MICRORNAS REGULATE SEA URCHIN GUT SPECIFICATION AND DEVELOPMENT BY TARGETING COMPONENTS OF THE WNT SIGNALING PATHWAY

by

Priya A. Nigam

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Priya A. Nigam

Approved: _____

Jia L. Song, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Randall L Duncan, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____

George H. Watson Ph.D.
Dean of the College of Arts and Sciences

Approved: _____

James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

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ABSTRACT

microRNAs (miRNAs) are established regulators of cellular signaling and biological processes such as differentiation, apoptosis and proliferation. Previously, our laboratory has demonstrated that miRNAs may regulate endodermal and mesodermal specification (Song et al., 2012). The canonical Wnt signaling pathway plays a highly conserved role in endodermal specification (Logan *et al*., 1999; Wikramanayake *et al.*, 1998) and proper gut formation (Theodosiou and Tabin, 2003). Therefore, this study tested the hypothesis that miRNAs regulate endodermal specification and gut development in part by regulating conserved components of the canonical Wnt signaling pathway. Using luciferase reporter assays and site-directed mutagenesis of reporter constructs, we demonstrate that miRNAs directly regulate both *β-catenin* and *Dishevelled* (*Dsh/Dvl*) post-transcriptionally. Blocking miRNA-mediated regulation of *β-catenin* by **t**arget **p**rotector morpholino anti**s**ense oligonucleotide (miRNA TP) resulted in: (1) increased β-catenin protein levels, (2) upregulation of β-catenin responsive genes that are involved in endodermal specification, and (3) a structurally and functionally defective gut tissue. In addition, we also observed that inhibition of miRNA-mediated regulation of *Dsh/Dvl*, an upstream regulator of β-catenin, by use of miRNA TP resulted in an even more severe

phenotype of a morphologically thinner gut than treatment with the β-catenin miRNAT TP. In summary, we identified two key components of Wnt signaling to be directly regulated by miRNAs. Importantly, we demonstrated that removal of miRNA-mediated regulation of either *β-catenin* or *Dsh/Dvl* in the developing embryo led to defects in endodermally derived gut tissue.

Chapter 1

INTRODUCTION

1.1 Synthesis and Function of miRNAs

miRNAs are non-coding regulatory RNA molecules that are approximately 21-23 nucleotides in length. miRNAs are capable of regulating thousands of genes that are involved in diverse biological processes, including cellular differentiation and development (He and Hannon, 2004). Because a single miRNA regulates so many gene targets, dysregulation of miRNAs is associated with various pathologies such as cancer (Iorio and Croce, 2012), autoimmune (Zhu *et al.*, 2013) and developmental disorders (Bian and Sun, 2011).

miRNAs are encoded by genomic DNA. It had been reported that there are approximately 238 miRNAs in *Drosophila melanogaster*, 1,872 in humans and around 223 in worms *Caenorhabditis elegans* (as per miRBase, v 20, June 2013). Most miRNA genes in the genome are located in regions that are distant from previously annotated genes, suggesting that they are in itself an independent transcription unit having a promoter of their own (Lagos-Quintana *et al.*, 2001). A minority of miRNAs are derived from the intronic regions of the pre-mRNAs transcribed from the protein coding genomic sequences, implying that these miRNAs are dependent on the

promoter region of the associated gene and the mRNA splicing mechanisms (Kim and Kim, 2007). Furthermore, some miRNA genes are also found clustered in the genome, indicating their transcription as a multi-cistronic primary transcript (Hayashita *et al.*, 2005).

Majority of miRNAs are transcribed as primary miRNA (pri-miRNA) transcripts by the RNA polymerase II, although some are transcribed by RNA polymerase III as well (Bartel, 2004). Most pri-miRNAs are initially processed by Drosha and its cofactor DGCR8 (Han *et al.*, 2006;Lee *et al.*, 2003) in the nucleus to form the pre-miRNAs which are exported into the cytoplasm (Fig. 1.1). Here, the pre-miRNAs are further processed by Dicer, leading to formation of double-stranded RNA fragments (20-25bp) (Lund and Dahlberg, 2006; Zhang *et al.*, 2002). One strand of the resulting miRNA:miRNA* duplex, also known as the guide strand, is then loaded on to the RNA-induced silencing (RISC) complex (Schwarz *et al.*, 2003), forming mature miRNAs that are able to recognize and bind their target mRNAs. The mechanism of strand selection had been reported to be dependent upon the relative free energies of the duplex ends, as the small RNA whose 5'-end exhibits the less stable end is preferentially maintained in the mature silencing complex (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). Nevertheless, the passenger strand denoted with an asterisk (miRNA*) is either degraded or in some cases, may become functional miRNA that can target different mRNA (Okamura *et al.*, 2008). A recent study provides some concrete evidence that miRNA* species are bona fide trans-regulatory RNA molecules

with demonstrable endogenous regulatory effects in *D. melanogaster* (Okamura,*et al.*, 2008). It was also shown that the miRNA* species are often present at physiologically relevant levels and can associate with Argonaute proteins, like their complementary miRNA guide strand. Moreover, using functional assays the inhibitory activity of miRNA* species in both cultured cells and transgenic animals also validated their functions (Okamura *et al.*, 2008).

In animals, binding of miRNA to the **m**iRNA **r**ecognition **e**lement (MRE) in the target mRNA is facilitated by the "seed" or the "core" sequence. The seed sequence is a 6-8 nucleotide stretch present at the 5' end of the miRNA from positions 2 to 8 and is generally considered necessary and sufficient for miRNA function (Shukla *et al.*, 2011). However, the importance of miRNA "anchor" sequence (nucleotides 13-16) in the process of target recognition had also been emphasized by several groups (Bartel, 2004; Lee *et al.*, 2002; Lund *et al.*, 2004). It should also be noted that a single target mRNA (3'untranslated region (3'UTR) in particular) can have more than one MREs that can bind to different miRNAs, and a single miRNA can also regulate multiple target mRNAs by binding to its corresponding MRE in the 3'UTR of those mRNAs (Shukla *et al.*, 2011).

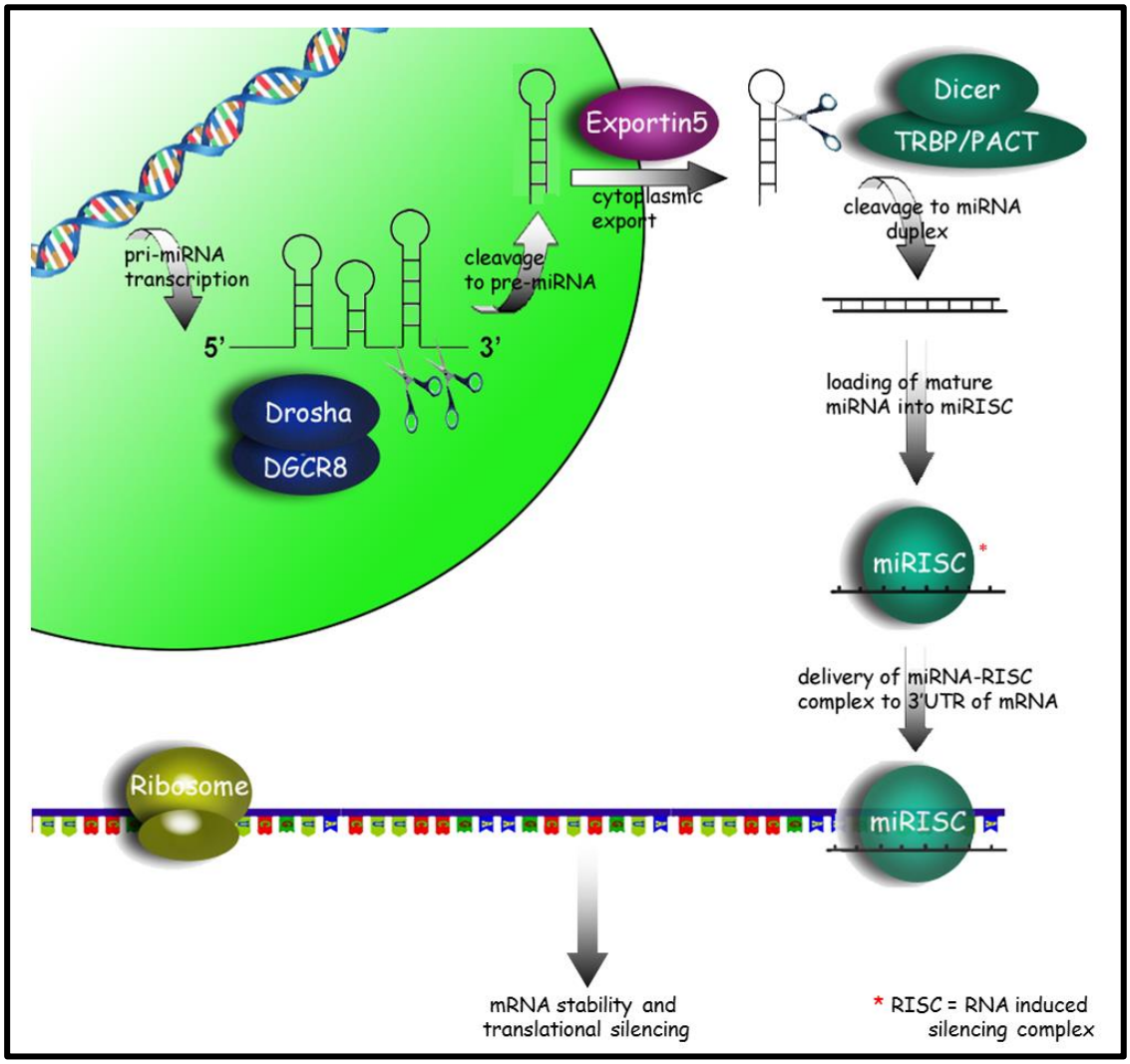

Fig. 1.1. Biogenesis of miRNAs. miRNAs are encoded in the DNA and undergo sequential processing to reach mature state. The newly synthesized transcripts are processed by enzyme Drosha and its cofactor DGCR8 and then transported outside of the nucleus. They are further processed by enzyme Dicer. One arm of the resulting miRNA duplex is then loaded to the RNA-induced silencing (RISC) complex. In association with the RISC complex, miRNAs are able to recognize their target mRNAs. The binding of miRNA to the target leads to the translational repression and/or degradation of the target mRNA (illustration modified from www.micrornaworld.com).

1.1.1 miRNAs in developmental processes

Results from various studies have implicated the role of miRNAs in animal development (Alvarez-Garcia and Miska, 2005; Ambros, 2004; Bushati and Cohen, 2007; Wienholds and Plasterk, 2007). Dicer, a key enzyme in the processing of miRNA maturation, is essential for mouse oocyte maturation (Murchison *et al.*, 2007). Loss of *Dicer1* is lethal to early mouse development and defects become apparent as early as embryonic day 7.5 (Bernstein *et al.*, 2003). Furthermore, target-selected inactivation of *dicer1* gene in zebra fish results in developmental arrest around day 10 (Wienholds *et al.*, 2003). Also, global depletion of miRNAs by knockdown of Dicer and Drosha in the sea urchin embryo resulted in early developmental defects, including gastrulation failure and embryonic lethality (Song *et al.*, 2012).

miRNAs have also been established as key regulators of cell fate and differentiation (Ivey and Srivastava, 2010). miRNAs may function both as a switch and a fine-tuner of the gene regulatory network. Importance of miRNA *lin-4* as a developmental switch during normal temporal regulation of post-embryonic developmental events in *C. elegans* is well established (Lee *et al.*, 1993; Reinhart *et al.*, 2000). miRNA *lin-4* mediates translational repression of its target mRNA *lin-14* which facilitates switching of *C. elegans* larva from stage L1 to L2 and then to L3. Furthermore, during developmental transitions where a tissue-specific miRNA is upregulated and its pool of target genes are downregulated, the effective concentration

of the miRNA could greatly increase (Brennecke *et al.*, 2005). The transcription factors of the developmental gene regulatory network, whose protein exerts a switch-like response, may be suppressed in the first place through molecular repression by its regulatory miRNA. In this manner, miRNAs may precisely and promptly fine-tune gene expression thresholds which are an important feature of cell fate decisions.

Studies have also shown that miRNAs play an important role in regulation of cell proliferation and apoptosis during development (Jovanovic and Hengartner, 2006). *Drosophila melanogaster* (dme) miRNA dme-miR-*bantam* promotes tissue growth by targeting the apoptic gene *hid* (Brennecke *et al.*, 2005), and the dme-miR-2 family targets the proapoptotic genes *Grim* and *Reaper* (Stark *et al.*, 2003).

1.1.2 miRNAs in Echinoderms

The sea urchin gene regulatory network (GRN) is one of the best described in all animals examined. Despite our advanced understanding about the gene transcriptional networks, little is known about post-transcriptional regulation by miRNA in developmental pathways in echinoderms. In a seminal work, Pasquinelli *et al.* examined the expression of the highly conserved *let-7* miRNA in 14 species from 8 phyla, and found that only sea urchin embryos lacked mature transcripts for the miRNA (Pasquinelli *et al.*, 2000). Although, depending on temporal expression pattern, the let-7 miRNA may control late temporal transitions during development

across animal phylogeny, in sea urchin such transitions may be independent of let-7 (Pasquinelli *et al.*, 2000). Recently, it was shown that the main genes involved in the RNA interference (RNAi) pathway are expressed in sea urchin embryos (Song and Wessel, 2007). Employing an algorithm miRDeep2 that identifies miRNA genes, 49 miRNAs were annotated to be expressed in sea urchins (Song *et al.*, 2012). miRBase (v. 20, June 2013) contains 61 sequences for *S. purpuratus* miRNAs. Furthermore, in a species of star fish *H. sanguinolenta* 42 miRNAs were found (Wheeler *et al.*, 2009). Recently 38 miRNAs were identified in sea star *P. miniata* during early development of which 32 of them were common to those found in sea urchin *S. purpuratus* (Kadri *et al.*, 2011). Unlike vertebrates that have hundreds of miRNAs in multiple families, the sea urchin lacks multiple miRNA families and functional redundancies (Song *et al.*, 2012). Thus, the sea urchin relatively small pool of miRNAs makes it an attractive model to investigate single miRNA function.

1.2 Purple Sea Urchin as a Model for Developmental Studies

The purple sea urchin, *Strongylocentrotus purpuratus,* is one of the preferred model organisms for studying embryonic development, morphogenesis and evolution. The sea urchins belong to the phylum echinodermata (invertebrate deuterosomes) that share a common ancestor with the chordates (including humans) and thus, echinoderms are the closest invertebrate outgroup to the chordates (Turbeville *et al.*, 1994). The GRN that drives cell fate determination and specification of the three germ

layers in embryonic development of the sea urchin is one of the best understood GRN (Davidson et al., 2002; McClay, 2011; Su, 2009). The early developmental process is relatively rapid in sea urchins. Development begins when the sperm and egg fertilize externally forming zygote, which develop relatively fast, ideal for early developmental studies (Fig. 1.2). The sea urchin embryos are transparent that allows one to observe phenotypic changes from experimental manipulations. These embryos can withstand manipulations such as transplantations and microinjections of loss-of-function or gain-of-function reagents, thereby allowing us to phenotype following experimental perturbations (Hardin, 1994). For these reasons, the sea urchin has become a preferred model for studying morphogenetic movements and cell interactions, the changes in cell biology, molecular interactions of regulatory molecules, and developmental biology.

Sea urchins shed gametes directly into the marine environment. Fertilization of two gametes restores diploidy and produces a genetically unique zygote which subsequently undergoes radial holoblastic cleavage (Gilbert, 2010). By the end of third cleavage, an embryo having eight similar size blastomeres, also known as mesomeres, is produced with an arrangement of two layers with four cells each forming the animal and the vegetal tier. The fourth cleavage is different than others, as it produces 8 similar sized mesomeres from 4 cells of animal tier, whereas, the 4 cells of vegetal tier undergoes an unequal cleavage producing 4 macromeres and 4 micromeres (Fig.

1.3.A). The 16-celled embryo, thus formed undergoes 3 more cleavages ending-up into a 128-celled blastula by 24 hours post fertilization (Gilbert, 2010).

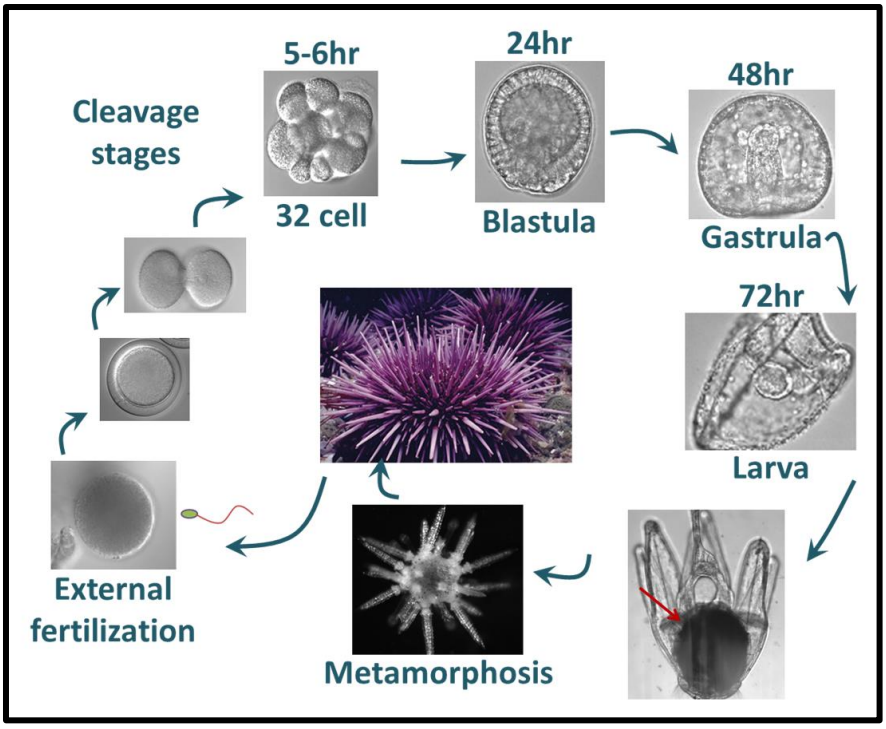

Fig. 1.2. Life cycle of the purple sea urchin. Purple sea urchin produces large number of gametes that are externally fertilized to produce transparent embryos that undergo rapid early embryonic development reaching the larval stage in 72 h. Finally, the larva grows in size and undergoes metamorphosis forming a young sea urchin in approximately two years (Courtesy of Dr. Song).

Fig. 1.3B illustrates a cell fate map based on the 60-cell embryo. Cells in the animal half (an_1 and an_2) of the embryo give rise to the ectodermal tissue that eventually forms the larval skin and neurons. The cells of veg_1 layer produces either the ectodermal or endodermal organs, whereas, cells of the veg_2 layer gives rise to the endodermally derived gut (which is divided into fore-, the mid- and the hindgut), the coelom (body wall), and the secondary mesenchyme (pigment cells, immunocytes, and muscle cells derived from mesoderm). The first tier of micromeres produces the primary mesenchyme cells that form the larval skeleton, while the second tier of micromeres contributes cells to the coelom.

By 24 hour post fertilization (hpf), a blastula is formed, which consist of a hollow sphere of cells surrounding a central cavity called blastocoel. The cells of blastula are united by the tight junctions to form a unified layer of epithelial cells. Rapid invariant cell cleavages continue till the ninth or tenth cell division, depending upon the sea urchin species, after which (a) the embryo enters the mid-blastula stage, (b) synchrony of cell division comes to an end, (c) new set of genes are expressed and (d) many non-dividing cells start to develop cilia on their outer surface (Gilbert, 2010). The cells of the vegetal pole of ciliated embryo soon start to thicken forming the vegetal plate (Gilbert, 2010).

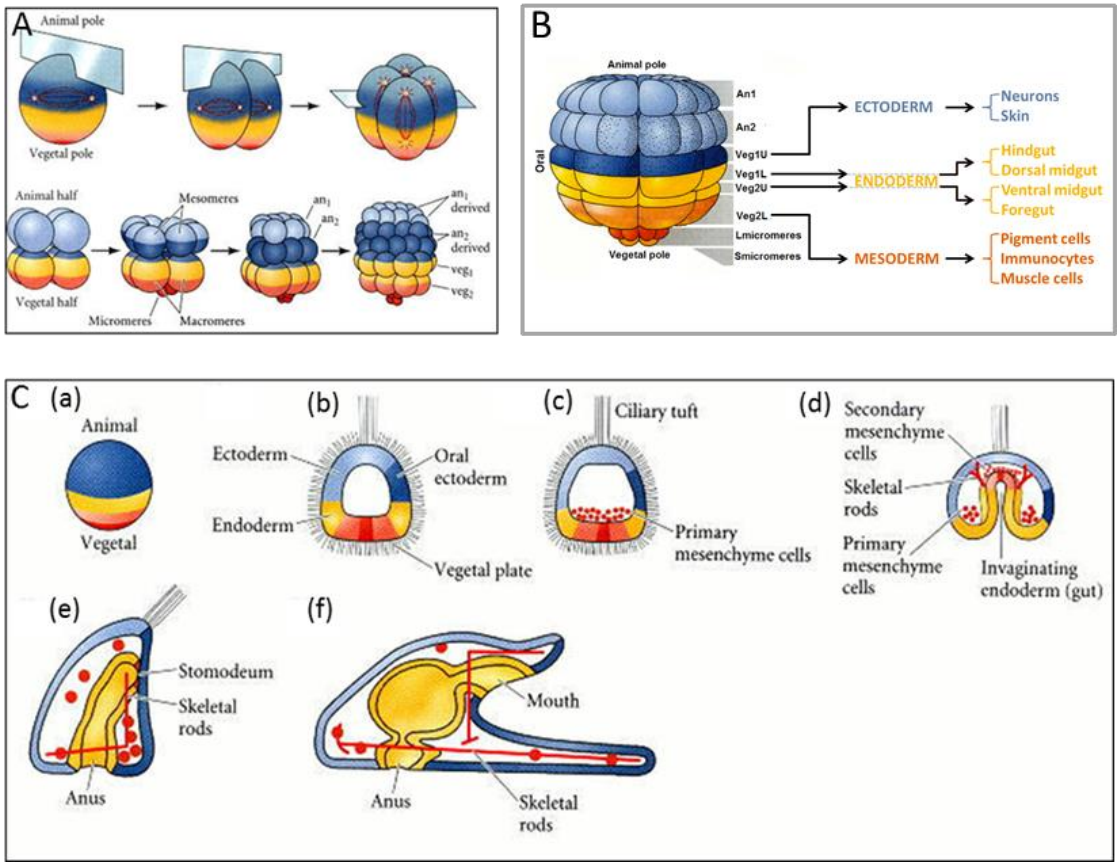

Fig. 1.3 Sea urchin embryonic development. (A) Cleavage in the sea urchin. Planes of cleavage in the first three divisions and the formation of tiers of cells in divisions 3 to 6. (B) Fate map and cell lineage of *S. purpuratus* is shown in a 60-cell embryo. (C) Normal sea urchin development. Fates of the zygote cytoplasm can be followed through the color pattern. (a) Zygote. (b) Late blastula with ciliary tuft and flattened vegetal plate. (c) Blastula with primary mesenchyme. (d) Gastrula with secondary mesenchyme. (e) Prism stage larva. (f) Pluteus larva (adapted from Gilbert, 2010).

At the mid-blastula stage, the embryo secretes hatching enzyme and is able to exit the fertilization envelope. Next is the process of gastrulation that occurs in two temporally distinct phases. First, the primary mesenchyme cells ingress at the center of the vegetal plate that is soon followed by invagination of the archenteron. The secondary mesenchyme cells in the center of the vegetal plate initiate invagination by changing shape to produce an inward bending sheet of cells. The archenteron then elongates to extend across the blastocoel by recruitment of endoderm cells into the archenteron, by elongation via convergent extension movements of cells and by extension of thin filopodia by the secondary mensenchyme cells to help pull the archenteron toward the animal pole. The archenteron reaches a target site near the animal pole and, shortly thereafter, the oral ectodermal epithelium invaginates to form the stomodeum (future mouth), which then fuses with the foregut epithelium (McClay, 2011). As the embryo progresses towards formation of pluteus larva, the gut subdivides into the foregut, midgut and hindgut, with cardiac muscular sphincter forming between foregut and midgut and a pyloric sphincter forming between midgut and the hindgut. Coelomic pouches, derived from secondary mesenchymal cells and small micromeres, develop on either side of foregut. As the larva grows, the rudiment of the adult grows from the left coelomic pouch. The pluteus feeds on plankton and upon metamorphosis, the larva transforms into the juvenile sea urchin (Hardin, 1994; McClay, 2011).

1.3 Wnt Signaling Pathways and their Regulation by miRNAs

Wnt signaling can be classified as (a) canonical β-catenin-dependent Wnt pathway, and (b) non-canonical β-catenin-independent Wnt/Planar Cell Polarity (Wnt/PCP) and Wnt/Ca^{2+} pathways. Canonical Wnt signaling had been shown to control diverse biological processes and functions including cellular proliferation and differentiation (Teo and Kahn, 2010), survival (Lin *et al.*, 2006), cell fate decisions (Martin and Kimelman, 2012), stem cell maintenance and somatic cell reprogramming (Miki *et al.*, 2011). Furthermore, canonical Wnt signaling has been shown to be involved in many important embryological events, including axis specification (Hikasa and Sokol, 2013), gastrulation (Lee et al., 2006), and breast (Boras-Granic and Wysolmerski, 2008), limb (Gao and Yang, 2013), heart (Gessert and Kühl, 2010), neural (Mulligan and Cheyette, 2012), or bone (Day and Yang, 2008) development. The non-canonical Wnt pathways regulate processes such as establishment of tissue polarity (Goh *et al.*, 2012) and neuroectoderm formation (Range *et al.*, 2013), and cell morphogenesis (Gao and Chen 2010). Table 1.1 below illustrates various Wnt ligands and Frizzled receptors known in sea urchin and their respective combinations including information from other species.

Wnt signaling begins when secretory protein Wnt ligand binds to its cognate cell surface receptor Frizzled (Fz). Secretion of newly synthesized Wnt is a tightly regulated process and requires the protein Wntless (Wls) (Port *et al.*, 2008). Wls was

initially identified in fruit flies and nematodes as a cargo protein that functions in the secretion of the Wnt ligand (Port *et al.*, 2008). Blocking Wls expression or function by various methods resulted in phenotypes that corresponds to Wnt signaling failure (Adell *et al.*, 2009; H. Kim *et al.*, 2009). The Wls is directly targeted by miR-8 as demonstrated by using *lacZ* reporter construct fused to the 3'UTR of the Wls (Kennell *et al.*, 2008). In general, Wnt binding to Fz can trigger signaling that can either propagate through canonical or non-canonical pathways, depending on the binding of specific Wnt ligands to their corresponding Frizzled receptors. For instance, ligand Wnt4 had been reported to trigger both canonical and non-canonical Wnt signaling pathway (Lyons *et al.*, 2004; Chang *et al.*, 2007).

Table 1.1: Wnt and Frizzled isoforms in Wnt signaling pathways.

Families	Genes	Sea Urchin *S. purpuratus* (Number of isoforms; x means absence)	Receptor (*Species*; Reference) [G = *Gallus gallus* (chick embryo); H = *Homo sapiens* (humans); D = *Danio rerio* (zebrafish); M = *Mus musculus* (mouse); X = *Xenopus laevis* (frog)]	Wnt pathways [Canonical - β-catenin dependent / Non-canonical - β-catenin independent / Other]	Reference
Wnt	Wnt1	1	Fz10 (G; Galli *et al*., 2014)	Canonical - β-catenin dependent	Sato, 2012
	Wnt2/13	x	Fz9 (H; Zola *et al*., 2007)		
	Wnt3	1	Fz3 (H; Endo *et al., 2008*); Fz10 (G; Galli *et al*., 2014)		
	Wnt7	1	Fz9 (H; Zola *et al*., 2007)		
	Wnt8	1	Fz9 (D; Momoi *et al*., 2003)		
	Wnt10	1	?		
	Wnt4	1	Fz6 (M; Lyons *et al*., 2004)		Lyons *et al.*, 2004
	Wnt5	1	Fz2 (M; Li *et al.*, 2013)		Li *et al.*, 2013
	Wnt16	1	?		Mazieres *et al.*, 2005
	Wnt2	x	?	Non-canonical - β-catenin independent	Onizuka *et al.*, 2012
	Wnt4	1	?		Chang *et al*., 2007
	Wnt5	1	Fz5 (M; Li *et al*., 2011a)		Shimuzu *et al.*, 2011
	Wnt6	1	Fz5 (X; Schulze, 2012)		Schmidt *et al.*, 2007
	Wnt7	1	Fz7 (H; Grand *et al.*, 2009)		Grand et al., 2009
	Wnt11	x	Fz4 (X; Gorny *et al*., 2013)		Pandur *et al.*, 2002
	WntA	1	?		Croce *et al.*, 2006
	Wnt9/14/15 or Wnt9/9a/9b	1		Other Wnt pathways* (Fz independent)	Zhang *et al.*, 2012
Fz	Fz1/2/7	1		Canonical - β-catenin dependent	Hering and Sheng, 2002
	Fz3/6	x			Deardorff *et al.*, 2001; Golan *et al.*, 2004
	Fz9/10	1			Karasawa *et al.*, 2002; Fukukawa *et al.*, 2009
	Fz4	1		Non-canonical - β-catenin independent	Robitaille *et al.*, 2002
	Fz7	1			Grand *et al.*, 2009
	Fz5/8	1			Byrum *et al.*, 2009

** Other Wnt pathway here refers to Wnt/MUSK pathway in muscle cells*

In canonical Wnt pathway (Fig. 1.4), the absence of the ligand Wnt leads to rapid phosphorylation of cytoplasmic β-catenin by glycogen synthase kinase 3β (GSK3β) at Ser33, Ser37 and Thr41 (Yost *et al.*, 1996) and by casein kinase Ia (CK Ia) at Ser45 (Liu *et al.*, 2002). GSK3β is found as a part of the destruction complex which also consists of Axin and APC (MacDonald *et al.*, 2009). The post-translational phosphorylation of β-catenin targets it for ubiquitinylation and subsequent proteasomal degradation (Hart *et al.*, 1999), preventing its nuclear accumulation. However, binding of canonical Wnt ligand (*e.g.,* Wnt1; Grumolato *et al.*, 2010) to its corresponding transmembrane receptor Frizzled (Fz1; Cossu and Borello, 1999) as well as co-receptor LRP5/6 (Cadigan and Liu, 2006), results in recruitment of cytoplasmic protein Dishevelled (Dsh/Dvl) to the cell membrane due to interaction between the Dsh PDZ domain and Fz cytoplasmic domain (Wong *et al.*, 2003). Concomitantly, Dsh/Dvl is activated via phosphorylation. The binding of Fz to the activated Dsh/Dvl recruits Axin and GSK3β to cell membrane, thereby, dismantling the destruction complex and inhibiting phosphorylation of β-catenin (Wong *et al.*, 2003). This results in increased stability of β-catenin, facilitating its nuclear accumulation where β-catenin interacts with Tcf/Lef family of transcription factors to regulate transcription of several genes involved in diverse biological processes such as cellular proliferation, apoptosis, and differentiation (Teo and Kahn, 2010; Miki *et al.*, 2011).

Besides functioning as a transcriptional co-activator, β-catenin is also a component of the adherens junction (Orsulic *et al.*, 1999). β-catenin links the cadherin molecules to the α-catenin, leading to strong cadherin-mediated cell adhesion (Heuberger and Birchmeier, 2010). The level of β-catenin in the adhesion complex at the plasma membrane affects the availability of β-catenin functioning as a transcription co-activator in the nucleus (Heuberger and Birchmeier, 2010). This is demonstrated with experiments in which perturbation of cadherin complexes has an effect on Wnt/β-catenin regulated processes. For example, overexpression of cadherins in *Xenopus* embryos results in inhibited dorsal axis formation because binding of cadherin to endogenous β-catenin antagonizes its role as a nuclear transcription co-activator (Fagotto *et al.*, 1996; Heasman *et al.*, 1994). Moreover, E-cadherin knockout embryonic stem cells showed accumulation of β-catenin/Lef1 in the nucleus and activation of a Wnt reporter, which could be reversed by expression of E-cadherin (Orsulic *et al.*, 1999). Furthermore, post-translational phosphorylation or dephosphorylation status of components of cadherin adhesion complex, β-catenin destruction complex, or of β-catenin effectors involved in gene transcription, have also been shown to regulate the crosstalk between adhesion and Wnt signaling (Heuberger and Birchmeier, 2010). Recently it was revealed that in human HEK293 cells, miR-25 targets and inhibits human β-catenin (Anton et al. 2011). Moreover, the mammalian *β-catenin* gene was also shown to under the regulation of miR-200a (Huang *et al.*, 2010).

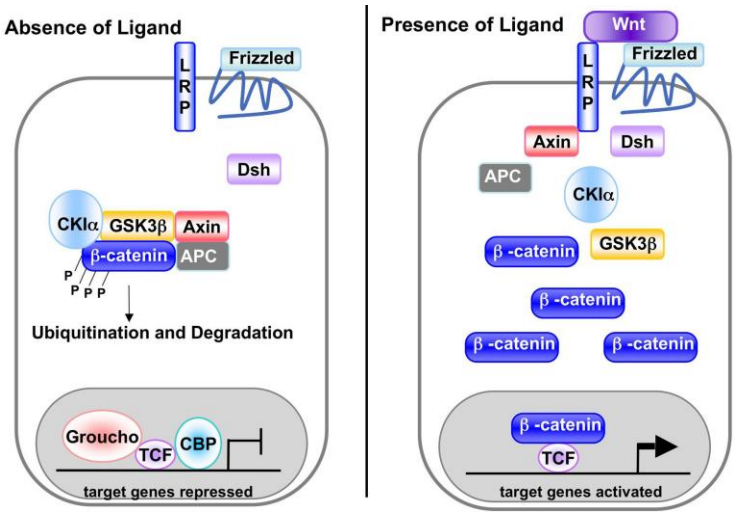

Fig. 1.4. Canonical Wnt signaling pathway. Absence of signal leads proteasome-mediated β-catenin degradation. However, Wnt binding to its receptor Frizzled and co-receptor LRP-5/6 complex results in stabilization of β-catenin, its nuclear accumulation, and subsequent transcriptional activation of downstream genes (adapted from www.wormbook.com).

Several reports have implicated canonical Wnt ligands and Frizzled (Fz) receptor to be under direct regulation of miRNAs. For instance, Wnt1 is a direct target of miR-34a and miR-let-7e during human monocyte derived dendritic cell (MDDC) differentiation (Hashimi *et al.*, 2009). miR-199a-5p was upregulated in human dystrophic muscles and regulate Wnt2 and Fz-4 of canonical Wnt pathway (Alexander *et al.*, 2013). In addition, Fz-4 had also been shown to be directly regulated by miR-493 (Ueno *et al.*, 2012). Overexpression of miR-493 resulted in decreased protein levels of Fz-4, leading to reduced motility and migration of T24 and J82 transfected bladder cancer cell lines (Ueno *et al.*, 2012). Wnt3a of the canonical Wnt pathway, known to promote several tumorigenic features such as survival, proliferation and invasion, was reported to be targeted by two miRNAs *viz.* miR-15a and miR-16-1

(Bonci *et al.*, 2008). While investigating the human trabecular meshwork (HTM) cells, it was revealed that the canonical Fz-1 3'UTR sequence was directly targeted by miR-204 (Li *et al.*, 2011b). In colon cancer cells, an association is established between canonical Fz-7 and its direct regulator miR-23b (Zhang *et al.*, 2011). Moreover, the effects Colon cancer cells overexpressing miR-23b had diminished ability of tumorigenesis and metastasis to lungs (Zhang *et al.*, 2011), suggesting miR-23b to have tumor suppressor activity.

In addition to targeting extracellular secretory ligands (Wnt) and their cell surface cognate receptors Fz, miRNAs also target cytoplasmic effectors of the canonical Wnt signaling pathway. Using human mesenchymal stem cells (hMSCs) and human embryonic kidney 293 (HEK293) cells, it was revealed that the 3'UTR of Axin-2 is a direct target of miR-Let-7f (Egea *et al.*, 2012) and miR-1 (Anton *et al.*, 2011) respectively. Axin-2 is also targeted by miR-34 in colorectal cancer (Kim et al. 2013). Another effector of Wnt signaling, APC is targeted by different miRNAs such that it was found to be the target of miR-135 family in colorectal cancer (Nagel *et al.*, 2008), miR-155 in papillary thyroid carcinoma (Zhang et al. 2013) and miR-27 in promoting differentiation of odontoblastic cells (Park et al. 2014). In many colorectal cancer samples, miRNAs belonging to miR-135 family were highly upregulated. miR-135a and miR-135b both targeted APC 3'UTR and induce its mRNA degradation rather than translational repression (Nagel *et al.*, 2008). GSK3β, yet another member

of the β-catenin destruction complex, was found to be targeted directly by miR-155 (Feng *et al.*, 2013).

Dsh/Dvl transduces Wnt ligand activation of both canonical Wnt/β-catenin and the non-canonical Wnt signaling pathways (Gao and Chen, 2010). Dsh/Dvl is a multifunctional phosphoprotein that has three known homologues (Dsh/Dvl1, 2 and 3) in humans. In sea urchins, however, only one Dsh/Dvl isoform is identified (http://spbase.org; Cameron *et al.*; 2009). A large number of Dsh/Dvl interacting proteins had been reported so far (for complete list refer Gao and Chen, 2010). The Dsh/Dvl protein is capable of converging extracellular signals and directing different intracellular outcomes based on its interacting proteins (Gao and Chen, 2010).

Dsh/Dvl protein has three highly conserved domains (Fig. 1.5A), an N-terminal DIX (**Di**shevelled, A**x**in) domain of 80 amino acids, a central PDZ (**P**ostsynaptic density 95, **D**iscs Large, **Z**onula occludens-1) domain of about 90 amino acids, and a C-terminal DEP (**D**vl, **E**gl-10, **P**leckstrin) domain of 80 amino acids (Gao and Chen, 2010). Also, it has two more conserved regions, the basic region and the proline-rich region, that are implicated to mediate protein–protein interaction and protein phosphorylation.

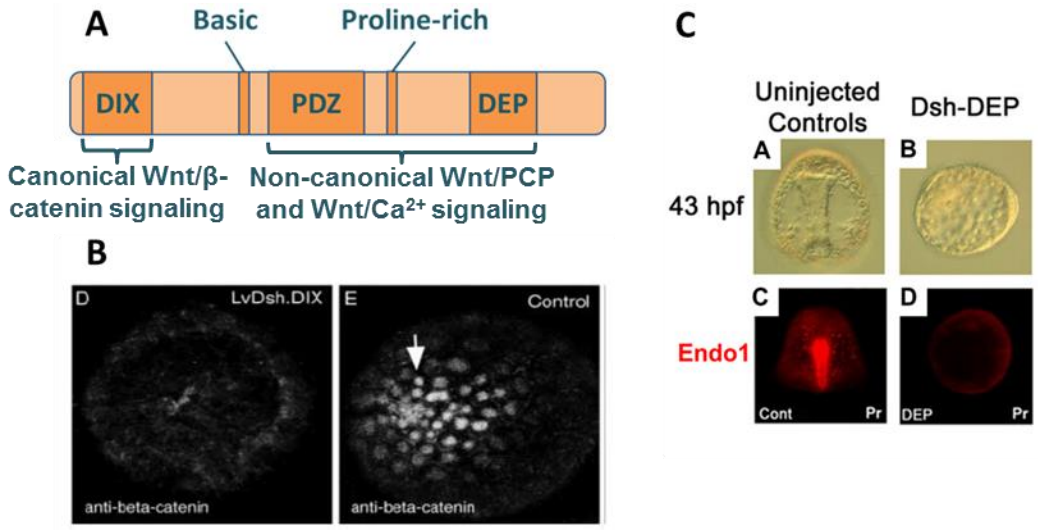

Fig. 1.5. Three main protein domains in Dishevelled and their roles. (A) Dsh/Dvl protein has a conserved N-terminal DIX domain, a basic and serine/threonine-rich region, a PDZ domain, a proline-rich region and a C-terminal DEP domain. (B) Overexpression of Dsh/Dvl DIX domain has dominant negative effect inhibiting Dsh/Dvl interaction with Axin and thus preventing disintegration of destruction complex, thereby, resulting in degradation of β-catenin (modified from Weitzel *et al.*, 2004). (C) Overexpression of Dsh/Dvl DEP domain blocks non-canonical Wnt signaling which is required for endoderm formation (modified from Byrum *et al.* , 2009).

Transmission of Wnt/β-catenin signaling via Dsh/Dvl had been shown to be dependent on dynamic dimerization of Dsh/Dvl which is mediated by the DIX domain (Gao and Chen, 2010). Dsh/Dvl can form (A) a homodimer due to interaction between the DIX domains of the two Dsh/Dvl molecules (Miller et al., 1999), or (B) a heterodimer due to interaction of DIX domains of Dsh/Dvl and Axin protein (Fiedler *et al.*, 2011). The Dsh/Dvl DIX domain and its proximal region are important for Dsh/Dvl oligomerization which is required for relay of signal and subsequent stabilization of β-catenin (Schwarz-Romond *et al.*, 2007). The DIX domain interacts

with PIP5KI thereby generating PIP_2 which is required for Wnt dependent clustering and phosphorylation of LRP6, which is a key step in Wnt/β-catenin signal transduction (Gao and Chen, 2010). Overexpression of the DIX domain in sea urchin embryos have resulted in a dominant negative effect on endogenous Dsh/Dvl, leading to no nuclear accumulation of β-catenin in vegetal cells in the embryo (Fig. 1.5.B, pg. 20) (Weitzel *et al.*, 2004), disrupting endomesodermal specification.

The Dsh/Dvl-DIX is believed to be dispensable in the non-canonical Wnt/PCP and Wnt/Ca^{2+} signaling pathways (Byrum *et al.*, 2009; Veeman *et al.*, 2003; Wharton, 2003). The PDZ domain is a modular protein interaction platform that has a peptide-binding cleft (Cheyette *et al.*, 2002). Dsh/Dvl makes use of its PDZ domain for directly interacting with Fz at the cell membrane (Wong *et al.*, 2003) and thus, relaying signal from the membrane receptor Fz to downstream effectors. Dsh/Dvl had been reported to interact via its PDZ and DEP domains with the DAD domain of Daam1 (Gao and Chen, 2010). This association results in disruption of the intramolecular inhibition leading to Daam1 activation (Gao and Chen, 2010). Both Dsh/Dvl and active Daam1 can interact with protein **w**eak **s**imilarity **G**EF (WGEF) (Habas *et al.*, 2001), a Rho guanine nucleotide exchange factor hence resulting in RhoA activation. It has been shown that Dsh/Dvl interaction with N-terminal WGEF is dependent on Dsh PDZ domain (Habas *et al.*, 2001). Furthermore, using *Xenopus* embryos, it was shown that expression of truncated Dsh/Dvl possessing only PDZ and DEP domain relayed Wnt signaling predominantly via Wnt/Ca^{2+} cascade (Komiya and

Habas, 2008) as seen in elevated levels of intracellular Ca^{2+} and activation of Ca^{2+}-dependent enzymes such as PKC and Calcineurin (Komiya and Habas, 2008). An overexpression of Dsh-DEP domain in sea urchin embryos manifests a dominant negative effect over endogenous Dsh/Dvl and blocks specifically non-canonical Wnt/PCP and Wnt/Ca^{2+} signaling with developmental defects including a lack of endodermal markers and organized skeleton (Byrum *et al.*, 2009) (Fig. 1.5.B, Pg. 20). However, alterations in the primary mesenchyme cells and pigment cells in Dsh-DEP overexpressing sea urchin embryos were not observed (Byrum *et al.*, 2009). This study, along with work on other bilaterians (Axelrod *et al.*, 1998; Tada and Smith, 2000; Tada *et al.*, 2002) indicate that PDZ and DEP domains of the Dsh/Dvl protein are both required for non-canonical Wnt/PCP and Wnt/Ca^{2+} pathway and the non-canonical Wnt pathway regulates early cell specification.

Similar to other components of the Wnt signaling pathway, direct regulation of mammalian *Dsh/Dvl* isoform *Dvl2* by miR-221 had been reported in human prostate cancer cell lines (Zheng et al., 2012). Its interacting protein, Daam1, which directs Dvl signaling to RhoA following non-canonical Wnt/PCP signaling pathway, is regulated by miR-490 (5p and 3p) in thrombocythemia platelets (Xu *et al*. 2012).

The canonical Wnt signaling pathway is a highly conserved mechanism for germ layer specification in all metazoans. Besides this, Dsh/Dvl has been shown to be required for animal-vegetal axial patterning, β-catenin stabilization and endodermal

specification in bilaterians (including echinoderms and chordates) and non-bilaterians (such as starlet sea anemone in the Cnidaria phylum) (Lee *et al.*, 2007). Activation of canonical Wnt signaling results in β-catenin stabilization and its nuclear accumulation. Previously, it has been shown that both Dsh/Dvl (Weitzel *et al.*, 2004) and β-catenin (Logan *et al.*, 1999) have vegetal pole localization. This is similar in invertebrate ascidian *Halocynthia roretzi* (Kawai *et al.*, 2007), starfish *Asterina pectinifera* (Miyawaki *et al.*, 2003) and *Xenopus laevis* (Larabell *et al.*, 1997). The differential stability of β-catenin along the animal-vegetal axis of the sea urchin embryo is mediated by Dvl/Dsh (Peng and Wikramanayake, 2013; Weitzel *et al.*, 2004). In the sea urchin embryo, an accumulation of the protein β-catenin in the micromeres at the vegetal end of the embryo results in activation of transcription factor *Pmar1,* which subsequently represses the ubiquitously expressed transcriptional repressor *HesC* (Fig. 1.6). This relieves the HesC dependent repression of transcription factors crucial for the activation of endoderm and mesodermal specification. For example, the mesodermally derived larval skeleton results from β-catenin activation of *Alx1, Tbr, Ets1* (Logan *et al.*, 1999; Weitzel *et al.*, 2004). The rest of the embryo that lacks β-catenin nuclear localization is prevented from the activation of *Pmar1*, leading to the activation of the repressor *HesC* and thus, repression of skeletogenic fates (Fig. 1.6).

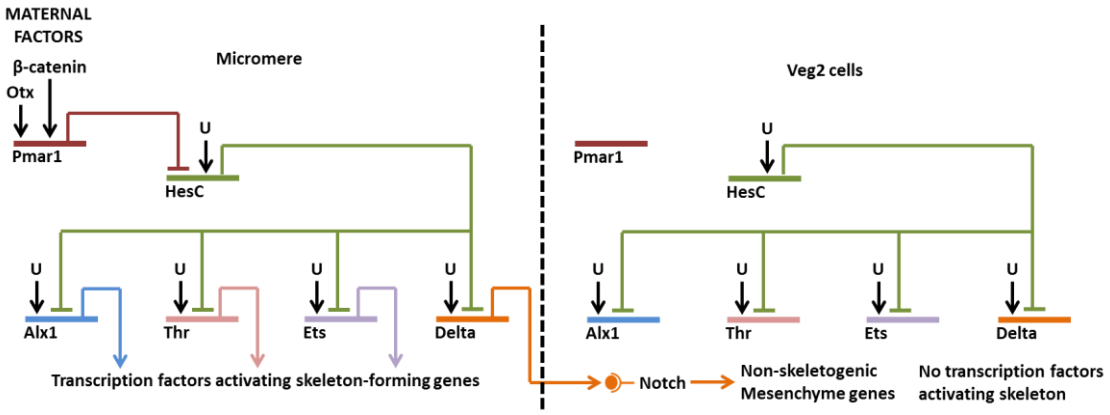

Fig. 1.6 Overall GRN for specification of the skeletogenic and non-skeletogenic micromere lineage. *β-catenin* activation in micromeres leads to activation of transcription factor (TF), *pmar1,* which in turn suppresses the repressor *HesC*. This double repression causes the activation of TFs that control skeletogenesis. Contrary to this at the vegetal cells (VEG2) layer, absence (or lesser concentration) of nuclear localized *β-catenin* leads to the repression of TF *Pmar1* and consequent activation of the repressor *HecC* and thus the repression of genes with skeletogenic fates (adapted from Gilbert, 2010). U=ubiquitous.

The non-canonical Wnt pathways or the β-catenin independent Wnt signaling includes the extensively studied Wnt/Planar Cell Polarity (PCP) pathway and the Wnt/Ca^{2+} pathway. In the Wnt/PCP signaling pathway, non-canonical Fz occupancy by cognate non-canonical Wnt ligands activates the signaling which is then transduced to Dsh/Dvl, which relays the signal via two parallel pathways stimulating RhoA and Rac GTPase respectively (Fig. 1.7.A) (Komiya and Habas, 2008). In sea urchin, Fz5/8 is known to relay Wnt signaling via Wnt/PCP pathway but not canonical Wnt/β-catenin signaling (Byrum *et al.*, 2009). Wnt/PCP signaling has been shown to be important for the initiation of archenteron invagination in sea urchin embryo by activating RhoA (Beane *et al.*, 2006; Croce *et al.*, 2006). Activation of Rho GTPase

leads to the activation of the Rho-associated kinase (ROCK), which in turn results in dynamic cytoskeletal rearrangement (Komiya and Habas, 2008) leading to formation of lamellopodia and filopodia-like structures associated with the leading edge during cell migration (Komiya and Habas, 2008). ROCK phosphorylates Myosin Light Chain (MLC) Phosphatase leading to its inactivation which results in accumulation of phosphorylated form of MLC. Active phosphorylated form of MLC is associated with acto-myosin contractility and bundling of actin filaments forming stress fibers (Heng and Koh, 2010). Inhibition of RhoA results in failure to initiate invagination movements (Fig. 1.7.B); whereas, constitutively active RhoA induced precocious invagination of the archenteron (Beane *et al.*, 2006). Similarily, inhibition of ROCK also results in blocking gut invagination (Beane *et al.*, 2006). The second branch of Wnt/PCP signaling activates the Rac GTPase which in turn stimulates c-Jun N-terminal Kinase (JNK) activity also known to be involved in modification of actin cytoskeleton (Tada *et al.*, 2002). Moreover, JNK activation downstream of RhoA, but independent of ROCK, has been shown to essential during *Xenopus* convergent extension movements (Kim and Han, 2005). Pharmacological inhibition of JNK blocks archenteron elongation but not blastopore invagination or skeletogenesis (Fig. 1.7.B) (Croce *et al.*, 2006). Combined inhibition of both JNK and ROCK gave rise to embryos with pigment cells but lacking skeletons and blastopores (Fig. 1.7.B) (Croce *et al.*, 2006). It has been shown that JNK phosphorylates a focal adhesion adaptor protein paxillin thus, promoting cellular migration (Huang *et al.*, 2003). Paxillin and its interacting proteins including focal adhesion kinase, c-Src, vinculin, Erk, p38

MAPK, MEK had all been implicated in cell migration (Mierke, 2013). Besides this JNK also phosphorylates FOXO which in nucleus can regulate transcription of several genes involved in diverse cellular processes including metabolism and cell death (Glauser and Schlegel, 2007).

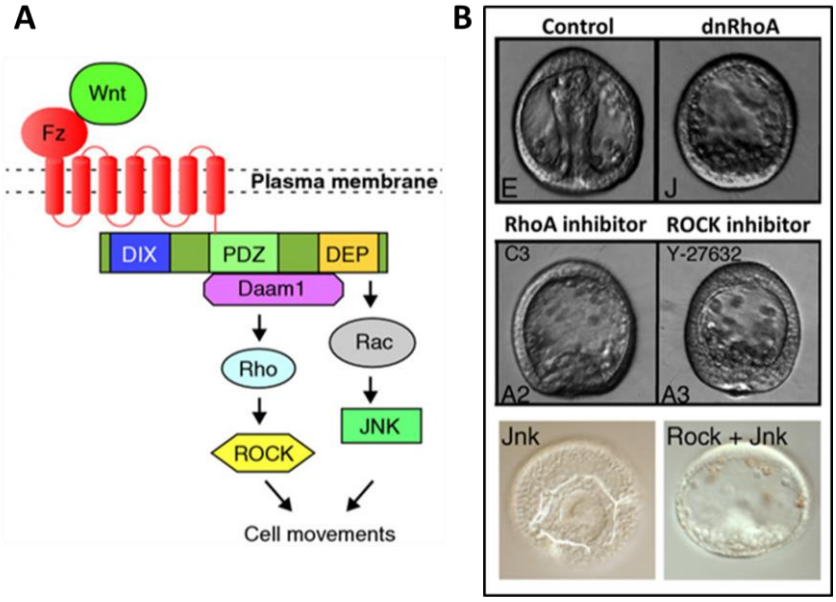

Fig. 1.7. Wnt/PCP pathway in sea urchin embryonic development. (A) Schematic diagram of the Wnt/PCP non-canonical signaling pathway. (B) Inhibition of Wnt/PCP pathway by overexpression of dominant negative form of RhoA (dnRhoA) or by use of pharmacological inhibitors (as indicated) leads to developmental defects (modified from Beane *et al.*, 2006; Croce *et al.*, 2006).

The non-canonical Wnt/Ca^{2+} pathway (Fig. 1.8) has been reported to further modulate canonical signaling for dorsal axis formation and PCP signaling for gastrulation cell movements (Komiya and Habas, 2008). Binding of non-canonical Wnt to its cognate

non-canonical receptor Fz results in activation of phospholipase C (PLC) via a heterotrimeric G-protein. This activation of PLC is coupled with short-lived increase in concentration of intracellular second messengers like inositol 1,4,5-triphosphate (IP_3), 1,2-diacylglycerol (DAG), and Ca^{2+} with the consequences of rapid alteration of the cellular functions. Elevated levels of intracellular Ca^{2+} is sufficient for activation of calcium-sensitive enzymes such as calmodulin dependent protein kinase II (CaMKII), protein kinase C (PKC) (Sheldahl *et al.*, 2003), calcinurin (Cn; protein phosphatase) (Rao and Kühl, 2010) and calpain (a non-lysosomal cysteine protease) (De, 2011). Activated CaMKII, PKC as well as Cn are already established in mediating gene transcription via NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), CREB (cAMP response element-binding protein) and NFAT (nuclear factor of activated T cells) (Komiya and Habas, 2008) transcription factors. NFAT has been shown to regulate transcription of heavy chains of several isoforms of motor protein Myosin (Delling *et al.*, 2000), implicating its role in regulation cellular movements by controlling actomyosin contractility (Rao et al., 1997). NFκB was shown to target protein kinase A gene (Kaltschmidt *et al.*, 2006); whereas, CREB targets the $GABA_B$ receptor gene (Steiger *et al.*, 2004) in murine neuronal cells. In sea urchin it has been shown that pharmacological inhibition of PKC (a component of Wnt/Ca^{2+} pathway) results in tripartite gut formation and ingression of PMCs, however, skeletogenesis was inhibited (Croce *et al.*, 2006).

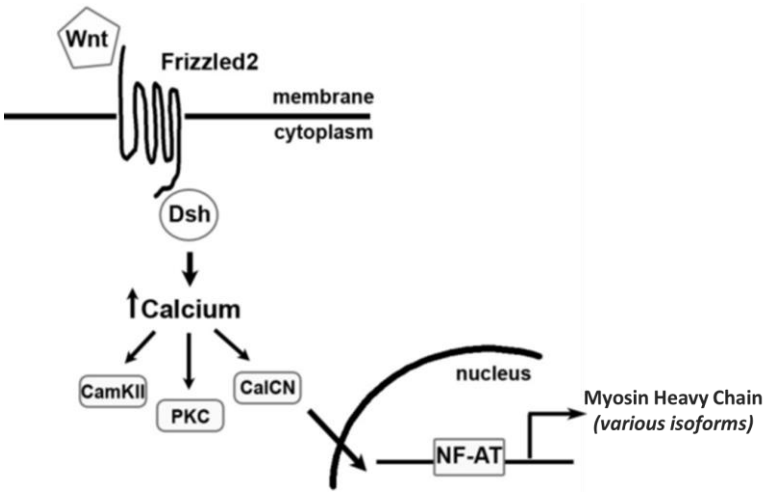

Fig. 1.8. Non-canonical Wnt/Ca^{2+} Pathway. Wnt signaling via this pathway results in elevation of intracellular calcium, activation of calcium-dependent proteins and induction of gene expression. CamKII - calcium/calmodulin-dependent protein kinase; CalCN - calcineurin; PKC - protein kinase C (modified based on Delling *et al.*, 2000).

Besides playing regulatory roles in canonical Wnt pathway, miRNAs also regulate non-canonical Wnt pathways. Wnt4, a non-canonical Wnt ligand regulating actin reorganization and cell motility, was found to be under direct regulation of miR-24 (De Menna *et al.*, 2012). In addition, while studying epithelial-to-mesenchymal transition and metastasis of breast cancer cells, it was revealed that miR-374a directly targets non-canonical Wnt5a (Cai *et al.*, 2013). Moreover, components of Wnt/PCP pathway such as RhoA has been shown to be a direct target of miR-31 (Valastyan *et al.*, 2009). In hepatocellular carcinoma cells (HCC), miR-142-3p was shown to directly target Rac1, which remodels actin, thus inhibiting invasion and migration potential of HCC (Wu *et al.*, 2011).

1.4 Goal of the Study

By treating the newly fertilized eggs with *Dicer* morpholino, a key enzyme in miRNA biogenesis pathway, our laboratory had previously demonstrated the importance of miRNAs in endomesoderm formation in sea urchin embryos (Fig. 1.9, Song *et al.*, 2012). Besides this, role of β-catenin in endomesodermal specification in various organisms including sea urchin (Fig. 1.10) had also been established (Logan *et al.*, 1999; Wikramanayake *et al.*, 1998). Furthermore, miRNA-mediated regulation of various components of Wnt signaling, as discussed in previous sections, are also documented based on the research on various vertebrate physiological and pathophysiological systems. Taking all this together, we test the hypothesis that components of the Wnt signaling pathway is under miRNA regulation during sea urchin development. For this, I tested the direct regulation of two key components of Wnt signaling pathway, Dsh/Dvl and β-catenin, by miRNAs using luciferase reporter constructs and site-directed mutagenesis. I will investigated the effect of blocking miRNA-mediated regulation of *Dsh* and *β-catenin* using miRNA target protector morpholinos (miRNA TP).

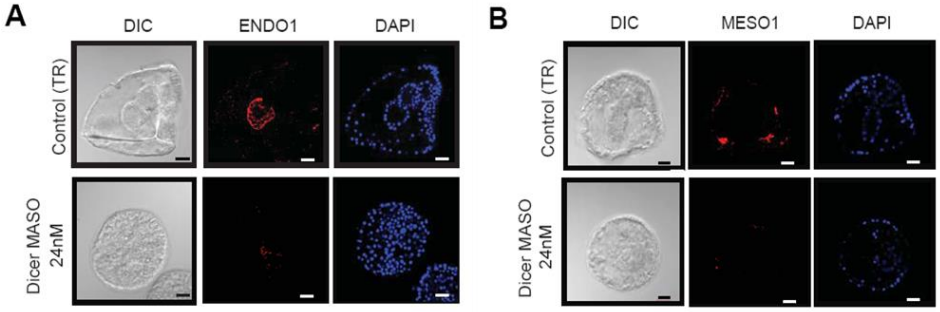

Fig. 1.9 miRNAs regulate endomesoderm formation. Embryos at 48 hpf were immunolabeled with (A) Endo1 and (B) Meso1 monoclonal antibodies to identify cell type specific differentiation markers of endoderm and mesoderm, respectively. Scale bar is 20 µm (adapted from Song *et al.*, 2012). Dicer MASO treated embryos have much less endodermal and mesodermal staining.

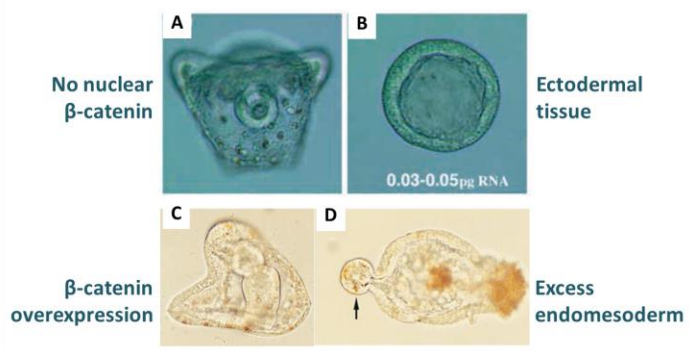

Fig. 1.10 β-catenin is essential for endomesodermal specification. Nuclear localization of β-catenin is important for (A) normal endomesoderm specification and inhibition of β-catenin nuclear accumulation results in (B) synthesis of ectodermal tissue only (modified from Logan et al., 1999). (C) Control embryo (modified from Wikramanayake *et al.*, 1998). (D) overexpression of β-catenin leads to excessive endomoesodermal tissue compared to control embryo at pluteus larva stage.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

Adult *Strongylocentrotus purpuratus* were obtained from Point Loma Marine Company, California. Adult males and females were given 0.5M KCl intracoelomic injections for obtaining sperm and eggs. In-vitro fertilization and embryo development was carried out in filtered natural sea water (collected from Indian River Inlet; University of Delaware Lewes campus) or artificial sea water at 15°C.

2.2 Cloning of Luciferase Reporter Constructs

Previously, *β-catenin* 3'UTR was cloned downstream of the eGFP coding sequence (in pGEMT vector background) (Siddam, 2012). For making *Renilla* luciferase-*β-catenin* 3'UTR reporter construct, the eGFP coding sequence was removed using *Xho*1 and *Sal*1 restriction enzymes to yield *β-catenin* 3'UTR in pGEMT background. *Renilla* luciferase (Rluc) vector (from psiCHECK vector, Promega, Madison, WI) was used as a template for PCR amplification of Rluc CDS. The primers were designed to insert *Xho*1 and *Apa*1 restriction sites flanking Rluc CDS. The primers used were 5'AACGCTCGAGATCCAGATGTACCAAGCCAA3'

and 5'GGCACTAGTAGGAACTACAAGAAAGTCTC3' (restriction sites are underlined). The PCR reaction contained 0.2 mM of both forward and reverse primers, 1x Green Flexi buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.025 units of Go Taq Hot Start polymerase (Promega, Madison, WI). PCR conditions used were step 1: 95°C for 2 minutes; step 2: 95°C for 1 minute; step 3: 60°C for 1 minute; step 4: 72°C for 1 minute (steps 2-4 repeated 35 times) and finally, step 5: 72°C for 5 minutes. Subsequently, the PCR product was digested with Xho1 and Apa1 restriction enzymes and the *β-catenin* 3'UTR in pGEMT background was phosphatase-treated with TSAP (Promega, Wisconsin, WI) for 45 minutes at 37°C, followed by inactivation at 74°C for 15 minutes. These two preparations were separately purified with the PB buffer (5 M Guanidine-HCl, 30% ethanol, 10 mM Tris-HCl pH 6.6) at 5x the volume as the initial volume (of digestion mixture or TSAP treatment reaction mixture volume). The contents were spun at 15,000 RPM for 1 minute and the flow through was discarded. 500 µl PE Buffer was added and spun at 15,000 RPM for 1 minute and flow through was discarded. Columns were spun for additional 1 minute and lastly the DNA was eluted with 30 µl of molecular grade water (Mediatech, Inc., Manassas, VA). Next, the Rluc CDS with flanking sticky restriction sites was ligated upstream of *β-catenin* 3'UTR to generate the desired luciferase reporter construct which was used to transform the DH5-α cells (Invitrogen, Carlsbald, CA). Firefly luciferase from psiCHECK vector was PCR amplified with primers, 5'GCATAGATCT ATGGCCGATGCTAAGAACATT3' and 5'ATAGCTCGAGATACCACATTTG

TAGAGGTTTTA3' with restriction enzymes sites underlined, and cloned into the pSP6T4. Positive clones were sequenced (Genewiz, Inc., South Plainfield, NJ).

For generating Dsh/Dvl 3'UTR luciferase reporter constructs, Dsh/Dvl 3'UTR was amplified from sea urchin cDNA library generated from total mRNA extracted from embryos at 6 hpf. Subsequent to amplification, the PCR product was subjected to restriction digestion using *Xho*1 and *Not*1 restriction enzymes. The Rluc CDS containing vector was digested with *Xho*1 and *Not*1. After restriction digestion the Rluc CDS containing vector were TSAP treated and then PB purified as discussed above. Subsequently, the Dsh/Dvl 3'UTR was ligated downstream of Rluc CDS to generate the desired luciferase reporter construct which was transformed into the DH5-α cells (Invitrogen, Carlsbald, CA). Firefly luciferase to be used as loading control was generated as mentioned above and all the positive clones were confirmed by DNA sequencing (Genewiz, Inc., South Plainfield, NJ).

2.3 Site-directed Mutagenesis

The miR-2007 seed sequence was identified to have two binding sites within the *β-catenin* 3'UTR. The miR-Deep 2-35240 seed sequence was identified to have one binding site within the *β-catenin* 3'UTR. All seed sequences were mutated at positions 3 and 5 within the miRNA seed sequence. miR-2007 seed sequence 5'CTGAAAT 3' was modified to 5' CTCACAT 3' and miRDeep2-35240 was

modified from 5’ GTGCAAT 3’ to 5’ GTCCTAT 3’. Mutageneic primers were designed according to the primer design program at www.agilent.com/genomics/qcpd. Mutations in the miR-2007 (single and double mutations) and miR Deep 2-35240 seed regions within the *β- catenin* 3’UTRs were generated using the QuikChange Lightning according to manufacturer’s instructions (Agilent Technologies, Santa Clara, CA). Positive clones were sequenced (Genewiz, Inc., South Plainfield, NJ). Primer sequences used for site directed mutagenesis for miR-2007 at position +922 are: 5’-CAGGCCTCGTCAAGATTATATCTCACATAGATATCTCATGATTGGCTAC-3’ and 5’-GTAGCCAATCATGAGATATCTATGTGAGATATAATCTTGACGAGGCCTG-3’ The second set of primers for miR-2007 at position +2647 are: 5’-AATCTAAGCTACTTCCATTTTTCATGTCTTAGATAAACTGAATACATTCTTTAAGATTG-3’ and 5’-GCAATCTTAAAAGAATGTATTCAGTTTATCTAAGACATGAAAAATGGAAGTAGC TTAGAT-3’. The primers used for miRDeep2-35240 are 5’-GCATATTGATGCCTCTTAAAAAGGTCCTATAAAGAGTACAATATGCAACAATC-3’ and 5’-GATTGTTGCATATTGTACTCTTTATAGGACCTTTTTAAGAGGCATCAATATGC-3’. Altered seed sequences are underlined.

For Dsh/Dvl, miR-153* seed sequence 5’-AAAATG-3’ was mutated to 5’-AGAAGG-3’ and miRDeep-2-35240 was modified from 5’-GTGCAAT-3’ to 5’-GCGCGAT-3’. The mutagenic primers are: (1) miR-153* primers - 5'-GCTGTGGTAGGAAGTACAGCTTTCAAAAAAAGAAGGCTAGATGATGTAAC A-3' and 5'-TGTTACATCATCTAGCCTTCTTTTTTTGAAAGCTGTACTTCCTA

CCACAGC-3'; and (2) miRDeep-2-35240 primers - 5'GATGTAACATAGAGCTT CTTGCGCGATGTTGATTGATCTGGAGAGA-3' and 5'TCTCTCCAGATCAATC AACATCGCGCAAGAAGCTCTATGTTACATC-3'. Altered seed sequences are underlined.

2.4 *In vitro* Transcription.

The reporter constructs were linearized prior to *in vitro* transcription. β-catenin luciferase, Dsh/Dvl luciferase and firefly reporter constructs were linearized with *EcoR*I enzyme. Linearized reporter constructs were *in vitro* transcribed using mMessage machine kit with either T7 (for *β-catenin* and *Dsh/Dvl* mRNAs) or Sp6 (firefly luciferase mRNAs) RNA polymerases (Ambion, Carlsbad, CA) according to the manufacturer's instructions with the following modifications: DNAse treatment step during *in vitro* transcription reaction was carried out for 40 minutes at 37°C instead of 15 minutes. mRNAs were purified by using Qiagen microRNeasy kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA) and purified mRNAs were loaded onto the Millipore spin columns to further clean the mRNAs (Millipore, Billerica, MA).

2.5 Microinjections

Microinjections were performed as previously described (Song *et al.*, 2012) with modifications. For luciferase assays, 100 ng of *Renilla* luciferase and 60 ng of

firefly luciferase were prepared in 2.5 µl of injection solution consisting of 0.5 µl of 100% glycerol and 0.5 µl of Texas Red dextran (Molecular Probes, Carlsbad, CA). Approximately 1-2 picoliter (pl) was injected into each newly fertilized egg. For miRNA TP, oligonucleotides were diluted in injection solutions containing 20% sterile glycerol, 2 mg/ml 10,000 MW Texas Red lysine charged dextran (Molecular Probes, Carlsbad, CA). For β-catenin studies 3µM, 30µM and 300µM of miRNA TP was injected, whereas for Dsh/Dvl studies 2µM of respective miRNA TP were injected. Approximately 2 µM of control miRNA-TP was injected into each zygote. Eggs from *S. purpuratus* were collected and dejellied in acidic sea water (pH 5.15) for 10 minutes on ice, followed by two sea water washes. Dejellied eggs were rowed onto 60 × 15 mm petri dishes that were previously coated with protamine sulfate (4% w/v). Eggs were fertilized with sperm in the presence of 1 mM 3-amino-triazol (Sigma, St. Louis, MO). Injections were performed using the Femto Jet injection system (Eppendorf; Hamberg, Germany). Vertical needle puller PL-10 (Narishige, Tokyo, Japan) was used to pull the injection needles 1x90 mm glass capillaries with filaments (Narashige; Tokyo, Japan).

2.6 Dual Luciferase Quantitation

All dual luciferase quantitation was performed using the Promega™ Dual-Luciferase™ Reporter (DLR™) Assay Systems with the Promega™ GloMax™ 20/20 Luminometry System (Promega, Madision, WI). 50 embryos at the 32-cell stage were

collected at 5-6 hpf in 22 µl 1X lysis buffer and vortexed for 1 minute. Embryonic lysates were either stored at -80°C or processed immediately. Prior to luciferase readings, 100 µl of the Luciferase Activating Reagent II (LAR-II) was added to each well of the 96-well plate. 20 µl of the embryonic lysates was then added and luciferase reading for the firefly was obtained. Subsequently, 100 µl of the Stop and Glow solution was added to quench *Firefly* luciferase signal and the *Renilla* luciferase reading was obtained. *Renilla* luciferase readings were subtracted from corresponding initial reading to obtain firefly reading which was used to calculate the RLuc/FLuc ratio. Finally, the values of mutants were normalized to that of wild type.

2.7 Western Blotting

200 each of control and β-catenin miRNA-TP treated embryos were pelleted by spinning the embryos at 15,000 RPM for 30 seconds and resuspended in 15 µl of 4X sample buffer (1 mM Tris HCl, 0.1 mM sucrose, 4% SDS and 10 mM EDTA) and vortexed for 30 seconds and heated at 100°C for 10 minutes. Samples were stored at -80°C until later use. 1mM DTT was added to the protein sample and heated for 10 minutes at 100°C immediately prior to loading samples onto Tris-Glycine 4-20% gradient gel (Bio-Rad, Hercules, CA) and ran at 120 V for 1 hour in Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine at pH 8.3, 0.1% SDS). Proteins were transferred onto PVDF membranes (Bio-Rad, Hercules, CA) at 250 AMP for 2 hours at 4°C. Membranes were blocked with Blotto (0.05 mM Tris at pH 7.5, 0.18 M

NaCl, 3% dry milk and 0.05% Tween 20) for 2 hours at RT and then incubated with β-catenin antibody at 1:250 (generously gifted by Dr. Athula Wikramanyake, University of Miami) in Blotto overnight at 4°C, followed by three TBST (0.05 M Tris, 0.18 M NaCl, 0.05% Tween) washes. The blot was incubated with secondary antibody goat anti-rabbit HRP (Jackson Immuno Research, West Grove, PA) diluted to 1:3000 in Blotto for 45 minutes at RT. The blots were washed 3 times with TBST and signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions and exposed using Kodak CareStream Imager 4000R system.

2.8 Real time, Quantitative PCR (qPCR)

80-100 Dicer morpholino, control morpholino, or *β-catenin* miRNA TP-injected embryos were collected at various time points. Total RNA was collected using Qiagen microRNeasy kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA). cDNA was synthesized using TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster city, CA). qPCR was performed using two embryos equivalent for each reaction with the Applied Biosystems power SYBER Green PCR Master Mix (Invitrogen) in the 7300 Real-Time PCR cycler system (Applied Biosystems, Foster City, CA). Results were normalized to the mRNA expression of housekeeping gene ubiquitin and shown as fold changes compared to control embryos that were injected with the control morpholino using the ΔΔCt method. At least three

biological replications were conducted. Statistical significance was calculated using the 2 tailed unpaired Student t-test (Goni *et al.*, 2009).

2.9 Immunofluorescence

Embryos at a specific time point were fixed in 4% paraformaldehyde (16% stock; EMS, Hatfield, PA) in artificial sea water for 10 minutes at room temperature. Four 10 minute PBS-Tween washes were performed, followed by 1 hour of blocking with 4% sheep serum (Sigma Aldrich, St. Louis, MO). Primary antibody incubation was performed with Endo1 antibody (Wessel and McClay 1985) at 1:200 overnight at 4°C. Embryos were washed three times for 15 minutes each with PBS-Tween followed by goat anti-mouse Alexa488 (Invitrogen, Carlsbad, CA) conjugated secondary antibody at 1:300. After two more PBS-Tween washes, embryos were incubated with Hoechst dye at 1:1000 for 5 minutes. Two more PBS-Tween washes were performed. The immunolabeled embryos were then imaged with confocal microscope LSM 780 (Carl Zeiss Incorporation, Thorwood, NY) and data analysis was performed using the Zen software.

2.10 Detection of Endogenous Alkaline Phosphatase

Three day old larvae were fixed in MOPs paraformaldehyde fixative (4% paraformaldehyde, 100 mM MOPS pH 7.0, 2 mM $MgSO_4$, 1 mM EGTA, and 0.8 M NaCl) for 10 min at room temperature. Embryos were washed thrice with alkaline

phosphatase buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20), followed by staining until desired color development with the staining solution (0.1 M Tris pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl, 1 mM Levimasole, 10% Dimethylformamide, 75 mg/mL NBT and 50 mg/mL BCIP). The staining step is stopped with washes with MOPS buffer (0.1 M MOPS pH 7.0, 0.5 M NaCl, and 0.1% Tween-20). Images were acquired with Nikon D90 digital camera with the Zeiss Axiovert Observer Z1 microscope.

Chapter 3

RESULTS

3.1 MiRNAs Directly Regulate β-catenin Post-transcriptionally.

We have previously observed that knockdown of Dicer results in increased mRNA transcripts of *β-catenin* (manuscript submitted). This suggests that miRNAs regulate the transcript stability of *β-catenin*.

Bioinformatic analysis revealed that *β-catenin* 3'UTR (3034 bp in length) had 100 percent seed match to several potential miRNA binding sites (Table 3.1) (Song et al., 2012). From amongst the 16 miRNA predicted sites, only 2 miRNAs, *viz.* spu-miRDeep2-30364-35240 and spu-miR-2007, have more than 1,000 normalized deep sequence reads during initial stages of the sea urchin embryonic development (Song *et al.*, 2012). Hence, we tested if spu-miRDeep2-30364-35240 and spu-miR-2007 regulate *β-catenin* post-transcriptionally. The 3'UTR of *β-catenin* obtained from previous analysis (Samanta *et al.*, 2006; Sodergren *et al.*, 2006) was cloned downstream of *Renilla* luciferase reporter constructs. The potential miRNA binding sites corresponding to seed sequences for miR-2007 and miRDeep2-30364-35240 were altered using site directed mutagenesis. miR-2007 has two binding sites (at positions +922 bp and +2647 bp downstream of the stop codon at +1 bp) and

miRDeep2-30364-35240 has one binding site (at position +2401 bp) within the *β-catenin* 3'UTR.

Table 3.1 Potential miRNA Binding Sites in β-catenin 3'UTR.

microRNA	Seed Sequence	Frequency of Seed Sequence	Stage					
			Ovaries	Egg	32-cell	24 hours	48 hours	72 hours
spu-miRDeep2-30364 spu-35240	GTGCAAT	1	2003	1794	591	907	1875	2390
spu-miR-2007	CTGAAAT	2	2255	3441	789	1648	9380	8290
spu-miR-2007*	CAACAGG	1	0	1	17	43	11	72
spu-miR-252b*	TAGTACAG	1	0	0	33	8	3	44
spu-miR-2011*	AGTGACT	1	1	0	10	30	47	90
spu-miR-34*	AAGCTGG	1	4	0	27	31	21	83
spu-miR-2009*	CAAAAAC	1	1	1	51	139	50	245
spu-miR-2002*	TTGGTCT	1	2	0	14	28	3	47
spu-miR-124*	TGAACAC	1	0	0	0	5	0	5
spu-miR-200*	CAGTATT	1	147	37	40	70	59	503
spu-miR-9*	AGCTTTA	1	2	0	13	33	11	63
spu-miR-137*	GAATACC	1	0	0	0	1	0	1
spu-let-7*	CTGTATA	2	0	0	0	0	0	0
spu-miR-153*	AAAAATG	2	0	0	0	0	0	0
spu-miR-2004*	ACAGAAA	1	0	0	0	0	0	0
spu-miR-1*	AAGTATG	2	57	5	1	51	225	104

(*) denotes the passenger strand of the miRNA duplex. (adapted from Song *et al.*, 2012).

The third and fifth base pairs of the seed sequences were mutated to disrupt the miRNA target recognition (Fig. 3.1 A) (Gregory *et al.*, 2008). Various mutants designed using this strategy are: (1) Single mutants in which either of the three sites were mutated, (2) double mutant in which both the miR-2007 sites were mutated, and (3) triple mutant in which all three miRNA binding sites were mutated (Fig. 3.1 A). Newly fertilized cells were co-injected with *in vitro* transcribed *Renilla* luciferase reporter constructs fused to the WT or mutant *β-catenin* 3'UTR. Firefly luciferase

reporter constructs were used to adjust for injection volume differences for *Renilla* luciferase quantitation.

The results show that miR-2007 at +922 bp and miRDeep2-30364-35240 at +2401 bp are functional miRNA binding sites at the 32-cell stage (Fig. 3.1 B, C). The double and triple mutants containing miR-2007 and miRDeep2-30364-35240 have additional increase in luciferase reading, indicating additive regulatory effects by multiple miRNAs. Overall, these results indicate that *β-catenin* 3'UTR contains at least three functional miRNA regulatory sites.

3.2 Endogenous miRNA Modulate *β-catenin* Expression.

To test if the miRNA binding sites within the *β-catenin* 3'UTR are important for regulating its protein translation in the developing embryo, we designed miRNA **t**arget **p**rotector morpholinos (miRNA TP) against the three miRNA binding sites within the *β-catenin* 3'UTR (Fig. 3.2 A) (Staton and Giraldez, 2011). These antisense morpholino sequences are unique to the *β-catenin* 3'UTR, as BLASTN searches in the sea urchin genome only identified these sequences to be within the *β-catenin* 3'UTR. We found that embryos receiving miRNA TP have a 2.5 fold increase of β-catenin protein, (Fig. 3.2 B).

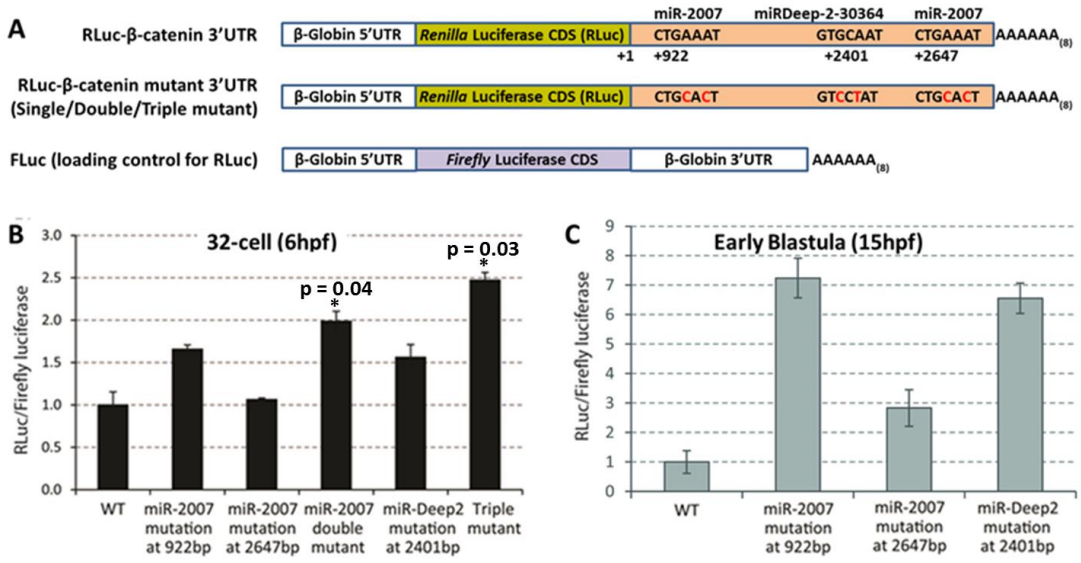

Fig. 3.1 Direct regulation of *β-catenin* by miRNAs. (A) *β-Catenin* 3'UTR was cloned downstream of *Renilla* luciferase coding sequence (RLuc). The three miRNA binding sites (seed sequences in particular) were mutated using site-directed mutagenesis to generate various mutant constructs. *Firefly* luciferase (FLuc) was used as a loading control for RLuc. WT and mutant RLuc constructs as well as control FLuc constructs were transcribed *in vitro*. The mRNAs were co-injected in newly fertilized eggs and embryos were collected (B) at 32-cells or at (C) early blastula stages and were subjected to dual luciferase assay.

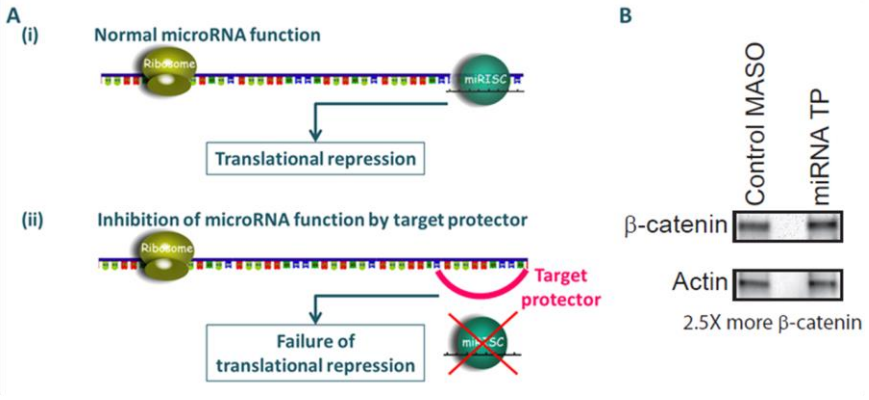

Fig. 3.2 Endogenous miRNAs modulate *β-catenin* expression. (A) Illustration of miRNA target protector morpholino is used to block functional miRNA regulatory sites. (i) Binding of miRNA to its target site in the 3'UTR of the mRNA results in translational repression. (ii) miRNA target protector morpholino (miRNA TP) is used to disrupt miRNA target binding.(B) Blocking miRNA regulation with miRNA TP results in increased β-Catenin protein at 32-cell stage embryos (2 biological replicates).

3.3 Blocking miRNA-mediated Regulation of *β-Catenin* Results in Increased Transcripts of Endodermal Regulatory Genes

Previously, it has been reported that β-catenin is important for endoderm and mesoderm specification during early embryonic development in sea urchin (Logan *et al.*, 1999; Wikramanayake *et al.*, 1998). Therefore, we next examined if the increase in β-catenin protein in miRNA TP treated embryos has an impact in early development at the molecular level. We assayed the transcript level changes of Wnt responsive genes, including transcription factors and signaling molecules that regulate germ layer specification. Our results demonstrate that *Blimp1b*, *Krl*, *Eve, FoxA* (endodermal genes), and *FoxQ2* (ectodermal gene) were increased at least two-fold in embryos treated with miRNA TPs as compared to embryos treated with the control miRNA TP (Fig. 3.3). This is consistent with the conserved role of Wnt signaling pathway in regulating genes controlling endodermal specification and cell fate decisions.

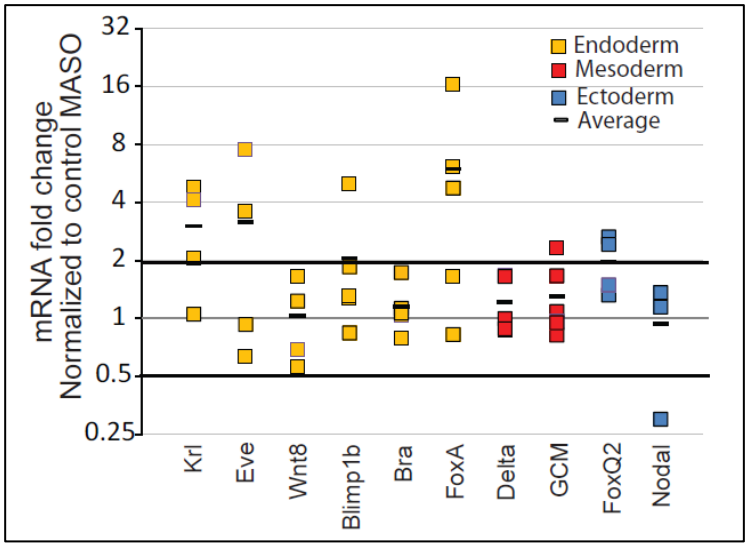

Fig. 3.3 Inhibition of miRNA mediated regulation of β-catenin results in increase in transcripts of Wnt responsive genes. Real time, quantitative PCR (QPCR) was used to measure the transcript levels of genes involved in the specification of endoderm, mesoderm, and ectoderm in control and miRNA TP-injected embryos. Embryos were collected at the early blastula stage at 15 hpf and total mRNA was isolated and subjected to QPCR analysis (Jia L. Song and Nadezda Stepicheva).

3.4 Elimination of miRNA-mediated Regulation of *β-catenin* Results in Morphological Changes in Gut Structure.

We next evaluated the impact of miRNA TP induced increase of β-catenin levels in early development. We observed that embryos injected with the β-catenin miRNA TP have gut developmental defects in that they have narrower gut width than the control (Fig. 3.4 B). Furthermore, the embryos injected with control miRNA TP were larger than the embryos treated with miRNA TP (Fig. 3.4 C). Approximately 18% of the miRNA TP injected embryos have aberrant hindgut structure, suggesting potential defects in gut development (Fig. 3.5A).

3.5 Impaired Endodermal Specification in *β-catenin* miRNA TP-Treated Embryos.

Next we wanted to investigate if this apparent defect in gut morphology (Fig. 3.4A and 3.5A) translates into defective gut function. For this we evaluated the digestive function of the larval gut by detecting the presence of endogenous digestive enzyme alkaline phosphatase (Annunziata *et al.*, 2014). In the sea urchin larvae, expression of alkaline phosphatase is restricted to gut epithelium and is considered as a marker of differentiated endoderm. β-catenin miRNA TP treated embryos showed reduced alkaline phosphatase stain compared to the control (Fig. 3.5B,C). In addition, a significant number of miRNA TP treated embryos had negligible alkaline phosphatase staining in the hindgut (intestine) as compared to the control (black arrow), indicating potential defective intestinal role during digestion (Fig. 3.5B,C).

Therefore, the smaller embryo size observed (Fig. 3.4C and 3.5D) can be explained due to defect in digestion and malnutrition suffered by growing embryo.

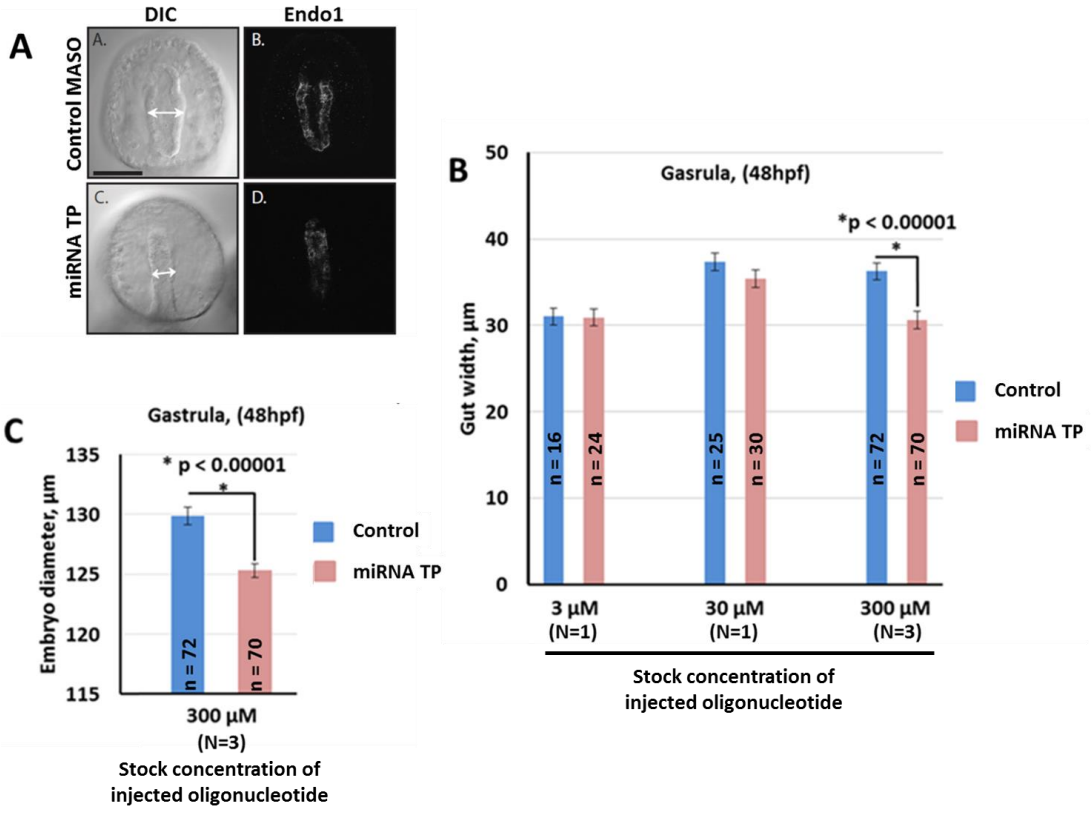

Fig. 3.4 Inhibition of miRNA-mediated regulation of *β-catenin* results in morphological differences. (A) Representative images of control and miRNA TP treated embryos (300µM) using differential interference contrast microscopy. Scale bar = 50µm. **(B and C)** Bar charts depicting gut width and embryo diameter (in µm), respectively, of control and miRNA TP treated embryos at indicated concentration (n = total number of embryos; N = number of biological replicates; Panel A: Courtesy Nadezda Stepicheva).

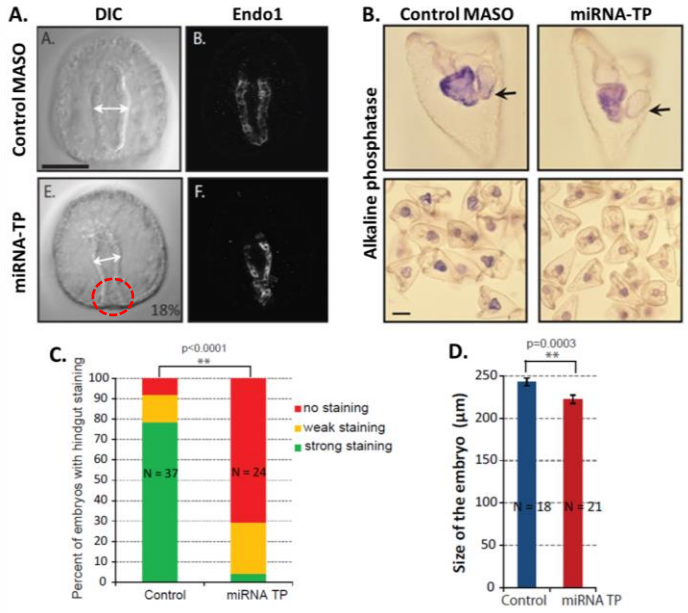

Fig. 3.5. Defective hindgut morphology and impaired digestive function in *β-catenin* miRNA TP-treated embryos. (A) Treatment with *β-catenin* miRNA TP results in trapezoidal morphology of hindgut compared to control. (B and C) Alkaline phosphatase (AP) is a marker for differentiated endoderm. Control embryos have more intense AP staining than the *β-catenin* miRNA TP-treated embryos. Many of the embryos exposed to the *β-catenin* miRNA TP lack AP staining in the hindgut (arrows). (D) Smaller embryo size upon miRNA TP treatment potentially due to improper hindgut digestive function (Courtesy: Nadezda Stepicheva).

3.6 Dishevelled is Directly Regulated by miRNAs.

We examined another key component of the Wnt signaling pathway, the *Dishevelled* gene, which is a key regulator of both canonical as well as non-canonical Wnt signaling (Gao and Chen, 2010). Bioinformatic analysis of 3'UTR of *Dsh/Dvl* mRNA revealed two putative miRNA binding sites. In order to determine the functionality of these sites we used the strategy similar to that of *β-catenin*. Wild-type *Dsh/Dvl* 3'UTR was cloned downstream of RLuc CDS. The miRNA binding seed

sequences were mutated using site-directed mutagenesis to abolish endogenous miRNA binding to the target sites in the 3'UTR of reporter constructs (Fig. 3.6 A)

Our results show that miR-153* and miRDeep-2-35240 are functional regulatory miRNAs of *Dsh/Dvl*. The double mutant resulted in increased luciferase reading when normalized to wild type, but not as much as the single mutants (Fig. 3.6.B).

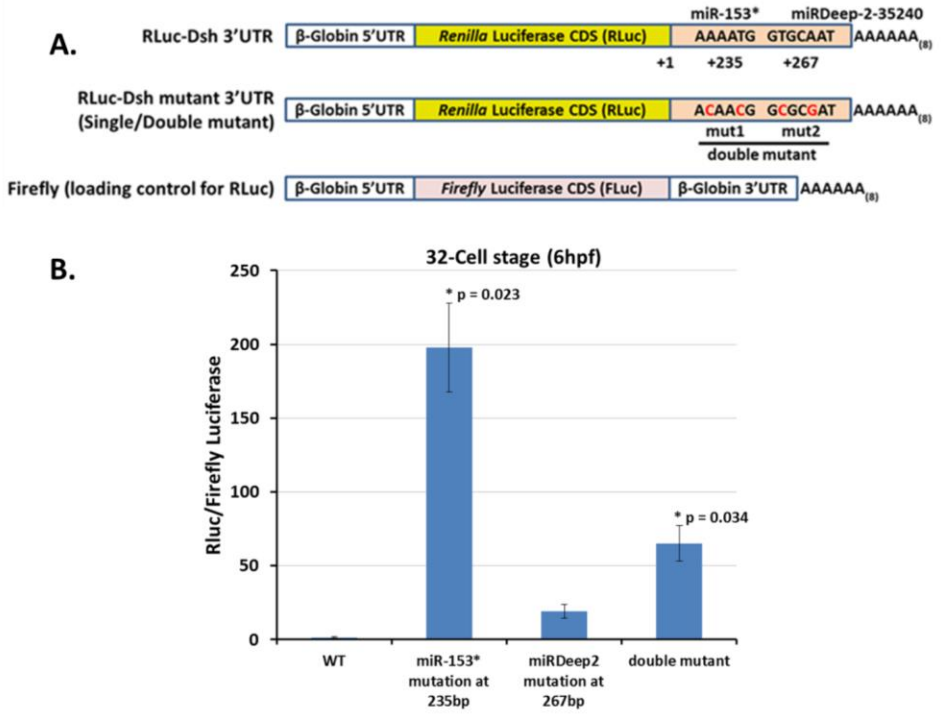

Fig. 3.6 Direct regulation of *Dishevelled* by miRNAs. (A) *Dsh/Dvl* 3'UTR was cloned downstream of *Renilla* luciferase coding sequence (Rluc). The two miRNA binding seed sequences were mutated using site-directed mutagenesis to generate various mutant constructs. *Firefly* luciferase (Fluc) was used as a loading control for Rluc. Rluc constructs containing wild type or mutated seed sequences as well as control Fluc constructs were transcribed *in vitro*. (B) The mRNAs were co-injected in newly fertilized eggs and embryos were collected at 32-cell stage and subjected to dual luciferase assay. (3 biological replicates).

3.7 Blocking miRNA-mediated Regulation of *Dsh/Dvl* Results in Thinner Gut and Smaller Embryos.

Next we examined the effect of blocking the miRNA regulation of *Dsh/Dvl* on the development of sea urchin embryo. Embryos treated with *Dsh/Dvl* miRNA TP showed an early invagination of gut (29 hpf) as compared to control (Fig. 3.7A). Previously, we have seen that blocking miRNA regulation of *β-catenin* using miRNA TP results in thinner gut formation (Fig. 3.4). In line with this, we observe that elimination of miRNA regulation of *Dsh*, an upstream regulator of β-catenin, also results in thinner gut phenotype when compared to control miRNA TP treated embryos (Fig. 3.7A, B). Furthermore, the embryos injected with *Dsh/Dvl* miRNA TP were significantly smaller than embryos injected with control morpholino (Fig. 3.7 C).

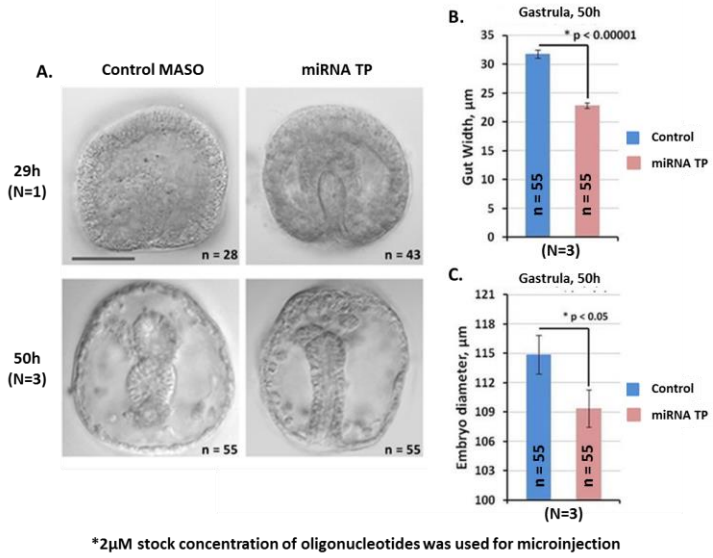

Fig. 3.7 Blocking miRNA regulation of *Dsh/Dvl* affects gut morphology. (A) Representative image showing earlier invagination (29 h) and overall thinner gut phenotype in miRNA TP treated embryos compared to control. (B) Decrease in average gut width of miRNA TP treated embryo compared to control miRNA TP treated embryos. (C) Reduction in embryo size upon treatment with miRNA TP (n = total number of embryos; N = number of biological replicates; scale bar = 50µm).

Chapter 4

DISCUSSION

Our results demonstrate for the first time that miRNAs modulate *β-catenin* and *Dishevelled*, two key components of the Wnt signaling pathway, during early sea urchin embryonic development. β-catenin is a highly conserved transcriptional coactivator of the canonical Wnt signaling pathway that regulates endoderm specification in metazoans (Ettensohn 2006). β-catenin is known to be regulated at transcriptional, translational, and post-translational levels (Huang et al. 2010; Zhao et al. 2011). We show that miRNAs directly regulate *β-catenin* expression at the post-transcriptional level, in addition to its regulation by GSK3β through phosphorylation (Weitzel et al. 2004). We show that the 3'UTR of sea urchin *β-catenin* contains at least three functional miRNA binding sites that are targeted by two different miRNAs: Spu-miR2007 and Spu-miRDeep-2-30364. This is supported by our luciferase reporter data (Fig. 3.1) and by our observation that the level of β-catenin protein at the 32-cell stage is significantly increased in miRNA TP-treated embryos compared to the control (Fig. 3.2).

In the sea urchin, the cells of the veg1 and veg2 lineage (Fig. 1.3B) are known to give rise to the endodermal tissue (Logan and McClay 1997; Ransick and Davidson

1998). The veg2 cells form the endodermally derived foregut as well as ventral midgut, whereas, veg1 cells produce the hindgut and the dorsal midgut (Logan and McClay 1997; Ransick and Davidson 1998). Besides these structures, veg2 cells also form the future non-skeletogenic mesodermal cells, while veg1 cells contribute to the formation of future ectodermal tissue (Logan and McClay 1997; Ransick and Davidson 1998). Localized canonical Wnt signaling along with Otx in the vegetal blastomeres induces the veg2 cells to become the endoderm by activating endodermal regulatory genes such as *Blimp1b*, *Krl*, and *Eve* (Arenas-Mena et al. 2006; Howard et al. 2001; Peter and Davidson 2010; Peter and Davidson 2011; Smith et al. 2008). In turn, these genes activate more endodermal regulators, including *Brachyury* and *FoxA* (Peter and Davidson 2011; Smith et al. 2008). *Eve* regulates the veg1 endodermal GRN by inducing synthesis of transcription factor *Hox11/13b* (Peter and Davidson 2011). We observed a dramatic increase in the FoxA transcript upon *β-catenin* miRNA TP treatment (Fig. 3.3). FoxA, a highly conserved forkhead transcription factor that regulates endoderm formation in many bilaterians and cnidarians (Boyle and Seaver 2008; Burtscher and Lickert 2009; Friedman and Kaestner 2006; Hiruta et al. 2005; Oliveri et al. 2006), was found to be restricted to the β-catenin expressing Veg2/endoderm lineage cells and is repressed in mesodermal precursor cells (de-Leon and Davidson 2010; Peter and Davidson 2011). Our QPCR results demonstrate elevated transcript levels of several endodermal regulators in response to enhanced β-catenin protein levels that result from upon treatment of *β-catenin* miRNA TP. Thus, these data support function of β-catenin as a highly conserved transcriptional co-

activator that regulates endoderm specification in metazoans (Ettensohn 2006; Holstein et al. 2011; Petersen and Reddien 2009).

Dishevelled, another key component of canonical Wnt signaling, was found to have two miRNA binding sites in its 3'UTR that were targeted by miR-153* and miRDeep2-30364-35240. Interestingly, miRDeep2-30364-35240 also targets *β-catenin* mRNA. Literature on miRNA regulation of *Dsh/Dvl*, is scarce with only one report showing direct regulation of mammalian *Dsh/Dvl* isoform *DVL2* by miR-221 in human prostate cancer cell lines (Zheng et al., 2012).

Blocking miRNA regulation of either *Dsh/Dvl* or *β-catenin* resulted in significantly narrower gut in the embryos at the gastrula stage compared to control (Fig. 3.7 and 3.4, respectively). This suggests that removal of miRNA regulation of *Dsh/Dvl* or *β-catenin* results in compromised gut morphogenesis and gut functions. Besides the narrower gut, we had also observed that about 18 percent of the *β-catenin* miRNA TP-treated embryos had trapezoidal hind gut morphology which was absent in control miRNA TP treated embryos. Digestive enzyme alkaline phosphatase, expressed in the sea urchin gut epithelium, serves as a marker for differentiated endoderm and the digestive function of the gut. Our data from alkaline phosphatase (AP) staining assays where we observed diminished AP staining in the hindgut region of *β-catenin* miRNA TP treated embryos suggesting impaired intestinal (hindgut) digestive functions, is thus consistent with the phenotypic defect we observed in *β-*

catenin miRNA TP treated embryos at the gastrula stage. Furthermore, this supports our other observation that *β-catenin* as well as *Dsh/Dvl* miRNA TP treated embryos are smaller in size compared to control potentially due to poor nutritional intake as a result of impaired digestive function.

Moreover, we observed that elimination of miRNA-mediated regulation of *Dsh/Dvl* results in early invagination of gut in Dsh/Dvl miRNA TP treated embryos compared to control. RhoA, a downstream effector of non-canonical Wnt/PCP pathway, had been associated with initiation of gut invagination (Beane *et al.*, 2006). It was demonstrated that inhibition of RhoA by use of dominant negative RhoA resulted in failure of archenteron invagination, whereas, constitutively active RhoA induced precocious invagination of the archenteron (Beane *et al.*, 2006). Our observation is in line with these as blocking miRNA regulation of Dsh/Dvl can result in increased activity of Wnt/PCP pathway due to increased RhoA leading to early invagination phenotype (29h; Fig. 3.7A).

We also observed a thickened gut lining of *Dsh/Dvl* miRNA TP treated embryos (Fig. 4.1) that is absent in control morpholino treated embryos. We speculate that such structures might be extracellular matrix proteins such as proteoglycans, chondroitin sulfate (CS) that are a result of downstream Wnt activation (Beane *et al.*, 2006).

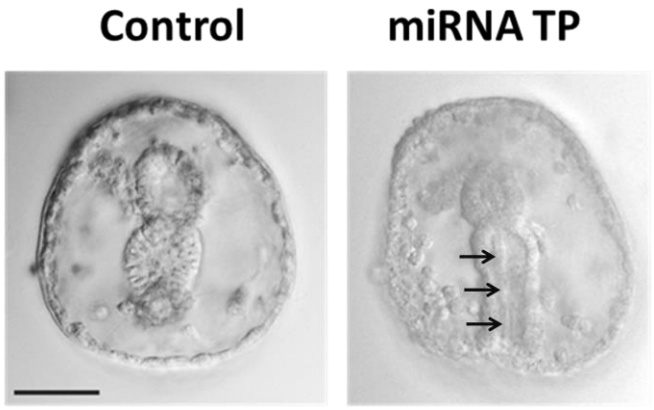

Fig. 4.1 Structural difference in the gut lumen of Dsh/Dvl miRNA TP-treated embryos. Note the linear structure running in middle of the gut tube in miRNA TP treated embryos compared to control (arrow). Scale bar is 50μm.

To summarize, we have: (1) identified specific miRNAs that regulate *β-catenin* and *Dishevelled* post-transcriptionally, (2) demonstrated that blocking miRNAs that regulate *β-catenin* results in an increase in β-catenin protein levels and a subsequent increase in β-catenin target genes transcripts, (3) inhibition of miRNA mediated regulation of *β-catenin* or *Dishevelled* results in narrower gut and smaller overall embryo, and (4) *β-catenin* miRNA TP-treated embryos have impaired digestive function as demonstrated by reduced endogenous alkaline phosphatase staining. This study contributes to an improved understanding of the regulatory role of miRNAs on components of Wnt signaling that are critical for proper specification of the endoderm during early development.

Chapter 5

FUTURE PERSPECTIVE

β-catenin is known to regulate gene regulatory network involved in endoderm and mesoderm specification (Logan *et al.*, 1999; Wikramanayake *et al.*, 1998). Furthermore, recent study shows involvement of non-canonical Wnt pathways in patterning of ectodermal tissue (Range *et al.*, 2013). In this study we have shown that blocking miRNA-mediated regulation of *β-catenin* results in increased transcripts of Wnt responsive endoderm regulatory genes. We will use QPCR to assay for transcript levels of genes downstream of the Wnt/β-catenin, Wnt/PCP, and Wnt/Calcium pathway in *Dvl* miRNA TP treated embryos (Fig. 5.1) With this approach we will begin to understand the molecular mechanism of the Dsh/Dvl miRNA TP induced phenotypes.

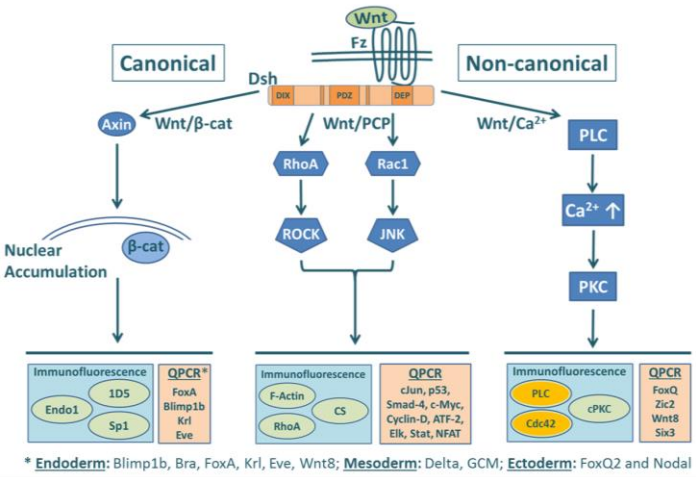

Fig. 5.1 Schematic of QPCR and immunofluorescence analysis to identify the molecular mechanism of Dsh/Dvl miRNA TP induced phenotypes.

Also, based on our result in *Dsh/Dvl* miRNA TP treated embryos, where we see thinner gut compared to controls, we want to check the level of expression of endodermal markers such as Endo1 and alkaline phosphatase (AP) using immuno-fluorescence or AP staining. Particularly AP staining will be helpful in evaluating digestive function of the larval gut which in turn will shed light on the reasons for smaller embryo size in Dsh miRNA TP treated embryos compared to control. We also expect to see differences in immunostaining pattern of primary mesenchymal cells (PMCs, using antibody against 1d5) as well as pigment cells (using antibody against Sp1 antigen). This is primarily because Wnt/PCP signaling regulates morphogenesis and gut tube invagination and migration of PMCs and pigment cells (Miller and McCrea, 2010). Moreover, immunofluorescence may prove instrumental in characterizing the ECM proteins like cross-linked collagen (Wessel and McClay, 1987), fibronectin (Spiegel *et al.*, 1980), chondroitin sulfate proteoglycan (Beane *et al.*, 2006) that we speculate to be part of the gut lumen that were altered in Dsh/Dvl miRNA TP embryos.

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