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Identification and Characterization of the Molecular Determinants of the Anterior-Posterior Axis in the Sea Urchin Embryo

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IDENTIFICATION AND CHARACTERIZATION OF THE MOLECULAR DETERMINANTS OF THE ANTERIOR-POSTERIOR AXIS IN THE SEA URCHIN EMBRYO

By

Lingyu Wang

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Identification and Characterization of
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How the embryonic body axis emerges from a seemingly symmetrical egg and is subsequently patterned during embryogenesis is one of the most fundamental questions in developmental biology. It is now clear that localized activation of the Wnt/β-catenin signaling pathway plays a conserved role in primary body axis formation and/or endoderm/endomesoderm specification during embryo development of phylogenetically diverse animal species. In sea urchin embryos, recent experimental evidence has shown that Dishevelled (Dsh), a central hub protein in the Wnt/β-catenin pathway, is asymmetrically enriched and differentially modified in a vegetal cortical domain (VCD) of the unfertilized egg. Experimental evidence has also shown that during embryonic development the VCD-enriched Dsh plays a critical role in locally activating Wnt/β-catenin to specify the primary body axis and to activate the endomesodermal gene regulatory network. Hence, elucidating the molecular mechanisms that regulate the accumulation of Dsh in the VCD, mediate its activity in the Wnt/β-catenin pathway will provide key insight into how the sea urchin egg becomes an embryo. In this dissertation, I successfully used two complementary approaches, RNA-seq and protein co-immunoprecipitation, to identify key candidate molecules that may participate in regulating the spatiotemporal activity of Dsh and the Wnt/β-catenin pathway. Functional

analysis of one particular candidate, DIXDC1, has shown that it may play an important role in regulating nuclear β-catenin and endomesoderm formation; two other candidate proteins were shown to be co-localized with Dsh, suggesting that they may have potentially novel functions in interacting with and regulating Dsh in sea urchin embryos. This dissertation has provided a valuable resource of candidate RNAs and proteins to study the regulation of Wnt/β-catenin pathway in early embryo development in sea urchins. Studies on these candidates in other phylogenetically important species will further provide useful insight into the regulation and evolution of the Wnt/β-catenin pathway in metazoans.

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LIST OF ABBREVIATIONS

AP	Anterior-posterior
AV	Animal-vegetal
Ani	Animal pairs
APC	Adenomatous polyposis coli
AP-MS	Affinity purification–mass spectrometry
ASW	Filtered artificial seawater
ATA	3-amino-1, 2, 4-triazole
BCV	Biological coefficient of variation
BSA	Bovine serum albumin
CELSR	Cadherin EGF LAG seven-pass G-type receptor
CFSW	Calcium free seawater
CK1	Casein kinase 1
CK2	Casein kinase II
Co-IP	Co-Immunoprecipitation
Cor	Cortices
CPM	Counts per million
DE	Differentially expressed/differential expression
DN	Dominant-negative
Dsh	Dishevelled
Dvl	Dishevelled
EEF2	Eukaryotic translation elongation factor 2
EEF2K	EEF2 kinase
EGF	Epidermal growth factor

EMS	Endomesodermal blastomere
FDR	False discovery rate
Fmi	Flamingo
Fz	Frizzled
GFP	Green fluorescent protein
GLMs	Generalized linear models
GO	Gene ontology
GPCR	G protein-coupled receptors
GRN	Gene regulatory networks
GSK-3β	Glycogen synthase kinase-3β
HEM	Hyaline extraction medium
LAG	Laminin G
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LEF	Lymphoid enhancer-binding factor 1
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
Mic	Micromeres
MS	Mesodermal
N16	Normal 16-cell stage embryos
PBS	Phosphate-buffered saline
PC	Principal component
PCA	Principal component analysis
p-EEF2	Phospho-Eukaryotic Elongation Factor2
QL	Quasi-likelihood

RINs	RNA integrity numbers
S16	Sucrose gradient treated 16-cell stage
SFRP	Secreted frizzled-related protein
TCF	T cell-specific transcription factor
VCD	Vegetal cortical domain
Veg	Vegetal pairs
WBA	Wnt/β-catenin asymmetry

Chapter 1: Wnt/β-catenin signaling pathway in germ layer specification and body axis formation during early animal embryo development

Introduction

How the embryonic body axis emerges from a seemingly symmetrical egg and is subsequently patterned during embryogenesis is one of the most fundamental questions in developmental biology. Data from diverse animals spanning from non-bilaterians (sea anemone and jelly fish) to protostomes (snail, ribbon worm and nematode) to deuterostomes (sea urchin, hemichordate, ascidian, zebrafish and frog) suggest that the Wnt/β-catenin signaling pathway controls germ layer specification and/or primary body axis (animal-vegetal or anterior-posterior axis) formation. Specifically, in many bilaterians, Wnt/β-catenin signaling is selectively activated at the vegetal/posterior end of the embryos to specify the endoderm/endomesoderm and to determine future posterior identity. In contrast, in cnidarians, this pathway is activated at the animal pole where it specifies endoderm in this group of animals. Despite this conserved role of the Wnt/β-catenin pathway in specifying early embryonic polarity across multiple phylogenetic lineages, the upstream molecular mechanisms regulating spatiotemporal Wnt/β-catenin signaling activity remain unclear in most metazoans where this process has been studied. Hence, a better understanding of how the Wnt/β-catenin pathway is regulated during early development will help us to understand germ layer specification and body axis formation and the evolution of such regulation in different groups of animals.

In this dissertation, I will first review the current understanding of the role of Wnt/β-catenin signaling in endoderm/endomesoderm specification and animal-vegetal/anterior-posterior axis formation during early embryogenesis in animal species spanning a wide phylogenetic range, and, especially, point out the important but unanswered questions. To

address these questions, I describe two approaches I have used to identify potential new candidate genes that are involved in regulating Wnt/β-catenin activity in early sea urchin embryos. Finally, I will provide evidence that some of these genes either play a direct role in this process or deserve more intense studies in the future.

The Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling pathway, also known as the canonical Wnt signaling pathway, participates in a myriad of developmental events during embryogenesis (Logan and Nusse 2004, MacDonald, Tamai et al. 2009, Petersen and Reddien 2009, Clevers and Nusse 2012). The signaling cascade is usually initiated by the Wnt family of secreted glycolipoproteins (Wnt ligands) and transduced via the transcriptional coactivator β-catenin to control the gene expression. In the absence of the Wnt ligand, a cytoplasmic destruction complex containing Adenomatous Polyposis Coli (APC), Axin, casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β) constitutively binds and phosphorylates β-catenin, causing β-catenin to be degraded by the proteosome. When the Wnt ligand binds to the seven-pass transmembrane receptor, Frizzled (Fz) and its co-receptor low-density lipoprotein receptor-related protein 5/6 (LRP5/6), the signaling pathway is activated by recruitment of the scaffolding protein Dishevelled (Dsh or Dvl) to inhibit the destruction complex. As a result, β-catenin is stabilized in the cytoplasm and can accumulate in nuclei. Nuclear β-catenin then binds to transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to activate Wnt target gene transcription in various biological contexts during animal development.

A brief animal phylogeny

Based on phylogenetic studies, all living animals (metazoans) can be grouped into five monophyletic clades: Placozoa, Porifera, Ctenophora, Cnidaria and Bilateria (Dunn, Giribet et al. 2014, Dunn and Ryan 2015, Telford, Budd et al. 2015). Animals belonging to the former four clades are also called non-bilaterians and most of them do not exhibit a bilaterally symmetrical body plan. The Bilateria, which is the largest clade, contains more than 99% of the estimated over one million living animal species (Niehrs 2010). Within Bilateria, there are three major clades: Deuterostomia, Spiralia and Ecdysozoa, where Spiralia and Ecdysozoa are also grouped as Protostomia (Dunn, Giribet et al. 2014, Dunn and Ryan 2015, Telford, Budd et al. 2015).

Members of the Wnt/β-catenin signaling pathway can be found in the genome of all metazoan clades but not in unicellular eukaryotes, fungi or plants. Choanoflagellates are unicellular protists that are considered to be the closest relatives of living multicellular animals (metazoans). Genome analysis shows that the majority of the Wnt signaling pathway components are absent in its genome except for GSK-3β (King, Westbrook et al. 2008). Wnt genes are also absent from other protists and fungi (Sebé-Pedrós, de Mendoza et al. 2011, Holstein 2012). These genome comparison studies suggest that the Wnt/β-catenin signaling pathway is a metazoan-specific innovation.

The Wnt/β-catenin signaling pathway in non-bilaterians: Genomic repertoire and pathway functions in early development

The Wnt/β-catenin signaling pathway appeared during early metazoan evolution, hence elucidating the role of the pathway in embryo development of early branching metazoans is of great significance for understanding its origin, evolution and ancestral functions.

However, due to the difficulty in accessing embryos of most non-bilaterians and the lack of tools available for manipulating more advanced model organisms, such as transgenic models, very little is known about the role of this pathway outside of the Bilateria except for a couple of species.

Placozoa

Placozoans are small, disk-like animals and the only species described in this phylum is *Trichoplax adherens*. *Trichoplax* has a simple three-layered organization with only six cell types described (Schierwater 2005, Srivastava, Begovic et al. 2008, Smith, Varoqueaux et al. 2014). *Trichoplax* has a simple body plan without any evident body axis other than top-bottom and center-rim axes. Yet, most key components of Wnt/β-catenin signaling pathway—used for anterior-posterior axis specification in bilaterians and cnidarians (see below)—have been identified in the *Trichoplax* genome. At least three Wnt ligands, a Frizzled (Fzd) receptor, a Secreted frizzled-related protein (SFRP), a Dishevelled (Dsh), an Axin, a GSK3-β, a β-catenin and a T cell-factor/lymphoid-enhancer-factor (Tcf/Lef) family member are present in the genome draft (Srivastava, Begovic et al. 2008). Since *Trichoplax* reproduces by fission in culture and its embryonic development has not been described, it is unclear what the function of Wnt signaling is in its body axis formation. Nevertheless, with such a simple body plan but a relatively complete and complex Wnt/β-catenin pathway, *Trichoplax* is a key species to unravel the role of Wnt/β-catenin pathway during early metazoan evolution.

Porifera

As one of the oldest multicellular lineages of extant animals, poriferans (sponges) are aquatic, mostly marine, sessile animals with low degree of organization. Sponges show astonishing diversity in embryogenesis and the phylogentic position of this phylum remains uncertain (reviewed by Ereskovsky 2010, Adamska, Degnan et al. 2011, Worheide, Dohrmann et al. 2012, Ereskovsky, Renard et al. 2013). Adult sponges have a unique filter feeding system but they do not possess a distinct gut, or nervous and muscular systems (Ereskovsky 2010). Sponges do not display evident anterior-posterior polarity as adults, but the larvae swim directionally. In the species *Amphimedon queenslandica*, key components of Wnt/β-catenin signaling pathway have been identified, including Wnt, Fzd, SFRP, Lipoprotein receptor-related protein-5/6(Lrp5/6), Dsh, Axin, APC, GSK3-β, β-catenin, Groucho and Tcf/Lef. *In situ* hybridization studies have shown many of these genes are expressed asymmetrically along the anterior-posterior axis (as defined by the larval swimming direction) and the asymmetries first appear at the "gastrula" stage during embryo development (Adamska, Degnan et al. 2007, Adamska, Larroux et al. 2010). The *in situ* hybridization studies alone are insufficient to determine the exact role of this pathway in embryo development, especially β-catenin's role, therefore functional studies of these genes in sponge models would be of great benefit. In one study using the species *Ephydatia muelleri*, activation of Wnt/β-catenin pathway by lithium or alsterpaullone caused ectopic oscula (chimney, vent), suggesting that the Wnt/β-catenin pathway is involved in body axis formation in sponges (Windsor and Leys 2010). However, there are no experimental studies on the potential role of Wnt/β-catenin signaling during embryogenesis in *E. muelleri*. But due to their key position as an early branching

metazoan, sponges are one taxon that will be important to determine the role of Wnt/β-catenin in the evolution of the mechanisms specifying the polarity of the embryo.

Ctenophora

Ctenophores, commonly known as comb jellies, are a group of gelatinous marine zooplankton, which are the largest animals to move entirely by means of cilia (Pang and Martindale 2008). They show a stereotypical cleavage program during embryogenesis and the adults have an oral-aboral axis as the main body axis with biradial symmetry. Ctenophores have only two germ layers, endoderm and ectoderm. Most key components of Wnt/β-catenin pathway have been identified in the ctenophore *Mnemiopsis leidyi* but the RNA *in situ* hybridization expression patterns of these genes do not show any polarity before gastrulation. The Wnt mRNAs are detected at a relatively late stage and Frizzled and Dishevelled are maternally loaded without clear asymmetric localization (Pang, Ryan et al. 2010). These results, however, cannot rule out the role of maternal proteins in embryogenesis, as we will discuss later. Also, a recent study shows that 11 members of the Wnt/β-catenin pathway are strongly expressed in the oral region in the adult ctenophore *Pleurobrachia pileus*, indicating the Wnt/β-catenin pathway may participate in the adult oral-aboral axis formation (Jager, Dayraud et al. 2014). Whether the Wnt/β-catenin pathway also participates in the embryonic axis formation in ctenophores remains to be characterized.

Cnidaria

Cnidarians compose a diverse group of aquatic (predominantly marine) animals with a relatively simple body plan (Daly, Brugler et al. 2007a, Technau and Steele 2011). The

distinguishing trait of this phylum is a stinging cell, the cnidocyte (also known as the nematocyte or the chida) (Daly, Brugler et al. 2007b). Cnidarians display an external radial symmetry, yet many groups have an internal asymmetry or bilateral symmetry (Technau and Steele 2011). Cnidarians have a blind gut and two germ layers. In cnidarians, the primary body axis is the oral-aboral axis, where the oral end of the planula arises from the animal pole of the egg, which is opposite to most bilaterians (Martindale 2005, Martindale and Hejnol 2009, Petersen and Reddien 2009). Since cnidarians are the sister group of all bilaterians, comparing these groups can provide insights about the ancestral function of the Wnt/β-catenin pathway in AP body axis formation. The role of the Wnt/β-catenin pathway during embryogenesis has been functionally examined in cnidarians.

In the anthozoan sea anemone *Nematostella vectensis*, studies have shown that endogenous β-catenin or GFP-tagged β-catenin is differentially stabilized along the oral-aboral axis and translocated only into nuclei in the cells at the animal pole, where gastrulation occurs and endoderm is specified (Wikramanayake, Hong et al. 2003). Treating embryos with lithium chloride to activate ectopic nuclear β-catenin signaling causes an expansion of endoderm (Wikramanayake, Hong et al. 2003). Conversely, down regulation of nuclear β-catenin by overexpressing cadherin (Wikramanayake, Hong et al. 2003) or by expressing a dominant-negative form of the Dsh protein (Lee, Kumburegama et al. 2007) results in defects in endoderm specification in *Nematostella* embryos. Furthermore, studies have shown that the maternal protein Dsh is enriched at the animal pole in *Nematostella* eggs and it is required for nuclear β-catenin regulation (Lee, Kumburegama et al. 2007). For the first time, these studies demonstrated that the Wnt/β-

catenin pathway plays a fundamental role in establishing embryonic polarity and endoderm segregation in a non-bilaterian clade.

Similarly, in the hydrozoan jellyfish *Clytia hemisphaerica*, nuclear β-catenin is also restricted to the animal pole at the early embryonic stages (Momose and Houliston 2007). More interestingly, studies have shown that three localized maternal mRNAs regulate the activity of nuclear β-catenin along the animal-vegetal axis (Momose and Houliston 2007, Momose, Derelle et al. 2008, Amiel and Houliston 2009). In the animal half of *C. hemisphaerica* eggs, *in situ* hybridization analyses have shown that *CheFz1* mRNA is enriched in the cytoplasm, whereas *CheWnt3* mRNA is localized to the cortex. By contrast, *CheFz3* mRNA is localized to the vegetal egg cortex. Blocking the translation of *CheFz1* or *CheWnt3* mRNA using morpholino antisense oligos results in the loss of nuclear β-catenin and the downregulation of endodermal gene expression in embryos. Conversely, CheFz3-MO causes an expansion of nuclear β-catenin and the expansion of endodermal genes towards the vegetal pole. Based on these observations, Houliston and colleagues have proposed a model for reciprocal regulation between maternal factors from the animal pole and vegetal pole for germ layer segregation and oral-aboral axis formation in *Clytia* (Momose and Houliston 2007, Momose, Derelle et al. 2008, Amiel and Houliston 2009). This is the second example of a maternal Wnt ligand that is required for activation of the canonical Wnt pathway during early embryogenesis, after *Xenopus* Xwnt11, which we will discuss later.

These studies in cnidarian embryos clearly show that the Wnt/β-catenin pathway is activated at the animal pole to specify the endoderm at that pole and to establish the primary embryonic axis in this clade. Despite the work in *Clytia*, the upstream molecular

mechanisms remain elusive in other cnidarian species. None of the Wnt ligands in the *Nematostella* genome are localized in the unfertilized egg (Lee, Pang et al. 2006). Dsh is a good candidate for regulating the spatiotemporal activity of nuclear β-catenin because its role in basal deuterostome sea urchin embryos is consistent with that in *N. vectensis* (Weitzel, Illies et al. 2004, Peng and Wikramanayake 2013). Additional work on Dsh in other non-bilaterian species, however, is required before drawing a concrete conclusion. Similarly, since the localized mRNAs in *C. hemisphaerica* have not been shown to have conserved localization or functions in other organisms yet, more comprehensive studies are required. Another interesting perspective is that in bilaterians, unlike cnidarians, the Wnt/β-catenin pathway is selectively activated at the vegetal pole and in several species this pathway also specifies endoderm/endomesoderm at this vegetal pole (see below). This major difference suggests a significant shift of the site of endoderm/endomesoderm specification may have taken place in the bilaterians lineage during evolution (Lee, Kumburegama et al. 2007, Peng and Wikramanayake 2013). Therefore, careful comparison of the molecular mechanisms regulating the asymmetric activity of Wnt/β-catenin pathway in non-bilaterians and bilaterians will provide more insight into germ layer and body axis evolution.

The Wnt/β-catenin pathway regulates anterior-posterior axis formation and patterning in diverse bilaterian phyla

Most animals, except the early branching metazoan clades such as the placozoans, sponges, ctenophores and cnidarians, display bilateral symmetry and are grouped together as the Bilateria. The bilaterians are a group of very successful animals in evolution exhibiting diverse body plans and living in wide-ranging habitats. All bilaterians have three germ layers, endoderm, mesoderm and ectoderm. Most bilaterians display bilateral

symmetry along a polarized anterior-posterior (AP) body axis. They also display a key feature—cephalization, where the central nervous system, sense organs and feeding structures are concentrated at the anterior end. Evidence from most of the bilaterian embryos examined has shown that Wnt/β-catenin pathway plays a crucial role in endoderm/endomesoderm specification and/or primary body axis formation in this clade. Bilaterians are further split into three major clades, spiralians, ecdysozoans and deuterostomes. Together, the spiralians and ecdysozoans constitute the protostomes.

Spiralia

The spiralian clade constitutes one of the three major branches of the Bilateria and it includes invertebrate groups like the molluscs, nemerteans and annelids. Embryos from these phyla exhibit a highly stereotypical spiral cleavage mode: the cell geometry and spindle orientation during early cleavage stages, as well as the general cell fates of individual blastomeres are highly conserved (Wilson 1898, Henry and Martindale 1999, Lambert 2010, Henry 2015). Spiralia is very large and diverse clade but the role of nuclear β-catenin in early embryo development has only been examined in a small number of species.

Mollusca

Mollusks are one of the largest and most diverse animal phyla in terms of species and morphology. They exhibit varied modes of development, body plans and life history strategies (Henry 2015). Despite this fact, the role of Wnt/β-catenin pathway in early embryogenesis has only been studied in one species, the gastropod *Crepidula fornicata*.

In *C. fornicata* embryos, a 4d cell (mesentoblast cell) forms at the 25-cell stage. Fate maps show that this cell contributes to the majority of adult mesoderm (Henry and Martindale 1999). Moreover, the 4d mesentoblast acts as the key embryonic organizer, which controls the development of the dorso-ventral axis and cell fate specification in adjacent quadrants (Henry, Perry et al. 2006). Recent work has shown that GFP-tagged β-catenin protein is distributed in all cells of the early *C. fornicata* embryo prior to the 25-cell stage. Starting at the 25-cell stage, however, β-catenin is selectively stabilized only in the 4d mesentoblast, which gives rise to key mesodermal and endodermal cell fates (Henry, Perry et al. 2010). Blocking the translation of β-catenin mRNA using morpholino antisense oligos results in failure of gastrulation (Henry, Perry et al. 2010), suggesting that the Wnt/β-catenin pathway may play an important role in the germ layer specification and body axis formation in *C. fornicata* embryos, but additional functional analysis is required.

Nemertea

Nemerteans, also known as ribbon worms, are a phylum of mostly marine worms, specialized with a structure called a proboscis to capture prey. They exhibit equal spiral cleavage pattern for the first two cell cycles (Henry and Martindale 1998). In the nemertean worm, *Cerebratulus lacteus*, GFP-tagged β-catenin is differentially stabilized along the animal-vegetal axis and translocated only into nuclei in the four vegetal-most cells at the 64-cell stage, which give rise to endoderm. Morpholino-mediated knockdown of β-catenin produces embryos do not undergo gastrulation, and the endoderm fails to form. Moreover, the epithelial cells display long slow-beating cilia (Henry, Perry et al. 2008). Similarly, blocking nuclear β-catenin activity by over-expressing the cytoplasmic domain of cadherin or a β-catenin-engrailed repressor fusion protein generates the same phenotype (Henry,

Perry et al. 2008). These phenotypes are remarkably reminiscent of the phenotype produced when nuclear beta-catenin is blocked in sea urchins (Wikramanayake, Huang et al. 1998b, Henry, Perry et al. 2008). By contrast, gain of function of β-catenin by over-expressing a constitutively activated β-catenin or a dominant-negative form of GSK3-β produces a phenotype where nearly all cells are converted to endodermal fates (Henry, Perry et al. 2008). These results indicate that in *C. fornicata* β-catenin is both necessary and sufficient for endoderm cell fate specification, consistent with the signaling role of this protein during early development in many other species.

Annelida

Annelids, also known as segmented worms, are a large phylum that contains species occupying a wide range of habitats from terrestrial to freshwater to marine. Animals in this group include polychaetes, leeches and earthworms, most of which are segmented worms. The feature of segmentation is only shared with two other phyla, the arthropods and the chordates (Seaver 2003, Ferrier 2012). Like many other spiralians, annelids undergo stereotypical spiral cleavage and the species *Platynereis dumerilii* has been used to studying the role Wnt/β-catenin in early embryo development. Using immunostaining Schneider and Bowerman (2007) showed that beginning at the 8-cell stage nuclear β-catenin is asymmetrically localized with higher levels in the vegetal-pole blastomeres. In every subsequent cell division and in all the daughter cell pairs during early spiral-cleavage stages, nuclear β-catenin follows the same pattern: higher levels in the vegetal-pole daughter cells but lower in the animal-pole daughter cells. Ectopic activation of β-catenin in the animal-pole daughter cells using GSK3-β inhibitors including lithium, Alsterpaullone or 1-azakenpaullone, causes them to adopt the more vegetal cell fate

(Schneider and Bowerman 2007). These results indicate that in *P. dumerilii* embryos, the reiterative pattern of β-catenin localization mediates binary cell-fate specification along the entire animal-vegetal axis (Schneider and Bowerman 2007). However, whether this binary pattern of nuclear β-catenin is related to endoderm or endomesoderm specification remains to be determined by further experiments.

Ecdysozoa

The Ecdysozoa clade is another major branch of the Bilateria and it comprises molting animals like nematodes, arthropods and relatives. Given the species rich nature of this clade, only a small subset have been sampled to study the function of nuclear β-catenin in early embryo development.

Nematoda

Unlike most of the model organisms, which have only one β-catenin gene, the nematode *Caenorhabditis elegans* has four β-catenin paralogs; HMP-2, BAR-1, WRM-1 and SYS-1. SYS-1 shares only about 11% amino acid sequence identity to human β-catenin, but it functions with the *C. elegans* TCF homolog POP-1 as the bona fide member of β-catenin family (Kidd, Miskowski et al. 2005, Liu, Phillips et al. 2008). *C. elegans* β-catenin paralogs have diverged and have complicated functions. BAR-1 binds POP-1 and activates Wnt signaling target genes (Lin, Thompson et al. 1995, Lin, Hill et al. 1998, Korswagen, Herman et al. 2000, Herman 2001, Natarajan, Witwer et al. 2001), but it mainly functions in postembryonic stages (Eisenmann, Maloof et al. 1998, Maloof, Whangbo et al. 1999, Eisenmann and Kim 2000). HMP-2 mainly interacts with cadherin for cell adhesion (Costa, Raich et al. 1998, Korswagen, Herman et al. 2000, Natarajan,

Witwer et al. 2001) but as described below, it has also been reported to function in Wnt signaling directly to regulate endoderm specification during early embryo development (Putzke and Rothman 2010, Sumiyoshi, Takahashi et al. 2011).

C. elegans embryos develop rapidly, and cell fates are specified starting at very early cleavage stages. In four-cell stage embryos, a Wnt signal from the posterior blastomere P2 instructs the endomesodermal blastomere (EMS) to divide asymmetrically, generating a posterior endodermal (E) founder cell and an anterior mesodermal (MS) cell (reviewed by Jackson and Eisenmann 2012). In the MS cell, high levels of POP-1 in the nucleus interact with repressors to keep the target gene expression off, promoting mesoderm cell fate. In the E cell, WRM-1 binds and activates a Nemo-like kinase to export POP-1 from the nucleus and SYS-1 interacts with low level of POP-1 in the nucleus to turn on target gene expression, inducing endodermal cell fates. Perturbation of the nuclear levels of these components, directly or indirectly, will alter the fate of cells and produce extra endoderm or mesoderm (Lin, Thompson et al. 1995, Kaletta, Schnabel et al. 1997, Rocheleau, Downs et al. 1997, Thorpe, Schlesinger et al. 1997, Lin, Hill et al. 1998, Miskowski, Li et al. 2001, Park and Priess 2003, Kidd, Miskowski et al. 2005, Nakamura, Kim et al. 2005, Takeshita and Sawa 2005, Huang, Shetty et al. 2007, Phillips, Kidd et al. 2007, Owraghi, Broitman-Maduro et al. 2010, Yang, Karhadkar et al. 2015). Recent results also suggest HMP-2 can contribute to endoderm induction under certain conditions (Putzke and Rothman 2010, Sumiyoshi, Takahashi et al. 2011). This Wnt/β-catenin asymmetry (WBA) pathway, mediated by β-catenin and TCF homologs, is widely used to regulate many asymmetric cell divisions during *C. elegans* embryonic and larval development.

Arthropoda

Arthropoda is the largest metazoan phylum with the most diverse animals. Several key components of Wnt/β-catenin pathway, including β-catenin, were first discovered in a well-studied model organism of this phylum, *Drosophila melanogaster*. However, *Drosophila* axis formation is highly derived (Niehrs 2010). *Drosophila* adopts the long germ band mode of embryogenesis, in which the diffusion of transcription factors between the syncytial nuclei instructs the embryonic patterning and segmentation takes place almost simultaneously; as a result, Wnt/β-catenin signaling plays a different role at early stages of embryogenesis compared with that of ancestral arthropods (Stjohnston and Nussleinvolhard 1992, Riechmann and Ephrussi 2001, Davis and Patel 2002, Oberhofer, Grossmann et al. 2014). By contrast, in short germ band insects, the posterior segments form sequentially from a posterior growth zone and Wnt/β-catenin signaling pathway plays an essential role to pattern the posterior end of the embryos in the species examined (Oberhofer, Grossmann et al. 2014). In the red flour beetle *Tribolium castaneum* (Bolognesi, Beermann et al. 2008, Bolognesi, Farzana et al. 2008, Bolognesi, Fischer et al. 2009, Beermann, Pruhs et al. 2011, Oberhofer, Grossmann et al. 2014), the centipede *Strigamia maritima* (Hayden, Schlosser et al. 2015), the common house spider *Achaearanea tepidariorum* (McGregor, Pechmann et al. 2008) and the cricket *Gryllus bimaculatus* (Miyawaki, Mito et al. 2004), there is evidence that perturbation of Wnt/β-catenin pathway components cause defects in developmental processes at the posterior end of the embryo consistent with other phyla described above. Additional studies are required to examine the role of Wnt/β-catenin in the body axis formation and germ layer specification in arthropod embryo development.

Deuterostomia

Echinodermata

Echinoderms are the second largest deuterostome phylum that includes well-known marine invertebrates such as sea urchins, sea stars, sand dollars. Many species in this phylum were among the first organisms used for comparative and experimental embryology studies (McClay 2011). The sea urchin embryos are the best studied model organism in Echinodermata, with a rich and distinguished history for studying the molecular mechanisms of embryonic development. In most species, the embryos undergo radial and holoblastic cleavage. After the first three equal cleavages, the fourth cleavage is unequal in the vegetal half, producing three tiers of blastomeres from the animal pole to the vegetal pole: eight mesomeres, four macromeres and four micromeres. These blastomeres are different in size as well as in cell fates: mesomeres will give rise to the majority of the embryonic ectoderm and the macromeres and micromeres to all the embryonic endomesoderm and a small part of the ectoderm. The blastula is hollow and the presumptive endomesoderm is located at the vegetal plate, where gastrulation initiates and the archenteron forms (McClay 2011).

Classic embryological studies in sea urchins have demonstrated that the determinants that induce endomesoderm are localized at the vegetal pole of the unfertilized egg (Hörstadius 1973, Maruyama, Nakaseko et al. 1985). Work from three labs provided the initial evidence that these determinants either directly or indirectly regulate the Wnt signaling pathway (Emily-Fenouil, Ghiglione et al. 1998, Wikramanayake, Huang et al. 1998b, Logan, Miller et al. 1999). For example, work done by Emily-Fenouil, Ghiglione et al. showed that knockdown of GSK3-β activity by a dominant-negative form of this protein causes vegetalization (production of excess endomesodermal tissues), whereas

overexpression of wild-type GSK3-β leads to animalization (complete loss of endomesoderm and ectopic neural cell fates), suggesting that the Wnt/β-catenin pathway plays an important role in endomesoderm specification in sea urchin embryos (Emily-Fenouil, Ghiglione et al. 1998). Additionally, depleting nuclear β-catenin by overexpressing C-cadherin caused animalized embryos and overexpression of a constitutively active form of β-catenin caused vegetalized embryos providing the direct evidence that β-catenin is both necessary and sufficient to specify endomesoderm in sea urchin embryos (Wikramanayake, Huang et al. 1998b). Beta-catenin antibody staining showed that this protein accumulates in the nuclei at the vegetal pole starting at the 16-cell stage and the perturbations using lithium or cadherin actually acts via affecting nuclear β-catenin levels. Furthermore, dissociation experiments have shown that β-catenin is nuclearized cell autonomously (Logan, Miller et al. 1999). Perturbations of other key components of the Wnt/β-catenin pathway, including TCF, Dishevelled, and Groucho, have also produced animalized or vegetalized phenotypes in sea urchin embryos consistent with the effect of each manipulation on nuclear β-catenin levels (Huang, Li et al. 2000, Vonica, Weng et al. 2000, Weitzel, Illies et al. 2004, Range, Venuti et al. 2005).

In sea star embryos, nuclear β-catenin localizes in vegetal cells at the 16-cell stage and this pattern is maintained at a high level in vegetal pole blastomeres at early stages (Miyawaki, Yamamoto et al. 2003). Furthermore, McCauley, Akyar *et al.* provided evidence that endomesoderm specification by nuclear β-catenin at the vegetal pole is dose-dependent: low and intermediate levels of nuclear β-catenin drive endoderm specification while high levels drive the mesoderm specification in sea star embryos (McCauley, Akyar et al. 2015). In summary, these studies have shown that the Wnt/β-catenin pathway plays

an essential role in endomesoderm specification at the vegetal pole in sea urchins and sea stars. However, the maternal determinants that asymmetrically activate this pathway at the vegetal/posterior end of these embryos have not been identified.

Hemichordata

Hemichordates are the sister group to echinoderms and closely related to chordates. They are exclusively marine organisms and can be further divided into two major groups: the colonial tube-dwelling pterobranchs and the solitary enteropneusts (the acorn worms) (Rottinger and Lowe 2012). The embryology of enteropneusts is relatively well studied compared to pterobranchs. Embryonic development of enteropneusts is very similar to that of echinoderm sea urchins, with a radial and holoblastic cleavage pattern, a hollow blastula with a thickened vegetal plate which is the region where gastrulation initiates and the archenteron forms (Rottinger and Lowe 2012). Similar to echinoderms, the endomesoderm in hemichordates is specified at the vegetal pole of the embryo (Darras, Gerhart et al. 2011, Rottinger and Lowe 2012).

Recent studies have shown that nuclear β-catenin is both necessary and sufficient to specify endomesoderm at the vegetal/posterior part during embryonic development of the enteropneust worm *Saccoglossus kowalevskii*, (Darras, Gerhart et al. 2011). First, fluorescent protein-tagged β-catenin protein was observed in the nuclei at the vegetal cells at the blastula stage. Knockdown of β-catenin by siRNA causes loss of endomesoderm, whereas early activation of β-catenin by the GSK3-β inhibitor 1-azakenpaullone leads to ectopic formation of endomesoderm (Darras, Gerhart et al. 2011). Furthermore, the animal-vegetal polarity in *S. kowalevskii* was set up after fertilization via a "vegetal contraction" that may be mediated by actin filaments (Darras, Gerhart et al. 2011). This feature makes

the *S. kowalevskii* embryo a good model to study the molecular mechanisms that regulate the initial steps of animal-vegetal polarity formation. Since hemichordates are a sister group of echinoderms, one perspective is to examine the localization and activity of Dsh in *S. kowalevskii* embryo before and after fertilization.

Tunicata

The tunicates (urochordates) and vertebrates are sister taxa and together they form two major clades within the chordate phylum. Ascidians, also called sea squirts, represent the most speciose group of tunicates and include several developmental models (Lemaire 2011). In the ascidian *Ciona savignyi* embryos, lithium treatment converts mesodermal notochord cells into endodermal cells, suggesting that Wnt/β-catenin pathway plays a role in endoderm specification (Yoshida, Marikawa et al. 1998). In the ascidians *C. intestinalis* and *C. savignyi* embryos starting at 8- to 16-cell stage, antibody staining has shown that β-catenin protein starts to accumulate in the nuclei in vegetal blastomeres, where the endoderm and mesoderm are specified (Imai, Takada et al. 2000, Kawai, Iida et al. 2007, Hudson, Kawai et al. 2013). Overexpression of β-catenin induces ectopic endodermal cells, whereas knockdown of β-catenin by overexpressing cadherin leads to defects in endoderm formation (Imai, Takada et al. 2000). The dynamic patterns of nuclear β-catenin give rise to different germ layers during embryogenesis. From the 16- to 32-cell stage, the endodermal precursors (the E cell lineage) with a nuclear β-catenin sequence initially on and then maintained in the 32 cell stage (ON-to-ON) specifies endoderm, the notochord and neural precursors (the NN cell lineage) with ON-to-OFF specifies margin cells (mesoderm and ectoderm) and animal pole cells with OFF-to-OFF adopts ectoderm cell fate (Hudson, Kawai et al. 2013). These results indicate that β-catenin plays a similar role

in ascidian embryos as that in sea urchins, hemichordates, nemerteans and cnidarians to specify endoderm/endomesoderm cell fate and regulate the body axis formation.

Vertebrata

Asymmetrically localized nuclear β-catenin in early embryos was first discovered in the vertebrates *Xenopus* (frog) and *Danio* (zebrafish) (Schneider, Steinbeisser et al. 1996). During *Xenopus* oogenesis, the maternal β-catenin stabilizing factors are localized at the vegetal pole. Following fertilization, and driven by cortical rotation, these factors are transported towards the equatorial region, which becomes the future dorsal side. As a result, the animal-vegetal asymmetry determines the dorsal-ventral axis in *Xenopus* (reviewed by Heasman 2006, Houston 2012). Antibody staining has shown that β-catenin accumulates in the nuclei at the future dorsal side at the blastula stage in *Xenopus* and zebrafish embryos (Schneider, Steinbeisser et al. 1996). Later, this dorsally localized β-catenin protein was observed as early as the 16- to 32-cell stage in *Xenopus* embryos (Larabell, Torres et al. 1997). *Xenopus* embryos dorsalized by lithium have increased localization of β-catenin to the nuclei, whereas embryos ventralized by UV light abolishes the dorsal-ventral pattern of nuclear β-catenin (Schneider, Steinbeisser et al. 1996). Many other Wnt/β-catenin components, including Wnt11b, Frl1/Tdfg1, Wnt5a, Dkk1, Frizzled7, Lrp6, Axin1, Dishevelled2, Dishevelled3, TCF1, TCF3, Bcl9, Pygo1, Frat1, JNK1, Ext1 and Tpst1, have been demonstrated to regulate the abundance of nuclear β-catenin or have an effect on dorsal-ventral axis formation (reviewed by Houston 2012). In particular, Dsh homologs, DVL2 and DVL3, are thought to be the key determinants in regulating nuclear β-catenin in *Xenopus* oocytes and early embryos, but this remains controversial (Tao, Yokota et al. 2005, Houston 2012). Moreover, work has shown that maternal Wnt11 and

Wnt5 act in the same complex to regulate the activity of nuclear β-catenin (Tao, Yokota et al. 2005, Cha, Tadjuidje et al. 2008). These studies provide very useful insights into the molecular mechanism of how asymmetric nuclear β-catenin is regulated in a spatiotemporal manner. More importantly, the studies in *Xenopus* embryos provide a valuable resource of candidate genes to be examined in other model organisms. The comparison between *Xenopus* and other model organisms will be extremely useful to understand the molecular mechanisms and evolution of Wnt/β-catenin signaling pathway regulation in embryonic development.

Discussion and Conclusion

After reviewing studies on animal species ranging from non-bilaterians to vertebrates, it is clear that Wnt/β-catenin signaling plays a critical role in endoderm or endomesoderm specification and/or anterior-posterior body axis formation. In animals such as the cnidarians *N. vectensis*, and *C. hemisphaerica*, the nemertean *C. lacteus*, the nematode *C. elegans*, echinoderm sea urchins, the hemichordate *S. kowalevskii* and the vertebrates *Xenopus* and zebrafish, it is clear that nuclear β-catenin accumulates in one-half of the embryo at the right time and in the right place for endoderm/endomesoderm specification and/or anterior-posterior body axis formation. It also has been demonstrated that nuclear β-catenin is both necessary and sufficient for endoderm/endomesoderm specification or body axis (especially anterior-posterior/animal-vegetal axis) formation in many species. The results suggest the conserved role of Wnt/β-catenin pathway in the common ancestor of cnidarians and bilaterians.

In other animals such as the mollusk *C. fornicata*, the arthropods *T. castaneum*, *S. maritima*, *A. tepidariorum,* and *G. bimaculatus*, there is evidence that correlates the

expression pattern of nuclear β-catenin to its conserved role in endoderm/endomesoderm specification. In some cases, partial functional experimental evidence supports the idea that nuclear β-catenin is required for the posterior development in embryos, but additional functional studies or expression analyses are needed to draw any conclusion.

At the same time, denser sampling of many more metazoans across the phylogenetic tree is required to make more rigorous comparisons as well as to distinguish special cases to fully understand the functions the Wnt/β-catenin pathway plays in embryo development. Currently, models from the deuterostomes (chordates such as mouse, zebrafish, frog *Xenopus*) and ecdysozoans (especially the fruit fly *Drosophila*, nematode *C. elegans*) have received relatively more attention than other models. Generalization of the results discovered only from this small group of species could be biased. Additional studies on other diverse but highly underrepresented metazoan lineages, such as animals in spiralia, ecdysozoa (other than *Drosophila* and *C. elegans*) and non-bilaterians (especially ctenophores) are needed to obtain a better overview of the function of the Wnt/β-catenin pathway in early embryo development and the evolution of embryonic patterning. In contrast to the most prevalent model that β-catenin only accumulates in the nuclei in one half of the embryo, are the results coming from the nematode *C. elegans* and the annelid *P. dumerilii. In these systems*, nuclear β-catenin is distributed in a binary pattern in almost all sister blastomeres along the primary body axis. To determine whether these scenarios are just species-specific cases or can be applied to a broader group of animals, additional studies in other developmental contexts are needed.

Finally, if the nuclear β-catenin is the conserved molecular mechanism to control endoderm/endomesoderm specification and primary body axis formation, there are other

interesting questions that remain to be answered: What are the upstream molecular mechanisms that control β-catenin nuclear accumulatione in the nuclei in the right place at the right time? Are those mechanisms also conserved across metazoans or are there lineage specific mechanisms?

In the cnidarian *C. hemisphaerica*, three maternal mRNAs encoding two Fz receptors (Fz1 and Fz3) and one Wnt ligand (Wnt3) are localized in animal or vegetal halves of the oocyte to regulate the nuclear β-catenin (Momose and Houliston 2007, Amiel and Houliston 2009). However, this strategy is not found to be conserved in other species thus far. In *Xenopus*, maternal mRNA *Wnt11* is necessary and sufficient to activate nuclear β-catenin for axis formation (Tao, Yokota et al. 2005) but, again, the mechanism mediated by Wnt11 has not been found in other models. Another promising candidate for regulating early Wnt/β-catenin pathway is the asymmetrically enriched Dsh protein. In both the cnidarian *N. vectensis* and echinoderm sea urchins, maternal Dsh protein is localized at the animal cortex or vegetal cortex, respectively. Nuclear β-catenin is activated in the blastomeres inheriting these Dsh-containing cortices (Lee, Kumburegama et al. 2007, Peng and Wikramanayake 2013). Dsh homologs are also thought to be the key determinants to activate nuclear β-catenin in *Xenopus* embryo development, but it remains controversial (Tao, Yokota et al. 2005, Houston 2012). As a result, understanding how upstream molecular mechanisms regulate the β-catenin activation spatiotemporally in early embryo is another fascinating question.

In the remaining Chapters, I will focus on the Dsh protein and the potential maternal factors that may regulate asymmetric localization and "activation" of Dsh and hence control asymmetric nuclear entry of β-catenin in sea urchin embryos. Ultimately, these

findings may provide useful resources for future studies of the Wnt/β-catenin signaling pathway in both sea urchin embryos as well as other phylogenetically important organisms to unveil the role of this pathway in metazoan body plan evolution.

Chapter 2: Identification of differentially distributed RNAs along the animal-vegetal axis of the sea urchin egg and embryo using RNA-seq

Background

How the embryonic body axis forms and is subsequently patterned along the axis from a seemingly symmetrical egg is a central question in biology. For over 100 years, sea urchin embryos have been an important model system for addressing this question and more recent studies have focused on elucidating the underlying molecular mechanisms (Ernst 2011, McClay 2011). The primary body axis of sea urchin embryos, the animal-vegetal (AV) axis, is established during oogenesis, with the protrusion of polar bodies marking the animal pole. After fertilization and following the three equal cell cleavages, the fourth cleavage is unequal at the vegetal pole, resulting in three tiers of embryonic cells that form along the AV axis at the 16-cell stage (Fig. 2.1C). These cells are different both in terms of size and cell fates. At the animal pole, the eight intermediate-sized mesomeres are primarily fated to ectoderm tissues. At the vegetal pole, the four small-sized micromeres and the four large-sized macromeres in between are primarily fated to endomesodermal tissues. The molecular mechanisms regulating this germ layer specification are not fully understood yet. While it is clear that the axial determinants are located at the vegetal pole (Hörstadius 1973) or, more specifically, the vegetal cortex (Croce, Range et al. 2011), their molecular identities are not known.

Recent work has shown that the protein Dishevelled (Dsh), a key regulator of the Wnt/beta-catenin pathway, is required for activation this pathway in vegetal cells during early embryonic development (Weitzel, Illies et al. 2004, Peng and Wikramanayake 2013). Dsh is required for the initiation of the Wnt/β-catenin pathway in the 16-cell stage micromeres and the subsequent endomesoderm specification at the vegetal pole

(Wikramanayake, Huang et al. 1998b, Logan, Miller et al. 1999, Weitzel, Illies et al. 2004). Intriguingly, this protein was shown to be differentially post-translationally modified and highly enriched in the cortex at the vegetal pole and this region is described as the vegetal cortical domain (VCD) (Peng and Wikramanayake 2013). However, the cellular and molecular mechanisms that modify and anchor Dsh in the VCD, and activate the Wnt/β-catenin pathway asymmetrically at the vegetal pole remain elusive. This information is critical to gain a comprehensive knowledge of how the AV axis is initially specified and subsequently patterned during early embryonic development. Thus, identification of the maternal molecules differentially expressed in the VCD and micromeres will provide insights into these questions.

In a broad spectrum of organisms, including ascidians, fruit flies, frogs, zebrafish and jellyfish, maternal RNAs have been shown to be asymmetrically localized in oocytes or eggs. These RNAs dictate local protein synthesis or play structural roles, both of which are essential for body axis formation, embryonic germ layer specification and germline determination during embryogenesis (reviewed by (King, Messitt et al. 2005, Kloc and Etkin 2005, Prodon, Yamada et al. 2007, Sardet, Paix et al. 2007, Houston 2012, Kumano 2012, Medioni, Mowry et al. 2012, Houston 2013, Weil 2014)). In sea urchin eggs, although a couple of maternal mRNAs have been reported to be localized along the AV axis (Dicarlo, Romancino et al. 1994, Di Carlo, Montana et al. 2004), there is no evidence that they play a role in AV axis formation and subsequent cell fate specification. A comprehensive and unbiased screen is needed to identify these maternal localized mRNA, if any, as axial determinant candidates.

With the advances in high-throughput RNA-sequencing technologies, it is now possible to explore the whole transcriptomes of different blastomeres and even subcellular structures to identify candidate RNAs that are differentially expressed and localized. Here, I performed transcriptome studies on isolated sea urchin egg cortices and micromeres, with stringent experimental controls. After correcting for the strong batch effects that existed in the data with rigorous analytical methods, promising candidate RNAs, including the ones related to Wnt/β-catenin pathway, have been successfully identified. The functional analysis of these candidate molecules will likely provide useful insight into the molecular mechanisms that regulating asymmetric activation of Wnt/β-catenin pathway in the vegetal hemisphere during sea urchin embryo development.

Materials and Methods

Animal handling and sample collection

Adult sea urchins *Strongylocentrotus purpuratus* were obtained from Marinus, Garden Grove, CA, and maintained in a seawater aquarium at 15°C. Gametes and embryos were obtained as previously described (Peng and Wikramanayake 2013).

Four biological replicates from four separate matings (families) for the seven types of samples were prepared (Table 1): egg (Egg), cortices (Cor), normal 16-cell stage embryos (N16), sucrose gradient treated 16-cell stage as control for blastomere isolation (S16), micromeres (Mic), animal pairs (Ani) and vegetal pairs (Veg).

Egg cortices were isolated as previously described (Peng and Wikramanayake 2013). Briefly, dejellied eggs were washed with calcium free seawater (CFSW) 5 times and placed on poly-L-lysine-coated 35-mm petri dishes in a density to saturate the bottom of each dish. Cortical lawn isolation buffer (0.8 M mannitol, 50 mM HEPES, 50 mM PIPES, 5 mM

EGTA, 2.5 mM $MgCl_2 \cdot 6H_2O$, pH 6.5) was squirted from a rinse bottle to lyse the eggs, only leaving the isolated cortices attached to the petri dish. Cortices isolated from ten petri dishes were dissolved in TRI Reagent (Molecular Research Center) for RNA extraction. Approximately 10,000 intact eggs were collected from each female sea urchin for each RNA sample.

Animal (mesomeres) and vegetal (macromere/micromere) blastomeres were isolated using a previously described procedure (Wikramanayake, Brandhorst et al. 1995), with a few modifications. Eggs were first fertilized in filtered artificial seawater (ASW) with 0.5 mM of fresh 3-amino-1, 2, 4-triazole (ATA) (Sigma). Fertilization envelopes were removed by passing the embryos through a 70 μm mesh size cell strainer (BD Falcon). Embryos were cultured until the 8-cell stage at 15°C and incubated in hyaline extraction medium (HEM, 0.3 M glycine, 0.3 M NaCl, 10 mM KCl, 10 mM MgS04, 10 mM Tris, 2 mM EGTA, pH 8.0) (McClay 1986) for 5 minutes in a 24-well plate. HEM was removed and 2 ml of CFSW was added to the embryos. Embryos were pipetted up and down gently using a glass pasteur pipette under a stereo microscope until all the cells were dissociated. Dissociated cells were cultured until the cells divided and two distinct patterns of cell pairs were evident. These blastomere pairs are easily distinguishable because the mesomere pairs from the animal pole are of equal size whereas the vegetal cells divide to produce unequal large macromere and smaller micromere pairs (Fig. 2.1F). About 2,000 animal pairs and vegetal pairs each were collected separately using a mouth pipette and dissolved in TRI Reagent for RNA extraction.

Micromeres from 16-cell stage embryos were collected using a sucrose gradient as previously described (Sweet, Amemiya et al. 2004). At the fourth cell division, 16-cell-

stage embryos were sequentially incubated in 30 ml HEM and 30 ml CFSW for 15 minutes each. After the incubation in CFSW, 25 ml of the solution was carefully aspirated and embryos were re-suspended in 5 ml of CFSW. The embryo suspension was then sucked in and out of a 10 ml plastic pipette for 5 to 7 times until most of the embryos were dissociated. A 1 ml volume of the suspension of dissociated embryos was layered over a discontinuous sucrose gradient solution consisting of 7.5 ml 4% sucrose solution at the top and 20 ml 8% sucrose solution at the bottom of a 50 ml glass centrifuge tube. Dissociated blastomeres were allowed to settle down in the sucrose gradient solution at 1 × g for one hour on ice. After 1 hour, micromeres formed a layer at the interface between 4% and 8% sucrose solution, while mesomeres, macromeres and undissociated embryos remained in the 8% sucrose solution. The layer of micromeres was then carefully collected with a pipette and pelleted in a 1.5ml centrifuge tube for RNA extraction.

Approximately 10,000 normal 16-cell stage embryos and 10,000 16-cell stage embryos treated with the same solutions used to dissociate the embryos and isolate the micromeres were collected separately as controls for RNA-seq.

RNA Extraction

Total RNA was extracted from the TRI Reagent using the Direct-zol RNA MiniPrep kit (Zymo Research) followed by in-column DNase I digestion according to the manufacturer's instructions. The RNA concentration was determined by Qubit (Thermo Fisher Scientific) and RNA quality was assessed by electrophoresis on denaturing agarose gels prior to submitting the samples for library preparation and RNA sequencing.

Library preparation and sequencing

Libraries were prepared and sequenced by the Beijing Genomics Institute (BGI, Shenzhen, China). rRNA was first depleted using the Ribo-Zero Magnetic Kit (Human/Mouse/Rat) (Illumina). Libraries were constructed using the TruSeq RNA Sample Prep Kit (Illumina) with average insert size of 200bp. Clusters were generated using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) and sequenced on Illumina Hiseq 2000 on three lanes using a high output model to generate 91-bp paired-end reads. Standard quality checks for material degradation and measuring concentrations were done using Bioanalyzer 2100 (Agilent Technologies) and Qubit (Thermo Fisher Scientific) respectively, and the analyses were done before and after library construction (Table 2.1). After sequencing, adapters were removed from the raw reads. Reads in which unknown bases were more than 10% and the percentage of low quality bases was over 50% were also removed to get the clean reads for downstream analysis (Table 2.1).

Short Read mapping

After checking the quality of the clean reads using FastQC version 0.11.2 (Andrews 2010), reads were aligned to the sea urchin transcriptome (Tu, Cameron et al. 2012, Tu, Cameron et al. 2014) downloaded from EchinoBase (www.echinobase.org) using BWA mem version 0.7.10-r789 (Li and Durbin 2009) with default parameters and converted to bam files and sorted using SAMtools version 1.1 (Li, Handsaker et al. 2009). Aligned reads were counted using HTSeq version 0.6.1 (Anders, Pyl et al. 2015) using the following options: htseq-count --format=bam --stranded=no --type=CDS --order=pos --idattr=Name. The gff3 annotation file required for HTSeq counting was generated from the reference

transcriptome sequences using the gmod_fasta2gff3.pl script from the Generic Model Organism Database (gmod.org).

Exploratory analysis

Before running the differential expression analysis, gene counts were loaded using the R Bioconductor package EdgeR version 3.12.0 (Robinson, McCarthy et al. 2010) for exploratory analysis. Low count values with counts per million (CPM) values lower than 5 were removed. After normalization, the relationship of all samples was examined using principal component analysis (PCA). Batch effects associated with four families of adult sea urchins were corrected using the removeBatchEffect() function in EdgeR.

Differential expression analysis

Using the seven different types of samples, I performed 13 different pairwise comparisons (Table 2). In each pairwise comparison, the design matrix was constructed using model.matrix(~ Family + Type), where the Family term specifies which family each of the samples came from and the Type term specifies the sample type of interest to make the comparison. The preferred generalized linear models (GLMs) with a quasi-likelihood (QL) F-test was applied to test the differential expression, which is considered to be more robust and reliable for experiments when the number of replicates is small. To control for the false discovery rate (FDR), the multiplicity correction was performed by applying the Benjamini-Hochberg method on the *P* values. Transcripts with default parameters of *P* values < 0.05 and FDR < 0.05 were considered to be statistically significant. Venn diagrams of different sets of differentially expressed (DE) genes were produced using R

package VennDiagram (version 1.6.16) and heat maps were produced using R package pheatmap (version 1.0.8).

To validate the results from EdgeR using another software package, the differential expression analysis was also performed using R Bioconductor package DESeq2 version 1.10.1 (Love, Huber et al. 2014) using the same design matrix to control the batch effects associated with the adult sea urchin families. In DESeq2, removing low count genes was done by the default option independentFiltering=TRUE in the results() function, which maximizes the number of DE genes by examining different thresholds automatically. The default settings of DESeq2 were applied to the DE analysis.

Gene annotation and gene ontology analysis

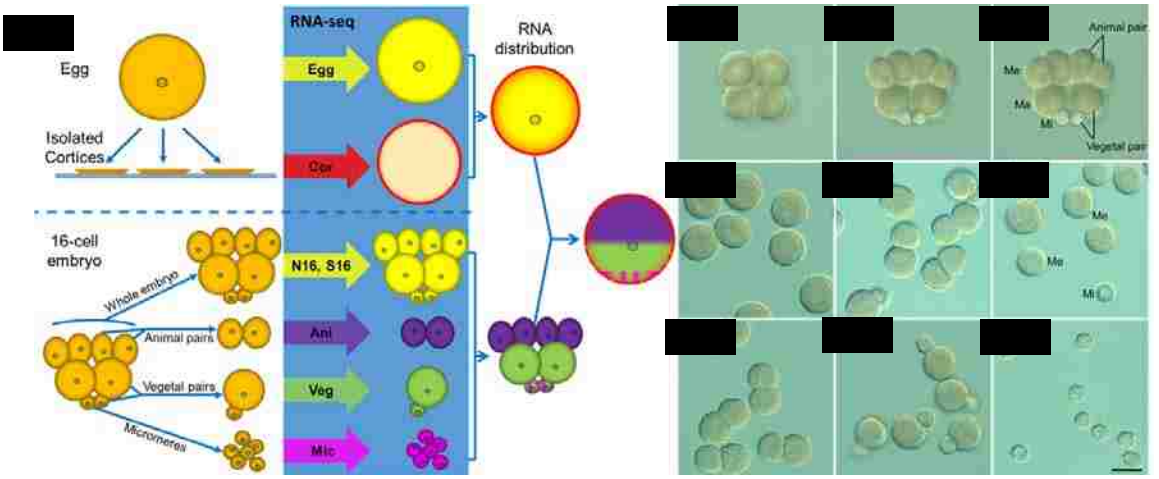
Genes were annotated using EchinoBase (Tu, Cameron et al. 2012, Tu, Cameron et al. 2014) to retrieve SPU identifiers. Gene lists of interest were uploaded to the Gene Ontology Consortium to perform gene ontology (GO) analysis. The reference gene list included genes that were monitored in the DE analysis genes after removing low count genes. Bonferroni correction was applied to control the number of false positives.

Results

RNA-seq analysis of differential RNA expression along the animal-vegetal axis in sea urchin eggs and embryos

To characterize the differential distribution of RNAs along the animal-vegetal axis in sea urchin eggs, especially the RNAs that are enriched in the VCD, I developed a strategy to perform RNA-seq on seven different types of samples (Fig. 2.1A). I predicted that RNA-seq of Egg and Cor samples followed by differential expression (DE) analysis would reveal

transcripts enriched in the cortex of the egg. I further predicted that RNA-seq followed by differential expression analysis of the rest of the five types of samples (N16, S16, Mic, Ani and Veg) would identify transcripts differentially expressed along the animal-vegetal (AV) axis at the 16-cell stage. Finally, I predicted that by combining the DE analysis of these two sets of results, I would be able to identify RNAs differentially distributed along the



AV axis in the egg, and importantly, those RNAs enriched in the VCD at the extreme vegetal pole.

Table 2. 1 Summary of the sequenced three biological replicates from four families.

Sample Type	Family 1	Family 2	Family 3	Family 4
Eggs (Egg)	28.3 M (9.2)	28.0 M (9.4)	31.4 M (9.4)	NS (9.6)
Cortices (Cor)	28.2 M (9.5)	31.5 M (9.5)	32.9 M (9.5)	NS (9.3)
Normal 16-cell embryos (N16)	29.6 M (9.1)	31.6 M (8.9)	28.4 M (9.6)	NS (9.1)
Treated 16-cell embryos (S16)	33.2 M (9.2)	29.5 M (9.5)	30.4 M (9.4)	NS (9.5)
Micromeres (Mic)	30.6 M (9.9)	31.3 M (9.4)	29.9 M (9.5)	L (9.6)
Animal pairs (Ani)	27.8 M (9.9)	26.4 M (9.8)	25.4 M (9.1)	NS (9.9)
Vegetal pairs (Veg)	24.9 M (10.0)	27.9 M (9.4)	L (7.9)	23.9 M (9.9)

Three biological replicates for seven different types of samples from four families have been sequenced. Numbers indicate the millions of reads that have been produced. Numbers in brackets are RNA Integrity Numbers (RINs) of the samples. M: million reads; L: low amount of RNA and not sequenced. NS: not sequenced.

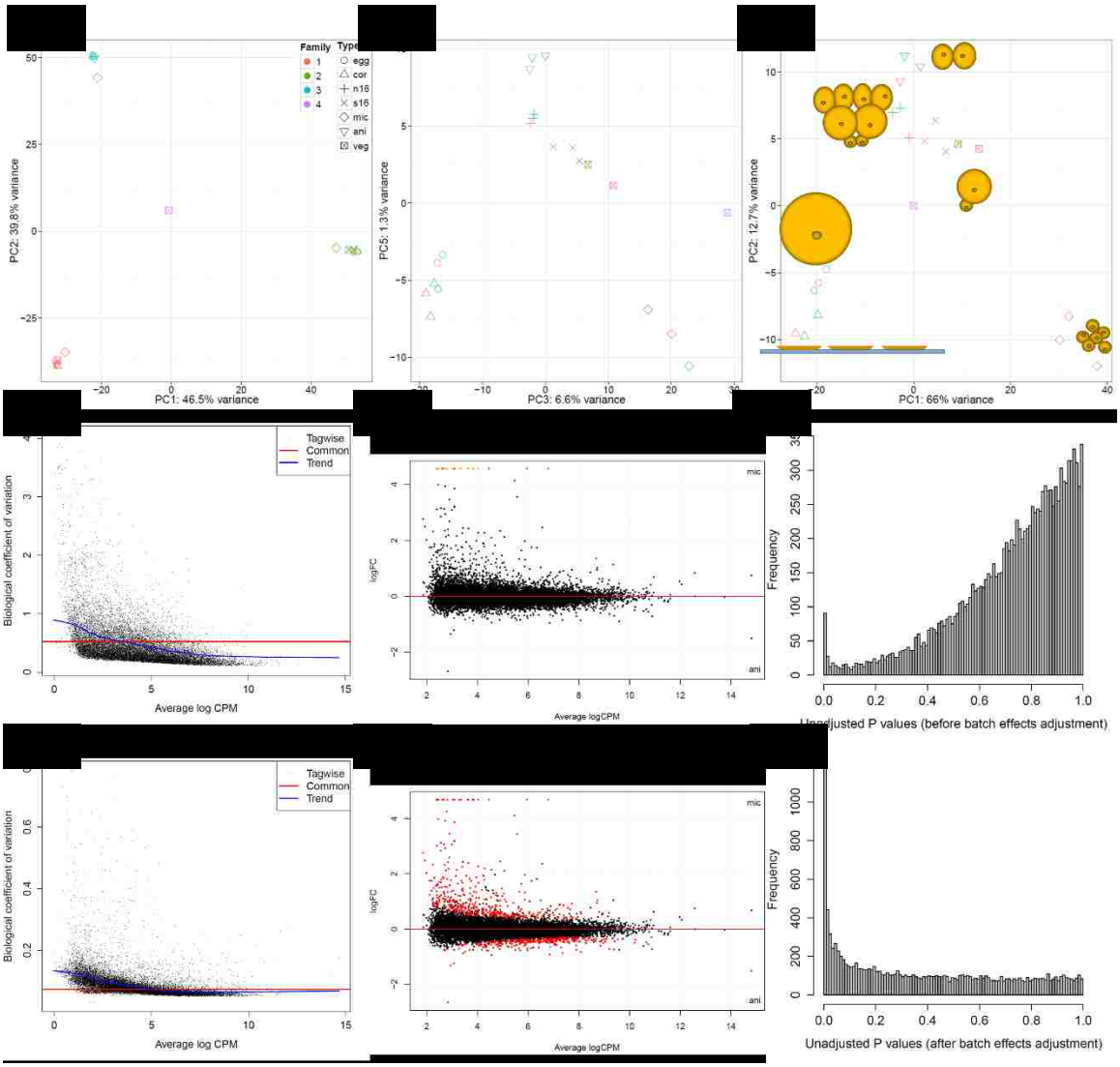
I collected four biological replicates from each of four families of adult sea urchins, respectively, for each of the seven types of sample (Table 2.1) and chose three replicates for each type of sample based on assessment of both high RNA concentration and quality for library preparation. Note that out of 21 samples, 20 of them are from the first three families whereas the third biological replicate of Veg sample was from the fourth family (Table 2.1). After filtering low quality reads, I obtained 611 million high quality 91-bp paired-end reads, for an average of 29 ± 2.6 million reads per sample (Table 2.1). Reads were mapped to the previously reported sea urchin transcriptome (Tu, Cameron et al. 2012, Tu, Cameron et al. 2014) using BWA mem (Li and Durbin 2009), with an average of 13.7

Figure 2. 2 **Principal component analysis and correction of batch effects**
(A) Principal component analysis (PCA) plot of all 21 samples showing that samples from the same family are clustered together, indicating large batch effects could be caused by the variations between different families. The two greatest PCs account for 86.3% (46.5% + 39.8%) of the variance. **(B)** PCA plot showing PC3 and PC5 are associated with the biological differences of interest, i.e. the difference across different types of sample. But the percent variance explained is low (7.9%, PC3 + PC5). **(C)** PCA plot after batch effects correction showing the samples of the same type are clustered together, with the two greatest PCs accounting for 78.7% (66.0% + 12.7%) of the variance. Symbols of different sample types are shown in the plot. **(D)** and **(G)** Plots of biological coefficient of variation (BCV) of all samples, before and after batch effects correction, respectively. After batch effects correction, the value of BCV decreases to a reasonable level. **(E)** and **(H)** MA plots of a pairwise comparison, Mic vs Ani, before and after batch effects correction, respectively, showing that after batch effects correction, the number of statistically significantly differentially expressed genes, color-coded in red, increases dramatically. **(F)** and **(I)** Histograms of unadjusted *P* values from (E) and (H), respectively, showing an expected peak at the left side and a uniform distribution of all null *P* values between 0 and 1 after batch effects correction, indicating that the statistical tests have worked successfully.

± 1.7 million reads mapped to the transcriptome. The values were expected because the available reference transcriptome was based on sequencing of only poly(A)+ RNA, where poly(A)- RNA was absent from the reference. Because I used the rRNA depletion method to prepare the library, where both poly(A)+ and poly(A)- RNA were present, only the reads from poly(A)+ could be mapped to the available reference transcriptome.

Strong batch effects associated with variations across sea urchin families and its correction for RNA-seq data analysis

To uncover the main source of variation, I performed principal component analysis (PCA) on normalized and log transformed counts per million values (CPM). Strikingly, the top principal components (PCs) were associated with the adult sea urchin families rather than the sample types, indicating that transcriptome heterogeneity in the sample is dominated by variations across different adult sea urchin pairs. The two greatest PCs account for 86.3% (46.5% + 39.8%) of the variance (Fig. 2.2A). PC3 and PC5 were



associated with the sample types, but they only accounted for 7.9% of variation (Fig. 2.2B). The batch effects were successfully corrected by applying the removeBatchEffect() function in the EdgeR package (Robinson, McCarthy et al. 2010), and the resulting PCA plot showed a very similar pattern with that in Fig. 2.2B and two greatest PCs accounting for 78.7% (66.0% + 12.7%) of the variance, indicating that the batch effects were successfully corrected (Fig. 2.2C).

Biological coefficient of variation (BCV) plots of all samples showed that after the batch effects correction, the BCV decreased to a reasonable value with a common BCV of 0.07 (Fig. 2.2D and G). After batch effects correction, more DE genes were identified by EdgeR, compared to the DE genes identified prior to the correction. MA plots (plots log-fold change versus log-concentration (or, M versus A) for count data) of a typical example of a pairwise comparison between Mic sample and Ani sample are shown in Fig. 2.2E and H. Post-differential analysis sanity check using the histogram of unadjusted *P* values of Mic vs Ani also showed the expected pattern after batch effects correction, with a peak close to zero and a uniform distribution of all null *P* values between zero and one (Fig. 2.2F and I). These results together indicate that the batch effects had been successfully controlled and additionally, that the DE genes could be confidently identified.

Table 2. 2 Summary of significantly differentially expressed/distributed transcripts.

Pairwise Comparison	Up	Down	Total
Cor vs Egg	431	268	699
Mic vs N16	1,212	1,502	2,714
Mic vs S16	576	606	1,182
Mic vs Ani	413	303	716
Mic vs Veg	457	522	979
Veg vs N16	37	0	37
Veg vs S16	37	54	91
Veg vs Ani	30	35	65
Ani vs N16	15	6	21
Ani vs S16	42	94	136
S16 vs N16	14	0	14
N16 vs Egg	107	115	222
S16 vs Egg	53	98	151

Cor: Cortex; N16: Normal 16-cell embryos; S16: Sucrose treated 16-cell embryos; Mic: Micromeres; Ani: Animal pairs; Veg: Vegetal pairs.

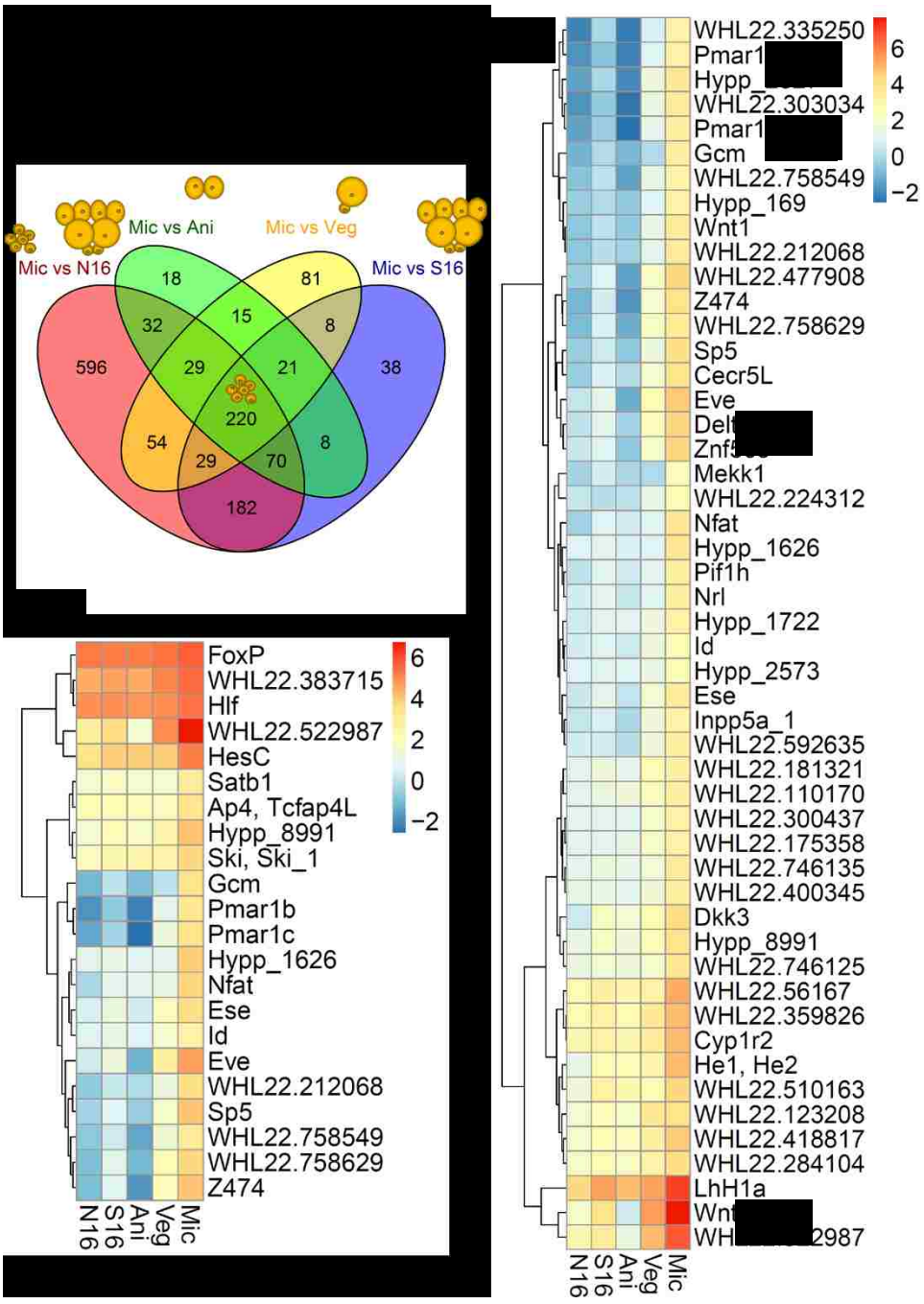
After performing the batch effects correction, I carried out 13 pairwise comparisons with threshold of *P* values < 0.05 and false positive rate (FDR) < 0.05 across the seven

types of samples and the results are summarized in Table 2.2. In general, S16 vs N16 gives only 14 DE genes, indicating that the blastomere isolation procedure does not substantially alter blastomere transcriptomes. From the pairwise comparisons between the different sample types, most of the DE genes seen in the analyses came from comparison of Mic samples with other samples.

The differential expression in micromeres is consistent with the known rapid activation of beta-catenin-dependent zygotic transcription in the 16-cell stage micromeres, and the central role of these cells in endomesoderm specification (Oliveri, Carrick et al. 2002, Wikramanayake, Peterson et al. 2004, Oliveri, Tu et al. 2008, Peter and Davidson 2010, Peter and Davidson 2011). I also identified DE genes in the Ani sample, indicating that there may be low differential transcription activity in the animal half at the 16-cell stage. My analysis also identified 431 transcripts that are potentially enriched in the sea urchin egg cortex in comparison to the whole egg.

Transcripts differentially expressed in micromeres at the 16-cell stage.

The 16-cell stage micromeres act as a signaling center to specify endomesoderm in sea urchin embryos (Ransick and Davidson 1993, Ransick and Davidson 1995, Oliveri, Carrick et al. 2002). Moreover, there is evidence that the signaling activity of the micromeres depends on nuclear beta-catenin (Logan, Miller et al. 1999). Hence, one expectation from this analysis was that I would identify genes that are early targets of nuclear beta-catenin with potential roles in endomesoderm specification. Consistent with my expectations, I identified 220 genes that were significantly upregulated in the micromeres (Fig. 2.2A). Additionally, these 220 upregulated transcripts include those previously reported to play key roles in micromeres to regulate the endomesoderm gene regulatory networks (GRN),


Mic vs Ani
Mic vs Veg
Mic vs N16
Mic vs S16
18
81
15
32
8
596
29
21
38
220
54
8
29
70
182
FoxP
WHL22.383715
Hlf
WHL22.522987
HesC
Satb1
Ap4, Tcfap4L
Hypp_8991
Ski, Ski_1
Gcm
Pmar1b
Pmar1c
Hypp_1626
Nfat
Ese
Id
Eve
WHL22.212068
Sp5
WHL22.758549
WHL22.758629
Z474
N16
S16
Ani
Veg
Mic
6
4
2
0
−2
WHL22.335250
Pmar1
Hypp_
WHL22.303034
Pmar1
Gcm
WHL22.758549
Hypp_169
Wnt1
WHL22.212068
WHL22.477908
Z474
WHL22.758629
Sp5
Cecr5L
Eve
Del
Znf
Mekk1
WHL22.224312
Nfat
Hypp_1626
Pif1h
Nrl
Hypp_1722
Id
Hypp_2573
Ese
Inpp5a_1
WHL22.592635
WHL22.181321
WHL22.110170
WHL22.300437
WHL22.175358
WHL22.746135
WHL22.400345
Dkk3
Hypp_8991
WHL22.746125
WHL22.56167
WHL22.359826
Cyp1r2
He1, He2
WHL22.510163
WHL22.123208
WHL22.418817
WHL22.284104
LhH1a
Wnt
WH
2987
N16
S16
Ani
Veg
Mic
6
4
2
0
−2

such as *delta* (Sweet, Hodor et al. 1999, Sweet, Gehring et al. 2002), *pmar1* (Oliveri, Carrick et al. 2002) and *wnt8* (Wikramanayake, Peterson et al. 2004), indicating our RNA-seq screen was effective at identifying early regulators of the micromere gene regulatory network. Additionally, gene ontology (GO) analysis showed that multiple GO terms are enriched in this list of 220 micromere DE genes (Table 2.3). Some of the enriched terms are Positive regulation of protein modification process (GO:0031401), Regulation of cellular metabolic process (GO:0031323), Regulation of transcription, DNA-templated (GO:0006355) and Transcription factor (PC00218), which are consistent with the

Table 2. 3 Gene ontology (GO) analysis of DE genes in micromeres

GO biological process	Count in ref. list	Count in data set	Expected count	Fold enrichment	Bonferroni corrected P-value
Positive regulation of JUN kinase activity (GO:0043507)	3	3	0.05	63.76	1.68E-02
Positive regulation of transferase activity (GO:0051347)	36	6	0.56	10.63	2.64E-02
Positive regulation of protein modification process (GO:0031401)	49	7	0.77	9.11	1.49E-02
Regulation of transferase activity (GO:0051338)	68	9	1.07	8.44	1.64E-03
Regulation of cellular metabolic process (GO:0031323)	608	30	9.54	3.15	2.56E-05
Regulation of nucleobase-containing compound metabolic process (GO:0019219)	437	21	6.85	3.06	5.96E-03
Regulation of transcription, DNA-templated (GO:0006355)	401	19	6.29	3.02	2.07E-02
Regulation of nucleic acid-templated transcription (GO:1903506)	413	19	6.48	2.93	3.09E-02
PANTHER GO-Slim Molecular Function	**Count in ref. list**	**Count in data set**	**Expected count**	**Fold enrichment**	**Bonferroni corrected P-value**
Nucleic acid binding transcription factor activity (GO:0001071)	384	18	6.02	2.99	5.92E-03
Sequence-specific DNA binding transcription factor activity (GO:0003700)	369	17	5.79	2.94	1.21E-02
PANTHER Protein Class	**Count in ref. list**	**Count in data set**	**Expected count**	**Fold enrichment**	**Bonferroni corrected P-value**
Transcription factor (PC00218)	407	19	6.38	2.98	4.67E-03

micromeres' role as an early organizing center in the embryo. The differential expression pattern of the top 50 micromere DE genes and all transcription factors annotated by EchinoBase and PANTHER (Mi, Poudel et al. 2016) are shown in Fig. 2.2B and C.

Transcripts differentially enriched in the vegetal cortical domain.

The egg cortex is an essential subcellular region for maternal RNA localization and embryo development in *Xenopus* and ascidian embryos (Sardet, Paix et al. 2007, Houston 2012). By performing DE analysis on Cor and Egg samples, I have identified 431 transcripts that are enriched in the cortex (*P* values < 0.05, FDR < 0.05) (Table 2.1). GO analysis shows that terms such as Microtubule motor activity (GO:0003777), Developmental process (GO:0032502), Membrane-bound signaling molecule (PC00152), Microtubule binding motor protein (PC00156) and Signaling molecule (PC00207) are significantly enriched (Table 2.4), consistent with the expected molecular functions or involved biological processes of the molecules enriched in the egg cortex. The top 50 of these transcripts are listed in Fig. 2.4A, in descending order of fold change. Venn diagram analysis showed that eight genes are common with the set of micromere DE genes, providing evidence for possible maternal RNAs that could be enriched in the vegetal cortical domain (VCD) (Fig. 2.4B). To further confirm this finding made using EdgeR, I also performed the DE analysis using the DESeq2 package (Love, Huber et al. 2014) with the same design matrix and default settings. Five of these eight transcripts were also identified using DESeq2, giving me more confidence that these transcripts are enriched in the VCD (Fig. 2.4C). These five RNA transcripts are *Bcl9, Herc1, Alms1L* and two different transcripts from *Polq*. Plots of normalized counts across all seven types of samples for these five transcripts are shown in Fig. 2.4D to H, where two peaks at the Cor and Mic samples can be observed.

Table 2. 4 Gene ontology (GO) analysis of DE genes in the cortex

PANTHER GO-Slim Molecular Function	Count in ref. list	Count in data set	Expected count	Fold enrichment	Bonferroni corrected P-value
microtubule motor activity (GO:0003777)	20	6	0.8	7.49	2.68E-02
PANTHER GO-Slim Biological Process	**Count in ref. list**	**Count in data set**	**Expected count**	**Fold enrichment**	**Bonferroni corrected P-value**
immune system process (GO:0002376)	124	15	4.96	3.02	3.36E-02
developmental process (GO:0032502)	276	26	11.05	2.35	1.19E-02
PANTHER Protein Class	**Count in ref. list**	**Count in data set**	**Expected count**	**Fold enrichment**	**Bonferroni corrected P-value**
membrane-bound signaling molecule (PC00152)	61	12	2.44	4.91	1.66E-03
microtubule binding motor protein (PC00156)	49	9	1.96	4.59	3.60E-02
signaling molecule (PC00207)	214	22	8.57	2.57	1.29E-02

Discussion

It has long been appreciated that localized RNAs are essential for embryonic axis formation and cell fate specification in multiple species (Medioni, Mowry et al. 2012). The work reported in this chapter represents the first transcriptomic study on isolated cortices and blastomeres from the 16-cell stage embryos to identify differentially expressed or distributed RNAs along the AV axis in sea urchins. The results of this study offers the opportunity to unravel the fundamental localized RNA determinants shared among many species, as well as those uniquely localized RNAs specific to sea urchins.

The first rRNA depletion RNA-seq study in sea urchins

In the sea urchin *S. purpuratus,* about 85% of maternal RNAs are rRNAs (Goustin 1981), which, if not removed from the sample, will hamper RNA-seq data analysis. In the

[redacted]

Gene name	Fold Change	FDR
Hypp_442	1.9	1.7E-03
Glul	1.6	1.3E-02
MifL1	1.6	7.9E-08
GtpbpL2	1.6	3.0E-04
WHL22.341657	1.6	1.4E-02
Herc2	1.6	5.3E-04
Herc1	1.6	4.2E-03
WHL22.576938	1.5	5.3E-03
Flot1	1.5	7.4E-03
Herc1L_2	1.5	3.1E-02
Ddx51	1.5	3.9E-02
Magt1	1.5	4.1E-07
WHL22.647930	1.5	2.3E-02
Axndhch	1.5	4.3E-02
Mpdz	1.5	9.0E-05
Talpid3	1.5	6.5E-03
Nup98	1.5	8.8E-03
WHL22.248691	1.4	5.6E-05
WHL22.556319	1.4	1.6E-02
Lzic	1.4	4.3E-03
Fras1	1.4	6.7E-03
Cpsf6L	1.4	5.9E-04
Thr, Thrb	1.4	1.1E-02
Ttf	1.4	3.6E-03
WHL22.232024	1.4	2.6E-07
WHL22.434992	1.4	1.2E-02
Cbfb	1.4	1.4E-02
WHL22.435005	1.4	2.6E-03
WHL22.697134	1.4	3.3E-05
Pric2	1.4	3.6E-03
WHL22.280925	1.4	6.4E-04
Gnn	1.4	1.4E-02
Flnb	1.4	2.1E-02
WHL22.434995	1.4	3.2E-03
Nwd1	1.4	4.2E-02
Sptlc2_1	1.4	1.1E-06
Sfrs1L	1.4	3.9E-06
Mpdz	1.4	3.2E-03
Nbc	1.4	7.0E-03
Ahctf1L	1.4	3.7E-02
MifL2	1.4	4.0E-04
WHL22.556449	1.4	3.5E-04
Trrap_3	1.4	3.3E-02
Hypp_278	1.4	1.3E-03
WHL22.136737	1.4	1.0E-02
Lama5Lf	1.4	1.3E-02
Axndhch	1.3	1.1E-02
Dnah1_1	1.3	1.5E-02
WHL22.209793	1.3	4.0E-03
WHL22.617358	1.3	4.4E-02

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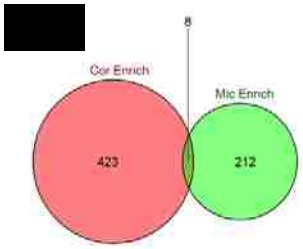

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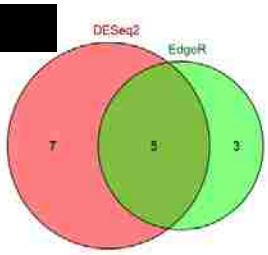

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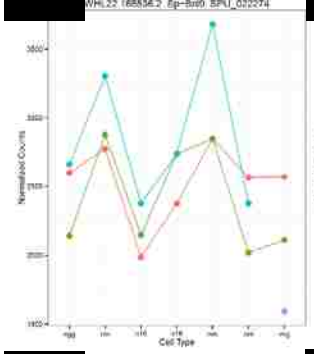

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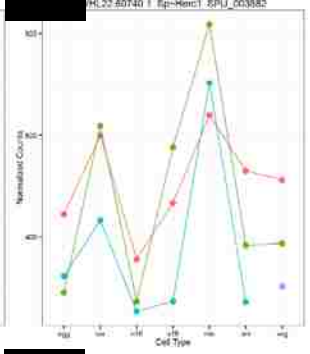

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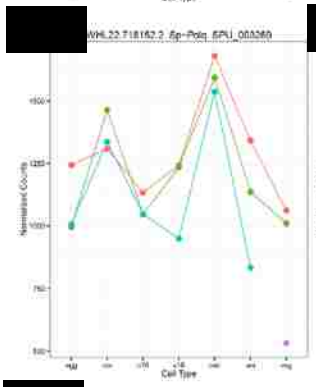

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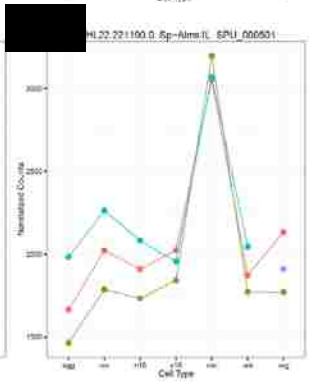

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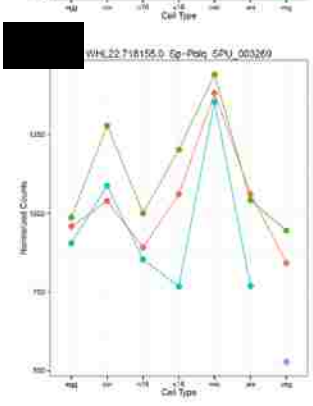

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sea urchin *Tripneustes gratilla*, it has been observed that more than half of maternal RNAs by mass are non-polyadenylated mRNAs (Duncan and Humphreys 1981) suggesting that using the popular poly-A selection method for library preparation may lead to a substantial loss of non-polyadenylated mRNAs. Therefore, to identify any non-rRNA molecules differentially distributed along the primary egg axis, I depleted rRNAs while keeping non-polyadenylated mRNAs, as well as other non-polyadenylated RNAs for the RNA-seq analysis.

Of the total of 611 million reads, only 7 million (2.4%) reads were mapped to an rRNA precursor transcript WHL22.598028 (data not shown), which encodes the 18S, 5.8S and 28S rRNAs, indicating that the majority of the rRNAs were successfully depleted by using the Ribo-Zero (Human/Mouse/Rat) kit. Besides the transcriptome comparisons done in the current study, this set of data would also be valuable for the study of maternal non-polyadenylated mRNAs.

Correcting strong batch effects associated with transcriptomes of sea urchin families

Aiming to obtain robust unbiased transcriptomes from different samples, I designed the experiment prudently and took precautions to avoid experimental variation. Hence, to limit variation associated with time or developmental stage, embryos were collected at the same stage, with all RNAs from each of four families collected at the same time and with multiplexed samples from the same family distributed to different lanes when possible. Still, unexpectedly strong batch effects were detected and found to be associated with the different families of adult sea urchins (Fig. 2.2A), resulting in high variation, few DE genes and problematic histogram of *P* values (Fig. 2.2D-F). To the best of our knowledge, similar strong batch effects have not been reported in RNA-seq studies with embryos from sea

urchins or other animals. One possible explanation is that each female adult sea urchin deposits excess maternal RNAs and other components to the oocytes during oogenesis and the levels of the components can vary as long as they meet the minimum requirement for the offspring embryos to develop. Considering that we are using wild-caught sea urchins, other factors, such as the high level of variation across different individuals, ages and environmental factors, may also contribute to the high batch effects. High-throughput sequencing technologies are becoming the preferred approach to identify DE genes but very little attention has been paid to the various factors that can cause batch effects. Fortunately, after careful data analysis using both EdgeR and DESeq2, I was able to correct the batch effects and identify several interesting DE genes. Our data have provided a useful case to study the source of batch effects and to identify ways to correct for unavoidable sources of natural variation.

New candidate players in the endomesoderm gene regulatory network (GRN)

The micromeres in sea urchin embryos play two fundamental roles in the endomesoderm GRN: they induce the macromere lineage to form endomesodermal tissues through signaling and they give rise to the skeletogenic mesenchyme cells that produce the spicules of the larva. In addition, at the 5th cell division, the micromeres divide to give rise to the large micromeres, and the small micromeres which contribute to the germline in sea urchins. My analysis successfully captured some of the known early key regulators to these processes, including the Wnt/β-catenin target genes *wnt8*, *pmar1* and *delta*. *delta* plays an essential role in the development of non-skeletogenic mesoderm and it was previously considered to be transcribed a few cleavages later after 16-cell stage (Sweet, Gehring et al. 2002). It seems that our RNA-seq approach is more sensitive than RT-PCR and *in situ*

hybridization to detect low level of zygotic transcription in micromeres. Furthermore, some of the identified DE genes, especially those encoding transcriptional factors and/or signaling factors, are potentially involved in the endomesoderm GRN. The gene *sp5*, for example, has been reported to be a direct target of the Wnt/β-catenin pathway and regulates patterning in Zebrafish and *Xenopus* embryos and embryonic stem cell self-renewal in mouse embryos (Weidinger, Thorpe et al. 2005, Park, Seo et al. 2013, Ye, Zhang et al. 2016). Functional analysis of this gene, together with other transcription factors or signaling genes, will definitely provide useful insights into the upstream regulation of the early endomesoderm GRN.

Asymmetrically distributed maternal RNAs in the VCD as potential determinants

Of the 5 transcripts identified to be potentially VCD enriched by both EdgeR and DESeq2, the most exciting one is *Bcl9*. The function of *Bcl9* was first characterized in *Drosophila*. The *Drosophila* homolog *legless* is a segment polarity gene and its product interacts with β-catenin physically to activate Wnt target gene transcription (Kramps, Peter et al. 2002). In mammals, there are two orthologs of *Bcl9*, *Bcl9* and *Bcl9-2*. The product of *Bcl9-2* is reported to act as a switch between β-catenin's adhesive function and transcriptional function (Brembeck, Schwarz-Romond et al. 2004). Prior to accumulating in nuclei of micromeres at the 16-cell stage in sea urchin embryos, β-catenin is ubiquitously distributed at adherens junctions (Miller and McClay 1997, Logan, Miller et al. 1999). Hence, it is tempting to speculate that the product of VCD-enriched *Bcl9* is involved in regulating the temporal accumulation of β-catenin in micromeres. This could potentially be achieved by a control of its translation timing. Additional work will be required to

further examine this possibility and the role of other VCD enriched maternal RNAs in regulating early patterning in the sea urchin.

herc1 encodes a large protein with an E3 ubiquitin ligase homology to E6AP carboxyl terminus (HECT) domain. E3 ligases usually dictate the specificity of ubiquitylation for protein degradation as well as regulating trafficking of many transmembrane proteins such as receptors, channels and transporters (Rotin and Kumar 2009). In *Xenopus*, the mRNA of a HECT subclass of E3 ubiquitin ligases, *smurf1*, was identified to be localized at the animal pole of the egg and regulate BMP signaling and the patterning of the embryos (Zhu, Kavsak et al. 1999). *herc1* is the first identified localized E3 ligase mRNA in early sea urchin embryos. Based on these data, I speculate that the Herc1 E3 ligase could regulate the stability or trafficking of certain key proteins at the vegetal pole, contributing to embryo polarity.

Taken together, our transcriptome data have provided useful insights into transcripts differentially expressed in the micromeres and candidate determinants enriched in the vegetal cortex of the egg. Moreover, our data will be a valuable resource for the sea urchin community to study the maternal RNAs and early input for the endomesoderm gene regulatory networks.

Chapter 3: Identification of Dsh-interacting proteins in sea urchin eggs and embryos, and their potential roles in regulating Dsh "activity"

Background

Animal-vegetal (AV) axis specification and patterning has been an area of interest to embryologists for over a century because of the fascinating molecular mechanisms that underlie these processes. In most animal embryos, the cells derived from the animal pole give rise to ectoderm and the cells derived from the vegetal pole give rise to the endomesoderm, so that in general the AV axis determines the AP axis in the adult (Martindale 2005, Martindale and Hejnol 2009, Petersen and Reddien 2009, Niehrs 2010, Wikramanayake 2013). Recent work has established that the Wnt/β-catenin pathway plays a conserved role in specifying the vegetal/posterior end of the embryos. For example, as described in Chapter 1, Wnt/β-catenin signaling is required for the development of the vegetal/posterior end of embryos in the protostome worm *Cerebratulus lacteus* (Henry, Perry et al. 2008), and in the deuterostomes including echinoderms (Wikramanayake, Huang et al. 1998b, Logan, Miller et al. 1999, McCauley, Akyar et al. 2015), and hemichordates (Darras, Gerhart et al. 2011) as well as many other animals (reviewed in Petersen and Reddien 2009, Niehrs 2010, Hikasa and Sokol 2013). How this conserved process is precisely regulated spatiotemporally, however, remains poorly understood in most animals.

Sea urchin embryos have a rich history as a model system to study the molecular mechanisms underlying AV axis specification. In 1892, Herbst first found that lithium chloride can enhance vegetal cell fates in sea urchin embryos along the AV axis (Herbst 1892). We now known that lithium activates Wnt signaling by inhibiting the GSK-3β, a negative regulator of the pathway (Klein and Melton 1996). Following that, lithium has

been shown to strongly influence embryo development in diverse animals, including various cnidarians, tunicates, zebrafish and *Xenopus* (Kao, Masui et al. 1986, Hassel, Albert et al. 1993, Stachel, Grunwald et al. 1993, Yoshida, Marikawa et al. 1998, Wikramanayake, Hong et al. 2003).

The sea urchin embryo has provided a useful model to study how early cell fates are specified by Wnt/β-catenin pathway during early embryogenesis, and to understand how this pathway is differentially regulated along the primary axis. Several years ago, work done in three laboratories demonstrated the importance of the Wnt/β-catenin pathway for endomesoderm specification in early sea urchin embryos (Emily-Fenouil, Ghiglione et al. 1998, Wikramanayake, Huang et al. 1998b, Logan, Miller et al. 1999). In particular, Wikramanayake, Huang *et al.* demonstrated that Wnt/β-catenin signaling is required for AV axis patterning and endomesoderm specification at the vegetal pole. They demonstrated that over-expression of a constitutively active form of β-catenin vegetalized the embryos, resulting in excess endomesodermal cells, while blocking nuclear β-catenin with C-cadherin over-expression animalized the embryos, causing complete loss of endomesoderm, suggesting that nuclear β-catenin is both required and sufficient to regulate the endomesoderm specification in the early sea urchin embryo (Wikramanayake, Huang et al. 1998b). Furthermore, Logan, Miller *et al.* showed that the transcriptional co-activator, β-catenin, started to accumulate in the nuclei of blastomeres at the vegetal pole (Logan, Miller et al. 1999). Recent work described below has provided key insight into how the Wnt/β-catenin pathway is selectively activated at the vegetal/posterior pole during early development.

The Dishevelled (Dsh) protein is an important mediator of Wnt signaling (reviewed by Gao and Chen 2010). Recent work in sea urchin embryos has established that this protein plays a central role in regulating localized Wnt/β-catenin signaling at the vegetal pole. Interestingly, while over-expression of the dominant-negative form of Dsh blocks nuclear β-catenin at the vegetal pole, the ectopic expression of full length Dsh in animal-half blastomeres fails to drive β-catenin nuclear entry, suggesting that Dsh itself is required but not sufficient for localized activation of Wnt signaling (Weitzel, Illies et al. 2004). One hypothesis is that the Dsh protein at the animal pole, either overexpressed or endogenous, is not active and consequently, there must be other molecules involved in activating Dsh locally at the vegetal pole (Weitzel, Illies et al. 2004). In accordance with this hypothesis, a recent study has shown that Dsh is highly enriched at the vegetal cortical domain (VCD) and that this the VCD-enriched pool of Dsh is differentially post-translationally modified (Peng and Wikramanayake 2013). The VCD and the post-translationally modified pool of Dsh is inherited by those cells that activate Wnt/β-cat signaling at the vegetal pole (Peng and Wikramanayake 2013). This modified form of Dsh could be responsible for its localization and local regulation of nuclear β-catenin activity. The nature and the molecular mechanism of the modification, however, remain unknown. Based on the observations from these two studies, it is necessary to identify the molecules that interact with and regulate Dsh before nuclear β-catenin is activated.

In this study, I proposed to use Dsh co-immunoprecipitation (Co-IP) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) to identify the proteins that interact with Dsh, both directly or indirectly. The roles of interaction proteins in regulating Dsh localization and/or activity will be examined during early sea urchin embryo

development. Using these procedures, I have identified 326 proteins from egg and 80 proteins from cortex that were co-immunoprecipitated with Dsh. I have identified both known Dsh-interacting proteins and potentially novel proteins (yet to be validated). Follow-up functional studies provided evidence that one Dsh-interacting protein, DIXDC1, may play a role in endomesoderm specification via regulating nuclear β-catenin. I also discovered two other proteins, Fmi and p-EEF2, that co-localize with Dsh at the vegetal cortical domain (VCD), providing promising candidates for future studies.

Materials and Methods

Animal handling, gamete collection and microinjection

Adult *Strongylocentrotus purpuratus* were obtained from Marinus, Garden Grove, CA, and maintained in a seawater aquarium at 15°C. Gametes and embryos were obtained as previously described (Peng and Wikramanayake 2013).

For microinjection, 100 to 200 dejellied eggs were placed in a row on a 1% protamine sulfate-coated 60 mm plastic petri dish lid. The seawater had a final concentration of 0.5 mM amino-3-4 triazole (ATA) to prevent fertilization envelopes from hardening. Synthesized mRNAs were prepared to the required concentrations containing 20% to 40% glycerol (v/v). Eggs were then fertilized and microinjected as previously described (Bince and Wikramanayake 2008).

Dsh Co-Immunoprecipitation (Co-IP)

Dsh Co-IP was done using the Pierce Crosslink IP Kit (Catalog number: 26147; Thermo Fisher Scientific) following the manufacturer's instructions with the following modifications. Between 20 µg to 100 µg rabbit anti-Dsh-DIX antibody (Peng and

Wikramanayake, 2013) was used for crosslinking per column, and between 20 µg to 100 µg rabbit normal IgG was used for each control column. About 20 mg to 50 mg eggs or embryos were used for the lysate that was applied per column. For cortex Co-IP, egg cortices isolated from 20 to 30 35-mm petri dishes were solubilized using lysis buffer and applied per column. After column pre-clearing and antigen immunoprecipitation, bound proteins were eluted with 50 to 100 µl Elution Buffer provided by the Pierce Crosslink IP Kit for further analysis.

For the RNase A treatment experiment to investigate potential protein-RNA interactions, RNase A (Catalog number: EN0531; Thermo Fisher Scientific) at a final concentration of 5 µg/µl was added to the lysis buffer.

SDS-PAGE, silver staining and Western blot analysis

One tenth of the Co-IP eluate was mixed with Sample Buffer (Thermo Fisher Scientific), boiled for 5 minutes and resolved on 0.75 mm gels using SDS-PAGE followed by silver staining or Western blot analysis. Silver staining was done using the Pierce Silver Stain Kit (Catalog number: 24612; Thermo Fisher Scientific) following the manufacturer's instructions. Western blot analysis was done as previously described (Peng and Wikramanayake 2013).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of Dsh Co-IP samples

Dsh Co-IP samples resolved using SDS-PAGE and visualized with silver staining were assessed for quality control. Only the samples that showed clear, differential bands in control and Co-IP groups were used for the next step. The eluates were then trichloroacetic

acid (TCA) precipitated using the ProteoExtract Protein Precipitation Kit (Catalog number: 539180-1KIT; EMD Millipore) and sent to the Taplin Biological Mass Spectrometry Facility at Harvard University for LC-MS/MS.

Immunostaining and image analysis

Immunostaining and image analysis was done as previously described (Peng and Wikramanayake 2013). The mouse anti-DIXDC1 monoclonal antibodies were custom antibodies generated by Abmart (Shanghai, China), using the synthetic peptides GGGKEGSNSWEE and AHYKPSSVKQAS as immunogens and affinity purified. The mouse anti-Fmi monoclonal antibody was generated against the Fmi cytoplasmic domain. Rabbit anti-*Hemicentrotus pulcherrimus* β-catenin polyclonal antibody was a gift from the Yazaki lab (Yazaki, Tsurugaya et al. 2015). Rabbit anti-phospho-EEF2(pThr56) was purchased from Thermo Fisher Scientific (PA5-17839). Antibody concentrations used for this study are: anti-DIXDC1 (1:200), anti-β-catenin (1:10,000) (Yazaki, Tsurugaya et al. 2015), anti-Fmi (1:300), anti-phospho-EEF2 (1:200).

For cortex immunostaining, cortices were first isolated on a poly-L-lysine coated coverslip and fixed in 4% paraformaldehyde for 15 minutes on ice in a glass coplin staining jar. After three washes (10 minutes each) with ice cold blocking buffer (Phosphate-buffered saline (PBS) containing 0.01% bovine serum albumin (BSA)) the samples were incubated with the antibodies diluted in ice cold blocking buffer for 1 hour on ice. For double labeling, rabbit anti-Dsh antibody was mixed with mouse anti-Fmi or mouse anti-p-EEF2 antibody. After another three washes with blocking buffer, samples were incubated with 1:1000 Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 568 conjugate (Thermo Fisher Scientific, A11011) and 1:1000 Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa

Fluor® 488 conjugate (Thermo Fisher Scientific, A11001) in blocking buffer for 45 minutes on ice. After three washes with ice cold PBS, samples were mounted on slides and imaged using a Leica TCS SP5 Confocal Laser Scanning Microscope.

Whole-mount embryo staining was done as previously described (Peng and Wikramanayake 2013, Yazaki, Tsurugaya et al. 2015)

cDNA construction and RNA synthesis

DIXDC1 full length coding sequence was PCR-amplified using egg cDNA and cloned into the pCS2+ vector at the BamH1 and EcoR1 sites using following primers:

Forward: 5'-GCGGATCCACCATGACAACTAGGGCATC

Reverse: 5'-CGGAATTCAGCCCGTGTCTTCATCC

The C-terminal DIX domain of DIXDC1 alone without the N-terminal has been shown to function as a dominant-negative form of DIXDC1 (Shiomi, Uchida et al. 2003). The DIXDC1 dominant-negative (DN) construct was PCR-amplified using egg cDNA and cloned into the pCS2+GFP vector at BamH1 and EcoR1 sites using the following primers:

Forward:5'-GTGCGGATCCATGAATAATAACCTATCCCAGCC

Reverse: 5'-TCACATCGATATCCTCCGCCCGTGTCTTCATCCACC

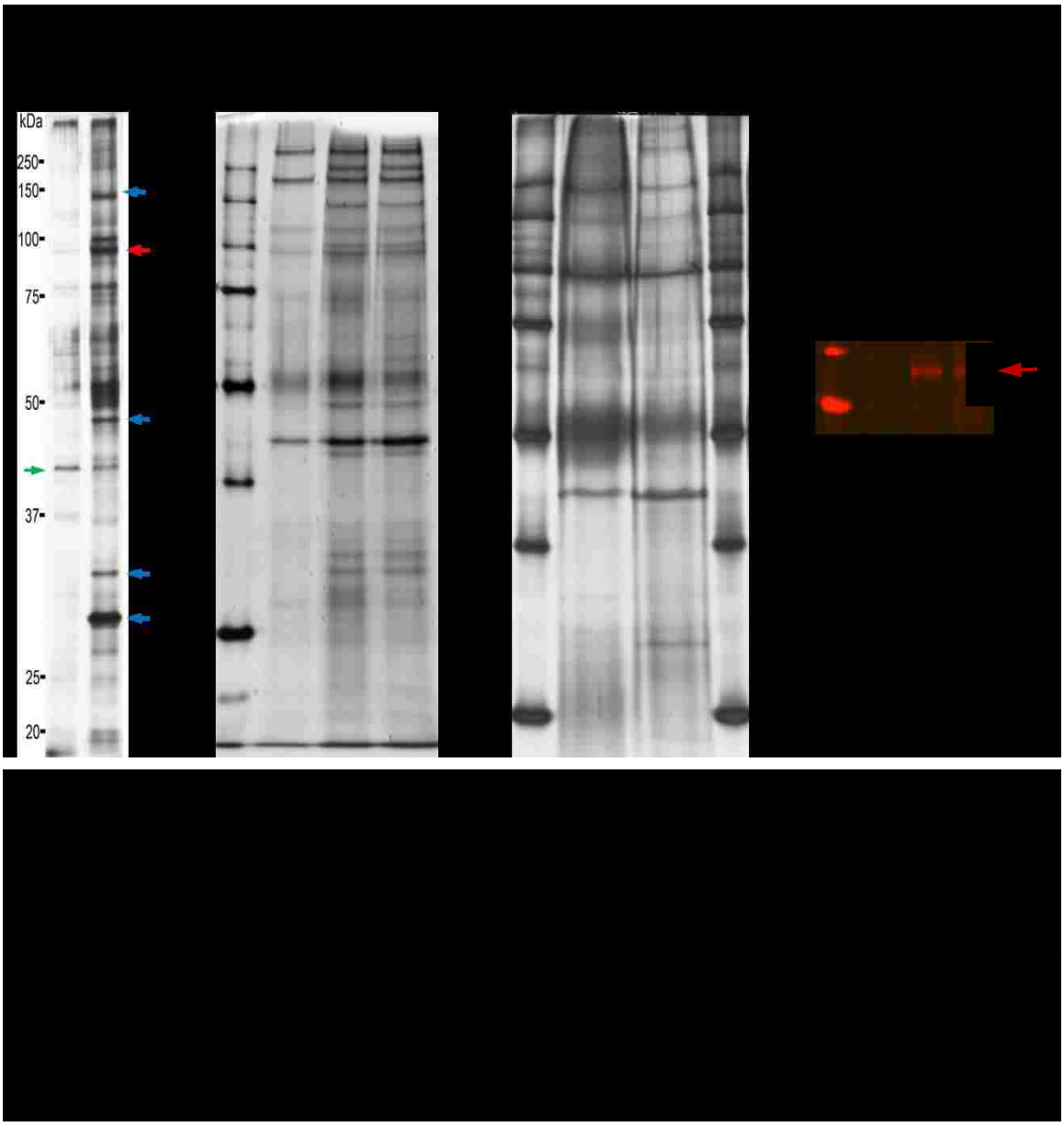
All constructs were sequenced to validate the fidelity of the cloning.

pCS2/DIXDC1 and pCS2/DIXDC1-DN plasmids were linearized with Not1 and used as a template for RNA synthesis using the mMESSAGE mMACHINE SP6 Transcription Kit following the manufacturer's instructions (Catalog number: AM1340; Thermo Fisher Scientific). The concentrations of mRNA used for microinjections are: 1.8 µg/µl for *DIXDC1*, 0.4 µg/µl for *DIXDC1-DN* and 0.4 µg/µl for control *GFP*.

Results

Successful capture of Dsh and its interacting proteins from egg and egg cortex lysates

In initial experiments, egg and egg cortex lysates were subjected to Dsh Co-IP and the eluates were resolved using SDS-PAGE. Fig. 3.1A shows a typical result from an egg Co-IP experiment. Compared to the control sample where lysates were incubated with non-immune normal IgG, SDS-PAGE analysis showed that there were multiple extra polypeptide bands in the eluate from Dsh Co-IP of egg lysates. Those bands could be either



Dsh protein itself or proteins interacting with Dsh, directly or indirectly, or false positives. An anti-Dsh antibody detected one band at the expected size for Dsh on Western blots (Fig. 3.1D), confirming that the endogenous Dsh protein was successfully immunoprecipitated. Two separate egg and isolated cortex lysates were subjected to Dsh Co-IP and up to 1/10th volume of these samples were resolved using SDS-PAGE followed by silver staining (Fig. 3.1B and C). After confirming that the quality of these two batches met the requirement for LC-MS/MS analysis, the remaining eluates were TCA precipitated and submitted for tandem MS analysis. The results below show that I was successful in performing endogenous Dsh Co-IP to identify potential Dsh interacting proteins in *S. purpuratus* eggs and isolated egg cortices.

326 and 80 potential Dsh-interacting proteins were identified from Co-IP, using eggs and egg cortices as input, respectively

LC-MS/MS analysis of the Dsh Co-IP samples showed that many proteins were pulled down and these proteins potentially interact with Dsh directly or indirectly through other Dsh-interacting proteins. Proteins with two or more fragments detected by the LC-MS/MS analysis were considered to be candidate Dsh-interacting proteins. After subtracting proteins identified from the control samples and removing the repeats in the protein annotation, 326 unique proteins from egg Co-IP and 80 unique proteins from cortex Co-IP samples were identified.

A selection of interesting proteins identified from egg lysate and cortex lysate Dsh Co-IP experiments is listed in Table 3.1. Consistent with the results from Western blot analysis, Dsh was among the top detected proteins, indicating that the Co-IP experiments were successful. The number of Dsh fragments detected in the cortex IP sample is considerably

higher than that in the egg sample, indicating that Dsh is enriched in the cortex, which is consistent with the immunostaining results previously reported (Peng and Wikramanayake 2013). Several previously known Dsh interacting proteins such as DIXDC1, CK1δ/ε and CK2α were identified from the Co-IP experiment (Table 3.1), reinforcing the reliability of this approach. These proteins provide a valuable resource of candidate molecules that may provide further insight into regulation of Dsh and Wnt/β-catenin signaling in sea urchin embryos.

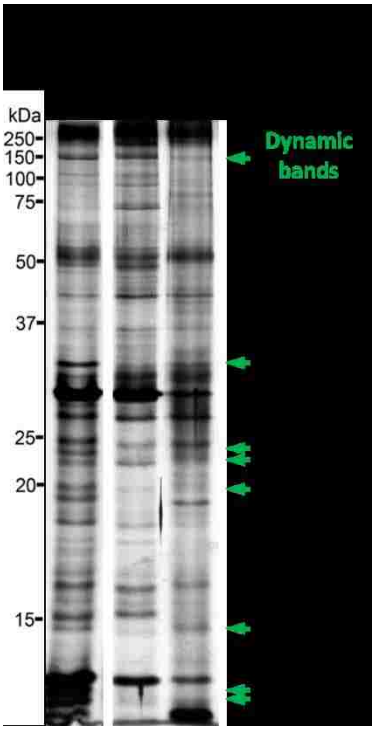
Table 3. 1 List of selected proteins identified for egg IP and cortex IP.

Egg IP	Cortex IP	Protein	Annotation	Note
19	38	Dsh	Dishevelled	Indicates IP worked
25	6	Tubgcp6	tubulin, gamma complex associated protein 6	Gamma-tubulin complex
10	7	Git1	G protein-coupled receptor kinase interacting ArfGAP 1-1	Kinase or Kinase related
3	5	EIF4G2	Translation initiation factor 4 gamma 2, DAP5, p97, NAT1	Translation
13	4	DIXDC1	DIX domain containing 1-like	Known Dsh interacting
13	4	Tubgcp2	tubulin, gamma complex associated protein 2	Gamma-tubulin complex
8	3	Tubgcp5	tubulin, gamma complex associated protein 5	Gamma-tubulin complex
6	4	Eif3m	eukaryotic translation initiation factor 3, subunit M	Translation
5	3	Pgk1	Phosphoglycerate kinase 1	Kinase or Kinase related
3	3	EIF2S2	Translational Initiation Factor 2 subunit 2,	Translation
6	3	CK2α	casein kinase II alpha subunit	Known Dsh interacting
6	3	Tubgcp3	tubulin, gamma complex associated protein 3	Gamma-tubulin complex
6	3	Mcm5	MCM5, Minichromosome maintenance protein 5	MCM complex
4	2	Ddx6	ATP dependent RNA helicase p54, rck oncogene	RNA binding
10	2	TUBG2	tubulin, gamma 2	Gamma-tubulin complex
4	2	Mcm2	MCM2, Minichromosome maintenance protein 2	MCM complex
3	2	EFTUD2	elongation factor Tu GTP binding domain containing 2	Translation
4	3	Rbm14	RNA binding motif protein 14; RRM-containing coactivator	RNA binding
0	5	CK1δ/ε	casein kinase I delta/epsilon subunit	Known Dsh interacting

Temporal dynamics of Dsh interacting proteins

To determine if there are stage-dependent Dsh protein interaction dynamics, I performed Co-IP with some key stages of sea urchin embryo development where the Wnt/β-catenin pathway is inactive or active. From a preliminary experiment using sea urchin eggs, 16-cell stage embryos and gastrula stage embryos (Fig. 3.2), multiple dynamic bands were identified on silver stained gels, indicating that there are stage-dependent,

transient protein-protein interactions with Dsh. These bands could be associated with the different state, function of Dsh and activity of Wnt/β-catenin signaling in each stage. Identification of the nature and functions of these proteins could provide useful insights into the molecular mechanisms that regulate Dsh and Wnt/β-activity in different stages of sea urchin development.



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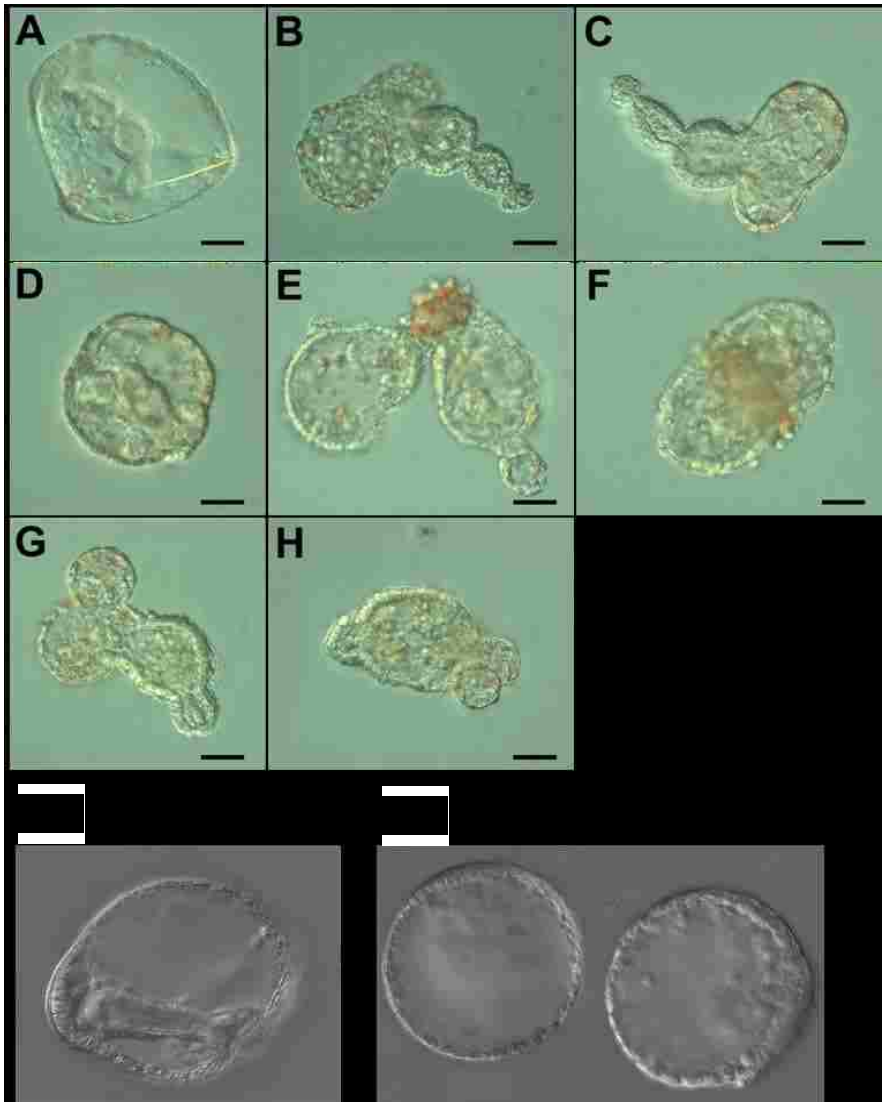
The role of DIXDC1 in early sea urchin embryo development

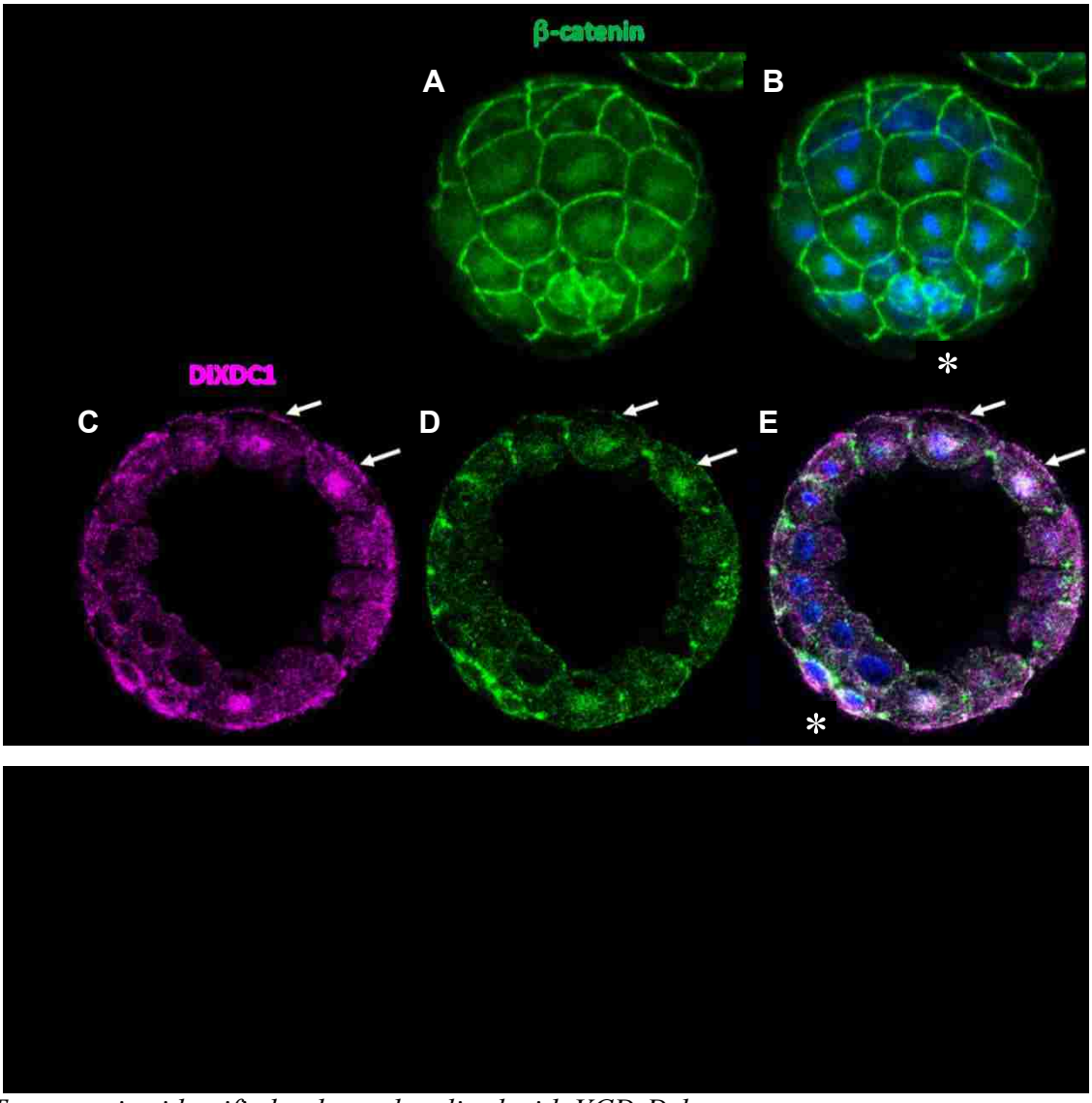
LC MS/MS analysis of Dsh Co-IP samples from isolated egg cortex lysates revealed 80 potential Dsh-interacting proteins. I selected DIXDC1 for further analysis because of its previously described roles in the Wnt pathway. Interestingly, DIXDC1 has homologs in all bilaterians except *Drosophila* and *C. elegans*, and moreover, it is present in all the non-

bilaterians, namely ctenophores, sponges, placozoans, and cnidarians, indicating it may have conserved functions in the Wnt/β-catenin signaling pathway. For example, DIXDC1 has been reported to directly interact with Dvl and regulate Wnt/β-catenin activity in HEK293T and Simian COS7 cells (Liu, Dan et al. 2011). However, compared to other key components in the Wnt pathway, relatively little is known about the role of DIXDC1 in the regulation of this pathway, particularly in the context of early embryonic development.



To begin to understand the function of DIXDC1 during sea urchin development, I carried out gain-of-function and loss-of-function studies using mRNA injection (Fig. 3.3). When full length DIXDC1 was over-expressed by injection of 1.8 µg/µl mRNA, most of the embryos formed a huge ectopic gut and very little ectoderm (Fig. 3.3B-H), resembling the phenotype induced by overexpression of constitutively activated β-catenin (Wikramanayake, Huang et al. 1998a). Work done in zebrafish has shown that the DIX domain of DIXDC1 without the N-terminus can act as a dominant-negative inhibitor (Shiomi, Uchida et al. 2003). To determine if DXIDC1 was required for endomesoderm formation, I overexpressed the dominant-negative form of the protein by mRNA injection into zygotes. These experiments showed that dominant-negative DIXDC1 expression in early embryos led to embryos that failed to gastrulate (Fig. 3.3 I-J). It will be necessary to thoroughly analyze these embryos using various molecular markers to determine the role that DIXDC1 plays in regulating pattern formation along the AV axis.

To investigate if over-expression of DIXDC1 affects the level of nuclear β-catenin, I performed the β-catenin immunostaining on 60-cell stage embryos injected with *DIXDC1* full length RNA (Fig. 3.4). In the GFP-mRNA injected control embryos, immunostaining showed that β-catenin only accumulated in the nuclei of the cells at the vegetal half of the embryo at the 60-cell stage. However, when DIXDC1 full length mRNA was injected, it was clear that β-catenin accumulated in the nuclei of cells at the animal half, indicating DIXDC1 is sufficient to induce ectopic nuclear β-catenin. This result is consistent with the vegetalization phenotype observed when DIXDC1 was overexpressed (Fig. 3.3 B-H).

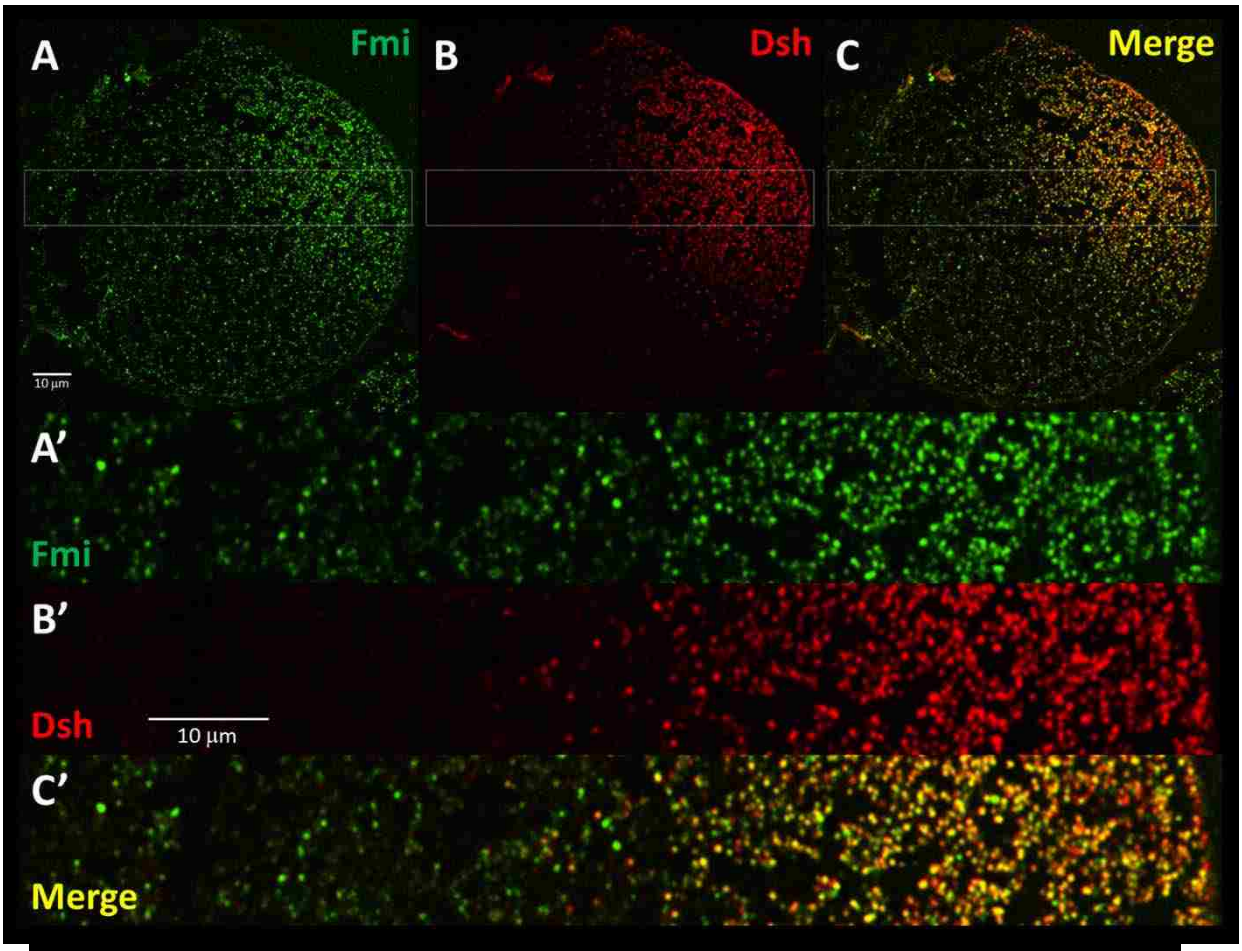


Two proteins identified to be co-localized with VCD-Dsh puncta

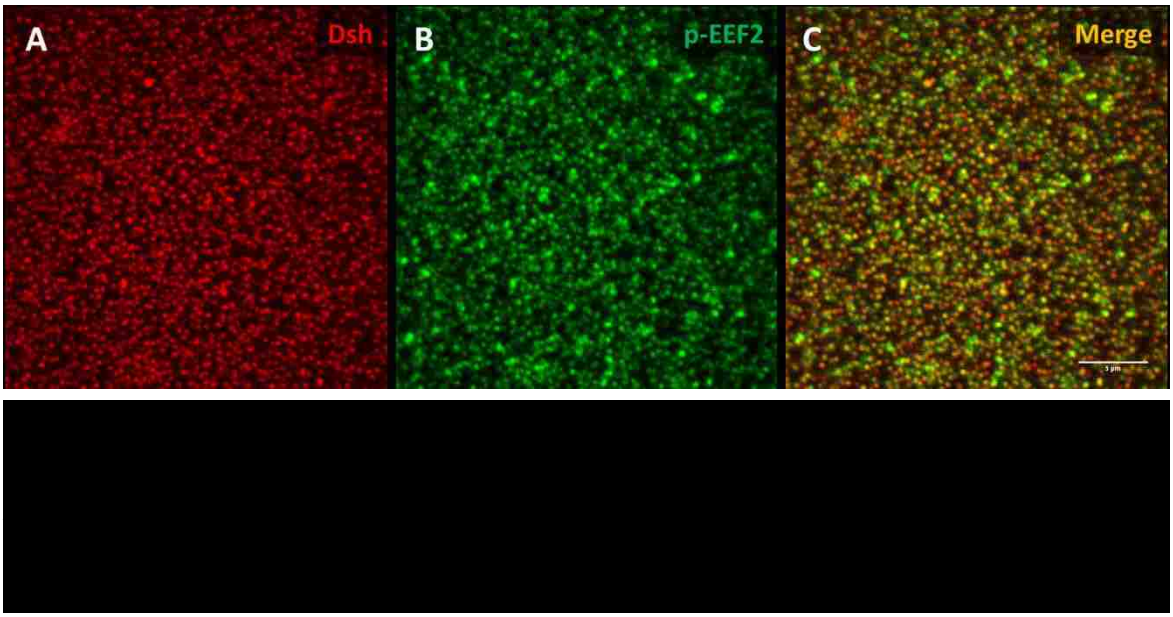
Besides the candidates mentioned above, two other candidate proteins, Flamingo (Fmi) and phosphorylated eukaryotic translation elongation factor 2 (p-EEF2), also show interesting patterns when they are double stained with Dsh on the isolated cortices.

Flamingo (Fmi) is a conserved non-canonical Wnt signaling receptor and a study in the cnidarian *Nematostella vectensis* has shown that Fmi plays an important role in regulating both cell fate specification and morphogenesis (Wijesena 2012). It also has been shown that Fmi and Dsh co-localize at the distal cell edge of the *Drosophila* wing (Shimada, Usui

et al. 2001). To investigate the localization of this protein in sea urchin eggs, I performed double staining of Fmi with Dsh on isolated cortices (Fig. 3.5). Both proteins showed a punctate pattern in the isolated cortices and co-localized at the VCD. The co-localization pattern in the egg cortex suggests Fmi and VCD-Dsh may interact directly but it is important to notice Fmi has a broader distribution than Dsh at the vegetal cortex.



Since many translation machinery components were identified from the Co-IP experiment (Table 3.1), it is tempting to consider that the Dsh puncta in the VCD may play a role in regulating local translation activity. To test this idea, I picked one highly conserved protein, Eukaryotic translation elongation factor 2 (EEF2), to examining its localization on the cortex. I used a commercial antibody, anti-phospho-EEF2 (anti-p-EEF2), which can recognize the phosphorylated form of EEF2 at residue Thr56. Phosphorylated EEF2 acts as a translation elongation inhibitor. p-EEF2 showed a similar puncta pattern as VCD-Dsh and they were co-localized at the egg cortex (Fig. 3.6). Whether this p-EEF2 plays a role in regulating Dsh or other polarity related processes, however, remain to be examined.



Discussion

The Wnt/β-catenin pathway plays a conserved role in specifying the endomesoderm in embryo development in sea urchins but the molecular mechanisms regulating the spatiotemporal activity of this pathway are not well understood. Studies on the vegetal cortical domain (VCD)-enriched upstream component Dishevelled (Dsh) protein have shed

light on how β-catenin nuclear entry is restricted to vegetal pole blastomeres in sea urchin embryos. Hence, elucidating how Dsh is regulated at the VCD would provide key insight into this process. In this study, using endogenous Dsh co-immunoprecipitation (Co-IP) followed by mass spectrometry techniques, I have provided evidence that a set of candidate proteins may interact with Dsh and contribute to the regulation of Dsh and Wnt/β-catenin activities. Functional studies on these proteins would be crucial to elucidate the nature of Dsh's post-translational modification, the mechanisms of Dsh anchoring at the VCD and a possible fertilization-mediated regulation of local translation at the VCD to regulate nuclear β-catenin in sea urchin embryos.

Endogenous Co-IP and MS is a powerful approach to identify candidate protein-protein interactions

Affinity purification–mass spectrometry (AP-MS) is one of the most widely used methods to capture protein complexes and identify novel protein-protein interactions. It usually serves as an entry point for subsequent functional studies to elucidate the role of the protein-protein interactions in the regulation of activity of the target protein as well as the corresponding pathway (Kocher and Superti-Furga 2007, Malovannaya, Li et al. 2010, Dunham, Mullin et al. 2012, Morris, Knudsen et al. 2014, Marcon, Jain et al. 2015). Moreover, of the two major strategies, endogenous Co-IP has many advantages over Co-IP with overexpressed proteins. Massively overexpressed proteins may cause protein misregulation or mislocalization, leading to increases in both false-positives and false-negatives (Dunham, Mullin et al. 2012). So theoretically, endogenous Co-IP is an excellent strategy to identify protein-protein interactions with Dsh aiming to elucidate their roles in

the regulation of Dsh localization and activity as well as the regulation of the Wnt/β-catenin pathway.

The proposed approach has worked successfully to capture Dsh protein and identify both known and novel protein-protein interactions with Dsh (Table 3.1). In our hands, when carrying out Dsh Co-IP, we found that the target antigen, Dsh protein was placed at the top of the list, indicating that the antibody works very well for Co-IP (Marcon, Jain et al. 2015). Analysis of the data from the Dsh Co-IP revealed that several previously identified Dsh interacting proteins, such as DIXDC1, CK1δ/ε and CK2α were pulled down with Dsh from sea urchin egg and cortex lysates. CK1δ/ε and CK2α have been reported to interact with Dsh directly to phosphorylate the protein in various contexts (Willert, Brink et al. 1997, Peters, McKay et al. 1999, Sakanaka, Leong et al. 1999, Song, Sussman et al. 2000, Kishida, Hino et al. 2001, Cong, Schweizer et al. 2004, Gao and Wang 2006, Bryja, Schulte et al. 2007, Bernatik, Ganji et al. 2011). In particular, CK2α phosphorylates Dsh in a constitutive manner and then CK1δ/ε phosphorylates Dsh upon Wnt stimulation to mediate the activation of the Wnt/β pathway (Bernatik, Ganji et al. 2011). CK1δ/ε can further modify Dsh to a phosphorylated and shifted form to inactivate the Wnt/β-catenin pathway (Bernatik, Ganji et al. 2011). Since the Dsh protein in the sea urchin egg VCD and micromeres is differentially modified and this modification is very likely to be phosphorylation, it is possible that the differentially modified form of Dsh may play essential roles in the regulation of spatiotemporal activity of the Wnt/β-catenin pathway (Peng and Wikramanayake 2013). Hence, studying the function of these two candidate proteins will be important to understand the nature and regulation of Dsh post-translational

modification and its role in regulating Wnt/β-catenin activity during early embryonic development.

The role of DIXDC1 in germ layer specification in the sea urchin embryo

DIXDC1 is a conserved protein and it acts synergistically with Dsh to regulate the activity of the Wnt/β-catenin pathway (Liu, Dan et al. 2011). Homologs of DIXDC1 are present in non-bilaterians such as cnidarians, sponges and ctenophores as well as in the major bilaterian clades, but interestingly this gene is absent in the genomes of the *Drosophila* and *C. elegans*. DIXDC1, Dsh and Axin all contain a conserved DIX domain and they each play distinct roles in regulating Wnt/β-catenin pathway. In mammalian cells, when one of the three Dsh homologs, Dvl2, is overexpressed, the protein forms large cytoplasmic puncta and the Wnt/β-catenin pathway is activated (Liu, Dan et al. 2011). When DIXDC1 is co-overexpressed with Dvl2, the Dvl2 puncta are converted into an evenly distributed form and the Wnt/β-catenin pathway is synergistically activated (Liu, Dan et al. 2011). X-ray crystallography studies also show that the DIX domains of these two proteins can form "head-to-tail" heterodimers, suggesting that they work together to regulate the Wnt/β-catenin pathway (Liu, Dan et al. 2011).

The Co-IP and MS results in my study also support the notion that DIXDC1 and Dsh interact with each other in sea urchin embryos. The functional studies provide more insights into their role in regulating Wnt/β-catenin activity. The vegetalization phenotype of the DIXDC1 full length protein overexpression (Fig. 3.3) is reminiscent of that caused by activated β-catenin (Wikramanayake, Huang et al. 1998b), suggesting that DIXDC1 over-expression may directly or indirectly, lead to activation of nuclear β-catenin. This hypothesis is also supported by the observation that DIXDC1 overexpression in the sea

urchin embryo leads to ectopic activation of nuclear β-catenin at the animal pole (Fig. 3.4). However, overexpression of the dominant-negative DIXDC1 construct does not completely reproduce the severe animalization phenotype produced by blocking nuclear β-catenin with C-cadherin overexpression or GSK-3β overexpression (Fig. 3.3; Emily-Fenouil, Ghiglione et al. 1998, Wikramanayake, Huang et al. 1998b). It is possible that maternal DIXDC1is not completely blocked with the DIXDC1 dominant-negative and the activity of Wnt/β-catenin pathway may not be knocked down low enough to cause the extreme phenotype seen in C-cadherin overexpressing embryos.

Additional questions need to be addressed to elucidate the role of DIXDC1 during early sea urchin embryo development. First, does DIXDC1 act via Dsh to regulate nuclear β-catenin? To address this question, the best experiment is reciprocal Co-IP using the DIXDC1 antibody. If Dsh can be detected from DIXDC1 Co-IP, it will provide strong evidence for their interactions. Another experiment needed is the overexpression of DIXDC1 followed by Dsh immunostaining to examine the distribution of Dsh. This experiment would tell us if and how DIXDC1 regulates Dsh to control Wnt/β-catenin pathway during early sea urchin development. Second, in the phenotypes generated by full length DIXDC1 and DIXDC1 DN overexpression, are there any changes in endomesoderm cell fate specification? This question can be addressed by examining various germ layer markers using *in situ* hybridization. Third, since DIXDC1 is sufficient to activate ectopic nuclear β-catenin at the animal pole, it would be interesting to investigate if DIXDC1 alone can activate nuclear β-catenin and the endomesoderm GRN in isolated animal halves, where VCD-Dsh is absent. This would show whether DIXDC1's regulation independent of VCD-Dsh. In sum, these experiments would provide evidence that DIXDC1 is a key

regulator of endomesoderm specification during sea urchin embryogenesis. The answers to these questions can further our understanding about the role of DIXDC1 in early sea urchin development and may also allow us to gain a better understanding of how this protein interacts with other components of the Wnt pathway.

The role of other promising candidate proteins

Flamingo (Fmi)

Fmi was selected for custom antibody generation and localization studies because of its important role in polarity formation and maintenance. Besides its essential role in planar cell polarity (Hale and Strutt 2015, Sokol 2015), Fmi is also reported to control neurite polarity during neuronal development in *Drosophila* and *C. elegans* (Lee, Clandinin et al. 2003, Senti, Usui et al. 2003, Shen 2004, Steimel, Wong et al. 2010, Berger-Mueller and Suzuki 2011, Shimizu, Sato et al. 2011, Najarro, Wong et al. 2012, Najarro and Ackley 2013). It was surprising to find that Fmi is co-localized with Dsh at the vegetal cortex (Fig. 3.5) and because of this, it is tempting to speculate that Fmi may play a key role in recruiting Dsh or anchoring Dsh to the VCD during oogenesis. But the difference in the size of Fmi and Dsh distributing domains suggest that the mechanism may not be that simple. Other molecules in the Wnt signaling pathway, like extracellular Wnt ligands, trans-membrane (co-receptors and/or intracellular components, may contribute to the regulation of the patterns of Fmi and Dsh at the VCD. Additional functional studies are required to test Fmi's role in the formation and maintenance of the egg polarity in sea urchin.

Phospho-Eukaryotic Elongation Factor2 (p-EEF2)

The co-localization of pEEF2 with Dsh at the vegetal pole is intriguing and raises the possibility that p-EEF2 may be involved in local translational regulation at the VCD. VCD-mRNAs that are bound to p-EEF2 will be translationally be suppressed due to the inhibitory nature of p-EEF2 (Proud 2015). The potential local translation regulation may contribute to polarity formation, maintenance and local Wnt/β-catenin pathway activation. The key to study the function of EEF2/p-EEF2 lies in its kinase, EEF2 kinase (EEF2K). EEF2K belongs to the α-kinase family and it is highly specific, with EEF2 as its only known substrate (Kenney, Moore et al. 2014). Studies in mammalian cells has shown that the activity of EEF2K depends on cytoplasmic pH: the activity is low at pH of 7.2 to 7.4 and is increased by seven-fold at pH of 6.6-6.8, which leads to more inhibitory form of p-EEF2 and subsequently strong inhibition of protein synthesis (Dorovkov, Pavur et al. 2002). Considering the fact that after fertilization, the intracellular pH increases from 6.84 to 7.26 (Shen and Steinhardt 1978), it is tempting to speculate that some species of VCD-mRNAs, which are inhibited for translation, will be translated after fertilization and this could be a mechanism contributing to the spatiotemporal regulation of nuclear β-catenin. EEF2K/EEF2 has been reported to regulate such localized protein synthesis in neuron cells (Verpelli, Piccoli et al. 2010, Weatherill, McCamphill et al. 2011, Heise, Gardoni et al. 2014). Therefore, it is definitely worth investigating what RNAs are bound and regulated by the p-EEF2/EEF2K pathway in the VCD, what role the product of these RNAs have in early embryo development, and whether this mechanism has a conserved role in different lineages of animals.

In conclusion, in Chapter 3 of my dissertation, I have successfully applied co-immunoprecipitation in combination with liquid chromatography tandem-mass

spectrometry to identify endogenous protein-protein interactions with Dsh in sea urchin eggs and egg cortices. Some functional studies or localization characterizations have provided useful insights into the regulation of Dsh localization and activity, as well as the activity of Wnt/β-catenin pathway. The novel interactions from this study will serve as a valuable resource for future studies in both sea urchins and other evolutionarily important species.

Chapter 4: Speculations and models for future research

Summary of the findings

Understanding how Dsh is polarized and how nuclear β-catenin is spatiotemporally regulated is the key to elucidate the mechanisms for endomesoderm specification and animal-vegetal axis formation in the early sea urchin embryo. To address these questions, my dissertation has focused on understanding how a sub-population of Dsh protein is tethered initially to the vegetal pole, and how this vegetal cortex-enriched Dsh is differentially modified to locally activate Wnt/β-catenin signaling. I have used approaches that focus on the level of RNA and protein, to identify candidate molecules that may play a role in these processes.

From the results presented in chapters 2 and 3, VCD-enriched *Bcl9* RNA and p-EEF2 protein may provide a mechanism to regulate the asymmetry of nuclear β-catenin. In chapter 2, the RNA-seq study, *Bcl9*, together with a few other transcripts, were identified as enriched in the vegetal cortical domain (VCD), where Dsh and p-EEF2 are localized. *Bcl9* encodes a protein that can physically interact with β-catenin and convert the adhesive population of β-catenin to the transcriptional population in the nuclei of vertebrates (Kramps, Peter et al. 2002, Brembeck, Schwarz-Romond et al. 2004). Considering that p-EEF2 is also localized at the VCD, it would be very interesting to test if p-EEF2 binds to the *Bcl9* mRNA and inhibits its translation before fertilization until the right time during development. To test the role of *Bcl9* in the temporal control of nuclear β-catenin in sea urchin embryos, additional work is required, for example, whether p-EEF2 binds to *Bcl9* mRNA.

Going forward, the transcriptome study will also provide a valuable resource for the sea urchin community to study the early endomesoderm gene regulatory networks (GRNs)

downstream of early Wnt/β-catenin activation. A few transcripts that encode transcription factors, including *Sp5*, were also identified to be differentially expressed in the micromeres where the β-catenin starts to accumulate in the nuclei. Transcription factor Sp5 is known to be a Wnt/β-catenin target gene and regulates germ layer specification in multiple organisms (Weidinger, Thorpe et al. 2005, Park, Seo et al. 2013, Ye, Zhang et al. 2016). Therefore, it is very likely that Sp5 acts downstream of Wnt/β-catenin signaling in endomesoderm specification in sea urchin embryos, but functional tests are required.

At the protein level, endogenous Dsh immunoprecipitation (IP) has identified dozens of candidate proteins that may regulate the localization and activity of Dsh and thus the asymmetric activation of nuclear β-catenin. For example, CK1δ/ε and CK2α may be responsible for the post-translational modification and activation of Dsh at the vegetal pole; the γ-tubulin complex might contribute to the transportation of Dsh; the translation machinery components may work with Dsh to regulate local translation activity to specify the polarity.

One candidate selected for functional studies is DIXDC1, which turns out to play an important role in endomesoderm formation in sea urchin embryos. There is also evidence that over-expression of DIXDC1 leads to ectopic nuclear β-catenin at the animal pole, suggesting this protein contributes to the spatial regulation of nuclear β-catenin along the AV axis. The remaining question is whether DIXDC1 regulates β-catenin entry into the nucleus via interacting with Dsh directly. If it does, then it would be important to identify the underlying mechanism since it would be of general relevance to those studying the Wnt pathway in other organisms. Axin, Dsh and DIXDC1, key proteins in the Wnt/β-catenin signaling pathway, are also the only proteins that share a DIX domain. Therefore, it would

be fascinating to study how these three proteins work together to regulate the activity of the pathway. A lot of work is required to further elucidate their roles in the Wnt/β-catenin pathway.

I have identified two proteins, Fmi and p-EEF2, that are co-localized with VCD-Dsh puncta and they have provided useful insight to understand mechanisms regulating axial polarity. The co-localization of Fmi with VCD-Dsh suggests that non-canonical Wnt signaling pathway may contribute to AV polarity formation and/or maintenance. p-EEF2 co-localization may provide a mechanism to control the timing of the asymmetrical activation of Wnt/β-catenin pathway. Their exact functions remain to be characterized.

A working model and future directions

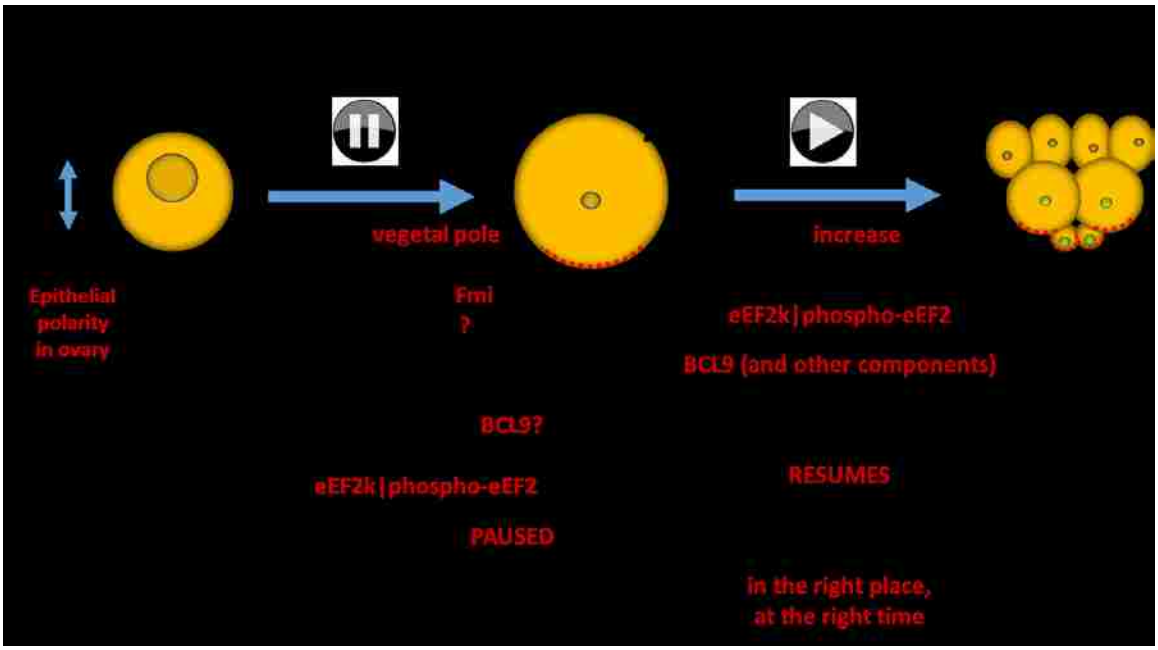
Since the animal-vegetal Dsh polarity in sea urchins is already established in the early oocyte (Peng and Wikramanayake 2013), it is crucial to look at the early stages during oogenesis to study how it is established. During oogenesis in echinoderms, the polarity of oocytes is tightly associated with the apical-basal polarity of the germinal epithelium (Frick, Ruppert et al. 1996, Wikramanayake 2013). The oocyte is initially part of the germinal epithelium and is attached to the basal lamina only at the vegetal pole. After the oocyte enters the vitellogenic phase and begins to enlarge, the basal lamina increases its coverage on the growing oocyte continuously except for an apical region, termed the oocyte apex. The oocyte apex bears a flagellum and becomes future animal pole of the egg, which is also marked by the formation of polar bodies. The other end, which contacts with basal lamina initially, becomes the future vegetal pole (Frick, Ruppert et al. 1996). It seems the initial interaction with the basal lamina is important for the AV axis formation for the

oocyte. What happens in the oocyte at the molecular level during the interaction with the basal lamina might be the key to unveil how the oocyte polarity forms.

Fmi protein, also called cadherin epidermal growth factor (EGF) laminin G (LAG) seven-pass G-type receptor (CELSR) in mammals, is co-localized with VCD-Dsh at the vegetal pole. Fmi belongs to a group of adhesion G protein-coupled receptors (GPCR) and coincidentally, it has a large extracellular region containing domains like Laminin-type epidermal growth factor-like domain. The Laminin G domain is able to mediate the signaling and adhesion functions of Fmi. A recent study in mouse neural tube has identified a novel distribution of Fmi at the extreme basal surface of the neuroepithelial cells, termed as endfeet, which are anchored to the pial basement membrane (Formstone, Moxon et al. 2010). The basement membrane is considered to consist of basal lamina. Furthermore, the Fmi homolog CELSR is found to be spatially restricted to the basement membrane in the mouse lung epithelium (Yates and Dean 2011). Thus, Fmi appears to mediate the interaction between the cell surface and basal lamina in certain biological contexts. Whether Fmi also mediates the interaction between oocyte and the basal lamina and whether this interaction contributes to VCD-Dsh polarity formation during oogenesis should be tested.

Another finding in accord with this hypothesis is that the transcript encoding laminin subunit beta-2, a member of the laminin family proteins and one of the major components of basal lamina, is enriched in the VCD according to my RNA-seq. Whether the product encoded by this RNA is involved in the processes described above remains to be characterized.

Based on findings from this dissertation and observations from other studies, I propose the following working model (Fig. 4.1).



In particular, during oogenesis, the apical-basal polarity of the germinal epithelium may influence the animal-vegetal polarity of the developing oocytes. Due to the interaction with the basal lamina mediated by Fmi at the basal end, or perhaps mediated by another unknown signal such as canonical or non-canonical Wnts at the basal end, Dsh accumulates at the future VCD region. But this signaling process pauses at a certain step without leading to nuclear β-catenin. The pause could be caused by the pH-dependent and localized EEF2K/p-EEF2, which inhibits the translation of certain key components required for the nuclear activity of β-catenin. One good candidate that may be inhibited in translation is *Bcl9*. Also CK1δ/ε (not shown in the figure) may be required to modify VCD-Dsh differentially to keep it localized and active.

After fertilization, the pH increases dramatically, resulting in the subsequent low activity of EEF2K and the high activity of translation at the VCD. Components such as *Bcl9* are likely to be transcribed and promote the accumulation nuclear β-catenin. Vegetal pole restricted Dsh, together with other proteins such as DIXDC1 and other Dsh interacting proteins restrict nuclear β-catenin to the vegetal pole.

This model is highly speculative but, like all good models, it generates testable predictions. A couple of experiments that can be done in the future to validate or falsify this model include:

1. Dsh Co-IP or p-EEF2 Co-IP followed by RNA-seq to identify transcripts inhibited by p-EEF2 when the pH is low; examine if *Bcl9* is included in the p-EEF2 binding RNAs. Double labeling of *Bcl9* by *in situ* hybridization and VCD-Dsh or p-EEF2 by antibody on isolated cortices would tell us directly whether *Bcl9* is co-localized with pEEF2. But this will be a tough experiment.

2. Perturbation of the timing and location of *Bcl9* translation by mopholino anti-sense oligos, and overexpression of dominant-negative or wild type Bcl9 protein would also provide insight whether this protein plays an important role in nuclear β-catenin regulation.

3. The perturbation of EEF2K/p-EEF2 is a good strategy to determine the role of VCD-specific translation regulation in nuclear β-catenin regulation.

4. Since the pattern of VCD-Dsh is formed during oogenesis and the interaction between the oocyte and basal lamina mediated by Fmi might be required for VCD-Dsh polarity formation, the perturbation of the interaction between Fmi and basal lamina in early oocyte stages, if possible, will be informative. Also, blocking the canonical or non-

canonical Wnt ligands during oogenesis will provide insight into whether this early AV axis formation is Wnt-dependent or Wnt-independent.

5. Studies from other systems including *Xenopus* oogenesis, *Drosophila* oogenesis and neuron polarity regulation will also provide useful information to identify conserved mechanisms. The same cellular and molecular mechanisms could be co-opted to establish cell polarity in different contexts during evolution.

I will end this dissertation with a final question: which came first, the chicken or the egg? In this case we ask instead: where does the animal-vegetal axis of the embryo come from? It comes from the mother, the mother's apical-basal polarity in germinal epithelium. Where does the apical-basal polarity in germinal epithelium come from? It comes from the embryo during development. And these two processes influence each other and form a closed circle.

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