PATTERNING EMBRYOS AND HEALING ADULTS: ANALYSIS OF CNIDARIAN DEVELOPMENT AT DIFFERENT LIFE CYCLE STAGES.

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By

Timothy R. DuBuc

Dissertation Committee:

Mark Q. Martindale, Chairperson
Michael G. Hadfield
Steven Robinow
Elaine Seaver
Yusuke Marikawa

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ABSTRACT

The primary goal of the following dissertation was to gain a greater understanding to the origins of animal diversity. Key transitions like the emergence of triploblasty and Hox genes are thought to be crucial innovations that allowed for the radiation of bilaterians. Using early animals such as placozoans and cnidarians, the following dissertation aims at understanding developmental phenomenon associated with patterning embryogenesis and regeneration. This study looks at the evolution, timing of activation and function of the first Hox genes in relation to different stages of development. These findings suggest that the first Hox genes likely patterned aspects of early development rather than the late developmental role often compared in bilaterians. In this dissertation, these results are compared with new evidence as to how placozoans pattern their adult body form and the emergence of anterior-posterior and dorsal-ventral patterning. Finally, this study describes the molecular and cellular processes involved with cnidarian wound healing. These results provide insight into how to initiate a regenerative response and may provide clues as to why regeneration is lost in many bilaterian lineages.
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INTRODUCTION

Arguably, one of the greatest mysteries of biology pertains to the beginning of animal life. Since the beginning of mankind, we have questioned where life began, accepted Darwinian evolution, discovered the role of DNA, and have started to reassess the origin of the first animals. Now, advances in DNA sequencing technologies have provided a unique opportunity to analyze extant animals to understand the genotype associated with early-branching animals in relation to morphological and functional innovation across the tree of life.

When comparing the morphological and genetic aspects of development, the first stages of embryonic development are thought to share a number of similarities among distantly related taxa. Every sexually produced animal begins life when a sperm fertilizes an egg and subsequent embryogenesis commences to form a complex multicellular individual. After fertilization, maternal RNAs guide the initial cleavae events until zygotic transcription takes over, patterning the remaining embryonic process. Most changes in the early embryonic program result in death, demonstrating that tight regulation of the first steps of development is crucial for survival. An increase in genotypic redundancy (e.g. gene copy number) is one way to buffer ‘survivability’ during development and may allow for continued innovation by releasing constraints on the normal function of individual genes. While this is a rather intuitive scenario, nature also has other ways of changing developmental programs through regulation of gene products.

The field of developmental biology is devoted to understanding the cellular and molecular regulation of normal develop. If we then compare normal development among
a diverse set of animals, we may be able to retrace the evolutionary history of genetic change(s) that allowed for the morphological diversity of today. Deleterious genetic change can result in disease formation and developmental abnormalities, therefore, it is important to understand the evolutionary and embryological origin of a specific tissue (e.g. muscle, neural, epithelial), to be able to assess and correct problems to the development. Knowing the evolutionary relationship of a tissue allows for direct comparison of species with varying properties (e.g. regenerative vs. non-regenerative) and provides clues as to how a condition may have been lost in one lineage compared to another (e.g. the direction of evolutionary change).

**The origin of Hox genes, from EvoDevo to early branching metazoans**

The field of evolutionary developmental biology (EvoDevo) was founded on the idea that the evolutionary history of metazoans can be resolved by comparing aspects of embryonic development. Within EvoDevo biological studies, gene content and regulation are instrumental to understanding the origin of morphological structure with respect to phylogenetic position. Using these tools we can hypothesize and systematically test how genotypic changes result in a change to form and function during metazoan evolution.

Bilateral symmetry is thought to have emerged after cnidarians (e.g. corals, sea anemones, jellyfish) split from their bilaterian relatives. The evolution of the Bilateria is closely associated with the emergence of triploblasty, or the separation of embryonic tissue into three germ layers (ectoderm, endoderm and mesoderm). The cnidarians are the phylogenetic sister taxa to bilaterians and have two tissue layers (diploblast), the ectoderm and endoderm. Genes associated with mesodermal lineages in bilaterians are
often found in the endodermal tissue in cnidarians (Magie, Daly, & Martindale, 2007; Magie, Pang, & Martindale, 2005; Martindale, Pang, & Finnerty, 2004; Scholz & Technau, 2003), and this layer is often referred to as the endo-mesoderm. It is unclear how the segregation of these tissue layers evolved, but the phylogenetic position and associated gene content of cnidarians provides hope that studies will help resolve this mystery.

The Hox genes, a class of homeobox transcription factors, are key regulators of anterior and posterior cellular fates during bilaterian development. Hox genes were first identified in Drosophila because phenotypes associated with Hox mutants exhibit an extreme homeotic transformation of structures along the anterior-posterior axis (Lewis, 1978; Struhl, 1981). Subsequent research on the genes associated with these drastic mutations, later called ultrabithorax (ubx) and antennapedia (antp), suggested that minor changes to DNA could result in macroevolutionary morphological innovation. When Hox genes were found to be highly conserved across broad taxonomic groups (mice and flies), details surrounding the functionality of these genes as master regulators of anterior/posterior fates brought great interest to the field of EvoDevo biology. Most importantly, the discovery of Hox genes helped stress the importance of understanding how genes pattern different regions of the body during development.

Since their discovery, Hox genes have been found in nearly every metazoan investigated (Moroz et al., 2014; Ryan et al., 2013; Ryan, Pang, Mullikin, Martindale, & Baxevanis, 2010; Srivastava et al., 2008, 2010; Swalla, 2006). Hox genes in vertebrates diversified to not only regulate spatial patterning along the anterior-posterior axis, but were co-opted throughout developmental time and pattern to pattern a host of structures
ranging from limbs to reproductive structures. The broad role of Hox genes in vertebrate development likely evolved as by-product of having four nearly-complete copies of the Hox cluster associated with total genome duplication events. More recently it was found that, in a wide array of vertebrates, Hox gene expression appears to begin patterning during gastrulation, well before their role in axial patterning (Forlani, S. Lawson, K. Deschamps, 2003; Iimura & Pourquié, 2006; Wacker, Jansen, McNulty, Houtzager, & Durston, 2004). Fly (Driever, Siegel, & Nüsslein-Volhard, 1990; Stauber, Jäckle, & Schmidt-Ott, 1999), mammalian (Paul, Bridoux, Rezsöhazy, & Donnay, 2011) and cnidarian (Chiori et al., 2009; Yanze, Spring, Schmidli, & Schmid, 2001) studies suggest that Hox gene function may begin as a maternally loaded protein, prior to fertilization. In flies, the maternally expressed Hox gene, bicoid, is an important regulator of anterior fates during Drosophila development. Bicoid protein is necessary for head and thorax development and functions at the earliest stage of development to designate anterior zones. Although expression analysis of vertebrate Hox genes suggests a potential maternal role during development, functional analysis of the early role of these genes has not been studied extensively. The vertebrate Hox ‘code’, or segregated anterior-to-posterior expression of the Hox cluster, is first activated during gastrulation in vertebrates and their deuterostome relative, the sea urchin. When comparisons of the presumptive conserved expression domains were assessed across all animals, including cnidarians which have both anterior and posterior Hox genes, it had been assumed that the ancestral role of these genes was for ‘late’ stage developmental patterning events. For example, the patterning of larval and adult structures in both cnidarian (anemone and jellyfish) and Drosophila development were thought to be comparable to aspects of
head, trunk and limb elements in vertebrate development. While there is some overlap between potential patterning domains across these three taxonomic groups, earlier stages of Hox gene deployment takes place may represent the true ancestral patterning role of the first Hox genes.

The first Hox genes arose in cnidarians (corals, anemones and jellyfish) (Chiori et al., 2009; Chourrout et al., 2006; DuBuc, Ryan, Shinzato, Satoh, & Martindale, 2012; Finnerty, Pang, Burton, Paulson, & Martindale, 2004; Ryan et al., 2007) although a protoHox-like gene is present in Placozoa (Jakob et al., 2004; Srivastava et al., 2008). Early bilaterians are thought to have all three classes of Hox genes, termed anterior, central and posterior based on their phylogenic relatedness and which often recapitulates the mRNA localization during development. The cnidarian-bilaterian ancestor likely had both anterior and posterior class Hox genes that were once found in a single cluster (Chiori et al., 2009; Chourrout et al., 2006; DuBuc et al., 2012; Ryan et al., 2007). Previous research has shown that Hox genes are expressed during various stages of cnidarian development (Gauchat D, Mazet F, Berney C, Schummer M, Kreger S, Pawlowski J, 2000; Jakob & Schierwater, 2007; Ryan et al., 2007; Yanze et al., 2001) although fragmentation of the cluster in diverse cnidarian lineages along with novel life history stages (jellyfish) has complicated identifying the ancestral function of these genes. The anthozoan cnidarians have a highly intact cluster of Hox genes relative to the hydrozoan Hydra (Chourrout et al., 2006; DuBuc et al., 2012; Ryan et al., 2007). Understanding the timing of activation, expression domain and regulation of anthozoan Hox genes will help resolve the ancestral role of these genes. Broadly, using this data will tell us more about the cnidarian-bilaterian ancestor and may provide
insight into how body plans in Hydrozoans have diversified compared to their anthozoan relatives.

Although true Hox genes first emerged in Cnidaria, the placozoans have a protoHox-like gene (Trox-2). Placozoans have a morphologically simplified body plan relative to their distant cnidarian relatives. Consisting of only six different cell-types, these enigmatic animals are thought to secondarily morphologically simplified. Few studies have addressed the growth and patterning behaviors associated with the normal biology of these creatures. Since their extensive analysis by Karl Grell, placozoans are thought to be composed of a dorsal (top) and ventral (bottom) epithelial layer. The dorsal epithelium has been proposed to contain predatory defense-related cells, shiny sphericals, while the ventral side is thought to serve as a feeding and motile surface. The genome of Trichoplax adhaerens, although other species are likely present, suggests that elements of dorsal-ventral patterning may have existed (e.g. presence of bmp and chordin-like genes). The biology and developmental patterning of Placozoa (including embryogenesis) is relatively unknown and studies could easily shape the way we think about animal evolution.

Wound healing and regeneration, aspects of post-embryonic development

If an animal is fortunate enough to make it through early development, the aging process continues and new tasks such as continued growth and maintenance take place. Cells within the body are also tasked with protection and identification of injury, and with extensive damage the cells would need to process signals to induce regeneration. In most vertebrates, including mammals, the ability to regenerate lost structures is greatly diminished. On the contrary, invertebrates like
cnidarians (Bergmann & Steller, 2010; Bibb & Campbell, 1973; Chera et al., 2009; Gierer, Berking, & Bode, 1972; Holstein, Hobmayer, & Technau, 2003; Passamaneck & Martindale, 2012; Shenk, Bode, & Steele, 1993), acoels (Perea-Atienza et al., 2013; Sikes & Bely, 2010; Srivastava, Mazza-Curll, van Wolfswinkel, & Reddien, 2014), planarians (Altincicek & Vilcinskas, 2008; Gaviño & Reddien, 2011; Tasaki et al., 2011; Wenemoser, Lapan, Wilkinson, Bell, & Reddien, 2012) and annelids (Bely & Sikes, 2010; Giani, Yamaguchi, Boyle, & Seaver, 2011; Novikova, Bakalenko, Nesterenko, & Kulakova, 2013) all possess a high capacity for regeneration. Regeneration among animals with high regenerative capacity is thought to share one common defining feature, the wound epithelium (Brockes & Kumar, 2008). When an injury occurs, an epithelial layer soon forms a new boundary between the outer environment and inner layers of the animal. This cell layer is thought to provide the necessary signals to initiate a regenerative response.

Defective wound healing or formation of the wound epithelium, has been linked to the inability to regenerate lost tissue in even highly regenerative animals (Mescher, 1976; Newman, 1974). It is therefore important to understand what signals are generated during wound healing to reinitiate embryonic programs of development (if a similar program is used) to regenerate lost structures. In particular, the cnidarian model system, Nematostella vectensis, is one of the few systems in which both embryonic development AND regeneration can be studied. Therefore, understanding the wound healing program in Nematostella, may provide clues as to how regeneration is initiated relative to the embryonic development of the same structure. Also, identification of a
shared wound healing response specific transcriptome among highly regenerative animals may help dissect the regulatory network necessary for proper regeneration.

The following dissertation research utilizes both stages of development (embryogenesis and adult regeneration) to address the origin and activation of developmental programs that regulate axially patterning in early metazoans.
Coral comparative genomics reveal expanded Hox cluster in the cnidarian-bilaterian ancestor

ABSTRACT

The key developmental role of the Hox cluster of genes was established prior to the last common ancestor of protostomes and deuterostomes and the subsequent evolution of this cluster has played a major role in the morphological diversity exhibited in extant bilaterians. Despite 20 years of research into cnidarian Hox genes, the nature of the cnidarian-bilaterian ancestral Hox cluster remains unclear. In an attempt to further elucidate this critical phylogenetic node we have characterized the Hox cluster of the recently sequenced Acropora digitifera genome. The A. digitifera genome contains two anterior Hox genes (PG1 and PG2) linked to an Eve homeobox gene and an Anthox1A gene, which is thought to be either a posterior or posterior/central Hox gene. These data show that the Hox cluster of the cnidarian-bilaterian ancestor was more extensive than previously thought. The results are congruent with the existence of an ancient set of constraints on the Hox cluster and reinforce the importance of incorporating a wide range of animal species to reconstruct critical ancestral nodes.
INTRODUCTION

*Hox* genes are homeobox transcription factors that play a critical role in developmental patterning (McGinnis, Levine, Hafen, Kuroiwa, & Gehring, 1984) and have been identified in every extant phylum outside of Porifera, Ctenophora, and Placozoa (placozoans have *ParaHox* but not *Hox* genes (Jakob et al., 2004; Ryan et al., 2010). The last common ancestor of protostomes and deuterostomes, which gave rise to 99% of all described animal species (Zhang, 2011), is thought to have had an extensive cluster of *Hox* genes. Furthermore, since the expression of these genes in the body of many protostomes and deuterostomes is correlated with their position within the cluster, this ancestral condition was likely important for regulation of transcription (reviewed by (Akam, 1989)).

Cnidarians (e.g. corals, sea anemones, hydroids, and medusae) are the only non-bilaterian phyla with *Hox* genes, and therefore critical to our understanding of the early evolution of the *Hox* cluster. The exact relationship of cnidarian *Hox* genes with those of the protostomes and deuterostomes has been difficult to establish and has been debated (Chiori et al., 2009; Chourrout et al., 2006; Finnerty & Martindale, 1997; Gauchat et al., 2000; Jakob & Schierwater, 2007; Kamm, Schierwater, Jakob, Dellaporta, & Miller, 2006; Ryan et al., 2007; Yanze et al., 2001). In addition, the genomic arrangement of *Hox* genes from sequenced cnidarian genomes have only been analyzed in *Nematostella vectensis* and *Hydra magnipapillata*. The *H. magnapapillata* genome shows no *Hox* cluster (Chapman et al., 2010) and the clustering in *N. vectensis* is limited to anterior *Hox* genes (Chourrout et al., 2006; Putnam et al., 2007; Ryan et al., 2007).
Most studies agree that cnidarians possess representatives of phylogenetically anterior Hox genes including paralogous group 1 (PG1) and paralogous group 2 (PG2) (Chiori et al., 2009; Chourrout et al., 2006; Finnerty & Martindale, 1997; Ryan et al., 2007). For example, the anthozoan Nematostella vectensis has PG1 (Anthox6 and Anthox6a) and PG2 genes (Anthox7, Anthox8a, and Anthox8b) (Chourrout et al., 2006; Finnerty & Martindale, 1997; Ryan et al., 2007) and orthologs of these genes have been identified in the medusozoan Clytia hemisphaerica (Chiori et al., 2009).

The relationship of the other Hox genes in cnidarians is more controversial. For example, N. vectensis has a set of paralogs (Anthox1 and Anthox1a) that have often been affiliated with posterior Hox genes (PG9-14) (Figure 1.1) (Chourrout et al., 2006; Finnerty & Martindale, 1997; Ryan et al., 2007). Three similar genes are found in C. hemisphaerica (Chiori et al., 2009). Our analyses, along with those of others (e.g., (Chiori et al., 2009; Chourrout et al., 2006)), show that these genes share almost equal phylogenetic affinity with central Hox genes (PG4-8) as they do with posterior Hox genes (Figure 1.1). Therefore, either cnidarians have lost the ancestral central Hox gene, or the cnidarian-bilaterian ancestor possessed a single gene that gave rise to the bilaterian central and posterior genes as well as the cnidarian genes related to Anthox1 and Anthox1a (Ryan et al., 2006).

In addition to disagreements as to the phylogenetic affinity of cnidarian Hox genes, there are differences in opinion as to the extent and nature of the cnidarian-bilaterian Hox cluster. Some authors have suggested, based on variations of expression domains in distant cnidarian species, that the cnidarian-bilaterian ancestor lacked bona fide Hox genes (Kamm et al., 2006; Schierwater & Kamm, 2010). Others have asserted that the
*Hox* cluster of the last common ancestor of cnidarians and bilaterians consisted of a maximum of two anterior-like *Hox* genes (Chourrout et al., 2006).

The criteria used by Kamm and coauthors to define what they call a “*Hox* system” are: 1) *Hox* genes are closely linked within the genome along the same chromosome; and 2) The *Hox* genes are primarily responsible for patterning structures along the primary body axis of a developing embryo (Kamm et al., 2006). In *N. vectensis*, the expression of *Hox* genes is restricted along the primary body axis (Martindale et al., 2004; Ryan et al., 2007), but with only a partial cluster, it is perhaps difficult to assess these criteria *in toto*. However, the number of cnidarians examined to date and the extent of experiments involving cnidarian *Hox* genes are both far too few to rule out the existence of a functional *Hox* cluster in the cnidarian-bilaterian ancestor.

Recently, the genome of the staghorn coral *Acropora digitifera* was published (Shinzato et al., 2011). *A. digitifera* and *N. vectensis* are both members of the class Anthozoa however, it is estimated that these two lineages diverged from each other some 500 million years ago (Shinzato et al., 2011). An analysis in another coral, *Acropora formosa*, was the first to show linkage between the *Hox*-related gene *Eve* and the PG1-related *Hox* gene *Anthox6* in a cnidarian (D. Miller & Miles, 1993). In this paper, we characterize the *Hox* cluster of *A. digitifera* and show that the *Hox* cluster in the last common ancestor of cnidarians and bilaterians was more elaborate than previously documented. This conserved synteny is consistent with an ancient functional constraint present in this ancestral *Hox* cluster.
Figure 1.1 - Phylogenetic analysis of Hox, ParaHox, Hox-related homeodomains using genomic data from Acropora digitifera. Support values at nodes are based on 100 bootstraps. Nematostella vectensis taxa are prefixed with Nv and are in orange. Acropora digitifera taxa are prefixed with Ad and are in red. Clytia hemisphaerica taxa are prefixed with Ch and are in purple. All other taxa represent bilaterian homeodomains (Bf = Branchiostoma flordiae, Hs = Homo sapiens, Dm = Drosophila melanogaster, Ct = Capitella telata, Cs = Cupiennius salei). *Anthox7, 8, and 8a have been most often associated with bilaterian Hox2 (e.g., Chourrout et al. 2006 and Ryan et al. 2007), but this new analysis suggests the possibility (low bootstrap support notwithstanding) that Hox2 was lost in the cnidarian lineage and Anthox7/8 was lost in the Bilateria.

MATERIALS AND METHODS

Homeodomains from Nematostella vectensis, Clytia hemisphaerica, Acropora digitifera, Branchiostoma flordiae, Homo sapiens, Drosophila melanogaster, Capitella telata, and Cupiennius salei were aligned by eye. See Table 1 for accession numbers. We used ProtTest (AIC criteria) to determine that the LG+Gamma model best fit our multiple sequence alignment (Abascal, Zardoya, & Posada, 2005). We ran RAxML version 7.2.8 with the following command line: raxmlHPC-PTHREADS -T 6 -n hox -s hox.phy -m PROTGAMMALG -k -f a -N 100 -x 43241 (Stamatakis, Hoover, & Rougemont, 2008). The resulting tree is shown in Figure 1.1. We used version 2.5.5 of Augustus (Stanke, Tzvetkova, & Morgenstern, 2006) to predict genes on the A. digitifera scaffold (DF093930) with the following command line: augustus --species=human -- AUGUSTUS_CONFIG_PATH= augustus.2.5.5/config DF093930.fa.

RESULTS

The Acropora digitifera genome (Shinzato et al., 2011) has the most extensive Hox cluster of all reported cnidarians. Phylogenetic analysis of twelve homeobox genes from the genome of Acropora digitifera revealed a total of six Hox, one ParaHox, three Mox, one Eve, and one HlxB9 gene (Figure 1.1, Table 1). Two of the identified Hox genes
(Anthox6 and Anthox7/8) along with the homeobox genes Hlx9 and Eve were found to be in the same 5’-3’ scaffold orientation as in N. vectensis (Figure 1.2A/B). Contrary to N. vectensis, the 3’-end of the A. digitifera Hox cluster contains the central/posterior Hox gene Anthox1a. Interestingly, Anthox1a in N. vectensis is flanked by the pseudogene Anthox9, suggesting additional genes may have been present in the cnidarian-bilaterian cluster (Figure 1.2).

Table 1 - Acropora digitifera homeodomain sequence information and alignment with other taxa. A) Sequences are organized by their linkage in the genome, where genes sharing the same color are found on the same scaffold. Genes in white were not linked to other Hox or Hox-related genes. (* denotes gene having an intron) B) Allignment of homeodomain sequences.

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<th>Strand</th>
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Figure 1.2 - The anthozoan complement of Hox genes and the implications of the evolution of the Hox cluster. Comparing the genomic linkage of Hox genes in the sea anemone Nematostella vectensis and the staghorn coral Acropora digitifera confirms that cnidarians once had a Hox cluster that contained both anterior and posterior/class Hox genes. (A) The Hox cluster of Nematostella vectensis includes the anterior Hox genes Anthox6 (PG1), Anthox8b (PG2), Anthox8a (PG2), and Anthox7 (PG2) as well as the Eve homeobox gene. (B). The Hox cluster of Acropora digitifera includes the anterior Hox genes Anthox6 (PG1) and Anthox7/8 (PG2), and the posterior/class Hox gene Anthox1a (PG4-14), as well as the Eve homeobox gene. Another gene HlxB9 (also named MNX) is found upstream of Anthox6 in the Hox cluster of both genomes (data not shown). (C) The metazoan tree of life with inferred ancestral Hox clusters. The ancestor to protostomes and deuterostomes is thought to have had two anterior class Hox genes (Hox1 and Hox2), one paralogous group 3 gene (Hox3), three central class genes (Hox4, Hox5, and Hox6-8), one posterior class Hox gene (Hox9-14), and one Eve homeobox gene. Because of the extended cluster in Acropora digitifera, we can now say that the cnidarian-bilaterian ancestor had, at least, two anterior class Hox genes (Anthox6 and Anthox7/8), a central/posterior class Hox gene (Anthox1/1a), and the Eve homeobox gene. It is unclear at what point the genomic rearrangement involving the Eve homeobox gene occurred. The origin of the PG3 Hox genes also is not clear. * Anthox7/8 has been categorized as a PG2 Hox gene in previous publications, but it is possible, based on our current phylogenetic analysis, that Anthox7/8 descended from a Hox gene that was lost in bilaterians. Based on the genomic orientation of these genes, we also believe the ancestor likely had a fourth Hox gene potentially related to Anthox9. Abbreviations: PG = paralogous group, Ax = Anthox.
Additionally, a single ParaHox gene (Gsx) was identified in *A. digitifera*, where *N. vectensis* has two clustered ParaHox genes (Gsx and a gene related to *Cdx* and/or *Xlox*) (Ryan et al., 2007). A Mox cluster was also identified and contains three genes, where *N. vectensis* has four genes. *A. digitifera* and *N. vectensis* have similar Hox and Hox-related gene complements, but the three randomly-linked *N. vectensis* PG2-related genes (*Anthox7, 8, and 8a*) are represented by only a single gene (*Anthox7/8*) in *A. digitifera*. This is congruent with the assertion that the *Anthox7, 8, and 8a* genes were most likely the result of recent duplications in the *N. vectensis* lineage (Ryan et al., 2007).

The Hox cluster of *A. digitifera* is situated on the end of a 270-kilobase scaffold. We predict 16 genes downstream of the *A. digitifera* Hox cluster (Figure 1.3A). Of these, only *Rac3, Dars, Psma2*, and the Hox-related *HlxB9* are linked to the cluster in both anthozoans (Figure 1.3). *Dars* and *Psma2* are adjacent to each other in both genomes, but are on opposite sides of the cluster. Seven of the 16 downstream genes appear not to be linked to the *N. vectensis* cluster (Figure 1.3C).
Figure 1.3 - Genes associated with the Hox cluster of Acropora digitifera and their location in the Nematostella vectensis genome. (A) The Hox cluster of Acropora digitifera occurs on the scaffold with the NCBI accession DF093930 (total size = 271,156 base pairs). Genes on this scaffold were predicted with the Augustus gene finder (Stanke et al., 2006). If the resulting protein had a reciprocal best BLAST hit with a human RefSeq, the gene name associated with that RefSeq is used in the figure; if not, the genes were numbered from g1 to g16. Predictions and spatial relationships are roughly to scale. (B) The Hox cluster of Nematostella vectensis occurs on the scaffold with the JGI ID# 61 (total size = 1,073,712 base pairs) in v1.0 of the JGI assembly. This scaffold includes three non-homeobox genes (DARS, PSMA2, and RAC3) that are also associated with the A. digitifera Hox cluster. N. vectensis genes not associated with the A. digitifera Hox cluster are not displayed. The broken line and broken box indicate that the distance between the HlxB9 gene and the rest of the scaffold is not to scale. (C) Those genes in the A. digitifera cluster not on scaffold 61 in N. vectensis are listed along with the corresponding JGI scaffold number. The coordinates below the A and B panels indicate the scaffold coordinates of the region shown. Genes in red are Hox and Hox-related genes. Genes in blue are non-homeobox genes that are associated with the Hox cluster in both A. digitifera and N. vectensis.

DISCUSSION

For the first time, we show that a cnidarian Hox gene (i.e., the A. digitifera Anthox1a gene) related to the central/posterior Hox genes of the Bilateria is linked to a cluster of genes that includes anterior Hox genes and an Eve gene (Figure 1.2B). This new evidence and the genomic linkage between the Anthox1a and Anthox9 genes of N. vectensis suggest that a larger cluster almost certainly existed in the cnidarian-bilaterian ancestor (Figure 1.2C). The N. vectensis Anthox9 is thought to be a highly derived pseudogene and is unstable in phylogenies making it difficult to know its true identity (Kamm et al., 2006; Ryan et al., 2007). An ortholog of Anthox9 was not identified in the A. digitifera genome. These data show that the Hox cluster of the cnidarian-bilaterian ancestor consisted of at least two anterior-related Hox genes, one central/posterior-related Hox gene, an Eve homeobox, and perhaps another gene related to Anthox9 (Figure 1.2C).
Despite many examples of rearrangements and breakages, the persistence of a Hox cluster in disparate bilaterian lineages is attributed to constraints on the developmental regulation of these genes. The absence of Hox clustering in the H. magnipapillata genome (Chapman et al., 2010) and the partial clustering in N. vectensis, a genome remarkable for its large-scale conserved synteny with vertebrate genomes (Putnam et al., 2007), presented the possibility that these regulatory constraints were established after cnidarians and bilaterians diverged. Nevertheless, the presence of an extensive Hox cluster in a third cnidarian lineage suggests that this regulatory constraint dates back prior to the last common cnidarian-bilaterian ancestor. The conservation of the clusters in the two anthozoan lineages despite the many genomic events that appear to have occurred in the region (Figure 1.3) reinforces this view.

Our current understanding of the early evolution of the Hox cluster is still at an early stage. As more cnidarian genomes are sequenced, and as experimental techniques are established for these new model systems (including A. digitifera), the structure and function of the cnidarian-bilaterian ancestor’s Hox cluster will become even clearer. A better understanding of the similarities and differences between the Hox clusters of the cnidarian-bilaterian ancestor and the protostome-deuterostome ancestor will help explain the origin of bilaterian-specific complexities. Furthermore, more comprehensive surveys into the independent variation of Hox genes in cnidarian lineages will lead to a better understanding of the role these genes have played in establishing the vast diversity of body plans exhibited in the Cnidaria.
CHAPTER 2

Identifying the evolutionary role of Hox genes: clustering, duplications, axis specification and gastrulation

ABSTRACT

The emergence of Hox genes in the cnidarian-bilaterian ancestor is thought to be a crucial innovation for bilaterian evolution. The ancestral role of the first Hox genes has often been thought to have little role in patterning the anterior-posterior axis in cnidarians. In fact, the orientation and presence of a ‘true’ anterior-posterior axis in cnidarians is highly debated. If the phylogenetic relationship of Hox genes also dictates which genes pattern anterior, central and posterior (as in bilaterians) then the oral pole, also the site of gastrulation in anthozoans, is the anterior end of the animal. Conversely, if the swimming direction of the larva corresponds to the anterior region, then the localization of anterior Hox genes around the site of gastrulation becomes confounding. The following research uses the cnidarian model system, Nematostella vectensis, to understand the role of the first Hox genes. This study compares the timing of expression of Hox genes with two other cnidarians, Acropora digitifera and Fungia scutaria, to assess the level of conservation among cnidarians. Functional knockdown of Nematostella Hox genes suggests a much earlier role of developmental patterning prior to gastrulation, and suggest that anterior (Ax6) and posterior (Ax1) genes may pattern presumptive ectodermal and endodermal regions during blastula formation. Interestingly, paralogs of these genes become highly involved in the asymmetric expression of Hox genes during gastrulation and suggests that multiple waves of activation pattern different developmental time points of cnidarian development.
INTRODUCTION

Discovery and properties of bilaterian Hox

Arguably one of the most thoroughly studied group of bilaterian genomic innovations, the Hox genes are a class of homeobox transcription factors involved with patterning anterior-posterior domains in nearly all bilaterally symmetrical metazoans examined (Hejnol & Martindale, 2009; McGinnis & Krumlauf, 1992; Ryan et al., 2007; Swalla, 2006). Hox genes are a distinct family of genes derived from the NK homeobox superclass (Holland, 2013; Larroux et al., 2007) and have undergone tandem duplications in different animal lineages (Pascual-Anaya, D’Aniello, Kuratani, & Garcia-Fernández, 2013). Three features were thought to characterize the functionality of Hox genes: 1) conserved linkage of genes on the same chromosome within the genome, 2) expression in a sequential order, temporally and/or spatially that corresponds to their position in the genomic cluster, and 3) their role in patterning cell fates along the anterior/posterior axis. Generally, invertebrate bilaterians have 7-11 Hox genes that have been phylogenetically organized into anterior, central and posterior groups (Figure 2.1A) (Swalla, 2006). Through whole genome duplications, vertebrates have an even more complex repertoire of Hox genes with mammals having four and teleost fish possessing as many as seven copies of each (Duboule & Morata, 1994; Krumlauf & Hill, 1994).

As more genomic data became available from a wider taxonomic range, the features that once defined Hox functionality were found to be an oversimplification of the level of variation found in nature (Monteiro, Ferrier, & Duboule, 2007). Insect studies alone have
shown a great diversity in the organization of the Hox cluster, with mosquitos and beetles having a more intact cluster than well studied Drosophila species (Powers et al., 2000). Recent findings also now suggest that the spatial expression of Hox genes can be independent of clustering (Kmita & Duboule, 2003; Wacker et al., 2004). Although it is now apparent that a high level of variability exists concerning the layout and gene copy number of Hox genes among diverse taxa, a common theme has remained, that suggests Hox genes function to specify tissue fates along the anterior posterior axis based on their phylogenetic relatedness. In other words, phylogenetically anterior genes pattern more anterior regions of animals, while central and posterior class Hox genes are found to pattern more central and posterior domains.

Although the Hox genes pattern anterior to posterior regions during development, the timing and tissue localization is quite different between flies and vertebrates. In Drosophila, collinear expression of Hox genes is ectodermally derived and appears during segment formation, after gastrulation. Prior to transcription of the Hox cluster, gap and pair rule genes compartmentalize the embryo into distinct zones. Interestingly, an unlinked gene derived from the homolog of Hox3 in vertebrates (bicoid) (Stauber et al., 1999), is maternally expressed in the anterior region of the animal prior to the axial patterning role of the Hox cluster (Driever et al., 1990; St Johnston & Nüsslein-Volhard, 1992). Bicoid protein is necessary for proper head and thorax formation in Drosophila, and has a broader range of activity in other flies (Stauber, Taubert, & Schmidt-Ott, 2000). Although the Hox model, as identified by Drosophila research may provide insight into establishment of anterior posterior patterning, Hox expression in vertebrates appears much earlier in development.
In vertebrates, \textit{Hox} genes pattern a number of ectodermal and endodermal structures throughout developmental time, although studies have often compared aspects of late embryonic patterning to the collinear expression of \textit{Hox} genes in flies (McGinnis & Kuziora, 1994; Pearson, Lemons, & McGinnis, 2005). During vertebrate gastrulation, \textit{Hox} genes are transcriptionally activated around the blastopore region of the animal and are shuttled in with the developing mesoderm in an anterior to posterior fashion (Forlani, S. Lawson, K. Deschamps, 2003; Iimura & Pourquié, 2006; Wacker et al., 2004). \textit{Hox} genes are known to pattern mesodermal regions (axial, paraxial and lateral plate mesoderm) around the neural tube (Burke, Nelson, Morgan, & Tabin, 1995; Carapuço, Nóvoa, Bobola, & Mallo, 2005; Durston, 2012). It has been proposed that Hox proteins shuttled in during gastrulation provide the initial positional identity of their regions, and that signals are then transferred to the neuroectoderm (by retinoic acid signaling), another region patterned by \textit{Hox} genes (Lloret-Vilaspasa et al., 2010; Wacker et al., 2004).

Furthermore, \textit{Hox} genes can even be expressed maternally during development. In mice and bovine oocytes, anterior, central and posterior class genes are detectable by quantitative pcr (qPCR) during oocyte maturation, prior to fertilization (Paul et al., 2011). The early activation of \textit{Hox} genes during embryogenesis (\textit{bicoid} in flies, during gastrulation in vertebrates) could represent a taxonomically restricted specialization of \textit{Hox} genes, but a deeper understanding to the activation and function of \textit{Hox} genes during embryogenesis is required for understanding the origin of \textit{Hox} patterning.
Figure 2.1 – Distribution of cnidarian Hox-related genes in comparison to the protostome-deuterostome ancestor. A) The linkage of Hox genes in the protostome-deuterostome ancestor consisted of at least seven Hox genes in close proximity to the homeobox gene even-skipped (eve). B) The cnidarian bilaterian ancestor had both anterior (yellow/green) and posterior-related (light blue-grey) genes found in the Hox cluster. C) Acropora digitifera had a Hox complement more conserved with the ancient cnidarian-bilaterian cluster. D) Nematostella vectensis has a Hox cluster with independent duplications (Ax8b, Ax8a and Ax7) and is fragmented relative to the cnidarian-bilaterian ancestor.

Origin of Hox genes

Genomic resources from four early-branching animal groups have shed light into the origin of Hox genes. Studies on Porifera (sponges) and Ctenophora (comb jellies) have independently concluded that both taxonomic groups lack Hox genes (Ryan et al., 2013; Srivastava et al., 2010). There is evidence that parahox-like genes were the sister family to Hox genes and may have been present in the ancestor of the first animals (Fortunato et al., 2014). Phylogenetic studies suggest these two phyla are the earliest branching animals, basal to both Placozoa and Cnidaria (corals, anemones and jellyfish) (Collins, 1998; Dunn et al., 2008; Hejnol et al., 2009; Ryan et al., 2013). These
results also suggest that placozoans diverged from other metazoan lineages prior to cnidarians, and that cnidarians are the sister group to bilaterians. The first protoHox-like gene, *Trox-2*, was identified in Placozoa and is an ortholog of the cnidarian genes *Cnox2/Anthox2/GSX* (Hayward et al., 2001; Jakob et al., 2004; Ryan et al., 2007; Yanze et al., 2001). It has been proposed that a protoHox-like gene may have given rise to the elaborated *Hox* cluster found in cnidarian lineages (Chiori et al., 2009; Chourrout et al., 2006; Ryan et al., 2007). *Trox-2* expression is localized to the oral/feeding surface of the animal (see Chapter 3), although embryonic expression has not been determined. If *Trox-2* expression is still representative of the ancestral condition, this expression suggests that the first *Hox* genes may have had a role in patterning oral/digestive regions in the placozoan-cnidarian common ancestor. Orthologous cnidarian genes of *Trox-2* are expressed along the oral and endomesoderm region of anthozoan cnidarians (corals and anemones) and may be involved with patterning sensory cells (Hayward et al., 2001; Ryan et al., 2007). In two hydrozoan cnidarians, *Hydractinia* and *Hydra*, expression is found along both oral and aboral domains (Cartwright, Bowsher, & Buss, 1999; Shenk et al., 1993) and may have a broader patterning role.

The cnidarian model system, *Nematostella vectensis*, has been an instrumental tool for identifying and characterizing the repertoire of genes present in the cnidarian-bilaterian ancestor (Matus, Pang, et al., 2006; Putnam et al., 2007; Eric Röttinger, Dahlin, & Martindale, 2012; Saina, Genikhovich, Renfer, & Technau, 2009; Wikramanayake et al., 2003). In the lineage of animals that gave rise to cnidarians and bilaterians, the genomic developmental toolkit expanded to include true fibroblast growth factor (FGF) domains (Bertrand, Iwema, & Escriva, 2014), a wingless (*wnt*)
complement similar to bilaterians (Kusserow et al., 2005), and *Hox* genes belonging to both anterior and posterior classes (Figure 2.1B) (Chiori et al., 2009; Chourrout et al., 2006; DuBuc et al., 2012; Ryan et al., 2007). Due to the genomic resources (Putnam et al., 2007), ability to knockdown/misexpress genes (Michael J Layden, Röttinger, Wolenski, Gilmore, & Martindale, 2013), amenability to stable transgenics (Nakanishi, Renfer, Technau, & Rentzsch, 2012; Renfer, Amon-Hassenzahl, Steinmetz, & Technau, 2010) and the ability to screen entire genomic expression profiles (Dubuc, Traylor-Knowles, & Martindale, 2014; Eric Röttinger et al., 2012) has pushed *Nematostella* to the forefront of cnidarian developmental biological studies. The *Hox* cluster found in *Nematostella* consists of more anterior-like *Hox* genes (Chourrout et al., 2006; Ryan et al., 2007). More recently, sufficient evidence emerged indicating that the cnidarian-bilaterian ancestor had a *Hox* cluster consisting of both anterior and posterior *Hox* genes (DuBuc et al., 2012).

The chromosomal organization of *Hox* genes in the cnidarian-bilaterian ancestor remains unclear. A comparison of the *Hox* cluster found in the coral *Acropora digitifera* and *Nematostella* showed that an independent duplication event occurred with the *Hox2* class of genes, giving rise to three genes in *Nematostella* (*Ax8b, Ax8a* and *Ax7*) from a single ortholog in *A. digitifera* (*Ax7/8*) (Figure 2.1C-D). Interestingly, the breakage between anterior and posterior genes (that are still linked in *A. digitifera*) in the *Nematostella* cluster occurs immediately after these duplicated genes (Figure 2.1D). Both clusters are flanked by the homeobox gene *hixB9* (Figure 2.1C-D) and a number of other conserved synteny regions have been found between cnidarians (DuBuc et al., 2012).
We have suggested a close relationship between the homeobox gene even-skipped (eve/evx) among vertebrate clusters, and an ortholog of eve is found between the anterior Hox1 genes (AdAx6 and NvAx6) and the Hox2-related genes AdAx7/8 or NvAx8b (Figure 2.1C-D). A posterior-like gene Ax1a is present in each genome and is linked to the Hox cluster in A. digitifera. Interestingly, a paralog exists for both the anterior (Ax6) and posterior (Ax1a) genes (named Ax6a and Ax1), and are unlinked to the genomic cluster in each species (Figure 2.1C-D) (Chourrout et al., 2006; DuBuc et al., 2012; Ryan et al., 2007). In total, Nematostella has five true anterior class genes (Hox1 homologs - Ax6, Ax6a; Hox2 homologs - Ax8b, Ax8a and Ax7), along with two posterior-like genes (Ax1 and Ax1a) (Figure 2.1D). Thus, utilizing other cnidarian species have allowed us to make more definitive predictions about the ancestral cnidarian/bilaterian Hox cluster.

Previous studies on the expression of NvAnthox genes suggested that the function of these genes may be involved in larval patterning, primarily endomesodermal patterning (Ax8b, Ax8a, Ax7, Ax1a and Ax6a), including oral/pharynx development (Ax6) and ectodermal apical organ development (Ax1) (Finnerty et al., 2004; Matus, Pang, et al., 2006; Ryan et al., 2007). The endodermally derived Hox genes (Ax8b, Ax8a, Ax7, Ax1a and Ax6a) exhibit asymmetric spatial restriction along the directive axis in Nematostella larvae, opposite of components of bone morphogenetic protein (bmp) signaling (Finnerty et al., 2004; Matus, Thomsen, & Martindale, 2006; Matus, Pang, et al., 2006; Rentzsch et al., 2006; Ryan et al., 2007; Saina et al., 2009). The anterior (Ax6) and posterior (Ax1) Hox genes found in Nematostella are expressed at opposite poles along the oral (Ax6) and aboral (Ax1) domains of the planula larva.
(Finnerty et al., 2004; Ryan et al., 2007). This relationship suggests that the anterior portion of the animal is the oral side, and that Ax1 defines the posterior region of the animal, a finding that is consistent with fate mapping studies (Freeman, 1983). Alternatively, studies have disregarded the anterior posterior relationship of Hox expression in Nematostella and suggest that the larval swimming direction (aboral to oral) as a function for defining anterior posterior axis of the animal. These studies have utilized gene expression analysis of markers of neural circuitry, involved in brain patterning in bilaterians, to further support this hypothesis (H. Marlow et al., 2014; Sinigaglia, Busengdal, Leclère, Technau, & Rentzsch, 2013; Steinmetz et al., 2010; Steinmetz, Kostyuchenko, Fischer, & Arendt, 2011). Recently, studies have analyzed the function of Ax1 through morpholino knockdown and have shown that Ax1 is required for apical organ development along the aboral pole of the animal (Rentzsch, Fritzenwanker, Scholz, & Technau, 2008). Although these expression domains and functional perturbations suggest Anthox genes pattern along the oral-aboral and directive axis respectfully, they contrast the traditional view that Hox genes pattern ectodermal regions during development. Instead, the larval patterning role of Anthox genes could be a secondarily derived function depending on the exact timing and activation relative to development. Hox genes have also been found expressed during gastrulation in multiple vertebrate species (Forlani, S. Lawson, K. Deschamps, 2003; Iimura & Pourquié, 2006; Wacker et al., 2004), and may even have patterning roles prior to zygotic transcription (Driever et al., 1990; Paul et al., 2011). However, little evidence has suggested a dynamic role in patterning prior to larval development in cnidarians.
Understanding that closely related species often exhibit variation in the genomic organization and content pertaining to the Hox cluster, the following research takes a multi-species comparative approach to defining the first Hox genes. We utilized expression analysis from three anthozoan cnidarians, Nematostella vectensis, Acropora digitifera and Fungia scutaria, to determine the localization of Hox genes during development. Quantitative PCR, in situ hybridization and gene perturbation of Nematostella Hox genes shows an ancient role in oral-aboral patterning, activated prior to gastrulation. These data suggest that waves of Hox expression are found during different life history stages, and that Hox expression in larval and adult stages may be representative of secondarily derived patterning events. We propose that the first Hox genes had a patterning role during early embryogenesis prior to and during gastrulation.

MATERIALS AND METHODS

Animal collection and husbandry

*Acropora digitifera*

Adult specimens were collected and maintained in aquaria at the Sesoko Station at the Tropical Biosphere Research Center (TBRC) at the University of the Ryukyus. Embryos were collected from spawned animals at TBRC and processed for RNA or *in situ* hybridization as described below.

*Fungia scutaria*

Adult specimens were collected from the Hawaii Institute of Marine Biology (HIMB), Coconut Island, in Kaneohe Bay, Hawaii. Animals were spawned in sea water tanks at
HIMB and transferred to the Kewalo Marine Lab, Honolulu, Hawaii for processing of material.

*Nematostella vectensis*

Adult animals were raised in 1/3X seawater in glass bowls in constant darkness at 16°C. Animals were fed oyster two weeks before spawning and otherwise were fed artemia. Individual groups were spawned by placing them on a light-box for ten hours. Individual animals were spawned every four to five weeks. Embryos were collected and the outer jelly layer was removed by washing eggs in 4% cysteine dissolved in 1/3X seawater (pH 7.4) for approximately fifteen minutes. Eggs were then washed 3X with 1/3X seawater and then all experiments were conducted on animals raised at 16°C.

**RNA extraction, cDNA synthesis, DNA extraction and sequencing**

RNA from *Acropora digitifera* was collected from the following developmental stages: egg, prawnchip, donut, planula, polyp and colonial adult. Samples were a collection of hundreds of individuals pooled into a single replicate. For the adult stage, a single branch was processed from a colony.

RNA from *Fungia scutaria* was collected from a mix of developmental stages of early cleavage, blastula, gastrula, early and late planula. These samples were pooled into a single sample to gather a complete library of transcripts during development. cDNA was constructed using the Advantage RT-PCR kit (Cat.# 639506, Clontech).

RNA for the identification of the full length Hox coding region in *Nematostella vectensis* was collected from various stages including: early cleavage, blastula, gastrula, planula and early polyp stages. RACE cDNA, for the identification of complete Hox coding regions, was constructed using the SMARTer RACE 5'/3' kit (Cat.# 634858,
Clontech). RNA for cloning of full length transcripts was processed into cDNA using the Advantage RT-PCR kit (Cat.# 639506, Clontech). cDNA stages utilized for our time series experiment were previously described in detail (Eric Röttinger et al., 2012). DNA for the identification and cloning of the Ax1 promoter region was extracted from adult polyps using the DNAeasy kit (Cat.# 69581, Qiagen) according to manufacturers protocol.

Total RNA for each species was stored in TRIzol (Cat.#15596-026, Invitrogen) at -80°C until processed. RNA processing methods have been previously described (Dubuc et al., 2014; M. J. Layden, Boekhout, & Martindale, 2012; Eric Röttinger et al., 2012).

**Identification of full-length NvAnthox coding regions and Fungia scutaria genes**

Each *N. vectensis Anthox* gene was raced out using SMARTer RACE 5'/3' template cDNA made from mixed stage embryos. Five prime and three prime dna fragments were sequenced and sequences were aligned with genomic data to identify the presumptive start and stop codons.

Gene fragments identified from Fungia scutaria were cloned using standard degenerate *Hox* primers (Hejnol & Martindale, 2009) used when genomic resources are unavailable. Identified sequences were raced in the 5'/3' direction and sequenced for proper identification.

**Morpholino and mRNA injection**

All methods related to morpholino and mRNA injection were taken from previous described methods (Michael J Layden et al., 2013). Morpholinos and mRNA were utilized at a range of 0.1-1.0 µM. Each construct was thoroughly tested for toxicity.
concerns by testing at least three different concentrations within this range and assessed on survivability.

**Quantitative PCR and in situ hybridization**

qPCR samples were standardized with NvGADPH and NvRiboPro and primers for other genes were designed using MacVector (www.macvector.com) to amplify 75-150 base-pair fragments of the desired gene. These primers were then back-blasted against the *Nematostella* genome to make sure they only will amplify a single region from the genome. We checked each primer efficiency with a dilution curve (10^-1-10^-5) to make sure their range was within the negligible value of 1.9-2.0. A total of three biological replicates consisting of 100 embryos per sample were analyzed. Relative fold change values were calculated in Microsoft Excel and were standardized against our reference genes based on formulas from Livak and Schmittgen (Livak & Schmittgen, 2001).

All *in situ* hybridizations were based off of the previous protocol for *Nematostella vectensis* (Pang, Matus, & Martindale, 2004). Fixations were done in 1% gelatin coated dishes to prevent tissue from sticking to the plastic (sticking to plastic causes tissue damage and non-specific staining). Embryos were fixed in ice cold 4% paraformaldehyde with 0.2% glutaraldehyde in 1/3x seawater for two minutes, followed by 4% paraformaldehyde in 1/3x seawater for one hour at 4°C. DIG-labeled probes, ranging from 550-1200 base pairs, were hybridized at 64°C for two days and developed with the enzymatic reaction of NBT/BCIP as substrate for the alkaline phosphatase-conjugated anti-DIG antibody (Cat.#11093274910, Roche, Inc.). Samples were developed for an equal amount of time and if no expression was visible, a subset of samples remained in developing solution to determine if any expression was present.
**Imaging and phenotype quantification**

All in situ photos were first imaged using a Zeiss Axio Imager Z1 using a Hamamatsu (Orca-ER) camera with Volocity 5 software ([www.improvision.com](http://www.improvision.com)). Fluorescently labeled embryos used in phenotype analysis were imaged using a Zeiss 710 scanning laser confocal. To measure different dimensions within the embryo, z-stack images were compiled. Using ImageJ, measurements were taken along the oral-aboral axis, directive (axis perpendicular to the oral-aboral), and the total surface area was calculated by tracing the outer dimensions of the embryo. To standardize our measurements, we took measurements approximately half way through each sample by first setting the z-stack range to encompass the two surfaces of the embryo. The central position of the embryo was calculated by using the image capture setting for the central position (median value between the two manually set z-stack values).

**RESULTS**

**Anthox genes exhibit temporal collinearity during gastrulation, relative to the ancestral cnidarian-bilaterian Hox cluster.**

The ancestral cnidarian-bilaterian Hox cluster had both phylogenetically defined anterior genes and a posterior-like Hox gene (Chiori et al., 2009; Chourrout et al., 2006; DuBuc et al., 2012; Ryan et al., 2007). The described expression patterns found in both anthozoan and hydrozoan cnidarians have provided little evidence for collinear Hox expression of cnidarian gene clusters (Cartwright et al., 1999; Chiori et al., 2009; Hayward et al., 2001; Kamm et al., 2006; Ryan et al., 2007; Shenk et al., 1993; Yanze et al., 2001). In fact, expression analysis is often restricted to larval and adult stages,
which may not represent the ancestral function of these genes. Previous studies have shown that \textit{Hox} genes are activated in collinear fashion during vertebrate gastrulation (Forlani, S. Lawson, K. Deschamps, 2003; Iimura & Pourquié, 2006; Wacker et al., 2004), therefore we used the highly sensitive approach of quantitative PCR (qPCR) to determine if \textit{Anthox} genes are active during gastrulation in \textit{Nematostella}.

The cnidarian-bilaterian ancestor had a linkage of \textit{Hox} and homeobox-related genes (Figure 2.1B) (DuBuc et al., 2012). Two genes (Ax1, Ax6A) are present in the \textit{Nematostella} genome, and are paralogs of Ax1a and Ax6 from within the ancestral cluster (Chourrout et al., 2006; Ryan et al., 2007). We designed qPCR primers for \textit{Hox} and \textit{Hox}-related genes present in the \textit{Nematostella} genome to determine when mRNA of these genes is turned on during development. In contrast to previous in situ hybridization reports, mRNA transcripts could be detected for both Ax1 and Ax6a during early cleavage events (2-10 hours after fertilization) (Figure 2.2). Surprisingly, during early blastula formation, mRNA of the homeobox gene \textit{hlxB9} and Ax6 (\textit{Hox1}) were first detected prior to earlier reports (Ryan et al., 2007). During this time of development, the embryo has clear animal-vegetal polarity, detectable by nuclear b-catenin along the animal pole (Wikramanayake et al., 2003). During the blastula to gastrula transition, we see the activation of orthologs of vertebrate \textit{eve (evx)} and \textit{Hox2} class of genes (Ax8b, Ax8a and Ax7). At twenty-four hours after fertilization, during the invagination of the endomesoderm, we find mRNA transcripts of Ax1a. These results show that \textit{Hox} expression in \textit{Nematostella} is activated prior to gastrulation, and subsequent expression is temporally activated in collinear fashion, including both \textit{Hox} and \textit{Hox-related} genes associated with the cluster.
Figure 2.2 – *Nematostella Anthox* genes are temporally activated during the blastula to gastrula transition. Quantitative PCR of Hox-related genes from *Nematostella* reveals that the activation of the first Hox-related gene (*hlxB9*) is found during the early blastula development, coinciding with activation of the anterior-like Hox gene, *Ax6*. The hox-related gene *eve* is sequentially activated after *hlxB9* and *Ax6*, followed by three *Hox* 2 class genes (*Ax8b, Ax8a* and *Ax7*). The final *Hox* gene that is conserved in the cnidarian-bilaterian cluster (*Ax1a*) is transcribed during early gastrulation. Paralogs of both *Ax6* and *Ax1a* (*Ax6a* and *Ax1*) can be found maternally expressed and present throughout development.

**Multiple waves of expression localize to both ectodermal and endomesodermal tissue in *Nematostella*.**

Previous studies on the spatial localization of *Anthox* genes in *Nematostella* showed clear restriction of expression along one half of the endomesoderm during the larval stage of development, with only *Ax1* expressed during cleavage stages (Ryan et al., 2007). More recently, the *Hox-related* genes *hlxB9* and *evx* were found to be expressed along the oral/anterior pole of the animal during gastrulation (Eric Röttinger et al., 2012). We constructed probes encoding the full-length transcript (confirmed by race pcr) of each *Hox* gene and conducted *in situ* hybridization during the transition from early blastula to gastrula (12-48 hours after fertilization). Compared to the cnidarian-bilaterian cluster, *Nematostella* has a unique gene duplication of an ancestral cnidarian gene.
Ax7/8 (found in corals) (DuBuc et al., 2012) that gave rise to three different paralogs, Ax8b, Ax8a and Ax7 (Figure 2.3A). We found early expression of Ax6 and eve on the animal hemisphere side of the embryo beginning at twelve hours after fertilization (Figure 2.3B). Expression was continuously present during early development and could be found along the blastopore prior to gastrulation (24 hrs). During gastrulation, the patterning becomes localized into a salt and pepper pattern within the endomesoderm (48 hrs). Expression of Hox2 class genes (Ax8b, Ax8a and Ax7) exhibited expression along the aboral pore (Ax8b, Ax8a) and both the oral/aboral poles (Ax7). Ax7 appears to exhibit higher asymmetric expression along one side of the blastopore, likely opposite of chordin-bmp2/4 expression (Matus, Pang, et al., 2006; Ryan et al., 2007; Saina et al., 2009). If these genes had a common origin, we may expect the ancestral gene to exhibit both oral/aboral domains.

The first expression of the posterior-like Hox gene (Ax1a) and the anterior-like gene (Ax6a) can be faintly expressed at the site of gastrulation (24 hrs). During gastrulation, each gene increases expression along one side of embryo (48 hrs). Cloning and expression analysis, by qPCR or in situ hybridization, for Ax9 (a proposed pseudo gene) (Ryan et al., 2007) was unsuccessful. These results suggest that during early development, Anthox genes are spatially restricted to different domains of ectoderm along the animal-vegetal axis prior to gastrulation, and that a second wave of expression is initiated during formation of the endomesoderm (Ryan et al., 2007).
Figure 2.3 – *Nematostella* Hox-related genes exhibit temporal activation prior to gastrulation. A) The *Nematostella* Hox cluster consists of the same genes as the found in the cnidarian bilaterian ancestor, except has an independent duplication of *Ax7/8* that became three genes (*Ax8b, Ax8a* and *Ax7*). Additionally *Ax1a* is unlinked from the cluster in *Nematostella*, and is flanked by a proposed pseudo gene *Ax9*. B) *Ax6* and *eve* are both found expressed on one side of the embryo during the early blastula stage (12Hr). This expression is restricted to a salt and pepper pattern with the endomesoderm during gastrulation (48hr). The paralogs (*Ax8b, Ax8a* and *Ax7*) of Hox 2 class of genes are simulatenously activated prior to gastrulation and remain localized to the aboral pole during gastrulation. *Ax1a* and *Ax6a* are both expressed in the blastula and appear asymmetrically expressed along side of the endomesodermal during gastrulation. The oral pole/site of gastrulation is indicated by a yellow star.

Posterior-like *anthox1* is spatially restricted to the aboral (vegetal) pole, coinciding with the timing of the invagination of the endomesodermal plate on the opposite side of the embryo.
The aboral pole of cnidarian larvae consists of a dense set of ciliated cells called the apical tuft. Fibroblast growth factors (FGF), FGF receptors and the homeobox gene six3/6 are expressed in this region during larval development and have been shown to pattern the apical tuft (Rentzsch et al., 2008; Sinigaglia et al., 2013). These studies have also shown that the posterior-like Hox gene (Ax1) is also involved in patterning the apical domain. Quantitative PCR analysis of Ax1 during development suggests that this gene is maternally expressed and may have a broader role in development, than strictly setting up apical territories (Figure 2.2). We found Ax1 to be asymmetrically expressed along the vegetal pole (presumptive aboral/posterior pole) of the embryo early in development, prior to the activation of the Hox cluster (Figure 2.4A). At the late blastula phase of development (18hrs), Ax1 expression is localized to apical half of the animal. As tissue along the oral pole thickens, prior to gastrulation (24 hrs), Ax1 expression is found throughout the non-invaginating tissue. At the time of gastrulation (48 hrs), Ax1 is segregated to the aboral pole of the animal. Co-labeling in situ hybridization experiments with the nuclear marker Hoechst, shows that this apical restriction coincides with the establishment of the endomesodermal layer (Figure 2.4B). Four days after fertilization (96 hrs), Ax1 continues to be restricted to the site in which the apical tuft forms.

Expression of mRNA in subsets of cells does not always mean functional protein exists in those cells. We used microinjection of the 5' promoter region of Ax1 and a partial region of endogenous first exon of Ax1, driving the expression of the fluorescent protein mCherry (Renfer et al., 2010) to determine the localization of Ax1 protein during development (Figure 7C). We found that the Ax1-mCherry fusion protein localized to the
aboral pole prior to gastrulation (24 hrs, Figure 2.4D). At the larval stage of
development, Ax1-mCherry is highly expressed in cells at the aboral pole (Figure 2.4E),
yet protein persists in some cells along the middle of the body. This suggests that Ax1
may pattern cells other than those restricted to the apical pole of the animal, because
this construct was originally injected into every cell of the embryo prior to cleavage.

In total, we found that two genes, Ax6 and Ax1 (the phylogenetically anterior and
posterior Anthox genes), are expressed in opposite domains during early development.
Double in situ hybridization was performed to determine their distribution over time
(Figure 2.4F). Prior to gastrulation (24 hrs), Ax6 and Ax1 are expressed in opposite
domains and encompass the entire area of the blastula. After gastrulation (48 hrs), Ax6
is localized to the endomesoderm while Ax1 is again restricted to the aboral pole. This
expression is maintained during early larval development (96 hrs), while Ax6 has been
shown to further restrict to a ring around the pharyngeal endoderm (Finnerty et al.,
2004; Ryan et al., 2007). Our data indicate that Ax1 is the first of the Hox genes
maternally transcribed (Figure 2.4G) and localizes to the aboral pole at the blastula
stage of development. Prior to zygotic activation of the Hox cluster, Ax1 is absent in
domains where Ax6 is first expressed (Figure 2.3A, 10 hrs). By both qPCR (Figure 2.2)
and in situ hybridization (Figure 2.3B), the Hox cluster appears to be activated in a
semi-collinear fashion, with paralogous genes (Ax8b, Ax8a and Ax7) being activated
simultaneously.
Figure 2.4 – The posterior-like Hox gene, Anthox1 is highly expressed at the aboral pole of development and is spatially restricted during gastrulation.

A) In situ hybridization of Ax1 throughout early blastula, gastrula and planula development. Expression is asymmetrically expressed on the aboral pole of the animal and becomes spatially restricted during the onset of gastrulation (48 hrs). C) Diagram of the transgenic reporter construct utilized to visualize Ax1-mCherry protein expression during development. D) Ax1-mCherry localized to the aboral pole during early gastrula formation, and becomes spatially restricted at the planula stage of development (E). Double in situ hybridization of both Ax6 (light blue) and Ax1 (dark blue) shows that coexpression at opposite poles of the embryo during early development. Expression of each gene become restricted over developmental time, Ax6 to the endomesoderm and Ax1 to the aboral ectoderm. G) Schematic representation of the activation of NvAnthox genes during early development. The oral pole/site of gastrulation is indicated by a yellow star.
**Hox expression in related coral species suggests a deep level of temporal and spatial conservation.**

When studies first began assessing the mRNA localization of Anthox genes in *Nematostella*, the highest levels of expression were found during the planula phase of development (Finnerty et al., 2004; Ryan et al., 2007), leading many to question whether these genes actually function similar to bilaterian Hox genes, due to their asymmetric endomesodermal expression. Due to the unique duplications and breaks in the *Nematostella* cluster, we set out to compare gene expression in other anthozoan species. We analyzed sequencing data from the coral *Acropora digitifera* during different life cycle stages of development. Similar to *Nematostella*, AdAx1 is maternally expressed within uncleaved egg samples (Figure 2.5A) and persists throughout development. *A. digitifera* has a single representative of the Hox2 class of genes found in *Nematostella* (Ax8b, Ax8a and Ax7) given the name AdAx7/8 (DuBuc et al., 2012). Interestingly, we found NvAx8a and NvAx8b to exhibit overlapping domains with NvAx1 during blastula formation (Figure 2.3B). Our sequencing expression data from *A. digitifera* shows a low level of AdAx7/8 present during egg and prawnchip phases of development, suggesting that this gene may have a unique maternal function. Similar to the first mRNA studies in *Nematostella*, the highest levels of mRNA expression were found during planula development and were also identified in subsequent polyp and colonial adult stages (Figure 2.5A).

Next, we conducted *in situ* hybridization experiments on a number of genes from *A. digitifera*, and from Anthox-related genes found in the mushroom coral *Fungia scutaria*. Currently, no genomic resources exist for *F. scutaria*, so genomic fragments were
cloned through degenerate PCR. In *A. digitifera*, *AdAx1* localizes to the aboral domain in both planula and polyp stages of development (Figure 2.5B). The paralog of *AdAx1*, *AdAx1a* was faintly expressed along one side of the planula larva (within the endomesoderm) and appears segregated to a single position within the polyp (Figure 2.5C). Three *Fungia* genes were cloned, (*FsAx1*, *FsAx7/8* and *FsAx6a*) and expression analysis was conducted at gastrula and planula stages of development (Figure 2.5D). Again, *FsAx1* localizes to the aboral pole of the animal during both gastrula and planula stages. *FsAx7/8-like* was asymmetrically expressed during planula development, although we do not know how many paralogs of this gene are present in *Fungia*. Similar to *Nematostella*, *FsAx6a* was highly asymmetrically expressed in both gastrula and planula stages. Concurrently, we found expression of asymmetrically expressed *Nematostella* genes to exhibit similar patterns as previously identified (Finnerty et al., 2004; Ryan et al., 2007). Thus, for the data available, there is almost a complete agreement in the spatio-temporal expression of these genes during larval development in three different anthozoan species.
Figure 2.5 – Temporal and spatial expression of other Anthozoan Hox genes. A) Transcriptomic genome analysis shows Acropora digitifera Anthox genes are expressed throughout developmental time. Similar to early findings in Nematostella, the highest levels of expression are found during planula development. B-D) In situ hybridization of Hox genes in other anthozoan cnidarians. B) Expression of AdAx1 in the coral Acropora digitifera is spatially restricted to the aboral pole during larval and adult development. C) Faint expression of AdAx1a could be found along a single side in the planula larvae and polyp forms. D) Expression of Fungia scutaria Anthox genes are localized to the aboral pole (FsAx1), a single side in the endomesoderm of the planula (FsAx7/8, FsAx6a) and a single side of the endomesoderm in gastrula stages (FsAx6a). E) Planula stages of Nematostella also exhibit asymmetric expression of select Hox genes in the
endomesoderm, as previously described in Ryan et al. (2007). The oral pole/site of gastrulation is indicated by a yellow star.

**Disruption of Nematostella Hox results in gastrulation defects.**

Functional disruption of cnidarian Hox genes give rise to local structural defects such as loss of the apical tuft (Rentzsch et al., 2008), axial duplications/abnormalities and tentacle deformities (Jakob & Schierwater, 2007). We now know that Anthox genes are transcriptionally activated prior to gastrulation and may play a role in establishing domains during this time. We utilized microinjection techniques to knockdown (translation blocking morpholinos) and mis/over-express (mRNA of the full length transcript tagged with the fluorescent protein venus) of select Anthox genes during embryonic development. Experiments were visualized at three time points during develop (24, 48 and 96 hours after fertilization) to determine if any genes resulted in patterning defects during gastrulation. Samples were preserved and stained using phallacidin-FL to visualize cellular boundaries, and Hoechst to label nuclei for quantification. Control samples, injected with dextran only, form a blastula by twenty-four hours after fertilization at 16°C (Figure 2.6A) and by forty-eight hours, the endomesoderm completed the invagination process (Figure 2.6A, white arrowheads). During larval development, the pharyngeal ectoderm becomes established and is visible by day four (96 hours, white arrowheads). Disruption of Ax1 during gastrulation does not compromise endoderm formation, but appears to form animals that are shorter along the oral aboral axis (Figure 2.6B). Knockdown of the anterior-like gene Ax6 results in a disorganization of the boundary between the pharyngeal ectoderm and endomesoderm in the planula larva (Figure 2.6C, white arrowheads). Over-expression of Ax6, results in
animals having ectopic oral tissue outside the body cavity (Figure 2.6D, white arrowheads), which may arise during early gastrulation stages. The results from Ax1 and Ax6 suggest that these two opposed genes may broadly effect the distribution or allocation of tissue with oral and aboral domains.

The anterior-like gene, Ax6a, first appears by in situ at the onset of gastrulation (Figure 2.3B) although it is detectable by qPCR much earlier (Figure 2.2). Morpholino knockdown of this gene results in a dramatic delay in gastrulation (Figure 2.6E) in which tissue along the blastopore thickens without proper invagination (48 hours, white arrowhead). At four days after injection, planula stage animals have an abnormally formed endomesoderm, and animals appear shorter along the oral-aboral axis. Over-expression of this gene in all cells does not result in premature gastrulation (Figure 2.6F), yet a high rate of mortality during the gastrula-planula transition did not allow us to quantify planula stages for this treatment. Due to the extreme phenotypes produced during gastrulation, we set out to quantify these changes through two types of measurements including, length along the oral-aboral and directive axis (this value is the length perpendicular to the oral-aboral axis) and quantification of the total number of ectodermal nuclei standardized to the total surface area (based on the circumference) (Figure 2.7A). Analysis of embryo dimensions show that both knockdown of endomesodermal Hox genes (Ax6 and Ax6a) significantly increases the length of the directive axis or a dimension that is perpendicular to the oral-aboral axis during gastrulation (48 hrs), while Ax6a knockdown significantly increases the length of the directive axis in each time point (Figure 2.7B). The increase in the length of the directive axis by Ax6a knockdown is correlated with a loss in length along the oral-aboral axis.
Analysis of the difference in length between the oral-aboral axis and directive axis, shows the average length of the oral aboral axis is greater that the length of the directive axis (in controls) during gastrulation and in the planula larva (Figure 2.8). Morpholino knockdown of Ax6 and Ax6a causes the length of the directive axis to be larger than the oral-aboral axis (negative value) during gastrulation (Figure 2.8). The length of the directive axis remains larger in Ax6a treated animals during the larval stage. Interestingly, Ax6 RNA over expression significantly reduces the length of the directive axis (Figure 2.7B) and nearly doubles the difference between the two axis (Figure 2.8). In total, these results suggest that disruption of oral/anterior Anthox genes may disrupt normal elongation of the embryo, and could be a product of defective gastrulation.

Prior to gastrulation, an embryo must regulate the amount of tissue allocated as ectodermal versus tissue that will invaginate to form the endomesoderm. To determine if any of these genes affect this segregation, we quantified the total number of nuclei within the first sixty microns of the lateral ectodermal portion of the embryo (Figure 2.9, see materials and methods for measurement technique). These data were standardized to the total surface area of the embryo (Figure 2.10), determined by the measurement of the circumference. During blastula formation (24 hrs), knockdown of Ax1 resulted in a larger proportion of ectodermal nuclei relative to controls (Figure 2.9). During gastrulation (48 hrs), Ax6 knockdown resulted in a lower number of nuclei relative to controls. Interestingly, knockdown of Ax1, Ax6 and Ax6a all produced lower numbers of ectodermal cells relative to controls at the planula phase of development. Ax6a exhibited the greatest loss of cell numbers and may be associated with the inability to
properly gastrulate; this treatment does not result in high mortality rates and will be discussed in detail below. These results suggest that throughout development Ax1 and Ax6 may regulate the allocation of ectodermal and endodermal fates. Further quantification of the rate of proliferation and apoptosis may provide insight into this discrepancy.
Figure 2.6 – Disruption of Anthox genes results in gastrulation defects. A) Control embryos injected with dextran. B,C,E) Animals injected with translation blocking morpholinos (MO) against Ax1 (B), Ax6 (C) and Ax6a (E). D,F) Animals in which mRNA was overexpressed during early development for Ax6 (D) and Ax6a (F). Animals were stained with Phalloidin-FL (green) or Hoerchst (white) to visualize actin cellular boundaries (green) or nuclei (white). The oral pole/site of gastrulation is indicated by a yellow star.

Figure 2.7 – Disruption of Anthox genes alters the distribution of tissue along the oral-aboral and directive axis. A) Schematic representation of the positions measured. B) Quantification of linear measurements from different developmental stages.
Figure 2.8 – Difference values between linear measurements from the oral-aboral and directive axis. Difference values were calculated by subtracting the linear measurement of the directive axis from the linear measurement along the oral-aboral axis.

Figure 2.9 – Knockdown Anthox genes changes the number of ectodermally derived cells.
Figure 2.10 – Average surface area of embryos through developmental time. Values were collected by measuring the circumference of each embryo.

**Loss of Ax6a changes endomesodermal fates to ectoderm**

Prior to gastrulation, a boundary is formed which designates tissue that will go on to form the ectoderm and endomesoderm of the animal. This boundary has been shown to be established by the onset of nuclear beta-catenin along the animal half of the embryo (Wikramanayake et al., 2003), which gives rise to the future site of gastrulation and the mouth of the animal. We found that knockdown of Ax6a disrupts the normal onset of gastrulation (Figure 2.6E) and changes the dimensions of the animal to have a longer directive axis than normal (Figure 2.7B). Knockdown of Ax6a appears to have a concentration dependent effect on the progression of gastrulation (Figure 2.11A). At high concentration (1 µM) Ax6a treated embryos do not invaginate at the same time as
controls. At a lower concentration (0.5 µM) animals appear to progress further along in the process of gastrulation, however, the endomesodermal plate fails to make contact with the aboral ectoderm. As we showed previously, at four days after fertilization, Ax6a morpholino treated animals form an inner layer of cells, resembling a gastrula at 48 hours (Figure 2.6E) and these embryos do not change significantly after another 24 hours (5 day old) (Figure 2.11A).

To determine the broad effects from our treatment, we conducted in situ hybridization on three ectodermally derived genes (foxA, wnt2 and fgf1a) found in discreet regions along the oral aboral axis. FoxA has been proposed to define the boundary between the ectoderm and endomesoderm in Nematostella (Magie et al., 2007). Prior to gastrulation (24 hrs), foxA is localized to the blastopore lip (Figure 2.11B, top row) and remains present along this domain throughout gastrulation. In late stage planula larvae (7 days), foxA is present in the ectodermal portion of the pharynx. In Ax6a morpholino injected animals (Figure 2.11B, bottom row), foxA appears reduced and does not progress internally compared to controls. Interestingly, in the planula larva, foxA expression is found highly expressed throughout the endomesoderm, suggesting this tissue has taken on an ectodermal fate.

A second gene, wnt2, is first present along the prospective oral ectoderm prior to gastrulation (Figure 2.11C, top row). The positioning of wnt2 shifts during gastrulation to encompass a region along the middle portion of the outer ectoderm and a similar expression remains in the planula larva. Interestingly, this shift in pattern domain correlates with the onset of gastrulation and appears to be expressed in a gradient of lower to higher expression from oral to the aboral pole. Although wnt2 does not exhibit
direct spatial overlap with Ax6a, we see reduced expression of wnt2 throughout gastrulation in Ax6a treated embryos. Additionally, wnt2 was expressed at the oral pole (Figure 2.11B, indicated by the yellow star) and within one side of the endomesoderm (white arrowhead) at the planula stage of development. These results suggest that wnt2 expression along the middle region of the ectoderm, may be activated by signals generated during gastrulation, specifically the migration of endomesodermal tissue along the inner ectodermal layer.

In contrast to the above results, we found that little change occurs to the aboral pole expression of fgf1a after Ax6a morpholino injection. This gene is expressed in a wide domain during the blastula stage of development (Figure 2.11D, top row) and becomes spatially restricted to the aboral pole after gastrulation. Expression remains in a small zone where the apical tuft develops in the planula larva. In Ax6a morpholino treated animals (Figure 2.10D, bottom row), the spatial expression appears wider in embryos that have not completed contact between the endomesodermal and the aboral pole relative to controls (36 and 48 hrs). Fgf1a appears normally restricted in the planula larva (7 days). These results suggest that restriction of apical territories could be controlled by the timing or signaling (between the two layers, prior to mesoglea formation) generated during gastrulation.
Figure 2.11 – Disruption of Ax6a delays gastrulation and the migration of ectodermal signals to a more aboral position. A) Knockdown of Ax6a is concentration dependant. At 48 hours, control animals have underwent proper gastrulation. Ax6a knockdown at both 1 and 0.5 μM inhibit proper gastrulation. Animals kept for 5 days after injection appear to have unusual gastrulation. B) FoxA expression is localized to the blastopore lip and developing pharyngeal ectoderm during development. Knockdown of Ax6a initially reduces the expression of FoxA, followed by expanding expression to encompass the entire endoderm (7 days). C) Wnt2 is expressed along the oral pole of the animal and becomes spatially restricted to a central domain between the oral and aboral domains. In Ax6a knockdown animals, wnt2 is highly reduced and expands to both oral and endodermal domains after seven days. D) Fgfa1 is broadly expressed in the aboral pole, and undergoes spatial restriction to the aboral domain over time. In Ax6a treated embryos, the inhibition of gastrulation appears to stop the constriction of fgfa1 until gastrulation has finished.

Additional Hox-related phenotypes suggest control of oral aboral territories

We have documented small territorial changes during the onset of gastrulation (Figure 2.6) and it has been established that the loss of the posterior-like Hox gene (Ax1) results in a loss of the apical tuft (Rentzsch et al., 2008; Sinigaglia et al., 2013). Independently, we confirmed that the loss of Ax1 results in a loss of the apical tuft (Figure 2.12A). Additionally, we found that the loss of Ax1 expands wnt2 territories along the oral-aboral axis (Figure 2.12B). Similar to experiments conducted on apically restricted fgf ligands and receptors (Rentzsch et al., 2008), the loss of Ax1 results in animals that remain in the larval phase of development for a prolonged period of time (Figure 2.12C). After ten days, a number of animals formed long tube-like animals, often with reduced oral structures (Figure 2.12D). Larvae that were kept for approximately twenty-one days after fertilization showed a higher rate of tube-shaped animals (Figure 2.12E). Conversely we documented increased oral territory associated with the overexpression of the anterior-like Hox gene (Ax6) during larval development (Figure 2.6D). These animals exhibit a large amount of ectopic tissue around the oral region of
the animal after metamorphosis (Figure 2.12F-G). Although we have not identified the origin of this tissue, ectopic tissue at the larval stage appears to be a continued outgrowth of the ectodermal region around the mouth of the animal (Figure 2.6D). These results, although limited to morphological assessment, will be crucial for identifying how anterior-posterior Hox genes regulate domains along the oral-aboral axis of the animal.
Figure 2.12 – Disruption of Anthox genes alters the distribution of tissue along the oral-aboral and directive axis. A-B) Ax1 knockdown inhibits apical tuft formation. View of the aboral pole of planula larvae stained with alpha-tubulin (red) phallacidin-FL (green) and Hoerchst (blue). C-D) In situ hybridization of wnt2 in planula larvae treated with Ax1 MO. Expression of wnt2 expands in both the oral and aboral domains as a result of Ax1 knockdown. E) Quantification of Ax1 phenotypes 10 days after injection. Phenotype analysis was based on at least 100 injected embryos. F-G) Long-term (21 days) phenotypes from Ax1 treated embryos that eventually metamorphosed. H-I) Phenotype associated with metamorphosed polyps treated with Ax6 MO.

DISCUSSION

Timing and transcriptional activation of the cnidarian Hox cluster

In bilaterian animals, Hox genes are expressed in an anterior to posterior fashion, with phylogenetically-related anterior genes generally being expressed near the head or anterior location of the animal. A “posterior” group of Hox genes are involved in patterning the regions at the back end of the embryo, including the posterior animal trunk (Favier & Dollé, 1997). Sea urchins are also members of the deuterostomes lineage, which have a complex larval to adult metamorphosis, are now considered to have both anterior and posterior genes expressed along the oral aboral axis of the animal during gastrulation (Morris & Byrne, 2014; Peter & Davidson, 2014). Similar to early work in Drosophila, annelid Hox genes were found to exhibit spatial and temporal colinearity (Fröbius, Matus, & Seaver, 2008; Irvine & Martindale, 2000; Kulakova et al., 2007) and are also involved in regeneration (Novikova et al., 2013). The early-branching acoel bilaterian, Convolutriloba longifissura, also exhibits anterior-posterior spatial segregation during development (Hejnol & Martindale, 2009). Thus, nearly all bilaterian lineages that have been analyzed have a spatial segregation of Hox genes during
development. In fact, the collinear expression system has been co-opted in other developmental contexts, such as vertebrate limb and digit formation (Dolle P, Izpisua-Belmonte JC, Falkenstein H, Renucci A, 1989; Favier & Dollé, 1997; Montavon, Le Garrec, Kerszberg, & Duboule, 2008; Zakany J, Fromental-Ramain C, Warot X, 1997; Zakany & Duboule, 2007).

The variability in Hox expression domain location is most apparent when comparing adult structures across various taxonomic groups (Swalla, 2006). These highly diversified body plans may have retained the anterior to posterior orientation of Hox expression, yet the structures they pattern may be a result of evolutionary novelties found in a clade specific manor. Recent work has shown that the first activation of Hox expression in vertebrates is much earlier during development compared to all the findings on larval and late embryonic patterning. Hox genes are first activated during the onset of gastrulation in vertebrates (Forlani, S. Lawson, K. Deschamps, 2003; Iimura & Pourquié, 2006; Wacker et al., 2004) and are found to exhibit temporal and spatial colinearity during that time. These findings have not been thoroughly tested among invertebrate taxa, although previous results in cnidarian studies have hinted at an early embryonic role.

Expression analysis of Hox genes in the hydrozoan cnidarian, Podocoryne carnea, showed both anterior (PdCnox1) and posterior (PdCnox4) genes localized at opposite poles during cleavage stages and larval of development (Yanze et al., 2001). A study in another hydrozoan species, Clytia hemisphaerica, identified two posterior like genes (CheHox9-14A, CheHox9-14B) expressed in uncleaved eggs and at the blastula stage of development (Chiori et al., 2009). A third hydrozoan study used RNAi to inhibit Hox
gene expression in *Eleutheria dichotoma* (Jakob & Schierwater, 2007). This study showed changes to axial properties and tentacle morphology in adults, but revealed little about the embryonic expression of these genes, while studies in *Hydra* have been restricted to the adult stage (Gauchat D, Mazet F, Berney C, Schummer M, Kreger S, Pawlowski J, 2000; Shenk et al., 1993). Additionally, the genomic organization of *Hox* genes in hydrozoans is only known from *Hydra*, which has a highly fragmented set of genes (Chourrout et al., 2006). Due to the complex life history stages in hydrozoan cnidarians, primarily the presence of a jellyfish stage or lack there of (*Hydra*), embryonic studies may reveal a higher rate of conserved developmental properties relative to anthozoan cnidarians, which have a biphasic life cycle. Conversely, the study of *Hox* genes in these diversified body plans may give insight into the radiation of this unique life history stage. In total, the early embryonic expression within this group of cnidarians has been overlooked and must be addressed in future studies.

Due to the relatively high expression level of *Anthox* genes during larval development, the first studies of *Nematostella Hox* genes indicated that their primary function was to patterned structures during the larval phase of development (Finnerty et al., 2004; Matus, Pang, et al., 2006; Ryan et al., 2007). We have clearly shown by quantitative PCR and *in situ* hybridization that *Hox* and *Hox*-related genes are activated prior to gastrulation in overlapping domains with future ectodermal and endomesodermal domains (Figure 2.2-2.3). Although we have not studied how the first genes in the cluster become transcriptionally activated, the loss of *Ax1* expression along the oral domain correlates with the onset of cluster zygotic transcriptional activation (Figures 2.2-2.4). Interestingly, during gastrulation in vertebrates, posterior-like *Hox*
genes also appear to be the driving factor for expression of anterior-like Hox genes (Iimura & Pourquié, 2006). This phenomenon, known as posterior prevalence, was originally found to explain why loss of anterior Hox genes gave rise to more posterior structures (Duboule & Morata, 1994; Gibson & Gehring, 1988; González-Reyes & Morata, 1990; Lufkin T, Mark M, Hart C, Dolle P, LeMeur M, 1992; Mann & Hogness, 1990; Struhl, 1983). Recent work has drawn a close link to the posterior prevalence hypothesis and control of anterior Hox genes by microRNAs (Yekta, Tabin, & Bartel, 2008). A single microRNA is found between Ax8a and Ax7 in the Nematostella cluster (Moran et al., 2014) and further analysis may provide insight into why Ax8b and Ax8a are expressed in overlapping domains with Ax1 prior to gastrulation (Figure 2.3). A detailed comparative analysis between the functional knockdown and overexpression of Ax1 may reveal a change in the activation of the Hox cluster.

Analysis of two other anthozoan species, Acropora digitifera and Fungia scutaria, have again highlighted the expression Hox genes during early development as well as larval and adult stages. As previously stated, expression during larval development appears to be the easiest to discern, although we now know a first wave of Hox expression is likely present. These results help support the idea that two dynamic waves of Hox expression occur during development in cnidarians. The first wave of expression is likely controlled by the anterior and posterior Hox genes, Ax6 and Ax1, because these are the first two genes transcriptionally activated during early development (Figure 2.2), and have been confirmed to localize on opposite poles in blastula by in situ hybridization (Figure 2.3-2.4). We have attempted to identify expression from Ax6a, which appears maternally expressed (Figure 2.2), but have yet
to visualize its expression by *in situ* hybridization. If the transcriptional control of the *Hox* cluster progresses from *Ax6* towards *Ax7* (Figure 2.13A-B), which appears likely based on our quantitative and spatial data, we suspect the first wave of expression aids in patterning broad territories of the blastula. We find the activation of paralogs *Ax6a* and *Ax1a* (paralogs of *Ax6* and *Ax1*) activated during the onset of gastrulation (Figures 2.2-2.3). Knowing that *Ax6a* dramatically effects the onset of gastrulation (Figure 2.6), we suggest that a second wave of *Hox* expression may be activated in a reverse collinear fashion to drive asymmetric expression of *Hox* genes within the endomesoderm (Figure 2.13A,C). *Ax6a* is broadly expressed among overlapping domains with *Ax8a*, *Ax8b*, *Ax7* and *Ax1a* during larval development (Ryan et al., 2007) and is expressed early on, prior to the activation of these genes within the endomesoderm (Figures 2.2-2.3). The phenomenon of reverse colinearity is not foreign to *Hox* functionality and has been attributed to specification of hand and foot digits in vertebrates (Nelson et al., 1996). In this case, multiple waves of *Hox* expression pattern during limb bud formation, while the final stage of digit specification translates information from a posterior to anterior directionality. Although we have not analyzed the regulatory interactions between *Nematostella Hox* genes, the paralogous relationship of *Ax6* and *Ax6a* may suggest they both could be ‘activators’ of genes downstream of the cluster.

**Phenotypes and functionality of Anthox genes**

Through functional perturbation of *Anthox* genes, we have shown both changes during blastula, gastrula, planula and adult stages of development (Figures 2.6-2.7). Disruption of anterior (*Ax6*) posterior (*Ax1*) genes appear to disrupt localized structures around the mouth and apical tuft respectively. Maintaining *Ax1* injected animals for long
periods of time first results in a lack of settlement, but is then translated into elongated body wall tissue, upon metamorphosis. This shift in tissue allocation can be seen during the planula phase of development, in which \textit{wnt2} domains are highly expanded along the oral-aboral axis of \textit{Ax1} injected animals. Conversely, over expression of the anterior gene, \textit{Ax6}, expands oral domains around the mouth, or could be interpreted as overproduction of endomesodermal components that does not allow pharyngeal tissue to form internally. Future analysis of target genes that exhibit spatial segregation along the oral aboral axis (primary \textit{wnt} genes) will aid in describing these phenotypes.

Asymmetrically expressed genes along the directive axis of the animal are first activated during the onset of gastrulation (Matus, Thomsen, et al., 2006; Rentzsch et al., 2006; Saina et al., 2009). It has been proposed that \textit{chordin-bmp} signaling is responsible for specification of the directive axis in \textit{Nematostella} (Saina et al., 2009). \textit{Chordin-bmp} signaling is found on the opposite side of \textit{Hox} expression during endomesodermal formation (Finnerty et al., 2004; Matus, Thomsen, et al., 2006; Matus, Pang, et al., 2006; Saina et al., 2009). Knockdown of components of \textit{chordin-bmp} signaling radialize planula larvae, but do not inhibit gastrulation (Saina et al., 2009). This research also showed that radialization through knockdown of components of \textit{chordin-bmp} signaling resulted in a loss of expression of \textit{Ax1a} (HoxE), although expression analysis was not performed on \textit{Ax6a}. We have not resolved the interplay between \textit{Ax6a} and components of \textit{chordin-bmp} signaling, but our results suggest that the side opposite of \textit{chordin-bmp} signaling (where \textit{Hox} genes are expressed) may be necessary and/or responsible for initiation of endomesodermal formation. Interestingly, animals kept for prolonged periods of developmental time (7 days after \textit{Ax6a} morpholino
injection) appear to have an endomesoderm composed of ectodermal derivatives, highlighted by foxA expression (Figure 2.11B).

Finally, we found correlative evidence that signaling may occur between the endomesoderm and outer ectoderm during gastrulation, prior to mesoglea formation. First, Ax1 expression is locally restricted to the aboral pole of the animal during the onset of gastrulation (Figure 2.4). Second, disruption of Ax6a delays the internalization of endomesoderm toward the aboral pole and prolongs the normal restriction of fgfa1 (normally restricts to the apical pole along with Ax1) until the completion of gastrulation (Figure 2.11D). Third, disruption of Ax6a effects the expression of wnt2 that does not exhibit overlapping boundaries with Ax6a (Figure 2.11C). Fourth, previous results show that knockdown of components of chordin-bmp signaling cause ectopic localization of endomesodermal components within the aboral ectoderm. These ectopic expression domains may suggest that signals generated in the endoderm pass to overlying ectoderm, by trans epithelial signaling. Therefore, loss of the normal protein, by knockdown of the endogenous mRNA in the endoderm, may block the passage of signal to the outer ectoderm, resulting in cells transcriptionally activating the endomesodermal genes in areas of need (ectoderm) as a regulatory mechanism. Although these results are highly correlative, it has been previously suggested that Hox genes carried into the mesoderm during gastrulation of vertebrates may transfer their collinear positional information to neural ectoderm (Wacker et al., 2004). This transfer of positional information would provide an anterior-posterior framework for neural ectoderm development.
In conclusion, we have provided strong evidence that Hox genes are expressed much earlier in cnidarian development than previously believed. Analysis of Hox gene expression across different invertebrate lineages, including hydrozoan cnidarians, has confounded the identity of the ancestral role of Hox genes in bilaterian lineages. It is our current understanding that a level of functional conservation may occur during patterning events prior to and during gastrulation between cnidarians and vertebrates. A close relationship to the onset of gastrulation and endomesodermal production may have provided the foundation for an elaborated split of the endomesoderm into two distinct layers in Bilateria. If this is true, the primary purpose of the first Hox genes may have been to coordinate the timing and allocation of tissue during gastrulation in an anterior posterior fashion. Furthermore, this would suggest that animals that lack Hox genes (sponges, ctenophores and arguably placozoans) might have highly divergent forms of gastrulation, although additional work is required to test this hypothesis.
Figure 2.13 – Hox expression may be induced in two waves during Nematostella development. A-B) The first wave of Hox expression begins during early embryonic development with the maternally expressed Ax1. During early blastula formation (12 hrs), transcription of the anterior Hox gene (Ax6) begins and appears to follow colinearity with the remaining portion of the intact cluster. These initial expression domains are localized to the ectoderm. A-C) A second wave of expression (#2) begins during endomesodermal formation and hypothetically could follow a reverse collinear orientation based on the timing and activation of Ax7, Ax8a and Ax8b.
CHAPTER 3

Asymmetric expression of developmental regulatory genes in the placozoan, *Trichoplax adhaerens*

ABSTRACT

The phylum Placozoa is arguably the most mysterious and understudied animal group with a published genome. The first descriptions of these unusual animals suggested that the two distinct epithelia layers, compromising the top and bottom surfaces of the “adult” animals, exhibit dorsal/ventral polarity. Since this time, little progress has been made to identify how these morphologically simplistic animals pattern the primary axis of their body. With improved methods for fixation and *in situ* hybridization, we describe mRNA expression patterns for the developmental regulatory genes: *chordin*, *bmp3/admp*, *gdf5-like*, *snail*, *Trox-2*, *ELAV*, and *Beta-actin*.

The single *Trichoplax* chordin-like gene (*TaChordin*) lacks CHD domains, but has four CR domains that are phylogenetically conserved with CR domains from bilaterian *chordin* genes. We found that *TaChordin* is expressed in a small domain in the bottom epithelia of the animal. An ortholog of *bmp3/admp* genes (*TaBmp3*) is asymmetrically expressed along a lateral side of the animal and suggests cryptic patterning along the periphery of the animal. Expression of other genes such as *snail*, *Trox-2*, *gdf5-like* and *ELAV* (a neural marker) can be found on the lower epithelial surface. Our results suggest that the top layer of the animal contains ectodermal derivatives, while the bottom layer exhibits properties of a primitive digestive (absorptive) surface with endomesodermal-like gene expression.

This study suggests that there are at least four distinct patterning regions present
within the adult *Trichoplax*. These regions can be separated into upper and lower epithelial layers, the lateral edge epithelia (boundary between upper/lower regions) and the inner fiber layer. Once thought to exhibit simple morphological organization, *Trichoplax* may have functionally distinct regions masked by a lack of morphologically distinct cell types. These animals sit on the cusp of a great genomic radiation event, where the transition to a more cnidarian-like animal brought with it the advent of true *Hox* genes, FGF signaling and gene copy numbers more similar to bilaterians. Placozoans demand more attention to understand their basic biology in relation to their genomic complexity.

INTRODUCTION

The majority of phylogenetic studies recognize the phylum Placozoa as one of four early-branching animal phyla, along with Cnidaria (corals, sea anemones and jellyfish), Porifera (sponges) and Ctenophora (comb jellies). Placozoans once were proposed to be at the base of the animal tree of life (Dellaporta et al., 2006; Schierwater et al., 2009), but recent molecular evidence places them as the closest outgroup to the cnidarian-bilaterian ancestor (Dunn et al., 2008; Hejnol et al., 2009; Srivastava et al., 2008) (Figure 3.1A). Based on the original descriptions by Grell and recent ultrastructural analyses, placozoans were found to consist of an upper and lower epithelium, with a fibre cell layer in the middle (K. G. Grell & Benwitz, 1981; K. Grell, 1971, 1972; Guidi, Eitel, Cesarini, Schierwater, & Balsamo, 2011; Rassat & Ruthmann, 1979). The lower epithelium is composed of flagellated, cylindrical cells and scattered aflagellated gland cells, while the upper layer consists of ciliated epithelial cells interspersed with autofluorescent ‘shiny sphericals’ of unknown cell type (Figure 3.1B).
Numerous small, ovoid cells (theorized to be stem cells) are positioned at the margin between upper and lower epithelia (along the lateral edge) and have been described in detail by ultrastructural analyses (Guidi et al., 2011). Overall, the morphological simplicity (four to five distinct cell types) and the high level of genomic conservation (Srivastava et al., 2008) make placozoans an interesting taxon for studying the emergence of developmental regulatory interactions such as chordin/bmp gradients and Hox genes, both thought to be absent in ctenophores and sponges (Ryan et al., 2013; Srivastava et al., 2010).

Placozoans are widely distributed geographically, and while the phylum currently only consists of one described species, *Trichoplax adhaerens*, molecular analysis indicates there is likely much greater species diversity (M Eitel & Schierwater, 2010; Guidi et al., 2011; Voigt et al., 2004). Sexual reproduction and subsequent complete embryonic development remain unknown, while lab cultured animals appear to represent the ‘adult’ form and reproduce via lateral fission or budding of “swarmers” from the upper portion of the animal (Michael Eitel, Guidi, Hadrys, Balsamo, & Schierwater, 2011; Thiemann & Ruthmann, 1990, 1991; Thiemann, M Ruthmann, 1988). Following collection from the field, placozoans are maintained on glass slides in bowls of seawater, and are thought to ingest food via their lower epithelium (Grell, KG Benwitz, 1971; Ruthmann, A Behrendt, 1986; Wenderoth, 1986). The thin upper epithelium contains “shiny spherical” cells which are hypothesized to be involved with predator defense (Jackson & Buss, 2009). Animals move along their lower epithelial layer by gliding along the substrate through ciliary beating, exhibit shape change in response to increased nutrient concentrations (Ueda, Koya, & Maruyama, 1999) and
are described as exhibiting amoeboid-like movement (Supplemental Figures 3.1-3.2). Although animals appear to sense nutritional cues, no preferred direction of locomotion has been described. Placozoans appear to have distinct oral and aboral regions (corresponding to the lower and upper epithelia, respectively), responsible for two basic functions necessary for survival (food intake and predatory defense). The lateral edge of the animal consists of birefringent granules (Pearse, Uehara, & Miller, 1994) and cells that both RFamide antibody (Schuchert, 1993) and PaxB mRNA expression (Hadrys, DeSalle, Sagasser, Fischer, & Schierwater, 2005a) appear to recognize. Overall, placozoans appear to have four distinct regions including: upper and lower epithelia, lateral edge boundary zone and the inner fiber cell layer.

Placozoans exhibit a limited amount of morphological complexity and potentially limited cellular function, therefore we have to ask why these creatures have many gene families present in cnidarians and triploblastic animals. One of the key gene families to emerge in the placozoan-cnidarian common ancestor, ParaHoxozoa (Ryan et al., 2010), was a proto-Hox/ParaHox-like gene Trox-2. It has been proposed that a proto-Hox like gene(s) could have given rise to Hox and ParaHox genes in cnidarians (Chourrout et al., 2006; Ryan et al., 2007). The orthogous gene to Trox-2 in cnidarians is the ParaHox-like gene Cnox2/Anthox2/GSX (Hayward et al., 2001; Ryan et al., 2007; Yanze et al., 2001). Expression of this gene is typically found in the oral region of the animal and in the endomesodermal portion of the body (Hayward et al., 2001; Ryan et al., 2007; Yanze et al., 2001), although it is also found in both oral and aboral regions in some species of hydzoan cnidarians (Cartwright et al., 1999; Shenk et al., 1993). Expression of Trox-2 in placozoans has been described to be localized in two different
regions of the animal, cells along the outer edge of the animal (Martinelli & Spring, 2004) and internal cells separating the upper and lower regions of the animal (Jakob et al., 2004). Functional experiments suggest that knock down of Trox-2 results in a loss of growth and asexual reproduction over time in cultures (Jakob et al., 2004).

Trox-2 is by far the most studied gene from the Trichoplax genome although a total of only four other papers have investigated the expression of seven different genes by in situ hybridization (Hadrys et al., 2005a; Jakob et al., 2004; Martinelli & Spring, 2003, 2004). Aside from actin, where expression is detected homogeneously throughout the animal, there are broad similarities in the spatial expression patterns for each of the other genes. Each gene is expressed at the outer periphery of the animal, either in a few cells or groups of cells (PaxB, Brachyury), at the periphery of attached animals (Tbx2/3), in a broader domain (Trox-2, Secp1), or in the body folds (Not) (Hadrys, DeSalle, Sagasser, Fischer, & Schierwater, 2005b; Jakob et al., 2004; Martinelli & Spring, 2003, 2004). Cells expressing mRNA were identified to be in all three layers (Secp1), between the upper and lower epithelia (Trox-2, PaxB), primarily in the upper layer (Tbx2/3) or of undetermined position (Brachyury). Orthologs of developmental patterning genes such as wnt, bmp and a chordin-like gene are present in the genome of Trichoplax adhaerens (Srivastava et al., 2008), but to date little advancement has been made to determine if any of these genes function in regional patterning of these enigmatic animals.

The primary goal of this study was to improve the RNA in situ hybridization technique in Trichoplax. Minor changes to the fixation/preservation and more stringent hybridization conditions has allowed us to assay the expression patterns of a number of
axial patterning and other evolutionarily conserved genes. In this study, we chose to look at key genes involved in dorsal-ventral patterning to determine if Grell’s original axial orientation of top (dorsal) and bottom (ventral) can be verified with molecular techniques.

Figure 3.1 – Current phylogenetic relationship of early-evolved animal lineages in relation to bilaterians. A) Recent findings suggest that placozoans are the sister taxa to cnidarians (Hejnol et. al 2009). B) Schematic diagram of the top, middle and bottom tissue layers of placozoans (Grell, 1972). The upper layer consists of a thin ciliated epithelial layer, with interspersed ‘shiny spherical’ cells thought to be specific to placozoans. The middle layer has fiber like cells (blue) of unknown functionality. The bottom layer is a thick ciliated epithelial layer interspersed with gland cells.

MATERIALS AND METHODS

Collection/culture of *Trichoplax*

All animals were collected from an outdoor flow-through sea water system at the Kewalo Marine Laboratory (Honolulu, HI). Seawater tanks were seeded with heavily biofouled rocks and algae samples from the nearby Kewalo basin. Animals were collected from glass slides that were placed in a metal slide rack and submerged with the rocks/algae for 3-5 weeks. Tanks were kept under constant low flow rates in full sunlight. Animals were typically found creeping along slides covered with a dense
bacterial biofilm that also had microalgae and diatoms present (Figure 3.2A, Supplemental Figure 3.1). Specimens were removed from the surface of the slide by gentle pipette pressure (Figure 3.2B) and transferred to gelatin-coated plastic dishes. Gelatin coated dishes were prepared by dissolving gelatin (1x) into distilled water and covering each petri dish (47mm) with approximately 2 ml of liquid. Excess gelatin was then removed and dishes were dried overnight. After transfer, animals were kept for twenty-four hours to allow for acclimation to the dish.
**Figure 3.2 – Habitat and collection of *Trichoplax adhaerens*.** A) Animals adhere to the benthic environment along the thick lower epithelial layer, thought to be their digestive zone (indicated by red dots). The substrate in which they are found is often covered with microalgae, bacteria and diatoms among other microscopic material. The upper layer of the animal consists of “shiny sphericals” thought to provide anti-predatory defense is exposed to the surrounding environment. B) To remove animals from their environment, we would gently pipette bursts of water along the lateral edge of the animal to lift them from the surface. Often, one side of the animal appeared to adhere to the surface of the substrate while the opposite side could be lifted. During transfer, a number of animals received damage along a single side as it was removed from the substrate. These animals were given twenty-four hours to heal and resettle in our new culture dishes before processing.

**Latex bead experiments**

Animals were kept for longer periods of time (e.g., bead feeding experiments) in glass bowls and provided a drop of Micro Algae Grow (http://florida-aqua-farms.com) every 2-3 days. We set out to determine if the ventral side has absorptive properties. We used latex beads of 0.5 and 2 microns in diameter (Cat.# L3280 & L4530, Sigma Aldrich) to cover the surface of plastic petri dishes filled with seawater. Beads were suspended at a dilution of 1:100 from its stock in seawater, then two droplets from both bead samples were added to each dish and left to settle on the bottom overnight. The next day, dishes were washed with seawater to remove excess floating beads. Animals were then added to each dish and allowed to come in contact with the beads along the surface of the dish for four hours. After four hours of exposure to beads, animals were moved to glass slides for visualization by microscopy.

**Sample Imaging**

All experiments, except those involving fluorescent beads, were visualized using an Axioscope 2 compound microscope with an AxioCam (HRc) camera. Images were compiled using Axiosvision software (Zeiss Inc, Jena, Germany). Autofluorescence of
shiny sphericals could be seen using a FITC filter cube and was visualized using 488 wavelength settings. For fluorescent bead experiments, live animals were placed on glass slides and visualized using a Zeiss 710 scanning laser confocal. Images were cropped and assembled using Adobe Photoshop and Illustrator CS6.

**RNA, cDNA and Gene Isolation**

We collected approximately 100 animals from biofilmed slides and transferred animals to gelatin coated dishes. Any debris transferred with animals was removed periodically to eliminate foreign contaminants from our samples. Animals were starved overnight and washed with seawater again prior to RNA collection. All animals were transferred to a 1.5 ml tube and centrifuged (3000g) to the bottom of the tube. All remaining seawater was removed and animals were suspended in Trizol (Cat.# 15596-026, Invitrogen) and vortexed to lyse the tissue. Total RNA was extracted using the manufacturers protocol. Samples were DNAse (Cat.# 79254, Quiagen) treated to remove DNA contamination (15 minutes at 37°C) and then an Supplemental phenol chloroform extraction was used to clean and precipitate the RNA. cDNA was constructed using a SMARTer® RACE cDNA Amplification Kit (Cat.# 634923, ClonTech) for RACE cDNA and Advantage® 2 PCR Kit (Cat.#639206, ClonTech) for cDNA to be used for general PCR.

A number of desirable genes for analysis were identified using the genome (Srivastava et al., 2008) through the Joint Genomic Institute website (http://genome.jgi-psf.org/Triad1/Triad1.home.html). Nucleotide sequences were gathered and primers designed using MacVector (www.macvector.com). A list of primers used in this study can be found in Supplemental Figure 3.3. Fragments of approximately 600-1100 bases
were cloned using the pGEM® T Easy System (Cat.# A1360, Promega). Probes were synthesized using the MEGAscript® T7/SP6 Transcription Kits (Cat.# AM1334, AM1330, Invitrogen) and were stored at -20°C.

**Phylogenetic Analysis**

Protein sequences from a diverse set of taxa were collected from the NCBI protein database (http://www.ncbi.nlm.nih.gov). A list of the different species used in our phylogenetic analyses can be found in Supplemental Figure 3.4. Genomic resources of *Mnemiopsis leydei* (Ryan et al., 2013), *Acropora digitifera* (Shinzato et al., 2011), *Capitella teleta*, *Lottia gigantica*, *Helobdella robusta* (Simakov et al., 2013), *Daphnia pulex* (Colbourne et al., 2011) and *Branchiostoma floridae* (Putnam et al., 2008) were utilized in this study. Protein coding domains were assessed by SMART protein prediction (Schultz & Milpetz, 1998) and all sequences were aligned using MUSCLE (Edgar, 2004). Trees were constructed with MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) using five independent runs, consisting of 5,000,000 generations of “mixed” models. A second tree was constructed through maximum-likelihood analysis using RaxML (version 7.2.8) as described (Ryan et al., 2013). Maximum-likelihood tree bootstraps were based on 100 replicates. Trees were imported and edited in FigTree (version 1.4.0) (Rambaut, 2007).

**Whole mount in situ hybridization**

Animals were transferred from glass slides to gelatin-coated dishes using a glass pasteur pipette (Cat.#CLS7095D5X, Sigma-Aldrich) and allowed to settle on the bottom of the dish overnight. Fixation was achieved by gently adding ice-cold fix (4% PFA, 0.2% Glutaraldehyde in high salt seawater; 0.5g NaCl/50mL seawater) to the dish for 90
seconds. Following this, the solution was gently removed and ice-cold 4% PFA (in high salt seawater) was added and dishes placed at 4°C for 1 hour. Fix was removed and animals were washed three times in ice-cold diethylpyrocarbonate (DEPC)-treated H₂O, then dehydrated to 100% methanol over several steps (25% methanol:DEPC-H₂O for 5 minutes, 50% methanol:DEPC-H₂O for 5 minutes and 75% methanol:DEPC-H₂O for 5 minutes). Animals were washed in 100% methanol for one hour at 4°C and then used immediately for *in situ* hybridization.

Animals were transferred to a 96-well plate (1 animal per well), and rehydrated into 1x PBS pH7.4 + 0.1% Tween-20 (PTw) using a series of washes (150 µl per wash; 75% methanol:25% PTw, 50% methanol:50% PTw, 25% methanol:75% PTw), before washing 5 times in PTw. Proteinase K was added (0.01mg/mL) for 5 minutes before digestion was stopped by two, 5-minute washes in PTw+2mg/mL glycine. Animals were then washed twice (5 minutes each time) in 0.1% triethanolamine:PTw. This solution was substituted with 0.1% triethanolamine:PTw with 3µL/mL acetic anhydride added for 5 minutes, followed by a 5 minute wash in 0.1% triethanolamine:PTw with 6µL/mL acetic anhydride added. Animals were then washed 2 times for 5 minutes each in PTw and refixed in 4% PFA in PTw at 4°C for 1 hour. Fix was removed and animals washed 5 times in PTw, and then 2 times (10 minutes each wash) in hybridization buffer (50% deionized formamide, 5x SSC pH 4.5, 50g/mL heparin, 0.5% Tween-20 freshly made, 1% SDS, 100g/mL salmon sperm DNA). Animals were prehybridized in hybridization buffer for 3 hours to overnight at hybridization temperature. One crucial difference in our protocol, compared to previously published data, is that we hybridized our probes at 64°C, where all other published works used 50°C. Digoxigenin (DIG)-labelled anti-
sense RNA probe was added to a final concentration of 1ng/L and left to incubate at hybridization temperature for 48 hours. Following hybridization, probe was removed and a series of washes conducted at hybridization temperature; 10 minutes in 100% hybridization buffer, 20 minutes in 100% hybridization buffer, 20 minutes in 75% hybridization buffer:25% 2x SSC, 20 minutes in 50% hybridization buffer:50% 2x SSC, 20 minutes in 25% hybridization buffer:75% 2x SSC, 3 x 20 minute washes in 2x SSC and 3 x 10 minute washes in 0.05x SSC. A series of wash steps at room temperature were then conducted; 5 minutes in 75% 0.05x SSC:25% PTw, 5 minutes in 50% 0.05x SSC:50% PTw, 5 minutes in 25% 0.05x SSC:75% PTw and 3 x 10 minute washes in PTw. Samples were incubated in 1x Roche Blocking reagent:maleic acid buffer (Cat.# 11096176001) overnight (at 4°C?), then incubated in anti-DIG-AP antibody (catalog number 11093274910, 1:5000) overnight at 4°C. The following day, tissue was washed in at least 10 x 30 minute washes in PTw at room temperature, followed by 3 x 5 minute washes in PBS. Animals were then washed for 2 x 5 minute washes in AP-M buffer (0.1M NaCl, 0.1M Tris pH9.5, 0.05% Tween-20), then 2 x 5 minute washes in AP buffer (0.1M NaCl, 0.1M Tris pH9.5, 0.05% Tween-20, 0.1M MgCl₂). Visualization of probe was achieved by adding 3.3μL/mL NBT (US biological N2585; 75mg/mL in 70% dimethylformamide) and 3.3μL BCIP (US biological B0800; 50mg/mL in dimethylformamide) to AP buffer and incubating at room temperature until the desired level of staining intensity was reached. The reaction was stopped by washing in PBS or PTw 3-5 times, and animals mounted in 70% glycerol:PBS for analysis.
RESULTS AND DISCUSSION

Top vs. bottom and the biology of *Trichoplax*

One of the biggest mysteries concerning placozoan biology is how the animal thrives and survives in nature. Early studies by Grell and Benwitz (K. G. Grell & Benwitz, 1981; Grell, KG Benwitz, 1971) have suggested that the bottom epithelial layer has absorptive properties and that food may be stored in fiber cells. Animals are found in the wild on heavily biofouled surfaces and in culture are often raised on single species microalgae with dissolved organic media (Michael Eitel et al., 2011). While the bottom surface of the animal appears to have phagocytic capabilities, the top shiny spherical side is thought to serve as a protective layer (Jackson & Buss, 2009). Feeding experiments comparing whole placozoans vs. shiny spherical-free tissue showed that ingestion of shiny sphericals causes paralysis in the cnidarian *Podocoryna carneae* (Jackson & Buss, 2009). When observing the animal with transmitted light, the shiny sphericals glisten along the top of the animal (Figure 3.3A). We confirmed that when animals were exposed to FITC wavelength of light (excitation 488) the shiny sphericals along the top surface were easily identifiable by auto-fluorescence (Figure 3.3B) as documented (D. J. Miller & Ball, 2005). We utilized this property to distinguish the top layer during the remaining experiments. To test the absorptive properties of the bottom layer, we created an artificial substrate of carboxylate-modified latex beads and exposed animals to this bead-coated surface (see Materials and Methods). After a few hours of incubation, latex bead particles could be seen localized intracellularly within the epithelial layer that contacts the substrate (Figure 3.3C, white arrow indicates particles). Video analysis where we adjusted the focal plane during filming, revealed that the latex
beads were localized on the opposite side of the shiny sphericals (Supplemental Figure 3.5). The shiny sphericals along the top surface are approximately 4-5 microns in size (Figure 3.3D). Scanning confocal analysis of the lower epithelium shows that both sizes of beads (0.5 and 2 microns) could be found in clusters and appear compartmentalized along the lower surface. These results help confirm that the lower epithelial surface of the animal exhibits absorptive capabilities necessary for digestion of food.

Sexual reproduction and embryonic development of Trichoplax remain unknown (Michael Eitel et al., 2011), and laboratory cultured animals reproduce via lateral fission or budding of “swarmers” from the top portion of the animal (K. Grell, 1974; Schulze, 1891; Thiemann & Ruthmann, 1991; Thiemann, M Ruthmann, 1988). Swarmers found in our cultures appear to have differentiated top and bottom surfaces reminiscent of adults (Figure 3.3F-H), and have been reported to have all three cell layers (Thiemann & Ruthmann, 1991). We could not confirm if the two distinct surfaces exist when a swarmer is born or if this patterning occurs after it has budded from the parent. Overall, the morphologically distinct upper layer, and the phagocytotic lower layer (which appears much thicker) are easily distinguishable and these characteristics were used to determine top from bottom after in situ hybridization.
Figure 3.3 – *Trichoplax* has morphologically distinct upper and lower epithelial layers. A) A view of a single whole animal under transmitted light reveals the ‘shiny sphericals’ along the upper layer of the animal. B) Animal exposed to FITC wavelength of light, showing the distribution of auto-fluorescent ‘shiny sphericals’ along the top tissue layer. C) A second photo from B, showing a distribution of green particles along the lower epithelial layer. Animal in B-C were exposed to a layer of charged fluorescent beads that get absorbed and accumulate along this lower surface. D) High magnification image of the ‘shiny spherical’ surface along the upper layer. E) Confocal microscopy image of the lower epithelial layer shows that animals exposed to beads accumulate both two and one-half micron beads. (Beads false colored blue to show
contrast with ‘shiny spherical’ images). F-H) Asexually produced ‘swarmer’ has an asymmetric distribution of ‘shiny sphericals’ along one side of the animal.

*Endomesodermal targets are expressed along the bottom layer of the animal.*

Several genes thought to play a role in endomesodermal formation in cnidarians have been reported present in the genome of *Trichoplax*. The homeobox gene *forkhead* (*foxA*) and the zinc-fingered transcription factor *snail* are expressed along the developing endomesodermal region of the cnidarian, *Nematostella vectenesis* (Magie et al., 2007) and within endodermal (*foxA*) or mesodermal (*snail*) tissue of the hemichordate *Saccoglossus kowalevskii* (Green, Norris, Terasaki, & Lowe, 2013). *Trichoplax* has a single ortholog each of *forkhead*, *snail* and *scratch* (Kerner et al., 2009; Srivastava et al., 2008), a relative of *snail* typically associated with nervous system development in protostomes (Roark et al., 1995). We found *TaSnail* to be highly expressed along the bottom surface of the animal, and exhibiting a salt-and-pepper pattern along the top layer of the animal (Figure 3.4A).

Two other homeobox genes, *brachyury* and *tbx2/3* were previously analyzed by *in situ* hybridization in *Trichoplax* (Martinelli & Spring, 2003). *Brachyury* is often associated with endomesodermal fates in cnidarians (Scholz & Technau, 2003), echinoids (Gross & McClay, 2001) and hemichordates (Green et al., 2013) and was previously shown to be expressed in a ring around *Trichoplax* (Martinelli & Spring, 2003) (Figure 3.4B). Although the authors did not confirm the position of expression (top vs. bottom), we feel that based on the location and distribution of signal that this area is the “ring” around the lower epithelial layer. T-box transcription factor *tbx2/3* is a marker of dorsal tissue in both sea urchins (Lapraz, Besnardeau, & Lepage, 2009; Saudemont et al., 2010) and
hemichordates (Lowe et al., 2006). In the ctenophore, *Mnemiopsis leidyi*, *MITbx2/3* localizes to both oral and aboral domains during gastrulation and is found along the region where ectodermal comb-rows and the apical sensory organ develop in the cydippid larva (Yamada, Pang, Martindale, & Tochinai, 2007) (Figure 3.4B). For comparative measures, we conducted *in situ* hybridization of the ortholog of *tbx2/3* from the cnidarian sea anemone, *Nematostella vectensis* (Yamada et al., 2007). We found *NvTbx2/3* to be expressed within both oral and aboral domains during gastrulation and at the planula stage of development (Supplemental Figure 3.6). Interestingly, *TaTbx2/3* was reported to localize to both top and bottom layers with greater expression on the top layer of the animal (Martinelli & Spring, 2003) (Figure 3.4B). It appears *tbx2/3* was first involved in patterning both oral and aboral domains, and was restricted to dorsal domains in deuterostomes.

A fourth homeobox gene belonging to the *hox/parahox* class of transcription factors, *Trox-2*, was previously reported to be expressed in a ring around the top side of the animal [37] or in a subset of cells between the two layers (Jakob et al., 2004). Knockdown of *Trox-2* by soaking in RNAi or a morpholino inhibits growth and asexual reproduction with cultured animals (Jakob et al., 2004). Contrary to the previously published findings, we found *Trox-2* to be highly expressed along the entire lower half of the animal (Figure 3.4C). In one sample, the bottom layer had been destroyed during the fixation process (red dotted square) and the shiny sphericals are easily visible (red arrow) on the opposite side of *Trox-2* expression. The ortholog of *Trox-2* in cnidarians is primarily expressed along the oral pole and within the endomesoderm of both corals and anemones (Hayward et al., 2001; Ryan et al., 2007) while ctenophores and
sponges lack \textit{Hox/ParaHox} like genes (Ryan et al., 2013; Srivastava et al., 2010) (Figure 3.4B). Taking together the different expression patterns collected here and among other publications, a growing amount of evidence suggests that the bottom layer of the \textit{Trichoplax} may share characteristics of a primitive oral and/or endomesodermal tissue. These findings also suggest that homeobox genes, which are found in plants, fungi and animals, may have similar territorial patterning functionality between early animals, in the absence of true hox genes which emerged in the cnidarian-bilaterian ancestor (Chourrout et al., 2006; DuBuc et al., 2012; Ryan et al., 2007, 2013; Srivastava et al., 2010).

One of the more interesting questions pertaining to placozoan biology is why they appear to lack a true nervous system while ctenophores and cnidarians have a nerve net? Based on the phylogenetic position and gene complement found in ctenophores, it has been proposed that the nervous system in ctenophores may have independently evolved after the emergence of placozoans and cnidarians or may have been secondarily reduced in sponges and placozoans (Ryan et al., 2013). One interesting shared trait among ctenophores, placozoans and cnidarians is the presence of RF-amide positive cells, suggesting neuropeptide signaling is present between cells in each animal (Girosi et al., 2005; C. J. Grimmelikhuijzen, 1983; C. Grimmelikhuijzen, 1985; Jager et al., 2011; Mackie, Singla, & Stell, 1985; H. Q. Marlow, Srivastava, Matus, Rokhsar, & Martindale, 2009; Schuchert, 1993). In ctenophores and cnidarians, localization of FMRFamide antibody positive cells appears in neuron-shaped cells with cellular projections (Girosi et al., 2005; C. J. Grimmelikhuijzen, 1983; C. Grimmelikhuijzen, 1985; Jager et al., 2011; Mackie et al., 1985; H. Q. Marlow et al.,
2009), while staining in *Trichoplax* is found in round cells, lacking projections (Schuchert, 1993). It has also been suggested that this region overlaps with expression of the homeobox gene *PaxB* (Hadrys et al., 2005b), orthologous to vertebrate Pax 2/5/8 genes and potentially Pax 4/6 genes (Matus, Pang, Daly, & Martindale, 2007), a gene family closely related to visual sensory system development. We investigated a third neuronal related gene, embryonic lethal abnormal vision (*ELAV*) previously found expressed in neuronal tissue in the cnidarian *Nematostella* (H. Q. Marlow et al., 2009; Nakanishi et al., 2012) and a wide variety of bilaterians (H. C. Park et al., 2000; Perron, Furrer, Wegnez, & Th??odore, 1999; Robinow, Campos, Yao, & White, 1988). The *Trichoplax* genome has a single *ELAV* gene which is closely related to *NvELAV1* from *Nematostella* (Nakanishi et al., 2012). During the larval and adult stage of *Nematostella* development, *NvELAV1* mRNA is found within the endoderm of the animal (H. Q. Marlow et al., 2009), while protein localizes to post-mitotic neurons of ectodermal and endodermal origin (Nakanishi, Yuan, Jacobs, & Hartenstein, 2008). Expression of *TaELAV* is faintly expressed along the bottom layer of the *Trichoplax* (Figure 3.4D). Although *TaELAV* is likely not patterning nervous system tissue in *Trichoplax*, it raises the possibility that the bottom layer may have sensory capabilities.

Due to the broader expression domain of *Trox-2* and the higher abundance of transcripts localized to the bottom layer of the animal (this manuscript) or in a ring (previous publications), we wanted to determine if areas within the animal are prone to develop non-specific staining. We used a probe developed for beta-actin, which is generally considered a “house-keeping” gene, expressed in all cells. We found that beta-actin is indeed found in all cells (Figure 3.4E) and appears to exhibit greater
density in animals that were preserved in a contracted state (Figure 3.4E, left). The lower layer of the animal generally appears to exhibit lighter expression than the upper layer (Figure 3.4E, right).

Figure 3.4 – Endomesodermal targets localize to the lower epithelial layer of *Trichoplax*. A) *In situ* hybridization of adult *Trichoplax*, showing the distribution of
*TaSnail* primarily along the lower epithelial layer. Along the top surface of the animal, *TaSnail* is distributed in a ‘salt-n-pepper’ fashion, where in the lower epithelial layer it is broadly expressed throughout. B) Previously reported expression domains of targets found to exhibit endomesodermal expression. Only *MlBrachyury* and *MlTbx2/3* have been identified in ctenophores, while ctenophores lack *hox*-related genes (*Trox-2/GSX*). C) Expression of the *hox*-related gene, *Trox-2* localizes to the entire lower epithelial layer of the animal. D) Expression of the RNA-binding protein, *ELAV*, localized primarily to the lower epithelial layer of the animal. E) *Beta-actin* expression is found throughout the animal, and appears highly expressed in animals that are in a contracted state (left) and exhibit lower expression along the lower epithelial layer (right). *In situ* results were based off a total of twelve samples. The total sample number (N) is indicated in each panel series. Values indicate how many animals exhibited the same expression domain and total numbers that were less than twelve indicated the remaining samples did not show any expression.

**Chordin - tgfβ signaling and the dorsal/ventral axis**

Coordinated patterning of the dorsal-ventral axis through chordin-bmp signaling, as known in bilaterians is thought to have originated in the cnidarian-bilaterian ancestor (Matus, Thomsen, et al., 2006; Matus, Pang, et al., 2006; Rentzsch et al., 2006; Saina et al., 2009). Ctenophores appear to lack *chordin (chd)* and bone morphogenetic protein 2/4 (*bmp2/4*) (Pang, Ryan, Baxevanis, & Martindale, 2011; Ryan et al., 2013), two of the main components associated with dorsal-ventral patterning in bilaterians (Lowe et al., 2006). In *Trichoplax*, it has previously been stated that the oral-aboral axis (lower-upper layer) corresponds to ventral-dorsal layers of other animals (K. G. Grell & Benwitz, 1981). To date, few genes have been analyzed by *in situ* hybridization to test this hypothesis (Hadrýs et al., 2005b; Jakob et al., 2004; Martinelli & Spring, 2003, 2004; D. J. Miller & Ball, 2005). Placozoans have four bmp-like genes and a single chordin-like gene (Srivastava et al., 2008). The chordin-like gene however lacks chordin (CHD) domains (Richards & Degnan, 2009) and is composed of four Von Willebrand factor repeats or CR-domains (Figure 3.5). We gathered chordin protein sequences from a
number of taxa to compare conservation of both chordin (CHD) and cysteine-rich (CR) domains found throughout metazoa (Supplemental Figures 3.7-3.8). It appears that the cnidarian-bilaterian ancestor had a chordin protein consisting of four cysteine-rich Von Willebrand domains (CR1-4) and four chordin domains (CHD1-4), although a truncated chordin-like gene was found in the coral *Acropora digitifera* (Figure 3.5). Interestingly, blast analysis did not reveal a true *chordin* gene from annelids as reported (Kuo & Weisblat, 2011), and chordin genes from mollusks, nematodes and some arthropod genes appear fragmented or missing CHD domains.

CHD domains are easily resolved into four different families, and appear to be well conserved among different taxa (Supplemental Figure 3.9). Studies in *Xenopus* have shown that chordin CR-domains are primarily responsible for dorsalization of tissue during development (Larraín et al., 2000), although CR-domains from closely-related genes also show dorsalizing activity (Matsui, Mizuseki, Nakatani, Nakanishi, & Sasai, 2000). A comparison of *Trichoplax* chordin CR domains to cnidarian and bilaterian chordin CR domains shows that *Trichoplax* chordin contains each one of the four CR domains present in other taxa (Figure 3.6). A single CR domain-containing gene was identified from the ctenophore *Mnemiopsis leidyi* and the sponge *Amphimedon queenslandica*, while a second gene in *A. queenslandica* contains three CR domain repeats (Supplemental Figure 3.10). The two CR domain-containing genes from ctenophores and sponges do not clearly associate with the four domains in other taxa (Figure 3.6), and the additional *A. queenslandica* (three CR domain) gene does not clearly resolve into distinct groups (1-4) found in other taxa (data not shown). Additional chordin-like genes (lacking CHD domains) from *Nematostella* and *Hydra* were identified
but appear to group closely with only CR2 domains of *chordin* (Figure 3.6), suggesting these are not true *chordin* genes or are highly divergent. Urchin, hemichordate and vertebrate *chordin*-like genes (genes containing 3 CR repeats) appear to be closely related to CR1, 2 and 4 of other taxa (Figure 3.6, Supplemental Figure 3.10), but have been reported to be related to CR1, 2 and 3 of vertebrates (Coffinier, Tran, Larraín, & De Robertis, 2001). Our results suggest that annelids and *Hydra* may have lost true chordin genes, while ctenophores and sponges diverged prior to the evolution of this gene family all together. Two *chordin*-like genes, *kielin*-related, are present in the ctenophore genome (data not shown) and a single gene has previously been documented in *Hydra* (Rentzsch, Guder, Vocke, Hobmayer, & Holstein, 2007). The *kielin* genes, which contain many CR repeats, have dorsalizing activity in vertebrates but are not sufficient to cause ectodermal neurulation (Matsui et al., 2000). If a dorsal-ventral axis, comparable to bilaterians, existed in ctenophores and sponges, a *kielin*-like gene may function in establishing or maintaining that axis during development (Garcia Abreu, Coffinier, Larraín, Oelgeschläger, & De Robertis, 2002).

Because CR domains are found in a number of different genes, we performed genomic synteny analysis between *Nematostella chordin* and the four CR repeats in *Trichoplax* to determine if other conserved elements support a common origin. We found that four genes (two on either side) are highly conserved between the two scaffolds containing *chordin* in each group (Supplemental Figure 3.11), although each gene is distantly spaced over each scaffold. One of the conserved genes, *suppressor of hairless* (SuH), was previously shown to interact with *notch* signaling in *Nematostella* (H. Marlow, Roettinger, Boekhout, & Martindale, 2012). Together with the phylogenetic
relatedness of CR domains and the conserved genomic synteny, there is compelling evidence that *Trichoplax* has a *chordin* gene ortholog that lacks the CHD domain.

One of the core features of dorsal-ventral patterning in bilaterians is the interaction between the inhibitor *chordin* and *bmp* signaling. *Trichoplax* has four *bmp-related* genes in the genome, therefore we decided to determine the phylogenetic relationship of these genes to counterparts in cnidarians, ctenophores and sponges. Recent work on ctenophores showed a clear ortholog of *bmp5/8* and *bmp3/admp* in *Mnemiopsis leidyi* (Pang et al., 2011) and transcriptomic analysis of various sponge species identified additional tgfβ-ligands (Riesgo, Farrar, & Windsor, 2014). We aligned sequences from a number of invertebrate taxa including ctenophores, sponges, placozoans and cnidarians to determine the origin of *bmp-related* genes in *Trichoplax* (Supplemental Figures 3.12-15). A summary of the different phylogenetic conditions along with the number of resolved *bmp-like* tgfβ ligands was constructed (Figure 3.7). This table is based on both maximum likelihood (white triangle) and bayesian (grey triangle) phylogenetic analysis taken from Supplemental Figures 3.16-23. In each scenario, our trees resolved a *bmp3/admp-like* gene as well as a *bmp2/4* ligand. We found that including all sponge and ctenophore sequences along with *nodal* allowed us to resolve only two *bmp* ligands (Figure 3.7). If *nodal* sequences are removed, an additional gene, *gdf5*, is suggested to be present in *Trichoplax*. Limiting the number of sponge, ctenophore and nodal sequences helped resolve *bmp5/8* in *Trichoplax*, which is also present in ctenophores (Pang et al., 2011). Although *bmp5/8* appears resolved, our *gdf5-like* gene becomes closely related to the *bmp2/4* lineage, and to date early branching animals and most invertebrates are thought to only have a single *bmp2/4* ligand. Our phylogenetic
analysis (although variable) may suggest that Trichoplax has a bmp-like content more similar to cnidarians, composed of bmp3/admp, bmp5/8, bmp2/4 and a gdf5-like gene.

Due to the variability within our phylogenetic analysis, we also performed synteny analysis (compared to Nematostella) of bmp-related genes found in the Trichoplax genome. TaBmp3/admp did not exhibit synteny with related NvBmp3/admp, however TaBmp2/4 shared close proximity to a total of four genes with NvBmp2/4, including an ABC transporter immediately adjacent to the coding region of bmp2/4 in both animals (Figure 3.8). A notch gene from Nematostella (H. Marlow et al., 2012) is closely associated with bmp2/4 in both genomes, and likely duplicated in Trichoplax (Figure 3.8). The conservation of both SuH and notch-like genes in both taxa may imply an ancient relationship existed between regulatory elements of notch signaling and chordin/bmp patterning in early animals. Further analysis also shows a large amount of synteny between TaBmp2/4 and NvChordin containing scaffolds (Supplemental Figure 3.24), suggesting that the common ancestor of placozoans and cnidarians may have had a genomic cluster where the two antagonistic genes were found adjacent to one another. A case in which this is still present in an animal genome was identified only in Caenorhabditis elegans and Caenorhabditis briggsae and involved the chordin-like gene crm-1 and a bmp2/4 ortholog dbl-1, which were found to be present on the same chromosome (data not shown).

Synteny analysis of bmp5/8-related genes in Nematostella and Trichoplax showed a larger conservation of genes over a larger scaffold distance (Figure 3.9). A single gene along the bmp5/8 scaffold in Trichoplax appears to have undergone duplication (itm1). TaGdf5 is found on the same scaffold as TaBmp5/8, although this is not the case in
Nematostella. NvGdf5 is located on a small scaffold containing only 137,328 bases (Supplemental Figure 3.25), and a single gene (epidermal retinol dehydrogenase) from this scaffold exhibited synteny with the Trichoplax genome. Interestingly, a gene (mutS) located immediately adjacent to this shared region exhibits synteny with the scaffold containing NvBmp5/8, suggesting that this gene once belonged among this group of genes (Supplemental Figure 3.25). Taking in account both phylogenetic and synteny analysis, we believe that the common ancestor of placozoans and cnidarians had four bmp-ligands related to bmp2/4, bmp5/8, bmp3/admp and gdf5. With the inclusion of more sponge and previously identified ctenophore sequences (Pang et al., 2011), we found that sponges and ctenophores had at least bmp5/8 and bmp3/admp. Future studies may resolve these groups to also have a gdf5-related family of genes, yet a true bmp2/4 ligand has never been identified through phylogenetic analysis.

We performed in situ hybridization on TaChordin, TaBmp2/4, TaGdf5 and TaBMP3/admp in Trichoplax (Figure 3.10). Expression of TaChordin is localized to a small spot on the bottom layer of the animal and overlaps with TaBmp2/4 expressing cells (Figure 3.10A-C). When both probes were added, a central domain (TaChordin) and a broad domain (TaBmp2/4) can be seen together (Figure 3.10C). TaGdf5 localizes to a ring around the lower epithelial layer and may exhibit patches of lower expression within this ring (indicated by arrows) (Figure 3.10D). To our surprise, TaBmp3/admp was asymmetrically localized to the lateral half of the animal and exhibited expression on both surfaces (Figure 3.10E). Due to the lack of morphologically distinguishable cell-types in the region of TaBmp3/admp expression, our current opinion is that this expression suggests underlying cryptic patterning, which may not be visible by
morphological structures. During collection, animals could easily be lifted off the substrate by pipetting streams of water towards their lateral edge. Often, one side of the animal exhibited greater adhesion and would take more pipette propulsion to remove them from the substrate. These correlative results hint that animals may have a region of attachment to allow them to remain on the substrate.

Expression analysis of *chordin* in *Nematostella* shows localization along one half of the blastopore lip during gastrulation (Figure 3.10F), and functional analysis suggests *chordin* regulates the directive axis (along with *bmp2/4*) of the cnidarians (Saina et al., 2009). *Chordin* is expressed along the dorsal ectoderm in vertebrates (Sasai et al., 1994) and ventral ectoderm in echinoids (Lapraz et al., 2009), hemichordates (Lowe et al., 2006; E Röttinger & Martindale, 2011), flies (Biehs, Francois, & Bier, 1996) and beetles (van der Zee, Stockhammer, von Levetzow, Nunes da Fonseca, & Roth, 2006). Our expression analysis of *TaChordin* and *TaBmp2/4* suggest the bottom surface could have ventral patterning components (Figure 3.10G), but more molecular markers and functional data is needed to test this hypothesis.

**Regionalized expression domains of placozoans**

With our current findings, coupled with previously published expression patterns, it appears that placozoans have at least four distinct molecular patterning zones. Although only a few genes have been tested, components involved in endomesodermal formation in cnidarians are often expressed along the lower epithelial layer of *Trichoplax* (Figure 3.11A), an area thought to be an absorptive/digestive layer (Figure 3.2A, Figure 3.3). Interestingly, a *chordin*-like gene is expressed in the lower layer of the animal and overlaps *bmp2/4* expression. If we think of the bottom surface of *Trichoplax* as a giant
mouth, *TaChordin* may function in a similar way to cnidarians, urchins and hemichordates which also exhibit chordin expression around the presumptive mouth. Undoubtedly, many of these details could be resolved by solving the ‘black box’ that is *Trichoplax* embryonic development.

Our findings, although suggestive, require greater analysis and further study to fully understand what genetic components allowed for the radiation and increase in morphological complexity of cnidarians and bilaterians. For example, another pathway of great interest to animal-vegetal patterning is wingless (*wnt*) signalling. *Wnt* ligands in *Nematostella* are expressed in stripes along both ectodermal and endodermal regions of the animal (Kusserow et al., 2005; P. N. Lee, Pang, Matus, & Martindale, 2006) (Figure 3.11B). *Trichoplax*, has three *wnt* ligands, related to *wnts* 2, 5 and 8 of *Nematostella*. With our current understanding of *Trichoplax* patterning domains, we could predict to find the three *wnt* genes in discrete domains along the upper and lower epithelial layers (Figure 3.11C). Unfortunately, the fiber layer of the animal has been overlooked in all studies to date and it may require sectioning to completely identify transcripts localized to this region. We hope that this study can help pioneer future studies to gain a greater understanding of the functional toolkit present in the last common ancestor of parahoxozoa.
Figure 3.5 – Protein structure of *chordin* genes among diverse taxa. Domain analysis of chordin domains among many taxonomic groups suggests that most chordin genes consist of four cysteine-rich (CR) domains with a variable number of chordin (CHD) domains. A single *chordin-like* gene consisting of four CR-repeats is present in the genome of *Trichoplax adhaerens.*
Figure 3.6 – Domain analysis of *chordin* CR-domains suggests that *TaChordin* has phylogenetically similar domains as other metazoan chordins. A) Predicted domain structure of *chordin* genes in a variety of metazoan taxa. Most *chordin* genes have both CHD and CR domains, while *Acropora digitifera* appears to have two truncated versions of *chordin*. *TaChordin* is composed of four CR-domain repeats. B) Maximum likelihood analysis of CR-domains suggests that *Trichoplax* has a single representative of each CR-domain (indicated by red box) found in true *chordin* genes and that these domains appear to be highly conserved over evolutionary time. (See materials and methods for information on tree construction). *Chordin-like* genes (ChdL) containing three CR-repeats are found in many taxa, yet only deuterostome genes could be resolved into distinct clades.
Figure 3.7 – Distribution of tgfβ ligands in Trichoplax based on multiple phylogenetic trees. Trichoplax appears to have representatives of bmp3/admp and bmp2/4, while representatives of bmp5/8 and gdf5 are less resolved. Table is based on maximum likelihood (white triangle) and bayesian phylogenetic analysis (grey triangle). Relationships were constructed from Supplemental figures 16-23.

Figure 3.8 - Synteny analysis of scaffolds containing bmp2/4 genes of Nematostella and Trichoplax genomes. (Green dotted line shows that synteny occurred between two separate regions of the scaffold).
Figure 3.9 - Synteny analysis of genomic scaffolds containing a \textit{bmp5/8} gene of \textit{Nematostella} and \textit{bmp5/8} and \textit{gdf5} of \textit{Trichoplax}. (Green dotted line shows that synteny occurred between two separate regions of the scaffold).
Figure 3.10 – Distribution of *chordin-bmp* signaling components involved in patterning of *Trichoplax*. A) Expression of *TaChordin* localizes to the lower epithelial layer of the animal in a discrete population of cells. B) *TaBmp2/4* expression along the oral surface of the animal. C) Co-expression of both *TaChordin* and *TaBmp2/4*. D) *TaGdf5* is expressed in a ring along the lower epithelial layer of the animal, and appears to be differentially expressed along this ring. E) *TaBmp3/Admp* is found on one side of the animal and may be involved with regeneration of the animal. D) Distribution of dorsal-ventral patterning genes found during gastrulation of *Nematostella vectensis*. The oral pole / site of gastrulation is indicated by an asterisk. E) Patterning domains in *Trichoplax*.

**Figure 3.11 – *Trichoplax* likely has four discrete patterning domains found along their primary axis.** A) A summary of known expression domains for key regulators of tissue specification in cnidarians and bilaterians. To date, no expression data exists for the central fiber layer of the animal, but currently three domains exist along the upper and lower epithelial layers. We suggest that the upper and lower epithelia, the lateral edge and the fiber cells compromise four distinct patterning zones in *Trichoplax*. B) Summary of wnt orthologs during gastrulation along the oral-aboral axis of *Nematostella vectensis*. C) We predict that the three wnt orthologs in *Trichoplax* may also pattern the three distinct outer regions of placozoans.
CHAPTER 4

Initiating a regenerative response, cellular and molecular features of wound healing in the cnidarian *Nematostella vectensis.*

ABSTRACT

Wound healing is the first stage of a series of cellular events that are necessary to initiate a regenerative response. Defective wound healing can block regeneration even in animals with a high regenerative capacity. Understanding how signals generated during wound healing promote regeneration of lost structures is highly important, considering that virtually all animals have the ability to heal but many lack the ability to regenerate missing structures. Cnidarians are the phylogenetic sister taxa to bilaterians and are highly regenerative animals. To gain a greater understanding of how early animals generate a regenerative response, we examine the cellular and molecular components involved during wound healing in the anthozoan cnidarian *Nematostella vectensis.*

Pharmacological inhibition of notch blocks regeneration in *Nematostella*, while inhibition of MAPK (ERK) signaling blocks regeneration and wound healing. We characterized early and late wound healing events (in comparison to ERK treated samples) through genome-wide microarray analysis, quantitative PCR, *in situ* hybridization and confocal microscopy to identify potential wound healing targets. We identified a number of genes directly related to the wound healing response in other animals (metalloproteinases, growth factors, transcription factors) and suggest that glycoproteins (mucins and uromodulin) play a key role in early wound healing events. This study also identified a novel cnidarian specific gene, a thiamine biosynthesis
enzyme (vitamin B synthesis) that may have been incorporated into the genome by lateral gene transfer from bacteria and now functions during wound healing. Lastly, we suggest that ERK signaling is a shared element of the early wound response for animals with a high regenerative capacity.

This research describes the temporal events involved during Nematostella wound healing, and provides a foundation for comparative analysis with other regenerative and non-regenerative species. We have shown that the same genes that heal puncture wounds are also activated after oral-aboral bisection, indicating a clear link with the initiation of regenerative healing. This study demonstrates the strength of using a forward approach (microarray) to characterize a developmental phenomenon (wound healing) at a phylogenetically important crossroad of animal evolution (cnidarian-bilaterian ancestor). Accumulation of data on the early wound healing events across numerous systems may provide clues as to why some animals have limited regenerative abilities.

INTRODUCTION

Wound healing is the process of cellular contraction, movement and re-adhesion immediately after injury and is the precursor to regeneration of lost structures. During these cellular dynamics, other components such as immunity, cell-death/proliferation, or nervous system inputs all interact during the process of scar-free healing (Gurtner, Werner, Barrandon, & Longaker, 2008). Among animals with high regenerative capabilities, a unifying theme has emerged suggesting that the cells that re-epithelialize the wound provide the signals necessary to initiate regeneration (Brockes & Kumar,
Defects in the wound healing program, including excessive scar formation or mechanically manipulating the wound, can block regeneration, even in animals with a high regenerative capacity (Mescher, 1976; Newman, 1974; Silver & Miller, 2004). Understanding the process of wound closure in diverse animal groups that vary in regenerative capacity, may help reveal factors correlated with the loss of regeneration.

The process of wound healing exists widely throughout the animal kingdom, yet after a significant loss of tissue, few animal groups can faithfully regenerate the entire complement of original tissue. The cnidarians (corals, jellyfish, sea anemones, etc.) are diploblastic animals, consisting of ectodermal and endodermal tissue (Hyman, 1940). Many studies have demonstrated that cnidarians are a powerful model for understanding the evolution of bilaterians, based on their phylogenetic position (sister to bilaterians), and because they are more similar in terms of genomic content and organization to deuterostomes than other model systems (Hejnol et al., 2009; Matus, Pang, et al., 2006; Philippe et al., 2009; Putnam et al., 2007; Saina et al., 2009). As adults, cnidarians exhibit a high regenerative capacity with few limitations (Holstein et al., 2003). The medusazoan model system, Hydra, has long been a comparative model for regenerative study, bridging the gap between early animals, planarians, flies and vertebrates. The anthozoan cnidarian, Nematostella vectensis, is widely known as a comparative system for embryological studies, yet following bisection through their major longitudinal axis (the oral-aboral axis) both halves can regenerate into normal animals (Bosser, Dunn, & Thomsen, 2013; Passamaneck & Martindale, 2012; Reitzel, Burton, Krone, & Finnerty, 2007; Stefanik, Friedman, & Finnerty, 2013; Trevino, Stefanik, Rodriguez, Harmon, & Burton, 2011; Tucker, Shibata, & Blankenship, 2011).
Unlike *Hydra*, anthozoans like *Nematostella* do not appear to have an I-cell population of precursor stem cells [20]. Instead, cell proliferation is required for the completion of the regenerative process in *Nematostella* and is first active eighteen hours after injury (Holstein, Hobmayer, & David, 1991; H. Park, Ortmeyer, & Blankenbaker, 1970; Passamaneck & Martindale, 2012). Interestingly, regeneration experiments where wound healing was allowed to proceed, but cellular proliferation was chemically blocked, can be rescued by re-injuring the same untreated tissue, triggering mitosis and regeneration (Passamaneck & Martindale, 2012). This suggests that wound healing acts as an initiator of regeneration in *Nematostella*, where the onset of proliferation may serve as an important transition between wound healing and a regenerative response (Figure 1A). Regeneration of lost oral structures takes approximately 72 hours (Figure 4.1B), yet little is known about the timing and transition from wound healing to regeneration in *Nematostella*. A genomic survey of stress response genes suggests that homologs of many vertebrate genes previously associated with wound healing are also present in the *Nematostella* genome, although a quantitative assessment of gene expression is lacking (Reitzel, Sullivan, Traylor-Knowles, & Finnerty, 2008). Overall, the high regenerative capacity and key phylogenetic position of cnidarians provide a unique opportunity to study the basic mechanism underlying animal wound repair. This type of study is of great interest in comparison to other highly regenerative animals (to see similarities) and in comparison to animals that lack the capacity to regenerate (to see differences). Studying new systems with forward approaches also provides unique opportunities for gene discovery.
The map-kinase (MAPK) signaling pathway is present in all eukaryotic genomes and functions in a wide range of cellular processes including immune system regulation, proliferation, apoptosis, cell signaling and movement. A subset of the pathway, ERK signaling, regulates initial events of *Drosophila* wound closure by regulating actin dynamics around the site of injury (Wang et al., 2009). In vertebrate cell culture, scratch assays show that ERK is localized in contractile cells around the wound margin (Matsubayashi, Ebisuya, Honjoh, & Nishida, 2004). ERK signaling is also a key regulator of the *Grainyhead* gene family, a group of genes known for their role in establishment of the epithelial layer and their role in wound healing across animals (Kim & McGinnis, 2011). Activation of ERK is also linked with the innate immune response in a number of animals (Furler & Uittenbogaart, 2010; Odendall et al., 2012; Surachetpong, Singh, Cheung, & Luckhart, 2009) where MAPK signaling is likely the main signaling system for host/parasite or symbiont/host interactions. Among the results reported here, we found that the inhibition of ERK signaling blocked both wound healing and regeneration in *Nematostella*. Using a diverse set of approaches we describe many of the components involved during wound healing in *Nematostella* and show that a universal set of genes are activated during different types of wound healing, prior to regeneration.
Figure 4.1 - Wound healing, a necessary precursor to regeneration. A) Wound healing and regeneration are separable developmental processes that may involve different gene cascades. B) Head regeneration is a rapid process in *Nematostella* where structural integrity is renewed approximately seventy-two hours after injury. C) Schematic representation of the experimental setup of regeneration and wound healing experiments using *Nematostella* juveniles. In regeneration experiments, animals are cut just below the pharynx region (red line). In wound healing experiments, animals are punctured at the central position between the mesenteries and the aboral pole. Puncture wounds span both sides of the animal creating two similar wounds.

MATERIALS AND METHODS

Animal care, cutting and puncture assays

*Nematostella* polyps raised in 1/3x seawater (Hand & Uhlinger, 1994) were collected at day fourteen after fertilization. Animals underwent two feedings of *Artemia* between day seven and day fourteen after fertilization, and then were starved for seven days to minimize non-specific staining due to food particles in the body cavity. Juvenile polyps were used due to their small size and ease of visualization and because they can regenerate lost structures similar to adults. Animals were cut in two millimeters thick
silicon-coated dishes (SYLGARD–184, Dow Corning, Inc.). The silicon-coated dish was used to create a cutting/poking surface that doesn’t damage the tools used. Glass needles for puncture assays were formed from capillaries (World Precision Instruments – TW100F4) using a needle puller (Model P97, Sutter Instrument Co.). All transverse cuts were done with a cornea scalpel and were preformed near the base of the pharynx. Cutting experiments were done in all regeneration experiments; puncture experiments were used to assay components of wound healing. A standardized protocol for injuring animals was developed, similar to puncture assays in *Drosophila* (Wang et al., 2009), to allow for easy visualization of cellular events during wound healing. Punctures were centered between the base of the mesenteries and aboral tip of the animal (Figure 4.1B). We chose this area to assay wound healing targets because of the thin tissue layer that can be easily visualized microscopically. This area is also highly regenerative in *Nematostella* (Reitzel et al., 2007), being the approximate location of bud formation and serves as a comparative point with other cnidarians such as *Hydra*.

Animals used in drug treatment experiments were soaked for one hour prior to injury. The drugs U0126 (Cat. #U120, Sigma, Inc.) and DAPT (Cat. #D5942, Sigma, Inc.) were both used at 10µM concentration dissolved in 1% dimethyl-sulfoxide (DMSO). These two drugs have been shown to knockdown components of MapK (U0126) and Notch (DAPT) signaling in *Nematostella* (H. Marlow et al., 2012; Rentzsch et al., 2008). For experiments lasting longer than twelve hours, drugs were replaced every twelve hours until the termination of the experiment. All experiments were conducted at 25°C in the dark to prevent degradation of the inhibitors. For recovery experiments, animals were washed three times with 1/3x seawater, and then placed back in the dark at 25°C.
**Tunel assay for apoptosis**

We used the DeadEnd Colorimetric Tunel kit (Cat.#G7130, Promega, Inc.) to determine the distribution of apoptotic cells over time. Although the manufacturer’s protocol was designed for tissue sections, we made the following changes for whole mount preparations: 1) Animals were relaxed by gently adding 7% MgCl₂ to the 1/3x seawater. 2) Animals were fixed with 4% paraformaldehyde in 1/3x seawater for one hour at room temperature. 3) Animal tissue was washed five times with phosphate buffered saline (PBS) with 0.2% Triton-X100 (PBT). 4) Fixed polyps were permeabлизed with proteinase K for twenty minutes as specified in the protocol. 5) Tissue was then washed (2x) with PBS. 6) Refixed with 4% paraformaldehyde in 1/3x seawater for one hour at room temperature. 7) Washed (5x) in PBS. 8) The manufacturers protocol was followed for equilibration, biotinylation and SSC washes of the tissue. 9) We added two extra 0.3% hydrogen peroxide washes (3x total for 15 minutes each) to help quench endogenous peroxidase activity. 10) Washed (2x) in PBS. 11) Streptavidin HRP antibody was incubated overnight at 4°C. 12) Due to the larger amount of tissue used (rather than thin tissue sections), a larger volume of developing solution was needed, therefore signal was developed using DAB (Cat. #11718096001, Roche, Inc.) rather than kit components. The developing reaction was stopped by washing with PBS. Samples were then cleared in an 80% glycerol solution containing Hoechst (Cat.#H1399, Life Technologies) to label nuclei. Samples were quantified by measuring a 50 x 50 µm area centered around the site of injury (aboral region in controls) and the number of nuclei in this region was compared to the number of DAB positive cells (Supplemental Figure 4.1).
**Mucus staining**

We attempted to stain *Nematostella* mucus with the histological stains alcian blue (acidic mucins) and periodic acid schiff reagent (neutral mucins). These stains were utilized to visualize mucins present in mucosal tissue in vertebrates (Cohen, Varki, Jankowski, & Gagneux, 2012). Samples were compared over time and against individuals that were exposed to U0126. Samples were gathered one, four and twelve hours after oral bissection and compared to uninjured animals to determine if there is an increase of mucus after injury, and to determine if the drug U0126 has any effect on mucus production. Samples were pre-incubated in gelatin coated dishes with 1% DMSO (controls) or 10µM U0126 (experimental) dissolved in 1/3x filtered sea water for one hour prior to injury. Juvenile polyps were cut along the oral-aboral axis (Figure 4.1C) and incubated at 25°C in the dark. Prior to fixation, samples were relaxed with 7% MgCl$_2$ to the 1/3x seawater. Samples were fixed by washing polyps with ice-cold 100% Methanol (3x) for 1 hour at 4°C. Samples were rehydrated immediately after fixation in 60% methanol in distilled water (1X) and then 30% (1X) methanol and finally PBT solution for (5x) washes. Samples were transferred to glass 3-spot dishes for subsequent staining. At this time samples were soaked in alcian blue solution (pH 2.5) for ten minutes, then washed (3x) in distilled water. Samples were soaked in 1% periodic acid solution for ten minutes, then washed (3x) with distilled water. Schiff’s reagent was added to each sample and was stained for approximately twenty minutes. Samples were then washed (10x) with distilled water, then dehydrated through a methanol series: 50% (2x), 75% (1x), 85% (1x), 95% (1x) and 100% (2x). Samples were further cleared (3x) in Murray clear (1:2 benzyl alcohol:benzyl benzoate).
**Phallacidin, Hoechst and phosphorylated ERK antibody**

We found that after six hours, puncture wounds were no longer visible by transmitted light and therefore we utilized confocal microscopy to determine the timeline of events leading up to the completion of the wound healing process (Figure 4.2). Samples were relaxed and fixed the same as our apoptosis protocol (above). We used Biodypy FL phallacidin diluted 1:100 (Cat.#B607, Life Technologies) in PBT to visualize F-actin especially along cell boundaries. Nuclei were visualized by incubation in Hoechst diluted 1:500 in PBT. Samples were incubated in a mixture of phallacidin and Hoechst overnight at 4°C. Samples were washed (3x) in PBT then cleared with 80% glycerol.

We utilized an antibody against phosphorylated-ERK, (Cat.#4377, Cell Signalling Technology) to identify if p-ERK is activated during wound healing and regulated by U0126 (Figure 4.3G,H). In these samples, animals were punctured in the aboral region and allowed to rest for one hour prior to fixation. To maintain the phosphorylated activity, all phosphate buffers were avoided, and instead animals were washed (5x) in Tris-buffered saline with 0.1% Tween20 (TBST buffer). Specimen were blocked in 5% Normal Goat Serum, in TBST buffer, overnight at 4°C. Samples were incubated in p-ERK antibody at 1:200 overnight at 4°C. Antibody was removed and samples were quickly washed (3x) in TBST buffer, followed by three additional washes of ten minutes each. Specimen and antibodies were again pre-blocked for one hour at 4°C. Then a secondary antibody Alexa Fluor® 488 Goat anti-rabbit (Cat.#A11008, Life Technologies) was used at 1:250 and placed in 5% NGS in TBST buffer overnight at 4°C. The secondary antibody was removed and samples were washed (3x) in TBST, then cleared in 80% glycerol containing
Imaging

Many different techniques were attempted to immobilize living animals for visualization during wound healing, including: increasing the viscosity of the media, creating small chambers for enclosure, and deciliation, but the best method was using negative pressure with one or two suction pipettes to hold animals in a fixed position. We used small capillaries (Cat. #TW100F4, World Precision Instruments, Inc.) attached to a small transfer pipette to gently create suction on the side of the polyp (see Supplemental Figures 4.2-4.4). With this method, we were able to hold animals in a similar viewing plane for up to eight hours at a time. These animals were mounted under cover slips and sealed with Vaseline to prevent water evaporation during live imaging. Acridine orange was used as a counter stain in our experiments to help visualize structures that were not visible using transmission light microscopy. We prepared a 1µM acridine orange solution (in 1/3x sea water) incubated animals for five minutes, and then washed the animals (3x) with 1/3x sea water before experimentation.

Time series images of regenerating head structures (Figure 4.1B) were taken on a Zeiss Axio Imager Z1 using a Hamamatsu (Orca-ER) camera with Volocity 5 software (www.improvision.com). Photos of drug treated animals (Figure 4.3A-D) and apoptosis images (Figure 4.2E) were taken on an Axioscope 2 compound microscope using an AxioCam (HRc) camera with Axiovision software (Zeiss Inc, Jena, Germany). The mucus labeling experiment (Supplemental Figure 4.5), time series of puncture wound healing (Figure 4.2A-D) and all additional videos were taken on a Zeiss 710 scanning laser confocal. Z-stacks images and time-series (videos) were compiled with Zen software (Zeiss Inc, Jena, Germany). Images from the in situ hybridization experiments
were captured using a Zeiss Axio Imager M2 using an AxioCam (HRc) camera and processed using Zen software. All figures were created using Adobe Illustrator (CS4).

**RNA and cDNA Handling**

A total of 300 polyps (for microarray) or 100 polyps (for qpcr) were used for one biological replicate for each assay. RNA extraction techniques and cDNA synthesis, were the same as described in Layden et al. (M. J. Layden et al., 2012) and Röttinger et al. (Eric Röttinger et al., 2012). To maximize the abundance of wound healing transcripts, multiple puncture wounds were created in animals used for microarray and qpcr analysis. These animals received three puncture wounds along the oral-aboral axis. Therefore, these transcripts likely incorporate wound-related genes regardless of the body position. A single wound was formed in animals that were used for *in situ* hybridization and our other imaging studies.

**Microarray**

Our 4-plex Nimblegen (Inc.) microarray chip consists of 72,000 features, covering the complete *Nematostella* genome with three replicate oligonucleotide probes per gene. Samples were normalized and fold change calculations were produced using Nimblegen (Inc.) software according to previous work (Bolstad, Irizarry, Astrand, & Speed, 2003; Irizarry et al., 2003). All associated microarray files were uploaded to ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under the accession numbers A-MEXP-2380 (design file) and E-MTAB-2341 (protocol and data file). Treatments included: uninjured animals (in 1% DMSO), animals one and four hours after puncture injury (in 1% DMSO), and one and four hour animals in U0126 (in 1% DMSO) at a concentration of 10µM. A total of two biological replicates per time-point and treatment
(DMSO vs. U0126) were analyzed at 300 polyps sample. Due to the large number of polyps utilized and the laborious nature of each wound experiment, we chose to analyze only two biological replicates by microarray treatment and confirm these results by quantitative PCR and in situ hybridization. In all drug treatment experiments, animals were pre-soaked for one hour prior to injury. A total number of 1434 significant expression values exhibited a fold change of 2.5x or greater (Supplemental Figure 4.6). From this dataset we analyzed a total 830 protein sequences from the Nematostella genome via the Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html). Each sequence was manually blasting against NCBI’s protein BLAST database (http://blast.ncbi.nlm.nih.gov) and we recorded the top blast hit, species, e-value and any predicted domains. To extract the maximum amount of data for each gene, we also gathered gene description information (column NvJGI Description – conserved domain, Figure 4.4) from the JGI website. All genes were also analyzed with Blast2Go (http://www.blast2go.com/b2ghome) software, and added to our Supplemental Figure 4.6 (RawBlast2GoData).

**Quantitative PCR**

qPCR samples were standardized with NvGADPH and NvRiboPro (see Supplemental Figure 4.7). Primers for other genes were designed using MacVector (www.macvector.com) to amplify 75-150 base-pair fragments of the desired gene. These primers were then back-blasted against the Nematostella genome to make sure they only will amplify a single region from the genome. We checked each primer efficiency with a dilution curve (10^-1-10^-5) to make sure their range was within the negligible value of 1.9-2.0. A total of three biological replicates consisting of 100 polyps
per sample were analyzed. Relative fold change values were calculated in Microsoft Excel and were standardized against our reference genes based on formulas from Livak and Schmittgen (Livak & Schmittgen, 2001).

**in situ hybridization**

All in situ hybridizations were based off of the previous protocol for *Nematostella vectensis* (Pang et al., 2004). Fixations were done in 1% gelatin coated dishes to prevent tissue from sticking to the plastic (sticking to plastic causes tissue damage and non-specific staining). Animals were fixed in ice cold 4% paraformaldehyde with 0.2% glutaraldehyde in 1/3x seawater for two minutes, followed by 4% paraformaldehyde in 1/3x seawater for one hour at 4°C. DIG-labeled probes, ranging from 550-1200 base pairs, were hybridized at 64°C for two days and developed with the enzymatic reaction of NBT/BCIP as substrate for the alkaline phosphatase-conjugated anti-DIG antibody (Cat.#11093274910, Roche, Inc.). Samples were developed for an equal amount of time and if no expression was visible, a subset of samples remained in developing solution to determine if any expression was present.

**RESULTS**

**Cellular events orchestrated during wound healing of *Nematostella vectensis***

Punctures were formed by passing a glass needle through ectodermal and endodermal layers of the aboral portion of animals (Figure 1C) and take approximately six hours to heal (Figure 4.2A). Immediately after injury, tissue in the aboral portion of the animal becomes deflated. This is primarily due to the loss of water within the gastrovascular system, and the inability to stop water flow from exiting the wound. This
compacted form lasts approximately four hours, until animals are capable of holding water again (Supplemental Figure 4.2). At two hours after injury (Figure 4.2B, left), an enrichment of actin is seen around the injury site, but a hole is still visible that does not contain nuclei (Figure 4.2B, right). This suggests that cells immediately surrounding the injury site stretch actin filapodia towards the central portion of the wound and create connections to pull the wound closed. A small subset of animals were found to make long actin strings that stretched from one side of the animal to the opposite side (Figure 4.2C). This is likely due to the tissue having injuries on both sides and cells on each side stretching to fill the wound, while potentially coming in contact with one another. Overall scar-free wound healing finishes approximately 6-8 hours after injury, unless substantial damage to retractor muscles inadvertently occurred during injury. These structures were not repaired at six hours and may need proliferation to regrow (Passamaneck & Martindale, 2012; Renfer et al., 2010).

During the early stages of wound healing, mesentery structures plug the wound for short periods of time (Figure 4.2D, left; Supplemental Figures 4.3-4.4). This behavior could provide signals from the endoderm to the outer ectoderm that an injury has occurred. A similar phenomenon is found during regeneration, if the aboral regenerate has retained mesentery tissue after bisection (data not shown). Interestingly, a sticky mucus-like material is excreted from the wound (Figure 4.2D, right; Supplemental Figure 4.5) and contains cellular debris. We used alcian blue and periodic acid Schiff’s reagent (PAS) to identify potential areas of mucin production within Nematostella. We found potential regions of mucus synthesis / secretion along the pharynx (Supplemental Figure 4.5A) the outer epithelium (Supplemental Figure 4.5B) and the base of the
mesenteries (Supplemental Figure 4.5C), while little mucus is visible along the tentacles (Supplemental Figure 4.5D). When we looked at mucus production during regeneration, we found that wild-type animals appear to have a greater amount of mucus along the endodermal tissue compared to U0126 animals (Supplemental Figure 4.5E-E’). At one hour after injury, mucus can be found throughout tissue along the site of injury, with little staining in U0126 animals (Supplemental Figure 4.5F-F’). By four hours, extensive mucus staining was visible within the endoderm of wild-type animals and appears less abundant in U0126 animals (Supplemental Figure 4.5G-G’). Again at twelve hours mucus staining appears diminished in both controls and U0126 treated animals, with slightly elevated levels in U0126 samples (Supplemental Figure 4.5H-H’). We did not identify and areas enriched with alcian blue staining, except the tips of the tentacles (data not shown).

Apoptosis initiates head regeneration in Hydra, and wounding along the body column can create new buds (Chera et al., 2009). In Nematostella, we found that apoptosis is also activated upon injury along the ectodermal surface (Figure 4.2E). Expression of apoptotic signal appears greatest minutes after injury but a small amount can be found as late as six hours after injury. We quantified the number of DAB positive cells around the site of injury compared to the total number of nuclei within close proximity of the wound (Supplemental Figure 4.1A). A ratio of tunel positive cells / total nuclei suggests immediately after injury there is eight times the number of cells undergoing apoptosis than in uninjured animals (Supplemental Figure 4.1B). This ratio ranges from 16% immediately after injury to 9% after six hours.
MAPK (ERK) signaling is crucial for proper wound healing

Previous studies suggest a link between activation of apoptosis and the onset of proliferation during regeneration in vertebrates and invertebrates alike (Bergmann & Steller, 2010). Apoptosis is driven through a number of MAPK signaling components and has known roles in Hydra regeneration without affecting wound healing (Chera et al., 2009). We chose to conduct a pharmacological inhibitor screen to see what other signaling pathways have a potential role in wound healing and regeneration. During regeneration, the wnt-signaling pathway is suggested to play a key role in axis formation in both Hydra and Nematostella (Chera et al., 2009; Trevino et al., 2011). Inhibitors for notch, TGFβ, and MAPK were tested to determine if we could differentially affect wound healing or regeneration.

Inhibition of notch signaling using the λ-secretase inhibitor, DAPT, blocks head reformation (Supplemental Figure 4.9) with no morphological changes in wound healing. TGFβ signaling is one of the first pathways activated and a known regulator of vertebrate wound healing (Penn, Grobbelaar, & Rolfe, 2012). Surprisingly, we did not see any phenotypic change in wound healing or regeneration with the TGFβ inhibitor SB431542. Neither inhibitor was analyzed further because they did not exhibit morphological changes to wounded animals.

The MAPK inhibitor of ERK signaling (U0126) blocked regeneration with dramatic wound healing defects in both head regeneration (Figure 4.3A-B) and aboral regeneration (Figure 4.3C-D). In aboral regenerates, the site of injury appears wrinkled with the wound opening still visible (data not shown) while oral regenerates often have excess tissue that does not reintegrate into the animal (Figure 4.3D). Interestingly, the
loss of regenerative ability occurred in animals that were exposed to U0126 prior to injury (Figure 4.3A-D) and when drug was added at eight hours after injury (data not shown). This suggests that ERK signaling may have multiple functions spanning the whole process of wound healing and regeneration. We set forth to determine the role of ERK signaling during wound healing because it was the only pathway that caused wound healing defects after injury.

Using our puncture assay methodology, we found that inhibition of ERK signaling by U0126 caused aboral puncture wounds to remain open after six hours, the normal time for wounds to heal (Figure 4.3E-F) and eliminates local phosphorylation of ERK at one hour after injury (Figure 4.3G-H). Incubation of animals in U0126 did not result in a loss of apoptotic signal immediately after injury (Figure 4.3I), suggesting that apoptosis alone cannot initiate regeneration. Long-term exposure to U0126 during wound healing results in animals that remain in a compressed state (Figure 4.3J). Washing out U0126 after twelve hours is sufficient to reinitiate wound healing (Figure 4.3K), and these animals appear to be morphologically similar to wild-type animals, six hours after injury (Figure 4.3L). These data show that U0126 is capable of blocking wound healing, without affecting the normal apoptotic program. This suggests that cellular movement and adhesion could be primary targets of ERK signaling, because proliferation is not active until much later during regeneration (Passamaneck & Martindale, 2012). In fact, U0126 treated animals did not show large amounts of actin around the site of injury (Figure 4.3F) as seen in Figure 4.2C. Overall, inhibition of ERK signaling by U0126 is a reversible process that is necessary for Nematostella wound closure.
Transcriptional component of wound healing as revealed by microarray

To determine the transcriptional input necessary for stimulating a wound healing response, we used a *Nematostella* genome-wide microarray (Nimblegen, Inc.) to identify target genes involved in wound healing. We isolated mRNA from uninjured polyps, as well as injured animals from one hour (early response genes) and four hours (late response genes). To determine how ERK signaling effects mRNA transcription during wound healing, we also collected U0126 treated animal mRNA at one and four hours after injury for comparison. Genes organized in Figure 4.4 were identified in our microarray analysis and found to exhibit higher levels of expression due to injury activation and/or drug treatment. These genes are a subset of genes from Supplemental Figure 4.6, and represent genes that were found among multiple comparisons (temporally and/or drug treated). A complete set of genes with every fold change comparison can be found in Supplemental Figure 4.6 (RawBlast2GoData), along with analyzed Blast2Go results (GO prediction and top BLAST hit). Genes in Figure 4.4 are organized in relation to the highest fold change expression, and each gene is identifiable by it’s protein identification number from the JGI *Nematostella* genome website (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html). From here forward, genes will be referred to by their protein identification number.

The genes with the highest up-regulation after injury and during wound-healing appear to be dominated by peptidase activity (148396, 32224, 112683, 107554) or moderators of MAPK signaling including alpha-integrin (196726) and g-protein signaling (128258, 206698, 72442). The primary transcription factors activated by injury are Fox O-related (138488, 150900), Sox E1 (235335), and Runt (129231). A number of growth
factor-related genes are also activated, including Wnt2 (242584), platelet-derived growth factor (239536) and sprouty (29671). Interestingly a number of genes related to mucus proteins or mucosa tissue are also highly up-regulated, including maltase-glucoamylase (224255) and mucin-1/2 precursors (74775, 3 others in the 'Complete Dataset’- Supplemental Figure 4.6). This cohort of genes occupies the full range of MapK signaling components from the immediate sub-nuclear activation (through phosphorylation) of MAPK specific transcription factors to the extracellular activity of pepidases and across the membrane bound regulators like G-proteins and integrins.

**Quantitative PCR reaffirms microarray findings**

A total number of seventeen genes were analyzed by quantitative PCR and/or by *in situ* hybridization to confirm microarray results. Genes were selected for qPCR based on their genomic sequence size and if the desired PCR primer conditions could be met for qPCR. The seven genes shown in Figure 4.5, represent genes that exhibited high fold change levels from the array due to U0126 treatment and/or temporal variation. The Fos-like gene (232694) exhibits increased expression at one hour after injury and appears to be up-regulated at four hours by U0126, where all other genes in Figure 4.5, are greatly reduced in relation to U0126. Seven additional genes (138488, 86916, 140525, 238642, 39805, 37059, and 98391) were characterized by qPCR,(data not shown) and followed similar up/down regulation patterns as described by microarray analysis. Only a single gene (170407) identified as a potential candidate by microarray analysis exhibited little change by qPCR. This gene was only identified in our comparison between four hour injured animals vs. uninjured animals and had a relatively low fold change (2.9x).
In situ hybridization of target genes confirm microarray results and localize around the site of injury

Uromodulin (39872), also known as Tamm-Horsfall glycoprotein, is normally expressed in the aboral pole of uninjured animals (Figure 4.6, row 1, left). At one hour after injury, uromodulin is highly expressed around the wound ectoderm and expression continues to expand in four hour animals (Figure 4.6, row 1, middle, right). The wild-type localized expression of this gene is not reduced following treatment with U0126 (Figure 4.6, row 1 left), U0126 appears to block the activation of uromodulin around the wound site, without reducing the endogenous expression around the aboral pole (Figure 4.6, row 1, middle, right).

SoxE1 (235335) belongs to the homeobox class of transcription factors and is normally expressed along the aboral ectodermal walls of the body column (Figure 4.6, row 2, left). Incubation with U0126 causes a slight reduction of expression in uninjured animals (Figure 4.6, row 2, left). Wounding causes broad ectodermal patterning to become reduced, and local expression near the injury site becomes present in both animals examined at one and four hours after puncture (Figure 4.6, row 2, middle, right). Exposure to U0126 during wound healing thus reduces local expression (Figure 4.6, row 2, middle, right).

The thiamine biosynthesis enzyme (248223) is only expressed after injury and is localized in the ectodermal layer cells immediately around the site of injury (Figure 4.6, row 3). This gene also appears to be controlled by ERK signaling because expression during injury is down-regulated in U0126 animals (Figure 4.6, row 3, middle, right). We did not detect any endogenous expression in uninjured animals (Figure 4.6, row 3, left).
The inhibitor of matrix metalloproteinase gene (238195) is found broadly expressed throughout the endoderm and is drastically reduced in U0126 uninjured animals (Figure 4.6, row 4, left). Expression is localized in the body column and tentacle endoderm as well as the mesenteries. In both one and four hour old injured animals, expression is localized in a circle within the endoderm, around the injury site and is also up-regulated in the mesenteries (Figure 4.6, row 4, middle, right). U0126 reduces local expression around the injury site, but some expression remains within the mesenteries.

The gene encoding a maltase-like enzyme (224255) is found lightly expressed compared to injured animals, and localizes to the aboral endoderm, tentacle tip endoderm, and near the mouth (Figure 4.6, row 5). Puncture injury to the aboral region induces expression throughout the mesenteries and locally at the site of injury (Figure 4.6, row 5, middle, right). Expression in the mesenteries appears to be greatest at one hour after puncture, where expression around the injury looks higher at four hours after injury. U0126 treatment appears to dissipate staining in both the mesenteries and locally at the site of injury.

Animals recover gene expression and wound healing upon drug removal.

We determined that removal of U0126 allows for injured animals to begin healing (Figure 4.3F), therefore we wanted to know if drug removal also restores normal expression of wound healing targets. We exposed punctured animals to U0126 for four hours after injury, then washed and incubated for another four hours without drug in 1/3x seawater. Removal of U0126 reinitiates the normal expression of target genes (Figure 4.6B). Both SoxE1 (235335) and the maltase enzyme (224255) exhibited expression domains comparable with wild-type punctured animals at four hours after
injury. SoxE1 expression was greater within the endoderm in these washout experiments, but normal ectodermal staining around the wound was exhibited. Expression of the maltase enzyme was also found around the site of injury and mildly expressed within the mesenteries, comparable to wild-type animals. These experiments demonstrate the level of morphological and transcriptional recoverability after drug removal, and help suggest that these target genes are necessary for wound healing.

Microarray targets also activated prior to oral/aboral regeneration

Our findings through microarray analysis, qPCR and in situ hybridization suggests that we have identified several genes utilized during aboral wound closure. To determine if our identified targets also play a role in wound healing before regeneration, we conducted in situ hybridization of animals that underwent biscection along the oral-aboral axis in comparison to animals impaired by U0126 (Figure 4.7). Similar to results obtained by our puncture assay, the uromodulin-like gene is expressed in the aboral domain immediately after injury and expression appears expanded by four hours after injury (Figure 4.7A). This appears to be slightly delayed from the timing of puncture experiments. In oral halves expression is found immediately around the base of the tentacles, similar to puncture experiments. Overtime, expression in both the regenerating oral and aboral halves expands from a localized expression (aboral pole or base of the tentacles) to a broad expression encompassing most of the ectoderm except the tentacles. Treatment with U0126 results in maintained endogenous expression of the gene along the aboral pole or base of the tentacles, and similarly to puncture assays limits the expansion of gene expression associated with injury.
Expression of uromodulin was maintained through the first twenty-four hours when proliferation is known to begin (Passamaneck & Martindale, 2012).

The four other genes: SoxE1, thiamine enzyme, MMP inhibitor and the maltase enzyme all exhibited expression immediately after injury in both oral and aboral halves (Figure 4.7B). SoxE1 was expressed along the ectoderm at the site of injury in both oral and aboral regenerates. The gene encoding for thiamine enzyme also localized to the ectoderm immediately surrounding the site of injury. The MMP inhibitor and maltase enzyme were found expressed in the endoderm of regenerates. The MMP inhibitor exhibits a ring-like expression around the wound (Figure 4.7B, red arrow) similar to puncture experiments (Figure 4.6A). The maltase enzyme was highly expressed within the endoderm at the site of injury. Each of the four genes were analyzed at four, twelve and twenty-four hours but did not exhibit drastic changes in localization domain, but exhibited less expression at twenty-four hours (data not shown). Samples that were incubated with U0126 resulted in a decreased or absence of expression.

DISCUSSION

Comparative embryological studies using *Nematostella vectensis* have shown that many molecule components utilized during early embryogenesis of deuterostomes were present in the cnidarian-bilaterian ancestor and remain today (Martindale et al., 2004; Saina et al., 2009; Wikramanayake et al., 2003). This suggest that the signals patterning during early embryonic development have an ancient origin and exhibit a strong level of conservation over evolutionary time. The healing of the wound in the epithelial layer after injury is a necessary process where disruption of this process is
known to inhibit regeneration (Brookes & Kumar, 2008; Mescher, 1976; Newman, 1974; Silver & Miller, 2004). Therefore, to gain a better understanding of the regenerative potential between species we must first understand what signals activate wound healing and allow for the step-wise activation of regeneration. Characterization of the wound response in a diverse set of animals, will provide clues into why so many animal lineages have lost the ability to regenerate as a result of defective wound repair.

Previously, in *Nematostella* it was shown that an injury is capable of initiating regeneration (Passamaneck & Martindale, 2012). In the current study, the wound healing response in *Nematostella* was characterized, to determine the cellular and molecular components necessary for activating a regenerative response. Our findings suggest that the wound healing response consists of an early cellular response and a late growth response that leads into the proliferative response necessary for regeneration.

**The early cellular response during wound healing**

**Peptidase activity and their inhibitors, regulators of cellular dynamics during wound healing**

Growth factors play an instrumental role in the activation of wound healing (Werner & Grose, 2003). The first signals present after injury in vertebrates are growth factors distributed to the site of injury through the release of blood and platelets. In *Nematostella*, we see an activation of similar growth factors (fibroblast, epidermal and vascular endothelial/platelet derived), but this activation appears hours after the initial injury. We have identified a number of early up-regulated matrix metalloproteinases (MMPs) that may act in releasing these growth factors to initiate synthesis. MMPs are a
subset of peptidase enzymes that utilize calcium or zinc ions for activation and are known for their ability to release adhesion complexes between cells. Together with tissue inhibitors of metalloproteinases (TIMPs), which block MMP activity, cells have the ability for coordinated cell movement through regulation of adhesion molecules (Gill & Parks, 2008). MMPs can also function to release growth factors or modify growth factor receptors during wound healing, where TIMPs are also thought to block pathogen MMP activity to prevent infection (Montagnani, Le Roux, Berthe, & Escoubas, 2001; Mott & Werb, 2004). Similar to this study, MMPs have been identified in wound healing studies on *Hydra* (Shimizu et al., 2002), planarians (Altincicek & Vilcinskas, 2008) axolotyl (Campbell et al., 2011), mouse (Cooper, Johnson, Burslem, & Martin, 2005) and human skin (Cole, Tsou, Wallace, Gibran, & Isik, 2001). Our findings suggest that numerous metalloproteinases are activated upon injury, while NvTIMP is expressed locally around the injury site (Figure 4.6-7). NvTIMP may suppress MMP activity around the wound to solidify the damaged tissue, creating a gradient of mechanical stiffness. A localized rigid cellular environment can act as a signal to promote cellular migration, otherwise called durotaxis, or the migration of cells towards an area of greater rigidity (Harland, Walcott, & Sun, 2011; Plotnikov & Waterman, 2013). Although NvTIMP could be used for pathogen-host prevention, it may serve as a signal for cellular migration in *Nematostella*. Our microarray results suggest that metalloproteinase activity is a major component to wound healing in many different animals with various levels of regenerative capacity.

The primary events taking place during early wound healing of *Nematostella* appear to be cellular shape change, migration, adhesion and death. After any injury, a number
of stimuli (e.g., stress, mechanical forces, bacterial invasion, fluid loss) become factors in the regulation of the wound healing process. ERK signaling is known to play an important role in the regulation of apoptosis and bud formation in *Hydra* (Chera, Ghila, Wenger, & Galliot, 2011; Fabila, Navarro, Fujisawa, Bode, & Salgado, 2002; Manuel, Reynoso, Gee, Salgado, & Bode, 2006), and is necessary to establish blastema formation during head regeneration in Planarians (Tasaki et al., 2011). We have established that ERK signaling is activated immediately after injury in *Nematostella* and is necessary for proper wound healing. Although ERK signaling acts as an early response element among these three taxa, several novel genetic components to the *Nematostella* wound healing program may provide a significant advantage compared to other systems that lack a high regenerative capacity, other than simply having highly proliferative I-cells (*Hydra*) or neoblast cells (Planarians).

**Apoptosis is active throughout all phases of *Nematostella* wound healing**

Apoptosis is the known driver of future proliferation in a number of species after an injury occurs (Bergmann & Steller, 2010). Cell death appears active throughout the early and late injury response in *Nematostella* (Figure 4.2E, Figure 4.8B). *Hydra* studies show that inhibition of apoptosis is known to inhibit regeneration without wound healing defects, and ectopic activation of apoptotic signals can induce a second head to grow (Chera et al., 2009, 2011). Contrary to the findings of Chera et al. (2011), we did not see a decrease in apoptotic signal due to inhibition of ERK signaling. Interestingly, blocking Notch signaling in *Hydra* does not inhibit head regeneration (Münder et al., 2010), where inhibition during embryogenesis of *Nematostella* causes tentacle deformities (H. Marlow et al., 2012) and loss of tentacles during regeneration (this
study). This suggests that although there is a conserved cell behavior necessary for regeneration, that the modes of regeneration may be quite different even within the same phylum. Furthermore, activation of apoptotic signals during wound healing could be necessary for initiating proliferation, but inhibition of normal wound healing by U0126 did not result in a loss of apoptosis, suggesting other signals along with apoptosis initiate a regenerative response.

**Glycoproteins play an important role in *Nematostella* wound healing**

Immediately after injury, animals exhibit a “deflated collapsed state” due to failure to maintain positive pressure in the gastrovascular cavity (Bossert et al., 2013). This collapsed state may help push damaged areas together, allowing for rapid closure of the wound. (Figure 4.8A). During these early hours after injury, the endodermally derived mesentery structures often act to plug the wound (Figure 4.2B; Supplemental Figures 4.3-4.4; Figure 4.8A). It was also found that a sticky mucus-like substance was excreted from the wound (potentially from mesentery interactions) and could aid in closing the wound (Figure 4.2B; Supplemental Figure 4.5). We know that puncture injury inhibits peristaltic movements down the oral-aboral axis immediately around the site of injury (Supplemental Figure 4.2). Peristaltic movement likely provides nutrients and oxygen exchange throughout the body and inhibition leaves animals in a compromised state. Therefore the interaction of mesenteries and/or mucus could help animals temporally stop water from leaking from gastrovascular cavity, and may act as a cue to initiate local wound healing around damaged tissue.

A number of mucin-related glycoprotein genes are activated early during *Nematostella* wound healing (Figure 4.4). Mucins are classified into two groups,
transmembrane or secreted gel-forming mucins and phylogenetic analysis suggests *Nematostella* has primarily gel-forming mucins (Lang, Hansson, & Samuelsson, 2007). Gel-forming mucins are thought to provide a protective layer for underlying mucosal epithelial cells, are known to be expressed after damage to mucosal tissue, and mucin (muc-2) deficient mice show impaired gastric healing (Ikezawa et al., 2004; Wallace, Vong, Dharmani, Srivastava, & Chadee, 2011). Future experiments may reveal an interesting link between mucosal tissue and the role of mucins in regenerative animals, and the possibility of discovering other genes capable of accelerating wound healing (Ho et al., 2006).

Additionally we identified one uromodulin-like glycoprotein in *Nematostella*, an ortholog which was also found to be highly up-regulated in the regenerating epithelial layer of axolotyl limb regenerates (Campbell et al., 2011). Expression of this gene was restricted to the wound ectoderm in *Nematostella*, and appeared to spread laterally away from the wound over-time. Interestingly, wild-type expression of this gene is localized to the aboral portion of the animal (Figure 4.6A) the position of the apical tuft during larval development. The apical tuft region of the animal is often associated with habitat selection and potentially bacterial recognition in corals during metamorphosis (Tran & Hadfield, 2011). In mammalian systems, uromodulin protein is found in urine and is locally synthesized in epithelial cells of the kidney. Uromodulin knockout mice have a great susceptibility to bacterial infection and this gene is thought to regulate inflammatory signals to allow healing (Devuyst, Dahan, & Pirson, 2005; El-Achkar et al., 2013). Although these data are all based on studies of the kidney, this gene may be a
part of a conserved pathway of wound healing that operates for regenerative and non regenerative species.

**Signals from the endoderm direct wound healing events**

A number of conditions suggest that the endoderm in cnidarians may act as the driving force for wound healing. During wound healing of *Hydra*, the endoderm closes prior to the ectodermal layer (Bibb & Campbell, 1973). In *Nematostella*, two genes analysed by *in situ* hybridization (MMPI and maltase enzyme) exhibited mesentery staining during wound healing (Figure 4.6A), and are localized to the endoderm during regeneration (Figure 4.7B), suggesting signals generated here may help guide wound healing. Sox genes have been found in all animals and SoxE-related genes are localized in developing endoderm during gastrulation in *Nematostella* and *Acropora millepora* (Magie et al., 2005; Shinzato et al., 2008). A single SoxE-like gene is also expressed during early wound healing events suggesting that some of the same genes utilized during gastrulation movements could be reutilized during healing. SoxE expression shows a conical shape of tissue within the endoderm suggesting these cells are contracting towards the injury site (Figure 4.6A). Sox9 genes (group E members) have been implicated in neural crest development, a process often compared to gastrulation and wound healing (Y.-H. Lee et al., 2004; Sakai, Suzuki, Osumi, & Wakamatsu, 2006). Sox9 is also a stem cell marker in epidermal cells and is activated during healing and regeneration in mice (Mardaryev et al., 2011).

We found that cells around the wound exhibit increased levels of actin at two hours after injury (Figure 4.2B). It appears that the actin cytoskeleton is recruited to close the wound through filapodial extensions into the wound margin, rather than the actin purse-
string model described in *Drosophila* (Wang et al., 2009). In a number of samples, actin filapodia could be seen connecting the two injury sites (Figure 4.2C). It has been noted that actin filapodia extend into the blastocoel during *Nematostella* gastrulation (Magie et al., 2007) and both wound healing and gastrulation are halted by inhibition of ERK signaling. Overall, several lines of evidence suggest that wound healing could share many cellular and molecular behaviors and genes also used during gastrulation.

**A bacterial derived thiamine synthesis enzyme is highly expressed post-injury**

This study identified a cnidarian specific gene, (thiamine synthesis enzyme) an enzyme utilized during the synthesis of vitamin B, where deficiency has been linked to wound healing defects (Brown & Phillips, 2010). This gene is found on scaffold 466 in the *Nematostella* genome, was represented in expressed sequence tag (EST) resources utilized in genome construction (Putnam et al., 2007), and was confirmed to be highly up-regulated after puncture injury by qPCR and in situ hybridization (Figure 4.4-6). However, thiamine enzymes are not known in animals, and BLAST analysis only finds similar representatives of this gene in bacteria and *Acropora digitifera* (Supplemental Figure 4.10). This gene appears to only be activated after injury, and BLAST analysis did not reveal other components of vitamin B synthesis (data not shown). Together, these data suggest that this gene likely incorporated into the *Nematostella* genome through lateral gene transfer and may aid in production of vitamin B by symbiotic bacteria within the animal, or alternatively have a novel wound healing function independent of vitamin B. Functional analysis of this gene may reveal an interesting novelty that has provided an evolutionary advantage for cnidarian healing and regeneration.
Late wound healing response

A single $\alpha$-integrin is upregulated in response to wound healing

At four hours after injury, we begin to see components of cell membrane signaling (integrins, g-proteins and a wnt ligand) as well as activation of growth factors. Integrins are mediators of focal adhesion complexes and numerous alpha and beta subunits are activated or up-regulated during vertebrate wound healing (Margadant, Charafeddine, & Sonnenberg, 2010). *Nematostella* and closely related coral species have fewer numbers of integrins than vertebrates (Knack et al., 2008). We identified a single alpha-integrin up-regulated at four hours after injury, with significant expression change in relation to the MEK inhibitor (U0126). Although we do not know the location of expression, we predict that it will be found in a similar location to SoxE because these two genes are known to be in overlapping regions of the presumptive endoderm during gastrulation in *Acropora millepora* (Knack et al., 2008; Shinzato et al., 2008). Due to functional redundancy and lethality of integrin knockouts in vertebrates (Margadant et al., 2010), *Nematostella* could be a valuable system to discern the functional relationship of integrins and wound healing.

The activation of integrin is closely associated with regulation of calcium and ionic balance (Becchetti & Arcangeli, 2010) and a number of calcium related proteins (86027, 216996, 100701) were activated at four hours in *Nematostella*. In *Hydra* and axolotl the EF-hand calcium-binding domains have been associated with the wound response (Altincicek & Vilcinskas, 2008; Campbell et al., 2011) and are thought to function during
cnidarian morphogenesis (Reyes-Bermudez et al., 2009). EF-hand motif containing genes regulate filapodia formation in migratory cells (Goh Then Sin et al., 2011), and are a cellular component known to participate in *Nematostella* gastrulation (Magie et al., 2007). Although we have made several comparisons between the potential parallels of gastrulation and wound healing, a direct comparison of the transcriptional repertoire of genes utilized in both gastrulation and wound healing (in relation to U0126 treatment) would need to be done to confirm our findings.

**Expression of growth factors is delayed in the *Nematostella* wound healing process**

Based on the timeline of gene activation (Figure 4.8B) our results suggest that MMPs may have a conserved role in cell movement and may act in releasing growth factors to initiate synthesis. The growth factor PDGF (239536) has not been characterized in detail in *Nematostella*, but other FGF-related genes are thought to regulate axial patterning and cell specification (Matus, Thomsen, & Martindale, 2007; Rentzsch et al., 2008; Sinigaglia et al., 2013). Interesting, a single transforming growth factor beta (TGF-β) gene was identified to be down-regulated in one hour wild-type animals compared to U0126 treated individuals. Tight regulation of TGF-β and downstream targets is suggested as a possible means to resolve excess scar formation (Penn et al., 2012). Data found in this paper suggest that there is a delay in growth factor initiation, unlike many vertebrate systems in which growth factors arrive immediately after injury through the circulatory system (Werner & Grose, 2003).

Comparing how growth factors are activated in different model systems may be crucial for understanding how wound healing varies in animals with different regenerative
capacities. Based on the timing and activation of proliferation during regeneration, it is
likely that this later stage of wound healing prepares the tissue for proliferation (Figure
4.8B). Future studies will be necessary to determine which genes are functionally
responsible for the initiation of regeneration.

Summary

Wound healing and regeneration are separable developmental processes. Here we
suggest that healing from a puncture wound takes roughly six hours after injury to allow
for injured tissue to become functional again. Some time between twelve-eighteen
hours, signals from healed tissue activate a program, initiating proliferation and
regeneration (Passamaneck & Martindale, 2012). We have shown that the same genes
that heal puncture wounds also are activated after oral-aboral bisection. Apoptosis is
activated immediately after injury and could provide signals for head regeneration,
although ectopic activation of apoptotic signals in the aboral zone do not induce a
second axis. Interestingly, notch signaling only effects head regeneration, where
inhibition of ERK signaling is capable of blocking healing and regeneration (Figure
4.8B). We propose that in Nematostella, the key transition from wound healing to a state
of regeneration is the activation of cell proliferation. ERK signaling is necessary for the
initiation of the early wound healing response in Nematostella and is closely linked to
the activation of proliferative cells in other highly regenerative invertebrate model
systems (Arvizu, Aguilera, & Salgado, 2006; Fabila et al., 2002; Manuel et al., 2006;
Tasaki et al., 2011). After the first wave of ERK generated signals, later stages appear
to prepare the tissue for proliferation of lost structures. This study uncovered a
potentially important role of glycoprotiens (mucins) during wound healing and found a
novel anthozoan-specific thiamine biosynthesis enzyme utilized during healing. We have demonstrated that whole genomic microarray analysis is a powerful way to identify new targets of developmental processes. Our study is the first to identify and characterize genes involved during wound healing in *Nematostella* and is relatable to multiple model systems of regenerative biology.

**Figure 4.2 – Biological events during *Nematostella* wound healing.** A) Timeline of morphological events during the first six hours after injury. Filamentous actin, a core component of the extracellular matrix and muscle fibers, was labeled with Phallacidin-FL (false-colored black). A comparison of uninjured animals (far left), to injured animals shows that after six hours (far right), the wound is unidentifiable. The yellow box around the aboral side of the uninjured animal designates the zone of injury throughout our study. At one hour, the animal exhibits a deflated collapsed state due to water loss from the gastrovascular cavity, and the mesentery can be seen extending towards the wound
(yellow M). B) By hour two, high concentrations of actin are found in the cells along the margin of the wound (left), with nuclei positioned outside the wounded area (right), suggesting these are actin filapodial projections into the wounded area. C) In a small percentage of animals, long actin filapodia can be found connecting the two parallel wounds. D) Punctured live animals (stained with acridine orange) often plug their wound with mesentery structures (left) and secrete a mucus-like material from the injury site (right). E) Time-series of potential apoptotic cells during puncture wound healing, as revealed by the DeadEnd tunel assay (labels cells with DNA damage). Control samples show no signal immediately after injury, where the highest concentration of signal is seen immediately (15 minutes) after injury and the signal diminishes over time. (Negative control did not have rTdT enzyme - as specified from the protocol). Counterstaining with DAPI (image 2) designates that the kit is identifying cells with extensive DNA damage (apoptotic). (All red arrow heads or white stars indicate the position of the injury site).

**Figure 4.3 - Nematostella wound healing is mediated by ERK signaling.**
Inhibition of ERK signaling by U0126 disrupts normal wound healing and regeneration. A) Animals that undergo oral-aboral bisection regenerate oral structures by 72 hours, where B) animals soaked for three days in 10 μM U0126 (MAPK inhibitor) do not regenerate lost heads. C-D) Heads that have to regenerate lost aboral structures exhibit wound healing defects when soaked in 10 μM U0126 for three days. E-F) Aboral view of phallicidin stained animal tissue exposed to 10uM U0126 for six hours after injury lack proper wound closure. G-H) Puncture wounds cause local activation of phosphorylated-
ERK around the site of injury, where incubation with U0126 blocks phosphorylated activity of ERK around the site of injury. I) Exposure of injured animals to U0126 does not block the activation of apoptosis after injury. Animals pre-soaked in U0126 for one hour, injured and analyzed with the DeadEnd tunnel assay for apoptosis. J-L) Scanning laser confocal images of injured animals that were soaked in U0126 for twelve hours (J), twelve hours then U0126 was removed and animals were placed in 1/3x seawater for an additional six hours (K), and wild-type injured animals at six hours (L). Animals exposed to U0126 for prolonged periods of time (J) remain in a compacted state as a result of wounds never healing. Removal of U0126 at twelve hours allows animals to reinitiate wound healing (K) and is morphologically similar to animals at six hours (L). (Red arrow head in all images is used to identify the position of the wound). Black color in E-F and the white color in J-L is from PhallAcidin-FL, while the blue color in G-H and J-L is created from DAPI - labeled nuclei. (Wild-type = wt, U0126 = UO).

Table 1 - Comparison of genes up-regulated by injury and/or down-regulated by U0126.

Color Coding
- Gene also significantly regulated among 3 or more other treatments
- Gene significantly regulated among 1 hour and 4 hour treated samples
- Gene significantly regulated among 1 hour and 4 hour U0126 treated samples
- Gene significantly regulated among 4 hour and 6 hour U0126 treated samples
- Gene significantly regulated among 1 hour U0126 and 4 hour U0126 treated samples
- Gene also analyzed by quantitative PCR and/or in situ hybridization

Figure 4.4 – Comparison of genes identified through microarray analysis that are up-regulated by injury and/or down-regulated by U0126.
Figure 4.5 - Relative fold change quantification of mRNA transcript levels confirms targets identified by microarray analysis. Quantitative pcr of seven
representative genes showing a similar affinity to U0126 as identified by microarray analysis. Genes were standardized against the house keeping gene NvGADPH and confirmed with a second gene, NvRiboPro. Values <1 or >-1 are insignificant.

Figure 4.6 - Multiple signals are derived from different tissues during wound healing. A comparison of different genes identified by microarray analysis and their expression profile over time and in relation to U0126 treated samples. A) Uromodulin
(Row 1) is always expressed at the aboral pole and expands expression as a result of injury. Drug treatment appears to block the expansion of expression over time. SoxE1 (Row 2) is expressed in the endoderm around the injury site, appears down-regulated by U0126 and is expressed broadly in the endoderm, and potentially salt-n-pepper in cells within the ectoderm of wild-type uninjured individuals. The thiamine enzyme (Row 3) is expressed primarily in the ectoderm of only injured animals. The matrix metalloproteinase inhibitor (MMP inhibitor, Row 4) is expressed within the mesenteries as well as the endoderm surrounding the site of injury. Wild-type expression is found throughout the endoderm and is reduced in all U0126 treated samples. The maltase enzyme (Row 5) is also restricted to the mesenteries and endoderm of injured animals. Wild-type expression is lowly expressed in the endoderm, mesenteries and tentacle tips. B) Removal of U0126 reinitiates gene expression at the site of injury. U0126 was removed after four hours, then animals were then allowed to heal for another four hours before fixation. Expression of SoxE1 and Maltase enzyme look strikingly similar to four hour animals in A. All drug treated samples showed reduced expression. Inset pictures compare control vs. drug treated samples. (Red arrow head indicates the site of injury).

Figure 4.7 – Similar activation of wound healing transcripts during head regeneration. All five genes identified by puncture assays are also upregulated during wound healing, prior to regeneration. A) Time series of expression levels of uromodulin transcripts during oral and aboral wound healing events. One hour after injury,
uromodulin localizes to the aboral pole of tissue that is regenerating oral structures, and around the base of the tentacles in tissue regenerating aboral structures. U0126 blocks the normal activation of uromodulin during regeneration. Uromodulin appears to be highly expressed in the aboral ectoderm during both oral and aboral regeneration. B) All four genes exhibit the same expression domains as previously identified during puncture analysis. SoxE1 and the thiamine enzyme are both expressed along the ectoderm at the injury site in both oral and aboral regenerates. Similar to puncture assays, the MMP inhibitor is highly expressed in the endoderm at the site of injury in a circle pattern. The maltase enzyme also localizes to the endoderm in both oral and aboral regenerates. Incubation of tissue in U0126 appears to reduce expression in all samples. All time-points contain a lateral view of the aboral regenerate, an oral-aboral view of the oral regenerate, and a lateral view of an oral regenerate. (Red star indicates the position of the oral side of the animal, red line indicates circular expression).
Figure 4.8 – Summary of the signals, pathways and cellular behavior of *Nematostella* wound healing. Potential tissue interactions that initiate the transition from wound healing to regeneration (A) followed by a timeline of events leading to a regenerative response (B). A) Puncture wound healing involves collapsing of tissue due to the compromised tissue walls, resulting in cells becoming compacted in local proximity to the wound. Tissue like the mesenteries could provide local signals to aid in initiating wound healing as it comes in contact with the injury site in both puncture assays and decapitated animals. Wound healing related gene expression is found locally around the site of injury and along the mesenteries during healing. Apoptosis is a known regulator of future proliferation during regeneration and is active during *Nematostella* wound healing. B) Wound healing and regeneration in *Nematostella* appear to be developmentally separable processes, where wound healing initiates regeneration. Apoptosis is activated immediately after injury, where proliferation may be the key transition for a regenerative response. MAPK-ERK signaling spans over the whole regenerative process, potentially regulating cellular movement and/or recognition during wound healing and proliferation during regeneration. We did not find any evidence that Notch signaling effects the wound healing process, but rather inhibited head regeneration. The Wnt planar cell polarity pathway is likely to effect wound healing and is known to regulate axis formation during regeneration.
CONCLUSIONS

The future of Hox studies

The evolution of bilateral symmetry likely had a profound impact on the radiation of animals. The ancestor of bilaterians is thought to resemble a cnidarian-like animal with radial symmetry along the oral-aboral axis. One of the primary axes that scientists agree is present in bilaterian taxa and is thought to have emerged in early animals. In bilaterians, the orientation of the anterior-posterior axis can be clearly visualized with a head-like structure on the anterior end, and a through-gut that leads to the more posterior end of the animal. Generally, anterior and posterior boundaries can be identified by which types of Hox genes are expressed, with phylogenetically anterior genes being expressed closer to the oral end of the animal.

In cnidarians, the first animals with definitive anterior and posterior Hox genes, the orientation and presence of an A/P axis is highly debated. Anthozoaan cnidarians have a biphasic lifecycle and expression of anterior-related Hox genes are closely associated with the site of gastrulation, which goes on to form the larval and adult mouth. The medusazoan cnidarians, which often have other derived life history stages (e.g. jellyfish stage), may have little resemblance of the ancestral cnidarian-bilaterian condition. Regardless of the phylogenetic position of these two classes of cnidarians, anterior and posterior Hox genes have been found in both branches of the tree, with anthozoan cnidarians having a more intact cluster than the medusazoans (Chourrout et al., 2006; Ryan et al., 2007). A greater genomic sampling of cnidarian taxa and establishing chromosomal resolution of already public cnidarian genomes will tell wonders about the diversification of the first Hox clusters. Many questions remain pertaining to cnidarian
cluster evolution. Does having more posterior Hox genes correlate to evolution of the unique jellyfish life history stage? Has *Hydra* lost this life history stages due to its highly fragmented cluster? Will better genomic assemblies reveal hidden secrets about cnidarian cluster evolution? These are only a few of the stories that will continue to emerge as we continue to understand the first Hox genes.

**Regeneration, a renewed frontier**

It is often hard to imagine how a sea anemone could help decipher the code for unlocking mammalian regeneration, but wound healing and regeneration studies in regenerative and non-regenerative systems can have direct implications for our own health. Some of the shared features of wound healing in diverse animals may include processes closely associate gastrulation. This association supports the idea that components of embryonic development are reutilized during regeneration. If a cell can only move and take shape in a number of ways, it makes sense that gastrulation and wound healing have common features. Therefore, to adapt, survive and evolve over the course of history, cells had to take on new functions to deal with environmental stress. In this study, we found two examples of genes that function during wound healing in *Nematostella* that highlight why a sea anemone can help decipher mammalian regeneration.

First, the study invertebrate models like *Nematostella* can offer alternative approaches to solving a problem. This study identified an anthozoan specific bacterial gene that appears to function after injury. This gene is most closely related to thiamine biosynthesis enzymes in bacterial but vitamin synthesis is not thought to occur in animals. This discovery could provide a unique perspective on the role of bacteria in
during animal development, but also has interesting implications as a target for gene therapy. Regardless, it is also very interesting how and why this gene became closely associated with the wound healing response in *Nematostella*.

When scientists are swamped with numerous amounts of data from modern day sequencing and expression techniques, it is difficult to identify targets to pursue. A number of glycoproteins, including mucins, were identified in this study and similar genes are also activated during wound healing in the highly regenerative vertebrate model axolotl. Utilizing microarray technology to identify wound healing related genes, this research identified glycoprotein targets, but without similar studies in axolotl, this shared feature could have been overlooked in each system. Glycoproteins are often found along the cell surface, but generally are extremely long proteins that could easily activate many targets during a stressful event like injury due to their size and distribution. Interestingly, mucins are the primary component of mucus found in many invertebrates as well as the ectoderm of axolotl. Mucus in the ectoderm was likely lost in most vertebrate taxa when animals moved primarily to land. If mucus, which is found all along the damaged tissue in both animals, plays an important role in wound healing and regeneration, the loss of ectodermal mucus cells may help explain the low regenerative potential of mammals. Although these correlations sound drastically ‘hand-wavy’ to why mammalians cannot regenerate, it may have been discovered when a sea anemone was poked, analyzed and shared with an axolotl specialist.
SUPPLEMENTAL FIGURES

Supplemental Figure 3.1 – Time-lapse movie of a single placozoan moving along its' natural substrate.

Supplemental Figure 3.2 – Time-lapse movie of a placozoan, immediately after being placed on a slide. Video shows the animal settling and moving around the surface of the slide. ‘Shiny sphericals’ can be seen clearly along the upper epithelial layer.

Supplemental Figure 3.3 – Table of primers used for cloning genes utilized in this study.

<table>
<thead>
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<th>Gene</th>
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Supplemental Figure 3.4 – Table of species abbreviations used for phylogenetic analysis in this study.

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<th>Species Name</th>
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Supplemental Figure 3.5 – Time-lapse movie of a bead-treated animal showing the auto-fluorescent ‘shiny sphericals’ along the upper layer and an accumulation of fluorescent signal along the lower layer. Video is taken by adjusting the focal plane to show the distribution of signal across both halves of the animal.

Supplemental Figure 3.6 – Expression of NvTbx2/3 during embryonic and early planula development. Expression localized to both oral and aboral domains and is often salt-n-pepper during planula development within the outer ectoderm.

Supplemental Figure 3.7 – Alignment of chordin CHD domains. Text figure containing sequence alignments of CHD domains.

Supplemental Figure 3.8 – Alignment of chordin CR domains. Text figure containing sequence alignments of CR domains.
Supplemental Figure 3.9 – Maximum likelihood analysis of chordin CHD domains across metazoan. Our results suggest that CHD domains are highly conserved, although many taxa are missing certain CHD domains.

Supplemental Figure 3.10 – Supplemental CR containing genes.
Supplemental Figure 3.11 – Synteny analysis of *chordin* from scaffolds in the *Nematostella* and *Trichoplax* genomes.

Supplemental Figure 3.12 – Alignment of *bmp* ligands containing all sponge, ctenophore and *nodal-like* sequences. Text figure containing sequence alignments of *bmp* ligands.

Supplemental Figure 3.13 – Alignment of *bmp* ligands containing all sponge and ctenophore sequences without *nodal-like* sequences. Text figure containing sequence alignments of *bmp* ligands.

Supplemental Figure 3.14 – Alignment of *bmp* ligands containing a limited number of sponge and ctenophore sequences without *nodal-like* sequences. Sequences were removed that exhibited multiple copies of a particle gene (e.g. 3 sponge genes that all grouped with *bmp5/8* were reduced to a single sponge gene). Text figure containing sequence alignments of *bmp* ligands.

Supplemental Figure 3.15 – Alignment of *bmp* ligands containing just cnidarian and placozoan sequences with *nodal-like* sequences. Text figure containing sequence alignments of *bmp* ligands.
Supplemental Figure 3.16 – Maximum likelihood analysis of aligned bmp ligands from Supplemental figure 12.
Supplemental Figure 3.17 – Bayesian analysis of aligned bmp ligands from Supplemental figure 12.
Supplemental Figure 3.18 – Maximum likelihood analysis of aligned *bmp* ligands from Supplemental figure 13.
Supplemental Figure 3.19 – Bayesian analysis of aligned *bmp* ligands from Supplemental figure 13.
Supplemental Figure 3.20 – Maximum likelihood analysis of aligned bmp ligands from Supplemental figure 14.
Supplemental Figure 3.21 – Bayesian analysis of aligned bmp ligands from Supplemental figure 14.
Supplemental Figure 3.22 – Maximum likelihood analysis of aligned bmp ligands from Supplemental figure 15.
Supplemental Figure 3.23 – Bayesian analysis of aligned *bmp* ligands from Supplemental figure 15.
Supplemental Figure 3.24 - Synteny analysis between scaffolds containing *NvChordin* and *TaBmp2/4.*
Supplemental Figure 3.25 - Synteny analysis of the \textit{NvGdf5} scaffold shows a single gene related to scaffold 37 in \textit{Trichoplax}. In scaffold 37, a mutS homolog is found to be highly conserved with scaffold 22 of the \textit{bmp5/8} scaffold in \textit{Nematostella}. This suggests that \textit{NvGdf5} may have been found on scaffold 22 at some point during evolution.
Supplemental Figure 4.1 – Maintained apoptotic regulation during wound healing. A) Quantification of apoptosis and nuclei count of five different stages of juvenile polyps before and after injury. Samples exhibit relatively equal numbers of tunel labeled cells, where uninjured animals express the lowest number. B) Ratio of apoptosis compared to total nuclei count shows maintained apoptosis throughout early puncture events.
**Supplemental Figure 4.2 – Time-lapse movie from 2-6 hours after injury.** Photos were taken every fifteen seconds. Movies are played at four times the normal speed. This movie highlights the behavioral change in peristaltic movement during early aboral puncture wound healing. Over time, the wound closes and normal peristalsis can resume.

**Supplemental Figure 4.3 – Time-lapse movie over the first hour after injury.** Photos were taken every fifteen seconds. Movies are played at four times the normal speed. Animals were stained with acridine orange and fluoresced with 488 nm argon laser (with only 10% power). This movie shows the interaction between mesentery structures and the puncture wound immediately within the first hour of injury.

**Supplemental Figure 4.4 – Time-lapse movie over the first three hours after injury.** Photos were taken every fifteen seconds. Movies are played at four times the normal speed. Animals were stained with acridine orange and fluoresced with 488 nm argon laser (with only 10% power). This movie shows the interaction of mesentery structures over the course of the first three hours after injury.
**Supplemental Figure 4.5 – Mucin analysis during regeneration.** Animals were stained with periodic acid and Schiff’s reagent which contains the fluorescent compound fuchsin. A) Scanning laser confocal images of the head (oral region) show large round bundles of cells that are heavily stained with fuchsin (white arrow). B) Structures similar to those found in the pharynx are found throughout the ectoderm in the aboral part of the animal (white arrow). C) The brightest concentration of staining occurs at the base of the mesenteries, and little to no staining was found along the tentacles (D). E-E‘) Uninjured animals appear to have less fluorescent labeling found throughout the animal when individuals are exposed to U0126. F-H‘) Time series of mucus staining during wound healing after head removal. F-F‘) One hour after injury fluorescent staining appears greatest near the wound epithelium. G-G‘) A brighter amount of staining appears present at four hours after injury, while U0126 animals show wound healing defects and less staining. H-H‘) By twelve hours, little mucin staining is visible in controls, where U0126 animals still exhibit wound healing defects, but appear to have elevated mucin levels.

**Supplemental Figure 4.6 -** This file contains all microarray data. Each document is separated by a different tab. This is the excel file used to create Figure 4 in the paper. A KEY designates what the colors mean in Figure 4. Complete Dataset is the accumulated amount of genes that showed expression greater than 2.5 fold change and were identified due to U0126 treatment or injury. Each subsequent tab (1Hr-Uninjured, 1Hr-1HrUO, 4 Hr-Uninjured, 4 Hr-4 HrUO) are individual comparisons of temporal and drug-interactions with injured and non-injured animals. The RawBlast2GoData tab is a full dataset of every comparison, including (1Hr-4Hr, 1Hr-4HrUO, 1HrUO-4Hr, 1Hr-4HrUO, 1HrUO-Uninjured, and 4HrUO-Uninjured). This dataset includes expression data from the array as well as GO predictions and the top blast hit for each protein. The last two tabs are compiled from the “Complete Dataset” and contain a pie chart and the data used to make it. This pie chart demonstrates the relatedness of the wound healing response genes to their BLAST. Although it may be bias by the number on genomes in the NCBI database, it suggests that 40% of the genes are closely related to those of chordates. Interestingly 10% are related to bacteria, and 12% belong to other deuterostomes (hemichordates and echinoderms). From a blast perspective, over half the genes activated have a common potential relative in deuterostomes, although a more detailed phylogenetic analysis needs to be performed on a gene-by-gene basis.
Supplemental Figure 4.7 – Primer information for cloning.

Supplemental Figure 4.8 – Time-lapse movie over fifteen minutes during the first hour after injury. Photos were taken every ten seconds. Movies are played at two times the normal speed. Animals were stained with acridine orange and fluoresced with 488 nm argon laser (with only 10% power). This movie shows a sticky mucus-like residue left from the injury site as the animal migrates out of the focal view.

Supplemental Figure 4.9 – Inhibition of notch signaling disrupts head regeneration. Exposure of regenerating polyps to the notch inhibitor, DAPT, prevents head from developing new oral regions.
Supplemental Figure 4.10 - The thiamine enzyme from *Nematostella* is likely a cnidarian specific gene, derived from bacteria. This gene is found on scaffold 466 and spans positions 44637:52229.

A) *Acropora digitifera* genes closely related to the thiamine enzyme identified in *Nematostella vectensis* and their associated homology. B) Alignment of *Nematostella* and *Acropora* sequences.


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of Nodal and BMP2/4 revealed by gene regulatory network analysis in an echinoderm. *PLoS Genetics*, 6(12), e1001259. doi:10.1371/journal.pgen.1001259


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