissue samples, the chitin-digesting enzyme chitinase was applied. Chitinases degrade chitin molecules by breaking glycosidic bonds and CBD histochemistry and subsequently be used for assaying its efficacy. For these experiments, chitin was digested utilizing a chitinase isolated from the nematode *Brugia malayi* (New England Biolabs). Samples were permeabilized with PBS + 0.2% Triton X-100 and equilibrated for 1 hour at room temperature to chitinase buffer: 20 mM $Na_2HPO_4$ pH 6.0, 200 mM NaCl 1mM EDTA, 500 µg/mL BSA. Chitinase was added to a concentration of 5µL enzyme per 100µL buffer. Samples were incubated with chitinase at 37°C for approximately 16 hours and then thoroughly washed in PTw prior to CBD affinity histochemistry. Tissue that was incubated in buffer without chitinase enzyme served as negative controls.

**PCR Protocol**

Target genes were amplified from genomic DNA or cDNA using the following polymerase chain reaction mixture: <1µg DNA, 200µM each dNTP, 1µg/µL primer stock, 1-unit house-made *Taq* polymerase, and 10x PCR buffer containing $MgCl_2$. PCRs were carried out on a Mastercycler Gradient thermal cycler (Eppendorf).

Most target genes were amplified utilizing the following protocol for 35-42 cycles:

| Cycle | Temperature | Time (minutes) |
|---|---|---|
| Initial Denature | 94 | 3 |
| Denature | 94 | 30sec |
| Anneal | 58 | 30sec |
| Extend | 72 | 30sec per kb |
| Final Extension | 72 | 10 |
| Soak | 4 | Hold |

**Imaging**

Stereoscope imaging was done on a Leica M205FA fluorescent stereoscope equipped with a DFC360FX monochrome CCD camera and a DFC425C color CCD camera. Epifluorescent images were taken using a Leica DMR upright epifluorescent microscope equipped with a SPOT RT Slider cooled 1.4 megapixel color/monochrome CCD camera and an Insight 4 megapixel color CCD camera (Diagnostic Instruments). Confocal images were obtained with a Leica TCS SP5 laser scanning confocal microscope. Brightness and contrast adjustments, gamma correction, background subtraction, and confocal image smoothing was performed using Fiji (a.k.a. ImageJ, National Institutes of Health freeware).

## 0.5 Introduction and General Methods – References Cited


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## Chapter 1 – Chitin prevalence and diversity of *chitin synthase* genes across Cnidaria

### 1.0 Abstract

The phylum Cnidaria is a clade of primarily marine aquatic animals displaying a diversity of body plans and ecological roles. All members of this clade possess an evolutionarily unique cell type – the cnidocyte – which produces the explosive harpoon-like organelle that gives cnidarians their famous sting. Cnidarians are usually gelatinous, being primarily composed of an extracellular gel-like collagenous tissue, the mesoglea. Some cnidarian species possess known chitinous structures such as the endoskeletons of some anthozoans or holdfast-like coatings on hydrozoan polyp stalks. However, genes coding for enzymes involved in chitin synthesis were recently identified in two cnidarian taxa with no known hard chitinous structures.

In this chapter, I explore the prevalence and diversity of genes for the chitin-assembling enzyme, *chitin synthase*, across Cnidaria using genomic and transcriptomic data. I find that *chitin synthase* is present and expressed in all cnidarian classes, including in taxa or life stages with no known chitinous structures. Cnidarian *chitin synthase* genes display diverse domain organizations even within species, suggesting multiple roles for these enzymes. Chitin affinity histochemistry experiments provide validation that chitin is present in the mesoglea and other tissues of scyphozoan and hydrozoan medusae, and in the hydrozoan polyp *Hydra vulgaris*. Chitin is also present in the tubule of scyphozoan *Aurelia* nematocysts and the capsules of *H. vulgaris* nematocysts. The genetic diversity of chitin synthesizing enzymes and widespread presence of the molecule chitin suggests an important and hitherto unknown role for chitin in the biology of cnidarians.

## 1.1 Introduction

*Relationships of Cnidaria*

Cnidarians are a phylum of animals comprising over 11,000 described species, with a highly diverse array of life cycles, morphologies, developmental programs, and ecological niches (Appeltans et al., 2012; Zapata et al., 2015). Cnidarians (i.e. medusae, corals, sea anemones, cubomedusae) are widely accepted to be the sister taxon to Bilateria and are one of the most ancient animal lineages, with an estimated origin over 700 million years ago in the Ediacaran (Park et al 2012). The phylum Cnidaria is united by a unique cell type: the cnidocyte. These cells contain a large, single-use explosive organelle, the cnida (pl. cnidae), that is synthesized in a large post-Golgi apparatus vesicle (Adamczyk et al 2010). The cnida is comprised of a collagenous capsule containing a tightly coiled tubule under immense pressure (David et al 2008; Ozbek et al 2009). When triggered with mechanical, chemical, or electrical stimuli the cnida "fires" and the tubule penetrates the dermis of the target organism like a harpoon and any associated venoms are then injected into the target. These organelles, with everted tubules accelerating at incredibly high speeds, give cnidarians their infamous stinging capabilities.

Cnidarian venom often targets nerves or muscle tissue. The composition of cnidarian venom is an area of active research, both for treatment of stings as well as potential therapeutic uses to treat neurological diseases (Mariottini et al 2013; Mariottini et al 2015; Morabito et al 2017). Venom composition is diverse among cnidarians, with some taxa synthesizing metalloproteases, pore-forming proteins (porins), digestive enzymes, and voltage-gated ion channels (reviewed in Jouaiei et al 2015). Cubozoa pore-forming proteins are especially potent, with the potential to cause cardiac arrest within one minute of exposure in rats (Brinkman et al 2014).

The two major clades in Cnidaria are the Anthozoa (exclusively polyps, such as anemones) and the Medusozoa (polyps and jellyfish-like medusa) (Figure 1.1). Medusae are ambulatory and planktonic, whereas polyps are typically benthic and mostly sessile. Both polyp

and medusae bauplans have the same basic blueprint – an oral-aboral axis, with a mouth surrounded by stinging tentacles at the oral end. The mouth leads to a sac-like gut in most taxa (hydrozoan medusae, anthozoans, scyphozoans, staurozoans), where ingested food is digested in the gastrointestinal cavity (or coelenteron), and any refuse is expelled back through the mouth. Much of the volume of cnidarian bodies is composed of a tissue layer called mesoglea. This gelatinous tissue is primarily composed of collagen and is largely acellular, though it is traversed by muscle cells (myoepithelial cells), neurons, and motile cell types. It is likely that the basal lamina of cnidarians is composed of laminin and collagen-IV and that the mesoglea also contains proteoglycans and glycoproteins (Fowler et al 2000; Tucker and Erickson 1986; Zhang et al 2002). The tensile strength of the mesoglea allows for the jet propulsion swimming of medusae, though the organization of the mesoglea, the size and orientation of mesogleal fibrils, and stiffness or elasticity varies between taxa (Megill et al 2005). The organization of the mesoglea can also differ within the body of an individual (Tucker et al 2011).

Because some lineages (e.g. Hydrozoa) contain taxa displaying both the polyp and medusal body plans, there was historical uncertainty about the timing of emergence of the medusa form. The polyp body form is presumed to be the ancestral cnidarian bauplan and the swimming medusa body form likely arose once within Cnidaria – at the base of the Anthozoa-Medusozoa split (Collins et al 2006; Daly et al 2007; Kayal et al 2013). The subgroup Medusozoa contains the classes Scyphozoa (true jellies), Staurozoa (stalked jellies – medusal body plans with a holdfast-like stalk at the aboral end), Cubozoa (the highly venomous sea wasps or box jellies), and the diverse Hydrozoa. The class Hydrozoa (about 3500 species) contains the widest variation of body plans and life cycles, from single polyps (e.g. the model *Hydra*) to colonial siphonophores with specialized zooids, to individual medusae (e.g. *Aequorea victoria* of GFP fame). Hydrozoa also contains a variety of freshwater and marine taxa. In colonial hydrozoan species, individual members of the colony – zooids – may be so specialized in function that they are not capable of feeding themselves (e.g. siphonophore gonozooids). Zooids are connected to

one another via gastrovascular canals, and nutrients are distributed to individuals throughout the colony. The Endocnidozoa, a group of parasitic cnidarians, deviates entirely from the general cnidarian morphotype with a simple highly reduced body plan. Endocnidozoans (best known for the clade Myxozoa) do not have a mouth, mesoglea, or tentacles, but they do possess cnidae. For decades, myxozoans were considered protists but recent cellular and genomic data place them within the Cnidaria as an outgroup to Medusozoa (Chang et al 2015).

Class Anthozoa is the most taxonomically diverse cnidarian subgroup, containing over 7,000 species (Daly et al 2007; Zapata et al 2015). Anthozoans exhibit the polyp bauplan exclusively and individuals may be single polyps (anemones) or colonial (most corals, sea pens, zoanthids). Anthozoa is divided into two well-established groups – Hexacorallia and Octocorallia. Hexacoralids have six-point symmetry in their tentacles and mesentery structure, whereas octocorals have eight-fold symmetry.

*Chitin in Cnidarians*

Though the mesoglea or hydrostatic support are common sources of structural form and stability in cnidarian tissues, some taxa possess specific skeletal structures. Some anthozoans (exclusively hexacorallians) secrete endoskeletons made of calcium carbonate (e.g. scleractinians or stony corals) or the chitinous and proteinaceous material in antipatharians (black corals). It has been suggested that cnidarians synthesize α-chitin (Mendoza-Becerril et al 2016). It has been shown that chitin is present in the organic component of scleractinian coral skeletons and has even been proposed to serve as a chemical substrate for the deposition of aragonite from seawater (Wainwright 1963). Antipatharians have rigid endoskeletons similar to scleractinians, but their skeleton is composed of proteinaceous material, rather than calcified carbonate. Black coral endoskeletons also contain a relatively large proportion of chitin (10% or greater), that is complexed with proteins and lipids (Shapeero 1968; Bo et al 2012). Octocorallians (soft corals such as sea fans) have not been found to contain chitin.

Some species of sea anemone (Anthozoa-Hexacorallia-Actinaria) may synthesize a chitinous coating on their basal disc, where the aboral end attaches to the substrate (Dunn 1980; Dunn and Liberman 1983). The basal disc has undergone interesting modifications in some clades. Deep sea anemones of the genera *Adamsia* and *Strobilates* have a symbiotic relationship with hermit crabs. These anemones settle on the gastropod shells housing the crabs and eventually replace the original mineralized gastropod shell with a chitinous shell-shaped structure of their own secretion, called a carcinoecium (Dunn and Liberman 1983; Crowther et al 2011).

Outside of the anthozoans, cnidarian taxa do not have chitinous or other hard endoskeletons. However, some do have chitinous coatings that help give support to morphological structures that are anchored to the substrate. The presence of chitin in parts of hydrozoan colonies has been well documented: *Hydractinia* colonies consist of polyps connected to each other by a network of "vessels" called stolons; these tubes are coated in chitinous material that helps the stolons maintain structural integrity (Collcutt, 1897; Dunn and Liberman 1983). Thecate hydrozoan polyp colonies (i.e. hydroids) are often coated by rigid structure called the perisarc. This covering is composed of polysaccharides including chitin, which overlies a proteinaceous layer (Knight, 1968, 1970a, b; Chapman, 1974; Campbell 1999). The only description of chitin in Scyphozoa has been in the orders Rhizostomidae (but exclusively in suborder Dactyliophorae) and Semaeostomae, where it encapsulates podocysts. Podocysts are small spherical structures containing a nutrient reserve, which positioned under the pedal discs of polyps or strobilae in some taxa (Arai 2008; Ikeda et al 2011). Chitin has not been reported in the tissues or structures of adult scyphozoans or in hydrozoan medusae.

Though putative *chitin synthase* genes have been identified in the two model cnidarians, anthozoan *Nematostella vectensis* and the hydrozoan polyp *Hydra vulgaris* (Zakrzewski et al., 2014), distribution of chitin and *chitin synthase* genes throughout the Cnidaria has not been explored. In this chapter, I assess the diversity and relationships of *chitin synthase* genes across

the phylum Cnidaria and examine how soft-bodied cnidarians without endoskeletons may be utilizing chitin in novel, non-skeletal ways.

## 1.2 Chapter-Specific Materials and Methods

### *1.2.1. Animal collection and sample preparation*

Cnidarian taxa were collected from Puget Sound using a scoop at the end of a PVC pipe (a.k.a. a dipper) (Table 1.1). Animals were maintained in 32 ppt seawater (Instant Ocean) at 4°C. Medusae and hydroid polyps were fixed either in 4% PFA or Lavdovski's fixative (ethanol:formaldehyde:acetic acid:ddH2O; 50:10:4:36) for 1 hour at 4°C or 16 hours at 4°C, depending on the fragility of the taxon's tissues. Samples were thoroughly washed with PTw and stained for chitin as described in Methods (see Section 0.2).

### 1.2.2. Embedding and cryosectioning of cnidarians

To understand internal distribution of chitin within cnidarian tissues, individuals were sectioned using a cryotome– a microtome that finely sections frozen tissues before staining. Because the cnidarians have bodies that are largely gelatinous, sectioning the animals at low temperatures preserves the integrity of internal structures. Fixed medusae were equilibrated in 15% sucrose in PBS overnight at 4°C, then incubated with 15% sucrose/7.5% gelatin in PBS for three hours at 37°C followed by incubation with 20% gelatin in PBS overnight at 37°C. Fresh gelatin solution was added and samples were embedded using plastic molds. Embedded samples were mounted onto a cryotome block with Tissue-Tek O.C.T. (Optimal Cutting Temperature) compound (VWR), frozen at -80°C for at least 1 hour, and sectioned at depths of ~8 µm on a cryotome. Sections were mounted on Superfrost Plus slides (VWR) and allowed to sit undisturbed for 24 hours prior to processing. Gelatin and Tissue Tek were removed from sections with 0.3% gelatin in 50% ethanol for 10-30 minutes, and completely air dried before further processing. Chitinase treatments were performed as described in the Methods section (Section 0.2).

1.2.3. Isolation of nematocysts

*Scyphozoa*

Tentacles were snipped from a live moon jellies (*Aurelia*) and placed in isolation solution for 1 hour at -20°C (Isolation solution: 50% Percoll, 10% sucrose, 0.003% Triton X-100 in PBS). The tissue was thawed for 10 minutes on ice. Dissociated tissue was centrifuged at 8200rpm at 4°C for 10 minutes, the supernatant removed, and pellet containing nematocysts was resuspended in cold Lavdovski's fixative. Suspended nematocysts were plated on slides coated with Matrigel (1:15 in PBS; Corning) and fixed overnight at 4°C. Slides were gently rinsed with PBS+0.5% Triton X-100, blocked with TBS blocking solution (ThermoFisher Scientific), and stained with CBD-546 (1:40 in TBS blocking). Slides were gently washed in PBS, mounted with Vectashield (Vector Laboratories), and imaged.

*Hydrozoa*

Polyps were fixed in 4% PFA and dehydrated into methanol, where they were stored at -20°C. Samples were gradually rehydrated into PTw at room temperature. Whole individuals were placed in isolation solution (see previous section) for 1 hour at -20°C and thawed on ice. Nematocyst isolation and chitin staining proceeded as described above.

1.2.4. Identification and isolation of chitin synthase genes in cnidarian transcriptomes and bioinformatic pipeline

*Homolog Identification*

Cnidarian and outgroup transcriptomic datasets used for analyses and accession numbers of identified homologs are available in Table S1.1. Sixty-seven ssembled and translated cnidarian transcriptomes were kindly provided by David Platchetzki; these sequences and assemblies were included in a previously-published dataset (Zakrzewski et al 2014; Kayal et al 2018). Thirty-five outgroup taxa were included (NCBI). The cnidarian datasets were scanned for putative chitin

synthase sequences using HMMER version 3.2.1. Three *chitin synthase* Pfam (Finn et al. 2016 ; PF01644, PF03142, and PF08407) Markov models were compressed by applying *hmmpress* with default program settings. Employing *hmmsearch* under default inclusion criterion, sequences containing any of the three compiled chitin synthase models were isolated and passed to OrthoFinder (version 2.2.6; Emms and Kelly 2015) for orthology group prediction and clustering. OrthoFinder was executed with the dependency parameters DIAMOND (Buchfink et al. 2015) for all-vs-all BLAST analyses (-S option); tree from multiple sequence alignment (-M msa option); MAFFT (Nakamura et al. 2018) for multiple sequence alignment (-A option); FastTree for tree-building (-T option; Price et al. 2009). Following orthology group (OG) prediction, each OG was annotated with Pfam domains using HMMER's *hmmscan* (default settings). This process identified OGs representing canonical chitin synthase domain architectures, in addition to OGs representative of putative chitin synthase domain-containing proteins with novel domain architectures.

*Phylogenetics*

The orthology group containing canonical chitin synthase proteins (n = 141) was supplemented with 39 additional chitin synthase proteins identified on the NCBI sequence repository (see Table 1.S1). Multiple sequence alignment was performed using MAFFT's L-INS-I protocol (Nakamura et al. 2018). Bayesian Information Criterion (BIC) was employed to find the best-fit substitution model using ModelFinder in the IQ-TREE 1.6.6 distribution package (-m MFP; Kalyaanamoorthy et al. 2017). The best-fit model was LG+R8 (R models: relaxed gamma-distributions for rate heterogeneity; 8 categories). Following model inference, IQ-TREE was used to infer a maximum-likelihood topology of chitin-synthases (Nguyen et al. 2015) and perform ultrafast bootstrapping for node support (Hoang et al. 2017). Trees were visualized with FigTree (version 1.4.3; Rambaut et al 2016; http://tree.bio.ed.ac.uk/software/figtree).

## 1.3 Results

*1.3.1. Predicted homologs for the enzyme* chitin synthase (CHS) *are present in all cnidarian classes*

Using the newly available transcriptomic and genomic data available for model and non-model cnidarian taxa (Kayal et al 2018; Zakrzewski et al 2014; Table 1.1), we applied our bioinformatics pipeline to search for *chitin synthase* genes in sixty-seven cnidarian species. Fifty-three taxa passed the initial HMMer search, and 365 putative *CHS* homologs were passed to OrthoFinder. 193 genes were placed in to orthogroups, with 141 genes places into the high-confidence *CHS* orthogroup. 180 total sequences (including outgroups) were included in the alignment, with a matrix size of 5669 and 1209 invariant sites.

All classes had representatives that possess *chitin synthase* genes in their genomes or transcriptomes. It is possible that some species surveyed do possess *chitin synthase* genes, but these genes are not expressed in the tissues or life stages from which the transcriptomes were provided. Domain organization of *chitin synthase* genes can vary within and between cnidarian taxa (Fig. 1.2), suggesting that CHSs may serve multiple functions in an individual. In addition to the well-conserved glycosyltransferase region that is present in all *CHS* genes, many cnidarian chitin synthases also contain sterile alpha motifs (SAM) and other domains. A largely complete scleractinian *CHS* from the coral *Acropora digitifera* has several predicted transmembrane regions (Fig. 1.2), consistent with expected localization of the chitin synthase enzyme to the cell membrane.

A phylogenetic tree of relationships of cnidarian *chitin synthase* (CHS) genes with those of diverse metazoan taxa are shown in Figure 1.3. All classes of Cnidaria contain taxa that express CHSs, and in many cases have multiple homologs (Table 1.1). Surprisingly, transcriptomic data show that several cnidarian lineages that have no previous reports of chitin in their tissues (e.g. Octocorallia, Cubozoa) express *chitin synthase* genes. Octocorals have been

explicitly described as not having chitin in the tissues of any taxa examined; however, all species assayed here possessed at least one *chitin synthase* gene.

Most cnidarian *chitin synthase*s are clustered into two clades – an anthozoan clade and a medusozoan clade. However, several octocorals have *CHS* genes that are sister to the protostome *CHS Type I* clade. Some cnidarian taxa appear to have undergone lineage-specific expansions of *CHS* genes, particularly the sea anemone *Aiptasia* (Anthozoa-Hexacorallia-Actinaria) and the coral *Montastrea cavernosa* (Anthozoa-Hexacorallia-Scleractinia), which have four and five predicted *chitin synthases* in their genomes, respectively (Table 1.S1). Cnidarian taxa that possess more than one predicted *CHS* gene do not always have orthologs that cluster together (Fig. 1.2), suggesting that the taxa that have several *CHS* orthologs did not have these genes arise through duplication events or lineage-specific expansions. It appears that many orthologs share common origins based on domain or sequence homology and that these clustered orthologs may serve similar functions or in similar capacities in multiple taxa.

### *1.3.2. Chitin is present in the mesoglea and other tissues of diverse scyphozoan and hydrozoan species*

There is a wide distribution of chitin in hydrozoan and scyphozoan tissues, shown with fluorescent affinity histochemistry using our lab's chitin-binding-domain (CBD) probe (Figure 1.4 A-C; Figure1.5). Multiple tissues, individual cells, and the mesoglea in *Phacellophora camtschatica* tentacle, *Aequeorea victoria* bell tissue, and *Catablema nodulosa* tentacle show chitin labeling. Much of the staining appears to be acellular, consistent with the canonical process of secretion of the chitin molecule from chitin-producing cells (Figure 1.5). In confocal images, some cells show chitin labeling in the cell periphery (Figure 1.5, white arrows), potentially corresponding to the cell membrane, indicating that these cells may be synthesizing chitin. Chitin synthesis usually occurs in the membrane, as the enzyme chitin synthase is a membrane-bound protein (see General Introduction).

To confirm that the chitin binding domain (CBD) probe was binding to chitinous structures, tissue was incubated with the chitin-digesting enzyme, chitinase. If the CBD probe is binding chitin preferentially, fluorescent labeling should be diminished in tissue exposed to chitinase. Chitinase digest experiments show that the CBD-probe was binding specifically to chitin in the hydrozoan medusa *Aequorea victoria*, as labeling was significantly reduced in enzyme-treated samples (Figure 1.6 A, B) as compared to controls (Figure 1.6 C, D). This chitin labeling reduction is consistent with results from chitin digests in whole and cryosections of the anthozoan *Nematostella vectensis* (see Chapter 2).

*Hydra vulgaris* has two *chitin synthase* orthologs in its genome, and polyps have widely distributed chitin labeling (Figure 1.7; Figure S1.1). Based on DropSeq data, *Hv-CHS1* (g10332) is expressed at high levels in endodermal tissue and scattered other cell types; *Hv-CHS2* expression is found in the foot ectoderm and female germline (Celina Juliano, personal communication). Chitin labeling is also present in the capsule of *Hydra* stenotele nematocysts (Figure S1.2)

Colonial hydrozoan polyps (*Obelia* sp.) have thecae (outer coverings) composed of chitin (Figure S1.3). Though the zooid itself is not present, the chitinous outer coating (the perisarc) remained and this structure labeled with CBD. Chitin has been described in the composition of hydroid perisarcs (Gravili *et al* 2011), though specific chitin labeling experiments have been performed on relatively few taxa.

### *1.3.3. Chitin partially comprises the nematocyst tubule of scyphozoan* Aurelia *sp*.

Isolated nematocysts from the oral arms of the scyphozoan moon jelly *Aurelia aurita* show chitin labeling. The fluorescent-conjugated chitin binding domain (CBD) probe labels part of the nematocyst tubule (Fig. 1.8). The presence of chitin in any component of nematocysts has not been previously described. Chitin labeling appears as a lattice-like pattern along the tubular

harpoon of the nematocyst – the rest of the nematocyst tubule is likely comprised of mini-collagens (Beckmann and Ozbek 2012).

## 1.4 Discussion

The molecular toolkit for chitin synthesis is present in far more cnidarian taxa than previously realized. Only two species of cnidarians had previously been described has having *chitin synthase* (*CHS*) genes – the anthozoan model anemone *Nematostella vectensis* (Hexacorallia-Actinaria) and the model hydrozoan solitary polyp *Hydra vulgaris* (formerly *H. magnipapillata*) (Zakrzewski et al 2014). In a recent study of *CHS* genes across Fungi, Choanoflagellata, and Metazoa, it was shown that poriferan and cnidarian *CHS* belonged to the metazoan Type-1 *chitin synthase* (Zakrzewski et al 2014). This clade contains *CHS* genes that have sterile alpha motif (SAM) domains in their protein sequence. While not all cnidarian *CHS* genes exhibit this domain in our analysis, most do. SAM domains are involved in protein-protein interactions and also in RNA binding in a wide array of proteins that are involved in a suite of biological processes (Kim and Bowie 2003). It is unclear what roles SAM domains are playing in metazoan chitin synthesis, or what the domain binding targets may be. The diversity of domains in cnidarian *chitin synthase* genes suggests that *CHS* may be interacting with different proteins under different physiological states to serve multiple functions.

The presence of chitin has been described in various developmental or young life history stages of some cnidarian lineages, particularly in taxa with a reproductive polyp phase and an adult medusa phase. While chitin has been seen in various reproductive or embryological support tissues in hydrozoan and scyphozoan species – where a polyp-like form is anchored to substrate and either metamorphoses into a medusa or buds off medusa-like larvae – adult jellies have not been shown to synthesize or possess chitin. The medusa body plan has no known endo- or exoskeletal structures however affinity histochemistry shows that chitin is present in tissues of adult medusae, and transcriptomic data confirm that the genes for chitin synthesis are expressed

in these life stages. It is unclear how exactly chitin is being deployed in soft tissues, what it may be complexed with, and what cell types are synthesizing it.

Early works describing chitin in Scleractinia hypothesized that the zooxanthellae – symbiotic dinoflagellate algae – of corals was the source of chitin in coral tissue, thus allowing for deposition of the endoskeleton (Wainwright 1963). We show here that stony coral species (i.e. *Acropora*, *Montastraea*) possess the molecular toolkit needed to synthesize chitin endogenously. Previous research has examined octocorals species for presence of chitin, but it appeared to be absent (Bo et al 2012), unlike in Hexacorallia, where chitin presence is relatively widespread). Here we show that several species of octocorals (soft corals such as sea pens and sea fans) both possess and express the enzyme *chitin synthase*.

The presence of chitin in nematocyst tubules (scyphozoan *Aurelia*) and capsules (hydrozoan polyp *Hydra*) is intriguing; these data are the first descriptions of chitin in cnidocysts of any cnidarian taxon. Cnidarian-specific collagens – termed “mini-collagens” – are known to compose the majority of the nematocyst capsule and tubule (Adamczyk et al 2008; 2010. Cnidarian mini-collagens are named as such because their triple helices (characteristic of collagens) are short compared to collagen fibrils in other taxa (Holstein et al 1994; Beckmann and Ozbek 2012). In addition to mini-collagens, cnidocysts can also be composed of the proteins NOWA and spinalin (reviewed in Beckmann 2012). Also complexed with cnidocyst-specific proteins is glycosaminoglycan chondroitin (non-sulfonated), which has been proposed to add stability to the tubule structure (Adamczyk et al 2010). Given that chitin is water insoluble and often found in robust morphological structures across Metazoa, especially when complexed with proteins or minerals (e.g. crustacean exoskeletons), it is perhaps not surprising that chitin may play a role in the structure of the cnidarian stinging cell.

Though it has been well-established that thecate hydrozoans (order Leptothecata) can have chitinous structures, our analyses show that athecate hydrozoans possess genes for the enzyme *chitin synthase* as well. It is unclear what the distribution of the chitin molecule may be in

myxozoans, given their highly derived and reduced body plans. Endocnidozoans are exclusively parasitic and were thought to be protists for many years – it is possible that chitin plays a role in the structural support of the body wall, though given the presence of chitin in some varieties of cnidocysts (see also: Chapter 2), it could be that parts of the myxozoan nematocyst are chitinous.

It has been demonstrated that the hydrozoan polyp *Hydractinia* expresses *chitinases* in the stolons of its colonies, with proposed roles for the chitinase enzyme in both immunity and patterning of chitinous structures (Mali et al 2004). However, genes coding for *chitin synthase*s had not been previously identified in this taxon. Chitinases have also been found in the nematocysts of *Hydra* (Weber et a; 1987), indicating that chitinase is deployed to digest away exoskeletons of crustacean prey. Several species of hydractinians have had their chitinous structures described in detail, with attention also being given to the ecology of chitin in these animal's community. Bacteria that feed on chitinous structures have been found in association with hydroids and likely contribute to carbon cycling in the oceans (Gravili et al 2011).

Previous works have noted that the absence of detectable chitin alone (usually by chemical assays or histochemistry) is insufficient to confirm that an organism is unable to synthesize it (Mendoza-Becerril et al 2016). The presence of the molecular toolkit for chitin synthesis is required to confirm an organism's ability or inability to produce chitin (Wagner 1994). Genetic resources for Cnidaria have been limited until very recently, especially in non-model species. Here we show that numerous cnidarian taxa which have no previous descriptions of chitin do possess the genes needed to synthesize chitin, and affinity histochemistry confirms the presence of chitin in cnidarian soft tissues. Expression studies and functional analyses will be informative in determining the precise roles of chitin in soft-bodied cnidarians. However, the presence of the molecular machinery for chitin assembly in every major cnidarian taxonomic clade and the existence of endogenous chitin in tissues of multiple lineages suggests an important and unexpected role for chitin in cnidarian biology.

## 1.5 Chapter 1 Tables

Table 1.5.1 – Species collected from Puget Sound, WA for chitin labeling experiments

| Class | Species | Body Plan |
|---|---|---|
| **Anthozoa** | *Urticina grebelnyi* | Solitary polyp |
| | *Balanophyllia elegans* | Solitary polyp |
| | *Metridium* sp. | Solitary polyp |
| **Scyphozoa** | *Aurelia* sp. | All species are medusae |
| | *Phacellophora camtschatica* | |
| | *Cyanea capillata* | |
| | *Cassiopeia* sp. | |
| **Hydrozoa** | *Mitrocoma cellularia* | Medusa |
| | *Obelia* sp. | Colonial polyp |
| | *Manania handi* | Colonial medusae, mixed zooids |
| | *Euphysa* sp. | Medusa |
| | *Catablema nodulosa* | Medusa |
| | *Aequorea victoria* | Medusa |

## 1.6 Chapter 1 Figures

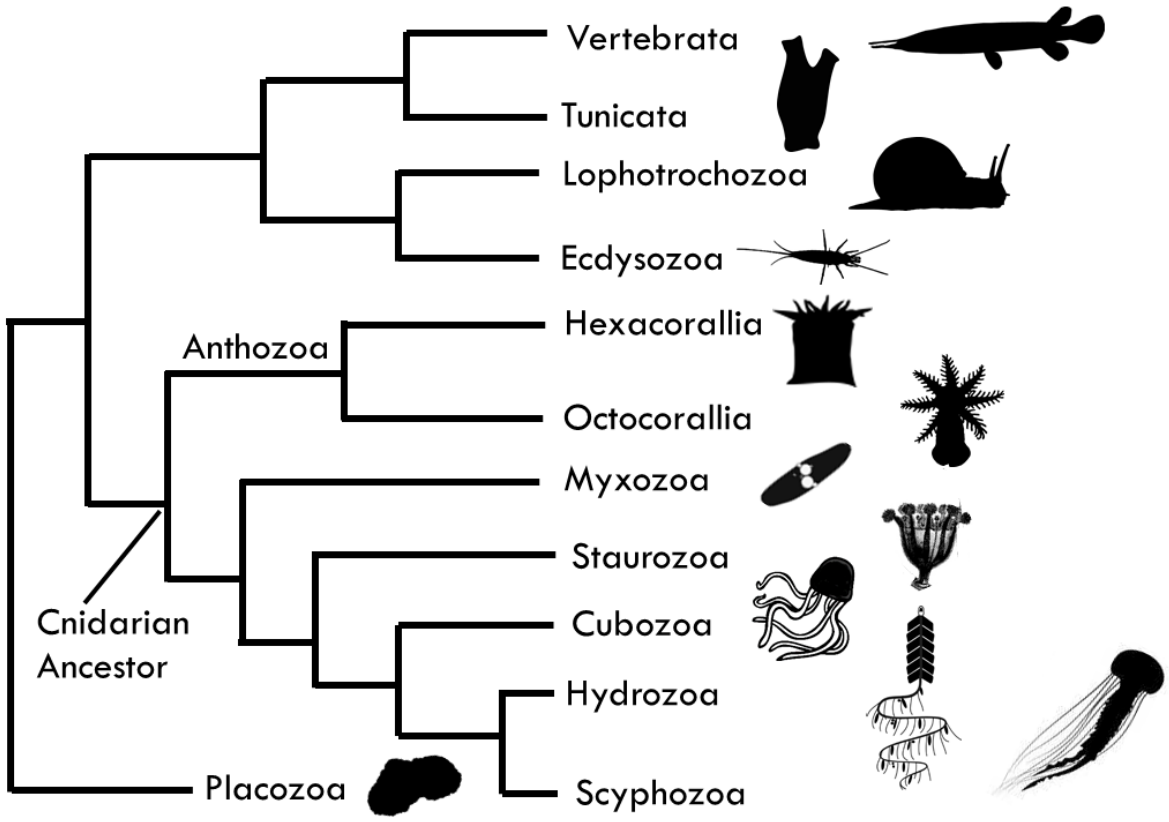


**Figure 1.1 – Relationships of the Cnidaria (after Zapata et al 2015).** Cnidarians are the sister phylum to Bilateria, which consists of deuterostomes (chodates, echinoderms, and hemichordates) and protostomes (ecdysozoans, lophotrochozoans). Anthozoans (corals, anemones) are the earliest-diverging cnidarian class. Medusozoa is the clade comprising classes that contain taxa that have medusa stages, though not all taxa do. Myxozoans are a reduced, worm-like parasitic clade, though they still possess cnidae. Animal silhouettes are available from PhyloPic (phylopic.org)

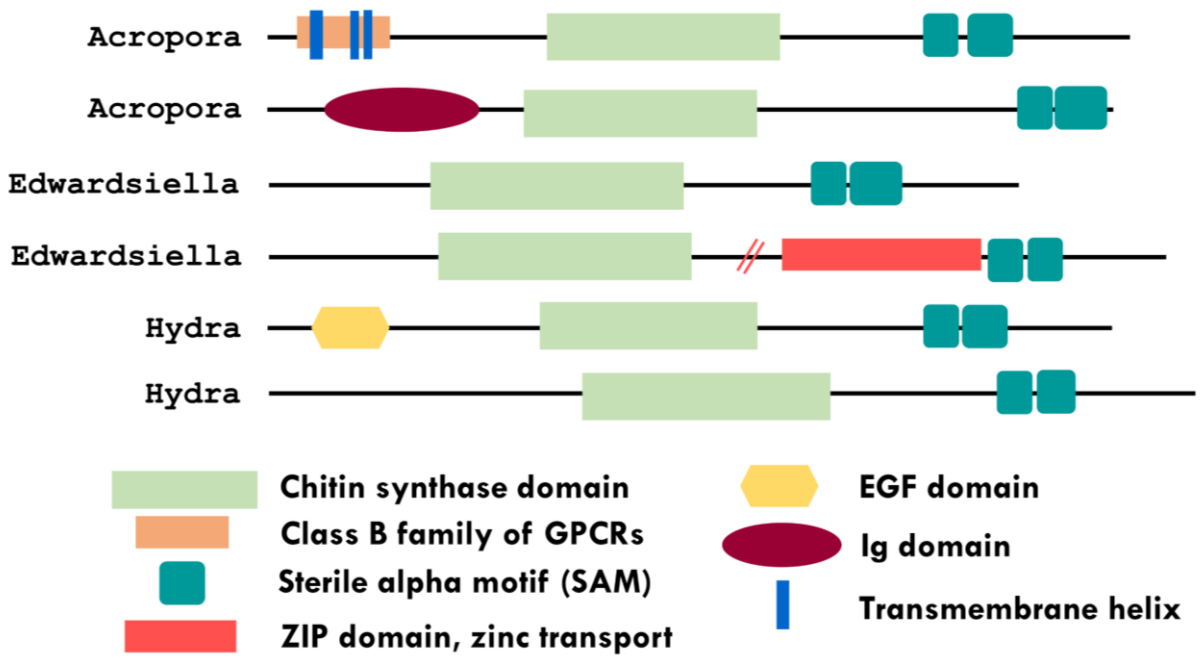


**Figure 1.2 – *Chitin synthase* domain diversity in Cnidaria.** Almost all full-length sequences contain at least one protein-binding or RNA-binding sterile alpha motif (SAM) domain. Within taxa, *CHS* genes have different domains, which may indicate multiple functions for chitin synthases within the animal. Red dashes indicate removal of non-domain-containing sequence due to space restrictions.

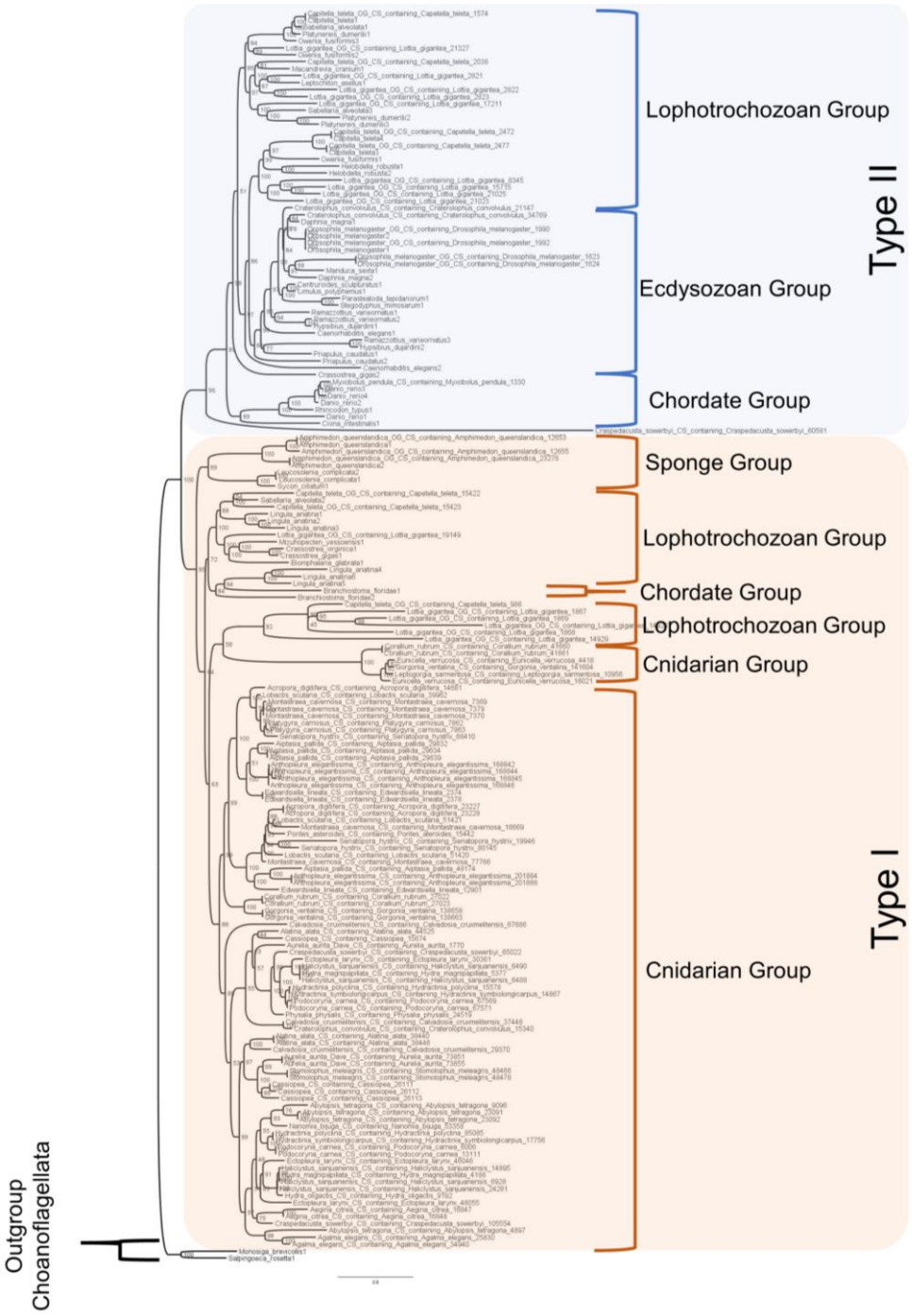


**Figure 1.3 – Relationships of cnidarian + other metazoan chitin synthases as determined by Maximum Likelihood (ML) analyses.** Outgroup is choanoflagellate CHS. While most cnidarian CHS genes are contained either in an anthozoan clade or a medusozoan clade, a few Stauromedusae, a Hydrozoan, and Myxozoan gene cluster with protostome Type I CHSs. For sequence references and any species abbreviations, see Table S1.

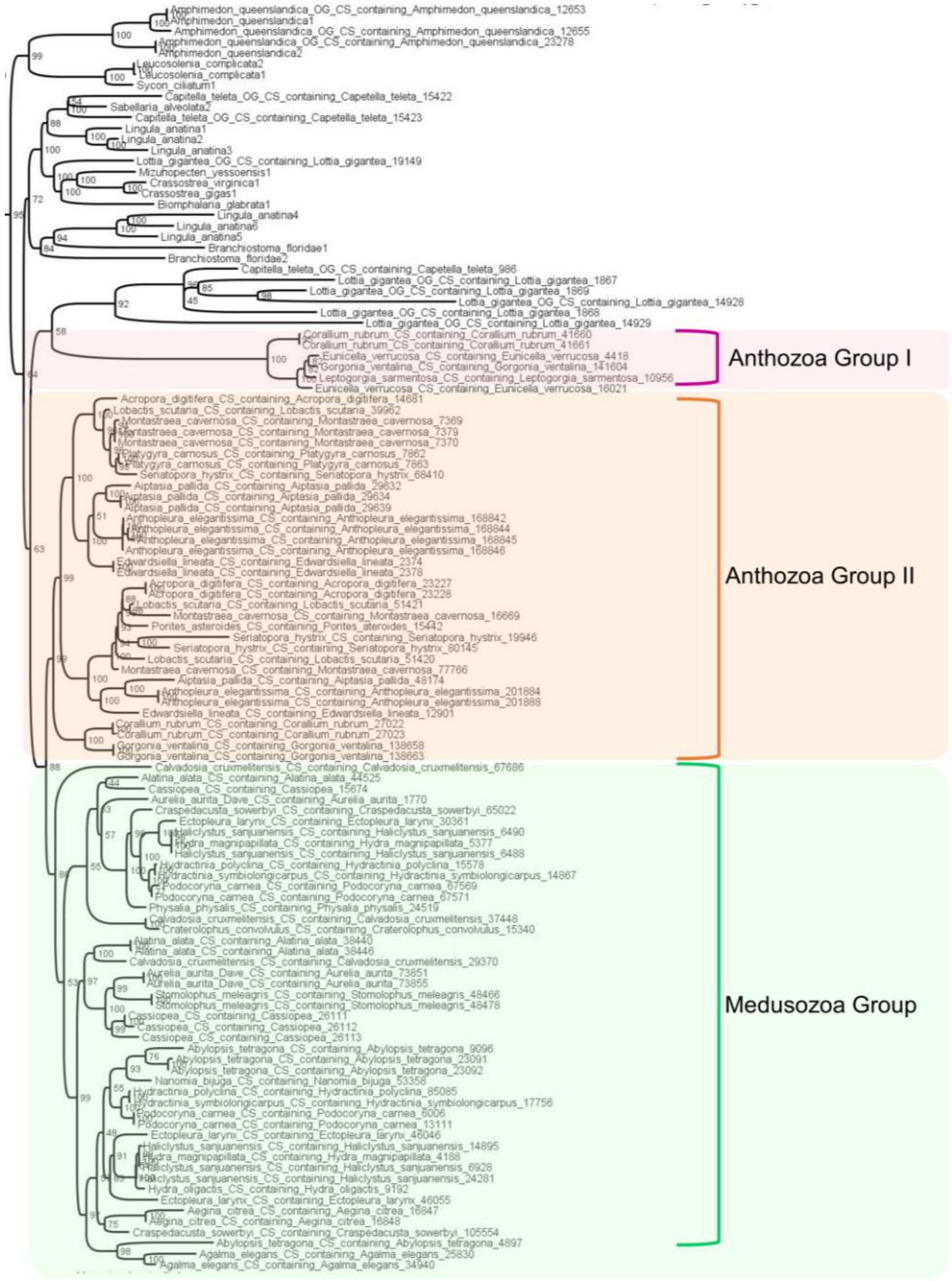


**Figure 1.4 – Relationships of Type I metazoan chitin synthases as determined by Maximum Likelihood (ML) analyses.** While most cnidarian CHS genes are contained either in an anthozoan clade or a medusozoan clade, a few Stauromedusae, a Hydrozoan, and Myxozoan gene cluster with protostome Type I CHSs. Some species (e.g. the anthozoan *Anthopleura*) show lineage-specific expansions. For sequence references and any species abbreviations, see Table S1.

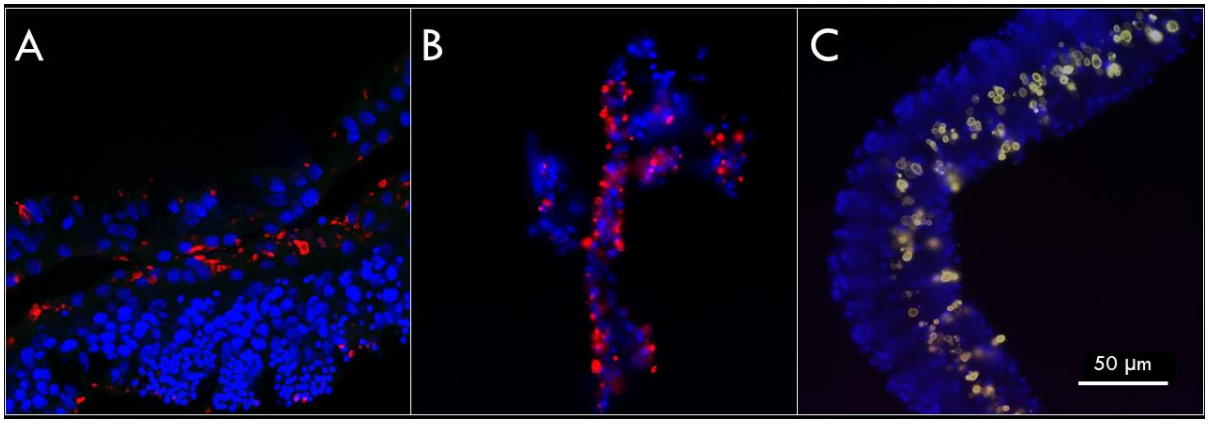


**Figure 1.5 – Chitin is found in the adult tissues of Scyphozoan and Hydrozoan medusae.** Nuclei (blue, DAPI). Chitin is either red or yellow (CBD-546). Chitin appears as circular blobs or rings in the tissue. A.) Cryosection of bell tissue of hydrozoan medusa *Aequorea victoria*. Chitin labeling is observed in the velum and mesoglea. (confocal image). B.) Scyphozoan (*Phacellophora camtschatica*) tentacle. Chitin is widely present in the dermis throughout the tentacle. C.) Tentacle of hydrozoan medusa *Catablema nodulosa*. Chitin is present in the internal tissue of the tentacle. Scale bar is 50 µm.

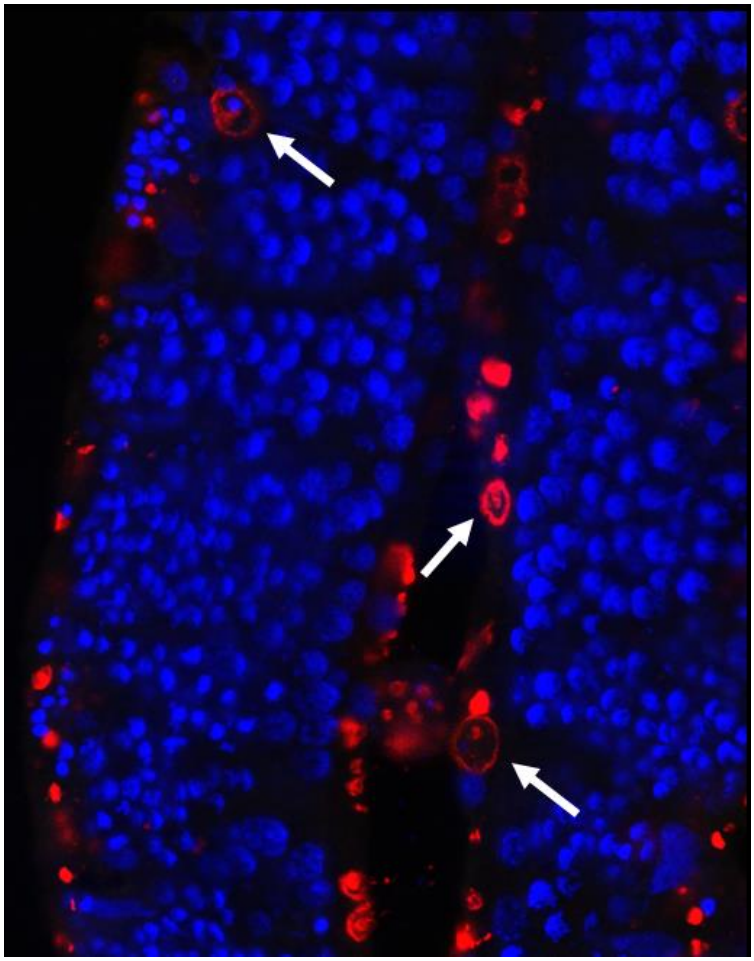

**Figure 1.6 – Detail of hydrozoan medusa *Aequorea victoria* stained for chitin.** Nuclei are blue (DAPI). Chitin is labeled in red (CBD-546). Confocal image of a cryosection shows chitin is widely present in the bell tissue of *A. victoria*. Some chitin staining is acellular, though several cells show chitin labeling within them. Arrows point to individual cells that appear to be producing chitin – chitin signal appears to be localized to the membranes of these cells.

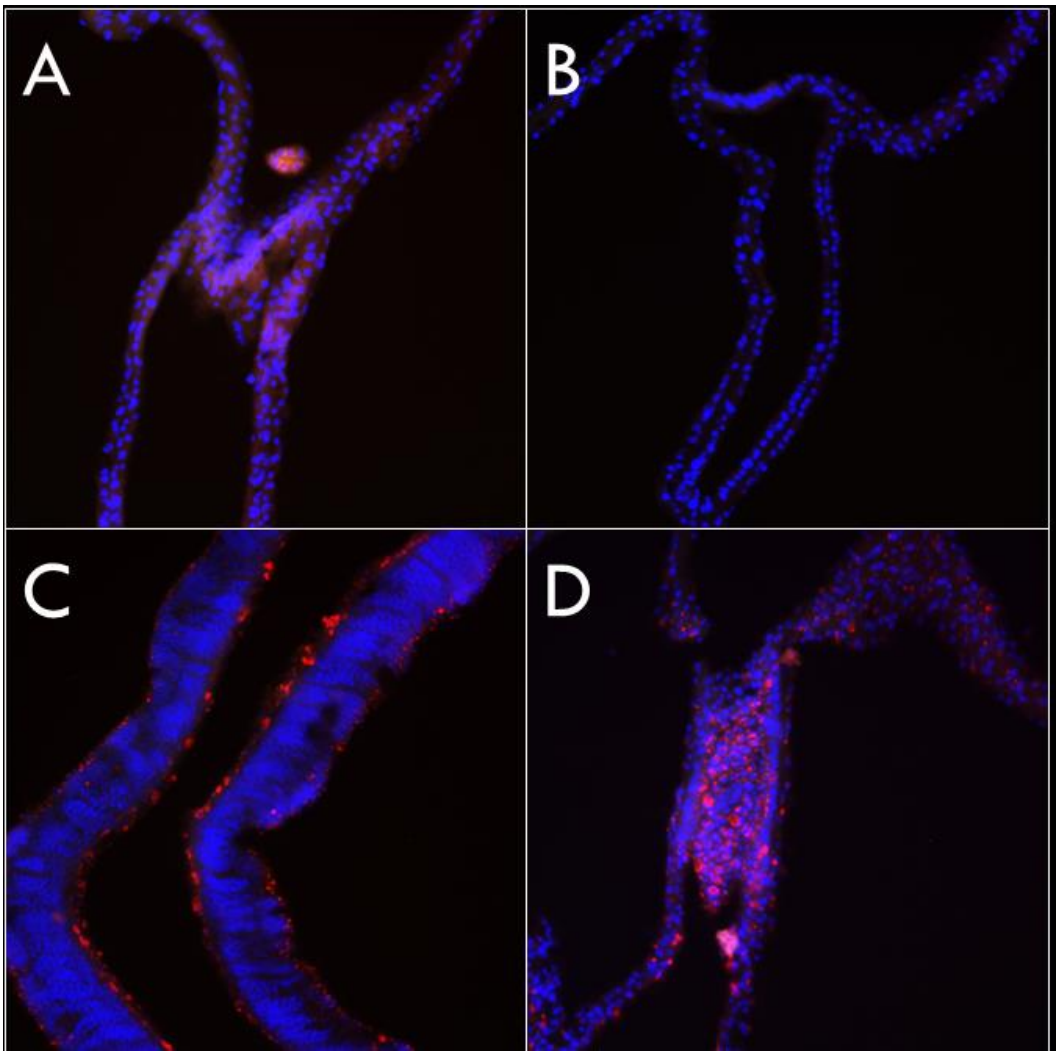


**Figure 1.7 – Chitinase treatment of scyphozoan tissue.** Nuclei, blue (DAPI); chitin labeling is red (CBD-546). A.) and B.) are chitinase-treated tissue sections. C.) and D. were incubated with buffer alone. Chitinase-treated samples show depleted chitin labeling, indicating that the chitin binding domain probe (red fluorescent signal) is specifically labeling chitin.

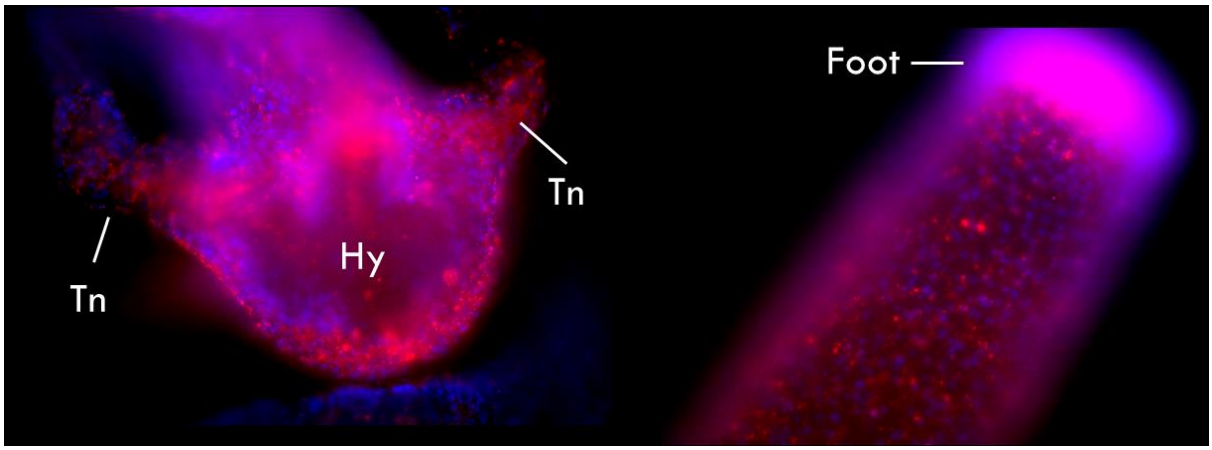


**Figure 1.8 – Chitin labeling in whole *Hydra vulgaris*.** Nuclei, blue (DAPI); chitin labeling is red (CBD-546). Hy – hypostome (oral region); Tn – tentacle. Both the dermis and endoderm show widespread chitin labeling in the oral part of the animal, as well as the trunk (left). At the aboral pole, the distal-most portion of the foot is strongly labeled for chitin. This corroborates transcriptomic data from foot tissue.

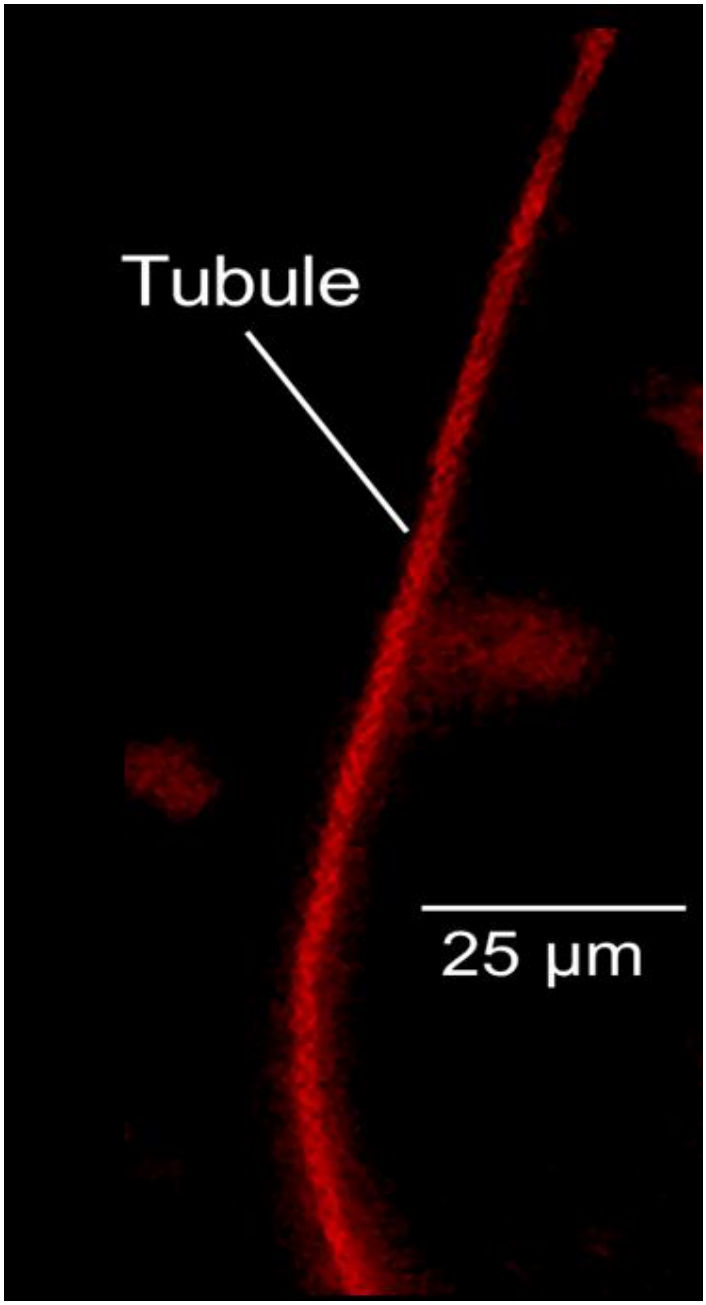


**Figure 1.9 – Chitin labeling in nematocysts of a scyphozoan.** Isolated cnidocysts from the scyphozoan *Aurelia* were labeled with CBD-546 to probe for chitin. The tubular "harpoon" of the nematocysts show chitin signal, which appears as a lattice-like pattern across the tubule. Chitin signaling was absent from the nematocyst capsules in *A. aurita*.

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**Chapter 1 Supplemental Data**

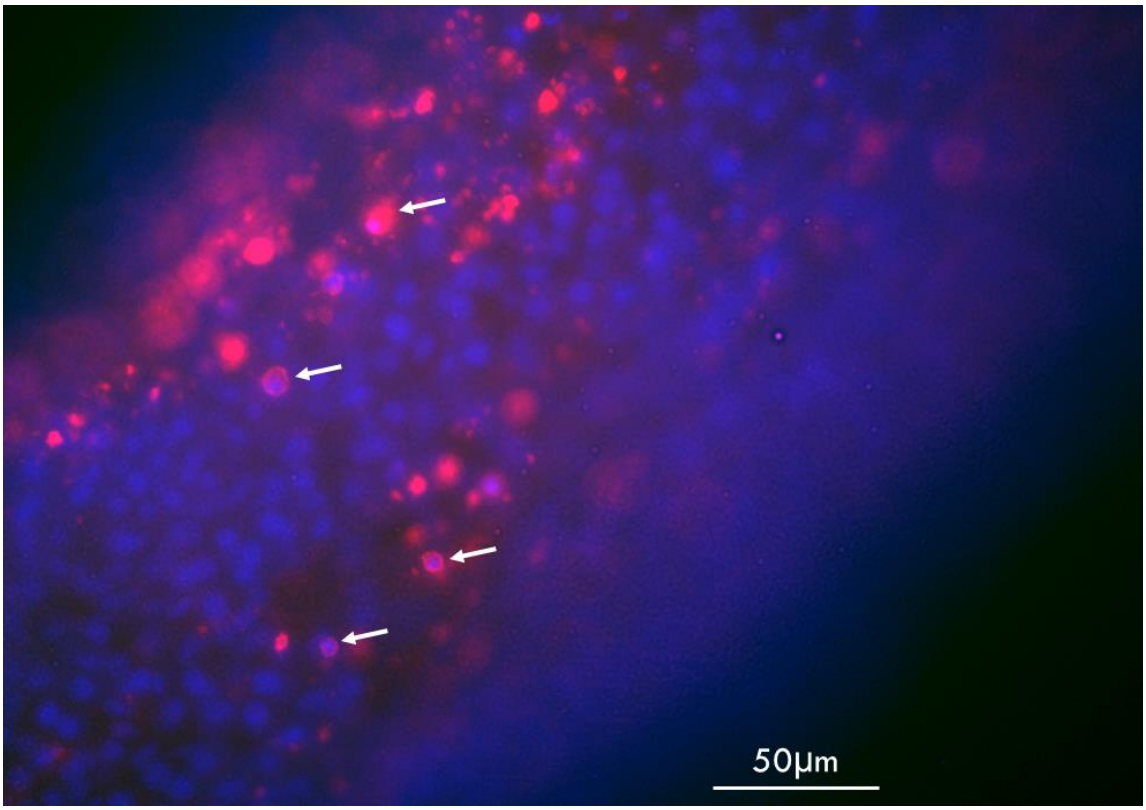


**Figure S1.1 – Detail of chitin staining in *Hydra vulgaris* trunk epidermal tissues.** Much of the chitin labeling appears to be acellular, though there are some cells (white arrows) that show chitin labeling within them. It is possible that these cells are synthesizing chitin, given the staining pattern. Chitin is usually assembled near the membrane and subsequently secreted.

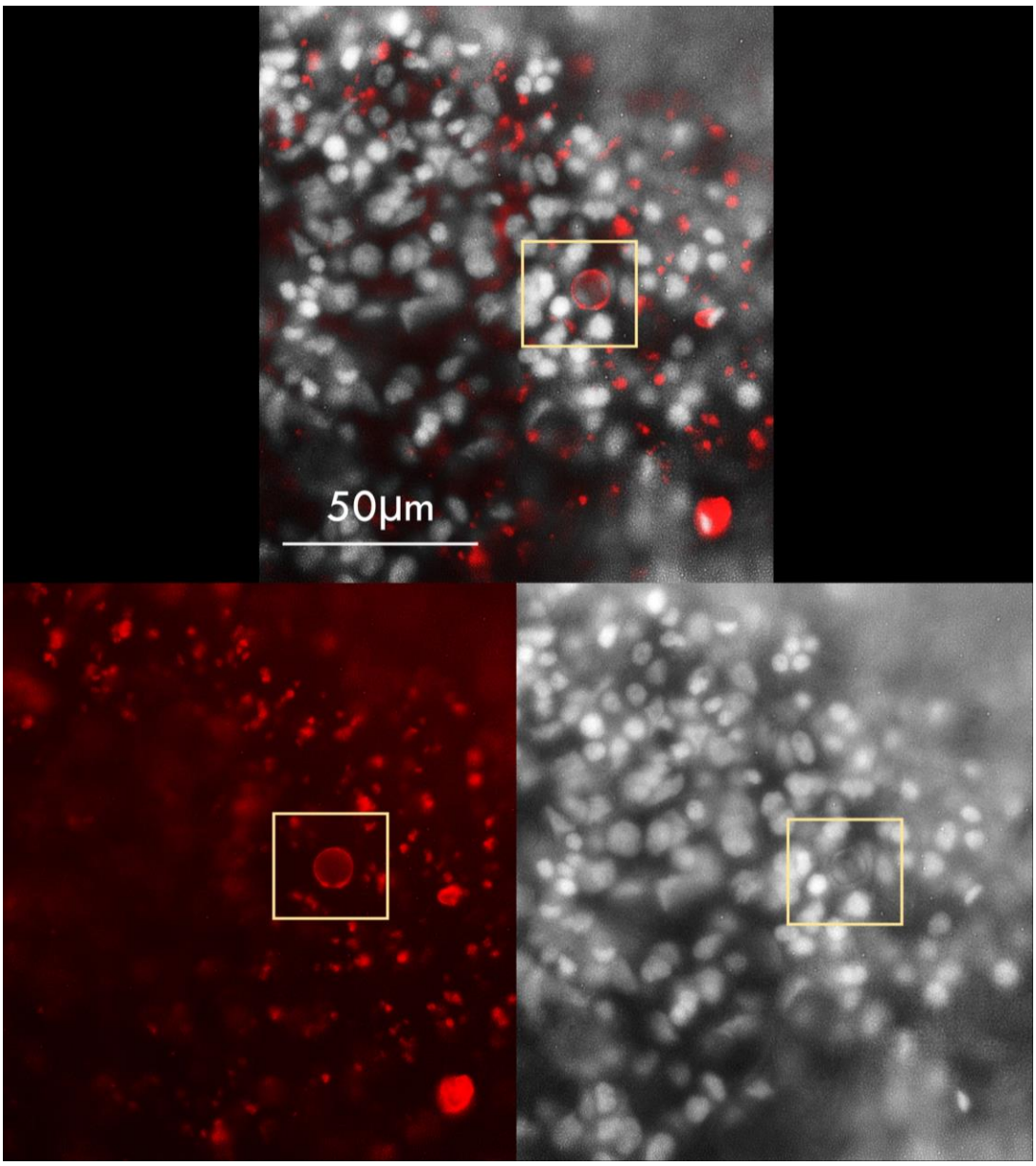


**Figure S1.2 – Detail of chitin staining in *Hydra magnipapillata* trunk epidermal tissues and some nematocysts.** Nuclei are gray (Hoescht); chitin is red (CBD-546). CBD labeling shows broad distribution of chitin in *Hydra* epidermis. Nematocysts are also visible (bottom right, yellow box). The capsule of some nematocysts appear to be chitinous (yellow boxes in all panels).

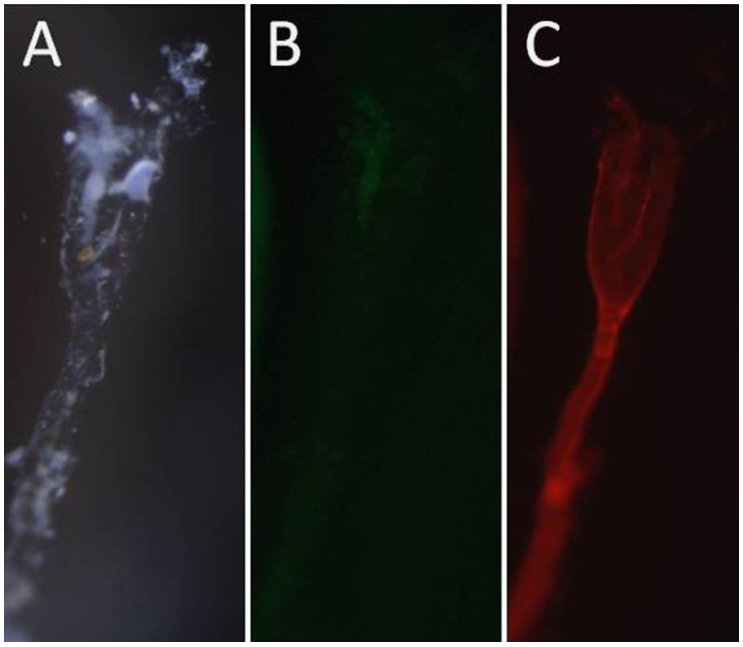


**Figure S1.3 – Hydrozoan theca composed of chitin.** Chitin – red fluorescence (CBD-546). A.) Brightfield image of thecate hydrozoan zooid (*Obelia* sp.) attached to an intertidal sponge. The zooid is no longer present, but the theca is preserved. B.) Autofluorescence of hydrozoan theca (green). C.) Chitin labeling of the theca – the cup and stem of the theca appear to be chitinous.

Supplementary Table S1.1 – List of taxa used in *chitin synthase* analyses and source material.

| Taxonomic Clade | Species name | Numbers of CHS | Sequence Source |
|---|---|---|---|
| **Cnidaria - Anthozoa** | | | |
| **Subclass Hexacorallia** | | | |
| Actiniaria | *Aiptasia pallida* | 4 | Zapata et al. 2014 |
| Actiniaria | *Anthopleura elegantissima* | 5 | SRR1645256 |
| Actiniaria | *Edwardsiella lineata* | 3 | Stefanik et al. 2014 |
| Actiniaria | *Nematostella vectensis* | 3 | Nordberg et al. 2014; this thesis |
| Scleractinia | *Acropora digitifera* | 3 | Shinzato et al. 2011 |
| Scleractinia | *Lobactis scutaria* | 3 | SRR2300562 |
| Scleractinia | *Montastraea cavernosa* | 5 | SRR2306543 |
| Scleractinia | *Platygyra carnosus* | 2 | SRR402974-5 |
| Scleractinia | *Porites asteroides* | 1 | Kenkel et al. 2013 |
| Scleractinia | *Seriatopora hystrix* | 3 | SRR2300678 |
| **Subclass Octocorallia** | | | |
| Alcyonacea | *Corallium rubrum* | 4 | SRR1552944 |
| Alcyonacea | *Eunicella verrucosa* | 2 | SRR1324944 |
| Alcyonacea | *Gorgonia ventalina* | 3 | SRR935083 |
| Alcyonacea | *Leptogorgia sarmentosa* | 1 | SRR1324968 |
| **Cnidaria - Endocnidozoa** | | | |
| Myxozoa | *Myxobolus pendula* | ? | SRR2472984;SRR2472987;SRR2472989 |
| **Cnidaria - Cubozoa** | | | |
| Carybdeida | *Alatina alata* | 3 | Zapata et al. 2014 |
| **Cnidaria - Staurozoa** | | | |
| Stauromedusae | *Calvadosia cruxmelitensis* | 3 | Kayal et al. 2018 |
| Stauromedusae | *Craterolophus convolvulus* | 3 | Kayal et al. 2018 |
| Stauromedusae | *Haliclystus sanjuanensis* | 3 | Kayal et al. 2018 |
| **Cnidaria - Hydrozoa** | | | |
| Anthothecata | *Hydractinia polyclina* | 2 | SRR923509 |
| Anthothecata | *Hydractinia symbiolongicarpus* | 2 | Zapata et al. 2014 |

| | | | |
|---|---|---|---|
| Anthothecata | *Podocoryna carnea* | 4 | Zapata et al. 2014 |
| Aplanulata | *Ectopleura larynx* | 3 | Zapata et al. 2014 |
| Aplanulata | *Hydra vulgaris* | 2 | Celina Juliano, personal comm. |
| Siphonophorae | *Abylopsis tetragona* | 4 | Zapata et al. 2014 |
| Siphonophorae | *Agalma elegans* | 2 | Zapata et al. 2014 |
| Siphonophorae | *Nanomia bijuga* | 1 | Zapata et al. 2014 |
| Siphonophorae | *Physalia physalis* | 1 | Zapata et al. 2014 |
| Trachylinae | *Aegina citrea* | 2 | Zapata et al. 2014 |
| Trachylinae | *Craspedacusta sowerbyi* | 3 | SRR923472 |
| **Cnidaria - Scyphozoa** | | | |
| Discomedusae | *Aurelia aurita* | 3 | Brekhman et al. 2015 |
| Discomedusae | *Cassiopea xamachana* | 4 | Kayal et al. 2018 |
| Discomedusae | *Stomolophus meleagris* | 2 | SRR1168418 |
| **Outgroups** | | | |
| **Annelida** | | | |
| Errantia | *Platynereis dumerilii* | *3* | Zakrzewski et al 2014 |
| Sedentaria | *Capitella teleta* | 10 | Simakov et al. 2013 |
| Sedentaria | *Owenia fusiformis* | *3* | Zakrzewski et al 2014 |
| Sedentaria | *Sabellaria alveolata* | 4 | Zakrzewski et al 2014 |
| **Arthropoda** | | | |
| Insecta | *Drosophila melanogaster* | 4 | Gramates et al. 2017 |
| Insecta | *Manduca sexta* | *1* | Zhu et al 2002 |
| Arachnida | *Centruroides sculpturatus* | *1* | BioProject: PRJNA422877 |
| Arachnida | *Parasteatoda tepidariorum* | *1* | BioProject: PRJNA316108 |
| Merostomata | *Limulus polyphemus* | *1* | BioProject: PRJNA238073 |
| Crustacea | *Daphnia magna* | *2* | Colbourne et al 2011 |
| **Brachiopoda** | | | |
| Inarticulata | *Lingula anatina* | 6 | Luo et al 2015 |
| Articulata | *Macandrevia cranium* | *2* | Zakrzewski et al 2014 |
| **Choanoflagellata** | | | |
| | *Monosiga brevicollis* | *1* | Kuo et al 2007 |

| | | | |
|---|---|---|---|
| | *Salpingoeca rosetta* | *1* | Russ et al 2015 |
| **Chordata** | | | |
| Cephalochordata | *Branchiostoma floridae* | *2* | Putnam et al 2008 |
| Tunicata | *Ciona intestinalis* | *1* | BioProject: PRJNA187185 |
| Vertebrata | *Danio rerio* | 4 | Tang et al 2015 |
| Vertebrata | *Rhincodon typus* | *1* | |
| **Mollusca** | | | |
| | *Biomphalaria glabrata* | *1* | BioProject: PRJNA290623 |
| | *Crassostrea gigantus* | 1 | |
| | *Crassostrea virginica* | 1 | |
| | *Leptochiton asellus* | 1 | Zakrzewski et al 2014 |
| | *Lottia gigantea* | 13 | Simakov et al. 2013 |
| | *Mizuhopecten yessoensis* | 1 | BioProject: PRJNA390633 |
| **Nematoda** | | | |
| | *Caenorhabditis elegans* | *2* | |
| **Porifera** | | | |
| | *Amphimedon queenslandica* | 5 | Srivastava et al. 2010 |
| | *Leucosolenia complicata* | 2 | Zakrzewski et al 2014 |
| | *Sycon ciliatum* | 1 | Zakrzewski et al 2014 |
| **Priapulida** | | | |
| | *Priapulus caudatus* | 2 | PRJNA303167 |
| **Rotifera** | | | |
| | Helobdella robusta | 2 | Simakov et al 2013 |
| **Tardigrada** | | | |
| | *Hypsibius dujardini* | *2* | Yoshida et al 2017 |
| | *Ramazzottius varieornatus* | *3* | Hashimoto et al 2016 |

## Chapter 2: Unexpected presence of citin in the soft-bodied model sea anemone *Nematostella vectensis*

*2.0 Abstract*

Chitin is a highly abundant polysaccharide best known for its structural roles in the exoskeletons of arthropods. Recent data suggest that chitin is found in a much more diverse range of animals than previously known – including vertebrates – and that it may be utilized in roles other than formation of hard structures. Many cnidarians, such as the model sea anemone *Nematostella vectensis*, lack an endoskeleton or other obvious hard parts. Intriguingly, *Nematostella* has three predicted *chitin synthase* genes in its genome, though whether these genes are expressed is unknown, and the distribution of chitin has not been described. In this chapter, I assess the biology of chitin in *Nematostella* and investigate the distribution and possible functions of chitin in these soft-bodied cnidarians. Our data also show that chitin appears to be a component of the tubular "harpoons" of some populations of cnidarian stinging cells (cnidae) in *Nematostella*. All three *chitin synthase* genes are expressed in *Nematostella* tissues and chitin itself is widely present throughout the anemone, suggesting an important and non-skeletal role for chitin in these gelatinous organisms.

*2.1 Introduction*

The starlet sea anemone, *Nematostella vectensis* (Anthozoa; Actinaria; Edwardsiidae), lives in estuaries where it burrows its trunk into the mud and sits with tentacles exposed to catch passing prey (Figure 2.1). This species is dioecious and free-spawning – gametes are released into the water column to be fertilized. Once embryogenesis completes, free-swimming planula larvae hatch from the eggs and swim upward into the water column. Over the last twenty years, *N. vectensis* has emerged as a model for exploring early metazoan evolution and development, comparative genomics, emergence of novel structures, and the origins of Bilateria (Darling et al., 2005; Genikhovich and Technau, 2009; Ikmi et al., 2014; Stefanik et al., 2013). These animals are easily maintained in the laboratory with no specialized equipment and were the first non-bilaterian taxon to have their genome sequenced (Putnam et al., 2007; genomic resources reviewed in Genichovich and Technau 2009).

Like most cnidarians, *Nematostella*'s main body axis is the oral-aboral plane, with the mouth serving as the single opening to the body cavity. The mouth is surrounded by 16-20 tentacles that are densely covered in stinging cells termed cnidocytes. The epidermis and pharynx are composed of ectodermally-derived tissue, with cnidocytes present in both the pharynx and along the body wall. Endodermally-derived tissue (gastrodermis or endo-mesoderm) comprises the eight mesenteries; mesenteries are a multipurpose organ that contain digestive cells, gonads, cnidocytes, and myoepithelial cells. Nematostella can contract dramatically along its oral-aboral axis, and these muscle movements are driven by the myoepithelial cells of the mesenteries. Though cnidarians have traditionally been described as possessing radial symmetry in their body plans, it has been argued in recent years that Cnidaria are bilaterally symmetrical, in part due to the organization of the mesentery retractor muscles (Berking 2007; Layden et al 2016).

In *Nematostella* the endoderm and ectoderm contain specialized, fully-differentiated cell types. These include neurons, cnidocytes, gland cells, and epithelial cells. Endodermally-derived tissue also contains absorptive cells that serve a digestive function. Like most cnidarians,

*Nematostella* has an ectodermal and endodermal nerve net, and lacks cephalization. However, differences in neuronal gene expression across the animal show that there may be undescribed complexity and functional specificity in different populations of neurons (Marlow *et al* 2009; Layden *et al* 2012; Layden and Martindale 2014). Members of the genus *Nematostella* also possess a unique tissue type – nematosomes. Nematosomes are unattached ciliated balls of tissue that are free-floating within the *Nematostella* body cavity. They are packed with cnidocytes and help subdue ingested prey within the gastrovascular cavity (Babonis and Martindale 2014; Babonis *et al* 2016). Nematosomes also contain cells with phagocytic abilities and may serve in removing unwanted particles from the body cavity (Babonis et al 2016).

While some anthozoan taxa possess endoskeletons that may be chitinous (Bo *et al* 2012), *Nematostella* lacks internal or external skeletal structures or other obvious hard parts. Recently, it was shown that the *Nematostella vectensis* genome contains multiple predicted genes for the enzyme that synthesizes the molecule chitin – *chitin synthase* (*CHS*) (Zakrzewski et al. 2014). However, whether these genes were expressed, if chitin was actually present and what its potential role might be in this soft-bodied species is unknown. In this chapter, I sought to determine the distribution and function of chitin in *Nematostella* tissues and assess the pattern of *chitin synthase* expression. Using the CBD peptide probe, I performed affinity histochemistry experiments on whole animals, tissue cryosections, and dissociated cnidocysts to ascertain whether chitin is present and to examine its cellular localization. My data suggest that chitin is being used in novel structural and nonstructural ways in this soft-bodied sea anemone.

Description of nematostella tissue and glossary of terms

*Cnidocyte* – The cell type that synthesizes the explosive organelle, the cnidocyst.

*Gastrodermis/Endomesoderm* – Specific to cnidarians, this bifunctional tissue type has properties of digestive tissue and contractile tissue.

*Mesentery* – A tissue comprising most internal structures in *Nematostella*. Mesenteries have cell types that are both endodermally- or ectodermally-derived, and function in reproduction, digestion, and movement.

*Mesoglea* – largely acellular collagenous layer separating endodermal and ectodermal tissues

*Nematocyst* – Specialized projectile organelles used in defense and prey capture. Nematostella possess two varieties of nematocyst – bastitrichs and mastigophores. Nematocysts can contain neurotoxins and their tubules are barbed or spiny.

*Nematosome* – Ciliated balls of free-floating tissue present in the *Nematostella* gastrovascular cavity; they are composed of cnidocytes and phagocytic cells. Nematosomes function in prey-wrangling and removal of particles from the body cavity and are found exclusively in members of the genus *Nematostella*.

*Physa* – aboral-most portion of the *Nematostella* trunk. This area is often buried in mud or sand

*Spirocyst* – A variety of cnidocyst that is specific to Anthozoa. There are no toxins known to be associated with spirocysts, the tubules are longer than those of nematocysts and they lack spines.

## 2.2 Chapter-Specific Materials and Methods

### *2.2.1 Embedding and cryosectioning of adult Nematostella*

To understand internal distribution of expression patterns within adult tissues, individuals were sectioned using a cryotome – a microtome that finely sections frozen tissues. Because the anemones have bodies that are gelatinous, sectioning the animals at low temperatures preserves the integrity of internal structures and morphology. Fixed and re-hydrated adult nematostella were equilibrated in 15% sucrose in PBS overnight at 4°C, then incubated with 15% sucrose/7.5% gelatin in PBS for three hours at 37°C followed by incubation with 20% gelatin in PBS overnight at 37°C. Fresh 20% gelatin solution was added and samples were embedded using plastic molds. Embedded samples were mounted onto a cryotome block with Tissue-Tek O.C.T. (Optimal Cutting Temperature) compound (VWR), frozen at -80°C for at least 1 hour, and sectioned at ~6

μm on a cryotome. Sections were mounted on Superfrost Plus slides (VWR) and allowed to rest for 24 hours prior to processing. Gelatin andTissue Tek were removed from sections with 0.3% gelatin in 50% ethanol for 10-30 minutes, and completely air dried before further processing.

*2.2.2. Isolation of cnidocysts*

Adult animals were relaxed in 3% $MgCl_2$ in 1/3 FSW for 15 minutes and placed in isolation solution for 1 hour at -20°C (Isolation solution: 50% Percoll, 10% sucrose, 0.003% Triton X-100 in PBS). The tissue was thawed for 10 minutes at room temperature or until liquid. Dissociated tissue was centrifuged at 8200rpm at 4°C for 10 minutes, the supernatant removed, and pellet containing cnidocysts resuspended in Lavdovski's fixative (ethanol:formaldehyde:acetic acid:ddH2O; 50:10:4:36). Suspended cnidocytes were plated on slides coated with Matrigel (1:15 in PBS; Corning) and fixed overnight at 4°C. Slides were gently rinsed with PBS+0.5% Triton X-100, blocked with TBS blocking solution (ThermoFisher Scientific), and stained with CBD-546 (1:40 in TBS blocking). Slides were gently washed in PBS, mounted with Vectashield (Vector Laboratories), and imaged.

*2.2.3. Identification and isolation of* chitin synthase *genes in* Nematostella vectensis

The *N. vectensis* genome contains three predicted homologs of *chitin synthase* (*CHS*) genes – two partial homologs were reported in Zakrzewski *et al* 2014, and a third I cloned by initially utilizing degenerate primers. Using RNA from whole animals, I synthesized cDNA through reverse transcription (RT PCR), and subsequently cloned and sequenced the *CHS* genes predicted in the *N. vectensis* genome (GenBank IDs: XM_001627169.1, XP_001633545.1, XP_001637059.1). A full transcript sequence of XP_001637059.1 was kindly provided by Leslie Babonis (Whitney Marine Laboratories, Marineland, FL). These cDNA templates were subsequently used to synthesize digoxigenin-labeled (DIG) RNA probes for the alkaline phosphatase system of probe detection (Table 2.1).

## 2.3 Results

### *2.3.1* Nematostella *possesses three* chitin synthase *genes in its genome, each with unique domain architecture*

There are three genes coding for the enzyme chitin synthase in the *Nematostells vectensis* genome (Figure 2.2). These genes each contain a conserved glycosyl transferase type 2 (GTA) domain, which is enzymatically active site of chitin chain formation (Supp. Fig. S2.1). Beyond the GTA region, each chitin synthase has a unique domain signature. Nv-CHS1 and Nv-CHS3 contain sterile alpha motif (SAM) domains – these domains consist of approximately 70 amino acids. SAM domains are found widely in fungi and metazoan proteins, and have diverse functions (protein-protein interactions, RNA binding, membrane interactions, etc). It is unclear what functions this domain has in *Nematostella* chitin synthase enzymes. Also present in Nv-CHS3 is a DXD motif, which is a short domain commonly found in a variety of glycosyltransferase-possessing enzymes that require metallic cations to synthesize glycopolymers.

The orthologs Nv-CHS1 and Nv-CHS2 are more similar to each other than to other cnidarian or metazoan *chitin synthase*s (see Chapter 1), indicating a possible gene duplication within the *Nematostella* lineage. All three *Nematostella chitin synthase* genes fall within the broader cnidarian clade (Chapter 1). Each *chitin synthase* ortholog contains a unique protein domain composition, indicating that the orthologs may be deployed in different physiological or developmental contexts. Previously published sequence descriptions of *Nv-CHS1* and *Nv-CHS3* were incomplete (Putnam et al 2007; Zakrzewski et al 2014) and have been updated here (Figure 2.2; Supp. Fig. S2.1).

Beyond the synthesis of chitin, the *Nematostella* genome contains a diverse repertoire of other protein domains or gene families involved in chitin metabolism. *Nematostella* posesses seven putative *chitinase* genes containing a variety of domains beyond the conserved glycosyl hydrolase and chitin binding domains (CBDs) (Figure 2.3; Supp. Fig S2.2-S2.5). When comparing

*Nematostella* chitinase genes to those of other cnidarians, and more broadly across Metazoa, *Nematostella* orthologs are scattered throughout the cnidarian chitinase clade (Supp. Figure S2.2, S2.3). It's likely that different orthologs are utilized in different biological processes – chitinases can be involved in digestion, immunity, and the maintenance of endogenous chitinous structures. The *Nematostella* genome also contains a variety of other proteins that include predicted CBDs. CBDs are cysteine-rich sections of a protein that specifically bind to chitin.

Intriguingly, several *mucin* genes contain chitin binding domains (Fig. 2.4). Mucins are glycoproteins that are a major component of mucous and are usually produced by epithelial cells. Several other taxa, such as flies, also have mucin sequences that contain CBDs. Chitin has been hypothesized to be involved in the formation of gel-like substances in animals (Syed et al 2008; CT Amemiya, unpublished data).

### *2.3.2 Chitin is broadly distributed throughout* Nematostella *tissues*

Fluorescent histochemistry using the chitin-binding-domain (CBD) probe shows wide distribution of chitin in both endodermally- and ectodermally-derived tissues across the *Nematostella* adult polyp (Fig. 2.5 A-D). The staining pattern around the pharyngeal region appears to be largely acellular, consistent with the known biosynthetic process of secretion of the chitin molecule from chitin-producing cells (Fig. 2.5 A, B, D). Chitin labeling is associated with oblong cells in the pharynx, as well as on fine filamentous structures protruding into the lumen of the pharynx (panel B). These thread-like structures are the tubules of fired cnidocysts.

Chitinase experiments confirmed that the CBD-probe was binding specifically to chitin, as labeling was significantly reduced in chitinase-treated samples (Figure 2.6). Were chitin labeling not affected by the digest of the tissue with chitinase, the CBD probe would likely be binding nonspecific targets. The chitinase enzymes has a high specificity for the dismantling of chitin molecules, and the depleted chitin labeling in tissues treated with chitinase indicate that the staining patterns observed are accurate.

### *2.3.3 Chitin comprises the tubules of* Nematostella *spirocysts*

Chitin histochemistry shows that chitin is present in some cnidocysts (Figure 2.7 A-D). CBD-labeling of dissociated cnidocysts shows that there is chitin present in the tubules of spirocysts, but not in nematocyst tubules (Figure 2.7. B, D). Chitin labeling is present in the tubule of both fired and unfired spirocysts (Figure 2.7 A,C), though spirocyst capsules appear to not be chitinous. Previous studies have indicated that cnidocyst capsules and tubules are composed of minicollagens and chondroitin (Engel 2001; Adamczyk et al 2008; Zenkert et al 2011; Beckmann and Ozbek 2012), but the presence of chitin in these stinging organelles has never been reported. It is unclear whether chitin is complexed with collagen to make this structure – electron microscopy utilizing tagged collagen and chitin probes together would be useful in assessing detailed molecular composition of the spirocyst tubule.

### *2.3.4 Each* Nematostella chitin synthase *gene has a unique pattern of expression in adult tissue*

The three predicted *chitin synthase* (*CHS*) paralogs in the *Nematostella* genome are all expressed in adult tissues with unique patterns of expression (Fig. 2.8). *Chitin synthase* expression is not observed in tissues or areas of tissue that lack chitin labeling, and chitin labeling is not present in tissue type that do not show *CHS* expression. Due to the relative impermeability and density of *Nematostella* tissues, special expression assays can be challenging in adult stages. Using a DIG-probe system for RNA expression, *in situ* hybridization data reveal that there is broad expression of *Nv-CHS1* and *Nv-CHS3* in adult tissues (Fig. 2.8 A-B, E), while *Nv-CHS2* expression is concentrated in ectodermal tissue in the tentacles in adults. There is wide ectodermal expression of *Nv-CHS1* (Fig. 2.8A), with concentrated expression in the tentacle tips (Fig. 2.8 A-B). Expression of *Nv-CHS3* is widespread, with endodermal and ectodermal expression. *Nv-CHS2* is specific to tentacle tissue (Fig 2.8D). A representative sense probe (negative control) of *Nv-CHS1* shows a lack of nonspecific DIG labeling (Fig. 2.8 C).

## 2.4 Discussion

Due to a lack of rigid internal or external anatomical structures, the presence of the glycomolecule chitin in the sea anemone *Nematostella vectensis* tissues is unexpected. It is unclear exactly what types of cells are producing chitin across the animal; it may be presumed that cnidocytes are expression *chitin synthase-2,* as chitin is present in some popuations of cnidocysts. Though *Nematostella* is an established model for cnidarian development and the evolution of Bilateria, much of its cellular biology remains unexplored. The widespread distribution of chitin within endodermally- and ectodermally-derived tissues, combined with the unique expression patterns of each of the *chitin synthase* orthologs, suggests that multiple cell types are synthesizing and excreting chitin. It is possible that chitin is working in concert with, or is complexed to, other structural molecules to help provide form and stability to *Nematostella* tissues in the absence of a specific hard skeletal structure. The arrangement of chitin in a globular, widespread pattern seen in *Nematostella* is similar to chitin staining in other cnidarians (see Chapter 1). Expression of all three *Nematostella chitin synthase* genes correspond to anatomical areas in which chitin labeling was observed. Conversely, chitin labeling was not observed in tissues that lack expression of at least one *CHS* gene.

We show that chitin is present in the stinging cells of the sea anemone *Nematostella vectensis*, although this role likely does not involve mineralized chitin (as found in arthropod exoskeletons). These data are the first description of chitin presence in cnidarian stinging cells. Cnidarian-specific mini-collagens have been shown to comprise the capsule and tubules of cnidocysts, along with proteoglycans such as chondroitin (Adamczyk et al 2008; Adamczyk et al., 2010; Ozbek, 2011). However, the chitin molecule has not been discovered in previous analyses, which have usually consisted of targeted antibody assays. Preliminary data also suggest that chitin is present in the cnidocysts of scyphozoans (Chapter 1). Because of their unique function, structure and toxicity, cnidocytes are of interest for venom toxicology, in addition to gene

regulatory network studies associated with novel cell type evolution, and novel methods of drug delivery (Babonis and Martindale, 2014; Jouiaei et al., 2015; Shaoul et al., 2012). Gaining further insight into the cellular processes of cnidocyst synthesis and cnidocyte structural composition may help further explorations of cnidocyte biology as a tool in medicine or bioengineering. Cnidocytes are among the most complex eukaryotic cell types known (Anderson and Bouchard 2009; Platchetzki et al 2012), and chitin's role as a component in this evolutionarily unique cell type suggests that the ability to synthesize chitin may help facilitate the evolution of novel structures.

Cellulose, another glycopolysaccharide, comprises a unique structure in animals – the urochordate tunic (Nakashima et al 2004). Urochordates, or tunicates, obtained the *cellulose synthase* gene (an enzyme similar to chitin synthase) through horizontal gene transfer from Actinobacteria (Sasakura et al 2005), and the tunicate clade is defined by the presence of this cellulose-based structure (Lemaire and Piette 2015). If the cellular behavior, such as migration or differentiation, can be facilitated using a polysaccharide such as chitin (or cellulose), this is another means by which animals can evolve innovative structures.

These data together suggest a hitherto unknown role for the chitin molecule in cnidarian taxa that do not possess hard morphological structures. While some anthozoans possess chitinous bases for attachment to substrate (some actinarian species such as *Anthopleura xanthogammica*) or chitinous endoskeletons (e.g. antipatharian corals), the function of chitin in a species lacking those features is enigmatic. Further investigations of the role of chitin will need to include functional experiments in *Nematostella*. Given the widespread distribution of the chitin molecule in soft *Nematostella* tissues, we predict that if chitin production were abrogated, cnidocyte structure would be affected and that there may be other morphological defects.

## Chapter 2 Tables

Table 2.1 – Primers used for *Nematostella vectensis* gene amplification and/or subsequent RNA probe synthesis

| Gene | Primer Sequence |
|---|---|
| Actin | F-CTATCCAGGCCGTACTCTCCC |
| | R-TAGTGGAACCACCAGACAAGA |
| CHS-1 | F- TTCATGGTGGCTGCACTGAT |
| | R- CACTCGGCTCCGTATAGCTG |
| | F- CCTCGTCCGTGACGATCAAA |
| | R- GTACACCATACCGCCCACAA |
| | F- GTACGGGCTCATTTGCCTCT |
| | R- TCTGCGGTAGAGTGTTACGC |
| | F- GCTGGCGTTTGATGTACTGC |
| | R- TTGTAGACACGGTGGGTGTG |
| CHS-2 | F-GATGCAGTTCAGGTGGCGTC |
| | R-GTTGTCACACCGGTAGCGT |
| | F- GGCTGCGCGAAATTCTCTAAA |
| | R- CAGCCCTTCCCAGAAATCAAA |
| | F- ATTTGGAAAAGAGGGAGGCTG |
| | R- ATGATACTCCGTCTGCAACTACT |
| CHS-3 | F-ACCGGAGTTACCCATCCAGA |
| | R- GCTGATTTGCCTCGTGCATT |
| | F- TCCTTCGATCGTGTTGGGTG<br>R- GACTAGTGCCAGCGAGACAG |
| | F- CTGGGAATACTTCGTGCGGA |
| | R- TGGAGCTTTGCTCAGTCGAG |

2.6 Chapter 2 Figures

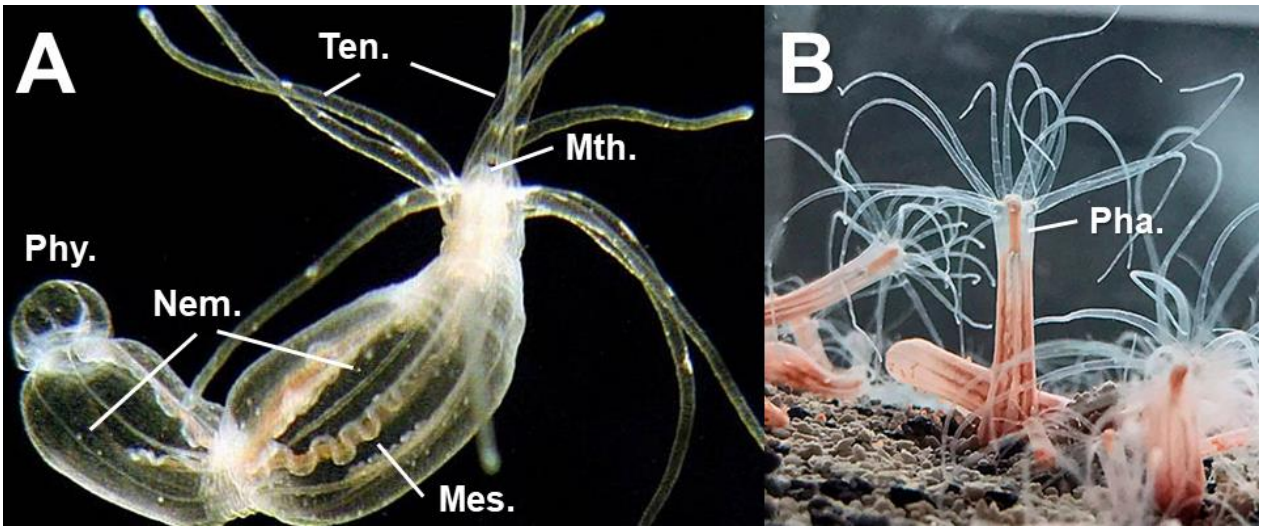


Figure 2.1: A.) Live adult *Nematostella vectensis* with major tissues and organs labeled. Source: *Uri Gat.* B.) Nematostella individuals buried in muddy substrate with feeding tentacles exposed. *Stowers Institute*. Mth – mouth; Mes – mesenteries; Nem – nematosomes; Pha – pharynx; Phy – physa; Ten – tentacle

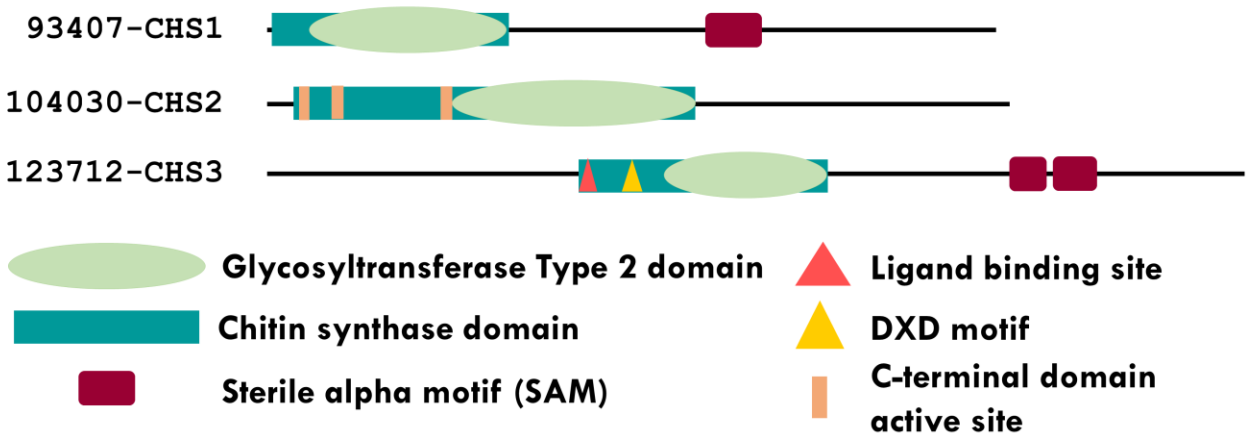


Figure 2.2: Overview of protein domain architecture of *chitin synthase* genes in the *Nematostella vectensis* genome. All chitin synthase genes share a conserved glycosyltransferase domain Domains were identified using BLAST and Interpro. Predicted domains with e-value support greater than $10^{-5}$ were not included in this figure or other analyses. DXD motifs are a domain specific to glycosyltransferases that utilize nucleoside diphosphate sugars as donors and need metal cations (such as manganese) to function. Numerical identifiers correspond with protein ID labels in the genome (Putnam *et al*. 2007; https://genome.jgi.doe.gov/Nemve1/Nemve1.home.html). Domains and transcripts are to scale.

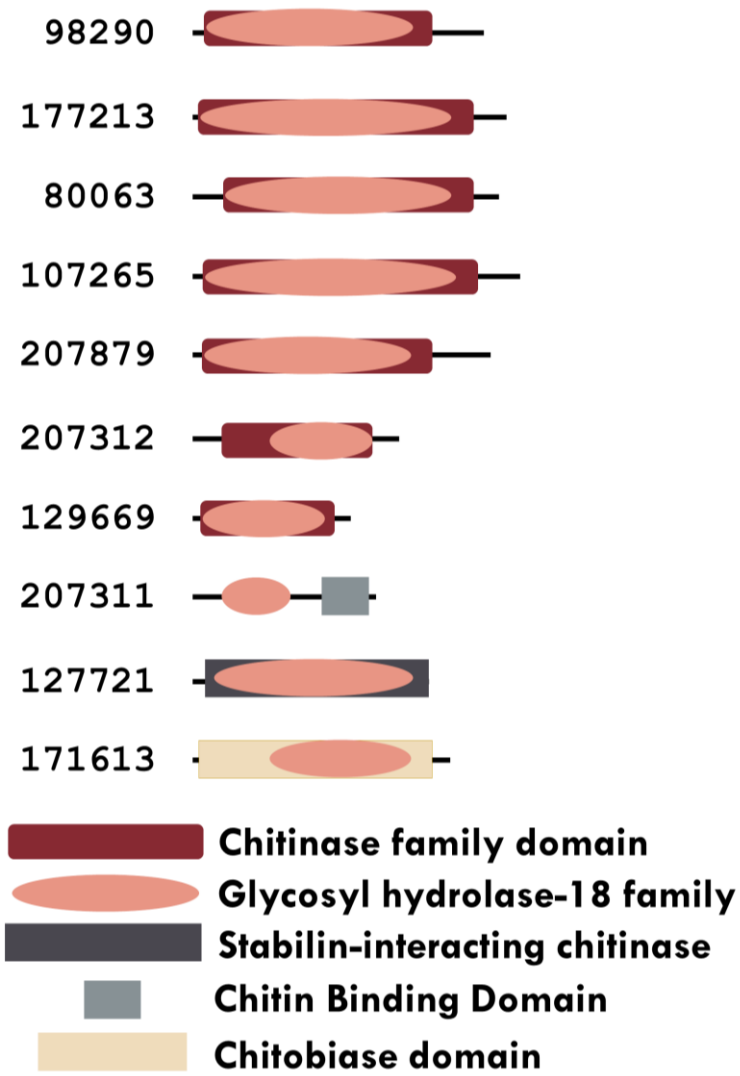


Figure 2.3: Overview of protein domain architecture of predicted *chitinase* genes in the *Nematostella vectensis* genome. Chitinases are chitin-digesting enzymes that have roles in digestion, immunity, and the maintenance of chitinous morphological structures. *Nematostella vectensis* has seven predicted chitinases. Domains were identified using BLAST and Interpro. Predicted domains with e-value support greater than $10^{-5}$ were not included in this analyses. Numerical identifiers correspond with protein ID labels in the genome (Putnam *et al*. 2007; https://genome.jgi.doe.gov/Nemve1/Nemve1.home.html). Domains and transcripts are to scale.

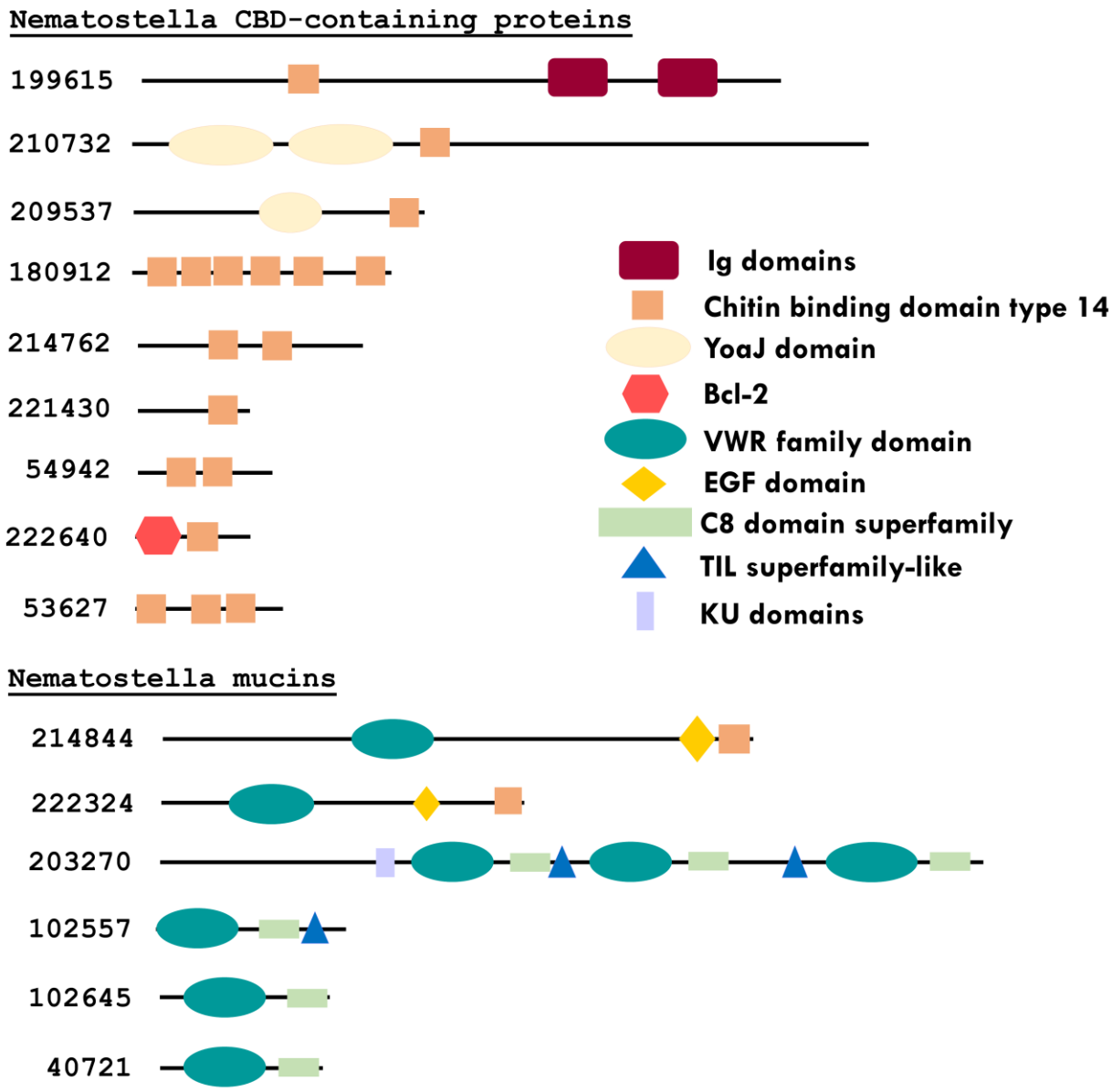


Figure 2.4: Overview of protein domain architecture of predicted mucins and chitin binding domain (CBD)-containing genes in the Nematostella vectensis genome. Domains were identified using BLAST and Interpro. Predicted domains with e-value support greater than $10^{-5}$ were not included in this figure or other analyses. Numerical identifiers correspond with protein ID labels in the genome (Putnam *et al*. 2007; https://genome.jgi.doe.gov/Nemve1/Nemve1.home.html). Domains and transcripts are to scale.

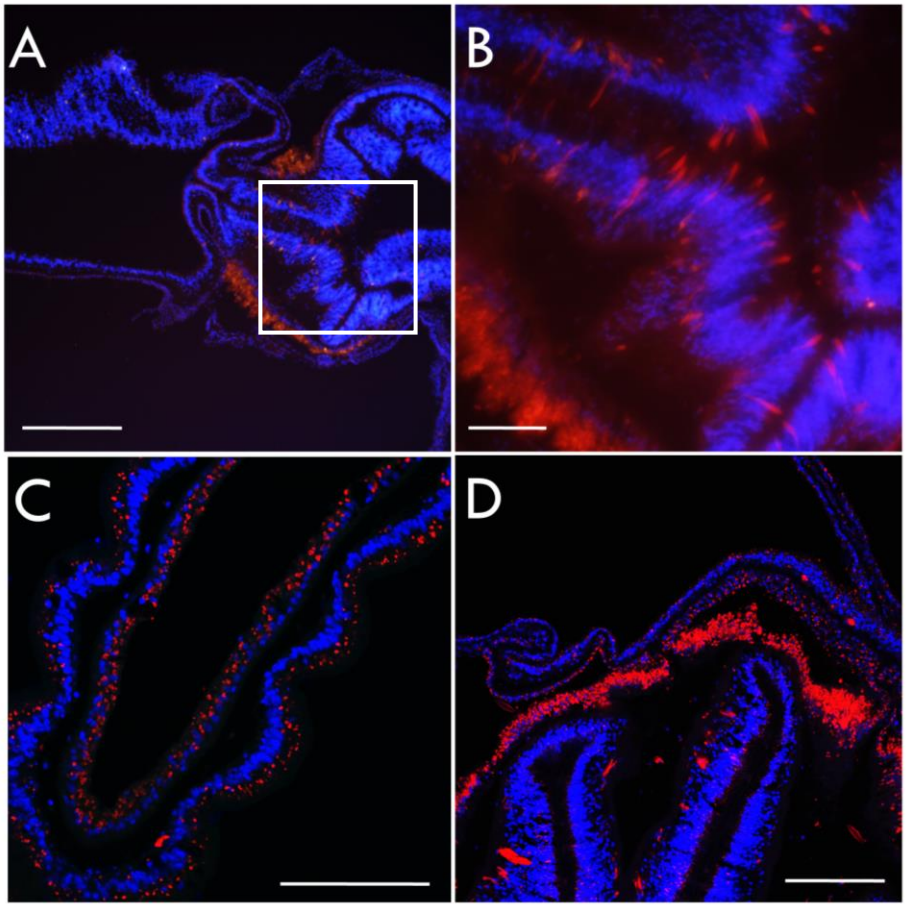


Figure 2.5: Chitin histochemistry in adult *Nematostella* cryosections. Red: chitin (CBD-546 probe). Blue: DAPI (nuclei). A.) Chitin labeling is widely present throughout *Nematostella* tissues, including the tentacles, pharynx, body wall, and mesoglea. Mouth is pointing left. Scale bar - 200µm. B.) Magnified view (white box) in (A), showing chitin labeling in the pharynx. Cnidocysts label positively with the chitin probe and there is labeling in the acellular mesoglea as well as scattered pharyngeal cells. Scale bar – 50µm C.) 100x view of mesenteries showing chitin labeling in endo- and ectodermal layers. Scale bar – 50µm. D.) Oral region of a polyp. Chitin labeling is present in the endoderm and ectoderm. Scale bar – 100µm.Panels A-B: compound microscope; Panels C-D: confocal microscope images.

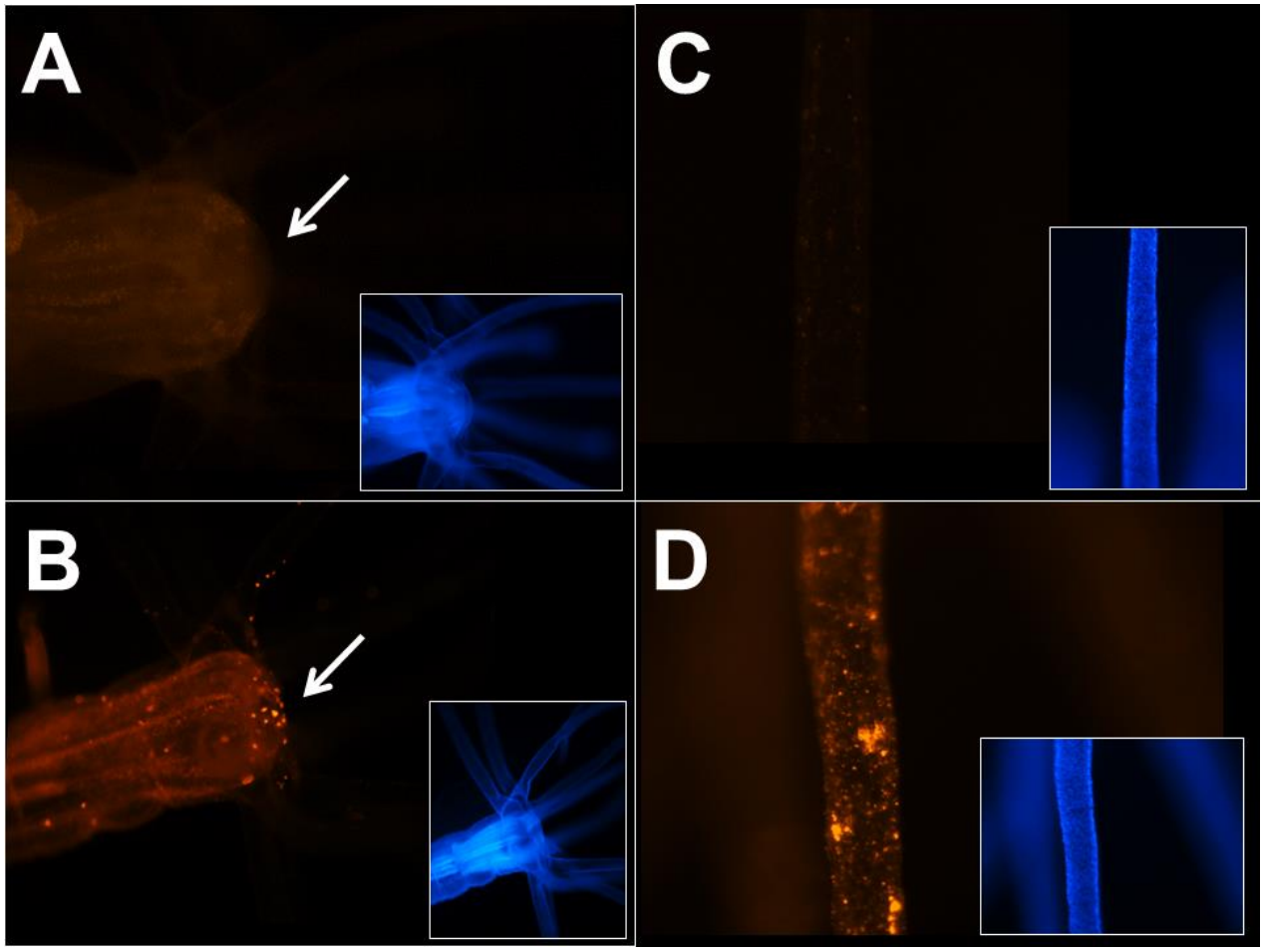


Figure 2.6: Stereoscope images of chitinase digests and subsequent CBD probe labeling of adult whole *Nematostella*. Insets are labeled nuclei of samples (DAPI). Arrows point to the location of the mouth. Compare fluorescent signal in A and B, and C and D. Samples in A and C were treated with chitinase enzyme to digest away any chitin present in the samples. Samples in B and D were incubated in buffer alone, without enzyme. A.) Oral end (mouth, pharynx, tentacles) of *Nematostella* incubated with chitinase enzyme. Chitin labeling is depleted. B.) Oral end of *Nematostella* showing preserved chitin labeling. C.) Magnified tentacle of specimen digested with chitinase. Chitin histochemical labeling is absent. D.) Magnified tentacle of a specimen with chitin intact showing chitin labeling.

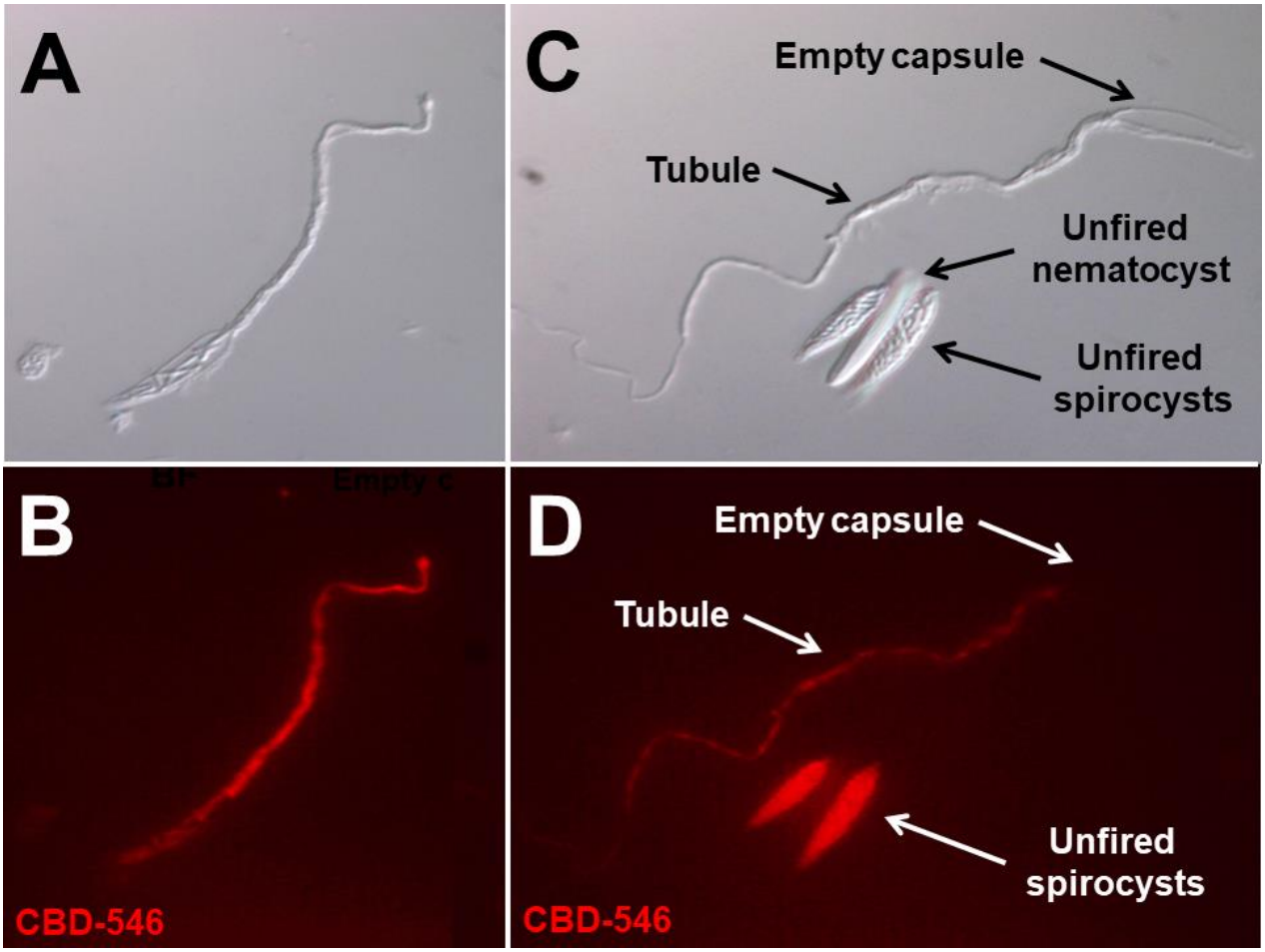


Figure 2.7: Isolated *Nematostella* cnidocysts labeled with chitin binding domain (CBD-546) probe. A.) Spirocyst with everted tubule (fired) – DIC image, 100x B.) Spirocyst from panel (A) showing labeling with fluorescent chitin probe. The tubule is staining for chitin. C.) DIC image of isolated cnidocysts – a fired spirocyst (top) and two unfired spirocysts flanking a nematocyst (bottom of image). D.) Fluorescent image of panel (C) – spirocysts show clear chitin labeling (red), but the nematocyst is not labeling with chitin probe.

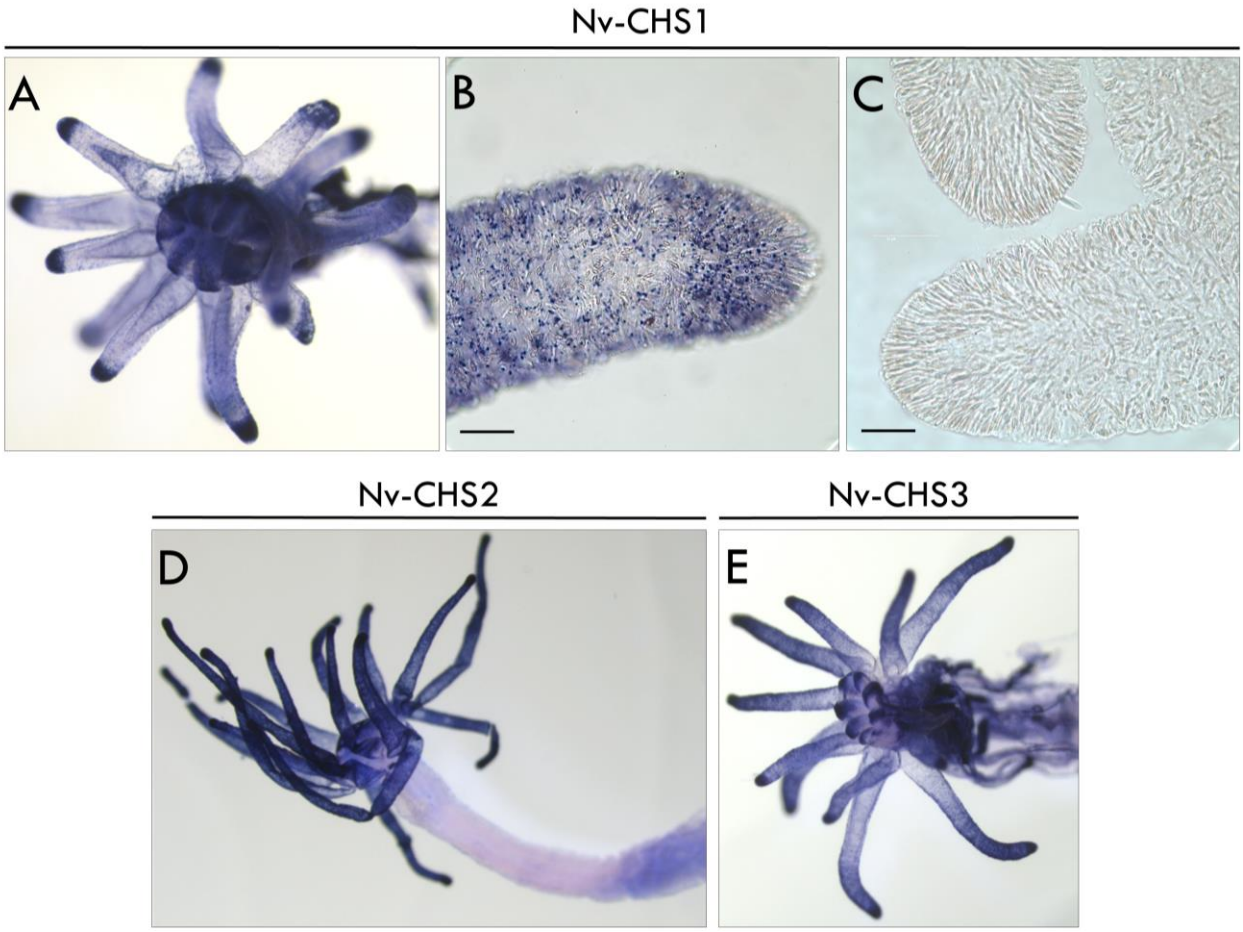


Figure 2.8: *In situ* hybridization data of *Nematostella* chitin synthase genes in adults or juvenile polyps. A.) Expression of *Nv-CHS-1* (JGI protein ID – 93407). There is broad expression throughout ectodermally- and endodermally-derived tissues, with expression concentrated in the tips of tentacles. B.) CHS-1 expression in nematostella tentacles, showing cellular-level labeling. C.) Sense probe (negative control) of *CHS-1*. D.) Pattern of expression of *Nv-CHS-2* (JGI protein ID – 104030). Expression is localized to the tentacles. E.) Pattern of expression of *Nv-CHS-3* (JGI protein ID – 123712). Expression appears to be widespread in ectodermally-derived and endodermally-derived tissues. A, D, E – stereoscope images. B-C – compound microscope images. Scale bar – 50µm.

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**Chapter 2 Supplemental Data**

Figure S2.1: Amino acid alignment of the three predicted *chitin synthase* orthologs encoded in the *Nematostella vectensis* genome. The numerical identifiers correspond with genomic sequence IDs (https://genome.jgi.doe.gov/Nemve1/Nemve1.home.html). Amino acid sequences were aligned using MAFFT under the L-INS-i model.

```
93407_NvCHS-1   MV------------------------------------------------------------
104030_NvCHS2   M------------------------------------------------------------
123712_NvCHS3   MSAEEIKYTVKQPSKITRLSERPKDPRPSAWISGVKWVFAFFLSMSLLICIVSSKISLLS
                *

93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   ------------------------------------------------------------
123712_NvCHS3   IAMYFNATRLPTGVTHPEYGEQSQPETVFVMLILALMIPFALTFLRSCWVSLFRSSHLWP


93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   ------------------------------------------------------------
123712_NvCHS3   SNSALFWCVVSSVIEVAALCYFTIIPLMYTSLPATYCILISNSVFCFPIAWHLWKLWRNF


93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   ------------------------------------------------------------
123712_NvCHS3   NENRRLFFATLASLILELGSLGVLAYKVSSVLNTMGMSTHKSASIMVLLPASMVLLSIAW


93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   ------------------------------------------------------------
123712_NvCHS3   SPKVQKKMLEPRGDQYDELDAREASMRTTAGLPGSSEGDPAPPVFTPQTAAHLLLSDGKN


93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   ------------------------------------------------------------
123712_NvCHS3   ARGKSAIITSLIKLIITPIIAGLIAWPYGVLENLGDVLSKGFGAFTFNHLAFPHFLAQIF


93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   ------------------------------------------------------------
123712_NvCHS3   TSVVGYILGWLACSMCLQKIAYALPLILATPVSLALVFIPKICASDRVPMKCEYDDGSEL


93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   ----------------------------------------------LEQNLLLNRRNEVT
123712_NvCHS3   YYIVVLAACLWLAQLLWTGCFIWKEQTFIMARESSLFWMPLYNGVFLEQNLLLNRKNVIT


93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   DESHVTQKVLKRDTHVFICTTMYHEADYEMEQLLNSLHDVDRYRRKARRHIESHVFFDGC
123712_NvCHS3   DEFHVNQREIAKNACVYICTTMYHETEEEMEQLLHSLHDIDTARAASKRQFESHVFFDGA
```

```
93407_NvCHS-1   ------------------------------------------------------------
104030_NvCHS2   IKSDELNSYILQLVSLVESTLRIPLNKCTKMKTPYGMQLRWKLPGGMPFTIHLKDNNKVK
123712_NvCHS3   VKGEVLNDFVLQLIALVPKTLKVKVEAVMKIKTPYGMQFRWRLPGGMPFHIHLKDNLKVK


93407_NvCHS-1   ----------------------------------------SDIRLRHRS-------LVH
104030_NvCHS2   NKKRWSQVMYMSYVPHSFFYLFNEMYFCLVRDDQTFILTTDADVNFKHESMEALIDYMLQ
123712_NvCHS3   NKKRWSQVMYMSYI------LDYKEGTMGSTDENTYILATDADVRFTHGDVQALLDLLMR
                                                :*:.: * .       :::

93407_NvCHS-1   DKQQRISRTGTPKAGNPTTILIQDNIATIYNYASLNQLQ-VANHMFGSVLCCPGCFSLYR
104030_NvCHS2   DEKVGAVCGRTHPMGKGPLVWYQ-----IFDYAIGHWFQKVANHMLGSVLCCPGCFSLYR
123712_NvCHS3   DPRVGAVCGRTHPLGVGPIVWYQ-----VFDYAIGHWFQKTANHVLGTVLCSPGCFSAYR
                * :       *   *  . :  *     :::**  : :* .***::*:***.***** **

93407_NvCHS-1   CQAVRDILPTYSTTVNEAFEFLTKDMGEDRWMCTLMVQAGWRLMYCAAAEDVTYCPMNFD
104030_NvCHS2   CKAVKDILPEYSTNVNEASEFLTKDMGEDRWMCTLMVQAGWRLLYCAAAEDVTYCPTTFD
123712_NvCHS3   CLAVRDVLPTYATGVTTSTDFLTKDMGEDRWFCTLMVQSGWRLEYCAAAEDSTFCPDNFD
                * **:*:** *:* *. : :***********:******:**** ******* *:** .**

93407_NvCHS-1   EFYKQRRRWMPSTLANLALLISEWRVTLGNNEHISVPFIIFQLFLLVSTIIGPSTVILVI
104030_NvCHS2   EFYNQRRRWMPSTLANLILLISEWRLVVTNNDYISVPFIIFQLILLLATVIGPSTVILVI
123712_NvCHS3   EFFRQRRRWIPSTLANLLQLISQWRVTTENNDMVSIWFILYQALNVVATLICPATVILII
                **:.*****:*******  ***:**:.  **: :*: **::* : :::*:* *:****:*

93407_NvCHS-1   VGGMVY-GVGA-NENTSVILLSLITLTYGLICLFMSQDFQLKTAKFLTFVFAVIMILVTI
104030_NvCHS2   TGGMNYVGIAT-NEVTTVTIMSLITLAFGLICLFTSQSFQLLIAKLLTFAFSIVMLMVAV
123712_NvCHS3   SGALHYAHLGVEDELIPAVLLVLVSIGYALVCLYTKQEFQLKVAKLLTIIFALIMAFVVV
                 *.: *  :.. :*  .. :: *::: :.*:**: .*.***  **:**: *:::* :*.:

93407_NvCHS-1   GVAAQIANELEVRRIDKQT---------PTIPPNSXXATPTVSTSVELM-----------
104030_NvCHS2   GVAVQISKDLEKREAARNSLN----------------STSTSELVV--------------
123712_NvCHS3   GMAAQIAIDLQLRSTPKPTTSPVPTALVPTTQATTPMSTSTAKTAVDRLLAAITSSSTPG
                *:*.**: :*: *   : :                  :*.* .  *

93407_NvCHS-1   ----------------------LPAGISTLYLGGLAGIFMVAALMHPTEAYCLINGLWYL
104030_NvCHS2   ----------------------LPASVSTLYLG---GIFVLAALLHPKEAYCLLNGIFYL
123712_NvCHS3   PTAPSGTSSPLPTTSYVPLKDRLPADVTSLYLGGLVGIFLIAALLHPFEAFCLVHGMWYL
                                    ***.:::****   ***::***:** **:**::*::**

93407_NvCHS-1   LCLPSGYLLLTIYSVVNMTDRSWGNEHDLADFESSRELLAPGER----HSTVPSKAACE-
104030_NvCHS2   LCLPSGYLLLTVYSIVNITDRSWG-----------------------KSLLPFSAPTD-
123712_NvCHS3   LCLPSGYLLLIIYSVCNVTDRSWGT-RETKEKKSGAEVPWYEELWKTIKYVFTCCRPAEP
                ********** :**: *:******                        :  ..   . :

93407_NvCHS-1   YPGPRAKRNSAPPDLQPTTSNGNSLSKQSASQESAYVSEDSRNTLPQMSSGSIAGNGGRR
104030_NvCHS2   NPNHLAPRCACHPLQKP----DDDYLGRARTNRSRSLDDIDSP-----------------
123712_NvCHS3   NPDPQAP-ATNNQTDNP----PSQYEDSSSSETNGVLSDEEQPPLP-LSDSSIQSAAPPE
                 *.  *   :     :*     ..    : :: .  :.: .

93407_NvCHS-1   ----HTMQSSMTAP---SVDELR---TYDLD----------------------------MI
104030_NvCHS2   -----------------QVINLR-------------------------------------
123712_NvCHS3   VMIDETHSPSATPPMPKSVLKLRKAASLDADSPEAPKWRFPGERKVRFDTRLPKLLAEMP
                          .* :**
```

```
93407_NvCHS-1   VEEWLKGNMK-VYIPLFKEHGYDNLKFISTMTEKDLETIGITTRGHRQKLSRHLKKIPKL
104030_NvCHS2   ------------------------------------------------------------
123712_NvCHS3   VEEWLPGELKGLYLKNFKDNGYDNVSFIAGMTNKDLRAIGISTRGHRMQLVYAIERIPHV


93407_NvCHS-1   EIDEGVPDNVQQWLEDLSLGQYWPHFVDNSYTEPSDLEDVKSMGRDLVQETFR----VHK
104030_NvCHS2   ----DIHCNLGTWDARFGFQ-----------------------------------------
123712_NvCHS3   TLNADVPESVEEWLHELGLECYWEYFVRNGYNEPRVLEDLKAMDFTTLKQTLKADLNINK
                    .:  .:  *   :.:

93407_NvCHS-1   MAHINILWQAIRKLQYPNTGQVKIRHARRQLDRC-RTIDLEVDNSDDGAEYDFWDGLREA
104030_NvCHS2   ---------------------------QQIKKEAKVVA--------DGVSFDFWEGLRRI
123712_NvCHS3   AGHLTKMISAIKKLQYPTPSEREIRRARELVKEAGHVIDMEKSNQDDGMEFKFWKGLREA
                                         :.  ...  .         ** .:.**.***.

93407_NvCHS-1   CLKPELAGFDGNTELKDKLEDLRNTTLMVFLISNVLWMIVIMVLVKHSNLKLLGVDFVGL
104030_NvCHS2   SLEPEAAAFSTSSELKEKLTSLRNSVLVIFFVANVLWMLLIMVLARHQSLKVLGVDVIGL
123712_NvCHS3   CLLPESAIFGAVTELKEKLEDLRDSILMVFAMTNAIWMILMVTLTKTPQLKVLGTDALGL
                .* ** * *.  :***:** .**:: *::* ::*.:**::::.*.:  .**:**.* :**

93407_NvCHS-1   MFLSVYGTIILFQFIAMLAHRLTTLIHLLARAPYICGVQ---------------EETTVV
104030_NvCHS2   GFLIVYGVIIIVQFLTMVCHRLATMIHVLARAPWTCG-----------------KDSPIE
123712_NvCHS3   AFLAIYGFIIVLQFLTLIWHRLSTWCHMIARAPWTRGPYKMAWAFSDENLVPEPTQADLE
                 ** :** **:.**:::: ***:*  *::****:  *                 :: :

93407_NvCHS-1   QVDNLNEQHLTLSSPETV-------------------------------------------
104030_NvCHS2   KVRTTIRKRTLRKTPRRVGARN-----------------------------------------
123712_NvCHS3   RVRRLRHKRRRRSRHSSSDAHHALLYNSTEQSSTSNLEHSGHTNYGATSYGNSLTPNNNY
                :*    .::   .

93407_NvCHS-1   EDCETINEC------
104030_NvCHS2   ------NRVVETR--
123712_NvCHS3   KDSLTLNKAGSSFVV
```

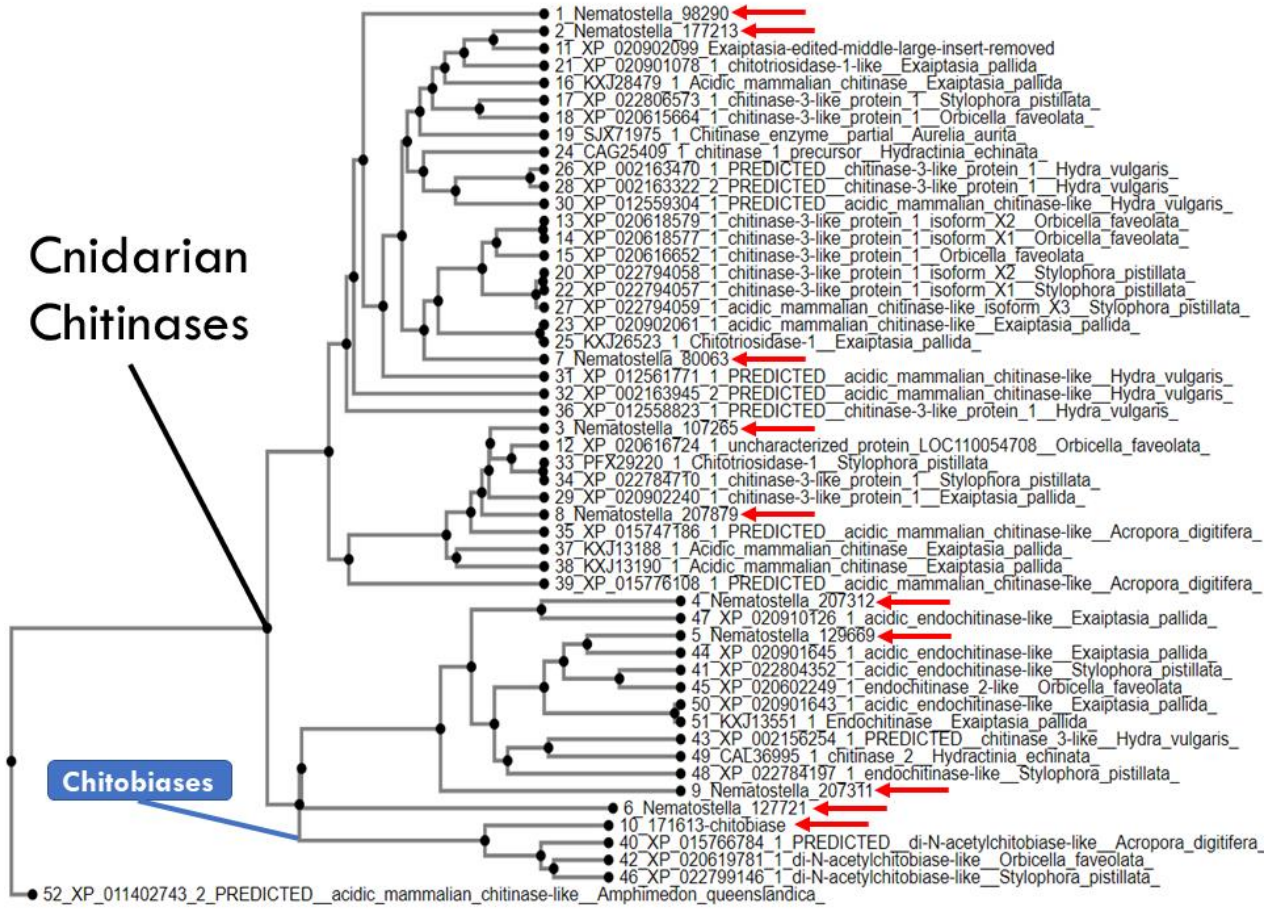


Figure S1.2: Neighbor-joining gene tree of predicted chitinases in several cnidarian transcriptomes: *Nematostella* (Anthozoa-Actinaria); *Exaiptasia pallida* (Anthozoa-Actinaria); *Stylophora pistillata* (Anthozoa-Scleractinia); *Orbicella faveolata* (Anthozoa-Scleractinia); *Aurelia aurita* (Scyphozoa- Semaeostomeae); *Hydractinia echinata* (Hydrozoa-Anthoathecata); *Hydravulgaris* (Hydrozoa-Anthothecata); *Acropora digitifera* (Anthozoa-Scleractinia); outgroup *Amphimedon queenslandica* (Porifera-Demospongiae). Several functional clades are present. *Nematostella vectensis* has 10 predicted *chitinase* and *chitobiase* genes in its genome. Red arrows represent location of *Nematostella vectensis* chitinase genes.

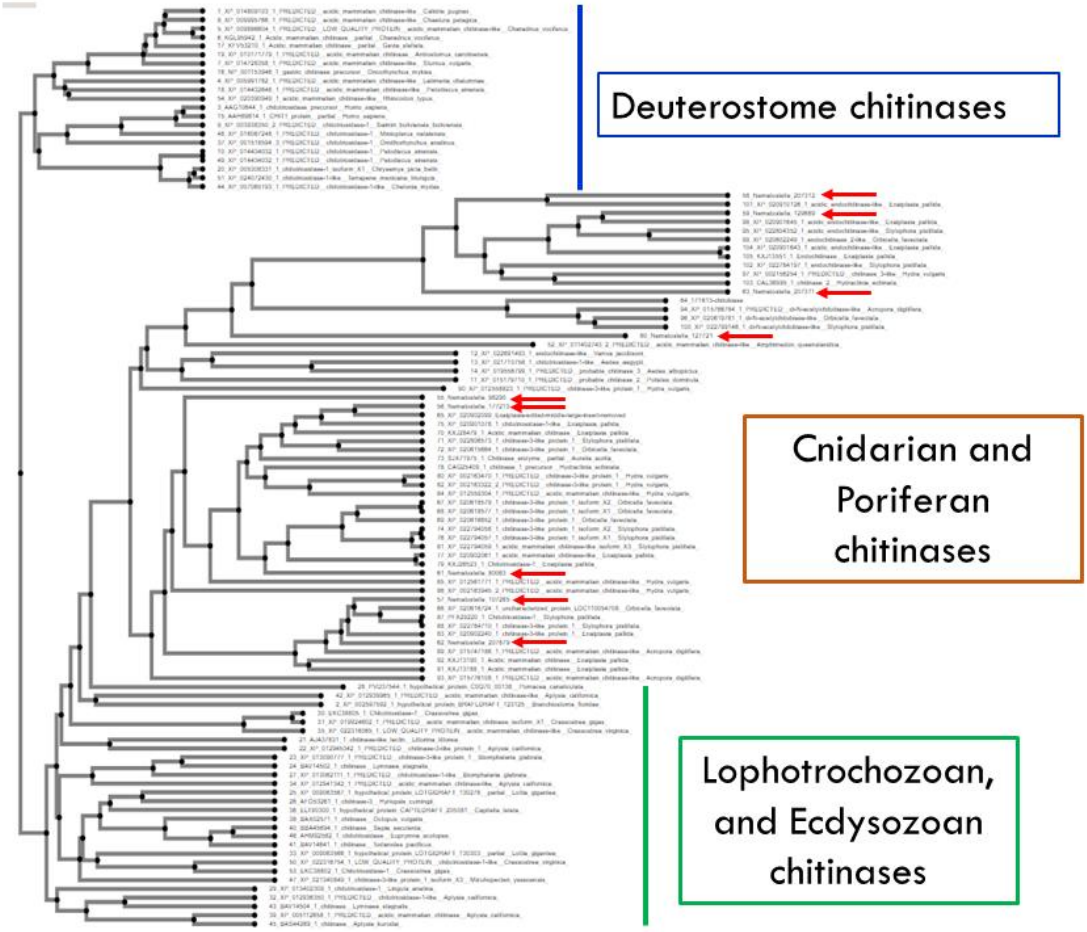


Figure S1.3: Neighbor-joining gene tree of predicted chitinases from metazoan transcriptomes. Cnidarian chitinases form a clade. Red arrows represent location of *Nematostella vectensis* chitinase genes.

Supplemental Figure S1.4 – alignment of chitinase amino acid sequences from the *Nematostella vectensis* genome.

**MAFFT-E-INS-i**

CLUSTAL format alignment by MAFFT (v7.402)

```
98290           M-------------------------------------------------LPLILL
177213          ---------------------------------------------------MQLL
107265          M---------------------------------------------------KSLL
207879          M---------------------------------------------------ARVL
80063           -----------------PQPSDLCSTSFFISFLHIIRCRTLAE---------ASIKMRSL
207311          ------------------------------------------------------
129669          ------------------------------------------------------
207312          M-SEYGYVNTAVMTVETPQ----------------KSAGLFSASIALGNIGAMMTVVQL
127721          NQQAVSNVITRGLVTETPRIRD------------ILKHHSKYYKNVSLQN----------
171613-chitobia M-------------------------AFFKRWL-------------------AVICVFSF

98290           PLLVV------------------------------------------------------
177213          TLLVL------------------------------------------------------
107265          LLAVL------------------------------------------------------
207879          AILIL------------------------------------------------------
80063           AFLLL------------------------------------------------------
207311          ------------------------------------------------------
129669          ------------------------------------------------------
207312          ALVLVIASLLSCASASTKRSL--PSNRQLLVGYWGQNAAGPTFGRLNYERDLRHFCNNYD
127721          ------------------------------------------FEGDV--------
171613-chitobia AISLTLCRKCPCKEESLCEPITRPPGKECLV----------------------------

98290           -------------------PSSLFASASYFRVCYYTNWAQYR-GLAKYTPDNIDPSL--C
177213          -------------------AVAACATGNYVRVCYHTNWSQYRQGRAKFWPEDIPADL--C
107265          ------------------GCALSVTASEYVRGCYYTNWSQYRPKGGTFWPEDIDPFL--C
207879          -------------------CFVPFAVSEYVRVCYYTNWSQYRPKGGTFWPEDIDPHL--C
80063           -------------------VCSCSLVAAYHRVCYFTNWAQYRPDPVKFLPKDIDPLL--C
207311          ------------------------------------------------------
129669          ----------------SAFPSGELPALNFAFHCKDSVSTDY-------------PYLLRC
207312          FDIYVIAFVHRLFDVRNKGPDPKLPGMNFAHHCSYVPDANY-------------PGIYEC
127721          -------------------------------LAYVTPWNSHGYDVAKAFGAKF-TYV--C
171613-chitobia ------------------------------------WTTT-------------PNVWRK



98290           THIV---YAFAKMNADNSKLAMFEWND-AAMYKKVNDLKS-SSNMKTL--LAVGGWNMGS
177213          THLM---YSFAKINQKN-ELAMYEWND-DKLYPRFNALKQKNPELKTL--LAVGGWNHEN
107265          THIL---FSFAKVNQTTHKLDIYEEND-HELYQRINALKKINPKLKTQ--IAVGGWTHEE
207879          THVI---HSFSKVNLTTHVMEKYEKND-FDLYKRINALKKINPKLKTQ--IAVGGWTHEE
80063           THIV---YAFAKIDPATNKIGTYEWND-DRLYKEINDLKLKNPSLKTL--LAVGGWNHES
207311          ---------MAEGRQRSRRIDY----------------------KAL--NNVSSADFN-
129669          PE-------IENGIKECQKMGK----------------------KVL--ISVGGATGD-
207312          AT-------IGGGIRDCQKMGK----------------------KVI--ISLGGDTCD-
127721          PV-------WLQIKRRY-KFALEGSHDIDKGW--LEDVKKSNDKAKILPRVLFDQWTYND
171613-chitobia TDFNAPGYDWSKIT----TIGMFREWD-DELFCTAHS--------KGVRVVLVSGFPVE-
                                    :                       *     ..
```

```
98290           ---GPFQDMVSTASRRKIFIDDAILFLRNHMF-----------DGLDLDWEYPGARG-SP
177213          AN-SPFSRMVKTAATRKVFIDSVIRILRTWGF-----------DGLDLDWEYPATRGGSP
107265          KN-SPFSKMVATKEKRAIFIKSAIETLRTHGF-----------DGLDLDWEYPGMRGGSP
207879          KN-SPFSKMVATKEKRAIFIKSAIETLRTNGF-----------DGLDLDWEYPGMRGGSP
80063           GPVSPFSQMVASKSNRTTFISSLLQLSDKYDF-----------DGFDLDWEYPASRGNSP
207311          ---FDFQRKRKYKSGSKVY-----------------------------------------
129669          ---GTLPSPAKAKELANTFYDLFLGGSRFDGTTNLRPFGRLVMVGIDLNIQ---------
207312          ---GTLGSAANARALAYNIWNMFLGGQDLPGK---RPFLSAVLDGVDLDIE---------
127721          -----FHIVLSSEPALTSLVDVVTSFCQNHGF-----------DGVVLEVWSQYS-----
171613-chitobia --------KLSNSQVRKDWIKNKTTEAKENFA-----------DGINIDIEGPIEKG---

98290           PEDKHRFTLLCQELLAAFETEAANTGKPRLLLTAAVS-----------AGETTVKNGY--
177213          PEDKQRFTVLCQELMNTFIAEGKQTGKPRLLLTAAVS-----------AGKNTIDKAY--
107265          KSDKGRFTLLCRELREAFEAEAKASGKERMLLTAAVA-----------AGLWTIKGAY--
207879          KSDKGRFTLLCQELRDAFEAEAKDSGKERLLLTAAVA-----------AGLWTIKDAY--
80063           PQDKQHFTILCEEMLDAFKRKAADTDKPRMLLTAAVS-----------AGHGTVDAAY--
207311          ------------DVERIISQRITS----RNRLEGE------------------FDYLFIQ
129669          AGSGQYYEHLIREMRRLMDADLSR----EYLITGAPQCPYPDHYLGPGAGTELVDHLYIQ
207312          VGSYKYYPDFVREIRQLMRTDSSR----QYLITAAPQCPFPDKWMGPQTPGSALEGLY--
127721          GDTKEGLYLLVSKLADALHKAKMKL---ILVIPPPIH-----------AGYETNKRGT--
171613-chitobia SKEVTLLTKLVNNVAKAFRKEFKHT---QISFDAA------------------------
                           .:   :             :

98290           -------------EISALGQALDWINLMSYDLHGDWEDTTGHHAAMDSSTGDPLTVTHA-
177213          -------------EVEKIGKILDLINLMAYDLRGKWEPHTGHHTSLEGPPGEILTVKFA-
107265          -------------DIADIAEPLDWINLMTYDLHGTWEYKTGHHTAM-GPDGDKLTLPFA-
207879          -------------DIEGISKPLDWINVMTYDLHGTWEPKTGHHTAM-GPDGDKLTLPFA-
80063           -------------EVHKLAGILDWINLMTYDLHGPWEPYTGHHTALVGPPGDKLTVSYA-
207311          FYNNYCYPGSS-YFVSVMDTWLNWANGIT-----------------NGP-----------
129669          FYNNFCHTGAGNDFYKSLNKWLDFANKRY----------------PRGP-----------
207312          -------GGRKEGKRKS-----------------------------GK-----------
127721          FDKN---------DFELLFPVLDGFSLMTYDYSSPGRP---------GP-----NAPIA-
171613-chitobia -WSPDCIDGRC-YDLKAIVDIVDFMVIMAYDQQSQIKE---------GPCIAKANSPYSQ
                                                                .

98290           ----VDLW-----------IAGGMPSNKIALGIPLYGRSFTLKTANKT------------
177213          ----TQYW-----------IDMGAPPSKIALGLGTYGRSFKLTFKEKN------------
107265          ----IWYWMNNRDTWEKPGIRKGMPANKIVLGLATYGRAFGLPSPATN------------
207879          ----IWYWMNNRDTWEKPGIRNGMPANKIVLGLGTYGRAFGLESAGNN------------
80063           ----VKYW-----------MEKGMPCGKIALGMANYGHAFELSDPTKT------------
207311          --------------------------KIILGLPA-------------------------
129669          --------------------------LIFVGLPA-------------------------
207312          ------------------------------------------------------------R
127721          -------WVNDCVLAL--SPEAGKQRSKIMMGLNFYGFDYSLPQNASH------------
171613-chitobia TLEGVQQY-----------LKLNISNNKLVLGLPWYGYDYPCEEIDEKNDICFIKKVPFR

98290           -LDAPATKG--GQGPYTKEAGYIAYFEIC----KMGLSVTRDP-VLVSPYGVDVN----N
177213          GIGAPANGNP-PRGPYTRESGFLSYYEICDMMPKMKTMKTDQS-EVRAPYGYVKQDWA-D
107265          GLDSARDYQYTPKGKYTDAEGFLAYYEIC----KRGLTVVHKN-KANAPYGYKG-----K
207879          GLDA-----------------------IC----KMGLTVVENN-KAKAPYGYKG-----H
80063           ALGAPANVNK-GRS--------YPYYELC----KLPLTKVTDN-PAKAPYGYHG-----S
207311          GTGAAGKPH-----------------------------------------------------
129669          ATGGASDAQ-----------------------------------------------------
207312          KHGERKDGE-----------------------------------------------------
127721          --------------------MYVTLSRYLELLEKNKDGKLTWE-KTSAEHLMKFSSAGGD
171613-chitobia GVNCSDAAG-----------TQIPYATVNDLLMTRTDTGRKWSLKSLSPYFWYSDHVGNP
```

```
98290           QWVGYDDVTSVQEKV-NYIKKKSLLGAMFWAMDLDDF---KGD-CGQGSYPLMTAVKTGL
177213          VWVGYDDERSLQLKVEEVIKAKGLAGAMFWALDLDDF---DGSSCGKGNYPLMNAVKKYL
107265          DWIGFDDPNSLVYKIDNVVKKNQLRGVMFWAIDLDDF---SGEHCGQGKYPLMSAVKNYL
207879          DWVGFDNPKSLIYKIDNVVKKNQLRGVMFWAIDLDDF---SGEHCGQGKYPLMSAVKNYL
80063           QWIAYDDVTSLGRKV-ELIKKENLLGAMFWAIDLDDF----GNVCGQGAHPLMGAVRYML
207311          ---YYFTPEKLAEKYKLIRDKPGVSGIMLWDCSWAQNNKPNGNHYSEHAFKLVSG-----
129669          ---FYRPPSELRTMY-QVS----------W-----------MERAGDGK-----------
207312          ------------------------------------RKRGRRIGNGSRRKSEG-----
127721          HMVWYPTLKSIQARL-DLARDLGV-SIAIWEI-------------GQG------------
171613-chitobia HQVWYDDPSSLTFKY-RIANMLDLKGVAIWNADLLDY---SG---------LMRA-----

98290           TGVAP---------------DPTPAP-----------------------------------
177213          GGYVPPPP--PTGTLPP---PPVTPPPGPETPPP----------------------PDTPA
107265          IDGVQPTF--PPATAPPQTLPPATDPPSATTAAPI---------PPSPGTTQDPPPVTTA
207879          TNGVRPTF--PPATAPPQTLPPATDPPSATTVA---------------------------
80063           EGG--------------GVMPPTLAPGVPTTIKP--------------------------
207311          -GTVKPPPVHPTTNAPPQ--PPA------TTKAPLGTDKVTTTTTTTTTTTTTKAPVTTS
129669          ------------------------------------------------------------
207312          ------------------------------------------------------------
127721          ------------------------------------------------------------
171613-chitobia -NVQTPDMWNKLHQAMPK-------------------------------------------

98290           NPLPLTDA--VASSN---KMTCTVKPGFA--IITDEWCQQ----ICDNNYCY-EKSCDCV
177213          PPVPPTEAPPTAPPT---GGSCVSKPPYKS-ANMDGWCAT----NCAVGFCP-ETHCKC-
107265          APRPPTTA-PGGGGT---SGNCVATGAWAGYANMDAWCAS----NCALGNCP-STHCKC-
207879          ----PTTG-GGGGGG---SGGCKATGAWAGDANMDQWCKD----NCAVGYCP-TSMCVC-
80063           --------------------TCKAAGVWAGNVGMDSWCVN----NCALGNCP-PTICVC-
207311          QPSGPVSCSSLGDGTHPDPNDCSKFVMCAGGISYPNSCPAGLLYNKKTKNCDWPSNVTC-
129669          ------------------RGG-----------------------------------------
207312          ------------------------------------------------------------
127721          ---------------------------------LDYFYDL--------------------
171613-chitobia --SGTFGC----------VGQC-----------------------------------------
```

# Chapter 3: Distribution of chitin and *chitin synthase* expression during *Nematostella vectensis* development

## 3.0 Abstract

Many developmental studies predominantly focus on transcriptional regulation as a major basis for evolutionary innovations in metazoan body plans. However, it has also been suggested that polysaccharides may be utilized in the development of novel anatomical structures. For example, tunicates have acquired an anabolic *cellulose synthase* gene through horizontal gene transfer and utilize it to construct their evolutionarily unique tunic. The roles of polysaccharides in animal development have not been widely explored, outside of non-arthropod lineages. In this chapter, I ask whether chitin might play a hitherto unknown developmental role in the sea anemone *Nematostella vectensis*. *Nematostella* has *bona fide* genes for *chitin synthase* (*CHS*) but lacks obvious structures that might be chitinous, such as an endo- or exoskeleton. A recent study showed that genes involved with chitin metabolism are differentially expressed in regenerating anemones, though *CHS* expression patterns and chitin's role in *Nematostella* developmental processes has not been explored. Given that chitin has a potential role in development in diverse animal taxa, expression of *chitin synthase*s during development in *Nematostella* would provide additional evidence for an expanded role for chitin in animals.

## 3.1 Introduction

*Chitin in animal development*

Though many developmental studies predominantly focus on transcriptional regulation and genetic mutation as major bases for evolutionary innovations in metazoan body plans (Carroll et al., 2005), it has also been suggested that polysaccharides may be utilized in the development of novel anatomical structures. For example, tunicates, the only animals known to possess the enzyme cellulose synthase, acquired this gene through horizontal gene transfer and utilize cellulose synthase and cellulase to build and maintain a novel structure: the tunic (Satoh, 2014). Tunicates are the only metazoans to synthesize cellulose and have clearly coopted its chemical properties for building its body. Chitin is a biological polymer made from repeating N-acetyl glucosamine molecules linked in a β(1,4) configuration and is found in abundance as a structural component of invertebrate exoskeletons (Merzendorfer, 2006), mollusk radula (Peters, 1972), the chaetae of annelids (Ruppert et al., 2004), and fungal cell walls (Lenardon et al., 2010) (see Thesis Introduction for more detail).

Chitin was recently discovered to be endogenously produced in fishes and amphibians and is thought to have roles in developmental processes (Tang et al 2015). In the zebrafish *Danio rerio*, disruption of chitin production with morpholinos targeting *chitin synthase* leads to morphological defects of the eye and body trunk (Tang et al 2015). It was also proposed that chitin may serve a scaffolding role in the developing intestinal lining in zebrafish, similarly to how chitin is deployed in insect peritrophic membranes (Tang et al 2015). Additional recent work by the Amemiya lab (unpublished) has shown that chitin is likely found in other developmentally- and physiologically-important anatomical structures in bony and cartilaginous fishes.

In the protostome clade Ecdysozoa, arthropods have exoskeletons or cuticles that are composed of mineralized chitin, and these animals also use chitin in their internal structures. Nematodes and insects have been shown to possess two genes for *chitin synthase* (Zhang et al

2005; Zakrzewski et al 2014). Though it has been known that chitin production is required for maintenance of chitinous exoskeletons, it has been shown that the chitin molecule is also essential for the morphogenesis of internal structures (like insect tracheae) and is likely stabilizing for the requisite tissue during growth or cell migration (Devine et al 2005).

Differential exon usage by some taxa has been shown to produce different isoforms of *chitin synthases* that are deployed in varied developmental contexts, indicating that seemingly limited genetic toolkits can still serve diverse functions (Arakane et al 2004; Zhang et al 2005). The beetle *Tribolium* has two *chitin synthase* genes (*TcCHS1*, *TcCHS2*) that are expressed in different developmental stages and tissues (embryonic/pupal vs. late larva and adult). In the beetle, *TcCHS1* expression is localized to cuticle-forming tissue whereas *TcCHS2* appears to function in the formation of the peritrophic membrane (Arakane et al 2004). Because chitin synthases can have a variety of domains beyond the conserved glycosyltransferase region (see Chapter 1), it is likely that these enzymes serve different roles in different tissue contexts.

Nematostella *development*

Cnidaria is the outgroup to the bilaterians and has been extensively utilized to study the evolution of bilaterian characters such as the emergence of mesodermal tissue layers and secondary body axes (Wikramanayake et al 2003; Martindale et al 2004; Tulin et al 2013). Developmental biologists have explored cnidarian embryogenesis since the late 1800s (Metschnikoff 1874). The sea anemone *Nematostella vectensis* (Cnidaria – Anthozoa) has been a useful model for studying the early evolution of animals, the emergence of bilaterian traits, and evolution of tissue complexity. Though the hydrozoan polyp *Hydra vulgaris* has been widely deployed as a model for studying the evolution and development of animals, anthozoan models (e.g. *Acropora, Nematostella*) may be more suitable for evo-devo studies; the *Hydra* lineage has a highly derived body plan compared to other cnidarians, unusual life history and reproduction, and has undergone significant gene loss (Chapman et al 2010; Technau and Steele 2011).

Cnidarians are considered diploblastic and possess an endo-mesoderm. However, cnidarians have been shown to express genes that are essential for mesodermal patterning, indicating that the mesoderm may have evolved from endoderm after the Cnidaria-Bilateria split (Fritzenwater et al 2004; Martindale et al 2004; Tulin et al 2013). The establishment of major body axes in the evolution of metazoan bauplans has been an area of intensive research in cnidarian model taxa. Whether the cnidarian oral-aboral axis is homologous to the dorsal-ventral or anterior-posterior axes in bilaterians is a critical component of understanding early animal development. Members of genetic networks involved in patterning orthogonal body axes in bilaterians are also expressed asymmetrically in *Nematostella vectensis* (Dubuc et al 2018). The parallels between cnidarian and bilaterian major body axes, in terms of both gene expression and tissue layer definition, remain unclear (Dubuc et al 2018; reviewed in Rentzsch and Holstein 2018).

Although cnidarians appear to have less "complex" body plans than many bilaterian taxa, the suite of major developmental gene regulatory networks they possess is surprisingly sophisticated. For example, *Nematostella vectensis* expresses the entire suite of *Wnt* genes found in bilaterians (with the exception of *Wnt9*) (Lee et al 2006, reviewed in Rentzsch and Holstein 2016). Canonical Wnt signaling defines the oral pole in gastrulating *N. vectensis* embryos (it defines the posterior axis in bilaterians). It has been proposed that *Wnt* signaling, rather than *Hox* gene expression, regulates major body axis formation in cnidarians. However, whether cnidarian *Wnts* are mirroring bilaterian *Hox*-like functions in body axis specification is debated (Technau and Steele 2011).

Developmental processes in *Nematostella* have been well documented (Layden et al 2016). The species is dioecious, with males and females free spawning into the water column where fertilization and development occur. The female pronucleus marks the site of the animal pole, where cleavage is initiated. The animal pole will become the oral end of the developing anemone. Early cleavages are described as "chaotic" and are asynchronous between embryos (Fritzenwanker et al 2007). Cleavages produce uneven cell sizes, are initiated in a variety of cell

axes, and vary widely between individuals – cells can also re-fuse together, losing visible cell boundaries as they merge. After several rounds of cleavages, an organized spherical blastomere forms, though cells may still vary in size between individual embryos. Gastrulation is initiated at approximately 20 hours post-fertilization. Nematostella embryos, like other cnidarians, gastrulate at the animal pole – in contrast to bilaterians which gastrulate in the vegetal hemisphere of the embryo (Leclere et al 2016). Gastrulation in *Nematostella* occurs via invagination (Magie et al 2007), though cnidarians as a whole vary in the types of cellular movement that drive their modes of gastrulation. The bilaterian mesodermal gene *brachyury* is expressed in the "central ring" – the area marking the site of the oral pole – during gastrulation in *Nematostella*. When *Nv-brachyury* is disrupted, embryos still gastrulate but do not form a pharynx or organized endoderm, and development arrests (Servetnick et al 2017).

Though it has been proposed that *Hox* genes do not specify oral-aboral patterning in cnidarians (expression of *Hox* genes defines the anterior-posterior axis in bilaterians), *N. vectensis* does have several likely *Hox* genes (*H*ox1, *Hox2*, *Hox9*+), as well as a *Parahox* gene (Finnerty et al 2004; Ryan et al 2007; reviewed in Technau and Steele 2011). In *Nematostella*, cnidarian *Hox* genes are expressed either in endoderm or ectoderm, and some are localized to lateral hemispheres of the developing planula larva (Finnerty et al 2004; Ryan et al 2007).

Orthologs of the BMP family and chordins, which establish the doral-ventral axis in bilaterians, are also expressed asymmetrically in *Nematostella*. BMP2/4 and chordin orthologs are both expressed around the *Nematostella* gastropore, in contrast to bilaterian taxa in which expression of each form opposing gradients (Rentzsch et al 2006; Saina et al 2009). Later in development, the *Nematostella* BMP2/4 ortholog is expressed in the mesenteries, where it is thought to drive differentiation of retractor muscles in these structures (Finnerty et al., 2004; Saina and Technau, 2009). Though homology of axis formation in cnidarians vs. bilaterians is still an active arena of debate, both lineages deploy the same suite of gene regulatory networks to define body axes during development.

At approximately one day post-fertilization (24 hpf), the *Nematostella vectensis* gastrula organizes into an early planula larva. Planula larvae are ciliated oval-shaped, free-swimming, nonfeeding larvae with an apical sensory tuft at the aboral end (Chia and Koss 1979). Apical organs are common in invertebrate marine larvae (though the structures may not be homologous in cell-type composition or gene expression), and the planula swims in the direction of the tuft. It is adorable. It is possible that this apical tuft has a sensory function, and cells are patterned by expression of neuronal patterning genes – *Fox* and *FGF* (Rentzsch et al 2008). In *Nematostella*, neurons begin to develop from the ectoderm during the early gastrula stage. Like many bilaterian taxa, *Nematostella* embryos express neuronal-specific *Sox* genes in neural progenitor cells, as well as *achaete-scute* BHLH family homologs (Nakanashi et al 2011; Layden et al 2012). In the late gastrula, neurons arise in both endodermal and ectodermal tissue and express neuronal markers such as *Elav* (Nakanashi et al 2011), and it is thought that the mechanisms for neuronal specification occur similarly in both tissue layers (Layden et al 2016).

Cnidocytes (cnidarian-specific stinging cells) appear at approximately 48 hours post-fertilization (hpf), based on expression data, though cnidocysts are not yet present. Markers for neuronal progenitors also label cnidocyte precursors; for example, the paired-box gene *PaxA* is found in cnidocytes (Matus et al 2007) and disruption of the gene disrupts proper cnidocyte formation (Babonis and Martindale 2018). Cnidocytes are considered to be a neuronal derivative because they possess sensory mechanisms (chemical or mechanic stimuli) and their behavior is mediated by calcium flux (Kass-Simon et al 2002; Babonis and Martindale 2014). Cnidocytes are cell type unique to the phylum Cnidaria. Though cnidocyte-specific progenitor cells have yet to be identified in *Nematostella* (Babonis and Martindale 2014), it is known that their specification employs a number of classical neuronal pathways that are also present in neurons (e.g. Notch, SoxB, COUP-TP) (Galliot and Quiquand 2011; Marlow et al 2012) and evolutionarily novel genes, such as cnidarian-specific minicollagens (Beckmann and Ozbeck 2012).

During larvalgenesis, cell proliferation occurs throughout the animal (Fritz et al 2013). As the planula larva ages, the mesenteries form via continued inward migration of endoderm and ectoderm. The mesenteries are comprised of cells from both tissue layers. Tentacle tissue organizes at the oral pole of the planula, and four projections of tissue – tentacle buds – form around the site of the mouth. The tentacle buds and body column gradually elongate and the planula settles onto the substrate and metamorphoses into a polyp. Primary polyps initially possess only four tentacles (adults have 16-20) and two mesenteries (adults have 8). During larval development, the structures of *Nematostella* form in a predictable order: the mouth develops first, followed by synchronous development of the pharynx, tentacles, mesenteries, and physa.

The role of polysaccharides in animal development has not been widely explored. In this chapter, I examine whether chitin might play a developmental role in the sea anemone *Nematostella vectensis*. Using semi-quantitative PCR and *in-situ* hybridization, I show that the three Nv-CHS homologs are differentially expressed during development. The *Nematostella* genome contains three chitin synthase orthologs (see Chapter 2), each with its own expression pattern in adult tissues. We analyze gene expression of *chitin synthases* through development in wild type *Nematostella* embryos and larvae. The CRISPR/Cas9 system has recently been optimized for *Nematostella* (Ikmi et al 2014) and has been utilized to disrupt developmental genes like brachyury (Servetnick et al 2016). To explore the function of chitin in *Nematostella*, we disrupted genes coding for the enzyme that assembles the chitin molecule – chitin synthase – utilizing the CRISPR/Cas-9 system of genome editing. I show that disruption of chitin production leads to morphological defects in *Nematostella* larvae.

## 3.2 Chapter-Specific Methods

### *3.2.1. Spawning and sample collection*

Adult animals were maintained in 1/3 seawater and separated by sex based on prior

spawning events. To obtain a synchronous batch of developing embryos, timing of fertilization is critical, and mixed male/female populations do not allow for this. One day before spawning, adults were fed pieces of oyster, which is has a high nutritional content and helps induce spawning. After spawning was induced via a light-dark cycle, eggs were collected and fertilized. The fertilized egg masses were de-jellied with a 4% cysteine solution in 1/3 FSW. Zygotes were transferred to plastic dishes for microinjection or allowed to develop in 1/3 SW for later collection.

Embryos and larvae used for *in-situ* hybridization or chitin histochemistry were collected at specific time-points that coincide with developmental milestones (early planula, late planula, tentacle bud, primary polyp, etc). Animals were relaxed in 3% $MgCl_2$ in FSW for 15 minutes. Embryos and larvae were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in PTw for 1 min at room temperature, and then in 4% paraformaldehyde for 1 h at 4°C on a rotating platform. Fixative solution was removed and embryos were thoroughly washed in PTw, dehydrated into methanol and stored at -20°C to await further processing.

### *3.2.2. RT PCR and amplification of* CHS *genes*

Using 1 µg of cDNA, each *chitin synthase* gene was amplified from various life stages of *Nematostella vectensis* (1 dpf – 15 dpf, and adults) using semi-quantitative PCR (Table 3.1). Actin was also amplified to normalize band intensity and ensure that expression of CHSs could be compared between developmental stages. Band intensity was calculated using FIJI software (https://imagej.net/Fiji; Schindelin et al 2012) and graphed in Excel.

### *3.3.3. Guide RNA design*

Target sites for guide RNAs were identified using CCTop's genome browser (Stemmer et al. 2015; https://crispr.cos.uni-heidelberg.de/). This service was also used to identify potential off-target binding sites (OTSs) for the gRNAs. Guide RNAs with 20-base pairs and more than four OTSs were selected for each target gene (CHS1, CHS2, CHS3) to avoid nonspecific DNA editing.

With these templates, guide RNAs were synthesized by Synthego (Menlo Park, CA, USA). Three guide RNAs were designed for each gene; two sgRNAs to flank or disrupt the glycosyl transferase (GT) region, and one at the 3' UTR of the gene (targeting total gene excision) or the 3' end of the GT region (Table 3.S1; Fig. 3.S3-S5).

*3.3.4. Guide RNA microinjection*

While eggs were being fertilized, the guide RNA/Cas-9 mixture was prepared fresh for each gene target. Lyophilized Cas9 (PNA Bio, Thousand Oaks, CA) was reconstituted in 50% glycerol and 0.1 mM DTT. We injected a mixture containing gRNAs (total concentration 300 ng/µl, consisting of equal concentrations of each of the three gRNAs for each *CHS* gene), Cas9 (1 µg/µl), and dextran-red (0.2 µg/µl; Molecular Probes, Eugene, OR, USA). Embryos were injected in a chilled room as described previously (Layden et al. 2013; Servetnick et al 2017). In triple-knockdown samples, all nine guide RNAs were combined (3 sgRNAs per *CHS* gene) and injected. Control injections were performed; these conditions were Cas9 enzyme injected alone (with no sgRNAs), and sgRNAs injected alone (no Cas9).

*3.2.5. Analysis of genomic DNA from CRISPR-manipulated embryos and controls*

Individual embryos were allowed to develop for 24 hours before they were transferred to PCR tubes. Genomic DNA was extracted as described in the General Methods (Section 0.2). *Chitin synthase* genes were amplified from this genomic DNA using PCR primers that flanked the target regions (Table 3.S1; Fig. 3.S3-S6).

## 3.3 Results

*3.3.1. Genes for* chitin synthase *are differentially expressed during* Nematostella vectensis *development*

Total RNA show that transcript abundance for all three *CHS* genes increases through development, with the highest expression levels being in the primary polyp (15 days post-fertilization, or dpf) and adult (Figure 3.1, A-B). Chitin synthase-1 (Nemve1|93407) is faintly detectable at 24 hpf, and expression increases at 4dpf through adult stage. Chitin synthase-2 (Nemve1|104030) expression is detectable at 24hpf and gradually increases; this gene appears to have the lowest expression of the three *CHS* orthologs. Chitin synthase-3 (Nemve1|123712) expression is detectable at 48hpf, with strong expression through the rest of development and high expression levels in the adult. Expression of chitin synthase genes were normalized to actin expression (Figure 3.1A, bottom panel).

The *Nv-chitinsynthase1* ortholog is widely expressed in larval tissues

*In situ* hybridization of wild type embryos shows broad and dynamic mRNA expression of *CHS1* in both the endoderm and ectoderm of developing *N. vectensis* embryos and larvae (Fig. 3.2). This expression pattern is consistent with probe labeling in adult *Nematostella* tissues (Chapter 2), where chitin histochemical data also show the presence of chitin in diverse endo- and ectodermally-derived tissues. Expression is first detected in the early gastrula stage (Fig. 3.2A-C). Individual cells can be seen expressing *CHS1* in the early gastrula (Fig. 3.2B). As the gastrulation continues, *CHS1* is expressed in overlapping endodermal and ectodermal domains (panels C-I). *CHS1* is expressed in the ectodermal portion of the pharynx, (panels D-F) and in the body wall at mid-planula stage (panels F-G). By late planula stage, expression is concentrated at the oral pole, with strong expression around the body wall (Fig. 3.2H). During tentacle bud stage, expression is concentrated at the tentacle emergence zone, at the aboral-most pole of the larva, and at the distal end of the pharynx (Fig. 3.2I). Once metamorphosis has completed in early polyps, *Nv-CHS1* expression concentrates to the lateral endoderm of the mesenteries (Fig. 3.2J-K). However, by primary polyp stage expression boundaries appear to expand throughout the endoderm (Fig. 3.2K).

It is unclear what cell type is expressing *CHS-1* or synthesizing chitin in these tissues. Sense probe (negative controls) for all *CHS* genes showed no specific labeling and low background (Fig. 3.S1).

The *Nv-chitinsynthase2* gene is expressed in developing cnidocytes

*Nv-CHS2* appears to be expressed in cnidocyte precursors in developing larvae and polyps (Fig. 3.3 A-H, Fig 3.S2). Spatial mRNA expression of *Nv-CHS2* is first detectable at approximately 96 hpf, where it is expressed in a punctate pattern across the ectoderm of the early planula (Fig. 3.3 A-B). *Nv-CHS2* has similar expression patterns to cnidocyte markers such as *minicollagen-3* and *PaxA*; punctate labeling reveals cells in the process of synthesizing components of cnidocysts. In the late planula stage, *CHS2*-positve cells begin to either migrate to the oral pole or may differentiate there (Fig. 3.3 C-D). Individual cells expressing *CHS2* can be seen in the ectoderm throughout the larva, with expression concentrated at the oral pole (the future site of the tentacles). At the tentacle bud stage, no trunk expression is seen and *CHS2*-positive cells are exclusively localized to the four developing tentacles (panels E-F). Primary polyps show expression in the ectoderm of the tentacles (Fig. 3.3G-H). Cnidocytes are heavily concentrated in this region.

*Nv-chitinsynthase3* has widespread ectodermal expression

*In situ* hybridization of wild type embryos shows that *Nv-CHS-3* has broad expression in most ectodermal tissues (Fig. 3.4 A-C). Expression is detectable in early planula larvae (Fig. 3.4 A,A') and remains highly expressed throughout development. Punctate labeling can be seen in shallow views of early planula tissue (panel A'), showing expression in individual cells. In the late planula, there is widespread expression throughout the ectoderm, with expression concentrated at the oral pole (Fig. 3.4 B,B'). In tentacle bud larvae, expression concentrates at the oral end, where strong signal is observed under the developing tentacle bulbs, and at the aboral pole (Fig. 3.4 C,C'). A

mesh-like network of expression can be seen on the surface ectoderm of tentacle bug larvae (Fig. 3.4 C').

*3.3.4. Chitin labeling is distributed throughout the developing planula and primary polyp*

Chitin is not detectable by histochemistry prior to late planula stage (approximately 144 hpf; Fig. 3.5A), possibly due to the reduced sensitivity of the CBD probe compared to other detection methods we have applied to assay chitin synthesis (RT PCR, *in situ* hybridization). In planula stages, the CBD probe labels extracellular areas and scattered cells (Fig. 3.5A, B). Chitin labeling is prevalent in the endoderm of the body wall and in the pharynx (ectoderm) of the tentacle bud larva (Fig. 3.5C, C'). There is concentrated chitin labeling under the budding tentacles (Fig. 3.5C, arrows). There is widespread chitin labeling in the endodermal and ectodermal tissues of primary polyps (Fig. 3.5D, D'); this labeling pattern is similar to that seen in adult *Nematostella* tissues, where chitin labeling was fairly ubiquitous in mature animals (see Chapter 2).

*3.3.5 Disruption of chitin synthesis results in disorganized tissue and abnormal development*

We disrupted genomic sequences for each of the three *chitin synthase* (*CHS*) genes encoded in the *N. vectensis* genome in two separate experiments: single-gene disruptions for which three guide RNAs (sgRNAs) were deployed per gene (example: CHS1-3sgRNA/Cas9) or disruption of all three *CHS* genes utilizing the guide RNAs for each gene together (9sgRNA/Cas9). We opted to use three guide-RNAs per *chitin synthase* gene: one sgRNA in the 3' UTR, which also flanks the enzymatically active site – the glycosyl transferase (GT) region; one sgRNA targeting the 5' side of the GT region; one sgRNA targeting the 5' UTR. Genome editing of each *chitin synthase* ortholog was confirmed with PCR amplification of target gene areas for wild type controls and knock-down samples (sgRNA/Cas-9 injected, N = 16) (Supp. Fig. S3.3, S3.4, S3.5). Amplification of actin was used as a positive control for genomic DNA quality, and almost all samples amplified normally (Fig. S3.6). In *CHS* triple-gene knockdowns, our target was to remove

or severely limit the animals' ability to synthesize any chitin.

Morphological and gene expression analyses of *CHS*-sgRNA/Cas9-injected *Nematostella*

**Disruption of chitin synthesis leads to morphological defects in *N. vectensis* larvae and polyps**

Individuals that had been injected with the cocktail of guide RNAs targeting all three *chitin synthase* genes (9sgRNA/Cas9) have a high prevalence of disorganized anatomy, including reduced or absent tentacles, poor body column structure, and deformity of the mesenteries (Fig. 3.6). Some sgRNA/Cas-9 polyps display wild type morphology (e.g. injected sample in Fig. 3.6A, A'), while others show severe anatomical deformities (Fig. 3.6B-E). Some samples produced structures that appear normal (a tentacle in Fig. 3.6B) in an otherwise highly disorganized polyp. In some samples, it was difficult to discern the polarity of the animal or identify specific morphological structures (Fig. 3.6E). Out of 82 triple CHS knock-down (9sgRNA/Cas9) polyps examined, 31% of individuals had broadly wild type morphology (N=26) and 69% had obvious physical anomalies (N=56). Chitin labeling in 9sgRNA/Cas9 samples is reduced compared to wild type but is not eliminated entirely. This could be due to mosaicism – the phenomenon in which some embryonic cells are affected by gene editing and others are not. Mosaicism of genotype or phenotype was expected in a least some individuals due to the lag in Cas9 cleaving of genomic DNA during progressive, rapid cell divisions in the early embryo.

The F0 population (individuals what had been injected with 3sgRNAS/Cas9) did not survive in culture beyond 6 weeks post-fertilization. Some larvae did not develop into polyps, or polyps that displayed abnormal tentacle or mesentery morphology. Young polyps seemed to be unable to feed when presented with a slurry of shrimp and eventually died. *Nematostella vectensis* larvae raised in the chitin synthesis-inhibiting pesticide diflubenzuron (20 µM/mL in 1/3 FSW) also display similar abnormal morphologies. The pesticide acts at the protein level, prohibiting proper function of the chitin synthase enzyme. Animals raised with this drug do not elongate into the planula shape (Fig. S3.7 B-C) and the larvae do not swim. Similar effects were observed in

embryos injected with a morpholino targeting *CHS-1* (data not shown).

**Expression analyses of single-gene-targeted knockdowns**

*Chitin synthase-1* single-gene knockdown larvae show delayed development and ectopic or reduced expression of *Nv-CHS1* (Fig. 3.7A-F) compared to wild type larvae (see Fig. 3.2 for comparison). Expression is increased in some samples (panels A-E), possibly due to upregulation of the *CHS1* gene as a feedback result of mutant mRNA transcripts failing to produce a viable chitin synthase enzyme (protein product). Internal structures appear to be disorganized (panels C,D). Mesenteries look abnormal in some samples (panel D). Expression of *minicollagen-3* (*mCol3*), a universal cnidocyte marker, was used as a metric for ectodermal patterning, cell differentiation, and cell migration. In *CHS1*-3sgRNAS/Cas9 samples, *mCol3* expression appeared to be broadly normal (Fig. 3.7 H-J). Late planula larva show punctate expression throughout the ectoderm (panels H, H'). Tentacle bud individuals also displayed wild type expression, with scattered cnidocyte expression throughout the body column and concentrated labeling at the oral pole in the tentacle buds (panels I-J).

Excision of *Nv-CHS2* disrupts development of some cnidocyte populations. In CHS2-3sgRNA/Cas9 single-gene knockdown larvae, expression boundaries of *NvCHS2* are not well defined (Fig. 3.8A-E). Some punctate expression can be seen in a subset of samples (e.g. Fig. 3.7A'), though in contrast to wild type samples expression is also observed in endodermal cells (compare to late planula in Fig. 3.3). Ectopic expression of *Nv-CHS2*, which is expressed exclusively in the ectoderm in wild type animals, may be due either to ectopic gene expression in cells which do not normally express the gene or, more likely, due to the inability of cells that do express the gene to properly differentiate or migrate. *MCol3* expression is disrupted in many CHS2-3sgRNA/Cas9 mutants (Fig. 3.8F-K), with some individuals displaying mosaic *mCol3* expression, with no labeling in some portions of the trunk (Fig. 3.8 G, I). Some larva lack expression entirely (panel H). Many larvae displayed morphological abnormalities (e.g. panels H,

K) though a worm-like larva with no tentacles maintained a normal expression pattern (Fig. 3.8K).

CHS3-3sgRNA/Cas9 single-gene knockdown larvae expression is localized to endodermal tissue (Fig. 3.9 A-F); in wild type larvae, *CHS3* expression is seen exclusively in the ectoderm (Fig. 3.4, all panels). Some individuals show delayed development (panels A-D, F) and internal structures appear to be disorganized (A,D) or truncated (short mesenteries in panel B). *Minicollagen-3* expression appears to be broadly normal (Fig. 3.9 G-H).

**Analyses of expression patterns of *chitin synthase*s in triple-knockdown 9sgRNA/Cas9 larvae**

Abnormal expression patterns of all three *Nv-CHS* genes were observed in triple-knockdown embryos and larvae, indicating that these genes and chitin synthesis were broadly disrupted. Larvae collected at 144hpf and assayed for *CHS1* expression show delayed development – gastrula or early planula mutants vs. late planula/tentacle bud in wild type at that timepoint (Fig. 3.10 A-I). Expression is seen in the ectoderm and endoderm of some samples (panels A-E), though expression boundaries are not well defined, and some larva are displaying morphological abnormalities (e.g. panels C, E, F). Some larva showed no probe labeling (panels G-H) and seem to have arrested in early development. Some individuals displayed normal gastrula-stage expression (Fig. 3.10 I).

Expression of *Nv-CHS2* is depleted or disorganized in 9sgRNA/Cas9 samples (Fig. 3.11 A-F). Samples were fixed at 240hpf and developing anemones should be in the late planula or tentacle bud stage at this timepoint, though many 9sgRNA/Cas9 larvae display delayed or arrested development. Cells expressing *Nv-CHS2* are localized to the endoderm in some individuals (panels A, D); expression is punctate and exclusive to ectodermal tissues in wild type larvae (Fig. 3.3, all developmental stages). Panel C shows an individual that is expressing *CHS2* in a punctate pattern, but it is distributed throughout the body column rather than localizing to the oral end as it does in wild type tentacle bud larvae (Fig. 3.11 C, compare to Fig. 3.3). *CHS2*

expression is nearly absent in some individuals (e.g. Fig. 3.8E). Similar ectopic expression was seen in larvae with single-gene knockdowns for *Nv-CHS2* (Fig. 3.8).

Triple *CHS* knockdown larvae also show ectopic or reduced expression of *Nv-CHS3* compared to wild type (Fig. 3.12 A-L). Uninjected individuals have wild type expression in the ectoderm of the trunk, physal region (aboral pole), and tentacle buds (panel A). Many 9sgRNA/Cas9 individuals display a variety of morphological defects including reduced numbers of tentacles or a short trunk, although expression patterns appear broadly normal in the trunk (panels B-D). Many individuals show delayed developmental progress (Fig. 3.12 F-L), and depleted or absent expression of *CHS3*.

### *3.3.6. Disruption of* chitin synthase *leads to aberrations in* minicollagen-3 *expression, and disrupts spirocyst production*

*Minicollagen-3* (Nv-mCol3) is a cnidocyte marker and is expressed in both nematocysts and spirocysts in wild type larvae (Fig. 3.S8). Triple-knockdown planula larvae show reduced or disorganized expression of cnidocyte marker *mCol3* (Fig. 3.13). There is reduced or mosaic expression in the body wall compared to wild type (panel A), with scattered cells expressing *mCol3* but others tissue areas lacking labeling (panels B-H). While some 9sgRNA/Cas9 samples display limited *Nv-mCol3* expression in the tentacles of primary polyps (Fig. 3.14), spirocysts were not observed in the tentacles of 9sgRNA/Cas9 samples (Fig. 3.14). This data suggests an essential role for chitin synthesis in the production of these organelles. Though there are some nematocysts visible in the tentacle tip (Fig. 3.14), spirocysts are not visible. Cnidocyst abundance was generally depleted in the tentacle of 9sgRNA/Cas9 polyps (Fig. 3.14A-B).

## 3.4 Discussion

The presence of the glycomolecule chitin has not been previously described in the sea anemone *Nematostella vectensis,* and thus its role in the biological processes of this species are

undescribed. This species does not contain rigid anatomical structures that would be obviously chitinous; however, we have shown that the chitin molecule is present in diverse tissues throughout the adult anemone, and that *N. vectensis* possesses the molecular toolkit to produce chitin (see Chapter 2). Here we show that genes for the enzyme responsible for synthesizing the chitin molecule (*chitin synthase*) are differentially expressed during *N. vectensis* development. Intriguingly, disruption of chitin production in *Nematostella* (with genetic manipulation or by chemical means) causes morphological defects, loss of cnidocyte populations, and abnormal cell migration.

Given the widespread expression of *CHS1* and *CHS3* in *Nematostella* tissues, the broad distribution of chitin itself, the morphological abnormalities displayed by knockdowns, it is possible that chitin has a role in the extracellular matrix (ECM) of these animals. While some studies conflate cnidarian mesoglea with extracellular matrix (Tucker et al 2011), these two areas have distinct molecular compositions and cellular associations. Extracellular matrices are amalgams of proteins and other biomolecules secreted by epithelial cells into the extracellular environment; ECMs provide structural support to overlaying cells and are also involved in intracellular or other chemical signaling. The *Nematostella vectensis* ECM has been proposed to contain laminin, thrombospondins, heparan sulfate proteoglycan 2 (HSPG2)/perlecan, and collagen IV (Tucker et al 2011; Tucker et al 2012; Warren et al 2015). *Nematostella* has four *thrombospondin* orthologs, each with a distinct but widespread pattern of expression in the ECM of endodermal and ectodermal tissues and three of which have predicted integrin binding capabilities (Tucker et al 2012).

The mesoglea in *N. vectensis* is less voluminous than in other cnidarians (e.g. scyphozoan bells) and is located between endodermal and ectodermal tissues (Layden et al 2016). *Nematostella* mesoglea contains ECM components as well as neurons and migratory amoebocytes of unknown function (Tucker et al 2011; Nakanishi et al 2011). The *Nematostella CHS3* paralog has wide expression in adult and larval ectoderm, similar to *perlecan* expression

domains (Warren et al 2015). *CHS1* is widely but dynamically expressed in developing anemones, with both ectodermal and endodermal expression in some developmental stages, before becoming localized to the endoderm of the primary polyp mesentery. It is unclear whether distinct cell types are expressing *CHS* genes or ECM genes in *Nematostella*, as many cells appear to be multifunctional, particularly in the interior of the body wall and in the gastrodermis (Tucker et al 2011).

We find that *CHS2* has a distinct expression boundary in *Nematostella* and has similar patterns of expression to other cnidocyte markers both through development and in adults. Cnidocyte development takes place continuously in the ectodermal tissues of *N. vectensis* (Babonis and Martindale 2017), with synthesis of cnidae occurring in the tentacle dermis, pharynx, body wall, and ectodermally-derived mesentery tissue. Babonis and Martindale also discovered that cnidocytes are present at the aboral-most pole of the polyp, termed the physal pore. Cnidocytes differentiate and begin to appear early in development (late gastrula stage), with mature fully-formed nematocysts detectable at late planula stages (Babonis and Martindale 2017). *Minicollagen-3* (mCol3, a cnidarian-specific collagen) is a universal cnidocyte marker (Zenkert et al 2011; Babonis and Martindale 2017) and is expressed in developing cnidocytes in the early planula – well before cnidocysts themselves are visible.

Expression of *Nv-mCol3* is scattered throughout the body column through development, but later in development minicollagen-1 and minicollagen-4 expression concentrates in the tentacle region at the oral pole of the larva (Zenkert et al 2011; Babonis and Martindale). In planula larva, bastitrich nematocysts comprise the majority of stinging cells (90%) (Zenkert et al 2011). Given the high level of *chitin synthase-2* expression in early planula larvae, it is possible that *Nv-CHS2* is expressed in most cnidocytes at that stage. *Nv-mCol3* is expressed throughout the ectoderm of early planula larva in a punctate pattern similar to *Nv-CHS2* expression. Later in development, *Nv-CHS2* expression localizes to the tentacle buds, indicating that this gene (and its enzyme product) becomes involved in the synthesis of a specific cnidocyst type. It is plausible

that a *chitin synthase-2* is expressed specifically by spirocytes later in development and into adult stages; fluorescent chitin histochemistry labeled these populations of cnidocysts specifically, and expression of *Nv-CHS2* corresponds with spirocyst-specific mCol4 gene expression (Zenkert et al 2011). These results confirm data from chitin affinity histochemistry performed in Chapter 2, in which the fluorescent chitin probe labeled the tubules of spirocysts in *N. vectensis* but no component of nematocysts (capsules, spines, or tubules) (Fig. 2.7).

*Chitin synthase* triple knock-down and *Nv-CHS2* Cas9-treated samples show reduced or disorganized distribution of *Nv-mCol3*, indicating that cnidocyte cell migration or cell type differentiation requires *chitin synthase* or the presence of chitin itself. Histochemical chitin labeling shows that chitin is widely distributed throughout endodermal and ectodermal tissues of *Nematostella* larvae and adults. The disruption of *chitin synthase* genes, particularly when all three paralogs are targeted simultaneously, results in reduced chitin production in the larva and widespread morphological defects. Given the diversity of expression patterns observed in developing anemones, it is likely that the *chitin synthese*s present in the *N. vectensis* genome have diverged to serve different functions in the animal. Protein-level disruption of chitin synthase enzymes using pharmacological drugs (e.g. the chitin synthase inhibitor diflubenzuron) also results in widespread morphological abnormalities. This drug should target all isoforms of chitin synthase enzymes in the anemone, and morphological effects of this drug are similar to what was observed in triple *CHS* knockdown samples.

Although *Nematostella vectensis* is a commonly-used model cnidarian, its cellular biology has not been well characterized, and cell culture techniques are not established for this taxon. It is thus unclear what cell types are expressing *chitin synthase*s or producing chitin, or to what chitin is complexed in the extracellular environment. Given the pattern of chitin labeling in *Nematostella*, it is possible that chitin is an integral component of the extracellular matrix in these animals. Hyaluronic acid (HA), another glycomolecule, is a major element of the extracellular matrix in vertebrates and is molecularly similar to chitin (Morganti 2015). Hyaluronan is composed

of alternating units of β1-4-linked GlcNAc and β1-3-linked glucuronic acid; like chitin, it is assembled with UDP-based donor nucleotides. It was demonstrated that the enzyme hyaluronan synthase is able to synthesize chitin molecules when provided with GlcNAc-UDP molecules (Weigel et al 2017). The tails and trunks of zebrafish (*Danio rerio*) fail to properly elongate when chitin production is disrupted (Tang et al 2015). Given our findings that chitin is widely distributed throughout the extracellular matrix (ECM)/mesoglea of cnidarians, it is possible that chitin is complexed with structural proteins to stabilize the ECM, similar to the function of chitin in the insect peritrophic membranes (reviewed in Hegedus et al 2009). Transcriptomic data from regenerating *Nematostella* show that *chitin synthase-1* (*Nv-CHS1*) is dramatically upregulated (Schaffer et al 2016), though the presence of chitin was not demonstrated in that study. Mucins, another type of glycomolecule, are also upregulated during *Nematostella* regeneration. Chitin may therefore serve as a potential stabilizing molecule for mucosal ECMs.

The role of glycopolysaccharides in animal development and regeneration has not been widely explored, particularly in non-arthropod lineages. Though the sea anemone lacks hard skeletal structures, expression assays and functional experiments demonstrate that chitin and its assembly enzyme *chitin synthase* have essential roles in the development and maintenance of *Nematostella vectensis* tissues. It is unclear what other molecules chitin may be complexed with *Nematostella* extracellular matrix or other structures. We show here that chitin has an essential role in the proper formation of the evolutionarily novel cnidarian stinging cell, and although *N. vectensis* is soft-bodied, the chitin molecule serves a structural function. Chitin's function in developmental processes is not well understood however prior work suggests that glycopolymers may have a widespread role in cell behavior and migration. The expression of *chitin synthase* and detection of chitin in cnidarian stinging cells and soft tissues suggests an expanded role for chitin in animals.

## 3.5 Chapter 3 Tables

Table 3.1 – Primers used for *Nematostella vectensis* RT PCR gene amplification and/or subsequent RNA probe synthesis

All primer sequences are listed 5'-3'.

| Gene | Primer Sequence |
|---|---|
| Actin | F- CTATCCAGGCCGTACTCTCCC |
| | R- TAGTGGAACCACCAGACAAGA |
| CHS-1 | F- TTCATGGTGGCTGCACTGAT |
| | R- CACTCGGCTCCGTATAGCTG |
| | F- CCTCGTCCGTGACGATCAAA |
| | R- GTACACCATACCGCCCACAA |
| | F- GTACGGGCTCATTTGCCTCT |
| | R- TCTGCGGTAGAGTGTTACGC |
| | F- GCTGGCGTTTGATGTACTGC |
| | R- TTGTAGACACGGTGGGTGTG |
| CHS-2 | F- GATGCAGTTCAGGTGGCGTC |
| | R- GTTGTCACACCGGTAGCGT |
| | F- GGCTGCGCGAAATTCTCTAAA |
| | R- CAGCCCTTCCCAGAAATCAAA |
| | F- ATTTGGAAAAGAGGGAGGCTG |
| | R- ATGATACTCCGTCTGCAACTACT |
| CHS-3 | F- ACCGGAGTTACCCATCCAGA |
| | R- GCTGATTTGCCTCGTGCATT |
| | F- TCCTTCGATCGTGTTGGGTG |
| | R- GACTAGTGCCAGCGAGACAG |
| | F- CTGGGAATACTTCGTGCGGA |
| | R- TGGAGCTTTGCTCAGTCGAG |

## 3.6. Chapter 3 Figures

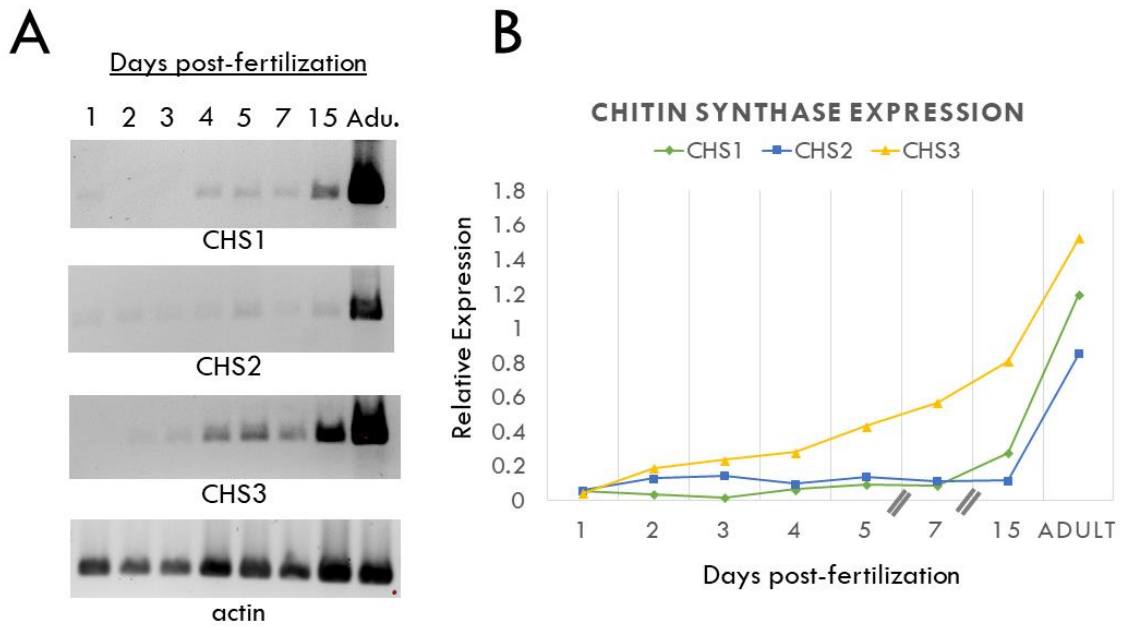


**Figure 3.1** – **Semi-quantitative RT PCR expression of the three predicted *chitin synthase* genes in *Nematostella vectensis* during development.** A.) Electrophoresis gels showing amplified *Nv-CHS* genes during *Nematostella vectensis* development. Temporal expression profiles of each *Nematostella chitin synthase* ortholog through development (1 day-post fertilization through 15 days-post fertilization), as determined by RT-PCR. Concentrations of cDNA were normalized, and *actin* was amplified as a control for expression. *Nv-CHS3* is expressed throughout development at high levels compared to other orthologs. Adu. – adult stage. B.) Normalized semi-quantitative PCR expression of three predicted *chitin synthase* genes during *Nematostella vectensis* development. *Nv-CHS* expression was normalized to actin expression using Image J. Raw quantitative data may be found in the chapter Supplement.

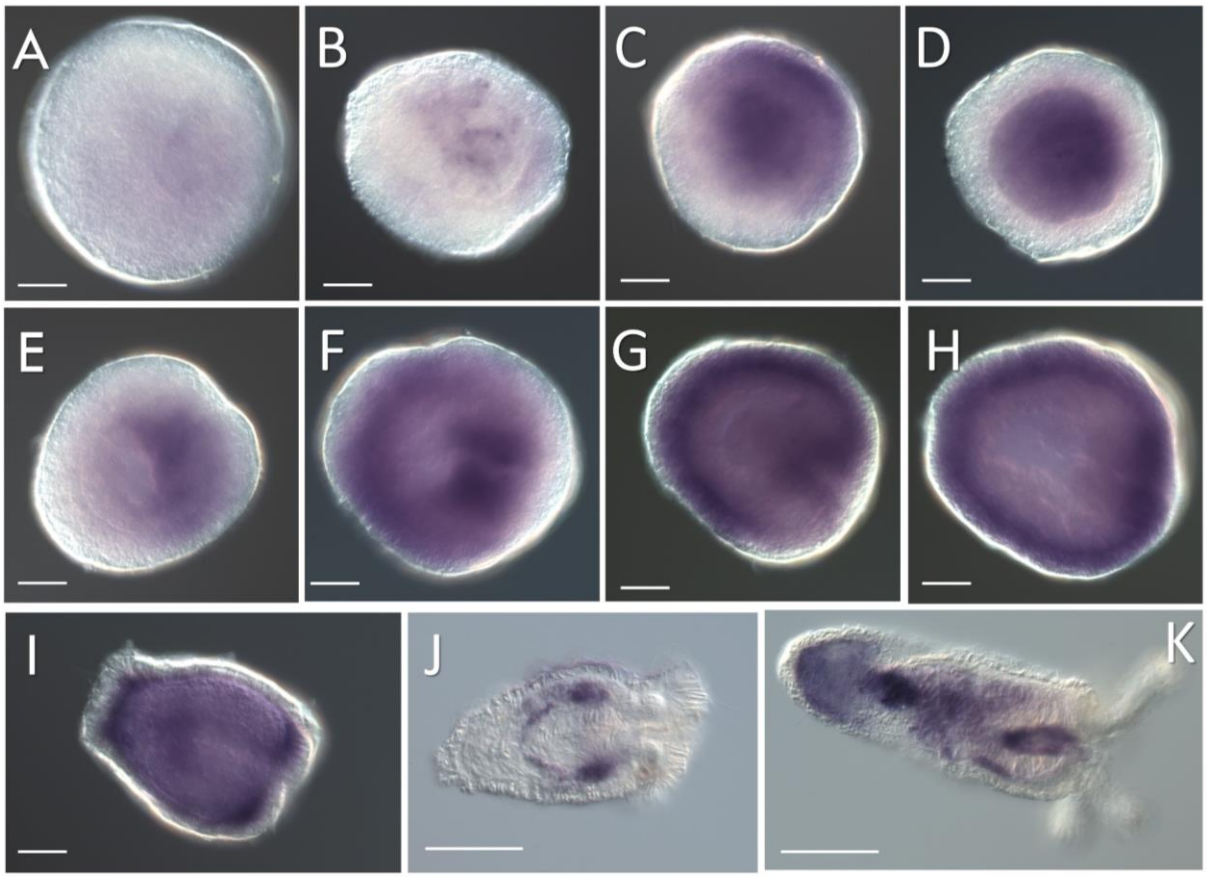


**Figure 3.2 – Wild type expression of *Nv-CHS1*.** Spatial expression patterns of *chitin synthase-1* ortholog as determined by *in situ* hybridization. A.) Expression is not detected in the blastula. B.) Individual cells can be seen expressing *CHS1* in the early gastrula. C.) and D.) show sequential development of the late gastrula. E.) & F.) Early planula larva express *CHS1* in the endoderm of the body wall and pharynx. G.) Mid-planula larva showing widespread endodermal staining. H.) Late planula showing expression across the body wall and concentration of expression at the oral pole. I.) Expression is concentrated in the aboral pole, at the tentacle emergence boundary, and some mesentery endoderm in the tentacle bud stage J.) After metamorphosis, the early primary polyp showing expression of *CHS1* in the lateral portion of the endoderm in the developing mesenteries. K.) Endodermal tissues of the directive mesenteries show expression (facing out).

Scale bar is 50µm in panels A-I; scale bar is 100 µm in panels J-K.

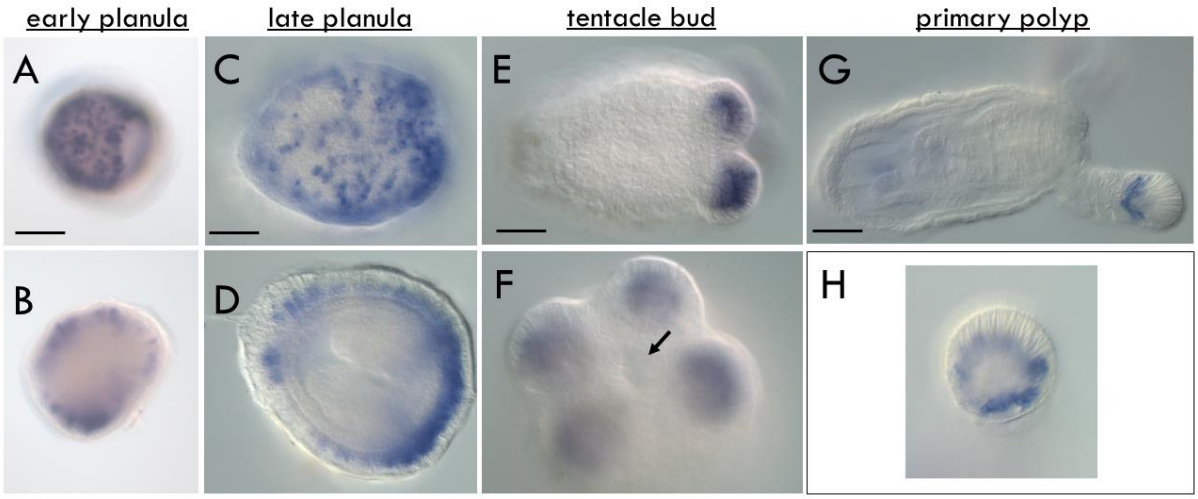


**Figure 3.3 – Wild type expression of *Nv-CHS-2*.** Spatial expression patterns of *chitin synthase-2* ortholog as determined by in situ hybridization. A-G: Lateral views, mouth to the right, apical pole to the left. A.) *Nv-CHS2* expression is first detectable in the early planula, with widespread punctate labeling in the ectoderm (surface view). B.) Deep plane view of early planula. C.) Surface view of late planula, with widespread expression throughout the ectoderm, though expression is beginning to concentrate around the oral region. D.) Deep plane view of *Nv-CHS2* expression. E.) Lateral view of a tentacle bud larva, with expression localized to the developing tentacles. F.) Oral view of tentacle buds (4) showing expression. Arrow points to the mouth. G.) Expression is localized to the tentacle tips in the primary polyp. H.) Frontal view of a tentacle tip, showing individual cells expressing *Nv-CHS2*.

Scale bar is 50µm in all panels.

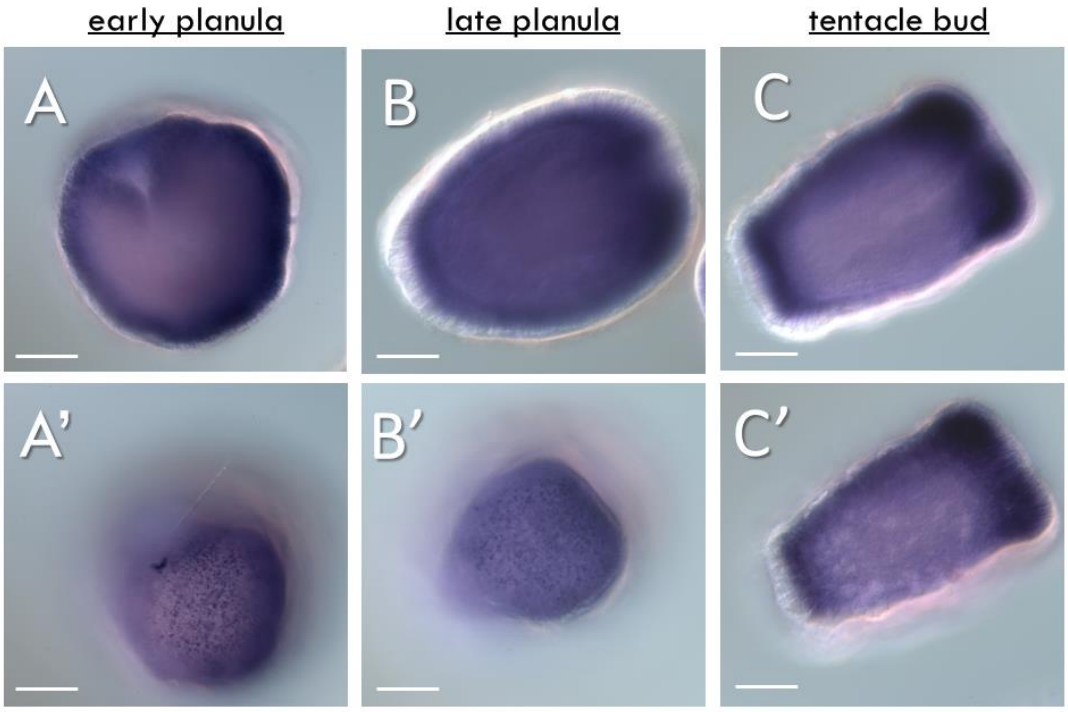


**Figure 3.4 – Wild type expression of *Nv-CHS-3* through *N. vectensis* development**. Deep-tissue views (plain letters) and surface tissue (marked with an apostrophe) of the same larva. A.) Broad expression is seen in early planula larvae and is localized to the ectoderm. A' shows dermal tissue, and individual cells (punctate labeling) are visible. B.) Expression begins to concentrate at the oral pole of the planula (facing right), but expression remains throughout the ectoderm. Individual cells are visibly expressing *CHS3* (B'). C.) Expression has condensed in the oral and aboral poles of the tentacle bud larva, though broad expression remains throughout the ectoderm. Individual cells are no longer visible, though a web-like network can be seen in shallow tissue views (C').

Scale bar is 50µm in all panels.

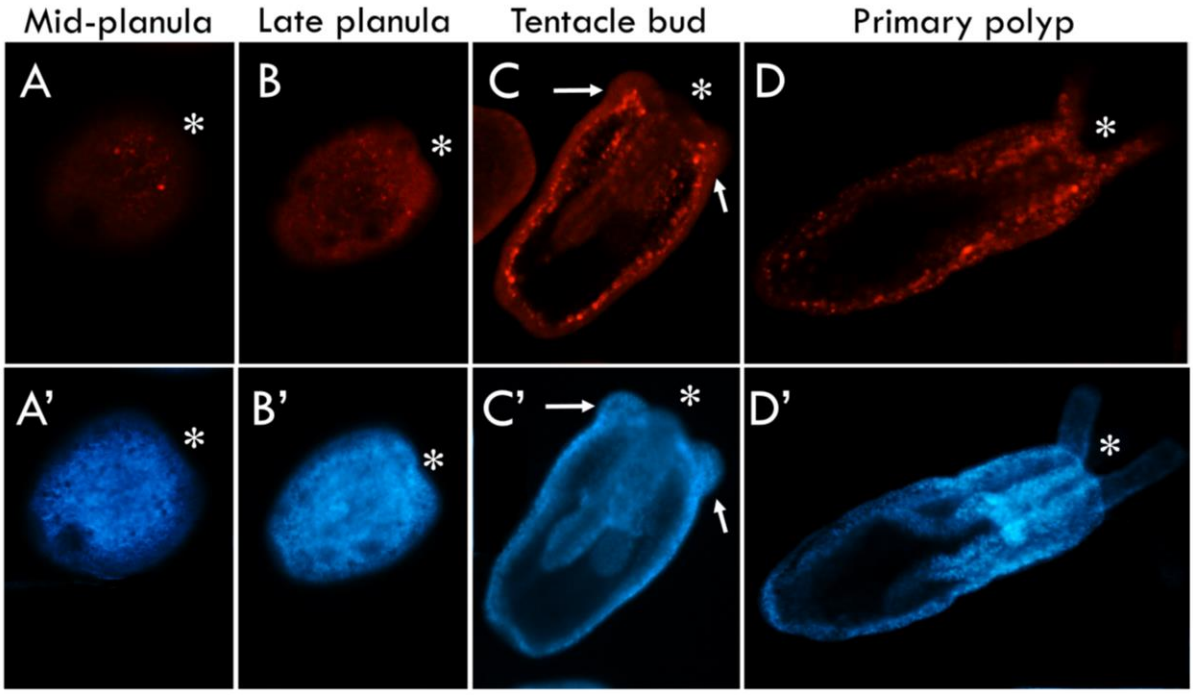


**Figure 3.5 – Chitin histochemistry in wild type *Nematostella* development.** Confocal images of chitin labeling at sequential developmental stages. All panels – chitin (red, chitin binding domain CBD-546 probe), nuclei (‘) (blue, Hoechst). Scattered cells or areas of extracellular tissue label with chitin in early larval stages (A, B). In the tentacle bud stage (C, C’) chitin labeling is abundant in the endoderm along the body wall and beneath the budding tentacles (arrows). Chitin is widely distributed in the primary polyp stage (D, D’) in both endodermal and ectodermal tissues. Oral end labeled with an asterisk (*).

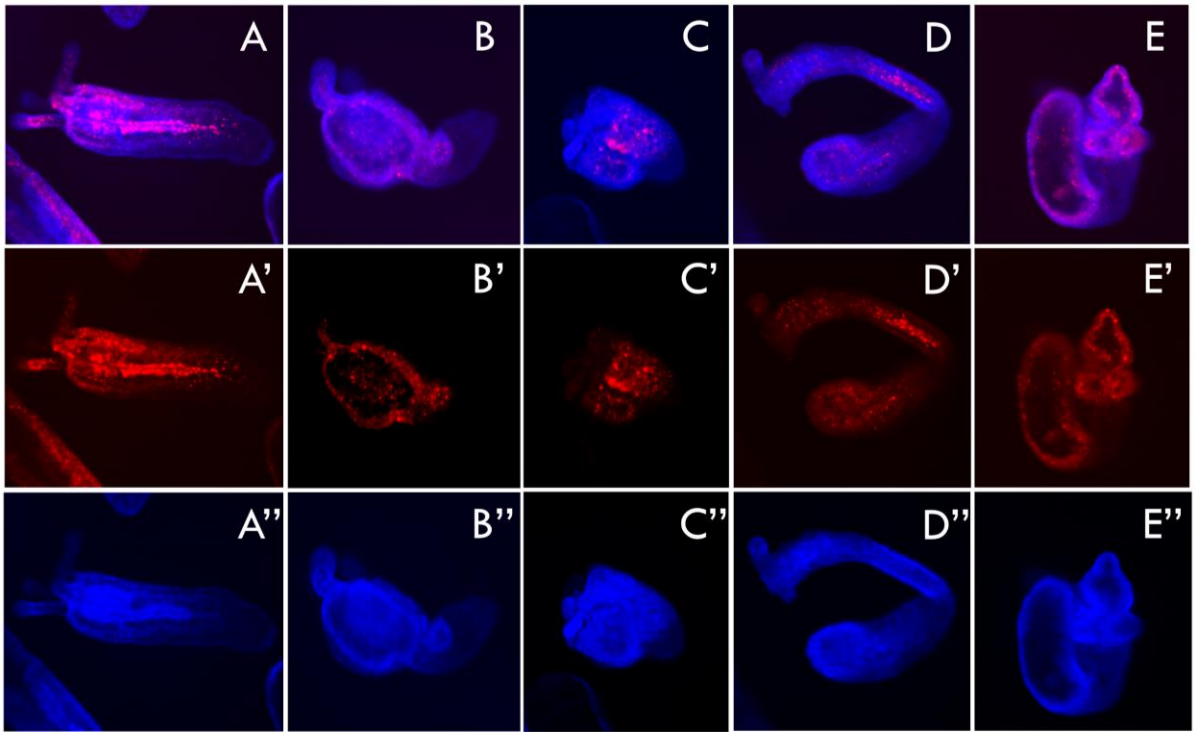


**Figure 3.6 – Chitin histochemistry in CRISPR/Cas9 9sgRNA triple-knockdown polyps.** Confocal images. In all panels, (X) is merged image, X' is chitin labeling (red, CBD-546), and X'' is nuclear staining (blue, Hoechst). Tentacles, where present, pointing to the left. A.) Wild type polyp exhibiting normal chitin staining patterns and displaying normal morphology (four primary tentacles, two mesenteries, fusiform body column). B.) – E.) 9sgRNA-injected knockdowns. Some chitin labeling is present but reduced compared to wild type. Individuals are displaying a variety of morphological defects including reduced or absent tentacles, poorly-formed or absent mesenteries, and disorganized trunk tissue.

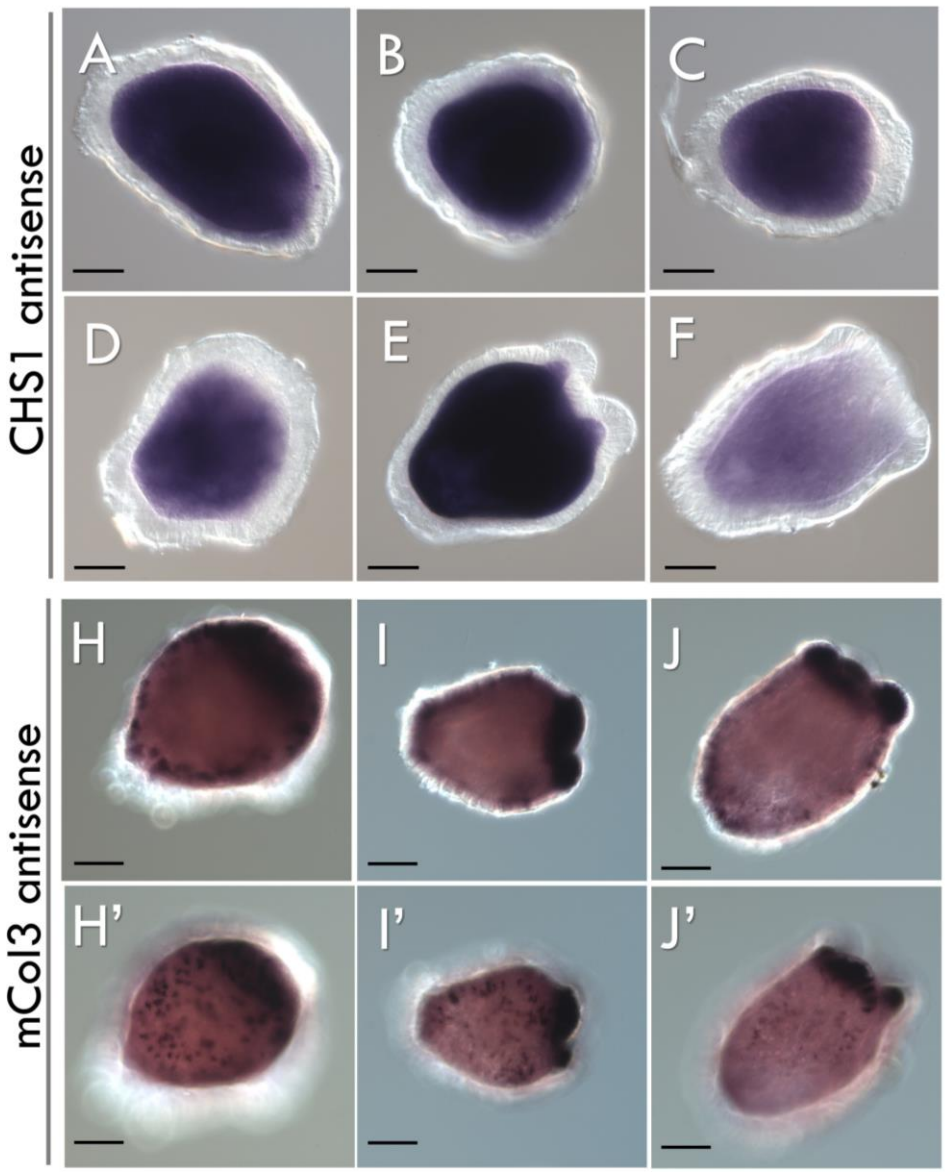


**Figure 3.7 – Varieties of expression of *chitin synthase-1* and *mCol-3* in NvCHS1-3sgRNA/Cas9 larvae.** All panels are 240 hpf. A.) – F.) Individuals showing delayed development (panels A-D) and disorganized expression in the endoderm. Expression levels appear to be high. F.) Tentacle bud phase showing disperse staining. H.) – J.) Deep plane (top row) and shallow plane views (bottom row) of *minicollagen-3* expression. Though some individuals are displaying morphological defects (panel J.), *mCol-3* expression appears normal.

Scale bar is 50µm in all panels.

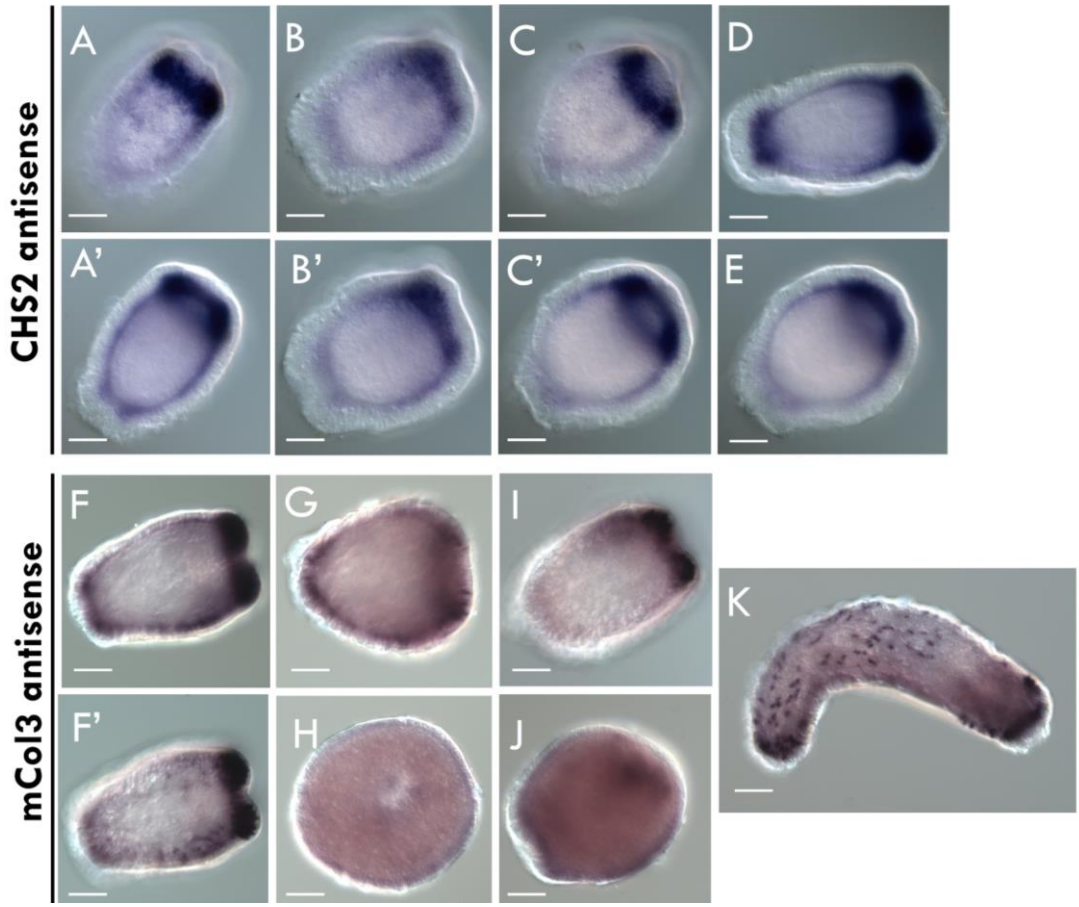


**Figure 3.8 – Varieties of expression of *chitin synthase-2* and *mCol-3* in NvCHS2-3sgRNA/Cas9 larvae.** *CHS-2* samples (top) are 240 hpf, *mCol3* samples (bottom panels) are 96 hpf. A-C shallow plane view of the ectoderm. Some punctate labeling is visible, though it is disorganized compared to wild type. A-C', E-F) Deep plane views of larvae; there is no punctate labeling and expression boundaries are not well defined. F-F'.) An injected individual displaying broadly wild type expression of minicollagen. G. – J.) Larvae in various stages of development; pools of injected larvae were not synchronous in development, and it appears that some have arrested (H.). G.) and I.) appear to be displaying mosaicism for *mCol3* expression. K.) A larva with disorganized morphology and no tentacles, though *mCol3* expression appears to be intact.

Scale bar is 50µm in all panels.

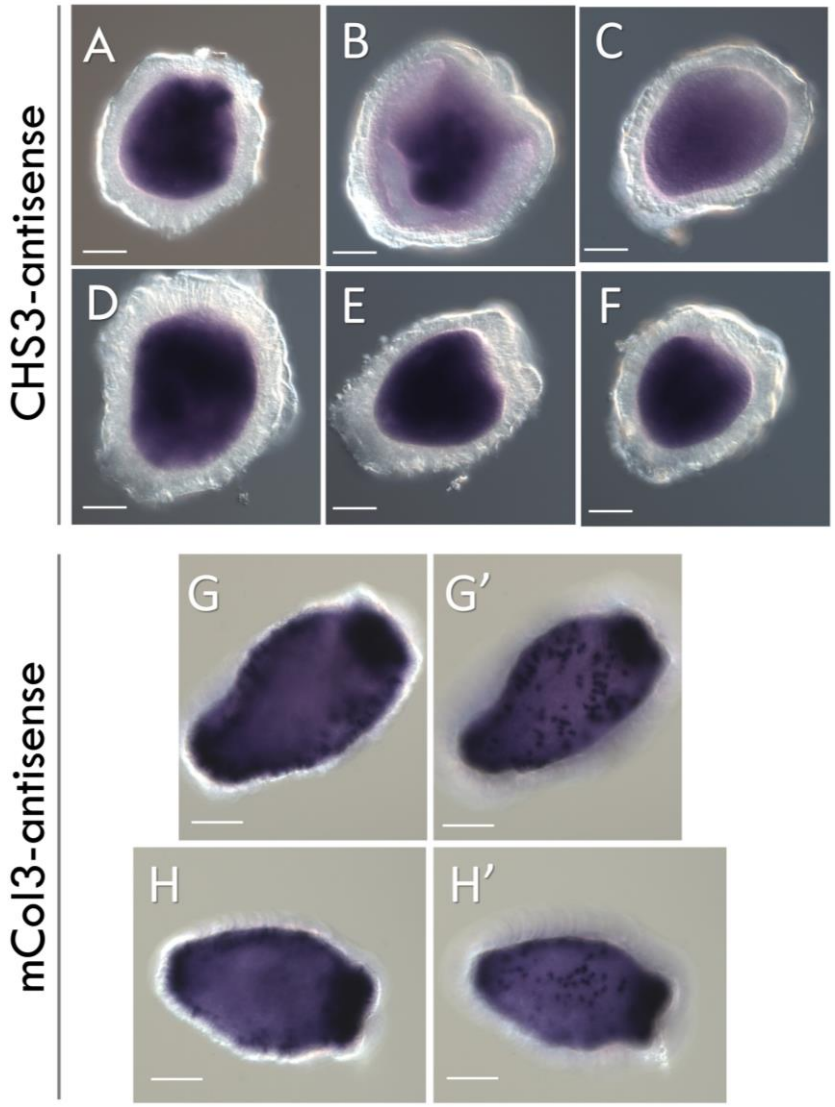


**Figure 3.9 – Varieties of expression of *chitin synthase-3* and *mCol-3* in NvCHS3-3sgRNA/Cas9 240 hpf larvae.** A.) – F.) show ectopic expression of *Nv-CHS3*, which is expressed in the ectoderm in wild type embryos and larva. Development appears to be delayed in some samples (e.g. panels A-D, F). G.) Deep plane view of *mCol-3* expression in cnidocytes. G'.) Shallow plane view of panel G. H.) Individual displaying wild type expression of *mCol3* (deep view). H'.) Surface ectoderm view of the individual.

Scale bar is 50µm in all panels.

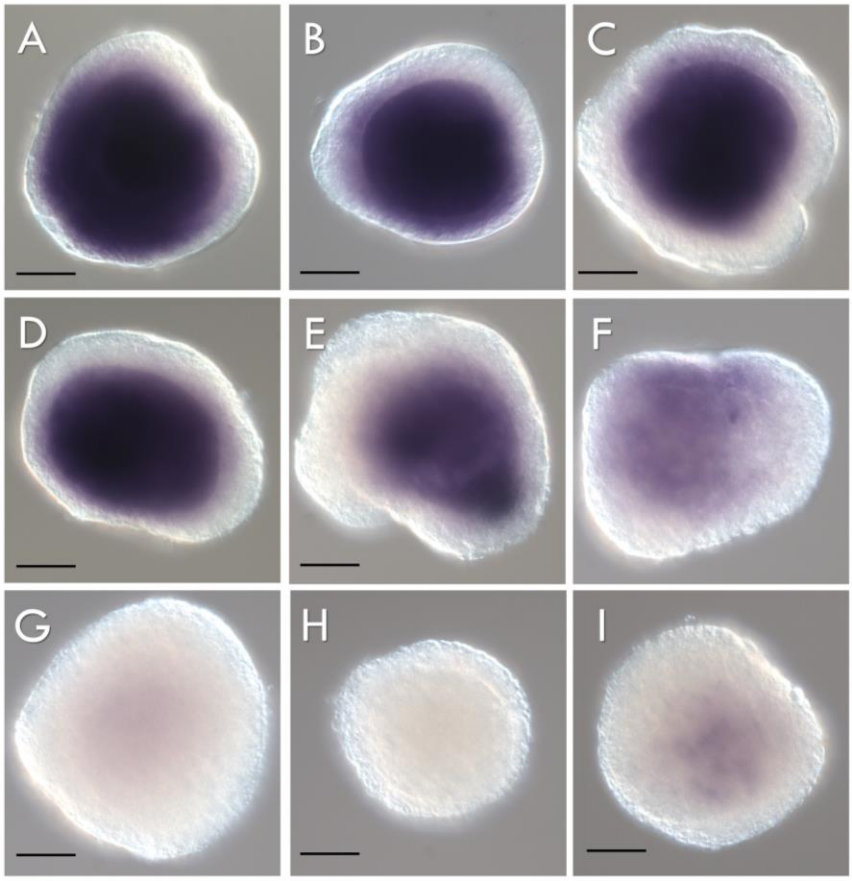


**Figure 3.10 – Varieties of expression patterns of *Nv-CHS1* in triple-knockdown 9sgRNA/Cas9 larvae (144 hpf).** Knockdown larvae show ectopic or reduced expression of *Nv-CHS1* compared to wild type and delayed development. A.) An individual showing increased labeling (dark purple) in the aboral ectoderm and throughout the endoderm. B.) – D.) Larva showing high levels of expression in the endoderm, with some labeling in ectoderm. F.) A larva with disorganized morphology showing endodermal and ectodermal labeling. G.) – H.) Individuals displaying no expression and delayed development. I.) Some expression is seen in individual cells, similarly to gastrula-stage wild type embryos.

Scale bar is 50µm in all panels.

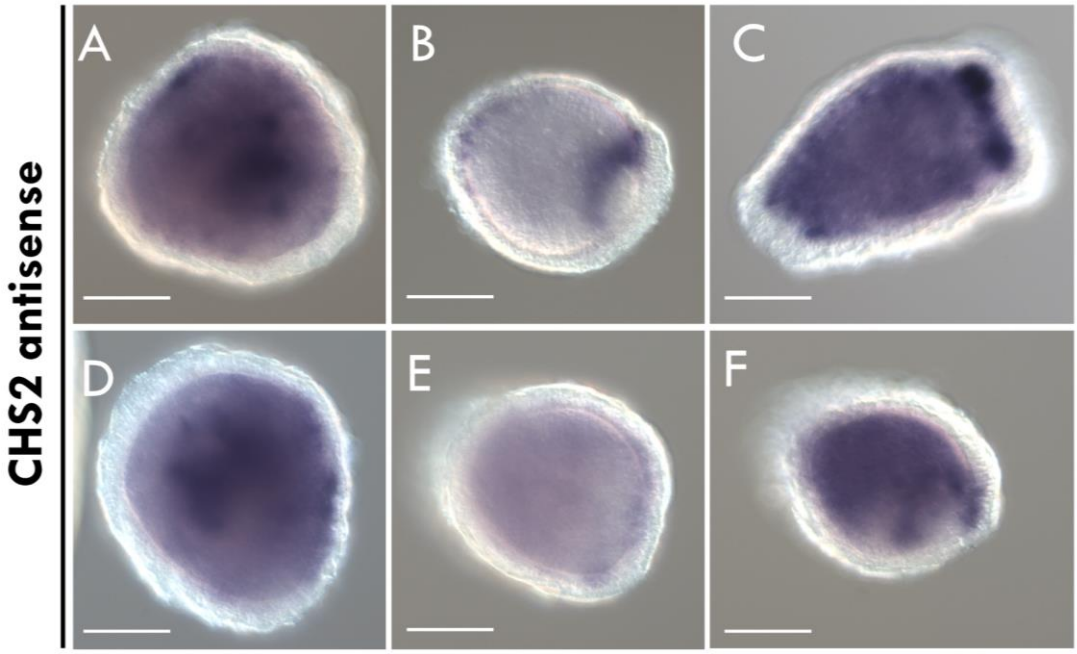


**Figure 3.11 – Varieties of expression patterns of *Nv-CHS-2* in triple-knockdown 9sgRNA/Cas9 larvae (240 hpf).** Knockdown larvae show delayed development and ectopic or reduced expression of *Nv-CHS2* compared to wild type. A.) An early planula larva with some ectodermal labeling but ectopic endodermal expression. B.) An individual displaying overall wild type expression in the oral region, though the body wall is missing *Nv-CHS2*-positive cells. C.) Punctate expression is visible, though the expression boundaries are not as well defined as in wild type. Expression is still visible throughout the animal; at this stage in wild type, cells expressing *Nv-CHS2* are concentrated in the oral region, with little labeling in the trunk. D.) – F.) Larvae show a variety of mosaic expression patterns, with scattered punctate labeling.

Scale bar is 50µm in all panels.

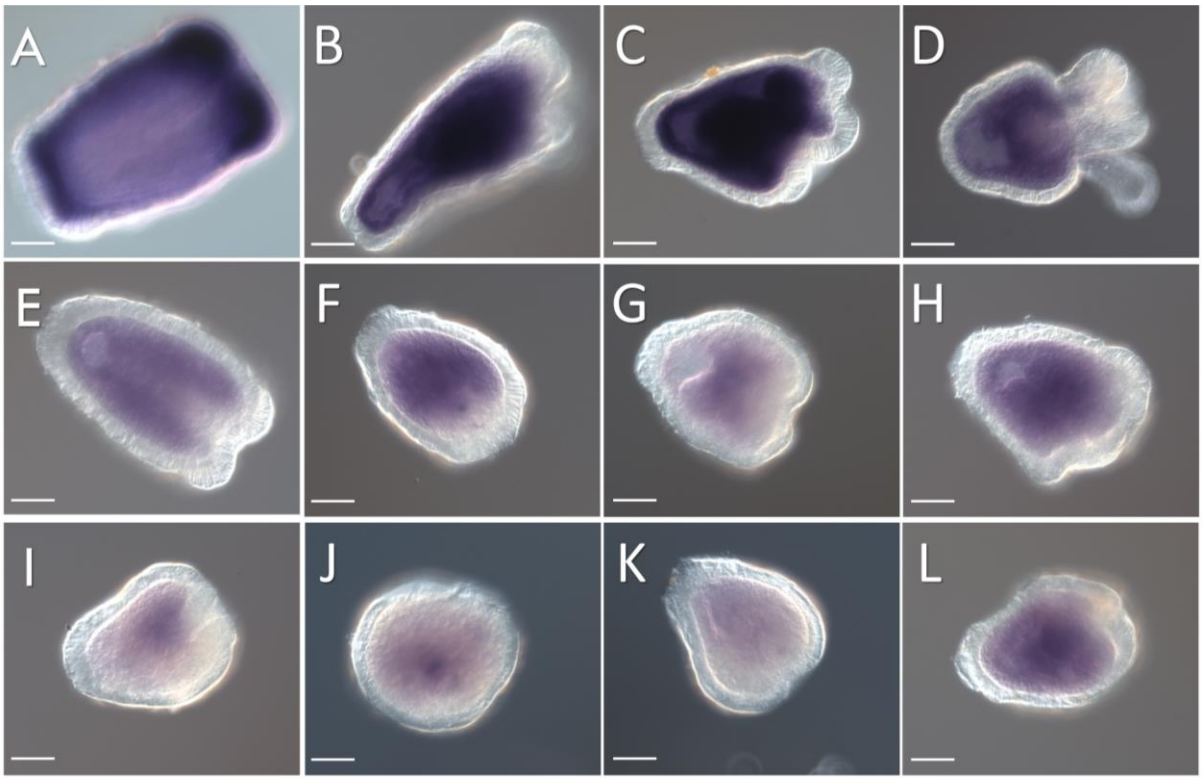


**Figure 3.12 – Varieties of expression patterns of *Nv-CHS3* in triple-knockdown 9sgRNA/Cas9 larvae.** Knockdown larvae show ectopic or reduced expression of *Nv-CHS3* compared to wild type. All panels, 240 hpf (late planula/tentacle bud). A.) An uninjected sample demonstrating wild type expression. B.) – D.) Individuals displaying a variety of morphological defects including reduced numbers of tentacles or a short trunk, although expression patterns appear broadly normal in the trunk. E.) A tentacle bud larva showing reduced expression but relatively normal morphology. F.) – L.) Planula larva showing reduced or disorganized expression patterns and abnormal morphology.

Scale bar is 50µm in all panels.

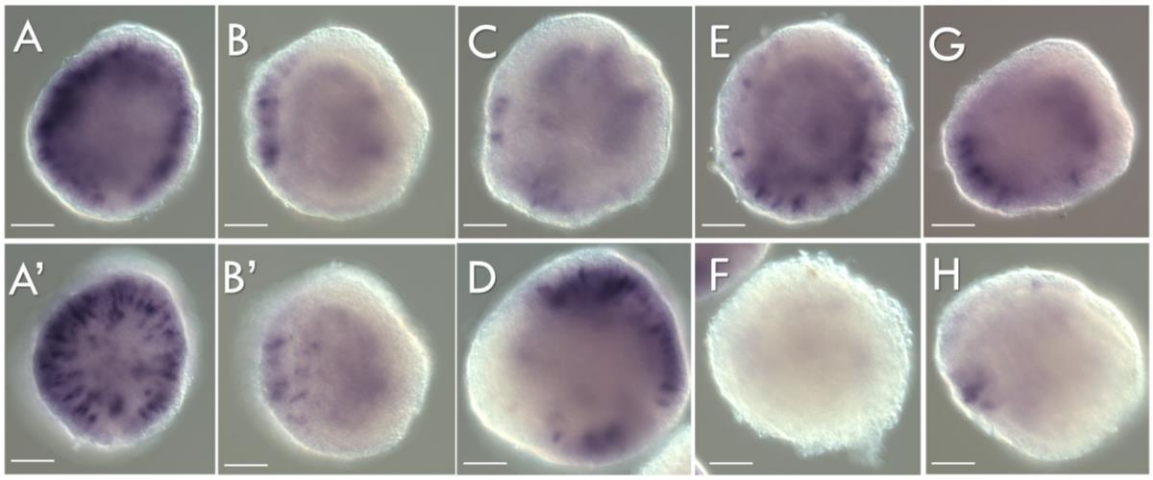


**Figure 3.13 – Expression of *Nv-mcol3* in *Chitin synthase*-9sgRNA/Cas9 planulae.** Triple-knockdown planula larvae show reduced or mosaic expression of cnidocyte marker minicollagen-3. A.) Deep view of an injected individual displaying broadly wild type *mCol3* expression. A'.) Shallow plane view of larvae in panel A, displaying typical punctate labeling of cnidocytes. B.) Deep tissue view of an individual showing reduction in mcol3-positive cells in the body wall B'.) Shallow plane view of larvae in panel A, displaying some punctate labeling of cnidocytes. C.) – H.) Individuals displaying a variety of mosaic expression patterns of *mCol3*. Some larvae lack expression (e.g. panel F).

Scale bar is 50µm in all panels.

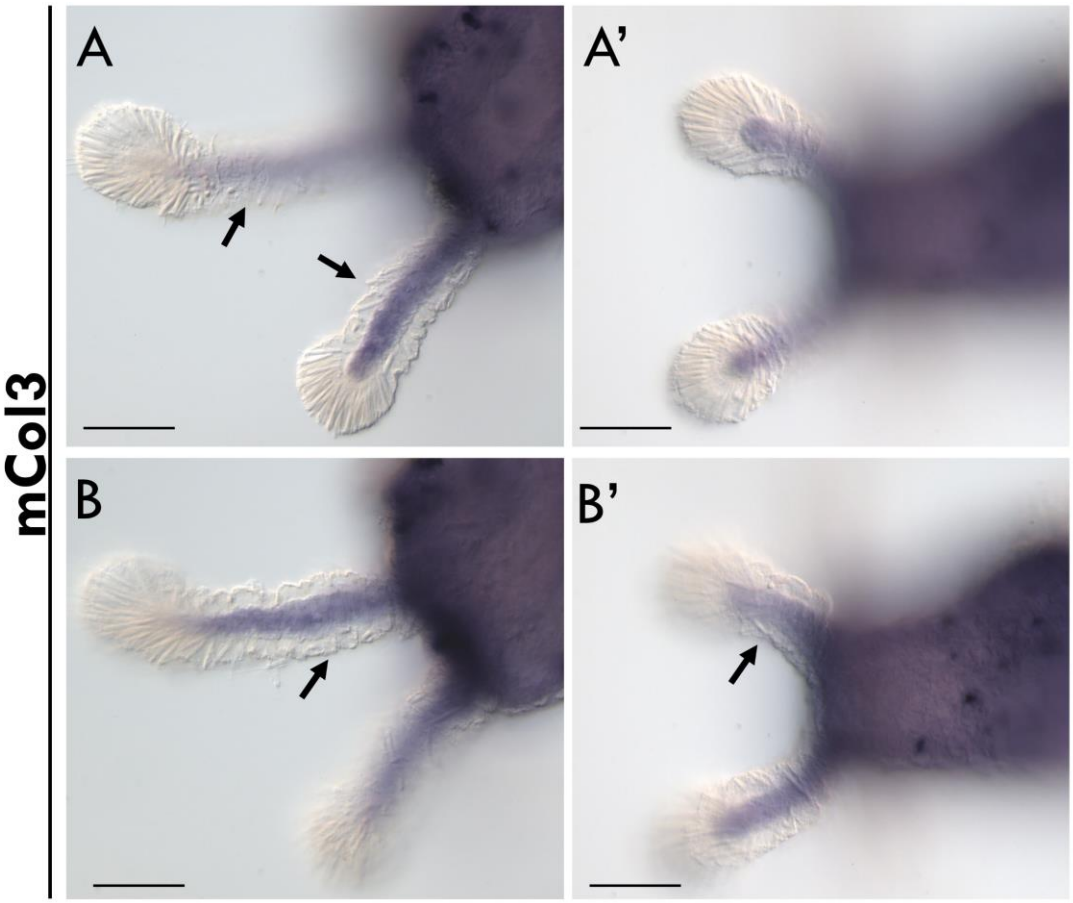


**Figure 3.14 – *Chitin synthase*-9sgRNA/Cas9 primary polyps lack spirocysts.** Triple-knockdown polyps show reduced or chaotic expression of cnidocyte marker minicollagen-3. Spirocysts are absent, though there are some nematocysts in the tentacle tip. Cnidocysts (both spiro- and nematocysts) are depleted in the tentacle (black arrows). All images 40x DIC. Scale bar is 50µm in all panels.

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## Chapter 3 – Supplemental Data

### Supplemental Table 3.S1 – Guide RNAs targeting *chitin synthase* genomic sequences

| Guide RNA Name | Sequence |
|---|---|
| CS93407_5CDS | CGUAUGGAAUGCAGCUGCGG |
| CS93407_CDST30 | UGACAGUGGUUGAAUAAGUC |
| CS93407_ CDS | CACGCCUAGAAGUUUCAAAU |
| CS104030_5UTR | AGUCCUAUUAAUAACUAUCU |
| CS104030_3UTR | GCGCCGAAUCUCUCUCGAAC |
| CS104030_T9 | GCCCUUCACAAUUCACCUCA |
| CS123712_5UTR | CAAAGCUUUCCCCGCGGGAC |
| CS123712_ T35 | UUCAGGUGGCGUCUGCCUGG |
| CS123712_T14 | AACUGUACCUAGCACAUGAU |

**Supplemental Table 3.S2 – Primer sets used to amplify *Nematostella chitin synthase* genes targeted with CRISPR/Cas-9 modification**

All primer sequences are listed 5'-3'.

| Gene Target | Primer Name | Primer Sequence |
|---|---|---|
| Nv-CHS1 | Nv-93407-CRISPRchk-4F | CGCCACAAATACGCCTTAGC |
| Nv-CHS1 | Nv-93407-CRISPRchk-4R | ATACGTTCGCAGCTCGTCAA |
| Nv-CHS1 | Nv-93407-CRISPRchk-24F | CTGGCGTTTGATGTACTGCG |
| Nv-CHS1 | Nv-93407-CRISPRchk-24R | TTTAGCACGCTCGTCGGTTA |
| Nv-CHS2 | Nv-1040-CRISPRchk-14F | GCCATTGTCTTGCGAGCG |
| Nv-CHS2 | Nv-1040-CRISPRchk-14R | GTTGTTCCGAGCACCAACAC |
| Nv-CHS2 | Nv-1040-CRISPRchk-28F | CACGCCACCAGAGTCTCAAG |
| Nv-CHS2 | Nv-1040-CRISPRchk-28R | AGCGCTAATCGGCTTTTCCAG |
| Nv-CHS2 | Nv-1040-CRISPRchk-1F | TGAAGACGCCGTATGGGATG |
| Nv-CHS2 | Nv-1040-CRISPRchk-1R | CCCGATGGCGTAGTCAAAGA |
| Nv-CHS2 | Nv-1040-CRISPRchk-5F | CATGTGGGTAGGGCAGTTCC |
| Nv-CHS2 | Nv-1040-CRISPRchk-5R | TACGGCACTGTAGTTGTGGC |
| Nv-CHS3 | Nv-1237-CRISPRchk-17F | ATATCGGTGCCTTGTCGTGG |
| Nv-CHS3 | Nv-1237-CRISPRchk-17R | TATACCCCTCGTGCCCCTTT |

**Supplemental Table 3.S3 – raw band intensity used to calculate relative expression levels of *Nv-CHS* genes through *N. vectensis* development.**

| Lane | Actin | Nv-CHS-1 | Nv-CHS-2 | Nv-CHS-3 |
|---|---|---|---|---|
| 1 | 23033.38 | 1199.678 | 1333.092 | 918.021 |
| 2 | 17404.258 | 585.385 | 2213.213 | 3276.284 |
| 3 | 17986.551 | 303.899 | 2574.506 | 4229.062 |
| 4 | 34207.907 | 2209.698 | 3268.799 | 9596.024 |
| 5 | 30220.057 | 2782.941 | 4118.406 | 12999.702 |
| 6 | 29617.856 | 2550.163 | 3274.042 | 116778.525 |
| 7 | 44574.128 | 46530.773 | 5158.234 | 36061.057 |
| 8 | 44191.492 | 6500.678 | 37724.359 | 67449.844 |

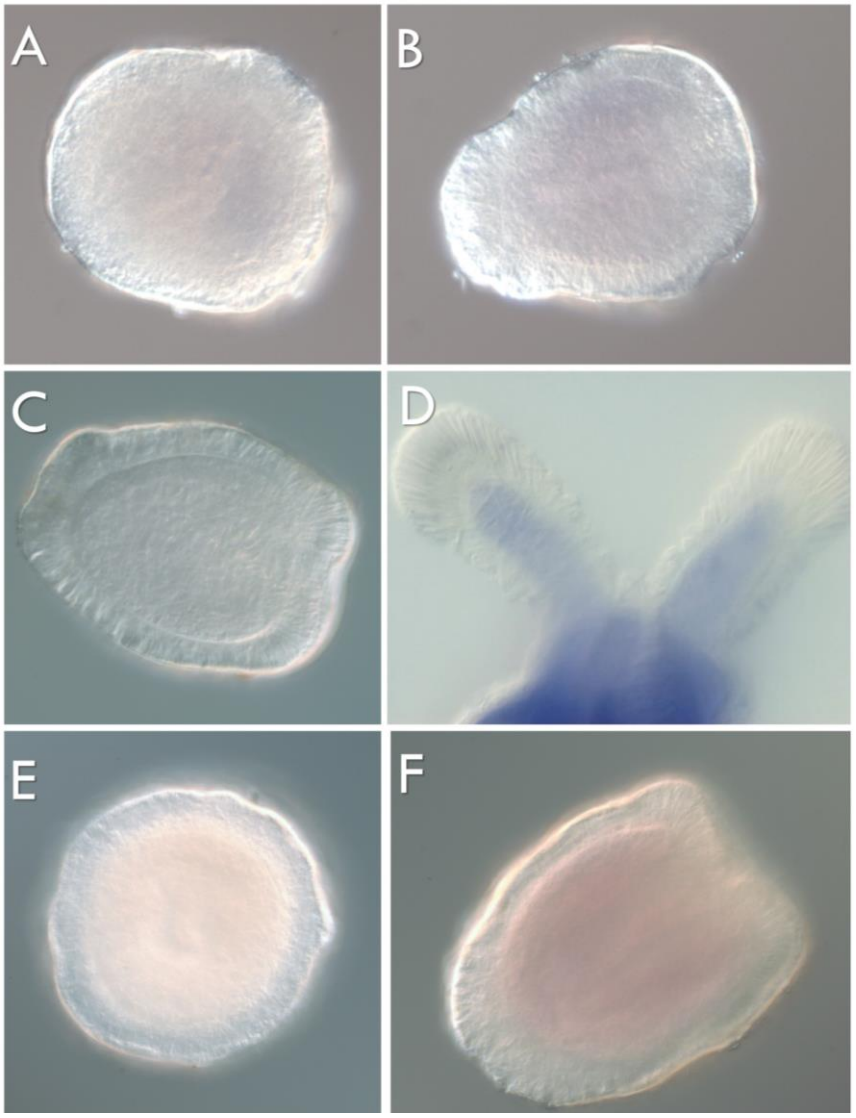


**Figure 3.S1 – Sense control probes for *chitin synthase* genes.** Nonspecific labeling was not observed. A.) Nv-CHS1 sense probe: mid-planula stage larva. C.) *Nv-CHS1* sense probe: late stage planula. D.) Nv-CHS2 sense probe: primary polyp stage. Some background nonspecific labeling was observed in primary polyps (diffuse purple), but staining patterns did not overlap with those of the antisense probe. E.) *Nv-CHS1* sense probe: early planula larva. B.) *Nv-CHS1* sense probe: late stage planula/early tentacle bud larvae. Little background nonspecific labeling was observed in larvae or other stages (diffuse pink), but staining patterns did not overlap with those of the antisense probe.

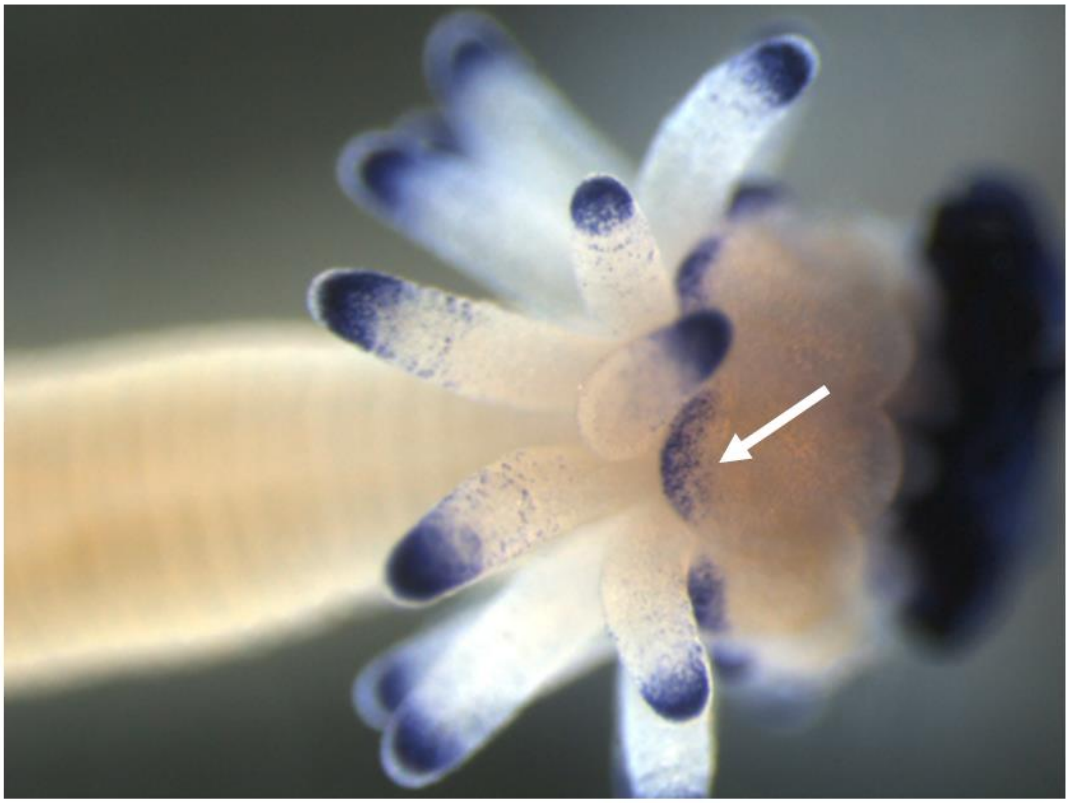

**Figure 3.S2 – Expression of Nv-CHS2 in adolescent wild type polyp.** *In-situ* hybridization of *chitin synthase-2* in a juvenile polyp. Primary polyps have four tentacles and adults have up to 20.

Expression is localized to the tentacles and can be seen in the emerging boundary of a tentacle bud (arrow). Labeling of individual cells (punctate purple) can be seen.

Stereoscope image.

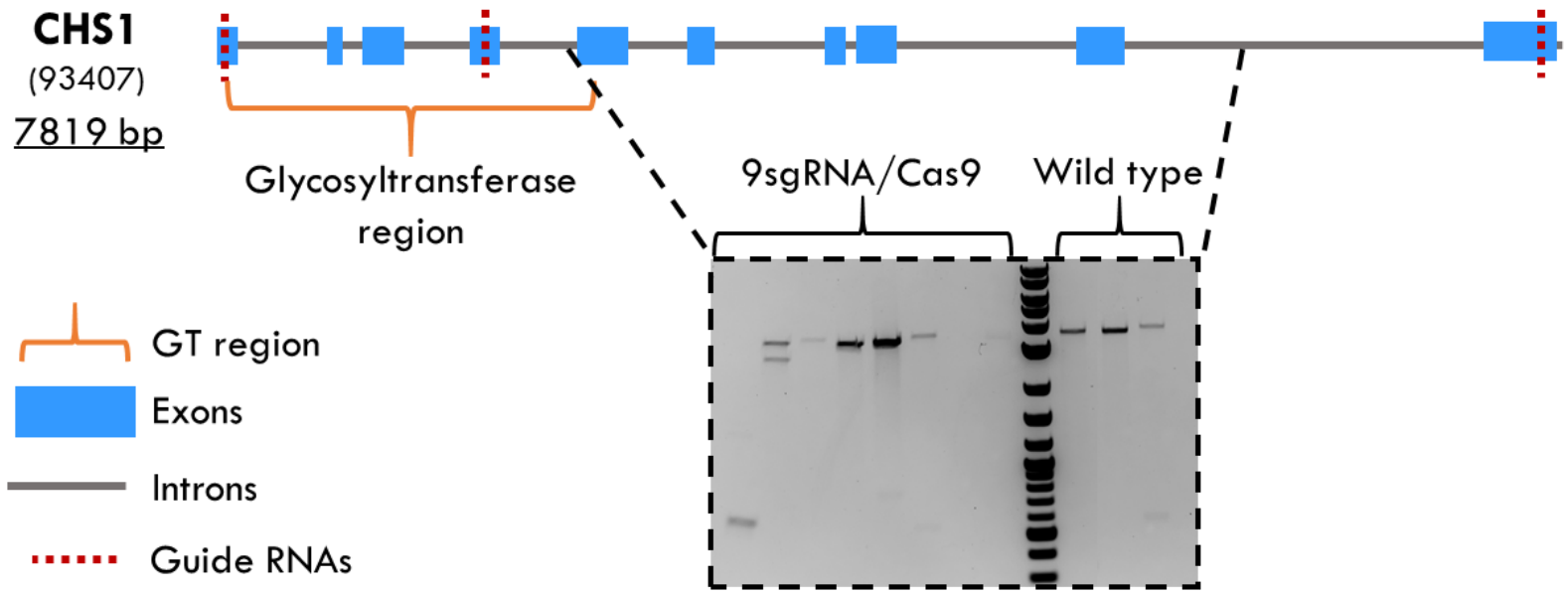


**Figure 3.S3 – CHS1 sgRNA design and PCR gels of Cas9-injected samples.** Wild type (WT) samples show expected PCR band sizes (approximately 3,600 bp) whereas sgRNA/Cas9-injected samples are varied in whether they produced a PCR product, or the size of the product (lanes left of ladder). Multiple bands indicate potential mosaicism in samples, where some alleles may be much smaller in sequence than others due to cutting with Cas9.

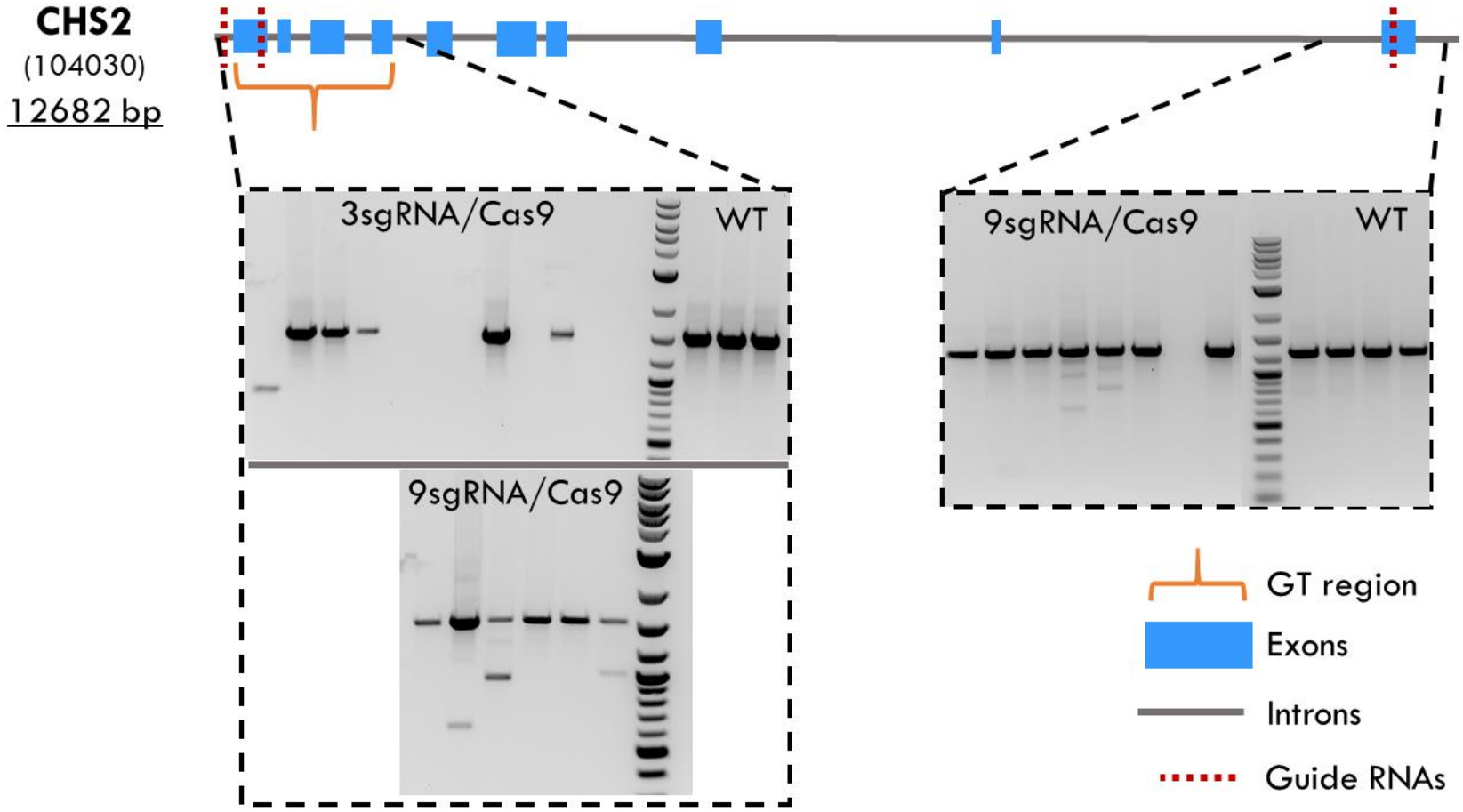


**Figure 3.S4 – CHS2 sgRNA design and PCR gels of sgRNA/Cas9-injected samples.** Wild type (WT) samples show expected PCR band sizes (approximately 2,000 bp, left; 1,800 bp, right) whereas sgRNA/Cas9-injected samples are varied in whether they produced a PCR product, or the size of the product (lanes left of ladder, all gels). Multiple bands indicate potential mosaicism in samples, where some alleles may be much smaller in sequence than others due to cutting with Cas9.

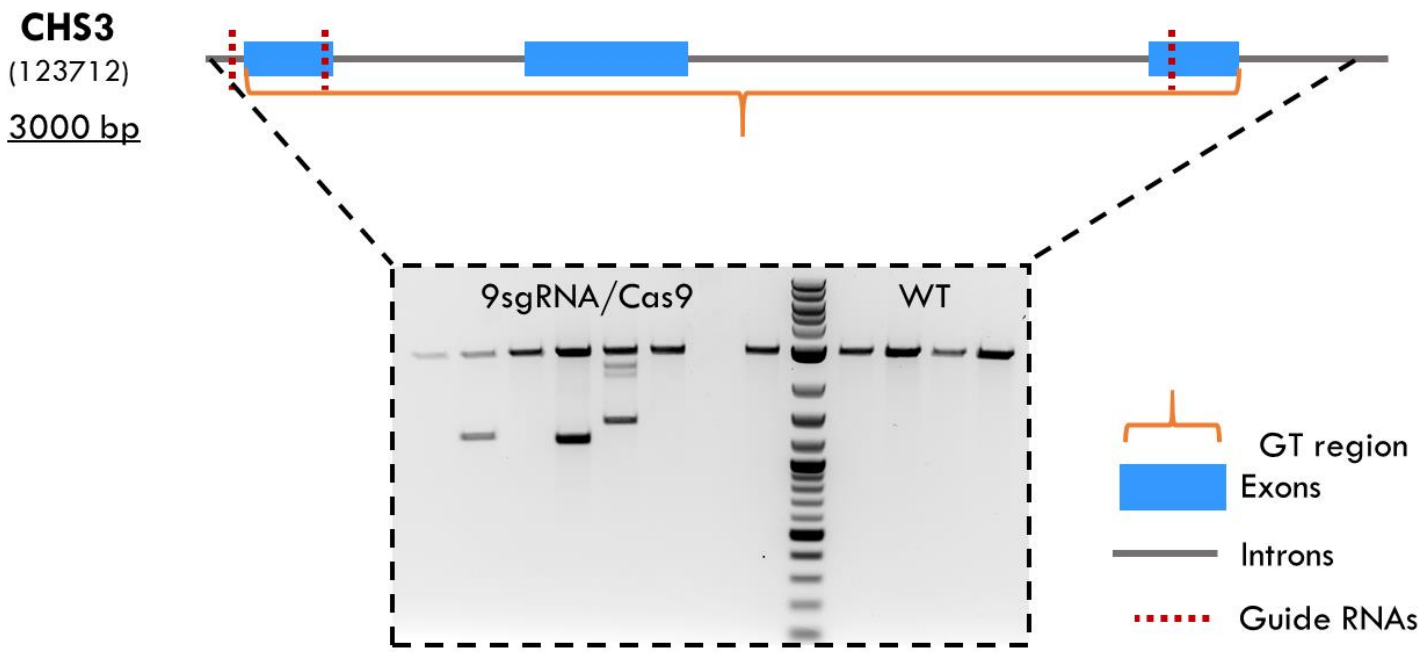


**Figure 3.S5 – CHS3 sgRNA design and PCR gels of Cas9-injected samples.** Wild type (WT) samples show expected PCR band sizes (approximately 3,000 bp) whereas sgRNA/Cas9-injected samples are varied in whether they produced a PCR product, or the size of the product (lanes left of ladder). Multiple bands indicate potential mosaicism in samples, where some alleles may be much smaller in sequence than others due to cutting with Cas9.

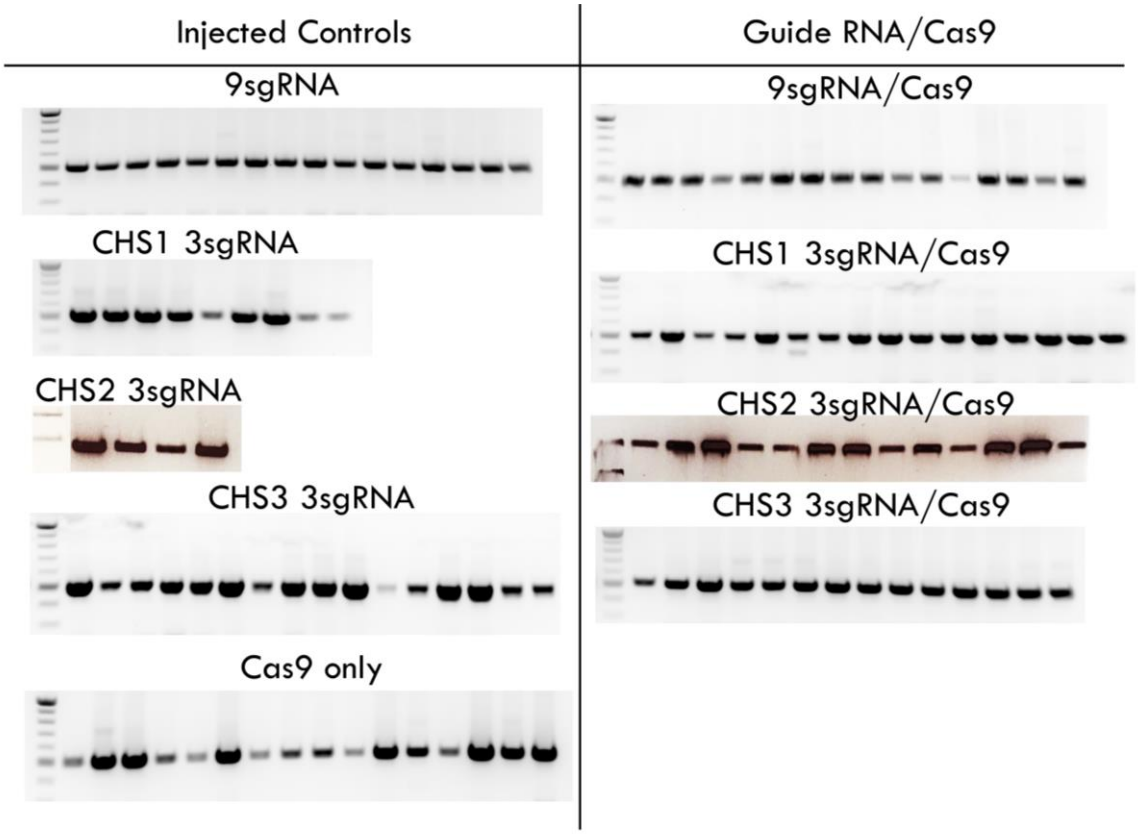


**Figure 3.S6 – Actin PCR gels of Cas9-injected samples and negative controls.**

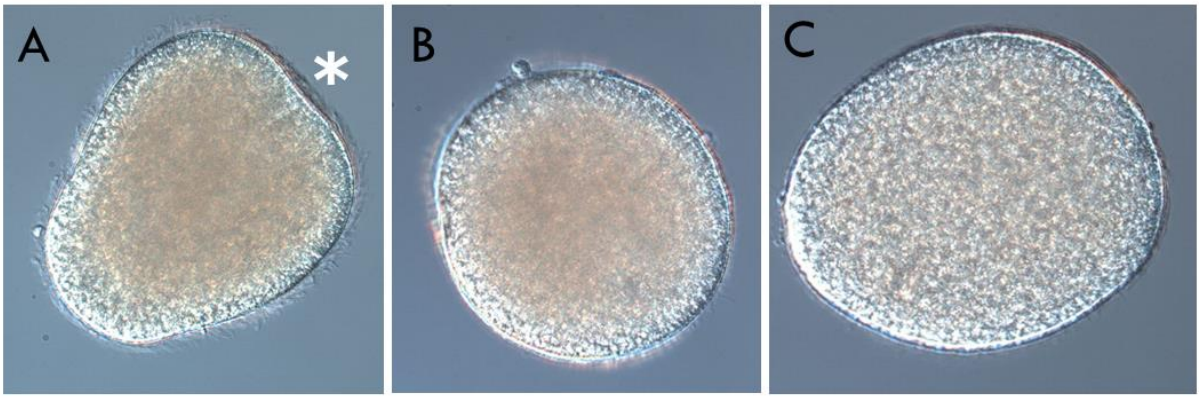


**Figure 3.S7 – Chitin synthesis inhibition with diflubenzuron.** All panels 7dpf larvae. A.) Late stage planula larva, raised in 1/3 seawater with DMSO (negative control). Larvae behaved normally, swimming in the water column. Asterisk marks site of mouth. B.) and C.) Larvae raised in 10ng/µL diflubenzuron. Larvae did not elongate into the typical fusiform planula shape and did not swim.

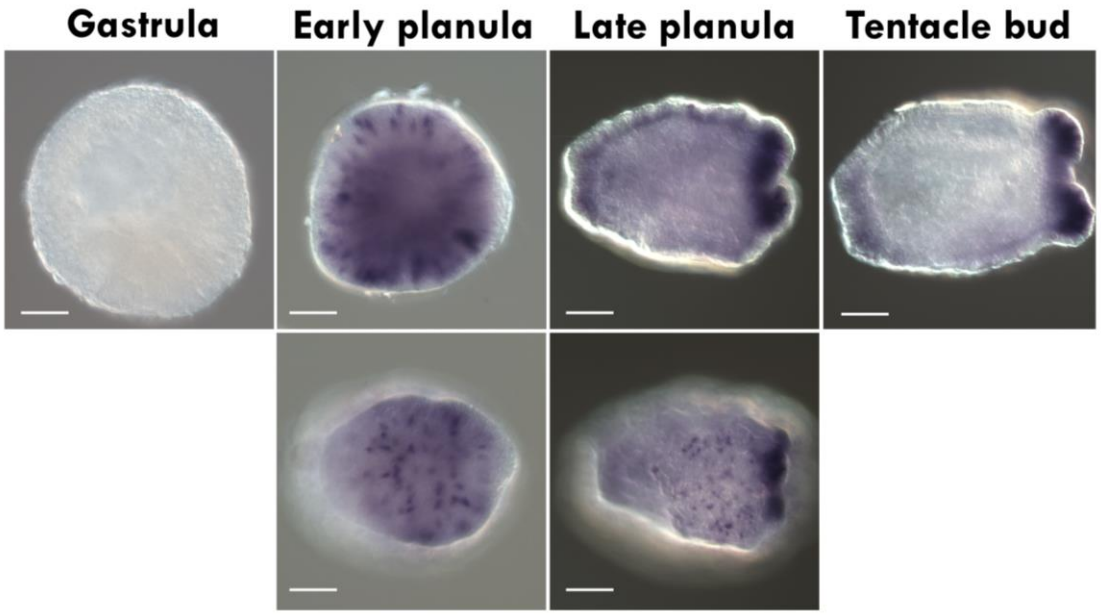


**Figure 3.S8 – Wild type expression of minicollagen-3, a cnidocyte marker.** Deep planes, top row; shallow surface views of dermis, bottom row. Expression appears in early planula stages in the ectoderm. Though development, some expression stays in the body column ectoderm but also concentrates at the oral pole and in the developing tentacles. Scale bar is 50µm in all panels.

## Appendix A1 – Chitin in Ctenophores? Chitinophores.

### *A1.1. Introduction*

Ctenophora (“comb bearing”, in Greek) is a phylum comprised of an estimated 100-200 extant species (Ward Appletans et al 2012; Whelan et al 2017) and are one of the most ancient metazoan lineages. Ctenophores are exclusively marine; they are found in all oceans, from the tropics to the poles, and at all depths. Most ctenophores (comb jellies) are planktonic predators and feed on other zoo- and gelatinous plankton (usually other ctenophores). Ctenophores, like cnidarians, have bodies that are primarily composed of gelatinous mesoglea; ctenophore mesoglea largely consists of collagen and is mostly acellular. Neurons and muscle cells transverse the mesoglea, along with motile stellate cells that may have immune function (William Browne, personal communication). Comb jellies get their name from the eight ctenes, or comb plates, that traverse their bodies along the oral-aboral axis. Long, fused cilia are attached to these plates. Ctenophores beat the cilia to move and are the largest taxon to exclusively utilize cilia for locomotion.

Like cnidarians, the oral-aboral axis is the major body plane in ctenophores, with a mouth at the oral pole and an apical sensory organ at the aboral pole. The apical organ consists of a statolith connected to four balancing cilia. Each balancing cilia is connected to nerve bundles that run along two adjacent comb rows. The apical organ allows ctenophores to sense their swimming direction in the water column (Brusca and Brusca 2002; Tamm 2014). Ctenophores exhibit bi-radial symmetry around their oral-aboral axis in contrast to the radial symmetry of Cnidaria, which ctenophores superficially resemble. Where cnidarians have specialized cell types used in hunting (nematocysts) that spear the dermis of prey, ctenophores also possess a unique cell type – the colloblast. Most species of ctenophore (aside from the class Nuda) possess two tentacles, and these tentacles are covered with colloblasts. Colloblasts do not penetrate tissue but have

adhesive properties and stick to prey. Prey (small crustaceans, larval fish) get trapped by the colloblasts and the tentacles ferry the pray to the mouth.

A recent debate over the phylogenetic relationships of early metazoans has resulted in several hypotheses. Ctenophora has a limited and controversial fossil record (Conway Morris and Collins 1996, Shu et al 2006) making it difficult to discern exactly when the lineage appeared. Because ctenophores have bodies that are primarily composed of gelatinous mesoglea, they are not prone to fossilization and it is possible that no ctenophore fossils exist. Some recent molecular studies have grouped the two gelatinous non-bilaterian phyla – Ctenophora and Cnidaria – into the historical clade Coelenterata (Philippe et al., 2009; Pick et al., 2010; Schierwater et al., 2009). However, genomic evidence from developmental genes such as Wnt (of which cnidarians have thirteen *Wnt* genes, but ctenophores have only four) provide clues that ctenophores diverged before the duplication events that created multiples of Wnt genes shared by cnidarians and bilaterians (Kusserow et al., 2005; Moroz et al., 2014; Pang et al., 2010; Ryan et al., 2013). A hypothesis that has gained support as more genomic data has become available places ctenophores as the sister group to the rest of Metazoa (Dunn et al. 2008, Ryan et al., 2013; Whelan et al 2015; Whelan et al 2017). Other studies have placed Porifera as the earliest-diverging and the sister group to all other metazoans (Srivastava et al., 2010).

Within Ctenophora, relationships between taxonomic clades has been challenging to elucidate, with multiple topologies proposed (Hernandez-Nicaise and Franc 1994; Podar et al 2001; Simion et al 2014; Whelan et al 2017). There are currently 9 accepted ctenophore orders (with 27 total families), though some clades have few known species and are rarely observed or collected (Mills 2012). The most widely accepted orders are Cydippida (which is paraphyletic), Lobata, Platyctenida; Beroida, and Cestida. Though the best-known body plan is the cydippid form – eight prominent ctenes, two tentacles with branching tentillae covered in colloblasts, almost spherical body shape – ctenophore bauplans are diverse.

The ancestral bauplan is assumed to be the cydippid, though taxa displaying the cydippid form have been found to be paraphyletic with genomic analyses (Simion et al 2014; Whelan et al 2017), in contrast to conclusions about inter-Ctenophora relationships proposed by morphology alone (Hernandez-Nicaisse and Franc 1994, and others). Beroida (two genera, *Beroe* and *Neis*) lack a tentacle apparatus and colloblasts, are football-shaped, and engulf their prey whole; they usually eat other ctenophores. Lobate ctenophores have two large extensions of the body wall (lobes), surrounding the mouth. Tentacles line the interior of the lobes, and the tentacle bulbs are reduced compared to those of cydippids. Platyctenids are a clade of benthic species; platyctenes have reduced comb rows and do not swim after metamorphosis. Superficially resembling nudibranchs, these platyctenes crawl along corals and rocks with their tentacles extended to snare prey.

Ctenophores regenerate their tentacles continuously in response to damage during prey capture and maintain a population of stem cells or stem cell-like cell populations in the tentacle bulbs (Alie et al., 2011). During embryological development, tentacle bulbs begin to develop at about 9-10 hours post-fertilization and the tentacle apparatus (tentacle sheath, bulb, and the colloblasts) continues to develop over the course of a day, with tentacles appearing in about 30 hpf cydippid larvae (Martindale and Henry, 1999; Pang et al., 2011). In adults, tentacle tissue emerges from the tentacle bulb from muscle cell, nerve, and colloblast progenitors (Dayraud et al 2012). The tentacle tissues emerge covered in colloblasts. At the surface, the tentacle bulb appears to be a largely homogeneous and featureless structure. However, within the tentacle bulb interior gene expression data reveals discrete tissue zones that produce the muscle of the tentacle core, tentillae (branches of the tentacle), nerves, and colloblasts (Alie et al 2010; Dayraud et al 2012).

Chitin (and/or its cognate *chitin synthase* gene) has been found in two of the other non-bilaterian metazoan phyla (Porifera and Cnidaria) and in choanoflagellates. The existence of chitin in ctenophores has been dismissed and it is thought that synthesis of this molecule was lost in

the ctenophore lineage (Hyman, 1966; Willmer, 1990; Ehrlich et al 2007 a, b; Bo et al 2012; Ehrlich et al 2013; this thesis). However, we have collected intriguing new data that show that chitin is likely present in adult tissues of the cydippid ctenophore *Pleurobrachia bachei* and the lobate ctenophore *Mnemiopsis leidyi*.

## A1.2. Methods

### *A1.2.1. Animal Collection and Care*

Cydippid ctenophore species *Pleurobrachia bachei* were collected from Puget Sound using a scoop at the end of a pole. Animals were maintained in seawater at 4°C. Lobate ctenophore species *Mnemiopsis leidyi* were collected in Daytona Beach, FL and maintained in seawater at room temperature.

### *A1.2.2. Sample collection, embedding, and tissue sectioning*

Whole adult *Pleurobrachia* were fixed in PFA 4% PFA at 4°C for one hour and gradually dehydrated into methanol. Samples were stored at -20°C. Whole animals were gradually rehydrated into PTw and thoroughly washed in PBS (3 x 10 minutes) before embedding. Tissue was equilibrated in 15% sucrose in PBS for three hours at room temperature then in 15% sucrose/7.5% gelatin in PBS at 37°C for three hours. Samples were infiltrated with 20% gelatin in PBS overnight at 37°C and embedded in fresh 20% gelatin in PBS using plastic molds. Embedded samples were mounted onto a cryotome chuck with Tissue-Tek O.C.T. (optimum cutting temperature) compound and frozen at -80°C overnight. Samples were allowed to come up to cryotome temperature (-17°C) for one hour prior to sectioning. Embedded samples were sectioned at ~7 µm on a Cryostat cryotome, and mounted on charged Superfrost Plus slides. Slides were allowed to rest at room temperature for 24 hours prior to further processing.

### *A1.2.3. Chitin Histochemistry*

**Whole *P. bachei* adults**

Animals were gradually rehydrated from methanol into PTw. All washes and incubations were performed on a rotating platform. Tissues were permeabilized with 0.2% Triton X-100 in PBS (three 15-minute washes) and blocked in Protein-Free T20 (TBS) blocking buffer overnight at 4°C. Slides were then incubated with CBD-546 (1:40) and DAPI (1:1000) (4′,6-diamidino-2-phenylindole) or Toto-3 (1:100) in TBS blocking buffer overnight at 4°C. Samples were thoroughly washed in PTw, mounted in VectaShield, and imaged.

**Isolated *M. leidyi* tentacles**

Tentacle bulbs were dissected from live *Mnemiopsis* and utilized immediately for staining. Bulbs were fixed in 4% PFA at 4°C for one hour and washed thoroughly with PTw. Staining was carried out as described above.

*A1.2.4. In situ hybridization*

*Pleurobrachia bachei* adults were processed for in situ hybridization as previously described (Dayraud et al 2012) with some modifications. Samples were digested with Proteinase-K for 25 minutes, digestion was stopped with washes of 4mg/mL glycine. Tissues were incubated in triethanolamine as described (Servetnick et al 2016) and post-fixed in 4% PFA before proceeding with hybridization steps.

*A1.2.5. Cloning of partial* chitin synthase *gene from Pleurobrachia bachei*

Because genomic or transcript sequences for chitin synthases in ctenophores are present in available genetic resources, degenerate primers were designed using the j-CODEHOP BLOCKS tool (https://virology.uvic.ca/virology-ca-tools/j-codehop/). *CHS* sequences from other non-bilaterian taxa were used to design degenerate primers around the conserved glycosyl transferase region of the gene (Supp. Data A1.S1). Primers deployed to amplify genes from *P.*

*bachei* cDNA are listed in Table A1.1. RNA isolation, cDNA synthesis, gene cloning, and RNA probe synthesis were carried out as described in Methods (Section 0.2).

## A1.3. Results

### *A1.3.1. Isolation of a partial* chitin synthase *gene from* Pleurobrachia bachei

A section of a putative *chitin synthase* gene was cloned from adult whole *P. bachei* using degenerate primers (amino acid sequence: Supplemental Data A1.S1). This 131 amino acid fragment has is a glycosyl transferase domain with high support of homology ($e^{-44}$) and BLASTs to cnidarian and other animal *chitin synthases*. RACE PCR failed to yield the full gene (results not shown), and southern blot data were inconclusive to confirm the presence of this putative *CHS* gene in *P. bachei* genomic DNA (results not shown).

### *A1.3.2. Chitin Staining in Ctenophore Tentacle Bulbs*

Chitin was detected in the tentacle bulbs of adult *Pleurobrachia bachei*, where it appears to surround the area in which the tentacle connects to the bulb, as well as the portions of the tentacle itself that are closest to the bulb (Fig. A1.2 B-D; Fig A1.3 B-D). There is no fluorescent signal in control samples (Fig. A1.3 A). Chitin signals are punctate both within what looks to be boundaries of individual cells (Fig. A1.2 D), and is present extracellularly. Colloblasts and interior-facing portions of the tentacle bulb do not show chitin labeling.

Lobate ctenophore *Mnemiopsis leidyi* has reduced tentacle bulbs compared to cydippid taxa like *P. bachei*, but the outward-facing portion of the tentacle bulbs also show chitin labeling (Fig. A1.4 A, C). Nuclei labeling (DAPI) show that, similarly to *P. bachei,* the entirety of the *M. leidyi* tentacle bulb does not label with chitin probe (Fig. A1.4 B, D). Chitin labeling appears to be specific to the region where the tentacle is emerging from the tentacle bulb.

### *A1.3.3. Putative Pb-CHS gene is expressed in the tentacle bulbs*

*In situ* hybridization expression data shows that the putative *chitin synthase* gene cloned from *P. bachei* is widely expressed in the tentacle bulbs (Fig. A1.5). Expression is not seen in other anatomical structures (ctenes, mesoglea, gastrovascular canals). The signal appears to be specific, as dioxygenin labeling is absent in negative controls (sense probe; Fig. S1.1). While chitin histochemistry shows chitin labeling at the outer boundary of the ctenophore tentacle bulb, expression of *CHS* is more widespread.

## A1.4 Discussion

Chitin has been described as absent in the Ctenophora (Hyman 1966) and given its presence in other basal taxa (cnidarians, sponges), the ability to synthesize chitin was assumed secondarily lost in this lineage. Here we show that chitin is likely present in ctenophores and may have a role in structural support of the adult tentacle apparatus. Examining the tentacle bulbs of both model ctenophore species – cydippid *Pleurobrachia bachei* and lobate *Mnemiopsis leidyi* – is informative because the tentacle morphology of these species is very different, and the two taxa are not closely related (Whelan et al 2017). In previous works regarding gene expression and descriptions of cell types in the *Pleurobrachia* tentacle bulb, a distinct orange-colored material can be seen in Brightfield images at the distal external portion of the bulbs (Alie et al 2010; Dayraud et al 2012). This area matches closely with the chitin signal seen with the chitin binding domain probe.

Intriguingly, we were unable to identify any potential orthologs of *CHS* or chitin synthase-specific glycosyl transferase domains in any of the available ctenophore genomes or transcriptomes (data from Whelan et al 2017). However, a partial *chitin synthase* putative ortholog was repeatedly cloned from whole adult *P. bachei* cDNA with the use of degenerate primers. It is unclear whether the *Pleurobrachia bachei* individuals themselves were expressing this gene, or if a symbiont or other organism associated with the ctenophore tentacle bulb is responsible for

the presence of this transcript. *In-situ* hybridization data, using RNA probes designed from the partial *CHS* gene, suggest that the expression of the *chitin synthase* transcript is genuine.

Chitin histochemistry demonstrates the likely presence of chitin in a portion of ctenophore tentacle bulbs in two divergent species, and *in-situ* hybridization shows expression of the partial putative *CHS* gene localized to the tentacle bulbs as well. With the inability to reconcile the absence of a well-supported *chitin synthase* ortholog in transcriptomic and genomic data with positive chitin labeling and expression of a partial *chitin synthase* gene it remains unclear whether ctenophores produce chitin endogenously. If ctenophores do synthesize chitin, the genes for chitin metabolism may be too divergent to identify with current bioinformatics methods. Ctenophores are strikingly divergent with respect to the genetic components of major physiological systems, most notably nerves and muscles, which may have arisen independently in the ctenophore lineage (Ryan et al 2013; Moroz et al 2014). Given the unusual aspects of other ctenophore molecular pathways, it is possible that any endogenous chitin synthesis that may be occurring is doing so via proteins containing divergent domains.

## A1.5 Tables

Table A1.1 – Degenerate Primers Deployed to Clone *Chitin Synthase* from ctenophore *P. bachei*

| Primer | Sequence |
|---|---|
| BasalCSBLOCK-A1 (forward primer) | 5'AGCGGTGGTCCCAGGTNATGTAYATG3' |
| BasalCSBLOCK-E1 (reverse primer) | 5'CCGGTCCTCGCCCATRTCYTT3' |

## A1.6 Figures

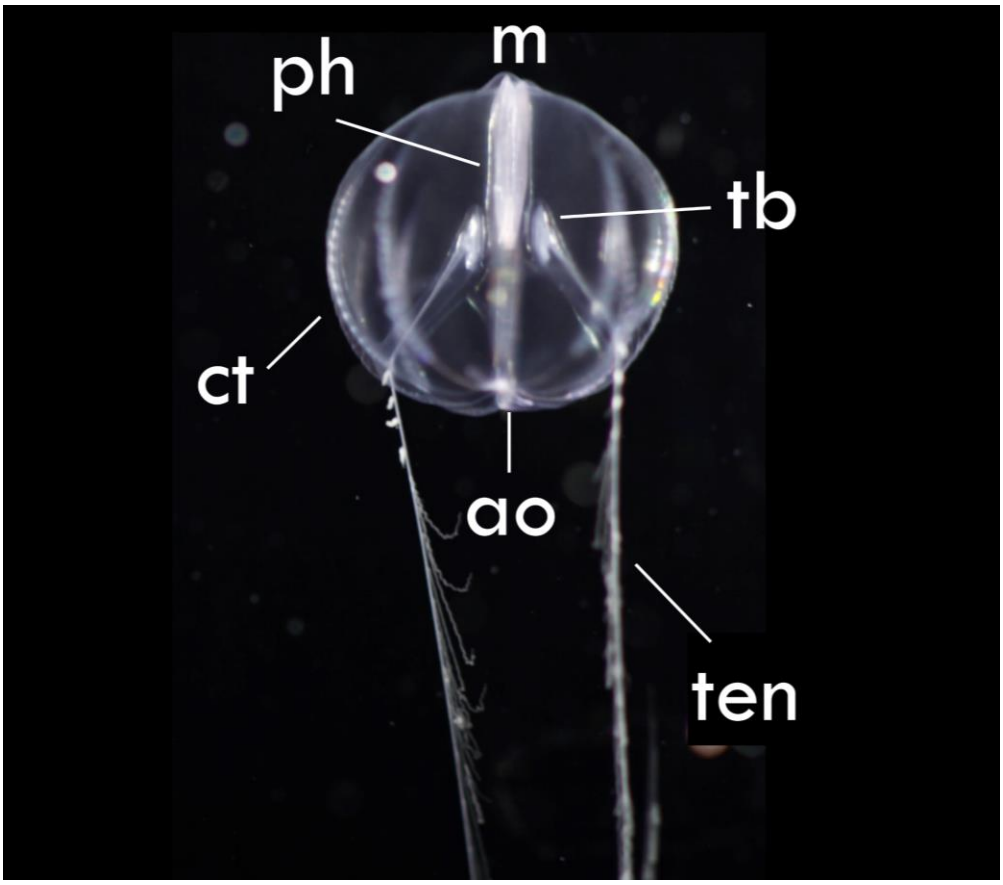


**Figure A1.1 – Live cydippid ctenophore *Pleurobrachia bachei*.** The main body plane is the oral/aboral axis. Ctenophores swim by beating cilia along their ctenes, or comb rows. These structures appear iridescent from light refracting off the comb plate.

ao – apical organ, ct – ctene, m – mouth, ph – pharynx, ten – tentacle, tb – tentacle bulb

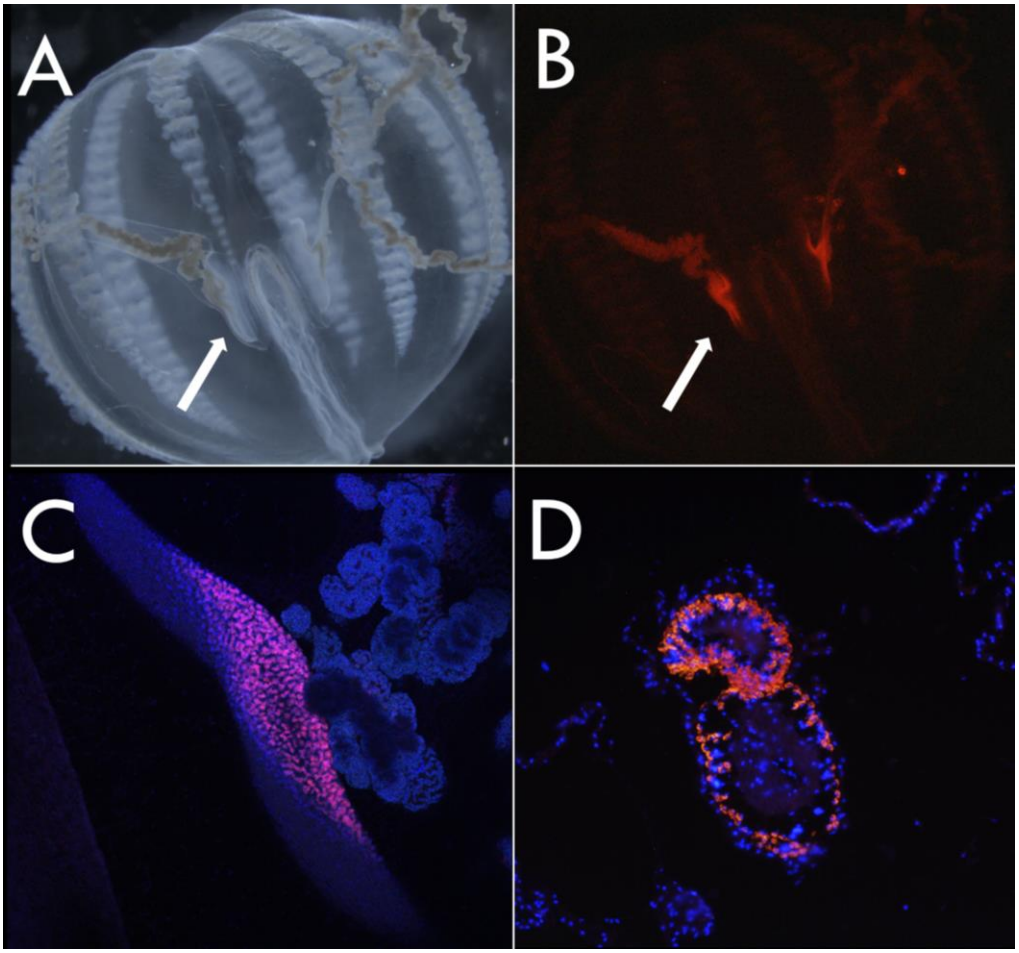


**Figure A1.2 – Chitin affinity histochemistry in ctenophore *Pleurobrachia bachei*.** A.) Brightfield stereoscope image of a whole *Pleurobrachia*. The comb rows, pharynx, tentacles, and tentacle bulbs (white arrow) are clearly visible. B.) Stereoscope image of fluorescent chitin affinity histochemistry using a chitin binding domain probe complexed to Alexa-546. Chitin labeling (red) is present in the tentacle bulbs (white arrow) and emerging tentacle. C.) Confocal image composite stack (10x) of a tentacle bulb stained for chitin (red) and nuclei (blue). The outer-facing portion of the tentacle bulb is labeled with chitin. D.) DMR microscope image (20x) of a sectioned *Pleurobrachia* tentacle bulb. Chitin labeling (red) appears to be largely extracellular.

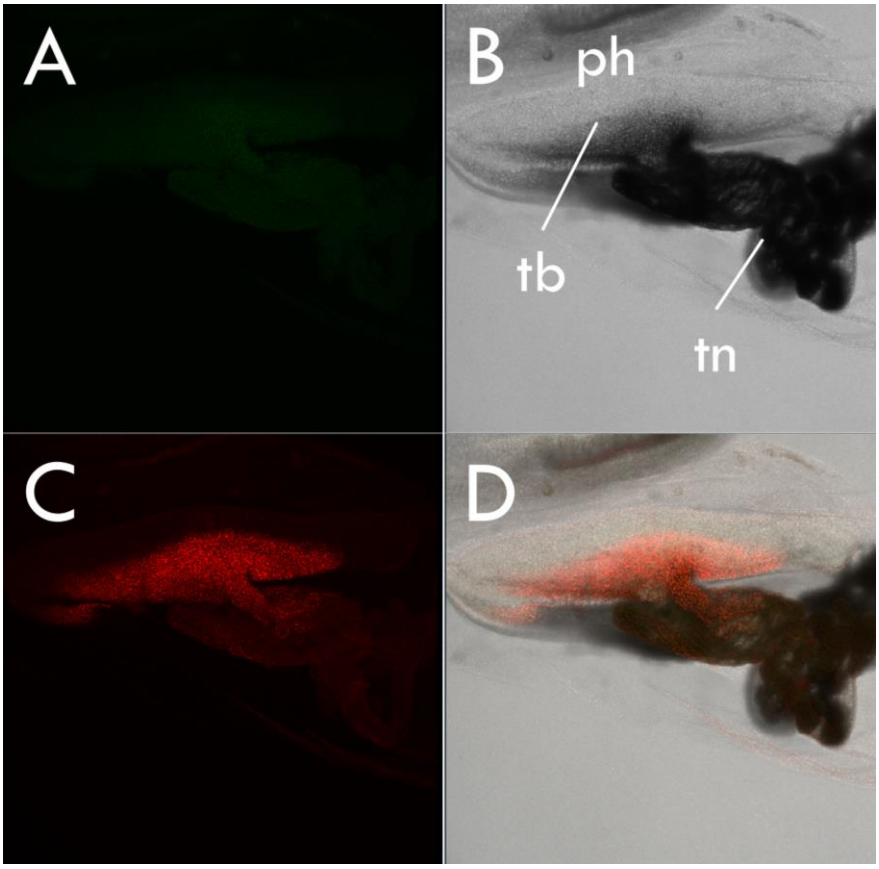


**Figure A1.3 – Confocal image of isolated tentacle bulbs and viscera of *Pleurobrachia bachei*.** A.) Autofluorescence (green) of *P. bachei* tentacle bulb and tentacle. B.) Brightfield image of the pharynx (ph), tentacle bulb (tb), and tentacle (tn). C.) Stack (10x) of ctenophore viscera labeled with CBD-546. Chitin labeling (red) is present in the outer-facing portion of the tentacle bulb and on the surface of the emerging tentacle. D.) Composite DMR microscope image (20x) of a sectioned *Pleurobrachia* tentacle bulb. Chitin staining does not occur in all areas of the tentacle bulbs, but is concentrated on the side from which the tentacle emerges and connects.

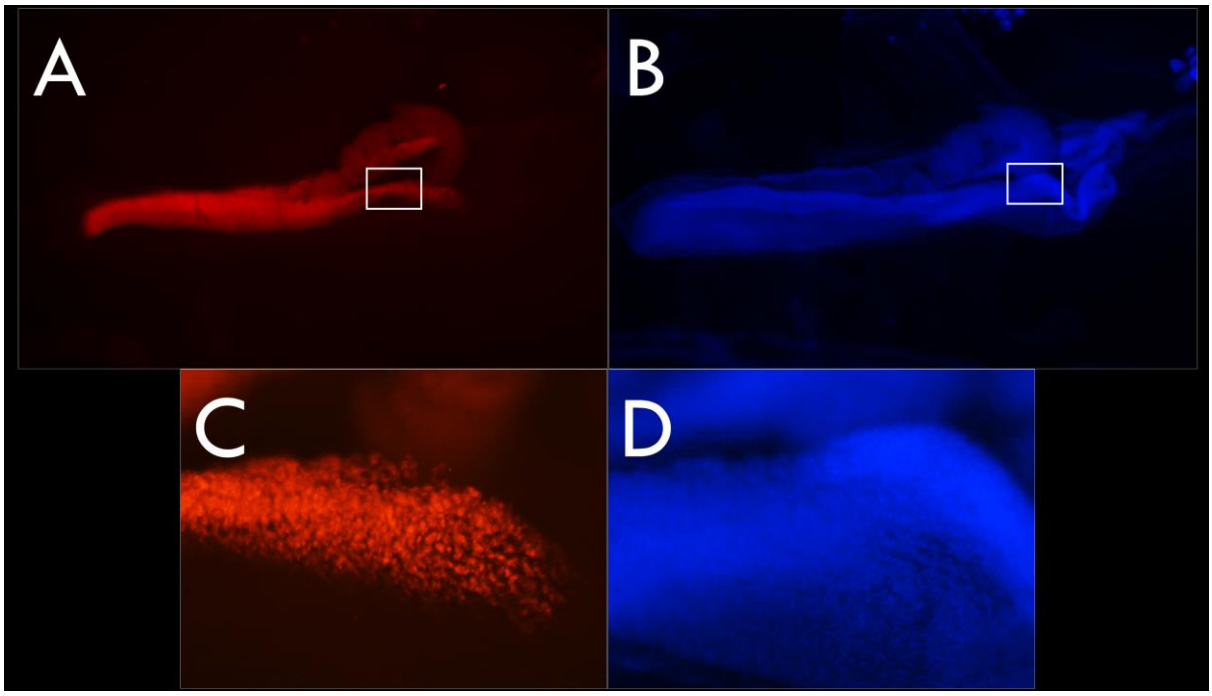


**Figure A1.4 – Fluorescent chitin histochemistry of *Mnemiopsis leidyi tentacle bulb*.** Chitin staining does not occur in all areas of the tentacle bulb, but is concentrated on the side from which the tentacle emerges and connects.

A.) Chitin labeling (red; CBD-546) in isolated *M. leidyi* tentacle. Chitin labeling is present along the outward-facing portion of the bulb, where the emerging tentacle connects to the tentacle bulb. B.) Nuclei staining (blue; DAPI) of *Mnemiopsis leidyi* tentacle bulb, showing the morphology of the bulb and the attached tentacle. C.) Inset from panel A (white box) – showing punctate chitin staining. D.) Inset from panel B (white box).

Images taken on a stereoscope.

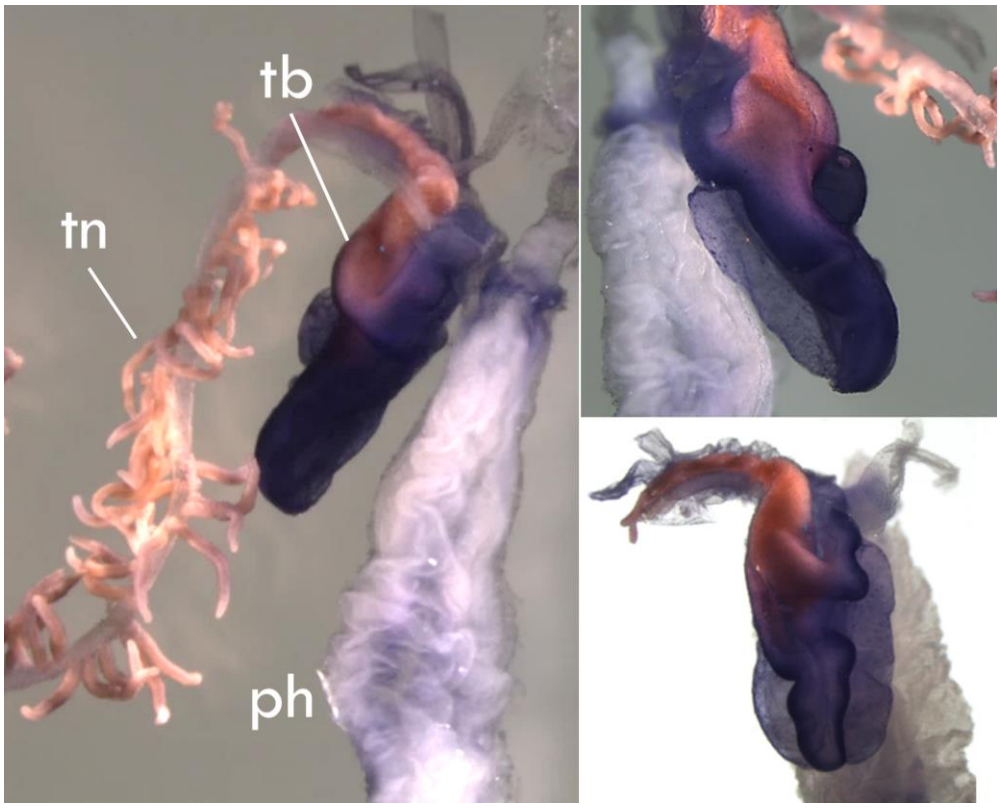


**Figure A1.5 – Expression of putative *chitin synthase* in the ctenophore *Pleurobrachia bachei*.** Antisense probe showing widespread labeling (purple) in the *Pleurobrachia* tentacle bulb. Labeling is concentrated in the lateral ridges of the bulb, which contain emerging tentillae, muscles, and other (unknown) cell types. No expression is seen in the tentacle or emerging tentacle structure. Images were taken on a stereoscope.

Ph – pharynx, tb – tentacle bulb, tn – tentacle.

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## Appendix A1 Supplemental Data

Supplemental Data A1.S1 – Degenerate primer design

Block A, width = 46

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          Amphemido1   637 KWKTPYGYRFQWKIGTSKMPFVVHFKDNLKVRNKKRWSQVMYMKYI
          Amphemido2   637 KQKTPYGYRFMWLIDNCXMPFMIHLKDNHKVRNKKRWSQVMYMKFI
          Hydramagni   637 KLETPYGLQLKWRLPNGXMPFIIHLKDTVKVKSKKRWSQVMYMSYI
          Nematoste1   637 KMKTPYGMQLRWKLPGGXMPFTIHLKDNNKVKNKKRWSQVMYMSYV
          Nematoste2   637 KVKTPYGMQLRWKLPGGXMPFTVHLKDNTKVKNKKRWSQVMYMSYV
```

Block unknown__A

```
K  W  K  T  P  Y  G  M  Q  F  M  W  K  I  P  N  C  K  M  P  F  M  I  H  L  K
D  N  H  K  V  K  N  K  K  R  W  S  Q  V  M  Y  M  S  Y  I
```

**AGCGGTGGTCCCAGgtnatgtayatg -3'  Core: degen=8 len=12  Clamp: score=79, len=14 temp= 60.0**

```
AGCGGTGGTCCCAGgtnatgtayat -3'  Core: degen=8 len=11  Clamp: score=79, len=14
temp= 60.0

AAGAAGCGGTGGTCCcargtnatgta -3'  Core: degen=8 len=11  Clamp: score=74, len=15
temp= 60.2

GGAAGATCCCCAACTGCaaratgccntt -3'  Core: degen=8 len=11  Clamp: score=60,
len=17 temp= 61.0
```

Block E, width = 51

```
Amphemido1  (179)  1031 IFLTTAILHFSEGFALIHSLWYLLGLPAGYLFLLIFSTANLNDRSWGTREA
Amphemido2  (179)  1031 VFVTAAILHFFEGFALFQCLWYLLGLPSGYLLLLIYSTANLNDRRWGTREA
Hydramagni  (179)  1031 IFILTAILHGSEAPYVFHGVWYLLCLPSGYLLLTIYSVCNLTDRSWGTREV
Nematoste1  (179)  1031 IFVLAALLHPKEAYCLLNGIFYLLCLPSGYLLLTVYSIVNITDRSWGKXXX
Nematoste2  (179)  1031 IFMVAALMHPTEAYCLINGLWYLLCLPSGYLLLTIYSVVNMTDRSWGNEHD
```

Complement of Block unknown__E

```
A  M  E  F  L  M  K  D  M  G  E  D  R  W  M  C  T  L  M  V  E  A  G  W  R  L
E
ttyctrtacccGCTCCTGGCC -5'  Core: degen=4 len=11  Clamp: score=76, len=10
temp= 61.7
ctrtacccnctCCTGGCCACCTACACG -5'  Core: degen=8 len=11  Clamp: score=80,
len=16 temp= 62.6
tacccnctyctGGCCACCTACACGTGG -5'  Core: degen=8 len=11  Clamp: score=79,
len=16 temp= 63.6
```

Figure A1.S1 – Nucleotide and Amino Acid Sequences of Partial *P. bachei* putative *chitin synthase* fragment

> Pbachei_CHS_nucleo411bp
AGCGGTGGTCCCAGGTAATGTACATGTCCTATGTTCTTGATTACAAGCAGCGTTGTCTTG
GAGTTAACGATGATCAGACATTCATCTTAACAACTGATGCAGATGTCAACTTCACTCACG
ATTCAATGGAAGCACTGATCGGCTACATGGTGCAAGATGATAAAGTTGGTGCTGTGTGTG
GAAGGACACACCCAATGGGTAATGGCCCCCTGGTCTGGTACCAAGTATTCGACTACGCTG
TTGGTCACTGGTTCATGAAGGTGGCGAATCATGTATTTGGCTCAGTGCTGTGCTGTCCTG
GCTGCTTTTCCCTGTACCGGTGCAGAGCTGTAAGAAGTGTTCTTAGAGAATATTCTACCA
ATGTGAACGAAGCTTTTGAATTTTTAACCAAAGATATGGGCGAGGACCGG

>Pbachei_CHS_aa
MYMSYVLDYKQRCLGVNDDQTFILTTDADVNFTHDSMEALIGYMVQDDKVGAVCGRTHPM
GNGPLVWYQVFDYAVGHWFMKVANHVFGSVLCCPGCFSLYRCRAVRSVLREYSTNVNEAF
EFLTKDMGEDR

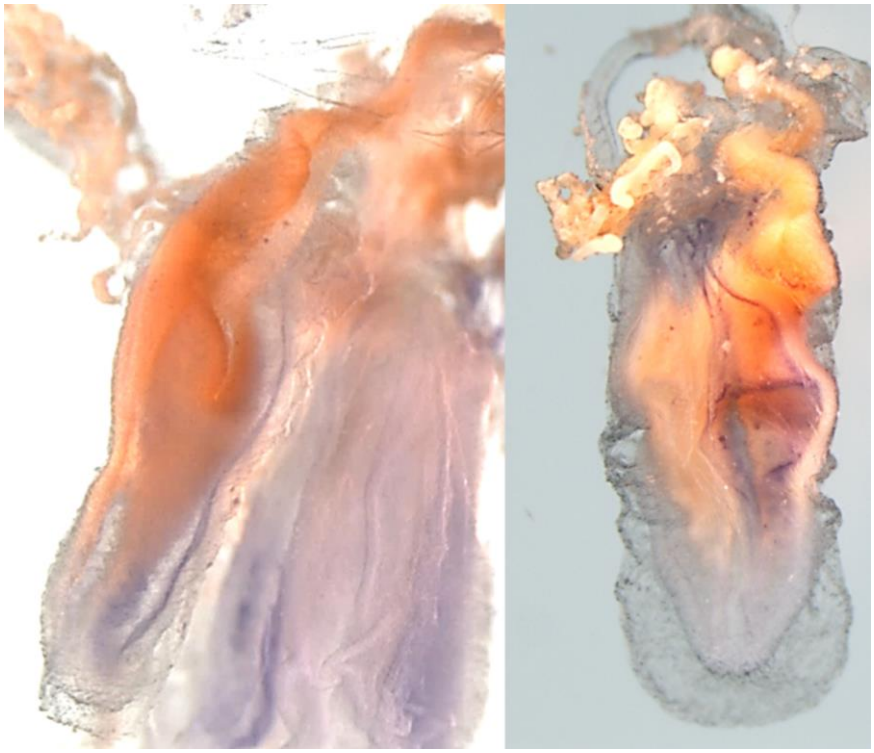

**Figure A1.S2 – Sense probe control of putative *Pb-CHS*.** Sense probe (negative control) incubation in *P. bachei* tentacles and tentacle bulbs. Lateral view of viscera and tentacle apparatus (left) shows low background (light purple). External (tentacle-out) view of tentacle bulb apparatus (right). Stereoscope images.

## 4.1 Conclusions and Speculation

The polysaccharide chitin is endogenously produced by more animal taxa than previously realized, including species with no rigid morphological structures. We believe that chitin can be used in structurally malleable tissues – such as cnidarian mesoglea – and that it can be complexed with a number of biological compounds (e.g. proteins or other glycomolecules) to achieve a diversity of structural presentations that do not involve firm fibrous chitin strands or mineralization. Cnidarians are deploying chitin in a variety of disparate morphological contexts – both in their evolutionarily unique stinging apparatus, and broadly in their soft tissues. While body plans in Cnidaria are diverse, most cnidarians are known for their gelatinous bodies and few taxa possess hard structures. Here I demonstrate that chitin, best known as a structural molecule in animals with hard endo- or exoskeletons, is widely present across Cnidaria and it occurs in diverse tissue types in multiple taxa.

Within cnidarian soft tissues, chitin appears to be in present in an unusual form – not as fibrils or string-like bundles complexed with minerals, but as discrete small masses in our formalin-fixed specimens. All cnidarian clades surveyed appear to possess the molecular capacity to synthesize chitin, as assessed by their possession of cognate *chitin synthase* homologs. Many cnidarian taxa express multiple paralogs of *chitin synthase*, indicating diverse roles and integral for chitin in their biological processes. I demonstrated that three *chitin synthase* paralogs are differentially expressed in the soft-bodied anemone *Nematostella vectensis* and that chitin is widespread in its tissues and some populations of stinging cells (cnidocytes). When chitin production is disrupted in developing anemones, the resulting larvae display a variety of morphological defects, suggesting that chitin is essential for tissue stability and cell mobility, as well as proper cnidocyte development and migration. Chitin appears to comprise a portion of the ctenophore tentacle bulbs, perhaps adding stability to the area in which the newly formed tentacle emerges. These findings center on an essential but as yet undescribed role for the polysaccharide

chitin in the biology of *Nematostella vectensis per se*, and more broadly in the Cnidaria and Ctenophora.

Our lab has also found that chitin is abundantly present in animal hydrogels, such as the gel found in Chondrichthyes electro-sensing organs (Ampullae of Lorenzini) (Chris Amemiya and Molly Phillips, unpublished data). We have also detected the presence of chitin in gastropod mucous; gastropod "slime" maintains its physical properties (i.e. sliminess) even when submerged in water, and it is not known what chemical characteristic of the slime allows it to maintain its form. It is possible that chitin, which is usually water-insoluble, helps this material retain its composition when complexed with proteoglycans such as mucin and other glycoproteins.

My findings and those of the Amemiya laboratory clearly show that chitin and its cognate *chitin synthase* genes are far more prevalent in metazoans than previously known, and suggest that chitin plays an important, hitherto unknown role(s) in the life histories of diverse animal taxa. Specifically, "soft chitin" has not been previously characterized in any animal and offers a new avenue for biological inquiry. I posit that soft chitin may be part of a larger biological complex involving proteoglycans and other biomolecules that could be applied to a variety of biological functions (e.g. mucosal secretions, structural components in tissues, electrical conduits, and components of the extracellular matrix).

The anomalies in embryonic development observed in the sea anemone *Nematostella* and in zebrafish when chitin synthesis is genetically disrupted (Tang et al 2015; this thesis) are consistent with a role for chitin in the extracellular matrix and its necessity for cellular processes – including cell migration. These aspects of cellular behavior must occur together with requisite gene regulatory networks to carry out their designated physiological processes. Thus, chitin and *chitin synthase* genes may have a much greater influence on biodiversity and evolution than previously known. Moreover, because we are finding chitin to be so prevalent across Metazoa, this biomolecule may have a larger role than appreciated with respect to large-scale marine biomass and nutrient cycling. The broader potential impacts of chitin-inhibiting pesticides on the

ecosystem should be assessed in non-ecdysozoan taxa. Lastly, the fact that soft chitin is used so widely in the biosphere may bode well for its use as a novel structural molecule for biomedicine and biotechnology.