Wnt Signaling in the Cnidarian Nematostella vectensis: Insights into the Evolution of Gastrulation

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WNT SIGNALING IN THE CNIDARIAN *NEMATOSTELLA VECTENSIS*: INSIGHTS INTO THE EVOLUTION OF GASTRULATION

By

Naveen M. Wijesena

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2012
A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

WNT SIGNALING IN THE CNIDARIAN _NEMATOSTELLA VECTENSIS_: INSIGHTS INTO THE EVOLUTION OF GASTRULATION

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The genesis of gastrulation was arguably a key evolutionary innovation that enabled metazoan diversification. The developmental mechanisms that induced archenteron formation and endodermal cell fate specification during gastrulation are unknown, but one crucial step was likely the co-option of a localized molecular asymmetry that was present in ancient embryos. One ancient polarity present in most metazoan eggs is the animal vegetal (AV) or primary egg axis, which is established by the asymmetric localization of maternal factors such as RNA, proteins and organelles during oogenesis. In bilaterians, which account for a majority of animal taxa, the AV axis predicts the axial properties of the embryo and the adult with the animal pole giving rise to the anterior of the organism, and the vegetal pole giving rise to the endomesoderm and the site of gastrulation. In contrast, in early diverging non-bilaterian taxa such as ctenophores and cnidarians, the animal pole gives rise to the endoderm and the site of gastrulation. These and other observations have led to the hypothesis that endoderm specification and gastrulation evolved at the animal pole, and moreover, that the mechanisms that regulate these processes were moved down to the vegetal pole in the bilaterian lineage. This idea is supported at the molecular level by the observation that endoderm specification in both
bilaterians and non-bilaterians is regulated by nuclear beta-catenin signaling indicating a role for the Wnt signaling pathway in the evolution of gastrulation. In the cnidarian *Nematostella vectensis*, the Dishevelled (NvDsh) protein, a critical component of Wnt signaling, is enriched at the animal pole of eggs and embryos and is required for Wnt/beta-catenin signaling mediated endodermal cell fate specification but not for primary archenteron invagination. Primary archenteron invagination in *Nematostella* is mediated by the localized activation of Wnt/PCP signaling at the animal pole. The mechanisms coordinating Wnt signaling dependent endoderm cell fate specification and primary archenteron invagination at the animal pole in *Nematostella* embryos are not known, but elucidation of these mechanisms may provide critical insight into the evolution of gastrulation.

There is increasing evidence to suggest that a highly conserved group of core proteins functioning in the Wnt/PCP signaling pathway play a critical role in regulating gastrulation through the asymmetric localization of key components of signaling cascades in both vertebrates and invertebrates. For my dissertation research, I focused on the role of core Wnt/PCP proteins in establishing the embryonic polarity that leads to the asymmetric activation of Wnt signaling in the blastomeres at the animal pole in *Nematostella*. In *Nematostella*, the core PCP genes *NvStrabismus* (*NvStbm*) and *NvFlamingo* (*NvFmi*) are expressed maternally and are localized to the animal pole from the egg stage throughout early development. The Frizzled homologs *NvFrizzled1* (*NvFz1*), *NvFrizzled4* (*NvFz4*) and *NvFrizzled5* (*NvFz5*) are expressed maternally in dynamic expression patterns during early development. *NvFz10* is expressed in the early
gastrula stage at the animal pole and is restricted to the invaginating cells of the presumptive endoderm. \textit{NvPrickle (NvPk)} is asymmetrically expressed at the blastula stage and at the late gastrula stage it is expressed in the pharynx and on one side of the embryo in the presumptive ectoderm. Inhibition of NvFz1 function during early development of \textit{Nematostella} blocked endodermal cell fate specification but not primary archenteron invagination. In contrast, loss of NvFz10 function blocked primary archenteron invagination without affecting endodermal cell fate specification. This experimental uncoupling of Wnt/PCP signaling mediated initial archenteron invagination from Wnt/beta-catenin mediated endoderm specification in \textit{Nematostella} provides further evidence for the independent evolution of these two processes during early metazoan evolution.

The localized expression of NvFmi to the animal pole of eggs and embryos indicated a possible role for this core Wnt/PCP protein in coordinating the activity of the two Wnt pathways at the animal pole. This idea was tested by disrupting NvFmi function using morpholinos and dominant-negative approaches. Downregulation of NvFmi function blocked both Wnt/PCP signaling mediated archenteron invagination and Wnt/beta-catenin signaling mediated endoderm cell fate specification, while overexpressing the cytoplasmic carboxy terminus of the NvFmi protein selectively disrupted endodermal gene expression. These observations indicates that NvFmi regulates both Wnt pathways and that it may function as a scaffold to coordinate both Wnt/\(\beta\)-catenin and Wnt/PCP signaling to drive primary archenteron invagination and endoderm cell fate specification in \textit{Nematostella} through the asymmetric localization of Wnt pathway components to the animal pole. Overall, this study has provided experimental evidence as to how different branches of Wnt signaling mediate primary
archenteron invagination and cell fate specification through NvFz10 and NvFz1 respectively during gastrulation in *Nematostella* and possible mechanisms of coordination of these two processes in time and space through the function of NvFmi.
Acknowledgments

First and foremost I owe my most sincere gratitude to my advisor Dr Athula Wikramanayake for giving me the opportunity to work and learn in his laboratory and for his time, ideas and encouragement. I would also like to thank the rest of my committee Dr. Julia Dallman, Dr. Alex Wilson, Dr. James Baker and Dr. Mark Q. Martindale for their support and guidance.

I would also like to thank all past and present members of the Wikramanayake lab for their support and friendship. A special thanks goes to Shalika Kumburegama, with whom I had the good fortune of collaborating on several projects, during which time I gained valuable training and knowledge on both molecular biological and embryological techniques.

I would also like to take this opportunity to thank the faculty and staff at the Department of biology for all their support and encouragement during my time in Miami. Also, a big thank you to all the past and present biology graduate students for being wonderful friends, without you all, my graduate student experience would not be the same.

Finally, I want to thank my family. I’m grateful to my parents for all the guidance and encouragement and then for letting me go. Most of all, I would like to thank my loving wife, Bhagya for her constant support, patience and companionship and also my son, Mevan for making all this more meaningful. Thank you.
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Chapter 1: Wnt signaling and the evolution of embryonic polarity

Gastrulation is the first major morphogenetic process that occurs during embryonic development in metazoans, and it leads to the segregation of the primary germ layers and the formation of a primitive gut. In most bilaterally symmetrical animals (bilaterians), the initiation of major organogenesis events takes place during or following gastrulation (Martindale, 2005). It has been proposed that the genesis of gastrulation was a pivotal event during metazoan evolution, as it paved the way for the evolution of the diversity of metazoan forms that we know of today from a hollow, ciliated, radially symmetrical ancestor (Wilmer, 1990; Martindale, 2005). Gastrulation facilitated the interaction of different germ layers leading to the induction of new cell types, complex structures and organs, which has been the key to the diversification of metazoan taxa (Martindale, 2005). Despite its critical role during development of metazoan embryos, the developmental mechanisms that induced initial gastrulation movements are unknown. One crucial step leading to the onset of gastrulation was likely the co-option of a molecular asymmetry present in ancient embryos that led to the localized activation of specific gene regulatory networks regulating cell fate specification and morphogenesis.

Primary egg axis and the adult axial properties of metazoans

Most animal ova have a primary polarity termed the animal-vegetal (AV) axis, and this ancient molecular asymmetry may have played a role in the evolution of gastrulation. The animal pole is defined by the site of polar body release during meiosis (Goldstein and Freeman, 1997 and Martindale and Hejnol, 2009). But it has also been
shown that in oocytes of many taxa the AV polarity corresponds to the apical-basal polarity of the germinal epithelium with the animal pole corresponding to the apical pole of the oocyte and the basal pole corresponding to the vegetal pole (reviewed in Wourms, 1987). In almost all investigated animal taxa, the animal vegetal axis predicts the axial properties of both the embryo and the adult (reviewed in Martindale and Hejnol, 2009). For example in bilaterians, endomesoderm is generated from vegetal pole derived blastomeres (reviewed in Martindale and Hejnol, 2009). However, in order to gain insights into key events in metazoan body plan evolution, it is important to look at out-group taxa to the bilaterian clade at the base of the metazoan tree. There are only a few extant basal, non-bilaterian taxa and these include the cnidarians, the ctenophores, the sponges and the placozoans. Out of these taxa, the cnidarians and the ctenophores are the most informative groups as they include the most basal metazoans with true epithelia and nerve cells and have readily available embryos that can be used for embryological and molecular analyses. In addition, all recent phylogenomic analyses show that the cnidarians are the sister group to all bilaterians and in some cases, ctenophores have been shown to be the earliest branching metazoan lineage (Dunn et al., 2008; Martindale & Hejnol, 2009).

Ctenophores and cnidarians share several cellular/developmental features that distinguish them from the bilaterians. Both ctenophores (Freeman, 1977; Martindale and Henry, 1999) and cnidarians (Freeman 1981; Momose and Schmid, 2006; Schlwany and Pfannenstiel, 1991) undergo unipolar cleavage, and the first cleavage furrow forms at the animal pole marking the site of gastrulation as well as the future oral pole. Animal blastomere derivatives will give rise to the endodermal tissues Also, it has been shown
experimentally in both ctenophores and cnidarians, that displacing the zygotic nucleus from the animal pole to an ectopic site by centrifugation specifies a new site of first cleavage and entrains a new oral-aboral axis (Freeman, 1977; Freeman, 1981; reviewed in Martindale and Hejnol, 2009). These results show that unlike in most bilaterians, in which the definitive embryonic and organismal axial properties are established maternally in an irreversible fashion, ctenophores and cnidarians display a relationship between the AV axis and the axial properties of the embryo which only establish the conditions for the formation of the first cleavage furrow (Martindale & Hejnol, 2009). In both ctenophores and cnidarians, the mouth that forms at the oral pole has been shown to be homologous to both the protostome and deuterostome mouths, as they express the same genes that are expressed at the oral openings of most investigated bilaterian taxa (Goldstein and Freeman, 1997; Nielsen, 1999; Neilsen, 2005; Holland and Holland, 2007; Henry et al., 2001). The mouth of all non-chordate metazoans being homologous suggests that gastrulation and endoderm formation might have evolved at the animal pole leading to the formation of a gut with a single opening at the animal pole in the metazoan ancestor. However, the development of endomesoderm from vegetal pole derived blastomeres in the bilaterians suggests that there was a switch in the site of gastrulation and endomesoderm specification from the animal pole in the ctenophores and the cnidarians to the vegetal pole in the last common ancestor of all bilaterians (Lee et al., 2007; Martindale and Hejnol, 2009). Experimental evidence for the embryonic and adult axial properties not being irreversibly determined maternally in ctenophores and cnidarians suggests that such a switch in the site of endoderm specification would have been possible in the lineage leading up the bilaterians.
The hypothesis that there was a switch in the site of gastrulation from the animal pole of non-bilaterians to the vegetal pole of bilaterians is supported by the molecular evidence for a deeply conserved role for the Wnt/beta-catenin pathway in endoderm specification. For example, the site of gastrulation in bilaterians, such as echinoderms (Logan et al., 1999), a spiralian protostome (Henry et al., 2008), a hemichordate (Darras et al., 2011), a mollusk (Henry et al., 2010) and non bilaterians such as cnidarians (Wikramanayake et al., 2003; Momose et al., 2008) is marked by the site of nuclearization of the protein beta-catenin which is a downstream effector of the Wnt/beta-catenin signaling pathway. Strikingly, nuclear beta-catenin marks the vegetal pole of these bilaterian embryos whereas in cnidarians, it marks the animal pole and as discussed earlier, corresponds to the site of endoderm specification (Momose et al., 2008; Wikramanayake et al., 2003).

**The Wnt signaling pathways**

Signal transduction pathways are a critical aspect of the development of multicellular organisms. Of these pathways, the evolutionarily conserved Wnt signal transduction pathway regulates multiple developmental processes in animal embryos, and moreover, it is also critical for tissue homeostasis in adults (Holstein, 2012). Misregulation in Wnt signaling during embryogenesis therefore results in severe developmental defects whereas misregulation of the pathway in adults leads to various degenerative diseases and cancer (reviewed in Logan and Nusse, 2004). Secreted Wnt signals can be transduced through one of the three branches of the Wnt signaling
pathway, the Wnt/β-catenin pathway, the β-catenin-independent Wnt/planar cell polarity (PCP) pathway and the Wnt/Ca\(^{2+}\) pathways (Holstein, 2012).

The Wnt/β-catenin pathway, which leads to the stabilization of β-catenin upon activation, is the most extensively studied branch of the Wnt signaling pathway. It has been shown to be involved in processes ranging from cell proliferation and cell fate specification to axis formation and endomesoderm patterning in both vertebrates and invertebrates (Holstein, 2012). In the absence of a Wnt ligand, a multi-protein complex referred to as the “destruction complex” containing Axin, GSK-3β and APC (adenomatous polyposis coli) phosphorylates β-catenin, targeting it for ubiquitylation and proteasome-mediated degradation. When a Wnt ligand is present, it binds to a receptor complex containing Frizzled (Fz) and LRP (lipoprotein receptor-related protein) 5/6 found on the target cell surface. This ligand-receptor complex in turn “activates” the cytoplasmic protein Dishevelled (Dsh) via a still poorly understood mechanism, and activated Dsh inhibits the destruction complex from phosphorylating β-catenin. This inhibition of β-catenin phosphorylation and degradation leads to the accumulation of β-catenin in the cytoplasm and later its nuclearization. Once in the nucleus, β-catenin binds to the transcription factors LEF/TCF (lymphoid enhancer factor/T cell factor) and either activates or represses Wnt target genes (Holstein, 2012).

The Wnt/PCP pathway was first identified in genetic studies aimed at elucidating the molecular basis for generating planar cell polarity in Drosophila (Wong and Adler, 1993). These studies showed that the Wnt/PCP pathway functions independently of β-catenin, but involved several components common to the Wnt/beta-catenin pathway such as Fz and Dsh (Shulman et al, 1998; Strutt, 2008). Together with a set of proteins referred
to as the “core” PCP genes including Flamingo/Starry night (Fmi), Strabismus/Van Gogh (Stbm), Dachous, Fat, Diego and Prickle (Pκ), Fz and Dsh regulate cell polarity through cytoskeleton organization. This regulation of polarity is believed to be carried out mainly through the asymmetric localization of Fz protein. In Drosophila, PCP signaling is involved in polarizing the sensory bristles and ommatidia of the eye (reviewed in Strutt, 2008; Strutt et al., 2011). PCP signaling is also important for convergent extension movements during gastrulation in vertebrates and in establishing polarity of sensory structures in the mammalian ear (reviewed in Gray et al., 2011; Wallingford, 2012).

The third branch of the Wnt signaling pathway, the Wnt/Calcium pathway functions through heterotrimeric G proteins activating cGMP phosphodiesterase, calmodulin dependent calcium kinase II and protein kinase C to upregulate calcium release in cells. Experimental evidence has shown that a subset of Wnt and Fz proteins, together with Dsh, play an important role in activating this pathway in cells (reviewed in Holstein, 2012).

_Nematostella vectensis as a model system_

As it was discussed above, studies aimed at reconstructing ancestral traits consider the basal groups to be of great importance. When considering the origins of critical characters important for the evolutionary diversification of the bilaterians, the cnidarians which are at the base of the metazoan tree provide an ideal outgroup. Within the phylum Cnidaria, there are several well-established and new model systems such as Eleutheria, Hydra, Hydractinia, Clytia and Podocoryne that have made important contributions to evolutionary studies (reviewed in Darling et al., 2005; Momose and Houlisten, 2007).
However, unlike most of these other cnidarian model systems, *Nematostella* represents a basal group within the cnidarians, which makes it even more suited for evolutionary studies. *Nematostella* belongs to the order Actinaria in class Anthozoa. Several studies provide evidence for the basal position of Anthozoa within the phylum Cnidaria and its relation as a sister group to the other three cnidarian classes, Hydrozoa, Scyphozoa and Cubozoa (Bridge et al., 1992; Bridge et al., 1995).

First recognized as a useful model system for the study of cnidarian biology Cadet Hand (Hand and Uhlinger, 1992), *Nematostella* has become a powerful model system for studies of not only cnidarian biology, but also in various aspects of metazoan evolution and more recently, for ecological and environmental studies (Darling et al., 2005; Finnerty, 2007). Other than its important phylogenetic position in the metazoan tree, which has made it an ideal system for evolutionary studies, the development of *Nematostella* as a model system was due to several other biological characteristics of this animal. *Nematostella* is found in brackish water habitats from Nova Scotia to Louisiana in the East and Gulf coast of North America, while in the West coast it is found in Washington, Oregon and California. *Nematostella* is also recorded from the United Kingdom. Maintaining *Nematostella* cultures in the laboratory is extremely easy and it is also relatively easy to access wild populations. These animals have separate sexes and synchronous spawning of males and females can be induced year round by simply manipulating the feeding and light cycles in the laboratory. The eggs are released embedded in a gelatinous matrix and have a rapid developmental period where they go from egg to larvae in a period of about 48 hours (Finnerty, 2005; Hand and Uhlinger, 1992) A fertilized egg becomes a hollow blastula by undergoing a chaotic cleavage
program by 4-6 hour post fertilization (hpf). The embryos begin to gastrulate around 12-15 hpf and by 24 hpf, become a swimming planula larva (Lee et al., 2007). The eggs are relatively large with a diameter of around 200-250 μm and eggs and embryos can be easily manipulated in embryological experiments using classical methods as well as modern molecular techniques such as micro injections of synthetic mRNAs and antisense morpholino oligonucleotides. In addition to all these characteristics that have contributed to its success as a model system, *Nematostella* has a readily available sequenced genome, which has enhanced the utility of this system for comparative studies in evolutionary genomics (Putnam et al., 2007).

**Wnt signaling and gastrulation in Nematostella**

Gastrulation in *Nematostella* is initiated by primary archenteron invagination at the animal pole (Magie et al., 2007). The invagination of the gut is induced by an apical constriction of cells at the future blastopore leading to bottle cell formation and subsequent buckling of the blastula wall at the animal plate (Kumburegama et al., 2011; Magie et al., 2007). Bottle cells play an important role in initiating gastrulation in diverse taxa and components of the beta-catenin independent Wnt/PCP pathway appear to play an important role in regulating apical constriction of these cells (Sawyer et al., 2010). Concomitant or subsequent activation of Wnt/beta-catenin signaling in the animal pole blastomeres specifies endodermal fates in the invaginating cells of the presumptive endoderm (Kumburegama et al., 2011; Lee et al, 2007).

Recent studies have shown that *Nematostella* has at least eleven of the twelve
known Wnt gene subfamilies that are found in bilaterians, and this unexpected diversity of Wnt genes in a basal non-bilaterian suggests that the Wnt signaling factors evolved early during metazoan evolution (Kusserow et al., 2005). Despite the crucial role played by Wnt signaling in germ layer segregation and gastrulation and the diversity of Wnt ligands in the *Nematostella* genome, exactly how this pathway is activated in *Nematostella* embryos is yet to be explained (Lee et al., 2007). The mechanism of Wnt pathway activation in *Nematostella* is further complicated by the fact that no Wnt ligands have been found to be expressed in the egg or during cleavage stages in *Nematostella* (Kusserow et al., 2005; Lee et al., 2006). Therefore, in *Nematostella*, the Wnt signaling pathways might be activated in a ligand independent manner during gastrulation. Looking at other upstream components of the Wnt pathway might be critical for identifying how this pathway is activated in *Nematostella*. For example, studies in *Xenopus* and sea urchins have shown that the cytoplasmic protein Dsh is critical for the asymmetric activation of the Wnt/beta-catenin pathway (Miller et al., 1999; Weitzel at al., 2004). Similar studies in *Nematostella* have also shown that Dsh is required for endoderm specification (Lee et al., 2007). Even though *Dsh* mRNA is expressed ubiquitously during *Nematostella* early development, Dsh protein is localized to the animal pole from the unfertilized egg stage (Lee et al., 2007). However, the mechanisms responsible for this asymmetric localization of Dsh are also unknown. Therefore, studies aimed at identifying mechanisms regulating Dsh localization in *Nematostella* would provide insights into the evolutionary origins of embryonic polarity and germ layer segregation in the metazoa.

As described earlier, extensive studies on how PCP signaling is involved in
regulating tissue polarity in *Drosophila* and vertebrates has shown that there are around six “core” PCP proteins that play critical roles in regulating asymmetric localization of cellular determinants that generate tissue polarity (Gray et al., 2011; Strutt, 2008; Wallingford, 2012). Additionally, recent studies in vertebrates such as *Xenopus* and zebrafish have shown the importance of these core PCP proteins in regulating morphogenetic movements such as convergence and extension during gastrulation (Carreira-Barbosa et al., 2009; Darken et al., 2002; Park and Moon, 2002). These studies imply that it may be possible that these core PCP pathway genes are playing important roles in regulating the asymmetric localization of Wnt pathway components to the animal pole of *Nematostella* eggs and embryos, resulting in the selective activation of Wnt signaling at the animal pole of these embryos. This selective activation of Wnt signaling at the animal pole in turn would activate specific gene regulatory networks driving cell fate specification and morphogenesis in *Nematostella* embryos.

Thus, the major objective of my dissertation research is to gain insights into how core PCP proteins might be involved in mechanisms that regulate embryonic polarity leading to the asymmetric activation of Wnt signaling in the blastomeres at the animal pole in the cnidarian *Nematostella*. This early activation of Wnt signaling then leads to germ layer segregation and gastrulation morphogenesis. A better understanding of the mechanisms regulating these pathways in a basal cnidarian species would provide valuable insights into the evolution of embryonic polarity and the evolution of gastrulation in the metazoans. The results of this study, which is the first comprehensive survey and functional analysis of PCP signaling in any cnidarian, are arranged in the following four chapters emphasizing the importance of core PCP proteins in regulating
Chapter 2: Core Planar Cell Polarity genes in *Nematostella*

**BACKGROUND**

Planar polarization of epithelial cells, or polarization of cells along the axis orthogonal to the epithelial cell sheet was first described in insects (Adler, 1992; Gubb and Garcia-Bellido, 1982; Wong and Adler, 1993). In insects, planar polarization of epithelial cells plays an important role in the orientation of wing and abdomen bristles, the organization of ommatidia in the compound eye into a crystalline structure and epidermal wound healing (Gubb and Garcia-Bellido, 1982; Nubler-Jung et al., 1987; Wong and Adler, 1993; Yang et al., 2002). Also, planar polarization of cells is critical for morphogenetic events such as gastrulation in insects, as seen in the case of *Drosophila*, where the germ band is elongated through the intercalation of cells in a polarized manner (Zallen and Wieschaus, 2004). Later studies in vertebrates have shown that planar polarization of cells can be seen in a variety of tissue types, from mammalian inner ear cells (Curtin et al., 2003; Dabdoub and Kelly, 2005) to mesodermal cells during gastrulation (Carreira-Barbosa et al., 2009; Gong et al., 2004; Goto and Keller, 2002) and the multi-ciliated cells of the airway, to cells of the developing long bones (reviewed in Wallingford, 2012). In most of the instances mentioned above, a conserved set of key proteins encoded for by the PCP genes are required for the control of cell polarization. However, it is important to note that all cases of planar polarization of epithelial cells does not require the function of these conserved core planar cell polarity proteins. For example, the planar polarized cell movements during germ band extension in *Drosophila* do not involve the function of PCP proteins (Zallen and Wieschaus, 2004). PCP signaling
refers to the overall molecular function of the core PCP proteins which does not involve an on or off outcome, as is the case in most signaling events (reviewed in Wallingford, 2012). Instead, PCP proteins play a critical role in setting up an asymmetric platform through the differential localization of the core PCP proteins within the cell to regulate various cell behaviors (Chen et al., 2008; Shimada et al., 2001; reviewed in Simons and Mlodzik, 2008; Usui et al., 1999).

Pioneering studies of planar polarity were first carried out on the cuticle of various insects by Katharina Nubler-Jung and colleagues (reviewed in Strutt, 2008). The findings of these initial studies provided the basis for more detailed experimental studies of planar polarity in the fruit fly *Drosophila*, specifically on the cuticle in the form of ordered patterns of trichomes, in the cells of the wing and the ommatidial units in the hexagonal facets of the compound eye (Adler, 1992; Gubb and Garcia-Bellido, 1982; Wong and Adler, 1993). These studies led to the identification of a common set of genes that are required for the generation of planar cell polarity in a variety of tissue types, and the description of the Planar Polarity Pathway (Wong and Adler, 1993). These investigators identified Fz and Dsh proteins to be functioning at the top of the pathway, which identified its link to Wnt signaling (Wong and Adler, 1993). However, to date, there has been no clear evidence of a graded Wnt ligand that activates this pathway to generate planar cell polarity (reviewed in Strutt, 2008). Further studies in both invertebrates and vertebrates has resulted in the characterization of the core set of proteins which include Flamingo/Starry night (Fmi) (Curtin et al., 2003; Usui et al., 1999), Strabismus/Van Gogh (Stbm) (Darken et al., 2002; Goto and Keller, 2002; Wolf and Rubin, 1998), Diego (Dg) (Feiguin et al., 2001; Simons et al., 2005), Prickle (Pk)
(Carreira-Barbosa et al., 2003; Gubb et al., 1999; Wong and Adler, 1993), Fz (Vinson et al., 1987; Wallingford, 2001; Wang et al., 2006; Wong and Adler, 1993) and Dsh (Tada and Smith, 2000; Thiesen et al., 1994; Wallingford et al., 2000; Wong and Adler, 1993) to be involved in regulating cell/tissue polarity (reviewed in Strutt, 2008) and these proteins are referred to as the “core” PCP genes (Gray et al., 2011). In the absence of a Wnt ligand, the regulation of polarity is believed to be carried out mainly through the asymmetric localization of Fz protein, which acts in concert with Stbm and Fmi to regulate cell-cell communication and ensure that neighboring cells adopt the coordinated polarities (Chen et al., 2008; reviewed in Klein and Mlodzik, 2005; Strutt, 2008; Usui et al., 1999). More recent work has led to the proposal that Wnt/PCP signaling is in fact a three-tiered linear pathway (reviewed in Tree et al., 2002). Upstream global cues for regulating PCP are generated by the proteins Fat-jointed, Daschous and Fat, which are characterized as parallel signaling molecules for planar polarity (Adler et al., 1998; Zeidler et al., 1999). These global cues in turn regulate the sub-cellular localization of Fz (Yang et al., 2002) and subsequently, the asymmetric localization of Fz-Dsh and Stbm-Pk protein complexes. This process is facilitated by the intercellular communication between these proteins themselves. Then, these localized proteins interact with tissue specific effector molecules such as Fuzzy (Collier and Gubb, 1997), Inturned (Park et al., 1996), RhoA (Strutt et al., 1997) and Drok (Winter et al., 2001) to bring about morphogenetic changes (reviewed in Strutt, 2008).

Recent studies in vertebrates such as Xenopus and zebrafish have shown the importance of the core PCP genes in regulating cell movement during convergence and extension (CE) (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000)
and cell fate specification through the antagonism of the Wnt/beta-catenin pathway (Park and Moon, 2002). Inhibition of PCP activity during vertebrate development results in CE defects such as widening of the neural plate and neural tube malformations in frogs and fish (Ciruna et al., 2006; Tawk et al., 2007; Wallingford and Harland, 2002), and/or an open neural tube from hindbrain to tail (craniorachischisis) in mice (Curtin et al., 2003; Kibar et al., 2001). Moreover, mutations in Stbm homologs Vangl1 and Vangl2 in humans are linked to defects in neural tube closure (Kibar et al., 2007; Lei et al., 2010).

In addition to their function in regulating CE in zebrafish, recent studies have shown that PCP genes are required for the localization of microtubular organizing centers (MTOC) within cells engaged in CE (Sepich et al., 2011). During embryogenesis, processes of embryonic axis determination and morphogenesis have to be precisely coordinated, suggesting that morphogenetic behaviors of cells or groups of cells might be synchronized with axial patterning. Being an evolutionarily conserved regulator of morphogenetic movements such as gastrulation and neurulation, there is increasing evidence to support the idea that in vertebrates, PCP signaling provides the link between global anterior-posterior and dorso-ventral patterning information to individual cells, bringing about coordination of morphogenetic movements of cells and embryonic polarity (reviewed in Gray et al., 2011).

The extensive use of Wnt/PCP signaling to generate diverse embryonic polarity events in metazoans hints that PCP signaling may have had an ancestral role in the evolution of embryonic polarity. While the various regulatory functions of PCP signaling in bilaterians have been studied extensively, relatively little is known about the role of PCP signaling in non-bilaterians (Holstein, 2012). In order to gain insights into the
evolution of embryonic polarity in the metazoans, and to gain insights into possible ancestral functions of PCP genes in generating these polarities, it is essential to look at how these genes function in animals at the base of the metazoan tree of life, namely, the non-bilaterians. Therefore, in this chapter, I have focused on the spatial and temporal expression patterns of the core PCP pathway genes in the cnidarian, *Nematostella* to begin to understand the role of these proteins in regulating embryonic polarity in non-bilaterians.

**MATERIALS AND METHODS**

**Spawning *Nematostella***

Adult *N. vectensis* were kept at 17°C in 1/3 seawater. The animals were fed either brine shrimp or finely chopped mussels or clams 24 hours before spawning. The animals were then rinsed with fresh 1/3 seawater and placed in a 24°C incubator with a halogen light source on a timer. The timer was set so that the animals were subjected to a light cycle of 12-hours of darkness followed by 12-hours of light. Following the 12-hour light cycle the lights were turned off and the animals would begin to spawn approximately 2 hours later.

**De-jellying *Nematostella* eggs**

Spawned egg masses were transferred into 15 ml conical plastic tubes, taking care to minimize the amount of 1/3 seawater in the tube without exposing the eggs to air. A 4% cysteine (Sigma, Cat # C7352) solution made in 1/3 seawater was added to the tube and the eggs were gently agitated in this solution for 5-8 min by inverting the tube slowly
to dissolve the jelly mass to release the eggs. Once all the jelly was dissolved, the eggs were allowed to settle to the bottom of the tube. The cysteine solution was removed by pipetting out as much of the solution as possible and then washing the eggs at least four times with fresh 1/3 seawater to remove all traces of cysteine. (Fritzenwanker and Technau, 2002)

**Whole mount RNA in situ hybridizations**

Embryos were fixed in 0.2% glutaraldehyde (Electron Microscopy Sciences, PA), 3.7% formaldehyde (EMD, NJ) in 1/3 seawater for 2 minutes and post-fixed in 3.7% formaldehyde in 1/3 seawater for 1 hour at room temperature. The fixed embryos were then washed 5X in PTw (0.1% Tween-20 in 1X PBS made with DEPC-treated water), 1X in 100% methanol and stored in 100% methanol at -20˚ C. Whole mount RNA *in situ* hybridization of *N. vectensis* embryos was carried out as previously described (Martindale et al., 2004). Digoxigenin-labeled RNA probes for *NvStbm, NvFmi, NvFz1, NvFz4, NvFz5, NvFz10 and NvPrk* were synthesized using the MegaScript Transcription Kit (Ambion, Austin, TX). Hybridization of the DIG-labeled RNA probes (1 ng/l) was carried out at 60-65˚ C. The labeled probes were visualized using NBT/BCIP as substrate for the alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Bioscience). Embryos were imaged using a Zeiss Axiovert 200 inverted microscope equipped an Axiovision camera and a Nikon CoolPix 990 digital camera.
Phylogenetic analyses of core PCP pathway genes in *Nematostella*

Amino acid sequence alignments including core PCP pathway proteins Stbm, Fmi, Fz1, Fz4, Fz5, Fz10 and Pk from *Nematostella* and other representative vertebrate and invertebrate taxa were generated using MacClade. Bayesian Phylogenetic analysis was carried out using MrBayes 3.1 with a run of one million generations sampled every 100 generations. The summary consensus tree was generated in MrBayes using the last 7,500 trees and the posterior probabilities were calculated for this consensus tree. The posterior probability values for the consensus tree are shown.

**RESULTS**

Cloning and phylogenetic analysis of core Wnt/PCP pathway gene orthologs from *Nematostella*

Known bilaterian core Wnt/PCP pathway gene sequences that included *Fz*, *Stbm*, *Fmi* and *Pk* were used to search an assembly of the *Nematostella* genome (Sullivan et al., 2006 and Joint Genome Institute) and this resulted in the identification of several putative coding sequences for Stbm, Fmi, four Fz homologs and Pk in *Nematostella*. Predicted sequences were used to design PCR primers to amplify a full length Stbm sequence and partial sequences for Fmi, Pk and Fz 1,4,5 and 10 from cDNA made from mRNA collected from mixed stage *Nematostella* embryos. Phylogenetic analysis confirmed that the *Nematostella* core PCP pathway gene orthologs *NvStbm*, *NvFmi*, *NvFz1,4,5* and 10 and *NvPk* are closely related to cnidarian, placozoan and bilaterian homologs, and that they are most likely *Nematostella* orthologs of these genes (Figure 2.1-2.4).
NvStbm shows between 31%-48% identity to various metazoan Stbm proteins and contains the four hydrophobic transmembrane domains and the highly conserved PDZ binding motif at the carboxy terminus. Searching available databases for sponge and ctenophore genomes did not result in the identification of orthologs of Stbm in these groups. There is increasing evidence that ctenophores and sponges are the earliest branching metazoan taxa (Dunn et al., 2008; Ryan et al., 2010) and this data indicates that a Stbm homolog was present in the last common ancestor to placozoans, cnidarians and bilaterians.

NvFmi contains the characteristic seven hydrophobic transmembrane domains and the conserved cadherin repeats in the extra cellular domain. The C-terminal cytoplasmic domain is less conserved compared to the other regions of the protein except for the functionally important SE/D domain (Carreira-Barbosa et al., 2009). The failure to identify other related sequences in additional searches of the Nematostella genome sequences suggests that there is only one Fmi homolog in Nematostella. As in the case of NvStbm, no homologs of this gene were identified from the sponges or ctenophores using the available databases.

As previously mentioned, genome searches of the Nematostella genome assembly lead to the identification of four Fz homologs in Nematostella and phylogenetic analyses which included Fz gene sequences from other representative taxa with fully annotated genomes showed strong support for the Nematostella Fz genes grouping in the Fz 1/2/7, Fz 5/8 and Fz 4/9/10 clusters. The genome searches resulted in the identification of two other Fz sequences in Nematostella but these were not characterized in this study.
Expression of *NvStbm* during *Nematostella* development

Expression of core PCP pathway genes in *Nematostella* using whole-mount in situ hybridization (WMISH) revealed that the *Nematostella Stbm* ortholog is expressed in an intriguing pattern which corresponds to the site of primary archenteron invagination and bottle cell formation. *NvStbm* mRNA is broadly expressed in unfertilized eggs with a consistent slight asymmetry towards one side (Figure 2.5 A). After fertilization and during early embryogenesis *NvStbm* transcripts show a striking asymmetric expression pattern (Figure 2.5 B-D), and by the early gastrula stage (12 to 14 hours post fertilization, hpf) the transcripts accumulate around the blastopore, suggesting a role in gastrulation (Figure 2.5 E). The lack of morphological landmarks during the early cleavage and the blastula stage made it difficult to determine if *NvStbm* expression is strictly restricted to the animal pole blastomeres prior to gastrulation initiation. However the localization of *NvStbm* to the animal pole at the gastrula stage made it likely that the expression is restricted to the animal pole blastomeres. In sum, these results showed that *NvStbm* is maternally expressed at the animal pole after fertilization, suggesting a role for *NvStbm* in regulating gastrulation.

Expression of *NvFmi* during *Nematostella* development

Using a partial fragment of *NvFmi*, whole-mount in situ hybridization was performed to examine the spatial and temporal expression pattern of *NvFmi* during early development in *Nematostella*. Similar to *NvStbm*, *NvFmi* was also found to be a maternally expressed mRNA, which displays an asymmetric expression pattern. However, one striking difference between *NvStbm* and *NvFmi* expression was observed
in the unfertilized egg. While *NvStbm* was more broadly expressed with a slight asymmetry in the unfertilized egg, *NvFmi* showed tightly localized expression in both unfertilized and fertilized eggs (Figure 2.6 A). At the cleavage and blastula stages *NvFmi* was expressed only in one pole of the embryo and at the early gastrula stage (12–14 hpf), expression was limited to the cells around the future blastopore (Figure 2.6 B-C). As in the case of *NvStbm*, it was difficult to determine whether *NvFmi* was asymmetrically expressed at the animal pole due to the lack of morphological landmarks, but based on the expression pattern at the early gastrula stage it is highly likely that the expression is restricted to the blastomeres at the animal pole (Figure 2.6 D). This strikingly asymmetric expression pattern leading up to primary archenteron invagination and gastrulation in *Nematostella* again suggests that maternally expressed *NvFmi* might be playing a key role in regulating gastrulation in *Nematostella*.

**NvFrizzled expression patterns**

Examination of mRNA expression patterns using whole mount in situ hybridization revealed that all four Frizzled homologs are expressed at the gastrula stage in localized domains. However, three of the homologs, *NvFz1, NvFz4* and *NvFz5* were maternal with broad expression patterns in eggs and cleavage stages. *NvFz1* was detected in both unfertilized and fertilized eggs but the transcripts were restricted to the non-cortical cytoplasm (Figure 2.7 A). Whereas, in the case of *NvFz4* and *NvFz5*, transcripts were expressed throughout the cytoplasm in both unfertilized and fertilized eggs (Figure 2.8 A and Figure 2.10 A). By the late blastula stage (6-8 hpf), the expression patterns of all three genes change where *NvFz1* and *NvFz4* expression gets restricted to the animal half
of the embryo (Figure 2.7 C and Figure 2.8 C) while the \textit{NvFz5} transcripts get restricted to the vegetal half of the embryo (Figure 2.10 C). By the early gastrula stage (12-14 hpf), \textit{NvFz1} and \textit{NvFz4} continue to be expressed in the animal half of the embryo including the blastopore and invaginating cells of the presumptive endoderm (Figure 2.7 D and Figure 2.8 D). At the same stage, \textit{NvFz5} transcripts are limited to the vegetal pole blastomeres of the presumptive ectoderm (Figure 2.10 C). \textit{NvFz10} is strikingly different from the other three \textit{Nematostella Frizzled} genes by lacking any maternal expression (Figure 2.9 A-B). \textit{NvFz10} is first detected in the late blastula stage in the blastomeres at the animal pole and by the early gastrula stage, \textit{NvFz10} transcripts are restricted to only the invaginating cells of the presumptive endoderm (Figure 2.9 C). Expression of \textit{NvFz1}, \textit{NvFz4} and \textit{NvFz10} at the animal pole from the late blastula stage to the early gastrula stage indicates a possible functional role in regulating gastrulation in \textit{Nematostella}. Moreover, even though \textit{NvFz5} expression is limited to the vegetal pole at later stages of development, its expression at the animal pole in eggs and early cleavage stages might result in \textit{NvFz5} also playing a regulatory role at the animal pole during early patterning of \textit{Nematostella} embryos.

\textbf{Expression of \textit{NvPk} in \textit{Nematostella}}

Whole mount in situ hybridization was performed using a partial fragment of \textit{NvPk} to examine the expression pattern of \textit{NvPk} during early development of \textit{Nematostella}. Expression was first detected in late blastula stage embryos, localized to one pole of the embryo (Figure 2.11 C). At the early to mid gastrula stage, \textit{NvPk} expression was restricted to the cells of the pharyngeal region of the developing embryo. However, at late gastrula stages a second tightly restricted expression domain was detected on one side of
the embryo in cells of the presumptive ectoderm (Figure 2.11 D). As in the case of all late blastula stage embryos, it was difficult to determine whether the expression of NvPk was at the animal pole. However, the expression in the pharyngeal region of the early and mid gastrula stage embryos suggests that again, NvPk expression is also likely to be restricted to the animal pole at the blastula stage. Different expression domains of NvPk might indicate to a dual role for Pk during Nematostella development, an earlier function during gastrulation and pharynx formation and a secondary function later on in determining the directive axis of the developing embryo.

**DISCUSSION**

While the Wnt ligands and the core components of the Wnt/beta-catenin pathway are well characterized in both bilaterians and non-bilaterians, relatively little is known about the core components of the Wnt/PCP pathway in non-bilaterians. To date, there have been a limited number of studies that have focused on core components that function in both Wnt/beta-catenin and Wnt/PCP pathways in non-bilaterians, which has lead to the characterization of two Fz genes in Clytia hemisphaerica (Momose and Houliston, 2007) and Dsh and Stbm in Nematostella (Kumburegama et al., 2011; Lee et al., 2007). In this study I have investigated the spatial and temporal expression patterns of all the core PCP genes except Diego/Inversin (Gray et al., 2011) in the non-bilaterian Nematostella.

The results confirm that Nematostella possesses all the core PCP genes that are present in bilaterians. More importantly, all the PCP genes considered in the current study are expressed very early in development, suggesting that they might be playing important roles in patterning the early development of these embryos. The functional
importance of Wnt/beta-catenin signaling during gastrulation and cell-fate specification in both bilaterians and non-bilaterians is well characterized and in cnidarians, components of the Wnt pathway have been shown to be involved in setting up some of the earliest molecular asymmetries in eggs and embryos (Kumburegama et al., 2011; Lee et al., 2007; Momose and Houliston, 2007; Momose et al., 2008). Work done in *C. hemisphaerica* has resulted in the identification of a maternally localized Wnt ligand (*CheWnt3*) (Momose et al., 2008) and two Frizzled receptors (*CheFz1* and *CheFz3*) (Momose and Houliston, 2007). In *Nematostella*, NvDsh has been shown to be a maternally localized protein regulating Wnt/beta-catenin mediated cell-fate specification during gastrulation (Lee et al., 2007). Even though NvDsh protein is localized asymmetrically at the animal pole of *Nematostella* eggs and embryos, NvDsh mRNA shows uniform expression during early development (Lee et al., 2007). Therefore, the first described molecular asymmetry at the mRNA level in *Nematostella* is set up by the two maternally localized mRNAs, NvStbm (Kumburegama et al., 2011) and NvFmi, from this study. One striking difference between the maternal expression of NvStbm and NvFmi can be seen at the unfertilized egg stage where NvStbm has a more broad expression domain with a slight asymmetry towards one pole and NvFmi mRNA is tightly localized to one pole of the unfertilized egg. However after fertilization, NvStbm transcripts show a much more restricted expression pattern, very similar to that of NvFmi, which might indicate a critical change during fertilization, which results in the tight localization of NvStbm mRNA to one pole of the zygote. During the cleavage and blastula stages leading up to the early gastrula stage, both these transcripts are tightly localized to the animal pole and accumulate around the blastopore, suggesting a role in
gastrulation. In *Drosophila*, both *Stbm* and *Fmi* have been shown to play critical roles in the asymmetric localization of core PCP pathway components into distinct regions of cells in the fly wing and the compound eye (Shimada et al., 2001; Taylor et al., 1998; Wang and Nathans, 2007). In vertebrates such as *Xenopus* and zebrafish, both *Stbm* (Darken et al., 2002; Goto and Keller, 2002) and *Fmi* (Carreira-Barbosa et al., 2009) have been shown to play key roles in regulating CE cell movements during gastrulation. One study carried out in cultured cells has shown that *Stbm* is able to antagonize Wnt/beta-catenin signaling, resulting in the inhibition of down stream target gene transcription (Park and Moon, 2002). Further work done in *Nematostella* has shown that *NvStbm* is required for the bottle cell formation and primary archenteron invagination during gastrulation morphogenesis (Kumburegama et al., 2011). Interestingly, downregulation of *NvStbm* had no effect on Wnt/beta-catenin mediated cell fate specification, suggesting that initial primary archenteron invagination and Wnt/beta-catenin signaling mediated endoderm cell fate specification in *Nematostella* can be uncoupled experimentally (Kumburegama et al., 2011). It is still not clear how these two processes, primary archenteron invagination and endoderm cell fate specification are coordinated in time and space in the developing embryo. However, the precise coordination of these two processes during gastrulation in *Nematostella* indicates that there is an upstream component that provides regulatory inputs to both the Wnt/beta-catenin and Wnt/PCP signaling cascades that are driving these processes. The tightly localized asymmetric expression pattern of *NvFmi* makes it an ideal candidate for such a regulatory role in both branches of the Wnt pathway. Also, work done in zebrafish has shown *Fmi* to be important in both epiboly and convergence and extension during gastrulation and serves
as a regulator of these two processes through different mechanisms involving cell cohesion in the case of epiboly, and Wnt/PCP signaling in the case of convergent extension (Carriera-Barbosa et al., 2009). Therefore, the functional role of NvFmi during gastrulation in Nematostella was experimentally tested and the results from those experiments are described in the fourth chapter.

Fz genes encode for receptors of Wnt ligands and therefore activate the down stream components of the Wnt signaling pathway through ligand binding (Huang and Klein, 2004). This study has revealed the presence of four different Fz homologs in Nematostella, which are all expressed during early development leading up to gastrulation. Like Dsh, Fz proteins are also upstream components of both Wnt/beta-catenin and Wnt/PCP signaling (Bhanot et al., 1996; Wong and Adler, 1993; reviewed in Huang and Klein, 2004). The first evidence found for Fz function in both Wnt/PCP signaling and Wnt/beta-catenin signaling was discovered in Drosophila (Bhanot et al., 1996; Wong and Adler, 1993). The asymmetric sub-cellular localization of Fz has been shown to be important in establishing cell polarity in a number of different patterning events in flies. In vertebrates, studies in Xenopus have indicated a regulatory role for Frizzleds in convergence and extension during gastrulation (Deardorff et al., 1998). Recent work in sea urchins has shown that a maternally expressed Frizzled SpFz1/2/7 is required for Wnt/beta-catenin mediated endoderm specification (Lhomond et al., 2011). In the cnidarian C. hemisphaerica, two maternally localized Frizzleds, CheFz 1 and CheFz3 have been shown to regulate Wnt/beta-catenin signaling during gastrulation (Momose and Houliston, 2007). Given the evidence for Frizzled function in other vertebrate and invertebrate systems and the expression patterns described in this study, it
is highly likely that Frizzled genes in *Nematostella* are playing important roles in regulating both cell fate specification and morphogenesis during gastrulation in *Nematostella*. The function of two *Nematostella* Frizzled genes that show asymmetric expression during early development was tested experimentally and the results from these experiments are described in the third chapter.

Pkd is also a core component of the Wnt/PCP pathway but compared to other genes of the pathway, the mechanism of Pkd function in generating polarity is less well known. In flies, Pkd has been shown to physically interact with Strabismus, resulting in the recruitment of Pkd to the membrane. Pkd is also known to interact with Dsh and therefore is capable of regulating both Stbm and Dsh localization (Jenny et al., 2003). Also, Pkd forms a functional complex with Stbm, which modulates Fz/Dsh activity (Jenny et al., 2003). In vertebrates, two *Pk* homologs in zebrafish have been shown to be required for normal CE movements during gastrulation by regulating Wnt/PCP signaling (Veeman et al., 2003). In *Nematostella*, *Pk* is not expressed until the late blastula stage (6-8 hpf) and its expression at the early gastrula stage is limited to the pharyngeal region. This exclusion of *Pk* transcripts in the early stages of development where all other core components of the PCP pathway such as *Fz*, *Dsh*, *Stbm* and *Fmi* are present might result in the absence of antagonistic interactions between the Fz/Dsh/Fmi and Stbm/Pkd/Fmi complexes. If the antagonistic interactions between Stbm and Fz/Dsh are mediated by Pkd, absence of Pkd during early patterning in *Nematostella* embryos might be allowing the asymmetric colocalization of all core components of the PCP pathway at the animal pole of the egg and the embryo unlike in the case of flies, where Fz/Dsh and Stbm/Pkd are localized to different regions of the cell. This hypothesis could be tested by over-expressing Pkd in the
egg and the early embryo to see if there are defects in the localization of Stbm and Fz/Dsh, which are linked to defects in gastrulation morphogenesis.
Figure 2.1: The summary consensus tree showing the gene orthology of *NvStbm* and its orthologs in representative species. The tree was generated in MrBayes using the last 7500 trees resulting from a run of one million generations sampled every 100 generations.
Figure 2.2: The summary consensus tree showing the gene orthology of NvFmi and its orthologs in representative species. The tree was generated in MrBayes using the last 7500 trees resulting from a run of one million generations sampled every 100 generations.
Figure 2.3: The summary consensus tree showing the gene orthology of four Nematostella Frizzled genes and their orthologs in representative species. The tree was generated in MrBayes using the last 7500 trees resulting from a run of one million generations sampled every 100 generations. The posterior probability values calculated for the consensus tree are shown.
Figure 2.4: The summary consensus tree showing the gene orthology of \textit{NvPk} and its orthologs in representative species. The tree was generated in MrBayes using the last 7500 trees resulting from a run of one million generations sampled every 100 generations. The posterior probability values calculated for the consensus tree are shown.
Figure 2.5: Expression of *NvStrabismus* during early embryogenesis, (A-E). *NvStbm* is more broadly expressed at the unfertilized egg stage (A) but is more tightly localized to the animal pole from the fertilized egg stage throughout early development leading up to the early gastrula stage (B-E).

Figure 2.6: Expression of *NvFlamingo* during early embryogenesis, (A-D). *NvFlamingo* (A, B, C) is localized to one pole in the egg, cleavage and blastula stages and is asymmetrically localized to the animal pole at the gastrula stage (D).

Figure 2.7: Expression of *NvFz1* during early embryogenesis, (A-D). *NvFz1* (A, B) is uniformly expressed in the egg and the cleavage stages. At the blastula and gastrula stages (C, D), *NvFz1* expression is restricted to the animal half of the embryo.
Figure 2.8: Expression of *NvFz4* during early embryogenesis, (A-D). *NvFz4* (A, B) is uniformly expressed in the egg and the cleavage stages. At the blastula and gastrula stages (C, D), *NvFz4* expression is restricted to the animal half of the embryo.

Figure 2.9: Expression of *NvFrizzled10* during early embryogenesis, (A-C). *NvFz10* is not expressed at the egg or cleavage stages (A-B). At the gastrula stage the expression is localized to the invaginating cells of the presumptive endoderm (C).
Figure 2.10: Expression of *NvFrizzled5* during early embryogenesis, (A-C). *NvFz5* is expressed uniformly at the egg and the cleavage stages (A-B). At the gastrula stage the expression is localized to the presumptive ectoderm at the vegetal pole (C).

Figure 2.11: Expression of *NvPk* during early embryogenesis, (A-C). *NvPk* is not expressed at the egg stage and the cleavage stages (A-B). It is expressed at one pole in the blastula stage embryo (C). At the gastrula stage the expression is localized to the pharyngeal region. Also there is diffuse expression on one side of the ectoderm (D).
Chapter 3: Regulation of early pattern formation in *Nematostella* by Frizzled signaling

**BACKGROUND**

First identified in *Drosophila*, the Frizzled family of proteins are seven pass transmembrane proteins that are upstream components of the Wnt signaling pathways (Wong and Adler, 1993; Vinson and Adler, 1987; reviewed in Huang and Klein, 2004, and Strutt, 2008). Frizzleds have been found in diverse metazoan taxa including both bilaterians and non-bilaterians. Vertebrates have at least ten Frizzled homologs and these fall into four main clusters based on sequence similarity. *Fz1, Fz2* and *Fz7* are clustered based on approximately 75% shared identity, *Fz5* and *Fz8* form a cluster with approximately 70% shared identity, *Fz4, Fz9, Fz10* form a cluster with approximately 65% identity, and *Fz3 and Fz6* are grouped based on approximately 50% shared identity (Huang and Klein, 2004). The amino terminus of the Fz protein is extracellular and contains a cysteine-rich domain (CRD). The intracellular carboxy terminal domain of variable length has no conserved domains between the different Fz family members (Dann et al., 2001; Umbhauer et al., 2000).

Fz receptors function in all three branches of the Wnt pathway. The role of Frizzled proteins in the Wnt/beta-catenin pathway was first identified in *Drosophila* and it has been shown to be important for Wnt/beta-catenin signaling in other invertebrates and vertebrates (Carron et al., 2003; Chen and Struhl, 1999; Umbhauer et al., 2000). Asymmetric sub-cellular localization of Fz proteins is critical for the establishment of planar cell polarity in a variety of cellular processes, from patterning wing hairs and
ommatidia in the compound eye of flies to inner ear patterning and gastrulation movements in vertebrates (Deardorff et al., 1998; Djiane et al., 2000; Usui et al., 1999; Vinson and Adler, 1987; Wallingford et al., 2001; Wong and Adler, 1993). During Wnt/PCP signaling, the regulation of polarity is believed to be carried out mainly through the asymmetric localization of Fz protein in response to a global cue, which is yet to be identified (Strutt, 2001; Yang et al., 2002). Coordination of Fz with the core PCP proteins Dsh, Stbm and Fmi to regulate cell-cell communication ensures that neighboring cells adopt coordinated polarities (Shimada et al., 2001; Strutt, 2008). Over expression studies in vertebrates suggest that different Fz homologs might be involved in both canonical Wnt/beta-catenin signaling and non-canonical Wnt/PCP signaling, and other homologs function specifically in one signaling cascade (Huang and Klein, 2004).

The mechanisms mediating Wnt ligand binding to the Fz receptor is fairly well characterized. Wnt ligands bind to Fz with high affinity through the CRD to activate the downstream signaling cascade (Bhanot et al., 1996; Dann et al, 2001; Hsieh et al., 1999). However, the mechanisms of transducing the signal to activate downstream signaling cascades after ligand binding are not well understood. It has been shown experimentally that the cytoplasmic protein Dsh binds to an internal peptide sequence of Fz through its PDZ domain (Wong et al., 2003) and the overexpression of Fz results in the recruitment of Dsh to the membrane (Kinoshita et al., 2003). Also, there is some experimental evidence to suggest that Fz interacts with Fmi to regulate localization of other core PCP proteins into specific domains (Carierra-Barbosa et al., 2009; Usui et al., 1999). Despite the lack of direct evidence for interactions between Fz proteins and other downstream components of the Wnt pathway, there is strong evidence to suggest that Fz protein plays
a critical role in the asymmetric localization of other core components of the signaling
cascade and activation of downstream signaling cascades during diverse cellular
processes (Strutt, 2001).

Therefore, in this chapter, I carried out functional analyses on three *Nematostella*
Fz homologs, Fz1, Fz10 and Fz5, which showed dynamic expression patterns during
early development. The results of these analyses indicated to functional roles for all three
Fz homologs during gastrulation in *Nematostella*. This study provided experimental
evidence for a functional role for NvFz1 in regulating endoderm cell fate specification
and a functional role for NvFz10 in initiating primary archenteron invagination. Also, it
provides evidence for a possible role for NvFz5 in the maintenance of a normal epithelial
gut, which requires further experimental characterization.

**MATERIALS AND METHOD**

**Antibody production**

To determine the NvFz10 protein expression pattern, affinity-purified
*Nematostella* anti-Fz10 (anti-NvFz10) polyclonal rabbit antibodies were made against a
selected amino acid region (NH2-DRLPKKGDPQAKSDPTK - COOH) (Bethyl Labs,
TX) of the NvFz10 protein.

**Immunohistochemistry**

Eggs and embryos at different stages of development were fixed as described in
Kumburegama et al. (2008). Fixed embryos were blocked using a 5% normal donkey
serum, 0.05% Tween-20 in PBS for 1 hour at room temperature. Then the embryos were
incubated in anti-NvFz10 rabbit polyclonal antibodies (1:100), diluted in blocking buffer at 4°C overnight. After rinsing in blocking buffer (5% normal donkey serum, 0.05% Tween-20 in PBS (10mM sodium phosphate, 150 mM sodium chloride, pH 7.8), embryos used for the substrate method were incubated with peroxidase-conjugated donkey anti-rabbit (1:500) (Jackson ImmunoResearch, Hatfield, PA) for one hour at room temperature. Embryos used for immunofluorescence were incubated with AlexaFluor 568 donkey anti-rabbit (1:250) secondary antibodies (Invitrogen) for 1 hour at room temperature. Eggs and embryos incubated with a peroxidase-conjugated secondary antibody were then incubated with DAB solution (Invitrogen, OR) for 5 minutes in the dark for color development. After washing the samples 3X in blocking buffer and once with PBS the eggs and embryos were dehydrated in isopropanol and cleared in BA:BB (1:2 Benzyl Alcohol: Benzyl benzoate). The cleared embryos were examined under a Zeiss Axiovert 200 inverted microscope. Eggs and embryos incubated with fluorescent secondary antibody were washed 3X in blocking buffer and once in PBS before they were cleared in BA:BB. The cleared embryos were observed using a Leica SP5 scanning confocal microscope.

cDNA constructs

NvFz1ΔC::GFP was constructed by PCR-amplifying an NvFz1 fragment that lacked the C-terminal domain using two gene specific primers (5'ATGCATTTCAGGTGAAATCTA 3' and 5'CCAAATCCAAAAATCCAGAGGTTA TTC 3') from cDNA prepared from gastrula stage embryos. The fragment was subcloned
into pCS2\(^+\) GFP to make the NvFz1\(\Delta\)C::GFP construct using standard molecular biology procedures.

**mRNA synthesis and microinjection**

The NvFz1\(\Delta\)C::GFP cDNA cloned into the pCS2\(^+\) vector was linearized and used as a template for SP6-dependent RNA transcription using mMessage mMACHINE (Ambion, Austin, TX) kit. NvFz1\(\Delta\)C::GFP mRNA was injected at a final concentration of 0.50 µg/µl in 40% glycerol. Embryos were observed using a Zeiss Axiovert 200 inverted microscope and digital images of the live embryos were taken using a Nikon Coolpix 990 digital camera.

**Morpholino anti-sense oligonucleotide microinjections**

Anti-sense morpholino oligonucleotides to NvFz10 (NvFz10-MO) (5’ AAGCTAAACGCTTAGCCCCCATATC 3’) and NvFz5 (NvFz5-MO) (5’ CCCGATCAAGTCTTCGAGTAGCCAT 3’) to block the translation of the endogenous protein were made along with control morpholinos (Control-MO) (5’ CCTCTTACCTCAGTTACAATTTATA 3’) (Gene Tools, Philomath, OR). The 3 mM stock solution of morpholinos was diluted to 750 µM and was injected into fertilized eggs. Embryos were allowed to develop and collected at 16-18 hpf and either fixed for scanning confocal imaging or whole mount RNA in situ hybridization.

**Whole mount RNA in situ hybridization**

Embryos were fixed in 0.2% glutaraldehyde:3.7% formaldehyde in 1/3\(^{rd}\) seawater for 2 minutes, post-fixed in 3.7% formaldehyde in 1/3\(^{rd}\) sea water for 1 hour at room
temperature, washed 5X in PTw, 1X in nuclease-free water, and stored in 100% methanol at -20˚ C. Whole mount in situ hybridization of N. vectensis eggs and embryos was carried out as previously described (Martindale et al., 2004). Digoxigenin-labeled RNA probes were synthesized using the MegaScript Transcription Kit (Ambion, Austin, TX). Hybridization of the DIG-labeled RNA probe (1 ng/µl) was carried out at 60-65˚ C. Visualization of the labeled probe was performed using NBT/BCIP as substrate for the alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Bioscience, Indianapolis, IN). Embryos were observed under a Zeiss Axiovert 200 inverted microscope equipped with epifluorescence optics, and digital images recorded using a Nikon CoolPix 990 digital camera or a Zeiss Axiovision camera.

RESULTS

The Frizzled 10 receptor is expressed asymmetrically during Nematostella embryogenesis

Whole mount in situ hybridization for NvFz10 showed a striking asymmetric expression pattern, where the NvFz10 transcripts were restricted to the presumptive endodermal cells of the invaginating archenteron. To better understand where the NvFz10 protein may function, a polyclonal antibody against NvFz10 was generated. Immunostaining of Nematostella eggs and embryos showed an asymmetric expression pattern for NvFz10, which corresponds to the expression pattern of its transcripts. There was no NvFz10 protein in the egg or cleavage stage embryos (Figure 3.1 A-B), but the protein was localized to the animal pole of the embryos at the onset of gastrulation (Figure 3.1 C) and continued to be expressed in the presumptive endoderm of later gastrula stage embryos (Figure 3.1 D).
**NvFz10 affects gastrulation movements in *Nematostella***

To determine the function of NvFz10 during early developmental events in *Nematostella*, I used an anti NvFz10 morpholino (NvFz10-MO) to knock down endogenous NvFz10 levels in developing *Nematostella* embryos. Fertilized eggs were injected with either NvFz10-MO or Control-MO and were allowed to develop until 12-14 hpf. The injected embryos were then collected, fixed and were immunostained with NvFz10 polyclonal antibody to test the efficacy of the NvFz10-MO. Analysis of the stained embryos showed that compared to the Control-MO injected embryos that showed the normal protein expression in the presumptive endoderm (Figure 3.2 A), the NvFz10-MO injected embryos had no detectable NvFz10 expression (Figure 3.2 B). Given that there is significant reduction of NvFz10 protein levels when injected with the NvFz10-MO, morphant and control embryos were stained for F-actin and nuclei and the morphology of the embryos were examined using scanning confocal microscopy to identify any defects in gastrulation in morphant embryos. Compared to the control morpholino injected embryos, which have undergone normal primary archenteron invagination (Figure 3.2 C), the NvFz-MO injected embryos failed to gastrulate and there was no indication of bottle cell formation or primary archenteron invagination in these embryos (Figure 3.2 D).

Whole mount RNA in situ hybridization for the endodermal marker *NvSnail* was carried out to determine whether cell fate specification was affected in these morphant embryos. Interestingly, despite their inability to gastrulate, whole mount in situ hybridization showed normal *NvSnail* expression in morphant embryos (Figure 3.3 B). Therefore, these results suggest that even though the NvFz10 morphant embryos were
unable to gastrulate, they were still undergoing normal endoderm cell fate specification. These results implicate NvFz10 in Wnt/PCP signaling mediated primary archenteron invagination but not Wnt/beta-catenin mediated cell fate specification.

To further examine the requirement of NvFz10 for normal primary archenteron invagination, I made an attempt to rescue the NvFz10-MO phenotype by co-injecting embryos with the NvFz10-MO together with either in vitro synthesized activated RhoA mRNA or RFP mRNA. RhoA is a downstream effector of Wnt/PCP signaling and over expression of a constitutively active form of the RhoA protein throughout developing embryos results in the abnormal movement of cells into the blastocoel. Embryos injected with NvFz10-MO and activated RhoA showed a similar phenotype to that seen when activated RhoA is overexpressed, where there is cell movement into the blastocoel even though there was no organized endodermal epithelium in these embryos (Figure 3.4 B). In contrast, embryos injected with NvFz10-MO and RFP mRNA showed a complete block of gastrulation, similar to the phenotype seen in embryos with reduced levels of NvFz10 protein (Figure 3.4 A). The partial rescue of the NvFz10 knockdown phenotype by over expressing activated RhoA, which is a downstream component of the Wnt/PCP pathway, provides further evidence for a functional role for NvFz10 in Wnt/PCP mediated primary archenteron invagination during gastrulation in Nematostella.

**NvFz1 is required for normal gastrulation morphogenesis in *Nematostella***

Frizzled 1/2/7 has been shown to be required for Wnt/beta-catenin signaling mediated endoderm specification in sea urchin embryos. Given the striking similarity
between Fz1/2/7 mRNA expression in sea urchins (Lhomond et al., 2012) and NvFz1 expression in *Nematostella* during early embryogenesis, functional studies were carried out to examine possible roles of NvFz1 during early development in *Nematostella*. To test the functional importance of NvFz1, I used a dominant-negative approach, where I over expressed mRNA of a truncated form of NvFz1 lacking the C-terminal domain (ΔC) of the protein. Zygotes were injected with either NvFz1ΔC::GFP or GFP mRNA and the injected embryos were allowed to develop at 25°C and fixed at 16-18 hpf. The fixed embryos were stained for F-actin and nuclei using phalloidin and propidium iodide or cytox green respectively, and were analyzed using scanning confocal microscopy to identify potential morphological changes in the developing embryos. Embryos injected with NvFz1ΔC::GFP and GFP mRNA were able to undergo primary archenteron invagination and a blastopore was present in both GFP and NvFz1ΔC::GFP over expressing embryos (Figure 3.5). However, the NvFz1ΔC::GFP overexpressing embryos failed to form a normal gut epithelium (Figure 3.5 B) whereas the GFP overexpressing embryos were able to form an epithelial gut (Figure 3.5 A). Since this phenotype was also similar to the one resulting from the inhibition of Wnt/beta-catenin signaling in *Nematostella* (Lee et al., 2007; Kumburegama et al., 2011), I carried out whole-mount *in situ* hybridizations for the endodermal marker *NvSnail* on embryos over expressing NvFz1ΔC::GFP and GFP. Preliminary analyses of the *in situ* hybridization data showed that compared to the GFP over expressing embryos where normal *NvSnail* expression was detected in a majority of the embryos (Figure 3.6 A), most of the NvFz1ΔC::GFP over expressing embryos lacked *NvSnail* expression in the invaginating cells of the archenteron (Figure 3.6 B). Therefore, these results indicate a functional role for NvFz1
in Wnt/beta-catenin mediated endoderm cell fate specification during gastrulation in *Nematostella* but not in Wnt/PCP mediated primary archenteron invagination.

**NvFz5 affects normal gut morphogenesis in *Nematostella* embryos**

*NvFz5* is expressed uniformly in both eggs and early embryos, and by the gastrula stage the expression becomes tightly localized to the vegetal pole. This dynamic expression pattern during early development raised the possibility that NvFz5 might be important for regulating early development in *Nematostella* embryos. To test the functional importance of NvFz5, I again used an anti-sense NvFz5 morpholino (NvFz5-MO) to knock down NvFz5 protein levels in developing *Nematostella* embryos. Zygotes were injected with either NvFz5-MO or a standard Control-MO and were allowed to develop at 25°C. The injected embryos were collected at 16-18 hpf and were fixed and stained for F-actin and nuclei. The stained embryos were examined using scanning confocal microscopy to identify any defects in normal development. This analysis showed that both NvFz5-MO injected and Control-MO injected embryos had undergone primary archenteron invagination and had normal looking blastopores (Figure 3.7). However, in embryos injected with NvFz5-MO the presumptive endoderm resulting from primary archenteron invagination did not form a normal gut epithelium and showed signs of disintegration (Figure 3.7 B). Considering the ubiquitous expression pattern of NvFz5 early in development, this phenotype suggests a possible role for NvFz5 in endoderm cell fate specification or the maintenance of a normal epithelial gut in the developing embryos.
**DISCUSSION**

This work shows that two Fz homologs in *Nematostella*, NvFz1 and NvFz10 are required for Wnt/beta-catenin signaling mediated cell fate specification and Wnt/PCP signaling mediated primary archenteron invagination respectively. Also, this study provides further evidence for the uncoupling of primary archenteron invagination and cell fate specification during gastrulation in *Nematostella* that was reported previously (Kumburegama et al., 2011). Therefore, these results support the model for the independent evolution of Wnt/PCP signaling mediated initial primary archenteron invagination and Wnt/beta-catenin mediated endodermal cell fate specification during the evolution of gastrulation in metazoa (Kumburegama et al., 2011). Also, results of this study indicate to a possible role for NvFz5 in maintenance of a normal epithelial gut in developing *Nematostella* embryos. However, further experimentation is needed to characterize the mechanisms of this potential role for NvFz5.

**NvFz1 and germ layer specification in *Nematostella***

Frizzled proteins are important components of Wnt signaling in all metazoan taxa from non-bilaterians such as sponges and cnidarians to bilaterians such as flies, nematodes and vertebrates. They function as receptors for Wnt ligands and activate downstream signaling cascades through mechanisms that are not well understood to date. There is also a diverse array of Fz homologs in most metazoans and there is evidence for functional specificity between these different homologs (Huang and Klein 2004). Fz 1/2/7 has been implicated in Wnt signaling in flies where it has been shown that expression of *Drosophila* frizzled 2 in *Drosophila* S2 cells makes these previously
Wingless insensitive cells now respond to this ligand (Bhanot et al., 1996). Recent work
done in sea urchins has shown that Fz1/2/7 is required for the regulation of Wnt/beta-
catenin signaling mediated endoderm specification in macromeres during embryogenesis
(Lhomond et al., 2012). In the current study, I show that the Nematostella homolog of
Fz1/2/7, NvFz1 is required for Wnt/beta-catenin mediated cell fate specification in
Nematostella. However, unlike in sea urchin embryos where Fz 1/2/7 is required for both
endoderm specification in the macromeres and normal archenteron formation, NvFz1 in
Nematostella is only required for Wnt/beta-catenin mediated cell fate specification.
Nematostella embryos overexpressing the truncated form of NvFz1 (NvFz1ΔC::GFP),
which would compete with the endogenous Fz1 protein resulting in a down regulation of
Fz1 signaling, were still able to undergo initial primary archenteron invagination. This
provides further evidence for the uncoupling of primary archenteron invagination and cell
fate specification in Nematostella. The Fz 1/2/7 ortholog in the hydrozoan cnidarian
Clytia hemisphaerica, CheFz1 has also been implicated in Wnt/beta-catenin signaling
during gastrulation (Momose and Houliston, 2007). In Clytia, loss of CheFz1 results in
both inhibition of Wnt/beta-catenin signaling and gastrulation morphogenesis. However,
one important difference in gastrulation morphogenesis between the more basal cnidarian
Nematostella and the derived hydrozoan Clytia is that Nematostella embryos gastrulate
through invagination while in Clytia, the cells of the presumptive endoderm move into
the blastocoel mainly through ingestion (Byrum, 2001; Momose et al., 2012). Therefore,
the mechanisms of regulating gastrulation morphogenesis in Clytia might be
fundamentally different from those that are regulating primary archenteron invagination.
During gastrulation morphogenesis, ingressing cells of the presumptive endoderm have to
undergo an epithelial-to-mesenchymal transition (EMT) prior to moving in to the blastocoel and there is strong experimental evidence indicating a regulatory role for Snail during EMT in different morphogenesis events from flies to vertebrates (reviewed in Shook and Keller, 2003). Snail expression is under the control of Wnt/beta-catenin signaling (Lee et al., 2007; Kumburegama et al., 2011) and thus, inhibition of Wnt/beta-catenin signaling would in turn block EMT and ingression in developing Clytia embryos. These results further confirms that the basal anthozoan cnidarian Nematostella represents the ancestral mode of gastrulation through invagination, which would have allowed the evolution of gastrulation morphogenesis independent of cell fate specification. However, with the utilization of more complex mechanisms of cell movements such as ingression during gastrulation morphogenesis these two processes became tightly linked in the more derived cnidian and bilaterians.

**NvFz10 and primary archenteron invagination in Nematostella**

Of the three different Nematostella Fz homologs considered in this study, NvFz10 is the only Fz homolog that is not maternally expressed. NvFz10 transcripts are not found in the eggs or the early developmental stages and at the early gastrula stage this gene is expressed in the invaginating cells of the presumptive endoderm. It is interesting that knock down of NvFz10 in developing Nematostella embryos blocked primary archenteron invagination but there was normal endodermal cell fate specification in these embryos. This provides further support for a model where primary archenteron invagination and cell fate specification evolving separately during the evolution of gastrulation in the metazoa (Kumburegama et al., 2011). However the expression of
NvFz10 in the presumptive endoderm raises some important questions about how this gene is regulated before and after the activation of Wnt/beta-catenin signaling. The inhibition of primary archenteron invagination in the absence of NvFz10 protein provides strong evidence for its involvement in Wnt/PCP signaling mediated gastrulation morphogenesis. However, inhibition of Wnt/beta-catenin signaling using a dominant negative form of Dsh in *Nematostella* results in the loss of NvFz10 expression in the presumptive endoderm in late gastrula stage embryos (Kumburegama et al., 2011). This suggests that NvFz10 expression might be controlled through different mechanisms before and after the activation of Wnt/beta-catenin signaling. One possible explanation for this inconsistency would be that the initial expression of NvFz10 is independent of Wnt/beta-catenin signaling and is required for primary archenteron invagination, but the maintenance of NvFz10 expression in the invaginating cells of the presumptive endoderm requires inputs from Wnt/beta-catenin signaling. Therefore further experimentation is required to better understand this apparent complex regulation of NvFz10 expression during gastrulation in *Nematostella*.

**NvFz5 and maintenance of an epithelial gut in *Nematostella***

*NvFz5* was found to be a maternal transcript that was expressed uniformly throughout eggs and early developmental stages. However, by the early gastrula stage, *NvFz5* expression gets restricted to the vegetal pole of the gastrulating embryo and this localized expression pattern was maintained throughout the later developmental stages. Knockdown of NvFz5 protein levels using anti-sense morpholino oligonucleotides had no detectable effects at the early gastrula stage. However, at the later gastrula stages when
the control embryos were forming a regular, epithelial gut, the NvFz5 morphant embryos had a much more disorganized gut which did not seem to be able to form an organized epithelium. These observations suggest that NvFz5 might be playing an important role in regulating the maintenance of an organized epithelial gut. However, it is not clear how NvFz5 is involved in this process. Given its dynamic expression pattern during early and late development, it could be an early function where NvFz5 is involved in cell fate specification through Wnt/beta-catenin signaling. Also, it is possible that NvFz5 has a later function in signaling from the vegetal pole to maintain an organized epithelial gut. Therefore, this study provides insight into a possible regulatory function for NvFz5 during gastrulation in Nematostella but characterization of the exact mechanisms of NvFz5 function requires further experimentation.

**Evolution of Fz receptors**

Fz proteins are of great importance for a number of different cellular processes ranging from embryonic development to tissue homeostasis due to their function in Wnt signaling as receptors for Wnt ligands. Presence of Frizzled-related genes in the social amoebae *Dictyostelium discoideum* indicates to a pre-metazoan origin for the Frizzled family of proteins. The *Dictyostelium* genome contains sixteen Fz-related genes encoding for a putative cysteine-rich (CRD) domain and two of these genes contain an additional motif required for the activation of the canonical Wnt pathway (Harwood, 2008). However, the genome of the choanoflagellate *Monosiga brevicolis*, does not contain any Wnt ligands or Fz-like Wnt receptors (King et al., 2008). Ctenophores and poriferans, the earliest branching metazoan taxa (Ryan et al., 2010), contain Fz genes in their genomes
The ctenophore *Mnemiopsis* genome contains two Fz homologs with the CRD motif but only one of these, MfFzA, contains the KTXXXW motif, which is required for canonical Wnt signaling. MfFzA in *Mnemiopsis* is expressed maternally in a uniform manner, indicating a role during early development. However, no Wnt ligands are expressed at this early stage of development. Therefore, it is not clear how this receptor might be involved in the activation of Wnt signaling (Pang et al., 2010). The genome of the sponge *Amphimedon queenslandica* also contains two Fz homologs that contain both the CRD and the KTXXXW motif and both these homologs are expressed from the earliest stages to the swimming larval stage (Adamska et al., 2010). However, in *Amphimedon*, as in the case of ctenophores, it is not clear how these Fz proteins are involved in Wnt signaling and the specific mechanisms of how the pathway is activated (Adamska et al., 2010). In the hydrozoan cnidarian *Clytia hemisphaerica*, there are two Fz homologs CheFz1 and CheFz3, whose expression is localized to the animal pole and the vegetal pole of the egg respectively. CheFz1 is required for Wnt/beta-catenin signaling mediated cell fate specification and CheFz3 was shown to antagonize CheFz1 function to inhibit Wnt/beta-catenin signaling at the vegetal pole (Momose and Houliston, 2007). Six Fz homologs have been identified in *Nematostella*, and in the current study I have shown that NvFz1 is required for cell fate specification while NvFz10 is required for primary archenteron invagination. These observations in basal metazoan groups indicate that Wnt pathway components were coopted early in metazoan evolution for early embryonic patterning events. The presence of functionally important motifs of the Fz receptors in the pre-metazoan *Dictyostilium* suggests that the metazoan Fz proteins might have evolved through domain shuffling to
produce the functional Wnt receptors. The lack of functional data on how Fz proteins affect early development in the basal metazoan groups such as ctenophores and poriferans make it difficult to understand how these receptors evolved into functional components of the Wnt signaling pathways. However, in *Nematostella*, the Fz gene family has undergone an expansion, shown by the presence of six Fz homologs as opposed to two in both *Mnemiopsis* and *Amphimedon* (Adamska et al., 2010; Pang et al., 2010). Also, in *Nematostella*, NvFz1 regulates cell fate specification independent of primary archenteron invagination and NvFz10 regulates primary archenteron invagination without affecting cell fate specification. These finding suggests that there are specific Fz receptors that function in either Wnt/beta-catenin signaling or Wnt/PCP signaling in *Nematostella*. The uncoupling of cell fate specification and gastrulation morphogenesis seen in *Nematostella* was lost in the lineages leading up to bilaterians, possibly due to the increasing complexity of gastrulation morphogenesis, which involved the function of other components that were under the control of different gene regulatory networks. However, evidence for the receptor specificity in Wnt signaling can still be seen in certain instances of bilaterian development. For example, Wnt5a, which is considered to be a non-canonical Wnt ligand, is capable of signaling in the Wnt/beta-catenin pathway if it is exposed to a specific receptor, Fz4 (Van Amerongen et al., 2008). These functional characteristics of Fz receptors suggest that Fz function in Wnt/beta-catenin signaling and Wnt/PCP signaling might have evolved independently during early metazoan evolution.
FIGURES

Figure 3.1: NvFz10 expression in egg and embryos. Frizzled 10 protein is not expressed in the early stages of development in *Nematostella* (A and B). The protein is expressed at the gastrula stage in the invaginating cells (C) and in the endoderm at the late gastrula stage (D).

Figure 3.2: Injection of an NvFz10 morpholino knocks down NvFz10 protein expression and blocks primary archenteron invagination. Compared to the control morpholino injected embryos (A) injection of NvFz10-MO completely blocked primary archenteron invagination in morphant embryos (B). Immunostaining of NvFz10-MO injected embryos with anti NvFz10 antibody (D) confirms the knockdown of Fz10 in morphant embryos by the lack of Fz10 staining compared to the control morpholino injected embryos (C).
**Figure 3.3:** NvFz10 is not required for endodermal cell-fate specification. Both Control-MO injected embryos (A) and NvFz10-MO injected embryos (B) showed *NvSnail* expression.

**Figure 3.4:** Rescue of NvFz10 knockdown phenotype with activated RhoA. Compared to the control embryos injected with NvFz10-MO and *RFP* mRNA (A) where there is no primary archenteron invagination, the embryos injected with NvFz10-MO and activated *RhoA::GFP* RNA showed cell migration, which resulted in cellular matter inside the blastocoel (B). The numbers on the figure indicate the number of embryos showing the respective phenotypes.
Figure 3.5: Over-expression of the dominant-negative NvFz1ΔC::GFP affects normal gastrulation and cell fate specification during development in *Nematostella*. Compared to the *GFP* mRNA injected control embryos (A) over-expression of NvFz1ΔC::GFP results in the embryos not forming an organized gut epithelium, despite undergoing primary archenteron invagination (B).

Figure 3.6: NvFz1 is required for endodermal cell-fate specification. Compared to the control embryos injected with *GFP* mRNA (A) where there is endodermal *NvSnail* expression, the *NvFz1ΔC* RNA injected embryos showed no detectable *NvSnail* expression (B).
Figure 3.7: Knockdown of Frizzled 5 protein using morpholino oligonucleotides. Even though embryos injected with NvFz5-MO undergo primary archenteron invagination (B) they are not able to maintain a normal organized epithelial endoderm compared to the control morpholino injected embryos (A).
Chapter 4: Flamingo function during early embryogenesis in *Nematostella*

BACKGROUND

In bilaterians, gastrulation morphogenesis and endodermal cell fate specification is tightly linked with germ layer specification preceding morphogenesis (reviewed in Heisenberg and Solnica-Krezel, 2008). Gastrulation in *Nematostella* involves cell shape changes leading to an epithelial buckling and invagination of a primary archenteron resulting from the activation of Wnt/PCP signaling at the animal pole of the embryo (Kumburegama et al., 2011; Magie et al., 2007). Concomitant or subsequent activation of the Wnt/beta-catenin pathway in the animal pole blastomeres results in the specification of endodermal cell fates in the archenteron (Kumburegama et al., 2011; Lee et al., 2007). Unlike in bilaterian embryos, in *Nematostella*, primary archenteron invagination and endodermal cell fate specification can be experimentally uncoupled by selectively blocking either Wnt/PCP signaling or Wnt/beta-catenin signaling respectively (Kumburegama et al., 2011). It is still not clear how primary archenteron invagination and endoderm cell fate specification are regulated in time and space in the developing embryo but the precise coordination of the two processes during gastrulation in *Nematostella* indicates the presence of a possible upstream regulator that provides inputs to both the Wnt/beta-catenin and Wnt/PCP signaling cascades. The tightly localized, asymmetric expression pattern of maternally expressed NvFmi (Chapter 2; Fig 2.6), makes it an attractive candidate for mediating such a regulatory role in both branches of the Wnt pathway.
Flamingo/Starry night (Fmi) is a seven-pass transmembrane protein initially identified in mutant screens carried out to identify tissue polarity genes in *Drosophila* (Usui et al., 1999). The *Drosophila* Fmi protein is relatively large with 3575 amino acids and is different from most other known cadherins in having seven transmembrane domains instead of one. As is the case in all other cadherins, it has nine extracellular cadherin repeats and in addition, the Fmi cadherins have three cysteine rich domains and two laminin A globular domains. Unlike classic-type cadherins, Fmi has no catenin binding domains in the cytoplasmic region, making it a non-classic-type protocadherin (Usui et al., 1999). Fmi is conserved from *C. elegans* to *Drosophila* to vertebrates and it is considered to be one of the “core” tissue polarity genes involved in the Wnt/PCP signaling (Carriera-Barbosa et al., 2009; Usui et al., 1999).

In flies, *Flamingo* is expressed in both the embryo and imaginal discs, mainly in epithelia and nerve tissue (Usui et al., 1999). Adult flies with *Fmi* mutations showed polarity defects in ommatida, sensory bristles and wing hairs (Usui et al., 1999). Additionally Flamingo is known to function in regulating tissue polarity through interactions with Frizzled and Disheveled proteins in *Drosophila*. Experimental evidence in the fly wing supports the possibility of Flamingo, Dishevelled and Frizzled forming a localized signaling complex that directs localized cytoskeletal reorganization (Shimada et al., 2001). It has also been shown that Fmi serves a dual molecular function during dendrite morphogenesis in flies where in the initial growing phase, Fmi functions as a receptor for an undefined ligand to limit dendrite branch elongation (Kimura et al., 2006). In later stages, homophilic interactions between Fmi bring about avoidance between dendritic terminals (Kimura et al., 2006).
There is also increasing evidence for the importance of Fmi in localization of other core PCP proteins within and between cells. For example, in Fmi mutant clones in the Drosophila wing, Fmi itself and other core PCP proteins fail to accumulate and polarize cells (Axelrod, 2001; Bastock et al., 2003; Shimada et al., 2001; Strutt and Strutt, 2007). However, the molecular mechanisms by which Fmi regulates the interactions between core PCP proteins are not well understood. Based on available data, Fmi can either function as a scaffold to localize other PCP proteins that activate localized signaling events or Fmi itself might be providing an instructive signal to regulate PCP signaling. One study in Drosophila shows that Fmi functions instructively to localize Fz and Stbm across cell borders in the wing to transmit PCP signals from cell to cell (Chen et al., 2008). Given the diversity of cellular processes that involve Fmi function, it is highly likely that Fmi operates through multiple mechanisms to provide instructive signaling inputs as well as permissive, scaffolding functions to localize other proteins.

More recent studies in vertebrates have also shown that Fmi is required for the regulation of a variety of biological processes from convergence and extension (CE) and cochlear hair cell orientation to axonal path finding and neuronal migration (Curtin et al., 2003; Formstone and Mason, 2005; Lee et al., 2003; Shima et al., 2004; Wada et al., 2006). There is extensive evidence to support a critical role for Fmi during zebrafish gastrulation where it regulates convergence and extension through its combinatorial activity with other core PCP proteins (Carriera-Barbosa et al., 2009). There is experimental evidence to suggest that Fmi functions in concert with Stbm and Wnt11 during zebrafish gastrulation to direct CE (Formstone and Mason, 2005). Additionally, there is evidence for a dual role for Fmi during zebrafish gastrulation where it is involved
in regulating epiboly through its role in mediating cell cohesion, and regulation of CE mediated by its role in Wnt/PCP signaling through its interaction with a Fz/Dsh complex (Carreira-Barbosa et al., 2009).

There is strong evidence for the importance of Flamingo function in regulating tissue polarity and specifically its functional importance in the asymmetric localization of other PCP proteins such as Dsh and Stbm during morphogenetic processes such as CE. Therefore, I hypothesized that NvFmi is an upstream regulator of both Wnt/beta-catenin signaling and Wnt/PCP signaling during early embryogenesis in *Nematostella*. NvFmi might be playing a critical role in cell fate specification through regulating localization/stabilization of Dsh at the animal pole leading to the selective activation of Wnt/beta-catenin signaling in animal pole blastomeres. Similarly, it may be required for the asymmetric localization of NvStbm to the animal pole, which in turn is required for the Wnt/PCP signaling mediated primary archenteron invagination. To test this hypothesis, I examined the functional role of NvFmi in both Wnt/beta-catenin and Wnt/PCP signaling during early development of *Nematostella*. This is the first study of Fmi function in a non-bilaterian metazoan and the results may provide valuable insights into the evolutionary origins of a functional archenteron in metazoans.
MATERIALS AND METHODS

Antibody production

To determine the NvFmi protein expression pattern, affinity-purified *Nematostella* anti-Fmi (anti-NvFmi) polyclonal rabbit antibodies were made against a selected amino acid region (NH2-AKYYPGNPELELARK - COOH) of the NvFmi protein (Bethyl Labs, TX).

Immunostaining

Eggs and embryos at different stages of development were fixed as described in Kumburegama et al. (2008). Fixed embryos were blocked using 5% normal donkey serum, 0.05% Tween-20 in PBS for 1 hour at room temperature. The embryos were then incubated in anti-NvFmi rabbit polyclonal antibodies (1:100), diluted in blocking buffer at 4°C overnight. After rinsing in blocking buffer [5% normal donkey serum, 0.05% Tween-20 in PBS (10mM sodium phosphate, 150 mM sodium chloride, pH 7.8)] embryos were incubated with AlexaFluor 568 donkey anti-rabbit (1:250) secondary antibodies for 1 hour at room temperature. After washing the samples 3X in blocking buffer, and once with PBS the eggs and embryos were dehydrated in isopropanol and cleared in BA:BB (1:2 Benzyl Alcohol: Benzyl benzoate). Embryos were examined under a Zeiss Axiovert 200 inverted microscope equipped with epifluorescence or a Leica SP5 scanning confocal microscope.
Anti-sense morpholino oligonucleotide microinjections

Anti-sense morpholino oligonucleotides to NvFmi (NvFmi-MO) to block the splicing of the endogenous transcript, resulting in a truncated protein and control morpholinos (Control-MO) (Gene Tools, Philomath, OR) were made. The 3 mM stock solutions of anti-sense NvFmi-MO (5’ CCTCTTACCTCAGTTACAATTTATA 3’) and Control-MO (5’ CCTCTTACCTCAGTTACAATTTATA 3’) were diluted to 1.0, 0.75 and 0.50 µM and the diluted morpholinos were injected into fertilized eggs. Embryos were allowed to develop and collected and either fixed for confocal imaging or for whole mount in situ hybridization.

DNA constructs

NvFmiC::GFP was constructed by PCR-amplifying an NvFmi fragment that lacked the extracellular domain and the trans-membrane domains of the protein using two gene specific primers (5’ ATGCAGGCCCTCCGCTCG 3’ and 5’ GTGCCGCTGCTGCCTTCTC 3’) from cDNA prepared from gastrula stage embryos. The fragment was subcloned into pCS2 + GFP to make the NvFmiC::GFP construct using standard molecular biology procedures.

mRNA synthesis and microinjection

Linearized constructs of NvFmiC::GFP, Nvβ-catenin::GFP that were cloned into pCS2 + vector were used as templates for SP6-dependent RNA transcription using the mMessage mMachine Kit (Ambion, Austin, TX). NvFmiC::GFP mRNA was injected at a final concentration of 0.30 µg/µl and 0.50 µg/µl in 40% glycerol. Co-injection of
NvFmi-MO, Nγβ-catenin::RFP and fluorescent dextran was carried out at a final concentration of 1 mM/µl, 0.3 µg/µl, and 0.2 µg/µl respectively in 40% glycerol. Embryos were allowed to develop in 1/3\textsuperscript{rd} seawater. Digital images of the live embryos were taken using a Nikon Coolpix 990 digital camera mounted on a Zeiss Axiovert 200 inverted microscope equipped with epifluorescence optics.

**Whole mount RNA in situ hybridization**

Embryos were fixed in 0.2% glutaraldehyde:3.7% formaldehyde in 1/3\textsuperscript{rd} seawater for 2 minutes, post-fixed in 3.7% formaldehyde in 1/3\textsuperscript{rd} sea water for 1 hour at room temperature, washed 5X in PTw, 1X in nuclease-free water, and stored in 100% methanol at -20˚ C until used. Whole mount RNA in situ hybridization of *N. vectensis* eggs and embryos was carried out as previously described (Martindale et al., 2004). Digoxigenin-labeled RNA probes were synthesized using the MegaScript Transcription Kit (Ambion, Austin, TX). Hybridization of the DIG-labeled RNA probe (1 ng/µl) was carried out at 60-65˚ C. Visualization of the labeled probe was performed using NBT/BCIP as substrate for the alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Bioscience, Indianapolis, IN). Embryos were observed under a Zeiss Axiovert 200 inverted microscope equipped with epifluorescence optics, and digital images were recorded using a Nikon CoolPix 990 digital camera.
RESULTS

*NvFmi* is an asymmetrically expressed maternal protein in *Nematostella* eggs and embryos

The spatial and temporal expression pattern of *NvFmi* mRNA was highly suggestive of a functional role for *NvFmi* during gastrulation morphogenesis and cell fate specification in *Nematostella* (Chapter 2; Fig 2.6). However, to test whether this localized expression pattern at the mRNA level was also reflected at the protein level, the subcellular localization of the NvFmi protein was examined using affinity-purified anti-NvFmi rabbit polyclonal antibodies. Immunostaining of *Nematostella* eggs and embryos using this polyclonal antibody confirmed that the NvFmi protein has an asymmetric expression pattern, which is congruent with *NvFmi* transcript expression. At the unfertilized and fertilized eggs stages, NvFmi is asymmetrically localized to one pole of the embryo and this pattern was maintained through cleavage and blastula stages (Figure 4.1 A-C). At the early gastrula stage, NvFmi is localized to the apical region of the cells at the blastopore (Figure 4.1 D). This expression pattern of NvFmi closely resembles the expression patterns of two other core PCP pathway proteins, NvStbm and NvDsh. However, one striking difference between NvFmi and NvStbm expression is seen at the unfertilized egg stage where like the NvStbm transcript, NvStbm protein has a broader expression domain compared to the more restricted expression of NvFmi (Figure 4.1 A). Moreover, while the NvDsh protein is enriched at the animal pole in unfertilized eggs, the *NvDsh* mRNA is uniformly expressed in eggs and early embryos (Lee et al., 2007).
NvFmi knockdown blocks primary archenteron invagination in *Nematostella*

To test whether NvFmi has a functional role in primary archenteron invagination during gastrulation in *Nematostella*, a splice-blocking NvFmi antisense morpholino (NvFmi-MO) was used to knock down NvFmi protein levels. The efficacy of the NvFmi-MO in knocking down NvFmi protein levels was confirmed by examining NvFmi protein expression in Control-MO and NvFmi-MO injected embryos at the late blastula stage using scanning confocal microscopy. The results showed that while the Control-MO injected embryos showed strong NvFmi protein expression (Figure 4.3 A), most of the NvFmi-MO injected embryos did not show any NvFmi expression (Figure 4.3 B).

To examine the effect of NvFmi knock down on the development of *Nematostella* embryos, fertilized eggs injected with three different concentrations of NvFmi-MO (0.5 mM, 0.75 mM and 1.0 mM) and Control-MO (1.0 mM) were collected at the mid gastrula stage (16-18 hpf) and fixed for phenotyping. Careful analysis of NvFmi-MO injected embryos using scanning confocal microscopy showed that knock down of NvFmi protein resulted in a disruption of normal primary archenteron invagination in morphant embryos. Compared to the Control-MO injected embryos (Figure 4.2 A), embryos injected with a lower concentration of NvFmi (0.5 mM) lacked an organized single layered epithelial endoderm despite the movement of cells into the blastocoel (Figure 4.2 B). Also, cells that moved into the blastocoel showed no coordinated movements and appeared to have lost their apical-basal polarity compared to the columnar shaped invaginating cells of normal embryos (Figure 4.2 B). These defects in shape and movement indicate that these cells have possible polarity defects. At the same time, the NvFmi-MO injected embryos showed defects in cilia organization, which might
be a further indication of defects in cell/tissue polarity (Fig. 4.2. At higher NvFmi-MO concentrations (0.75 mM and 1 mM) the knockdown of NvFmi completely blocked primary archenteron invagination in morphant embryos (Figure 4.2 C). These results demonstrated that reduced levels of NvFmi protein disrupted primary archenteron invagination in *Nematostella*.

**NvFmi knockdown affects beta-catenin nuclearization and endoderm cell fate specification**

The phenotype resulting from the knock down of NvFmi is similar to the inhibition of primary archenteron invagination resulting from NvStbm knock down. However, based on just the phenotype, it was not clear whether reduced levels of NvFmi affected Wnt/beta-catenin signaling and cell fate specification in *Nematostella*. To examine possible effects of NvFmi knock down on beta-catenin signaling, I analyzed embryos co-injected with *Nvbeta-catenin::RFP* mRNA (0.3 µg/ul) and NvFmi-MO or Control-MO (1.0 mM) for the presence of nuclear beta-catenin in the blastomeres at the animal pole. The injected embryos were analyzed at 6-8 hpf using fluorescence microscopy to detect the expression of the Nvbeta-catenin::RFP fusion protein. Compared to the control morpholino injected embryos in which nuclear Nvbeta-catenin::RFP expression was detected in blastomeres at one pole of the developing embryos (Figure 4.4 B), the NvFmi-MO injected embryos showed no detectable nuclear Nvbeta-catenin::RFP expression (Figure 4.4 D). These results suggest that NvFmi is required for the normal activation of Wnt/Beta-catenin signaling through nuclearization.
of beta-catenin in these embryos. Consistent with this observation, whole mount in situ hybridization for several endodermal markers revealed that compared to Control-MO injected embryos (Figure 4.5 A), the majority NvFmi-MO injected embryos showed no expression of the endodermal marker Snail (Figure 4.5 B), indicating failure of normal cell fate specification. These results indicated that knock down of NvFmi resulted in defects in both primary archenteron invagination and cell fate specification, suggesting that NvFmi might be an upstream regulator of both Wnt/beta-catenin signaling and Wnt/PCP signaling.

**NvFmi is required for the selective stabilization of core PCP proteins NvDishevelled and NvStbm at the animal pole**

Given the evidence for NvFmi function in both cell fate specification and primary archenteron invagination, it raises the question of how NvFmi is able to regulate both these processes in a coordinated fashion in time and space in developing embryos. Work done in flies and zebrafish has shown that Fmi is required for the asymmetric membrane localization of other core PCP pathway proteins, mainly, Dsh, Stbm and Fz (Chen et al., 2008; Shimada et al., 2001). In *Nematostella*, NvDsh is localized to the animal pole of both eggs and embryos throughout early development and disruption of this asymmetric localization of NvDsh results in defects of cell fate specification (Lee et al., 2007). This raised the possibility that NvFmi might be regulating beta-catenin mediated cell fate specification through the asymmetric localization of NvDsh to the animal pole.
To test this possibility, embryos injected with either NvFmi-MO or Control-MO and were collected at 4-6 hpf and were immunostained for NvDsh protein. The stained embryos were analyzed using scanning confocal microscopy to identify if NvFmi knockdown affected NvDsh localization to the animal pole. The results showed that the majority of the NvFmi morphants showed a disruption in NvDsh localization to the animal pole (Figure 4.6 B), compared to Cont-MO injected embryos (Figure 4.6 A). These data support a role for NvFmi in regulating beta-catenin mediated cell fate specification through the asymmetric localization of NvDsh to the blastomeres at the animal pole.

During early development of *Nematostella*, NvStbm protein is tightly localized to the animal pole blastomeres and is required for normal primary archenteron invagination through apical constriction of cells at the endodermal plate (Kumburegama et al., 2011). The inhibition of primary archenteron invagination resulting from the knock down of NvFmi protein levels in *Nematostella* closely resembles the morphogenesis phenotype resulting from reduced levels of NvStbm protein. Given the role Fmi plays in flies and vertebrates in the asymmetric localization of Stbm, these observations raised the possibility that NvFmi might be regulating apical constriction of endodermal plate cells and primary archenteron invagination through the localization of NvStbm to the apical end of animal pole blastomeres. This hypothesis was tested using two different experiments. In the first experiment zygotes were co-injected with either Fmi-MO or Control-MO in combination with *NvStbm::RFP* mRNA. The embryos were collected 2–4 hpf and the expression of NvStbm::RFP protein was analyzed using fluorescence microscopy. In the Control-MO injected embryos, NvStbm::RFP protein showed
asymmetric localization to one half of the embryo (Figure 4.7B) whereas in the NvFmi-MO injected embryos, NvStbm::RFP protein was expressed uniformly throughout the embryo (Figure 4.7D). In a separate experiment, zygotes were injected with either NvFmi-MO or Control-MO and the developing embryos were collected at 4–6 hpf and immunostained for NvStbm to determine the endogenous protein expression pattern. Analysis of the immunostained embryos revealed that most of the NvFmi-MO injected embryos showed no NvStbm protein expression (Figure 4.8B) compared to the Control-MO injected embryos that had endogenous NvStbm protein localized to one pole of the embryo (Figure 4.8A). These results support the idea that NvFmi regulates apical constriction of endodermal plate cells and primary archenteron invagination in *Nematostella* through the asymmetric localization of the NvStbm protein.

**Overexpression of NvFmi C-terminal domain disrupts Wnt/beta-catenin signaling mediated cell fate specification in *Nematostella***

Fmi is a relatively large, seven pass transmembrane protein with multiple, well defined extracellular domains and a C-terminal cytoplasmic tail (Usui et al., 1999). Compared to the extracellular region of the protein, the C-terminal cytoplasmic region is less well conserved between taxa and recent work in zebrafish has identified a single region of the intracellular domain of Fmi that is highly conserved between vertebrates and invertebrates (Carreira-Barbosa et al., 2009). This conserved SE/D domain in the C-terminal region of Fmi is required for the Fz induced membrane localization of Dsh, which leads to modulation of PCP activity during convergence and extension during
zebrafish gastrulation (Carreira-Barbosa et al., 2009). Sequence analyses comparing the C-terminal domain of Fmi from various taxa revealed the presence of the conserved SE/D domain in NvFmi (Figure 4.9 A), raising the question whether NvFmi function in beta-catenin mediated cell fate specification in *Nematostella* is mediated through the conserved SE/D domain of the protein. To determine the functional importance of the C-terminal domain, I overexpressed a truncated form of NvFmi, which included only the C-terminal region of the protein (Figure 4.9 B). I predicted that the truncated protein would compete with the endogenous protein to inhibit signaling through the C-terminal domain through a dominant-negative mechanism. The gene sequence that codes for the C-terminal domain of the NvFmi protein was cloned into the pCS2\(^+\)::GFP vector and this construct was used to synthesize the *NvFmiC::GFP* mRNA. Zygotes were injected with either *in vitro* synthesized *NvFmiC::GFP* mRNA or *GFP* mRNA. The injected embryos were collected at 16–18 hpf and stained with phalloidin and sytox green and then examined using scanning confocal microscopy for defects in normal development. Even though both the NvFmiC::GFP and GFP overexpressing embryos were able to undergo primary archenteron invagination, only the GFP overexpressing embryos were able to form a normal gut epithelium (Figure 4.10 A). The NvFmiC::GFP overexpressing embryos failed to form an epithelial gut and the cells that moved into the blastocoel through invagination appeared to be disintegrating, resulting in a compacted blastocoel cavity (Figure 4.10 B). Since this phenotype was similar to that resulting from blocking Wnt/beta-catenin signaling in *Nematostella*, the expression of endodermal and ectodermal markers were tested using whole mount in situ hybridization to detect any deficiencies in endodermal cell fate specification in the morphant embryos. Most of the
NvFmiC::GFP overexpressing embryos showed no expression of the endodermal marker *NvSnail* (Figure 4.11 B) compared to the GFP over-expressing embryos which had normal *NvSnail* expression (Figure 4.11 A). These results indicate a negative effect on Wnt/beta-catenin signaling when the C-terminal truncated form of NvFmi was over expressed, suggesting a possible function for the C-terminal domain of the NvFmi protein in regulating Wnt/beta-catenin signaling in *Nematostella*.

**DISCUSSION**

Results from this study show that in *Nematostella* the core PCP protein NvFmi is required for both Wnt/beta-catenin signaling mediated cell fate specification and Wnt/PCP signaling mediated primary archenteron invagination. This study also provides strong evidence to support the hypothesis that NvFmi regulates both morphogenesis and specification processes through the asymmetric localization of upstream components of the two signaling cascades to the animal pole of *Nematostella* eggs and embryos. Overall, these results support a model for the evolution of gastrulation in the last common ancestor to the eumetazoa where Fmi acted as a scaffold to localize Wnt pathway components to the animal pole to asymmetrically activate the two Wnt signaling pathways.

**NvFmi and regulation of primary archenteron invagination and germ layer specification in *Nematostella***

While primary archenteron invagination and germ layer specification is tightly coupled in bilaterians, these two events can be experimentally uncoupled during
*Nematostella* gastrulation (Kumburegama et al., 2011). Blocking Wnt/beta-catenin signaling in *Nematostella* using multiple reagents blocks germ layer specification but has no effect on primary archenteron invagination, while blocking primary archenteron invagination by knocking down NvStbm had no effect on germ layer specification (Kumburegama et al., 2011). However, during normal development of *Nematostella* embryos, these two processes must be coordinated temporally and spatially, and the exact mechanisms by which this coordination is brought about is not yet clear. However, one plausible scenario is the presence of an upstream regulator that provides inputs to both Wnt/beta-catenin and Wnt/PCP signaling to regulate cell fate specification and primary archenteron invagination during early development in the *Nematostella* embryo.

Several lines of evidence presented in this study support the idea that NvFmi coordinates Wnt/beta-catenin and Wnt/PCP signaling during endodermal cell fate specification and primary archenteron invagination respectively during gastrulation in *Nematostella*. Maternally expressed NvFmi is localized to the animal pole from the unfertilized egg stage and it is tightly localized to animal pole blastomeres at gastrulation. Knock down of NvFmi protein in developing embryos blocks endodermal cell fate specification and these embryos do not form bottle cells or undergo primary archenteron invagination. Fmi has been implicated in regulating cell polarity in both invertebrates (Chen et al., 2008; Usui et al., 1999) and vertebrates (Curtin et al., 2003) and CE movements during gastrulation in vertebrates (Carreira-Barbosa et al., 2009). How Fmi is regulating cell polarity during these processes is not well understood but there is increasing evidence to suggest that Fmi plays a critical role in the asymmetric localization of other core PCP proteins such as Dsh, Stbm, Fz and Pk (Chen et al., 2008;
Shimada et al., 2001; Usui et al., 1999). Dsh is a critical cytoplasmic component of both Wnt/beta-catenin signaling and Wnt/PCP signaling and during Nematostella development. Dsh protein is localized to the animal pole and is required for both Wnt/beta-catenin signaling (Lee et al., 2007) and Wnt/PCP signaling (Kumburegama, 2009). In zebrafish, Fmi has been implicated in the Fz mediated membrane localization of Dsh, which is required for normal CE during gastrulation (Carreira-Barbosa et al., 2009). Knock down of NvFmi protein levels in Nematostella results in the loss of asymmetric localization of NvDsh to the animal pole suggesting that NvFmi is required for the activation of Wnt-beta-catenin signaling at the animal pole through the asymmetric localization of NvDsh. On the other hand, reduced levels of NvStbm protein in Nematostella blocks bottle cell formation and primary archenteron invagination (Kumburegama et al., 2011). NvFmi knock down embryos showed no localized NvStbm at the animal pole indicating that NvFmi is required for the asymmetric localization of NvStbm. To the best of my knowledge, this is the only instance where Fmi has been implicated in Wnt/beta-catenin signaling and cell fate specification in metazoans and it is the only upstream regulator in Nematostella that has been shown to regulate both Wnt/beta-catenin and Wnt/PCP signaling during gastrulation. In summary, these findings may provide unique insight into the evolution of morphogenesis mediating archenteron formation and cell fate specification in the metazoa. However, these observations raise some important new questions about the mechanisms through which NvFmi is regulating these two processes, which would have to be addressed in future studies.
Mechanisms of NvFmi function during gastrulation in Nematostella

The only conserved domain in the C-terminal region of Fmi, the SE/D domain has been implicated in its critical interaction with the Fz/Dsh complex in regulating CE during zebrafish gastrulation (Carreira-Barbosa et al., 2009). In Nematostella, over-expression of a truncated form of NvFmi, which only had the C-terminal region of the protein, results in the inhibition of cell fate specification in these embryos, but they show no defects in primary archenteron invagination. It is not yet clear how this effect on cell fate specification is brought about but it raises new questions as to why the overexpression of the truncated protein has no effect on primary archenteron invagination. These results implicate the C-terminal region of NvFmi in regulating cell fate specification but the lack of any detectable defects in primary archenteron invagination in these embryos suggests that NvFmi might be regulating Wnt/PCP signaling through a different domain of the protein and a different mechanism. Disruption in asymmetric localization of NvDsh and NvStbm when NvFmi protein levels are knocked down is indicative of the importance of other core PCP proteins in the regulation of both Wnt signaling cascades by NvFmi. Investigating the localization patterns of NvDsh and NvStbm in embryos overexpressing the C-terminal region of NvFmi would provide further insights into the mechanisms by which NvFmi is regulating the localization of other core PCP proteins. While there is no evidence for direct interactions between Fmi and Dsh or Stbm, interactions between Fmi and other PCP proteins such as Fz has been reported in Drosophila (Chen et al., 2008). Therefore, regulation of NvDsh and NvStbm localization by NvFmi might involve other core PCP proteins and thus the importance of interactions between different core PCP pathway proteins cannot be over
looked in attempting to better understand the mechanisms of NvFmi function in *Nematostella*.

**Localization of Wnt pathway components to the animal pole and the evolution of gastrulation**

There is increasing evidence showing that there is an enrichment of Wnt pathway components at the animal pole of cnidarian eggs (Lee et al., 2007; Kumburegama et al., 2011; Momose and Houliston, 2007; Momose et al., 2008). This accumulation of Wnt pathway components at the animal pole results in the asymmetric activation of Wnt signaling at the animal pole of the embryo leading to primary archenteron invagination and cell fate specification. This work provides the first evidence for an upstream regulator of both Wnt/beta-catenin signaling and Wnt/PCP signaling at the animal pole. NvFmi regulates Wnt/beta-catenin signaling mediated cell fate specification and Wnt/PCP mediated primary archenteron invagination in time and space during early development of *Nematostella*. This study also provides insights into the possible mechanisms through which NvFmi might be regulating these two processes through the asymmetric localization of other Wnt pathway components. These results provide a much needed basis for future studies aimed at shedding more light on to the definitive interactions that leads to this asymmetric localization of core Wnt pathway components to the animal pole of cnidarian embryos and its evolutionary implications for the formation of a functional gut.

The function of Fmi as a scaffold to hold important components of Wnt signaling such as Dsh and Stbm at one pole of the embryo also provides valuable insights into the
evolution of germ layer segregation and gastrulation in metazoans. As was discussed earlier, the most striking change in the evolution of bilaterians from non-bilaterian ancestors is the change in the site of gastrulation and the origin of endomesodermal tissue (Martindale and Hejnol, 2009). In order for such a change in the site of gastrulation and endomesoderm specification to take place in the lineage leading up to bilaterians, the Wnt pathway components which were localized to the animal pole in non-bilaterians such as cnidarians would have to be moved to the vegetal pole in the bilaterians. Evidence for such a switch at the molecular level can be seen in the localization of Dsh in echinoids. In sea urchins, Dsh protein is highly enriched at the vegetal cortex and is required for Wnt/beta-catenin mediated endomesoderm specification at the vegetal pole (Weitzel et al., 2004). If Fmi were functioning as a scaffolding protein to hold Wnt pathway components at the animal pole in non-bilaterians such as Nematostella, moving the scaffolding protein from the animal pole to the vegetal pole in bilaterians would result in the relocation of all the Wnt pathway components that are associated with that scaffolding protein to the vegetal pole. Presence of such a scaffolding protein would make the switch in the site of gastrulation from non-bilaterians to bilaterians more plausible.

A recent study has shown that the nervous system of the cnidarian Nematostella is composed of sensory neurons of ectodermal origin and ganglion cells (neurons that provide connections in the nerve net) of endodermal origin (Marlow et al., 2009). Also, the largest concentration of different neuronal types in the cnidarian polyps is found associated with the pharyngeal nerve ring and it has been shown that these neurons are endodermally derived. These findings suggest that the oral neuronal structures in the non-
bilaterian cnidarians are homologous to the anterior neural structures of most bilaterians (Martindale & Hejnol, 2009). Unfortunately, there are no detailed studies of the ctenophore nervous system similar to the one carried out in cnidarians yet. However, a switch in the site of gastrulation during the evolution of bilaterians from non-bilaterian taxa would have separated the endomesodermal gene regulatory network from the neural gene network at the animal pole. This might have paved the way for the diversification and the specialization of the bilaterian nervous system leading to the evolution of cephalization and a central nervous system.
FIGURES

**Figure 4.1:** NvFmi localization in egg and embryos. NvFmi protein is tightly localized to one pole of the egg and blastula stages (A-B) leading up to the early gastrula stage where the protein is localized to the animal pole (C).

**Figure 4.2:** Knockdown of NvFmi protein using morpholino oligonucleotides confirms the importance of this protein for morphogenesis during development in *Nemastostella*. Compared to the control morpholino injected embryo (A) embryos injected with lower concentrations of NvFmi-MO showed defects in gastrulation movements and polarity defects in migrating cells (B). At high concentrations, injection of NvFmi-MO completely blocked primary archenteron invagination (C). The number of embryos showing the different phenotypes in the different treatments is shown in the graph (D).
Figure 4.3: Knockdown of NvFmi using morpholinos. Immunostaining of NvFmi-MO injected embryos with anti NvFmi antibody confirms the knockdown of Fmi in morphant embryos (B) by the lack of Fmi staining compared to the control morpholino injected embryos (A).

Figure 4.4: NvFmi is required for beta-catenin stabilization and nuclearization at the animal pole in Nematostella. Compared to the control morpholino injected embryos (A and B) where nuclear NvBeta-catenin::RFP expression was detected in one pole of the developing embryos, the NvFmi-MO injected embryos (C and D) showed no detectable NvBeta-catenin::RFP expression.
Figure 4.5: NvFmi is required for endodermal cell-fate specification. Compared to the control morpholino injected embryos (A) where there is endodermal *NvSnail* expression, the NvFmi-MO injected embryos showed no detectable *NvSnail* expression.

Figure 4.6: NvFmi is required for the asymmetric localization of NvDsh to the animal pole of the developing embryo. Compared to the control morpholino injected embryos (A) where there is asymmetric localization of NvDsh, in NvFmi-MO injected embryos NvDsh showed no asymmetric localization (B).
**Figure 4.7:** NvFmi is required for the asymmetric localization of NvStbm to the animal pole of the developing embryo. In control-MO injected embryos, NvStbm::RFP protein showed asymmetric localization to one half of the embryo (B), whereas in the NvFmi-MO injected embryos, NvStbm::RFP protein was expressed uniformly throughout the embryo (D).

**Figure 4.8:** NvFmi is required for the asymmetric localization of NvStbm to the animal pole of the developing embryo. Compared to the control morpholino injected embryos (A) where there is asymmetric localization of NvStbm, in NvFmi-MO injected embryos NvStbm showed no asymmetric localization (B).
Figure 4.9: Alignment of NvFmi C-terminal domain with C-terminal domains of Fmi homologs from four representative metazoan taxa. The alignment was generated using ClustalW. Conserved amino acid residues across all taxa are shown in red. The region highlighted in grey indicates the conserved SE/D domain (A). Domain structure of NvFmi and the dominant-negative construct with the C-terminal region linked to GFP. The conserved SE/D domain is shown in blue (B).
Figure 4.10: Over-expression of NvFmiC::GFP affects normal gastrulation and cell fate specification during early development in *Nematostella*. Compared to the *GFP* overexpressing control embryos (A) over-expression of NvFmiC::GFP results in the embryos not forming an organized gut epithelium, despite undergoing primary archenteron invagination (B). The number of embryos showing the different phenotypes in the different treatments is shown in the graph (C).
Figure 4.11: Overexpression of NvFmiC::GFP blocks expression of Snail. Compared to GFP over-expressing control embryos (A) where there is normal endodermal NvSnail expression, the NvFmiC::GFP mRNA injected embryos showed no detectable NvSnail expression.
Chapter 5: Wnt signaling, gastrulation and metazoan evolution

Gastrulation in Cnidarians

Simple multicellularity has evolved multiple times during the evolution of life on earth, but in contrast, complex multicellular organisms have evolved only six times in five different eukaryotic lineages (Knoll, 2011). Even within these limited number of complex multicellular lineages, it is only in the metazoans or multicellular animals that you see the greatest diversity of body plans and life history strategies among any eukaryotic lineage. There is strong empirical evidence to support the evolution of metazoans from a choanoflagellate like unicellular ancestor, through a radially symmetrical multicellular stage (King, 2004). The evolution of complex body plans in the metazoans from a simple, radially symmetrical multicellular stage is believed to be the result of the process of gastrulation (Martindale, 2005; Martindale and Hejnol, 2009; Kumburegama et al., 2011). Despite this morphogenetic process being the driving force behind the diversification of the metazoa, relatively little is known about the evolution of mechanisms regulating gastrulation (Martindale, 2005; Wilmer, 1990). Gastrulation mechanisms in basal metazoan taxa that diverged early in metazoan evolution would provide valuable insights into the evolutionary origins of gastrulation. Unfortunately, there are only a few basal taxa that can be considered in such investigations and these include the placozoans, the poriferans, the ctenophores and the cnidarians. Placozoans are represented by only one species worldwide and obtaining embryos from these organisms is extremely difficult (Srivastava et al., 2008). The sponges on the other hand, do not have germ layer segregation and gut formation during morphogenesis (reviewed in
Ereskovsky, 2010 and Martindale and Hejnol, 2009). Therefore, the only two basal
groups that can be used in comparative studies are the ctenophores and the cnidarians.
The cnidarians are the sister group to all bilaterally symmetrical animals (Dunn et al.,
2008; Phillipe et al., 2009) and is an ideal system for looking at the evolution of
gastrulation mechanisms.

Cnidarians have a diverse array of gastrulation mechanisms. These include unipolar and
multipolar ingression, epiboly, invagination and in some cases, a combination of these
different mechanisms (reviewed in Byrum and Martindale, 2004). The presence of
different forms of gastrulation even within the cnidarians makes it important to look at
the ancestral form of gastrulation within the Cnidaria.

In the class Anthozoa, which is considered to be the basal group within the
phylum (Finnerty et al., 2004), there is less diversity in gastrulation mechanisms and
most anthozoans gastrulate through invagination. Although it was believed that
gastrulation takes place via a combination of invagination and ingression movements in
*Nematostella vectensis* (Byrum and Martindale, 2004; Kraus and Technau, 2006), it has
been later shown that *Nematostella* gastrulates only through invagination (Kumburegama
et al., 2011; Magie et al., 2007). Gastrulation is initiated by cell shape changes in the
cells at the endodermal margins. The apical constriction in these cells results in the
bending of the endodermal plate at the animal pole initiating invagination (Magie et al.,
2007). At the same time the cells of the presumptive endoderm are internalized through
invagination, these cells gets specified as endoderm resulting the segregation of germ
layers, producing a diploblastic embryo with an outer ectoderm and an inner endoderm.
Uncoupling of primary archenteron invagination and endoderm specification

In bilaterians, where gastrulation has been studied extensively, the two processes of archenteron formation or internalization of the presumptive endomesoderm cells and the specification of these cells as endomesoderm are tightly linked (Heisenberg and Solinica-Krezel, 2008; Leptin, 2005; Rodaway and Patient, 2001). However, this work has contributed to showing that these two processes can be experimentally uncoupled during gastrulation in *Nematostella* by selective inhibition of either Wnt/beta-catenin pathway components or Wnt/PCP pathway components (Kumburegama et al., 2011). This striking difference in gastrulation mechanisms between bilaterians and the basal non-bilaterian *Nematostella* suggests that during the evolution of gastrulation, primary archenteron invagination could have occurred in the absence of cell fate specification in the basal non-bilaterians and leading up to the bilaterian lineage the two processes had become tightly linked. This coupling of primary archenteron invagination and cell fate specification in more derived taxa might be the result of incorporation of novel mechanisms of gastrulation morphogenesis. One example for this comes from the more derived hydrozoan cnidarian *Clytia hemisphaerica*, where gastrulation morphogenesis takes place through ingression. Ingression involves the detachment of cells from the blastula epithelium prior to their movement into the blastocoel (Leptin, 2005). This detachment of cells from an epithelium requires an epithelial to mesenchymal transition (EMT) and this process is regulated by the protein Snail (Shook and Keller, 2003). Snail is an endodermal marker whose transcription requires the cell to be specified as endoderm. Therefore, without cell fate specification, Snail would not be expressed, resulting in an inhibition of EMT and hence blocking ingestion of cells of the
presumptive archenteron. In Clytia, when endoderm specification is blocked through the inhibition of Wnt/beta-catenin signaling, the ingress of cells into the blastocoel is also blocked resulting in the inhibition of gastrulation morphogenesis (Momose and Houlston, 2007; Momose et al., 2008). This further suggests that the ancestral mechanism of gastrulation morphogenesis in metazoans was invagination. Since invagination does not require the function of endomesoderm specific proteins such as Snail, primary archenteron invagination could have evolved in the absence of cell fate specification.

**Wnt signaling and gastrulation**

Wnt/β-catenin signaling has been shown to be involved in regulating cell fate specification during gastrulation in both bilaterians and non-bilaterians (Darras et al., 2011; Henry et al., 2008; Henry et al., 2010; Logan et al., 1999; Momose et al., 2008; Wikramanayake et al., 1998; Wikramanayake et al., 2003). Also, recent work done in vertebrates such as Xenopus and zebrafish has shown that signaling though the Wnt/PCP pathway is important in regulating gastrulation morphogenesis through processes such as convergence and extension (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). These findings indicate the involvement of two different branches of the Wnt signaling pathways the Wnt/beta-catenin pathway and the Wnt/PCP pathway in regulating cell fate specification and morphogenesis during gastrulation respectively. Also there is evidence for the inhibition of one branch of Wnt signaling by the other during most signaling events that involve function of Wnt signaling (Park and Moon, 2002). This work provides further evidence for the involvement of Wnt/PCP signaling in regulating gastrulation morphogenesis through primary archenteron invagination in
*Nematostella* but there is no evidence to suggest that there are any inhibitory interactions between Wnt/beta-catenin signaling and Wnt/PCP signaling. During *Nematostella* gastrulation, Wnt/PCP signaling regulates primary archenteron invagination and Wnt/beta-catenin signaling regulates cell fate specification in the same cells at the animal pole indicating a tight coordination of these two signaling events during early development. One interesting difference in the molecules involved in these signaling events in *Nematostella* might provide a clue to the lack of inhibitory interactions between the two pathways. In flies during planar polarization of the wing epithelium, two different groups of proteins form complexes in the proximal and the distal ends of the cells (Zallen, 2007). The complex at the proximal end includes Flamingo (Fmi), Strabismus (Stbm) and Prickle (Pk) whereas the complex at the distal end includes Fmi, Fz and Dsh. These two complexes inhibit each other in the same cell resulting in the asymmetric localization of the two complexes (Zallen, 2007). However, Fmi is critical for the asymmetric localization of both these complexes (Shimada et al., 2001 Usui et al., 1999). One key molecule for the inhibitory interaction between the two complexes is Pk, which is found in the proximal complex in fly wings. During early gastrulation in *Nematostella* Fmi is localized to the animal pole of the embryo and is required for the asymmetric localization of both Dsh and Stbm to the animal pole. However, *NvPk* is not expressed during early development in *Nematostella* thus removing the potentially inhibitory interaction between the Fmi/Dsh and Fmi/Stbm complexes. Therefore, these observations in *Nematostella* provide support for the evolution of the different branches of Wnt signaling in the same cell without any inhibitory interactions. The later involvement of new components and the diversification of function of these different signaling cascades
might have resulted in the separation of these branches of Wnt signaling in time and space, possibly, through inhibitory interactions.

**Localization of Wnt signaling components and the evolution of gastrulation**

As discussed earlier, Wnt/PCP signaling mediated primary archenteron invagination and Wnt/beta-catenin mediated cell fate specification can be experimentally uncoupled in *Nematostella*. This separation of primary archenteron invagination and cell fate specification was first observed through the inhibition of either Stbm function in Wnt/PCP signaling or the inhibition of Dsh function in Wnt/beta-catenin signaling (Kumburegama et al., 2011). Work done in characterizing the function of Fz receptors during *Nematostella* gastrulation has further confirmed this separation where Fz1 is functioning to regulate cell fate specification while Fz10 is required for primary archenteron invagination. However, both these processes take place at the animal pole and there is evidence to show that there is enrichment of Wnt pathway components to the animal pole of cnidarian eggs (Kumburegama et al., 2011; Lee et al., 2007; Momose et al., 2008; Momose and Houliston 2007). The mechanisms of how these Wnt pathway components are asymmetrically localized to the animal pole of eggs and embryos and how primary archenteron invagination and cell fate specification are coordinated in these animal pole blastomeres was not well understood. This work on the characterization of Fmi function in *Nematostella* has provided insights into how both these processes might be aided by Fmi. Fmi is required for the asymmetric localization of both Dsh and Stbm to the animal pole during early development of *Nematostella*. Therefore, Fmi might be playing a key role as a scaffolding protein to localize other components of the Wnt
pathways to the animal pole of *Nematostella* eggs and embryos. By doing so, Fmi is also providing the regulatory input necessary for the coordination of Wnt/PCP mediated primary archenteron invagination and Wnt/beta-catenin mediated cell fate specification.

**Primary egg axis and the adult axial properties of metazoans**

One striking difference between Wnt/beta-catenin mediated cell fate specification between bilaterians and non-bilaterians is seen at the site of nuclear beta-catenin, which indicates the activation of the pathway. In bilaterians, beta-catenin is nuclearized in the blastomeres at the vegetal pole (Darras et al., 2011; Henry et al., 2008; Imai et al., 2000; Logan et al., 1999; Wikramanayake et al., 1998) as opposed to nuclearization of beta-catenin in the blastomeres at the animal pole in non-bilaterians (Momose et al., 2008; Wikramanayake et al., 2003). This observation raises the possibility that Wnt/beta-catenin mediated cell fate specification and gastrulation evolved at the animal pole in the ancestor to all metazoans and in the lineage leading up to bilaterians the site of gastrulation and cell fate specification switched to the vegetal pole (Kumburegama et al., 2011; Martindale and Hejnol, 2009).

In the non-bilaterians, the ctenophores and cnidarians, the first cleavage furrow forms at the animal pole and this marks the site of the future oral pole and endodermal tissue forms from the animal pole derived blastomeres (Freeman, 1980; Freeman 1981; Riverberi, 1971). However, it has been shown experimentally that in cnidarians and ctenophores, moving the zygotic nucleus, which is found at the animal pole, to an ectopic site, a new oral-aboral axis is specified with a new site of first cleavage (Freeman, 1981). This shows that unlike in most bilaterians, in which the definitive embryonic and
organismal axial properties are established maternally in an irreversible fashion (Goldstein and Freeman, 1997), in ctenophores and cnidarians, the relationship between the AV axis and the axial properties of the embryo only establishes the conditions for the formation of the first cleavage furrow (Martindale and Hejnol, 2009). In both ctenophores and cnidarians, the mouth that forms at the oral pole has been shown to be homologous to both the protostome and deuterostome mouths as they express the same genes that are expressed at the oral openings of most investigated bilaterian taxa (Martindale and Hejnol, 2009). The mouth of all non-chordate metazoans being homologous (Goldstein and Freeman, 1997; Henry et al, 2001; Holland and Holland, 2007; reviewed in Martindale and Hejnol, 2009) suggests that gastrulation and endoderm formation might have evolved at the animal pole leading to the formation of a gut with a single opening at the animal pole in the metazoan ancestor. However, the development of endomesoderm from vegetal pole derived blastomeres in the bilaterians indicates that there was a switch in the site of gastrulation and endomesoderm specification from the animal pole in the ctenophores and the cnidarians to the vegetal pole in the last common ancestor of all bilaterians. Experimental evidence for the embryonic and adult axial properties not being irreversibly determined maternally in ctenophore and cnidarians suggest that such a switch in the site of endoderm specification would have been possible in the lineage leading up the bilaterians.

Importance of Fmi functioning as a scaffolding protein that mediates the asymmetric localization of other Wnt pathway components to the animal pole in *Nematostella* also has implications for a switch in the site of gastrulation in the lineage leading up to the bilaterians. If Fmi is a scaffolding protein on which the localization of
other core components of the signaling cascade are dependent on, the movement of Fmi protein from one region of a cell to another will result in the re-localization of all other components that are associated with it. This provides us with an interesting possibility where the entire gastrulation morphogenesis and cell fate specification machinery at the animal pole in the non-bilaterian can be moved to the vegetal pole in bilaterians by the relocation of a scaffolding protein such as Fmi. This might have been one possible mechanism that resulted in the switch of site of gastrulation and cell fate specification during the evolution of metazoans.

The switch in the site of gastrulation and the evolution of the nervous system.

A recent study has shown that the nervous system of the cnidarian *Nematostella* is composed of sensory neurons of ectodermal origin and ganglion cells (neurons that provide connections in the nerve net) of endodermal origin (Marlow et al., 2009). Also, the largest concentration of different neuronal types in the cnidarian polyps is found associated with the pharyngeal nerve ring and these neurons are formed from the animal pole derived blastomeres (Nakanishi et al., 2012). These findings suggest that the oral neuronal structures in the non-bilaterian cnidarians are homologous to the anterior neural structures of most bilaterians (Martindale and Hejnol, 2009). However, a switch in the site of gastrulation during the evolution of bilaterians from non-bilaterian taxa would have resulted in the movement of the endomesodermal gene regulatory network to the vegetal pole of the embryo, resulting in its separation from the neural gene network at the animal pole. This might have paved the way for the diversification and the specialization of the bilaterian nervous system leading to the evolution of cephalization and a central
nervous system (Martindale and Hejnol, 2009), which are key characteristics of all bilaterian taxa that have contributed greatly to the diversification of the clade.

Conclusions and future directions

This work has contributed to the increased understanding of early development and gastrulation in the basal anthozaon cnidarian *Nematostella vectensis*. Specifically, it has provided experimental evidence as to how different branches of Wnt signaling are regulating primary archenteron invagination and cell fate specification during gastrulation in *Nematostella*. In doing so, this study has provided valuable insights into the evolution of gastrulation and Wnt signaling in metazoans. These findings can be used to support existing hypotheses on the evolution and diversification of the Wnt signaling pathways and how it has shaped the evolution of metazoans. Also, they might provide the basis for interesting new hypotheses to explain how important features of metazoan evolution such as a switch in the site of gastrulation might have taken place. Further experimentation along these lines would bring us closer to a better understanding of the evolutionary origins of gastrulation and cell signaling events that have resulted in the great diversification of the metazoan lineage.
**Figure 5.1:** A model for the evolution of germ layer segregation and gastrulation: A molecular asymmetry present in the unicellular last common ancestor of metazoans was co-opted as a scaffold to localize maternal factors regulating germ layer segregation and gastrulation to the animal pole in non-bilaterians. This scaffold was moved to the vegetal pole in the urbilaterian, triggering body plan changes in the bilaterian clade.
References


